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Effects of the Aromatase Inhibitor Fadrozole on Gene Expression in the Zebrafish brain

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Effects of the Aromatase Inhibitor Fadrozole on Gene Expression in the Zebrafish Brain

by

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Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the M.Sc. Biology Degree in the Ottawa-Carleton Institute of Biology

University of Ottawa

Ottawa, Ontario

September 2008

Your file Votre référence
ISBN: 978-0-494-52358-2
Our file Notre référence
ISBN: 978-0-494-52358-2

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Abstract

Aromatase is responsible for the conversion of androgens to estrogens in the brain and gonadal tissues. Teleosts have been characterized as having high levels of brain estrogen biosynthesis. Little is known about the effects of estrogens on brain function in teleosts. The main objective of this study was to assess the effects of fadrozole, a powerful aromatase inhibitor, on gene expression in the zebrafish brain. A fadrozole exposure leads to a decrease in estrogens and an increase in androgens. In the telencephalon, 235 genes were identified by Affymetrix GeneChip analysis as being differentially regulated. Real-time RT-PCR and in situ hybridization were used to validate the data obtained from the microarrays. Collectively, the results provide a better understanding of the effects of aromatase inhibitors on gene expression and also shed light on the underlying effects of sex hormone variation and their importance in the brain of teleosts.

Résumé

L'aromatase convertit les androgènes en estrogènes dans le cerveau et les tissus gonadiques. Les teleosts ont un haut niveau de biosynthèse d'estrogènes dans le cerveau. Peu d'information est connue au sujet de ce haut niveau d'estrogènes sur le fonctionnement du cerveau. L'objectif principal de cette étude était de déterminer les effets causés par le fadrozole, un inhibiteur d'aromatase, sur l'expression des gènes dans le cerveau du poisson-zèbre. Le fadrozole cause une diminution d'estrogènes et une augmentation d'androgènes. À l'aide d'analyse Affymetrix GeneChip, 235 gènes ont été identifiés comme ayant subi un changement d'expression dans le telencephalon. Des analyses par RT-PCR en temps-réel et par hybridation in situ ont été utilisées pour valider les données obtenues des microréseaux. Les résultats fournissent une meilleure compréhension des effets causés par les inhibiteurs d'aromatase sur l'expression génique et aident aussi à comprendre les effets associés avec les variations d'hormones sexuelles chez les teleosts.

1. Introduction

1.1 Aromatase

Aromatase is part of the cytochrome P450 superfamily, and is also referred to as cytochrome P450, family 19 (*cyp19*). Within this superfamily, aromatase is presently the sole member of gene family 19, designated *cyp19*. The number associated with this gene family refers to the fact that it is the C_{19} angular methyl group which is targeted by the aromatase enzyme (Simpson et al., 1994). The cytochrome P450 proteins are monooxygenases and catalyze numerous reactions including drug metabolism and steroid synthesis. In the majority of vertebrate species examined to date, aromatase expression is mostly found in the gonads and the brain (Simpson et al., 1993). Fish and avian species have all been found to express aromatase, as do most mammals. The aromatase protein contains a heme-binding region and is located on the endoplasmic reticulum where it catalyzes the last steps of estrogen biosynthesis which are three successive hydroxylations of androgens. Associated with the aromatase protein is a flavoprotein, nicotinamide adenine dinucleotide phosphate (NADPH) – cytochrome P450 reductase, responsible for transferring reducing equivalents from NADPH to any microsomal form of cytochrome P450 (Simpson et al., 1994).

The principal function of aromatase is to convert androgens to estrogens. To date, two reactions have been identified; the conversion of androstenedione to estrone and the conversion of testosterone to estradiol (E2) (Fig. 1.) (Horton and Tait, 1966; Longcope et al., 1969).

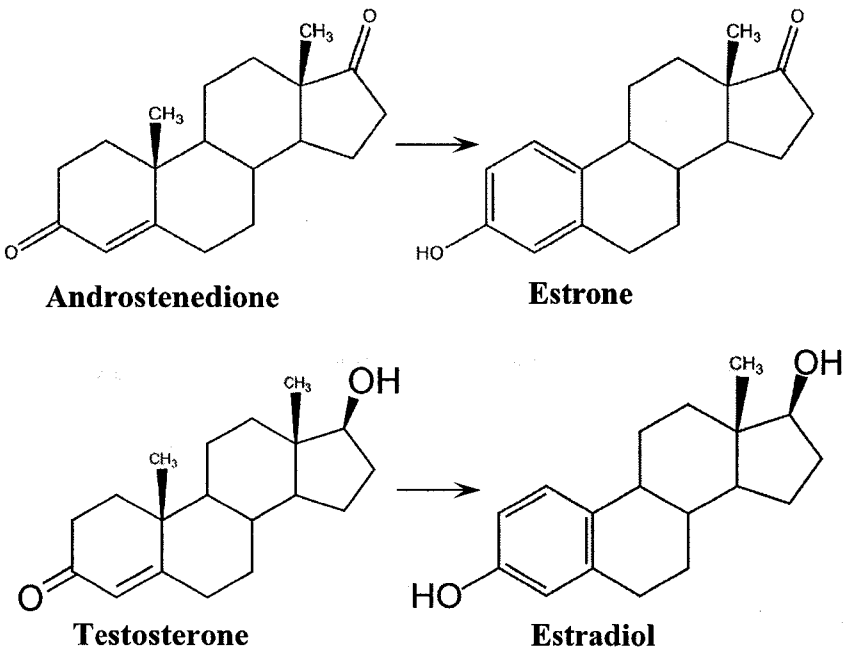


Figure 1. Conversion of andostenedione and testosterone into estrone and E2. Notice the aromatization of the A ring in the estrogens.

The aromatase reactions take place in the gonads and also in other aromatase expressing tissues, such as the central nervous system (Balthazart and Ball, 1998; Lephart, 1996). In the brain, multiple studies have shown that aromatase plays a crucial function in adults and in developing embryos (Bakker et al., 2002; Beyer, 1999; McCarthy et al., 2002; Milner et al., 2001; Wise et al., 2001). Most mammals and birds have a single aromatase gene, *cyp19*, with several tissue-specific promoters (Conley and Hinshelwood, 2001). Pigs have three active *cyp19* genes (Choi et al., 1997), and cattle have one active *cyp19* gene, plus a second *cyp19* gene, which is transcribed but is nonfunctional (Brunner et al., 1998). Most teleost fish, including zebrafish (*Danio rerio*), possess two aromatase genes due to a genome duplication in the actynopterygian lineage

(Blazquez and Piferrer, 2004). The two *cyp19* genes in teleosts have very distinct expression patterns. The *cyp19a* gene encodes for aromatase A and is mostly expressed in gonad tissues, while the *cyp19b* gene encodes for aromatase B, is mainly expressed in the brain (Chiang et al., 2001a; Kwon et al., 2001; Tchoudakova and Callard, 1998; Trant et al., 2001).

Multiple studies have shown that the brain of teleost fish exhibits very high aromatase activity (Gonzalez and Piferrer, 2003; Schlinger et al., 1999; Trant et al., 1997). Some studies have found that aromatase activity in teleosts can be 100 to 1000 times greater than that measured in mammals and birds (Callard et al., 1978; Pasmanik and Callard, 1985). The exact cellular location for this remarkable aromatase activity in the brain has been documented in a few teleost species. Aromatase activity in the brain of mammals and birds has been documented in neurons of the limbic and neuroendocrine systems (Balthazart and Ball, 1998). The anatomical distribution and cellular basis of aromatase activity was first described in 2001 by Forlano et al.. In zebrafish, and other teleosts, studies have shown that aromatase B is expressed exclusively in radial glial cells (Menuet et al., 2005). These aromatase B radial glial cells are mostly found through the forebrain bordering the ventricles of the anterior ventral regions (Menuet et al., 2005). In teleost species studied to date, aromatase B expression has been documented in the olfactory bulbs, the telencephalic area, the preoptic region, and the hypothalamic regions (Chiang et al., 2001a; Forlano et al., 2001; Menuet et al., 2003). This typical teleost expression pattern has also been confirmed in zebrafish using adult brains for whole-mount in situ hybridization with the aromatase gene, *cyp19b* (Chiang et al., 2001b).

In several teleost species, studies have shown that the aromatase B activity is up-regulated by E2, the product that it produces (Kishida and Callard, 2001; Kishida et al., 2001; Trant et al., 2001). Reverse transcription polymerase chain reaction (RT-PCR) showed that an E2 treatment of zebrafish embryos caused an over expression of the *cyp19b* transcript. Furthermore, a co-treatment using E2 and an estrogen receptor antagonist (ICI) blocked the *cyp19b* induction. This would suggest that estrogen receptors (ERs) are involved in the activation of *cyp19b* (Kishida and Callard, 2001). Additionally, studies in the goldfish and zebrafish have characterised estrogen response elements (EREs) which would suggest a direct link of ERs on the transcriptional regulation of *cyp19b* (Callard et al., 2001; Kazeto et al., 2001; Tchoudakova et al., 2001)

1.2 Estrogens

Estrogens, such as E2, are potent sex steroids which play an important role in development, differentiation and homeostasis of many target organs (Pellegrini et al., 2005). Estrogens are known to influence the expression of a wide variety of genes (Gruber et al., 2002). In the brain, E2 is essential for organizing and regulating effects on the neuroendocrine system which controls reproductive functions (Beyer, 1999). The role of E2 is not limited to reproductive functions. It had been well documented that E2 is also important for cognition, motor skills, memory, mood, and pain (Evrard and Balthazart, 2004). E2 is present in the brain of vertebrates from the developmental stages until adulthood and is known to also influence neuronal differentiation, survival and plasticity (Brann et al., 2007; Garcia-Segura et al., 2001; Suzuki et al., 2006; Toran-Allerand, 2004). Estrogens are considered as important neurotrophic and neuroprotective

factors (Behl, 2002). In other words, E2 plays an important role in the maintenance, survival and protection of neural tissues.

As previously mentioned, aromatase activity ultimately leads to the formation of estrogens. Therefore, aromatase directly participates in the regulation of processes controlled by estrogens (Ankley et al., 2002). Estrogens act through genomic mechanisms involving specific nuclear receptors, more specifically ERs. Teleost fish were found to possess a third ER. Most vertebrate have two ERs, *ERα* and *ERβ*, whereas teleosts have an additional ER, known as *ERγ* (Hawkins et al., 2000). Subsequent studies have shown that the third ER results from a duplication of the *ERβ* gene early in the teleost lineage (Bardet et al., 2002; Menuet et al., 2002). In the brain of teleosts, these receptors are expressed mostly in the neuroendocrine regions, such as the preoptic area in the hypothalamus and the ventral telencephalon (Andreassen et al., 2003; Anglade et al., 1994; Hawkins et al., 2000; Menuet et al., 2002). Regions with the highest expression of aromatase B also show the highest expression of ER (Menuet et al., 2003; Menuet et al., 2002). As a result, aromatase B-expressing radial cells in the telencephalon are ideally positioned to release E2 in proximity of these ER-expressing cells.

The zebrafish *cyp19b* promoter region has been recently characterized (Kazeto et al., 2001; Tong and Chung, 2003). A search for potential regulatory elements in the *cyp19b* gene promoter region identified an ERE at nt-349 identical to the perfect palindrome in *Xenopus laevis* vitellogenin A2 gene (Klein-Hitpass et al., 1988). Downstream of this ERE, an additional binding site containing an ERE half-site and a variant ERE were found (Tchoudakova et al., 2001). Further studies have demonstrated the functionality of these EREs and the direct implication of ERs (Pellegrini et al., 2005).

These studies have shown that estrogens directly control aromatase B expression via ERs and EREs in the *cyp19b* promoter region (Tchoudakova et al., 2001). However, the possibility that *cyp19b* expression can be modulated post-transcriptionally or indirectly by estrogens acting through other factors or pathways cannot be excluded.

E2 has long been considered to be the typical 'female' hormone, whereas testosterone is considered to be the 'male' hormone. However, these two hormones are present in both sexes. E2 it is now considered, in both males and females, as a hormone exhibiting neurotrophic and neuroprotective functions which are vital for neuronal development, survival and plasticity throughout life (Garcia-Segura et al., 2001; Garcia-Segura et al., 2003; Saravia et al., 2006). Therefore, sexual differences are not qualitative differences, but rather quantitative differences in hormone concentration and expression of steroid hormone receptors. In the male of many species, including humans, estrogens are present at low concentrations in the blood, but can be extraordinarily high in the semen, generally higher than the serum concentration of estrogens found in females (Free and Jaffe, 1979; Ganjam and Amann, 1976). Teleost fish, on the other hand, can produce and maintain incredible amounts of estrogens, particularly in the brain. This feature has been correlated with the high expression of aromatase B. Despite the fact that this has been well documented for quite some time, the significance of the high aromatase/estrogen activity is still not clearly understood (Pasmanik and Callard, 1985; Pellegrini et al., 2005).

Another interesting characteristic of the teleost brain is its enormous potential for neurogenesis. Adult neurogenesis in teleosts occurs in multiple regions, which are homologous to neurogenic regions in mammals. In addition, neurogenesis in teleosts can

also occur in numerous other regions throughout the brain (Adolf et al., 2006; Grandel et al., 2006; Pellegrini et al., 2007; Zupanc et al., 2005). These recent studies suggest that the high levels of estrogens, in teleost brains, could be involved in cell proliferation and neurogenesis. Radial glial cells have been characterized as the source of all brain neurons during development and in adults (Gotz and Barde, 2005). This would further support the role of aromatase and estrogens in neurogenesis since aromatase B expression in adults is only found in radial glial cells. These findings suggest that the brains of adult fish are capable of reparative neurogenesis and that aromatase and estrogens are directly involved in this process.

1.3 Androgens

Androgens, such as testosterone, play an essential role in vertebrates. Androgens are involved in many sexual processes such as sex differentiation, sexual maturation and spermatogenesis. Studies have shown that androgens act as neuroprotectants and neuromodulators during brain development and in the adult brain. The importance of androgens have also been shown on the systems involved in cognitive function, mood disturbance and central drive of sexuality (Janowsky, 2006; MacLusky et al., 2006). Androgens generally exert their actions through specific androgen receptors (ARs) (Takeo and Yamashita, 2000). The mechanism of ARs have been well documented in mammals but have only been described in a few teleost species (Olsson et al., 2005; Sperry and Thomas, 1999a; Sperry and Thomas, 1999b; Sperry and Thomas, 2000; Wilson et al., 2004).

In teleost fish, ARs reportedly mediate the actions of testosterone and 11-ketotestosterone. Similar to the ERs, ARs are able to bind and interact with DNA sequences called androgen response element (ARE), which are generally located upstream in promoter sequences of target genes (Berrevoets et al., 2002; Gobinet et al., 2002; Sultan et al., 2001). In some mammalian species, androgens have been found to regulate the expression of the AR transcript (Burnstein et al., 1995; Dai et al., 1996). Further studies found that the promoter and 5'-flanking sequences of the AR gene contained functional AREs (Blok et al., 1992). AREs were discovered and characterized more recently than EREs and consequently are not well studied. Similar to ERs and EREs, ARs and AREs may also influence the expression of genes through other mechanisms in absence of specific DNA binding either using receptor interaction with other transcription factors (Kallio et al., 1995) or through alterations in the messenger RNA stability (Liao et al., 1989).

1.4 Aromatase Inhibitors

Over the past few years, many concerns have been raised about chemicals found in the environment and their possible affects on the endocrine system of wildlife and even humans. In addition to regulating sexual functions and reproduction, the endocrine system is involved in regulating many other processes including development, metabolism and growth. Androgens and estrogens are key messengers in the endocrine system. The homeostatic balance between these two hormones is regulated by aromatase. Therefore, chemicals that affect aromatase activity can be detrimental to the endocrine

system. As previously mentioned, both males and females are susceptible to hormonal changes since androgens and estrogens are essential for both sexes.

Aromatase inhibitors (AIs) were first discovered in the 1970s as an active treatment for women with estrogen-dependent advanced breast cancer (Smith, 1999). The first AI produced was aminoglutethimide, although clinical trials concluded that it lacked specificity and had many adverse side effects. Since the arrival of this new drug, a series of much more potent and specific AIs have been developed. Some of these new drugs can be up to 10 000 times more potent than aminoglutethimide *in vivo*, with no evidence that other steroid pathways are affected (Smith, 1999). Two major groups of AIs were developed: inhibitors that utilize steroidal substrates that compete for the aromatase enzyme, and competitive inhibitors that are non-steroidal (Santen, 1993). One of the non-steroidal AI is fadrozole, which inhibits aromatase activity by direct binding to the heme group (Goss et al., 1986). Fadrozole is a derivative of imidazole, which is often used as building blocks for new, more complex molecules (Steele et al., 1987). Studies conducted in rats and humans, have shown that fadrozole is approximately 300 times more potent than its predecessor, aminoglutethimide (Steele et al., 1987).

Inhibiting aromatase activity has been shown to be an effective technique to reduce estrogens in post-menopausal women who have estrogen-dependent advanced breast cancer (Santen et al., 1990). Considering that over one-third of breast cancers are estrogen-dependent, reducing endogenous estrogens has become an effective second-line therapy in patients with ER positive breast cancer (Santen et al., 1981).

A study conducted in 2000 looked at the effects of commonly used pesticides on the aromatase activity in human placental microsomes (Vinggaard et al., 2000). They

were able to identify 7 pesticides out of the 22 tested, which significantly reduced the expression of aromatase. Various fungicides also have the ability to reduce aromatase activity (Mason et al., 1987). These results show that further research is needed to identify more potential endocrine disrupting chemicals and their negative effects on humans and wildlife. No studies, to date, have attempted to identify the presence of AIs in aquatic environments. The gaining popularity of AIs for breast cancer treatment and their use in pesticides and fungicides, however, would suggest that aromatase reducing compounds may be present, or may soon be introduced in aquatic environments.

Various studies have looked at the effect of AIs on fish. A group from Japan studied the effects of fadrozole in the gonadal development of juvenile zebrafish (Uchida et al., 2004). They observed gonadal masculinisation when fadrozole was administered to genetically female juveniles 40 days post-hatching. Sex-reversals were confirmed by identifying testes in all sex-reversed genetic females. Similar results have been observed in other fish species. In the Japanese flounder and tilapia, treatments of fadrozole during sex differentiation also caused a sex-reversal from genetic females to phenotypic males (Kitano et al., 2000; Kwon et al., 2000).

Another interesting study looked at the effects of fadrozole in a reproduction assay using fathead minnows (*Pimephales promelas*) (Ankley et al., 2002). Fish were exposed to various concentrations of fadrozole (2, 10 and 50 μg/L) for 21 days. The number of eggs spawned was monitored before and during the exposure. The authors observed a quick response for all concentrations of fadrozole. There was a significant decrease in the number of eggs produced, and in some cases, where higher concentrations of fadrozole were used, the fish completely stopped spawning.

Aromatase activity was measured in the brain tissues of the controlled and treated fish. An approximate 4-fold decrease in aromatase activity for male and female fish was observed. Hormones were also measured during the experiments. In the female fish exposed to fadrozole, a significant decrease in E2 was observed. In the male fish exposed to fadrozole, a significant increase in testosterone was observed (Ankley et al., 2002). The studies presented are a clear indication that AIs have a serious impact on hormonal levels, sexual development, sexual functions and reproductive processes.

1.5 Zebrafish Sex Determination and Differentiation

The sex of an organism is often established by the presence or absence of a sex specific chromosome. In some fish species, a sex specific chromosome system is present and plays an important role in sex determination. For example, the salmonid family has heterogametic males (XY) and homogametic females (XX) which is similar to the mammalian system (Phillips and Ihssen, 1985; Thorgaard, 1977; Thorgaard, 1978). Other fish species have homogametic males and heterogametic females which is similar to the sex determination system in birds (Volff and Schartl, 2001). Sex determination can also be influenced by the surrounding environment. Water temperature can directly influence the sex of certain fish species during the development of embryos (Conover and Heins, 1987). In zebrafish, the underlying mechanisms regulating sex determination and differentiation have not been characterized. However, a number of genes have been linked to the processes involved in sex determination and differentiation in zebrafish. These genes have not been shown to be sex-linked; therefore it is not likely that any of these genes is the lone factor involved in sex determination in zebrafish. Nevertheless,

the expression patterns and regulatory mechanism controlling these genes would seem to indicate that they are part of a complex system responsible for the sex determination in zebrafish.

One of the genes involved in sex determination and differentiation in zebrafish is the *fushi tarazu* factor-1 (*FTZ-F1*) gene (Chai and Chan, 2000). This gene was first identified in *Drosophila* and is the key regulator of the *fushi tarazu* gene expression, which acts as a central factor for segmentation (Lavorgna et al., 1991; Ueda et al., 1990). *FTZ-F1* homologues have been identified in teleost species including zebrafish (Chai and Chan, 2000; Liu et al., 1997; von Hofsten et al., 2002). Many teleosts have multiple copies of the *FTZ-F1* gene although its specific function is not completely understood. Studies conducted so far would seem to indicate that it is involved in the reproductive axis and also plays an essential role in steroidogenesis.

Steroidogenesis, sex determination and differentiation are directly associated to each other. The steroid biosynthesis pathway produces androgens and estrogens and the balance between these two sex hormones is very precise. Shifts in this balance leads to the development of the appropriate sex characteristics. As previously mentioned, the aromatase enzyme directly controls the balance between these two sex hormones. *Cyp19a* is expressed in the gonads whereas *cyp19b* is expressed mostly in the brain. This would suggest that *cyp19a* may be involved in gonadal development, whereas *cyp19b* may be involved in neuronal development. Both genes play important roles in estrogenic responses, suggesting that they somehow influence sex differentiation. The regulatory processes controlling *cyp19* in teleosts are not completely elucidated. As previously mentioned, *cyp19b* has been confirmed to be under the control of EREs. The regulatory

region for *cyp19a* was recently characterized, and was found to contain binding sites for *FTZ-F1* which would suggest an important function for this transcription factor in the regulation of *cyp19a* expression in the gonads (Tong and Chung, 2003; Watanabe et al., 1999). In many vertebrates, aromatase activity determines the sex of a organism during development (Jeyasuria and Place, 1998; Wibbels et al., 1998). Aromatase activity is influenced by temperature. Therefore, the temperature surrounding the developing embryos influences aromatase activity, ultimately leading to different variations in sex ratios (Bogart, 1987). Similar scenarios have been documented for many teleosts, including zebrafish, which would suggest that aromatase plays an important role during sex determination and differentiation.

1.6 17α-Ethinylestradiol

17α-ethinylestradiol (EE2) is a pharmaceutical estrogen which is the main active ingredient found in oral contraceptives and hormone replacement therapy. Many studies have documented its effects on wildlife. Even at the low concentrations detected in sewage effluent, EE2 has serious biological effects in fish, such as disrupting reproductive functions and inducing plasma vitellogenin (*vtg*) (Islinger et al., 2003; Miracle et al., 2006; Sumpter and Jobling, 1995). *Vtg* is an egg yolk precursor protein normally expressed in female fish. In the presence of estrogenic endocrine disruption chemicals this protein is up-regulated in both male and female fish.

In a recent study, researchers used environmentally relevant nominal concentrations of EE2 to study its effects on zebrafish gene expression in the liver and telencephalon (Martyniuk et al., 2007). In the liver, microarray analysis and real-time

RT-PCR identified typical biomarkers for an estrogenic exposure, such as *vtg1* and *3* and *ERα* as being significantly up-regulated. In the telencephalon, many regulated genes were identified by microarray analysis; however real-time RT-PCR did not statistically confirm the changes in expression of the selected genes. A possible explanation for this result is that the brains of teleost fish naturally have high levels of estrogens. Therefore, exposing fish to a low concentration of an estrogenic compound would not significantly effect gene expression in the brain simply because the effects of the estrogenic compound are being masked by the already high levels of estrogens normally present in the zebrafish brain. The authors were able to conclude that the EE2 exposure had significant effects on gene expression in the liver, but did not significantly effect gene expression in the telencephalon. This study further confirmed the deleterious effects of estrogenic endocrine disrupting chemicals. Additional studies are needed to identify the effects of endocrine disrupting chemicals on gene expression in the teleost brain.

1.7 Statement of Problem and Objectives

During the last few decades, it has become increasingly obvious that a number of environmental substances can cause adverse health effects to animals resulting from changes in endocrine functions. Most attention given to endocrine disrupting chemicals have focused on compounds that interact with ERs. However, it has been well documented that the endocrine system can also be altered by environmental substances through non-ER-mediated pathways and mechanisms. Proper functioning of the endocrine system can be effected by xenobiotics that, in turn, affect the metabolism of sex steroid hormones. The biosynthesis of hormones provides enzymatic targets for the

endocrine system, especially the steps catalyzed by the aromatase enzyme. As previously mentioned, alteration in steroidogenesis is associated with various harmful effects, particularly the disruption of the reproductive system.

The purpose of this study was to determine the effects of the AI and endocrine disrupting chemical, fadrozole, on gene expression in the zebrafish brain. AIs have long been studied for their detrimental effects on the endocrine system. The underlying effect on gene expression remains to be determined. To date, only one very recent study has examined the transcriptional response to an AI. This study will be further discussed in section 4. More specifically, the analysis will be conducted in the hypothalamus and the telencephalon, which contains the preoptic area. These regions have been found to contain the most abundant aromatase expressing cells, and are major targets for locally produced E2 which is most likely involved in controlling the expression of genes implicated in neuroendocrine regulations.

As previously documented, exposing fish to fadrozole will lead to a significant decrease in estrogens, notably E2, as well as a significant increase in androgens, notably testosterone. This exposure will ultimately produce the opposite effects of the EE2 exposure mentioned in section 1.6. Instead of increasing estrogens, fadrozole will deplete them. Other than assessing the transcriptional response to fadrozole, this exposure will also depict the importance and effects of aromatase expression and estrogens in the teleost brain. Even though it is well known that the brains of teleost fish contain surprisingly high levels of aromatase activity and estrogens, the exact purpose of these high levels are not fully understood. Some hypothesise that, in teleosts, they are associated with adult neurogenesis and brain repair. A recent study was conducted to

shed light on this subject. They were able to demonstrate for the first time, that aromatase-positive radial glial cells actively divided to generate new cells in various regions of the zebrafish forebrain (Pellegrini et al., 2007). They also later documented that some of the newly produced cells, derived from radial glial cells, ultimately differentiated into neurons.

Male zebrafish were used for the fadrozole exposure. Some species of teleost fish only breed once per year. Zebrafish were chosen because they have the ability to spawn at a few days interval. This is very advantageous for this type of study, allowing the experiments to be conducted at any time of the year. On the other hand, some fish, such as the goldfish, only spawn once per year. This would restrict the experiments to a small period of time where aromatase and sex hormones are at their peaks. Males were chosen for this study, to reduce the sex hormone variations within a gender. Male zebrafish have relatively constant levels of sex hormones and aromatase activity, whereas in female zebrafish, sex hormones and aromatase activity can have slight variations depending on age, ovulation and spawning (Kallivretaki et al., 2007).

The main objective of the research was to identify the genes affected by an exposure to an AI. Male zebrafish were exposed to fadrozole, at a concentration of 200 μg/L for a period of 10 days. This concentration and exposure period were determined after considering previous studies. The total RNA, isolated from the telencephalon, was used for an Affymetrix GeneChip zebrafish genome array experiment, to study gene expression of over 14 900 transcripts. These microarray experiments allowed the identification of genes that were unaffected by the treatment, and more interestingly, allowed the identification of genes that were up-regulated and down-regulated. Real-time

RT-PCR was used to validate the data obtained from the microarrays and to assess the transcriptional response in the hypothalamus.

An additional objective was to compare the expression patterns, within the brain, of some of the genes affected by the exposure. The fish were exposed to fadrozole and sacrificed. Their brain was then removed and sectioned using a cryostat. In situ hybridizations were preformed in order to visualise the spatial distribution, within the brain, of certain genes targeted by the fadrozole exposure. The effects of fadrozole on gene expression were also visualised by comparing the expression patterns of treated and control brains. Analysis through in situ hybridization strengthens the results obtained by real-time RT-PCR and ultimately further validates the microarray data.

Exposure to fadrozole leads to the opposite effects of an EE2 exposure, regarding E2 levels. It was therefore hypothesised that some of the regulated genes identified in the EE2 study would also be regulated for the fadrozole exposure. However, the genes identified in the fadrozole study would most likely be regulated in the opposite direction. This can be explained by the fact that E2 has the ability to act through ERs and regulate expression with the help of EREs. Consequently, if a particular gene is up-regulated when E2 levels are increased, this same gene should be down-regulated when E2 levels are decreased. For these reasons, it was further hypothesised that genes containing EREs or AREs in their promoter regions would be regulated by the fadrozole exposure.

This study enables the identification of genes that are directly and/or indirectly controlled by estrogens and androgens which help demonstrate the pronounced effects of endocrine disrupting chemicals on gene expression in the brain of teleosts. This study

also provides insight into the importance of estrogens and androgens, sex hormone-regulated genes, and the delicate balance that exists in sex hormone metabolism.

2. Materials and Methods

2.1 Oligonucleotides

2.1.1 Real-Time RT-PCR

Table 1. Primers for real-time RT-PCR.

Name	Targeted Gene	Sequence (5'→ 3')
SLC2A10 L	Solute carrier family 2 member 10	GGGTTTCGGGATTGTTAAGG
SLC2A10 R	Solute carrier family 2 member 10	AAGTGCATCGTTTCGTTGTG
CEL L	Carboxyl ester lipase tandem duplicate 2	TCTCGCTGGGAAAGTGAAG
CEL R	Carboxyl ester lipase tandem duplicate 2	TATCAAGGGTGGCAAAGAGG
FABP2 L	Fatty acid binding protein 2, intestinal	CCGCAATGAGAACTACGAGA
FABP2 R	Fatty acid binding protein 2, intestinal	AAGGTGACGCCCAGAGTAAA
CRY2B L	Cryptochrome 2b	TTCTTCCAGCAGTTCTTCCAC
CRY2B R	Cryptochrome 2b	GCGTTCCAGGGGTCATAA
OPS L	Opsin 1, long-wave-sensitive, 1	GGAAAGAAGGTGGATGATGG
OPS R	Opsin 1, long-wave-sensitive, 1	CAGTGTGGGTGAACAAGGA
HOXA11 L	Homeo box A11a	GATTCAGTCCACAAGGAGTGTT
HOXA11 R	Homeo box A11a	GCAGATGTCGTGTTCGATGT
OR22 L	Odorant receptor, family 2, member 2	CAAAAGATCGGCACCAGTTAG
OR22 R	Odorant receptor, family 2, member 2	CACACATTCAGTCCCAGCA
HOXA13 L	Homeo box A13b	GCGTCTTTACTCCTCCATTC

HOXA13 R	Homeo box A13b	ATTGCCTCCCACAAATCCT
ART L	Arrestin 3, retinal (X-arrestin)	CAGCGGACAAATACCACAAA
ART R	Arrestin 3, retinal (X-arrestin)	CAGAAGTGTTGAAGAAGCCAAG
CYP1B1 L	Cytochrome P450 family 1 subfamily B polypeptide 1	GACCCGACTTTGATTCATTC
CYP1B1 R	Cytochrome P450 family 1 subfamily B polypeptide 1	TCGCTTACGATGTGCTTCTC
PDC1 L	Phosducin 1	AGAAGAGAATGCCGATGTGG
PDC1 R	Phosducin 1	CTTGAGGTGTGCGTGTTTGT
CYP19A L	Cytochrome P450 family 19 subfamily A polypeptide 1b	TGGAATGTTAGGCCTGCTGTG
CYP19A R	Cytochrome P450 family19 subfamily A polypeptide 1b	CCTCATCGCCACTTTAGGTTT
RPL8 L	Ribosomal protein L8	TTGTTGGTGTTGTTGCTGGT
RPL8 R	Ribosomal protein L8	TCTCCTGATGGTTGAGGGTT

2.1.2 Probes for In Situ Hybridizations

Table 2. Primers for in situ hybridization.

Name	**Targeted Gene**	**Sequence (5'→ 3')**
CYP1B1 IN L	Cytochrome P450 family 1 subfamily B polypeptide 1	CTTCATTCCGGTTCGTGTCT
CYP1B1 IN R	Cytochrome P450 family 1 subfamily B polypeptide 1	AGTACTGCATCCAGGGCATA
CRY IN L	Cryptochrome 2b	CGTACCAAACGCTTCCAGACA
CRY IN R	Cryptochrome 2b	AAGCGAAGGTACGGACTCAA
CYP19A IN L	Cytochrome P450 family19 subfamily A polypeptide 1b	GCAGCCCAGACTGGTTACTC
CYP19A IN R	Cytochrome P450 family19 subfamily A polypeptide 1b	GGGTTACACCCCTACGCTTT

2.2 Experimental Design and Fadrozole Exposure

Adult zebrafish were purchased from a local pet shop. The fish were acclimated and observed for health for 3 months prior to the exposure. The temperature was kept at 28°C with a light-dark cycle of 14h:10h and the fish received a standard zebrafish diet (Westerfield, 1994). Adult males were sorted from females according to size, body shape and coloration. Adult males were placed in 39.5 L frameless glass exposure tanks (50.3 cm x 25.3 cm x 30 cm) (Hagen, Canada) containing 30 L of water oxygenated by an air stone. Each aquarium contained no more than 30 fish. A stock of fadrozole (Novartis Pharmaceuticals, Canada) was diluted in double distilled water (ddH_2O). Males were exposed to fadrozole at a concentration 200 µg/L for a period of 10 days. Control males received an equivalent volume of ddH_2O. Tanks were cleaned every 2 days and filled with clean water containing a dose of fadrozole or ddH_2O. After 10 days, zebrafish were removed from the exposure tanks and anaesthetized with MS222 (ethyl 3-aminobenzoate methanesulfonate salt; Sigma–Aldrich, St.Louis, MO). Blood was collected from the caudal artery/vein of each fish with a heparinised microhematocrit tube and pooled (approximately 20 samples/tube). Plasma was isolated by centrifugation at 7500 rpm at 4°C for 15 minutes and stored at -80°C until used for radioimmunoassay assay. The fish were then sacrificed, the sex was verified, and the telencephalon and hypothalamus were dissected and pooled (approximately 30 tissues/tube) and flash frozen on dry-ice. Samples were stored in RNA*later*® (Qiagen, Mississauga, Ont., Canada) overnight at -20°C. Tissues were then removed from the RNA*later*® and stored at -80°C until total RNA extraction.

2.3 Steroid Extraction and Radioimmunoassay

Steroids were extracted from plasma by shaking aliquots with 5 mL of diethyl ether. After freezing the aqueous phase in an acetone/dry ice bath, the organic phase was decanted and dried. The dry extracts were resolubilized in assay buffer and frozen at – 20°C for later use. A radioimmunoassay was used to measure the amount of steroids described by McMaster et al. (McMaster et al., 1992). Percent cross reaction of antigen with the steroid of interest was >99% (Medicorp Inc., Quebec, Canada). SigmaStat (Systat Sofware Inc.) was used to determine data distribution. Unpaired Student T-test was used to assess significant differences (p-value ≤ 0.05) between the control and treatment group.

2.4 RNA Extraction

Total RNA was extracted using RNeasy® Mini Kit (Qiagen, Mississauga, Ont., Canada) as per the manufacturer's protocol. The RNA samples were precipitated with sodium acetate/ethanol and resuspended in 10 µL of nuclease-free water and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA quality was assessed using a Bioanalyzer (Agilent). All samples had an rRNA ratio (28S/18S) greater than 2. RNA samples were then stored at -80°C.

2.5 Microarray Analysis

Affymetrix GeneChip® Zebrafish Genome Arrays containing approximately 15 000 *Danio rerio* gene transcripts were used. Probe sets on the arrays were designed with 16 oligonucleotide pairs to detect each transcript. Oligonucleotide probe lengths were

25-mer. Independent hybridizations of three biological replicates were performed for the control and treatment group. Approximately 10 μg of total RNA was converted to biotin-labelled cRNA using Gene Chip One-Cycle Target labelling kit and hybridized to the zebrafish genome array according to Affymetrix Standard Protocol (Affymetrix, Santa Clara, California, United States). Labelling and microarray hybridizations were performed by StemCore Laboratories (Ottawa Health Research Institute, Ottawa, Canada).

2.5.1 Data Analysis

Data analysis was done using GeneSpring GX (Agilent). Data normalization was done using per-chip-normalization where the signal of each gene is divided by the median intensity of the chip. Per-gene-normalization was also done, which accounts for the difference in the detection efficiency between spots. A cross gene error model (one-color data) was also used, which estimates measurement precision by combining variability of gene expression data. This model was based on "Deviation from 1" which assumes that a general-purpose array was used and that most genes have little biological variability. The data were then filtered on fold change with a fold difference cut off of 2. Statistical analysis was then performed using one-way analysis of variance (ANOVA). Genes were considered differentially expressed when the fold change was ≥ 2 with a p-value ≤ 0.05. Data was corrected for multiple comparisons using the Benjamini and Hochberg approach. Gene Ontology (GO) analysis for the differentially expressed genes was done using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/). Due to the lack of annotation of the zebrafish

transcripts, accession numbers were converted to mouse (*Mus musculus*) accession numbers for the DAVID analysis. This conversion was accomplished by the DAVID program and the protein domain composition were manually verified for each conversion. The biological rationale behind the conversion is that homologous proteins have similar functions and are also involved in similar biological processes.

2.6 Real-Time RT-PCR

Approximately 2 μg for each sample of DNAse-treated total RNA from the telencephalon and hypothalamus was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) for gene validation using real-time RT-PCR. Reverse transcription was carried out following the manufacturer's protocol. All primers were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Invitrogen. Optimal conditions for primers were specified in Primer 3, and included an amplicon of 100-250 bp, a primer size of 20-mer (±2), primer melting temperature of 60°C (±1), primer GC content of 50% (±5), maximal self complimentary of 3 and a maximal 3' self complimentary of zero. Primers used for real-time RT-PCR are listed in section 2.1.1 table 1. Amplicons for each primer set were cloned and sequenced to confirm specificity. Each real-time RT-PCR reaction contained the following: 25 ng first strand cDNA template, 1x Absolute QPCR SYBR Green (Thermo Fisher Scientific Inc.) which contained the Taq enzyme, 200 nM gene specific primer and 100 nM ROX reference dye, in a final volume of 25 μL.

The accumulation of PCR product was measured via SYBR Green fluorescence using the Mx3000P® QPCR System (Stratagene). The thermal cycling parameters were

an initial 1 cycle Taq activation at 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds, 58-60°C for 5 seconds, 72°C for 30 seconds, and a detection step at 80°C for 8 seconds. Dilutions of cDNA 1:10 to 1:12 500(50 ng to 0.016 ng) from each sample were used to construct a relative standard curve for each primer set. After the reaction was complete, a dissociation curve was produced starting from 55°C (+1°C/30 seconds) to 95°C. Data were analyzed using the MX4000 Software Package. Standard curves relating initial template copy number to fluorescence and amplification cycle were created and used to calculate the relative mRNA copy number in each sample.

Ribosomal protein L8 (*rpl8*) was used as the endogenous reference gene. This gene was validated as an appropriate internal control, housekeeping gene, for use in expression profiling using real-time PCR when studying the effects of environmental estrogens in fish (Filby and Tyler, 2007). The Filby and Tyler internal control study had been done in the liver and gonads. *Rpl8* expression in the telencephalon and the hypothalamus was verified in this present study and was not found to be significantly affected by the fadrozole treatment. Real-time RT-PCR data are expressed as mRNA levels relative to *rpl8*. SigmaStat (Systat Sofware Inc.) was used to determine data distribution. Unpaired Student T-test was used to assess significant differences (p-value ≤ 0.05) between the control and treatment group. The biological replicate for real-time RT-PCR was three and sample were ran in triplicates.

2.7 In Situ Hybridization

2.7.1 Preparation of Digoxygenin RNA Probe

All primers were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Invitrogen. Optimal conditions for primers were specified in Primer 3, and included an amplicon of 250-400 bp, a primer size of 20-mer (±2), primer melting temperature of 60°C (±1), primer GC content of 50% (±5). Primers used for in situ hybridizations probe generation are listed in section 2.1.2 table 2. Amplicons for each primer set were cloned and sequenced to confirm specificity. 10 μg of DNA (vector containing amplicon of interest) was linearized overnight in a final volume of 100 μL. Samples ran on a 1% agarose gel and were gel extracted using MinElute Gel Extraction Kit (Qiagen Mississauga, Ont., Canada). The DNA samples were then precipitated using sodium acetate/ethanol and resuspended in 1 μL of ddH_2O. The probe was synthesized for 2 hours at 37°C using 1 μg of linearized DNA, 1x Dig RNA labelling mix (Roche), 1x RNA transcription buffer (Roche), 20 units RNA polymerase T7 (Roche), 20 units RNAse inhibitor (Roche) in a final volume of 20 μL. DNA was removed using 2 units of DNase I (RNase-free) (Roche) for 15 minutes at 37°C. The probe was the precipitated using lithium chloride/ethanol and resuspended in 20 μL of nuclease-free water. Probe quality when the assessed using a 0.8% agarose gel.

2.7.2 Sample Preparation and Cryostat Sectioning

Whole brains were carefully removed from the exposed fish (described in section 2.2), and placed in cold 4% paraformaldehyde (PFA) / 1x phosphate buffered saline (PBS) overnight at 4°C. Brains were then removed and placed overnight in 10% sucrose

/ 1x PBS at 4°C. Brains were then carefully orientated and frozen in optimal cutting compound (OCT) embedding medium and sectioned at 10 μm onto Fisherbrand Superfrost/Plus slides. Slides were dried from approximately 30 minutes and stored and -20 °C.

2.7.3 Hybridization and Staining

Slides were removed from -20 °C and dried for approximately 15 minutes. The probe was diluted 1:200 in hybridization buffer (200 mM NaCl, 9 mM Tris HCl, 5 mM $NaH_2PO4 \cdot H_2O$, 2 mM Na_2HPO_4, 5 mM EDTA, 50% deionized formamide, 10% dextran sulphate, 1 mg/mL yeast tRNA and 1x Denhardts in ddH_2O). The diluted probe was then denatured at 70 °C for 15 minutes and added to each side. Slides were then covered with cover slips and hybridized overnight at 65 °C in a chamber containing 50% formamide in H_2O.

Slides were washed for 15 minutes at 65 °C in solution A (1x SSC, 50% formamide 0.1% tween 20) to allow cover slips to fall off. Slides were then washed 2x 30 minutes at 65 °C in solution A and 2x 30 minutes in 1x Tris-Buffered Saline Tween-20 (TBST) (1.4 M NaCl, 27 mM KCl, 0.25 M Tris HCl pH 7.5 and 1% tween 20 in ddH_2O) at room temperature. Blocking was done using 10% heat-inactivated calf serum in 1x TBST for 90 minutes at room temperature. Antibody staining was done using anti-Dig AP Fab fragment (Roche) diluted 1:2000 in 10% heat-inactivated calf serum in 1x TBST. Slides were then covered with cover slips and incubated overnight in a humidified chamber at 4°C.

Slides were washed 4x 20 minutes in 1x TBST at room temperature. Cover slips were removed after the first wash. Slides were then washed 2x 10 minutes in 1x NTMT (100 mM NaCl, 100 mM Tris HCl pH 9.5, 50 mM $MgCl_2$ and 0.1% tween 20 in ddH_2O) at room temperature. Staining was done in the dark at room temperature with 225 µg nitroblue tetrazolium in 70% dimethylformamide + 175 µg 5-Bromo-4-chloro-3-indolyl phosphate in dimethylfromamide / mL NTMT and 5 mM of levamisole. Staining time varied between 3 and 20 hours depending on the specific probe. Staining was stopped by washing twice in ddH_2O. Fixing was then done in 4% PFA / 1x PBS for 20 minutes at room temperature. Slides were then dried and mounted in Aqua Mount (VWR).

3. Results

3.1 Sex Hormone Analysis

After the 10-day fadrozole exposure, blood was collected from control and treated fish. A RIA was conducted in order to measure the plasma concentration of testosterone. RIAs are very sensitive and precise, utilizing radioactive antibodies to detect hormone levels in the blood. This procedure was done in order to assess whether the fadrozole exposure had the desired effect on the fish. As documented previously, a fadrozole exposure should lead to a significant increase in androgens (Ankley et al., 2002). This can be explained by the fact that aromatase converts androgens to estrogens. Therefore, inhibiting this enzyme leads to an accumulation of androgens and also leads to decrease of estrogens simply because it is no longer being produced by this pathway. As expected, male zebrafish exposed to fadrozole had a 3-fold increase in testosterone levels (Fig. 2). Average testosterone levels in the control group was 4.7 ng/mL and ranged between 4.0

and 4.9 ng/mL (n = 4). In males exposed to 200 μg/L of fadrozole, the average testosterone level was 13.9 ng/mL and ranged between 12.1 and 15.4 ng/mL (n = 4).

A fadrozole exposure should also lead to a decrease in estrogens (Ankley et al., 2002). This has been reported in female fish, but has never been observed in male fish because of the small volume of blood collected, the low concentrations of estrogens before treatment and the limitations of RIAs. Nevertheless, a RIA was conducted for E2; as anticipated, samples were below the limit of detection.

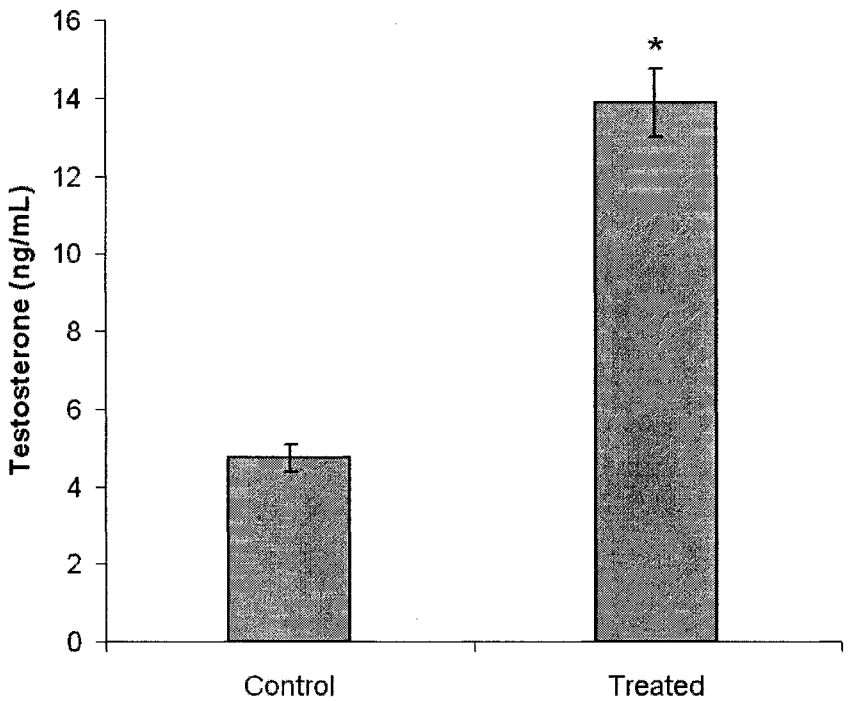


Figure 2. Plasma concentration of testosterone measured by radioimmunoassay in male zebrafish. Each tube for control (n = 4) and treated (n = 4), contained pooled plasma from approximately 20 fish. Means (± standard error) are presented and asterisks (*) denote a significant difference ($p < 0.05$; unpaired Student's T-Test).

3.2 Microarray Expression Analysis

After the 10-day fadrozole exposure, the brains from control and treated fish were removed. The telencephalon and hypothalamus were individually dissected to isolate the total RNA. The RNA from the control and treated fish was then used for microarray expression analysis. Microarrays consist of thousands of spotted oligonucleotides derived from the mRNA sequences of known genes. The control and treated RNA are transformed into cDNA and are hybridized to the microarrays allowing the identification of expressed genes. If a gene is over or under-expressed in the treated samples, the microarray will be able to detect differences in expression of this transcript and quantify them. By comparing the control and treated samples, genes that are affected by the fadrozole exposure will be identified and a negative or positive fold-change will be calculated.

Regulated transcripts, from the telencephalon, identified by microarray analysis are listed in Table 3 and Table 4. A total of 235 were found to be significantly regulated when compared to controls; 113 were found to be up-regulated whereas 122 were down-regulated. Genes were identified through ANOVA and were considered differentially expressed when the fold change was ≥ 2 with a p-value ≤ 0.05. Some transcripts were removed from the list due to the lack of annotation. After removing the unannotated transcripts, the final list contained 58 up-regulated genes (Table 3) and 48 down-regulated genes (Table 4)

Table 3. List of candidate genes identified as being up-regulated by microarray analysis in the telencephalon of male zebrafish after a 10 day exposure to fadrozole (p-value $\leq$ 0.05), fold change $\geq$ 2.00). Genes identified with "EST similar to" were inferred through nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Gene	Fold Change	Nucleotide Accession #
EST similar to host cell factor (HCF-1) (C1 factor)	12.49	CD605009
Opsin 1 (cone pigments), long-wave-sensitive, 1	8.67	NM_131175
EST similar to pseudouridylate synthase 1	7.98	BM777078
EST similar to troponin 1	7.52	AF539738
Cryptochrome 2b	6.80	NM_131792
EST similar to ZP1-related protein	6.67	BM775300
EST similar to TGF-beta inducible early protein	6.28	AI641738
EST similar to transposon helitron-like	6.23	CD599111
Rhesus blood group, B glycoprotein	6.00	BC049405
Fatty acid binding protein 2, intestinal	5.54	AF541953
FBXO11 protein	5.45	BI888954
UPF3 regulator of nonsense transcripts homolog B	5.29	BI473227
BCL2-associated athanogene 2	5.28	BC053303
Met proto-oncogene (hepatocyte growth factor receptor)	5.19	BQ262240
Xeroderma pigmentosum, complementation group C	4.83	AI959576
Thymosin beta b	4.67	BI709648
Fibrinogen, gamma polypeptide	4.50	BC045868

Pim-1 oncogene	4.39	NM_131539
EST similar to topoisomerase (DNA) II alpha	4.29	AL915830
Carboxyl ester lipase, tandem duplicate 2	4.23	AW116631
Phosphate regulating gene	4.21	BM095403
EST similar to ubiquitin A-52 residue ribosomal protein 1	4.19	BM081539
EST similar to fatty acid binding protein 6 (gastrotropin)	4.18	BQ479899
EST similar to major histocompatibility complex II DAB	4.17	BQ261450
EST similar to component of oligomeric golgi complex 7	4.14	AL920016
EST similar to DIX domain containing 1	4.03	BM316706
Embryonic shield mRNA	3.99	AF533659
Twist1	3.89	NM_130984
Lon peptidase 1, mitochondrial	3.78	AI584950
mRNA decapping enzyme	3.73	AF510720
EST similar to NIMA related kinase 7	3.71	BI980438
Protein O-fucosyltransferase 1	3.56	BM534004
Homeo box D12a	3.48	Y14547
Vang-like 2 (van gogh, Drosophila)	3.48	NM_153674
Zona pellucida glycoprotein 3	3.45	BQ617061
Thioredoxin interacting protein, like	3.40	AL726391
Solute carrier family 2 member 10	3.38	BM777825
EST similar to FK506 binding protein 9	3.32	AI943251
EST similar to Branchiostoma floridae Amphi Wnt7a	3.29	AI477500
Receptor-associated protein of the synapse, 43kD	2.82	NM_178305

EST similar to tubulin polymerization-promoting protein3	2.80	BE016153
EST similar to UDP-N-acetyl-alpha-D-galactosamine	2.79	AW076570
Novel alpha-type globin	2.78	AL721636
GINS complex subunit 3	2.77	BM571579
Rhomboid, veinlet-like 2 (Drosophila)	2.76	BC048048
Matrilin 1	2.76	BC045465
Homeo box (expressed in ES cells) 1	2.76	NM_131349
Piwi-like 1 (Drosophila)	2.70	AF336369
Plasminogen	2.61	AF515276
Egg envelope glycoprotein	2.59	BM140635
Transcription factor AP-2 beta	2.40	CD605856
Uridine phosphorylase, like	2.37	BC044525
PDZ and LIM domain 7	2.29	BQ615200
Neurolin-like cell adhesion molecule	2.23	AL719717
Apoptosis associated protein	2.16	BI889255
Angiopoietin 1	2.14	NM_131813
EST similar to vertebrate telomere-associated RIF1	2.13	BI889053
Far upstream element binding protein 1	2.10	BI891779

Table 4. List of candidate genes identified as being down-regulated by microarray analysis in the telencephalon of male zebrafish after a 10 day exposure to fadrozole. (p-value ≤ 0.05), fold change ≤ -2.00). Genes identified with "EST similar to" were inferred through nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Gene	Fold Change	Nucleotide Accession #
Phosducin 1	-8.85	BI671261
EST similar to type V collagen	-7.75	CB362946
Proteolipid protein 1a	-7.30	AL715267
Solute carrier family 35, member E1	-6.71	BC046896
Homeo box A13b	-6.29	NM_131194
EST similar to arpc3-like	-6.10	AL722539
Homeo box B5a	-5.95	NM_131101
Cytochrome P450, family 1, subfamily B, 1	-5.81	AL917535
Guanylate cyclase activator 1B	-5.75	NM_131871
N-acylsphingosine amidohydrolase 1	-5.65	CD052998
Bagpipe homeobox homolog 1 (NK3 homeobox 2)	-5.35	NM_178132
EST similar to ryanodine receptor RyR1	-5.24	AW232727
Arrestin 3, retinal (X-arrestin)	-5.15	BI879924
Odorant receptor, family 2, member 2	-5.08	NM_131592
N-acetylgalactosaminyltransferase	-4.95	BI983399
Muc2 protein	-4.85	BI673162
EST similar to Pim1	-4.72	BQ450046

Protein phosphatase 1, regulatory (inhibitor) 3B	-4.48	BC044421
Homeo box A11a	-4.44	NM_131544
EST similar to proteophosphoglycan 5	-4.31	AW343858
Vasodilator-stimulated phosphoprotein	-4.26	BE201011
Cytochrome P450, family 19, subfamily A, 1b	-4.05	NM_131642
AKAP 220	-4.03	AL912493
Distal-less homeobox gene 4b	-3.86	NM_131318
Fibroblast growth factor 2	-3.65	AY269790
EST similar to mannose-P-dolichol utilization 1a	-3.58	AI588415
Carnitine palmitoyltransferase 1A like	-3.32	BI475933
Dopa decarboxylase	-3.31	BG727262
EST similar to bromodomain adjacent to zinc finger	-3.29	BM026295
EST similar to alpha-2-macroglobulin-1	-3.27	BM071900
Activin receptor IIb	-3.25	BC044131
Cysteine conjugate-beta lyase	-3.21	BG883469
Farnesoid X receptor beta	-3.13	BI475825
Transferrin-a	-3.04	BM957464
Alpha 1 chain of type XII collagen	-2.68	BQ449552
EST similar to signal peptide, CUB	-2.61	BQ073932
Neurological oncogenic ventral antigen protein	-2.53	BE605952
Paired box gene 8	-2.49	AF072549
WD repeat domain 33	-2.40	CD605805
Neuron navigator 3	-2.35	BU670710

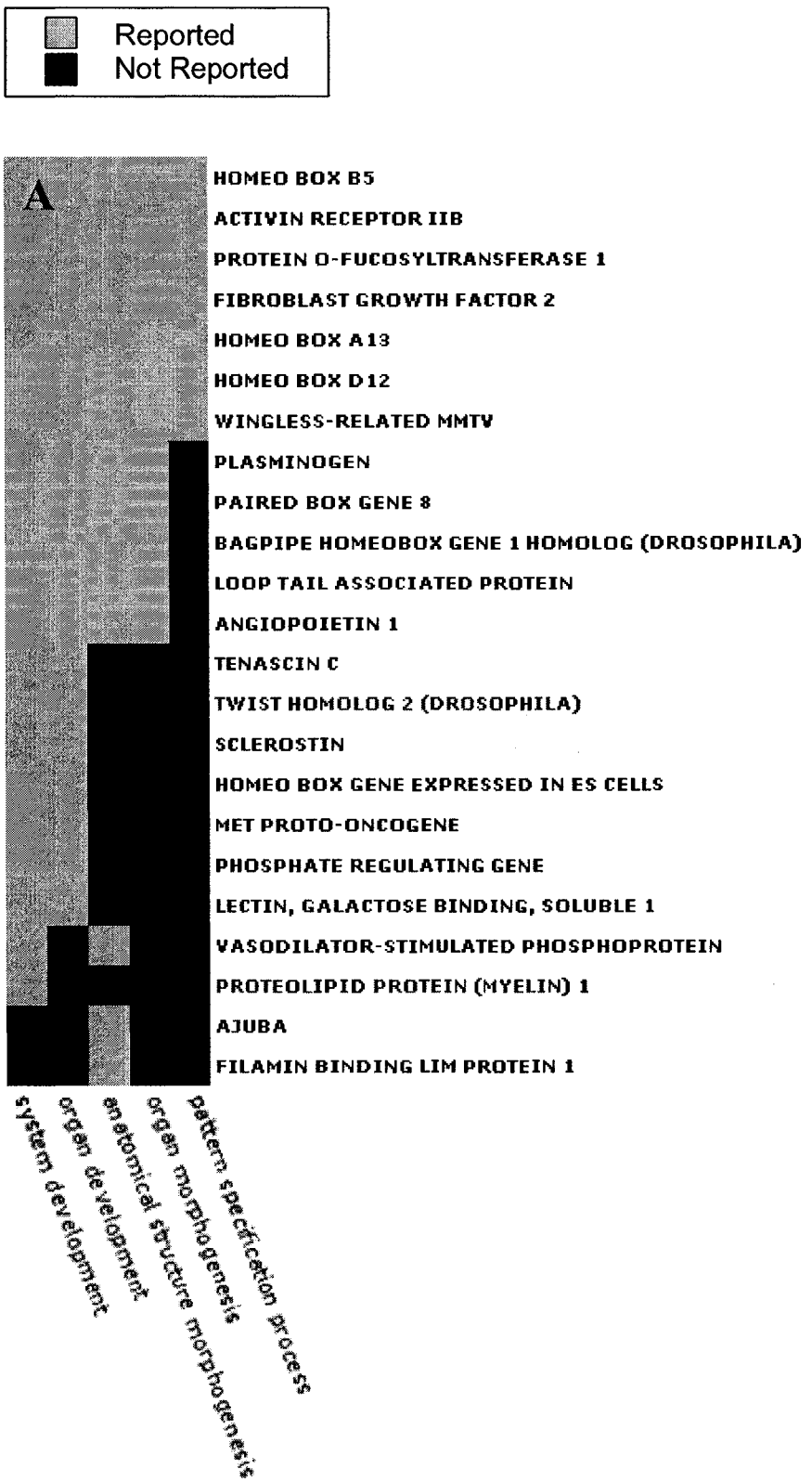
Lectin, galactoside-binding, soluble, 1 (galectin 1)-3	-2.31	BM181749
Transient receptor subfamily C, member 4, b	-2.24	BM186872
Cholinergic receptor, muscarinic 2	-2.19	NM_178301
Preproinsulin b	-2.16	AL922691
Myotubularin related protein 2	-2.10	AF073883
EST similar to ovary-expressed homeobox protein	-2.07	BQ285285
General transcription factor IIIC, polypeptide 4	-2.00	BI672460

3.3 Gene Ontology Analysis

Information on the biological function of differentially-expressed genes was obtained by GO analysis of the combined list of regulated genes. The DAVID functional annotation clustering uses a novel algorithm to measure relationships among the annotation terms. The end result is the grouping of similar, redundant, and heterogeneous annotation contents into various annotation groups. Using this tool, two annotation clusters based on biological processes were identified and one additional annotation clusters based on cellular components was identified. The first cluster mainly contained genes involved in morphogenesis and development (Table 5 and Fig. 3A). The second cluster mainly contained genes involved in sensory perception and the detection of stimuli (Table 5 and Fig. 3B). The third clusters mainly contained transcription factor genes (Table 5 and Fig. 3C)

Table 5. Gene ontology term associated with each annotation cluster. Annotation cluster 1 and 2 were produced using GO_TERM_BP_3 database, whereas annotation cluster 3 was produced using GO_TERM_CC_ALL database. The EASE score refers to the modified one-tail Fisher Exact Probability P-value used for gene-enriched analysis. An EASE score ≤ 0.05 is considered strongly enriched.

Gene Ontology Term	Genes	EASE Score
Annotation Cluster 1		
Organ morphogenesis	12	0.00068
Organ development	19	0.0045
Pattern specification process	7	0.0049
System development	21	0.0089
Anatomical structure morphogenesis	15	0.02
Annotation Cluster 2		
Response to radiation	6	0.00048
Detection of abiotic stimulus	4	0.0038
Detection of external stimulus	4	0.0068
Detection of stimulus during sensory perception	4	0.0075
Neurological system process	8	0.95
Annotation Cluster 3		
Organelle lumen	13	0.0047
Membrane-enclosed lumen	13	0.0047
Nuclear lumen	11	0.014
Transcription factor complex	8	0.017
Nucleoplasm part	9	0.022
Nucleoplasm	9	0.30
Nuclear part	11	0.13



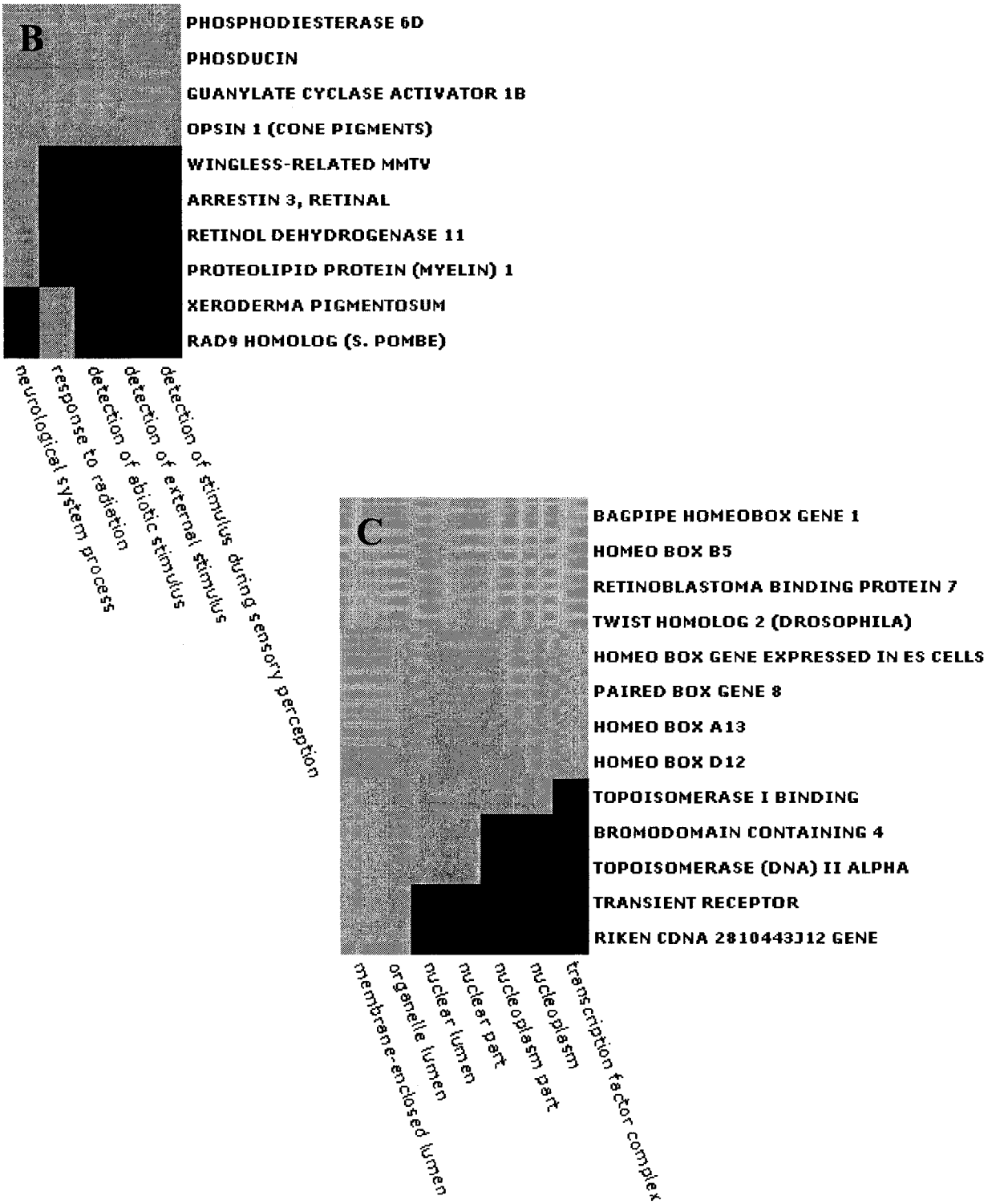


Figure 3. Enrichment analysis of Gene Ontology terms for differentially expressed genes in the telencephalon after a 10 day exposure to fadrozole using male zebrafish relative to control fish. Differentially expressed genes were identified by microarray (see Table 3 and Table 4). Annotation cluster 1(A), cluster 2(B) and cluster 3(C). Gray squares correspond to a gene-term association that has been reported; whereas black squares correspond to a gene-term association that has not been reported.

3.4 Real-Time RT-PCR Analysis

Real-time RT-PCR was used to validate some of the genes in the telencephalon that were identified as being differentially expressed by microarray analysis. This technique was also used to examine gene expression in the hypothalamus. Real-time RT-PCR monitors the amount of amplicon generated as the reaction occurs. The amount of product is directly related to the fluorescence of the reporter dye, SYBR green. Real-time RT-PCR provides a wide linear dynamic range, is highly sensitive and is exceptionally accurate for quantification. The quantification determined by this method is thought to be more accurate then the quantification achieved through microarray analysis. This is the reason why real-time RT-PCR is used to validate data obtained by microarray.

For all reactions the endogenous reference gene was *rpl8*. This gene was verified by real-time RT-PCR and was not regulated by the fadrozole exposure (data not shown). Genes selected for verification were chosen based on either the GO analysis or the previously mentioned EE2 study that looked at the affects of EE2 on gene expression in the goldfish telencephalon (Martyniuk et al., 2007). As hypothesised in section 1.7, fadrozole and EE2 cause opposite effects; therefore some of the genes identified in the

EE2 study should also be identified as being differentially regulated by the fadrozole exposure. Genes selected for real-time RT-PCR are presented in Table 6 along with a comparison to microarray data from the EE2 study. For example, the gene coding for fatty acid binding protein 2 was selected because the up-regulation in the fadrozole exposure corresponds to the down-regulation in the EE2 exposure.

Table 6. Comparison between fold changes observed in the fadrozole microarray analysis and EE2 microarray analysis. Genes with a fold change in the fadrozole column were verified by real-time RT-PCR, whereas genes with a fold change in the EE2 column were taken from a previous study by Martyniuk et al., 2007.

Gene	Fadrozole (Fold Change)	EE2 (Fold Change)
Opsin 1	8.7	
Cryptochrome 2b	6.8	
Fatty acid binding protein 2	5.6	-4.6
Carboxyl ester lipase	4.2	-3.2
Solute carrier 2, 10	3.4	
Solute carrier 34, 2		-3.3
Solute carrier 3, 2		-2.9
Phosducin 1	-8.9	
Homeo box A13b	-6.3	
Cytochrome P450, 1, B, 1	-5.8	
Arrestin 3	-5.2	
Odorant receptor 2, 2	-5.1	3.3
Homeo box A11a	-4.4	
Cytochrome P450, 19, A, 1b	-4.0	
Homeo box D10a		3.7
Homeo box D11a		2.6
Homeo box C4a		2.5
Homeo box A9a		2.3

The expression of opsin 1 (*ops1*), cryptochrome 2b (*cry2b*), fatty acid binding protein 2 (*fab2*), carboxyl ester lipase (*cel*) and solute carrier 2, 10 (*slc2,10*) was determined to be significantly up-regulated in the telencephalon by microarray analysis. Real-time RT-PCR confirmed the direction of all fold changes. Real-time RT-PCR confirmed *cry2b* and *fab2* as being significantly up-regulated in the telencephalon. *Cry2b* and *cel* were found to be significantly up-regulated in the hypothalamus. All other up-regulated genes verified by real-time RT-PCR were not identified as being significantly regulated in the telencephalon nor the hypothalamus (see Fig. 4 and Table 7).

The expression of phosducin 1 (*pdc1*), homeo box A13b (*hoxa13*), *cyp1b*, arrestin 3 (*art3*), odorant receptor 2, 2 (*or2,2*), homeo box A11a (*hoxa11*) and *cyp19b* was determined to be significantly down-regulated in the telencephalon by microarray analysis. Real-time RT-PCR identified the same direction of fold changes except for *pdc1* in both tissues, and *hoxa13* and *hoxa11* in the hypothalamus. Real-time RT-PCR confirmed *cyp1b* and *cyp19b* as being significantly down-regulated in the telencephalon and identified comparable results in the hypothalamus. All other down-regulated genes verified by real-time RT-PCR were not identified as being significantly regulated in the telencephalon nor the hypothalamus (see Fig. 5 and Table 7).

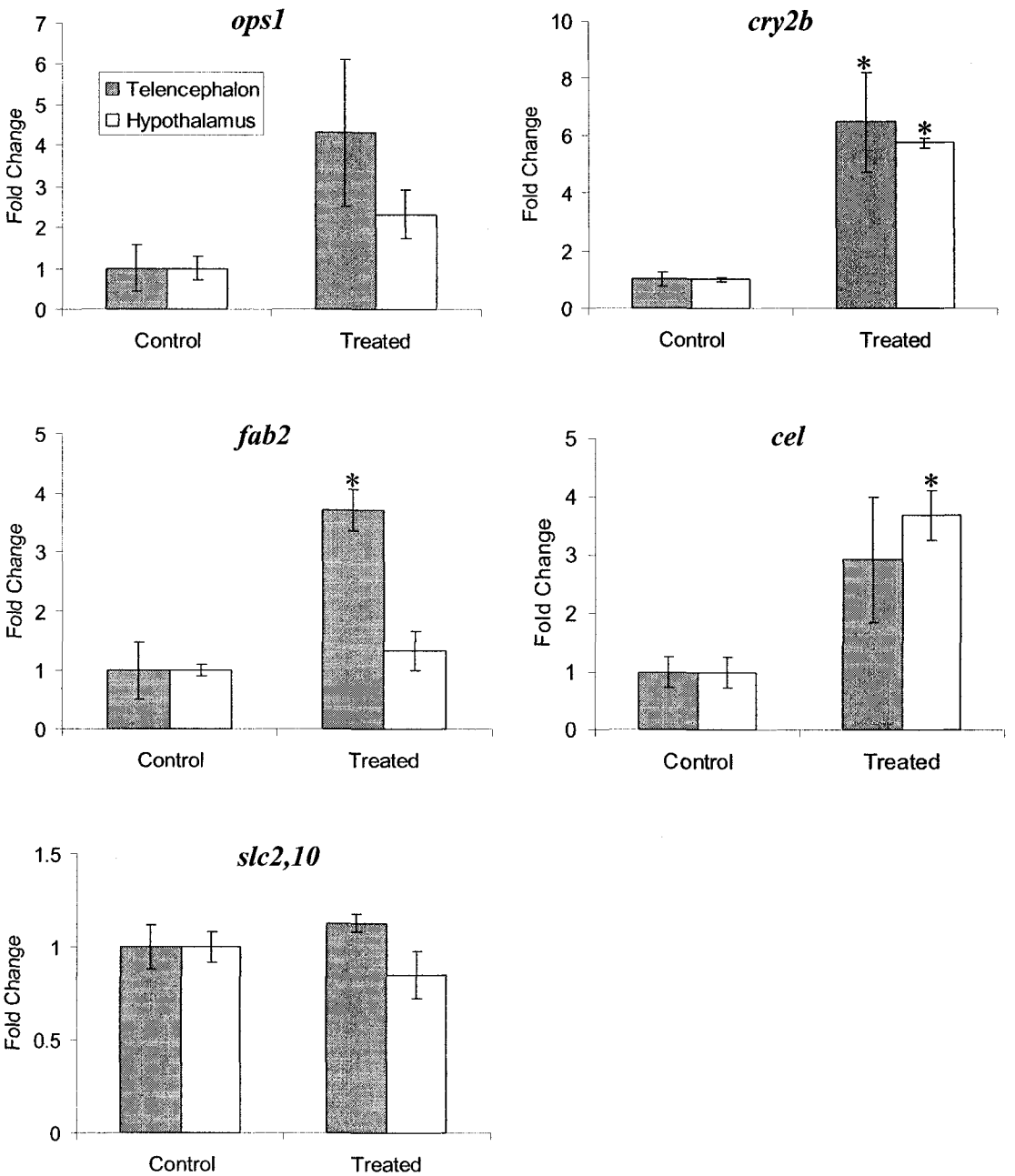
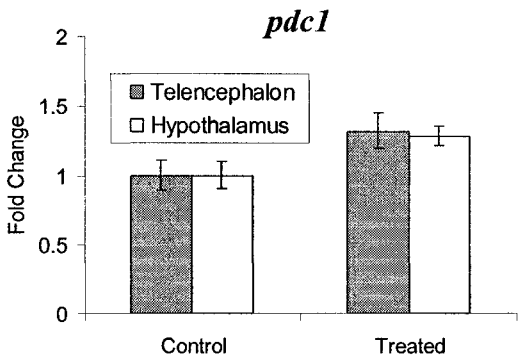
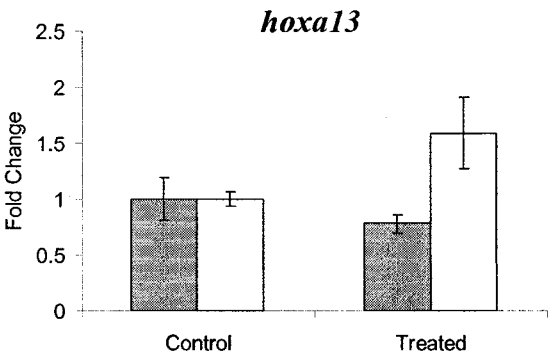
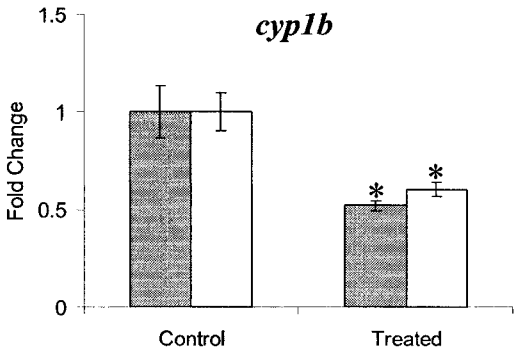
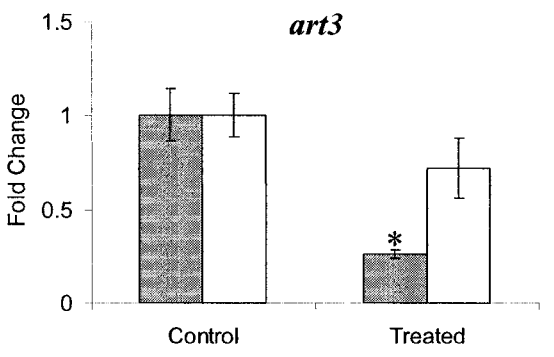


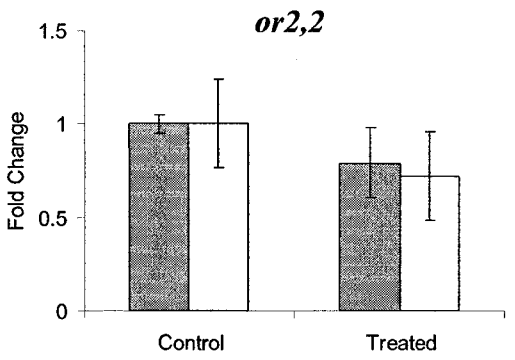
Figure 4. Effects of fadrozole on mRNA levels in male zebrafish, determined by real-time RT-PCR. Genes were identified as being up-regulated by microarray analysis. Relative fold changes (means ± standard error) are presented for the telencephalon (n = 3) and hypothalamus (n = 3). The expression levels of each gene were normalized with respect to *rpl8*. Asterisks (*) denote a significant difference between control and treated fish for the specific gene ($p < 0.05$; unpaired Student's T-Test).

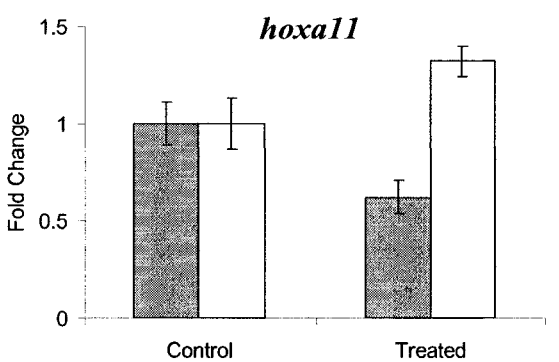












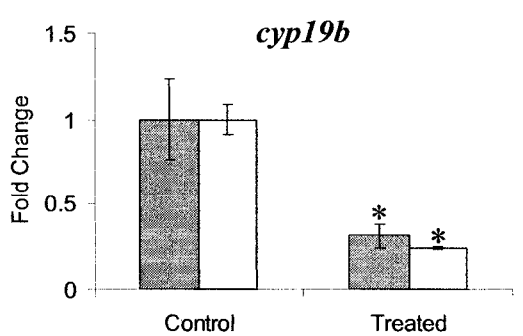


Figure 5. Effects of fadrozole on mRNA levels in male zebrafish. Genes were identified as being down-regulated by microarray analysis. Relative fold changes (means ± standard error) are presented for the telencephalon (n = 3) and hypothalamus (n = 3). The expression levels of each gene were normalized with respect to *rpl8*. Asterisks (*) denote a significant difference between control and treated fish for the specific gene ($p < 0.05$; unpaired Student's T-Test).

Table 7. Comparison between fold changes observed by microarray analysis and real-time RT-PCR analysis in the telencephalon (Tel.) and hypothalamus (Hyp.). Asterisks (*) denote a significant difference between control and treated fish for the specific gene ($p < 0.05$; unpaired Student's T-Test).

Genes	Gene Symbols	Microarray Fold Change[1]	Real-Time RT-PCR Fold Change	
		Tel.	Tel.	Hyp.
Opsin 1	*opn1lw1*	* 8.7	4.3	2.3
Cryptochrome 2b	*cry2b*	* 6.8	* 6.4	* 5.7
Fatty acid binding 2	*ifabp*	* 5.6	* 3.7	1.3
Carboxyl ester lipase	*cel.2*	* 4.2	2.9	* 3.7
Solute carrier 2, 10	*slc2a10*	* 3.4	1.1	-1.2
Phosducin 1	*pdc1*	* -8.9	1.3	1.3
Homeo box A13b	*hoxa13b*	* -6.3	-1.3	1.6
Cytochrome P450 1, B, 1	*cyp1b1*	* -5.8	* -1.9	* -1.7
Arrestin 3	*arr3*	* -5.2	* -3.9	-1.6
Odorant receptor 2, 2	*olfr2.2*	* -5.1	-1.3	-1.4
Homeo box A11a	*hoxa11a*	* -4.4	-1.6	1.3
Cytochrome P450 19, A, 1b	*cyp19b*	* -4.1	* -4.2	* -3.3

[1] Significant differences determined by ANOVA.

Table 8. P-values associated with real-time RT-PCR data presented in table 7 for the telencephalon (Tel.) and hypothalamus (Hyp.). P-values were determined by unpaired Student's T-Test.

Genes	Gene Symbols	Real-Time RT-PCR P-Values	
		Tel.	Hyp.
Opsin 1	*opn1lw1*	0.156	0.181
Cryptochrome 2b	*cry2b*	0.035	0.00002
Fatty acid binding 2	*ifabp*	0.019	0.167
Carboxyl ester lipase	*cel.2*	0.153	0.034
Solute carrier 2, 10	*slc2a10*	0.378	0.331
Phosducin 1	*pdc1*	0.132	0.079
Homeo box A13b	*hoxa13b*	0.345	0.215
Cytochrome P450 1, B, 1	*cyp1b1*	0.022	0.018
Arrestin 3	*arr3*	0.034	0.232
Odorant receptor 2, 2	*olfr2.2*	0.261	0.456
Homeo box A11a	*hoxa11a*	0.069	0.101
Cytochrome P450 19, A, 1b	*cyp19b*	0.037	0.002

3.5 In Situ Hybridization Analysis

In situ hybridization was preformed in order to visualise the spatial distribution, within the brain, of a selection of mRNAs affected by the fadrozole exposure. The effects of fadrozole on gene expression is visualised by comparing the expression patterns in treated and control brains. In situ hybridization is a commonly used technique that utilizes a complementary digoxigenin-labelled RNA probe to detect and localize a transcript in a given tissue (*in situ*). Three genes were selected; *cyp19b*, *cyp1b* and *cry2b*. These genes were chosen as good candidates because of their significant regulation in the telencephalon and hypothalamus determined by real-time RT-PCR. In addition, *cyp1b* was selected because it is part of the same superfamily as *cyp19b*. *Cyp1b* and *cyp9b* also share common molecular functions such as steroid metabolism. A negative control was done with a *cyp19b* sense probe; no expression was observed (data not shown). See Figure 6 for dorsal and lateral views of the zebrafish brain, including the position of each cross section found in Figure 7.

In the first set of in situ sections (Fig. 7, A-H) using an antisense probe against *cyp19b*, the transcript seems to be considerably more expressed in the control brain, especially throughout the telencephalon (Fig. 7, A-F), mostly throughout outer ridge of the median and lateral zone of the dorsal telencephalic area. Expression is also seen along the unpaired medial ventrical (section separating the left and right part of the brain) including the anterior part of the parvocellular preoptic nucleus and the nucleus of the dorsal and ventral telencephalic area. Further down into the brain in the optic tectum and hypothalamus (Fig. 7, G-H), the transcripts seem to be equally abundant in the control and treated brains. Expression can be seen in the periventricular gray zone of the optic

tectum, the torus longitudinalis, the valvula cerebelli, the posterior tuberal nucleus, the preglomerular nucleus and in the dorsal and ventral zone of the hypothalamus.

In the second series of in situ sections (Fig. 7, I-P) using an antisense probe against *cyp1b*, the transcripts appear to be slightly more expressed in the telencephalon of the control brain (Fig. 7, I-L), including the anterior part of the parvocellular preoptic nucleus and the nucleus of the ventral telencephalic area. Further down into the brain, at the more rostral regions of the optic tectum (Fig. 7, M-N), expression seems to be slightly more abundant in the control brain, especially in the ventral zone of the hypothalamus and the torus longitudinalis. Further down into the optic tectum (Fig. 7, O-P), expression in the control brain is very weak, whereas in the treated brain, expression can be seen in the periventricular gray zone of the optic tectum, the torus longitudinalis, the valvula cerebelli and very faintly in the dorsal zone of the hypothalamus.

In the third series of in situ sections (Fig. 7, Q-X) using an antisense probe against *cry2b*, the transcripts appear to be noticeably more expressed throughout the telencephalon of the treated brain (Fig. 7, Q-T) including the anterior part of the parvocellular preoptic nucleus and the nucleus of the ventral telencephalic area. Further down into the brain, at the more rostral regions of the optic tectum (Fig. 7, U-V), expression is very weak. However, the transcripts appear to be slightly more abundant in the treated brain. Further down into the optic tectum (Fig. 7, W-X), expression in the control brain appears to be stronger than the treated brain. Expression can be seen in the periventricular gray zone of the optic tectum, the torus longitudinalis, the posterior tuberal nucleus, the valvula cerebelli and in the dorsal and ventral zone of the hypothalamus.

The in situ hybridizations have allowed visualisation of the changes in expression patterns caused by the fadrozole exposure. The control brains, especially throughout the telencephalic area, showed apparently stronger expression for the genes that were identified as being up-regulated. For *cry2b*, which was identified as being down-regulated, the control brains show apparently weaker expression, again throughout the telencephalic area. These observations have helped to further validate the results obtained by the real-time RT-PCR analysis.

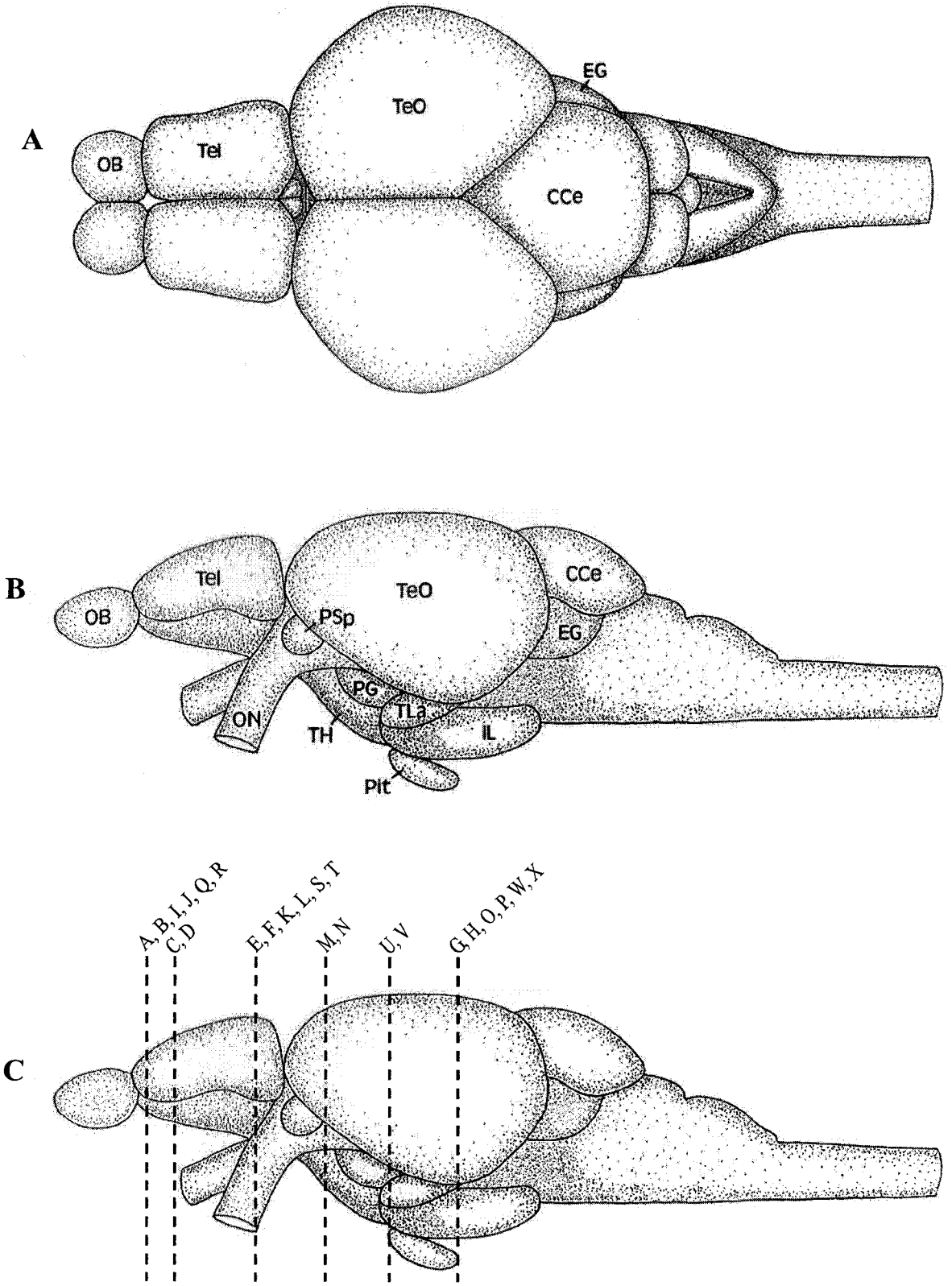


Figure 6. Dorsal (A) and lateral (B and C) views of the adult zebrafish brain. Figure C illustrates the position of each (A-X) cross section found in Figure 7. (modified from Wullimann et al., 1996)

Abbreviations:

CCe = corpus cerebelli

EG = eminentia granularis

IL = inferiror lobe of the hypothalamus

OB = olfactory bulb

ON = optic nerve

PG = preglomerular area

Pit = pituitary

PSp = parvocellular superficial pretectal nucleus

Tel = telencephalon

TeO = tectum opticum

TH = tuberal hypothalamus

TLa = torus lateralis.

Control	Treated

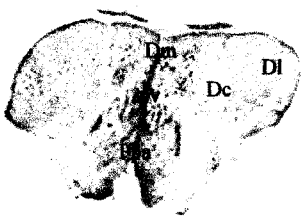
A

B

C

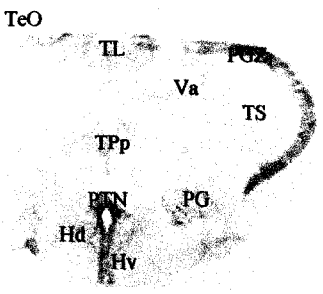
D

E

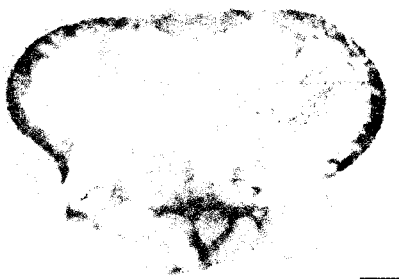


F

G



H



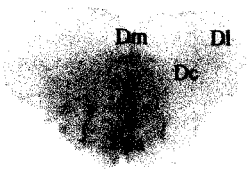
Control	Treated

Control	Treated

I

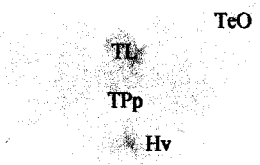
J

K



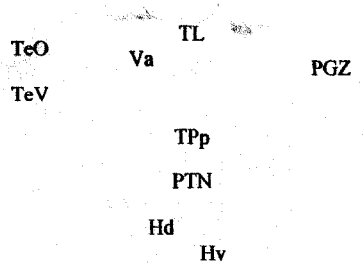
L

M



N

O



P

Control	Treated

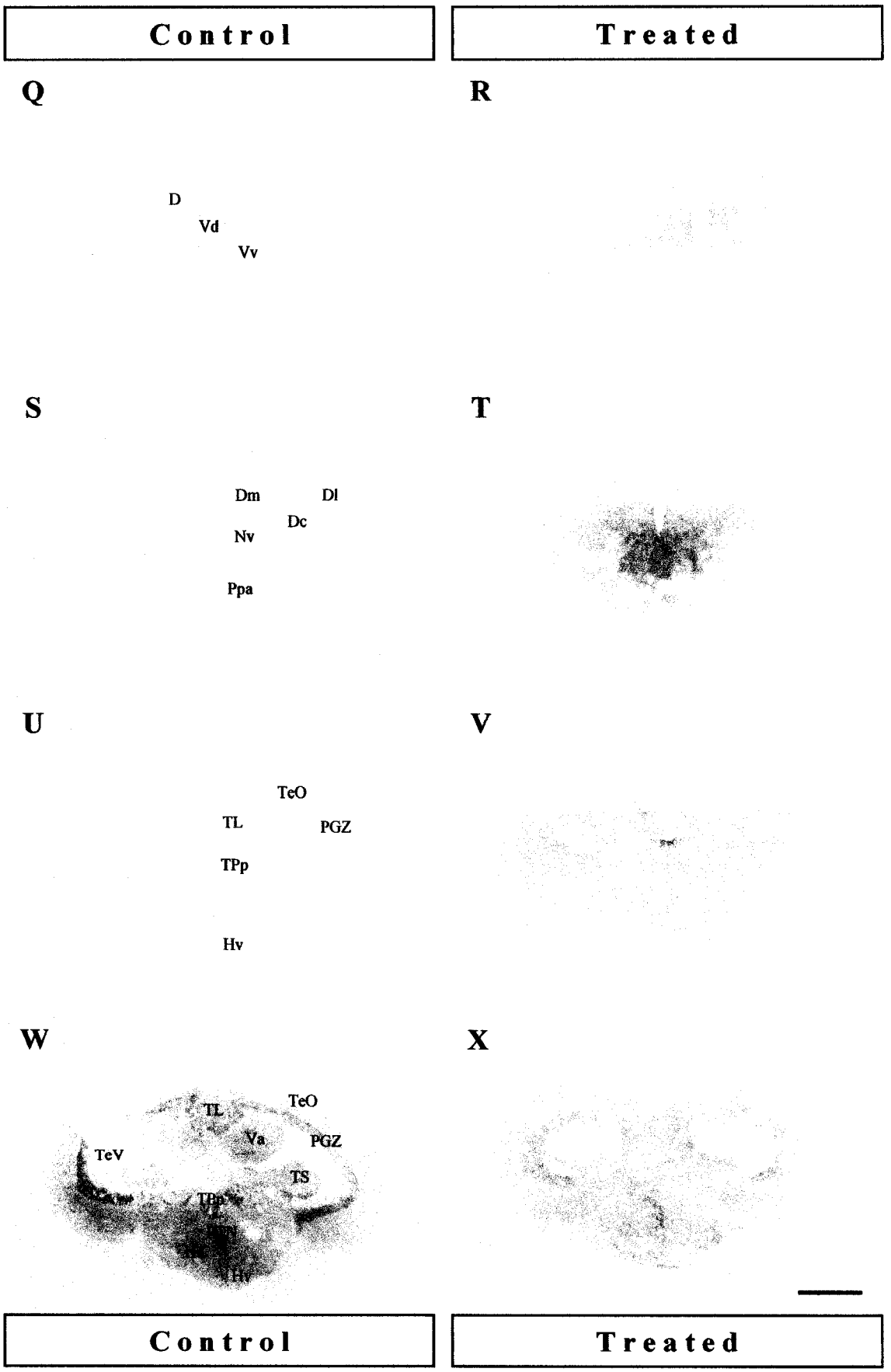


Figure 7. Visualisation of the effects of fadrozole on gene expression patterns in control and treated brains. First group of panels corresponds to the *cyp19b* antisense probe (A-H), second group of panels corresponds to the *cyp1b* antisense probe (I-P) and the third group of panels corresponds to the *cry2b* antisense probe (Q-X). Cross sections are 10μm thick and the black bar on each panel indicates a size of 500μm.

Abbreviations:

D = dorsal telencephalic area

Dc = central zone of D

Dl = lateral zone of D

Dm = medial zone of D

Hd = dorsal zone of the hypothalamus

Hv = ventral zone of the hypothalamus

Nv = nucleaus of the V

PG = preglomerular nucleus

PGZ = periventricular gray zone of the optic tectum

Ppa = anterior part of the parvocellular preoptic nucleus

PTN = posterior tuberal nucleus

TeO = tectum opticum

TeV = tectal ventricle

TL = torus longitudinalis

TPp = periventricular nucleus of the posterior tuberculum

TS = torus semicircularis

V = ventral telencephalic area

Va = valvula cerebelli

Vd = dorsal nucleus of V

Vv = ventral zone of V

4. Discussion

This is one of the first studies to examine the transcriptional response to an AI and contributes to our understanding of the importance of endogenous estrogens in the regulation of the vertebrate brain transcriptome. The AI fadrozole was used to conduct this assessment in the telencephalon and hypothalamus of the zebrafish brain. Testosterone plasma levels were assessed and showed a significant response to fadrozole. Microarray analysis of the telencephalon identified a total of 235 significantly regulated transcripts. GO analysis for the microarray data grouped common or similar annotations to reveal three annotation clusters based on biological processes and cellular components. Quantification through real-time RT-PCR was done to validate the microarray data, and to assess the transcriptional response in the hypothalamus. Collectively, these results provide a better understanding of the effects of AIs on gene expression. The results also shed light on the underlying effects of sex hormone variation and their importance in the brains of teleosts.

4.1 Increase of Plasma Testosterone

The fadrozole exposure, in the male zebrafish, caused a significant increase in the plasma concentration of testosterone. With a fadrozole concentration of 200 μg/L, an approximate 3-fold increase was observed, in comparison to control males. E2 concentrations were not assessed due to detection limits and volume limitations.

Similar results have been observed in other species including a teleost species. Male fathead minnows exposed to fadrozole at a concentration of 50 μg/L showed an approximate 2.5-fold increase in plasma testosterone (Ankley et al., 2002). The authors

mention that they were also unable to assess E2 concentrations in males due to volume limitations. Although, in female fathead minnows E2 plasma concentrations were detected and showed an approximate 20-fold decrease in response to fadrozole. Of note, in the female fathead minnows, testosterone levels appeared to be unaffected. The authors speculate that fadrozole could be inhibiting various mechanisms involved in androgen degradation or excretion, which is specific to males. Further observations of the exposed males revealed a concentration dependant enlargement of the seminiferous tubules accompanied by significant accumulation of sperm in the lumen, most likely caused by the increase in plasma testosterone.

Male Italian wall lizards (*Podarcis sicula*) injected with an AI showed comparable results (Cardone et al., 2002). The AI caused a significant increase in plasma testosterone while also causing a significant decrease in E2. Similar to the fathead minnow study, the authors observed an AI-induced spermatogenesis accompanied by a release of sperm into large lumens of the seminiferous tubules. In human males, AIs also cause a significant increase in plasma testosterone and a significant decrease in plasma E2 (Dowsett, 1999). Both hormones show an approximate 2-fold change in response to a low dose of AI.

EE2 studies, in teleosts, have shown variable results in response to the estrogenic compound. Generally testosterone levels decrease in response to EE2, whereas E2 levels can either increase or remain the same (MacLatchy et al., 2003; Martyniuk et al., 2007; Rinchard et al., 2001; Sisneros et al., 2004; Tilton et al., 2005). Further observations suggested the existence of a threshold effect of EE2. At low concentration EE2 stimulates the production of E2, whereas at higher concentrations, EE2 inhibits the

production of E2. This threshold concentration has been showed to be dependant of various factors, such as sex, age and the time of the season. EE2 studies using a range of concentration were able to examine this result. AI studies are relatively new and are not as abundant as EE2 studies. This threshold effect has not been observed with AIs, however additional studies using a wider range of concentrations are necessary to exclude this effect.

The significant increase of plasma testosterone observed in this present study, is a clear indication that the aromatase was inhibited. By inhibiting aromatase, it is assumed that testosterone concentrations would increase since it is no longer being converted into E2. The same would apply to E2 levels. Its concentration should decrease since it is no longer being produced. Although in female fathead minnows, testosterone levels do not seem to be significantly effected by AIs, (Ankley et al., 2002) suggesting a sex specific response. Other species have demonstrated that AIs can significantly decrease E2 in males, although this has not been reported in teleost males. One way of assessing low E2 levels in zebrafish males would be to pool numerous plasma samples to obtain greater volumes and to use an ultra-sensitive RIA protocol.

4.2 Differentially Regulated Genes in the Telencephalon

After the 10 day fadrozole exposure, microarray analysis of the telencephalon identified 113 up-regulated genes, whereas 122 genes were identified as being down-regulated. Changes in transcription levels following EE2 treatments have previously been reported to be relatively modest in the brain, when compared to other tissues such as the liver or gonads (Marlatt et al., 2008; Martyniuk et al., 2007; Martyniuk et al., 2006).

The relative fold-changes in the hypothalamus and/or telencephalon, for these mentioned studies, typically varied between 1.5 and 5. In the present study, using fadrozole, the relative fold-change in the telencephalon varied between 2 and 10 averaging approximately 4.3. Compared to previous studies using EE2, fadrozole would seem to have pronounced effects on gene expression. This could be explained by the fact that fadrozole inhibits both aromatase A in the gonads and aromatase B in the brain, which cause variations in both androgens and estrogens levels. On the other hand, EE2 mimics endogenous E2 and can have variable effects, such as a decrease in androgens accompanied by either an increase or a decrease in E2. Additionally, the previously mentioned EE2 exposure used notably low concentration varying between 10-300 ng/L, whereas the fadrozole exposure in this present study utilized a much higher concentration in the magnitude of μg/L. It is difficult to compare concentrations and relative changes in gene expression since these two drugs have very different mechanisms of action, and ultimately cause somewhat opposite effects. Even though, it is worth noticing the apparent pronounced effects of fadrozole in comparison to EE2.

A recent study examined the effects of fadrozole on gene expression in the brain and liver of fathead minnows (Villeneuve et al., 2008). This study exposed female fish for 7 days at a concentration of 60 μg/L. Sex hormones were not assessed and therefore cannot be compared to this study. Plasma *vtg* concentrations were determined and showed a significant decrease, thereby indicating a decrease in plasma E2 levels. The authors used a 2000 gene oligonucleotide microarray. A total of 27 genes in the brain and 86 genes in the liver were identified as being significantly regulated. Fold-changes in the brain varied between 1.2 and 2.7, whereas in the liver, fold-changes varied between

1.5 and 50. These fold-changes verify the previous statement regarding relatively modest transcriptional changes in the brain when compared to other tissues. The fold-changes obtained from the brain analysis in the fathead minnow study appear to be moderately lower than the present zebrafish study. There are four possible explanations for this. (a)The fadrozole concentration is considerably lower, approximately three time less concentrated than the compared study. (b)The exposure period in the fathead minnow study is slightly shorter. (c)Each of the studies used different microarray platforms and are therefore subject to different platform sensitivities. (d)The microarray analyses were conducted with transcripts from the entire brain. This can significantly change the outcome in a microarray study. For example, if a transcript has a 2 fold-change down-regulation in the telencephalon, but is not affected in the remaining parts of the brain, the microarray analysis will not detect this gene as being significantly regulated. Previous studies have already identified the regions of the brain that contain very high aromatase B activity; those important regions are the telencephalon and hypothalamus. For these reasons, it is much more efficient, reliable and trustworthy to study the transcriptional response to an AI in these specific regions.

4.2.1 Annotational Clustering Using Gene Ontology

Regulated genes obtained from the microarray analysis were classified by biological processes and cellular components. Two annotation clusters were identified based on biological processes which included morphogenesis, pattern specification processes, development, sensory perception and the detection of stimulus. A third

annotation cluster was identified based on cellular components mainly containing genes involved with transcription factor complexes.

This is the first AI study to identify these differentially regulated annotation clusters. However some studies have previously observed the differential regulation of select *hox* genes in response to estrogenic compounds. *Hox* genes have been shown to be involved with pattern specification. Another interesting group of genes are those involved in the detection stimulus and sensory perception, notably *ops1*, *pdc1* and *art3*. These annotation clusters and groups of genes will be discussed in greater detail in subsequent sections.

4.2.2 *Cyp19b* is Down-Regulated

The *cyp19b* transcript was significantly down-regulated in the telencephalon. Microarray analysis identified a 4-fold decrease. Real-time RT-PCR statistically confirmed the direction and magnitude of the fold-change in the telencephalon and identified comparable results in the hypothalamus. Real-time RT-PCR showed a negative fold-change of 4.2 and 3.3 in the telencephalon and hypothalamus respectively. This gene is under the control of several transcriptional regulators including multiple EREs. Therefore the identification of the down-regulation of this transcript would indicate a decrease in estrogens in response to the fadrozole exposure.

EE2 studies in goldfish, using various low concentrations, have shown that the *cyp19b* transcript is significantly up-regulated in response to in increase in estrogens (Marlatt et al., 2008; Martyniuk et al., 2007; Martyniuk et al., 2006). Real-time RT-PCR

analyses have shown positive fold-changes between 1.5 and 3 in the telencephalon and yet again, positive fold-changes in the hypothalamus between 2 and 6.

The fathead minnow/fadrozole study identified a 2.7 fold-change down-regulation of the *cyp19b* transcript in the brain (Villeneuve et al., 2008), which is somewhat comparable to the fold-changes observed in the present study considering that the authors used whole-brain total RNA extracts. These analyses were conducted with microarrays. Real-time RT-PCR was not done to validate the data obtained from these transcripts; it is therefore difficult to compare these results to the present zebrafish study. Nevertheless, this present zebrafish study confirms that the *cyp19b* transcript is down-regulated in the telencephalon and hypothalamus in response to fadrozole.

4.2.3 *Cyp1b* is Down-Regulated

Microarray analysis of the telencephalon showed a significant decrease of the *cyp1b* transcript in response to the fadrozole exposure. Real-time RT-PCR confirmed the direction of the fold-change in the telencephalon and produced comparable results for the hypothalamus. A down regulation of 5.8 was observed with the microarray analysis of the telencephalon, whereas real-time RT-PCT showed a down regulation of 1.9 and 1.7 in the telencephalon and hypothalamus respectively.

Cytochrome P4501B1 is the only known member of CYP1B subfamily (Sutter et al., 1994) and is expressed in many human tissues, including breast and ovary (Muskhelishvili et al., 2001). *Cyp1b* is the most catalytically efficient E2 hydroxylase (Hayes et al., 1996). Expression of *cyp19* and *cyp1b* proteins have been detected in breast cancer by immunohistochemistry (McFadyen et al., 1999; Miki et al., 2007). In

addition, an up-regulation of *cyp19* and *cyp1b* transcripts has also been reported in breast cancer tissues when compared to normal tissues (Singh et al., 2005). This indicates the importance of these two enzymes in the development and progression of breast cancer. Both *cyp19* and *cyp1b* are efficient E2 hydroxylases (Hayes et al., 1996; Singh et al., 2005). This would indicate the potential for overlapping substrate and also the potential for overlapping inhibitor specificity. A recent study, using breast tumour epithelium, measured the inhibiting properties of various AIs against *cyp19* and *cyp1b* to determine whether *cyp1b* was also affected by AIs (Rahman et al., 2008). Using various AIs, including fadrozole, the authors observed that only one of the AIs, vorozole, caused a significant decrease in *cyp1b* activity. The general conclusion with the exception of vorozole, was that AIs are selective for *cyp19* relative to *cyp1b*.

The present zebrafish study has identified the down-regulation of *cyp1b* in response to fadrozole, contradicting the Rahman et al. study. Their study was conducted using *in vitro* experiments and human *cyp1b* transcripts and proteins, whereas the present study was conducted using *in vivo* experiments with a teleost species. *In vitro* experiments do not always necessarily reflect what happens in an organism. The results provided in the present study would suggest that *cyp1b* in teleosts, particularly in zebrafish, are susceptible to AIs. Further analysis of additional AIs would be required to strengthen this statement. However, the end result remains that fadrozole causes a significant down-regulation of the *cyp1b* transcript in the telencephalon and hypothalamus of zebrafish. A possible explanation for this down-regulation would be that the *cyp1b* gene contains EREs in its regulatory regions. Similar to the down-

regulation of *cyp19b*, the decrease in E2 would lead to a down-regulation of *cyp1b* since it is also under that control of EREs,

4.2.4 Differentially regulated *Hox* Genes

A number of *hox* genes were identified by microarray analysis as being significantly regulated in response to fadrozole. *Hox* genes, along with multiple other genes involved with transcription factor complexes, were identified through annotational clustering (see Fig. 3C and A). Notably, *hoxa13*, *hoxb5*, and *hoxa11* were down-regulated whereas *hoxd12* was up-regulated.

Hox genes have been well characterized for their role in embryonic development. They participate in controlling developmental processes and patterning of the body axes during embryogenesis (Krumlauf, 1994). More recently, *hox* gene expression has been documented in adults where they participate in functional differentiation. Very few studies have examined the regulatory mechanisms that drive *hox* expression in adults. However, hormones such as E2, progesterone and testosterone have been recently shown to possibly regulate *hox* gene expression, thus controlling development in embryos as well as functional differentiation in the adults (Kolon et al., 1999; Satokata et al., 1995; Taylor et al., 1998). Researchers are now proposing that *hox* genes could be responsible for the retention of developmental plasticity in certain adult tissues. For teleosts, this would mean that the high levels of estrogens drive the expression of *hox* genes in turn leading to adult brain plasticity allowing for neurogenesis and brain repair.

Multiple EREs were recently identified in the *HOXA10* regulatory regions of humans (Akbas et al., 2004). This *hox* gene was found to be regulated by estrogens and

is thought to be an important signalling molecule. Furthermore, endocrine disrupting chemicals have been shown to impair the normal development of embryos. Many researchers believe that this could be accomplished through the disruption of *hox* gene expression. Estrogenic compounds, such as diethylstilbestrol and methoxychlor have also been shown to alter the expression patterns of selected *hox* genes (Akbas et al., 2004; Fei et al., 2005).

The previously mentioned EE2 study has also identified differentially regulated *hox* genes. Their study found an up-regulation of *hoxa9*, *hoxc4*, *hoxd11*, *hoxd10* and a down-regulation of *hoxb9* (Martyniuk et al., 2007). Even though none of the *hox* genes identified in the EE2 study correspond to *hox* genes identified in the present fadrozole study, these combined results provide further evidence that sex hormones are implicated in the regulation *hox* genes in teleost brains. Further studies may elucidate the specific roles of the identified *hox* genes in adult teleost brain and demonstrate the specific influences of sex hormones and regulatory mechanisms.

4.2.5 Differential Regulation of Genes Involved in the Phototransduction Pathway

The GO analysis produced cluster B (see Fig. 3B) that identified multiple genes involved in sensory perception and the detection of stimulus. Notably, *ops1*, *pdc1* and *art3* were identified by the microarray analysis and were later grouped in this annotation cluster. In mammals, these genes are known to be expressed in retinal photoreceptor cells and pinealocytes, which are the cells that comprise the pineal glad (Stryer, 1986). The proteins coded by these three genes are known to play important roles in the

phototransduction pathway participating in the primary function of the photoreceptor cells.

The retinal photoreceptor cell and pineal gland are closely related in function as well as tissue specific expression patterns and development (Deguchi, 1979; Oksche, 1984). The pineal gland secretes melatonin and in some species, including fish, retinal photoreceptor cells are also able to synthesis and secrete melatonin (Pang and Rose, 1987). The exact functional role of melatonin is not quite understood, although it is believed that it regulates diurnal rhythmic and reproductive seasonal physiology in some species (Takahashi, 1995). Several studies have suggested that melatonin could be also synthesised and secreted by other non-pineal tissues, such as the brain. A study conducted in mice identified a small number of cell types in the brain that express high levels of *art* and *pdc* (Sunayashiki-Kusuzaki et al., 1997). This was the first study to identify these proteins within the brain cells of a species, although the functional role remains to be clarified. The authors speculated that the identified *art* and *pdc*-producing brain cells may be producing and synthesising melatonin.

The present zebrafish study suggests that *art3*, *pdc1* and possibly also *ops1* are specifically expressed in discrete regions of the telencephalon of zebrafish. This would suggest that, in the brain, these genes are possibly involved in regulating diurnal rhythmic and reproductive seasonal physiology and that they are under the control of aromatase and/or sex hormones. This group of genes could also have a different or unidentified function in the brain of teleosts. It is possible that some of the tissues from the pineal gland were mistakenly also removed during the dissections of the telencephalon, although the dissections were carried out very precisely under a dissection microscope.

Nevertheless, this is the first study to report the regulation of this group of genes by an AI. Further studies are needed to identify the exact expression patterns of these genes and to characterize their functional role and importance in the brains of teleosts.

4.2.6. *Cry2b* is Up-Regulated

Microarray analysis of the telencephalon identified the up-regulation of *cry2b*. Cryptochromes are photoreceptors that regulate the circadian clock in plants and animals (Lin and Todo, 2005). Cryptochromes were first identified in plants where they play an important role in regulating germination, elongation and photoperiodism. They were later identified in various species including insects and mammals where they also play a crucial role in generating and maintaining the circadian rhythm.

A previous study has evaluated the expression of *cry1* and *cry2* mRNAs in the brain of rats (Nakamura et al., 2001). They also explored the possibility that estrogens directly regulate clock genes involved in the circadian rhythm particularly *cry 1* and *cry2*. This study was developed because of the evidence showing that the circadian rhythm could be modified by estrogens (Albers, 1981). The authors discovered that *cry2* expression was significantly increased in estrogen-treated rats suggesting hormonal regulation of *cry2* in the brain.

This present zebrafish study confirms the findings that *cry2* can be, directly or indirectly, regulated by sex hormones. This study further adds to the previous section where a group of genes was identified that could also be involved in regulating the diurnal rhythm. The rat study identified an up-regulation of *cry2* in response to estrogens, whereas this zebrafish study has also identified an up-regulation in response to

an AI. The AI exposure is thought to cause a decrease in estrogens. Several aspects have indicated this decrease although it is worth noticing that a decrease in estrogens was not actually measured. The *cry2b* up-regulation identified in this study could be caused by E2, the increase in testosterone and/or the general inhibition of the aromatase enzyme. Further studies would be required to assess and understand the regulation of this gene in response to sex hormone variations and to better understand the role of sex hormones in the circadian rhythm of teleosts.

4.2.7 Comparison of Genes Regulated by Fadrozole and 17α-Ethinylestradiol in Zebrafish

Several genes identified by microarray, in this fadrozole study, were also identified as being differentially regulated in a recent EE2 study (Martyniuk et al., 2007). These genes are particularly interesting because the fold-change observed in the fadrozole study is in the opposite direction of the fold-change observed in the EE2 study (see Table 6). Fadrozole leads to variations in sex hormones that are to some extent opposite of the variations in sex hormones caused by EE2. It is therefore expected that some common genes would be identified in these two studies.

Fab2 and *cel* were both identified by the EE2 study as being significantly down-regulated. The present study identified an up-regulation for both genes in response to the fadrozole exposure. These genes are involved in lipid metabolism. The EE2 study determined that an increase in estrogens caused a down-regulation of these genes, whereas the present study has identified the opposite effect, where a decrease in estrogens

or an increase in androgens, causes a significant up-regulation of these genes involved in lipid metabolism.

Another regulated gene identified in the present fadrozole study is *slc2,10*. Along with the genes involved in lipid metabolism, this gene was found to be significantly up-regulated. Many solute carrier genes were also found to be regulated in the EE2 study. These genes are part of a large group of membrane transport proteins that are involved in transporting a variety of molecules including amino acids, sugars, salts and metal ions. Interestingly, *slc2,10* is involved in the transportation of carbohydrates such as glucose. Lipid metabolism is closely connected to the metabolism of carbohydrates since carbohydrates can subsequently be converted into fats. The identification of this differentially regulated solute carrier gene further adds to the genes involved in lipid metabolism. The up-regulation of *fab2*, *cel* and *cel2,10* indicates an increase in lipid metabolism possibly leading to an increase in energy production.

Or2,2 was identified by the microarray analysis as being significantly down-regulated. The EE2 study identified this same gene as being significantly up-regulated in the telencephalon. Odorant receptors are responsible for the perception and distinction of thousands of different odorants. To date, 143 odorant receptors have been identified in the zebrafish genome (Alioto and Ngai, 2005). Specific functional information on each receptor is not available. The evidence provided in the present fadrozole study and the EE2 study both show that the *or2,2* is under the control of sex hormones, possibly E2. An increase in E2 would appear to cause an up-regulation of this specific odorant receptor, whereas a decrease in E2 causes a down-regulation. Odorant receptors are usually expressed in nasal olfactory sensory neurons (Sullivan et al., 1996).

Identification of the *or2,2* in the telencephalon of teleosts indicates a possible novel function. Further studies would be needed to characterise its function in the brain of teleosts. Future studies may also be necessary in determining how and why sex hormones are able to regulate its expression.

4.3 Microarray Validation and Analysis of Hypothalamic Gene Expression

Real-time RT-PCR was used to verify selected genes in the telencephalon that were identified as being differentially expressed by microarray analysis. The direction of the fold-change was validated for the majority of the selected genes (11 out of 12 genes). However, only 5 out of 12 relative fold-changes, in the telencephalon, were found to be confirmed using statistical analysis (see Table 7). The small number of validated genes could be explained by the low number of biological replicates (n = 3). It is thought that a much larger number of the genes would have been validated by real-time RT-PCR had the biological replicates been larger.

Analysis of the hypothalamus provided similar results in comparison to the data obtained from the telencephalon analysis. The directions of the fold-changes for the hypothalamus were the same for 8 of the 12 selected genes, in comparison to the microarray data. When comparing the data between the two brain tissues, 4 of the 5 genes that were confirmed by statistical analysis in the telencephalon, were also found to be significant in the hypothalamus. Similar to the results obtained for the telencephalon, it is expected that a greater number of genes would have been validated by real-time RT-PCR had the biological replicates been larger. Nevertheless, the data obtained from the

telencephalon and hypothalamus produced very similar results in reference to the confirmation of the direction of the fold-changes and the relative magnitude of the fold-changes, suggesting that both brain regions respond similarly to the AI.

4.4 In Situ Hybridizations

Even though in situ hybridization is not a quantitative process, it can be a very interesting approach for the analysis of microarray data. It allows visualisation of the spatial distribution of gene expression and furthermore permits a comparative analysis of the expression patterns between treated and control tissues. Three genes were analyzed by in situ hybridization in this study. All were found to confirm the results obtained by microarray analysis and real-time RT-PCR. The *cyp19b* in situs (Fig. 7A-H) showed apparently stronger expression throughout the telencephalic area. The *cyp1b* in situs (Fig. 7I-P) also showed apparently stronger expression in the control sections particularly in the telencephalon, and *cry2b* in situs (Fig. 7Q-X) confirmed an apparent stronger expression in the treated brains, once more, throughout the telencephalic area.

These three genes are hard to compare to recent studies since most of them have not been characterised by in situ hybridization before, especially in the zebrafish adult brain. A recent study was able to identify aromatase-positive cells in the forebrain of zebrafish (Pellegrini et al., 2007). The identification of cells expressing the aromatase protein was accomplished using immunohistochemistry. The immunohistochemistry technique produced very similar and comparable results, in reference to the *cyp19b* in situs in this present study (see Fig. 7A-H). The authors observed expression throughout the outer ridge of the median zone of the dorsal telencephalic area. Expression was also

observed along the unpaired medial ventrical including the dorsal nucleus and ventral zone of the ventral telencephalic area. These observations are comparable to the results observed in Figure 7C. The authors also observed expression alongside the outer ridge of the lateral zone of the dorsal telencephalic area and along the unpaired medial ventricle including nucleus of the ventral telencephalic area and the anterior part of the parvocellular preoptic nucleus. These observations are similar to the results observed in Figure 7E. In the caudal regions of the brain, the authors observed expression in the periventricular gray zone of the optic tectum, the posterior tuberal nucleus, the periventricular nucleus of the posterior tuberculum and in the dorsal and ventral zone of the hypothalamus. These observations are comparable to the results observed in Figure 7G. The similar regions of expression reported in the Pellegrini et al. study have helped to validate the results for the *cyp19b* in situs.

In situ hybridization has allowed a visual comparison of gene expression in response to the fadrozole exposure. By comparing treated and control brain sections it is clear that fadrozole appears to have a more pronounced effect in the telencephalic areas while the more caudal regions of the brain would appear to be less influenced by the AI treatment. This is possibly due to the fact that, in the teleost brain, aromatase is almost exclusively expressed in the forebrain region.

4.5 Future Work and Conclusion

Additional work to follow-up this research project could include the examination of the response to an AI in telencephalon and hypothalamus of female zebrafish. This would identify sex specific transcriptional responses to AIs and would also determine the hormonal response in female zebrafish. Other projects could include exposure to lower doses of fadrozole and multiple shorter exposure periods. This would allow the identification of genes directly regulated by fadrozole and would also allow the detection of genes involved in dose-response relationships. The study of multiple tissues, such as the liver and gonads, could also improve understanding of the effects of AIs on aquatic organisms and would allow the identification of tissue specific responses. All of these approaches would require rigorous normalization and analysis of the microarray data. Many methods for microarray data analysis exist. Choosing the appropriate method would depend on the specifics of the given experiments (Xiong et al., 2008).

This research project could also expand by studying different organisms. Evaluating the effects of AIs in different organism would allow a broader understanding of the genes and pathways affects by these compounds and would also increase generalizability which would strengthen the conclusions and results obtained in this study. No studies to data have evaluated the presence of aromatase inhibitors in the environment. This type of study would be necessary to determine potential risks to wildlife and humans and to determine the proper use and regulation of AIs.

This is one of the first studies to examine the transcriptional response to an AI. Through microarray and real-time RT-PCR, this study has identified genes that are directly and/or indirectly controlled by estrogens and androgens. The identification of

these regulated genes has helped to demonstrate the pronounced effects of AIs on gene expression in the brain of teleosts. Through in situ hybridizations, this study was able to compare expression patterns within the brain, of genes effected by fadrozole, which has helped to demonstrate the importance of hormone metabolism and its delicate balance in zebrafish. Collectively, these results provide a better understanding of the effects of AIs on gene expression and also shed light on the underlying effects of sex hormone variation and their importance in the brain of teleosts.

Acknowledgments

I would like to express my deep and sincere gratitude to my supervisor, Marc Ekker and co-supervisor, Vance Trudeau. Thank you for giving me the opportunity to study in your laboratories. Thank you also for your constructive comments and encouraging guidance throughout my masters project.

I would also like to thank Carole Yauk and David Lean for being advisory committee members; Sally Ng for assisting with sectioning and in situ hybridizations; Emily Gerrie and Vishal Saxena for giving countless hours on the dissection days; Dapeng Zhang and Jason Popesku for helpful advice with microarray and real-time RT-PCR analyses; Kate Crump and Colin Cameron for helping with RIAs; members of the Ekker lab, especially Gary Hatch and Luc Poitras; and David Hartnett for editorial assistance.

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