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DOPAMINERGIC REGULATION OF GENE EXPRESSION IN THE NEUROENDOCRINE BRAIN OF THE GOLDFISH (*CARASSIUS AURATUS*)

JASON THEODORE POPESKU

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ABSTRACT

Dopamine (DA) is the single most potent inhibitor of luteinizing hormone (LH) release from the fish pituitary and is fundamental to the neuroendocrine control of reproduction in vertebrates. It exerts its functions through DA D1- and D2-specific post-synaptic receptors. In order for DA to be such a potent inhibitor of reproduction, I hypothesized that DA must inhibit multiple LH-stimulatory systems in the brain. I provide the first evidence that a specific DA receptor, the D2 receptor, is alternatively spliced in fish and discuss its significance in terms of the inhibitory tone of DA. A wide-scale assessment of the genes and proteins that are under the regulatory control of DAergic action is provided and begins to elucidate mechanistic pathways that DA may be modulating and showed that D1- and D2-receptor specific agonists modulate different biochemical pathways. It is then shown that DA, acting through the D1 receptor, may be modulating the effects of another neurotransmitter, glutamate, through AMPA-type receptors. Blockage of the D1 receptor resulted in a rapid and pronounced increase of activin βa transcription and subsequent stimulation with AMPA resulted in a significant increase in isotocin and chicken-type gonadotropin-releasing hormone, concurrently with increased circulating LH levels. The hypothalamic expression of a neuronal stem cell marker, DA cell markers and developmental factors are significantly increased in the days following injection with a specific DA neurotoxin. This provides evidence for DA neuron regeneration in the adult hypothalamus, and is indicative of an intrinsic regenerative capacity of the principle hypophysiotropic LH-inhibitory system for dynamic recovery and maintenance of function after injury. Comprehensive meta-analysis of microarray data identified 268 ESTs that are very likely to be transcriptionally-regulated by DA, indicating the major influence of DA on hypothalamic

function. The hypothesis that industrial pollution (pulp and paper mill effluents) inhibits reproduction in the fathead minnow was tested by determining effects on the hypothalamic gene expression. This thesis 1) characterizes the role of DA in neuroendocrine function in fish; 2) identifies a novel mechanism of LH release mediated through DA, and; 3) demonstrates an ecologically-relevant and industrial application of the findings of this thesis.

RÉSUMÉ

La dopamine (DA) est le plus puissant inhibiteur de la sécrétion de l'hormone lutéinisante (LH) par la glande hypophyse chez les poissons et est également fondamentale pour le contrôle de la reproduction chez les vertébrés. Elle agit en se liant aux récepteurs post-synaptiques spécifiques de DA : D1 et D2. C'est parce que la DA a un fort pouvoir d'inhibition de la reproduction que nous avons émis l'hypothèse que la DA devait inhiber la stimulation de la LH via des actions multiples dans le cerveau. Les résultats obtenus à partir d'une combinaison d'approches transcriptomiques et protéomiques supportent fortement cette hypothèse. Nous avons démontré pour la première fois qu'il y avait un épissage alternatif chez le récepteur D2 des poissons. Cet épissage alternatif pourrait expliquer les différentes réponses d'inhibition de la DA. De plus, nous avons étudié une multitude de gènes et protéines contrôlés par l'action de DA. Ces résultats ont permis de comprendre les actions de la DA sur différentes voies métaboliques ainsi que des agonistes spécifiques pour les récepteurs D1 et D2 peuvent moduler ces différentes voies biochimiques. Il a également été démontré que la DA en agissant sur le récepteur D1 pourrait modifier les effets d'un autre neurotransmetteur, le glutamate, en se liant aux récepteurs de type AMPA. En effet, le blocage du récepteur D1 a fait augmenter de manière rapide et prononcée la transcription de l'activine βa et une stimulation subséquente avec AMPA a fait augmenter l'expression de l'isotocine et de la gonadolibérine de type poulet. En parallèle, les niveaux de la LH dans le système circulatoire ont aussi augmenté. De plus, la transcription d'un marqueur des cellules souches de l'hypothalamus, d'un marqueur des cellules de DA et de facteurs développementaux ont augmenté significativement dans les jours suivant l'injection d'une neurotoxine spécifique à DA. Ceci supporte l'hypothèse qu'il y aurait une régénération des

neurones de la DA dans l'hypothalamus des adultes, et de plus, serait indicatif d'une capacité intrinsèque à agir sur le réseau d'inhibition de la DA de l'hypophyse qui permettrait le rétablissement dynamique et le maintient de la fonction après une blessure. Une méta-analyse des données de biopuces a permis d'identifier 268 marqueurs de séquence exprimés qui seraient possiblement contrôler par la DA soulignant l'influence majeure de la DA dans les fonctions de l'hypothalamus. Finalement, nous avons testé si la pollution industrielle (i.e. les effluents des usines de pâte et papier) peut inhiber la reproduction des poissons à tête-de-boule en évaluant leurs effets sur les gènes exprimés dans l'hypothalamus. Cette thèse a permis 1) de caractériser les rôles de la DA dans les fonctions neuroendocrines des poissons; 2) d'identifier un nouveau mécanisme de sécrétion de la LH arbitré par la DA, et 3) de démontrer des applications écologiques et industrielles des résultats de cette thèse.

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LIST OF ABBREVIATIONS

5HT	serotonin
αMPT	α-methyl-p-tyrosine; an inhibitor of TH
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; a glutamate agonist
ANOVA	analysis of variance
CCK	cholecystokinin
cDNA	complementary DNA derived from total RNA
Cer	cerebellum
cGnRH-II	chicken GnRH II
DA	dopamine
FSH	follicle stimulating hormone; previously gonadotropin-I (GTH-I)
GABA	γ-aminobutyric acid
GnRH	gonadotropin-releasing hormone
GPCR	G-protein-coupled receptor
Gria	glutamate receptor, ionotropic, AMPA
Hyp	hypothalamus
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
LH	luteinizing hormone; previously gonadotropin-II (GTH-II)
LY 171555	quinpirole; a D2-specific receptor agonist
mesDA	mesencephalic DA
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; a dopamine-specific neurotoxin
mRNA	messenger RNA
NE	norepinephrine; noradrenaline
OB	olfactory bulb
PD	Parkinson's disease
POA	preoptic area
SCH 23390	a D1-specific receptor antagonist
SCN	suprachiasmatic nucleus
sGnRH	salmon GnRH
SKF 38393	a D1-specific receptor agonist
SN	substantia nigra
sulpiride	a D2-specific receptor antagonist
Tel	telencephalon
TH	tyrosine hydroxylase; the rate-limiting enzyme in catecholamine biosynthesis
TPp	periventricular nucleus of posterior tuberculum

Chapter 1.

GENERAL INTRODUCTION*

1.1. INTRODUCTION

Teleosts represent more than half of all vertebrate species and are adapted to a wide range of marine and freshwater habitats (1). Numerous characteristics of the goldfish (*Carassius auratus*; reviewed in (2)) make this species an excellent model for understanding neuroendocrine signaling and the regulation of reproduction in vertebrates, including commercially important teleost fish.

In temperate climates in the northern hemisphere, goldfish reproduce annually in April and May. This reproductive cycle is primarily regulated through the release of luteinizing hormone (LH; previously called gonadotropin-II or GtH-II) which is structurally and functionally similar to mammalian LH (3). LH is important because it is an essential regulator of annual gonadal growth cycles, sex steroid and sex pheromone synthesis, and sperm production in males or ovulation in females during the breeding season. Failure of an environmental or endocrine signal to activate the neural LH release mechanisms at spawning may lead to reduced fertility. LH release is regulated by the stimulatory and inhibitory actions of multiple forms of gonadotropin-releasing hormone (GnRH) and dopamine (DA), respectively (3-5). GnRH and LH release are regulated by interactions between a multitude of classical neurotransmitters and neuropeptides (2) (Figure 1.1). In teleosts, the gonadotrophs, as well as other endocrine cells in the anterior pituitary, are directly innervated (6). Interestingly, the teleost pituitary is highly regionalized, with gonadotrophs being

* *Modified from*: 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

clustered in the proximal pars distalis in association with somatotrophs (6), which allows for the precise determination of the preoptic telencephalic and hypothalamic origins of hypophysiotropic inputs to the pituitary using tract-tracing methods (7). This is in contrast to mammals and other tetrapods, which have a hypothalamic-hypophyseal blood portal system, and for which multiple neurotransmitter and neuropeptidergic inputs converge at the median eminence. It is thus very difficult to determine the origins of the hypophysiotropic neurons in tetrapod models. As a consequence, recent work has taken advantage of the regionalized distribution of cells in the fish pituitary to demonstrate a unique reciprocal paracrine relationship between gonadotrophs and somatotrophs that is mediated by LH and growth hormone (GH) (8).

The interactions between the neuropeptide GnRH, the catecholamine DA and amino acid γ-aminobutyric acid (GABA) (Figure 1.1) form the central core of our understanding of the integrated control of LH in fish, which has been compared previously to parallel systems in the rat (2). The multiplicity of peptides and receptors and the central role of GnRH has been reviewed rather extensively (9-11), and will not be covered here. Numerous other neuropeptides and neurotransmitters are involved (2, 12), and with the exception of the indoleamine serotonin (5HT), our understanding of their roles in fish is rather limited. Here we will review the roles of DA, GABA and 5HT, and advance the hypothesis that modulation of these systems is at the foundation of complex control of LH release, and thus reproduction in a vertebrate. Recent advances in transcriptomic analysis reveal that neurotransmitters not only control pituitary hormone release, but have rapid and profound receptor-mediated effects to regulate gene expression in the neuroendocrine brain.

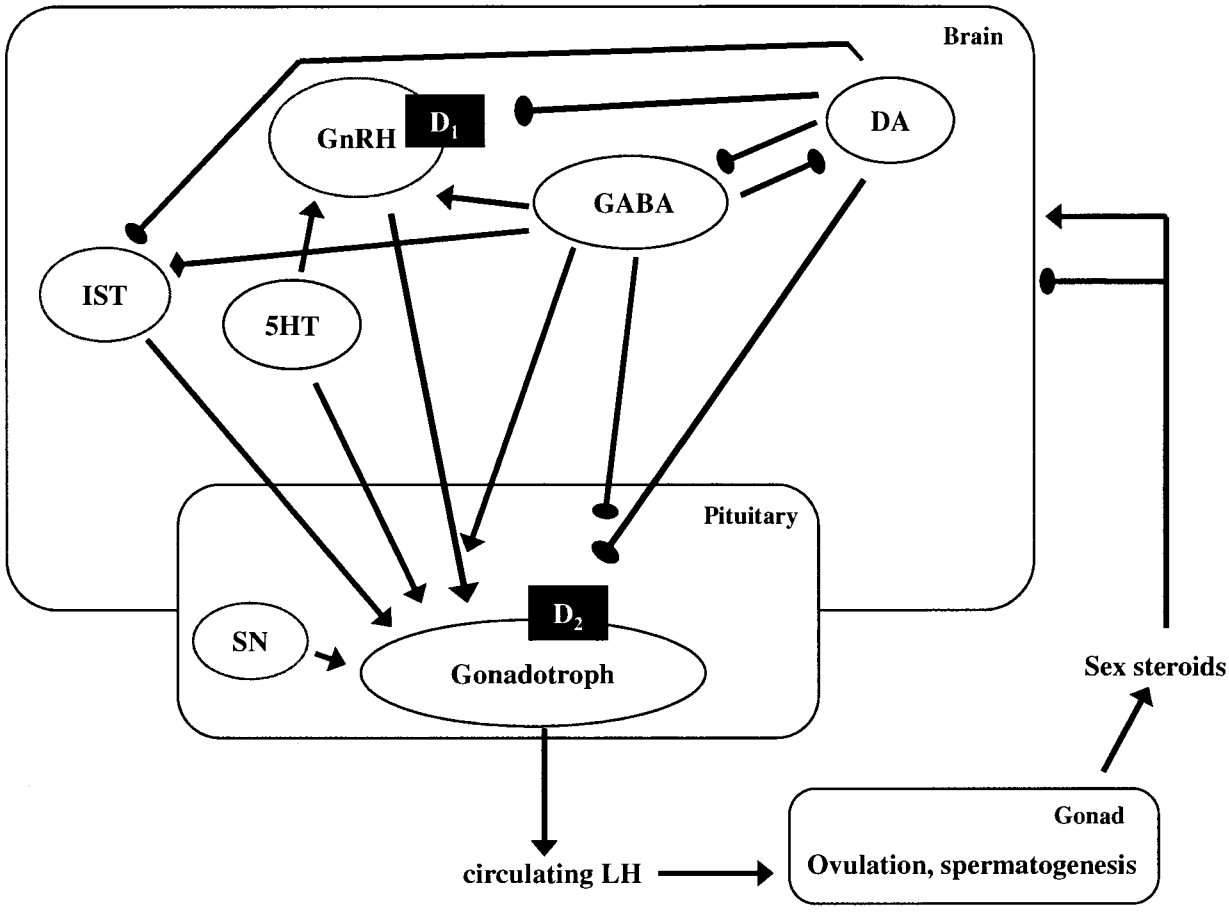


Figure 1.1: Schematic representation of the reproductive neuroendocrine axis in the brain of the goldfish. Arrows indicate stimulation. Bulbous arrows indicate inhibition. The diamond-tipped arrow indicates a speculated pathway. See text for abbreviations.

1.2. THE GOLDFISH MODEL SERVES MULTIPLE DISCIPLINES

The availability of model organisms with unique characteristics as well as a wide range of research reagents and tools drive research discoveries in the biological sciences. The common goldfish has served this purpose for more than 3 decades. As a member of one of the largest vertebrate families, the *Cyprinidae*, goldfish are related to important ecological and genetic models, for example fathead minnows and zebrafish, and to economically important cultured carp species. Perhaps the most significant scientific advances resulting from research on goldfish are largely related to neuroendocrine signaling and how the brain regulates growth, feeding, reproduction, pituitary and gonadal physiology, sex pheromones and behaviour, and stress response (13-20) (and references therein). Indeed, basic discoveries on reproductive neuroendocrine signaling in goldfish by R.E. Peter's group led to the development of an internationally successful commercial spawning kit named OVAPRIM® and marketed to the aquaculture industry by Syndel Inc. (www.syndel.com) in Vancouver, BC.

Goldfish also serve as useful model organisms in the fields of cell biology, immunology, toxicology, endocrine disruption, molecular evolution and comparative genomics, neurobiology, olfaction, learning and memory, vision, and taste (21-30).

1.3. DOPAMINE (DA) IS THE KEY INHIBITOR OF LH RELEASE

The catecholamines, dopamine (DA), norepinephrine (NE), and epinephrine (E) are all produced from tyrosine in a sequential manner. DA is synthesized from the hydroxylation and subsequent decarboxylation of tyrosine through the action of the enzymes tyrosine hydroxylase (TH) and DOPA decarboxylase (DDC; also known as aromatic amino acid decarboxylase, AAADC), respectively. DA can then be converted into NE through

further hydroxylation by dopamine β-hydroxylase (DBH), and subsequently, into E through the methylation of NE via phenylethanolamine N-methyltransferase (PNMT). TH catalyzes the rate-limiting step in the process (31) and is regulated by a variety of different mechanisms (reviewed by (32)). It was recently shown that goldfish possess olfactory sensitivity to dopamine and epinephrine as well as their 3-O-methoxy derivatives, metadrenaline and 3-O-methoxytyramine (33), raising the intriguing possibility that catecholamines and/or their metabolites may be used in external chemical communication. DA is the only identified inhibitor of LH release in goldfish and is well studied and will be addressed further. In contrast, the role of NE and E are less clear and will not be discussed.

Dopamine is involved in 4 main pathways in the vertebrate CNS: the mesolimbic (pleasure and reward, addiction), the mesocortical (learning and memory), the nigrostriatal (movement, and hence Parkinson's disease), and the tuberoinfundibular pathways. Neuroanatomical features of the catecholaminergic systems in goldfish and zebrafish are well described (34-42). The area dorsali telencephali, pars centralis has been identified as being the striatum based on the presence of TH and lack of DBH immunoreactivity (43). There is still considerable debate to whether fish possess a defined nigrostriatal pathway. Based on hodologic and anatomical studies in the Senegal bichir (44) and zebrafish (37), the periventricular posterior tuberculum (TPp) is a good candidate of the ventral tegmental area and substantia nigra of tetrapods. However, based on functional and neurochemical studies by dopamine depletion via 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; a selective dopaminergic neurotoxin) in goldfish (45-48), it appears that the telencephalic nucleus pars dorsalis is also a good candidate as the fish homolog of the tetrapod substantia nigra. A more recent study in the zebrafish (34) provides support to both tuberal and intrinsic telencephalic

nigrostriatal hypotheses; however, the authors of the study are more inclined towards the tuberal hypothesis based on their results. The lack of information of a definitive substantia nigra in fish is a disadvantage not only to the goldfish model, but to all fish models, for the study of diseases involving movement disorders such as Parkinson's disease.

While goldfish have been used to investigate the role of DA in motivation, memory, and learning (49-52), most research involving DA and fish has been in the area of reproduction and growth. However, the role of DA in reproduction is not limited to fish, as it is implicated in the reproduction of amphibians, birds, mammals including humans, and even crustaceans (53).

In goldfish, the gonadotrophs located in the proximal pars distalis (54) are directly innervated by GnRH neurons originating in the anteroventral pre-optic area and latero-basal hypothalamus (6, 55, 56). A surge of LH is responsible for ovulation (57) and it is clear that ovulation is due in part to the disinhibition of DA (58, 59). Two main TH-immunoreactive (TH-ir) tracts were traced from preoptic cells to the infundibulum in the goldfish (60). DA has been shown to inhibit LH release directly from the goldfish pituitary (5) as well as GnRH-stimulated LH release (61). Significantly, it is the only identified inhibitor of LH in this species (2, 4). Several intracellular signaling pathways are involved in LH synthesis and release in gonadotrophs, and DA may act to inhibit several points in these signal transduction cascades (62), including inhibition of GnRH-induced Ca^{2+} mobilization, cAMP generation, and protein kinase C-dependent mechanisms (63, 64). DA has also been shown to down-regulate goldfish pituitary GnRH receptors (61) and to inhibit the stimulatory actions of GABA (Figure 1.1; for review see (2, 12)). Levels of mRNA for the GABA synthetic enzyme glutamic acid decarboxylase 67 (GAD67) have been shown to be up-regulated in

goldfish telencephalon and optic tectum (65) by dopaminergic loss following MPTP injections, suggesting that DA inhibits the production of GABA. Furthermore, the tyrosine hydroxylase inhibitor, α-methyl-*p*-tyrosine (αMPT), has also been shown to significantly deplete DA levels in the goldfish brain and pituitary (66, 67) and to potentiate the response of LH to GnRH (4). DA has recently been found to be implicated in maintaining the European eel, *Anguilla anguilla*, in a pre-pubertal state for many years (53).

In order for DA to be such a potent inhibitor of LH secretion and reproduction, we propose that it must inhibit multiple LH-stimulatory systems. Various pharmacological manipulations reducing DA function also potentiate GABA-mediated LH release (68). DA depletion increases GABA synthesis [68] supporting our working hypothesis of reciprocal DAergic and GABAergic inhibition (Figure 1.1) as an element in the control of LH release (69). Moreover, this inhibition of DA synthesis by GABA is at least partially mediated by rapid effects to decrease the expression of TH (70). There are numerous other neurohormones that stimulate LH release in goldfish (2, 12). Dopaminergic inhibition of these multiple other stimulatory signaling pathways is suspected but remains to be fully substantiated experimentally. There is, however, one example that serves to illustrate this point. Secretogranin-II (SGII) is a large secretory vesicle protein and well characterized marker of the regulated secretory pathway that undergoes processing by prohormone convertases to produce the bioactive peptide secretoneurin (SN). SN is a 34 amino acid peptide that stimulates LH release in goldfish pretreated with a DA receptor antagonist (71, 72), very much a corollary to the situation with GnRH-stimulated LH release at some periods of the reproductive cycle (2, 4).

Dopamine exerts its effects via seven-transmembrane domain, G-protein-coupled receptors, which are separated into the D1 and D2 receptor classes (73). The 2 classes differ both structurally and functionally (Table 1.1; reviewed in (74, 75)), as well as pharmacologically (reviewed in (76)). The current hypothesis is that D1 and D2 receptors arose through convergent evolution and independently evolved the ability to bind DA (75).

Table 1.1: Comparison of the D1 and D2 receptor classes in the goldfish
(derived from Callier *et al.* (74))

DA receptor class	D1	D2
Activity	stimulate AC[a]	inhibit AC; modulate VGCC[b] and VGKC[c]
Subtypes	D1A/D1, D1B/D5, D1C	D2, D3, D4
Third cytoplasmic loop	short	long
C-terminal	long	short
G protein	G_s/G_{olf} class of G_α proteins	G_i/G_o class of G_α proteins
Genomic DNA	intronless	several large introns

[a] adenylate cyclase
[b] voltage-gated calcium channel
[c] voltage-gated potassium channel

Generally, the D1 class of receptors stimulate cAMP production whereas the D2 class of the receptors inhibit cAMP production (73), and also modulate the activity of voltage-gated calcium and potassium channels. The actions of dopamine receptors are achieved via the coupling of a G-protein. The various G-proteins coupled to dopamine receptors has been reviewed (77).

The D1 class can be subdivided into the D1A/D1, D1B/D5, and D1C receptor subtypes in fish (reviewed in (78)). The D2 receptor class can be further categorized into the D2, D3, and D4 subtypes. In mammals, two alternately-spliced D2 receptors have been identified as having short (D2S) or long (D2L) third cytoplasmic loops, differing by 29 amino acids (79). There is currently no evidence that fish or amphibians contain an alternatively spliced variant of the D2 receptor (80-84), suggesting this post-transcriptional modification occurred recently in the vertebrate lineage (82). Partial DNA sequences for DA receptors, DA transporter (DAT) as well as for the biosynthetic enzymes for DA and NE have been identified in goldfish (Table 1.2).

Many factors contribute to the development of DAergic neurons. This has been most studied in the zebrafish, and include regulation by numerous transcription factors and signaling pathways including the Orthopedia homeodomain (85), neurogenin1 (86), pax6 (87), tof/fezl (88), as well as Shh, FGF8, Nodal/TGF, and retinoic acid (reviewed in (89)). It was recently found that the D2 receptors are expressed developmentally before the D1 receptors in zebrafish (90). In what has been termed D1/D2 synergism (91), some effects of dopamine action are observed, in mammals, only if both D1 and D2 receptors are stimulated concurrently (reviewed in (92)). Further experimentation in fish is needed to establish if this

occurs in lower vertebrates and whether this receptor synergism has a role to play in the control of reproduction.

Plasma membrane transporters modulate the action of neurotransmitters by neurotransmitter reuptake by the presynaptic axon and surrounding glial processes (93). The DA receptors and transporter are the primary targets in the treatment of schizophrenia and Parkinson's disease, and therefore have a rich assortment of effective pharmacological agents (for review see (74, 76, 94-96)). Domperidone, a selective D2 receptor antagonist, has been shown to decrease DA in the goldfish pituitary without affecting DA levels in the hypothalamus or telencephalon, as it does not cross the blood-brain barrier (97). Quinpirole, a selective D2 receptor agonist, has been shown to reduce the mRNA level of the tilapia GnRH receptor (98). Quinpirole and SKF 38393, a selective D1 receptor agonist, which are both able to cross the blood-brain barrier, were used separately to examine their effects on gene expression in the hypothalamus of goldfish, compared with other neurotransmitter systems (Table 1.3, Figure 1.2).

Table 1.2: Partial coding sequences for DA receptors, transporter, and biosynthetic and degradation enzymes cloned from the goldfish brain

Gene	Function	Accession Number
D_{1B}	receptor	EF377327
D_{1C}	receptor	EF396233
D_2	receptor	EF382625
D_3	receptor	EF382624
D_4	receptor	EF640988
DAT	transporter	EF371919
TH	anabolic enzyme	AY644727
DDC	anabolic enzyme	EF371918
DBH	catabolic enzyme	EF396232

Note: the D_{1A} receptor had previously been cloned from the goldfish retina (99), and was subcloned by our laboratory

Table 1.3: Descriptions of the experiments used in the cluster analysis

Experiment	Mechanism	Date	GSI[a] (%)	Description	Exposure	Sex
MPTP + αMPT	DA depletion	early-May	4.7 ± 0.6	i.p.-injected 50 µg/g MPTP on Day 0, i.p.-injected 240 µg/g αMPT on Day 5	24h after final injection	Female
SKF 38393	D_1 agonist	mid-May	4.5 ± 1.3	i.p-injected 40 µg/g	5h	Female
Quinpirole	D_2 agonist	mid-May	4.5 ± 1.3	i.p-injected 2 µg/g	5h	Female
Fluoxetine	SSRI[b]	mid-Dec	N.D.[c]	i.p.-injected 5 µg/g twice a week for 17 days, for a total of five injections	24h after final injection	Female
Muscimol	$GABA_A$ agonist	late-Aug	N.D.[d]	i.p.-injected with 1 µg/g	6h	Female
Baclofen	$GABA_B$ agonist	early-Sep	N.D.[d]	i.p.-injected with 10 µg/g	6h	Female

[a] Gonadosomatic index
[b] Selective serotonin reuptake inhibitor
[c] not determined; expected GSI for mid-Dec is approximately 2.5% (100).
[d] not determined; expected GSI for this time of year is approximately 2% (100). A separate experiment conducted in mid-Sept confirmed a GSI for female goldfish of 2.1 (± 0.1)% (unpublished).

Studies using the goldfish have demonstrated that intraperitoneal (i.p.) administration of MPTP causes a selective depletion of DA and NE, without altering the serotonergic system (45-48, 65) and induces a parkinsonian syndrome which parallels that of mammals (48). A light and electron microscopic study on the effect of MPTP on DAergic neurons in the goldfish brain showed progressive irregularities occurring on the contours of the neuronal nuclei and progressive granularization of nucleoplasm, suggesting an apoptotic mechanism of cell damage (45). These results led Pollard *et al.* (101) to propose the goldfish as a model for drug discovery in Parkinson's disease research. While MPTP is an irreversible neurotoxin in mammals, including primates and humans (102-104), goldfish appear to completely recover to basal DA and NE levels after 6 weeks (105). The study was unable to determine whether catecholamine recovery was due to regenerative processes or by metabolic compensation of the surviving neurons. Nevertheless, these results are suggestive of a degree of plasticity in DAergic systems in the adult fish brain as is the case in the fish optic system (106, 107). We have used a similar MPTP-treated goldfish model, in combination with αMPT, to explore the effects of DA depletion on LH release and regulation of hypothalamic gene expression (Table 1.3 and Figure 1.2).

Dopamine neurons may also mediate steroid negative feedback on LH release. The inhibitory action of DA on LH varies during the seasonal reproductive cycle in goldfish (66). Reducing the DAergic inhibition of LH, for example, by blocking DA action through specific DA antagonists, has been shown to significantly enhance LH secretion in the goldfish, particularly in late ovarian recrudescence (4, 97, 108, 109) suggesting that the tone of DA inhibition increases in parallel with gonadal development and increased steroid secretion (reviewed by (2)). Indeed, testosterone and estradiol increase pituitary dopamine

turnover rates, and the serum LH response to DA antagonist injections relative to sexually regressed fish without supplemental steroids (66). While this has yet to be demonstrated conclusively, this is likely a direct effect of the sex steroids on DA neurons. A neuroanatomical study of the trout demonstrated that TH-positive DA neurons express the estrogen receptor-α (ERα) protein (110) whereas trout GnRH neurons do not express the ERα (111). In marked contrast, the positive feedback actions of the sex steroids do not appear to involve changes in DA function, but rather are a result of increased pituitary sensitivity to GnRH, and increased GABAergic activity (66, 68). The co-activation of both the positive and negative sex steroid feedback mechanisms during the seasonal reproductive cycle allows for dynamic and sensitive control of LH release and thus gonadal development and function (2, 3).

Clearly, DA serves a key inhibitory role in reproduction through multiple mechanisms, and is intricately controlled through the actions of other neurotransmitters (e.g. GABA) and sex steroids.

1.4. DETERMINING POTENTIAL GENOMIC INTERACTIONS AND PATHWAYS REGULATED BY DA, GABA AND 5HT

From multiple microarray experiments (Table 1.3), we collected raw data and performed unsupervised, average-linkage hierarchical clustering (112) to investigate the interactions of different neurotransmitter systems on gene expression in the female goldfish hypothalamus (Figure 1.2; see caption for method). Clustering was performed in two ways. First, by clustering genes across all of the treatments, co-expressed groups can be delineated, many of whom share common functional properties or participate in coherent biological pathways or processes. Secondly, by clustering between treatments it is possible to use the

extraordinarily rich datasets from the array to identify which treatments are most closely related to other treatments in terms of the overall gene expression profile. Thus, it may be possible to identify shared regulatory sequences by bioinformatic methods such as Gibbs sampler (113) in the sets of co-upregulated or co-downregulated genes.

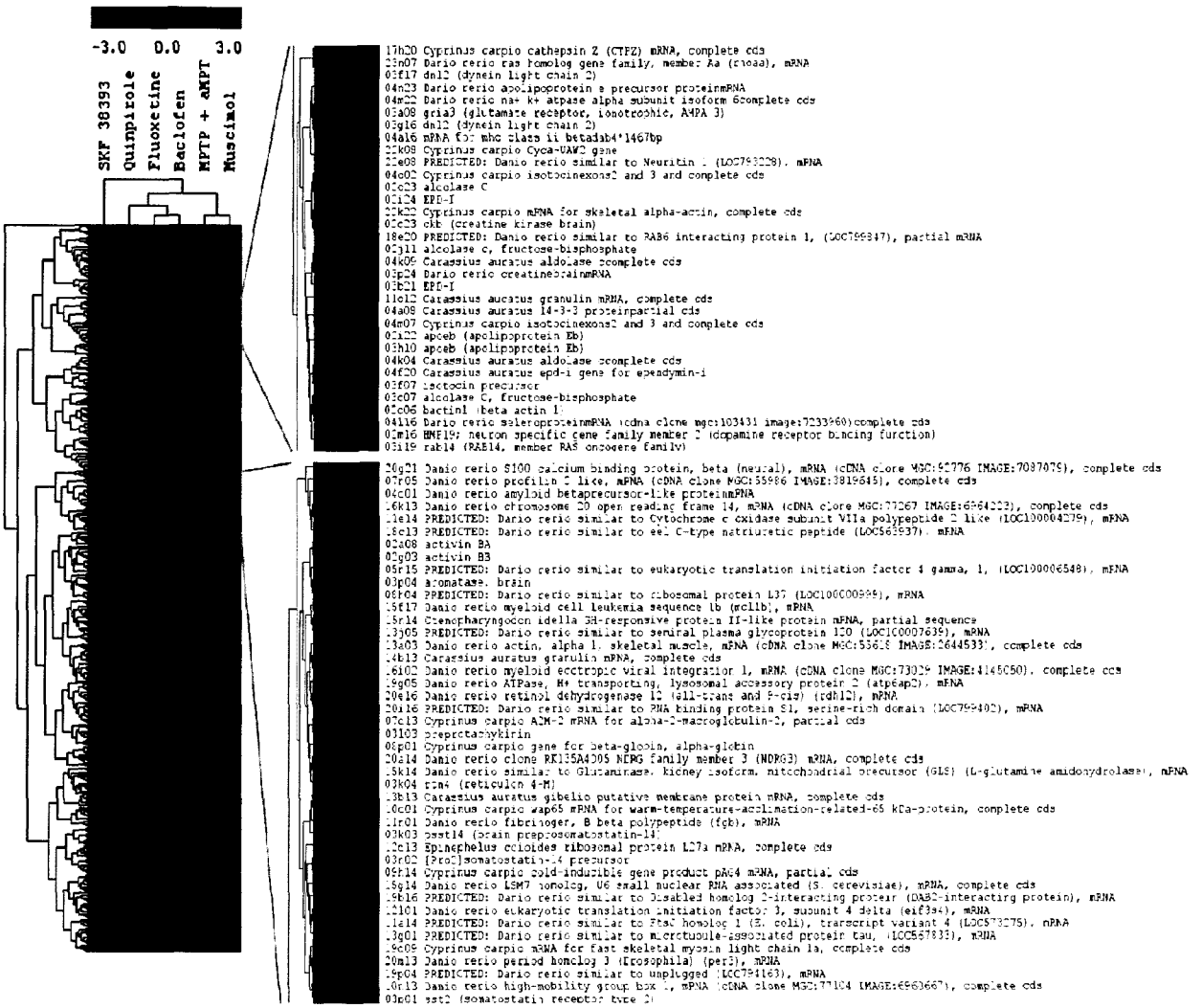


Figure 1.2: Cluster diagram of multiple microarray experiments on hypothalamic tissues from female fish injected with various pharmaceuticals. Microarray data was retrieved from six different studies (Table 1.3) subjected to goldfish brain cDNA microarray. The microarrays of experiment were hybridized using the common reference design (114). For each study, signals were normalized using a locally weighted scatterplot-smoothing regression (Lowess; (115)) to remove intensity dependent noise. Modified t tests with q values (false-discovery rate) management were used for statistical analysis. The sets of cDNAs were ranked based on q values and FDR threshold for the identification of differentially expressed genes was set to 0.05 (116). 1932 cDNA genes were selected with differential expression at least in one study (q values<0.05 and fold-change >1.5), and were further reduced in number by removing unclassified and/or unannotated ESTs (1415). The resultant 517 annotated genes were used to perform unsupervised, average-linkage hierarchical clustering (112). The complete colour image is available in the Supplementary Material online (69).

Somewhat surprisingly, the quinpirole, fluoxetine, and baclofen experiments clustered together, suggesting similarities in the effects of these drugs on gene expression in the hypothalamus. However, these 3 pharmaceuticals all target G-protein-coupled receptors either directly (quinpirole and baclofen) or indirectly (fluoxetine). An additional unexpected result was that the muscimol experiment clustered with the catecholamine depletion experiment (MPTP + αMPT). We speculate that this is indicative of the inhibitory effects that GABA has on both DA (Figure 1.1) and NE systems in goldfish brain (68, 117). The D1 agonist experiment (SKF 38393) did not cluster with any of the other experiments, reflecting a distinct pharmacological profile and differential distribution of D1 versus D2 receptors in the brain. These data demonstrate that the carp-goldfish microarray (15) coupled with cluster analysis can distinguish between pharmacological manipulations. There are genes both commonly and differentially regulated by these treatments (Figure 1.3).

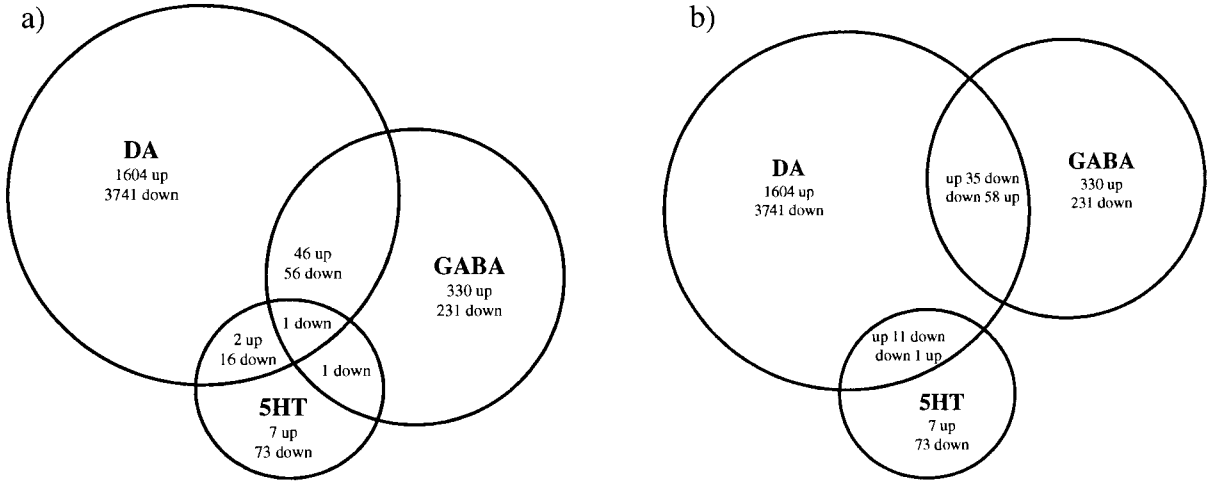


Figure 1.3: Number of mRNAs identified by cluster analysis as being a) commonly regulated or b) differentially regulated by different neurotransmitters in the hypothalamus of female goldfish.

While it is not our intention to describe in detail all the results of the cluster analysis, there are several interesting transcripts that warrant further discussion here. Modulation of the DA, GABA and 5HT systems all lead to effects on isotocin gene expression (Table 1.4). This is intriguing since isotocin is a reproductive neuropeptide and the fish homolog of mammalian oxytocin (see section 1.5). Activin subunits were also identified by microarray cluster analysis and will be discussed further (see section 1.6).

Table 1.4: Isotocin mRNA expression following various neurotransmitter system modulations in the hypothalamus of female goldfish as determined using cDNA microarray.

Experiment	Effect on mRNA
MPTP + αMPT (DA depletion)	Up
SKF 38393 (D_1 agonist)	Up
Quinpirole (D_2 agonist)	Down
Fluoxetine (SSRI[a])	Down
Muscimol ($GABA_A$ agonist)	Up
Baclofen ($GABA_B$ agonist)	Down

[a] selective serotonin reuptake inhibitor

1.5. ISOTOCIN IS A REPRODUCTIVE NEUROPEPTIDE REGULATED BY MULTIPLE NEUROTRANSMITTER SYSTEMS

Isotocin (IST) is a nine amino acid neuropeptide synthesised in magnocellular and parvocellular neurons of the preoptic area, which project widely in the brain, especially in the telencephalon, hypothalamus and to the neural lobe of the pituitary (118). It is the teleost homologue of mammalian oxytocin (OXT) and has roles in a variety of physiological functions, for example, ion homeostasis at the level of the gill (119, 120). Importantly, there

is accumulating evidence for its role in fish reproduction. Pickford *et al.* (121) first described the stimulating effect of injected IST on spawning in killifish, and circulating IST levels have been found to vary seasonally in female three-spined sicklebacks, with the highest levels occurring in July, the period of reproduction for this long-day breeder (122). A similar peak was found in brain IST mRNA expression in female masu salmon, in May, correlating with high estradiol levels (123). The IST gene has been shown to be estrogen-responsive, in that it possesses 3 estrogen response elements (ERE) in teleosts (124). The number of IST-immunoreactive neurons was found to be sexually dimorphic in medaka and decrease significantly after spawning in females (125). Some preoptic neurons in the dwarf gourami, *Colisa lalia*, stained positively for both IST and GnRH (126), further supporting a role of IST in reproduction. In rainbow trout, both chicken GnRH-II and salmon GnRH immunopositive nerve-fibers have been found to be in close proximity to IST neurons and application of either GnRH form generated synchronous Ca^{2+} pulses in IST neurons (118). IST is also involved in sex specific vocal courting behaviours in plainfin midshipman fish, *Porichthys notatus* (127), and changes during socially induced sex-change in blue banded goby, *Lythrypnus dalli* (128). Effects of OXT on LH (129-131) and steroid (132-134) release have been described for mammalian models *in vivo* and *in vitro*. Similarly, in rainbow trout, IST has been found to stimulate testosterone release at the level of the testis *in vitro* (135). Furthermore, a stimulatory effect of mammalian OXT on sperm release has been shown in the African catfish (136). The IST receptor mRNA has been quantified in different tissues of the reproductive axis in the white sucker, *Catostomus commersoni*, for example, brain and ovary (137), further substantiating its status as a reproductive neuropeptide.

MPTP, in combination with αMPT, reduced DA and NE levels by 70% in telencephalon and by 80% and 87%, respectively, in the hypothalamus of goldfish, relative to saline-injected controls as determined by high-performance liquid chromatography (Figure 6.1). This caused a statistically significant ($p<0.05$) 1.8-fold decrease and a 1.3-fold increase of IST in the telencephalon and hypothalamus, respectively, as determined by real-time RT-PCR (unpublished). This result in the hypothalamus was confirmed in our microarray experiment (Figure 1.2, Table 1.4). The differences in the IST response in these tissues may possibly be due to dopaminergic action through different receptor classes in the hypothalamus compared to the telencephalon.

The other neurotransmitter-active drugs studied in this meta-analysis also had effects on IST. A two-week fluoxetine treatment resulted in a 6-fold decrease of IST mRNA in the hypothalamus (138). The $GABA_B$ receptor agonist, baclofen, had an inhibitory effect on IST (down ~1.5-fold) whereas the $GABA_A$ receptor agonist, muscimol, had a stimulatory effect on IST (up ~1.3 fold) (Figure 1.2). It is unclear at this time whether, or which, neurotransmitter(s) are having direct effects on IST release. However, electrophysiological studies in the rat support the hypothesis that DA neurons directly innervate OXT neurons as they express functional D2 receptors (139), and TH-ir fibres are present in the anterior part of the parvocellular preoptic nucleus of the zebrafish brain (34). Furthermore, *in situ* hybridization studies in the rainbow trout revealed a strong signal for the D2 receptor mRNA in the parvocellular preoptic area, but no signal was found in the magnocellular preoptic area (84). As no 5HT-ir fibres were found in close proximity of the magnocellular or parvocellular preoptic areas of the zebrafish (34), the effects of 5HT on IST mRNA expression are hypothesized to be indirect, potentially by modulation of DAergic neurons.

There is currently no evidence for the co-localization of GABA receptors on IST neurons in fish; however there is strong evidence for direct GABAergic regulation of OXT neurons in the rat (140). It is speculated that GABA in goldfish may be affecting IST mRNA levels both directly and indirectly by modulating DAergic neurons (Figure 1.1).

1.6. ACTIVINS ARE GROWTH FACTORS IMPLICATED IN NEUROENDOCRINE CONTROL AND NEURONAL REPAIR

The activins, members of the transforming growth factor β (TGF-β) superfamily (141), are homo- or heterodimeric proteins. They are composed of 2 βa subunits (activin A), 2 βb subunits (activin B) or 1 βa and 1 βb subunit (activin AB). Activins were originally isolated because of their abilities to stimulate FSH release from the mammalian pituitary. In goldfish, activin B stimulates FSHβ mRNA expression while inhibiting LHβ mRNA expression *in vitro* (142). Furthermore, the activin binding protein follistatin was found to have the opposite effect; it inhibits FSHβ while stimulating LHβ expression in the goldfish pituitary (143). Activin βa, βb, as well as activin receptor subunits IB and IIB are also expressed in the goldfish brain (144). Activin, once bound to the Type II receptor, forms a complex along with the Type I receptor which activates the Smad family of transcription factors (145). Full-length cDNAs for Smads2 and 3 (regulatory, or R-Smads), Smad4 (co-Smad), as well as Smad7 (an inhibitory, or I-Smad) have recently been cloned in goldfish (144). Although the authors focused on these transcription factors in goldfish pituitary, it is interesting to note that they are also expressed in the brain. In rat brain, activins and their receptors are expressed in neurons (146). The effect of activins and the role of neurotransmitter-mediated modulation of activin expression on the fish brain are a matter of

speculation but are likely to be involved in LH release by enhancing GnRH production in the brain (70, 147, 148).

The activins are implicated in many processes, including development, reproduction, behaviour, neuronal stem cell differentiation and neuronal repair (146, 149-151). Given that DA is a major neurotransmitter and is implicated in many of the same functions, it may be that DA receptor-mediated modulation of activin βa expression is also important for maintenance of neuronal function in addition to a role in neuroendocrine signaling in the adult brain.

1.7. CONCLUSIONS

Endocrine systems regulate reproduction, development and growth, and exist for the purpose of the organism to propagate their genomes. Model systems are essential to the advancement of biology and the goldfish has featured prominently in numerous fundamental discoveries concerning neuroendocrine regulation of reproduction. The central role of DA as the potent inhibitor of LH release, and GnRH and a multitude of other stimulatory neurohormones has helped to advance the concept of inhibitory-stimulatory duality of control mechanisms in the brain and pituitary. Part of the utility of the model resulted from the pioneering work of R.E. Peter and his brain atlas (152) and classic electrolytic lesioning studies delineating the brain regions important for neuroendocrine signalling (4). Many reagents are now available, including recombinant hormones and cytokines and there are numerous fields employing the goldfish model.

Recently, we initiated an expressed sequence tag (EST) sequencing project to address one of the main limitations of the goldfish model: a lack of gene sequence information.

Increasing availability of ESTs for goldfish, together with the ~40K ESTs available for the common carp, *Cyprinus carpio* (Cossins, A., unpublished sequences and (153)), and the recent development of a goldfish bacterial artificial chromosome library (26) is beginning to address this limitation. Similar efforts for genes in the goldfish immune system are underway (154). We have also demonstrated the utility of microarray analysis and associated bioinformatics for research on neuroendocrine signaling. With these techniques, we have found that multiple genes involved in reproduction are differentially regulated by various neurotransmitter systems in the brain, and have begun to elucidate the complexity of the neural pathways involved in reproduction. It will soon be possible to study the concept of neuroendocrine-immune interactions in fish (23, 154, 155). Moreover, there are numerous other gut, pituitary and brain peptide genes being sequenced, and certainly the goldfish model will continue to contribute significantly to our understanding of neuroendocrine control of growth and feeding (18, 156).

Highly complementary resources are available for the common carp (carpBASE; http://legr.liv.ac.uk), a closely related species. While zebrafish are without doubt excellent models for developmental biology and toxicology, they remain a challenging organism for basic physiological studies because of their small size and lack of pituitary hormone assays. Thus physiological genomic analysis of reproduction is somewhat limited in zebrafish. Importantly, however, the vast resources for zebrafish as well as Fugu and medaka models make it relatively easy to categorize goldfish ESTs using comparative genomic approaches. Meta analysis of expression data from numerous neuropharmacological experiments in goldfish helps to focus attention on key neuroendocrine systems, for example IST (127) and activins (142), whose functions in the teleost brain are only partially characterized. What we

have discovered is that hundreds of goldfish ESTs derived from brain cannot be found in the other fish genomes. The 1415 unidentified ESTs derived from both goldfish and carp that respond to neuroendocrine manipulations in the goldfish brain may be derived from the highly divergent untranslated regions of mRNAs already isolated. Others may be unique sequences from coding regions that we cannot yet characterize, representing new genes. They may also represent non-protein coding transcriptional products that participate more widely in biological regulation through RNA/RNA, RNA/DNA and RNA/protein interactions. Together these uncharacterised products provide rich biological material for future analysis of neuroendocrine signaling in the vertebrate brain.

1.8. THESIS OUTLINE AND RATIONALE

While there is considerable knowledge of DAergic regulation of LH release at the level of the pituitary in fish, there is a general lack of knowledge of gene expression regulation by DA in higher brain centres in the context of reproduction. This thesis begins to address that limitation by investigating the role of DA on gene expression in the hypothalamus and telencephalon of the goldfish. Together, the hypothalamus and the telencephalon make up the neuroendocrine brain and it is hypothesized that these brain regions control the coordinated events of reproduction by integrating signals from internal and external sources.

1.8.1. Hypotheses

1) DA modulates novel genes in the neuroendocrine brain related to reproduction

2) DA modulates novel systems in the neuroendocrine brain related to reproduction

To address these hypotheses I investigated the underlying molecular mechanisms involved in the inhibitory tone of DA on reproduction in the goldfish neuroendocrine brain.

This thesis is a collection of manuscripts that are either published (Chapter 1) or are in preparation for publication (Chapters 2-6 and Appendix A). There was an effort to reduce redundancies throughout the thesis where and if possible. Chapter 2 describes the identification of an alternatively-spliced isoform of the D2 receptor that has long-been hypothesized to exist but has never been shown in fish, despite active efforts. This D2 short isoform is discussed in the context of its fundamental role in creating a high inhibitory tone of DA prior to ovulation. Chapter 3 demonstrates the wide-scale genomic and proteomic effects of DA in the hypothalamus and allowed us to hypothesize, based on the results, that DA, acting through the D1 receptor, may play a role in the brain by inhibiting LH release from the pituitary. The implication of D1 involvement in reproductive processes in fish was dismissed many years ago (157). Nevertheless this hypothesis was tested in Chapter 4 and shows that indeed, by blocking the D1 receptor when fish are in the spawning season, circulating LH levels are significantly increased when challenged with a glutamate mimic, but alone, neither treatment had any effect. In association with this increase circulating LH levels, an increase in IST, activin βa, and cGnRH-II mRNA levels was observed. The findings from this study suggest a novel mechanism of LH release from the pituitary of the goldfish.

Chapter 5 provides evidence suggesting that DAergic neurons in the hypothalamus spontaneously regenerate following chemical lesioning using a DA-specific neurotoxin. While this was hypothesized by Poli *et al.* (105) years ago, it has never been examined until now. The findings from this study may provide insights into novel methods of treatments for

Parkinson's disease in humans. Furthermore, these findings suggest that DA neuronal populations in the hypothalamus, some of which project to the pituitary (43), may be important in modulating the inhibitory tone of DA throughout the reproductive cycle in fish.

In Chapter 6, a number of microarray datasets collected during the tenure of this thesis were compared to identify mRNAs that are very likely to be regulated by DA. This novel approach reduced the complexity of analyzing large volumes of data by identifying approximately 300 out of the 8000 sequences on a microarray as regulated by DA. The findings of that study provide a rich platform for future investigations of the DAergic regulation of gene expression in the neuroendocrine brain and signify a major advance in studying neuroendocrine processes in fish. Taken together, this thesis has characterized the DAergic system in goldfish to gain a better understanding of the role of DA in neuroendocrine function and reproduction. Furthermore, it provides a platform for understanding the modes of action on the brain by endocrine-disrupting chemicals. An example of this is provided in Appendix A.

Chapter 2.

EVIDENCE FOR ALTERNATIVE SPLICING OF THE DOPAMINE D2 RECEPTOR IN THE GOLDFISH NEUROENDOCRINE BRAIN[†]

2.1. INTRODUCTION

Dopamine (DA) is an important neurotransmitter in both vertebrates and invertebrates. In the vertebrate central nervous system, DA is involved in four main pathways: the mesolimbic (pleasure, reward, and addiction), the mesocortical (learning and memory), the nigrostriatal (movement), and the tuberoinfundibular (hormonal regulation) pathways. In humans, DA is the main inhibitor of prolactin synthesis and release (158) and DA agonists are the first treatment choice in hyperprolactinemia (159). DA is also very important in neurodegenerative (e.g. Parkinson's disease (160)) and neuropsychiatric disorders (e.g. schizophrenia (161)). In the goldfish, it was discovered more than 20 years ago that DA is the single most potent inhibitor of LH release from the pituitary (59). This inhibitory control of LH by DA is also important in many other teleosts, amphibians, and mammals including sheep, rabbits, and humans (162).

DA exerts it its effects via 2 classes of 7-transmembrane domain G-protein-coupled receptors: the D1-class and D2-class receptors. The D2-class of receptors can be separated into the D2, D3, and D4 DA receptors, all of which contain multiple introns (74). In mammals, 2 splice variants of the D2 receptor exist (79), where Exon VI (87 nucleotides or 29 amino acids) is spliced out resulting in a D2 receptor with a shorter third intracellular loop (D2S) relative to the unspliced transcript (D2L). A D2 splice variant has also been identified

[†] Popesku, J.T., Navarro-Martín, L., Trudeau, V.L. Evidence for alternative splicing of the dopamine D2 receptor in goldfish neuroendocrine brain. *In preparation.*

in the domestic turkey, *Maleagris gallopavo* (163, 164). However, in lower vertebrates, such as fish (80-82, 165, 166) and frogs (83), no evidence of the D2 receptor alternative splice variant has been found, leading others to conclude that splicing of the D2 receptor is an evolutionarily recent development (82). Here we propose that D2 receptor splicing must have developed earlier because we present evidence for splicing in the goldfish, a model teleost species.

Goldfish are an amenable model for vertebrate neuroendocrine signaling and over the past 30 years have been used to advance our basic knowledge about how the brain regulates growth, feeding, reproduction, pituitary and gonadal physiology, sex pheromones and behavior, and the stress response (69). In teleosts, the gonadotrophs, as well as other endocrine cells in the anterior pituitary, are directly innervated by a multitude of hypophysiotropic neurons (167). This is in contrast to the mammalian hypothalamo-pituitary system whereby the median eminence and portal system delivers neurohormones to the target anterior pituitary cells. The system of direct innervation of anterior pituitary cells is a highly derived rather than primitive condition because sharks and tetrapods share common anatomical features (42). The advantage of teleost models in this regard is that it has allowed the precise determination of the phenotype and source of the direct multiple catecholaminergic and neuropeptidergic inputs controlling anterior pituitary function, which is much more difficult in tetrapod models with a median eminence. Importantly, through numerous functional and anatomical studies, the anteroventral telencephalic DAergic neurons are the well-characterized source of the direct inhibitory control of LH release in the goldfish (7). Terminals of goldfish DAergic neurons have been visualized with electron microscopy to make direct contact with gonadotrophs and somatotrophs (56, 168, 169). The

teleost pituitary is highly regionalized, with gonadotrophs being clustered in the proximal pars distalis (within the anterior pituitary) in association with somatotrophs (6) where DA inhibits LH release via the D2 receptor and stimulates GH release via the D1 receptor (157, 170-172). Additionally, DA inhibits GnRH release through a D1-class receptor (167). In many fish, DA is the single most important inhibitor of LH release, the potency of which is such that it is able to keep eels in a pre-pubertal state for many years (53). Goldfish are a representative member of the *Cyprinidae*, one of the largest families of extant vertebrates with over 2400 species (1). The common goldfish (*Carassius auratus*) is a tetraploid (4n = 100) organism (26), and could potentially possess up to 8 copies of a gene. This is the result of a fish-specific genome-duplication event that occurred around 235 million years ago (mya), after the teleosts diverged from the tetrapods around 450 mya (173). Such duplication events have generated diversity in numerous genes in fish (78, 174), and presents the possibility to uncover novel DA receptors.

In this study we provide evidence for the existence of a D2 alternative splice variant in the goldfish and its seasonal variation and auto-regulation. Furthermore, we provide evidence of an additional distinct D2 paralog that does not appear to be alternatively spliced in adult goldfish.

2.2. MATERIALS & METHODS

2.2.1. Animals

Common goldfish (24.0 ± 5.6 g) were purchased from a commercial supplier (Aleong's International Inc., Mississauga, ON, Canada) and maintained at 18°C under a natural simulated photoperiod on standard flaked goldfish food. Fish were kept in 70L tanks (18 fish/tank). All procedures used were approved by the University of Ottawa Protocol

Review Committee and followed standard Canadian Council on Animal Care guidelines on the use of animals in research. Goldfish were anaesthetized using 3-aminobenzoic acid ethylester (MS222; Aquatic Eco-Systems, Apopka, FL) for all handling, injection, and dissection procedures.

2.2.2. In vivo experiments and sampling procedures

For the tissue distribution of the gfD2 receptor isoforms, 1 male and 1 female sexually maturing (March) goldfish each were sacrificed by spinal transaction and brain tissues were rapidly dissected and frozen on dry ice. Brain tissues (olfactory bulbs, Tel, Hyp, cerebellum, optic tectum, vagal lobes, medulla, and the "remainder" including diencephalic/mesencephalic areas) were rapidly dissected and immediately frozen on dry ice. In addition, pituitary, heart, interrenal gland, gonad (ovary or testis), gill, liver, and intestine were also rapidly dissected and frozen on dry ice. Following rapid freezing on dry ice, all samples were stored at -80°C for later use.

For investigation of the seasonal regulation of the gfD2 receptor isoforms, male and female fish (n=40 for each sex) were sampled as above in May and November. Brain tissue samples were pooled (5 brain parts/tube) and pituitary samples were pooled (10 pituitaries/tube) to increase RNA yield prior to RNA isolation.

For investigation of the regulation of the gfD2 receptor isoforms by dopamine antagonists, DA D_1-specific antagonist SCH 23390 and DA D_2-specific antagonist sulpiride were purchased from Tocris (Ballwin, MO, USA). The antagonists were first dissolved in a minimal amount of dimethylsulfoxide (DMSO), and subsequently diluted with 0.6% saline. The final concentration of DMSO was 0.099%. Sexually mature female goldfish (June; GSI

2.9 ± 0.4 %; n=18 each) received injections at 5 μL/g body weight of either SCH 23390 or sulpiride to give a dose of 40 μg/g or 2 μg/g body weight of fish, respectively, or saline containing an equivalent amount of DMSO. After 5 hours, fish were sacrificed as described above. Brain tissue samples were pooled (3 samples/tube) to increase RNA yield prior to RNA isolation.

The experimental procedure for the fadrozole exposure is explained elsewhere (Zhang *et al.*, *in press*). Briefly, female goldfish were exposed to waterborne fadrozole (50 μg/L), renewed every 3 days, for a total of 40 days during April-May. Brain tissues and pituitaries were dissected and pooled as described above.

2.2.3. RNA extraction and cDNA synthesis

Tissues were homogenized using stainless steel beads in an MM301 Mixer Mill (Retsch, Newton, PA, USA) at 20 Hz for 2 min. RNA was isolated using the RNeasy Mini kit (Qiagen, Mississauga, ON) as described in the manufacturer's protocol, including the on-column DNase I treatment. RNA quantity was evaluated using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific). RNA quality was determined using the Agilent 2100 BioAnalyzer and RIN numbers were determined to be 8.1-9.4; above the minimum value considered as "perfect total RNA" for further analysis (175). cDNA was prepared from either 1 μg (0.5 μg for female medulla) total RNA for tissue distribution or 2 μg of total RNA for all other experiments. To the RNA was added 200 ng of random hexamer primers (Invitrogen) with Superscript II RNase H^- reverse transcriptase as described by the manufacturer (Invitrogen). cDNA synthesis was performed separately for the whole brain cloning, tissue distribution, antagonist auto-regulation, and seasonal profiling. The "no

reverse transcriptase" (NoRT) reaction was included for each cDNA synthesis run where RNase/DNase-free water was added to the reaction *en lieu* of Superscript II.

2.2.4. PCR Cloning and sequence analysis

Degenerate primers based on the conserved nucleotide sequences of DA D2 receptors in GenBank were designed using Primer3 (Table 2.1) following ClustalW2 alignment (http://www.ebi.ac.uk/Tools/clustalw2). The primers were designed such that they spanned the alternative splice region found in humans. Species and accession numbers for the sequences used in the alignment were eel D2A (DQ789976) and D2B (DQ78997), trout D2R1 (AJ347728) and D2R2 (AJ347728), tilapia D2 (AY673985), zebrafish D2a (NM_183068) and D2b (NM_197936), carp D2 (Y14632), and human D2 short (NM_016574) and D2 long (NM_000795). Only the coding sequence was used in the alignment.

Table 2.1: Primers used in this study

Primer	Purpose	Forward (5' → 3')	Reverse (5' → 3')	Amplicon size
Degenerate	Cloning	SCAGWCCACCACCAATTACC	AKGAARAASGGYARCCAGCA	~1000 bp
Spanner	Tissue dist.	CCCYGCTTTTGTTGTCTACT	TYCTCTTGCTCATGGTTTTGG	458 or 545 bp
D2b1	Seasonal	ATTCGCTATTTCCTGCCCTTTGTTG	GTAACGTCCACCCTCACATCA	384 bp
D2b2	Seasonal	TTCACTATTTCCTGCCCCCTGCTTT	CCTGCCACGATGTCCACTCCAA	389 bp
D2L specific	real-time	TGTCATTCTACGTGCCCTTC	AACCCCCGTTGGTCTTAATG	201 bp
D2S specific	real-time	CTAAGCCCCCTCTCAAGCA	TGCCCATTTTTTATCATCTCCA	207 bp
β-actin	Tissue dist.	ACTACTGGTATTGTGATGGACTCC	CGGTCAGGATCTTCATCAGGTAG	142 bp

Platinum® Taq DNA Polymerase High Fidelity, and dNTPs were purchased from Invitrogen. Each 25 μL PCR reaction tube contained ~200 ng whole brain cDNA and ~200 ng pituitary cDNA from a mixed pool of male and female adult fish, 2.5 μL HiFi buffer (10X), 1 μL $MgSO_4$ (50 mM), 0.6 μL dNTPs (10 mM), 1 μL each of forward and reverse primers (10 μM), and 0.1 μL HiFi Taq. The thermal cycling parameters were an initial 1

cycle Taq activation at 95 °C for 2 min, followed by 40 cycles of 95°C for 45s, 56°C for 30s, and 72 °C for 1 min, followed by a final elongation step of 72 °C for 10 min. The PCR products (Figure 2.1a) were resolved on a 1.3% agarose gel, gel purified using the QIAquick Gel Extraction kit (Qiagen), and subcloned into the pCR4-TOPO vector in the TOPO® TA Cloning Kit for Sequencing (Invitrogen). The ligated vector was transformed into One Shot® Chemically Competent TOP10 *E. coli* cells according to the manufacturer's protocol (Invitrogen). After miniprep purification using the QIAprep Spin Miniprep Kit (Qiagen), plasmids were sent for sequencing at the Centre for Advanced Research in Environmental Genomics' DNA Sequencing Service (University of Ottawa). A total of 49 positive clones were sequenced in the sense and antisense directions. The resulting cDNA sequences were compared by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) to known vertebrate sequences available in GenBank. Transmembrane (TM) domain prediction on the deduced amino acid sequence from Expasy's Translate Tool (http://www.expasy.ch) was performed using TMHMM (http://www.cbs.dtu.dk). Post-translational modification sites were analyzed with ScanProSite (http://www.expasy.org/prosite/prosite_ref_html). Alignments with known or suspected D2 receptors from vertebrates and invertebrates available in GenBank and Ensembl were performed using ClustalW2. Phylogenetic analysis using phyML (176) was performed with bootstrap resampling of 1000 replicates.

2.2.5. Tissue distribution by RT-PCR

Nested primers, based on the cloned gfD2b sequences were designed (Figure 2.1d) to investigate expression of the receptors in various tissues. The primers were designed to be specific to all 3 D2b sequences outlined in this study, while excluding D2a and D3 receptors. For convenience, we called this primer set "Spanner" . The conditions and thermocycler

program were identical as those for cloning, with the following exceptions: 50 ng of cDNA was used in each reaction, and the annealing temperature was 58°C. Concurrently, a PCR reaction was run on the same cDNA dilutions for β-actin (Table 2.1) at an annealing temperature of 60°C. All samples were visualized on the same 1.3% agarose gel.

2.2.6. Real-time RT-PCR assays

To investigate the expression of the putative alternative splice, specific primers were designed using Primer3 (Table 2.1). The specific gfD2S primer set was designed such that the forward primer spanned the exonIV-exonVI boundary, and the specific gfD2L primer set was designed such that the reverse primer bound to the exon that was spliced out (Figure 2.1d). It was not feasible to design real-time RT-PCR-quality primers that differentiated between gfD2b1 and gfD2b2 while also precluding D2b1S, thus the specific gfD2L primer set is common to both gfD2b1 long and gfD2b2. Primers were tested by regular PCR and single bands were visualized on a gel. Amplicons from these primer sets were sequenced and their identity was confirmed.

The Mx3005 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA) was used to amplify and detect the transcripts of interest. Each PCR reaction contained the following final concentrations: 25 ng first strand cDNA template, 1× QPCR buffer, 3 mM MgCl2, 150 nM each F & R primers, 0.25× SYBRGreen (Invitrogen), 200 μM dNTPs, 1.25U HotStarTaq (Invitrogen), and 100 nM ROX reference dye, in a 25 μL reaction volume. The thermal cycling parameters were an initial 1 cycle Taq activation at 95°C for 10 min, followed by 40 cycles of 95°C for 30s, 59°C for 45s, and 72°C for 30 s. After the reaction was complete, a dissociation curve was produced starting from 55°C (+1°C /30 s) to 95°C. Dilutions of cDNA (1:10 to 1:31,250) from all samples were used to construct a

relative standard curve for each primer set, relating initial template copy number to fluorescence and amplification cycle. For each PCR reaction, negative controls were also introduced including a no-template control (NTC) where RNase-free water was added to the reaction instead of the template (cDNA) and NoRT. The SYBR green assay for each target gene was optimized for primer concentration and annealing temperature to obtain, for the standard curve, an $R^2 > 0.99$, amplification efficiency between 90-110% and a single sequence-specific peak in the dissociation curve. No amplification was observed in the NoRT or NTC controls indicating no genomic or reagent contamination. Results were normalized to RNA concentration as determined by the NanoDrop ND-1000. Data were analyzed with the MxPro v4.01 software package.

2.2.7. Dispersed pituitaries

Pituitaries from 100 male and female goldfish were collected and immediately put in ice-cold dispersion. Pituitaries were dispersed as described by Chang *et al.* (177). Pituitaries were washed with dispersion medium at room temperature 3 times, before pituitaries were diced into 1 mm fragments. Fragments were then dispersed in medium containing 2.5 mg/mL trypsin II for 35 min and shaken at room temperature, followed by 5 min in dispersion medium containing trypsin inhibitor (2.5 mg/mL) and 5min in dispersion medium containing 10 g/mL DNase II. Fragments were washed in calcium-free medium containing progressively less EGTA. Cells were then filtered through a nylon mesh and centrifuged at 1500rpm for 10 min. Cells were resuspended in calcium-free medium and counted using a hemocytometer. Cells were distributed in n=6 wells and kept in plating medium at 28°C and 5% CO_2 under saturated humidity. Prior to the incubation cells were

transferred to testing medium and incubated for 12h. Following incubation, plates were centrifuged and supernatant taken for subsequent RNA isolation.

2.2.8. Statistical analysis

All gene expression data were assessed using the two-tailed Mann-Whitney U rank sum test (http://elegans.swmed.edu/~leon/stats/utest.html) with significance considered at $p<0.05$, except for the antagonist experiment. The antagonist experiment was analyzed using the Kolmogorov-Smirnov test for normality and Levene's test of homogeneity of variance using SPSS v17.0. Data was found to be normally distributed and homoscedastic. A 1-way ANOVA was performed with significance considered at $p<0.05$, followed by Tukey's HSD post hoc test.

2.3. RESULTS

2.3.1. Cloning and characterization of the goldfish D2 receptors

A partial coding sequence (348 bp) for the goldfish dopamine (DA) D_2 receptor had already been isolated by us (GenBank Accession EF382625). However, in an attempt to clone a larger fragment from a mixed pool containing male and female goldfish whole brain cDNA using degenerate primers, we observed the expected band at ~1 kb but also a band at ~920 bp (Figure 2.1a). Furthermore, the 2 bands resulted in 5 distinctly different sequences: 4 with nucleotide length 1005 bp, and 1 with nucleotide length 918 bp (Table 2.2).

Following database searches and phylogenetic analysis, it was revealed that all 5 sequences belong to the D_2-class of dopamine receptors (Figure 2.1c). Here we have named the goldfish sequences (Table 2.2) according to their clustering with the available zebrafish sequences.

Table 2.2: GenBank Accession numbers of the cloned goldfish D2-class receptors obtained in this study.

Goldfish Sequence length[a]	Named	GenBank Accession #
1005/986	D2a	FJ793516
1005/986	D2b1	FJ793517
918/899	D2b1S	FJ793518
1005/986	D2b2	FJ793519
1005/986	D3	FJ793520

[a] The second number indicates the sequence length following removal of the degenerate section of the primers.

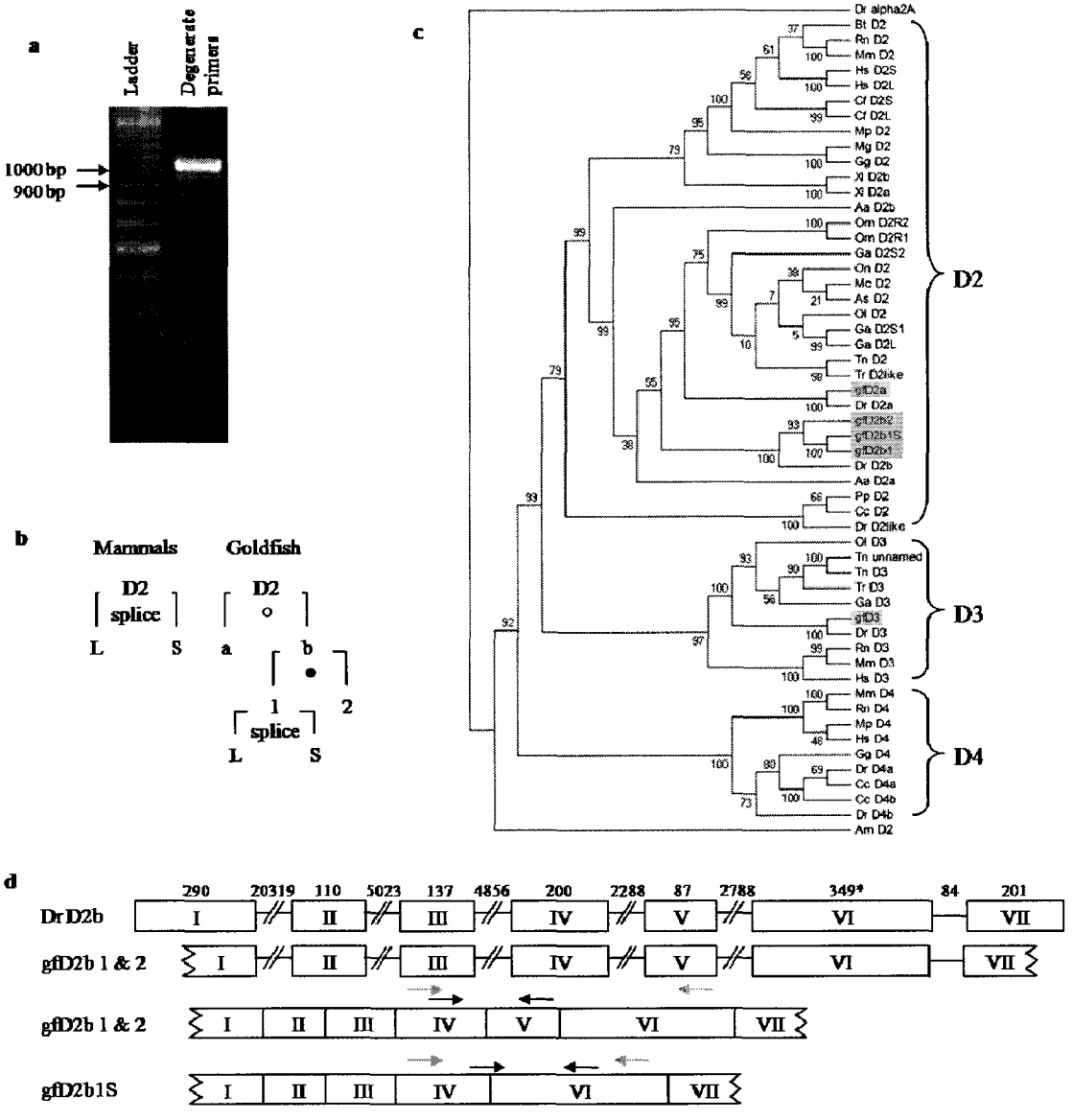


Figure 2.1: Sequence analysis of the goldfish D2-class receptors. a) Photo of the 1.3% agarose gel showing the expected band at ~1kb and the unexpected band at ~930 bp. b) A comparison of goldfish D2 receptors to mammalian D2 receptors. A fish-specific genome duplication (○) resulted in 2 paralogs of the D2 gene. A putative gene duplication (●) of the D2b receptor in goldfish gave rise to an additional D2 paralog. c) A phylogenetic distance tree of D_2-class receptors built using PhyML (176) was made and rooted on *Danio rerio* α2-adrenergic receptor. The JTT matrix-based model of amino acid substitution (178) was used and replicate trees in which the associated sequences clustered together in the bootstrap test (1000 replicates) are shown next to the branches (179) as a percentage. The consensus tree from bootstrapping 1000 replicates was visualized using MEGA4.1 (180). The goldfish sequences obtained in this study are shown in grey. Aa = *Anguilla anguilla*; Am = *Apis mellifera*; As = *Acanthopagrus schlegelii*; Bt = *Bos taurus*; Cc = *Cyprinus carpio*; Cf = *Canis familiaris*; Dr = *Danio rerio*; Ga = *Gasterosteus aculeatus*; Gg = *Gallus gallus*; Hs = *Homo sapiens*; Mc = *Mugil cephalus*; Mg = *Meleagris gallopavo*; Mm = *Mus musculus*; Mpf = *Mustela putorius furo*; Ol = *Oryzias latipes*; Om = *Oncorhynchus mykiss*; On = *Oreochromis niloticus*; Pp = *Pimephales promelas*; Rn = *Rattus norvegicus*; Tn = *Tetraodon nigroviridis*; Tr = *Takifugu rubripes*; Xl = *Xenopus laevis*. d) The gene structure of zebrafish (Dr) D2b aligned with the presumptive goldfish gene structure of D2b1 & 2 and D2b1S. Black arrows indicate the approximate positions of real-time RT-PCR primers and grey arrows indicate the approximate positions of the "Spanner" primer set (Table 2.1). Roman numerals indicate exons. Note: In Exon VI, 3 nucleotides (TCC) are missing from the goldfish sequence compared to zebrafish, which, in zebrafish code for Pro at position 313.

The 4 deduced partial gfD2 amino acid sequences are 70-74% identical to human D2 and the predicted D3 amino acid sequence is 60% identical to human D3 (Figure 2.2). The 5 gfD2 sequences were submitted to GenBank (Table 2.2). We focused our attention to the multiple D2b isoforms and did not further investigate the distribution or regulation of D2a or D3. Figure 2.3 shows partial coding sequences of the D2b cDNAs obtained.

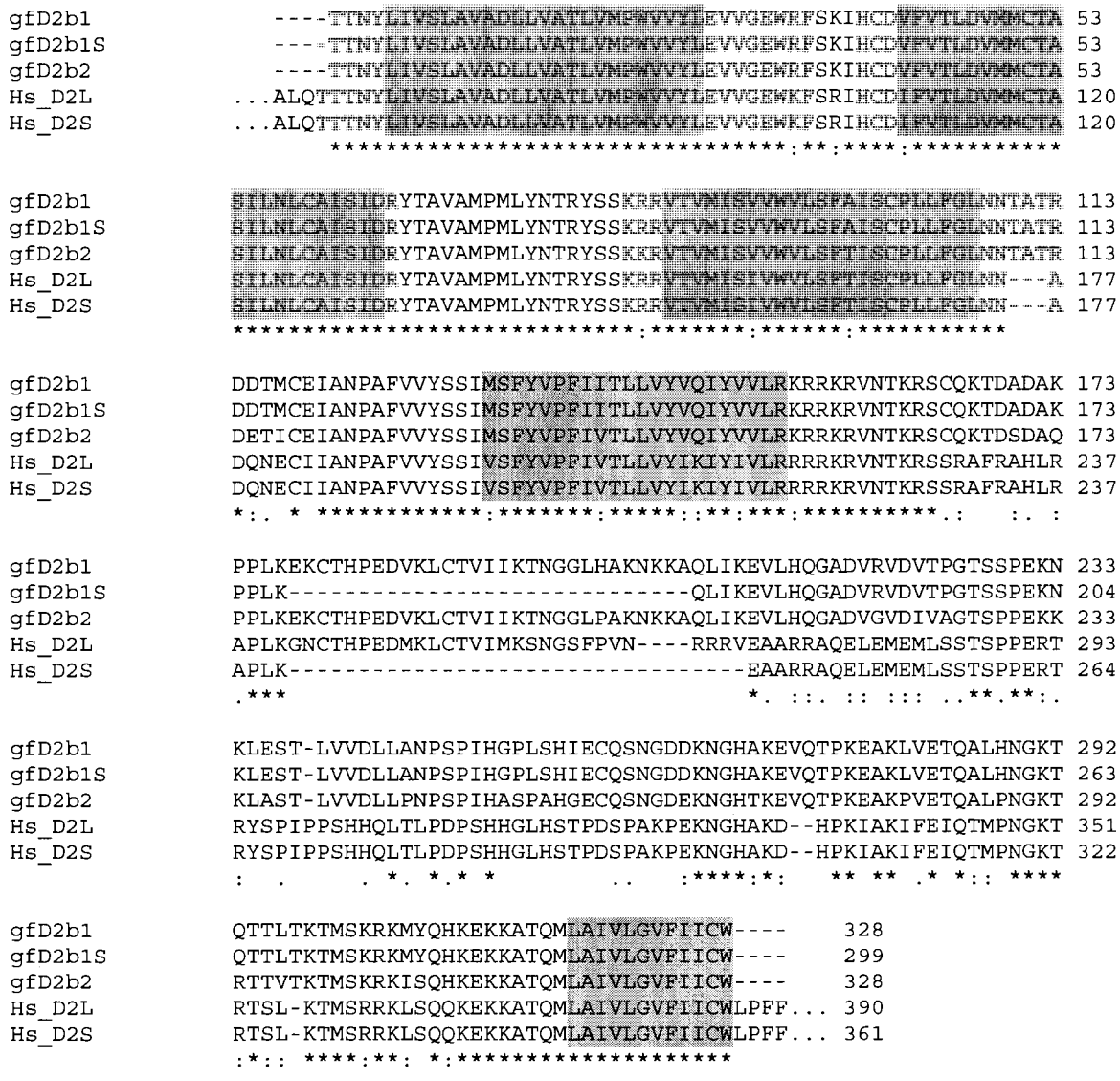


Figure 2.2: ClustalW2 alignment of goldfish D2b predicted amino acid sequences with human (Hs) D2 amino acid sequences. Transmembrane domains are shown in grey. The human sequence is trimmed and presented only as the region aligning with the goldfish sequences.

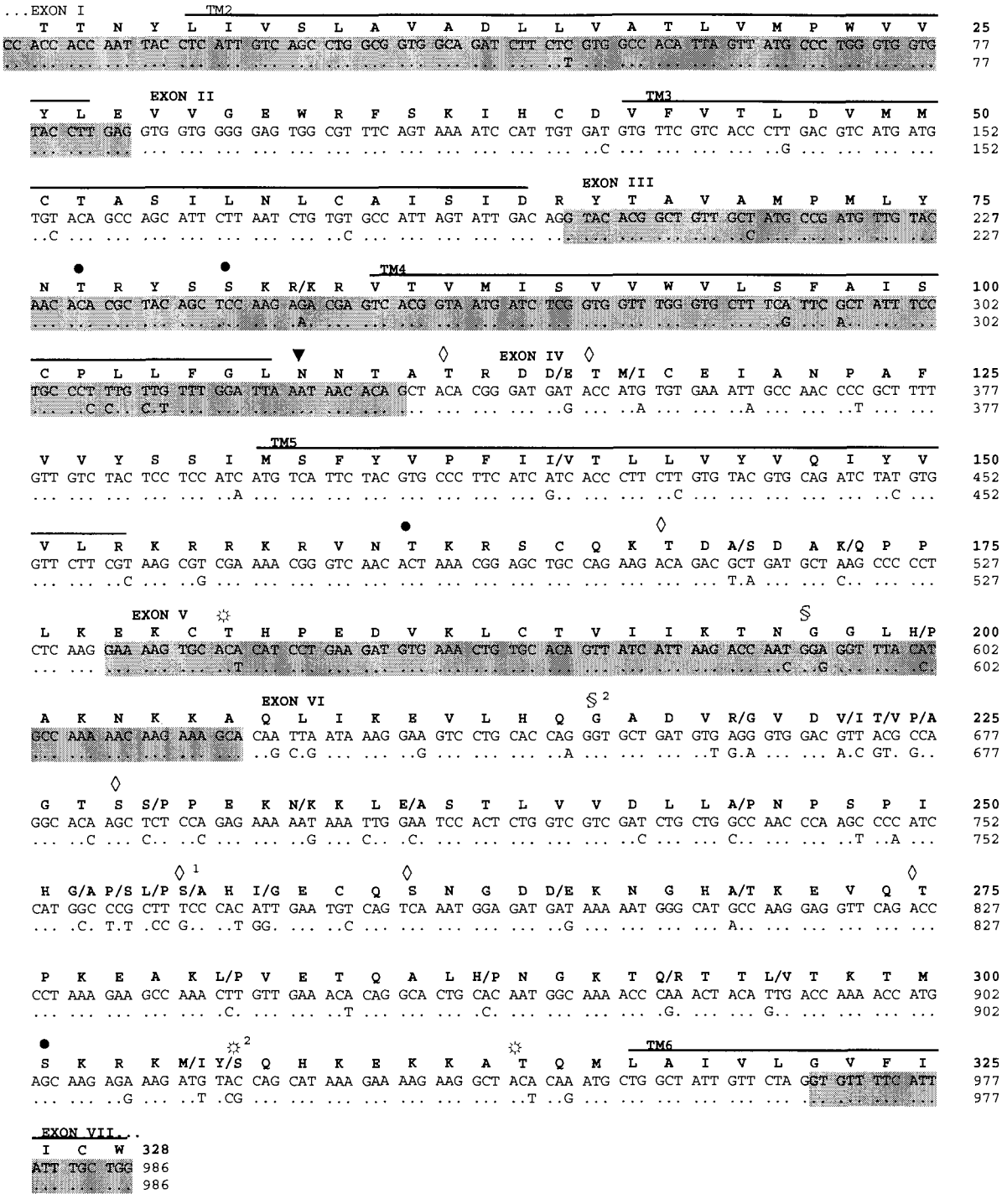


Figure 2.3: Partial nucleotide and deduced amino acid sequences of the goldfish DA D2b cDNAs. The partial sequence of D2b1 is shown. Differences in the nucleotide sequence of D2b2 are shown underneath the D2b1 sequence. The deduced amino acid sequence of D2b1 is shown above the nucelotide sequence and differences in D2b2 are shown after the "/". The seven putative transmembrane domains (TM) are indicated as bars above the sequences. The putative PKC (●), PKA (☼) and CK-2 (◊) phosphorylation sites, as well as putative N-glycosylation (▼) and N-myristoylation (§) sites are indicated. These sites are present on all D2b sequences except: 1 present on D2b1 only; and 2 present on D2b2 only. Exons based on the zebrafish gene and cDNA are shown as alternating in grey/white. Note: D2b1 S is 100% identical to D2b1 except that Exon V is missing in D2b1 S. The nucleotide sequences have been deposited in GenBank (Table 2.2).

Intron/exon boundary predictions of the gfD2b sequences were determined by comparing the zebrafish D2b genomic and mRNA sequences (GenBank GeneID 378719). The sequence region for both gfD2b1 and gfD2b2 spanned ~75% of the coding sequence for zebrafish D2b and included 7 exons and 6 introns (Figure 2.1d).

2.3.2. Tissue distribution of D2b receptors by RT-PCR

Using the "Spanner" primer set to investigate the tissue distribution of the gfD2b, we found no detectable expression in either male or female heart, intestine, or gill, and very little, if any, expression was observed in the interrenal gland or ovary (Figure 2.4a). On the other hand, expression of the long isoform was observed in the testis. Both sexes express D2b (long) in the olfactory bulb, medulla, and vagal lobes, but neither sex appear to express the short isoform in those brain regions. Male cerebellum (Figure 2.4a) expressed no observable D2b, while the short isoform of D2b was observed in female cerebellum. A further sexual dimorphism is observed in the rest of the brain, where the female expresses both long and short D2b isoforms in the optic tectum, Tel, Hyp, pituitary and "remainder", which includes the mesencephalic areas.

We focused our attention to the neuroendocrine brain (Hyp and Tel) and the pituitary because of their central importance to the control of reproduction. Primers designed to differentiate between the D2b1 and D2b2 genes (Table 2.1) were tested on pooled (n=8) Hyp, Tel, and pituitary samples in male and female fish for May, when fish are sexually mature, and November, when fish are sexually inactive, but at the beginning of the seasonal gonadal redevelopment phase. Figure 2.4b shows that the short isoform originates from D2b1, whereas D2b2 does not seem possess a similar splice variant, at least in adult fish.

2.3.3. Seasonal variation of D2b1 short

To examine the seasonal expression of the long and short isoforms of gfD2 in the Hyp, Tel, and pituitary, real-time RT-PCR primers were designed (Table 2.1). As D2b1 and D2b1S are 100% identical except for the spliced-out exon, it is not currently feasible to design real-time RT-PCR-quality primers that differentiate between D2b2 and the long isoform of D2b1 while precluding D2b1S. Thus, the D2S primers were specific for D2b1S by spanning the alternative splice, whereas the D2L primers encompassed both D2b1 long and D2b2 and excluded D2b1S (Figure 2.1). In male fish (Figure 2.4c), both the Hyp and Tel expressed ~2.3-fold higher levels of D2S mRNA in November relative to May. D2L mRNA expression was 1.3-fold higher ($p=0.031$) in Tel in male fish in November relative to May. In females, a 3.5-fold increase ($p=0.021$) in pituitary D2S mRNA expression was observed in November compared to May (Figure 2.4d). Furthermore, a 1.8-fold increase ($p=0.020$) in D2L mRNA expression was observed in the pituitary of female fish in November when compared to May.

2.3.4. Autoregulation of D2b1 S

Using the real-time RT-PCR primers described above, we investigated whether the newly-identified D2b isoforms are auto-regulated. Relative D2 receptor mRNA levels were measured in the Hyp or Tel of female fish 5h post-injection with either SCH 23390, a selective D1-receptor antagonist, or sulpiride, a selective D2-receptor antagonist. The results demonstrate that both antagonists decrease the expression of D2S by 4 fold ($p<0.05$), but not D2L, in the telencephalon (Figure 2.5a). The results in the Hyp exhibit a similar trend, but did not reach statistical significance.

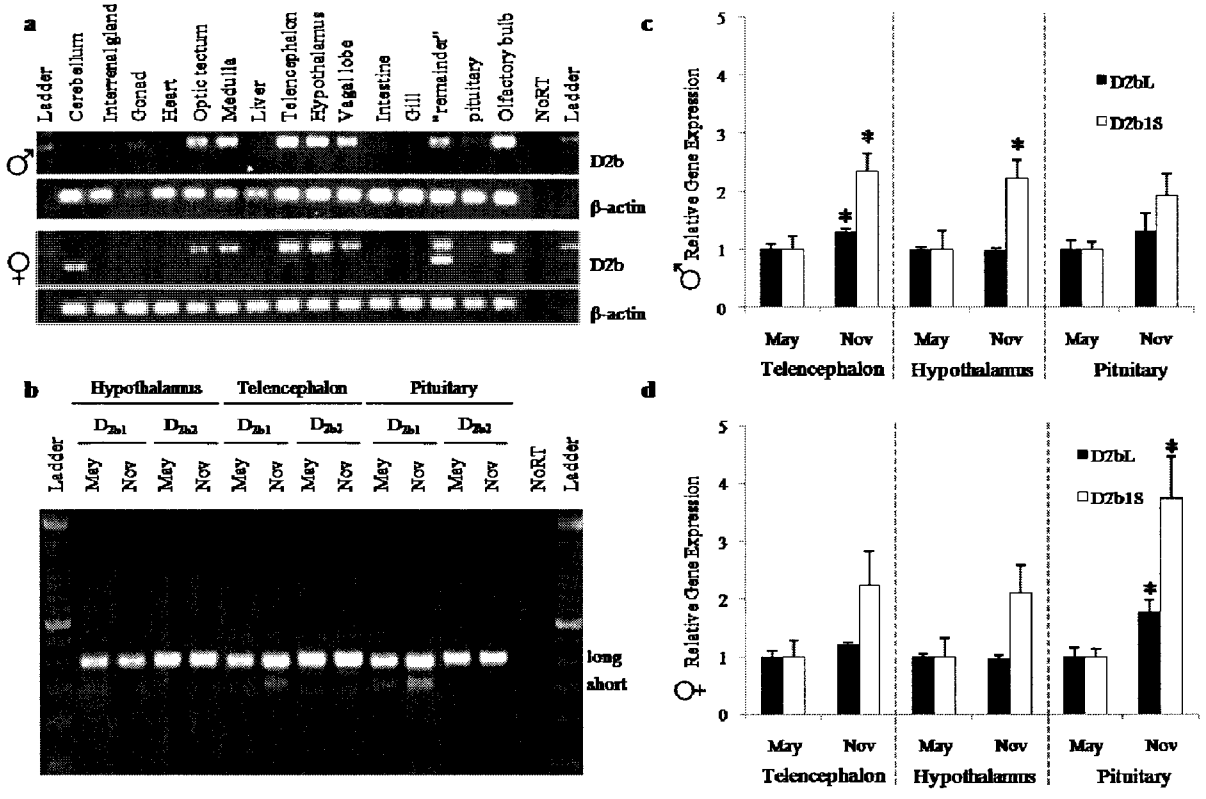


Figure 2.4: Characterization of gfD2b cDNAs a) Tissue distribution performed with RT-PCR using a primer set that spanned the putative alternative splice in various tissues in male and female goldfish. Note: "remainder" includes the rest of the brain once the telencephalon, hypothalamus, cerebellum, optic tecta, medulla, vagal lobes, and olfactory bulbs have been removed. b) Differentiation between D2b1 and D2b2. Pools (n=8) of either Hyp, Tel, or pituitary tissues were made. Both males and females were investigated and the results were identical; results for female fish are presented. Real-time RT-PCR of the seasonal expression of D2S and D2L in the Tel and Hyp (n=8) and pituitary (n=4) in c) male and d) female goldfish. Two-tailed Mann-Whitney U test was performed with significance considered at 0.05. * $p < 0.05$ relative to the corresponding tissue in May.

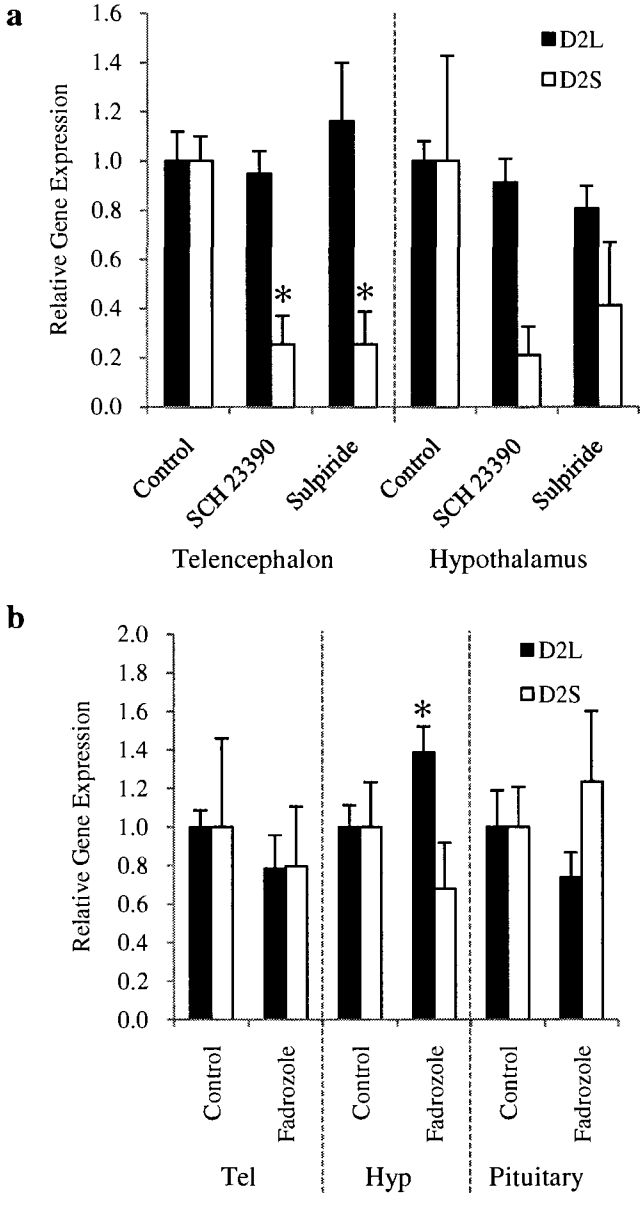


Figure 2.5: Autoregulation of and effect of fadrozole on D2S. Real-time RT-PCR of D2S (D2b1 short) and D2L (both D2b1 long and D2b2) in the telencephalon, hypothalamus, or pituitary (n=4-6 each) in response to a) SCH 23390, a selective dopamine D1 receptor antagonist, and sulpiride, a selective dopamine D2 receptor antagonist or b) fadrozole. One-way ANOVA was performed with significance considered at 0.05 for the antagonist experiment. * $p < 0.05$ relative to saline-injected control fish. Two-tailed Mann-Whitney U rank sum test was performed with significance considered at 0.05 for the fadrozole experiment. *$p<0.05$ relative to unexposed control fish.

2.3.5. Estradiol regulation of D2b

We examined the role of estrogens on the expression of D2 receptor in the neuroendocrine brain and pituitary of goldfish by exposing mature pre-spawning female goldfish to waterborne fadrozole, an aromatase inhibitor for 40 days. Serum E2 levels were reduced by 50% ($p<0.05$) in exposed fish relative to unexposed fish (D. Zhang, personal communication). The results presented in Figure 2.5b show that only D2L expression increased significantly (1.4-fold; $p=0.027$) in the Hyp, but not in the Tel. Inhibition of aromatase by fadrozole had no effect on gfD2S in any of the tissues examined.

2.3.6. Dispersed pituitaries vs. whole pituitaries

Owing to the fact that the goldfish adenohypophysis receives direct neural innervation, we used dispersed pituitary cells that are free of nerve terminals and cellular debris (177) to investigate whether the mRNAs encoding the short isoform are found in hypophysiotropic neuronal elements (i.e., axons or terminals) or in the non-neuronal endocrine cells of the pituitary. Very little, if any, gfD2b1S was observed in dispersed pituitaries compared to whole pituitaries (Figure 2.6), even after 55 cycles of PCR (not shown). In contrast, both D2b1 and D2b2 were clearly amplified from cDNA originating from dispersed pituitary cells or whole pituitaries which contain both the hypophysiotropic axons and terminals, and all endocrine elements.

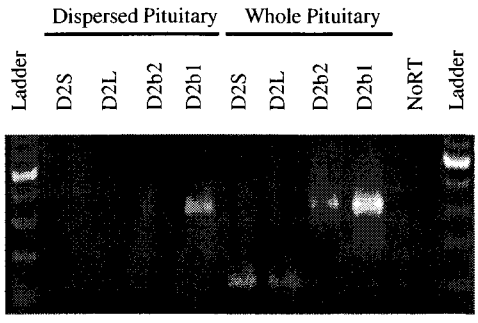


Figure 2.6: gfD2b expression in dispersed or whole pituitaries. Photo of the gel is shown after 35 cycles. An additional 20 cycles were run, but no expression of D2S was observed in dispersed pituitaries (not shown). The NoRT negative control reaction contained the gfD2S primer set.

Table 2.3: Pairwise distance matrix estimates of evolutionary divergence between sequences. The number of amino acid substitutions per site from analysis between sequences is shown . All results are based on the pairwise analysis of 11 sequences. Analyses were conducted using the Poisson correction method in MEGA4 (180, 181). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 251 positions in the final dataset.

		Human (Hs)			Zebrafish (Dr)			Goldfish (Ca)			
		D2L	D2S	D3	D2a	D2b	D3	D2a	D2b1	D2b1S	D2b2
Hs	D2L										
	D2S	0.000									
	D3	0.549	0.549								
Dr	D2a	0.305	0.305	0.598							
	D2b	0.247	0.247	0.563	0.237						
	D3	0.528	0.528	0.407	0.563	0.535					
Ca	D2a	0.294	0.294	0.598	0.016	0.227	0.563				
	D2b1	0.273	0.273	0.549	0.258	0.074	0.535	0.247			
	D2b1S	0.273	0.273	0.549	0.258	0.074	0.535	0.247	0.000		
	D2b2	0.258	0.258	0.549	0.258	0.053	0.522	0.247	0.083	0.083	
	D3	0.522	0.522	0.396	0.570	0.515	0.066	0.570	0.515	0.515	0.502

2.4. DISCUSSION

In the present study, we identified partial cDNAs encoding 5 goldfish D2-class receptors, which, based on their phylogenetic relationships to zebrafish and their pairwise comparisons to zebrafish and human D2 receptors, were named D2a, D2b1, D2b1S, D2b2, and D3. Other groups have reported 2 separate cDNAs in trout (84), 2 distinct genes in eel (166), and 3 different genes in zebrafish (165) for D2 receptors, all of which were included in our phylogenetic analysis. As has been shown in other teleost species (81, 166), the third intracellular loop of the gfD2b receptors shows the highest variation in amino acid sequence, while the transmembrane domains are very well conserved. The identification of 3 different D2b sequences has not previously been reported, and, as such, we focused our attention to these 3 D2 isoforms. It appears that, following the fish genome duplication event (174) producing D2a and D2b, the goldfish D2b gene was duplicated again to produce D2b1 and D2b2 paralogs (Figure 2.4b). This is similar to what is likely to have happened with goldfish estrogen receptors (182).

We also report, to the best of our knowledge, the first evidence for the alternative splicing of a dopamine D2 receptor in a lower vertebrate and its autoregulation, similar to what has been shown in mammals (183). Cloning of the D2 receptor in tilapia (81), eel (166), Fugu (82), carp (80), zebrafish (165), rainbow trout (84), and *Xenopus* (83) did not provide any evidence of a splice variant, leading some authors to suggest that such a splice variant does not exist in lower vertebrates (82). We mined the fish genomes in Ensembl and found that no splice variants for the D2 receptor have thus far been elucidated in medaka (*Oryzias latipes*) or green pufferfish (*Tetraodon nigroviridis*). On the other hand, our data mining revealed that stickleback (*Gasterosteus aculeatus*) may have a D2 splice variant that

is similar to the short isoform we isolated from goldfish. Both the goldfish and the stickleback possess a splice that, when translated lacks 29 amino acids in the third intracellular loop. The stickleback splice variant, however, is a predicted protein coding gene based on its genome sequencing project and nothing, as far as we know, has been verified experimentally nor presented in the literature. The fact that other groups have found no evidence of an alternative splice of the D2 receptor may reflect the polyploidy of fish, particularly as we identified a D2 paralog that does not show evidence of alternative splicing.

Tissue distribution as determined with RT-PCR of the gfD2 receptors corroborates the distribution of D2 receptors recently reported in tilapia (81) and eel (166). Otth *et al.* (184) identified the D2 receptor in male rat germ cells and suggested that DA signalling is involved in testicular and sperm physiology. We observed expression of the gfD2L in testis, but not the ovary, which supports this hypothesis. No expression of gfD2S was observed in any tissue in male fish, which may be due to very low expression at this time of year (March), as varying levels of gfD2S were observed in the Hyp, Tel, and pituitary at other times of the year (May and November).

We further examined the seasonal expression of the goldfish D2b cDNAs in the Hyp and Tel. In the goldfish, the Tel contains the pre-optic area (POA) where numerous hypophysiotropic neurons involved in the control of LH and GH, including GnRH, γ-aminobutyric acid (GABA), and secretoneurin, some of which are inhibited by DA (69). Furthermore, the D2 receptor is highly expressed in the telencephalon of female rainbow trout (84). In general, in both sexes gfD2b1S is more highly expressed in November when seasonal gonadal development is starting compared to May during the breeding season (April to mid-May) of goldfish. The lower expression in May suggests that gfD2b1S is important

in regulating the inhibitory tone of DA during the reproduction cycle, particularly as *in vitro* studies in rat striatal slices indicate that stimulation of D2S inhibits tyrosine hydroxylase (TH), the rate-limited enzyme in DA synthesis (185, 186). Therefore, we hypothesize that the increased expression of D2b1S in November decreases DA biosynthesis and release to keep DA levels in check (negative feedback). But in May, with lower expression of D2b1S expression, the negative feedback on TH is reduced, allowing for high DAergic inhibitory tone of LH until an internal cue is met (see below). Alternatively, since D2b1S is autoregulated, similarly to mammalian D2S (183), the seasonal expression of D2b1S may be the result, and not the cause, of varying levels of DA throughout a reproductive cycle (187). This seasonal regulation may also play an important role in the release of other hormones from the anterior pituitary. For example, the D2 receptor has been shown to be important in GH release in mice (188) but this has yet to be shown in fish.

It has been suggested that E2 may increase the inhibitory tone of dopamine during gametogenesis and a subsequent decrease in E2 would result in a release of dopaminergic inhibition and contribute to generation of the LH surge in fish (162). Is it possible that these events are regulated by, or regulate, the splicing event of the goldfish D2 receptor? Guivarch'h *et al.* showed that the D2L/D2S alternative splicing event is modulated by sex steroids *in vitro* in the rat MMQ prolactin cell line (189) and *in vivo* in rat brain (190). More recently, Oomizu *et al.* (191) examined the effect of E2 on the D2 splicing event in the anterior pituitary of ovariectomized rats. As a percentage of total D2, they observed a 5% decrease in D2S in Fischer-344 rats and a ~15% decrease in D2S in cultured anterior pituitary cells, respectively, due to E2. We did not observe a statistically significant change in gfD2S (D2b1S) mRNA in this study, but hypothalamic D2L mRNA levels were

significantly increased following fadrozole treatment indicating that estrogens regulate this D2 receptor mRNA.

In our study we found that the deduced amino amino acid sequence for gfD2b1S is missing the identical 29 amino acid region of the protein as human D2S. We suggest that the gf D2b1 receptors should behave similarly to their mammalian counterparts with respect to their interaction with G-proteins, although this remains to be shown experimentally. In mammalian systems, D2S is missing the third intracellular loop and interacts differently with inhibitory G-proteins than does the D2L isoform. (192). Moreover, D2S and D2L possess similar pharmacological profiles (193) but differentially affect extracellular signal-regulated kinases (194). Lindgren *et al.* (195) showed that the D2S isoform regulates the phosphorylation state and activity of TH while D2L is mainly involved in the phosphorylation state of DARPP-32 (dopamine- and cAMP-regulated phosphoprotein 32 kDa), an important integrator of multiple signals (196).

One puzzling observation is the amplification of D2S from cDNA produced from whole goldfish pituitary, but its conspicuous absence in primary cultures of endocrine cells derived from dispersion of whole pituitaries from more than 100 fish of both sexes. This is in marked contrast to the high expression of D2L in the same cell cultures, suggesting that the splicing event does not occur in goldfish pituitary cells. Axons and nerve terminals of a multitude of classical neurotransmitters and neuropeptides are present in whole goldfish pituitary, as would be any potential mRNAs in axons and terminals. Importantly, cell dispersion and culture eliminates the heavy innervation of the posterior and anterior pituitary, and any associated elements or debris (177). This leads us to conclude that D2S mRNA is found in axons and/or terminals of hypophysiotropic neurons in the goldfish. In adult rhesus

monkey substantia nigra and ventral tegmental areas, the D2S receptor protein is located mainly on pre-synaptic axon while the D2L isoform is found mainly on post-synaptic dentrites (197). Although the concept of axonal mRNA translation was proposed decades ago, it has been largely ignored despite compeling evidence (198). There have been multiple reports of RNAs found in the distal part of the axon in a variety of organisms including rat, chicken, snail and squid, although this seems to be an exception, rather than a rule, particularly with respect to mature neurons (reviewed in (199)). In developing neurons, however, regenerating goldfish retinal axons support the hypothesis that local protein synthesis occurs (200). In goldfish brain *in vivo*, BC1 RNA, a noncoding RNA polymerase III transcript, is specifically transported along goldfish Mauthner axons (201), similar to what has been previously found in rat hypothalamo-neurohypophyseal axons *in vivo* (202). Furthermore, rat hypothalamo-neurohypophysial axons express both oxytocin and vasopressin mRNAs (203-205). It is unknown whether the neurons innervating the adult fish pituitary are mature or rather in a state of constant developmental flux throughout the reproductive cycle. However, it should be noted that adult teleosts have a high capacity for neurogenesis, and many neurons arising from the development of aromatase-positive radial glial cells will be hypophysiotropic (206).

In conclusion, we provide strong evidence for the alternative splicing of a D2 receptor gene in the goldfish, its seasonal regulation during the reproductive cycle and by rapid DAergic autoregulation as shown with our *in vivo* injection of DA receptor antagonists. The finding that goldfish splice D2L to potentially produce D2S, similarly to mammals, highlights the importance of this event in the evolutionary conservation of molecular signalling mechanisms. Therefore, the hypothesis that D2 splicing is a relatively recent

innovation in higher tetrapods (82) is not supported by our discovery in goldfish. That we also identified *in silico* a putative splice product in the stickleback genome database indicates that this splicing of the D2L can be found in at least 2 highly different fish families, *Gasterosteidae* and *Cyprinidae*. The discovery of alternative splicing of the D2 receptor, and the similarities between goldfish and some mammalian species in the dopaminergic regulation of reproduction and growth (162, 171), GABA synthesis (207), and responses to the DAergic neurotoxin MPTP are all supportive of the use of teleost fish as models to elucidate Parkinson's disease in humans (208) and the evolution of neuroendocrine function in vertebrates (69).

Chapter 3.

RAPID DOPAMINERGIC MODULATION OF THE NEUROENDOCRINE TRANSCRIPTOME AND PROTEOME OF THE GOLDFISH[‡]

3.1. INTRODUCTION

The relationship between gene expression and protein changes *in vivo* are poorly understood. In this study, we investigated the rapid transcriptomic and proteomic responses in the goldfish hypothalamus to injection of dopamine (DA) D1 or D2 receptor agonists, providing a step towards understanding systems neuroendocrinology. Few studies link genomic, proteomic, and physiological responses, particularly in fish. We chose to characterize the response to DA due to its central inhibitory role in teleost reproduction (69) and its link to numerous major neurological disorders in humans (209-211).

Our "non-genome" organism model of choice was goldfish (69) because i) goldfish are amenable to physiological manipulation; ii) the role of DA as a central regulator of reproductive processes is best-described in the goldfish; iii) a goldfish EST project has been initiated; and iv) a goldfish-carp cDNA microarray has been developed and validated.

In this paper, we present a novel method of transcriptomic and proteomic comparison in a non-genome organism. Furthermore, we provide insights into the hypothalamic processes that are under the regulation of DA, a potent inhibitor of LH release in the goldfish (reviewed in (69)), as well as many other species, including humans (162).

[‡] Popesku, J.T., Martyniuk, C.J., Denslow, N.D., Trudeau, V.L. Rapid dopaminergic modulation of the neuroendocrine transcriptome and proteome of the goldfish. *In preparation.*

3.2. MATERIALS & METHODS

3.2.1. Experimental animals and design

Common adult female goldfish were purchased from a commercial supplier (Aleong's International Inc., Mississauga, ON, Canada) and maintained at 18°C under a natural simulated photoperiod on standard flaked goldfish food. Goldfish were anaesthetized using 3-aminobenzoic acid ethylester (MS222) for all handling, injection, and dissection procedures. All procedures used were approved by the University of Ottawa Protocol Review Committee and followed standard Canadian Council on Animal Care guidelines on the use of animals in research. Sexually mature, pre-spawning (mid-May; GSI 4.5 ± 1.3%) female goldfish (15-40 g) were injected intraperitoneally with either SKF 38393 (D_1 agonist; SKF; 1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol) or LY 171555 (D_2 agonist; LY; (-)-Quinpirole hydrochloride) purchased from Tocris (Ballwin, MO, USA). LY was dissolved in physiological saline (0.6% NaCl) to yield a dose of 2 μg/g body weight of fish. SKF was first dissolved in a minimal amount of dimethylsulfoxide (DMSO), and subsequently diluted to 40 μg/g body weight of fish with physiological saline (0.6% for fish). The final concentration of DMSO was 0.099%; DMSO up to 0.1% does not affect basal GH or LH levels (212). While 0.1% DMSO may (213) or may not (214) affect gene expression, all of our gene expression work is relative to saline-injected control fish which received an equivalent amount of DMSO. The fish received 2 i.p. injections at 5 μL/g body weight according to the schedule shown in Table 3.1.

Table 3.1: Injection schedule for the administration of dopamine agonists used in this study.

Treatment	i.p. Injection 1	i.p. Injection 2	# fish injected
Control	0.1% DMSO/saline	0.6% saline	13
SKF	SKF 38393 40 μg/g	0.6% saline	14
LY	0.1% DMSO/saline	LY 171555 2 μg/g	11

After 5 hours, blood was sampled (400-600 μL) by puncture of the caudal vasculature via a 25-gauge needle attached to a 1-mL syringe. The fish were sacrificed by spinal transection and hypothalami and telencephali tissues were rapidly dissected and immediately frozen on dry ice. Brain samples were pooled (2-3 hypothalami or telencephali/tube) to increase RNA yield prior to RNA isolation. Serum was collected by centrifuging the blood at 4,000g at 4°C for 10 minutes. Serum was stored at -80°C until used for the radioimmunoassay.

3.2.2. Radioimmunoassay for Luteinizing Hormone

The double antibody radioimmunoassay (RIA) protocol of Zhao *et al.* [15] was used to analyze circulating LH levels from serum samples. Data was tested for normality using SPSS v16 and was determined not to be normally distributed. Data was log-transformed, determined to be normally distributed and one-way ANOVAs were used to test for significant differences ($p<0.05$).

3.2.3. RNA isolation and quality

RNA was isolated with the TRIzol method (Invitrogen, Burlington, ON, Canada) per the manufacturer's protocol. Samples were treated with DNase on-column in an RNeasy Mini Plus kit (Qiagen, Mississauga, ON, Canada). RNA quantity was evaluated using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA integrity was evaluated using the BioAnalyzer (Agilent); RIN for each sample was > 8.4.

3.2.4. Microarray hybridizations

We previously described and validated the production and use of our goldfish-carp cDNA microarray (15, 138, 215), and a detailed description of the microarray is available

(216). Four microarray hybridizations were performed for each hypothalamic and telencephalonic tissue for both D1 and D2 agonists (total of 16 arrays) to screen for the effects of the agonists in the neuroendocrine brain. Three separate pools of RNA from treated fish were hybridized to the microarrays, and a fourth hybridization was a replicate dye-reversal of one of the three RNA pooled samples. Hybridizations were carried out relative to a common pool of control samples (~30 control fish) for each tissue, which decreases technical variation as only one reference is utilized while maintaining biological variation of the treatment samples (217). All cDNA synthesis, labeling, and hybridizations were performed using the Genisphere 3DNA Array 900MPX kit according to the manufacturer's protocol (Genisphere, Hatfield, PA). Hybridizations and scanning protocols were described previously (15, 138, 215). Briefly, microarrays were scanned at full-speed 10-μm resolution with the ScanArray 5000 XL system (Packard Biosciences/PerkinElmer, Woodbridge, ON, Canada) using both red and blue lasers. Images were obtained with ScanArray Express software using automatic calibration sensitivity varying photomultiplicator (PMT) gain (PMT starting at 65% for Cy5 and 70% for Cy3) with fixed laser power at 80% and the target intensity set for 90%. Microarray images were analyzed with QuantArray (Packard Biosciences/Perkin Elmer), and raw signal intensity values were obtained for duplicate spots of genes. Raw intensity values for all microarray data and microarray platform information have been deposited in the NCBI Gene Expression Omnibus database (platform accession no. GPL3735; SuperSeries accession no. GSE15855). Generalized Procrustes Analysis (218) was used for normalization of the array data and the Significance Analysis of Microarrays (SAM) method (116) was used for microarray analysis.

3.2.5. Protein quantitation and database search using iTRAQ® labeling

Approximately 20 mg of hypothalamic tissue was collected and mechanically disrupted in 500 uL RIPA Lysis and Extraction Buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% nonyl phenoxylpolyethoxylethanol-40, 1% sodium deoxycholate and 0.1 % SDS) (Pierce, Rockford, IL., USA) in the presence of 10 μg protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) to prevent any non-intentional protein degradation. We performed three separate iTRAQ® labeling experiments. Each labeling reaction consited of a single hypothalamus for control (label 114), LY 171555 (D_2 agonist; label 115), and SKF 38393 (D_1 agonist; label 117) (total n=9). Proteins were precipitated with cold acetone at 5x volume of the sample. Precipitated proteins were reconstituted in 20 μl dissolution buffer (iTRAQ® kit) and water sonicated for 5 min. Total protein concentration was determined using Coomassie Plus Better Bradford Assay™ Reagent (Pierce). 100 μg total protein/sample was used for labeling reactions according to the manufacturer's protocol (Applied Biosystems Inc, Foster City, CA). After reducing with 50 mM Tris-(2-carboxyethyl)-phosphine and blocking the thiol groups using methyl methanethiosulfonate, proteins in buffer were digested with 10 μg trypsin (Sigma-Aldrich) for 16 h at 37 °C. Samples from one iTRAQ® experiment were pooled and desalted on a Vydac Silica C18 (The Nest Group Inc, Southboro, MA). The eluted labeled peptides were dried, resuspended in 100 μl buffer A (75% 0.01M ammonium formate and 25% acetonitrile) and placed onto an off-line polysulfoethylA column (100 x 2.1 mm, 5 μm, 300 Å) for strong cation exchange. Peptides were eluted using a linear gradient of 0-20% buffer B (75% 0.5M ammonium formate, 25% ACN) over 40 min, followed by a gradient of 20-100% buffer B for 5 min. Peptide peaks were monitored by UV at 280 nm. Each individual iTRAQ® experiment was fractionated into 20 peptide fractions and pooled into four fractions of relatively equal protein abundance

for mass spectrometry analysis. Each sample fraction was injected onto a capillary trap LC Packings PepMap (DIONEX, Sunnyvale, CA) and desalted for 5 min with a flow rate of 20 μL/min of 3% ACN, 0.1% acetic acid, 0.01% trifluoroacetic acid) onto an in line LC Packing C18 Pep Map HPLC column (300 μM x 5 mm) connected to a hybrid quadrupole-TOF mass spectrometer QSTAR XL (Applied Biosystems) at a flow rate of 200 nL/min. The elution gradient of the nano-HPLC column started at 3% solvent B and finished at 60% solvent B and proceeded for 2 h. Solvent B consisted of 0.1% acetic acid, 96.9% ACN. The focusing potential and ion spray voltage was set to 275 V and 2600 V, respectively. The information-dependent acquisition (IDA) mode of operation was employed in which a survey scan from *m/z* 400–1200 was acquired followed by collision induced dissociation (CID) of the three most intense ions. Survey and MS/MS spectra for each IDA cycle were accumulated for 1 and 3 s, respectively.

In order to better assign peptides to proteins, a ray-finned fish database containing trypsin digested peptides was created by extraction of protein sequence information from NCBI (08/07/2008; # entries = 121756) and searched using MS/MS data interpretation algorithms within Protein Pilot™ (Paragon™ algorithm, v 2.0, Applied Biosystems). The Paragon algorithm from Protein Pilot™ was set up to search iTRAQ® 4-plex samples as variable modifications with methyl methanethiosulfonate as a fixed modification (219). The Protein Pilot™ algorithm was selected to search automatically for biological modifications such as homocysteines. The confidence level for protein identification was set up to 1.3 (95%) which is the default for the detected protein threshold in a Paragon™ method.

To calculate a false-discovery rate (FDR) for peptide-protein assignments, Proteomics System Performance Evaluation Pipeline (ProteomicS PEP, Applied Biosystems)

in Protein Pilot™ was used to create a reversed ray-finned fish database in which to search. When searching the ProteomicS PEP reverse database, 621 proteins were identified with an FDR below 5%, thus there is high confidence (>95%) in the proteins identified in this study.

The differential expression ratios for protein quantitation were obtained from Protein Pilot™ which calculates protein ratios using only ratios from the spectra that are distinct to each protein, excluding the shared peptides of protein isoforms. Peptides with low spectral counts were also excluded from the calculation of averages by setting the intensity threshold for the sum of the signal-to-noise ratio for all the peak pairs at >9. All the quantitative ratios were then corrected for bias automatically by Protein Pilot™. Each protein that was quantified was identified by a minimum of three spectra. To calculate differential expression ratios, all identified spectra from a protein were used to obtain an average protein ratio relative to the control label (i.e. fold change). The error factor (EF) is a measure of the variation between the different iTRAQ® ratios (the greater the variation, the greater the uncertainty) and represents the 95% uncertainty range for a reported ratio. The p-value is calculated based on the 95% confidence interval.

3.2.6. Bioinformatics

Protein sequences from proteins identified by iTRAQ analysis as differentially expressed were downloaded from NCBI using extracted GI numbers with the following BioPerl script:

```
#!/usr/bin/perl -w

use Bio::DB::GenBank;
use Bio::SeqIO;

print "Path and filename containing the accession numbers: ";
chomp($data_file = <>);
open (FILEHANDLE, $data_file) || die("Could not open input file!");
@raw_data=<FILEHANDLE>;
close(FILEHANDLE);

$gb = new Bio::DB::GenBank;
foreach $accession (@raw_data)
{
  chop($accession);
  my $seq = $gb->get_Seq_by_acc($accession);
  my $seqout = new Bio::SeqIO(-format => 'Fasta');
  $seqout->write_seq($seq);
}
```

The protein sequences were converted into a blastable database using formatdb. All of the nucleotide sequences identified as being differentially expressed (q<5%) from the agonist experiment were compared (blast-2.2.19) against the above database through Blast2GO. A graphical depiction of the workflow is presented in Figure 3.1.

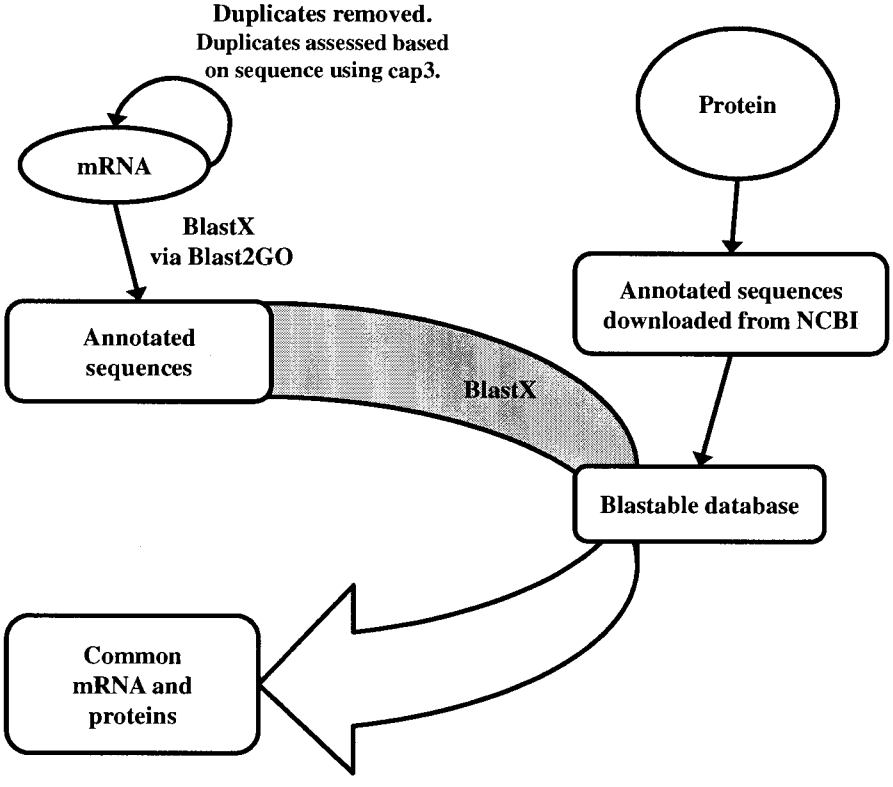


Figure 3.1: Information workflow diagram.

3.3. RESULTS/DISCUSSION

To confirm that our DA agonist injection protocol was effective in inhibiting LH release, circulating serum LH was determined by radioimmunoassay (RIA; Figure 3.2). It is well-known that DA via the D2 receptor is the primary inhibitor of LH release in goldfish and numerous other teleosts (55, 59, 162, 220-227). Here we show that LY rapidly reduced circulating LH levels to 25.0% of control. Surprisingly, we also found that the DA-D1 agonist SKF also decreased LH by 42.5%, which is a novel finding for DA regulation of LH release in fish. However, this is in contrast to other studies in goldfish where DA-D1 agonists had little effect on basal or GnRH-stimulated LH (170). Based on these results we hypothesize that the D1-R is important for controlling LH by inhibiting the stimulatory effects of glutamate. The most plausible explanation for this difference is that other studies were performed at a time in the seasonal breeding cycle when D2 responses were dominant over D1 responses, or that the *in vivo* dose and time of blood sampling were sufficently different that the D1 inhibitory responses on LH release were not detected. Nevertheless, our treatements produced relevant physiological responses in female goldfish.

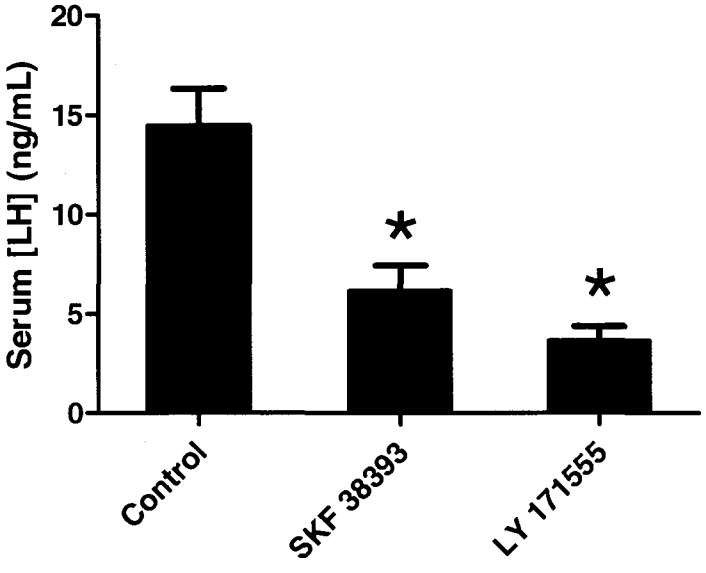
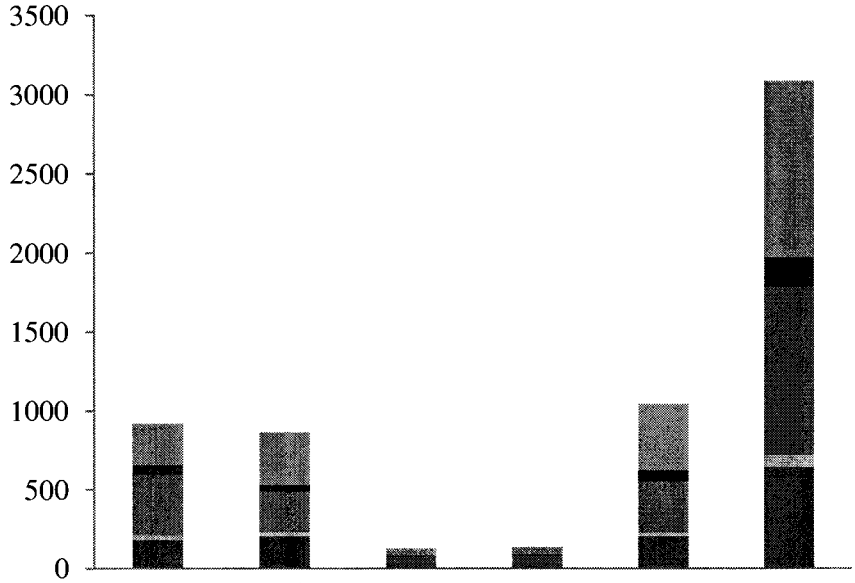


Figure 3.2: Serum LH concentration following DA agonist injections. Mean (± SE) serum LH concentration in control and injected (40 μg/g SKF 38393 or 2 μg/g LY 171555) female goldfish (n=23-26 each). Results presented are a combination/average of 2 identical but independent experiments that showed similar results. Data were log-transformed to approximate normality and a one-way ANOVA was performed in SPSS v16 with significance considered at $p<0.05$, followed by Tukey's HSD multiple comparisons as data were homoscedastic. * signifies $p<0.001$ relative to control.

3.3.1. Transcripts identified in the goldfish hypothalamus as differentially regulated by dopamine

In total, 3088 ESTs were identified as being statistically ($q<5\%$) significantly differentially expressed following either D_1- or D_2-receptor stimulation in the hypothalamus. Many of these are as yet unidentified (Figure 3.3). A large proportion (Figure 3.4) of the annotated ESTs (1042) were involved in "regulation of biological process" (13%) and "signal transduction" (10%) and were "nucleotide binding" (19%). Furthermore, 29% of the cDNAs were localized to a protein complex.



	No Blast	No Blast Hits	No Mapping	No Annot	Annot	Total
SKF 38393 Up	265	336	48	49	419	1117
Shared between LY Up and SKF Up	63	39	8	6	68	184
LY 171555 Up	382	262	45	48	330	1067
Shared between LY up and SKF Down	30	22	1	2	20	75
SKF 38393 Down	179	205	25	31	205	645

Figure 3.3: Number of ESTs identified by microarray analysis as being statistically (q<5%) differentially regulated by dopamine agonists in the hypothalamus of female goldfish 5h post-i.p.-injection. The data distribution is shown as output from Blast2GO. Duplicates were removed. Overlapping ESTs (i.e. ESTs regulated by more than 1 agonist) are indicated as "Shared between...".

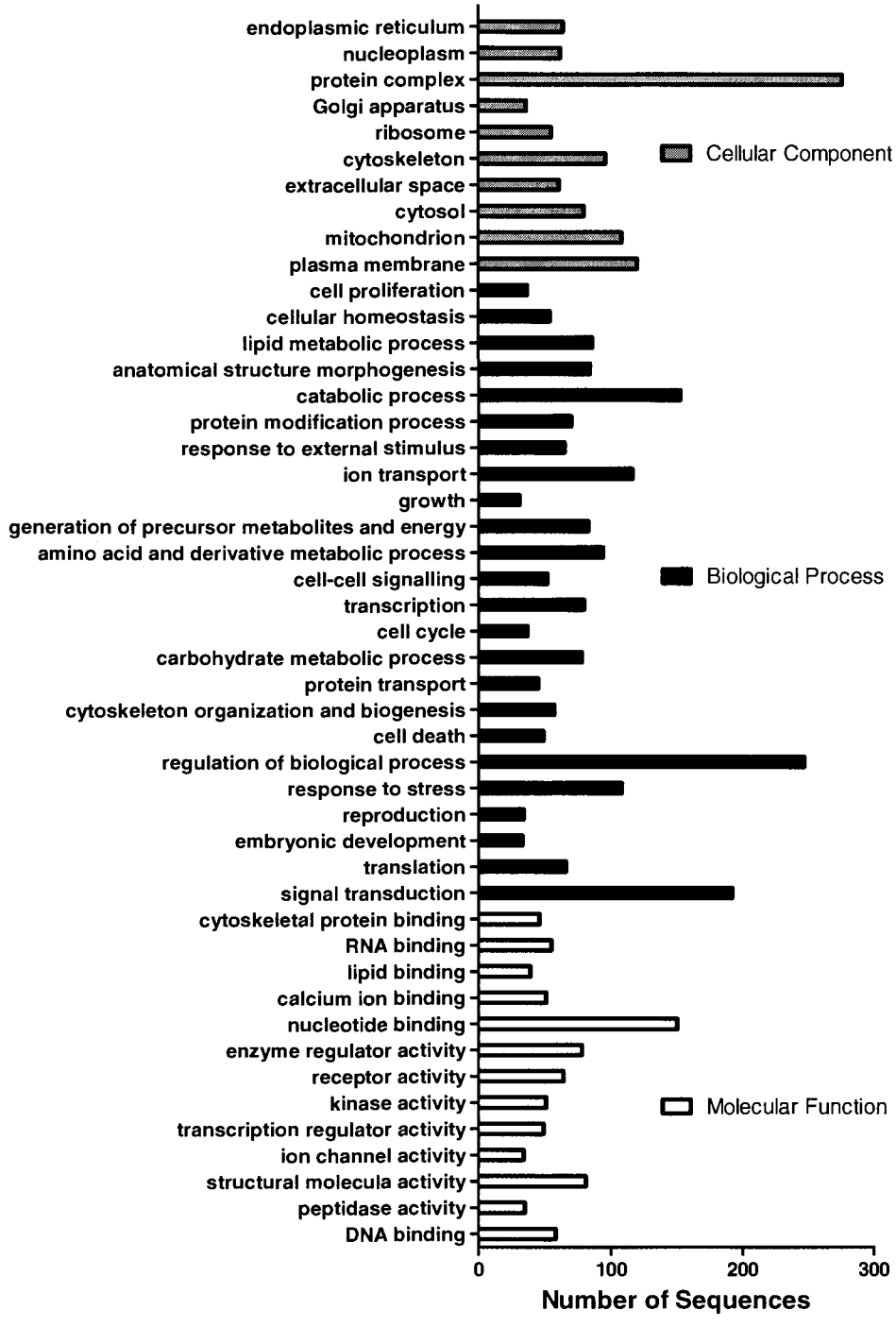


Figure 3.4: Multilevel Gene Ontology categorization of the 1042 annotated ESTs affected by DA agonists. Annotations were first converted to GO-Slim annotations (goslim_generic.obo) and the multilevel chart was constructed using a sequence convergence cutoff of 30 to reduce the complexity of the chart. Both agonists and both up- and down-regulated genes (q<5%) are included in this analysis.

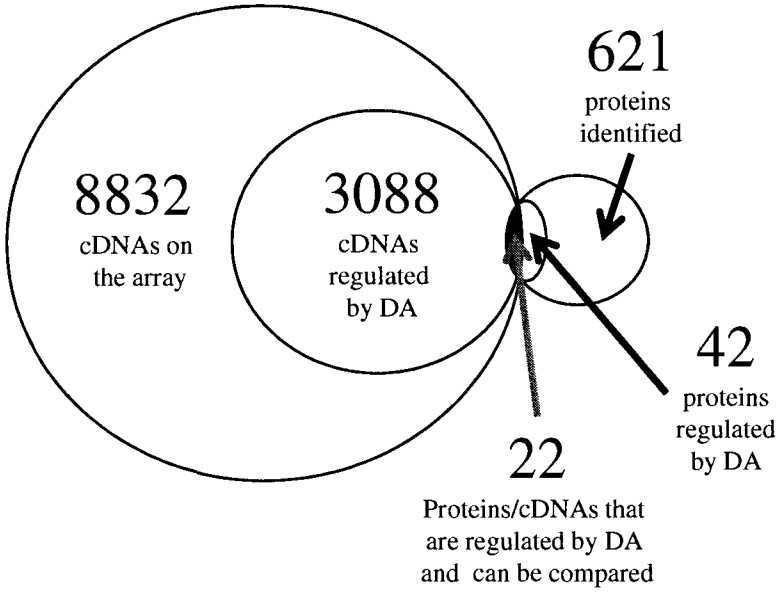


Figure 3.5: Venn Diagram summarizing the number of cDNAs (q<5%) and proteins (FDR-adj p<0.05) regulated by DA in the hypothalamus of female goldfish. Duplicate cDNAs were removed; cDNAs and proteins were counted once regardless if they were regulated by both agonists. The complete listing of cDNAs (Table C.1) and proteins (Table C.2) is presented in the supplemental material (Appendix C).

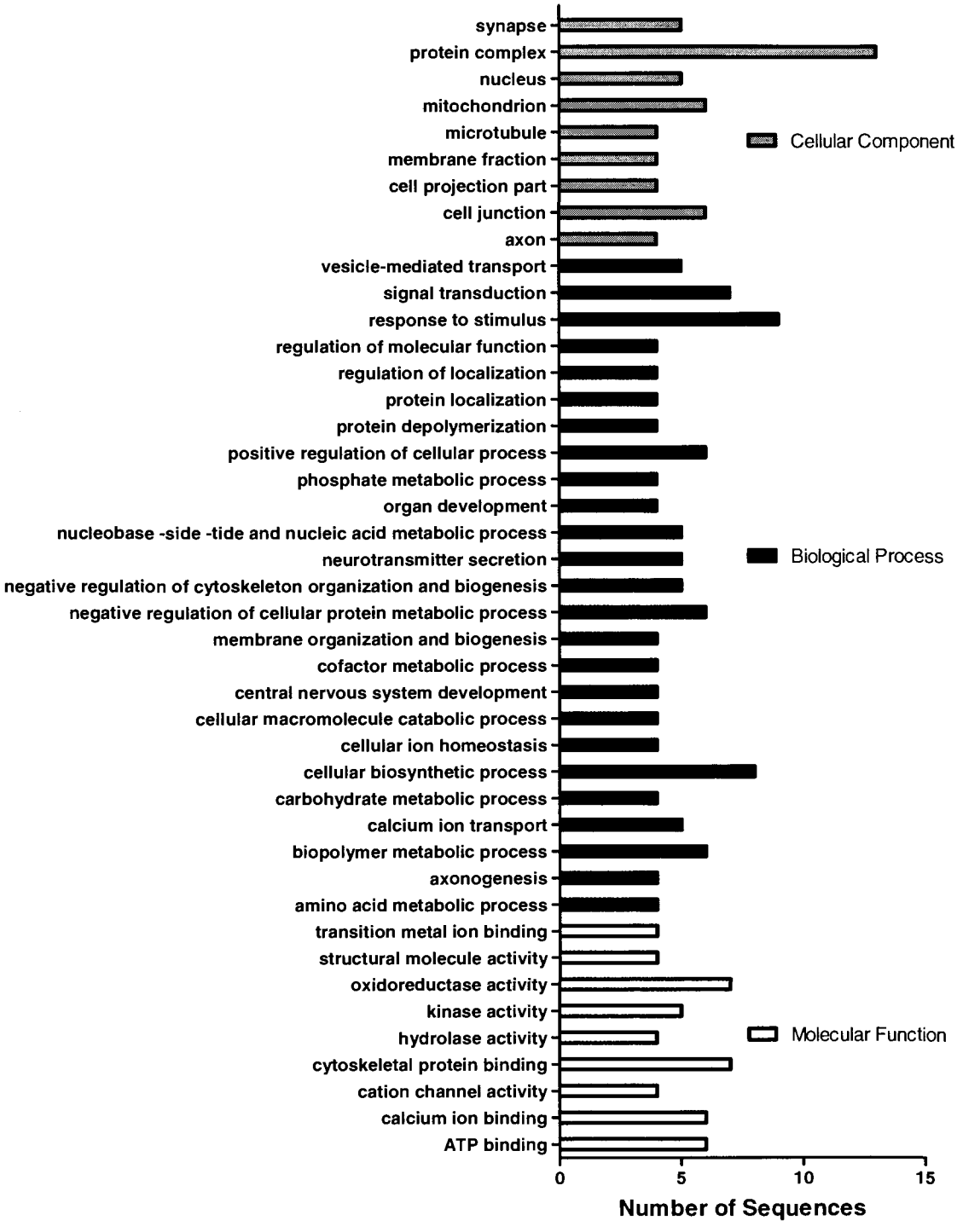


Figure 3.6: Differentially expressed proteins identified by iTRAQ (42; $p<0.05$) binned into multilevel GO categorizations with a sequence cutoff of 3. Both treatments (D1 and D2) and both directions are included in this analysis but are counted only once if the protein is common to both treatments.

A recent review by Altar *et al.* (228) summarized targets for the identification of central nervous system (CNS) diseases using transcriptional profiling. Many of the transcriptional targets reported by Altar *et al.* (228) were also differentially regulated by DA in the goldfish hypothalamus. For example, mRNAs for GAD1, microtubule-associated protein tau, serpin A, malate dehydrogenase, regulator of G-protein signaling, transferrin, s100 calcium binding protein, glutathione-S-transferase, calmodulin, AMPA receptors, GFAP, NMDA receptor 1, glutamate transporter, calbindin, alpha enolase, peroxiredoxin, fructose-bisphosphate aldolase c, glutamine-ammonia ligase (aka glutamine synthetase), and dopamine receptors and transporter were all identified as being differentially regulated (see Appendix C; Table C.1) and are identified as targets for CNS diseases such as Alzheimer's disease, Parkinson's disease, aging, and schizophrenia. Furthermore, many mRNAs in the ubiquitination pathway were affected, but UCHL1, a gene important in Parkinson's disease, was not on the array. More cDNA fragments related to these diseases and identified by Altar *et al.* (228) were previously not on the goldArray 1.0, such as BDNF, GDNF, GAP43, UCHL1 and nurr1, and have subsequently been cloned or subcloned by our lab and will appear on future versions of the array.

3.3.2. Proteins identified in the goldfish hypothalamus

The experiment was repeated on the same date the following year in an identical experimental design. The levels of LH were determined by RIA and were found to be similarly reduced in both agonist treatments as in the previous year. Thus, data from both years were combined (Figure 3.2). The hypothalami from this repeated experiment were subjected to iTRAQ proteomic analysis.

There were 621 proteins identified in this study using the ray-finned fish database (see Appendix C; Table C.2). This table also includes the NCBI protein accession number and protein name, % coverage of protein sequence, ray-finned species identified as showing the highest homology to the query, ratios of labels 115 (D2) and 117 (D1) to control (114), p value for all protein fold changes, and the error factor. All raw peptide data are also present in Table C.2 and includes the peptide sequence and the confidence (%) of the peptide-protein identification, information indicating whether or not the peptide was used in the quantitation of the protein, and all modifications identified for a peptide. The number of distinct peptides detected in this study was 4779 and the total number of spectra detected was 8569. Of the spectra identified, 59.7% could be assigned to a protein, leaving 40% of the spectra unidentified by homology searches of other ray-finned fishes.

Of the 621 identifiable proteins, 42 were determined as being significantly (FDR-adj *p*-value <0.05) differentially regulated each by SKF 38393 (D1 agonist) or LY 171555 (D2 agonist) (Figure 3.5). The protein dataset (for both D_1 and D_2 results combined) was analyzed using Blast2GO and binned into their corresponding GO terms (Figure 3.6). A significant number of proteins being affected are localized to the protein complex and are involved in a wide variety of biological processes, including signal transduction, response to stimulus, and positive, as well as negative, regulation of cellular process. Of interest are the biological processes of neurotransmitter secretion and calcium ion transport. Calcium/calmodulin-dependent kinase II α subunit (CaMKIIα), calbindin 2, neuronal calcium-binding protein 2, and plasma membrane calcium ATPase 4 proteins were significantly affected by at least 1 of the dopamine agonists (Table 3.2).

Calmodulin is expressed in the hypothalamus and pituitary of goldfish (229). In the same study, goldfish pituitary cells were subjected to either D1 or D2 agonists to examine the effect on CaM transcript levels. Only the D2 receptor agonist decreased CaM mRNA levels in these cells, with the D1 receptor agonist having no effect (229), suggesting that pituitary CaM transcript regulation is mainly under the regulation of the D2 receptor rather than the D1 receptor. Our results suggest that, in the hypothalamus, CaM expression is also D2 receptor-mediated, and the mechanism of action may be the same as that in the pituitary. Interestingly, a recent meta-analysis by Zhang *et al.* (230) using data from control samples from multiple goldfish hypothalamic microarray experiments, including the data presented in this study, identified CaM mRNA levels as being relatively highly expressed in May, when fish are sexually mature, compared to August or December, when fish are sexually regressed or recrudescing, respectively. This information, coupled with the changes in mRNA and protein levels of CaM (this study), suggests that CaM may be important for the DA inhibition of LH and may provide insights into the signalling mechanism of DAergic action in the hypothalamus with respect to reproductive processes. CaMKIIα protein levels, whose transcript levels follow the same seasonal profile as CaM (high in May, low in August and December) (230), were increased in both D1- and D2-agonist treated fish suggesting that, as for CaM, CaMKIIα may be important in the hypothalamic reproductive signaling. Indeed, CaMKII phosphorylates cAMP response binding element (CREB) protein, thereby inhibiting its function (231), which may lead to downstream transcriptional repression of genes involved in reproduction. Furthermore, CaMKII positively regulates the D2 receptor promoter in rats (232), suggesting a possible feedback mechanism. We observed a decrease in hypothalamic Apo-14 protein expression with both D1- and D2- receptor agonists. Apo-14 seems to be specific to fish (233), is mainly expressed in liver and brain of adult orange-

spotted groupers and has been suggested to play a role in neuronal growth and repair (234), similar to ApoE (235). Vitale and Carbajal (236) demonstrated that DA induces substantial remodelling of cytoskeleton in rat lactotrophs *in vitro*. The decrease in Apo-14 microtubule-associated protein tau, along with an increase in microtubule-associated protein 1A suggests that DA may also have remodelling effects on the cytoskeleton of cells in the goldfish hypothalamus. The high energy requirement of such remodelling is supported by an increase observed in the proteins for mitochondrial creatine kinase, cytochrome c oxidase subunit IV, and malate dehydrogenase.

3.3.3. A comparison of the differentially expressed transcriptome to the differentially expressed proteome in response to DA agonists

The protein dataset was further compared to the microarray dataset by extracting the Gene Identifier (GI) numbers from the protein results. A Perl script was then written and used to obtain the corresponding amino sequences from GenBank, which were converted into a BLASTable database. The microarray nucleotide sequences were then BLASTed (BLASTx) against this differentially-expressed protein database. The results (Table 3.2) show directional correlation in some mRNAs and proteins (for example, kainate receptor α subunit and 14kDa apolipoprotein for D1 and liver-basic fatty acid binding protein for D2), while others are inversely correlated (for example, stathmin 1/oncoprotein 18 in either agonist). The mRNAs and proteins exhibiting discordant directional change following agonist treatments also indicate that particular pathways and processes are regulated. Differences in the direction of change between transcript and protein is likely related to our single sampling time-point, as the time-series relationship between changes in transcript versus protein *in vivo* are poorly understood in fish (237). Furthermore, the regulatory

mechanisms of the genome and proteome are very different and turnover and stability of mRNA levels is very important (238). For example, if the mRNA is decreased, but the protein is increased, it is possible that the mRNA has already begun to be degraded. Conversely, if the mRNA is increased, but the protein is decreased, there may be regulation in the sequence of events involved in translation. The interest here, however, lies with those mRNAs and proteins that share a common direction: in the D_1-agonist-treated fish, there are 8 out of the 14 common mRNAs/proteins (57%) whereas there is only 1 out of 7 of the common mRNAs/proteins (14%) for the D_2-agonist-treated fish that share a common direction (Table 3.2). These results are comparable to what was recently shown in the rat colon mucosa where only 16% direction identity between the transcriptome and the proteome was found (239). Furthermore, that study included the development of a TRIzol®-based method to analyze both the transcriptome and the proteome from the same sample, which should reduce variation in gene-protein correlation. Our results in this study show that we can achieve a comparable directional correlation from independent animals and experiments. This is significant, as it shows that the method is valid for a "non-genome" organism, such as the goldfish.

While some of the proteins identified as differentially expressed had corresponding changes in mRNA/cDNA, many cDNAs encoding the proteins that were regulated were not represented on our array. For example, the Nj-synaphin 1 (also known as complexin 1) protein was identified as being down-regulated 2.2-fold in response to the D_2-agonist (Table 3.2) but the complexin cDNA was not on the array. However, both N-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment protein- (SNAP-) 25 were affected by the D2 agonist. This is interesting, as these proteins are major players in exocytosis of

neurosecretory vesicles (240). Current evidence (241, 242) indicates that complexin holds the vesicle in a "ready-to-release" state near the membrane, while preventing the spontaneous zippering of the t- and v-SNAREs and thus spontaneous fusion of the vesicle to the membrane. Upon introduction of Ca^{2+}, which binds to synaptotagmin, complexin is "knocked off" and the membranes fuse, resulting in exocytosis. Since complexin expression is decreased, our results suggest that DA is stimulating exocytosis in the hypothalamus via the D2 receptor. However, as SNAP-25 protein levels are also being reduced, this may not be the case and requires further examination. Perhaps it is a homeostatic mechanism to control the over-secretion of neurotransmitters/neurohormones. Furthermore, N-ethylmaleimide-senstive factor (NSF) protein levels increased in response to D2 agonist. NSF transcript levels were initially found to be decreased in schizophrenic patients (243) but this was not observed in subsequent studies (228). Similarly to CaM, the meta-analysis by Zhang *et al.* (230) identified NSF transcripts as being relatively highly expressed in May when goldfish are sexually mature. This information, coupled with the increase in NSF mRNA (D1) or protein (D2) found in this study, suggest that the DAergic tone of inhibition on LH release may be dependent upon NSF expression and may provide further insights into the signaling mechanism of DAergic action in the hypothalamus.

Many of the proteins identified as differentially regulated by dopamine are involved in a disease state. For example, malate dehydrogenase, glutathione-S-transferase, calmodulin, glutamine synthetase, transferrin, tyrosine-3-monooxygenase/tryptophan-3-monooxygenase activation protein epsilon (YWHAE), microtubule-associated protein tau and beta-synuclein are among proteins identified that are known to be involved in neurodegenerative and/or psychiatric diseases (228).

There is a discrepancy between the number of mRNAs and proteins that were identified as differentially regulated that must be addressed. It is interesting to note that many mRNAs for ribosomal proteins are induced, and some of the corresponding proteins were detected, but did not change. This may be due to a limitation in the use of "snapshot" time frame studies. For example, while the steady-state mRNA levels for many ESTs were increased by 5h, the translational machinery may require additional time to efficiently produce the corresponding proteins. Importantly, concordance in the mRNA and protein changes despite the recognized limitations were well within the ranges seen with better characterized vertebrate systems (239, 244, 245). We show that it is possible to compare and characterize the hypothalamic transcriptome and proteome for a organism whose genome has yet to be sequenced. Future studies aimed at examining the temporal correlation between the hypothalamic transcriptome and proteome of the goldfish should reveal further relationships and critical pathways regulated by DA.

Table 3.2: List of proteins determined by iTRAQ as being significantly (FDR-adj p<0.05) differentially regulated in the hypothalamus of female goldfish treated with either SKF 38393 (pSKF) or LY 171555 (pLY) agonists. This list also shows those cDNAs corresponding to the proteins that were significantly (q<5%) affected by the same treatment (mSKF or mLY). Negative values indicate a decrease relative to control. Absent values indicate no change detected or that the corresponding cDNA was not present on the array. ESTs were annotated using Blast2GO's Blast Descriptor Annotator with default values except the Blast ExpectValue was changed from 1.0E-3 (default) to 1.0E-5. Following the Mapping step, the Annotation Configuration E-Value-Hit-Filter was changed from 1.0E-6 (default) to 1.0E-8 to increase the likelihood of correct GO annotation. Duplicates were assessed on the basis of sequence comparison and removed if a similar expression was observed. In the case where different expression profiles were seen (*), both ESTs were included, as it is possible that the sequences correspond to separate genes. pLength (aa) = the length in amino acids of the protein corresponding to the given Protein Accession, mLength (aa) = the length of the predicted protein sequence based on the cDNA corresponding to the given Blast2GO-annotated name, min eValue = the minimum ExpectValue from the BlastX result, Sim = the similarity of the cDNA sequence corresponding to the given Blast2GO-annotated name.

Protein Accession	Protein identified	Fold Change				Accession	Blast2GO annotated from mRNA (NCBI)	pLength (aa)	mLength (aa)	min. eValue	Sim
		mSKF	pSKF	mLY	pLY						
AAW82445	14kDa apolipoprotein*	-1.6	-1.3			CA967592	14 kda apolipoprotein	141	211	1.0E-69	96%
AAW82445	14kDa apolipoprotein*			1.9	-1.5	CF662502	14 kda apolipoprotein	141	226	1.0E-69	96%
CAG00145	25 kDa synaptosomal-associated protein			1.4	-1.5	CA969142	synaptosomal-associated protein 25	299	142	1.0E-35	75%
NP_956213	adaptor-related protein complex 2, beta 1 subunit		1.3					951			
AAH83251	Atp2b4 protein				1.4			346			
AAZ38450	beta thymosin-like protein				-1.3			52			
AAF79948	brain-type fatty-acid binding protein; B-Fabp				-1.4			132			
CAK04737	calbindin 2, like		-1.2					271			
NP_001017741	calcium/calmodulin-dependent protein kinase II alpha		2.3		1.8			478			
Q71UH6	Calmodulin			1.4	-1.2	CA969795	calmodulin variant 1	149	96	1.0E-33	98%
CAF92971	Creatine kinase, brain				-1.4			381			
NP_942096	creatine kinase, mitochondrial 1				1.2			417			
CAK10905	cytochrome c oxidase subunit IV isoform 1		1.2					169			
AAV52802	glutamine synthetase	1.9	1.4		-1.3	FG393017	glutamine synthetase	230	184	1.0E-77	97%
ABD67511	glutathione S-transferase rho	-1.6	-1.3		-1.2	CA964231	glutathione S-transferase rho	226	139	1.0E-16	89%
AAV52803	glyceraldehyde-3-phosphate dehydrogenase	1.6	-1.4			DY231775	GAPDH	210	323	1.0E-63	87%
AAA21578	kainate receptor alpha subunit	1.4	1.8		1.3	FG392717	kainate receptor alpha subunit	459	97	1.0E-34	94%
AAH63955	Krt5 protein				2.6			553			
AAM21708	liver-basic fatty acid binding			1.4	3.6	CA968596	fatty acid binding	122	156	1.0E-55	90%

Protein Accession	Protein identified	Fold Change				Accession	Blast2GO annotated from mRNA (NCBI)	pLength (aa)	mLength (aa)	min. eValue	Sim
		mSKF	pSKF	mLY	pLY						
	protein*						protein liver basic				
AAM21708	liver-basic fatty acid binding protein*	-1.5	8.6			CA970443	fatty acid binding protein liver basic	122	159	1.0E-46	95%
NP_956241	malate dehydrogenase 1a, NAD (soluble)	2.0	1.2	1.3		CA964750	malate dehydrogenase nad	305	216	1.0E-95	89%
ABC69306	myoglobin isoform 2		-1.4	1.4		CA968088	myoglobin	147	139	1.0E-60	98%
NP_958898	N-ethylmaleimide-sensitive factor	1.4			1.3	FG392958	n-ethylmaleimide-sensitive factor	744	283	1.0E-146	98%
CAN88379	novel protein sim to vert EF hand calcium binding protein 2 (EFCBP2)			-1.1				428			
CAK05381	parvalbumin			1.5	-1.1	CA969705	parvalbumin	109	240	1.0E-47	97%
ABF57553	Pi-class glutathione S-transferase		-1.7		-1.6			208			
BAA78376	polypeptide elongation factor 1 alpha	-1.8	1.3			CA967511	eukaryotic translation elongation factor 1 alpha 1	461	148	1.0E-51	98%
XP_001340376	PREDICTED: myelin basic protein isoform 3	2.0	-3.9			CA967910	myelin basic protein	170	172	1.0E-27	52%
CAF98839	PREDICTED: similar to germinal histone H4 gene				-1.1			234			
XP_001338014	PREDICTED: similar to microtubule-associated protein 1 A				1.4			3632			
XP_696230	PREDICTED: similar to microtubule-associated protein tau	1.9	-1.1			CA967834	microtubule-associated protein tau	285	193	1.0E-47	85%
XP_001335551	PREDICTED: similar to Myelin basic protein		-1.5		-1.5			121			
XP_691535	PREDICTED: similar to Nj-synaphin 2				-2.2			161			
CAF95822	Putative histone cluster 1, H2bb		-1.6		-1.5			258			
AAG14350	putative oncoprotein nm23	-1.6	-1.3			CA964203	non-metastatic cells 2, protein (NM23B)	153	211	1.0E-50	94%
DAA04576	RTN1	1.8				DY231940	reticulon 4	212	231	1.0E-57	81%
AAI14255	short chain dehydrogenase/reductase		-1.9	1.3	-1.5	CA968680	dehydrogenase reductase sdr family member 12	320	154	1.0E-45	82%
NP_001091958	spectrin alpha 2	1.4	1.3			FG392760	spectrin alpha 2	2480	205	1.0E-111	100%
NP_001017850	stathmin 1/oncoprotein 18*			1.4	-1.3	CA969799	stathmin 1 oncoprotein 18 variant 8	149	182	1.0E-44	90%

Protein Accession	Protein identified	Fold Change				Accession	Blast2GO annotated from mRNA (NCBI)	pLength (aa)	mLength (aa)	min. eValue	Sim
		mSKF	pSKF	mLY	pLY						
NP_001017850	stathmin 1/oncoprotein 18*	1.5	-1.5			CA966170	stathmin 1 oncoprotein 18 variant 8	149	191	1.0E-73	95%
NP_001018488	synuclein, gamma b (breast cancer-specific protein 1)		-1.5					122			
AAM90972	transferrin variant A1	1.9	1.5			CA968595	transferrin variant c	666	143	1.0E-22	97%
AAM90973	transferrin variant B1				-1.9			661			
NP_705954	triosephosphate isomerase 1b	1.8	-1.3		-1.2	CA968504	triosephosphate isomerase 1b	248	144	1.0E-70	96%
NP_997770	tyrosine 3-/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	1.1						255			
AAQ94569	ubiquitin C		-1.3					235			

Chapter 4.

DOPAMINE D1 RECEPTOR BLOCKAGE POTENTIATES AMPA-STIMULATED LH RELEASE IN THE GOLDFISH (*CARASSIUS AURATUS*) [§**]

4.1. INTRODUCTION

Dopamine (DA) is a potent inhibitor of LH release in the goldfish, as well as in many other fish, amphibians, birds, and mammals. The mechanisms of inhibitory action of DA in the goldfish are multi-fold: a) DA inhibits GnRH release from GnRH neurons through the D1 receptor (246); b) DA directly inhibits LH release from gonadotrophs in the anterior pituitary through the D2 receptor (4, 5); c) DA decreases the expression of GnRH receptor mRNA in the pituitary (98, 247); and, d) DA inhibits the synthesis of GABA (65, 207), a stimulator of LH release (70).

Previous work in our laboratory suggested that glutamate may be involved in stimulating LH release and that dopamine (DA) may be inhibiting its effect. For example, a previous study by Trudeau *et al.* (12) showed that injection of 2.5 μg/g AMPA, but not other glutamate agonists, significantly increased circulating LH levels in sexually regressed male goldfish 30 minutes post-injection. AMPA exerts its effects through ionotropic glutamate receptors (Grias). In mammals, there are 4 Grias (Gria1-4) whereas zebrafish, owing to the teleost-specific genome duplication (248), possess 8 copies of the receptor (Gria1-4a and b) (249). In persistently estrus rats, Gria expression was increased in GnRH neurons (250), and Garyfallou *et al.* (251) showed that Gria2 and Gria4 are expressed in immortalized hypothalamic GnRH neuronal cells (GT1-1, GT1-7 and Gn10). In a separate study,

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[§] Preliminary results of this chapter were presented at the *41st Annual Meeting of the Society for the Study of Reproduction.* Kailua-Kona, HI, USA. May 27-30, 2008. The refereed conference abstracts are published in the 2008 Special Issue of *Biol Reprod.*

[**] Popesku, J.T. Chang, J.P., Trudeau, V.L. Dopamine D1 receptor blockage potentiates AMPA-stimulated LH release in the goldfish. *In preparation.*

microarray analysis of the hypothalamus identified the mRNA for Gria2a subunit as being significantly ($q<5\%$) increased 5h post-injection with the DA D1-receptor-specific agonist SKF 38393 in sexually mature female goldfish (Chapter 3). With these observations, we hypothesized that DA may be having an inhibitory effect on the glutamate system in the context of reproduction.

In a previous study by our laboratory, activin βa mRNA levels were identified as being significantly affected by SKF 38393 (Chapter 3). The activins are members of the transforming growth factor β (TGFβ) superfamily and are homo- or heterodimeric proteins. Activin A is comprised of 2 βa subunits, whereas activin B is made up of 2 βb subunits and activin AB is made up of 1 βa and 1 βb subunit. Activin A is a potent stimulator of FSH release from the rat pituitary *in vitro* (252) and is able to stimulate LH release when injected intracerebroventricularly (i.c.v.) into the rat hypothalamus *in vivo* (253) and from goldfish perifused pituitary fragments *in vitro* in a dose-dependent manner (147). Activin is also capable of stimulating GnRH release in the GnRH-secreting GT1-7 neuronal cell line (254). Activin βa immunoreactivity is localized in neuroendocrine areas, including the telencephalon, and in nerve fibers in close contact with gonadotrophs in the pituitary of the thin-lipped grey mullet (255). Both activin A and B stimulate expression of FSHβ but decrease expression of LHβ subunit mRNA levels in goldfish pituitary (142, 143).

DA is also a stimulator of growth hormone release from the pituitary in goldfish (157, 171, 172, 256) as well as in other fish species (257-260) acting through the D1 receptor at the level of the pituitary. There is also evidence that DA may also stimulate food intake in fish (172, 257, 261, 262). Cholecystokinin (CCK), cocaine- and amphetamine-regulated transcript 1 (CART-1), and Neuropeptide Y (NPY) are peptides that are strongly involved in

food intake (reviewed in (263)). CCK and NPY act by decreasing and increasing food intake, respectively. CART-1 also acts as an anorexigenic factor by inducing satiety and increasing energy consumption (264). Tachykinin (PPT) may also be an anorexigenic factor, as PPT levels increase following feeding (265). Other studies in our lab indicate that isotocin (IST) may also inhibit food intake in the goldfish (J. Mennigen, personal communication). Furthermore, CCK, NPY, and IST are all involved in LH release in the goldfish (69, 266-269) and CART-1s colocalized with NPY in the pituitary and various regions of the brain of catfish (270).

With the known impacts of DA on LH and GH release, we investigated, in the present study, the effect of pre-treatment with either a D1- or D2-specific antagonist followed by treatment with AMPA on LH and GH release in the sexually regressing goldfish.

4.2. Materials & Methods

4.2.1. Animals

This subsection is identical to Section 2.2.1 in this thesis.

4.2.2. Experimental and sampling procedures

The DA D_1-specific antagonist SCH 23390, DA D_2-specific antagonist sulpiride, and the glutamate agonist AMPA were purchased from Tocris (Ballwin, MO, USA). AMPA was dissolved in 0.6% saline to give a dose of 2.5 μg/g body weight of fish. The antagonists were first dissolved in a minimal amount of dimethylsulfoxide (DMSO), and subsequently diluted with 0.6% saline. The final concentration of DMSO was 0.099%. Sexually regressing (June; gonadosomatic index, GSI 3 ± 0.4 %; n=18 each) or sexually regressed

(September; GSI 2 ± 0.1%; n=18 each) female goldfish received injections at 5 μL/g body weight of either SCH 23390 or sulpiride to give a dose of 40 μg/g or 2 μg/g body weight of fish, respectively, or saline containing an equivalent amount of DMSO. After 4.5 hours, fish received a second injection of either saline or 2.5 μg/g AMPA. Thirty minutes following the second injection, blood was sampled by puncture of the caudal vasculature via a 25-gauge needle attached to a 1-mL syringe. Brain tissues were obtained as described in Section 2.2.2 of this thesis. Blood was stored 4°C over night. Serum was collected by centrifuging the blood at 7,500 rpm at 4°C for 15 mins and stored at -20°C until used for the radioimmunoassay (RIA).

4.2.3. Radioimmunoassay (RIA)

The double antibody RIA protocol of Zhao *et al.* (72, 271) was used to analyze serum LH levels. For all LH RIAs, n=14-16 (June) or n=17-18 (Sept) per treatment group. GH levels were determined by RIA as previously described by Cook *et al.* (272). For all GH RIAs, n=11-15 (June) and 17-18 (Sept) per treatment group.

4.2.4. RNA extraction and cDNA synthesis

See Section 2.2.3 of this thesis.

For microarray analysis, cDNA was synthesized from 2 μg total RNA according to the Genisphere 3DNA Array 900MPX kit according to the manufacturer's protocol (Genisphere, Hatfield, PA).

4.2.5. Microarray hybridizations

Four independent microarray hybridizations were performed for each hypothalamic and telencephalonic tissue for both D1 and D2 antagonists alone (total of 16 arrays) to screen

for the effects of the antagonists on the neuroendocrine brain. Hybridizations were carried out relative to a common pool of control samples (~30 control fish) for each tissue, which allows for less technical variation as only one reference is handled while maintaining biological variation of the treatment samples (217). All labeling and hybridizations were performed as described in Section 3.2.4 of this thesis. Raw intensity values for all microarray data and microarray platform information were deposited in the NCBI Gene Expression Omnibus database (platform accession no. GPL7056; SuperSeries accession no. GSE15763). Generalized Procrustes Analysis (9) was used for normalization of the array data and the Significance Analysis of Microarrays (SAM) method (10) was used for microarray analysis.

Table 4.1: Primers for real-time RT-PCR used in this study

Amplicon	Forward primer (5' → 3')	Forward primer (5' → 3')	Accession
L8	AACTACGCCACAGTCATCTCC	CCAGCAACAACACCAACAAC	EU313780
eF1α	GATTGTTGCTGGTGGTGTTG	GCAGGGTTGTAGCCGATTT	AB056104
activin βa[a]	TTTAAGGACATCGGGTGGAG	TGATTGATGACGGTGGAATG	AF169032
activin βb[a]	GATGGAAAAGCGTGTGGAGT	CAGGAATGGACGGTGTGAG	AF004669
sGnRH[a]	CTGGTCATACGGTTGGCTTC	CATCAGCATCCACTTCATTCAC	AB020242
cGnRH-II[b]	TACGATTCCTCAGAGGTTTCAG	CATCCAGCACTATTGTCTTCAG	U30386
Gria2a	CACTGAGGAGTTTGAGGATGG	TTAGCCGTGTAGGAGGAGATG	AM886310
Gria2b	TGGTCAGTCGTTTCAGTCCA	AACCACCAGACTCCTCCAAC	DY231932[†]
Gria3b	TGAGGAAATGGACAGGAGACA	CTGATGTTGGTAAAGCCCAGA	DY231931[†]
Gria4	TGGACCGAAGACAGGAGAAG	TCCCAGGTTAGCGATGATGT	U12018
NPY[c]	CTGGGGATGGGACTCTGTTT	TTCGTCTGCTTGGGAACTCT	M87297
CART-1[c]	CCATGGAGAGCTCCAAACTC	TCTTGACCCTTTCCTGATGG	AF288810
preprotachykinin[c]	GGTTCAGGATGAGTGCCTTC	CCTCTTGGCTCCTTTTACCCC	U61272
CCK	AACGCTGGAATCTGTGTGTG	GGGGCTCTCATCATCCTCT	U70865
Isotocin[d]	ATCTTGGCTACTGGCAGCTT	GTATCTGCTGTGGTGAAGGT	AF322651
DARPP-32	AGGCAACAGAACCGACTCA	GGCTTGAGGACACCATTTTC	FJ855221
Endozepine	GAAAGCAGCAGAGGAGGTCA	TTAGATTCCGTATTTCCCCTTCA	L77976

[†] primers were based on the AURATUS EST project. In all cases, PCR amplicons were sequenced to confirm identity.
Superscript letters indicate primers that have previously been published: [a] (70), [b] (273), [c] (274), [d] (138)

4.2.6. Real-time RT-PCR assays

Real-time RT-PCR was performed as described in Section 2.2.6 except that 2 ng first strand cDNA template was used instead of 25 ng. Primers used in this study are listed in Table 4.1. Results for the telencephalon were normalized to ribosomal L8 and results for the hypothalamus were normalized to elongation factor 1α (eF1α), as it was determined these genes did not change in these tissues for any of the given treatments. For all real-time RT-PCR reactions, n = 4-6 per treatment.

4.2.7. Statistical analysis

All statistics for real-time RT-PCR and RIA were performed using Systat 12. Data was assessed using the Kolmogorov-Smirnov test for normality and Levene's test of homogeneity of variance. In the cases where data was not normally distributed, the data was log-transformed. In the cases were log-transformed data was still not normally distributed, a square-root transformation was able to achieve normality. A two-way ANOVA was performed with significance considered at $p<0.05$, followed by Tukey's HSD pairwise comparisons if data was homoscedastic or Dunnett's T3 pairwise comparisons in cases where data was heteroscedastic. For GH data in June, data could not be transformed to be made normal; therefore, a two-way non-parametric test was used.

4.3. RESULTS

4.3.1. LH and GH

Serum LH and GH levels were measured by RIA (Figure 4.a). Neither D1-specific nor D2-specific antagonist alone, nor AMPA alone, had any effect on circulating LH levels relative to saline-injected controls. However, with pre-treatment of the DA D1-specific receptor antagonist, SCH 23390, AMPA provided a synergistic increase (289%; $p<0.0005$)

in circulating LH. The combination of the D2-specific antagonist, sulpiride, with AMPA, provided a moderate, but not statistically significant increase (180%) in serum LH levels. Neither DA antagonist had any statistical effect on GH levels at this time of year. AMPA, however, provided a general decrease on GH levels ($p=0.022$), but the multiple comparisons were not statistically significant.

The experiment was repeated in September, when goldfish are sexually regressed (Figure 4.1b). In contrast to fish treated in June, all of the treatments, alone or in combination, significantly increased serum LH levels ($p \leq 0.003$). Both SCH 23390 and sulpiride increased circulating LH levels by 210% and 213%, respectively, relative to controls. Furthermore, injection of AMPA alone increased serum LH levels by 415% relative to saline-injected controls. The combined treatments of SCH 23390 or sulpiride with AMPA increased serum LH levels by 332% and 349%, respectively, relative to control fish. There were no statistical differences between any of the treated groups relative to each other. SCH 23390 alone or in combination with AMPA, reduced GH levels to 57% or 48%, respectively, of control levels. Sulpiride alone, or in combination with AMPA, had no effect on circulating GH levels.

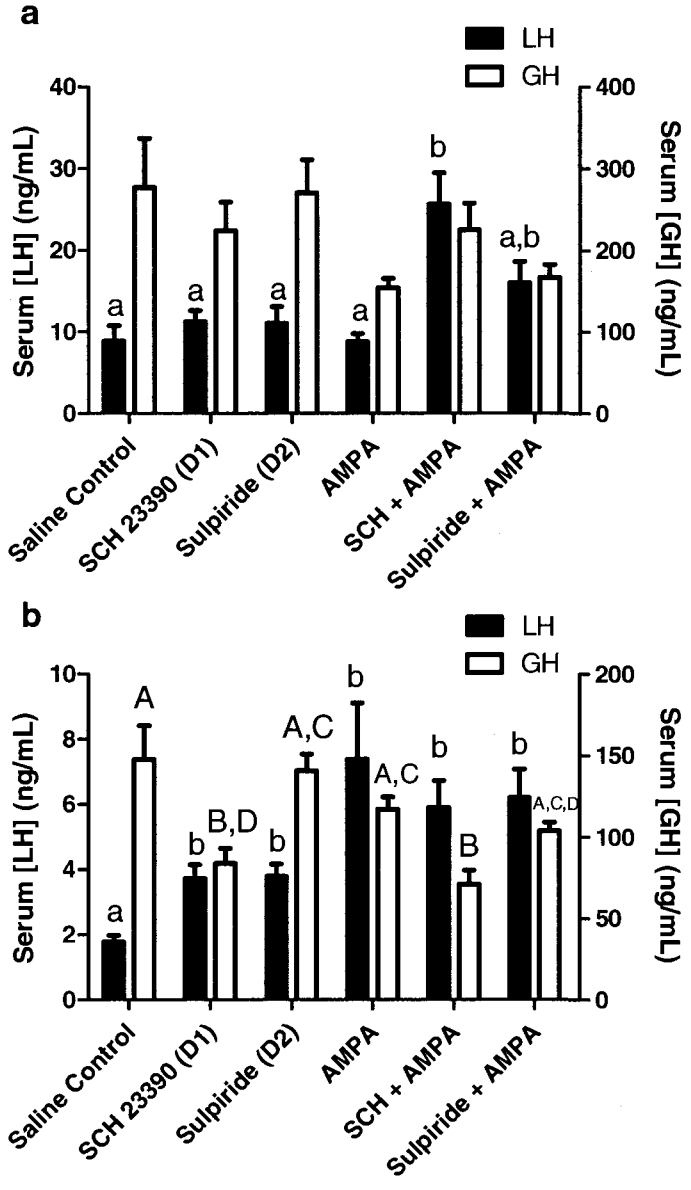

Figure 4.1: Serum LH and GH levels determined by RIA following DA antagonists alone or combined with AMPA. The experiment was completed in a) mid-June and repeated in b) September. Letters indicate statistical differences between treatments (capital letters for GH and non-capital letters for LH). In June, there was a general effect of AMPA on GH (p=0.022).

4.3.2. Microarray analysis

Microarray analysis was performed to determine potential transcripts that would prime the animal to becoming stimulated by AMPA. cDNA derived from total RNA isolated from Hyp and Tel tissues for the SCH and sulpiride groups were submitted to microarray analysis. This revealed very few (<20) mRNAs being affected with the exception of the Hyp SCH group, which showed 78 mRNAs being up-regulated and 447 mRNAs being down-regulated. The Hyp SCH dataset (up & down combined; q<5%, duplicates removed) was binned into their corresponding GO terms (Figure 4.2) using Blast2GO. The results show that processes such as protein modification, regulation of transcription, and signal transduction are being affected by the D1 antagonist in the hypothalamus. Specific mRNAs being affected are listed in Table C.3 (Appendix C). In particular, NPY, IST and endozepine mRNA levels were identified as being decreased, while CCK, CART-1, and PPT mRNA levels were increased. We selected these transcripts for validation using real-time RT-PCR.

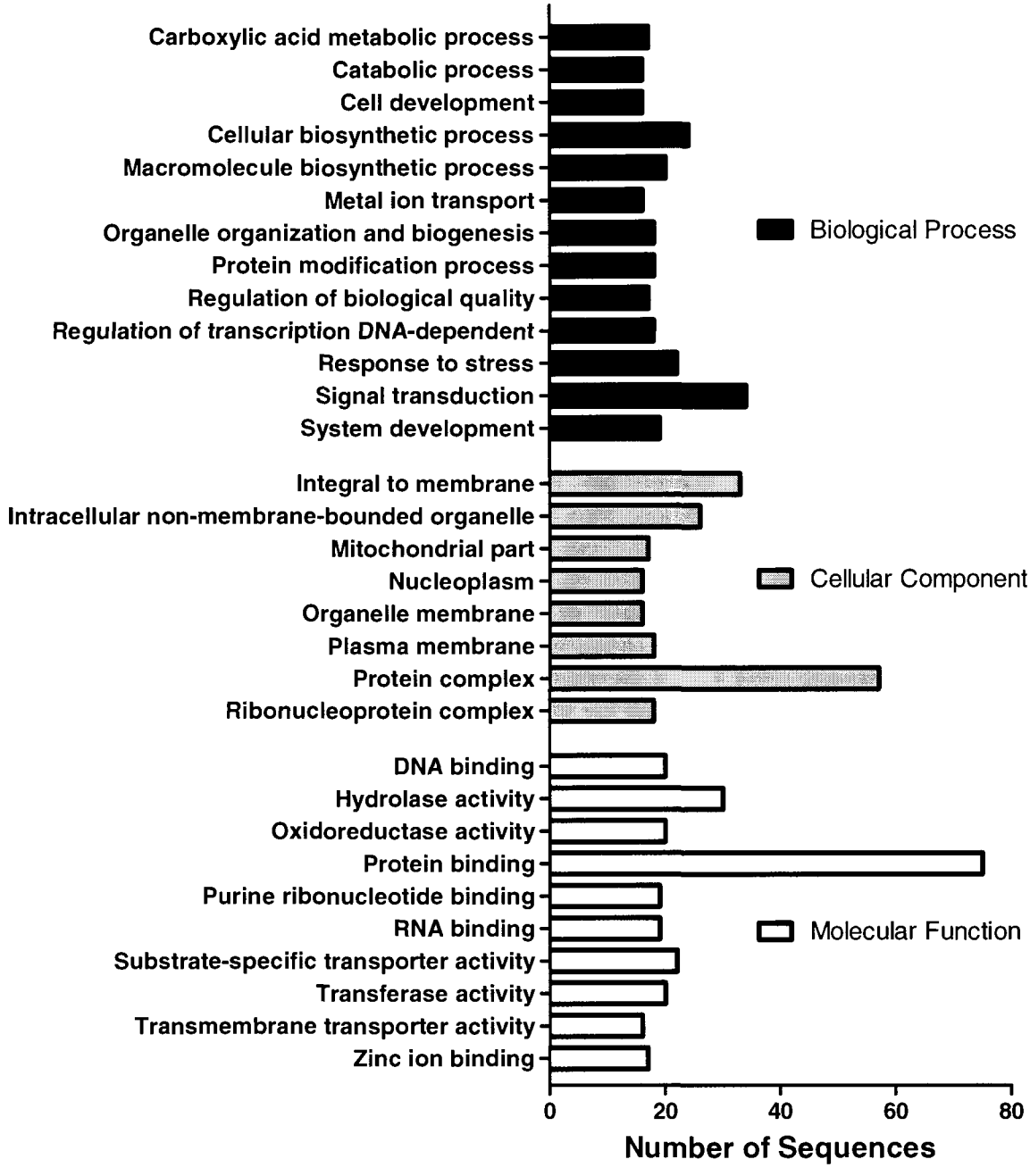

Figure 4.2: Gene Ontology categorization of mRNAs that are statistically (q<5%) differentially expressed (both increased and decreased combined) in the hypothalamus of female goldfish 5h post-injection with SCH 23390.

4.3.3. Real-time RT-PCR

As we were testing the hypothesis that DA is inhibiting the glutamate system, we investigated the expression of different AMPA receptor subunits by real-time RT-PCR. Steady-state mRNA levels of the Gria2a subunit in the hypothalamus were decreased 1.8-fold ($p=0.028$) in response to AMPA, but this response was blocked when fish were pre-treated with SCH 23390 (Figure 4.3a). In the telencephalon, the results are similar to those obtained for Gria4 (Figure 4.3b), but did not reach statistical significance (data not shown).

An interaction ($p=0.006$) was observed between DA antagonists and AMPA for Gria4 mRNA levels in the hypothalamus. Steady-state mRNA levels for Gria4 were decreased 1.9-fold ($p=0.02$) in the hypothalamus of fish pre-treated with SCH 23390 relative to control fish (Figure 4.3b). In fish treated with AMPA alone, Gria4 steady-state mRNA levels only marginally increased 1.4-fold ($p=0.055$). However, in fish pre-treated with SCH 23390 and subsequently injected with AMPA, relative mRNA abundance of Gria4 was unchanged relative to control fish. Sulpiride alone or in combination with AMPA had no effect on Gria4 mRNA levels in the hypothalamus. In the telencephalon, Gria4 mRNA levels were not statistically affected by any of the treatments alone.

Relative mRNA abundance of the Gria2b and Gria3b receptor subunits were not statistically affected by any of the treatments in either hypothalamus or telencephalon (data not shown).

As we did not perform microarray analysis on all of the treatments, we investigated the expression of the GnRHs known to be expressed in goldfish brain: cGnRH-II and sGnRH. cGnRH-II mRNA levels were unaffected by SCH 23390, sulpiride, or AMPA

alone, as well as a sulpiride + AMPA combination, relative to controls. However, in fish pre-treated with SCH 23390 and subsequently challenged with AMPA, an increase (12-fold; p=0.015) in cGnRH-II mRNA levels was observed (Figure 4.4), relative to control fish. None of the treatments had any statistically significant effect on sGnRH steady-state mRNA levels.

Relative expression of IST mRNA levels in the telencephalon (Figure 4.5) of treated fish followed a similar, albeit not as pronounced, profile as those of cGnRH-II: there was little change except for the combined SCH 23390 + AMPA treatment. This combined treatment induced a 2.8-fold increase (p=0.002) in isotocin mRNA levels relative to control fish. An interaction (p=0.001) was observed between DA antagonists and AMPA for IST in the telencephalon.

Activin βa mRNA levels were significantly induced in both the hypothalamus (10-fold; p=0.001) and telencephalon (4-fold; p=0.011) in SCH 23390 treated fish relative to control fish (Figure 4.6). There were no effects of sulpiride alone or in combination with AMPA on activin βa mRNA levels. Steady-state mRNA levels for activin βa were similarly increased in both the hyp (8.8-fold; p=0.001) and the tel (4.1-fold; p=0.007) in the combined SCH 23390 + AMPA treatment relative to control fish. Activin βb mRNA levels remained unaffected by any of the treatments (data not shown).

Real-time RT-PCR was performed on select genes identified by the microarray as being differentially expressed in the hypothalamus. Endozepine (aka diazepam-binding inhibitor; DBI), cholecystokinin (CCK), neuropeptide Y (NPY), cocaine- and amphetamine-regulated transcript 1 (CART-1), preprotachykinin (PPT) were selected. A 4.4-fold decrease

in NPY, a 1.9-fold increase in CCK, 1.9-fold increase in CART-1, and 1.4-fold increase in PPT mRNAs was observed using real-time RT-PCR (Figure 4.7). This was similar to the results observed in the microarray analysis: a 1.5-fold decrease in NPY, a 1.5-fold increase each in CCK and PPT, and a 1.4-fold increase in CART-1 mRNAs (Table C.3 in Appendix C). Furthermore, similar to the microarray result for DBI (-1.5-fold; Table C.3), a 1.4-fold decrease in DBI mRNA levels in the hypothalamus was observed with real-time RT-PCR; however, this did not reach statistical significance (data not shown). Nevertheless, PCR analysis confirmed the direction of gene expression in all cases, although this did not reach statistical significance.

A partial coding sequence for goldfish dopamine- and cAMP-regulated phosphoprotein, 32 kDa (DARPP-32, aka protein phosphatase 1, regulatory subunit 1b or PPP1R1B) was cloned and its expression was examined by real-time RT-PCR. No statistical difference in DARPP-32 mRNA levels was observed with any of the treatments in either the Hyp or the Tel (data not shown). The partial coding sequence for goldfish DARPP-32 was submitted to GenBank and assigned an accession number (FJ855221).

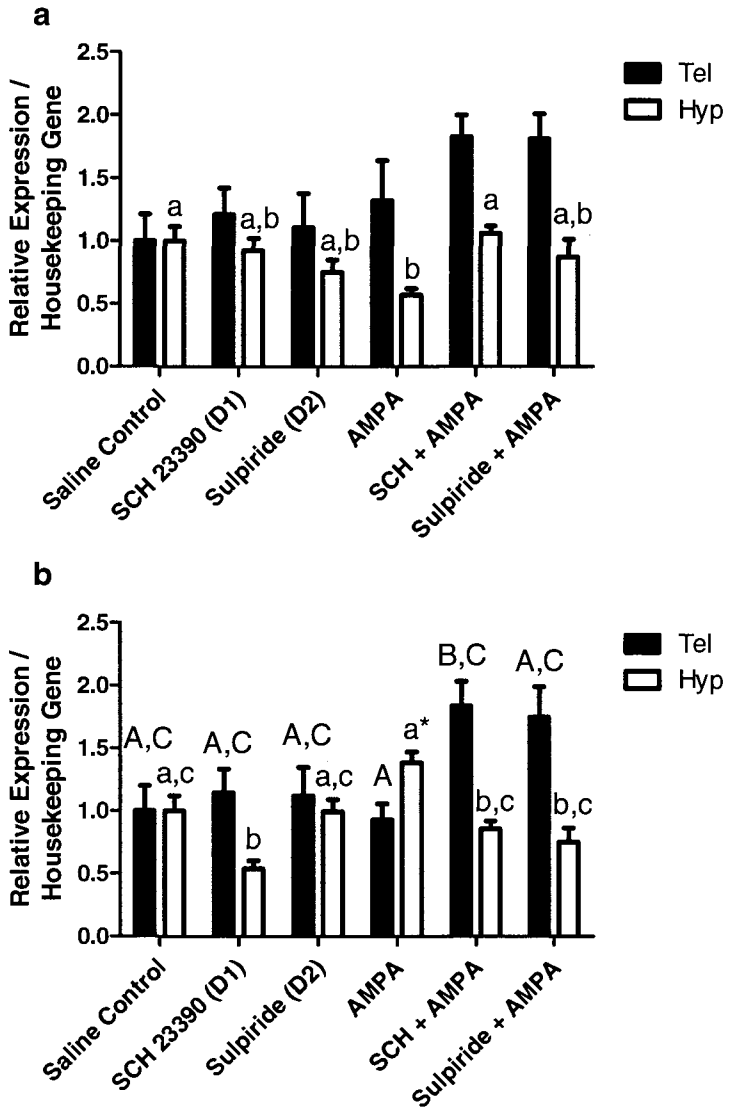

Figure 4.3: Relative expression of a) Gria2a and b) Gria4 in the hypothalamus of female goldfish 5h post-injection with either SCH 23390 or sulpiride with or without AMPA. Letters indicate statistical differences between treatments (capital letter for Tel and non-capital letters for Hyp). There was no statistical difference in Gria2a mRNA levels in the telencephalon. * indicates marginal significance (p=0.055).

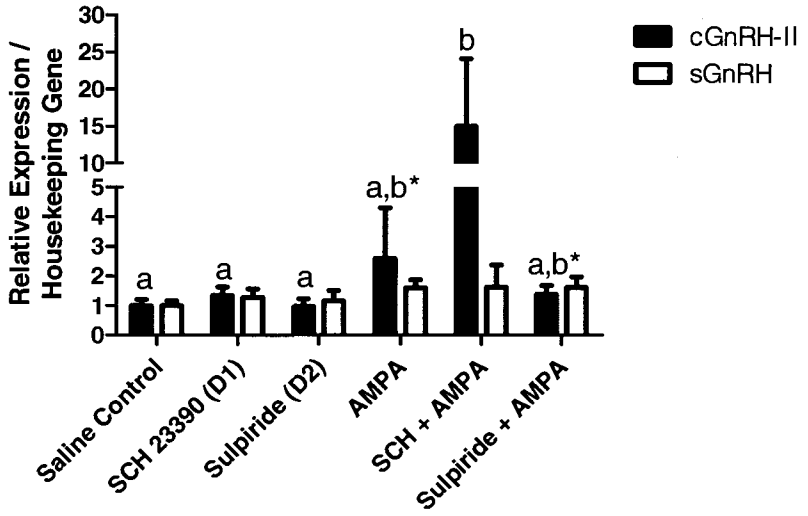

Figure 4.4: Relative expression of cGnRH-II and sGnRH mRNAs in the telencephalon of female goldfish 5h post-injection with either SCH 23390 or sulpiride with or without AMPA. Letters indicate statistical differences between treatments. * indicates marginal significance ($0.052<p<0.059$).

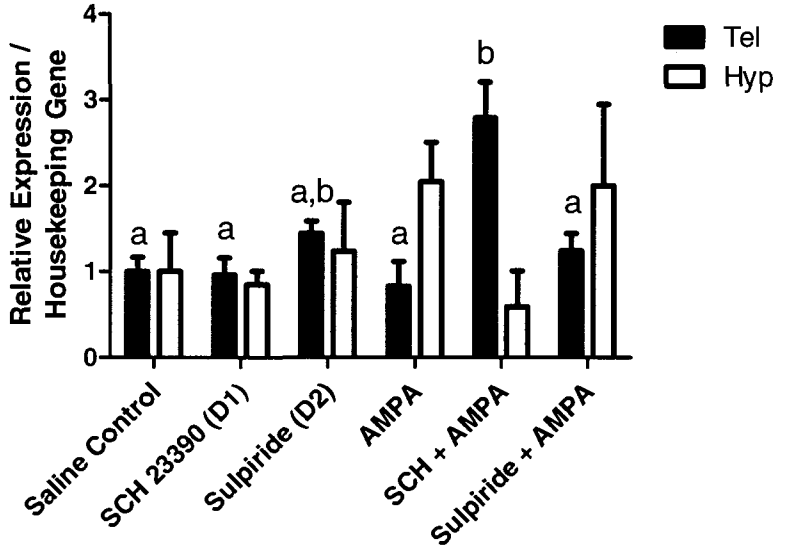

Figure 4.5: Relative expression of IST mRNA in the telencephalon and hypothalamus of female goldfish 5h post-injection with either SCH 23390 or sulpiride with or without AMPA. Letters indicate statistical differences between treatments. There were no statistical differences in IST mRNA levels in the hypothalamus.

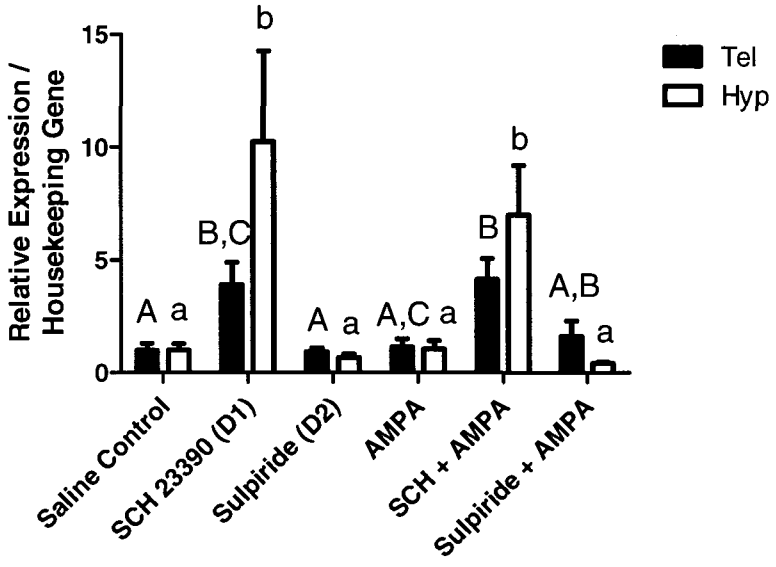

Figure 4.6: Relative expression of activin βa mRNA in the telencephalon and hypothalamus of female goldfish 5h post-injection with either SCH 23390 or sulpiride with or without AMPA. Letters indicate statistical differences between treatments (capital letters for Tel and non-capital letters for Hyp).

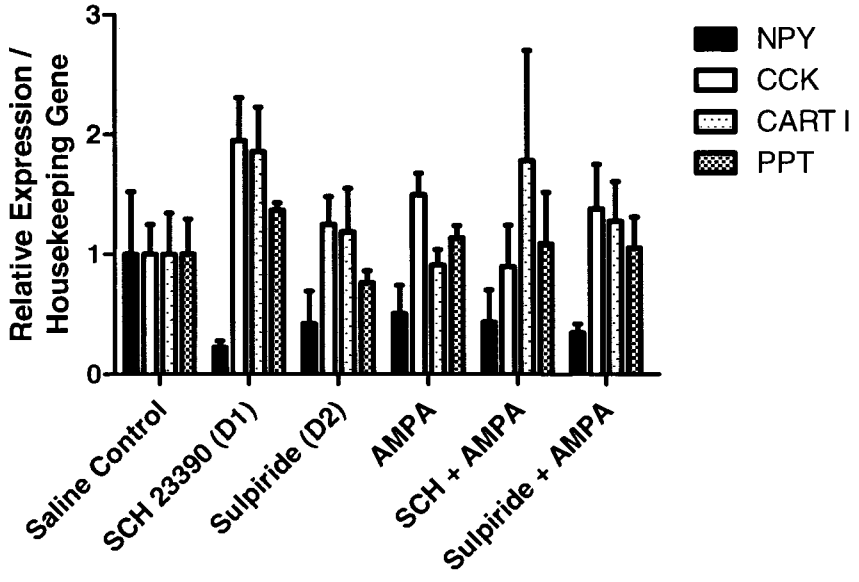

Figure 4.7: Real-time RT-PCR of feeding peptide mRNAs in the hypothalamus of female goldfish 5h post-injection with saline, SCH 23390 and sulpiride alone or in combination with AMPA.

4.4. DISCUSSION

In this study, we show for the first time, that inhibition of DA action primes goldfish for AMPA-stimulated LH release. A previous study by Trudeau *et al.* (12) suggested that AMPA may have stimulatory effects on LH release in the goldfish. That information, coupled with our previous study investigating the effects of DA agonists on gene expression in the goldfish neuroendocrine brain (Chapter 3), led us to hypothesize that DA is inhibiting the glutamate system.

Grodum *et al.* (275) investigated the effects of the D1 antagonist NNC 01-0687 on pituitary hormone release in human males. They found no change in the release of multiple hormones from the pituitary, concluding that D1 is not activated during basal conditions. Our results corroborate their findings, in that SCH 23390, a D1-specific receptor antagonist alone, had no effect on circulating LH. However, a combined treatment of SCH 23390 with AMPA, a glutamate agonist, manifested in a synergistic increase in serum LH levels. It is unclear whether this effect is the result of an interaction at the level of the pituitary, but specific D1 agonists or antagonists have no effect on LH release from goldfish pituitary cells *in vitro* (170), suggesting this effect may be due to interactions in higher brain centres.

High variability in cGnRH-II expression was observed, similar to the SA group in LH response. This is likely due to fish being in different physiological states at that time of year and also suggests that there is another factor modulating the response; potentially GABA (12). Nevertheless, the cGnRH-II expression profile across treatments mirrored the circulating LH levels in those same treatments. Interestingly, the expression profile for isotocin (IST) in the telencephalon also mirrored circulating LH levels. Gria3 protein has been found to be co-localized with oxytocin in rat and monkey hypothalamic magnocellular

neurons (276). In fish, this area is located in the preoptic area, where IST is synthesized in magnocellular and parvocellular neurons and project widely in the brain (118). Following real-time RT-PCR, IST mRNA was decreased 1.6-fold ($p=0.016$) in sexually mature female goldfish hypothalami 5h post-injection with SKF 38393, a D1-specific agonist (Popesku, unpublished). The fact that high IST expression was observed in the telencephalon, coinciding with high serum LH levels in the combined D1-antagonist + AMPA, supports IST as a regulator of LH release (reviewed in (69)). Taken together, increased expression of cGnRH-II and IST may explain the observed increase in serum LH levels in SCH 23390 + AMPA-treated fish.

Activin βa mRNA levels were significantly increased in SCH 23390-treated fish, with or without the addition of AMPA in both the hypothalamus and the telencephalon. Activin βa subunit mRNA was previously determined by real-time RT-PCR to be decreased 5.0-fold ($p=0.049$) in the hypothalamus 5h post-injection with SKF 38393, a D1-specific agonist (Popesku, unpublished). These 2 treatments conclusively demonstrate that activin βa expression is under the regulatory control of DA acting through the D1 receptor. Martyniuk *et al.* (70) showed that baclofen, a $GABA_B$ receptor agonist, but not the $GABA_A$ receptor agonist muscimol, increased activin βa mRNA expression in the goldfish *in vivo*. It is known that GABA and DA inhibit each other, but it is currently unclear as to whether it is DA or GABA, or both, that are directly affecting activin βa expression. The decrease in expression of endozepine, a naturally-occurring benzodiazepine that binds to $GABA_A$ receptors and prolongs the opening of the chloride ion channel, by DA antagonists supports the hypothesis that GABA and DA inhibit one another. In addition, immortalized hypothalamic GnRH neuronal cells have been shown to express $GABA_A$ receptors (251). Studies are currently

underway in our laboratory to further examine activin expression and neurotransmitter involvement.

The mRNAs for CCK and CART-1 were increased while NPY mRNA was decreased in response to blockage of the D1 receptor, suggesting that the fish are limiting food intake and hence limit growth. This is in line with other studies showing that DA, acting through the D1 receptor in the pituitary, stimulates GH release in goldfish (171, 172). Interestingly, NPY has been shown to stimulate both LH and GH release from goldfish pituitary fragments *in vitro* (269). CCK has also been shown to stimulate LH release, in a dose-dependent manner, in pituitary fragments from sexually regressed, but not recrudescing, goldfish (266). There is already an established link between reproduction and feeding (reviewed in (277)) and Matsuda *et al.* (278) showed that i.c.v.-injections cGnRH-II, but not sGnRH, decreased food intake in a dose-dependent manner. Furthermore, Hoskins *et al.* (273) confirmed that i.c.v.-injections of cGnRH-II decreased food intake and also showed that orexin mRNA expression decreased in the hypothalamus in goldfish. Conversely, they also showed that i.c.v.-injections of orexin decreased cGnRH-II mRNA expression in the hypothalamus and optic tectum of goldfish. The fact that the mRNAs encoding orexigenic peptides are decreased and anorexigenic peptides are increased strongly suggests that the fish limit food intake in response to blockage of the D1 receptor. This, coupled with the decrease in GH implicates DA in energy balance in goldfish as in rodents (279, 280).

DARPP-32 is known to integrate signals from dopamine and glutamate in mammals (196, 281, 282) and we hypothesized that the mechanism of action observed in this study may be through transcriptional regulation of DARPP-32. We cloned a partial coding sequence for goldfish DARPP-32 and examined its expression in the treatment groups. We

did not observe any significant change in goldfish DARPP-32 mRNA levels, but we cannot reject the hypothesis that DARPP-32 is involved in LH release, because it acts at the level of protein and may be outside of the scope of transcriptional regulation. Further experimental evidence investigating the localization of DARPP-32 and the phosphorylation state of DARPP-32 is required.

Seasonal effects of neuropeptides and neurotransmitters on LH are common in the goldfish model (108, 266, 268, 283), which are likely due to the cyclicity of circulating sex steroids (100, 283). We repeated our experiment in September, when goldfish are sexually regressed. These results in female goldfish corroborate the results of Trudeau *et al.* (12), who showed an increase in LH release resulting from AMPA injection at the same time of year in male goldfish. In contrast to the experiment conducted in sexually mature/regressing fish in June, all of the treatments significantly increased circulating LH levels in September. This suggests that the DAergic tone is important in modulating the response of AMPA-stimulated LH release. Indeed, the recent discovery of a DA D2-receptor alternative splice, its seasonally dimorphic expression, and its implication in maintaining the DAergic tone (Chapter 2) may alter telencephalonic and hypothalamic responses to DA, which, in turn, may modify the cellular response of other neurotransmitters, such as AMPA. Alternatively, estrogen and/or testosterone levels, which cycle throughout the year and peak in April/May (100, 283), may be important in modulating the responses observed in this study. Further investigation into the factors mediating the response observed in this study is warranted.

Taken together, the results presented in this study suggest that blockage of the D1 receptor primes the goldfish for AMPA-stimulated LH release and suggests a novel mechanism of LH release in vertebrates. The mechanism of action is currently unknown, but

is speculated to be through decreased food intake with a concurrent increase in activin βa initialized by blockage of D1 receptors. This primes the fish for an AMPA-stimulated induction of cGnRH-II and IST and a subsequent release of LH from the pituitary. Future studies aimed at investigatating the seasonality and mechanism of action of the response observed in this study may provide insights into vertebrate reproductive processes.

Chapter 5.

DOPAMINERGIC NEURONS SPONTANEOUSLY REGENERATE IN THE GOLDFISH HYPOTHALAMUS FOLLOWING MPTP INJECTION[††]

5.1. INTRODUCTION

Adult teleost fish in general have a good capacity for neuronal regeneration (206, 284-287). Dopaminergic (DAergic) neuron regeneration is most extensively studied in the goldfish retina (106, 288, 289). Goldfish can also regenerate axons projecting to the optic tectum from the thalamus and brainstem following removal of the tectal lobe (290). Adult zebrafish are able to regenerate motor neurons as evidenced by an increase in the proliferation and expression of olig2$^+$ ependymo-radial glial progenitor cells (287). There is a report of DAergic neurogenesis in the substantia nigra (SN) of adult mice (291), but this finding is challenged (292). In mammals, adult neurogenesis is known to occur in only two discrete regions of the central nervous system (CNS): the hippocampus and olfactory bulb (293, 294). In amphibians, however, it has recently been reported that adult red spotted newts are able to completely regenerate mesencephalic and diencephalic DAergic neurons following chemical lesioning with 6-hydroxydopamine (6-OHDA) (295). Both 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are specific DAergic neurotoxins that selectively ablate DA neurons. However, MPTP is the most widely used and potentially best DA neurotoxin available (296).

MPTP induces a parkinsonian syndrome in goldfish similar to that of higher vertebrates (45-48, 105) and the goldfish MPTP model has been proposed for studying

[††] Popesku, J.T., Xia, X., Anisman, H., Trudeau, V.L. Dopaminergic neurons spontaneously regenerate in the goldfish hypothalamus following MPTP injection. *In preparation.*

Parkinson's disease (PD) (208). PD is characterized by DAergic neuron loss in the SN pars compacta (SNc) with subsequent loss of DAergic input to the striatum (297). In mammals including humans (104, 298), primates (299, 300) and rodents (103, 301), the effect of MPTP is irreversible. In contrast, Poli *et al.* (105) reported spontaneous complete recovery of catecholamine levels in goldfish brain regions after 6 weeks following intraperitoneal (i.p.) injections of 10 μg/g of MPTP on 3 consecutive days. The authors noted, however, that their results did not determine whether DAergic neurons regenerate or if there is metabolic compensation provided by the surviving neurons. Possible compensatory mechanisms include increase synthesis of TH, increased DA turnover and release, and decreased DA uptake. In monkeys treated with MPTP, surviving striatal TH-positive fibers were found to increase their branching and thickness with a concomitant increase in growth-associated protein 43 (GAP-43) suggesting a compensatory sprouting mechanism of extracellular DA normalization (302), similar to what has been suggested in mouse SN DAergic neurons (303).

To act as a neurotoxin, MPTP must first be converted to MPP^+, its oxidized congener, by monoamine oxidase B (MAO-B) in astrocytes, which is then selectively taken up by DAergic neurons through the DA transporter (DAT) (304-306). Deprenyl, a MAO-B inhibitor, counteracts the effects of MPTP in both mammals (307, 308) and goldfish (45, 105, 309). MPP^+ accumulates in the mitochondria where it inhibits complex I of the respiratory chain leading to anoxia, anaerobic respiration, and cell death (310, 311). Moreover, i.p. injection of MPP^+ into goldfish replicates the effect of MPTP-induced DA decrease in the hypothalamus, telencephalon, and pituitary (312). Light and electron microscopic studies on the effect of a single injection of MPTP on DAergic neurons in the

goldfish telencephalon showed progressive irregularities occurring on the contours of the neuronal nuclei and progressive granularization of nucleoplasm, suggesting an apoptotic mechanism of cell damage (45, 48). Pollard *et al.* (48) reasoned that if parkinsonism developed following MPTP-induced DAergic neuron loss in the goldfish, goldfish could provide a good model for human PD that would be a relatively inexpensive system to screen potential therapeutic drugs. Studies using the goldfish have demonstrated that i.p. administration of MPTP causes a selective depletion of the catecholamines, DA and norepinephrine (NE), without affecting serotonin (5HT) content (46, 105), and induces a parkinsonian syndrome which parallels that of higher vertebrates (48).

The striatum has been identified in the goldfish and likely resides in the telencephalon in the area dorsalis telencephali, pars centralis/medialis (Dc) based on immunocytochemical evidence (43) and DA depletion with MPTP (45). However, it is currently unclear as to where the cell population in fish homologous to the mammalian SN resides (reviewed in (69)). It is likely not in the midbrain, as no TH-immunoreactive (TH-ir) cells bodies are observed in that region in the goldfish (43, 60). Studies in zebrafish identify the periventricular posterior tuberculum (TPp) as the most likely candidate based on immunohistochemical and tract-tracing evidence (34, 37). Indeed, immunocytochemical studies in the goldfish identify the same region as containing densely-stained TH-ir cell bodies (43, 60), but lacking dopamine β-hydroxylase (DBH) immunoreactivity (43, 313). In the goldfish, the TPp (or nucleus posterior tuberis; NPT) lies within the hypothalamus (43, 45, 152). Goping *et al.* (45) have suggested that, based on substantial degradation of DAergic neurons in the nucleus telencephali, pars medialis (NPM) that they observed following MPTP injections, and citing previous investigations of tract-tracing with

horseradish peroxidase (HRP) by Echteler and Saidel (314), the teleost homologue of the mammalian SN is the NPM. However, the results from the retrograde tract-tracing of Echteler and Saidel are inconclusive, as the HRP was placed on the surface of the areas dorsalis telencephali, pars medialis (Dm) and pars lateralis (Dl), some distance from the Dc.

In this study, we cloned partial coding sequences for a variety of DAergic markers and show that their expression in the hypothalamus is significantly increased in the days following a single injection of MPTP in the goldfish, corresponding with an initial decrease and subsequent recovery of hypothalamic DA levels. These data indicate that goldfish possess an intrinsic ability to spontaneously regenerate damaged DAergic neurons in the hypothalamus and may provide insights in neurodegenerative diseases in humans.

5.2. MATERIALS & METHODS

5.2.1. Animals

Adult common goldfish (16.3 ± 1.0 g) were purchased from a commercial supplier (Aleong's International Inc., Mississauga, ON, Canada) and maintained at 18°C under a natural simulated photoperiod on standard flaked goldfish food. Fish were kept in a single 270L tank and acclimatized over several weeks. All procedures used were approved by the University of Ottawa Protocol Review Committee and followed standard Canadian Council on Animal Care guidelines on the use of animals in research. Goldfish were anaesthetized using 3-aminobenzoic acid ethylester (MS222; Aquatic Eco-Systems, Apopka, FL) for all handling, injection, and dissection procedures.

5.2.2. *Experimental and sampling procedures*

The dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was purchased from Sigma-Aldrich (Oakville, ON, Canada), and dissolved in 0.6% saline to give a dose of 50 μg/g body weight of fish at 2 μL/g. Sexually recrudescent female goldfish (October; GSI 1.5 ± 0.2%; n=30 total) received a single injection of MPTP. This time of year was chosen because the somatic and linear growth rates of goldfish are low (315). The treatment schedule used in this study is identical to that used by Pollard's group (45, 47). For time t_0, fish (n=5) were injected and immediately sacrificed by spinal transection, and the hypothalamus was rapidly dissected and frozen on dry ice. All other fish were randomly sampled (n=5) at 2, 4, 7, 14 days post-injection. All dissections were performed at the same time of day. Samples were stored at -80°C until neurotransmitter levels were determined by HPLC.

The following year at exactly the date and time, the experiment above was repeated identically as described. This time, 70 fish received a single injection of MPTP and were kept together in a single 270L tank. For time t_0, fish (n=10) were injected and immediately sacrificed by spinal transection, and hypothalami were rapidly dissected and frozen on dry ice. Hypothalami were pooled (2 samples/tube) to increase RNA yield prior to RNA isolation. All other fish were randomly sampled (n=10) at 2, 4, 7, 14, 28, and 51 days. All dissections were performed at the same time of day.

5.2.3. *High-Performance Liquid Chromatography (HPLC)*

The HPLC protocol described by DiBattista *et al.* (316) was used to assess neurotransmitter levels in goldfish hypothalami. Individual frozen hypothalami (n=2-10 for each time point) were sonicated in a homogenizing solution comprising 0.1 mM Na_2EDTA,

0.3 M monochloracetic acid, 10% methanol, and 12.5 pg/μL DHBA (the internal standard). Protein levels were determined with the BCA protein assay reagent kit (Pierce, Rockford, IL) and an ICK Multiscan/MCC 348 (Titertek, Huntsville, AL). Brain monoamines were then quantified by HPLC using electrochemical detection. The HPLC consisted of a solvent delivery system (Waters590/WaterPump, Mississuaga, ON, Canada), an autoinjector (Waters712WISP), a reverse-phase column (8·mm x 100·mm, Waters, NovaPak, 4 μm) kept at 30°C and a 5100A Coulochem detector (ESA, Bedford, MA, USA) with two electrodes at oxidizing potentials of –330 mV and +350 mV. The mobile phase consisted of 1.3 g/L heptanesulphonic acid sodium salt, 0.1 g/L disodium ethylene tetracycline, and 7.3 ml triethyloamine adjusted to pH 2.45 with orthophosphoric acid. Sample monoamine levels were indexed to standard solutions of known concentration (Sigma-Aldrich), corrected for recovery of the internal standard, and expressed relative to total tissue protein content. HPLC analysis was performed on 2 independent, but identically designed, experiments. The results obtained from each experiment were not significantly different and were thus combined. The monoamines measured were 5-hydroxytryptamine (5-HT) and DA, as well as their major metabolites, 5-hydroxyindolacetic acid (5-HIAA) and 3,4-dihydroxyphenylacetic acid (DO) and homovanillic acid (HVA).

5.2.4. RNA extraction and cDNA synthesis

Tissues were homogenized using stainless steel beads in an MM301 Mixer Mill (Retsch, Newton, PA, USA) at 20 Hz for 2 min. RNA was isolated using the RNeasy Mini kit (Qiagen, Mississauga, ON) as described in the manufacturer's protocol, including the on-column DNase I treatment. RNA quantity was evaluated using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific). RNA quality was determined using the Agilent 2100

BioAnalyzer and RIN numbers were determined to be 8.3-9.4; above the minimum value considered as "perfect total RNA" for further analysis (175). For real-time RT-PCR and microarray analysis, cDNA was prepared as described in Section 2.2.3.

5.2.5. Real-time RT-PCR assays

Real-time RT-PCR was performed as described in Section 2.2.6. Primers used in this study are listed in Table 5.1. For 18S, only 0.1 ng cDNA template was used in the reaction. A panel of housekeeping genes were analyzed using real-time RT-PCR including ribosomal protein L8, β-2-microglobulin, 18S, and β-actin. Results were normalized to ribosomal 18S as it was determined to be the most stable reference gene (geNORM; http://medgen.ugent.be/~jvdesomp/genorm/). For all real-time RT-PCR reactions, n = 4-5 per time point.

5.2.6. Statistical analysis

All statistics for real-time RT-PCR and HPLC were performed using Systat 12. Data was assessed using the Kolmogorov-Smirnov test for normality and Levene's test of homogeneity of variance. In the cases where data were not normally distributed, the data were log-transformed, which achieved normality. For HPLC data, a Bonferroni-corrected t-test was performed comparing treatment time against time zero; significance was taken at $p<0.015$. For real-time RT-PCR, a one-way ANOVA was performed with significance considered at $p<0.05$, followed by Tukey's HSD pairwise comparisons if data was homoscedastic or Dunnett's T3 pairwise comparisons in cases where data was heteroscedastic.

Table 5.1: Primers used in this study

Amplicon	Forward primer (5' → 3')	Forward primer (5' → 3')	Accession
L8	GTGATGTTTCGTGACCCGTACC	AGCTGAGCTTTCTTGCCACAG	EU313780
18S	AAACGGCTACCACATCCAAG	CACCAGATTTGCCCTCCA	AF047349
β-actin	ACTACTGGTATTGTGATGGACTCC	CGGTCAGGATCTTCATCAGGTAG	AB039726
β2M	GCCCTGTTCTGTGTGCTGTA	AAGGTGACGCTCTTGGTGAG	L05536
GAP-43	CAGCTTCCGTGGACACATC	GGCTGGTTTGCTCTCCTCT	M26250
GFAP	CCTACGGCAAGAAGCAGAAA	TCCTTCAGGGCAGTGGTTAG	L23876
TH	AGCACACTGGTCAGCTCTCTC	ATCATCTTCCGGCGTTTTC	AY644727
DAT	TGGTGATCGCTGGGATGCC	GAAGCCCACACCTTTGAAGAGG	EF371919
D2S	CTAAGCCCCCTCTCAAGCA	TGCCCATTTTTTATCATCTCCA	FJ793518
cdk5[a]	ACAGCCGAAATGTTCTCCAC	TGTACCACAGCGTCACCAC	EU286947
otx2[a]	AAACGGAGGCCAGAACAAG	TGCAGGAGGAGGAGGTAGAG	EU286948
nurr1[a]	AGAACCTACCCCATACCGAAC	GGAGACTGCTTGAACGAGAAC	EU125352
DJ1[a]	AGGTCTGGCGGGTAAAGAG	GATACCGTGTGCCAAGAGTG	EF392843
pitx3[a]	ATGGAGAAAGCGTGAAAGGA	ATGTTCATGGAAGGGATGGA	EU531569
PINK1[a]	AGGGACGCACTTATCAGGAA	ACCCTTCAGCAGCACCAC	EF396231
olig2[a]	AACAGCCGCGAAAGAAAA	AGCCGTTTCATCTCCTCCA	EU346689
parkin[a]	CGCTGGTGGGCTACTCTC	TGGAGCGGGACACAGAAC	EU169139
GDNF	ATGGACTCAGGAACCAAGCA	GTCTGTGACGTTGAGGTGGA	EU828244
uchL1[a]	CTTGTGGAACTGTGGGTTTG	AACTTTGTCTGGCTCTGGTTG	DQ917576
nestin[a]	GAACCAGGCCAAAGCTAACA	GCTGTCCTTCTCCATCTCCA	FJ855222

[a] partial coding sequences were cloned in this study and submitted to GenBank
DAT: Dopamine transporter; D2S: dopamine D2 short isoform; TH: tyrosine hydroxylase; β2M: β2-microglobulin; cdk5: cyclin-dependent kinase 5; otx2: homolog of *Drosophila* orthodenticle 2; GAP-43: growth-associated protein 43; nurr1: nuclear receptor subfamily 4, group A, member 2 (NR4A2); pitx3: paired-like homeodomain transcription factor 3; DJ1: oncogene DJ1; PINK1: pten-induced putative kinase 1; olig2: oligodendrocyte lineage transcription factor 2; GFAP: glial fibrillary acidic protein; GDNF: glial-derived neurotrophic factor; uchL1: ubiquitin carboxyl-terminal esterase L1; L8: ribosomal protein L8; 18S: ribosomal protein 18S

5.3. RESULTS

5.3.1. General observations

Consistent with previous studies by others (47), MPTP-injected goldfish displayed bradykinesia and ataxia which reached its maximum by 3-4 days. These symptoms were not noticeable by 7-8 days (data not shown). Average fish length (nose to tail-fork) remained unchanged (9.6 ± 0.2 cm) throughout the 51 days of the experiment. Average fish mass also remained unchanged (final average mass = 16.4 ± 1.7 g). Gonadosomatic index (GSI) was not statistically affected during the treatment period.

5.3.2. *MPTP temporarily decreases hypothalamic DA, NE, and* 5HT *levels with no effect on DA turnover rates*

Hypothalamic levels of DA were decreased by 24% ($p= 0.011$) after 4 days post-injection with MPTP and recovered to baseline (t_0) levels by 7 days (Figure 5.1a). There was no change observed in levels of DOPAC and HVA, DA metabolites (Figure 5.1b). Furthermore, there was no statistically significant change in the ratios of DA to its metabolites (DA/DOPAC, DA/HVA, and DA/DOPAC+HVA) suggesting that DA turnover rates were unaffected (data not shown). NE levels were significantly reduced by 44%, 52%, and 37% ($p<0.001$) after 2, 4, and 7 days, respectively, before returning to baseline levels by 14 days (Figure 5.1a). Serotonin (5HT) levels were reduced by 53% ($p=0.0014$) after 2 days but returned to baseline levels after 4 days (Figure 5.1c). No statistical effect was observed in 5-HIAA, a 5HT metabolite.

5.3.3. *Real-time RT-PCR*

Partial coding sequences for goldfish genes involved in DAergic neuron development, Parkinson's disease, and DA functioning were cloned, submitted to GenBank (Table 5.1) and their expression was examined by real-time RT-PCR.

5.3.3.1. *DA system and synthesis*

Steady-state mRNA levels for TH, DAT, and the short isoform of the D2 receptor were significantly ($p<0.0005$) increased by 9.8-, 8.5-, and 9.3-fold, respectively, 4 days after injection of MPTP compared to T_0 levels (Figure 5.2). D2S mRNA levels returned to t_0 levels by 7 days post-injection (dpi) and remained similar to t_0 levels. TH mRNA levels were subsequently reduced by 28 dpi to being not statistically different than t_0 levels and

then increased slightly by day 51. DAT mRNA levels remained high throughout the 51 days following MPTP injection.

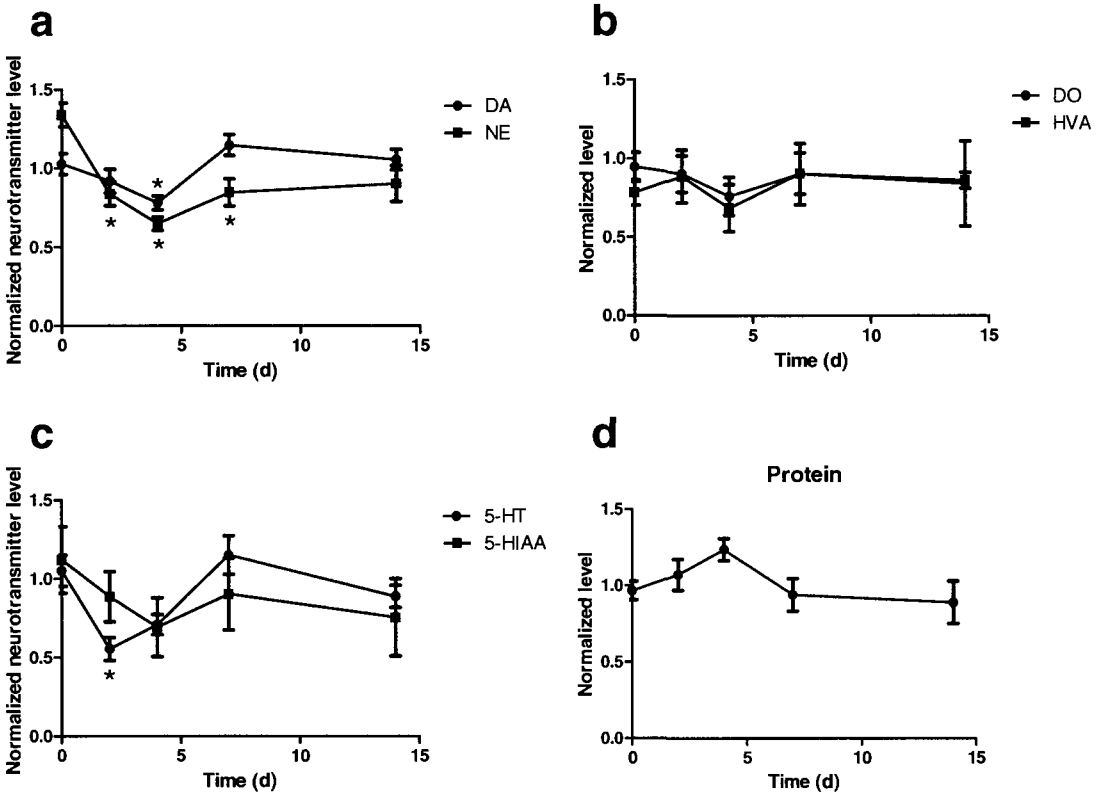

Figure 5.1: HPLC analysis of neurotransmitter and neurotransmitter metabolites in the hypothalamus of female goldfish injected (i.p.) with MPTP at Time 0. * indicates $p<0.015$ relative to t_0 levels. For time points 0, 2, 4, 7, 14d, n=10, 9, 6, 8, 2, respectively.

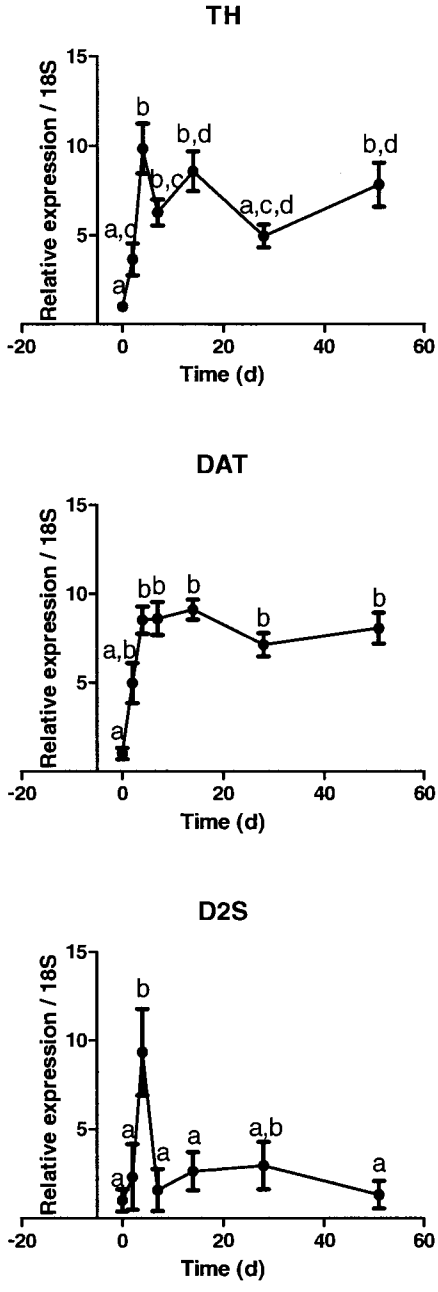

Figure 5.2: Real-time RT-PCR analysis of DA system gene expression in the hypothalamus of female goldfish injected (i.p.) with MPTP at t_0. One-way ANOVA was performed with significance considered at $p<0.05$, followed by Tukey's HSD (or Dunnet's T3 if data was heteroscedastic) multiple comparisons test. n=4-5 for each time point, representing 8-10 female hypothalami

5.3.3.2. Neuronal differentiation factors

Steady-state mRNA levels for nestin were significantly ($p<0.0005$) increased 6.2-fold 4 dpi and increased further to 7.2-fold increase over t_0 levels by day 14 before decreasing slightly, albeit still statistically higher than t_0 levels, by day 28 (Figure 5.3a).

No statistically significant changes were observed in the expression of GAP-43 (Figure 5.3b).

Steady-state mRNA abundance for Nurr1 was significantly ($p=0.003$) increased 4.6-fold over t_0 levels 4 dpi and slowly returned to baseline levels by 51 dpi (Figure 5.3c). Similarly, mRNA levels for pitx3 (Figure 5.3d) were increased but did not reach statistical significance ($p=0.013$) until 14 dpi (2.7-fold), at which point they slowly returned to baseline levels by day 51.

Levels of otx2 mRNA levels (Figure 5.3e) increased over time and peaked at day 7 (6.8-fold over t_0 levels; $p=0.002$) before decreasing slightly to being not statistically different than t_0 levels by day 28.

GDNF mRNA levels (Figure 5.3f) were increased 1.9-fold ($p=0.009$) over t_0 levels by day 7 returned to levels not statistically different than t_0 levels by day 14.

GFAP (Figure 5.3g) and olig2 (Figure 5.3h) mRNA levels follow the same trend: both are increased by day 4 (5.4-fold $p<0.0005$ and 4.0-fold $p=0.006$, respectively) and remained high until day 14, at which point they began to return to t_0 levels and were not statistically different than t_0 levels by day 28. However, GFAP mRNA levels began to increase again by day 51 (4.6-fold; $p=0.005$ relative to t_0).

5.3.3.3. Genes implicated in Parkinson's disease

Steady-state mRNA levels for PINK1, cdk5, DJ-1, and uch-L1 (Figure 5.4a-d) followed similar patterns to those of the neuronal differentiation factors. No statistical change in parkin mRNA expression was observed (Figure 5.4e). β-actin mRNA expression (Figure 5.4f) was dramatically increased 17.4-fold ($p<0.0005$) by day 7 and decreased to 3.0-fold (over t_0; $p<0.0005$) by day 28.

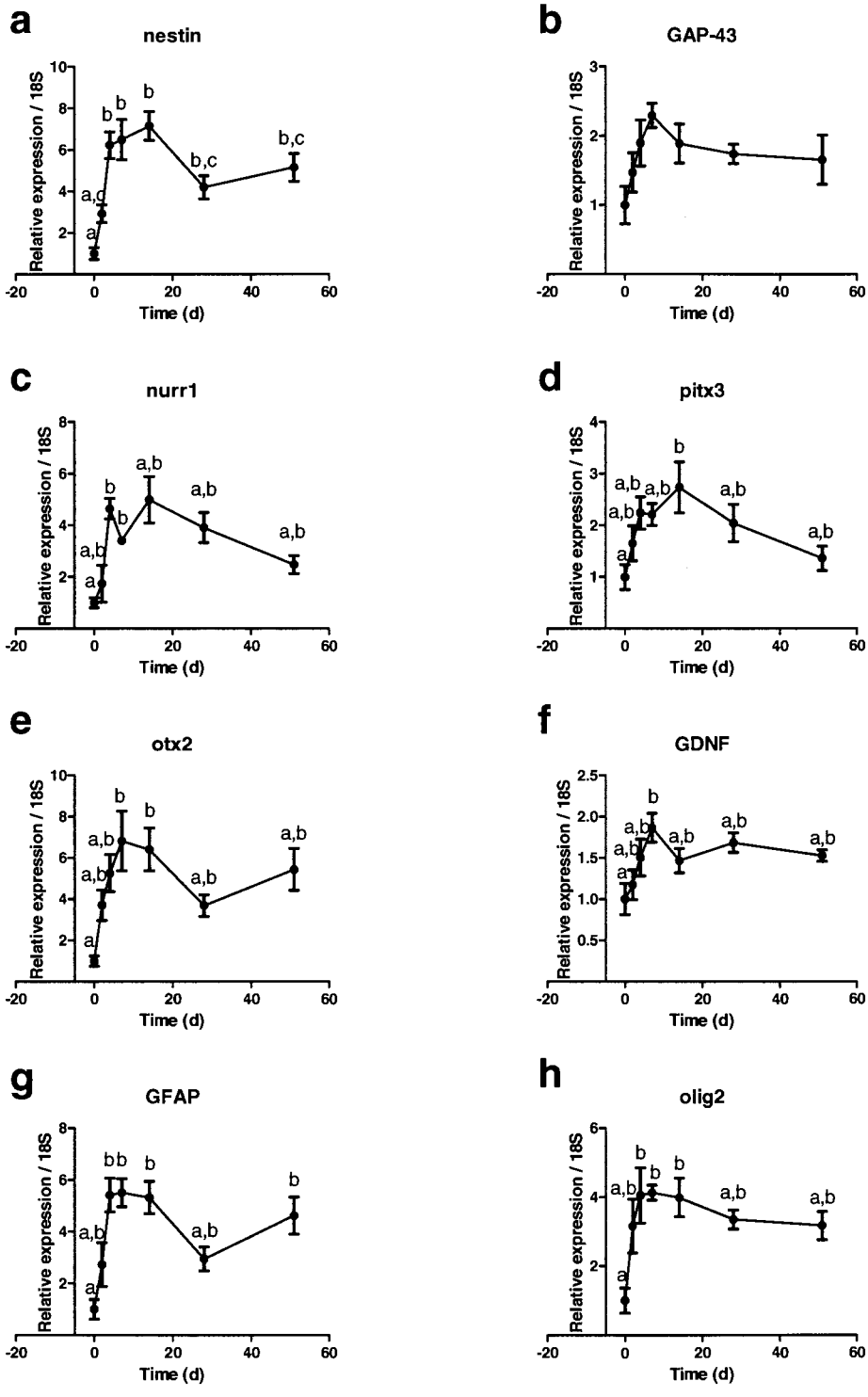

Figure 5.3: Real-time RT-PCR analysis of neuronal differentiation factor gene expression in the hypothalamus of female goldfish injected (i.p.) with MPTP at t_0. One-way ANOVA was performed with significance considered at $p<0.05$, followed by Tukey's HSD (or Dunnet's T3 if data was heteroscedastic) multiple comparisons test. n=4-5 for each time point, representing 8-10 female hypothalami.

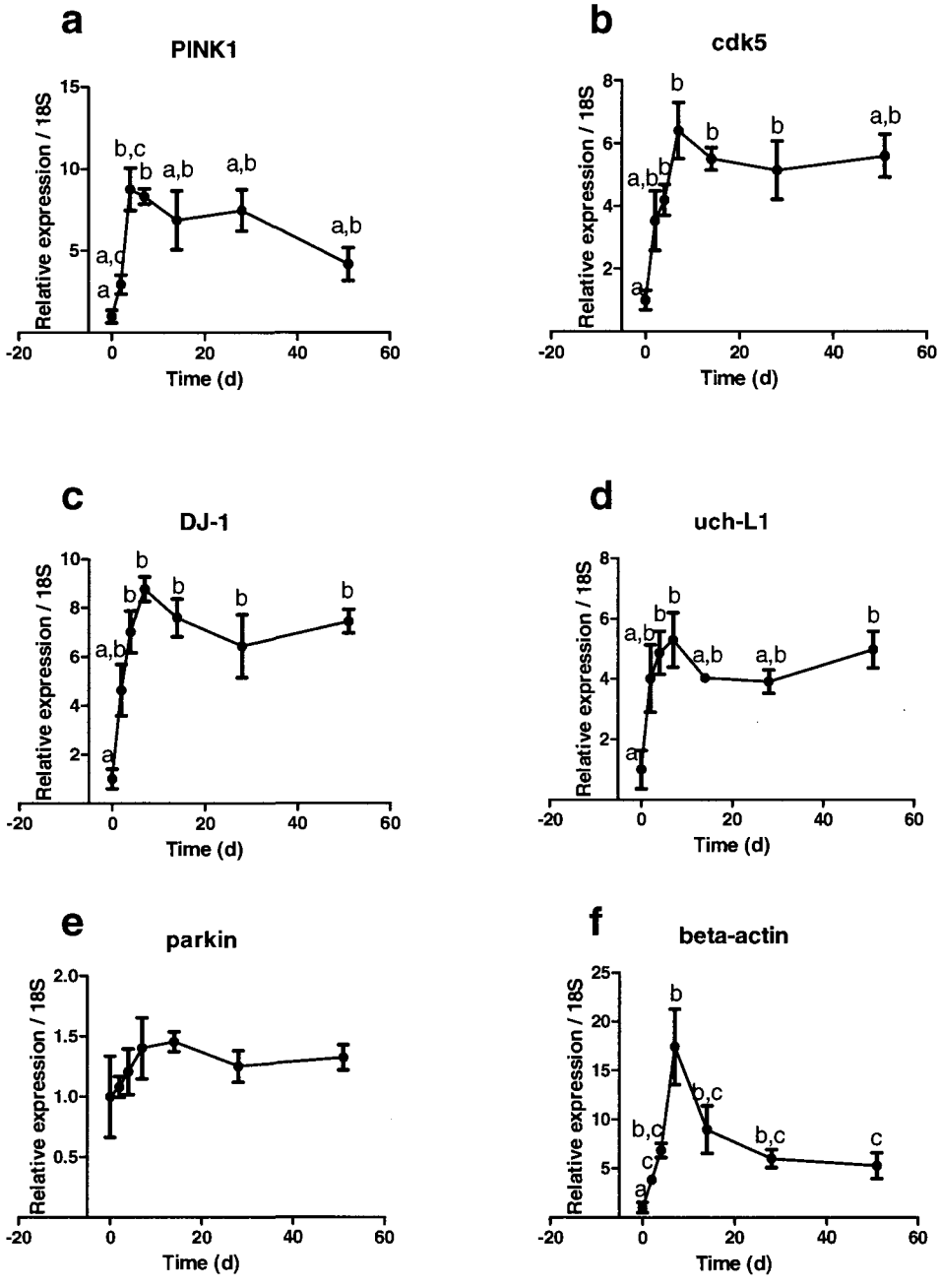

Figure 5.4: Real-time RT-PCR analysis of Parkinson's disease gene expression in the hypothalamus of female goldfish injected (i.p.) with MPTP at t_0. One-way ANOVA was performed with significance considered at $p<0.05$, followed by Tukey's HSD (or Dunnet's T3 if data was heteroscedastic) multiple comparisons test. n=4-5 for each time point, representing 8-10 female hypothalami.

5.4. DISCUSSION

We report here, for the first time, evidence for the spontaneous regeneration of hypothalamic DAergic neurons in an adult fish following a single injection of MPTP. There was an initial decrease in hypothalamic DA levels reaching a minimum at day 4, followed by full recovery by day 7. Our results corroborate those of Pollard *et al.* (47), who showed full recovery of DA levels in the brain of goldfish after 8 days using the same dose of MPTP (50 μg/g). Poli *et al.* (105) previously showed spontaneous recovery of DA and NE levels in the goldfish telencephalon, diencephalon, and medulla after 6 weeks following injection of MPTP at a lower dose (10 μg/g) for 3 consecutive days. Neither of these studies, however, conducted any gene expression analyses as presented here. The decrease and subsequent recovery of DA levels coincided with an increase in hypothalamic gene expression of nestin, nurr1, pitx3, otx2, TH, DAT, D2S, GDNF, GFAP, and olig2, PINK1, cdk5, DJ-1, uch-L1, and β-actin by 4-7 days post-injection with MPTP.

The increased expression of gfNestin following MPTP injection suggests that goldfish neural stem cells are proliferating. Nestin is an intermediate filament protein and gene marker for neural stem cells in mammalian systems that give rise to neurons and glia (317). Nestin was recently cloned in zebrafish and determined that its expression is similar to that of mammals and thus may also serve as a marker for stem cells in the nervous system of fish (318).

Increased expression of otx2, pitx3, and nurr1, all factors important in the normal development and maturation of DAergic neurons in the mammalian mesencephalon (319-326) or zebrafish ventral diencephalon (327), was observed. A number of factors play

important roles in the differentiation and survival of mesencephalic DA (mesDA) neurons in mammals.

Nurr1 is required for expression of TH (328) and mice lacking nurr1 do not develop midbrain DAergic neurons (326, 329, 330). Interestingly, nurr1 may also protect DAergic neurons from degeneration in PD (331). Nurr1 has been cloned in medaka and its expression is co-localized with TH, suggesting that nurr1 function is conserved in fish (332). Increased expression of TH and DAT, markers for DAergic neurons, was observed, in contrast to SN neurons of the mouse (333, 334) and monkey (335, 336) where MPTP significantly decreased the expression of DAT and TH. The substantial increases in gene expression observed in this study suggest that hypothalamic DAergic neurons are being regenerated.

In mice treated with MPTP, mRNA levels for uch-L1 and parkin have been shown to be decreased (334). We report here an increase in uch-L1 mRNA with no change in parkin expression. We also show that, following MPTP injection, olig2 mRNA levels are increased. In zebrafish, olig2 was recently shown to be expressed in diencephalic DA progenitor cells, but its expression is extinguished prior to terminal DA differentiation (337).

In contrast to Poli *et al.* (46, 105), we observed a significant decrease in hypothalamic 5HT levels after 2 days, which returned to baseline levels by 7 days. This is likely the result of different injection schedules: we injected a single relatively high dose of MPTP, whereas Poli *et al.* injected a much lower dose once each day for 3 consecutive days. Indeed, exposure of zebrafish larvae to 1000 μM MPTP resulted in a 40% reduction in 5HT-ir cells in the intermediate paraventricular organ, whereas exposure to 100 μM MPTP had no effect on these cells (338).

It is still unknown precisely where the goldfish equivalent of the mammalian SN resides. The most likely candidate is the posterior tuberculum in the hypothalamus based on dense TH-ir immunoreactivity (43, 60) and DBH-ir immunonegativity (43, 313), as well as evolutionarily related similarities to the zebrafish (34, 37). Nevertheless, the decrease, followed by full recovery, of DA levels in the hypothalamus of the goldfish reported here and by others (105), coupled with the gene expression data presented in this study, suggests that goldfish hypothalamic DAergic neurons are being regenerated. Future studies aimed at localizing these regenerating hypothalamic DAergic neurons using double-labeled (nestin and TH) immunohistochemical techniques are planned. Time-course analysis of transcriptomic changes using the goldfish-carp microarray (15, 138, 215, 216) are also planned to determine if full recovery is indeed happening. The findings presented in this study strongly support the goldfish model of Parkinson's disease (208) and may provide insights into novel methods of treatment for PD in humans.

Chapter 6.

General Discussion[‡‡]

In this last chapter, a final analysis is performed, this time limited strictly to microarray data generated in this thesis: the DA agonists (Chapter 3), antagonists (Chapter 4), and a neurotoxin + TH inhibitor (MPTP + αMPT) experiment (Popesku, unpublished). This novel approach is an effort to identify, for the first time, a group of genes that are very likely regulated by DA. The principle behind the analysis is that genes commonly affected in one direction by severe catecholamine depletion (MPTP + αMPT) and/or DA antagonists will also be affected by DA agonists but expression changes will be in the opposite direction. In this way, the main findings in the thesis will be summarized to allow for speculation on DAergic mechanisms of action in the goldfish neuroendocrine brain. This also serves to set the stage for future work on DAergic regulation of neuroendocrine gene expression.

The DAergic neurotoxin MPTP, and the TH-inhibitor αMPT, were injected into female goldfish (see Figure 6.1 caption for methods). To ensure that the MPTP + αMPT treatment effectively decreased DA levels in the brain, Hyp, Tel, and cerebellum (Cer) tissues were analyzed for catecholamine content using HPLC. Following injections of MPTP (-6 day) and αMPT (-1 day), DA levels were decreased by 69.6% and 70.9% in the Hyp and Tel, respectively, and by 88.2% in the Cer relative to saline-injected controls (Figure 6.1). Norepinephrine (NE) levels were also reduced in the Hyp (79.4%), Tel (87.5%), and Cer (90.4%). We focused our attention on the neuroendocrine regions of the brain (Hyp and Tel) as central regulators of LH release.

[‡‡] Popesku, J.T., Trudeau, V.L. A meta-type analysis of dopaminergic effects on gene expression in the neuroendocrine brain of female goldfish. *In preparation.*

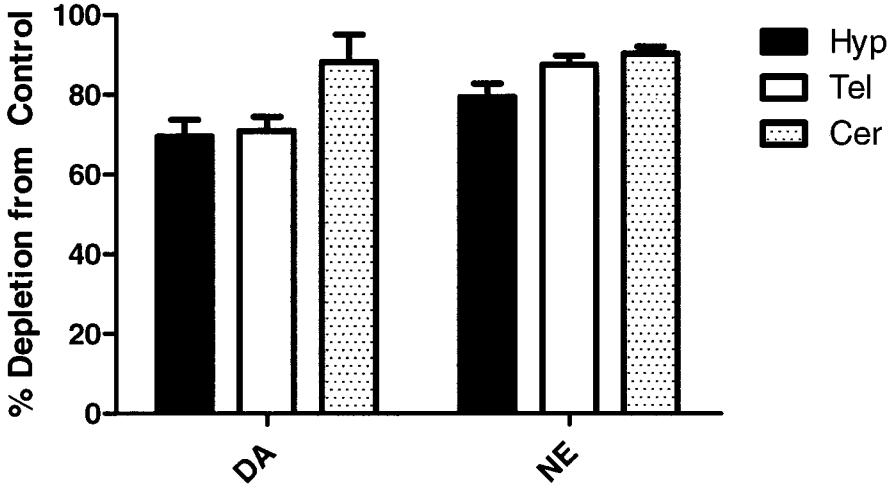

Figure 6.1: Percent DA depletion in different brain tissues relative to saline-injected control 6d post-injection with MPTP and 1d post-injection with αMPT ($p<0.01$ in all cases, relative to control). Sexually mature (June) female goldfish (n=5 each) were injected with MPTP (50 μg/g; day 0) and αMPT (240 μg/g; day 5) or saline (control) in order to severely deplete dopamine. On day 6, telencephali, hypothalami, and cerebelli were collected along with blood samples from each fish as previously described in Chapters 2-5. Catecholamine levels in brain tissues were determined on alumina-extracted samples (100 μl) using HPLC with electrochemical detection (339). The HPLC incorporated a Varian ProStar 410 solvent delivery system (Varian Chromatography Systems, Walnut Creek, CA, USA) coupled to a Princeton Applied Research 400 electrochemical detector (EG & G Instruments, Princeton, NJ, USA). Concentrations were calculated relative to appropriate standards, using 3,4-dihydroxybenzalamine hydrobromide (DHBA) as an internal standard. (Special thanks to B. McNeill and S.F. Perry for performing the HPLC).

Using the microarray datasets from previous experiments (Chapters 3 and 4), along with the microarray data from this study, a meta-analysis of genes likely regulated by DA was performed (see Table 6.1 caption for method and results). A total of 268 genes/ESTs were identified in the hypothalamus as being regulated by DA, while only 4 were identified in the telencephalon. Of the 268 genes/ESTs identified in the hypothalamus, only 41% are annotated (Figure 6.2). The others currently have no known biological function, are not similar to any sequences in GenBank, or are lacking sequence information. The relatively high number of sequences affected by DA in the hypothalamus, the majority of which are acting through the D1 receptor, highlights the importance of this receptor in this tissue. The annotated sequences were binned into their corresponding GOSlim terms, using Blast2GO as described in Chapters 3 and 4 (Figure 6.3). As expected, the GO term profile is similar to those in Chapters 3 and 4.

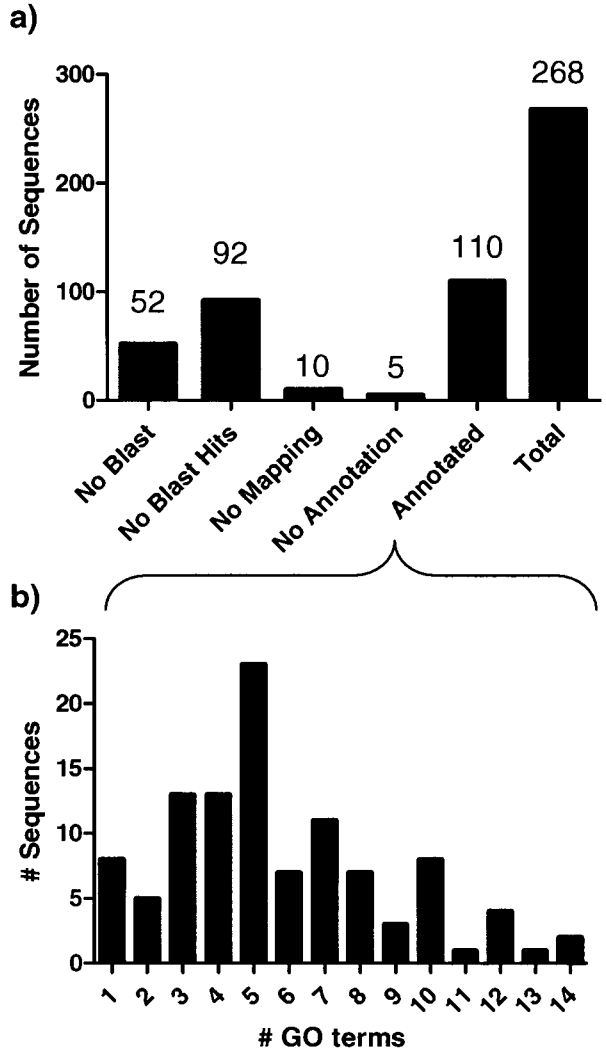

Figure 6.2: a) Data distribution of the 268 ESTs identified as being differentially regulated (q<5%) by DA in hypothalamus. b) Annotation distribution of the 110 annotated ESTs. In the telencephalon (not shown), a total of 4 sequences were found of which 3 had blast hits, and only 2 were annotated. Total RNA from Hyp and Tel tissues was isolated with the TRIzol® method as per the manufacturer's protocol (Invitrogen, Burlington, ON, Canada). Samples were treated with DNase on-column in an RNeasy Mini Plus kit (Qiagen, Mississauga, ON, Canada). RNA quantity and quality was assessed with the NanoDrop ND-1000 and Agilent BioAnalyzer, respectively, as previously described. Microarray hybridizations were performed using the Hyp and Tel tissues (n=4 each) as previously described (Chapters 3 and 4). Real-time RT-PCR was performed on aromatase b as previously described (Chapters 2, 4, and 5).

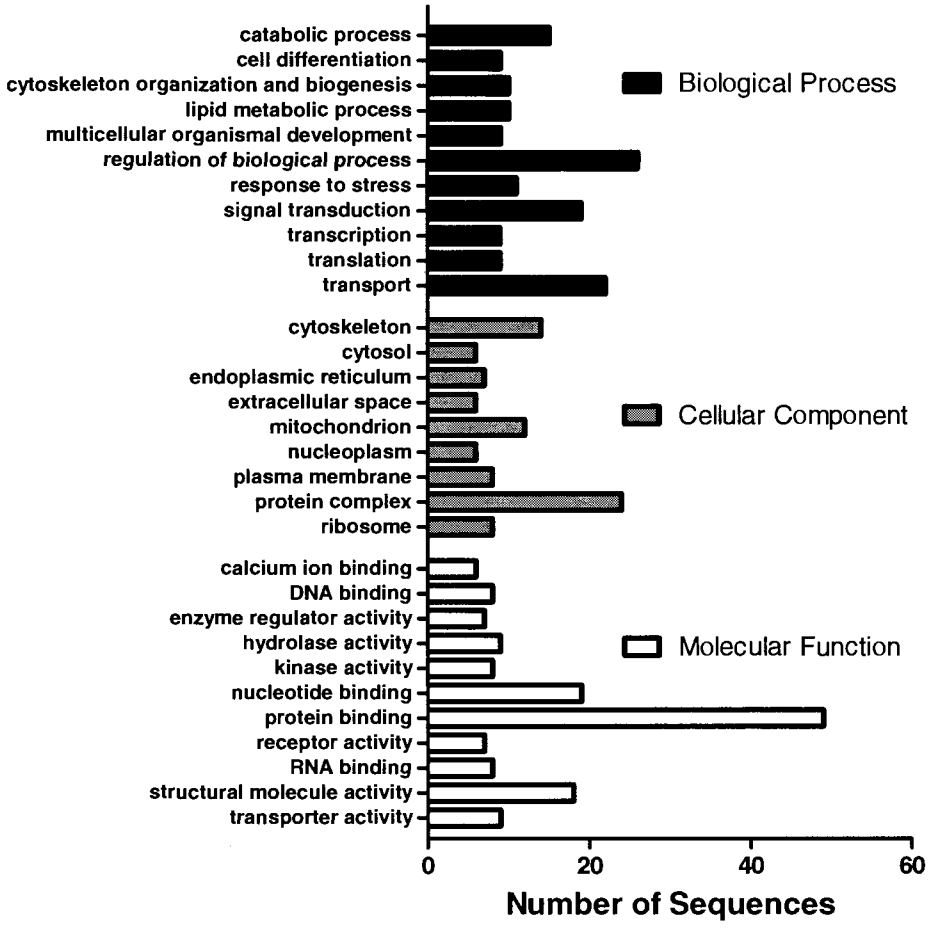

Figure 6.3: Multilevel Gene Ontology categorization of 110 annotated ESTs into their corresponding Biological Process, Cellular Component, and Molecular Function terms. GO Annotations were first converted to GO-Slim annotations (goslim_generic.obo) and the multilevel chart was constructed using a sequence convergence cutoff of 5 (7 for Biological Process) to reduce the complexity of the chart.

Some of the genes/ESTs identified in this analysis were previously discussed (e.g. calmodulin and 14kDa apolipoprotein) and will not be discussed further. Furthermore, it is not my intention to examine all of the genes/ESTs listed in Table 6.1, nor to reiterate what was discussed in preceding chapters. Only a select few genes/ESTs identified by the current analysis will be discussed in the context of the entire thesis. For example, the identification of ependymin and vimentin in the hypothalamus highlights the significance of neuronal regeneration related to DA (Chapter 5). Ependymin is an extracellular glycoprotein and neurotrophic growth factor involved in optic nerve regeneration, synaptic plasticity, and long-term potentiation in *Cypriniformes* (340-343). Moreover, ependymin was shown to be overexpressed in regenerating echinoderms (344). Ependymin-related proteins were identified in amphibians and mammals (345) and Shashoua *et al.* (346) showed that a short fragment of goldfish ependymin was able to activate the AP-1 transcription factor in neuroblastoma and primary rat brain cortical cultures. Similarly, vimentin is an intermediate filament and was shown to increase during cerebellar regeneration in the brown ghost knifefish, *Apteronotus leptorynchus* (347). At least 2 forms of vimentin exist in goldfish (348), and while the current analysis is unable to resolve the form(s) of vimentin regulated by DA, it is likely that both of the sequences listed in Table 6.1 correspond to the same form, as they share nearly identical expression patterns in response to DA. Both vimentin and ependymin, along with α- and β-actin and tubulins (Table 6.1) were decreased in response to DA, supporting the role of DA in synaptic plasticity and tissue remodeling (349).

Cytoskeletal remodeling is hypothesized to be important for hormone secretion from the anterior pituitary in mammals (350). Furthermore, Ravindra and Grosvenor (351) demonstrated that domperidone, a D2-specific antagonist that does not cross the blood-brain

barrier but can act on the pituitary, increased prolactin (PRL) levels as well as pituitary polymerized tubulin levels, similar to levels seen in suckling rats. This response, the authors observed, was blocked by bromocriptine, a D2-specific agonist supporting a role for DA in changes observed in the tubulin system in the anterior pituitary. This is relevant because, while Chapter 5 was written with respect to Parkinson's disease and not in the context of reproduction, it should be noted that DAergic neurons in the mediobasal hypothalamus (e.g. posterior tuberculum) project to the pituitary (i.e. are hypophysiotropic) (7, 43). This is important as it suggests the need for maintaining DA neuronal populations throughout seasonal reproductive period. The examination of ependymin and vimentin mRNA levels by real-time RT-PCR in the MPTP Time Course study (Chapter 5) is planned.

The granulins are conserved growth factors and are able to stimulate the proliferation of macrophages in goldfish (23). Furthermore, granulin has protease inhibitor activity in invertebrates (352) and cysteine protease activity in plants (353). Granulin was shown to be relatively lowly expressed in the brain of goldfish (23) and tilapia (354). It appears as though DA, acting through the D1 receptor, stimulates expression of granulin the hypothalamus of female goldfish. In the developing rat hypothalamus, it was demonstrated that both estrogen and androgen induced granulin expression (355) and that estrogen induced granulin expression in the dentate gyrus (hippocampus) of adult rats (356). Furthermore, in hippocampal rat tissue *in vitro*, estradiol enhanced neural progenitor cell proliferation and this response was blocked by a granulin-specific antibody (356). Although speculative, this is relevant, as hydroxysteroid (17β) dehydrogenase (17β-HSD) was identified as being increased in response to DA (current study), which inverconverts 17β-estradiol with estrone and 16-α-hydroxyestrone with estriol, as well as androstenedione and testosterone (357),

suggesting that sex steroids influence the DAergic regulation of granulin or, alternatively, the DA influences granulin expression by estradiol. Granulin mRNA levels were also identified as being decreased 4.2-fold in the telencephalon by 0.1 μM thyroid hormone (T_3) following a 2-day waterborne exposure (S. Wiens, personal communication). This is worth noting because the current study identified transthyretin (TTR) mRNA levels as being significantly increased in response to DA. TTR is a thyroid hormone-binding and transport protein and is necessary for maintaining normal levels of circulating thyroid hormone in plasma (358). Furthermore, TTR protein levels are increased in the cerebrospinal fluid (CSF) of rats with degenerating nigrostriatal neurons (359). Future studies aimed at examining the potential interaction between T_3 and DA are warranted, particularly as microarray analysis identified increases in mRNA levels of iodothyronine deiodinase type I in the hypothalamus of female fish in response to SKF 38393LY and 171555 (D1- and D2-specific agonists, respectively) (Table C.1; Appendix C).

The identification of U2 small nuclear RNA auxiliary factor 1 (U2AF1) mRNA levels as being increased by DA acting through the D1 receptor and potentially the D2 receptor (Table 6.1) is relevant. There are currently 5 known small nuclear ribonucleoproteins (snRNPs) that make up the spliceosome (360). LSM7 protein, whose mRNA levels were also increased in both DA agonist treatments (Table C.1, Appendix C), also forms part of the spliceosome complex (361). The increase in both of these factors in response to either DA agonist allows us to speculate that blockage of either of these receptors would inhibit transcription of particular components of the spliceosome, and thus decrease splicing activity, thereby decreasing the amount of a particular splice variant. The observed decrease of the D2 short isoform splice variant in response to both D1 and D2 antagonists (Chapter 2)

supports this hypothesis. It remains to be seen whether the function of goldfish D2S isoform is able to stimulate phospholipase D (PLD) activity, as shown in rat pituitary cells *in vitro* (362), but a partial coding fragment encoding a goldfish PLD-like protein (GenBank Accession EU295895) was cloned during the tenure of this Ph.D. project.

Multiple genes/ESTs identified as being regulated by DA are involved in the lipid and fatty acid metabolic process or transport (Figure 6.3). For example, 3-ketoacyl-CoA reductase type B (KAR; down), high density lipoprotein binding protein (HBP; up), vitellogenin 2 (vtg2; up), cubulin (up), sh3-domain grb-like 2 (up), and StAR-related lipid transfer (START) domain containing 4 (StarD4; down), and sterol-c5-desaturase homolog (SC5D; up) were identified as being regulated by DA. SC5D is involved in the biosynthesis of cholesterol (363). KAR reduces 3-ketoacyl-CoA to 3-hydroxyacyl-CoA in the second step of fatty acid elongation (364). *In vivo* studies in zebrafish demonstrated that HBP is not affected by the insulin family or growth hormone, but it is hypothesized that HBP is involved in food intake or lipid transfer, based on its high expression in the liver and ovary (365). Cubilin is a high-density lipoprotein receptor (366) and StarD4 is hypothesized to facilitate transport of a cholesterol precursor (367). Vtg is typically thought of as being an egg protein synthesized in the liver of female fish and deposited in the ovary (368, 369). However, Vtg is a lipid transport molecule and its identification in this study in the hypothalamus as being differentially regulated by separate experiments suggests that other roles for vitellogenin exist and present an avenue of exploration. The implication of the above mRNAs in steroid biosynthesis is supported by the identification of the transcriptional levels for aromatase b (cytochrome P450b or AroB), the key enzyme in converting testosterone into estradiol, as inhibited by DA. AroB mRNA levels were further examined by real-time RT-PCR as

described earlier in this thesis. Injection of SKF 38393 resulted in a 4.7-fold decrease ($p=0.025$) in hypothalamic AroB mRNA levels (Figure 6.4). The combined MPTP + αMPT treatment resulted in a 1.3-fold increase in hypothalamic AroB mRNA levels but did not reach statistical significance. Nevertheless, both real-time RT-PCRs confirm the direction of AroB expression in response to different DA treatments, suggesting that the transcriptional regulation of AroB is under the influence of DA. Indeed, at least some AroB-ir neurons in the medial preoptic area (POA) of the Japanese quail brain respond to DA (370) and some AroB-ir neurons in the POA of the bluehead wrasse are co-regionalized with TH (371). Moreover, some TH-ir neurons in the POA of rainbow trout express estrogen receptors (110) and testosterone and estradiol increase goldfish pituitary DA turnover rates as measured following αMPT-induced catecholamine depletion (66). More importantly, DA was shown to reduce aromatase enzyme activity in quail POA homogenates *in vitro* (372). These studies, including the current one, clearly demonstrate the next logical step from this thesis: examination of the influence of sex steroids on the inhibitory tone of DA, as suggested by Dufour *et al.* (162), and which is a major theme in this thesis.

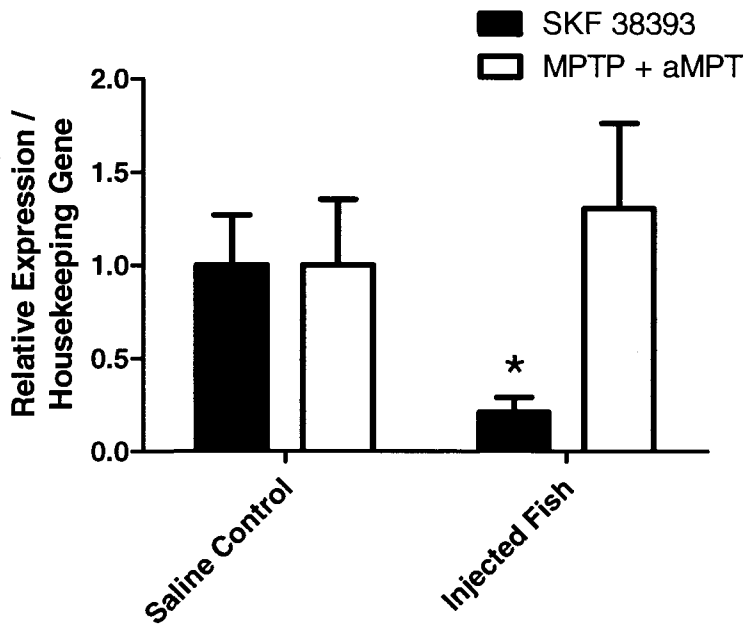

Figure 6.4: Real-time RT-PCR of aromatase b mRNA levels in the hypothalamus of SKF 38393-injected fish after 5h or MPTP + αMPT-injected fish after 24h. SKF 38393 data were normalized to β-actin and MPTP + αMPT data were normalized to 18S as they were determined to be the most stable for the respective experiments. A Mann-Whitney U Rank Sum test was performed on injected vs. control fish with significance (*) considered at $p<0.05$ (two-tailed).

Only 3 annotated genes/ESTs were identified in the telencephalon and were all increased in response to D2 receptor agonists and decreased in response to D2 receptor blockage or DA depletion, suggesting that, indeed, DA, acting through the D2 receptor, stimulates the expression of these genes/ESTs. Two of the genes/ESTs in the telencephalon identified as regulated by DA are leucine-rich ppr-motif containing protein (LRPPRC) and solute carrier family 2 (facilitated glucose fructose transporter) member 5 (SLC2A5; glucose transporter 5; GLUT5). LRPPRC is a core nucleoid protein (373) and is hypothesized to have a regulatory role in the integration of the cytoskeleton with vesicular trafficking, nucleocytosolic shuttling, transcription, chromosome remodelling, and cytokinesis based on its interactions with other proteins by yeast 2-hybrid analysis (374).

The increase in CCAAT/enhancer binding protein beta (C/EBPβ) in the telencephalon in response to DA is particularly interesting. CaMKII phosphorylates C/EBPβ (375), which, in turn, activates transcription factor-1 (ATF1) (376), among other things. Methamphetamine administration to mice caused a dose-dependent increase in ATF1 and CREB DNA-binding activities (377). As CaMKIIα protein levels were increased in response to DA agonists (Chapter 3), a working hypothesis of DAergic regulation of gene expression in the neuroendocrine brain of goldfish through the increase in ATF1 can thus be put forth.

6.1. General Conclusions and Perspectives

6.1.1. Significance of thesis

I investigated the neuroendocrine regulation of gene expression by DA using separate molecular and bioinformatic techniques. The major objective of this thesis was to determine which higher brain centres are being influenced by DA, especially within the context of the neuroendocrine control of reproduction. Chapter 1, in addition to containing original data,

examined the little-explored concept of neurotransmitter-regulated transcription of neuroendocrine genes. It was a "meta-type" analysis of multiple experiments and lays the foundation for a large database describing the fish transcriptome. Results from this bioinformatic analysis formed the basis of this thesis as well as those of other M.Sc. and Ph.D. projects in the laboratory (e.g. D. Zhang, S. Wiens, J. Mennigen, W. Lado, K. Crump).

Despite years of unsuccessful investigation by others, I uncovered an alternative splice of D2 receptor in a teleost. This splicing event is influenced by the reproductive cycle as well as by DA itself (Figure 6.5). As this splice variant is missing the identical 29 amino acid region as human D2S, I suggest that the short isoform of goldfish D2 behaves similarly to its mammalian counterpart with respect to its interaction with G-proteins and signalling mechanisms. In particular, as *in vitro* studies in rat striatal slices indicate that stimulation of D2S inhibits tyrosine hydroxylase (TH), the rate-limited enzyme in DA synthesis, it suggests that this alternative splice is a crucial requirement for the high DAergic tone in the spawning season. Therefore, I hypothesize that the increased expression of the short isoform of the D2 receptor in November decreases DA biosynthesis and release to keep DA levels in check by a negative feedback mechanism. In May there is lower expression of D2b1S expression which would be predicted to result in reduced negative feedback on TH, allowing for high DAergic inhibitory tone on LH until ovulation. Thus, this discovery provides a basis of the inhibitory tone of DA throughout the reproductive cycle, rather than referring to it as a type of "black box". Moreover, the hypothesis that D2 splicing is a relatively recent innovation in higher tetrapods is not supported by the results presented in Chapter 2 of this thesis and underscores the importance of such a splicing event in the evolutionary conservation of molecular signaling mechanisms by DA.

The relationship between gene expression and protein changes *in vivo* are poorly understood, particularly in teleosts. To address this general lack of knowledge, I developed a novel method for the comparison of transcriptomic and proteomic data obtained from *in vivo* experiments designed to study the neuroendocrine actions of DA (Chapter 3). This method is novel because while it is still difficult to do this type of comparison in better-characterized systems such as yeast, it has been, until now, it is even more challenging to do so in an organism whose genome has yet to be sequenced. The results demonstrate the applicability of advanced high-throughput genomic and proteomic analyses in the goldfish; an organism whose genome has yet to be sequenced. Furthermore, I demonstrate that DA rapidly decreases LH levels through both D1- and D2-type receptors and regulates multiple hypothalamic pathways and processes that are also known to be involved in human pathologies of the central nervous system. In particular, the identification of both mRNAs and proteins involved in calcium signalling and synaptic vesicle secretion further highlight the evolutionary conservation of molecular signaling mechanisms by DA. Importantly, despite the recognized limitations outlined in the chapter, the level of concordance in the mRNA and protein changes in the goldfish brain are well within the ranges seen with better characterized vertebrate and yeast systems.

Results from Chapter 3 led to the hypothesis that DA, acting through the D1 receptor, is influencing the glutamate system through transcriptional regulation of AMPA receptors. Indeed, Chapter 4 demonstrates that blockage of the D1 receptor primes the goldfish for AMPA-stimulated LH release to occur and suggests a novel mechanism of LH release in vertebrates. The mechanism of action is currently unknown, but is speculated to be through an increase in activin βa initialized by blockage of D1 receptors, which primes the fish brain for an AMPA-stimulated induction of cGnRH-II and IST and a subsequent release of LH

from the pituitary. Interestingly, this appears to be a seasonally-dependent mechanism as a different LH profile was observed at a different time of the year suggesting that the DAergic tone is important in modulating the response of AMPA-stimulated LH release. Indeed, the alternatively-spliced D2 receptor discovered in this thesis may alter telencephalonic and hypothalamic responses to DA, which, in turn, may alter the cellular response of other neurotransmitters, such as AMPA. Alternatively, estrogen and/or testosterone levels, which cycle throughout the year and peak in April/May, may be important in modulated these responses. This was evident in the data as aromatase b mRNA levels were found to be regulated by DA (current chapter).

In addition to its importance in neuroendocrine control, DA is central to the control of numerous other processes, especially locomotion. Indeed, the goldfish treated with MPTP, a specific DAergic neurotoxin, has recently been proposed as a model to investigate the pathology of Parkinson's Disease (208). The potential regeneration of DAergic neurons in the hypothalamus, some of which are likely to be hypophysiotropic while others involved in locomotor control and other processes, was examined in Chapter 5. What is not currently clear is whether the genes usually associated with stem cells and regeneration are active at particular times of the year (e.g. to account for the major physiological and environmental changes a temperate poikilotherm such as cyprinid fishes experience over the year) or whether they also have a role to maintain DAergic neurons rather than to induce neuronal differentiation. In other words, it is currently unknown whether DAergic neuron atrophy and regrowth is a normal process throughout the reproductive season of the fish or if it is strictly a pathological condition in response to DAergic neuronal damage.

Another line of investigation was to apply the results and the methods developed in this thesis to a real-life problem: that of exposure of fish to chemicals that affect

reproduction. Appendix A examined the effect of pulp and paper mill effluents on a species closely related to the goldfish, the fathead minnow. This additional chapter shows that mRNA levels for urocortin, which has been shown to stimulate GnRH release in mammals, and for CCK, which has been shown to inhibit DA action in mammals, are both decreased following chronic exposure to PPMEs. These results provide evidence that PPMEs are neuroactive and may be acting through some of the pathways modulated by DA. The identification of some of the neuroendocrine systems being affected by effluent exposure will help with future work in the industry-driven goal of reducing the ecological impact of the pulp and paper mill industry.

Finally, the current chapter identifies specific gene targets that are very likely to be regulated by DA, which was only possible through the work performed in this thesis. While this chapter contains new data, it was crucial for contrasting the DA agonist experiment as both of these studies were performed at the same time of year (May). It is evident from this thesis that seasonality is very important in the effect of DA on the molecular neuroendocrinology of the goldfish.

Together, the results of this thesis indicate that the DAergic system in the goldfish neuroendocrine brain is dynamic and seasonally regulated, and has intrinsic regenerative potential. The basic science presented in this thesis may further the understanding of vertebrate reproduction and provide insights into the effects of endocrine-disrupting chemicals in fish.

6.1.2. Concluding remarks

I investigated the molecular mechanisms underlying the inhibitory action of central DA on pituitary LH release. Since DA is such a potent inhibitor of LH release, it was hypothesized from the outset that DA modulates the expression of novel genes and systems

in the neuroendocrine brain related to reproduction. Indeed, the findings in this thesis demonstrate that DA regulates the expression of isotocin and activin βa, among others, as well as modulating the inhibitory tone of itself and the stimulatory tone of glutamate. Based on these studies, I propose an expansion of the model for the neuroendocrine regulation of pituitary LH release (Figure 6.5).

Although it was not discussed in the thesis, I also coordinated the expansion of a brain enriched cDNA microarray. As such, there are an additional 2373 ESTs that have been deposited on NCBI's dbEST and will appear on future generations of the AURATUS microarray platform. The power of a microarray is that it allows new hypotheses to be proposed about underlying genomic mechanisms of physiology. Indeed, this is evident from Chapter 3, in which multiple Gria receptors were identified as being regulated by DA agonists, and led to the hypothesis generated and tested in Chapter 4 using DA antagonists.

In future studies, in addition to what has been outlined in the individual chapters of this thesis, I would investigate the potential synergism of D1 and D2 receptor activation (reviewed in (378)) on the molecular neuroendocrinology of LH release. For example, in the caudate-putamen (dorsal striatum) of rats administered with high doses of either of the same D1 or D2 receptor agonists used in this thesis, Fos-like immunoreactivity was induced to a limited degree, but when both agonists were administered concurrently, patches of intensely-stained immunoreactive nuclei were visible (91). Both D1 and D2 antagonists were shown to reduce the expression of the alternatively-spliced D2 receptor demonstrated in this thesis supporting the hypothesis that D1/D2 activation synergism is important in the physiology of teleosts.

To conclude, this thesis 1) characterizes the role of DA in neuroendocrine function in fish; 2) identifies a novel mechanism of LH release mediated through DA, and; 3) demonstrates an ecologically-relevant and industrial application of the findings of this thesis.

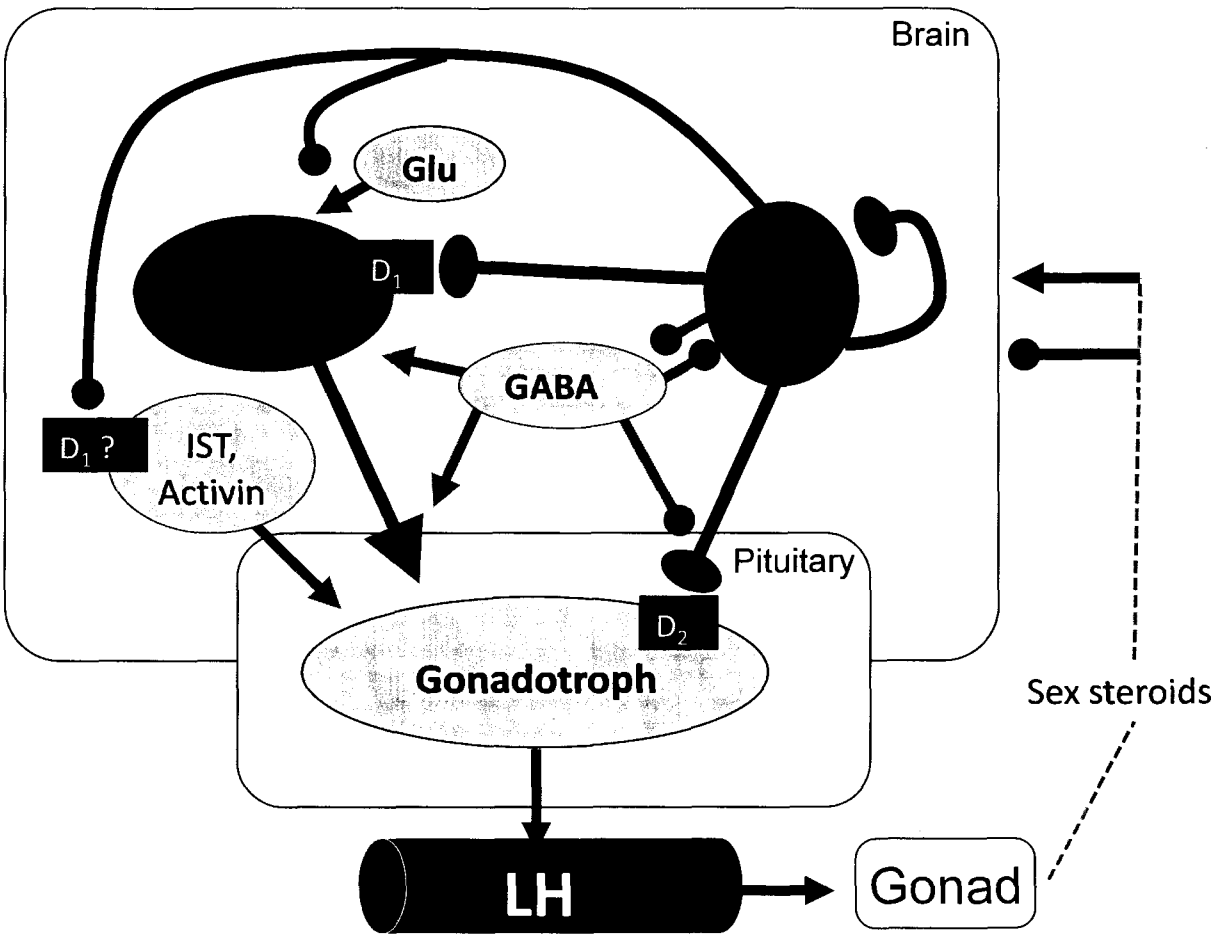

Figure 6.5: A model for the inhibitory tone of central DA on pituitary LH release in sexually mature female goldfish based on studies presented in this thesis. Pointed arrows indicate stimulation and bulbous arrows indicate inhibition.

Table 6.1: ESTs were manually selected based on identical AURATUS GeneIDs and on the basis of differential regulation in opposite directions for MPTP or the antagonists vs. agonists, or in the same direction for MPTP vs. antagonists. All ESTs were identified as being differentially regulated (q<5%) in all treatments. Only those with BLAST hits (NCBI), obtained with Blast2GO, are shown. Duplicate names may exist in the list, but were not identified by sequence overlap (cap3) and may represent separate genes or individual isoforms. The median "minimum ExpectValue" = 1.9E-57 and the average "mean similarity" = 84.8% ± 1%. Aromatase b was selected for validation with real-time RT-PCR. In the case where a suitable BlastX hit was unavailable, the best BlastN hit is used and is listed in the complete table in the supplemental data (Table C.4; Appendix C)

Tissue	AURATUS ID	Best Blast Hit	DA depletion or receptor blockage			DA mimic		Accession
			MPTP + aMPT	SCH 23390	sulpiride	SKF 38393	LY 171555	
Hyp	08j13	14 kDa apolipoprotein	-1.5			1.7		CF662566
Hyp	08b22	17-beta hydroxysteroid dehydrogenase type 12B, 3-ketoacyl-CoA reductase type B	1.4			-1.7		CA968619
Hyp	16j14	26s protease regulatory subunit 4	-1.4			1.4		CA966407
Hyp	08e14	40S ribosomal protein S27	-1.5			1.7		CA968660
Hyp	07f01	abhydrolase domain containing 12	-1.6			1.8		CA967283
Hyp	22n08	adenylate kinase 3-like 1	1.3			-1.5		CA969490
Hyp	08k20	aldehyde dehydrogenase 7 family, member A1	-1.3				1.3	CA968758
Hyp	03h23	aldolase C	1.4			-1.6		DY231930
Hyp	05f06	alpha-2-macroglobulin-1	-1.6			1.5	2.1	CF662428
Hyp	22i24	alpha-actin	1.4			-1.5		CA969403
Hyp	09p02	angiotensinogen	-1.5			1.8	1.3	CA964907
Hyp	09j02	apolipoprotein a-iv	-1.5			1.7		CA966743
Hyp	16n14	apolipoprotein e	-1.3			2.4		CF662778
Hyp	**04a17**	**aromatase b**	**1.3**			**-1.7**		**FG392770**
Hyp	14k14	arp2 actin-related protein 2 homolog	-1.3			2.3	1.3	CA964468
Hyp	12l13	asf1 anti-silencing function 1 homolog b (cerevisiae)	-1.3			1.9		CA966040
Hyp	16l15	atp-binding sub-family f member 2	-1.3			1.6		CA966450
Hyp	16o14	BC-10 protein	-1.3			1.9		CA966992
Hyp	03o22	beta-actin	1.3			-1.6		DY232011
Hyp	22l24	branched chain ketoacid dehydrogenase kinase		1.6		-1.8		CA969461
Hyp	02a23	calmodulin 1b	1.2			-1.7		FG392553
Hyp	14g01	claudin 23	-1.4			1.8		CA964745
Hyp	14k02	coiled-coil domain containing 47	-1.3			2.1		CA964457

Tissue	AURATUS ID	Best Blast Hit	DA depletion or receptor blockage			DA mimic		Accession
			MPTP + aMPT	SCH 23390	sulpiride	SKF 38393	LY 171555	
Hyp	19a04	cold shock domain-containing protein e1		-1.4		1.5	1.3	CA964993
Hyp	08o15	complement C3-H2	-1.4			1.6		CA970421
Hyp	08b20	complement component q subcomponent-like 4	-1.3				1.3	CA968617
Hyp	02c23	creatine kinase b variant 1	1.3			-1.6		DY231608
Hyp	02n10	creatine testis isozyme	1.2			-1.5		DY231690
Hyp	21l19	C-type lectin	1.5	-1.7		-1.7		CA969207
Hyp	19a14	cubilin (intrinsic factor-cobalamin receptor)	-1.4			1.4		CA964997
Hyp	17g09	cxxc finger 1 (phd domain)	1.3			-1.7		CA964951
Hyp	06d13	cytochrome p450 like	-1.4				1.6	CA965416
Hyp	05l01	cytokine induced apoptosis inhibitor 1	-1.4			2.3		CA966987
Hyp	03f23	deoxyribonuclease 1-like 3	1.5			-1.5		DY231911
Hyp	23k24	e3 ubiquitin protein ligase	1.6			-1.6		CA968074
Hyp	02i24	ependymin	1.3			-1.6		DY231713
Hyp	03o21	ependymin	1.4			-1.7		DY232010
Hyp	24a12	eph receptor a7	1.6			-2.1		CA969719
Hyp	15a10	equilibrative nucleoside transporter 1	1.3			-1.6		CA965545
Hyp	07b01	eukaryotic translation elongation factor 1 gamma	-1.5			1.7		CA966738
Hyp	20j14	eukaryotic translation initiation factor 2, subunit 1 alpha	-1.3	-2.0		2.3		CA966561
Hyp	09e01	fibronectin 1b	-1.3			2.0	1.3	CA964120
Hyp	24j21	fk506-binding protein 1a	1.3			-1.5		CA966789
Hyp	03o09	fructose-bisphosphate aldolase c	1.4			-1.6		FG392624
Hyp	10m11	g protein-coupled family group member c	1.3			-1.6		CA967701
Hyp	17n11	gamma-glutamyl cyclotransferase	1.3			-1.7		CA965786
Hyp	03i20	glutamine synthetase	1.2			-1.5		DY231974
Hyp	10d04	glutathione peroxidase 3	1.4			-1.5		CA964192
Hyp	23o12	glyceraldehyde 3-phosphate dehydrogenase	2.0			-2.1		CA968103
Hyp	08h01	glyceronephosphate o-acyltransferase	-1.6			2.2		CA968696
Hyp	14b13	granulin 1	-1.3			1.5		CA964295
Hyp	19m14	h2a histone member y2	-1.4			1.6		CA965061
Hyp	14k03	heat shock protein 90 beta	-1.3			1.7		CA964458

Tissue	AURATUS ID	Best Blast Hit	DA depletion or receptor blockage			DA mimic		Accession
			MPTP + aMPT	SCH 23390	sulpiride	SKF 38393	LY 171555	
Hyp	14i04	HECT domain containing 1		-1.4		1.5		CA964417
Hyp	24o12	hexokinase 1	1.6			-1.9		CA969997
Hyp	08g14	high density lipoprotein binding protein	-1.4			1.6		CA968690
Hyp	19d02	hydroxysteroid (17-beta) dehydrogenase 10	-1.3			2.2	1.3	CA965806
Hyp	03i10	immunoglobulin mu heavy chain	1.5			-1.5		FG392590
Hyp	04j23	jumonji domain containing 3	1.3			-1.5		FG392963
Hyp	13o14	latexin		-1.7		1.6		CF662717
Hyp	22g07	leucine rich repeat (in flii) interacting protein 1	1.2			-1.7		CA969350
Hyp	11p01	leucine rich repeat containing 58	-1.3			2.2		CF662658
Hyp	19f13	loc548392 protein	-1.4			2.0		CA969104
Hyp	14m01	malate dehydrogenase 1, NAD (soluble)	-1.3			1.8	1.3	CA964750
Hyp	12k14	male-specific protein	-1.3			1.9		CA970272
Hyp	22o11	map microtubule affinity-regulating kinase 4	1.5			-2.0		CA969512
Hyp	21l16	membrane palmitoylated	1.9			-1.6	1.3	CA966525
Hyp	09p22	methylcrotonoyl-coenzyme a carboxylase 2	1.5			-1.8		CA964915
Hyp	22k08	MHC class I antigen	1.4			-2.0		CA969424
Hyp	08a03	mid1 interacting g12-like protein	-1.3			1.6		CA970376
Hyp	09k02	mid1 interacting g12-like protein	-1.4			1.7		CA964854
Hyp	08l01	middle subunit	-1.4			2.5		CA965449
Hyp	03k10	midkine-related growth factor b	1.4			-1.5		FG392604
Hyp	12n01	mitochondrial ribosomal protein l19	-1.6			1.5		CA966046
Hyp	19p16	mitochondrial ribosomal protein l20		-1.4		2.0		CA967272
Hyp	11j11	mitogen-activated protein kinase kinase kinase 7 interacting protein 3	1.4			-1.7		CF662634
Hyp	12p13	m-phase phosphoprotein 6	-1.5			2.1		CA966058
Hyp	06g06	myelocytomatosis oncogene b	1.3			-2.7		CF662485
Hyp	14n02	myosin regulatory light chain	-1.3			1.6		CA964520
Hyp	24b19	nck adaptor protein 2	1.4			-1.5		CA969746
Hyp	19l18	negative elongation factor d		-1.8		1.5		CA965844
Hyp	03i12	nel-like protein 2	1.3			-1.7		FG392591
Hyp	16k15	nlr card domain containing 3		-1.3		1.8		CF662774

Tissue	AURATUS ID	Best Blast Hit	DA depletion or receptor blockage			DA mimic		Accession
			MPTP + aMPT	SCH 23390	sulpiride	SKF 38393	LY 171555	
Hyp	18c18	nol1 nop2 sun domain member 2		-1.5		-1.5		CA964613
Hyp	08o01	novel protein	-1.4			1.5		CA968809
Hyp	11d07	novel protein	1.3			-1.5		CF662614
Hyp	15i06	novel protein (zgc:136439)		-1.6		1.6		CA965636
Hyp	15b13	novel protein lim domain only 3 (rhombotin-like 2) (zgc:110149)	-1.4			2.0		CA965552
Hyp	11e15	novel sulfotransferase family protein	-1.3			1.9		CA965939
Hyp	19e01	nuclear receptor subfamily group member 2	-1.4			2.1		CA966183
Hyp	15e23	phosducin-like 3		1.5		-1.6		CA966723
Hyp	12l11	plasma retinol-binding protein 1	1.3			-1.5		CA966039
Hyp	03k09	poplar cdna sequences	1.3			-1.5		FG392603
Hyp	08g04	prostaglandin h2 d-isomerase	-1.5			1.5		CA968684
Hyp	22p03	proteasome (macropain) 26s non- 4	1.4			-1.6		CA969527
Hyp	12b01	proteasome (macropain) alpha 5	-1.5			2.0	1.3	CA965983
Hyp	12i01	purine nucleoside phosphorylase	-1.5			1.6		CA967769
Hyp	22b23	response gene to complement 32	1.3			-2.0		CA969259
Hyp	22g21	ribosomal protein l13	1.5			-1.7		CA969362
Hyp	08o16	ribosomal protein l27a	-1.3			1.5		CA968817
Hyp	12d13	ribosomal protein l27a	-1.5			1.6		CA965998
Hyp	09o01	serine incorporator 1	-1.4				1.5	CA964172
Hyp	21a01	sh3-domain grb2-like 2	-1.5			1.7	1.3	CA967895
Hyp	09g14	si:ch211- protein	-1.4			1.8		CA964823
Hyp	24i19	StAR-related lipid transfer (START) domain containing 4	1.4			-2.1	1.6	CA969885
Hyp	09n02	sterol-c5-desaturase (fungal delta-5-desaturase) homolog (cerevisae)	-1.3			2.5		CA964885
Hyp	12p21	surfeit 4		-1.5		1.6		CA966062
Hyp	20o02	tetraspanin 9	-1.6			1.6		CA965906
Hyp	24i22	transaldolase 1	1.4			-1.6		CA969888
Hyp	15f10	translocon-associated protein subunit delta precursor	1.3			-1.8		CA965601
Hyp	12f01	transthyretin precursor	-1.3			3.0		CA966004
Hyp	07h01	triosephosphate isomerase	-1.3			1.8		CA968504
Hyp	14f24	troponin c type 2	1.4			-2.1		CA964383

Tissue	AURATUS ID	Best Blast Hit	DA depletion or receptor blockage			DA mimic		Accession
			MPTP + aMPT	SCH 23390	sulpiride	SKF 38393	LY 171555	
Hyp	21g17	troponin c type 2		-1.5		1.6		CA967929
Hyp	22g09	tubulin alpha 8 like 4	1.3			-1.8		CA969352
Hyp	03o23	tubulin beta 2c	1.4			-1.5		FG392672
Hyp	17j23	tubulin beta-2c chain	1.4			-1.5		CA965774
Hyp	14f02	u2 small nuclear rna auxiliary factor 1		-1.7		2.4	1.3	CA964363
Hyp	22l09	vacuolar protein sorting 13c	1.3			-1.5		CA969449
Hyp	20j02	vacuolar protein sorting 4a	-1.5			1.7	1.6	CA966560
Hyp	14j12	vimentin	1.4			-1.5		CA964445
Hyp	24i24	vimentin	1.4			-1.7		CA969890
Hyp	12i13	vitellogenin 2	-1.3			1.4		CA967775
Hyp	19o08	zinc and double phd fingers family 2		-1.5		1.5		CA965067
Hyp	23a24	zinc finger ccch-type containing 7a	2.0			-2.3		CA967982
Hyp	15i14	zinc finger protein 782	-1.3			2.0		CA965639
Hyp	20c13	zona pellucida glycoprotein		-1.6		1.7		CA966260
Tel	12o17	ccaat enhancer-binding protein beta			-1.6		1.7	CA967804
Tel	12e10	leucine-rich ppr-motif containing	-1.8				1.6	CA970240
Tel	14f04	solute carrier family 2 (facilitated glucose fructose transporter) member 5			-1.3		1.9	CA964365

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Appendix A.

GENE EXPRESSION PROFILING OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*) NEUROENDOCRINE BRAIN IN RESPONSE TO PULP AND PAPER MILL EFFLUENTS[§§]

A.1. INTRODUCTION

Pulp and paper mill effluents (PPMEs) have negative effects on their surrounding aquatic ecosystems (379-381). Given the crucial role of fish within ecosystems of affected rivers and estuaries, the long-term and short-term effects on fish survivability in these systems is of utmost concern. While dramatic reductions in acute PPME toxicity have been attained (382), they are still resulting in decreased reproductive success of fish populations and research has now shifted to the long-term effects of effluent exposure on non-lethal endpoints. For example, in fathead minnows (FHM) exposed to PPME, short term exposures decrease egg production, while developmental exposures can increase male-biased sex ratios (383). Similar studies have also demonstrated a male-biased population of eelpout (*Zoarces viviparous*) in rivers containing mills (384) and a temporary recovery of sex ratios coinciding with a temporary closure of the mill (385). Most studies examine physiological endpoints such as growth, development, liver function, gonad size, secondary sex characteristics, fecundity and spawning behaviour (386, 387), while few examine brain responses to PPME exposure. The reproductive neuroendocrine has been well studied in cyprinids, particularly in the goldfish (2, 3, 12, 69, 388-390). Signals from stimulatory (e.g. gonadotropin-releasing hormone; GnRH) or inhibitory (e.g. dopamine; DA) neurons are integrated in the brain where nerve terminals project to and directly innervate the anterior pituitary to control the release of luteinizing hormone (LH) as well as other hormones and

[§§] Popesku, J.T., Tan, E.Y.Z., Martel, P.H., Kovacs, T.G., Rowan-Carroll, A., Williams, A., Yauk, C. Trudeau, V.L. Gene expression profiling of the fathead minnow (*Pimephales promelas*) neuroendocrine brain in response to pulp and paper mill effluents. *In preparation.*

neuropeptides. In all vertebrates studied thus far, a surge of LH is required for ovulation to occur and it is thought that the LH surge, and in numerous fish species, is the result of a disinhibition of DA. Conversely, increased DAergic action can result in decreased plasma LH levels (69) and DA is shown to keep eels in a prepubertal state for many years (53). Indeed, administration of specific DA D1-receptor or D2-receptor agonists were both able to rapidly and significantly reduce circulating LH levels in sexually mature female goldfish (Chapter 3).

The fathead minnow is a member of one of the largest vertebrate families, the *Cyprinidae*, and has a broad geographic distribution across North America. They are an amenable model in ecotoxicological studies for assessing the effects of Canadian PPMEs (391). The frequent spawning period of approximately 3 days, with an average egg production of 85 eggs/spawn (391), allows easy reproductive endpoints to be assessed. Short-term studies aimed at examining the effects of effluent on reproductive indices often look at egg production, egg viability and offspring viability methods to profile effluent effects. Indeed, fish spawning is one of the most ecologically relevant indicators of endocrine disruption (392). Furthermore, according to Hall *et al.* (393), egg count is often the most sensitive bioindicator in effluent exposure tests, supporting the use of FHM in ecotoxicological studies.

An endocrine-disrupting chemical (EDC), as defined by Kavlock *et al.* (394), is "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes." In particular, EDCs can alter normal reproductive processes (395). PPMEs are sources of concentrated chemicals that are

released into rivers and other aquatic systems. The compositions of the effluents are difficult to characterize due to their heterogeneous nature and phytochemical origin; however, several biologically active chemical families are identified. For example, plant by-products such as phytosterols (e.g. β-sitosterol), stillbenes and retenes are released during the wood extraction process in paper-making (379), and β-sitosterol induces plasma vitellogenin levels, a biomarker for estrogenic compounds, in sexually immature rainbow trout (396).

Exposure of separate groups of FHM to 5 different PPMEs (100%) resulted in the immediate inhibition of spawning to varying degrees (44-100%) as determined by female egg production relative to unexposed fish. Using Agilent-manufactured oligo microarrays based on the EcoArray platform, we examined the effects of 3 of these effluents, which covered the range of spawning inhibition observed, on gene expression in the hypothalamus of female fish. We then validated a select subset of the genes identified by microarray analysis using real-time RT-PCR in the hypothalamus and telencephalon while extending this to all of the treatment groups as well as to male fish in all treatments groups. We hypothesized that, owing to the rapidity and degree of spawning inhibition, the DAergic system is being activated, which is supported from previous work in our lab with radioligand binding assays showing that PPMEs resulted in 20-40% increase in D2 receptor binding in goldfish pituitaries (397). We cloned partial coding fragments for the dopamine transporter (DAT) and tyrosine hydroxylase (TH), which were not represented on the microarray. The findings from this study strongly suggest that the PPMEs are acting through different mechanisms that manifest in decreased spawning in FHM and that at least one of the PPMEs may be acting through the DA system.

This approach identifies key neuroendocrine systems which may be implicated in spawning inhibition in PPMEs in the industry-driven efforts to reduce the ecological impact of effluents on the environment. Given the severe impact on aquatic ecosystems, this study provides information to potentially alleviate the environmental footprint of the pulp and paper mill industry in order to build a more sustainable industry.

A.2. Materials & Methods

A.2.1. Animals, experimental conditions, and sampling procedures

Male (n=48) and female (n=96) fathead minnow (FHM; *Pimephales promelas*) were raised in the Forest Product Innovations-Paprican Division (Pte. Claire, QC) laboratory in 25 ± 1°C water and a photoperiod of 16:8 light:dark cycles. Final biotreated mill effluents from five separate sources were collected and coded (DSF, AKN, ABC, DWN and EBE) by FPInnovations.

A five-day exposure to all five effluent treatments at 100%-strength and one control (water) was carried out with 2 males and 4 females in each tank in quadruplicate. Egg counts were taken each day, and for each tank were calculated as the number of eggs/female/day.

Following exposure, fish were anaesthetized using 3-aminobenzoic acid ethylester (MS222) and sacrificed by spinal transaction. Morphological measurements were made (fork length, weight and gonad weight) and brain tissues were rapidly dissected and frozen on dry ice. Hypothalami and telencephali from each sex and tank were collected and pooled (n=4 per tissue per sex) to increase RNA yield prior to RNA isolation.

A.2.2. RNA extraction and cDNA synthesis

RNA was extracted as described in Section 2.2.3 of this thesis. RNA quality was determined using the Agilent 2100 BioAnalyzer. All RIN values, with the exception of three samples, were above 8.2 with the mean value for all samples being 8.9. The 3 samples with lower RIN numbers were not used in microarray analysis. Furthermore, results from these 3 samples from real-time RT-PCR assays were not different than those from their respective groups.

cDNA was prepared as described in Section 2.2.3.

A.2.3. Microarray analysis

To obtain global gene expression profiles of systems likely affected by exposure to PPMEs, microarray analysis was performed using Agilent-manufactured arrays built on the EcoArray FHM Platform (EcoArray Inc, Gainesville, FL). Female hypothalami (n=4 each) from Control, DSF-, ABC-, and AKN-exposed fish were hybridized against a common universal reference RNA pool containing equal amounts of all male and female samples from all treatments.

A.2.4. Real-time RT-PCR assays

To analyze the expression of the TH, DAT, Per4, RevErbβ, CCK and urotensin I genes, specific primers were designed using Primer3 (Table A.1) and ordered from Invitrogen. Primers were tested by PCR on FHM whole brain cDNA and single bands were visualized on a gel. Amplicons from these primer sets were sequenced to confirm specificity.

Table A.1: Real-time RT-PCR primers used in this study.

Gene	Primers (5' – 3')	Sequence Source	Annealing Temp (^{O}C)	Amplicon Lengths (bp)
TH	F: AAAGGACGGCTTGGAGGA R: TTTGACCTCTTTTGTGGTTTTG	FJ621331	60	124
DAT	F: GAGCAGCAGGAGTCTGGAA R: GCGTGTCGTTGAGCAGAG	FJ859182	59	222
Per4	F: TTGAACCGTACCATCAAAGAGA R: TCCAAGACAACTGGAAGTCAAA	EcoArray Inc.	59	204
RevErbβ	F: TGGAACACCAACACCAAACA R: CCAGGAAACATGGAAAGGAA	EcoArray Inc.	60	157
CCK	F: ACCAGCCTCACCCTCAAAC R: AAATCCATCCAGCCCACA	EcoArray Inc.	60	185
urotensin I	F: TGTGGCATCAGGGTTATGTG R: CCTGCACAGCGTTTAGAGGT	EcoArray Inc.	60	213

Real-time RT-PCR was performed as described in Section 2.2.6 of this thesis. Results were normalized to total RNA as determined by the NanoDrop ND-1000.

A.2.5. Bioinformatic meta-analysis of microarray data

The microarray dataset for DSF-exposed fish provided the largest number of genes identified as significantly differentially expressed. Additionally, the DSF-exposed fish were completely inhibited from spawning. Thus, the microarray dataset for the DSF-exposed FHM was compared (BLAST) against microarray datasets generated from multiple DAergic pharmaceutical-injection microarray experiments in the goldfish (*Carassius auratus*), a closely-related species, using Blast2GO. Only those genes/ESTs identified as being statistically significantly regulated (FDR-adj p-value 0.05 or $q<5\%$) in the hypothalamus of female fish were considered. Results were then manually confirmed.

A.2.6. Statistical analysis

All real-time RT-PCR data were assessed using the Kolmogorov-Smirnov test for normality and Levene's test of homogeneity of variance in SPSS v15.0. In the cases where data was not normally distributed, the data was log-transformed and determined to be normally-distributed. A one-way ANOVA was performed with significance considered at $p<0.05$, followed by a two-tailed t-test with Bonferroni correction against control.

A.3. RESULTS

A.3.1. Egg production and GSI

Effluent exposure significantly reduced the number of eggs produced in all effluent treatments relative to control (Table A.2). Relative to control, DSF completely inhibited (100%) egg production whereas AKN (44%), ABC (90%), DWN (59%), and EBE (91%) all had varying levels of inhibition of egg production. Furthermore, the number of spawning events was reduced to zero in DSF-exposed fish.

The gonadosomatic index (GSI) of female FHM exposed to the DSF effluent was significantly higher than that of unexposed fish (Table A.3). No other statistically different GSI values were observed.

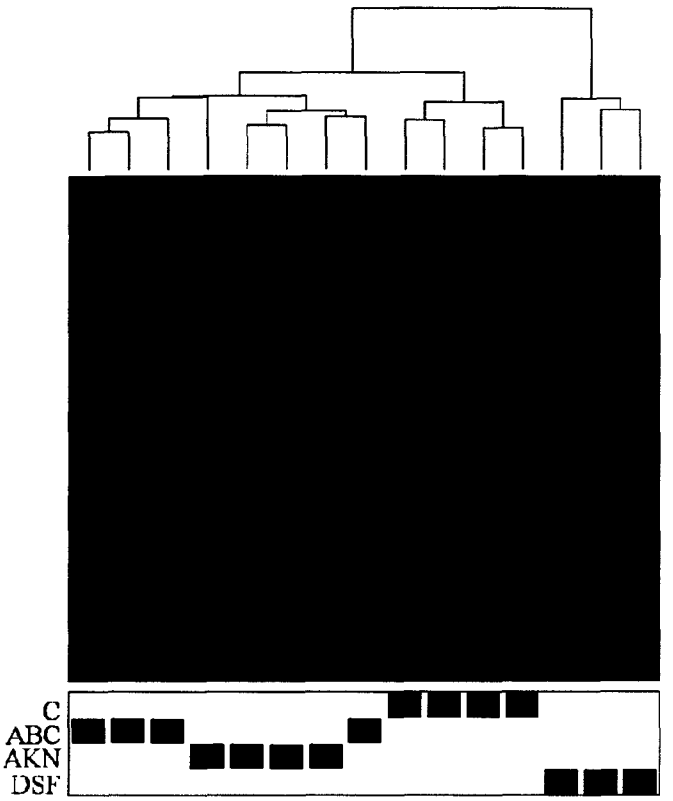
Table A.2: Egg production and number of spawning events for fathead minnows before and after exposure to final biotreated mill effluents. Results are expressed as means (standard error). The bracketed numbers represent the range. * indicates a statistically significant difference from the control (Kruskal-Wallis, $p < 0.05$)

Treatment	Eggs/female/day	Number of spawning events
Control	81 (7.4) [64-98]	4.5 (0.5) [3-5]
DSF	0* (0.0) [0-0]	0* (0.0) [0-0]
AKN	45* (4.8) [36-57]	3.3 (0.3) [3-4]
ABC	8* (3.8) [1-16]	2.3 (0.8) [1-4]
DWN	33* (8.1) [11-47]	3.3 (0.5) [2-4]
EBE	7* (2.8) [2-14]	2.3 (0.6) [1-4]

Table A.3: Morphometric parameters and gonad somatic index for male and female fathead minnows exposed to final biotreated mill effluents. Values are expressed as means (standard error). The bracketed numbers represent the range.* indicates a statistically significant difference from the control (ANOVA, $p < 0.05$)

Treatment		Fork Length (mm)	Body weight (g)	Condition factor	Gonad weight (mg)	GSI (%)
Control	Females	55 (0.9) [49-63]	2.2 (0.1) [1.5-3.6]	1.3 (0.0) [1.0-1.7]	265 (28) [131-636]	12 (0.7) [8.6-18]
	Males	70 (1.7) [61-76]	5.4 (0.3) [4.3-6.6]	1.6 (0.1) [1.4-1.9]	66 (10) [31-113]	1.2 (0.1) [0.5-1.7]
DSF	Females	56 (0.7) [50-60]	2.4 (0.1) [1.6-3.5]	1.3 (0.0) [1.0-1.7]	382* (38) [78-697]	16* (1.0) [3.3-20]
	Males	71 (1.0) [67-75]	4.9* (0.3) [3.7-6.4]	1.4 (0.1) [1.1-1.6]	70 (7) [48-98]	1.5 (0.1) [1.0-2.0]
AKN	Females	56 (0.7) [50-62]	2.3 (0.1) [1.5-3.5]	1.3 (0.0) [1.1-1.6]	287 (33) [183-713]	13 (0.7) [8.6-21]
	Males	73 (1.8) [66-81]	6.1 (0.4) [4.3-7.6]	1.6 (0.1) [1.3-1.9]	96 (12) [49-146]	1.5 (0.1) [1.2-2.0]
ABC	Females	55 (0.5 [53-59]	2.3 (0.1) [1.8-3.1]	1.3 (0.0) [1.1-1.8]	309 (29) [148-506]	13 (0.9) [6.8-18]
	Males	73* (1.1) [70-80]	5.8 (0.2) [5.0-6.6]	1.5 (0.0) [1.3-1.6]	85 (10) [54-129]	1.4 (0.1) [1.0-2.1]
EBE	Females	57 (1.2) [50-66]	2.4 (0.2) [1.6-4.1]	1.3 (0.0) [1.2-1.4]	328 (37) [169-788]	14 (0.8) [8.7-19]
	Males	69 (1.3) [62-73]	5.0 (0.2) [3.9-5.8]	1.5 (0.1) [1.3-2.0]	72 (11) [18-116]	1.4 (0.2) [0.4-2.0]
DWN	Females	56 (1.0) [49-65]	2.4 (0.1) [1.6-3.5]	1.3 (0.0) [1.1-1.5]	262 (20) [149-406]	11 (0.6) [6.3-15]
	Males	73 (1.4) [66-76]	6.0 (0.2) [5.0-6.6]	1.6 (0.1) [1.3-2.2]	87 (13) [34-148]	1.4 (0.2) [0.7-2.3]

a)



b)

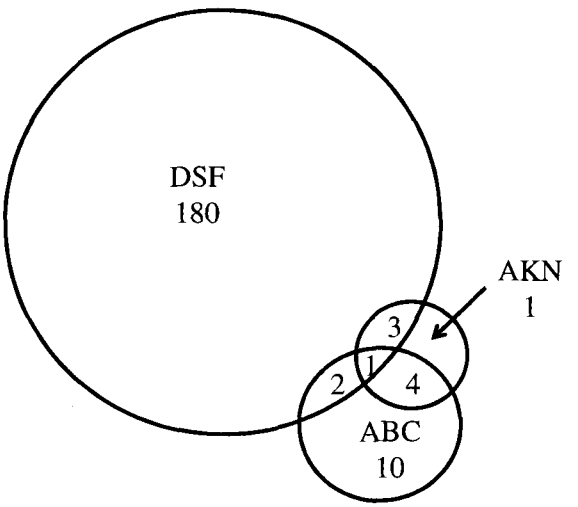


Figure A.1: a) Cluster diagram showing the grouping of the individual arrays. b) Total number of mRNAs affected by at least one effluent (FDR-adj p-value < 0.05)

A.3.2. Microarray analysis

Hypothalami from control, DSF-, AKN-, and ABC-exposed female fish were submitted for microarray analysis on Agilent-manufactured EcoArray-based oligo arrays (Figure A.1a). Of the 15 000 genes profiled, the vast majority were not affected by effluent exposure; however, the relative abundance of 201 mRNA transcripts were significantly (FDR-adj p-value<0.05) changed by at least 1 effluent treatment, with the majority of these changes observed in the DSF-exposed fish (Figure A.1b, Table A.4).

Table A.4: Abbreviated list of genes/ESTs identified by microarray analysis as being statistically significantly regulated (FDR-adjusted p-value < 0.05) following 5d-exposure to PPMEs. DSF, ABC, and AKN are coded for individual PPMEs. Gene Names were annotated by EcoArray, Inc. Only those discussed in this chapter are shown; the full table is available in the supplementary data (Table C.5). * Urotensin I was marginally statistically significant (FDR-adjusted p-value = 0.058); we chose to validate it using real-time RT-PCR because it was decreased in all three treatments. See text for details. Negative values indicated a decrease in mRNA levels.

Gene Name	DSF	ABC	AKN	EcoArray ID
Period Homolog 4	2.9	-2.9	-2.3	EA_Pp_56149a
RevErb Beta 2		-2.3	-1.9	EA_Pp_55899a
Cholecystokinin	-2.1			EA_Pp_63088a
Urocortin (Urotensin I)*	-1.8	-1.5	-1.3	EA_Pp_70043a
Corticotropin-releasing factor	-1.5			EA_Pp_56548a
Homer homolog 1	-1.5			EA_Pp_54413a
SMAD6	1.6			EA_Pp_56618a
YWHAB	-1.6			EA_Pp_58408a

A.3.3. Microarray validation using real-time RT-PCR

CCK, Per4, RevErbβ, and urotensin I were selected for validation using real-time RT-PCR for female hypothalamic tissues. Furthermore, we extended the analysis to include a second neuroendocrine tissue (telencephalon), and EBE-, and DWN-exposed fish as well as male neuroendocrine tissues. Per4 transcript levels were not affected by any effluent exposure in Hyp or Tel in either sex (data not shown). Figure A.2 shows the results for CCK, RevErbβ, and urotensin I. For fold-changes reported here, significance was considered at $p<0.01$. Transcript levels for CCK and RevErbβ in the telencephalon of male fish (Figure A.2b and 2f, respectively), were below detection limits. A 1.6-fold decrease in CCK mRNA levels was observed in the female hypothalamus of DSF-treated fish (Figure A.2a), confirming the result of the microarray (Table A.4). In male fish, relative mRNA abundance of CCK was decreased 5.0-fold, 5.3-fold, and 4.0-fold in the hypothalamus of AKN-, EBE-, and DWN-exposed fish, respectively, relative to unexposed fish (Figure A.2b).

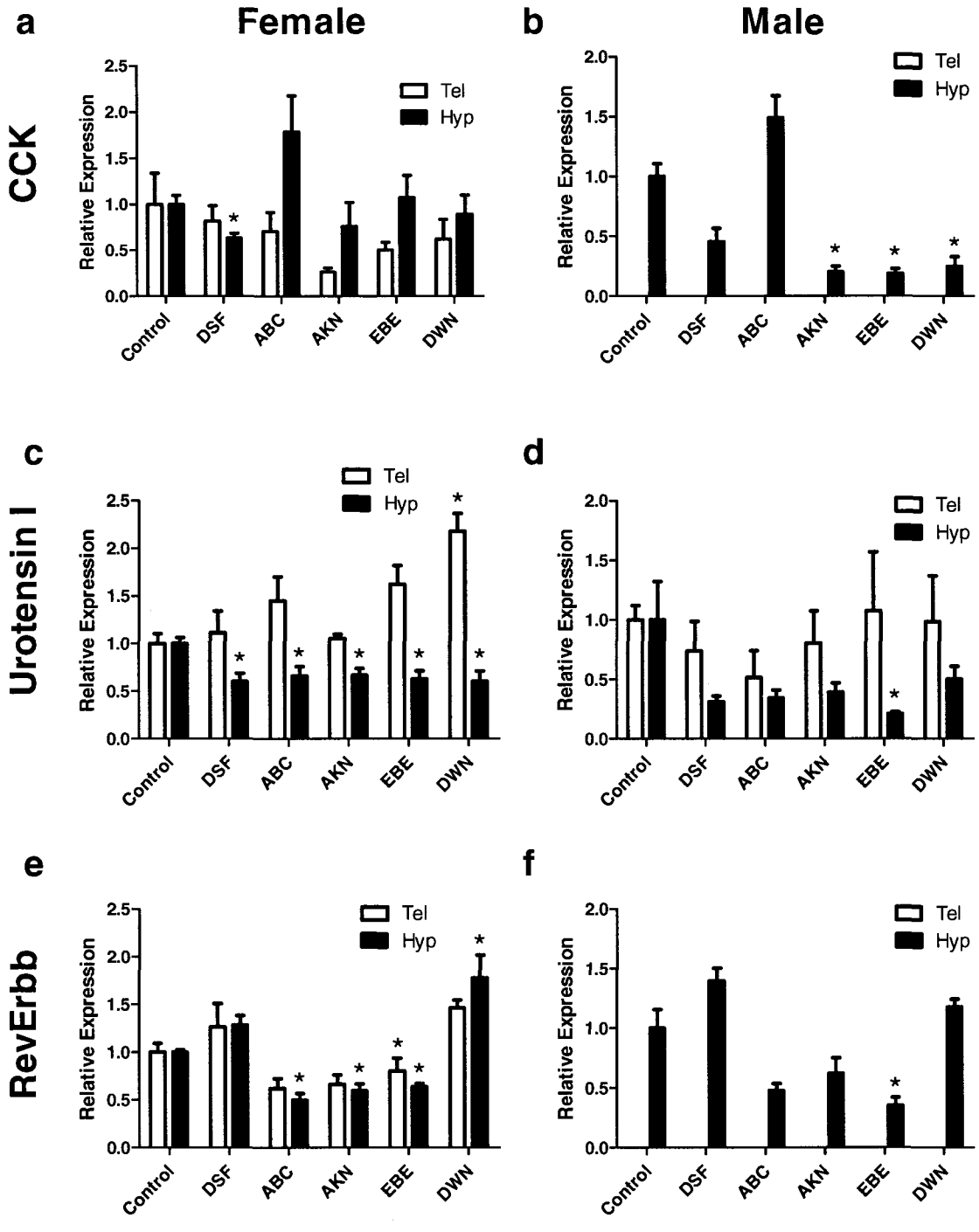


Figure A.2: Real-time RT-PCR of steady-state mRNA levels for cholecystokinin in a) female and b) male, urotensin I in c) female and d) male, and RevErbβ in e) female and f) male fathead neuroendocrine brain tissues following 5d exposure to 100% biotreated secondary effluent. Tel = telencephalon; Hyp = hypothalamus. * indicates $p<0.01$ relative to the corresponding control tissue. n=4 /sex/tissue/treatment. Note: CCK and RevErbβ mRNA levels were below detection limits in the male telencephalon.

Urotensin I mRNA levels were significantly decreased 1.5-1.7-fold in the hypothalami of all effluent-exposed female fish relative to unexposed fish (Figure A.2c). In male fish, urotensin I mRNA levels were similarly-affected, but due to high variability in the control, only EBE-exposed fish were statistically significant and were decreased 4.8-fold relative to control (Figure A.2d). Urotensin I mRNA levels were significantly increased in the telencephalon of DWN-exposed female fish but remained unaffected in the telencephali of all male fish.

RevErbβ mRNA levels were significantly decreased 1.6-fold in the hypothalamus of both ABC- and AKN-exposed female fish, but not DSF-treated female fish (Figure A.2e), relative to control, again confirming the microarray results (Table A.4). Furthermore, female fish exposed to EBE had significantly decreased RevErbβ mRNA levels (1.2-fold) while those exposed to DWN had a 1.5-fold increase in RevErbβ mRNA levels relative to control. The results for the female telencephalon were similar to those in the hypothalamus, but only those in the EBE-exposed group were statistically significant (1.6-fold decrease). In the male hypothalamus (Figure A.2), the results similar to those obtained for the female hypothalamus, but again, only the EBE-exposed group met statistical significance compared to control (2.8-fold decrease).

Tyrosine hydroxylase (TH) and the dopamine transporter (DAT) were not represented on the array. TH is the rate-limiting enzyme in DA biosynthesis (31) and DAT is necessary to remove DA from the synaptic cleft (96). We cloned partial coding fragments for these two genes and submitted the sequences to GenBank (Accession Numbers shown in Table A.1). We designed real-time RT-PCR primers for DAT and TH using Primer3 and examined their relative mRNA expression levels in effluent-exposed fish. Neither DAT nor TH mRNA

levels were affected by any of the effluents in either Hyp or Tel in either sex (data not shown).

A.3.4. Bioinformatic meta-analysis

A total of 26 common genes/ESTs were identified as being differentially regulated in FHM exposed to PPMEs or to goldfish injected with DAergic pharmaceuticals, 17 of which were common with the goldfish D1 receptor experiments and 9 of which were common with the goldfish D2 receptor, respectively. Only sequences with length > 500 bases provided Blast hits. This is presumably due to limited sequence information as neither FHM nor goldfish have had their genomes sequenced and thus rely on comparison to EST projects of other species. EST projects typically generate short sequences (398) and therefore insufficient overlap exists to achieve a valid Blast hit. Nevertheless, the 26 co-identified genes/ESTs provide significant information (Table A.5). There is a 65% agreement in direction (17 of the 26) between the DSF-exposed FHM dataset and DA stimulation datasets (i.e. in the same direction for DA agonists and the opposite direction for DA antagonists).

Table A.5: A comparison of the microarray dataset obtained in the current study for hypothalamus of DSF-treated female FHM with hypothalamic microarray datasets obtained in previous studies in our laboratory from female goldfish 5h post-injection with different DAergic pharmaceuticals. The FHM data set was compared to the goldfish datasets using BlastN and are thus based on sequence similarity. Annotations used are from EcoArray, Inc.

Gene Name (EcoArray)	DSF Fold Change	Goldfish DA treatment	Goldfish DA Fold Change	FHM Sequence Length	Mean Similarity	Min eValue	AURATUS ID
Ba1 Globin	2.5	D1-antagonist	-1.4	679	91%	1.50E-157	61i09
Similar To Cyclic-AMP-dependent Transcription Factor ATF-5	2.2	D1-agonist	-1.5	1186	91%	1.80E-130	24f05
Fibrinogen Gamma Chain	-2.2	D2-agonist	1.2	544	93%	1.80E-119	05a10
Zgc:65809	1.9	D2-agonist	1.5	1495	90%	0	12b15
Zgc:91870	1.9	D1-agonist	-2.0	960	89%	9.80E-135	22b23
Tubulin, Beta 2B	-1.8	D1-agonist	-1.5	1835	85%	1.00E-143	03o23
Formyl Peptide Receptor 1	1.8	D1-agonist	1.5	1399	88%	9.20E-99	19f03
Zgc:65987	1.7	D2-agonist	1.5	1187	91%	0	08l19
Solute Carrier Family 31, Member 2	1.7	D1-agonist	1.5	1599	88%	5.00E-76	11f06
Chaperonin Containing TCP1, Subunit 7	-1.7	D2-agonist	1.3	1319	91%	0	11g16
Zgc:92903	1.7	D2-agonist	1.4	699	92%	4.40E-133	09d18
Tubulin, Beta 2C	-1.6	D1-agonist	1.6	635	89%	3.80E-130	04p15
Tubulin, Alpha 1 Tubulin, Alpha 8 Like 4	-1.6	D1-agonist	-1.8	1606	94%	0	22g09
Lymphocyte Cytosolic Protein 1	1.5	D1-agonist	1.5	2692	86%	7.00E-98	07m16
Pl10	-1.5	D1-agonist	-1.7	3591	93%	3.10E-119	20o09
Zgc:64123	1.5	D1-agonist	1.8	843	89%	2.70E-79	11e02
Tubulin, Beta 2A	-1.5	D2-agonist	1.5	624	93%	2.20E-159	20e09
Corticotropin Releasing Hormone	-1.5	D1-agonist	1.7	837	94%	0	02a14
AMP Deaminase 3	1.5	D2-agonist	1.6	1046	92%	5.50E-155	21p13
C-terminal Binding Protein 2	-1.5	D1-agonist	-1.6	2417	91%	1.30E-83	19o06
Chaperonin Containing TCP1, Subunit 5	1.5	D2-agonist	1.3	1320	89%	0	06a12
Brain Protein I3	1.4	D1-agonist	1.4	1815	88%	1.10E-52	07k01
Sterol-C5-desaturase Homolog	-1.4	D1-agonist	2.5	1540	93%	5.60E-108	09n02
ATPase, H+ Transporting, Lysosomal 70kDa, V1 Subunit A	-1.4	D1-agonist	2.1	1679	93%	9.60E-161	04d02
Cathepsin L, A	1.4	D1-antagonist	-1.5	1473	92%	0	23g24
Postsynaptic Protein CRIPT	1.3	D2-agonist	1.3	1291	91%	2.70E-117	21a16

A.4. DISCUSSION

In this study, we examined the effects of PPMEs on gene expression in the neuroendocrine brain of an ecotoxicological model, the fathead minnow. We hypothesized that, owing to the rapidity and extent of spawning inhibition in effluent-exposed fish, the DA system is being affected. The increase in GSI observed in female fish and corresponding complete inhibition of spawning in the DSF-treated group suggests that female fish remain capable of producing eggs internally, but are incapable of ovulating. This further suggests that follicle-stimulating hormone (FSH) levels are relatively unaffected, but luteinizing hormone (LH) levels are reduced. It is the surge release of LH that induces ovulation in vertebrates. It has been shown that dopaminergic (DAergic) antagonists increase circulating levels of LH but have no effect on circulating FSH levels in female rainbow trout (399). Thus, the increased GSI, coupled with abolished spawning in the DSF-exposed fish, supports our hypothesis.

The hypothalamus is a central integrator of signals from throughout the brain. We submitted cDNA derived from hypothalamic RNA from DSF-, ABC-, and AKN-exposed female FHM for microarray analysis to obtain gene expression profiles for the widest range of spawning inhibition observed in this study. From the results, it is clear that the DSF effluent is affecting the brain of FHM differently than the ABC or AKN effluents. This is likely due to different chemical compositions of the effluents because the processes used to make final products (e.g. newsprint or bleached-kraft paper) or different raw materials (i.e. tree species) are mill-specific. We validated select genes identified by the microarray as being differentially regulated by different effluents, including cholecystokinin (CCK), urotensin I and RevErbβ.

There is an established relationship between food intake/energy balance and reproduction (277, 400-403). In general, stimuli and physiological cues that increase metabolic intake decrease reproductive processes (401). Hormones such as CCK (401) and urotensin I (13, 404) play a role in long-term metabolism and energetic regulation. These neuropeptides not only play a role in feeding behaviour but studies have shown that they may even directly affect GnRH and LH release (405-407). CCK is a peptide hormone that is released in the gut and the brain to inhibit food intake (401). CCK can also stimulate GnRH release in monkeys (408) and LH release in goldfish pituitary fragments *in vitro* (266). Furthermore, in mice, increased CCK levels in the brain have been shown to inhibit DA release via the CCK_B receptor (405). Urotensin I, the fish homologue of human urocortin (404, 409), also plays a role in appetite-suppression. It is a member of the corticotropin-releasing factor-related (CRF-related) protein family and plays a role in the stress response (409, 410). However, urotensin I can also stimulate GnRH expression in mouse cells (407). Thus, both CCK and urotensin I may stimulate LH release from the pituitary and have stimulatory effects on reproductive processes. As both CCK and urotensin I mRNA levels are decreased following exposure to PPMEs in the present study, our hypothesis that PPMEs may inhibit spawning through actions on the brain is supported and may provide insights into a possible method of action.

While somewhat speculative, some of the FHM microarray results are potentially indicative of a disruption of physiological rhythms. Environmental cues play a large role in regulating physiological functions in all organisms; these exogenous signals may be in the form of countless stimuli including light (411). The detection and reaction to the rhythm of daylight (photoperiod) is likened to an internal clock, termed the circadian rhythm. This

phenomenon – mediating external cues and the internal 'clock' – is present in virtually all organisms from complex mammals to unicellular cyanobacteria (412). The circadian rhythm plays a central role in maximizing the success of behaviours such as feeding and reproduction through the coordinated rhythmic expression of Period, RevErb, Bmal, and CLOCK genes, among others (413-415). Since the advent of microarrays, profiling temporal fluctuations in genes known to regulate circadian rhythm has revealed the complex interplay between environment, physiology and behaviour (412). The influence on the hypothalamus has profound implications on hormonal oscillations in blood cortisol, growth hormone and melatonin levels in humans (416). In fish, it has been shown that seasonal photoperiod is a key factor in physiological changes such as gonad size and fecundity, sex steroid level, LH level as demonstrated with female mummichogs (417). This study goes on to find that manipulation of photoperiods was able to artificially induce the spawning schedule of these fish with variable success. Similar experiments have been performed with trout to establish the importance of circannual rhythms (yearly patterns) in breeding (413). More recently, in an unpublished M.Sc. thesis, the role of photoperiod is such a strong determinant factor in reproductive success that unnatural and artificial 15-minute pulses of light were able to significantly reduce the spawning success in FHM (418). This experiment illustrates the fragile relationship between the light-dark cycle and the endogenous clock suggesting that both elements need to be in coordination to maximize spawning success in FHM. Although this physical phenomenon has been well characterized, the molecular aspects of the circadian rhythm remain unclear. The proteins encoded by RevErbα and β are nuclear receptors that play a role in the transcriptional regulation of other circadian genes such as Bmal1 (419, 420) and may have redundant functions (421). Regulation of Bmal1 is involved in the expression cycle of several genes within the circadian rhythm; consequently, its own expression follows

a cyclic pattern (421). Several studies have pointed to the apparent regulatory or pace-making role of RevErb in the circadian rhythm suggesting that it is not necessary for circadian function but only regulatory in nature (421-423). Therefore the RevErb genes may not be essential to the maintenance of a rhythm but nonetheless plays a role in phase-shifting or adjusting the system. Nonetheless, disruption of genes relating to circadian rhythm may have negative effects on reproduction. RevErbβ mRNA levels were decreased in some of effluent treatments (ABC, AKN, and EBE) but increased in DWN-exposed fish. Given the importance of the oscillatory levels on circadian rhythm genes such as RevErbβ, we hypothesize that any disruption (i.e. irregular expression) could be a possible mechanism of spawning inhibition.

We were unable to validate the result for Period homolog 4 (Per4), which requires some attention. The Per genes are a multi-gene family and Per1-4 have been found in teleosts (424, 425). Given the polyploidy of fish (248), and the nature of EST projects, it is possible that the EST identified as Per4 by the microarray analysis is, in fact, a different isoform or homolog, particularly given the degree of validation obtained with CCK, urotensin I, and RevErbβ.

It is well known that DA, acting through the D2 receptor at the level of the pituitary, is a potent inhibitor of LH release in fish (2, 3, 12, 69, 388-390). Previous studies by Popesku *et al.* (Chapters 3 and 4) suggest that DA, acting through the D1 receptor in the hypothalamus, may also have inhibitory effects on LH release and hence, reproductive success. We hypothesized that one or more effluents may be acting as a DA mimic by potentially causing a decrease in the expression of DAT, which would effectively allow for prolonged post-synaptic receptor stimulation by DA as it is not cleared from the synapse

(426) or by increasing the biosynthesis of DA through an increase in the expression of TH, the rate-limiting enzyme in the biosynthetic pathway (31). We thus cloned partial coding fragments for these genes in the FHM and examined their expression in effluent-exposed fish. However, there were no statistically significant changes in the expression levels of DAT or TH. Although these findings do not conclusively show any direct effect of effluents on TH and DAT, it does not rule out the possibility of DAergic disruption. The expression of these two genes simply represents two areas of the DA pathway (synthesis and transport); expression of other parts of signalling such as DA metabolism/catabolism, DA vesicularization, DA release and DA receptors may provide more insight into possible changes in the DA signalling. As well, analysis of neuroendocrine response five days from the onset of exposure may only reveal part of the response. Any response and/or recovery from PPME exposure prior to the fifth day may not be captured by these experimental methods. Future work may warrant a time-course exposure to determine the longitudinal responses to PPME exposure. Nevertheless, the results from our novel comparative genomics analysis provide strong evidence that PPMEs may contain substances modulating DA systems in the brain. We compared the microarray dataset for DSF-exposed FHM obtained in the current study against microarray datasets from previous experiments in our laboratory that investigated the actions of DA D1- and D2-specific agonists and antagonists. Interestingly, mRNAs encoding tubulins were found to be regulated by the same amount both treatments, although some in opposite directions. Chapter 5 discussed the effects of DA on the cytoskeletal structure. Steady-state mRNA levels for tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, β isoform (YWHAB), a member of the 14-3-3 family of proteins, were also affected in female fish exposed to the DSF effluent. This family of proteins mediates signal transduction by binding to

phosphoserine-containing proteins, which may lead to future work elucidating the mechanisms of PPME action on the neuroendocrine brain. Furthermore, an mRNA for ATF5-like transcription factor was identified as being increased in fish exposed to DSF. The role of DA on ATF1 was discussed in Chapter 5.

Taken together, the results presented in this study provide evidence that the PPMEs may be acting through some of pathways modulated by the catecholaminergic neurotransmitter DA. The identification of the neuroendocrine systems being affected by effluent exposure, including CCK, urotensin I, and RevErbβ, will help with future work with the industry-driven goal of reducing the ecological impact of the pulp and paper mill industry.

APPENDIX B

LIST OF MANUSCRIPTS NOT PRESENTED IN THIS THESIS

PUBLISHED OR ACCEPTED FOR PUBLICATION

Zhang, D., Xiong, H., Mennigen, J., **Popesku, J.T.**, Marlatt, V., Martyniuk, C.J., Crump, K., Cossins, A.R., Xia, X., Trudeau, V.L. Defining global neuroendocrine gene expression patterns associated with reproductive seasonality in fish. *Physiol Genomcis* 2009.

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Croteau, M.C., Davidson, M., Duarte, P., Wade, M., **Popesku, J.T.**, Wiens, S.C., Lean, D.R.S., Trudeau, V.L. Assessment of thyroid system disruption in *Rana pipiens* tadpoles chronically exposed to UVB radiation and 4-*tert*-octylphenol. *Accepted in Aquat Toxicol.*

Nagahuedi, S., **Popesku, J.T.**, Trudeau, V.L., Weber, J.-M. 2009. Mimicking the natural doping of migrant sandpipers in sedentary quails: effects of dietary n-3 fatty acids on muscle membranes and PPAR expression. *J Exp Biol* **212**:1106-1114.

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

Martyniuk, C.J., Gerrie, E.R., **Popesku, J.T.**, Ekker, M., Trudeau, V.L. 2007. Microarray analysis in the zebrafish (*Danio rerio*) liver and telencephalon after exposure to low concentration of 17alpha-ethinylestradiol. *Aquat Toxicol.* **84**:38-49.

APPENDIX C

SUPPLEMENTAL MATERIAL

The following supplemental files are on the enclosed CD with this thesis:

Table C.1: Chapter 3; List of mRNAs

Table C.2: Chapter 3; List of proteins

Table C.3: Chapter 4; List of mRNAs

Table C.4: Chapter 5; List of mRNAs

Table C.5: Appendix A; List of mRNAs