uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

Susanna Claire Wiens
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

Ph.D. (Biology)
GRADE / DEGREE

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

Thyroid Hormone Regulation of the Reproductive Neuroendocrine Axis of the Goldfish (*Carassius auratus*)

TITRE DE LA THÈSE / TITLE OF THESIS

V. Trudeau
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

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

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

K. Gilmour

S. Perry

S. Kennedy

K. Storey

D. MacKenzie

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

Thyroid hormone regulation of the reproductive neuroendocrine axis of the goldfish (*Carassius auratus*)

by

Susanna Claire Wiens

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

Thèse soumise à Faculté des etudes supérieurs et postdoctorales, Université d'Ottawa en vue de l'obtention du maîtrises L'Institut de biologie d'Ottawa-Carleton.

Your file Votre référence
ISBN: 978-0-494-61387-0
Our file Notre référence
ISBN: 978-0-494-61387-0

Abstract

This thesis explores thyroid hormone (TH) effects on adult neuroendocrine brain and pituitary in goldfish (*Carassius auratus*) to address the hypothesis that THs regulate the reproductive neuroendocrine axis through effects on γ-aminobutyric acid (GABA), a neurotransmitter that stimulates gonadotropin release in fish. The experimental approach was to increase circulating levels of the TH 3,5,3′-triiodothyronine (T_3) in goldfish through waterborne exposures, and measure effects on gene expression, enzyme activity and hormone levels in comparison to controls. In sexually regressed males, T_3 exposure decreased gene expression of GABA-synthetic and degradative enzymes (glutamic acid decarboxylase and GABA-transaminase, respectively) in telencephalon in a time- and dose-dependent manner but not in hypothalamus. GABA-transaminase activity was not affected by this T_3 challenge. In sexually mature males and females, T_3 treatment resulted in increased follicle stimulating hormone beta subunit expression in pituitary in females only, and had no effect on luteinizing hormone (LH) beta subunit expression or circulating LH levels in either sex. Further experiments with GABA agonists indicated that T_3 exposure had no effect on GABA-mediated LH release. TH receptor expression was affected in a tissue-specific manner by T_3 exposure, appearing to be autoregulated in pituitary, but unaffected in neuroendocrine brain regions. TH deiodinase expression in brain and pituitary and thyroid stimulating hormone beta subunit expression in pituitary was affected in a manner reflective of negative feedback by T_3 to reduce T_3 levels in tissues and in circulation to a set-point. Gene expression profiling with cDNA microarrays indicated a large-scale decrease in gene expression in adult male goldfish telencephalon in response to T_3. This included genes encoding proteins with functions central to general metabolic activity including mRNA

splicing and proteasomal protein turnover, as well as neuroendocrine signalling relevant to reproduction and neurogenesis. In conclusion, THs have the potential to regulate the reproductive neuroendocrine axis through minor effects on GABA function in neuroendocrine brain and sex-dependent effects on glycoprotein hormone subunit gene expression, except for LH beta, in pituitary. Moreover, gene expression in adult teleost brain is responsive to TH, which is critical to understanding the potential for TH regulation of the reproductive neuroendocrine axis in teleosts.

Resumé

Cette thèse explore les effets neuroendocriniens des hormones thyroïdiennes (HT) sur le cerveau et la glande hypophyse chez le poisson rouge (*Carassius auratus*) adulte. L'hypothèse testée est que les HT contrôlent l'axe reproducteur neuroendocrinien par l'entremise de l'acide γ-aminobutyrique (GABA), un neurotransmetteur qui stimule le relâchement des gonadotrophines chez les poissons. L'approche expérimentale était d'élever les niveaux de l'HT 3,5,3′-triiodothyronine (T_3) dans le système circulatoire du poisson rouge via une exposition aquatique afin de mesurer l'expression génique, l'activité enzymatique et les concentrations d'hormones par rapport aux animaux témoins. Chez les poissons mâles en régression sexuelle, l'exposition au T_3 a diminué l'expression génique des enzymes de synthèse et de dégradation de GABA (l'acide glutamique décarboxylase et le GABA-transaminase, respectivement) dans le télencéphale en fonction du temps et de la concentration, aucun effet n'a été détecté dans l'hypothalamus. L'activité GABA-transaminase n'a pas été affectée par le traitement au T_3. Lors du traitement de mâles et femelles sexuellement matures au T_3, l'expression de la sous-unité béta de l'hormone folliculostimulante dans l'hypophyse a augmenté pour les femelles seulement, et aucun effet n'a été observé sur l'expression et sur la concentration dans le sang de la sous-unité béta de l'hormone lutéinisante (LH) chez aucun des deux sexes. De plus, des expériences complémentaires utilisant les agonistes de GABA, ont indiqué que le traitement au T_3 n'avait également aucun effet sur le relâchement de LH via GABA. L'expression des récepteurs HT a été affectée par T_3 de manière spécifique selon les organes, les récepteurs semblent être autorégulés par l'hypophyse, mais pas chez les autres parties neuroendocrines du cerveau. Les expressions des déiodinases dans le cerveau et l'hypophyse ainsi que l'expression de la

sous-unité béta de l'hormone thyrotropine dans l'hypophyse ont été affecté de manière à refléter une régulation négative produite par T_3 afin de réduire les concentrations de T_3 dans les organes et le système circulatoire à un niveau prédéterminé. Des analyses de biopuces ont démontré que l'expression de plusieurs gènes diminue suite au traitement au T_3 incluant les gènes qui codent pour des protéines ayant des fonctions centrales et des fonctions reliées à l'activité métabolique générale dont l'épissage de l'ARN et le renouvellement des protéines protéasomales ainsi que les signaux neuroendocriniens, la reproduction et la neurogenèse. En conclusion, les HT peuvent réguler l'axe reproducteur neuroendocrinien par l'entremise de mineurs effets sur la fonction de GABA dans le cerveau neuroendocrinien et par des effets sur l'expression génique de sous-unités d'hormones glycoprotéines, exceptée pour LH-beta dans l'hypophyse. De plus, l'expression génique chez le cerveau des adultes téléostéens répond aux HT ce qui permet de mieux comprendre la régulation des HT dans l'axe reproducteur neuroendocrinien chez les téléostéens.

Acknowledgements

Thank you to my primary supervisor, Vance Trudeau, for the opportunity to do this work, and to my co-supervisor Marc Ekker and my advisory committee, Steve Perry and Ken Storey, for their time and insight, which helped shape this thesis.

I gratefully acknowledge the individuals who have directly contributed to the data presented in this thesis. Francina Jackson assisted in the May fish exposure and carried out the majority of real-time RT-PCR analysis and statistical analysis for that exposure presented in Chapter 3 as her honours thesis research under my supervision. Robert Zhao carried out the LH radioimmunoassays. Huiling Xiong carried out the data normalization and statistical analysis of the microarray data under the supervision of Dr. Xuhua Xia. Jason Popesku provided the biostatistical support required to do gene ontology analysis of the microarray data. Joel Cahn contributed to cloning the thyroid hormone receptors.

My research was greatly assisted by members of TEAM.Endo who contributed by giving me technical and statistics training, by assisting with fish dissections, and through thoughtful discussion on experimental design and interpretation of data: Rosalie Awad, Kate Crump, Chris Martyniuk, Emily Gerrie, Linhui Yao, Vicki Marlatt, Natacha Hogan, Paula Duarte, Valérie Langlois, Dapeng Zhang, Jan Mennigen, Rob Zhao, Mandy Woodhouse, Caroline Mimeault, Maxine Croteau, Wudu Lado, Colin Cameron and many summer students including Nick Potvin, Henrietta Parnas, Linda Jewel, Jennifer Watt, Elvin Tan and Ed Harris. I also pay tribute to the memory of Christian Doyon whose presence in the lab in my first years here enriched that experience and taught me a lot about how to live a life.

I thank Bill Fletcher in the animal care facility, Phillip Pelletier in the common molecular facility of CAREG, Drs. Gilmour, Moon, Aranson and Walsh for the use of spectrophotometers in their labs, and Valérie Langlois for French translation of the abstract.

For the third thesis in a row, I thank my partner Brian Eves for his vast supply of technical support (reference manager, formatting) and emotional support in completing this thesis. I also thank my family and friends for their unwavering support and patience.

I acknowledge the financial support of NSERC, OGS, and OGSST (Tito Scaiano). In particular I thank Tito Scaiano for the opportunity to have a fifth year of funding to help complete my doctoral work.

Table of Contents

List of Tables

List of Figures

Abbreviations

ALS	amyotrophic lateral sclerosis
BLAST	Basic Local Alignment Search Tool
BTEB	basic transcription element binding protein
BZ	benzodiazepine
cDNA	complementary DNA
CRH	corticotropin releasing hormone
C_t	threshold cycle
Cy3	Cyanine 3
Cy5	Cyanine 5
D1	Deiodinase Type 1
D2	Deiodinase Type 2
D3	Deiodinase Type 3
DA	dopamine
DNA	deoxyribonucleic acid
EIA	enzyme immunoassay
FABP7	fatty acid binding protein 7
FSH	follicle stimulating hormone
FWER	family-wise error rate
FSHβ	follicle stimulating hormone beta subunit
FTLD	frontotemporal lobar degeneration
GABA	γ-aminobutyric acid
GABA-T	GABA-transaminase
GAD	glutamic acid decarboxylase
GAT	GABA transporter
GH	growth hormone
GnRH	gonadotropin releasing hormone
GO	gene ontology
GPHα	glycoprotein hormone alpha subunit
GSI	gonadosomatic index
HPG	hypothalamo-pituitary-gonadal
HPT	hypothalamo-pituitary-thyroid
KLF13	Kruppel-like factor 13
LH	luteinizing hormone
LHβ	luteinizing hormone beta subunit
MBP	myelin basic protein
MCT	monocarboxylate transporters
mGLUR	metabotropic group I glutamate receptor
mOD	milli optical-density

NADH	reduced nicotinamide adenine dinucleotide
NCBI	The National Center for Biotechnology Information
NoRT	no-reverse-transcriptase-control
NPY	neuropeptide Y
NTC	no template control
OATP1C1	organic anion-transporting polypeptide 1C1
PACAP	pituitary adenylate cyclase activation polypeptide
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PEBP	phosphatidylethanolamine binding protein
PLP	pyridoxal-5'-phosphate
POMC	proopiomelanocortin
PTU	propylthiouracil
RAR	retinoic acid receptor
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
rT_3	3,3′,5′-reverse T_3
RT-PCR	reverse transcriptase-PCR
RXR	9-cis retinoic acid receptor
snRNP	small nuclear ribonucleoprotein
SSDH	succinate semialdehyde dehydrogenase
T_3	3,5,3′-tri-iodothyronine
T_4	thyroxine
TH	thyroid hormone
TMS	tricaine methanesulfonate
TR	thyroid hormone receptor
TRα-t	TRα-truncated
TRE	thyroid response element
nTRE	negative thyroid response element
pTRE	positive thyroid response element
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
TSHβ	thyroid stimulating hormone beta subunit
TTR	transthyretin
UPS	ubiquitin-proteasome system

CHAPTER 1

General Introduction

(Sections 1.4-1.8 are adapted from Wiens and Trudeau, 2006)

1.1 Thesis rationale

The reproductive neuroendocrine axis refers to the structures and signalling systems that ultimately control reproductive processes, starting with neuronal and hormonal inputs from brain regions to the pituitary gland. These brain regions are referred to as neuroendocrine brain regions, and in goldfish (*Carassius auratus*) are hypothalamus and the preoptic region of the telencephalon. The neuroendocrine inputs to the pituitary control synthesis and release of the gonadotropin hormones from the gonadotropes in the pituitary, follicle stimulating hormone (FSH) and luteinizing hormone (LH), which act on the gonads to stimulate steroidogenesis, vitellogenesis, spermatogenesis, ovulation and sperm release.

In goldfish, gonadotropin releasing hormone (GnRH) and dopamine (DA) are the main neuroendocrine stimulatory and inhibitory effectors of gonadotropin release, respectively (Trudeau, 1997), with numerous other neurotransmitters and neuromodulators modulating these effects, including steroid hormone feedback (Chang et al., 2009; Popesku et al., 2008; Trudeau, 1997). The neurotransmitter γ-aminobutyric acid (GABA) has clear roles in stimulating LH release in goldfish, in a sex- and season-dependent manner, through both enhanced GnRH release and by inhibiting DA neurons thus lifting tonic inhibition by DA on gonadotropes, and also by direct action on gonadotropes (reviewed by Popesku et al., 2008; Trudeau et al., 2000b). GABA also stimulates FSH release in trout (Mañanos et al., 1999).

For seasonal breeders, the neuroendocrine reproductive axis has critical roles in transducing physiological signals, such as changes in circulating hormone levels, and environmental signals, such as photoperiod and ambient temperature, into changes in reproductive status. In several species, including sheep and Japanese quail, thyroid hormones (THs) have been shown to have critical, though permissive, roles in photoperiod-induced changes in reproductive function through actions in hypothalamus that are necessary for gonadotropin release and gonadal growth (Hanon et al., 2008; Nakao et al., 2008b). In fish species, it is well established that the thyroid and reproductive systems interact (Cyr and Eales, 1996; Swapna and Senthilkumaran, 2007); however little attention has been given to TH effects on neuroendocrine signalling in fish species.

Given the effects of neurotransmitters including GABA on gonadotropin synthesis and release in goldfish, and the importance of THs on reproduction in fish and other vertebrates, one possible route for TH regulation of reproduction in goldfish is through effects on neuroendocrine signalling systems in brain, including that of GABA. A thorough review of the existing and disparate literature revealed strong evidence that THs regulate the GABA system in rats, mice and humans, with no research published on non-mammalian species (Wiens and Trudeau, 2006). The GABA system components identified as TH-responsive in the mammalian literature are present in goldfish, with high gene sequence similarities to humans e.g. GABA receptor subunits, transporters and synthetic and degradative enzymes (Martyniuk et al., 2005). Furthermore, studies on regulation of the GABA system in goldfish by GABA (Martyniuk et al., 2005) and estradiol (reviewd by Trudeau, 1997) indicate some conservation in regulation of the GABA system between rats and goldfish. Thus, THs have the potential to regulate GABA function in goldfish.

Although TH action in developing brain has been extensively studied, and is known to be critical to brain development (Bernal, 2002), the importance and mechanisms of TH action in adult brain have not been well addressed in the scientific literature for any species. Large scale gene expression profiling to define TH action in the central nervous systems of a non-mammalian species has yet to be done.

The goldfish is an excellent organism for studying neuroendocrine control of reproduction, given the extensive knowledge base on this system in this species resulting from over thirty years of research (reviewed by Chang et al., 2009; Popesku et al., 2008; Trudeau, 1997). It belongs to one of the largest vertebrate families, Cyprinidae, which includes fish used as models for ecotoxicology research (fathead minnow), genetic and developmental research (zebrafish), and fish that are a major food source world-wide (carp).

1.2 Hypothesis and Objectives

This thesis explores the effects of the transcriptionally active TH, 3,5,3′-triiodothyronine (T_3), on adult neuroendocrine brain and pituitary in goldfish to address the hypothesis that THs regulate the reproductive neuroendocrine axis through effects on GABA.

To this end, I had three main objectives: 1) to determine whether T_3 regulates gene expression and enzyme activity of the GABA system in neuroendocrine brain; 2) to determine whether T_3 regulates gene expression and release of gonadotropins, LH and FSH, in the pituitary and whether GABA is involved in these effects; 3) to obtain a gene expression profile for T_3 action in adult neuroendocrine brain in a teleost. A secondary objective was to determine the involvement of TH receptor and deiodinase expression in the mechanism of T_3 action in neuroendocrine brain and pituitary.

The knowledge gained from fulfilling these objectives will help elucidate mechanisms of neuroendocrine control of reproduction, identify potential sites of endocrine disruption by thyroid disrupting chemicals in the environment, and identify potential pathways through which hyperthyroid disorders result in neuroendocrine changes. Moreover, it will provide insight on the importance of TH action in adult brain.

1.3 Thesis outline

In support of the hypothesis that THs have the potential to regulate reproduction through actions on the GABA system, in the remaining sections of the General Introduction (sections 1.4-1.8), I present a survey of the existing and disparate literature on clinical evidence and experimental effects of THs on GABA function and GABA effects on the thyroid system. This provides strong evidence of reciprocal regulation of thyroid and GABA systems in mammals. I begin with brief overviews of the thyroid system and GABA system in vertebrates with details on fish, where relevant. The remainder of the thesis is organized into three data chapters followed by a General Discussion (Chapter 5). The general experimental approach for each data chapter was to expose adult goldfish to waterborne T_3 using two doses and two time-points to elicit an acute and chronic response to both a physiological and supraphysiological increase in circulating T_3 levels, and measure brain or pituitary gene expression, or brain enzyme activities, and circulating T_3 and LH levels for comparison to controls. In Chapter 2, I exposed sexually regressed, male goldfish to T_3 and measured gene expression of the enzymes responsible for synthesis and degradation of GABA in neuroendocrine brain regions, and one accompanying enzyme activity, in neuroendocrine and non-neuroendocrine brain regions. I also tested whether there were rapid, direct *in vitro* effects of T_3 on this enzyme activity in brain region homogenates. In

Chapter 3, I exposed sexually mature male and female goldfish to T_3 under the same regime as in Chapter 2, and measured gene expression of LH and FSH subunits in pituitary. I performed a second experiment in Chapter 3 in which males and females exposed to T_3 for one dose and time point were then injected with GABA receptor agonists, to determine whether T_3 exposure affected GABA-mediated LH release. In Chapter 4, I used telencephalon samples from the male fish exposed in Chapter 2 to determine a gene expression profile for T_3 action in adult neuroendocrine brain, using the brain-enriched goldfish-carp microarray developed in the Trudeau lab (Martyniuk et al., 2006).

1.4 The thyroid system

Thyroxine (T_4) and, to a much lesser degree, T_3 are secreted to the blood stream from the thyroid gland in response to thyroid stimulating hormone (TSH), produced by thyrotropes in the pituitary. TSH is released in response to thyrotropin releasing hormone (TRH) from the hypothalamus in mammals, and depending on the fish species, TRH or corticotropin releasing hormone (CRH) (MacKenzie et al., 2009). Once secreted into the blood stream, THs are reversibly bound to plasma binding proteins (thyroxine-binding globulin, thyroxine-binding prealbumin or transthyretin, and albumin), which facilitate TH transport and possibly entry into tissues including brain and pituitary. The mechanisms by which THs cross cell membranes, the blood-brain barrier and blood-cerebral spinal fluid barrier are not fully understood and have been a matter of some controversy (reviewed by Oppenheimer and Schwartz, 1997; Visser et al., 2008). The majority of TH entry into cells is through TH transporters. Several families of TH transporters have been identified, but only three transporters have high affinity or specificity for THs: the monocarboxylate transporters (MCT) MCT8 and MCT10 and organic anion-transporting polypeptide 1C1

(OATP1C1)(Visser et al., 2008). Furthermore, a multi-step mechanism has been proposed for TH crossing of the blood-brain barrier: T_4 and T_3 enter astrocytes through OATP1C1 and T_3 enters neurons from the astrocytes through MCT8 (Visser et al., 2008).

T_3 is the transcriptionally active TH, which is produced intracellularly from circulating T_4 by the process of deiodination. Deiodination is the stepwise enzymatic removal of iodine from the TH molecule by enzymes known as deiodinases (Figure 1.1) (reviewed by Eales and Brown, 1993; St Germain et al., 2009). T_3 may act in the cell or tissue where it is produced, or it may re-enter the circulation and be transported to target tissues. Although some T_3 enters the brain and pituitary from the circulation, T_3 is also produced in the brain by deiodination of T_4. T_3 is produced by removal of iodine from the outer phenolic ring of T_4. Deiodinase Type 2 (D2) is responsible for this activating deiodination activity in brain (reviewed by Bernal et al., 2003; Courtin et al., 2005). T_4 and T_3 are deiodinated respectively to the relatively inactive THs 3,3′,5′-reverse T_3 (rT_3) and 3,3′-diiodothyronine (T_2) by removal of an iodide from the inner tyrosil ring. Deiodinase Type 3 (D3) is responsible for this inactivating deiodination activity in brain (Bernal et al., 2003; Courtin et al., 2005). Deiodinase Type 1 (D1) has also been characterized in vertebrates including fish, and it has been shown to have both deiodinating activities (St Germain et al., 2009). The relative activity of each deiodinase determines the amount of T_3 available for action in cells. Depending on species, developmental stage and hormonal state, the brain and pituitary may be poised for T_3 and T_4 degradation or T_3 generation to maintain intracellular T_3 at a set-point (Bernal et al., 2003; Eales and Brown, 1993).

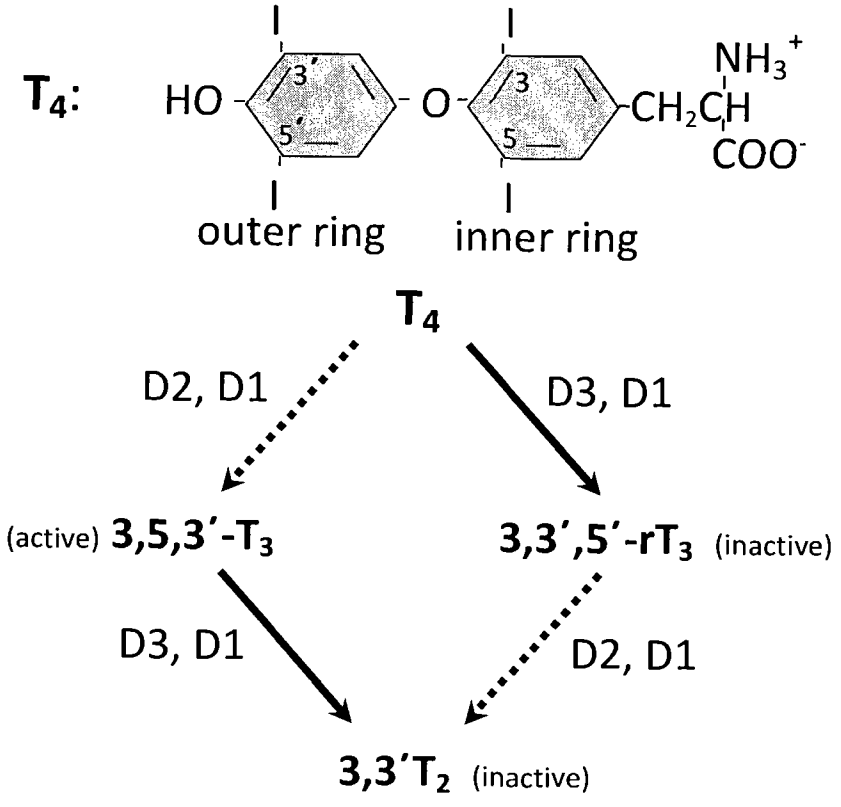

Figure 1.1 Deiodination is the stepwise enzymatic removal of iodine from the TH molecule by enzymes known as deiodinases. Iodine removal from the outer phenolic ring is catalyzed by D2 and D1 (dashed arrows), and iodine removal from the inner tyrosil ring is catalyzed by D3 and D1 (solid arrows). D1, Deiodinase Type 1; D2, Deiodinase Type 2; D3, Deiodinase Type 3; T_4, thyroxine; T_3, triiodothyronine; rT_3, reverse T_3; T_2, diiodothyronine (Adapted from: Eales and Brown, 1993; St Germain et al., 2009).

Thyroidal status, or a TH set-point in tissues or circulation, is tightly self-regulated by THs at several levels of the thyroid axis in many species including fish. First, there is negative feedback of T_3 or T_4 at the level of hypothalamus or pituitary, to reduce TRH (or CRH) or TSH release as TH levels increase (MacKenzie *et al.*, 2009). Second, THs regulate expression and activity of deiodinases in a homeostatic, autoregulatory fashion: an experimental T_3 challenge results in a homeostatic decrease in D2 expression and activity, and simultaneous increase in D3 activity, which would reduce T_3 production from T_4, and increase breakdown of T_3 to T_2 and T_4 to inactive rT_3 (Bres et al., 2006; Eales and Brown, 1993). Thyroid hormone receptors (TRs) belong to the nuclear hormone receptor superfamily (reviewed by Flamant and Samarut, 2003; Jones et al., 2003; Nelson and Habibi, 2009; Yen, 2001). In mammals there are two main TR subtypes, TRα and TRβ, which are encoded by different genes, NR1A1 and NR1A2 respectively (Jones et al., 2003). There is one form of TRα that binds T_3, designated TRα-1, but there are three forms of TRβ that bind T_3, TRβ-1, TRβ-2 and TRβ-3, generated by alternate promoter use (Flamant and Samarut, 2003). Both TRα and TRβ also have multiple isoforms generated by alternate splicing that do not bind TH but do bind to thyroid response elements (TREs) in the promoter of thyroid-responsive genes and may act as antagonists to T_3 action (Flamant and Samarut, 2003). TRs have been identified in several fish species including goldfish, in which four TR isoforms have been identified: TRβ, TRα-1, TRα-2 and TRα-truncated (TRα-t) (Nelson and Habibi, 2006). TRβ and TRα-1 were characterized as the classical TRβ and TRα-1 of mammals, and TRα-2 and TRα-t were characterized as novel TRs likely to be splice-variants of TRα-1 either with an incomplete ligand binding domain (TRα-2) or completely lacking a ligand binding domain (TRα-t) and therefore likely do not bind T_3 (Nelson and Habibi, 2006).

Once in the cell, T_3 moves to the nucleus and binds to intracellular TRs with TH-binding capacity, which then bind, or are already bound, to TREs The TR binds the TRE generally as a heterodimer with a 9-cis retinoic acid receptor (RXR). Unliganded TR/RXR bound to a positive TRE represses transcription by recruitment of co-repressor molecules, whereas once ligand binds to the TR/RXR, transcription is activated by recruitment of co-activator molecules (Paul et al., 2005; Yen, 2001). The opposite effect occurs on negative TREs (Yen, 2001).

T_3 has been shown to upregulate the expression of TRs, a process known as feed-forward receptor autoregulation, which is beneficial as a regulatory mechanism in that the ligand, T_3, modifies expression of thyroid-responsive genes in a manner reflective of its own intracellular abundance (Buchholz et al., 2006; Tata, 1994). This process is known to occur with TRβ in *Xenopus laevis* during metamorphosis, and is likely through direct action of T_3 and TRs on the TRE in the promoter of TRβ (Buchholz et al., 2006; Tata, 1994). TREs have also been identified in the promoters of the genes encoding human TRα (Chatterjee et al., 1989b) and TRβ1 (Suzuki et al., 1994), and it has been proposed that human TRβ1 is autoregulated (Sakurai et al., 1992).

The importance of TH action in the adult brain is seen from human clinical studies that indicate that hypothyroidism or hyperthyroidism are associated with neurological and psychiatric disorders (Bauer et al., 2008; Joffe and Sokolov, 1994; Simon et al., 2002) and from experimental studies on rat brain (reviewed by Calzà et al., 1997). Long-term embryonic or neonatal hypothyroidism has several irreversible effects on brain development that affect behaviour and performance, for example reduced myelination, and abnormal cell migration and differentiation (Bernal, 2002). Several genes have been identified that are regulated by TH during brain development both in mammals (Bernal, 2002; Thompson,

1996) and in amphibians undergoing metamorphosis (Denver et al., 1997). Recent large-scale gene expression analysis studies have also identified TH-responsive genes in adult mouse and rat brain (Diez et al., 2008; Haas et al., 2004; Miller et al., 2004).

THs also exert actions by mechanisms independent of the classical T_3 action through their receptors in the nucleus, referred to as extranuclear or nongenomic actions of THs (reviewed by Bassett et al., 2003; Davis et al., 2008). Recently, integrin $\alpha_V\beta_3$ has been identified as a cell surface receptor for TH, which preferentially binds T_4 over T_3 (Bergh et al., 2005). It is now thought that the majority of extranuclear actions of THs observed to date are likely mediated through integrin $\alpha_V\beta_3$ or through actions on nuclear receptors outside of the nucleus. The resulting signal transduction can culminate, in some cases, in effects on transcription in the nucleus.

T_4 and rT_3 have extranuclear actions in astrocytes (Farwell and Dubord-Tomasetti, 1999; Farwell et al., 1995) and elongating neuronal processes (Farwell et al., 2005) essential for neuronal migration and axonal projection necessary for normal brain development. T_4 regulates the interaction of the extracellular neuronal guidance molecule laminin with the cell surface adhesion molecule integrin by control of intracellular F-actin filament polymerization (Farwell et al., 1995). In the catfish *Heteropneustes fossilis*, T_3 was shown to stimulate tyrosine hydroxylase activity in telencephalon and hypothalamus *in vitro*, an effect that may not have been dependent on nuclear TRs (Chaube and Joy, 2005). This suggests that THs may also have extranuclear actions to affect neuronal enzyme activities in adult brain.

There is also a large body of work showing that thyroid-disrupting compounds in the environment have detrimental effects on neurodevelopment (reviewed by Colborn, 2004; Howdeshell, 2002), showing yet another link between thyroid function and brain function.

There is a growing list of environmental contaminants which have been found to have specific effects on the thyroid system and to cause impairments to brain function (reviewed by Brucker-Davis, 1998; Howdeshell, 2002). Main examples are industrial chemicals including polychlorinated biphenyls (PCBs) and plasticizers such as phthalates, and pesticides including dichlorodiphenyltrichloroethane (DDT).

1.5 GABA system components

The main synthetic pathway for GABA is the enzymatic conversion of glutamate to GABA by glutamic acid decarboxylase (GAD) in GABAergic neurons. Three GAD isoforms have been identified. GAD65 and GAD67 are expressed in most vertebrates (Lariviere et al., 2002; Trudeau et al., 2000b) and GAD3 has been identified in deep sea armed grenadier (*Coryphaenoides (Nematonurus) armatus*) and goldfish (Trudeau et al., 2000a). The different GAD isoforms have distinct functions as indicated by a range of evidence, from distinct subcellular localization of each isoform, to GAD isoform knockout studies (Ji et al., 1999; Soghomonian and Martin, 1998; Tian et al., 1999). GABA can also be formed from ornithine, as ornithine decarboxylase converts ornithine to putrescine, which is then converted to GABA (Eliasson et al., 1997). This is a minor route for GABA formation and is likely only significant early in development, before GAD activity appears (Eliasson et al., 1997). GABA is metabolized to succinate in GABAergic neurons and in glial cells, by sequential actions of GABA-transaminase (GABA-T) and succinate semialdehyde dehydrogenase (SSDH).

GABA is released from the neuron into the synapse by depolarization-induced, Ca^{+2}-dependent exocytosis. GABA then binds to GABA receptors on the post-synaptic membrane to exert its effects. There are three types of GABA receptors, ionotropic $GABA_A$ and

$GABA_C$ receptors and metabotropic $GABA_B$ receptors (Mehta and Ticku, 1999). $GABA_A$ receptors are ligand-gated channels permeable to Cl^- and HCO^-_3, formed from five receptor subunits with the channel at the centre. Although there are at least seven classes of $GABA_A$ receptor subunits (α, β, γ, ε, π,θ, δ), $GABA_A$ receptors are most commonly composed of α, β, and γ subunits, with the GABA binding site at the interface of the α and β subunits (reviewed by Wisden and Stephens, 1999). Although GABA is the major inhibitory neurotransmitter in developed vertebrate brain, it is now clear that GABA-gated channel opening can result in either hyperpolarization or depolarization of the post-synaptic neuron in both developing and developed brain, resulting in either inhibitory or excitatory effects (reviewed by Marty and Llano, 2005). $GABA_B$ receptors are coupled to Ca^{2+} or K^+ channels by G-proteins and second messenger systems, and upon GABA binding, cause postsynaptic membrane hyperpolarization or inhibit neurotransmitter release from presynaptic membranes. $GABA_C$ receptors are directly associated with the Cl^- channel but are pharmacologically distinct from $GABA_A$ receptors in that they are likely homo-oligomers of the ρ subunit and have a restricted distribution, being highly expressed in the retina.

To terminate GABA signalling, GABA is taken up rapidly from the synapse into the presynaptic neuron or glial cells by GABA transporters (GAT1-3), which are membrane-bound, Na^+/Cl^--dependent proteins (Gadea and López-Colomé, 2001). In neurons, GABA can be re-packaged into vesicles and re-used for neurotransmission, and in both neurons and glial cells, GABA is degraded by metabolic pathways dictated by the metabolic needs of the cells.

1.6 GABA system function is affected by thyroid hormones

The possibility that TH affects the GABAergic system was first recognized in the late 1960's (Ramírez de Guglielmone and Gómez, 1966). Since it was first determined that congenital hypothyroidism disrupts normal brain development (resulting in mental retardation, known as cretinism) there has been ongoing research on the role THs play in brain development. Included in this body of work are experimental studies on effects of TH deficiency or excess on development of neurotransmitter systems in developing brain (Ford et al.; Patel et al., 1980a). In particular, it appears that the γ-aminobutyric acid (GABA) system is sensitive to THs. Underscoring the possible significance of the experimental evidence that THs regulate brain function by modulating GABA function (sections 1.6.1-1.6.6, below), there is clinical evidence to suggest that some human nervous disorders involving alterations in GABAergic neurotransmission are related to thyroid dysfunction, i.e. hyperthyroidism or hypothyroidism. Such disorders include anxiety disorder and panic disorder (Kragie, 1993; Simon et al., 2002), epilepsy and and seizure susceptibility (Olsen et al., 1999), and mood and cognitive impairment (Rizzo et al., 2008).

Various aspects of GABAergic function in response to TH *in vivo* and *in vitro* have been examined in numerous studies (Table 1.1); however, rarely have the different components necessary for GABAergic activity been considered simultaneously in one study. Moreover, differences in experimental design pose a significant challenge in comparing results between studies to develop a consensus opinion on TH-GABA interactions. For example, in the studies that will be summarized below, *in vivo* studies vary in that hypothyroidism was induced in many different ways, either by thyroidectomy or administration of any number of thyroid inhibitors by several different administration methods, with different time courses and doses. There is similar variety in methods for

inducing hyperthyroidism. Furthermore, rats and mice of different ages and sexes have been used. Often between studies, the same variable is measured but in different brain regions or by a different method. Thus, these differences must be carefully considered when interpreting existing data.

Table 1.1 Effects of altered thyroidal status *in vivo* or TH addition *in vitro* on GABA-system components in developing and adult brain. Data are from studies on rats unless otherwise indicated.

	Observation/Effect	Reference
GABA-ergic circuit formation		
Development		
Hypothyroid	Reduced parvalbumin immunoreactivity *in neocortex*	Berbel et al., 1996
TRα knockout mouse	Reduced parvalbumin and GAT-1 immunoreactivity *in hippocampus (no effect in neocortex)*	Guadaño-Ferraz et al., 2003
GAD Activity		
Development		
Hypothyroidism	Reduced	
	In frontal, prefrontal and visual cortex (no change in hippocampus, striatum, cerebellum , superior colliculus or spinal cord)	Virgili et al., 1991
	In basal forebrain (striatum, septum, thalamus, hypothalamus)	Patel et al., 1988
	Reduced developmental increase	
	In cerebral cortex	Balázs et al., 1968
	In cerebral cortex and cerebellum	Garcia Argiz et al., 1967
	Increased *in corpus striatum*	Kalaria and Prince, 1985
Hyperthyroidism	No effect	
	In frontal, prefrontal and visual cortex, hippocampus, striatum, cerebellum , superior colliculus or spinal cord)	Virgili et al., 1991
	In basal forebrain (striatum, septum, thalamus, hypothalamus)	Patel et al., 1988
T_4-replacement	Rescued to control levels *in basal forebrain (striatum, septum, thalamus, hypothalamus)*	Patel et al., 1988
In vitro T_3 addition	Accelerated developmental increase	
	In fetal brain cell culture	Honneger and Lenoir, 1980
	In fetal cortical neuronal culture in presence of insulin only	Aizenman and deVellis, 1987
Adult		
Hypothyroidism	Reduced *in visual cortex (no effect in striatum)*	Kalaria and Prince, 1986

GABA-T/SSDH Activity		
Development		
Hypothyroidism	Reduced developmental increase	
	In cerebral cortex and cerebellum	Garcia Argiz et al., 1967
	In cerebral cortex neurons and glia	Pesetsky and Burkart, 1977
T_3 replacement	Rescued to control levels if administered early enough	
	In cerebral cortex and cerebellum	Krawiec et al., 1969
Glutamate and GABA levels		
Development		
Hypothyroidism	Reduced (transient) *in whole brain*	Ramirez de Guglielmone and Gomez, 1966
Hyperthyroidism (mouse)	Reduced or no effect *in cerebellum*	Messer et al., 1989
Adult		
Hypothyroidism	Increased	
	In whole brain	Chapa et al., 1995-1
	In cortex (GABA only), hypothalamus (GABA only)	Upadhyaya and Agrawal, 1993
	Reduced	
	In thalamus (glutamate only), cortex (glutamate only)	Upadhyaya and Agrawal, 1993
Hyperthyroidism	Increased	
	In hypothalamus (glutamate only), thalamus (glutamate only)	Upadhyaya and Agrawal, 1993
	Reduced	
	In hypothalamus (GABA only), thalamus (GABA only)	Upadhyaya and Agrawal, 1993
	No effect	
	In cortex	Upadhyaya and Agrawal, 1993
	In whole brain	Chatterjee et al., 1989a
GABA release		
Adult		
In vitro T_3 addition	Increased *in cerebral cortex synaptosomes*	Hashimoto et al., 1991

GABA re-uptake		
Development		
Hypothyroidism	Increased *in corpus striatum homogenates*	Kalaria and Prince, 1985
Adult		
Hypothyroidism	Increased *in cerebral cortex homogenates*	Mason et al., 1987
	No effect *in corpus striatum homogenates*	Kalaria and Prince, 1986
Hyperthyroidism	No effect *in cerebral cortex homogenates*	Mason et al., 1987
In vitro TH addition	Decreased *in cerebral cortex homogenates*	Mason et al., 1987
Muscimol binding sites		
Development		
Hypothyroidism	No effect on number *in corpus striatum*	Kalaria and Prince, 1985
	Delayed developmental increase in number *in cerebellum (no effect in forebrain)*	Patel et al., 1980
Hyperthyroidism	Increased developmental increase in number *in cerebellum (no effect in forebrain)*	Patel et al., 1980
T_4-replacement	Rescued number to hyperthyroid levels *in cerebellum (no effect in forebrain)*	Patel et al., 1980
Adult		
Hypothyroidism	Decreased number *in visual cortex*	Kalaria and Prince, 1986
Hyperthyroidism	Decreased number *in cerebral cortex*	Sandrini et al., 1991

Benzodiazepine binding sites		
Development		
In vitro T_3&T_4 addition	Decreased number *in neuron-enriched primary cultures from neopallium*	Go et al., 1988
Adult		
Hypothyroidism	No change *in hippocampus or cerebral cortex*	Sandrini et al., 1993
	Increased number *in cerebral cortex*	Medina and De Robertis, 1985
	Decreased number *in medial amygdala*	Ortiz-Butron et al., 2003
Hyperthyroidism	No change *in hippocampus or cerebral cortex*	Sandrini et al., 1993
	Decreased number *in cerebral cortex*	Medina and De Robertis, 1985
In vitro T_3 addition	Decreased number and binding affinity *in cerebral cortex membrane preparations*	Medina and De Robertis, 1985
	Decreased number and binding affinity *in mouse whole brain minus cerebellum membrane preparations*	Nagy and Lajtha, 1983
GABA receptor function		
***In vitro* TH addition**	Inhibited GABA-stimulated Cl^- currents	
	In forebrain membranes and human embryonic kidney-293 cells expressing recombinant $GABA_A$ receptor subunits	Martin et al., 1996
	In Xenopus *oocytes expressing recombinant $GABA_A$ receptors*	Chapell et al., 1998 Thompson et al., 1998
	In cerebellum and cerebral cortex synaptoneurosomes	Martin et al., 2004

1.6.1 TH affects GABAergic circuit formation

Thyroidal status influences the development of inhibitory cortical GABAergic circuits (Berbel et al., 1996). Rats were rendered hypothyroid by administration of methimazole to mothers in their drinking water from embryonic day 14 until birth, followed by thyroidectomy. In hypothyroid rats from postnatal days 80 to 95, there was reduced parvalbumin immunostaining in the neocortex in comparison to controls. Parvalbumin is a calcium-binding protein that is associated with a subpopulation of inhibitory GABAergic neurons and therefore is used to identify these neurons. The reduced immunostaining indicated either that the hypothyroid condition resulted in impaired axonal development, or there were fewer parvalbumin-positive cells in the neocortex. Regardless, these data suggest that THs are required for normal development of the parvalbumin-positive subpopulation of neocortical GABAergic neurons in the developing rat brain (Berbel et al., 1996).

Supporting this study, TRα knockout mice show a major reduction in parvalbumin positive GABAergic terminals that are also immunoreactive for the GABA transporter GAT-1, on pyramidal cells in the hippocampus in comparison to wild-type mice (Guadaño-Ferraz et al., 2003). It was concluded that this was likely due to a reduction in axonal arborizations of basket and chandelier cells rather than a reduction in cell number, because the total number of parvalbumin-positive cells was similar in knockout and wild-type mice. This impairment of GABAergic circuit formation was correlated with changes in behaviour that reflected hippocampal abnormalities, specifically reduced exploratory behaviour in open field tests, and increased freezing response in contextual fear conditioning tests. The authors discounted the possibility that the slightly hypothyroid state of TRα knockout mice caused

the brain structure changes, as opposed to the absence of TRα, since brain T_4 levels were not different, and T_3 levels were only slightly higher than wild-type levels.

1.6.2 TH affects enzymes involved in synthesis and metabolism of GABA

Neonatal hypothyroidism, induced by various methods, results in decreased GAD activity in various brain regions of rats in several studies (Balázs et al., 1968; García Argiz et al., 1967; Patel et al., 1988; Virgili et al., 1991) while resulting in increased GAD activity in one study (Kalaria and Prince, 1985). Virgili et al. (1991) found that hypothyroidism induced by methimazole injection on postnatal days one to 27 resulted in lower GAD activity measured on days 14 and 28 in frontal cortex, prefrontal cortex and visual cortex compared to controls, but no differences in hippocampus, striatum, cerebellum or superior colliculus during the injection period. Patel et al. (1988) found that ten-day old rats made hypothyroid by PTU administration to mothers two days before they gave birth, had lower basal forebrain GAD activity than controls. Balázs et al. (1968) found that rats rendered hypothyroid on the day of birth by administration of ^{131}I had 15% lower GAD activity in cerebral cortex grey matter compared to controls from days 17 to 32 after birth, a period in which GAD activity was increasing. GAD activities in both groups were similar after day 32 and continued to increase up to day 46 (Balázs et al., 1968). Garcia Argiz et al. (1967) studied the effects of ^{131}I–induced hypothyroidism on the developmental rise in GAD activity in cerebellum and cerebral cortex in neonatal rats. Similar to the results of Balázs et al. (1968), the developmental rise in GAD activity was depressed in hypothyroid rats in comparison to controls after postnatal day 20. For cerebellum, GAD activity was approximately 25% lower only from day 20 until day 30, whereas for cerebral cortex, GAD activity was lower by 20-53% from day 20 until the end of the study, at day 50 (García Argiz et al., 1967). Kalaria

and Prince (1985) induced hypothyroidism in newborn rats by feeding propylthiouracil (PTU) to mothers two to three days after birth until weaning at four to five weeks, then feeding the young rats PTU in food. In contrast to the above studies, they found a small but significant increase in corpus striatum GAD activity at three and six weeks of age. They suggest that in studies that induced hypothyroidism with ^{131}I, the reduced GAD activity might be related to the resultant reduced food intake rather than hypothyroidism per se. This however does not explain the results of Virgili et al. (1991) or Patel et al. (1988), who used thyroid inhibitors and observed decreases in GAD activity. This is in marked contrast to results presented by Kalaria and Prince (1985) who used similar methods. In another study on adult rat brain, hypothyroidism was induced by administration of PTU in food for ten to 12 weeks (Kalaria and Prince, 1986). Visual cortex GAD activity was slightly higher in hypothyroid rats in comparison to controls and there was no difference in the corpus striatum (Kalaria and Prince, 1986).

Taken together, the data indicate that experimentally induced hypothyroidism reduces GAD activity in neonatal brain but not in adult brain. Therefore, there is a likely role for TH in controlling GAD activity during development. Experimental evidence on TH supplementation also supports this proposal. In an *in vitro* study using fetal rat brain cell cultures that mimic normal *in vivo* brain development, T_3 application accelerated the developmental increase in GAD activity (Honegger and Lenoir, 1980). As *in vivo* (e.g. Balázs et al., 1968), GAD activity *in vitro* increased with development, peaking at day 33 in culture. The addition of T_3 resulted in GAD activity peaking at a similar level to control cultures, but at day 20 instead of day 33 (Honegger and Lenoir, 1980). In cultured fetal cerebral cortex neurons, T_3 application accelerated the developmental increase in GAD activity (Aizenman and de Vellis, 1987). T_3 treatment also resulted in a higher specific

activity of GAD at the end of the exposure period, six days in culture, but this effect was dependent on the presence of insulin in the culture medium (Aizenman and de Vellis, 1987). Estradiol and hydrocortisone were tested without effect, suggesting that this action to increase GAD specific activity is specific to TH (Aizenman and de Vellis, 1987). Supporting these two *in vitro* studies, TH replacement to hypothyroid neonatal rats by subcutaneous T_4 injections rescued GAD activity to control levels compared to the depression in GAD activity measured in hypothyroid animals ten days after birth (Patel et al., 1988).

Together with the data on hypothyroidism in neonatal rats, it seems possible that TH levels regulate GAD activity, with decreased TH levels resulting in lower GAD activity or slower development of GAD activity, and higher TH levels resulting in higher GAD activity or accelerated development of GAD activity. However, in two studies that measured neonatal brain GAD activity in response to *in vivo* TH administration to euthyroid animals (*i.e.* experimentally induced hyperthyroidism), there was no effect of excess TH on GAD activity (Patel et al., 1988; Virgili et al., 1991). This suggests that whereas a minimum amount of TH is required for normal GAD development in brain, excess levels will not affect development of GAD activity.

THs also affect activities of other enzymes involved in the GABA metabolism pathway. GABA-T and SSDH sequentially convert GABA to succinate. GABA-T is specific to the pathway and thus could be used as an indicator of changes in GABA metabolism. In addition to measuring GAD activity in thyroidectomized neonatal rats as reported above, Garcia Argiz et al. (1967) also measured GABA-T and SSDH activities over 50 days after birth. Both enzymes showed increases in activity over that developmental time period in hypothyroid and control rats. In cerebral cortex, both enzymes had lower activities

in hypothyroid animals compared with controls over the 50 day exposure. In cerebellum, GABA-T activity was lower over the entire 50 day exposure, but SSDH activity was only lower from day 15 to 30, having reached control levels by day 40 (García Argiz et al., 1967). In a subsequent study, T_3 replacement to hypothyroid rats restored GABA-T and SSDH activities back to control levels, when administered early enough (day ten) (Krawiec et al., 1969). Bovine growth hormone administration was able to rescue enzyme activities to control levels equally to T_3 replacement, suggesting that effects of early hypothyroidism on brain development may not be due to direct effects of T_3 but due to some indirect mechanism (Krawiec et al., 1969). Histochemical studies in cerebral cortex of hypothyroid rats also show that decreased TH levels result in transient decreases in GABA-T and SSDH activities in most cells (Pesetsky and Burkat, 1977). Rats were rendered hypothyroid by PTU administration in food to mothers in the last week of gestation until they were weaned, followed by PTU in their food until 100 days of age. In neuronal cells and some glial cells, GABA-T and SSDH activities were lower than controls in hypothyroid rats from days ten to 26 after which they were similar to controls. In Bergmann glia, however, there were no apparent differences in GABA-T and SSDH activities between hypothyroid animals and controls until day 40, and this late depression of enzyme activities persisted until the end of the study at day 100.

These studies indicate that THs modulate normal activity of degradative enzymes that regulate GABA levels. Since, in developing brain, hypothyroidism appears to result in lower activity of enzymes that synthesize GABA as well as degrade it, and TH replacement rescues enzyme activities to control levels, TH may be a general regulator necessary for maintaining gene expression and therefore activity of all of these enzymes and not a specific regulator that decreases or increases GABA levels.

1.6.3 TH affects glutamate and GABA levels in brain

GABA and glutamate levels have also been measured as indicators of *in vivo* changes in the GABAergic system in response to changes in thyroidal status in neonatal rats (Messer et al., 1989; Ramírez de Guglielmone and Gómez, 1966) and adult rats (Chapa et al., 1995; Chatterjee et al., 1989a; Upadhyaya and Agrawal, 1993). In none of these studies was activity of enzymes associated with GABA synthesis and metabolism simultaneously measured. Neonatal rats rendered hypothyroid by ^{131}I injection within 2 hours of birth had reduced whole brain glutamate and GABA concentrations compared with controls from days 16 to 30 after birth, after which point they had control, adult concentrations (Ramírez de Guglielmone and Gómez, 1966). Thus, neonatal hypothyroidism results in overall decreased GABAergic function in developing rat brain. It is tempting to infer that the effects of hypothyroidism to decrease enzyme activities and neurotransmitter levels indicate a positive correlation between TH levels and GABAergic function. However, in studies in which mice were made mildly hyperthyroid by T_4 supplementation in the mother's food, glutamate and GABA levels were either slightly reduced or not affected at two to four weeks of age (Messer et al., 1989). This suggests that the mechanism by which THs regulate GABA levels in developing brain is not a direct positive correlation between GABAergic function and TH levels.

In adult rats, thyroidectomy followed by ^{131}I injection resulted in higher whole brain glutamate and GABA levels (Chapa et al., 1995), the opposite of what was observed in neonatal rats in response to ^{131}I injection (Ramírez de Guglielmone and Gómez, 1966). In another study on adult rats, hypothyroidism induced by intraperitoneal carbimazole injection resulted in higher GABA levels in cortex and hypothalamus and lower glutamate levels in the cortex and thalamus (Upadhyaya and Agrawal, 1993). The increased GABA levels

measured in these two studies is consistent with the increase in GAD activity in visual cortex of adult rats in response to hypothyroidism (Kalaria and Prince, 1986), suggesting that hypothyroidism has opposite effects in developing versus adult rat brain.

The effects of hyperthyroidism on glutamate and GABA levels in adult brain are more difficult to interpret. In one study, hyperthyroidism induced by intraperitoneal T_4 injection resulted in higher glutamate and lower GABA levels in hypothalamus and thalamus (Upadhyaya and Agrawal, 1993) but in another study, there was no effect on whole brain GABA or glutamate levels (Chatterjee et al., 1989a). In control rats there was a significant positive correlation between plasma T_3 levels and GABA levels in the hypothalamus and thalamus (Upadhyaya and Agrawal, 1993). This discounts the possibility that the experimental trends observed for adult rats (that lower TH levels result in higher GABA levels and higher TH levels result in lower GABA levels) are due to a simple negative correlation between circulating TH levels and GABAergic function.

1.6.4 TH affects GABA release and reuptake

GABA is released from the neuron into the synapse by the process of depolarization-induced, Ca^{2+}-dependent exocytosis. Using *in vitro* preparations of synaptosomes from adult rat cerebral cortex, low nanomolar concentrations of T_3, but not T_4 or rT_3, increased depolarization-induced GABA release by a direct, nongenomic mechanism (Hashimoto et al., 1991). It was suggested that this effect could be due to increased Ca^{2+} uptake, which had been observed in a previous study as a direct, extranuclear effect of T_3 on synaptosomes (Mason et al., 1990).

One mechanism of GABA inactivation is its removal from the synapse by neuronal or glial cell plasma membrane GABA transporters. Homogenates of corpus striatum from

young rats made hypothyroid by PTU treatment from birth to six weeks of age had slightly increased GABA uptake and increased GAD activity (Kalaria and Prince, 1985). These significant increases, however, were lost when the values were expressed per striatum rather than per unit mass of tissue, suggesting that the effects were due to increases in cell packing rather than to increased GABA uptake or GAD activity within each cell (Kalaria and Prince, 1985).

The majority of studies on GABA reuptake have been performed using adult brain tissues. *In vitro*, T_3, T_4, and rT_3 (in order of decreasing potency) competitively inhibited uptake of GABA by cerebral cortex homogenates of adult male rats by a nongenomic mechanism (Mason et al., 1987). Relatively high concentrations (micromolar) were required to have an effect. Therefore, the physiological relevance of this effect was questioned by the authors (Mason et al., 1987). The effects of *in vivo* hypothyroidism and hyperthyroidism on *in vitro* GABA uptake were also studied. Homogenates from rats rendered hypothyroid by surgical thyroidectomy demonstrated greater GABA uptake than controls, whereas homogenates from hyperthyroid rats demonstrated no difference in GABA uptake (Mason et al., 1987). This effect of long-term hypothyroidism was assumed to reflect a genomic action of TH that was reduced due to lower TH levels. Perhaps THs inhibit GABA transporter synthesis, thus lower TH levels resulted in an increased number of transporters in neuron membranes and therefore greater GABA uptake by brain homogenates. In contrast to these results, using a similar *in vitro* method there was no difference in GABA uptake by cerebral cortex homogenates in adult rats made hypothyroid by PTU administration in food for ten to 12 weeks in comparison to controls (Kalaria and Prince, 1986).

1.6.5 TH affects GABA receptors

Several studies have examined the effects of hypothyroidism and hyperthyroidism on $GABA_A$ receptor agonist (muscimol) binding and benzodiazepine (BZ) binding to indicate changes in receptor density or binding affinity. The $GABA_A$ receptor complex binds GABA with high affinity, but it also has several specific binding sites for other compounds, including ethanol and BZs. BZs are a class of anxiolytic drugs that includes Valium, and they increase the affinity of the GABA binding site for GABA, and thus increase the frequency of Cl^- channel opening in response to GABA (Barnard et al., 1998). Endogenous hormones, including TH, may also act at the BZ site in the physiological control of anxiety (Kragie, 1993; Skolnick and Paul, 1983).

In developing rat brain, in one study, PTU-induced hypothyroidism had no effect on density of muscimol binding sites in corpus striatum (Kalaria and Prince, 1985), whereas in a study on cerebellum and forebrain, neonatal hypothyroidism resulted in delayed developmental increases in density of GABA-binding sites on day 12 after birth in cerebellum only (Patel et al., 1980b). T_4 replacement to these hypothyroid animals resulted in an increase in binding site density above control levels in cerebellum (Patel et al., 1980b). T_4 treatment of normal animals also increased the density of GABA binding sites on day 21 after birth in cerebellum but not forebrain (Patel et al., 1980b).

Hyperthyroidism in adult male rats, induced by seven days of subcutaneous injection of T_3 *in vivo*, resulted in fewer GABA binding sites in the cerebral cortex, determined by *in vitro* binding of muscimol (Sandrini et al., 1991). However, hypothyroidism in adult male and female rats, induced by PTU in food for ten to 12 weeks, had the same effect as hyperthyroidism in the study of Sandrini et al. (1991), resulting in fewer GABA binding sites in the visual cortex (Kalaria and Prince, 1986).

Neither hypothyroidism nor hyperthyroidism altered the number of BZ binding sites of the $GABA_A$ receptor complex in adult rat hippocampus or cerebral cortex (Sandrini et al., 1993). In contrast, in male and female adult rats, hyperthyroidism induced by two weeks of subcutaneous injection of T_3 resulted in fewer BZ binding sites with no change in binding affinity in cerebral cortex (Medina and De Robertis, 1985). Two weeks of surgical thyroidectomy-induced hypothyroidism in male and female adult rats resulted in an increased number of BZ sites with no change in binding affinity in cerebral cortex (Medina and De Robertis, 1985). In contrast to that result, in female rats made hypothyroid by methimazole in drinking water, BZ binding decreased by 24% in the medial amygdala (Ortiz-Butron et al., 2003). It is clear from these studies that thyroidal state has an effect on the number of GABA and BZ binding sites on $GABA_A$ receptors, suggesting that TH may modulate transcription or translation of the $GABA_A$ receptor subunits. However, there does not appear to be a clear pattern of stimulation or depression of GABA receptor number in rat brain by changes in TH levels.

Several *in vitro* studies have indicated that THs also have direct, rapid, nongenomic effects at the level of the membrane to decrease the number of BZ binding sites or alter binding affinities in brains of embryonic (Go et al., 1988)and adult rats (Medina and De Robertis, 1985) and also adult mice (Nagy and Lajtha, 1983). Despite these effects on binding in adult rats, neither chronic nor acute hyperthyroidism *in vivo* had effects on normal BZ site function, as indicated by seizure threshold and effects of BZ site ligands on seizure threshold (Atterwill and Nutt, 1983). The authors concluded that although T_3 binds to BZ sites, it is not a BZ receptor agonist or antagonist, and therefore suggested that conclusions on *in vivo* pharmacology of THs cannot necessarily be made from *in vitro* binding studies (Atterwill and Nutt, 1983).

There is evidence to suggest that THs have direct nongenomic effects on the $GABA_A$ receptor complex to affect normal GABA activity. Martin et al. (1996) determined that T_3 inhibited GABA-stimulated Cl^- currents in rat forebrain membranes and both T_3 and T_4 inhibited GABA-stimulated Cl^- currents in human embryonic kidney-293 cells expressing recombinant $GABA_A$ receptor subunits. These effects did not depend on the BZ binding site (Martin et al., 1996). In a subsequent study on recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes, it was determined that T_3 inhibited GABA-induced Cl^- currents in a non-competitive manner, suggesting a channel blocking mechanism (Chapell et al., 1998). It was also determined that it is the α_1 receptor subunit that imparts T_3 sensitivity to the $GABA_A$ receptor. In the absence of GABA, however, T_3 had a different effect: T_3 directly gated the Cl^- channel, inducing a Cl^- current. The $GABA_A$ receptor antagonist bicuculline did not block this T_3-induced current, therefore T_3 did not exert its current-inducing effect through this receptor subtype (Chapell et al., 1998). In a different study on the characteristics of human recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes, T_3 inhibited GABA-induced Cl^- current by 65% (Thompson et al., 1998). Most recently, Martin et al. (2004) have further characterized the rapid actions of THs on $GABA_A$ receptors by studying the effects of several TH metabolites on muscimol-stimulated uptake of $^{36}Cl^-$ by synaptoneurosomes of cerebellum and cerebral cortex. Cerebral cortex is more sensitive to T_3 than cerebellum. The most effective Cl^- uptake inhibitors are T_3 and T_4, with the degree of inhibition generally decreasing with number of iodine residues on TH metabolites (Martin et al., 2004).

There is also intriguing toxicological evidence that compounds that interfere with thyroid function also interfere with $GABA_A$ receptors. Polychlorinated biphenyls (PCBs) have been shown to decrease circulating T_3 levels and affect peripheral TH metabolism in

fetal and neonatal rats (Morse et al., 1993). In primary cultures of rat neocortical cells, application of the PCB mixture Arolclor 1254 inhibited GABA-stimulated increases in intracellular Ca^{2+} concentration and inhibited Cl^- influx (Inglefield and Shafer, 2000). This suggests that PCBs could potentially affect $GABA_A$ receptor function *in vivo*, possibly through a similar mode of action as TH or by interfering with normal TH action at the level of the $GABA_A$ receptor. This could play a role in the effects of PCBs on nervous system development that have been observed in humans, such as delayed development of motor control and cognitive function (Inglefield and Shafer, 2000). Such a hypothesis awaits rigorous testing.

1.7 Thyroid system function is affected by GABA

Although GABA is prominent in the brain, it is also present in some peripheral tissues. The rat thyroid gland contains GABA, and has both GAD (Crailsheim and Gebauer, 1980) and GABA-T activities (Gebauer and Crailsheim, 1981), suggesting that GABA is produced and metabolized there. The thyroid gland is also able to accumulate exogenously administered GABA *in vivo*, and thyroid slices take up GABA *in vitro*, mainly into follicle cells, by a high-affinity mechanism structurally specific for GABA, and also by a more general low-affinity mechanism (Gebauer, 1981; Gebauer and Pabst, 1981). Moreover, GAD activity, GABA-T activity and high-affinity GABA uptake were all up-regulated by long-term experimental hyperthyroidism induced by T_3 injection, and were down-regulated by long-term hypothyroidism induced by PTU injection (Crailsheim and Gebauer, 1980; Gebauer and Crailsheim, 1981; Gebauer and Haas, 1980). Together these data suggest that GABA has some function in the thyroid gland, possibly in negative feedback for TH homeostasis (Figure 1.2).

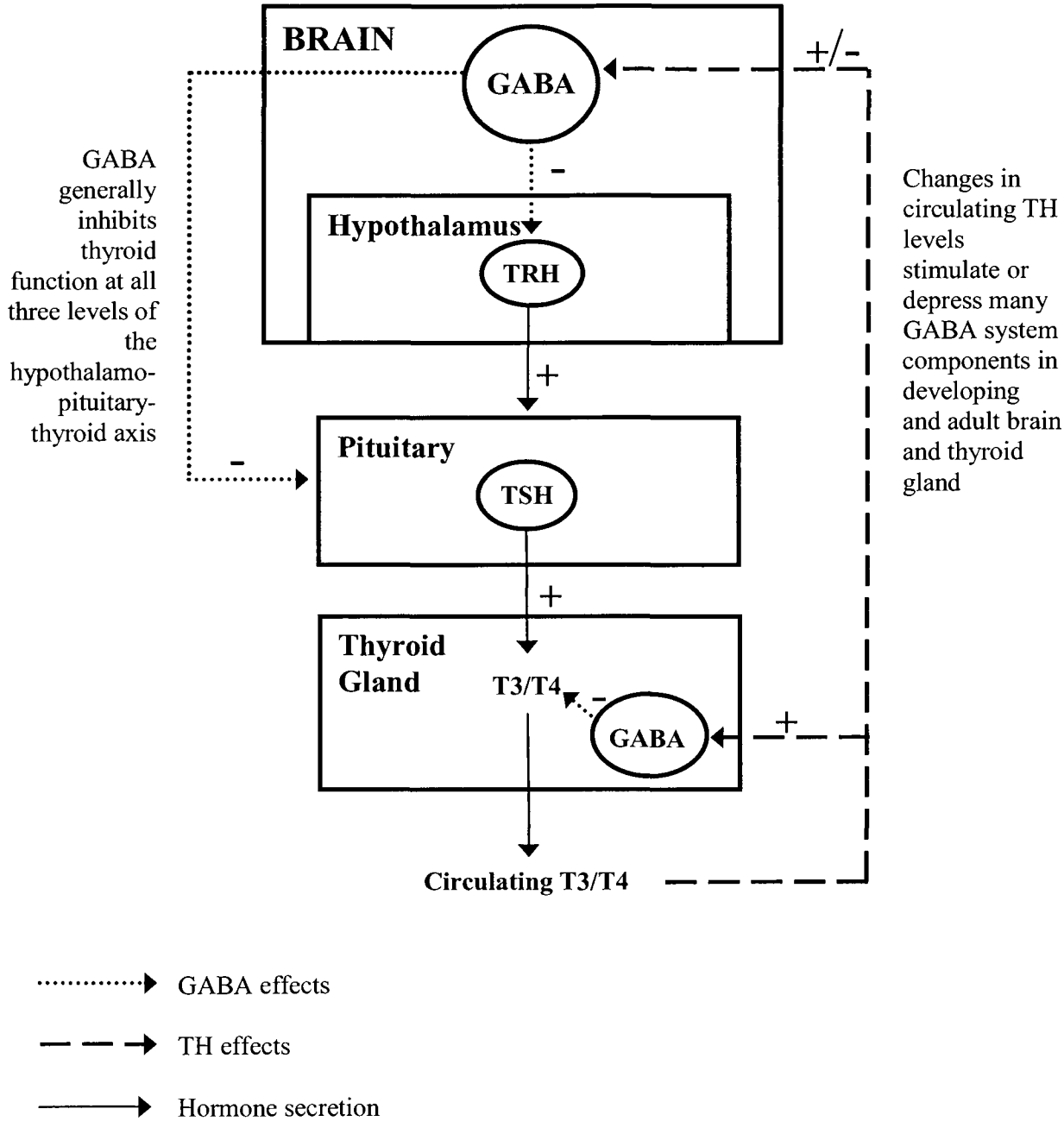

Figure 1.2 Schematic representation of the possible interactions between the thyroid and GABA systems in vertebrates. GABA, γ-aminobutyric acid; TRH, thyroropin releasing hormone; TSH, thyroid stimulating hormone; T_3, triiodothyronine; T_4, thyroxine. The + and – symbols indicate stimulation and inhibition, respectively. Please refer to main text for details

1.7.1 GABA inhibits TSH-stimulated TH release from thyroid gland

Effects of GABA on basal and TSH-stimulated TH release from the thyroid gland have been studied in adult female mice (Ahrén, 1989). GABA levels were increased *in vivo* by either intravenous administration of GABA or the GABA-T inhibitor γ-vinyl GABA. These treatments to increase GABA levels had no effects on basal TH release. However, TSH-stimulated TH release was lower than in controls. Moreover, the $GABA_A$ receptor antagonist bicuculline completely abolished the effect of GABA, implicating the $GABA_A$ receptor in the inhibition of TSH-stimulated TH release (Ahrén, 1989).

1.7.2 GABA affects TSH secretion from the pituitary

GABA affects TSH secretion by thyrotropes in the pituitary. In clinical experiments on normal and hypothyroid humans, the GABA-T inhibitor valproic acid resulted in decreased TRH-stimulated TSH release (Elias et al., 1981). It was postulated that this could either be due to direct inhibition of TRH-stimulated TSH release from pituitary by GABA acting directly on the pituitary, or due to indirect inhibition by production of a hypothalamic TSH-inhibitory factor in response to the increased GABA levels (Elias et al., 1981). *In vitro* studies on perifused rat pituitaries have demonstrated a biphasic response of TRH-stimulated TSH-release to GABA (Tapia-Arancibia et al., 1987). GABA affected TRH-stimulated TSH release, but not basal TSH release (Tapia-Arancibia et al., 1987). At the lowest concentrations tested (≤10 nM), GABA enhanced TRH-induced TSH release, but at higher concentrations, it inhibited TRH-induced TSH release (Tapia-Arancibia et al., 1987). The pharmacology of the response was investigated, and it was suggested that both the GABA effects on TRH-stimulated TSH release occurred through the $GABA_A$ receptor, but through

two different GABA binding sites, one a low-affinity binding site, the other a high-affinity binding site.

There is also striking evidence from studies in rats that GABA inhibits TSH release by inhibition of hypothalamic TRH release (Fekete et al., 2002; Jordan et al., 1983; Vijayan and McCann, 1978). Injection of GABA or the GABA-ergic drug gamma-amino-beta-hydroxybutyric acid into the third brain ventricle of rat rapidly decreased serum TSH levels, and this effect was abolished by the $GABA_{A/C}$ receptor antagonist picrotoxin (Jordan et al., 1983). *In vitro*, GABA did not inhibit basal TSH release from anterior rat pituitary (Jordan et al., 1983). Furthermore, picrotoxin and semicarbazide, a GAD inhibitor, administered separately to normal rats both caused an increase in the basal nocturnal TSH levels usually observed, lending strength to the suggestion that GABA plays a role in maintaining the homeostatic TH setpoint (Jordan et al., 1983). In a similar study, intraventricular GABA injection, but not intravenous injection or *in vitro* GABA application to pituitaries, reduced plasma TSH levels in a dose-dependent manner within ten minutes, indicating that the GABA effect was likely mediated through TRH (Vijayan and McCann, 1978). This effect was completely blocked by the $GABA_A$ receptor antagonist bicuculline, implicating this receptor subtype in the GABA effect. However, bicuculline administered without GABA had no effect on plasma TSH levels, leading the authors to question the physiological significance of the GABA-inhibition of TSH levels (Vijayan and McCann, 1978). Finally, neuroanatomical evidence indicates that GAD-immunoreactive axons make extensive contact with hypophysiotrophic proTRH-immunoreactive neurons. Therefore GABA could act as a central regulator of the hypothalamo-pituitary-thyroid axis (Fekete et al., 2002).

The hypothalamo-pituitary-thyroid axis is strongly regulated by TH negative feedback at the level of the hypothalamus and pituitary in mammals (reviewed by

Nikrodhanond et al., 2006; Shupnik, 2000), and there is mounting evidence supporting such negative feedback in fish (MacKenzie et al., 2009). The evidence presented in this review that THs affect GABA function in the brain coupled with GABA's effects to down-regulate the thyroid axis suggests that TH negative feedback may be in part mediated by effects on the GABA system. However, critically missing are experiments where GABAergic function is pharmacologically manipulated during periods of TH negative feedback.

1.8 Conclusion

The accumulated evidence from divergent clinical and experimental studies indicates that the thyroid and GABAergic systems interact in mammals (Figure 1.2). THs have effects on production and metabolism of GABA, levels of GABA and glutamate in brain, release and reuptake of GABA by neurons, and function of GABA receptors. It appears that THs have differential effects on the GABA system in developing versus adult brain. THs generally act to stimulate GABA function, or development of GABA function, in developing brain, and to inhibit GABA function in adult brain. There is also good evidence that GABA regulates the function of the thyroid system. GABA has direct effects on the hypothalamus to inhibit TRH release, as well as inhibiting TRH-stimulated TSH release from the pituitary and TSH-stimulated TH release from the thyroid gland. Despite a few conflicting reports it is likely that TH-GABA interactions are physiologically relevant, especially given the correlations between dysthyroidism and human neurological disorders with an underlying GABAergic component. The effects of THs on the GABAergic systems in non-rodent, and non-mammalian model organisms have yet to be investigated. Alternative animal models could be particularly useful in examining the TH-GABA connection given the fundamental role of TH in the control of amphibian central nervous system development and

metamorphosis (Barale et al., 1996; Denver, 1998), as well as in profound seasonal variations in metabolism and reproduction in mammals (Billings et al., 2002; Pompolo et al., 2003; Yoshimura, 2006), birds (Yoshimura, 2006) and fish (Cyr and Eales, 1996; Trudeau, 1997).

CHAPTER 2

Effects of T_3 exposure on GABA-system gene expression and enzyme activity in neuroendocrine brain of adult male goldfish

2.1 Introduction

The neurotransmitter gamma-aminobutyric acid (GABA) has clear roles in stimulation of luteinizing hormone (LH) release and therefore reproduction in goldfish (*Carassius auratus*) (Trudeau et al., 2000b). There is compelling evidence from clinical and experimental studies in mammals that thyroid hormones (THs) have effects on GABA function in developing and adult brain (Wiens and Trudeau, 2006; Chapter 1, Fig. 1.2). In mammals and birds, the transcriptionally active TH, T_3, plays a critical, permissive role in adult hypothalamus to bring about seasonal changes in reproductive state (Nakao et al., 2008b). THs also have effects on reproduction in fish and one target of TH action could be on the neuroendocrine signalling systems in brain regions regulating reproduction (Cyr and Eales, 1996). Given the roles of GABA in LH release in goldfish, and the potential of THs to affect GABA function, one possible route for TH regulation of reproduction in goldfish is through modulation of GABA system function. Although THs have critical roles in brain development in mammals, little research has focussed on TH action in adult brain in any species, including fish. In order to fully understand the hormonal mechanisms regulating GABA function in goldfish, including its role in neuroendocrine control of reproduction, it is critical to determine whether GABA function is responsive to THs.

GABA is synthesized from glutamate by the enzyme glutamic acid decarboxylase (GAD) in GABAergic neurons. Three GAD isoforms have been identified. GAD65 and GAD67 are expressed in most vertebrates (Lariviere et al., 2002; Trudeau et al., 2000b) and

GAD3 has been identified in deep sea armed grenadier and goldfish (Trudeau et al., 2000a). In mammals, a range of evidence, including subcellular location of each isoform and knockout studies, indicates that GAD65 and GAD67 have distinct functions (Ji et al., 1999; Soghomonian and Martin, 1998; Tian et al., 1999). This has not been well studied in non-mammalian vertebrates. GABA is metabolized to succinate in GABAergic neurons and in glial cells, by sequential actions of GABA-transaminase (GABA-T) and succinate semialdehyde dehydrogenase (SSDH).

The GADs and GABA-T therefore tightly regulate the amount of GABA available in brain cells. There is evidence in goldfish that GAD65, GAD67 and GABA-T are differentially expressed and have different levels of activity both among different brain regions, and among subregions of neuroendocrine brain regions (Martyniuk et al., 2007a). Furthermore, the two GADs are differentially regulated, as seen from experiments with GABA agonists (Martyniuk et al., 2007b) and on catecholamine depletion (Hibbert et al., 2004). Expression of GADs and their responsiveness to sex steroids are also dependent on sex and sexual state (Lariviere et al., 2005). THs have effects on transcription of thyroid-responsive genes through TH receptors (TRs) that act as ligand-activated transcription factors by binding to a TRE in the promoter of a gene (Yen, 2001). THs also exert extranuclear actions (Davis et al., 2008), and in the catfish *Heteropneustes fossilis*, T_3 stimulates activity of tyrosine hydroxylase, an enzyme involved in dopamine (DA) synthesis, in neuroendocrine brain regions possibly through an extranuclear mechanism (Chaube and Joy, 2005). It is possible, then, that GAD and GABA-T expression and activity could be sites of TH control over GABA function.

In rats, effects of altered thyroidal status on GAD and GABA-T enzyme activities, but not gene expression, have been studied, mostly in developing brain (for review see

Wiens and Trudeau, 2006; Section 1.6.2, Table 1.1). These studies indicate that, in developing brain, hypothyroidism (subnormal circulating TH levels) appears to result in lower activity of both GAD and GABA-T, and TH replacement restores both enzyme activities to control levels. In the one study in adult brain, hypothyroidism also resulted in reduced GAD activity (GABA-T not measured) in visual cortex but not striatum. Thus in rat brain, TH may be a general regulator necessary for maintaining gene expression and therefore activity of all of these enzymes and not a specific regulator that decreases or increases GABA levels.

Effects of THs on GABA function have not been studied in non-mammalian models, to my knowledge. In this chapter, my objective was to determine whether THs regulate GABA function in adult goldfish neuroendocrine brain through the enzymes GAD and GABA-T, by measuring gene expression and GABA-T activity in response to a T_3 challenge *in vivo*, and by measuring effects of short-term *in vitro* T_3 exposure on GABA-T activity. Adult male goldfish were exposed to a T_3 challenge, and expression of GAD65, GAD67 and GABA-T was measured in telencephalon and hypothalamus to compare thyroid-responsiveness of the neuroendocrine brain regions. Expression of the TR isoforms TRα-1 and TRβ were measured to help elucidate a mechanism of action for T_3 effects on GAD and GABA-T, if any. Expression of the TH deiodinases D2 and D3 was also measured as a positive control for efficacy of the T_3 challenge in affecting brain gene expression, since they are known to be thyroid responsive in other fish species. In target tissues, D2 catalyzes the activation of the prohormone T_4 to active T_3, and D3 catalyzes conversion of T_3 to inactive T_2, and to a lesser degree in the brain, inactivation of T_4 to rT_3 (Figure 1.1). In fish, a T_3 challenge is expected to result in a homeostatic decrease in D2 activity, and an increase in

D3 activity, to reduce intracellular T_3 levels (Bres et al., 2006; Eales and Brown, 1993). In order to measure deiodinase expression, it was first necessary to clone goldfish D2 and D3. GABA-T activity was measured in the neuroendocrine brain regions (telencephalon and hypothalamus) as well as non-neuroendocrine brain regions (cerebellum and optic tectum), to test the regional specificity of TH effects. Finally, *in vitro* exposures to T_3 were done on homogenates of all four brain parts, to determine whether T_3 may have rapid, direct effects on GABA-T activity.

2.2 Materials and Methods

2.2.1 Experimental protocol

Fish holding methods and experimental protocols were conducted in accordance with the principles and guidelines of the Canadian Council on Animal Care and approved by the University of Ottawa Protocol Review Committee.

2.2.1.1 Experimental animals

Four- to five-inch male and female common goldfish (*Carassius auratus*) were purchased from Aleong's International Inc. (Mississauga, ON, Canada) in 2005. Body mass ranged from 12 – 44 g. Fish were acclimatized for several months in 70-L cylindrical fibre-glass tanks, with flow-through dechloraminated Ottawa city tap water at 18 °C, with a light:dark cycle paralleling the natural Ottawa photoperiod, and fed a diet of Martin Mills classic floating fish food (3PT regular), *ad libitum*. In September, 2005, approximately two weeks prior to the start of exposure, fish were acclimatized to a maintenance diet of 0.1 g food per fish per day (~0.5 % bw).

2.2.1.2 T_3 exposures

Goldfish were exposed to static waterborne T_3 at nominal concentrations of 0.02 and 0.1 μM for two and six days for gene expression analysis, and for six days at 0.1 μM for GABA-T

activity analysis, from September 20th-28th, 2005. The two doses and two time-points were chosen to elicit an acute and chronic response to both a physiological and supraphysiological increase in circulating T_3 levels. Several pilot exposures were carried out to develop an appropriate exposure protocol including unsuccessful attempts to lower circulating T_3 levels with thyroid inhibitors (pilot exposure protocols and effects on circulating T_3 levels are summarized in Appendix A). The 0.1 μM T_3 dose chosen was based on a protocol for short-term T_3 immersion of killifish (*Fundulus heteroclitus*) carried out by García-G et al. (2004) and the 0.02 μM T_3 dose was chosen because it resulted in a lower, physiological increase in circulating T_3 levels. On day zero of exposure, water-flow to tanks without fish was turned off, and tanks were aerated with air-stones. T_3 sodium salt (SIGMA) was dissolved in 0.05N NaOH (vehicle), and delivered in 1.4 ml to 70-L tanks for a final vehicle concentration of ~0.002 %. Tank-water was mixed manually. Fish were then transferred to the day zero exposure tanks. Fish and air-stones were transferred to tanks with fresh, static water and T_3 and/or vehicle every two days, with the last transfer 48 h before euthanasia and dissection. Fish were fasted for 24 h prior to tank transfer and fed directly after.

Exposures for gene expression analysis were done at a density of 18 fish per tank, with two tanks of fish per treatment, for a final n of 36 fish per treatment. For GABA-T activity analysis, exposures were done at a density of 12 fish per tank with two tanks of fish per treatment, for a final n of 24 fish. An equal number of predicted males and females were assigned to each treatment tank randomly; however subsequent dissection revealed that the sample size of actual females was not big enough to warrant tissue analysis, so data are presented for males only.

2.2.1.3 Tissue sampling

Fish were last fed 24 h before sampling. Fish were anaesthetized in tricaine methanesulfonate (TMS), body mass measured, and blood taken by puncture of the caudal vasculature with heparinized syringes. Fish were then euthanized by decapitation. Sex was noted and gonads were removed and weighed for calculation of gonadosomatic index (GSI) as a measure of sexual maturity. Brain regions were dissected out, pooled two per tube, flash-frozen on dry ice and stored at -80 °C until total RNA isolation (hypothalamus and telencephalon) or GABA-T activity assays (hypothalamus, telencephalon, cerebellum and optic tectum). Blood was centrifuged at 5100 *g* for 5 min at room temperature to separate plasma from red blood cells; then plasma was removed and stored at -80 °C until the enzyme immunoassay.

2.2.2 Plasma T_3 enzyme immunoassay

To determine the effect of T_3-treatment on circulating T_3 levels, total plasma T_3 concentrations were measured using a spectrophotometric human serum T_3 enzyme immunoassay kit (MP Biomedicals, Orangeburg, NY) following the manufacturer's protocol, using a SPECTRAmax 340PC (Molecular Devices). A dilution curve of a plasma sample showed parallelism to the kit standard curve, demonstrating that heparin in plasma samples did not interfere with the assay. Samples from 0.02 μM and 0.1 μM T_3-treated groups were serially diluted 1 in 4 and 1 in 32 respectively with 0 ng/ml standard from the kit, in order for values to fall within the standard curve. The standard curve was plotted as the logit value of absorbance of each standard concentration (average of duplicates) as a function of the log value of standard concentration in ng/ml. Sample concentrations in ng/ml were then interpolated from the sample absorbances (average of duplicates) using the standard curve and corrected for dilution.

2.2.3 Plasma LH radioimmunoassay

To compare reproductive state between two-day and six-day exposure groups and to determine any effect of T_3 exposure on basal LH levels, plasma LH levels were determined by a double antibody LH radioimmunoassay in 96-well plates following the procedure of Zhao *et al.* (2006) adapted from Peter *et al.* (1984). Plasma [LH] was measured for the gene expression analysis group only, using a subset of 12 of 16 plasma samples for each treatment group.

2.2.4 Gene expression analysis for telencephalon and hypothalamus

2.2.4.1 Cloning deiodinases

Partial sequences for D2 and D3 were obtained via homology cloning from goldfish brain or liver using degenerate primers based on sequences from species of fish, amphibian and mammal (Table 2.1). Primers were designed using Primer3 (http://frodo.wi.mit.edu/) and synthesized by Invitrogen. Each PCR reaction (final volume 25 µl) contained 1 µl cDNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2mM dNTPs, 0.4 mM forward and reverse primers, and 0.02 U Taq® Polymerase (all reactants and primers from Invitrogen). PCR Amplification was carried out in a thermocycler under the following time and temperature cycle: initial denaturation at 95 °C for 5 min, followed by 35 to 40 cycles of denaturation at 95 °C for 45 s, annealing at 58.7-60 °C for 45 s (Table 2.1), and elongation at 72 °C for 60 s, followed by a final elongation step at 72 °C for 10 min. After sequence length confirmation via agarose gel electrophoresis, each amplification product was ligated directly into TOPO® II vector. One Shot TOPO® 10 chemically competent *E. coli* were then transformed with the vector. The cells were plated on LB-agar plates with ampicillin and X-gal and grown up over night. Positive colonies were selected and grown overnight in LB broth containing ampicillin and then plasmids containing the amplicon were isolated using the Miniprep kit (QIAGEN).

Sequencing was carried out for three cultures of each amplicon at Canadian Molecular Research Services (Ottawa, ON). A consensus sequence was deduced from the three sequences and submitted to Genbank (accession numbers in Table 2.1).

Table 2.1

Primer sequences and specifications for homology cloning of goldfish Deiodinase Type 2 and Deiodinase Type 3.

gene	fwd primer (5' to 3') rev primer (5' to 3')	tissue cloned from	sequence length (bp)	annealing temperature (°C)	Genbank Accession Number
D2	WGAGTGGCAGCGCATGYT RTCGATGTAGACCAGCAGGAA	liver	322	58.7	EU313786
D3	TGTGGCTCCTGGAYTTCYT ATGAAYGGNGGTCAGGWG	brain	316	60	EU313787

D2, Deiodinase Type 2; D3, Deiodinase Type 3

2.2.4.2 RNA isolation and quantification, and cDNA synthesis

Total RNA was isolated and genomic DNA removed from brain region samples using RNeasy® Plus Mini kits (QIAGEN, Mississauga, ON) according to the manufacturer's protocol for microcentrifuge with the following specifications. Each sample was homogenized using stainless steel beads (5 mm) in a MM301 Mixer Mill (Retsch, Pennsylvania, USA). RNA was resuspended in 30 µl of nuclease-free water. RNA quality was verified and concentrations were quantified spectrophotometrically using a GeneQuant RNA/DNA Calculator (Amersham Pharmacia Biotech, Piscataway, NJ). Hypothalamus samples from the six-day exposure were found to be too dilute for cDNA synthesis; therefore samples were lyophilized in a VIRTIS freeze mobile 8EL (The Virtis Company, Gardener, NY) and resuspended in 11 or 15 µl of nuclease-free water. The new RNA concentrations were quantified spectrophotometrically using a NanoDrop® Spectrophotometer ND-1000, with ND-1000 software, version 3.3 (NanoDrop Technologies, Inc.).

For each sample, cDNA was reverse-transcribed from 2 µg total RNA in a final volume of 20 µl using Superscript II® Reverse Transcriptase with 200 ng random hexamer primers (Invitrogen, Burlington, ON) following the manufacturer's protocol. cDNA synthesis was performed simultaneously on all samples of any one brain part within one time point, allowing for statistical comparisons within that group. For each cDNA synthesis run, one sample was run in parallel under the same reaction conditions but with water in place of reverse transcriptase as a control to demonstrate the absence of genomic DNA contamination (no-reverse-transcriptase-control, NoRT). Samples were stored at -20 °C until used for real-time reverse transcriptase-PCR (real-time RT-PCR).

2.2.4.3 Real-time reverse transcriptase PCR

Real-time RT-PCR assays with the double-stranded DNA-binding dye SYBR® Green were used to measure changes in brain part gene expression relative to control levels. In telencephalon, expression of TRα1, TRβ, D2, D3, GAD65, GAD67 and GABA-T was measured. Hypothalamus gene expression was measured only for D2, D3, GAD65, and GAD67.

Primers were designed using Primer3 (http://frodo.wi.mit.edu/) and synthesized by Invitrogen, except for GAD65 and GAD67 primers which had been previously designed (Martyniuk et al., 2006) (Table 2.2). Primers were based on the consensus sequences obtained by cloning in this study, or previous cloning in the Trudeau lab or other labs (Table 2.2). Primer sets were verified to be specific to the amplicon of choice by running a PCR on whole brain cDNA under standard conditions, and sequencing the resulting amplicon.

For each gene of interest, the samples of one brain part within one time point were run in triplicate on one 96-well plate allowing for statistical comparisons within that group. cDNA (with an original RNA input of 2μg in 20 μl) was diluted for each plate in order for sample values to fall within the range of the standard curve. Each 25 μl reaction contained RNase/DNase-free water (GIBCO), 1X buffer (QIAGEN), 3.5 mM $MgCl_2$ (QIAGEN),100-175 nM forward and reverse primer (Table 2.2), 0.25X SYBR Green I dye (Invitrogen), 200 μM dNTPs (Invitrogen), 1.25 U HotStarTaq (QIAGEN), 100 nM ROX reference dye (Stratagene), and 5 μl diluted sample cDNA.

Table 2.2
Real-time RT-PCR primer sequences and assay conditions for SYBR Green I assays for relative quantification of genes in goldfish brain regions.

gene	fwd primer (5' to 3') rev primer (5' to 3')	amplicon length (bp)	[primer] (nM)	annealing temperature (°C)	accession number of template
TRα-1	TGGAGGGTTAGGAGTGGTGT GAGATGTTGTGCTTGCGATG	216	150	58	AY973629
TRβ	CCGTGATTTTGCTTTCCTCT ACCTTGTGCTTGCGGTAGTT	118	150	58	AY973630
D2	TCCAAGGTGGTGAAGGTTG AGCAGATGGCACTCGTTGT	77	100	58	EU313786
D3	CTTGAAGAAGTCCAGCTTGTG ACGAAGATGCGCGAGAG	126	175	58	EU313787
*GAD65	GGATACGTGCCGTTCTTTGT CTCGACTCCATTCAGCTTCC	180	150	58	AF045594
*GAD67	CCAAAGGCATGTCTGTAGCA CCCTTCTGTTTGGCATCAAT	187	150	58	AF045595
GABA-T	ATGTTGGAGCGGTTCATTTC GGATGGAGGAGATTTGTGTGT	111	150	58	AY640231

*primers designed by Martyniuk *et al.*, 2006

The relative standard curve method was used to interpolate relative mRNA levels of target genes within each sample as described by Martyniuk et al. (2006). On each plate, a relative standard curve consisting of four to seven points was generated from relative standards run in triplicate in parallel with the experimental samples. The relative standard samples were generated by serial dilution of a cDNA pool of representative samples from the plate. Real-time RT-PCR conditions were optimized to produce standard curves with an R^2 of ≥ 0.99 and a slope representing 90-110% reaction efficiency. Two controls were run in duplicate on each plate. A no template control (NTC) containing water in place of cDNA was used to detect amplification not due to sample cDNA. A NoRT, made in parallel with cDNA synthesis, was used to detect amplification due to genomic DNA contamination in cDNA samples.

The real-time RT-PCR assays were run in an MX-3000® and MX-4000® Multiplex Quantitative PCR system (Stratagene, La Jolla, CA). The thermocycle program began with an initial 15-minute enzyme activation step at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 5 s, extension at 72 °C for 30 s, and fluorescence detection at 80 °C for 8 s. Thus, the accumulation of PCR product was measured in real time as the increase in SYBR Green I fluorescence. These 40 amplification cycles were followed by denaturation at 95 °C for 1 min and then 40 cycles of detection for 30 s starting at 55 °C and increasing 1 °C per cycle to 95 °C to produce a product dissociation curve for detection of the number of double-stranded products.

The resulting data were analyzed using MX-Pro 3000/4000 software (Stratagene). The threshold cycle (C_t) of fluorescence detection of each standard was plotted as a function of initial quantity of total RNA. For each sample, triplicate C_t values were averaged, and using the standard curve, initial template quantities were interpolated. These values are

effectively normalized against input RNA amount (Bustin, 2000), since each cDNA sample was synthesized from 2 μg RNA. Relative gene expression level was then calculated by dividing each treatment sample quantity by the average of control sample quantities.

2.2.5 GABA-T activity assay

2.2.5.1 In vivo samples

GABA-T enzyme activity was determined for each sample using a high-throughput spectrophotometric assay modified from Jung *et al.* (1977) as previously reported by Martyniuk *et al.* (2007a). Each sample pool of two brain parts was homogenized on ice by sonication, in 6 volumes of 10 mM dipotassium hydrogen orthophosphate buffer, pH 6.8, containing 20 % glycerol, 0.13 % Triton X-100, 0.1 mM reduced glutathione, 0.1 mM pyridoxal-5'-phosphate (PLP) and 1 mM Na_2EDTA. The homogenates were centrifuged at 2000 *g* for 20 min at 4 °C, and the enzyme-containing supernatant was used for the assay. Protein content of each supernatant was determined by the Bradford method (Bradford, 1976). In a 96-well plate, each sample was run in triplicate, with GABA and without GABA as substrate. On ice, in each well, 20 μl of sample homogenate supernatant or buffer blank was combined with 180 μl incubation medium, pH 8.6, containing 100 mM potassium pyrophosphate, 5 mM α–ketoglutarate, 4 mM nicotinamide adenine dinucleotide, 3.5 mM 2-mercaptoenthanol, and 0.01 mM PLP. 20 μl of water or 115 mM GABA was added to each well for a final [GABA] of 10 mM per well, and the plate was then incubated at 30 °C for 1.5 h in a SpectraMax M5 spectrophotometer, and readings taken at 340 nm every 30 s for the duration of incubation to detect NADH production. Maximum reaction rate (mOD/min) for each sample was determined from a 10 minute linear portion of the NADH production curve, using SoftMax Pro Software version 4.8 (Molecular Devices Corporation, Sunnyvale, Calif.). The average of triplicate reaction rate values with and without GABA were corrected for

background absorbance (buffer and water blank), and the without-GABA values were subtracted to correct for endogenous substrate. This corrected reaction rate and sample protein concentration were then used to calculate specific activity for each sample, in mU·mg protein^{-1} (mU = nmol·min^{-1}). All treatment and control samples for one brain part were run on one plate (n = 6 per treatment).

2.2.5.2 In vitro T_3-exposures

A study in the catfish *Heteropneustes fossilis*, on enzyme preparations from hypothalamus and telencephalon, has indicated a likely extranuclear action of T_3 to stimulate tyrosine hydroxlase activity *in vitro* (Chaube and Joy, 2005); therefore I investigated the effects of short-term T_3 exposure *in vitro* on brain GABA-T activity as a preliminary exploration of potential extranuclear actions of T_3 on GABA-T activity. The assay described above was used, with modifications similar to those reported by Awad *et al.* (2007) for testing *in vitro* effects of compounds. In March, 2006, hypothalamus, telencephalon, cerebellum and optic tectum were collected from 40 untreated male goldfish (sexually recrudescent) and pooled for each brain part. Each brain part pool was homogenized in six volumes of buffer as above except for cerebellum, which was homogenized in four volumes of buffer. Incubation medium was as above except with varying concentrations of T_3 for final well concentrations of 0, 0.31, 0.62, 1.25, 2.5, 5, and 10 ng/ml. Incubation medium with T_3 was made by dissolving T_3 sodium salt in 0.05N NaOH, and diluting this in incubation medium for a final NaOH level in the medium of 0.001 % or less, and pH of 8.6 for each T_3 concentration. For each brain part pool, each T_3 concentration was tested in triplicate on two different plates (n = 6), with and without GABA as above. As a preliminary test of *in vitro* T_3 effects at higher concentration ranges, T_3 concentrations of 20, 100 and 1000 ng/ml were tested in triplicate on one plate only (n = 3). Each plate was incubated at 30 °C for 40 min

after addition of GABA, and the reaction rate was calculated from readings from minutes 25-35 of incubation (10 min of the linear range). The time lapse between addition of homogenate to the T_3-containing incubation medium on ice and the beginning of the 30 °C incubation varied between 15 and 30 min.

2.2.6 Statistical analyses

For each variable measured, data were tested for normality with the Kolmogorov-Smirnov test, and for homoscedasticity with the Levene test. If these assumptions were not met, the data were $\log_{10}$- or reciprocal-transformed. If transformation failed to make the data meet the assumptions of normality and homoscedasticity, nonparametric one-way ANOVA (Kruskall-Wallis test), nonparametric two-way ANOVA (Scheirer-Ray-Hare extension of the Kruskall-Wallis test) or the Mann-Whitney U test was used. Where post-hoc tests were needed on nonparametric data, Tukey's test on ranked data was used. Significance level was set at 0.05 for all statistical tests (significant p-values from ANOVA and t-tests are reported in the text). All statistical analyses were carried out using S-Plus or Systat software.

For the gene expression analysis group of fish, for plasma [T_3], mass, GSI, and plasma [LH], the two-way ANOVA model was used to determine differences both due to T_3 dose and exposure time. If two-way ANOVA indicated a significant main effect of dose or a significant interaction, then Tukey's post-hoc test was carried out. Gene expression data for each gene were analyzed by one-way ANOVA, within one exposure time, to determine effects of T_3 dose. If one-way ANOVA revealed a significant effect of T_3 dose, Tukey's post-hoc test was used to identify significant differences among the doses.

For the GABA-T activity group of fish, for plasma [T_3], mass and GSI, Mann-Whitney U tests were used to compare control and 0.1 μM T_3-treated fish. GABA-T activity

data from the *in vivo* exposure were analyzed by Student's t-tests for each brain part, to compare GABA-T activities in control and T_3-treated fish.

For the *in vitro* T_3 exposures, GABA-T activity was analyzed by one-way ANOVA for each brain part followed by Tukey's test if ANOVA revealed significance, to determine which doses were significantly different from control.

2.3 Results

2.3.1 Plasma T_3 concentrations

Water-borne T_3 exposure resulted in significantly higher plasma [T_3] than controls at both doses and exposure times for the gene expression analysis group with no significant differences between the two-day and six-day exposure (Figure 2.1) (non-parametric two-way ANOVA, main effect of dose, $p < 0.001$, followed by Tukey's test on ranks). Control levels of plasma T_3 were similar in two-day and six-day fish (2.4 ± 0.1 and 2.8 ± 0.3 ng/ml, respectively). 0.02 μM T_3 exposure for two days resulted in higher plasma [T_3] than six-day exposure (23.6 ± 7.4 ng/ml, 10-fold higher than two-day control vs. 6.6 ± 1.1 ng/ml, 2.4-fold higher than six-day control), but this difference was not statistically significant. 0.1 μM T_3 for two days resulted in slightly higher plasma [T_3] than six-day exposure, but this difference was not significant (101.0 ± 27.7 ng/ml, 43-fold higher than two-day control vs. 80.0 ± 12.6 ng/ml, 29–fold higher than six-day control).

For the GABA-T activity analysis group, 0.1 μM T_3 exposure for six days resulted in plasma [T_3] approximately 26-fold higher than control levels (93.5 ± 23.8 ng/ml vs. 3.6 ± 0.2 ng/ml, data not shown, Mann-Whitney U test, $p < 0.001$).

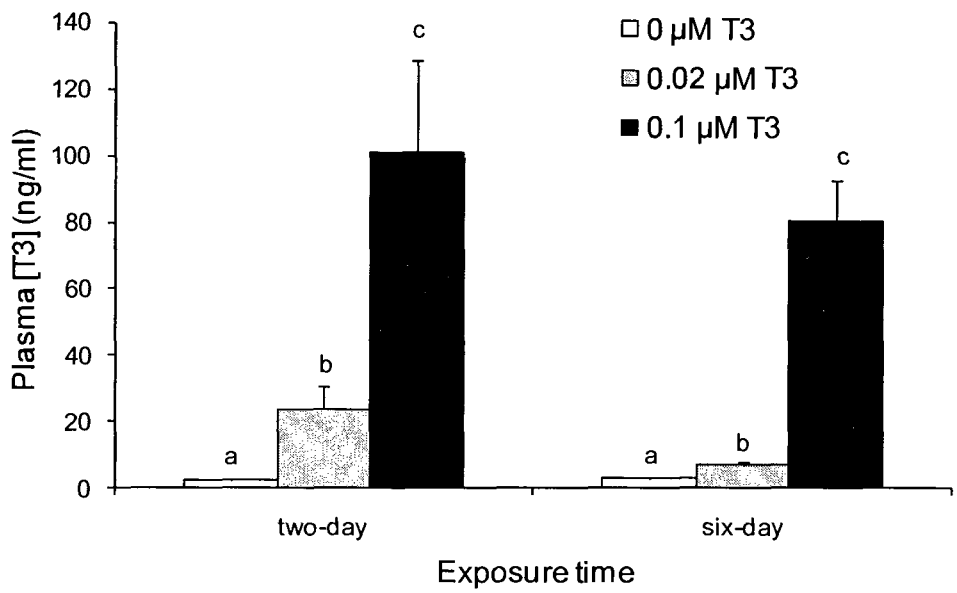

Figure 2.1 Plasma T_3 levels, measured by enzyme immunoassay, in male goldfish in September after two-day and six-day water-borne exposure to 0, 0.02 and 0.1 µM T_3 (for gene expression analysis). Data are presented as mean + SEM and were analyzed by non-parametric two-way ANOVA, followed by Tukey's post-hoc comparisons on ranks. Significant differences among T_3 doses across both exposure times are denoted by different letters ($p \leq 0.05$). $n = 16$. T_3, tri-iodothyronine.

2.3.2 Body mass, gonadosomatic index, and plasma LH concentrations

Body mass was similar among all T_3 doses and time points, both in the gene expression analysis group and the GABA-T activity group (Table 2.3). There were no significant differences in GSI or plasma [LH] due to T_3 dose; however both were slightly but significantly lower in the six-day group than the two-day group (Table 2.3) (two-way ANOVA, main effect of exposure time, $p = 0.02$ for GSI, $p = 0.003$ for LH).

2.3.3 Gene expression in the telencephalon

There were no effects of T_3 exposure on TRα-1 or TRβ expression in telencephalon for either exposure time (Figure 2.2 a, b). There were time- and dose-dependent effects of T_3 exposure on gene expression for both deiodinases (Figure 2.2 c, d). D2 expression was 1.6-fold lower than control for 0.1 μM only, after two-day exposure (one-way ANOVA on log10-transformed data, $p = 0.01$, followed by Tukey's test), with no differences after six-day exposure. D3 expression was not affected by two-day T_3 exposure, but it was 2.1-fold higher than control for 0.1 μM only, after six-day exposure (one-way ANOVA on log10-transformed data, $p < 0.001$, followed by Tukey's test).

For GAD65, GAD67 and GABA-T expression in telencephalon, T_3 exposure had a similar time- and dose-dependent effect (Figure 2.2 e-g). For two-day exposure only, the 0.1 μM group had lower expression of GAD 65, GAD67 and GABA-T than respective controls by 1.7-, 2.3- and 2.1-fold respectively, with no effects of the lower T_3 dose (0.02 μM) (for each gene, one-way ANOVA, $p = 0.002$, 0.005, 0.004, respectively, followed by Tukey's test).

Table 2.3

Body mass, gonadosomatic index and plasma LH levels of sexually regressing male goldfish exposed to T_3 for two days and six days (for gene expression analysis) and six days only (for GABA-T activity analysis) in September.

Analysis	Exposure time	T_3 dose (μM)	n	Body mass (g)	GSI (%)	*Plasma [LH] (ng/ml)
Gene Expression	two-day	0	16	26.4 ± 1.6	1.24 ± 0.21[a]	10.9 ± 4.9[a]
		0.02	16	24.0± 1.2	1.13± 0.12[a]	8.3 ± 2.3[a]
		0.1	16	26.2 ± 1.7	1.56 ± 0.17[a]	8.4 ± 1.6[a]
	six-day	0	16	23.0 ± 1.3	1.00 ± 0.12[b]	4.4 ± 1.8[b]
		0.02	16	25.6 ± 1.8	1.06 ± 0.13[b]	5.1 ± 1.8[b]
		0.1	16	24.9 ± 1.0	1.12 ± 0.09[b]	6.6 ± 3.0[b]
GABA-T Activity	six-day	0	12	25.6 ± 1.1	1.19 ± 0.16	nm
		0.1	12	24.0 ± 1.7	1.74 ± 0.34	nm

Data are presented as mean ± SEM; for the gene expression analysis group, two-way ANOVA was used to determine differences due to dose and exposure time (different superscripted letters denote statistical differences within each variable, $p \leq 0.05$; *plasma [LH]was measured in a subset of 12 samples; for the GABA-T activity analysis group, Mann-Whitney *U* test was used, alpha = 0.05; GSI is gonadosomatic index, gonad mass as percentage of body mass; LH is luteinizing hormone; nm is not measured.

Figure 2.2 Telencephalon gene expression, measured by real-time RT-PCR, in male goldfish in September after two-day and six-day water-borne exposure to 0, 0.02 and 0.1 μM T_3. Data are presented as a proportion relative to control expression level for each exposure time (mean + SEM). Data within each exposure time were analyzed by one-way ANOVA followed by Tukey's post-hoc comparisons where ANOVA revealed significance ($p \leq 0.05$). Significant differences among doses within each exposure time are denoted by different letters. n = 8 except for D2, six-day, 0.1 μM and GAD67, six-day, 0 μM and 0.1 μM, n = 7. TR, thyroid hormone receptor; D, iodothyronine deiodinase; GAD, glutamic acid decarboxylase; GABA-T, GABA-transaminase; T_3, tri-iodothyronine.

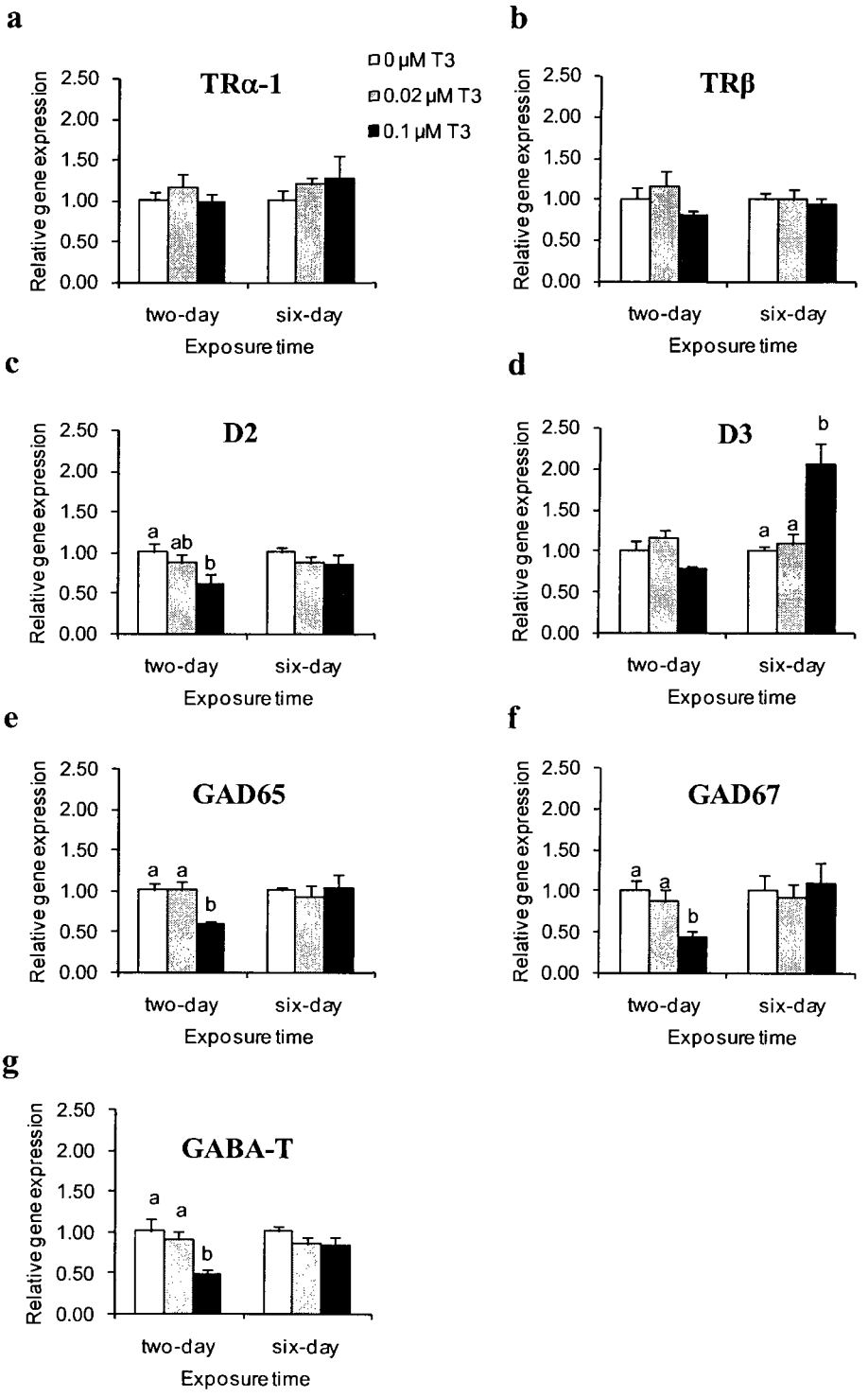

Figure 2.2 (caption on previous page)

2.3.4 Gene expression in the hypothalamus

Gene expression of D2, D3, GAD65 and GAD67 in hypothalamus was also measured (Fig 2.3) in order to compare it to T_3-responsiveness in the telencephalon. There was no effect of T_3 treatment on D2 expression. D3 expression after two-day exposure to 0.02 μM and 0.1μM T_3 was 1.4- and 1.5-fold higher than control, respectively (one-way ANOVA, $p = 0.03$, followed by Tukey's test). Similarly, D3 expression after six-day exposure to 0.02 μM and 0.1μM T_3 was 1.4- and 1.7-fold higher than control, though this was only statistically significant for 0.1 μM (one-way ANOVA, $p = 0.006$, followed by Tukey's test). There was no T_3 effect on GAD65 or GAD67expression in hypothalamus.

2.3.5 GABA-transaminase activity

2.3.5.1 In vivo samples

GABA-T activity of male goldfish was not affected in any brain region by six-day water-borne exposure to 0.1 μM T_3 (Figure 2.4). Control activity in optic tectum was highest (1.79 ± 0.05 mU·mg protein^{-1}), followed by hypothalamus (1.50 ± 0.05 mU·mg protein^{-1}), with slightly lower levels of activity in telencephalon (1.22 ± 0.06 mU·mg protein^{-1}) and considerably lower levels in cerebellum (0.42 ± 0.04 mU·mg protein^{-1}) (Figure 2.4).

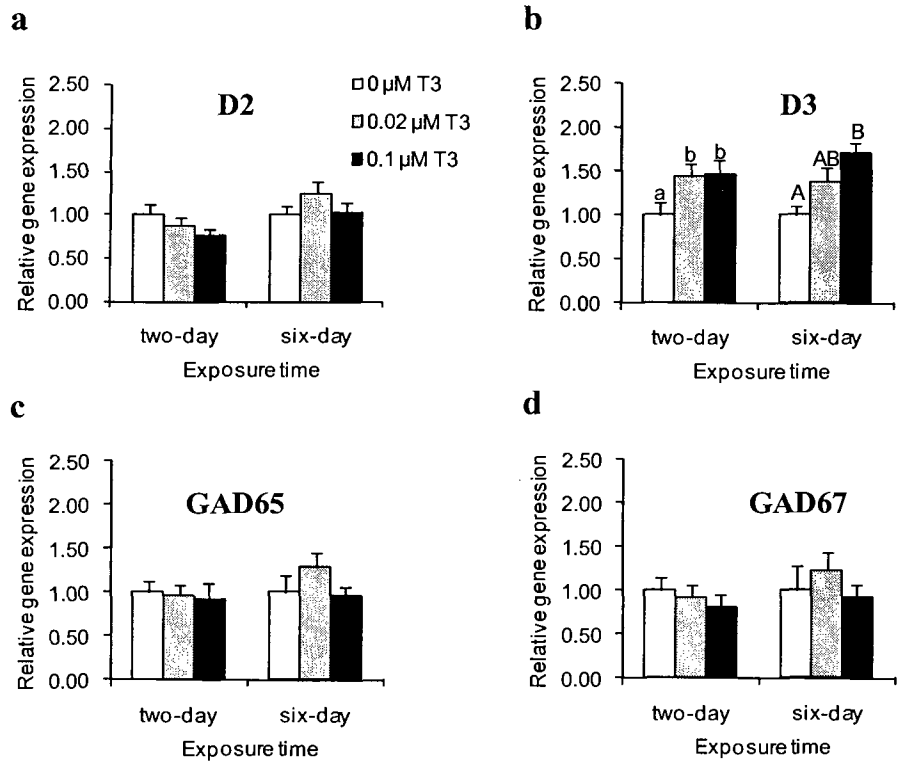

Figure 2.3 Hypothalamus gene expression, measured by real-time RT-PCR, in male goldfish in September after two-day and six-day water-borne exposure to 0, 0.02 and 0.1 µM T_3. Data are presented as a proportion relative to control expression level for each exposure time (mean ± SEM). Data within each exposure time were analyzed by one-way ANOVA followed by Tukey's post-hoc comparisons where ANOVA revealed significance ($p \leq 0.05$). Significant differences among doses within the two-day exposure time are denoted by different lower-case letters and within the six-day exposure time are denoted by upper-case letters. For 0 µM, 0.02 µM and 0.1 µM T_3 doses, respectively, n = 8, 8, 7 for 2 d exposure, and n = 7, 7, 8 for 6 d exposure. D, iodothyronine deiodinase; GAD, glutamic acid decarboxylase; T_3, tri-iodothyronine.

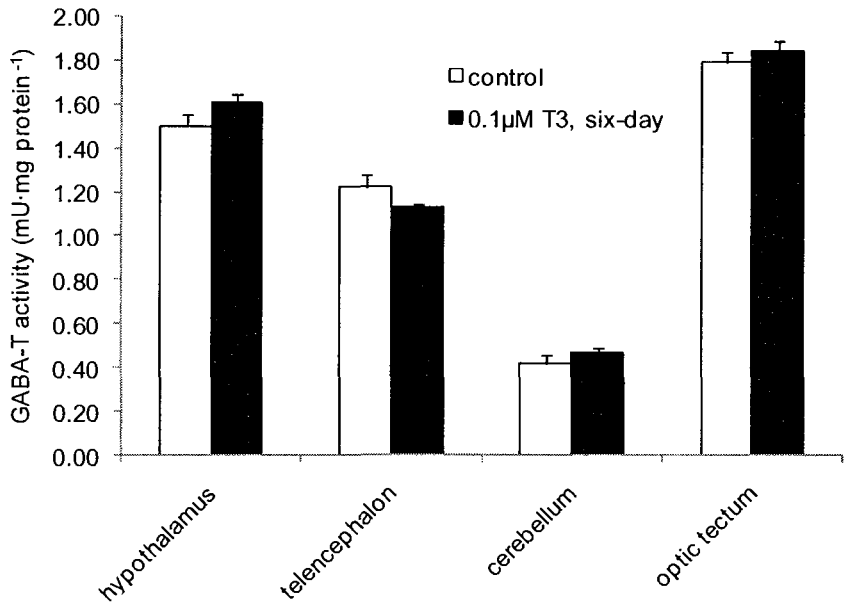

Figure 2.4 GABA-transaminase activity in brain parts of male goldfish exposed to 0 (control) or 0.1 µM T_3 in tank water for six days in September (sexually regressing). No statistical differences were observed between control and treatment for each brain part (Student's t-test, alpha = 0.05). n = 6. GABA-T, GABA-transaminase; mU, nmol/min.

2.3.5.2 In vitro exposures

Relative GABA-T activity between brain part pool homogenates was similar to the pattern seen in the homogenates from the *in vivo* T_3 exposure (Figure 2.5). In hypothalamus, cerebellum, and optic tectum, but not telencephalon, at least one T_3 concentration inhibited GABA-T activity *in vitro* in comparison to control, to a small but statistically significant extent (one-way ANOVA; $p = 0.013$, 0.003, and 0.017, respectively) (Figure 2.5). In hypothalamus, the 1.25 ng/ml dose resulted in 14 % lower GABA-T activity than control. In cerebellum, the 0.31 ng/ml, 0.62 ng/ml and 2.5 ng/ml doses resulted in 35 %, 37 % and 34 % lower GABA-T activities than control, respectively. In optic tectum, the three highest doses of T_3, 2.5 ng/ml, 5 ng/ml and 10 ng/ml, resulted in 18 %, 18 % and 17 % lower GABA-T activities than control, respectively.

The only effect of the higher doses of T_3 tested in triplicate for telencephalon, cerebellum and optic tectum (20 ng/ml, 100 ng/ml and 1000 ng/ml; data not shown) was in the telencephalon at the 100 ng/ml dose, for which GABA-T activity was 21 % higher than controls (one-way ANOVA, $p < 0.001$; Tukey's test only, $p \leq 0.05$).

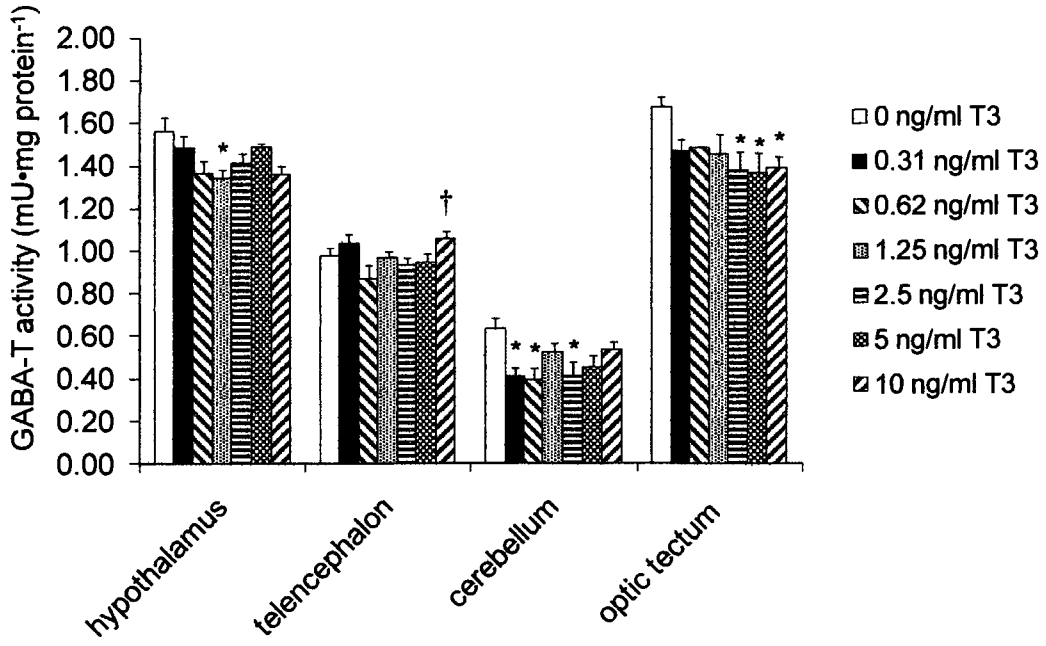

Figure 2.5 Effects of short-term *in vitro* T_3 exposure on GABA-transaminase activity in brain part homogenate pools of 40 male goldfish in March (sexually recrudescent). For each brain part * indicates a statistically significant difference from control (0 ng/ml T_3) and † indicates statistically significant difference from 0.62 ng/ml T_3 (one-way ANOVA followed by Tukey's test, $p \leq 0.05$). n = 6 except the 5 ng/ml and 10 ng/ml doses in optic tectum, n = 5. GABA-T, GABA-transaminase; mU, nmol/min.

2.4 Discussion

Mean plasma T_3 levels in T_3-exposed fish were between 2.4- and 43-fold above the time-matched controls. Over the course of the seasonal cycle, goldfish have been shown to have a cycle of T_3 and T_4 levels, with the maximal levels being 3- to 4-fold higher than minimal levels for T_3, and 5- to 6-fold higher than minimal levels for T_4 (Sohn et al., 1999a). Therefore the low, 0.02 μM T_3 doses in this study, especially for six days, raised plasma T_3 within the physiological range of goldfish under normal conditions. The high, 0.1 μM T_3 doses raised plasma T_3 to supraphysiological levels that may mimic levels that are caused by pathological hyperthyroidism, exposure to thyroid-disrupting chemicals in the environment, or ingestion of the thyroid gland of another animal. The latter source of a T_3 challenge in nature is common for piscivorous fish, and is less likely to occur in goldfish, except to some degree in eating decomposing matter.

Of the two neuroendocrine brain regions studied, GAD expression was affected by T_3 exposure in telencephalon, but not hypothalamus, for the high dose after two days only. This twofold lower GAD65 and GAD67 expression in telencelphalon was accompanied by twofold lower GABA-T and D2 expression. The physiological significance of these simultaneous decreases in GAD and GABA-T expression in telencephalon is unknown, but theoretically could have resulted in a change in local GABA levels depending on the relative kinetics of the two enzymes. It is also possible that T_3 caused a decrease in expression in one of the two enzymes, resulting in a homeostatic decrease in expression of the other enzyme to bring GABA synthesis back to a set-point. A time-course study between zero and two days could help address this hypothesis. On the other hand, such a high dose of T_3 could be having a general effect to depress gene expression of this neurotransmitter system. This is a similar

result to the effects of hypothyroidism in developing rat brain, in that both GAD and GABA-T enzyme activities were depressed (reviewed in Wiens and Trudeau, 2006).

The only significant effect on telencephalon gene expression seen after six days of exposure was twofold higher D3 expression. Since D3 breaks down T_3 to inactive T_2, and T_4 to inactive rT_3 (Figure 1.1) it is plausible that the increased D3 expression was a homeostatic response resulting in increased enzyme activity to return intracellular T_3 levels to a set-point, thus allowing GAD and GABA-T expression levels to return to normal. The lower D2 expression after two days also suggests a homeostatic response, since this would decrease local T_3 synthesis from T_4; however D2 activity may not be high enough in telencephalon for a twofold decrease to return intracellular T_3 levels to a set-point, especially given the degree to which the high dose raised circulating T_3 levels.

In hypothalamus, D2 expression was not affected by T_3 treatment on either day but D3 expression was higher than control on both days and, to some degree, at both doses and there were no effects on GAD expression. It is possible that the increase in D3 after two-day exposure prevented T_3 effects on GAD expression by maintaining set-point T_3 levels in hypothalamus despite the T_3 challenge, or it is possible that there is some other mechanism preventing T_3 effects on GAD expression. Measuring GABA-T expression in hypothalamus will be useful in order to get a complete picture of the tissue-specificity of the response seen in telencephalon. The effect of T_3 to decrease GAD65, GAD67 and GABA-T expression in telencephalon but not affect GAD65 and GAD67 in expression in hypothalamus (GABA-T was not measured) is similar to the effect of treatment of sexually regressed female goldfish with a $GABA_A$ receptor agonist, muscimol (Martyniuk et al., 2007b). In that study, GAD65 and GABA-T (but not GAD67) expression was decreased in telencephalon with no effect in hypothalamus. Given the similarity in tissue specificity of the $GABA_A$ receptor agonist and

T_3 on GAD and GABA-T gene expression, it is tempting to speculate that the T_3 effects to decrease GAD and GABA-T expression in telencephalon could be through T_3 effects on $GABA_A$ receptors. *In vivo* studies in rat brain and *in vitro* studies on human cell-lines and *Xenopus* oocytes indicate that thyroid hormones may affect $GABA_A$ receptor function both by modifying numbers of receptors, and through non-genomic effects to affect GABA-stimulated chloride currents (for review, see Wiens and Trudeau, 2006).

The absence of a T_3 effect on TR expression (tested in telencephalon only) indicates both that TR autoregulation is not likely in play to mediate T_3 effects on GAD, GABA-T and deiodinase expression in telencephalon, and that TR expression is not involved in the homeostatic response in this brain region, at least not in the time-frames tested. T_3 effects on TR expression in hypothalamus should also be measured to complete the picture of T_3 action on TRs in goldfish neuroendocrine brain.

In the current study, GSI and plasma [LH] were slightly lower in the fish for the six-day exposure compared to the two-day exposure. Since all variables were measured at the end of the exposures, and the six-day exposure ended three to four days after the two-day exposure, it is possible that these differences were due to continued regression of sexual state over those three to four days. It is also possible that the fish randomly selected for the six-day exposure were in a slightly more regressed state than the two-day fish at the start of the exposure. Either way, differences between two-day and six-day gene expression in response to T_3 exposure could be due either to slight differences in sexual state or to the difference in exposure time. The absence of effect of T_3 exposure on GSI or plasma [LH] indicates that a T_3 challenge for two and six days does not affect these measures of reproductive state, in male, sexually regressed (late September) goldfish. It is of interest also to test this in both sexes at different stages of the reproductive cycle (e.g. sexual maturity, Chapter 3).

The relative GABA-T activity between brain parts measured in the current study showed a similar pattern to that seen in female goldfish by Martyniuk *et al.* (2007a), with low activity in cerebellum compared to telencephalon, and higher activity in hypothalamus and optic tectum compared to telencephalon, suggesting that this pattern is conserved between the sexes and of importance.The GABA-T activity measured in the present study was approximately ten-fold higher than that previously reported for female goldfish (sexual state not specified; Martyniuk et al., 2007a). This difference could be due to sex or sexual maturity.

GABA-T activity was not affected by *in vivo* T_3 exposure in neuroendocrine brain regions (telencephalon, hypothalamus) or non-neuroendocrine brain regions (cerebellum or optic tectum). This reiterates the lack of effect of T_3 on GABA-T expression in neuroendocrine brain parts after six-day exposure to 0.1 μM T_3. Since there was lower GABA-T expression in telencephalon after the two-day exposure, it is of interest to measure GABA-T activity at that time-point. It should be noted that since entire brain regions were used for this assay, rather than specific areas or cell-types within brain regions, T_3 effects on specific hypothalamic or telencephalic regions or cell-types could have been masked. Since different telencephalon and hypothalamus regions have different GABA-T activities (Martyniuk et al., 2007a), it suggests that each sub-region is separately regulated, and thus a hormone such as T_3 could affect GABA-T activity and expression in each sub-region differently.

To my knowledge, no previous studies have determined possible *in vitro* effects of T_3 on GABA-T activity, however T_3 has been shown to have non-genomic effects on tyrosine hydroxylase activity (involved in dopamine synthesis) in the catfish *Heteropneustes fossilis* (Chaube and Joy, 2005). Various doses of T_3 inhibited GABA-T activity in all brain parts

except for telencephalon, although in hypothalamus only one dose had a significant effect, whereas in cerebellum and optic tectum, three doses had effects. *In vitro* T_3 treatment increased GABA-T activity in telencelphalon at a high dose. This suggests that *in vitro* T_3 effects are tissue-specific and dose-specific, and that the non-neuroendocrine brain regions may be more responsive. GABA-T activity in cerebellum was inhibited by a greater degree than in hypothalamus or optic tectum. It is important to consider the physiological significance of a decrease in GABA-T activity of 14-37 % that theoretically could cause an increase in available GABA levels due to decreased break-down. GABA synthesis rate has been altered by ~30 % in goldfish hypothalamus in response to physiologically relevant treatments, and where measured, was accompanied by paralleled changes in serum LH (Hibbert et al., 2005). This suggests that the magnitude of change in GABA-T activity in response to *in vitro* T_3 treatment measured here could have physiological effects if present *in vivo*, including modulation of LH release. These studies are preliminary, and should be repeated to determine V_{max} and Km of the enzyme in response to T_3 treatment. It would also be interesting to extend this study to females, and different sexual states.

In conclusion, water-borne T_3 exposure affects telencephalic and hypothalamic gene expression in adult male goldfish brain, suggesting that T_3 is crossing the blood-brain barrier, and that adult brain is responsive to T_3, which produces effects on GABA-system gene expression. From the current results, I conclude that T_3 affects GAD and GABA-T gene expression in telencephalon but not hypothalamus, and that these effects could have a physiologically significant effect on GABA function. It is of interest to extend this study to females and to different sexual states, to determine the specificity of this response. It is also of interest to determine whether T_3 exposure affects GABA-stimulated LH release (Chapter 3), since in this current study, there was no effect on basal LH levels. Measuring expression

and activity of other components of the GABA system, such as GABA-transporters and GABA receptors, is also key to understanding how thyroid hormones affect GABA system function. In Chapter 4, I use microarray analysis to identify such T_3 targets.

CHAPTER 3

Effects of T_3 exposure on gonadotropin gene expression in pituitary and luteinizing hormone release in goldfish

3.1 Introduction

The gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), belong to the pituitary glycoprotein hormone family, which also includes thyroid stimulating hormone (TSH). The pituitary glycoprotein hormones are heterodimeric, with each hormone composed of an alpha subunit common to all three hormones (glycoprotein hormone alpha subunit, GPHα), and a beta subunit that imparts hormonal specificity through its distinct protein sequences among the three hormones (LHβ, FSHβ, TSHβ) (Pierce and Parsons, 1981).

Seasonal reproduction in goldfish is regulated by the hypothalamo-pituitary-gonadal (HPG) axis, with the gonadotropins as the main drivers of gonadal steroidogenesis and maturation (Trudeau, 1997). In teleosts, which lack a median eminence, preoptic and hypothalamic hypophysiotropic neurons producing a multitude of neurotransmitters and neuropeptides directly innervate the gonadotropes, which are usually either LH- or FSH-producing. The gonadotropins are secreted from the pituitary into general circulation in response to gonadotropin releasing hormone (GnRH) and other neuroendocrine factors from the hypothalamus, and act on the gonads. LH stimulates gonadal growth, gametogenesis, and ovulation or sperm release (Trudeau, 1997). The roles of FSH are less well studied than those of LH in goldfish, however recent studies on recombinant goldfish FSH indicate that it likely has roles in stimulating steroidogenesis and possibly also ovulation and milt production (Hayakawa et al., 2008; Kobayashi et al., 2006; Morita et al., 2003).

In goldfish, both forms of GnRH present (salmon GnRH and chicken GnRH-II) stimulate gene expression of the three gonadotropin subunits (GPHα, LHβ, FSHβ) (Huggard-Nelson et al., 2002; Klausen et al., 2001), although these effects depend on sexual maturity (Sohn et al., 2001) and possibly on sex. Both GnRH forms also stimulate LH release in goldfish (Trudeau, 1997). Effects and mechanisms of GnRH on FSH release are unknown due to the lack of FSH detection assays for goldfish (Chang et al., 2009). Beyond direct GnRH effects, gene expression of the gonadotropin subunits and gonadotropin release are dynamically regulated by interplay of stimulatory and inhibitory neuroendocrine signals from the preoptic region and hypothalamus, paracrine and autocrine signals from pituitary, and steroid hormone feedback (for reviews see Chang et al., 2009; Popesku et al., 2008; Trudeau, 1997).

GABA is an amino acid neurotransmitter that stimulates LH release in goldfish in a seasonal and sex-specific manner through both enhanced GnRH release and by inhibiting dopamine (DA) neurons thus lifting tonic inhibition by DA on gonadotropes (reviewed by Popesku et al., 2008; Trudeau et al., 2000b). GABA-stimulated LH release is mediated by both ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors (Martyniuk et al., 2007b; Trudeau et al., 2000b). GABA receptor activation also has profound effects on expression of several genes involved in GABA function and gonadotropin secretion in neuroendocrine brain, with different effects in telencephalon and hypothalamus (Martyniuk et al., 2007b; Popesku et al., 2008). GABA has also been shown to stimulate FSH release in rainbow trout (Mañanos et al., 1999).

Other hormone systems also have effects on the HPG axis thus having roles in regulation of reproduction. Thyroid hormones play roles in mediating the transition in and

out of breeding season in many species including fish (Cyr and Eales, 1996; Nakao et al., 2008b; Swapna and Senthilkumaran, 2007). In goldfish, four thyroid hormone receptors (TRs) have been identified, and all four are expressed in brain and pituitary, with differential expression between breeding and non-breeding season (Nelson and Habibi, 2006), suggesting that regulation of TR levels plays a role in reproductive processes. In birds and mammals, it has been elegantly demonstrated that changes in T_3 levels in hypothalamus, through changes in deiodinase expression, are necessary for regulating the HPG axis in response to seasonal changes in photoperiod, including changes in gonadotropin expression and secretion (Hanon et al., 2008; Nakao et al., 2008b). In quail, it is hypothesized that T_3 affects secretion of GnRH through morphological changes of the GnRH neuron and glial processes (Nakao et al., 2008b). Such a mechanism has not been reported in mammals, and it is suggested that T_3 in hypothalamus could be affecting any of the hypothalamic factors that regulate gonadotropins (Hanon et al., 2008). TSH is also emerging as an important regulator of reproduction. Recent studies show that it is TSH in the pituitary that conveys changes in day-length into changes in hypothalamic T_3 levels through effects on deiodinase expression in hypothalamus (Hanon et al., 2008; Nakao et al., 2008a). TSH also likely has other novel, undiscovered roles on reproduction in fish, given studies showing expression of TSH receptors (Kumar and Trant, 2001) and TSHβ and GPHα in fish gonads (von Schalburg et al., 2005; Wang et al., 2004).

In order to address whether thyroid hormones regulate the reproductive neuroendocrine axis in goldfish, and whether GABA is involved in such regulation, in the current chapter my objectives were to determine: 1) whether T_3 regulates gene expression of the glycoprotein hormone subunits in the pituitary and basal LH release; 2) whether T_3

modulates GABA-mediated LH release. Two main experiments were done to address these objectives, using adult male and female goldfish in order to determine sex differences in response. For the first experiment, to determine T_3 effects on gene expression in pituitary, the T_3 exposures done in Chapter 2 were repeated on sexually mature male and female goldfish in late May, and LHβ, FSHβ, TSHβ and GPHα1 expression in pituitary was measured. TR and deiodinase expression were also measured, because this expression has been shown to be thyroid hormone-responsive in fish (Bres et al., 2006; Liu et al., 2000; Manchado et al., 2009; Nelson and Habibi, 2008) and therefore can be used as positive controls for efficacy of T_3 exposure in affecting pituitary gene expression, while also helping to elucidate mechanisms of T_3 action and feedback in pituitary. Circulating LH levels were also determined. For the second experiment, to determine T_3 effects on GABA-mediated LH release, I did two additional T_3 exposures. Sexually regressing and regressed male and female fish were exposed to a T_3 challenge, and then treated with $GABA_A$ or $GABA_B$ agonists, and circulating LH levels were measured.

3.2 Materials and Methods

3.2.1 Experimental protocol

Fish holding methods and experimental protocols were conducted in accordance with the principles and guidelines of the Canadian Council on Animal Care and approved by the University of Ottawa Protocol Review Committee.

3.2.1.1 Experimental animals

Four- to five-inch male and female common goldfish (*Carassius auratus*) were purchased from Aleong's International Inc. (Mississauga, ON, Canada). Fish were acclimated in 70-L, cylindrical, fibre-glass tanks, with flow-through dechloraminated Ottawa

city tap water at 18 °C, with a light:dark cycle paralleling the natural Ottawa photoperiod, and fed a diet of Martin Mills classic floating fish food (3PT regular), *ad libitum*. Approximately one week prior to the start of exposure, fish were moved to their exposure tanks with an equal number of predicted males and females assigned to each treatment tank randomly, and acclimated to a maintenance diet of 0.1 g food per fish per day (~0.5 % bw).

3.2.1.2 Experiment to determine T_3 effects on gene expression in pituitary

Sexually mature male and female goldfish ranging in mass from 12-42 g were exposed to static, waterborne T_3 at nominal concentrations of 0.02 and 0.1 μM for two and six days, from May 22nd-29th, 2007, using the exposure protocol described in section *2.2.1.2*. Exposures were carried out at a density of 15 fish per tank, with two tanks of fish per treatment (*i.e.* n = 30 per treatment). On day zero of exposure, water-flow to tanks without fish was turned off, and tanks were aerated with air-stones. The T_3 sodium salt (SIGMA) was dissolved in 0.05N NaOH (vehicle), and delivered in 1.4 ml to 70 L tanks for a final vehicle concentration of ~0.002 %. Tank-water was mixed manually. Fish were then transferred to the day zero exposure tanks. Fish and air-stones were transferred to tanks with fresh, static water and T_3 and/or vehicle every two days, with the last transfer 48 h before euthanasia and dissection. Fish were fed 24 h prior to tank transfer and fed directly after.

Fish were last fed 24 h before sampling. Fish were anaesthetized in TMS, body mass measured, and blood taken by puncture of the caudal vasculature either with heparinized syringes for plasma for T_3 EIA analysis, or with unheparinized syringes for serum for LH RIA anlaysis (approximately half of each treatment group for each analysis). Fish were then euthanized by decapitation. Sex was noted and gonads were removed and weighed for calculation of GSI as a measure of sexual maturity. Pituitaries were dissected out, flash-frozen on dry ice and stored at -80 °C until total RNA isolation for gene expression analysis

with real-time RT-PCR. Blood was centrifuged at 5100 *g* for 5 min at room temperature to separate plasma or serum from red blood cells then plasma or serum was removed and stored at -80 °C until enzyme immunoassay or radioimmunoassay (blood for serum radioimmunoassay analysis was kept overnight at 4 °C before centrifugation).

3.2.1.3 Experiment to determine T_3 effects on GABA-stimulated LH release

In order to determine whether a T_3 challenge affects GABA-stimulated LH release, two additional T_3 exposures were performed on male and female goldfish at two different stages of sexual maturity, followed by administration of GABA receptor agonists. The T_3 exposure followed the protocol above (3.2.1.2) except it was limited to 0.1 μM T_3 for six days, on sexually regressing fish from July 5th-12th, 2007 (body mass range: 13-33 g), and on sexually regressed fish from October 24th-31st, 2007, (body mass range: 9-39 g). Exposures were done at a density of 12 fish per tank, with two tanks of fish per treatment (*i.e.* n = 24 per treatment). Following six-day control or T_3 exposure, fish were exposed to GABA agonists for six hours following the procedures of Martyniuk et al. (2007b). Fish were injected intraperitoneally with saline vehicle (0.6 % NaCl), the $GABA_A$-receptor agonist muscimol (1 μg/g bw) or the $GABA_B$-receptor agonist baclofen (10 μg/g bw). Muscimol dissolved completely in saline, and baclofen was injected as a fine suspension in saline. Injections were staggered so that fish in each treatment group were sampled and euthanized approximately six hours after injection.Fish and tissues were sampled as described in 3.2.1.2 above, except the only tissues collected for analysis were gonads for GSI calculation and blood; unheparinized syringes were used, and the resulting serum was used for both T_3 enzyme immunoassays and LH radioimmunoassays. LH levels were measured in all samples, and T_3 concentrations were measured in a subset of approximately nine samples.

3.2.2 T_3 enzyme immunoassay

T_3 concentrations in plasma or serum were measured using the spectrophotometric human serum T_3 EIA kit as described in section 2.2.2 with the following modifications to sample dilution. Samples were serially diluted with steroid-free serum (MP Biomedicals, Orangeburg, NY, USA). For the gene expression in pituitary experiment (May), samples from 0.02 μM and 0.1 μM T_3-treated groups were diluted 1 in 4 and 1 in 64 respectively. For the GABA-stimulated LH release experiment (July and October) samples from 0 μM T_3 (control) and 0.1 μM T_3-treated groups were diluted 1 in 2 and 1 in 32 respectively, except for 0.1 μM T_3-treated males from the July exposure, which were diluted 1 in 16.

3.2.3 LH radioimmunoassay

Serum LH levels were determined by a double antibody LH radioimmunoassay in 96-well plates following the procedure of Zhao *et al.* (2006) adapted from Peter *et al.* (1984).

3.2.4 Gene expression analysis

3.2.4.1 RNA isolation and quantification, and cDNA synthesis

The Qiagen RNeasy® Micro Kit with DNase treatment (QIAGEN, Mississauga, ON) was used to isolate pituitary total RNA according to the manufacturer's protocol for microcentrifuge with the following specifications. In order to obtain a sufficiently high RNA concentration for cDNA synthesis, two pituitaries were pooled thereby halving the sample size. Each sample was homogenized using stainless steel beads (5 mm) in a MM301 Mixer Mill (Retsch, Pennsylvania, USA). RNA was resuspended in 14 μl of nuclease-free water. RNA was quantified and its quality assessed using a NanoDrop® Spectrophotometer ND-1000, with ND-1000 software, version 3.3 (NanoDrop Technologies, Inc.).

For each sample, cDNA was reverse-transcribed from 2 μg total RNA in a final volume of 20 μl using Superscript II® Reverse Transcriptase with 200 ng random hexamer

primers (Invitrogen, Burlington, ON) following the manufacturer's protocol. cDNA synthesis was performed simultaneously on all male and female pituitary samples within one time point allowing for statistical comparisons within that group. For each cDNA synthesis run, one sample was run in parallel under the same reaction conditions but with water in place of reverse transcriptase as a control to demonstrate the absence of genomic DNA contamination (no-reverse-transcriptase-control, NoRT). Samples were stored at -20 °C until used for real-time RT-PCR.

3.2.4.2 Real-time reverse transcriptase PCR

Real-time RT-PCR assays with the double-stranded DNA-binding dye SYBR® Green were used to measure differences in pituitary gene expression relative to control levels, following the procedures detailed in section 2.2.4.3 with the modifications described below. Gene expression was measured for TRα1, TRβ, the deiodinases D2 and D3, the glycoprotein hormone beta subunits TSHβ, FSHβ, LHβ and the glycoprotein hormone alpha subunit GPHα1.

Primers were designed using Primer3 (http://frodo.wi.mit.edu/) based on goldfish gene sequences obtained from GenBank, and synthesized by Invitrogen (Table 3.1). Primer sets were verified to be specific to the amplicon of choice by running a PCR on brain or pituitary cDNA under standard conditions, and sequencing the resulting amplicon.

Table 3.1
Real-time RT-PCR primer sequences and assay conditions for SYBR Green I assays for relative quantification of genes in goldfish pituitary.

gene	fwd primer (5' to 3') rev primer (5' to 3')	amplicon length (bp)	[primer] (nM)	annealing temperature (°C)	[$MgCl_2$] (mM)	accession number of template
TRα-1	CATCGCAAGCACAACATCTC TTCTGTGGGACACTCCACTTT	123	125	58	3.5	AY973629
TRβ	CCGTGATTTTGCTTTCCTCT ACCTTGTGCTTGCGGTAGTT	118	150	58	2.5	AY973630
D2	TCCAAGGTGGTGAAGGTTG AGCAGATGGCACTCGTTGT	77	100	58	3.5	EU313786
D3	CTTGAAGAAGTCCAGCTTGTG ACGAAGATGCGCGAGAG	126	185	58	3.5	EU313787
TSHβ	GGTTTAGCAGTTTGCGTCTGT CTGGGACTTCACCTCTCGTT	112	150	58	1.5	AB020320
FSHβ	ATGCGCTTCGTTGTTATGGT AGCTGCCACATTCCTCACTT	121	100	58	3.5	D88023
LHβ	GAGGGCTGTCCAAAATGTCT AGCCACAGGGTAGGTGATGT	195	100	58	3.5	D88024
GPHα1	TGAACTTTGGATGTGAGGAGTG AGCATGTAGCTTCGGATGTG	171	100	58	3.5	AY800266.1

For each gene of interest, the male and female pituitary samples within one time point (two-day or six-day) were run in triplicate distributed equally across two 96-well plates allowing for statistical comparisons between sexes within a time point.

cDNA (with an original RNA input of 2μg in 20 μl) was diluted 1 in 100 in order for sample values to fall within the range of the standard curve. Each 25 μl reaction contained RNase/DNase-free water (GIBCO), 1X buffer (QIAGEN), 1.5-3.5 mM MgCl2 (QIAGEN; Table 3.1),100-185 nM forward and reverse primer (Table 3.1), 0.25X SYBR Green I dye (Invitrogen), 200 μM dNTPs (Invitrogen), 1.25 U HotStarTaq (QIAGEN), 100 nM ROX reference dye (Stratagene), and 5 μl diluted sample cDNA.

D3 expression levels in the majority of samples in the control and 0.02 μM T_3 exposure groups were lower than the lowest point of the standard curve. Their values could not be accurately interpolated from the standard curve, and therefore were replaced with the "detection limit" value of the initial quantity of total RNA in the lowest standard curve point.

Relative gene expression level (normalized for input RNA amount) was calculated as a proportion relative to male control expression level for each exposure time, by dividing each treatment sample quantity for both males and females by the average of control male sample quantity.

3.2.5 Statistical analyses

For each variable measured, data were tested for normality with the Kolmogorov-Smirnov test, and for homoscedasticity with the Levene test. If these assumptions were not met, the data were $\log_{10}$- or reciprocal-transformed. If transformation failed to make the data meet the assumptions of normality and homoscedasticity, nonparametric two-way ANOVA (Scheirer-Ray-Hare extension of the Kruskall-Wallis test) or the Mann-Whitney U test was

used. Where post-hoc tests were needed on nonparametric data, Tukey's test on ranked data was used. Significance level was set at 0.05 for all statistical tests (significant p-values from ANOVA and Mann-Whitney U tests are reported in the text). All statistical analyses were carried out using S-Plus software.

Two-way ANOVA was used for the gene expression experiment, with T_3 treatment and sex as main factors. Two-day and six-day exposures were analyzed separately. If there was a significant main effect of T_3 treatment but no interaction, this was followed by Tukey's test between T_3 dose groups across both sexes. If there was significant interaction, ANOVA was followed by Tukey's test among T_3 treatment groups within a sex and between sexes within T_3 treatment groups.

Two-way ANOVA was used for the GABA-agonist experiment with agonist treatment and T_3 pre-treatment as factors. Males and females were analyzed separately, for both the July and October exposures. If there was a significant main effect of agonist treatment but no interaction, this was followed by Tukey's test between agonist treatment groups across T_3 exposures. If there was significant interaction, ANOVA was followed by Tukey's test among agonist treatment groups within a T_3 exposure and between T_3 exposures within agonist treatment groups. The Mann-Whitney U test was used to compare average GSIs between the July and October exposure groups.

3.3 Results

3.3.1 Experiment to determine T_3 effects on gene expression in pituitary

3.3.1.1 Plasma T_3 concentrations

For both male and female goldfish, water-borne T_3 exposure for two days and six days at both doses resulted in significantly higher plasma T_3 concentrations than controls; this effect was dose-dependent, with no significant differences between males and females

(two-way ANOVA followed by Tukey's test for each time point, main effect of T_3 treatment: two-day, $p < 0.001$; six-day, nonparametric, $p < 0.001$) (Figure 3.1). In fish treated for two days with 0.02 μM and 0.1 μM T_3, male T_3 levels were 10- and 71-fold higher, respectively, than controls (1.8 ± 0.3 ng/ml) and female T_3 levels were 8.5- and 141-fold higher, respectively, than controls (1.7 ± 0.2 ng/ml) (Figure 3.1 a). In fish treated for six days with 0.02 μM and 0.1 μM T_3, T_3 levels were 14- and 84-fold higher, respectively, than controls (1.4 ± 0. 1 ng/ml) and female T_3 levels were 15- and 130-fold higher, respectively, than controls (1.5 ng/ml ±0.3 ng/ml)(Fig 3.1 b).

3.3.1.2 Body mass and gonadosomatic index

There were no differences in body mass among T_3-treatments and sexes for both two-day and six-day exposures (Table 3.2). For GSI, there were also no differences among T_3 treatments for both exposures, however female GSI was significantly greater than male GSI in the six-day exposure only (main effect of sex, $p = 0.029$). Subsequent two-way ANOVA comparison of female GSI between two-day and six-day exposures indicated that female GSI was significantly greater in the six-day exposure, with no effect of T_3-treatment (main effect of exposure time, nonparametric, $p = 0.019$).

3.3.1.3 Serum LH concentrations

T_3 exposure had no effect on serum LH levels at either dose or exposure duration for males and females (Figure 3.2). There were also no sex differences in serum LH.

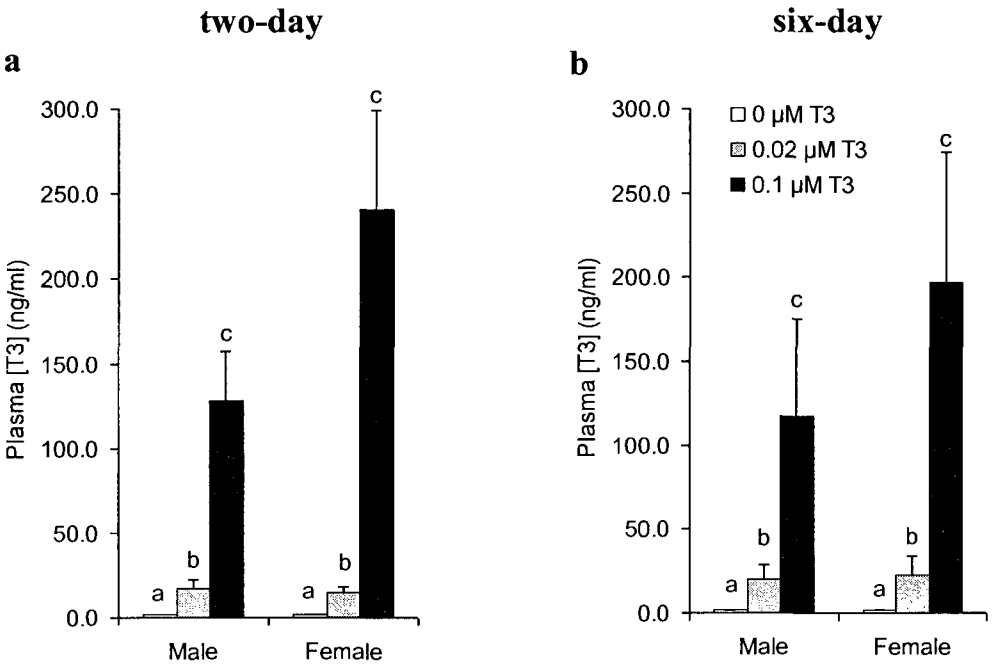

Figure 3.1 Plasma T_3 levels (ng/ml), measured by enzyme immunoassay, in sexually mature male and female goldfish in May after two-day and six-day water-borne exposure to 0, 0.02 and 0.1 µM T_3. Data are presented as mean + SEM; n = 6-10. Data within each exposure time were analyzed by two-way ANOVA (nonparametric for six-day) with main factors as T_3 exposure and sex, followed by Tukey's test where there was a significant effect of T_3 exposure ($p \leq 0.05$). Main T_3-treatment effects across sexes are indicated by the letters a, b, c. T_3, tri-iodothyronine.

Table 3.2
Body mass and gonadosomatic index of sexually mature male and female goldfish in May, after two-day and six-day water-borne exposure to 0, 0.02 and 0.1 μM T_3.

Exposure time	sex	T_3 dose (μM)	n	Body mass (g)	GSI (%)
two-day	male	0	15*	20.1 ± 0.9	2.60 ± 0.28
		0.02	16	18.2 ± 1.2	2.25 ± 0.27
		0.1	16	19.7 ± 1.0	2.36 ± 0.34
	female	0	15	19.5 ± 1.1	2.80 ± 0.59
		0.02	13	21.1 ± 1.7	3.33 ± 0.85
		0.1	13	18.8 ± 1.4	3.05 ± 0.76
six-day	male	0	15	20.7 ± 1.1	2.90 ± 0.39^x
		0.02	15	20.0 ± 1.2	2.63 ± 0.27^x
		0.1	12	20.1 ± 2.3	1.86 ± 0.30^x
	female	0	15*	19.1 ± 0.7	4.48 ± 1.07^y
		0.02	15	19.3 ± 1.8	5.94 ± 1.27^y
		0.1	18	20.5 ± 0.9	5.27 ± 1.11^y

Data are presented as mean ± SEM; *n = 14 for GSI; data within each exposure time were analyzed by two-way ANOVA (nonparametric for six-day GSI) with main factors as T_3 exposure and sex, alpha = 0.05; main sex effects across T_3 treatments are indicated by the superscripted letters x, y; GSI, gonadosomatic index; T_3, tri-iodothyronine.

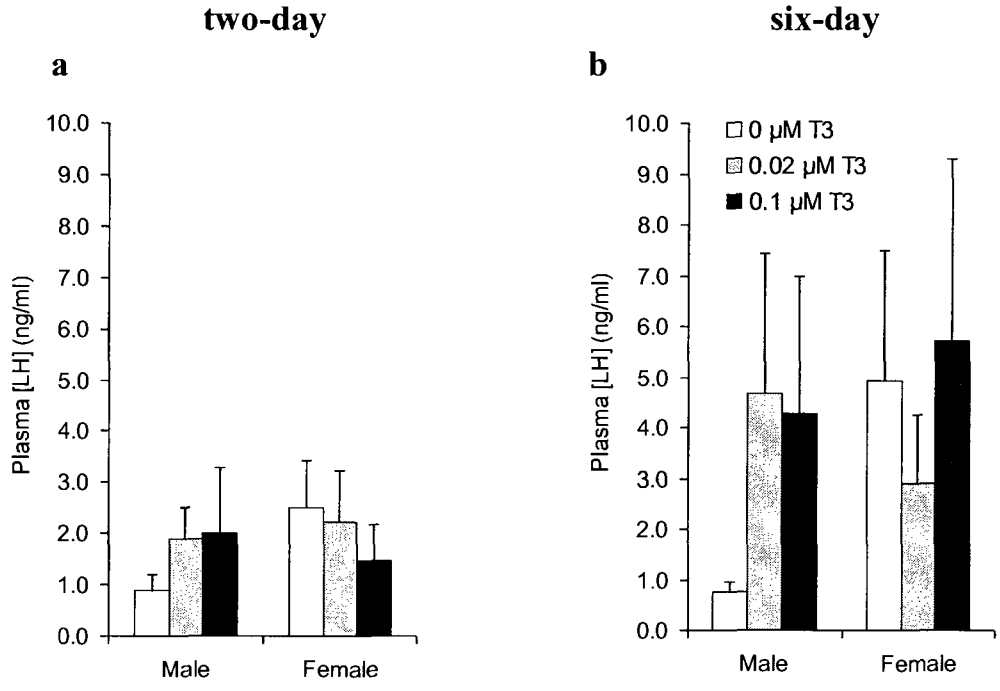

Figure 3.2 Serum LH levels (ng/ml), measured by radioimmunoassay, in sexually mature male and female goldfish in May after two-day and six-day water-borne exposure to 0, 0.02 and 0.1 µM T_3. Data are presented as mean + SEM; n = 6-8. Data within each exposure time were analyzed by nonparametric two-way ANOVA with main factors as T_3 exposure and sex, alpha = 0.05. There were no significant effects.

3.3.1.4 Gene expression in pituitary

Pituitary TRα-1 and TRβ expression had similar responses to T_3-challenge, with two-day T_3 treatment at both doses resulting in higher expression levels than controls; this effect was dose-dependent, independent of sex, absent after six days of treatment, and more pronounced in TRβ than TRα-1 (Figure 3.3). For TRα-1 after two-day treatment with 0.02 μM and 0.1 μM T_3, male expression levels were 1.4- and 2.2-fold higher than male controls, and female expression levels were 2.1- and 3.6-fold higher than female controls (main effect of T_3, $p < 0.001$, followed by Tukey's test) (Figure 3.3 a). For TRβ after two-day treatment with 0.02 μM and 0.1 μM T_3, male expression levels were 2.0- and 4.0-fold higher than male controls, and female expression levels were 3.3- and 5.2-fold higher than female controls (main effect of T_3, $p < 0.001$, followed by Tukey's test) (Figure 3.3 c).

D2 expression decreased and D3 expression increased in response to T_3 treatment, in a sex-, dose- and time-dependent fashion (Figure 3.4). Two-day treatment with T_3 had an effect on D2 expression in males, with 3.3- and 4.4- fold lower expression in 0.02 μM and 0.1 μM T_3 treatment groups, and no significant effects in females (main treatment effect, $p = 0.003$; significant interaction, $p = 0.016$; followed by Tukey's test) (Figure 3.4 a). Six-day treatment with 0.1 μM T_3 had significant effects on both male and female D2 expression, with male levels 3.9-fold lower than male controls, and female levels 2.9-fold lower than female controls, but the 0.02 μM dose did not have significant effects (main effect of T_3 treatment, $p = 0.026$, followed by Tukey's test) (Fig 3.4 b). For both time points, female control expression levels of D2 were approximately 2-fold lower than male control levels, but this was not statistically significant.

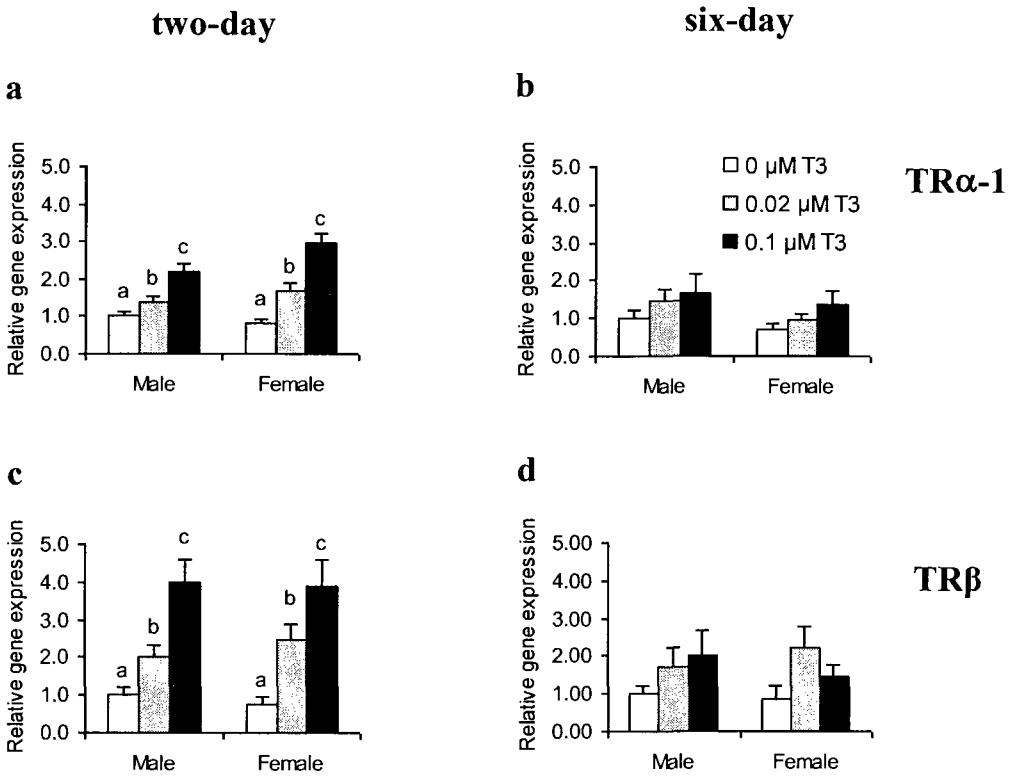

Figure 3.3 Relative pituitary gene expression, measured by real-time RT-PCR, of the thyroid hormone receptors TRα-1 (a, b) and TRβ (c, d), in sexually mature male and female goldfish in May after two-day and six-day water-borne exposure to 0, 0.02 and 0.1 μM T_3. Data are presented as a proportion relative to male control expression level for each exposure time (mean + SEM; n = 4-9). Data within each exposure time were analyzed by two-way ANOVA with main factors as T_3 exposure and sex, followed by Tukey's test where there was a significant effect of T_3 exposure ($p \leq 0.05$). Main T_3-treatment effects across sexes are indicated by the letters a, b, c.

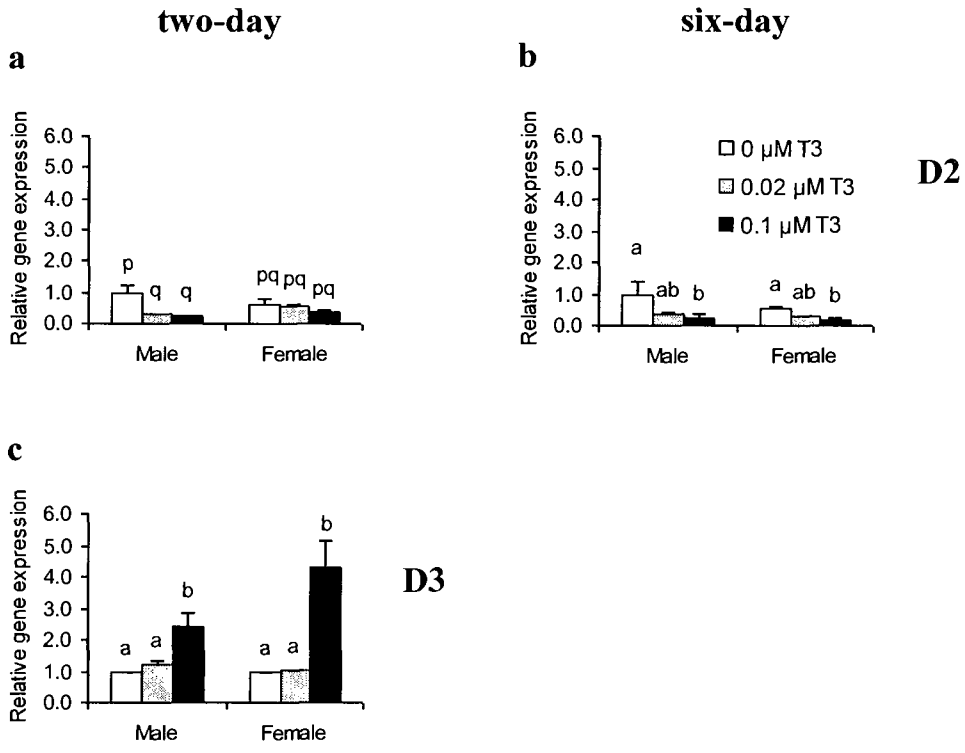

Figure 3.4 Relative pituitary gene expression, measured by real-time RT-PCR, of the deiodinases D2 (a, b), and D3 (c), in sexually mature male and female goldfish in May after two-day and six-day (D2 only) water-borne exposure to 0, 0.02 and 0.1 µM T_3. Data are presented as a proportion relative to male control expression level for each exposure time (mean + SEM; n = 4-9). Data within each exposure time were analyzed by two-way ANOVA (nonparametric for D3) with main factors as T_3 exposure and sex, followed by Tukey's test if there was a significant effect of T_3 exposure or interaction ($p \leq 0.05$). Where there was no interaction between agonist treatment and T_3-treatment, main T_3-treatment effects across sexes are indicated by the letters a, b, c. Where there was significant interaction, different letters p, q, r indicate significant differences among T_3-treatments within a sex and between sexes within an agonist treatment.

D3 expression was only measured for the two-day exposure (Figure 3.4 c). D3 expression levels were below the detection limit in the control and 0.02 μM T_3 treatment groups, and 2.4- and 4.3-fold higher than this detection limit in the 0.1 μM dose, in males and females, respectively (main effect of T_3 treatment, nonparametric, $p < 0.001$, followed by Tukey's test on ranks).

Expression of the glycoprotein hormone subunits TSHβ, FSHβ, and GPHα1 were all thyroid hormone-responsive in pituitary, in a dose-, sex- and time-dependent manner, but LHβ was not (Figure 3.5). For TSHβ, two-day treatment with T_3 had a dose-dependent effect on expression in males, with 3.6-fold lower expression in the 0.02 μM T_3 group and 12-fold lower expression in the 0.1 μM T_3 treatment group than male controls, but no significant differences between control and T_3 treatments in females (main treatment effect, $p < 0.001$; significant interaction, $p = 0.031$; followed by Tukey's test) (Figure 3.5 a). Six-day treatment with T_3 had a significant effect on both male and female TSHβ expression, but only with the 0.1 μM T_3 dose, with male expression levels 5.7-fold lower than male controls, and female expression 2.6-fold lower than female controls (main effect of T_3 treatment, $p = 0.002$, followed by Tukey's test) (Figure 3.5 b).

FSHβ expression was only affected by T_3 treatment in females after two-day T_3 treatment with 0.1 μM T_3, where expression was six-fold higher than female control levels (main effect of T_3 treatment, $p = 0.041$; significant interaction, $p = 0.005$; followed by Tukey's test) (Figure 3.5 c,) with no effects after six-day exposure (Figure 3.5 d). FSHβ is the only glycoprotein hormone subunit that increased in response to T_3 treatment.

Figure 3.5 Relative pituitary gene expression, measured by real-time RT-PCR, of the glycoprotein hormone subunits TSHβ (a, b), FSHβ (c, d), LHβ (e, f) and GPHα1 (g, h), in sexually mature male and female goldfish in May after two-day and six-day water-borne exposure to 0, 0.02 and 0.1 μM T_3. Data are presented as a proportion relative to male control expression level for each exposure time (mean + SEM; n = 4-9). Data within each exposure time were analyzed by two-way ANOVA (nonparametric for GPHα1) with main factors as T_3 exposure and sex, followed by Tukey's test if there was a significant effect of T_3 exposure or interaction ($p \leq 0.05$). Where there was no interaction between agonist treatment and T_3-treatment, main T_3-treatment effects across sexes are indicated by the letters a, b, c, and main sex effects across T_3-treatments are indicated by the letters x, y. Where there was significant interaction, different letters p, q, r indicate significant differences among T_3-treatments within a sex and between sexes within an agonist treatment.

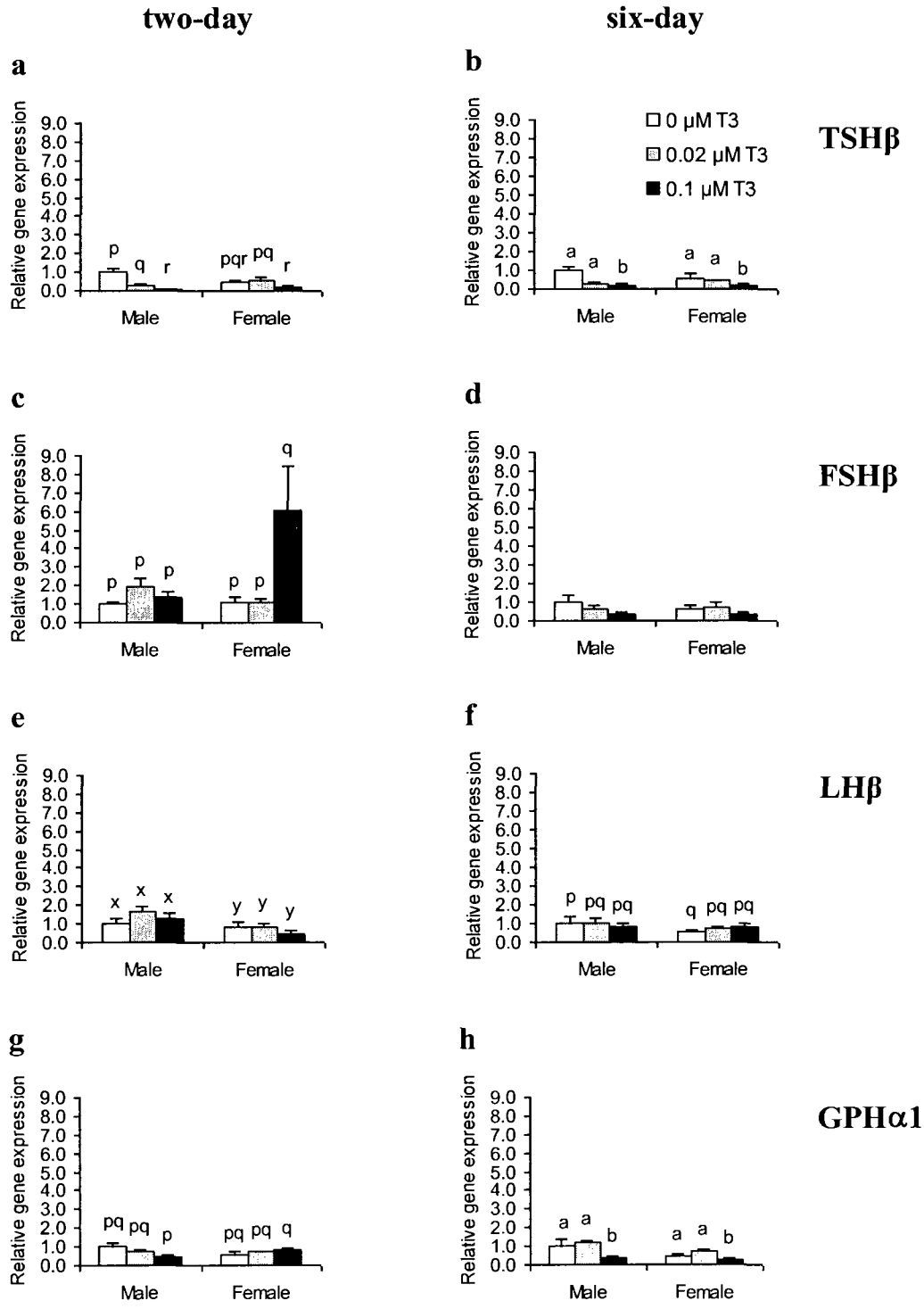

Figure 3.5 (caption on previous page)

LHβ expression was not significantly affected by T_3 exposure. However, females had lower expression than males in both the two-day and six-day exposures. In the two-day exposure, female levels were on average 1.8-fold lower than male levels (main effect of sex, $p = 0.010$) (Figure 3.5 e). In the six-day exposure, female control levels were exactly half that of male control levels (significant interaction, $p = 0.031$, followed by Tukey's test) (Figure 3.5 f).

GPHα1 expression was significantly affected by T_3 treatment after six-day treatment with 0.1 μM T_3, where male and female expression levels were 2.8- and 1.8-fold lower than their respective controls (main effect of T_3 exposure, nonparametric, $p = 0.004$, followed by Tukey's test) (Figure 3.5 h). Although there were no significant main effects of two-day T_3 exposure, there was significant sex*treatment interaction (nonparametric, $p = 0.017$), and Tukey's test revealed that for the 0.1 μM dose, male levels were 1.8-fold higher than female levels (Figure 3.5 g).

3.3.2 Experiment to determine T_3 effects on GABA-mediated LH release

3.3.2.1 Serum T_3 concentrations

Water-borne exposure to 0.1 μM T_3 for six days resulted in significantly higher levels of serum T_3 in males and females in both sexually regressing fish (July exposure) and sexually regressed fish (October exposure), although levels were raised to a lower level on average in October (Figure 3.6). In males in July (Figure 3.6 a), T_3 levels were 86- to 100-fold higher in T_3-treated fish than in control, vehicle-injected fish (1.2 ± 0.1 ng/ml), with no significant differences among GABA agonist treatments (main effect of T_3 treatment, nonparametric, $p < 0.001$). In females in July (Figure 3.6 b), T_3 treatment had a similar significant effect (main effect of T_3 treatment, nonparametric, $p < 0.001$, followed by Tukey's test) except that although T_3 levels in vehicle- and baclofen-injected females were

80- to 85-fold higher in the T_3-treated groups than control, vehicle-injected females (1.6 ± 0.1 ng/ml), T_3 levels were only 29-fold higher in T_3-treated, muscimol-injected females than controls, although this difference was not statistically significant. In males in October (Figure 3.6 c), T_3-treated fish injected with vehicle, muscimol and baclofen had T_3 levels that were respectively 41-, 58-, and 31-fold higher than in control, vehicle-injected fish (1.5 ± 0.3 ng/ml), although there were no significant differences among agonist treatments (main effect of T_3 treatment, nonparametric, $p < 0.001$). In females in October (Figure 3.6 d), T_3-treated fish injected with vehicle, muscimol and baclofen had T_3 levels that were respectively 65-, 39-, and 116-fold higher than in control, vehicle-injected fish (1.3 ± 0.2 ng/ml), although there were no significant differences among agonist treatments (main effect of T_3 treatment, nonparametric, $p < 0.001$).

3.3.2.2 Body mass and gonadosomatic index

For each sex within both the July exposure and the October exposure, in general there were no differences in body mass or GSI between control and T_3-treated animals or between animals injected with different agonists (Table 3.3). The only minor but statistically significant differences were in July, where mass of muscimol-injected males was slightly lower than mass of vehicle- or baclofen-injected males (nonparametric two-way ANOVA, $p = 0.024$, followed by Tukey's test on ranks), and GSI of T_3-treated females was lower than GSI of control females (two-way ANOVA, $p = 0.046$).

Figure 3.6 Serum T_3 levels (ng/ml), measured by enzyme immunoassay, in male and female goldfish in July (**a**, **b**; sexually regressing) and October (**c**, **d**; sexually regressed) pre-treated for 6 d with water-borne exposure to 0 μM T_3 (control) or 0.1 μM T_3, followed by 6 h exposure to saline vehicle, the $GABA_A$-receptor agonist muscimol (1 μg/g bw) or the $GABA_B$-receptor agonist baclofen (10 μg/g bw) via intraperitoneal injection. Data are presented as mean + SEM; n = 7-13. Data within each month and sex were analyzed by nonparametric two-way ANOVA with main factors as T_3 exposure and agonist treatment, alpha = 0.05. There were no significant effects of agonists. Main T_3-treatment effects are indicated by the letters x, y. There was no interaction between agonist and T_3-treatment. T_3, tri-iodothyronine.

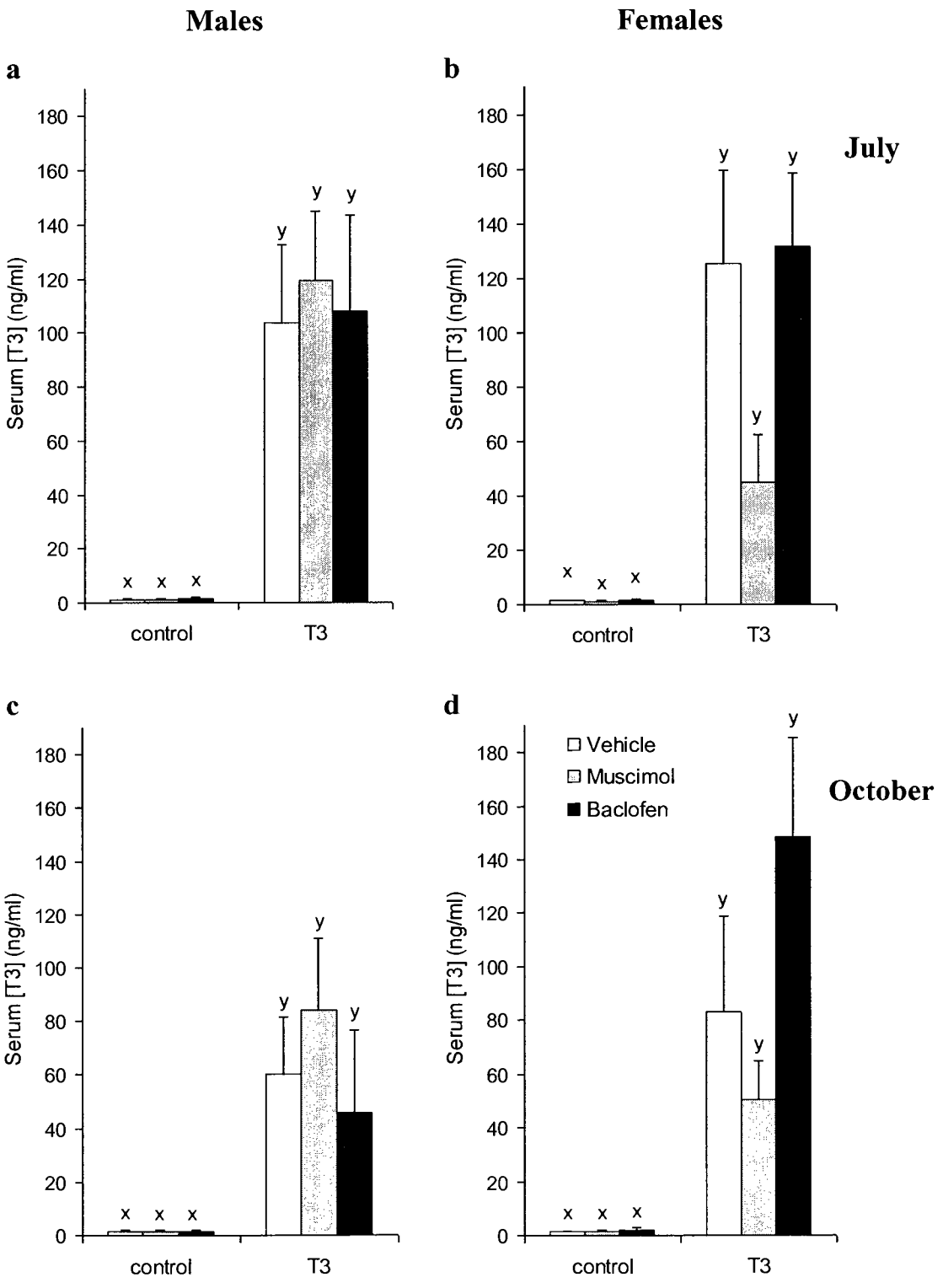

Figure 3.6 (Caption on previous page)

Table 3.3

Body mass and gonadosomatic index (GSI) of male and female goldfish in July (sexually regressing) and October (sexually regressed) pre-treated for six days with water-borne exposure to 0 or 0.1 μM T_3, followed by six hours exposure to saline vehicle, the $GABA_A$-receptor agonist muscimol (1 μg/g bw) or the $GABA_B$-receptor agonist baclofen (10 μg/g bw) via intraperitoneal injection.

Month of Exposure	sex	T_3 dose (μM)	agonist	n	Body mass (g)	GSI (%)
July	male	0	vehicle	11	21.2 ± 1.4^a	1.78 ± 0.40
			muscimol	9	20.1 ± 1.4^b	1.64 ± 0.29
			baclofen	13	22.8 ± 1.5^a	1.69 ± 0.25
		0.1	vehicle	15	22.3 ± 0.9^a	2.61 ± 0.31
			muscimol	10	18.1 ± 0.6^b	1.66 ± 0.43
			baclofen	16	21.5 ± 0.7^a	1.90 ± 0.26
	female	0	vehicle	13	21.7 ± 1.1	5.43 ± 1.45^x
			muscimol	14	19.7 ± 1.1	2.95 ± 0.67^x
			baclofen	11	20.0 ± 0.8	6.17 ± 1.31^x
		0.1	vehicle	9	18.9 ± 1.8	2.88 ± 1.02^y
			muscimol	14	21.2 ± 1.1	2.98 ± 0.51^y
			baclofen	8	21.6 ± 1.8	3.27 ± 1.05^y
October	male	0	vehicle	10	18.2 ± 0.9	1.10 ± 0.25
			muscimol	6	21.1 ± 2.3	0.72 ± 0.18
			baclofen	7	17.1 ± 2.6	0.67 ± 0.19
		0.1	vehicle	13	18.5 ± 1.3	1.18 ± 0.28
			muscimol	9	18.9 ± 1.9	1.02 ± 0.21
			baclofen	6	17.8 ± 1.9	0.88 ± 0.19
	female	0	vehicle	12	18.5 ± 1.9	2.04 ± 0.55
			muscimol	14	18.1 ± 1.3	1.83 ± 0.318
			baclofen	14	18.4 ± 0.9	1.68 ± 0.125
		0.1	vehicle	8	17.8 ± 1.9	1.62 ± 0.12
			muscimol	12	19.4 ± 2.1	1.61 ± 0.17
			baclofen	14	19.1 ± 1.3	1.60 ± 0.18

Data are presented as mean ± SEM; for each exposure (July and October) data within each sex were analyzed by two-way ANOVA (nonparametric on ranks for male mass in July) with main factors as T_3 exposure and agonist treatment, alpha = 0.05; male mass in July was followed by Tukey's test on ranks because there was a significant effect of agonists; main agonist treatment effects across T_3 treatments are indicated by superscripted letters a, b, c, and main T_3-exposure effects across agonist-treatments are indicated by superscripted letters x, y; GSI, gonadosomatic index; T_3, tri-iodothyronine.

Comparison of average GSI between the July exposure and October exposure for males and females indicates that gonad size was greater in July than October for both males (1.92 ± 0.13 % vs. 0.98 ± 0.10 %; Mann-Whitney U test, $p < 0.001$) and females (3.96 ± 0.44 % vs. 1.68 ± 0.11 %; Mann-Whitney U test, $p < 0.001$). This confirms that the fish were more sexually regressed in October than July.

3.3.2.3 Serum LH concentrations

For both sexually regressing (July) and sexually regressed (October) males and females, serum LH levels in muscimol-injected fish were statistically significantly higher than vehicle-injected fish, with larger differences in October than in July (Figure 3.7). Although baclofen-injected fish also appeared to have higher LH levels than vehicle-injected fish in all exposure groups, these differences were smaller, and this was only statistically significant in females in October (Fig 3.7). Moreover, T_3-pre-treatment did not significantly affect GABA-receptor agonist elevation of serum LH levels (Fig 3.7). For males in July (Figure 3.7 a), serum LH levels in muscimol-injected fish were 1.9- and 1.6-fold higher than in vehicle-injected fish in control and T_3-treated groups, respectively, and also significantly higher than serum LH in baclofen-injected fish (main effect of agonist, $p < 0.001$, followed by Tukey's test). For females in July (Figure 3.7 b), serum LH levels in muscimol-injected fish were 1.6- and 1.5-fold higher than in vehicle-injected fish in control and T_3-treated groups, respectively, and not statistically different from LH levels in baclofen-injected fish (main effect of agonist, $p = 0.024$, followed by Tukey's test). For males in October (Figure 3.7 c), serum LH levels in muscimol-injected fish were 2.6- and 2.8-fold higher than in vehicle-injected fish in control and T_3-treated groups, respectively, and also significantly higher than serum LH in baclofen-injected fish (main effect of agonist, $p < 0.001$, followed

by Tukey's test). For females in October (Figure 3.7 d), serum LH levels in muscimol-injected fish were 1.9- and 3.7-fold higher than in vehicle-injected fish in control and T_3-treated groups, respectively, and significantly higher than LH levels in baclofen-injected fish, which were 1.5- and 1.7-fold higher than in vehicle-injected fish in control and T_3-treated groups, respectively (main effect of agonist, nonparametric, $p < 0.001$, followed by Tukey's test).

3.4 Discussion

In this chapter, I showed that an experimental increase in circulating T_3 levels in goldfish had a sex-specific effect to increase gene expression of the gonadotropin subunit FSHβ in pituitary in females but not males, with no effects on LHβ. TSHβ and GPHα1 expression, which has been previously shown to be TH-responsive (MacKenzie et al., 2009; Yen, 2001), decreased in response to T_3 treatment in both sexes. The raised T_3 levels also affected expression of the TRs and deiodinases measured, all of which are known to be thyroid-responsive in fish (Bres et al., 2006; Liu et al., 2000; Manchado et al., 2009; Nelson and Habibi, 2008). This confirms that gene expression in the pituitaries of the fish used in this study was thyroid-responsive, and thus validates the conclusions on T_3 action on gonadotropin subunit expression drawn from this study.

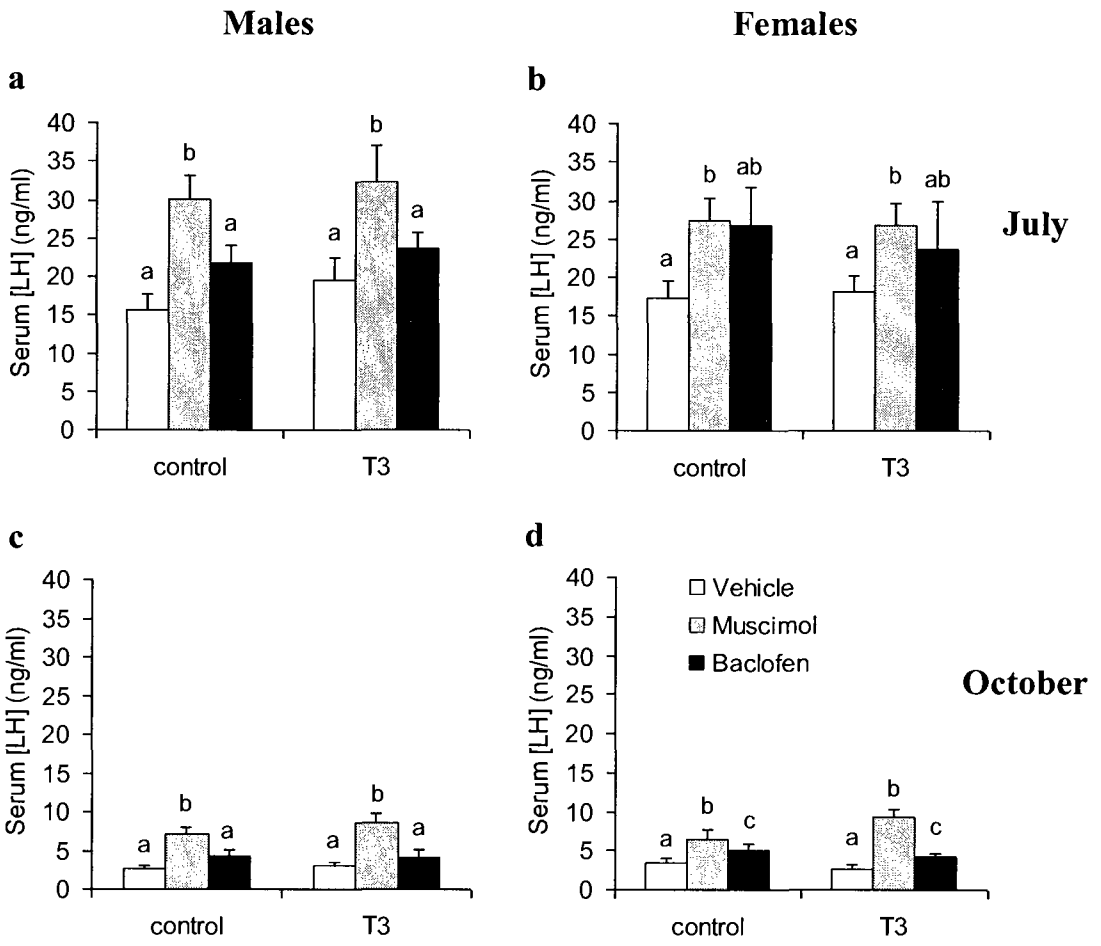

Figure 3.7 Serum LH levels (ng/ml), measured by radioimmunoassay, in male and female goldfish in July (**a**, **b**; sexually regressing) and October (**c**, **d**; sexually regressed) exposed for six days to 0 or 0.1 μM T_3, followed by six hours exposure to saline vehicle, $GABA_A$- or $GABA_B$-receptor agonists (muscimol, 1 μg/g bw and baclofen, 10 μg/g bw, respectively) via intraperitoneal injection. Data are presented as mean + SEM; n = 8-16. Data within each sex were analyzed by two-way ANOVA (**a**-**c**) or nonparametric two-way ANOVA (**d**) with main factors as T_3 exposure and agonist treatment, followed by Tukey's test (on ranks for **d**) (alpha = 0.05). Main agonist effects are indicated by the letters a, b, c. There were no main T_3-treatment effects, and no interaction between agonist and T_3-treatment. LH, lutenizing hormone; T_3, tri-iodothyronine.

A previous study has explored the effects of a two-week T_4 challenge or thyroid inhibition by thiourea on glycoprotein hormone subunit expression in sexually mature goldfish, but in males only, using Northern blot analysis (Yoshiura et al., 1999). In that study, T_4 treatment increased plasma T_4 but not T_3, and caused a decrease in expression of TSHβ to 30 % of control levels, but had no effects on FSHβ, LHβ, or GPHα. This absence of effect of raised thyroid hormone levels on male expression of FSHβ and LHβ is consistent with the results presented here. It is worth noting that in the fish treated with thiourea alone and thiourea plus T_4 replacement, plasma T_3 levels were lower than controls, and FSHβ expression was 1.5-fold higher than controls (Yoshiura et al., 1999). Although the authors interpret this as a pharmacological effect of thiourea (Yoshiura et al., 1999), it seems possible that this could have been an effect of lowered T_3 levels on male FSHβ expression, which would imply an inhibitory effect of T_3 on male FSHβ expression, the opposite of the effect of T_3 on female FSHβ expression observed in this chapter.

The novel effect of T_3 to increase FSHβ expression in females only, reported in this chapter, suggests that T_3 could be involved in a female-specific mechanism of regulation of the HPG axis in goldfish. Despite extensive research on regulation of gonadotropins in fish, with goldfish as a main model organism (reviewed by Chang et al., 2009; Popesku et al., 2008), T_3 has not been identified as an important regulator, largely because its effects have not been extensively assessed. A study tracking seasonal expression of the glycoprotein hormone subunits in pituitary of male and female goldfish indicated a sex difference in FSHβ expression in goldfish (Sohn et al., 1999a). Whereas seasonal LHβ expression patterns in males and females were similar, FSHβ levels in males did not increase as steeply as in females from November until the end of spawning in May, nor did they decrease after

spawning (Sohn et al., 1999a). This suggests that FSH expression in males and females is controlled differently, supporting the sex difference in T_3 effect on FSHβ expressed observed in the current study.

In the same study, Sohn *et al.* (1999a) also determined the seasonal profiles of plasma T_3 and T_4 levels. Female T_3 levels increased sharply after spawning, when FSH levels were dropping off, whereas male levels decreased. This inverse correlation between plasma T_3 and FSH expression in females does not support the hypothesis that circulating T_3 levels are driving the seasonal increase in FSHβ expression in pituitary in females. There is convincing evidence in birds and mammals that seasonal change in photoperiod alters T_3 levels in hypothalamus without changing circulating TH levels, and that this change in hypothalamic T_3 level is necessary for transitions in and out of breeding season (Hanon et al., 2008; Nakao et al., 2008b). Thus, the absence of positive correlation between plasma T_3 levels and FSHβ expression in pituitary over the seasonal reproductive cycle does not preclude the possibility that T_3 may play a role in regulating FSH levels in female goldfish.

T_3 could have affected FSHβ expression either by direct or indirect action in the pituitary, or indirectly by effects on the signalling systems in neuroendocrine brain regions that regulate FSH expression, such as GnRH or pituitary adenylate cyclase-activating polypeptide (PACAP; Chang et al., 2009). Direct actions in pituitary could include a direct effect on FSHβ transcription through TRs acting on a positive TRE in the promoter of the FSHβ gene. Although the upregulation of both TRα1 and TRβ indicate that TRs in pituitary were responsive to T_3 treatment, a TRE has not been identified in regulatory regions of FSHβ in any species, to my knowledge, including goldfish, despite characterization of its promoter in fish (reviewed by Yaron et al., 2003). This suggests that such a direct action is unlikely. T_3

could also act on autocrine or paracrine regulators of FSHβ expression in pituitary. Activin, a protein of the transforming growth factor β superfamily, differentially regulates goldfish FSHβ and LHβ expression, acting within the pituitary to stimulate FSHβ expression while simultaneously inhibiting LHβ expression (Yam et al., 1999; Yuen and Ge, 2004). Follistatin, the binding protein for activin, has the opposite effects on FSHβ and LHβ expression and overrides activin effects on gonadotropin expression (Yuen and Ge, 2004). These studies have not determined if sex differences exist. Thus, an increase in activin or decrease in follistatin levels could be involved in the T_3 action to increase FSHβ expression in female goldfish pituitary. To determine whether T_3 acts directly on the pituitary to increase female FSHβ gene expression, and whether it has effects on activin subunit and follistatin expression, *in vitro* exposures of pituitary tissue to T_3 should be conducted.

A recent study from our laboratory has shown that six-hour treatment with the $GABA_B$ agonist baclofen, but not the $GABA_A$ agonist muscimol, resulted in a three to four-fold increase in expression of activin subunit βa in female telencephalon and hypothalamus (pituitary expression was not measured) while simultaneously stimulating LH release (Martyniuk et al., 2007b). Although the effects of changes in activin expression in brain regions on pituitary gene expression are not known, it is possible that $GABA_B$-receptor-mediated effects on activin expression could have effects on FSHβ expression in pituitary. To test this hypothesis, the GABA agonist experiments should be repeated on fish pre-treated with T_3 for two days (the exposure time at which female FSHβ was increased), measuring activin subunit gene expression in neuroendocrine brain regions and pituitary, and FSHβ expression in pituitary, both to determine GABA-mediated effects on activin and FSHβ expression, and also to determine whether T_3 modulates these effects.

Since an assay for FSH detection has yet to be developed for goldfish (Chang et al., 2009), and most other teleosts species, I could not determine whether the increase in FSHβ expression in female pituitary observed here resulted in increased circulating FSH levels, or affected possible GABA-mediated FSH release. Furthermore, increased FSHβ expression does not necessarily reflect increased FSH hormone levels in pituitary, since FSHβ must dimerize with GPHα to form the mature hormone. At the time point and dose at which FSHβ expression was increased in females (two-day exposure to the high dose of T_3), there was no significant effect of T_3 on GPHα1 expression; however in both males and females after six days of 0.1 μM T_3 treatment, GPHα1 expression was significantly lower than controls. That expression pattern in response to T_3 treatment was similar to TSHβ, and not FSHβ. This suggests that T_3 effects on GPHα1 expression would not be contributing to an increase in FSH levels in pituitary; however, since all pituitary cell types were included in each RNA sample in this study, it is possible that thyrotrope- or gonadotrope-specific differences in GPHα1 response to T_3 treatment were masked. Furthermore, there are two GPHα transcripts in goldfish, GPHα1 and GPHα2, that are encoded by separate genes (Kobayashi et al., 1997), but only GPHα1 expression was measured in this study. Therefore, to get a complete picture of T_3 effects on GPHα expression, GPHα2 levels should also be measured. Furthermore, to understand how T_3 effects on GPHα expression affect glycoprotein hormone synthesis in pituitary, in situ hybridization studies on all four subunits, and immunohistochemistry studies on relative quantities of the hormones in response to T_3 treatment, should be carried out.

The effect of T_3 to decrease expression of both GPHα1 and TSHβ is consistent with bodies of knowledge showing that THs negatively regulate the expression of these two genes

i.e. that presence of TH results in repressed transcription of the gene (MacKenzie et al., 2009; Yen, 2001; Zhang et al., 2002). This is the first time TH has been shown to decrease expression of GPHα in goldfish. In contrast to the decrease in GPHα1 expression observed in the present study, Yoshiura *et al.* (1999) found no effect of a two-week T_4 challenge on GPHα expression in sexually mature male goldfish; however their GPHα probe equally hybridized with GPHα1 and GPHα2 transcripts (Yoshiura et al., 1999). Therefore, a specific effect of T_4 treatment on GPHα1 may have been masked by the presence of GPHα2. Alternatively, the administration of T_4 rather than T_3 and the longer exposure time could be responsible for the differences in results. A negative TRE or other regulatory elements that result in transcriptional repression in response to T_3 have been identified in mammalian GPHα (Brent et al., 1991; Zhang et al., 2002) and TSHβ of mammalian species (Nagayama et al., 2008) and goldfish (Sohn et al., 1999b). TSHβ expression level in pituitary of male goldfish exposed to T_4 for two weeks was 30 % that of control fish (Yoshiura et al., 1999), and *in vitro* exposures of goldfish pituitary fragments to T_3 resulted in decreased expression TSHβ (Sohn et al., 1999b). Thus the T_3 effect to decrease TSHβ expression in pituitary in the current study could have been due to direct action through TRs at the negative TRE of the TSHβ gene.

Expression in pituitary of TRs measured, TRα-1 and TRβ, increased in a dose-dependent manner in both males and females in response to T_3 treatment, indicating autoregulation of these receptors by their ligands. This indicates that T_3 plays important roles in regulation of gene expression in the adult goldfish pituitary through TRs. Dose-dependent decreases in TSHβ and GPHα expression occurred simultaneously with, or following, dose-dependent increases in TRα1 and TRβ, in males and females with some sex-specific

differences. This suggests that TR autoregulation in goldfish pituitary may be involved in inhibition of TSHβ and GPHα expression. In addition to studies on goldfish, there is evidence from several other teleost species that T_3 exerts negative feedback inhibition on TSH expression in pituitary, a mechanism of central hypothalamo-pituitary-thyroid (HPT) axis regulation that is well established in mammals but has been under debate for teleosts (MacKenzie et al., 2009).

Peripheral regulation of TH delivery to cells through deiodination activity in target tissues has been proposed as an alternative to the central, negative-feedback model of thyroidal status regulation for teleost fish (Eales and Brown, 1993). It is therefore interesting to note that in the current study, expression of D2, the deiodinase that synthesizes T_3 from T_4, decreased, and simultaneously the expression of D3, the deiodinase that breaks down active T_3 to T_2 and T_4 to inactive rT_3, increased. These simultaneous, opposing effects of T_3 on deiodinase expression should theoretically result in an overall decrease in T_3 in pituitary cells, indicating a homeostatic response to return intracellular T_3 levels to a set-point. Such a response of D2 and D3 expression and their respective deiodination activities has been observed in rainbow trout in response to a T_3 challenge (Bres et al., 2006) and in D3 expression in goldfish hepatocytes (Nelson and Habibi, 2008). The results presented here are a significant contribution in demonstrating that a T_3 challenge simultaneously has negative feedback effects on gene expression in pituitary to maintain thyroidal status at a set-point both through the central HPT model of regulation (TSHβ and GPHα expression) and the deiodination model of regulation.

There was no effect of T_3 treatment on basal circulating LH levels, nor on LHβ expression in pituitary of sexually mature male and female goldfish, nor on GABA-mediated

LH release in both sexually regressing and sexually regressed males and females. In Chapter 2, I also showed that T_3 exposures on sexually regressed males had no effect on basal, circulating LH levels. Thus, it appears that LH expression and release are not thyroid responsive in goldfish; however since LH expression and GABA-stimulated LH release were not measured in all of these experiments, nor was a time-course or dose-response tested for T_3 effects on GABA-mediated LH release, possible T_3 effects on LH cannot yet be completely ruled out. Although there were no effects of T_3 on GABA-mediated LH release, a trend was noted for sexually regressing females that serum T_3 levels in both the control and T_3-treated fish injected with the $GABA_A$ agonist muscimol were lower than saline-injected controls (Figure 3.6 b). GABA has been shown to inhibit TSH release from pituitary in rats and humans and TSH-stimulated thyroid hormone release from thyroid gland in mice, and the $GABA_A$ receptor has been implicated in these effects (see Section 1.7, Figure 1.2). This suggests that GABA might be an inhibitor of the thyroid system in goldfish, which has not been previously shown in teleosts (MacKenzie et al., 2009).

In conclusion, I have found a female-specific effect of *in vivo* T_3 treatment to increase expression of the gonadotropin subunit FSHβ in sexually mature goldfish, indicating that thyroid hormones may have roles in regulating female reproduction. LHβ expression and LH release were not found to be thyroid responsive. I also demonstrated that a T_3 challenge simultaneously has negative feedback effects on gene expression in pituitary to maintain thyroidal status at a set-point both through down-regulation of TSHβ and GPHα, and by shifting deiodinase expression to decrease local, intracellular T_3 levels. Furthermore, I have demonstrated TR autoregulation in goldfish pituitary for the first time, and hypothesize that this is involved in the transcriptional repression of TSHβ, GPHα and D2 expression by T_3

observed in this study. This research contributes both to understanding possible interactions of the reproductive and thyroid axes, but also to understanding thyroid axis regulation in an important teleost model species.

CHAPTER 4

Gene expression profiling of T_3 action in adult male goldfish neuroendocrine brain

4.1 Introduction

Thyroid hormones have critical roles in brain development. There is a large body of scientific research on TH action in developing, mammalian brain including detailed examination of TH effects on gene expression across different brain regions at different stages of development (Bernal, 2002). This has indicated that the majority of the TH-responsive genes that are critical to development are only responsive to TH during a restricted time-window, and are unresponsive in adult brain. Outside of these studies on genes implicated in development, very few studies have characterized TH responsiveness of gene expression in adult brain, despite the impact of hyper- and hypothyroidism on adult neurological function (Joffe and Sokolov, 1994; Simon et al., 2002). Indeed, there is mounting evidence that THs affect metabolic activity in adult, mammalian brain and affect mood and cognitive function (Bauer et al., 2008) as well as being implicated in neurodegenerative disorders such as Alzheimer's disease (Tan and Vasan, 2009).

Obtaining a gene expression profile for T_3 action would be very useful first step for determining potential roles T_3 in adult brain function. A limited number of microarray studies have been carried out in rats and mice to determine expression profiles of altered thyroidal states and T_3 treatment in adult brain (Diez et al., 2008; Haas et al., 2004; Miller et al., 2004) with contrasting results, but no such studies have been published in fish or any other non-mammalian vertebrate.

There is evidence in fish that T_3 has important functions in the adult brain through effects on gene expression, and that these functions may be related to reproduction. In goldfish, all four TR isoforms were expressed in adult neuroendocrine brain with no differences between males and females, but significant differences in expression between sexual maturity and sexual recrudescence (Nelson and Habibi, 2006). A microarray study profiling sex differences in whole brain gene expression between male and female zebrafish revealed that that the T_3-metabolizing enzyme D2 is more highly expressed in male than female brain, suggesting sex differences in regulation of TH levels in brain (Santos et al., 2008). Gene expression studies have also shown that thyroid disrupting chemicals have effects on important thyroid-response genes in fish brain (Nakayama et al., 2008) accompanied in some cases by compromised reproductive function (Lema et al., 2008). It is thus critical to determine what roles THs play in regulating reproduction through actions in brain, to understand how thyroid-disrupting environmental chemicals could impact reproduction.

Goldfish is an excellent species for studying neuroendocrine signalling, including neuroendocrine control of reproduction (Popesku et al., 2008). The AURATUS.CA carp-goldfish cDNA microarray (Martyniuk et al., 2006; Williams et al., 2008), contains brain-enriched goldfish sequences, including several sequences specific to neuroendocrine signalling that regulates reproduction, such as estrogen receptors and genes involved in GABA-ergic, dopaminergic and serotonergic signalling (Popesku et al., 2008). It is therefore an excellent tool for determining gene profiles for effects of specific hormones, neurotransmitters and drugs in neuroendocrine brain (Popesku et al., 2008).

To further address the hypothesis that THs regulate reproduction through effects on the GABA system as explored in a limited capacity by real-time RT-PCR in Chapter 2, the

AURATUS.CA microarray was used to determine a gene expression profile for T_3 effects on male, adult, neuroendocrine brain in goldfish, using telencephalon RNA from the six-day, 0 and 0.1 μM T_3 exposures described in Chapter 2. In addition to determining effects of T_3 on GABA-system genes, and thyroid-system genes cloned and added to the array in this study, this approach allowed the identification of genes involved in other neuroendocrine signalling systems that affect reproduction, and genes involved in other general cellular and metabolic processes and functions, indicating that adult goldfish brain is highly TH-responsive.

4.2 Materials and methods

4.2.1 Experimental protocol

The samples used for microarray analysis and array validation in this chapter are from the water-borne T_3 exposure of sexually regressed male goldfish in September, described in detail in section 2.2.1. For microarray analysis, only the telencephalon samples from the six-day, 0.1 μM T_3 exposure gene expression analysis group were used to screen for candidate TH-responsive genes (details below). Real-time RT-PCR validation of candidate genes was extended to include the 0.02 μM T_3 and 0.1 μM T_3, six-day telencephalon samples, and for genes selected by enrichment analysis, array validation was done for both doses and two-day and six-day samples from both telencephalon and hypothalamus.

4.2.2 Plasma T_3 enzyme immunoassay

In Chapter 2, total T_3 concentrations in plasma were determined by T_3 enzyme immunoassay for the same fish used here, to determine the effect of T_3-treatment on circulating T_3 levels, and is described in detail in section 2.2.2.

4.2.3 RNA isolation and quantification

In Chapter 2, total RNA was isolated from brain region samples and quantified for the same fish used here, and is described in detail in section 2.2.4.2. RNA quality was determined prior to use in microarray analysis. The integrity of a representative sample was assessed with an Agilent Bioanalyzer (Agilent Technologies, California, USA) and found to be of high quality.

4.2.4 Microarray production, hybridization and analysis

The AURATUS.CA carp-goldfish cDNA microarray, version 1.1, was used to obtain an expression profile for T_3 action in the telencephalon for the six-day, 0.1 μM T_3 exposure. The production of this goldfish brain-enriched microarray platform is described in detail by Martyniuk *et al.* (2006) and Williams *et al.*(2008), and has been validated in several studies (Marlatt et al., 2008; Martyniuk et al., 2006; Mennigen et al., 2008). There are 8089 carp (*Cyprinius carpo*) and goldfish cDNA sequences spotted in duplicate on the microarray. Approximately 1100 of these are goldfish cDNA sequences obtained by subtractive suppressive hybridization or through homology cloning. I cloned partial sequences of several thyroid system-related genes for inclusion on the microarray by homology cloning, following the protocol described in Chapter 2, section 2.2.4.1 (primers and PCR annealing temperatures are listed in Table 4.1). These include the three deiodinase types (D1, D2, D3), two of four goldfish TH receptors (TRα-1, TRβ), retinoic acid receptor (RAR) subtypes, retinoid X receptor (RXR) subtypes, pre-pro thyrotropin-releasing hormone (TRH), the TH binding protein transthyretin (TTR), the thyroid-responsive transcription factor Kruppel-like factor 13 (basic transcription element binding protein (BTEB) 3), and a potential normalization gene, 60S ribosomal protein L8 (L8).

Table 4.1

Thyroid system-related genes cloned and included on the AURATUS.CA carp-goldfish 1.1.microarray.

gene	fwd primer (5' to 3') rev primer (5' to 3')	tissue cloned from	sequence length (bp)	annealing temperature (°C)	Genbank Accession Number
D1	VCTGGTKCTGAGTTTYGGAAG CATYTCATCCACMACCACA	liver	256	52	EU313785
D2	WGAGTGGCAGCGCATGYT RTCGATGTAGACCAGCAGGAA	liver	322	58.7	EU313786
D3	TGTGGCTCCTGGAYTTCYT ATGAAYGGNGGTCAGGWG	brain	316	60	EU313787
TRα-1	GCATGGCCATGGACYTGGTGC TCTCCTGRCACTTCTCGATCTT	brain	674	60	NA*
TRβ	GAAGCCTTCAGTCAGTTTACAA CAGGTCYGTCACCTTCATCA	brain	485	54	NA*
TTR	TCTGCWCCAGTGGSTWTTCA ASCTGGTGRAAAGGTGTRCG	brain	281	55.4	EU313781
TRH	AAGCATCGAGGAGCTGTGTT TGTGGAGCAGCATCAGGTAG	brain	541	58.1	NA**
KLF13	TATGGCAAATCGTCCCATCT TGCATGTTTSRTCAGGTGRT	brain	207	50	EU313778
RARα-a	YACCATCGCHGACCAGATCA TGBGGTTTGTGDGGCCTYCT	brain	360	55.4	EU313782
RARα-b	GACATCCTGATVCTSCGRAT RACAGATGGCGCTRAGHAGC	brain	201	58.1	EU313783
RARγ	CTGYCAGATYAACAARGTCA ATGCAYTTGGTGGARAGCTC	brain	324	54	EU313784
RXRβ-b	GGMTGYAARGGCTTCTTCAA GCTCTGTCARMACYCTGTCA	brain	622	54	EU313779
L8	AGCACAGAAAAGGTGCTGCT ACCAGCAACAACACCAACAA	brain	452	52.7	EU313780

*The TRα-1 and TRβ sequences cloned here BLAST to AY973629 and AY973630, previously cloned in goldfish and deposited in Genbank by Nelson and Habibi (2006-Mol. Cell. Endocrinol. 253 (1-2), 83-95).

**The TRH sequence cloned here BLASTs to AB179819, previously cloned in goldfish and deposited in Genbank by Aoki *et al.* (2005).

NA, not applicable; D, deiodinase; TR, thyroid hormone receptor; TTR, transthyretin; TRH, pre-pro thyrotropin-releasing hormone; KLF13, Kruppel-like factor 13; RAR, retinoic acid receptor; RXR, retinoid X receptor; L8, 60S ribosomal protein L8.

Microarray hybridizations were performed using the Genisphere Array 900MPX cDNA microarray labelling kit (Genisphere, Pennsylvania, USA) following the manufacturer's hybridization protocol (http://www.genisphere.com/pdf/Array900MPX_20July2007.pdf). Using this kit, cDNA was synthesized from 2 μg total RNA and indirectly labeled with the fluorescent dye Cyanine 3 (Cy3) or Cyanine 5 (Cy5) to distinguish control cDNA from treatment cDNA. Four microarrays (biological replicates) were run in total. From the six-day time point, equal amounts of telencephalic total RNA from all eight control sample-pools (two individuals per pool) were combined (16 animals) into a single reference control pool before cDNA synthesis. Labelled cDNA (2 μg RNA) from one of four independent treatment sample-pools (two individuals per pool) from the six-day, 0.1 μM T_3 dose was combined with the dye-labeled reference control cDNA pool (2 μg RNA) and hybridized to a goldfish-carp microarray. To minimize dye bias, for two of the arrays, control cDNA was labeled with Cy3 and treatment cDNA labeled with Cy5, and for the other two arrays, control cDNA was labeled with Cy5 and treatment cDNA labeled with Cy3. To reduce array background and some types of nonspecific binding, the array prewashing procedure and array prehybridization with bovine serum albumin were carried out. The 2X formamide-based hybridization buffer was used for prehybridization, the 2X enhanced DNA hybridization buffer was used for the tagged DNA hybridization step, and 2X SDS-based hybridization buffer was used for the capture reagent (3DNA) hybridization step.

Microarray scanning followed the procedure of Martyniuk *et al.* (2006). In brief, microarrays were scanned with the ScanArray 5000XL system (Perkin Elmer, Massachusetts, USA) at 543 nm (Cy3) and 645 nm (Cy5). Spots were identified, those of

poor quality were flagged for exclusion, and raw Cy3 and Cy5 signal intensity was quantified for the remaining spots using QuantArray software (Perkin Elmer).

Microarray data were normalized and assessed for significant differential expression by Ph.D. candidate Huiling Xiong, under the supervision of Dr. Xuhua Xia (Department of Biology, University of Ottawa) following the procedures described in Martyniuk *et al.* (2006). In brief, microarray data were normalized by an intensity-dependent Lowess normalization (Yang et al., 2002). Significant differences in gene expression between control and treatment groups were assessed using the Significance Analysis of Microarrays approach (Tusher et al., 2001). The acceptable false discovery rate was set as a q-value of ≤ 5 %. Candidate-genes were only further considered if the fold-change resulting from T_3 exposure was 1.5 or greater.

Jason Popesku, a Ph.D. candidate in the Trudeau lab, has developed a database containing the goldfish-carp array sequence information and he provided the bioinformatics support to assign putative gene identities to differentially expressed sequences and to do Gene Ontology (GO) analysis using BLAST2GO (Conesa et al., 2005). BLAST2GO utilizes the GO database (Ashburner et al., 2000), to provide functional annotation of sequence information and incorporates statistical tests. Within this application, enrichment analysis using the GOSSIP package was performed (Fisher's Exact Test) to determine whether particular GO terms were significantly over- or under-represented in the list of differentially expressed genes in comparison to the other genes on the array (Blüthgen et al., 2005). Results were filtered by family-wise error rate (FWER) $\leq$ 0.05 (an adjusted p-value for multiple testing).

4.2.5 Real-time RT-PCR

Real-time RT-PCR was used to validate selected candidate genes identified as differentially expressed by microarray analysis on four telencephalon samples from the six-day, 0.1 μM T_3 exposure. For all genes selected for validation, real-time RT-PCR was extended to six-day, 0.02 μM T_3 dose telencephalon samples, and for those genes identified by enrichment analysis, real-time RT-PCR was done on telencephalon and hypothalamus samples from two-day and six-day, 0.02 μM and 0.1 μM exposures (n = 6-8). cDNA synthesis from these telencephalic and hypothalamic RNA samples is described in detail in section 2.2.4.2. Real-time RT-PCR primer design and assays were carried out as described in section 2.2.4.3. Primers and real-time RT-PCR conditions are detailed in Table 4.2.

4.2.6 Statistical analysis of real-time RT-PCR data

Real-time RT-PCR data for each gene within a time point within a brain region were analyzed by one-way ANOVA to determine effects of T_3 dose on gene expression. If one-way ANOVA revealed a significant effect of T_3 dose, Tukey's post-hoc test was used to identify significant differences among the doses.

First, data were tested for normality with the Kolmogorov-Smirnov test and for homogeneity of variance with the Levene test. If the assumptions of normality and homoscedasticity were not met, the data were $\log_{10}$- or reciprocal-transformed. If transformation failed to make the data meet the assumptions of normality and homoscedasticity, non-parametric one-way ANOVA was used (Kruskall-Wallis test). Where post-hoc tests were needed on non-parametric data, Tukey's test on ranks was used. Significance level was set at 0.05 for all statistical tests (significant p-values from one-way ANOVAs are reported in the text). All statistical analyses were carried out using S-Plus software.

Table 4.2

Real-time RT-PCR primer sequences and assay conditions for SYBR Green I assays for relative quantifiation of genes in goldfish telencephalon for validation of microarray results.

gene	fwd primer (5' to 3') rev primer (5' to 3')	amplicon length (bp)	[primer] (nM)	annealing temperature (°C)	accession number of template
AMPA2a*	CACTGAGGAGTTTGAGGATGG TTAGCCGTGTAGGAGGAGATG	198	100	58	AM886310
1CLP2	AGATTCCTCTACTCCCAAACCA CAGTCAGTCTTGTCCCAGCA	144	300	58	AB098610
NPY	TTCGTCTGCTTGGGAACTCT TGCCATACCTCTGCCTTGTT	142	150	58	M87297
Rab5b	ACTGGCAACAACAGCATCAC GCGTCTCTAAAAGCAGCAACTC	93	175	58	array gene id 03h01
SG1	ATCGTGTCCTGGCTCTTCTC CGTAGTTACAGGCGTTCTGGT	124	125	58	array gene id 15f16
beta actin*	ACTACTGGTATTGTGATGGACTCC CGGTCAGGATCTTCATCAGGTAG	142	100	58	AB039726
ARP	TCCCATCAGGTTGTTGTGTT ATGGTCTGCATCTGCTTGG	122	100	58	AY686702.1
L8	AACTACGCCACAGTCATCTCC CCAGCAACAACACCAACAAC	119	150	58	EU313780
$GABA_A\gamma 2$	AGCTGGCACTCTGCATCAA CTGCGTCTCAACAGCAACA	173	125	58	AY640227
GnRH rec A	CGGATGAAGACGCTGAAGA ACGAAGAGCAGGTGATGGA	146	125	58	AF121845
MBP	GACCACCAAACAGAGCAACA AAATGAGCCCTTTCCCTTTC	155	125	58	array gene id 60i02

E2-C	CCACCCAAATGTTGATGAGA GACAATAGAATGGAGCGGACA	92	100	58	array gene id 39m06
granulin	AGGAGACCCAGACAGCAAGA GGGCAGCAGTACCAAATACC	204	100	58	DQ369750.1
SNRPB/B′	CGGTCGGTAAGAGCAGTAAGA CCACGGTCATAGAAACCAGA	243	100	58	array gene id 22o19
Pβ7	CTCGTGCGACTGAAGGAATG CTGTGTGGTCATCTCGGTGT	119	150	58	array gene id 24h19

*Primers designed by others in Trudeau lab; array gene id refers to sequences on AURATUS.CA version 1.1; AMPA2a, AMPA receptor subunit 2a, flip variant; ICLP2, invariant chain like protein 2; NPY, neuropeptide Y; SG1, synaptogyrin 1; ARP, acidic ribosomal protein; L8, 60S ribosomal protein L8; $GABA_A\gamma 2$, $GABA_A\gamma 2$ receptor subunit; GnRH recA, gonadotropin releasing hormone receptor type A; MBP, myelin basic protein; E2-C, cyclin-selective ubiquitin carrier protein E2-C; SNRPB/B′, small nuclear ribonucleoprotein polypeptides B and B'; Pβ7, proteasome subunit beta type 7.

4.3 Results

4.3.1 Plasma T_3 concentrations

Plasma T_3 concentrations for this exposure are presented in detail in section 2.3.1, Figure 2.1. In brief, water-borne T_3 exposure resulted in significantly higher plasma [T_3] than controls at both doses and exposure times. For the exposure groups used for microarray analysis (six-day control and 0.1 μM T_3), plasma [T_3] was 29-fold higher in the T_3-treated group than control.

4.3.2 Identification of differentially expressed genes by microarray analysis

Of the 8089 cDNA sequences on the goldfish-carp microarray, 633 were statistically differentially expressed (q-value ≤ 5 %) in male telencephalon between control and T_3-treated fish (six days, 0.1 μM T_3), with a 1.5-fold or greater difference in expression levels. Decreased expression (1.5- to 4.5-fold) was observed for 621 sequences, while increased expression (1.5- to 1.6-fold) was observed for only 12 sequences. Of the 621 sequences whose expression decreased, 482 had significant similarity to nucleotide sequences in the NCBI database (BLASTN hits), and are listed in Appendix B under their putative gene identities. Of these, 267 sequences were fully GO annotated and therefore used in BLAST2GO gene ontology analysis. Of the 12 sequences whose expression increased, eight had significant similarity to nucleotide sequences in the NCBI database and are listed in Table 4.3 as putative genes. Seven of these were fully GO annotated and therefore included in BLAST2GO analysis.

Table 4.3
List of the eight putative genes that increased in expression in response to T_3 treatment.

Gene Name	array fold-change	*PCR fold-change	E-value
AMPA receptor subunit 2a, flip variant	+1.5	**	1e-170
ATPase, Ca^{++} transporting, cardiac muscle, fast twitch 1 like	+1.5		1e-170
cytokine receptor-like factor 3	+1.5		1e-88
invariant chain like protein 2	+1.5	-1.1 ± 0.2	1e-170
neuropeptide Y	+1.5	-1.1 ± 0.2	1e-170
PREDICTED: similar to synaptogyrin 1	+1.5	**	1e-135
Rab5b, member RAS oncogene family	+1.6	**	1e-131
reticulon 4-M	+1.5		1e-170

*PCR fold-change refers to real-time RT-PCR validation for selected genes within this list, and is presented as mean fold-change ± SEM; effects of the 0.02 μM T_3 dose for six days was simultaneously tested by real-time RT-PCR, but the results are not presented; one-way ANOVA revealed no significance, $\alpha = 0.05$.
**Real-time RT-PCR validation results are presented for these genes in Figure4.4
E-value is maximum expectation value from BLASTN.

4.3.3 Sequence distributions among common GO terms

BLAST2GO analysis annotates each sequence with gene ontology terms from three ontologies: biological process, molecular function, and cellular component. Multilevel GO analysis was used to produce sequence distributions of the most common biological processes, molecular functions and cellular components of the products of genes affected by T_3 treatment (Figure 4.1 and 4.2). Multilevel GO groups sequences by GO terms from any GO level for each ontology. For the 267 GO annotated sequences that decreased in expression, the sequence distributions are presented for GO terms at the lowest level of a GO branch at which there were more than 20 sequences (Figure 4.1). The most common biological process for the genes with decreased expression was signal transduction (14 %), followed by cellular biosynthetic processes (10 %) (Figure 4.1 a). The most common molecular function was protein binding (33 %) followed by hydrolase activity (11 %) (Figure 4.1 b). The most common cellular component was protein complex (26 %) followed by membrane (18 %) (Figure 4.1 c). For the seven GO annotated sequences that increased in expression, the sequence distributions are shown for GO terms at the lowest level of a GO branch at which there was more than one sequence (Figure 4.2). Several biological processes are equally represented by these seven genes, including synaptic transmission, intracellular signalling cascade, and protein transport (Figure 4.2 a). The most common molecular function is protein binding (23 %), followed equally by five other functions that include various transporters, enzyme and receptor activities (Figure 4.2 b). The cellular components to which these gene products are localized include membranes (29 %), synapses, cell junctions, macromolecular complexes and endoplasmic reticulum (Figure 4.2 c).

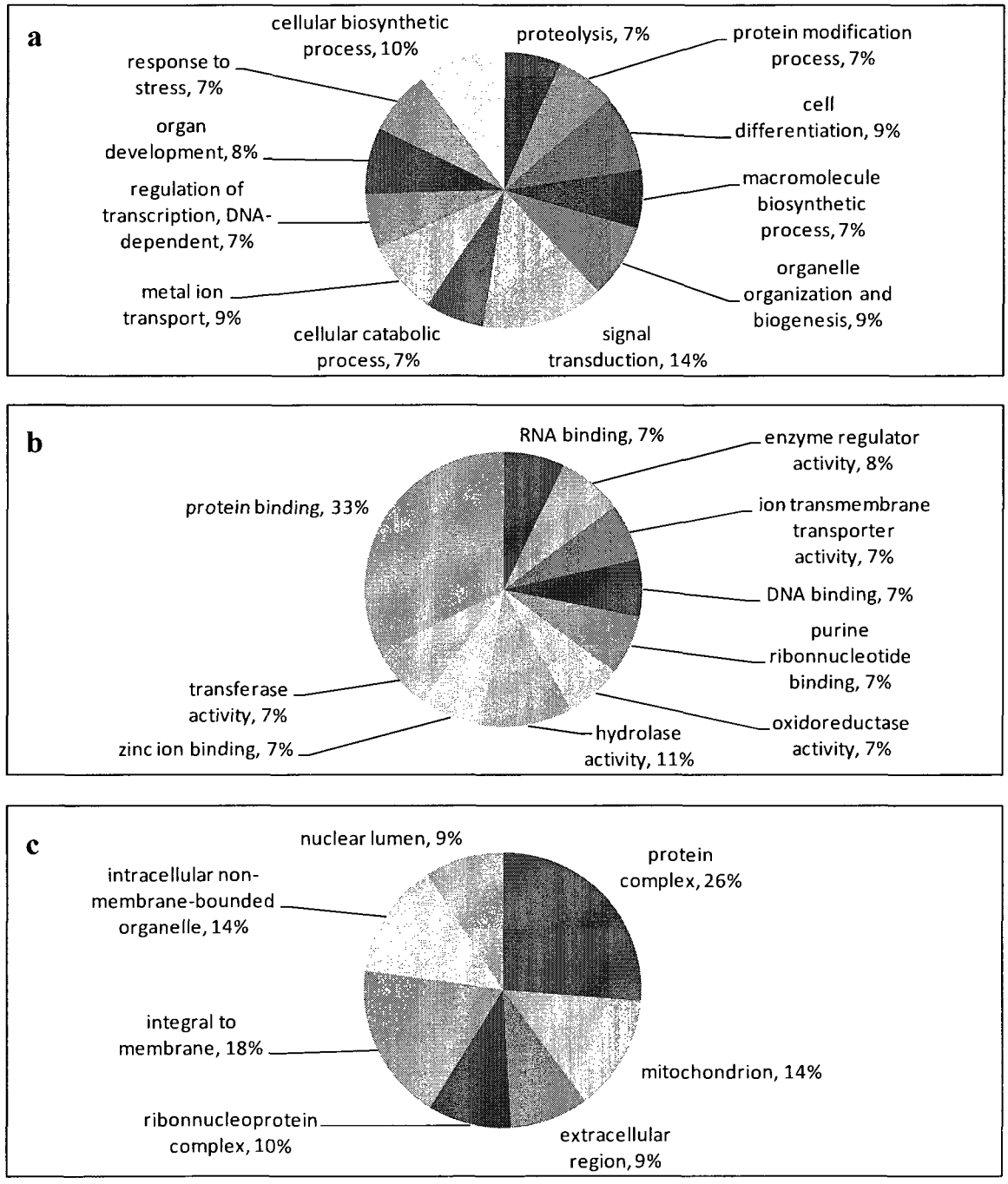

Figure 4.1 Sequence distributions representing the most common GO terms within each ontology for the 267 GO annotated genes that decreased in expression in response to a T_3 challenge: a) biological process b) molecular function and c) cellular component. Distributions derived by Multilevel GO analysis in BLAST2GO (cutoff = 20 sequences; see text for details). GO, gene ontology.

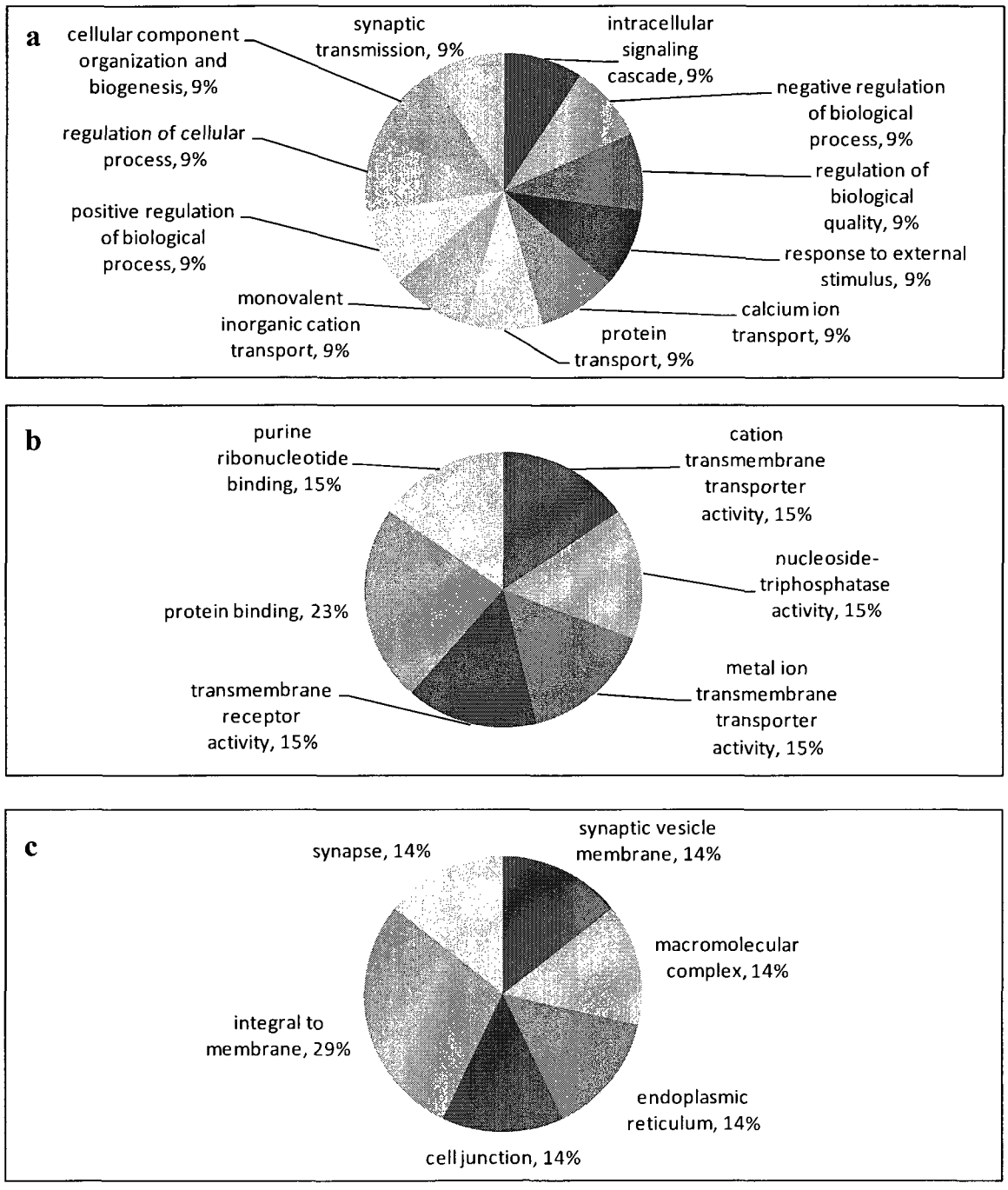

Figure 4.2 Sequence distributions representing the most common GO terms within each ontology for the seven GO annotated genes that increased in expression in response to a T_3 challenge: a) biological process b) molecular function and c) cellular component. Distributions derived by Multilevel GO analysis in BLAST2GO (cutoff = 1 sequence; see text for details). GO, gene ontology.

4.3.4 GO terms and candidate genes identified by enrichment analysis

Since the sequence distributions presented above could simply reflect the GO annotation distribution for genes expressed in male telencephalon, GO enrichment analysis was used to determine statistically whether groups of genes associated with particular functions were responsive or unresponsive to a T_3 challenge. The analysis determines whether any GO term is statistically significantly over- or under-represented in the list of differentially expressed genes in comparison to the genes on the microarray that did not change in expression. Enrichment analysis was carried out separately for genes that decreased and that increased in expression in response to T_3.

In the group of 267 GO annotated genes that decreased in expression, two molecular function GO terms and one biological process GO Term were over-represented in the group (Table 4.4). Protease inhibitor activity was represented by 15 sequences, and 14 of those, excluding granulin, have endopeptidase inhibitor activity, a lower classification level of that GO branch (Table 4.5). The proteins these genes encode include those whose primary functions are identified as endopeptidase inhibitors *e.g.* cystatin (a cysteine-type endopeptidase inhibitor), latexin (a metallocarboxypeptidase inhibitor), and serine protease inhibitor, Kunitz type 1-like, or have secondarily been identified to have protease inhibitor function *e.g.* phosphatidylethanolamine binding protein (PEBP; serine protease inhibitor; Hengst et al., 2001). There are also two endopeptidases on this list (cathepsin L and trypsin II precursor) and three proteasome subunits, which participate in protein degradation. These five proteins have all been shown to have the potential for autolytic degradation, hence their categorization as having endopeptidase inhibitor activity. The category of mRNA metabolic process was represented by 16 sequences (Table 4.5). These included genes encoding proteins of the small nuclear ribonucleoprotein (snRNP) complexes that comprise the

spliceosome, including the Sm spliceosomal core proteins (*e.g.* SNRPD2, D3, G, B/B′) and the snRNP-specific proteins (*e.g.* U5 snRNP-associated 102 kDa protein).

In the group of seven GO annotated genes whose expression increased, three related cellular component GO terms were over-represented by three genes, and one biological process GO term was under-represented by one gene (Table 4.4 and 4.5). Rab5b, AMPA receptor subunit 2a, flip variant (AMPA2a), and synaptogyrin 1 localize to cytoplasmic membrane-bound vesicles, and specifically, AMPA2a and synaptogyrin 1 localize to synaptic vesicle membranes (Table 4.5). Rab5b belongs to a family of small GTPases with roles in endocytosis, cell-surface molecule recycling, and regulation of intracellular vesicle transport (Baskys et al., 2007; Hirota et al., 2007). AMPA2a is an ionotropic glutamate receptor subunit and synaptogyrin 1 is an integral membrane protein associated with presynaptic vesicles in neurons. The significantly under-represented GO term for the seven GO annotated genes that increased in expression was metabolic process (Table 4.4), represented by a cardiac muscle ATPase (Table 4.3).

For each of the three over-represented GO terms in the group of genes that decreased in expression, one gene was chosen for real-time RT-PCR validation: granulin, proteasome subunit beta type 7 (Pβ7) and SNRPB/B′ (Figure 4.3). For the three over-represented GO terms in the group of genes that increased in expression, all three genes were chosen for real-time RT-PCR validation of the array results: Rab5b, AMPA2a and synaptogyrin 1 (Figure 4.4). These real-time RT-PCR experiments were not only used to validate the array results for six-day, 0.1 μM T_3 effects on telencephalon, but also to determine the time-, dose- and tissue-specificity of the effects by extending the analysis to both doses and time points and to hypothalamus, using all six to eight brain pools per treatment, rather than only the four used for the microarray.

Table 4.4
GO terms identified by GO enrichment analysis as over- or under-represented in the differentially expressed gene sets (decreased and increased).

Direction of expression change	Gene Ontology terms	Representation	Differentially expressed genes with this GO term		Unaffected genes with this GO term	FWER
			count	%	%	
decreased	Molecular Function					
	protease inhibitor activity	over	15	5.4	1.5	0.02
	endopeptidase inhibitor activity	over	14	5.1	1.4	0.03
	Biological Process					
	mRNA metabolic process	over	16	5.8	1.8	0.04
increased	Cellular Component					
	membrane-bound vesicle	over	3	43	2.0	0.04
	cytoplasmic membrane-bound vesicle	over	3	43	1.9	0.03
	synaptic vesicle membrane	over	2	29	0.2	0.02
	Biological Process					
	metabolic process	under	1	14	57	0

FWER, family-wise error rate.

Table 4.5

The putative genes within each GO term identified by GO enrichment analysis as over- or under-represented in the differentially expressed gene sets (decreased and increased).

Gene Ontology Term and Gene Name	fold-change	E-value
Molecular Function		
protease inhibitor activity		
granulin	-1.8	1e-109
endopeptidase inhibitor activity		
alpha 4 subunit of 20S proteasome	-1.5	1e-170
cathepsin L preproprotein	-1.5	1e-170
cystatin	-1.6	1e-108
cystatin	-1.8	1e-052
PREDICTED: similar to cystatin B (stefin B)	-1.9	3e-39
hypothetical protein LOC567341	-1.9	1e-101
latexin (endogenous carboxypeptidase inhibitor)	-1.5	3e-29
phosphatidylethanolamine binding protein	-1.6	1e-170
PREDICTED: similar to proteasome subunit alpha type 1	-1.5	1e-120
** proteasome (prosome, macropain) subunit, beta type, 7*	-2.1	1e-170
serine protease inhibitor, Kunitz type 1-like (spint1l)	-2.0	1e-015
trypsin II precursor	-2.0	1e-056
trypsin II precursor	-1.8	1e-106
trypsin II precursor	-1.7	1e-106
Biological Process		
mRNA metabolic process		
heterogeneous nuclear ribonucleoprotein L	-1.5	1e-060
hypothetical protein LOC415140	-1.6	1e-076
hypothetical protein LOC436976	-1.6	1e-110
KH domain containing RNA binding signal transduction associated 1 like	-1.5	1e-124
LSM7 homolog, U6 small nuclear RNA associated (S. cerevisiae)	-1.8	1e-110
PREDICTED: similar to peptidylprolyl isomerase domain and WD repeat containing 1	-1.8	1e-115
probable C->U editing enzyme APOBEC-2	-2.1	1e-91
probable C->U editing enzyme APOBEC-2	-1.6	6e-58
RNA binding motif protein 4.2	-1.6	1e-089
Sjogren syndrome antigen B (autoantigen La)	-2.1	1e-139
small nuclear ribonucleoprotein D3 polypeptide, like	-1.9	1e-108
small nuclear ribonucleoprotein G (snRNP-G) (Sm protein G)	-1.5	1e-25
** small nuclear ribonucleoprotein polypeptides B and B'*	-2.2	1e-102
PREDICTED: similar to small nuclear ribonucleoprotein D2	-1.8	1e-045
SYF2 homolog, RNA splicing factor (S. cerevisiae)	-1.5	1e-079
U5 snRNP-associated 102 kDa protein	-2.1	1e-170
Cellular Component		
membrane-bound vesicle		
cytoplasmic membrane-bound vesicle		
**Rab5b*	+1.6	1e-123
synaptic vesicle membrane		
**AMPA receptor subunit 2a, flip variant*	+1.5	1e-170
**PREDICTED: similar to synaptogyrin 1*	+1.5	1e-087

*Real-time RT-PCR validation results are presented in Figure 4.3 or 4.4.
E-value is maximum expectation value from BLASTN.

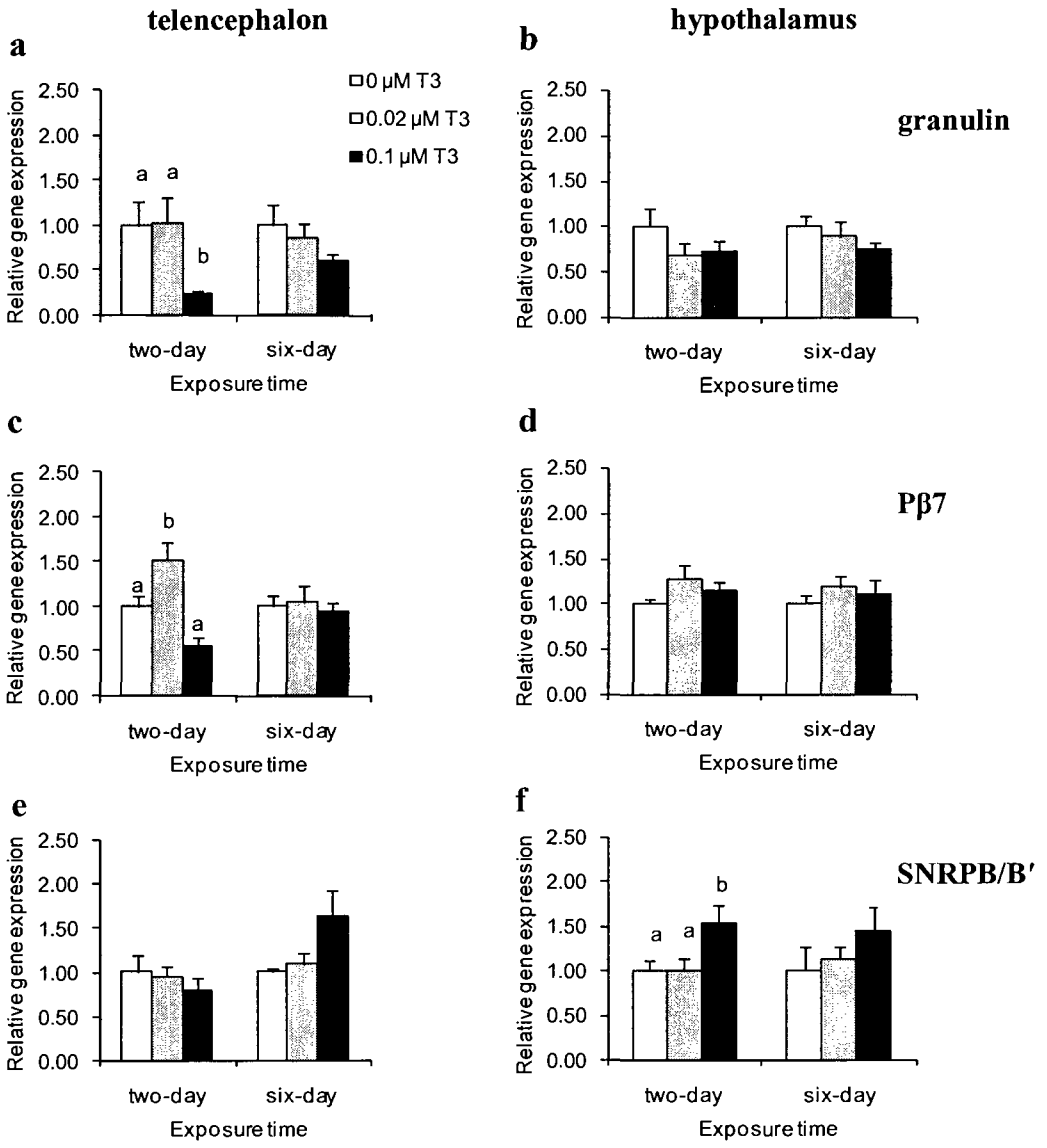

Figure 4.3 Real-time RT-PCR validation of candidate genes identified by enrichment analysis from the group of genes that decreased in expression on the microarray (six-day, 0.1 μM T_3, telencephalon). Relative gene expression in telencephalon (a, c, e, g) and hypothalamus (b, d, f, h) was determined in male goldfish after two-day and six-day water-borne exposure to 0, 0.02 and 0.1 μM T_3 in September. Data are presented as a proportion relative to control expression level for each exposure time (mean + SEM, n = 7-8). Data within each exposure time were analyzed by one-way ANOVA followed by Tukey's post-hoc comparisons where ANOVA revealed significance ($p \leq 0.05$). Significant differences among doses within each exposure time are denoted by different letters. SNRPB/B', small nuclear ribonucleoprotein polypeptides B and B'; Pβ7, proteasome subunit beta type 7.

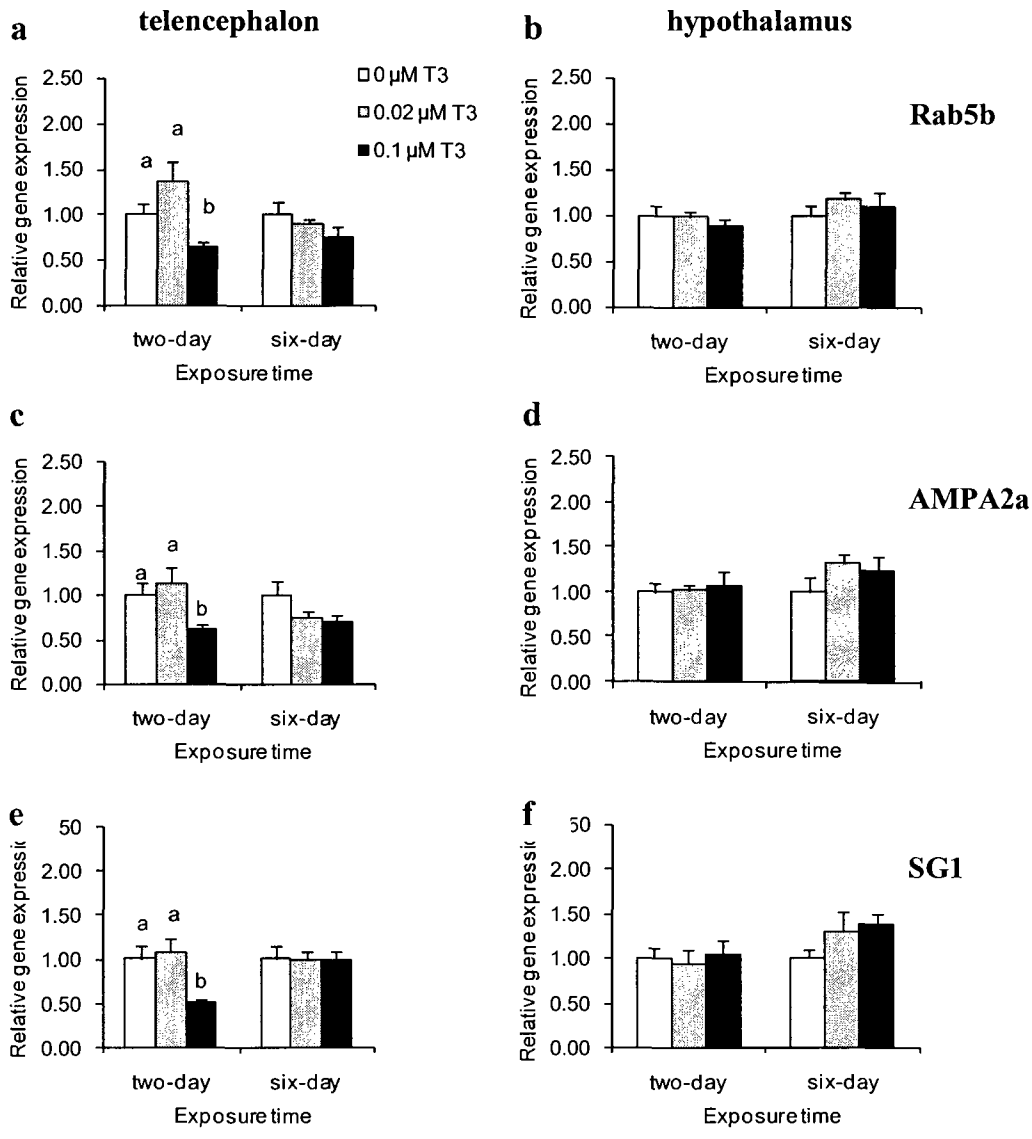

Figure 4.4 Real-time RT-PCR validation of candidate genes identified by enrichment analysis from the group of genes that increased in expression on the microarray (six-day, 0.1 µM T_3, telencephalon). Relative gene expression in telencephalon (a, c, e, g) and hypothalamus (b, d, f, h) was determined in male goldfish after two-day and six-day water-borne exposure to 0, 0.02 and 0.1 µM T_3 in September. Data are presented as a proportion relative to control expression level for each exposure time (mean + SEM, n = 7-8). Data within each exposure time were analyzed by one-way ANOVA followed by Tukey's post-hoc comparisons where ANOVA revealed significance ($p \leq 0.05$). Significant differences among doses within each exposure time are denoted by different letters. AMPA2a, AMPA receptor subunit 2a, flip variant; SG1, synaptogyrin 1.

Of the six genes tested in the six-day, 0.1 μM T_3 telencephalon group, direction and magnitude of change in gene expression determined by the microarray was only confirmed for granulin (1.7-fold decrease from real-time RT-PCR analysis, Figure 4.3 a, 1.8-fold decrease from microarray analysis). The only statistically significant effect of T_3 treatment on granulin expression was a 4.2-fold decrease after two-day, 0.1 μM T_3 treatment (one-way ANOVA, $p = 0.003$, followed by Tukey's test). Granulin expression also decreased in hypothalamus, although not significantly, at both doses and time points (Figure 4.3 b). The only statistically significant effect on Pβ7 was a 1.5-fold increase in expression in response to two-day exposure to the 0.02 μM T_3 dose (one-way ANOVA, $p = < 0.001$, followed by Tukey's test), with a non-significant 1.8-fold decrease at the 0.1 μM dose after two days (Figure 4.3 c) and no effect on hypothalamus (Figure 4.3 d). For SNRPB/B′, the major trend was the opposite to that observed in the array, with the 0.1 μM dose resulting in a 1.4 to 1.6-fold increase in expression in six-day telencephalon, and both two-day and six-day hypothalamus, although this was only significant for two-day hypothalamus (one-way ANOVA, $p = 0.02$, followed by Tukey's test) (Figure 4.3 e-f).

Of the three genes with increased expression on the microarray representing enriched GO terms analyzed by real-time RT-PCR, there was a significant effect of the 0.1 μM dose after two-day treatment in telencephalon for all three genes to decrease expression, rather than increase it (Figure 4.4 a, c, e). Rab5b expression was 1.5-fold lower than control (one-way ANOVA, $p = 0.001$, followed by Tukey's test). AMPA2a expression was 1.7 fold lower than control (nonparametric one-way ANOVA, $p = 0.01$, followed by Tukey's test on ranks). Synaptogyrin 1 expression was 2.0 fold lower than control (one-way ANOVA, $p = 0.001$, followed by Tukey's test). There were no effects of T_3 treatment on expression of these three genes in hypothalamus (Figure 4.4 b, d, f).

4.3.5 Additional candidate genes with roles in neuroendocrine signalling and brain development

In addition to identifying and grouping differentially expressed genes by GO enrichment analysis, I have also identified several additional putative genes of interest from the 482 sequences that decreased in expression in response to a T_3 challenge and grouped them in three main categories of interest (Table 4.6). Several genes were identified that have roles in reproduction and neuroendocrine signalling. They include hormones (isotocin, growth hormone (GH), secretogranin V), and hormone receptors (GnRH receptor Type A, pituitary adenylate cyclase activation polypeptide (PACAP) type 1 receptor precursor). Proteins that are related to GABA function were also included in this category, such as $GABA_A$ receptor γ2-subunit ($GABA_A\gamma2$), pyridoxal kinase (which phosphorylates pyridoxal to generate PLP, the co-factor necessary for GAD action), endozepine (which modifies $GABA_A$ receptor function through the benzodiazepine binding site), and parvalbumin (associated with GABA-interneurons), and calcium binding proteins (calmodulin 1b, ependymin II, sorcin, parvalbumin). Genes with vesicle transport functions were identified and included in this grouping because of the key role of vesicle transport in neuroendocrine signalling, including several genes related to Rab5b.

Several differentially expressed genes involved in brain development and neurogenesis were identified. These genes included myelin basic protein, two proteins related to epidermal growth factor (Delta/Notch-like EGF-related receptor and betacellulin), reticulon 1a, fatty acid binding proteins, and a nerve growth factor receptor (neurotrophic tyrosine kinase receptor type 1).

Table 4.6
List of selected putative genes that decreased in expression in telencephalon of male goldfish, as identified by microarray analysis, in response to a T_3 challenge of 0.1 μM T_3 for six days.

Grouping and gene name	array fold-change	*PCR fold-change	E-value
Reproduction and Neuroendocrine Signalling			
growth hormone	-1.8		1e-017
isotocin neurophysin	-1.7		1e-113
secretogranin V	-1.5		1e-54
gonadotropin releasing hormone receptor type A	-1.9	-1.6 ± 0.2	1e-92
pituitary adenylate cyclase activating polypeptide type 1 receptor precursor	-1.5		1e-009
$GABA_A\gamma 2$ receptor subunit	-2.0	-1.1 ± 0.1	1e-170
endozepine	-1.8		1e-091
PREDICTED: similar to G protein-coupled inwardly rectifying potassium channel Kir3.4	-1.8		1e-050
PREDICTED: similar to taurine transporter	-1.9		1e-046
pyridoxal (pyridoxine, vitamin B6) kinase, like	-1.8		1e-028
calmodulin 1b	-2.2		1e-104
ependymin ii	-1.5		1e-176
parvalbumin	-2.7		1e-170
parvalbumin isoform 1d	-1.6		1e-150
PREDICTED: similar to parvalbumin, transcript variant 2	-1.7		1e-170
sorcin	-2.3		1e-170
PREDICTED: similar to Ras and Rab interactor 2	-1.7		1e-054
PREDICTED: similar to RAB3D, member RAS oncogene family	-1.7		3e-54
RAB18B, member RAS oncogene family	-1.7		1e-034
ras homolog gene family, member Ab (rhoab)	-1.6		1e-180
vesicle transport through interaction with t-SNAREs homolog 1A	-2.3		1e-161
Brain Development and Neurogenesis			
PREDICTED: similar to Delta/Notch-like EGF-related receptor	-2.5		1e-026
betacellulin, epidermal growth factor family member	-2.4		1e-076
PREDICTED: neurotrophic tyrosine kinase, receptor, type 1	-1.9		1e-088
fatty acid binding protein 2, intestinal	-1.9		1e-136
fatty acid binding protein 7, brain, b	-2.5		1e-023
myelin basic protein	-1.6	+1.1 ± 0.2	1e-170
myelin basic protein, transcript variant 3	-1.7		1e-28
reticulon 1a	-1.5		1e-51
Protein synthesis and breakdown			
40S ribosomal protein S11	-1.5		1e-130
60S ribosomal protein L8	-2.2	-1.3 ± 0.1	1e-079
acidic ribosomal protein P1	-1.5	1.0 ± 0.1	1e-041
PREDICTED: similar to ribosomal protein L13a	-1.9		1e-009
PREDICTED: similar to ribosomal protein L5b, transcript variant 2	-1.5		1e-171
ribosomal protein L41	-2.0		1e-154
ribosomal protein L6	-1.6		1e-048
ribosomal protein S19	-2.5		1e-016
ribosomal protein S24 isoform 1	-1.6		1e-170
ribosomal protein S30 (fau)	-1.5		1e-087
ribosomal protein S5	-1.6		1e-170

ubiquitin C-terminal hydrolase L1	-1.5		1e-170
ubiquitin fusion degradation 1-like	-1.6		1e-066
ubiquitin-conjugating enzyme E2N-like	-1.5		1e-170
cyclin-selective ubiquitin carrier protein E2-C	-4.5	-2.1 ± 0.2	1e-167
Other			
beta actin	-1.5	-1.6 ± 0.2	1e-117

*PCR fold-change refers to real-time RT-PCR validation for selected genes within this list, and is presented as mean fold-change ± SEM; effects of the 0.02 μM T_3 dose for six days was simultaneously tested by real-time RT-PCR, but the results are not presented; one-way ANOVA revealed no significance, $\alpha = 0.05$.
E-value is maximum expectation value from BLASTN.

Several genes that function in protein synthesis and breakdown were also identified. These included several key genes involved in the ubiquitin-proteasome system (UPS). Also included were several ribosomal proteins that have been traditionally used as reference genes in real-time RT-PCR assays. Finally, beta actin is included on the list since it is another commonly used reference gene for real-time RT-PCR assays in neuroendocrine research.

From this list (Table 4.6), real-time RT-PCR validation was carried out on the following seven candidate genes in telencephalon, for the six-day exposure only, at both 0.02 μM T_3 and 0.1 μM T_3 doses: ribosomal protein L8, acidic ribosomal protein, beta actin, GnRH receptor type A, $GABA_A\gamma2$, myelin basic protein (MBP), and cyclin-sensitive ubiquitin carrier protein E2-C (E2-C). The gene expression fold-changes determined by real-time RT-PCR for the 0.1 μM dose only are shown in Table 4.6. Of these seven candidate genes, direction of gene expression change (decreased) was confirmed for the six-day, 0.1 μM T_3 exposure for all except $GABA_A\gamma2$ and MBP. There was no statistical significance, and no effects of the 0.02 μM T_3 dose (one-way ANOVA).

Real-time RT-PCR validation was also carried out in six-day telencephalon samples on two additional putative genes of the eight that increased in expression: neuropeptide Y (NPY), which has roles in reproductive neuroendocrine signalling, and invariant chain like protein 2 (ICLP2), which is involved in the immune response. Direction was not confirmed for 0.1 μM T_3, nor were there any significant effects at the lower dose. The gene expression fold-changes determined by real-time RT-PCR for the 0.1 μM T_3 dose only are shown in Table 4.3.

4.4 Discussion

This is the first study to use a transcriptomic approach to explore effects of altered thyroidal status on brain gene expression in a fish species, and moreover on adult brain gene expression in a non-mammalian model organism. I show that adult neuroendocrine brain of male goldfish is responsive to a supraphysiological increase in circulating T_3 levels, with the vast majority of genes decreasing in expression. Moreover, this demonstrates that T_3 regulates metabolic activity of adult goldfish brain.

In the few microarray studies on T_3 effects on adult brain gene expression in rats and mice, in general the majority of differentially expressed genes have increased in expression (Diez et al., 2008; Haas et al., 2004; Miller et al., 2004), in contrast to my results. Furthermore, in the two studies in which euthyroid animals were treated with T_3, very few genes were thyroid-responsive in adult male brain: eight increased and three decreased in expression in whole rat brain (Haas et al., 2004) and 18 increased and three decreased in expression in mouse cerebellum (Miller et al., 2004). This is in contrast to a study in which T_3 was administered to hypothyroid male adult rats where the single, high dose of T_3 resulted in 149 genes increased and 88 decreased in expression in striatum, indicating that adult hypothyroid striatum was highly thyroid-responsive (Diez et al., 2008). Although the contrasting results between the two rat studies could be attributed to insensitivity of euthyroid animals to increased T_3 levels in comparison to hypothyroid animals, the use of whole-brain samples in the T_3 exposure of euthyroid rats by Haas *et al.* (2004) likely masked important changes in gene expression in specific brain regions and cell-types.

Although these gene expression profiles of T_3 action in rat and mouse brain indicate that T_3 treatment results in increased gene expression more than decreased expression, there is microarray data showing that in known thyroid-responsive tissues such as white adipose

tissue and heart (Miller et al., 2004) and liver (Feng et al., 2000), the main effect of pharmacologic hyperthyroidism was suppression of gene expression. This supports my results of a large-scale decrease in gene expression in adult male goldfish telencephalon in response to a supraphysiological increase in T_3 levels. Several well-characterized thyroid-response genes are negatively regulated, *i.e.* T_3 represses their transcription, and this negative regulation plays a key role in processes such as TH negative feedback to the brain and pituitary to maintain a homeostatic TH set-point (MacKenzie et al., 2009; Yen, 2001), and differential regulation of thyroid response genes during development (Morkin, 2000).

One mechanism of transcriptional repression by T_3 is through a negative thyroid response element (nTRE) in the promoter of a gene (Yen, 2001). It has been demonstrated in some studies that binding of unliganded TRs to nTREs results in transactivation of transcription, and binding of T_3 to a TR on a nTRE represses transcription (Sanchez-Pacheco and Aranda, 2003), the opposite of what occurs at positive TREs (pTREs)(Yen, 2001). nTREs have been identified in several negatively regulated thyroid-response genes (Brent et al., 1991; Morkin, 2000; Sanchez-Pacheco and Aranda, 2003; Shen et al., 2004; Shen et al., 2005), including in goldfish (Sohn et al., 1999b), although in general, fish TREs have not been well characterized (Nelson and Habibi, 2009). At least two of the goldfish genes identified as decreasing in expression by the microarray analysis are known to have an nTRE in the promoter of their mammalian counter parts: GH (Sanchez-Pacheco and Aranda, 2003), and β-myosin heavy chain (Appendix B)(Morkin, 2000) although the significance of myosin heavy chain expression in brain is unknown. This suggests that at least one of the mechanisms of T_3 action to decrease gene expression in this study was a direct action through nTREs of responsive genes; however, recently a few different mechanisms of transcriptional repression by T_3 have been identified, including T_3 effects on co-activators

(Zhang et al., 2002) and transcription factors (Matsushita et al., 2007). This illustrates that indirect T_3 effects, i.e. not through a TRE, can also alter gene expression, and were likely in effect to bring about the large-scale decrease in gene expression observed in this study.

Two such indirect mechanisms could have been mediated by changes in expression of two of the groups of differentially expressed genes identified in this study: genes involved in the ubiquitin-proteasome system (UPS) and genes with mRNA metabolism functions involved in the spliceosome. The UPS targets specific proteins for degradation through enzymatic tagging with ubiquitin, and degrades the ubiquinated proteins through protease activity of the proteasome (reviewed by Korhonen and Lindholm, 2004). Enrichment analysis identified several proteasome subunits that decreased in expression in telencephalon of T_3-treated goldfish, and other components of the UPS were also identified as decreased in expression in response to T_3 treatment. There is an accumulation of evidence that the UPS is an integral part of the mechanism of nuclear hormone receptor signalling, and that proteasomal protein turnover is required for receptor-mediated transcription, including that of TRs, likely through degradation of co-repressors, co-activators, and receptors themselves and that this is stimulated by binding of hormone to its receptor (reviewed by Nawaz and O'Malley, 2004). Therefore, another mechanism of T_3 to cause large-scale decreases in gene expression in goldfish telencephalon could have been through the observed decreased expression of UPS components, resulting in decreased proteasomal protein turnover and gene expression, assuming positive regulation of target genes by T_3. In support of this hypothesis, T_3 has been shown to modulate TR function by controlling its protein level through rapid UPS degradation in growth-hormone producing GC cells *in vitro*, and this T_3 action was necessary for the T_3-mediated transcriptional activation of GH which is observed under control conditions in this cell-line (Dace et al., 2000). Although speculative, decreased

UPS activity is an alternate potential mechanism through which GH expression decreased by 1.8-fold in response to T_3 treatment in the current study (yet to be verified by real-time RT-PCR), and could therefore also be a mechanism through which the decrease in expression of other genes on the array occurred.

The spliceosome is composed of snRNP complexes, and is responsible for production of mature mRNA from the primary transcript, through excision of introns and ligation of exons (Patel and Bellini, 2008). The reduced expression of the splicing machinery responsible for the production of mature mRNA observed from the microarray analysis could therefore be another mechanism through which T_3 resulted in overall decreases in transcript levels, which would then actually represent decreased production of mature mRNA, *i.e.* decreased post-transcriptional modification, rather than decreased transcription. The real-time RT-PCR validation of SNRPB/B′ did not confirm a decrease in expression in telencephalon, and indicated a significant increase in expression in hypothalamus after two-day treatment with 0.1 μM T_3; however, SNRPB/B′ is alternatively spliced to produce the Sm spliceosomal core proteins SmB or SmB′ and the primers used for real-time RT-PCR validation may have not been specific to only the sequence on the microarray. TH has previously been identified as controlling genes that are splicing regulators in mammalian brain (Bernal, 2002; Cuadrado et al., 1999; Gerrelli et al., 1994) and has been shown to increase protein levels of the core protein SmN (Gerrelli et al., 1994), encoded by SNRPN which is very similar in sequence to SNRPB/B′ and replaces it in mammalian brain (Gray et al., 1999). Together with the results shown here that T_3 has effects on expression of many spliceosomal genes, this suggests that regulation of RNA splicing may be a conserved TH action in vertebrate brain.

The identification by enrichment analysis of groups of genes central to spliceosome and proteasome function as thyroid-responsive underscores the finding in this study that adult male goldfish telencephalon was metabolically responsive to T_3 treatment. This is an important contribution to the growing recognition that TH is an important modulator of adult brain function (Bauer et al., 2008), and has identified key pathways that are T_3 targets. It is worth noting that several groups of metabolically important genes that are considered to be house-keeping genes (e.g. ribosomal proteins and beta actin) were all found to be highly thyroid responsive. This indicates that use of traditional house-keeping genes as internal control or reference genes for gene expression analysis is not advisable for studies on thyroid hormone effects.

In addition to the role of the UPS-mediated protein turnover in the mechanism of nuclear hormone receptor signalling, the UPS is also responsible for degradation of proteins involved in essential cellular processes including regulation of the cell cycle, cell survival, cell proliferation and apoptosis (Adams, 2003). The cyclin-selective ubiquitin carrier protein E2-C had a notably greater fold decrease than any other gene on the array (4.5- vs. 1.5 to 3-fold). E2-C is believed to mediate cyclin-B ubiquination and subsequent breakdown, a process necessary for transition from mitosis to interphase of the cell cycle in mammals as well as goldfish (Tokumoto et al., 1999). Such a large decrease in expression of E2-C in response to T_3 treatment suggests an inhibition of cell division by T_3 at this high dose in adult male goldfish telencephalon. Another key function of UPS protein turnover is as a quality control mechanism, in that it facilitates degradation of damaged or misfolded proteins (Korhonen and Lindholm, 2004). Dysfunction of the UPS has been implicated in neurodegenerative disorders, including Huntington's disease and Parkinson's disease

(Korhonen and Lindholm, 2004). This emphasizes the potential significance of the T_3 action to depress UPS function in adult brain as reported here.

In addition to the proteasome subunits, enrichment analysis identified several other genes encoding proteins with protease inhibitor activity, including some proteases. The interplay between proteases and protease inhibitors is critical for proper protease function in all tissues including the nervous system (Hengst et al., 2001). For example, interaction of serine proteases and their inhibitors modulates neurite outgrowth and astrocyte stellation *in vitro* (Hengst et al., 2001). PEBP, which decreased in expression in response to T_3 in this study, has been identified as a serine protease inhibitor in brain of mice (Hengst et al., 2001). Another gene in this enrichment group is cathepsin L, which in mice has major roles as an endopeptidase in production of NPY in secretory vesicles in neuroendocrine brain (Funkelstein et al., 2008a) and of the proopiomelanocortin (POMC)-derived pituitary hormones adrenocorticotropic hormone, beta-endorphin, and α-melanocyte stimulating hormone in secretory vesicles of pituitary (Funkelstein et al., 2008b). Thus, T_3 effects on telencephalon gene expression may decrease protease and protease inhibitor activities that are critical to brain-specific processes, including neuroendocrine signalling. Supporting this hypothesis are studies showing negative regulation by T_3 of prohormone convertases PC1 and PC2, which have endopeptidase activity to activate neuropeptides and pro-hormones in brain and pituitary, in rat hypothalamus and cerebral cortex (Shen et al., 2004; Shen et al., 2005).

Granulin expression was clearly and consistently decreased by T_3 treatment as observed through real-time RT-PCR confirmation of the microarray result. The significant 4.2-fold decrease after two days of T_3 treatment at the higher dose of 0.1 μM T_3 in

telencephalon, in contrast to the non-significant 1.8 fold decrease after six days of T_3 treatment suggests that a homeostatic mechanism was in place to return granulin expression levels closer to control levels by day six of treatment. Although granulin has been demonstrated to have protease inhibitor activity in invertebrates (Hong and Kang, 1999) and cysteine protease activity in plants (Chen et al., 2006), to my knowledge, these activities have not been demonstrated in vertebrates. In vertebrates, granulins (or epithelins) were first identified as a family of growth factors with similarities to the epidermal growth factor/transforming growth factor alpha family of regulatory proteins (Bateman and Bennett, 1998). Goldfish granulin was identified and cloned from macrophages, and was expressed mainly in kidney and spleen with little expression in the brain (Hanington et al., 2006). Granulin expression was induced by GH treatment in tilapia liver suggesting a role in GH-regulated growth (Chen et al., 2007); however, to my knowledge, granulin has not previously been identified as thyroid-responsive in any species.

In rats, granulin has critical roles related to estrogen action in developing and adult brain. Granulin expression was induced by both androgen and estrogen administration in developing hypothalamus, and was critical in male hypothalamus during the period of sexual differentiation of brain for normal sexual behaviour as adults (Suzuki and Nishiahara, 2002). In adult hippocampus, estrogen induced expression of granulin *in vivo*, and furthermore increased neural progenitor cell proliferation *in vitro*, an effect which was dependent on the presence of granulin protein (Chiba et al., 2007). Granulin mutations have been implicated in a human neurodegenerative disease, ubiquitin-positive frontotemporal lobar degeneration (FTLD), and it has been suggested that granulin is also involved in related neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS) (Gijselinck et al., 2008). Granulin levels were reduced in cerebrospinal fluid

of FTLD patients with the granulin mutation, and granulin was seen to enhance neuronal survival and neurite outgrowth of motor and cortical rat neurons *in vitro*, indicating that granulin is a neurotrophic factor with possible roles in neural development and neurodegeneration (Van Damme et al., 2008). The functions of granulin in mammalian brain and the modulation of granulin function by hormones underscore the potential importance of T_3 action to decrease granulin expression by 4.2-fold in male goldfish telencephalon. Moreover, clinical studies and experimental studies indicate some association between TH and neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and ALS (Chinnakkaruppan et al., 2009; Tan and Vasan, 2009) suggesting that T_3 effects on granulin expression could be involved in these diseases.

Of the six genes chosen for real-time RT-PCR confirmation from the enrichment groups, direction and magnitude of expression change was only confirmed for granulin; however, statistically significant effects of T_3 exposure outside of the six-day, 0.1 μM T_3 telencephalon samples were found for each gene. This discordance between the microarray results and the real-time RT-PCR results for an individual gene from an enrichment group does not invalidate the statistical significance of the enrichment analysis, which identifies that an entire pathway is affected; however it does suggest that some of these data should be interpreted with caution. Given tetraploidy of goldfish, there are likely multiple paralogs of these genes, and the primers used for real-time RT-PCR validation may have not been specific to the sequences on the microarray. Experiments to further explore the thyroid-responsiveness of these genes are warranted.

In addition to genes identified by enrichment analyses being implicated in neurogenesis, neurodegeneration, synaptic function and hormone signalling as discussed above, several other genes on the microarray that were affected by T_3 treatment were

identified to have similar functions. This lends support to the hypothesis that T_3 may have regulatory roles in these processes in adult fish brain. Mammalian fatty acid binding protein 7 (FABP7) has roles in neuronal and glial cell differentiation, including axonal guidance, and may have roles in Notch signalling (reviewed by Storch and Corsico, 2008) and both FABP7 and a Delta/Notch-like EGF-related receptor in the current study decreased in expression in response to T_3 treatment. MBP is a known thyroid-responsive gene with a positive TRE identified in mammals, and is critical for neonatal brain development through axon myelination (Bernal, 2002). Reticulons, one isoform of which went down in expression and one which went up in expression in this study, have been shown to be neurite growth inhibitors in mammals, and likely prevent axonal regeneration in adult mammals, and in fish have structural differences that may be implicated in the axonal regeneration capability of fish axons (Diekmann et al., 2005). Taken together, the effect of T_3 on expression on these genes or related genes in the current study suggests that T_3 has the capability to regulate processes related to neurogenesis, regeneration or axonal degeneration, which is intriguing, given the critical roles of TH in neurogenesis in developing brain. This may be especially important given the relationships shown between thyroid dysfunction and neurodegenerative disorders like Alzheimer's disease (Tan and Vasan, 2009) and mood disorders through effects on hippocampal neurogenesis (Montero-Pedrazuela et al., 2006).

One of the main goals of this microarray analysis was to determine whether THs regulate expression of neuroendocrine genes implicated in the control of reproduction, and in particular whether GABA-system genes other than the GADs are affected by T_3. Microarray analysis identified several differentially expressed genes that have roles in GABA function, including one GABA receptor subunit, a taurine transporter which can transport GABA, pyridoxal kinase, which synthesizes the cofactor necessary for GAD function, and

parvalbumin. Parvalbumin, GAD activity, GABA-receptor subunits and other indicators of GABA-function have been previously shown to be affected by thyroid hormones (reviewed by Wiens and Trudeau, 2006). The neuropeptides PACAP and NPY are important regulators of gonadotropin subunit expression in fish, although NPY does not affect FSHβ (Yaron et al., 2003).

Genes encoding other proteins involved in neuroendocrine signalling were also identified as thyroid-responsive in this study, including calcium signalling molecules and several Rabs. Rab proteins are small GTPases with roles in endocytosis, cell-surface molecule recycling, and regulation of intracellular vesicle transport (Baskys et al., 2007; Hirota et al., 2007). Rab5b specifically has been shown to have a role in metabotropic group I glutamate receptor (mGLUR)-dependent synaptic plasticity and neuroprotection (Baskys et al., 2007). Rabs were also identified as thyroid responsive in the microarray study on T_3 action in adult striatum, where 90 % of cells are GABA-ergic neurons (Diez et al., 2008), indicating that T_3 may have a conserved action in vertebrates to regulate vesicle function in brain, including GABA neurons.

Several genes encoding hormones or hormone receptors were also identified as thyroid responsive in the current study. Isotocin is a reproductive neuropeptide that has been shown to be regulated by multiple neurotransmitter pathways in goldfish neuroendocrine brain, including GABA and dopamine (Popesku et al., 2008). Although GnRH receptors and GH were originally characterized for their presence and significance in pitutitary, both have been identified in brain (Harvey et al., 2003; Peter et al., 2003). Neural GH has been found in rats, mice and many bird species, likely having autocrine or paracrine effects there, and moreover, it plays key roles in neurogenesis (Harvey et al., 2003). GnRH receptors are

distributed throughout goldfish brain, including telencephalon although their function there is not known (Peter et al., 2003).

In conclusion, I have shown that adult male goldfish neuroendocrine brain is highly responsive to TH, with a supraphysiological dose of T_3 resulting in a large-scale decrease in gene expression indicating that T_3 affects metabolic activity of the adult brain. Genes encoding proteins central to mRNA splicing and proteasomal protein turnover were highly thyroid-responsive, and both groups of genes could be involved in the T_3 mechanism to decrease transcript levels on such a large-scale. T_3 had effects on a wide diversity of genes implicated in neuroendocrine signalling and reproduction in telencephalon of male goldfish, including some genes related to GABA function. Many of the differentially regulated genes are implicated in neurogenesis, brain development, and neurodegenerative disorders, indicating that TH is critical to adult brain function.

CHAPTER 5
General Discussion

5.1 Thesis summary

This thesis explores TH effects on adult neuroendocrine brain and pituitary in goldfish to address the hypothesis that THs regulate the reproductive neuroendocrine axis through effects on GABA. As my first objective, I was interested in determining whether THs have effects on GABA function, given known effects of GABA on LH release in goldfish, and evidence from mammalian models that THs have effects on multiple aspects of GABA function. In sexually regressed males, I determined that T_3 exposure decreased gene expression of the GABA synthetic and degradative enzymes, GAD and GABA-T, in telencephalon but not hypothalamus with no effect on basal LH levels. Furthermore, I found that T_3 can modulate GABA-T activity in both neuroendocrine and non-neuroendocrine brain regions, although this effect was only seen with *in vitro* treatment of brain homogenates with T_3, and not in response to the *in vivo* exposures. T_3 had no effect on TR expression in neuroendocrine brain but decreased D2 expression and increased D3 expression in a tissue-specific manner indicative of a negative feedback effect to decrease local levels of T_3. Together, these results indicated that T_3 has the potential to affect GABA function in the adult neuroendocrine brain of teleosts.

My second main objective was to determine whether THs regulate gene expression and release of gonadotropins, LH and FSH, in the pituitary and whether GABA is involved in these effects. In an exposure of sexually mature males and females, T_3 treatment resulted in increased expression of the gonadotropin subunit FSHβ in females only, indicating that THs may have roles in regulating female reproduction. LHβ expression and both basal and

GABA-mediated LH release were not found to be thyroid responsive in either sex. A T_3 challenge resulted in decreased expression in pituitary of TSHβ, GPHα, and D2 and increased expression of TRα-1, TRβ, and D3. These results indicate negative feedback by T_3 in pituitary to regulate local T_3 levels through deiodination, and systemic T_3 levels through TSH signalling. I hypothesize that TR autoregulation in pituitary, demonstrated in goldfish for the first time here, is involved in the transcriptional repression by T_3 of TSHβ, GPHα and D2 and the transcriptional stimulation by T_3 of D3.

My third main objective was to establish a gene expression profile for T_3 action in adult goldfish neuroendocrine brain. cDNA microarray analysis indicated a large-scale decrease in gene expression in male telencephalon in response to T_3 including genes central to cellular metabolism, indicating that the adult brain was TH-responsive. Groups of genes encoding proteins with functions in mRNA splicing and proteasomal protein turnover were identified as TH-responsive, and are hypothesized to be involved in facilitating the large-scale decrease in gene expression observed. T_3 had effects on genes implicated in neuroendocrine signalling and reproduction, neurogenesis, brain development, and neurodegenerative disorders. What is not known is what proportion of genes found to be regulated by T_3 are directly responsive, that is to say whether consensus functional thyroid response elements are located in promoter regions.

5.2 TH effects on GABA function

This thesis provides the first evidence that THs regulate GABA function in a non-mammalian vertebrate. The most convincing evidence comes from the real-time RT-PCR studies on GAD and GABA-T expression, which decreased in response to T_3 treatment in a tissue-, dose- and time-specific manner. Preliminary results also indicate inhibiton of GABA-

T activity by *in vitro* T_3 exposure, especially in non-neuroendocrine brain regions. The microarray analysis indicated that several other genes with supportive roles in GABA function also decreased in response to T_3 treatment. Several of the key genes in GABA function were included on the array, such as GABA receptor subunits, GABA transporters and GAD and GABA-T themselves. Very few of these were identified as differentially expressed on the array; however, given that the real-time RT-PCR analysis carried out in Chapter 2 indicated decreased expression of GAD65, GAD67 and GABA-T in telencephalon after two days of T_3 exposure but no effects after six days of exposure, it is very possible that the choice of the six-day time point and 0.1 μM T_3 dose chosen for the microarray analysis missed some of the potential early effects of T_3 on key GABA system genes. Finally, although I found no effects of T_3 on GABA-mediated LH release in regressing and regressed goldfish, it is possible that the response would be different in either sexually recrudescent or mature fish. Identifying T_3 as a regulator of the GABA system is an important scientific contribution since GABA is a major neurotransmitter in the vertebrate central nervous system acting through both ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors to regulate neurotransmission in numerous systems.

5.3 TH effects on reproduction

In this thesis, I show that T_3 exposure results in increased FSHβ expression in pituitary and both decreased and increased expression of genes with functions in neuroendocrine signalling, indicating that T_3 has roles in regulating the reproductive neuroendocrine axis of the goldfish. Although previous studies investigated T_4 effects on gonadotropin subunit expression in male goldfish pituitary and found no effects, females had not been studied, nor had T_3 exposures been conducted (Yoshiura et al., 1999). Therefore,

my finding that T_3 results in increased FSHβ expression in females but not males is a significant contribution to understanding sex specific mechanisms of regulation of gonadotropin expression. The mechanism of action warrants further study, including T_3 effects on known regulators of FSHβ expression such as PACAP, activin and follistatin. Given the T_3 effects observed on GABA function in telencephalon in this thesis, and $GABA_B$ receptor-mediated effects on activin expression observed in telencephalon in a previous study in the Trudeau lab (Martyniuk et al., 2007b), I hypothesize that GABA is involved in the T_3 effect to increase FSHβ expression in female goldfish. An experiment in which gene expression in neuroendocrine brain regions and pituitary are measured simultaneously in response to a T_3 challenge, and in response to GABA receptor agonists after a T_3 challenge, is an important next step in determining connections between T_3 effects on GABA function in brain and on gonadotropin expression in pituitary.

The absence of effects of T_3 exposure on basal LH levels and GABA-mediated LH-release in males and females in different reproductive states, and on LHβ expression in pituitary in both males and females, indicates that LH release and expression are likely not important TH targets. A previous study on T_4 effects on male goldfish LHβ expression and circulating LH support this conclusion (Yoshiura et al., 1999).

Finally, the effect of T_3 to decrease TSHβ and GPHα expression in pituitary is another hypothetical mechanism of regulation of reproduction, given studies showing expression of TSH receptors (Kumar and Trant, 2001) and TSHβ and GPHα in fish gonads (von Schalburg et al., 2005; Wang et al., 2004). Thus, T_3 regulation of a thyroid system gene may directly affect reproduction at the level of the gonads. This possibility warrants further study.

5.4 Regulation of the thyroid-axis

In addition to determining T_3 effects on GABA function and reproduction, I have provided detailed description of T_3 regulation of TR and deiodinase expression in hypothalamus, telencephalon and pituitary in male and female goldfish. I have shown tissue-specific effects of T_3 on these key TH system genes, with some sex differences. This could be due to different rates of entry of T_3 or differences in sensitivity to T_3 between tissues and suggests important differences in roles of T_3 between telencephalon, hypothalamus and pituitary.

My results indicate that peripheral and central control mechanisms are in play simultaneously to regulate thyroidal status in goldfish, which is an important contribution to the continued debate on regulation of thyroid hormone function in teleost fish (MacKenzie et al., 2009). My observations for brain regions and pituitary that a T_3 challenge resulted in decreased D2 expression or increased D3 expression or both simultaneously supports the peripheral model of regulation of thyroidal status for teleosts by Eales and Brown (Eales and Brown, 1993). In that model, tissue levels of T_3 are regulated at the level of the target tissues through effects on deiodination activity, rather than through negative feedback on the central thyroid axis. My observation that a T_3 challenge also resulted in decreased expression of TSHβ and GPHα in pituitary indicates that T_3 does exert negative feedback through the central axis.

The observation in Chapter 3 that the $GABA_A$ receptor agonist muscimol decreased circulating T_3 levels suggests that GABA may be a regulator of thyroidal status in goldfish. There is evidence for this in mammals, where GABA has been shown to inhibit release of TRH from hypothalamus, TSH from pituitary, and TH from thyroid gland (Wiens and Trudeau, 2006; Fig. 1.2). It has been proposed for nearly four decades that there is a TSH-

inhibitory factor in goldfish that is a central inhibitor of TSH release, although none has been identified (MacKenzie et al., 2009). Therefore it is an intriguing hypothesis, given the observation in Chapter 3, that GABA could be this TSH-inhbitory factor.

Finally, to gain a full understanding of T_3 effects on neuroendocrine brain and pituitary, it is necessary to also study the roles of all four TR subtypes in goldfish, including the two not studied in this thesis, TRα-2 and TRα-t. Both have been identified in goldfish brain and pituitary and are differentially expressed between different reproductive states (Nelson and Habibi, 2006). TRα-t, which likely does not bind T_3, has been shown to play novel roles in regulating D3 expression in liver of goldfish. This underscores the fact that much remains to be discovered in how T_3 and TRs function, especially in teleosts.

5.5 Significance of TH effects on adult brain of fish

I have demonstrated that gene expression and enzyme activity in adult brain of goldfish are responsive to T_3, with implications for novel mechanisms of control of gene expression, metabolic function, and neuroendocrine signalling with downstream effects on reproduction. This complements the growing interest in TH functions and mechanisms of action in adult mammalian brain that are implicated in the neurological foundations of symptoms associated with thyroid disorders.

5.6 Concluding remarks

In conclusion, I have shown that the adult brain of a teleost fish is responsive to TH, that THs have a generally inhibitory effect on GABA function in neuroendocrine brain, and that THs have effects on gene expression in pituitary and neuroendocrine brain that impact the reproductive neuroendocrine axis. These contributions will help elucidate mechanisms of neuroendocrine control of reproduction and thyroid function in teleosts, identify potential

sites of endocrine disruption by thyroid disrupting chemicals in the environment, and identify evolutionarily conserved pathways through which dysthyroid states result in neurological side effects.

Appendix A

The following tables summarize the pilot exposures carried out to determine an exposure protocol to raise and lower circulating T_3 levels in goldfish. Plasma or serum T_3 levels were determined in ng/ml by enzyme immunoassay (EIA), and are presented as mean ± standard deviation. For T_3 immersion, T_3 was delivered in 1.4 ml NaOH vehicle for the final, nominal water T_3 levels indicated. For all studies, the first day of exposure was called day zero.

Pilot 1: Effects of six intraperitoneal injections of the thyroid inhibitor 6-n-propyl-2-thiouracil (PTU) dissolved in DMSO at dose indicated (μg /g body weight) or DMSO alone (control) every second day over 10 days, and fish euthanized on day 12, November 13 - 25, 2002, on plasma T_3 levels of sexually regressed male goldfish. T-test indicated no significant decrease, $p = 0.16$.

Treatment	n	[T_3] (ng/ml)
Control	15	1.66 ± 0.51
PTU (20μg/g)	14	1.43 ± 0.37

Pilot 2: Effects of three intraperitoneal injections of PTU or tri-iodothyronine (T_3) in DMSO at doses indicated (μg /g body weight) or DMSO alone (control) every second day over four days and fish euthanized on day six, May 20 - 27, 2003, on serum T_3 levels of female and male goldfish.

	females		males	
Treatment	n	[T_3] (ng/ml)	n	[T_3] (ng/ml)
Control	9	2.44 ± 0.60	3	2.35 ± 0.69
PTU (20μg/g)	13	2.52 ± 0.61	3	2.56 ± 1.46
T_3 (4μg/g)	15	978.26 ± 359.99		N/A

Pilot 3: November 2004 Injection Protocol Test. Males and females pooled. n = 5 unless otherwise indicated. Injected every day for six or ten days at doses indicated (μg /g body weight), euthanized on day six and ten, 24 h after last injection.

	Plasma [T_3] (ng/ml)	
Treatment	six days	ten days
Saline Control	2.57 ± 0.23 (n=3)	4.04 ± 0.67
Vehicle Control (1.7:1 DMSO:saline)	2.78 ± 0.89	3.53 ± 0.48 (n=4)
PTU High (20μg/g)	2.45 ± 0.86	2.54 ± 0.67 (n=4)
PTU Low (2μg/g)	2.29 ± 0.59 (n=4)	2.73 ± 0.65 (n=4)
T_3 (0.2 μg/g)	35.74 ± 23.67	28.12 ± 5.37 (n=4)

Pilot 4: Thiourea (TU) and T_3 immersion experiment with mix of males and females (sex not determined) at nominal doses indicated for six days, January 29 - February 4, 2005. n = 6. Water was replaced fully every second day with the last transfer 48 h before euthanasia and dissection on day six.

Treatment	Plasma [T_3] (ng/ml)
Control (1.4ml 0.01N NaOH in tank)	3.80 ± 0.70
TU (0.06%)	4.18 ± 1.64
T_3 (0.1 μM + 1.4 ml 0.01N NaOH)*	620.36 ± 299.23

Notes: The 0.06% TU dose used in Pilots 4-5 was based on the protocol of Chaube and Joy (2003) for the catfish *Heteropneustes fossilis*. The 0.1 μM T_3 dose used in Pilots 4-7 was based on the protocol of García-G et al. (2004) for killifish (*Fundulus heteroclitus*).
*0.1 μM (67.3 ng/ml) was the theoretical water concentration. One water sample was analyzed by EIA, indicating water [T_3] of ~10ng/ml in the T_3-treatment tanks. Based on this level, T_3 was concentrated in goldfish blood from water by a factor of ~60.

Pilot 5: Three-week thiourea (TU) and T_3 immersion experiment with mix of males and females at nominal doses indicated. n = 5. Water was replaced fully every day for one, two or three weeks, February 9 - March 2, 2005

	Plasma [T_3] (ng/ml)		
Treatment	1 week	2 weeks	3 weeks**
Control (1.4ml 0.01N NaOH in tank)	3.55±0.56	3.40±1.28	2.89±0.99
TU (0.06%)	4.36±1.24	2.69±0.29	2.63±0.65
T_3 (0.1 μM + 1.4 ml 0.01N NaOH)*	388.42±198.20	313.30±93.56	Not msd.

Notes: *0.1 μM (67.3 ng/ml) was the theoretical water concentration in T_3-treatment tanks. One water sample was analyzed by EIA, indicating water [T_3] of ~16ng/ml in the T_3-treatment tanks. Based on this level, T_3 was concentrated in goldfish blood from water by a factor of ~24 after one week, and by ~38 after two weeks.
**three-week plasma and water samples were not analyzed for T_3-treated fish.

Pilot 6: Two- and six-day T_3 immersion experiment with mix of males and females at nominal doses indicated, May 10 -16, 2005. n = 6 except for two-day, 0.0025 μM and 0.005 μM, n = 5. Water replaced fully every day, with the last transfer 24 h before euthanasia and dissection on days two and six.

	Plasma [T_3] (ng/ml)	
Treatment	two days	six days
Control (1.4ml 0.01N NaOH in tank)	2.78 ± 0.77	3.05 ± 1.48
0.0025 μM T_3 (+ 1.4 ml 0.01N NaOH)	4.54 ± 0.98	5.80 ± 3.45
0.005 μM T_3 (+ 1.4 ml 0.01N NaOH)	4.42 ± 1.08	6.98 ± 5.81

Pilot 7: Two- and six-day T_3 immersion experiment with mix of males and females at nominal doses indicated, July 3 - 9, 2005. n = 5 except for two-day, 0.01 μM, n = 4. Water replaced fully every second day, with the last transfer 48 h before euthanasia and dissection on days two and six.

	Plasma [T_3] (ng/ml)	
Treatment	two days	six days
Control (1.4ml 0.05N NaOH in tank)	2.16 ± 0.45	3.17 ± 0.88
0.01 μM (+ 1.4 ml 0.05N NaOH)	60.39 ± 32.57	6.57 ± 4.21
0.02 μM T_3 (+ 1.4 ml 0.05N NaOH)	36.77 ± 48.11	10.75 ± 7.80
0.05 μM T_3 (+ 1.4 ml 0.05N NaOH)	158.70 ± 207.62	131.26 ± 88.57
0.1 μM T_3 (+ 1.4 ml 0.05N NaOH)	103.61 ± 144.86	280.56 ± 241.68

Appendix B

List of the 482 putative genes out of 621 cDNA sequences that decreased in expression in telencephalon of male goldfish in response to six-day 0.1 μM T_3 waterborne exposure.

Gene Name	Fold-Change	E-value
40S ribosomal protein S11	1.5	1e-130
60S ribosomal protein L8	2.2	1e-079
acidic ribosomal protein P1	1.5	1e-041
adaptor-related protein complex 2, beta 1 subunit	1.6	1e-010
alpha & ccdb lethal fusion protein	1.5	1e-127
alpha 4 subunit of 20S proteasome	1.5	1e-170
alpha globin	2.2	1e-170
alpha globin	1.8	1e-148
annexin A1a	1.5	1e-032
annexin A1c	1.6	1e-119
anonymous nuclear locus 039 marker sequence	1.8	1e-007
apolipoprotein A-I	2.2	1e-051
apolipoprotein A-IV	1.5	1e-11
arginine methyltransferase 1	1.9	1e-029
arginine-glutamic acid dipeptide repeats	1.6	1e-170
aspartic acid-rich protein aspolin1	1.8	1e-112
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1	1.5	1e-170
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit G	1.9	1e-148
ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	1.7	1e-157
basic transcription factor 3	1.7	1e-170
beta actin	1.5	1e-117
betacellulin, epidermal growth factor family member	2.4	1e-076
beta-globin	2.3	1e-031
cadherin 1, epithelial	1.6	1e-031
calmodulin 1b	2.2	1e-104
carbonic anhydrase	1.5	1e-069
carboxypeptidase, vitellogenic-like	2.0	1e-177
catenin, beta like 1	1.5	1e-150
cathepsin L preproprotein	1.5	1e-170
CCAAT/enhancer binding protein, beta	1.9	1e-082
cell division cycle 42	1.5	1e-170
class I metallothionein (MT)	1.8	1e-170
Claudin 10 like	1.6	1e-097
clone carpf-C114	1.7	1e-006
clone DKEY-175A17 in linkage group 15	1.9	1e-012
clone IMAGE:7052580	1.7	1e-113
clone IMAGE:7151632	1.8	1e-086
clone IMAGE:7421217	1.6	1e-011
clone IMAGE:7426884	1.9	1e-032
clone IMAGE:8777863	1.7	1e-132
clone MGC:158292	1.8	1e-082
clone MGC:162304 IMAGE:8775377	1.9	1e-111

clone MGC:162613	2.1	1e-032
clone:OVRM10039C08	2.0	1e-029
connexin 43	1.7	1e-156
creatine	1.5	1e-170
creatine kinase M2-CK	2.0	1e-170
creatine kinase, brain	1.7	1e-170
C-type lectin	2.0	1e-094
cyclin-selective ubiquitin carrier protein E2-C	4.5	1e-167
cystatin	1.6	1e-108
cystatin	1.8	1e-052
DEAH (Asp-Glu-Ala-His) box polypeptide 16	2.2	1e-091
death associated protein 1a	1.7	1e-005
DJ-1 protein	1.5	1e-170
dual specificity phosphatase 5	2.0	1e-170
E12 transcription factor	1.7	1e-078
zona pellucida glycoprotein 3	1.9	1e-170
egl nine homolog 3 (C. elegans)	1.9	1e-022
electron-transfer-flavoprotein, beta polypeptide	1.6	1e-170
endothelial differentiation-related factor 1	1.8	1e-126
endozepine	1.8	1e-091
ependymin ii	1.5	1e-176
erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked)	2.0	1e-054
eukaryotic translation initiation factor 4E binding protein 3	1.8	1e-105
farnesyltransferase, CAAX box, beta	2.0	1e-028
fast skeletal myosin light chain 1a	1.5	1e-170
fast skeletal myosin light chain 3	1.6	1e-070
fast skeletal myosin light chain 3	1.5	1e-170
fatty acid binding protein 2, intestinal	2.0	1e-015
fatty acid binding protein 2, intestinal	1.9	1e-136
fatty acid binding protein 7, brain, b	2.5	1e-023
fatty acid desaturase 2	1.5	1e-9
F-box protein 32	1.6	1e-149
FK506 binding protein 3	1.5	1e-037
from clone BUSM1-219H16 in linkage group 7	1.8	1e-009
from clone carpf-118	1.7	1e-013
from clone carpf-C114	1.5	1e-007
from clone CH1073-48M5 in linkage group 22	1.7	1e-108
from clone CH211-10K1 in linkage group 14	1.8	1e-062
from clone CH211-128E9 in linkage group 15	1.7	1e-007
from clone CH211-147L19 in linkage group 21	2.2	1e-014
from clone CH211-149B20 in linkage group 9	1.6	1e-009
from clone CH211-14L9 in linkage group 16	1.6	1e-021
from clone CH211-150G2	2.0	1e-028
from clone CH211-151O1	1.9	1e-063
from clone CH211-152F23 in linkage group 3	1.5	1e-073
from clone CH211-158M24 in linkage group 5	2.0	1e-023
from clone CH211-161H23 in linkage group 1	2.0	1e-087
from clone CH211-173L10 in linkage group 18	1.6	1e-020
from clone CH211-173P18 in linkage group 19	1.7	1e-058
from clone CH211-178H11	1.9	1e-058

from clone CH211-190L11 in linkage group 12	1.6	1e-026
from clone CH211-194C3 in linkage group 13	1.9	1e-115
from clone CH211-198A16 in linkage group 20	1.8	1e-046
from clone CH211-202D4 in linkage group 22	1.6	1e-085
from clone CH211-204J16 in linkage group 19	1.7	1e-063
from clone CH211-212J22 in linkage group 23	1.7	1e-087
from clone CH211-214G8 in linkage group 20	2.1	1e-005
from clone CH211-214J24 in linkage group 4	1.6	1e-025
from clone CH211-218K21 in linkage group 16	2.0	1e-019
from clone CH211-220F13 in linkage group 2	1.7	1e-011
from clone CH211-222M18 in linkage group 18	1.5	1e-006
from clone CH211-225P5 in linkage group 4	2.1	1e-008
from clone CH211-236P22 in linkage group 18	1.8	1e-031
from clone CH211-239H19 in linkage group 4	1.5	1e-008
from clone CH211-239N21 in linkage group 3	1.8	1e-065
from clone CH211-262M14 in linkage group 15	1.9	1e-006
from clone CH211-266M11 in linkage group 17	1.5	1e-010
from clone CH211-276A17 in linkage group 6	2.2	1e-053
from clone CH211-62B4 in linkage group 5	2.5	1e-050
from clone CH211-67C24 in linkage group 23	1.8	1e-015
from clone CH211-87N9 in linkage group 5	1.8	1e-047
from clone CH211-91P22 in linkage group 18	1.9	1e-009
from clone CH73-36G21 in linkage group 17	1.9	1e-017
from clone CH73-44A21 in linkage group 7	1.5	1e-048
from clone DKEY-103K21 in linkage group 12	1.6	1e-027
from clone DKEY-106G10 in linkage group 7	1.7	1e-010
from clone DKEY-108D22 in linkage group 4	1.5	1e-033
from clone DKEY-11K2 in linkage group 7	2.1	0.0001
from clone DKEY-11K4 in linkage group 18	1.5	1e-047
from clone DKEY-13A3 in linkage group 14	1.9	1e-022
from clone DKEY-146H10 in linkage group 19	2.1	1e-010
from clone DKEY-14A21 in linkage group 12	1.9	1e-028
from clone DKEY-14I8 in linkage group 7	1.8	1e-033
from clone DKEY-14M15 in linkage group 13	2.1	1e-104
from clone DKEY-14M15 in linkage group 13	2.0	1e-043
from clone DKEY-14M15 in linkage group 13	1.9	1e-049
from clone DKEY-14M15 in linkage group 13	1.7	1e-094
from clone DKEY-14M15 in linkage group 13	1.7	1e-051
from clone DKEY-14M15 in linkage group 13	1.5	1e-094
from clone DKEY-161L11 in linkage group 2	2.2	1e-076
from clone DKEY-18P14 in linkage group 5	1.9	1e-048
from clone DKEY-20F24 in linkage group 6	2.0	1e-040
from clone DKEY-237E17 in linkage group 17	1.5	1e-025
from clone DKEY-242H9 in linkage group 18	1.6	1e-011
from clone DKEY-245F17 in linkage group 17	1.9	1e-011
from clone DKEY-246K9 in linkage group 20	1.9	1e-016
from clone DKEY-25J19 in linkage group 7	2.0	1e-007
from clone DKEY-269E10 in linkage group 7	1.9	1e-012
from clone DKEY-270K22 in linkage group 2	1.6	1e-008
from clone DKEY-274N8 in linkage group 3	2.1	1e-021

from clone DKEY-284M18 in linkage group 20	1.7	1e-119
from clone DKEY-30E9 in linkage group 15	1.7	1e-120
from clone DKEY-31B16 in linkage group 23	2.0	1e-016
from clone DKEY-33L16 in linkage group 22	1.5	1e-013
from clone DKEY-34B22 in linkage group 5	1.7	1e-042
from clone DKEY-46i9 in linkage group	1.6	1e-68
from clone DKEY-4E7 in linkage group 9	1.9	1e-006
from clone DKEY-4G17 in linkage group 17	1.6	1e-126
from clone DKEY-66D18 in linkage group 4	1.7	1e-018
from clone DKEY-69P21 in linkage group 2	2.0	1e-022
from clone DKEY-6B12 in linkage group 21	2.0	1e-043
from clone DKEY-7B6 in linkage group 12	1.7	1e-007
from clone DKEY-81J5	1.7	1e-016
from clone DKEYP-101F3 in linkage group 6	2.0	1e-005
from clone DKEYP-33B5 in linkage group 17	1.8	0.0001
from clone DKEYP-35B8 in linkage group 2	2.2	1e-054
from clone MGC:158567	2.3	1e-116
from clone RP71-1P14 in linkage group 21	1.7	1e-026
$GABA_Ag2$ subunit	2.0	1e-170
gamma-glutamyl cyclotransferase	1.6	1e-51
gamma-glutamyl cyclotransferase	1.5	1e-53
gamma-glutamyl cyclotransferase	1.5	1e-47
gamma-glutamyl cyclotransferase	1.5	1e-32
GINS complex subunit 3	2.0	1e-058
glutamate oxaloacetate transaminase 2	1.9	1e-170
glutathione S-transferase alpha	2.0	1e-170
glyoxalase 1	1.5	1e-170
gonadotropin releasing hormone receptor type A	1.9	1e-92
granulin	1.8	1e-109
growth arrest and DNA damage 45 gamma	1.9	1e-134
growth hormone	1.8	1e-017
heat shock protein Hsp70	1.6	1e-170
HECT domain containing 1	1.5	1e-119
heterogeneous nuclear ribonucleoprotein A/B	1.6	1e-068
heterogeneous nuclear ribonucleoprotein U	1.7	1e-057
hypothetical protein LOC100002920	1.6	1e-030
hypothetical protein LOC324723	1.9	1e-122
hypothetical protein LOC325979	1.7	1e-074
hypothetical protein LOC327248	1.5	1e-024
hypothetical protein LOC334244	1.6	1e-170
hypothetical protein LOC335335	1.5	1e-110
hypothetical protein LOC336303	1.5	1e-149
hypothetical protein LOC393443	1.6	1e-042
hypothetical protein LOC393492	1.6	1e-011
hypothetical protein LOC393539	1.9	1e-058
hypothetical protein LOC393720	1.9	1e-019
hypothetical protein LOC393720	1.8	1e-170
hypothetical protein LOC393720	1.5	1e-070
hypothetical protein LOC402840	2.0	1e-074
hypothetical protein LOC406325	2.0	1e-136

hypothetical protein LOC415140	1.6	1e-076
hypothetical protein LOC415221	1.6	1e-093
hypothetical protein LOC415237	2.0	1e-051
hypothetical protein LOC422344	1.9	1e-048
hypothetical protein LOC422385	2.0	1e-090
hypothetical protein LOC431737	1.8	1e-146
hypothetical protein LOC436721	1.5	1e-022
hypothetical protein LOC436846	1.5	1e-067
hypothetical protein LOC436910	2.1	1e-088
hypothetical protein LOC436976	1.6	1e-110
hypothetical protein LOC445104	1.5	1e-170
hypothetical protein LOC445187	1.6	1e-091
hypothetical protein LOC445486	1.8	1e-065
hypothetical protein LOC445489	1.5	1e-087
hypothetical protein LOC450011	2.0	1e-145
hypothetical protein LOC492329	1.7	1e-039
hypothetical protein LOC492499	1.9	1e-039
hypothetical protein LOC503606	1.8	1e-105
hypothetical protein LOC541417	1.8	1e-159
hypothetical protein LOC550358	1.8	1e-048
hypothetical protein LOC553527	1.5	1e-041
hypothetical protein LOC553678	1.9	1e-048
hypothetical protein LOC553678	1.5	1e-112
hypothetical protein LOC553701	2.0	1e-068
hypothetical protein LOC555178	1.6	1e-154
hypothetical protein LOC560478	1.8	1e-091
hypothetical protein LOC561162	1.5	1e-59
hypothetical protein LOC564838	1.6	1e-170
hypothetical protein LOC566205	1.5	1e-110
hypothetical protein LOC567341	1.9	1e-101
hypothetical protein LOC571620	1.8	1e-060
hypothetical protein LOC606666	2.0	1e-065
hypothetical protein LOC641490	1.5	1e-087
hypothetical protein LOC678599	1.8	1e-061
hypothetical protein LOC723999	1.5	1e-103
hypothetical protein LOC751640	2.3	1e-067
hypothetical protein LOC751673	1.7	1e-106
hypothetical protein LOC797937	1.8	1e-033
immediate early response 3-interacting protein 1	1.5	1e-058
immunoglobulin light chain	1.6	1e-020
inhibitor of DNA binding 1	1.7	1e-160
insulin induced gene 1	1.5	1e-170
isotocin neurophysin	1.5	1e-024
isotocin neurophysin	1.7	1e-113
isotocin neurophysin	1.6	1e-39
isotocin neurophysin	1.6	1e-111
isotocin neurophysin	1.6	1e-132
KH domain containing RNA binding signal transduction associated 1 like	1.5	1e-124
kiaa0550 protein	1.6	1e-56
latexin (endogenous carboxypeptidase inhibitor)	1.5	3e-29

LSM7 homolog, U6 small nuclear RNA associated (S. cerevisiae)	1.8	1e-110
lysozyme g	1.8	1e-170
map-kinase activating death domain	1.5	1e-102
MARCKS-like 1	1.5	1e-027
matrix metalloproteinase 13	1.9	1e-028
matrix metalloproteinase 13	1.7	1e-076
MGC69008 protein	2.1	1e-092
MHC class I antigen	2.1	1e-074
microsatellite J42	1.5	1e-014
microtubule-actin crosslinking factor 1, isoform CRA_g	1.8	1e-170
mitochondrial trifunctional protein, beta subunit	1.6	1e-010
mitogen-activated protein kinase kinase 1	1.6	1e-077
muscle-specific beta 1 integrin binding protein 2	1.5	1e-098
muscle-specific beta 1 integrin binding protein 2	1.5	1e-015
myelin basic protein	1.6	1e-170
myelin basic protein, transcript variant 3	1.7	1e-28
myeloid cell leukemia sequence 1b	1.5	1e-046
myeloid cell leukemia sequence 1-like	1.8	1e-147
myosin heavy chain, partial cds	1.5	1e-070
myosin, light polypeptide 9, like	1.5	1e-019
N-acetyltransferase 5	1.7	1e-170
NAD(P)H dehydrogenase quinone 1	1.6	1e-011
NADH dehydrogenase (ubiquinone) Fe-S protein 1	1.7	1e-170
novel protein similar to human titin (TTN)	1.6	1e-098
nuclear factor, interleukin 3 regulated	1.6	1e-100
nucleobindin 2b	1.5	1e-164
nucleoside phosphorylase	1.8	1e-070
nucleosome assembly protein like 1	1.8	1e-57
odd-skipped related 1 (Drosophila)	1.5	1e-106
ornithine decarboxylase antizyme 1	1.5	1e-117
parvalbumin	2.7	1e-170
parvalbumin	2.0	1e-170
parvalbumin	1.9	1e-170
parvalbumin	1.8	1e-170
parvalbumin isoform 1d	1.6	1e-150
PDZK1 interacting protein 1, like	2.0	1e-021
peptidylprolyl isomerase E	1.5	1e-027
phenylalanine hydroxylase	1.6	1e-101
phosphatidylethanolamine binding protein	1.6	1e-170
pituitary adenylate cyclase activating polypeptide type 1 receptor precursor	1.5	1e-009
pleckstrin homology-like domain, family A, member 3	1.5	1e-040
PREDICTED: alpha-2,8-sialyltransferase ST8Sia I/V/VI-r2	1.6	1e-050
PREDICTED: heterogeneous nuclear ribonucleoprotein L, transcript variant 2	1.5	1e-068
PREDICTED: hypothetical LOC556254	2.0	1e-049
PREDICTED: hypothetical LOC557419	1.8	1e-063
PREDICTED: hypothetical LOC558108, transcript variant 1	1.9	1e-149
PREDICTED: hypothetical LOC564572	1.6	1e-013
PREDICTED: hypothetical LOC565869	2.0	1e-027
PREDICTED: hypothetical LOC566559, transcript variant 1	1.6	1e-011
PREDICTED: hypothetical LOC569538 (LOC569538)	1.6	1e-091

PREDICTED: hypothetical LOC569905	1.5	1e-080
PREDICTED: hypothetical protein LOC100000476	1.8	1e-023
PREDICTED: hypothetical protein LOC100001412	1.9	1e-038
PREDICTED: hypothetical protein LOC100003272	1.7	1e-020
PREDICTED: hypothetical protein LOC100003669	1.6	1e-078
PREDICTED: hypothetical protein LOC100003679	1.8	1e-170
PREDICTED: hypothetical protein LOC100005694	1.6	1e-066
PREDICTED: hypothetical protein LOC100006568	1.6	1e-005
PREDICTED: hypothetical protein LOC100008025	1.5	1e-025
PREDICTED: hypothetical protein LOC553467	2.2	1e-162
PREDICTED: hypothetical protein LOC553689	2.1	1e-115
PREDICTED: hypothetical protein LOC556114	1.9	1e-096
PREDICTED: hypothetical protein LOC556556	1.5	1e-152
PREDICTED: hypothetical protein LOC558153	2.3	1e-058
PREDICTED: hypothetical protein LOC558162	1.8	1e-011
PREDICTED: hypothetical protein LOC558162	1.6	1e-010
PREDICTED: hypothetical protein LOC565507	1.5	1e-170
PREDICTED: hypothetical protein LOC565822, transcript variant 1	1.8	1e-041
PREDICTED: hypothetical protein LOC569534	1.5	1e-021
PREDICTED: hypothetical protein LOC569721	1.7	1e-018
PREDICTED: hypothetical protein LOC570492	1.8	1e-176
PREDICTED: hypothetical protein LOC571546	1.5	1e-111
PREDICTED: hypothetical protein LOC664768	2.3	1e-044
PREDICTED: hypothetical protein LOC791539	1.9	1e-099
PREDICTED: hypothetical protein LOC791577	1.6	1e-124
PREDICTED: hypothetical protein LOC791650	1.7	1e-109
PREDICTED: hypothetical protein LOC791696	1.9	1e-049
PREDICTED: hypothetical protein LOC791733	1.7	1e-015
PREDICTED: hypothetical protein LOC791766	1.9	1e-067
PREDICTED: hypothetical protein LOC791777	1.7	1e-049
PREDICTED: hypothetical protein LOC791849	1.6	1e-006
PREDICTED: hypothetical protein LOC791868	2.0	1e-045
PREDICTED: hypothetical protein LOC791868	2.3	1e-045
PREDICTED: hypothetical protein LOC792219	1.7	1e-110
PREDICTED: hypothetical protein LOC793764	2.1	1e-056
PREDICTED: hypothetical protein LOC794081	1.5	1e-080
PREDICTED: hypothetical protein LOC794882	1.8	1e-033
PREDICTED: hypothetical protein LOC796865	1.9	1e-170
PREDICTED: hypothetical protein LOC797224	1.7	1e-057
PREDICTED: hypothetical protein LOC798068	2.0	1e-095
PREDICTED: hypothetical protein LOC799154	1.6	1e-100
PREDICTED: hypothetical protein LOC799499	1.7	1e-035
PREDICTED: hypothetical protein LOC799896	1.8	1e-027
PREDICTED: hypothetical protein LOC799896	2.2	1e-027
PREDICTED: hypothetical protein LOC799921	1.6	1e-018
PREDICTED: neurotrophic tyrosine kinase, receptor, type 1	1.9	1e-088
PREDICTED: si:ch211-214j24.9	1.7	1e-058
PREDICTED: si:dkey-34f16.1	1.6	1e-017
PREDICTED: similar to AF1q	1.6	1e-034
PREDICTED: similar to A-kinase anchoring protein AKAP350	1.5	1e-057

PREDICTED: similar to Anaphase promoting complex subunit 1	1.8	1e-082
PREDICTED: similar to ATP-binding cassette, sub-family C, member 8	1.7	1e-022
PREDICTED: similar to carnitine O-palmitoyltransferase I	1.5	1e-011
PREDICTED: similar to CC chemokine SCYA106	2.1	1e-005
PREDICTED: similar to cystatin B (stefin B)	1.9	3e-39
PREDICTED: similar to Delta/Notch-like EGF-related receptor	2.5	1e-026
PREDICTED: similar to dynein, axonemal, heavy polypeptide 9 isoform 2	1.6	1e-047
PREDICTED: similar to EFHA2 protein	1.6	1e-063
PREDICTED: similar to enhancer of rudimentary homolog	1.7	1e-128
PREDICTED: similar to family with sequence similarity 65, member B	1.5	1e-49
PREDICTED: similar to G protein-coupled inwardly rectifying potassium channel Kir3.4	1.8	1e-050
PREDICTED: similar to GTP binding protein like isoform 1	1.9	1e-179
PREDICTED: similar to H+ transporting F1 ATP synthase epsilon subunit	1.9	1e-030
PREDICTED: similar to histocompatibility 28	1.6	1e-007
PREDICTED: similar to kinesin-related microtuble-based motor protein	1.5	1e-170
PREDICTED: similar to liver bile salt export pump	1.5	1e-170
PREDICTED: similar to LOC495350 protein	1.7	1e-156
PREDICTED: similar to LOC569147 protein	1.8	1e-118
PREDICTED: similar to membrane-associated nucleic acid-binding protein, transcript variant 2	2.0	1e-008
PREDICTED: similar to methionyl aminopeptidase 2 like	1.6	1e-124
PREDICTED: similar to Nebulin	1.5	1e-108
PREDICTED: similar to parvalbumin, transcript variant 2	1.7	1e-170
PREDICTED: similar to peptidylprolyl isomerase domain and WD repeat containing 1	1.8	1e-115
PREDICTED: similar to potassium channel, subfamily K, member 13	1.9	1e-040
PREDICTED: similar to PP2C	1.6	1e-077
PREDICTED: similar to proteasome subunit alpha type 1	1.5	1e-120
PREDICTED: similar to putative tumor suppressor	1.8	1e-038
PREDICTED: similar to Ras and Rab interactor 2	1.7	1e-054
PREDICTED: similar to Rbm4l protein, transcript variant 1	1.6	1e-014
PREDICTED: similar to rhamnose-binding lectin OLL	1.7	1e-027
PREDICTED: similar to ribosomal protein L13a	1.9	1e-009
PREDICTED: similar to ribosomal protein L5b, transcript variant 2	1.5	1e-171
PREDICTED: similar to Serine/threonine kinase 3	1.9	1e-032
PREDICTED: similar to small nuclear ribonucleoprotein D2	1.8	1e-045
PREDICTED: similar to Solute carrier family 15 (oligopeptide transporter), member 1	1.9	0.0001
PREDICTED: similar to Solute carrier family 23, member 1	1.5	1e-061
PREDICTED: similar to solute carrier family 25 member 5 protein	2.1	1e-025
PREDICTED: similar to Sympk protein	1.5	1e-127
PREDICTED: similar to RAB3D, member RAS oncogene family	1.7	3e-54
PREDICTED: similar to taurine transporter	1.9	1e-046
PREDICTED: similar to tetratricopeptide repeat domain 15	2.0	1e-057
PREDICTED: similar to WD repeat and FYVE domain containing 3	1.7	1e-174
PREDICTED: similar to protein-tyrosine phosphatase, type 4A, 3	1.7	1e-005
PREDICTED: similar to zinc finger and BTB domain containing 41	1.8	1e-099
PREDICTED: similar to zinc finger protein 648	1.9	1e-164
PREDICTED: wu:fi13g07	1.9	1e-005
PREDICTED: zgc:100870 protein	2.1	1e-043

probable C->U editing enzyme APOBEC-2	2.1	1e-91
probable C->U editing enzyme APOBEC-2	1.6	6e-58
prohibitin	2.1	1e-091
proteasome (prosome, macropain) 26S subunit, non-ATPase, 4	2.0	1e-101
proteasome (prosome, macropain) subunit, beta type, 7	2.1	1e-170
proteasome26snon-10	1.8	1e-170
putative membrane protein	2.1	1e-016
putative sodium-potassium-chloride cotransporter	2.0	1e-028
pyridoxal (pyridoxine, vitamin B6) kinase, like	1.8	1e-028
pyrophosphatase (inorganic) 1	2.0	1e-170
pyruvate dehydrogenase (lipoamide) alpha 1	1.7	1e-011
RAB18B, member RAS oncogene family	1.7	1e-034
RAB18B, member RAS oncogene family	1.7	1e-017
ras homolog gene family, member Ab (rhoab)	1.6	1e-180
RD RNA binding protein	1.7	1e-062
repetitive sequence, clone pCchr-3	1.5	1e-052
requiem, apoptosis response zinc finger gene	1.8	1e-170
reticulon 1a	1.5	1e-51
retinol binding protein 2a, cellular	1.7	1e-138
retinol dehydrogenase 12 (all-trans and 9-cis)	1.6	1e-054
ribosomal protein L41	2.0	1e-154
ribosomal protein L6	1.6	1e-048
ribosomal protein S19	2.5	1e-016
ribosomal protein S24 isoform 1	1.6	1e-170
ribosomal protein S30 (fau)	1.5	1e-087
ribosomal protein S5	1.6	1e-170
ribosome associated membrane protein 4	2.0	1e-071
ring-box 1	2.1	1e-125
RNA binding motif protein 4.2	1.6	1e-089
S100 calcium binding protein B	1.5	1e-48
sarcoma amplified sequence	2.1	1e-011
scavenger receptor class B, member 2	1.8	1e-015
SEC13 homolog (S. cerevisiae)	1.9	1e-127
secretogranin V	1.5	1e-54
selenoprotein W1	1.6	1e-120
serine protease inhibitor, Kunitz type 1-like (spint1l)	2.0	1e-015
SET translocation (myeloid leukemia-associated) B	2.0	1e-027
short-chain dehydrogenase/reductase	1.7	1e-115
si:dkey-30j22.11	1.5	1e-098
Sjogren syndrome antigen B (autoantigen La)	2.1	1e-139
small nuclear ribonucleoprotein D3 polypeptide, like	1.9	1e-108
small nuclear ribonucleoprotein G (snRNP-G) (Sm protein G)	1.5	1e-25
small nuclear ribonucleoprotein polypeptides B and B'	2.2	1e-102
solute carrier family 4, sodium bicarbonate cotransporter-like, member 10	1.5	1e-015
sorcin	2.3	1e-170
splicing factor, arginine/serine-rich 3 isoform 2	1.7	1e-138
stromal cell-derived factor 1a precursor	1.9	1e-170
succinate dehydrogenase complex, subunit D, integral membrane protein	1.5	1e-129
superoxide dismutase 1, soluble	1.7	1e-090
surfeit gene 4, like	1.7	1e-151

SYF2 homolog, RNA splicing factor (S. cerevisiae)	1.5	1e-079
T cell antigen receptor alpha chain C region	2.3	1e-179
TBP-like 1	1.6	1e-066
t-complex polypeptide 1	1.7	1e-072
TH1-like (Drosophila)	2.0	1e-092
trafficking protein particle complex 6b-like	1.5	1e-170
transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C)	1.6	1e-133
transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C)	1.6	1e-134
translocase of outer mitochondrial membrane 20 homolog	1.9	1e-106
translocase of outer mitochondrial membrane 22 homolog	1.9	1e-137
transmembrane emp24 protein transport domain containing 7	1.7	1e-091
transposase	1.6	1e-050
triosephosphate isomerase 1a	1.5	1e-170
troponin C, fast skeletal	2.0	1e-170
trypsin II precursor	2.0	1e-056
trypsin II precursor	1.8	1e-106
trypsin II precursor	1.7	1e-106
U5 snRNP-associated 102 kDa protein	2.1	1e-170
ubiquinol-cytochrome c reductase binding protein-like	1.7	1e-025
ubiquitin C-terminal hydrolase L1	1.5	1e-170
ubiquitin fusion degradation 1-like	1.6	1e-066
ubiquitin-conjugating enzyme E2N-like	1.5	1e-170
ubiquitin-conjugating enzyme E2N-like	1.5	1e-170
vacuolar protein sorting 28	1.7	1e-170
ventricular myosin heavy chain	2.0	1e-122
vesicle transport through interaction with t-SNAREs homolog 1A	2.3	1e-161
villin 1 like	1.9	1e-157
WD repeat domain 43, like	1.7	1e-170
wd repeat domain 68	1.5	1e-68
Zebrafish DNA sequence from clone CH211-125O16 in linkage group 17, complete sequence	1.8	1e-024
Zebrafish DNA sequence from clone DKEY-71L10 in linkage group 8	1.6	1e-081
zgc:109719 protein	1.5	1e-017
zgc:110631	1.8	1e-058
zgc:152944	1.7	1e-048
zgc:154022	1.6	1e-008
zgc:158381	1.8	1e-010
zgc:55573	1.9	1e-028
zgc:56219	1.5	1e-032
zgc:73136	1.5	1e-170
zgc:77820	1.7	1e-012
zgc:85717	1.5	1e-147
zinc finger protein 330	1.7	1e-170
zinc finger, RAN-binding domain containing 2	1.7	1e-033

References

Adams, J., 2003. The proteasome: structure, function, and role in the cell. Cancer Treatment Reviews 29 Suppl 1, 3-9.

Ahrén, B., 1989. GABA inhibits thyroid hormone secretion in the mouse. Thyroidology / A.P.R.I.M 1, 105-108.

Aizenman, Y., de Vellis, J., 1987. Brain neurons develop in a serum and glial free environment: effects of transferrin, insulin, insulin-like growth factor-I and thyroid hormone on neuronal survival, growth and differentiation. Brain Research 406, 32-42.

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, M. A., Hill, D. P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M., Sherlock, G., 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nature Genetics 25, 25-29.

Atterwill, C. K., Nutt, D. J., 1983. Thyroid hormones do not alter rat brain benzodiazepine receptor function in-vivo. Journal of Pharmacy and Pharmacology 35, 767-768.

Awad, R., Levac, D., Cybulska, P., Merali, Z., Trudeau, V. L., Arnason, J. T., 2007. Effects of traditionally used anxiolytic botanicals on enzymes of the gamma-aminobutyric acid (GABA) system. Canadian Journal of Physiology and Pharmacology 85, 933-942.

Balázs, R., Kovács, S., Teichgräber, P., Cocks, W. A., Eayrs, J. T., 1968. Biochemical effects of thyroid deficiency on the developing brain. Journal of Neurochemistry 15, 1335-1349.

Barale, E., Fasolo, A., Girardi, E., Artero, C., Franzoni, M. F., 1996. Immunohistochemical investigation of gamma-aminobutyric acid ontogeny and transient expression in the central nervous system of Xenopus laevis tadpoles. Journal of Comparative Neurology 368, 285-294.

Barnard, E. A., Skolnick, P., Olsen, R. W., Mohler, H., Sieghart, W., Biggio, G., Braestrup, C., Bateson, A. N., Langer, S. Z., 1998. International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. Pharmacological Reviews 50, 291-313.

Baskys, A., Bayazitov, I., Zhu, E., Fang, L., Wang, R., 2007. Rab-mediated endocytosis: linking neurodegeneration, neuroprotection, and synaptic plasticity? Annals of the New York Academy of Sciences 1122, 313-329.

Bassett, J. H. D., Harvey, C. B., Williams, G. R., 2003. Mechanisms of thyroid hormone receptor-specific nuclear and extra nuclear actions. Molecular and Cellular Endocrinology 213, 1-11.

Bateman, A., Bennett, H. P., 1998. Granulins: the structure and function of an emerging family of growth factors. Journal of Endocrinology 158, 145-151.

Bauer, M., Goetz, T., Glenn, T., Whybrow, P. C., 2008. The thyroid-brain interaction in thyroid disorders and mood disorders. Journal of Neuroendocrinology 20, 1101-1114.

Berbel, P., Marco, P., Cerezo, J. R., DeFelipe, J., 1996. Distribution of parvalbumin immunoreactivity in the neocortex of hypothyroid adult rats. Neuroscience Letters 204, 65-68.

Bergh, J. J., Lin, H.-Y., Lansing, L., Mohamed, S. N., Davis, F. B., Mousa, S., Davis, P. J., 2005. Integrin alphaVbeta3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis. Endocrinology 146, 2864-2871.

Bernal, J., 2002. Action of thyroid hormone in brain. Journal of Endocrinological Investigation 25, 268-288.

Bernal, J., Guadaño-Ferraz, A., Morte, B., 2003. Perspectives in the study of thyroid hormone action on brain development and function. Thyroid 13, 1005-1012.

Billings, H. J., Viguié, C., Karsch, F. J., Goodman, R. L., Connors, J. M., Anderson, G. M., 2002. Temporal requirements of thyroid hormones for seasonal changes in LH secretion. Endocrinology 143, 2618-2625.

Blüthgen, N., Brand, K., Cajavec, B., Swat, M., Herzel, H., Beule, D., 2005. Biological profiling of gene groups utilizing Gene Ontology. Genome Informatics. International Conference on Genome Informatics 16, 106-115.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Brent, G. A., Williams, G. R., Harney, J. W., Forman, B. M., Samuels, H. H., Moore, D. D., Larsen, P. R., 1991. Effects of varying the position of thyroid hormone response elements within the rat growth hormone promoter: implications for positive and negative regulation by 3,5,3'-triiodothyronine. Molecular Endocrinology 5, 542-548.

Bres, O., Plohman, J. C., Eales, J. G., 2006. A cDNA for a putative type III deiodinase in the trout (Oncorhynchus mykiss): influence of holding conditions and thyroid hormone treatment on its hepatic expression. General and Comparative Endocrinology 145, 92-100.

Brucker-Davis, F., 1998. Effects of environmental synthetic chemicals on thyroid function. Thyroid 8, 827-856.

Buchholz, D. R., Paul, B. D., Fu, L., Shi, Y. B., 2006. Molecular and developmental analyses of thyroid hormone receptor function in Xenopus laevis, the African clawed frog. General and Comparative Endocrinology 145, 1-19.

Bustin, S. A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 25, 169-193.

Calzà, L., Aloe, L., Giardino, L., 1997. Thyroid hormone-induced plasticity in the adult rat brain. Brain Research Bulletin 44, 549-557.

Chang, J. P., Johnson, J. D., Sawisky, G. R., Grey, C. L., Mitchell, G., Booth, M., Volk, M. M., Parks, S. K., Thompson, E., Goss, G. G., Klausen, C., Habibi, H. R., 2009. Signal transduction in multifactorial neuroendocrine control of gonadotropin secretion and synthesis in teleosts-studies on the goldfish model. General and Comparative Endocrinology 161, 42-52.

Chapa, F., Künnecke, B., Calvo, R., Escobar del Rey, F., Morreale de Escobar, G., Cerdán, S., 1995. Adult-onset hypothyroidism and the cerebral metabolism of (1,2-13C2) acetate as detected by 13C nuclear magnetic resonance. Endocrinology 136, 296-305.

Chapell, R., Martin, J., Machu, T. K., Leidenheimer, N. J., 1998. Direct channel-gating and modulatory effects of triiodothyronine on recombinant GABA(A) receptors. European Journal of Pharmacology 349, 115-121.

Chatterjee, A. K., Dalal, B. B., Sadhu, U., 1989a. Hormone mediated changes of brain glutamic acid system in amino acid imbalance. Hormone and Metabolic Research 21, 116-119.

Chatterjee, V. K., Lee, J. K., Rentoumis, A., Jameson, J. L., 1989b. Negative regulation of the thyroid-stimulating hormone alpha gene by thyroid hormone: receptor interaction adjacent to the TATA box. Proceedings of the National Academy of Sciences USA 86, 9114-9118.

Chaube, R., Joy, K. P., 2003. Thyroid hormone modulation of brain in vivo tyrosine hydroxylase activity and kinetics in the female catfish *Heteropneustes fossilis*. Journal of Endocrinology 179, 205-215.

Chaube, R., Joy, K. P., 2005. Cyclic AMP-protein kinase a and protein kinase C mediate in vitro T activation of brain tyrosine hydroxylase in the female catfish *Heteropneustes fossilis*. Journal of Neuroendocrinology 17, 91-96.

Chen, H.-J., Huang, D.-J., Hou, W.-C., Liu, J.-S., Lin, Y.-H., 2006. Molecular cloning and characterization of a granulin-containing cysteine protease SPCP3 from sweet potato (Ipomoea batatas) senescent leaves. Journal of Plant Physiology 163, 863-876.

Chen, M. H.-C., Li, Y.-H., Chang, Y., Hu, S.-Y., Gong, H.-Y., Lin, G.-H., Chen, T. T., Wu, J.-L., 2007. Co-induction of hepatic IGF-I and progranulin mRNA by growth hormone in tilapia, Oreochromis mossambiccus. General and Comparative Endocrinology 150, 212-218.

Chiba, S., Suzuki, M., Yamanouchi, K., Nishihara, M., 2007. Involvement of granulin in estrogen-induced neurogenesis in the adult rat hippocampus. Journal of Reproductive Development 53, 297-307.

Chinnakkaruppan, A., Das, S., Sarkar, P. K., 2009. Age related and hypothyroidism related changes on the stoichiometry of neurofilament subunits in the developing rat brain. International Journal of Developmental Neuroscience 27, 257-261.

Colborn, T., 2004. Neurodevelopment and endocrine disruption. Environmental Health Perspectives 112, 944-949.

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics (Oxford, England) 21, 3674-3676.

Courtin, F., Zrouri, H., Lamirand, A., Li, W. W., Mercier, G., Schumacher, M., Goascogne, C. L., Pierre, M., 2005. Thyroid hormone deiodinases in the central and peripheral nervous system. Thyroid 15, 931-942.

Crailsheim, K., Gebauer, H., Glutamate decarboxylation in the rat thyroid gland. In: M. Brzin, et al., (Eds.), Synaptic Constituents in Health and Disease. Pergamon Press, Oxford, 1980, pp. 176-176.

Cuadrado, A., Bernal, J., Munoz, A., 1999. Identification of the mammalian homolog of the splicing regulator Suppressor-of-white-apricot as a thyroid hormone regulated gene. Brain Research. Molecular Brain Research 71, 332-340.

Cyr, D. G., Eales, J. G., 1996. Interrelationships between thyroidal and reproductive endocrine systems in fish. Reviews in Fish Biology and Fisheries 6, 165-200.

Dace, A., Zhao, L., Park, K. S., Furuno, T., Takamura, N., Nakanishi, M., West, B. L., Hanover, J. A., Cheng, S., 2000. Hormone binding induces rapid proteasome-mediated degradation of thyroid hormone receptors. Proceedings of the National Academy of Sciences USA 97, 8985-8990.

Davis, P. J., Leonard, J. L., Davis, F. B., 2008. Mechanisms of nongenomic actions of thyroid hormone. Frontiers in Neuroendocrinology 29, 211-218.

Denver, R. J., 1998. The molecular basis of thyroid hormone-dependent central nervous system remodeling during amphibian metamorphosis. Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology & Endocrinology 119, 219-228.

Denver, R. J., Pavgi, S., Shi, Y. B., 1997. Thyroid hormone-dependent gene expression program for Xenopus neural development. Journal of Biological Chemistry 272, 8179-8188.

Diekmann, H., Klinger, M., Oertle, T., Heinz, D., Pogoda, H.-M., Schwab, M. E., Stuermer, C. A. O., 2005. Analysis of the reticulon gene family demonstrates the absence of the neurite growth inhibitor Nogo-A in fish. Molecular Biology and Evolution 22, 1635-1648.

Diez, D., Grijota-Martinez, C., Agretti, P., De Marco, G., Tonacchera, M., Pinchera, A., de Escobar, G. M., Bernal, J., Morte, B., 2008. Thyroid hormone action in the adult brain: gene expression profiling of the effects of single and multiple doses of triiodo-L-thyronine in the rat striatum. Endocrinology 149, 3989-4000.

Eales, J. G., Brown, S. B., 1993. Measurement and regulation of thyroidal status in teleost fish. Reviews in Fish Biology and Fisheries 3, 299-347.

Elias, A. N., Valenta, L. J., Szekeres, A. V., Grossman, M., 1981. Effect of di-N-propylacetic acid (valproic acid) on the TSH response to TRH--a presumptive role for gamma aminobutyric acid. Metabolism: Clinical and Experimental 30, 1021-1023.

Eliasson, M. J., McCaffery, P., Baughman, R. W., Dräger, U. C., 1997. A ventrodorsal GABA gradient in the embryonic retina prior to expression of glutamate decarboxylase. Neuroscience 79, 863-869.

Farwell, A. P., Dubord-Tomasetti, S. A., 1999. Thyroid hormone regulates the extracellular organization of laminin on astrocytes. Endocrinology 140, 5014-5021.

Farwell, A. P., Dubord-Tomasetti, S. A., Pietrzykowski, A. Z., Stachelek, S. J., Leonard, J. L., 2005. Regulation of cerebellar neuronal migration and neurite outgrowth by thyroxine and 3,3',5'-triiodothyronine. Brain Research. Developmental Brain Research 154, 121-135.

Farwell, A. P., Tranter, M. P., Leonard, J. L., 1995. Thyroxine-dependent regulation of integrin-laminin interactions in astrocytes. Endocrinology 136, 3909-3915.

Fekete, C., Wittmann, G., Liposits, Z., Lechan, R. M., 2002. GABA-ergic innervation of thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus of the rat. Brain Research 957, 251-258.

Feng, X., Jiang, Y., Meltzer, P., Yen, P. M., 2000. Thyroid hormone regulation of hepatic genes in vivo detected by complementary DNA microarray. Molecular Endocrinology 14, 947-955.

Flamant, F., Samarut, J., 2003. Thyroid hormone receptors: lessons from knockout and knock-in mutant mice. Trends in Endocrinology and Metabolism 14, 85-90.

Ford, D. H., Cramer, E. B., Grave, G. D., Developing nervous system in relation to thyroid hormones. Thyroid Hormones and Brain Development. Raven Press, New York, 1977, pp. 1-17.

Funkelstein, L., Toneff, T., Hwang, S.-R., Reinheckel, T., Peters, C., Hook, V., 2008a. Cathepsin L participates in the production of neuropeptide Y in secretory vesicles, demonstrated by protease gene knockout and expression. Journal of Neurochemistry 106, 384-391.

Funkelstein, L., Toneff, T., Mosier, C., Hwang, S.-R., Beuschlein, F., Lichtenauer, U. D., Reinheckel, T., Peters, C., Hook, V., 2008b. Major role of cathepsin L for producing the peptide hormones ACTH, beta-endorphin, and alpha-MSH, illustrated by protease gene knockout and expression. Journal of Biological Chemistry 283, 35652-35659.

Gadea, A., López-Colomé, A. M., 2001. Glial transporters for glutamate, glycine, and GABA III. Glycine transporters. Journal of Neuroscience Research 64, 218-222.

García-G, C., Jeziorski, M. C., Valverde-R, C., Orozco, A., 2004. Effects of iodothyronines on the hepatic outer-ring deiodinating pathway in killifish. General and Comparative Endocrinology 135, 201-209.

García Argiz, C. A., Pasquini, J. M., Kaplún, B., Gómez, C. J., 1967. Hormonal regulation of brain development. II. Effect of neonatal thyroidectomy on succinate dehydrogenase and other enzymes in developing cerebral cortex and cerebellum of the rat. Brain Research 6, 635-646.

Gebauer, H., 1981. GABA transport in the rat thyroid. Naunyn-Schmiedeberg's Archives of Pharmacology 317, 61-66.

Gebauer, H., Crailsheim, K., 1981. Gamma-aminobutyrate transaminase in the rat-thyroid. Acta Endocrinologica 97, 171.

Gebauer, H., Haas, K., 1980. Gamma-aminobutyric acid transport in thyroids of triiodothyronine-treated and propylthiouracil-treated rats. Hoppe-Seylers Zeitschrift fur Physiologische Chemie 361, 1284.

Gebauer, H., Pabst, M. A., 1981. Autoradiographic localization of 3H-GABA uptake in the thyroid gland of the rat. Cell and Tissue Research 220, 873-879.

Gerrelli, D., Huntriss, J. D., Latchman, D. S., 1994. Antagonistic effects of retinoic acid and thyroid hormone on the expression of the tissue-specific splicing protein SmN in a clonal cell line derived from rat heart. Journal of Molecular and Cellular Cardiology 26, 713-719.

Gijselinck, I., Van Broeckhoven, C., Cruts, M., 2008. Granulin mutations associated with frontotemporal lobar degeneration and related disorders: an update. Human Mutation 29, 1373-1386.

Go, T., Ito, M., Okuno, T., Mikawa, H., 1988. Effect of thyroid hormones on benzodiazepine receptors in neuron-enriched primary cultures. Journal of Neurochemistry 51, 1497-1500.

Gray, T. A., Smithwick, M. J., Schaldach, M. A., Martone, D. L., Graves, J. A., McCarrey, J. R., Nicholls, R. D., 1999. Concerted regulation and molecular evolution of the duplicated SNRPB'/B and SNRPN loci. Nucleic Acids Research 27, 4577-4584.

Guadaño-Ferraz, A., Benavides-Piccione, R., Venero, C., Lancha, C., Vennström, B., Sandi, C., DeFelipe, J., Bernal, J., 2003. Lack of thyroid hormone receptor alpha1 is associated with selective alterations in behavior and hippocampal circuits. Molecular Psychiatry 8, 30-38.

Haas, M. J., Mreyoud, A., Fishman, M., Mooradian, A. D., 2004. Microarray analysis of thyroid hormone-induced changes in mRNA expression in the adult rat brain. Neuroscience Letters 365, 14-18.

Hanington, P. C., Barreda, D. R., Belosevic, M., 2006. A novel hematopoietic granulin induces proliferation of goldfish (Carassius auratus L.) macrophages. Journal of Biological Chemistry 281, 9963-9970.

Hanon, E. A., Lincoln, G. A., Fustin, J. M., Dardente, H., Masson-Pevet, M., Morgan, P. J., Hazlerigg, D. G., 2008. Ancestral TSH mechanism signals summer in a photoperiodic mammal. Current Biology 18, 1147-1152.

Harvey, S., Kakebeeke, M., Murphy, A. E., Sanders, E. J., 2003. Growth hormone in the nervous system: autocrine or paracrine roles in retinal function? Canadian Journal of Physiology and Pharmacology 81, 371-384.

Hashimoto, H., Walker, C. H., Prange, A. J., Mason, G. A., 1991. The effects of thyroid hormones on potassium-stimulated release of 3H-GABA by synaptosomes of rat cerebral cortex. Neuropsychopharmacology 5, 49-54.

Hayakawa, Y., Morita, T., Kitamura, W., Kanda, S., Banba, A., Nagaya, H., Hotta, K., Sohn, Y. C., Yoshizaki, G., Kobayashi, M., 2008. Biological activities of single-chain goldfish follicle-stimulating hormone and luteinizing hormone. Aquaculture 274, 408-415.

Hengst, U., Albrecht, H., Hess, D., Monard, D., 2001. The phosphatidylethanolamine-binding protein is the prototype of a novel family of serine protease inhibitors. Journal of Biological Chemistry 276, 535-540.

Hibbert, B., Fung, I., McAuley, R., Lariviere, K., MacNeil, B., Bafi-Yeboa, N., Livesey, J., Trudeau, V., 2004. Increased GAD67 mRNA levels are correlated with in vivo GABA synthesis in the MPTP-treated catecholamine-depleted goldfish brain. Brain Research. Molecular Brain Research 128, 121-130.

Hibbert, B., Fung, I., McAuley, R., Samia, M., Trudeau, V., 2005. Catecholamine depletion modulates serum LH levels, GAD67 mRNA, and GABA synthesis in the goldfish. General and Comparative Endocrinology 140, 176-183.

Hirota, Y., Kuronita, T., Fujita, H., Tanaka, Y., 2007. A role for Rab5 activity in the biogenesis of endosomal and lysosomal compartments. Biochemical and Biophysical Research Communications 364, 40-47.

Honegger, P., Lenoir, D., 1980. Triodothyronine enhancement of neuronal differentiation in aggregating fetal rat brain cells cultured in a chemically defined medium. Brain Research 199, 425-434.

Hong, S. J., Kang, K. W., 1999. Purification of granulin-like polypeptide from the blood-sucking leech, Hirudo nipponia. Protein Expression and Purification 16, 340-346.

Howdeshell, K. L., 2002. A model of the development of the brain as a construct of the thyroid system. Environmental Health Perspectives 110 Suppl 3, 337-348.

Huggard-Nelson, D. L., Nathwani, P. S., Kermouni, A., Habibi, H. R., 2002. Molecular characterization of LH-beta and FSH-beta subunits and their regulation by estrogen in the goldfish pituitary. Molecular and Cellular Endocrinology 188, 171-193.

Inglefield, J. R., Shafer, T. J., 2000. Perturbation by the PCB mixture aroclor 1254 of GABA(A) receptor-mediated calcium and chloride responses during maturation in vitro of rat neocortical cells. Toxicology and Applied Pharmacology 164, 184-195.

Ji, F., Kanbara, N., Obata, K., 1999. GABA and histogenesis in fetal and neonatal mouse brain lacking both the isoforms of glutamic acid decarboxylase. Neuroscience Research 33, 187-194.

Joffe, R. T., Sokolov, S. T., 1994. Thyroid hormones, the brain, and affective disorders. Critical Reviews in Neurobiology 8, 45-63.

Jones, I., Srinivas, M., Ng, L., Forrest, D., 2003. The thyroid hormone receptor beta gene: structure and functions in the brain and sensory systems. Thyroid 13, 1057-1068.

Jordan, D., Poncet, C., Veisseire, M., Mornex, R., 1983. Role of GABA in the control of thyrotropin secretion in the rat. Brain Research 268, 105-110.

Jung, M. J., Lippert, B., Metcalf, B. W., Böhlen, P., Schechter, P. J., 1977. gamma-Vinyl GABA (4-amino-hex-5-enoic acid), a new selective irreversible inhibitor of GABA-T: effects on brain GABA metabolism in mice. Journal of Neurochemistry 29, 797-802.

Kalaria, R. N., Prince, A. K., 1985. Effects of thyroid deficiency on the development of cholinergic, GABA, dopaminergic and glutamate neuron markers and DNA concentrations in the rat corpus striatum. International Journal of Developmental Neuroscience 3, 655-666.

Kalaria, R. N., Prince, A. K., 1986. Decreased neurotransmitter receptor binding in striatum and cortex from adult hypothyroid rats. Brain Research 364, 268-274.

Klausen, C., Chang, J. P., Habibi, H. R., 2001. The effect of gonadotropin-releasing hormone on growth hormone and gonadotropin subunit gene expression in the pituitary of goldfish, Carassius auratus. Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology 129, 511-516.

Kobayashi, M., Kato, Y., Yoshiura, Y., Aida, K., 1997. Molecular cloning of cDNA encoding two types of pituitary gonadotropin alpha subunit from the goldfish, Carassius auratus. General and Comparative Endocrinology 105, 372-378.

Kobayashi, M., Morita, T., Ikeguchi, K., Yoshizaki, G., Suzuki, T., Watabe, S., 2006. In vivo biological activity of recombinant goldfish gonadotropins produced by baculovirus in silkworm larvae. Aquaculture 256, 433-442.

Korhonen, L., Lindholm, D., 2004. The ubiquitin proteasome system in synaptic and axonal degeneration: a new twist to an old cycle. Journal of Cell Biology 165, 27-30.

Kragie, L., 1993. Neuropsychiatric implications of thyroid hormone and benzodiazepine interactions. Endocrine Research 19, 1-32.

Krawiec, L., García Argiz, C. A., Gómez, C. J., Pasquini, J. M., 1969. Hormonal regulation of brain development. 3. Effects of triiodothyronine and growth hormone on the biochemical changes in the cerebral cortex and cerebellum of neonatally thyroidectomized rats. Brain Research 15, 209-218.

Kumar, R. S., Trant, J. M., 2001. Piscine glycoprotein hormone (gonadotropin and thyrotropin) receptors: a review of recent developments. Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology 129, 347-355.

Lariviere, K., MacEachern, L., Greco, V., Majchrzak, G., Chiu, S., Drouin, G., Trudeau, V. L., 2002. GAD(65) and GAD(67) isoforms of the glutamic acid decarboxylase gene originated before the divergence of cartilaginous fishes. Molecular Biology and Evolution 19, 2325-2329.

Lariviere, K., Samia, M., Lister, A., Van Der Kraak, G., Trudeau, V. L., 2005. Sex steroid regulation of brain glutamic acid decarboxylase (GAD) mRNA is season-dependent and sexually dimorphic in the goldfish Carassius auratus. Brain Research. Molecular Brain Research 141, 1-9.

Lema, S. C., Dickey, J. T., Schultz, I. R., Swanson, P., 2008. Dietary exposure to 2,2',4,4'-tetrabromodiphenyl ether (PBDE-47) alters thyroid status and thyroid hormone-regulated gene transcription in the pituitary and brain. Environmental Health Perspectives 116, 1694-1699.

Liu, Y. W., Lo, L. J., Chan, W. K., 2000. Temporal expression and T3 induction of thyroid hormone receptors alpha1 and beta1 during early embryonic and larval development in zebrafish, Danio rerio. Molecular and Cellular Endocrinology 159, 187-195.

MacKenzie, D. S., Jones, R. A., Miller, T. C., 2009. Thyrotropin in teleost fish. General and Comparative Endocrinology 161, 83-89.

Mañanos, E. L., Anglade, I., Chyb, J., Saligaut, C., Breton, B., Kah, O., 1999. Involvement of gamma-aminobutyric acid in the control of GTH-1 and GTH-2 secretion in male and female rainbow trout. Neuroendocrinology 69, 269-280.

Manchado, M., Infante, C., Rebordinos, L., Canavate, J. P., 2009. Molecular characterization, gene expression and transcriptional regulation of thyroid hormone receptors in Senegalese sole. General and Comparative Endocrinology 160, 139-147.

Marlatt, V. L., Martyniuk, C. J., Zhang, D., Xiong, H., Watt, J., Xia, X., Moon, T., Trudeau, V. L., 2008. Auto-regulation of estrogen receptor subtypes and gene expression profiling of 17beta-estradiol action in the neuroendocrine axis of male goldfish. Molecular and Cellular Endocrinology 283, 38-48.

Martin, J. V., Padron, J. M., Newman, M. A., Chapell, R., Leidenheimer, N. J., Burke, L. A., 2004. Inhibition of the activity of the native gamma-aminobutyric acid A receptor by metabolites of thyroid hormones: correlations with molecular modeling studies. Brain Research 1004, 98-107.

Martin, J. V., Williams, D. B., Fitzgerald, R. M., Im, H. K., Vonvoigtlander, P. F., 1996. Thyroid hormonal modulation of the binding and activity of the GABAA receptor complex of brain. Neuroscience 73, 705-713.

Marty, A., Llano, I., 2005. Excitatory effects of GABA in established brain networks. Trends in Neurosciences 28, 284-289.

Martyniuk, C. J., Awad, R., Hurley, R., Finger, T. E., Trudeau, V. L., 2007a. Glutamic acid decarboxylase 65, 67, and GABA-transaminase mRNA expression and total enzyme activity in the goldfish (*Carassius auratus*) brain. Brain Research 1147, 154-166.

Martyniuk, C. J., Chang, J. P., Trudeau, V. L., 2007b. The effects of GABA agonists on glutamic acid decarboxylase, GABA-transaminase, activin, salmon gonadotrophin-releasing hormone and tyrosine hydroxylase mRNA in the goldfish (*Carassius auratus*) neuroendocrine brain. Journal of Neuroendocrinology 19, 390-396.

Martyniuk, C. J., Crawford, A. B., Hogan, N. S., Trudeau, V. L., 2005. GABAergic modulation of the expression of genes involved in GABA synaptic transmission and stress in the hypothalamus and telencephalon of the female goldfish (*Carassius auratus*). Journal of Neuroendocrinology 17, 269-275.

Martyniuk, C. J., Xiong, H., Crump, K., Chiu, S., Sardana, R., Nadler, A., Gerrie, E. R., Xia, X., Trudeau, V. L., 2006. Gene expression profiling in the neuroendocrine brain of

male goldfish (*Carassius auratus*) exposed to 17alpha-ethinylestradiol. Physiological Genomics 27, 328-336.

Mason, G. A., Walker, C. H., Prange, A. J., 1990. Depolarization-dependent 45Ca uptake by synaptosomes of rat cerebral cortex is enhanced by L-triiodothyronine. Neuropsychopharmacology 3, 291-295.

Mason, G. A., Walker, C. H., Prange, A. J., Bondy, S. C., 1987. GABA uptake is inhibited by thyroid hormones: implications for depression. Psychoneuroendocrinology 12, 53-59.

Matsushita, A., Sasaki, S., Kashiwabara, Y., Nagayama, K., Ohba, K., Iwaki, H., Misawa, H., Ishizuka, K., Nakamura, H., 2007. Essential role of GATA2 in the negative regulation of thyrotropin beta gene by thyroid hormone and its receptors. Molecular Endocrinology 21, 865-884.

Medina, J. H., De Robertis, E., 1985. Benzodiazepine receptor and thyroid hormones: in vivo and in vitro modulation. Journal of Neurochemistry 44, 1340-1344.

Mehta, A. K., Ticku, M. K., 1999. An update on GABAA receptors. Brain Research. Brain Research Reviews 29, 196-217.

Mennigen, J. A., Martyniuk, C. J., Crump, K., Xiong, H., Zhao, E., Popesku, J., Anisman, H., Cossins, A. R., Xia, X., Trudeau, V. L., 2008. Effects of fluoxetine on the reproductive axis of female goldfish (*Carassius auratus*). Physiological Genomics 35, 273-282.

Messer, A., Eisenberg, B., Martin, D. L., 1989. Effects of mild hyperthyroidism on levels of amino acids in the developing Lurcher cerebellum. Journal of Neurogenetics 5, 77-85.

Miller, L. D., McPhie, P., Suzuki, H., Kato, Y., Liu, E. T., Cheng, S.-y., 2004. Multi-tissue gene-expression analysis in a mouse model of thyroid hormone resistance. Genome Biology 5, R31.

Montero-Pedrazuela, A., Venero, C., Lavado-Autric, R., Fernandez-Lamo, I., Garcia-Verdugo, J. M., Bernal, J., Guadano-Ferraz, A., 2006. Modulation of adult hippocampal neurogenesis by thyroid hormones: implications in depressive-like behavior. Molecular Psychiatry 11, 361-371.

Morita, T., Yoshizaki, G., Kobayashi, M., Takeuchi, T., 2003. Production of biologically-active recombinant goldfish gonadotropins in transgenic rainbow trout. Fish Physiology and Biochemistry 28, 473-474.

Morkin, E., 2000. Control of cardiac myosin heavy chain gene expression. Microscopy Research and Technique 50, 522-531.

Morse, D. C., Groen, D., Veerman, M., van Amerongen, C. J., Koëter, H. B., Smits van Prooije, A. E., Visser, T. J., Koeman, J. H., Brouwer, A., 1993. Interference of polychlorinated biphenyls in hepatic and brain thyroid hormone metabolism in fetal and neonatal rats. Toxicology and Applied Pharmacology 122, 27-33.

Nagayama, K., Sasaki, S., Matsushita, A., Ohba, K., Iwaki, H., Matsunaga, H., Suzuki, S., Misawa, H., Ishizuka, K., Oki, Y., Noh, J. Y., Nakamura, H., 2008. Inhibition of GATA2-dependent transactivation of the TSHbeta gene by ligand-bound estrogen receptor alpha. Journal of Endocrinology 199, 113-125.

Nagy, A., Lajtha, A., 1983. Thyroid hormones and derivatives inhibit flunitrazepam binding. Journal of Neurochemistry 40, 414-417.

Nakao, N., Ono, H., Yamamura, T., Anraku, T., Takagi, T., Higashi, K., Yasuo, S., Katou, Y., Kageyama, S., Uno, Y., Kasukawa, T., Iigo, M., Sharp, P. J., Iwasawa, A., Suzuki, Y., Sugano, S., Niimi, T., Mizutani, M., Namikawa, T., Ebihara, S., Ueda, H. R., Yoshimura, T., 2008a. Thyrotrophin in the pars tuberalis triggers photoperiodic response. Nature 452, 317-322.

Nakao, N., Ono, H., Yoshimura, T., 2008b. Thyroid hormones and seasonal reproductive neuroendocrine interactions. Reproduction 136, 1-8.

Nakayama, K., Sei, N., Oshima, Y., Tashiro, K., Shimasaki, Y., Honjo, T., 2008. Alteration of gene expression profiles in the brain of Japanese medaka (Oryzias latipes) exposed to KC-400 or PCB126. Marine Pollution Bulletin 57, 460-466.

Nawaz, Z., O'Malley, B. W., 2004. Urban renewal in the nucleus: is protein turnover by proteasomes absolutely required for nuclear receptor-regulated transcription? Molecular Endocrinology 18, 493-499.

Nelson, E. R., Habibi, H. R., 2006. Molecular characterization and sex-related seasonal expression of thyroid receptor subtypes in goldfish. Molecular and Cellular Endocrinology 253, 83-95.

Nelson, E. R., Habibi, H. R., 2008. Functional significance of a truncated thyroid receptor subtype lacking a hormone-binding domain in goldfish. Endocrinology 149, 4702-4709.

Nelson, E. R., Habibi, H. R., 2009. Thyroid receptor subtypes: structure and function in fish. General and Comparative Endocrinology 161, 90-96.

Nikrodhanond, A. A., Ortiga-Carvalho, T. M., Shibusawa, N., Hashimoto, K., Liao, X. H., Refetoff, S., Yamada, M., Mori, M., Wondisford, F. E., 2006. Dominant role of thyrotropin-releasing hormone in the hypothalamic-pituitary-thyroid axis. Journal of Biological Chemistry 281, 5000-5007.

Olsen, R. W., DeLorey, T. M., Gordey, M., Kang, M. H., 1999. GABA receptor function and epilepsy. Advances in Neurology 79, 499-510.

Oppenheimer, J. H., Schwartz, H. L., 1997. Molecular basis of thyroid hormone-dependent brain development. Endocrine Reviews 18, 462-475.

Ortiz-Butron, R., Pacheco-Rosado, J., Hernández-Garcia, A., Briones-Velasco, M., Rocha, L., 2003. Mild thyroid hormones deficiency modifies benzodiazepine and mu-opioid receptor binding in rats. Neuropharmacology 44, 111-116.

Patel, A. J., Balázs, R., Smith, R. M., Kingsbury, A. E., Hunt, A., Thyroid hormone and brain development. In: C. Di Benedeatta, et al., (Eds.), Multidisciplinary Approach to Brain Development, Developments in Neuroscience. Elsevier/North-Holland, Amsterdam, 1980a, pp. 261-277.

Patel, A. J., Hayashi, M., Hunt, A., 1988. Role of thyroid hormone and nerve growth factor in the development of choline acetyltransferase and other cell-specific marker enzymes in the basal forebrain of the rat. Journal of Neurochemistry 50, 803-811.

Patel, A. J., Smith, R. M., Kingsbury, A. E., Hunt, A., Balázs, R., 1980b. Effects of thyroid state on brain development: muscarinic acetylcholine and GABA receptors. Brain Research 198, 389-402.

Patel, S. B., Bellini, M., 2008. The assembly of a spliceosomal small nuclear ribonucleoprotein particle. Nucleic Acids Research 36, 6482-6493.

Paul, B. D., Fu, L., Buchholz, D. R., Shi, Y.-B., 2005. Coactivator recruitment is essential for liganded thyroid hormone receptor to initiate amphibian metamorphosis. Molecular and Cellular Biology 25, 5712-5724.

Pesetsky, I., Burkat, J. F., Role of thyroid hormones in development of GABA-metabolic enzymes in cerebellar neurons and glia: loss of enzymatic activity in Bergmann cells of hypothyroid rats. In: G. D. Grave, (Ed.), Thyroid Hormones and Brain Development. Raven Press, New York, 1977, pp. 93-104.

Peter, R. E., Nahorniak, C. S., Chang, J. P., Crim, L. W., 1984. Gonadotropin release from the pars distalis of goldfish, Carassius auratus, transplanted beside the brain or into the brain ventricles: additional evidence for gonadotropin-release-inhibitory factor. General and Comparative Endocrinology 55, 337-346.

Peter, R. E., Prasada Rao, P. D., Baby, S. M., Illing, N., Millar, R. P., 2003. Differential brain distribution of gonadotropin-releasing hormone receptors in the goldfish. General and Comparative Endocrinology 132, 399-408.

Pierce, J. G., Parsons, T. F., 1981. Glycoprotein hormones: structure and function. Annual Review of Biochemistry 50, 465-495.

Pompolo, S., Pereira, A., Kaneko, T., Clarke, I. J., 2003. Seasonal changes in the inputs to gonadotropin-releasing hormone neurones in the ewe brain: an assessment by conventional fluorescence and confocal microscopy. Journal of Neuroendocrinology 15, 538-545.

Popesku, J. T., Martyniuk, C. J., Mennigen, J., Xiong, H., Zhang, D., Xia, X., Cossins, A. R., Trudeau, V. L., 2008. The goldfish (Carassius auratus) as a model for neuroendocrine signaling. Molecular and Cellular Endocrinology 293, 43-56.

Ramírez de Guglielmone, A. E., Gómez, C. J., 1966. Influence of neonatal hypothyroidism on amino acids in developing rat brain. Journal of Neurochemistry 13, 1017-1025.

Rizzo, V., Crupi, D., Bagnato, S., Quartarone, A., Benvenga, S., Bartolone, L., Ghilardi, M. F., Trimarchi, F., Girlanda, P., Battaglia, F., 2008. Neural response to transcranial magnetic stimulation in adult hypothyroidism and effect of replacement treatment. Journal of the Neurological Sciences 266, 38-43.

Sakurai, A., Miyamoto, T., DeGroot, L. J., 1992. Cloning and characterization of the human thyroid hormone receptor beta 1 gene promoter. Biochemical and Biophysical Research Communications 185, 78-84.

Sanchez-Pacheco, A., Aranda, A., 2003. Binding of the thyroid hormone receptor to a negative element in the basal growth hormone promoter is associated with histone acetylation. Journal of Biological Chemistry 278, 39383-39391.

Sandrini, M., Marrama, D., Vergoni, A. V., Bertolini, A., 1991. Effects of thyroid status on the characteristics of alpha 1-, alpha 2-, beta, imipramine and GABA receptors in the rat brain. Life Sciences 48, 659-666.

Sandrini, M., Vergoni, A. V., Bertolini, A., 1993. No modifications of GABAA and benzodiazepine receptors following experimental dysthyroidism in rats. Pharmacological Research 28, 47-52.

Santos, E. M., Kille, P., Workman, V. L., Paull, G. C., Tyler, C. R., 2008. Sexually dimorphic gene expression in the brains of mature zebrafish. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 149, 314-324.

Shen, X., Li, Q. L., Brent, G. A., Friedman, T. C., 2004. Thyroid hormone regulation of prohormone convertase 1 (PC1): regional expression in rat brain and in vitro characterization of negative thyroid hormone response elements. Journal of Molecular Endocrinology 33, 21-33.

Shen, X., Li, Q. L., Brent, G. A., Friedman, T. C., 2005. Regulation of regional expression in rat brain PC2 by thyroid hormone/characterization of novel negative thyroid hormone response elements in the PC2 promoter. American Journal of Physiology - Endocrinology and Metabolism 288, E236-E245.

Shupnik, M. A., 2000. Thyroid Hormone Suppression of Pituitary Hormone Gene Expression. Reviews in Endocrine & Metabolic Disorders 1, 35-42.

Simon, N. M., Blacker, D., Korbly, N. B., Sharma, S. G., Worthington, J. J., Otto, M. W., Pollack, M. H., 2002. Hypothyroidism and hyperthyroidism in anxiety disorders revisited: new data and literature review. Journal of Affective Disorders 69, 209-217.

Skolnick, P., Paul, S. M., 1983. New concepts in the neurobiology of anxiety. Journal of Clinical Psychiatry 44, 12-20.

Soghomonian, J. J., Martin, D. L., 1998. Two isoforms of glutamate decarboxylase: why? Trends in Pharmacological Sciences 19, 500-505.

Sohn, Y. C., Kobayashi, M., Aida, K., 2001. Regulation of gonadotropin beta subunit gene expression by testosterone and gonadotropin-releasing hormones in the goldfish, Carassius auratus. Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology 129, 419-426.

Sohn, Y. C., Yoshiura, Y., Kobayashi, M., Aida, K., 1999a. Seasonal changes in mRNA levels of gonadotropin and thyrotropin subunits in the goldfish, Carassius auratus. General and Comparative Endocrinology 113, 436-444.

Sohn, Y. C., Yoshiura, Y., Suetake, H., Kobayashi, M., Aida, K., 1999b. Isolation and characterization of the goldfish thyrotropin beta subunit gene including the 5'-flanking region. General and Comparative Endocrinology 115, 463-473.

St Germain, D. L., Galton, V. A., Hernandez, A., 2009. Minireview: Defining the roles of the iodothyronine deiodinases: current concepts and challenges. Endocrinology 150, 1097-1107.

Storch, J., Corsico, B., 2008. The emerging functions and mechanisms of mammalian fatty acid-binding proteins. Annual Review of Nutrition 28, 73-95.

Suzuki, M., Nishiahara, M., 2002. Granulin precursor gene: a sex steroid-inducible gene involved in sexual differentiation of the rat brain. Molecular Genetics and Metabolism 75, 31-37.

Suzuki, S., Miyamoto, T., Opsahl, A., Sakurai, A., DeGroot, L. J., 1994. Two thyroid hormone response elements are present in the promoter of human thyroid hormone receptor beta 1. Mol Endocrinol 8, 305-314.

Swapna, I., Senthilkumaran, B., 2007. Thyroid hormones modulate the hypothalamo-hypophyseal-gonadal axis in teleosts: Molecular insights. Fish Physiology and Biochemistry 33, 335-345.

Tan, Z. S., Vasan, R. S., 2009. Thyroid function and Alzheimer's disease. Journal of Alzheimer's Disease 16, 503-507.

Tapia-Arancibia, L., Roussel, J. P., Astier, H., 1987. Evidence for a dual effect of gamma-aminobutyric acid on thyrotropin (TSH)-releasing hormone-induced TSH release from perifused rat pituitaries. Endocrinology 121, 980-986.

Tata, J. R., 1994. Autoregulation and crossregulation of nuclear receptor genes. Trends in Endocrinology and Metabolism 5, 283-290.

Thompson, C. C., 1996. Thyroid hormone-responsive genes in developing cerebellum include a novel synaptotagmin and a hairless homolog. Journal of Neuroscience 16, 7832-7840.

Thompson, S. A., Bonnert, T. P., Whiting, P. J., Wafford, K. A., 1998. Functional characteristics of recombinant human GABA(A) receptors containing the epsilon-subunit. Toxicology Letters 100-101, 233-238.

Tian, N., Petersen, C., Kash, S., Baekkeskov, S., Copenhagen, D., Nicoll, R., 1999. The role of the synthetic enzyme GAD65 in the control of neuronal gamma-aminobutyric acid release. Proceedings of the National Academy of Sciences USA 96, 12911-12916.

Tokumoto, M., Nagahama, Y., Tokumoto, T., 1999. Molecular cloning of cDNA encoding a cyclin-selective ubiquitin carrier protein (E2-C) from Carassius auratus (goldfish) and expression analysis of the cloned gene. FEBS Letters 458, 375-377.

Trudeau, V. L., 1997. Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, Carassius auratus. Reviews of Reproduction 2, 55-68.

Trudeau, V. L., Bosma, P. T., Collins, M., Priede, I. G., Docherty, K., 2000a. Sexually dimorphic expression of glutamate decarboxylase mRNA in the hypothalamus of the deep sea armed grenadier, Coryphaenoides (Nematonurus) armatus. Brain, Behavior and Evolution 56, 269-275.

Trudeau, V. L., Spanswick, D., Fraser, E. J., Larivière, K., Crump, D., Chiu, S., MacMillan, M., Schulz, R. W., 2000b. The role of amino acid neurotransmitters in the regulation of pituitary gonadotropin release in fish. Biochemistry and Cell Biology 78, 241-259.

Tusher, V. G., Tibshirani, R., Chu, G., 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proceedings of the National Academy of Sciences USA 98, 5116-5121.

Upadhyaya, L., Agrawal, J. K., 1993. Effect of L-thyroxine and carbimazole on brain biogenic amines and amino acids in rats. Endocrine Research 19, 87-99.

Van Damme, P., Van Hoecke, A., Lambrechts, D., Vanacker, P., Bogaert, E., van Swieten, J., Carmeliet, P., Van Den Bosch, L., Robberecht, W., 2008. Progranulin functions as a neurotrophic factor to regulate neurite outgrowth and enhance neuronal survival. Journal of Cell Biology 181, 37-41.

Vijayan, E., McCann, S. M., 1978. Effects of intraventricular injection of gamma-aminobutyric acid (GABA) on plasma growth hormone and thyrotropin in conscious ovariectomized rats. Endocrinology 103, 1888-1893.

Virgili, M., Saverino, O., Vaccari, M., Barnabei, O., Contestabile, A., 1991. Temporal, regional and cellular selectivity of neonatal alteration of the thyroid state on neurochemical maturation in the rat. Experimental Brain Research 83, 555-561.

Visser, W. E., Friesema, E. C., Jansen, J., Visser, T. J., 2008. Thyroid hormone transport in and out of cells. Trends in Endocrinology and Metabolism 19, 50-56.

von Schalburg, K. R., Rise, M. L., Brown, G. D., Davidson, W. S., Koop, B. F., 2005. A comprehensive survey of the genes involved in maturation and development of the rainbow trout ovary. Biology of Reproduction 72, 687-699.

Wang, Y., Zhou, L., Yao, B., Li, C. J., Gui, J. F., 2004. Differential expression of thyroid-stimulating hormone beta subunit in gonads during sex reversal of orange-spotted and red-spotted groupers. Molecular and Cellular Endocrinology 220, 77-88.

Wiens, S. C., Trudeau, V. L., 2006. Thyroid hormone and gamma-aminobutyric acid (GABA) interactions in neuroendocrine systems. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 144, 332-344.

Williams, D. R., Li, W., Hughes, M. A., Gonzalez, S. F., Vernon, C., Vidal, M. C., Jeney, Z., Jeney, G., Dixon, P., McAndrew, B., Bartfai, R., Orban, L., Trudeau, V., Rogers, J., Matthews, L., Fraser, E. J., Gracey, A. Y., Cossins, A. R., 2008. Genomic resources and microarrays for the common carp *Cyprinus carpio*. Journal of Fish Biology 72, 2095-2117.

Wisden, W., Stephens, D. N., 1999. Towards better benzodiazepines. Nature 401, 751-752.

Yam, K. M., Yoshiura, Y., Kobayashi, M., Ge, W., 1999. Recombinant goldfish activin B stimulates gonadotropin-Ibeta but inhibits gonadotropin-IIbeta expression in the goldfish, Carassius auratus. General and Comparative Endocrinology 116, 81-89.

Yang, Y. H., Dudoit, S., Luu, P., Lin, D. M., Peng, V., Ngai, J., Speed, T. P., 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Research 30, E15.

Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Elizur, A., Levavi-Sivan, B., 2003. Regulation of fish gonadotropins. International Reviews in Cytology 225, 131-185.

Yen, P. M., 2001. Physiological and molecular basis of thyroid hormone action. Physiological Reviews 81, 1097-1142.

Yoshimura, T., 2006. Molecular mechanism of the photoperiodic response of gonads in birds and mammals. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 144, 345-350.

Yoshiura, Y., Sohn, Y. C., Munakata, A., Kobayashi, M., Aida, K., 1999. Molecular cloning of the cDNA encoding the ? subunit of thyrotropin and regulation of its gene

expression by thyroid hormones in the goldfish, Carassius auratus. Fish Physiology and Biochemistry 21, 201-210.

Yuen, C. W., Ge, W., 2004. Follistatin suppresses FSHbeta but increases LHbeta expression in the goldfish - evidence for an activin-mediated autocrine/paracrine system in fish pituitary. General and Comparative Endocrinology 135, 108-115.

Zhang, X., Grand, R. J., McCabe, C. J., Franklyn, J. A., Gallimore, P. H., Turnell, A. S., 2002. Transcriptional regulation of the human glycoprotein hormone common alpha subunit gene by cAMP-response-element-binding protein (CREB)-binding protein (CBP)/p300 and p53. Biochemical Journal 368, 191-201.

Zhao, E., Basak, A., Trudeau, V. L., 2006. Secretoneurin stimulates goldfish pituitary luteinizing hormone production. Neuropeptides 40, 275-282.