Developmental ethanol exposure and its impact on behaviour and HPI axis activity of zebrafish

Baiamonte, Matteo

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Matteo Baiamonte

Submitted in partial fulfillment of the requirements of the Degree of Doctor of Philosophy

September 2014

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Abstract

Ethanol exposure during pregnancy is one of the leading causes of preventable birth defects, leading to a range of symptoms collectively known as fetal alcohol spectrum disorder (FASD). More moderate levels of prenatal ethanol exposure (PNE) lead to a range of behavioural deficits including aggression, poor social interaction, poor cognitive performance and increased likelihood of addiction in later life.

Current theories suggest that adaptation in the hypothalamic-pituitary-adrenal (HPA) axis and neuroendocrine systems contributes to mood alterations underlying behavioural deficits and vulnerability to addiction. This has led to the suggestion that corticotrophin-releasing factor (CRF) antagonists and glucocorticoid (steroid) inhibitors may be potential therapeutics to address the deficits of PNE and for the treatment of addiction.

The zebrafish (*Danio rerio*) has several advantages over mammalian models, such as low cost of maintenance, short life cycle, easy embryological manipulation and the possibility of large-scale genetic screening. By using this model, our aim is to determine whether developmental ethanol exposure provokes changes in the HPA axis (HPI axis in fish), as it does in mammalian models, therefore opening the possibilities of using zebrafish to elucidate the mechanisms involved, and to test novel therapeutics to alleviate deleterious symptoms. Thus this thesis focuses solely on the effect of developmental ethanol exposure on the functioning of the HPI axis in zebrafish.

Stress-reactivity in zebrafish larvae ethanol-treated 1-9 days post

fertilisation (dpf) was assessed using thigmotaxis and thigmotaxis following air-stress. In both tests, lower stress-related responses were obtained with ethanol treated animals, in that they spent less time at the edges of the apparatus ($P<0.01$, n=3). They also showed lower total body cortisol *(*$P=0.04$, n=14). Larvae also showed the same behaviour pattern two weeks after ethanol exposure, (23dpf) *(*$P=0.04$, n=3), again with reduced total cortisol *(*$P=0.03$, n=4).

HPI-related gene transcription was also assessed in 9dpf ethanol treated zebrafish larvae, by qRT-PCR. Revealing up-regulation of CRH, CRHBP and CRHR2, normalized against β-Actin, Elav1 and Gap43 housekeeping genes. In situ hybridization revealed no spatial changes in CRH, CRH-BP and POMC with animals at the same stage.

Behavioural stress-reactivity differences in 6-months old adults that had been exposed developmentally to ethanol were assessed using novel tank diving and thigmotaxis. Both assays indicated a decrease in stress-like behaviour due to early ethanol exposure compared to controls ($P<0.05$, n=5 both).

Finally, cortisol levels were assayed from 9dpf larvae and 6-month-old adults that had been treated with ethanol during early development showed a significant reduction in cortisol output when air-exposed stressed compared to controls ($P=0.04$, n=5).

Conclusion: Early ethanol exposure produced significant changes in cortisol, HPI gene mRNA expression and stress-reactive behaviour in 9dpf animals. Changes in cortisol and behaviour were still detected in 6-months old adults, developmentally treated with ethanol, indicating that early ethanol exposure has permanent effects on the HPI axis.

As our data contradicts the findings in mammalian literature where early ethanol exposure increases stress-like behaviour in later life, it is also possible that more permanent effects of PNE in mammals may arise through maternal-offspring interactions, during and post gestation, such as breastfeeding and maternal grooming of the offspring, which are absent in the zebrafish model.

This thesis is dedicated to my family

Acknowledgements

I want to thank both my supervisors, Dr. Caroline Brennan and Prof. Gavin Vinson for allowing me to do this Ph.D, for their inspirational scientific philosophies, for their insightful and invaluable advices, to endure with me through the good and bad times of this project and for letting me conduct this research with the outmost freedom a student can have.

I thank Dr. John Puddefoot for introducing me to the research field with an undergraduate final year project and for all his support thereafter. I thank Dr. Matthew Parker for his valuable discussions and support. I am thankful to Dr. Ruth Rose, David Malley, Dr. Michaela Egertova, Dr. Rachel Ashworth, Dr. Rui Oliveira, Ana Felix, Ana Faustino, Ricardo de Padua, Dr. Wilson de Paula, Dr. Iskander Ibrahim, Mollie Millington and Fraser Combe for their valuable support in many aspects of my research. Also thankful to my colleagues; Aryeh Sudwarts, Dean Semmens, Ryan Graves and Alistair Brock for our insightful discussions and to Daniel Caires, Flavia D'avila and Ellen Kris Fantossi for their support during this project.

I am grateful to my family; my mother, Grazia Baiamonte; my sister, Leticia Baiamonte; and my father, Matteo Baiamonte Filho; my grandfather, Matteo Baiamonte; my grandmother Apolonia Mancuso, and, my grandmother and grandfather that passed away during the completion of this research project, Jandira Baiamonte and Luigi Mancuso, for their support and encouragement to my scientific career. I acknowledge Queen Mary University of London and the BBSRC (grant: BB/F016913/1) for funding my research.

Abbreviations

ATP - Adenosine 5’-triphosphate

ADP – Adenosine 5’-diphosphate

BSA - Bovine serum albumin

cAMP – Cyclic Adenosine 5’-monophosphate

DNA – Deoxyribonucleic acid

Dpf – Days post-fertilization

Hpf – Hours post-fertilization

ISH – In situ hybridization

L – Litre

G – Gram

GABA – Gamma-Aminobutyric acid

µg – Microgram

µL – Microlitre

µM – Micromolar

mg – Milligram

mL – Millilitre

mM – Millimolar

mm - millimetre

NADH - Nicotinamide adenine dinucleotide

PCR – Polymerase chain reaction

qPCR – Quantitative polymerase chain reaction

PNE – Prenatal ethanol exposure

RNA – Ribonucleic acid

Rpm – Rotation per minute

v/v – volume per volume

Table of contents

List of figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Chapter 1: General Introduction

In this introduction, I will discuss the effects of ethanol at the systemic and cellular level, the function of the HPA axis and the impact early ethanol exposure has on this stress system. In the final sections I describe the possible links between the HPA axis and the mesolimbic system, focusing on the effects early ethanol has on both systems in order to influence vulnerability to addiction in later life.

1.1 Ethanol systemic action pathways

1.1.1 Ethanol as a drug of abuse

Human alcohol consumption has occurred for thousands of years, ranging as far back as the Neolithic period in Africa and Asia, presumably after sampling fermentation products by chance, following deliberate fermentation which lead to habitual ethanol consumption (McGovern, Zhang et al. 2004, O'Shea, Dasarathy et al. 2010).

Ethanol, like other drugs, induces pleasurable effects by activating the mesolimbic dopaminergic system in the central nervous system (CNS). Following ethanol administration, extracellular levels of dopamine (DA) in rat mesolimbic structures rise: Ventral tegmental area (VTA), nucleus accumbens (NAc) and prefrontal cortex (PFC) (Imperato and Di Chiara 1986, Schier, Dilly et al. 2013, Yorgason, Ferris et al. 2013).

Behavioural experiments reported increase in ethanol oral self-administration and even ethanol intracranial self-administration in the VTA region, implicating the reinforcing properties of ethanol in rats (Rodd-Henricks, McKinzie et al. 2000, Bertholomey, Verplaetse et al. 2013).

During the last century alcohol abuse has increase in several countries, increasing accidents and alcohol-related diseases, such as cirrhosis, hepatocellular carcinomas, alcoholic hepatitis, body growth defects, thyroid problems, bone disease, reproductive deficits, psychological and behavioural disorders (Mann,

Smart et al. 2003, Rachdaoui and Sarkar 2013). Alcohol consumption is a worldwide problem that is extensively researched, for its addiction and deleterious traits.

1.1.2 Ethanol action pathways

1.1.2.1 Ethanol effects on lipid membranes

Ethanol has long been thought to increase membrane fluidity. As early as 1977, Chin and Goldstein used electron paramagnetic resonance to demonstrate the ability of ethanol to increase membrane fluidity of rat erythrocytes plasma membranes in vitro (Chin and Goldstein 1977). Ethanol also affects the composition of lipid rafts, which are cholesterol and sphingolipid microstructures that confer high rigidity to the plasma membrane and also affect cell-signalling pathways (Szabo, Dolganiuc et al. 2007).

Later, erythrocytes from alcoholic patients were found to have less fluidity compared to controls, presenting an increase in microviscosity and an increase in lipid peroxidation following oxidative stress (Parmahamsa, Reddy et al. 2004). The opposite was found for rat liver cells, exhibiting more membrane fluidity compared to controls, with emphasis in the hydrophobic regions of the lipid bilayer. Similar findings were observed using human fetal hepatic cells exposed to ethanol chronically and acutely.

Ethanol treatment caused disorganization of acyl chains in the rat membrane and increased the cholesterol/phospholipid ratio, resulting in a higher fluidity

membrane compared to controls as assessed by fluorescence polarization (Yamada and Lieber 1984, Gutierrez-Ruiz, Gomez et al. 1995). Neural crest cells also present a dose-dependent increase in membrane fluidity after acute ethanol treatment, and this was inversely related to cell viability (Chen, Yang et al. 1996).

When rat hepatocytes were pre-treated with an inhibitor of ethanol metabolism, a reactive oxygen species (ROS) scavenger or a free radical antioxidant; ethanol-induced increase in the membrane fluidity was prevented, suggesting that ethanol metabolites and ROS are required for the damaged caused to lipid membranes (Sergent, Pereira et al. 2005).

Membrane stabilizing agents prevented fluidification of the membrane, ROS production, lipid peroxidation and apoptosis. Fluidizing components, such as tween 20, increased the membrane fluidity effect of ethanol and its oxidative stress (Sergent, Pereira et al. 2005). These findings indicate that as well as ROS and free radicals generated by ethanol exposure, the state of fluidity of the membrane will determine how vulnerable the cell will be to the ethanol insult, opening the possibility of the use of membrane stabilizing compounds as therapeutics.

1.1.2.2 Ethanol effects on ligand-gated ion channels

Ethanol is considered to be a promiscuous ligand, which is a ligand that modulates ligand-gated ion channels (LGIC) different states, over a wide range of ligand concentrations. Ethanol can induce LGIC receptor desensitization, of a number of ligand gated ion channels (LGIC) such as $GABA_A$, serotonin receptor

type 3 (5-HT_3) and nicotinic acetylcholine (nAchR) receptors over a wide range of concentrations and as such is considered a 'promiscuous ligand' (Lovinger and White 1991, Aguayo, Peoples et al. 2002, Dopico and Lovinger 2009).

Ethanol is thought to act mainly on ion channel post-translational modifications and subunit composition (Dopico and Lovinger 2009). Acute tolerance to ethanol is a classic example of desensitization, where desensitized naïve receptors will result in reduced receptor-mediated current, inducing a resistance seen in certain alcoholism phenotypes (Schuckit 2000).

Although ethanol is known to interact with a number of ligand gated ion channels its precise mode of action is not clearly understood: The binding of promiscuous ligands to LGIC and other receptors in the synaptic area is usually a very fast process ranging from seconds to milliseconds, but a slower effect on LGIC desensitisation, involving the lipid bilayer, has been proposed, which would explain the small variability in sensitivity to anaesthetic agents in humans and other animal species (Cantor 2003, Dopico and Lovinger 2009).

In addition, LGIC protein channels are associated with chaperones, making it challenging to identify the precise mode of ethanol action (Ueno, Lin et al. 2000, Harris, Trudell et al. 2008, Dopico and Lovinger 2009).

Desensitization studied by ionic current in a membrane following drug insult is composed of three variables: The channel unitary conductance (μ), its open probability (Po) and the number of channels present in the membrane (N). Acute ethanol exposure will not result in changes of μ or N, but it will change Po (Dopico and Lovinger 2009). Ethanol will induce a series of channel swelling states, the kinetic transition among them, resulting in differences in the Po.

Ethanol potentiates the function of several LGICs belonging to the Cys-loop family group, such as the previously mentioned $GABA_A$, serotonin receptor type 3 ($5\text{-}HT_3$) and nAchR receptors. At concentrations up to 100mM, ethanol increases the magnitude of the current in the presence of low concentrations of agonist, due to the ethanol-induced open state of these channels (Zuo, Nagata et al. 2004).

It also modulates the rate at which these receptors undergo desensitization/ resensitization with increasing and persistent agonist concentrations, resulting in observable current decay (Nagata, Aistrup et al. 1996, Zhou, Verdoorn et al. 1998, Dopico and Lovinger 2009).

Glutamate-activated LGIC are tetramer structures, made of three full transmembrane segments, a pore-loop ion pathway and an intracellular terminus with a carboxyl group (Collingridge, Olsen et al. 2009). Here the effect of ethanol is predominantly inhibitory (Woodward 2000, Moykkynen, Korpi et al. 2003, Lack, Ariwodola et al. 2008).

Extracellular ATP-gated ion channels (PSX) are also inhibited by ethanol (Li, Xiong et al. 2000). They are cationic trimeric receptors, with two transmembrane segments, a large extracellular loop where ATP binds and two amino and carboxyl ends in the cytosol (Vial, Roberts et al. 2004).

Calcium- and voltage-gated potassium channels (BK) do not belong to the LGIC superfamily, as they are rather triggered by biological signals than by ligand binding. Ethanol acts as an adjuvant of calcium, facilitating calcium-driven gating, potentiating the channel activity at low internal calcium concentration ($<10\mu M$) and inhibiting at higher ones ($>10\mu M$) (Liu, Vaithianathan et al. 2008).

1.1.2.3 Ethanol effects on G-protein coupled receptors

G protein-coupled receptors (GPCRs) constitute a large receptor family, also known as seven-transmembrane receptors (7TM receptors). Ligand binding elicits signal transduction, resulting a conformational change of the GPCR, leading to the activation of its associated G-protein by exchanging its bound guanosine diphosphate (GDP) for a guanosine triphosphate (GTP) (Oldham and Hamm 2008, Katritch, Cherezov et al. 2013).

When the G-protein is activated, its α subunit loses affinity for the βγ subunit complex, resulting into two signalling elements arising that can influence a variety of intracellular targets, such as altering ion channel functions and gene expression (Hille 1994, Rohrer and Kobilka 1998, Oldham and Hamm 2008, Katritch, Cherezov et al. 2013).

Three G-protein subclasses are associated with GPCRs, these are Gi/o, Gs and Gq. Gi/o activation results in an overall inhibitory effect through α subunit inhibition of adenylyl cyclase (AC), and activation of GIRK-type potassium channels (which inhibit neuronal activity) by the βγ subunit complex , βγ also inhibits CaV2 voltage-gated calcium channels, in neuronal cells, blocking neurotransmitter release and vesicle fusion (Wickman and Clapham 1995, Miller 1998, Dolphin 2003, Levitt, Purington et al. 2011).

Conversely, G-protein Gq subunits have an excitatory effect, activating ion channels that excite neurones and other cells, and inhibiting potassium channels

such as TREK-2, again leading to an increase in cell excitability (Kang, Han et al. 2006), and increased neurotransmitter or hormone release (Gyombolai, Pap et al. 2012, Weiss, Keren-Raifman et al. 2012). Gs G-protein activation stimulates AC and cyclic AMP (cAMP) production, enhancing cell activity (Hou, Suzuki et al. 2003, Rebois, Maki et al. 2012).

Ethanol has a general weak effect on GPCRs and G-proteins. Acute ethanol exposure stimulates an increase in cAMP (in mouse striatal cells and cervical cancer HeLa cells) due to actions on adenylyl cyclase 7 (AC7) (Luthin and Tabakoff 1984, Gupta, Qualls-Creekmore et al. 2013). Acute ethanol exposure has been implicated in an increase in neurotransmitter release, such as GABA, in a combined mechanism involving AC and protein kinase A (PKA) (Kelm, Criswell et al. 2008).

Ethanol inhibits the actions of three major GPCRs: Muscarinic ACh, serotonin type 2 and metabotropic glutamate receptors. In Xenopus laevis oocytes it reduces the ability of these GPCRs coupled with Gq G-proteins subunits to activate calcium-dependent chloride current (Minami, Gereau et al. 1998, Clayton and Woodward 2000). These GPCRs are structurally different, which implies different sites of action.

It has been suggested that ethanol and other volatile anaesthetics inhibit these GPCRs by promoting protein kinase C (PKC) mediated phosphorylation (Minami, Minami et al. 1997, Minami, Vanderah et al. 1997, Minami, Gereau et al. 1998, Costa and Guizzetti 2002, Shan, Hammarback et al. 2013).

1.1.2.4 Ethanol effects on protein kinases

Ethanol also has the potential to interact with signalling pathways contributing to the activation of protein kinases, which will lead to changes in gene transcription, protein phosphorylation and protein synthesis. Protein kinase C (PKC) is a well-studied target of ethanol modulation. PKC will phosphorylate receptors, ion channels, G-proteins and also other protein kinases (Kaczmarek 1987, Messing, Petersen et al. 1991, Zamponi, Bourinet et al. 1997, Mellor and Parker 1998, Stubbs and Slater 1999).

The effects of ethanol on PKC can be divided into direct or indirect effects. Directly, ethanol and other short chain alcohols (butanol, pentanol etc) moderately inhibit diacylglycerol-activated PKC activity, by acting at phorbol-ester binding sites of PKC (Pandey, Dwivedi et al. 1993, Stubbs and Slater 1999).

Acute ethanol exposure ranging from 25mM-200mM also results in translocation of PKC from the cytosol to the membrane in human astroglial cells and hepatocytes (Skwish and Shain 1990, DePetrillo and Liou 1993, Stubbs and Slater 1999). However, in rats, acute ethanol exposure inhibited PKC translocation to the membrane, in anterior pituitary cells (Steiner, Kirsteins et al. 1997). Ethanol has also been shown to reduce the cellular PKC-actin interaction, leading to reduced vesicle exocytosis in some cases (Stubbs and Slater 1999).

Other studies revealed that acute ethanol exposure has varying effects on rat PKC translocation and synthesis, according to the brain region and PKC isoform (Kumar, Lane et al. 2006). Prenatal ethanol exposure also induced a reduction in PKC expression in chick embryos, that varied with the PKC isoform and age of the embryo (McIntyre, Souder et al. 1999). Ethanol exposure has shown to promote

apoptosis in cerebellar granule cells by inhibiting PKC, a result mediated by reduction of phosphorylation of insulin-like growth factor 1 (IGF-1) that has an anti-apoptotic role (Zhang, Rubin et al. 1998, Stubbs and Slater 1999). Indirectly, ethanol effects on membrane phospholipid organization has shown to regulate different PKC isoform expressions (Slater, Ho et al. 1993).

Other examples of effects of ethanol on protein kinases include the reduction of extracellular signal-regulated kinase (ERK) phosphorylation (Sanna, Simpson et al. 2002, Chandler and Sutton 2005, Lee and Messing 2008), increase of activity and expression of protein kinase A (PKA) (Nagy, Diamond et al. 1991, Kumar, Ren et al. 2012, Carlson, Kumar et al. 2013), enhancement of cyclic dependent kinase 5 (CDK5) activity (Rajgopal and Vemuri 2001, Lee and Messing 2008), and, increased activity of Fyn kinases (Wang, Carnicella et al. 2007, Lee and Messing 2008).

1.2 HPA axis function and components

1.2.1 HPA axis

The Hypothalamic-Pituitary-Adrenal (HPA) axis is a major neuroendocrine system that evokes the main stress response of the organism, through the activation

of the hypothalamus, pituitary and adrenals resulting in the release of cortisol. Cortisol both regulates numerous systemic functions and negatively feeds back into the axis to prevent further cortisol release.

The HPA axis is also part of a complex and integrated system of multiple types of tissues and organs throughout the body. The HPA thus interacts with the function of many body tissues and peripheral organs apparently to minimize metabolic and other effects of stress, and to regain homeostasis (de Kloet, Joels et al. 2005, Hillhouse and Grammatopoulos 2006).

1.2.2 HPA axis mechanism

In this section, a brief summary of the HPA axis mechanism will be discussed, whereas the subject will be dealt with more depth in the HPA axis components and elements sections (figure 1.2).

In response to perceived stress, a specialized population of cells in the paraventricular nucleus (PVN) region of the hypothalamus, known as parvocellular neurones, secrete corticotropin-releasing hormone (CRH). CRH activates two distinct G- protein-coupled receptors (GPCRs), corticotropin-releasing hormone receptor 1 and 2 (CRHR1 and CRHR2), that share 70% amino acid identity and are located in different parts of the brain.

CRH binds with a higher affinity to CRHR1 in relation to CRHR2, whereas urocortin II and III, a second ligand for CRHR1, possess a higher affinity for

CRHR2 (Aguilera, Nikodemova et al. 2004, Hauger, Risbrough et al. 2006). CRH binds to CRHR1 on corticotroph cells of the anterior lobe of the pituitary gland, thus activating pre-proopiomelanocortin (POMC) gene transcription and translation (figure 1.3) (Autelitano, Lundblud et al. 1989, Refojo and Holsboer 2009).

Further post-translational modification yields adrenocorticotropic hormone (ACTH, corticotrophin) and other peptides. The most important action of ACTH is to increase the production and the release of corticosteroids such as cortisol, or corticosterone in rodents, from the adrenal cortex (Bornstein 1999).

Activated CRHR1 signalling occurs by Gαs coupling, resulting in adenylyl cyclase activation, generating cAMP which in turn activates protein kinase A (PKA) (Graf 2007). This results in phosphorylation of various nuclear and cytosolic targets, including CREB, inducing gene transcription (Nadhim Bayatti 2005).

Both CRH and arginine vasopressin (AVP) are released from neurones originating at the hypothalamic paraventricular nucleus into the hypophyseal portal system, stimulating the release and synthesis of ACTH from pituitary corticotrophs (de Goeij, Jezova et al. 1992, Ma, Levy et al. 1997).

AVP acts through the GPCR vasopressin receptor V1bR, resulting in a cross talk between the CRH generated cAMP-PKA and AVP generated protein kinase C (PKC) in mammalian corticotrophs (Young, Griffante et al. 2007, Cornett, Kang et al. 2013). FRET analysis in chicken corticotrophs cells suggested heterodimerization between the two receptors potentiates their effects (Marina V. Mikhailova 2007).

Pituitary corticotrophs produce POMC when CRHR1 is ligand-activated; CRH binding regulates POMC expression in mice, through the previously mentioned PKA activated CREB, and the transcription factor family Nur: Nur77,

Nurr1 and NOR-1 (figure 1.3) (Timpl, Spanagel et al. 1998, Maira, Martens et al. 2003).

PKA triggers calcium-dependent and calcium-independent pathways initiating a G-protein-linked cascade yielding the extracellular signal-regulated kinases ERK1 and ERK2, that regulate the activation of the Nur family transcription factors (Kovalovsky, Refojo et al. 2002).

ACTH is the main hormone stimulating glucocorticoid biosynthesis and release from the adrenal gland. ACTH binds to melanocortin receptor 2 (MC2R), a class A G-protein-coupled receptor, initiating steroidogenesis (Simpson and Waterman 1988). ACTH signals via adenyl cyclase to regulate cortisol in humans (corticosterone in rodents) and aldosterone.

Steroidogenic acute regulatory (StAR) is induced by ACTH and is responsible for shuttling cholesterol from the outer to the inner mitochondrial membrane, where conversion to pregnenolone takes place, catalyzed by CYP11A (P450scc) (Nishikawa, Sasano et al. 1996). This is the rate-limiting step in acute stimulation of steroidogenesis in subsequent stages of the synthesis of glucocorticoids and aldosterone (figure 1.1).

Glucocorticoids present a pulsatile pattern of release, with varying frequency and amplitude under stress and are secreted in high amounts, up to 20mg/day in humans, under the control of ACTH. Binding of the glucocorticoid to the glucocorticoid receptor (GR, NR3C) in the cytosol activates the steroid-receptor complex, marked by the dissociation of heat-shock proteins and translocation to the nucleus, where the dimerized GR-ligand bind to glucocorticoid-response elements (GREs), in the promoter regions of target genes (Beato and Sanchez-Pacheco 1996).

Broadly, glucocorticoids affect the immune system and metabolic functions. It down-regulates the expression of pro-inflammatory proteins. It stimulates lipolysis and gluconeogenesis, and mobilizes amino acids for the latter as well as inhibiting the uptake of glucose by fat and muscle tissue to conserve glucose availability (Newton 2000), which will be discussed in greater depth later in this introduction.

Glucocorticoid binding to GR on hypothalamic CRH neurones and pituitary corticotrophs generates a negative feedback mechanism, reestablishing the HPA axis baseline levels (de Kloet, Joels et al. 2005). Negative feedback by glucocorticoids inhibits POMC transcription and ACTH release and in the corticotrophs. The transcription is disrupted by transrepression mechanisms between GR and Nur family transcription factors (Martens, Bilodeau et al. 2005).

Furthermore, AtT-20 cells (corticotroph tumor cells) were used to demonstrate that histone deacetylase 2 (HDAC2) and Brg1, a nuclear protein part of the chromatin remodeling complex, form a repression complex with GR at the POMC gene locus, based on the deficiency of these proteins in this cell line (Bilodeau, Vallette-Kasic et al. 2006).

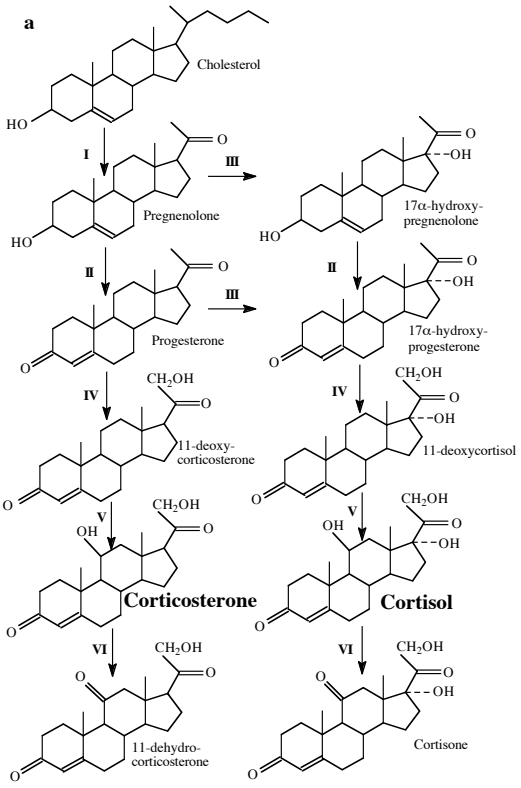


Figure 2.1: Steroidogenesis summary pathway.

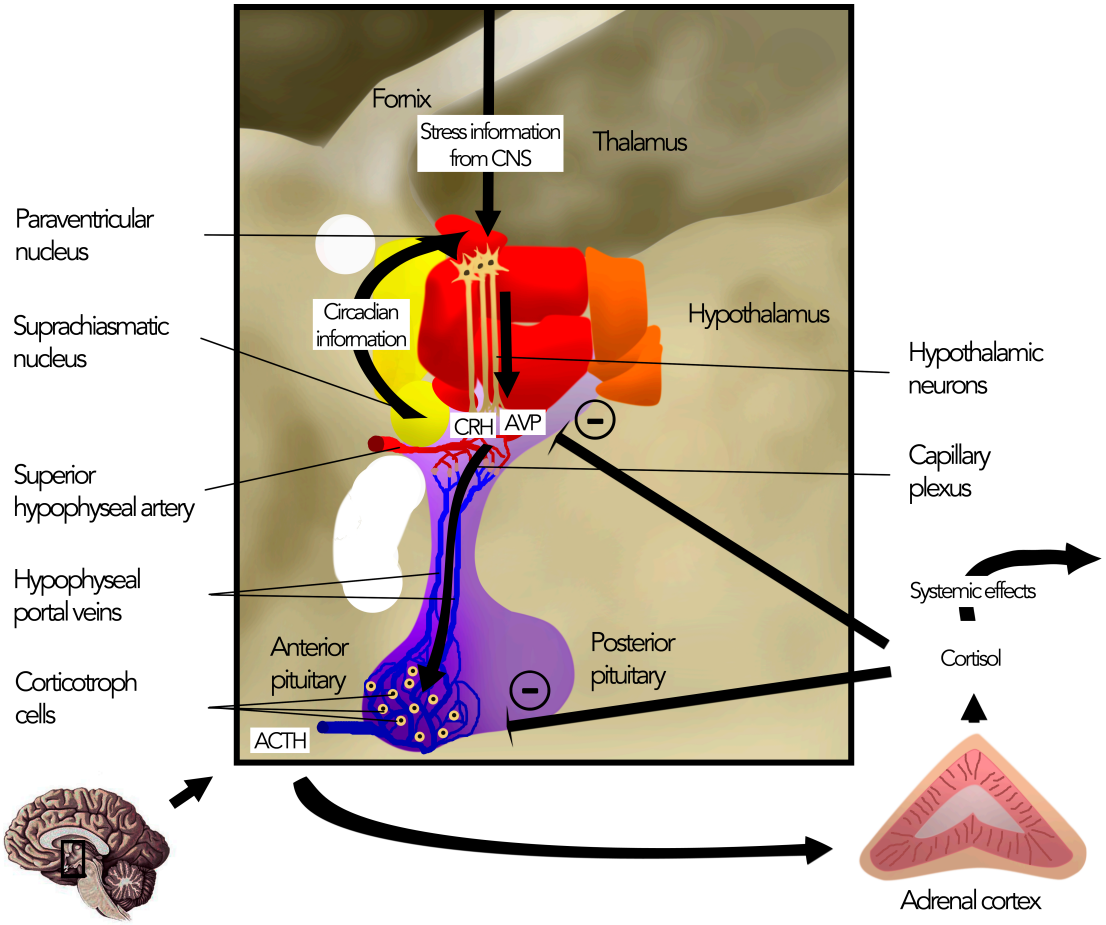


Figure 1.2: HPA axis diagram. Under circadian influences and stressors, corticotropin releasing hormone (CRH) is secreted from neurones originating at the paraventricular nucleus. Secretion of CRH is mainly into the capillary plexus of the hypophyseal portal system, travelling down the anterior pituitary through the hypophyseal portal veins and reaching the corticotroph cells. Adrenocorticotropin hormone (ACTH) is secreted from corticotroph cells in response to CRH and the synergistic action of arginine vasopressin (AVP). ACTH stimulates secretion of cortisol from the adrenal cortex, which will have systemic effects and a negative feedback into ACTH and CRH release.

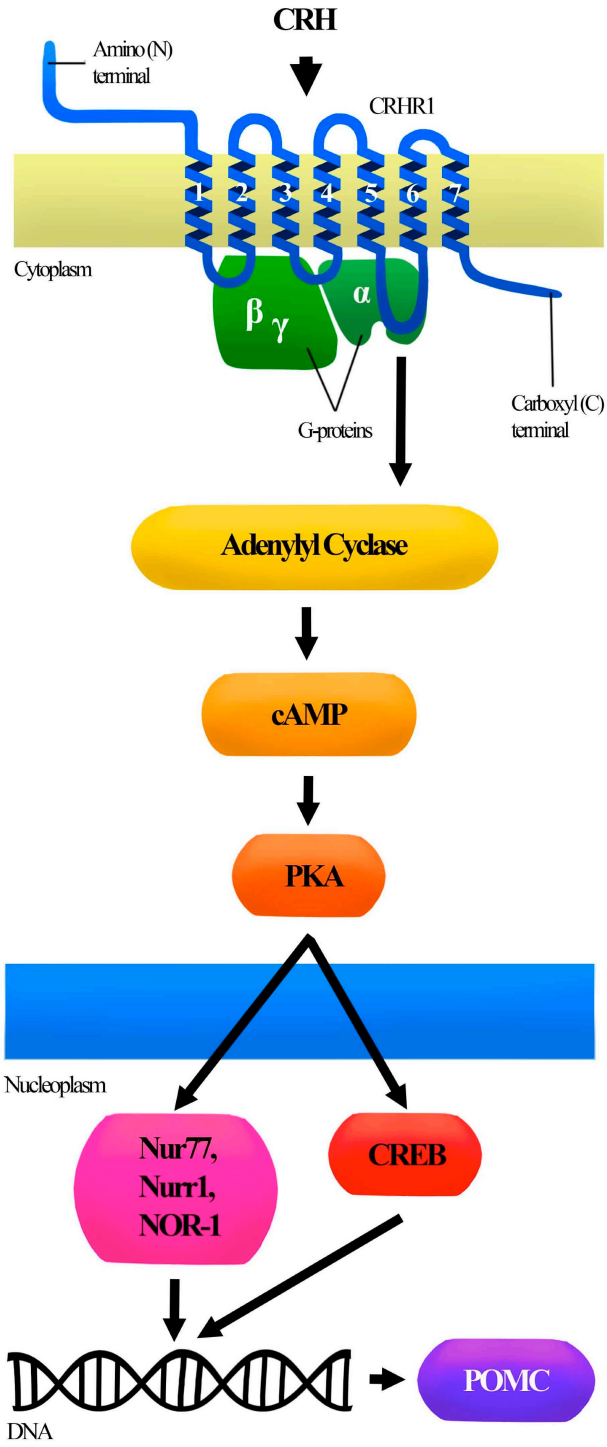


Figure 1.3: Mechanism of action of CRH on pituitary corticotrophs.

1.2.3 HPA axis components

1.2.3.1 Hypothalamus

A substantial portion of the field of neuroendocrinology is focused on the hypothalamic control over growth, development, reproduction and response to stress; a complex interaction between hormones and the nervous system that establishes and maintains homeostasis.

In humans, the hypothalamus is localized at the base of the forebrain, forming the walls and floor of the third ventricle. It is rostrally bounded by the optic chiasma and caudally by the midbrain tegmentum. Its central position in the brain favours the integration and processing of information from the forebrain, spinal cord, brain stem and other regions (Daniel 1976, Braak and Braak 1992).

In mammals and most vertebrates, the hypothalamus is involved in the control of blood flow, reproductive activity, body weight, food intake, metabolism, hepatic gluconeogenesis and response to threatening conditions.

Contextual information (originating from the cerebral cortex, amygdala and hippocampus) or sensory information (originating from visceral and somatic sensory pathways, chemosensory signals) received by the hypothalamus is compared to biological set points; and if homeostasis needs to be restored, neuroendocrine, visceral motor and somatic motor systems are activated accordingly (Brown-Grant and Raisman 1972, Daniel 1976, Grijalva and Novin

1990, Braak and Braak 1992, Coote 1995, Williams, Bing et al. 2001, Elmquist, Coppari et al. 2005, Lam, Pocai et al. 2005, Lam, Schwartz et al. 2005).

The hypothalamus contains a wide number of distinct nuclei, responsible for specific functions. These nuclei can be grouped into anterior, tuberal and latera-posterior regions (figure 1.4). The anterior group contains the suprachiasmatic nucleus that drives circadian rhythm from retinal input. This circadian information is also transmitted to the paraventricular nucleus, which then controls the HPA axis tone according the circadian cycle (figure 1.4) (Benarroch 2008).

In the same region, neurones distributed along the wall of the third ventricle secrete peptides able to regulate secretion of a variety of hormones by the anterior pituitary, via axons that project to the median eminence. The median eminence corresponds to junction between the hypothalamus and the pituitary, and the hypophysial portal system that feeds into the anterior portion of the gland (Daniel 1976, Flament-Durand 1980, Braak and Braak 1992) (figure 1.5).

The tuberal region is composed of the supraoptic nucleus and the paraventricular nucleus (PVN), whose axons extend to the posterior portion of the pituitary, and secrete the hormones oxytocin and vasopressin into the blood stream. Ventromedial and dorsomedial nuclei are also found in this region, and they are responsible for thermoregulation, water balance and reproductive behaviour, whose inputs originate from the limbic system and visceral sensory system (Daniel 1976, Flament-Durand 1980, Silverman, Hoffman et al. 1981, Braak and Braak 1992).

The PVN receives direct input from other hypothalamic regions as well as from the hippocampus, amygdala and portions of the cerebral cortex. Parvocellular neurosecretory cells from the PVN have axonal endings in the hypophysial portal

system, into which they release CRH, thyrotropin-releasing hormone (TRH), gonadotrophin releasing hormone (GnRH), vasopressin, oxytocin and neurotensin.

Other neurones from the PVN control sympathetic and parasympathetic divisions in the spinal cord and brainstem controlling visceral activity (Silverman, Hoffman et al. 1981, Ben-Jonathan, Arbogast et al. 1989, Toni and Lechan 1993, Tsigos and Chrousos 2002, Flak, Myers et al. 2014).

In mammals, neurones in the lateral-posterior region of the hypothalamus control behavioural arousal and attention to reproductive behaviours. The cells in this portion of the hypothalamus are not grouped into nuclei, but scattered among the fibers of the medial forebrain bundle, into structures such as mammillary body and posterior region (figure 1.3) (Brown-Grant and Raisman 1972, Mathews and Edwards 1977, Stolzenberg and Numan 2011). Overall, the hypothalamus plays a key role in regulating a wide array of physiological and behavioural functions.

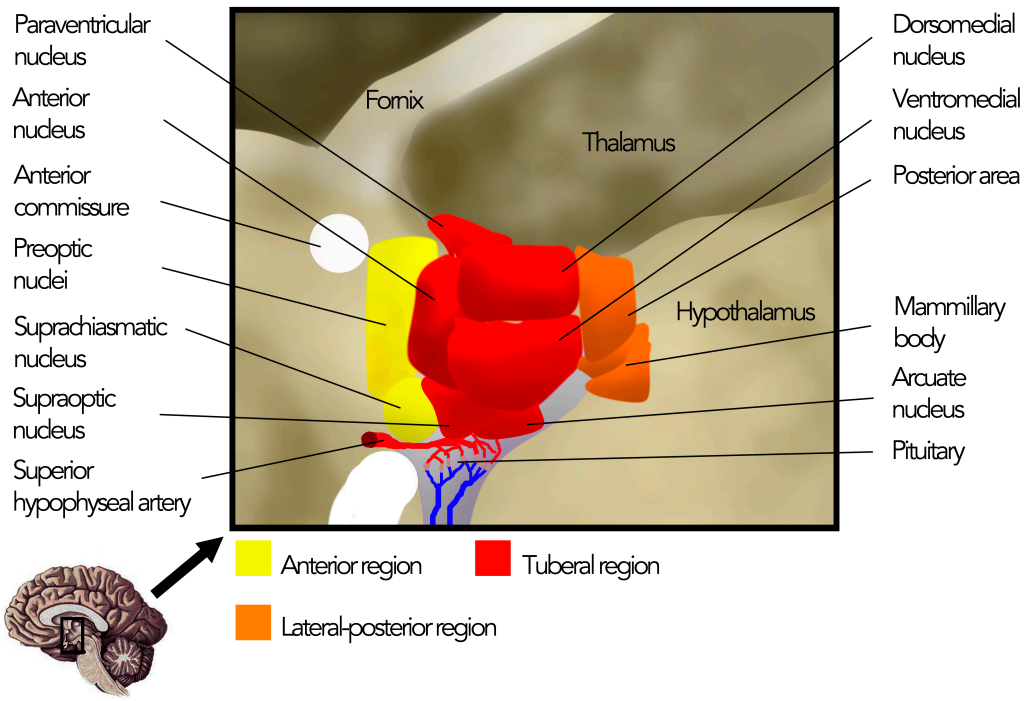


Figure 1.4: Schematic representation of hypothalamic anatomy. Main structures of the hypothalamus are colour-coded according to their region (anterior, tuberal and lateral-posterior).

1.2.3.2 Pituitary

In humans, the pituitary gland is divided into two portions: anterior and posterior pituitary. It is located at the center base of the skull, in a fibro-osseous compartment named hypophyseal fossa (figure 1.5). This gland is limited anteriorly, posteriorly, and inferiorly by bone formations named sella turcica. At the top of the gland, the diaphragma sellae has a variable size foramen named central aperture, which transmits the pituitary stalk and supply of blood (Kirgis and W 1972, Amar and Weiss 2003).

Developmentally, the pituitary gland, also known as hypophysis, originates from the Rathke's pouch in the bucco-pharygeal membrane and from a ventral

extension of the diencephalon, the infundibulum (Amar and Weiss 2003). In humans, the pituitary weighs around 100mg at birth, reaching 600mg in adulthood, with the female gland being around 20% heavier than the male one and being able to double its weight during pregnancy (Kirgis and W 1972).

The pituitary is regulated by the hypothalamus via axonal projections into the posterior pituitary, also known as neurohypophysis, and, by release of tropic hormones via the portal venous system, which irrigates the anterior pituitary. Portal veins run down the stalk into the parts distalis and pars tuberalis of the anterior pituitary, where hormones will be received, then capillaries will form the efferent laeral hypophyseal veins, which will carry the hormones originating into the anterior pituitary to the cavernous sinus following to the body (Amar and Weiss 2003).

The anterior pituitary, also known as the adenohypohysis begins functioning in the first trimester of pregnancy, expressing ACTH, β-endorphin, follicle stimulating hormone (FSH) and luteinizing hormone (LH) in the first weeks; whereas thyroid stimulating hormone (TSH), growth hormone (GH) and prolactin (PRL) start to be expressed at the second half of human gestation (figure 1.4) (Amar and Weiss 2003).

The posterior pituitary secretes oxytocin (OXY) and anti-diuretic hormone (ADH) (figure 1.5). These hormones are synthesized at the magnocellular neurones of the PVN and supraoptic nucleus of the hypothalamus, and then transmitted to the neurohypophysis via the hypothalamo-hypophyseal tracts, following release into the blood circulation (Amar and Weiss 2003, Clipperton-Allen, Lee et al. 2012).

In zebrafish as well as other teleosts, the pituitary structure is similar to mammals, except that the median eminence is shortened in fish (Lohr and

Hammerschmidt 2011). Besides the mammalian pituitary hormones listed above, the zebrafish pituitary will also secrete somatolactin.

Somatolactin is involved in gonadal maturation, blood acid-base balance, lipolysis control, gonadal steroidogenesis, and, in phosphate, sodium and calcium metabolism (Planas, Swanson et al. 1992, Rand-Weaver, Swanson et al. 1992, Rand-Weaver, Pottinger et al. 1993, Rand-Weaver and Swanson 1993, Zhu and Thomas 1995, Kakizawa, Kaneko et al. 1996, Mingarro, Vega-Rubin de Celis et al. 2002).

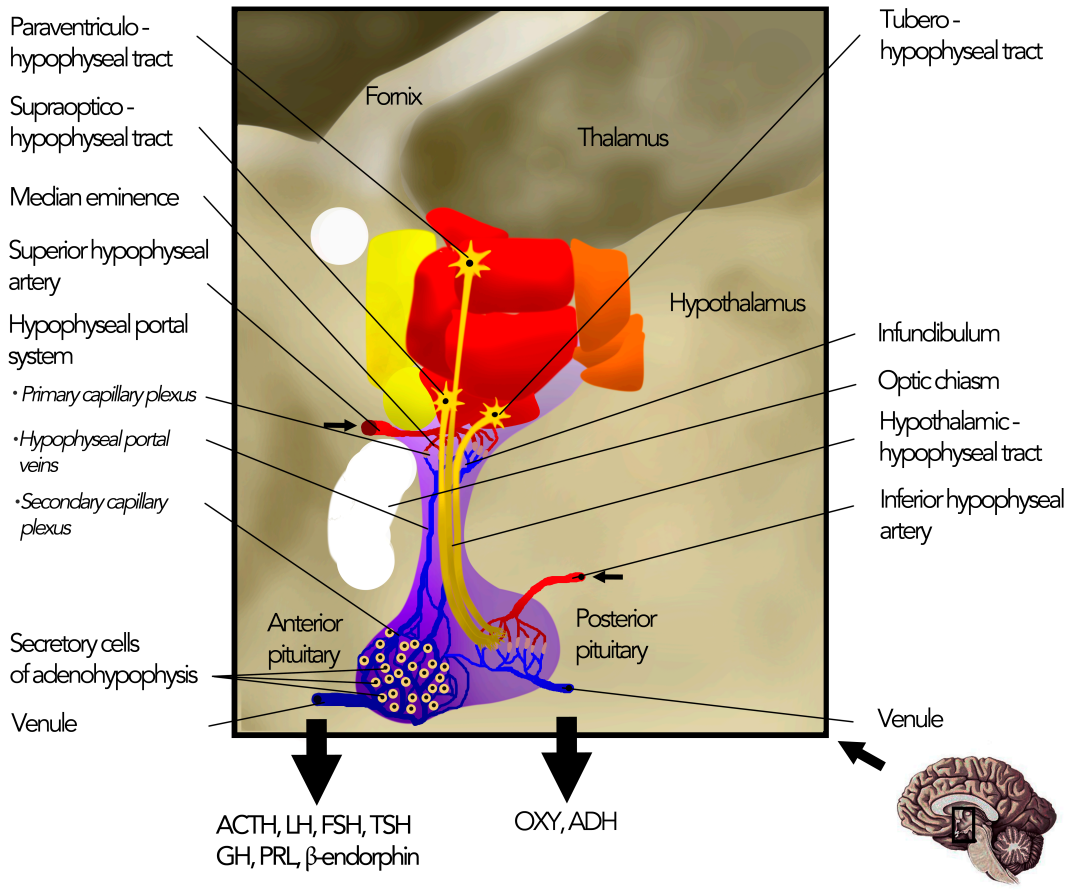


Figure 1.5: Schematic representation of pituitary anatomy and secreted hormones. Tracts connecting the hypothalamus to the posterior pituitary are also shown in the schematics (paraventriculo-hypophyseal tract, supraoptico-hypophyseal tract and tubero-hypophyseal tract). Hormones secreted by the anterior pituitary: adrenocorticotropin hormone (ACTH), β-endorphin, follicle stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH), growth hormone (GH) and prolactin (PRL). Hormones secreted by the posterior pituitary: oxytocin (OXY) and anti-diuretic hormone (ADH).

1.2.3.3 Adrenal glands/ Interrenal glands

In humans, the adrenal glands are endocrine glands located at the superior portion the kidneys and are responsible for releasing hormones in response to systemic stress. In mammals, this gland has two major components, the cortex and the medulla. The cortex, of mesodermal origin is responsible for releasing glucocorticoids, aldosterone and androgens; whereas the medulla, derived from the sympathetic nervous system, releases adrenaline and noradrenaline under sympathetic stimulation (figure 1.6) (Rosol, Yarrington et al. 2001).

Specialised cells in the medulla also secrete metenkephalin, neurotensin, neuropeptide Y and substance P. In mammals, the adrenal cortex is composed of three zones: zona glomerulosa (columnar cells that secrete aldosterone), zona fasciculata (polyhedral cells that secrete glucocorticoids) and zona reticularis (polyhedral cells that secrete glucocorticoids and androgens) (figure 1.5).

The gland is highly vascular, receiving blood from arterioles in the capsule which supply the extensive system of thinly walled sinuses penetrating the cortex. These in turn carry blood centripetally to the medulla. Blood charged with hormones leaves the gland via the medullary veins (Rosol, Yarrington et al. 2001).

The zebrafish adrenal glands equivalent are interrenal glands, located embedded to the anterior part of kidneys, with the right gland larger than the left one (Hsu, Lin et al. 2003). At 3dpf, interrenal cells are already enclosed by a capsule structure and already have steroidogenic potential, by possessing mitochondria with tubule-vesicular cristae (Hsu, Lin et al. 2003).

The organization of tissue types is different from mammalian adrenals. The interrenal cells are organized in layers of epithelial cells responsible for

steroidogenic production, with chromaffin cells interposed with these epithelial cells, which are responsible for adrenaline and noradrenaline production (figure 1.7). The tissues are associated with the posterior cardinal veins, responsible for irrigating and transporting hormones in this tissue (Hsu, Lin et al. 2003, Takahashi, Kobayashi et al. 2013).

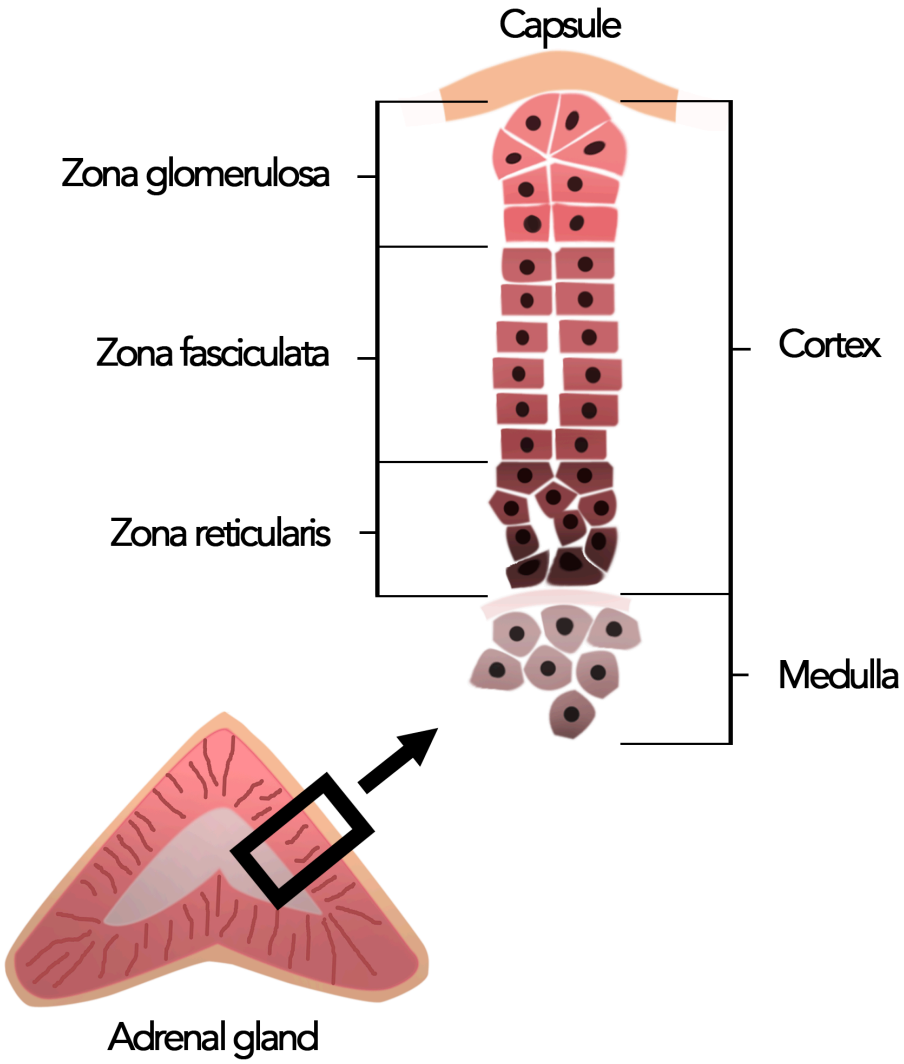


Figure 1.6: Schematic diagram of the human adrenal gland, depicting the cortex tissue organization (zona glomerulosa, zona fasciculata and zona reticularis) as well as the medulla.

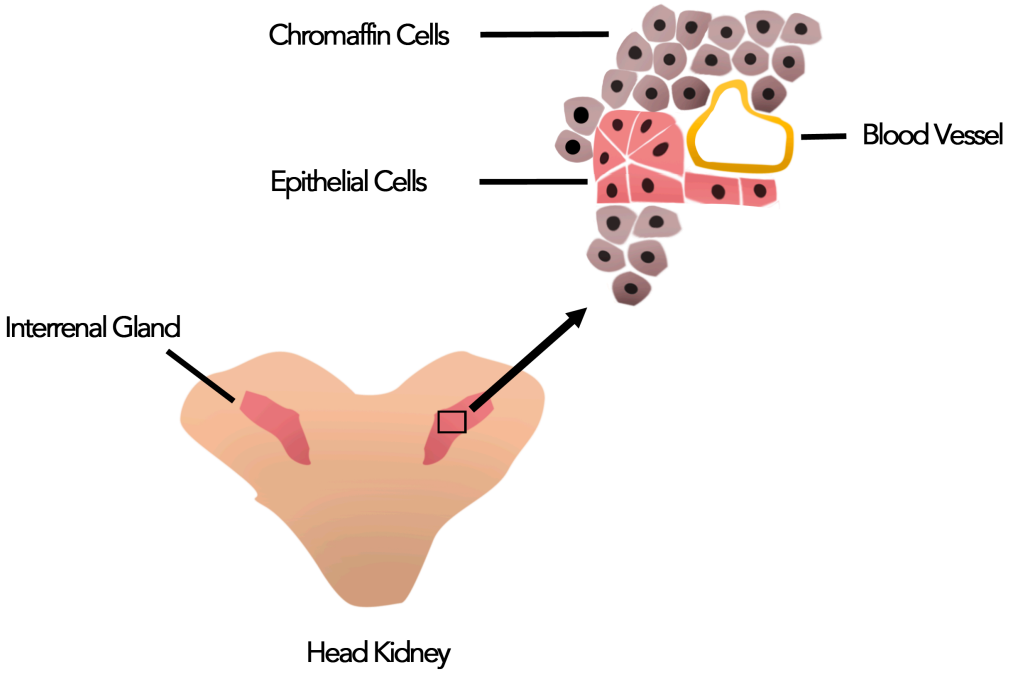


Figure 1.7: Schematic diagram of the zebrafish head kidney and the interrenal gland, depicting the tissue organization (chromaffin cells and epithelial cells).

1.2.4 HPA axis main neurotransmitters, hormones and receptors

1.2.4.1 Corticotropin releasing hormone (CRH)

In 1955, it was demonstrated that extracts from the hypothalamus were able to stimulate ACTH release. It was not until 1981 that the active compound was purified and characterized from ovine hypothalamic (Vale el al., 1981). CRH is a 41-amino acid peptide that has an important role in the adjustment of autonomic, neuroendocrine and behavioural adaptations to perceived stress (figure 1.8).

External environmental stress and disturbances to internal homeostasis are conveyed and processed at the central nervous system (CNS) and transmitted to the Hypothalamus. As mentioned earlier, parvocellular neurones from the paraventricular nucleus (PVN) secrete CRH in response to the stress inputs conveyed by the CNS. CRH activates adrenocorticotropin (ACTH) secretion from corticotroph cells residing at the anterior pituitary gland, which in turn will stimulate corticosteroid release from the adrenal gland (Smith et al., 2006).

CRH secreting neurones are stimulated by a large number of neuronal circuits, such as the limbic pathways carrying information from visceral and sensory stimuli via the brainstem pathways (de Kloet, Joels et al. 2005).

CRH is also widely distributed in other extra-hypothalamic circuits in the CNS. Antibody staining revealed distribution of CRH in the paraventricular nucleus and in the pituitary of adult rats as well as expression in the basal telencephalon and regions of the brain stem, responsible for autonomic responses (Swanson, Sawchenko et al. 1983). CRH was also expressed throughout the majority of the cerebral cortex, in interneurones, residing in the limbic regions such as the prefrontal cortex, cingulate gyrus and rhinal fissure (Swanson, Sawchenko et al. 1983).

In adult zebrafish, CRH mRNA expression is widespread throughout the forebrain, though focused more in the dorsal and ventral telencephalon, supracommissural nuclei and dorsal endopenduncular. In the midbrain, CRH is expressed in the preoptic area, parvocellular nucleus, magnocellular nucleus, suprachiasmatic nucleus, ventromedial thalamic nucleus, all the zones of the periventricular hypothalamus, dorsal posterior thalamic nucleus, and periventricular

optic tectum. Hindbrain CRH mRNA expression was only detected in the dorsal part of the trigeminal motor nucleus (Alderman and Bernier 2007).

Neurones expressing CRH in the neocortical limbic structures are responsible for other behaviours than stress activation, such as energy balance, food intake, weight gain, approach and avoidance behaviours, sleep patterns, memory consolidation, and, sexual and arousal behaviours (Brown-Grant and Raisman 1972, Grijalva and Novin 1990, Coote 1995, Cullen, Ling et al. 2001, Roozendaal, Brunson et al. 2002, Bale and Vale 2004, Heinrichs and Koob 2004).

CRH also acts peripherally, stimulating colonic motility through its actions on CRHR1 primarily and CRHR2, both found in the lining of the gastro-intestinal tract (Maillot, Million et al. 2000).

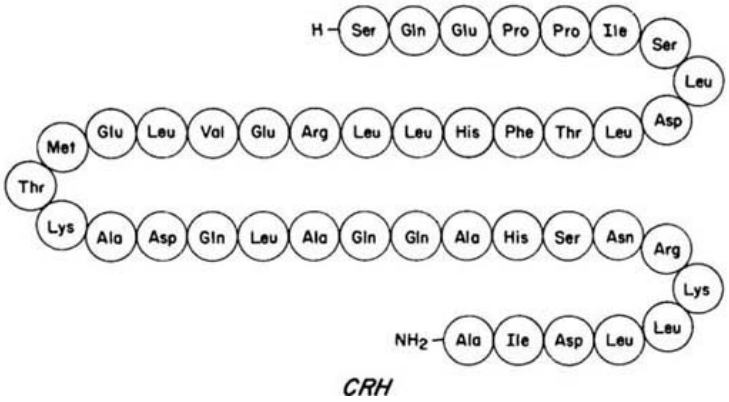


Figure 1.8: Human CRH primary structure. Reproduced from Stephen's physiology handbook (Sagar and Martin. 2011).

1.2.4.2 Corticotropin releasing hormone receptors (CRHR1 and CRHR2)

Both CRH receptors are class 2 (or B) subtype of G-protein-coupled receptors (GPCRs), encoded by different genes and sharing about 70% amino acid identity.

The third intracellular loop, responsible for the interaction with the G-proteins in most GPCRs is identical in all CRH receptors subtypes and isoforms (figure 1.9) (Perrin and Vale 1999, Arai, Assil et al. 2001).

These receptors predominantly activate adenylyl cyclase (AC) through the G-protein Gs, although other four G-proteins (Gi, Gq/11, Go and Gz) are activated by CRH, thus stimulating at least two distinct intracellular signaling cascades (Grammatopoulos, Randeva et al. 2001).

CRHR1 has seven subtypes: α, β, c, d, e, f, g and h, which have been detected in rodent and human tissues (Grammatopoulos and Chrousos 2002). The majority of the isoforms are non-functional; in binding properties or signaling functions (Grammatopoulos and Chrousos 2002). CRHR2 has three functional subtypes: α, β, and γ, (Dautzenberg and Hauger 2002).

CRHR2α and CRHR2β have been detected in both humans and rodents, but CRFR2γ exclusively detected in humans (Liaw, Lovenberg et al. 1996, Kostich, Chen et al. 1998, Kostich, Chen et al. 1998). Like the majority of teleosts, zebrafish possess two CRH receptor subtypes, CRHR1 and CRHR2 (Alderman and Bernier 2009).

Salmon is one of the few teleost species in which the receptors' amino acid identity has been compared to humans and other species. Salmon CRHR1 displays 82%, 83%, 81% identity, respectively to human, rat, and toad CRHR1. Salmon CRHR2 has 78 – 82%, 78 – 80%, 84% and 88% identity, respectively to the human (CRHR2α, CRHR2β and CRHR2γ), rat (CRHR2α and CRHR2β) and toad CRHR2 (Pohl, Darlison et al. 2001).

In our research, the protein sequence of zebrafish CRH receptors identity relative to humans and rats was compared. Zebrafish CRHR1 displays 82% and

88% identity to human and rat CRHR1α respectively, whereas zebrafish CRHR2 displays 77% and 84% identity to human and rat CRHR2α respectively (figure 1.10).

In catfish, a third CRH receptor has been identified, CRHR3, and it is the predominant CRH receptor type in the pituitary, which suggests it could be closely linked to POMC production in catfish (Arai, Assil et al. 2001). A phylogenic tree of the salmon CRHR1 and CRHR2 has been drawn to illustrate the similarity to human, rat, toad and catfish CRH receptors (figure 1.11).

The extracellular N-terminal domains (NT) (second and third), and, the N-terminus juxtamembrane regions are important in determining the specificity of ligand binding interaction (Assil, Qi et al. 2001, Hoare, Sullivan et al. 2005, Klose, Fechner et al. 2005). CRH is a high-affinity ligand for CRHR1, but has low affinity for CRHR2, which in turn binds urocortin II (UcnII, stresscopin-related peptide) and urocortin III (UcnIII, stresscopin) with higher affinity (Hauger, Risbrough et al. 2006).

Data from mice studies revealed that CRHR1 is highly expressed throughout the cerebral cortex, cerebellum, olfactory bulb, amygdala, hippocampus, medial septum, hypothalamic nuclei, anterior pituitary, neocortex, Purkinje cells, lateral dorsal tegmentum and in the limbic system, where it is involved in the feedback regulation of the HPA axis (Potter, Sutton et al. 1994, Muller, Zimmermann et al. 2003).

On the other hand, CRHR2 has a more limited expression compared to CRHR1 in the nervous system, appearing in the lateral septum, cortical nucleus of the amygdala, ventromedial hypothalamus and hippocampus, but it is widely

expressed in peripheral tissues, such as lung tissue, gastrointestinal, heart and skeletal muscle (Stenzel, Kesterson et al. 1995, Reul and Holsboer 2002).

Both receptors are equally expressed in the hippocampus. This diversification in CRH receptor expression throughout the brain is thought to mediate the different actions that CRH has in the CNS (Reul and Holsboer 2002, Reul and Holsboer 2002).

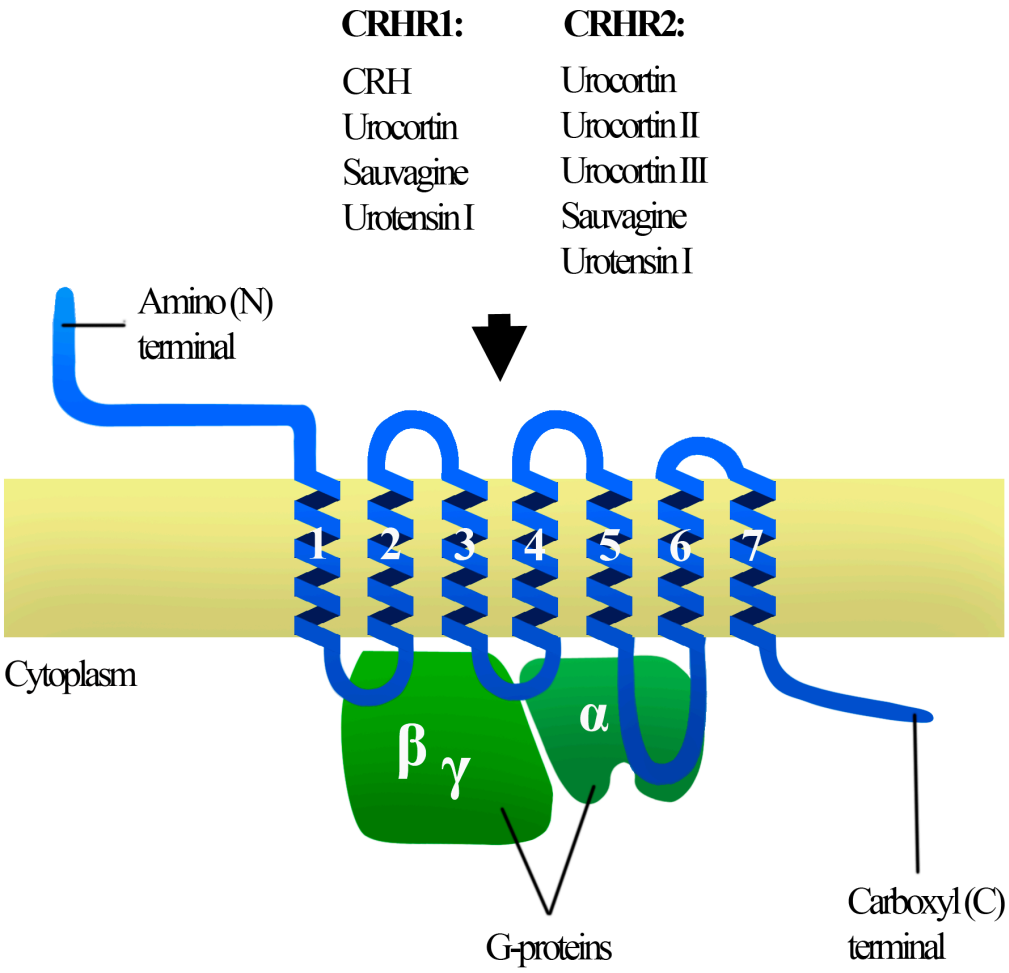


Figure 1.9: Schematic representation of human CRHR1 and CRHR2 secondary structure and their specific agonists. Transmembrane domains numbered 1-7 (blue) and G-proteins (green) also represented.

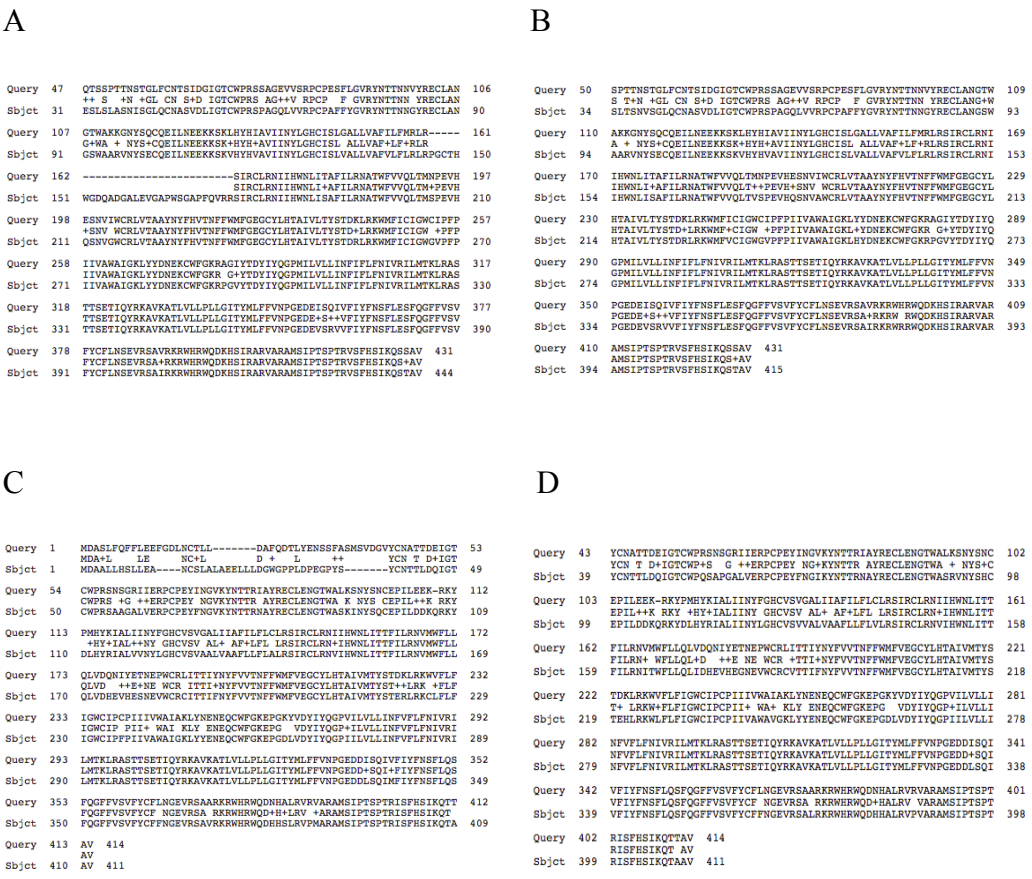


Figure 1.10: Protein amino acid identity comparison of zebrafish CRHR1 against human CRHR1α (A) and rat CRHR1α (B), and, zebrafish CRHR2 against human CRHR2α (C) and rat CRHR2α (D). Query represents zebrafish receptors' amino acid sequence and subject represents either human or rat receptors' amino acid sequence depending on the picture. Zebrafish CRHR1 displays 82% and 88% identity to human and rat CRHR1α respectively, and, zebrafish CRHR2 displays 77% and 84% identity to human and rat CRHR2α respectively. Protein alignment pictures obtained using BLAST alignment web software.

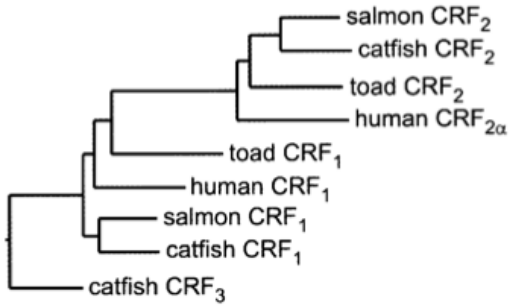


Figure 1.11: Phylogenic tree of CRH receptors. Salmon CRHR1 (CRF_1) and CRHR2 (CRF_2) similarity to human, rat, toad and catfish CRH receptors, generated using DNAstar software. Reproduced from Sigrun (Pohl, Darlison et al. 2001).

1.2.4.3 Corticotropin releasing hormone binding protein CRHBP

In humans, CRHBP is a 37-kDa N-linked glycoprotein composed of 322 amino acids, which has the ability to bind all members of the CRH ligand family (Cortright, Nicoletti et al. 1995, Seasholtz, Valverde et al. 2002). Protein and mRNA expression studies revealed CRHBP is mainly expressed in the cerebral cortex, amygdala, bed nucleus, stria terminalis, trigeminal systems, hypothalamus (dorsomedial and premammilary nuclei) and pituitary corticotrophs in rats (Potter, Behan et al. 1992).

In rats, dual staining revealed co-localized expression of CRH and CRHBP in the hippocampal formation (detate gyrus and Ammon's horn), olfactory bulb, septal nucleus, medial preoptic area, amygdala, suprachiasmatic nucleus, lateral dorsal tegmental nucleus and anterior pituitary corticotrophs (Potter, Behan et al. 1992).

Zebrafish CRHBP is expressed mainly in the preoptic area, periventricular hypothalamic and tectal regions, dorsal part of the trigeminal motor nucleus, olfactory bulbs, superior raphe nucleus and ventral nucleus of the ventral telencephalon, co-localized with CRH expression (Alderman and Bernier 2007). Human CRHBP has also been found in the plasma, placenta, fetal membranes, myometrium, endometrium, amniotic fluid and cervix (Klimaviciute, Calciolari et al. 2006).

By producing various fragments of human CRH and testing its affinity with CRHBP, it was found that CRH residues 9-28 are essential for binding (Sutton, Behan et al. 1995). CRHBP will bind with high affinity with human and rat CRH, urocortin I, sauvagine, and, will bind to low affinity to ovine CRH, however, when amino acids 22, 23 and 25 in ovine CRH are replaced by the same amino acids in

the human CRH structure, the binding affinity is increased (Sutton, Behan et al. 1995).

CRHBP dimerizes under certain circumstances, but the ligand binds exclusively as a monomer. By using photaffinity labelling in combination with mass spectrophotometry, amino acids Arg-23 and Arg-36 of the CRHBP were shown to be involved in ligand binding (figure 1.12) (Jahn, Eckart et al. 2002).

The protein has a disulfide arrangement, essential for the biding activity, composed of 10 cysteine residues forming five consecutive disulphide loops; which suggests that its highly folded structure is required for activity (Fischer, Behan et al. 1994).

The role of this binding protein is not entirely understood. Most researchers believe it plays an inhibitory role in the CRH signalling pathway, supported by numerous *in vitro* and *in vivo* studies. It binds to CRH and other CRH-like ligands with high affinity, sequestering the ligand and inhibiting its actions on the receptor; at synaptic and systemic levels, this will reduce CRH induced ACTH release, preventing inappropriate pituitary-adrenal stimulation, such as plasma CRH derived from the placenta during pregnancy (figure 1.13) (Potter, Behan et al. 1991).

Pituitary cell cultures transfected with vectors containing human and rat recombinant CRHBP decreases CRH-induced ACTH release over time (Potter, Behan et al. 1991). Other studies have also confirmed CRHBP-related inhibition of CRH induced ACTH release, including in teleosts (Turnbull and Rivier 1997, Riedel, Schlapp et al. 2002, Huising, Vaughan et al. 2008, Manuel, Metz et al. 2014).

CRHBP is also upregulated in the tail of tadpoles during metamorphosis, possibly to downregulate the thyroid and adrenal axes, or, to sequester CRH as it

might play role in supporting proliferation of tail cells, or, preventing tail cells' apoptosis (Brown, Wang et al. 1996).

Another theory is that CRHBP has the ability to be part of a signalling cascade when bound to CRH-related peptides. Intra-cerebroventricular administration of human/rat corticotropin-releasing factor (h/rCRH6-33), a specific ligand for CRH-BP, activates Fos expression in the isocortex, olfactory system, amygdala and brainstem (Chan, Vale et al. 2000).

A transgenic mice line that overexpresses CRHBP specifically in the pituitary, resulted in an 82% increase in CRH mRNA expression, apparently compensating for the abundance of CRHBP. The animals also had normal ACTH and glucocorticoid levels (Burrows, Nakajima et al. 1998). Another transgenic line of CRHBP deficient mice has normal ACTH and glucocorticoid levels, but has increased anxiogenic-like behaviour (Karolyi, Burrows et al. 1999). This further supports the idea the CRHBP has an inhibitory role.

Other possibilities are that CRHBP works as a clearance factor, terminating the activity of CRH and CRH-like ligands, or, that CRHBP increases the half-life of CRH, delivering it to the receptors, in that way acting as an enhancing agent. One last possibility is that the primary function of CRHBP is to signal via its own membrane receptor, since the majority of CRHBP in human and rat brain is bound to membrane, but this receptor has not been identified (Behan, De Souza et al. 1996).

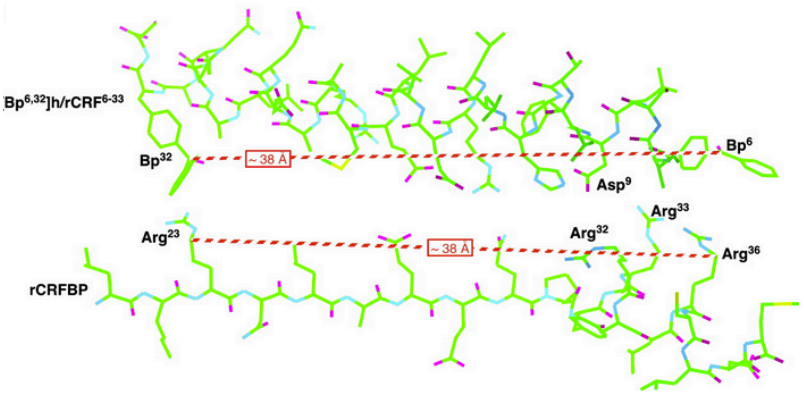


Figure 1.12: Schematics of CRH and CRHBP structure interaction. Frame model of photphore-containing human/rat CRH ([Bp6,32]h/rCRF6–33, above) interacting with the stretch of amino acids 20–40 of rat CRHBP (rCRFBP, bellow). Reproduced from Olaf (Jahn, Eckart et al. 2002).

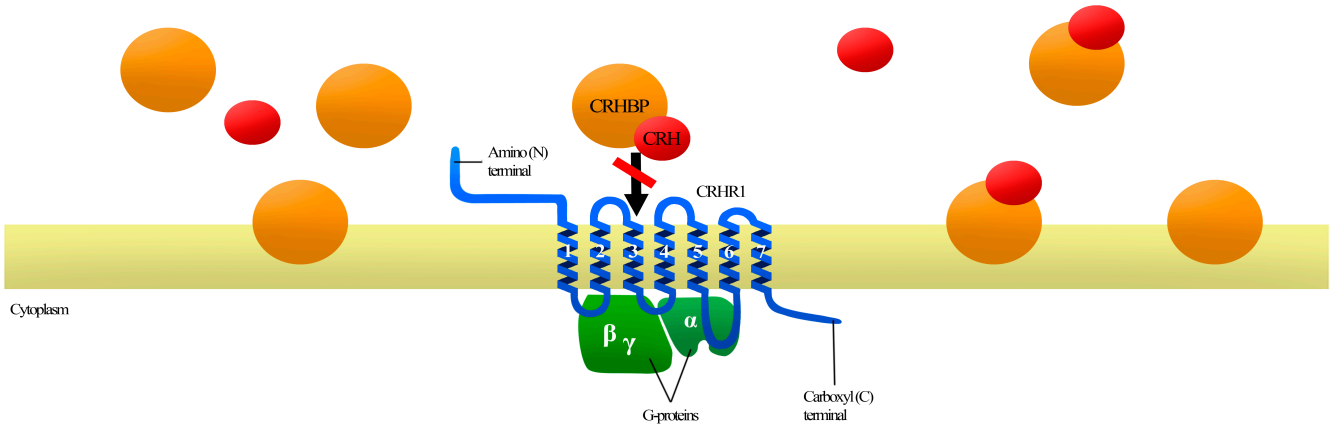


Figure 1.13: Schematics diagram of proposed CRH regulation by CRHBP on human corticotrophs. CRHBP binds to extracellular CRH and sequesters it, inhibiting CRH from binding to CRHR1 on pituitary cells.

1.2.4.4 Glucocorticoid receptor (GR)

Glucocorticoids are steroids capable of freely diffusing into the cytoplasm of target cells and bind to two distinct receptors: glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). In humans, GR is widely expressed in all tissues of the body with low affinity to endogenous glucocorticoids (10-fold lower then MR), therefore needing high concentrations of intracellular glucocorticoid to be fully activated (De Kloet, Vreugdenhil et al. 1998). Both receptors, GR and MR contribute to negative feedback of the HPA axis (Juruena, Cleare et al. 2006).

The human GR receptor is composed of either 777 (GRα) or 742 (GRβ) amino acids. GRα is the most abundant isoform. GRβ is non-ligand binding, but in some cases it may heterodimerise with ligand-bound GRα and thus inhibit its actions at the nucleus (de Castro, Elliot et al. 1996).

The transcriptional activation function 1 (AF1) of GR resides in the first 421 amino acids of the protein at the N-terminus, followed by 65 amino acids that make up the zinc finger DNA-binding domain (DBD), responsible for the dimerization of GR, DNA binding specificity and co-factor interactions.

The 250 amino acids in the C-terminus of the GR make up the ligand-binding domain (LBD) and the motif for transcriptional activation function 2 (AF2), also implicated in being involved in protein-protein interactions with other co-regulators (Hollenberg, Weinberger et al. 1985, Giguere, Hollenberg et al. 1986, Zhou and Cidlowski 2005).

In the absence of glucocorticoids in the cytoplasm, GR is bound into multiprotein heterocomplexes consisting of heatshock proteins, in the majority of cases Hsp90 and Hsp70, and, immunophilins, such as FKBP5, Cyp55, PP5, which

interact directly with the hormone binding domain (HBD) repressing the receptor in the absence of glucocorticoid (Sanchez, Toft et al. 1985, Pratt and Toft 1997).

Upon binding of glucocorticoid, conformational change in the receptor structure induces the dissociation of the heatshock proteins and immonophilins, and dimerization of the GR, association with nuclear co-activators and translocation to the nucleus takes place (Fig. 1.14) (Bledsoe, Montana et al. 2002).

Once activated, GR dimers bind to GREs in the promoter region of target genes and activate gene transcription. GR monomers can also bind to other nuclear factors and become transcriptionally inactive, in a process called transrepression. Studies pointed transrepression of GR occurring upon binding to nuclear factor kappa B (NFkB), Fos transcription facrors (not FosB), cyclic AMP response element (CREB), activator protein 1 (AP-1) and interferon regulatory factor 3 (IRF3) (Lucibello, Slater et al. 1990, Heck, Kullmann et al. 1994, McKay and Cidlowski 1998, Focking, Holker et al. 2003, De Bosscher and Haegeman 2009).

Some target genes mediated by the transrepression of GR include inflammatory cytokines, that will lead to the immunosuppressive properties of glucocorticoids, such as interleukins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-18), interferon-γ (IFN-γ), tumor necrosis factor α (TNFα), cyclooxygenase-2 (COX-2), intracellular adhesion molecule (ICAM), monocyte chemottractant protein 1 (MCP-1) and inducible NO-synthase (iNOS) (De Bosscher and Haegeman 2009). GR transrepression will also target the reduction in cell proliferation by glucocorticoids, by directly inhibiting the c-*jun* promoter when GR is associated with AP-1 (Wei, Inamdar et al. 1998).

Repression of gene transcription may also occur by GR dimer binding to negative GREs (nGREs). There are a number of cases of transcription repression,

but one of the most important ones is the repression of CRH gene transcription by glucocorticoid-dependent negative feedback at the hypothalamus. Mouse studies show that nGRE can mediate transcription activation by both cAMP and AP-1, or, transcriptional repression by glucocorticoids, by directly binding to GR as well as AP-1 or other related nucleoproteins (Malkoski and Dorin 1999).

A third mode of action of GR is through simultaneous interaction on DNA elements and other transcription factors. This dual interaction can lead to up-regulation or down-regulation of transcription. Toll-like receptor 2 (TLR2) is induced by treatment with dexamethasone and TNFα, causing protein-protein interaction between NFkB, STAT and GR, which then act synergistically on the promoter of the TLR2 gene (Hermoso, Matsuguchi et al. 2004).

In summary, GR can activate or repress a gene transcription by directly binding to the GRE, establishing a protein-protein interaction with nuclear transcriptional factors, or, by doing both tasks at the same time (Fig. 1.15).

GR is widely expressed in the brain, with the highest densities of cells expressing GR mRNA found in the cerebral cortex, olfactory cortex, amygdala, hippocampal formation, dorsal thalamus, hypothalamus, cerebellar cortex, nucleus raphe and locus coeruleus (Morimoto, Morita et al. 1996).

In humans, GRα mRNA abundance ($\times 10^6$ cDNA copies/μg total RNA) was as follows: brain (3.83 ± 0.80) > skeletal muscle > macrophages > lung > kidney > liver > heart > eosinophils > peripheral blood mononuclear cells (PBMCs) > nasal mucosa > neutrophils > colon (0.33 ± 0.04), and, GRβ mRNA was more scarce compared to GRα mRNA expression, ($\times 10^3$cDNA copies/μg total RNA) eosinophils (1.55 ± 0.58) > PBMCs > liver ≥ skeletal muscle > kidney > macrophages > lung >

neutrophils > brain ≥ nasal mucosa > heart (0.15 ± 0.08) (Pujols, Mullol et al. 2002).

Both GR isoforms have been detected in zebrafish. Immunohistochemistry and in situ hybridization revealed no spatial restriction on GRα and GRβ protein and mRNA expression, therefore being expressed in the whole body of 24-hour-old zebrafish embryos (Schaaf, Champagne et al. 2008, Schaaf, Chatzopoulou et al. 2009). There have been no GR localization studies in zebrafish brain.

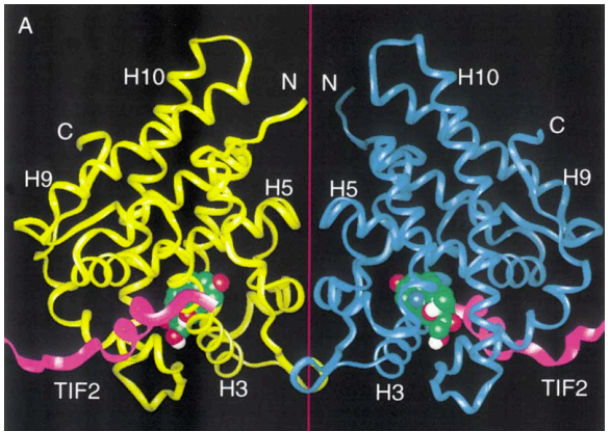


Figure 1.14: Arrangement of the GR lidang-binding domain dimer (yellow and blue ribbons) upon binding to the synthetic glucocorticoid dexamethasone (molecule: carbon, oxygen, and hydrogen colored in green, red, and white, respectively) and to the nuclear co-activator TIF2 (purple ribbon). The C2 symmetry axis shown in red. Reproduced from Randy (Bledsoe, Montana et al. 2002).

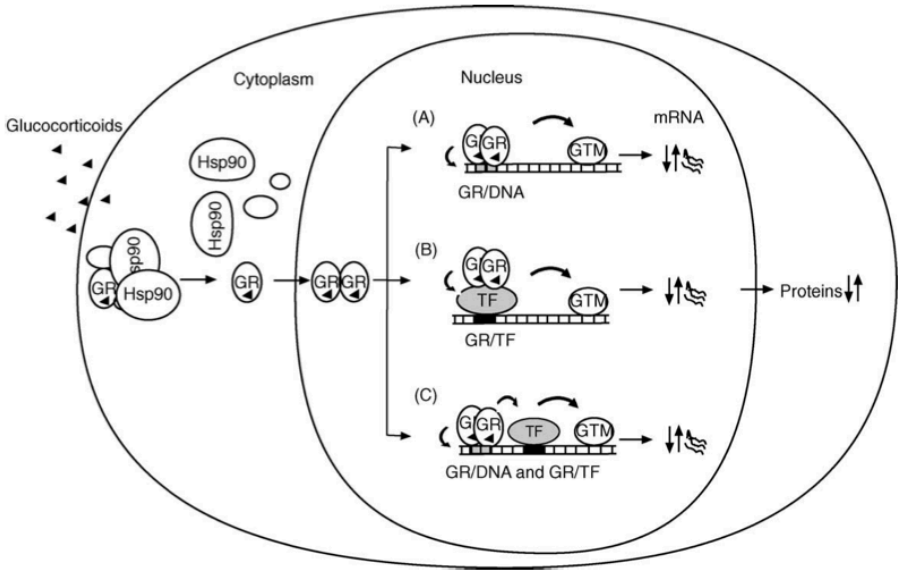


Figure 1.15: Signaling pathways of GR-mediated transcriptional regulation. Glucocorticoid binding leads to chaperone protein dissocation of the cytosolic GR and translocation to the nucleus. GR readily dimerizes and modulates gene target gene transcription by interaction with GREs and nGREs (A), protein-protein interaction such as AP-1 and NFkB (B), or both (C) resulting in modulation of the target gene expression by the general transcriptional machinery (GTM). Reproduced from Jungou (Zhou and Cidlowski 2005).

1.2.4.5 Mineralocorticoid receptor (MR)

Mineralocorticoid receptors (MR) were differentiated from GR when adrenalectomized rats injected with [^{3}H]corticosterone showed radioactive labeling in pyramidal and granular regions of the hippocampus. Rats injected with [^{3}H]dexamethasone showed labeling of other regions other than the hippocampus, staining the brain more broadly and concentrating more in the pituitary.

This indicated that both radioactive steroids were binding to different receptors. Then, [^{3}H]aldosterone labeling was retained in the hippocampus, hypothalamus and amygdala, similar to [^{3}H]corticosterone results, further confirming the presence of two distinct corticosteroid receptors in the brain (De

Kloet, Wallach et al. 1975, Birmingham, Stumpf et al. 1979, De Nicola, Tornello et al. 1981).

As mentioned in the GR section, MR has a 10-fold higher affinity for glucocorticoid compared to GR. Under low plasma corticosterone concentrations (<1.5 μg/100mL), MR receptors in rat brain are 80% occupied, whereas GR receptors are only 10% occupied. GR 50% occupancy only occurs under high corticosterone plasma concentrations (>10 μg/100mL) (Reul and de Kloet 1985).

In the rat brain, *in vitro* autoradiography studies revealed that MR receptor sites are almost entirely restricted to the septal-hippocampal complex, with the highest levels of MR observed in the granular layer of dentate gyrus and in the pyramidal layer of CA1 and CA2; with CA3 expressing lower MR density. All pyramidal cells (CA1-4) and granular neurones of the dentate gyrus expressed MR mRNA, while GR mRNA expression was restricted to CA1-2 and the dentate gyrus (Reul and de Kloet 1985, Reul and de Kloet 1986, Van Eekelen, Jiang et al. 1988).

Antibody staining of MR further confirmed the pattern observed by Reul and de Kloet, where the highest MR concentration is located at pyramidal neurones of CA1 and CA2, and, lower concentration the CA3 cell field (Reul, Gesing et al. 2000). MR is also expressed peripherally, in the kidney, colon, sweat and salivary glands, leukocytes, large blood vessels such as aorta and in the heart (Zennaro, Keightley et al. 1995).

Three isoforms of MR are known, MRα, MRβ and MRγ (rats only). All isoforms are found functionally in the hippocampus but MRα mRNA is the only one to be modulated under different stressful conditions at the rat hippocampus (Zennaro, Keightley et al. 1995).

Since 80% of MR are occupied at all stages of the normal diurnal glucocorticoid secretory pattern, it has been suggested that MR mediates a tonic inhibitory control of the HPA axis via the hippocampus. Treatment with the MR selective antagonist RU28318 increased basal corticosterone and ACTH levels in unstressed rats, which verifies this tonic inhibitory control (Ratka, Sutanto et al. 1989, Oitzl, van Haarst et al. 1995, De Kloet 2004).

Rats treated with RU28318 also had increased corticosterone levels when mildly stressed by being moved to a novel environment (Ratka, Sutanto et al. 1989), but not when restraint stress was used, suggesting MR is involved in the inhibitory process of the HPA axis during mild stress, but not (or to a lesser extent) during more robust stress (Pace and Spencer 2005).

MR are also dynamically controlled, able to adapt to different stressful conditions. Radiography assays revealed an increase in hippocampal MR concentration following intra-cerebroventricular CRH injection in adrenalectomized rats, but CRH antagonist injection caused no difference in MR levels after forced swimming stress (Gesing, Bilang-Bleuel et al. 2001).

MR is highly expressed in the hippocampus, and aldosterone activation of MR induces long-term potentiation (LTP), long-term memory formation and hippocampal plasticity in rats (Pavlides, Kimura et al. 1994). Cortisol, deoxycorticosterone and corticosterone are also able to modulate MR-related hippocampal LTP (Filioini, Gijsbers et al. 1991, Lupien, de Leon et al. 1998).

Like all members of nuclear receptor family, MR has a N-terminal domain (NTD), a DNA-binding domain (DBD) and a ligand-binding domain (LBD). Upon ligand binding, MR translocates to the nucleus, dimerises and binds to hormone

response elements (HRE). Similar to GR, MR is transrepressed by NFkB (Webster and Cidlowski 1999).

A non-genomic effect of aldosterone was found in rabbit cardiomyocytes. In those cells, aldosterone elevates $Na^+/K^+/2Cl^-$ co-transporter activity in less than 15 minutes, thus raising sodium intracellular concentration, and lowering Na^+/K^+ pump activity. Potassium canrenoate (aldosterone antagonist), and, MR antagonists, RU28318 and E-ring blocked this effect, indicating the participation of aldosterone and MR in this rapid response. This effect is also associated with PKCε, which is abolished when the latter is inhibited (Mihailidou, Mardini et al. 2004, Mihailidou and Funder 2005).

MR has also an important function in sodium homeostasis in epithelial tissues, regulated by aldosterone. Aldosterone bound to MR regulates the transcription of serum-regulated kinase and glucocorticoid-regulated kinase, which phosphorylates the ubiquitin ligase Nedd4-2, rendering it unable to bind to sodium channels, thus increasing the sodium influx in epithelial tissues (Fuller and Young 2005). This was further observed when MR knockout mice had a salt-wasting phenotype (Berger, Bleich et al. 1998).

1.2.4.6 Pro-opiomelanocortin (POMC)

The first evidence of a precursor molecule to ACTH was obtained in 1971, when a immunoreactive molecule to ACTH, of higher molecular weight than expected, was discovered (Yalow and Berson 1971). This molecule was later named

pro-opiomelanocortin, in recognition of its role as a precursor molecule to several hormones.

In pituitary corticotrophs, POMC mRNA is produced after the splicing of the primary transcript and the addition of a poly A+ tails, yielding a pre-POMC molecule, that has a sequence of 26 amino acid residues that signal the translocation of the peptide to the rough endoplasmic reticulum, where the protein is matured and secreted as a 241-amino-acid mature POMC molecule.

The human POMC gene is 7665bp long, made of three exons and two introns, giving rise to the N-terminal POMC peptide (NPP) or γ-MSH (Melanocyte-stimulating hormone), ACTH and β-lipotropin (β-LPH). Further cleavage will also happen depending on the cell type POMC is being expressed, resulting in the production of α-MSH and CLIP (corticotrophin-like intermediate peptide) from ACTH cleavage, and, β-endorphin, γ-lipotropin and β-MSH from β-lipotropin cleavage (Chang, Cochet et al. 1980, Solomon 1999, Raffin-Sanson, de Keyzer et al. 2003, Dores and Baron 2011, Harris, Dijkstra et al. 2014, Manso, Sanchez et al. 2014) (Figure 1.13)

In humans, rats and pigs, POMC mRNA is mostly transcribed in the anterior and intermediate sections of the pituitary, mostly in corticotrophs, but also expressed to a lesser extent in other parts of the brain, including the anterior hypothalamus, supraoptic nuclei, suprachiasmatic, preoptic areas, nucleus accumbens, periventricular stratum, parafascicular region of the thalamus and olfactory tubercle (Pintar, Schachter et al. 1984, Pilcher, Joseph et al. 1988, Kineman, Kraeling et al. 1989, Millington, Rosenthal et al. 1999).

Peripherally, POMC mRNA expression in humans, rats and pigs can be found in the uterus, heart, testis, ovary, adrenals, spleen, kidney, thyroid, colon, duodenum

and liver (Pintar, Schachter et al. 1984, DeBold, Menefee et al. 1988, Pilcher, Joseph et al. 1988, Kineman, Kraeling et al. 1989, Cliffton 1998, Millington, Rosenthal et al. 1999, Grigorakis, Anastasiou et al. 2000, Dores and Baron 2011).

In some peripheral tissues such as dorsal root ganglia, sciatic never and spinal cord, POMC mRNA is short or truncated, and, mostly non-functional (~800 mRNA nucleotides), compared to the pituitary functional POMC mRNA (~1200 mRNA nucleotides) (Plantinga, Verhaagen et al. 1992, Raffin-Sanson, de Keyzer et al. 2003).

After POMC peptide is synthesized in the endoplasmic reticulum it is transported to the trans-Golgi network for sorting and packaging into secretory granules via the regulated secretory pathway (RSP). Acid residues in the surface of POMC are responsible for binding to the trans-golgi network carboxypeptidase E receptor and activate the RSP (Loh, Maldonado et al. 2002). During this traffic, POMC undergoes a series of proteolytic cleavages to yield the POMC-derived peptides (figure 1.13).

POMC is cleaved by prohormone convertases 1 and 2 (PC1 and PC2) that target eight pairs, and one quadruplet, of basic amino acids. Corticotroph cells only express PC1, which cleaves POMC at four cleavage sites, all Arg-Lys residues sites, generating NT, joining peptide (JP), ACTH, β-LPH and γ-LPH (γ-Lipotropin) (Bertagna 1994, Tanaka 2003, Bohm and Grassel 2012).

Melanotrophs on the other hand express both PC1 and PC2, which results in the proteolysis of all cleavage sites, producing an array of smaller peptides such as γ-MSH (γ-Melanocyte-stimulating hormone), α-MSH, CLIP (corticotrophin-like intermediate peptide), β-MSH and β-Endorphin (Bertagna 1994, Perone, Chisari et

al. 1997, Tanaka 2003, Bohm and Grassel 2012). Equimolar amounts of these peptides are released in the blood stream by exocytosis.

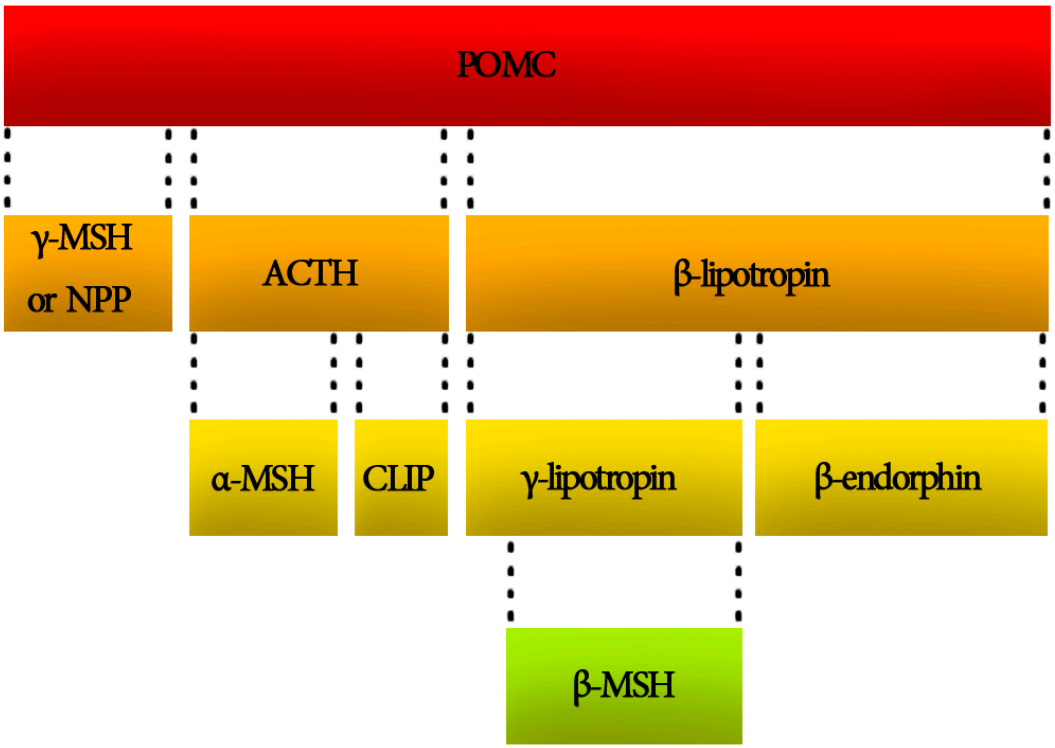


Figure 1.16: Schematic illustration of POMC cleavage and its derivate peptides.

1.2.4.7 Adrenocorticotropic hormone (ACTH)

Human ACTH is a polypeptide trophic hormone consisting of 39 amino acid residues. As it is a by-product of POMC, ACTH and POMC localization are the same. Its function in the adrenal cortex is to induce cAMP production, steroid synthesis (steroidogenesis) and secretion of glucocorticoids, mineralocorticoids and

androgens (Peytremann, Nicholson et al. 1973, Simpson and Waterman 1988, Cone, Lu et al. 1996, Hagen, Kusakabe et al. 2006).

Acute ACTH exposure increases the initial step in steroidogenesis, the mobilization of cholesterol to the mitochondria where it is converted to Δ^5-pregnenolone, via the action of CYP11A (cytochrome $P450_{scc}$ or P450 side chain cleavage) a cytochrome present in the inner mitochondrial membrane (Koritz and Kumar 1970, Farese 1971, J.A.Bermudez and M.B.Lipsett 1972, Stocco 2001, Raffin-Sanson, de Keyzer et al. 2003).

As mentioned previously, acute ACTH up-regulates the steroidogenic acute regulatory protein (StAR), a 30kDa phosphoprotein protein responsible for the shuttling of cholesterol from the outer to the inner mitochondrial membrane. StAR is a cycloheximide-sensitive mitochondrial protein and its expression regulation responds to both cAMP and PKA pathways.

Chronically, ACTH also increases transcription of genes encoding enzymes involved in steroidogenesis through their individual cAMP-response elements (CRE), which also binds to a variety of transcription factors (Nishikawa, Sasano et al. 1996, Waterman and Bischof 1996, Fleury, Ducharme et al. 1998, Stocco 2001, Sewer and Waterman 2003).

In the adrenal, there are five distinct steroid hydroxylases, which act in conjunction with steroid dehydrogenases, leading to the formation of cortisol, androgens and aldosterone. Hydroxylase enzymes comprise two microsomal enzymes: CYP17 and CYOP21 ($P450_{17}$ and $P450_{21}$) and three mitochondrial enzymes: CYP11A1, CYP11B1, CYP11B2 ($P450_{11A1}$, $P450_{11B1}$, $P450_{11B2}$).

Dehydrogenase enzymes involved are 3β-hydroxysteroid dehydrogenase (3β-HSD) and 11β-HSD. The enzyme CYP17 also has a lyase function, which

depending on the activity level of cytochrome B_5, leads to the formation of C_{19} steroids, such as androgens in the zona reticularis of the adrenal cortex and in the gonads (figure 1.17) (Sewer and Waterman 2003).

After Δ^5-pregnenolone is synthesized, it exits the mitochondrion and enters the microsomal compartment, where it is converted to progesterone by 3β-HSD, or, to 17-OH pregnenolone by CYP17. Progesterone and 17-OH progesterone are hydroxylated by CYP21 to produce deoxycorticosterone and 11-deoxycortisol, respectively, which are further hydroxylated by CYP1B1 to form corticosterone and cortisol. Aldosterone is formed through the action of CYP11B2, which occurs only in the zona glomerulosa of the adrenal cortex (figure 1.17) (J.A.Bermudez and M.B.Lipsett 1972, Cherradi, Defaye et al. 1994, Stocco 2001, Sewer and Waterman 2003).

As mentioned earlier, ACTH chronically modulates gene transcription of all of the enzymes involved in the steroidogenesis process. Binding of ACTH to the cell surface adrenocortical receptor activates adenylyl cyclase, elevating intracellular cAMP, which in turn activates PKA. PKA phosphorylates cholesterol ester hydroxylase, which in turn converts stored cholesterol esters to free cholesterol that are transported by StAR into the mitochondrion for the initiation of the steroidogenic pathway (Sewer and Waterman 2003).

Each gene transcription is activated in a different way by PKA, using know and unknown proteins and transcription factors. For instance, PKA will also trigger extracellular regulated kinase 1/2 (pathway ERK1/2), which interacts with steroidogenic transcription factor 1 (SF-1), specificity protein 1 transcription factor (SP-1) and co-regulatory CREB brinding protein p300 (CBP/p300) to activate

CYP11A1 gene transcription, leading to the expression of P45011A1 (Fleury, Ducharme et al. 1998, Sewer and Waterman 2003, Gómez, Gorostizaga et al. 2013).

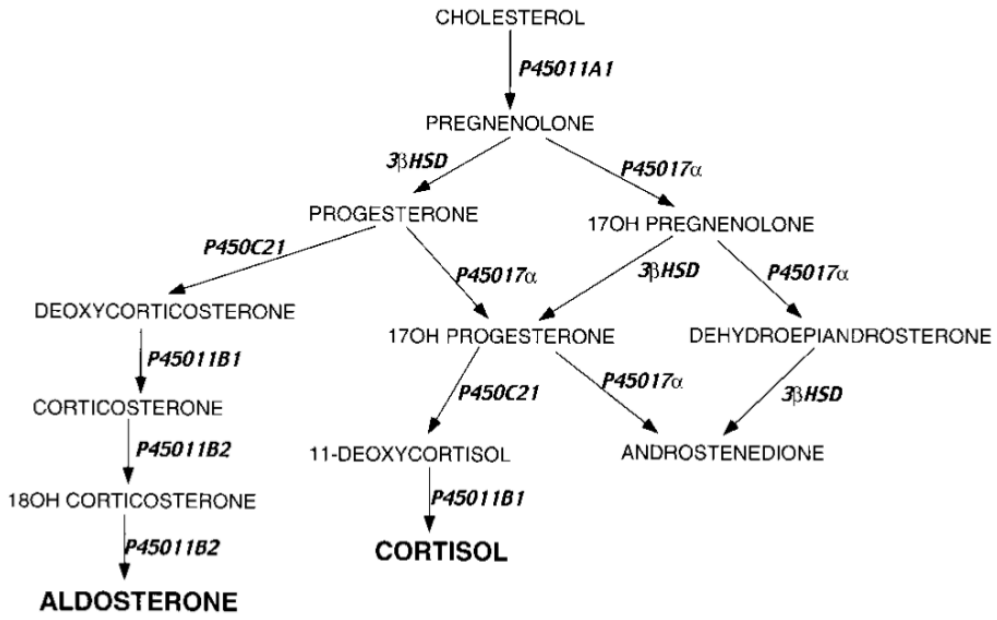


Figure 1.17: Human steroidogenesis pathway with emphasis on hydroxylases and dehydrogenases. Reproduced from Sewer (Sewer and Waterman 2003).

1.2.4.8 Melanocortin-2 receptors (MC2R)

In mammals and other vertebrates, the melanocortin-2 receptor (MC2R), a receptor specific for ACTH, is of key importance to the regulation of cortisol production and secretion from the adrenals. MC2R is a seven transmembrane G-protein coupled receptor, as all the other four melanocortin receptors (MCR), sharing around 50% homology with other MCRs (Mountjoy, Robbins et al. 1992, Gantz, Konda et al. 1993, Chen, Aprahamian et al. 2007).

MC2R is also present in teleost interrenal tissue, having 44% homology with

the human MC2R (Aluru and Vijayan 2008). MC2R expression is largely restricted to the adrenals, in the cortex region, and the expression of this receptor per cell is low compared to other MCRs (Rached, El Mourabit et al. 2005).

Truncated versions of ACTH revealed that the minimum size for MC2R activation is by using an ACTH molecule containing the first 16 amino acids (ACTH1-16), requiring the N-terminus of the peptide to be conserved. To bind to human MC2R, three key regions are necessary: ACTH1-5 for receptor activation, ACTH6-9 (His-Phe-Arg-Trp, sequence found you all melanocortins) for binding, and, ACTH14-16, which is specific for the activation of MC2R (Haskell-Luevano, Sawyer et al. 1996, Chen, Aprahamian et al. 2007).

Human MC2R has an ionic pocket composed of amino acid residues E80 in trans-membrane 2 (TM2) and D104 (amino acid residue believed to be essential for the specific ACTH binding) and D107 in TM3, as well as a second hydrophobic ligand binding pocket made of amino acid residues F235 and H238 in TM6 (figure 1.18) (Chen, Aprahamian et al. 2007).

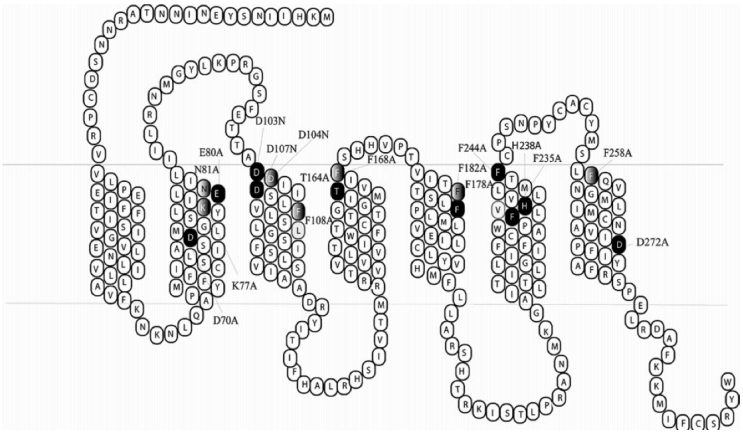


Figure 1.18: Human MC2R secondary structure, reproduced from Chen (Chen, Aprahamian et al. 2007).

1.2.4.9 Melanocortin receptors 4 (MC4R)

The central melanocortin system has been implicated in the control of energy homeostasis. It is composed mainly of: α- melanocyte-stimulating hormone (α-MSH) derived from proopiomelanocortin (POMC) as an agonist to the melanocortin-4 receptor (MC4R), and its antagonist, agouti-related protein (AgRP) (Farooqi and O'Rahilly 2004, Cone 2005).

There is a functional link between this central melanocortin system and the stress system, including POMC neurones situated in the mediobasal hypothalamus, also known as the arcuate nucleus, that in rats are activated by perceived stress, resulting in a rise of POMC and α-MSH expression (Liu et al. 2007; Yamano et al. 2004; Baubet et al. 1994).

In rats, injection of MC4R agonists, such as melanotan II, has the potential to activate the HPA axis, generating CRH transcription in the PVN following a rise in corticosterone and activation of anxiety behaviour (Lu, Barsh et al. 2003, Klenerova, Krejci et al. 2008). Conversely, administration of the MC4R selective antagonist HS014, reduces anxiety behaviour (Kokare, Dandekar et al. 2005). From these findings, it is possible to conclude that the activation of this melanocortin system is able to modulate behavioural and endocrine responses to stress.

1.2.4.10 Glucocorticoids

Glucocorticoids are part of a homeostatic system in the body, and are responsible for the classic response to stress, in particular controlling metabolism and immunity. Metabolic effects include, increased blood glucose concentration, and gluconeogenesis, mobilisation of amino acids and fatty acids, and additionally suppression of the immune system (Newton 2000, Bandsma, Grefhorst et al. 2004, Bandsma, van Dijk et al. 2004).

Gluconeogenesis is stimulated by activation of certain genes, in response to glucocorticoids and glucagon. CREB, induced by glucagon, activates expression of most genes involved in gluconeogenesis, through a combined action with nuclear receptor co-activator PGC-1 (Herzig, Long et al. 2001). PGC-1 is involved in the expression of hepatic Phosphoenolpyruvate carboxykinase (PEPCK) (Yoon, Puigserver et al. 2001).

PEPCK, the main rate-limiting enzyme in gluconeogenesis, which converts oxaloacetate into phophoenolpyruvate and carbon dioxide is up-regulated in the liver and kidney, but repressed in adipose tissue by GR protein-protein interaction (Hanson and Reshef 1997, Olswang, Blum et al. 2003).

Glucose-6-phosphatase, the enzyme responsible for the last step of gluconeogenesis, is also strictly regulated by glucocorticoids, possessing three GREs up-stream of its promoter in hepatocytes (Lange, Argaud et al. 1994, Maitra, Wang et al. 2000, Vander Kooi, Onuma et al. 2005). Tyrosine aminotransferase (TAT) converts tyrosine to 4-hydroxyphenylpyruvate, thus generating substrate for gluconeogenesis. TAT is regulated by GR (Barnabei and Sereni 1964, Jantzen, Strahle et al. 1987, Alexandrova 1994).

Activated GR suppresses the immune system by down-regulating, transcription of inflammatory cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-18, IFN-γ, TNFα, COX-2, ICAM, MCP-1 and iNOS) (De Bosscher and Haegeman 2009).

Glucocorticoids also induce redistribution of adipose tissue. They induce fatty liver development by increasing production and secretion of very low density lipoprotein (VLDL), inducing key lipogenic enzymes activities such as acetyl-CoA-carboxylase (ACC) and fatty acid synthase (FAS) and by receiving an influx of fatty acids originated from lipolysis of adipose tissue around the body. Ultimately this has detrimental effects to the liver, including impairment of insulin secretion pathway, by inhibiting PKC (Amatruda, Danahy et al. 1983, Mangiapane and Brindley 1986, Bugianesi, McCullough et al. 2005).

Adipose redistribution results in accumulating fat around the abdominal area and eliminating it from periphery depots, giving symptoms similar to those of Cushing's syndrome (Shibli-Rahhal, Van Beek et al. 2006). Glucocorticoids increase lipolysis of triacylglycerol from adipocytes by inducing hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) expression (Slavin, Ong et al. 1994, Jocken, Langin et al. 2007, Peckett, Wright et al. 2011).

As mentioned earlier in this introduction, glucocorticoids regulate the basal HPA axis activity tone and will terminate the stress response by acting on the hypothalamus, pituitary and other brain regions (Kanter, Wilkinson et al. 2001). A fast non-genomic mechanism of feedback occurs on glutamate release, specifically onto CRH-expressing cells of the PVN.

It occurs via membrane-bound glucocorticoid receptor that mediates the synthesis of endocannabinoids and retrograde activation of cannabinoid receptors in

the presynaptic region. This was further confirmed by blocking this negative feedback with the CB1 receptor antagonist AM-251 (Di, Malcher-Lopes et al. 2003, Malcher-Lopes, Di et al. 2006, Evanson, Tasker et al. 2010).

GR and MR both mediate effects of glucocorticoids in the brain and thus modulate glucocorticoid-related neuronal excitability of several brain regions, as well as the stress response and behavioural adaptation, depending on cellular context (De Kloet, Vreugdenhil et al. 1998).

1.3 Effects of developmental ethanol exposure on HPA axis plasticity and stress-related behaviour.

1.3.1 Developmental ethanol exposure effects on HPA axis plasticity

The components of the HPA axis are very susceptible to early environmental manipulation, in a process called early programming, in which developmental ethanol exposure can permanently alter some components of this system (Matthews 2000, Matthews, Owen et al. 2002).

In humans, 13-month-old infants exposed to maternal light-moderate drinking (<15mL of alcohol/day) during pregnancy had no difference in basal or post-stress (heelprick blood draw) salivary cortisol levels, however, infants exposed to heavy maternal drinking (>15mL of alcohol/day) during pregnancy had higher basal and higher post-stress cortisol levels (Jacobson 1999).

Another study in 5-7-month-old infants confirmed higher stressed cortisol salivary concentration, as well as increased heart rate, with prenatal ethanol intake (Haley, Handmaker et al. 2006). In another study, 2-month-old infants exposed to maternal moderate drinking showed higher basal salivary cortisol levels compared to control infants, but no difference when stressed by inoculation (Ramsay, Bendersky et al. 1996).

Several studies performed in rats also concluded that animals prenatally exposed to ethanol had elevated plasma corticosterone levels in response to acute and chronic stressors, such as morphine injection, cardiac puncture, restraint stress, shaking and electric footshock; independent of any variations on maternal nutrition due to the ethanol intake (Taylor, Branch et al. 1981, Taylor, Branch et al. 1982, Taylor, Branch et al. 1988, Weinberg 1988, Weinberg 1992). Higher basal plasma corticosterone levels were also found in ethanol prenatal exposed female mice, but not in males (Allan, Chynoweth et al. 2003).

Male and female rats developmentally exposed to ethanol also showed increases in plasma ACTH after stress, but there were no differences in baseline levels (Weinberg 1992, Osborn, Kim et al. 1996). These finding were further confirmed using a chronic stressor (cold stress) and an acute stressors (isotonic saline injection and pro-inflammatory cytokine injection), where plasma ACTH concentration was higher after stress in both female and male rats that had been developmentally exposed to ethanol (Kim, Giberson et al. 1999, Kim, Turnbull et al. 1999)

In rat offspring developmentally treated with ethanol during the second week of gestation, *crh* mRNA expression was increased, specifically in the PVN. (Lee, Imaki et al. 1990) (Glavas, Ellis et al. 2007). Another study with 60-day old males

prenatally exposed to ethanol also reported an increase in both *crh* and *pomc* transcription (Redei, Halasz et al. 1993). Basal levels of *crh* mRNA expression were also increased in PNE rat adults independent of their postnatal handling (Gabriel, Glavas et al. 2005).

There are no reported differences in *nr3c1* (GR) mRNA expression, but developmental ethanol exposure did reduce *nr3c2* (MR) mRNA transcription in females, but not male rats, in all sub-regions of the hippocampus (Sliwowska, Lan et al. 2008, Uban, Comeau et al. 2013).

Crhr1 mRNA expression is reduced in the pituitary, in early ethanol treated males, but not female rats; even under adrenalectomised conditions, with or without cortisol replacement (Glavas, Ellis et al. 2007). There are no reported studies that assess *crhr2* mRNA expression differences related to prenatal ethanol exposure.

It has been proposed that the catecholaminergic system may be affected during early ethanol exposure, as prenatal ethanol exposure reduces dopamine neurones in several parts of the brain, including the striatum and frontal cortex, and, noradrenaline transmission in other parts of the nervous system, including the hypothalamus (Detering, Collins et al. 1980, Druse, Tajuddin et al. 1990).

The catecholaminergic system is also known to be activated by stressors and are for being able to modulate HPA axis activity (Sawchenko, Cunningham et al. 1992). This could contribute to the hyperresponsiviness of the HPA axis in ethanol exposed animals. There was no difference between ethanol developmentally treated rats when injected with phenylephrine, an adrenergic receptor agonist; however there was an increase in activity of adrenergic neurones in the C1 region of the brain stem, a region known to project and modulate the PVN (Choi, Lee et al. 2008).

Serotonergic transmission has also been implicated in the modulation of the HPA axis in prenatal ethanol exposed animals. Early ethanol exposure affects the serotonergic system, by reducing the density of the serotonergic neurones in many brain regions, and retarding their migration and innervation during development (Tajuddin and Druse 1999, Sari and Zhou 2004, Zhou, Sari et al. 2005).

Early alcohol exposure leads to reduced attention and impulsivity, abnormalities that are linked to the modulation of the serotonergic system (Roebuck, Mattson et al. 1999, Huizink and Mulder 2006, Macrì, Spinelli et al. 2007).

Serotonin (5-HT) has long been thought to modulate the HPA axis, mostly at the pituitary and adrenal gland level in mammals (Fuller, Snoddy et al. 1976, Badgy 1989, Dinan 1996), and even in teleosts (Winberg, Nilsson et al. 1997). It is possible that developmental ethanol-related variations in this system could potentially affect the outcome on the HPA axis, but little work has been done on this subject.

An elegant experiment done by Weinberg and team found that prenatal ethanol exposed female rats, but not males, exhibited a decreased ACTH response when injected with serotonin receptor 5-HT_{1A} agonist 8-OH-DPAT [8-hydroxy-2-2(di-n-propylamino)tetralin], but, had an increased ACTH response when injected with serotonin receptor 5-$HT_{2A/C}$ agonist DOI [1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride]. This experiment established the first connection between the serotonin system and the HPA axis; modulated by early ethanol exposure (Hofmann, Ellis et al. 2007, Weinberg, Sliwowska et al. 2008).

Much of the effect of serotonin depends on the serotonin transporter (5-HTT) activity. 5-HTT transcription, synaptic density and reuptake are all decreased with a

short 5-HTT promoter allelic variation, s/s (Heinz, Mann et al. 2001, Glatz, Mossner et al. 2003). Rhesus monkeys prenatally exposed to ethanol and tested at 6 months of age, exhibited increased stress responsiveness (higher cortisol and ACTH plasma levels when separated from the mother) when carriers of the short polymorphism of 5-HTT (Kraemer, Moore et al. 2008). Serotonin has also been implicated in influencing the transcription of hippocampal GR mRNA during development, but not MR, which can further connect the two systems (Erdeljan, MacDonald et al. 2001)**.**

It has also been suggested that the GABAergic system interacts with the HPA axis differently following developmental ethanol exposure. GABA content decreased in the hippocampus (11%), thalamus (28%) and cerebellum (13%), but increased in the frontal cortex (14%) and amygdala (61%) (Ledig 1988).

There is also the possibility of variations in the developmental ethanol treatment affecting the outcome of the HPA axis hyperactivity. Based on other group's research data, by manipulating the gestational ethanol exposure period or ethanol concentration used during exposure, different HPA axis modulation outcomes can be achieved (Park, Dumas et al. 2004, Ouellet-Morin, Dionne et al. 2011, Xia, Shen et al. 2014).

In humans, prenatally alcohol exposed 19-month toddlers have lower basal cortisol levels compared to control children; more prominent in boys compared to girls; from mothers that drank less than 1 glass of alcoholic beverage a week during the gestational period (Ouellet-Morin, Dionne et al. 2011). This data contradicts the findings of Jaccobson (Jacobson 1999), however the children were at different ages when tested and they were also from different ethnic groups.

Another example occurred when rats exposed to ethanol from gestational day (GD) 11 until birth showed lower cortiscosterone basal levels compared to controls, and when chronically stressed, these differences disappeared (Xia, Shen et al. 2014). Another experiment with ethanol exposure during gestational weeks 2 and 3 yielded female rats that had higher corticosterone levels when chronically stressed (Taylor, Branch et al. 1982). A single day ethanol exposure was done in mice, GD9 exposure yielded corticosterone hyperresponsiveness in mature but not old mice when chronically stressed (Park, Dumas et al. 2004).

Female offspring of rats exposed to ethanol prenatally show greater changes in corticosterone and ACTH levels in response to short duration and acute stressors, compared to males (Taylor, Branch et al. 1988, Weinberg 1988, Kelly, Mahoney et al. 1991). PNE female rats also exhibit increased corticosterone levels in response to immune challenges (Kim, Turnbull et al. 1999).

Male offspring of rats exposed to alcohol developmentally are in contrast more stress reactive than females when the stressor is present for a prolonged time, assessed by corticosterone levels (Weinberg 1992, Kim, Giberson et al. 1999). Furthermore, both males and females developmentally exposed to ethanol exhibit higher mRNA levels of CRH, immediate early genes (IEGs) c-fos and NGFI-B in the PVN compared to untreated animals (Lee, Schmidt et al. 2000).

1.3.2 Developmental ethanol exposure effects on stress-like behaviour

Human infants that had been exposed to ethanol prenatally show changes in stress-reactivity, as early as 5-7 months, (Haley, Handmaker et al. 2006), but while

there have been many studies that focus on the effects of acute and chronic ethanol intake on the HPA axis and stress-like behaviour in humans, very few have addressed the effects of prenatal ethanol exposure, therefore, animal models are routinely used for this purpose.

Prenatal ethanol exposure in rats evokes detectable changes, increasing stress-like behaviours of male and female offspring, using tests such as the open field, in which findings correlate with corticosterone increases (Hellemans, Verma et al. 2008, Hellemans, Sliwowska et al. 2010, Hellemans, Verma et al. 2010).

Different results were achieved in mice. Mice prenatally treated with ethanol had no difference in time spent in different zones of the open field test, however, they were more hyperactive in their home cages and spent more time inspecting a novel object (Mothes, Opitz et al. 1996, Allan, Chynoweth et al. 2003).

Prenatal ethanol exposure also increases immobility in the forced swimming test (antidepressant-like behaviour test, in which the rodent behaviour is scored as active or passive when forced to swim in a cylinder, also can be used as a stress-reactive indicative) in female and male rats (Slone and Redei 2002, Carneiro, Diogenes et al. 2005, Slattery and Cryan 2012), and make fewer exploratory moves in the elevated plus maze (Osborn, Kim et al. 1998, Gabriel, Yu et al. 2006).

Sex-related differences in stress reactivity are heavily studied using corticosterone and ACTH as parameters, as discussed in the previous section, however, differences were also found in behaviour. Female offspring of rats exposed to ethanol prenatally consume more ethanol when stressed by footshock, and compared to males, PNE females show much more immobility compared to control females (Taylor, Branch et al. 1988, Cryan and Slattery 2007).

A range of behavioural tests for stress and anxiety, have also been developed for zebrafish, notably novel tank diving, thigmotaxis and scototaxis (Levin, Bencan et al. 2007, Bencan, Sledge et al. 2009, Champagne, Hoefnagels et al. 2010, Mathur and Guo 2011, Maximino, da Silva et al. 2011). Other tests address aggression, learning and memory (Norton and Bally-Cuif 2010).

1.4 Addiction, mesolimbic pathway and links to the HPA axis

1.4.1 Developmental ethanol exposure and addiction

Ethanol exposure during pregnancy is one of the leading causes of preventable birth defects, giving rise to a range of problems collectively termed Fetal Alcohol Spectrum Disorder (FASD). Children prenatally exposed to ethanol are at risk of developing cognitive deficits, behavioural problems and changes to their brain structure.

Behavioural problems range from hyperactivity, disruptiveness and impulsivity, to drug abuse and poor socialization and communication skills. Brain images have revealed structural changes in the corpus callosum, basal ganglia, cerebellum and hippocampus (Mattson, Schoenfeld et al. 2001, Burger, Goecke et al. 2011, Leigland, Ford et al. 2013).

The strong correlation in humans between prenatal ethanol exposure and increased drug abuse in later life (Baer, Barr et al. 1998, Yates, Cadoret et al. 1998,

Baer, Sampson et al. 2003, Foltran, Gregori et al. 2011) has been substantiated in animal studies (Bond and Di Giusto 1976, Hilakivi 1986, Lancaster and Spiegel 1989, Arias and Chotro 2005).

Most studies concluded that an exposure to ethanol during the two last weeks of gestation in the rat is enough to increase alcohol preference in the offspring, but two gestational periods seem to be critical, GD8 and GD17-20 when the fetus is vulnerable to the chemosensory as well as the toxic actions of the drug, (Molina, Hoffmann et al. 1987, Dominguez, Lopez et al. 1996, Dominguez, Lopez et al. 1998).

1.4.2 Mesolimbic pathway mechanism

Each class of psychoactive substances has its own pharmacological mechanism of action, but all activate the mesolimbic pathway. In humans, this pathway begins in the ventral tegmental area (VTA) of the midbrain and connects to the limbic system via the nucleus accumbens (NAcc), the amygdala, and the hippocampus as well as to the prefrontal cortex (PFC) (figure 1.19).

The VTA is important in drug dependence. It is rich in dopamine cell bodies projecting to the limbic system and to the forebrain. It signals survival stimuli such as those associated with feeding and reproduction, and many psychoactive drugs have an effect on the VTA, which may result in drug dependence (Di Chiara 1998, Koob and Le Moal 2001, Kelley and Berridge 2002).

Beneath the cortex are the amygdala (part of the limbic system) and the

nucleus accumbens, that when subjected to drugs of abuse trigger an increase in dopamine release. The limbic system connects to a series of structures, interacting with the cortex and the nucleus accumbens, and is important in relation to emotion, learning and motivation, playing a vital role in drug dependence (Di Chiara 1998, Koob and Le Moal 2001, Kelley and Berridge 2002, Everitt and Robbins 2005).

Today, the most accepted model of reward is that when an activity increases the levels of dopamine transmission in the mesolimbic system and in the nucleus accumbens, it is translated as motivational activity, which makes it prone to be reinforced and repeated (Di Chiara 1998, Koob and Le Moal 2001, Kelley and Berridge 2002, Everitt and Robbins 2005).

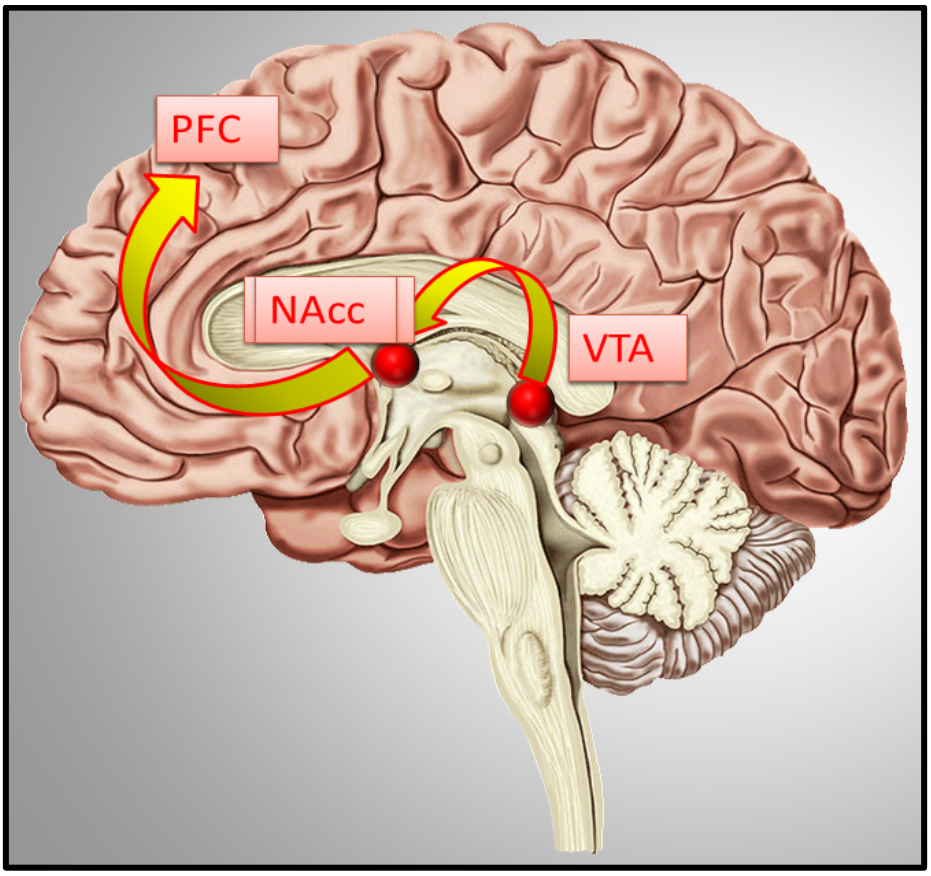


Figure 1.19: Schematic illustration of the mesolimbic system pathway. pathway begins in the ventral tegmental area (VTA) of the midbrain and connects to the limbic system via the nucleus accumbens (NAcc), the amygdala, and the hippocampus as well as to the prefrontal cortex (PFC).

1.4.3 HPA axis and Mesolimbic system links

The hypothalamic and extra-hypothalamic CRH pathways also target the brain motivational systems, such as the amygdala, that have high concentrations of CRH receptors (Shekhar, Truitt et al. 2005). Stress exposure and increased levels of glucocorticoids enhance dopamine release in the nucleus accumbens (Takahashi, Takada et al. 1998), and suppression of glucocorticoids by adrenalectomy gives reduced dopamine under basal conditions or in response to stress or psychostimulants (figure 1.17) (Barrot, Marinelli et al. 2000).

Conversely, chronic glucocorticoid inhibits dopamine synthesis and turnover in the nucleus accumbens (Pacak, Tjurmina et al. 2002). These findings indicate a correlation, though complex, between the HPA axis and glucocorticoids and the dopaminergic system. Stress also enhances glutamate activity in the VTA, further enhancing the activity of dopaminergic neurones by acting through CRF and glucocorticoids (figure 1.20) (Overton, Tong et al. 1996, Ungless, Singh et al. 2003).

Furthermore, in rats, corticosterone release following HPA axis activation is important for acquisition of drug self-administration behaviour (Goeders 2002, Goeders 2004). Corticosterone facilitates the psychomotor effects of morphine and cocaine; and corticosterone effects are reduced by glucocorticoid antagonists (Marinelli, Piazza et al. 1994, Marinelli, Aouizerate et al. 1998).

Given that both stress and psychoactive drugs activate the mesolimbic pathways in similar ways, it has been suggested that these activations can lead to synaptic adaptations in the VTA dopaminergic system and in the morphology of

dendritic spines in the nucleus accumbens and in the PFC (Robinson and Kolb 1999, Liston, Miller et al. 2006).

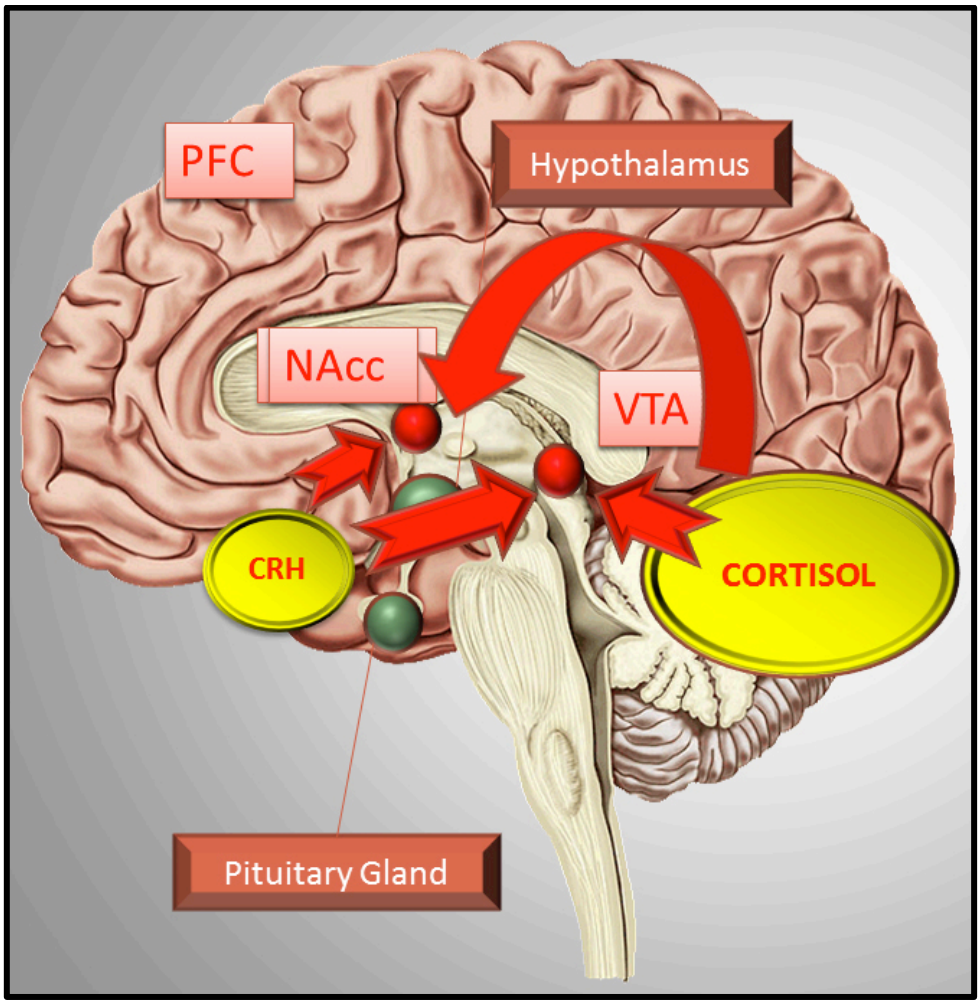


Figure 1.20: Schematic illustration of the links between the HPA axis and the mesolimbic system. Hypothalamus and pituitary represented by green dots and the nucleus accumbens and ventral tegmental area for red dots.

Aims of the study

- Discuss results of our experiments examining the effects of developmental exposure to ethanol on the zebrafish (Danio rerio) HPI axis, including zebrafish behaviour, cortisol output and gene expression.
- Compare these findings with rodent and mammalian models, currently used on this field of research, and assess the differences and similarities.
- Refine the use of zebrafish model to answer questions related to the effects of developmental ethanol exposure that other models couldn't.

Chapter 2: Methods and materials

2.1 Animal maintenance

Zebrafish (*Danio rerio*) adults from Tuebingen wild type (TUWT) line were kept on a constant 14h:10h light:dark cycle at 28°C and fed 3 times a day with flake food and brine shrimp. All fish were bred and reared in the aquarium facility at Queen Mary University of London, licenced by the UK Home Office. Fish water used was prepared by dissolving sodium bicarbonate (0.9mM), calcium sulphate (0.05mM) and marine salts (0.018g in 1L) in distilled water.

2.1.1 Embryo spawning and exposure to pharmaceuticals

Baskets filled with marbles were used to collect spawned eggs, they work by allowing accumulation of the eggs in the interior of the basket rather than in the bottom of the breeding tanks, therefore prevent damage to the embryos and adult fish from eating them.

These baskets were sunk into the breeding aquariums prior to the end of the photoperiod (8am-10pm) and collected the following day, 30-60 minutes after the start of the photoperiod. Alternatively, one male and one female were paired together in a breeding tank prior to the end of the photoperiod, and eggs were collected after the start of the photoperiod the following day

Embryos were separated from unfertilized ova and selected at the 8-cell stage to minimise the possibility of any differences in age. For further accuracy embryos were staged using parameters such as the head-trunk angle (HTA) and the optic vesicle length (OVL) at 24 hr. They were then grouped in Sterilin® petri dishes (catalogue No.: BS611) containing 50 embryos in 40ml of fish water, reared in an incubator set at 28°C. Each petri dish was considered to be a single batch unit, and each group was considered as a single replicate.

Larvae were fed with zmsystems® ZM-000 high protein food particle from 5dpf-10dpf, ZM-100 and paramecium from 11dpf-14dpf, and, ZM-200 and brineshrimp from 14dpf-30dpf. At one month of age, animals were transferred into the main system aquariums where they were fed zmsystems® flake food and brineshrimp.

For developmental ethanol exposure studies, treated larvae were exposed from 1-9dpf to 20mM and 50mM VWR® GPR ethanol (catalogue No.: 20824.321) concentrations and a handling control. For the characterization of thigmotaxis as a stress-reactive assay, 9dpf zebrafish larvae were exposed to psychoactive drugs at concentrations previously used in other studies.

These were diazepam, a benzodiazepine that modulates GABA type A receptors (Sigma-Aldrich, catalogue.: D0899), 0.05-0.7mg/L (Bencan, Sledge et al. 2009, Richendrfer, Pelkowski et al. 2012), and buspirone, a serotonin 5-HT1A receptor partial agonist (Sigma-Aldrich, catalogue No.: B7148-1G), 6.25 mg/L and 25mg/L (Bencan, Sledge et al. 2009, Steenbergen, Richardson et al. 2011).

Buspirone was dissolved directly into fish water and used immediately, whereas diazepam was dissolved into 100% VWR® dimethyl sulfoxide (DMSO)

(catalogue No.: 23500.297) to 100mg/mL concentration, immediately dissolved to 0.05-0.7mg/L in fish water or stored at −20°C. The corresponding DMSO concentrations were used as solvent in the control solutions during the behavioural assays.

2.2 Zebrafish larvae tissue ethanol intake, size and weight

2.2.1 Alcohol dehydrogenase analysis

The embryonic and larval tissue ethanol concentration was assessed using a method adapted from Mark Reimers (Reimers, Flockton et al. 2004). It is based on a reaction using *Saccharomyces cerevisiae* alcohol dehydrogenase (ADH, Sigma® Catalogue No.: A7011-7) 0.75mg/ml in distilled water, β-nicotinamide adenine dinucleotide (NAD+, Sigma® Catalogue No.: N7004-1G,1mg/ml in 0.5M Tris pH 8.8) and embryo and larval homogenate.

After treatment with ethanol, 25 live embryos or larvae (with intact chorions if applicable) were transferred into a Starlab® 1.5mL crystal clear microcentrifuge tube (catalogue No.: E1415-1500) and placed on ice. Animals were quickly rinsed twice with 500µl of ice-cold distilled water to remove any residual ethanol from the solutions they were in. An aliquot of 500µl of ice-cold 3.5% v/v Sigma® perchloric acid (catalogue No.: 311421-50ML) was added and the embryos were quickly homogenized with a microcentrifuge tube pestle on ice.

Samples were centrifuged at 4°C for 10 minutes at 12,000g and then stored in paraffin sealed tubes at 4°C until all samples were collected, or placed on ice and immediately used. Standard curves were constructed using six ethanol standards, ranging from 100mM to 3.125mM, yielding a non-linear quadratic polynomial function (r^2=0.99) (figure 2.1). Two replicates for each standard and samples were produced. The initial reaction mixture was: 870µl of the solution of NAD+ plus 43.5µl of the standard or the sample (in perchloric acid) in 1.5ml microcentrifuge tubes.

To start the reaction, 86.5µl of the ADH solution was added to the microcentrifuge tube, the cap was closed, and the content mixed by inverting the tube. Tubes were then incubated using a hot block at 37^{o}C for 10 minutes then transferred to a Starlab® 1.5mL semimicro cuvette (catalogue No.: E1412-4150). NADH production was calculated at 340nm wavelength. A blank reaction substituting the ethanol solutions for 3.5% v/v perchloric acid was used to calibrate the spectrophotometer initially.

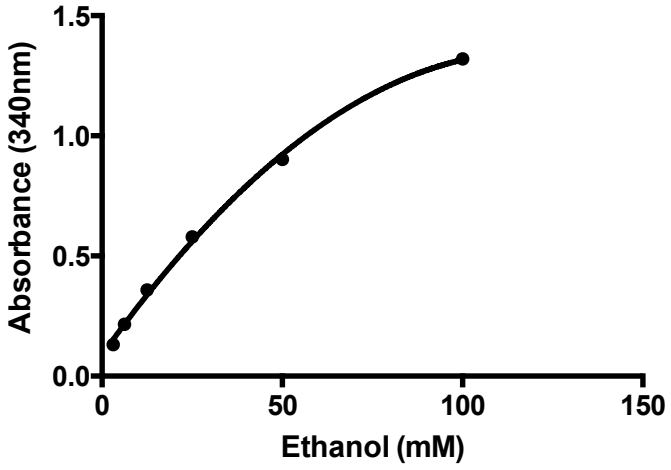


Figure 2.1: Alcohol dehydrogenase analysis ethanol standard curve. NADH production was calculated from its absorption at 340nm using ethanol standards: 100mM, 50mM, 25mM, 12.5mM, 6.25mM and 3.125mM. Each point is the mean of duplicates. r^2=0.99.

2.2.2 Larval size assessment

Larval size at 9dpf was determined using eLaborant, an automated image detection software made by eLaborant (Niels Bohrweg 1, Snellius, room 108, 2333 Leiden, The Netherlands). The method involves tracing a virtual line around the image of the animal, estimating both main axis length and the pixels contained within traced line perimeter (figure 2.2).

Animals treated with 20mM and 50mM ethanol solutions, as well as controls, were photographed under the same conditions in high resolution (4064 x 4064) using a Nikon D800 camera. The software analysed 60 larvae in each group and provided the pixel count/ central axis length per animal.

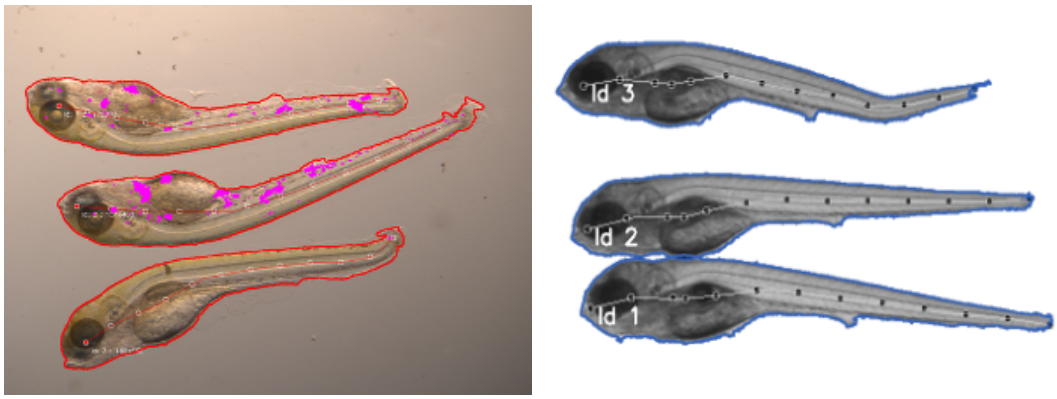


Figure 2.2: Zebrafish larvae size measurement mechanism by the eLaborant software**.** Demonstration images showing automated tracing of the zebrafish larvae, including central body axis measurement. Reproduced from eLaborant brochure.

2.2.3 Larvae dry weight assessment

Tissue dry weight was obtained by homogenizing 25 larvae in 1.5mL pre-weighed microcentrifuge tubes, using 500µl of ice-cold 3.5% v/v perchloric acid with a microcentrifuge tube pestle on ice. Samples were then evaporated in a Univapo 100H speed vac for 1 hour, using a Unijet II aspirator vacuum pump. Microcentrifuge tubes were weighed again using a precision analytical Sartorius 2006 MP scale, to obtain the dry weight of the samples.

2.3 Zebrafish larval and juvenile behavioural assays

2.3.1 Image collection

A high-throughput imaging system for automated analysis of zebrafish larval and juvenile thigmotaxis behavioural was used. It consists of a 15-megapixel infrared Imaginsource digital camera DMK21AF04 attached to the lower shelf of an acrylic cabinet to allow filming from below either of a 12-well plate (for 9dpf and 10dpf larvae) or a 6-well plate (for 23dpf juveniles) located in the upper shelf of the cabinet. Located in the upper portion of this cabinet there is a mounted infrared table.

Experiments using a predator simulation had a different arrangement; consisting of a 15-megapixel SONY digital camera mounted above to allow filming from above of a 12-well plate (9dpf and 10dpf larvae) or a 6-well plate (23dpf juveniles) plate attached to a 15" monitor, screening the predator presentation using PowerPoint.

Experiments requiring complete darkness such as the "darkness startle" stress stimulus required the acrylic cabinet to be covered by a box at the onset of this stimulus. Captured footage was automatically tracked using EthoVision® XT 10.

2.3.2 Thigmotaxis assay

Larvae or juveniles raised in petri dishes or tanks were placed in 6-well plates 1 hour prior to the behavioural assay with no more than 3 animals per well. Experimental animals were then drug treated for 6 minutes and transferred by pipetting individually to a 12-well plate (9dpf and 10dpf larvae) or a 6-well plate (used for 23dpf juveniles) containing the drug solution as appropriate, then immediately placed on the recording apparatus described above.

For air exposure challenge, 9dpf larvae and 23dpf juveniles, animals were removed from their home petri dishes (9dpf) or nursery tanks (23dpf) and placed in Falcon® 6-well plates (Catalogue No.: 08-772-1G) mounted with sieve inserts. Wells were filled with 10mL of either fish water or as specified in individual experiments.

Larvae were grouped 12 animals per well and juveniles 3 animals per well, then left to habituate for 1 hour in a lit and silent environment. Sieve inserts were lifted and placed in a paper towel at the desk for 1 minute, air exposing the subjects inside the sieve, then immediately placing them back to the wells. Control animals remained in the well.

2.3.3 Statistics

Thigmotaxis data were fitted to a linear mixed effects model that observes mixed effects like drug exposure, distances travelled and the time effect in the measured aspect of the assay, using R software. Distances travelled were entered as a covariate in order to control for immobility and darting periods. Dependent variable was time spent in the outer ring of the wells. The tests were evaluated with respect to type-1 error rate of 0.05.

2.4 Zebrafish adult behavioural assays

2.4.1 Image collection

A high-throughput imaging system for automated analysis of zebrafish adult was used. It consists of a 15-megapixel SONY digital camera attached to the ceiling, filming from above in stress-reactive assays and social behaviour assays,

with exception to novel tank diving, where the same camera was used in a tripod to film novel tank diving tanks laterally. Captured footage was also automatically tracked using EthoVision® XT 10.

2.4.2 Novel tank diving assay

Novel tank diving was assayed in trapezoid tanks (152mm height x 279mm top x 225mm bottom x 71mm width) filled with 1.5L of fish water. Diving was determined by time spent in the lower third of the tank (approx. 50mm). Zebrafish adults developmentally treated with ethanol and controls were pair-housed in the same tank for 2 weeks prior to the beginning of this assay to increase the power of the experiment.

Animals were carefully netted and transferred to the "novel" diving tanks and immediately filmed for 5 minutes. Testing was done during the light phase (9am-5pm). Ethanol treated and control groups were tested alternately in two identical tanks with order being counterbalanced. They were age and weight matched, in 3:2 females:males ratio.

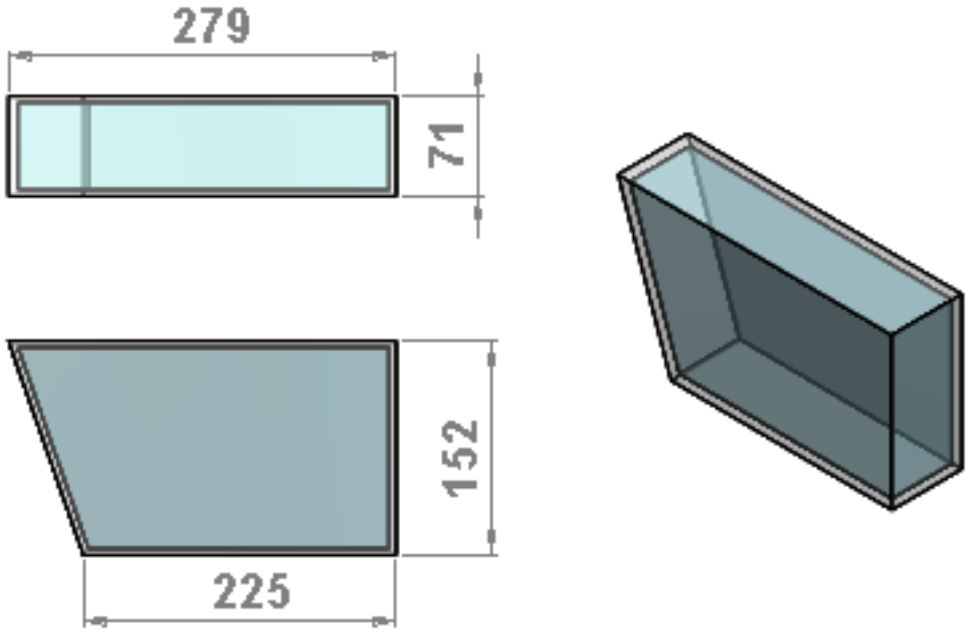


Figure 2.3: Novel tank diving tank used for stress-reactivity measured of 6-month-old zebrafish adults.

2.4.3 Thigmotaxis assay

Thigmotaxis was assayed using white opaque circular tanks (410mm height x 320mm diameter) filled with 2L of fish water. The outer zone was defined as the region 4cm (the average length of an adult fish) from the edge of the tank, and the time spent in this zone was determined. One zebrafish adult developmentally treated with ethanol and one zebrafish adult control were pair-housed together in the same tank for 2 weeks prior to the beginning of this assay, in order to increase the power of the experiment.

Animals were carefully transferred into the tanks and immediately filmed for 6 minutes. Testing was done during the light phase (9am-5pm). Ethanol treated and

control groups were tested alternately in four identical tanks with order being counterbalanced. They were age and weight matched, in 3:2 females:males ratio.

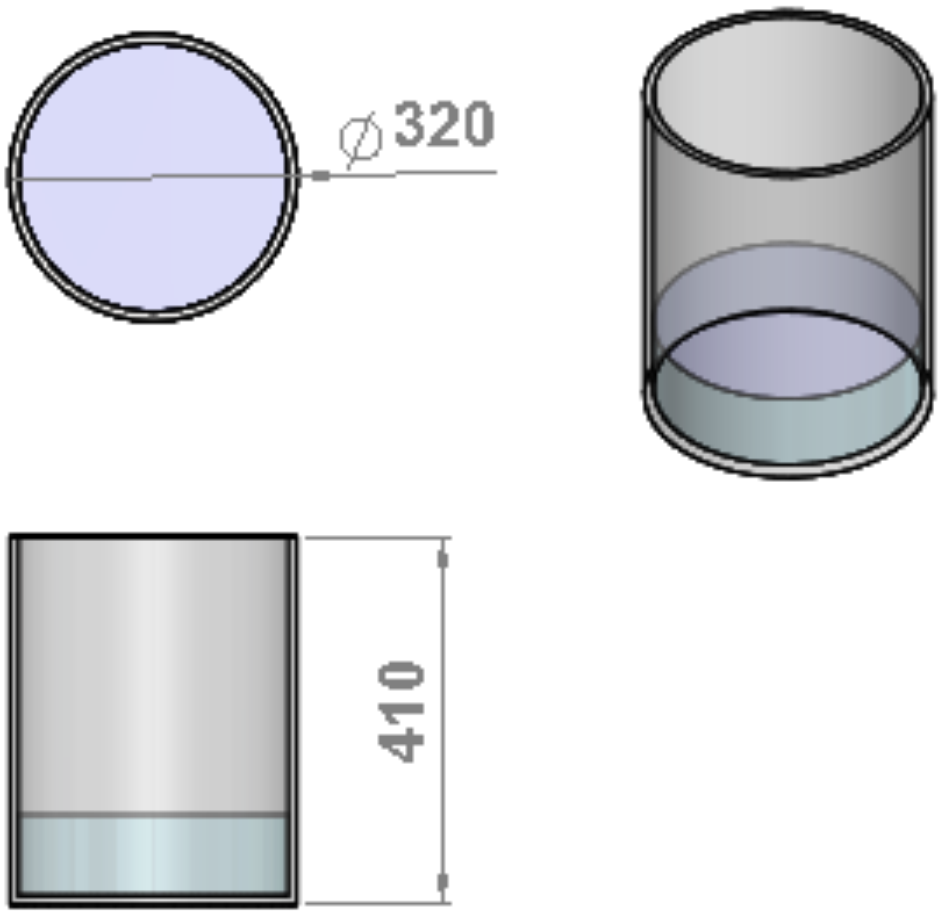


Figure 2.4: Thigmotaxis tank used for stress-reactivity measured of 6-month-old zebrafish adults. Tanks were opaque, translucent illustration to ease visualization.

2.4.4 Scototaxis

White opaque tanks (330mm length x 160mm width x 130mm height) containing 2L of fish water were used for scototaxis. They were divided into two compartments by a black opaque acrylic divider, with a square hole in the middle (50 x 50mm). One side of the tank was exposed to light where the animals were recorded and the time spent there was determined, and the other, darkened. Zebrafish adults developmentally treated with ethanol and controls were pair-housed in the same tank for 2 weeks prior to the beginning of this assay to increase the power of the experiment.

Animals were carefully transferred into the tanks and immediately filmed for 9 minutes in the bright side of the tanks. Ethanol treated and control groups were tested alternately in three two identical tanks with order being counterbalanced. They were age and weight matched, in 3:2 females:males ratio.

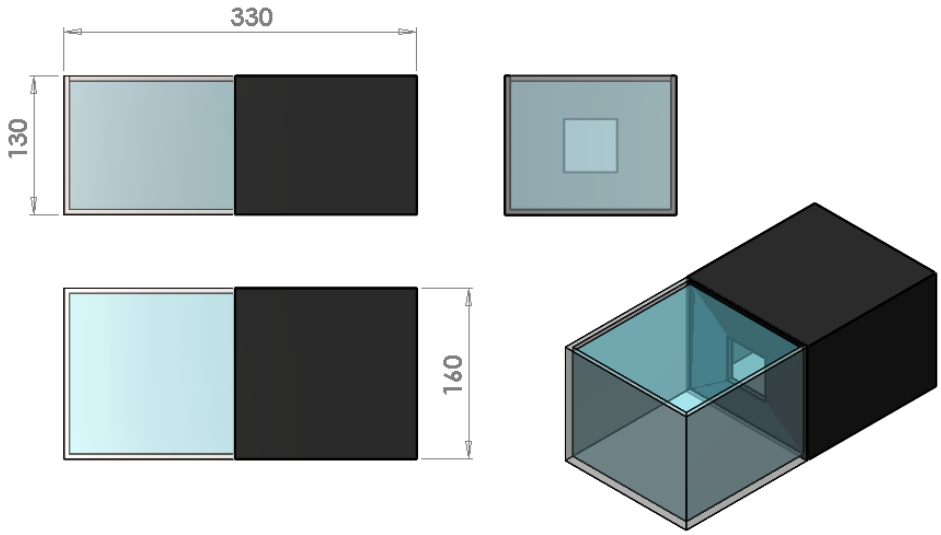


Figure 2.5: Scototaxis tank used for stress-reactivity measured of 6-month-old zebrafish adults. Tanks were opaque, translucent illustration to ease visualization.

2.4.5 Individual social behaviour

Individual social behaviour (also known as approach behaviour) is a measure of time spent by a single individual in a social zone of the tank (area near the acrylic perforated sheet), where they could respond to visual and olfactory cues of a group of 5 “strange” zebrafish adults swimming in the other portion of the tank through an acrylic perforated sheet (figure 2.6). They were tested in clear acrylic tanks (230mm width x 280mm top length x 250mm bottom length x 185mm height).

The apparatus consisted of a tank divided into two sections, a larger and a smaller one (figure 2.6). A group of 5 control fish was placed in the larger side of the tank. These fish were from an unrelated tank and had not previously been in contact with the fish to be tested. At the start of the experiment the fish to be tested was placed in the smaller side.

The fish behaviour was filmed over a 20-minute period. The time spent in the region defined as ‘social segment’ (figure 2.6) was determined in individual subjects. Testing was done during the light phase (9am-5pm). Ethanol treated and control groups were tested alternately in three identical tanks with order being counterbalanced. They were age and weight matched, in 3:2 females:males ratio.

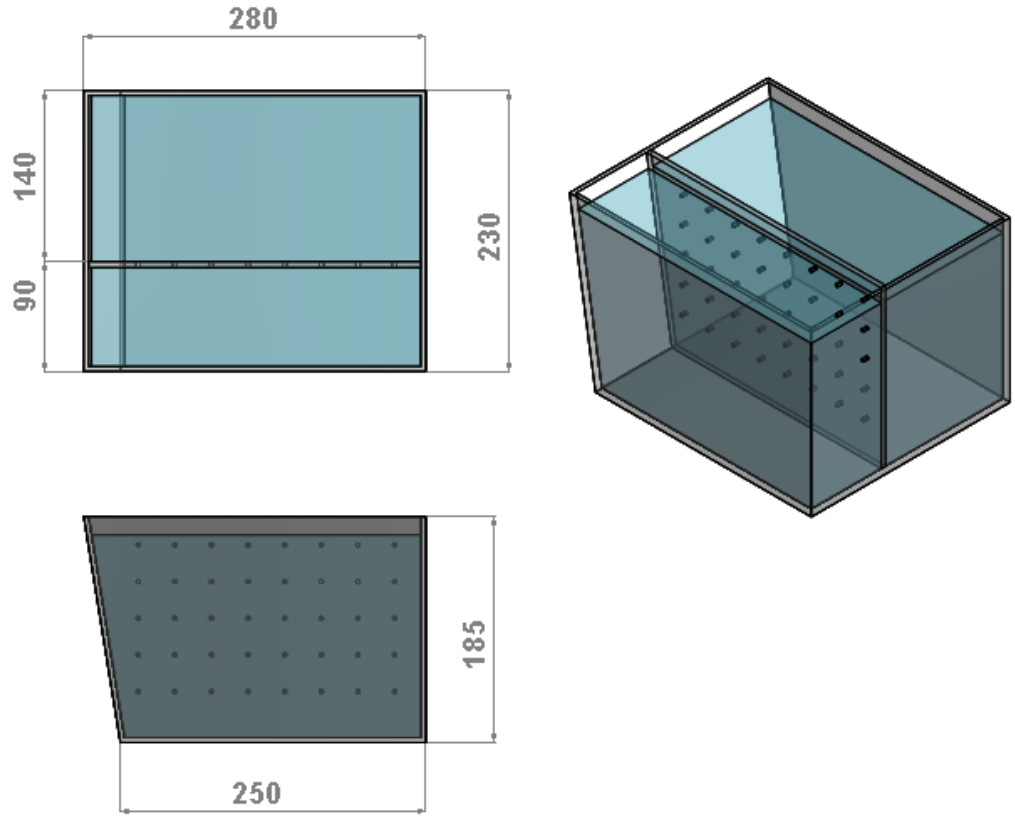


Figure 2.6: Individual social behaviour tank scheme used for social interaction measurement in 6-month-old zebrafish adults.

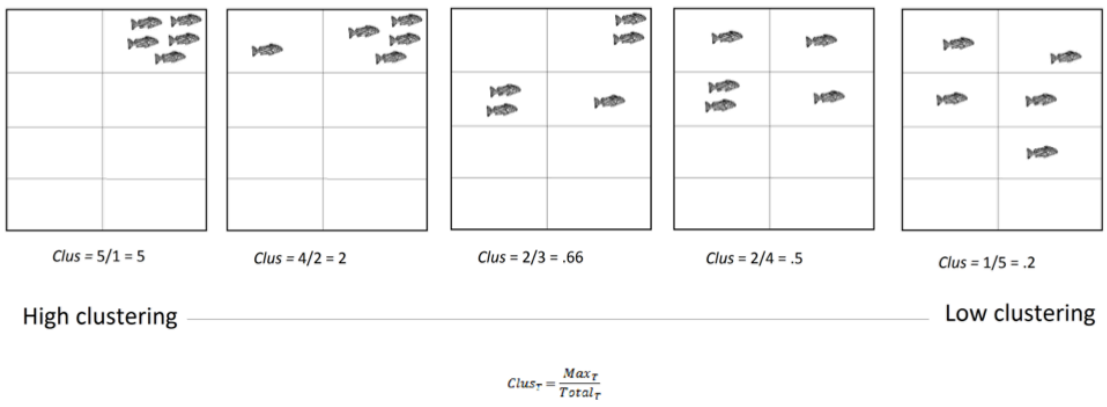
2.4.6 Shoaling

Shoaling is another way to determine social interaction; by measuring how "together" the fish shoal as a group. It works by attributing a cluster score to the group every 30sec, which consists in diving the highest number of animals in one of the eight sections of the tanks, per the total sections occupied by the animals (figure 2.6, A).

Shoaling assay was carried out in opaque white tanks (410mm width x 490mm length x 150mm height) containing 6L of fish water (figure 2.6, B). Animals were carefully transferred into the testing tanks and allowed to acclimatize for 5 minutes, then filmed for 10 minutes.

Testing was done during the light phase (9am-5pm). Ethanol treated and control groups were tested alternately in three identical tanks with order being counterbalanced. They were age and weight matched, in 3:2 females:males ratio. Every 30-second interval, still pictures of the recorded videos were taken, and the cluster score was calculated by dividing the maximum number of fish in one section of the tank by the total number of sections occupied by the animals (figure 2.6, A).

A



$$Clus_T = \frac{Max_T}{Total_T}$$

B

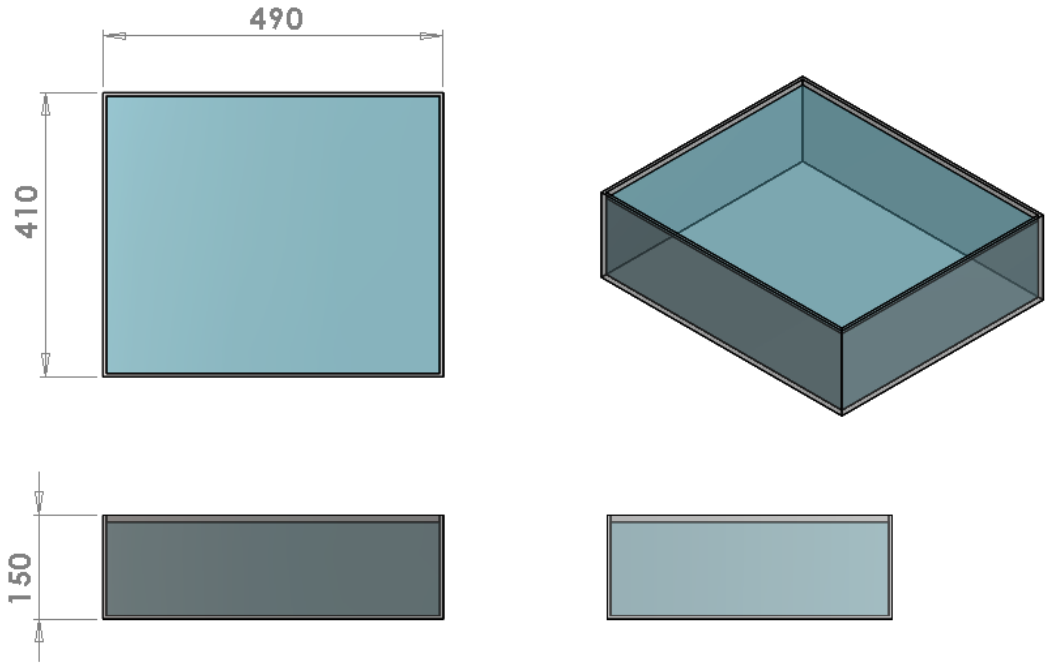


Figure 2.7: Shoaling tank scheme used for social interaction measurement in 6-month-old zebrafish adults. Method to obtain the clustering score (A) and shoaling tank measurements (B). Tanks were opaque, translucent illustration to ease visualization.

2.4.7 Statistics

For stress reactivity assays and individual social behaviour, data were fitted to a linear mixed effects model that observes mixed effects like drug exposure, distances travelled and the time effect in the measured aspect of the assay, using R software. Distances travelled were entered as a covariate in order to control for immobility and darting periods. Dependent variable were time spent in the designated zones. Anova and a series of student t-tests were used in shoaling social behaviour. The tests were evaluated with respect to type-1 error rate of 0.05.

2.5 Zebrafish larvae, juveniles and adults whole-body cortisol analysis

2.5.1 Air exposure challenge

Air exposure challenge in larvae and juveniles as described on 2.3.2 was used. Animals were then quickly pipetted into Starlab® 1.5mL crystal clear microcentrifuge tube (catalogue No.: E1415-1500), water was removed with the aid of a glass pasteur pipette and tubes were immediately flash frozen in liquid nitrogen and stored at -80^{o}.

Adult fish were individually netted from pair-housed tanks into white opaque tanks (42.5cm length x 16cm width x 17.5cm height) containing 2L of fish water. These tanks contained a smaller clear acrylic bottom-perforated tank, to allow easy and quick air exposure of the animals.

Tanks were covered with an opaque lid to prevent any motion perception from the researchers in the room and were left to habituate for 1 hour in a lit and silent environment. The animals were then air exposed by lifting the small acrylic tank and placed on the side for 30 seconds, then placed back into the larger opaque tank.

Animals were transferred 5 minutes later into an ice-cold water bucket for anaesthesia, dried on a paper towel, weighed and placed into 7ml Sterilin® polystrene sample containers (catalogue No.: 129B) and immediately flash frozen in liquid nitrogen, and stored at -80^{o}.

2.5.2 Larvae and juveniles whole body cortisol extraction

A modified version of the protocol of (Alderman and Bernier 2009) was used for homogenization and extraction. Microcentrifuge tubes were thawed on ice and samples were homogenized in 200µL of ice-cold 1x Sigma® PBS (catalogue No.: P4417-100TAB) using a Bandelin® Sonoplus UW2070 ultrasonicator for 10 seconds (Power 70%). following the addition of 200µL of ice-cold 1x PBS used to rinse the equipment's needle, in order to remove any remaining tissue, collected into the microcentrifuge tube A 50µL aliquot was pipetted into an additional microcentrifuge tube for protein quantification.

The ultrasonicator needle was then washed between homogenizations; once with 70% ethanol then once with distilled water, and dried with a paper towel. All samples were kept on ice during this process. Cortisol was extracted by adding 500µL of Fischer Chemical® ethyl acetate (catalogue No.: E/0850/17) to the homogenate. Samples were vortexed for 30 seconds, spun in a table-top centrifuge at 5000rpm for 10 miunutes and frozen at -80C^{o} for 25 minutes. The organic layer was then tipped off into a 10mL glass screw-top tube. Ethyl acetate was added again and the process was repeated twice more.

Tubes were placed in a rack inside a waterbath set at 60C^{o}, which was placed inside a fume hood. Nitrogen was gently blown into the tubes until all the ethyl acetate was evaporated (~1 hour). 200µL of ice-cold 1x PBS was added to the tubes, vortexed for 30 seconds, and kept at -20C^{o} until assessed with salimetrics human salivary cortisol kit (catalogue No.: 1-3002).

2.5.3 Larvae and juveniles whole body protein quantification

To quantify the protein contents of the homogenates, Bio-Rad bradford reagent was used (catalogue No.: B6916). Samples were thawed in ice, 50µL of ice-cold distilled water was added and microcentrifuge tubes were vortexed for 15 seconds. Standards were made with Sigma® bovine serum albumin (BSA) (catalogue No.: A9647-50g) ranging from 0.05mg/mL to 0.5mg/mL.

Samples were assessed in 10 µL duplicates using a Bio-Rad protein assay microtiter 96-well plate protocol according to the manufacturer's specifications. Absorbance was measured at 595nm wavelength in a plate reader. Sample protein content was calculated from the linear standard curve.

2.5.4 Adult whole body cortisol extraction

Modified from Allan Kalueff's adult zebrafish whole-body cortisol extraction (Egan, Bergner et al. 2009). Containers with fish were thawed on ice, the animals were weighed, and PBS added (2 X BW w/v). Animals were homogenized in pulses of 30 seconds and the rotor blade washed with an equal volume of PBS (2 X BW w/v), which was then added to the homogenate. The rotor blade was then washed between homogenizations; once with 70% ethanol then once with distilled water, and dried with a paper towel. All samples were kept on ice during this process.

Containers were quickly vortexed and 500µL of the homogenate was pipetted into microcentrifuge tubes. Cortisol was extracted by adding 500µL of ethyl acetate

to the homogenate in the microcentrifuge tube. Samples were vortexed for 30 seconds, spun in a table-top centrifuge at 5000rpm for 10 minutes and frozen at -80C^{o} for 25 minutes.

The organic layer was then tipped off into a 10mL glass screw-top tube. Ethyl acetate was added again and the process was repeated another 2X. Tubes were placed in a rack inside a waterbath set at 60C^{o}, which was placed inside a fume hood. Nitrogen was gently blown into the tubes until all the ethyl acetate was evaporated (~1 hour). 200µL of ice-cold 1x PBS was added to the tubes, vortexed for 30 seconds.

When homogenizing adult zebrafish tissue, a layer of lipids formed on the walls of the glass screw-top tube at the end of the nitrogen-blowing step. To minimise any cortisol differences caused by the lipid layer, a dissolving process was done. It consisted of adding 300µL of ice-cold 1x PBS to the existing 200µL of ice-cold 1x PBS added at the final step, following addition of 500µL of BDH® n-Hexane (catalogue No.: 203-777-6).

Glass tubes were vortexed for 30s and centrifuged at 2000rpm at 4C^{o} for 10 minutes. A glass Pasteur was used to transfer the bottom PBS layer into a new microcentrifuge tube, ready to etiher be used immediately in the salimetrics human salivary cortisol kit or stored at -20C^{o} until assessed.

2.5.5 Whole body cortisol assessment using Salimetrics kit

To quantify whole body cortisol extracts, ELISA was performed using a Salimetrics human salivary cortisol kit. Samples were thawed on ice and 50µL was used for each sample's quantification. The protocol was followed according to the manufacture's specifications. Absorbance was measured at 450nm wavelength in a plate reader.

Cortisol concentrations were calculated using a 4-parameter non-linear regression curve based on the absorbances of the standards provided. Standard curves were bound cortisol (B/Bo) plotted against log of the standards (ng/mL). B/Bo was calculated by dividing the optical density (OD) of the samples, by the OD of the "zero" (sample with no competitive enzyme conjugate binding) after subtracting absorbance of non-specific binding (NSB). In the case of larvae and juveniles, cortisol samples were further normalized against total protein of the animals.

2.5.6 Water-borne cortisol assessment

Zebrafish 9dpf larvae were pipetted in groups of 5 animals into glass petri dishes (90mm diameter x 16mm height) filled with 5mL. Animals were acclimatized in a quiet and lit environment 1 hour prior to the beginning of the assay. Animals were submitted to a mechanical swirling stress (100rpm). Water samples were collected and stored into 10mL glass screw-top tubes. Cortisol was extracted and assayed exactly as stated in Rui Oliveira's published protocol (Felix, Faustino et al. 2013).

2.6 Zebrafish larvae quantitative real-time PCR (qPCR) and in situ hybridization (ISH)

2.6.1 RNA isolation and cDNA synthesis

Embryos used for qPCR were collected in groups of 15 animals in Starlab® 1.5mL crystal clear microcentrifuge tubes (catalogue No.: E1415-1500) immediately after ethanol treatment, flash-frozen in liquid nitrogen and preserved at -80°C until required for RNA extraction.

Total RNA extraction was performed using 1ml of Sigma-Aldrich's TRI Reagent® (catalogue No.: T9424) following the manufacturer's specifications. RNA was treated with RNAse free DNAse I from NEB (catalogue No.: M0303S) for 10

minutes at 37^{o}C. The integrity of the RNA and genomic DNA contamination were checked by gel electrophoresis (1% gel, 50V for 40 minutes, 4^{o}C)

Upon verification of the RNA quality, 3µg of the total RNA extraction was reverse-transcribed using Invitrogen's Cloned AMV First-Strand Synthesis Kit (catalogue No.: 12328-040) according to the instructions provided by the manufacturer, in Starlab® 0.2mL crystal clear PCR tubes (catalogue No.: L1402-8100). Immediately after the reaction, 60µl of nuclease-free water was added to the 20µl then stored at -20^{o}C until analysis.

2.6.2 Absolute quantitative real-time PCR (qPCR)

The mRNA expression of the HPI and housekeeping genes were quantified.. First, to construct standard curves, large amounts of the PCR product for each gene were generated using the primers listed on figure 2.8, and purified using Qiagen's QIAquick PCR Purification Kit (catalogue No.: 28104).

DNA concentrations were assayed using a NanoDrop Spectrophotometer and dilutions were calculated from the PCR fragment size to produce a solution with 10^{11} gene copies per 2µl. This concentrated solution was subsequently diluted to form all the data points needed to produce the standard curve in the form of a linear plot of the Ct values supplied by the PCR cycler against the logarithm of known amount of fragments initially introduced in the mixture (Figure 2.7, A).

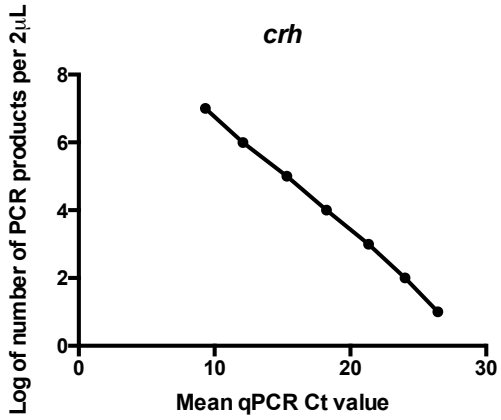
A mixture consisting of 10µL of *Power* SYBR® Green PCR Master Mix from Applied Biosystems (catalogue No.: 4368706), 6µL of double distilled water

(ddH_2O), 2µL of forward and reverse primers (10µM), and 2µL of cDNA (unknown or standard solution) was pipetted into each well of a Starlab® 96-well crystal clear PCR plate, non-skirted (catalogue No.: B1402-0595).

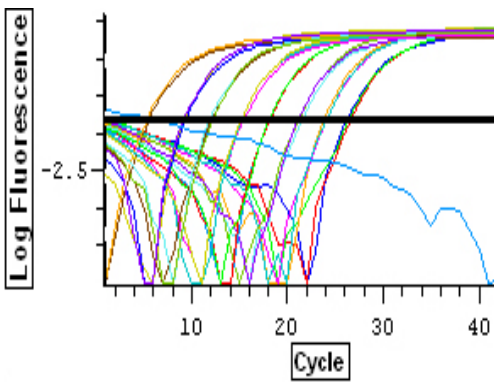
Quantitative real-time PCR was performed in a MJ Research PTC-200 DNA Engine cycler. The cycling parameters were as follows: 95°C for 10 minutes, followed by 40 cycles at 95°C for 10s and 61°C for 42s. Melting curve analysis was performed (0.2°C /sec increase from 61°C to 95°C with fluorescence readings taken every 1°C increment) to ensure that a single amplification was being achieved (Figure 2.7, C).

The absolute number of copies of the genes was calculated by matching the average Ct values of the unknowns (done in triplicates) with the standard curve Ct values. Normalization of gene expression was achieved dividing the absolute copy number of the target gene by the arithmetic average of all the three housekeeping genes.

A



B



C

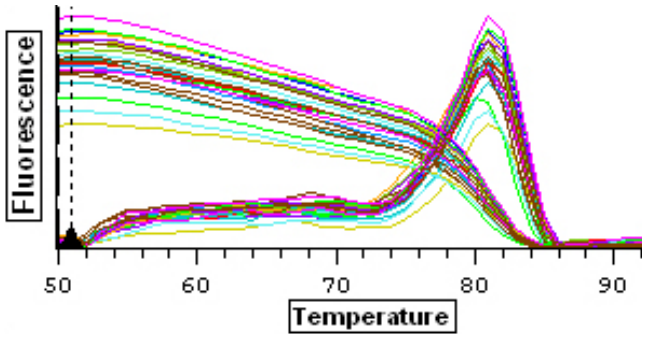


Figure 2.8: CRH standards data. Standards used were 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10 CRH PCR products per 2µL. Plot of the log of the number of PCR products initially introduced in the reaction against the provided Ct values (A). Standards reaching threshold fluorescence according to their initial concentration, 10^7 reaches at Ct=9.3 while 10 copies per 2µL reaches at Ct=26.4 (B). Melting curve indicating one type of fragment being amplified (C). Reactions were done in triplicates. R^2 = 0.9986.

Gene Symbol	Primers (5' > 3'), forward (**F**) and reverse (**R**)	Accession N°	Product Length (bp)
crh	**F:**ACGCACAGATTCTCCTCGCCAC **R:**CGCGGCTGGCTGATTGAAGC	NM_001007379.1	132
crhbp	**F:**GCCGACCTTCATTTGAGCTGGTCC **R:**CAAGAGGTCCATTCTTGGCTGCG	NM_001003459.1	139
crhr1	**F:**TACAGCTCGCACTTTAATCCTGTGG **R:**CTCCAGCACTGCTCCTGGGC	XM_691254.2	122
crhr2	**F:**GCGTCGATGGTGTCTATTGTAACGC **R:**TACTCCGGACACGGTCGCTCA	XM_002667848.2	100
nr3c1	**F:**ACAGCTTCTTCCAGCCTCAG **R:**CCGGTGTTCTCCTGTTTGAT	XM_005173120.1	116
nr3c2	**F:**CCCATTGAGGACCAAATCAC **R:**AGTAGAGCATTTGGGCGTTG	NM_001100403.1	106
β-actin	**F:**CGAGCAGGAGATGGGAACC **R:**CGAGCAGGAGATGGGAACC	NM_131031.1	102
gapdh	**F:**TGAGCTCAATGGCAAGCTTACTGGT **R:**TCAGCTGCAGCCTTGACGACT	NM_001115114.1	135
gap43	**F:**TGCTGCATCAGAAGAACTAA **R:**CCTCCGGTTTGATTCCATC	NM_131341.1	82
elavl1	**F:**CCATTGACATTTCTCTGTGGAAGT **R:**GAGGTACCAGTGATCATGTTCTTGA	XM_005173785.1	106

Figure 2.9: List of primers designed and used for real-time quantitative PCR experiments.

2.6.3 Competent cells production, bacterial transformations and purification of plasmid

To make competent cells, Invitrogen® TOP10 cell line (catalogue No.: C4040-10) was used. This cell line has an original transformation efficiency of $1x10^9$ colony-forming units/μg of plasmid DNA. TOP10 cells were struck into an LB agar plate (Sigma®, catalogue No.: 19344-500G-F) and incubated 37°C overnight.

Colonies were then selected using a sterile pipette tip and inoculated into a 50mL sterile Falcon® tube (catalogue No.: 734-0453) containing 5mL of LB broth (Sigma®, catalogue No.: L3022-1KG), and incubated overnight at 37°C. 1mL of this broth inoculated into a 250mL conical flask previously autoclaved with 100mL of LB broth and colonies were grown at 37°C in a shaker (100rpm) until reaching OD600, which took about 2 hours.

Cells were then chilled on ice for 15 minutes and centrifuged using 2 sterile 50mL tubes at 5000rpm for 30 minutes at 4°C. Supernatants were removed and 50mL of TFBI solution was added to the combined pellets (composition below) and left on ice for 15 minutes. Tubes were centrifuged at 5000rpm, 30 minutes, 4°C, then re-suspended in 4mL of ice-cold TFBII (composition below) and aliquots of 100μL were flash frozen in liquid nitrogen and stored at -80°C.

TFBI

0.294g Potassium acetate (VWR® GPR, catalogue No.: 437063N)
0.989g $MnCl2 *4H_2O$ (BDH®, catalogue No.: 101523J)
0.745g KCl (VWR® GPR, catalogue No.: 26759.291)
0.147g $CaCl *2H_2O$ (BDH®, catalogue No.: 100704Y)
15ml glycerol (VWR® GPR, catalogue No.: 24387.326)
85mL ddH_2O

TFBII

0.209g MOPS (Sigma® GPR, catalogue No.: M1254-25G)
1.1g $CaCl2 *2H_2O$ (BDH®, catalogue No.: 100704Y)
0.075g KCl (VWR® GPR, catalogue No.: 26759.291)
15ml glycerol (VWR® GPR, catalogue No.: 24387.326)
85mL ddH_2O

Both solutions were filtered through sterile 0.22μm filters.

For the bacterial transformation, heat shock was used. Cell aliquots were thawed on ice for 10 minutes and combined with 10μL of target plasmid in the 1.5ml microcentrifuge tube, following incubation on ice for another 30 minutes. Preparations were then heat shocked at 42°C for 45 seconds following incubation on ice for 2 minutes. LB media (900μL) was added to the tubes and incubated with shaking for 1 hour at 37°C.

Contents were plated on to an LB plate containing ampicillin 100μg/mL (Sigma®, catalogue No.: A-0166-5G). Colonies were selected the following day and incubated in 5mL of LB broth (Sigma®, catalogue No.: L3022-1KG) with ampicillin, overnight at 37°C. Samples were then mini-prepped using a Qiagen® kit according to the manufacturer's specifications to purify the plasmid (catalogue No.: 27104).

2.6.4 Larvae collection for in situ hybridization

To determine the patterns of *crh, crhbp and pomca* gene mRNA transcription in ethanol exposed zebrafish embryos, casper zebrafish larvae were collected at either 2dpf or 9dpf following 1-9dpf ethanol treatment. The casper mutant line lacks skin pigmentation, which makes it ideal for in situ hybridization (Wenner 2009, Parker, Brock et al. 2013). They were anaesthetised with Tricaine (160mg/L, Sigma® (catalogue No.: A-5040) and fixed in 4% Prolabo® paraformaldehyde (catalogue No.: 28 794.295)/PBS overnight at 4°C, then stored at -20°C in 100% Merck® methanol (catalogue No.: 607-089-00-0).

2.6.5 Probe synthesis for in situ hybridization

cDNA for *crh* and *crhbp* was received as a gift from Dr Giselbert Hauptmann from Stockholm University, cloned into Stratagene® pBluescript KS and Express Genomics® pExpress-1 respectively. *Pomca* was cloned in our facility using Promega® pGEM® T-Easy (catalogue No.: A1360) according to the manufacturer's specifications, following amplification by PCR using the *pomca* primers (Figure 7.2.4.1).

Antisense and sense riboprobes were generated using PCR products purified using Qiagen® QIAquick PCR purification columns (catalogue No.: 28104) obtained by setting a PCR reaction with M13 primers (figure 2.9) with templates of zebrafish *crh, crhbp* and *pomca*. The cycling parameters for the M13 primers were

as follows: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15s, 61°C for 30s and 67°C for 2 minutes. Final elongation done at 67°C for 5 minutes.

Probes were synthesized using a 20μL reaction in a PCR tube, using purified PCR DNA product (0.5μg in the reaction), a nucleotide mix containing digoxigenin labelled nucleotide, Digoxigenin-11-UTP (0.875 nmol in the reaction) (Roche, catalogue No.: 11573152910), and Roche's RNA polymerase T7 or SP6 (5 units in the reaction) (catalogue No.: 10881767001 and 10810274001 respectively) with RNA polymerase's respective transcription buffers (10X concentrated).

This reaction was incubated at 37°C for 4 hours. The integrity of the RNA was checked by gel electrophoresis (1% gel, 50V for 40 minutes, 4°C). RNA was precipitated using 3M sodium acetate, acetic acid and ethanol, then re-suspended in a solution of 50% BDH® formamide (catalogue No.: 103266T), 5x Sodium saline citrate (SSC) (Sodium chloride, Sigma®, catalogue No.: S3014-1KG; Sodium citrate, SAFC®, catalogue No.: W302600-1KG-K) and ddH_2O and stored at -20°C. The riboprobe sequences are listed below (figure 2.10).

M13 primers (5' > 3') forward (**F**) and reverse (**R**)	*pomca* primers (5' > 3') forward (**F**) and reverse (**R**), Accession: NM_181438.3, 582bp
F:GTTTTCCCAGTCACGAC **R:**CAGGAAACAGCTATGAC	**F:**TCAGAGACGAGCAAACGCAA **R:**AAAGGCATCTCTTCTTGCGGA

Figure 2.10: M13 primers used to amplify cloned sequences using plasmids as template, and, pomca primers used to amplify PCR product to be cloned on pGEM® T-Easy vector.

A

CRH Antisense Riboprobe (5' > 3')	CRH Sense Riboprobe (5' > 3')
UUUUUUUUUU UUUUUGUCUU UCAAAGUUUU AUUGAAAAAU AUAAAUAUAA GACAUUCUCU UGCACAACAA AAAUUAAUGG UUGCUCAUUA GUUUAUUAAC AAACUUAAAC AAUGUACUGU AUGUUACAAU GAGACUCAAG UCUGUCAGAU AAAAUAAUAC AGUACUGGAA AAUAUUGUCU UUUUAUUUUU UAAAGGAAAC CCAAACGUUU UUCAUCUACA GUUCUGAAUG AUCACUUAUA CAAUAACAUC GAUGGAAAGU GAUGACAGUG UUGCGCUUCU GAACACUUUG AUUCAUAUAC ACCUACUGCA CUCUAUUCGC CUUCCUUGAG AUAUCUACAA AUAAAACAAG UCACCGGUAU AAAUAGAUCA UGAUGGAAAA GCAGCACUAU GGUACAGAGU AUUCAUGUUU GUGCUAAAUG UAAAAAUAUC UUUGGCUGAU GGGUUCGCUC GUGGUUACUU CCCGAAUAUU UCCAUCAUUU UGCGGUUGCU GUGAGCUUGC UGGGCCAUUU GCUCGGCUCU GGCCAUCUCC AGUACUUCUC GUAGCAGAUG AAAGGUCAGA UCUAGGGAAA UCGGCGGCUC CUCCGACCUG CGCUCCCUCU CCAUUGAGUC GAGCGCCCGG AGCGCGUAAC UGCCAUCCAA GCGGCCGAUG UUUCCAACUU UCCCCUCCAA CAGACGCUGC GUUAACUGGA GCUGCAGCGC UCUUUUUGGA UACUGGGAUG UCUCGGGGAA GCUGUCGGCU GGAGAUCGGG GGAGAAGUCG GGUUUCUGUU GCCGAGCCGG AUGAAGUACU CCUCCCCCAA GCGUGCCAAA ACCGGCGGGG ACUGCCGCUC UCCAUCGGGG UCCGCGGCUG GCUGAUUGGA GCUGCUCUCG AUGGCUCUAC AUUCAUACGG	CCGUAUGAAU GUAGAGCCAU CGAGAGCAGC UCCAAUCAGC CAGCCGCGGA CCCCGAUGGA GAGCGGCAGU CCCCGCCGGU UUUGGCACGC UUGGGGGAGG AGUACUUCAU CCGGCUCGGC AACAGAAACC CGACUUCUCC CCCGAUCUCC AGCCGACAGC UUCCCCGAGA CAUCCCAGUA UCCAAAAAGA GCGCUGCAGC UCCAGUUAAC GCAGCGUCUG UUGGAGGGGA AAGUUGGAAA CAUCGGCCGC UUGGAUGGCA GUUACGCGCU CCGGGCGCUC GACUCAAUGG AGAGGGAGCG CAGGUCGGAG GAGCCGCCGA UUUCCCUAGA UCUGACCUUU CAUCUGCUAC GAGAAGUACU GGAGAUGGCC AGAGCCGAGC AAAUGGCCCA GCAAGCUCAC AGCAACCGCA AAAUGAUGGA AAUAUUCGGG AAGUAACCAC GAGCGAACCC AUCAGCCAAA GAUAUUUUUA CAUUUAGCAC AAACAUGAAU ACUCUGUACC AUAGUGCUGC UUUUCCAUCA UGAUCUAUUU AUACCGGUGA CUUGUUUUAU UUGUAGAUAU CUCAAGGAAG GCGAAUAGAG UGCAGUAGGU GUAUAUGAAU CAAAGUGUUC AGAAGCGCAA CACUGUCAUC ACUUUCCAUC GAUGUUAUUG UAUAAGUGAU CAUUCAGAAC UGUAGAUGAA AAACGUUUGG GUUUCCUUUA AAAAAUAAAA AGACAAUAUU UUCCAGUACU GUAUUAUUUU AUCUGACAGA CUUGAGUCUC AUUGUAACAU ACAGUACAUU GUUUAAGUUU GUUAAUAAAC UAAUGAGCAA CCAUUAAUUU UUGUUGUGCA AGAGAAUGUC UUAUAUUUAU AUUUUUCAAU AAAACUUUGA AAGACAAAAA AAAAAAAAAA

B

crhbp antisense riboprobe (5’ > 3’)	*crhbp* sense riboprobe (5’ > 3’)
UAGCUUUUUUUUUUUUUUUUUUUUU UUCAUGUUGACUGAUUUUAUUACAAAA GAUCUUUAAUACAGAUAAAUAAAUAAA GACAAAUACAUUUUCCGUACAUACUGGA UUUUAAUACUUGUGCAGUACCCCUCAUU UCUAAUAUGGGCUCUUCAAAAUGAAACA UGCUGAACCAAAAAAAUAAUUAAAAACA GUGAUGUGCACCAACACAAGUACAUCUU UUAUUUAACAUCAGCCACAGAUCUUACA AGUCAAUGAUAUUAAAAAGAACAGAAG AGUCAAACCAAAAGAUGAGGCCAAAAAU AAAUAAUUUUUUUAAUAAGGAAAAUAU ACAAGGGCCCGAAGGCUCCAACUCCAGU UAGUUUGGCAUCCCUCCUAACAAGAGCA GGGAAAUGGAGAGAUCUUAUGCUCUUA AACACACGUCUUCAACACUGUUGCCCUU CAUCUGCUGAAGCUCUUGGUGGCCCAGU AGCCGAUACUGGAAACUGACUCGAUUUA CGAACUUCCCGCUCGACACCAUUCUGAC CACAGUGUUAUCACAGCCGACCUUCAUU UGAGCUGGUCCAUUAAAGGAGUAGCAGA GAUCUGCCAUUGGGAACAUCUUAGACGU GUCCAUGCCGUUUCCACCAAGAAGCUCA ACAAAGUCUCCAGAACCAGCGCAGCCAA GAAUGGACCUCUUGAGAUCAUUGUGCUG UCCAAGGCUGAGCUCUCCAAUCUGGAUC UCCACUGGAUAGAUGAUGGAGAAGCUGC AGUUCCUGUGCUGCUGAGGAAUGAUCAU GGUGAAACUGCCCUCUGGGGUCUGAGAC ACAACAUUACAGGGGAAGGGAUUGAUG AGUUUGCGAAAUGUUACUGUGAAGCUGC UUCCUGAUUGGUGGAGCCUGAAGAACAG CAUGGCGACGUUCUGAGAGGAACGUACG AUUGGUCGAGACACUCCAGUUUCGCAGU AAUCAGUGUAACGCUCAUACAGAGGGAG AGGAUGAUCCUGCGAGCUGGGGAACUUC UCAC	GUGAGAAGUUCCCCAGCUCGCAGGAUCA UCCUCUCCCUCUGUAUGAGCGUUACACUG AUUACUGCGAAACUGGAGUGUCUCGACC AAUCGUACGUUCCUCUCAGAACGUCGCCA UGCUGUUCUUCAGGCUCCACCAAUCAGG AAGCAGCUUCACAGUAACAUUUCGCAAA CUCAUCAAUCCCUUCCCCUGUAAUGUUGU GUCUCAGACCCCAGAGGGCAGUUUCACCA UGAUCAUUCCUCAGCAGCACAGGAACUG CAGCUUCUCCAUCAUCUAUCCAGUGGAG AUCCAGAUUGGAGAGCUCAGCCUUGGAC AGCACAAUGAUCUCAAGAGGUCCAUUCU UGGCUGCGCUGGUUCUGGAGACUUUGUU GAGCUUCUUGGUGGAAACGGCAUGGACA CGUCUAAGAUGUUCCCAAUGGCAGAUCU CUGCUACUCCUUUAAUGGACCAGCUCAA AUGAAGGUCGGCUGUGAUAACACUGUGG UCAGAAUGGUGUCGAGCGGGAAGUUCGU AAAUCGAGUCAGUUUCCAGUAUCGGCUA CUGGGCCACCAAGAGCUUCAGCAGAUGA AGGGCAACAGUGUUGAAGACGUGUGUUU AAGAGCAUAAGAUCUCUCCAUUUCCCUG CUCUUGUUAGGAGGGAUGCCAAACUAAC UGGAGUUGGAGCCUUCGGGCCCUUGUAU AUUUUCCUUAUUAAAAAAAUUAUUUAUU UUUGGCCUCAUCUUUUGGUUUGACUCUU CUGUUCUUUUUAAUAUCAUUGACUUGUA AGAUCUGUGGCUGAUGUUAAAUAAAAGA UGUACUUGUGUUGGUGCACAUCACUGUU UUUAAUUAUUUUUUUGGUUCAGCAUGUU UCAUUUUGAAGAGCCCAUAUUAGAAAUG AGGGGUACUGCACAAGUAUUAAAAUCCA GUAUGUACGGAAAAUGUAUUUGUCUUUA UUUAUUUAUCUGUAUUAAAGAUCUUUUG UAAUAAAAUCAGUCAACAUGAAAAAAAA AAAAAAAAAAAAAAAAAGCUA

C

pomca antisense riboprobe (5’ > 3’)	*pomca* sense riboprobe (5’ > 3’)
CCGAACGACCGAGCGCAGCGAGUCAGUG AGCGAGGAAGCGGAAGAGCGCCCAAUAC GCAAACCGCCUCUCCCCGCGCGUUGGCC GAUUCAUUAAUGCAGCUGGCACGACAGG UUUCCCGACUGGAAAGCGGGCAGUGAGC GCAACGCAAUUAAUGUGAGUUAGCUCAC UCAUUAGGCACCCCCAGGCUUUACACUU UAUGCUUCCGGCUCGUAUGUUGUGUGGA AUUGUGAGCGGAUAACAAUUUCACACAG GAAACAGCUAUGACCAUGAUUACGCCAA GCUAUUUAGGUGACACUAUAGAAUACUC AAGCUAUGCAUCCAACGCGUUGGGAGCU CUCCCAUAUGGUCGACCUGCAGGCGGCC GCGAAUUCACUAGUGAUUAAAGGCAUCU CUUCUUGCGGAUAGUCGACCUCGUUAUU UGCCAGCUCGCGUCUCAUCUCUGCCGGA AGCGUUUCGGCGGAUUCCUCUUCCGCGC CGUUCGUGUACACCUUGAUGGGUCUGCG UUUGCGGCCGACCGGUUUGCCCCACCGG AAGUGCUCCAUGGAGUAGGAGCGCUUGU GGUCGUGUCGAGGGCCGGACUCCGGCUC UAUCUGUUCAGGGGCUGCGAGUGCUACA GGGGAAAGGUGUGCGAGGAGGUCGAUU UGCUCCGGCUCGGAGGGAGGCUGUAGAU GGCUUUCUCCAGGGUAGACGGGGGUUUC AUCUGUAAGUUCAGACCUGCAUAAUUGU AUGCAUUCCAAGAUGUUCUCCUCUGUGC UGAGGUCUCGACAGCGGGCAUUUUCCCA ACACUGAGCUCUGACUUCAGAUCCUCCU GCGCAGAGAACAGCCAGAGCCAAGAGCC AAGCAGGACACAACAUCCUCACUCCCCU CACCAUCUCUUUUGCACAGAAAGAAGUC AGUAACCAAAGUUGUUCUCUCUGAGAGU CUCGGAUGUGUCUGGUUGUCUUUGCGUU UGCUCGUCUCUGAAAUCGAAUUCCCGCG GCCGCCAUGGCGGCCGGAGCAUGC	GCAUGCUCCGGCCGCCAUGGCGGCCGCGG GAAUUCGAUUUCAGAGACGAGCAAACGC AAAGACAACCAGACACAUCCGAGACUCUC AGAGAGAACAACUUUGGUUACUGACUUC UUUCUGUGCAAAAGAGAUGGUGAGGGGA GUGAGGAUGUUGUGUCCUGCUUGGCUCU UGGCUCUGGCUGUUCUCUGCGCAGGAGG AUCUGAAGUCAGAGCUCAGUGUUGGGAA AAUGCCCGCUGUCGAGACCUCAGCACAGA GGAGAACAUCUUGGAAUGCAUACAAUUA UGCAGGUCUGAACUUACAGAUGAAACCC CCGUCUACCCUGGAGAAAGCCAUCUACAG CCUCCCUCCGAGCCGGAGCAAAUCGACCU CCUCGCACACCUUUCCCCUGUAGCACUCG CAGCCCCUGAACAGAUAGAGCCGGAGUCC GGCCCUCGACACGACCACAAGCGCUCCUA CUCCAUGGAGCACUUCCGGUGGGGCAAA CCGGUCGGCCGCAAACGCAGACCCAUCAA GGUGUACACGAACGGCGCGGAAGAGGAA UCCGCCGAAACGCUUCCGGCAGAGAUGA GACGCGAGCUGGCAAAUAACGAGGUCGA CUAUCCGCAAGAAGAGAUGCCUUUAAUC ACUAGUGAAUUCGCGGCCGCCUGCAGGU CGACCAUAUGGGAGAGCUCCCAACGCGU UGGAUGCAUAGCUUGAGUAUUCUAUAGU GUCACCUAAAUAGCUUGGCGUAAUCAUG GUCAUAGCUGUUUCCUGUGUGAAAUUGU UAUCCGCUCACAAUUCCACACAACAUACG AGCCGGAAGCAUAAAGUGUAAAGCCUGG GGGUGCCUAAUGAGUGAGCUAACUCACA UUAAUUGCGUUGCGCUCACUGCCCGCUU UCCAGUCGGGAAACCUGUCGUGCCAGCU GCAUUAAUGAAUCGGCCAACGCGCGGGG AGAGGCGGUUUGCGUAUUGGGCGCUCUU CCGCUUCCUCGCUCACUGACUCGCUGCGC UCGGUCGUUCGG

Figure 2.11: Sense and antisense riboprobe sequences for *crh* (A), *crhbp* (B) and *pomca* (C).

2.6.6 In situ hybridization

A standard 3-day-procedure in situ hybridization whole-mount protocol was used, adapted from Jowett’s in situ protocol (Jowett 1997) with probe hybridization

performed on day 1, following detection with a polyclonal anti-digoxigenin-AP antibody (Roche, catalogue No.: 11093274910) on day 2, and development with colorimetric AP substrate BM Purple (Roche, catalogue No.: 11442074001) on day 3.

Once the staining had developed to a desired extent, treatments were terminated by fixation in 4% Paraformaldehyde/PBS overnight at 4°C, preparations were then stored in 70% glycerol/PBS at 4^{o}C. Photographs were taken with a compound Leica microscope from the dorsal and lateral aspects using the same light intensity and conditions for all treatments.

Chapter 3: Differences in ethanol tissue concentration, size and weight of zebrafish larvae developmentally exposed to ethanol

3.1 Introduction

Ethanol consumption during pregnancy is known to produce a wide range of abnormalities to the fetus. Growth and facial defects, mental retardation and severe behavioural problems are the main traits observed on fetal alcohol syndrome (FAS) (Jones and Smith 1973, Colangelo and Jones 1982) and children from alcoholic mothers are more likely to become drug addicts in adolescence or in adulthood, and, to develop personality and psychotic disorders (O'Connor and Paley 2009, Evrard 2010).

This chapter focuses on determining the appropriate embryonic ethanol dose concentration to match the ethanol dose that may be experienced by the mammalian fetus during pregnancy. The plasma concentration of ethanol reached in the fetus in humans does not usually exceed 20mM, when the mothers consume alcohol regularly (Miller, Heather et al. 1991).

An alcohol dehydrogenase assay was used to measure whole body ethanol during chronic and acute ethanol treatment over 1-9dpf, the critical period for zebrafish neurogenesis. During the first hours of this ethanol exposure period, expression of the main HPI axis genes, *crh*, *crhbp*, *crhr1* and *crhr2* are already detectable in the zebrafish embryo (Alderman and Bernier 2009).

In the initial 24 hours of zebrafish development, CRH neurones are differentiated in the hypothalamus region; migrating and proliferating to the subpallium, posterior tuberculum and epiphysis by 2dpf and to the preoptic region, thalamus and retina by 3dpf (Chandrasekar, Lauter et al. 2007). By 4dpf, there is an intense proliferation of CRH-expressing neurones at the regions mentioned above (Chandrasekar, Lauter et al. 2007).

At 2dpf, *crhbp* mRNA expression is detected in the hypothalamus region, preoptic area, thalamus and medulla oblongata; proliferating in the same regions until 5dpf (Alderman and Bernier 2007). Other pathways are target for the 1-9dpf ethanol exposure, such as the cholinergic, dopaminergic, noradrenergic, serotonergic, glutamatergic and GABAergic pathways, as mentioned in the introduction.

These pathways' neurones differentiate in the initial 24 hours of zebrafish embryogenesis, and by 2dpf they vastly migrate and proliferate to different parts of the zebrafish larval brain (Wilson, Ross et al. 1990, Holzschuh, Ryu et al. 2001, Higashijima, Mandel et al. 2004, Wang, Takai et al. 2006, Schweitzer, Lohr et al. 2012).

By 5dpf, the cholinergic, dopaminergic, noradrenergic, serotonergic, glutamatergic and GABAergic neural pathways' patterns are mostly set in locations similar to the adult zebrafish brain, with neurones proliferating and expanding their main circuits (Kimmel 1993, Holzschuh, Ryu et al. 2001, Zirger, Beattie et al. 2003, Higashijima, Mandel et al. 2004, Kaslin, Nystedt et al. 2004, Kim, Nam et al. 2004, Arenzana, Clemente et al. 2005, Mueller, Vernier et al. 2006, Lillesaar, Stigloher et al. 2009, Sallinen, Torkko et al. 2009, Tay, Ronneberger et al. 2011, Yamamoto,

Ruuskanen et al. 2011, Schweitzer, Lohr et al. 2012).

Dopaminergic and noradrenergic neurones are proliferating in regions such as the preoptic area, subpallium, olfactory bulb, tuberculum, hypothalamus and medulla oblongata (Sallinen, Torkko et al. 2009, Tay, Ronneberger et al. 2011, Schweitzer, Lohr et al. 2012). Serotonin neurones are proliferating in the raphe nucleus, posterior tuberculum and other parts of the brain (Wang, Takai et al. 2006) while GABA neurones proliferate vastly in the subpallium, olfactory bulb, preoptic area, thalamus and hypothalamus (Mueller, Vernier et al. 2006).

Developmental ethanol exposure up to 9dpf caused differences in drug seeking behaviour in zebrafish (Parker, Evans et al. 2014). In order to assess if these differences in drug seeking were due to differences in the HPA axis caused by the ethanol treatment, animals used were exposed up to 9dpf as well. In the results following, zebrafish larvae were assessed for possible effects of exposure to ethanol in inducing developmental delay or morphological defects, since either would significantly influence subsequent behavioural and molecular analysis.

3.2 Methods

3.2.1 Animals

Larval zebrafish at 9df were age matched. Control fish and fish that had been exposed to ethanol from days 1-9 dpf as described in chapter 2 were used.

3.2.2 Alcohol dehydrogenase analysis

The embryonic and larval tissue ethanol concentration was assessed using an alcohol dehydrogenase assay from animals treated with 20mM or 100mM ethanol, acutely or chronically, from 1-9dpf with ethanol.

3.2.3 Larval size and dry weight

The larval size and dry weight was measured at the end of the chronic 1-9dpf ethanol treatment (20mM and 50mM). For the size measurement, an automated detection software was used, made by eLaborant (Niels Bohrweg 1, Snellius, room 108, 2333 Leiden,The Netherlands). For the weight, a precision analytical Sartorius scale was used.

3.2.4 Statistics

For size and dry weight measurements, ANOVA and student t-tests were used. The dependent variables were the size of the animal and axial differences in pixels, and, the dry weight. The tests were evaluated with respect to type-1 error rate of 0.05.

3.3 Results

3.3.1 Acute developmental ethanol exposure leads to differences in zebrafish embryonic tissue ethanol concentration according to time of exposure

Zebrafish embryos acutely developmentally exposed to ethanol for 24 hours showed differences in embryonic tissue ethanol concentration compared to the ethanol in the waterbath the animals were in. Their acute exposure periods were explored: 1-24hpf, 24-48hpf and 48-72hpf.

Exposure to 20mM and 100mM ambient waterbath ethanol concentrations from 1-24hpf, yielded embryonic tissue ethanol concentrations of 74% and 88% respectively. There was a significant decrease in the following days, dropping to 66% and 38% of the waterbath concentration when exposed from 24-48hpf, and stabilizing at 36% for both 20mM and 100mM ethanol waterbath concentrations, when acutely exposed from 48-72hpf (Figure 3.1).

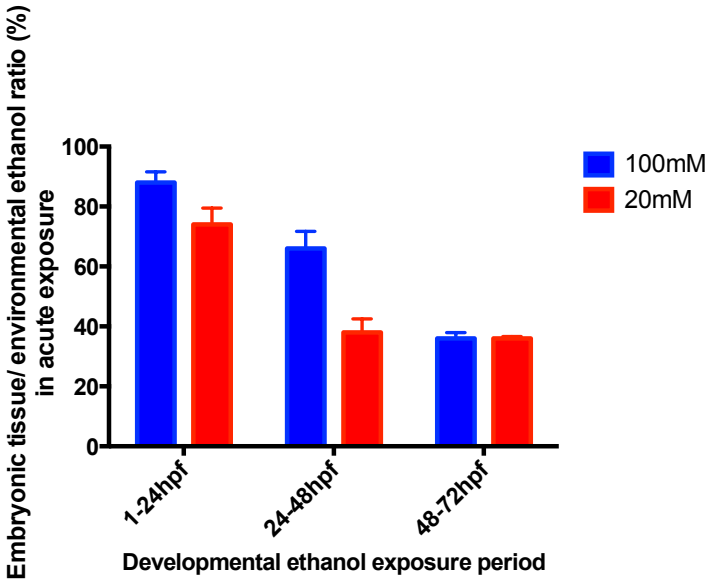


Figure 3.1: Assessment of embryonic tissue ethanol concentration compared to waterbath ethanol concentration in acute 24-hour ethanol exposure of zebrafish embryos. Alcohol dehydrogenase analysis was done in embryos developmentally exposed to an ethanol waterbath concentration of 100mM and 20mM for a period of 24 hours, using 1-24hpf, 24-48hpf and 48-72hpf periods. Three samples were used at each time period, 25 animals per sample.

3.3.2 Chronic developmental ethanol exposure impact on zebrafish larval tissue ethanol concentration from 4-9dpf.

Zebrafish larvae developmentally exposed to chronic ethanol (100mM) treatment 1-9dpf showed that tissue ethanol concentrations stabilises at 21% to 37% of the ethanol waterbath concentration (figure 3.2), consistent with the same range found on the last day assessed for short term exposure (48-72hpf period).

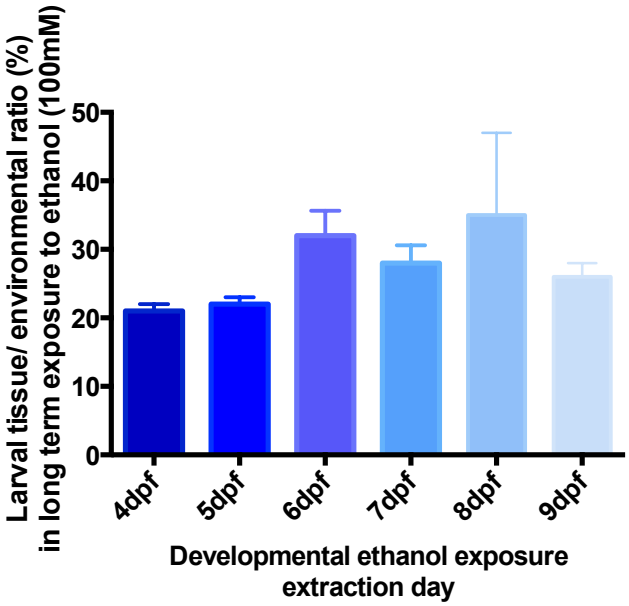


Figure 3.2: Assessment of larval tissue ethanol concentration compared to waterbath ethanol concentration in chronic 1-9dpf ethanol exposure of zebrafish larvae. Alcohol dehydrogenase analysis was done in larvae developmentally exposed to an ethanol waterbath concentration of approximately 100mM from 1-9dpf, with samples being collected from 4dpf. Internal ethanol concentration fluctuated from 21% to 37% of waterbath ethanol concentration. Three samples were used for each time period, 25 animals per sample.

3.3.3 Chronic developmental ethanol exposure leads to no differences in size in 9dpf zebrafish larvae

Zebrafish larvae developmentally exposed to chronic ethanol (20mM and 50mM) treatment 1-9dpf showed no difference in size compared to controls; assessed using eLaborant's software (figure 3.3) (P>0.5, ANOVA>0.5).

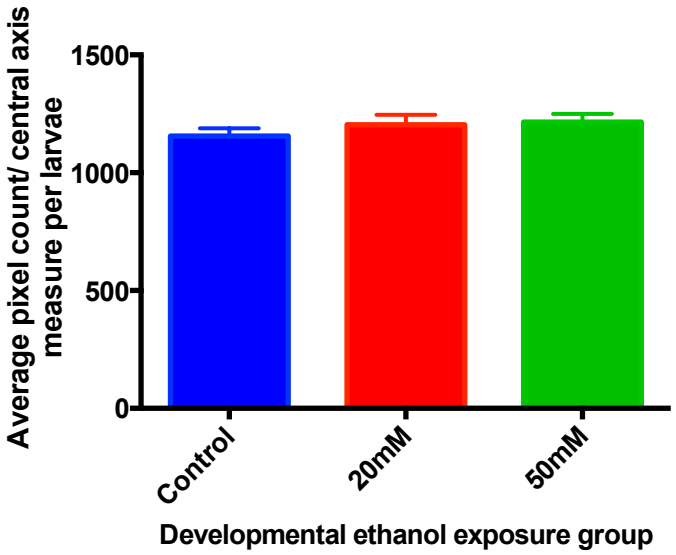


Figure 3.3: Ethanol exposed (1-9dpf) zebrafish 9dpf larvae size measured using eLaborant software. No difference in size due to early ethanol exposure. Three samples were used per group, with 20 animals per sample.

3.3.4 Chronic developmental ethanol exposure leads to no differences in dry weight in 9dpf zebrafish larvae

Zebrafish larvae developmentally exposed to chronic ethanol (50mM) treatment 1-9dpf showed no difference in dry weight compared to controls (figure 3.4) (P>0.5, ANOVA>0.5).

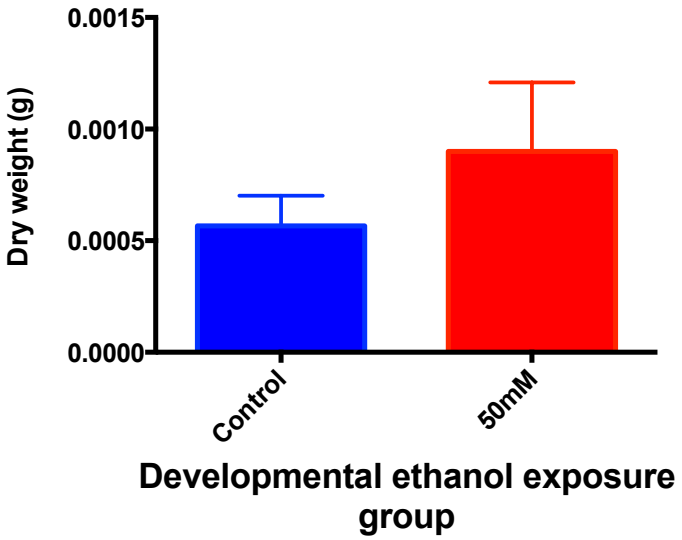


Figure 3.4: Developmental ethanol exposed (1-9dpf) zebrafish 9dpf larvae dry weight. There were no significant differences in dry weight. Three samples were used per group, with 25 animals per sample.

3.4 Discussion

Through the use of the modified alcohol dehydrogenase assay, we were able to assess the internal tissue embryonic or larval ethanol concentration in the animal homogenates and compare it to the ethanol concentration in the water they were treated in.

Early acute exposure revealed a fluctuation in the tissue ethanol concentration during the first three days of life of these animals. Internal tissue ethanol concentrations for the 1-24hpf, 24-48hpf and 48-72hpf periods were approximately 80%, 50% and 36% of the waterbath ethanol using 100mM and 20mM ethanol (figure 3.1).

To understand the observed differences in the ethanol concentration, it is

essential to know the temporal expression of enzymes responsible for the ethanol metabolism in the zebrafish. In mammals, ethanol is oxidised into acetaldehyde and then into acetic acid, followed by further metabolism via acetyl-CoA. Three enzymes can metabolise oxidise ethanol to acetaldehyde in mammals and although ADHs are the most important, catalase and cytochrome P-450 2E1 (CYP2E1) also catalyse the reaction.

Of the main ADHs in zebrafish, ADH8A and ADH8B were detected by qPCR as early as 24hpf, with increasing mRNA expression after 4dpf (Reimers, Flockton et al. 2004). ADH8A and ADH8B resemble mammalian ADH1 (more tissue specific) and ADH5 (more widespread expression) respectively.

In mice, ADH5 and ADH1 can be detected as early as 6.5 and 11.5 days postcoitum respectively (Ang, Deltour et al. 1996) and class I ADH in humans can begin to be detected at 18 weeks of gestation (Estonius, Svensson et al. 1996). CYP2E1 begins to be detected in humans between 16 and 24 weeks of gestation (Carpenter, Lasker et al. 1996) and in zebrafish from 36hpf onwards (Reimers, Flockton et al. 2004).

The zebrafish liver is considered to be fully mature and functional by 4dpf (Howarth, Passeri et al. 2011). Taken together with the temporal changes in expression of both ADH enzymes, it may be deduced that the zebrafish capability to metabolize ethanol is particularly low in the first 24 hours (hence the 80% ethanol concentration registered at 24hpf) and progresses with time.

Reimers also explored the embryonic tissue ethanol concentration (Reimers, Flockton et al. 2004). They found tissue ethanol concentration was 32% of the environmental when chronically exposed to 100mM ethanol from 3-48hpf, similar to the present results at this stage. However, they also found that exposure from 3-

24dpf yielded a tissue ethanol concentration of only 17% that of the waterbath, apparently very different from data given here (Figure 3.1), but this is possibly due to differences in the time frame of the exposure, here from 1-24hpf.

Chronic ethanol exposure of zebrafish larvae from 1-9dpf gave a smaller range of tissue ethanol fluctuation, which stabilized between 22%-37% of ambient (assessed from 3-9dpf) when 100mM ethanol was used (figure 3.2). In order to achieve the desired 20mM concentration and produce results in the same parameters as mammalian studies, we calibrated the dosage to 50mM ethanol, which 22% to 37% embryonic tissue absorption from the waterbath would yield 20mM ethanol, concentration necessary to replicate the parameters found in mammals. The 20mM ethanol concentration was used to provide a mid-range concentration for future comparisons.

In humans, developmental exposure to alcohol leads to a range of birth defects collectively known as fetal alcohol syndrome (FAS) (Jones and Smith 1973). It also causes a range of developmental defects in other models such as rodents, chickens and flies (Ranganathan, Davis et al. 1987, Becker, Diaz-Granados et al. 1996, Bupp Becker and Shibley 1998).

Developmental delays are seen in many cases of early alcohol exposure, such as delayed brain maturation in rodents (West, Hamre et al. 1984, Ranganathan, Davis et al. 1987) and delayed cognition maturation in monkeys (Clarren, Astley et al. 1988). It also causes behaviour problems, and developmentally delayed (intellectual disability) 36-month and 48-month old children were more aggressive, with higher social withdrawal and higher attention seeking behaviour (Baker, McIntyre et al. 2003).

Developmental defects and delays are also seen in the zebrafish model when exposed to early ethanol concentrations starting at 150mM ethanol, such as craniofacial, body structure, cardiac and brain defects (Baumann and Sander 1984, Bilotta, Barnett et al. 2004, Sylvain, Brewster et al. 2010).

Developmental delay, as well as developmental defects, could affect the outcome of the several tests done in the studies described here, therefore it was important to consider any ethanol-related impact on them. Size and dry weight of treated animals were used as indices of developmental delay, but ethanol exposure did not affect either.

Size measured automatically by eLaborant software calculated pixel count and any central axis differences between treated and untreated animals (figure 3.3). Visually, the treated animals did not have any craniofacial deformity or any body oedema compared to controls. These results are compatible with the literature, where embryos treated from 3-48hpf or 3-24hpf up to 150mM ethanol concentration did not exhibit any developmental delay, axial blistering, axial malformations, otolith defects, pericardial or yolk sac edemas at 5dpf (Reimers, Flockton et al. 2004).

It has been reported that only animals treated with ethanol concentrations above 150mM-170mM ethanol will exhibit developmental defects and delays, such as delayed motor and muscle fibre development, yolk sac and pericardial edema and axial malformations (Reimers, Flockton et al. 2004, Sylvain, Brewster et al. 2010).

A more chronic exposure study using Acridine Orange (AO) staining to quantify cell death in the brain and body of zebrafish larvae treated from 4hpf to

6dpf indicated that only ethanol concentrations above 100mM would increase the number of apoptotic cells in those animals (Carvan, Loucks et al. 2004).

As a second measurement for developmental progress, the dry weight of those animals at the end of the treatment again indicated no difference related to the ethanol exposure (figure 3.4). In humans, birth weights of new-borns from mothers that abused alcohol throughout the entire pregnancy were significantly lower compared to mothers that did not (Smith, Coles et al. 1986), however, mothers that drank moderately gave birth to children with the same birth weight as mothers that did not drink at all (Tennes and Blackard 1980).

In mice, ethanol exposure during pregnancy also reduced the birth weight of pups (Abel 1978). Dry weight has been used as a developmental indicative in zebrafish larvae, in a study with chronic 1-10dpf nicotine exposure (Parker and Connaughton 2007). There was also a further validation that these animals were in the same developmental stage at the end of the ethanol treatment done in chapter 7, in which the absolute copy number of structural and neuronal housekeeping genes was calculated by quantitative PCR, exhibiting no difference related to ethanol treatment.

Chapter 4: Differences in stress-reactivity behaviour in zebrafish larvae and juvenilles developmentally treated with ethanol

4.1 Introduction

This thesis explores the hypothesis that developmental exposure to ethanol leads to altered function of the HPI axis, which contributes to changes in stress-related behaviour and vulnerability to psychiatric disease, including addiction. In mammals there is clear evidence of altered stress-reactivity following prenatal ethanol exposure. In this chapter the effect of developmental ethanol exposure on behavioural measures of stress reactivity in larval zebrafish will be described.

Testing for fear, anxiety, aggression, learning and memory is performed in adult zebrafish (Norton and Bally-Cuif 2010), and a growing interest has recently focused on the use of zebrafish larvae for testing of certain behaviours. The advantages include rapid maturation into free-swimming larvae and the quantity of animals that can be used at this stage.

By 5dpf, zebrafish larvae exhibit a range of behaviours. They will avoid a bouncing ball presentation, regardless of its colour and size, moving to an area in the assay plate where this stimulus is absent (Pelkowski, Kapoor et al. 2011). They exhibit a spatial behaviour known as phototaxis, consisting of a group of manoeuvres and swimming speeds to navigate towards a brighter environment (Burgess, Schoch et al. 2010).

In adult zebrafish, thigmotaxis and novel tank diving have been used to assess anxiety (Levin, Bencan et al. 2007, Bencan, Sledge et al. 2009, Champagne,

Hoefnagels et al. 2010, Mathur and Guo 2011). Thigmotaxis has also been validated for zebrafish larval use as an index of anxiety and stress (Richendrfer, Pelkowski et al. 2012, Schnorr, Steenbergen et al. 2012).

This assay has a high potential of becoming a valuable tool for testing novel drugs and screening genetic factors linked to anxiety and stress disorders. We used thigmotaxis with an array of different stressors to assess stress-reactivity differences in ethanol developmentally treated zebrafish larvae and juvenilles.

4.2 Methods

4.2.1 Animals

Zebrafish 9dpf and 10dpf larvae, and, 23dpf juveniles were used in this chapter. Animals were reared, and developmentally treated with ethanol or drug treated according to chapter 2 methods. Animals were size and age matched for all experiments.

4.2.2 Stress-reactivity measurement in zebrafish larvae and juveniles

To assess differences in stress reactivity in these animals, thigmotaxis was used with no stressor, and, following stressors, such as light-dark startle, air-exposure and predator simulation. Immobility frequency was also used as a measure

of stress-reactivity. Full methods for the assays, recordings and statistical analysis found in chapter 2.

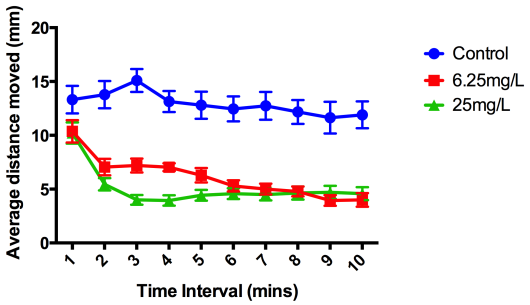
4.3 Results

4.3.1 Characterization of thigmotaxis as a larval indicator of stress-reactivity

For the characterization of larval thigmotaxis as an indicator of stress-reactivity, thigmotaxis behaviour was first determined in control animals before and after exposure to the psychoactive drugs, buspirone and diazepam. Buspirone severely decreased zebrafish larvae locomotion over a 15-minute exposure period (Figure 4.1, A) whereas diazepam did not for the same parameters, as indicated by calculated average distance moved (Figure 4.1, B).

Diazepam did not affect locomotion until past the first 50 minutes of drug exposure (Figure 4.1, C). Based on these findings, buspirone was discarded and diazepam was used for the remaining validation tests.

A



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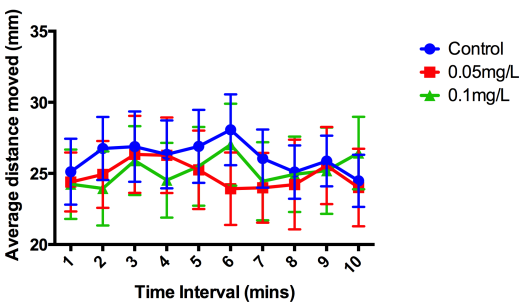

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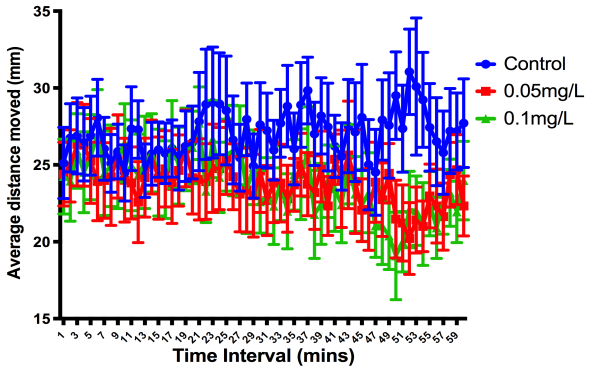


Figure 4.1: Zebrafish 9dpf larvae locomotion, treated with buspirone (A) and with diazepam (B and C). Animals treated with buspirone moved significantly less compared to control ($P<0.01$). No difference between treated and untreated groups with diazepam (B). Extended analysis of recordings of diazepam locomotion effects seen in B revealed that the drug does not start affecting distance travelled of treated animals until 50 minutes of drug exposure (C). Three batches of animals used on both experiments.

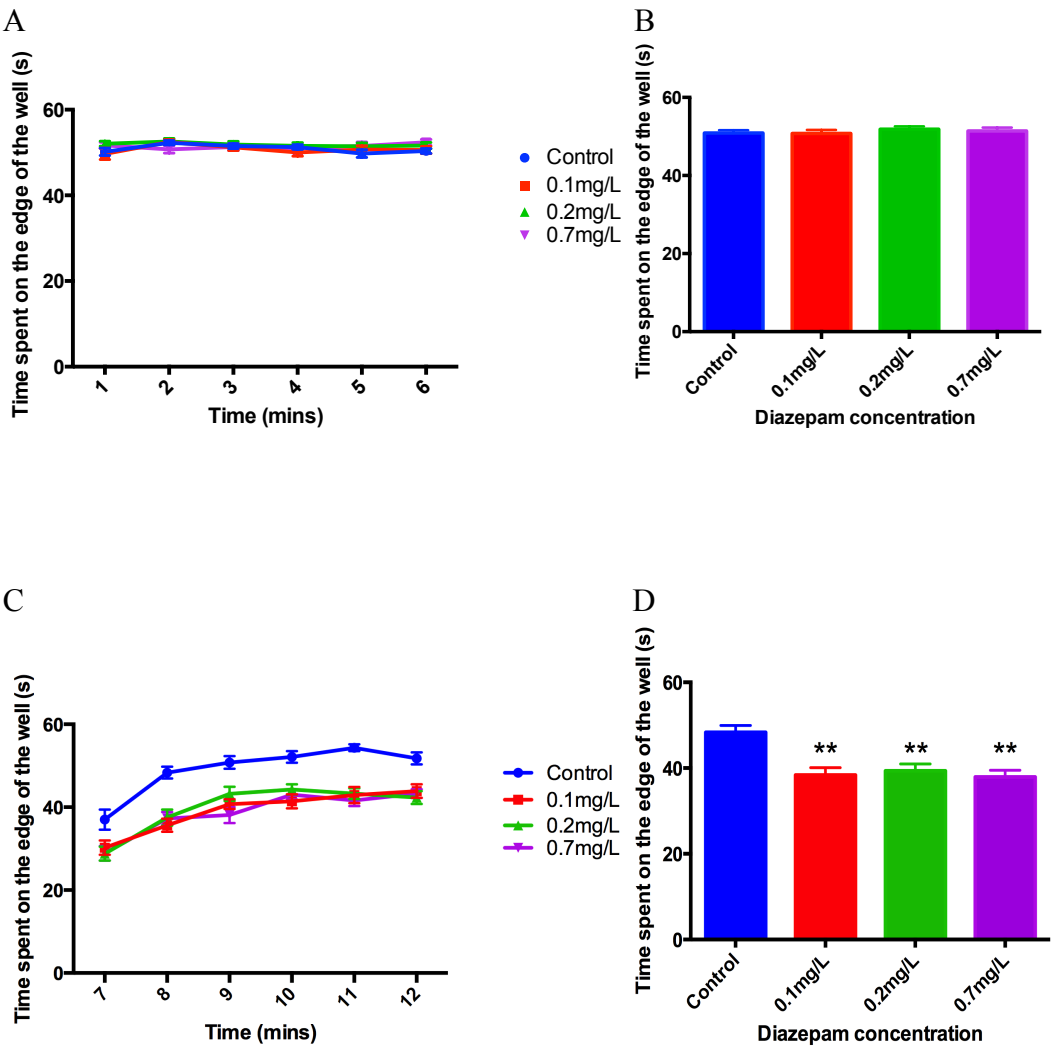
Larvae were forced to engage robust locomotor activity by challenging them with either light-dark "startle" or air exposure stimuli for 1min. The effects of diazepam were then compared with unchallenged controls. Thus, untreated larvae (9dpf) and larvae treated by incubation in diazepam 0.1mg/L up to 0.7mg/L were subjected to an initial 6 min period in a lit environment (figure 4.2, A-B), then switched to complete darkness and recorded for an additional 6 minutes (figure 4.2, C-D).

Diazepam decreased the thigmotaxis response after the light-dark startle but not before it, controls remained swimming for roughly 50 secs per minute in the outer region before and after the darkness switch. Diazepam treated animals exhibited reduced thigmotaxis after the darkness switch.

As a second measure to induce stress reactivity, untreated and diazepam (0.1mg/L) treated larvae were held for an initial 5min in a lit environment then

experimental groups were air exposed for 1 min followed by recording and compared with unstressed controls (figure 4.2, E-F). Air exposure induced a significant increase in thigmotaxis, whether animals were treated or not with diazepam compared to unstressed groups (P<0.01, ANOVA <0.01) (figure 4.2, E-F).

Air exposed controls also exhibited more thigmotaxis compared to air exposed diazepam treated larvae (P<0.01, ANOVA <0.01) (figure 4.2, E-F). Unstressed animals treated with diazepam also exhibited lower thigmotaxis compared to unstressed controls (P<0.01) (figure 4.2, E-F).



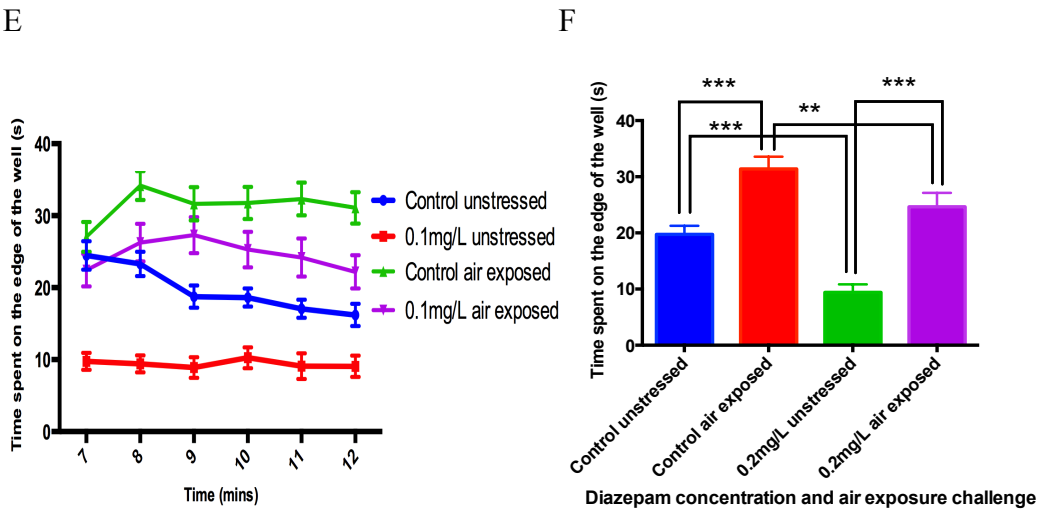


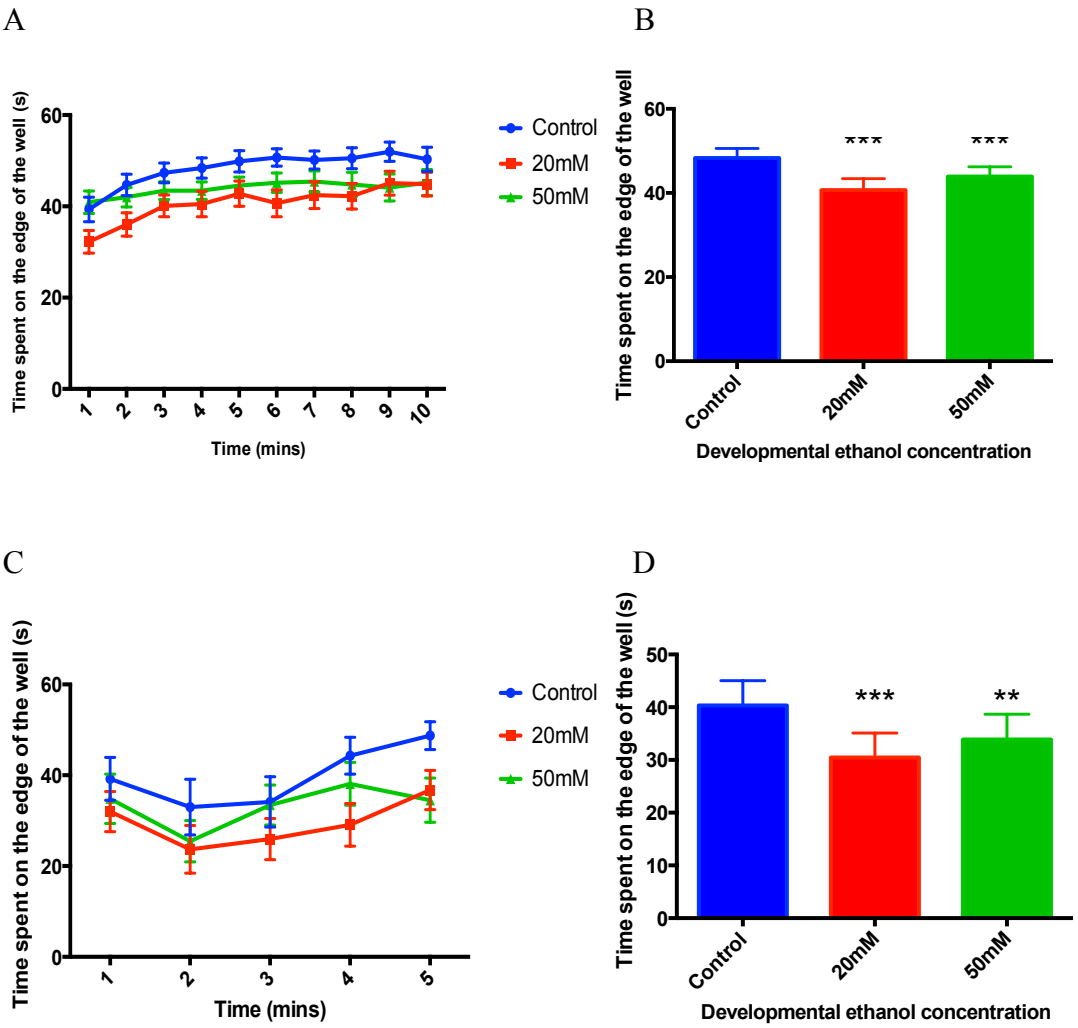
Figure 4.2: Characterization of thigmotaxis using zebrafish larvae (9dpf) treated with diazepam. Before light-dark startle (A-B), after light-dark startle (C-D) and air exposure (E-F). Time course on average time spent at the edge of the apparatus (A, C, E), Absolute average of time spent at the edge of the apparatus (B, D, F). Animals treated with diazepam from 0.1mg/L up to 0.7mg/L exhibited no differences before the switch to darkness (A-B), and exhibited lower thigmotaxis compared to control after the darkness switch challenge (P=0.01) (C-D). Animals treated with diazepam 0.1mg/L solution, unstressed or air exposed exhibited lower thigmotaxis compared to controls, in unstressed or air exposed (P<0.05) (E-F). There was also a difference between controls, unstressed and air exposed animals, and Diazepam treated unstressed and air exposed larvae (P<0.01). Four batches of animals were used.

4.3.2 Developmental ethanol exposure reduces stress-related behaviour in zebrafish larvae and juveniles measured by thigmotaxis in the absence of a stress challenge

Zebrafish developmentally treated were tested at 9dpf, 10dpf and 23dpf stages for differences in thigmotaxis in the absence of a stress challenge. The use of the 23dpf stage was used to observe any differences in stress-reactivity two weeks after the ethanol treatment had ended; therefore, any differences in thigmotaxis would be an effect of the ethanol treatment in the stress-response system of the animals and not an effect of withdrawal or anxiolytic properties of the ethanol.

The previous section revealed that the use of thigmotaxis without any

challenge can be used as a measure of stress-reactivity, as diazepam treated unstressed animals exhibited less thigmotaxis compared to control unstressed animals (figure 4.3, E-F). Results indicate a decrease in stress reactivity related to early developmental ethanol exposure in all three stages chosen (P<0.01, ANOVA <0.01) (figure 4.3, A-F).



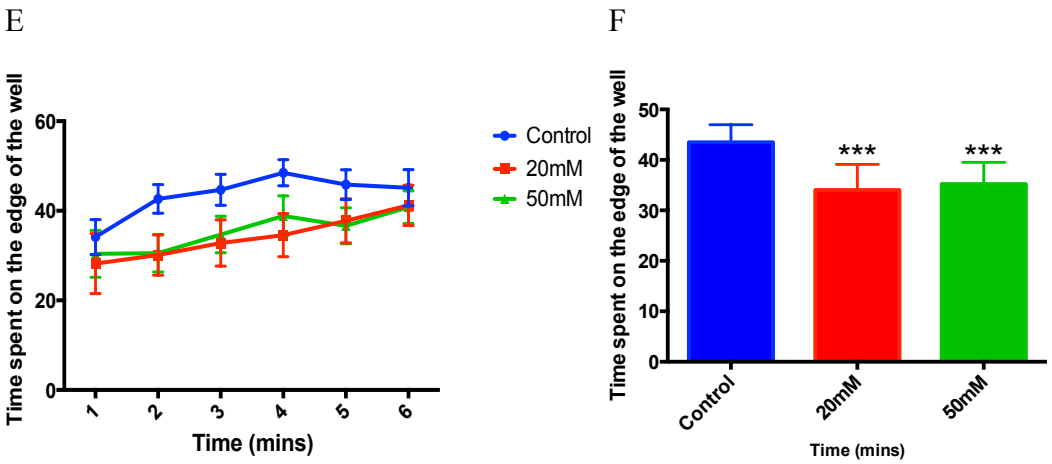


Figure 4.3: Stress-related behavious measured by thigmotaxis in zebrafish larvae 9dpf (A-B), 10dpf (C-D) and 23dpf juveniles (E-F) without a stress challenge. Time course on average time spent at the edge of the apparatus (A, C, E), Absolute average of time spent at the edge of the apparatus (B, D, F). Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and a handling control. Once animals reach 9dpf, 10dpf and 23dpf they were tested for thigmoptaxis. 9dpf and 10dpf Developmental ethanol exposure decreased thigmotaxis, with the greterest difference between 20mM ethanol treatment and the control (P<0.01, ANOVA <0.01). Siblings of the same animals were raised for another 2 weeks and tested as juveniles (23dpf). These juveniles exhibited a similar thigmotaxis response as they exhibited at 9dpf, with decreased thigmotaxis in ethanol treated animals compared to controls (P<0.01, ANOVA <0.01). Three batches of animals were used.

4.3.3 Developmental ethanol exposure reduces stress-reactivity in zebrafish larvae and juveniles measured by thigmotaxis following air exposure challenge

To confirm the findings of the previous section, zebrafish embryos and juveniles were submitted to thigmotaxis following air exposure, as a stress-reactive assay. Developmentally ethanol exposed zebrafish were air exposed for 1 minute prior to thigmotaxis recording. At both stages studied, 9dpf larvae (figure 4.4, A-B) and 23dpf juveniles (figure 4.4, C-D), there was a decrease in thigmotaxis behaviour related to developmental ethanol exposure, indicating less stress-reactivity (P<0.01, ANOVA <0.01).

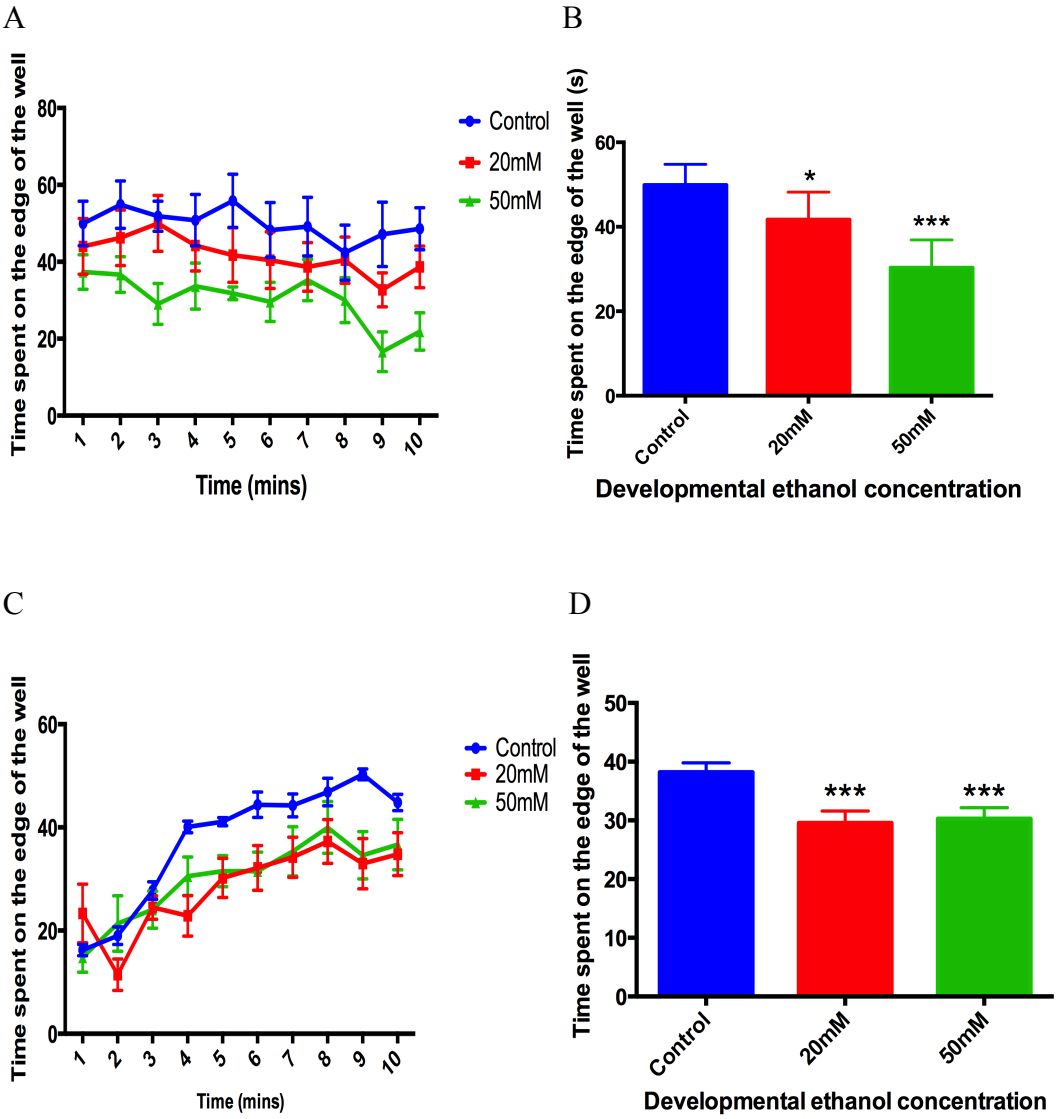


Figure 4.4: Stress-reactivity measured by thigmotaxis after air exposure, in developmentally ethanol treated and control zebrafish 9dpf larvae (A-B) and 23dpf juveniles (C-D). Time course on average time spent at the edge of the apparatus (A and C), Absolute average of time spent at the edge of the apparatus (B and D).9dpf zebrafish larvae showed decreased thigmotaxis with increasing ethanol concentration exposure during development, with the greatest difference between 50mM ethanol treatment and tcontrol (P<0.01, ANOVA <0.01). Siblings of the same animals were kept for another 2 weeks and tested as juveniles (23dpf). These juveniles exhibited a similar thigmotaxis response as they had exhibited at 9dpf, with decreasing thigmotaxis in ethanol treated animals compared to controls (P<0.01, ANOVA <0.01). Three batches of animals were used.

4.3.4 Withdrawal of chronic ethanol exposure measured by thigmotaxis in zebrafish larvae developmentally treated from 1-9dpf

There are no studies on the effects of alcoholic withdrawal on zebrafish larvae. Results obtained from assessing thigmotaxis behaviour of 9dpf larvae treated with ethanol indicate that treated animals exhibit a significant increase in stress-related behaviour when abruptly moved to an environment without the drug, compared to siblings tested in the continued presence of ethanol (figure 4.5). Siblings tested 24hrs later (10dpf) in control fish water showed similar post-ethanol treatment thigmotaxis responses seen in previous sections, causing a decrease in thigmotaxis associated with early ethanol exposure (figure 4.6).

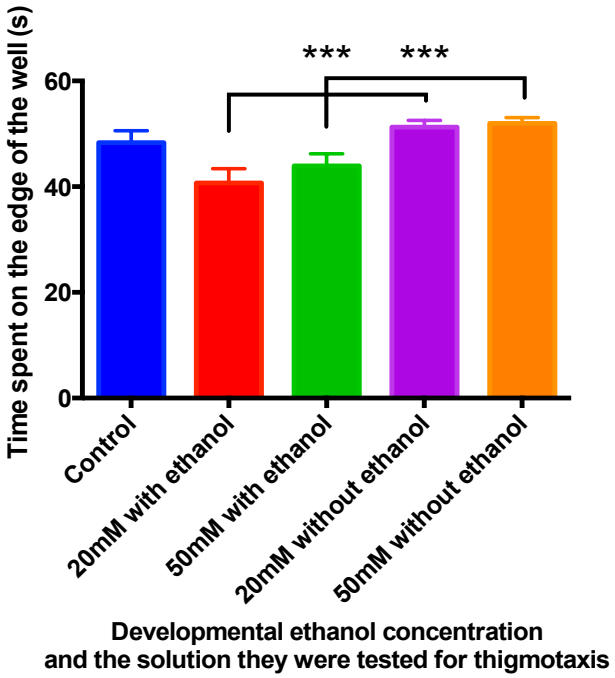


Figure 4.5: Stress-reactivity measured by thigmotaxis of 9dpf zebrafish larvae. Animals were tested in the presence of continued ethanol exposure, or in after withdrawal. Thigmotaxis was measured without a stress challenge. Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and compared with similarly handled controls without

ethanol exposure. Zebrafish larvae showed increased thigmotaxis with increasing ethanol concentration exposure during development, when tested in an environment without ethanol. This is statistically different from testing the animals in the continued presence of ethanol (P<0.01, ANOVA <0.01, at both ethanol concentrations used). This indicates that withdrawal at this larval stage has a detectable impact immediately after ethanol withdrawal. Three batches of animals were used.

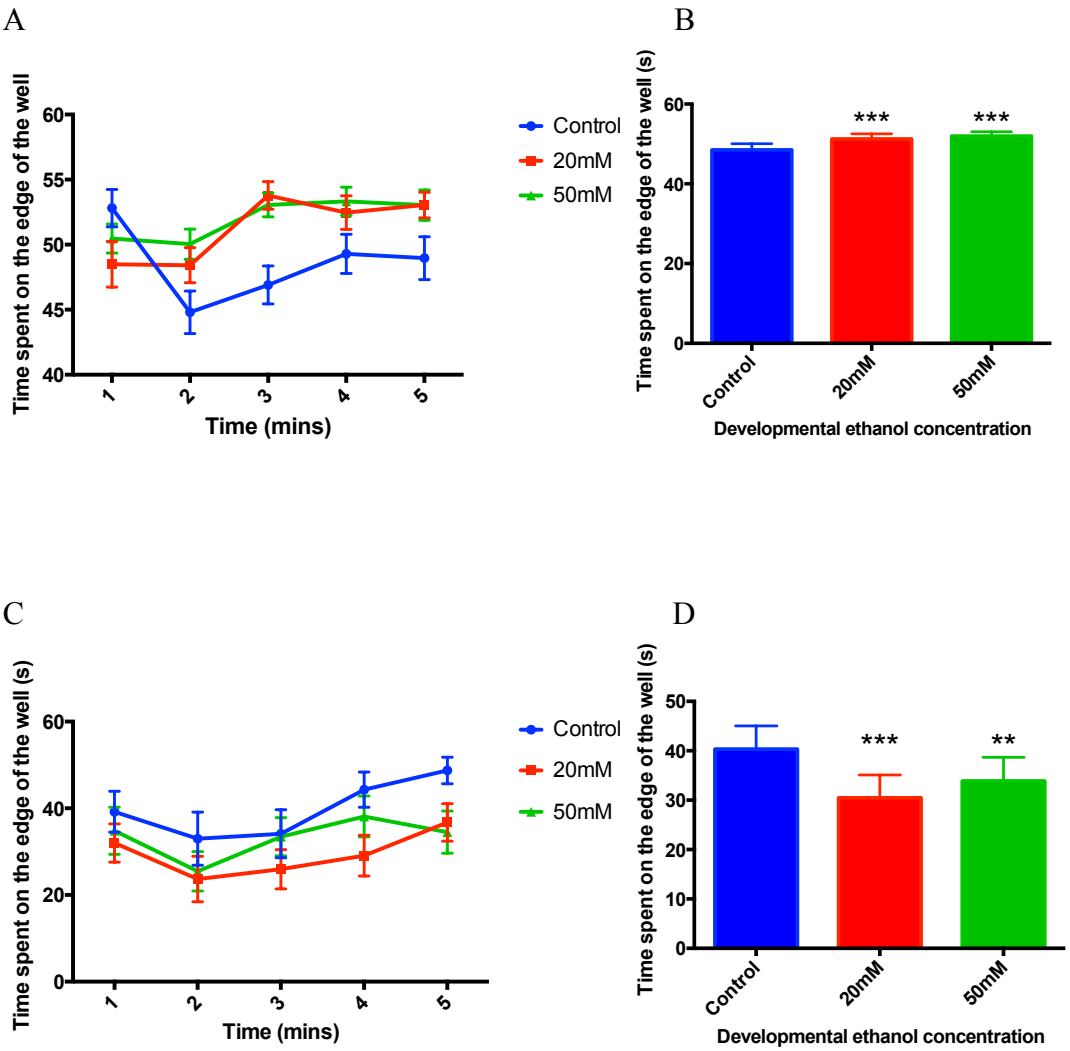
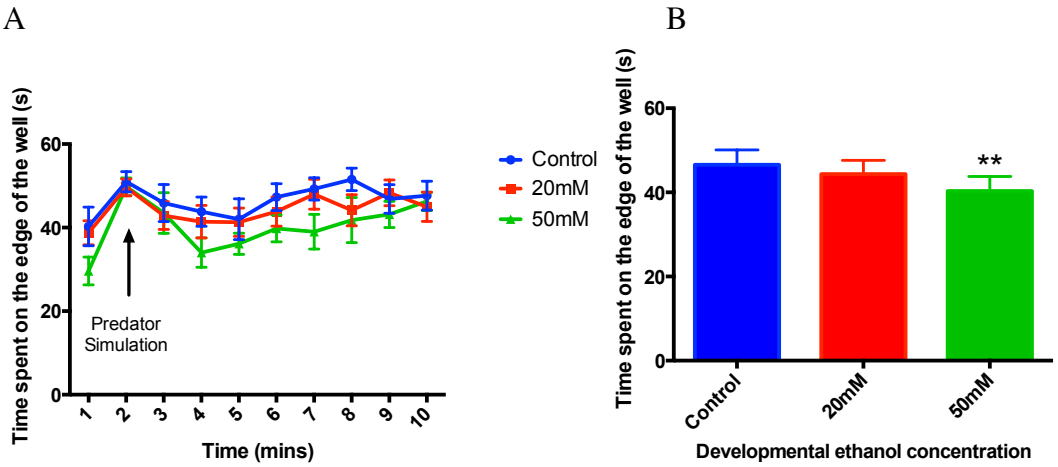


Figure 4.6: Stress-reactivity measured by thigmotaxis to assess withdrawal. 9dpf zebrafish larvae were assayed at the beginning of withdrawal (A-B) and 24hrs later (C-D). Time course on average time spent at the edge of the apparatus (A and C), Absolute average of time spent at the edge of the apparatus (B and D). Thigmotaxis was measured without a previous challenge. Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and compared with a similarly handled control. . Zebrafish larvae showed increased thigmotaxis at the beginning of withdrawal with increasing ethanol concentration exposure during development, with a maximum at 50mM ethanol treatment. (P<0.01, ANOVA <0.01) (A-B). After a further 24hrs, the 10dpf larvae exhibited a similar thigmotaxis response to that seen in previous,sections with maximum decreased stress-reactivity after 20mM ethanol treatment (P<0.01, ANOVA <0.01) (C-D). Three batches of animals were used.

4.3.5 Developmental ethanol exposure decreases thigmotaxis stress-reactivity after a simulated predator challenge in zebrafish juveniles, but not in larvae

It has been demonstrated that larvae exposed to a stimulus that resembled a predator attack, can exhibit thigmotaxis behaviour, as a measure of anxiety (Richendrfer, Pelkowski et al. 2012). Using a similar approach, developmentally ethanol treated larvae were exposed to a simulation of a predator threat in the form of a PowerPoint presentation, generating a pulsating red ball at the centre of the well.

At 23dpf, the predator challenge resulted in changes in thigmotaxis though to a lesser extent than achieved by air exposure. Only animals treated developmentally with 50mM ethanol exhibited a thigmotaxis difference compared to control *P*=0.02, ANOVA=0.03 (figure 4.7, A-B). At 9dpf, alcohol treated larvae exhibited no difference compared to controls based on thigmotaxis behaviour (figure 4.7, C-D).



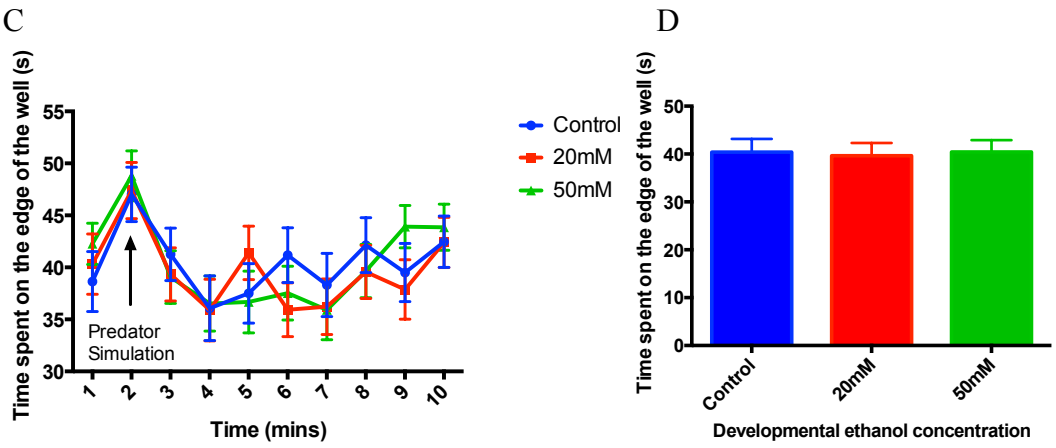


Figure 4.7: Stress-reactivity measured by thigmotaxis following predator simulation challenge of 23dpf (A-B) and 9dpf (C-D) zebrafish juveniles and larvae. Time course on average time spent at the edge of the apparatus (A and C), Absolute average of time spent at the edge of the apparatus (B and D). Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and compared with similarly handled controls. Animals were tested at 9dpf or 2 weeks later (23dpf larvae). Stressed 23dpf zebrafish larvae exhibited decreases in thigmotaxis following 50mM developmental ethanol exposure compared to controls (P=0.02, ANOVA=0.03). 9dpf larvae exhibited no changes among the groups. Three batches of animals were used.

4.3.6 Developmental ethanol exposure decreases stress-reactivity in zebrafish larvae and juveniles measured by frequency of periods of immobility (freezing)

Acute stress exposure causes an array of behaviours in adult zebrafish as described in the introduction. Other responses include reduced exploration, erratic swimming behaviour and frequent periods of immobility or “freezing”. These are also frequently used as measures of anxiety (Egan, Bergner et al. 2009, Cachat, Stewart et al. 2010, Pittman and Ichikawa 2013).

To test this, periods of immobility were counted in larvae after air exposure, and using diazepam as a validation tool. It was concluded that this equates to

freezing behaviour in zebrafish adults. Diazepam significantly reduced the immobile frequency of zebrafish 9dpf larvae compared to controls (figure 4.8, C).

At 9dpf and 23dpf stages, animals exposed to ethanol developmentally exhibit less immobile frequency then controls, leading to the conclusion that they are less stress-reactive (figure 4.8, A-B). It was also observed that zebrafish juveniles "freeze" more frequently than larvae (figure 4.8, A-B).

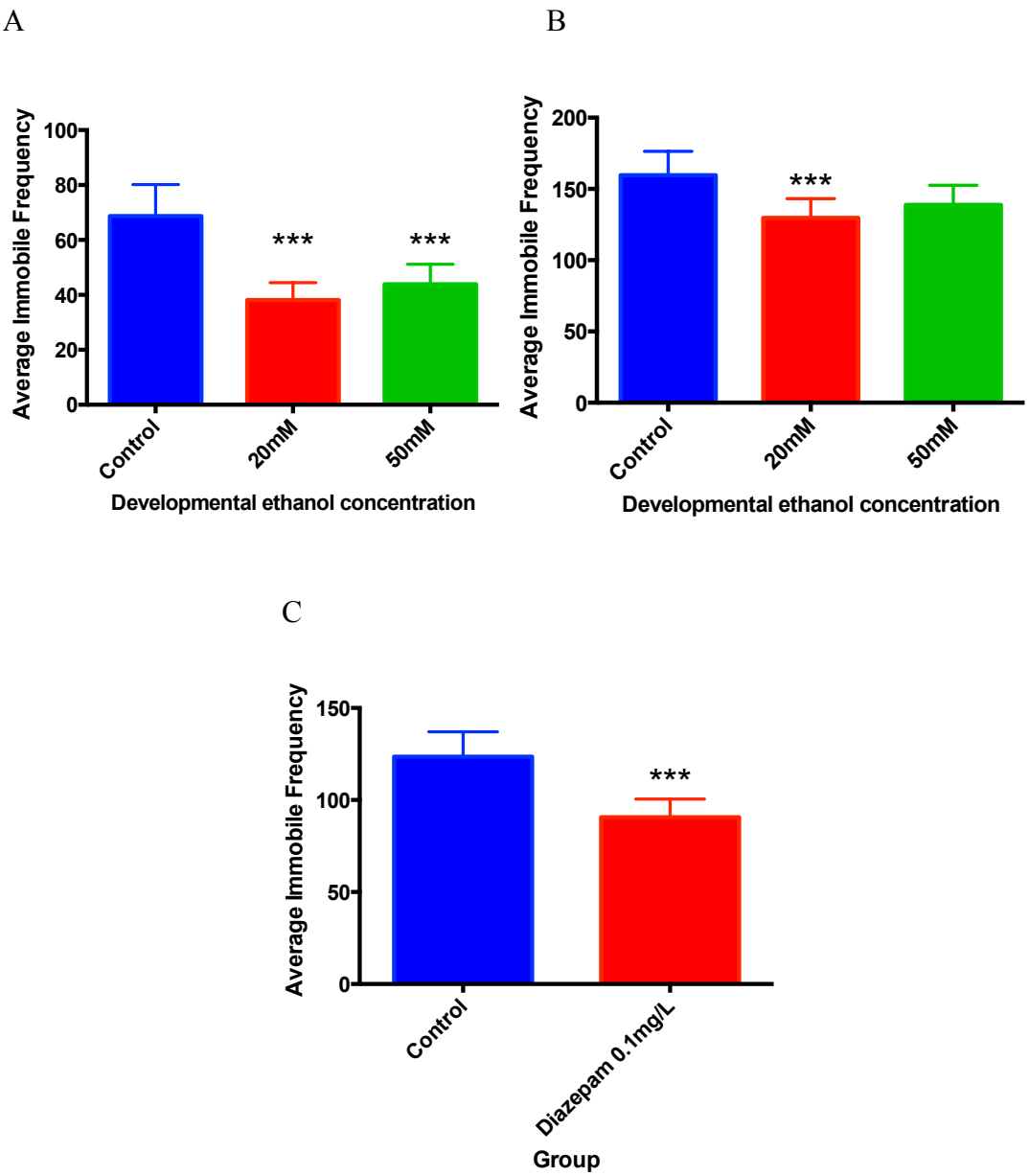


Figure 4.8: Zebrafish stress-reactivity measured by frequency of immobility at 9dpf (A) or 23dpf (B) following air exposure challenge. Effects of diazepam on frequency of immobility of zebrafish 9dpf

larvae (C). Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and compared with controls. Animals were tested at 9dpf or 23dpf. 9dpf ethanol treated larvae (A) exhibited significant less immobile frequency compared to controls (P<0.01, ANOVA <0.01). 23dpf zebrafish larvae (B) also exhibited decrease in immobile frequency with 20mM ethanol concentration exposure during development compared to controls (P<0.01, ANOVA<0.01). Diazepam validation of immobile frequency (C) was also significant (P<0.01, ANOVA<0.01). Three batches of animals were used for all experiments.

4.4 Discussion

Thigmotaxis and avoidance behaviours have been suggested to be a measure of anxiety in zebrafish larvae (Colwill and Creton 2011, Colwill and Creton 2011, Richendrfer, Pelkowski et al. 2012). To characterize the use of thigmotaxis in our labarotary as a measure of anxiety, we exposed 9dpf zebrafish larvae to the anxiolytic drugs buspirone and diazepam. A pilot study was performed to assess whether these drugs affect locomotor activity in the larvae at this stage, because differences in locomotion may greatly affect thigmotaxis assessment.

A 6-minute incubation with buspirone significantly decreased locomotion at 6.25mg/L and 25mg/L, whereas diazepam had no effect at 0.05mg/mL and 0.1mg/mL concentrations (Figure 4.1). Buspirone was therefore discarded and validation continued using diazepam alone.

Animals were tested on different days for both drugs and different distance travelled for controls were obtained according to the day they were tested (Figures 4.1 A and 4.1 B). Since they those differences were averaged among the tested days, and drugged animals were tested at the same time as controls, the experiment was still considered valid.

To trigger thigmotaxis, light-dark "startle" was first used. Upon exposure to darkness, the larvae exhibit a burst in locomotor activity, known as the visual motor response, resulting in an enhanced exploration of the environment they are in (Emran, Rihel et al. 2008, MacPhail, Brooks et al. 2009, Irons, MacPhail et al. 2010, Schnorr, Steenbergen et al. 2012).

The initial 6 minutes in the light and the following 6 minutes in the dark revealed no changes in thigmotaxis with the control group, but it exhibited a decreased in thigmotaxis on diazepam treated animals (Figure 4.2 A-D). The fact that the control group exhibited no change even after the light-dark "startle" suggests that this assay may not reflect a stress response, and the differences in the diazepam treated larvae are an effect of the drug alone.

Another group that explores thigmotaxis in zebrafish larvae validated the use of ligh-dark startle as a stressor for thigmotaxis. However, they have not recorded the animals in the light phase as we did, therefore only analysing the larvae behaviour when they already were 6 minutes immersed into the drug, giving the notion that the light-dark startle was able to induce differences in their thigmotaxis behaviour (Schnorr, Steenbergen et al. 2012). This assumption would also have been concluded if this part of the experiment was missing in our approach as well (Figure 4.2 C-D).

Thigmotaxis was then assayed without any stress challenge, and diazepam treated larvae again exhibited a decrease in thigmotaxis compared to controls (Figure 4.2 E-F). To confirm if a different stress challenge would increase the thigmotaxis response, air exposure challenge was also used (Figure 4.2 E-F). Air exposure is a more conventional fish stressor and routinely used in zebrafish adults (Fuzzen, Van Der Kraak et al. 2010, Alderman and Vijayan 2012).

Air exposure significantly increased thigmotaxis in control and diazepam treated animals (Figure 4.2 E-F). Diazepam also decreased the thigmotaxis response in air exposed animals and in unstressed animals (Figure 4.2 E-F). These findings confirm that larval thigmotaxis could be used as a measure of stress-reactivity if performed with air-exposure challenge or without any stressor.

Developmentally ethanol treated animals were then tested for stress-reactivity by thigmotaxis following air-exposure and by thigmotaxis without stressor. Both approaches showed that early ethanol exposure does decrease stress reactivity in 9dpf animals (figure 4.3 A-B and figure 4.4 A-B).

To ensure that these findings were an effect of ethanol on the stress system of the subjects, 23dpf juveniles (14 days post ethanol treatment) were also used, in order to verify that changes in thigmotaxis were attributable to the effects of ethanol treatment on the stress-response system, and not an acute effect of withdrawal or anxiolysis.

Zebrafish 23dpf juveniles exhibited the same thigmotaxis response as 9dpf larvae, showing that the effects of early ethanol treatment do indeed persist after treatment (figure 4.3 E-F and figure 4.4 C-D). Ethanol treated animals also shows lower frequency of periods of immobility, which in adults is also used as a stress-reactive measure (figure 4.8 A-B). Use of diazepam, validated the use of immobility frequency in zebrafish larvae as a measure of stress reactivity (figure 4.8 C).

The acute effect of ethanol withdrawal was also studied. At the end of the early ethanol treatment (1-9dpf) they were tested for thigmotaxis in the continued presence of ethanol and on its absence. Developmentally ethanol treated animals tested for thigmotaxis without a stressor in the absence of ethanol exhibited an increase in thigmotaxis compared to animals tested in its presence (figure 4.5).

These differences were no longer present 24 hours later, and ethanol treated animals at 10dpf exhibited thigmotaxis responses similar to those of 9dpf animals tested in the continued presence of ethanol (Figure 4.6 C-D). These findings match other studies, where zebrafish adults in alcohol withdrawal exhibited a reverse preference for lit environments when tested for scototaxis (Holcombe, Howorko et al. 2013).

Finally, the use of another challenge for thigmotaxis, predator simulation, was explored. Developmentally treated animals at 23dpf exhibited lower thigmotaxis than controls (figure 4.7 A-B), but there was no difference at 9dpf, suggesting perhaps that larvae do not perceive this simulation as a threat (figure 4.7 C-D).

The three measures of stress reactivity used, thigmotaxis without a stressor, thigmotaxis following air exposure and immobility frequency all indicate that zebrafish larvae and juveniles that are developmentally exposed to ethanol 1-9dpf have decreased behavioural stress responses. An additional measurement of thigmotaxis following predator simulation in developmentally treated juveniles also showed decreased thigmotaxis compared to controls.

These findings contradict mammalian studies that show that early ethanol exposure increases stress-reactivity and anxiety (Osborn, Kim et al. 1998, Carneiro, Diogenes et al. 2005, Hellemans, Sliwowska et al. 2010, Hellemans, Verma et al. 2010). To verify if these stress-reactivity changes are long-lasting in the zebrafish model, siblings were tested at 6 months post-fertilization in chapter 5.

Chapter 5: Differences in stress-reactivity and social behaviour in zebrafish adults developmentally treated with ethanol

5.1 Introduction

Humans exposed to ethanol prenatally show changes in stress-reactivity (Haley, Handmaker et al. 2006) and social skills (Keil, Paley et al. 2010, Rasmussen, Becker et al. 2011). Prenatal ethanol exposure in rodents evokes similar changes in stress-reactivity and social behaviour (Hellemans, Sliwowska et al. 2010, Hellemans, Verma et al. 2010).

The results described in chapter 4 showed that following developmental ethanol exposure, changes in larval zebrafish behaviour suggested reduced stress-reactivity compared to sibling controls. The aim of this chapter is to determine whether stress-reactive and social behaviour differences persist into adulthood in zebrafish as a consequence of early ethanol exposure.

There is also a correlation between stress and social interactions in children. Cortisol levels are closely related to shyness, aggression and social competence (Tout, de Haan et al. 1998). These data suggests that stress and social interactions are both affected by early ethanol exposure and possibly interlinked later in life.

In rodents, elevated plus maze (Sidor, Rilett et al. 2010) and open field (Britton and Britton 1981) assays are used to test for fear and anxiety. These tests were translated for adult zebrafish use into novel tank diving and thigmotaxis respectively (Levin, Bencan et al. 2007, Bencan, Sledge et al. 2009, Champagne,

Hoefnagels et al. 2010, Mathur and Guo 2011). Behaviours such as fear and anxiety, aggression, learning and memory are routinely assessed in the adult zebrafish model nowadays (Norton and Bally-Cuif 2010).

Novel tank diving is an extensively used assay for anxiety in zebrafish and other teleost species. It consists of introducing the animal to a novel tank where they tend to spend around 85% of the first minute in the bottom third of this tank then gradually exploring the rest of the novel tank for the remaining time of the test (Maximino, de Brito et al. 2010, Parker, Annan et al. 2014).

Anxiolytic drugs such as diazepam, buspirone, fluoxetine, citalopram, desipramine, ethanol and nicotine have shown to reduce the time spent at the bottom third of the novel tank; without having sedative effects or increasing the locomotion of these animals (Bencan, Sledge et al. 2009, Egan, Bergner et al. 2009, Maximino, de Brito et al. 2010, Sackerman, Donegan et al. 2010). Caffeine and alarm pheromone have shown anxiogenic properties by increasing time spent at the bottom third of the novel tank (Egan, Bergner et al. 2009).

In zebrafish, thigmotaxis assay, or also called open-field as its rodent assay equivalent, is another measure of anxiety-like behaviour. It assays time spent swimming near the walls of the tank. Diazepam reduced thigmotaxis behaviour, whilst LSD, cocaine withdrawal and FG-7142 (benzodiazepine receptor inverse agonist) increased this behaviour in adult zebrafish (Lopez-Patino, Yu et al. 2008, Grossman, Utterback et al. 2010, Maximino, de Brito et al. 2010).

Finally, scototaxis was used as a third stress-reactive assay to confirm the findings obtained with novel tank diving and thigmotaxis. This behaviour task will assess the animal's preference for protected dark areas (crypsis) and the motivation

to explore new environments (Maximino, de Brito et al. 2010, Blaser and Rosemberg 2012, Holcombe, Howorko et al. 2013).

Pharmacological validations of this assay included the use of fluoxetine, benzodiazepines (chlordiazepoxide, clonezapam and diazepam), buspirone and ethanol yielding an anxiolytic effect by increasing time spent at the bright side of the tank. Caffeine was used as an anxiogenic, decreasing time spent at the bright side of the tank (Maximino, de Brito et al. 2010, Maximino, da Silva et al. 2011).

On the social behaviour aspect, offspring developmentally exposed to alcohol had been studied for years, implicating deficits from social bonding skills to hyperactivity in children (Keil, Paley et al. 2010, Rasmussen, Becker et al. 2011). These deficits persist into adulthood and adolescence, also increasing the risk factor of the subjects developing drinking problems (Streissguth, Aase et al. 1991, Baer, Sampson et al. 2003).

Animal models have been used to gain insight into possible neurological mechanisms underlying developmental ethanol exposure and social behaviour deficits later in life. Using a rodent model, it was demonstrated that prenatal ethanol exposure resulted in social changes in adulthood, such as increased wrestling behaviour towards other males and decreased social investigation, suggested to be due to structural and synaptic changes in the frontal cortex regions (Hamilton, Akers et al. 2010).

Structural and synaptic changes in other regions of the brain due to early ethanol exposure have also been suggested to affect social behaviour, such as a reduction in the number of neurones in the trigeminal-associated cranial nerve nuclei, a region required for social interaction (Hamilton, Akers et al. 2010, Mooney and Varlinskaya 2011).

Zebrafish is a social species, exhibiting an array of social behaviours, such as shoaling, social rewarding, social environment learning and individual social interaction (Hall and Suboski 1995, Engeszer, Barbiano et al. 2007, Al-Imari and Gerlai 2008, Fernandes and Gerlai 2009, Parker, Annan et al. 2014).

The adaptive function of the shoaling response in zebrafish is still under debate. It is said to be an anxiety direct response to predator avoidance, confusing the predator with synchronized group movement and not allowing it to focus on a single target (Olst and Hunter 1970, Landeau and Terborgh 1986, Maximino, de Brito et al. 2010, Gerlai 2014).

Shoaling behaviour has not been extensively validated pharmacologically, as the other stress-reactive assays have. Ethanol, nicotine and dizocilpine (NMDA receptor antagonist) all exerted anxiolytic effects by reducing shoaling cohesion in zebrafish (Maximino, de Brito et al. 2010, Miller, Greene et al. 2013). Another theory being discussed is that shoaling behaviour facilitates finding food, and that it increases mating likelihood (Miller and Gerlai 2011, Gerlai 2014).

5.2 Methods

5.2.1 Animals

Zebrafish 6 months old animals, age and weight matched, in 3:2 females:males ratio. Control fish and fish that had been exposed to ethanol from days 1-9dpf as described in chapter 2 methods were used. Following ethanol

treatment larvae were transferred to the aquarium facility and reared to adulthood in the absence of ethanol. Fish of each group were maintained in six separate tanks.

5.2.2 Assessing stress reactivity of adult fish.

Three methods were used to assess stress reactivity of adult fish; novel tank diving, thigmotaxis, and scototaxis as described in full detail in chapter 2 methods. All the following assays including social behaviour assays used the same animals, six different batches; with exception to scototaxis, which was done with different animals and in three different batches, but treated identically.

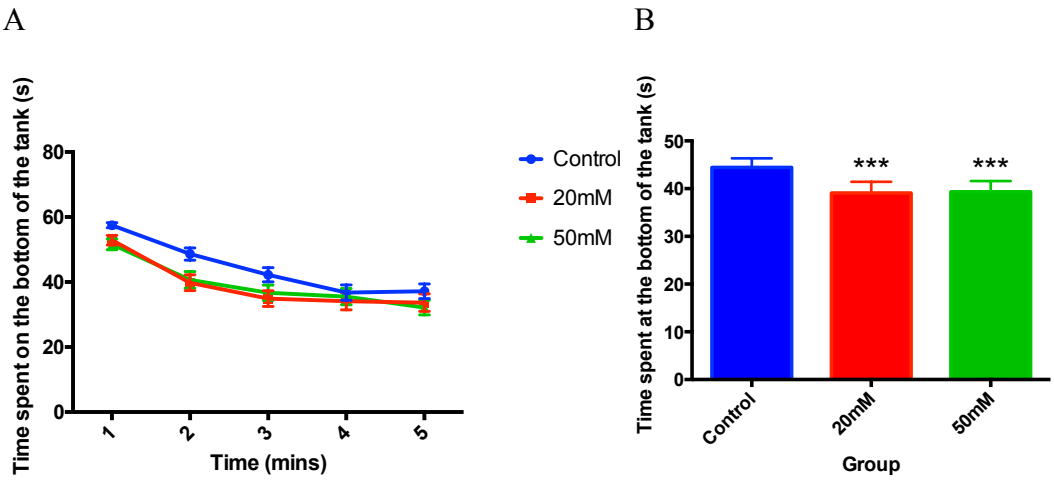
5.2.3 Assessing social interaction.

To assess social behaviour two tests were used: individual social behaviour and shoaling, as described in full detail in chapter 2 methods.

5.3 Results

5.3.1 Developmental ethanol exposure reduces stress-reactivity in zebrafish adults measured by novel tank diving

To assess if the stress-reactive differences measured with zebrafish larvae and juveniles treated developmentally with ethanol (chapter 4) still persist in adulthood, the novel tank diving assay was used. Animals treated with ethanol during development exhibited decreased bottom dwelling in the novel tank (figure 5.1) (P<0.01, ANOVA <0.01).



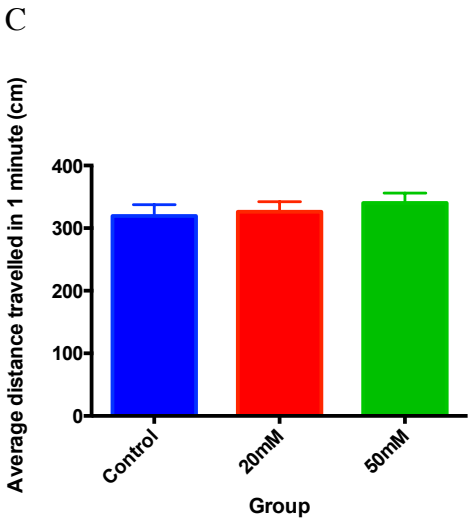


Figure 5.1: Stress-reactivity measured by novel tank diving of zebrafish 6-month-old adults (A and B). Distance travelled during novel tank diving (C). Time course on average time spent at the bottom of the tank (A) and absolute average of time spent at the bottom of the tank (B). Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and a handling control, siblings of animals used for larval and juvenile thigmotaxis in chapter 4. Adult zebrafish subjects exhibited decreased bottom dwelling with ethanol exposure during development, reaching a difference between 50mM and 20mM ethanol treatment and the control (P<0.01, ANOVA <0.01) (A-B). No differences in distance travelled among the groups (C). Six batches of animals were used.

5.3.2 Developmental ethanol exposure reduces stress-reactivity in zebrafish adults measured by thigmotaxis

To confirm the findings obtained from the novel tank diving assay, thigmotaxis was used. Adult zebrafish that had been treated developmentally with 20mM ethanol exhibited a decrease in time spent at the edge of the circular tank compared to controls (figure 5.2) (P<0.05), however, animals treated with 50mM showed no change in time spent at the edge of the tank compared to controls.

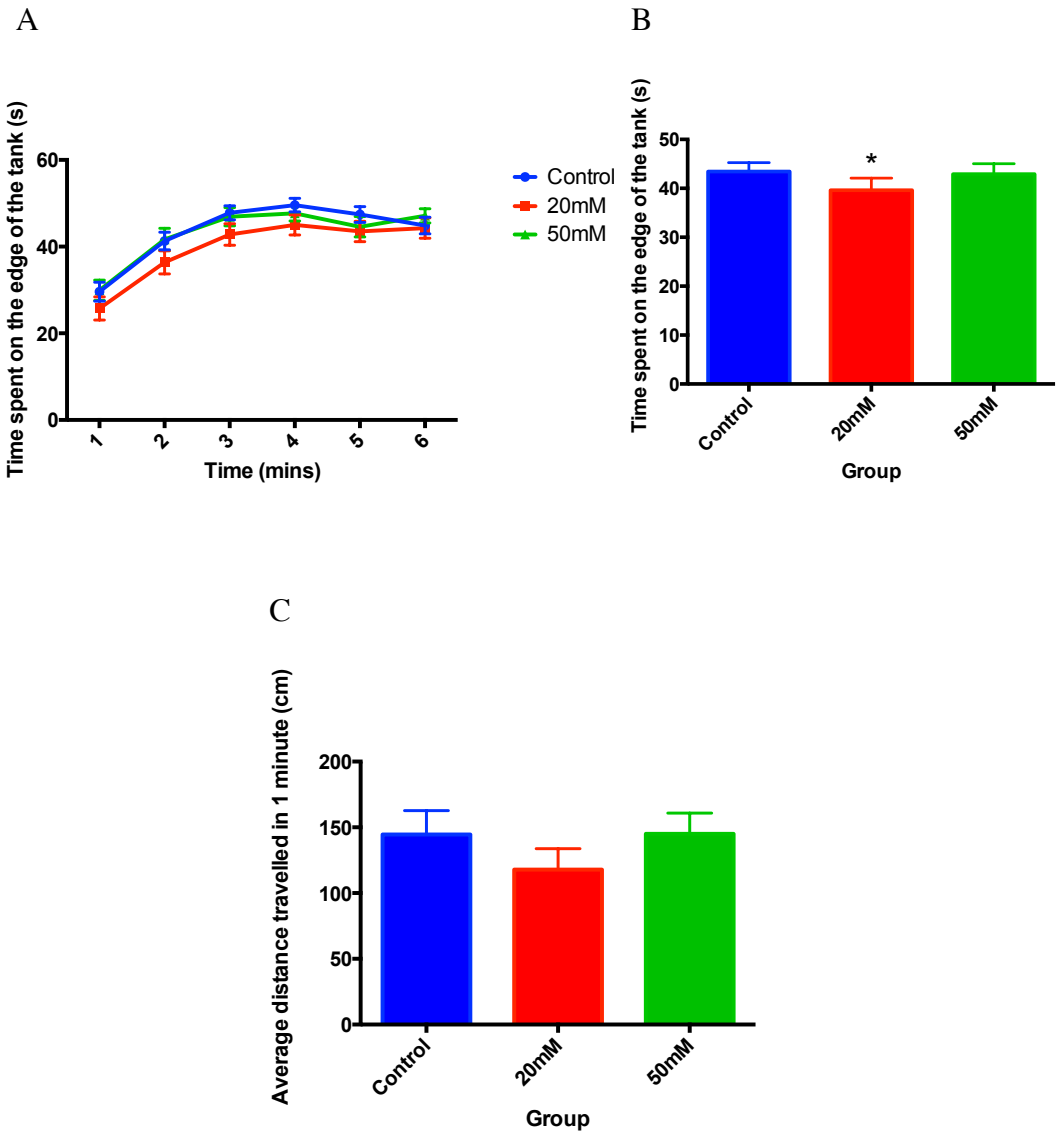


Figure 5.2: Stress-reactivity measured by thigmotaxis of zebrafish 6-month-old adults (A and B). Distance travelled during novel tank diving (C). Time course on average time spent at the edge of the apparatus (A) and absolute average of time spent at the edge of the apparatus (B). Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and a handling control, siblings of animals used for larval and juvenile thigmotaxis in chapter 4. Adult zebrafish subjects exhibited decreased time spent on the edge of the tank related with ethanol exposure during development, reaching a difference between 20mM ethanol treatment and the control (P<0.05, ANOVA <0.05) (A-B). No differences in distance travelled among the groups (C). Six batches of animals were used.

5.3.3 Developmental ethanol exposure reduces stress-reactivity in zebrafish adults measured by immobility in novel tank diving and thigmotaxis

Immobility or "freezing" was previously used in chapter 4 as a measure for larval stress-reactivity. In novel tank diving, developmental ethanol exposure reduced the time the animal spends immobile during the assay (figure 5.3) (P<0.05, ANOVA <0.05). There was no significant change in thigmotaxis.

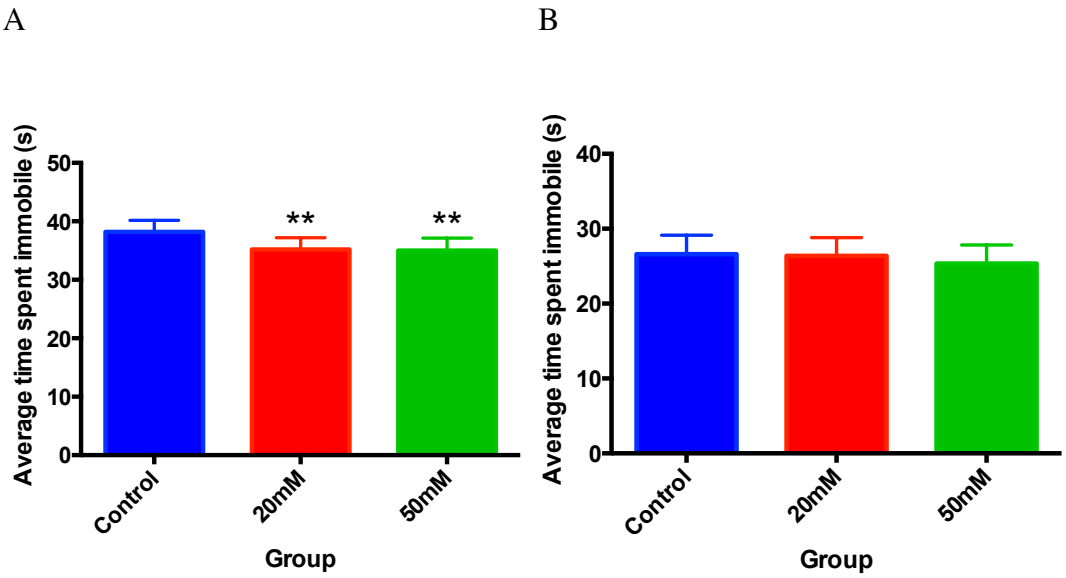


Figure 5.3: Stress-reactivity measured by immobility of novel tank diving (A) and thigmotaxis (B) of zebrafish 6-month-old adults. Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and a handling control, siblings of animals used for larval and juvenile thigmotaxis in chapter 4. Adult zebrafish subjects exhibited decreased time spent immobile related with ethanol exposure during development, reaching a difference between 50mM and 20mM ethanol treatment and the control (P<0.05, ANOVA <0.05) using novel tank diving assay. No differences were found using thigmotaxis. Six batches of animals were used.

5.3.4 Developmental ethanol exposure reduces stress-reactivity in zebrafish adults measured by scototaxis

Scototaxis was used as a third assay to confirm the changes in stress-reactivity produced by early ethanol exposure on the zebrafish model. Early ethanol exposure caused an increase in time spent on the bright side of the tank compared to controls. This effect was more prominent with animals exposed to 20mM ethanol (figure 5.4) (P<0.01, ANOVA <0.01). Time spent on the bright side (open side) opposite to time spent on the dark side (enclosed side) of the tank can be an indication of less stress in those animals.

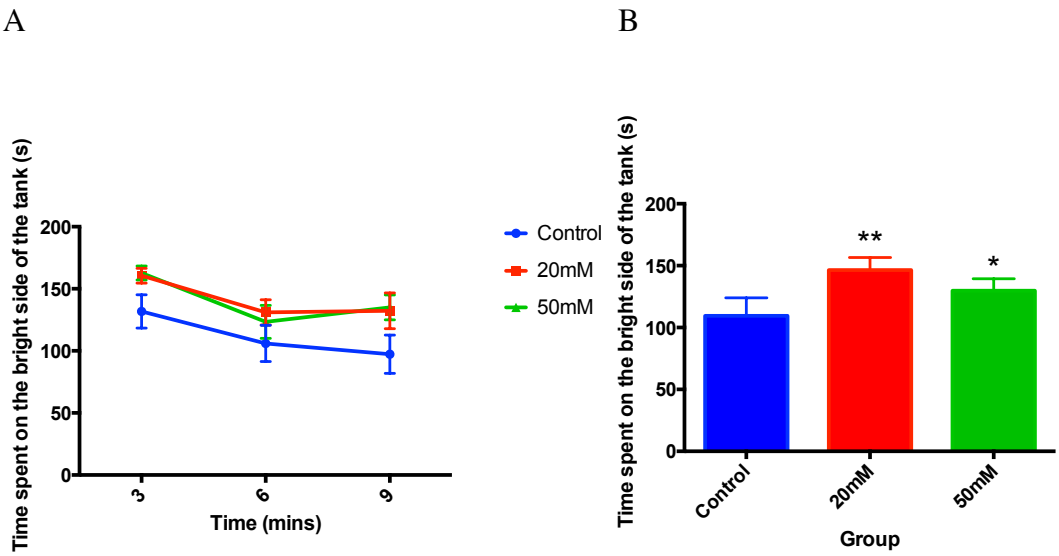


Figure 5.4: Stress-reactivity measured by scototaxis of zebrafish 6-month-old adults (A and B). Time course on average time spent at the bright side of the apparatus (A) and absolute average of time spent at the bright side of the apparatus (B). Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and a handling control. Adult zebrafish subjects exhibited an increase in time spent on bright side of the tank related with ethanol exposure during development, reaching a greater difference between 20mM ethanol treatment and the control (P<0.01). Animal treated with 50mM also exhibited a difference (P<0.05, ANOVA <0.01) (A-B). Three batches of animals were used.

5.3.5 Sex differences in stress reactivity responses to developmental ethanol exposure, assessed by novel tank diving and thigmotaxis.

In animals developmentally exposed to 20mM ethanol, males showed less stress reactivity than females (figure 5.5) (P<0.01) when assessed by novel tank diving. Using thigmotaxis, females treated with 20mM ethanol developmentally spent less time on the edge of the tanks compared to males treated with the same ethanol concentration (figure 5.5) (P<0.01). Controls and 50mM treated animals showed no significant differences.

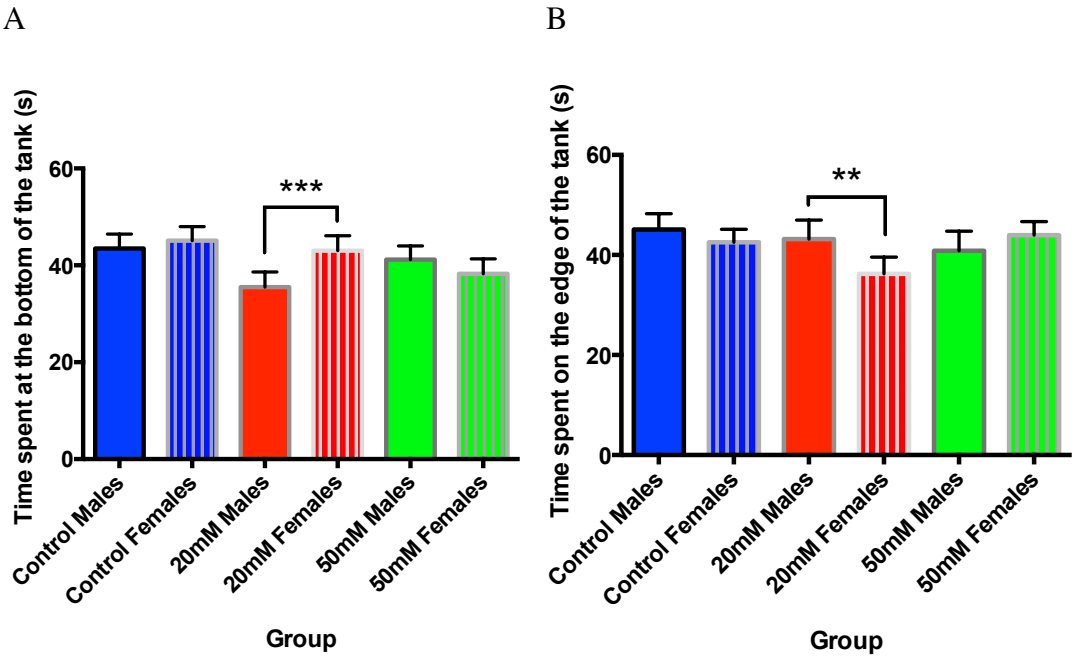


Figure 5.5: Sex differences in stress-reactivity measured by novel tank diving (A) and by thigmotaxis (B) of zebrafish 6-month-old adults. Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and a handling control, siblings of animals used for larval and juvenile thigmotaxis in chapter 4. Adult zebrafish subjects exhibited sex differences when treated at 20mM. Females spent more time at the bottom of the tank compared to males in tank diving, and spent less time at the edge of the tanks in thigmotaxis (P<0.01). Six batches of animals were used.

5.3.6 Developmental ethanol exposure increases social bonding in zebrafish adults measured by individual social behaviour, but not in shoaling.

As a way to measure social interaction behaviour of developmentally treated zebrafish adults, individual social behaviour and shoaling behaviour were assayed. In individual social behaviour, tested subjects were placed in a tank with five strange zebrafish adults in another compartment. Time spent close to a divider where the tested subject could observe and "smell" the strange animals (the social zone) was recorded and tracked.

Animals developmentally treated with ethanol exhibited higher social interaction individually, by spending a significant higher amount of time on the social zone of the tanks compared to controls (figure 5.6) (P<0.01, ANOVA <0.01). When social interaction was assessed by the shoaling assay, early ethanol treatment produced no difference in the proximity the animals swam together (clustering score) as a group compared to controls (figure 5.7).

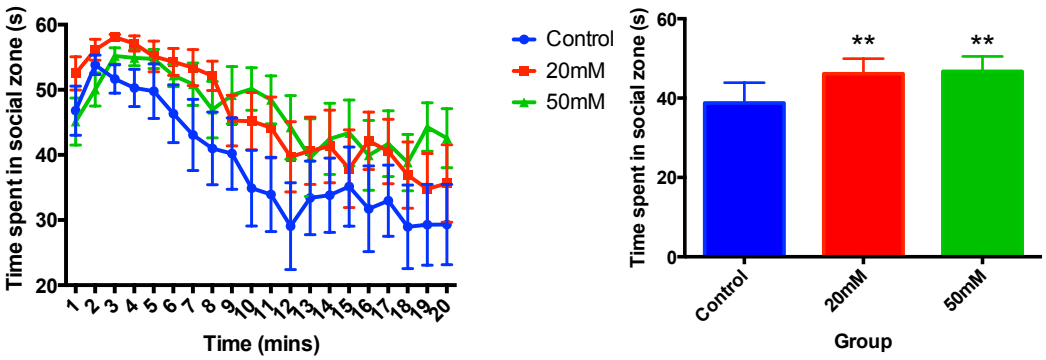


Figure 5.6: Individual social behaviour measured in zebrafish 6-month-old adults. Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and a handling control, siblings of animals used for larval and juvenile thigmotaxis in chapter 4.

Adult zebrafish subjects exhibited an increase in social interaction related to ethanol exposure during development, reaching a difference between the 20mM and 50mM groups compared to the controls (P<0.01, ANOVA <0.01) (top). Six batches of animals were used

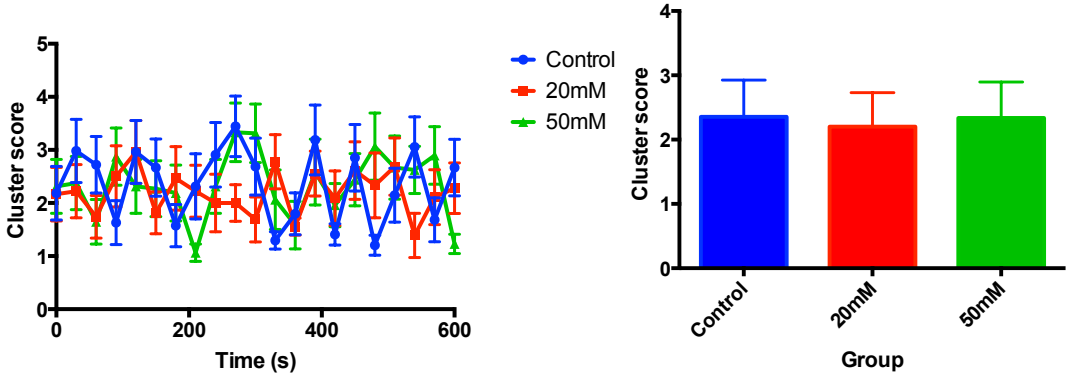


Figure 5.7: Shoaling social behaviour measured in zebrafish 6-month-old adults. Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and a handling control, siblings of animals used for larval and juvenile thigmotaxis in chapter 4. Adult zebrafish subjects exhibited no difference in social clustering related with ethanol exposure during development. Six batches of animals were used.

5.3.7 Developmental ethanol exposure combined with methylene blue eliminates stress-reactivity differences in zebrafish adults measured by novel tank diving and thigmotaxis

An original pilot experiment where animals were treated in presence of the fungicidal methylene blue yielded a different set of results compared to the previous one. All the differences in stress reactivity seen with ethanol treated adults were eliminated (figure 5.8). This suggests a possible counter effect that this drug might have in the early ethanol exposure of zebrafish embryos.

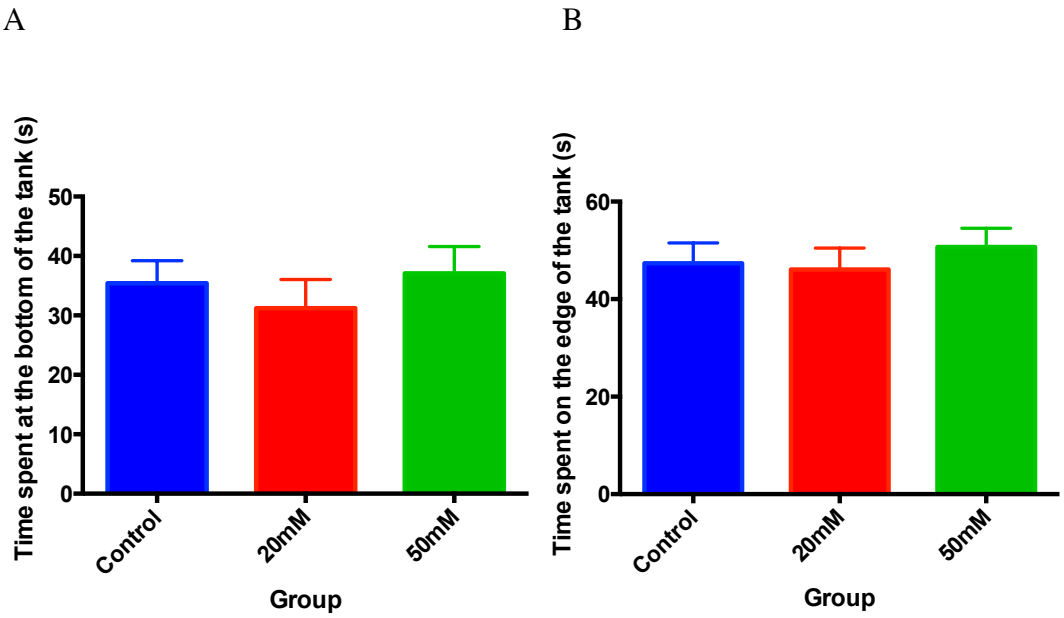


Figure 5.8: Stress-reactivity of animals treated with methylene blue. Stress-reactivity was measured by novel tank diving (A) and thigmotaxis (B) of zebrafish 6-month-old adults. Animals were developmentally treated with ethanol and methylene blue 0.01% from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and a handling control. Adult zebrafish subjects exhibited no difference in bottom dwelling or time spent on the edge of the tanks. Three batches of animals were used.

5.4 Discussion

In human and other mammalian models, developmental exposure to alcohol leads to stress-handling and social behavioural deficits. In this chapter, the impact of early ethanol exposure on social behaviour and stress were analysed in the zebrafish model.

Initially, the use of novel tank diving as a stress reactivity measurement was characterised. Novel tank diving as an assay to measure anxiety-like behaviour in zebrafish has been extensively used (Mathur and Guo 2011, Blaser and Rosemberg 2012, Parker, Millington et al. 2012, Parker, Annan et al. 2014). Zebrafish adults exposed to ethanol from1-9dpf exhibited significant decreases in the time spent at the bottom of the tank, therefore being less stress-reactive compared to controls (figure 5.1).

Scototaxis and thigmotaxis, two other assays routinely used to assess anxiety-like behaviour, were used to confirm the findings observed with novel tank diving. Animals developmentally exposed to ethanol spent more time at the bright side and less time in the edge of the tanks respectively (figure 5.2 and 5.4).

Observing changes in mobility, early ethanol exposure also decreased the average time these animals stay immobile or "freeze" in novel tank diving and thigmotaxis, and this may also be interpreted as a measure of stress-reactiveness or boldness of these animals (figure 5.3).

These findings match the previous findings obtained with zebrafish larvae and juveniles developmentally exposed to ethanol. It contradicts data previously been described in other studies, indicating that early ethanol exposure increases stress-

reactivity and anxiety in mammalian models (Osborn, Kim et al. 1998, Carneiro, Diogenes et al. 2005, Hellemans, Sliwowska et al. 2010, Hellemans, Verma et al. 2010).

In the present study, the animals tested as adults were siblings of the larvae and juveniles used in the studies illustrated in chapter 4, leading to the conclusion that early ethanol exposure causes persistent, and possibly permanent changes in the stress handling system of these animals.

Sex differences in behaviour were also observed using novel tank diving and thigmotaxis. Males exposed to 20mM developmentally were less stress reactive compared to females using novel tank diving, and the opposite result was seen using thigmotaxis (figure 5.5). Since no sex differences were seen in animals developmentally exposed to 50mM ethanol, it seems that the sex related differences in stress reactivity were potentiated by the 20mM ethanol treatment.

These sex related differences in stress reactivity have been studied in rodent models and the sexual dimorphic response varies with the intensity and type of stressor. Female offspring of rats exposed to ethanol prenatally show greater changes in corticosterone and ACTH levels in response to short duration and acute stressors, compared to males (Taylor, Branch et al. 1988, Weinberg 1988, Kelly, Mahoney et al. 1991). Female rats also exhibit increase corticosterone levels in response to immune challenges (Kim, Turnbull et al. 1999).

Male offspring of rats exposed to alcohol developmentally are in contrast more stress reactive than females when the stressor is present for a prolonged time, assessed by corticosterone levels (Weinberg 1992, Kim, Giberson et al. 1999). Furthermore, both males and females developmentally exposed to ethanol will

exhibit higher mRNA levels of CRH, immediate early genes (IEGs) c-fos and NGFI-B in the PVN compared to untreated animals (Lee, Schmidt et al. 2000).

Accordingly, the sexual dimorphic difference obtained in novel tank diving and thigmotaxis, when 20mM treated animals were tested, is possibly due to the nature of the stress stimulus in these assays and how it is perceived by the animals.

It was also observed that a developmental ethanol treatment of zebrafish embryos 1-9dpf in the presence of methylene blue (0.01%) resulted in animals with no stress-reactivity differences (figure 5.8). Methylene blue is a biological active compound in zebrafish, used for a variety of pharmacological function, such as as fungicidal to prevent mould forming in the ethanol-containing solutions.

A possible explanation why it obliterates the early effects of ethanol in stress reactivity is that methylene blue is also a highly redox active dye (Heydrick, Reed et al. 2007), therefore possibly preventing the oxidative stress caused by ethanol, which was extensively discussed in the introduction.

Lastly, social behaviour differences were observed in developmental ethanol exposed animals. Individual social behaviour indicated that ethanol treated animals are more socially interactive compared with controls, spending significantly more time in the social segment of the tanks (figure 5.6). By using shoaling as a social behaviour measurement, ethanol treated animals and controls had no significant difference (figure 5.7).

Shoaling assay does not necessarily answer the same question as individual social behaviour, as it observes animals that are housed together for 6 months swimming as a group. For most of the time these animals shoal, they are following a leader, matching its movements and speed. This can be observed from the recorded videos in the present experiments. It may thus be more indicative of how well the

subjects follow the movements of the leader rather than a social interaction measurement.

It is also important to mention that the order in which these experiment were done could have had an effect. Individual social behaviour following shoaling was done first, with a one-week gap between them. Immediately after shoaling was done, animals were pair housed and novel tank diving was done two weeks later, following thigmotaxis one week after novel tank diving. This order was used in every adult animal and was not randomized; therefore it is possible that animal exposure to social assays could have lead to differences in stress-reactivity performed weeks later.

Chapter 6: Differences in whole-body cortisol concentration of zebrafish larvae, juveniles and adults developmentally exposed to ethanol

6.1 Introduction

In the previous chapters behavioural analysis indicated differences in stress-reactivity of zebrafish larvae, juveniles and adults that had been developmentally exposed to ethanol. Overall, zebrafish exposed to ethanol during development exhibited lower stress reactivity, as assessed by a number of behavioural tests. The aim of this chapter is to elucidate whether this is associated with changes in HPI function. To achieve this, whole-body cortisol was assayed in control and developmentally ethanol-treated animals.

Stress is a term that comprises experiences that are physiologically and emotionally challenging to the organism. The stress response is a cascade of physiological events following the perception of a threat, comprising the activation of autonomic nervous system and the hypothalamo-pituitary-adrenal (HPA) axis in order to achieve allostasis (stability by active means) (McEwen 2007).

In teleost fish, the primary stress response factor is cortisol (Schreck, Contreras-Sanchez et al. 2001). Cortisol is a hormone that is typically extracted from the plasma of larger fish species such as the tilapia (Kammerer, Cech et al. 2010), whereas whole-body cortisol is more routinely used in smaller species, such as zebrafish, with more details in 2.5.2 and 2.5.4 (Ramsay, Feist et al. 2006). Most recently a non-invasive method using water-borne cortisol from zebrafish holding-

water was used, based on the principle that steroids are released by passive diffusion through the gills of the adult zebrafish (Felix, Faustino et al. 2013).

In fish, cortisol regulates many systems, including osmoregulation, glucose metabolism, behaviour and immunological functions (Wendelaar Bonga 1997). Due to lack of aldosterone synthase (essential for aldosterone production) it has been suggested that cortisol controls mineralocorticoid functions too, stimulating Na^+ and Cl^- transport across membranes and essential for hydromineral control in marine and fresh water environments (Wendelaar Bonga 1997). Cortisol is synthesized and secreted in the fish interrenal steroidogenic cells, found in the interrenal tissue, the structural analogue of the mammalian adrenal cortex (Wendelaar Bonga 1997).

Developmental ethanol exposure has been implicated to modulate glucocorticoid activity later in life. In humans, prenatally alcohol exposed (PAE) 19-month children have lower basal cortisol levels compared to controls though this is more prominent in boys compared to girls (Ouellet-Morin, Dionne et al. 2011). Female PAE rats exposed to ethanol during gestational weeks 2 and 3 had higher corticosterone levels when chronically stressed in adulthood (Taylor, Branch et al. 1982).

Although little work has been done on the effects of developmental ethanol exposure on cortisol in the fish model, early-life stress experienced by fish larvae still in their eggs has been demonstrated to reduce cortisol response in 5-month-old rainbow trout (Auperin and Geslin 2008). In this chapter, the evaluation of a human salivary cortisol kit for use in the zebrafish and the effects of developmental ethanol exposure on whole-body cortisol in zebrafish larvae, juveniles and adults are presented.

6.2 Methods

6.2.1 Animals

Larval zebrafish at 9df, juveniles at 23dpf and 6 month old adults, age and weight matched, (3:2 females:males ratio) were used. Control fish and fish that had been exposed to ethanol from days 1-9 dpf, as described in chapter 2, were used. Following ethanol treatment, larvae were transferred to the aquarium facility and reared to adulthood in the absence of ethanol. Fish of each group were maintained in six separate tanks.

6.2.2 Characterization of Salimetrics human salivary cortisol for zebrafish cortisol analysis

First, the Salimetrics human salivary kit was validated for zebrafish whole-cortisol use. Sensitivity, precision, accuracy and parallelism were evaluated and the tests used and data obtained are presented in Results section 6.3.1.

6.2.3 Whole-body cortisol extraction

To test for differences in response to stress, animals were exposed to air for 30sec (adults) or 1min (larvae and juveniles). Animals were then flash frozen 5mins later. Cortisol was extracted and assayed using the Salimetrics human salivary cortisol kit 1-3002 validated for zebrafish use (6.3.1). Zebrafish larvae and juvenile protein quantification was performed using the Bradford reagent, as described in chapter 2.

6.2.4 Water borne cortisol extraction

To test for differences in water borne cortisol levels of zebrafihs 9dpf larvae, an extraction procedure was performed in the labatory of Prof Rui Oliveira, Lisbon, Portugal according to his published protocol (Felix, Faustino et al. 2013). Larvae were acclimatized for 1 hour in glass petri dishes filled with 5mL of fish water prior to a mechanical swirling stress (100rpm). Water-borne cortisol was then extracted using solid-phase extraction cartridges and analysed using a Cayman cortisol EIA kit. Protein quantification was obtained by using the Lowry protocol, as described in chapter 2.

6.2.5 Statistics

For cortisol and stress reactivity assays, ANOVA and student t-tests were used. For characterization of parallelism of standard curves in 6.3.1.5, a non-linear model analysed the slope of both curves to determine if they were parallel. The dependent variables were whole-body cortisol concentration and time spent in the bottom of the tank in the novel tank diving assay. The tests were evaluated with respect to type-1 error rate of 0.05.

6.3 Results

6.3.1 Validation of Salimetrics 1-3002 human salivary cortisol kit for zebrafish whole-body cortisol

6.3.1.1 Sensitivity of Salimetrics 1-3002

The lower limit of sensitivity was determined by comparison of the mean optical density obtained with zero cortisol with that obtained with low levels of added cortisol. The minimal concentration of cortisol that is distinguishable from 0 is 0.12 ng/mL, obtained from a kit standard or 0.17ng/mL from a zebrafish adult cortisol extraction ($P<0.05$) (figure 6.1).

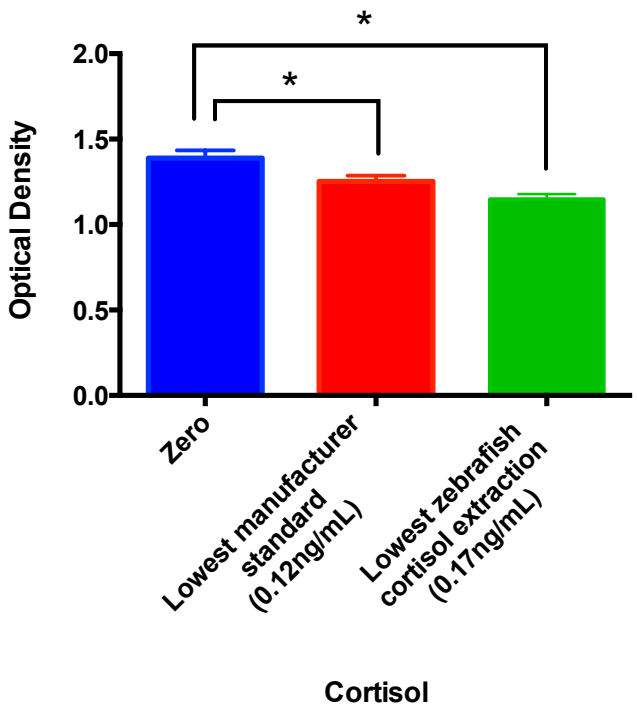


Figure 6.1: Sensitivity of Salimetrics cortisol assay, calculated by the mean of six duplicate samples on 4 different days. Cortisol concentration 0.12ng/ml is the minimum detectable value distinguishable from zero (P<0.05), as well as the lowest zebrafish cortisol extraction, 0.17ng/mL. N=4.

6.3.1.2 Specificity of antiserum, Salimetrics 1-3002

Cross reactivity with other related compounds provided by Salimetric (figure 6.2).

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in Salivary Cortisol EIA
Prednisolone	100	0.568
Prednisone	1000	ND
Cortisone	1000	0.130
11-Deoxycortisol	500	0.156
21-Deoxycortisol	1000	0.041

17α-Hydroxyprogesterone	1000	ND
Dexamethasone	1000	19.2
Triamcinolone	1000	0.086
Corticosterone	10,000	0.214
Progesterone	1000	0.015
17β-Estradiol	10	ND
DHEA	10,000	ND
Testosterone	10,000	0.006
Transferrin	66,000	ND
Aldosterone	10,000	ND

Figure 6.2: Specificity of Salimetrics cortisol assay antiserum. Provided by the company. ND = None detected (<0.004).

6.3.1.3 Precision of Salimetrics 1-3002

Intra-assay precision was determined from six replicates samples from three adult zebrafish whole-body cortisol extracts. The coefficient of variation did not exceed 6% (figure 6.3, A). Inter-assay precision was determined from twelve replicates samples from two adult zebrafish whole-body cortisol extracts (samples 1 and 3 used for intra-assay precision) run in two different ELISA plates on three different days (figure 6.3, B). Coefficient of variation did not exceed 5%.

A

Sample	N	Mean (ng/mL)	Standard Deviation (ng/mL)	Coefficient of Variation (%)
1	6	4.85	0.08	1
2	6	0.91	0.05	6
3	6	0.45	0.02	5

B

Sample	N	Mean (ng/mL)	Standard Deviation (ng/mL)	Coefficient of Variation (%)
1	12	4.7	0.24	5
3	12	0.45	0.02	4

Figure 6.3: Precision of Salimetrics cortisol assay. Intra-assay (A) and inter-assay (B) precision using samples extracted from adult zebrafish. Intra-assay was calculated by the mean of six replicates of three different adult zebrafish samples. Inter-assay precision was calculated fom the mean of twelve replicates of two different adult zebrafish samples (samples 1 and 3 used for intra-assay precision) ran in two different ELISA plates in three different days. Coefficient of variation did not exceed 5%.

6.3.1.4 Accuracy of Salimetrics 1-3002

Three adult zebrafish whole-body cortisol extract samples containing different levels of endogenous cortisol were spiked with a known quantity of cortisol (3ng) and assayed. Two replicates per sample were used. Recovery was between 101% and 104% and a strong correlation (R^2=0.94, P<0.0001) (figure 6.4).

A

Sample	Endogenous (ng/mL)	Added (ng)	Expected (ng/mL)	Observed (ng/mL)	Recovery (%)
1	0.42	3	3.41	3.47	101
2	0.24	3	3.24	3.30	102
3	0.49	3	3.49	3.65	104

B

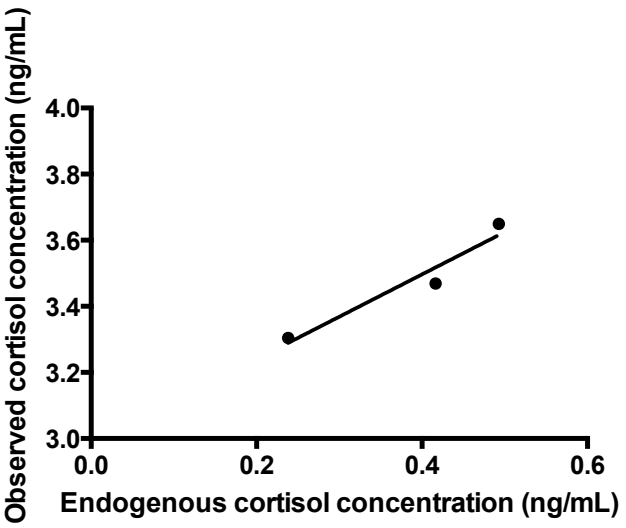


Figure 6.4: Accuracy of Salimetrics cortisol assay using samples extracted from adult zebrafish. Table (A) and correlation graph (B) of samples. 3ng of cortisol was added to known endogenous cortisol samples from zebrafish adult extracts. Recovery was between 101% and 104%. Two replicates per sample were used. R^2=0.94, P<0.0001.

6.3.1.5 Parallel standard curves of Salimetrics 1-3002

Parallel standard curves were generated by taking a high value zebrafish adult whole-body cortisol extract sample, serially diluted and assayed. Resultant curve is parallel to the salimetrics standard curve over that range. Using a comparison of fits in a non-linear model, the curves were shown to be parallel with a similar slope (P=0.3) (Figure 6.5).

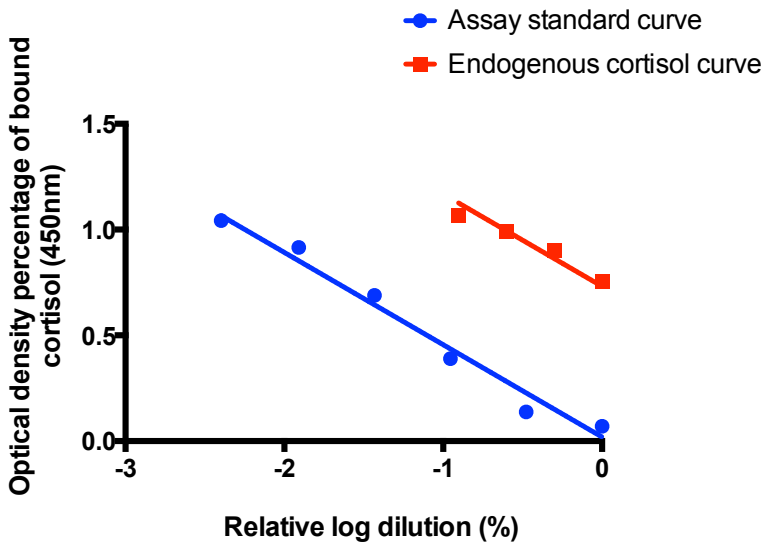


Figure 6.5: Parallelism of Salimetrics cortisol assay standard curve against a standard curve made from a sample extracted from adult zebrafish. Three replicates per sample were used. Using a comparison of fits in a non-linear model, the curves were parallel with a similar slope between them, *P*=0.3.

6.3.1.6 Characterization of water-borne cortisol extraction for zebrafish larvae use

Cortisol can be extracted from adult zebrafish water. To test whether the same is true for young zebrafish larvae, cortisol was extracted from larval (9dpf) fish water. Experimental animals were exposed to a mechanical swirling stressor and left for 1 hour. Stress significantly increased water-borne cortisol with time (*P*=0.01) (figure 6.6).

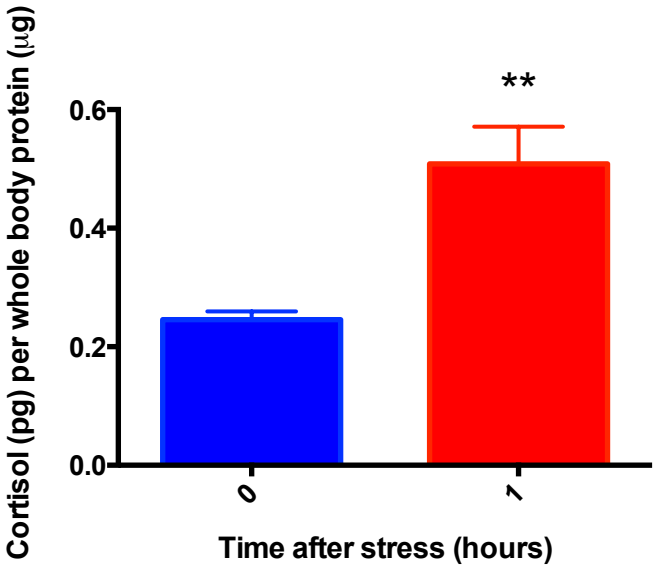


Figure 6.6: Stress-reactivity measured by water-borne cortisol concentration of zebrafish 9dpf larvae. Animals were acclimatized fore 1 hour prior to mechanical swirling, and water was extracted from the petri dishes immediately or 1 hour after the stress challenge. Zebrafish larvae showed increased water-borne cortisol concentration with time (P=0.01). Three batches of animals were used.

6.3.2 Developmental ethanol exposure reduces cortisol output in zebrafish larvae following air exposure challenge

To assess whether the stress reactivity differences measured using the thigmotaxis assay in 9dpf larvae (chapter 4) are associated with changes in whole-body cortisol, siblings of the same animals used for the thigmotaxis assays (chapter 4) were used. There were no differences in cortisol between unstressed control and unstressed ethanol treated animals.

However, cortisol in air exposed developmentally treated animals was decreased (50mM ethanol group), compared with controls (P<0.05, ANOVA <0.01) (figure 6.7). There was also a significant difference between unstressed and air exposed stressed controls and 20mM treated groups, but no difference with 50mM

treated animals (figure 6.7) (P<0.01, controls; P<0.05, 20mM group).

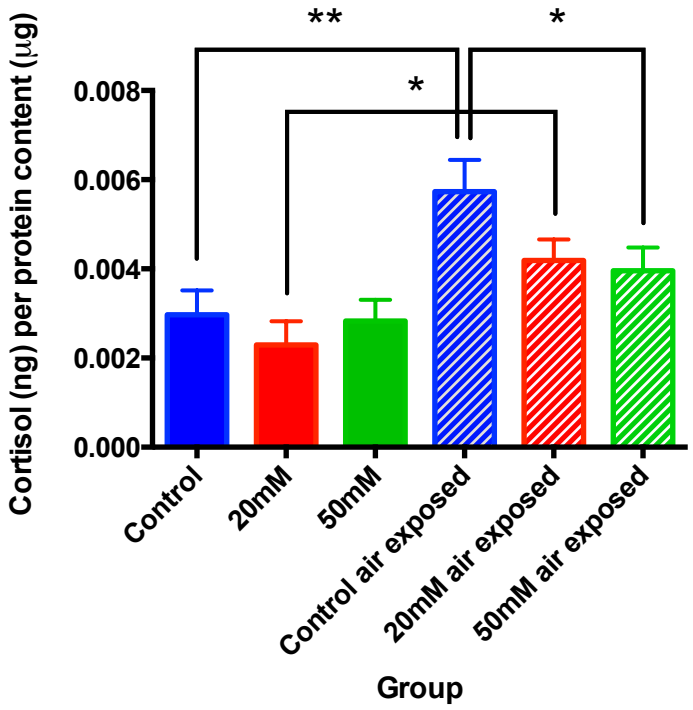


Figure 6.7: Stress-reactivity measured by whole-body cortisol concentration of zebrafish 9dpf larvae. Experimental animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations. Once animals reached 9dpf they were tested for stress-reactivity based on their cortisol output. Animals were either flash frozen immediately or air exposed and frozen 6 minutes later. Air stressed animals showed decreased cortisol concentration with increasing ethanol concentration exposure during development, reaching a higher difference between 50mM ethanol treatment and the control, when air exposed (P<0.05, ANOVA <0.01). There was also a difference between control and 20mM unstressed, and, control and 20mM stressed (P<0.01 and P<0.05 respectively). No other differences were found among the ethanol treated animals. Six batches of animals were used.

6.3.3 Developmental ethanol exposure reduces cortisol output in zebrafish juveniles following air exposure challenge or not

Zebrafish 23dpf juveniles were studied in chapter 4 to verify that the developmental effects of ethanol in the stress-reactivity of these animals was due to

long term changes to the HPI axis rather than an anxiolytic effect of the drug itself. Whole-body cortisol concentration of the siblings was assessed.

Animals treated with 50mM ethanol during development exhibited decreased whole-body cortisol compared to unstressed and stressed controls (figure 6.8) (P<0.05, ANOVA <0.05). No difference was found between unstressed and air exposed stressed controls (figure 6.8).

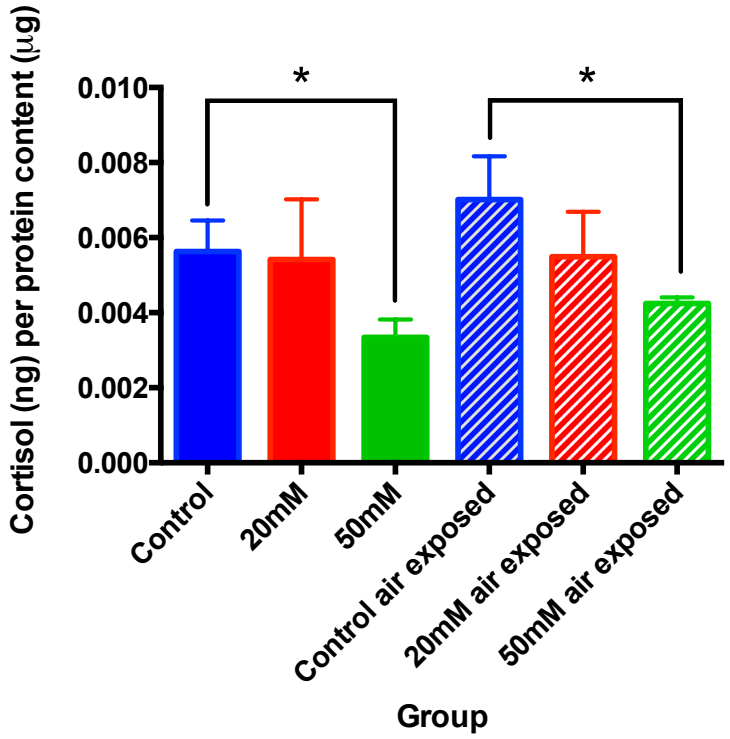


Figure 6.8: Stress-reactivity measured by whole-body cortisol concentration of zebrafish 23dpf juveniles. Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and a handling control. Once animals reached 23dpf they were tested for stress-reactivity based on their cortisol output. Animals were either flash frozen immediately or air exposed and frozen 6 minutes later. 23dpf zebrafish juveniles showed decreased cortisol concentration with increasing ethanol concentration exposure during development, reaching a higher difference between 50mM ethanol treatment and the control when unstressed and when air exposed (P<0.05 both, ANOVA <0.05). No other differences were found between the controls and ethanol treated animals unstressed and air exposed. Four batches of animals were used.

6.3.4 Developmental ethanol exposure reduces cortisol output in zebrafish adults following air exposure challenge

Zebrafish 6-month-old adults were studied in chapter 5 to further verify that the developmental effects of ethanol in the stress-reactivity of these animals was indeed due to long term changes to the HPI. In these animals,a pilot study to verify the effects of air stress on cortisol concentration treatment was performed. In this experiment, air exposure time was varied, and an acute exposure of previously untreated animals to 1% alcohol was also used to test its anxiolytic effect.

Whole-body cortisol concentration following 1min air exposure was not different from that obtained from unstressed control animals (figure 6.9). A 30-second air exposure yielded a cortisol concentration difference (figure 6.9) and was therefore adopted as standard. The animals tested were those used in the novel tank diving and thigmotaxis tests described in chapter 5.

Animal treated with 50mM ethanol during development exhibited decreased whole-body cortisol compared to controls when air stressed (figure 6.10) ($P<0.05$, ANOVA <0.05). No difference was found between unstressed animals (figure 6.10). There was a difference between unstressed and air exposed stressed controls 20mM and 50mM ethanol pretreated animals, in which the response to stress was present (figure 6.10) ($P<0.001$, controls and 20mM groups; $P<0.05$, 50mM group).

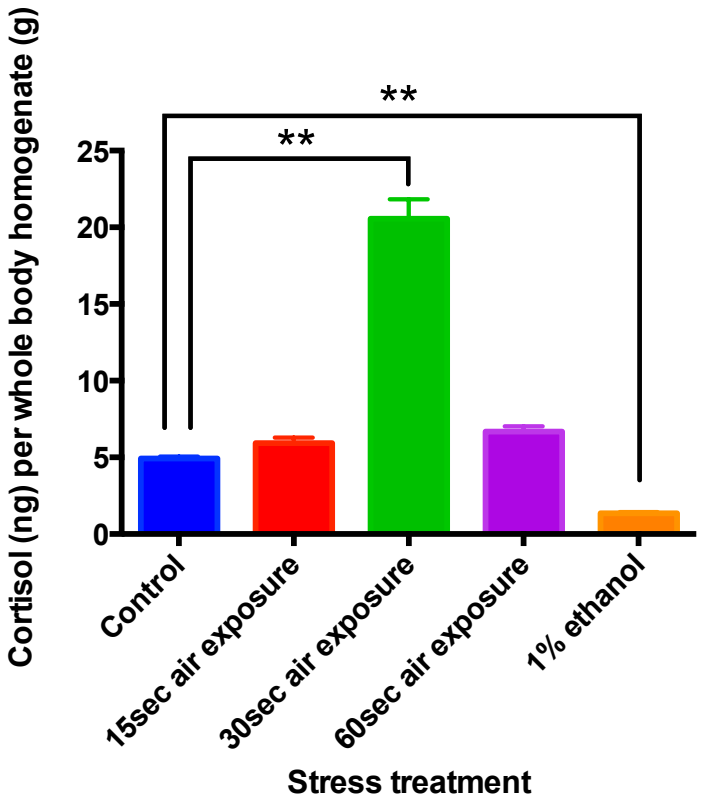


Figure 6.9: Stress-reactivity measured by whole-body cortisol concentration of zebrafish 6-month-old adults. Animals were either flash frozen or stressed, either by air exposure at a specific time or acute exposure to ethanol (1%), followed by freezing 6 minutes later. Zebrafish adults showed increased cortisol concentration when air exposed for 30 seconds and decreased cortisol output when acutely exposed to 1% ethanol compared to controls (P<0.01, ANOVA <0.01). Three batches of animals were used.

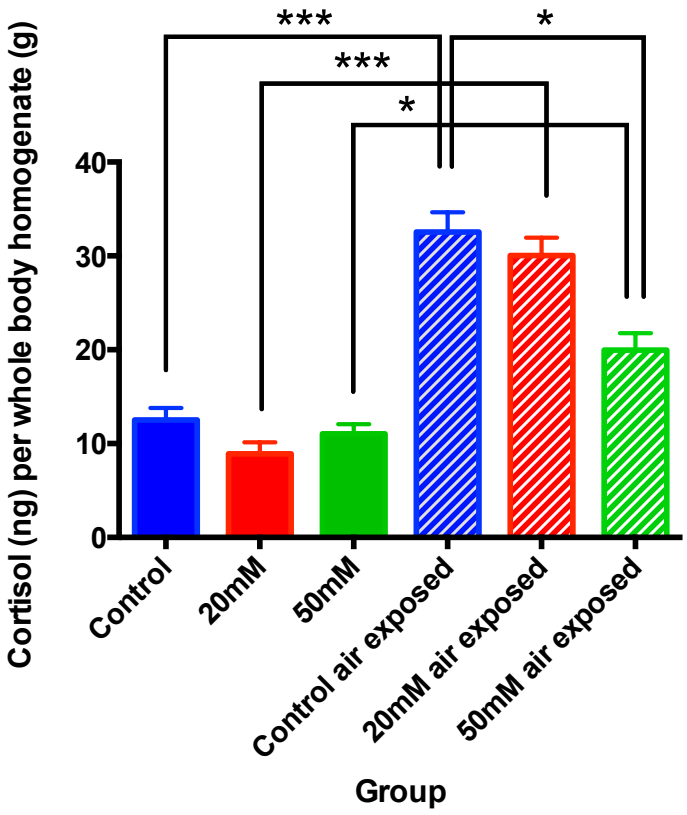


Figure 6.10: Stress-reactivity measured by whole-body cortisol concentration of developmentally treated zebrafish 6-month-old adults. Animals were developmentally treated with ethanol from 1dpf-9dpf with 20mM and 50mM ethanol concentrations and a handling control. Once animals reached 6 months of age they were tested for stress-reactivity based on their cortisol concentration. Animals were either flash frozen immediately or air exposed and frozen 6 minutes later. Zebrafish adults showed decreased cortisol output with increasing ethanol concentration exposure during development, reaching a higher difference between 50mM ethanol treatment and the control when when air exposed (P<0.05, ANOVA <0.01). There was also a significant difference between unstressed and air exposed groups (P<0.01 control and 20mM, P<0.01 50mM). Nine batches of animals were used.

6.3.5 Stress-reactivity in zebrafish adults measured by thigmotaxis correlates with whole-body cortisol concentration

As an indirect validation of thigmotaxis as an assay of the zebrafish stress response, the basal whole-body cortisol levels of male zebrafish adults that had

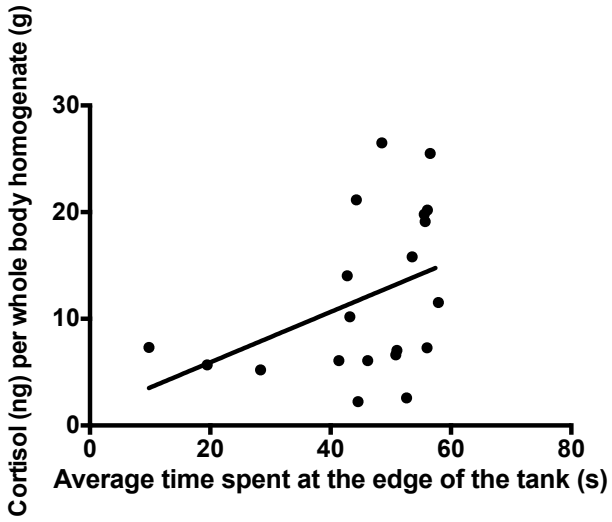
previously been used for novel tank diving and thigmotaxis (chapter 5) were assayed. Thigmotaxis data and whole-body cortisol concentrations were positively correlated in all groups, as it can be seen in the table and an overall correlation graph (figure 6.11, A-B). A strong positive Pearson's correlation (r) was seen in all groups (figure 6.11, A).

Animals with high and low whole-body cortisol were then grouped together, using the mean cortisol values from control and 20mM and 50mM ethanol pretreated animals as thresholds: 3.22ng/mL, 2.89ng/mL and 2.84ng/mL respectively (figure 6.11, C). Animals with basal whole-body cortisol levels higher than the mean for that group spent more time at the edge of the tank compared to animals with basal whole-body cortisol level lower than the mean (figure 6.11, C). The effect was more significant with animal treated with 20mM ethanol during development (figure 6.11, C) (P<0.001, ANOVA <0.05).

A

Developmental Treatment	r
Control	0.39
20mM Ethanol	0.6
50mM Ethanol	0.57

B



C

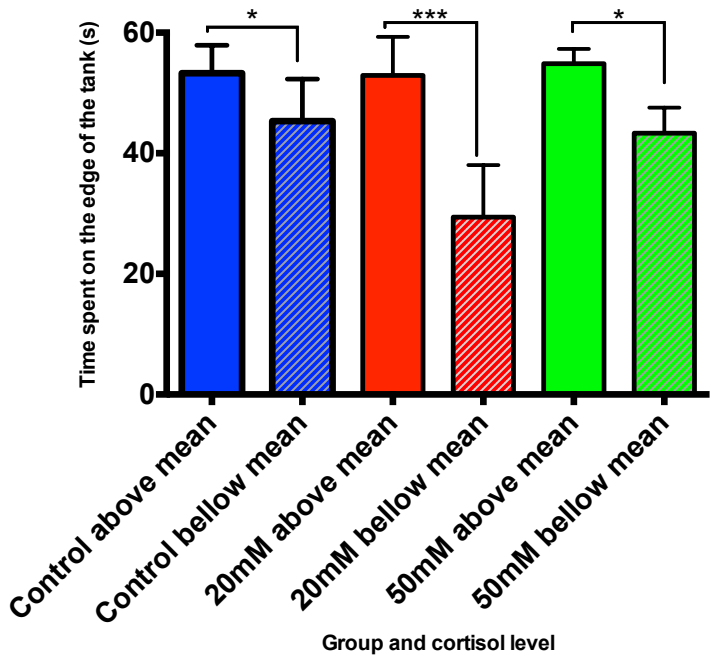


Figure 6.11: Stress-reactivity measured by thigmotaxis and correlated with whole-body cortisol cortisol of zebrafish 6-month-old adults. Pearson's correlation table of control, 20mM and 50mM groups (A) and overall graph containing control, 20mM and 50mM groups together (B). A positive correlation was found between thigmotaxis and whole-body cortisol concentration (A-B). The overall correlation graph also exhibited a strong Pearson's correlation value (r=0.39) (B). An association by grouping whole-body basal cortisol concentrations above and below the mean values with thigmotaxis was also done (C). Animals were developmentally treated with ethanol from 1dpf-

9dpf with 20mM and 50mM ethanol concentrations and a handling control solution, siblings of animals used for larval and juvenile thigmotaxis in chapter 4. Adult zebrafish subjects exhibited decreased time spent on the edge of the tank related with lower whole-body cortisol concentration (C). Zebrafish treated with 20mM exhibited a greater difference, between animals with whole-body cortisol levels higher and lower than the mean (P<0.001) (E). Controls and 50mM ethanol treated animals also exhibited a difference (P<0.05, ANOVA <0.05) (E). This further characterizes thigmotaxis as a stress-reactivity assay, correlating time spent on the edge of the tank with cortisol output of the subjects. Six batches of animals were used.

6.3.6 Characterization of novel tank diving as an assay to measure stress-reactivity

Novel tank diving was used in chapter 5 as a measure of stress reactivity. Here the acute effects of ethanol (for these purposes assumed to be predominantly anxiolytic) were used in adults to further validate the interpretation of cortisol values as indicators of stress and its association with novel tank diving. Fish acutely treated with ethanol showed decreased bottom dwelling compared to controls (P<0.01) (figure 6.12, B). Fish acutely exposed to ethanol also exhibited a decrease in whole-body cortisol concentration compared to controls (P<0.01) (figure 6.12, A).

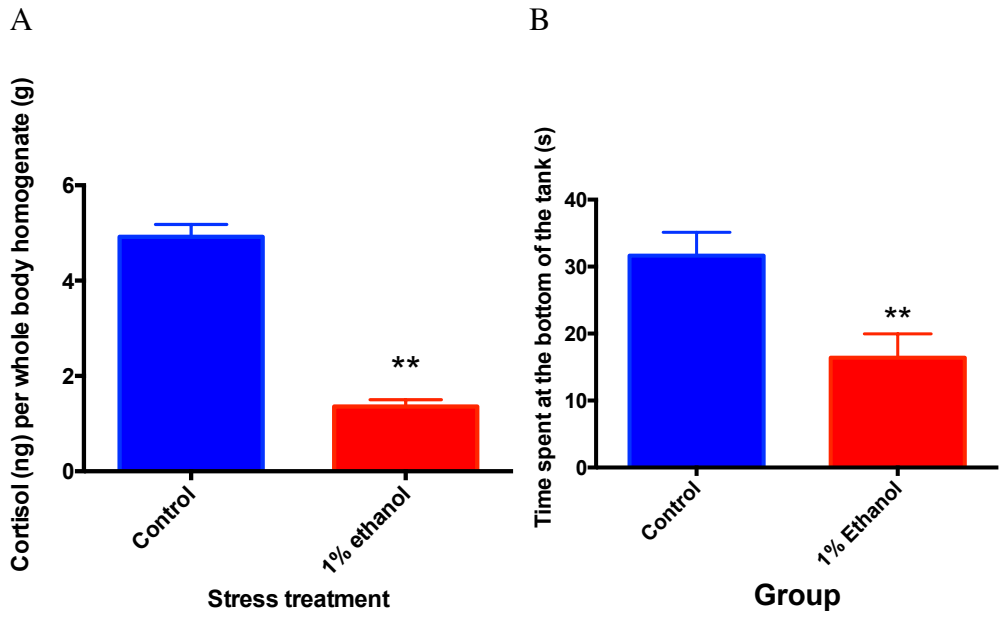


Figure 6.12: Characterization of novel tank diving as an assay to measure stress reactivity. Stress reactivity of zebrafish 6-month-old adults acutely exposed to 1% ethanol using whole-body cortisol (A) and novel tank diving (B). Animals were either treated with ethanol 1% or control fish water. Adult zebrafish subjects exhibited decreased bottom dwelling with ethanol anxiolytic exposure (P<0.01). Three batches of animals were used.

6.4 Discussion

In this chapter, the impact of early ethanol exposure on whole body cortisol was analysed in the zebrafish model. Following the findings of previous chapters in which developmental ethanol exposure decreased stress reactivity in larvae, juveniles and adults, and increased social interaction in adult zebrafish; cortisol was assayed to confirm whether these findings were indeed linked to altered HPI function.

Initially, a characterization of the Salimetrics human salivary cortisol kit was performed. Although this ELISA kit has not previously been fully validated for

zebrafish use before, it is extensively used as a reliable cortisol assay in the zebrafish community (Egan, Bergner et al. 2009, Cachat, Canavello et al. 2010, Grossman, Utterback et al. 2010, Griffiths, Schoonheim et al. 2012).

Here, sensitivity was assessed by determining whether the minimum cortisol standard concentration (0.12ng/mL) provided in the kit, or, 0.17ng/mL from a zebrafish adult cortisol extraction, would be statistically different from zero cortisol concentration (figure 6.1).

Precision (intra and inter assay variation), accuracy and parallelism were all assessed using adult zebrafish fish extracts (figures 6.3-5). Intra and inter assay precision yielded a coefficient of variation that did not exceed 6%. Recovery was observed to be between 101% and 104% with a strong correlation between added cortisol concentration and observed cortisol concentration (R^2=0.94) (figure 6.4). Others have stated that their similar extraction protocol using the same salimetrics kit achieves around 90% recovery (Egan, Bergner et al. 2009).

Parallelism was achieved by comparing a serial dilution of an adult fish sample with the standard curve, and using a non-linear model, the curves were in a similar slope at a specific range, statistical calculations revealed no difference between the two curves (P=0.3), therefore being parallel (figure 6.5). A parallel displacement of serially diluted standard and sample solutions is used in order to determine if the antibody-binding characteristics between samples and standards are similar (Mills, Mourier et al. 2010).

The manufacturer provided specificity data. It showed compounds in human samples that can cross-react with the antibody (figure 6.2). There are no available data about steroids in zebrafish whole-body extracts that can cross-react with the antiserum of this kit, however, a cross-reactivity study has been performed using

rainbow trout and lake trout plasma with a cortisol monoclonal antibody microtitre plate.

Steroids found in the plasma of these telosts had the following stress-reactivities: 11-deoxycortisol (6.2%), cortisone (5%), corticosterone (0.7%), 11-deoxycorticosterone (0.3%), progesterone (0.2%), 17α-hydroxyprogesterone (0.3%), 11-KT (<0.1%) (Barry 1993). The cross-reactivities of the main steroids listed are larger than the ones listed for the salimetriscs assay, which uses a mouse monoclonal antibody (figure 6.2).

Since the Salimetrics ELISA kit has satisfied the strict criteria of precision, accuracy and parallelism using adult zebrafish extracts (figures 6.3-5), it can be assumed that if there are any compounds that can cross-react with the Salimetrics antiserum, they have a minor effect.

In an attempt to reduce the number of animals used for whole-body cortisol extraction, a pilot study was done to extract water-borne cortisol from 9dpf zebrafish larvae fish water, at a laboratory in Lisbon, Portugal. Cortisol extracted from water in which larvae were stressed and left for 1 hour was more concentrated compared to water in which larvae have just been stressed (figure 6.6).

This concludes that water-borne cortisol extraction used with adult zebrafish can also be reliably used with a small group of zebrafish 9dpf larvae as a cortisol variation indicative. However, due to impractical reasons, this technique was not applied in our UK facilities, therefore using whole-body cortisol analysis for subsequent analysis.

Once the use of the Salimetrics human salivary cortisol kit was shown to be valid for zebrafish whole-body cortisol analysis, it was used to assess differences in stress-reactivity of developmentally treated animals with ethanol. Air stressed but

not unstressed (basal) cortisol values for 9dpf larvae developmentally treated with 50mM ethanol were lower than controls (figure 6.7).

There was also a significant difference between the unstressed (basal) and air exposed challenged controls and 20mM treated animals, indicating a strong cortisol response to stress, but no difference between unstressed and air exposed 50mM ethanol pretreated groups (figure 6.7).

Zebrafish adults whole-body cortisol response was very similar to 9dpf larvae, animals subjected to 50mM ethanol exposure during development also exhibited decreased whole-body cortisol compared to controls when air exposed stressed, but, there were whole-body cortisol differences between unstressed and air exposed in all groups, and not just in controls and 20mM treated animals, as seen in 9dpf larvae (figure 6.10).

This suggests that in zebrafish 9dpf larvae, exposure to early 50mM ethanol treatment has the potential to eliminate, at least in part, the cortisol stress response. As discussed in chapter 4, it is believed that most of the stress behavioural response observed in 9dpf treat larvae is due to the impact of ethanol in early plasticity and programming of the HPA axis; it is possible that part of this effect is also due to the acute anxiolytic effects of ethanol, as these animals were tested in ethanol solution.

It is possible that at larval stages, responses to stress are naturally partly suppressed as in human infants (Gunnar, Brodersen et al. 1996). This effect could have played a role in all groups when stressed, being more visible in the 50mM treated groups, diminishing the stress response to undetectable levels. Juveniles at 23dpf treated with 50mM ethanol during development also exhibited decreased whole-body cortisol compared to controls when air exposed, and, when unstressed as well.

Another difference compared to adults and larvae, is that 23dpf juveniles showed no cortisol response to stress, either in ethanol pretreated animals, or untreated controls (figure 6.8). Similar findings were reported in 13-month-old infants, prenatally exposed to ethanol. The subjects exposed to an acute stressor (blood draw) did not exhibit a difference between baseline and post-stress salivary cortisol levels (Jacobson 1999). This could be due to the dampening in adrenocortical response as mentioned earlier, or, that the blood draw in those infants was not stressful enough to elicit a stress response (Gunnar, Brodersen et al. 1996, Jacobson 1999)

It is also possible that they should have been air exposed for different time periods. An air exposure time titration similarly done with adults (figure 6.9) was not done with juveniles as they showed differences in stress-reactive behaviour with 1 min air exposure (figure 4.4 C-D), therefore a titration on the severity of the air exposure should be considered in the future when dealing with zebrafish juveniles.

Zebrafish adults also exhibited a correlation between their whole-body cortisol concentration and the time they spent at the edge of the tank during the thigmotaxis assay (chapter 5). Thigmotaxis and whole-body cortisol concentration correlate positively by performing a Pearson's correlation (figure 6.11, A-B). Animals with basal whole-body cortisol level higher than the mean (~ 3ng/mL) spent more time on the edge of the tank compared to animals with a basal whole-body cortisol level lower than the mean (figure 6.11, C).

These findings match a study peformed in rats in which the level of corticosterone was positively correlated with the number of visits to different arms in the Y-maze, which is an indicator of anxiety-like behaviour (Vallee, Mayo et al. 1997). There was also another indicator of anxiety-like behaviour from this same

study, in which corticosterone was negatively correlated with the time spent in open arms of the elevated plus-maze (Vallee, Mayo et al. 1997).

Novel tank diving has been validated as a measure of stress reactivity by other research teams, with the use of anxiogenics and anxiolytics. Fluoxetine chronic treatment reduced time spent at the bottom of novel tanks and was associated with whole-body cortisol reduction (Egan, Bergner et al. 2009). Acute ethanol exposure has also been used as an anxiolytic in novel tank diving, however, it has not previously been associated with cortisol changes (Egan, Bergner et al. 2009, Parker, Millington et al. 2012).

Adult fish acutely treated with 1% ethanol exhibited decreased bottom dwelling compared to controls when introduced in a novel tank ($P<0.01$) (figure 6.12, B). Animals treated identically with 1% ethanol showed a decrease in whole-body cortisol concentration compared to controls ($P<0.01$) (figure 6.12, A). These findings further support the use of novel tank diving as a reliable measurement to assess stress-reactivity in zebrafish adults.

The larval and adult data presented here matches the mammalian literature; in the sense that at basal conditions, prenatally ethanol exposed (PNE) rats show no difference in plasma corticosterone levels compared to controls (Weinberg, Taylor et al. 1996). However, in contrast to the present data, when exposed to an array of acute and chronic stressors, PNE rats exhibit a higher or prolonged corticosterone response (Nelson, Taylor et al. 1986, Angelogianni and Gianoulakis 1989, Weinberg, Taylor et al. 1996, Kim, Giberson et al. 1999, Kim, Turnbull et al. 1999).

Although the majority of the mammalian literature supports the hyperresponsiveness of the HPA axis due to PNE in mammalian models; there are a few cases in which a hyporesponsiveness was registered. PNE rats exposed from

gestational day (GD) 11 until birth showed lower basal corticosterone levels compared to controls, and when chronic stressed, these differences disappear (Xia, Shen et al. 2014). A single day ethanol exposure done in mice at GD9 yielded corticosterone hyperresponsiveness in mature but not in old mice when chronically stressed (Park, Dumas et al. 2004).

Adolescent rats exposed to prenatal stress (PS) also exhibited a blunted HPA axis response to a moderate dose of ethanol (Van Waes, Enache et al. 2006). Another effect that has a high impact in the outcome in stress reactivity of PS rats is the postnatal handling effect. PS rats had a lower plasma cortisol level when adopted by a foster mother in comparison in being raised by their biological mother (Maccari, Piazza et al. 1995).

Adoption itself reduced corticosterone levels in unstressed rats, which was believed to be due to the increase in care to the pups by their foster mothers, with increased devoted attention compared to biological mothers (Maccari, Piazza et al. 1995).

There may be other prenatal effects that happen in the mother-infant relationship in mammals that do not apply to fish. Cortisol can pass from mother to fetus through the placenta during stress. Acute stress increased 11β-hydroxysteroid-dehydrogenase enzyme (responsible for protecting the fetus by converting maternal cortisol to cortisone) activity in the placenta by 160%, but chronic stress did not affect the basal levels of this enzyme and even reduced its activity by 90% in the event of an acute stressor (Welberg, Thrivikraman et al. 2005).

A strong correlation was also found between maternal and amniotic fluid cortisol concentration (r=0.32), with higher correlation in anxious mothers (r=0.59) (Glover, Bergman et al. 2009). Increased in utero cortisol concentration is

associated with lower cognitive scores in 17-month-old infants (Bergman, Sarkar et al. 2010). Amniotic cortisol was also negatively correlated with gestational age and birth age, and in turn, birth weight was associated with 3-month-old temperament imbalance, assessed by infant behaviour questionnaire (IBQ) (Baibazarova, van de Beek et al. 2013).

From an epigenetics perspective, prenatal stress due to intimate partner violence (IPV) during pregnancy was associated with glucocorticoid receptor (GR) promoter methylation in teenagers (Radtke, Ruf et al. 2011). Methylation of this receptor is associated with higher cortisol responses to stress. IPV before or after gestation was not associated with GR promoter methylation (Radtke, Ruf et al. 2011).

Depression during the third trimester of gestation was also associated with increased neonatal methylation of the GR promoter, and most importantly, methylation of CpG3 in new-borns was associated with increased stress reactivity, measured by salivary cortisol (Oberlander, Weinberg et al. 2008).

The zebrafish model lacks the mother-offspring relationship, and their interplay prenatally and postnatally. When stressed, ethanol treated larvae, juveniles and adults exhibit a decrease in whole-body cortisol concentration. It is possible that the reduced, glucocorticoid response to stress consequent of early ethanol exposure as opposed to the increased stress reactivity found in most mammalian studies might be due in part to the lack of mother-offspring interaction in zebrafish, in which the “fetal programming “ factor of the HPI axis is absent.

Chapter 7: Differences in expression of genes coding for HPI axis components in zebrafish larvae developmentally exposed to ethanol

7.1 Introduction

In the previous chapters behavioural and cortisol analysis indicated differences in stress-reactivity of zebrafish larvae developmentally exposed to ethanol. Overall, zebrafish exposed to ethanol during development exhibited lower stress reactivity assessed by behavioural assays and lower whole-body cortisol. The aim of this chapter is to elucidate whether these changes are due to differences in key HPI axis gene mRNA transcription. To achieve this, transcripts of selected HPI genes in zebrafish 9dpf larvae were assayed by absolute quantitative real-time polymerase chain reaction (qPCR) and by in situ hybridization (ISH).

In mammals, the components of the HPA axis are very susceptible to early environmental manipulation, in a process called early programming, reported in many species (Matthews, 2002). For example, mRNA coding for corticotropin-releasing hormone (CRH) is increased in the paraventricular nucleus (PVN) in rodent pups prenatally exposed to ethanol (Lee et al., 1990; Gabriel et al., 2005) and in male adults (Redei et al., 1993).

In the present study the effects of chronic ethanol exposure on the mRNA expression of some components of the fish HPI axis were investigated, these were: CRH, CRH-R1, CRH-R2, CRH-BP), GR, MR and POMCa. Other genes such as MC2R and MC4R were also going to be tested, but due to experiment limitations

during sample preparations and qPCR machine readings, only 8 genes quantitative transcriptions could be assessed.

7.2 Methods

7.2.1 Animals

Larval zebrafish were size and age matched at 9df. Control fish and fish that had been exposed to ethanol from 1-9 dpf were used. To facilitate visualisation following in situ hybridisation, the Casper mutant line was used, which lacks skin pigmentation (Wenner 2009, Parker, Brock et al. 2013). Results obtained with Caspers were comparable with results seen in TU wild type larvae with no noticeable differences so the majority of ISH experiments were done using this line.

7.2.2 Whole-body absolute quantitative real time PCR

Ethanol treated zebrafish 9dpf were studied and compared with controls. Animals were flash frozen, RNA extracted and reverse-transcribed into cDNA, then quantified.

7.2.3 Whole-body in situ hybridization

Casper zebrafish 9dpf, treated with ethanol and controls were used for this experiment. Animals were fixed with paraformaldehyde (PFA) after the end of the ethanol treatment and hybridized with antisense and sense riboprobes to identify the spatial distribution of mRNA transcripts.

7.2.4 Statistics

For qPCR, ANOVA and student t-tests were used. The dependent variable was number of copies of target gene divided by the number of copies of the housekeeping genes. The tests were evaluated with respect to type-1 error rate of 0.05.

7.3 Results

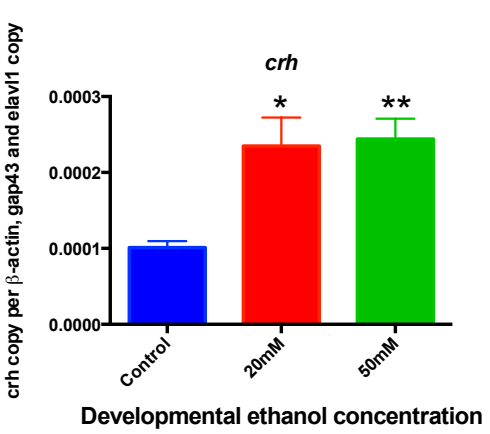
7.3.1 Developmental ethanol exposure increases *crh*, *crhbp* and *crhr2* mRNA expression in zebrafish 9dpf larvae, assayed by qPCR.

Zebrafish 9dpf larvae developmentally treated with ethanol showed differences in *crh* and *crhbp* mRNA expression as determined by qpcr analysis. The expression of *crh* mRNA was increased in 20mM (2.3-fold) and 50mM (2.4-fold) groups compared to controls ($P<0.05$ and $P<0.01$ respectively, ANOVA=0.01) (figure 7.1, A). *crhbp* mRNA expression was also increased in 20mM (3.4-fold) and 50mM (3.7-fold) groups compared to controls ($P<0.001$ and $P<0.05$ respectively, ANOVA<0.01) (figure 7.1, B).

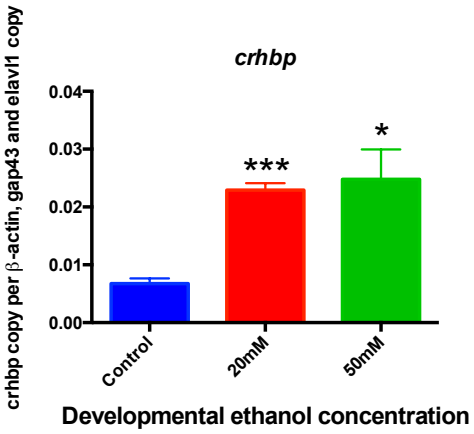
There were no differences in mRNA transcripts for the remaining tested genes (figure 7.1, C-F) between ethanol treated and control animals, however, when both 20mM and 50mM groups were combined, a difference was seen in *crhr2* mRNA expression (figure 7.2). No differences were found in housekeeping gene mRNA expression due to ethanol treatment ($P>0.1$, ANOVA>0.1) (figure 7.3).

As the expression of *crh* mRNA goes up with ethanol treatment, so does *crhbp* and *crhr2* mRNA expression (figure 7.4) at the same proportion. There are a constant 0.03 copies and 0.06 copies of *crh* mRNA transcript for each *crhbp* and *crhr2* mRNA transcript copy respectively. No differences were found in due to ethanol treatment ($P>0.5$, ANOVA>0.5) (figure 7.4).

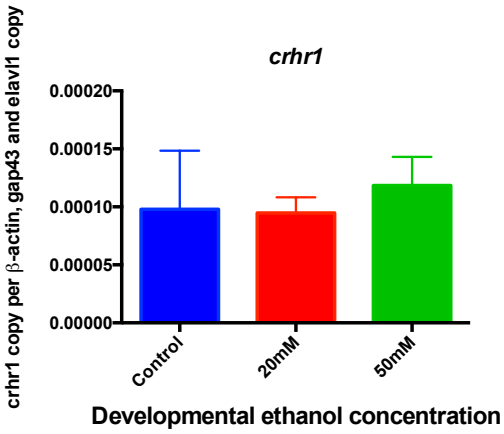
A B


crh
crh copy per β-actin, gap43 and elavl1 copy
0.0003
0.0002
0.0001
0.0000
*
**
Control
20mM
50mM
Developmental ethanol concentration

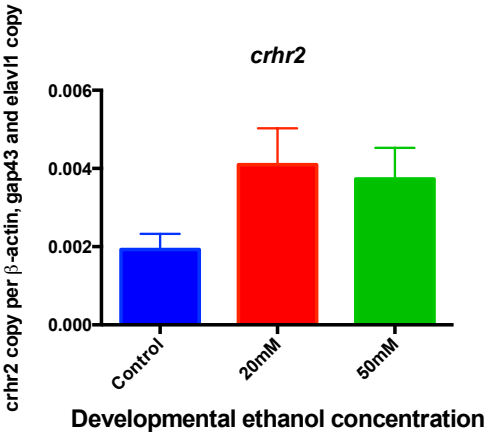
C


crhbp
crhbp copy per β-actin, gap43 and elavl1 copy
0.04
0.03
0.02
0.01
0.00
***
*
Control
20mM
50mM
Developmental ethanol concentration

D


crhr1
crhr1 copy per β-actin, gap43 and elavl1 copy
0.00020
0.00015
0.00010
0.00005
0.00000
Control
20mM
50mM
Developmental ethanol concentration

E


crhr2
crhr2 copy per β-actin, gap43 and elavl1 copy
0.006
0.004
0.002
0.000
Control
20mM
50mM
Developmental ethanol concentration

F

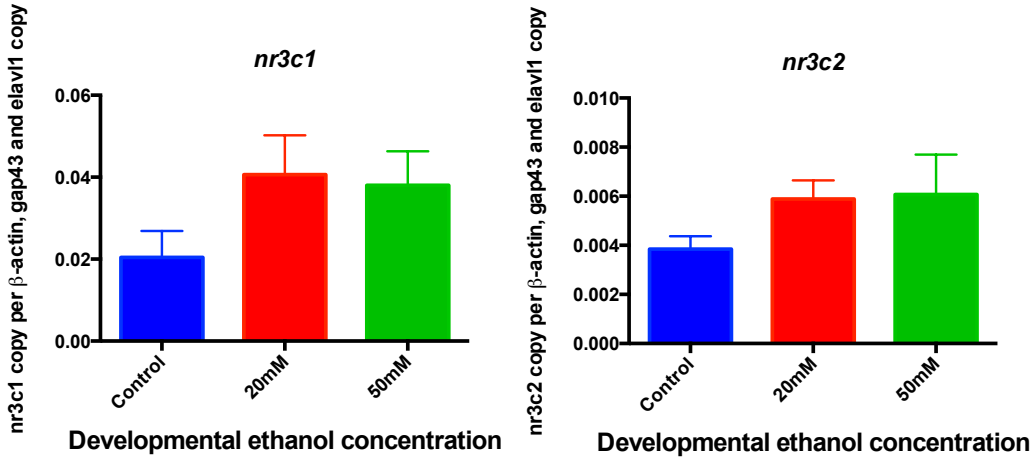


Figure 7.1: Mean (±SE) mRNA expression of zebrafish HPI axis genes assessed by real-time PCR. Absolute copy number of *crh* (A), *crhbp* (B), *crhr1* (C), *crhr2* (D), *nr3c1* (E) and *nr3c2* (F) genes were quantified and normalized against absolute copy number of *gap43*, *β-actin* and *elavl1* housekeeping genes. Samples were extracted from developmentally treated with ethanol 9dpf unstressed zebrafish larvae (1dpf-9dpf). were found In ethanol treated animals there was up-regulation of mRNA transcription of *crh* ($P<0.05$ for 20mM and $P<0.01$ for 50mM groups, ANOVA=0.01) and *crhbp* ($P<0.001$ for 20mM and $P<0.05$ for 50mM groups, ANOVA<0.01). Ethanol treatment did not affect expression of *crhr1*, *crhr2*, *nr3c1* and *nr3c2* genes. Three batches of samples were used, 15 animals in each sample.

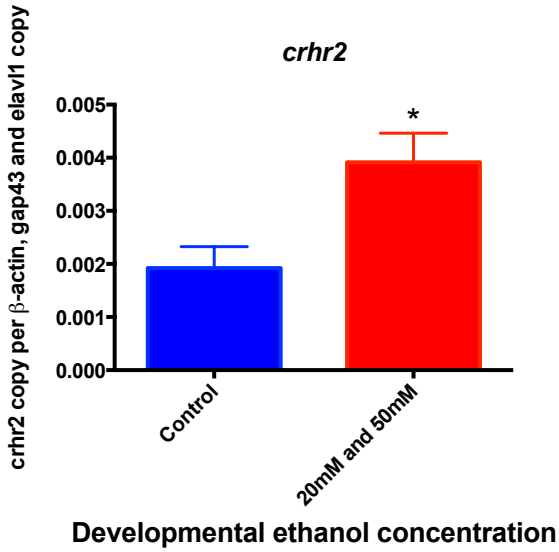


Figure 7.2: mRNA transcription of crhr2 assessed by real-time PCR (means± SE). Absolute copy number of *crhr2* was quantified and normalized against absolute copy number of *gap43*, *β-actin* and *elavl1* housekeeping genes. Samples were extracted from zebrafish 9dpf developmentally treated with ethanol (1dpf-9dpf) and 9dpf controls: crhr2 transcription was upregulated in ethanol treated animals, assessed using t-test ($P<0.05$). Three batches of samples were used, 15 animals in each sample.

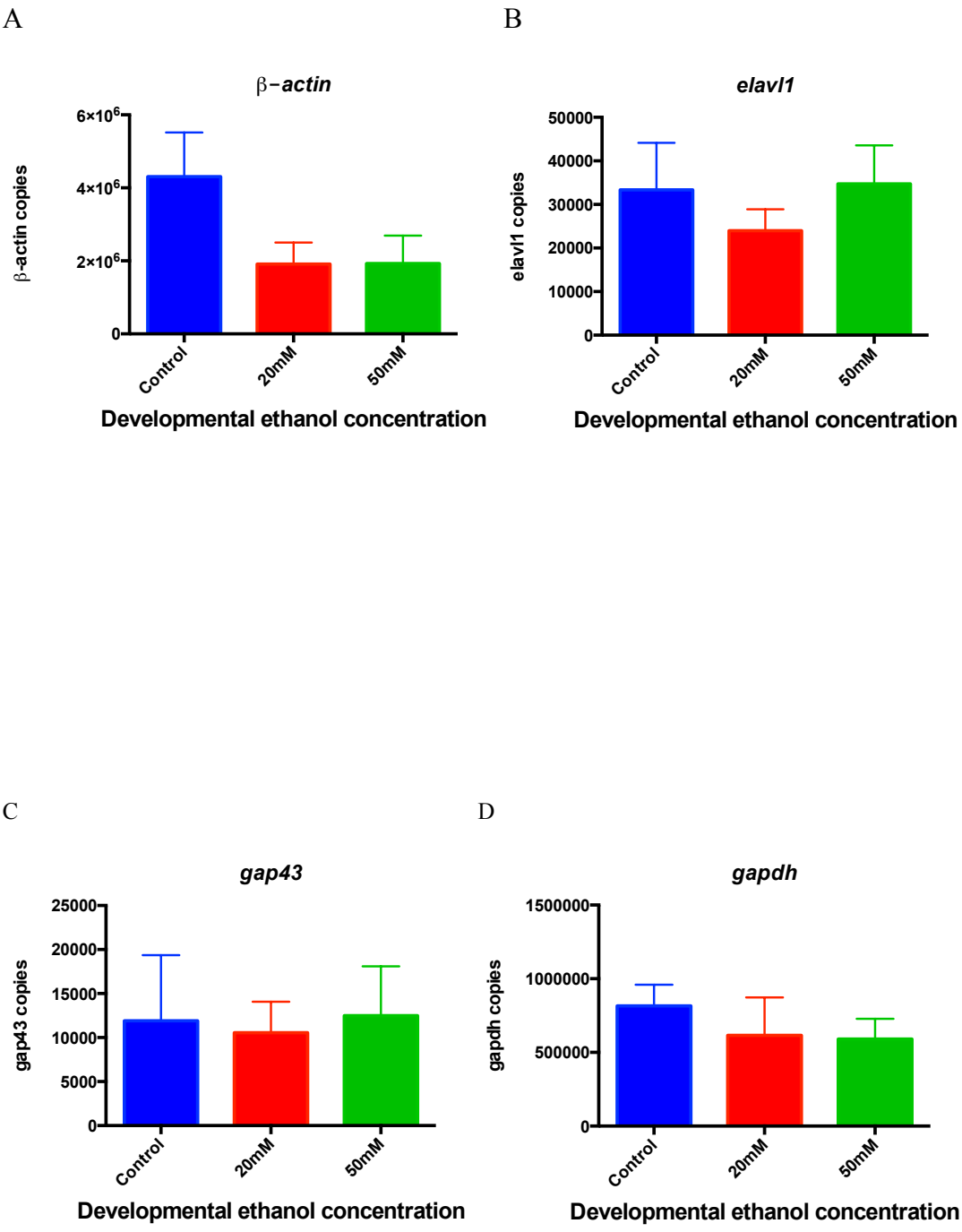


Figure 7.3: mRNA trasncription of zebrafish housekeeping genes assessed by real-time PCR (means ± SE). Absolute copy numbers of *β-actin* (A), *elavl1* (B), *gap43* (C) and *gapdh* (D) genes were quantified. Samples were extracted from zebrafish larvae (9dpf) developmentally treated with ethanol (1dpf-9dpf) (A-C), and from 2dpf larvae developmentally treated with ethanol 1-2dpf (D). No change was seen in gene transcription with ethanol treatment (P>0.15, ANOVA>0.1, all). Three batches of samples were used, 15 animals in each sample.

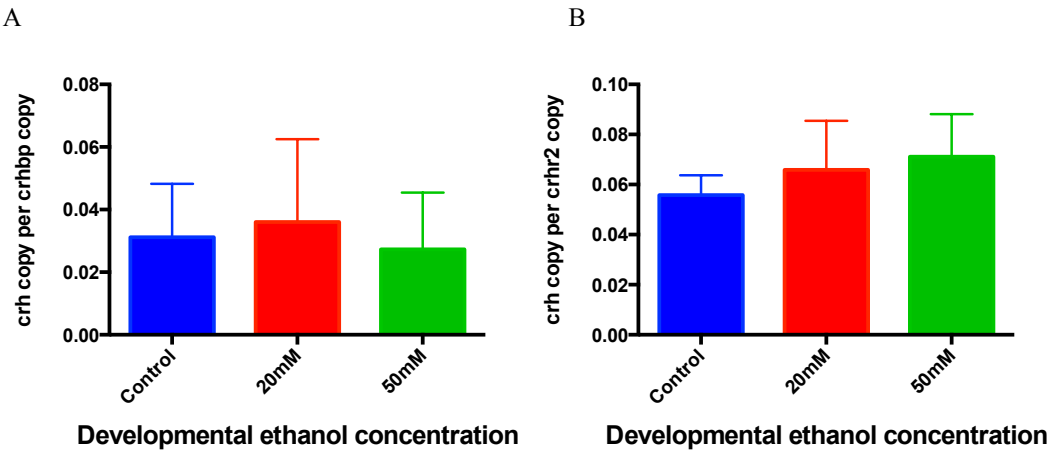


Figure 7.4: Mean (±SE) mRNA expression of zebrafish HPI axis genes assessed by real-time PCR. Absolute copy number of *crh* normalized against absolute copy number of *crhbp* (A) and *crhr2* (B). Samples were extracted from developmentally treated with ethanol 9dpf unstressed zebrafish larvae (1dpf-9dpf). As *crh* is up-regulate, so it is *crhbp* and *crhr2*, at equal proportions among treated and untreated animals, therefore showing no difference (P>0.5, ANOVA>0.5, both). Three batches of samples were used, 15 animals in each sample.

7.3.2 Developmental ethanol has no effect on *crh* mRNA expression in zebrafish 2dpf larvae

Zebrafish 2dpf larvae developmentally treated with ethanol showed no differences in *crh* mRNA expression (P>0.05, ANOVA>0.05) (figure 7.5).

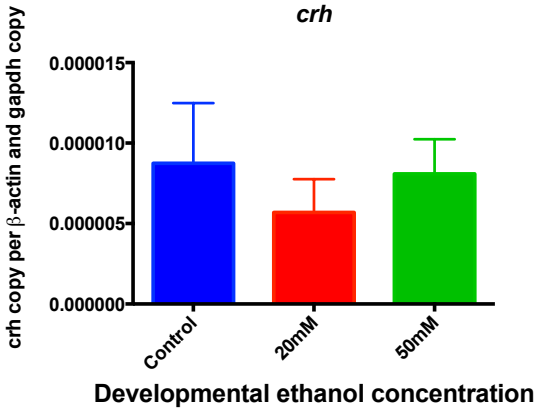


Figure 7.5: Transcription of zebrafish crh assessed by real-time PCR. Absolute copy numbers of *crh* gene transcripts were quantified and normalized against absolute copy numbers of *β-actin* and *gapdh* housekeeping genes. Samples were extracted from zebrafish larvae 2dpf developmentally treated with ethanol 1dpf-2dpf. No differences were found. Three batches of samples were used, 15 animals in each sample.

7.3.3 Developmental ethanol has no effect on *crh*, *crhbp* and *pomca* mRNA spatial distribution in zebrafish 9dpf larvae

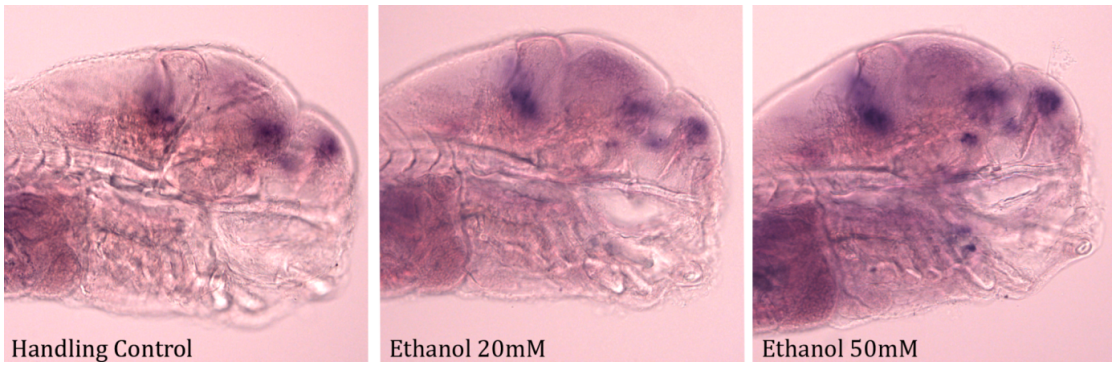
Zebrafish 9dpf developmentally treated with ethanol showed no spatial distribution differences in *crh*, *crhbp* and *pomca* mRNA expression compared to controls (figure 7.6). The *crh* mRNA was found in the forebrain of these animals in the subpalladium (sp) and pre-optic region (po) regions, with a high clustering of stained neurones (figure 7.7, A). Cell clusters were also seen in the midbrain hypothalamus (hy), thalamus (th) and epiphysis (ep) (figure 7.7, A).

crhbp mRNA co-localized with *crh* mRNA in some places, such as the pre-optic region (po), thalamus (th) and hypothalamus (hy) (figure 7.7, B). Staining in the thalamus region extended to the tuberculum (ptv), and heavy clustering was also detected in the hindbrain rhombomeres (rh) (figure 7.7, B). Corticotroph cells with *pomca* mRNA were distributed in a placodal fashion at the anterior edge of the developing brain, near the oropharyngeal tract (figure 7.7, C)

Due to the intensity of staining, it is often difficult to assess precisely the extent of the localization in some regions, such as the thalamus. The dorsal portion of the thalamus is stained and possibly the ventral portion too, similarly, the

hypothalamus occupies a large portion of the base of larval brain, and its rostral portion and possibly its intermediate portion show *crh* mRNA expression.

A

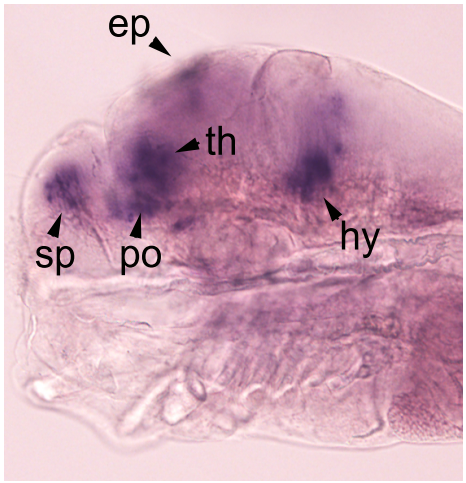


B

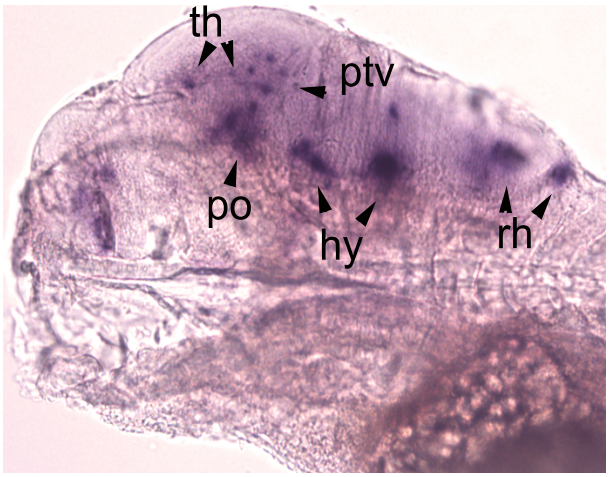
C

Figure 7.6: In situ hybridization pictures (ISH), lateral view, of *crh* (A), *crhbp* (B) and *pomca* (C) mRNA (dark purple staining) in 9dpf zebrafish larvae developmentally treated with ethanol 1-9dpf. No differences in spatial distribution were detectable among the groups. Three batches of animals were used.

A



B



C

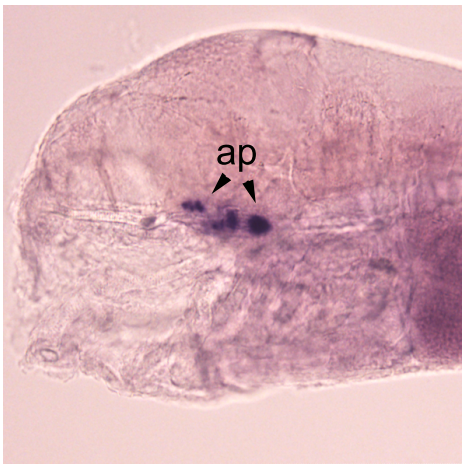
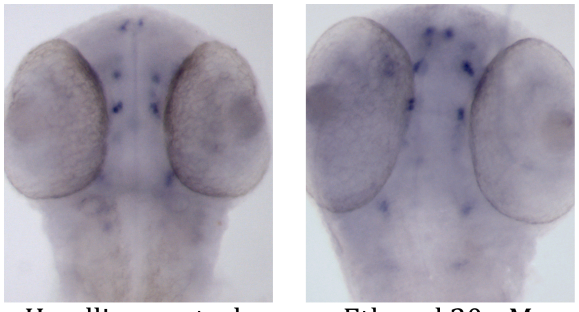


Figure 7.7: In situ hybridization pictures with brain structure labelling. Lateral view, of *crh* (A), *crhbp* (B) and *pomca* (C) mRNA (dark purple staining) in 9dpf zebrafish larvae. Stained *crh* (A) neurones were found in subpalladium (sp), pre-optic region (po), thalamus (th), epiphysis (ep) and hypothalamus (hy). *crhbp* (B) mRNA expressing neurones were located in the pre-optic region (po), thalamus (th), ventral part of posterior tuberculum (ptv), hypothalamus (hy) and rhombomeres (rh). Expression of *pomca* (C) was limited to corticotroph cells at the anterior pituitary (ap). Three batches of animals were used as a comparison for the stained regions.

7.3.4 Developmental ethanol has no effect on *crh* mRNA expression spatial distribution in zebrafish 2dpf larvae

Zebrafish 2dpf developmentally treated with ethanol showed no spatial distribution differences in *crh* mRNA compared to controls (figure 7.8, A). *crh* mRNA in the forebrain of these animals was found in the subpalladium (sp), posterior tuberculum (pt), thalamus (th), epiphysis (ep) and rhombomeres (R1-4) (figure 7.8, B).

A



Handling control Ethanol 20mM

B

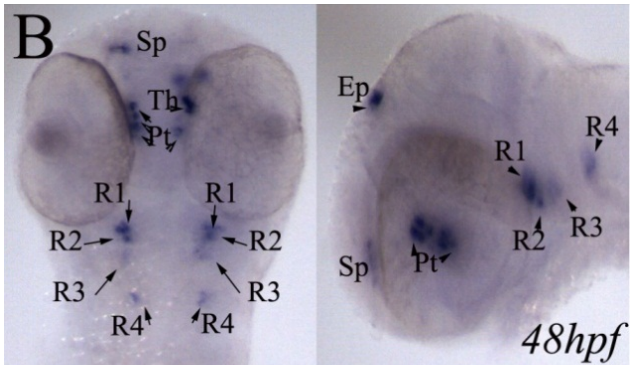


Figure 7.8: In situ hybridization pictures of 2dpf zebrafish larvae. Dorsal view comparison (A) and dorsal and lateral view (B), of *crh* mRNA expression (dark purple staining) in 2dpf zebrafish larvae developmentally treated with ethanol 1-2dpf. No differences in spatial distribution were detectable among the groups (A). Stained *crh* (B) neurones were found in subpalladium (sp), thalamus (th), posterior tuberculum (pt), epiphysis (ep) and rhombomeres (R1-4).Three batches of animals were used.

7.4 Discussion

In humans and other mammalian models, developmental exposure to alcohol leads to a hyperresponsiviness of the HPA axis in the majority of cases, but as noted in chapter 6 (discussion), in some cases, rats developmentally exposed to ethanol lead to a hyporesponsiviness of the HPA axis (Park, Dumas et al. 2004, Xia, Shen et al. 2014).

Following the results described in the previous chapters (4, 5 and 6) using developmentally ethanol treated zebrafish, in which a hyporesponsiveness of the HPI axis was seen in cortisol and behavioural responses to stress, the effect of early ethanol exposure on key HPI axis genes transcription was examined by absolute quantitative real-time PCR (qPCR) and in situ hybridization (ISH).

Traditional PCR detects amplification products at the end of the reaction, qPCR on the other hand allows the detection and amplification to be observed simultaneously, rendering a technique that is quick, automated and can detect the absolute number of transcript copies initially placed in that reaction. To normalize the HPI axis genes mRNA copy numbers, zebrafish housekeeping genes that were stable under treatment conditions were used in this study.

According to the literature, *gap43*, *β-actin* and *elavl1* were stable under chronic ethanol treatment up to 6dpf zebrafish larvae (ethanol values ranging from 0.005% to 1%) (McCurley and Callard 2008, Fan, Cowden et al. 2010). The present data extended these findings, with no difference in total copy number of the genes attributable to chronic ethanol treatment from 1-9dpf (figure 7.3).

However, although *gapdh* is a stable gene in the initial days of embryonic development, its mRNA expression was affected by ethanol exposure from 2-4dpf (McCurley and Callard 2008). Here *gapdh* was only used for the acute 1-2dpf ethanol exposure, and, under these conditions, there were no differences in *gapdh* mRNA expression due to the early acute ethanol exposure (figure 7.3).

McCurley and Callard (2008) also made this *gapdh* gene expression in a time course manner, but in terms of Ct values assessing the stability of expression, and, by using absolute copy number, our results were comparable to theirs in the 1-2dpf interval. Other relatively stable genes under the same conditions are synapsinII and *sonic hedgehog 2*, which were not used on this project (Fan, Cowden et al. 2010).

When normalized against *gap43*, *β-actin* and *elavl1* gene expression, an increase in *crh*, *crhbp* and *crhr2* transcripts was observed in 9dpf zebrafish larvae chronically exposed to ethanol (figure 7.1-2) compared to controls. Expression of *crh*, *crhbp* and *crhr2* mRNA transcripts also increased in a constant proportion, independent of the ethanol treatment used (figure 7.4).

In many analyses of the responsiveness of the HPA axis, *crh* gene expression is commonly assayed alongside with cortisol. For example, in 3-week-old rats that had been treated with ethanol during the second week of gestation, increased mRNA coding for CRH was found, in the paraventricular nucleus (PVN) of the hypothalamus, as assessed by northern blot and in situ hybridization (Lee, Imaki et al. 1990).

Lee et al (2000) also found *crh* heteronuclearRNA (hnRNA) was enhanced in the PVN of 60-day old rats developmentally exposed to ethanol, when they received mild electrofootshocks (Lee, Schmidt et al. 2000). Another study with 60-day old male rats prenatally exposed to ethanol also reported an increase in *crh* mRNA

expression and *pomc* compared to controls (Redei, Halasz et al. 1993). Therefore our results are comparable to rodents.

Using ISH, *crh* mRNA was also found in adrenalectomized male and female rats submitted to early ethanol exposure (Glavas, Ellis et al. 2007) and basal levels of *crh* mRNA expression were also increased in prenatal ethanol exposed adult rats independent of their postnatal handling (Gabriel, Glavas et al. 2005).

All these findings are reflected in the present results, in which *crh* mRNA expression was increased in larvae as a result of early ethanol exposure (figure 7.1, A). Early acute ethanol exposure in the other hand did not result in any differences in *crh* mRNA expression (figure 7.5), suggesting that at the concentrations used induction of *crh* mRNA expression requires chronic exposure.

In male and female rats, there is no evidence of differences in *nr1c3* (GR) gene mRNA expression between ethanol exposed animals and controls in the hippocampus. There is however an increase in *nr1c3* expression during chronic variable stress in male rats (not in females), but no correlation with ethanol exposure (Sliwowska, Lan et al. 2008, Uban, Comeau et al. 2013). Expression of the *nr2c3* (MR) gene mRNA on the other hand is reduced in prenatally ethanol exposed females but not males, in all sub-regions of the hippocampus (Sliwowska, Lan et al. 2008, Uban, Comeau et al. 2013).

Ethanol exposure had no effect on either GR or MR in the present experiments (figure 7.1, E-F). In the rat studies, differences in MR gene expression were only found in females, and since the zebrafish larvae used were both male and female, the effect of sex was not assessed.

From a functional perspective; there are also sex differences in the effects of blocking the MR and GR in PNE rats with antagonists (spironolactone and

RU38486 respectively) also resulted in different HPA axis feedback results. There was an increase in ACTH levels in females injected with either spironolactone or RU38486 at rest in prenatal alcohol exposed females compared to controls (Glavas, Yu et al. 2006). MR and GR blockade also increased corticosterone plasma concentration in prenatal alcohol exposed females during restraint stress and during recovery respectively (Glavas, Yu et al. 2006).

In the rat studies, another sex-related difference is in the mRNA expression of *crhr1*, in which PNE males but not females have lower pituitary mRNA expression of this receptor, even when adrenalectomised, with or without cortisol replacement (Glavas, Ellis et al. 2007). No differences in *crhr1* mRNA in ethanol treated and control 9dpf zebrafish larvae (figure 7.1, C-D) were detected, but, as noted previously, in these experiments the sexes were not separated, as sexing 9dpf is unviable under these procedural conditions.

As there is not much data related to prenatal ethanol exposure on this subject, prenatal stress can be used as reference, since ethanol is also a stressor when acutely administered early in development. Prenatal stress during GD 12-20 also decreased significantly *crhbp* mRNA expression in males and females, assessed by qPCR; females also had a higher *crh* and a lower *crhr2* mRNA expression compared to controls (Zohar and Weinstock 2011). PS animals also exhibited an increase in anxiety behaviour, assessed by an elevated plus maze (Zohar and Weinstock 2011).

By studying knockdowns of CRHR1 and mice that had defective copies of this receptor, it was possible to confirm that CRHR1 is responsible for the activation of the HPA axis and its anxiogenic effects in this species whereas CRHR2 has an anxiolytic role (Heinrichs, Lapsansky et al. 1997, Smith, Aubry et al. 1998). (Bale, Contarino et al. 2000). .

From the zebrafish data it may be conjectured that the increase in *crhr2* mRNA due to developmental ethanol exposure results in an increase number of membrane CRHR2, therefore lowering the anxiety-like behaviour and cortisol of 9dpf zebrafish larvae, as seen in chapter 4 and 6. Another important factor that might contribute to the reduced anxiety-like behaviour and cortisol of 9dpf zebrafish larvae is the increased *crhbp* mRNA expression we found in developmentally treated animals (figure 7.1, B).

As mentioned earlier, PS rats exhibit increase anxiety-like behaviour and decreased *crhr2* mRNA expression (Zohar and Weinstock 2011). CRH-BP is able to specifically inhibit CRH and Urocortin-1 activation of CRHR1 (Turnbull and Rivier 1997, Riedel, Schlapp et al. 2002, Huising, Vaughan et al. 2008). A study using carp as model has confirmed the inhibition of CRH and Urocortin-1 by the binding protein as well (Manuel, Metz et al. 2014). CRH has only 40% affinity with CRHR2, and Urocortin 2 and 3 bind with a much higher affinity to this receptor (Lewis, Li et al. 2001, Reyes, Lewis et al. 2001).

Although there is an increase in *crh* mRNA expression as a result of ethanol treatment, it is possible that increased CRHBP may neutralise some or all of its actions, thereby inhibiting activation of CRHR1 and decreasing anxiogenic-like behaviour. Possibly, the increase in membrane CRHR2, due to the increase in *crhr2* gene expression, complements the anxiolytic effect, by activating other pathways, explained in more depth at the main introduction.

To determine whether the changes obtained with *crh* and *crhbp* mRNA expression also resulted in different patterns of expression in different areas of the zebrafish brain, antisense CRH and CRHBP riboprobes were produced, as well as an additional probe for another component of the HPI axis, POMC-a.

It was possible to localize the expression of these genes and their distribution was comparable with published work (Hansen, To et al. 2003, Herzog, Zeng et al. 2003, Alderman and Bernier 2007, Chandrasekar, Lauter et al. 2007, Herget, Wolf et al. 2014). In situ hybridization pictures revealed no change in the spatial distribution of *crh*, *crhbp* and *pomca* mRNA expression due to early ethanol exposure (figure 7.6, A-C). It did apparently increase their expressions at those locations, supporting the qPCR findings.

Chapter 8: General discussion

8.1 Summary

During the course of this project, developmental ethanol exposure of zebrafish has led to three main findings: early ethanol exposure of this fish model induces a stress-like behavioural hyporesponsiveness; a dampened whole-body cortisol response to stress; and mRNA over-expression of certain key HPI axis regulatory genes (figure 8.1). These findings indicate that this early ethanol exposure affects early plasticity of the HPI axis, creating a stress hyporesponsive phenotype that is detectable at early stages and in adulthood.

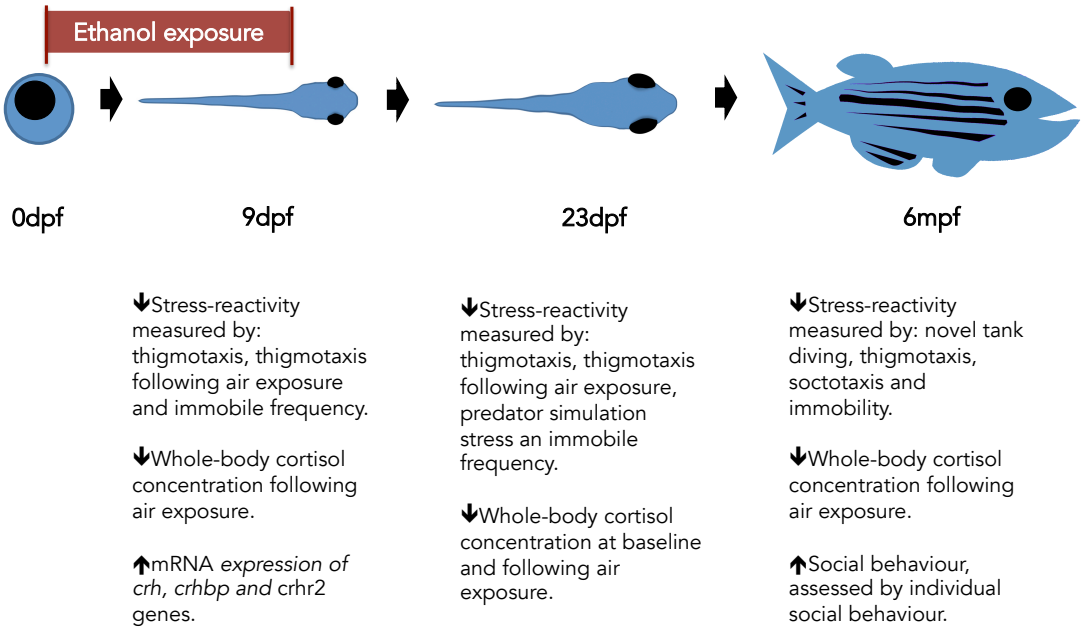


Figure 8.1: Summary diagram of findings in zebrafish 9dpf larvae, 23dpf juveniles and 6-month-old (6mpf) adults; related to developmental ethanol 1-9dpf exposure on zebrafish larvae.

8.2 Final conclusions

After successfully characterising the use of thigmotaxis, thigmotaxis following air-exposure, and immobile frequency as measures of stress reactivity in zebrafish larvae using diazepam, these tests revealed that developmental ethanol exposure from 1-9dpf decreased stress reactivity in 9dpf zebrafish larvae (figures 4.3 A-B, 4.4 A-B and 4.8 A). These changes were consistent even 2 weeks after the end of the ethanol treatment (figures 4.3 E-F, 4.4 C-D and 4.8 B).

To further exclude any possibility that the ethanol was acting as an anxiolytic drug rather than affecting the brain wiring and HPA axis plasticity at an early age, siblings of the same animals were tested 6 months later and confirmed to be less stress-reactive in four different assays: tank diving, thigmotaxis, scototaxis, and immobility (figures 5.1, 5.2, 5.3 and 5.4). These ethanol treated animals were also more sociable, assayed by individual social behaviour assay (figure 5.6).

As stated in previous chapters, developmental ethanol exposure in humans and other mammals leads to a hyperresponsiveness of the HPA axis in the majority of cases (Osborn, Kim et al. 1998, Carneiro, Diogenes et al. 2005, Haley, Handmaker et al. 2006, Hellemans, Sliwowska et al. 2010, Hellemans, Verma et al. 2010); however, manipulation of when and how rats are developmentally exposed to ethanol can also lead to a hyporesponsiveness of the HPA axis in certain cases (Park, Dumas et al. 2004, Xia, Shen et al. 2014).

To compare the findings of this study, the most similar experiment conducted to date was realised by another member of this research group. A different time

frame of ethanol exposure (20mM ethanol, 2-9dpf) was used and it yielded a different stress-reactive behaviour in adult zebrafish, increasing stress reactivity in early ethanol-treated animals compared to controls. Differences in stress-reactivity were assessed by the same method: novel tank diving. These treated animals were also less sociable, assessed by individual social behaviour (Parker, Annan et al. 2014).

As discussed in the introduction to this thesis, prenatal ethanol exposure alters the plasticity of dopamine, noradrenaline, serotonin and GABA neurones (Detering, Collins et al. 1980, Ledig 1988, Druse, Tajuddin et al. 1990, Tajuddin and Druse 1999, Sari and Zhou 2004, Zhou, Sari et al. 2005, Hofmann, Ellis et al. 2007, Kraemer, Moore et al. 2008, Weinberg, Sliwowska et al. 2008).

By exposing these animals from 2-9dpf, the long-pec stage was targeted, which is believed to be a key stage in the monoaminergic neural development (Guo, Brush et al. 1999, Parker, Annan et al. 2014). The 1-9dpf ethanol exposure also targets the same monoaminergic neuron development, in addition to earlier proliferation and differentiation of this and other pathways that occur from the 1-2dpf stage of development.

For instance, cholinergic neurones differentiate in the initial 24 hours of zebrafish embryogenesis, found at specific brain regions such as the telencephalon, diencephalon, postoptic region, optic stalk, pituitary, epiphysis early analogue regions, nucleus medial fasciculus, cerebellum and spinal cord. By 2dpf, they proliferate and reach a higher concentration of neurones with many additional axons in those regions, but the majority of those are added to pre-existing tracts instead of originating from new neurones (Wilson, Ross et al. 1990).

The dopaminergic neurones targeted with an in situ riboprobe against tyrosine hydroxylase (*th*) and dopamine transporter (*dta*) revealed expression mainly in the locus coeruleus, olfactory bulb and prectum at 1dpf, following further expression in the thalamus and hypothalamus at 2dpf (Holzschuh, Ryu et al. 2001). Serotonergic neurones targeted with an in situ riboprobe against serotonin transporter A (*serta*) exhibited expression in the pineal organ at 1dpf, spreading to raphe nuclei and posterior tuberculum at 2dpf (Wang, Takai et al. 2006).

Glutamatergic neurones proliferation from 1-2dpf in the zebrafish occurs at the mediodorsal expression domain of the spinal cord, assessed using in situ hybridization with a riboprobe against vesicular glutamate transporter 2 (VGLUT2) (Higashijima, Mandel et al. 2004).

Proliferation of GABA neurones at this stage was similar to glutamate neurones, in the mediodorsal expression domain (lower expression compared to glutamate neurones) and in the ventral (Kolmer-Agduhr cells) region of the spinal cord, assessed using a riboprobe against glutamic acid decarboxylase 65/67 (GAD65/67) (Higashijima, Mandel et al. 2004).

This substantial proliferation of dopamine, acetylcholine, glutamate and GABA neurones from 1-2dpf is a potential target affected by the early ethanol exposure executed in this research, which could interact with the HPI axis and yield differences in stress reactivity depending on the time frame of exposure.

Analysing the HPI axis directly, developmental ethanol exposure from 1-9dpf reduced whole-body cortisol concentration in 9dpf zebrafish larvae, 23dpf juveniles and in 6-month-old adults when air-exposed stressed, and at basal levels in 23dpf animals (figures 6.7, 6.8 and 6.10).

In adults, there was also a positive correlation between basal whole-body cortisol concentration and stress reactivity, measured by thigmotaxis (figure 6.11). Gene expression following developmental ethanol exposure resulted in an increase of *crh*, *crhbp* and *crhr2* mRNA expression in 9dpf larvae (figures 7.1 and 7.2).

Another aspect that could have yielded those behavioural differences seen in Parker et al are the gene expression changes of those and other zebrafish HPI axis genes affected differently from this 1-9dpf ethanol exposure. That study focused on differences in gene expression of social genes after early ethanol exposure, such as oxytocin receptor and serotonin transporter (Parker, Annan et al. 2014), but unpublished data has indicated an increase in *crh* gene expression, but not a 2.5-fold change as observed in this research.

Proliferation of CRH-expressing neurones in the zebrafish embryo from 1-2dpf is a very extensive process. Starting with a small cluster of cells in the hypothalamic region at 25hpf, and by 2dpf the cell clusters in the hypothalamus increased in number and expanded to the thalamus, pretectum, posterior tuberculum, postoptic commissure, epiphysis, midbrain tegmentum and rhombomeres in the hindbrain, confirmed by in situ staining done in this research (figure 7.8) (Chandrasekar, Lauter et al. 2007). Besides the change in the time of exposure, one must also consider that manual handling of the animals could also have had an impact in the outcome of stress reactivity of those subjects.

It was also found that CRHBP and CRHR2 expression goes up with CRH expression in ethanol treated animals (figures 7.1). Arguably, this has never been assessed before in any model. Theoretically, the CRHBP production is sequestering the circulating CRH, therefore yielding this stress-hyporeactive phenotype. In

addition, the increase in membrane CRHR2 possibly contributes to the anxiolytic effect.

As zebrafish exposed to ethanol under such experimental conditions yielded a stress-hyporeactive phenotype, thus contradicting most of the mammalian studies. The absence of the mother-offspring relationship must also be considered, as well as the pre and postnatal interplay of this relationship.

This aspect was discussed in depth in chapter 6, with several factors originating from the maternal-offspring bond that can potentially contribute to the early plasticity of the HPA axis, such as control of steroidogenic enzymes expressions (Welberg, Thrivikraman et al. 2005) and epigenetic control of the GR promoter (Oberlander, Weinberg et al. 2008, Radtke, Ruf et al. 2011).

Even postnatal epigenetic effects have been associated with HPA axis plasticity. Differences in offspring methylation of the GR promoter were associated with different levels of maternal care in mice (Weaver, Cervoni et al. 2004) and recently, hypomethylation of the CRH promoter was associated with early maternal separation, resulting in an increased CRH transcriptional expression in adulthood, also in mice (Chen, Evans et al. 2012). Methylation was also suggested to be the reason in CRHBP expression differences observed in children exposed to childhood trauma (Roy, Hodgkinson et al. 2012).

Prenatal ethanol exposure also has the potential to alter the expression profile of 84 genes during neurulation in mice, by increasing and decreasing methylation of metabolic and developmental genes such as *Cyp4f13, Elavl2, Nlgn3, Sox21* and *Sim1* (Liu, Balaraman et al. 2009). A recent study conducted on rats identified hypermethylation of the POMC promoter at the hypothalamus as an effect of prenatal ethanol exposure (Bekdash, Zhang et al. 2013). To date, little is known

about the effects of alcohol exposure on the epigenetics of the HPA axis gene transcription.

Zebrafish is a valuable model to assess if the epigenetics control of HPA axis components is comparable or not to the rodent model, when developmentally exposed to ethanol. It would confirm whether the mother-offspring interaction with early ethanol exposure does affect the epigenetics control of the HPA axis. Epigenetics could be linked to the detected overexpression of *crh*, *crhbp* and *crhr2* mRNA.

By further using the zebrafish model, it is possible to shed a light on whether the mother-offspring interaction, prenatally or postnatally, as well as the time frame of the ethanol exposure, are indeed important in the hyperactivity of the HPA axis observed in mammalian models.

It is also important to mention that bold behaviour might influence part of the stress-reactive outcome detected in adults and even in larvae and juveniles. Boldness is classified as a personality trait, associated with the animals' coping mechanisms, in which they can be proactive (animals that react actively in threating situations, confronting a predator for example) or reactive (animals that react passively in threating situations) (Dahlbom, Lagman et al. 2011).

Also called the aggression-boldness behaviour for its correlation, it has a mechanism that is not entirely understood. Causes behind this behaviour are: hormonal imbalance, genetics and optimal environmental adaptation (Landeau and Terborgh 1986, Dahlbom, Lagman et al. 2011, Miller and Gerlai 2011, Norton, Stumpenhorst et al. 2011, Miller, Greene et al. 2013, Gerlai 2014). Recently, disruptive expression of fibroblast growth factor receptor 1a (*fgfr1a*) has been

associated with increased boldness, aggression and exploration in zebrafish (Norton, Stumpenhorst et al. 2011).

Fibroblast growth factor receptor 1a is greatly expressed in the paraventricular nucleus, a region important in the HPA axis regulation as mentioned in the introduction and also associated with aggressive behaviour in teleosts (Topp, Stigloher et al. 2008, Norton, Stumpenhorst et al. 2011).

It is known that ethanol disrupts the effect of fibroblast growth factor in astrocytoma cell culture, reducing proliferation, possibly by the action on fibroblast growth factor receptors (Luo and Miller 1996, Luo and Miller 1998). As far as this study is concerned, it is still unknown whether ethanol has an effect on fibroblast growth factor receptor 1a, which could provide a link between boldness and prenatal ethanol exposure.

To observe whether developmental ethanol exposure does lead to differences in boldness, the best approach would be to use a novel object inspection assay. The procedure involves a similar tank as those used on shoaling with a novel object in it.

Research groups that use this assay measure time spent by the animal near the novel object, as well as velocity and immobile periods (Wright, Nakamichi et al. 2006, Maximino, de Brito et al. 2010, Norton, Stumpenhorst et al. 2011). Due to project limitations, it was not possible to test this using the ethanol-exposed animals.

8.3 Future directions

Based on the findings described in this thesis, in this section a few suggestions for the continuation of this research topic are listed.

8.3.1 Titration of air exposure stress in zebrafish juveniles

As discussed in chapter 6, a 30-second air exposure was not sufficient to elicit a change in whole-body cortisol concentration in 23dpf juveniles. A titration of air exposure time periods, as it was done in adults, should answer if these animals at this stage are irresponsive to stress or if the stress procedure itself was not sufficient to initiate the stress response resulting in the fluctuation of cortisol.

8.3.2 Use of waterborne cortisol extraction for zebrafish larvae and juvenile use

Waterborne cortisol extraction in larvae and juveniles can be applied in any experiment that requires multiple samples from the same animals without having to sacrifice them. We pioneered in this technique, assessing that cortisol fluctuation in the water can be detected form stressed animals, but to be used routinely a full validation procedure needs to be done.

8.3.3 Assessment of transcriptional expression of HPI axis genes by qPCR in developmentally exposed zebrafish juveniles and adults

It would be valuable to assess if the changes in *crh*, *crhbp* and *crhr2* gene expression detected in developmentally treated animals, do persist as the animals grow older, as well as changes in the other HPI axis gene expressions. As seen in chapter 4, 5 and 6, we have a complete behavioural and cortisol analysis of zebrafish adults and juveniles, and gene expression data would enrich our findings.

8.3.4 Assessment of transcriptional expression of other HPI axis genes by ISH in developmentally exposed zebrafish larvae, juveniles and adults

Functional antisense riboprobes for CRH, CRHBP and POMC mRNA expression were generated and utilized to define the expression patterns in developmentally treated 9dpf zebrafish larvae. To do an ISH staining using those probes in zebrafish juveniles and adults would enhance the behavioural and whole-body cortisol we have. Other antisense riboprobes for CRHR1, CRHR2, GR, MR and MC2R were generated but never yielded specific staining during the trial experiments, therefore making these probes to work would also enrich this research.

8.3.5 Assessment of HPI axis protein expression in developmentally exposed zebrafish larvae, juveniles and adults

This would be a fundamental step to correlate with the mRNA expression data we obtained, and verify if *crh*, *crhr2* and *crhbp* gene overexpression in ethanol

treated animals also results in an increase of systemic CRH, CRHR2 and CRHBP proteins. Preliminary tests with monoclonal anti-CRH antibody were conducted, unfortunately the antibody generated in the 1970's was not properly stored and yielded no specific staining.

8.3.6 Assessment of neural pathways in developmentally exposed zebrafish larvae, juveniles and adults

This would also be a fundamental step, to assess any further differences in the development of dopamine, noradrenaline, serotonin, glutamate and GABA neurones, at 9dpf, 23dpf and 6-month-old animals treated animals. This would be complementary to our findings and possibly shed some light on the reasons why these treated animals are more sociable compared to controls.

8.3.7 Assessment of boldness in zebrafish adults developmentally treated with ethanol

As mentioned on the final discussion, although our findings indicate that ethanol treated animals are less stress reactive; there is still the possibility that the behaviour results can be influenced in part by the boldness behaviour of the animals. To begin to verify this, differences in boldness can be assayed using the novel object inspection assay, described in more depth at the general discussion.

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