Impact of the antidepressant venlafaxine on the hypothalamus-pituitary-interrenal axis function in rainbow trout

by

Nataliya Melnyk-Lamont

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AUTHOR'S DECLARATION

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

STATEMENT OF CONTRIBUTIONS

Chapter 2 of this thesis has been submitted for publication in:

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Coauthor contributions: experiment conducted by NML and CB; CB contributed plasma measurements and enzyme activity data; NML contributed GR protein expression and interrenal transcript abundance data; GM contributed Na^+K^+ATPase protein expression data.

[*Ex vivo* head kidney study in Chapter 3 (conducted and analyzed by NML) was not part of this publication submission].

Abstract

Over the recent years, venlafaxine has become the predominant antidepressant drug detected in municipal wastewater effluents (MWWE) and aquatic systems. However, very little is known about the effect of this drug in the aquatic environment on non-target organisms, including fish. Venlafaxine is a pharmaceutical compound designed to inhibit serotonin and norepinephrine reuptake, thereby increasing the synaptic availability of these neurotransmitters. In teleosts, the key aspect of stress adaptation involves the activation of the hypothalamus-pituitary-interrenal (HPI) axis, leading to the production of cortisol. Given that monoamine neurotransmitters (serotonin, norepinephrine, and dopamine) are involved in the regulation of a wide range of neuroendocrine responses, including stress axis function, my primary hypothesis was that venlafaxine acts as a neuroendocrine disruptor impacting the functioning of the corticosteroid stress axis in rainbow trout (*Oncorhynchus mykiss*). This hypothesis was tested through a series of *in vivo* exposure studies, as well as *in vitro* experiments, using environmentally relevant levels of venlafaxine, in order to tease out potential mode of action of this drug on target tissues involved in HPI axis functioning.

The results suggest that venlafaxine alters monoamine neurotransmitter levels and their turnover rates in a region-specific manner in trout brain, and that the midbrain is the prime target. The monoamine changes may be responsible for the downstream effects on neuroendocrine responses coordinated in the hypothalamus, as this region receives monoaminergic inputs from the midbrain. The functional relevance of the above finding was confirmed by showing that venlafaxine exposure disrupted the neuroendocrine responses associated with social stress and appetite regulation. Functional downstream effects of HPI axis dysfunction were further confirmed by subjecting the fish to a handling disturbance, which revealed that the highly conserved cortisol and glucose responses to stressors were disrupted by venlafaxine. Also, there were tissue-specific effects of venlafaxine exposure on metabolic capacities, including enhanced gluconeogenesis and amino acid catabolism in the liver (a key glucose producing tissue), and alterations in the glycolytic capacity and sodium potassium ATPase activity in the gill (a key glucose utilizing tissue).

The results suggest that the mode of action of venlafaxine may involve disruption of each target tissue involved in the HPI axis functioning. *In vitro* mechanistic studies indicated that hypothalamus functioning is disrupted by venlafaxine and this may involve effects mediated by serotonergic pathways. The reduced phosphorylation of cAMP response element binding protein (CREB) suggests that venlafaxine may impact downstream signalling cascades that are CREB-dependent. The transcript changes observed with venlafaxine in the hypothalamus include changes in mRNA levels of key genes involved in appetite regulation and stress response, including corticotropin releasing factor (CRF) and neuropeptide Y (NPY). At the pituitary level, venlafaxine impaired adrenocorticotropic hormone (ACTH) production, and this involved disruption of corticotropin releasing factor-receptor type 1 (CRF-R1), which is a key sensor for CRF stimulation. At the interrenal tissue level, the responsiveness of steroidogenic cells to ACTH stimulation was altered by venlafaxine and the mode of action appears to involve pathways upstream of the intracellular cAMP production. Also, cortisol biosynthetic capacity was disrupted by venlafaxine and this was accompanied by changes in transcript abundances of steroidogenic acute regulatory protein and cytochrome P450 side chain cleavage in the interrenal tissue.

Taken together, the results demonstrate for the first time that the antidepressant venlafaxine, a human pharmaceutical contaminating aquatic systems, disrupts neuroendocrine responses and affects stress, feeding and metabolic responses in rainbow trout. The mode of action may include disruptions in brain monoamine levels and pathways involved in CREB signalling, while the exact mechanism of action remains to be elucidated. Exposure of fish to this pharmaceutical drug adversely affects the highly conserved adaptive responses that are essential to cope with subsequent stressors, and may translate into reduced fitness over the long-term. The findings underscore the necessity to understand the mechanisms of action of chemicals present in MWWE, and develop and utilize effective risk management strategies aimed at minimizing discharge of pharmaceuticals into the aquatic environment.

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To my family, I am very grateful for your endless love, moral support and encouragement. Most importantly, I will forever be thankful to my husband and my best friend, Ryan Lamont, for giving me strength and endless reassurance.

Dedication

To my parents, who guided me in life and have always been my source of strength and motivation

Table of Contents

List of Figures

CHAPTER 4

CHAPTER 5

CHAPTER 6

Appendix A

Appendix B

Appendix C

List of Tables

List of Abbreviations

3,4-dihydroxyphenylacetic acid (DOPAC)

5-hydroxyindoleacetic acid (5-HIAA)

5-hydroxytryptamine or serotonin (5-HT)

Adrenocorticotropic hormone (ACTH)

Alanine aminotransferase (AlaAT)

Aspartate aminotransferase (AspAT)

Cerebellum (Cer)

Corticotropin-releasing factor (CRF)

Corticotropin-releasing factor binding protein (CRF-BP)

Corticotropin-releasing factor receptor (CRF-R)

Cyclic AMP response element binding protein (CREB)

Cytochrome P450 side-chain cleavage (P450scc)

Dopamine (DA)

Elongation factor 1α (EF1α)

Glucocorticoid receptor (GR)

Glucocorticoid response element (GRE)

Glucokinase (GK)

Glucose transporter type 2 (Glut2)

Hexokinase (HK)

Hindbrain (HB)

Hypothalamus-pituitary-adrenal (HPA)

Hypothalamus-pituitary-interrenal (HPI)

Hypothalamus (Hyp)

Lactate dehydrogenase (LDH)

Melanocortin 2 receptor (MC2R)

Midbrain (MB)

Mineralocorticoid receptor (MR)

Municipal wastewater effluent (MWWE)

Neuropeptide Y (NPY)

Norepinephrine (NE)

Nucleus preopticus (NPO)

Optic tectum (OT)

Phosphoenolpyruvate carboxykinase (PEPCK)

Pituitary (Pit)

Preoptic area (POA)

Pro-opiomelanocortin (POMC)

Protein kinase A (PKA)

Pyruvate kinase (PK)

Sodium-potassium adenosinetriphosphatase (Na^+-K^+ ATPase)

Steroidogenic acute regulatory protein (StAR)

Telencephalon (Tel)

Venlafaxine (VEN)

Chapter 1

General Introduction

1.1 Overview

Over the last two decades, pharmaceuticals became recognized as an important source of environmental contamination. Many drugs are only partially removed by wastewater treatment process, and often remain biochemically active upon reaching the ecosystem. Human consumption of antidepressant drugs has been on the rise, and venlafaxine has become a predominant antidepressant detected in aquatic environments across North America and Europe. Main therapeutic properties of this psychotropic drug are based upon modulation of important monoamine neurotransmitters, serotonin and norepinephrine, in the human brain. Many drug targets, including neurotransmitter systems and neuroendocrine pathways, are highly conserved among vertebrates, suggesting that fish are likely to be affected by environmental drug exposure. Monoamine neurotransmitters are intimately interconnected with the neuroendocrine pathways that regulate various physiological and behavioural stress response mechanisms. Stress response is an essential adaptive mechanism that allows an animal to meet increased energy demands associated with stressor exposure and to regain homeostasis. In fish, a non-target organism for human pharmaceuticals, alterations of monoamine levels by antidepressants, including venlafaxine, may disrupt neuroendocrine components of the hypothalamus-pituitary-interrenal (HPI) axis, leading to impact on downstream physiological and/or behavioural responses. This chapter provides insights into the main concepts addressed throughout this thesis in order to establish potential links between environmental exposure to this pharmaceutical and stress physiology in fish, and highlight a relative lack of knowledge in this area.

1.2 Pharmaceuticals in the aquatic environment

Municipal wastewater effluent (MWWE) is a primary source introducing pharmaceutical contamination in the aquatic environment (Chambers *et al.,* 1997). Inadequacy of wastewater treatment methods combined with low environmental degradability leads to environmental pollution with pharmaceutical compounds and their metabolites, which often remain biochemically active upon reaching the ecosystem (Corcoran *et al.,* 2010).

The issue of environmental contamination with pharmaceutical agents did not receive much attention until the 1990s (Henschel *et al.,* 1997; Purdom *et al.,* 1994), when it was

discovered that many compounds can exhibit biological effects and have an ability to interfere with ecosystems in trace concentrations, as low as few nanograms per litre (Halling-Sørensen *et al.,* 1998). Development of analytical methods for detection and quantification of pharmaceutical residues in environmental samples (Gros *et al.,* 2006; Ternes, 2001) allowed conducting accurate assessments of contamination levels. The rise in pharmaceutical consumption world-wide has resulted in an increasing deposition of these drugs into the aquatic environment (Corcoran *et al.,* 2010). Pharmaceutical contamination is of major concern in regards to its impact on ecosystem health, especially because so little is known about its effect on non-target organisms, including fish.

Human pharmaceutical groups that are commonly detected in the environment include analgesics, antibiotics, β-blockers, lipid-regulators, anti-inflammatories, contraceptives and psychiatric drugs (e.g. anxiolytics, sedatives, antidepressants) (Jones *et al.,* 2001). In most cases pharmaceuticals are characterized by highly specific modes of action to reduce potential toxicity, a high degree of stability and prolonged activity to minimize dosing requirements and ensure sufficient therapeutic activity before elimination (Winter *et al.,* 2010).

A large number of potential target sites for pharmaceutical agents are evolutionarily conserved across the animal kingdom (e.g. neurotransmitter systems, metabolic pathways, signalling pathways, etc.), therefore, many compounds that are designed to affect humans or livestock are likely to be biologically active in non-target wildlife species (Gunnarsson *et al.,* 2008; Corcoran *et al.,* 2010). As a result, inadvertent exposure of non-target organisms, such as fish, to active pharmaceutical components may lead to adverse effects that may translate into reduced fitness and reproduction (Santos *et al.,* 2010). From an ecotoxicological standpoint, it is important to study the effects of the MWWE mixtures on aquatic organisms in order assess overall exposure impact. In addition, it is also essential to investigate effects of individual pharmaceutical components of such complex effluent mixtures in order to elucidate their mechanisms of action in non-target species.

1.2.1 Antidepressant drugs

Over the last two decades, antidepressant drugs have become a significant source of aquatic contamination due to a constantly increasing human consumption. Antidepressant

drugs are in the top ten list of pharmaceutical classes ranked by global sales, and antidepressants comprise a large portion of worldwide medicinal prescriptions (IMS Health, 2010). Selective serotonin reuptake inhibitors (SSRIs) and serotonin norepinephrine reuptake inhibitors (SNRIs) are the most common classes of antidepressants that are used to treat depression, anxiety, obsessive-compulsive disorders, and panic disorders (Silverstone, 2004). SSRIs (e.g. fluoxetine, sertraline, citalopram) selectively inhibit presynaptic neuronal reuptake of serotonin (5-HT), while SNRIs (e.g. duloxetine, venlafaxine) block 5-HT and norepinephrine (NE) reuptake, thus increasing synaptic availability of these neurotransmitters (Horst and Preskorn, 1998) (Fig. 1-1). Interestingly, antidepressant effects of drugs with 5-HT reuptake inhibiting properties appear only after 2-4 weeks of administration, despite the fact that reuptake inhibition happens immediately (Celada *et al.*, 2004; Gardier *et al.*, 1996). Acute administration of SSRIs leads to a rapid elevation of extracellular 5-HT levels due to reuptake inhibition, and is then followed by activation of an intrinsic negative feedback mechanism that controls presynaptic neuronal firing. This mechanism involves somatodendritic 5-HT_{1A} autoreceptors on presynaptic neurons. However, chronic treatment leads to desensitization of these autoreceptors and normalizes firing rate of presynaptic 5-HT neurons, while postsynaptic 5-HT_{1A} receptors become sensitized in the forebrain areas (Blier and Abbott, 2001; Hjorth *et al.*, 2000). Consequently, long-term treatment with SSRIs results in amplification of 5-HT neurotransmission and onset of the antidepressant effects. However, there is no conclusive evidence confirming 5-HT autoreceptors desensitization by SNRI drugs (Roseboom and Kalin, 2000).

History of the antidepressant drugs [reviewed in (Janowsky, 2004)] goes back to the late 1950s. Introduction of monoamine oxidase inhibitors (MAOIs) and the tricyclic antidepressants revolutionized the treatment of depression. Due to undesirable side effects of MAOIs (e.g. induction of hypertensive crisis), their popularity decreased, while tricyclic antidepressants (e.g. imipramine, desipramine) gained popularity. In the late 1980s SSRIs were introduced on the market (beginning with fluoxetine) and soon after, commercialization of similar compounds followed (paroxetine, sertraline, fluvoxamine). SSRIs soon replaced tricyclic antidepressants due to their characteristics, which offered reduced side effects in comparison to the tricyclic compounds (i.e. lack of lack of anticholinergic effects, reduced toxicity in overdose). In the mid-1990s, SRNIs were introduced (serotonergic and

noradrenergic agents), and they also attained high popularity as effective antidepressant agents.

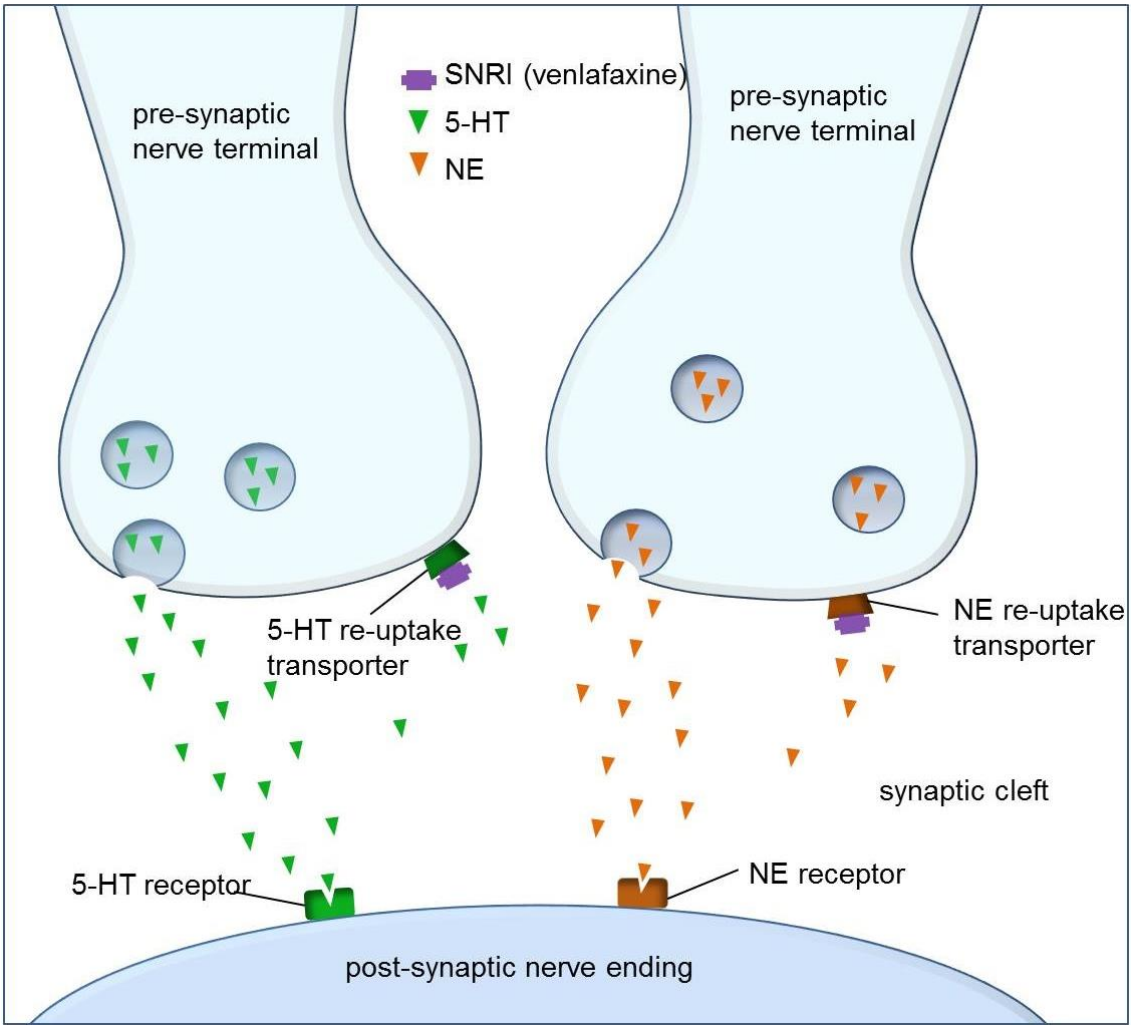


Figure 1-1. Diagram representing pharmacological mechanism of serotonin and norepinephrine reuptake inhibitors (SNRIs), such as venlafaxine. When pre-synaptic nerve terminals of serotonergic and noradrenergic neurons are depolarized, serotonin (5-HT) and norepinephrine (NE) are released into the synaptic cleft and subsequently bind to their corresponding receptors on a post-synaptic cell. Venlafaxine blocks 5-HT and NE re-uptake transporters, which take up these neurotransmitters back into the presynaptic terminals, leading to increasing concentrations of these neurotransmitter in the synapse.

1.2.2 Pharmacological profile of venlafaxine

Over the last decade, venlafaxine, also known under the brand name Effexor (first introduced by Wyeth Pharmaceuticals, Inc.), became one of the most frequently prescribed antidepressant drugs due to its faster therapeutic onset and fewer side effects in patients (Horst and Preskorn, 1998). According to IMS Health (2011), venlafaxine was number seven on the list of most dispensed medications in 2010 in Canada. Although it is a dual reuptake inhibitor, it has a stronger effect on 5-HT reuptake compared to NE (Roseboom and Kalin, 2000). This drug also has a relatively weak effect on dopamine (DA) reuptake (Roseboom and Kalin, 2000). Venlafaxine can modulate DA levels in a region-specific manner, although the underlying mechanism may be a reflection of alteration in NE levels, since noradrenergic neurons modulate mesolimbic and mesocortical DA systems (Muneoka *et al.*, 2009; Liprando *et al.*, 2004). Venlafaxine undergoes first-pass metabolism in liver, particularly by the CYP2D6 isoenzyme (Thase and Sloan, 2009). Its major metabolite is O-desmethylvenlafaxine, which has a similar potency to the parent compound for inhibiting neurotransmitter reuptake, and it contributes significantly to the overall antidepressant efficacy of the drug (Roseboom and Kalin, 2000). Renal elimination of venlafaxine and its metabolites is the primary route of excretion for these compounds; clearance rate O-desmethylvenlafaxine (10 h half-life) is slower than that of venlafaxine (4 h half-life) (Thase and Sloan, 2009).

1.2.3 Environmental presence of venlafaxine

Environmental values of venlafaxine were reported as high as 2.19 μg/L in MWWE (St. Paul, MN, USA) and 1.31 μg/L in the effluent-dominated creek (Pecan Creek, Denton, TX) (Schultz and Furlong, 2008). A recent study reported the occurrence of these compounds in different German rivers and streams at concentrations ranging from 18-122 ng/L for venlafaxine, and 23-743 ng/L for O-desmethylvenlafaxine (Rúa-Gomez and Püttmann, 2012).

Metcalfe *et al.* (2010) reported that locally, in the Grand River watershed (ON, CAN), venlafaxine and its metabolite were present in significantly higher concentrations compared to other antidepressants/metabolites in the untreated and treated MWWE, as well as in the receiving waters as far as 17 km downstream of a municipal wastewater treatment

plant. The removal rates for these compounds from the MWWE were around 40%. Concentrations of venlafaxine and O-desmethylvenlafaxine were measured at 0.253 µg/L and 0.486 µg/L, respectively, in MWWE collected downstream of the tertiary sewage treatment plant in Guelph, ON, CAN (Ings, 2011). In general, removal rates for venlafaxine and its major metabolite were shown to be inefficient, leading to their continual discharge in waters (Rúa-Gomez and Püttmann, 2012; Schultz *et al.*, 2010; Metcalfe *et al.*, 2010). Indirect photodegradation appears to be the dominant degradation process responsible for removal of these pharmaceuticals from the environment, and the half-lives of venlafaxine and O-desmethylvenlafaxine determined from the field experiment were 51 and 18 h, respectively (Rúa-Gómez and Püttmann, 2013).

1.2.4 Effects of antidepressants on non-target species

Despite the overwhelming evidence of environmental contamination with antidepressant drugs, relatively little research has been carried out to elucidate their potential effects as well as their mechanisms of action on non-target animals, including fish. The majority of research in this field has been focused on fluoxetine (also known under the trade name Prozac, originally marketed by Eli Lilly & Co.), which was marketed as the first SSRI drug in 1987 (Mennigen *et al.*, 2011). It remains one of the most commonly prescribed antidepressants and frequently detected in the environment (Oakes *et al.*, 2010). Fluoxetine and norfluoxetine show a high degree of bioaccumulation in fish relative to other antidepressants, especially in the neural tissue (Schultz *et al.*, 2010). Studies demonstrated that fluoxetine reduces fish growth and feeding in fathead minnows (*Pimephales promelas*) (Stanley *et al.*, 2007), increases circulating estradiol levels in females of Japanese medaka (*Oryzias latipes*) (Brooks *et al.*, 2003), affects ovarian steroid hormone levels and gene expression in zebrafish *(Danio rerio)* (Lister *et al.*, 2009), decreases food intake, weight and disrupts reproductive physiology in goldfish (*Carassius auratus*) (Mennigen *et al.*, 2010; Mennigen *et al.*, 2009), and diminishes aggressive behaviours in Siamese fighting fish (*Betta splendens*) (Dzieweczynski and Hebert, 2012). Another environmental concern regarding pharmaceutical exposure arises from the fact that some compounds may have synergistic or additive effects due to similarities in their pharmacological modes of action. For example, Christensen *et al*. (2007) have demonstrated that several SSRIs produced additive toxic

effects on the tested algae and crustacean species. However, there is no data available on possible additive or synergistic effects of antidepressants from the SNRI class, such as venlafaxine and duloxetine.

Despite the growing environmental relevance of venlafaxine, only a small number of studies have examined the effect of this drug in fish. In trout hepatocytes *in vitro*, high concentrations of venlafaxine (100 and 1000 nM) attenuated the epinephrine-stimulated glucose production and this involved inhibition of β-adrenoreceptor signalling (Ings *et al*., 2012). Genome analysis in fathead minnows exposed to pharmacological concentration of venlafaxine revealed upregulation of several genes involved in neural development and signalling (Thomas *et al.,* 2012). Painter *et al*. (2009) demonstrated that environmentally relevant concentrations of venlafaxine adversely affect predator avoidance behaviour in larval fathead minnows. Assessments of venlafaxine levels in the neural tissue of fish suggest that this drug does not bioaccumulate during environmental exposures (Schultz *et al.,* 2011; Schultz *et al.,* 2010). Fathead minnows exposed to environmentally relevant concentration (305 and 1104 ng/L) of venlafaxine suffered significant mortality after a 21-day exposure (Schultz *et al*., 2011). A study based on a 6-day exposure of hybrid striped bass (*Morone saxatilis x Morone chrysops*) to venlafaxine (35-500 μg/L range) showed a significant reduction in total brain 5-HT levels and increased length of time needed to capture pray (Bisesi *et al.,* 2014). Together, the picture that is emerging from these studies is that venlafaxine may be acting as a neuroendocrine disruptor in fish. As corticosteroid response to stressor is a highly conserved adaptive response in vertebrates to reestablish homeostasis, any impact on this neuroendocrine stress response by the drug may lead to reduced fitness.

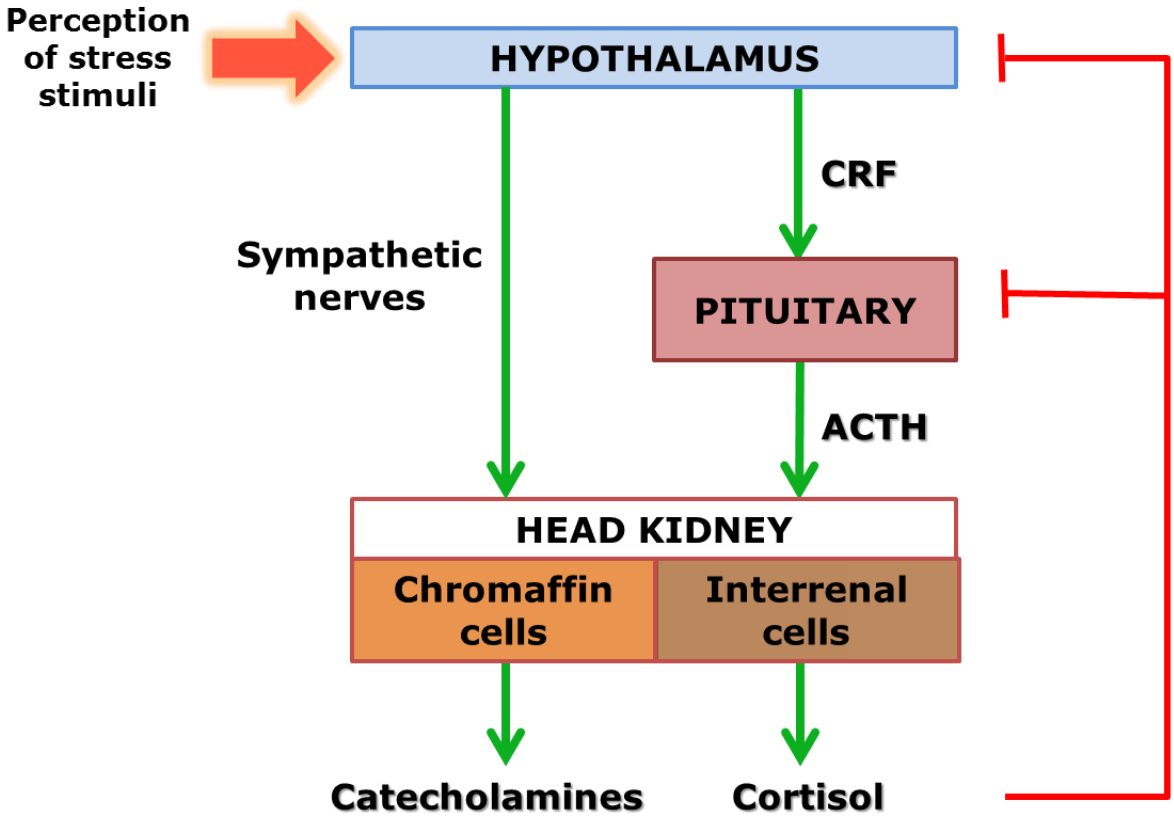
1.3 Stress response in fish

In fish, as in other vertebrates, stress involves disturbance of the homeostatic equilibrium of the organism by stressors, which may include intrinsic or extrinsic stimuli (chemical, physical, or biological) (von Holst, 1998; Vijayan *et al*., 2010). In order to regain homeostasis, fish exhibit an adaptive response that consists of a suite of coordinated physiological and behavioural reactions, referred to as the stress response (Vijayan *et al.,* 2010). Such adjustments ultimately result in altered digestive, respiratory, metabolic, osmoregulatory, immune and reproductive functions in order to re-establish the internal

milieu (Sapolsky *et al.,* 2000). Physiological responses to acute stress are generally categorized into the primary (neuroendocrine/endocrine activation), secondary (biochemical and physiological adjustments) and tertiary responses (population level changes).

The primary response (Fig. 1-2) involves a rapid increase in tone of the sympathetic nervous system and activation of the hypothalamic-sympathetic-chromaffin cell axis (HSC), also known as the fight-or-flight response (Mommsen *et al*., 1999). HSC axis activation leads to the release of catecholamines, epinephrine (E) and norepinephrine (NE), into the general circulation from the chromaffin cells (Vijayan *et al.,* 2010). Chromaffin cells are clustered within the anterior (head) kidney region around post-cardinal vein along with glucocorticoid-producing interrenal cells (Hontela and Vijayan, 2009; Wendelaar Bonga, 1997). Release of catecholamines is essential for adjustment of cardiovascular and respiratory systems for enhanced blood oxygen transport, and mobilization of energy substrates to meet increased energy demands that accompany stress (Reid *et al.,* 1998; Wendelaar Bonga, 1997). The activation of the hypothalamus-pituitary-interrenal axis (HPI), which is analogous to mammalian hypothalamic-pituitary-adrenal axis (HPA), is delayed (within minutes) relative to catecholamine secretion. Activation of the HPI axis leads to the release of cortisol, the primary glucocorticoid hormone in teleosts, which is synthesized by the steroidogenic cells dispersed within the interrenal tissue (Vijayan *et al.,* 2010). Fish, unlike mammals, do not have a discrete adrenal gland and the interrenal tissue is distributed mostly in the head kidney region (Wendelaar Bonga, 1997; Mommsen *et al*., 1999). Cortisol mediates essential physiological adaptations to stress, including regulation of energy metabolism, hydromineral balance, and immune functions (Wendelaar Bonga, 1997; Mommsen *et al.,* 1999).

Figure 1-2. The stress axis in teleost fish. Upon perception of stress stimuli, immediate neuroendocrine response is activated. Rapid activation of the hypothalamic-sympathetic-chromaffin cell (HSC) axis leads to increased sympathetic nerve firing and release of catecholamines (epinephrine and norepinephrine) from chromaffin cells in the head kidney region into the general circulation. Activation of the hypothalamus-pituitary-interrenal axis (HPI) involved release of corticotropin-releasing factor (CRF) from hypothalamus, which stimulates corticotroph cells of the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) into the general circulation. ACTH acts on the interrenal cells of the head kidney to increase cortisol production and output into the bloodstream. Cortisol release is regulated by a negative feedback mechanism. Overall, the stress hormones (catecholamines and cortisol) will initiate a series of physiological adjustments that allow an animal to re-establish homeostasis. Green arrows indicate secretion; red lines indicate negative feedback inhibition. Adapted from Wendelaar Bonga (1997).



- Plasma glucose
- Glycogenolysis
- Lipolysis
- Cardiovascular changes
- Respiratory changes
- Hydromineral balance

- Plasma glucose
- Gluconeogenesis
- Proteolysis
- Lipolysis
- Hydromineral balance
- Immune functions
- Reproduction
- Growth

The secondary stress response mediated by catecholamines and glucocorticoids results in a series of physiological adjustments (e.g. metabolism, cellular changes, acid-base and hydromineral balance, immune function), enhancing delivery of energy substrates to tissues with higher energy demands (Barton, 2002; Mommsen *et al.,* 1999). In addition, tertiary response may occur, involving changes at the whole-animal and populations levels (Barton, 2002; Iwama, 1998). For instance, prolonged stress response may lead to exhaustion associated with reallocation of energy resources away from critical processes to cope with stress. This may lead to negative consequences, including decreased growth and reproduction, increased disease susceptibility and mortality (Barton, 2002; Iwama, 1998).

1.4 Hypothalamus-pituitary-interrenal axis (HPI)

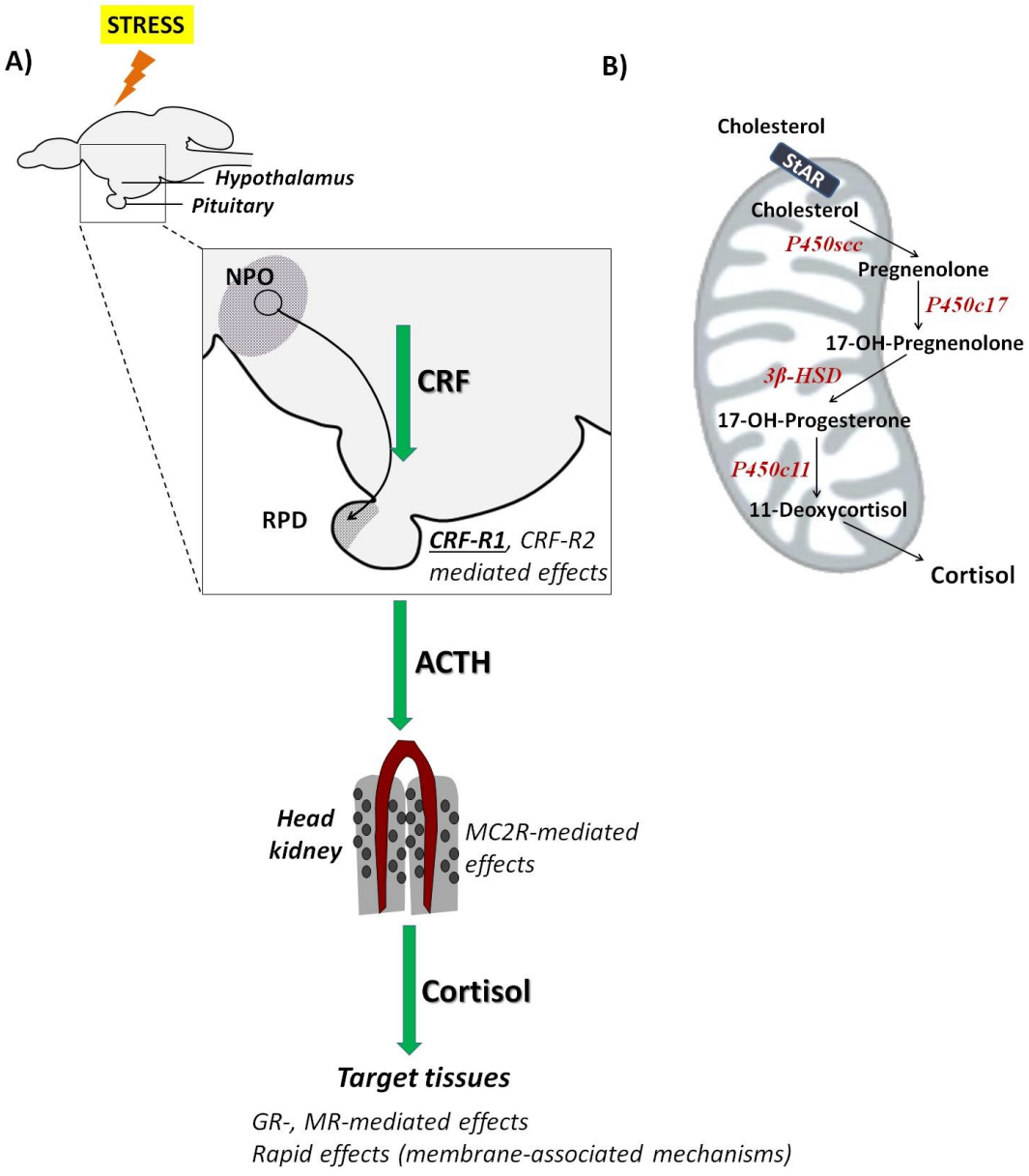
As stated earlier, activation of the HPI axis, resulting in the synthesis and release of glucocorticoid hormone cortisol, is an integral component of stress response (Fig. 1-3A). The following section describes in detail elements of the teleost HPI axis, as well as the neuroendocrine and endocrine responses involving the hypothalamus, pituitary and interrenal tissue.

1.4.1 Hypothalamus

Activation of the HPI axis is initiated at the level of hypothalamus, a brain region that receives and integrates inputs transmitted from central and peripheral nervous systems. The first step in the HPI axis activation leads to the release of the neuropeptide, corticotropin releasing factor (CRF), from the hypothalamic neurosecretory cells into the anterior pituitary (Fig. 1-3A). CRF stimulates secretion of the adrenocorticotropic hormone (ACTH) by corticotroph cells in the anterior pituitary (rostral pars distalis) (Mommsen et al., 1999; Wendelaar Bonga, 1997). In mammals, CRF is delivered to the anterior pituitary via intrinsic hypophyseal circulatory portal system (Chrousos, 1998). Teleosts, in contrast, do not have such hypophyseal portal system and axon projections of the hypothalamic neurosecretory cells make direct synaptic contacts with the pituitary corticotrophs (Bernier *et al.,* 2009). CRF-producing neurons originating in the nucleus preopticus (NPO), analogous to the mammalian paraventricular nucleus (PVN), are known to play a pivotal role in stress-mediated ACTH secretion (Flik *et al.,* 2006). CRF bioavailability is regulated by CRF binding protein (CRF-BP) (Bernier *et al.,* 2008; Bernier, 2006). This neuropeptide mediates

its effects via two types of receptors in teleosts: CRF-R1 and CRF-R2 (Pohl *et al.*, 2001). CRF-R1 plays an important role in the stress axis, while CRF-R2 is involved in a broad range of physiological and behavioural responses (e.g. feeding, learning, vascular tone) (Flik *et al.*, 2006). Even though CRF is a major player of stress-induced ACTH secretion in fish, other hypothalamic neuropeptides, including arginine vasotocin and urotensin, are also involved in ACTH secretion (Bernier *et al.*, 2009).

Figure 1-3. Overview of the major components of the teleost hypothalamus-pituitary-interrenal axis (HPI) and associated signalling cascades **(A)**. Sagittal view of the brain shows enlarged preoptic and hypothalamic brain areas along with pituitary gland. Corticotropin-releasing-factor (CRF)-producing neurons originating from the nucleus preopticus (NPO) make synaptic connections with the corticotroph cells in the rostral pars distalis (RPD) of the pituitary. CRF release from NPO-originating neurons leads to activation of CRF-R1 and CRF-R2 (receptor 1 and 2) signalling in the corticotrophs, leading to secretion of adrenocorticotropic hormone (ACTH) into the bloodstream. In the interrenal cells (in head kidney region), ACTH initiates cortisol synthesis via melanocortin type 2 receptor (MC2R) signalling. Upon reaching target tissues, cortisol mediates its genomic effects via glucocorticoid and mineralocorticoid receptors (GR and MR, respectively), and rapid non-genomic action by membrane-associated mechanisms. Representation of cortisol biosynthetic pathway (taking place in the interrenal cells) showing enzymatic reactions occurring within the mitochondrial compartment (inside shaded area) and cytosolic compartment (outside shaded area) **(B)**. Once cholesterol is transported to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR), it gets converted to pregnenolone by cytochrome P450 side chain cleavage enzyme (P450scc), which serves as a substrate for a series of reactions catalyzed by 17-α-hydroxylase (P450c17), 3β-hydroxysteroid dehydrogenase (3β-HSD), steroid 21-hydroxylase (P450c21), and 11-β-hydroxylase (P450c11), producing cortisol. Adapted from Mommsen *et al.* (1999).


STRESS
A)
Hypothalamus
Pituitary
NPO
CRF
RPD
CRF-R1, CRF-R2
mediated effects
ACTH
Head
kidney
MC2R-mediated
effects
Cortisol
Target tissues
GR-, MR-mediated effects
Rapid effects (membrane-associated mechanisms)
B)
Cholesterol
StAR
Cholesterol
P450scc
Pregnenolone
P450c17
17-OH-Pregnenolone
3β-HSD
17-OH-Progesterone
P450c11
11-Deoxycortisol
Cortisol

1.4.2 Pituitary gland

Pituitary corticotroph cells produce ACTH from a precursor peptide pro-opiomelanocortin (POMC) and release it into the general circulation (Mommsen *et al.*, 1999; Wendelaar Bonga, 1997). In vertebrates, POMC is a precursor for several biologically active peptides, including the opioid, β-endorphin, and the melanocortin-related peptides, ACTH, α-melanocyte stimulating hormone (α-MSH), β-MSH, and γ-MSH (Tanaka, 2003). Proteolytic cleavage of large POMC peptide is catalyzed by prohormone convertases (PC) in a tissue-specific manner. In the corticotrophs, the major products of POMC cleavage by PC1 include ACTH and β-lipotrophic hormone. Salmonids possess two copies of POMC genes, POMC A and POMC B, which arose from a genome duplication event (Salbert *et al.*, 1992). Both gene products are found in the pituitary, and expression of the different POMC transcripts could vary with life-history stage or in response to environmental conditions (Leder and Silverstein, 2006).

1.4.3 Interrenal tissue

The sequence of events in the interrenal tissue involves the binding of ACTH to melanocortin 2 receptor (MC2R), leading to the activation of cortisol biosynthetic pathway (Aluru and Vijayan, 2008). MC2R is a G-protein-coupled receptor belonging to the family of melanocortin receptors (MCRs). ACTH binding to MC2R activates adenylate cyclase and cAMP signalling cascade, leading to initiation of cholesterol delivery to the inner mitochondrial membrane, followed by a series of enzymatic reactions (Vijayan *et al.*, 2010). Steroidogenic acute regulatory protein (StAR) mediates transport of cholesterol to the inner mitochondrial membrane (Sierra, 2004), and this step is often considered as the rate-limiting step in steroid synthesis. Also, peripheral-type benzodiazepine receptor (PBR) is known to facilitate mitochondrial cholesterol trafficking (Papadopoulos, 2004), but its precise role in fish remains unclear. The first enzymatic reaction involves conversion of cholesterol to pregnenolone by cytochrome P450 side chain cleavage (P450scc) and pregnenolone serves as a precursor for all biological steroids. A detailed pathway of cortisol biosynthesis is presented in Fig. 1-3B. Although several pituitary and extra pituitary hormones (e.g. α-MSH, β-endorphin) are involved in regulation of cortisol secretion, ACTH is the primary

secretogogue for cortisol secretion in fish (Vijayan *et al*., 2010; Bernier *et al*., 2009; Wendelaar Bonga, 1997; Mommsen *et al.*, 1999).

1.4.4 Plasma cortisol response

Cortisol response to acute stressor is a well-established physiological response and commonly used as an indicator of stress in fish. Acute stressor exposure causes a transient surge in plasma cortisol concentrations, followed by a decline to basal levels over a 24 h period (Vijayan *et al.,* 2010). Plasma levels of cortisol are dependent upon the net effect of its production and clearance, and hormonal clearance is usually determined by a combination of several factors, including binding proteins, target tissue receptors, uptake and catabolism (Mommsen *et al.,* 1999). Mammals are known to have cortisol binding globulin (CBG) regulating the amount of free plasma cortisol (i.e. bioavailable form) (Gayrard *et al.,* 1996), while there is no evidence of CBG in the plasma of fish (Mommsen *et al*., 1999). Both sensitivity of the stress axis and amplitude of cortisol output are influenced by genetic, developmental and environmental factors, as well as the intensity and duration of the stressors (Vijayan *et al.*, 2010; Barton, 2002). For example, strain differences in cortisol responsiveness to stressors have been demonstrated and used to establish high- and low-responsive rainbow trout lines by selective breeding (Pottinger and Carrick, 1999). Under non-stressed conditions, basal cortisol levels are influenced by circadian rhythms for maintenance of normal physiological functions (Wendelaar Bonga, 1997). In addition, seasonal rhythms, reproductive state and gonadal steroids also affect basal and stress-induced cortisol levels (Norris, 2000). Measurement of plasma cortisol levels provides a great tool for assessment of acute stress in fish. Since catecholamine response is very rapid and occurs immediately when animals are handled, measuring resting (basal) plasma levels is very difficult. In contrast, stress-induced cortisol release is slightly delayed, and resting levels can be accurately measured when animals are anaesthetised and sampled quickly (Vijayan *et al.,* 2010).

1.4.5 Cortisol signalling

Genomic signalling of cortisol and its negative feedback regulations are mediated by intracellular corticosteroid receptors, glucocorticoid (GR) and mineralocorticoid (MR), which are ligand-dependent transcription factors (Aluru and Vijayan, 2009). In brief,

classical GR signalling (Fig. 1-4) is initiated by binding of cortisol to cytosolic GR. Upon binding, molecular chaperones that are associated with GR in the cytoplasm are released. A homodimer of activated GR forms in the cytoplasm, and following translocation into the nucleus, it binds to a glucocorticoid response element (GRE) within a promoter region of a glucocorticoid-responsive gene leading to alterations in gene expression (Prunet *et al.*, 2006). In addition to its ability to bind to GR, cortisol also has high affinity for MR (10-fold higher than GR) (Greenwood *et al.*, 2003). Mammals have another natural ligand for MR, aldosterone, a hormone involved in the control of hydromineral balance. Cortisol is usually inactivated in MR-specific tissues by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), permitting aldosterone binding and mediation of its effects (Pascual-Le Tallec and Lombes, 2005). Teleosts do not produce aldosterone and it is not clear whether MR has another natural ligand, although there is some evidence that another circulating corticosteroid, 11-deoxycorticosterone, may be a candidate (Sturm *et al.*, 2005).

Rainbow trout, like most teleosts, possess two GR isoforms, encoded by GR1 and GR2 genes (Alsop and Vijayan, 2008). These receptor isoforms have different properties: GR2 has greater transactivational activity and higher binding affinity for cortisol than GR1. GR2 is sensitive to lower cortisol concentrations than GR1, and the latter receptor type is thought to be activated by stressor-induced levels of cortisol (Sturm *et al.*, 2011; Prunet *et al.*, 2006). Suppression of cortisol secretion by the negative feedback mechanisms is an important component of proper HPI axis functioning. Prolonged elevation of glucocorticoids can result in detrimental effects, including suppression of reproduction, immune response, and growth (Norris, 2000; Wendelaar Bonga, 1997). This neuroendocrine feedback loop involves cortisol inhibition of hypothalamic CRF and pituitary ACTH secretion. In fish, a recent study demonstrated that the central and peripheral GR signalling is essential for the negative feedback regulation of plasma cortisol levels (Alderman *et al.*, 2012).

In addition to the classical genomic signalling mechanisms, all steroid hormones, including glucocorticoids, are known to exhibit rapid effects on various physiological processes (Borski, 2000). These rapid effects are known to be independent of gene transcription and are mediated by membrane-associated mechanisms. For example, it has been shown that *in vitro* exposure to cortisol rapidly alters fluidity, surface topography, and elasticity of hepatic plasma membranes in rainbow trout (Dindia *et al.*, 2012). Cortisol-

mediated membrane alterations have been shown to be associated with activation of intracellular stress signalling pathways, including increased phosphorylation of extracellular signal-related kinases as well as several putative protein kinase A (PKA) and protein kinase C (PKC) substrate proteins (Dindia *et al.*, 2013). However, most of the studies on cortisol signalling in fish have focused on genomic signalling by GR activation, while the nongenomic action of cortisol is still in its infancy (Dindia *et al*., 2013; Aluru and Vijayan, 2009).

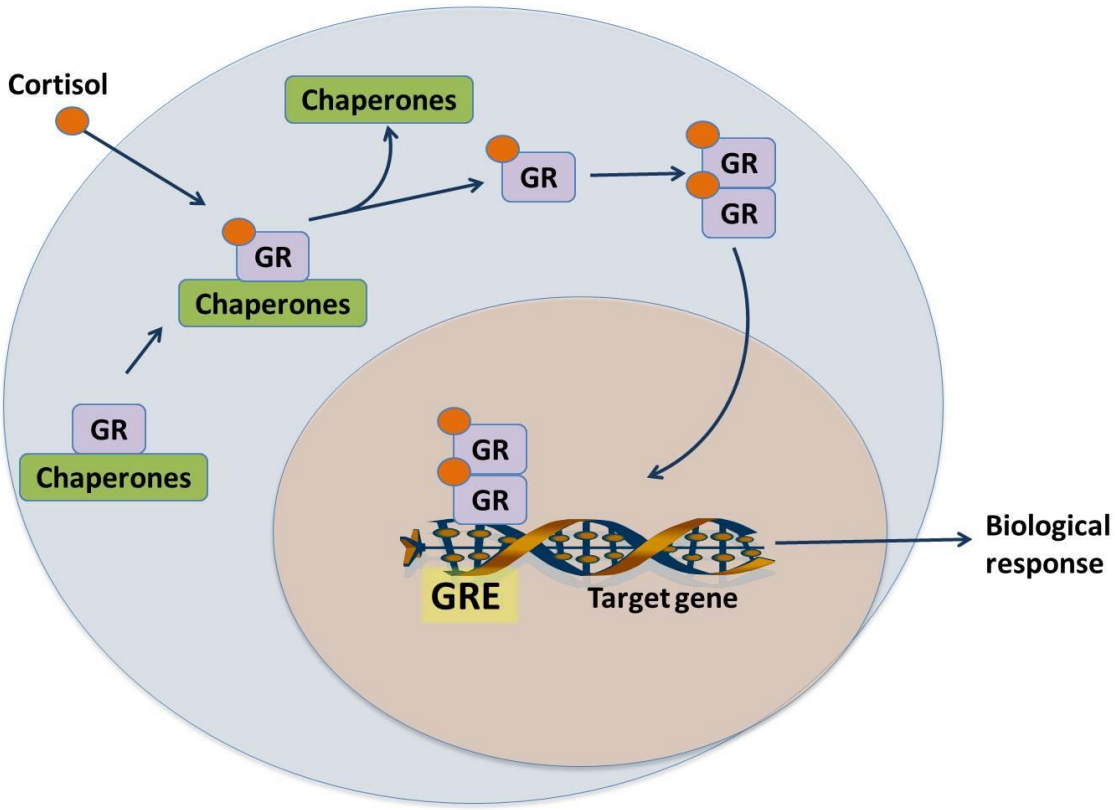


Figure 1-4. Schematic diagram of classical (genomic) glucocorticoid receptor (GR) signalling. Cortisol diffuses through plasma membrane due to its lipophilic nature. In cytosol, cortisol binds GR, which is thought to be bound by molecular chaperones in teleosts. Cortisol binding leads to disassociation of molecular chaperones and subsequent homodimerization of the activated receptor. This complex then translocates into the nucleus, where it acts as a transcription factor regulating expression of target genes via binding to the glucocorticoid response elements (GREs).

1.4.6 Metabolic effects of cortisol

Stress in energy demanding, and the primary role of cortisol is to mobilize energy substrates to fuel metabolic demands associated with stress (Mommsen *et al*., 1999). While catecholamines are involved in the fight-or-flight response by increasing glucose production via glycogenolysis (breaking down glycogen stores), cortisol plays a role in the longer-term glucose maintenance (Vijayan *et al.,* 2010; Mommsen *et al.,* 1999). The metabolic effects of cortisol during stress are aimed at increasing rates of gluconeogenesis − production of glucose from non-carbohydrate substrates, glycolysis, which may be critical to cope with increased liver energy demand, and proteolysis and lipolysis (Vijayan *et al.,* 2010; Mommsen *et al.,* 1999). Previous studies in fish have demonstrated that both stress and cortisol elevate transcript abundances and/or enzymatic activity of the key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK), which catalyzes the rate-limiting step in gluconeogenesis (conversion of oxaloacetate into phosphoenolpyruvate) and the enzymes involved in amino acid catabolism, alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT), providing important source of substrates for liver gluconeogenesis (Gravel and Vijayan, 2007; Wiseman *et al.,* 2007; Vijayan *et al.,* 2003; Vijayan *et al.,* 1994). In addition, cortisol has been associated with upregulation of the glycolytic enzymes, including hexokinase, pyruvate kinase, and lactate dehydrogenase (Gravel and Vijayan, 2007; Vijayan *et al.,* 1994). Stress-induced glucose response is an essential evolutionarily conserved physiological mechanism that allows an organism to regain homeostasis. Impairment of this adaptive response by exogenous means, such as exposure to certain water contaminants, may affect energy metabolism and result in decreased capacity to cope with other environmental stressors.

1.5 Neural inputs regulating stress axis activation

In contrast to mammals, the sensory components of the stress axis are less studied in fish, although there seems to be a high degree of homology in the brain circuitry and neurotransmitter mechanisms regulating stress axis in all vertebrates. In mammals, neuronal circuits involved in activation and regulation of the HPA axis have been well documented (Carrasco and Van de Kar, 2003; Herman and Cullinan, 1997). Multiple brain structures and a variety of neurotransmitters are involved in coordination of the stress responses. Interaction

between the stress-sensitive pathways and the neuroendocrine neurons of the hypothalamic PVN plays a key role in the HPA axis activation (Van de Kar and Blair, 1999). For example, secretion of CRF is known to be regulated by ascending aminergic input from the locus coeruleus by NE and raphe nuclei by 5-HT, extra hypothalamic neurons secreting dopamine (DA) from the medial zona incerta of the subthalamus, GABAergic neurons originating from the posterior bed nucleus of the stria terminalis (Choi *et al.,* 2007; Herman and Cullinan, 1997; Van de Kar and Blair, 1999). Monoamine neurotransmitters (5-HT, NE and DA) play an important role in integrating autonomic, behavioural and neuroendocrine pathways coordinating stress response (Cerdá-Reverter and Canosa, 2009; Winberg *et al.,* 1997; Ortega *et al.,* 2005).

1.6 Monoamines

Monoamines are important neurotransmitters and neuromodulators in the central nervous system of vertebrates involved in the mediation of physiological and behavioural stress responses. They consist of the indoleamine, 5-HT, and the catecholamines, DA and NE (Purves *et al.,* 2008). The catecholamines are synthesised from the amino acid precursor tyrosine, while 5-HT is synthesised from tryptophan. Effects of monoamine neurotransmitters are terminated following the reuptake into presynaptic nerve terminals or surrounding glial cells. Monoamine oxidases (MAOs) play an important role in the inactivation of monoamine compounds (Purves *et al.,* 2008). The major metabolite of 5-HT catabolism is 5-hydroxyindoleacetic acid (5-HIAA) (Winberg and Nilsson, 1993; Øverli, 2001). In DA metabolism, 3,4-dihydroxyphenylacetic acid (DOPAC) is the main deamination product, which can also be processed into 3-methoxytyramine (3-MT) and homovanilic acid (HVA), depending on species-specific distribution of the metabolising enzymes (Winberg and Nilsson, 1993). Major metabolite of NE is considered to be 3-methoxy-4-hydroxyphenylglycol (MHPG) (Øverli, 2001; Winberg and Nilsson, 1993). Ratios of monoamine metabolites to their respective parent compounds are often used as index of neural activity, since decreased metabolite production suggests decreased turnover of the parent neurotransmitter (Fillenz, 1993; Winberg and Nilsson, 1993).

The monoamines act on a variety of pre- and postsynaptic receptors in the brain and specificity of their function is determined by differences in distribution of receptor subtypes.

In vertebrates, at least five DA receptor subtypes have been identified, which are usually referred as D_1-like (D_1, D_5) and D_2-like (D_2, D_3, D_4) (Callier *et al.*, 2003). Also, five adrenergic receptors have been characterized in mammals: α_1, α_2, β_1, β_2 and β_3 (Purves *et al.*, 2008). Serotonergic system shows the highest receptor divergence. Seven families of 5-HT receptors have been identified (5-HT_1-5-HT_7), comprising 14 structurally and pharmacologically distinct subgroups (Hoyer *et al.*, 2002). Relatively little is known about monoamine receptor subtypes in the teleost brain. Overall, neuroanatomical organization of most monoaminergic pathways is well conserved in vertebrates. Monoaminergic neurons compose a very small fraction of neurons in the vertebrate brain; however, their divergent projections establish strong influence in many brain areas.

1.6.1 Role of monoamines in HPI axis regulation

While the mechanisms leading to the hypothalamic activation during stress in teleosts are not well understood, monoamine neurotransmitters, 5-HT, NE and DA, play a central role in the regulation of the stress axis (Cerdá-Reverter and Canosa, 2009; Øverli *et al.*, 2005; Winberg *et al.*, 1997; Winberg and Nilsson, 1993). In the vertebrate brain, a variety of neuronal inputs converge on the CRF-neurosecretory cells. The brain noradrenergic system was shown to be involved in the stress response in mammals, and numerous stressors may induce the NE-dependent release of CRF (Dunn *et al.*, 2004; Pacak *et al.*, 1995). It is well established that 5-HT induces CRF release from the hypothalamic neurosecretory cells (Chaouloff, 1993). Evidence suggests that in fish 5-HT also plays a key role in the HPI axis activation and downstream cortisol production (Winberg *et al.*, 1997). The stimulatory role of NE and 5-HT on CRH release *in vitro* has been reported in tilapia (*Oreochromis mossambicus*) (Pepels *et al.*, 2004). Studies suggest that DA may be involved in the inhibitory modulation of pituitary corticotrophs, for instance, in goldfish injections with DA antagonists induced hypertrophy of corticotroph cells and stimulated ACTH release (Olivereau *et al.*, 1988). Physiological evidence from *in vitro* superfusion study with pituitary pars distalis of common carp (*Cyprinus carpio*) suggested that CRF can only stimulate ACTH release in the presence of mild dopaminergic inhibitory tonus (Metz *et al.*, 2004).

1.6.2 Role of serotonin subtype 1A receptor (5-HT$_{1A}$) in HPI axis regulation

Involvement of 5-HT in the HPA axis regulation has been relatively well studied in mammals. The 5-HT$_{1A}$ receptor has long been identified as a key link mediating serotonergic effects on the stress axis (Goel *et al.,* 2014; Pitchot *et al.,* 2001; Lorens and Van de Kar, 1987). Relatively little is known about 5-HT receptor subtypes in the teleost brain; however, studies done in Arctic charr (*Salvelinus alpinus*) provided evidence for the presence of a multitude of 5-HT receptors in the brain, especially one that has a pharmacological profile strikingly similar to 5-HT$_{1A}$ in mammals (Winberg and Nilsson, 1996). In addition, several studies in teleost fish provided substantial evidence demonstrating involvement of 5-HT$_{1A}$ receptor in the HPI axis regulation axis (Medeiros *et al.,* 2014; Höglund *et al.,* 2002; Winberg *et al.,* 1997). Work conducted on cannulated rainbow trout showed a dose-dependent increase in plasma cortisol levels with the administration of a selective 5-HT$_{1A}$ receptor agonist, 8-hydroxy-N,N-dipropyl-2-aminotetralin (8-OH-DPAT) (Winberg *et al.,* 1997) . Interestingly, Höglund *et al.* (2002) observed that 8-OH-DPAT had a stimulatory effect on the HPI axis activation in unstressed fish (equipped with a permanent i.p. catheter), whereas stressed fish (handled for i.p. injections) showed a suppressive effect of 8-OH-DPAT on the HPI axis. The authors suggested that 5-HT$_{1A}$ receptors may act both as post-synaptic receptors and pre-synaptic autoreceptors in fish (Höglund *et al.,* 2002). A study conducted in the Gulf toadfish (*Opsanus beta*) with cortisol-releasing oil implants provided evidence of cortisol-mediated downregulation 5-HT$_{1A}$ transcripts via GR-mediated mechanisms (Medeiros and McDonald, 2013). Collectively, this evidence demonstrates that 5-HT$_{1A}$ receptor is the key player involved in mediation of serotonergic effects on the HPI axis regulation in teleosts.

1.6.3 Serotonin in the head kidney

Presence of 5-HT in the adrenal gland has been demonstrated (immunohistochemically and biochemically) in various vertebrate species (Contesse *et al*., 2000). 5-HT has also been localized in the head kidney of several fish species (Reid *et al*., 1998). In rainbow trout, 5-HT is stored in high concentrations in the anterior region of the post-cardinal vein within the head kidney and it can mediate release of catecholamines from the chromaffin cells (Fritsche *et al*., 1993). Similarly, in Atlantic hagfish (*Myxine glutinosa*),

evidence suggests that 5-HT is stored in a separate population of cells within the head kidney region, whereas Atlantic cod (*Gadus morhua*) and European eel (*Anguilla anguilla*) release serotonin from the chromaffin cells (Bernier and Perry, 1996; Reid *et al*., 1995). Moreover, 5-HT can induce corticosteroid secretion from the adrenocortical cells via paracrine action in many vertebrate species (Contesse *et al*., 2000). Recent studies in teleost fish showed that 5-HT can directly stimulate cortisol secretion from the interrenal tissue, and most likely several 5-HT receptor subtypes are involved in this response (Lim *et al*., 2013; Medeiros and McDonald, 2012). Therefore, findings of the above mentioned studies suggest that cells within the head kidney region are involved in paracrine interactions, where 5-HT possibly plays a role in a local positive feedback mechanism facilitating endocrine responses of the catecholamine- and cortisol-secreting cells during acute stress.

1.7 Behavioural links to monoamines and stress

In fish, like in other vertebrates, behavioural and physiological stress responses are intimately linked by a central control mechanism in the brain, where monoamine neurotransmitters play a central role in coordination of these responses (Øverli *et al.*, 2005). For example, monoamines affect behavioural responses involved in feeding and aggression, which are usually associated with social rank establishment (Huber, 2005; Winberg and Nilsson, 1993). It is not surprising to see that exposure to antidepressant drugs alters behavioural responses in fish, including changes in aggressive behaviour or predator avoidance, due to effects exerted upon monoaminergic systems (Painter *et al.*, 2009; Perreault *et al.*, 2003).

Salmonid fish tend to form social hierarchies in groups of two or more (Gilmour *et al.*, 2005b; Øverli *et al.*, 2005). Dominant individuals display agonistic behaviours and are able to acquire more food. Behavioural differences in coping styles are associated with important differences in physiological and neuroendocrine responses. For instance, subordinate fish have been characterized by increased brain serotonergic activity and higher plasma cortisol levels, whereas dominant fish have increased brain dopaminergic activity and lower cortisol levels (Doyon *et al.*, 2003; Pottinger and Carrick, 2001; Sloman *et al.*, 2000; Summers *et al.*, 2005). Behavioural responses attributed to specific monoaminergic profiles undoubtedly demonstrate a link between monoamines and the stress axis. Changes in

monoaminergic activity result in altered functioning of the HPI axis, reflected in changes in CRF and POMC gene expression levels (Doyon *et al.,* 2003; Winberg and Lepage, 1998), interrenal activity and cortisol production (DiBattista *et al.*, 2005; Øverli *et al.*, 2004; Noakes and Leatherland, 1977). Overall, behavioural responses and their associated stress effects constitute prominent targets for antidepressant-mediated effects in fish.

1.8 Food intake control and interactions with the stress axis

Regulation of food intake in vertebrates is a complex process involving interactions between brain neuronal circuits and peripheral systems. The brain produces key factors that either induce (orexigenic) or inhibit (anorexigenic) food intake (Volkoff *et al.,* 2005). For instance, neuropeptide Y (NPY), orexins, galanin and β-endorphin have orexigenic properties, while CRF-related peptides, 5-HT, bombesin, cholecystokinin, tachykinins have anorexigenic properties (Lin *et al.,* 2000; Volkoff *et al.,* 2005). Hypothalamus plays a central role in food intake control and a complex neuronal network integrates sensory information arising from peripheral signals of appetite, satiation, and long-term energy-balance (Kalra *et al.,* 1999). The HPI axis interacts with appetite regulation and exposure to environmental, social or physical stressors inhibits feeding behaviour in fish (Bernier and Peter, 2001). Evidence from mammalian studies demonstrated that substances that stimulate serotonergic neurotransmission inhibit food intake (Dryden *et al.,* 1996; Anelli *et al.,* 1992). 5-HT appears to have similar properties in fish. For example, administration of a 5-HT-releasing agent induced a short-term inhibition of food intake in rainbow trout (Ruibal *et al.,* 2002). CRF has been shown to be an important mediator of appetite-suppressing effects associated with stress. CRF-administration inhibited food intake in a dose-related manner in goldfish (Bernier and Peter, 2001). Moreover, pharmacologically removing negative feedback action of cortisol by treatment with the GR antagonist, RU-486, or cortisol synthesis inhibitor, metyrapone, led to a dose-dependent food intake reduction (Bernier and Peter, 2001). In mammals, the important appetite regulator NPY is involved in complex interactions with the HPA axis (Krysiak *et al.,* 1999). In fish, evidence suggests that NPY may participate in activation of the HPI axis in trout, possibly through interaction with CRF (Doyon *et al.,* 2003). Overall, these findings demonstrate that there are reciprocal interactions between

neuroendocrine responses associated with the appetite regulation and mediators of the stress response in fish.

1.9 Thesis objectives

The major objectives of this thesis were to examine the impact of environmental levels of venlafaxine exposure on fish stress axis functioning and to identify possible modes of action of this drug. The research objectives were based on the primary hypothesis that Venlafaxine is a neuroendocrine disruptor in juvenile rainbow trout (*Oncorhynchus mykiss)*, impairing the functioning of downstream responses including stress and feeding responses. Testing of this hypothesis was accomplished through a series of *in vivo* exposure studies in a controlled laboratory setting, as well as *in vitro* experiments to examine the mode of action of this drug on target tissues involved in HPI axis functioning.

The specific research objectives were to assess whether:

1. Venlafaxine exposure disrupts brain monoamine levels and neuroendocrine responses to stress in rainbow trout (Chapter 2).
2. Exposure to venlafaxine impacts the metabolic capacity of rainbow trout to a secondary acute stressor (Chapter 3).
3. Venlafaxine exposure impacts the functioning of the HPI axis to a secondary stressor (Chapter 4).
4. Venlafaxine affects multiple neuroendocrine components of the HPI axis at the tissue level (Chapter 5).

Overall representation of the main biological aspects examined in this thesis is presented in Fig. 1-5.

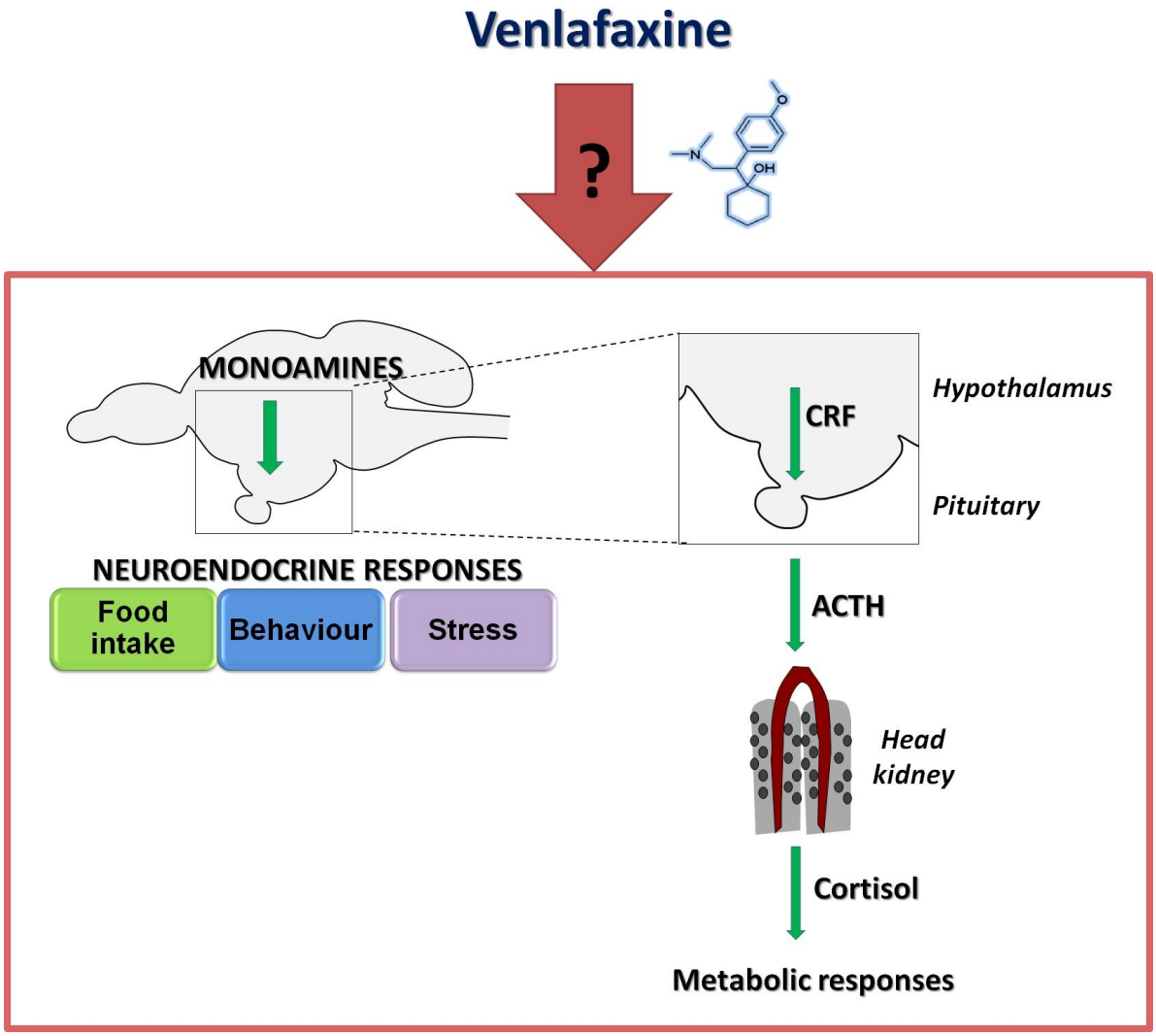


Figure 1-5. Graphic representation of the main biological aspects examined in this thesis in order to assess impact of waterborne venlafaxine exposure on juvenile rainbow trout. Image on the left demonstrates neuroendocrine responses that are regulated by monoamines. Image on the right shows tissue components and mediators involved in the HPI axis functioning.

Chapter 2

The antidepressant venlafaxine disrupts brain monoamine levels and neuroendocrine responses to stress in rainbow trout

2.1 OVERVIEW

Venlafaxine, a serotonin-norepinephrine reuptake inhibitor, is a widely prescribed antidepressant drug routinely detected in the aquatic environment. However, little is known about the impact that this pharmaceutical may have on the physiology of non-target organisms. We tested the hypothesis that venlafaxine perturbs brain monoamine levels and disrupts molecular responses essential for stress coping and feeding activity in fish. Juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to waterborne venlafaxine (0.2 and 1.0 μg/L) for seven days. This drug elevated brain norepinephrine, serotonin and dopamine levels in a region-specific manner. There were also region-specific elevations in expression levels of genes involved in stress and feeding responses, including corticotropin releasing factor, pro-opiomelanocortin B, and glucose transporter type 2 in the brains of venlafaxine-exposed fish. We also conducted a behavioural experiment following venlafaxine exposure with paired trout demonstrating that plasma cortisol levels were higher in the subordinate fish from the drug-exposed group compared to the control group. Total feed consumed per day was reduced in the venlafaxine group, although there were no significant drug treatment effects within each social rank. Collectively, our results demonstrate that venlafaxine is a neuroendocrine disruptor that can impact feeding response and response to a social stressor in rainbow trout. We propose the midbrain region of the trout brain is a key target for venlafaxine impact and the mode of action involves alterations in monoamine content.

2.2 INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) are the most common classes of antidepressants that are used to treat depression, anxiety, obsessive-compulsive disorders, and panic disorders (Silverstone, 2004). They selectively inhibit presynaptic neuronal reuptake of serotonin (5-HT) and/or norepinephrine (NE), thereby increasing synaptic availability of these monoamines. One of the most frequently prescribed antidepressant drugs in recent years is the SNRI venlafaxine (Horst and Preskorn, 1998). Consequently, this drug along with its major metabolite O-desmethylvenlafaxine is detectable in our waterways at parts per million levels (Metcalfe *et al.,* 2010). Municipal wastewater effluent (MWWE) is the primary source introducing

pharmaceuticals, including venlafaxine, in the aquatic environment (Chambers *et al.*, 1997), but little is known about the impact of this drug on non-target aquatic species, including fish.

The mode of action of venlafaxine has not been established in non-target organisms, but this drug is primarily designed to modulate brain 5-HT and NE signalling (Horst and Preskorn, 1998). In teleosts, brain 5-HT, NE and dopamine (DA) play a very important role in the regulation of motivated behavioural responses, including establishing social hierarchies and feed acquisition (Huber, 2005; Winberg and Nilsson, 1993). For instance, trout form social hierarchies in groups of two or more (Øverli *et al.*, 2005; Gilmour *et al.*, 2005b), and dominant individuals usually acquire more food, display agonistic behaviours and have lower plasma cortisol levels compared to subordinate fish (Doyon *et al.*, 2003; Pottinger and Carrick, 2001; Sloman *et al.*, 2000). These behavioural responses are associated with altered monoaminergic activity, leading to altered hypothalamus-pituitary-interrenal (HPI) axis functioning and cortisol production (DiBattista *et al.*, 2005; Øverli *et al.*, 2004; Winberg and Nilsson, 1993; Noakes and Leatherland, 1977).

In teleosts, the HPI axis activation involves the release of corticotropin releasing factor (CRF) by hypothalamic neurons, which stimulates the corticotroph cells of the pituitary to secrete adrenocorticotropic hormone, ACTH, a peptide produced post-translationally from pro-opiomelanocortin (POMC) (Mommsen *et al.*, 1999). ACTH stimulates cortisol biosynthesis in the interrenal cells, and the secreted cortisol mediates its physiological actions via glucocorticoid and mineralocorticoid receptors (GR and MR) (Vijayan *et al.*, 2010). While the mechanism leading to the neuroendocrine regulation of stress in teleosts is not well understood, studies suggest a role for 5-HT, NE and dopamine (DA) in the regulation of the cortisol response to stressors (Cerdá-Reverter and Canosa, 2009). For instance, serotonergic activity stimulates the release of CRF from the hypothalamus and activates the HPI axis function leading to cortisol production in fish (Winberg *et al.*, 1997). There is also evidence of NE and DA involvement in the control of HPI axis activity (Pepels *et al.*, 2004; Olivereau *et al.*, 1988), although the mechanisms are less clear.

Given that venlafaxine may impact brain monoamine levels, we hypothesized that environmentally relevant levels of this drug will perturb the molecular response to stress and

appetite regulation in rainbow trout (*Oncorhynchus mykiss*). To this end, trout were exposed to waterborne venlafaxine at environmentally relevant levels for seven days and brain monoamine levels, including 5-HT, DA and NE and their major metabolite levels (only of 5-HT and DA) were measured in different brain regions. Also, the molecular markers of stress and appetite regulation, including CRF and its binding protein, CRF-BP (Bernier, 2006; Flik *et al.,* 2006), POMC (Xu *et al.,* 2011; Leder and Silverstein, 2006), glucose transporter type 2 (GLUT2) (Polakof *et al.,* 2007; Polakof *et al.,* 2011), and neuropeptide Y(NPY) (Volkoff *et al.,* 2005; Lin *et al.,* 2000), were analyzed in different brain regions. The functional impact of venlafaxine exposure on behaviour and stress response was monitored by carrying out a social pairing experiment and measuring food acquisition as marker of appetite, as well as plasma glucose and cortisol levels as indicators of stress response in the dominant and subordinate fish.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Immature rainbow trout were obtained from Rainbow Springs Hatchery (Thamesford, ON, CAN). Fish were maintained at the University of Waterloo Aquatic Facility (Waterloo, ON, Can) in a continuous supply of aerated well water under a 12 h-light and 12 h-dark cycle in a 2000L tank at ~13.5 °C. Fish were fed once daily to satiety with commercial trout chow (Martin Feed Mills, Elmira, ON, CAN). All experimental protocols were approved by the University of Waterloo Animal Care Committee and conducted in accordance with the Canadian Council for Animal Care guidelines.

2.3.2 Experimental protocol: brain responses

Groups of 10 trout (89 ± 6 g) were randomly assigned to three 120 L tanks. They were acclimated for two weeks to static water conditions with 40% water exchange daily. Fish were fed once daily as mentioned above. Following acclimation, fish were exposed to three experimental treatments for 7 days: control (no drug), 0.2 μg/L and 1.0 μg/L venlafaxine (venlafaxine hydrochloride; Sigma Aldrich, St. Louis, MO, USA). Venlafaxine was dissolved in water and its experimental dosage was maintained by re-administering the drug with each tank water exchange. Exposure concentrations were chosen to approximate

environmental levels of venlafaxine (Metcalfe *et al.,* 2010; Schultz and Furlong, 2008). Food was withheld during the 7-day drug exposure period. Water samples were collected daily from each treatment tank after a 2 h equilibration period in 20 mL glass scintillation vials and frozen at -20°C until further analysis of venlafaxine concentrations.

On the day of sampling, trout were euthanized with an overdose of 2-phenoxyethanol (1:1000 dilution; Sigma Aldrich, St. Louis, MO, USA). Fish were bled by caudal severance into sterile centrifuge tubes containing 40 µl of 10 mg/mL EDTA in physiological saline. Plasma was collected following blood centrifugation (6000 × g for 10 min) for cortisol and glucose analyses. Each brain was quickly removed, placed on a chilled petri dish and dissected into seven regions, including hypothalamus (Hyp), telencephalon (Tel), preoptic area (POA), optic tectum (OT), midbrain (MB), cerebellum (Cer) and hindbrain (HB) (Fig. A1-A; Appendix A); pituitaries (Pit) were also collected. All samples were snap frozen on dry ice and then stored at -80 °C until processed for further analyses.

2.3.3 Experimental protocol: behavioural response

A total of 24 trout (30 ± 2 g) were divided equally among four 30 L tanks. Following acclimation, two tanks received control treatment and two tanks received 1.0 µg/L venlafaxine treatment, administered as above, for a 7-day period. Fish were food-deprived during the exposure period. Following exposure, trout were transferred into 10 L flow-through tanks (no drug exposure) in approximately size-matched pairs for a total of six tanks of paired fish per treatment. Either the dorsal or ventral caudal fin tip was clipped to distinguish between individuals of each pair for visual tracking. Each pair of fish were fed 10 pellets (21.5 ± 1.0 mg) 4 h after transfer (day 1) and were observed live and video recorded (Sony Handycam HDR-CX550V) for 10 min. Fish were given all 10 pellets at once and the share eaten by each fish was noted. At the end of 10 min, any uneaten food was removed from the tank. This feeding protocol was repeated again on days 2 and 3 for a total of three feeding trials. Additionally, agonistic interactions were also assessed by counting the number of attacks by the dominant fish, and food acquisition and agonistic interactions were used as markers to separate the dominant and subordinate fish (Gilmour *et al.,* 2005a; Øverli *et al.,* 2005). The day following the last feeding trial, all pairs were euthanized with an

overdose of 2-phenoxyethanol as above, and plasma was collected for cortisol and glucose analyses.

2.3.4 Venlafaxine analysis

Water samples from the first exposure experiment were analysed to ensure that nominal drug concentrations were maintained in our static exposure system. We selected six samples from both venlafaxine tanks (from days 2-7 of the exposure period for 0.2 and 1.0 µg/L treatments), and three samples from the control tank (from days 2, 5, and 7) for analysis of venlafaxine concentrations. Solid phase extraction (SPE) was used to extract venlafaxine from the water samples (15 mL) using Oasis HLB cartridges (6 mL, 500 mg, Waters Corporation, Millford, MA,USA) as previously described (Rahman *et al.,* 2010). Samples were spiked with 100 µL of 100 µg/L of deuterated venlafaxine prior to extraction to compensate for possible matrix effects from the analysis. Dried extracts were reconstituted with 500 µL of methanol plus internal standards (Lorazapam and Chloramphenicol) and stored at -20°C until analysis.

Analysis of SPE extracts was completed using a 1200 Agilent liquid chromatograph with a Sciex API 3200 QTRAP mass spectrometer with electrospray ionization using multiple reaction monitoring (MRM) as previously described (Rahman *et al.,* 2010). The ion transition monitored in MRM was 278/58 for venlafaxine and 284/64 for d6-venlafaxine. The method detection limit (DL) was 1 ng/L. Venlafaxine calibration curve was analyzed to confirm peak shape, retention time and peak area. Each sample was injected once at a volume of 20 µL. Matrix spikes were completed and showed recoveries between 106-117%. Method blanks were also completed and showed no analyte.

2.3.5 Plasma analysis

Plasma cortisol levels for the brain response experiment were measured using ^{3}H cortisol radioimmunoassay as described previously (McMaster *et al.,* 1995). Cortisol antibody was obtained from MP Biomedicals (Solon, OH, USA) and tritiated cortisol was purchased from GE Healthcare (Waukesha, WI, USA). Plasma cortisol levels for the behavioural response study were measured using a commercially available cortisol ELISA (Neogen, Lexington KY, USA) as described previously (Nesan and Vijayan, 2012). Plasma

glucose levels were measured enzymatically by the hexokinase and glucose-6-phosphate dehydrogenase enzymatic method (Bergmeyer *et al.*, 1974).

2.3.6 Brain monoamine analysis

Fish (n = 5 per treatment) from the brain response study were used for monoamine analyses. Seven rainbow trout brain regions (Hyp, POA, Tel, MB, HB, OT) and Pit (Fig. A1-A; Appendix A) were analysed for 5-HT, DA, their major oxidative metabolites 5-HIAA and DOPAC, respectively, and NE contents. High performance liquid chromatography with electrochemical detection (HPLC-EC) was used as previously described (Gesto *et al.*, 2006) with some modifications and HPLC system specifications were identical. The mobile phase was composed of 63.9 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 0.80 mM sodium 1-octanesulfonate and 15.3 % (v/v) methanol (pH 2.95), and was filtered (0.20 µm filter, Millipore, Bedford, USA) and degasified at vacuum before use. Analytical run time was 15 minutes at an isocratic flow rate of 1.0 $mL \cdot min^{-1}$ at room temperature. Detection limit for the amines and their metabolites was between 0.5 and 1.5 pg per injection, with a signal-to-noise ratio of 2. Acquisition and integration of chromatograms were performed using the ChromNAV version 1.12 software (Jasco Corp., Tokyo, Japan).

Tissues were weighed and then homogenized by ultrasonic disruption in 0.15 mL (Pit), 0.5 mL (Hyp, Tel, POA), or 1 mL (Cer, OT, MB, HB) of mobile phase. Homogenates were then centrifuged (16000 × g, 10 min) and supernatants diluted with mobile phase prior to HPLC analysis. Dilutions were 1:1 (supernatant: mobile phase) for Hyp, Tel and Pit; 1:2 for POA, Cer, MB and HB; 1:4 for OT. Data were normalized to the protein content of the samples, which was measured with the bicinchoninic acid method (Smith *et al.*, 1985). The 5-HIAA/5-HT and DOPAC/DA ratios were calculated since they are often used as indices of the turnover rates of their respective parent neurotransmitters, 5-HT and DA.

2.3.7 Transcript abundance

As most changes in the brain monoamine concentrations were seen in the 1.0 µg/L venlafaxine group compared to control, quantitative real-time RT-PCR (qPCR) analysis was carried out only in this venlafaxine group (0.2 µg/L group omitted). Total RNA was extracted from the brain regions and pituitary glands of the fish used in the brain response experiment

(n = 5 per treatment) using TRIzol reagent (Invitrogen Corp., San Diego, CA, USA) according to the manufacturer's instructions. One μg RNA was treated with DNase I (Qiagen, Mississauga, ON, CAN) to remove genomic DNA contamination prior to cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' protocols.

Transcript levels of some key genes involved in the HPI axis function and energy homeostasis were measured by qPCR in triplicates using gene-specific primers (Table 2-1) as previously described (Aluru and Vijayan, 2007), with one modification of PCR conditions for NPY (Conde-Sieira *et al.,* 2010). A relative standard curve method was used to determine relative transcript abundances according to established protocols (Sathiyaa and Vijayan, 2003). All data were normalized to EF1α expression since it was unchanged by treatments. All gene expression levels are shown as percent of the control of the Hyp region.

Transcript abundances of CRF and CRF-BP were analyzed in the Hyp and POA, two brain regions involved in the HPI axis regulation and primary expression areas for CRF and CRF-BP (Bernier *et al.,* 2008; Flik *et al.,* 2006). POMCA and POMCB gene expression levels were examined in the Pit (Leder and Silverstein, 2006), and additionally in the Hyp, MB and HB, areas associated with food intake/energy homeostasis (Grill and Hayes, 2012; Kalra *et al.,* 1999). GLUT2 and NPY expression levels were analyzed in the Hyp, HB, and MB due to the same selection criteria as stated earlier.

2.3.8 Statistical analysis

All statistical analyses were performed using Sigmaplot 11 software (Systat Software Inc., San Jose, CA, USA) and data were expressed as mean ± standard error of mean (SEM). Data were log transformed, when necessary, to meet the assumptions of homogeneity of variance. For the monoamine analyses, treatment effects of venlafaxine in different brain regions were determined by one-way analysis of variance (ANOVA) followed by post-hoc Holm-Sidak test. In our transcript analyses, significant differences between control and 1.0 μg/L venlafaxine treatments were assessed by Student's *t*-test in selected regions. For the behavioural response experiment, the sum of the number of pellets eaten by each treatment group per day (12 fish) and by each social rank (6 fish) in each treatment group were determined for each feeding trial (n = 3 trials). The mean ± SEM of these values were used

for statistical analyses by Student's *t*-test and two-way ANOVA with Tukey's post-hoc test, respectively. Agonistic interactions were analyzed by Mann-Whitney rank sum test. Cortisol levels due to social stress were measured on the third day of social pairing experiment and were analyzed by two-way ANOVA followed by Tukey's post-hoc test. In all cases, statistical significance level was set at $\alpha = 0.05$.

Table 2-1. Sequences (forward and reverse), product sizes, accession numbers and annealing temperatures for oligonucleotide primers used in quantitative real-time RT-PCR.

Gene	Primer sequence	Accession No.	T_{anneal} (^{o}C)	Product size (bp)
EF1α	F: 5'-CATTGACAAGAGAACCATTGA-3' R: 5'-CCTTCAGCTTGTCCAGCAC-3'	AF498320.1	56	95
CRF	F: 5'-ACAACGACTCAACTGAAGATCTCG-3' R: 5'-AGGAAATTGAGCTTCATGTCAGG-3'	NM_001124286.1	60	54
CRF-BP	F: 5'-GGAGGAGACTTCATCAAGGTGTT-3' R: 5'-CTTCTCTCCCTTCATCACCCAG-3'	NM_001124631.1	60	51
POMCA	F: 5'-AGGGTTGAGGGAGGAAGAGA-3' R: 5'-TGTCAGAGGACATGGCTTTT-3'	NM_001124718.1	60	116
POMCB	F: 5'-CCAGAACCCTCACTGTGACGG-3' R: 5'- CCTGCTGCCCTCCTCTACTGC-3'	NM_001124719.1	60	199
GLUT2	F: 5'-GGCCATCTTCCTGTTTGTGT-3' R: 5'-TGAAGTTGCTGGTCCAGTTG-3'	AF321816	60	140
NPY	F: 5'-CTCGTCTGGACCTTTATATGC-3' R: 5'-GTTCATCATATCTGGACTGTG-3'	NM_001124266	58	247

EF1α, elongation factor 1α; CRF, corticotropin-releasing factor; CRF-BP, corticotropin-releasing factor binding protein; POMCA and POMCB, pro-opiomelanocortin A and B; GLUT2, glucose transporter type 2, and neuropeptide Y, NPY.

2.4 RESULTS

2.4.1 Brain response study

Venlafaxine concentrations

The average water venlafaxine concentrations were 0.2583 ± 0.0075 µg/L and 1.0233 ± 0.0141 µg/L (n = 6) for the nominal 0.2 µg/L and 1.0 µg/L treatments, respectively. Therefore, drug concentrations were accurately maintained in our static exposure system. No venlafaxine was detected in the control treatment (< DL, n = 3).

Plasma cortisol and glucose levels

Venlafaxine treatment did not significantly affect plasma cortisol levels. Cortisol levels were 0.37± 0.1 ng/mL in the control group, and 0.57± 0.24 and 0.35 ± 0.15 ng/mL in the 0.2 µg/L and 1.0 µg/L venlafaxine groups, respectively. Plasma glucose levels were not significantly different in the control (3.94 ± 0.16 mM) and venlafaxine groups (3.67 ± 0.15 and 3.61 ± 0.21 mM in 0.2 µg/L and 1.0 µg/L groups, respectively).

Brain monoamine levels

Levels of 5-HT, 5-HIAA, DA, DOPAC and NE were measured in seven brain regions and pituitary glands of rainbow trout. The ratios of 5-HIAA/5-HT and DOPAC/DA were used as indices of the turnover rates of 5-HT and DA. Statistically significant elevation of 5-HT levels was detected in the MB of the 1.0 µg/L venlafaxine group, while no significant changes were detected in other brain regions (Fig. 2-1A). No significant changes were detected in the levels of 5-HIAA in any of the brain regions (Fig. A1-B; Appendix A). Treatment with 1.0 µg/L venlafaxine significantly reduced the 5-HIAA/5-HT ratio in the MB, but not other brain regions (Fig. 2-1B).

Significant changes in the DA levels were also detected in the MB, where 1.0 µg/L venlafaxine group had higher DA than 0.2 µg/L venlafaxine, but neither venlafaxine group was found to be different from the control (Fig. 2-1C). There were no significant changes in DOPAC levels across the brain regions (Fig. A1-C; Appendix A). However, there were significant changes in DOPAC/DA ratio in the HB and MB regions (Fig. 2-1D). In the HB, both venlafaxine treatments decreased the ratio in comparison to the control. In the MB, 1.0

μg/L venlafaxine group had lower DOPAC/DA ratio than 0.2 μg/L venlafaxine treatment, but neither venlafaxine group was found to be different from the control.

There was a significantly higher NE level in the MB region in 1.0 μg/L but not the 0.2 μg/L venlafaxine treatment group (Fig. 2-1E). NE was not significantly different due to venlafaxine exposure in any of the other brain regions.

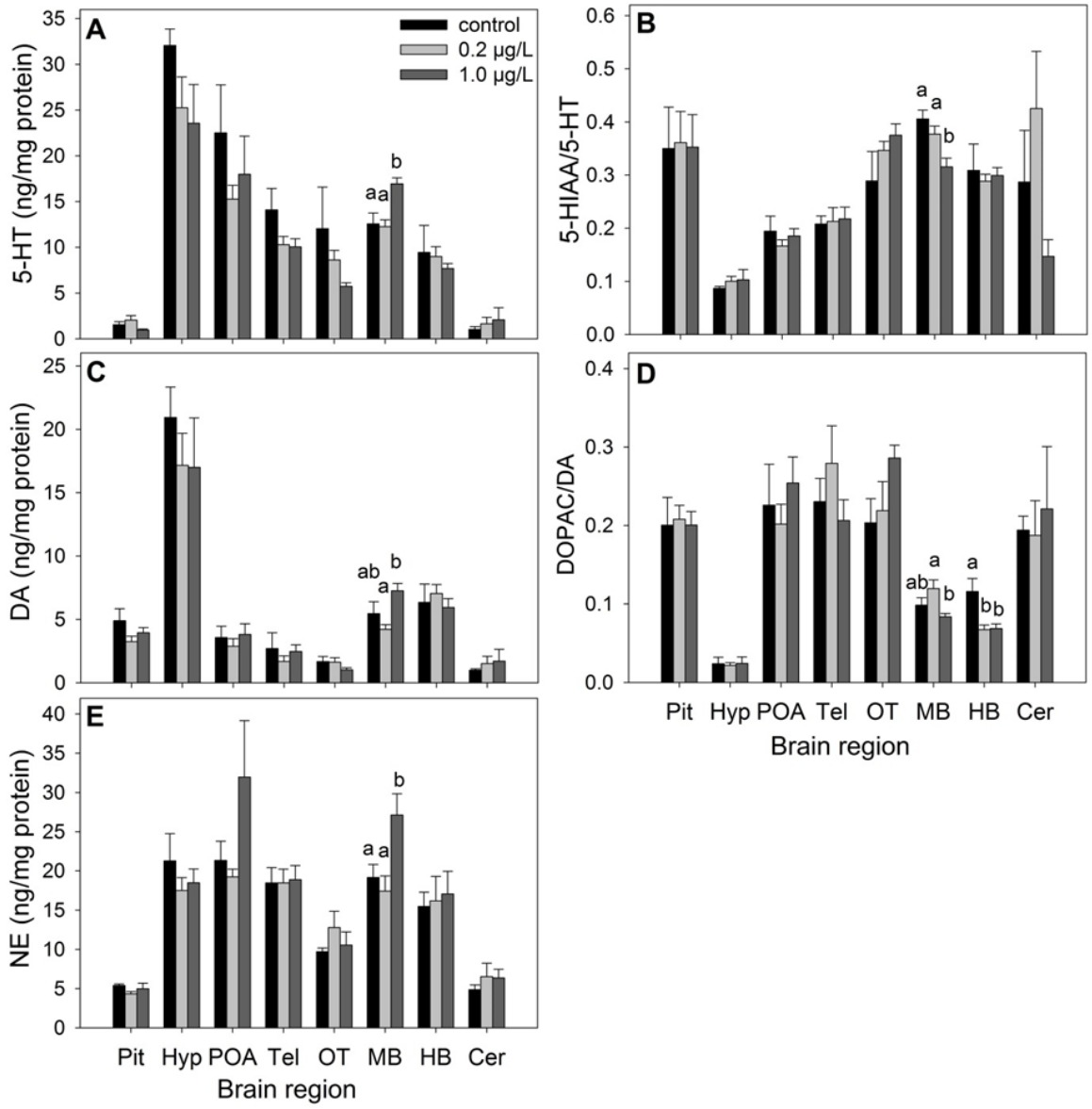


Figure 2-1. Brain monoamine levels: concentrations of 5-hydroxytryptamine, 5-HT (A) and its turnover rate (represented by the ratio of 5-hydroxyindoleacetic acid, 5-HIAA/5HT) (B), dopamine, DA (C), and its turnover rate (represented by the ratio of 3,4-dihydroxyphenylacetic acid, DOPAC/DA) (D), and norepinephrine, NE (E) in rainbow trout brain following a 7-day venlafaxine exposure. Values represent means ± SEM (n = 5). Different letters indicate significant treatment effects within a given region (one-way ANOVA; $P < 0.05$). Abbreviations: hypothalamus (Hyp), telencephalon (Tel), preoptic area (POA), optic tectum (OT), midbrain (MB), cerebellum (Cer), hindbrain (HB), and pituitary (Pit).

Transcriptional response

CRF mRNA abundance was significantly higher in 1.0 μg/L venlafaxine group compared to the controls in the Hyp but not POA (Fig. 2-2A). There was no significant difference in CRF-BP transcript levels with venlafaxine in the Hyp or POA (Fig. A2-A; Appendix A). Exposure to venlafaxine significantly increased GLUT2 transcript levels in the MB, but not Hyp or HB, compared to the control group (Fig. 2-2B). Venlafaxine exposure significantly increased POMCB mRNA abundance in the HB, but not in the Pit, Hyp, or MB (Fig. 2-2C). There was no significant effect of venlafaxine exposure on mRNA levels of POMCA (Fig. A2-B; Appendix A) in the brain regions examined (Pit, Hyp, MB, and HB). Transcript abundance of NPY was not affected by venlafaxine in the Hyp, MB, or HB (Fig. A2-C; Appendix A).

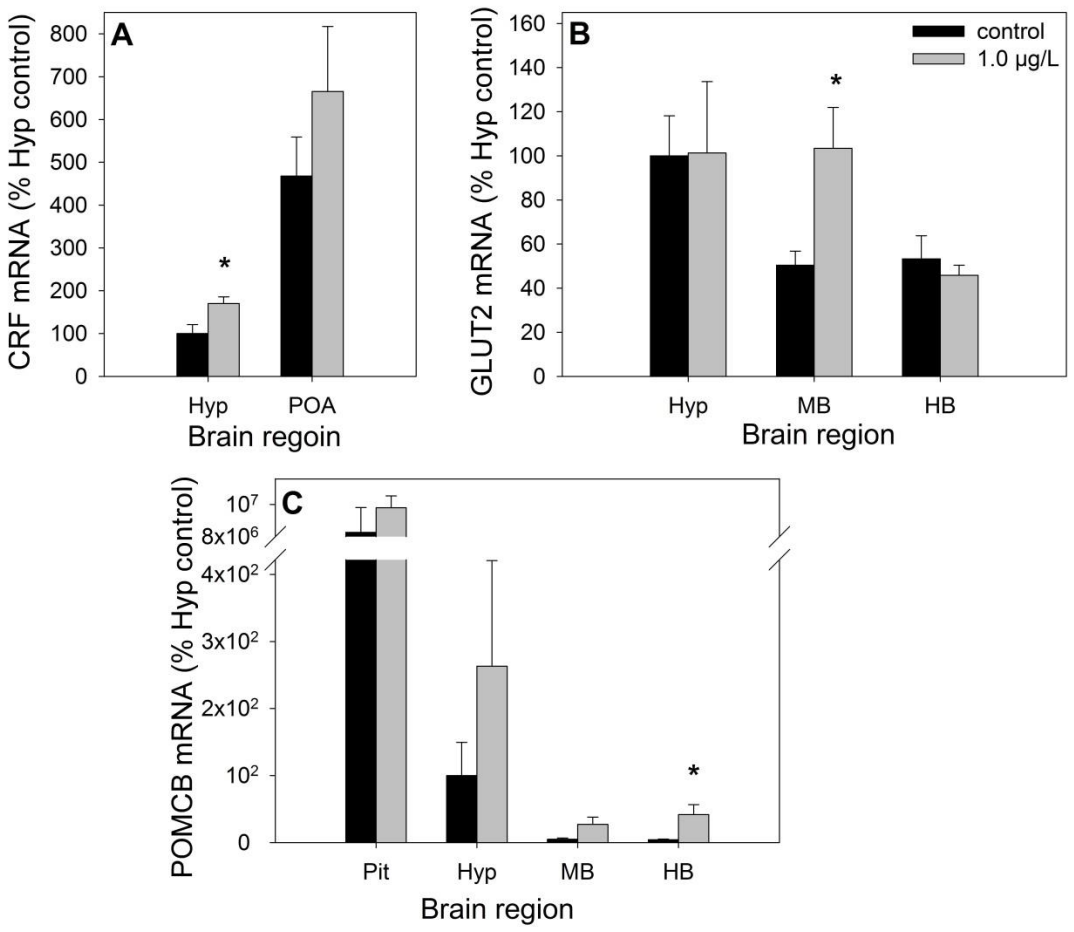


Figure 2-2. Spatial brain gene expression: mRNA abundances of corticotropin-releasing factor, CRF (A), glucose transporter type 2, GLUT2 (B), and pro-opiomelanocortin B, POMCB (C) in different brain regions of rainbow trout brain following a 7-day venlafaxine exposure. Values were normalized to EF1α and represent percent hypothalamus control. Bars show means ± SEM (n = 5); asterisk indicates a significant treatment effect within a given region (Student's t-test; $P < 0.05$). Abbreviations: hypothalamus (Hyp), preoptic area (POA), midbrain (MB), hindbrain (HB), and pituitary (Pit).

2.4.2 Behavioural response study

Food acquisition and agonistic interactions

Trout held in pairs formed social hierarchies, and food intake, agonistic behaviour and cortisol levels (assessed at the end of the pairing experiment) were used to assign social status. The dominant fish obtained majority of the food, displayed more agonistic behaviours and also had lower plasma cortisol levels compared to the subordinate fish. Agonistic interactions within pairs were limited in most cases. Although there was no significant differences in agonistic behaviours on day 1 (4 h post-transfer) or day 2 (24 h post-transfer) (Table 2-2), the highest frequency of attacks occurred on day 3 (or 48 h post-transfer), where the dominant fish exhibited higher number of attacks compared to the subordinates in both control and venlafaxine treatment groups, although no significant drug treatment effect was detected in this study.

Table 2-2. Agonistic behaviours displayed by each rainbow trout rank (dominant or subordinate) on days 1-3 of social pairing experiment conducted after a 7-day exposure to 1.0 µg/L venlafaxine. Days 1, 2 and 3 correspond to 4, 24 and 48 h post-transfer from venlafaxine exposure system, respectively. Values in the table represent number of agonistic attacks, shown as means ± SEM. Significant differences between social ranks are indicated by different letters ($P < 0.05$, Mann-Whitney Rank Sum Test). No significant venlafaxine exposure effects were observed in this study.

	Control		**1.0 µg/L venlafaxine**	
	Dominant (*n* = 6)	**Subordinate (*n* = 6)**	**Dominant (*n* = 5)**	**Subordinate (*n* = 5)**
Day 1	0.0 ± 0.00	0.0 ± 0.00	0.4 ± 0.40	0.6 ± 0.40
Day 2	0.2 ± 0.17	0.2 ± 0.17	4.4 ± 4.15	0.6 ± 0.40
Day 3	6.2 ± 3.59 ^{A}	0.3 ± 0.21 ^{B}	14.6 ± 8.32 ^{A}	0.0 ± 0.00 ^{B}

Overall, food intake was reduced by venlafaxine, which is demonstrated by a significantly lower amount of total food pellets eaten by fish in the drug exposed group compared to the control over the 3-day feeding trial (Fig. 2-3A). When food acquisition data were examined by each social rank (dominant and subordinate) within each treatment group over the 3-day feeding trial period (Fig. 2-3B), there was no significant treatment effect on food acquisition.

Plasma cortisol and glucose levels

Analysis of plasma cortisol levels measured 24 h after the final feeding session detected a significant interaction between venlafaxine treatment and social rank (Fig. 2-3C). In both treatment groups plasma cortisol levels were significantly higher in the subordinate individuals compared to the dominant individuals. Within the dominant social rank, there was no significant effect of drug exposure on plasma cortisol levels, whereas within the subordinate rank, venlafaxine exposure resulted in significantly higher circulating cortisol levels compared to the control group. Plasma glucose levels were measured 24 h following the final feeding session. No significant drug treatment or social rank effects were identified in the present study (data not shown).

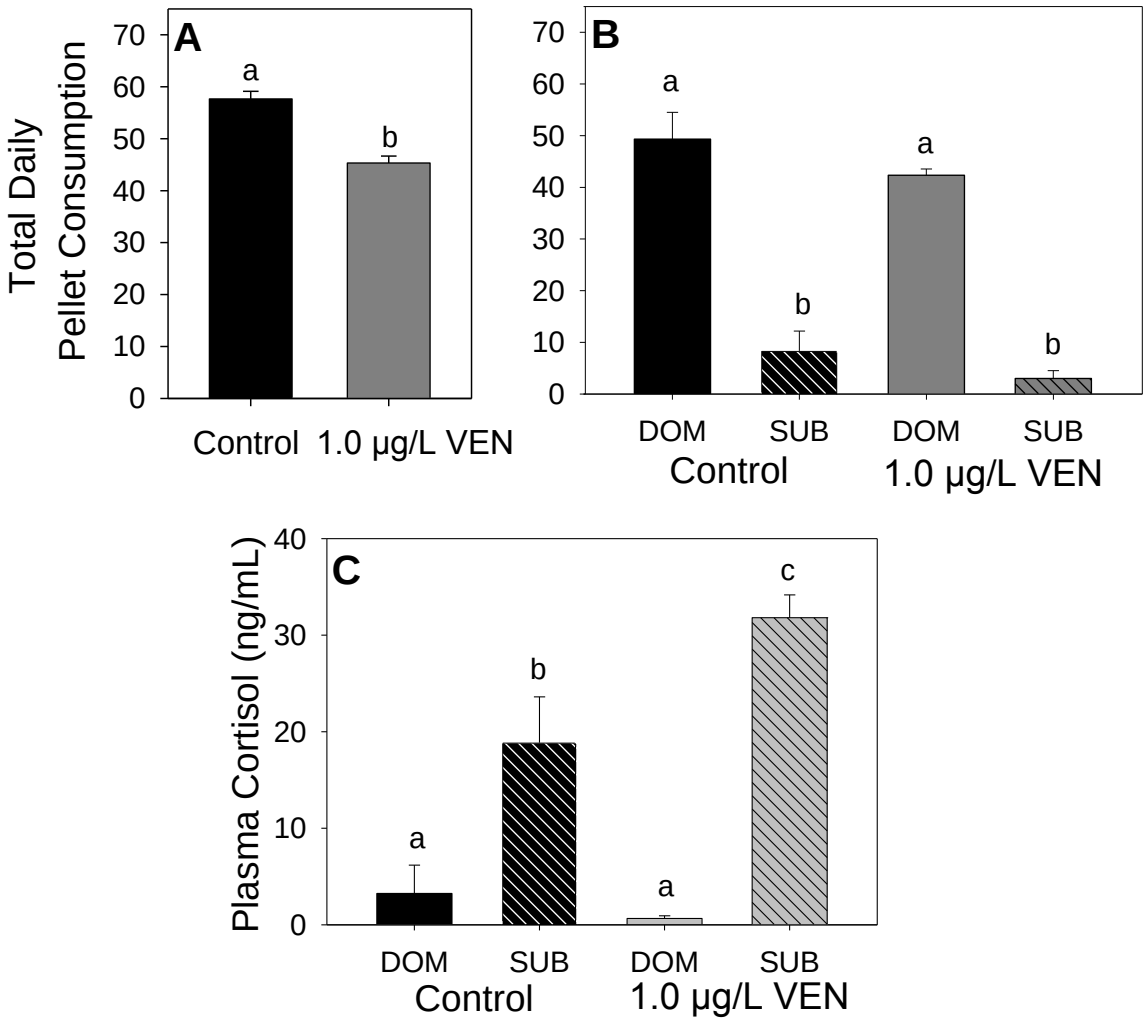


Figure 2-3. Behavioural response over a 3-day social pairing experiment in rainbow trout following a 7-day exposure to 1.0 µg/L venlafaxine (VEN). Food consumption by trout, represented as the total number of food pellets consumed per day by all fish in each treatment group (12 fish/group) (**A**); different letters indicate significant differences ($P < 0.05$, *t*-test). Food consumption in established social dyads shown as the total number of pellets consumed per day by the dominant (DOM) and subordinate (SUB) fish per day in each treatment group (**B**); different letters indicate significant differences ($P < 0.05$, 2-way ANOVA). In both food intake graphs bars represent means ± SEM, $n = 3$ feeding trials. Plasma cortisol levels in the DOM and SUB fish measured on the last day of experiment (day 3) (**C**); bars represent means ± SEM ($n = 5$-6 fish) and different letters indicate significant differences ($P < 0.05$, 2-way ANOVA).

2.5 DISCUSSION

Our results demonstrate for the first time that environmentally relevant concentrations of venlafaxine affect brain monoamine levels in rainbow trout. Since brain monoamines have hypophysiotropic functions and play important roles in integrating autonomic, behavioural and neuroendocrine responses (Cerdá-Reverter and Canosa, 2009; Winberg *et al.,* 1997), the disruption in 5-HT, NE and DA levels by venlafaxine may affect several downstream pathways leading to disturbances in homeostasis. For instance, our findings demonstrate that venlafaxine exposure reduces food intake, and leads to an elevation in plasma cortisol levels in the socially stressed trout. This along with changes in mRNA abundances of genes involved in HPI axis functioning and appetite regulation suggests that neuroendocrine pathways involved in stress response and feeding are targets for venlafaxine impact in trout.

There was a clear spatial difference in 5-HT, DA and NE levels suggesting that the venlafaxine effect on the brain is region-specific. Our results suggest that the MB and HB regions are key targets for venlafaxine impact in rainbow trout, while the mechanism is unknown. The higher 5-HT and NE levels in the MB region in trout is in agreement with mammalian studies confirming these neurotransmitters as primary drug targets for venlafaxine. For instance, a 4-d treatment with venlafaxine increased 5-HT levels in the MB region and NE levels in the ventral tegmental area (associated with the MB) in rats (Muneoka *et al.,* 2009). In our study, 1.0 μg/L venlafaxine treatment increased 5-HT levels in the MB without alterations in the concentration of its main metabolite, 5-HIAA. This suggests that venlafaxine treatment increased 5-HT production rate rather than its catabolism, thus lowering the turnover rate of this neurotransmitter, indicated by reduced 5-HIAA/5-HT ratio. For instance, 5-HIAA is formed in the presynaptic neurons after 5-HT reuptake by serotonin transporters (Winberg and Nilsson, 1993), so any disruption in the monoamine uptake will lead to changes in metabolite levels and 5-HIAA/5-HT ratio. In addition to a direct effect of venlafaxine on serotonergic neurons, the higher levels of NE may also modulate 5-HT levels. This is supported by the modulation of 5-HT levels in the mammalian MB, including raphe nuclei, by NE (Adell *et al.,* 2002). It remains to be seen whether the observed changes in NE levels in the present study in the MB due to venlafaxine disrupt downstream serotonergic

pathways. The observed changes in DA levels in the MB region between the high and low venlafaxine group along with reduced turnover in the HB region suggest that DA dynamics is also disrupted by venlafaxine exposure in trout. It should be noted that evidence from mammalian studies suggest that venlafaxine can have a relatively weak effect on dopamine (DA) reuptake (Roseboom and Kalin, 2000), modulating DA levels in a region-specific manner; however, the underlying mechanism may be a reflection of alteration in NE levels, since noradrenergic neurons modulate certain DA systems (Muneoka *et al.,* 2009; Liprando *et al*., 2004).

The MB and HB regions are abundant in cell bodies of 5-HT (Nieuwenhuys, Ten Donkelaar, and Nicholson 1998) and NE (Ekström *et al.,* 1986) producing neurons, and their nerve projections are very divergent and reach most of the brain regions, including dense innervations to the POA and HYP. Also, the soma of dopaminergic neurons are contained mainly in the hypothalamus, telencephalon and pretectal areas, and in medulla oblongata, while their projections are widely distributed with the highest density of terminals in the Tel, POA and HYP regions (Meek *et al.,* 1989). As serotonergic, noradrenergic and dopaminergic pathways innervate key areas of the brain involved in the integration of endocrine and metabolic signals to coordinate stress response and food intake, any disruption in the dynamics of these neurotransmitters may lead to homeostatic disturbances, including stress axis dysfunction (Pepels *et al.,* 2004; Winberg *et al.,* 1997).

Indeed, our gene expression data indicate changes to transcript levels of key markers of HPI axis functioning and appetite regulation in response to venlafaxine exposure. CRF is a neuroendocrine marker of stress activation (Flik *et al.,* 2006) and appetite suppression (Bernier, 2006; Volkoff *et al.,* 2005). Our study showed that CRF mRNA abundance was increased by venlafaxine in the HYP, the region of the brain that plays a central role in appetite regulation. This, along with the higher abundance of POMCB mRNA, encoding peptides associated with energy homeostasis and appetite suppression (Xu *et al.,* 2011; Volkoff *et al.,* 2005), in the HB suggests a role for venlafaxine in impacting appetite regulation and stress response in trout. Furthermore, the higher transcript levels of GLUT2, a bidirectional glucose transporter that is a key component of the brain glucosensory system

and appetite regulation (Polakof *et al.*, 2007), in the MB of trout by venlafaxine points to a dysregulation in feeding response. The change in GLUT2 mRNA level was not accompanied by concomitant changes in plasma glucose levels leading to the proposal that the disruption may be brain-specific. Together, the transcript changes observed in the brain suggests an anorexigenic role for venlafaxine, mediated in part by alterations in the brain monoamine levels. The impact of venlafaxine on appetite suppression, as indicated by the transcript changes, was confirmed by our feeding trials that clearly showed a reduced feed intake after a 7-d drug exposure in trout.

Brain monoamine neurotransmitters are thought to play a key role in the establishment of social ranks in fish (Winberg and Nilsson, 1993). For instance, subordinate fish have increased brain serotonergic activity along with higher cortisol levels and lower appetite compared to the dominant fish that have higher brain dopaminergic activity, lower cortisol levels and increased appetite (Summers *et al.*, 2005). When we subjected the venlafaxine-exposed fish to a social stressor, the dominant and subordinate hierarchies were clearly established as in the control group. As expected, the dominant fish consumed significantly more food than the subordinate fish in both treatment groups. However, the lack of venlafaxine effect on feed consumed based on social ranking may be due to the limited number of feeding trials. Interestingly, plasma cortisol level in the subordinate response to a social stressor was amplified in the drug exposed group. It is well known that there is a strong interplay between the serotonergic system and the stress axis. For instance, an acute stressor induces very rapid increases in brain serotonergic activity in trout, as well as in lizards, supporting the involvement of the serotonergic system as a primary activator of the stress response (Gesto *et al.*, 2013; Emerson *et al.*, 2000). Furthermore, elevation in cortisol levels increases serotonergic activity (DiBattista *et al.*, 2005) and administration of a 5-HT receptor agonist significantly elevates cortisol levels (Winberg *et al.*, 1997). This relationship also holds true during social stress in fish, as an increase in brain serotonergic activity corresponds to a reduction in aggressive behaviour, resulting in lower social rank (Winberg and Nilsson, 1993). For example, in the study based on a territorial reef fish species, aggression was reduced following a treatment with the SSRI fluoxetine due to modulation of

the serotonergic system by this drug (Perreault *et al.*, 2003).

Our finding that venlafaxine treatment leads to a regional increase in serotonergic activity combined with the fact that subordination stress itself leads to higher serotonergic activity, suggest that drug-exposed subordinate fish may experience hyperactivation of the HPI axis. Subordinate individuals are affected by chronically elevated cortisol levels leading to compromised health (Gilmour *et al.*, 2005b), and our results suggest that such effects may be amplified in fish exposed to venlafaxine in the aquatic environment. Although aquatic species are usually exposed to numerous chemical compounds in complex mixtures, including MWWE, it is important to investigate the effects of these pharmaceutical drugs individually in order to elucidate their mechanisms of action on non-target species. For instance, inadvertent exposure of non-target organisms to active pharmaceutical compounds may lead to adverse effects that may translate into reduced fitness and reproduction. To this end, exposures to environmentally relevant concentrations of venlafaxine, fluoxetine and bupropion (individually or in mixtures) adversely affected predator avoidance behaviour in larval fathead minnows suggesting performance dysfunction (Painter *et al.*, 2009). Consequently, the potential implications of venlafaxine and other relevant antidepressants on fish fitness do emphasize their ecological relevance and the necessity for a thorough understanding of their mode of action on non-target aquatic organisms for environmental effects monitoring.

2.6 CONCLUSIONS

Collectively, results of this study suggest that exposure to environmentally relevant venlafaxine concentrations affect monoamine and neuroendocrine responses pertaining to stress and appetite regulation in trout. Our findings indicate that the midbrain region may be a key target for venlafaxine impact and the mode of action may involve changes in monoamine content. Since this region contains numerous nuclei and nerve tracts that interconnect with rostral and caudal brain regions, any impact on the midbrain region may lead to downstream effects on endocrine and behavioural responses. Indeed, venlafaxine modulated the functioning of the HPI axis in the socially stressed rainbow trout resulting in

amplified cortisol response in the subordinate fish. Our results underscore a role for this antidepressant as a neuroendocrine disruptor in trout, and its exposure may lead to several downstream adverse effects in non-target animals.

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Coauthor contributions: NML conducted brain response study and analysis, CB conducted behavioural study and analysis; GM performed monoamine measurements.

Chapter 3

Environmental levels of the antidepressant venlafaxine impact the metabolic capacity of rainbow trout

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3.1 OVERVIEW

The antidepressant venlafaxine is detected at parts per billion levels in tertiary-treated municipal wastewater effluent. However, the impact of this serotonin-norepinephrine reuptake inhibitor (SNRI) on non-target aquatic animals is poorly understood. We tested the hypothesis that environmentally relevant levels of venlafaxine disrupt the highly conserved cortisol and glucose response to stress in rainbow trout (*Oncorhynchus mykiss*). Juvenile trout were exposed to venlafaxine (0, 0.2 and 1.0 µg/L) in a static system with daily renewal for seven days. The fish were then subjected to an acute handling disturbance and sampled either prior to (0 h) or 1, 4 and 24 h after stressor exposure. Venlafaxine exposure did not affect the stressor-mediated transient elevation in plasma cortisol levels or target tissue glucocorticoid receptor expression. The drug exposure disrupted the interrenal steroidogenic capacity, including altered stressor-mediated changes in mRNA abundances of steroidogenic acute regulatory protein and cytochrome P450 side chain cleavage. Also, venlafaxine at the higher concentration increased the ACTH-induced cortisol production by head kidney tissue *ex vivo*. The stressor-induced transient elevations in plasma glucose levels were significantly reduced in the venlafaxine-exposed fish. This was not accompanied by changes in liver glycogen content, glucose transporter 2 mRNA levels or the glycolytic capacity, whereas the capacity for gluconeogenesis and amino acid catabolism were enhanced. Venlafaxine also brought about changes in the gill of trout, including enhanced lactate dehydrogenase activity and Na^+-K^+ ATPase protein expression, while the Na^+-K^+ ATPase enzyme activity was reduced. Collectively, our results demonstrate that venlafaxine at levels detected in the aquatic environment impacts tissue metabolic capacities and may compromise the adaptive responses to an acute stressor in rainbow trout.

3.2 INTRODUCTION

Over the last two decades, pharmaceutical compounds have become a prominent source of environmental contamination and they are constantly being discharged into the aquatic environment through treated sewage effluent, leaching, and direct disposal (Daughton and Ternes, 1999). Pharmaceuticals are frequently detected in municipal wastewater effluents

(MWWEs) and receiving waters (Corcoran *et al*., 2010; Metcalfe *et al*., 2010). Among antidepressant drugs, fluoxetine, a selective serotonin reuptake inhibitor (SSRI), is the most commonly studied in the aquatic environment and it has been shown to affect fish performance (Mennigen *et al*., 2011). Recently, the use of venlafaxine, a serotonin-norepinephrine reuptake inhibitor (SNRI) antidepressant with improved therapeutic action and fewer side effects than fluoxetine (Horst and Preskorn, 1998) has been on the rise. At present, venlafaxine and its active metabolite O-desmethylvenlafaxine are among the highest detected antidepressants in tertiary-treated MWWEs (Metcalfe *et al*., 2010; Schultz *et al*., 2010). Despite this, very little is known about the impact of this pharmaceutical drug on non-target aquatic organisms, including fish. A recent study showed that exposure to tertiary-treated MWWE, that also contains venlafaxine, impairs the cortisol and metabolic responses to an acute stressor in rainbow trout (*Oncorhynchus mykiss*) (Ings *et al*., 2011).

The cortisol stress response is part of an evolutionarily conserved adaptive response that allows animals to restore homeostasis when challenged by stressors. To that end, cortisol and catecholamines induce several metabolic alterations to help to overcome the threat. Briefly, after exposure to a stressor, the primary fight or flight response involves the stimulation of the sympathetic division of the autonomic nervous system and results in increased production of catecholamines, especially epinephrine and norepinephrine, within seconds of stressor perception (Vijayan *et al*., 2010; Fabbri *et al*., 1998; Reid *et al*., 1998). Catecholamines activate alpha- and beta-adrenergic signalling leading to rapid cardiovascular adjustments and glycogen breakdown to enhance oxygen and glucose delivery to tissues, respectively (Vijayan *et al*., 2010; Reid *et al*., 1998). The activation of the hypothalamus-pituitary-interrenal (HPI) axis lags behind the sympathetic response and begins with the release of corticotropin releasing factor (CRF) from the hypothalamus triggering adrenocorticotropic hormone (ACTH) release from the pituitary, which stimulates cortisol secretion from the interrenal cells of the head kidney (Vijayan *et al*., 2010). ACTH binding to melanocortin 2 receptors (MC2R) activates cAMP-signalling cascade, resulting in the initiation of cortisol biosynthetic pathway. The key rate determining steps in steroidogenesis involve the steroidogenic acute regulatory protein (StAR), which transports cholesterol from

the outer to the inner mitochondrial membrane, followed by conversion of cholesterol to pregnenolone by cytochrome P450 side chain cleavage (P450scc) (Aluru *et al*., 2005). The elevated cortisol levels in response to stress mediate the target effects, including energy substrate mobilization, by activating glucocorticoid receptor (GR) and/or mineralocorticoid receptor (MR) signalling (Bury and Sturm, 2007; Prunet *et al*., 2006).

We have previously demonstrated that venlafaxine at environmental concentrations modulates levels of monoamine neurotransmitters in the midbrain and hindbrain regions of the rainbow trout brain [Chapter 2; Melnyk-Lamont *et al*. submitted]. Monoamine neurotransmitters (serotonin, norepinephrine and dopamine) are known to be involved in the regulation of the neuroendocrine components of the HPI axis (Cerdá-Reverter and Canosa, 2009; Winberg *et al.,* 1997). Consequently, venlafaxine-mediated changes in brain monoamine levels may lead to downstream effects on the HPI axis functioning, but this has not been tested before.

In this study, the hypothesis tested was that venlafaxine at environmentally realistic levels impairs the adaptive cortisol and glucose responses to an acute stressor in fish. Rainbow trout were exposed to waterborne venlafaxine (0.2 and 1.0 µg/L) for seven days. The drug exposure was considered as the primary stressor, and these fish were then subjected to a handling disturbance, which constituted the secondary stressor. The concentration of venlafaxine used was based on the levels detected in the aquatic environment (Metcalfe *et al*., 2010). The impact of the drug on basal and stressor-stimulated steroid biosynthetic capacity in the interrenal tissue as well as target tissue metabolic capacities was assessed. At the interrenal level, StAR and P450scc transcript levels were measured along with plasma cortisol levels to assess cortisol biosynthetic capacity, as these are targets for xenobiotic impact (Sandhu and Vijayan, 2014; Arukwe, 2008; Aluru *et al*., 2005). In addition, we examined the capacity of head kidney tissue from venlafaxine-exposed fish to respond to ACTH challenge *in vitro*. Tissue metabolic capacity was assessed by measuring plasma glucose levels and liver glycogen content, glucose transporter type 2 (GLUT2) transcript levels and activities of enzymes involved in key metabolic pathways, including glycolysis [hexokinase (HK), glucokinase (GK), pyruvate kinase (PK) and lactate dehydrogenase

(LDH)], gluconeogenesis [phosphoenolpyruvate carboxykinase (PEPCK)] and amino acid catabolism [alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT)] (Vijayan *et al*., 2006). Also, metabolic capacity of the gill, a key target tissue for glucose utilization during stress, was assessed by measuring Na^+-K^+ ATPase activity and its protein expression, as well as activities of key glycolytic enzymes, including HK, PK and LDH (Soengas *et al*., 1995; Mommsen, 1984). In addition, brain and liver GR protein expression was used as a marker of target tissue responsiveness to cortisol (Vijayan *et al*., 2005).

3.3 MATERIAL AND METHODS

3.3.1 Animals

Juvenile rainbow trout (average body mass 8.5 ± 2.5 g) were obtained from Rainbow Springs Hatchery (Thamesford, ON) and maintained in flow-through well water (13ºC, 400 mg $CaCO_3$/L, pH 8.0, Alsop *et al*. 2009). Fish were acclimated for two weeks in 2000 L tanks and lighting was on a 12 h day/night cycle. Fish were fed to satiety daily during the acclimation period with a commercial trout chow (Martin Feed Mills, Elmira, ON).

3.3.2 Experimental design

The study involved exposing trout to different concentrations of venlafaxine over a period of seven days. There were duplicate tanks (30 L) for each treatment and 16 fish in each tank. The tanks were continuously aerated and their water temperature maintained by leaving them in a larger tank (2000 L) with flow-through well water exactly as mentioned above. Fish were acclimated in the experimental setup tanks for one week prior to the exposure. During acclimation, the fish were fed to satiety (once daily) and the tanks were cleaned and 40% water replenished (12 L) daily. Fish were exposed to either 0 (control), 0.2 or 1.0 µg/L nominal venlafaxine concentrations (venlafaxine hydrochloride*;* Sigma Aldrich, St. Louis, MO; dissolved in water). Food was withheld for the duration of the experiment and 40% of water was replaced daily and supplemented with venlafaxine at the appropriate concentrations before addition to the fish tanks. Previous work in our laboratory demonstrated that this static exposure system with such daily water changes did accurately

maintain the expected nominal concentrations of venlafaxine [Chapter 2; Melnyk-Lamont *et al*., submitted]. Another study also conducted in a static system showed that venlafaxine concentrations remain stable over the exposure period similar to that used in the present study (Bisesi *et al*., 2014).

Following the 7-d exposure, an initial group of fish were sampled (0 h) while the rest were subjected to an acute stressor (consisting of 30 s netting and 5 min crowding stressor at 3 fish /L) and sampled at 1, 4 and 24 h post-handling disturbance. Sampling consisted of quickly netting four fish from each replicate tank (8 per treatment) at the respective time-points. Fish were euthanized with an overdose of 2-phenoxyethanol (1:1000 dilution; Sigma-Aldrich), weighed and bled by caudal severance into heparinized capillary tubes. The whole procedure took less than 5 minutes for each tank. The plasma was collected after centrifugation of the capillary tubes at approximately 5000 x *g* for four minutes and was stored in microfuge tubes at -80°C for later hormone and metabolite assay. Liver, gill, brain, and head kidney were removed and snap frozen on dry ice and were stored at -80 °C for later analysis. The experimental protocol was approved by the University of Waterloo Animal Care Committee and was conducted in accordance with the Canadian Council for Animal Care guidelines for the use of fish in research.

3.3.3 *Ex vivo* head kidney study

A follow-up study was conducted using head kidney tissues pieces from rainbow trout (average body mass 78 ± 3 g) *in vivo* exposed to venlafaxine treatments for a 7 day period as described earlier (control, 0.2 μg/L and 1.0 μg/L venlafaxine). The exposure was conducted in 120 L tanks to accommodate larger fish size (tank set-up as described in Chapter 2). At the end of the exposure period, fish were quickly sampled and the head kidney region (containing the interrenal cells) was quickly dissected from each fish (n = 6 independent fish/ treatment group), and tissues were prepared using a well-established method (Sandhu and Vijayan, 2011; Aluru and Vijayan, 2006). Briefly, tissues were finely minced (~1 mm^3 pieces) in chilled Petri dishes with modified Hank's medium (136.9 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_20$, 0.33 mM $Na_2HPO_4 \cdot 7H_2O$, 0.44 mM KH_2PO_4, 5.0

mM HEPES, 5 mM HEPES·Na, 5mM $NaHCO_3$, 5mM glucose, pH 7.63) and cleaned from blood clots. After rinsing, the tissue from each fish was distributed equally among treatment wells (500 µL of buffer with ~35-40 mg of tissue) in a sterile 24-well culture plate (Sarstedt, Newton, NC, USA). Following pre-incubation period for 2 h at 13 °C, medium was replaced with fresh aliquots containing the following treatments: control, 0.5 IU/mL ACTH (porcine $ACTH^{1–39}$; Sigma-Aldrich), 0.5 mM 8-Bromo-cAMP (Sigma-Aldrich), and the tissues were incubated for 4 h as described previously (Sandhu and Vijayan, 2012). ACTH is the primary cortisol secretogogue in fish, while 8-Br-cAMP (analogue of cAMP) was used to determine if venlafaxine effect was upstream of cAMP signalling. Concentration of ACTH for *in vitro* treatment and the incubation period were chosen based on previous studies (Sandhu and Vijayan, 2011; Aluru and Vijayan, 2006). At the end of incubation period, samples were collected, quickly centrifuged (13,000 x g for 1 min), and the supernatant was collected and stored at -30^{o}C for later cortisol determination.

3.3.4 Plasma glucose and cortisol levels

Plasma glucose was measured colorimetrically according to Hancock *et al*. (2004). Briefly, samples and standards were loaded onto a 96-well plate, followed by 0.1 M, pH 7.4 monobasic sodium phosphate buffer with phenol (1 mM) and 4-aminoantipyrine (2.5 mM). Glucose oxidase (10 U/ml) and peroxidase (10 U/ml) were added to initiate the reaction. Following 45 min incubation in the dark at room temperature, absorbance was read at 500 nm using a microplate reader (VersaMax, Molecular Devices LLC, Sunnyvale, CA, USA). Plasma cortisol levels were determined using a commercially available competitive ELISA kit (Neogen, Lexington, KY, USA) according to the manufacturer's instructions. Media cortisol concentrations from the head kidney *ex vivo* study were measured using ^{3}H cortisol radioimmunoassay as described previously (Sandhu and Vijayan, 2011; Aluru and Vijayan, 2006).

3.3.5 Tissue preparation

Brain, liver and gill tissues were sonicated in ice cold 50 mM Tris buffer (pH 7.5) with protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). Protein

concentrations of the samples were determined using the bicinchoninic acid (BCA) reagent (Sigma-Aldrich) with bovine serum albumin as the standard. For the enzyme assays, portions of liver and gill homogenates were aliquoted and stored in 50% glycerol buffer (50% glycerol, 21 mM Na_2HPO_4, 0.5 mM EDTA-Na, 0.2% BSA, 5 mM β-mercaptoethanol, pH 7.5) at -20°C as described previously (Sandhu *et al*., 2014). For protein analysis, a portion of the homogenate was prepared for western blot analysis as described before (Dindia *et al*., 2013).

3.3.6 Glycogen and Enzyme Activity Measurements

Glycogen content was determined in liver tissue, and the activity of several enzymes was measured in liver and gill tissue. Glycogen content was determined by measuring glucose levels in the homogenate before and after hydrolysis by amyloglucosidase (Sigma-Aldrich) as described previously (Vijayan *et al*., 2006). Enzyme activities in liver and gill were determined by continuous UV spectroscopy at 340 nm at 22°C using a microplate reader as described previously (Ings *et al*., 2011; Gravel and Vijayan, 2007). Both glycogen and enzyme activity data were normalized to protein content of the tissue and the enzyme activity was expressed as μmol per minute (U) per gram protein. The individual enzyme assay conditions were as follows (final concentration in a well shown, all in 50 mM imidazole buffer, pH 7.4):

- **Hexokinase** (HK: EC 2.7.1.1): 1 mM glucose, 5 mM $MgCl_2$, 10 mM KCl, 0.24 mM NADH, 2 mM phosphoenolpyruvate (PEP), 20 U/mL lactate dehydrogenase (LDH) and 2.5U/mL pyruvate kinase (PK); reaction started with 1mM ATP.
- **Glucokinase** (GK: EC 2.7.1.2): 20 mM glucose, 5mM $MgCl_2$, 10mM KCl, 0.25 mM NADH, 2 mM PEP, 20 U/mL LDH and 2.5 U/mL PK; reaction started with 1 mM ATP.
- **Pyruvate kinase** (PK: EC 2.7.1.40): 30 mM KCl, 10 mM $MgCl_2$, 0.12 mM NADH, 2.5 mM ADP, 10 U/mL LDH; reaction started with 2.5 mM PEP.
- **Lactate dehydrogenase** (LDH: EC 1.1.1.27): 0.12 mM NADH and reaction initiated with 1 mM pyruvate Na.

- **Phosphoenolpyruvate carboxykinase** (PEPCK: EC 4.1.1.32): 20 mM $NaHCO_3$, 1 mM $MnCl_2$, 0.5 mM PEP,and 0.12 mM NADH; reaction started with 0.2 mM deoxyguanosine diphosphate.
- **Alanine aminotransferase** (AlaAT: EC 2.6.1.2): 0.12 mM NADH, 200 mM L-alanine, 0.025 mM pyridoxal 5-phosphate, and 12 U/mL LDH; reaction started with 10.5 mM α-ketoglutarate.
- **Aspartate aminotransferase** (AspAT: EC 2.6.1.1): 7 mM α-ketoglutarate, 0.025 mM pyridoxal 5-phosphate, 0.12 mM NADH,and 8 U/mL malate dehydrogenase; reaction started with 40 mM aspartic acid.
- **Sodium-Potassium ATPase** (NKA: EC 3.6.3.9): 45 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$, 2 mM PEP, 0.5 mM ATP, 0.16 mM NADH, 1 mM NaN_3, 1 mM EGTA, 5 U/ml PK, 5 U/ml LDH. For inhibition, add 0.5 mM ouabain.

3.3.7 SDS-PAGE and immunodetection

SDS-PAGE and western blotting were used to examine brain and liver GR and gill Na^+-K^+ ATPase expression. The procedure for SDS-PAGE and western blotting followed established protocols (Boone *et al*., 2002). In brief, samples containing 40 µg of total protein were separated on 8% or 10% polyacrylamide gel (for GR and Na^+-K^+ ATPase, respectively) with Precision Plus protein molecular marker (BioRad, Hercules, CA, USA). The proteins were transferred onto a nitrocellulose membrane (20 V for 25 min) in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine and 10% (v/v) methanol) using a semidry transfer unit (BioRad). Membranes were blocked for one hour at room temperature in 5% skim milk with 0.05% sodium azide in TTBS (20 mM Tris pH 7.5, 300 mM NaCl, 0.1% (v/v) Tween 20). This was followed by incubation with either polyclonal rabbit anti trout GR antibody (Sathiyaa and Vijayan, 2003, 1:1000 dilution) or mouse anti- Na^+-K^+ ATPase (Wilson *et al*., 2007; 1:1000 dilution) for one hour at room temperature. Subsequently, membranes were washed in TTBS (3× for 10 min) and probed with secondary antibody (goat anti-rabbit IgG for GR or goat anti-mouse IgG for Na^+-K^+ ATPase) conjugated to horseradish peroxidase (1:3000; BioRad) for one hour at room temperature. Membranes were washed 3x10 min with

TTBS and washed 1x10 min with TBS (20 mM Tris pH 7.5, 300 mM NaCl). Band detection was carried out using ECL+ Western Blotting Detection System and imaged with a Typhoon scanner (GE Healthcare Life Sciences, Piscataway, NJ, USA). Bands were quantified with AlphaEase software (Alpha Innotech, CA, USA) and equal loading was confirmed by probing the blots with β-actin (Cy3-coupled monoclonal antibody from mouse 1:1000; Sigma-Aldrich).

3.3.8 RNA isolation and cDNA synthesis

Total RNA was extracted from liver (0 h time point, prior to acute stressor) and head kidney (all time points) with TRIzol reagent (Invitrogen Corp., San Diego, California, USA) according to the manufacturer's instructions. Briefly, samples were lysed with the reagent, chloroform was added, and cellular RNA was precipitated by isopropanol. After washing with 70% ethanol, the RNA pellet was dissolved in nuclease-free water. RNA was quantified by NanoDrop spectrophotometer (Thermo Scientific, Nepean, ON, CAN) at 260 nm. RNA samples were DNase treated to avoid genomic contamination. 1 µg of total RNA per sample was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol.

3.3.9 Quantitative real-time polymerase chain reaction (qPCR)

Gene-specific primers designed for rainbow trout StAR, P450scc, GLUT2, and EF1α cDNA sequences were used to amplify ~100 bp products in real-time quantitative PCR. EF1α was used as housekeeping gene for data normalization because its relative abundance was unchanged between treatment groups. The primer sequences and their accession numbers are presented in Table 3-1. Briefly, a master mix containing 2.5 µL of cDNA, 2.5 µL of 10 µM primer pair, 40 µL of SYBR green supermix (BioRad) and 35 µL of nuclease-free water was prepared for each sample and 25 µL was added to three wells (all samples assayed in triplicate). The following conditions were used for amplification: initial denaturation for 2 min at 94°C, followed by 40 cycles of 20 s at 94°C, 20 s at annealing temperature (Table 3-1); 95°C for 1 min; 55°C for 1 min followed by melt curve analysis starting at 55°C and increasing in 0.5°C increments to 95°C every 10 s. Threshold cycle

values were calculated using iCycler iQ real-time detection software (BioRad). Relative standard curve method was used to determine relative transcript abundance exactly as described previously (Aluru and Vijayan, 2004).

3.3.10 Statistical Analysis

As there were no significant tank effects, the fish from duplicate tanks for each treatment were pooled for statistical comparison. A Student's *t*-test was used to determine significant treatment effect after a 7-d exposure to venlafaxine for gill sodium-potassium ATPase activity and protein expression, while a two-way analysis of variance (ANOVA) was used to determine significant treatment (control, 0.2 and 1 µg/L), time (0, 1, 4 and 24 h after stressor exposure) and interaction effects (treatment x time) after stressor exposure. Tukey's post-hoc test was used to identify significant differences. Data from our *ex vivo* head kidney experiment was analyzed with one-way repeated measures ANOVA to assess effects of *in vitro* stimulation on cortisol production in control tissues. Effect of *in vivo* venlafaxine exposure on *in vitro*-stimulated cortisol production was analyzed with two-way repeated measures ANOVA (one factor repetition). Holm-Sidak post hoc was used to identify significant differences. All data were log transformed when necessary to meet the assumptions of normality and equal variance; non-transformed data are shown in the tables and figures. All statistical analyses were performed using Sigmaplot 11 software (Systat Software). Significance level was set to P-value < 0.05 in all cases.

Table 3-1. Primers used in real-time quantitative RT-PCR. Sequences (forward and reverse), accession numbers, annealing temperatures and product sizes are indicated. Primers include elongation factor 1 alpha (EF1α), steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage (P450scc), and glucose transporter 2 (GLUT2).

Primer	Sequence	Accession No.	Annealing Temp (^{0}C)	Product size (bp)
EF1α	F: 5'-CATTGACAAGAGAACCATTGA-3' R: 5'-CCTTCAGCTTGTCCAGCAC-3'	AF498320.1	56	95
StAR	F: 5′-TGGGGAAGGTGTTTAAGCTG-3′ R: 5′-AGGGTTCCAGTCTCCCATCT-3′	AB047032	60	101
P450scc	F: 5′-GCTTCATCCAGTTGCAGTCA -3′ R: 5′-CAGGTCTGGGGAACACATC -3′	S57305.1	60	140
GLUT2	F: 5'-GGCCATCTTCCTGTTTGTGT-3' R: 5'-TGAAGTTGCTGGTCCAGTTG-3'	AF321816	60	140

3.4 RESULTS

3.4.1 Plasma glucose and cortisol levels

A 7-d venlafaxine exposure did not significantly affect basal plasma cortisol levels (Fig. 3-1A; 0 h time point) in the present study. Handling disturbance significantly elevated plasma cortisol at 1 h, after which the levels dropped significantly at 3 h, but did not return to basal levels, and this cortisol level was maintained over the 24 h period post-stressor exposure (Fig. 3-1A). There was no significant effect of venlafaxine on the secondary stressor-induced plasma cortisol levels (Fig. 3-1A).

Venlafaxine exposure also did not significantly affect basal plasma glucose levels (Fig. 3-1B; 0 h time point). There was an interactive effect between venlafaxine treatment and time post-stressor exposure. The temporal glucose profile in the control and 1.0 µg/L venlafaxine groups showed significant elevation at 1 and 4 h compared to 0 and 24 h post-stressor exposure (Fig. 3-1B; see inset), while in the 0.2 µg/L venlafaxine group the glucose level was significantly higher only at 1 h but not at other time-points post-stressor exposure (Fig. 3-1B; see inset). While a secondary stressor significantly increased glucose levels within each treatment group following the acute secondary stressor challenge, the magnitude of response at 1 h was significantly lower in the 1.0 µg/L, but not in the 0.2 µg/L, venlafaxine group compared to the control (Fig. 3-1B). At 4 h post-stressor, the 1.0 µg/L group maintained their 1 h plasma glucose levels while the levels dropped significantly lower in the 0.2 µg/L group compared to the controls (Fig. 3-1B). At 24 h, glucose returned to basal levels and there were no significant differences between treatment groups (Fig. 3-1B).

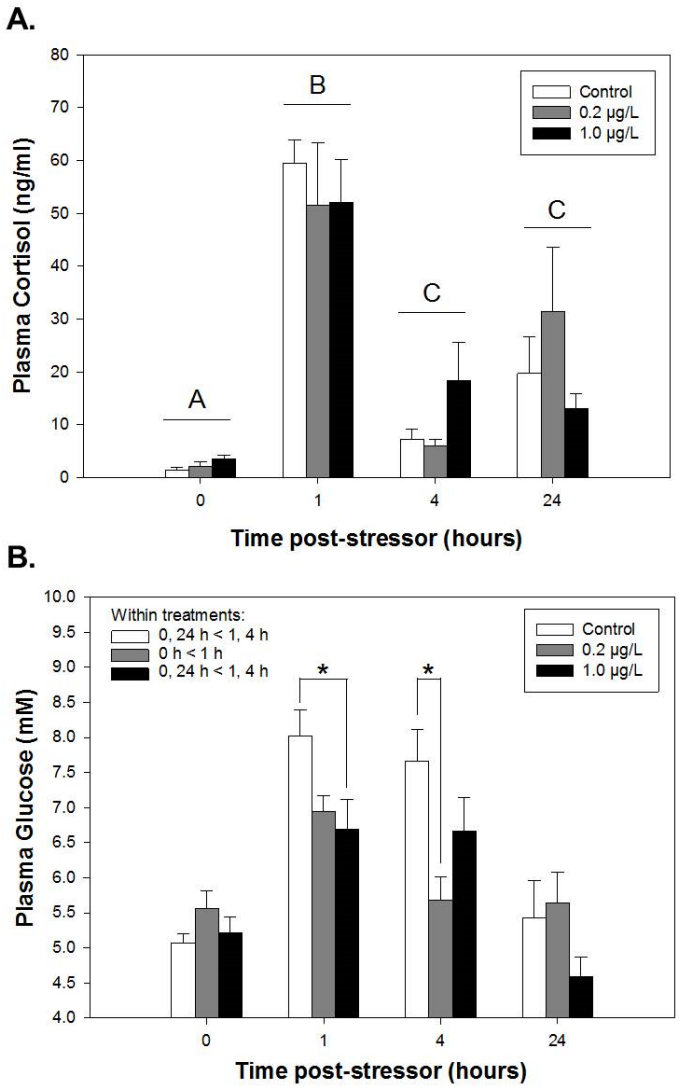

Figure 3-1. Plasma cortisol and glucose levels. Effect of a 7-day venlafaxine exposure on plasma cortisol (**A**) and glucose (**B**) levels prior to an acute stressor (0 h) and 1, 4 and 24 h after stressor exposure. Bars represent means ± SEM (n = 8 fish). There were only time effects, but no treatment effects for cortisol (indicated by different uppercase letters), while for glucose a significant interaction was detected: treatment effects within a time point are indicated with asterisks and time effects within treatments are shown inset (within treatments) ($P < 0.05$, two-way ANOVA).

3.4.2 Head kidney steroidogenic capacity

Exposure to 1.0 µg/L venlafaxine significantly increased the basal (0 h time point) P450scc mRNA abundance compared to the control and 0.2 µg/L treatment groups (Fig. 3-2A). Additionally, exposure to 0.2 µg/L but not 1.0 µg/L venlafaxine increased P450scc mRNA abundance compared to the control at 24 h post-stressor exposure (Fig. 3-2A). Temporally, stressor exposure affected the P450scc mRNA abundance in the venlafaxine groups but not in the control group (Fig. 3-2A). In the 0.2 µg/L venlafaxine group the P450scc mRNA levels were significantly higher at 24 h compared to all other time points (Fig. 3-2A), while in the 1.0 µg/L venlafaxine group the levels were significantly lower at 1,4 and 24 h post-stressor exposure compared to the pre-stress levels (Fig. 3-2A). There was a significant venlafaxine treatment effect on StAR mRNA abundance in the present study (Fig. 3-2B). Both concentrations of venlafaxine were significantly lower than the control regardless of time post- stressor exposure (Fig. 3-2B). There was also a time effect, independent of treatment effects, with the pre-stress StAR mRNA levels significantly higher when compared to 1, 4 and 24 h post-stressor values (Fig. 3-2B).

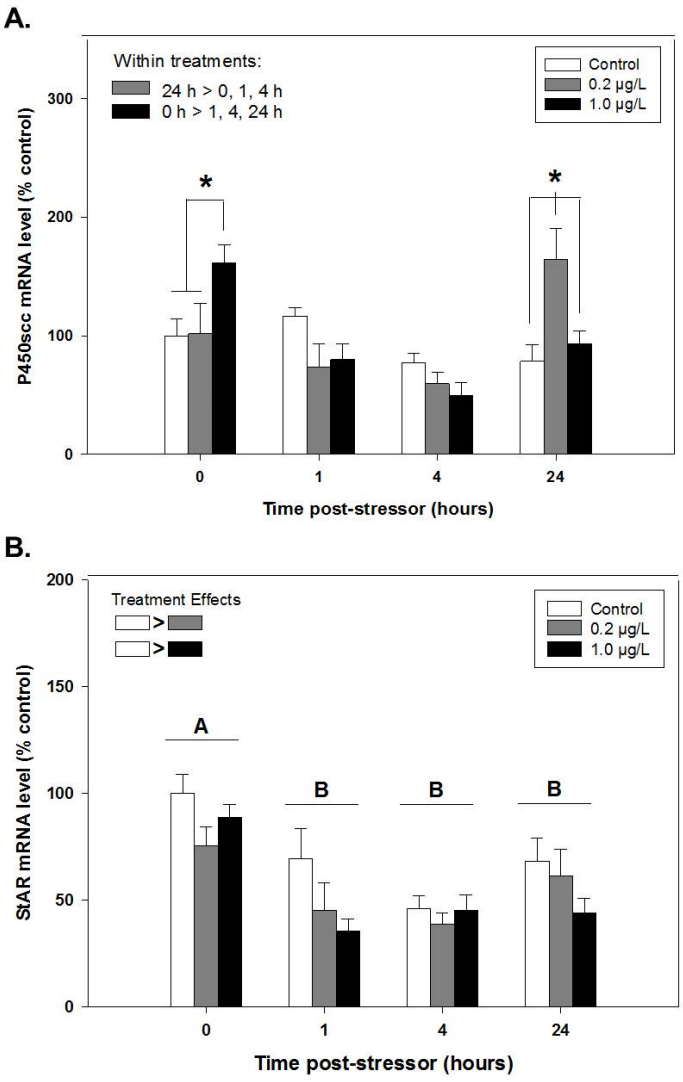

Figure 3-2. Head kidney steroidogenic capacity. Effect of a 7-day venlafaxine exposure on head kidney cytochrome P450 side chain cleavage (P450scc; **A**) and steroidogenic acute regulatory protein (StAR; **B**) mRNA levels prior to an acute stressor (0 h) and 1, 4 and 24 h after stressor exposure. Bars represent means ± SEM (n = 6 fish). A significant interaction was detected for P450scc: treatment effects within a time point are indicated with asterisks and time effects within treatments are shown as inset (within treatment). Significant overall treatment effects for StAR are shown as inset, while overall time effects are indicated by different uppercase letters ($P < 0.05$, two-way ANOVA).

3.4.3 *Ex vivo* head kidney study – cortisol production

As expected, both 8-Br-cAMP and ACTH stimulation significantly increased cortisol production in kidney pieces from the control fish (no drug) (Fig. 3-3A). There was ~ 20-fold increase in cortisol production in response to these treatments compared to the unstimulated cortisol production (Fig. 3-3A).Venlafaxine treatment *in vivo* did not significantly affect the interrenal tissue response to 8-Br-cAMP stimulation compared to the control fish (Fig. 3-3B). However, ACTH-stimulated cortisol production was significantly higher (~47%) in the head kidney tissues from fish exposed to 1.0 μg/L venlafaxine compared to the control and 0.2 μg/L venlafaxine groups (Fig. 3-3B). While for the control and 0.2 μg/L venlafaxine groups there were no differences between 8-Br-cAMP and ACTH stimulated responses, amplitude of cortisol response was significantly higher with ACTH treatment than 8-Br-cAMP in the tissues from fish exposed to 1.0 μg/L venlafaxine (Fig. 3-3B).

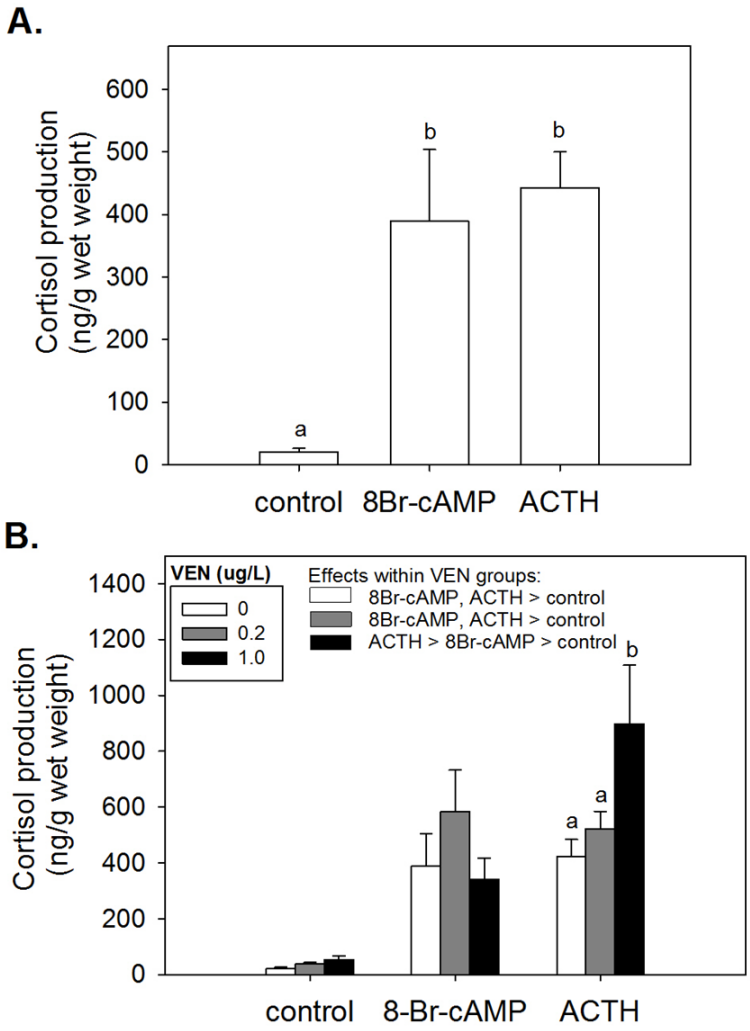

Figure 3-3. *In vitro*-stimulated cortisol production. Stimulation of cortisol secretion by ACTH and 8-Br-cAMP in the control head kidney tissue (**A**) and effect of a 7-day *in vivo* venlafaxine (VEN) exposure on *in vitro* cortisol production in response to ACTH and 8-Br-cAMP stimulation in the head kidney tissue slices of rainbow trout (**B**). Data shown as means ± SEM (n = 5-6 fish). In (**A**), different letters indicate significant differences between *in vitro* treatments ($P < 0.05$, one-way repeated measures ANOVA). In (**B**), insets indicate *in vitro* treatment effect for each VEN group, and different letter indicate differences between venlafaxine exposure groups within an *in vitro* treatment group ($P < 0.05$, two-way repeated measures ANOVA, Holm-Sidak *post hoc*).

3.4.4 Liver metabolic capacity

Venlafaxine treatment had no significant effect on liver glycogen content (Table 3-2). There was a temporal drop in liver glycogen content, irrespective of venlafaxine treatment, with the levels at 24 h significantly lower than the values prior to stress (Table 3-2). Venlafaxine treatment did not significantly alter the activities of the glycolytic enzymes in the present study (Table 3-2). However, there were time effects observed in enzyme activities, independent of venlafaxine treatment, in response to secondary stressor exposure. For instance, the pre-stressor HK and GK activities were significantly higher compared to all subsequent time points (Table 3-2). PK and LDH activities were significantly lower at 1 h compared to 24 h post-stressor exposure, but not at any other time points (Table 3-2).

Basal activity of PEPCK, a key gluconeogenic enzyme, was not affected by a 7-d exposure to venlafaxine. However, there was a significant interaction between venlafaxine treatment and time after stressor exposure on this enzyme activity (Table 3-2). At 4 h post-stressor exposure the PEPCK activity was significantly higher in the venlafaxine groups compared to the control group (Table 3-2). There was also a temporal change in PEPCK activity to stressor exposure that was treatment dependent. In the control, this enzyme activity significantly dropped at 1 and 4 h but not at 24 h post-stressor exposure compared to the pre-stress values (Table 3-2). The 0.2 µg/L venlafaxine group also showed a PEPCK response similar to that of the control group except that the reduced activity recovered at 4 h post-stressor exposure (Table 3-2). There was no time effect on PEPCK activity in the 1.0 µg/L venlafaxine group in the present study (Table 3-2).

Enzymes involved in amino acid catabolism were also affected by venlafaxine. Fish exposed to 1.0 µg/L of venlafaxine, but not 0.2 µg/L venlafaxine, had significantly higher AlaAT activity compared to the control group (Table 3-2). There was also a significant time effect, regardless of treatments, with the activity significantly lower at 1, 4 and 24 h post stressor exposure compared to the pre-stress values (Table 3-2). Venlafaxine exposures did not significantly affect basal or stress-mediated AspAT activity (Table 3-2). There was a significant time effect with the activity lower at 1 h compared to either the pre-stress or 24 h post-stressor values (Table 3-2).

Table 3-2. Liver metabolic capacity. Changes seen in glycogen content (µmol glucosyl units/g protein) and enzyme activities (µmol/min/g) of hexokinase (HK), glucokinase (GK), pyruvate kinase (PK), lactate dehydrogenase (LDH), phosphoenolpyruvate carboxykinase (PEPCK), alanine aminotransferase (AlaAT), and aspartate aminotransferase (AspAT) in response to an acute stressor (0, 1, 4, and 24 h post-stressor) in trout exposed to venlafaxine concentrations (0, 0.2, 1.0 µg/L). Values are mean ± SEM (n = 7-8 fish); significant treatment (**bold**) and time effects are shown in the last column. For significant interactions, an asterisk (*) indicates a significant difference from the control within a time point while different letters indicate time effects within a treatment group ($P < 0.05$, two-way ANOVA).

	Venlafaxine (µg/L)	Post-acute stressor exposure (h)				Significance P < 0.05
		0	1	4	24	
Glycogen	**0**	4160±269	2714±430	2622±188	2112±145	0 h > 24 h
	0.2	3007±414	2686±338	3270±345	2942±362	
	1.0	3441±451	2973±327	3215±336	2059±390	
HK	**0**	12.9±0.6	8.5±0.9	7.7±0.7	7.5±0.9	0h > 1h, 4h, 24h
	0.2	12.2±0.6	9.0±0.6	8.5±0.7	8.1±0.8	
	1.0	11.6±0.9	9.6±0.8	9.1±0.5	9.3±0.8	
GK	**0**	13.3±0.5	9.5±0.8	8.2±0.6	8.4±1.1	0h > 1h, 4h, 24h
	0.2	12.8±0.6	9.7±0.7	8.3±1.0	8.2±0.7	
	1.0	12.5±0.8	9.7±0.9	9.7±0.5	9.5±0.9	
PK	**0**	7.92±0.62	5.17±1.22	7.31±0.89	7.11±1.04	24h > 1h
	0.2	8.18±1.32	5.15±0.32	8.85±1.02	8.34±0.70	
	1.0	6.85±0.52	6.41±1.05	6.75±0.68	7.70±0.93	
LDH	**0**	323±20	281±21	322±13	274±26	24h > 1h
	0.2	330±10	272±31	329±19	353±16	
	1.0	317±30	307±19	316±16	368±9	
PEPCK	**0**	0.43±0.04^{a}	0.24±0.04^{b}	0.19±0.01^{b}	0.21±0.02^{ab}	significant interaction
	0.2	0.38±0.03^{a}	0.22±0.02^{b}	0.34±0.04^{a}*****	0.26±0.03^{ab}	
	1.0	0.35±0.03^{a}	0.26±0.03^{a}	0.33±0.02^{a}*****	0.25±0.04^{a}	
AlaAT	**0**	43±6	24±5	18±2	17±2	0h > 1h, 4h, 24h **1.0 > 0**
	0.2	41±4	19±2	29±4	25±4	
	1.0	39±4	25±3	30±5	26±2	
AspAT	**0**	52±5	44±4	41±3	43±6	0h, 24h > 1h
	0.2	48±3	36±4	42±3	52±3	
	1.0	50±2	43±3	46±2	56±4	

3.4.5 Gill glycolytic capacity

Venlafaxine exposure for a 7-d period did not significantly affect either basal or stressor-mediated HK and PK activities (Table 3-3). While the pre-stress LDH activity was also not significantly affected by venlafaxine exposure, there was a significantly higher activity in the 1.0 µg/L of venlafaxine group at 24 h post-stressor exposure compared to the control group (Table 3-3). There was a significant time effect, regardless of treatments, on HK and PK activities post-stressor exposure. HK activity was significantly higher at 24 h compared to 0, 1 and 4 h post-stressor exposure, while PK activity was significantly lower at 24 h compared to the other time-points (Table 3-3). The PK activity at 4 h post stressor exposure was also significantly higher than the pre-stress values (Table 3-3).

Table 3-3. Gill metabolic capacity. Changes seen in enzyme activities (µmol/min/g) of hexokinase (HK), pyruvate kinase (PK), and lactate dehydrogenase (LDH) in response to an acute stressor (0, 1, 4, and 24 h post-stressor) in trout exposed to venlafaxine concentrations (0, 0.2, 1.0 µg/L). Values are mean ± SEM (n = 6-8 fish); significant time effects are shown in the last column. For significant interactions, an asterisk (*) indicates a significant difference from the control within a time point ($P < 0.05$, two-way ANOVA).

	Venlafaxine (µg/L)	Post-acute stressor exposure (h)				Significance P < 0.05
		0	1	4	24	
HK	0	17.0±0.8	17.0±0.6	16.5±0.7	17.2±0.6	24 > 0, 1, 4
	0.2	16.2±1.1	15.3±0.5	14.8±1.0	18.4±1.1	
	1.0	14.9±0.7	16.1±0.8	14.0±0.7	18.9±1.5	
PK	0	114±8	114±5	137±6	87±2	0, 1, 4 > 24 4 > 0
	0.2	107±6	130±12	136±10	95±5	
	1.0	114±10	123±6	127±9	119±13	
LDH	0	56±4	53±1	57±2	43±1	significant interaction
	0.2	50±2	51±2	56±4	46±2	
	1.0	51±3	57±2	52±3	54±4*	

3.4.6 Gill sodium-potassium ATPase

Na^+-K^+ ATPase activity and protein expression in the gills were measured only in the 1.0 µg/L venlafaxine exposure group prior to the acute stressor challenge (Fig. 3-4). Activity of this enzyme was significantly lower in the venlafaxine group compared to the control (Fig. 3-4A). Gill Na^+-K^+ ATPase protein expression was significantly higher in the 1.0 µg/L venlafaxine treatment group compared to the control group (Fig. 3-4B).

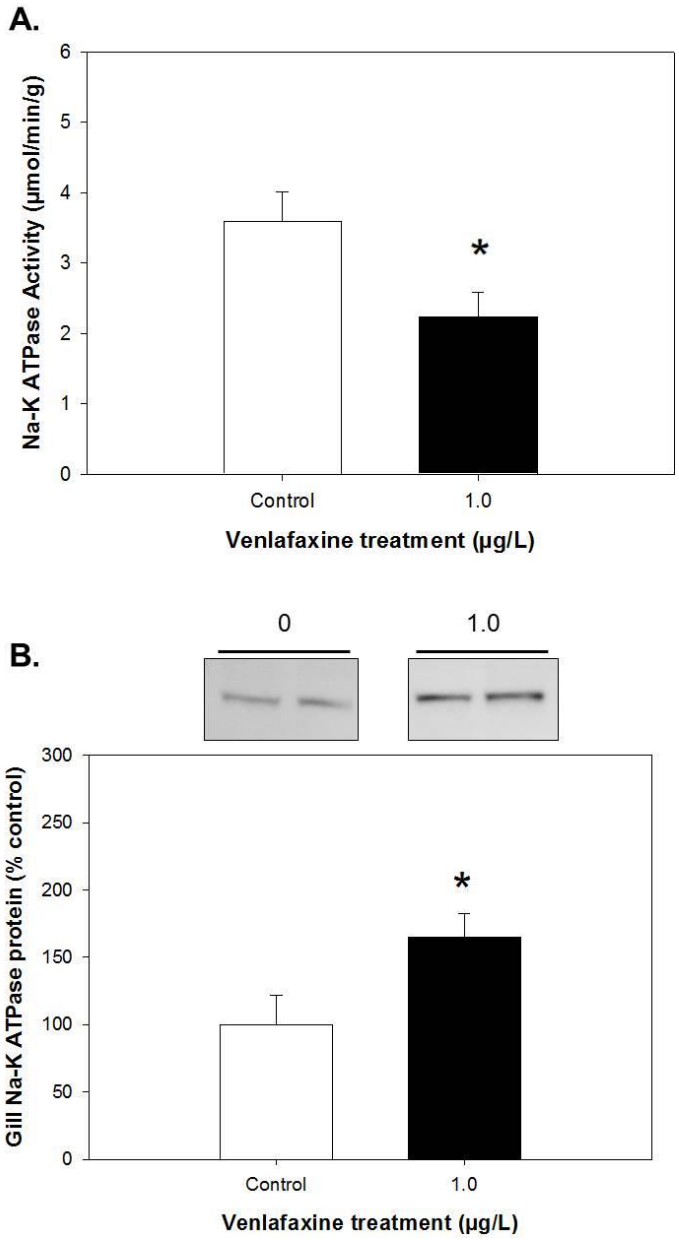

Figure 3-4. Branchial Na^+-K^+ ATPase activity and protein expression. Effects of a 7-d venlafaxine exposure on Na^+-K^+ ATPase activity (µmol/min/g; **A**) and protein expression (**B**). A representative western blot is shown for protein expression and the values are shown as % control in the bar graph (**B**). Bars represent means ± SEM (n = 6-8 fish). Asterisks indicate significant differences ($P < 0.05$, Student's t-test).

3.4.7 Brain GR protein expression

Brain GR protein expression was not affected following 7-d venlafaxine exposure (Fig. B1-A; Appendix B).

3.4.8 Liver GR protein expression and GLUT2 mRNA abundance

GR protein expression was not altered following a 7-d venlafaxine exposure in the liver tissue (Fig. B1-B; Appendix B). GLUT2 mRNA abundance (Fig. B2; Appendix B) was also unchanged.

3.5 DISCUSSION

The results demonstrate that environmentally relevant concentrations of venlafaxine can compromise the adaptive metabolic response to an acute stressor in rainbow trout. An essential component of the metabolic response is an elevation in circulating glucose levels to provide the fuel required to drive the energy demanding processes that are essential to regain homeostasis. Our results suggest that nominal levels of venlafaxine detected in MWWEs have the potential to disrupt metabolic performances essential to cope with stress in fish.

Circulating plasma glucose levels were transiently elevated in response to the netting and crowding stressor and this is a conserved adaptive response that allows the animal to metabolically cope with stress (Vijayan *et al*., 2010; Wendelaar Bonga, 1997; Iwama *et al*., 2006). The liver is an essential organ for glucose regulation, critical for the synthesis, storage and release of glucose, especially in response to stress (Mommsen *et al*., 1999). Hepatic glycogen is a major source for rapid glucose output during acute stress, which involves catecholamine-mediated glycogenolysis (Vijayan *et al*., 2010). The drop in liver glycogen content at 24 h supports the mobilization of this substrate as a contributing factor for the observed hyperglycemia after stressor exposure in trout (Wiseman and Vijayan, 2011; Gravel and Vijayan, 2007; Andersen *et al*., 1991).

The elevation in plasma glucose levels following stress was attenuated in venlafaxine-exposed fish suggesting a disruption in liver metabolism. One possible mode of action of venlafaxine in suppressing stressor-mediated glucose output may involve

modulation of target tissue responsiveness to catecholamines. The rapid mobilization of glucose from the liver after acute stressor exposure is primarily mediated by the phosphorylation of glycogen phosphorylase and subsequent glycogenolysis by epinephrine-mediated activation of beta-adrenoreceptor signalling (Fabbri *et al*., 1998). Any disruption in catecholamine synthesis and/or activation of beta-adrenergic signalling will lead to a reduction in stressor-induced acute plasma glucose response (Perry *et al*., 1988; Wright *et al*., 1989). Recently, venlafaxine (1 µM) exposure *in vitro* was shown to inhibit epinephrine-stimulated, but not basal, glucose release in isolated trout hepatocytes (Ings *et al*., 2012). This suggests that the venlafaxine impact on stressor-induced plasma glucose elevation may involve inhibition of epinephrine signalling. Whether this is a direct effect of venlafaxine acting as a beta-blocker (Ings *et al*., 2012) and/or an indirect effect through the alteration of brain monoamine levels [Chapter 2; Melnyk-Lamont *et al.*, submitted] needs to be determined. Another possibility may involve venlafaxine-mediated beta-adrenoreceptor desensitization in fish, since in mammalian studies it is established that chronic administration of some noradrenergic antidepressant causes a desensitization of the beta-adrenoreceptor coupled adenylate cyclase system (Manier *et al.*, 2002).

The higher liver PEPCK and AlaAT activities suggest that environmental levels of venlafaxine exposure may increase the metabolic demand in response to stress, and this is exacerbated given that the fish were food-deprived during the experimental period. Also, the increased liver gluconeogenic capacity at 4 h post-stressor exposure may be due to the reduced plasma glucose levels, as studies have shown that lower extracellular glucose will enhance gluconeogenesis in fish liver (Plagnes-Juan *et al*., 2008). The lack of changes in the glucose transporter 2 (GLUT2), a low-affinity transporter (Polakof *et al*., 2007; Krasnov *et al*., 2001), along with hexokinase and glucokinase activities suggests that the liver capacity for glucose import and export may not be impacted by venlafaxine exposure. Consequently, a reduction in stress-induced blood glucose may not be attributed to compromised transport from liver, suggesting an enhanced liver metabolic demand and increased endogenous use of fuel associated with venlafaxine exposure.

While catecholamines are responsible for the rapid hyperglycemia in acutely stressed trout, cortisol is thought to be a key player in maintaining the fuel in circulation for longer periods and for repletion of the liver glycogen stores (Vijayan *et al*., 2010; Mommsen *et al*., 1999). This cortisol response, at least in part, is due to GR-mediated gluconeogenesis (Vijayan *et al*., 2010; Aluru and Vijayan, 2007). Venlafaxine exposure did affect the liver metabolic capacity of trout in the present study. Specifically, the drug increased the activity of liver PEPCK, a key enzyme in gluconeogenesis (Mommsen *et al*., 1999). This was further supported by increased activity of AlaAT, an enzyme that converts amino acids into C3 precursors (i.e. alanine into pyruvate) for gluconeogenesis in fish (Mommsen *et al*., 1999). Stress-induced cortisol plays a role in increasing liver gluconeogenic capacity by increasing the activities of PEPCK and transaminases (Mommsen *et al*., 1999). In this study, venlafaxine exposure did not modify either plasma cortisol levels or the target tissue GR expression in the liver or in the brain, where it mediates negative feedback actions of cortisol on the HPI axis, suggesting other possible factor(s) modulating circulating glucose levels. A similar lack of effect on cortisol response to stress was also observed in trout exposed to environmentally relevant levels of fluoxetine (Stroud, 2012).

Despite a lack of effects on basal plasma cortisol levels, venlafaxine exposure disrupted the interrenal cortisol biosynthetic capacity as evidenced by the reduced transcript levels of StAR, a key rate-limiting step in steroidogenesis. Also, the transcript levels of the key rate-limiting enzyme in steroid biosynthesis, P450scc (orthologous to mammalian CYP11A1), was elevated prior to and at 24 h after stressor exposure suggesting disturbances in interrenal steroidogenic capacity. Indeed, studies have shown that StAR and P450scc are targets for impact by xenobiotics, including AhR agonists, heavy metals, salicylate, pesticides, and municipal wastewater effluent (Ings *et al*., 2011; Sandhu and Vijayan, 2011; Gravel and Vijayan, 2007; Aluru *et al*., 2005; Walsh *et al*., 2000). Our results add venlafaxine to the list of contaminants with the potential to disrupt steroidogenesis. However, the changes in mRNA levels of steroidogenic genes by venlafaxine did not translate into modulation of basal or stressor-stimulated plasma cortisol levels in trout *in vivo*. While the reason for this is unclear, previous studies have also shown a similar mismatch between

steroidogenic gene expression and corticosteroid output in trout (Aluru *et al*., 2005; Geslin and Auperin, 2004; Kusakabe *et al*., 2002).

Interestingly, the results of our *ex vivo* head kidney experiment indicate that venlafaxine increases the magnitude of cortisol response to *in vitro* challenge with ACTH, suggesting that at the interrenal level, tissue sensitivity to stressors may actually be increased. Stimulation of *ex vivo* head kidney tissues with 8-Br-cAMP, a cAMP analogue that can stimulate membrane signal cascades while bypassing MC2R receptor ligand binding and adenylate cyclase activation in the interrenal cells, did not reveal differences in cortisol production response between the tissues from different venlafaxine exposure groups. This leads us to propose that venlafaxine effect on cortisol production may be mediated by mechanism upstream of intracellular cAMP signalling cascade. Although the acute stressor challenge *in vivo* did not reveal significant effects of venlafaxine exposure on stressor-mediated plasma cortisol response in the present study, we have shown previously that this drug exposure significantly amplified cortisol response to a social stress in trout [Chapter 2, Melnyk-Lamont *et al*., submitted]. Consequently, effects of venlafaxine exposure on plasma cortisol response may vary with different types of stressors and/or the nutritional/metabolic state of the animal and this warrants further investigation.

While liver is a key organ for glucose production, the gill is a key target for glucose utilization in fish (Hemre and Kahrs, 1997; Soengas *et al*., 1995). An increase in LDH activity in the gill at 24 h in the 1.0 µg/L group suggests enhanced glycolytic capacity associated with drug exposure. The gill is essential for gas exchange and hydromineral homeostasis (Wendelaar Bonga, 1997) and the sodium pump activity is critical in maintaining ion gradients required for gill function (Morgan *et al*., 1997). The Na^+-K^+ ATPase capacity in the gill was decreased in venlafaxine-exposed trout. Gill Na^+-K^+ ATPase is located on the basal membrane of the chloride cells and actively maintains ion balance by exporting sodium ions into the blood in exchange for potassium ions (Wendelaar Bonga, 1997). In brook trout brain synaptosomes, activity of this ATPase is downregulated by SSRIs, which is in agreement with our results but the mechanism is far from clear (Lajeunesse *et al*., 2011). The higher Na^+-K^+ ATPase protein expression in response to

venlafaxine exposure suggests a compensatory mechanism to offset the reduced activity, but this remains to be tested. The changes in sodium pump expression and activity along with changes in LDH activity suggest a dysregulation in gill metabolism in response to venlafaxine exposure. We propose that exposure to venlafaxine may disrupt the osmo- and iono-regulatory capacity of fish and this warrants further investigation.

From an ecotoxicological standpoint, it is important to assess the effects of pharmaceutical contaminant exposure at environmentally realistic levels in order to estimate the impact on the organisms exposed in the wild. Since little is known about effects of venlafaxine on fish, exposure studies utilizing individual drug as opposed to complex effluent mixtures can elucidate its mechanism of action in these non-target animals. Overall, frequent or chronic aquatic contaminant exposures may lead to longer-term repartitioning of energy resources away from vital processes important to fitness, including growth, proper immune function, and reproduction (Iwama *et al*., 2006). As venlafaxine is typically found amid a cocktail of pharmaceuticals in municipal wastewater effluents along with other antidepressants that often have similar modes of action, the potential for additive, synergistic or antagonistic effects is an important consideration for environmental risk assessment.

3.6 CONCLUSIONS

Overall, this study demonstrates for the first time that environmental concentrations of venlafaxine can perturb the metabolic response to stress in rainbow trout. The importance of this work is underscored by using environmentally realistic concentrations for assessment of the impact of venlafaxine on stress physiology of fish. The reduced plasma glucose response to a secondary stressor suggests disruption in target tissue metabolic capacities. Indeed, the metabolic capacity of the liver, a key glucose producing tissue, was disrupted by venlafaxine exposure and this was evidenced by increased gluconeogenic and transaminase activities. The metabolic capacity of gills, a key glucose utilizing tissue, was also impacted by venlafaxine and this involved enhanced glycolytic capacity as well as decreased Na^+-K^+ ATPase activity and increased protein expression of this enzyme. Though no changes were

seen in cortisol levels or GR expression in the brain or liver, the capacity for steroidogenesis in interrenal tissue (transcript levels of StAR, P450scc) was altered by venlafaxine. Moreover, *ex vivo* head kidney tissues from venlafaxine-exposed fish exhibited increased production of cortisol in response to ACTH stimulation *in vitro*. Our results suggest that venlafaxine impacts the metabolic adjustments that are essential to cope with stress and re-establish homeostasis in fish. The overall picture of venlafaxine effects on the functioning of the HPI axis and the disruption of cortisol response appears complex and warrants further study.

3.7 ACKNOWLEDGEMENTS

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Coauthor contributions: experiment conducted by NML and CB; CB contributed plasma measurements and enzyme activity data; NML contributed GR protein expression and interrenal transcript abundance data; GM contributed Na^+K^+ATPase protein expression data.

[*Ex vivo* head kidney study in Chapter 3 (conducted and analyzed by NML) was not part of this publication submission].

Chapter 4

Attenuation of stressor-induced cortisol response by venlafaxine in rainbow trout

4.1 OVERVIEW

Venlafaxine, a commonly prescribed antidepressant drug that is frequently detected in aquatic systems across North America, impacts aspects of stress physiology in rainbow trout (*Oncorhynchus mykiss*), although effects on the functioning of the hypothalamus-pituitary-interrenal (HPI) axis are far from clear. Utilizing a flow-through exposure system and maintaining a regular feeding schedule, we mimicked an environmentally relevant exposure scenario and tested the hypothesis that venlafaxine disrupts the functioning of the HPI axis. Fish were subjected to an acute stressor following a seven day exposure to 1.0 μg/L venlafaxine. We examined plasma parameters associated with stress physiology and gene expression levels of molecular markers associated with the HPI axis. Venlafaxine exposure led to an attenuated stressor-induced cortisol response. The interrenal capacity for cortisol production in response to ACTH-stimulation was not altered by this drug. However, mRNA levels of steroidogenic acute regulatory protein (StAR) and cytochrome P450 side chain cleavage (P450scc) were upregulated at the interrenal level. At the hypothalamus, venlafaxine exposure did not affect corticotropin releasing factor (CRF) or CRF binding protein transcript levels. At the pituitary, the drug exposure significantly reduced CRF receptor type 1 (CRF-R1), but not pro-opiomelanocortin (POMC) mRNA levels. There was no significant effect of the drug on plasma ACTH levels or on the mRNA levels of corticosteroid receptors, which are involved in mediation of negative feedback effects by cortisol in the pituitary and hypothalamus. Transcript levels of neuropeptide Y (NPY), an appetite-stimulating peptide that is also known to interact with the HPI axis, were upregulated by venlafaxine exposure. No significant changes were seen in the mRNA abundance of glucose transporter type 2, GLUT2, another player involved in energy homeostasis, in the hypothalamus. Collectively, these findings suggest that venlafaxine perturbs the highly conserved and adaptive cortisol response to stressor challenge in rainbow trout. We propose that the mode of action of this drug may involve disruption of HPI axis activity in rainbow trout.

4.2 INTRODUCTION

Stressor-mediated increase in plasma corticosteroids is an evolutionarily conserved response mechanism that allows animal to re-establish homeostasis (Sapolsky *et al.,* 2000). Cortisol is a principal corticosteroid hormone in teleosts and it plays an essential role in the physiological adjustments associated with stress, including regulation of energy metabolism, hydromineral balance, and immune functions (Vijayan *et al*., 2005; Mommsen *et al*., 1999; Wendelaar Bonga, 1997). Plasma cortisol elevation in response to stressor in teleosts is regulated by the functioning of the hypothalamus-pituitary-interrenal (HPI) axis (Vijayan *et al.,* 2010; Barton, 2002). Briefly, upon perception of stress stimuli, corticotropin releasing factor (CRF) is released form the hypothalamic neurosecretory cells in the nucleus preopticus (NPO) (Bernier *et al.,* 2009). CRF stimulates cells in the anterior pituitary to produce adrenocorticotropic hormone (ACTH) from a precursor peptide pro-opiomelanocortin (POMC), and this hormone is released into the general circulation (Mommsen *et al*., 1999; Wendelaar Bonga, 1997). CRF mediates its actions via two types of receptors, CRF-R1 and CRF-R2 (Pohl *et al.,* 2001). In teleosts, CRF-R1 is known to play a pivotal role in the stress response, while the second type is thought to be associated with a broad range of physiological and behavioural responses, including appetite regulation (Flik *et al.,* 2006). ACTH is the primary cortisol secretogogue in teleosts and activates the melanocortin 2 receptors (MC2R), a G-protein coupled receptor, present on the steroidogenic cells in the head kidney region (interrenal tissue), leading to cortisol biosynthesis (Aluru and Vijayan, 2008). The first step in steroid biosynthesis involves delivery of cholesterol from the outer to the inner mitochondrial membrane, and this process is mediated by the steroidogenic acute regulatory protein (StAR) (Stocco, 2000). In the inner mitochondrial membrane, cytochrome P450 side chain cleavage (P450scc) converts cholesterol to pregnenolone and this is first rate-limiting enzymatic reaction for steroid biosynthesis (Payne and Hales, 2004). Cortisol mediates its actions in target tissues by binding to corticosteroid receptors, which are ligand-activated transcription factors. There are multiple isoforms of corticosteroids receptors, including two isoforms of glucocorticoid receptors, GR1 and GR2, and one mineralocorticoid receptor (MR) in trout (Aluru and Vijayan, 2009).

Disruption of HPI axis functioning reduces the organism's ability to cope with subsequent stressors and may lead to reduced fitness (Ings *et al*., 2011). The cortisol stress axis is a key target for endocrine disruptors, including polychlorinated biphenyls, metals and pharmaceuticals (Hontela and Vijayan, 2009). In rainbow trout, exposure to municipal wastewater effluent (MWWE), which exhibits a complex mixture of chemicals, impacts the cortisol stress response (Ings *et al.*, 2011). Over the recent years, venlafaxine became a predominant antidepressant drug detected in the MWWE.

Venlafaxine, a serotonin and norepinephrine reuptake (SNRI) inhibitor, is a commonly prescribed antidepressant drug for treatment of many depressive disorders (Roseboom and Kalin, 2000; Thase and Sloan, 2009). Due to its increased human consumption over the last decade and inadequacy of removal from the wastewater effluent (Corcoran *et al.*, 2010), its detection levels in the aquatic systems are usually one of the highest among antidepressants, ranging from low ng/L to over 1000 ng/L (González Alonso *et al.*, 2010; Metcalfe *et al.*, 2010; Schultz and Furlong, 2008; Schultz *et al.*, 2010). Our earlier acute stressor study conducted in a static experimental system revealed that venlafaxine exposure results in attenuated stressor-induced plasma glucose response [Chapter 3; Best *et al*., accepted], while no effect on stressor-induced plasma cortisol response was detected *in vivo*, although at the level of the interrenal tissue, cortisol response to *in vitro* ACTH stimulation was amplified in the tissues from venlafaxine-exposed fish [Chapter 3].

Static experimental systems utilizing daily water changes are commonly used in the fields of aquatic toxicology. However, during the acclimation period, habituation may occur to repeated handling associated with water exchanges and the animal may not respond to an additional stressor (Cyr and Romero, 2009). Repeated exposures to mild stressors can desensitize fish and attenuate neuroendocrine responses to subsequent stressors (Barton, 2002). Also, the fish in the static system were not fed during the experimental period and this may have led to changes in the metabolic demand of the animal, leading to an altered response. To test this, and to determine whether changes in the physiological stress response by VEN exposure involve alterations in HPI axis functioning, we exposed juvenile rainbow

trout to 1 μg/L venlafaxine in a flow-through water system with daily feed for seven days, followed by subjecting the fish to an acute stressor challenge.

To test the hypothesis that exposure to environmental levels of venlafaxine disrupts functioning of the HPI axis, we examined plasma parameters of stress response (cortisol, ACTH, glucose, lactate) along with GR1, GR2 and MR transcript levels in the hypothalamus and pituitary, where cortisol mediates negative feedback regulation of the HPI axis (Aluru and Vijayan, 2009), following an acute stressor challenge. The drug exposure was considered as the primary stressor, and these fish were then subjected to a handling disturbance, which constituted the secondary stressor. Also, transcript levels of key molecular markers involved in HPI axis function, including CRF and its binding protein, CRF-BP, in the hypothalamus, pro-opiomelanocortin, POMCA and POMCB, and CRF-R1 and CRF-R2 in the pituitary and StAR, P450scc and MC2R in the interrenal tissue were measured to indicate central and peripheral endocrine disruption by venlafaxine. In addition, we investigated mRNA abundance of genes involved in appetite regulation, including hypothalamic neuropeptide Y (NPY) and bidirectional glucose transporter type 2 (GLUT2) (Polakof *et al.*, 2011;Volkoff *et al.*, 2005), and their modulation by acute stressor given that venlafaxine appeared to play a role in feed intake and energy homeostasis [Chapter 2; Melnyk-Lamont *et al.*, submitted].

4.3 MATERIALS AND METHODS

4.3.1 Animals and experimental protocol

Juvenile rainbow trout were purchased from Rainbow Springs Trout Hatchery (Thamesford, ON) and were kept at the aquatics facility at Wilfrid Laurier University in 200 L polyethylene tanks with flowing fresh water (dechlorinated city tap water) at 700 ml/min at 12°C. Trout were fed 2% of body weight with commercial fish food (Bio Oregon Protein Inc., Warrenton, OR) once daily and a photoperiod (16 light : 8 dark) was maintained throughout the experiment. All experimental protocols were approved by the University of Waterloo and the Wilfrid Laurier University Animal Care Committees and conducted in accordance with the Canadian Council for Animal Care guidelines.

Rainbow trout (112 ± 6 g) were randomly distributed into four 200 L polyethylene tanks (32 fish per tank) where they were acclimated for two weeks prior to start of the experiment. In our drug exposure set-up, trout were exposed to either 0 (control) or 1.0 μg/L venlafaxine for seven days in a flow-through system. Venlafaxine concentration of 1.0 μg/L was chosen to approximate environmental levels (Metcalfe *et al.*, 2010; Schultz and Furlong, 2008) and this concentration was shown to affect brain monoamine levels [Chapter 2; Melnyk-Lamont *et al*., submitted]. Venlafaxine exposure involved metering (QG6 pump, Fluid Metering Inc., Oyster Bay, NY) of a concentrated stock solution (venlafaxine hydrochloride*;* Sigma Aldrich, St. Louis, MO; dissolved in water) into a mixing head tank before delivery to duplicate fish tanks (180 L volume of water in each tank). Exposures were initiated by spiking the tanks with appropriate volumes of concentrated stock solution of venlafaxine to achieve the target exposure concentration. Header and fish tanks were supplied with continuous aeration to ensure mixing. Fish were fed once daily throughout the exposure period and the feeding stopped 24 h prior to acute stressor exposure.

Following the drug exposure period, pre-stress fish from each tank were sampled (represented by 0 h time point). Following the 0 h sampling, the remaining fish were exposed to a secondary acute stressor consisting of a 5 min handling disturbance as previously described (Ings et al., 2011) followed by sampling at 1, 4 and 24 h post-stressor exposure. At each sampling point, 8 fish (4 fish per replicate exposure tank) were sampled.

4.3.2 Collection of tissue samples

Fish were euthanized with an overdose of tricaine methanesulfonate (MS-222; Syndel Laboratories Ltd., Vancouver, BC; 0.6 g/L buffered with sodium bicarbonate) and were bled, by severing the caudal peduncle, into 1.5 ml centrifuge tubes containing 40 μl of 10 mg/mL EDTA in physiological saline as an anticoagulant. Blood samples were immediately centrifuged at 10,000 x g for 2 min and plasma was collected and stored at -30 °C for later analysis of cortisol, ACTH, glucose and lactate levels. Each brain was quickly removed and placed on a chilled petri dish. Hypothalamic tissue along with preoptic region (containing NPO) was dissected and pooled as one region. Trout brain regionalization diagram is shown

in [Fig. A1-A; Appendix A]. Pituitaries and head kidney tissue were also collected from all fish. All tissue samples were flash frozen in dry ice and stored at -80^{o}C for later analysis of mRNA levels.

4.3.3 Plasma measurements

Plasma cortisol levels were measured by radioimmunoassay (RIA) using ^{3}H cortisol as described previously (McMaster *et al.,* 1995). Cortisol antibody was obtained from MP Biomedicals (MP Biomedicals, Irvine, CA, USA) and ^{3}H cortisol was purchased from GE Healthcare (Waukesha, WI, USA). Plasma ACTH concentrations were measured using a double antibody (hACTH) ^{125}I RIA kit (MP Biomedicals) as per the manufacturer's instructions. This kit has been previously validated for ACTH measurements in trout plasma (Doyon *et al.*, 2006; Craig *et al.*, 2005). Plasma glucose was measured by the hexokinase and glucose-6-phosphate dehydrogenase enzymatic method (Bergmeyer *et al.*, 1974), and plasma lactate was determined by lactate dehydrogenase and nicotinamide adenine dinucleotide enzymatic method (Gutmann and Wahlefeld, 1974) using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

4.3.4 Quantification of gene expression

Total RNA was isolated from head kidney, pituitary, hypothalamic tissues using the RNeasy Mini Kit (Qiagen, Mississauga, ON) according to manufacturer's instructions. RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Nepean, ON) at 260/280 nm and 1 μg of total RNA (DNase treated) was used for first-strand cDNA synthesis using high capacity cDNA reverse transcription kit (Applied Biosystems, Streetsville, ON, CAN) in 20 μl reaction volume. Real-time quantitative PCR (qPCR) was used for quantification of transcript levels using iCycler real-time PCR detection system (Bio-Rad, Hercules, CA). cDNA samples were used as template for amplification of target genes using gene-specific primers (Table 4-1). cDNA from the hypothalamic region was used for analysis of CRF, CRF-BP, GLUT2, NPY, GR1, GR2 and MR transcript expression (genes involved in HPI axis and energy balance regulation); the pituitary cDNA was used for expression analysis of CRF-R1 and CRF-R2, POMCA, POMCB, GR1, GR2, MR (genes involved in

HPI axis); and the head kidney cDNA was used for StAR, P450scc, and MC2R analysis (genes involved in cortisol biosynthesis).

A master mix containing 2.5 μL of cDNA, 2.5 μL of 10 μM primer pair, 40 μL of SYBR green supermix (Bio-Rad) and 35 μL of nuclease-free water was prepared for each sample and run in triplicates. The following PCR conditions were used for amplification: initial denaturation for 3 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 20 s at annealing temperature (see Table 4-1); 95 °C for 1 min; 55 °C for 1 min followed by melt curve analysis starting at 55 °C and increasing in 0.5 °C increments to 95 °C every 10 s. Only PCR conditions for NPY amplification differed from the above stated, and were described previously (Conde-Sieira *et al.,* 2010). Threshold cycle values were calculated using iCycler iQ real-time detection software (Bio-Rad). All data were normalized to relative expression of a housekeeping gene EF1α since its expression did not change across treatments or time points. A relative standard curve method was used to determine relative transcript abundances according to established protocols (Sathiyaa and Vijayan, 2003). All transcript levels are shown as percent change from the control group of 0 h time-point.

4.3.5 Statistical analysis

All statistical tests were performed using *Sigmaplot 11* (Systat Software Inc., San Jose, CA, USA). For plasma measurements and transcript levels, two-way analysis of variance (ANOVA) was used to examine venlafaxine treatment and time post-stressor exposure effects, and their interactions (treatment × stress), followed by Holm-Sidak *post hoc* test. Data were transformed when necessary to meet the assumptions of normality and homogeneity of variance, although non-transformed data are shown in figures. A significance level of $\alpha = 0.05$ was used in all cases.

Table 4-1. Sequences (forward and reverse), amplicon size, accession number and annealing temperature for primers used in quantitative real-time RT-PCR.

Gene	Primer sequence	Accession No.	T_{anneal} (^{o}C)	Amplicon (bp)
EF1α	F: 5'-CATTGACAAGAGAACCATTGA-3' R: 5'-CCTTCAGCTTGTCCAGCAC-3'	AF498320.1	56	95
CRF	F: 5'-ACAACGACTCAACTGAAGATCTCG-3' R: 5'-AGGAAATTGAGCTTCATGTCAGG-3'	NM_001124286.1	60	54
CRF-BP	F: 5'-GGAGGAGACTTCATCAAGGTGTT-3' R: 5'-CTTCTCTCCCTTCATCACCCAG-3'	NM_001124631.1	60	51
CRF-R1	F: 5'-TCACACCCAGCAATGTC-3' R: 5'-GCAGTGCTCTTTGGCCAGC-3'	AJ277157	60	82
CRF-R2	F: 5'-CCAAGTTGAGAGCTTCTACC-3' R: 5'-AACAGCATGTAGGTGATCCC-3'	AJ277158	60	103
GLUT2	F: 5'-GGCCATCTTCCTGTTTGTGT-3' R: 5'-TGAAGTTGCTGGTCCAGTTG-3'	AF321816	60	140
NPY	F: 5'-CTCGTCTGGACCTTTATATGC-3' R: 5'-GTTCATCATATCTGGACTGTG-3'	NM_001124266	58	247
GR1	F: 5'-TTCCAAGTCCACCACATCAA-3' R: 5'-GGAGAGCTCCATCTGAGTCG-3'	Z54210	60	115
GR2	F: 5'-GGGGTGATCAAACAGGAGAA-3' R: 5'-CTCACCCCACAGATGGAGAT-3'	AY495372.1	60	140
MR	F: 5'-ACCAACAACATGAGGGCTTC-5' R: 5'-AGTTCACTAGCAGGGCTGGA-3'	NM_001124740.1 NM_001124483.1	60	131
StAR	F: 5′-TGGGGAAGGTGTTTAAGCTG-3′ R: 5′-AGGGTTCCAGTCTCCCATCT-3′	AB047032	60	101
P450scc	F: 5′-GCTTCATCCAGTTGCAGTCA -3′ R: 5′-CAGGTCTGGGGAACACATC -3′	S57305.1	60	140
MC2R	F: 5'-GAGAACCTGTTGGTGGTGGT-3' R: 5'-GAGGGAGGAGATGGTGTTGA-3'	EU119870	60	105
POMCA	F: 5'-AGGGTTGAGGGAGGAAGAGA-3' R: 5'-TGTCAGAGGACATGGCTTTT-3'	NM_001124718.1	60	116
POMCB	F: 5'-CCAGAACCCTCACTGTGACGG-3' R: 5'- CCTGCTGCCCTCCTCTACTGC-3'	NM_001124719.1	60	199

Gene abbreviations: EF1α, elongation factor 1α; CRF, corticotropin-releasing factor; CRF-BP, corticotropin-releasing factor binding protein; CRF-R1 and CRF-R2, corticotropin releasing factor receptor 1 and 2, respectively; GLUT2, glucose transporter type 2; NPY, neuropeptide Y; GR1 and GR2, glucocorticoid receptor 1 and 2, respectively; MR, mineralocorticoid receptor; StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 side chain cleavage; MC2R , melanocortin 2 receptor, POMCA and POMCB, pro-opiomelanocortin A and B, respectively.

4.4 RESULTS

4.4.1 Plasma measurements

As expected, stressor-induced plasma cortisol response was affected by a significant stressor effect ($P < 0.001$, two-way ANOVA). There was a significant increase at the 1 h post-stressor time point (Fig. 4-1A), followed by a significant decrease at the 4 h time point and returning to basal (pre-stress) levels at 24 h post-stressor exposure Also, a significant treatment effect was present ($P < 0.05$, two-way ANOVA), demonstrating significant attenuation of plasma cortisol levels in the venlafaxine-exposed group compared to the control (Fig. 4-1A). Attenuation was most prominent at 1 h post-stressor time point, where cortisol levels were reduced by approximately 40% in the venlafaxine group compared to the control. There was no significant treatment effect on plasma ACTH levels (Fig. 4-1B), although a significant stressor exposure effect was detected ($P < 0.05$, two-way ANOVA), demonstrating that ACTH levels were significantly elevated at 24 h post-stress relative to 1 or 4 h time-points regardless of treatments (Fig. 4-1B). Stress-induced glucose levels showed a profile similar to that of cortisol, with significant elevation at 1 h post-stressor and returning to pre-stress levels at 24 h post-stressor (Fig. 4-1C). There was no significant treatment effect on plasma glucose levels (Fig. 4-1C). Stressor exposure significantly increased plasma lactate levels at 1 h post-stressor exposure and the levels dropped to pre-stress levels by 4 h post stressor exposure (Fig. 4-1D). There was a significant increase in plasma lactate levels at 24 h compared to the pre-stress levels. Venlafaxine exposure did not significantly affect plasma lactate levels in the present study (Fig. 4-1D).

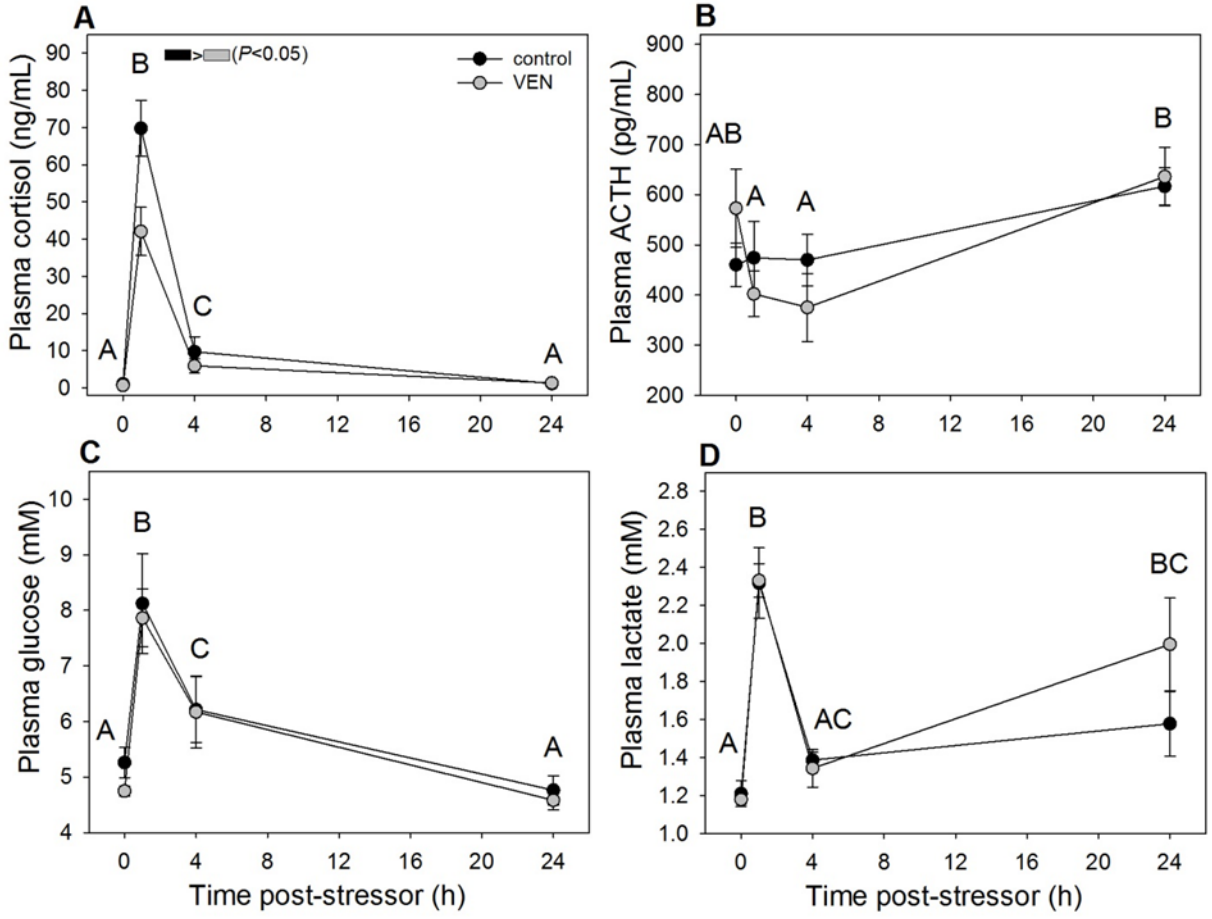


Figure 4-1. Effect of a 7-day venlafaxine exposure (1.0 µg/L) on plasma cortisol (**A**), adrenocorticotropic hormone, ACTH (**B**), glucose (**C**), and lactate (**D**) in rainbow trout prior to (0 h) and after an acute stressor (1, 4 and 24 h). Values represent mean ± SEM (n = 7-9 fish). Capital letters indicate significant differences between stress time points, and insets show significant treatment effects. Data were analysed by two-way ANOVA, followed by Holm-Sidak *post hoc* test when significant effects were detected ($P < 0.05$).

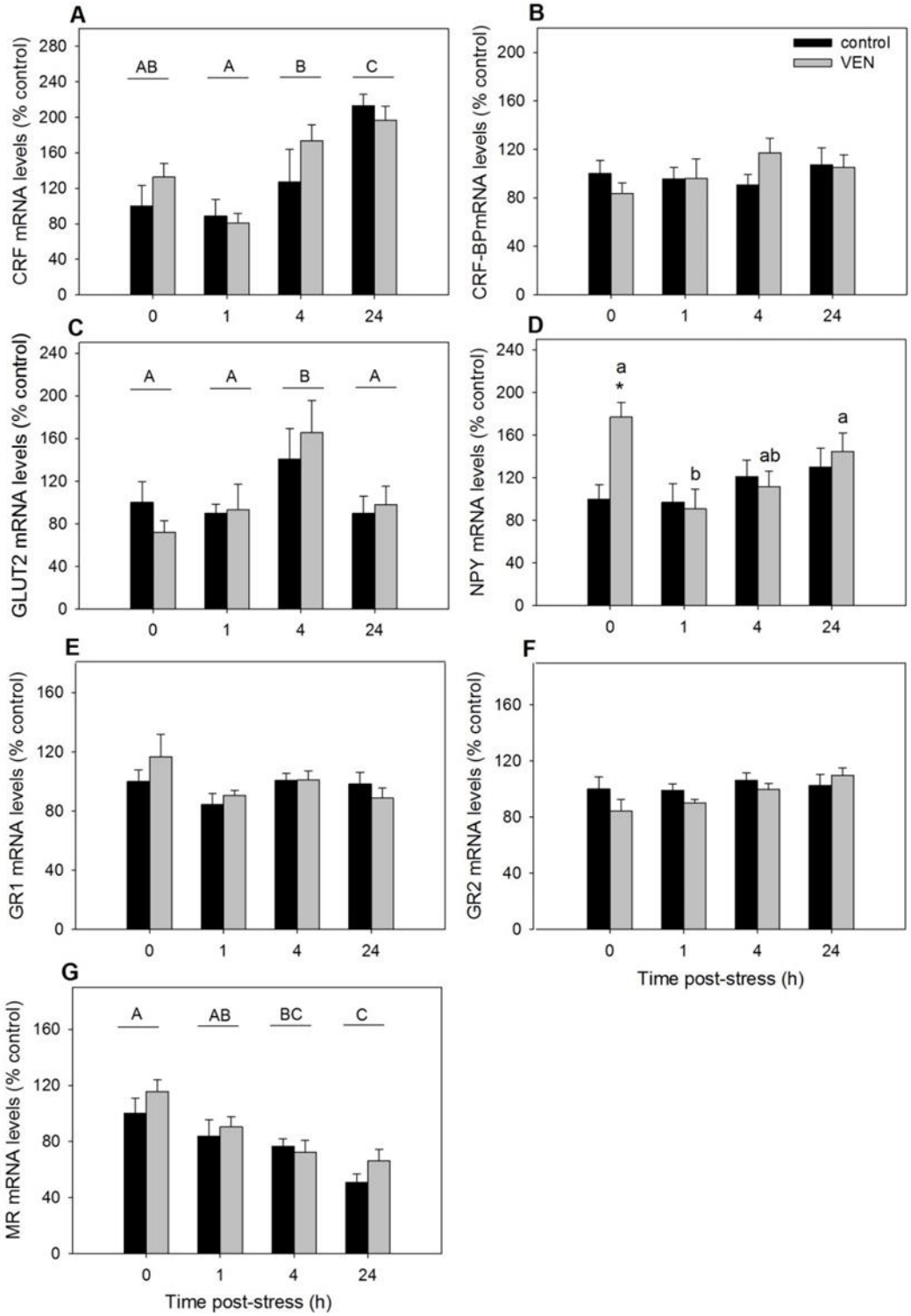
4.4.2 Transcript levels - hypothalamic region

In the hypothalamic region (including the preoptic area), overall CRF mRNA abundance was affected by stressor application ($P < 0.05$, two-way ANOVA), where transcript levels were significantly elevated at 4 h and 24 h relative to 1 h post-stress time point (Fig. 4-2A). No venlafaxine treatment effect on CRF mRNA abundance was present (Fig. 4-2A). Expression of CRF-BP, a binding protein that regulates CRF bioavailability, was not affected by either stressor application or venlafaxine exposure in this study (Fig. 4-2B).

Analysis of transcript levels of the glucose transporter, GLUT2, detected a significant effect due to stressor application ($P < 0.05$, two-way ANOVA), where transcript levels were elevated at 4 h post-stress relative to other time points (Fig. 4-2C). No venlafaxine treatment effect on GLUT2 expression was observed (Fig. 4-2C). Statistical analysis of mRNA expression of NPY detected a significant interaction ($P = 0.05$, two-way ANOVA) between the drug treatment and stress factors. Comparison between the control and venlafaxine groups within each time point revealed that drug exposure significantly elevated NPY transcript levels (~1.8-fold) before acute stressor application at the 0 h time point, although no treatment effect was present at post-stress time points (Fig. 4-2D). There was an effect of stressor application on NPY expression in the control group; however, venlafaxine-exposed fish demonstrated a significant time effect, where expression levels were reduced 1h post-stress relative to pre-stress levels (Fig. 4-2D).

Expression levels of the corticosteroid receptor genes, important in the negative feedback regulation of the HPI axis, were also examined in the hypothalamic region. Transcript abundances of GR1 and GR2 genes were not affected by either venlafaxine treatment or stressor application (Fig. 4-2E and 4-2F, respectively). There was no treatment effect on MR mRNA levels, although a significant stressor effect was present ($P < 0.001$, two-way ANOVA), showing a gradual reduction in expression levels after acute stressor application (Fig. 4-2G).

Figure 4-2. Effect of a 7-day venlafaxine exposure (1.0 μg/L) on the hypothalamic (including preoptic area) mRNA levels of corticotropin-releasing factor, CRF (**A**), its binding protein CRF-BP (**B**), glucose transporter type 2, GLUT2 (**C**), neuropeptide Y, NPY (**D**), glucocorticoid receptors, GR1 (**E**) and GR2 (**F**), and mineralocorticoid receptor, MR (**G**) in rainbow trout prior to (0h) and after an acute stressor (1, 4, and 24 h). All mRNA levels were normalized to EF1α and values are shown as percent of the control group (0 h), bars represent means ± SEM (n = 6 fish). Capital letters indicate significant differences between stress time points. When significant interaction was detected (treatment × stress), small letters indicate differences between stress time points for an individual treatment group, and asterisks indicate significant treatment effect within a time point. Transcript abundances were analyzed with two-way ANOVA, followed by Holm-Sidak *post hoc* test when significant effects were detected ($P < 0.05$).


A
CRF mRNA levels (% control)
AB
A
B
C
B
CRF-BPmRNA levels (% control)
control
VEN
C
GLUT2 mRNA levels (% control)
D
NPY mRNA levels (% control)
a
*
b
ab
a
E
GR1 mRNA levels (% control)
F
GR2 mRNA levels (% control)
G
MR mRNA levels (% control)
BC
Time post-stress (h)

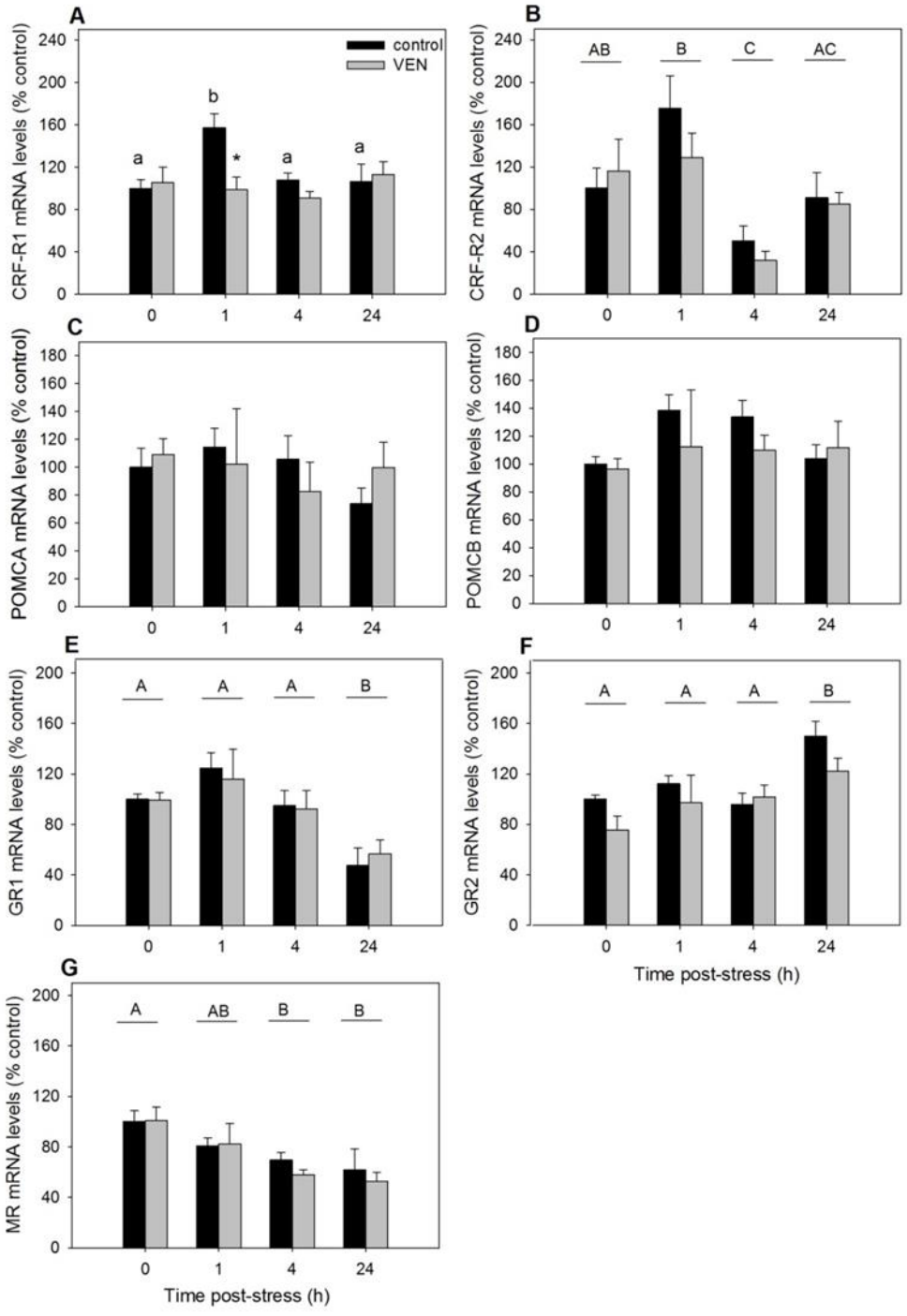
4.4.3 Transcript levels - pituitary

In the pituitary, transcript levels of the two CRF receptor genes, CRF-R1 and CRF-R2, were examined. Statistical analysis of CRF-R1 gene expression detected a significant interaction between venlafaxine treatment and stressor factors ($P = 0.031$, two-way ANOVA). Comparison between the control and venlafaxine-exposed groups, detected a significant reduction in CRF-R1 transcript abundance (~1.6 fold-change) in the venlafaxine group 1 h following stressor exposure (Fig. 4-3A). No treatment effect was detected within 0, 4, or 24 h time-points. There was a significant stressor effect present in the control group, where CRF-R1 transcripts were significantly elevated 1 h post-stressor relative to other time points, whereas the venlafaxine-exposed group did not exhibit stress effects on CRF-R1 expression (Fig. 4-3A). There was no treatment effect on CRF-R2 expression (Fig.4-3B), although a significant stressor effect was detected ($P < 0.001$, two-way ANOVA) demonstrating a reduction in CRF-R2 mRNA levels at the 4 and 24 h time-points relative to the 0 and 1 h time points (Fig. 4-3B).

Analysis of the pituitary expression profiles of the genes encoding the pro-opiomelanocortin peptides, POMCA and POMCB, did not identify significant effects of venlafaxine treatment or stressor exposure for either of the genes (Fig. 4-3C and Fig. 4-3D, respectively).

Transcript abundances of GR1 and GR2 (Fig. 4-3E and 4-3F, respectively) were influenced by significant stressor effects ($P < 0.05$ in both cases, two-way ANOVA), where 24 h post-stressor time point exhibited reduced GR1 (Fig. 4-3E) and elevated GR2 (Fig. 4-3F) mRNA abundances in comparison to other time-points, although neither of the GR isoforms were affected by venlafaxine treatment. MR expression (Fig. 4-3G) was significantly affected by stressor application ($P < 0.001$, two-way ANOVA), demonstrating reduced expression at 4 and 24 h post-stress relative to the pre-stress levels. There was no treatment effect on MR mRNA abundance.

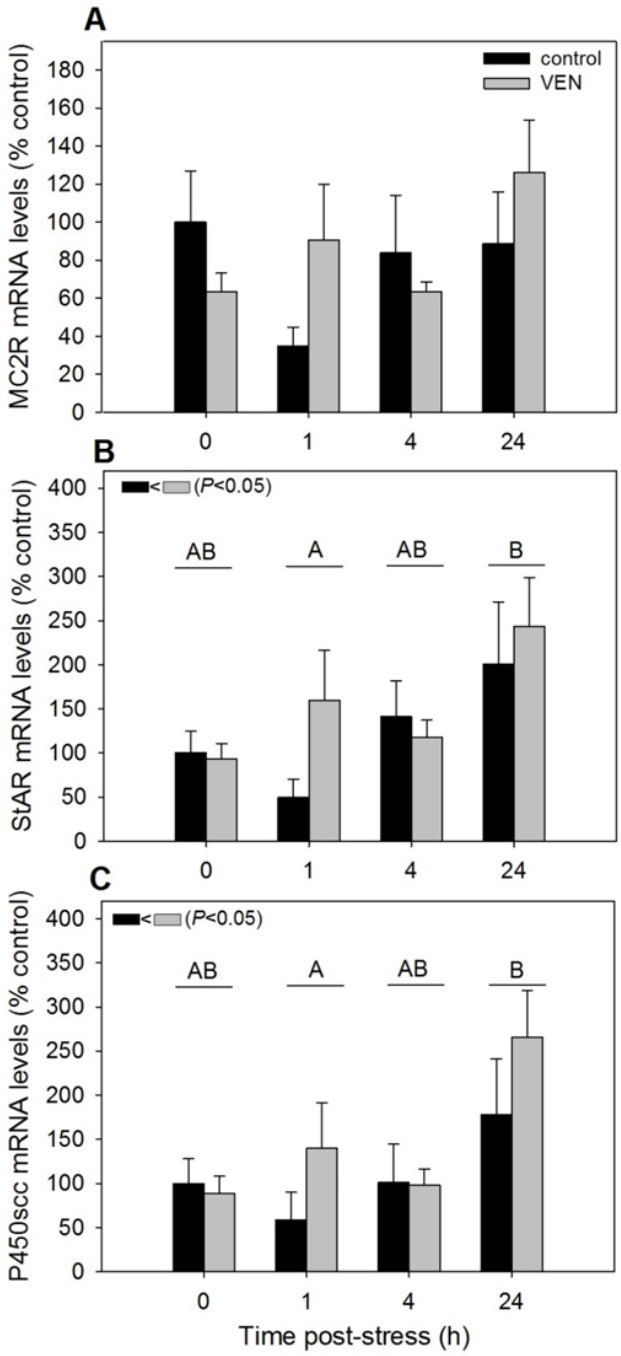
Figure 4-3. Effect of a 7-day venlafaxine exposure (1.0 μg/L) on the pituitary mRNA levels of corticotropin-releasing factor receptors, CRF-R1(**A**) and CRF-R2 (**B**), pre-opiomelanocortin genes, POMCA (**C**) and POMCB (**D**), glucocorticoid receptors, GR1 (**E**) and GR2 (**F**) and mineralocorticoid receptor, MR (**G**) in rainbow trout prior to (0 h) and after an acute stressor (1, 4, and 24 h). All mRNA levels were normalized to EF1α and values are shown as percent of the control group (0 h), bars represent means ± SEM (n = 6 fish). Capital letters indicate significant differences between stress time points. When significant interaction was detected (treatment × stress), small letters indicate differences between stress time points for an individual treatment group, and asterisks indicate significant treatment effect within a time point. Transcript abundances were analysed with two-way ANOVA, followed by Holm-Sidak *post hoc* test when significant effects were detected ($P < 0.05$).


A
control
VEN
CRF-R1 mRNA levels (% control)
a
b
*
a
a
B
CRF-R2 mRNA levels (% control)
AB
B
C
AC
C
POMCA mRNA levels (% control)
D
POMCB mRNA levels (% control)
E
GR1 mRNA levels (% control)
A
A
A
B
F
GR2 mRNA levels (% control)
A
A
A
B
G
MR mRNA levels (% control)
A
AB
B
B
0
1
4
24
Time post-stress (h)

4.4.4 Transcript levels - interrenal tissue

Statistical analysis of the gene expression levels of MC2R did not detect any significant treatment or stressor exposure effects (Fig. 4-4A). There were significant main treatment and stressor effects detected on the expression of the two steroidogenic genes – StAR and P450scc (Fig. 4-4B and 4-4C, respectively). Venlafaxine-exposed fish had higher expression of StAR and P450scc compared to the control fish ($P < 0.05$; two-way ANOVA). Transcript levels for both genes showed significant elevations at 24 h relative to 1 h post-stressor exposure ($P < 0.05$; two-way ANOVA).

Figure 4-4. Effect of a 7-day venlafaxine exposure (1.0 μg/L) on the head kidney mRNA levels of melanocortin 2 receptor, MC2R (**A**), steroidogenic acute regulatory protein, StAR (**B**), and cytochrome P450 side chain cleavage, P450scc (**C**) in rainbow trout prior to (0 h) and after an acute stressor (1, 4, and 24 h). All mRNA levels were normalized to EF1α and values are shown as percent of the control group (0 h), bars represent means ± SEM (n = 6 fish). Capital letters indicate significant differences between stress time points, and insets show significant treatment effects. Transcript abundances were analysed with two-way ANOVA, followed by Holm-Sidak *post hoc* test when significant effects were detected (P < 0.05).


A
control
VEN
MC2R mRNA levels (% control)
0
20
40
60
80
100
120
140
160
180
0
1
4
24
B
< (P<0.05)
AB
A
AB
B
StAR mRNA levels (% control)
0
50
100
150
200
250
300
350
400
C
< (P<0.05)
AB
A
AB
B
P450scc mRNA levels (% control)
Time post-stress (h)

4.5 DISCUSSION

Our study demonstrates that sub-chronic exposure to an environmentally relevant concentration of venlafaxine perturbs the organismal cortisol response in rainbow trout, which is a highly conserved mechanism essential for the metabolic adjustments required for re-establishing homeostasis (Aluru and Vijayan, 2009). Reduced capacity to evoke a proper physiological cortisol response may impair the animal's stress performance, which can translate into reduced fitness in fish exposed to environmental levels of venlafaxine.

Venlafaxine exposure did not affect basal cortisol levels after the seven day exposure period, and this is in agreement with our previous findings [Chapters 2, 3]. However, our results demonstrate that venlafaxine impaired the ability to respond to a secondary stressor (handling disturbance), as the amplitude of the stressor-induced cortisol response was reduced by the drug exposure. So what is the mechanism leading to the attenuation of stressor-included cortisol response by venlafaxine?

Analysis of plasma ACTH, a primary secretagogue for cortisol in fish produced by the pituitary gland, did not provide evidence for its involvement in attenuation of the cortisol response. Typically application of an acute stressor (e.g. handling, confinement, heat stress) leads to elevation of plasma ACTH in conjunction with elevation of plasma cortisol levels (Aluru and Vijayan, 2008; Balm and Pottinger, 1995; Sumpter *et al.*, 1986). In the present study, we did not observe a peak in plasma ACTH concentrations at the 1 h time point following the acute stressor. Plasma ACTH concentrations may peak as early as 5 min following stressor application (preceding the rise in plasma cortisol), and the duration and amplitude of the response may be stressor-specific (Sumpter *et al.*, 1986; Cook *et al.*, 1973). Thus, it is possible that in our experimental animals the stressor-induced ACTH levels may have peaked prior to the 1 h sampling point.

Signalling of CRF and CRF-related peptides is mediated by two main G-protein coupled receptor types, CRF-R1 and CRF-R2, which are widely expressed in the central nervous system, as well as peripherally (Pohl *et al.*, 2001). CRF-R1 is known to be associated with the stress axis activation, while CRF-R2 is involved in a wide range of

physiological and behavioural responses (Flik *et al.,* 2006). In the pituitary corticotrophs, CRF-R1 is the major subtype and is responsible for regulating the synthesis and secretion of ACTH (Kageyama and Suda, 2009). In the present study, the control fish exhibited a significant elevation of the pituitary CRF-R1 transcripts 1 h after the acute stressor challenge and returned to the resting levels at the later time points, and this is in agreement with the finding that CRF stimulation of pituitary CRF-R1 leads to subsequent downregulation of CRF-R1 mRNA levels (Kageyama and Suda, 2009; Rabadan-Diehl *et al.,* 1996). Interestingly, the venlafaxine-exposed group did not exhibit the same temporal profile, demonstrating significantly lower transcript levels of this receptor type 1 h post-stressor exposure. This suggests that venlafaxine exposure may reduce the sensitivity of the pituitary corticotroph cells to CRF stimulation, but this remains to be tested. Despite the reduced CRF-R1 transcript levels post-stressor in the venlafaxine group, there was no significant effect on the expression levels of either of the two pro-opiomelanocortin encoding genes, POMCA and POMCB, although the venlafaxine group exhibited a high degree of variation at this time point for both genes. POMC is a precursor molecule for several biologically active peptides and it undergoes post-translational processing in the corticotrophs, resulting in the production of ACTH and teleost fish have two gene isoforms due to a genome duplication event in salmonids (Salbert *et al.,* 1992). Although there was no significant downstream effect on POMC transcript abundance due to alteration in CRF-R1 transcript levels by venlafaxine, it remains to be determined whether the drug affected the translational/post-translational processing and secretion of this peptide in trout pituitaries.

While examining the most upstream element of the HPI axis, the hypothalamic region, we did observe an overall upregulation of CRF transcripts in the hypothalamic area at 4 and 24 h, but not at 1 h time-points, consistent with the idea that stress-induced CRF gene expression increases in response to depletion of the stored CRF neuropeptide (Imaki *et al.,* 1992). Venlafaxine treatment affected neither CRF mRNA abundance nor CRF-BP transcripts, encoding the protein that regulates CRF bioavailability (Flik *et al.,* 2006). Our previous findings [Chapter 2; Melnyk-Lamont *et al*., submitted] demonstrated that venlafaxine exposure significantly increased basal CRF mRNA in the hypothalamus, but not

in the preoptic area. In this current study, the preoptic area was pooled together with the hypothalamic region and this may have a played a role in the lack of change in CRF transcript levels with venlafaxine, as the brain responses are very region-specific. In the fish brain, CRF-neurons from nucleus preopticus (in the preoptic area) and hypothalamic nucleus tuberalis are thought to be involved in the regulation of pituitary ACTH production and secretion (Yulis and Lederis, 1987). However, nucleus preopticus plays a more pivotal role in stressor-mediated ACTH regulation compared to the tuberal hypothalamus in fishes (Bernier *et al.,* 2008; Flik *et al.,* 2006).

While the hypothalamic brain region is an integral neuroendocrine element of the HPI stress axis, this region also plays a central role in the control of food intake, integrating sensory information from peripheral signals of appetite, satiation, and long-term energy-balance (Kalra *et al.,* 1999). The pre-stress levels (0 h) of NPY transcript were elevated due to venlafaxine treatment and this observation can be related to the feeding behaviour. Similar to mammals, NPY is an orexigenic peptide in fish involved in energy balance regulation by stimulating appetite (Volkoff *et al.,* 2005). Previous studies demonstrated that fasted fish exhibit higher expression of NPY transcripts in the hypothalamus and preoptic area (MacDonald and Volkoff, 2009; Silverstein *et al.,* 1998). Our results from behavioural study [Chapter 2; Melnyk-Lamont *et al*., submitted] suggested that fish exposed to venlafaxine consumed less food compared to the control fish. If a similar venlafaxine effect on food intake occurred in the present study (although food consumption was not evaluated), it might have resulted in reduced nutritional status in the venlafaxine-exposed fish leading to transcriptional activation of the appetite-stimulating NPY. Thus, the elevated levels of hypothalamic NPY mRNA observed in the venlafaxine group may be related to the feeding behaviour. In addition to its role in appetite regulation, NPY is also involved in complex interactions with the hypothalamus-pituitary-adrenal (HPA) axis based on mammalian studies, and NPY is known to stimulate ACTH release in mammals [reviewed in (Krysiak *et al.,* 1999)]. Studies in fish also suggest that NPY may participate in the activation of the HPI axis in trout, possibly through interaction with CRF (Doyon *et al.,* 2006; Doyon *et al.,* 2003).

Consequently, we propose that venlafaxine-mediated changes in NPY transcript levels may also be involved in the impaired HPI axis regulation, in addition to appetite-related effects.

Another potential mechanism associated with altered cortisol production may involve changes in the cortisol-mediated negative feedback regulation. However, no significant changes were seen in the mRNA levels of genes encoding corticosteroid receptors GR1, GR2, MR by venlafaxine exposure in either the hypothalamic tissue or the pituitary arguing against perturbation in the negative feedback regulation. Investigation of steroidogenic capacity at the interrenal level did show interesting results. Overall, venlafaxine-exposed fish demonstrated higher transcript levels of the steroidogenic genes StAR and P450scc, while the mechanism leading to this effect remains unknown. As mentioned earlier, the stimulation of steroid biosynthetic pathway in the interrenal tissue involves binding of ACTH to MC2R receptor, which leads to increased adenylate cyclase activity, leading to elevation of intracellular levels of cAMP and activation of protein kinase A (PKA), which in turn phosphorylates protein targets including StAR (Stocco, 2000). While some researchers have shown an increase in the expression of these genes to ACTH or a handling disturbance stressor (Aluru and Vijayan, 2008; Aluru and Vijayan, 2006), others saw no change in transcript levels of these genes following ACTH stimulation or stressor (Alderman *et al.,* 2012; Gravel and Vijayan, 2007). Transcript response of the steroidogenic genes in the interrenal appears to be related to the magnitude of cortisol response. For instance, high intensity stressor resulted in elevation of StAR and P450ssc transcripts in the interrenal tissue of trout; however, no change in expression of these genes was present following a low intensity stressor (Geslin and Auperin, 2004). Moreover, transcriptional regulation of these genes seems to be rapid and transient− for instance, transcripts of the steroidogenic genes showed a significant elevation 10 min of *in vitro* ACTH stimulation of the head kidney tissue and dropped back at the 30 min time point (Conde-Sieira *et al.,* 2013). We hypothesize that the decreased transcript levels of StAR and P450scc in the control fish may reflect higher turnover of pre-existing mRNA pools to support higher cortisol output. Previous *in situ* hybridization results showed presence of substantial StAR mRNA pool in the interrenal cells in non-stressed trout that may be readily translatable (Kusakabe *et al.,* 2002).

In the present study, the reduced cortisol response to a handling disturbance in the venlafaxine exposure group did not translate into effects at the metabolic level. To this end, we examined plasma glucose and lactate levels post-stressor exposure and they did not show any significant treatment effects. However, in the previous study [Chapter 3; Best *et al*., accepted], when fish were held in static water system and food was withheld during the experiment, venlafaxine exposure caused attenuation of stressor-mediated plasma glucose levels, but not plasma cortisol. Nutritional status has been shown to modulate stress response in salmonids (Barton *et al.,* 1988); moreover, food deprivation in rainbow trout is associated with more severe metabolic effects to acute handling stress (Vijayan and Moon, 1992). In the current study, fish were fed for the duration of the experiment. The discrepancy in our findings from previous and current studies may stem from the fact that nutritional status can influence the rate of metabolic recovery, offsetting the effect of venlafaxine on the stressor-mediated physiological cortisol response. Also, timing, duration and magnitude of these plasma indicators of stress vary with animal model and stressor type and intensity (Iwama *et al.,* 2006; Barton, 2002).

4.6 CONCLUSIONS

Our study provides evidence that venlafaxine is a neuroendocrine disruptor in fish and interferes with the physiological stress response. Although a significant attenuation of stressor-mediated cortisol response was evident in the venlafaxine-exposed fish, no treatment effects were detected in either glucose or lactate concentrations, suggesting a lack of metabolic effects. Examination of individual components of the HPI axis at mRNA level revealed that expression of CRF-R1, a key receptor type involved in stress response, was reduced by venlafaxine in the pituitary, suggesting that venlafaxine exposure may lead to changes in ACTH production and/or secretion. No treatment effects were seen on hypothalamic CRF or pituitary POMC gene expression levels, although venlafaxine exposure significantly increased basal expression of hypothalamic NPY – a neuropeptide involved in interaction with the HPI axis and appetite regulation. At the interrenal tissue level, venlafaxine treatment resulted in higher transcript levels of StAR and P450scc, while this

corresponded with attenuation in stressor-induced cortisol response. Taken together, venlafaxine is an endocrine disruptor in trout and impairs the highly conserved cortisol response that is essential to allow animal to cope with stress. We propose that venlafaxine may exert its effect centrally and peripherally, at the level of the interrenal tissue, but the mechanisms remains to be elucidated.

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Chapter 5

Venlafaxine disrupts the hypothalamus, pituitary and interrenal functioning *in vitro* in rainbow trout

5.1 OVERVIEW

There is an increasing body of evidence suggesting that venlafaxine acts as a neuroendocrine disruptor affecting the cortisol stress axis in rainbow trout. Venlafaxine is a pharmaceutical compound that is widely distributed in the aquatic environment due the high rates of human consumption for treatment of depressive disorders. However, the mode of action of this selective serotonin and norepinephrine reuptake inhibitor in non-target organisms like fish is less understood. We tested the hypothesis that venlafaxine directly affects the functioning of the hypothalamus, pituitary and interrenal tissues in rainbow trout. We utilized an *in vitro* approach using hypothalamus, pituitary and head kidney tissues from rainbow trout (*Oncorhynchus mykiss*) to tease out the mode of action of venlafaxine in disrupting neuroendocrine/endocrine function. Venlafaxine exposure *in vitro* disrupted hypothalamus and pituitary function in trout. These tissues were exposed to 0, 0.3 and 3 nM venlafaxine either in the presence or absence of a specific 5-HT_{1A} receptor antagonist, NAN-190, to determine whether serotonin signalling is involved in venlafaxine-mediated endocrine/neuroendocrine disruption. In the hypothalamus, venlafaxine reduced expression of phosphorylated form of cAMP response element binding protein (CREB). Also, venlafaxine treatment disrupted the transcript abundance of genes involved in hypothalamus-pituitary-interrenal axis functioning and appetite regulation, including CRF, NPY, glucocorticoid receptors (GR1 and GR2), pro-opiomelanocortins (POMCA and POMCB), neuropeptide Y (NPY), and glucose transporter type 2 (Glut2). The venlafaxine effect on CRF and NPY transcript abundance may involve disruption in serotonin signalling. In the pituitary, higher venlafaxine concentration resulted in a significant increase in basal ACTH production, but reduced the CRF-stimulated ACTH production. At the head kidney level, neither unstimulated nor ACTH-induced cortisol production were altered in response to tissue exposure to a range of venlafaxine concentrations (0.1 – 1000 nM), although our result from *ex vivo* study demonstrated that venlafaxine significantly increased interrenal cortisol response to ACTH stimulation in the presence of serotonin. Collectively, our findings demonstrate that venlafaxine is a neuroendocrine disruptor in fish, primarily affecting key genes in the hypothalamus and pituitary involved in stress axis and appetite regulation. We

propose that the mode of action of venlafaxine, at least in some aspect of HPI axis function and appetite regulation, may involve disruption in serotonin signalling.

5.2 INTRODUCTION

Vertebrates have evolved complex adaptive physiological mechanisms that enable them to maintain their normal homeostatic state in a challenging environment. In teleosts, a key aspect of stress response involves activity of the neuroendocrine stress axis − the hypothalamus-pituitary-interrenal (HPI) axis, with cortisol as its primary end product in teleosts (Vijayan *et al.,* 2010; Wendelaar Bonga, 1997). Over the last decade, evidence has accumulated demonstrating the presence of endocrine disrupting chemicals in the aquatic environments that compromise the functioning the HPI axis in fish (Norris, 2000; Hontela and Vijayan, 2009). Any alteration or disruption of the HPI axis activity due to contaminant exposure may be maladaptive and can undermine the stress performance, leading to reduced fitness in fish (Ings *et al*., 2011).

Upon perception of stress stimuli, corticotropin releasing factor (CRF) is released from the hypothalamus (Mommsen *et al.,* 1999). Several intracellular signalling cascades are known to participate in the regulation of CRF gene transcription, and the most significant one involves the activation of cAMP-dependant protein kinase A (PKA), resulting in the phosphorylation of cAMP response element binding protein (CREB) at a conserved serine residue and this occurs within minutes following stressor exposure (Denver, 2009). CRF stimulates corticotroph cells in the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), which is produced from a precursor peptide pro-opiomelanocortin (POMC) (Vijayan *et al.,* 2010; Wendelaar Bonga, 1997), encoded by two gene isoforms in trout, POMCA and POMCB (Leder and Silverstein, 2006). ACTH is released into the circulation and activates melanocortin 2 receptor (MC2R) leading to cortisol biosynthesis and secretion from the steroidogenic cells dispersed in the head kidney region of teleosts (Vijayan *et al.,* 2010). In the target tissues, cortisol mediates its effects via glucocorticoid (GR) and mineralocorticoid (MR) receptors, which are also involved in the negative feedback regulation of the HPI axis at the level of hypothalamus and pituitary (Aluru and Vijayan,

2009). Similarly to most teleost species, rainbow trout have one MR and two GR genes (GR1 and GR2) (Alsop and Vijayan, 2008).

In mammals, it is well established that the serotonergic system is involved in the HPA (hypothalamus-pituitary-adrenal) axis regulation leading to cortisol production (Chaouloff, 1993). In fish, as in mammals, studies suggests that serotonin (5-hydroxytryptamine; 5-HT) plays a key role in HPI axis activation and downstream cortisol production, and evidence suggests that 5-HT_{1A} receptor is the primary mediator of serotonergic effects on the HPI axis (Medeiros *et al.*, 2014; Höglund *et al.*, 2002; Winberg *et al.*, 1997). In addition to its neurotransmitter role, 5-HT can induce corticosteroid secretion from the adrenocortical cells (analogous to fish interrenal cells) via paracrine action in many vertebrate species (Contesse *et al.*, 2000) and the presence of 5-HT in the head kidney has been demonstrated in fish species (Reid *et al.*, 1998).

Venlafaxine is a drug designed to modulate levels of the brain monoamines 5-HT and norepinephrine (NE) by blocking their respective reuptake transporters, thus increasing synaptic availability of these neurotransmitters, with a stronger effect on 5-HT reuptake (Roseboom and Kalin, 2000), and it is a commonly prescribed antidepressant. In recent years, it has become a predominant antidepressant detected in urban wastewater effluent and aquatic systems (Rúa-Gomez and Püttmann, 2012; Metcalfe *et al.*, 2010; Schultz *et al.*, 2010). Although detection of pharmaceuticals, including antidepressants, in the aquatic environment has received a lot of attention, their effects on non-target animals, including fish, are not very clear. We have previously demonstrated that rainbow trout (*Oncorhynchus mykiss*) exhibit an attenuated plasma cortisol response to an acute handling stressor following exposure to environmentally realistic levels of venlafaxine [Chapter 4]. However, the mechanism involved in this attenuation of stressor-induced cortisol response by this drug remains unclear. We hypothesized that the impact of venlafaxine on the HPI axis activity involves disruption of neuroendocrine/endocrine responses at multiple levels, including the functioning of the hypothalamus, pituitary and internal tissue in rainbow trout. This was tested using an *in vitro* approach with head kidney, pituitary and hypothalamic tissues exposed to venlafaxine. Specifically our objective was to determine the mode of action of

this drug in disrupting hypothalamus, pituitary and interrenal function in trout. This was carried out by assessing the phosphorylation of CREB and the transcript levels of genes involved in HPI axis functioning and appetite regulation, including CRF and its binding protein (CRF-BP), pro-opiomelanocortin (POMCA and POMCB), glucocorticoid receptors (GR1, GR2), mineralocorticoid receptor (MR), glucose transporter type 2 (Glut2), in the hypothalamus in response to venlafaxine exposure. Also, the drug effect on pituitary function was ascertained by measuring either the unstimulated or CRF-stimulated ACTH content in the tissue and medium. We used a specific 5-HT_{1A} antagonist NAN-190 in our studies with hypothalamic and pituitary tissues in order to determine whether the effects of venlafaxine exposure are mediated by this receptor. Similarly, the effect of venlafaxine on interrenal function was determined by measuring either the unstimulated or ACTH-stimulated cortisol production in trout head kidney pieces *in vitro*. In order to test whether this drug can alter responsiveness of the interrenal tissue to stimulation in the presence of 5-HT, we used isolated head kidney tissues from trout exposed to venlafaxine *in vivo* and examined ACTH-stimulated cortisol production in the presence of 5-HT.

5.3 MATERIAL AND METHODS

5.3.1 Animals

Sexually immature rainbow trout (average body weight ~200 g) were used for experiments in this study. Fish were purchased from Alma Aquaculture Research Station (Alma, ON, CAN) and maintained at the University of Waterloo Aquatic Facility. Fish were acclimated for a month in 2000-L tanks with continuous running well water at 13 °C and 12-h light: 12-h dark photoperiod before experimentation. The fish were fed to satiety once daily with a commercial trout chow (Martin Feed Mills, Elora, ON, CAN). Experimental protocols were approved by the Animal Care Committee at the University of Waterloo and conducted in accordance with the Canadian Council for Animal Care guidelines. During sampling, fish were netted quickly and euthanized with an overdose of 2-phenoxyethanol (1:1000 dilution; Sigma-Aldrich, St. Louis, MO). Fish that were sampled for the head kidney tissue (containing interrenal cells) were bled and then the tissue was immediately dissected. Fish

used for collection of pituitaries and hypothalami were rapidly decapitated, brains and pituitaries were immediately removed.

5.3.2 Treatments

Venlafaxine hydrochloride (VEN) and serotonin hydrochloride (5-HT) were initially dissolved in water, and NAN-190 hydrobromide (5-HT_{1A} receptor antagonist) was dissolved in 1:10 solution of DMSO and then further diluted in water (vehicle). NAN-190 control group represents vehicle control. The above listed chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Modified Hank's medium (136.9 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_20$, 0.33 mM $Na_2HPO_4 \cdot 7H_2O$, 0.44 mM KH_2PO_4, 5.0 mM HEPES, 5 mM HEPES·Na, 5mM $NaHCO_3$, 5mM glucose, pH 7.63) was used in our all tissue incubations. Peptide hormones used in this study were porcine $ACTH^{1–39}$ (Sigma-Aldrich) and tilapia (*Oreochromis mossambicus*) CRF, which was a kind gift form Dr. D.A. Lovejoy (University of Toronto). All stock solutions of experimental treatments were diluted in modified Hank's medium to achieve the appropriate working concentrations. Concentration of 5-HT was 100 nM (10^{-7} M), in mammalian studies 5-HT in range 10^{-12} - 10^{-7} M have stimulatory role on CRF release (Nakagami *et al.,* 1986), and a preliminary experiment with rainbow trout hypothalami incubations demonstrated that this concentration upregulated CRF mRNA after 2 h incubation (Fig. C1-B, Appendix C). Concentration of CRF (10 nM) for stimulation of ACTH secretion was selected based on a previous study, where human/rat CRF was effective in 1-1000 nM dose range on ACTH secretion by trout pituitary (Baker *et al.,* 1996). ACTH concentration (0.5 IU/mL) was based on previous work in our laboratory (Sandhu and Vijayan, 2011; Aluru and Vijayan, 2008). Venlafaxine concentrations for experiments with the pituitary and hypothalamic tissues were 0, 0.3 and 3 nM. We selected 3.0 nM venlafaxine concentration since it approximates 1.0 μg/L concentration used in our previous studies, and 0.3 nM – an order of magnitude lower. Tissue viability was determined by measuring the release of lactate dehydrogenase (LDH) into the media according to established protocols (Boone *et al.,* 2002).

5.3.3 *In vitro* experiment with hypothalami

The hypothalamic region from each brain was separated by making transverse cuts immediately anterior to the optic chiasm, posterior to saccus vasculosus, followed by longitudinal cuts along the boundary of the optic tectum (Fig.C1-A; Appendix C), in order to include nucleus preopticus and nucleus lateral tuberalis − the major nuclei of CRF-ergic neurons that are thought to be involved in the regulation of pituitary ACTH production and secretion (Yulis and Lederis, 1987). This fragment was minced in a Petri dish with ice-cold modified Hank's medium and transferred into a sterile 24-well culture plate (Sarstedt, Newton, NC, USA) containing 500 µl of modified Hanks buffer (hypothalamic region from 1 fish per well). Our experimental set-up included three venlafaxine exposure groups (0, 0.3 and 3 nM), and each either in the absence or presence of 5-HT_{1A} antagonist NAN-190 (1 µM). In addition, to assess whether the changes seen with VEN are mediated by 5-HT, we also had control groups (not exposed to venlafaxine) exposed to either 5-HT (100 nM) alone or in combination with NAN-190. Six hypothalami were used (n = 6, independent samples) per treatment group. Initially, the tissues were equilibrated for 1 h with gentle rocking at 13^{o}C. Next, the tissues were incubated in fresh buffer containing venlafaxine treatments for 1 h. Antagonist treatments (control or NAN-190) were added to the appropriate wells and tissues were allowed to incubate for 30 min. Then 5-HT treatments were added to the appropriate wells and the tissues were allowed to incubate for 2 h in order to assess treatment effects on hypothalamic transcript levels. At the end of the incubation period, samples were quickly collected, centrifuged at 9,000 x g for 20 s and then the supernatant an the tissues were separated, snap-frozen on dry ice and stored at -80^{o}C for further analysis. This experiment was repeated exactly as described above with identical treatments groups, where the final incubation point (after 5-HT addition) was 10 min instead of 2 h, for assessment of rapid phosphorylated CREB expression.

5.3.4 *In vitro* experiment with pituitaries

After tissue collection, each pituitary was rinsed with ice-cold modified Hanks, sliced into 4 fragments and transferred into a well of a sterile 96-well plate with 75 µl of modified

Hank's buffer supplemented with 0.5% bovine serum albumin (BSA). Our experimental set-up included three venlafaxine exposure groups (0, 0.3 and 3 nM), and each either in the absence or presence of 5-HT_{1A} antagonist NAN-190 (1 μM). One set of tissues was treated with CRF (10 nM), and one set did not receive CRF stimulation (no CRF). In addition, to assess whether the changes seen with venlafaxine mimic 5-HT-mediated effects, we also had control groups (no venlafaxine treatment) exposed to 5-HT (100 nM), with either absence or presence of CRF (10 nM CRF or no CRF). Initially, the tissues were equilibrated for 1 h with gentle rocking at 13^{o}C. After equilibration, the tissues were incubated in fresh buffer containing venlafaxine treatments (0, 0.3, 3 nM) for 1 h. This was followed by addition of antagonist (control or 1 μM NAN-190) and allowing the tissues to incubate 30 min. Then, CRF treatments were added (10 nM CRF or no CRF). After a 4 h incubation period, media and pituitaries were collected separately, snap-frozen on dry ice, weight of the tissues recorded, and stored at -30^{o}C for later ACTH analysis.

5.3.5 *In vitro* experiment with head kidney tissue

Effect of *in vitro* venlafaxine exposure on cortisol production was determined using a well-established head kidney tissue preparation method (Sandhu and Vijayan, 2011; Aluru and Vijayan, 2006). Head kidney tissue was finely minced (~1 mm^3 pieces) in a Petri dish with ice-cold modified Hank's medium and cleaned of blood clots. After rinsing, tissue was distributed equally (500 μL of buffer with ~50 mg of tissue per well) into 24-well culture plates (Sarstedt, Newton, NC, USA). The tissues were left to equilibrate for 2 h at 13°C with gentle rocking. After 2 h, the buffer was replaced with fresh medium containing a range of venlafaxine concentrations (0, 0.1, 1, 10, 100, 1000 nM). After 1 h incubation period, media was collected for determination of basal cortisol levels, and replaced with fresh aliquot with appropriate venlafaxine concentration containing either no ACTH (control) or 0.5 IU/mL ACTH. The experiment was repeated with tissues from six independent fish (n = 6). At the end of the incubation period, samples were collected, quickly centrifuged (13,000 x g for 1 min), and the supernatant was collected and stored at -30^{o}C for later cortisol determination.

5.3.6 *Ex vivo* head kidney study with serotonin

Head kidney tissues were isolated from rainbow trout exposed to venlafaxine *in vivo* for a 7-day period (control or 1.0 μg/L venlafaxine) exactly as previously described [Chapter 4]. Head kidney tissue preparation was done exactly as described above. Tissue were pre-incubated for 2 h at 13 °C, then medium was replaced with fresh medium with control, low (10^{-8} M) and high (10^{-7} M) 5-HT treatments in combination with ACTH stimulation (0.5 IU/mL) or in the absence of stimulation (no ACTH). At the end of the 4 h incubation period, media and tissues were collected as described above.

5.3.7 Cortisol analysis

Media cortisol levels were measured from the head kidney experiments by radioimmunoassay (RIA) using ^{3}H cortisol as previously described [Chapter 4]. Cortisol antibody (rabbit antiserum) and scintillation cocktail (Universol™) were obtained from MP Biomedicals (Irvine, CA, USA) and ^{3}H cortisol was purchased from GE Healthcare (Waukesha, WI, USA). All cortisol samples were measured in one assay with intra-assay variability of 3.6%.

5.3.8 ACTH analysis

ACTH concentrations in the incubation media and the tissue samples were measured using a double antibody (hACTH) ^{125}I RIA kit (MP Biomedicals) as described previously [Chapter 4]. Tissue homogenates were prepared by sonication of pituitary fragments in 50 μL of ice-cold 50 mM Tris buffer (pH 7.5) with protease inhibitor cocktail (Roche, Mannheim, Germany). The validity of this RIA kit, previously established for trout plasma (Craig *et al.*, 2005; Doyon *et al.*, 2006), was confirmed by observation of parallel dilution curves for media and tissue homogenate samples. Media and homogenate samples were analyzed in two RIA runs; the intra-assay variation was 4.7% and 3.8%, respectively.

5.3.9 Quantification of gene expression

Total RNA was isolated from the hypothalamic tissue using Ribozol RNA extraction reagent (Amresco, Solon, OH, USA) according to manufacturer's instructions. Briefly, 500

µl of Ribozol was added to 30-50 mg of frozen tissue. Samples were homogenized on ice, chloroform was added (100 µl) and the aqueous phase (containing RNA) was collected following centrifugation at 12,000 x g for 15 min. RNA was recovered by isopropanol precipitation and reconstituted in nuclease-free water. RNA was then DNase-treated as per manufacturer's instructions (Fermentas, Pittsburgh, PA, USA) and one microgram of total RNA was reverse-transcribed with high capacity cDNA reverse transcription kit (Applied Biosystems, Streetsville, ON, CAN). Hypothalamic cDNA samples were used for transcriptional analysis by quantitative real-time PCR exactly as described earlier [Chapter 2, 3, 4]. Expression levels of the following genes were investigated: corticotropin releasing factor (CRF) and its binding protein (CRF-BP), glucocorticoid receptors 1 and 2 (GR1 and GR2), mineralocorticoid receptor (MR), pre-opiomelanocortin A and B (POMC A and B), neuropeptide Y (NPY) and glucose transporter type 2 (Glut2). All gene expression data is shown as percent change from control of venlafaxine control (0) group for presentation purposes. Specific information for the primers used in this study was previously listed in Table 5-1.

5.3.10 Immunoblotting

Hypothalamic tissues were homogenized in ice-cold lysis buffer (50 mM Tris-Cl, 5 mM EDTA, 150 mM NaCl, 5 mM NaF, 5 mM NEM, 2 mM Na_3VO_3, 1% SDS, 1% Nonidet-P40, 1x protease inhibitor cocktail (Roche). Protein concentration was measured using the bicinchoninic acid (BCA) method using BSA as the standard. Samples were diluted in Laemmmli's sample buffer (1 M Tris-HCl, pH 6.8, 60 mM, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, 0.1% bromophenol blue) and 40 µg of total protein was separated on a 10% SDS-PAGE at 180 V. Proteins were transferred onto 0.45 µM nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) in a semi-dry transfer unit (Bio-Rad) with transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol) at 20 V for 25 min with using. Following 1 h blocking with 5% solution of non-fat dry milk in 1xTTBS (2 mM Tris, 30 mM NaCl, 0.01% Tween, pH 7.5) at room temperature, membranes was incubated overnight at 4^oC with either monoclonal rabbit phospho-CREB (ser133) or total CREB (Cell Signalling technology, Beverly, MA) antibodies diluted 1:1000 in 5% BSA in TTBS.

Following washing, blots were probed with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad, diluted 1:3000 in 5% skim milk in TTBS). Protein bands were detected with Luminata Crescendo Western HRP Substrate (Millipore, Billerica, MA, USA) and imaged using Pharos FX molecular Imager (Bio-Rad). Total band intensity was quantified using AlphaEase Software (Alpha Innotech, Santa Clara, CA, USA) and densitometric values were normalized to a reference sample ran on each blot for inter-blot comparisons. All membranes were also probed with Cy3-conjugated monoclonal mouse β-actin antibody (1:1000; Sigma-Aldrich) for 1 h at room and protein loading was normalized based on β-actin expression.

5.3.11 Statistics

All analyses were conducted using *Sigmaplot 11* (Systat Software Inc., San Jose, CA, USA). For the hypothalamus experiment, one-way ANOVA was used for comparison between treatment groups in the control tissues (not exposed to venlafaxine), and two-way ANOVA was used for venlafaxine exposed tissues (venlafaxine and NAN-190 treatments). For the pituitary experiment, two-way ANOVA was used for non-stimulated (no CRF) and CRF-stimulated groups (venlafaxine and NAN-190 treatments), for control and NAN-190 groups (CRF and venlafaxine treatments). For the *in vitro* head kidney experiment, paired *t*-test was used to assess effect of ACTH stimulation in the control tissues, and one-way repeated measures analysis of variance (ANOVA) was used to assess effect of venlafaxine treatments on basal and ACTH-stimulated cortisol production. For *ex vivo* head kidney study, two-way repeated measures ANOVA was used to analyse cortisol response (5-HT and venlafaxine variables). Significant differences between treatment groups were compared using Holm-Sidak *post hoc* test. Data were log transformed when necessary to meet the assumptions of normality and homogeneity of variance, although non-transformed data are shown in figures (mean ± SEM). A significance level of $\alpha = 0.05$ was used in all cases.

Table 5-1. Sequences (forward and reverse), amplicon size, accession number and annealing temperature for primers used in quantitative real-time RT-PCR.

Gene	Primer sequence	Accession No.	T_{anneal} (°C)	Amplicon (bp)
EF1α	F: 5'-CATTGACAAGAGAACCATTGA-3' R: 5'-CCTTCAGCTTGTCCAGCAC-3'	AF498320.1	56	95
CRF	F: 5'-ACAACGACTCAACTGAAGATCTCG-3' R: 5'-AGGAAATTGAGCTTCATGTCAGG-3'	NM_001124286.1	60	54
CRF-BP	F: 5'-GGAGGAGACTTCATCAAGGTGTT-3' R: 5'-CTTCTCTCCCTTCATCACCCAG-3'	NM_001124631.1	60	51
GLUT2	F: 5'-GGCCATCTTCCTGTTTGTGT-3' R: 5'-TGAAGTTGCTGGTCCAGTTG-3'	AF321816	60	140
NPY	F: 5'-CTCGTCTGGACCTTTATATGC-3' R: 5'-GTTCATCATATCTGGACTGTG-3'	NM_001124266	58	247
GR1	F: 5'-TTCCAAGTCCACCACATCAA-3' R: 5'-GGAGAGCTCCATCTGAGTCG-3'	Z54210	60	115
GR2	F: 5'-GGGGTGATCAAACAGGAGAA-3' R: 5'-CTCACCCCACAGATGGAGAT-3'	AY495372.1	60	140
MR	F: 5'-ACCAACAACATGAGGGCTTC-5' R: 5'-AGTTCACTAGCAGGGCTGGA-3'	NM_001124740.1 NM_001124483.1	60	131
POMCA	F: 5'-AGGGTTGAGGGAGGAAGAGA-3' R: 5'-TGTCAGAGGACATGGCTTTT-3'	NM_001124718.1	60	116
POMCB	F: 5'-CCAGAACCCTCACTGTGACGG-3' R: 5'- CCTGCTGCCCTCCTCTACTGC-3'	NM_001124719.1	60	199

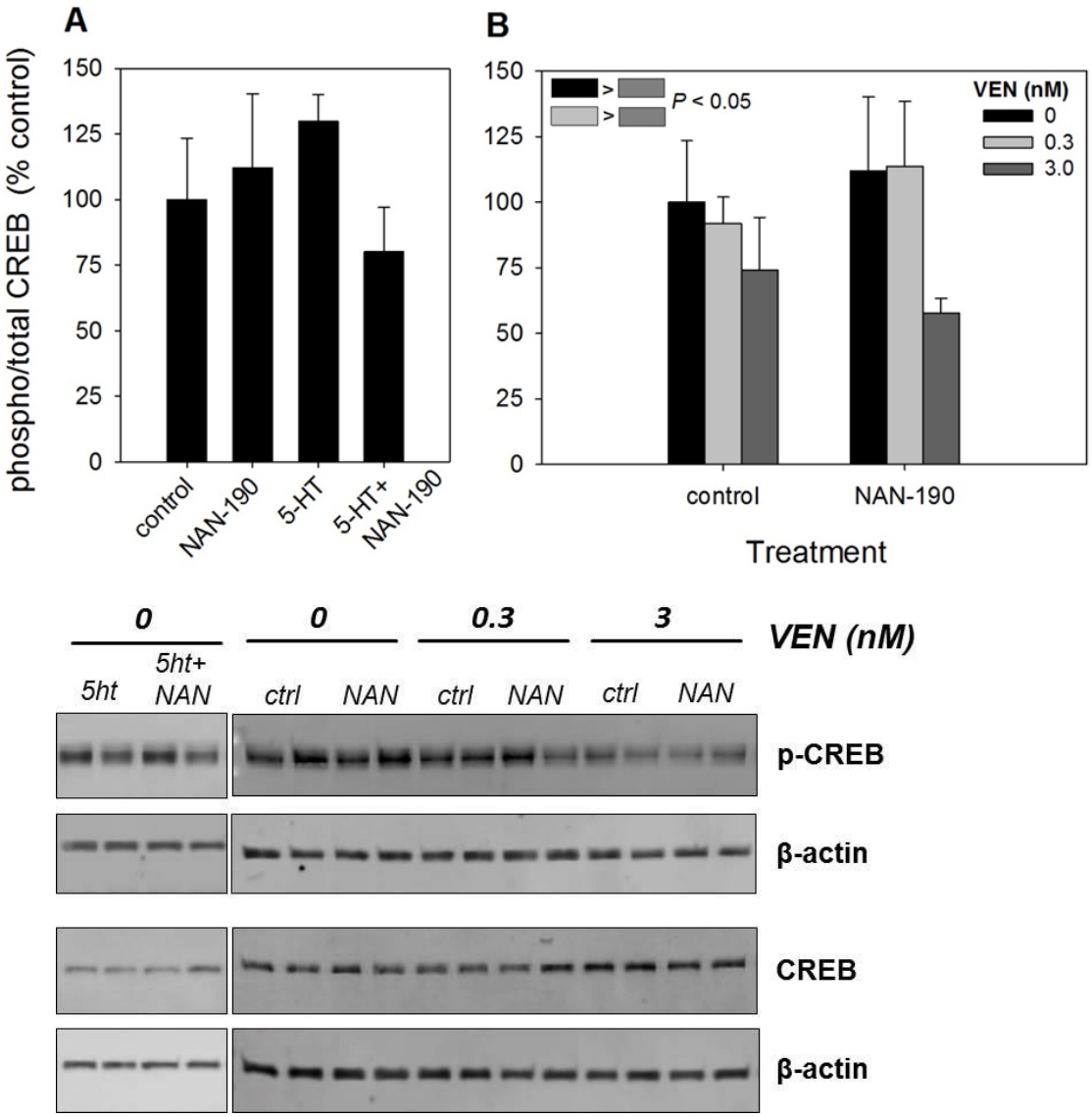
Gene abbreviations: EF1α, elongation factor 1α; CRF, corticotropin-releasing factor; CRF-BP, corticotropin-releasing factor binding protein; Glut2, glucose transporter type 2; NPY, neuropeptide Y; GR1 and GR2, glucocorticoid receptor 1 and 2, respectively; MR, mineralocorticoid receptor; POMCA and POMCB, pro-opiomelanocortin A and B, respectively.

5.4 RESULTS

5.4.1 *In vitro* experiment with hypothalami: phosphorylation of CREB

In our experiment involving *in vitro* incubations of trout hypothalamic tissues, treatments with either 5-HT, NAN-190 or a combination of both did not significantly affect CREB phosphorylation in the present study (Fig. 5-1A). In the tissues exposed to venlafaxine treatments (Fig. 5-1B), 3.0 nM venlafaxine significantly reduced phosphorylation of CREB (expressed as the ratio of phosphorylated to total CREB protein) compared to 0 or 0.3 nM venlafaxine groups ($P < 0.05$, two-way ANOVA), but there was no significant effect in response to NAN-190 exposure.

Figure 5-1. Rapid phosphorylation of cAMP response element binding protein (CREB) in rainbow trout hypothalami (not exposed to venlafaxine; VEN) in response to response to incubation with serotonin, 5-HT (100 nM), 5-HT_{1A} receptor antagonist, NAN-190 (1 μM), or combination of NAN-190 and 5-HT (**A**). Effect of *in vitro* venlafaxine exposure on phospho-CREB response in hypothalami treated with NAN-190 or vehicle control (**B**). Samples (40 μg of protein) were probed with monoclonal rabbit phospho-CREB (133) and total-CREB antibodies (Cell Signalling Technology, Beverly, MA) and protein loading was confirmed by normalizing to β-actin (monoclonal mouse antibody, Sigma, St. Louis, MO). Values plotted represent mean ± SEM of the ratio of phosphorylated to total CREB densitometric values and are shown as percent control (n = 6 independent fish), insets indicate significant venlafaxine effects ($P < 0.05$, two-way ANOVA).


A
B
phospho/total CREB (% control)
150
125
100
75
50
25
0
control
NAN-190
5-HT
5-HT+ NAN-190
P < 0.05
VEN (nM)
0
0.3
3.0
control
NAN-190
Treatment
0
0
0.3
3
VEN (nM)
5ht
5ht+ NAN
ctrl
NAN
ctrl
NAN
ctrl
NAN
p-CREB
β-actin
CREB
β-actin

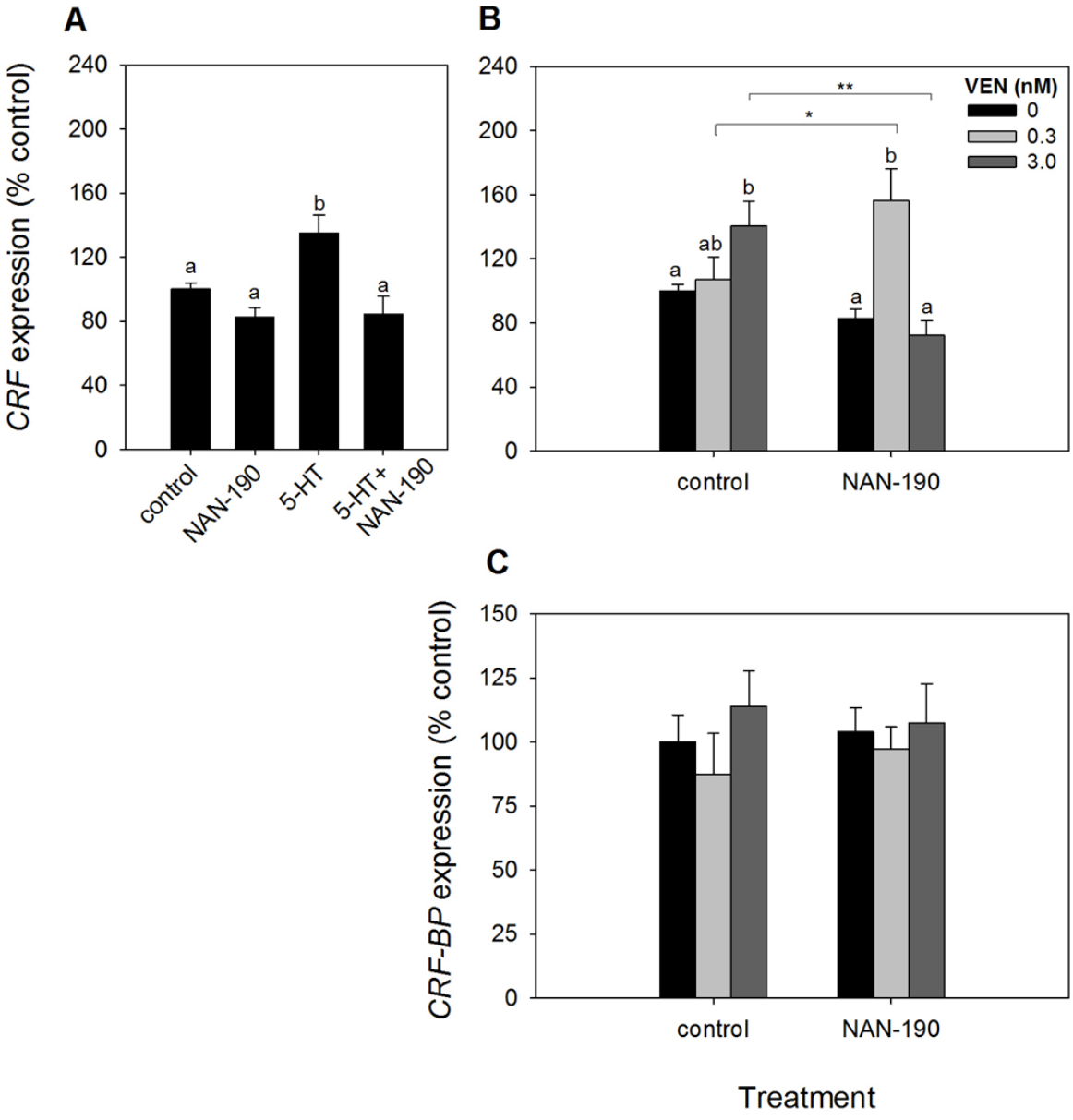
5.4.2 *In vitro* experiment with hypothalami: transcript abundances

Transcript responses in the control tissues (not exposed to venlafaxine) for all the genes examined, with the exception of CRF, were not significantly affected by 5-HT, NAN-190 or their combination (Fig. C2; Appendix C).

CRF and CRF-BP

5-HT significantly increased CRF transcript abundance (1.3-fold increase from the control) in the present study and this effect was completely abolished in the presence of NAN-190 (Fig. 5-2A). In the tissues exposed to venlafaxine, a significant interaction was detected between venlafaxine and antagonist treatments ($P < 0.001$; two-way ANOVA). Venlafaxine at 3.0 nM concentration significantly increased CRF transcript abundance compared to venlafaxine control (0) group (1.4-fold) but not the 0.3 nM venlafaxine concentration (Fig. 5-2B). This effect of 3.0 nM venlafaxine on CRF mRNA level was completely abolished by NAN-190. In the 0.3 nM VEN group, NAN-190 exposure significantly increased CRF mRNA levels compared to the control (1.5-fold) (Fig. 5-2B). Analysis of CRF-BP mRNA levels (Fig. 5-3C) did not reveal any significant effects of any *in vitro* venlafaxine or antagonist treatments.

Figure 5-2. Transcript levels of corticotropin releasing factor, CRF, in rainbow trout hypothalami in response to incubation with serotonin, 5-HT (100 nM), 5-HT_{1A} receptor antagonist, NAN-190 (1 μM), or combination of NAN-190 and 5-HT (**A**). Effect of *in vitro* venlafaxine (VEN) exposure on CRF (**B**) and its binding protein, CRF-BP (**C**), transcript levels in hypothalami treated with NAN-190 or vehicle control. In (**A**), different letters indicate significant differences ($P < 0.05$, one-way ANOVA) between treatments. In (**B**), significant interaction was detected ($P < 0.05$; two-way ANOVA) between venlafaxine and antagonist treatments. Letters indicate significant differences between venlafaxine groups within control or NAN-190 treatments, and asterisks demonstrate differences between control and NAN-190 treatments for individual venlafaxine groups. All values were normalized to EF1α and represent percent control. Bars show means ± SEM ($n = 6$ independent fish).


A
CRF expression (% control)
240
200
160
120
80
40
0
a
a
b
a
control
NAN-190
5-HT
5-HT+ NAN-190
B
VEN (nM)
0
0.3
3.0
**
*
a
ab
b
a
b
a
control
NAN-190
C
CRF-BP expression (% control)
150
125
100
75
50
25
0
control
NAN-190
Treatment

GR1, GR2 and MR

Examination of transcript abundances of the corticosteroid receptor genes revealed similar overall effects of venlafaxine exposure on GR1 and GR2 mRNA levels (Fig. 5-3A and 5-3B, respectively), where 0.3 nM and 3.0 nM venlafaxine groups had significantly attenuated transcript levels (approximately 1.5-fold change) in comparison to venlafaxine control (0) group ($P < 0.001$ in both cases, two-way ANOVA). There were no differences between the control and NAN-190 treatments. MR mRNA levels were not affected by venlafaxine exposure or antagonist treatments (Fig. 5-3C).

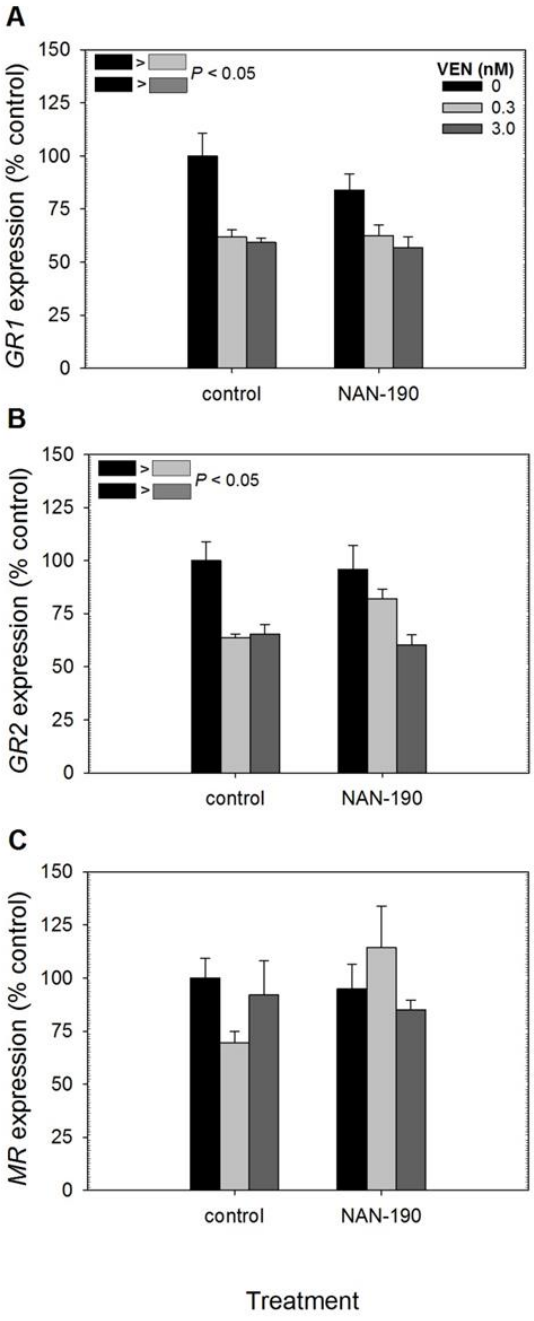


Figure 5-3. Effect on *in vitro* venlafaxine (VEN) exposure on mRNA levels of glucocorticoid receptor 1 and 2, GR1 and GR2 (**A** and **B**, respectively) and mineralocorticoid receptor, MR (**C**) in rainbow trout hypothalami in response to incubation with either control or 5-HT_{1A} receptor antagonist (NAN-190; 1 μM) treatments. Insets indicate significant venlafaxine effects ($P < 0.05$; two-way ANOVA). All values were normalized to EF1α and represent percent control. Bars show means ± SEM ($n = 6$ independent fish).

POMCA and POMCB

Expression analyses of the pro-opiomelanocortin encoding genes determined main venlafaxine exposure effects ($P < 0.05$ in both cases, two-way ANOVA). POMCA mRNA levels (Fig. 5-4A) were approximately 3- and 2.3-fold reduced in 0.3 and 3.0 nM venlafaxine groups, respectively, compared to venlafaxine control (0). POMCB expression (Fig. 5-4B) was strongly diminished in 0.3 and 3.0 nM venlafaxine groups by approximately 9.6- and 7.2-fold, respectively, relative to venlafaxine control (0) group. There was no overall effect of NAN-190 treatment on either POMCA or POMCB mRNA levels.

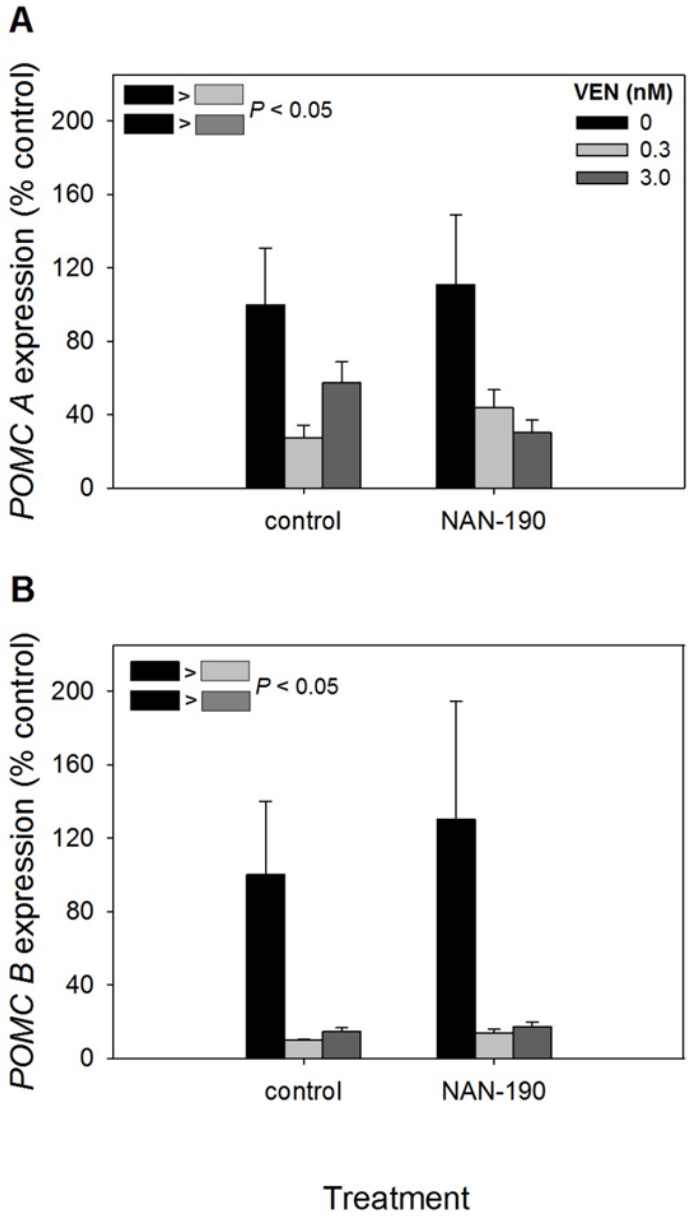


Figure 5-4. Effect on *in vitro* venlafaxine (VEN) exposure on mRNA levels of pro-opiomelanocortin genes A and B, POMCA and POMCB (**A** and **B**, respectively) in rainbow trout hypothalami in response to incubation with either control or 5-HT_{1A} receptor antagonist (NAN-190; 1 μM) treatments. Insets indicate significant venlafaxine effects ($P < 0.05$; two-way ANOVA). All values were normalized to EF1α and represent percent control. Bars show means ± SEM ($n = 6$ independent fish).

Glut2 and NPY

Examination of Glut2 transcript levels detected a significant main effects of *in vitro* venlafaxine exposure (Fig. 5-5A), where 0.3 and 3.0 nM venlafaxine groups had significantly reduced transcript levels (approximately 8.8-fold) in comparison to venlafaxine control (0) group ($P < 0.001$, two-way ANOVA), although there was no differences between the control and NAN-190 treatments. Analysis of NPY mRNA levels (Fig. 5-5B) revealed a significant interaction between venlafaxine and 5-HT_{1A} antagonist treatments ($P < 0.05$; two-way ANOVA). Within the control group, exposure to 3.0 nM venlafaxine reduced NPY transcripts relative to venlafaxine control (0) group by 2.2-fold, although no venlafaxine effects were present within the NAN-190 treatment group. Comparison between the control and NAN-190 treatments within each venlafaxine group did not reveal any significant effects.

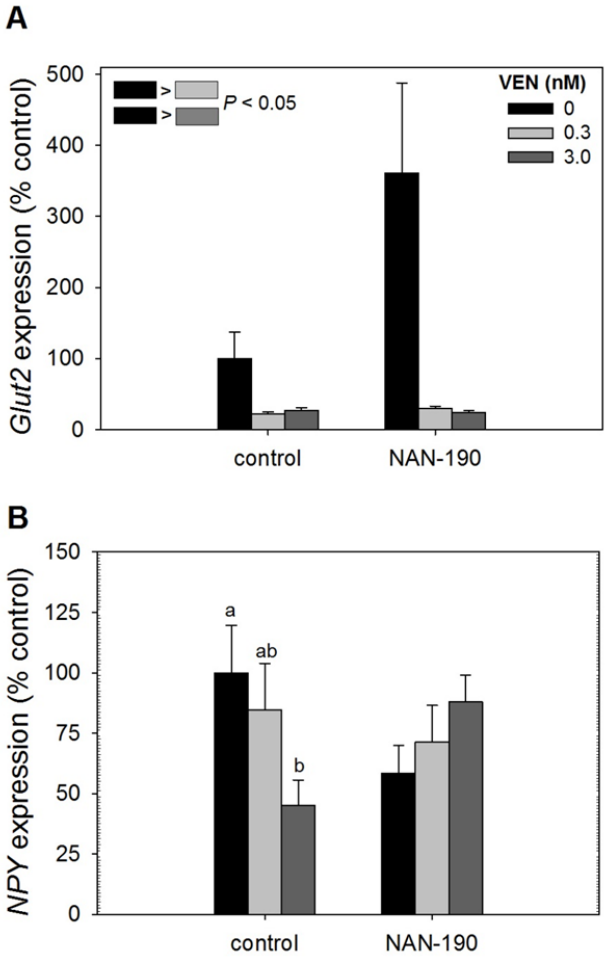


Figure 5-5. Effect on *in vitro* venlafaxine (VEN) exposure on mRNA levels of glucose transporter type 2, Glut2 (**A**) and neuropeptide Y, NPY (**B**) in rainbow trout hypothalami in response to incubation with either vehicle control or 5-HT_{1A} receptor antagonist (NAN-190; 1 μM) treatments. Insets indicate significant venlafaxine effects ($P < 0.05$; two-way ANOVA). Significant interaction was detected for NPY ($P < 0.05$; two-way ANOVA); letters indicate significant differences between venlafaxine groups within control treatment. All values were normalized to EF1α and represent percent control. Bars show means ± SEM ($n = 6$ independent fish).

5.4.3 *In vitro* experiment with pituitaries: tissue ACTH levels

In the control pituitary tissues (not exposed to venlafaxine), CRF stimulation significantly increased (3.8-fold) ACTH levels in trout pituitary pieces (Fig. 5-6A). Neither 5-HT nor NAN-190 had any significant effect on unstimulated ACTH levels in trout control pituitaries. However, both 5-HT and NAN-190 completely abolished the CRF-induced ACTH production (Fig. 5-6A). Analysis of ACTH content in the pituitaries exposed to venlafaxine demonstrated that in the absence of CRF stimulation, 3.0 nM venlafaxine resulted in significantly higher ACTH content compared to 0 or 0.3 nM venlafaxine (5.4-fold change from venlafaxine control (0), and this was abolished by NAN-190 (Fig. 5-6B). Production of ACTH in the tissues stimulated with CRF did not demonstrate significant effects of venlafaxine exposure, however NAN-190 reduced ACTH production in the venlafaxine control (0) group (Fig. 5-6B).

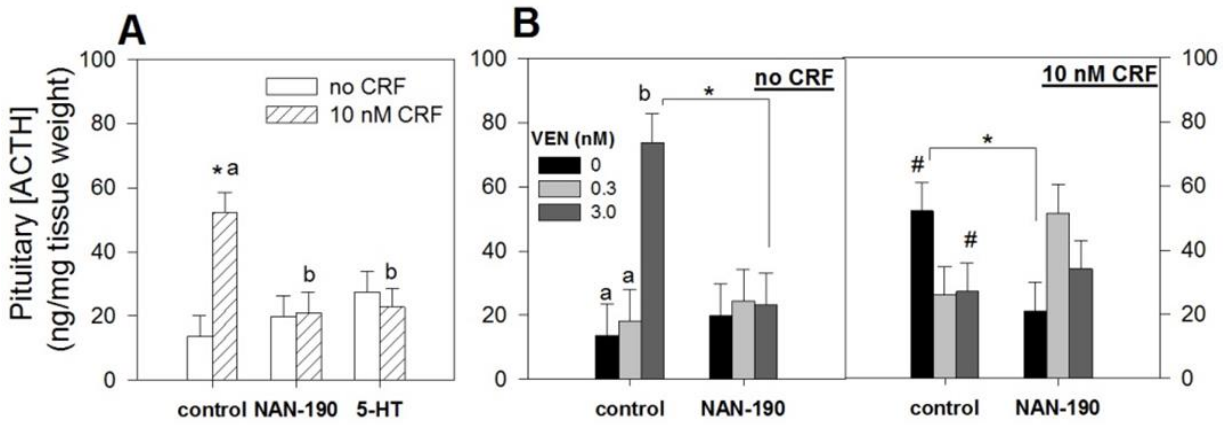


Figure 5-6. Tissue ACTH levels (*in vitro* static incubation experiment with rainbow trout pituitaries). Effect of CRF stimulation on ACTH levels in pituitaries treated with 5-HT_{1A} receptor antagonist (NAN-190; 1 μM) and serotonin (5-HT, 100 nM) (**A**). Effect of *in vitro* venlafaxine (VEN) exposure and NAN-190 treatment on ACTH production without (no CRF) or with 10 nM CRF stimulation (**B**). In (**A**), asterisks show a significant CRF effect within a treatment group and different letters indicate a significant treatment effects for individual CRF group. In (**B**), different letters indicate significant venlafaxine effects, asterisks indicate significant NAN-190 effects for individual venlafaxine group, and hash signs demonstrate a significant change resulted by CRF stimulation for individual venlafaxine treatments ($P < 0.05$). All values were normalized to the tissue weight and bars show means ± SEM (n = 5-6). Tilapia CRF peptide was used for stimulations (4 h), incubations were performed in modified Hank's medium supplemented with 0.5% bovine serum albumin.

5.4.4 *In vitro* experiment with pituitaries: ACTH levels in incubation medium

ACTH analysis in the medium from the non-exposed (not exposed to venlafaxine) pituitary incubations did not detect any effect of either CRF stimulation or NAN-190 or 5-HT treatments (Fig. 5-7A). Media for non-stimulated (no CRF) tissues did not show changes with venlafaxine exposure or NAN-190 treatment (Fig. 5-7B). No venlafaxine effects were detected in in the absence of CRF. Analysis of ACTH content in the medium from the stimulated (10 nM CRF) incubations demonstrated ACTH levels were higher in 0.3 nM venlafaxine relative to 0 and 3.0 nM venlafaxine in the control group (2.3-fold change from venlafaxine control), and this effect was abolished with NAN-190 treatment. NAN-190 treatment had an opposite effect in 3.0 nM venlafaxine group, where ACTH levels were elevated by the antagonist. Stimulation with CRF produced significant effect only for 0.3 nM venlafaxine group, elevating ACTH secreted by 2.3-fold.

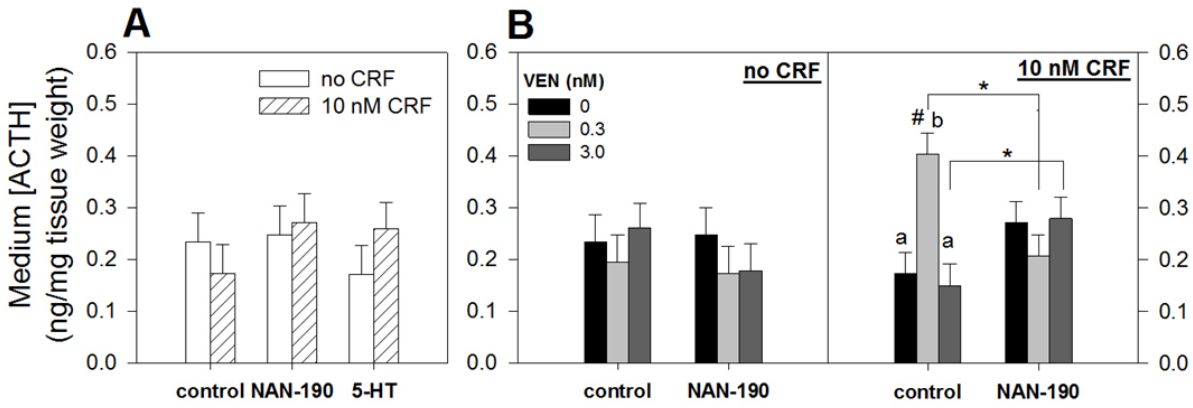


Figure 5-7. Medium ACTH levels (*in vitro* static incubation experiment with rainbow trout pituitaries). Effect of CRF stimulation on ACTH released in medium by pituitaries treated with 5-HT_{1A} receptor antagonist (NAN-190; 1 μM) and serotonin (5-HT, 100 nM) (**A**). Effect of *in vitro* venlafaxine (VEN) exposure and NAN-190 treatment on ACTH secretion without (no CRF) or with 10 nM CRF stimulation (**B**). In (**B**), different letters indicate significant venlafaxine effects, while asterisk indicate significant NAN-190 effects for individual venlafaxine group, and hash sign demonstrates a significant change resulted by CRF stimulation for individual venlafaxine treatment ($P < 0.05$). All values were normalized to the tissue weight and bars show means ± SEM (n = 5-6). Tilapia CRF peptide was used for stimulations (4 h), pituitaries were incubated in modified Hank's medium supplemented with 0.5% bovine serum albumin.

5.4.5 *In vitro* experiment with head kidney: cortisol production

In this experiment we examined whether *in vitro* venlafaxine exposure effects basal or ACTH-stimulated cortisol production. ACTH stimulation resulted in approximately 11-fold increase (paired *t*-test, $P < 0.05$) in cortisol production in the control trout head kidney tissues (Fig. 5-8A). Venlafaxine concentrations (0.1, 10, 100, or 1000 nM) did not significantly affect unstimulated cortisol production compared to the venlafaxine control (0) group (Fig. 5-8B). There was also no significant effect of venlafaxine on ACTH-induced cortisol production in the head kidney tissue (Fig. 5-8C).

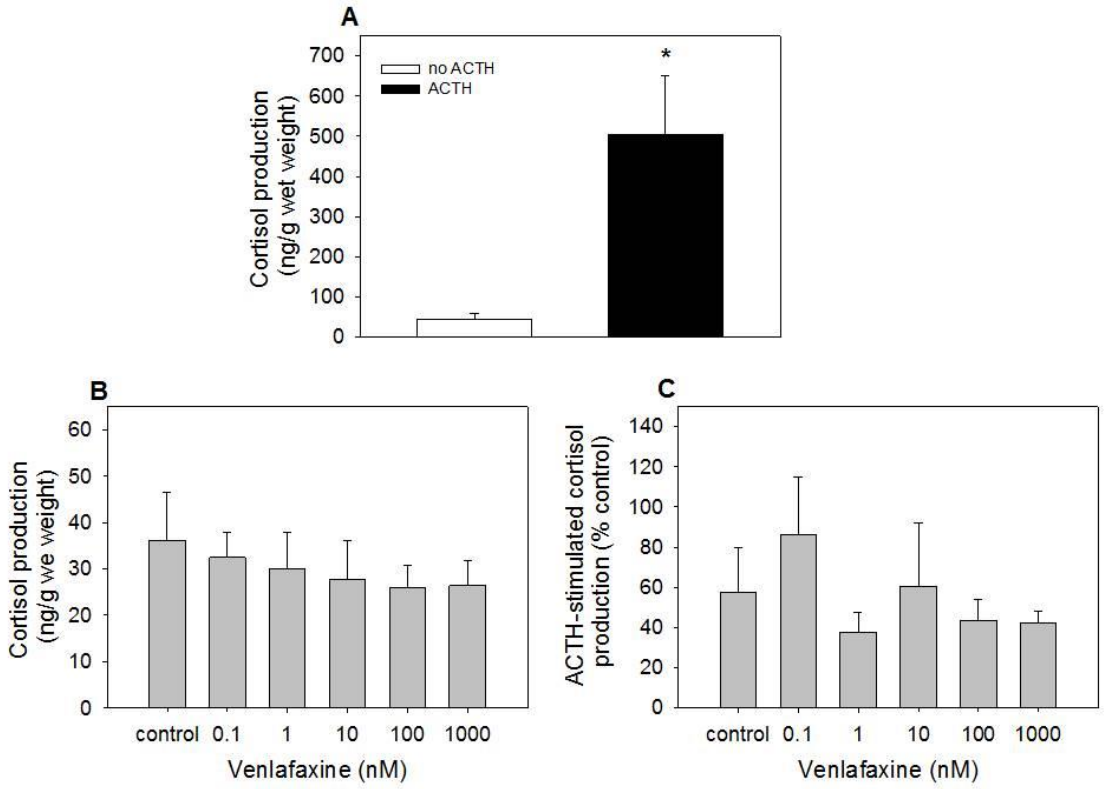


Figure 5-8. *In vitro* cortisol production in rainbow trout head kidney tissues. Basal and ACTH-stimulated (0.5 IU/mL) cortisol production in the control head kidney tissues (**A**). Effect of *in vitro* venlafaxine exposure (0.1, 10, 100, 1000 nM) on basal cortisol production (**B**) and ACTH-induced cortisol production − expressed as percent control of the control group (**C**). Data are shown as mean ± SEM (n = 5 fish); asterisk indicates a significant increase compared to no ACTH group (paired t-test, $P < 0.05$).

5.4.6 *Ex vivo* head kidney study: cortisol production

Cortisol production by the interrenal tissue from venlafaxine exposed fish was examined to determine whether basal or stimulated (ACTH) response was affected by 5-HT. Unstimulated (basal) cortisol production was not affected by 5-HT treatments (Fig. 5-9A). Stimulated cortisol levels (Fig. 5-9B) revealed a significant interaction between 5-HT and venlafaxine variables ($P < 0.005$, two-way repeated measures ANOVA). In presence of the higher 5-HT concentration, cortisol output was approximately 4-fold elevated in the tissues from the venlafaxine exposed fish relative to the control tissues. Presence of 5-HT did not alter cortisol levels in the control tissues.

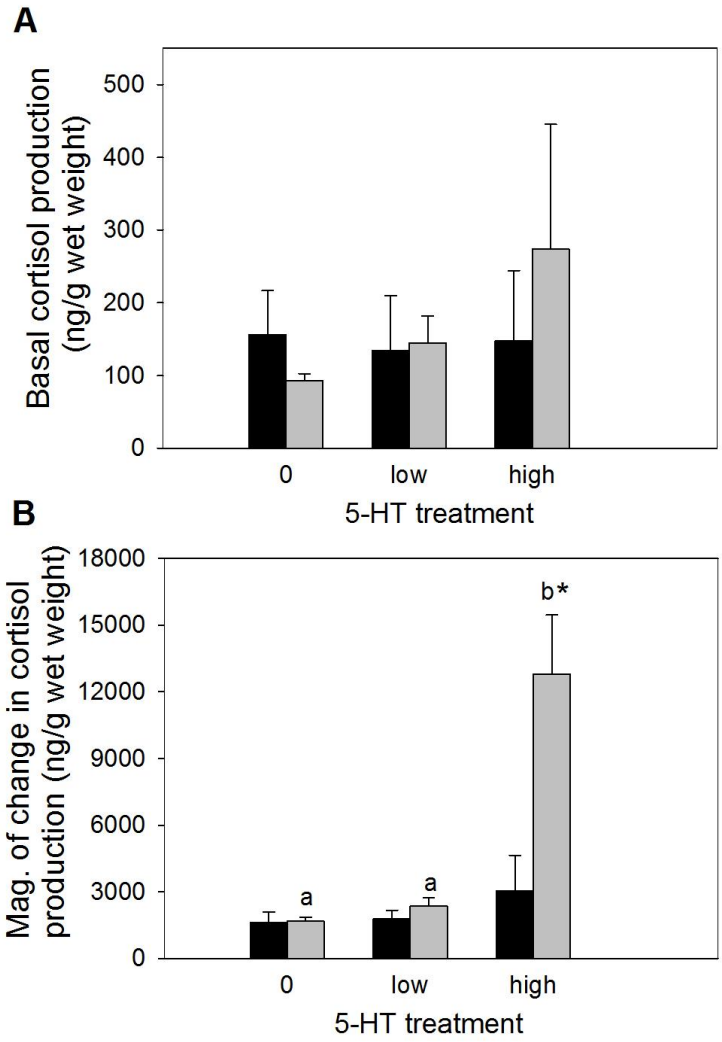


Figure 5-9. Effect of *in vitro* serotonin (5-hydroxytryptamine, 5-HT) treatments on basal (**A**) and ACTH-stimulated (shown as magnitude of change) (**B**) cortisol production in the head kidney tissues exposed to venlafaxine *in vivo* (1.0 μg/L) for 7-day period. For 5-HT treatments, low corresponds to 10^{-8} M and high 10^{-7} M. Values are shown a mean ± SEM, (*n* = 8-6 independent fish from each drug exposure group). An asterisk shows a significant effect of venlafaxine exposure on cortisol production within individual 5-HT group, while different small letters indicate a significant 5-HT effect within individual venlafaxine exposure group ($P < 0.05$, two-way repeated measures ANOVA).

5.5 DISCUSSION

This study demonstrates that venlafaxine can act as a neuroendocrine disruptor at the upstream elements of the HPI axis in rainbow trout. Specifically, this drug disrupts transcript levels of key genes involved in HPI axis function and appetite regulation in the hypothalamus, disrupts activation of CREB transcription factor in the hypothalamus, affects production of ACTH in the pituitary, and may alter interrenal cortisol response. This is in agreement with our previous finding from an *in vivo* study where venlafaxine exposure disrupted plasma cortisol response to an acute stressor [Chapter 4]. Overall, our results demonstrate that venlafaxine exposure can lead to disruption of endocrine pathways involved in cortisol stress axis functioning in fish. Disruption of any elements of the HPI axis may result in the impairment of physiological stress response, which may translate into reduced ability of an organism to cope with subsequent stressors. In fish, 5-HT plays a key role in the HPI axis activation and downstream cortisol production (Winberg *et al.,* 1997). 5-HT_{1A} receptors are primary mediators of 5-HT effects on HPI axis regulation (Medeiros *et al.,* 2014; Höglund *et al.,* 2002; Winberg *et al.,* 1997), especially at the level of the hypothalamus and/or pituitary (Höglund *et al.,* 2002). Our results suggest that the mode of action of venlafaxine in impacting stress axis function and appetite regulation may at least in part involve disruption in 5-HT signalling.

Hypothalamus

Results of our *in vitro* experiment with hypothalamic fragments demonstrated that 5-HT treatment increases expression of CRF mRNA, and pre-treatment with 5-HT_{1A} antagonist abolishes this 5-HT effect, thus supporting the role of 5-HT_{1A} receptors in stress activation at the level of hypothalamus. This is also in agreement with a recent study in Gulf toadfish (*Opsanus beta*), demonstrating that *in vivo* administration of 5-HT_{1A} receptor agonist (8-OH-DPAT) resulted in increased CRF mRNA abundance in the hypothalamic region (Medeiros *et al.,* 2014). In the hypothalamic tissues exposed to 3.0 nM venlafaxine, a similar increase in CRF transcripts was observed in the presence of 5-HT and this was abolished in the presence of 5-HT_{1A} antagonist NAN-190, suggesting that the venlafaxine effect may be mediated by

changes in 5-HT levels. On the other hand, 0.3 nM venlafaxine by itself had no effect on CRF mRNA abundance, but in the presence of the antagonist NAN-190, CRF transcript levels were elevated, suggesting that venlafaxine may also exert an inhibitory effect on CRF synthesis but this remains to be verified. Monoamine analysis in our previous study demonstrated that hypothalamic and preoptic areas (both pooled as one region for the present study) in the trout brain have relatively high 5-HT content [Chapter 2; Melnyk-Lamont *et al*., submitted]. Hypothalamic tissues have endogenous 5-HT, thus venlafaxine may inhibit cellular reuptake of this monoamine after it has been leaked into the incubation media. In the present study with hypothalamic fragments, we did not utilize additional *in vitro* treatment with 5-HT in the tissues exposed to venlafaxine based on the reasoning that the hypothalamic tissues contain sufficient endogenous 5-HT, in order to keep experimental conditions physiologically relevant.

CRF is a 41-amino acid peptide with a remarkably conserved sequence similarity in mammals, fish, and amphibians (Hauger *et al*., 2006; Lovejoy and Balment, 1999). Cyclic AMP response element binding protein (CREB) is known to be one of the mediators of stress-induced increases in CRF gene expression, and the promoter region of CRF gene contains cAMP response element (CRE), which regulates its transcription (Yao and Denver, 2007). Transcription activity of CREB is determined by phosphorylation at the conserved serine 133 residue in response to variety of neuronal stimuli (Denver, 2009). In this study, 3.0 nM venlafaxine treatment significantly decreased phosphorylation of CREB (pCREB) in the hypothalamus *in vitro*. The mechanism responsible for venlafaxine effect on phosphorylation of CREB remains unclear. In addition, significantly reduced pCREB was detected in nuclear lysates of cortex after chronic but not acute venlafaxine administration in rats (Rossby *et al*., 1999), although another study demonstrated that chronic low dose (but not high dose) of venlafaxine increased expression of pCREB in the hippocampus of rats exposed to chronic unpredictable stress (Li *et al*., 2011). Venlafaxine effect on CREB phosphorylation observed in our experiment is intriguing since this transcription factor strongly influences expression of various genes that contains CREs in their regulatory

regions, and it has diverse functions in various tissues. Whether venlafaxine affects CREB phosphorylation levels in other fish tissues remains to be elucidated.

Our gene expression results from *in vitro* study with hypothalamic tissue provided more evidence that venlafaxine may act as a neuroendocrine disruptor. Physiological effects of cortisol on target tissues are mediated by corticosteroid receptors – glucocorticoid and mineralocorticoid types (GR and MR, respectively). Cortisol can mediate negative feedback regulation of the HPI axis activity via corticosteroid receptors at the level of hypothalamus and pituitary. Our results suggest that venlafaxine (both 0.3 nM and 3.0 nM treatments) significantly reduced transcript abundances of GR1 and GR2 (but not MR). In our *in vivo* exposure study with acute stressor challenge [Chapter 4], venlafaxine did not alter GR1, GR2 or MR mRNA expression in hypothalamus or pituitary. Also, another *in vivo* exposure study [Chapter 3, Best *et al*., accepted] did not detect venlafaxine effects on GR protein expression in the whole brain or liver. Taken together, these finding suggest that the effect of *in vitro* venlafaxine treatment on GR gene expression is a short-term/acute exposure effect, although reduction in GR1 and GR2 in hypothalamus could lead to decreased tissue sensitivity to the negative feedback regulation by cortisol. It should be noted that in humans, many antidepressants are known to modulate the HPA axis hyperactivity, which is often associated with depressive symptoms, by modulating GR expression (Anacker *et al.*, 2011). In rats, it has been shown that even a short-term treatment with venlafaxine affects both GR and MR expression in the hippocampus (Yau *et al.*, 2001).

Our gene expression analyses in the hypothalamus also demonstrated that venlafaxine exposure alters the expression of several molecular markers involved in regulation of food intake, adding to our *in vivo* observations that venlafaxine exposure reduced food intake in rainbow trout [Chapter 2, Melnyk-Lamont, *et al*. submitted]. The mRNA levels of two pro-opiomelanocortin encoding genes, POMCA and POMCB were significantly reduced by venlafaxine exposure. POMC undergoes tissue-specific post-translational processing and serves as a precursor molecule for several biologically active peptides, including the opioid, β-endorphin, and the melanocortin-related peptides, ACTH, α-melanocyte stimulating

hormone (α-MSH), β-MSH, and γ-MSH (Tanaka, 2003). In addition to its role in stress response, hypothalamus also plays a central role in energy homeostasis, integrating peripheral signals of appetite, satiation, and long-term energy-balance and coordinating food intake responses (Kalra *et al.,* 1999). Hypothalamus contains neurons that produce numerous appetite-stimulating and inhibiting neuropeptides, including neuropeptide Y (NPY) and POMC-derived β-endorphin with orexigenic properties, and POMC-derived α-MSH is an anorexin (Volkoff *et al.,* 2005; Lin *et al.,* 2000) . Thus, reduction in POMC transcripts may lead to a reduction in the formation of POMC-derived peptides by venlafaxine.

We also detected a reduction of NPY transcripts in the hypothalamic tissues exposed to 3.0 nM venlafaxine. As mentioned earlier, NPY is a known appetite stimulator in both mammals and fish. Also, NPY is involved in complex interactions with hypothalamus-pituitary-adrenal (HPA) based on mammalian studies [reviewed in Krysiak *et al.* (1999)]. Doyon *et al.,* (2006) suggested that NPY may participate in activation of the HPI axis, possibly through interaction with CRF in trout. It should be noted that venlafaxine effect on NPY mRNA expression was not present in NAN-190 treatment group, but only in the control group. In mammals, many feeding related peptides are known to be modulated by 5-HT, including NPY, and there is an antagonistic relationship between 5-HT and NPY (Halford *et al.,* 2007). 5-HT_{1A} receptor immunoreactivity has been directly demonstrated in the hypothalamic NPY-producing neurons, suggesting that this receptor type is involved in mediation of 5-HT effects on the release of this orexigenic peptide (Collin *et al.,* 2002). Therefore, the observed downregulation of NPY transcript by venlafaxine may be related to alterations in 5-HT signalling similar to that seen with CRF gene expression. We propose that the venlafaxine mediated increase in 5-HT levels negatively regulate NPY expression and this involves 5-HT_{1A} signalling in trout.

Reliance of the brain on glucose for meeting energy demands suggest that central glucosensing mechanisms play a vital role in energy homeostasis. Hypothalamus contains glucosensing neurons, and their firing rates can be either excited or inhibited by circulating glucose concentration (Polakof *et al.,* 2011). Glut2 plays a crucial role in glucosensing, and a role for Glut2 in feeding regulation has also been suggested (Stolarczyk *et al.,* 2010).

Reduced hypothalamic transcript abundance of Glut2 in response to venlafaxine treatment may also be playing a role in the drug effect on appetite regulation in trout.

Pituitary

In our *in vitro* experiment with rainbow trout pituitaries, whole pituitaries were used (fragmented) as opposed to using only pituitary pars distalis (containing corticotrophs) or dispersed pituitary cell culture in order to preserve as much physiological integrity as possible. For example, the pituitary neurointermediate lobe contains endogenous arginine vasotocin (AVT), which can potentiate response of corticotrophs to CRF stimulation (Baker *et al.,* 1996). The purpose of this study was to study the effects on *in vitro* venlafaxine exposure on ACTH production and release in the pituitary. In the pituitary corticotrophs, CRF stimulates pro-opiomelanocortin transcription and ACTH secretion through the cAMP-protein kinase A (PKA) pathway, and although CRF stimulates secretion of ACTH via its CRF-R1 receptor, the mechanism of ACTH secretion via the receptor is not fully understood (Moriyama *et al.,* 2005).

Stimulation of rainbow trout pituitary fragments with 10 nM tilapia CRF did significantly elevate tissue ACTH levels, but did not show any corresponding changes in ACTH released into the incubation medium. Static *in vitro* incubation systems using pituitary tissue fragments are widely used to characterize endocrine physiology (Tsai and Wang, 1997; Baker *et al.,* 1996; Yu and Peter, 1992; Yu *et al.,* 1991). However, a potential drawback of this system arises due to receptor desensitization as a result of continuous contact with the released peptides. This could account for the lack of an effect of CRF on ACTH release in the present study. Overall, venlafaxine did affect ACTH production in the trout pituitary, although concentration effects or effects of ACTH released in to the medium was unclear. This experiment demonstrated some evidence that 5-HT_{1A} receptor may be involved in mediation of some of the observed venlafaxine effects. This *in vitro* venlafaxine effect at the level of the pituitary corresponds with our previous results from *in vivo* exposure study where expression of CRF-R1 was altered in the pituitaries of venlafaxine exposed fish following a

stressor challenge [Chapter 4], suggesting reduced sensitivity of corticotrophs to CRF stimulations due to drug exposure.

Interrenal

Neither basal nor ACTH-induced cortisol production were altered in head kidney pieces exposed to a range of venlafaxine concentrations (0.1 – 1000 nM). Such *in vitro* exposure model represents short-term or acute effects of drug exposure on tissue suggesting that venlafaxine exposure does not affect interrenal cortisol biosynthetic capacity in response to ACTH stimulation. The results of our *ex vivo* head kidney revealed that ACTH-induced cortisol response was significantly elevated in the presence of high 5-HT (10^{-7} M) in the venlafaxine exposed fish. 5-HT is known to play a prominent role as a paracrine factor in peripheral tissues involved in various aspects of fish physiology. Recent studies revealed a direct stimulatory effect of 5-HT on cortisol secretion by the interrenal tissue in fish (Lim *et al.,* 2013; Medeiros and McDonald, 2012). Although in our study 5-HT treatments did not significantly stimulate cortisol response, the observed elevation of ACTH-stimulated cortisol production in the tissues from venlafaxine exposed fish with high 5-HT treatment suggests that this drug may impact endocrine/paracrine interactions that control corticosteroid biosynthesis, but this needs further validation. In addition, our previous *ex vivo* head kidney study [Chapter 3] demonstrated the venlafaxine can alter responsiveness of steroidogenic cells to ACTH stimulation, and the mode of action appears to involve pathways upstream of the intracellular cAMP signalling cascade. A potential link between venlafaxine and paracrine or paracrine/endocrine interactions in the head kidney should be investigated in more detail in future studies. Taken together, venlafaxine exposure may alter the functioning of the interrenal tissue by eliciting effects on the upstream regulatory elements of the HPI axis.

5.6 CONCLUSIONS

Overall, this study demonstrated several lines of evidence suggesting that venlafaxine is a neuroendocrine disruptor in fish, and it directly affects the functioning of the

hypothalamus, pituitary and interrenal tissues in rainbow trout. Venlafaxine reduced phosphorylation of CREB transcription factor, which is involved in the regulation of a wide array of genes involved in cellular metabolism and hormone production. Venlafaxine altered expression of key molecular markers involved in HPI axis regulation, including CRF and GR transcript levels. Moreover, mRNA abundances of several peptides involved in energy homeostasis/food intake regulation, including POMC, Glut2, and NPY, were also affected by venlafaxine exposure. Effects of 3.0 nM venlafaxine treatment on CRF and NPY involve disruption in serotonergic activation. Venlafaxine also disrupted CRF-mediated ACTH production in trout pituitaries. Interrenal cortisol biosynthesis capacity was not affected by *in vitro* venlafaxine exposure in the present study, although finding of our head kidney *ex vivo* study indicated that interrenal tissues from venlafaxine exposed fish have higher cortisol production in response to ACTH stimulation in the presence of serotonin. Altogether, venlafaxine impacts the functioning of each component of the HPI axis and the mode of action involves disruption in serotonin signalling.

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Chapter 6

General conclusions

Summary of key findings

The overall goal of this thesis was to characterize the impact of environmentally relevant venlafaxine exposure on the stress performance in rainbow trout (*Oncorhynchus mykiss*), as well as to investigate the mechanisms of action of venlafaxine in disrupting the functioning of the hypothalamus pituitary interrenal (HPI) axis. Altogether, the findings support our primary hypothesis that venlafaxine at environmentally relevant concentrations is a neuroendocrine disruptor in rainbow trout. Specifically, the studies provide evidence for the first time that venlafaxine exposure:

1. Alters monoamine levels and their turnover rates, with significant changes occurring in the midbrain and hindbrain regions of the trout brain [Chapter 2].
2. Impacts appetite regulation [Chapter 2] and this involves changes in the expression levels of key neuropeptides associated with food intake/energy homeostasis [Chapter 2, 4, 5].
3. Alters liver and gill metabolic capacities [Chapter 3].
4. Disrupts adaptive stressor-mediated plasma cortisol and glucose responses [Chapter 2 , 3, 4], where specific effects on these plasma parameters are influenced by several factors, including type of stress (e.g. social or handling), overall sensitivity/responsiveness state of the HPI axis, and nutritional status of animals [Chapter 2, 3, 4].

These findings are summarized in Fig. 6-1.

In an attempt to elucidate potential mechanisms involved in disruption of the HPI axis functioning, our studies established that venlafaxine targets multiple neuroendocrine/endocrine components involved in its functioning (summary presented in Figure 6-2). In the hypothalamus, venlafaxine alters expression of the key neuropeptides involved in the HPI axis regulation (corticotropin releasing factor, CRF, and neuropeptide Y, NPY). In the pituitary, ACTH response to CRF stimulation is disrupted and this may involve alteration in CRF receptor type 1 (CRF-R1) transcript levels. At the interrenal tissue level,

the responsiveness of steroidogenic cells to ACTH stimulation appears to be affected by venlafaxine and the mode of action appears to involve disruption of pathways upstream of cAMP production. Also, our results suggest the venlafaxine effects on the interrenal tissue may be mediated via interactions with paracrine factors in the head kidney. In general, the mode of action of this drug may include disruptions of specific responses via serotonergic actions and pathways involved in CREB signalling, while the exact mechanism of action remains to be elucidated.

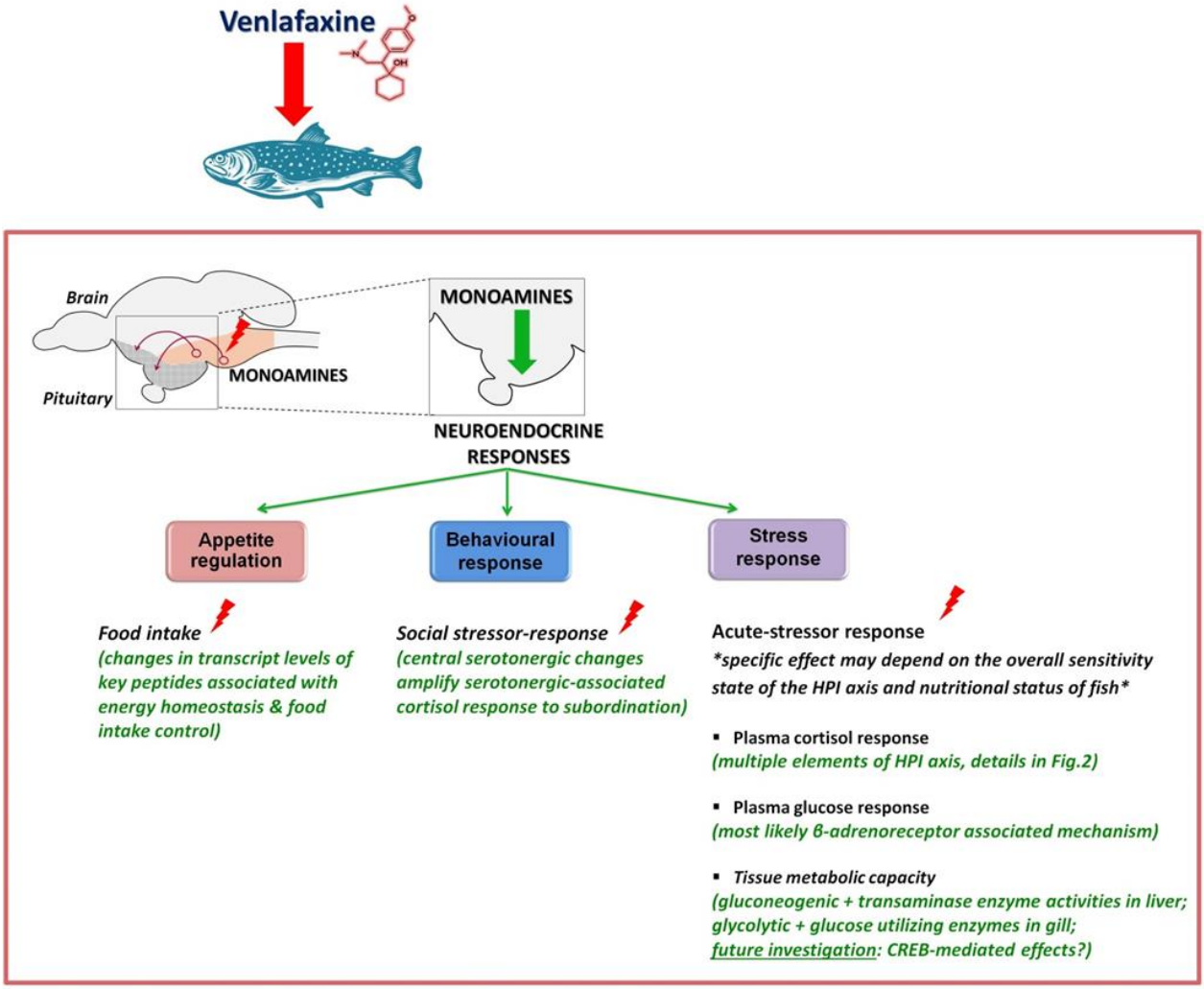

Figure 6-1. Overview of adaptive responses affected by venlafaxine exposure in rainbow trout, as demonstrated over the course of the studies included in this thesis (affected responses indicated by red bolts). Monoamine levels were mostly altered in the midbrain and hindbrain regions (highlighted in orange). Hypothalamic region (highlighted in grey) participates in regulation of various neuroendocrine responses, and receive monoaminergic innervation (represented by arrows) from the affected areas. Trout brain is in sagittal view; enlarged window shows hypothalamic region (with preoptic area) and pituitary. Abbreviations: HPI – hypothalamic pituitary interrenal axis, CREB – cAMP response element binding protein.

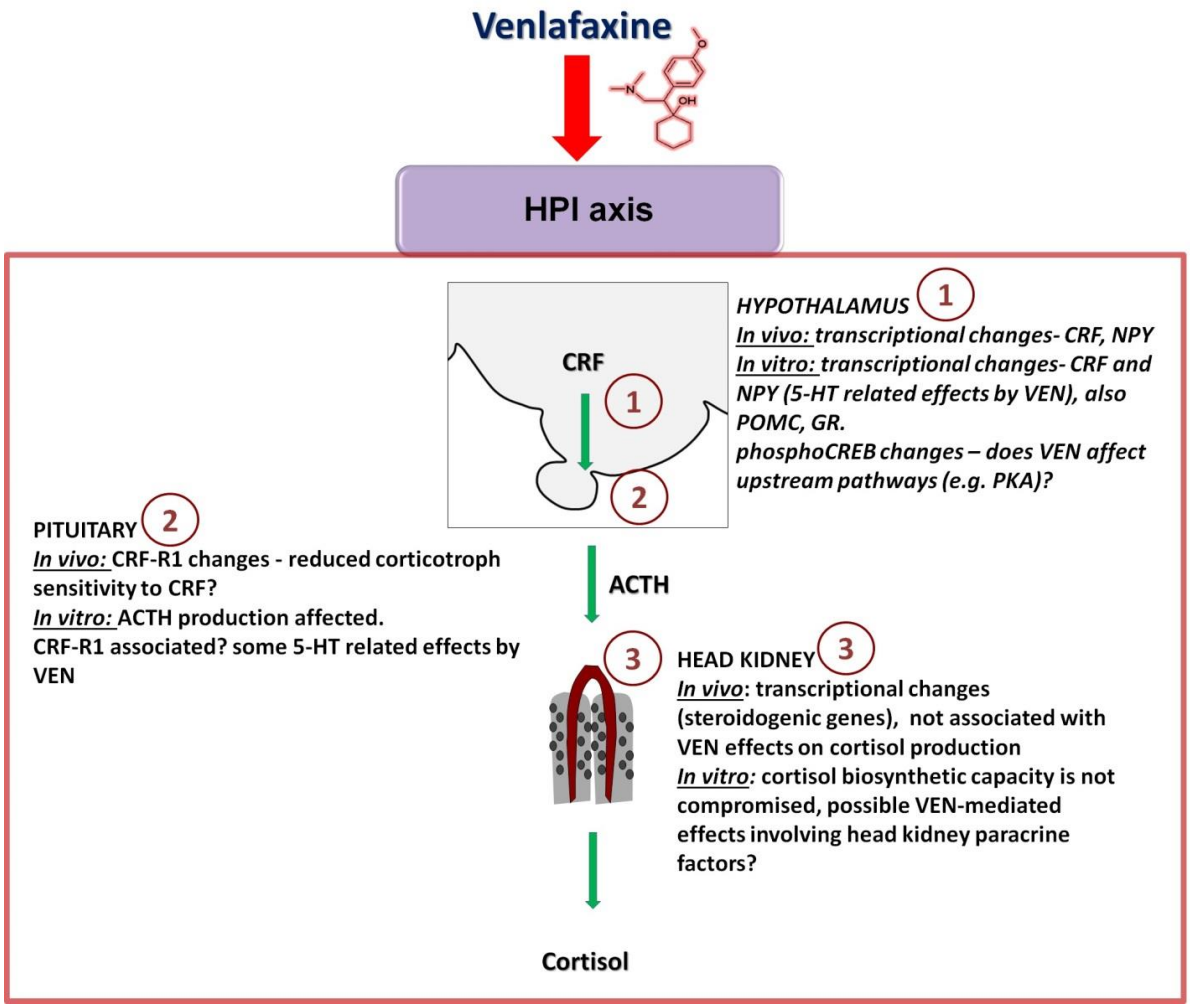

Figure 6-2. Overview of venlafaxine exposure effects on individual components of the hypothalamus-pituitary-interrenal (HPI) axis in rainbow trout, as demonstrated by our *in vivo* and *in vitro* studies presented this thesis. Abbreviations: VEN – venlafaxine, CRF- corticotropin releasing factor, ACTH – adrenocorticotropic hormone, CREB – cAMP response element binding protein, 5-HT- serotonin. Numbers: 1- hypothalamic region, 2 - pituitary, and 3 - head kidney tissue in rainbow trout.

Limitations and future directions

Results from our *in vivo* exposure study with acute stressor challenge [Chapter 4] demonstrated that venlafaxine-exposed fish exhibited reduced transcript abundance of CRF receptor CRF-R1, which is the primary type involved in regulation of the HPI axis. While no changes in mRNA levels of CRF were detected, it is not known whether CRF protein levels remained unchanged. CRF has been shown to downregulate CRF-R1 mRNA levels via a cAMP-PKA mediated mechanism (Moriyama *et al.,* 2005). Thus, further investigation into how venlafaxine affects hypothalamic CRF protein levels and cAMP-PKA signalling cascades in the pituitary will help to elucidate mechanisms of venlafaxine effects on the pituitary corticotrophs.

CREB is a key transcriptional regulator of many genes that function in cellular metabolism. For instance, CREB has been proposed to regulate hepatic enzymes involved in gluconeogenesis including phosphoenol pyruvate carboxykinase (PEPCK) (Mayr and Montminy, 2001). Our past results from *in vivo* exposure study [Chapter 3, Best *et al*., accepted] demonstrated that venlafaxine exposure increased activity of liver PEPCK. Thus, future investigation into how venlafaxine affects rapid CREB phosphorylation in liver could provide a link to a possible mechanism responsible for effect of this drug on glucose production in response to stress in trout.

Our *in vitro* studies with the pituitary fragments were performed in a static system and this may have inhibited the ACTH secretion response, possibly due to desensitization of secretion responses mediated by CRF. Therefore, perfusion studies with trout pituitaries would offer a better system for evaluation of venlafaxine effect on ACTH secretion. In addition, it would be interesting to investigate whether venlafaxine exposure affects cortisol mediated negative feedback on the hypothalamus and/or pituitary using *in vitro* approach with glucocorticoid receptor antagonists.

Work presented in this thesis was mainly aimed at teasing out serotonin-associated venlafaxine effects, and investigation of potential norepinephrine and dopamine mediated effects due to venlafaxine exposure remains a subject for future studies. Specifically, a

potential focus of future studies can be directed towards elucidating venlafaxine effects on stressor-induced rapid catecholamine responses.

Relevance / significance

Most aquatic biomonitoring studies involving controlled exposures are conducted with concentrations that are greatly exceeding environmentally realistic levels in order to increase chances of detecting and quantifying exposure effects. To our knowledge, very few studies have looked at the effects of venlafaxine exposure at environmentally relevant levels, which range from low ng/L to low μg/L, and none have evaluated the effects on the physiology of stress response. The work presented in this thesis underscores the need to conduct detailed studies in non-target species involving individual pharmaceutical components associated with wastewater effluent mixtures. This will assist in extrapolating potential effects of a wide range of pharmaceutical compounds that share similar pharmacological modes of action. From an ecotoxicological standpoint, it is important to study the effects of the wastewater effluent and its components on aquatic organisms in order to estimate their impact on the ecosystem. In addition to contributing to the body of literature on neuroendocrine disruptive compounds affecting stress physiology of fish, the results from our studies underscore the need to develop and employ effective risk management strategies to minimize environmental contamination with pharmaceuticals. For example, since overall pharmaceutical consumption is not likely to be reduced with growing population levels and increasing prescription trends, employment of drug disposal programs and public education in proper disposal would help to reduce entry of unused medicines into the environment. Development of advanced wastewater treatment processes with higher pharmaceutical removal efficiencies and investment in wastewater infrastructure is also important. Another additional risk management approach that should gain more emphasis in the near future is development of "green pharmacy". In accordance with its principles, drug design should not only be based on the functionality of a chemical necessary for its therapeutic application, but also ensure fast degradability after its use, taking into account the full life cycle of the chemical (Kümmerer, 2009).

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Appendix A

Chapter 2 Supplemental Figures

Melnyk-Lamont, N.; Best, C.; Gesto, M.; Vijayan, M.M. The antidepressant venlafaxine disrupts brain monoaminergic levels and neuroendocrine responses to stress in rainbow trout. Submitted to *Environmental Science & Technology* (2014). Manuscript ID: es-2014-00830q.

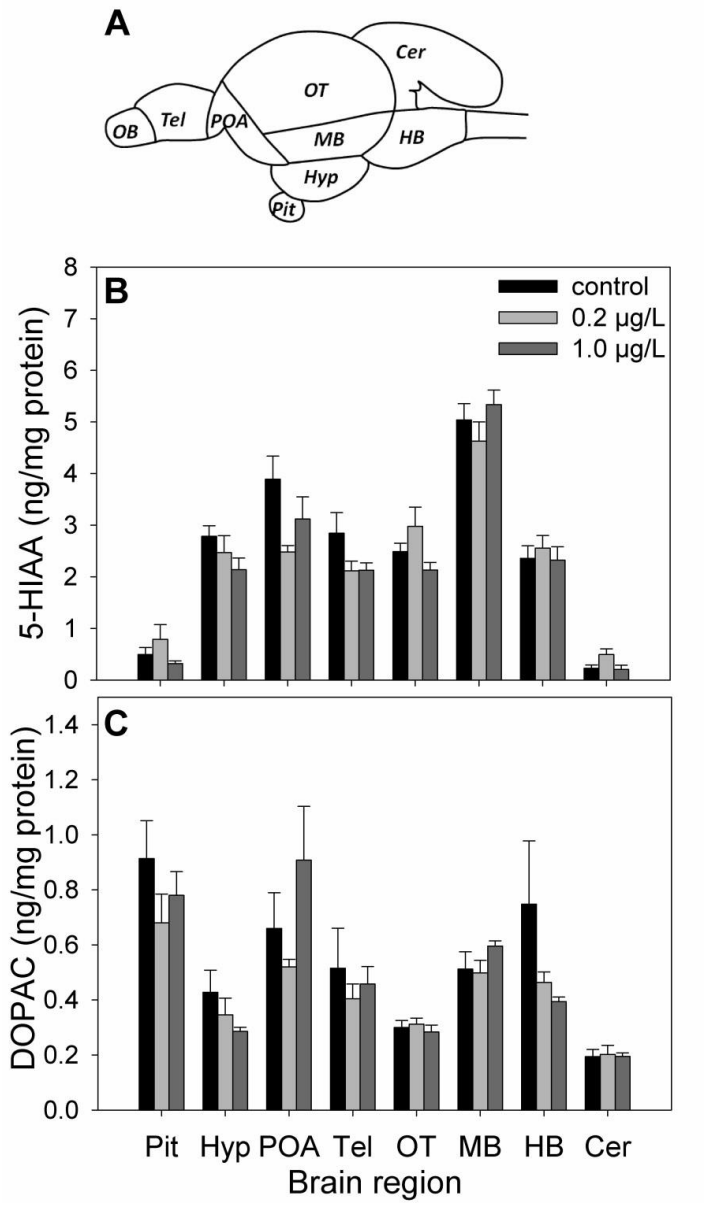

Figure A1. Schematic representation of the rainbow trout brain showing different regions [hypothalamus (Hyp), telencephalon (Tel), preoptic area (POA), optic tectum (OT), midbrain (MB), cerebellum (Cer) and hindbrain (HB), olfactory bulbs (OB), and pituitary (Pit)] (**A**), and levels of 5-hydroxyidndoleacetic acid, 5-HIAA (**B**) and 3,4- dihydroxyphenylacetic acid, DOPAC (**C**) in these brain regions (excluding OB) after a 7-day exposure to venlafaxine. Values represent means ±SEM (n = 5).

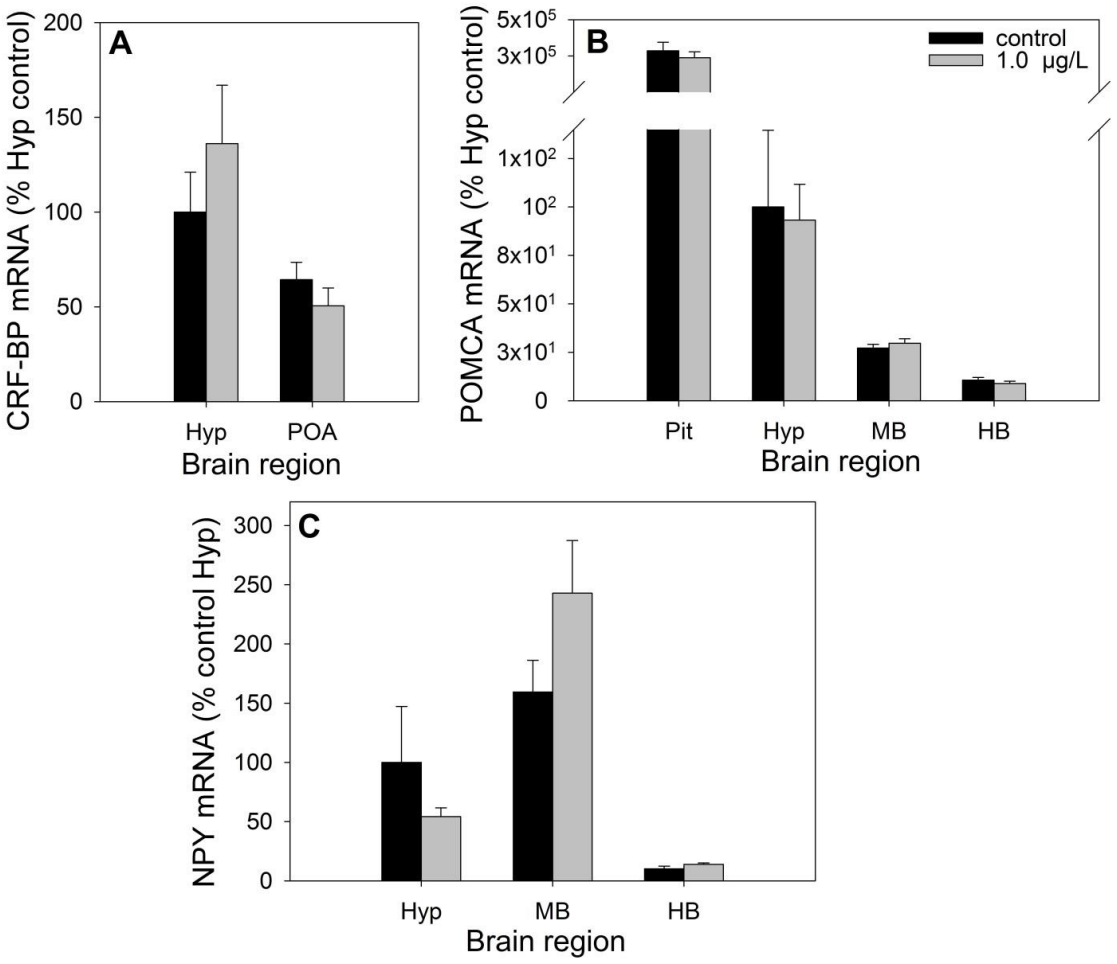

Figure A2. Transcript levels of corticotropin-releasing factor binding protein, CRF-BP (**A**), pro-opiomelanocortin A, POMCA (**B**), and neuropeptide Y, NPY (**C**) in selected regions of rainbow trout brain (see methods for details) following a 7-day venlafaxine exposure. Values were normalized to EF1α and represent percent hypothalamus control; bars represent means ± SEM (*n* = 5). Abbreviations: hypothalamus (Hyp), preoptic area (POA), midbrain (MB), hindbrain (HB) and pituitary (Pit).

Appendix B

Chapter 3 Supplemental Figures

Best, C.; Melnyk-Lamont, N.; Gesto, M. Vijayan, M.M. Environmental levels of the antidepressant venlafaxine impact the metabolic capacity of rainbow trout. Accepted to *Aquatic Toxicology* (2014). Manuscript ID: AQTOX-D-14-00153.

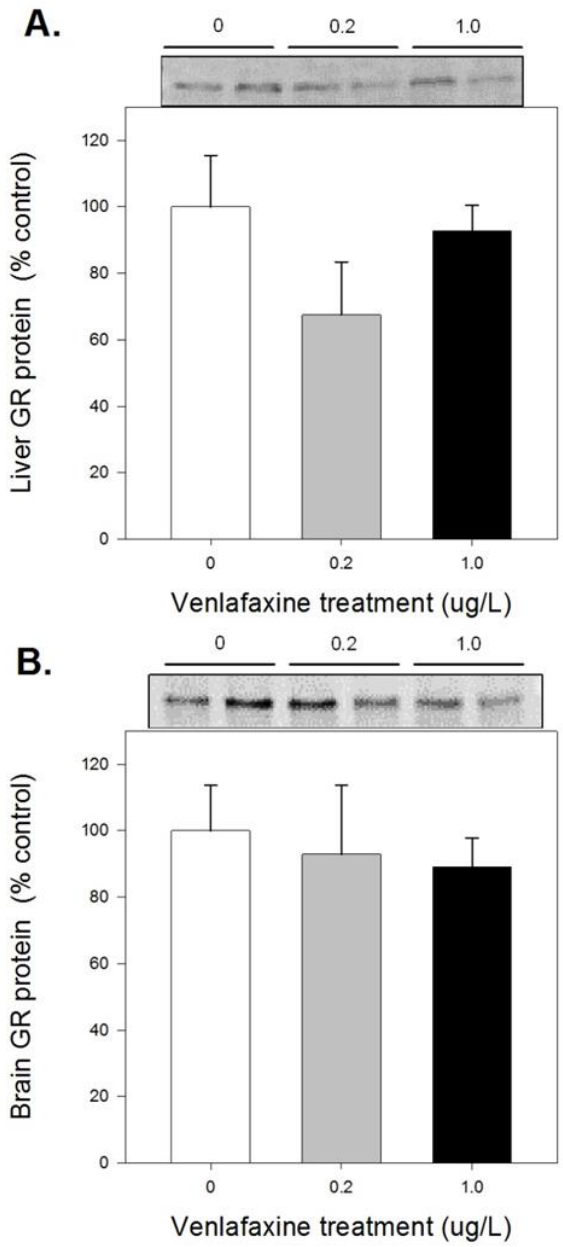

Figure B1. Glucocorticoid receptor (GR) protein expression. Effect of 7 day venlafaxine exposure on GR protein expression levels in liver (**A**) and brain (**B**) of rainbow trout. A representative western blot image of GR expression is shown above the bar graph. Values are shown as % control (normalized to β-actin) and represent means ± SEM (n = 6 fish). There were no significant differences between treatments ($P > 0.05$, one-way ANOVA).

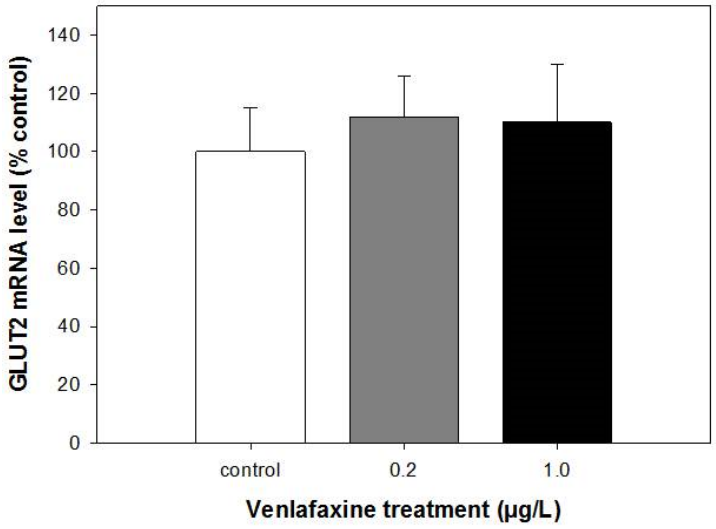

Figure B2. Liver glucose transporter 2 (GLUT2) mRNA abundance. Effect of a 7-day venlafaxine exposure on liver GLUT2 mRNA abundance in rainbow trout. Values are shown as % control (normalized to EF1α) and represent means ± SEM (n = 6 fish). There were no significant differences between treatments ($P > 0.05$, one-way ANOVA).

Appendix C

Chapter 5 Supplemental Figures

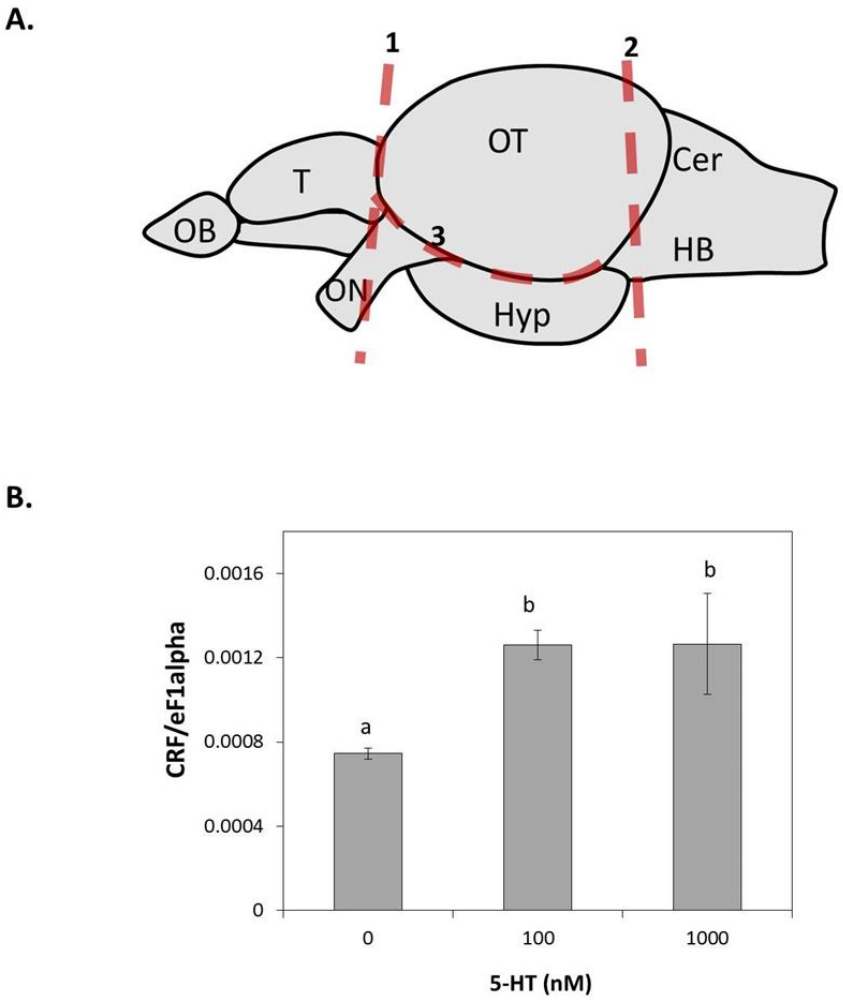

Figure C1. Schematic representation of rainbow trout brain in sagittal view, depicting the way the brain was sectioned to separate hypothalamic region from the rest of the brain. Dashed lines indicate where the cuts were made, and numbers represent their order (**A**) (Abbreviations: OB - olfactory bulb, ON - optic nerve, T - telencephalon, OT - optic tectum, Cer - cerebellum, HB - hindbrain, Hyp - hypothalamus). Effect of serotonin treatments (5-hydroxytryptamine, 5-HT) on transcript abundance of corticotropin releasing factor (CRF) in hypothalamic tissue (**B**) following 2 h incubation period. Bars represent means ± SEM (n = 5 independent samples), values were normalized to elongation factor 1 alpha (eF1alpha). Different letters indicate significant differences between treatments ($P < 0.05$; one-way ANOVA).

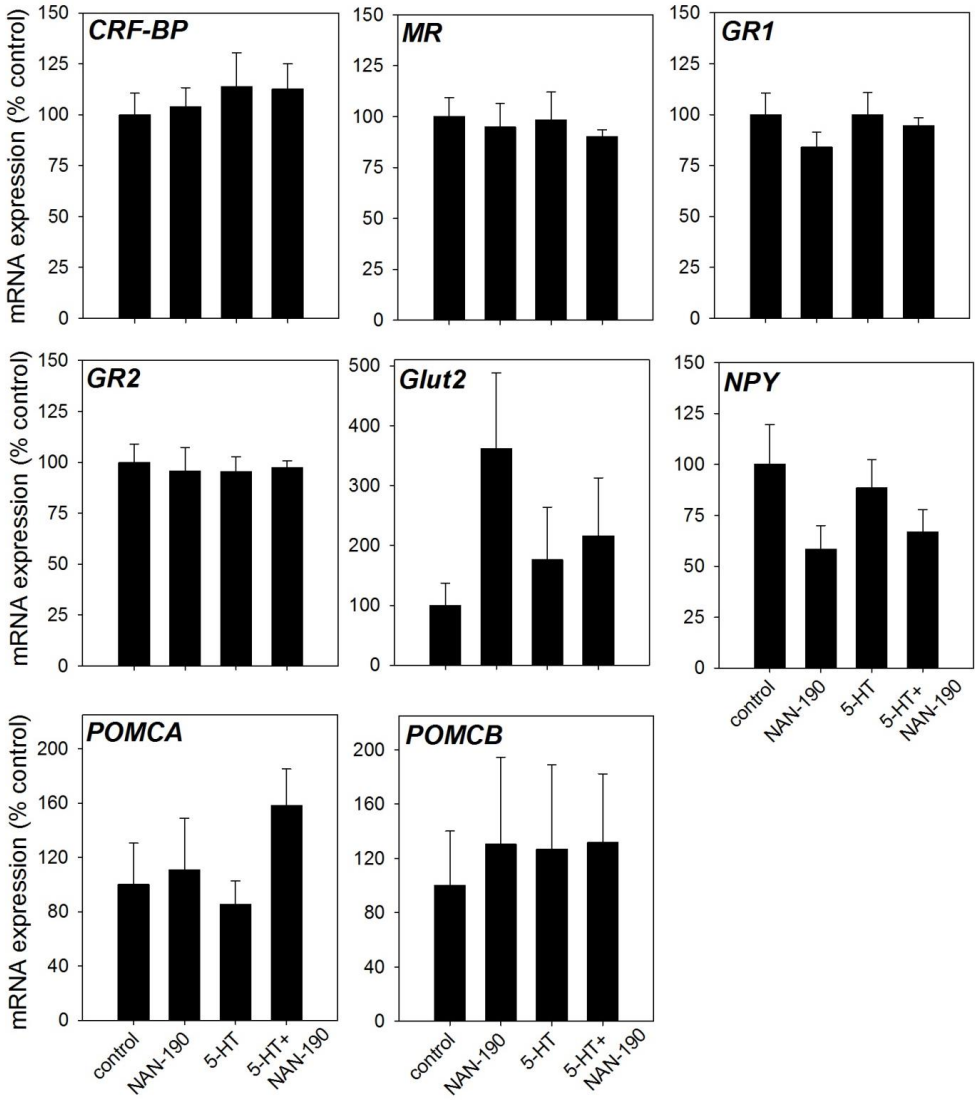

Figure C2. Transcript levels in rainbow trout hypothalami in response to incubation with serotonin, 5-HT (100 nM), 5-HT_{1A} receptor antagonist, NAN-190 (1 μM), or combination of NAN-190 and 5-HT. All values were normalized to EF1α and represent percent change from the control group. Bars show means ± SEM (n = 6 independent fish). Gene abbreviations: EF1α, elongation factor 1α; CRF-BP, corticotropin-releasing factor binding protein; Glut2, glucose transporter type 2; NPY, neuropeptide Y; GR1 and GR2, glucocorticoid receptor 1 and 2, respectively; MR, mineralocorticoid receptor; POMCA and POMCB, pro-opiomelanocortin A and B, respectively. No significant treatment effects were detected on transcript abundances of the above mentioned genes ($P > 0.05$, one-way ANOVA).