The Effect of Neuroinflammation on Oxidative Stress and Reproductive Neuropeptides in The Hypothalamus in Adult Male Zebrafish

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Abstract

Inflammation and oxidative stress are known to be one of the many causes of infertility. While most research in this field highlight the effects of inflammation and oxidative stress in the gonad level of the hypothalamic-pituitary-gonadal (HPG) axis, it is of interest to explore whether similar effects can be observed in the hypothalamus and the pituitary, and whether this affects fertility the same way.

This study aimed to fill this gap by looking into the effects of neuroinflammation on key hypothalamic reproductive neuropeptides in adult male zebrafish. Inflammation was induced via intraperitoneal injection of lipopolysaccharide (LPS) at different doses (1, 5 and 10 ug/uL) for 5 consecutive days. The gene expression profiles of several pro- and anti-inflammatory cytokines were measured in the diencephalon, which consists of the hypothalamus. A significant upregulation of pro-inflammatory cytokines such as TNFα and anti-inflammatory cytokines such as IL-10 suggests an inflammatory response being stimulated in the hypothalamus. mRNA levels of MAPKs were also measured upon induction of inflammation such that the genes encoding for p38, ERK and JNK were significantly dysregulated compared to the control group. The expression of *gnrh3*, *gnih*, *kiss2* and *spx2* were also significantly dysregulated upon treatment with LPS. However. The expression of *smim20*, which encodes for phoenixin (PNX) remained unchanged. In addition, the gene expression profiles within the pituitary were also measured where it was found that there was no significant inflammatory response upon treatment with LPS. The mRNA levels of *lhb* and *fshb* were also unaffected by the induction of inflammation. Furthermore, there was no significant change in sexual activity of the zebrafishupon treatment with LPS.

This study to identified the change in gene expression of certain reproductive neuropeptides upon the induction of inflammation. However, there was no significant change found in genes of the pituitary and sexual behaviour. This study sets a basis for future work by establishing the interplay between neuroinflammation, oxidative stress and reproductive neuropeptides.

Declaration

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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ARC – arcuate nucleus

ARE – antioxidant response element

AVPV – anteroventral periventricular

BBB – blood brain barrier

CNS – central nervous system

DAMP – damage associated molecular pattern

ERK – extracellular signal-regulated kinases

FSH – follicle stimulating hormone

GnIH – gonadotropin inhibitory hormone

GnRH – gonadotropin releasing hormone

GSH - antioxidant tripeptide glutathione

HPG – hypothalamic-pituitary-gonadal

IKK – inhibitory κB kinase

IL – interleukin

IP – intraperitoneal injection

JNK - c-Jun N-terminal kinases

KISS - kisspeptin

LH – luteinising hormone

LPS – lipopolysaccharide

ME – median eminence

NFκB - nuclear factor-κB

Nrf2 - nuclear factor erythroid 2-related factor 2

OB – olfactory bulb

PAMP – pathogen associated molecular pattern

POA – preoptic area

PVN – paraventricular nucleus

RNS – reactive nitrogen species

ROS – reactive oxygen species

TEL – telencephalon

TLR – toll-like receptor

TN – terminal nerve

TNF – tumour necrosis factor

1 Introduction

Infertility is a global health issue affecting both the male and female populations. According to the World Health Organization, half of the individuals diagnosed with infertility were associated with men (Inhorn and Patrizio, 2015, Agarwal et al., 2015). Unlike female infertility, male infertility is not well reported, leading to inaccuracy in statistics, as men who are further examined in fertility clinics are not representative of the greater population of infertile men (Agarwal et al., 2015). Hence, studies on how male infertility can be identified and treated are also lacking. This sets the basis for this current study being conducted on male reproductive function. Research on fertility indicates that it is affected by both lifestyle as well as disease related factors, one of which includes inflammation, of which most research mainly focus on the direct effects of inflammation on the gonads (Fijak et al., 2018) The possible effects could eventually lead to fertility issues through the alteration of sperm quality in males (Azenabor et al., 2015). While there are numerous research on this aspect, there is a gap in researching the effect of inflammation at different levels of the hypothalamic-pituitary-gonadal (HPG) axis.

The HPG axis is the central regulator of reproduction and sexual development across all species (Acevedo-Rodriguez et al., 2018). Reproductive function and fertility are regulated by several hormones. Gonadotrophs in the anterior pituitary secrete luteinising hormone (LH) and follicle-stimulating hormone (FSH) as a response to the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus (Janes, 2016) (Meethal and Atwood, 2005). The hypothalamus secretes other the key hypothalamic neuropeptides such as gonadotropin-inhibitory hormone (GnIH), kisspeptin, spexin and PNX, which will be explored in this study.

Neuroinflammation is an inflammatory response that is within the spinal cord or brain. In its transient form, neuroinflammation is largely protective; however, mounting evidence from clinical and preclinical investigations indicates that prolonged or maladaptive neuroinflammation is the key pathological driver of many neurological diseases. This includes neurodegenerative diseases, psychiatric illnesses, pain syndromes, stroke, and traumatic brain injury (DiSabato et al., 2016). (Libby, 2007) It has been proven to have several repercussions that can affect the immune system which leads to biochemical and physiological changes.

The relationship between neuroinflammation and oxidative stress has been well established such that neuroinflammation can induce oxidative stress and vice versa through several mechanisms (Azenabor et al., 2015, He et al., 2020). Oxidative stress is known as an imbalance between the

production and accumulation of reactive oxygen species (ROS) in cells and tissues and the ability for it to be cleared from the system through detoxification of these reactive products. This affects several physiological roles including cell signalling. It is evident that ROS plays a crucial pathophysiological role and that its accumulation increases the susceptibility of damage in the brain tissues (He et al., 2020).It has also been shown that different neurons are vulnerable to oxidative stress at varying extents (Cenini et al., 2019).

Given that neuroinflammation and oxidative stress can be damaging to the brain, and that there is a distinct relationship between the two parameters, it is of interest to explore whether they have any effect on reproductive function. There is yet a distinct link between neuroinflammation and its effects on reproductive neuropeptides involved in the HPG axis (figure 1).

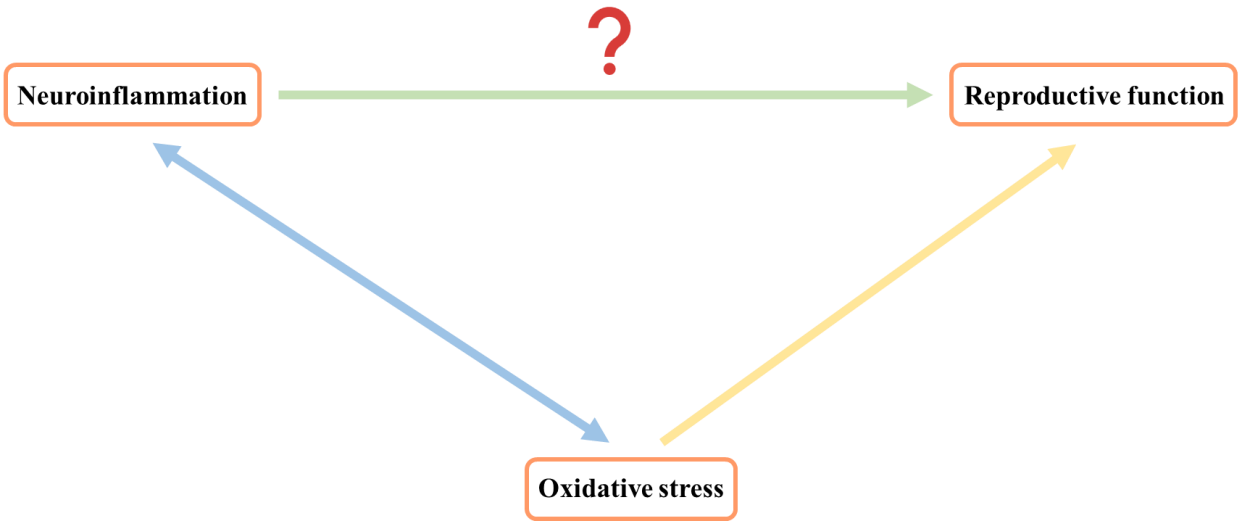


Figure 1 The relationship between neuroinflammation, oxidative stress and reproductive function. Neuroinflammation and oxidative stress have a well-established connection. The effects of oxidative stress on reproductive function are also well studied. However, there are limited studies exploring the relationship between neuroinflammation and reproductive function.

While many studies dive into infertility from aspects relating to the reproductive function of both males and female, they are mainly focused on the direct effects of reproductive issues/diseases on the gonads and their functions. To date, there are limited studies exploring the roles of the HPG axis at multiple levels onreproductive dysfunction (Barabás et al., 2020). Although studies looking into the effects of inflammation on reproductive function, they are focused more specifically on the key reproductive regulator GnRH (Bidne et al., 2018). Furthermore, these studies are largely conducted on female animal models *in vitro* or *in vivo* in which its results are not representative of the male species (Barabás et al., 2018, Sarchielli et al., 2017).. Hence this study aims to fill that gap

using male adult zebrafish as an animal model to study the effects of inflammation on the reproductive axis, and whether oxidative stress play a role in it.

1.1 Research Question

Does neuroinflammation affect the hypothalamic reproductive neuropeptide expression through oxidative stress?

1.2 Hypothesis

We hypothesise that neuroinflammation alters the expression of reproductive neuropeptides via oxidative stress.

1.3 Objectives

1.3.1 General Objective

To elucidate the interplay between neuroinflammation, oxidative stress and hypothalamic neuropeptides in reproduction in an animal model of LPS-induced oxidative stress.

1.3.2 Specific Objectives

1. To evaluate the effect of neuroinflammation on hypothalamic reproductive neuropeptides and oxidative stress assessed by quantitative real time PCR of genes of interest
2. To understand the effect of systemic inflammation on the reproductive axis evaluated by sexual activity study of zebrafish.

2 Literature Review

2.1 Infertility

Infertility is a global health issue affecting both the male and female populations. According to the World Health Organization, half of the individuals diagnosed with infertility were associated with men. This male factor accounts for 7% of the men population worldwide (Inhorn and Patrizio, 2015). It affects around 15% of couples globally, whereby females are sole contributors to 50% of infertility cases and males are found to be solely responsible for 20-30% of cases of infertility and contribute to 50% of overall cases (Agarwal et al., 2015). The increasing trend of infertility in men has reached 50% and is expected to continue in the upcoming years (Harris et al., 2011).

Several factors that are gender specific and non-gender-specific could lead to infertility. To list a few, some of the factors affecting both genders include lifestyle-related factors/diseases, infection, and systemic diseases. In female, such factors include endometrial polyps, endometriosis, premature ovarian insufficiency, polycystic ovary syndrome and uterine fibroids. While in male, testicular deficiency and post-testicular impairment may affect their fertility (as reviewed by (Vander Borght and Wyns, 2018). Male fertility is directly dependant on the uninterrupted completion of spermatogenesis. A disruption in this process leads to no or incompetent spermatozoa produced. Fertility is at stake when sperm count is low, poor semen quality, decreased sperm motility and damage to its DNA (Aitken, 1999). Several factors account for this including high levels of ROS and oxidative stress (Aitken and Clarkson, 1987, Armstrong et al., 1999). Such factors have been proven to affect spermatogenesis. Spermatogenesis is a physiological process in which spermatogonial stem cells (SSCs), which are diploid progenitors, undergo mitotic and meiotic divisions before the final synthesis of haploid gam-etes. It has been documented that different human spermatozoa subsets produce ROS at various phases of maturation (Gil-Guzman et al., 2001). It was observed that ROS production is lowest in mature spermatozoa and immature germ cells and is highest in immature spermatozoa with irregular head morphology and cytoplasmic retention. When spermatids go through spermiogenesis, their membranes are modified and their cytoplasmic volume decreases by up to 70%. When spermiation occurs, the remaining body is released and phagocytosed by Sertoli cells. Therefore, spermatozoa with retained cytoplasmic droplets represent a major source of ROS when spermiogenesis or spermiation is dysregulated (Aitken and Baker, 2002).. There are also mounting evidence suggesting the link between oxidative stress and inflammatory conditions in the testis (Reddy et al., 2006a). Male obesity has also been identified as one of the factors associated with low

sperm quality through changes to hormone levels and direct changes to sperm function and sperm molecular composition (Palmer et al., 2012).

2.1.1 Inflammation and infertility

Inflammation is a defensive immune response that is initiated by the innate immune system against infection, injury, and other environmental challenges. One of the factors that can cause an inflammatory response is gram-negative bacteria of which its cell wall contains lipopolysaccharide (LPS). LPS can stimulate an immune response as it is considered to be endotoxic. An inflammatory response is triggered by the recognition of pattern-associated molecular pattern (PAMP) or damage-associated molecular pattern (DAMP) by receptors on circulating immune cells (Brusselle and Bracke, 2014, Gudkov and Komarova, 2016). This subsequently sets off the release of cytokines, chemokines, and inflammatory mediators, ultimately resulting in inflammation (Czerkies and Kwiatkowska, 2014). Inflammation is naturally switched off through the balance of pro- and anti-inflammatory cytokines. However, more often than not this does not happen. This results in tissue damage, causing prolonged stimulation of immune response which leads to collateral damage. This prolonged inflammation is also known as chronic or low-grade inflammation, hence increasing susceptibility to illnesses and diseases.

Different bacterial infections affect male infertility in different ways as the site of infection often varies from one bacterium to another. Urogenital tract infections are common amongst males, resulting in the inflammation of the reproductive tract (Cutolo et al., 1988). This includes areas such as the testis, epididymis, seminal vesicle, urethra, or prostate. Tissue damage and inflammation caused by bacterial infections can lead to infertility as the maintenance of male's reproductive function is dependent on effective spermatogenesis and the synthesis of testosterone (Schuppe et al., 2008). Inflammation increases local blood flow, microvascular permeability as well as the recruitment of leukocytes to the site of infection (Sarkar et al., 2011). Additionally, various cytokines such as tumour necrosis factors (TNF) and interleukins (IL) mediate inflammation, which affects the reproductive organs. Specifically, TNF α, IL-1β and IL-6 can inhibit the synthesis of testosterone in Leydig cells and induce apoptosis of spermatogenic cells (Agarwal and Saleh, 2002, Reddy et al., 2006b). These infections could consequently affect spermatogenesis, sperm motility and morphology, sperm DNA damage and orchitis, all of which could lead to infertility (Pellati et al., 2008, Nunez-Calonge et al., 1998, Abusarah et al., 2013, Gallegos et al., 2008).

2.1.2 Oxidative stress and infertility

Oxidative stress refers to a state where an abundance ROS is produced in cells and tissues where it is unable to be balanced out by antioxidants. As a result of internal aerobic metabolism, living organisms continuously produce both ROS and reactive nitrogen species (RNS) (Krumova and Cosa, 2016). They are also generated by by-products of cellular metabolism via the electron transport chain, cytochrome P450 and NADPH oxidases. It is also produced in response to infection, UV light, ionising radiation, cellular respiration, inflammation, and certain drugs amongst others. In healthy humans, production of ROS/RNS can play both positive and potentially damaging roles. When the balance of pro- and antioxidants shift in favour to pro-oxidants, it results in oxidative stress. As a whole, it is not just about the imbalance, but also the disruption of redox signalling as well (Tauffenberger and Magistretti, 2021). Uncontrolled/excessive ROS leads to potential damage to all biomolecules – most susceptible being proteins, DNA, lipid membrane, leading to functional impairment and cell death. Free radical damage to DNA leads to mutations and strand breaks (Nissanka and Moraes, 2018). Effects on protein can affect processing and clearance leading to an accumulation of ROS in the brain and tissues.

In the male reproductive system, high amounts of ROS can eventually lead to the immobilisation of the sperm as a result of compromised axonemal protein phosphorylation due to a reduction in intracellular ATP (Walrand et al., 2003). As briefly mentioned, in semen, inflammation is linked to oxidative stress. This is observed in infertile men, whereby high levels of ROS in the semen showed an increase in pro-inflammatory cytokine levels as well as infiltration of leukocyte (D'agata et al., 1990). Although the invasion of bacteria on its own can lead to the production of ROS, leukocytes can also amplify this as it is also one of the sources of ROS in semen (Pasqualotto et al., 2000). Research has shown the imbalance between oxidants and antioxidants was more drastic in infertile male prior to induction of inflammation (Sanocka et al., 2004). This suggests that inflammation induced by oxidative burst could also play a vital role in male infertility.

2.2 Neuroinflammation

Neuroinflammation is an inflammatory response that is within the spinal cord or brain. In a general perspective neuroinflammation is largely protective; however, mounting evidence from clinical and preclinical investigations indicates that prolonged or maladaptive neuroinflammation is a key pathological driver of many neurological diseases. This includes neurodegenerative diseases, psychiatric illnesses, pain syndromes, stroke, and traumatic brain injury (Libby, 2007). In chronic

neurological diseases, neuroinflammation becomes persistent with the subsequent damage to neuronal cells. Several studies have suggested that neuroinflammation is a principal pathology in neurodegenerative and other central nervous system (CNS) diseases.

Neuroinflammation involves the activation of microglia and astrocytes, release of cytokines and chemokines, production of ROS, and often the infiltration of peripheral leukocytes into the CNS. The blood brain barrier (BBB) has been considered impenetrable for many years. However, it has since been discovered that there are several pathways connecting the peripheral system and the CNS. This allows peripheral inflammation to induce an inflammatory response within the CNS. The BBB contains astrocytes, endothelial cells, and tight junction (Förster, 2008). The activated T and B cells interact with the endothelial system and pass through the BBB which results in its damage. This in turn activates an immune response within the CNS (Klein et al., 2019). The innate immune capacity of the CNS is mediated mainly by the release of cytokines and chemokines from the microglia. Neuroinflammation may also involve secondary messengers such as NO, prostaglandins as well as reactive ROS. This leads to a cascade of events that eventually enhances transcription factors such as nuclear factor-κB (NFκB) resulting in the release of several inflammatory cytokines as well as triggering a B and T-cell mediated response (Pasparakis et al., 2006).

Cytokines are a category of signalling molecules that are involved in the immune response as well as inflammation and haematopoiesis. It triggers a cascade of events by binding to its receptor on a responsive target cell, causing various responses (Turner et al., 2014). Cytokines are further categorised into either pro-inflammatory or anti-inflammatory cytokines. As briefly mentioned earlier, it is the balance between them that determines the net effect of an inflammatory response. Pro-inflammatory cytokines are predominantly produced by macrophages and are involved in the upregulation of inflammatory reactions. Some major pro-inflammatory cytokines include IL-1α, IL-1β, IL-6 and TNFα. In contrast, anti-inflammatory cytokines are immunoregulatory molecules that function to control the response of pro-inflammatory cytokines. They act with specific cytokine inhibitors and soluble cytokine receptors to regulate the overall immune response. The main anti-inflammatory cytokines include IL-4, IL-10, IL-11 and IL-13.

Table 1 Highlights the effects and function of vital inflammatory markers that is further explored in the current research

Inflammatory marker	Effects	Function	Reference
IL-1β	Pro-inflammatory	Stimulation of APCs and T cells, acute phase response, haematopoiesis	(Ren and Torres, 2009)
IL-6	Pro- and anti-inflammatory	Acute phase response, B cell proliferation, stimulation of T cells, microglial and astrocytic activation	(Klein et al., 1997, Zhang and An, 2007)
IL-10	Anti-inflammatory	Inhibits cytokine production, promotes B cell proliferation and antibody production	(Lobo-Silva et al., 2016)
TLR4	Pro-inflammatory	Activated by LPS or DAMPs, triggers the production of pro-inflammatory cytokines	(Molteni et al., 2016)
TNFα	Pro-inflammatory	Involved in pain, inflammation, and cell death	(Idriss and Naismith, 2000)
TNFβ	Pro-inflammatory	phagocytosis, NO production, cell death	(Zhang and An, 2007)

2.3 Oxidative Stress

Oxidative stress is a concept that is often defined as the "i*mbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption in redox signalling and/or molecular damage"* (Jones and Sies, 2007). It can be further classified into different types of oxidative stress by looking into its intensity, ranging from physiological oxidative stress, known as eustress, to toxic oxidative burden leading to damaged biomolecules, known as distress (Niki, 2016, Sies, 2017). The production of various oxidants by different endogenous and exogenous sources are often controlled removal reactions. A low oxidant exposure would trigger redox signalling, addressing specific targets (oxidative eustress). In contrast, high exposure to oxidants would result in the disruption of this signalling and/or damage to biomolecules (oxidative distress). Oxidative eustress generally contributes to one's physiology and health. In contrast, oxidative distress contributes to pathophysiology and disease processes (Jones and Sies, 2007).

One of the most crucial features of oxidative challenge is the initiation of a stress response that is mediated via molecular redox switches that activates the expression of specific genes of the defence system to counterbalance this challenge. A salient feature in this process is the activation of

transcriptions of several antioxidant genes including nuclear factor erythroid 2-related factor 2 (Nrf2) has proved to be the key transcription factor that binds to several genes via the antioxidant response element (ARE) in the promoter region (Guerrero-Hue et al., 2017). This subsequently triggers the production of antioxidative enzymes and cytoprotective proteins. The nuclear translocation of Nrf2 requires the activation of a few transduction pathways such as mitogen-activated protein kinase (MAPK) as a result of antioxidant response. The activation of this pathway leads to the activation of many transcription factors including that of extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38. Another one of the major regulators is the NF-κB/inhibitor of κB (IκB) system, which results in the involvement of inflammatory, immune, and acute phase responses (Schreck et al., 1992).

2.3.1 Neuronal vulnerability to oxidative stress in the brain

It is evident that ROS plays a crucial pathophysiological role and that its accumulation increases the susceptibility of damage in the brain tissues (Campese et al., 2004). The exact sequence of events that occurs within the CNS leading to oxidative stress-induced cognitive and behavioural decline can be looked into in several levels. Studies have shown that different neurons are vulnerable to oxidative stress at varying extents. For instance, the hippocampus, amygdala and cerebellar granule cells have been proven to be most susceptible to oxidative stress in several studies and therefore are the first to undergo any functional decline (Wang and Michaelis, 2010). Physiological stress has the ability to disrupt the balance between oxidants and antioxidants within the brain, impairing the function of antioxidant enzymes. This leads to a depletion in glutathione, increasing oxidative stress. Furthermore, the occurrence of glutamate toxicity, along with that of calcium imbalance and impaired mitochondria all further intensifies oxidative stress, resulting in biochemical distress in the brain. This eventually results in behavioural and cognitive deficits through the disruption of neurocircuitry and weakened hippocampal, amygdalar and cortical connections (Salim, 2017).

2.3.2 Hypothalamic vulnerability to oxidative stress

Several studies have looked into the effects of oxidative stress on the hypothalamus in relation to feeding behaviours and diet. The primary part of the brain that regulates energy balance is the hypothalamus. The hypothalamus is one of the first tissues to display signs of damage after exposure to a high-fat diet (HFD) (Lumeng and Saltiel, 2011), according to mounting data. For instance, it has been demonstrated that HFD causes hypothalamus inflammatory responses that interfere with the control of glucose homeostasis and energy balance (White et al., 2009, Rother et al., 2012). Numerous

mechanisms, such as mitochondrial dysfunction, ROS, and endoplasmic reticulum stress linked to unfolded protein response, have been proposed to explain diet-induced inflammation (Dandona et al., 2004, Hotamisligil, 2006). A chronic imbalance between the generation of ROS and the body's own antioxidant defence system is referred to as oxidative stress (Maritim et al., 2003).

The activation of reactive oxygen species (ROS) in the hypothalamus during fuel utilisation is critical for maintaining energy homeostasis (Åberg et al., 2001). Thus, suppressing ROS in the hypothalamus reduces proopiomelanocortin (POMC) cell activation while enhancing neuropeptide Y (NPY) neuron activity and feeding, whereas ROS activates POMC neurons and decreases feeding (Diano et al., 2011). Furthermore, glucose-utilising POMC neurons are fired, resulting in an increase in ROS levels in hypothalamic cells during positive energy balance. NPY neurons that use fatty acids, on the other hand, are activated, but their ROS levels do not increase during negative energy balance (Andrews et al., 2008, Leloup et al., 2006). At physiological low levels, ROS function as "redox messengers" in intracellular regulation, whereas excess ROS induce oxidative modification of cellular macromolecules, inhibit protein function, and promote cell death (Circu and Aw, 2010).

Oxidative stress in the pituitary gland is often associated with the formation of tumours. A different tactic based on information about the role of mitochondria during the development of pituitary tumours is a newly examined approach (Sabatino et al., 2018). In addition to having a significant impact on intracellular signalling and being able to affect homeostasis in a number of interrelated ways, mitochondria are dynamic structures that dynamically fluctuate, influencing how cells function and adapt. The detrimental cellular effects of chronic stress, such as oxidative stress, inflammation, telomere shortening, epigenetic dysregulation, altered gene expression, and cellular senescence, can all be caused by mitochondrial malfunction (Duchen, 2004). The generation of ROS may rise as a result of metabolic processes (Balaban et al., 2005). Oxidative stress and damage to biomolecules (proteins, DNA, and RNA) may occur when the generation of ROS exceeds the antioxidant capacity of the cell, and this may play a causal role in the development of cancer (Jackson and Loeb, 2001).

2.4 Neuroinflammatory processes and oxidative stress

Neuroinflammation is also known to induce oxidative stress, likewise the constant exposure to oxidative stress may also prompt an inflammatory response if the mitochondria are compromised. The relationship between oxidative stress and inflammation has been well established with evidence suggesting its pathogenic role in chronic inflammatory diseases (Scholz and Woolf, 2007). Oxidative stress is associated with many diseases including neurodegenerative diseases like Parkinson’s and

Alzheimer's, and other such as diabetes, and cardiovascular disease. In the brain, oxidative stress is often associated with neuroinflammation, cell death, neurodegeneration and memory loss. These processes may occur as a result of the generation of ROS in the brain tissues, regulating both synaptic and nonsynaptic communication between neurons. Oxidative damage to the glia can produce excessive proinflammatory cytokines, which results in the membrane receptors of neuronal cells to activate inflammatory pathways, causing inflammation (Myers et al., 2006). When toll-like receptors (TLR)/ nucleotide-binding oligomerization domain (NOD)-like receptors (NLR)/ receptor for advanced glycation end products (RAGE) bind PAMPs/DAMPS, it activates transcription factors for pro-inflammatory genes – co-stimulation of several TLRs in the presence of cytokine imbalance results in ROS generation. Macrophages use oxidative stress to eliminate pathogens by inducing cell death via caspase activation and creating imbalance in glutathione equilibrium (Kayagaki et al., 2011). Lower levels of the antioxidant tripeptide glutathione (GSH) result in higher levels of ROS, leading to an imbalanced immune response and inflammation (Mytilineou et al., 2002).

One of the ways oxidative stress can cause inflammation is through NF-κB, activator pritein-1 and MAPK pathways (Poli et al., 2004). ROS activates inhibitory κB kinase (IKK) which results in the phosphorylation of IKK and labels it for ubiquitination mediated proteasomal cell death. The release of free NF-κB heterodimer from this process allows it to cross the nuclear membrane and binds to the kappa region of the genome. This transcriptional facilitation of this part of the genome leads to the production of inflammatory cytokines such as TNFα and IL-6 and IL-1β.

2.5 The hypothalamic-pituitary-gonadal axis

The HPG axis is the central regulator of reproduction and sexual development across all species (Couse et al., 2003). It is responsible for releasing both centrally and peripherally produced hormones whereby a disturbance in this could affect in the endocrine and immune system. The HPG axis plays a crucial role in developmental phases such as prenatal and neonatal phases, puberty as well as adulthood. Gonadotrophs in the anterior pituitary secretes LH and FSH as a response to the release of GnRH from the hypothalamus (Meethal and Atwood, 2005). These gonadotrophins in turn stimulate sex steroid production and gametogenesis in the gonads. Oestrogen released from the gonads in females and various tissues in males create a negative feedback loop by suppressing the synthesis of GnRH in the hypothalamus (figure 2). In males, LH is mainly responsible for the stimulation of Leydig cells in the testes to produce testosterone (Ramaswamy and Weinbauer, 2014). Meanwhile, in females, LH plays a crucial role in controlling the menstrual cycle as well as triggering

ovulation through the production of oestrogen and progesterone from the ovaries. In contrast, FSH is important for the regulation of seminiferous tubule and spermatogenesis in males via action on the Sertoli cells. In females, FSH stimulates follicular growth in the ovaries as well as the secretion of oestrogen from developing follicles. In addition, it stimulates the division and function of granulosa cells that surround and nurture the developing oocyte in the follicle (Richards, 1994).

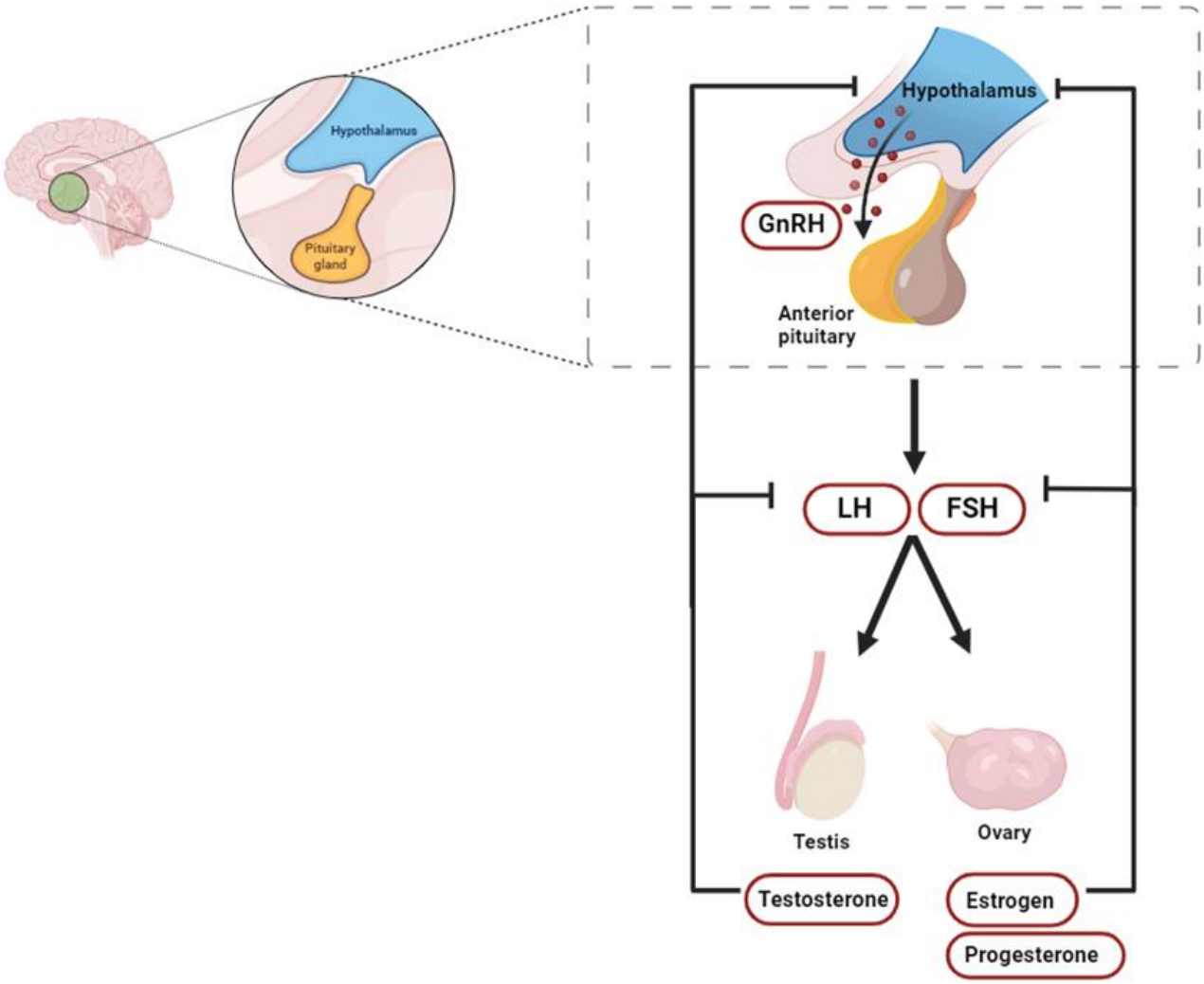


Figure 2 illustrates the hypothalamic-pituitary-gonadotrophin (HPG) axis. Gonadotrophin releasing hormone (GnRH) is released from the hypothalamus to the anterior pituitary. This stimulates the synthesis and release of luteinising hormone (LH) and follicle stimulating hormone (FSH) which acts on the gonads to release testosterone from the testis and oestrogen and progesterone from the ovary. These hormones contribute to the feedback mechanism by acting on hypothalamus by suppressing the synthesis of GnRH and anterior pituitary by suppressing the release of LH and FSH.

2.5.1 Cellular signalling in the hypothalamus and pituitary

The brain's diencephalon contains the hypothalamus, which is situated anterior and inferior to the thalamus. It produces and secretes a large number of hormones and serves both neurological and endocrine purposes. Additionally, the pituitary gland and the hypothalamus share anatomical and physiological relationships (or hypophysis).The anterior and posterior pituitaries, which make up the

pituitary gland, have different origins, structures, and functions. The posterior pituitary develops from neural ectoderm and is made up of pituicytes, specialised glial cells that resemble astrocytes and neuronal projections of neurosecretory cells from the hypothalamus that generate vasopressin (AVP) and oxytocin (Scanes, 2022). The non-secretory folliculostellate cells and six lineages of secretory cell types, which can be divided into three groups, are produced by the anterior pituitary, which develops from oral ectoderm. The first group is made up of two cell types that express the proopiomelanocortin gene (*Pomc*), which cleaves POMC protein in different ways: corticotrophs from the anterior lobe produce adrenocorticotropic hormone while melanotrophs from the intermediate lobe produce -melanocyte-stimulating hormone and -endorphin. The members of the second group include gonadotrophs, secreting luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and thyrotrophs, secreting thyroid-stimulating hormone (TSH) (Stojilkovic, 2018).

Somatostatin and dopamine inhibit electrical activity; however all secretory pituitary cells are excitable and can fire action potentials on their own or in response to hypothalamic neurohormones such GHRH, TRH, CRH, AVP, and GnRH (Zemková and Stojilkovic, 2018). Action potentials are followed by calcium influx through voltage-gated calcium channels, increasing intracellular calcium concentration both locally and globally. In turn, this results in the activation of effectors in the plasma membrane, cytosol, and nucleus that regulate a variety of cellular functions, such as electrical activity, gene expression, and exocytosis (Vazquez-Borrego et al., 2018).

2.5.2 The HPG axis and neuroinflammation

Physical and physiological stressors can suppress the HPG axis by inhibiting the activity of GnRH in both males and females (Liu et al., 2017). In addition to its role in fertility, the HPG axis is also known to act in accordance with the immune system, controlling immune functions. Changes in the immune function may affect the function of HPG axis. Likewise, gonadal hormones too influence the immune system. This interaction is primarily mediated by their shared receptors and mediators like IL-1, TNFα and IL-10 (Segner et al., 2017). As mentioned earlier, the imbalance in production of such pro- and anti-inflammatory cytokine can lead to neuroinflammation.

Previous study has shown that GnRH neuron express various cytokine receptors. Thus, neuroinflammation is postulated to affect the *gnrh* expression. Previously the regulation of HPG axis is focused on GnRH (Lainez and Coss, 2019). Nonetheless, recent findings have shown that *gnrh* is regulated by several hypothalamic reproductive neuropeptide including kisspeptin, spexin, PNX and GnIH (Harter et al., 2018, Tran et al., 2021, Tsutsui et al., 2018, Yuan et al., 2017). In addition, the effect of neuroinflammation on these reproductive neuropeptides remained elusive.

2.5.3 Reproductive neuropeptides

Neuropeptides are small proteins that act as signalling molecules in the CNS (Larhammar, 2009). They coexist in the neurons with one or more classical transmitters. Research has demonstrated its involvement in the regulation of reproductive function among others (Crown et al., 2007). Neuropeptides are produced by ribosomes in cell bodies and are replaced after their release by axonal transport from cell bodies to nerve endings. It is generally released in conditions where there are high, excessive, or pathological neuronal activities resulting in modulatory effects and trophic actions (Hökfelt et al., 2000). Neuropeptides have recently been shown to be involved in the pathogenesis of fertility disorders (Gołyszny et al., 2022). Several key reproductive neuropeptides are able to influence the activity of the HPG axis in different ways in which its alterations may add to the cause of infertility. Some key neuropeptides reviewed in this study includes GnRH, GnIH, kisspeptin, spexin and PNX, covering its functions and localisation across different species, with special focus on teleost.

Table 2 List of key hypothalamic reproductive neuropeptides

Neuropeptide	Function	Reference
GnRH	responsible for the release of the FSH and LH from the anterior pituitary	(Marques et al., 2022)
GnIH	regulates reproduction by inhibiting GnRH and pituitary LH and FSH	(Teo et al., 2021)
Kisspeptin	activation of GnRH neurons, which the stimulates the release of GnRH	(Zeydabadi Nejad et al., 2017)
Phoenixin	regulates the expression of kisspeptin, GnRH, GnRH receptors and LH	(Clarke and Dhillo, 2019)
Spexin	regulates function at different levels, affecting GnRH, kisspeptin and galanin neurons	(Lv et al., 2019)

2.5.4 Gonadotropin-releasing hormone

What is commonly known today as the central regulator of the reproductive axis, the gonadotropin-releasing hormone (GnRH) is a decapeptide that was first discovered in mammalian

hypothalami as (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly·NH2) (Matsuo et al., 1971, Schally et al., 1971). Over 25 primary structures of GnRH can be found across species, including vertebrates to invertebrates. Despite the differences, all the variants are decapeptides with at least 50% sequence identity (Dubois et al., 2002), suggesting its early development and evolutionary relationship between each variant. Although most vertebrate species have two or three forms of GnRH in the brain, because GnRH3 has only been confirmed in fish and amphibians, mammals are known to only produce GnRH1 and GnRH (Millar, 2005). In addition, each variant play slightly different roles where GnRH1 is known to stimulate the release of pituitary gonadotropins which ultimately regulates gonadal maturation. GnRH2, on the other hand, modulates sexual and feeding behaviour while GnRH3 is thought to exert neuromodulatory functions (Okubo and Nagahama, 2008).

Table 3 List and function of different GnRH types and its respective functions

Type	Function
GnRH1	stimulate the release of pituitary gonadotropins
GnRH2	modulates sexual and feeding behaviour
GnRH3	exert neuromodulatory functions

GnRH1 is commonly known as hypothalamic form because of its distribution in the hypothalamus in mammals. The hormone is widely expressed across the brain, scattered from the olfactory bulbs to the medial basal hypothalamus (Clifton and Steiner, 2009). However, the distribution of each GnRH variant differs slightly across different species. Soon after the first discovery of GnRH1 in seabream, the brain distribution of all three GnRH was revealed (Powell et al., 1994). As illustrated in figure3, *gnrh1* neurons were distributed in the preoptic area while *gnrh2*-producing cells were found in the midbrain tegmentum. *gnrh3*-expressing neurons were observed in the ventromedial olfactory bulbs as well as the terminal nerve (Gothilf et al., 1996). This was later elucidated in other teleost too, including the European sea bass (González-Martínez et al., 2001, Zohar et al., 2010). GnRH2 is generally localised exclusively in the dorsal synencephalon/midbrain tegmentum. GnRH1 and GnRH3 however are found across several regions including overlaps in the olfactory bulbs/terminal nerve (OB/TN), ventral telencephalon (vTEL) as well as the preoptic area (POA) (Muñoz-Cueto et al., 2020). In species where only two paralogs of GnRH exist, the distribution

is still the same as those with all three isoforms. In scenarios where fish species lack GnRH1 such as zebrafish, its functions are assimilated by *gnrh3*-expressing neurons in the POA (Abraham et al., 2010, Steven et al., 2003). Similarly, catfish and eels lack *gnrh3*, hence the function of the GnRH3 system is compensated by *gnrh1*-expressing neurons in the OB/TN (Montero et al., 1994, Zandbergen et al., 1995).

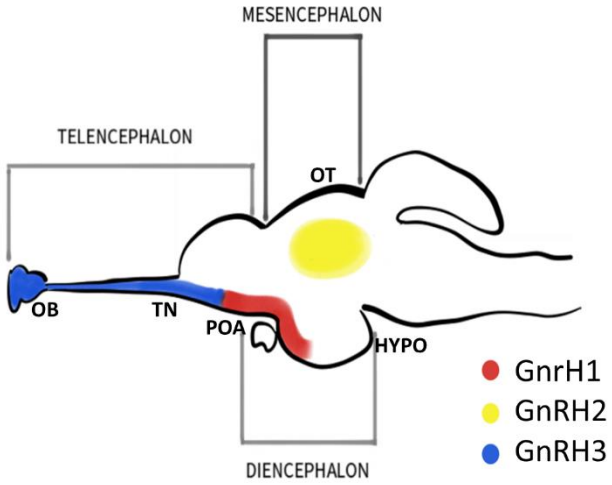


Figure 3 Shows a diagram of a teleost brain and the distribution of the different forms of GnRH across the brain. GnRH1 are distributed in the preoptic area; GnRH2 in the midbrain tegmentum and Gnrh3 in the ventromedial olfactory bulbs as well as the terminal nerve. Hypo = hypothalamus; OB = olfactory bulb; OT = optic tectum; POA = preoptic area; TN = terminal nerve.

For the longest time, GnRH was known as the sole regulator of this axis. However, in more recent years, kisspeptin was found to be the neuromodulator of GnRH release and so also controls the activity of gonadotropins and the HPG axis (Lehman et al., 2010). GnIH is also a regulator of the HPG axis in physiological condition by suppressing the release of GnRH in a pulsatile manner (Tsutsui et al., 2000). It is also worth noting that the GABAergic system (the main inhibitory neurotransmitter system) can also inhibit the synthesis of GnRH. Conversely, one of the main excitatory neurotransmitter systems, the glutamatergic system, causes an increase in GnRH secretion (Clarkson and Herbison, 2006). These polarising effects suggests a large field in the regulation of the HPG axis via GnRH, which can be linked to fertility disorders.

2.5.5 Gonadotrophin-inhibitory hormone

Since the discovery of GnRH, it was known to be the sole hypothalamic regulator of pituitary gonadotropins until Tsutsui et. al. discovered the presence of a hypothalamic neuropeptide that had inhibitory effects on the release of pituitary gonadotropins (Tsutsui et al., 2000). First isolated in Japanese quail, an avian species, GnIH is a dodecapeptide that regulates reproduction in vertebrates by decreasing gonadotropins through the GnRH system and the anterior pituitary gland. The precursor for GnIH encodes for one GnIH and two GnIH-related peptides possessing LPXRFamide (X = L or Q) motif at the C-terminus in all avian species studied. These two peptides are known as GnIH-RP-1 and GnIH-RP-2. This was found to be conserved across mammals, primates, fish, as well as avian species (Tsutsui, 2009, Sawada et al., 2002, Ukena et al., 2003a). Goldfish was found to possess three GnIH peptides LPXRFa-1, -2 and -3 which have both inhibitory and stimulatory effects on gonadotropin synthesis and releases, depending on the reproductive conditions (Moussavi et al., 2012, Moussavi et al., 2013, Qi et al., 2013). Similarly, zebrafish peptide xfLPXRF-3 also has an inhibitory effect on gonadotropin release (Zhang et al., 2010).

As briefly mentioned, GnIH can act directly on GnRH1 in the hypothalamus, resulting in a decrease in the activity of pituitary gonadotrophs. The effects of *in vivo* administration of GnIH were observed across different species. Peripheral administration in Syrian hamsters caused a decline in LH release, which was also the case in central administration of GnIH in avia, hamsters and rats (Kriegsfeld et al., 2006, Ubuka et al., 2012, Johnson et al., 2007). Alternatively, it is also able to affect the HPG axis through directly regulating gonadal activity as GnIH receptors are expressed in steroidogenic cell and germ cells in gonads in both mammals and birds.

GnIH is known to be localised in the hypothalamus of mammals and primates (Kriegsfeld et al., 2006, Ubuka et al., 2009, Ubuka et al., 2012, Ukena et al., 2002). In the brain of quail, GnIH-immunoreactive (-ir) neurons was found to be localised specifically in the paraventricular nucleus (PVN) in the hypothalamus while its fibres are widely distributed across the diencephalic and mesencephalic regions. The most prominent fibres were observed in the median eminence (ME) of the hypothalamus and the dorsal motor nucleus of the vagus in the medulla oblongata (Tsutsui et al., 2000, Ukena et al., 2003b). GnIH cell populations are also widely distributed across the brain in different fish species ranging from the olfactory bulb, ventral telencephalic area, hypothalamus as well as mesencephalic *tegmentum* in goldish (Sawada et al., 2002), sea bass (Paullada-Salmerón et al., 2016), Indian major carp (Biswas et al., 2015), cichlids, zebrafish (Corchuelo et al., 2017), sole (Aliaga-Guerrero et al., 2018)and pejerrey (Pahí-Rosero et al., 2018). Different fish species had

different distributions, however in all fish species studied, GnIH cells are definitely present in the nucleus posterior periventricular nucleus (NPPv) of the caudal preoptic area.

2.5.6 Kisspeptin

Kisspeptin is a neuropeptide that is encoded buy *KISS1/Kiss1* gene. In humans, kisspeptin neurons are distributed in the infundibular/arcuate nucleus within the hypothalamus, often co-expressed by neurokinin B and dynorphin (KNDy) neurons (Skorupskaite et al., 2014). The regulation of pulsatile kisspeptin secretion acts via neurokinin B receptor as well as kappa opioid peptide receptor. Across all species, kisspeptin is a potent stimulation of the HPG axis as it signals directly to GnRH neurons via kisspeptin receptors. This results in the release of GnRH into the circulation which in turn triggers the secretion of LH and FSH from the anterior pituitary. In rodents, kisspeptin neurons are distributed in the arcuate nucleus (ARC) and the antroventral periventricular (AVPV). ARC-kisspeptin neurons are involved in the negative feedback regulation of gonadotropin secretion in females while AVPV-kisspeptin neurons participate in preovulatory gonadotropin surge (Dungan et al., 2006).

The *KISS1/Kiss1* gene in mammals codes for a polypeptide that is 145 amino acids long. This can be cleaved into endogenous fragments resulting in polypeptides with different numbers of amino acids residues (Kotani et al., 2001). Most mammalian genomes only consist of one form of *kiss1* with the exception of monotreme platypus, which has both *Kiss1* and *Kiss2* genes (Lee et al., 2009). The paralogous gene *kiss2* was first reported in zebrafish and medaka(Kitahashi et al., 2009, Van Aerle et al., 2008), and it was since discovered that non-mammalian vertebrates possess two or more kiss genes (Felip et al., 2009, Li et al., 2009). The functional role of the two types of kisspeptin in reproduction have been characterised in several fish species. Central administration of Kiss2 peptides in European sea bass, striped sea bass and zebrafish has shown to exhibit a more potent stimulatory effect on the secretion of gonadotropin as compared with Kiss1 (Zmora et al., 2015, Espigares et al., 2015). However, the opposite effect was found in goldfish and medaka (Selvaraj et al., 2013, Li et al., 2009).

Different species of fish have slightly varying distributions of both Kiss1 and Kiss2 gene. Kiss1 gene is expressed in the habenula in the brain of zebrafish, medaka and goldfish (Kanda et al., 2008, Mitani et al., 2010, Wang et al., 2013, Ogawa et al., 2014). It is also found to be expressed in the preoptic-hypothalamic regions of medaka and goldfish, but not zebrafish. Kiss2 neurons, however, is mainly expressed in the preoptic area and hypothalamic nuclei and its neural processes are widely distributed

across the brain (Kitahashi et al., 2009, Song et al., 2015). Therefore, the function of the two kisspeptins in controlling reproduction could be independent of the kisspeptin type. Instead, it may depend on their site of action as well as characteristics of the cells expressing the respective Kiss receptor types (Parhar et al., 2012).

2.5.7 Phoenixin

PNX is a neuropeptide that is a product of cleaved Smim20 protein. It was recently discovered to exists in two isoforms: PNX-14 and PNX-20, comprising of 14 and 20 amino acids respectively. Although PNX-14 is expressed across the CNS, it is found to be mainly expressed in the hypothalamic nuclei. Its biological functions are known to be mediated by the receptor GPR173 as it plays a crucial role in the CNS. PNX of a hypothalamic origin is important in controlling the oestrous cycle and stimulates LH (Yosten et al., 2013). PNX-20 was observed to trigger GnRH-stimulated secretion of LH in vitro. In addition, the same study also observed that PNX was increased the expression of GnRH receptors in the pituitary and potentiated the expression of GnRH receptor expression induced by GnRH. Interestingly, PNX had no influence in the release of LH from male pituitary cells (Yosten et al., 2013). A contrasting finding in rats was observed by whereby administration of PNX-20 via intracerebroventricular (icv) injection induced an increase in LH secretion as well as serum concentrations of both FSH and testosterone (Stein et al., 2016). These studies showed polarising effects of PNX in vitro as compared to in vivo. However, it can still be concluded that this peptide is involved in the control of gonadotropin secretion. PNX is also involved in other aspects of the CNS such as anti-inflammatory and cell protective effects (Yosten et al., 2013, Yang et al., 2020).

2.5.8 Spexin

Spexin is also known are neuropeptide Q (NPQ). It is expressed in the CNS within the hypothalamic PVN, supraoptic nucleus (SON) as well as in the anterior pituitary gland (Porzionato et al., 2010) . Spexin is known to bind to galanin receptor to elicit its effects (Kim et al., 2014). It also plays various roles in relation to stress as well as reproductive function through the inhibition of cell proliferation in the adrenal gland (Rucinski et al., 2010).

Several studies have been performed on various species of fish that demonstrates the role of spexin in the regulation of the HPG axis through the suppression of both LH and FSH secretion (Cohen et al., 2020). Spexin possess an inhibitory action on the reproductive axis whereby a low level of spexin results in a high level of LH during breeding season. The expression of spexin in the brain

can be regulated by gonadal hormones in a feedback manner. A study supporting this hypothesis observed an increase in hypothalamic *spx* expression after castration in goldfish. It was also found that oestrogen replacements could reverse the effects of the up-regulated hypothalamic *spx* expression (Liu et al., 2013). As most studies were carried out in fish, further investigations are required in other species to identify whether the effects of spexin are similarly or differently regulated in the HPG axis of other.

2.6 Zebrafish as an animal model

Model organisms are generally used to replicate metabolic symptoms and associated disease developments in humans, with mice being the leading experimental animal model used. This has allowed for the development, diagnosis, and treatment of metabolic syndromes via biomedical research. However, findings made from such animal models are not fully comparable to that of humans due to some differences in lifestyle, dietary requirements, and microbiomes. Hence the use of other animal models in parallel to further understand human pathogenesis of metabolic diseases (Santoro, 2014). One of many species that adds value to biomedical research is zebrafish.

First introduced in the early 1980s, zebrafish are often used in genetic studies as an animal model (Streisinger et al., 1981). Around 70% of human genes have at least one obvious zebrafish ortholog, which is comparable to that of human and mouse (at approximately 80%) (Howe et al., 2013). This fact has aided further understanding of human genetics through the use of zebrafish. They also possess similar metabolic characteristics to humans that complement data obtained from other animal models (Nakayama et al., 2018). Zebrafish is often used in biomedical research to study mental disorders, developmental disorders and the communication between the brain and different organs. Many studies utilise zebrafish to better understand factors affecting the function of the HPG axis, it was deemed appropriate to utilise zebrafish in the current study as they are advantageous in studying the reproductive system due to the short cycle of reproductive period (Akhter et al., 2016, Ivan Bassi and Yoav Gothilf, 2016). Its high similarity in its system in regulating reproductive hormones to humans also makes it a favourable organism for research (Laan et al., 2002). Courtship behaviour has also been established in zebrafish, adding more value in the field of fertility research (Darrow and Harris, 2004).

3 Methodology

3.1 Experimental design

3.1.1 Phase 1

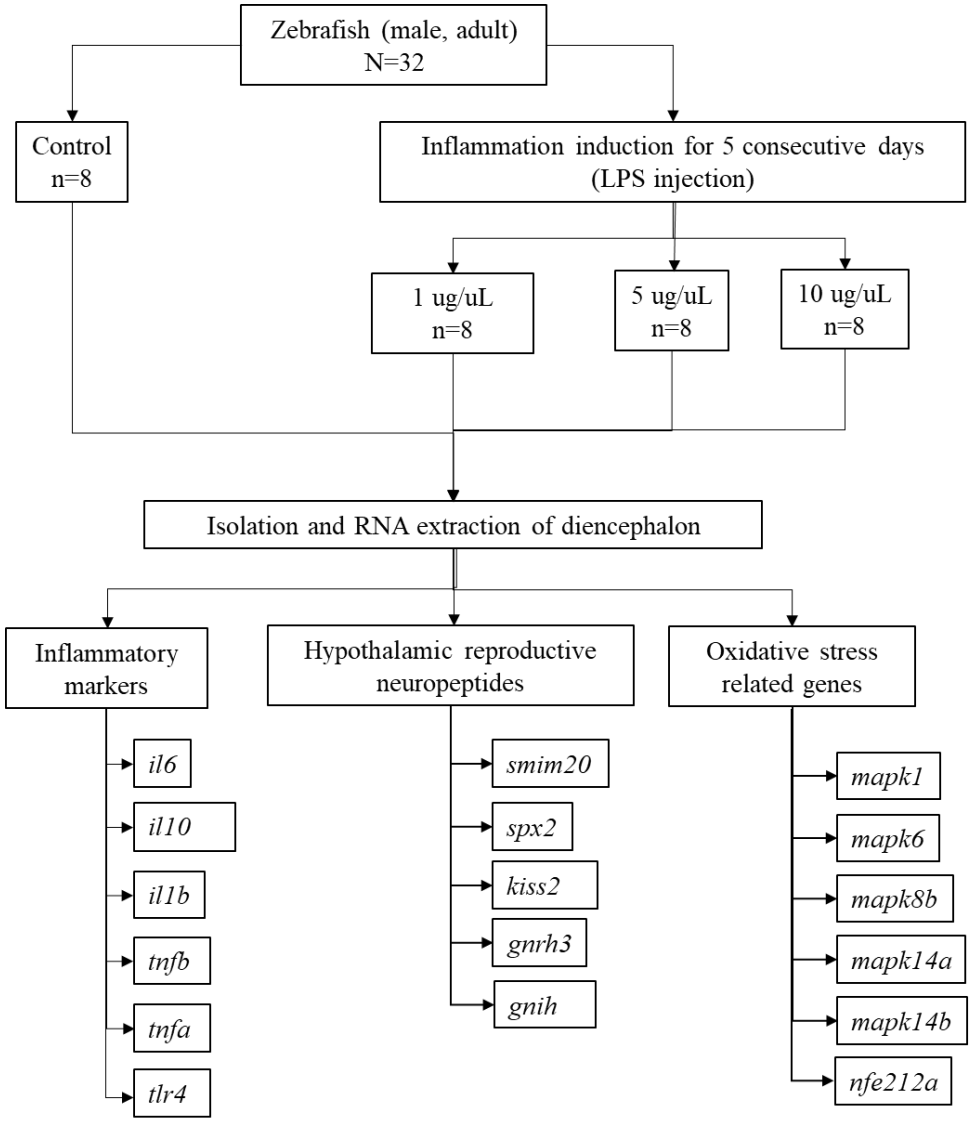


Figure 4 The workflow of phase 1 consists of induction of inflammation and gene expression profiling.

3.1.2 Phase 2

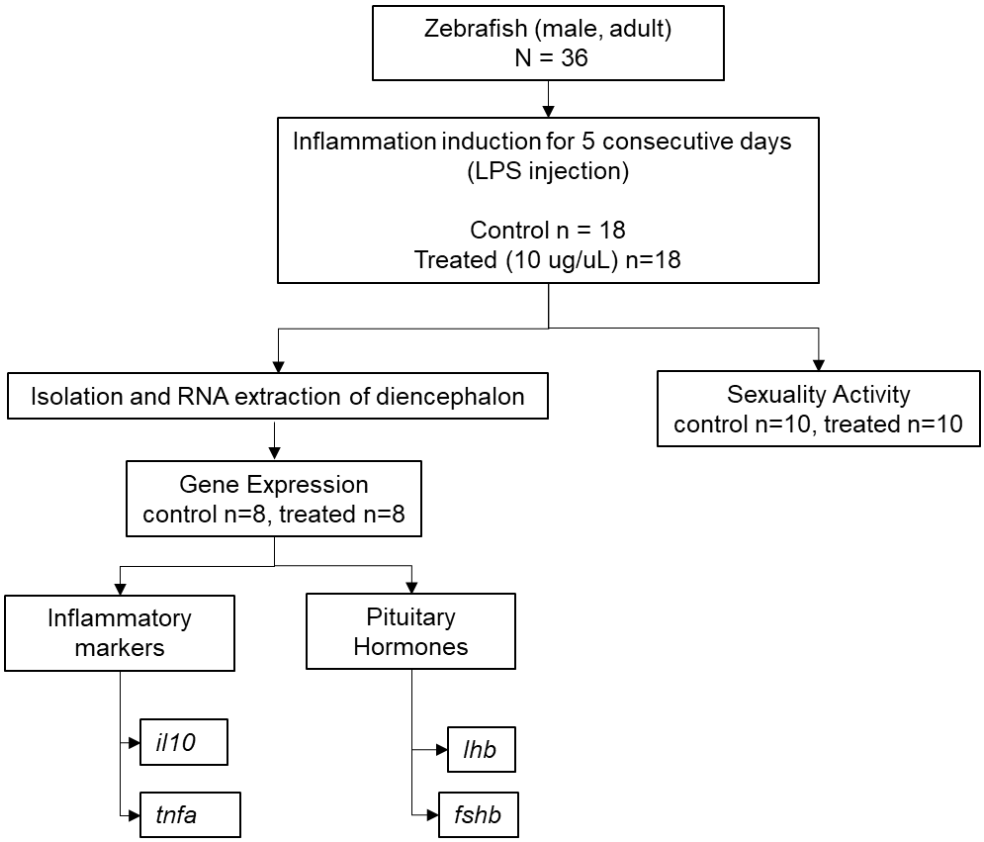


Figure 5 The workflow of phase 2 consists of another round of induction of inflammation followed by gene expression profiles of genes in the pituitary and sexual activity study.

3.2 Animal handling

A total of 68 adult (3-6 months), male wildtype zebrafish (bought from local pet stores). All experimental procedures were conducted humanely, food and water will be given *ad libitum*. Zebrafish were fed with pellet (I-bus MICRO PELLET) twice a day. Zebrafish were supplemented with food flakes (SISO Tropical Flake) or brine shrimps every 2-3 days. Zebrafish were housed in circulating tanks with 14:10 hour light dark cycle. The study design of this project approved by Monash University Animal Ethic Committee (approved AEC ethics number: 2022-26966-71970).

3.3 Induction of inflammation

The zebrafish were randomly distributed into control and the different treatment groups with Lipopolysaccharide from *Escherichia coli* O111:B4 (Sigma-Aldrich, Saint Louis, MO, USA). In phase 1 (figure 4), 32 zebrafish were divided randomly into different groups; control (n=8) and treated (n=24). The treated group were further divided into different treatment groups at 1 ug/uL (n=8), 5 ug/uL (n=8) and 10 ug/uL (n=8) of LPS. The control group were injected with PBS only. Each zebrafish was placed in water with 30% benzocaine to anesthetize before intraperitoneal (IP) injection was performed. The treatment was carried out for 5 consecutive days, after which the diencephalon was extracted within one hour of the final administration of LPS.

During phase 2 of the study (figure 5), a total of 32zebrafish were separated into two groups for gene expression study and sexual activity study. For the analysis of gene expressions, zebrafish were separated into control (n=8) and treatment (n=8) groups. IP injection of LPS at high dose was carried out for 5 consecutive days, after which the pituitary was extracted for gene expression. A further 10 zebrafish was also treated with 10 ug/uL of LPS for the sexual activity study, compared to 10 control zebrafish. This specific dose was selected for further study in phase 2 as it was the dose of LPS that caused significant increase in both pro- and anti-inflammatory markers in the diencephalon during phase 1 of the study. Hence it is of interest to observe whether these effects will be mirrored in the pituitary.

3.4 Isolation of diencephalon

Zebrafish were euthanised in 30% benzocaine solution, followed by decapitation for brain isolation. The brain was isolated and further dissected to obtain the diencephalon as shown in the illustration in figure 6. The dissected brain tissue was immediately placed in ice-cold cold FavorPrep™ Tri-RNA Reagent (Favorgen Biotech Corp., Ping Tung,Taiwan) and immediately stored at -80 °C.

The harvest of the brain was limited to only the diencephalon as it is the origin of all the reproductive neuropeptides of interest in this study, that was earlier identified as the key players in regulating the reproductive system. These neuropeptides are distributed within the area circled in figure 3 (b).

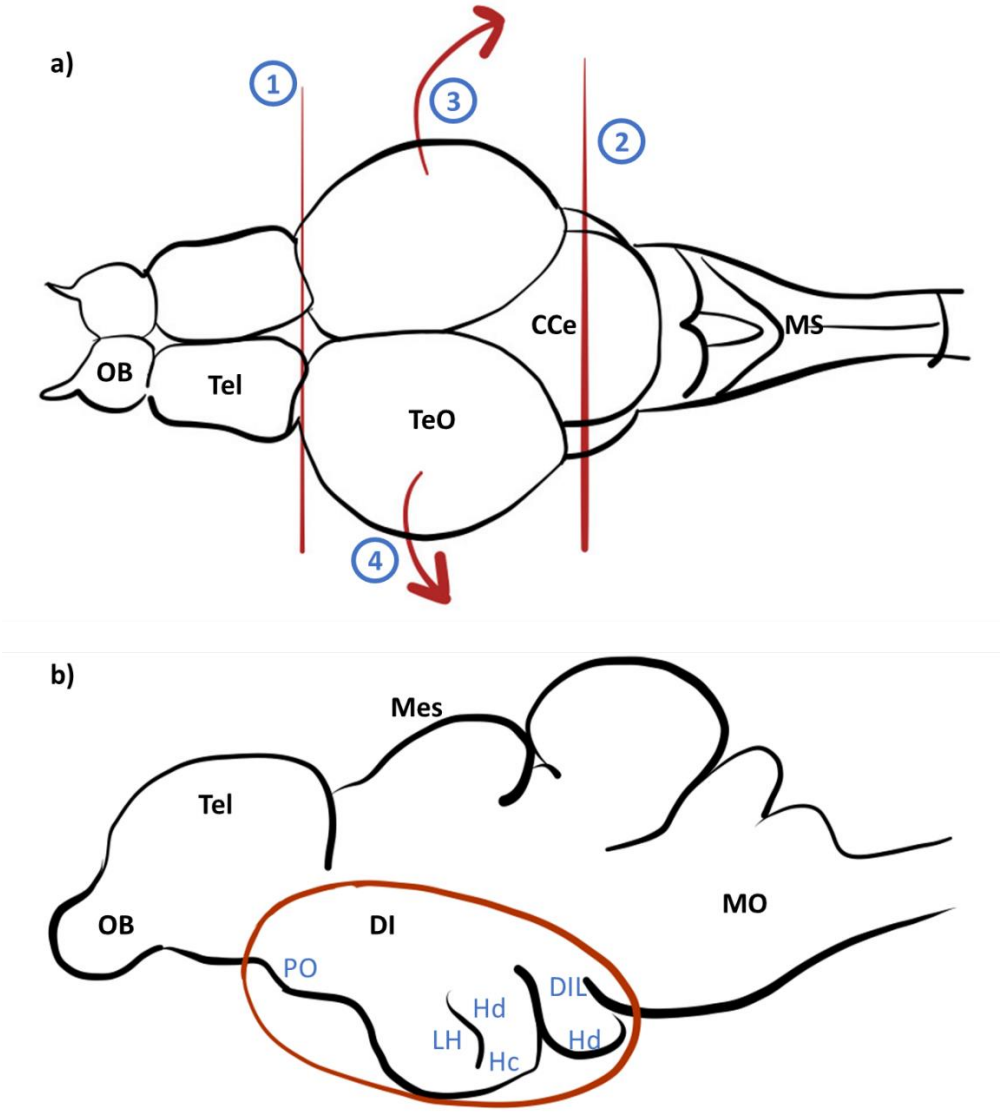


Figure 6 a) dorsal view of adult zebrafish brain. Red lines indicate the sections that were dissected off in order to isolate the diencephalon in its respective order. **b)** sagittal plane illustrating the region obtained for sampling. CCe: corpus cerebellum; Di: diencephalon; Mes: mesencephalon; MO: medulla oblongata; MS: medulla spinalis; OB: olfactory bulb; Tel: telencephalon; TeO: Tectum opticum. The diencephalon was isolated which contains the diffuse nucleus of the inferior lobe of the hypothalamus (DIL), caudal zone of periventricular hypothalamus (Hc), dorsal zone of the ventricular hypothalamus (Hd), lateral hypothalamic nucleus (LH) as well as the pre-optic area (PO).

3.5 RNA extraction

The diencephalon of each zebrafish brain was then homogenised to permit complete dissociation of nucleoprotein complex, using a homogeniser. 40 µL of chloroform (chloroform: TRIzol = 1:5) was added to each tissue tube which was then vortexed to ensure complete mix and incubated at room temperature for 3 minutes. The tubes were centrifuged at 12,000g, 4°C for 15 minutes. The colourless aqueous phase was transferred into fresh ethanol precipitation tube where 100 µL of isopropyl alcohol (isopropyl alcohol:TRIzol = 1:2) was added. The solution was mixed properly by gentle tapping the tube with fingers. The tubes were incubated at room temperature for 10 minutes. The tubes were then centrifuged at 12,000g, 4°C for 15 minutes and the supernatant was discarded. 200 µL of 75% ethanol was added to rinse the pellet by centrifuging the tubes at 7,500g, 4°C for 5 minutes. The supernatant was completely removed, and tubes were left to airdry. The pellets were then resuspended in 20 µL of Ultrapure Milli-Q. The concentration of total RNA was measured based on the A260 value using NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the purity of each sample was verified by determining the A260/A280 ratio.

3.6 cDNA synthesis

Synthesis of cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermofisher, UK) as described by the manufacturer where the cDNA concentration of each sample was normalised to 200 ng/µL. The master-mix was prepared according to table 4 to make up a 20 µL reaction, and 6.7 µL was loaded into PCR tubes followed by 13 of RNA and Ultrapure Milli-Q mixture. All tubes were mixed properly by gentle tapping, after which they were spun down using a microcentrifuge and loaded into a thermocycler (Eppendorf Mastercycler ® nexus GX2) according to the steps in table 5. All samples were stored at -20°C until the next usage.

Table 4 Reagents and volumes required for 20 µl cDNA synthesis reaction . Multiscribe Reverse Transcriptase and RNAse Inhibitor were added last. RNA and Ultrapure Milli-Q were excluded from the master mix.

Reagents	**Volume (µL)**
10X RT Buffer	2.0
25X dNTP mix	0.8
10X Random primer	2.0
Multiscribe Reverse Transcriptase	1.0
RNAse Inhibitor	0.9
RNA + Ultrapure Milli-Q*****	13.3

*****The volume of RNA was added according to the standardised RNA concentration of 200 ng/µL. Ultrapure MilliQ was topped up to amount to 13.3 µL

Table 5 Conditions of cDNA conversion using a thermocycler

Temperature (°C)	**Duration**
25	10 min
37	2 hours
85	5 min
4	Hold

3.7 Quantitative Real-Time PCR

The genes of interest and their respective forward and reverse primer sequences are listed in the Table 6. FASTA sequence of the genes of interest were obtained from NCBI GenBank for the species *danio rerio.* The primers were designed by using NCBI Primer-BLAST to target selected genes. Primer Express was also used to analyse each primer for hairpin loops, self-dimers as well as cross-dimers.

Table 6 Primer Sequence for the Quantitative Reverse Transcription PCR used in this Study

Gene Name	Forward primer (5' to 3')	Reverse Primer (3' to 5')	Product length (bp)	GeneBank accession number
fshb	cagatgaggatgcgtgtgc	acccctgcaggacagcc	281	NM_205624.1
gnih	gctaagtgaagttacggctctc	agctggttttggtattataggatg	205	NM_001082949.1
gnrh3	atggaggcaacattcaggatgt	cctttcagaggcaaaccttca	131	NM_182887.2
il10	gtcatgaacgagatcctgca	atcccgcttgagttcctgaa	129	NM_001020785.2
il1b	gacttcgcagcacaaaatga	tcacttcacgctcttggatg	100	NM_212844.2
il6	agaccgctgcctgtctaaaa	tttgatgtcgttcaccagga	129	NM_001261449.1
kiss2	gcctatgccagaccccaaa	tttactgcgtgctagtcgatgttt	154	NM_001142585.1
lhb	ggtgtcttctttctcttctc	cgggctcttgtaaacgggat	186	NM_205622.2
mapk1	gttgaagacgcagcacttga	acaggtttgatggcttcagg	122	AY922320
mapk14a	actcgcattccaagcagact	ttcgaggcggtggagttgt	147	AB030897
mapk14b	cggagcggtaccagaattta	cgggagagtttcttcactgc	109	AB030898
mapk6	cagggctttgacttcgactc	tgtgaaacggaggtgttca	107	DQ360074
mapk8b	acaggaataagcgcgagaaa	tggtcatacgctgagcagac	133	AB030900
nfe2l2a	gacaaaatcggcgacaaaat	ttaggccatgtccacacgta	165	AB081314
smim20	gtcctcgagtgaggtgaaa	tcagaccagaccttcacacc	253	NM_001302624.1
spx2	ctcgcaggggttgttattg	tgcaatgagactctgtcacc	195	XM_005162991.1
tlr4	QuantiTect Primer Assay Dr_tlr4ba_va.1_SG (QT02198539)		95	NM_001131051
tnfa	QuantiTect Primer Assay Dr_tnfa_1_SG (QT02097655)		81	NM_212859
tnfb	ttcctcagaccacggaaaag	aacccatttcagcgattgtc	140	NM_001024447.1

Quantitative real-time PCR was carried out using the Sensifast SYBR Hi-Rox kit (Bioline, Meridian Bioscience, UK). The master mix was prepared according to table 7, making up a 10 µL reaction. The cDNA of each sample was placed individually into each tube of the PCR strip.

Table 7 Lists the reagent and volumes of each reagent required to make up a reaction of 10 µL.

Reagents	Volume (µL)
SYBR Green	5.0
Forward primer	0.2
Reverse primer	0.2
Template (c DNA)	1.0
Ultrapure Milli-Q	3.6

Both a negative and positive control was run for each gene. Each reaction was performed in duplicate samples and was run concurrently with the gene of interest and the housekeeping gene (*ef1a*). e*f1a* was selected as a housekeeping gene as it has proven to be one of the more stable genes expressed during development as well as upon hormone or toxicant treatment in zebrafish (McCurley and Callard, 2008). PCR strips were loaded into 7500 Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, US). PCR amplification was conducted on according to the run method in table 8.

Table 8 Lists the cycling conditions for real-time PCR. The cycling stage was repeated for 40 cycles.

Stages	Steps	Temperature (°C)	Duration
Holding	Step 1	50	20 sec
	Step 2	95	10 min
Cycling	Step 1	95	15 sec
	Step 2	60	1 min
Melt curve	Step 1	95	15 sec
	Step 2	65	1 min
	Step 3	95	30 sec
	Step 4	65	15 sec

3.8 Statistical analysis

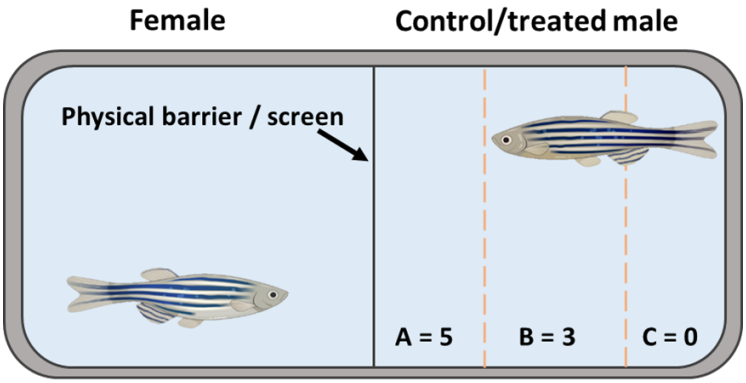
The relative expression levels of all genes were calculated using the $2^{-\Delta\Delta Ct}$ method. GraphPad Prism 9.3.1. was used to perform all statistical analysis. The data were presented as means ± standard error

of the mean (SEM). Outliers were removed from all data sets using the ROUT methods (Q = 0.1%). Ordinary one-way ANOVA were then conducted with Tukey's multiple comparison test, with a single pooled variance. The equality of group variances was based on the Brown-Forysthe test and Barlett's tests. Statistical differences with $p < 0.05$ were considered s significant.

3.9 Sexual activity

The treatment was carried out for five consecutive days as previously described for both the control and treated group. Ten control zebrafish and ten treated zebrafish were then analysed around 15 hours after the final administration of LPS. Zebrafish swimming behaviours were filmed using a camera set on a tripod according to an established protocol (Zemková and Stojilkovic, 2018). The recording was scheduled for early morning, from 07:30 to 08:00 AM, when natural mating occurs. In the evening before filming, the male and female zebrafish (either a control or LPS-treated fish) were transferred to the mating tank (13 cm width×7 cm depth×7 cm height). Each tank hosted one male and one female zebrafish (untreated), divided into two sections by a clear screen inserted in the middle of the cage. The screen permitted water flow but separated the male and female fish, preventing them from making physical contact. The male half of the tank was further partitioned into three sections by lines marked on a paper that was placed underneath the transparent tank to indicate the areas relative to the divider in the middle of the mating tank (figure 7). On the day of the experiment, immediately after light on-set in the morning, the zebrafish were filmed for 30 min.

After filming, the swimming behaviours of the male zebrafish were analysed to determine their tendency to mate (Liu et al., 2021). Their desire for mating was quantified by several measures to indicate their sexuality levels. The locations of where the zebrafish swam, and the speed of its swimming were analysed at 15 second intervals throughout the 30 min recording and scored according to figure 7. The number of times the zebrafish attempted to cross the middle of the cage (e.g., the number of times the zebrafish approached the screen) was also valued. Each time it swam against the screen (i.e., physical contact), the zebrafish received an additional 5 points. Total scores were summed for individual zebrafish to indicate its sexuality levels. All behaviour videos were analysed by researchers who have no knowledge on the origins or condition of the fish.



Speed of zebrafish	Score
Fast swimming (>3.0 cm/s)	2
Normal swimming (1.0–3.0 cm/s)	1
Slow swimming (<1.0 cm/s)	0

Figure 7 Illustrates the setup of the tank for sexual activity observation. The male half of the tank was divided into three sections by placing paper underneath the transparent tank to indicate its location in relation to the female half of the tank. The sexual activity of the zebrafish were scored based on their location every 15 seconds in their compartment according to area A, B and C which scores 5, 3 and 0 point respectively. The speed at which the zebrafish swims is also contributes to the sexual activity scoring where fast swimming scores 2 points, normal swimming scores 1 point and slow swimming costs 0 points. An additional 5 point is awarded when the zebrafish comes in physical contact with the screen.

4 Results

4.1 Phase 1 - Gene Expression within the Diencephalon

4.1.1 The effects of LPS on the expression of inflammatory markers

Administration of LPS via intraperitoneal injection showed to induced an inflammatory response within the hypothalamus. There was a significant upsurge in the expression of several inflammatory markers that were studied when the treatment groups were compared to the control (figure 4).

il1b showed no significant increase in its expression across all three doses of LPS treatment when compared control (figure 4a). Although there was a slight increase in its expression levels at 5 ug/uL and 10 ug/uL of LPS treatment, the difference was not significant compared to the control. However, there is a significant difference compared between these two treatment groups compared to treatment at 1 ug/uL of LPS, in which 5 and 10 ug/uL has a P value ≤ 0.01 and ≤ 0.05 respectively.

il6 on the contrary showed a significant upsurge in its expression when treated with 10 ug/uL of LPS compared to the control group ($p \leq 0.001$) as well as treatment with 1 and 5 ug/uL of LPS with $P \leq 0.01$ (figure 4b).

Similarly, *il10* expression also increased significantly when treated with LPS (figure 4c). There is no significant difference between the control group and that treated with 1 ug/uL. However, there is a significant difference in its level of expression when treated with 5 and 10 ug/uL of LPS compared to that of control ($p \leq 0.0001$) and treatment with 1 ug/uL of LPS ($p \leq 0.0001$).

The expression levels of *tlr4* also increased upon treatment with LPS (figure 4d). Compared to the control group, treatment with LPS showed a significant difference at the different doses where 1 ug/uL had a P value ≤ 0.05, and treatment with 5 and 10 ug/uL had P values ≤ 0.001. Additionally, there was also a significant difference in *tlr4* expression when treated with 1 ug/uL of LPS compared to treatment with 5 ug/uL ($p \leq 0.001$) and 10 ug/uL ($p \leq 0.01$) of LPS.

When treated with LPS, a significant difference in *tnfa* was observed at 1 ug/uL ($p \leq 0.01$), 5 ug/uL ($p \leq 0.0001$) and 10 ug/uL ($p \leq 0.0001$). There was also significant difference it is expression when comparing treatment with 1 ug/uL of LPS and that with 5 and 10 ug/uL of LPS ($p \leq 0.05$) (figure 4e).

Tnfb showed an increase in expression levels in some treatment groups compared to control (figure 4f). Treatment with 5 ug/uL and 10 ug/uL showed a significant increase in expression with P values ≤ 0.001 and ≤ 0.0001 respectively. Similarly, a significant increase was also observed when 5 ug/uL ($p \leq 0.05$) and 10 ug/uL ($p \leq 0.001$) are compared to that with treatment of 1 ug/uL of LPS.

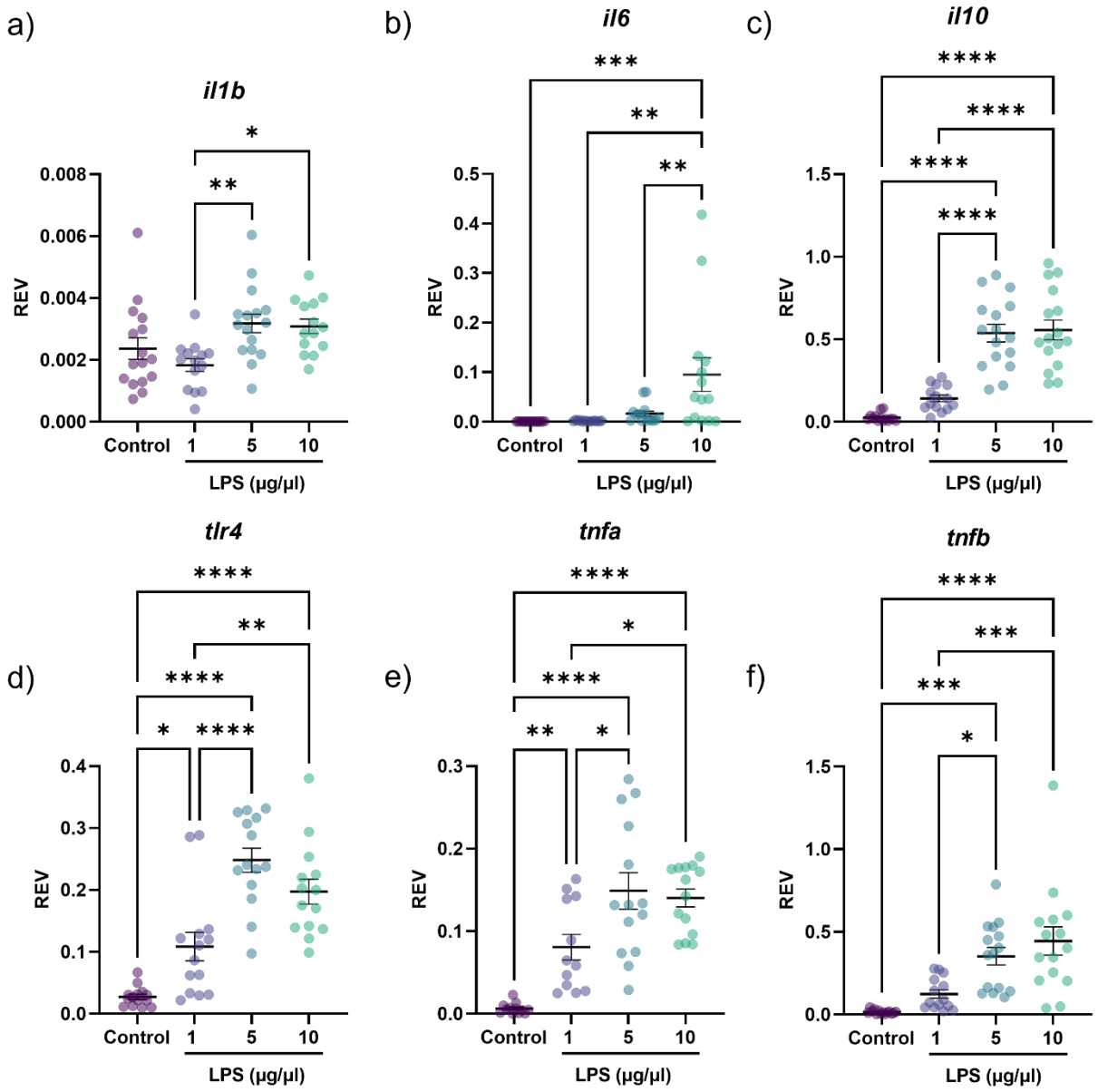


Figure 8 Real-time quantitative PCR analyses of the mRNA levels of a) il1b b) il6 and c) I il10 d) tlr4 e) tnfa and f) tnfb comparing the control group with groups treated with 1, 5 and 10 ug/uL of LPS. Each group consisted of 7 to 8 different samples (n=7-8). P values that are significantly different from the control are indicated by asterisks (one-way ANOVA *$p \leq 0.05$; **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$). Values are the means of two determination on each of the duplicate treatment and are presented as mean ± SEM.

4.1.2 The effects of LPS on the expression of hypothalamic reproductive neuropeptides

Treatment with LPS showed a change in the expression of various reproductive neuropeptides in the diencephalon (figure 5).

Expression levels of *gnih* showed a significant change between that of control group and those treated with LPS (figure 5a). There was no significant difference in its levels when treated with 1 ug/uL of LPS compared to the control group. However a significant increase was observed when the control group was compared to treatment with 5 ug/uL($p \leq 0.0001$) and 10 ug/uL ($p \leq 0.01$). There is also a significant upsurge in *gnih* levels at 5 ug/uL ($p \leq 0.0001$) and 10 ug/uL ($p \leq 0.05$) compared to treatment with 1 ug/uL of LPS. However, a significant decrease ($p \leq 0.01$) in *gnih* is observed when zebrafish were treated with 10 ug/uL compared to 5 ug/uL.

A Complementary pattern was observed in the expression of *gnrh3* whereby a significant upsurge ($p \leq 0.0001$) was found when treated with 1 ug/uL of LPS compared to control (figure 5b). However, there was a significant drastic decrease in *gnrh3* when treated with 5 ug/uL and 10 ug/uL ($p \leq 0.0001$) compared to treatment with 1 ug/uL of LPS. There is no significant change in its expression between these two treatment groups and the control group.

Similarly, expression levels of *kiss2* also showed a similar trend whereby there was significant upsurge in its levels when treated with 1 ug/uL of LPS ($p \leq 0.01$). However, there is no significant change when treated with 5 ug/uL and 10 ug/uL of LPS compared to control. Similar to that of *gnrh3* expression, the level of *kiss2* significantly decreased ($p \leq 0.01$) from 1 to 10 ug/uL (figure 5c).

In contrast, the relative expression levels of *spx2* showed a significant upsurge when treated with 10 ug/uL of LPS, with P values ≤ 0.0001 between the control group and treatment with 1 and 5 ug/uL of LPS (figure 5d). However, no significant change was observed in its expression level when comparing treatment with 1 and 5 ug/uL of LPS to the control group.

The only reproductive gene that did not show any significant change it in its expression levels when treated with LPS was *smim20* (figure 5e).

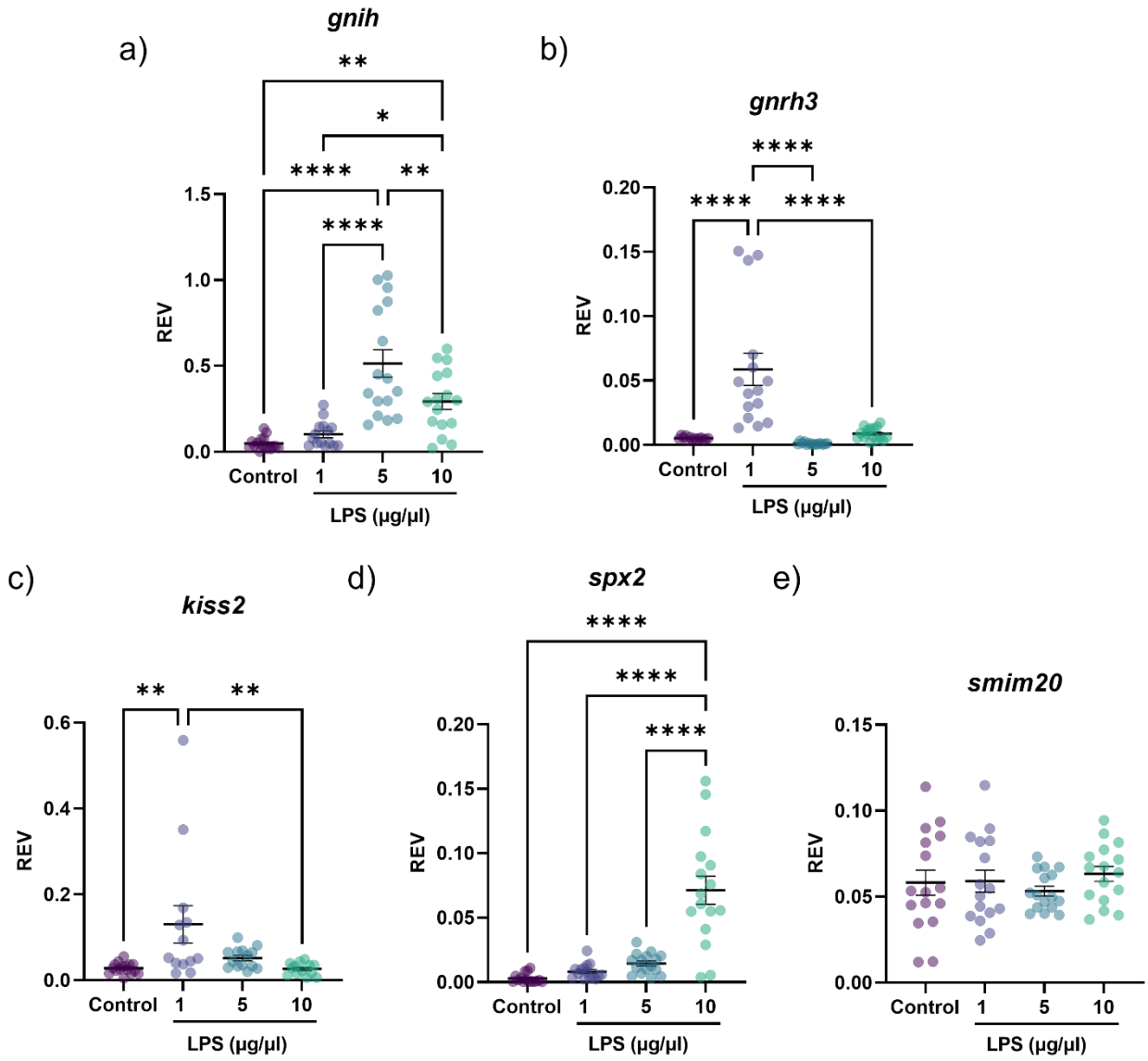


Figure 9 Real-time quantitative PCR analyses of the mRNA levels of reproductive genes a) gnih b) gnrh3 and c) kiss2 d) spx2 and e) smim20 comparing the control group with groups treated with 1, 5 and 10 ug/uL of LPS. Each group consisted of 7 to 8 different samples (n=7-8). P values that are significantly different from the control are indicated by asterisks (one-way ANOVA *p ≤ 0.05; **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001). Values are the means of two determination on each of the duplicate treatment and are presented as mean ± SEM.

4.1.3 The effects of LPS on the expression of oxidative stress-related genes

Administration with LPS also showed a change in levels of some genes related to oxidative stress, namely those involved in the MAPK pathway (figure 6).

There was a significant upsurge in expression levels of *mapk1* when zebrafish was treated with 1 ug/uL ($p \leq 0.0001$) of LPS compared to control group (figure 6a). However, there is no significant difference between the control and those treated with 5 and 10 ug/uL of LPS. Treatment with 5 ug/uL resulted in a significantly higher ($p \leq 0.0001$) expression of *mapk1* compared to treatment with 1 and 10 ug/uL of LPS.

A similar pattern was observed in the expression of *mapk6* where treatment with 5 ug/uL showed in a significantly increase in expression compared to the control group ($p \leq 0.001$) and treatment with 10 ug/uL of LPS ($p \leq 0.005$). There was no significant change in its expression between the other treatment groups and the control (figure 6b).

In contrast *mapk8b* expression levels showed the opposite effect when treated with LPS (figure 6c). A significant decrease in its levels were observed when treatment with 1 ug/uL ($p \leq 0.05$), 5 ug/uL ($p \leq 0.0001$) and 10 ug/uL ($p \leq 0.0001$) of LPS was compared to the control group. This significant chage was also observed when treatment with 1 ug/uL of LPS was compared to treatment with 5 ug/uL and 10 ug/uL of LPS ($p \leq 0.0001$).

There was also a significant upsurge in levels of *mapk14a* when the treatment with 5 ug/uL and 10 ug/uL of LPS ($p \leq 0.0001$) was compared to the control (figure 6d). However, treatment with 1 ug/uL of LPS showed no significant change compared to control. But when compared to treatment with 5 ug/uL ($p \leq 0.0001$) and 10 ug/uL of LPS ($p \leq 0.05$). It also worthy to note that *mapk14a* levels decreased significantly ($p \leq 0.001$) from with 5 ug/uL and 10 ug/uL of LPS treatment.

Interestingly, the expression of *mapk14b* showed a slight decreasing trend whereby treatment with 1 ug/uL ($p \leq 0.05$), 5 ug/uL ($p \leq 0.05$) and 10 ug/uL ($p \leq 0.0001$) of LPS were significantly lower compared to the control group (figure 6e). However, across the different dose of LPS administration, no significant difference was observed in *mapk14b* levels.

Lastly, *nfe212a* showed a significant upsurge in its levels when treated with 5 ug/uL ($p \leq 0.01$) of LPS compared to the control (figure 6f). Although there is a slight increase in its levels when treated with 1 and 10 ug/uL of LPS, statistically this change was not significantly different.

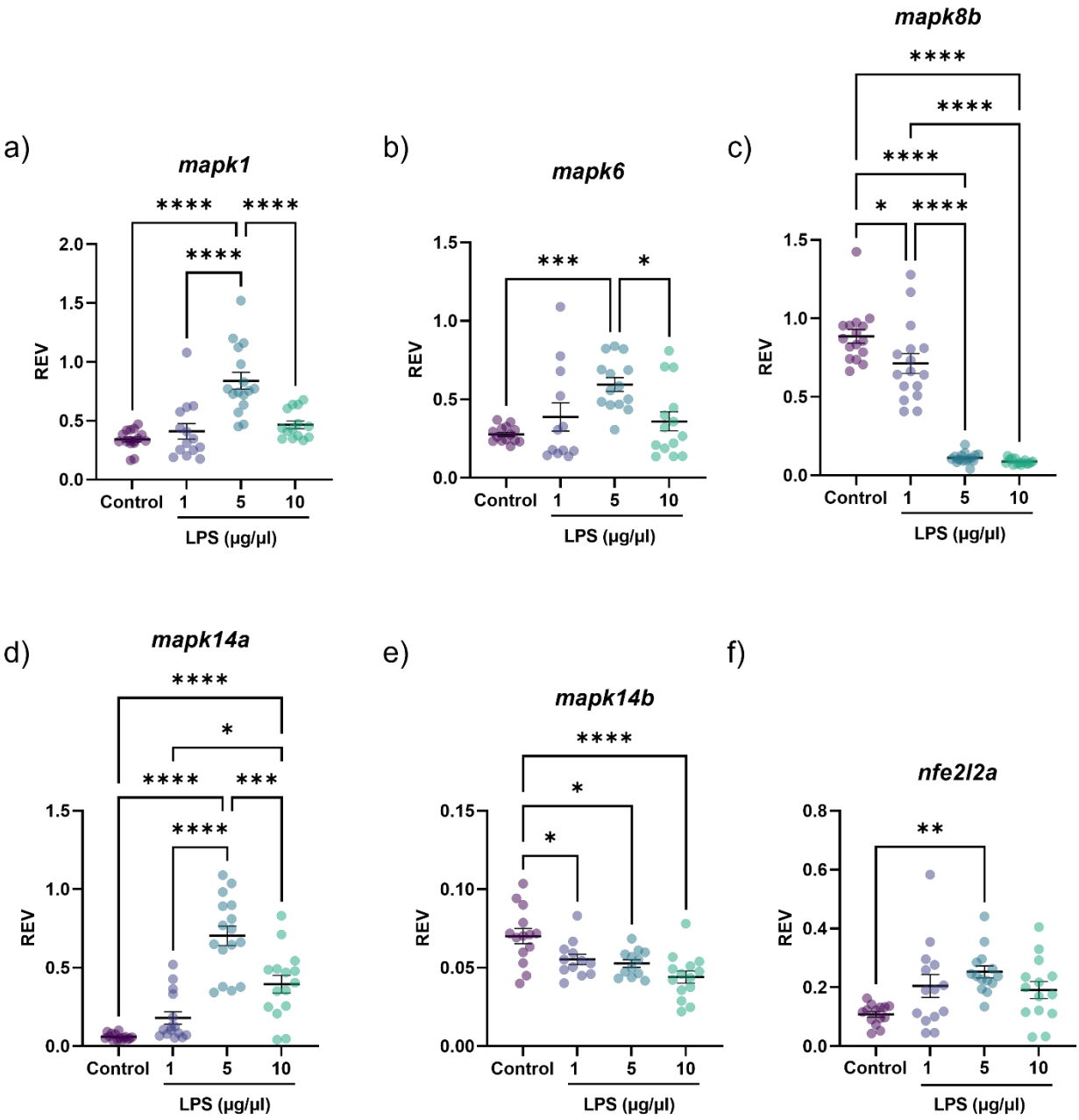


Figure 10 Real-time quantitative PCR analyses of the mRNA levels of oxidative stress related genes a) mapk1 b) mapk6 and c) mapk8b d) mapk14a e) mapk14b and f) nfe212a comparing the control group with groups treated with 1, 5 and 10 ug/uL of LPS. Each group consisted of 7 to 8 different samples (n=7-8). P values that are significantly different from the control are indicated by asterisks (one-way ANOVA *p ≤ 0.05; **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001). Values are the means of two determination on each of the duplicate treatment and are presented as mean ± SEM.

4.2 Phase 2 – Gene expressions within the pituitary gland and sexual activity

4.2.1 The effects of LPS on the expression of inflammatory markers

A pro- and an anti- inflammatory marker in the pituitary were also measured to observe if administration of LPS via intraperitoneal injection induces inflammation in the pituitary (figure 11). The expression level of *il10* showed a slight increase in the group of zebrafish that were treated with 10 ug/uL of LPS compared to the control. However, this change was not considered of significant difference statistically. Similarly, the expression of *tnfa* between the control and treated group also showed no significant change.

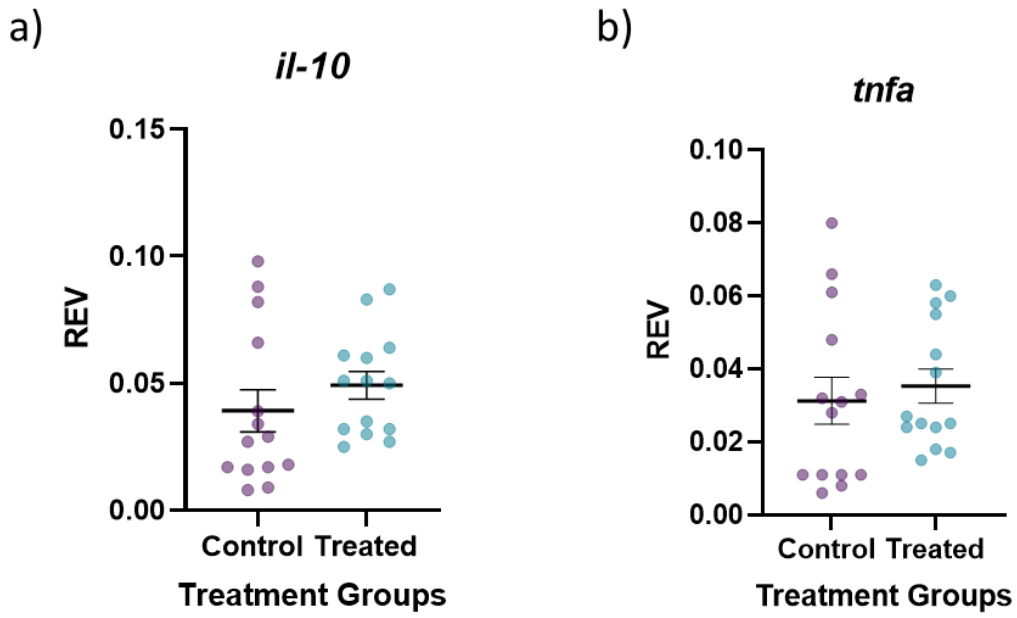


Figure 11 Real-time quantitative PCR analyses of the mRNA levels of inflammatory markers a) *il10* and b) *tnfa* comparing the control group with a treated group, treated with 10 ug/uL of LPS. Each group consisted of 7 to 8 different samples (n=7-8). P values that are significantly different from the control are indicated by asterisks (two-tailed unpaired t-test *$p \leq 0.05$; **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$). Values are the means of two determination on each of the duplicate treatment and are presented as mean ± SEM.

4.2.2 Reproductive axis-related genes

The expression level of two key reproductive genes in the pituitary were also analysed (figure 12). It was found that although there was a slight increase in expression of both *lhb* and *fshb*,in the treated group of zebrafish, neither of these change are significantly different compared to control.

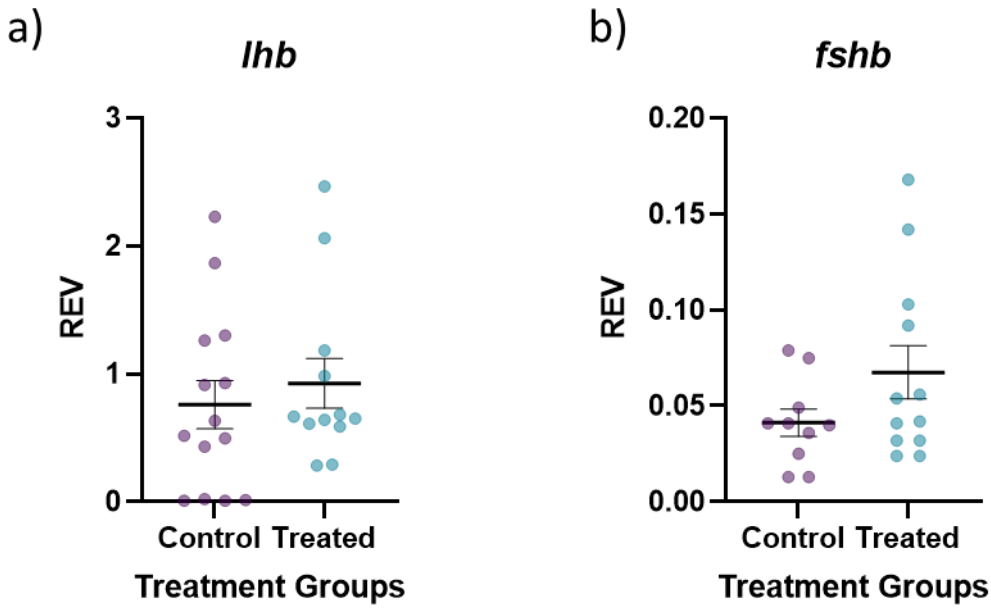


Figure 12 Real-time quantitative PCR analyses of the mRNA levels of reproductive axis related genes a) *lhb* and b) *fshb* comparing the control group with a treated group, treated with 10 ug/uL of LPS. Each group consisted of 7 to 8 different samples (n=7-8). P values that are significantly different from the control are indicated by asterisks (two-tailed unpaired t-test *$p \leq 0.05$; **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$). Values are the means of two determination on each of the duplicate treatment and are presented as mean ± SEM.

4.2.3 The effects if LPS on sexual activity of zebrafish

The sexuality scores of the zebrafishwere scored based on their swimming behaviour such as their affinity for areas close to the screen, their speed of swimming and the number of times the zebrafishbumped into the screen. A comparison between control and treated group (10 ug/uL of LPS) showed no significant difference in the sexual behaviour of control and treated group (i.e., their tendency to mate).

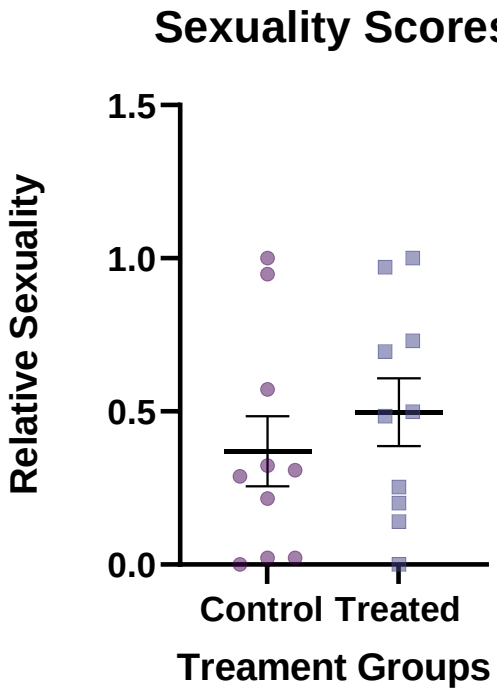


Figure 13 Sexuality scores of control (n=10) and treated (n=10) male zebrafish. treated with 10 ug/uL of LPS. The sexuality levels were normalised to 1. P values that are significantly different from the control are indicated by asterisks (two-tailed unpaired t-test *p ≤ 0.05; **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

5 Discussion

5.1 Neuroinflammation induced by LPS administered via intraperitoneal injection

The present study confirmed that treatment with LPS via intraperitoneal injection induced inflammation in the hypothalamus. This means that systemic inflammation can induce neuroinflammation, as clearly established in various animal models (Kettenmann et al., 2011). There are several ways as to how a systemic inflammation causes neuroinflammation. One of which is through circumventricular organs where cytokines freely diffuse into the brain parenchyma from the blood as these regions of the brain lack an intact BBB. They then interact with macrophages within the brain to induce neuroinflammation. An alternative means, in contrast, involves an intact BBB. The endothelium of the BBB can be activated by cytokines, signalling the perivascular macrophages located adjacent to the endothelial cells. This allows the perivascular macrophage to communicate with the microglia, the resident macrophage of the brain parenchyma, stimulating an inflammatory response (Williams et al., 2001). Following the induction of inflammation in the peritoneal cavity, another route stimulating neuroinflammation involves sensory afferents of the vagus nerve and its communication with neuronal populations within the brain stem (Dantzer et al., 2000). Alternatively, cytokines can also pass through the BBB through active transport, possibly affecting cognitive processes (Banks et al., 2002). The causal relationship between systemic inflammation and neuroinflammation is important in the context of fertility as well. Several diseases are known to cause systemic inflammation while also causing infertility. For instance, male obesity has been identified as one of the factors associated with infertility caused by low sperm quality through changes to hormone levels, as well as direct changes to sperm function and sperm molecular composition (Palmer et al., 2012). Obesity is a state of hyperinsulinemia, hyperlipidaemia, hyperleptinemia, and chronic inflammation subsequently causing inflammation in the brain (Lainez and Coss, 2019). Hence the effects of neuroinflammation on the HPG axis is important to explore, setting a basis for the rest of this study.

LPS is a component of Gram-negative bacterial cell wall that once induced, is recognised by the immune system and elicits a proinflammatory response (Zhang and Ghosh, 2000). Neuroinflammation has been found to be induced by the central or peripheral administration of LPS (Rivest, 2003). However, it is important to note that the neuroinflammatory effects of LPS is both time and dose-dependent (Lopes, 2016, Lewis et al., 2016). Activated microglia within the CNS has shown to increase inflammatory mediators including cytokines. This provides the basis for the

treatment of LPS to be induced for 5 consecutive days as the preliminary study conducted prior to this showed significant dysregulation in inflammatory markers.

5.2 Possible oxidative stress signalling pathways involved in the dysregulation of inflammatory markers in the hypothalamus upon treatment with LPS

This study found a significant increase in *il10* upon administration of LPS across different doses. IL-10 is produced by almost all leukocytes in response to different stimuli, resulting in a highly regulated mechanism in controlling the effects of il10 in a tissue specific manner. IL-10 is able to inhibit the production of pro-inflammatory cytokines by the microglia, protecting the astrocytes from excessive inflammation (Ledeboer et al., 2002). Similarly, IL-10 also acts on astrocytes to stimulate the production of transforming growth factor–β (TGFβ), causing anti-inflammatory effects on the microglia (Norden et al., 2014). Amongst others, IL-10 is also crucial for regulating adult neurogenesis and cellular survival through its receptor signalling (Pereira et al., 2015, Zhou et al., 2009). IL-10 binds to IL-10 receptors to activate the IL-10/JAK1/STAT3 cascade. It has been shown that both IL-10 and STAT3 are essential for an anti-inflammatory response and are irreplaceable by other cytokines or transcription factors (Murray, 2006). STAT3 heterodimers are phosphorylated in response to the receptor and translocate to the nucleus to activate the expression of target genes (Hutchins et al., 2013). This regulates several aspects of the immune response by decreasing the gene expression of cytokines which in turn down-regulates the expression major histocompatibility complex class II (MHC-II) and thus the presentation of antigens to T-cells (Ledeboer et al., 2002, Moore et al., 2001).

Several intracellular signalling cascades regulate IL-10. One of its regulators include the mitogen-activated protein kinase (MAPKs) extracellular signal-regulated kinase (ERK) and p38. They act as positive regulators of IL-10 which signal downstream of pattern recognition receptor activation. TLR4 stimulation of microglial cells by some IL-10 enhancers induces the production of IL-10, which is associated with the activation of ERK. In this study, the gene expression of *tlr4* showed a significant increase across treatment with different doses of LPS, compared to the control. This explains the surge in expression of *il10*, with similar patterns. As for ERK, its gene expression was also measured, showing a significant increase in the gene expression level of *mapk1* and *mapk6*, which encodes for the proteins that are commonly known as ERK2 and ERK3 respectively. The spike in levels of *mapk1* and *mapk6* was observed particularly when treated with 5 ug/uL of LPS. The ERK signalling pathway is commonly related to cell survival and proliferation during oxidant injury (Meloche and Pouysségur, 2007). A study looking into negative regulations of IL-10 production

proved that by blocking glycogen synthase kinase-3 (GSK-3), which functions to inhibit IL-10 production, confirms the role of ERK and p38 in enhancing IL-10 production (Huang et al., 2009).

The mRNA expression for *mapk14a* and *mapk14b*, otherwise known as *p38a* and *p38b* respectively, were also measured in this study. Interestingly, the expression pattern of each of these genes differed from one another upon treatment with different doses of LPS. *p38a* showed a significant increase in its levels across treatment, with a particular upsurge in its levels when treated with 5 ug/uL of LPS, while *p38b* showed significant decrease in its level upon treatment with LPS, compared to the control. Conflicting functions have been concluded for the role of p38 in relation to the production of IL-10 by microglia. A study reported that the activation of p38 was shown to induce the expression of IL-10 in microglial cell line upon the administration of adenosine (Koscsó et al., 2012). This can be supported by the upsurge in *p38a* levels in this study. In contrast, another study utilising LPS-stimulated rats showed that downregulation of p38 was associated with increased IL-10 production (Liu et al., 2011). This can explain the downregulation of *p38b* in this study upon treatment with LPS. However, much is yet to be studied to determine the mechanism in which IL-10 is regulated by in this study. The difference in reports may be due to different models used or perhaps even the different stimuli used to induce inflammation, thus activating different pathways of regulation.

Another gene related to the MAPK pathway, *mapk8b*, which encodes the protein JNK1 was also observed in this study. Its mRNA levels significantly decreased upon treatment with LPS compared to the control group, indicating its suppression in expression during the onset of inflammation. JNK has been reported to be one of the possible players in regulating the expression of IL-10 in astrocytes activated by TLR3 (Park et al., 2006). JNK is also known to enhance the transcriptional activity of AP-1, a binding site that exists in the promoter regions of several genes including that of IL-8, a pro-inflammatory cytokine (Hibi et al., 1993, Mukaida et al., 1989). The reason for the suppression of *mapk8b* upon induction of inflammation is yet to be elucidated. It may suggest that the inflammatory response in place in the current study may be regulated through other aspects of the MAPK pathway, such as ERK or p38.

The mRNA levels of tumour necrosis factors-(TNF) α and β were both analysed in this study. Both cytokines are similar such that they bind to common receptors present in both neurons and glia and exhibit similar biological activities as they both possess pro-inflammatory properties (Feghali and Wright, 1997). TNFα is able to potentiate secondary inflammatory effects by stimulating the synthesis of IL-6 in several cell types, which was observed in this study (Warren, 1990). A significant increase in *tnfa* upon treatment with different doses of LPS was observed. Similarly, the mRNA levels

of *il6* also significantly increased upon treatment with LPS, of which treatment with 10 ug/uL showed the most significant increase. IL-6 is a pro-inflammatory cytokine responsible for differentiation and cytokine production (Pearson et al., 2001). It also plays a central role in the neuronal reaction to nerve injury as evidence shows its contribution to the development of neuropathic pain behaviour after the onset of a peripheral nerve injury (Ramer et al., 1998). TNFβ, also known as lymphotoxin, functions to regulate apoptotic pathways and NF-κB activation of inflammation (Chu, 2013). Its mRNA levels were also observed to significantly increase upon treatment with LPS at different doses, compared to the control group.

Additionally, it was observed that there was no significant change in levels of *il1b* gene expression. IL-1β is a pro-inflammatory cytokine that is primarily released by monocytes and macrophages. Its expression has been found to be upregulated following an injury to peripheral nerve and after trauma in microglia astrocytes (Copray et al., 2001). Since LPS treatment was conducted via intraperitoneal injection, there was no direct damage done to the CNS, hence why the expression levels of this gene were not affected. Furthermore, IL-1β has been found to produce hyperalgesia following intraperitoneal injection (Yan et al., 1992). This can occur when there is damage to the nerves or chemical changes to the nerve pathways that are involved in sensing pain, suggesting that such event did not occur in this study.

The mRNA expression of *nfe2l2a*, which encodes for the protein Nrf2, was found to be significantly upregulated when treated with 5 ug/uL of LPS, compared to the control. Nrf2 is a critical transcription factor that binds to the antioxidant response element (ARE) in the promoter region of several genes that encodes for antioxidative enzymes and cytoprotective proteins (Nguyen et al., 2009). Hence it is denoted that Nrf2 is crucial in the involvement for cells to combat oxidative stress that is generated from exposure to exogenous and endogenous chemicals (Niture et al., 2010). This upregulation indicates the involvement oxidative stress in mediating the neuroinflammatory response induced by the administration of LPS. A recent study showed the involvement of Nrf2 in the suppression of pro-inflammatory cytokine transcription, such as IL-6 and IL-1β (Kobayashi et al., 2016). However, the upsurge in *nfe2l2a* expression in this study was not significant enough to suppress that of *il6* or *il1b*.

Among pro-inflammatory cytokines, IL-1β happens to be the most potent inhibitor of the GnRH-LH system (Watanobe and Hayakawa, 2003), which is not reflected in this current study, suggesting that the system may be regulated by other factors instead. It was found that IL-1β might be responsible for most of the effects that LPS exerts when administered through intracerebroventricular injection (Herman et al., 2012). However, in this current study, LPS was administered via intraperitoneal

injection, where a systemic inflammation is induced first, subsequently inducing neuroinflammation. When bacterial infections disrupt the BBB, the circulating immune cells such as lymphocytes (B and T cells), granulocytes and monocytes are able to enter the brain parenchyma with a supply of cytokines. Cytokines can also be secreted locally in the brain parenchyma, the BBB and choroid plexus cells to induce further inflammatory response within the CNS, known as neuroinflammation (Chen et al., 2017, Förster, 2008, Johnson et al., 2018). Hence, this may explain the reason why the expression levels of IL-1β remains unchanged across the treatment with all doses of LPS.

The changes in the level of expressions of inflammatory marker and oxidative stress related genes within the hypothalamus in this study suggests the involvement of several signalling pathways in neuroinflammation. It can also be interpreted that neuroinflammation may cause the changes in the level of expression of oxidative stress related genes. At this point of the study, the causal relationship between the two parameters in zebrafish hypothalamus cannot be deduced.

5.3 Interplay between neuroinflammation, oxidative stress and their involvement in affecting the expression of key reproductive neuropeptides in LPS-induced zebrafish

The hypothalamic reproductive neuropeptides play a vital role in the regulation of the HPG axis. This study aims to explore whether neuroinflammation affects the expression of several genes in the hypothalamus including GnRH, GnIH, spexin, PNX and kisspeptin. Since gonadotropin secretion is controlled by the brain's primary output, GnRH, its neuronal activities are continuously altered by a variety of stimulatory and inhibitory signals. There is compelling evidence to suggest that GnIH directly inhibits GnRH neuronal activity. A subpopulation of GnRH neurons fire less often after being exposed directly to GnIH in hypothalamic brain slices (Ducret et al., 2009). to The impulses from GnRH neurons are transmitted to GnRH neurons in the hypothalamus and pituitary gonatropes. GnIH acts upon its target cells via Gαi-coupled GPR147 or GPR74. GnRH neurons are activated by kisspeptin or vasoactive intestine peptide (VIP) The kisspeptin/Gq-coupled GPR54-induced Ca2+ or PKC pathway are not directly inhibited by GnIH. GnIH does not directly target the kisspeptin/GPR54 pathway, but it can still influence kisspeptin neuronal activity through direct fibre contact as GnIH receptors that are expressed in kisspeptin neurons. GnIH, on the other hand, effectively inhibits on the adenylate cyclase (AC)/cAMP/protein kinase A (PKA)-dependent pathway to suppress the VIP/Gs-coupled VPAC2-induced pathway. GnIH selectively inhibits the signalling of the G-coupled GnRH receptor in gonadotropes by acting as an inhibitor via the AC/cAMP/PKA pathway (Son et al., 2019).

The expression of GnRH upon treatment with LPS was intriguing in such a way that its levels significantly increased at treatment with low dose of LPS, after which it was downregulated at treatment with medium and high dose. This pattern of gene expression was complemented by that of GnIH upon treatment with LPS. GnIH is one of the main inhibitors of GnRH, and this was reflected in this study. Where levels of GnRH3 was upregulated (i.e., at treatment with 1 ug/uL of LPS), GnIH was downregulated. Conversely, where levels of GnRH3 was downregulated (i.e., at treatment with 5 and 10 ug/uL of LPS) GnIH was upregulated respectively. Although this trend is complementary between GnRH3 and GnIH, the reason as to why the levels of both these genes were downregulated and upregulated respectively at medium and high dose treatment of LPS is of question. A study was conducted in which the expression of GnRH was measured following induction inflammation via intravenous LPS administration in ewes (Herman and Tomaszewska-Zaremba, 2010). It was found that GnRH and its receptor mRNA levels in the POA and ME decreased Another study conducted on juvenile female pigs found that low dose of LPS (500 ng/kg) from Salmonella enteritidis dysregulated the expression of GnRH peptide. This subclinical dose of LPS caused an increase in GnRH level across the hypothalamus, mammillary bodies, median eminence as well as the ovary, without clinical symptoms (Mikołajczyk and Złotkowska, 2019). This finding is in line with that of this study as GnRH mRNA levels surged at treatment with low dose of LPS, suggesting that even an asymptomatic infection is able to disrupt homeostasis and cause reproductive dysfunctions. This is supported by a study that found the integrity of the HPG axis to be altered even in a less severe immune challenge (Barabás et al., 2018).

A similar trend was observed in the mRNA expression of *kiss2* whereby a significant upregulation was observed when treated with 1 ug/uL of LPS, compared to the control group. Recent studies suggest that kisspeptin is sensitive to inflammation. A study conducted on primary cultures of human fetal hypothalamic cells showed that TNFα reduced the secretion of GnRH through the downregulation of kisspeptin signalling (Iwasa et al., 2014). Another study showed a decrease in *kiss1* mRNA expression in the hypothalamus of female rats upon systemic endotoxin injection with LPS, which subsequently suppressed serum LH levels (Sarchielli et al., 2017). The findings of this study are in line with that of recent data, however, it is not sufficient to conclude the mechanism as to how kisspeptin regulates GnRH secretion or expression in this study. Hence, further research is needed to establish a model demonstrating the mechanisms of how neuroinflammation affects each neuropeptide in zebrafish.

Spexin is a novel hypothalamic neuropeptide that exerts inhibitory effects on reproduction. Upstream kisspeptin and galanin neurons also control GnRH. These hypothalamic neurons may be

directly or indirectly modulated by SPX to regulate reproduction and energy homeostasis. However, research on the exact neurons that SPX targets is currently lacking. In this study, *spx2* was significantly upregulated upon treatment with LPS in a dose-dependent manner. A study was conducted using a model of GnRH neurons; the mHypoA-GnRH/GFP cell line to explore the effects of a high-fat diet on the expression of *spx* and its receptors, inducing neuroinflammation through sodium palmitate and LPS from E. coli (Wang et al., 2020). It was found that the expression of *spx* and its receptors were elevated, which is in line with this study. They also delved into the possible molecular pathways involved in the regulation of spexin in this cell line TLR4 was discovered to play a role in the palmitate-mediated induction of *spx* and *galanin receptor 3* (*Galr3*) mRNA, but not *galanin receptor 2* (*Galr2*). The induction of all three genes was mediated by ERK1/2. Protein kinase c (PKC) was involved in *spx* and *Galr2* mRNA induction, whereas p38 MAPK and JNK were only involved in *Galr3* mRNA induction (Tran et al., 2020, Wang et al., 2020).This indicates that spexin may play an important role in reproduction at a hypothalamic level, leading to reproductive dysfunction that may be caused by obesity or systemic inflammation.

This study also looked into the expression of *smim20*, which encodes for PNX. PNX is currently known to bind and signal via GPR173, but this may not be its only receptor. GPR173 was discovered using a ligand-binding assay and was found to be critical for PNX's effects on GnRH, Kiss1, and normal estrous cycling (McIlwraith and Belsham, 2018). However, it was found that treatment with LPS did not cause any significant change in the expression of *smim20*, suggesting that neuroinflammation does not affect PNX. This is supported by a study that also showed no changes in *pnx* mRNA levels upon treatment with LPS, even though there were changes in neuroinflammatory signalling (Shi et al., 2006). However, a contrasting study found that PNX possesses a regulatory effect in alleviating LPS-induced activation of NLRP3 inflammasome, achieved through the modulation of neuroinflammation (Zeng et al., 2020, Sun et al., 2020). Although PNX is linked to a broad range of function, its regulation remains unclear due to conflicting and negative results (McIlwraith et al., 2021).

These results demonstrate the regulation of the expression of key reproductive neuropeptide genes by various inflammatory markers in the hypothalamus. The involvement of oxidative stress in this process has also been postulated within this study. It is difficult to determine whether these changes are caused by difference in expression of inflammatory markers or the oxidative stress related genes remains to be elucidated at this stage.

5.4 The effects of LPS treatment on the expression of genes within the pituitary gland

In the second phase of this study, we explored whether the dysregulations of these neuropeptides within the hypothalamus would have any effect on other aspects of the HPG axis. *Tnfα* and *il10* expression levels were measured in the pituitary. Due to limited sample, only two inflammatory markers were selected to indicate an inflammatory response in the pituitary. Specifically, *il10* and *tnfa* were selected for the second phase of the study as the expression of these pro- and anti-inflammatory markers were significantly affected by the induction of LPS. Although there was a slight increase in the expression of *il10* and *tnfa*, in the group of zebrafish that was treated with 10 ug/uL of LPS, there was no significant change. These data suggest that systemic inflammation did not stimulate an inflammatory response within the pituitary.

Similarly, the same observation was made for *lhb* and *fsh* in the pituitary. LPS has been shown to affect GnRH and LH secretion by binding directly to Toll-like receptors (TLR2/4) found in both the hypothalamus and pituitary, proving that inflammation disrupts the expression of GnRH in the hypothalamus (Haziak et al., 2014, Haziak et al., 2018). This finding was reflected in the current study for GnRH, however the gene expression of *lhb* remains unchanged upon treatment with LPS. A possible reason for this can be explained by research that showed IL-1β to be a potent inhibitor of the GnRH-LH system, whereas TNFα was less effective in doing so, and the involvement of IL-6 was deemed irrelevant (Watanobe and Hayakawa, 2003, Rivier and Wylie, 1990). Since there was no significant change in the expression of *il1b* in this study, it can be postulated that the expression of *gnrh3* in this study is not regulated by this specific pro-inflammatory cytokine, hence the expression of *lhb* remains unaffected. As for FSH, one of the many regulators of the gene expression of *fshb* includes the pulsatile frequency of GnRH (Dalkin et al., 2001). One of the pathways involved in this includes the MAPK signalling pathway, whereby an activation in this leads to stimulation of ERK 1/2 (Liu et al., 2002). As this study found no significant increase in the expression of *mapk*, which encodes for ERK2, upon treatment with 10 ug/uL of LPS compared to the control, it is possible that this could be the reason why *lhb* levels remain unchanged.

5.5 The effects of LPS treatment on sexual activity of zebrafish

It was also of interest to observe whether the sexual activity of the zebrafish would be affected by the induction of inflammation. Sexuality scores showed no significant difference in the sexual activity of zebrafish treated with 10 ug/uL of LPS compared to the control. This suggests that

neuroinflammation did not impact the sexual activity of the fish according to the current study. To date, there are no studies looking into the effects of neuroinflammation on sexual activity, which sets a good basis for future studies to further investigate the possible effects. Sexual behaviour is generally controlled by circulating concentrations of gonadal steroid hormones such as androgens (testosterone), estrogens (oestradiol) and progesterone. Examining the levels of these hormones would provide a better explanation on the effects that neuroinflammation has on sexual behaviour. Additionally, GnRH2 is the variant of GnRH that controls sexual behaviour in zebrafish. Hence, it would be valuable to observe the changes in it its mRNA expression in the midbrain upon treatment with LPS.

6 Conclusions

This study serves as a preliminary study establishing the interplay between neuroinflammation, oxidative stress and key reproductive neuropeptides of the hypothalamus. Neuroinflammation was induced by systemic administration of LPS as the expression of crucial pro- and anti-inflammatory markers were observed to be upregulated along with significant changes in the expression of genes involved in the MAPK pathway were also observed upon induction of inflammation. Oxidative stress may play a role in these changes due to significant upregulation of Nrf2. These changes may collectively lead to the dysregulation of the gene expression of several key reproductive neuropeptides including *gnrh3*, *gnih*, *kiss2* and *spx2*. These findings are summarised in figure 14.

The induction of systemic inflammation did not stimulate an inflammatory response in the pituitary. The gene expression of reproductive axis related genes in the pituitary, including *lhb* and *fshb*, were unaffected by the dysregulation of the regulatory neuropeptides as a result of inflammatory response within the hypothalamus. Furthermore, sexual scores showed no significant change in sexual activity of zebrafish that have been treated with LPS.

Phase 1: Gene expression in the hypothalamus relative to untreated zebrafish

LPS treated adult male zebrafish	Inflammatory markers	Reproductive neuropeptides	Oxidative stress related genes
1 ug/uL	↑ *tlr4* ↑ *tnfb* ↑ *tnfa*	↑ *gnrh3* ↑ *kiss2*	↓ *mapk8b* ↓ *mapk14b*
5 ug/uL	↑ *tnfb* ↑ *il10* ↑ *tlr4*	↑ *gnih*	↑ *mapk1* ↑ *mapk6* ↑ *mapk14a* ↓ *mapk8b* ↓ *mapk14b* ↑ *nfe2l2a*
10 ug/uL	↑ *tlr4* ↑ *il6* ↑ *tnfa* ↑ *il10* ↑ *tnfb*	↑ *gnih* ↑ *spx2*	↓ *mapk8b* ↑ *mapk14a* ↓ *mapk14b*

Phase 2: - *Gene expression levels of inflammatory markers in the pituitary as well as reproductive genes did not show a significant change upon treatment with 10 ug/uL of LPS*
- Sexual activity study also showed no difference in sexuality score upon treatment with LPS

Figure 14 summarises the findings of this study. The following oxidative stress related genes encodes for their respective proteins: *mapk1*:ERK2, *mapk6*:ERK3, *mapk8b*: JNK1, *mapk14a*:p38a, *mapk14b*:p38b, *nfe2l2a*;Nrf2.

7 Future Directions

Although this study identified the changes caused by the induction of inflammation in the hypothalamus, pituitary and in reproductive behaviour, the main limitation is that a conclusion on the causal effect of changes in each parameter can be drawn. This study sets a basis for future work by establishing the interplay between neuroinflammation, oxidative stress and reproductive neuropeptides. There are several ways in which this research can be expanded to provide more fruitful data.

One of the ways this study can be improved is if the strain of zebrafish was uniformed. However, due to the movement control order during the pandemic, the supply for zebrafish was halted, hence why unknown strains bought from local pet stores were used in this study.

A possible way to determine the causal relationship of each parameter is by treating the zebrafishwith antagonists of each parameter (i.e., the inflammatory or oxidative stress pathway). With this, it can be concluded whether the inflammatory response or the oxidative stress is causing the dysregulation in the reproductive neuropeptides of interest. Furthermore, the ratio of reduced/oxidised glutathione (GSH:GSSG) could be measured to provide more reliable data on the redox state of the hypothalamus.

In regard to LH and FSH, measurement of their serum concentration would provide a clearer understanding whether the key reproductive neuropeptides' dysregulation affects these hormones on a protein level. In addition to this, it should be of interest to also look into other hormones regulating LH and FSH such as testosterone, oestradiol and progesterone levels in the gonads, as they are one of the main regulators of their expression.

A more thorough behaviour study could also be conducted to draw solid conclusions on whether the changes hormone levels affect its mating behaviour as judging solely based on sexual score does not provide a fair representation of the fish’s desire to mate. The ways in which their behaviours were characterised (i.e., fast swimming, proximity to female zebrafishand the number of times the zebrafishcomes in contact with the screen) is not limited to just the portrayal of sexual activity as similar behaviour can also be characterised for aggression. Hence, in addition to the sexual behaviour study, the mating rate of zebrafishtreated with LPS should be compared to the control. With this, the number of zebrafishused in each behaviour study could also be increased to improve reliability.

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