

STUDIES ON THE ROLE OF FORMYL PEPTIDE RECEPTORS IN
PITUITARY AND ADRENAL FUNCTION.

By

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Declaration of Originality

I hereby declare that all work submitted within this thesis is my own, and where I have received help or collaborated, I have referenced and acknowledged this accordingly.

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Abstract

The mechanism by which glucocorticoids (GCs) achieve negative feedback on the release of hypothalamo-pituitary-adrenal (HPA) axis hormones has long been under investigation. Annexin A1 (ANXA1) is a key mediator of the effects of GCs within the neuroendocrine and immune systems. Previous research suggested the negative feedback effect of the GC-ANXA1 system on the pituitary release of adrenocorticotrophic hormone (ACTH) was mediated via a member of the formyl peptide receptor (Fpr in rodents, FPR in humans) family. The present work tested the hypothesis that specific Fpr/FPR subtypes modulate both pituitary and adrenal function in terms of stress hormone (ACTH and corticosterone) secretion (following exposure to secretagogues CRH and ACTH or the inflammogen LPS in rats), and their tissue distribution (mice and rats) will itself be upregulated following inflammogenic stress (LPS in mice and rats, multiple sclerosis in human tissue). This was achieved through 1) immunohistochemical detection of FPR1, FPR2/ALX, and FPR3 in human anterior pituitary sections, and of green fluorescent protein (GFP) in HPA axis tissues from Fpr2/3^{-/-}, GFP^{+/+} mice; 2) measurement of basal and stimulated ACTH and GC release from dispersed rat anterior pituitary and adrenal cells, incubated with agonists and antagonists for FPR1, FPR2/ALX and FPR3, namely, formyl-Met-Leu-Phe (fMLF), ANXA1 amino-acetylated peptide (ANXA1_{Ac2-26}), Trp-Lys-Tyr-Met-Val-*D*-met (WKYMVm), haem-binding protein fragment (F2L), aspirin-triggered lipoxin A₄ (15-epi-LXA₄), and Trp-Arg-Trp-Trp-Trp-Trp (WRW₄). These ligands were found to elicit a range of modulatory, concentration-dependent effects on pituitary ACTH and adrenal GC secretion. Evidence was found for the spatial distribution of: FPR3 and FPR2/ALX within the human anterior pituitary, and GFP (as a surrogate for Fpr2/3) colocalised with ACTH, growth hormone (GH), and prolactin (PRL) within the Fpr2/3^{-/-}, GFP^{+/+} mouse pituitary. The findings contribute evidence in support of a role of Fpr/FPR subtypes and their ligands as modulators of pituitary and adrenal hormone release.

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Chapter One: **Introduction**

1.1 Background

In 1929, Walter Cannon coined homeostasis as a term for the “coordinated physiological reactions which maintain most of the steady states in the body” (Cannon 1929) that were first observed and precisely described by Claude Bernard in 1878. Cannon also described the acute effects of stress on physiological systems, noting they were similar to injections of the adrenal gland hormone adrenin (epinephrine), under what is now commonly known as the ‘fight-or-flight response’ (Cannon 1914; 1915). Stress as a biological concept, however, was first described by Hans Selye as “a non-specific response to a variety of threatening situations” (Selye 1936). After four decades of researching the physiological effects of stress, he updated the nomenclature thusly:

“Stress is the nonspecific response of the body to any demand. A stressor is an agent that produces stress at any time. The general adaptation syndrome (GAS) represents the chronologic development of the response to stressors when their action is prolonged. It consists of three phases: the alarm reaction, the stage of resistance and the stage of exhaustion” (Selye 1976).

Since then, scientific research of stress has been prolific, and the term is in common parlance. Despite this, a disparity between high public awareness and poor biological definitions of stress, is being exploited by an industry offering pseudo-scientific diagnoses and treatments worth 18 billion dollars per annum in the USA alone (Patmore 2006; Weissmann 2007). Apart from these controversies, there is peer-reviewed evidence to indicate stress can increase the risk of numerous health problems. Sepsis, for instance, is caused by bacterial cells, toxins or other components that enter the blood or other tissues distal to an infection site and if poorly managed can result in multiple organ failure and death. Stressors such as surgery, blood loss, and unhealed wounds increase both the risk and exacerbate the development of sepsis. This is thought to be due to bacterial proliferation that is unchecked by the suppressed immune system that typically follows stress. Similar risks are evident in patients receiving immunosuppressive agents such as anti-rejection drugs or analogues of steroids released during stress. Interestingly, increased doses of steroid therapy are required to counter inflammation and tissue damage that can occur during severe stress. Invasive surgery, for instance, is a major stressor and approximately 1 in 5 cases (19.5 per cent) of sepsis following operations in US hospitals between 1998-2006 resulted in death, versus 1 in 10 deaths (11.7 per cent) for sepsis without surgery (Eber *et al.* 2010). Sepsis adds 8.1 billion dollars in

added hospitalisation costs per annum, and these costs are likely to rise if challenges of antibiotic resistance, natural population increases, and clinical management of patients under severe stress remain unaddressed. This underscores the importance of further research to improve our understanding of stress. For the purposes of understanding the present work, however, the terms stress, stressor, and stress response, will have distinct meanings.

Stress (verb) is any challenge beyond an organism's current capacity to coordinate the physiological reactions that maintain steady states in the body through homeostasis.

A stressor (noun) is any cue that can cause stress.

A stress response (noun) is an engagement of adaptive systems following any challenge beyond an organism's current homeostatic capacity.

An organism that is under stress (stressed) has been exposed to a stressor and is being challenged, either by an on-going stressor (e.g. predation), or by the effects of a preceding stressor (e.g. blood loss). Stress, therefore, is a problem that cannot be solved by inertia—whether physiological or behavioural—and is most severe when the problem is an urgent matter of survival. The first description of stress by Selye, however, was based on observations of a given stressor applied to a largely homogenous population of rats in a controlled laboratory environment (Selye 1936). From these experiments, Selye found that stress followed three stages: alarm, resistance, and exhaustion. The early, alarm stage was characterised by adrenal hypertrophy but with depleted carbon skeletons for cortical steroid and medullary catecholamine synthesis; hepatic and lymphoid atrophy; thymic, pleural and peritoneal oedema, and gastrointestinal ulceration. The stage of resistance included: adrenal repletion of cortical lipids, reduced oedema, upregulated pituitary basophilic cells, thyroid hyperplasia, curbed body growth, gonadal atrophy and cessation of lactation (Selye 1936). Finally, unresolved stress leads to the stage of exhaustion and ultimately, death. These observations seemed to indicate stress might somehow induce a response that prioritises the mobilisation of energy stores over functions that are not of immediate importance for survival.

Stressors are varied in terms of type (tangible or intangible), origin (external or internal) and scale (magnitude and duration). These include, but are not limited to tangible cues, such as fighting, sexual

assault, or pathogens; or intangible cues, such as negative thoughts and social subordination. Since stressors are innumerable in type, origin, and scale, then there are many ways in which stress can be induced. The specificity of a response to a given stressor can also depend on numerous factors described later (Section 1.2.5).

An appropriate stress response is timely and of a scale commensurate with the level of threat. The hypothalamo-pituitary-adrenal (HPA) axis is a critical, self-regulating, neuroendocrine system allowing vertebrates to respond to stress through i) enabling adaptation to survive the changes imposed by stress, and ii) restoration and regulation of homeostasis once stress is over. The timely release of stress hormones from the HPA axis enables adaptive responses to stress and also regulates a wide range of homeostatic functions, not limited to growth, metabolism, and reproduction.

Conversely, an inappropriate stress response might be asynchronous (early or late) or disproportionate to the level of threat. If an inappropriate stress response continues, this may result in acute or chronic tissue damage, and might even contribute to the aetiology of autoimmune diseases such as multiple sclerosis (MS) (Mason *et al.* 1990; Gold *et al.* 2012), or provide immunosuppressive conditions conducive for the proliferation of pathogens and development of infections and sepsis, for instance after traumatic brain injury (Ayala *et al.* 1994; O'Sullivan *et al.* 1995). In these conditions, the exogenous administration of synthetic stress hormones such as hydrocortisone, is associated with an increase in the production of anti-inflammatory cytokines and amelioration of some symptoms (van der Poll *et al.* 1996; Gayo *et al.* 1998). The evidence indicates the timing and concentration of stress hormones is influential in the aetiology and recovery from inflammatory stress, but our understanding of this relationship is still complicated and necessitates further research in order to simplify and find safe, efficacious drugs with fewer side effects than hydrocortisone (Schäcke *et al.* 2002).

Having described the basic nomenclature, the following sections will describe: the physiology of the HPA axis in terms of its structure, function, and regulation in humans and rodents; how the stress response differs depending on the source of stress; the self-regulatory nature of the HPA axis and its role in host defence, and the molecular mediators of feedback regulation.

1.2 Anatomy and physiology of the HPA axis

After a brief overview of the axis, this section focuses mainly on the key effectors of HPA axis functions, namely, the glucocorticoids (GCs), and describes their effects, modes of action, biosynthesis in the adrenal cortex, and the role of adrenocorticotrophic hormone (ACTH) in their regulated release. The focus then moves to the neurohumoral mechanism of controlling pituitary ACTH secretion involving the hypothalamic hormones arginine vasopressin (AVP) and corticotrophin-releasing hormone (CRH), and their roles during stress and quiescence. Finally, an outline of the negative feedback system illustrates how GCs regulate HPA axis activity and their own release.

1.2.1 Overview of the HPA axis

The HPA axis is a major system of neuroendocrine interactions between the hypothalamus, pituitary, and adrenal glands, whose hormones regulate many homeostatic processes as well as the stress response. Hypothalamic CRH and AVP synergistically stimulate the anterior pituitary to secrete ACTH that stimulates the adrenal cortex to produce and release GCs (Gillies *et al.* 1982). The GCs have widespread, tissue-specific effects that enable homeostatic mechanisms during quiescence and periods of stress, after which they effectively turn off their own release via feedback inhibition of pituitary ACTH secretion, and hypothalamic CRH and AVP secretion.

The hypothalamus, extending sagittally from the optic chiasm to the mammillary bodies, comprises four major zones (pre-optic, anterior, middle, and posterior) and is situated above the third cerebral ventricle. Most hypothalamic cells have specialised neural and secretory activity, and are either organised in nuclei or scattered within the tissue. Distinct nuclei and neuronal sub-populations are also organised into systems with separate functions. The magnocellular neurons (>30 μm diameter) are situated in the paraventricular (PVN) and supraoptic (SON) nuclei where they synthesise oxytocin and AVP to be released from axonal projections at fenestrated capillary beds within the posterior pituitary gland (Stopa *et al.* 1993). Release of oxytocin is triggered by neural reflexes activated during lactation and parturition, whereas osmotic stimuli trigger magnocellular AVP release where it functions mainly as an antidiuretic hormone via renal V2 receptors (Manning *et al.* 2008). Parvocellular neurons (< 10 μm diameter) also produce AVP, along with CRH, but this AVP mainly modulates HPA axis function and does so in

synergy with CRH (Gillies *et al.* 1982). In fact, the release of the six hormones of the anterior pituitary gland is controlled by different parvocellular neurosecretory neurons in the hypothalamus. The medial parvocellular region of the hypothalamic paraventricular nucleus (PVNmp) contains neurosecretory neurons that regulate ACTH secretion from the anterior pituitary gland. On each side of the PVNmp in rats, there are around 2000 CRH neurons with fusiform bodies and bipolar dendrites, and axons terminating in the median eminence. Parvocellular AVP is synthesised and packaged into large dense core vesicles (LDCVs) with CRH in approximately half of these neurons, particularly in the dorsal PVNmp (Swanson *et al.* 1983; Whitnall *et al.* 1985). The rate of synthesis of parvocellular peptides is typically related to the rate of secretion, but also depends on feedback signals. Parvocellular CRH and AVP synthesis and release are sensitive to feedback inhibition by GCs although to different extents. Increased release of CRH and AVP—following adrenalectomy and loss of GC feedback, for instance—is related to an increased expression of CRH and AVP genes. Synthesis of these peptides follows gene transcription and translation events before processing into LDCVs for axonal transport (6-8 mm/h) to neurosecretory terminals in the median eminence. Neurosecretion of CRH and AVP is unidirectional and dependent on depolarisation and Ca^{2+} influx induced by action potentials generated in the soma. The Ca^{2+} induces membrane docking of LDCVs and exocytosis of their contents into the hypophyseal portal vasculature, that perfuses the cells of the anterior pituitary with hypothalamic hormones (Jacobsson & Meister 1996).

The hypophyseal portal system services the whole pituitary gland for vascular communication between the endocrine and nervous systems (Popa & Fielding 1930; Harris 1948). A direct link from the hypothalamus to the anterior lobe, for instance, begins with the internal carotid arteries extending into three pairs of hypophyseal arteries. The superior hypophyseal arteries enter the median eminence, breaking into a plexus surrounding hypothalamic nerve endings, and then long portal veins down the pars tuberalis into the capillaries of the anterior lobe. The middle and inferior hypophyseal arteries supply the posterior lobe but also have short portal vessels to the anterior lobe. This allows the cells of the anterior lobe to respond to their respective parvocellular hormones and other mediators to regulate the secretion of hormones into systemic circulation, whereupon they have access to target tissues, including the adrenal glands.

The pituitary gland sits in the sella turcica of the sphenoid bone beneath the hypothalamus. It comprises two embryologically distinct lobes: the epithelial anterior lobe, and the neuro-glial posterior lobe. The anterior lobe can be further sub-divided in three: pars distalis, pars intermedia, and pars tuberalis. The pars distalis and intermedia contain numerous endocrine cell types in proportions (percentage of total) that vary slightly between species and are distinguished by the types of hormones they secrete. In humans, the largest proportion is made up of the large ovoid somatotrophs (growth hormone, GH; 50 per cent), then lactotrophs (prolactin, PRL; 25 per cent), corticotrophs (ACTH; 20 per cent), gonadotrophs (follicle-stimulating hormone (FSH) and luteinising hormone (LH); 10 per cent), and thyrotrophs (thyroid-stimulating hormone (TSH); less than 5 per cent) (Stevens & Lowe 2011).

The adrenal glands are located above the kidneys and comprise two distinct systems for hormone biosynthesis and secretion: i) the neuroendocrine adrenal medulla for catecholamines, and ii) the adrenal cortex for steroid hormones and their precursors, with several relevant to HPA axis function. Enclosed between the fibrous external capsule and the adrenal medulla, the adrenal cortex contains three histologically and functionally distinct zones, namely, the zona glomerulosa (ZG), the zona fasciculata (ZF), and zona reticularis (ZR). The sub-capsular ZG is a thin, unevenly distributed layer of cells that secrete mineralocorticoids (mainly aldosterone and deoxycorticosterone). The ZF is the largest cortical layer and contains rectangular, vacuolated cells with lipid-rich cytoplasm and is the main site of GC production, and secretion in response to ACTH. The ZR is the second largest cortical layer with eosinophilic cells containing the pale-brown pigment lipofuscin, in columnar arrangement around capillaries, and is the main androgenic site secreting dehydroepiandrosterone (DHEA). Conversely, the chromaffin cells of the adrenal medulla contain the catecholamines, epinephrine, norepinephrine, and dopamine, as well as the monoamine, serotonin (5-hydroxytryptophan; 5-HT) (Holzwarth & Brownfield 1985). The adrenal gland thus produces numerous, and chemically diverse hormones, but most of the effects of the HPA axis, however, are achieved through the widespread, tissue-specific actions of its chief end products—the GCs.

1.2.2 Role of glucocorticoids

GCs are steroid hormones common to all vertebrate species. In mammals, the main site of GC synthesis is the adrenal cortex. Adrenal steroidogenesis is a biosynthetic reaction series, and starts with the rate-

limiting step of converting cholesterol to pregnenolone. Thirty or more GCs have been isolated from the mammalian cortex, but (in most mammalian species) many of these are not released into the circulation as they are intermediaries for the production of the main endogenous GCs—cortisol and corticosterone. The ratio in which these are released over 24 hours varies between mammalian species. Whereas cortisol predominates corticosterone 3:1 in humans, the inverse applies to rodents (1:2) (van der Vies 1961; Nishida *et al.* 1977).

The GCs have wide ranging effects on the body, and give rise to net catabolic effects by: stimulating gluconeogenesis; decreasing cellular concentrations of glucose and protein (except in the liver); mobilising fatty acids, glucose, and amino acids. The GCs also have profound anti-inflammatory effects that will be described later (Section 1.2.2.2), but it is helpful to first look at the common mechanisms of GC actions.

1.2.2.1 Molecular mechanisms of GC action

As steroid hormones, GCs exhibit molecular mechanisms that are similar to the classical steroid hormone receptor pathway, but they also have other mechanisms that are perhaps unique to GCs. The next subsection will describe the actions of GCs in terms of i) differences in the structure, expression, transcriptional regulation, activation by ligands, and modes of action of their receptor subtypes; ii) their pre-receptor metabolism; and iii) genomic and non-genomic mechanisms and target proteins.

1.2.2.1.1 Glucocorticoid receptors

Receptors for GCs can be one of two known major subtypes: mineralocorticoid receptors (MRs), and glucocorticoid receptors (GRs) (de Kloet *et al.* 1998). Both MRs and GRs are members of the ligand-regulated nuclear receptor family. The MR has a high and similar affinity (K_d 0.5–2 nmol.l⁻¹) for cortisol, corticosterone, and the mineralocorticoid, aldosterone (Mogilewsky & Raynaud 1980). It is expressed in certain tissues including those that depend on aldosterone to regulate Na⁺/K⁺ balance, namely, in the distal renal tubule, sweat glands, parotid, colon, but also the limbic, entorhino-cortical, and hypothalamic regions of the brain. The GR has ten-fold lower affinity (K_d 10-20 nmol.l⁻¹) for GCs, but is GC-selective with negligible aldosterone affinity. Unlike MRs, GRs are expressed ubiquitously, and mediate the majority of GC actions *in vivo*. The GR structure comprises functionally specialised domains for ligand

binding, transactivation, and DNA binding (Giguère *et al.* 1986). Constitutive alternative splicing of GR mRNA results in two receptor isoforms—GR α and GR β . GR β has a low affinity for GCs, and can act as a dominant repressor over GR α activation (Hollenberg *et al.* 1985; Oakley *et al.* 1996). In addition to the differential affinities above, ligand binding also depends on pre-receptor metabolism.

1.2.2.1.2 Pre-receptor metabolism of GCs

Pre-receptor metabolism of GCs by the 11 β -hydroxysteroid dehydrogenase isozymes (11 β -HSD1 and 11 β -HSD2) governs the availability of active GCs for binding to GRs and MRs (Funder *et al.* 1988). The distribution of these isozymes is tissue-specific. 11 β -HSD2 is evident mainly where mineralocorticoid effects predominate (kidney, colon) and inactivates cellular GCs through conversion of cortisol to cortisone, thus freeing up the MRs for aldosterone (White *et al.* 1997). The effects of the 11 β -HSD isozymes on GC concentration and activity are local, and have no effect on circulating GC concentrations (Kershaw *et al.* 2005). The 11 β -HSD1 isozyme is particularly abundant within the endoplasmic reticular lumens of hepatic and adipose tissues. As an oxidoreductase, 11 β -HSD1 catalyses opposing reactions that are each limited by co-factor availability (Agarwal *et al.* 1990); however, reductase activity predominates in most tissues *in vivo* given adequate production of nicotinamide adenine dinucleotide phosphate (NADPH) by hexose 6-phosphate dehydrogenase (H6PDH) (Bujalska *et al.* 2005; Lavery *et al.* 2006). Interestingly, 11 β -HSD1 appears to be essential for the target tissue conversion of orally administered cortisone and prednisone to their active forms—cortisol and prednisolone, respectively (Stewart *et al.* 1999; Rask *et al.* 2001).

1.2.2.1.3 Genomic mechanisms

Unbound GRs reside in the cytosol in association with heat shock proteins (hsp90 and hsp70) (Beato *et al.* 1996). Ligand binding to the GR induces conformational change, and the resulting dissociation permits the translocation of GR via nuclear pores. The DNA binding domain interacts with specific GC response elements (GREs) to modulate the transcription of GC-responsive genes through transactivation or transrepression. The classical mechanism of transactivation is well understood, and requires the formation of GR homodimers about a binding domain, specifically, the second zinc finger motif (Beato *et al.* 1989). Although the mechanism is less clear, some have posited that transrepression could occur via

negative GREs (nGREs) and so might explain the negative feedback action of GCs on the proopiomelanocortin (POMC) promoter and its proteolysed products, including ACTH (Charron & Drouin 1986). Upon binding of GR to specific GREs, further modulation of transcription is afforded by the recruitment of corepressor and coactivator molecules, many of which have native histone acetyltransferase actions that unwind and accelerate the transcription of downstream DNA. Such transcription factors include cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), cAMP binding protein (CBP), and p300. In particular, CBP can also recruit activator protein-1 (AP-1), nuclear factor kappa B (NF- κ B), and members of the signal transducer and activator of transcription (STAT) family. AP-1 is a Fos and Jun oncoprotein heterodimer that (as well as interacting with GR) exerts regulation of inflammatory processes via its own response element. NF- κ B is a key regulator of inflammation, and often amplifies pro-inflammatory pathways when not interacting with GR in the cytosol (De Bosscher *et al.* 2003) and will be discussed in more detail later (Section 1.5). Other GR coactivators include steroid receptor coactivator 1 (SRC-1), transcription factor intermediary factor 2 (TIF-2), GR-interacting protein 1 (GRIP-1), and p300/CBP co-integrator-associated protein (p/CIP). The inflammatory activity of many of these factors is limited in part by their interaction with GR. Conversely, transcriptional corepressors have native histone deacetylase activity and some (e.g. RAP46) are able to limit GC-induced, GR-mediated transactivation by binding to the GR hinge region (Kullmann *et al.* 1998; Kivimäki *et al.* 2002). The stoichiometry of these interactions with the GR before the transcription of inflammatory or anti-inflammatory genes is a determinant of net cellular inflammation.

1.2.2.1.4 Non-genomic mechanisms

The non-genomic actions of GCs are more rapid in relation to those that require transcription of genes. With regard to the immunosuppressive and anti-inflammatory effects of GCs, there are at least three hypotheses for how the rapid actions occur via i) GC-induced changes in membrane structure, ii) membrane-bound GRs (mGRs), and iii) cytoplasmic GRs.

First, there is some evidence that high concentrations of GCs can intercalate with membrane phospholipids and proteins and reduce Na⁺-K⁺-ATPase and Ca²⁺-ATPase activity. In activated immune cells such as lymphocytes, a reduction in energy-dependent inflammatory pathways is evident shortly after exposure to high GCs, and is thought to occur to conserve energy (Schmid *et al.* 2000).

Secondly, evidence of membrane-bound GRs (mGRs) came first in cells from amphibians, and were later found to be upregulated in human peripheral blood mononuclear cells (PBMCs) stimulated with lipopolysaccharide (LPS) *in vitro* or isolated from rheumatoid arthritis sites (Bartholome *et al.* 2004).

Third, the rapid effects of GCs on phospholipase A2 (Croxtall *et al.* 2002) and nitric oxide synthase release (Limbouurg *et al.* 2002) have—because they are blocked by GR antagonists but not transcriptional inhibitors—been suggested to occur via cytoplasmic GRs without nuclear translocation (Limbouurg & Liao 2003).

These hypothetical mechanisms have been bolstered to different extents by evidence over the years, but can be considered speculative, as they are not predictive of the major anti-inflammatory effects of GCs. An additional non-genomic mechanism of GC action, however, involves the mediation of GC effects via GC-induced proteins, and, given the substantial body of evidence, this deserves its own section (see Section 1.4).

1.2.2.2 Cellular mechanisms of GC action

The following subsection describes the mechanisms through which GCs regulate cellular function, with special reference to the discovery of their anti-inflammatory effects that paved the way for understanding their cellular actions via GC-inducible proteins and interactions with other mediators.

1.2.2.2.1 Reconciling anti-inflammatory effects with molecular mechanisms

The GCs are renowned for their anti-inflammatory and immunosuppressive effects, and have been for decades. Despite this, the use of GCs is not without risks or side effects, and questions regarding their precise mode of action are still a fertile area of research. Before looking at the details and remaining questions for elucidating these anti-inflammatory mechanisms, it is worthwhile to consider the key milestones of research achievements to date.

The importance of GCs to human survival was established by Dunlop's review of Addison's disease patient outcomes between 1928 and 1962 (Dunlop 1963), noting the landmark availability of cortisone in 1948 (Hench *et al.* 1949b). Prior to this point ('before cortisone'), patient life expectancy and survival rates post-diagnoses were mediocre for those receiving mineralocorticoids (2 - 5 years), but prior to 1939

(‘before corticosteroids’), the prognoses were abysmal (median 1 year; maximum 2 years). By 1930, however, it became clear that adrenalectomised animals were much more susceptible to stressors in general (e.g. infectious, psychological) whereupon the adrenal cortex in intact animals responds with two classes of steroid, namely, mineralocorticoids and glucocorticoids (Hartman & Brownell 1930; Rowntree *et al.* 1930; Swingle & Pfiffner 1930). These terms were coined by Hans Selye in observation of their principal actions on salt and carbohydrate metabolism respectively (Selye 1946). Further investigation, however, ascertained that of the two steroids, the general stress-resistance actions were ascribed to the GCs (Hench *et al.* 1949a).

The mode of action and effects of GCs have been under continuous research since cortisone was first used therapeutically in 1948 (Hench *et al.* 1949a). Early hypotheses that cortisone had little affinity for GC receptors and conversion to cortisol was necessary for its GC function in patients were vindicated empirically. Not all of these early hypotheses were to be as propitious, however. Nobody researching GCs in the 1940s had predicted their suppressive or anti-inflammatory effects and some, hypothesised quite the opposite. It was thought Selye’s syndrome could lead to ‘diseases of adaption’ such as rheumatoid arthritis, when excessive ACTH and GCs were released (Selye 1946). Kendall recited Selye’s hypothesis that “administration of ACTH would not relieve the symptoms of rheumatoid arthritis; rather, it would cause an exacerbation of symptoms” (Kendall 1971). It came as a surprise, therefore, when high doses of either ACTH or GCs in fact led to the relief of rheumatoid arthritis (Hench *et al.* 1949a; Kendall 1950). These effects were charged as being merely pharmacological phenomena, partly because of the high doses required, but also because they suppressed inflammation—with the premise that inflammation is a defence mechanism—and so were seemingly at odds with evidence that GCs conferred stress-resistance (Sayers 1950; Baxter 1976).

The charge was countered by arguments based on emerging knowledge of GC receptor expression and function. Indeed, shortly after their discovery (Munck & Brinck-Johnsen 1968; Schaumburg & Bojesen 1968), evidence emerged that GC receptors are common in both their expression within virtually all nucleated cell types, and in their basic molecular mechanism for effecting GC actions (Baxter 1976; Munck & Leung 1977). Further experiments demonstrated the anti-inflammatory actions were steroid-specific, concentration-dependent, and blocked by protein and RNA synthesis inhibitors or GC receptor

antagonists, and were therefore consistent with other (less contentious) physiological actions via the GR (Danon & Assouline 1978; Russo-Marie *et al.* 1979; Hirata *et al.* 1980). As with other amphipathic steroids— β -methasone (Lewis & Piper 1978), for instance—the non-specific effects of GCs were found to be antecedent (Homo *et al.* 1980) and inverse to receptor binding (Munck & Brinck-Johnsen 1968), and so could be attributed to membrane binding (Munck 1976; Fahey *et al.* 1981).

There was no difference in the primary mode of action between GCs, whether exogenous or endogenous, or stimulated or basal (Tsurufuji *et al.* 1979; 1980). On this basis, it was argued that there was: first, a common mechanism for GC actions whether anti-inflammatory or stress-resistant; and second, therefore, no empirical basis for labelling the anti-inflammatory effects as being pharmacological (Fahey *et al.* 1981; Guyre *et al.* 1982).

Once the false premises of the actions of GCs being pharmacological and pro-inflammatory had been countered, it became necessary to approach further research questions under a new hypothesis. Alan Munck and colleagues proposed that:

“(1) the physiological function of stress-induced increases in glucocorticoid levels is to protect not against the source of stress itself, but against the normal defence reactions that are activated by stress; and (2) the glucocorticoids accomplish this function by turning off those defence reactions, thus preventing them from overshooting and themselves threatening homeostasis” (Munck *et al.* 1984).

This was substantiated by the observation that most effects of GCs were either suppressive by direct action on target cells (Araki *et al.* 1981; Rabbani *et al.* 1981), or they induced enzymes and other mediators which brought about similar effects. The mediation of the effects of GCs, and their regulatory actions on a known network of intercellular mediators (Fahey *et al.* 1981; Guyre *et al.* 1982), was central to this new hypothesis.

1.2.2.2.2 Glucocorticoid mediators and inducible proteins

From the inception of Munck’s hypothesis, there was already much evidence that GCs inhibited the production, and often the actions, of numerous intercellular mediators, namely: (1) phospholipase A2 and the production of arachidonic acid (Ney *et al.* 1967; Rabbani *et al.* 1981) and its metabolites

(prostaglandins, thromboxanes, and leukotrienes) (Gemzell *et al.* 1951); (2) signalling proteins and peptides such as bradykinin and cytokines such as IL-1 and IFN- γ that stimulate Fc-receptor augmenting factor (FRAF), macrophage activating factor (MAF), natural killer (NK) cells, and colony stimulating factor (CSF) (Newcombe *et al.* 1977); (3) neuroendocrine hormones such as ACTH (Guillemin *et al.* 1977), CRH (Vale *et al.* 1981), AVP, and POMC (Roberts *et al.* 1982) and its cleavage products such as the opioid β -endorphin (Guyre *et al.* 1982); (4) neurotransmitters such as serotonin and histamine (Gryglewski *et al.* 1975); and (5) neutral proteases such as plasminogen activator and collagenase (Seifert 1978; Crutchley *et al.* 1981).

Overall, these mediators shared several features in that they were: regulated by GCs in a common mechanism; well characterised with clear roles in host defence, vascular permeability, coagulation, and oedema; and secreted in response to stress. On this basis, then the GCs were viewed as suppressors of defensive or restorative inflammatory mechanisms.

1.2.3 Regulation of glucocorticoid release

This subsection focuses on the role of ACTH in the regulation of GC release, but begins with a primer on the biosynthesis of GCs and other steroids in the adrenal gland, and how these processes are regulated by local factors. The circadian and ultradian rhythms of GC secretion are summarised before the hypothalamic control of ACTH secretion.

1.2.3.1 Glucocorticoid biosynthesis

As with all other steroids, the precursor of GCs is cholesterol. The majority (90% (Plump *et al.* 1996)) of adrenal cholesterol is selectively transported into the adrenal cortex upon binding of high-density lipoproteins (HDL) (Gwynne *et al.* 1976) to adrenocortical scavenger receptor BI (SR-BI) (Rigotti *et al.* 1996; Wang *et al.* 1996; Temel *et al.* 1997), and the remainder is from low density lipoproteins (LDL) or synthesised *de novo*. Esters of cholesterol are stored in lipid droplets until a hydrolase produces free cholesterol for sterol carrier proteins to shuttle cholesterol into the mitochondria. Here, enzymatic side-chain cleavage by cholesterol desmolase of the cytochrome P450 superfamily (P450_{scc}) converts cholesterol to pregnenolone, which is then transported to the smooth endoplasmic reticulum (Constantopoulos & Tchen 1961). As well as subcellular compartmentation, the production of different

classes of steroids is compartmented across the adrenal cortex, with each zone containing steroidogenic enzymes acting at similar carbon positions (Ishimura & Fujita 1997). Within the zona glomerulosa (ZG), some pregnenolone is dehydrogenated forming progesterone. From these two steroids, the final secreted product depends on which cortical zone any further reactions take place. Within the ZG, progesterone is serially converted to 11-deoxycorticosterone, corticosterone, 18-hydroxycorticosterone, and ultimately, aldosterone. Alternatively, some of the pregnenolone and progesterone can be transported to the zona fasciculata (ZF) by the trans-zonal enzyme 17 α -hydroxylase (P450c17; CYP17), producing 17 α -hydroxypregnenolone and 17 α -hydroxyprogesterone, respectively. Staying with the enzymes of the ZF, these products are converted to form the secretable steroids—11-deoxycortisol, and cortisol. Alternatively, the trans-zonal enzyme CYP17 converts them into DHEA, and androstenedione, respectively, within the zona reticularis (ZR) (Pelletier *et al.* 2001).

There are numerous local mechanisms that regulate adrenal steroidogenesis governed by interacting cell types and communication systems, including: the juxtaposition of cortical cells with chromaffin cells of the medulla; adrenal vasculature and endothelial cell products; an intra-adrenal CRH/ACTH system; as well as adrenal immune cells, cytokines, and peptide growth factors. In mammals such as humans (Bornstein *et al.* 1994) and rats (Gallo-Payet *et al.* 1987), whilst being largely confined to the cortex and medulla, the steroidogenic and catecholaminergic cells can also be interspersed within islets or single cells within both compartments, but particularly in the cortical ZG (Palacios & Lafarga 1975). These areas of colocalisation are not separated by extracellular matrices or fluids, and thus provide direct, cortico-chromaffin, paracrine interactions. This is particularly evident between the ZR and the medulla, where splanchnic nerve innervation maintains diurnal variations in GC production (Ottewiller & Meier 1982; Dijkstra *et al.* 1996) and enhanced ACTH sensitivity in response to splanchnic epinephrine (Bornstein *et al.* 1990a; Guse-Behling *et al.* 1992). Interestingly, basal GC secretion in response to ACTH₁₋₂₄ is higher in cultures containing both cortical and chromaffin cells, thus highlighting the importance of such interactions (Haidan 1998). Evidence of other steroidogenic regulators, such as 5-HT, have been found within chromaffin cells of the mouse (Ritzen *et al.* 1965) and rat (Holzwarth & Brownfield 1985), and within mast cells of the human adrenal, where 5-HT stimulates GC production (Lefebvre *et al.* 2001).

The vasculature of the adrenal gland provides a rich source of modulators of GC secretion and release. The adrenal gland is well perfused and receives a high proportion of cardiac output relative to its mass; the rat adrenal gland, for instance, comprises 0.02% of total body mass, yet receives 0.14% of cardiac output (Sapirstein & Goldman 1958). Adrenal blood flow at rest is known to be stimulated by ACTH, and this has been attributed to ACTH-stimulated release of vasoactive histamine and 5-HT from sub-capsular, degranulation of mast cells native to the adrenal (Sapirstein & Goldman 1958). This vasodilatory effect of ACTH can thus be abolished by disodium cromoglycate (mast cell stabiliser) (Hinson *et al.* 1989; 1991a). Studies of whole adrenals from dogs (Frank *et al.* 1955; Urquhart 1965), rats (Urquhart 1965), and calves (Jones *et al.* 1990), have demonstrated a positive relationship between adrenal blood flow and GC secretion that is not dependent on the plasma concentration of ACTH (Sibley *et al.* 1981; Hinson *et al.* 1986), but rather the rate of its presentation to the adrenal (Jones *et al.* 1990). In contrast, these flow-induced changes are absent in collagenase-dispersed cells superfused on a column, which prompted the suggestion that a factor within the vasculature contributes to these changes (Hinson *et al.* 1986). Endothelin-1, for instance, acts via endothelin receptor-B (ETB) to stimulate GC secretion from rat and human adrenals (Hinson *et al.* 1991b; Belloni *et al.* 1996). Similarly, endogenous nitric oxide synthesis is dependent on substrate availability of L-arginine, which, when limited, leads to decreased basal secretion of GCs (ACTH response unaffected) (Cameron & Hinson 1993).

There is evidence of an intra-adrenal CRH-ACTH system that works independently of the central hypothalamo-pituitary system. This is substantiated by morphometric observations and functional data. Immunoreactive ACTH and CRH has been found within discrete adrenal medulla cells from rats (Bagdy *et al.* 1990; Mazzocchi *et al.* 1993; Venihaki *et al.* 1997) and humans (Suda *et al.* 1984), with a similar pattern evident in cattle and dogs (Bruhn *et al.* 1987a, b; Minamino *et al.* 1988). This ACTH and CRH immunoreactivity is particularly abundant at the cortico-medullary junction (Bruhn *et al.* 1987a, b), and is increased in response to splanchnic nerve stimulation (Edwards & Jones 1988; Jones & Edwards 1990), haemorrhage (Bruhn *et al.* 1987b), nicotine (Venihaki *et al.* 1997), AVP (Mazzocchi *et al.* 1997), and IL-1 (Andreis *et al.* 1991a; Mazzocchi *et al.* 1993; Venihaki *et al.* 1997). Experiments involving peripheral administration of CRH provided functional evidence of the intra-adrenal CRH-ACTH system. Intravenous CRH (1 mg/kg) increased the ratio of cortisol to ACTH secretion in healthy adult humans (Azar *et al.* 1992). Similarly high concentrations of CRH led to increased adrenal steroidogenesis, mass,

and ACTH-stimulated corticosterone secretion (De Souza & Van Loon 1984; van Oers *et al.* 1992) in rats (Bornstein *et al.* 1990b) and calves (Jones & Edwards 1992), despite hypophysectomy. This ostensibly direct, stimulatory effect of CRH on GC secretion, however, is blocked *in vivo* by anti-CRH antibodies, without affecting plasma ACTH (van Oers & Tilders 1991), and *in vitro* by corticotrophin-inhibiting peptide (Andreis *et al.* 1991b; Andreis 1992), suggesting the CRH requires intra-adrenal ACTH for its stimulation of GC release. Further support for the existence of the intra-adrenal CRH-ACTH system comes from evidence it can compensate for the removal of the central CRH-ACTH system. In hypophysectomised animals, for instance, the intra-adrenal content of CRH and ACTH increase as a function of time since surgery (Bagdy *et al.* 1990), but this is prevented by ACTH infusion (Edwards & Jones 1988; Mazzocchi *et al.* 1994). In rats, GC treatment reduces adrenal ACTH secretion after 2 days, and adrenal CRH content after 7 days (Bagdy *et al.* 1990). This suggests that as with the central system, the intra-adrenal CRH-ACTH system is subject to feedback inhibition from adrenal GCs and ACTH.

1.2.3.2 Role of ACTH

ACTH secreted from the anterior pituitary regulates the basal and stress-induced release of GCs from the adrenal cortex. ACTH constitutes a single polypeptide chain of 39 amino acids, with residues of serine-1 to tyrosine-23 forming the hormonally active part of the molecule, and residues 24-39 forming a stabilising tail that varies slightly between species by substitution of amino acids (Li 1972; Clark *et al.* 1986; Hanukoglu *et al.* 1990). These differences generally affect neither the activity nor the half-life of ACTH *in vivo*, which in humans is 8 – 24 minutes (Gemzell *et al.* 1951; Besser *et al.* 1971; Willoughby *et al.* 1977; Park *et al.* 2012), and in rats, 8 minutes (López & Negro-Vilar 1988; Carnes *et al.* 1990; Windle *et al.* 1998; Tomlinson *et al.* 2004; Atkinson *et al.* 2006), but they can contribute to antigenicity in heterologous species. The half-life of ACTH₁₋₂₄ is lower than full-length ACTH, at around 5 minutes (Makara *et al.* 1970; Loudon *et al.* 1994; Darmon *et al.* 1999; Waite *et al.* 2012).

ACTH binds to its receptor on the membrane of the GC-secreting, adrenocortical cells of the inner two zones of the adrenal cortex (ZF and ZR). These type 2 melanocortin receptor (MC₂R) is coupled with the stimulatory G-protein (G_s) that activates adenylyl cyclase to produce cyclic adenosine monophosphate (cAMP). The cAMP then binds and activates protein kinase A (PKA) which upregulates the activity of cholesteryl ester hydrolase (CEH) by phosphorylating it. As a result, more cholesterol is liberated and

converted to pregnenolone, supplying a biosynthetic reaction series that produces the steroid hormones, including corticosterone and cortisol, which are then secreted. Prolonged periods of ACTH activity (> 2 h) also result in upregulated expression of the P450 enzymes involved in steroid biosynthesis (Hanukoglu *et al.* 1990).

Models that remove ACTH have substantiated the importance of its effects on both the adrenal rate of GC secretion and sensitivity to further stimulation. Hypophysectomy in dogs results in a decline in GC secretion within 1 hour to very low levels (Scharrer 1954). ACTH also increases the sensitivity of the adrenal to repeated doses of ACTH. This is evident in patients with hypopituitarism or hypophysectomised animals where within 24 hours of surgery, a single administration of ACTH is no longer able to increase GC secretion, and repeated injections are required to progressively restore adrenal sensitivity to ACTH (Ney *et al.* 1967). ACTH, therefore, is a vital effector molecule for eliciting an adequate circulating concentration of GCs, whether their release is basal or stimulated, for their permissive and stress-resistant effects, respectively.

1.2.3.3 Patterns of GC release

An ultradian rhythm—a pulsatile secretion of GCs at approximately hourly intervals, punctuated by periods of very low secretion—has been observed in each of these mammalian species: rats (Windle *et al.* 1998), hamsters (Loudon *et al.* 1994), horses (Cudd *et al.* 1995), goats (Carnes *et al.* 1992), monkeys (Tapp *et al.* 1984), and humans (Henley *et al.* 2009). This secretory pattern varies with adolescence (Evuarherhe *et al.* 2009), sex (Seale *et al.* 2004), and lactation (Windle *et al.* 1997). Alterations to this pattern have also been observed in several disease states, with changes in the frequency and amplitude of pulses commonly reported in mood disorders and following chronic stress (Yehuda *et al.* 1996).

In investigating the origin of the pulsatile pattern of GC release, Spiga and coworkers detected GC pulses within 5 minutes of an ACTH pulse, which after 15 minutes were followed by upregulated transcription of steroidogenic acute response (StAR) and P450 side chain cleavage enzymes (Spiga *et al.* 2011a, b). In similar experimental models, parallel changes have been detectable because of advances in telemetry devices that allow continuous measurement of circulating GCs in free-living animals (Waite *et al.* 2012). A major upshot of this work is that since the secretion of GCs alternates *between* periods of HPA axis

activation and stress-sensitivity, and periods of inhibition and stress-refractivity, then GC secretion following a stressor will be lower during a refractive period than in a sensitive one.

The pulsatile release of GCs at least seems to continue following hypothalamic deafferentation, with evidence for this from sheep (Engler *et al.* 1990), and rats (Makara *et al.* 1970). Despite this, the origin of GC pulsatility was not clear until the following strands of evidence were obtained. First, evidence that ultradian corticosterone pulsatility does not depend on circadian variations, but rather, nadir levels are increased when circadian inputs are inhibited (Waite *et al.* 2012). Second, mathematical modelling highlighted several interesting relationships, the simplest being the delay in pituitary-adrenal stimulation and the rapid, non-linear, negative feedback of GCs from the adrenals (Keenan *et al.* 2001). In particular, the model revealed pituitary-adrenal oscillations (corroborating the deafferentation studies) suggesting a supra-pituitary (hypothalamic) pulse generator is not required for driving ultradian GC secretion. Although hypothalamic CRH does, however, contribute to the amplitude of ACTH and GC release, it need not be pulsatile to do this.

Circadian rhythms of GC secretion, unlike their ultradian rhythm, require supra-pituitary pulse generation in the suprachiasmatic nuclei (SCN). Evidence for this comes from experiments which have disrupted SCN inputs to the HPA axis through lesions to the SCN (Buijs *et al.* 1993; 1999) or constant light exposure for instance, in rats (Park *et al.* 2012). The human circadian rhythm of cortisol secretion reaches nadir around midnight and after 2-3 hours of sleep it increases to a peak at around 9 am, and, as in rats, is controlled by the SCN. The rhythm encompasses 15-18 pulses of ACTH at different amplitudes, and pulses of CRH are released from the parvocellular CRH/AVP neurons of the PVN (Carnes *et al.* 1990). The PVN receives multiple direct connections from the SCN along with many indirect inputs, for instance, via the dorsomedial hypothalamus (Roland & Sawchenko 1993; Dai *et al.* 1998; Aston-Jones *et al.* 2001). The anterior pituitary corticotrophs then secrete ACTH following binding of CRH/AVP to their receptors. In addition, the SCN has direct connections to the adrenal cortex where it can stimulate adrenal GC release directly and before CRH release is increased (Watts 2003; Thorn *et al.* 2004). Whilst the SCN is the site of the pulse generator for HPA axis activity, it is the hypothalamus that provides the neuroendocrine mechanisms through which the rest of the body is synchronised with such rhythms.

1.2.4 Hypothalamic control of ACTH secretion

This subsection will focus on the role of the hypothalamus—including the neurohumoral mechanism and synergistic actions of CRH and AVP—on the secretion of ACTH from the anterior pituitary gland.

1.2.4.1 Neurohumoral control

The posterior lobe of the pituitary gland, as part of the neurohypophysis, has an extensive network of nervous tissue and is dependent on neural connections to the hypothalamus for its secretory activity. The anterior pituitary gland, however, although it too depends on neural activity, lacks a widespread neural network. To explain this, some hypothesised a neurohumoral mechanism of pituitary control whereby nervous stimuli from the hypothalamus reach the median eminence of the neurohypophysis, and are then humorally transmitted via the portal system across the pars distalis of the anterior pituitary (Green & Harris 1947; Harris 1948). This hypothesis had foundations in several strands of preceding evidence. Popa and Fielding contributed an anatomical description of the portal system of the blood vessels in the pituitary stalk that seemingly led to the hypothalamus (Popa & Fielding 1930; 1933). Their conclusions were partly supplanted by those of Wislocki and King (1936) that suggested a unidirectional flow of blood from the median eminence down to the pars distalis of the anterior pituitary. The first functional evidence for the neurovascular control of anterior pituitary secretions came from the link between ovulation and brain stimulation in the hypothalamus-pituitary-gonadal axis in rabbits (Harris 1937). Building on this, and the emerging evidence for a role of ACTH during stress, the Harris lab investigated whether such neurohumoral control could be applicable to the HPA axis too. Stimulation of the mammillary body or the tuber cinereum in rabbits led to reduced blood lymphocyte count (lymphopenia) and lymphoid tissue mass (thymic involution). These effects were analogous to those of stress or injections of ACTH, but were specific to stimulation of certain hypothalamic areas (De Groot & Harris 1950). The tuber cinereum, for instance, was the only location for anterior pituitary grafts to effectively revascularise and restore adrenal weight, structure, and HPA function in hypophysectomised rats (Harris & Jacobsohn 1952). Once the neurohumoral mechanism for mediators of ACTH release was established in evidence, interest in the interplay of these mediators opened up, particularly for CRH and AVP.

1.2.4.2 Synergistic actions of CRH/AVP

This subsection will describe the physiological role of CRH and AVP on the regulation of pituitary ACTH secretion, the evidence for their synergistic actions, and the site, dynamics, and mechanisms of these actions.

The sequencing of CRH revealed that it is a 41-amino acid peptide (Vale *et al.* 1981) whereas AVP is a 9-amino acid peptide. Each circulates unbound in the blood, with a plasma half-life of between 4 and 15 min in humans (Schürmeyer *et al.* 1984). The physiological effects of these peptides are mediated via different receptor subtypes. Whereas CRH acts on Class B G-protein coupled receptors (GPCRs), namely CRH-R1 and CRH-R2, the physiological effects of AVP are mediated via three subtypes of Class A GPCRs, namely, V1a, V1b, and V2 receptors (Lolait *et al.* 2007; Young *et al.* 2007). Of these, the CRH-R1 and V1b receptor subtypes are of most relevance to their effects on ACTH secretion. The V1b receptor is coupled to phospholipase C (PLC) and signal pathways that stimulate ACTH secretion (Guillon *et al.* 1987). This effect is evinced by observations that V1b receptor knockout mice have lower ACTH secretion (Lolait *et al.* 2007) and show less aggressive behaviour (Wersinger *et al.* 2002) than wildtype mice. The upregulatory effects of CRH on HPA axis activation, ACTH secretion, and anxiety-relevant behaviour are mediated via CRH-R1 (Liebsch *et al.* 1999), which at nanomolar concentrations of CRH activates the adenylyl cyclase pathway (Grigoriadis *et al.* 2006).

Hypothalamic nuclei are the major endogenous sites of CRH and AVP synthesis. Prior to the sequencing of these peptides, it was known that AVP potentiates the stimulatory effect of CRH on the anterior pituitary gland secretion of ACTH *in vivo* (Yates *et al.* 1971). Despite this, there was still some debate as to whether the ACTH releasing hormone from the hypothalamus was a single peptide (Schally *et al.* 1973) or a complex of peptides (Pearlmutter *et al.* 1975; Sayers *et al.* 1980). It became evident from several studies of adrenalectomised rats that the parvocellular and magnocellular cell bodies within the PVN contained both CRH and AVP with terminals in the median eminence, compared with the supraoptic nucleus that contained only AVP (Roth *et al.* 1982; Tramu *et al.* 1983; Kiss *et al.* 1984; Sawchenko *et al.* 1984). Analysis of PVN neurons by electron microscope revealed that half of their secretory granules had CRH and AVP colocalised within them (Whitnall *et al.* 1985; 1987). The synergistic actions of CRH and AVP became evident when their co-application at low concentrations elicited a substantial release of ACTH whereas individually they were much less effective (Gillies *et al.*

1982). Hypothalamic CRH and AVP directly stimulate the anterior pituitary gland to secrete ACTH, and AVP enhances the effect of the more potent secretagogue CRH, rather than the other way round (Gillies *et al.* 1982; Turkelson *et al.* 1982; DeBold *et al.* 1984). This effect of CRH on ACTH secretion is also potentiated by factors other than AVP, including, oxytocin (Antoni *et al.* 1983), angiotensin II (Abou-Samra *et al.* 1986; Schoenenberg *et al.* 1987), epinephrine and norepinephrine (Vale *et al.* 1983). Elsewhere, the synergy of CRH with AVP also enhances the release of insulin from pancreatic beta cells (O'Carroll *et al.* 2008), and thus demonstrates biological coherence as a mechanism.

Once the anterior pituitary gland was established as the major site of their synergistic actions, the mechanism by which AVP potentiates the effect of CRH was then investigated. The prevailing hypotheses explored to date are that the synergy between AVP and CRH is explained by 1) crosstalk of second messengers downstream of their receptors, and/or 2) allosteric regulation by mutual receptor dimerisation. Potential levels of crosstalk between CRH-R1 and V1b receptor signal pathways include the second messengers diacylglycerol (DAG) and inositol phosphate (IP) downstream of the V1b receptor and the accumulation of cAMP downstream of CRH-R1. Collectively, the actions of these signals converge to increase cytosolic Ca^{2+} concentration and upregulate the ACTH secretory mechanism. Early work into this hypothesis found that AVP increases CRH-induced cAMP accumulation in corticotrophs (Giguère & Labrie 1982; Labrie *et al.* 1984). This was recapitulated by synthetic protein kinase C (PKC) agonists (phorbol esters) (Cronin *et al.* 1986; Abou-Samra *et al.* 1987) that mimic the actions of DAG on PKC activation (Nishizuka 1984). Although fluorescence/bioluminescence resonance energy transfer (FRET/BRET) studies have obtained evidence for the dimerisation of receptors for CRH and AVP, the precise mechanism behind their synergistic action requires further investigation (Mikhailova *et al.* 2007; Albizu *et al.* 2010; Murat *et al.* 2012). Interestingly, pharmacological blockade of the V1b receptor using an antagonist (Org) reduced the LPS-induced release of ACTH, but not GCs, without affecting basal HPA axis activity *in vivo*, suggesting that AVP is perhaps more instrumental during stress than at rest (Spiga *et al.* 2009). Whatever the molecular details of this mechanism, the fact that a potent secretagogue of ACTH release (CRH) can be substantially enhanced by AVP and multiple other mediators has important implications for the stress response.

1.2.5 The stress response

This subsection will focus on how the HPA axis responds to stress, through a mechanism that involves ascending and descending neural pathways, and blood-borne factors impinging on CRH/AVP neurons that form the start of a final common pathway leading to increased GC release.

1.2.5.1 CRH/AVP neurones—the final common pathway leading to GC secretion

The increased secretion of GCs following stress is largely achieved by a convergence of factors on the hypothalamus that trigger the release of the two major ACTH-releasing hormones from their eponymous CRH/AVP neurons. CRH and AVP are: i) synthesised by the neurons of the PVNmp that project to the median eminence (Sawchenko *et al.* 1984); ii) released into the hypothalamo-hypophyseal portal blood vessels that perfuse the anterior pituitary gland; iii) synergistic in their stimulation of ACTH release (Gillies *et al.* 1982), which in turn stimulates the adrenal cortex to synthesise and release GCs. The nature of the stimulus also plays a major part in the relative contribution of nerve pathways, blood-borne factors, and local mediators to the final activation of the CRH/AVP neurones. These receive, for instance, noradrenergic inputs from the brain stem during hypotensive stress, C-fibre inputs during cold stress, and inputs from the limbic system during emotional stress. Some of the most severe stressors, however, are those that pose a threat to homeostasis by challenging the immune system (Section 1.3). Tangible stressors such as blood loss, and cold temperature involve the rapid recruitment of brainstem and hypothalamic regions, whereas intangible stressors such as social subordination and deadlines involve the engagement of higher brain centres associated with emotion (amygdala and prefrontal cortex (PFC)), learning and memory (hippocampus) and decision-making (PFC). Whilst the stress response will typically involve the secretion of adrenal GCs, driven by the release of ACTH from pituitary corticotrophs, these cells are also modulated by stressor-specific combinations of release-controlling factors secreted by neurons into the hypothalamo-hypophysial portal circulation.

There are three main neural pathways with excitatory inputs to the CRH/AVP neurons; some connect directly, others co-opt multi-synaptic afferents of the basal forebrain and hypothalamic nuclei. Firstly, interoception of physiological stressors (hypotension, hypoglycaemia, and pain, for instance) is conveyed by afferents converging on the PVN from the nucleus tractus solitarius (NTS) including A2/C2

noradrenergic groups, the ventral medullary A1/C1 noradrenergic groups, the locus coeruleus (LC), the serotonergic dorsal raphe nuclei, and the cholinergic lateral dorsal tegmental nuclei (LDT) and pedunculopontine nucleus (PPN). Secondly, blood-borne signals such as blood osmolarity, cytokines, and hormones, are conveyed to the PVN via angiotensinergic projections from circumventricular organs, namely, the subfornical organ (SFO) and the organum vasculosum of the lamina terminalis (OVLT). Thirdly, complex signals for intangible stressors—such as social defeat or isolation—are integrated in the sensory and association cortex, passed to the amygdala, and lateral septum, which then activate the PVN indirectly via bisynaptic glutamate-glutamate or γ -aminobutyric acid (GABA)-GABA connections. The amygdala and lateral septum signal by glutamate-glutamate, or GABA-GABA bisynaptic connections with the PVN. The basal forebrain, certain hypothalamic nuclei, and the multi-synapses peripheral to the PVN, constitute the final neurons with projections to the PVN and the HPA axis. Overall, the key sites for the evaluation of stressors are the extra-hypothalamic structures of the hippocampus and amygdala, with direct and indirect inputs into the hypothalamic parvocellular system of CRH/AVP neurons for the integration of multiple signals as the final pathway for the HPA axis stress response. These are elicited through a system of descending and ascending neural pathways described below.

1.2.5.2 Descending and ascending neural pathways

The CRH/AVP neurones receive inputs from multiple ascending and descending nerve pathways, primarily those of the ascending noradrenergic tracts, but also the largely GABAergic pathways descending from the hypothalamus, preoptic area (PoA), and bed nucleus of the stria terminalis (BNST), as well as the cortico-limbic areas, including the raphe nuclei (RN), the hippocampus, and the amygdala.

The descending neural pathways are predominantly involved with inhibitory or disinhibitory inputs to the PVN. Stressors that can be anticipated to some extent, for instance, social defeat stress, are transduced by sensory pathways and are evaluated in the medial forebrain by the medial PFC, hippocampus, and ventral subiculum (vSub). These then inhibit the PVN via excitation of bisynaptic glutamate-GABA pathways and thus inhibit three local GABAergic PVN-projecting regions posterior to the anterior commissure: the BNST, hypothalamic nuclei (medial preoptic area, dorsomedial hypothalamus, anterior hypothalamic area, SCN), and to the peri-PVN zone of interneurons. This circuitry is also sensitive to GC negative feedback (Section 1.2.6).

The main ascending pathways with projections to the mpPVN can be divided into those originating from the brainstem, and those from the circumventricular organs, including lamina terminalis areas. Retrogradal labelling of neurons, for instance, within the PVN-projecting nuclei, and direct neurotransmitter labelling of the PVN fibre plexus, allowed identification of the key brain areas involved in the stress response. Within the brainstem, these include: the norepinephrine (NE)-positive A2 regions and NTS; the epinephrine-positive C2 regions; the acetylcholine (ACh)-positive laterodorsal tegmental nucleus; and the 5-HT-positive raphe nucleus (Schwaber *et al.* 1982). Within the circumventricular organs, the key areas include the angiotensin II (AII)-positive SFO, areas of the OVLT and median preoptic areas (McKinley *et al.* 1996).

Ascending catecholaminergic pathways from the brainstem to the PVN are critical for the HPA response to stress (Plotsky *et al.* 1989). This was demonstrated by local administration of electricity or NE causing an increased release of CRH akin to those induced by stress (Plotsky 1987; Szafarczyk *et al.* 1987). Following stress, the HPA axis is activated, and NE is released from the PVN (Pacák *et al.* 1995), whereas local damage to the PVN attenuates HPA activation following stress (Szafarczyk *et al.* 1987). Interestingly, the nature of the stressor leads to differential recruitment of certain ascending catecholaminergic pathways and modulations in the pattern of CRH, ACTH and GC secretion (Gaillet *et al.* 1991). For instance, damage to the ventral noradrenergic bundle blocks GC release in response to ether but not restraint stress whereas the removal of all ascending medullary pathways blocks cFos-indexed PVN activation in response to IL-1 β but not footshock (Li *et al.* 1996). In other words, the nature of the stress response depends on the nature of the stressor, which in turn induces specific neuronal pathways.

Other ascending pathways modulate activity of the PVN (and thus the HPA axis), but lack direct connections. The central amygdaloid nucleus (CeA), for instance, increase PVN activity via projections to the NTS (Schwaber *et al.* 1982), which have noradrenergic and non-catecholaminergic connections to the medial parvocellular PVN (Horst & Luiten 1987). The LC appears to exert trans-synaptic PVN activation (Ziegler & Herman 2002) via the amygdala, prefrontal cortex and hippocampus (Grant & Redmond 1981) as no direct connections to the PVN have been found (Cunningham & Sawchenko 1988). The neurotransmitters 5-HT and ACh activate the HPA axis *in vivo* (Plotsky 1987; Ohmori 1995; Jørgensen *et*

al. 1998) and upregulate CRH, ACTH and GC release when administered to the PVN—despite containing minimal and sparse 5-HT (Sawchenko *et al.* 1983) and ACh (Mesulam *et al.* 1983) inputs.

The circumventricular organs, including the SFO and the OVLT, provide the medial parvocellular PVN with rich innervation. The SFO promotes CRH biosynthesis and secretion (Aguilera *et al.* 1995) via ATII-ergic projections and likely permits the HPA axis response to osmotic stress (Kovács & Sawchenko 1993). Other circumventricular organs may exert indirect, trans-synaptic modulation on HPA activity, including the area postrema via the NTS.

Collectively, these ascending brainstem pathways convey rapid transmission of reactive stimuli for interoception, arousal, and nociception, and can be co-opted by descending cortico-limbic pathways for trans-synaptic modulation of PVN, and hence, HPA activities.

1.2.5.3 Limbic system inputs

Early suggestions that the hippocampus was involved in the stress response were based on its role in learning, memory, and emotional evaluation as part of the limbic system, and evidence of its projections to the hypothalamus (Nauta 1956). The hippocampus has since been shown to influence HPA activity in terms of the stress response, and circadian rhythm, but primarily as a site of GC feedback inhibition (McEwen *et al.* 1968; Canny 1990). Within the hippocampal formation, the hippocampus and subiculum have major projections to the BNST, cerebral cortex, mammillary complex, nucleus accumbens, anterior olfactory nucleus, the anterior and lateral thalamic nuclei, and ventromedial hypothalamic nuclei (VMH) (Swanson & Cowan 1977). The major source of hippocampal formation inputs to the hypothalamic nuclei appears to be the ventral subiculum (Herman *et al.* 1998). Transection of the fornix reduces the sensitivity of CRH, AVP, and oxytocin to GC feedback (Sapolsky *et al.* 1989), and hippocampectomy increases CRH and AVP gene expression (Herman *et al.* 1989). These measures do not completely abolish GC feedback however, suggesting other areas are involved. Elsewhere within the limbic system, the amygdala, for instance, has extensive afferents to the BNST that predominate those from the hippocampus (Cullinan *et al.* 1993). In terms of the route taken by the hippocampal efferents which modulate CRH/AVP neurons and thus ACTH release, most are seemingly indirect, although direct projections from the ventral subiculum to the PVN have been labelled by retrogradal but not anterogradal

transport of markers (Sawchenko & Swanson 1983). Less direct routes from the hippocampal formation to the PVN are via the fornix to the lateral septum and the BNST as well as via the medial corticohypothalamic tract to the VMH (Swanson & Sawchenko 1983). Overall the evidence for the mechanism of this role suggests the limbic structures such as the amygdala and hippocampus affect HPA axis activity through modulating the sensitivity of hypothalamic CRH/AVP neurons to GC feedback inhibition.

1.2.6 Negative feedback control of CRH/AVP and ACTH secretion

This subsection will describe the different phases of GC feedback on HPA axis activity, to permit a clear differentiation between the slow feedback inhibition of CRH/AVP and POMC synthesis, and the more rapid actions that inhibit the exocytosis of preformed ACTH and CRH/AVP from storage vesicles. The final paragraph alludes to evidence for one of the secondary mediators of feedback, but mechanistic details will be covered in later sections.

The feedback inhibition by GCs on CRH/AVP and ACTH secretion operates over at least two time domains (Beckford *et al.* 1983).

Rapid negative feedback effects occur within minutes (2 - 60 minutes) and are unaffected by inhibitors of transcription and protein synthesis, and therefore involve non-genomic actions of GCs (Keller-Wood & Dallman 1984). Rapid feedback involves the inhibition of exocytotic release of pre-formed ACTH and CRH/AVP from vesicles. One of the first observations of a rapid feedback effect of GCs was made by Dallman and Yates, whereby GCs injected into rats, from 15 seconds and up to 5 minutes prior to histamine injection, rapidly (10 – 12 minutes) inhibited the release of endogenous GCs whilst plasma GC concentrations were rising. Conversely, this effect of GCs was absent in rats that received either injections 15 minutes prior to, or 2 minutes after histamine (Dallman & Yates 1969; Jones *et al.* 1972), or a laparotomy (Yates *et al.* 1961), and the degree of inhibition was proportional to the rate at which GCs were administered (Dallman & Yates 1969; Besser *et al.* 1971; Abe & Critchlow 1977).

Delayed negative feedback effects occur over longer durations (more than 2 hours) and require classical genomic actions of GCs and their nuclear receptors to mediate changes in gene expression. The delayed feedback domain primarily involves suppressing the synthesis of CRH/AVP and ACTH. Initial

experiments investigating the ability of endogenous GCs to exhibit inhibitory effects found a delayed feedback window of between 45 and 120 minutes, outside of which the stimulatory effects of histamine on CRH/AVP were not inhibited (Dallman & Yates 1969). Different receptor subtypes for GCs are ostensibly in operation for each temporal domain of feedback. This is evident from observations that although 11-deoxycorticosterone and 11-deoxycortisol antagonise the fast feedback effect of corticosterone and cortisol, these are in fact effective mediators of delayed feedback (Jones *et al.* 1974). The magnitude and duration over which exogenous GCs exhibit delayed feedback effects depends on integrating factors including the total dose of GCs administered (Sirett & Gibbs 1969) and the circulating GC concentration achieved (Dallman & Yates 1969), which in turn determines the duration and latency of inhibition since administration (Abe & Critchlow 1980). Maximal inhibition occurs between 2 and 4 hours after administration (Takebe *et al.* 1971), and within this delayed feedback window the degree of inhibition is proportional to the dose of GCs (Fehm *et al.* 1979).

Within the hypothalamic PVN, negative feedback occurs over rapid and delayed domains, involving reduced stimulation of CRH neurosecretion (10 minutes) and downregulated CRH and AVP transcription (2 to 3 hours). The activity of neurons (when evoked) can be said to depend on the balance between excitatory and inhibitory neurotransmission. Rapid excitation of neurons is chiefly mediated by the transmission of glutamate, which acts via α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-Methyl-D-aspartate (NMDA) receptors, whereas GABA largely achieves inhibition of neuronal activity. The rapid feedback effects of GCs in the PVN are mediated via non-genomic actions of GCs interacting with extracellular, mGRs on the parvocellular CRH/AVP neurons. Exposure of these neurons to GCs *in vitro* was found to decrease glutamate release and although this was unaffected by GR or MR antagonists (Di *et al.* 2003), this rapid inhibition was abolished in GR knockout mice (Haam *et al.* 2010; Tasker & Herman 2011). Activation of the mGRs by GCs, induces a retrograde endocannabinoid signalling system across the synaptic cleft, activating presynaptic cannabinoid receptor-1 (CB1) and $G\alpha_s$ to inhibit the release of glutamate and CRH (Di *et al.* 2003; 2009). These effects are blocked by co-application of GCs with a CB1 receptor antagonist (Di *et al.* 2003; Wamsteeker *et al.* 2010).

Within the pituitary, rapid GC negative feedback effects on ACTH secretion has been demonstrated in experiments where the administration of GCs prior to application of a stressor or injection of CRH results

in rapid inhibition of ACTH release (Dayanithi & Antoni 1989; Cole *et al.* 2000; Hinz & Hirschelmann 2000). A second messenger protein induced by GCs has since been shown to mediate this inhibition (Taylor *et al.* 1993). The original GC second messenger hypothesis was conceived from observations of GCs repressing biosynthesis of prostaglandins within lung tissue from guinea pigs (Gryglewski *et al.* 1975). This occurred without affecting enzymes or transport (Lohrer *et al.* 2000), but rather, from inhibiting the production of arachidonic acid as a prostaglandin precursor (Lewis & Piper 1975). Since the cell membrane is a major source of phospholipids for arachidonic release, a GC second messenger with membrane activity was hypothesised as a simple explanation for the inhibition (Flower & Blackwell 1979). Annexin A1 (ANXA1; formerly macrocortin, renocortin, lipomodulin, and lipocortin-1), had such properties and was discovered following the elegant work of Blackwell and Flower in 1980 (Blackwell *et al.* 1980). Thus, ANXA1 emerged as a candidate for mediating the feedback effects of GCs and modulating the HPA axis response to inflammogenic stress (Section 1.4).

1.3 HPA axis responses to inflammogenic stress

All types of stress stimulate the HPA axis. This section will focus on inflammogenic stress caused by infections or pathogen associated molecular patterns (PAMPs) such as LPS, as these pose major challenges to the host defence system and overall homeostasis. In addition, several disease states that are exacerbated by inflammogens are also associated with HPA axis dysfunction, not limited to rheumatoid arthritis (Sternberg *et al.* 1989; Neeck *et al.* 1990), autoimmune thyroiditis (Schauenstein *et al.* 1987), multiple sclerosis (Levine *et al.* 1980; MacPhee *et al.* 1989), and sepsis (Marik *et al.* 2008).

1.3.1 The HPA-immune system

On noting that GC release is increased following all stressors, including infections, Besedovsky and colleagues substantiated the physiological role for glucocorticoids in regulating immune responses in the 1970s and 1980s (Besedovsky *et al.* 1975; 1981). They found that physiological concentrations of GCs had clear effects at multiple levels of immune system function, and argued that GCs helped to restrain the host-defence responses and thus prevented damage to the host. This gave way to the concept of an HPA-immune system, on the basis of the following strands of evidence.

First, immune responses were clearly shown to increase the release of GCs (Besedovsky *et al.* 1975).

Second, circulating concentrations of GCs are increased to similar levels whether in response to foreign antigens directly, through Newcastle Disease Virus (NDV), for instance, or to lymphokines generated by a previous immune reaction. This was demonstrated in rats, which at the peak of their immune response to sheep red blood cell exposure, had raised plasma ACTH and GC concentrations no different from rats injected with autologous lymphocytes stimulated *in vitro* (Besedovsky *et al.* 1975; 1981). The elevation in plasma GC concentrations, however, was abolished in hypophysectomised animals, underscoring the importance of pituitary ACTH, and thus demoting that of lymphoid or adrenal sources of ACTH at much lower concentrations (Besedovsky *et al.* 1985).

Third, physiological concentrations of GCs were associated with reduced splenic mass and volume of immunoglobulin-secreting B lymphocytes (Del Rey *et al.* 1984).

Fourth, antigenic specificity (where a given immune reaction is favoured over others) was facilitated by the raised, immunosuppressive, concentrations of GCs typical of primary immune responses. It was reasoned that GC-mediated antigenic specificity, achieved by non-specific immunosuppression, prevents unrestricted expansion of lymphocytes with “little or no affinity for antigen”. The authors acknowledged that this was analogous to lateral inhibition of surrounding neurons that increase the signal-to-noise ratio when a specific, discriminatory response is required (Besedovsky *et al.* 1979b).

Fifth, a feedback circuit between cells of the immune system and adrenal cortex was established. Immune cells released a Glucocorticoid Increasing Factor (GIF) that stimulated the adrenal cortex to produce and release GCs. The GIF was shown to require ACTH (at pituitary rather than lymphoid concentrations) for its steroidogenic effect (Besedovsky *et al.* 1985) and was identified as being IL-1 (Berkenbosch *et al.* 1987; Sapolsky *et al.* 1987).

Sixth, changes in hypothalamic norepinephrine turnover (Besedovsky *et al.* 1979a) and electrical activity (Besedovsky *et al.* 1977) were observed in parallel to peak plasma GC and lymphocyte concentrations in rats exposed to NDV and sheep red blood cells. This led to the suggestion that hypothalamic hormones were involved in the immunomodulation of GC release. CRH was shown to be stimulated by IL-1 (Berkenbosch *et al.* 1987; Sapolsky *et al.* 1987), and the means by which the immune response would drive HPA axis activity, increasing adrenal GC release, and in turn, increasing antigenic specificity.

1.3.2 Mechanisms of HPA activation during infection

The HPA axis is critical to homeostatic balance. The GCs modulate the stress response through molecular mechanisms, altering expression, transcription, and translation of a multitude of genes in diverse pathways. The overall effect is inhibition of inflammatory cell function through the inhibition of pro-inflammatory cytokines such as IL-1 and IL-6, and tumour necrosis factor- α (TNF- α). The central effectors of the stress response are the CRH and LC-NE/sympathetic systems. These systems are able to activate the stress response but can develop defects that have been associated with inflammatory diseases (e.g. rheumatoid arthritis, multiple sclerosis), and can be modulated by inflammatory mediators such as cytokines, as well as by hormones and neurotransmitters.

1.3.2.1 Role of cytokines

Cytokines are proteins released by cells upon activation by an antigen, and through cell-to-cell communication at membrane-bound receptors they modulate leukocyte function and immune responses. There are several types of cytokines released from different cell types, and include the interleukins (from leucocytes), lymphokines (from lymphocytes), interferons, and tumour necrosis factor.

1.3.2.1.1 Interleukin-1 (IL-1)

The work of Besedovsky and coworkers (1986) demonstrated a role for cytokines in mediating the neuroendocrine responses to NDV. The circulating concentrations of ACTH and GCs were raised in mice inoculated with NDV or cultures of NDV-stimulated leukocytes from human peripheral blood or mouse spleens. They ascribed this stimulation of ACTH and GC secretion to the release of proteins from the NDV-stimulated leukocytes, namely, IL-1, since its immuno-neutralisation blocked the stimulation (Besedovsky *et al.* 1986). In 1985, IL-1 α and IL-1 β were isolated from a human macrophage cDNA library, and were the first of 11 members of the IL-1 family of proteins to be cloned (March *et al.* 1985). IL-1 β is more potent than IL-1 α (in its HPA axis stimulation), and its release typically follows the stimulation of macrophages and monocytes by PAMPs, such as, LPS (Rivier *et al.* 1989; Quan *et al.* 1998). The mechanism by which IL-1 β increases plasma ACTH and GC concentration was believed to be by the direct IL-1-mediated stimulation of hypothalamic CRH neurons (Berkenbosch *et al.* 1987; Sapolsky *et al.* 1987) (despite only minimal [$<0.1\%$] IL-1 β crossing the blood brain barrier (Cocceani *et al.* 1988; Ferreira *et al.* 1988)). This was based on evidence that IL-1-stimulated ACTH release was blocked *in vivo* by anti-CRH immunoneutralisation and *in vitro* by application of IL-1 to dispersed pituitary cells without optimal concentrations of CRH. Indirect mediation by other hormones, or by IL-1-induced lymphocytes was ruled out since the IL-1 induced HPA axis activation was present in nude (T-cell- and therefore IL-2-deficient) mice (Besedovsky *et al.* 1986). Taken together, this suggested the neuroendocrine response to inflammogenic stress, elicited by the host defence and mediated by IL-1, was specific to the HPA axis hormones.

Several features of the HPA axis can feedback to inhibit the effects of IL-1 β , including those mediated by GCs (Wahl *et al.* 1975; Gillis *et al.* 1979; Snyder & Unanue 1982; Kelso & Munck 1984), and ANXA1 on IL-1-stimulated CRH release (Sudlow *et al.* 1996), and some can enhance them, including CRH (Karalis *et al.* 1991; Renner *et al.* 1998). These interactions will be described later (Section 1.4).

1.3.2.1.2 Interleukin-6 (IL-6)

Similar to IL-1, but less potent, persistent, and slower acting (Besedovsky *et al.* 1991), IL-6 has been shown to increase the secretion of ACTH in rats *in vivo* (Naitoh *et al.* 1988). IL-6 stimulates the *in vitro* release of CRH (Navarra *et al.* 1991), ACTH (Fukata *et al.* 1989), and GCs (Tominaga *et al.* 1991) from cells of the hypothalamus, pituitary gland, and adrenal cortex, respectively. The expression of IL-6 in the HPA axis and lymphoid tissues can be induced by inflammogenic PAMPs such as LPS (Muramami *et al.* 1993). IL-6 is essential for mediating the adrenal steroidogenic effect of bacterial and viral infections, and its actions are enhanced by ACTH in rats and humans (Bethin *et al.* 2000; Silverman *et al.* 2004; Zarkovic *et al.* 2008).

1.3.2.1.3 Tumour necrosis factor- α (TNF- α)

TNF- α , like IL-1 and IL-6, stimulates the HPA axis and increases GC secretion, with a similar potency to IL-1 and does so more rapidly than both IL-1 and IL-6 (Tracey *et al.* 1987; Sharp *et al.* 1989; Besedovsky *et al.* 1991). During infection, the level of TNF- α reaches picomolar concentrations in human blood plasma (Girardin *et al.* 1988; Michie *et al.* 2013), and 100 – 1000 picomolar TNF- α significantly raises plasma ACTH concentration in rats within 20 minutes, versus 60 minutes with equimolar IL-1 β (Sharp *et al.* 1989). The stimulatory effects of TNF- α , are thought to occur at a different site to those of IL- β (median eminence), as unlike IL-1 β , intracerebroventricular (i.c.v.) administration of TNF- α does not stimulate the HPA axis (Sharp *et al.* 1989). Rats exposed to LPS (0.03 $\mu\text{g/kg}$) by intraperitoneal injection exhibit a rapid (less than 20 minutes) rise in plasma TNF- α , ACTH, and GC levels (Waage 1987). This stimulation of the HPA axis is completely blocked by immunoneutralisation with anti-TNF- α antiserum, but not by IL-1 receptor antagonist (IL-1RA), except following higher doses of LPS (10 $\mu\text{g/kg}$) where both TNF- α and IL-1 β appear to stimulate the HPA axis (Ebisui *et al.* 1994).

In summary, the cytokines IL-1 α , IL-1 β , IL-6, and TNF- α , and their synergistic effects (Perlstein *et al.* 1993), are key to the immunomodulation and interaction of the HPA axis and immune system, and each is subject to upregulation by inflammogens such as LPS.

1.3.3 Evidence for inflammogen receptors within the pituitary gland

Lipopolysaccharides are constituents of gram-negative bacteria like *Escherichia coli*. The initial host defence against such bacterial infections involve toll-like receptors (TLR) that can detect and respond to LPS and other microbial ligands. The induction of cytokines is part of the initial response to LPS mediated by TLRs. There is evidence of TLR expression within the pituitary folliculostellate cells where cytokine production, for instance IL-6, can be induced by LPS, and impinges on signal cascades favouring ACTH secretion. This has been demonstrated in various pituitary models, including, the immortalised TtT/GF cell line (Lohrer *et al.* 2000), and mouse primary pituitary cultures (Renner *et al.* 1998). Toll-like receptor 4 (TLR4) is the main receptor for the mediating the effects of LPS, and is expressed in corticotroph-like AtT20 cells and human pituitary adenomas (Tichomirowa *et al.* 2005). In addition, cluster of differentiation 14 (CD14), which comprises the LPS-TLR4 receptor complex necessary for transducing the effects of LPS, is known to be expressed in TtT/GF cells (Lohrer *et al.* 2000).

The folliculostellate cells express S100 calcium binding protein b (S100b), CD14, CD45, CD54, major histocompatibility complex (MHC) class I and II antigens and complement receptors, making them replete with signalling molecules for inflammogens, particularly LPS (Baes *et al.* 1987; Whiteside *et al.* 2001; Solito *et al.* 2003). The CD14 expressed in folliculostellate cells enables LPS complexes with TLR4 to signal via NF- κ B to transcriptionally upregulate pro-inflammatory cytokines, such as, IL-6, IL-1 β , IL-1 receptor antagonist and TNF- α (Lohrer *et al.* 2000). LPS, IL-1 β and IL-6 are known to stimulate ACTH release *in vitro* and *in vivo* whereas TNF- α inhibits CRH-induced ACTH release (Solito *et al.* 2006).

1.3.4 Evidence for inflammogen receptors within the adrenal gland

LPS can induce cytokine expression in the adrenal cortex, and evidence that LPS receptors are expressed here supports the notion of locally mediated effects (Kanczkowski *et al.* 2011). Initially, LPS appears to stimulate GC release in a cyclooxygenase-2 (COX-2)-dependent mechanism (Vakharia & Hinson 2004; Zacharowski *et al.* 2006). There is a direct stimulatory effect of LPS on the production of GCs from human adrenocortical cells (Vakharia *et al.* 2002) but following this, the secretory response to LPS or

ACTH is attenuated (Liu *et al.* 2011). Receptors for LPS, TLR2 and TLR4 have been shown to be expressed in human adrenals (Fava *et al.* 1989; Traverso *et al.* 1998; Gerke & Moss 2002; Bornstein *et al.* 2004a), and over-expression of TLR4 (but not TLR2) has been shown to inhibit basal and stimulated GC secretion (Fava *et al.* 1989; Tsao *et al.* 1995; Rosengarth *et al.* 2001; Rosengarth & Luecke 2003; Davies *et al.* 2007; Kanczkowski *et al.* 2011). Counter-intuitively, TLR4 knockout mice also have impaired GC secretion despite enlarged adrenal glands and cortices (Zacharowski *et al.* 2006) whereas TLR2 knockout mice have elevated plasma ACTH concentrations (Bornstein *et al.* 2004b). Other TLRs, such as TLR9 are also implicated in adrenal GC release, and such activity is associated with cytokines TNF- α , IL-1 β , -6, -9, -12, in both mice and humans (Tran *et al.* 2007). There are a range of TLRs expressed within the human adrenal cortex (Kanczkowski *et al.* 2009) and different subtypes other than TLR4 might play a role in the inflammatory response, including, TLR9 for instance (Tran *et al.* 2007). There is also a degree of cross talk between TLRs and nuclear hormone receptors such as those for GCs (Ogawa *et al.* 2005).

There is growing evidence of a paradoxical impairment of steroidogenesis coupled with local inflammation within the adrenal gland following exposure to LPS. This has been demonstrated in murine models of endotoxaemia (Buss 2010; Mohn *et al.* 2011). Liu and colleagues found that pretreatment with LPS resulted in adrenocortical cells that were refractory both to further LPS challenge, but also to ACTH (Liu *et al.* 2011). This work substantiated the concept of adrenocortical tolerance to LPS. In investigating the mechanisms, they found that endotoxin treatment resulted in downregulated adrenocortical expression of both TLR4 and MC₂R (the ACTH receptor) and inhibited various enzymes in the corticosterone biosynthetic series. In addition, numerous cytokine networks are evident downstream of adrenal TLR4 receptors for LPS, but the precise mechanisms underpinning endotoxaemia-induced adrenal insufficiency, however, are yet to be fully elucidated (Engström *et al.* 2008). Despite this, there are several strands of evidence that suggest adrenal insufficiency is of pathological relevance to sepsis. There is very little *de novo* synthesis of cholesterol in the adrenal glands. The availability of cholesteryl ester obtained from LDL and HDL, therefore, is critical for adrenocortical steroidogenesis (Prigent *et al.* 2004). It has been shown repeatedly that both experimental endotoxaemia and clinical sepsis are associated with adrenocortical depletion of lipid droplets (Marik 2002; Buss 2010; Peng & Du 2010). In these circumstances, further steroidogenesis depends on adrenal uptake of cholesteryl ester from LDL and HDL molecules upon their binding to SR-BI (Temel *et al.* 1997). Unfortunately, during advanced

endotoxaemia and sepsis, there is an aberration in the SR-BI pathway and this coincides with hypocortisolemia (Cai *et al.* 2008). The development of adrenocortical insufficiency and tolerance to LPS is similar to the effects of the pro-inflammatory cytokines, TNF- α (Mikhaylova *et al.* 2007) and IL-6 (Zarkovic *et al.* 2008). Human adrenal cells exposed to TNF- α for 48 hours in culture, for instance, largely developed adrenocortical apoptosis and overall cortisol release was reduced. In the few cells still viable, however, cortisol release was increased (Mikhaylova *et al.* 2007). This adrenocortical inflammation and apoptosis has been replicated elsewhere (Kanczkowski *et al.* 2013). These inflammatory mediators may act as initial stimulants of GC release, but then ostensibly led to adrenocortical apoptosis, lipid depletion, and adrenocortical insufficiency. The adrenocortical resistance to ACTH during endotoxaemia might occur via one of several mechanisms. There is some evidence that neutrophil-derived corticostatins (ACTH receptor antagonists (Zhu & Solomon 1992)) may compete with ACTH for binding sites on MC₂R (Bornstein *et al.* 2008). Pathogens, such as the coronavirus that causes severe acute respiratory syndrome (SARS), can have areas of homology with the ACTH sequence, which could permit direct binding via MC₂R (Wheatland 2004). It is conceivable then, that an endogenous antibody against such pathogens might also immunoneutralise the ACTH in circulation, but this is speculative. The strongest evidence so far, however, is for adrenocortical insufficiency to take place via downregulation of MC₂R and TLR4 (Liu *et al.* 2011).

1.4 Role of annexin A1 in modulating HPA axis function

This section begins with a brief description of the basic biology of the annexin A1 protein and outlines the evidence for its anti-inflammatory actions and effects on the HPA axis, before focussing on its modulation of the HPA axis response to inflammogenic challenges. Finally, the description of its molecular mechanisms delineates the case for involvement of members of the formyl peptide receptor family.

1.4.1 Basic annexin A1 biology

Annexin A1 (ANXA1; formerly lipocortin 1) is a 37 kDa protein from the annexin superfamily of structurally related proteins. ANXA1 was the first of 13 members of the annexin family to be characterised by their ability to bind to acidic phospholipids in a Ca^{2+} -dependent mechanism, owing to their highly conserved carboxylic acid (C)-terminal core domain of 70 amino acids. The amino (N)-terminal domain of the annexin subtypes are unique, vary in length (44 amino acids in ANXA1) and in their composition of phosphorylation and glycosylation motifs (Btaouri *et al.* 1996; Buckingham 1996; Kalinec *et al.* 2009) that determine their functional specificity (Fava *et al.* 1989; Peers *et al.* 1993; Gerke & Moss 2002; Lu *et al.* 2007). The N-terminal domains undergo conformational change in line with increased Ca^{2+} concentration, resulting in ANXA1 being open to ligand binding, and thus influencing biological activity (Rosengarth *et al.* 2001; Rosengarth & Luecke 2003).

ANXA1 was first posited as being a mediator of GC actions in leukocytes of the innate immune system (Blackwell *et al.* 1980). Early investigations found that removal of GCs by adrenalectomy downregulates ANXA1 synthesis, and this is reversed by reintroduction of GCs to rat macrophages (Blackwell *et al.* 1982). Other researchers verified this relationship between the concentration of GCs and expression of ANXA1 in other cell types, both *in vitro* and *in vivo* (Baes *et al.* 1987; Fuller & Verity 1989; Vishwanath *et al.* 1992; Whiteside *et al.* 2001), including the hypothalamus and pituitary gland (Loxley *et al.* 1993; Smith *et al.* 1993; Taylor *et al.* 1993). These effects were blocked by mifepristone (GC receptor antagonist) (Peers *et al.* 1993; Taylor *et al.* 1993; Herkenham 2005) and unaffected by mineralocorticoid or sex steroids (Baes *et al.* 1987; Solito *et al.* 2001; Chapman *et al.* 2002), and thus substantiated the primacy of GCs in the regulation of ANXA1 expression. Given that secretion of GCs is increased

following inflammation and tissue injury (Taylor *et al.* 1993; Buckingham 1996; Castro-Caldas *et al.* 2001; Philip *et al.* 2001), and these sites attract leukocytes such as neutrophils expressing ANXA1 (Perretti *et al.* 1996), this partly explains why expression of ANXA1 is markedly upregulated at sites of inflammation.

The expression of ANXA1 in a variety of tissues has now been identified, including the brain (Smith *et al.* 1993), anterior pituitary gland (Ozawa *et al.* 2002), adrenal gland (Davies *et al.* 2007), neutrophils (Fava *et al.* 1989), cochlear Hensen cells (Kalinec *et al.* 2009), lung (Dreier *et al.* 1998), skeletal muscle and adipose tissue (Warne *et al.* 2006). Expression of ANXA1 is evident throughout the endocrine system. ANXA1 is abundantly expressed within the anterior pituitary gland, mainly along projections of non-endocrine folliculostellate cells, particularly those in apposition with endocrine cells (Christian *et al.* 1997; Ozawa *et al.* 2002). Expression of ANXA1 is also high within the hypothalamus, particularly within the median eminence (Buckingham 1996; Traverso *et al.* 1998; Solito *et al.* 2001; Hannon *et al.* 2003); the ependymal cells (Dreier *et al.* 1998) surrounding the third ventricle (Christian *et al.* 1997); in activated astrocytes; and small amounts in microglia (Hannon *et al.* 2003). There is also evidence of ANXA1 expression within the endocrine pancreas (Ohnishi *et al.* 1995), testis (Cover *et al.* 2002), ovary (Tsao *et al.* 1995), thyroid (Btaouri *et al.* 1996), thymus (Fava *et al.* 1989), placenta (Tsao *et al.* 1995), and the adrenal cortex (Davies *et al.* 2007).

1.4.2 Effects of ANXA1 within the HPA axis

The adrenal glands are the original source of the first annexin to be described, annexin A7 (Creutz *et al.* 1978), which was isolated from the chromaffin cells of the medulla, where ANXA1 was also observed later (Drust & Creutz 1991; Wang & Creutz 1992). Primary adrenal cell cultures derived from ANXA1^{-/-} mice were found to have a higher release of corticosterone in response to ACTH₁₋₂₄ than wildtype adrenals, and this effect occurred without any change in MC₂R expression, suggesting a short, ANXA1-mediated, negative feedback loop, controlling adrenal GC release (Omer *et al.* 2005). This was further substantiated by findings that adrenal extracellular ANXA1 was upregulated by GCs within 3 hours of exposure, and ACTH₁₋₂₄-stimulated corticosterone release was greater in ANXA1^{-/-} adrenals than wildtypes (Davies *et al.* 2007). This suggested that ANXA1 played a role in the adrenal responsiveness to ACTH₁₋₂₄ and possibly mediated short-loop feedback sensitivity to GCs.

ANXA1 is expressed within the hypothalamus, hippocampus, cortex, and other parts of the brain (Philip *et al.* 1997) but in lesser amounts than the anterior pituitary and adrenal. Within the hypothalamus, ANXA1 is concentrated within the median eminence, the ependymal cells surrounding the third ventricle, as well as the SON and PVN (Smith *et al.* 1993). Whilst ANXA1 is expressed within activated glial cells it is not evident in hypothalamic neurons, including those for CRH/AVP. As in the anterior pituitary, GCs regulate both the synthesis and the cellular disposition of ANXA1 in the hypothalamus, again, not in the endocrine cells but in the adjacent cells (Loxley *et al.* 1993). Here, ANXA1 suppresses the CRH neuron response to pro-inflammatory cytokines *in vivo* (Loxley *et al.* 1993). The findings that ANXA1 is not expressed by CRH/AVP neurons or corticotrophs, but is present in the non-endocrine activated microglia and folliculostellate cells, and is rapidly translocated to cell membranes in timings consistent with the feedback domains of GCs, prompted the hypothesis that ANXA1 mediates the inhibitory effects on CRH/AVP and ACTH release (Smith *et al.* 1993).

1.4.3 Mechanism of action of annexin A1 within the anterior pituitary gland

This section will deal with the mode of action of ANXA1 within the anterior pituitary gland, its localisation to non-endocrine folliculostellate cells, and its inducement by non-genomic steroid action and serine phosphorylation prior to translocation to the cell membrane, and the evidence for its externalisation via an ABCA1 transporter system.

1.4.3.1 Annexin A1 is a paracrine mediator of GC-inhibited ACTH release

Around the time of ANXA1's discovery, Deneff and coworkers began to investigate the possibility of paracrine communication between the various epithelial cell subtypes of the anterior pituitary gland (Deneff *et al.* 1989). By separating these cells based on their unit gravity of sedimentation (secretory granule diameter) and re-aggregating them at increasing ratios of endocrine to non-endocrine lineage, they found that preparations containing more non-endocrine (agranular) folliculostellate cells had reduced hormone secretion (Baes *et al.* 1987). The effect was paralleled by applying conditioned medium from folliculostellates to separate endocrine cells, and suggested the folliculostellate cells produced a paracrine factor.

A paracrine factor was not identified until the early 1990's, when Amanda Taylor and colleagues were exploring the mechanisms used by GCs to feedback and inhibit the exocytosis of ACTH following the stress response (Loxley *et al.* 1993; Smith *et al.* 1993; Taylor *et al.* 1993). Immunohistochemistry and cell fractionation studies provided evidence of ANXA1 expression within the non-endocrine, folliculostellate cells of the anterior pituitary, where it is compartmented between the cytoplasm, and the organellar and extracellular membranes (Philip *et al.* 1997; Traverso *et al.* 1999). This distribution and synthesis of ANXA1 is regulated by the action of GCs both within the anterior pituitary and the hypothalamus (Loxley *et al.* 1993; Taylor *et al.* 1993). The *de novo* synthesis of ANXA1 is induced by GCs via classic genomic actions and is slow in onset (more than 2 hours), whereas the translocation of cytoplasmic ANXA1 to the extracellular membrane is evident within 15 minutes of exposure to GCs whether *in vivo* or *in vitro* (Philip *et al.* 1997). These two methods of modulating ANXA1 expression and distribution are therefore consistent with the biphasic (rapid and delayed) feedback domains of GCs.

The absence of ANXA1 expression within CRH/AVP neurons or ACTH-positive corticotrophs, alongside abundant expression within adjacent S100b-positive folliculostellates, prompted the hypothesis that ANXA1 mediates the feedback inhibition of GCs on stress hormone release via a paracrine mechanism. Within the anterior pituitary, this was supported by five tessellating pieces of evidence. First, ANXA1 is predominately expressed within the folliculostellate cells and at high concentrations at points of contact with endocrine cells (Traverso *et al.* 1999). Second, the GC-induced release of ANXA1 to the extracellular membrane coincides with the rapid (<30 min) GC-induced inhibition of ACTH secretion (Loxley *et al.* 1993; Taylor *et al.* 1995c; Buckingham *et al.* 1996)—effects which are blocked by drugs that prevent protein transport (e.g. monensin), antagonise GR (McArthur *et al.* 2009), or inhibit PKC (John *et al.* 2002). Third, the corticotrophs have high-affinity, (K_d 14 nmol.l⁻¹) ANXA1-binding sites which, when enzymatically disrupted, impairs both GC- and ANXA1-mediated inhibition of ACTH release (Christian *et al.* 1997). Fourth, release of immunogold labelled ANXA1 is evident at focal points where folliculostellate projections are in apposition with endocrine cells (Chapman *et al.* 2002). Fifth, murine co-cultures of the ANXA1-positive, folliculostellate-like TtT/GF cell line, with the ANXA1-negative, corticotrophic AtT20 D1 cells, exhibited reduced CRH-stimulated ACTH release by GCs, and antisense oligodeoxynucleotides confirmed this effect was ANXA1-dependent (Tierney *et al.* 2003). Interestingly, AtT20 D1 cells cultured alone respond to ANXA1 and ANXA1_{Ac2-26} peptide (but not GCs),

whereupon CRH-induced ACTH release is inhibited dose-dependently (Tierney *et al.* 2003). This further substantiates the role of folliculostellates as a source of the ANXA1 required to mediate the feedback inhibition of ACTH release by GCs.

1.4.3.2 Annexin A1 translocation involves an ABCA1 transporter

The rapid nature of the GC-induced translocation of ANXA1 that was blocked by GC receptor antagonists but unaffected by inhibitors of transcription or translation (Philip *et al.* 1998), suggested a novel, non-genomic, GC receptor-mediated mechanism was involved. The translocation of ANXA1 to the extracellular membrane of the folliculostellates was also shown to be unaffected by inhibitors of Golgi-mediated exocytosis (namely, nocodazole and brefeldin A) and thus ruled out the release of ANXA1 within vesicles (Philip *et al.* 1997). Instead, analysis of membrane-bound ANXA1 found that phosphorylation by PKC was influential in this association, critically at serine residues, and to a lesser extent tyrosine residues (John *et al.* 2002; 2003). This was demonstrated by the fact that blockade of PKC prevented the GC-induced serine phosphorylation and translocation of ANXA1 to the extracellular membrane of primary folliculostellate cells, whereas inhibition of tyrosine phosphorylation was not critical. The role of serine phosphorylation was further substantiated by experiments using the human folliculostellate cell line (PDFS), whereby the actions of GCs on ANXA1 translocation were found to be mediated via a non-genomic pathway downstream of GRs, involving phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) in addition to PKC (Solito *et al.* 2003). To determine which serine residues specifically linked phosphorylation with membrane binding, site-directed mutagenesis of ANXA1 found that serine-27 and serine-45 were important (Solito *et al.* 2006; McArthur *et al.* 2009). The mechanism by which ANXA1 reaches the extracellular membrane, however, is less clear, although immunoreactive ANXA1 was evidently co-localised with the adenosine triphosphate (ATP)-binding cassette transporter protein type A1 (ABCA1) in primary folliculostellates, suggesting this protein might be involved (Chapman *et al.* 2003). In the same study, blockade of ABCA1 with glyburide prevented both the GC-induced translocation of ANXA1 and its inhibition of ACTH release from primary pituitary tissue. The caveat to these findings is that glyburide, as well as blocking ABCA1, also opens ATP-sensitive K⁺ channels which when blocked (by chromokalm for instance), have been shown to induce ANXA1 translocation in TtT/GF cells. Although the contribution of ABCA1 to the translocation

of ANXA1 might be less than that of ATP-sensitive K⁺ channels, the fact that glyburide prevented GC-induced ANXA1 translocation and inhibition of ACTH release, is consistent with the paracrine model of ANXA1 action (Chapman *et al.* 2003).

1.5 Annexin A1 signalling and the role of the formyl peptide receptor (Fpr/FPR) family

The paracrine model by which ANXA1 mediates the feedback inhibition of GCs on ACTH release via membrane-associated receptors on corticotrophs and other endocrine cells is well supported (Christian *et al.* 1997). In investigating the structure-activity relationship of ANXA1 action in different systems, evidence suggests the actions of ANXA1 could be mediated by more than one receptor subtype. Removal of the second repeat unit of the core domain (ANXA1₁₋₁₈₈) and most of the first repeat (ANXA1₁₋₅₀) result in peptides which inhibit ACTH release, but are significantly less potent, despite equal efficacy with full length ANXA1₁₋₃₄₆ (John *et al.* 2002). The N-terminal acetylated peptide ANXA1_{Ac2-26}, however, is less potent (10^5 times) and less efficacious than ANXA1₁₋₃₄₆, but equipotent to ANXA1₁₋₁₈₈, whereas shorter sequences have no effect whatever, indicating that the N-terminal is critical for the inhibition of ACTH release mediated by ANXA1 *in vitro*. This was corroborated by immunoneutralisation with antibodies against this N-terminal epitope, which blocked the inhibitory effect of GCs on ACTH release *in vivo* (Taylor *et al.* 1995c). In models of inflammation, however, ANXA1_{Ac2-26} has been shown to be as efficacious, but 100 times less potent than full length ANXA1₁₋₃₄₆ at reducing PMN infiltration, whereas ANXA1₁₋₁₈₈ is 10 times less potent (Perretti *et al.* 1996). Given the divergence in potency of ANXA1 peptides in different tissue systems, it seems likely that more than one receptor subtype mediates the endogenous actions of ANXA1. The discovery that ANXA1 interacted with a G-protein coupled formyl peptide receptor (Fpr in rodents, FPR in man) in leukocytes (Walther *et al.* 2000), opened up the possibility that ANXA1 actions within the HPA axis were mediated via an Fpr/FPR subtype. This research seeks to identify an Fpr/FPR subtype that modulates pituitary and adrenal stress hormone release because this could then be targeted with ligands that modulate HPA axis function without the use of GCs and their associated side effects (Schäcke *et al.* 2002).

1.5.1 Biology of the Fpr/FPR family

This subsection will describe the discovery of a family of G-protein coupled receptors, the formyl peptide receptors (Fpr in rodents, FPR in man); tabulate their tissue distribution; and describe their structural similarities and their function with regard to the relative affinities for agonists and antagonists, and signalling mechanisms.

Phylogenetic analysis of the different Fpr/FPR subtypes between species suggests that the Fpr/FPR family has undergone differential expansion (Figure 1.2). There are rodent-specific Fpr subtypes that have resulted from numerous gene duplication events, so that there are 8 functional mouse genes (Fpr1, Fpr2, Fpr3/Alx, Fpr-rs3, Fpr-rs4, Fpr-rs6, Fpr-rs7, and Fpr-rs8) on chromosome 17 A3.2, and 7 rat genes (Fpr1, Fpr2, Fpr2l/Alx, Fpr3, Fpr-rs3, Fpr-rs4, and Fpr-rs6) on chromosome 1q12, compared with only 3 human genes, FPR1, FPR2/ALX, and FPR3, on chromosome 19 (Gao *et al.* 1998; Rabiet *et al.* 2005).

The discovery of the first formyl peptide receptor, namely FPR1 began with the hypothesis that leukocytes are attracted to sites of infection through bacterial signals (chemotaxis). These signals were narrowed down to peptides containing the initiation sequence for prokaryotic protein synthesis, N-formyl methionine (Schiffmann *et al.* 1975a). After many compounds were tested, formyl-methionyl-leucyl-phenylalanine (fMLF) was found to be most potent in eliciting neutrophil chemotaxis, with a pEC₅₀ 10.05 - 10.16 (Showell *et al.* 1976; Freer *et al.* 1980). The identification of FPR1 itself was borne out of the cloning of a cDNA library which conferred transfected cells with high-affinity receptors for fMLF (Boulay *et al.* 1990). N-formyl-methionine, whilst highly potent, was found to be not necessary for ligand binding and responses via Fpr/FPR subtypes (Gao *et al.* 1994). In fact, collectively the Fpr/FPR are a family of highly promiscuous receptors, because they bind to a wide variety of chemically diverse ligands, typically with low selectivity (Hartt *et al.* 1999; Migeotte *et al.* 2006). On the basis that many of these ligands were present within bacteria, and because Fprs/FPRs are abundantly expressed on leukocytes, the Fpr/FPR family was thought to have evolved primarily to chemoattract leukocytes to the sites of bacterial infection (Prossnitz & Ye 1997). However, formylated peptides are also produced by mitochondria (Rabiet *et al.* 2005), and Fpr/FPR subtypes are expressed in a variety of cell types, including those of the chemosensory vomeronasal organ in rodents (Liberles *et al.* 2009; Rivière *et al.* 2009). These discoveries suggest that Fpr/FPR subtypes have diverse biological roles beyond leukocyte chemotaxis.

Each human FPR subtype will now be described in terms of their structural and functional similarities with the rodent (murine and rat) Fpr subtypes, the relative affinities of their most selective ligands and their binding domains, their regulation in terms of phosphorylation, internalisation, and desensitisation, and interactions with Fpr/FPR subtypes and other molecules such as β -arrestins. The signalling

mechanisms will be briefly covered in terms of their molecular mediators and how much of the evidence from leukocytes relates to pro-inflammatory (chemotaxis, superoxide generation, and degranulation events) or anti-inflammatory actions.

1.5.2 Formyl peptide receptor 1 (FPR1)

In terms of function, the human FPR1 and murine Fpr1 show high affinity for N-formyl peptides, especially fMLF, which is the most selective and potent ligand for human FPR1 and mouse Fpr1 with a pEC_{50} of 9.3 and 7.0, respectively (Koo *et al.* 1982; Hartt *et al.* 1999; He *et al.* 2000). Similarly, fMLF binding via the rat Fpr1 has a pK_d of 7.5 (Marasco *et al.* 1983) (see Table 1.3). It is on this basis, and their high nucleotide sequence identity (77%), that murine Fpr1 and rat Fpr1 are recognised as the structural and functional orthologues of human FPR1. Expression of human FPR1 is evident within: the endocrine system (adrenal, ovary, placenta); leukocytes (macrophages, monocytes, polymorphonuclear (PMN) cells, dendritic cells) and platelets; lymphoid tissues (bone marrow, spleen); and the cells (astrocytes and microglia) and tissues (hippocampus, hypothalamus) of the nervous system (see Table 1.2) (Becker *et al.* 1998; Czapiga *et al.* 2005; Ye *et al.* 2009). The expression of Fpr1, the murine orthologue of human FPR1, is broadly similar (Gao *et al.* 1999; Hartt *et al.* 1999; Dai *et al.* 2005; Mandal *et al.* 2005; Buss 2010). Although expression of the rat Fpr1 is evident within leukocytes such as neutrophils, its wider tissue distribution remains to be investigated (Marasco *et al.* 1985; Remes *et al.* 1991).

Several groups have attempted to determine the ligand binding domains of the Fpr/FPR subtypes. Receptor chimera studies in which the first and third extracellular loops of FPR2/ALX were replaced with those of FPR1, increased the affinity (K_d) for fMLF from 400 to 18 nmol.l^{-1} for FPR2/ALX, compared with 1 nmol.l^{-1} for FPR1 (Quehenberger *et al.* 1993). Further experiments which introduced positively charged amino acid residues at key positions, namely, Arg₈₄ and Lys₈₅, were able to increase the affinity of these chimeric receptors for fMLF to within 1.6 nmol.l^{-1} (Quehenberger *et al.* 1997). The fMLF binding region on FPR1 was confirmed by photolabelling experiments in which fMLF was visible between residues 83 and 87 encompassed by transmembrane domain 2 and the first extracellular loop (Mills *et al.* 2000). These findings are important because they demonstrate that the residues for high affinity fMLF binding are typically only found on FPR1, and explain the Fpr1/FPR1 selectivity of fMLF in human, mouse, and rat systems (see Table 1.3).

Upon binding to Fpr1/FPR1, fMLF is associated with NF- κ B-mediated transcription of chemokines and endoplasmic reticular Ca^{2+} release via G_β -mediated upregulation of phospholipase C- β (PLC- β), giving rise to diacylglycerol (DAG)-mediated stimulation of protein kinase C (PKC) (Naccache *et al.* 1979). This occurs alongside cytoskeletal actin reorganisation which follows the G_γ -mediated upregulation of phosphoinositide 3-kinase (PI3K) activity (Homma *et al.* 1985). In addition, fMLF upregulates Ca^{2+} mobilisation, superoxide anion production and ultimately cell degranulation in mouse neutrophils (Gao & Murphy 1993; Pivot-Pajot *et al.* 2010). Although these signalling events could be relevant to pituitary and adrenal stress hormone secretion, the available evidence suggests that fMLF and rat and mouse Fpr1 is not involved, but this does not exclude a role for human FPR1 (Section 1.5.5).

1.5.3 Formyl peptide receptor 2/ lipoxin receptor (FPR2/ALX)

Human FPR2/lipoxin receptor (ALX) shares 76% and 73% of its nucleotide sequence with murine Fpr2 and Fpr3/Alx respectively, compared with only 68% with Fpr1/FPR1. Similarly, FPR2/ALX has 77% and 74% sequence identity with rat Fpr2 and Fpr2l/Alx, but the rat Fpr1 is not far behind at 73% (Table 1.1). Despite the similarities in structure between FPR2/ALX and particularly Fpr2, attempts to discern which murine/rat Fpr subtype is the functional orthologue of human FPR2/ALX based on the effects of a ligand in a given system are fodder for scholarly debate. The most selective ligands for human FPR2/ALX and mouse Fpr2 are the peptide agonist, Trp-Lys-Tyr-Met-Val-*D*-met (WKYMVm) with a pEC_{50} of 12.0 and 9.3, respectively (Le *et al.* 1999; He *et al.* 2013a), and the peptide antagonist Trp-Arg-Trp-Trp-Trp-Trp (WRW₄) with a pIC_{50} of 6.7 and 6.6, respectively (Bae *et al.* 2004; Stenfeldt *et al.* 2007; Önnheim *et al.* 2008).

Receptor chimera experiments have been performed to determine which binding domains and residues are responsible for high affinity binding at FPR2/ALX. It was found that transmembrane domain 7 of human FPR2/ALX was responsible for binding the eicosanoid ligand, lipoxin A₄ (LXA₄) (Chiang *et al.* 2000). In addition, separate sites containing N-glycosylation domains at Asn₄ and Asn₁₇₉ were found to be responsible for binding of the peptide agonists MMK-1 and MHC that were blocked by deglycosylation, whereas LXA₄ activity was retained (Chiang *et al.* 2000). Given that bacteria and viruses manipulate glycosylation events to improve pathogenicity, the retention of ligand-specific activity via FPR2/ALX could have implications for modulation of the local response to infectious agents and inflammogens (Kim

& Cunningham 1993). Additional chimeric clones of FPR1 and FPR2/ALX found that the N-terminal region and the second extracellular loop form an anti-inflammatory ligand-binding domain required for ANXA1-mediated signalling, whereas LXA₄ signalling occurs via the third extracellular loop (Brancaleone *et al.* 2011; Bena *et al.* 2012). The ligand serum amyloid A also requires the second extracellular loop but instead mediates pro-inflammatory signalling via FPR2/ALX, ostensibly because it does not bind to the N-terminal region, whereas ANXA1 does (Bena *et al.* 2012). The identification of distinct ligand binding domains on FPR2/ALX is important, as they are predictive of the dichotomous ligand-specific effects via this receptor.

The phosphorylation of FPR2/ALX (as with FPR1) is ligand-dependent and is likely to involve the recruitment of alternative kinases depending on the agonist. Phosphorylation events are known to be influential in the internalisation of FPR1 and GPCRs in general, but these are yet to be fully elucidated for FPR2/ALX. Evidence obtained thus far suggests that ligand-bound FPR2/ALX (for instance, with WKYMVM) is internalised by clathrin-mediated endocytosis and recycled via the perinuclear endosome pathway. This can be blocked by siRNAs against clathrin or the knockout of β -arrestins 1 and 2, but can be restored by either β -arrestin (Ernst *et al.* 2004a; Huet *et al.* 2007). This suggests a level of redundancy in the molecular systems that facilitate the internalisation of Fpr/FPR subtypes.

Signalling via FPR2/ALX has been shown to elicit either pro-inflammatory or anti-inflammatory effects, depending on the ligand involved. FPR2/ALX was the first GPCR to be cloned that binds to both peptide and lipid ligands (Brink *et al.* 2003; Ye *et al.* 2009). The majority of the peptide agonists for FPR2/ALX upregulate pro-inflammatory signalling, including the nuclear translocation of NF- κ B, Ca²⁺ mobilisation, superoxide generation, and phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2), all of which facilitate leukocyte chemotaxis (Sodin-Semrl *et al.* 2004). The eicosanoid ligand LXA₄, and its stable analogues such as 15-epi-LXA₄, however, have been shown to elicit actin reorganization within monocytes, but not in neutrophils despite expression of FPR2/ALX (Maderna *et al.* 2002). High affinity binding of LXA₄ to FPR2/ALX cloned in Chinese hamster ovary cells led to the recruitment of G α_i proteins (Fiore *et al.* 1994). Whereas peptide ligands binding to FPR2/ALX activate pro-inflammatory signalling, LXA₄ did not lead to ERK1 or ERK2 phosphorylation even at high micromolar concentrations (Bae *et al.* 2003a, b). Upon FPR2/ALX binding, the partial agonist Quin-C1 promotes ERK2

phosphorylation events (Nanamori *et al.* 2004), yet whilst LXA₄ can induce ERK2 phosphorylation, it does so independently of FPR2/ALX and FPR3 (but possibly not FPR1) (Christophe *et al.* 2002). Interestingly, whole protein and peptide derivatives of recombinant and GC-generated endogenous ANXA1, were shown to interact with FPR2/ALX and mediate anti-inflammatory effects in human leukocytes (Perretti *et al.* 2002). Given the divergent effects elicited by ligands acting via FPR2/ALX and the evidence of distinct binding domains for the activation of pro-inflammatory or anti-inflammatory signalling, it is evident that FPR2/ALX is able to mediate ligand-specific effects. It remains to be determined whether mouse Fpr2 and Fpr3/Alx, and rat Fpr2 and Fpr2l/Alx share the divergent functions of FPR2/ALX and are relevant for modulating pituitary and adrenal stress hormone release.

1.5.4 Formyl peptide receptor 3 (FPR3)

The nucleotide sequence of human FPR3 is 63% and 60% identical to mouse Fpr2 and Fpr3/Alx, respectively, versus 56% with Fpr1. In rats, the subtypes Fpr2 and Fpr2l/Alx are each 77% identical to FPR3, whereas rat Fpr1 shares 68% sequence identity. FPR3 is expressed within the adrenal gland, placenta, testes, mature dendritic cells, macrophages, monocytes, spleen, hypothalamus, liver, and lung (Durstin *et al.* 1994; Yang *et al.* 2002; Ye *et al.* 2009). Unlike FPR1 and FPR2/ALX, neutrophils do not express FPR3 (Murphy *et al.* 1992). FPR3 is co-expressed with FPR2/ALX and FPR1 in monocytes, and the stoichiometry is subject to the route of monocyte differentiation. The dendritic cell route involves progressive down-regulation of FPR2/ALX (Yang *et al.* 2001) then FPR1 during maturation when FPR3 dominates (Yang *et al.* 2002; Migeotte *et al.* 2005). Compared with FPR1 and FPR2/ALX, FPR3 is constitutively phosphorylated in the absence of ligand binding (Christophe *et al.* 2001). It is still a matter of speculation, however, as to whether this relates to its internalisation and expression on cell membranes.

The most potent and selective ligand for FPR3 is the amino-acetylated haem-binding protein fragment (F2L) with a pEC₅₀ of 8.00, but F2L also has activity via FPR2/ALX, albeit with lower affinity with a pEC₅₀ of 6.3 (Migeotte *et al.* 2005). The only known antagonist for FPR3 is WRW₄ (Shin *et al.* 2006). FPR3 is also activated by the mitochondrial peptide agonist for FPR2/ALX, fMMY-ALF (Rabiet *et al.* 2005). In mice, the only receptor with F2L activity is Fpr2 with a pEC₅₀ of 6.4 (notably similar to human FPR2/ALX) (Gao *et al.* 2007). There are currently no data for F2L activity via rat Fpr subtypes.

1.5.5 Translation of rodent Fpr and human FPR biology

There is evidence that ANXA1 and its derivative peptide ANXA1_{Ac2-26} non-selectively binds to human FPR1 and FPR2/ALX, and murine Fpr1, Fpr2, and Fpr3/Alx, but also to rat Fpr2 (Table 1.3). Within the mouse and rat pituitary, there is evidence of Fpr2, Fpr3/Alx, and Fpr-rs6, but Fpr1 does not appear to be expressed and its germ line deletion had no effect on GCs/ANXA1-mediated ACTH inhibition (John *et al.* 2007). More selective Fpr/FPR ligands than ANXA1 have also been shown to inhibit ACTH release. These include LXA₄ (the selective agonist for FPR2/ALX, Fpr3/Alx, and Fpr2l/Alx in humans, mice and rats, respectively) but also the Fpr1-/FPR1-selective fMLF which inhibits ACTH release at high concentrations conducive for recruitment of Fpr2/FPR2/ALX but with lower affinity than Fpr1/FPR1 (Table 1.3) (John *et al.* 2007). The non-selective, Fpr1/FPR1 and Fpr2/FPR2/ALX antagonist, N-Boc-MLF (Table 1.4), can block the inhibition of ACTH release mediated by Fpr/FPR agonists, but also by GCs, giving further credence to the hypothesis that an Fpr/FPR subtype modulates stress hormone release (Taylor *et al.* 1993; 1997; 2000b; John *et al.* 2002). The identity of the relevant Fpr/FPR subtype for mediating these actions, and of the GCs and ANXA1 is still under investigation, however.

The rat Fpr1 was first characterised pharmacologically whereby tritiated-fMLF was found to bind Fpr1 in rat neutrophils with an antilog of the equilibrium dissociation constant (pK_d) of 7.5 which is similar to its murine and human counterparts (Marasco *et al.* 1983) (Table 1.3). Rat Fpr2 putatively mediates the anti-nociceptive effects of ANXA1 on the dorsal root ganglion response to inflammogenic complete Freund's adjuvant *in vivo* (Pei *et al.* 2011). Confocal analysis of primary rat glial cells provided evidence of Fpr2 co-localisation with ligands for the receptor for advanced glycation end products (RAGE), such as S100b, and the downstream production of cAMP and phosphorylated ERK1/2 was inhibited by the Fpr2-/FPR2/ALX-selective antagonist WRW₄, underscoring this relationship (Slowik *et al.* 2012). In an *ex vivo* rat cerebral cortex model of bacterial meningitis induced by *Streptococcus pneumoniae* and *Neisseria meningitides* conditioned media, the resulting cAMP-evoked activation of glial cells was associated with upregulated expression of Fpr2, and blocked by WRW₄ (Braun *et al.* 2011). It was the rat Fpr2l/Alx gene product, however, that was cloned and found to be responsive to LXA₄ like its human and mouse counterparts (Chiang *et al.* 2003; Motohashi *et al.* 2005). The remaining rat subtypes, Fpr3 and Fpr-rs3, Fpr-rs4, and Fpr-rs6, have been studied in the vomeronasal organ for their potential role in pheromone

detection (Liberles *et al.* 2009). Given this evidence, it seems plausible that the rat Fpr subtypes Fpr2 and Fpr2l/Alx are potential targets for modulating pituitary and adrenal stress hormone release.

Most of the mouse Fpr subtypes Fpr-rs3 to Fpr-rs8 are expressed within the vomeronasal organ and evidence points to their role in pheromone sensing in this rodent tissue (Liberles *et al.* 2009; Rivière *et al.* 2009; Bufe *et al.* 2012). Mouse Fpr1 is the structural and functional orthologue of human FPR1, whereas the nucleotide and amino acid sequences of Fpr2 and Fpr3/Alx are equally related to both FPR2/ALX and FPR3 (Gao & Murphy 1993; Takano *et al.* 1997; Gao *et al.* 1998; Wang & Ye 2002). In terms of their affinity for agonists, the mouse Fpr3 gene encodes the mouse lipoxin receptor (Fpr3/Alx), and since it shares this property with the human lipoxin receptor (FPR2/ALX), it can be said to be a functional orthologue (but not *the*). Whilst 15-epi-LXA₄ is certainly selective for Fpr3/Alx and FPR2/ALX, mouse Fpr2 appears to be a lower affinity receptor (Takano *et al.* 1997; Wang & Ye 2002). This is perhaps owing to the structural similarity between mouse Fpr3/Alx and Fpr2, and those with human FPR2/ALX (Vaughn *et al.* 2002). Other ligands which bind FPR2/ALX selectively, such as serum amyloid A and amyloid- β , also bind mouse Fpr2 (Ye *et al.* 2009). Conversely, the human FPR3-selective ligand F2L (Migeotte *et al.* 2005) binds specifically to mouse Fpr2 (Gao *et al.* 2007). Given this evidence, it seems plausible that the mouse Fpr subtypes Fpr2 and Fpr3/Alx are candidates for modulating pituitary and adrenal stress hormone release.

In summary, the structures of the Fpr/FPR subtypes are similar both within and between species, but this homology is not necessarily predictive of the functional orthologues of the human subtypes in rodents. Although ligand activities via a given human FPR subtype do, however, seem to be shared between multiple mouse subtypes. The inhibitory effects of GCs and ANXA1_{Ac2-26} on pituitary ACTH release, for instance, were the same in rat and mouse preparations, and these effects were unaffected by genetic knockout or pharmacological blockade of Fpr1 (John *et al.* 2007). Therefore, it would appear that pituitary ACTH release from glands derived from rats and mice at least, is modulated in a similar way by given Fpr/FPR ligands, particularly those which also bind human FPR2/ALX. This suggests the mechanism is conserved between species, in which case, finding ligands and Fpr subtypes that modulate stress hormone secretion in rodents could allow more specific targets to be investigated in human trials. The hypothesis that ANXA1 interacts with an Fpr/FPR subtype to regulate pituitary ACTH secretion has

not been refuted by any evidence to date. More research is needed, however, to delineate the primacy of the individual Fpr/FPR subtypes and whether their activity is predictive of pituitary ACTH and adrenal GC release. This is important because the Fprs/FPRs are targets for upregulation by inflammogens and HPA axis activators such as LPS (Section 1.6), and this research could therefore be of relevance to understanding the dissociation of pituitary-adrenal function evident in endotoxaemia or sepsis (Marik & Zaloga 2003; Mohn *et al.* 2011).

1.6 Effects of inflammogens on HPA axis activity, ANXA1, and the Fpr/FPR family

This section will describe the effect of experimental endotoxaemia on HPA axis function, and the mechanistic involvement of mediators such as ANXA1 and the Fpr/FPR family. This will build up to a discussion of the paradoxical effects of LPS on the expression of ANXA1 and Fprs/FPRs within the pituitary and adrenal gland, but not the hypothalamus and the hippocampus. Outside the HPA axis, there is also evidence for a similar effect of LPS within the host defence system.

Experimental models of endotoxaemia typically involve injection of an endotoxin such as LPS. Inflammogenic stressors such as LPS are known to induce cytokines like IL-6, TNF- α , and IL-1 β , as well as raising the circulating concentration of GCs (Spengler *et al.* 1990; Elenkov *et al.* 1995; Hasko *et al.* 1995). To investigate whether LPS-induced cytokines affect ANXA1 expression and activity, research groups have used animals with cytokines knocked out. In IL-6 knockout (IL-6 KO) mice, for instance, the increase in plasma GC concentration following LPS injection paralleled the wildtypes, but the IL-6 KO mice had higher TNF- α and IL-1 β levels. Recombinant ANXA1 administered to IL-6 KO mice before an LPS challenge induced less TNF- α release versus wildtypes, and recombinant IL-6 injections upregulated ANXA1 expression to wildtype levels. The authors concluded that ANXA1 is controlled by TNF- α and IL-6, and functions to resolve inflammation following systemic endotoxaemia through negative feedback on TNF- α release (de Coupade *et al.* 2001). This is supported by observations from ANXA1 KO mice, where LPS induced death within 48 hours unless low doses of exogenous ANXA1 were given. This lethality was preceded by impaired cytokine release, and increased leukocyte adhesion in the capillaries of ANXA1 KO mice (Damazo *et al.* 2005). Elsewhere, ANXA1 has been shown to inhibit LPS-induced cytokines (IL-6, TNF- α , IL-1 β) via glucocorticoid-induced leucine zipper (GILZ)-modulated signalling pathways. In addition, the expression of GILZ is in turn dependent on ANXA1 activity, which via GILZ, inhibits the activation of ERK, MAPK, and NF- κ B, leading to reduced translation of IL-6, TNF- α , and IL-1 β (Yang *et al.* 2009). Overall, the evidence suggests that the (moderately) FPR2/ALX-selective agonist ANXA1 mediates communication between the HPA axis and immune system in response to inflammogens such as LPS.

1.6.1 The role of pituitary ANXA1 and the Fpr/FPR family in response to an inflammogen

The effect of inflammogens such as LPS on pituitary function has been well characterised. The stimulatory action of LPS on pituitary ACTH secretion is mediated via the folliculostellate cells that release pro-inflammatory cytokines, namely, IL-6, which acts on neighbouring corticotrophic cells. Studies using a co-culture of two immortalised murine pituitary cell lines murine TtT/GF (folliculostellate-like cells) and AtT20 D16:16 (corticotroph-like), were used to determine the role of NF- κ B in mediating LPS-induced ACTH secretion. These cell lines express mRNAs for CD-14, TLR4, and TLR2, which are involved in LPS signalling. The stimulation of IL-6 and ACTH release, by both LPS and CRH, acting alone or synergistically, took place via an NF- κ B-mediated mechanism that was inhibited by GCs (Mehet *et al.* 2012). Following LPS treatment, ANXA1 is translocated to the cell surface and its cytoplasmic expression is later upregulated (Solito *et al.* 2006). The incubation of LPS with the folliculo-stellate-like TtT/GF cell line resulted in serine₂₇-phosphorylation of ANXA1 and its translocation to the extracellular membrane (Solito *et al.* 2006). Taken together, this suggests that LPS stimulates ACTH release via NF- κ B and IL-6 signalling, evoking increased secretion of GCs that inhibit ACTH release through ANXA1 phosphorylation.

1.6.2 The role of adrenal ANXA1 and the Fpr/FPR family in response to an inflammogen

The ANXA1-null mouse was used as a model to investigate the role of ANXA1 in adrenal steroidogenesis (Davies *et al.* 2007). The adrenal gland of wildtype littermates was found to contain an abundance of immunoreactive ANXA1 within the ZG of the outer cortex and the sub-capsular region, and was translocated to the extracellular surface by GC treatment for 3 h. Incubation of the cells with an N-terminal peptide portion of ANXA1 had an inhibitory effect on GC release, whereas the smaller adrenals from ANXA1-null mice had significantly higher ACTH-stimulated corticosterone release versus wildtype adrenals (Davies *et al.* 2007). In light of this work, this would suggest that ANXA1 is part of a mechanism that governs both adrenal size and overall steroidogenic capacity. During experimental endotoxaemia, however, LPS has interesting effects on the histological and functional characteristics of the adrenal gland of mice. As expected, the intraperitoneal administration of LPS induced an infiltration of leukocytes into the adrenal gland, but this was unaffected by deletion of either ANXA1 or Fpr/2/3 (Buss 2010). However, although LPS was associated with a loss of sterol vacuolation, this effect was

reduced in animals with a germ line deletion of ANXA1 or Fpr2/3 (Buss 2010). The loss of sterol vacuolation induced by LPS has been demonstrated elsewhere (Lomnitski *et al.* 2000; Kanczkowski *et al.* 2013), and also occurs following immobilisation stress (Pellegrini *et al.* 1998), possibly as a result of lipid peroxidation (Portoles *et al.* 1993). Taken together, these findings permit the hypothesis that blockade of ANXA1 and Fpr2/3 might prevent adrenal insufficiency during sepsis-related experimental endotoxaemia.

1.7 Hypothesis, aims, and objectives

While it is clear that the rodent Fpr family have a role in modulating pituitary-adrenal stress hormone responses to inflammogens such as LPS, relatively little is known about the individual roles of subtypes of this family. This project therefore aimed to advance knowledge on the expression of individual mouse/rat Fpr subtypes (with the closest structural and functional homology to the human FPR subtypes) within the pituitary-adrenal system and determine the functional significance of Fpr activation within pituitary and adrenal tissue.

Specifically, it tested the hypothesis, that formyl peptide receptor subtypes modulate both pituitary and adrenal function in terms of stress hormone (ACTH and corticosterone) secretion (following exposure to secretagogues CRH and ACTH₁₋₂₄ or the inflammogen LPS in rats), and their tissue distribution (mice and rats) will itself be upregulated following inflammogenic stress (LPS in mice and rats, multiple sclerosis in human tissue).

The principal objectives were to: 1) obtain morphometric evidence of the distribution of Fpr/FPR subtypes and stress hormones within the pituitary-adrenal axis from mouse, rat, and human tissues following inflammogenic stress; 2) perform tissue culture studies involving incubation of rat anterior pituitary and adrenal tissue with Fpr/FPR ligands to ascertain which Fpr/FPR subtypes modulate ACTH and corticosterone release; and 3) perform *in vivo* studies to determine whether an Fpr/FPR ligand modulates the effects of experimental endotoxaemia on cerebral, lymphoid, and pituitary-adrenal axis tissue morphology, and stress hormone secretion (see Figure 1.3)

Table 1.1 Percentage nucleotide sequence identity between subtypes of formyl peptide receptors/lipoxin receptors in humans (FPR/ALX), and mice and rats (Fpr/Alx).

	human FPR1	human FPR2/ALX	human FPR3
mouse Fpr1	77	68	56
mouse Fpr2	64	76	63
mouse Fpr3/Alx	59	73	60
rat Fpr1	77	73	68
rat Fpr2	71	77	71
rat Fpr2l/Alx	69	74	71
rat Fpr3	77	74	71

Table 1.2 Expression of formyl peptide receptors within human (FPR) and mouse (Fpr) tissues.

System	Tissue	FPR1	FPR2/ALX	FPR3	Fpr1	Fpr2	Fpr3/Alx	Fpr-rs3	Fpr-rs4	Fpr-rs6	Fpr-rs7	Fpr-rs8
Endocrine	adrenal	✓		✓	✓	✓	✓					
	anterior pituitary				✓	✓	✓					
	ovary	✓										
	placenta	✓	✓	✓								
	testis			✓						✓		
	pancreas										✓	
Immune	Dendritic cells (DCs)			✓	✓	✓						
	DCs - immature	✓	✓									
	macrophages	✓	✓	✓	✓							✓
	monocytes	✓	✓	✓								✓
	neutrophils	✓	✓		✓	✓	✓					✓
Lymphoid	bone marrow	✓	✓									✓
	spleen	✓	✓	✓	✓	✓	✓			✓	✓	✓
Nervous	hippocampus	✓			✓	✓	✓					
	hypothalamus	✓	✓	✓	✓	✓	✓					
	astrocytes	✓	✓									
	microglia	✓	✓		✓	✓	✓					
Other	liver	✓	✓	✓	✓		✓				✓	
	lung	✓	✓	✓	✓	✓	✓				✓	
	vomeronasal						✓	✓	✓	✓	✓	
References	(Becker <i>et al.</i> 1998; Czapiga <i>et al.</i> 2005) (Murphy <i>et al.</i> 1992; Durstin <i>et al.</i> 1994; Yang <i>et al.</i> 2002) (Durstin <i>et al.</i> 1999; Dai <i>et al.</i> 2005; Mandal <i>et al.</i> 2005; Buss 2010) (Gao <i>et al.</i> 1999; Hartt <i>et al.</i> 2005; Chen <i>et al.</i> 2010) (Iribarren <i>et al.</i> 1997; Gao <i>et al.</i> 1998) (Takano <i>et al.</i> 2009) (Rivière <i>et al.</i> 2009) (Liberles <i>et al.</i> 2002) (Wang & Ye 2002) (Wang & Ye 2002) (Tiffany <i>et al.</i> 2011)											

Table 1.3 Affinity data (pEC₅₀ / pK_d) for agonists binding to human (FPR) and rodent (Fpr) formyl peptide receptor subtypes.

	Human			Mouse			Rat		
	FPR1	FPR2 /ALX	FPR3	Fpr1	Fpr2	Fpr3 /Alx ¹	Fpr1	Fpr2	Fpr2l /Alx
fMLF	9.3 (Koo <i>et al.</i> 1982)	5.3 (Fiore & Serhan 1995)	x	7 (He <i>et al.</i> 2000)	5.3 (He <i>et al.</i> 2000)	x	7.5* (Marasco <i>et al.</i> 1983)		
WKYMVm	9 (Kang <i>et al.</i> 2005)	12 (Le <i>et al.</i> 1999)	8.5 (Christophe <i>et al.</i> 2001)	9.3 (He <i>et al.</i> 2013a)	10.1 (He <i>et al.</i> 2013a)	≈6 (He <i>et al.</i> 2013a)			
F2L	x (Migeotte <i>et al.</i> 2005)	6.3 (Migeotte <i>et al.</i> 2005)	8.2 (Migeotte <i>et al.</i> 2005)	x (Gao <i>et al.</i> 2007)	6.4 (Gao <i>et al.</i> 2007)	x (Gao <i>et al.</i> 2007)			
ANXA1	6.1 (Walther <i>et al.</i> 2000)	6.8 (Hayhoe <i>et al.</i> 2006)		6.6 (Perretti <i>et al.</i> 2001)	✓	✓		✓ (Pei <i>et al.</i> 2011)	
ANXA1_{Ac2-26}	5.9 (Hayhoe <i>et al.</i> 2006)	5.7 (Hayhoe <i>et al.</i> 2006)	5 ² (Ernst <i>et al.</i> 2004b)	✓	✓	✓		✓ (Pei <i>et al.</i> 2011)	6.1* (Chiang <i>et al.</i> 2003)
15-epi-LXA₄		7.8 (Chiang <i>et al.</i> 2006b)			✓ (Vaughn <i>et al.</i> 2002)	8.8* (Takano <i>et al.</i> 1997)			8.3* (Chiang <i>et al.</i> 2003)
pEC ₅₀ / pK _d	11+	9 – 10.9	7 – 8.9	5 – 6.9	< 5	* = pK _d	✓ = binding	x = no binding	= no data

¹ The murine lipoxin A₄ receptor (Alx) identified by (Takano *et al.* 1997) is identical to the product of Fpr3 (formerly, Fpr-rs1) identified by (Gao *et al.* 1998), whereas the LXAR-R (8C10) homologue identified by (Vaughn *et al.* 2002) is homologous to Fpr2 (formerly, Fpr-rs2) identified by (Gao *et al.* 1998).

² Authors used ANXA1_{Ac1-25}.

Table 1.4 Affinity data (pIC₅₀ / pK_d) for antagonists binding to human (FPR) and rodent (Fpr) formyl peptide receptor subtypes.

	Human			Mouse			Rat		
	FPR1	FPR2 /ALX	FPR3	Fpr1	Fpr2	Fpr3 /Alx ³	Fpr1	Fpr2	Fpr2l /Alx
WRW₄	≈5.1 (Stenfeldt <i>et al.</i> 2007)	6.7 (Bae <i>et al.</i> 2004; Stenfeldt <i>et al.</i> 2007)	< 5 (Shin <i>et al.</i> 2006)		6.6 (Önnheim <i>et al.</i> 2008)			6.9 (Braun <i>et al.</i> 2011)	
Boc1⁴	6.2 (Stenfeldt <i>et al.</i> 2007)	< 5 (Stenfeldt <i>et al.</i> 2007)							
Boc2	6.6 (Stenfeldt <i>et al.</i> 2007)	≈5.1 (Stenfeldt <i>et al.</i> 2007)		✓ (Gavins <i>et al.</i> 2003)	✓ (Gavins <i>et al.</i> 2003)				
Cyclosporin H	7.1 (Wenzel-Seifert & Seifert 1993)	< 5 (Stenfeldt <i>et al.</i> 2007)							
Cyclosporin A	6.1 (Yan <i>et al.</i> 2006)								
Spinorphin				≈4.5 (Liang <i>et al.</i> 2001)	X (Liang <i>et al.</i> 2001)	X (Liang <i>et al.</i> 2001)			
pIC ₅₀ / pK _d	11+	9 – 10.9	7 – 8.9	5 – 6.9	< 5	* = pK _d	✓ = binding	X = no binding	= no data

³ The murine lipoxin A₄ receptor (Alx) identified by (Takano *et al.* 1997) is identical to the product of Fpr3 (formerly, Fpr-rs1) identified by (Gao *et al.* 1998), whereas the LXAR-R (8C10) homologue identified by (Vaughn *et al.* 2002) is homologous to Fpr2 (formerly, Fpr-rs2) identified by (Gao *et al.* 1998).

⁴ Boc1 = Boc-Met-Leu-Phe (Boc-MLF), and Boc2 = Boc-Phe-Leu-Phe-Leu-Phe (Boc-FLFLF)

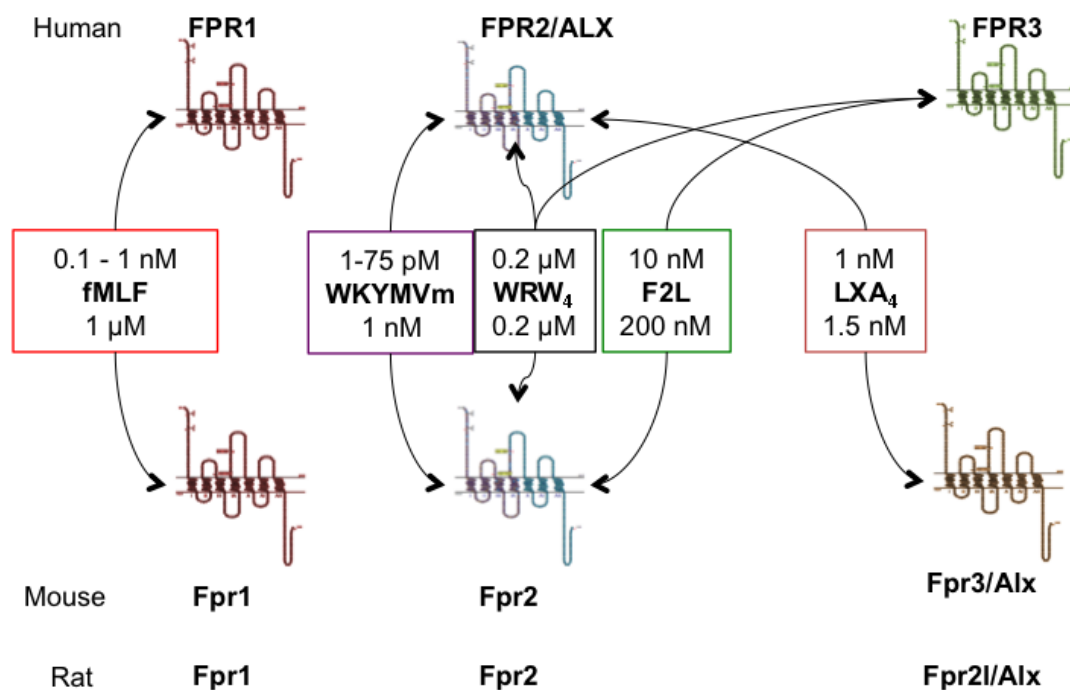


Figure 1.1 Pharmacology of the most selective ligands for the human formyl peptide receptor subtypes (FPR1, FPR2/ALX, and FPR3) and their closest functional counterparts in mice (Fpr1, Fpr2, Fpr3/Alx) and rats (Fpr1, Fpr2, Fpr2l/Alx).

The peptide formyl-methionyl-leucyl-phenylalanine (fMLF) is an FPR1/Fpr1-selective agonist with a 50% maximal effective concentration (EC_{50}) of 1 nmol.l^{-1} at FPR1 or 1 μmol.l^{-1} at Fpr1. The peptide tryptophan-lysine-tyrosine-methionine-valine-dextro-methionine (WKYMVm) is an agonist with an EC_{50} of $1\text{-}75 \text{ pmol.l}^{-1}$ at FPR2/ALX and 1 nmol.l^{-1} at Fpr2. The peptide tryptophan-arginine-tryptophan-tryptophan-tryptophan-tryptophan (WRW₄) is an antagonist with a 50% maximal inhibitory concentration (IC_{50}) of 0.2 μmol.l^{-1} for FPR2/ALX and Fpr2. The amino-acetylated haem-binding protein fragment (F2L) is an agonist with an EC_{50} of 10 nmol.l^{-1} for FPR3 and 200 nmol.l^{-1} for Fpr2. Lipoxin A₄ (LXA₄) is an eicosanoid agonist with an EC_{50} of 1 nmol.l^{-1} for FPR2/ALX and 1.5 nmol.l^{-1} for mouse Fpr3/Alx and similar for rat Fpr2l/Alx.

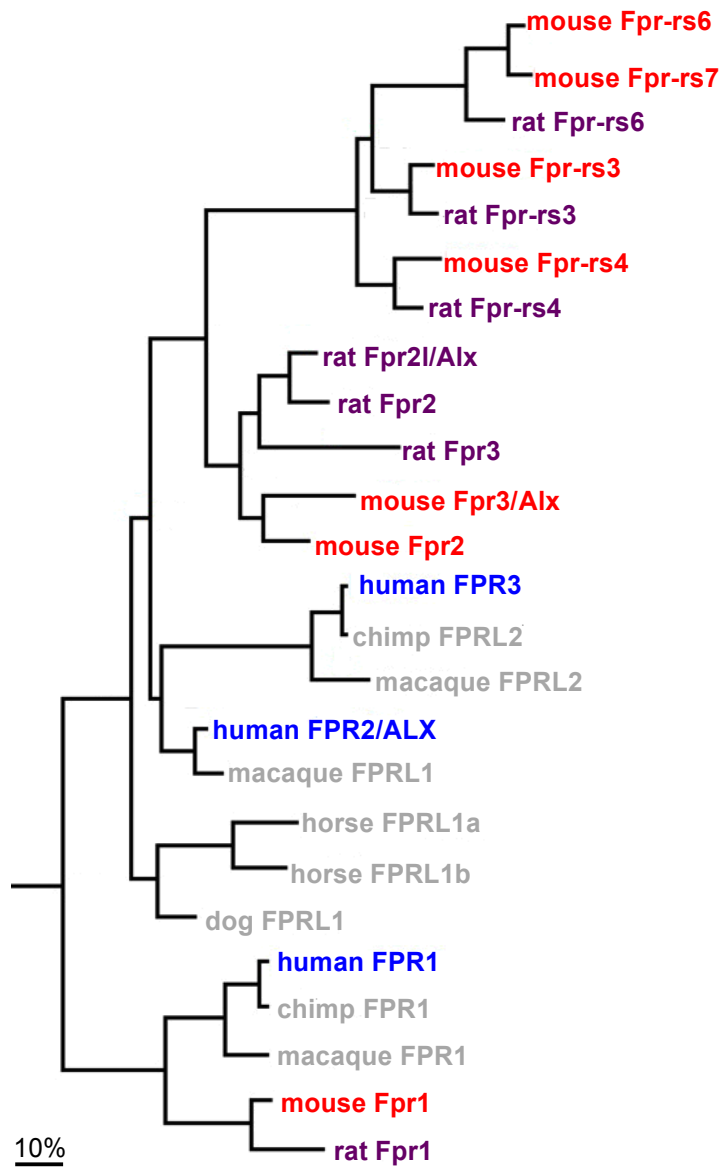


Figure 1.2 Phylogenetic tree of the mammalian formyl peptide receptor (Fpr/FPR) genes.

Branch points illustrate the evolution of genes through duplication or speciation events. Mouse Fpr subtypes are highlighted in red, rat in purple, and human FPR subtypes in blue. The upper rodent-specific branch is due to several duplication events. The scale bar length denotes the percentage of nucleotides substituted. Adapted from (Liberles *et al.* 2009).

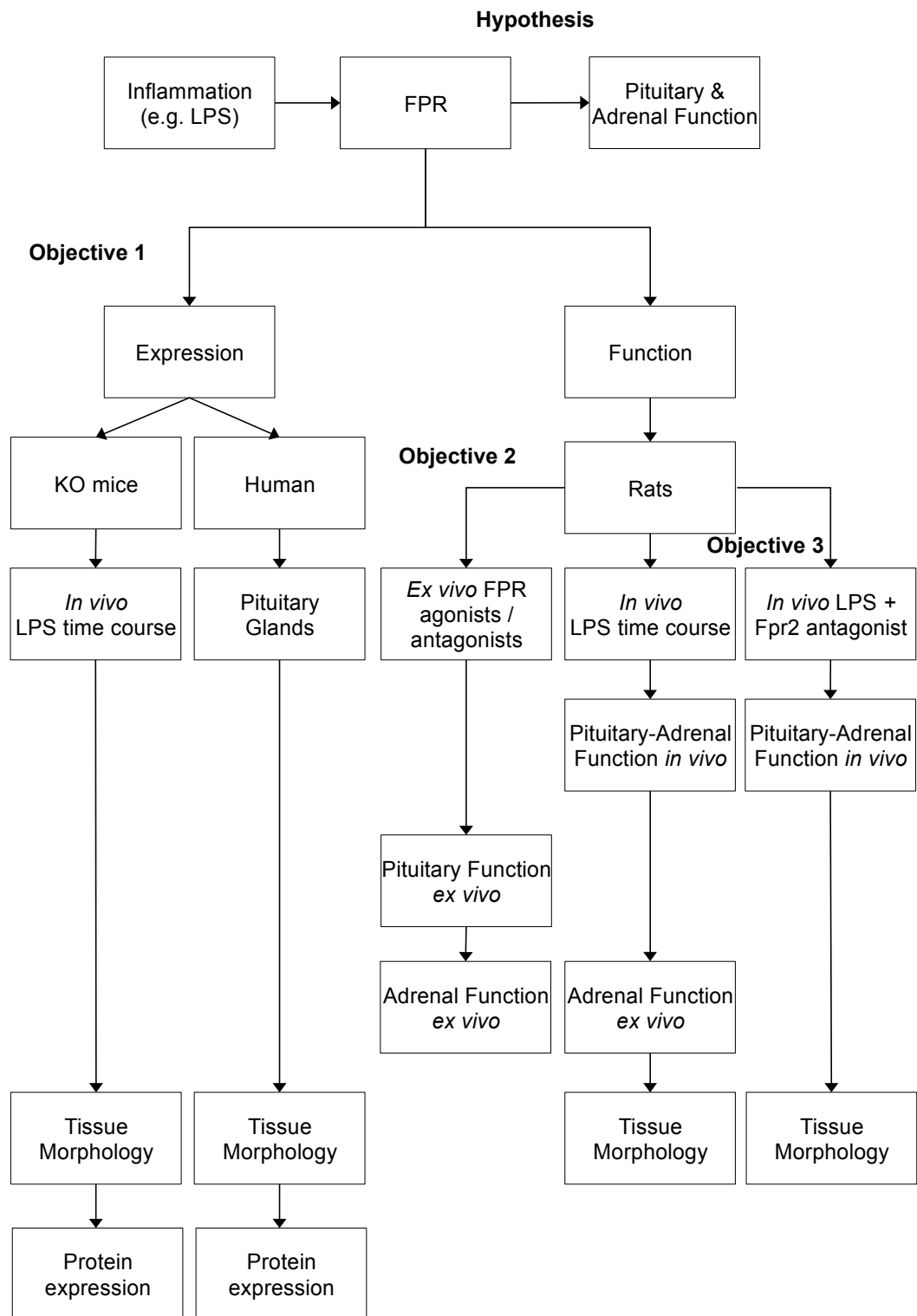


Figure 1.3 Overview of research objectives and the studies performed to meet them in addressing the hypothesis.

Chapter Two: **Materials and Methods**

2.1 Overview

Objective 1: Obtain morphometric evidence of the distribution of Fpr/FPR subtypes and stress hormones within the pituitary-adrenal axis from mouse, rat, and human tissues following inflammatory stress

In order to demonstrate whether proteins encoded at formyl peptide receptor 2 and 3 (Fpr2 and Fpr3) gene loci are translated within lymphoid (thymus, spleen), cerebral (hippocampal formation), and HPA axis tissues, green fluorescent protein (GFP)-transgenic (GFP^{+/+}), Fpr2 and Fpr3 knockout (Fpr2/3^{-/-}) mice were used. The Fpr2/3^{-/-} mice have a sequence encoding a pgk-neo cassette and GFP transgene inserted in-frame within the shared exon (exon 2) of the Fpr2 and Fpr3 gene loci (Dufton *et al.* 2011). In the absence of specific antibodies for murine Fpr2 and Fpr3, the detection of immunoreactive GFP translated from loci of the Fpr2/3 genes, provided a proxy indication of their translation within tissues from Fpr2/3^{-/-} mice. (Initial microscopic investigation of tissue sections from Fpr2/3^{-/-} mice found that native GFP fluorescence was not detectible). In order to determine whether an inflammogen induces such translation, and whether the genetic manipulation of the Fpr2/3 gene loci modulated the tissue response to the inflammogen, lipopolysaccharide (LPS) was injected intraperitoneally and tissues were examined histologically. LPS was the inflammogen of choice because it had been shown to upregulate the expression of Fpr2 and Fpr3 transcripts (Iribarren *et al.* 2005). Therefore, Fpr2/3^{-/-} mice and wildtype littermates received intraperitoneal (i.p.) injections of either LPS or vehicle (phosphate buffered saline, PBS) and killed 2, 4, and 6 hours later and perfusion fixed to preserve tissue morphology (Section 2.4.1). Wildtype littermates were used as a control for the genetic manipulation of the Fpr2/3^{-/-} mice so that differences in the tissue response to LPS could be attributed to these genes. Separate groups of mice receiving injections of PBS were used to control for the vehicle of LPS injections. Tissue sections from the wildtype littermates of Fpr2/3^{-/-}, and sections from an irrelevant GFP transgenic mouse were used as negative and positive controls for the immunohistochemical detection of GFP. The experimental design was done in collaboration with Bradley Spencer-Dene (London Research Institute) who also supervised the author, provided positive control tissue sections, and helped with the qualitative interpretation of slides.

To determine the distribution of human formyl peptide receptor (FPR) subtypes within the human anterior pituitary gland, such tissues were obtained from the United Kingdom Multiple Sclerosis (MS)

and Parkinson's disease (PD) tissue bank. All of the tissues were from diseased donors (MS and PD) as control tissues were not available at the time. The anterior pituitary glands were formalin fixed and embedded in paraffin, before sections were taken to enable immunohistochemical detection of FPR1, FPR2/ALX, and FPR3, and any colocalisation with adrenocorticotrophic hormone (ACTH) and S100 calcium binding protein b (S100b). The experimental design was done in collaboration with Bradley Spencer-Dene (London Research Institute) who also supervised the author, provided positive control tissues, and helped with the qualitative interpretation of slides. Federico Roncaroli (Imperial College London) also helped with the latter.

Objective 2: Perform tissue culture studies involving incubation of rat anterior pituitary and adrenal tissue with Fpr/FPR ligands to ascertain which Fpr/FPR subtypes modulate ACTH and corticosterone release

In order to ascertain which Fpr ligands modulate pituitary and adrenal function, glands were taken from male rats. Given the Home Office Project Licence restricted *in vivo* work on HPA axis function to rats and mice, rats were the species of choice primarily because they have larger glands than mice, and yield more dispersed cells with fewer animals. This was in keeping with the ethos of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs). Initial experiments involved pituitary glands from male Sprague-Dawley rats, but male Wistar rats were used in later pituitary and adrenal experiments that included the Fpr2-/FPR2/ALX-selective antagonist, Trp-Arg-Trp-Trp-Trp-Trp (WRW₄). This decision to change strain was made due to relocating from a lab that studied Sprague-Dawley rats to one that studied Wistar rats, and pituitary and adrenal glands were generally discarded in each lab. Therefore, for some pilot experiments, glands were obtained from untreated control animals studied by colleagues. Glands were dispersed enzymatically then cultured for between 2 and 72 hours, before 4 hours of contact with drugs. The dispersed rat anterior pituitary cells received corticotrophic releasing hormone (CRH) as a secretagogue for ACTH release, whereas the dispersed rat adrenal cells received ACTH₁₋₂₄ as a positive control for stimulated corticosterone (CORT) release. The cells were incubated (separately) with a range of chemically diverse Fpr agonists, alone, and in combination with secretagogue and/or WRW₄. The Fpr agonists were selected based on evidence of their relative affinity for structurally related human and mouse FPR/Fpr subtypes, and were the most selective

agonists available for these species (Section 2.2.1). At the time, however, little was known about the effect of these ligands on the rat Fpr subtypes but these were predicted to be structurally similar to the murine Fpr subtypes. Following 4 hours of contact with drugs, conditioned media were collected and frozen prior to radioimmunoassay (RIA, Section 2.6.1) and enzyme immunoassay (EIA, Section 2.6.6) of ACTH and corticosterone concentration, respectively.

Objective 3: Perform in vivo studies to determine whether an Fpr/FPR ligand modulates the effects of experimental endotoxaemia on cerebral, lymphoid, and pituitary-adrenal axis tissue morphology, and stress hormone secretion

Previous work had shown that the reduction in adrenal sterol vacuolation following LPS *in vivo* in wildtype mice was limited in Fpr2/3^{-/-} mice (Buss 2010). Therefore, in order to investigate whether pharmacological blockade of Fpr2 had any effect on the response to *in vivo* LPS, the Fpr2-/FPR2/ALX-selective antagonist WRW₄ was co-administered with LPS in male Wistar rats. To measure the effects of i.p. injections of LPS and WRW₄ on cerebral, lymphoid, and HPA axis tissue morphology, and pituitary and adrenal function, the animals were decapitated after 4 hours in order to collect tissues, namely, trunk blood (to assay plasma ACTH and corticosterone concentrations), and brain, thymus, spleen, and adrenal gland were collected, weighed, and post-fixed for histology. The four hour time-point was hypothesised to be the point at which maximal inhibition of adrenal function occurs *in vivo*, based on the inhibitory effect of LPS on release from dispersed rat adrenal cells *in vitro* after 4 hours in contact, and histological evidence of reduced sterol vacuolation 4 hours after LPS in Fpr2/3^{-/-} mice (Buss 2010).

To determine the effect of LPS on HPA axis activity as a function of time, rats received i.p. injections of LPS and were killed 0, 2, 4, and 24 hours later to obtain trunk blood for immunoradiometric (IRMA) assay and EIA of plasma ACTH and corticosterone concentration, respectively. Right adrenals were taken for primary culture to determine the effect of LPS *in vivo* on adrenal corticosterone release in response to graded concentrations of ACTH₁₋₂₄ *in vitro*. Remaining adrenals were collected along with thymus, brain, pituitary, and spleen for histological examination.

In order to determine whether pharmacological blockade of Fpr2 modifies the effect of LPS on HPA axis activity, rats received i.p. injections of the Fpr2-/FPR2/ALX-selective antagonist WRW₄, LPS, WRW₄

and LPS, or PBS, and were killed 4 hours later. Trunk blood was collected for ACTH and corticosterone determination. Adrenals were taken for primary culture, and the brain, thymus, and spleen were fixed for histology, following measurement of the mass of each tissue.

For detection of ACTH in plasma, IRMA is optimal for two-site detection of whole ACTH with negligible detection of fragments. Single-site RIA is sufficient for the detection of ACTH from *in vitro* experiments given that conditioned media contains fewer proteolytic enzymes such as pepsin, than in plasma or whole blood, therefore degradation of ACTH is less of an issue. Since RIA is well validated for detection of ACTH in conditioned media, whereas the IRMA kit was validated for detection of whole ACTH from plasma samples, each assay was used for these separate purposes.

2.2 Materials

2.2.1 Drugs

Corticotrophin releasing hormone (CRH) is a potent endogenous secretagogue of ACTH release, and a selective agonist for human CRH-receptor 1 (CRH-R1 $pEC_{50} = 9.6$), rat CRH-receptor 2 α (CRH-R2 α $pEC_{50} = 8.3$), and mouse CRH-receptor 2 β (CRH-R2 β $pEC_{50} = 8.5$) (Donaldson *et al.* 1996). CRH has an identical 41-amino acid sequence in humans, rats, and mice: Ser - Glu - Glu - Pro - Pro - Ile - Ser - Leu - Asp - Leu - Thr - Phe - His - Leu - Leu - Arg - Glu - Val - Leu - Glu - Met - Ala - Arg - Ala - Glu - Gln - Leu - Ala - Gln - Gln - Ala - His - Ser - Asn - Arg - Lys - Leu - Met - Glu - Ile - Ile -NH₂. Recombinant CRH (4757.45 g.mol⁻¹, Cat. C3042, Sigma, Poole, Dorset, UK) was diluted in water and stored at -20 °C in 10 μ mol.l⁻¹ aliquots, then used at 10⁻⁷ mol.l⁻¹ (pH 7.74).

N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF) is a potent peptide agonist for human FPR1 ($pEC_{50} = 9.3$ (Koo *et al.* 1982)), mouse Fpr1 ($pEC_{50} = 7$ (He *et al.* 2000)) and rat Fpr1 ($pK_d = 7.5$ (Marasco *et al.* 1983)). fMLF (437.55 g.mol⁻¹, Cat. F3506, Sigma, Poole, Dorset, UK) was diluted in 2.5% acetic acid and stored at -20 °C in 100 μ mol.l⁻¹ aliquots, then used at 10⁻⁵ mol.l⁻¹ (0.025% acetic acid, pH 6.88).

The amino terminal acetylated peptide fragment of haem-binding protein (1-21), F2L, is a selective agonist of human FPR3 ($pEC_{50} = 8.2$ (Migeotte *et al.* 2005)), and mouse Fpr2 ($pEC_{50} = 6.4$ (Gao *et al.* 2007)). There are no pharmacological data published for F2L and the rat Fpr subtypes. Recombinant human F2L (2478.99 g.mol⁻¹, Cat. 072-29, Phoenix Europe GmbH, Karlsruhe, Germany) with the following amino acid sequence: Ac - Met - Leu - Gly - Met - Ile - Lys - Asn - Ser - Leu - Phe - Gly - Ser - Val - Glu - Thr - Trp - Pro - Trp - Gln - Val - Leu was diluted in dimethyl sulphoxide (DMSO) and stored at -20 °C in 1200 nanomol.l⁻¹ aliquots, then used at 8 x 10⁻⁷ mol.l⁻¹ (0.4% DMSO, pH 7.81 for experiments including WRW₄; or 2.5% DMSO pH 8.31).

The hexapeptide NH₂-Trp-Lys-Tyr-Met-Val-D-Met-COOH (WKYMVm) is a synthetic agonist selective for human FPR2/ALX ($pEC_{50} = 12$ (Le *et al.* 1999)) and mouse Fpr2 ($pEC_{50} = 10.1$ (He *et al.* 2013a)). There are no pharmacological data published for WKYMVm and the rat Fpr subtypes. WKYMVm

(855.41 g.mol⁻¹, Cat. 1800, Tocris Bioscience, Abingdon, Oxfordshire, UK) was diluted in 2.5% acetic acid and stored at -20 °C in 100 µmol.l⁻¹ aliquots, then used at 10⁻⁶ mol.l⁻¹ (0.025% acetic acid, pH 6.88).

The nitrosyl terminal of annexin-A1 protein, ANXA1_{Ac2-26} is an agonist of human FPR2/ALX (pEC₅₀ = 6.8 (Hayhoe *et al.* 2006)) and FPR1 (pEC₅₀ = 6.1 (Walther *et al.* 2000)), and mouse Fpr1 (pEC₅₀ = 6.6 (Perretti *et al.* 2001)). There are no pharmacological data published for ANXA1_{Ac2-26} and the rat Fpr subtypes. ANXA1_{Ac2-26} (3089.46 g.mol⁻¹, Cat. 1845, Tocris Bioscience, Abingdon, Oxfordshire, UK) with the following amino acid sequence: Ac - Ala - Met - Val - Ser - Glu - Phe - Leu - Lys - Gln - Ala - Trp - Phe - Ile - Glu - Asn - Glu - Glu - Gln - Glu - Tyr - Val - Gln - Thr - Val - Lys, was diluted in water and stored at -20 °C in 30 µmol.l⁻¹ aliquots, then used at 3 x 10⁻⁵ mol.l⁻¹ (pH 7.74).

The carbon 15 epimer of lipoxin A₄, 15(R)-LXA₄, (5S,6R,15R)-5,6,15-Trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid (15-epi-lipoxin A₄), is a potent agonist of human FPR2/ALX (pEC₅₀ = 7.8 (Chiang *et al.* 2006b)), mouse Fpr3/Alx (pK_d = 8.8 (Takano *et al.* 1997)), and rat Fpr2 (pK_d = 8.3 (Chiang *et al.* 2003)). 15-epi-lipoxin A₄ (352.50 g.mol⁻¹, Cat. 437725, Calbiochem, Merck, Nottingham, UK) was diluted in 1.42% ethanol and stored at -20 °C in 1 µmol.l⁻¹ aliquots, then used at 10⁻⁷ mol.l⁻¹ (0.014% ethanol, pH 7.19).

Adrenocorticotrophic hormone 1-24 (ACTH₁₋₂₄) is a synthetic analogue of the endogenous 39-amino acid polypeptide ACTH and a full agonist that is selective for the mouse type 2 melanocortin receptor (pK_d = 9.1 (Kapas *et al.* 1996)). ACTH₁₋₂₄ is identical to the first 24 amino acids of ACTH in humans, rats, and mice: Ser₁-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro₂₄-Asn-**Val**₂₆-Ala-Glu-**Asn**₂₉-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe. The sequence of full-length human ACTH differs from these rodent species (Rodent → Human) only at amino acid positions 26 (Val → Gly) and 29 (Asn → Asp). ACTH₁₋₂₄, supplied as 1 mg.ml⁻¹ aliquots at 341 µmol.l⁻¹ (2933.44 g.mol⁻¹, tetracosactide acetate, Alliance Pharmaceuticals Ltd, Hammersmith Hospital Pharmacy, London, UK), was diluted in water immediately before use in culture medium (pH 7.74).

Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4, is an agonist for toll like receptor-4 (TLR₄, pEC₅₀ = 6.2 in the RAW 264.7 mouse macrophage cell line (Du *et al.* 1999)). LPS, purified by ion

exchange chromatography, (Cat. L3024, Sigma, Poole, Dorset, UK) was diluted in phosphate buffered saline and stored at -20 °C in 1 $\mu\text{mol.l}^{-1}$ aliquots, then used at 750 $\mu\text{g.kg}^{-1}$ (pH 7.74).

Trp-Arg-Trp-Trp-Trp (WRW₄) is a potent synthetic antagonist selective for human FPR2/ALX ($\text{pIC}_{50} = 6.7$ (Stenfeldt *et al.* 2007)), and mouse Fpr2 ($\text{pIC}_{50} = 6.6$ (Önnheim *et al.* 2008)). There are no pharmacological data published for WRW₄ and the rat Fpr subtypes. WRW₄ (1104.28 g.mol^{-1} , Cat. 2262, Tocris Bioscience, Abingdon, Oxfordshire, UK) was diluted in water and stored at -20 °C in 10 $\mu\text{mol.l}^{-1}$ aliquots, then used at 10^{-6} mol.l^{-1} (pH 7.74).

2.2.2 Human tissues

Human pituitary tissue samples were used to determine the immunohistochemical distribution and degree of colocalisation of FPR1, FPR2/ALX, and FPR3, with ACTH and S100b.

Human pituitary glands were donated under ethics committee approval by the UK Multiple Sclerosis (MS) Tissue Bank, funded by the Multiple Sclerosis Society of Great Britain and Northern Ireland (registered charity 207495) and supplied as formalin-fixed paraffin-embedded tissue blocks. All pituitaries were from diseased donors with either MS or Parkinson's disease (PD) (Table 2.1). Control tissues were requested but were not available at the time.

Table 2.1 Donor data for the human pituitary tissue samples

Case	Age y	Sex	Post-mortem delay h
MS469	52	F	47
MS476	67	M	31
PD404	84	M	48
PD418	70	M	22
PD419	83	M	36
PD420	77	M	32
PD422	75	M	39

2.3 Animals

All animals were used in accordance with the Animals (Scientific Procedures) Act 1986 under project licence PPL 70/6623 which permitted *in vivo* procedures on up to 500 rats and 500 mice. Optimal group sizes (n) were determined by power calculation (Equation 2.1), based on 95% power where SD = standard deviation, and Δ = expected difference to be observed, based on previous observations.

Equation 2.1

$$n = 2SD^2\left(\frac{13}{\Delta^2}\right)$$

2.3.1 *Fpr2* and *Fpr3* knockout (*Fpr2/3^{-/-}*) mice

2.3.1.1 Background

Fpr2 and *Fpr3* knockout (*Fpr2/3^{-/-}*) mice were generated as part of a collaborative project between Professors Roderick Flower and Mauro Perretti (William Harvey Research Institute, London), John Morris (University of Oxford), and Julia Buckingham (Imperial College London). The molecular biology behind these mice is reproduced below:

“Genomic clones containing *Fpr* sequences were isolated from a bacteriophage lambda library (129/SvJ; Stratagene, California, USA) by plaque hybridisation. Inserts from positive plaques were subcloned into pZero (Invitrogen, San Diego, California), end-sequenced and then aligned with the *Fpr* locus on Chromosome 17. A pgk-neo cassette was inserted into one of these clones (p2.1) just downstream of the ATG start codon for *Fpr2*, via site-specific recombination in bacteria. Using the plasmid pPGK-neo-FRT as a template for PCR with primers (forward 5'-TCA GAA GGA GCC AAA TAT CTG AGA AAT GGT TGT TTT TGA AAA CTT TCA GGT GCA GAC AAA ATG GCT AGC CCT TCT GCT TAA TTT GTG CCT GG, and reverse 5'-TGC TGT GAA AGA AAA GTC AGC CAA TGC TAG ATT CAG ATA CCA GAT AGT GGT GAC AGT GTG TGG CGT AGA GGA TCT GCL TCA TGT TTG AC) resulted in a 2.2 kilobase fragment containing the pgk-neo cassette in reverse orientation, flanked by 63 basepair arms with homology to *Fpr2*. This fragment was electroporated along with plasmid p2.1 into *E.coli* strain HS996 using the RED-ET subcloning kit supplied by Gene Bridges. The novel Nhe I site (gctage) located immediately after the ATG start codon in the forward primer was used for the subsequent in-frame insertion of GFP (Qbiogene, California, USA). All steps were confirmed by sequencing. The targeting vector was linearised by digestion with SnaBI and electroporated into ES cells (strain 129SvEv). Neomycin resistant colonies were selected and screened for correct insertion by Southern blotting using probes located beyond both the 5' and 3' ends of the vector arms, and also a probe for GFP. Clones showing homologous recombination into the *Fpr2* locus were

expanded, karyotyped by G-banding, and then injected into the blastocysts of C57Bl6 females (Caliper Life Sciences, Cambridge, MS). Male chimaeras showing greater than 95% agouti coat colour were paired with C57Bl6 females. F1 offspring were screened by PCR of tail clip DNA for germline transmission of the targeted allele using the Extract-N-Amp system (Sigma-Aldrich)" (Dufton *et al.* 2010).

Since publication of this molecular biology information, the official International Union of Basic and Clinical Pharmacology (IUPHAR) nomenclature of the human and mouse formyl peptide receptors was updated (Ye *et al.* 2009). This prompted the original report of the Fpr2 (formerly Fpr-rs2) knockout (Fpr2^{-/-}) genotype to be updated to a double knockout of both Fpr2 and Fpr3 (Fpr2/3^{-/-}) in a correction to the paper, in which their:

"targeting strategy would also delete Fpr3 itself because this gene also incorporates an exon found in Fpr2, and so insertion of the GFP reporter and the presence of the pgk-neo cassette in the cds (exon 2) of "Fpr-rs2" (as described in our paper) would prevent expression of this gene also. The transcription of the Fpr1 gene is unaffected" (Dufton *et al.* 2011).

2.3.1.2 Genotyping

To confirm the genotype of the animals, polymerase chain reaction (PCR) was performed on DNA isolates from ear clips (n = 6) taken from Fpr2/3^{-/-} mice and their wildtype littermates, and C57Bl6 mice. Purified DNA samples were then prepared using a DNA extraction kit (Nucleospin, Macherey-Nagel, Duren, Germany) and a PCR kit (AmpliTaQ Gold, Applied Biosystems, Foster City CA, Country), according to the manufacturer's instructions. Briefly, ear clips were lysed in 200 µl of 10% proteinase K/lysis buffer held in a water bath at 56 °C overnight. To each sample a further 200 µl of lysis buffer was added (70 °C, 10 min) plus 210 µl of 70% ethanol, before loading on to a column for centrifugation (11,000 rcf, 1 min). For each column, wash buffer was added twice, centrifuged, tube contents discarded, and centrifuged, and DNA eluted using elution buffer. The DNA concentration of each sample was checked by measuring the absorbance of ultraviolet light on a spectrophotometer (NanoDrop ND-1000 UV, NanoDrop Technologies).

The following primers were designed and manufactured (Sigma, Poole, UK): Fpr3 forward 5'-TAG AGG GAT CAT CAG GTT CA-3' and Fpr3 reverse 5'-TCT CTG GAG ACG AGA AGA CA-3'; Fpr2 forward 5'-ACT GTG AGC CTG GCT AGG AA and Fpr2 reverse 5'-CAG ACT TCA TGG GGC CTT TTA-3';

glyceraldehyde pyruvate dehydrogenase (GAPDH) forward 5'-AAG GTG AAG GTC GGA GTC AAC G-3' and GAPDH reverse 5'-GGC AGA GAT GAT GAC CCT TTT GGC-3'. On receipt, primers were reconstituted to 100 $\mu\text{mol.l}^{-1}$ in molecular grade water and stored (-20 °C) until diluted to 10 $\mu\text{mol.l}^{-1}$ stocks for PCR. For each PCR, 2 μl forward primer, 2 μl reverse primer, 25 μl AmpliTaq Gold PCR Mastermix, 1 μl DNA sample (0.1 μg), and molecular grade water were added to a total volume of 50 μl . Following a hot start Taq-polymerase activation step (5 min, 95 °C), there were 33 PCR cycles (30 s 92 °C, 30 s 54 °C, and 15 s 72 °C) followed by a final 10 min at 72 °C, and then held at 4 °C (Hybaid HBPX110 Thermal Cycler, Thermo Fisher Scientific, Loughborough, UK). 10 μl of each PCR product was then mixed with 3 μl Orange-G loading buffer, and 1 μl of 1-1000 kilobase ladder mixed with 9 μl water and 3 μl loading buffer. The samples and ladder were then loaded onto a 1% agarose gel containing ethidium bromide (1 $\mu\text{g/ml}$) in 0.5 mol.l^{-1} tris-borate-ethylenediaminetetraacetic acid (TBE) buffer and electrophoresed at 130 V for 45 min. The gel was then visualised using an ultraviolet gel documentation system.

2.3.1.3 Phenotyping

In order to confirm the genotyping, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot experiments were performed to detect Fpr2 in lung samples from Fpr2/3^{-/-} mice and wildtype littermates. The lung was chosen based on a report that Fpr2 was abundantly expressed in lung tissue (Dufton *et al.* 2010). The total tissue protein content was extracted and then quantified using the Bradford protein assay (Section 2.6.7). To detect Fpr2, an affinity-purified, rabbit polyclonal anti-human FPR2/anti-mouse Fpr2/3 IgG (rabbit anti-mouse Fpr2/3; sc-18191-R, Santa Cruz; Insight Biotech, Wembley, UK) was used in western blotting experiments following SDS-PAGE (Section 2.6.8).

2.3.2 Rats

Rats were used to determine the effects of: 1) an inflammogen and transcriptional upregulator of Fpr2 and Fpr3 expression, namely, LPS, on pituitary-adrenal axis activity *in vivo* (plasma ACTH and corticosterone concentration), the adrenal corticosterone response to ACTH₁₋₂₄ *in vitro*, and the histology of the brain, pituitary, adrenal, and lymphoid tissues (spleen and thymus), as a function of time (0, 2, 4, and 24 h); 2) a 4 h exposure to LPS and the Fpr2-/FPR2/ALX-selective antagonist WRW₄ on pituitary-

adrenal axis activity *in vivo* and histology of the brain, pituitary, adrenal, and lymphoid tissues; and 3) agonists with differential affinities for FPR1, FPR2/ALX, and FPR3, alone and in combination with the Fpr2-/FPR2/ALX-selective antagonist WRW₄ on secretagogue-stimulated and basal release of ACTH and corticosterone from dispersed anterior pituitary cells and adrenal cells *in vitro*.

During pilot experiments, adult male Sprague-Dawley rats were used to determine the effects of FPR1, FPR2/ALX, and FPR3 agonists on secretagogue-stimulated and basal release of ACTH from dispersed anterior pituitary cells. This initial choice of strain was based on three practical reasons. Firstly, Sprague-Dawley rats are conventionally used to investigate HPA axis function as they are thought to have low basal HPA axis activity in response to handling protocols versus the Fischer 344 strain, for instance (Dhabhar *et al.* 1993; Viau *et al.* 1993). Secondly, the supplier (Charles River Laboratories) offered a grant on this strain to graduates of the MRes Integrative Mammalian Biology course at Imperial College. Thirdly, at the time, the lab was affiliated with members of the Division of Cellular and Molecular Neuroscience whom mainly worked with Sprague-Dawley animals. The intention was to obtain pituitary and adrenal tissues from control animals of colleagues to reduce the number of animals required for *ex vivo* and *in vitro* work. The decision to switch to male Wistar rats was based on the lab relocating to the Section of Investigative Medicine where the majority of *in vivo* work was (is) with male Wistar rats.

Adult male Sprague-Dawley or Wistar rats weighing approximately 200 ± 20 g were used (Charles River Laboratories, Margate, Kent, UK). Upon arrival they were housed in groups of four per cage in a room with controlled lighting (lights on between 08:00 and 20:00) and temperature (21 ± 2 °C) with standard laboratory chow and water available *ad libitum* for at least 7 days prior to experiments. The same people handled rats each morning for a total of 5 days. *In vivo* experiments were always started between 08:00 and 09:30 to minimise circadian rhythm-associated variation in measurements. All procedures were carried out under license in accordance with the UK Animals (Scientific Procedures) Act, 1986.

2.4 In vivo methods

2.4.1 Time course of responses to intraperitoneal injection of lipopolysaccharide into male Fpr2/3^{-/-} mice and wildtype littermates

Male wildtype littermates and Fpr2/3^{-/-} mice (n = 4) received injections of either LPS (750 µg.kg⁻¹ i.p.) or saline (100 µl 0.9% NaCl i.p.) prior to CO₂ asphyxiation at 2, 4, and 6 hours later. The time course study design was adopted mainly to determine the time at which LPS maximally upregulated GFP expression. Transcardial injections of 50 ml PBS per mouse were given to clear the tissues of blood, and then perfusion-fixed with 50 ml of 4% paraformaldehyde (PFA, w/v PBS) per mouse. The cranial, peritoneal and cardio-thoracic cavities were exposed by dissection to allow rapid tissue penetration of fixative during whole-animal immersion in 250 ml of 10% formalin (CH₂O w/v ddH₂O) for 24 hours, after which the formalin was replaced with 70% ethanol (v/v ddH₂O). Tissues taken for embedding in paraffin included the brain, pituitary gland, thymus, spleen, and adrenal glands. Tissue sections (4 µm, performed by Bradley Spencer-Dene, Experimental Pathology, London Research Institute) were then taken on a microtome and placed onto slides ready for the IHC protocol (Section 2.5.1).

2.4.2 Time course of responses to intraperitoneal injection of lipopolysaccharide into male Wistar rats

Male Wistar rats (n = 4) received injections of LPS (750 µg.kg⁻¹ i.p.) using insulin myjector needles (Terumo, U-100 insulin, 0.5 ml syringe and needle), before decapitation at 2, 4, or 24 hours post-injection. Control rats (0 h) received no injection. The guillotine and sink below were washed before and after every decapitation to minimise the smell of blood in the procedure room. Blood (approximately 8 ml) was collected into EDTA-coated tubes and left on ice for up to 10 minutes. Tubes were centrifuged at 6000 rcf for 10 min to allow separation of the plasma (top layer) using disposable Pasteur pipettes. Plasma was then aliquoted into 3 Eppendorf tubes per sample (500 µl per tube) before storage at -80 °C for determination of ACTH and corticosterone concentration, by immunoradiometric assay (IRMA, Section 2.6.5) and EIA (Section 2.6.6), respectively. In addition to trunk blood, the following tissues were collected from each rat: brain, thymus, spleen, pituitary gland, and both adrenal glands, were weighed and masses recorded in grams to 3 decimal places (d.p.) along with body mass (grams, 2 d.p.). The left adrenal glands were placed into the buffer used for static incubation of dispersed adrenal cells with

ACTH₁₋₂₄ (Sections 2.6.2 and 2.6.2.2) and thus determine the variation in adrenal corticosterone release in response to ACTH₁₋₂₄ *in vitro* following i.p. LPS *in vivo* (Section 2.6.6). The thymus, spleen, and pituitary gland, were post-fixed in 10% neutral-buffered formalin. These tissues were then routinely formalin-fixed and paraffin-embedded (FFPE) into blocks (Paul Nya, Histopathology, Hammersmith Hospital) prior to taking 8 µm sections by microtome. The brains were mounted onto circular cork boards with OCT embedding compound (Bright Instruments, Huntingdon, UK), and held in freezing isopentane before snap-freezing in liquid nitrogen, after which they were stored at -80 °C until tissue sections (10 µm) were taken by cryostat.

2.4.3 Intraperitoneal injection of lipopolysaccharide and an Fpr2-/FPR2/ALX-selective antagonist (WRW₄) into male Wistar rats

Male Wistar rats (200 - 220 g, n = 6) were injected with LPS (750 µg.kg⁻¹ i.p.) or vehicle (PBS, i.p.), or the Fpr2-/FPR2/ALX-selective antagonist WRW₄ (250 µg.kg⁻¹ i.p.), or LPS and WRW₄ in combination. Needles and syringes were numbered with doses prepared for the body mass of a given rat, and volume-matched to 500 µl and refrigerated the night before administration. Drugs were dissolved in PBS and preparations were checked prior to the injection period to ensure the drugs had remained in solution. To minimise the influence of circadian variations, the experiment followed a schedule, with injections between 08:00 – 08:30 am, and tissue collection between 12:00 – 13:30 pm, accounting for a maximum lag of 90 minutes between the first and last injection. At 4 hours post-injection, the animals were decapitated. Adrenal glands were harvested into Earle's Balanced Salt Solution (EBSS) for primary culture. The brain, thymus, and spleen were isolated and placed into 4% paraformaldehyde (PFA) and then routinely formalin-fixed and paraffin-embedded (FFPE) into blocks (Paul Nya, Histopathology, Hammersmith Hospital) prior to taking 8 µm sections by microtome. Trunk blood was collected into EDTA-coated tubes, mixed, and centrifuged at 6000 rcf before separation of plasma (top layer) to determine the concentration of plasma ACTH (by IRMA, Section 2.6.5) and corticosterone (by EIA, Section 2.6.6).

2.5 Histological methods

2.5.1 *Fpr2/3^{-/-} mouse tissues: brain, pituitary, thymus, spleen, adrenal*

In order to determine the spatial distribution of Fpr2 and Fpr3 within mouse tissues, it was necessary to target the product of the green fluorescent protein (GFP) transgene inserted as a surrogate marker in the absence of the two receptors in Fpr2/3^{-/-} mice. Initial investigations found that the native fluorescence one would typically expect from GFP was either absent or negligible under microscopic analysis, and so this necessitated a means of signal amplification. For this reason, well-validated primary anti-GFP antibodies were used initially in non-fluorescent, immunohistochemical (IHC) experiments.

2.5.1.1 Immunohistochemical (IHC) staining for green fluorescent protein (GFP) within tissues from Fpr2/3^{-/-} mice and wildtype littermates

The formalin fixed tissues were embedded in a permanent preparation of agarose and paraffin before taking 4 µm sections on a sled microtome (Bright 8000 Microtome, Bright Instruments, Huntingdon, UK). Paraffin-embedded sections were then dewaxed in fresh xylene (3 x 3 min) then dehydrated in 100% industrial methylated spirit (IMS; 2 x 3 min) and rinsed in water (5 min) and then in PBS (2 x 5 min). The sections were microwaved (12 min, medium power, Panasonic NE1027 1000W model, Panasonic, Bracknell, UK) in boiling citrate buffer pH 6 (2.94 g tri-sodium citrate plus 22 ml 0.2 mol.l⁻¹ HCl in dd H₂O and cooled for 15 min at room temperature. The slides were placed briefly in PBS and then incubated sequentially with 1.6% H₂O₂ in PBS for 10 min (to block endogenous peroxidase) then in 0.3 % normal sera in PBS for 5 min before being subjected to three 5 minute washes in PBS then distilled water. The slides were drained and wiped, and the tissue sections were encircled with a hydrophobic fluid using a PAP pen, then placed into a humid incubator tray at room temperature. Normal serum (10% v/v in 1% BSA in PBS) from the species in which the secondary antibody was raised (e.g. donkey anti-rabbit required normal donkey serum) was applied to block non-specific background, and the slides were incubated for 60 min. The serum was then displaced without rinsing, before application of 20 - 200 µl primary antiserum to cover the section.

All antibodies were prepared in 1% BSA/PBS at varying dilutions (primary polyclonal goat anti-GFP antibody at 1/200 for 1 hour, Cat. ab6673, Abcam, Cambridge, UK). The antibody was drained from the

slides that were then washed three times in PBS for 5 minutes. Secondary antibody was then applied (donkey anti-goat-biotinylated antibody, 1/250 for 1 hour, Cat. 705-065-147, Jackson ImmunoResearch, Newmarket, UK). The avidin biotin complex (ABC, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, USA) solution was prepared and allowed to complex at room temperature for 30 min prior to use (one drop of A, one drop of B, in 5 ml PBS, where one drop = 35 μ l). Slides were drained of antibody and washed three times in PBS before addition of the ABC reagent and incubation for a further 30 min. The sections were then washed in PBS for 5 min. Diaminobenzidine (DAB) staining solution containing one 35 μ l drop of buffer, 70 μ l of DAB chromagen, and one 35 μ l drop of H₂O₂ in 2.5 ml PBS was prepared immediately prior to use. Sections were then incubated with DAB solution until observable stain intensity developed; typically after 2 - 5 min. Adjacent sections were then counterstained with a weak blue haematoxylin solution (1 min, room temperature) and mounted with DPX-coated coverslips.

2.5.1.2 Double immunofluorescence (IF) staining for GFP and cell lineage markers within pituitary glands from *Fpr2/3*^{-/-} mice and wildtype littermates

In order to map the GFP distribution to markers of specific cell lineages within the anterior pituitary gland, an immunofluorescence (IF) approach was taken because of the ease at which a multitude of IF probes can be detected simultaneously from complexes of secondary and primary antibodies bound to epitopes within a given tissue section, without compromising signal amplification. Thus, the IF approach permitted simultaneous staining of GFP (as a proxy of *Fpr2/Fpr3*) and markers of anterior pituitary cell lineage, namely, S100b (folliculostellates), ACTH (corticotrophic), GH (somatotrophic), LH (gonadotrophic), PRL (lactotrophic), and TSH (thyrotrophic).

Sections were stained using either a goat polyclonal anti-GFP (GxGFP; ab6673, Abcam, Cambridge, UK) or a rabbit polyclonal anti-GFP (RxGFP; ab6556, Abcam, Cambridge, UK), each used at 1 in 200 v/v in PBS, and counterstained adjacent sections with haematoxylin. In order to co-stain GFP with anterior pituitary hormones, antibodies for GFP (goat and rabbit) were raised in different species from the hormone antibodies, to minimise cross-reactivity (Table 2.2).

For each stain there were three slides, each with two pituitary sections, and each section requiring 100 μ l of primary antibody mixture, prepared as follows: for GH/GFP stains, a 300 μ l mixture of rabbit anti-GH

(1 in 250 v/v, 1.2 µl), goat anti-GFP (1 in 200 v/v, 1.5 µl) and 297.3 µl 1% BSA/PBS; for ACTH/GFP stains, a 300 µl mixture of rabbit anti-ACTH (1 in 1000 v/v, 0.3 µl), goat anti-GFP (1 in 200 v/v, 1.5 µl) and 298.2 µl 1% BSA/PBS; for PRL/GFP stains, a 300 µl mixture of rabbit anti-PRL (1 in 500 v/v, 0.6 µl), goat anti-GFP (1 in 200 v/v, 1.5 µl) and 297.9 µl 1% BSA/PBS; for LH/GFP stains, a 300 µl mixture of rabbit anti-LH (1 in 100 v/v, 3.0 µl), goat anti-GFP (1 in 200 v/v, 1.5 µl) and 295.5 µl 1% BSA/PBS; for TSH/GFP stains, a 300 µl mixture of guinea pig anti-TSH (1 in 100 v/v, 3.0 µl), rabbit anti-GFP (1 in 200 v/v, 1.5 µl) and 295.5 µl 1% BSA/PBS.

For single stains of GFP, rabbit anti-GFP only slides received 100 µl of antibody mixture (RxGFP, 1 in 200 v/v, 0.5 µl in 499.5 µl 1% BSA/PBS) and goat-anti-GFP only slides also received 100 µl of antibody mixture (GxGFP, 1 in 200 v/v, 0.5 µl in 499.5 µl 1% BSA/PBS). For no primary controls, each slide received 100 µl of the secondary antibody, and for no secondary controls, each slide received 100 µl of the antibody diluent (1% BSA/PBS).

Each primary incubation lasted 1 hours at room temperature and was followed by 3 x 5 min washes in PBS. Secondary antibodies were also prepared in 1% BSA/PBS and secondary incubations lasted 45 min at room temperature. Secondary antibody mixtures (1200 µl) for GFP/GH, GFP/ACTH, GFP/PRL, and GFP/LH were made up of donkey anti-rabbit⁴⁸⁸ (1 in 300 v/v, 4.0 µl) and donkey anti-goat^{biotin/streptavidin}⁵⁵⁵ (1 in 300 v/v, 4.0 µl) and 1196 µl 1% BSA/PBS. The secondary antibody mixture for GFP/TSH was made up of goat anti-guinea pig⁴⁸⁸ (1 in 300 v/v, 0.3 µl) and donkey anti-rabbit⁴⁸⁸ (1 in 300 v/v, 0.3 µl) and 199.4 µl 1% BSA/PBS.

Table 2.2 Antibodies used in immunofluorescence (IF) experiments with Fpr2/3^{-/-} mouse tissue and wildtype littermates.

Superscripts denote the fluorochromes Alexa Fluor⁴⁸⁸ (green), and Alexa Fluor⁵⁴⁶ and Alex Fluor⁵⁵⁵ (red).

Primary	Dilution	Secondary/tertiary complex	Dilution
Goat anti-GFP (ab6673, Abcam)	1/200	Donkey anti-goat ^{biotin} /streptavidin ⁵⁵⁵ (ab7124, Abcam; S21381, Invitrogen)	1/300
Rabbit anti-GFP (ab6556, Abcam)	1/1000	Donkey anti-rabbit ⁵⁴⁶ (A21206, Invitrogen)	1/300
Rabbit anti-ACTH (AFP6328031, NIDDK)	1/1000	Donkey anti-rabbit ⁴⁸⁸ (A21206, Invitrogen)	1/300
Rabbit anti-LH (AFP-10250C, NIDDK)	1/100	Donkey anti-rabbit ⁴⁸⁸ (A21206, Invitrogen)	1/300
Rabbit anti-PRL (AFP-131581570, NIDDK)	1/500	Donkey anti-rabbit ⁴⁸⁸ (A21206, Invitrogen)	1/300
Rabbit anti-GH (AFP5672099, NIDDK)	1/250	Donkey anti-rabbit ⁴⁸⁸ (A21206, Invitrogen)	1/300
Guinea pig anti-TSH (AFP329691Rb, NIDDK)	1/100	Goat anti-guinea pig ⁴⁸⁸ (ab6966, Abcam)	1/300
Rabbit anti-S100b (ab4066, Abcam)	1/70	Donkey anti-rabbit ⁴⁸⁸ (A21206, Invitrogen)	1/300

2.5.2 Human pituitary

Human pituitaries (n = 6) were formalin-fixed and paraffin-embedded before 4 µm sections were taken by microtome and placed onto slides. In order to immunostain the sections, the following antibodies and dilutions were used using a protocol similar to that described in Section 2.5.1.2:

- Mouse anti-ACTH monoclonal (ab21003, Abcam), 1/300, 60 min
- Mouse anti-S100b monoclonal (SAB1404351, Sigma), 1/35, 60 min
- Rabbit anti-human FPR1 (ab113531, Abcam), 1/200, 60 min
- Rabbit anti-human FPR2/ALX (HPA029154, Atlas) 1/75, 60 min
- Rabbit anti-human FPR3 (NLS3735 Novus Biologicals), 1/35, 60 min
- Donkey anti-mouse Alexa Fluor 488 (Invitrogen), 1/150, 60 min
- Donkey anti-rabbit Alexa Fluor 555 (Invitrogen), 1/150, 60 min

Confocal images (pinhole = airy 1) of the medial area between the lateral wings of the anterior pituitary (9 x 246 µm areas per section, minimum three sections per subject) were acquired on a confocal laser-scanning microscope (Leica SP5) with a 63.3 objective at 1024 by 1024 pixel resolution.

The anterior pituitary was reconstructed in 3D stacks of images (step size = 0.14 µm) and then analysed using Fiji is Just Image J software with the LOCI BioFormats Import plugin and the Just Another Colocalisation Plugin (JACoP). The confocal images were used to establish whether an individual corticotroph or folliculostellate was co-immunoreactive for lineage markers ACTH or S100b, with FPR1/FPR2/FPR3 subtypes, according to the following criteria: 1) a given corticotrophic cell had clear staining of ACTH and a nucleus; 2) a given folliculostellate cell had clear staining of S100b and a nucleus; 3) co-immunoreactive cells met criterion 1) or 2), and also had evidence of cytoplasmic or membranous staining of FPR1/FPR2/FPR3.

2.5.3 Wistar rat tissues: brain, pituitary, thymus, spleen, adrenal

Tissues were processed into FFPE tissue blocks by Paul Nya (Histopathology, Hammersmith Hospital). Sections (8 µm) of each tissue were taken by microtome and floated in water onto microscope slides before drying (37 °C, 20 min). Sections were deparaffinised in xylene for 5 min initially, and a further

three times (2 min). Sections were then dehydrated in 100%, 75%, and 50% ethanol, in series for 2 min each, and then washed in distilled water (3 min).

2.5.3.1 Haematoxylin & Eosin (H&E) Staining

Sections were covered with Gill's No1 haematoxylin for 75 s, then gently rinsed with (sections facing away from) running tap water for 10 min. Eosin Y solution (5% w/v dH₂O) was acidified with acetic acid (50 µl per 200 ml) until a brilliant red develops from dark red. Acidified Eosin Y solution was then added to the slides for 2 min to detect neutrophils, or 7 min to increase staining of eosinophils, and thus differentiate from other polymorphonuclear leukocytes (PMNs). Sections were then rinse in running tap water (4 s), dehydrated in 75% ethanol for 30 s, twice, then 100% ethanol for 30s, twice. Sections were then covered in xylene for 1 min, twice, before mounting coverslips to the slides with DPX. Slides were evaluated using a light microscope, whereby the cytoplasm is stained pink, nuclei dark blue, red blood cells orange, and fibrin deep pink.

2.5.3.2 Congo Red staining

By hydrogen bonding of amine groups to hydroxyl radicals within eosinophils, Congo Red stains cytoplasmic granules of eosinophils an orange-red colour (Meyerholz *et al.* 2009). Sections were covered in Gill's No1 haematoxylin for 75 s. Sections were rinsed with distilled water to remove excess haematoxylin. A solution of 0.5% Congo Red in 50% ethanol was prepared (pH 8.2). Sections were covered in the 0.5% Congo Red solution for 5 min, then rinsed with distilled water. Sections were then left to dry, and coverslips mounted with DPX. Slides were evaluated using a light microscope.

2.6 *In vitro* methods

2.6.1 Primary culture of dispersed anterior pituitary cells from rats

Male Sprague-Dawley or Wistar rats (200 – 220 g) were handled daily (11:00 am – 12:00 pm), to habituate them to a tight grip, and thus limit the effect of handling on the stress response at the time of culling ($t = 0$ h). Animals were decapitated individually and the skull was opened bilaterally using bone crushing forceps and the brain tilted back to expose the pituitary in the sphenoid bone posterior to the optic chiasm. Using sharp forceps, the posterior lobe of the pituitary was dissected *in situ* and discarded. By loosening the connective tissue that straddles the gland, the anterior pituitary was then collected into ice-cold culture medium (10 ml per 10 glands).

The sterile culture medium was prepared within a class II microbiological safety cabinet the day before culling with: 500 ml Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Paisley, UK) containing 4.5 g.l⁻¹ D-glucose and 44 mmol.l⁻¹ NaHCO₃, 111 μ mol.l⁻¹ sodium pyruvate and 4 mmol.l⁻¹ L-alanyl-L-glutamine (Invitrogen, Paisley, UK); 5 ml non-essential amino acids containing: 100 μ mol.l⁻¹ L-alanine, 98 μ mol.l⁻¹ L-asparagine, 8.8 μ mol.l⁻¹ L-aspartic acid, 10 μ mol.l⁻¹ L-glutamic acid, 1 μ mol.l⁻¹ glycine, 10 mmol.l⁻¹ L-proline, and 10 μ mol.l⁻¹ L-serine (Invitrogen, Paisley, UK); 50 ml (10% v/v working) fetal calf serum (FCS, Invitrogen, Paisley, UK); 5 ml penicillin-streptomycin (mixture of penicillin at 100 IU/ml) and streptomycin at 100 mg.l⁻¹ stock); 1.5 g (0.3% w/v working) bovine serum albumin (BSA); 5 ml insulin transferrin sodium selenite (ITS, mixture of 5 mg.l⁻¹ insulin, 5 mg.l⁻¹ transferrin, and sodium selenite 116 μ mol.l⁻¹), and 0.5 μ mol.l⁻¹ tri-iodo-L-thyronine sodium salt (T₃, 30 pmol.l⁻¹).

Blood was washed from the glands by aspiration in a shortened 1 ml pipette tip, after which small blocks of tissue (0.5 mm length) were produced using a sterile razor blade on the back of an autoclaved coplin lid. The pituitaries were centrifuged in culture medium (800 rcf 5 min, 4 °C, and the pellet resuspended in a dissociating medium containing trypsin (0.25% v/v culture medium) before agitation (37 °C, 800 rcf, 25 min). The medium was then supplemented with trypsin inhibitor (0.1 mg.ml⁻¹; and DNaseI (0.01% w/v; Sigma, Poole, UK) before centrifugation (700 rcf, 5 min, 4 °C). The pellet was resuspended in 50 ml ice-cold medium before filtration through a 40 μ m pore nylon mesh (BD, Franklin Lakes NJ, USA). A final centrifugation (700 rcf, 5 min, 4 °C) was followed by four medium changes, before taking a cell-viability

count using a haemocytometer (Kova Slide 10, Hycor Biomedical, Penicuik, UK) and trypan blue (0.4% v/v stock) to identify dead cells (Section 2.6.3). Cells were plated at an equal density (10^5 cells/ml) in 24-well or 96-well plates in medium and incubated at 95% O₂, 5% CO₂, 37 °C. At t = 24 hours, the medium was removed from the cells for washing in PBS (2 ml per well) before replacing with fresh medium until t = 48 hours when the cells were incubated with low serum medium (1% FCS in DMEM). At t = 72 hours, the medium was aspirated and the cells washed twice with PBS and were then incubated with drugs for 4 h.

2.6.1.1 Static incubation of dispersed anterior pituitary cells from untreated male Sprague-Dawley rats with CRH and graded concentrations of Fpr agonists

Dispersed anterior pituitary cells from untreated male Sprague-Dawley rats (n = 4 wells from a cell suspension of 20 pooled glands) were plated onto 24-well plates and incubated for 4 hours in the presence or absence of the ACTH secretagogue CRH (100 nmol.l⁻¹ after (Mehet 2007)) with or without one of the following drugs for each experiment: 1) the Fpr1-/FPR1-selective agonist fMLF (10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , and 10^{-5} mol.l⁻¹); 2) the Fpr2-/FPR2/ALX-selective agonist WKYMVm (10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , and 10^{-6} mol.l⁻¹); 3) the Fpr2-/FPR3-selective agonist F2L (0.5, 1, 2, 4, and 8×10^{-7} mol.l⁻¹), and 4) the Fpr3/Alx-/FPR2/ALX-selective agonist, 15-epi-lipoxin A₄ (10^{-10} , 10^{-9} , 10^{-8} , and 10^{-7} mol.l⁻¹). The conditioned media were collected into separate tubes and stored (-80 °C) prior to RIA of ACTH (Section 2.6.1).

2.6.1.2 Static incubation of dispersed anterior pituitary cells from untreated male Wistar rats with CRH and/or the Fpr2-/FPR2/ALX-selective antagonist WRW₄ and graded concentrations of Fpr agonists

Dispersed anterior pituitary cells from untreated male Wistar rats (n = 6 wells per condition, from a cell suspension of 20 pooled glands) were plated onto 96-well plates and incubated 4 hours in the presence or absence of the ACTH secretagogue CRH (100 nmol.l⁻¹) and/or the Fpr2-/FPR2/ALX-selective antagonist WRW₄ (10^{-6} mol.l⁻¹) with or without one of the following drugs for each experiment: 1) the Fpr1-/FPR1-selective agonist fMLF (10^{-7} , 10^{-6} , and 10^{-5} mol.l⁻¹); 2) the Fpr2-/FPR2/ALX-selective agonist WKYMVm (10^{-8} , 10^{-7} , and 10^{-6} mol.l⁻¹); 3) the Fpr2-/FPR3-selective agonist F2L (4, 40, and 400 nmol.l⁻¹); 4) the Fpr3/Alx-/FPR2/ALX-selective agonist, 15-epi-lipoxin A₄ (10^{-9} , 10^{-8} , and 10^{-7} mol.l⁻¹), and 5)

the non-selective Fpr agonist, ANXA1_{Ac2-26} (3×10^{-7} , 3×10^{-6} , and 3×10^{-5} mol.l⁻¹). The conditioned media were collected into separate tubes and stored (-80 °C) prior to RIA of ACTH (Section 2.6.1).

2.6.2 Static incubation of dispersed adrenal cells from rats

Adrenal glands from male Wistar rats were collected into 10 ml of Earle's Balanced Salt Solution (EBSS; with 2.2 g.l⁻¹ sodium bicarbonate, pH 7.4 (Cat. E3024, Sigma, Poole, Dorset, UK)), which had been pre-gassed with 95% O₂, 5% CO₂. EBSS provided a buffering system to maintain a physiological pH (7.2 - 7.6). One litre of EBSS also contained: .265 g CaCl₂.2H₂O, .098 g MgSO₄, .4 g KCl, 2.2 g NaHCO₃, 6.8 g NaCl, and 0.122 g NaH₂PO₄. Samples were kept at room temperature until dispersion, which was conducted immediately following sampling. The adrenals were cut into small pieces (1 mm length) using a razor blade and then transferred into 2 ml of EBSS with collagenase from *Clostridium histolyticum* (Collagenase, Cat. 2674, Sigma, Poole, Dorset, UK) used at 2 mg.ml⁻¹.

The end of a 1 ml pipette tip was cut short to facilitate dispersion of cells by aspiration every 10 minutes for 1 hour, along with gassing with 95% O₂, 5% CO₂. The cell suspension was filtered through a 100 µm gauze into 50 ml Falcon tubes. The filtrate was centrifuged at 1000 rcf for 15 minutes. The pellet was resuspended (5 ml) and centrifuged again as above. The pellet was resuspended in 5 ml prior to counting cells using a haemocytometer for a target of 10⁶ cells/ml (Section 2.6.3).

Cells were then plated into 96-well plates (200 µl per well) and incubated for 1 hour (95% O₂/5% CO₂, 37 °C), prior to the addition of drugs. Drugs were prepared in EBSS immediately prior to the end of this incubation period. Plates of cells were then centrifuged at 2000 rcf for 10 s, before inverting and blotting supernatant onto tissue paper. The drugs were diluted to working concentration in EBSS and were then added to each well (300 µl per well) for a contact period of 4 h. The plates were then centrifuged as above and conditioned media removed to assay corticosterone by EIA (Section 2.6.6) and stored at -20 °C.

2.6.2.1 Static incubation of ACTH₁₋₂₄ with dispersed adrenals from untreated male Wistar rats

Dispersed adrenal cells from untreated male Wistar rats (n = 8 wells per condition, from a cell suspension of 12 glands) were incubated for 4 hours in the presence or absence of graded concentrations of the corticosterone secretagogue ACTH₁₋₂₄ (10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} mol.l⁻¹). The conditioned

media were collected into separate tubes and stored (-80 °C) prior to EIA of corticosterone (Section 2.6.6).

2.6.2.2 Static incubation of ACTH₁₋₂₄ with dispersed adrenals from male Wistar rats injected with LPS (2, 4, 24 h)

Dispersed adrenal cells from male Wistar rats either injected with LPS (750 µg/kg i.p.) or untreated (n = 8 wells per condition, from a cell suspension of 6 glands) were incubated for 4 hours in the presence or absence of graded concentrations of the corticosterone secretagogue ACTH₁₋₂₄ (10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} , and 10^{-7} nmol.l⁻¹). The conditioned media were collected into separate tubes and stored (-80 °C) prior to EIA of corticosterone (Section 2.6.6).

2.6.2.3 Static incubation of ACTH₁₋₂₄ and Fpr agonists and the Fpr2-/FPR2/ALX-selective antagonist WRW₄ with dispersed adrenals from untreated male Wistar rats

Dispersed adrenal cells from untreated male Wistar rats (n = 8 wells per condition, from a cell suspension of 12 glands) were incubated for 4 hours in the presence or absence of the corticosterone secretagogue ACTH₁₋₂₄ (100 nmol.l⁻¹) and/or the Fpr2-/FPR2/ALX-selective antagonist WRW₄ (10^{-6} mol.l⁻¹) with or without one of the following drugs for each experiment: 1) the Fpr1-/FPR1-selective agonist fMLF (10^{-7} , 10^{-6} , and 10^{-5} mol.l⁻¹); 2) the Fpr2-/FPR2/ALX-selective agonist WKYMVm (10^{-8} , 10^{-7} , and 10^{-6} mol.l⁻¹); 3) the Fpr2-/FPR3-selective agonist F2L (4, 40, and 400 nmol.l⁻¹); 4) the Fpr3/Alx-/FPR2/ALX-selective agonist, 15-epi-lipoxin A₄ (10^{-9} , 10^{-8} , and 10^{-7}), and 5) the non-selective Fpr agonist, ANXA1_{Ac2-26} (3×10^{-7} , 3×10^{-6} , and 3×10^{-5} mol.l⁻¹). The conditioned media were collected into separate tubes and stored (-80 °C) prior to EIA of corticosterone (Section 2.6.6).

2.6.3 Cell counts and viability determination

One volume of culture medium containing primary cells was counted by mixing with one volume of 0.4% trypan blue and incubated for 3 minutes at room temperature. A 10 µl drop of the mixture was applied to a disposable haemocytometer (Kova Slide II; Cat. 87100, Hycor Biomedical, Edinburgh, UK) and analysed under a microscope. The unstained (viable) and blue-stained (nonviable) cells were counted separately, and the percentage of viable cells was always at least 95%, as calculated by (Equation 2.2).

Equation 2.2

$$\text{viable cells (\%)} = \frac{\text{total number of viable cells per ml of aliquot}}{\text{total number of cells per ml of aliquot}} \times 100$$

2.6.4 Radioimmunoassay of ACTH

ACTH from the *in vitro* studies was assayed by one-site radioimmunoassay (RIA) using reagents supplied by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda MD, USA) (Nicholson *et al.* 1984). The results were expressed as pictograms/ml in reference to a standard of human ACTH (AFP-2938C, NIDDK, Bethesda MD, USA). A standard curve was made by double dilution of 10000 pg.ml⁻¹ ACTH peptide down to 10 pg.ml⁻¹ in 200 µl assay buffer, containing: 12.7 mmol.l⁻¹ EDTA; 68.2 mmol.l⁻¹ disodium hydrogen phosphate (Na₂HPO₄); 3 mmol.l⁻¹ sodium azide (NaN₃); 1% Triton X-100; dithiothreitol (DTT); and 0.033% bovine serum albumin (BSA).

The ACTH to be used as the radiolabel was iodinated with Iodine-125 by colleagues (Prof. Mohammed Ghatei and Michael Patterson, Section of Investigative Medicine, Imperial College London) using the Iodo-Gen method, then purified on a G75 Sephadex column in assay buffer and finally used at 4000-6000 cpm per 100 µl (Salacinski *et al.* 1981). The antibody (rabbit anti-human ACTH) was used at a working dilution of 1 in 60,000 (v/v). Tubes were numbered and the following reagents added: 1-3 (Total) 100 µl label; 4-6 (NSB) 300 µl buffer + 100 µl label; 7-9 (Ref) 100 µl buffer + 200 µl antibody + 100 µl label; 10-42 (Standards) 100 µl standard + 200 µl antibody + 100 µl label; and remaining tubes contained 100 µl sample + 200 µl antibody + 100 µl label. After incubation (72 h, 4 °C), the bound antibody was separated from free tracer by further incubating samples (30 min, RTP) with horse serum/0.9% saline (50% v/v)

followed addition of polyethylene glycol (PEG, 18% w/v in 0.05 mol.l⁻¹ PO₄) before centrifugation (30 min, 2400 rcf, 4 °C) (Desbuquois & Aurbach 1971). The supernatant was aspirated and the bound fraction of Iodine-125 ACTH in the pellet was measured (counts per minute) for 120 s per tube (Cobra-II Auto-Gamma, Packard Bioscience, Pangbourne, UK).

2.6.5 Immunoradiometric assay of plasma ACTH

ACTH in plasma samples was assayed in triplicate using a double antibody immunoradiometric assay (IRMA) kit (Cat. 27065, DiaSorin, Stillwater, Minnesota, USA). The kit contained: polystyrene beads coated with polyclonal mouse anti-goat antibody; 125-Iodine (125-I) tracer containing polyclonal goat anti-ACTH and 125-I labelled-mouse monoclonal anti-ACTH diluted in buffered serum containing red dye and 0.1% sodium azide; wash solution containing concentrated buffered surfactant, diluted to 500 ml final volume in ultra-pure water; and controls of human plasma spiked with synthetic human ACTH (30, 109, and 387 pg.ml⁻¹), containing 0.1% sodium azide.

The IRMA detects the whole molecule of ACTH₁₋₃₉ by using two ACTH antibodies—each one specific for different regions of the ACTH molecule—ACTH₁₋₁₇ and ACTH₂₆₋₃₉. The two antibodies are raised against N- and C-terminal regions of the ACTH₁₋₃₉ peptide, thereby reducing the likelihood of the assay detecting ACTH fragments. The iodinated tracer contains a polyclonal goat antibody specific for ACTH₂₆₋₃₉, and an Iodine-125 labelled monoclonal antibody specific for ACTH₁₋₁₇. During incubation with the tracer and mouse anti-goat coated polystyrene beads, the ACTH-specific antibodies will bind to the ACTH₁₋₃₉ present in the sample. Any unbound radioactivity is washed away and the radioactivity bound to the bead is measured (counts per minute) over a 120 s counting period (Bloom & Long 2011) and is directly proportional to the ACTH₁₋₃₉ concentration in a given sample. Results are calculated by comparing the cpm of each sample to the cpm for the ACTH standard curve using logistic regression.

2.6.6 Enzyme immunoassay of corticosterone

Corticosterone was determined in the conditioned media from the primary dispersed adrenal cultures and plasma by a corticosterone enzyme immunoassay (EIA) kit (Cat. 500655-96 wells, Cayman Chemical, Ann Arbor, Michigan, USA). The kit contained a pre-coated rabbit anti-sheep IgG 96-well plate, Ellman's reagent, EIA buffer 10X-concentrate, wash buffer 400X-concentrate, corticosterone acetylcholinesterase

(AChE) tracer, sheep anti-corticosterone antiserum, and corticosterone standard. The EIA buffer was diluted with 90 ml of ultra-pure water. The wash buffer was diluted the 5 ml vial to 2 l and added 1 ml Tween 20. AChE tracer was reconstituted with 6 ml of EIA buffer. A bulk standard of 50 ng.ml⁻¹ corticosterone was prepared by diluting 500 ng.ml⁻¹ corticosterone 1/10 in ultra-pure water. A standard curve was prepared by diluting the bulk standard 1/10 in EIA buffer to make the top standard of 5,000 pg.ml⁻¹ (S1). This top standard was then serially diluted by 1/1.5 to prepare standards S2 - S8 of 2000, 800, 320, 128, 51.2, 20.5, and 8.2 pg.ml⁻¹ corticosterone. A 96-well plate was then prepared with wells to control for non-specific binding (NSB; containing 100 µl EIA buffer and 50 µl tracer); maximum binding (B₀; 50 µl EIA buffer, 50 µl tracer, and 50 µl antiserum); as well as blank wells, standards (S1 - S8; 50 µl standard, 50 µl tracer, 50 µl antiserum), and total activity (5 µl tracer).

Adrenal culture conditioned media samples were diluted 1/800, plasma samples were diluted 1/10 (50 µl diluted sample, 50 µl tracer, and 50 µl antiserum). The plate was then covered and incubated for two hours at room temperature (22 °C) with gentle shaking on a plate shaker. The plate was then washed using the supplied wash buffer and a multi-channel pipette. Then 5 µl tracer was added to the total activity (TA) well and 200 µl Ellman's reagent was added to all wells and the plate was covered and incubated in the dark for 90 min with gentle shaking. The plate was then read on a spectrophotometer at 415 nm wavelength light to measure absorbance and calculate the concentration of samples from the equation of the standard curve.

2.6.7 Bradford protein assay

Fragments of previously frozen (-20 °C mouse lungs (25-35 mg) from LPS-stimulated Fpr2/3^{-/-} mice and wildtype littermates (n = 4), were homogenised using an electric sonicator (Soniprep 150, MSE, London, UK) in ice-cold Radio-Immuno Precipitation Assay (RIPA)-based lysis buffer (100 µl per 50 mg tissue) containing sodium chloride 150 mmol.l⁻¹, Triton X-100 (1%), sodium deoxycholate (0.5%), sodium dodecyl sulphate (0.1%), and Tris pH 8.0 (50 mmol.l⁻¹). This also included a cocktail of protease and phosphatase inhibitors, namely, protease inhibitor (Complete LysisM tablets, Roche, Mannheim, Germany), serine/cysteine protease inhibitor (PMSF, 1 mmol.l⁻¹), tyrosine phosphatase inhibitor (Na₃VO₃, 0.5 mmol.l⁻¹), and serine/threonine phosphatase inhibitor (NaF, 0.05 mmol.l⁻¹). The protein concentration of the homogenate was determined using the Bradford assay (Bradford 1976) to allow

loading of 40 µg per well for each sample. This involved a standard curve from 20 to 100 mg/ml, and 4 µl of each standard in duplicate and 4 µl of sample with 4 replicates in a 96-well plate. Then 200 µl of Coomassie G250 solution (20% methanol, 10% phosphoric acid, 10% ammonium sulphate, 0.12% G-250) was added to each well. After 2 minutes in the dark, sample absorbance was recorded at 595 nm using a microplate spectrophotometer (Anthos HT2, Anthos Labtec, Salzburg, Austria).

2.6.8 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

10% SDS v/v polyacrylamide gels were prepared using the cassette manufacturers protocol (Invitrogen, Paisley, UK) and consisted of 10% w/v acrylamide, 0.3% w/v bisacrylamide, 0.001% w/v SDS, 0.1 mol.l⁻¹ Tris/bicine, 2% w/v ammonium persulphate, 0.1% w/v N,N,N',N' tetramethylethylenediamine (TEMED); stacking gel: 7.5% w/v acrylamide, 0.2% w/v bisacrylamide, ammonium persulphate (40 µl, 10% solution), and 0.66% TEMED (all from Sigma, UK). Samples were mixed 1:2 volumes with 3x working concentration of Laemmli sample buffer (Laemmli 1970) and boiled (100 °C, 5 min) before loading to 40 µg each well (Protein concentrations determined by Bradford Protein Assay, Section 2.6.7).

For electrophoresis, a tank was filled with 4% TRIS-glycine w/v dd H₂O buffer and the gel exposed to a current of 200 mA, 100 V until the ladder was vertically distributed across the gel (after approximately 90 min). The gel was transferred to a pre-wet (10 min in H₂O, 10 min in transfer buffer, 25% methanol v/v Tris-Glycine) nitro-cellulose membrane (0.45 µm pore size) using semi-dry transfer apparatus (Semi-Phor TE 70, General Electric, Amersham, UK) set at 5 V, 50 mA, 1 h. The membrane was then blocked with 5% skimmed milk v/v TBS-Tween for 1 hour, RTP. The membrane was then incubated in a heat-sealed polyethylene pouch overnight at 4 °C with the primary antibody (Fpr2/3 (N-20)-R, sc-18191-R, Santa Cruz; Insight Biotech, Wembley, UK) diluted at 1 in 200 volumes of TBS-Tween.

The secondary antibody was goat anti-rabbit HRP-conjugated IgG (7074, Cell Signalling, Danvers MA, USA) diluted at 1 in 1000 v/v of TBS-Tween, and rotated (1 h, RTP). The membrane was then blocked with 5% skimmed milk v/v TBS-Tween (1 h, RTP) to reduce secondary background noise. The blot was developed by enzymatic chemiluminescent (ECL) reagent (General Electric, Amersham, UK) for 1 minute in a dark room, prior to exposure to Amersham HyperFilm (General Electric, Amersham, UK) for 1 minute. A section of the membrane was incubated with mouse anti-GAPDH (MAB374, Millipore,

Town, Country) diluted at 1 in 300 volumes (1 h, RTP) followed by secondary horse anti-mouse (7076, Cell Signalling, Danvers MA, USA) diluted at 1 in 2000 v/v (1h, RTP).

The molecular weights of the bands of immunoreactive Fpr2/3 were determined by comparison with the migration of molecular weight markers (myosin 250kDa, phosphorylase 148 kDa, BSA 98 kDa, glutamic dehydrogenase 64 kDa, alcohol dehydrogenase 50 kDa, carbonic anhydrase 36 kDa, myoglobin red 22 kDa, lysozyme 16 kDa, aprotinin 6 kDa, and insulin B chain 4 kDa; SeeBlue Plus2 LC5925 molecular weight markers, Invitrogen, Paisley, UK). The blots were scanned using an HP Color LaserJet 9500 MFP (HP, Bracknell, UK).

2.7 Statistics

Data are presented as arithmetic mean $\pm$ standard error of mean (SEM). Kolmogorov-Smirnov tests were performed to ensure the data met the assumption of being normally distributed—a prerequisite for parametric statistics. Data were then analysed by either two-way analysis of variance (ANOVA) with *post hoc* Bonferroni correction, or three-way ANOVA with Bonferroni correction for multiple comparisons.

**Chapter Three: Study of formyl peptide
receptor distribution within the
hypothalamo-pituitary-adrenal axis**

3.1 Introduction

The following paragraphs will describe: the links between the hypothalamo-pituitary-adrenal (HPA) axis, the immune system and the formyl peptide receptor (Fpr/FPR) family and their relevance to inflammogenic disease; the expression of Fpr/FPR subtypes in terms of tissue distribution and cellular disposition and how these are affected by the inflammogen, lipopolysaccharide (LPS).

3.1.1 The HPA axis, immune system, and Fpr/FPR family

The relationship between the immune system and the HPA axis response to stress is a fertile area of research. Whilst the glucocorticoids (GCs) are among the most potent classes of immunosuppressant and anti-inflammatory drugs available, the nature of their actions and side effects remain poorly understood (Auphan *et al.* 1995). Dysfunctional release of GCs is implicated in numerous disease states, but is scarcely more critical than during systemic inflammatory response syndromes (SIRS) such as sepsis and septic shock following an infection. Severe organ damage during sepsis can lead to septic shock, defined as persistent hypotension of < 90 mmHg systolic blood pressure for > 1 h despite administration of fluids and vasopressors such as epinephrine. Whilst the actions of GCs are necessary to reverse septic shock, the relationship between patient mortality rates and circulating GC concentration is at best U-shaped, meaning an optimum dose is difficult to determine (Annane *et al.* 2009; Minneci *et al.* 2009; Sligl *et al.* 2009).⁵ In addition, whilst GCs are effective for treating inflammation within the central nervous system (CNS), their suppressive effects on neurogenesis (Gould *et al.* 1992) means they are contraindicated for treating inflammatory neurodegenerative diseases such as multiple sclerosis (MS), Alzheimer's disease (AD), and Parkinson's disease (PD).

Without understanding the nature of GC actions, it is unlikely that their unwanted side effects will be mitigated against or avoided. Increasing knowledge in this area, therefore, could help in the search for safe

⁵ Typically, under 3% of sepsis patients have absolute adrenal insufficiency, but up to 50% have relative adrenal insufficiency, with 82% mortality if >30 mcg/dL basal GCs and <9 mcg/dL increased GC secretion in response to cosyntropin (Annane *et al.* 2009).

and efficacious alternatives to GCs in the treatment of inflammogenic stress and disease. This highlights the need to contribute data on secondary mediators and mimetics of GC actions, particularly in terms of the function and expression of their target receptors within endocrine and lymphoid tissues. The Fpr/FPR family, for instance, mediate the effects of ligands which direct leukocytes and lymphocytes towards inflammatory sites (chemotaxis) (Hoshi & Ushiki 1999), as well as those which modulate the secretions of the anterior pituitary (Pompeo *et al.* 1997; John *et al.* 2007; McArthur *et al.* 2009) and adrenal glands (Buss 2010). Therefore, the Fpr/FPR family are potential mediators of the interactions between the immune system and HPA axis, and could be of relevance to endocrine and lymphoid tissue responses to inflammogenic stress. The contribution of individual Fpr/FPR subtypes to these systems, however, is not presently clear. The most relevant tissues in these systems will now be described in terms of their gross anatomy, links with the HPA axis and the response to stressors, particularly LPS, and evidence of Fpr/FPR expression.

3.1.1.1 Dentate gyrus of the hippocampal formation

The hippocampal formation is located in the medial temporal lobe of the brain. Within the hippocampal formation, the dentate gyrus (DG) is a site of prolific neurogenesis and hypothalamic afferents with a crucial role in learning and memory along with negative feedback effects on the HPA axis (Gould *et al.* 1999). From development into adulthood, neurogenesis occurs within the DG with neural stem/progenitor cells (NSPCs) forming within the hilus (Hil) and extending into the granule cell layer (GCL) and subgranular zone (SGZ). During the stress response the DG is exposed to increased plasma GCs, which inhibit granule cell production and proliferation via a mechanism involving an upregulation of glutamate release and N-Methyl-D-aspartate (NMDA)-receptor mediated excitation (Moghaddam *et al.* 1994; Cameron *et al.* 1995) and downregulated brain derived neurotrophic factor (BDNF). Stress-induced impairment in neurogenesis is observed within the DG following exposure to GCs or chronic stress, which reverses the increase in neurogenesis following adrenalectomisation (Gould *et al.* 1992; Rodriguez *et al.* 1998; Mineur *et al.* 2007). The inhibition of HPA axis activity is substantiated by the increase in corticotrophic releasing hormone (CRH) and arginine vasopressin (AVP) mRNA in the medial parvocellular neurons within the paraventricular nucleus of the hypothalamus upon hippocampectomy (Herman *et al.* 1989). During inflammogenic stress, such as that elicited by peripheral LPS, there is a subsequent upregulation of interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF- α) and activation of microglia within the DG, and anti-IL-6 neutralisation restores neurogenesis in co-cultures of DG progenitor cells and LPS-activated microglia *in vitro* (Monje *et al.* 2003). With regard to hippocampal expression of Fpr/FPR subtypes, mouse Fpr2 was shown to be upregulated in the DG of amyloid precursor protein/presenilin 1 (APP/PS1) transgenic mice, and the Fpr2-antagonist Trp-Arg-Trp-Trp-Trp-Trp (WRW₄) prevented amyloid beta 1-42 (A β 42) induced senescence of NSPCs (He *et al.* 2013b) and glial cells (Slowik *et al.* 2012). An *in situ* hybridisation study found evidence of transcripts for Fpr2 along with CRH-R1 and V1b within the DG and hippocampal formation, and this finding was validated by a qRT-PCR study which also found evidence of Fpr1, Fpr3 and annexin A1 (ANXA1), as well as Fpr2 (Buss 2010). It is not clear whether these hippocampal Fpr transcripts are translated into functional proteins, or if this process is subject to regulation by LPS in experimental models of inflammogenic stress.

3.1.1.2 Paraventricular nucleus of the hypothalamus

Within the anterior hypothalamus, the paraventricular nucleus (PVN) is adjacent to the third ventricle (3V) and encompasses the neurons for CRH/AVP that stimulate pituitary adrenocorticotrophic hormone (ACTH) release in response to stress. The release of CRH and AVP are regulated by bisynaptic glutamate-GABA connections under the control of nitric oxide (NO) and norepinephrine (NE) (Herman *et al.* 2002). A major input to the PVN comes from the brainstem locus coeruleus (LC)/NE catecholaminergic system, and blockade of this tract blunts the HPA axis response to LPS and IL-1 β (Givalois *et al.* 1995), which otherwise induce cFos mRNA and Fos protein within the nucleus tractus solitarius (NTS) and ventrolateral medulla (Day & Akil 1996). Increases in PVN NE turnover following LPS or IL-1 β are blocked by IL-1 receptor antagonist, suggesting IL-1 is involved in mediating the effects of LPS in the PVN (MohanKumar *et al.* 1999). Toll-like receptor 4 (TLR4) is expressed on LC/NE terminals, and within 2 hours of LPS injection, IL-1 β and nitric oxide synthase (NOS) mRNA transcription is induced and peaks between 4 and 6 hours within the PVN (Wong *et al.* 1996). Whilst the PVN is highly vascularised, it is largely protected by the blood-brain barrier (BBB), whereas the CRH/AVP neurons themselves have projections that extend beyond the BBB to the median eminence and the pituitary. Though it is debatable whether LPS crosses the BBB in significant amounts, NOS within the PVN is, however, induced by LPS which in turn leads to the transcription of CRH and AVP (Uribe *et al.* 1999), and an increase in NE turnover that parallels increases in ACTH and GC secretion (Francis *et al.* 2000). The Fpr/FPR ligand ANXA1 has also been shown to be expressed within key loci of the hypothalamus particularly the ependymal cells around 3V and the median eminence, including the PVN (Woods *et al.* 1990; Strijbos *et al.* 1991; Smith *et al.* 1993). It remains unclear whether Fpr/FPR subtypes are involved in the regulation of CRH or AVP release, but there is evidence that members of the Fpr/FPR family are expressed within the hypothalamus. A quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) study found evidence of Fpr1, Fpr2, and Fpr3/Alx transcripts expressed within the hypothalamus, whereas of these, only Fpr2 was evident within the hypothalamus upon *in situ* hybridisation (Buss 2010). It remains to be determined whether the transcripts from the Fpr/FPR genes are ultimately translated into functional proteins within the hypothalamus, and if so, whether these are relevant to HPA axis function.

3.1.1.3 Anterior pituitary gland

The anterior pituitary gland comprises the pars distalis, pars intermedia, and pars tuberalis. The anterior pituitary secretes ACTH in response to the hypothalamic secretagogues CRH and AVP. Within the medial region of the pars distalis, the corticotrophs synthesise and secrete ACTH and are polygonal cells of medium diameter (10-13 μm) that constitute between 15-20% of total cells within the anterior pituitary (see Krause 2005; Ross & Pawlina 2010; Stevens & Lowe 2011). There are several other endocrine and non-endocrine cell types within the anterior pituitary. The somatotrophs are oval cells of medium diameter (10-13 μm) that synthesise and secrete growth hormone (GH) and make up 40-50% of total cells, and are situated within the lateral wings of the pars distalis. The lactotrophs are large (14-20 μm), polygonal cells that synthesise and secrete prolactin (PRL), are uniformly scattered throughout the pars distalis and constitute 20-25% of total cells. The gonadotrophs are small (<10 μm), oval cells that synthesise and secrete luteinising hormone (LH)/follicle stimulating hormone (FSH), accounting for 10% of total cells and are uniformly scattered throughout the pars distalis. The thyrotrophs are large (14-20 μm), polygonal cells that produce thyroid-stimulating hormone (TSH) and are situated in the most anterior region of the gland, constituting 10% of total cells. The folliculostellates are small to medium diameter star-shaped cells accounting for 5-10% of total cells (Allaerts et al. 1996), and express S100 calcium binding protein b (S100b), as well as the Fpr/FPR ligand, ANXA1 (Traverso et al. 1999).

Several of the hormones of the anterior pituitary have receptors and functions on cells of the immune system. There are receptors for ACTH, GH, and PRL on T lymphocytes, B cells, natural killer (NK) cells, macrophages, and monocytes (Weigent & Blalock 1987). GH has been shown to maintain the competence of T and B cells and macrophages, and stimulate antibody production and NK cell activity; it also has haematopoietic and chemotactic roles within cells of the thymus, spleen, lymph nodes, and bone marrow (Welniak *et al.* 2002). On the other hand, PRL stimulates the clonal expansion of lymphoid cell populations and appears to have mitogenic activity on NK cells and macrophages, at least *in vitro* (Clevenger *et al.* 1998; Sun *et al.* 2004b). Administration of ACTH in humans affects the circulating concentration of leukocytes, increasing neutrophils and decreasing lymphocytes and eosinophils (Hills *et al.* 1948), and enhances T-cell mediated cytotoxicity (Johnson *et al.* 2005) but otherwise generally inhibits immune responses (Johnson *et al.* 1982).

In response to inflammogenic stress elicited by LPS, changes in the expression and secretion of pituitary hormones is subject to mediation by cytokines such as IL-1 β , IL-6, and TNF- α , as well as eicosanoids, and NO. The cells of the anterior pituitary, express a number of cytokines along with their receptors and other cellular mediators. In response to LPS or IL-1 β , pituitary cyclooxygenase expression is upregulated (Cover *et al.* 2001) and the production of eicosanoids in turn leads to increased IL-6 release by the folliculostellate cells which has been shown to stimulate ACTH release from the rat pituitary (Spangelo *et al.* 1991). This mechanism also involves Nf κ B-mediated transcription within the folliculostellates (Mehet *et al.* 2012) which in response to LPS or GCs release macrophage inhibitory factor (MIF) that limits the steroid-mediated inhibition of IL-6 release (Tierney *et al.* 2005). Conversely, the release of ACTH from the anterior pituitary has been shown to be inhibited by various Fpr/FPR ligands, including ANXA1 (Pompeo *et al.* 1997; John *et al.* 2007). ANXA1 is upregulated by LPS or GCs, and thus appears to function as a mediator of GC negative feedback for the pituitary-adrenal axis.

3.1.1.4 Thymus gland

The thymus gland is located within the mediastinum of the thoracic cavity, beneath the sternum and superior to the heart. The thymus gland is a primary lymphoid organ in which bone marrow-derived T cell precursors undergo differentiation and migration prior to maturity when they are circulated for secondary lymphoid organs such as the spleen, lymph nodes, Peyer's patches, and tonsils (Surh & Sprent 1994). The maturation process involves serial expression of clusters of differentiation (CD) and rearrangement of T cell receptor (TCR) genes and is compartmented to microenvironments conducive for the development of T cells as they migrate through the gland. The structure of the thymus can be visualised by routine haematoxylin and eosin (H&E) staining and consists of numerous lobules separated by trabeculae. Each lobule contains an outer thymic cortex and an inner medulla, separated by a cortico-medullary junction. Compared with the medulla, the thymic cortex is darkly stained by H&E and comprises branched thymic epithelial cells (TECs) in close association with immature thymocytes, as well as macrophages scattered throughout the cortex with roles in clearing apoptotic thymocytes (Billard *et al.* 2011). Thymocytes are apoptosed by default if their TCRs are not functionally expressed following its genetic rearrangement (Surh & Sprent 1994; Vacchio & Ashwell 1997). Conversely, thymocytes whose TCRs react with a major histocompatibility complex (MHC) on nonlymphoid cells are able to differentiate through positive or negative selection steps (Chiu *et al.* 1999; Doyle *et al.* 2003). This occurs under the influence of GCs (Surh & Sprent 1994; Sacedon *et al.* 1999) acting via GRs (Miller *et al.* 1998). The outer layer of the cortex is a site of immature thymocyte proliferation, whereas inner cortical thymocytes are predominantly immature T cells undergoing thymic selection. The thymic medulla encompasses mature thymocytes (single-positive for either CD4 or CD8 co-receptor), medullary epithelial cells, and bone-marrow derived macrophages and dendritic cells, and Hassall's corpuscles encapsulate sites of cell death (Williams & Felten 1981; King *et al.* 1995).

The interaction of the thymus gland with the HPA axis is achieved by neuroendocrine, humoral, and neural communication. The humoral link is maintained by reciprocal communication between thymic hormones such as thymulin, thymosin- α 1, and thymopoietin, and the hormones of the HPA axis, as well as pituitary PRL, GH, and LH. Thymulin, for instance, inhibits secretion of PRL (Hadley *et al.* 1997) but stimulates ACTH which also has a role in the release of thymulin (Buckingham *et al.* 1992), hence the

communication between the thymus and HPA axis is reciprocal. In 1936, Hans Selye described the involution of the thymus in animals exposed to stressors (Selye 1936). During the stress response, the thymus shrinks in proportion to the circulating concentration of GCs that suppress the formation of thymus (T)-lymphocytes that make up the bulk of thymic tissue prior to their secretion. In addition, because GCs inhibit IL-1 and interferon-gamma (IFN- γ) release, circulating T-lymphocytes are less responsive to pathogens but are susceptible to GC-induced apoptosis or clearance from the circulation towards lymphoid tissues or sites of infection (Dhabhar & McEwen 1996). Following exposure to LPS, for instance, T-lymphocytes undergo apoptosis and thymic atrophy occurs as a result (Zhang *et al.* 1993; Norimatsu *et al.* 1995). In TLR4-null mice, there is evidence of ANXA1 expression on apoptotic thymocytes (Weyd *et al.* 2013). In addition to thymic atrophy, the Fpr/FPR ligand lipoxin is upregulated and inhibits neutrophil functions (Billard *et al.* 2011). There is evidence of Fpr/FPR expression within the thymus, particularly mouse Fpr1 and Fpr2 (Rivière *et al.* 2009). Interestingly, there is evidence that formylated peptides are involved with the positive selection steps involving TCR binding with major histocompatibility complex (MHC) class Ib molecules H2-M3 (Chiu *et al.* 1999; Doyle *et al.* 2003). It remains unclear which Fpr/FPR subtype could be involved in mediating thymic function across the highly compartmented microenvironments and how this affects the response to inflammogenic stressors.

3.1.1.5 Zona fasciculata of the adrenal cortex

The adrenal cortex can be divided into three zones of cellular organisation. The zona glomerulosa (ZG) is the narrow outer zone and accounts for 15% of the total adrenocortical volume. The zona fasciculata (ZF) is a thick central zone of the cortex, constituting up to 80% of the total volume. The innermost zona reticularis (ZR) makes up between 5% and 7% of the cortical volume (Henrikson 1997). The ZF is the major site for the steroidogenesis of GCs. The cells of the ZF are large, polyhedral structures arranged in elongated mono- or bi-cellular cords separated by sinusoidal capillaries. Histologically, these cells typically have a lightly stained spherical nucleus or two nuclei, and the cytoplasm is acidophilic with numerous lipid droplets. These droplets may appear as sterol vacuolations following routine histological sections owing to dehydration steps extracting lipids. The lipid droplets contain fatty acids, cholesterol, and phospholipid precursors for steroidogenesis of GCs. The GCs have overall inhibitory effects on the immune and inflammatory responses, inhibiting wound healing. The GCs have receptors on T and B cells, neutrophils, monocytes, and macrophages, and have been shown to suppress IL-1 and IL-2 production by lymphocytes and macrophages. The GCs modulate the production of cytokines such as inhibition of IL-12 from antigen presenting cells (APCs) as well as inducing a shift from T_H1 to T_H2 cytokines (Elenkov *et al.* 1996; Miller *et al.* 1998). The GCs also upregulate the apoptosis of lymphocytes and inhibit the mitosis of transformed lymphoblasts.

3.2 Methodology

Based on the above evidence, it was hypothesised that 1) proteins translated from the transcripts of the Fpr2 and Fpr3 exons, will be expressed within cerebral, lymphoid, and HPA axis tissues following peripheral LPS injections; 2) the spatial distribution of green fluorescent protein (GFP) as a surrogate of Fpr2 and Fpr3 within the anterior pituitary of Fpr2/3-knockout, GFP-transgenic (Fpr2/3^{-/-}) mice will be evident within the endocrine cell types (expressing ACTH, GH, PRL, LH, TSH) but not the S100b-positive folliculostellates; and 3) immunoreactivity from the human FPR subtypes FPR2/ALX or FPR3 will be colocalised with ACTH- but not S100b-positive cells.

In the present work we used HPA tissues from Fpr2/3^{-/-}, GFP-positive mice to elucidate the distribution of GFP as a proxy of Fpr2/3 within the hypothalamus, pituitary, and adrenal, as well as thymus. In addition, we used antibodies to target epitopes on the human FPR1, FPR2/ALX, and FPR3 subtypes to map their distribution alongside ACTH and S100b-positive cells of the human anterior pituitary gland.

In order to test these hypotheses, mice with the Fpr2 and Fpr3 genes knocked-out and transgenic for GFP (Fpr2/3^{-/-}) were used. This model was selected because colleagues had recently generated the Fpr2/3^{-/-} mice and, at the time, there were no specific antibodies to detect the spatial distribution of murine Fpr2 or Fpr3 epitopes within tissues by IHC. Since measures of Fpr2 and Fpr3 gene transcripts had already been obtained (Buss 2010), it was important to advance this knowledge by determining whether these transcripts are translated into proteins, and if so, their spatial distribution within tissues. Therefore, to determine how peripheral administration of LPS *in vivo*, might affect the spatial distribution of GFP as a proxy of Fpr2 and Fpr3 protein expression within tissues, Fpr2/3^{-/-} mice and wildtype littermates received intraperitoneal injections of either LPS or vehicle over a brief time course of 2, 4, and 6 hours. Well-validated antibodies that target GFP were used in IHC experiments to detect GFP as a surrogate of Fpr2 and Fpr3 within murine tissues. Tissue sections from wildtype littermates were used as negative controls, and those from irrelevant GFP transgenic mice were used as positive controls, so that the staining pattern observed could be attributed to the presence of GFP signal above background autofluorescence (if any), and, therefore, indicative of the expression of Fpr2 and/or Fpr3 proteins within the tissue.

To determine which cell types of the mouse anterior pituitary gland contain translation products from the mouse Fpr2 and/or Fpr3 gene exons, formalin-fixed paraffin embedded anterior pituitaries from LPS or vehicle injected Fpr2/3^{-/-} mice or wildtype littermates were immunostained with antibodies for S100b, a marker of folliculostellate cells, and for each endocrine lineage (ACTH, GH, PRL, LH, and TSH) in conjunction with antibodies for GFP. Since the presence of GFP could only come from the translation of GFP transcribed from the GFP transgene inserted in the Fpr2/Fpr3 exon, this allowed inferences to be made about which cell types express Fpr2 and/or Fpr3.

To determine whether the genes for human FPR1, FPR2/ALX, and FPR3 are translated within the human anterior pituitary gland, commercially available antibodies specific to epitopes on each human FPR subtype were used along with those for ACTH and S100b. Fluorescently labelled secondary antibodies were added to allow detection of immunofluorescence from FPR subtypes and ACTH or S100b and to therefore quantify colocalisation by confocal microscopy.

3.3 Results

3.3.1 Genotyping of *Fpr2/3^{-/-}* mice

Figure 3.1 shows the results of genotyping experiments with primers specific for nucleotide sequences of DNA for the mouse genes *Fpr2*, *Fpr3*, and the housekeeping gene, glyceraldehyde 3-phosphate (*GAPDH*). Genotyping was performed prior to further studies in order to sort GFP transgenic, *Fpr2* and *Fpr3* knockout (*Fpr2/3^{-/-}*) mice and their wildtype littermates (WT) into experimental group allocations befitting their reported phenotypes (Dufton *et al.* 2010; 2011). From the collection of ear clips of *Fpr2/3^{-/-}*, WT, and positive control C57BL/6 mice, the genotyping experiments were performed blind to minimise the effect of bias (knowledge of the suspected genotype) on the results. Following DNA extraction and PCR, the products were separated by electrophoresis through an agarose gel containing ethidium bromide to resolve bands of DNA fragments by exposure to ultra-violet light. The size of the resulting amplicons were concordant with those predicted by the basic local alignment search tool (BLAST; <http://blast.ncbi.nlm.nih.gov/>) for primer alignment with gene sequences, whereby the expected size for amplicons of *Fpr2* was 650 base pairs (bp), *Fpr3* was 475 bp, and *GAPDH* was 363 bp. The results confirmed the *Fpr2/3^{-/-}* mice were all negative for the *Fpr2* gene, but in some mice the *Fpr3* gene was knocked-down but not completely knocked-out. In samples from these mice, a band for *Fpr3* was present but less concentrated than the *Fpr3* amplicons from WT and C57BL/6 mice. The presence of the housekeeping gene *GAPDH* was unaffected by the genetic modification at the *Fpr2* and *Fpr3* gene loci in the *Fpr2/3^{-/-}* mice. The water control confirmed the molecular grade water was not a source of contamination.

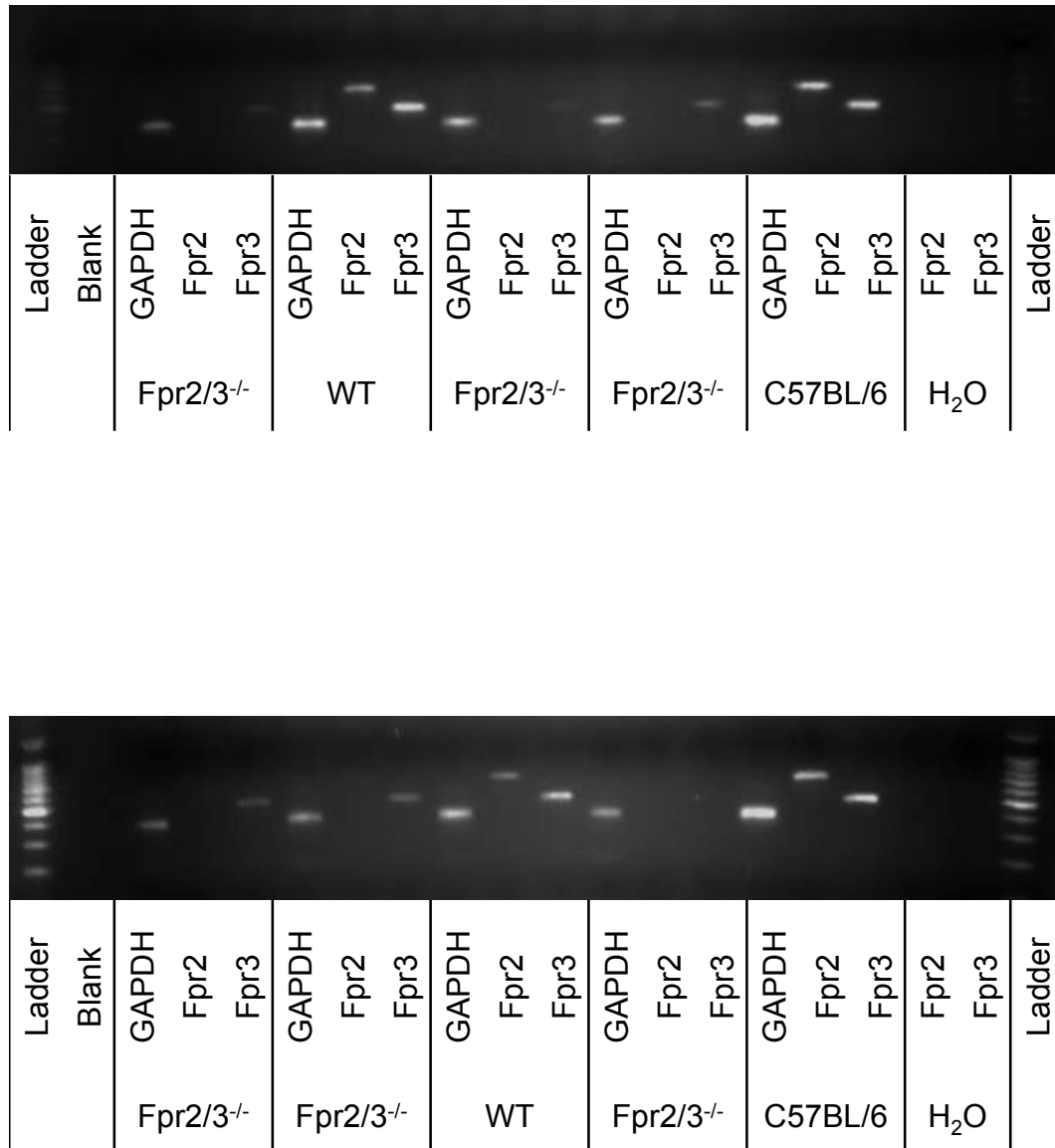


Figure 3.1: Agarose gel electrophoretogram of polymerase chain reaction (PCR) products of amplified DNA from formyl peptide receptor 2 and 3 knockout (Fpr2/3^{-/-}) mice, wildtype littermates (WT), and C57BL/6 positive control mice.

Ear clips were taken from Fpr2/3^{-/-}, WT, and C57BL/6 mice (n = 4) and DNA was extracted prior to performing PCR with specific primers for Fpr2, Fpr3, and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene. PCR products and loading buffer were loaded into each lane (except ladder and blank) on a 1% agarose gel containing ethidium bromide, before running the gel (140 V, 40 min). DNA bands were visualised on an ultraviolet gel documentation system. The experiment was performed blind.

3.3.2 Western blotting for Fpr2/3 in lung extracts of Fpr2/3^{-/-} mice and wildtype littermates

Figure 3.2 shows the result of the SDS-PAGE of protein extracts from the lung tissue taken from Fpr2/3^{-/-} and WT mice followed by western blot with a polyclonal rabbit anti-mouse Fpr2/3 antibody (sc-18191-R) immunoreactive with mouse Fpr2/3 and human FPR2. Molecular weight markers for alcohol dehydrogenase (39 kDa) and glutamic dehydrogenase (51 kDa) from the SeeBlue® Plus2 Prestained Standard ladder were used to determine the size of the immunoreactive epitope. The predicted monomeric size of Fpr3 is 351 amino acids (aa) or 39.5 kDa, and Fpr2 is 351 aa or 39.4 kDa. The result indicates the presence of an immunoreactive epitope of a molecular weight between 39 and 51 kDa present within the lung extracts of WT mice that is absent from Fpr2/3^{-/-} mice.



Figure 3.2: Immunoreactive formyl peptide receptor 2 (Fpr2) or 3 (Fpr3) in lung extracts from wildtype (WT) littermates of Fpr2 and Fpr3 knockout (Fpr2/3^{-/-}) mice, detected by western blot.

WT and Fpr2/3^{-/-} mice (n = 4) received an intraperitoneal injection of lipopolysaccharide (LPS; 750 µg/kg i.p.). Lung tissues were sonicated and extracted proteins then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a methanol-activated polyvinylidene fluoride (PVDF) membrane. This was then incubated with primary rabbit polyclonal anti-mouse Fpr2/3 (Fpr2/3 (N20)-R, Cat: sc-18191-R, Santa Cruz; Insight Biotech, Wembley, UK) diluted 1 in 200 v/v in tris-buffered saline (TBS)/Tween overnight at 4 °C. Molecular weight markers indicate the band in the WT lane is between 39 and 51 kDa.

3.3.3 Immuno-fluorescent detection of GFP within anterior pituitary sections taken from Fpr2/3^{-/-} mice and wildtype littermates

The following results are from a study in which sections of anterior pituitaries from Fpr2/3^{-/-} and WT mice were fluorescently immunostained for GFP and pituitary cell lineage markers, namely, ACTH, GH, PRL, and S100b. Mice were injected with LPS (750 µg/kg i.p.) and then killed by CO₂ asphyxiation 4 hours later, and perfusion fixed. Pituitary glands were then dissected out for formalin fixation and paraffin embedding. Immuno-fluorescent staining was performed on anterior pituitary sections from both Fpr2/3^{-/-} and WT mice, with the latter serving as a negative control to provide an indication of background and endogenous fluorescence without the presence of the GFP transgene in the Fpr2/3^{-/-} mice.

3.3.3.1 Adrenocorticotrophic Hormone

Figure 3.3 shows the immunofluorescence from antibodies detecting GFP and ACTH epitopes on anterior pituitary sections from *Fpr2/3^{-/-}* mice and wildtype littermates. Specifically, the red signal in Figure 3.3(d) indicates nuclear GFP immunoreactivity, and the green signal in Figure 3.3(b) indicates cytoplasmic ACTH immunoreactivity. Figure 3.3(f) shows the close association of the red and green signals within cellular structures, indicating GFP and ACTH co-immunoreactivity within cells. In contrast, the wildtype littermates lacked the red, nuclear GFP immunoreactivity altogether (Figure 3.3 c) but exhibited a similar pattern of cytoplasmic ACTH (Figure 3.3 a) as compared with the *Fpr2/3^{-/-}* mice. These results are enumerated in Figure 3.4, which shows the results of counting cells with either red GFP immunoreactivity, green ACTH signal, or both GFP and ACTH. No cells with GFP signal were observed in the wildtype mouse sections. In the *Fpr2/3^{-/-}* sections, cells with both ACTH+GFP outnumbered those of ACTH alone ($***P < .001$) and GFP alone ($P < .01$). These results indicate a significant colocalisation of ACTH and GFP within cells in the anterior pituitary of *Fpr2/3^{-/-}* mice, but not in wildtype littermates.

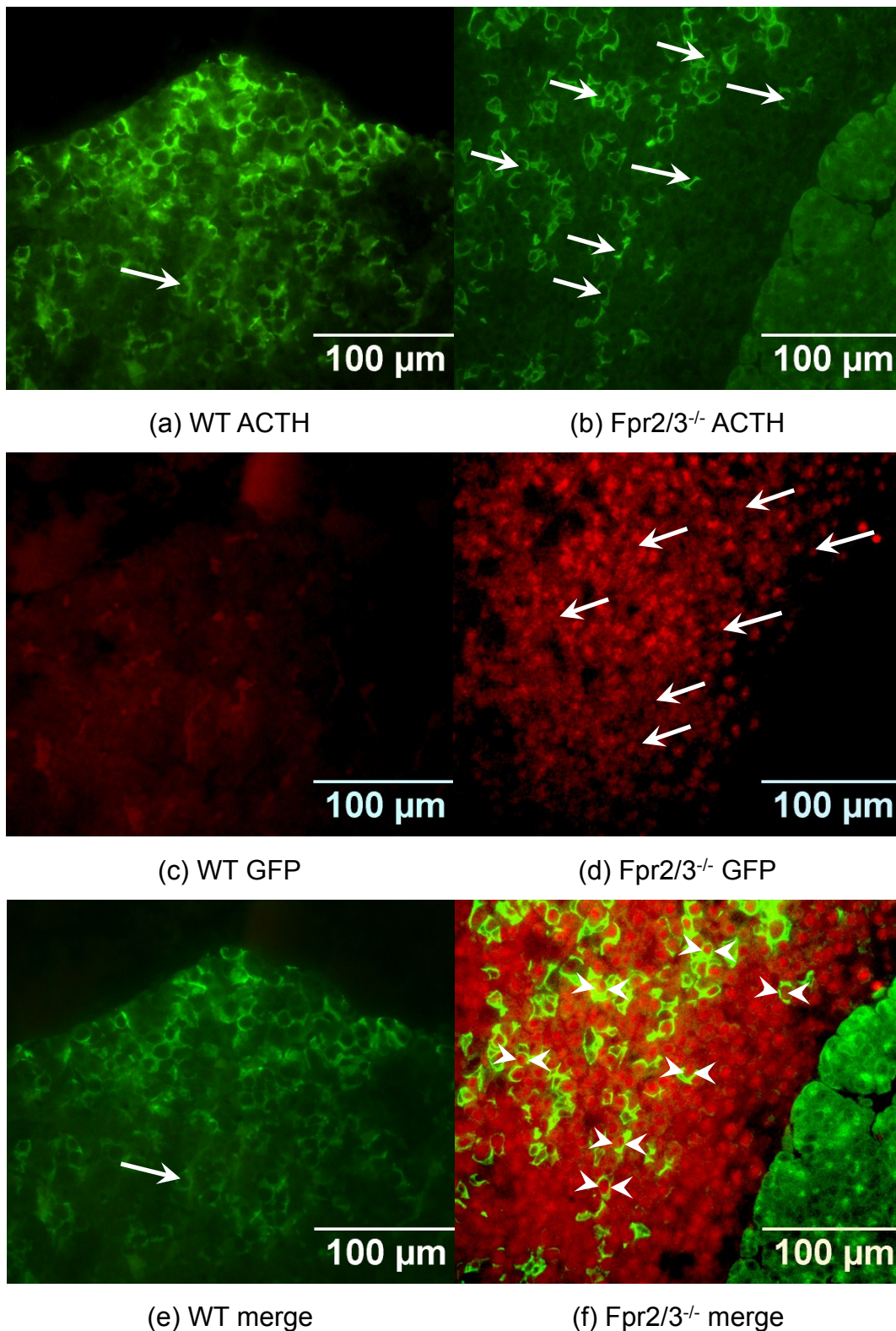


Figure 3.3: Immunofluorescence of adrenocorticotrophic hormone (ACTH) and green fluorescent protein (GFP) within the pars distalis of anterior pituitary sections from GFP transgenic, formyl peptide receptor 2 and 3 knockout (Fpr2/3^{-/-}) mice versus wildtype (WT) littermates.

Transverse sections (4 μm) of anterior pituitary glands taken from WT and Fpr2/3^{-/-} mice (n = 4) 4 h after intraperitoneal (i.p.) injection of lipopolysaccharide (LPS, 750 μg/kg i.p.) were stained with primary anti-ACTH and anti-GFP followed by secondary antisera tagged with the fluorochromes Alexa Fluor 488 (green, for ACTH) or biotin-streptavidin-Alexa Fluor 555 (red, for GFP), and counterstained with diamidino phenylindole (DAPI) to identify nuclei (not shown). The region between the lateral wings of the pars distalis and anterior to the pars intermedia was imaged by fluorescence microscopy (40x). Arrows denote immunofluorescence for cytoplasmic and membranous ACTH (a and e; b and f), perinuclear GFP (d), and arrowheads denote GFP and ACTH colocalisation (f).

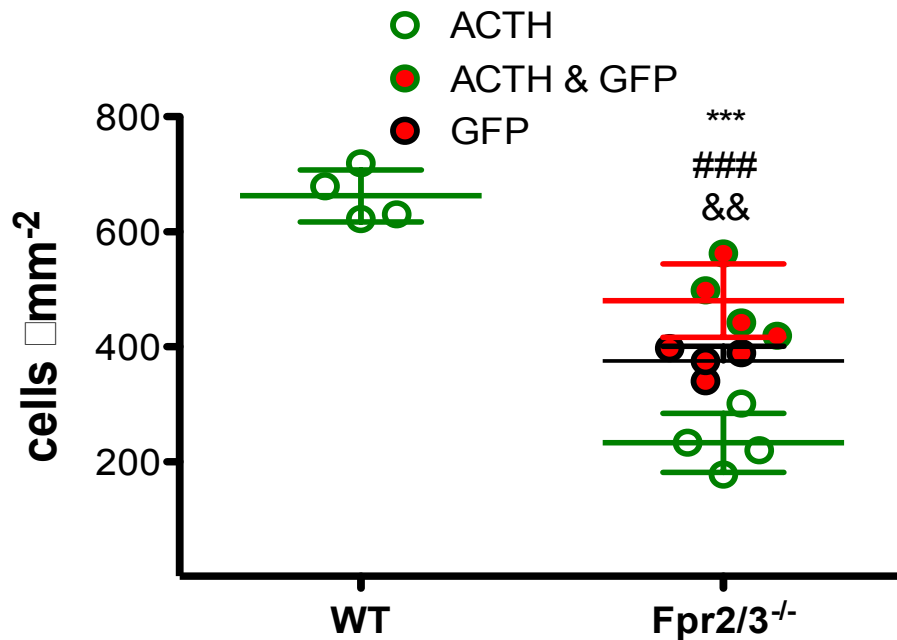


Figure 3.4: Number of cells with immunofluorescence for adrenocorticotrophic hormone (ACTH) and/or green fluorescent protein (GFP) within the pars distalis of anterior pituitary sections from GFP transgenic, formyl peptide receptor 2 and 3 knockout (Fpr2/3^{-/-}) mice versus wildtype (WT) littermates.

Transverse sections (4 μ m) of anterior pituitary glands taken from WT and Fpr2/3^{-/-} mice (n = 4) 4 h after intraperitoneal (i.p.) injection of lipopolysaccharide (LPS, 750 μ g/kg i.p.) were stained with primary anti-ACTH and anti-GFP followed by fluorescent secondary antisera tagged with the fluorochromes Alexa Fluor 488 (green, for ACTH) or biotin-streptavidin-Alexa Fluor 555 (red, for GFP), and counterstained with diamidino phenylindole (DAPI) to identify nuclei (not shown). The region between the lateral wings of the pars distalis and anterior to the pars intermedia was imaged by fluorescence microscopy (40x). Data are mean number of cells $\pm$ standard error of mean (n = 4 mice) counted per 1 mm² on the basis of being positive for ACTH, GFP, or both, and compared by two-way ANOVA: ACTH vs. ACTH+GFP ***P < .001; ACTH vs. GFP ###P < .001; and GFP vs. ACTH+GFP &&P < .01.

3.3.3.2 Growth Hormone

Figure 3.5 shows the immunofluorescence from antibodies detecting GFP and GH epitopes on anterior pituitary sections from *Fpr2/3^{-/-}* mice and wildtype littermates. Specifically, the red signal in Figure 3.5(d) indicates nuclear GFP immunoreactivity, and the green signal in Figure 3.5(b) indicates cytoplasmic GH immunoreactivity. Figure 3.5(f) illustrates an association of the red and green signals within cellular structures, indicating there was GFP and GH co-immunoreactivity within cells. In contrast, the wildtypes littermates lacked the red, nuclear GFP immunoreactivity altogether (Figure 3.5 c) but exhibited a similar pattern of cytoplasmic GH (Figure 3.5 a) as compared with the *Fpr2/3^{-/-}* mice. These results are enumerated in Figure 3.6, which shows the results of counting cells with either red GFP immunoreactivity, green GH signal, or both GFP and GH. No cells with GFP signal were observed in the wildtype mouse sections. In the *Fpr2/3^{-/-}* sections, cells with both GH+GFP outnumbered those of GH alone ($***P < .001$) and GFP alone ($***P < .001$). In addition, there were fewer cells with GFP alone than GH alone ($###P < .001$). These results indicate that in sections from the anterior pituitary of *Fpr2/3^{-/-}* mice (but not from wildtype littermates) the majority of GH-positive cells had colocalisation of GH and GFP within their structures. There also appeared to be a subset of GH-positive structures that were not GFP-positive.

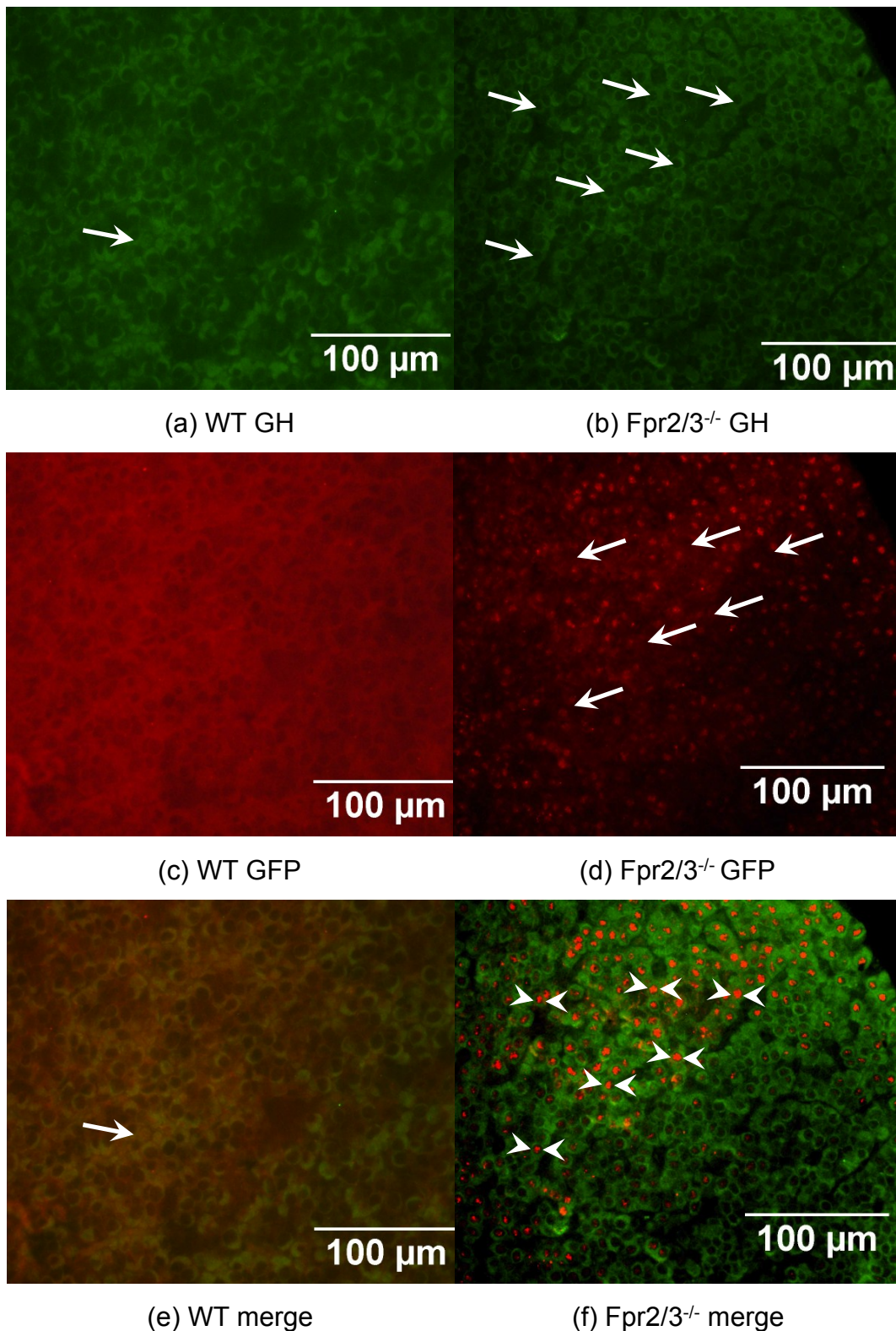


Figure 3.5: Immunofluorescence of growth hormone (GH) and green fluorescent protein (GFP) within the pars distalis of anterior pituitary sections from GFP transgenic, formyl peptide receptor 2 and 3 knockout ($Fpr2/3^{-/-}$) mice versus wildtype (WT) littermates.

Transverse sections (4 μ m) of anterior pituitary glands taken from WT and $Fpr2/3^{-/-}$ mice (n = 4) 4 h after intraperitoneal (i.p.) injection of lipopolysaccharide (LPS, 750 μ g/kg i.p.) were stained with primary anti-GH and anti-GFP followed by fluorescent secondary antisera tagged with the fluorochromes Alexa Fluor 488 (green, for GH) or biotin-streptavidin-Alexa Fluor 555 (red, for GFP), and counterstained with diamidino phenylindole (DAPI) to identify nuclei (not shown). The region within the lateral wing of the pars distalis was imaged by fluorescence microscopy (40x). Arrows denote GH staining (b), perinuclear GFP staining (d), and arrowheads denote GFP and GH colocalisation (f).

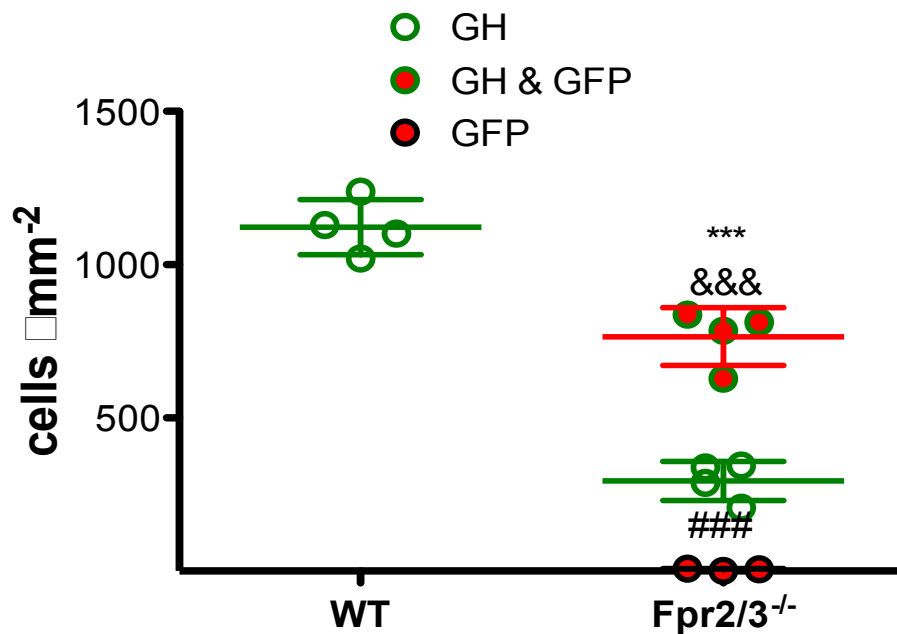


Figure 3.6: Number of cells with immunofluorescence for growth hormone (GH) and/or green fluorescent protein (GFP) within the pars distalis of anterior pituitary sections from GFP transgenic, formyl peptide receptor 2 and 3 knockout (Fpr2/3^{-/-}) mice versus wildtype (WT) littermates.

Transverse sections (4 μ m) of anterior pituitary glands taken from WT and Fpr2/3^{-/-} mice (n = 4) 4 h after intraperitoneal (i.p.) injection of lipopolysaccharide (LPS, 750 μ g/kg i.p.) were stained with primary anti-GH and anti-GFP followed by fluorescent secondary antisera tagged with the fluorochromes Alexa Fluor 488 (green, for GH) or biotin-streptavidin-Alexa Fluor 555 (red, for GFP), and counterstained with diaminidino phenylindole (DAPI) to identify nuclei (not shown). The region within the lateral wing of the pars distalis was imaged by fluorescence microscopy (40x). Data are mean number of cells $\pm$ standard error of mean (n = 4 mice) counted per 1 mm² on the basis of being positive for GH, GFP, or both, and compared by two-way ANOVA: GH vs. GH & GFP *** = P < .001; GH vs. GFP #### = P < .001; and GFP vs. GH & GFP &&& = P < .001.

3.3.3.3 Prolactin

Figure 3.7 shows the immunofluorescence from antibodies detecting GFP and PRL epitopes on anterior pituitary sections from *Fpr2/3^{-/-}* mice and wildtype littermates. Specifically, the red signal in Figure 3.7(d) indicates nuclear GFP immunoreactivity, and the green signal in Figure 3.7(b) indicates cytoplasmic PRL immunoreactivity. Figure 3.7(f) illustrates there was an association of the red and green signals within cellular structures, thus indicating co-immunoreactivity of GFP and PRL within cells. In contrast, the wildtype littermates lacked the red, nuclear GFP immunoreactivity altogether (Figure 3.7 c) but exhibited a similar pattern of cytoplasmic PRL (Figure 3.7 a) as compared with the *Fpr2/3^{-/-}* mice. These results are enumerated in Figure 3.8, which shows the results of counting cells with either red GFP immunoreactivity, green PRL signal, or both GFP and PRL. No cells with GFP signal were observed in the wildtype mouse sections. In the *Fpr2/3^{-/-}* sections, cells with both PRL+GFP outnumbered those of PRL alone ($***P < .001$) and GFP alone ($***P < .001$). In addition, there were more cells with GFP alone than PRL alone ($\#P < .05$). These results indicate that in sections from the anterior pituitary of *Fpr2/3^{-/-}* mice (but not from wildtype littermates) the majority of PRL-positive cells had colocalisation of PRL and GFP within their structures. There also appeared to be a subset of PRL-positive structures that were not GFP-positive.

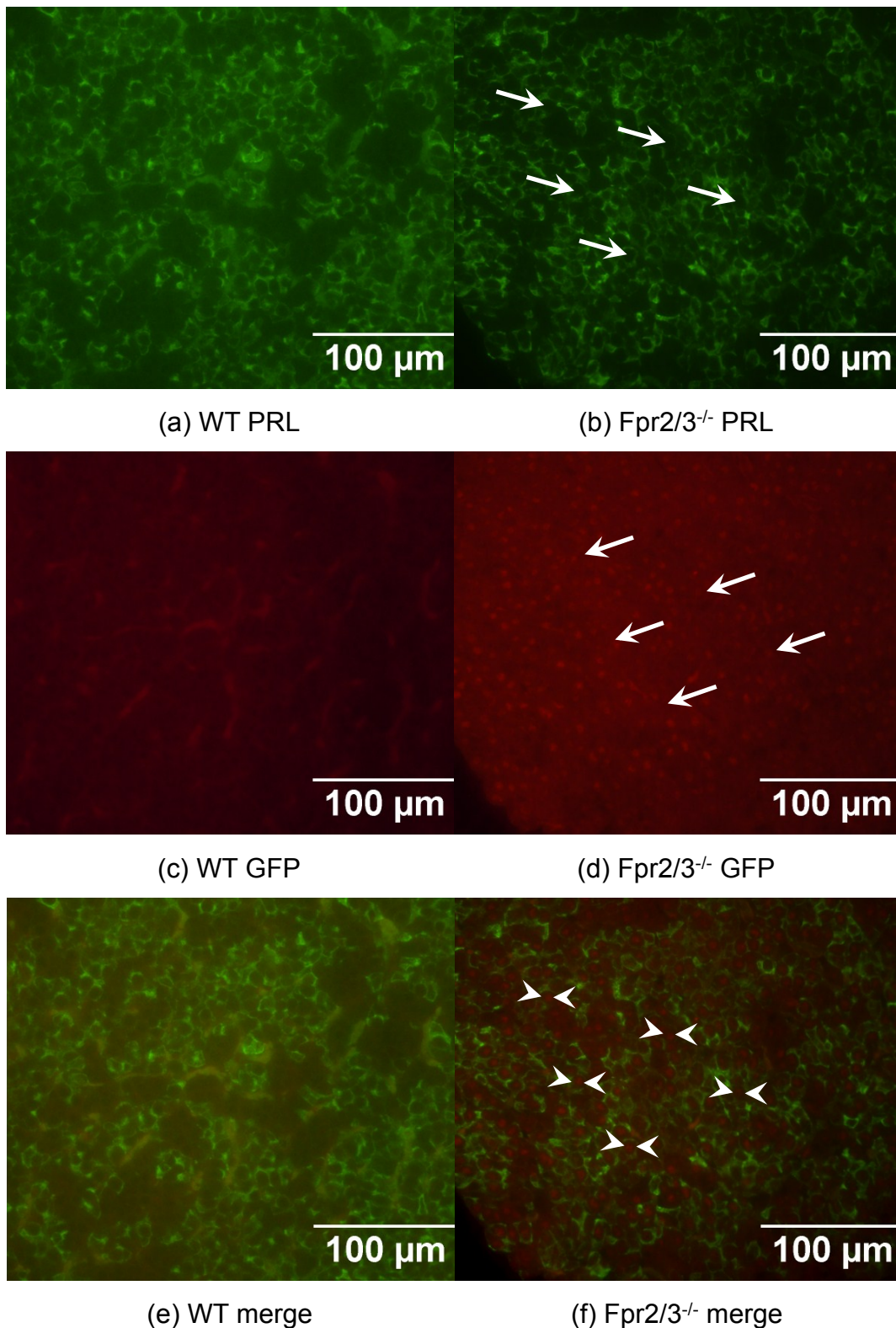


Figure 3.7: Immunofluorescence of prolactin (PRL) and green fluorescent protein (GFP) within the pars distalis of anterior pituitary sections from GFP transgenic, formyl peptide receptor 2 and 3 knockout ($Fpr2/3^{-/-}$) mice versus wildtype (WT) littermates.

Transverse sections (4 μ m) of anterior pituitary glands taken from WT and $Fpr2/3^{-/-}$ mice (n = 4) 4 h after intraperitoneal (i.p.) injection of lipopolysaccharide (LPS, 750 μ g/kg i.p.) were stained with primary anti-PRL and anti-GFP followed by fluorescent secondary antisera tagged with the fluorochromes Alexa Fluor 488 (green, for PRL) or biotin-streptavidin-Alexa Fluor 555 (red, for GFP), and counterstained with diamidino phenylindole (DAPI) to identify nuclei (not shown). The region between the lateral wings of the pars distalis and anterior to the pars intermedia was imaged by fluorescence microscopy (40x). Arrows denote PRL staining (b), perinuclear GFP staining (d), and arrowheads denote GFP and PRL colocalisation (f).

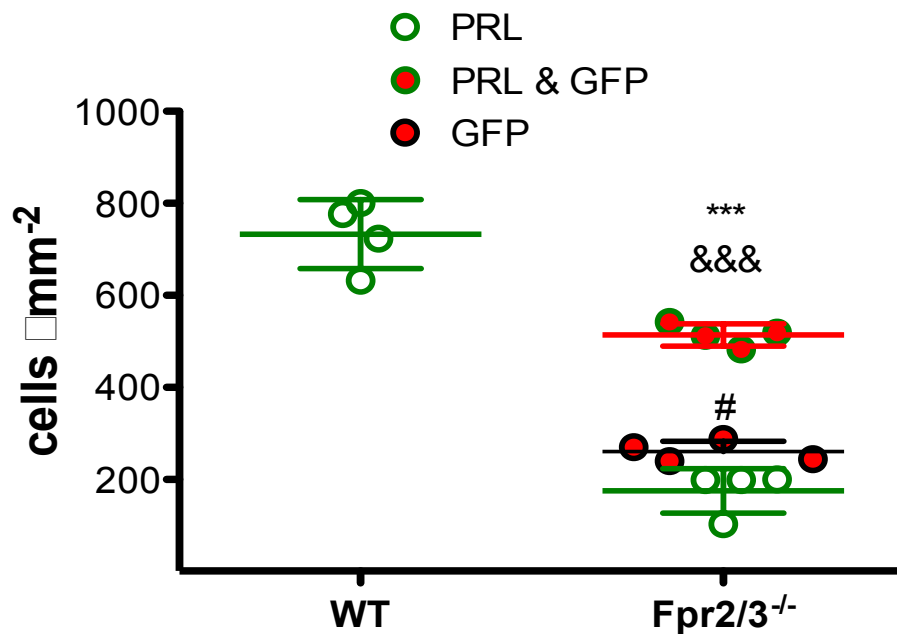


Figure 3.8: Number of cells with immunofluorescence for prolactin (PRL) and/or green fluorescent protein (GFP) within the pars distalis of anterior pituitary sections from GFP transgenic, formyl peptide receptor 2 and 3 knockout (Fpr2/3^{-/-}) mice versus wildtype (WT) littermates.

Transverse sections (4 μ m) of anterior pituitary glands taken from WT and Fpr2/3^{-/-} mice (n = 4) 4 h after intraperitoneal (i.p.) injection of lipopolysaccharide (LPS, 750 μ g/kg i.p.) were stained with primary anti-PRL and anti-GFP followed by fluorescent secondary antisera tagged with the fluorochromes Alexa Fluor 488 (green, for PRL) or biotin-streptavidin-Alexa Fluor 555 (red, for GFP), and counterstained with diamidino phenylindole (DAPI) to identify nuclei (not shown). The region between the lateral wings of the pars distalis and anterior to the pars intermedia was imaged by fluorescence microscopy (40x). Data are mean number of cells $\pm$ standard error of mean (n = 4 mice) counted per 1 mm² on the basis of being positive for PRL, GFP, or both, and compared by two-way ANOVA: PRL vs. PRL & GFP *** = P < .001; PRL vs. GFP # = P < .05; and GFP vs. PRL & GFP &&& = P < .001.

3.3.3.4 S100b

Figure 3.9 shows the immunofluorescence from antibodies detecting GFP and S100b epitopes on anterior pituitary sections from *Fpr2/3^{-/-}* mice and wildtype littermates. Specifically, the red signal in Figure 3.9(d) indicates nuclear GFP immunoreactivity, and the green signal in Figure 3.9(b) indicates cytoplasmic S100b immunoreactivity. Figure 3.9(f) indicates there was no association of the red and green signals within cellular structures, indicating there was zero GFP and S100b co-immunoreactivity within cells. In contrast, the wildtypes littermates lacked the red, nuclear GFP immunoreactivity altogether (Figure 3.9 c) but exhibited a similar pattern of cytoplasmic S100b (Figure 3.9 a) as compared with the *Fpr2/3^{-/-}* mice. These results are enumerated in Figure 3.10, which shows the results of counting cells with either red GFP immunoreactivity, green S100b signal, or both GFP and S100b. No cells with GFP signal were counted in the wildtype mouse sections. In the *Fpr2/3^{-/-}* sections, cells with GFP alone outnumbered those of S100b alone (####P < .001). These results indicate there was no colocalisation of S100b and GFP within cell structures in sections from the anterior pituitary of *Fpr2/3^{-/-}* mice, or from wildtype littermates.

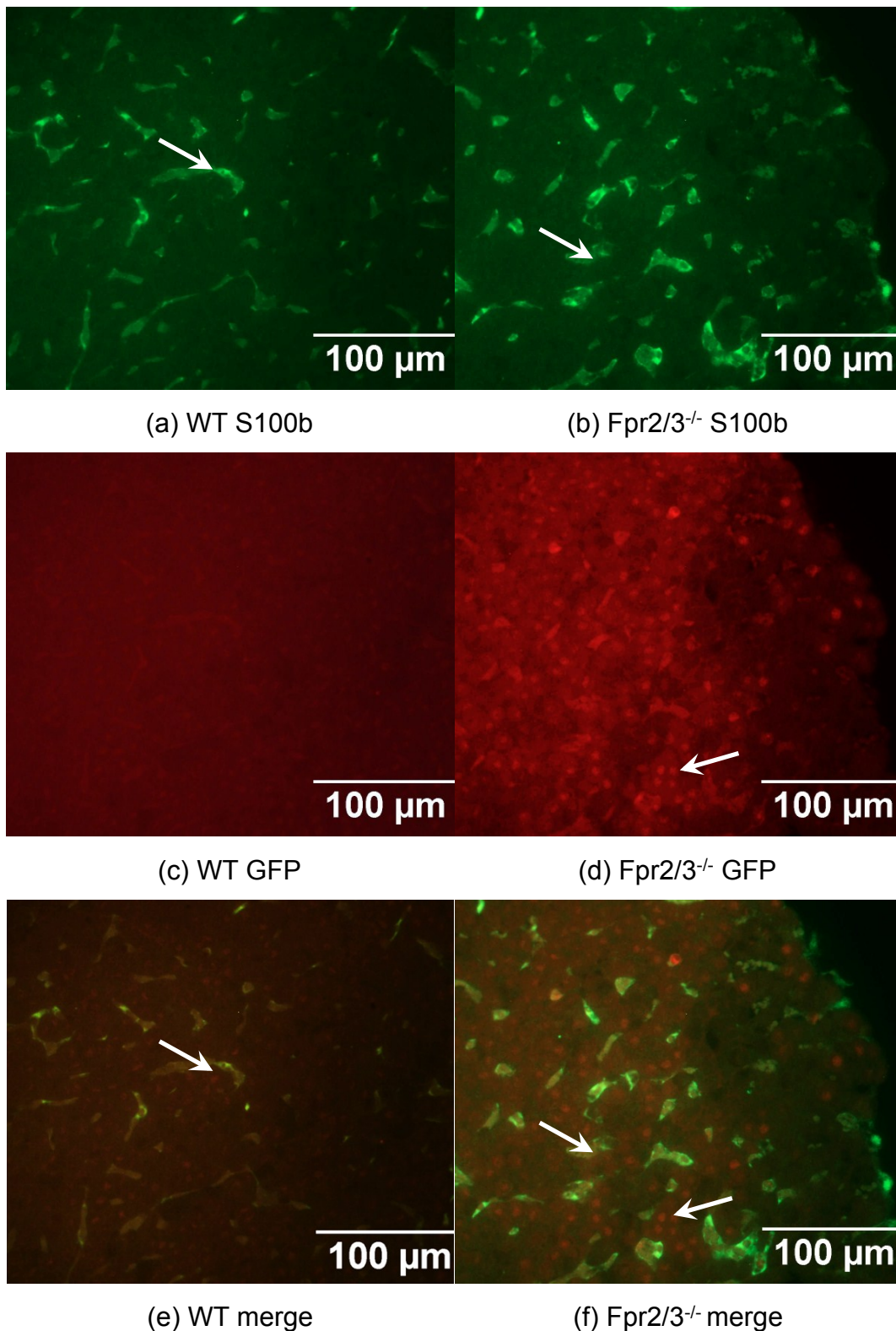


Figure 3.9: Immunofluorescence of S100b and green fluorescent protein (GFP) within the pars distalis of anterior pituitary sections from GFP transgenic, formyl peptide receptor 2 and 3 knockout (*Fpr2/3^{-/-}*) mice versus wildtype (WT) littermates.

Transverse sections (4 μ m) of anterior pituitary glands taken from WT and *Fpr2/3^{-/-}* mice ($n = 4$) 4 h after intraperitoneal (i.p.) injection of lipopolysaccharide (LPS, 750 μ g/kg i.p.) were stained with primary anti-S100b and anti-GFP followed by fluorescent secondary antisera tagged with the fluorochromes Alexa Fluor 488 (green, for S100b) or biotin-streptavidin-Alexa Fluor 555 (red, for GFP), and counterstained with diaminidino phenylindole (DAPI) to identify nuclei (not shown). The region between the lateral wings of the pars distalis and anterior to the pars intermedia was imaged by fluorescence microscopy (40x). Arrows denote S100b staining (b) and perinuclear GFP (d).

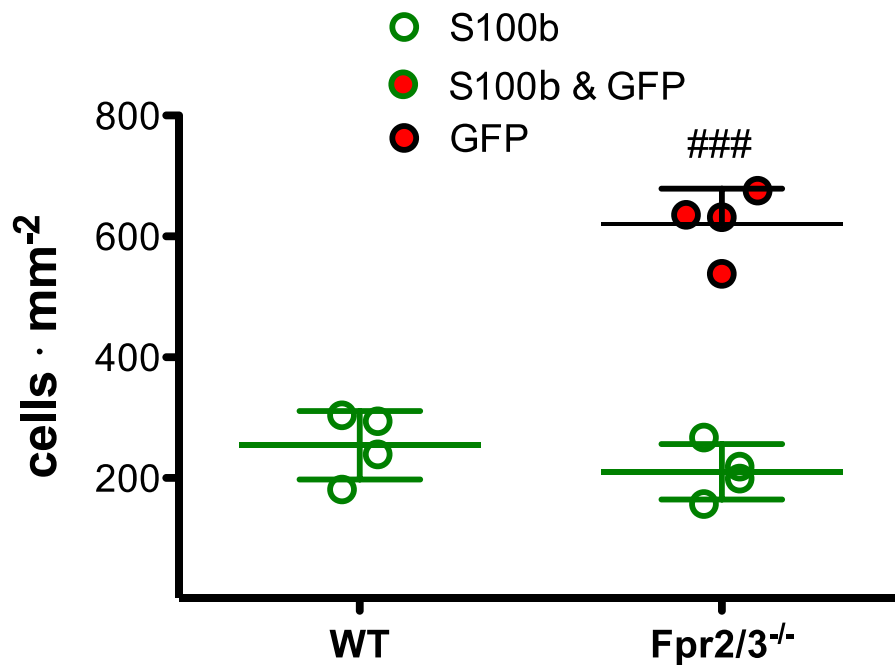


Figure 3.10: Number of cells with immunofluorescence for S100b and/or green fluorescent protein (GFP) within the pars distalis of anterior pituitary sections from GFP transgenic, formyl peptide receptor 2 and 3 knockout (Fpr2/3^{-/-}) mice versus wildtype (WT) littermates.

Transverse sections (4 μ m) of anterior pituitary glands taken from WT and Fpr2/3^{-/-} mice (n = 4) 4 h after intraperitoneal (i.p.) injection of lipopolysaccharide (LPS, 750 μ g/kg i.p.) were stained with primary anti-S100b and anti-GFP followed by fluorescent secondary antisera tagged with the fluorochromes Alexa Fluor 488 (green, for S100b) or biotin-streptavidin-Alexa Fluor 555 (red, for GFP), and counterstained with diamidino phenylindole (DAPI) to identify nuclei (not shown). The region between the lateral wings of the pars distalis and anterior to the pars intermedia was imaged by fluorescence microscopy (40x). Data are mean number of cells $\pm$ standard error of mean (n = 4 mice) counted per 1 mm² on the basis of being positive for S100b, GFP, or both, and compared by two-way ANOVA: S100b vs. GFP ### = P < .001.

3.3.4 Immuno-histochemical detection of GFP in key regions of the adrenal glands, thymus, and brains from Fpr2/3^{-/-} mice and wildtype littermates

The following results are from a study in which tissue sections of tissues from Fpr2/3^{-/-} mice and wildtype littermates were immunostained for green fluorescent protein (GFP). The tissue areas assessed were the dentate gyrus (DG) of the hippocampal formation, the paraventricular nucleus (PVN) of the hypothalamus, the follicles of the thymus gland, and the zona fasciculata (ZF) of the adrenal cortex.

3.3.4.1 Adrenal zona fasciculata

Figure 3.11 and Figure 3.12 show the effects of injections of LPS or PBS vehicle 2 hours or 6 hours previously, on the distribution of immunoreactive GFP observed within the ZF of the adrenal cortex from Fpr2/3^{-/-} mice and wildtype littermates (WT). The data indicate that in animals injected 2 hours previously, GFP immunoreactivity was evident in the LPS treated Fpr2/3^{-/-} mice (Figure 3.11 d), but was not observed in the wildtype littermates (Figure 3.11 c) or the vehicle (PBS) injected control mice of either genotype. In adrenals taken from animals injected 6 hours previously, GFP immunoreactivity was only scarcely evident in sections from LPS treated Fpr2/3^{-/-} mice (Figure 3.12 d) versus those injected 2 hours previously (Figure 3.11 d). There was no GFP observed in the wildtype littermates or the saline injected controls of either genotype.

Figure 3.13 shows the result of morphometric analyses of adrenal sections immunostained for GFP. The adrenal cortex was imaged by microscope and the ZF analysed by manually counting the total number of cells per square millimetre, the number of cells with brown, nuclear, GFP immunoreactivity, and the number of vacuolated cells across the section. Sterols are evident as circular vacuolations. In terms of total cell counts, there was a modest but significant reduction in the number of cells per square millimetre in the adrenal cortex from wildtype animals 2 hours after receiving i.p. LPS (550.0 ± 17.7) versus vehicle injected controls (575.3 ± 7.6 , $\dagger = P < .05$). This reduction in cell counts was also observed in wildtype animals 6 hours after receiving i.p. LPS (540.8 ± 14.6) versus vehicle injected controls (597.0 ± 7.7 , $\dagger = P < .05$). There was no significant difference in the number of cells counted within the ZF of the adrenal glands from any of the Fpr2/3^{-/-} mice ($ns = P > .05$).

In terms of the number of GFP-positive cells, sections of the adrenal cortex from most treatment groups did not show evidence of GFP immunoreactivity. The only two treatment groups to show evidence of GFP-positive cells were the $Fpr2/3^{-/-}$ mice that received i.p. injections of LPS then killed after 2 hours and 6 h. There were GFP-positive cells evident within the ZF of adrenal glands from the $Fpr2/3^{-/-}$ mice after 2 hours of receiving LPS (79.8 ± 5.2) and by comparison were significantly reduced in the $Fpr2/3^{-/-}$ mice injected with LPS in the six-hour group (9.0 ± 1.3 , *** = $P < .001$). With regard to sterol vacuolation, the LPS treatment significantly reduced the degree of sterol vacuolation in the adrenal cortex of the wildtype animals after 2 hours (68.0 ± 6.4), versus vehicle controls (207.8 ± 10.9 , ††† = $P < .001$). A similar effect was observed in the wildtype littermates receiving LPS after 6 hours (108.3 ± 4.7), versus PBS controls (221.8 ± 22.7 , ††† = $P < .001$). In the $Fpr2/3^{-/-}$ mice, however, there was no significant reduction in sterol vacuolation following LPS in either the two-hour or six-hour groups versus the respective vehicle injected controls (ns = $P > .05$).

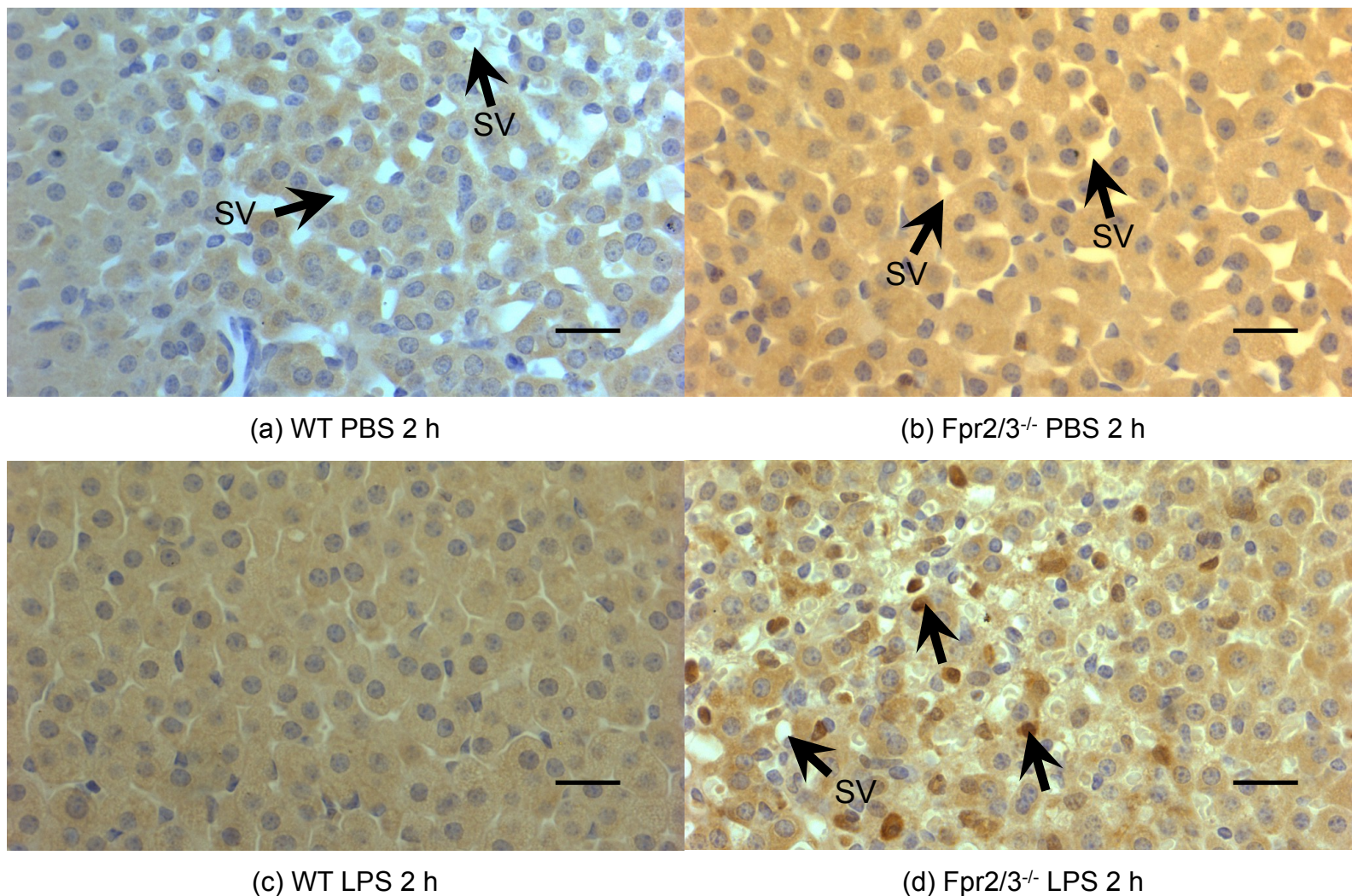


Figure 3.11: Immunostain for green fluorescent protein (GFP) within the zona fasciculata (ZF) of adrenal sections from GFP transgenic, formyl peptide receptor 2 and 3 knockout (*Fpr2/3^{-/-}*) mice versus wildtype (WT) littermates 2 h following intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) or phosphate buffered saline (PBS) vehicle.

Transverse sections (4 μm) of adrenal glands taken from WT and *Fpr2/3^{-/-}* mice ($n = 4$) 2 h after receiving LPS (750 $\mu\text{g/kg}$ i.p.) or PBS were stained with primary anti-GFP and secondary horseradish peroxidase (HRP)-conjugated antisera, then counterstained with diaminobenzidine (DAB, dark brown) and a weak haematoxylin solution (HX, blue). The medial region of the ZF within the cortex and axial to the medulla was imaged by light microscopy (20x, scale bar = 10 μm). Arrows denote sterol vacuolation (SV) or positive perinuclear staining for GFP.

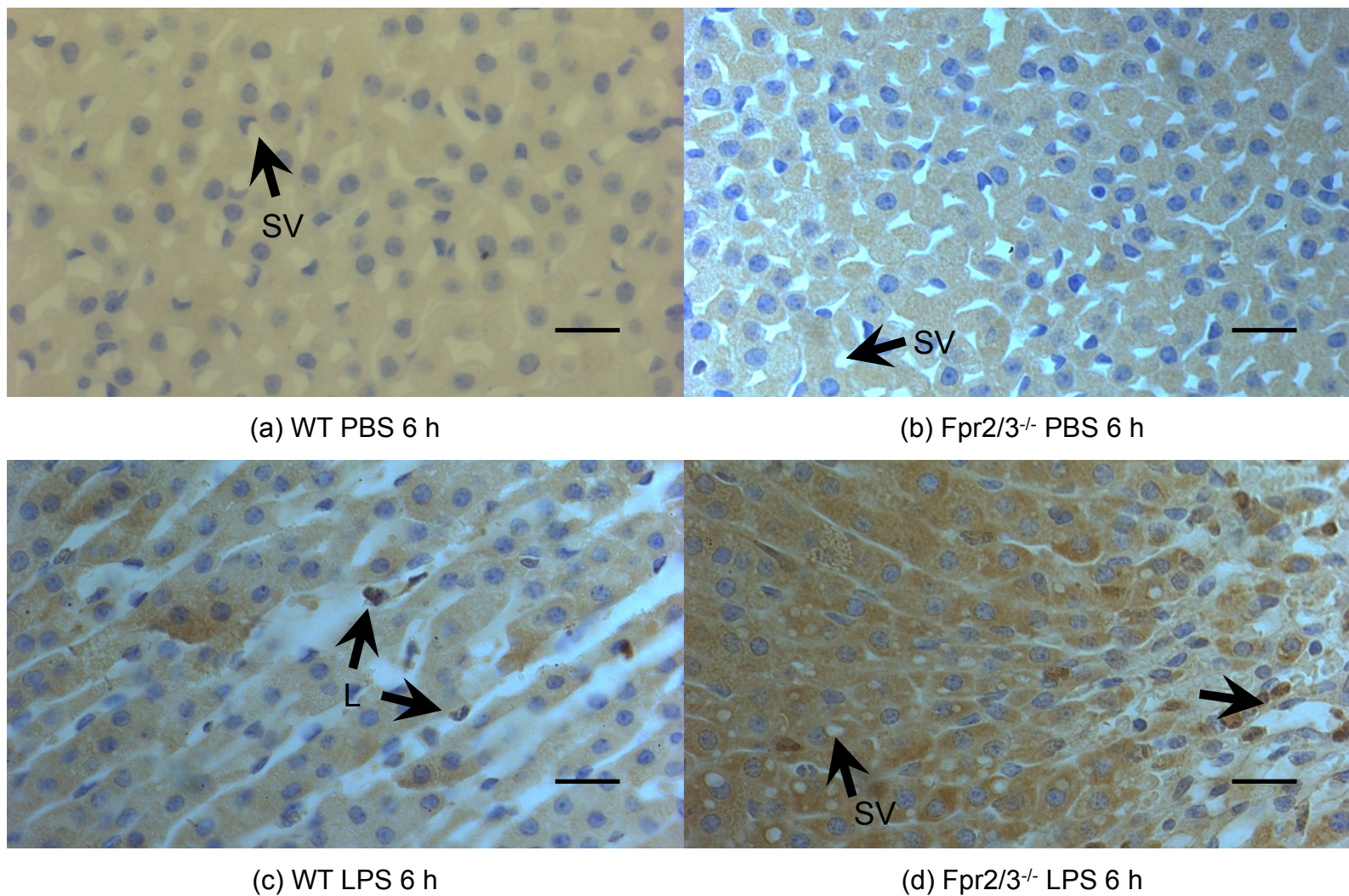


Figure 3.12: Immunostain for green fluorescent protein (GFP) within the zona fasciculata (ZF) of adrenal sections from GFP transgenic, formyl peptide receptor 2 and 3 knockout (*Fpr2/3^{-/-}*) mice versus wildtype (WT) littermates 6 h following intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) or phosphate buffered saline (PBS) vehicle.

Transverse sections (4 μ m) of adrenal glands taken from WT and *Fpr2/3^{-/-}* mice ($n = 4$) 6 h after receiving LPS (750 μ g/kg i.p.) or PBS were stained with primary anti-GFP and secondary horseradish peroxidase (HRP)-conjugated antisera, then counterstained with diaminobenzidine (DAB, dark brown) and a weak haematoxylin solution (HX, blue). The medial region within the ZF of the cortex and axial to the medulla was imaged by light microscopy (20x, scale bar = 10 μ m). Arrows denote sterol vacuolation (SV), polymorphonuclear leukocytes (L), or positive perinuclear staining for GFP.

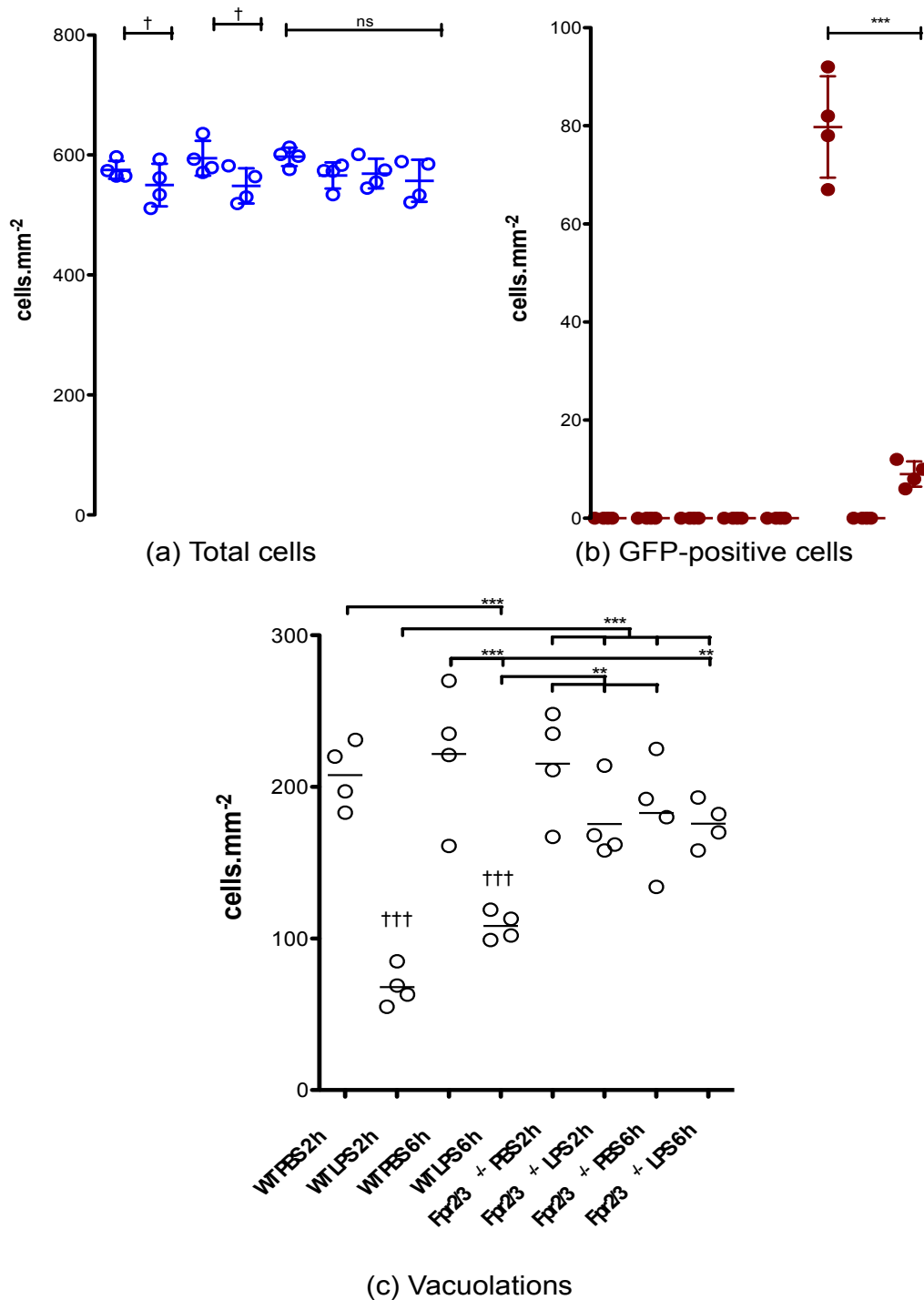
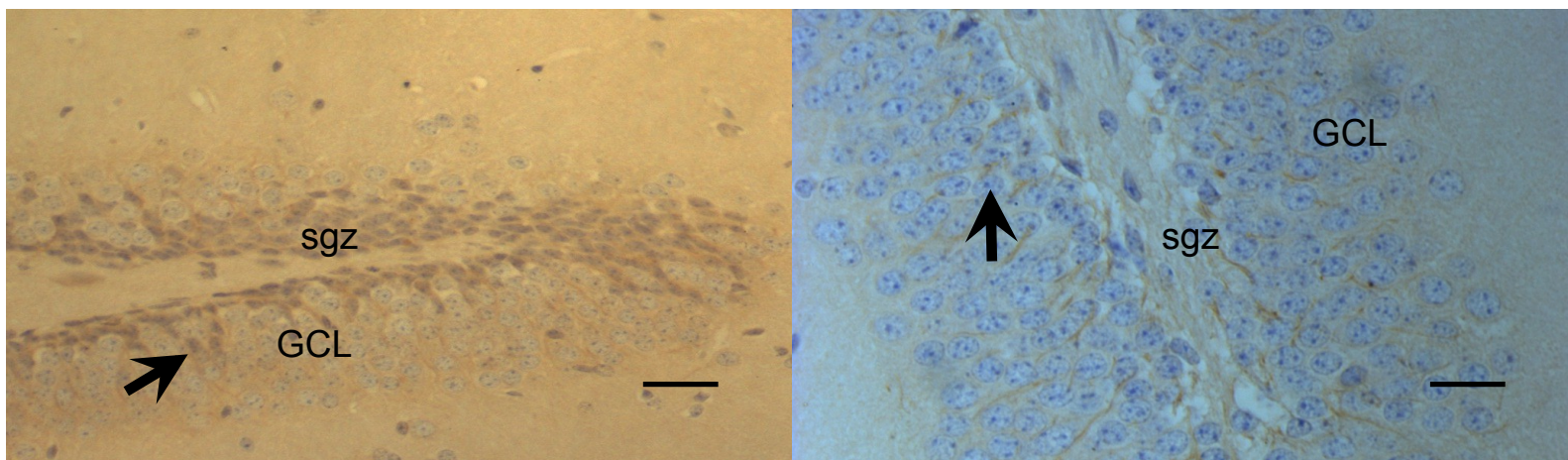


Figure 3.13 Morphometric analysis of immunostained green fluorescent protein (GFP) within the zona fasciculata (ZF) of adrenal sections from GFP transgenic, formyl peptide receptor 2 and 3 knockout (*Fpr2/3*^{-/-}) mice and wildtype (WT) littermates 2 h and 6 h following intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) or phosphate buffered saline (PBS) vehicle.

Transverse sections (4 μ m) of adrenal glands from WT and *Fpr2/3*^{-/-} mice (n = 4) 2 h and 6 h after receiving LPS (750 μ g/kg i.p.) or PBS were stained with primary anti-GFP and secondary horseradish peroxidase (HRP)-conjugated antisera, then counterstained with diaminobenzidine (DAB, dark brown) and a weak haematoxylin solution (HX, blue). The medial region within the ZF of the cortex and axial to the medulla was imaged by light microscopy (20x). Data are mean number of cells (n = 4 mice) $\pm$ standard error of mean counted per 1 mm² on the basis of being positive for blue HX-stained nuclei, dark brown DAB-stained GFP, or sterol vacuolation, and compared by two-way ANOVA: KO LPS 6 h vs. KO LPS 2 h * = P < .05, ** = P < .01, *** = P < .001; and LPS vs. PBS † = P < .05, ††† = P < .001.

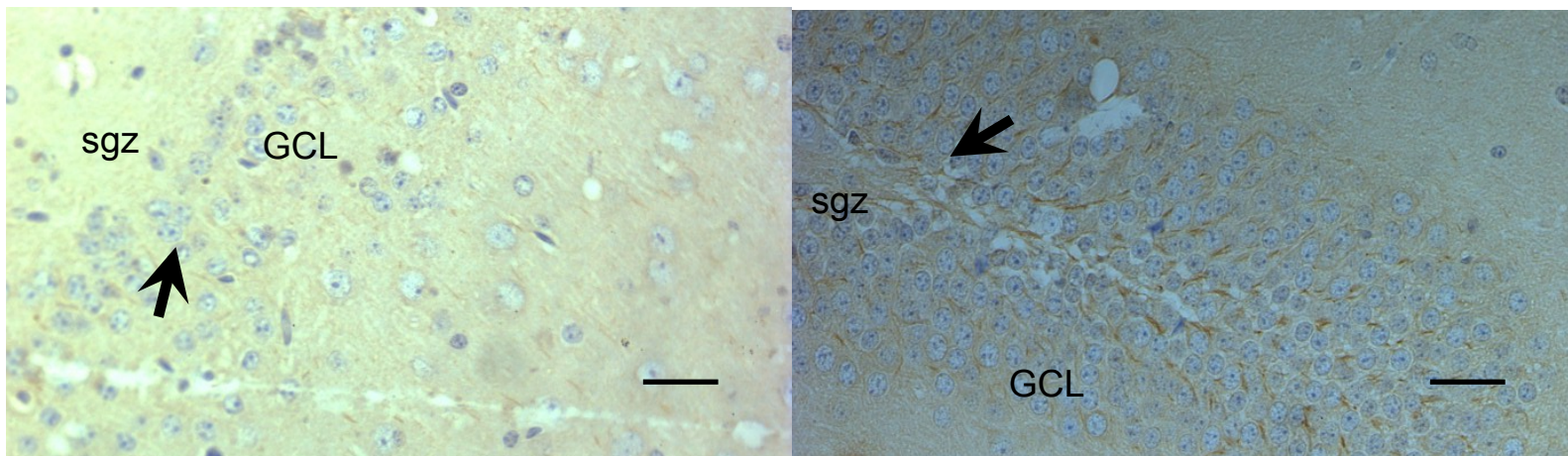
3.3.4.2 Dentate gyrus of the hippocampal formation

Figure 3.14 and Figure 3.15 show the results of immunostaining in coronal sections of the mouse brain, within the dentate gyrus (DG) of the hippocampus. Sections were taken between the septal nuclei rostrally and the temporal cortex caudally, where the DG had a characteristic banana-shape structure. Within the DG structure, the medial blade (mb) was observed for any changes in GFP immunoreactivity and in the thickness of the granule cell layer (GCL), subgranular zone (sgz), and the molecular layer (ML) in $Fpr2/3^{-/-}$ versus WT mice, 2 hours and 6 hours following intraperitoneal injections of LPS or PBS. In a region incorporating dendrites of dentate granule cells, as well as interneurons and perforant path fibres from the entorhinal cortex, the ML exhibited a typical thickness of around 60 μm . There were no obvious differences in the thickness of ML in the $Fpr2/3^{-/-}$ mice versus wildtypes, or following LPS or vehicle. The ML did not contain any immunoreactive staining for GFP in either the $Fpr2/3^{-/-}$ or the WT sections. The granule cell layer (GCL) includes the densely packed granule cells and other neuronal structures such as the pyramidal basket cells along the periphery and characteristically partly enclosed the polymorphic layer (PML). Taken together, the data indicate that in animals injected either 2 hours or 6 hours previously there was no observable GFP immunoreactivity in the ML, GCL or PML within the DG of the hippocampal formation of brain sections from $Fpr2/3^{-/-}$ mice or wildtype littermates, irrespective of LPS or saline treatment.



(a) WT PBS 2 h Hippocampus

(b) Fpr2/3^{-/-} PBS 2 h Hippocampus

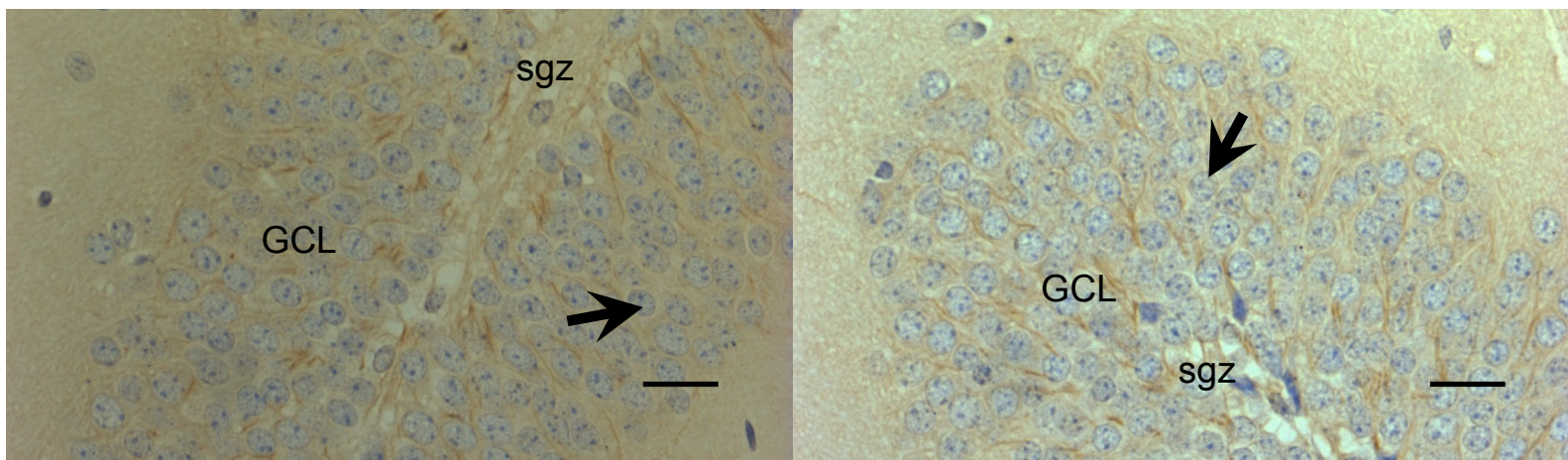


(c) WT LPS 2 h Hippocampus

(d) Fpr2/3^{-/-} LPS 2 h Hippocampus

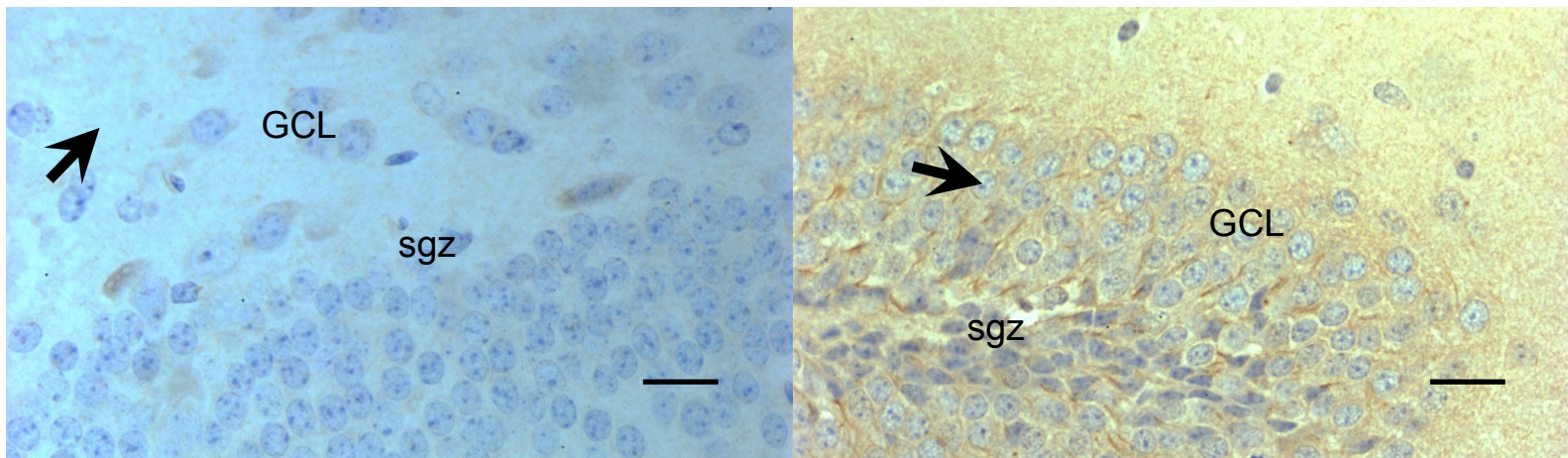
Figure 3.14: Immunostain for green fluorescent protein (GFP) within the hippocampus of sectioned brains taken from GFP transgenic, formyl peptide receptor 2 and 3 knockout (Fpr2/3^{-/-}) mice versus wildtype (WT) littermates 2 h following intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) or phosphate buffered saline (PBS) vehicle.

Coronal sections (4 μ m) of brains taken from WT and Fpr2/3^{-/-} mice (n = 4) 2 h after receiving LPS (750 μ g/kg i.p.) or PBS were stained with primary anti-GFP and secondary horseradish peroxidase (HRP)-conjugated antisera, then counterstained with diaminobenzidine (DAB, dark brown) and a weak haematoxylin solution (HX, blue). The medial blade of the dentate gyrus within the hippocampus was imaged by light microscopy (20x, scale bar = 10 μ m). Arrows denote granular neurons, and the granule cell layer (GCL) and subgranular zone (sgz) are annotated.



(a) WT PBS 6 h Hippocampus

(b) Fpr2/3^{-/-} PBS 6 h Hippocampus



(c) WT LPS 6 h Hippocampus

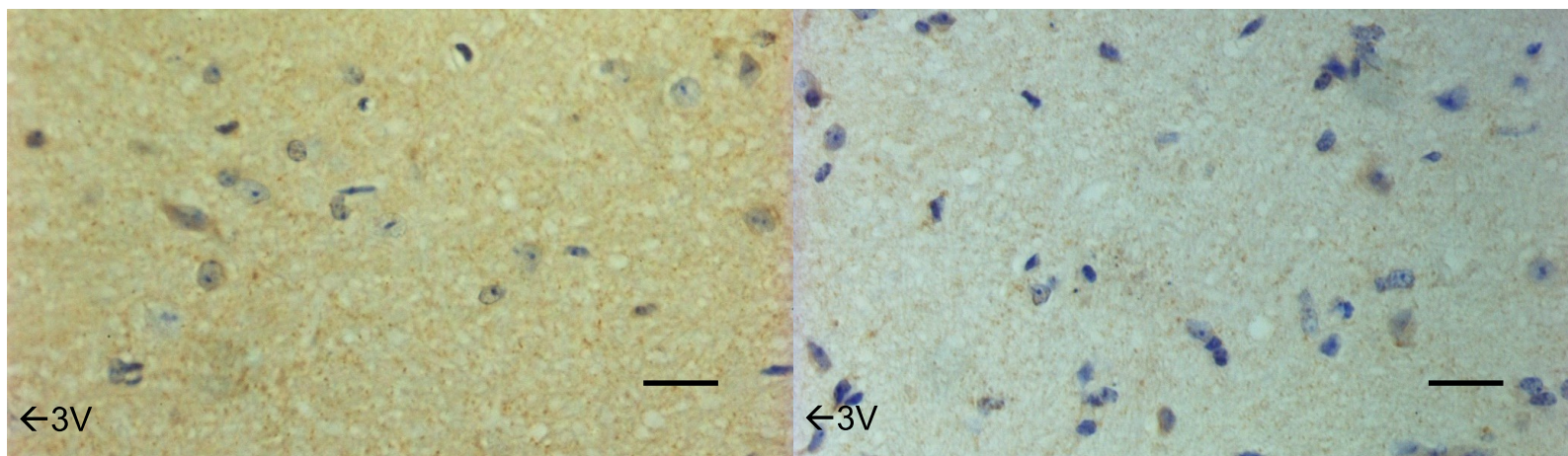
(d) Fpr2/3^{-/-} LPS 6 h Hippocampus

Figure 3.15: Immunostain for green fluorescent protein (GFP) within the hippocampus of sectioned brains taken from GFP transgenic, formyl peptide receptor 2 and 3 knockout (Fpr2/3^{-/-}) mice versus wildtype (WT) littermates 6 h following intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) or phosphate buffered saline (PBS) vehicle.

Coronal sections (4 μ m) of brains taken from WT and Fpr2/3^{-/-} mice (n = 4) 6 h after receiving LPS (750 μ g/kg i.p.) or PBS were stained with primary anti-GFP and secondary horseradish peroxidase (HRP)-conjugated antisera, then counterstained with diaminobenzidine (DAB, dark brown) and a weak haematoxylin solution (HX, blue). The medial blade of the dentate gyrus within the hippocampus was imaged by light microscopy (20x, scale bar = 10 μ m). Arrows denote granular neurons, and the granule cell layer (GCL) and subgranular zone (sgz) are annotated.

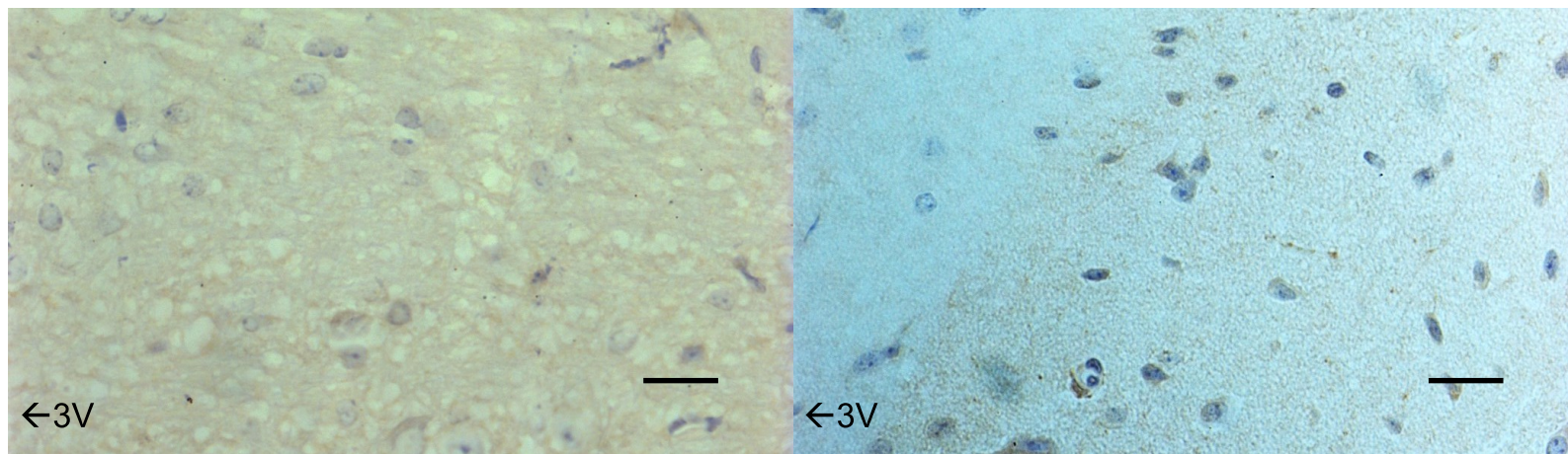
3.3.4.3 The paraventricular nucleus of the hypothalamus

Figure 3.16 and Figure 3.17 show the results of immunostaining in coronal sections of the hypothalamus. The area of assessment was the paraventricular nucleus (PVN) situated above the third ventricle and median eminence. The data indicate that in animals injected either 2 hours or 6 hours previously, there was no observable GFP immunoreactivity in sections from *Fpr2/3^{-/-}* mice or wildtype littermates, irrespective of LPS or saline treatment.



(a) WT PBS 2 h Hypothalamus

(b) $Fpr2/3^{-/-}$ PBS 2 h Hypothalamus

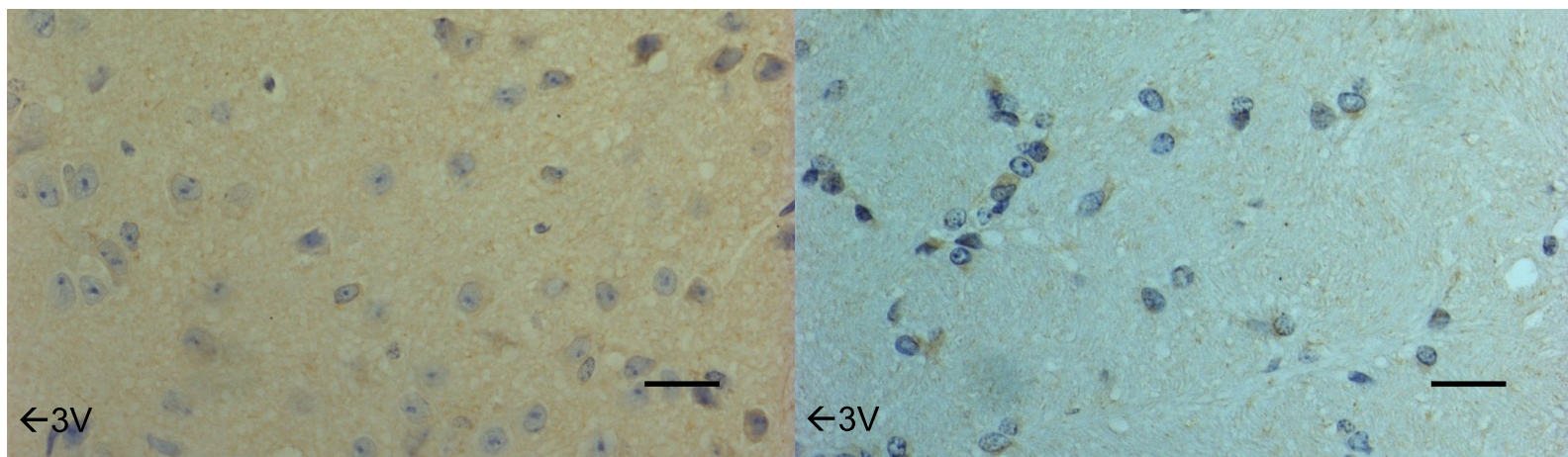


(c) WT LPS 2 h Hypothalamus

(d) $Fpr2/3^{-/-}$ LPS 2 h Hypothalamus

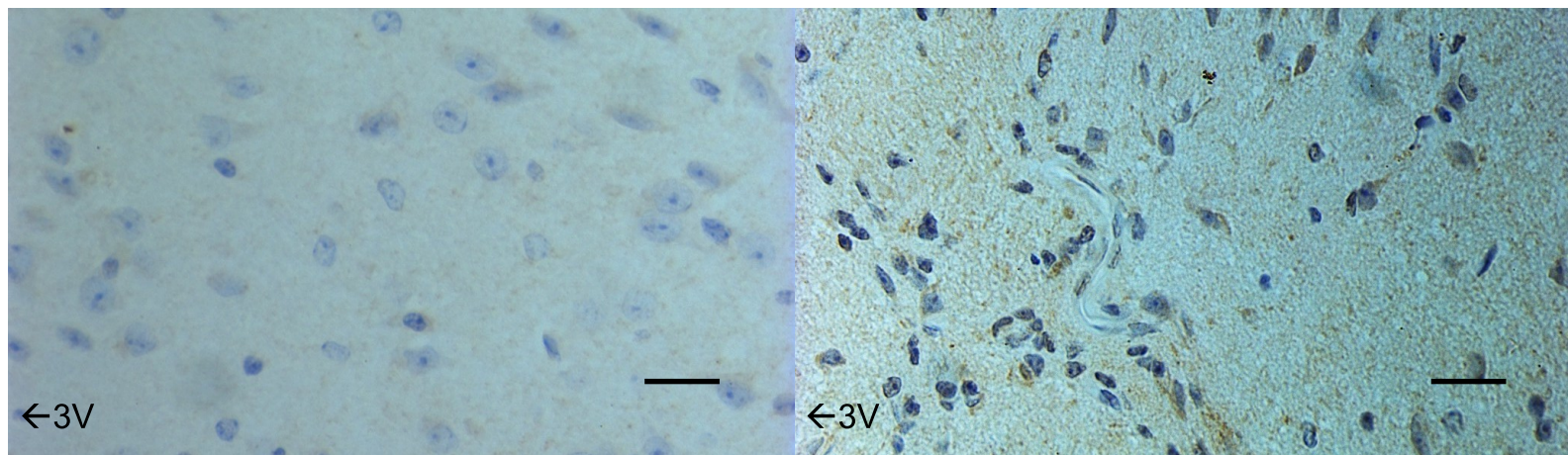
Figure 3.16: Immunostain for green fluorescent protein (GFP) within the hypothalamus of sectioned brains taken from GFP transgenic, formyl peptide receptor 2 and 3 knockout ($Fpr2/3^{-/-}$) mice versus wildtype (WT) littermates 2 h following intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) or phosphate buffered saline (PBS) vehicle.

Coronal sections (4 μ m) of brains taken from WT and $Fpr2/3^{-/-}$ mice (n = 4) 2 h after receiving LPS (750 μ g/kg i.p.) or PBS were stained with primary anti-GFP and secondary horseradish peroxidase (HRP)-conjugated antisera, then counterstained with diaminobenzidine (DAB, dark brown) and a weak haematoxylin solution (HX, blue). The region superior to the paraventricular nuclei (PVN) of the hypothalamus and lateral to the third ventricle (3V) was imaged by light microscopy (20x, scale bar = 10 μ m).



(a) WT PBS 6 h Hypothalamus

(b) Fpr2/3^{-/-} PBS 6 h Hypothalamus



(c) WT LPS 6 h Hypothalamus

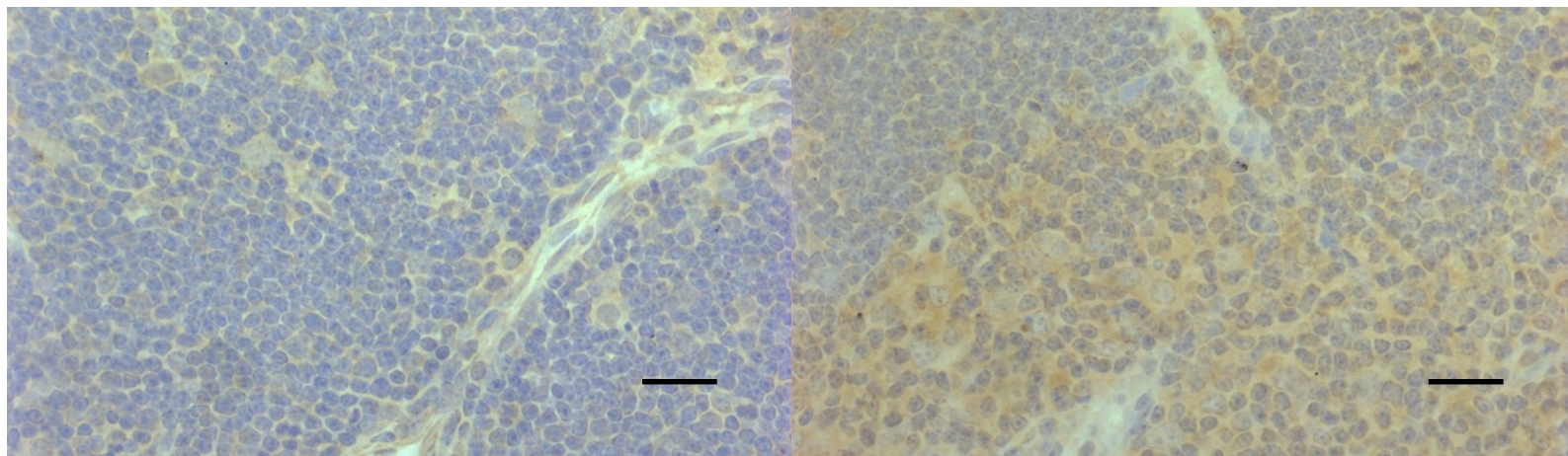
(d) Fpr2/3^{-/-} LPS 6 h Hypothalamus

Figure 3.17: Immunostain for green fluorescent protein (GFP) within the hypothalamus of sectioned brains taken from GFP transgenic, formyl peptide receptor 2 and 3 knockout (Fpr2/3^{-/-}) mice versus wildtype (WT) littermates 6 h following intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) or phosphate buffered saline (PBS) vehicle.

Coronal sections (4 μ m) of brains taken from WT and Fpr2/3^{-/-} mice (n = 4) 6 h after receiving LPS (750 μ g/kg i.p.) or PBS were stained with primary anti-GFP and secondary horseradish peroxidase (HRP)-conjugated antisera, then counterstained with diaminobenzidine (DAB, dark brown) and a weak haematoxylin solution (HX, blue). The region superior to the paraventricular nuclei (PVN) of the hypothalamus and lateral to the third ventricle (3V) was imaged by light microscopy (20x, scale bar = 10 μ m).

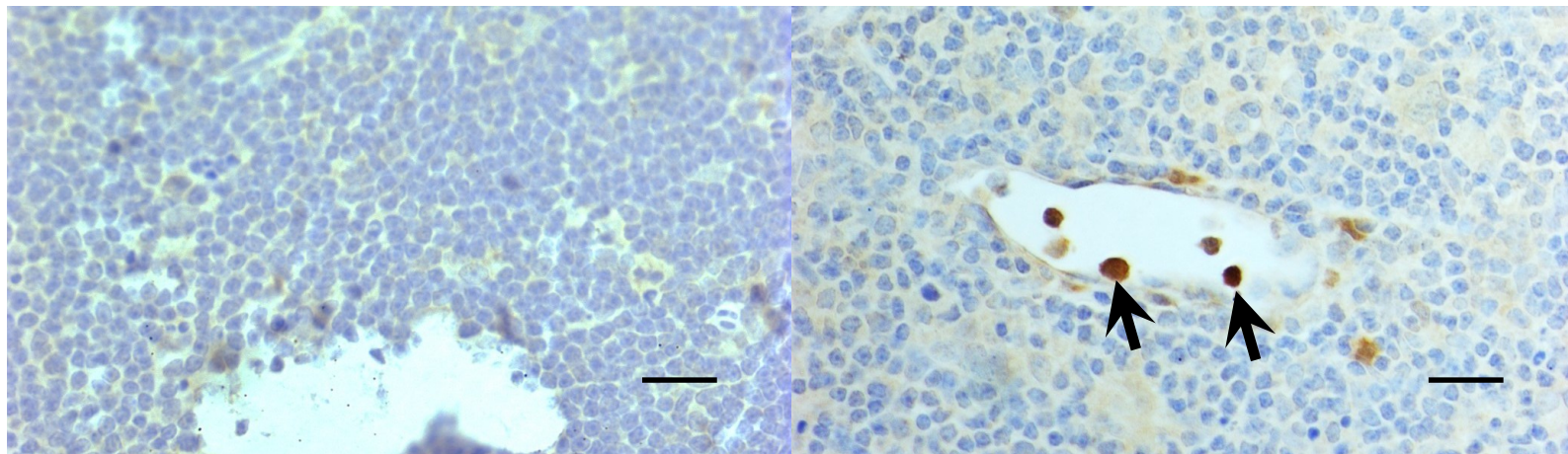
3.3.4.4 Thymic follicles

Figure 3.18 and Figure 3.19 show the results of immunostaining for GFP in sections of the thymus. The area of assessment was the thymic follicles within the cortex of the thymus. The thymus glands observed in the *Fpr2/3^{-/-}* and WT mice all exhibited a characteristic structure with an outer cortex surrounded by an external capsule, and an inner medulla region. The cortex encompassed multiple lobules separated by intralobular septa or trabeculae and contained blood vessels, thymocytes, and macrophages. As well as being central and having a lighter haematoxylin-staining pattern, the medulla was distinct from the cortex by the presence of Hassall's corpuscles. The data indicate that in animals injected 2 hours previously, GFP immunoreactivity was evident in the LPS treated *Fpr2/3^{-/-}* mice (Figure 3.18 d), but was not observed in the wildtype littermates (Figure 3.18 c) or the saline injected controls of either genotype. Conversely, in the animals injected 6 hours previously, GFP immunoreactivity was no longer evident in the LPS treated *Fpr2/3^{-/-}* mice (Figure 3.19 d) versus those injected 2 hours previously (Figure 3.18 d). But, as in the two-hour group, no GFP was observed in the wildtype littermates (Figure 3.19 c) or the saline injected controls of either genotype.



(a) WT PBS 2 h Thymus

(b) Fpr2/3^{-/-} PBS 2 h Thymus

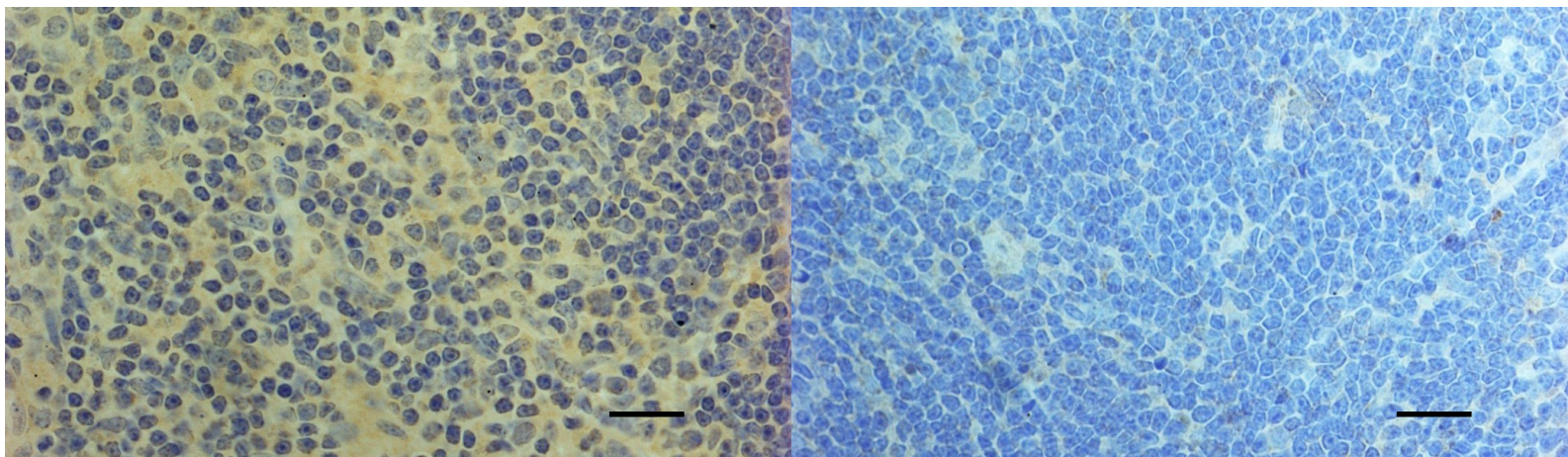


(c) WT LPS 2 h Thymus

(d) Fpr2/3^{-/-} LPS 2 h Thymus

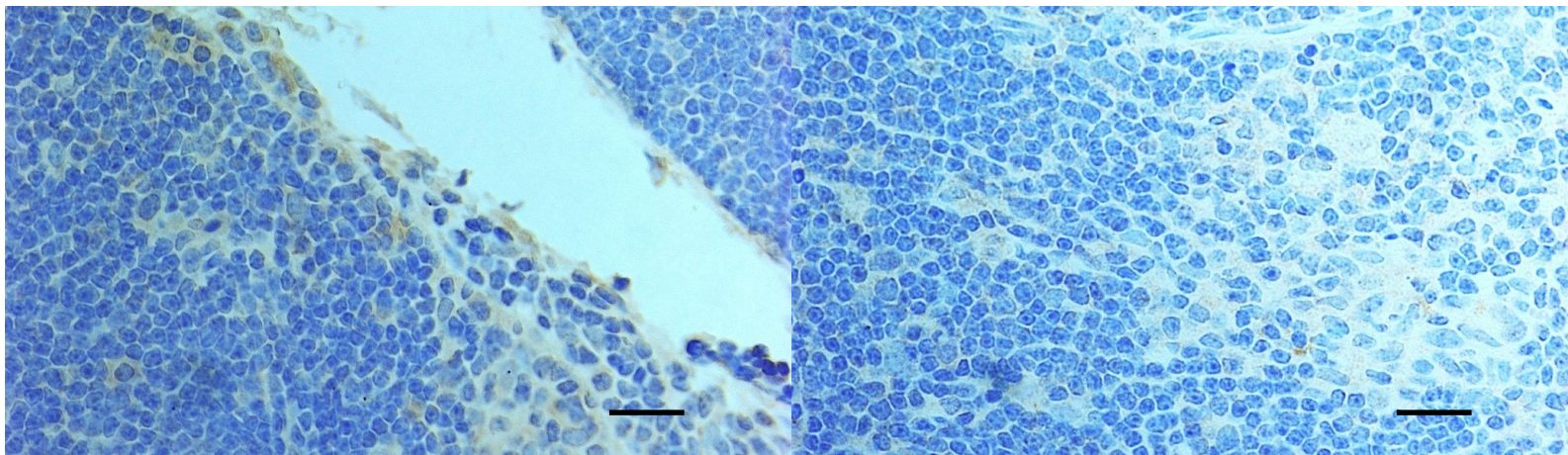
Figure 3.18: Immunostain for green fluorescent protein (GFP) within the thymus taken from GFP transgenic, formyl peptide receptor 2 and 3 knockout (Fpr2/3^{-/-}) mice versus wildtype (WT) littermates 2 h following intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) or phosphate buffered saline (PBS) vehicle.

Transverse sections (4 μ m) of the thymus taken from WT and Fpr2/3^{-/-} mice (n = 4) 2 h after receiving LPS (750 μ g/kg i.p.) or PBS were stained with primary anti-GFP and secondary horseradish peroxidase (HRP)-conjugated antisera, then counterstained with diaminobenzidine (DAB, dark brown) and a weak haematoxylin solution (HX, blue). The medial thymic follicles encompassing the cortex and medulla were imaged by light microscopy (20x, scale bar = 10 μ m). Arrows denote positive GFP staining.



(a) WT PBS 6 h Thymus

(b) $Fpr2/3^{-/-}$ PBS 6 h Thymus



(c) WT LPS 6 h Thymus

(d) $Fpr2/3^{-/-}$ LPS 6 h Thymus

Figure 3.19: Immunostain for green fluorescent protein (GFP) within the thymus taken from GFP transgenic, formyl peptide receptor 2 and 3 knockout ($Fpr2/3^{-/-}$) mice versus wildtype (WT) littermates 6 h following intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) or phosphate buffered saline (PBS) vehicle.

Transverse sections (4 μ m) of the thymus taken from WT and $Fpr2/3^{-/-}$ mice (n = 4) 6 h after receiving LPS (750 μ g/kg i.p.) or PBS were stained with primary anti-GFP and secondary horseradish peroxidase (HRP)-conjugated antisera, then counterstained with diaminobenzidine (DAB, dark brown) and a weak haematoxylin solution (HX, blue). The medial thymic follicles encompassing the cortex and medulla were imaged by light microscopy (20x, scale bar = 10 μ m).

3.3.5 Immunofluorescent detection of formyl peptide receptors (FPR1, FPR2/ALX, and FPR3) in the human anterior pituitary

The following results are confocal micrographs and colocalisation data, including cytofluorograms and cell counts, from experiments in which human anterior pituitary sections were immunostained with antibodies for ACTH or S100b, along with those for FPR1, FPR2/ALX, and FPR3. The cytofluorograms are scatterplots of red pixel intensities against green pixel intensities and are annotated with three coefficients of colocalisation generated by the Just Another Colocalisation Plugin (JACoP) in the ImageJ software from the National Institute of Health (Cordelieres & Bolte 2008). Pearson's correlation coefficient (r_p) enumerates the percentage of variability in channel 1 (red) that is equal to variability in channel 2 (green). The values for r_p range from -1 to +1, with -1 indicating total negative correlation, 0 indicative of random correlation, and +1 indicating total positive correlation. The Manders' colocalisation coefficient (M1) is the percentage of pixels above background in the first channel (red) that overlap pixels above background in the second channel (green), whereas M2 is the percentage of above-background pixels in the second channel that overlap those in the first channel (Manders *et al.* 1993). The background within images can be automatically subtracted using Costes' method, giving values for r_p , and M1 and M2 that avoid human bias, and analyses are thus consistent both within and between images. Costes' method involves a scatterplot of pixel intensities from the red and green channels, with a curve whose slope is determined by orthogonal regression with least-squares fit (Costes *et al.* 2004). The point of highest intensity on the curve (T) is used to calculate r_p for all pixels below T, and regression continues to reduce the value of T until r_p equals zero, indicating random correlation. This is then the threshold by which pixels are counted above background for each image. Costes' method also tests the values for r_p in each image against scrambled iterations of the second image to simulate random overlap, and the resulting P-value is the probability of real colocalisation in the original images at the 95% confidence level.

3.3.5.1 ACTH

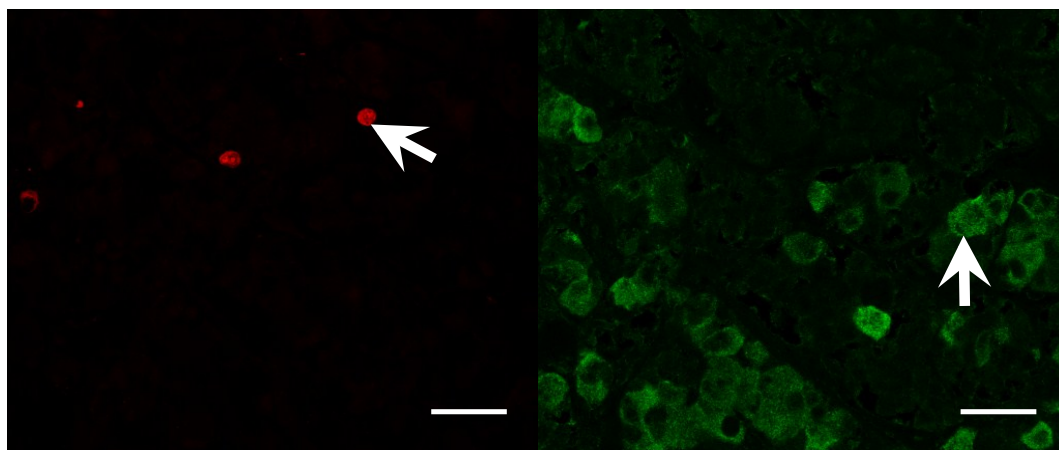
Figure 3.20, Figure 3.21, and Figure 3.22 show the confocal micrographs of immunostaining for ACTH and the three human formyl peptide receptors subtypes, FPR1, FPR2/ALX, and FPR3, respectively. In all cases, the stain for ACTH worked well and shows clear ACTH immunoreactivity (shown in green) compared with the no primary antibody stain (not shown). These results are also enumerated in Figure 3.26, which shows the results of manually counting cells with red FPR1, FPR2/ALX, or FPR3 immunoreactivity, green ACTH signal, or both FPR and ACTH. In all experiments, the number of ACTH-positive cells counted was the same (ns $P > .05$), irrespective of whether the sections were immunostained for FPR1, FPR2/ALX, or FPR3.

The experiments resulted in very clear positive staining for FPR1 immunoreactivity, although these cells were few in number, with between 1 and 10 cells in the field of view. Given their size and horse-shoe shaped nuclei these FPR1-positive cells were possibly indicative of monocytes (10 μm), or possibly macrophage infiltrates in the case of some larger cells (20 μm) with round nuclei (Krombach et al. 1997). Some larger, anucleated bodies of red fluorescence were observed, and these are likely to be from pituitary mucins which have immunoglobulin G (IgG)/fragment of crystallisation (Fc)-binding activity and thus permit non-specific binding of antibodies (Harada et al. 1997). The manual counting data indicated that there was no colocalisation of FPR1 immunoreactivity with ACTH, and the number of ACTH-positive cells was significantly greater than the number of FPR1-positive cells ($*** P < .001$). These data were corroborated by the cytofluorogram (Figure 3.20 d) for the representative image, and the overall results for the wider tissue area from several cases, as tabulated in Table 3.1.

In Figure 3.20 for instance, the values are .002 for M1 and .329 for M2. This indicates that, in the Costes' automatically thresholded images, .2% of the red pixels for FPR1 overlapped with the green pixels for ACTH, and that 32.9% of the green pixels for ACTH overlapped with the red pixels for FPR1. The value for r_p , however, is .000 indicating that there was no correlation between the variation in pixel intensities in the red and green channels. This was concordant with the overall findings across all samples for ACTH and FPR1 (Table 3.1) because Pearson's coefficient indicated zero correlation between ACTH and FPR1 pixel variation ($r_p = -.036 \pm .029$), and Costes' P value indicated this relationship was not significant at the 95% confidence level ($P = 72.44\% \pm 14.81$).

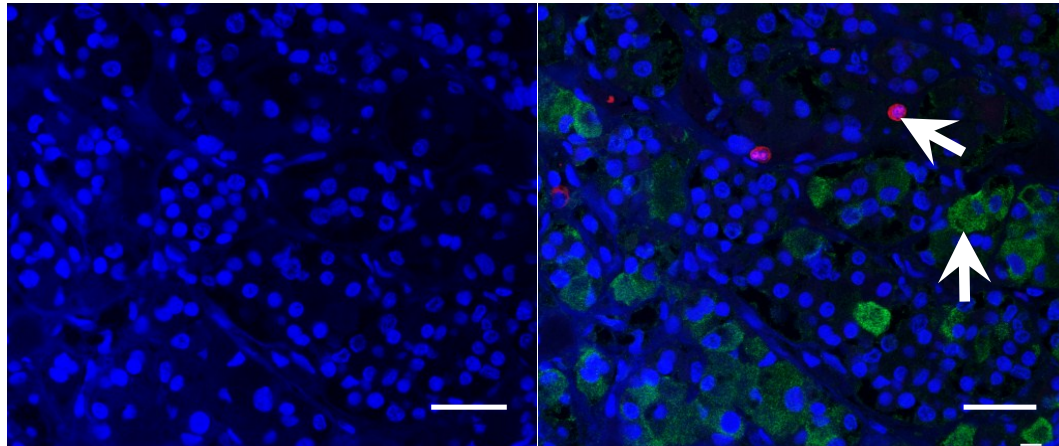
With regard to the immunostain of FPR2/ALX and ACTH, the manual cell count observed a number of cells that were positive for both ACTH and FPR2/ALX immunofluorescence (Figure 3.26), but the majority of ACTH-positive cells did not contain FPR2/ALX (** $P < .001$). In Figure 3.21, the Manders' coefficients ($M1 = .999$, $M2 = .624$) indicated that, in the Costes' background-subtracted images, 99.9% of the red pixels for FPR2/ALX overlapped with the green pixels for ACTH, and that 62.4% of the green pixels for ACTH overlapped with the red pixels for FPR2. The value for Pearson's correlation coefficient equals $-.113$, which indicated a negative correlation between the variation in pixel intensities for ACTH and FPR2/ALX immunofluorescence. This was concordant with the overall findings for ACTH and FPR2/ALX across all cases (Table 3.1) in which Pearson's coefficient indicated a negative correlation between ACTH and FPR2/ALX pixel variation ($r_p = -.024 \pm .018$), and Costes' P value indicated this relationship was not significant at the 95% confidence level ($P = 83.86\% \pm 11.61$).

The FPR3-positive cells were largely clustered into acini and were therefore suggestive of groups of corticotrophic, ACTH-positive cells. The manual cell count indicated there was little colocalisation of FPR3 with ACTH (Figure 3.26), but within the images obtained there is evidence of clustering of FPR3-positive cells neighbouring a body of ACTH-positive cells within acini (Figure 3.22). In fact, there were more FPR3-positive cells than ACTH-positive cells (** $P < .001$). In Figure 3.22, the Manders' coefficients indicated that, 99.9% of the red pixels for FPR3 overlapped with the green pixels for ACTH, and that 95.6% of the green pixels for ACTH overlapped with the red pixels for FPR3. The Pearson's correlation coefficient of $.544$ indicated a positive correlation between the variation in pixel intensities for ACTH and FPR3 immunofluorescence. This is concordant with the overall findings across all samples for ACTH and FPR3 (Table 3.1) in which Pearson's coefficient indicated a positive correlation between ACTH and FPR3 pixel variation ($r_p = .126 \pm .039$) and Costes' P value indicated that this relationship was significant at the 99.9% confidence level ($P = 100.00\% \pm 0.00$).



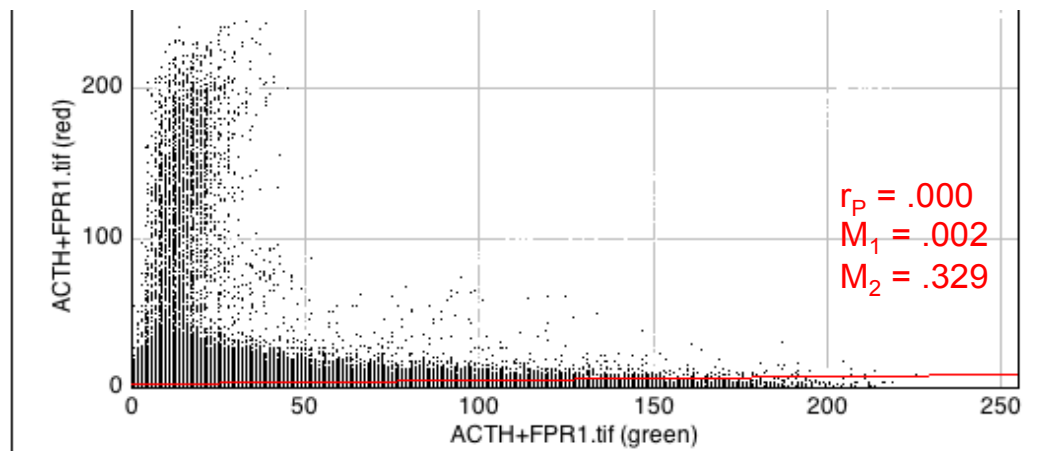
(a) FPR1

(b) ACTH



(c) DAPI

(d) Merge



(e) Cytofluorogram – FPR1 (red) against ACTH (green)

Figure 3.20: Confocal micrographs and cytofluorogram from an immunostain for formyl peptide receptor 1 (FPR1) and adrenocorticotrophic hormone (ACTH) within the pars distalis of sectioned anterior pituitary glands obtained *post mortem* from humans with multiple sclerosis (MS).

Transverse sections (4 μm) of anterior pituitary glands taken from deceased MS patients ($n = 6$) were stained with primary anti-FPR1 and anti-ACTH followed by secondary antisera tagged with Alexa Fluor 555 (red, for FPR1) or Alexa Fluor 488 (green, for ACTH), and counterstained with diamidino phenylindole (DAPI) to identify nuclei. Images are from one of nine adjacent fields of view analysed from one of six pituitary glands. The region between the lateral wings of the pars distalis and anterior to the pars intermedia was imaged by confocal fluorescence microscopy (63.3x, scale bar = 50 μm). Arrows denote immunofluorescence for perinuclear FPR1 (a) and cytoplasmic and membranous ACTH (b). The cytofluorogram (e) plots the pixel intensities from the red channel (FPR1, y-axis) versus green channel (ACTH, x-axis) and is annotated with Pearson correlation (r_P) and Costes' automatically thresholded Manders' colocalisation (M_1 and M_2) coefficients.

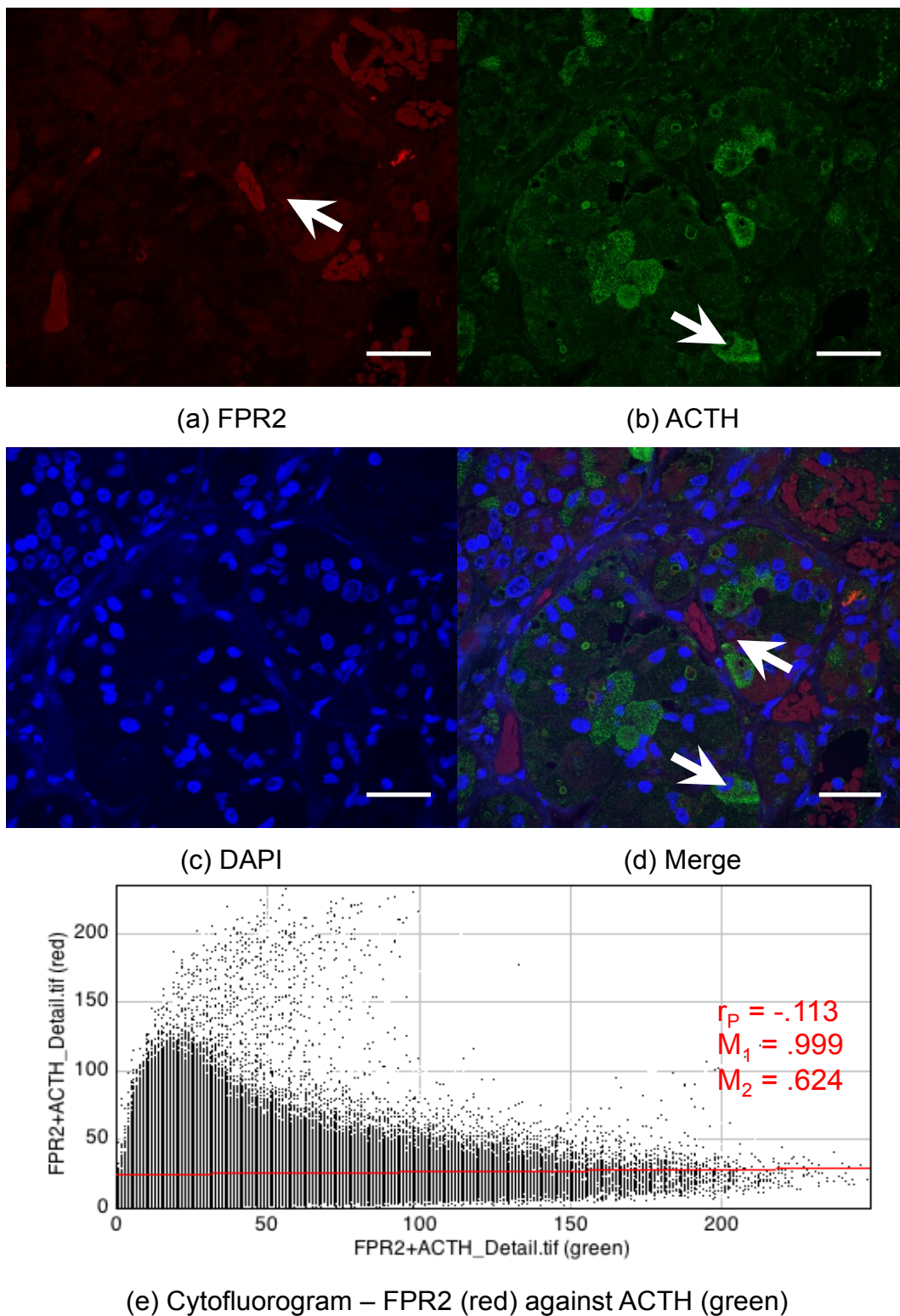


Figure 3.21: Confocal micrographs and cytofluorogram from an immunostain for formyl peptide receptor 2 (FPR2/ALX) and adrenocorticotrophic hormone (ACTH) within the pars distalis of sectioned anterior pituitary glands obtained *post mortem* from humans with multiple sclerosis (MS). Transverse sections (4 μm) of anterior pituitary glands taken from deceased MS patients ($n = 6$) were stained with primary anti-FPR2/ALX and anti-ACTH followed by secondary antisera tagged with Alexa Fluor 555 (red, for FPR2/ALX) or Alexa Fluor 488 (green, for ACTH), and counterstained with diamidino phenylindole (DAPI) to identify nuclei. Images are from one of nine adjacent fields of view analysed from one of six pituitary glands. The region between the lateral wings of the pars distalis and anterior to the pars intermedia was imaged by confocal fluorescence microscopy (63.3x, scale bar = 50 μm). Arrows denote immunofluorescence for perinuclear FPR2/ALX (a) and cytoplasmic and membranous ACTH (b). The cytofluorogram (e) plots the pixel intensities from the red channel (FPR2/ALX, y-axis) versus green channel (ACTH, x-axis) and is annotated with Pearson correlation (r_P) and Costes' automatically thresholded Manders' colocalisation (M_1 and M_2) coefficients.

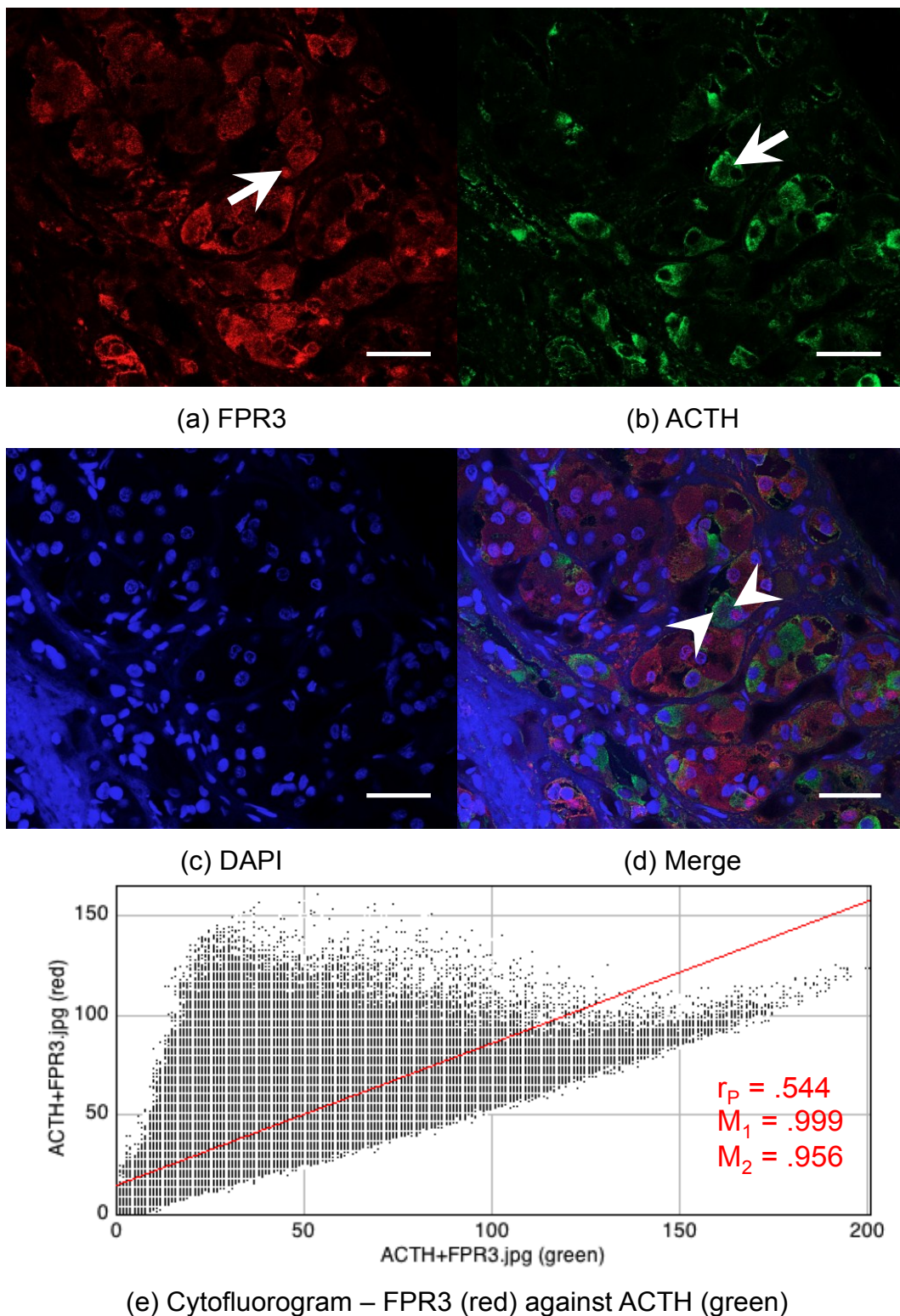


Figure 3.22: Confocal micrographs and cytofluorogram from an immunostain for formyl peptide receptor 3 (FPR3) and adrenocorticotrophic hormone (ACTH) within the pars distalis of sectioned anterior pituitary glands obtained *post mortem* from humans with multiple sclerosis (MS).

Transverse sections (4 μm) of anterior pituitary glands taken from deceased MS patients ($n = 6$) were stained with primary anti-FPR3 and anti-ACTH followed by secondary antisera tagged with Alexa Fluor 555 (red, for FPR3) or Alexa Fluor 488 (green, for ACTH), and counterstained with diamidino phenylindole (DAPI) to identify nuclei. Images are from one of nine adjacent fields of view analysed from one of six pituitary glands. The region between the lateral wings of the pars distalis and anterior to the pars intermedia was imaged by confocal fluorescence microscopy (63.3x, scale bar = 50 μm). Arrows denote immunofluorescence for perinuclear FPR3 (a) and cytoplasmic and membranous ACTH (b). The cytofluorogram (e) plots the pixel intensities from the red channel (FPR3, y-axis) versus green channel (ACTH, x-axis) and is annotated with Pearson correlation (r_P) and Costes' automatically thresholded Manders' colocalisation (M_1 and M_2) coefficients.

3.3.5.2 S100b

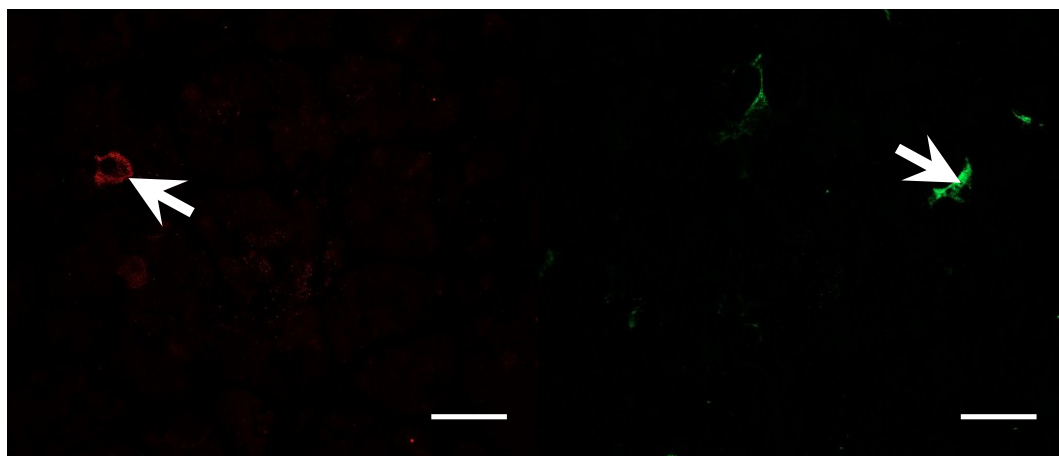
Figure 3.23, Figure 3.24, and Figure 3.25 show the results of immunostaining for S100b and the three human formyl peptide receptors subtypes, FPR1, FPR2/ALX, and FPR3. The results of the manual cell counts are enumerated in Figure 3.26, and these indicated that there was no colocalisation of FPR1, FPR2/ALX, or FPR3 with S100b immunoreactivity and there were no differences in the number of S100b-positive cells counted on separate sections stained for FPR1, FPR2/ALX, or FPR3 (ns $P > .05$).

With regard to FPR1 and S100b immunofluorescence evident in Figure 3.23, the Manders' coefficients indicated that 94.9% of the red pixels for FPR1 overlapped with the green pixels for S100b, and that 78.3% of the green pixels for S100b overlapped with the red pixels for FPR1. The Pearson's correlation coefficient of .067 indicated a slight correlation between the variation in pixel intensities for S100b and FPR1 immunofluorescence. In the overall results across all samples (Table 3.1), although the Pearson's correlation coefficient indicated a slight correlation between S100b and FPR1 pixel variation ($r_p = .055 \pm .016$), the Costes' P value, however, indicated that this relationship was not significant at the 95% confidence level ($P = 95.18\% \pm 5.57$).

With regard to FPR2/ALX and S100b immunofluorescence, Figure 3.24 shows that the Manders' coefficients of .983 for M1 and .604 for M2 indicated that, in the Costes' automatically thresholded images, 98.3% of the red pixels for FPR2/ALX overlapped with the green pixels for S100b, and that 60.4% of the green pixels for S100b overlapped with the red pixels for FPR2. The Pearson's correlation coefficient of .236 indicated a positive correlation between the variation in pixel intensities for S100b and FPR2/ALX immunofluorescence. In the overall results across all samples (Table 3.1), although the Pearson's correlation coefficient indicated a positive correlation between S100b and FPR2/ALX pixel variation ($r_p = .024 \pm .015$), this relationship was not significant as Costes' P value was lower than the 95% confidence level ($P = 67.49\% \pm 17.56$).

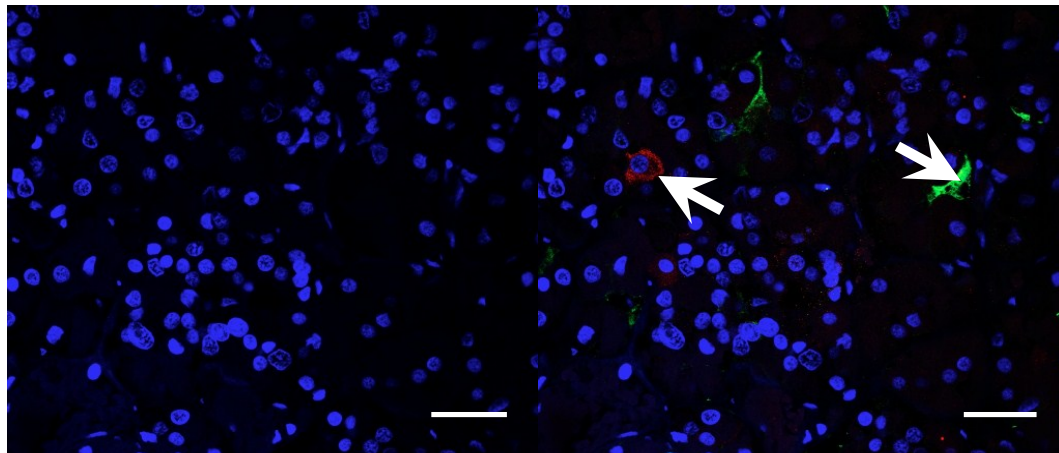
With regard to FPR3 and S100b immunofluorescence, Figure 3.25 shows that the Manders' coefficients of .000 for M1 and .000 for M2 indicated that, in the Costes' automatically thresholded images, 0.0% of the red pixels for FPR3 overlapped with the green pixels for S100b, and that 0.0% of the green pixels for S100b overlapped with the red pixels for FPR3. The Pearson's correlation coefficient of .000 indicated

there was no correlation between the variation in pixel intensities for S100b and FPR3 immunofluorescence. In the overall results across all samples (Table 3.1), although Pearson's correlation coefficient indicated a slight correlation between S100b and FPR2/ALX pixel variation ($r_p = .028 \pm .048$), the Costes' P value, however, indicated that this relationship was not significant at the 95% confidence level ($P = 90.97\% \pm 7.33$).



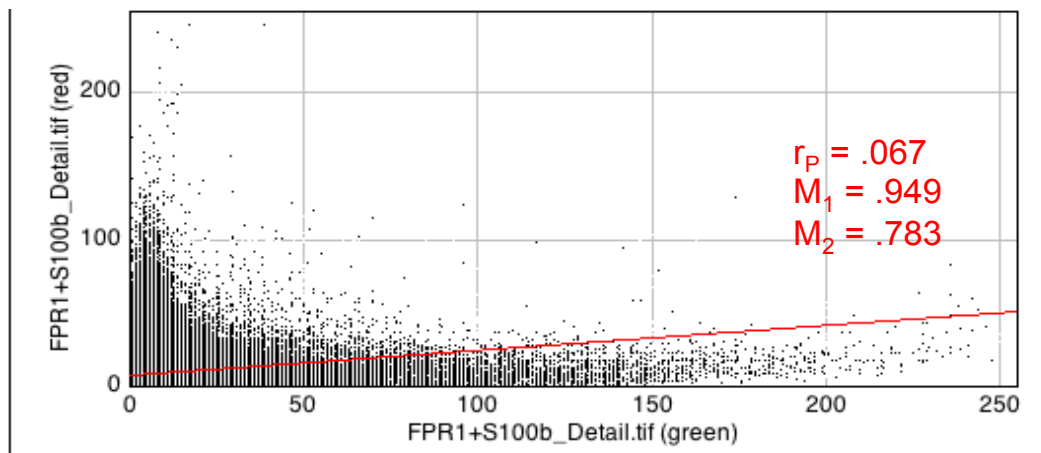
(a) FPR1

(b) S100b



(c) DAPI

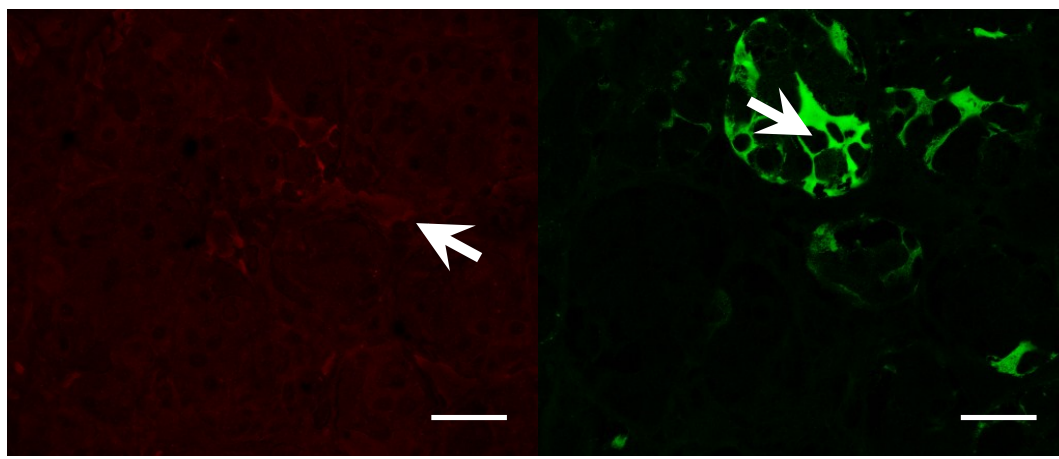
(d) Merge



(e) Cytofluorogram – FPR1 (red) against S100b (green)

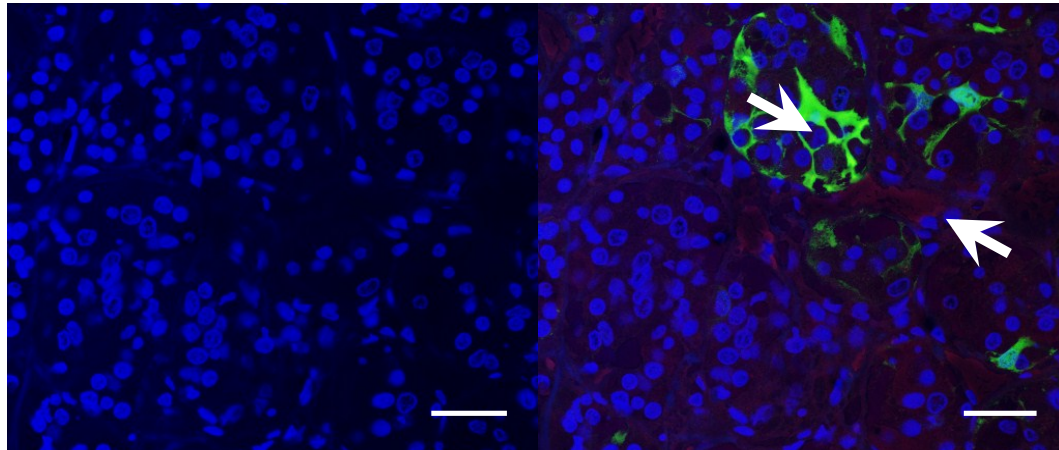
Figure 3.23: Confocal micrographs and cytofluorogram from an immunostain for formyl peptide receptor 1 (FPR1) and S100 calcium binding protein b (S100b) within the pars distalis of sectioned anterior pituitary glands obtained *post mortem* from humans with multiple sclerosis (MS).

Transverse sections (4 μm) of anterior pituitary glands taken from deceased MS patients ($n = 6$) were stained with primary anti-FPR1 and anti-S100b followed by secondary antisera tagged with Alexa Fluor 555 (red, for FPR1) or Alexa Fluor 488 (green, for S100b), and counterstained with diamidino phenylindole (DAPI) to identify nuclei. Images are from one of nine adjacent fields of view analysed from one of six pituitary glands. The region between the lateral wings of the pars distalis and anterior to the pars intermedia was imaged by confocal fluorescence microscopy (63.3x, scale bar = 50 μm). Arrows denote immunofluorescence for perinuclear FPR1 (a) and cytoplasmic and membranous S100b (b). The cytofluorogram (e) plots the pixel intensities from the red channel (FPR1, y-axis) versus green channel (S100b, x-axis) and is annotated with Pearson correlation (r_p) and Costes' automatically thresholded Manders' colocalisation (M_1 and M_2) coefficients.



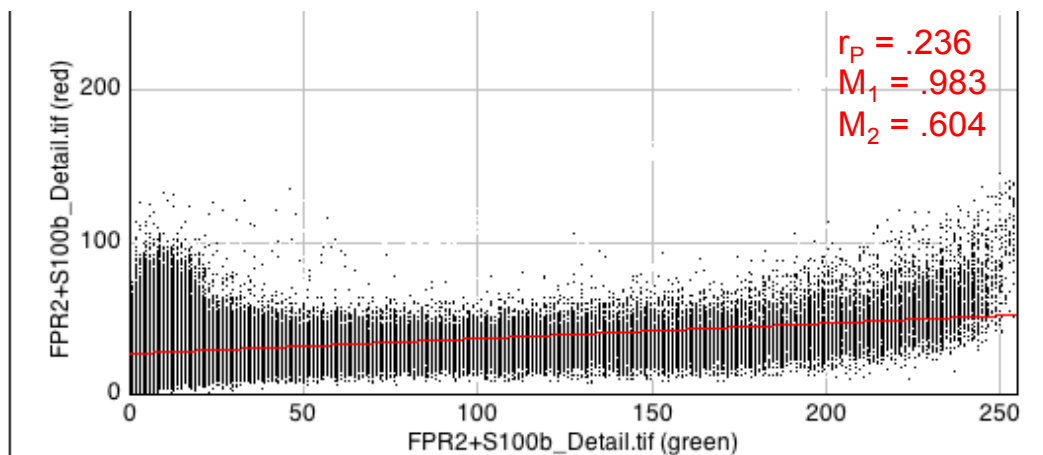
(a) FPR2

(b) S100b



(c) DAPI

(d) Merge



(e) Cytofluorogram – FPR2 (red) against S100b (green)

Figure 3.24: Confocal micrographs and cytofluorogram from an immunostain for formyl peptide receptor 2 (FPR2/ALX) and S100 calcium binding protein b (S100b) within the pars distalis of sectioned anterior pituitary glands obtained *post mortem* from humans with multiple sclerosis (MS). Transverse sections (4 μm) of anterior pituitary glands taken from deceased MS patients ($n = 6$) were stained with primary anti-FPR2/ALX and anti-S100b followed by secondary antisera tagged with Alexa Fluor 555 (red, for FPR2/ALX) or Alexa Fluor 488 (green, for S100b), and counterstained with diamidino phenylindole (DAPI) to identify nuclei. Images are from one of nine adjacent fields of view analysed from one of six pituitary glands. The region between the lateral wings of the pars distalis and anterior to the pars intermedia was imaged by confocal fluorescence microscopy (63.3x, scale bar = 50 μm). Arrows denote immunofluorescence for perinuclear FPR2/ALX (a) and cytoplasmic and membranous S100b (b). The cytofluorogram (f) plots the pixel intensities from the red channel (FPR2/ALX, y-axis) versus green channel (S100b, x-axis) and is annotated with Pearson correlation (r_p) and Costes' automatically thresholded Manders' colocalisation (M_1 and M_2) coefficients.

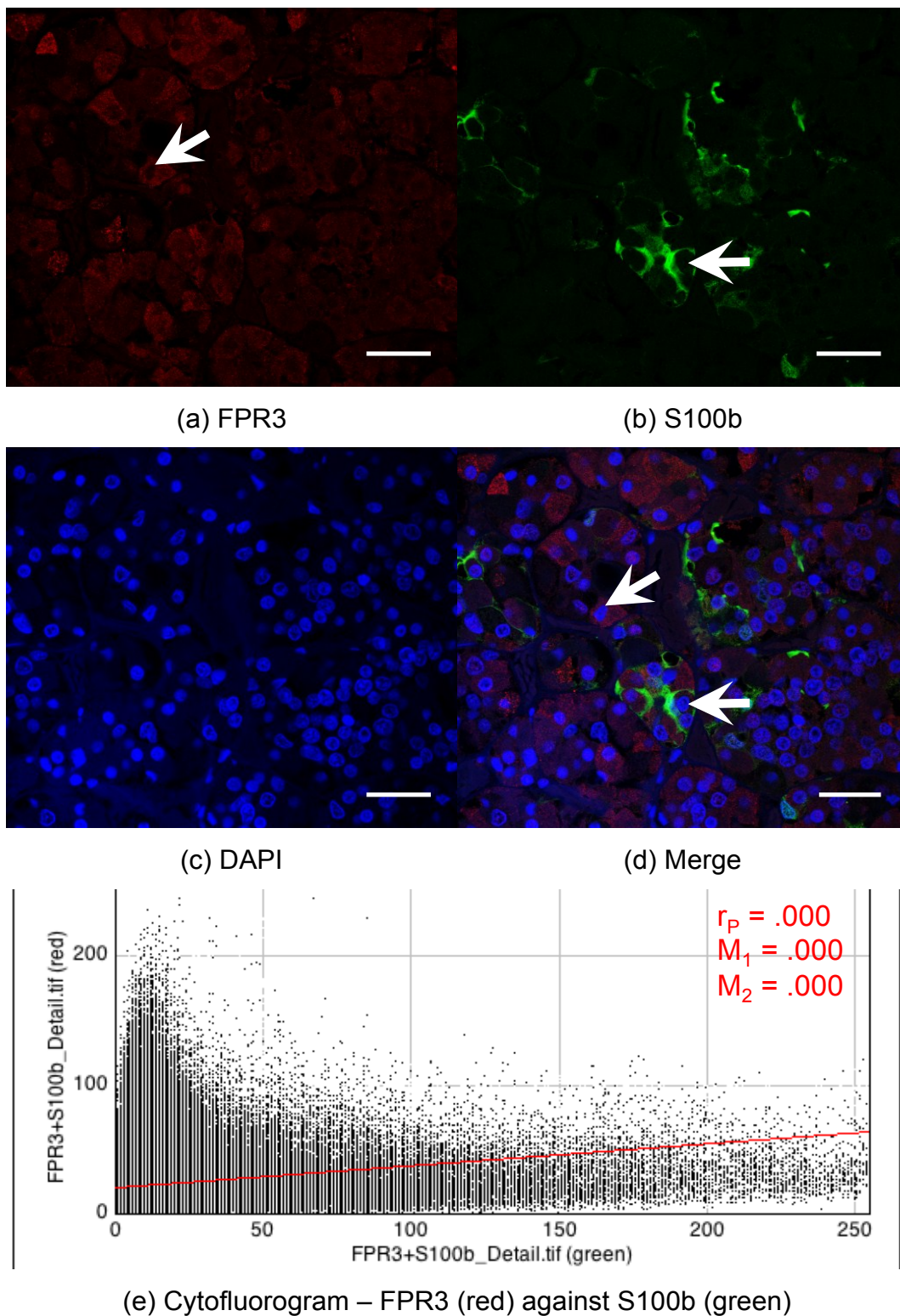


Figure 3.25: Confocal micrographs and cytofluorogram from an immunostain for formyl peptide receptor 3 (FPR3) and S100 calcium binding protein b (S100b) within the pars distalis of sectioned anterior pituitary glands obtained *post mortem* from humans with multiple sclerosis (MS).

Transverse sections (4 μm) of anterior pituitary glands taken from deceased MS patients ($n = 6$) were stained with primary anti-FPR3 and anti-S100b followed by secondary antisera tagged with Alexa Fluor 555 (red, for FPR3) or Alexa Fluor 488 (green, for S100b), and counterstained with diamidino phenylindole (DAPI) to identify nuclei. Images are from one of nine adjacent fields of view analysed from one of six pituitary glands. The region between the lateral wings of the pars distalis and anterior to the pars intermedia was imaged by confocal fluorescence microscopy (63.3x, scale bar = 50 μm). Arrows denote immunofluorescence for perinuclear FPR3 (a) and cytoplasmic and membranous S100b (b). The cytofluorogram (f) plots the pixel intensities from the red channel (FPR3, y-axis) versus green channel (S100b, x-axis) and is annotated with Pearson correlation (r_P) and Costes' automatically thresholded Manders' colocalisation (M_1 and M_2) coefficients.

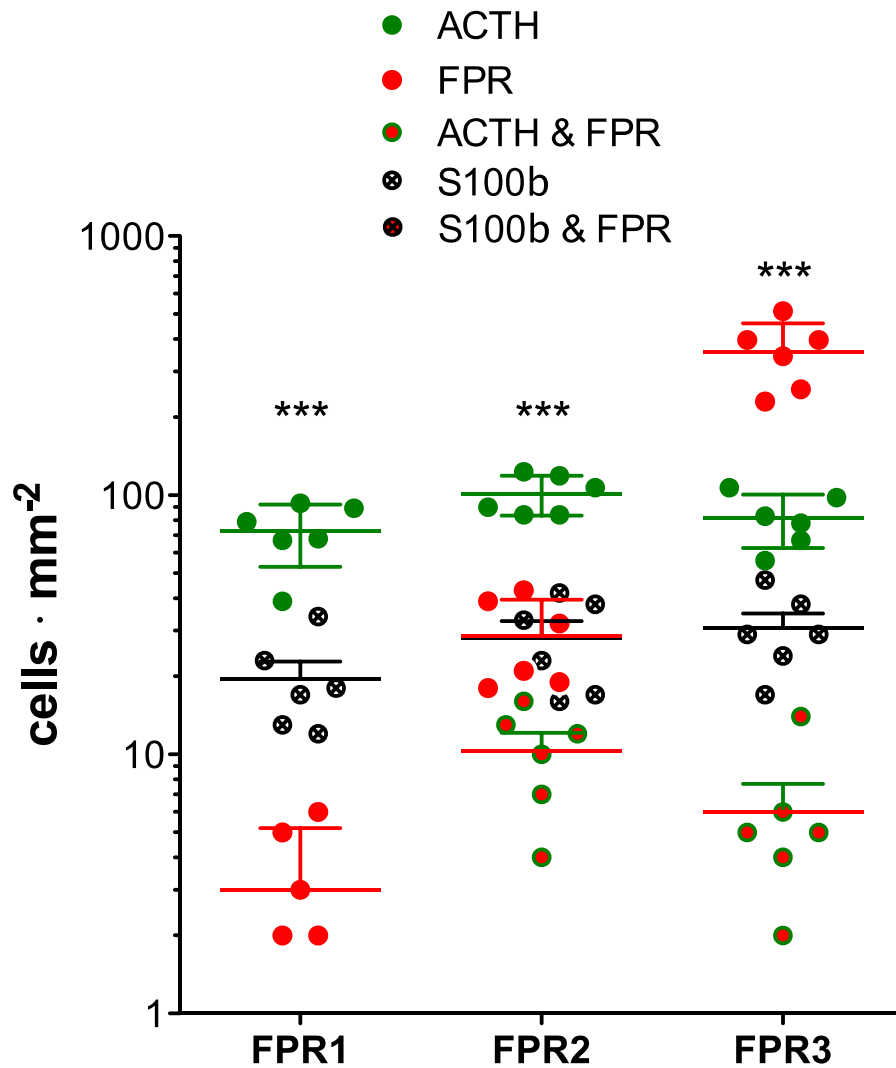


Figure 3.26: Morphometry of human anterior pituitary sections immunostained for formyl peptide receptors (FPR1, FPR2/ALX, FPR3) and adrenocorticotrophic hormone (ACTH), or S100 calcium binding protein b (S100b).

Data are representative of mean $\pm$ standard error of mean of $n = 6$ pituitaries, compared by two-way ANOVA: *** $P < .001$ = ACTH vs. FPR subtype.

Table 3.1 Results of computer automated colocalisation analysis of confocal images of sections of human anterior pituitary glands (n = 6) immunofluorescently stained for formyl peptide receptors (FPR1, FPR2/ALX, FPR3) detected in channel 2, and either adrenocorticotrophic hormone (ACTH) or S100 calcium binding protein b (S100b) in channel 1.

Pearson's coefficient (r_p), Manders' coefficients (M1 and M2), and Costes' P value (%) for significance at the 95% confidence level (***) = $P > 99\%$, ns = not significant) were calculated using Just another colocalisation plugin (JACoP) on Fiji is Just Image J (Fiji).

Channel 1	Channel 2	r_p	M1	M2	Costes' P value	
ACTH	FPR1	$-.036 \pm .029$	$.100 \pm .088$	$.171 \pm .053$	72.44 ± 14.81	ns
ACTH	FPR2	$-.024 \pm .018$	$.887 \pm .111$	$.592 \pm .075$	83.86 ± 11.61	ns
ACTH	FPR3	$.126 \pm .039$	$.978 \pm .017$	$.799 \pm .024$	100.00 ± 0.00	***
S100b	FPR1	$.055 \pm .016$	$.658 \pm .169$	$.599 \pm .143$	95.18 ± 5.57	ns
S100b	FPR2	$.024 \pm .015$	$.223 \pm .179$	$.414 \pm .132$	67.49 ± 17.56	ns
S100b	FPR3	$.028 \pm .048$	$.013 \pm .008$	$.135 \pm .055$	90.97 ± 7.33	ns

3.4 Discussion

The studies will be discussed in terms of: their main findings; the novel aspects of the study design; the consistency of the findings with previous research and reasons for discrepancies; the meaning of the findings and their implications for the stress response to inflammogens; limitations of the study design in terms of assumptions, precision, accuracy, and bias; and finally, a discussion of the areas for further work and future directions will be followed by a conclusion.

These studies sought to determine the spatial distribution of Fpr/FPR subtypes and stress hormones within the pituitary-adrenal axis from mouse and human tissues following inflammogenic stress, and in doing so achieved several main findings. First, the spatial distribution of GFP immunofluorescence—as a proxy of Fpr2/3 within the anterior pituitary gland of Fpr2/3^{-/-} mice—was colocalised with that of ACTH, GH, and PRL, but not S100b. Second, adrenal glands taken from wildtype mice after 2 and 6 hours following i.p. LPS had significantly fewer vacuolated cells than mice receiving only vehicle, but this effect of LPS was not observed in sections from the Fpr2/3^{-/-} mice. Third, GFP immunofluorescence was upregulated within the adrenal glands taken from Fpr2/3^{-/-} mice two hours after peripheral administration of LPS versus vehicle-injected controls, but the signal was significantly reduced after six hours. Fourth, automated analysis of confocal images of human pituitary gland sections from MS donors exhibited a spatial distribution of ACTH and S100b immunofluorescence that was not generally colocalised with FPR1, FPR2/ALX, or FPR3, except for a significant colocalisation of ACTH and FPR3.

3.4.1 Fpr2/3^{-/-} adrenals had reduced loss of sterol vacuolation but increased GFP following LPS

To the author's knowledge, this is the first report of the use of well-validated antibodies to map the spatial distribution of GFP within endocrine, cerebral, and lymphoid tissues taken from the Fpr2/3^{-/-} mouse. At the time of investigation, this novel approach was necessary owing to the lack of commercially available primary antibodies with sufficient specificity for murine Fpr2 or Fpr3 epitopes in spatial applications such as IHC. In this regard, the observation of immunoreactive GFP within the Fpr2/3^{-/-} adrenal ZF following intraperitoneal LPS is novel, whereas the reduced loss of sterol vacuolation versus WT animals is concordant with previous work. The histochemical methods of Buss (2010), for instance, used haematoxylin and eosin (H&E) staining to ascertain the effect of intraperitoneal LPS on the structure of

the adrenal cortex from *Fpr2/3^{-/-}* and WT mice. The WT adrenal gland sections had reduced sterol vacuolation in response to LPS versus vehicle, but this effect of LPS was not seen in the *Fpr2/3^{-/-}* mice (Buss 2010). Whilst the present study also used haematoxylin, in contrast with previous work, this study used primary antibodies to target epitopes of GFP and ascertain its spatial distribution as a proxy of *Fpr2/3*. In addition, the present study quantified vacuolated and GFP-positive cells as well as total cell counts, whereas the observations of Buss were recorded based on the number of animals affected. In this regard, the present study has put forward evidence of reduced sterol vacuolation following LPS that has been quantified and shown to be statistically significant.

Other studies in mice receiving intraperitoneal injections of LPS or saline, have observed both routine histochemical and terminal deoxyribonucleotidyl transferase (TDT)-mediated deoxyuridine triphosphate (dUTP)-digoxigenin nick end labeling (TUNEL) evidence of increased adrenocortical and medullary apoptosis and infiltration of inflammatory leukocytes, alongside increased ACTH and GC secretion within 3 hours of LPS treatment (Kanczkowski *et al.* 2013). There is also evidence that inflammogenic stress elicits a loss of steroidogenic cells within the adrenal glands from other species. In rats undergoing polymicrobial sepsis, there was evidence of complement C5a anaphylatoxin activation along with widespread apoptosis of adrenal cells (Flierl *et al.* 2008). In a study of rats that received an intraperitoneal injection of LPS or saline, there was immunohistochemical evidence that CD14, TLR-4, iNOS, and COX-II were upregulated especially within the ZG and ZF, however in this study, sterol vacuolation was not measured (Mohn *et al.* 2011). In a similar study, Liu and colleagues (2011) used confocal microscopy to detect evidence of LPS receptor expression in TLR4-positive cells and was colocalised with the MC₂ receptor for ACTH in steroidogenic cells marked with steroidogenic acute response (StAR) protein and cytochrome P450 11 β -hydroxylase (CYP11B1). In addition, it was found that a 24 hour pre-treatment of ZF and ZR cocultures with LPS led to a dose-dependent decrease in GC secretion in response to further LPS or ACTH treatment, along with reduced MC₂ receptor, TLR4, StAR, and CYP11B1 expression (Liu *et al.* 2011). This suggests that LPS is able to downregulate the steroidogenic capacity of the adrenal gland, leaving the gland refractory to either LPS or ACTH. Interestingly, *TLR4^{-/-}* mice have significantly larger adrenal glands, owing to a larger adrenal cortex than WT littermates, further implicating the LPS signalling cascade in modulating adrenal steroidogenesis (Zacharowski *et al.* 2006). The present finding of an LPS-induced loss of sterol vacuolation in the wildtype mouse adrenal, and that this effect of LPS is

attenuated in Fpr2/3^{-/-} mice, is therefore consistent with previous work. Taken together, this means that Fpr2/3 could be involved in the regulation of adrenal steroidogenic capacity in response to inflammogenic stress. This has implications for the understanding of inflammogenic stress and disease states such as sepsis.

3.4.2 The anterior pituitary gland of Fpr2/3^{-/-} mice contained GFP immunoreactivity within ACTH-, GH-, and PRL-positive cells

Prior to this work, it has been shown that the Fpr1 gene was not expressed within the pituitary of rats or mice (John *et al.* 2007). In addition, the Fpr2 and Fpr3 genes were shown to be expressed as mRNA transcripts within the mouse and rat pituitary, but it was not clear in which cell types these were expressed, or, whether the products of these gene loci were translated into immunoreactive proteins (John *et al.* 2007; Buss 2010). With regard to the distribution of the hormone cell types within the anterior pituitary gland, the observations of this study are consistent with previous work. The lateral wings of the pars distalis contained an abundance of GH-positive cells, the medial region between the wings contained ACTH-positive cells and also non-uniformly distributed PRL-positive cells (see Stevens & Lowe 2011). In this study of the spatial distribution of GFP as a surrogate marker for Fpr2/3 across the anterior pituitary, GFP was evidently distributed across several endocrine cell subtypes, although not within the glial-like S100b-positive cells, ostensibly the folliculostellate cells. Specifically, the fluorescence micrographs revealed punctate fluorescence from antibodies against GFP with a consistent nuclear expression. In addition, this was colocalised to a given cell with signals from antibodies raised against ACTH, GH, and PRL, each with cytoplasmic signals. To the author's knowledge, this is the first reported use of pituitary tissue from Fpr2/3^{-/-} mice with specific and well-validated antibodies against GFP and proteins specific to endocrine and non-endocrine pituitary cell subtypes, in order to determine the spatial distribution of Fpr2 and/or Fpr3 by proxy.

These findings are consistent with other data on the expression of the Fpr transcripts. There is evidence for the expression of Fpr2, Fpr3, Fpr-rs6, and Fpr-rs7, but not Fpr1, within the C57BL/6 mouse pituitary (John *et al.* 2007). This study has provided evidence towards elucidation of the distribution of Fpr2/3 within specific pituitary cell sub-populations identified by lineage markers, namely the corticotrophs (ACTH), somatotrophs (GH), and lactotrophs (PRL), whereas GFP was not expressed within the

folliculostellates (S100b). The lack of GFP signal, as a proxy for Fpr2/3, within the folliculostellate cells is consistent with previous data that suggests these cells are a source of Fpr/FPR ligands such as ANXA1 (Traverso *et al.* 1999). The folliculostellates show immunogold localisation of ANXA1 at sites in contact with endocrine cells (Christian *et al.* 1997), and the folliculostellate-like TtTGF cell line expresses S100b (Inoue *et al.* 1992; McArthur *et al.* 2009) and ANXA1 (Chapman *et al.* 2002), but do not possess the ANXA1 binding sites found on endocrine cells (Christian *et al.* 1997). The evidence of GFP immunoreactivity within the PRL-, GH-, and ACTH-positive cells of the anterior pituitaries from Fpr2/3^{-/-} mice is compatible with the evidence that putative Fpr ligands such as ANXA1, have been shown to inhibit the release of PRL (Taylor *et al.* 1995b), GH (Philip *et al.* 2001), LH, and TSH (Taylor *et al.* 1995b) *in vitro*, and also of ACTH *in vivo* (John *et al.* 2007). In summary, the present findings are concordant with the hypothesis that Fpr2 and Fpr3, and their ligands, could modulate the release of ACTH, GH, PRL, and possibly other hormones from the anterior pituitary. The observation that the inflammogen LPS upregulates the expression of GFP as a surrogate of Fpr2/3 distributed in ACTH-, GH-, and PRL-positive cells gives reason to investigate whether Fpr2/3 is involved in modulating the secretion of these hormones specifically. This could have implications for the pituitary stress response, particularly in conditions of inflammogenic stress such as sepsis.

3.4.3 No GFP immunoreactivity was observed within native cells of the hippocampus, hypothalamus, or thymus of Fpr2/3^{-/-} mice

The absence of immunoreactive GFP as a proxy for Fpr2/3 within the hippocampus is interesting. This finding is partly discordant, however, with previous research that found evidence of Fpr1/2 expression within the mouse hippocampus but from APP/PS1 transgenic mice. Briefly, Slowik and coworkers (2012) investigated whether Fpr2 and the receptor-for-advanced-glycation-end-products (RAGE) were involved in the response to inflammogenic stress of relevance to AD, by determining the gene expression and immunolocalisation of mouse Fpr1, Fpr2, and RAGE in APP/PS1 mice, rat primary microglia and astrocytes. Possible reasons for the discrepancy with the present findings could lie in the fact that the authors used an antibody that was raised against an epitope on human FPR2/ALX, but which nonetheless binds non-specifically to both murine Fpr2 and murine Fpr3. This was the same antibody that was used in the present study to perform a western blot on lung tissue from the Fpr2/3^{-/-} and WT mice and confirm the

genotyping results are applicable at the protein level, namely, anti-FPR2/anti-Fpr2/3 (Cat: sc-18191; Santa Cruz, Inc.). The affinity for the anti-FPR2/ALX antibody for murine Fpr2 and Fpr3 might well be greater than that of the rabbit anti-GFP used in the present study. An alternative explanation worthy of investigation would be that the inflammogen used in the present study, LPS, might not have passed through the BBB in sufficient quantities to initiate the NfκB-mediated transcription of the GFP transgene from the Fpr2/3 gene loci in Fpr2/3^{-/-} mice. Whilst LPS has been shown to upregulate the expression of Fpr2 in cell lines such as the murine microglial N9 cell line (Cui *et al.* 2002; Iribarren *et al.* 2003), other studies have found that expression of Fpr2 within microglia and macrophages native to tissues are not responsive to LPS treatment (Waechter *et al.* 2012).

The absence of immunoreactive GFP within the hypothalamic PVN in the present study is partly discordant with the work of Buss (2010), which found evidence of gene expression for Fpr1, Fpr2, and Fpr3/Alx within the hypothalamus of C57BL/6 mice. Specifically, the level of these transcripts were determined using quantitative PCR, which found that all three genes were expressed at levels at least two-fold higher than the GAPDH housekeeping gene. It is plausible that the discrepancy with the present work is because mRNA transcripts are not necessarily translated, and so the concentration of Fpr1, Fpr2, and Fpr3/Alx mRNA found by Buss would not necessarily correlate with the distribution of their proteins. In the present study, there was no effect of LPS on the spatial distribution of GFP within the hypothalamus of the Fpr2/3^{-/-} or WT mice at any of the time points observed (2 and 6 hours), versus saline. In this regard, the work of Buss (2010) also found that there was no effect of LPS on the gene expression of Fpr1, Fpr2, or Fpr3 at any of the time points observed (0, 2, 4, and 8 hours). This was also true of the hippocampus. The expression of all three genes was however upregulated by LPS in tissues that are not protected by the BBB, namely, the anterior pituitary and adrenal glands. Whether the protection afforded by the BBB could explain why the hypothalamic and hippocampal expression of Fpr subtypes was not modulated by LPS or detected as GFP in the present study, deserves further investigation.

The lack of immunoreactive GFP within native cells of the thymus requires further investigation. One possible reason why there were so few inflammatory infiltrates detected within the thymus of the LPS injected Fpr2/3^{-/-} mice versus those observed by Buss (2010), could be due to the differences in the histochemical methods used. The present study targeted immunoreactive GFP along with haematoxylin

counterstaining whereas Buss used H&E staining to detect general changes in tissue structure and inflammatory cells. It is plausible that the trade-off between detecting GFP and staining other cell types with eosin meant that it was not possible to detect all inflammatory infiltrates apart from those that were positive for GFP.

3.4.4 The $Fpr2/3^{-/-}$ genotype was consistent with previous work

The genotyping of the $Fpr2/3^{-/-}$ mice is consistent with the findings from previous work of Dufton and colleagues (2010) who initially generated and phenotyped the mouse colony, and who subsequently published a correction to their original paper (Dufton *et al.* 2011). They originally reported phenotypic data on what they thought were $Fpr2^{-/-}$ mice with a genetic background of the 129SvEv, and C57BL/6 inbred strains (Dufton *et al.* 2010). In their correction, however, they explain that it is more likely they were working with $Fpr2/3^{-/-}$ mice (Dufton *et al.* 2011). They reported that this arose partly out of the misidentification of the two “isoforms” of the $Fpr3$ gene (formerly $Fpr-rs1$), one being the renamed $Fpr3$ itself, and the other being the mouse lipoxin receptor (Alx). Since the human (FPR) and mouse (Fpr) IUPHAR nomenclature was updated by Ye and colleagues (2009), what were termed $Fpr-rs1$ and Alx are now recognised as being transcribed from the same $Fpr3$ gene and give rise to the same receptor. This is comparable with the human FPR2/ALX, with ALX denoting its lipoxin binding ability. In re-evaluating their gene targeting strategy, they describe that in wildtype littermates, the $Fpr2$ gene is transcribed from exons 1, 2, and 3, and the $Fpr3$ gene from the same exons 1 and 3, skipping exon 2. In their $Fpr2/3^{-/-}$ mice, however, a GFP sequence at intron 1 preceded a PGK-Neo cassette at exon 2 of the $Fpr2$ gene. As exon 2 is required for $Fpr3$ transcription, they conclude that this strategy resulted in loss of $Fpr3$ as well as $Fpr2$, as suggested by their PCR results (Dufton *et al.* 2011). For this reason, they believe they had different phenotypic results (including loss of LXA_4 responses) from those of Chen and coworkers (2010) who had developed an $Fpr2^{-/-}$ mouse. Taken together, the insertion of GFP in intron 1 and the PGK-Neo cassette at exon 2 of the $Fpr2$ gene in the $Fpr2/3^{-/-}$ mice has also resulted in $Fpr3$ alongside the $Fpr2$ gene being knocked out in place of the GFP transgene. In the context of the PCR results of Dufton and colleagues (2011), $Fpr2$ and $Fpr3$ were both knocked out versus wildtype littermates, and thus confirms the $Fpr2/3^{-/-}$ mouse genotype.

3.4.5 Colocalisation of FPR3 and ACTH detected within the human anterior pituitary gland

Prior to the present work, it was not known which FPR subtypes were expressed within the human anterior pituitary gland. To the author's knowledge, this is the first report of the spatial distribution of human FPR1, FPR2/ALX, and FPR3 within the human anterior pituitary gland. The human anterior pituitary glands were obtained from the UK Multiple Sclerosis (MS) and Parkinson's Disease (PD) tissue bank. The colocalisation of FPR subtypes with ACTH and S100b was quantified using both manual and automated methods. The manual method involved counting cells across a .464 mm² area of the pars distalis observed for evidence of immunolocalisation of FPR1, FPR2/ALX, and FPR3. Immunoreactive FPR1 was found in only a few cells, none of which contained immunoreactive ACTH or S100b and were possibly infiltrated leukocytes. This FPR1 staining, therefore, is probably a result of leukocytic infiltration, possibly monocytes or macrophages (Müller-Ladner *et al.* 1996). This is consistent with a lack of human FPR1 immunoreactivity in anterior pituitary tissue (Becker *et al.* 1998) and lack of Fpr1 expression within the mouse and rat anterior pituitary (John *et al.* 2007). Immunoreactive FPR2/ALX was not found in any S100b-positive cell, but was occasionally identified in ACTH-positive cells. However, ACTH-positive cells in general were largely FPR2-negative. The observation that FPR3-positive cells were the most numerous across the anterior pituitary, plus, the close apposition of FPR3-positive cells with ACTH-positive cells in clusters within acini of the pars intermedia, suggested that the single FPR3-positive cells are possibly GH-producing sommato-lactotrophic cells (Pantić 1995; Wheeler & Young 2006). The manual method of enumerating colocalisation indicated that ACTH-positive cells rarely contained FPR immunoreactivity of which between 1 and 10 were FPR3- or FPR2-positive cells, whereas the S100b-positive cells contained no immunoreactivity for any FPR subtype. As a whole, the FPR subtypes most abundantly distributed across the pituitaries were FPR3 >> FPR2/ALX > FPR1, and the automated method of quantifying colocalisation found a significant colocalisation of FPR3 with ACTH.

3.4.6 Biological significance and scope of findings

The finding that the ACTH-positive corticotrophs contain co-immunoreactivity for GFP—as a proxy for Fpr2/3 from mice with these genes knocked out—or FPR3 in the human pituitary is novel. This opens up avenues of research into whether Fpr2/3 or FPR3 ligands modulate pituitary ACTH release. This would be of particular importance in managing the response inflammogenic stress during acute conditions such

as sepsis and septic shock, or in chronic neurodegenerative conditions such as MS, PD, and AD. Although GCs are potent anti-inflammatory and immunosuppressive agents, they have undesirable side effects such as the acceleration of apoptosis within the hippocampus (Gould *et al.* 1992), and are thus contraindicated in neurodegenerative conditions. With regard to sepsis, the risk of patient mortality is still high even with GC treatment, underscoring the importance of research into alternative treatments (Sligl *et al.* 2009). Given the finding that LPS upregulated adrenal Fpr2/3 surrogated by GFP and reduced adrenal vacuolation of steroids including GCs, this implicates Fpr/FPR subtypes in the response to inflammogenic stressors such as LPS which are of relevance to sepsis. In context of wider literature, there is already evidence that the Fpr/FPR ligand ANXA1 is involved with the regulation of TLR4-mediated IFN- β production (Bist *et al.* 2013). Taken together, this highlights the importance of further research to increase our understanding of the expression and function of Fpr2/3 in rodents, and FPR3 in man. These findings have thus brought us a step closer to understanding the pituitary-adrenal response to inflammogenic stress, and could facilitate in the search for safer, efficacious alternatives to GCs in the treatment of conditions such as sepsis.

The study will now be discussed in terms of: the strengths and weaknesses of the study design; the limitations of the methodology, and the validity of assumptions in terms of precision, accuracy, and control of bias.

In terms of the study design, there are several features that enabled evidence to be obtained with optimal use of resources. A factorial study design, for instance, was adopted for the study of the Fpr2/3^{-/-} and WT mouse tissues by IHC. This meant that multiple factors were tested simultaneously: 1) genotype, with two levels (Fpr2/3^{-/-} and WT); 2) intraperitoneal injection of either LPS or PBS; and also 3) time after injection (0, 2, 4, and 6 h). The purpose of this was to obtain information on the spatial distribution of GFP as a surrogate for Fpr2/3 within tissues from the Fpr2/3^{-/-} mice, with WT mice serving as a negative control. The injection of LPS was expected to enhance the signal for GFP as LPS has been shown to enhance the expression of Fpr2 in other systems (Cui *et al.* 2002; Iribarren *et al.* 2003). The time course was chosen with the view to understand the dynamics of the signal for GFP to enable functional studies to be performed over an appropriate duration for comparison. If the work were to be performed again, it

would be beneficial to analyse the Fpr2/3^{-/-} and wildtype mouse pituitary sections by immunofluorescence confocal microscopy to allow for better comparison with the human pituitary staining.

The validity of the experiments will now be discussed in terms of the accuracy and precision of the methods used. With regard to the immunohistochemical detection of GFP and markers of pituitary cell subtypes, the choice of strategy was qualified by the specification and availability of the reagents at the time of investigation. The antibody for GFP was originally raised against epitopes from the jellyfish *Aequoria victoria*, and was supplied as a polyclonal anti-GFP preparation (ab6673, Abcam, Cambridge, UK). The sequences for GFP do not appear in any other known proteins or species other than the jellyfish, and genetically altered animals expressing the GFP transgene. The affinity and avidity of the anti-GFP for immunoreactive GFP is evident in the fact that it was able to detect GFP, and the LPS treatment enhanced the expression of GFP further, ostensibly via the mechanism by which LPS upregulates Fpr2 expression in microglia (Cui *et al.* 2002; Iribarren *et al.* 2003).

The precision of the immunostaining work is supported by the manufacturer's data on the antibodies used. The precision of the antibodies used is defined by the degree of cross-reactivity and the method of purification. The antibody for ACTH, for instance, is a well-characterised primary antibody that was raised in rabbit against human ACTH₁₋₃₉, (Cat: AFP6328031, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Torrance, CA, USA), with cross-reactivity at human, rat, mouse, and porcine ACTH, and was purified by immunoaffinity chromatography to be devoid of hormonal contaminants. The primary antibodies for GH, PRL, LH, and TSH were all from the same manufacturer and purified with similar immunoaffinity chromatography method and species cross-reactivity as for the anti-ACTH. The immunofluorescence staining for LH and TSH did not work and were not investigated further because they were not central to testing the hypothesis. The high similarity in amino acid sequences of the human anterior pituitary hormones with those of rats and mice enabled the use of the same antibodies raised against human epitopes to perform IHC in each of these species.

In terms of the human anterior pituitary confocal study, there were two methods used to quantify colocalisation. The first involved manual analysis of images and cell counts, whereas the second used an automated statistical approach to quantify pixel intensity. The manual approach is potentially confounded by random error and the bias of visual interpretation of patterns and structures. Manual, or visual

approaches can yield acceptable results if the compartments are spatially separated enough for the human eye to resolve and distinguish reproducibly. In order to eliminate bias, however, an automated approach was also used. The automated statistical approach provided three indices of colocalisation, Pearson's r , Manders' colocalisation coefficients (M1 and M2), and Costes' P-value for the colocalisation statistics. The Costes' automated statistical approach defines colocalisation as the degree to which spatial variation in pixel intensity in one channel is explained by another channel. Pearson's r provided a quantitative estimate of colocalisation that relates to the amount of colocalised signals in both channels in a nonlinear manner. Manders' coefficients quantify the colocalised fraction in one channel above a threshold of background noise, defined by Costes' automated method. The automated method is designed to reduce the frequency of type I errors or false positive for the existence of colocalisation. The strict conditions of Costes' method could have feasibly increased the rate of type II errors or false negatives for the existence of colocalisation of FPR1 and FPR2/ALX with either ACTH or S100b. Apart from this, the finding that only one FPR subtype, namely FPR3, was significantly colocalised with ACTH is plausible given the evidence of expression of Fpr subtypes in other species (John *et al.* 2007; McArthur *et al.* 2009).

The human anterior pituitary immunostaining for FPR subtypes along with ACTH and S100b involved the use of diseased tissue from donors who died with MS. It was not possible to obtain pituitaries from non-diseased donors, as these are more difficult to come by. It is noteworthy that both FPR and ANXA1 are upregulated in the CNS of MS patients (Elderfield *et al.* 1992; Müller-Ladner *et al.* 1996). With this in mind, it cannot be ruled out that the distribution of FPR subtypes and the overall lack of colocalisation with either ACTH or S100b, except for FPR3 and ACTH, might only be applicable within a population of MS sufferers.

3.4.7 Conclusion

These studies have provided evidence for a distribution of mouse Fpr2 and/or Fpr3, surrogated by GFP, within the mouse anterior pituitary that aligns with GH-, PRL-, and ACTH-immunoreactivity. This is complemented by a distribution of human FPR3 and FPR2/ALX immunoreactivity within the human anterior pituitary, albeit with fewer cells demonstrating co-immunoreactivity for ACTH and FPR2/ALX or FPR3, than for either FPR2/ALX or FPR3. Although these findings are novel, they are plausible given that the hypothesis was founded on existing evidence that Fpr transcripts are expressed within the mouse

and rat anterior pituitary gland, and also the mouse adrenal gland. These findings have implications for understanding the potential role of Fpr/FPR subtypes as targets of GC secondary mediators or mimetics with relevance to the management of response to inflammogenic stressors such as during sepsis and septic shock. Further research is needed and could take advantage of advances in three-dimensional cultures of human stem cells that have been shown to self-organise into functional networks similar to those found in intact pituitary glands (Suga *et al.* 2011). Combined with two-photon microscopy (Lafont *et al.* 2010), this would allow functional and spatial information to be obtained simultaneously, and on tissues that are relevant to the human system. Prior to this, it would be useful to expand on the present study with appropriate controls for the MS diseased pituitaries.

Chapter Four: Study of the effects of formyl peptide receptor ligands on adrenal function

4.1 Introduction

The following paragraphs describe the existing evidence for the role of formyl peptide receptor (Fpr/FPR) ligands in the modulation of adrenal function; the available experimental models for assessing adrenal function *in vitro*; the comparative physiology of the adrenal glands between humans, rats, and mice; and the basic regulation of adrenal glucocorticoid (GC) by adrenocorticotrophic hormone (ACTH) and its receptor.

4.1.1 Comparative anatomy and physiology of the adrenal gland

The adrenal gland is a composite organ that has evolved in all vertebrate species, and is derived from two embryological sources. The splanchnic mesoderm gives rise to the development of adrenocortical tissues that synthesises and secretes the mineralocorticoids and GCs in the adult. On the other hand, part of the neural crest develops into the chromaffin tissue within the adrenal medulla that produces the catecholamines epinephrine and norepinephrine. This development of the adrenal gland in humans is different from rodents in several features. During most of development *in utero*, up to 90% of the volume of the human adrenal is comprised of a fetal zone with a high steroidogenic capacity (Mesiano & Jaffe 1997). Surrounding this is the transitional zone that produces cortisol as the forerunner of the zona fasciculata, and this is enveloped by the definitive zone that later becomes the zona glomerulosa (Crickard *et al.* 1982). Following birth, the fetal zone regresses and the formation of the three layers of the adrenal cortex is apparent (Mesiano & Jaffe 1997). From 33 days post coitum (dpc), the adrenal gland differentiates and expression of the nuclear receptor, steroidogenic factor-1 (SF-1) is evident throughout the cortex (Hanley *et al.* 2001). In rodents, the adrenal glands (and gonads) are derived from the adrenogenital primordium (AGP) and this appears at 9 and 11.5 dpc in mice (Ikeda *et al.* 1994) and rats (Hatano *et al.* 1996) respectively, and marks the appearance of nerve cells that constitute the medulla, which becomes distinct from the cortex by 16 dpc. Despite the differences in the development of the adrenal gland in rodents and humans, they are very similar in form and function.

The main function of the adrenal gland is the production of two major classes of stress hormones. The adrenal medulla synthesises the catecholamines, and the adrenal cortex produces the GCs through steroidogenesis. Cholesterol is the substrate for steroidogenesis, but with very little *de novo* synthesis

within the cortex, the main cholesterol sources available to the adrenal are from circulating low-density and high-density lipoproteins (LDL and HDL), and local cholesterol stores. Steroidogenesis involves five isozymes of cytochrome P450 and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) (Koritz 1964; Basch & Finegold 1971) separated by the mitochondria and endoplasmic reticulum (Moustafa & Koritz 1975; Ishimura & Fujita 1997). Beginning with the conversion of cholesterol to pregnenolone by the P450 side-chain cleavage enzyme (P450_{scc}), the process of steroidogenesis is very similar in humans, rats, and mice (Hanukoglu *et al.* 1981). After this step, there are some key differences between the species. Firstly, in humans, pregnenolone is converted to 17 α -hydroxy-pregnenolone by 17 α -hydroxylase (P450C17) before conversion to 17 α -hydroxyprogesterone by 3 β -hydroxysteroid dehydrogenase (3 β -HSD). In rodents, however, pregnenolone is instead converted directly into progesterone by 3 β HSD (Brock & Waterman 1999; Gilep *et al.* 2011). From this point, the enzyme 21-hydroxylase (P450C21) produces 11-deoxycorticosterone in rodents or 11-deoxycortisol in humans (Dorfman & Hayano 1952; Kominami *et al.* 1980). These steroids are the final precursors for 11 β -hydroxylase to synthesise the principal GCs in rodents and man, corticosterone or cortisol, respectively.

Glucocorticoid release is primarily under the regulation of ACTH released from the pituitary gland. In humans, the 39 amino acid polypeptide is a full agonist for the type 2 melanocortin receptor (MC₂R) with a pK_d of 9.8, whereas the truncated synthetic form ACTH₁₋₂₄ has a lower affinity with a pK_d of 9.1 (Kapas *et al.* 1996). The human MC₂R is 297 amino acids long and the mouse and rat equivalents are 296 amino acids. The MC₂R is the main receptor for ACTH and is expressed predominantly within the zona glomerulosa and zona fasciculata cortex of the human (Chhajlani 1996), mouse (Xia & Wikberg 1996), and rat (Izumi *et al.* 2004) adrenal glands. The MC₂R was first cloned by Mountjoy and colleagues (1992) and responds exclusively to ACTH (Holder & Haskell-Luevano 2004), although ACTH also binds to the other four melanocortin receptor subtypes MC₁R, MC₃R, MC₄R, and MC₅R, along with α , β , or γ -melanocyte stimulating hormone (MSH) (Mountjoy *et al.* 1994).

The synthesis and release of adrenal GCs are under the control of ACTH. Upon binding to ACTH, MC₂R undergoes a conformational change that opens it up to interact with G proteins. This is followed by an exchange of GDP for GTP and dissociation of the heterotrimeric G proteins into an α subunit and a $\beta\gamma$ subunit. This increases the production of cAMP and this acts as a second messenger of ACTH to induce

protein kinase A (PKA) to phosphorylate cAMP response element binding protein (CREB) as a transcription factor for steroidogenic acute regulatory protein (StAR) and enzymes (Lehoux *et al.* 1998). The phosphorylation of serine and threonine residues by PKA have been shown to be necessary for steroidogenesis (Green & Orme-Johnson 1991). The intracellular Ca^{2+} is also increased by the inhibitory action of cAMP on potassium channels (Enyeart & Enyeart 1998). In humans, the expression of MC₂R is also upregulated by ACTH through a pathway dependent on cAMP (Sarkar *et al.* 2000) and PKA (Blondet *et al.* 2002). This also involves interactions of MC₂R with AP-1 and steroidogenic factor-1 (SF-1) binding elements (Naville *et al.* 1999) in humans, whereas in mice the SF-1 complex assembles on the Mc2r promoter (Winnay & Hammer 2006), and modulates the CREB-mediated activation of the rat steroidogenic enzymes (Zhang & Mellon 1996; Yan *et al.* 2006). Following activation by ACTH, the MC₂R internalises by clathrin-mediated endocytosis (Wenzel-Seifert & Seifert 1993; Kilianova *et al.* 2006), and a similar process occurs in the mouse (Ghatei *et al.* 1993; Baig *et al.* 2001), and presumably in rats.

Although the affinity and the capacity of the human adrenocortical MC₂R are very similar to those of the rat, there are however some differences between the species. Human adrenocortical cells are approximately 20 times more sensitive to ACTH than rat adrenocortical cells, possibly owing to a more efficient coupling of MC₂R with adenylate cyclase and subsequent steroidogenesis. This is substantiated by ACTH having a pK_d of 9.8 with MC₂R in human adrenocortical cells, but also has an EC_{50} for cAMP (120-140 pM) that is 20-fold lower, and an EC_{50} for steroidogenesis (3-8 pM) that is 720-times lower than its K_d (Kolanowski & Crabbe 1976; Catalano *et al.* 1986; Andreis *et al.* 1995). Conversely, in rats, the K_d for ACTH broadly equals its EC_{50} for cAMP, and the EC_{50} for steroidogenesis is only 40-times lower than its K_d (Sayers *et al.* 1971a; see Ramachandran 1987).

4.1.2 Adrenal function and expression of Fpr/FPR subtypes and their ligands

The Fpr/FPR subtypes known to be expressed within the adrenal gland are human FPR1 and FPR2/ALX (Becker *et al.* 1998) and mouse Fpr1, Fpr2, and Fpr3/Alx (Buss 2010). Very few of the hundred or so currently known ligands for these Fpr/FPR subtypes, however, have been studied for their effects on adrenal function. Of those that have, they were either not known as Fpr/FPR ligands at the time of the experiments, or they were studied by colleagues of the author. Beginning with the former, these include cyclosporine A, cyclosporine H, and pituitary adenylate cyclase activating protein (PACAP).

Cyclosporine A is a cyclic peptide obtained from the *Trichoderma polysporum* fungus and has immunosuppressive antibiotic properties. It was shown that Cyclosporine A administered to rats *in vivo* also has a suppressive effect on the steroidogenic capacity and impairs the stress response (Hirano *et al.* 1988; Rebuffat *et al.* 1989) of the adrenal gland where it can also accumulate (Niederberger *et al.* 1983; Ried *et al.* 1983). Since then, it has been shown that human FPR1 is antagonised by Cyclosporine A (Yan *et al.* 2006), and the related peptide, cyclosporine H is a potent antagonist for human FPR1 and FPR2/ALX (Wenzel-Seifert & Seifert 1993; de Paulis *et al.* 1996). At the time of writing, it is not known whether either of these antagonise the equivalent receptor subtypes in rat or mouse systems. Unlike the cyclosporines, PACAP is an endogenous peptide expressed within the adrenal medulla of rats, and humans (Sharma & Brush 1973; Ghatei *et al.* 1993), and possibly in mice, and has been shown to agonise human FPR2/ALX (Gazdar *et al.* 1990; Kim *et al.* 2006; Zein *et al.* 2008a). PACAP had no effect on basal GC secretion in dispersed cortical cells from rat or human adrenals, but micromolar PACAP stimulated GC release in rat adrenal slices that contained the medulla (Mountjoy *et al.* 1994; Andreis *et al.* 1995).

Work performed in this lab found evidence of ANXA1_{Ac2-26}-mediated inhibition of corticosterone release in dispersed mouse adrenal cultures, and ANXA1-knockout mice had higher basal corticosterone release than wildtype mice (Davies *et al.* 2007; Buss 2010). ANXA1_{Ac2-26} is the N-terminal peptide of ANXA1 which is expressed within the mouse (Buss 2010) and human adrenal cortex (Horvath *et al.* 2006). ANXA1_{Ac2-26} is a non-selective agonist with effects via human FPR1, FPR2/ALX, and FPR3, with pEC₅₀ values between 5.30 and 6.80 (Walther *et al.* 2000; Ernst *et al.* 2004b; Hayhoe *et al.* 2006). Similarly, fMLF, the synthetic variant of the bacterial prototypical Fpr/FPR ligand, was also shown to elicit

inhibition of adrenal GC release the same model (Buss 2010). It is not known, however, which receptor subtype mediated the effects of these ligands.

4.1.3 Models of adrenal function

Cell lines generally have some practical advantages over primary cultures. Once generated, the cells are more autonomous and have fewer requirements for growth.

The murine Y1 cell line is derived from an adrenocortical tumour in a male LAF1 (C57L x A/HeJ) mouse exposed to radiation. The tumour initially produced corticosterone in response to ACTH (Cohen *et al.* 1957), but lost this ability in sub-culturing steps (Bloch & Cohen 1960) due to a deficiency in 21-hydroxylase (P450C21) (Parker *et al.* 1985). Investigating whether ligands modulate corticosterone release would necessitate transfection with a P450C21 clone (Parker *et al.* 1985; Szyf *et al.* 1990).

There have been some attempts towards establishing a rat adrenocortical cell line through viral transformation. The transformed rat adrenocortical (TRA) cell line, for instance, was derived from a culture of an adrenocortical tumour of a virally infected Fischer rat (Auersperg *et al.* 1977). The TRA cells could not synthesise the 11- or 21-hydroxylated steroidogenic precursors of GCs, and had a low density of steroidogenic apparatus, namely, lipids, smooth endoplasmic reticulae, and mitochondria, versus untransformed cells (Auersperg 1978). Steroidogenic apparatus was also reduced in the Snell carcinoma-494 cell line versus normal adrenocortical cells from an Osborne-Mendel rat (Snell & Stewart 1959), and so this cell line produces only very low amounts of corticosterone (Sharma & Brush 1973).

The human NCI-H295R adrenocortical cell line is derived from an adrenocortical carcinoma (Gazdar *et al.* 1990), and although it expresses most steroidogenic enzymes, it exhibits only a limited response to ACTH. Most variant strains are totally resistant to ACTH in terms of cortisol production, possibly owing to the low expression of MC₂R (Mountjoy *et al.* 1994). Studies using this cell line therefore typically involve the use of forskolin or cAMP analogues.

Overall, the abnormal production of steroids or response to ACTH in terms of impaired or blocked ability to produce either corticosterone or cortisol limits the usefulness of currently available cell lines as a model for investigating whether Fpr/FPR ligands modulate GC release. Although primary tissue cultures typically have greater unsystematic variability versus cell lines, there are dispersed adrenal culture methods characterised by a high degree of reproducibility, mainly because of the elimination of inter-animal variation compared with adrenal slice cultures. The original dispersed rat adrenal bioassay

technique was performed by Swallow and Sayers (1969) and was 1000-fold more sensitive to ACTH than cultured adrenal quarters, and was in this regard comparable with radioimmunoassay technology at the time (Sayers *et al.* 1971b). The adrenals from several rats are dispersed into a pooled suspension of isolated cells, from which multiple aliquots are obtained. Primary dispersed adrenal protocols have been described for human, rat, and mouse adrenals (Armato *et al.* 1974; Buss 2010), but the smaller size of the mouse adrenal would necessitate killing more to achieve the same cell yield obtained from rats.

4.2 Methodology

Based on the above evidence, it was hypothesised that Fpr/FPR ligands will modulate adrenal function in basal or ACTH-stimulated conditions. In order to test this hypothesis, intact rats were killed by decapitation and the adrenal glands were pooled into Earle's balanced salt solution (EBSS). This was to provide a physiological buffer system for the adrenal cells prior to dispersal by cutting the glands with a razor blade in EBSS containing collagenase. Whilst not as effective at dispersing adrenal cells as trypsin (Sayers *et al.* 1971b), there is evidence that trypsin downregulates binding sites for Fpr/FPR ligands such as ANXA1 (Christian *et al.* 1997) but none for collagenase. Once dispersed, the adrenals were then aliquotted into 96-well plates prior to centrifugation, then allowed to equilibrate at 37 °C at 95% O₂/5% CO₂ for an hour. The cells were then incubated for 4 hours of contact with fresh EBSS containing Fpr/FPR ligands and/or ACTH₁₋₂₄, vehicle, or EBSS alone. The Fpr/FPR ligands included agonists with different relative affinities for each Fpr/FPR subtype, and the Fpr2-/FPR2/ALX-selective antagonist, WRW₄ with a pIC₅₀ for human FPR2/ALX, and mouse and rat Fpr2 of between 6.6 and 6.9, (Bae *et al.* 2004; Önnheim *et al.* 2008; Braun *et al.* 2011). The Fpr/FPR agonists tested were the Fpr1-/FPR1-selective agonist fMLF, the Fpr2-/FPR2/ALX-selective agonist WKYMVm, the Fpr2-/FPR3-selective F2L, the Fpr3/Alx-/FPR2/ALX-selective agonist 15-epi-LXA₄, and the non-selective Fpr agonist ANXA1_{Ac2-26}. The conditioned media were then removed and frozen at -20 °C, before being assayed for corticosterone using a proprietary enzyme-immunoassay (EIA) kit and a spectrophotometer.

The suppressive effect on pituitary-adrenal function remains one of the major drawbacks of long term GC treatment (Schlaghecke *et al.* 1992). Pituitary-adrenal function is also dissociated during sepsis-relevant endotoxaemia, and GC treatment alone is not sufficient to prevent death during sepsis (Marik 2009). The aim of these experiments therefore was to advance the knowledge on the modulation of adrenal GC release through Fpr/FPR ligands before further testing of whether such ligands protect adrenal steroidogenic capacity during sepsis-related endotoxaemia.

4.3 Results

4.3.1 The effect of ACTH₁₋₂₄ on corticosterone release from dispersed adrenal cells

Figure 4.1 shows the effects of graded concentrations $10^{-10} - 10^{-6}$ mol.l⁻¹ ACTH₁₋₂₄ (tetracosactide acetate, Alliance Pharmaceuticals) from dispersed adrenal cells from male Wistar rats *in vitro*. This resulted in concentration-related increases in corticosterone release with sub-maximal responses evident at the 10^{-7} mol.l⁻¹ concentration (* = P < .05 vs. drug-free). Therefore, this concentration of ACTH₁₋₂₄ was selected for all future experiments.

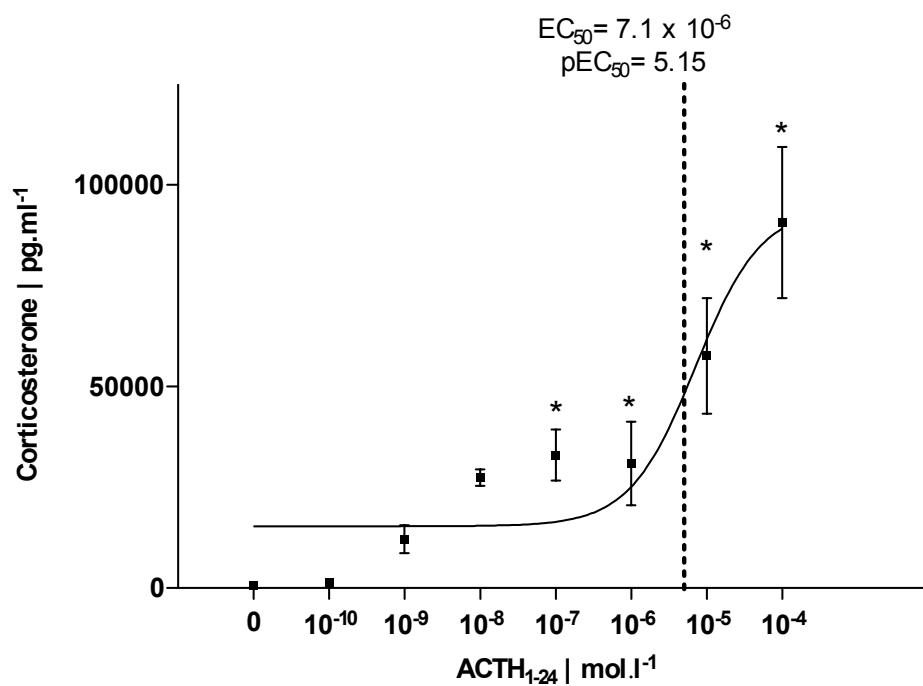


Figure 4.1: Effect of graded concentrations of adrenocorticotrophic hormone (ACTH₁₋₂₄) on the release of corticosterone from dispersed rat adrenal cells.

Points and error bars are arithmetic mean $\pm$ standard error of mean of $n = 8$ replicate wells. Data compared by one-way ANOVA with Dunett's *post hoc* test, * $P < .05$, vs. drug-free.

4.3.2 The effects of fMLF on corticosterone release from dispersed adrenal cells

Formyl-methionyl-leucyl-phenylalanine (fMLF) is a selective agonist for human FPR1, mouse Fpr1, and rat Fpr1. Figure 4.2 shows the effects of graded concentrations (10^{-7} , 10^{-6} , 10^{-5} mol.l⁻¹) of fMLF on the increases in corticosterone release induced by a sub-maximal concentration of ACTH₁₋₂₄ (10^{-7} mol.l⁻¹), in the presence and absence of a selective Fpr2 antagonist WRW₄ (10^{-6} mol.l⁻¹) from dispersed rat adrenal cells *in vitro*.

ACTH₁₋₂₄ (10^{-7} mol.l⁻¹) caused a significant increase in corticosterone release (* = $P < .05$ vs. basal). At all concentrations tested, fMLF reduced basal corticosterone release (***) = $P < .001$ vs. drug-free) and inhibited the steroidogenic response to ACTH₁₋₂₄ (***) = $P < .001$ vs. ACTH₁₋₂₄ alone). WRW₄ alone (10^{-6} mol.l⁻¹) or co-incubated with ACTH₁₋₂₄ had no effect on basal corticosterone release (ns = $P > .05$) but it reduced the response to ACTH₁₋₂₄ (††† = $P < .001$ vs. ACTH₁₋₂₄).

WRW₄ also reversed the inhibition of basal corticosterone release induced by the lower concentration of fMLF (10^{-7} mol.l⁻¹, *** = $P < .001$) but failed to modify the responses to the two higher concentrations of fMLF tested. WRW₄ appeared to partly reverse the inhibitory effects of fMLF on the secretory responses to ACTH₁₋₂₄ (# = $P < .05$ vs. fMLF + ACTH₁₋₂₄).

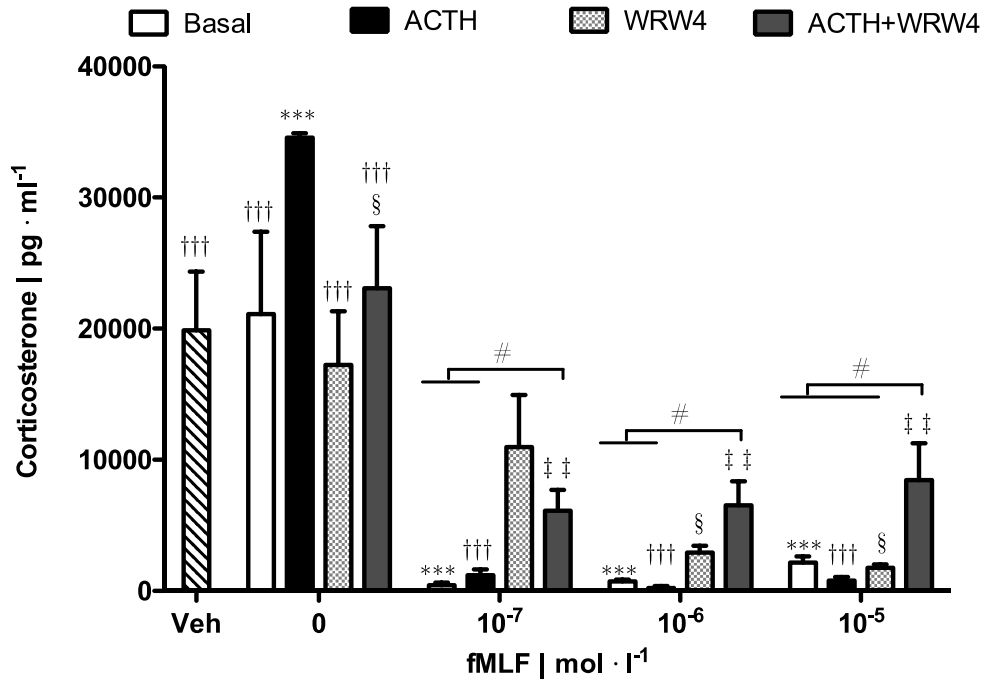


Figure 4.2: Effects of graded concentrations of the formyl peptide receptor 1 (FPR1)-selective agonist formyl-methionyl-leucyl-phenylalanine (fMLF) in the presence and absence of adrenocorticotrophic hormone (ACTH₁₋₂₄) and/or the FPR2/ALX-selective antagonist Trp-Arg-Trp-Trp-Trp-Trp (WRW₄) on corticosterone release from dispersed adrenal cells derived from male Wistar rats.

The cells were incubated at 37 °C in 95% O₂/5% CO₂ for 4 h with or without (basal) the ligands ACTH₁₋₂₄ (10⁻⁷ mol.l⁻¹), WRW₄ (10⁻⁶ mol.l⁻¹), and fMLF (10⁻⁷, 10⁻⁶, 10⁻⁵ mol.l⁻¹). The vehicle (Veh) for fMLF was .025% acetate. Data are arithmetic mean ± standard error of mean of n = 8 replicate wells, compared by three-way ANOVA with Bonferroni correction for multiple comparisons: *** = P < .001 vs. drug-free; ††† = P < .001 vs. ACTH₁₋₂₄; § = P < .05 vs. WRW₄; ‡‡ = P < .01 vs. ACTH₁₋₂₄+WRW₄; and # = P < .05 vs. fMLF ± ACTH₁₋₂₄ ± WRW₄.

4.3.3 The effects of WKYMVm on corticosterone release from dispersed adrenal cells

Figure 4.3 shows the effects of graded concentrations (10^{-8} , 10^{-7} , and 10^{-6} mol.l⁻¹) of the selective agonist for human FPR2/ALX and mouse Fpr2, Trp-Lys-Tyr-Met-Val-D-met (WKYMVm), on the increases in corticosterone release induced by a sub-maximal concentration of ACTH₁₋₂₄ (10^{-7} mol.l⁻¹), in the presence and absence of a selective Fpr2 antagonist WRW₄ (10^{-6} mol.l⁻¹) from dispersed rat adrenal cells *in vitro*.

ACTH₁₋₂₄ (10^{-7} mol.l⁻¹) caused a significant increase in corticosterone release (*** = $P < .001$ vs. basal). At all concentrations tested, WKYMVm reduced basal corticosterone release (*** = $P < .001$ vs. drug-free) and inhibited the steroidogenic response to ACTH₁₋₂₄ (*** = $P < .001$ vs. ACTH₁₋₂₄ alone).

WRW₄ (10^{-6} mol.l⁻¹) had no effect on basal corticosterone release (ns = $P > .05$), but it reduced the response to ACTH₁₋₂₄ (††† = $P < .001$). WRW₄ failed to modify the response to WKYMVm except at the highest concentration tested (10^{-6} mol.l⁻¹) where it reversed the inhibitory effects of WKYMVm, and thus increased corticosterone release (*** = $P < .001$ vs. 10^{-6} mol.l⁻¹ WKYMVm alone). In combination with WRW₄+ACTH₁₋₂₄, the inhibitory effect of low concentration WKYMVm (10^{-8} mol.l⁻¹) was partly reversed (# = $P < .05$).

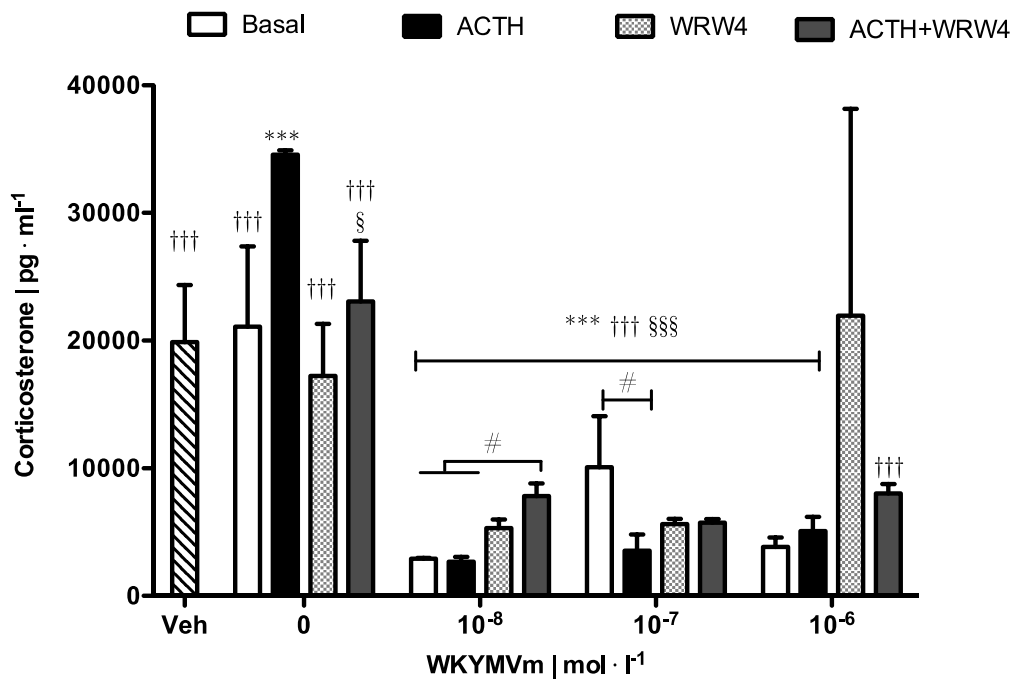


Figure 4.3: Effects of graded concentrations of the formyl peptide receptor 2 (FPR2/ALX)-selective agonist tryptophan-lysine-tyrosine-methionine-valine-*dextro*-methionine (WKYMVm) in the presence and absence of adrenocorticotrophic hormone (ACTH₁₋₂₄) and/or the FPR2/ALX-selective antagonist Trp-Arg-Trp-Trp-Trp-Trp (WRW₄) on corticosterone release from dispersed adrenal cells derived from male Wistar rats.

The cells were incubated at 37 °C in 95% O₂/5% CO₂ for 4 h with or without (basal) the ligands ACTH₁₋₂₄ (10⁻⁷ mol.l⁻¹), WRW₄ (10⁻⁶ mol.l⁻¹), and fMLF (10⁻⁷, 10⁻⁶, 10⁻⁵ mol.l⁻¹). The vehicle (Veh) for WKYMVm was .025% acetate. Data are arithmetic mean ± standard error of mean of n = 8 replicate wells, compared by three-way ANOVA with Bonferroni correction for multiple comparisons: *** = P < .001 vs. drug-free; †† = P < .01, ††† = P < .001 vs. ACTH₁₋₂₄; § = P < .05, §§§ = P < .001 vs. WRW₄; †††† = P < .001 vs. ACTH₁₋₂₄+WRW₄; and # = P < .05 vs. WKYMVm ± ACTH₁₋₂₄ ± WRW₄.

4.3.4 The effects of F2L on corticosterone release from dispersed adrenal cells

F2L is a selective agonist for human FPR3 and mouse Fpr2 derived from an amino-acetylated haem-binding protein fragment. Figure 4.4 shows the effects of graded concentrations (4×10^{-9} , 4×10^{-8} , and $4 \times 10^{-7} \text{ mol.l}^{-1}$) of F2L on the increases in corticosterone release induced by a sub-maximal concentration of ACTH₁₋₂₄ ($10^{-7} \text{ mol.l}^{-1}$), in the presence and absence of a selective Fpr2 antagonist WRW₄ ($10^{-6} \text{ mol.l}^{-1}$) from dispersed rat adrenal cells *in vitro*.

ACTH₁₋₂₄ ($10^{-7} \text{ mol.l}^{-1}$) failed to raise corticosterone release significantly (ns = $P > .05$ vs. basal). ACTH₁₋₂₄ increased corticosterone release when co-incubated with F2L ($\# = P < .05$ vs. F2L alone), except at $10^{-9} \text{ mol.l}^{-1}$ F2L where ACTH₁₋₂₄ inhibited release vs. F2L alone ($\### = P < .001$). At the concentrations tested, F2L had no effect on basal corticosterone release except at $4 \times 10^{-9} \text{ mol.l}^{-1}$ when it increased corticosterone release ($*** = P < .001$ vs. drug-free).

WRW₄ ($10^{-6} \text{ mol.l}^{-1}$) had no effect on basal or ACTH₁₋₂₄-stimulated corticosterone release (ns = $P > .05$), but in the presence of ACTH₁₋₂₄ and $4 \times 10^{-8} \text{ mol.l}^{-1}$ F2L, it potentiated the response to ACTH₁₋₂₄ ($\#### = P < .001$).

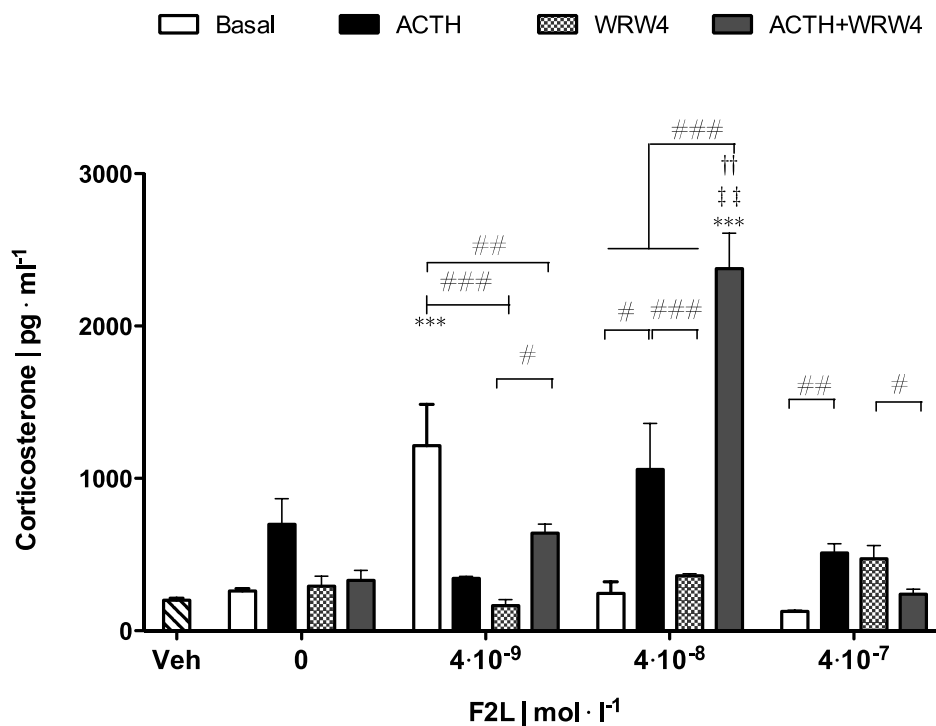


Figure 4.4: Effects of graded concentrations of the formyl peptide receptor 3 (FPR3)-selective agonist amino-acetylated haem-binding protein fragment (F2L) in the presence and absence of adrenocorticotrophic hormone (ACTH₁₋₂₄) and/or the FPR2/ALX-selective antagonist Trp-Arg-Trp-Trp-Trp (WRW₄) on corticosterone release from dispersed adrenal cells derived from male Wistar rats.

The cells were incubated at 37 °C in 95% O₂/5% CO₂ for 4 h with or without (basal) the ligands ACTH₁₋₂₄ (10⁻⁷ mol.l⁻¹), WRW₄ (10⁻⁶ mol.l⁻¹), and fMLF (10⁻⁷, 10⁻⁶, 10⁻⁵ mol.l⁻¹). The vehicle (Veh) for F2L was 0.4% dimethyl sulphoxide (DMSO). Data are arithmetic mean ± standard error of mean of n = 8 replicate wells, compared by three-way ANOVA with Bonferroni correction for multiple comparisons: *** = P < .001 vs. drug-free; †† = P < .01 vs. ACTH₁₋₂₄; § = P < .05, §§§ = P < .001 vs. WRW₄; ‡‡ = P < .01 vs. ACTH₁₋₂₄+WRW₄; and # = P < .05, ## = P < .01, ### = P < .001 vs. F2L ± ACTH₁₋₂₄ ± WRW₄.

4.3.5 The effects of 15-epi-LXA₄ on corticosterone release from dispersed adrenal cells, in the presence and absence of ACTH₁₋₂₄ and/or WRW₄

15-epi-lipoxin A₄ (15-epi-LXA₄) is a selective agonist for human FPR2/ALX, mouse Fpr3/Alx, and rat Fpr2l/Alx. Figure 4.5 shows the effects of graded concentrations (10^{-9} , 10^{-8} , and 10^{-7} mol.l⁻¹) of 15-epi-LXA₄ on the increases in corticosterone release induced by a sub-maximal concentration of ACTH₁₋₂₄ (10^{-7} mol.l⁻¹), in the presence and absence of a selective Fpr2 antagonist WRW₄ (10^{-6} mol.l⁻¹) from dispersed rat adrenal cells *in vitro*.

ACTH₁₋₂₄ (10^{-7} mol.l⁻¹) caused a significant increase in corticosterone release (***) = $P < .001$ vs. drug-free). At all concentrations tested, 15-epi-LXA₄ reduced basal corticosterone release (***) = $P < .001$ vs. drug-free) and inhibited the steroidogenic response to ACTH₁₋₂₄. WRW₄ had no effect on either basal corticosterone release (ns = $P > .05$) or the reduction in basal corticosterone release induced by each concentration of 15-epi-LXA₄ tested (***) = $P < .001$ vs. drug-free).

As in previous experiments, WRW₄ reduced the corticosterone response to ACTH₁₋₂₄. However, in the presence of the lowest concentration of 15-epi-LXA₄, WRW₄ did not affect the secretory response to ACTH₁₋₂₄ (10^{-7} mol.l⁻¹, ### = $P < .001$ vs. 15-epi-LXA₄ + ACTH₁₋₂₄), although in the presence of the two higher concentrations of 15-epi-LXA₄, WRW₄ again reduced the response to ACTH₁₋₂₄.

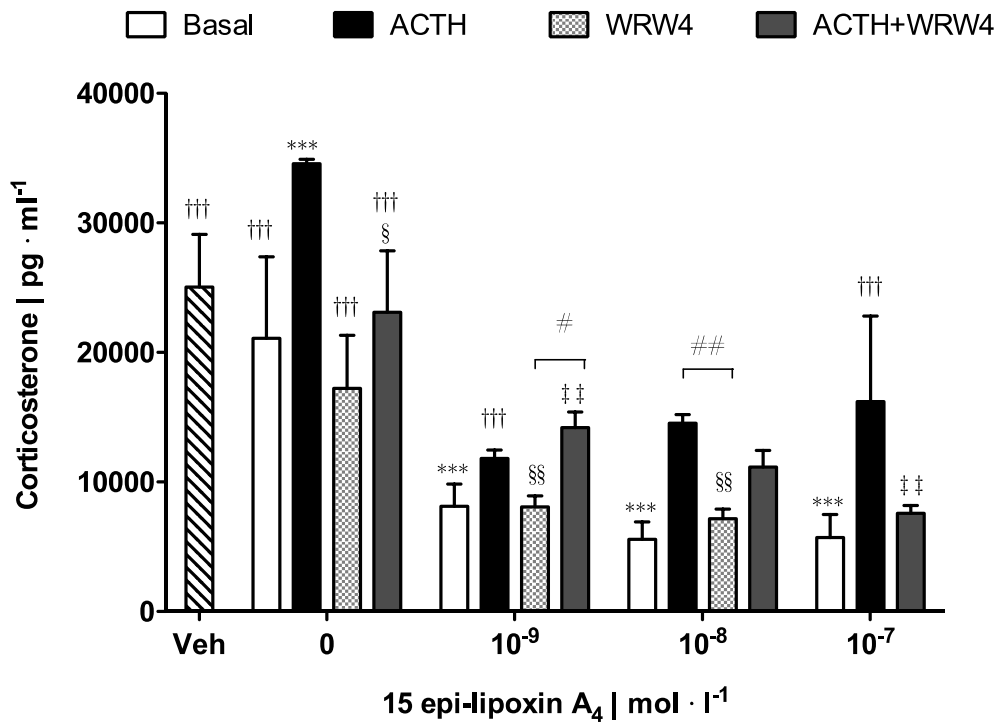


Figure 4.5: Effects of graded concentrations of the formyl peptide receptor 2 (FPR2/ALX)-selective agonist 15-epi-lipoxin A₄ (15-epi-LXA₄) in the presence and absence of adrenocorticotrophic hormone (ACTH₁₋₂₄) and/or the FPR2/ALX-selective antagonist Trp-Arg-Trp-Trp-Trp-Trp (WRW₄) on corticosterone release from dispersed adrenal cells derived from male Wistar rats.

The cells were incubated at 37 °C in 95% O₂/5% CO₂ for 4 h with or without (basal) the ligands ACTH₁₋₂₄ (10⁻⁷ mol.l⁻¹), WRW₄ (10⁻⁶ mol.l⁻¹), and fMLF (10⁻⁷, 10⁻⁶, 10⁻⁵ mol.l⁻¹). The vehicle (Veh) for 15-epi-LXA₄ was .014% ethanol. Data are arithmetic mean ± standard error of mean of n = 8 replicate wells, compared by three-way ANOVA with Bonferroni correction for multiple comparisons: *** = P < .001 vs. drug-free; ††† = P < .001 vs. ACTH₁₋₂₄; §§ = P < .01 vs. WRW₄; ‡‡ = P < .01 vs. ACTH₁₋₂₄+WRW₄; and # = P < .05, ## = P < .01 vs. 15-epi-LXA₄ ± ACTH₁₋₂₄ ± WRW₄.

4.3.6 The effects of ANXA1_{Ac2-26} on corticosterone release from dispersed adrenal cells, in the presence and absence of ACTH₁₋₂₄ and/or WRW₄

Annexin A1 acetylated peptide (ANXA1_{Ac2-26}) is a non-selective agonist of the mouse and human Fpr/FPR subtypes, but so far is only known to bind to Fpr2 of the rat subtypes.

Figure 4.6 shows the effects of graded concentrations (3×10^{-7} , 3×10^{-6} , and 3×10^{-5} mol.l⁻¹) of ANXA1_{Ac2-26} on the increases in corticosterone release induced by a sub-maximal concentration of ACTH₁₋₂₄ (10^{-7} mol.l⁻¹), in the presence and absence of a selective Fpr2 antagonist WRW₄ (10^{-6} mol.l⁻¹) from dispersed adrenal cells *in vitro*.

ACTH₁₋₂₄ (10^{-7} mol.l⁻¹) caused a significant increase in corticosterone release (* = $P < .05$ vs. drug-free). At all concentrations tested, ANXA1_{Ac2-26} reduced basal corticosterone release (***) = $P < .001$ vs. drug-free) and inhibited the steroidogenic response to ACTH₁₋₂₄ (***) = $P < .001$ vs. ACTH₁₋₂₄ alone).

WRW₄ alone (10^{-6} mol.l⁻¹) had no effect on basal corticosterone release (ns = $P > .05$) but reduced the steroidogenic response to ACTH₁₋₂₄ (††† = $P < .001$). However, WRW₄ did not affect the inhibition of basal corticosterone release induced by each concentration of ANXA1_{Ac2-26} (***) = $P < .001$ vs. drug-free). WRW₄ potentiated the steroidogenic response to ACTH₁₋₂₄ in the presence of ANXA1_{Ac2-26} (10^{-7} mol.l⁻¹) but not in the presence of the two higher concentrations of ANXA1_{Ac2-26} tested.

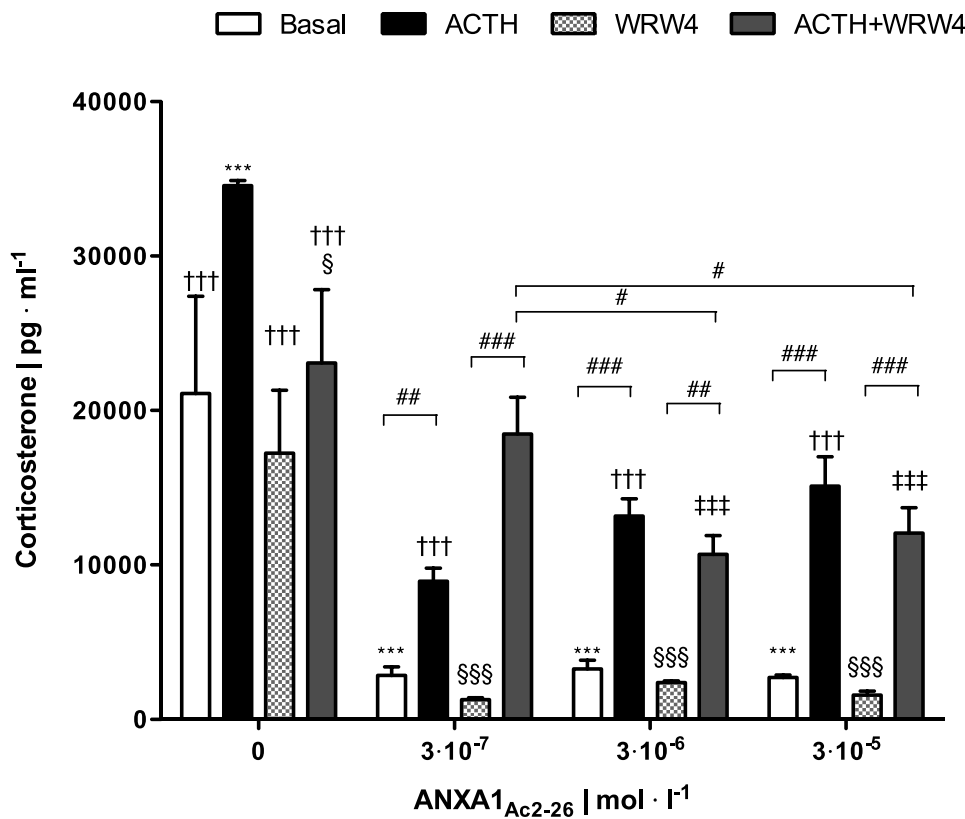


Figure 4.6: Effects of graded concentrations of the non-selective formyl peptide receptor (FPR) agonist annexin A1 amino-acetylated peptide 2-26 (ANXA1_{Ac2-26}) in the presence and absence of adrenocorticotrophic hormone (ACTH₁₋₂₄) and/or the FPR2/ALX-selective antagonist Trp-Arg-Trp-Trp-Trp (WRW₄) on corticosterone release from dispersed adrenal cells derived from male Wistar rats.

The cells were incubated at 37 °C in 95% O₂/5% CO₂ for 4 h with or without (basal) the ligands ACTH₁₋₂₄ (10⁻⁷ mol.l⁻¹), WRW₄ (10⁻⁶ mol.l⁻¹), and fMLF (10⁻⁷, 10⁻⁶, 10⁻⁵ mol.l⁻¹). Data are arithmetic mean ± standard error of mean of n = 8 replicate wells, compared by three-way ANOVA with Bonferroni correction for multiple comparisons: *** = P < .001 vs. drug-free; ††† = P < .001 vs. ACTH₁₋₂₄; §§§ = P < .001 vs. WRW₄; †††† = P < .001 vs. ACTH₁₋₂₄+WRW₄; and # = P < .05, ## = P < .01, ### = P < .001 vs. ANXA1_{Ac2-26} ± ACTH₁₋₂₄ ± WRW₄.

4.4 Discussion

The main findings can be drawn from (1) the efficacy of each agonist, compared at broadly the same concentration level, that is, around 10^{-7} mol.l⁻¹; (2) whether the given agonist is effective in basal and/or ACTH₁₋₂₄-stimulated conditions, and (3) the sensitivity of each agonist to the effects of the Fpr2-/FPR2/ALX-selective antagonist, WRW₄. It is evident, then, that fMLF elicited the most powerful inhibition of corticosterone release irrespective of whether ACTH₁₋₂₄ was present. Despite being generally Fpr1-/FPR1-selective elsewhere, fMLF was the only agonist to be at least partly inhibited by WRW₄. On the other hand, 15-epi-LXA₄ and ANXA1_{Ac2-26} were unaffected by WRW₄, although they each suppressed both basal and ACTH₁₋₂₄-induced corticosterone release. WKYMVm partly suppressed basal corticosterone release, and was unaffected by ACTH₁₋₂₄ or WRW₄. The effects of F2L cannot be interpreted with any accuracy, as the cells in this experiment were unresponsive to ACTH₁₋₂₄ (discussed later). Overall, a host of agonists with known activity via human FPR2/ALX and mouse Fpr2 or Fpr3/Alx, all suppressed basal or ACTH₁₋₂₄-induced corticosterone release from dispersed adrenal cells from rats. These effects were not as convincing, however, as the human FPR1 and mouse/rat Fpr1 agonist, fMLF, which was the only agonist tested to have effects which were sensitive to the Fpr2-/FPR2/ALX-selective antagonist, WRW₄. Thus, the potency of the tested FPR agonists for inhibition of basal and ACTH₁₋₂₄-stimulated corticosterone release can be summarised as: fMLF >>> WKYMVm > ANXA1_{Ac2-26} > 15-epi-LXA₄ >> F2L. The only agonist that was affected by WRW₄ was fMLF.

4.4.1 fMLF

Prior to this study, various published reports on the pharmacological actions of fMLF indicated that when it is used at low nanomolar concentrations, it is a selective agonist for human FPR1 and mouse Fpr1, with a pEC₅₀ value of around 9 (Freer *et al.* 1980; Koo *et al.* 1982; He *et al.* 2000). Whilst fMLF is selective for Fpr1/FPR1, it does not bind to these subtypes exclusively. Although it binds with five-times lower affinity than at Fpr1/FPR1, fMLF is an agonist for human FPR2/ALX with a pEC₅₀ of 4 (Schiffmann *et al.* 1975b; Mills *et al.* 2000), and for mouse Fpr2 with a pEC₅₀ between 5 and 6.37 (Quehenberger *et al.* 1997; Mills *et al.* 2000). Even when used at 100 micromolar concentrations, fMLF did not bind or elicit responses with either human FPR3 or mouse Fpr3, and so can be safely assumed to have negligible

efficacy via these receptors (Hartt *et al.* 1999; Liang *et al.* 2000). Thus the relative affinities of fMLF for mouse Fpr1 over Fpr2 and for human FPR1 over FPR2/ALX, suggests these subtypes are functionally similar, at least for fMLF. Unfortunately, there are no binding or efficacy data currently available for the effects of fMLF via the equivalent of Fpr1/FPR1 in rats, or indeed, any other rat receptors.

This study has shown that fMLF potently inhibits basal and ACTH₁₋₂₄-stimulated corticosterone release from dispersed rat adrenal cells *in vitro*. The concentrations of fMLF used here ($10^{-7} - 10^{-5}$ mol.l⁻¹) were chosen to match those of others researching its actions via human FPR1 and mouse Fpr1. The inhibition of fMLF on basal corticosterone release was partly reversed by a micromolar concentration of WRW₄, but only at the lowest tested concentration of fMLF (10^{-7} mol.l⁻¹). Given that WRW₄ is an Fpr2-/FPR2/ALX-selective antagonist (Bae *et al.* 2004; Önnheim *et al.* 2008), this finding suggests that WRW₄ antagonises the rat fMLF receptor/s.

To the author's knowledge, this is the first time that fMLF has been tested on dispersed adrenal cells from rats, and shown to inhibit both basal and ACTH₁₋₂₄-stimulated corticosterone release. To assess the plausibility of these findings, it is worthwhile comparing them to those of other studies using fMLF or other Fpr1-/FPR1-selective ligands. Similar experiments looking at the effects of fMLF alone or with ACTH₁₋₂₄ and WRW₄, on adrenal function have been performed on dispersed adrenal cells from mice. Corticosterone release was unaffected by fMLF when used at up to 100 micromolar concentrations, but was inhibited by as much as 70% when micromolar fMLF was incubated with ACTH₁₋₂₄ (Buss 2010). As is the case here, and consistent with its antagonist status, WRW₄ had no effect on corticosterone release either on its own or with ACTH₁₋₂₄ (Buss 2010). From the present data, it is not clear why the FPR1/Fpr1 agonist fMLF inhibited both basal and ACTH₁₋₂₄-stimulated corticosterone release from dispersed adrenal cells from rats, but only inhibited ACTH₁₋₂₄-stimulated release in the mouse model (Buss 2010). All conditions other than the source species and incubation period (4 hours here vs. 2 hours by Buss) were identical. At the time of writing, however, there were no other instances of fMLF being tested on dispersed adrenal cells. Therefore, to understand further the present results, they ought to be compared to the effects of different ligands that share receptors with fMLF.

Fpr1/FPR1 ligands other than fMLF have been shown to inhibit adrenal corticosteroid release. Cyclosporin A (CSA), for instance, is an Fpr1/FPR1 antagonist (Yan *et al.* 2006) that inhibits adrenal

steroidogenesis (Rebuffat *et al.* 1989). Having potent immunosuppressive effects on lymphocyte function, CSA is particularly useful as an anti-rejection drug following organ transplantation. In addition, CSA has been shown to inhibit the secretion of ACTH and corticosterone in Wistar rats under basal and stressed conditions (Hirano *et al.* 1988), and comparable observations have been made in humans (Oka *et al.* 1993). Other antagonists of Fpr1/FPR1 have similar effects. Boc2, for instance, is a synthetic compound (N-t-butoxycarbonyl-L-phenylalanyl-L-leucyl-L-phenylalanyl-L-leucyl-L-phenylalanine) that antagonises Fpr1/FPR1 when used at sub-micromolar concentrations (Wenzel-Seifert & Seifert 1993). Boc2 has been shown to inhibit corticosterone release in dispersed adrenal cells from mice when used at 10 micromolar concentrations. Again, this was only observed when ACTH₁₋₂₄ was applied with Boc2 (Buss 2010).

The present finding of an inhibitory effect of fMLF on adrenal corticosterone appears to be contradictory to the fact that antagonists selective for Fpr1/FPR1, namely, Boc2 and CSA, have been shown to inhibit adrenal steroidogenesis. Explanations for this can be drawn from what is known about the activation and signalling mechanisms of Fpr1/FPR1. One possibility is that the fMLF receptors in the dispersed rat adrenal cells are internalised following activation by fMLF, through a process known as homologous desensitisation. Alternatively, ligands binding to Fpr1/FPR1 have also been shown to internalise other GPCRs through heterologous or cross-desensitisation (Le *et al.* 2001b), as is the case with the chemokine receptors for IL-8 and CXCL8 (CXCR1 and CXCR2) or the two HIV co-receptors (Suvorova *et al.* 2004; Migeotte *et al.* 2005; Suvorova *et al.* 2009). Both mechanisms involve PKC activation, and subsequent phosphorylation of serine residues in the carboxylic acid terminals of the target GPCRs before internalisation (Christophe *et al.* 2001; Le *et al.* 2001b). If Fpr1, or the rat equivalent, were to exert tonic stimulation on adrenal corticosterone release as has been suggested by Buss (Boxio *et al.* 2005; Buss 2010), then fMLF might be inhibiting basal release by internalising the rat fMLF receptor, and inhibiting the ACTH₁₋₂₄-stimulated release through cross-desensitisation of MC₂R (Migeotte *et al.* 2005; 2006).

Taken together, the overall inhibitory effect of fMLF on corticosterone release in the study, at concentrations known to stimulate human FPR1 and mouse Fpr1, reaffirms the existing evidence that Fpr1 mediates inhibition of adrenal corticosterone release (Christophe *et al.* 2001; Buss 2010). Previous work with mouse adrenals co-incubated with WRW₄ and ACTH₁₋₂₄ found that WRW₄ had no effect on basal or ACTH₁₋₂₄-stimulated release of corticosterone (Boxio *et al.* 2005; Buss 2010). At the time of

writing, however, this is the first time that the Fpr2-/FPR2/ALX-selective antagonist WRW₄ has been co-incubated with both fMLF and ACTH₁₋₂₄ in any dispersed adrenal cell culture. The fact that micromolar WRW₄, an Fpr2-/FPR2/ALX-selective antagonist (Bae *et al.* 2004; Prat *et al.* 2006; Önnheim *et al.* 2008), when co-incubated with ACTH₁₋₂₄, attenuated the inhibitory effect of fMLF is worthy of further investigation. In this context, the combined findings from mouse (Bae *et al.* 2003b; Buss 2010) and rat adrenal studies, support the assertion that at least one of the rat receptors for fMLF is WRW₄-sensitive and coupled with signal cascades that inhibit corticosterone release in ACTH₁₋₂₄-stimulated and basal conditions. This effect of WRW₄ was surprising, as elsewhere, higher Fpr2-relevant 10⁻⁴ mol.l⁻¹ concentrations of fMLF (Schiffmann *et al.* 1975b; Mills *et al.* 2000), did not inhibit corticosterone release from mouse adrenals (Buss 2010). There are currently no pIC₅₀ data for WRW₄ in rat systems, and so this limits the scope of determining which receptors are blocked by WRW₄ in these dispersed rat adrenal studies.

4.4.2 WKYMVm

WKYMVm (Trp-Lys-Tyr-Met-Val-D-met-NH₂) is a synthetic hexapeptide (Migeotte *et al.* 2006) and selective agonist for human FPR2/ALX and mouse Fpr2 with a pEC₅₀ of 10.13 (Migeotte *et al.* 2005). WKYMVm also has activity through human FPR3 with a pEC₅₀ of 8.52 (Christophe *et al.* 2001) and mouse Fpr3 with a pEC₅₀ of 7.48 (Boxio *et al.* 2005). The present data reveal that WKYMVm, at all concentrations tested, caused a significant reduction in basal and ACTH₁₋₂₄-stimulated corticosterone release, except at micromolar WKYMVm, where a slight increase in corticosterone release occurred when WKYMVm was co-incubated with WRW₄. To the author's knowledge, this is the first recorded use of WKYMVm in dispersed adrenal cells from rats or any other species.

At all concentrations tested, WKYMVm caused a significant reduction in basal and ACTH₁₋₂₄-stimulated corticosterone release. At the micromolar concentration of WKYMVm, there was a slight increase in corticosterone release when co-incubated with WRW₄. The inclusion of WRW₄ reversed the inhibitory effects of higher concentrations of WKYMVm on basal corticosterone release. WKYMVm potently induces superoxide production, calcium cation mobilisation, and chemotaxis via human FPR2/ALX in neutrophils (Migeotte *et al.* 2005) and FPR3 in monocytes (Christophe *et al.* 2001), and via Fpr2 or Fpr3 in mouse neutrophils (Boxio *et al.* 2005). Similarly, incubations of HL-60 cells over-expressing Fpr2 with

micromolar WKYMVm result in upregulated calcium cation mobilisation traced by the Fluo3 marker (Prat *et al.* 2006). The role of calcium mobilisation in the promotion of secretory granule exocytosis by WKYMVm has been indexed by β -hexosaminidase activity in human FPR2-positive rat basophilic leukaemia cells (RBL-2H3), and by Fluo3 in mouse Fpr2-positive HL-60 cells, and is blocked by the calcium chelator BAPTA (Bae *et al.* 2003b).

Given that the evidence suggests that WKYMVm-induced effects are mediated via human FPR2/ALX (Migeotte *et al.* 2005) or FPR3 (Christophe *et al.* 2001), and mouse Fpr2 or Fpr3 (Boxio *et al.* 2005), it seems plausible that the inhibitory effect of WKYMVm on corticosterone release evident in the present studies could also be mediated via rat Fpr2 or Fpr3. Interestingly, WKYMVm is associated with ERK phosphorylation in macrophages (Kang *et al.* 2005) which is reduced in Fpr2/3-null mice (Dufton *et al.* 2010). However, ERK phosphorylation is associated with enhanced transcription of the StAR gene and increased corticosterone release from dispersed adrenal cells (Gyles *et al.* 2001; Ferreira *et al.* 2007; Hoeflich & Bielohuby 2008). This raises questions as to whether the inhibitory effects of WKYMVm seen here are mediated via Fpr2, Fpr3, or another receptor. This problem reflects the conclusions of Boxio and coworkers (2005) who showed that the stimulatory effects of WKYMVm on neutrophil granule release are reduced by 50% with a human FPR2/ALX antagonist termed C2 (WPLTHTLRHTIW) used at a concentration of 10^{-5} mol.l⁻¹. Despite this, they could not determine whether the effects were Fpr2 or Fpr3-mediated. In the present data, the inhibition of corticosterone release by micromolar WKYMVm was reversed partly by WRW₄, suggesting WKYMVm mediates its inhibition via a WRW₄-sensitive receptor. WRW₄ is a selective antagonist of human FPR2/ALX, mouse Fpr2, and rat Fpr2 (Braun *et al.* 2011). Therefore, further work is required to elucidate precisely which Fpr subtype, mediates these inhibitory effects of WKYMVm, and the reversal by WRW₄, in dispersed adrenal cells from rats. Given the non-endocrine effects of WKYMVm via mouse Fpr2 or Fpr3, it is reasonable to suggest that one or both of these receptors have equivalents that mediate the inhibitory effects in this rat system.

4.4.3 F2L

The present data show the effects of graded concentrations of the human FPR3-, mouse Fpr2-selective agonist, F2L, on corticosterone release from dispersed adrenal cells obtained from rats. F2L is an acetylated 21 amino acid oligopeptide derived from the amino terminal of the intracellular haem-binding

protein and has a pEC₅₀ for human FPR3 of 8.02 (Migeotte *et al.* 2005) and 6.4 for mouse Fpr2 (Gao *et al.* 2007). Unfortunately, the results from the F2L study cannot be interpreted with any accuracy, as the response to ACTH₁₋₂₄ was not significant. This is likely due to the CO₂ regulator supplying the incubator failing at the time of the experiment.

4.4.4 ANXA1_{Ac2-26} and 15-*epi*-LXA₄

The results show that ANXA1_{Ac2-26} and 15-*epi*-LXA₄ each suppressed both basal and ACTH₁₋₂₄-stimulated corticosterone release, but were unaffected by the Fpr2-/FPR2/ALX-selective antagonist WRW₄. To the author's knowledge, this is the first time that 15-*epi*-LXA₄ has been shown to inhibit corticosterone release from dispersed adrenal cells. ANXA1_{Ac2-26}, however, at 10⁻⁵ and 3 x 10⁻⁵ mol.l⁻¹ concentrations inhibited ACTH₁₋₂₄-stimulated corticosterone release from dispersed mouse adrenals (Buss 2010), and so is consistent with the present findings. Elsewhere, concentrations of ANXA1_{Ac2-26} ranging from 3 x 10⁻⁷ to 3 x 10⁻⁵ mol.l⁻¹ have inhibited ACTH₁₋₂₄-stimulated corticosterone release from dispersed mouse adrenals (Davies *et al.* 2007). ANXA1_{Ac2-26} is the N-terminal active peptide from ANXA1, and it elicits effects via human FPR1 with a pEC₅₀ of 5.30 (Walther *et al.* 2000), and the structurally similar peptide ANXA1_{Ac1-25} binds human FPR1, FPR2/ALX, and FPR3 with a pEC₅₀ between 5.00 and 6.80 (Ernst *et al.* 2004b). There are various data suggesting that effects of ANXA1_{Ac2-26} in mice *in vivo* are mediated via mouse Fpr1 or Fpr2 with approximate values for pED₅₀ of around 6.00 (Gavins *et al.* 2007), or more conservatively, 4.48 (Perretti *et al.* 1995). In HEK293 cells transformed with Fpr2l, the rat lipoxin receptor, concentrations of ANXA1_{Ac2-26} ranging from 10⁻⁸ to 10⁻⁵ have been shown to bind to Fpr2l with a pK_d of 6.09 (Chiang *et al.* 2003).

Biosynthesis of the eicosanoid 15-*epi*-LXA₄ is stimulated by aspirin (Clària & Serhan 1995; Gewirtz *et al.* 1999). As the epimeric form of lipoxin A4 (LXA₄), 15-*epi*-LXA₄ is a more potent and stable agonist for the human lipoxin receptor FPR2/ALX with a pEC₅₀ of 10.10 (Maddox *et al.* 1997; Chiang *et al.* 2000) versus LXA₄ with a pEC₅₀ of 9.10 (Maddox *et al.* 1997; Chiang *et al.* 2000). This relative stability has been attributed to 15-*epi*-LXA₄ being more resistant to the action of the 15-hydroxy prostaglandin dehydrogenase (Serhan *et al.* 1995). 15-*epi*-LXA₄ and LXA₄ bind to the mouse lipoxin receptor, Fpr3/Alx, with equal affinity and a pK_d of 8.82 (Takano *et al.* 1997). On the other hand, in terms of their anti-inflammatory effects, equimolar 15-*epi*-LXA₄ was found to be more potent than LXA₄, but broadly

similar in potency to dexamethasone (Takano *et al.* 1997), whose anti-inflammatory effects coincide with upregulated FPR2/ALX expression (Hashimoto *et al.* 2007). In rats, LXA₄ binds to the rat Fpr2l/Alx with a pK_d of 8.30 and this receptor has 74% and 84% sequence homology with human FPR2/ALX and mouse Fpr3/Alx respectively (Chiang *et al.* 2003).

To the author's knowledge, there are no other instances of 15-epi-LXA₄, the human FPR2/ALX and mouse Fpr3/Alx agonist, and rat Fpr2l/Alx agonist, inhibiting corticosterone from dispersed adrenal cell cultures. Given the similar effects of 15-epi-LXA₄ and ANXA1_{Ac2-26} in the present work, and the fact that they bind to the same rat Fpr2l/Alx (Chiang *et al.* 2003), it is reasonable to discuss the role of the rat Fpr2l/Alx in the context of the role of ANXA1_{Ac2-26} and full length ANXA1 in the adrenal glands. There is evidence of ANXA1 message and protein in the adrenal glands of mice (Davies *et al.* 2007). The adrenal glands respond to ACTH by upregulating the expression of steroidogenic enzymes and their glucocorticoid production. Within 3 hours of GC treatment, ANXA1 is upregulated, particularly within the cytosol of cells in the steroidogenic cortex and externalised in the sub-capsular zones (Davies *et al.* 2007). Consistent with the present findings, ANXA1_{Ac2-26} (3×10^{-9} mol.l⁻¹ - 3×10^{-4} mol.l⁻¹) was also shown to inhibit corticosterone release, and ANXA1-null mice have increased ACTH₁₋₂₄-stimulated corticosterone release (Davies *et al.* 2007). The present data showed that ANXA1_{Ac2-26} suppresses corticosterone release in both basal and ACTH₁₋₂₄-stimulated conditions. Taken together, this suggests ANXA1 may mediate negative feedback within the adrenal gland. Given the evidence of non-endocrine actions of ANXA1_{Ac2-26} and 15-epi-LXA₄ are mediated via high affinity rat Fpr2l/Alx (Chiang *et al.* 2003), it is not unreasonable to suggest that the pattern of inhibited corticosterone release shared by these ligands, is mediated by the common target of rat Fpr2l/Alx.

4.4.5 Biological significance and scope of findings

The finding that ligands such as fMLF, which are based on the prototypical Fpr/FPR ligand first obtained from gram negative bacteria, are able to modulate adrenal GC release has implications for our understanding of the adrenal insufficiency that can occur in up to 65% of patients with bacterial sepsis (Briegel *et al.* 1996; Marik 2002; Marik & Zaloga 2003). Interestingly, a modified form of fMLF has been found to function as an antimicrobial and opsonisation compound and facilitates the actions of the antibiotic erythromycin, and protects mice during sepsis-related endotoxaemia (Tsubery *et al.* 2005). It is

not presently clear from this work, whether adrenal function was involved in the protection afforded by fMLF. It is noteworthy that even LPS-primed macrophages are able to inhibit adrenal steroidogenesis (Mathison *et al.* 1983). Clearly, the subtypes of the Fpr/FPR family and their role in the dynamics between the immune and endocrine systems in response to sepsis-related endotoxaemia, are worthy of further investigation.

One key assumption in designing the study was that the selectivity of the ligands for the murine receptors is complemented by the rat receptors. Elsewhere, the anti-inflammatory effects of ANXA1 are evidently signalled through the rat Fpr2 subtype (Braun *et al.* 2011; Pei *et al.* 2011; Slowik *et al.* 2012), whereas the effects of LXA₄ and 15-epi-LXA₄ have been shown to be mediated via rat Fpr2/Alx which was upregulated in a rat model of endometriosis (Chiang *et al.* 2003; Motohashi *et al.* 2005).

The dispersed adrenal cell studies involved a factorial study design. In practice this meant testing multiple factors simultaneously: graded concentrations of an Fpr/FPR agonist, with or without the Fpr2-/FPR2/ALX-selective antagonist and the MC₂R agonist ACTH₁₋₂₄. The concentrations of the Fpr agonists used in these experiments were selected based on existing data on their potency (EC₅₀), or affinity (K_d) via the Fpr subtype for which they have the highest affinity. The three concentrations chosen straddled this EC₅₀ or K_d for each ligand. In hindsight, it would have been more beneficial to test the effects of the agonists over a wider range of concentrations, a minimum of six perhaps. This would have allowed calculation of an EC₅₀ for each Fpr ligand tested in this dispersed rat adrenal cell system. Only three concentrations were used because we sought to test the agonists in a factorial study design, involving the coincubation of the agonists with ACTH₁₋₂₄ and/or WRW₄. This was because WRW₄ is an Fpr2-/FPR2/ALX-selective antagonist (Braun *et al.* 2011) and much of the evidence at the time suggested that Fpr2 was involved in the regulation of GC release (John *et al.* 2007). To have both a factorial study design and a wider range of concentrations would have required more cells than could be viably generated in a single experiment (Carsia *et al.* 1996).

The supply of CO₂ to the incubator failed during the F2L experiment and could have affected viability. The viability of the cells was only checked before the addition of drugs and would benefit from checking the cells following the incubation period to ensure that the vehicle or the drug did not affect the release of corticosterone through the induction of cell death. Cell viability is typically between 80 – 95% in

conditions similar to the present work (Ramachandran & Suyama 1975). The adrenal gland undergoes atrophy following hypophysectomy or suppression of ACTH release through inhibition by exogenous GCs, and is partly reversed by exogenous ACTH (Ceccatelli *et al.* 1995). This atrophy is characterised by adrenocortical apoptosis, and the cortex size is markedly reduced in animals given GCs. Following hypophysectomy in rats, the adrenal cortex undergoes atrophy between 12 and 24 hours later, whereas cultured adrenal cells undergo apoptosis much earlier, around 1 hour following (Carsia *et al.* 1996).

Since the dispersed adrenal cells were generated from a single pool of multiple glands, the experiment would need to be repeated to confirm the reproducibility of the findings. Alternatively, a modified protocol for the experiments would involve multiple pools of dispersed glands, for instance, $n = 8$ pools of 2 glands each, per 96-well plate experiment. Such a protocol would have to be optimised to ensure that generating several pools rather than just one did not affect cell viability. Otherwise, multiple personnel would have to be involved and this was not an option available at the time of the experiments.

Some aspects of the experiments could be improved upon in future work. The adrenal glands were not decapsulated before incubation. This might have increased the mitotic and proliferative capacity of the adrenal cells versus a culture of dispersed cells from decapsulated glands (see Armato & Nussdorfer 1972; O'Hare & Neville 1973). Other dispersed adrenal cell monoculture protocols involve the removal of the surrounding fat and the adrenal capsule (Giroud *et al.* 1956), in order to take off the zona glomerulosa (Bankiewicz *et al.* 1968). The adrenal glands obtained were from control rats from *in vivo* experiments of colleagues. To reduce the number of animals required, one could consider using female rats, as their adrenals are larger and have a thicker cortex with respect to their body mass, and thus release around twice as much GCs (Kitay 1961; Biswas *et al.* 1967).

4.4.6 Conclusion

These studies have provided evidence in support of the hypothesis, that Fpr/FPR ligands are able to modulate GC release from dispersed adrenal cells *in vitro*, in basal and ACTH₁₋₂₄-stimulated conditions.

**Chapter Five: Study of the effects of formyl
peptide receptor ligands on pituitary
function**

5.1 Introduction

The following paragraphs describe the existing evidence for the role of formyl peptide receptor (Fpr/FPR) ligands in the modulation of pituitary function; the available experimental models for assessing pituitary function *in vitro*; the comparative physiology of the pituitary gland between humans, rats, and mice; and the basic regulation of pituitary adrenocorticotrophic hormone (ACTH) by corticotrophic releasing hormone (CRH), arginine vasopressin (AVP), and their receptors.

5.1.1 Comparative anatomy and physiology of the pituitary gland

As with the adrenal gland, the pituitary gland is a composite organ that has evolved in all vertebrate species, and is derived from two embryological sources. The posterior lobe of the pituitary gland is made from the neural tube that also forms the brain and nervous system, whereas the anterior lobe is made from Rathke's pouch in a folding of the stomodeum, which also forms the gut and mouth. The anterior lobe then differentiates into three major epithelial subdivisions: the pars distalis, pars intermedia, and the pars tuberalis. The developed pituitary gland in humans is a bean-sized hypophysis (under-growth) that sits within the sella turcica (Turkish saddle) of the sphenoid bone at the base of the brain above the nasal sinuses. The course of cell differentiation behind the diverse hormone secreting cells within the human anterior pituitary gland is similar in rats and mice. The effective differentiation of the endocrine cells of the anterior pituitary gland requires an exchange of signals with the developing hypothalamus. Explant studies of the Rathke's pouch taken from mice (Takuma *et al.* 1998; Treier *et al.* 1998) and rats (Nemeskéry *et al.* 1976; Dubois & Hemming 1991) demonstrated a critical period for this communication—between embryonic (E) day E12 and E13. After this period, the first hormone producing cells to develop are the corticotrophs expressing proopiomelanocortin (POMC), which begin to appear at E13.5 in mice (Japón *et al.* 1994) and rats (Watanabe 1982). This is followed by the appearance of the thyrotrophs (thyroid stimulating hormone- β (TSH β) positive) in the rostral tip at E14.5, followed by somatotrophs (growth hormone (GH) positive), lactotrophs (prolactin (PRL) positive), and gonadotrophs (luteinising hormone- β (LH β), or follicle stimulating hormone- β (FSH β) positive) at E15-17.5 (Burrows *et al.* 1999). One major difference in pituitary development between humans and rodents, however, is that the terminal differentiation of the anterior pituitary cell types is reached during the first trimester, whereas this extends into the perinatal period in rodents (Cushman *et al.* 2001; Polin *et al.* 2011). Indeed, the

pituitary-adrenal axis in rats (Schapiro *et al.* 1962) and mice (Schmidt *et al.* 2003) undergoes a clear period of postnatal hyposensitivity to stressors. To some extent, this might also occur during human infancy (Gunnar *et al.* 1989), but by adulthood, the basic mechanisms behind the pituitary-adrenal stress response in these species is broadly similar.

5.1.2 Pituitary function and expression of Fpr/FPR subtypes and their ligands

The secretion of pituitary ACTH is primarily under the control of CRH and AVP, whose actions are synergistic but each have different signalling mechanisms (Gillies *et al.* 1982). Upon reaching the corticotrophs, CRH activates the type 1 CRH receptor (CRH-R1) coupled with adenylate cyclase leading to the formation of cyclic adenosine monophosphate (cAMP) which triggers protein kinase A (PKA) activation and an influx of extracellular Ca^{2+} via L-type voltage-sensitive channels (Won & Orth 1990). AVP binds to its vasopressin receptor type 1b (V1bR) and activates phospholipase C (PLC) to generate inositol trisphosphate (IP_3) and diacylglycerol (DAG) that induces intracellular Ca^{2+} mobilisation and activation of protein kinase C (PKC) for a further influx of Ca^{2+} (Won *et al.* 1990). One of the main mechanisms of inhibiting ACTH secretion, however, involves the negative feedback effects of glucocorticoids (GCs). Although the precise mechanism of this feedback inhibition remains to be elucidated, GCs are known to induce the expression of the second mediator protein annexin A1 (ANXA1) within the pituitary, and elsewhere (Strijbos *et al.* 1991). In addition, ANXA1 mimics, while treatment with ANXA1 antisera attenuates the actions of GCs in models of inflammation, and hormone release (Taylor *et al.* 1997). Dartois and Bouton (1986) suggested an ANXA1-like protein is likely to be involved in the inhibition of ACTH release. Later, expression of ANXA1 was found to be localised within the folliculostellate cells of the anterior pituitary (Traverso *et al.* 1999) with ANXA1 binding sites evident on adjacent corticotrophs (Christian *et al.* 1997). These binding sites were suggested to be populated by an Fpr/FPR subtype on the basis that ANXA1 had been shown to mediate its effects via FPR expressed in leukocytes (Walther *et al.* 2000).

The potential for using Fpr/FPR ligands as tools to improve our understanding of the regulation of pituitary ACTH release, relates to the diversity of the currently known Fpr/FPR ligands, of which there are at least one hundred (Ye *et al.* 2009). This is partly restricted, of course, by the Fpr/FPR subtypes that are expressed within the pituitary gland. These are Fpr2, Fpr3, Fpr-rs6, and Fpr-rs7 within the mouse anterior pituitary (John *et al.* 2007; Buss 2010), and, based on the sequence homology with their mouse equivalents, Fpr2 and Fpr3 have been detected within the rat anterior pituitary (John *et al.* 2007). (The nucleotide sequences for the rat Fpr subtypes are still based on expressed sequence tags (ESTs) from cDNA libraries and so their predicted sequences have not yet been confirmed). Until the present work,

relatively little was known about the expression of FPR within the human pituitary, although FPR1 epitopes were not detectable (Becker *et al.* 1998). If an FPR were involved in the modulation of pituitary ACTH release, then this would leave only FPR2/ALX and FPR3 to investigate in the human case, as well as their equivalent Fpr subtypes in rodents, namely, Fpr2 and Fpr3 in both mice and rats.

Many of the ligands that mediate effects via human FPR2/ALX and FPR3 also exert effects via the structural equivalents of these receptors in rodents. The main endogenous ligands for FPR2/ALX and FPR3 include, the anti-inflammatory lipids lipoxin A4 (LXA₄) and 15-epi-LXA₄ (Fiore *et al.* 1994); resolvins (Norling *et al.* 2012); urokinase-type plasminogen activator (uPA) and its receptor (uPAR) (Resnati *et al.* 2002); amyloidogenic proteins such as serum amyloid A (SAA), beta amyloid (A β -42) and prion protein (Prp)106-125 (Su *et al.* 1999; Le *et al.* 2001a, c); humanin (Harada *et al.* 2004); ANXA1 and its peptide derivatives such as ANXA1_{Ac2-26} (Walther *et al.* 2000; Hayhoe *et al.* 2006); chemokines such as β -chemokine (Elagoz *et al.* 2004); as well as neuropeptides such as vasoactive intestinal peptide (VIP) (Zein *et al.* 2008b), and pituitary adenylate cyclase activating polypeptide (PACAP)-27 (Kim *et al.* 2006), and numerous mitochondrial peptides.

Of the endogenous Fpr/FPR ligands, those that have been detected within the pituitary gland include ANXA1, VIP, and PACAP-27. In rats, ANXA1 is expressed within the hippocampus, striatum, cortex, hypothalamus, and the pituitary gland (Smith *et al.* 1993), and has a similar distribution in humans (Dreier *et al.* 1998). Within the anterior pituitary, ANXA1 is expressed within the folliculostellate cells (Traverso *et al.* 1999) and has been shown to mediate the inhibitory effects of GCs on the secretion of ACTH, PRL, GH, and TSH (Taylor *et al.* 1993-b; 2000a). VIP and PACAP-27 are particularly evident within dispersed rat pituitary cells expressing ACTH, GH, PRL, or S100b (Vigh *et al.* 1993). Despite these similarities, the ligands differ in terms of their effects on pituitary function. PACAP-27, and full length PACAP-38 are synthesised within the hypothalamus and have been investigated along with VIP for their effects on pituitary-adrenal axis function. Unlike VIP, intravenous PACAP was able to stimulate ACTH release in humans (Chiodera *et al.* 1996), but the stimulatory effect takes up to 24 hours in dispersed rat pituitaries (Hart *et al.* 1992; Koch & Lutz-Bucher 1993). This suggested PACAP does not act directly on corticotrophs but possibly via upregulation of hypothalamic CRH expression (Grinevich *et al.* 1997).

The enzymes that produce the lipid Fpr/FPR ligands such as LXA₄, 15-epi-LXA₄, and resolvin D1, are all expressed within the pituitary gland (Won & Orth 1994; Ikawa *et al.* 1996) and elsewhere within the HPA axis (Cover *et al.* 2001). Interestingly, LXA₄ has been shown to prevent LPS-induced miscarriages in mice (Walker *et al.* 2011), and its serum concentration is inversely correlated with miscarriage in women (Xu *et al.* 2013). PACAP has also been shown to have an anti-inflammatory effect in sepsis (Martínez *et al.* 2002). The Fpr/FPR ligands are being investigated as candidate alternatives to corticosteroids, whose long-term use can suppress pituitary-adrenal function (Schlaghecke *et al.* 1992), or as tools to help us understand pituitary-adrenal function, that can often be dissociated in disease states, including sepsis (Marik 2009).

5.1.3 Models of pituitary function

A pituitary tumour from a LAF1 mouse gave rise to the corticotroph-like AtT20 D16:16 line of cells. These were serially selected and cultured for their production of ACTH and extended growth as non-adherent aggregates (Buonassisi *et al.* 1962). They express prohormone convertase-1 (PC-1) to process POMC into vesicles that secrete ACTH and β -endorphin in response to the secretagogues CRH, VIP, phorbol ester, forskolin, IL-1 and IL-6 (Făgărăsan *et al.* 1989; Fukata *et al.* 1989). Ligands that have later been shown to activate Fpr/FPR subtypes, including VIP and PACAP, have been shown to simulate the folliculostellate-like TtT/GF cells (Inoue *et al.* 1992) to release IL-6, which is known to induce secretion of PRL, GH, LH, FSH (Spangelo *et al.* 1989; Yamaguchi *et al.* 1990), and ACTH (Naitoh *et al.* 1988). Inhibitors of ACTH secretion have also been studied in the AtT20 D16:16 cell line. For instance, ACTH release was inhibited from AtT20 D16:16 cells incubated with ANXA1 (Pompeo *et al.* 1997), or when co-cultured with the TtT/GF line and incubated with GCs (Tierney *et al.* 2003). This prompted the hypothesis that ANXA1 induced by GCs inhibited the secretion of ACTH through a paracrine mechanism.

One major drawback to experimental setups of cell lines relates to their neoplastic and typically aneuploidy origin, and are thus indicative of either previous or ongoing chromosomal instability and defects in molecular signalling pathways (Levy & Lightman 2003). Conversely, static incubation of dispersed anterior pituitary cells can provide a convenient way of analysing the effects of numerous ligands on pituitary function that closely recapitulates that of the native gland *in vivo* (Cowell *et al.* 1991; Gillies & Buckingham 1995). In this regard, the larger rat pituitary gland is favoured over the mouse, because the yield of cells per animal is correspondingly greater.

5.2 Methodology

Based on the above evidence, it was hypothesised that Fpr/FPR ligands will modulate pituitary function in basal or CRH-stimulated conditions. In order to test this hypothesis, intact rats were killed by decapitation and the anterior pituitary glands were pooled into Dulbecco's modified Eagle's medium (DMEM). Once dispersed, the pituitaries were then aliquotted into 24-well plates then cultured at 37 °C at 95% O₂/5% CO₂ for three days. The cells were then incubated for 4 hours of contact with fresh DMEM containing Fpr/FPR ligands and/or CRH, vehicle, or DMEM alone. The 100 nmol.l⁻¹ concentration of CRH was selected on the basis that it has been shown to have a submaximal, stimulatory effect on the release of ACTH from dispersed pituitary cells (Cowell *et al.* 1991). The Fpr/FPR ligands included agonists with different relative affinities for each Fpr/FPR subtype, as well as the Fpr2-/FPR2/ALX-selective antagonist, WRW₄ with a pIC₅₀ value between 6.6 and 6.9 for Fpr2/FPR2/ALX in mouse, rat, and human cells (Bae *et al.* 2004; Önnheim *et al.* 2008; Braun *et al.* 2011). The Fpr/FPR agonists tested were the Fpr1-/FPR1-selective agonist fMLF, the Fpr2-/FPR2/ALX-selective agonist WKYMVm, the Fpr2-/FPR3-selective F2L, the Fpr3/Alx-/FPR2/ALX-selective agonist 15-epi-LXA₄, and the non-selective Fpr agonist ANXA1_{Ac2-26}. The conditioned media were then removed and frozen at -20 °C, before being assayed for ACTH by radioimmunoassay (RIA).

5.3 Results

5.3.1 The effects of fMLF on ACTH release from dispersed rat anterior pituitary cells, in the presence and absence of CRH

fMLF is a selective agonist for human FPR1, mouse Fpr1, and rat Fpr1. Figure 5.1 shows the effects of graded concentrations ($10^{-9} - 10^{-5} \text{ mol.l}^{-1}$) of fMLF on the increases in ACTH release induced by a submaximal concentration of CRH ($10^{-7} \text{ mol.l}^{-1}$) from dispersed rat anterior pituitary cells *in vitro*.

CRH ($10^{-7} \text{ mol.l}^{-1}$) caused a significant increase in ACTH release ($*** = P < .001$ vs. drug-free). fMLF ($10^{-9} - 10^{-5} \text{ mol.l}^{-1}$) had no effect on basal ACTH release ($ns = P > .05$ vs. drug-free), or on the secretory responses to CRH, except at a concentration of $10^{-6} \text{ mol.l}^{-1}$ fMLF when a small but significant in basal ACTH release occurred ($* = P < .05$ vs. drug-free) and at $10^{-5} \text{ mol.l}^{-1}$ when the response to CRH was attenuated ($\dagger = P < .05$ vs. CRH alone).

In summary, fMLF had no effect on basal or CRH-stimulated ACTH release, except at $10^{-5} \text{ mol.l}^{-1}$ where fMLF inhibited CRH-stimulated ACTH release.

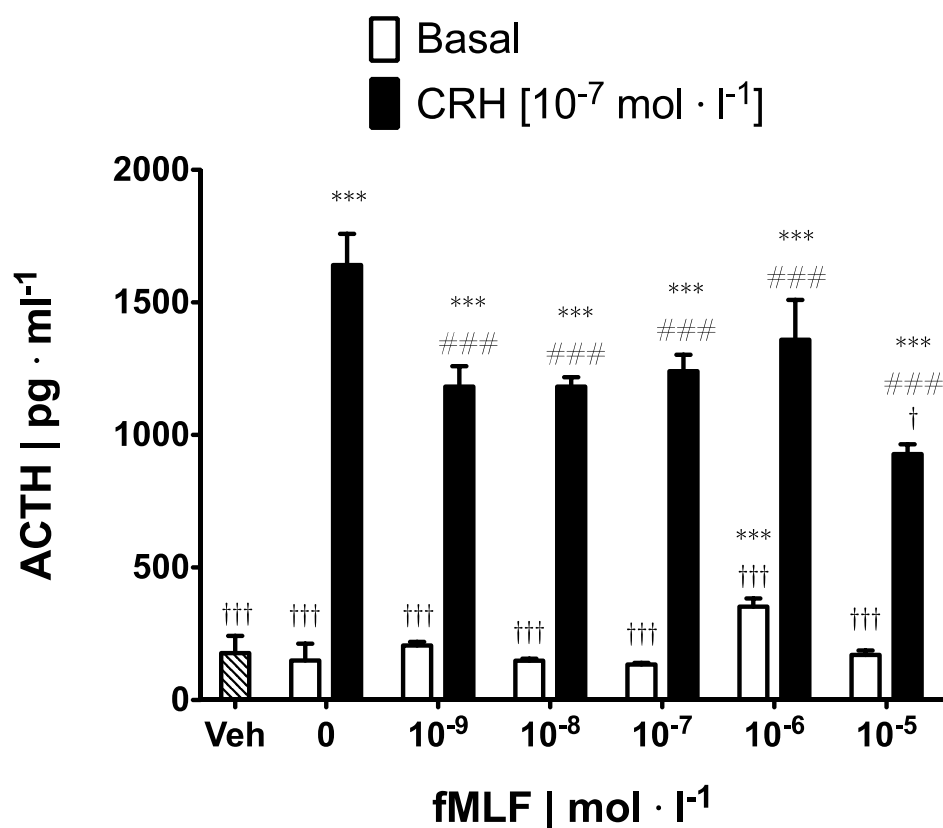


Figure 5.1: Effects of graded concentrations of the formyl peptide receptor 1 (FPR1)-selective agonist formyl-methionyl-leucyl-phenylalanine (fMLF) in the presence and absence of corticotrophic releasing hormone (CRH) on adrenocorticotrophic hormone (ACTH) release from dispersed anterior pituitary cells derived from male Sprague-Dawley rats.

The cells were incubated at 37 °C in 95% O₂/5% CO₂ for 4 h with the agonist fMLF (10⁻⁹ – 10⁻⁵ mol.l⁻¹), along with or without (basal) the secretagogue CRH (10⁻⁷ mol.l⁻¹). The vehicle (Veh) for fMLF was .025% acetate. Data are arithmetic mean ± standard error of mean of n = 6 replicate wells, compared by two-way ANOVA with Bonferroni correction for multiple comparisons: *** = P < .001, ** = P < .01, * = P < .05, vs. drug-free; ††† = P < .001, † = P < .05, vs. CRH alone; ### = P < .001 vs. fMLF alone.

5.3.2 The effects of WKYMVm on ACTH release from dispersed rat anterior pituitary cells, in the presence and absence of CRH

WKYMVm is a selective agonist for human FPR2/ALX, and mouse Fpr2, but with unknown activity via the rat Fpr subtypes. Figure 5.2 shows the effects of graded concentrations (10^{-10} – 10^{-6} mol.l⁻¹) of WKYMVm on the increases in ACTH release induced by a submaximal concentration of CRH (10^{-7} mol.l⁻¹) from dispersed rat anterior pituitary cells *in vitro*.

CRH (10^{-7} mol.l⁻¹) caused a significant increase in ACTH release (***) = $P < .001$ vs. drug-free). At all concentrations tested, WKYMVm stimulated basal ACTH release (***) = $P > .001$ vs. drug-free). No further increases in ACTH release were observed in the presence of WKYMVm when CRH was included in the medium.

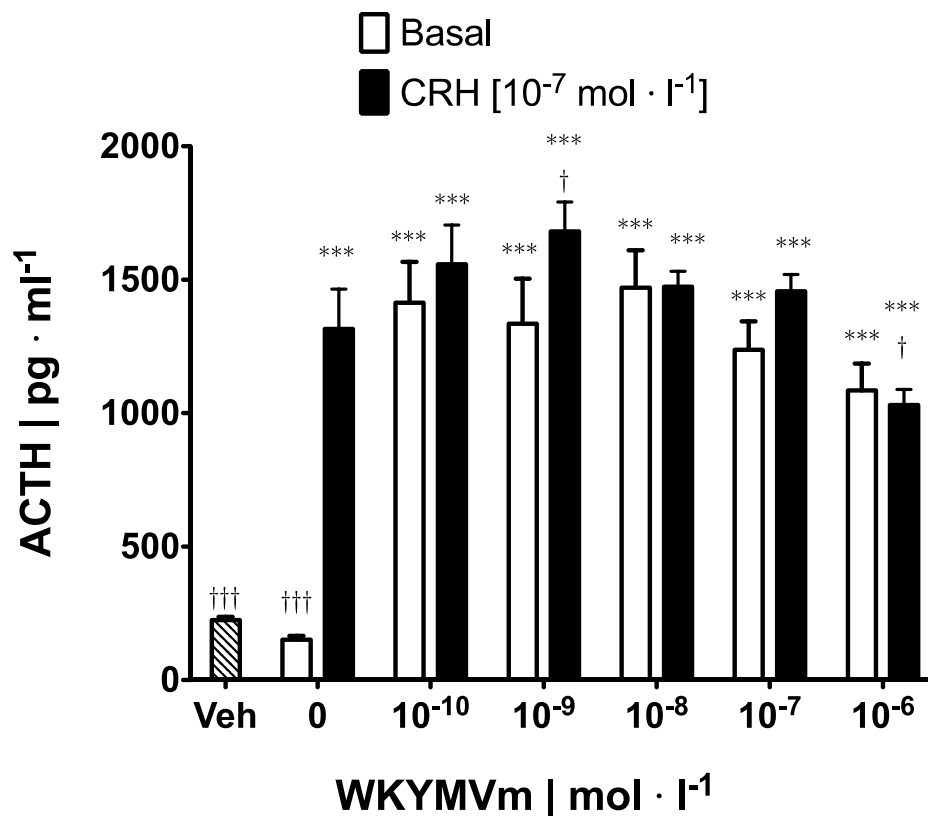


Figure 5.2: Effects of graded concentrations of the formyl peptide receptor 2 (FPR2/ALX)-selective agonist tryptophan-lysine-tyrosine-methionine-valine-*dextro*-methionine (WKYMVm) in the presence and absence of corticotrophic releasing hormone (CRH) on adrenocorticotrophic hormone (ACTH) release from dispersed anterior pituitary cells derived from male Sprague-Dawley rats.

The cells were incubated at 37 °C in 95% O₂/5% CO₂ for 4 h with the agonist WKYMVm (10⁻¹⁰ – 10⁻⁶ mol.l⁻¹), along with or without (basal) the secretagogue CRH (10⁻⁷ mol.l⁻¹). The vehicle (Veh) for WKYMVm was .025% acetate. Data are arithmetic mean ± standard error of mean of n = 6 replicate wells, compared by two-way ANOVA with Bonferroni correction for multiple comparisons: *** = P < .001, vs. drug-free; ††† = P < .001, † = P < .05, vs. CRH alone.

5.3.3 The effects of F2L on ACTH release from dispersed rat anterior pituitary cells, in the presence and absence of CRH

F2L is a selective agonist for human FPR3 and mouse Fpr2 derived from an amino-acetylated haem-binding protein fragment. Figure 5.3 shows the effects of graded concentrations ($0.5 \times 10^{-7} - 8 \times 10^{-7}$ mol.l⁻¹) of F2L on the increases in ACTH release induced by a submaximal concentration of CRH (10^{-7} mol.l⁻¹) from dispersed rat anterior pituitary cells *in vitro*.

CRH (10^{-7} mol.l⁻¹) caused a significant increase in ACTH release (***) = $P < .001$ vs. drug-free). At all concentrations tested, F2L increased basal ACTH release (***) = $P > .001$ vs. drug-free). However, at the lower concentrations tested ($0.5 \times 10^{-7} - 4 \times 10^{-7}$ mol.l⁻¹), F2L slightly reduced the CRH-induced increment in ACTH release, but the trend was not significant (ns = $P > .05$). At the highest concentration of F2L tested (8×10^{-7} mol.l⁻¹), co-incubation of F2L with CRH stimulated the release of ACTH to a level greater than that induced by CRH alone († = $P < .05$ vs. CRH alone).

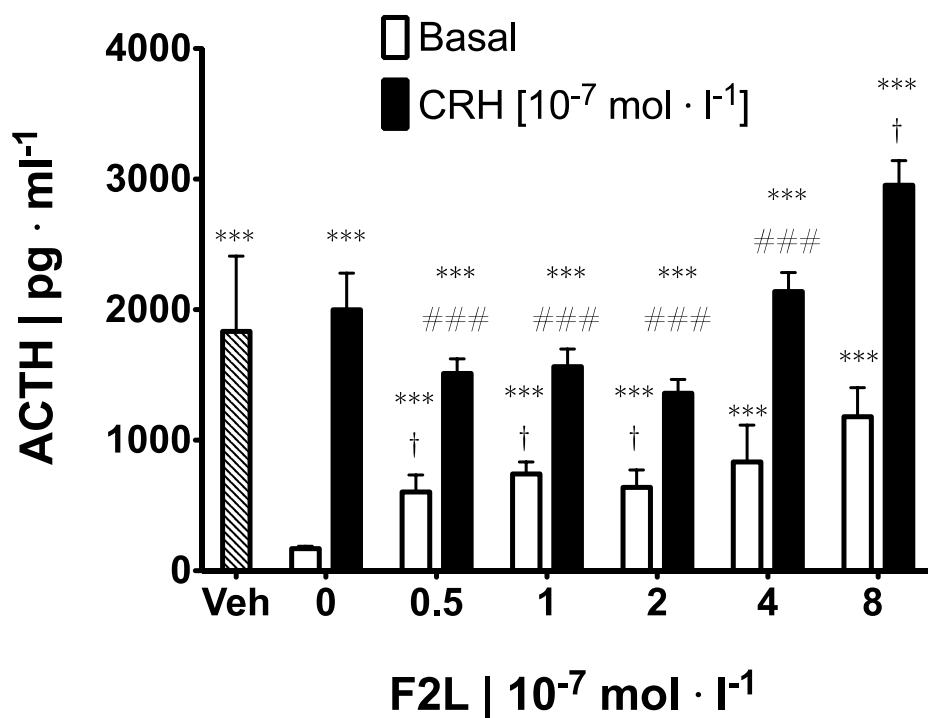


Figure 5.3: Effects of graded concentrations of the formyl peptide receptor 3 (FPR3)-selective agonist amino-acetylated haem-binding protein fragment (F2L) in the presence and absence of corticotrophic releasing hormone (CRH) on adrenocorticotrophic hormone (ACTH) release from dispersed anterior pituitary cells derived from male Sprague-Dawley rats.

The cells were incubated at 37 °C in 95% O₂/5% CO₂ for 4 h with the agonist F2L (0.5 x 10⁻⁷ – 8 x 10⁻⁷ mol.l⁻¹), along with or without (basal) the secretagogue CRH (10⁻⁷ mol.l⁻¹). The vehicle (Veh) for F2L was .4% dimethyl sulphoxide (DMSO). Data are arithmetic mean ± standard error of mean of n = 6 replicate wells, compared by two-way ANOVA with Bonferroni correction for multiple comparisons: *** = P < .001, vs. drug-free; † = P < .05, vs. CRH alone; ### = P < .001 vs. F2L alone.

5.3.4 The effects of 15-epi-LXA₄ on ACTH release from dispersed rat anterior pituitary cells, in the presence and absence of CRH

15-epi-lipoxin A₄ (15-epi-LXA₄) is a selective agonist for human FPR2/ALX, mouse Fpr3/Alx, and rat Fpr2l/Alx. Figure 5.4 shows the effects of graded concentrations (10^{-10} – 10^{-7} mol.l⁻¹) of 15-epi-LXA₄ on the increases in ACTH release induced by a submaximal concentration of CRH (10^{-7} mol.l⁻¹) from dispersed rat adrenal cells *in vitro*.

CRH (10^{-7} mol.l⁻¹) caused a significant increase in ACTH release (***) = $P < .001$ vs. drug-free). At the lower concentrations tested (10^{-10} and 10^{-9} mol.l⁻¹), 15-epi-LXA₄ caused a significant two-fold increase in basal ACTH release (***) = $P < .001$ vs. drug-free) but did not cause a further increase in ACTH release in the presence of CRH. By contrast, the two higher concentrations of 15-epi-LXA₄ (10^{-8} and 10^{-7} mol.l⁻¹) had no effect on basal ACTH release but significantly reduced the response to CRH (††† = $P < .001$).

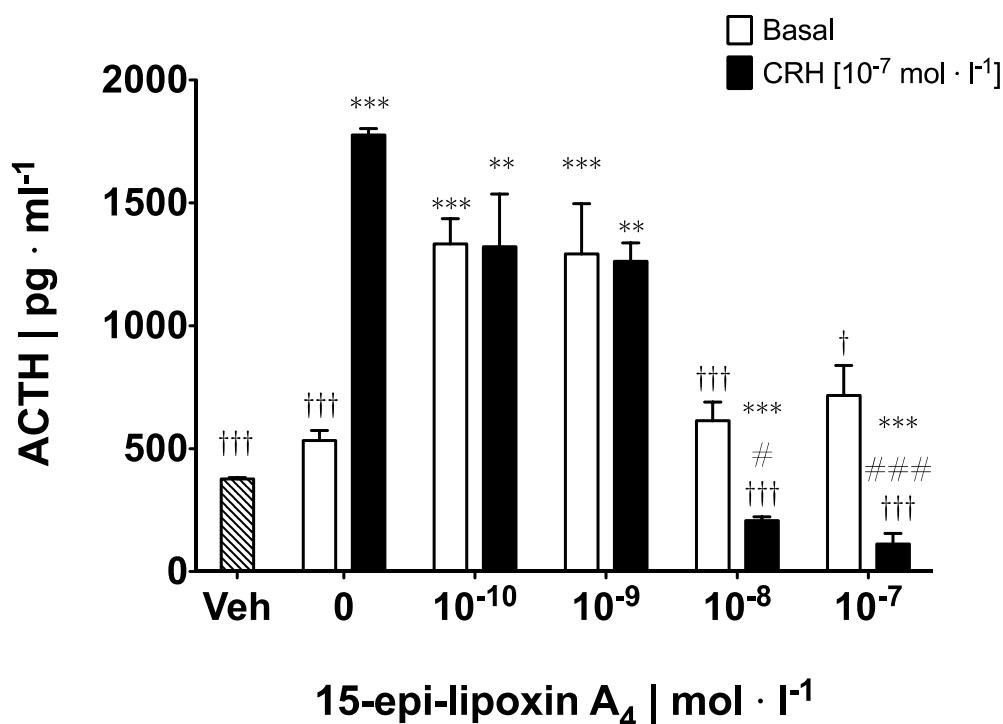


Figure 5.4: Effects of graded concentrations of the formyl peptide receptor 2 (FPR2/ALX)-selective agonist 15-epi-lipoxin A₄ (15-epi-LXA₄) in the presence and absence of corticotrophic releasing hormone (CRH) on adrenocorticotrophic hormone (ACTH) release from dispersed anterior pituitary cells derived from male Wistar rats.

The cells were incubated at 37 °C in 95% O₂/5% CO₂ for 4 h with the agonist 15-epi-LXA₄ (10⁻¹⁰ – 10⁻⁷ mol.l⁻¹), along with or without (basal) the secretagogue CRH (10⁻⁷ mol.l⁻¹). The vehicle (Veh) for 15-epi-LXA₄ was .014% ethanol. Data are arithmetic mean ± standard error of mean of n = 6 replicate wells, compared by two-way ANOVA with Bonferroni correction for multiple comparisons: *** = P < .001, ** = P < .01, vs. drug-free; ††† = P < .001, † = P < .05, vs. CRH alone; ### = P < .001, # = P < .05, vs. 15-epi-LXA₄ alone.

5.3.5 The effects of ANXA1_{Ac2-26} on ACTH release from dispersed rat anterior pituitary cells, in the presence and absence of CRH and/or WRW₄

Annexin A1 acetylated peptide (ANXA1_{Ac2-26}) is a non-selective agonist of the mouse and human Fpr/FPR subtypes, but so far is only known to bind to Fpr2 of the rat subtypes. Figure 5.5 shows the effects of graded concentrations (3×10^{-7} , 3×10^{-6} , and 3×10^{-5} mol.l⁻¹) of ANXA1_{Ac2-26} on the increases in ACTH release induced by a submaximal concentration of CRH (10^{-7} mol.l⁻¹), in the presence and absence of the selective Fpr2 antagonist WRW₄ (10^{-6} mol.l⁻¹), from dispersed rat anterior pituitary cells *in vitro*.

CRH (10^{-7} mol.l⁻¹) had no effect on ACTH release (ns = $P < .05$ vs. drug-free). At all concentrations tested, ANXA1_{Ac2-26} had no effect on basal ACTH release (ns = $P > .05$ vs. drug-free). Co-incubation of CRH with ANXA1_{Ac2-26} also had no effect on the release of ACTH, except for a slight increase at 3×10^{-6} mol.l⁻¹ ANXA1_{Ac2-26} (* = $P < .05$ vs. drug-free). Co-incubation of WRW₄ (10^{-6} mol.l⁻¹) with ANXA1_{Ac2-26} inhibited the release of ACTH (* = $P < .05$ vs. drug-free) at all but the highest concentration of ANXA1_{Ac2-26} (ns = $P > .05$ vs. drug-free). This inhibition of ACTH release was reversed when ANXA1_{Ac2-26} was co-incubated with WRW₄ and CRH to levels equivalent to basal (ns = $P > .05$).

In summary, micromolar ANXA1_{Ac2-26} inhibited ACTH release but only when co-incubated with WRW₄. This effect was reversed when ANXA1_{Ac2-26} was co-incubated with both WRW₄ and CRH, and lost altogether at ten micromolar ANXA1_{Ac2-26}.

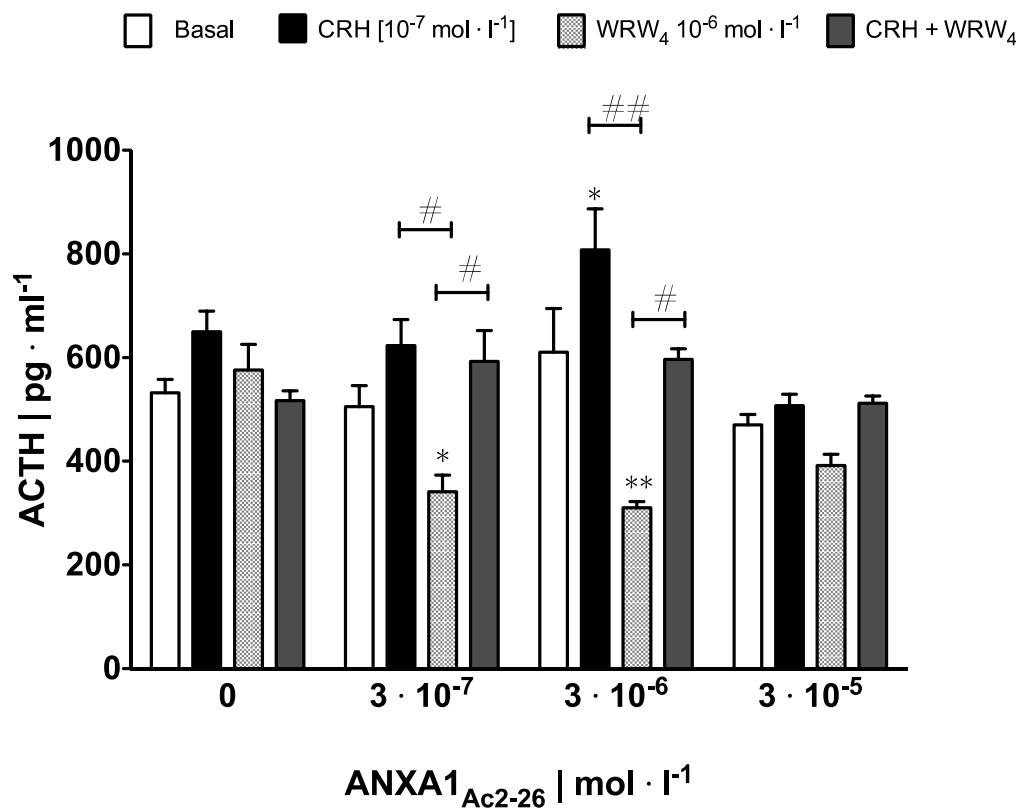


Figure 5.5: Effects of graded concentrations of the non-selective formyl peptide receptor (FPR) annexin A1 amino-acetylated peptide 2-26 (ANXA1_{Ac2-26}) in the presence and absence of corticotrophic releasing hormone (CRH) and/or the FPR2/ALX-selective antagonist Trp-Arg-Trp-Trp-Trp-Trp (WRW₄) on adrenocorticotrophic hormone (ACTH) release from dispersed anterior pituitary cells derived from male Wistar rats.

The cells were incubated at 37 °C in 95% O₂/5% CO₂ for 4 h with the agonist ANXA1_{Ac2-26} (3 x 10⁻⁷ – 3 x 10⁻⁵ mol.l⁻¹), along with or without (basal) the secretagogue CRH (10⁻⁷ mol.l⁻¹) and/or the antagonist WRW₄ (10⁻⁶ mol.l⁻¹). Data are arithmetic mean ± standard error of mean of n = 8 replicate wells, compared by three-way ANOVA with Bonferroni correction for multiple comparisons: ** = P < .01, * = P < .05, vs. drug-free; ## = P < .01, # = P < .05, vs. ANXA1_{Ac2-26} ± CRH ± WRW₄.

5.4 Discussion

The study of Fpr/FPR ligands and their effect on the release of ACTH from dispersed anterior pituitary cells led to several observations which are discussed below in terms of: the relative affinity of the ligands for both human (FPR) and rodent (Fpr) receptors; the evidence of their effects via high affinity receptor subtypes; and the literature on effects mediated via receptors with lower affinity.

To the author's knowledge, this is the first time that dispersed anterior pituitary cells from rats have been incubated with known Fpr/FPR ligands to investigate whether they modulate ACTH secretion in basal and CRH-stimulated conditions. Of the ligands tested, only F2L, 15-*epi*-LXA₄ and ANXA1_{Ac2-26} are endogenous mammalian Fpr/FPR ligands, whereas WKYMVm is a synthetic peptide agonist, and fMLF is a bacterial chemotactic peptide and the prototypical Fpr1/FPR1 ligand.

5.4.1 fMLF

fMLF is a selective agonist for the rat Fpr1, with a pEC₅₀ of 7.5 (Marasco *et al.* 1983). The lack of effect of fMLF on the release of ACTH from dispersed rat anterior pituitary cells suggests that Fpr1 is not involved in the modulation of ACTH release. This is consistent with evidence from slice cultures of anterior pituitaries, where neither Fpr1 gene deletion or sub-micromolar fMLF had any effect on ACTH release, and there was no evidence for Fpr1 expression (John *et al.* 2007). The lower range of concentrations of fMLF used here have been shown to activate the rat Fpr1 in neutrophils (Marasco *et al.* 1983), but these had no significant effect on the increase in ACTH release induced by CRH here, or by forskolin previously (John *et al.* 2007). Whilst fMLF does bind to receptors other than Fpr1 in rodents and FPR1 in man, these receptors have much lower affinity for fMLF. The small, but significant reduction in CRH-stimulated ACTH release elicited by fMLF at the ten micromolar concentration could be due to the activation of a rat Fpr subtype with lower affinity, possibly Fpr2 or Fpr3. This is comparable to the binding of supra-micromolar concentrations of fMLF to lower affinity receptors in other species, such as human FPR2/ALX (Fiore & Serhan 1995) or mouse Fpr2 (He *et al.* 2000) where fMLF has a similar affinity at each receptor, with a pK_d/pEC₅₀ of 5.3.

The lack of effect of fMLF at Fpr1-relevant concentrations can be further explained with the existing knowledge of its pharmacology. Firstly, compared with other ligands, the evidence for the relative affinity

of fMLF for human FPR1 is clear, surpassing other ligands such as cathepsin G and annexin I (Le *et al.* 2002; Sun *et al.* 2004a). This contrasts with the affinity of fMLF for human FPR2/ALX, where fMLF has perhaps the lowest known affinity of all the agonists this receptor binds, and has a pK_d of 7.65 (Koo *et al.* 1982). In systems involving the mouse Fpr1, there are some data that show fMLF elicits responses via mouse Fpr1 with a pEC_{50} of 9, and activates Fpr2 with 40 times lower potency, having a pEC_{50} of 5 (Mills *et al.* 2000). To date, functional responses of rat Fpr2 or Fpr3 to fMLF have yet to be reported, but the structurally similar mouse Fpr3 has been shown to not bind fMLF even at $100 \mu\text{mol.l}^{-1}$ concentrations.

Secondly, much of the functional data for fMLF is from studies of its chemotactic effects downstream of Fpr1, particularly within leukocytes. In this case, fMLF is associated with NF κ B-mediated transcription of chemokines and endoplasmic reticular Ca^{2+} release via G β -mediated up-regulation of phospholipase C- β (PLC- β) and activation of protein kinase C (PKC) (Naccache *et al.* 1979). This occurs alongside cytoskeletal actin reorganisation which follows the G γ -mediated up-regulation of phosphoinositide 3-kinase (PI3K) activity (Homma *et al.* 1985). In addition, fMLF upregulates superoxide anion production and ultimately cell degranulation in mouse neutrophils (Gao & Murphy 1993; Pivot-Pajot *et al.* 2010). If Fpr1 were present within the rat pituitary, such signalling mechanisms and Ca^{2+} mobilisation would be expected to facilitate an increase in ACTH secretion.

Finally, the remaining literature on fMLF is for its effects via receptors other than Fpr1. For instance, fMLF elicits responses via Fpr2 at supra-micromolar concentrations, where it has a pEC_{50} of 5 (Mills *et al.* 2000). Studies of HEK293 cells transfected with mouse Fpr3, in contrast with Fpr1 and Fpr2, have confirmed that fMLF does not elicit Ca^{2+} mobilisation via Fpr3, even at $100 \mu\text{mol.l}^{-1}$ when co-incubated with the Fura-2AM fluorescent Ca^{2+} indicator (Hartt *et al.* 1999; Liang *et al.* 2000). Given the nucleotide sequence homology of mouse Fpr3 and rat Fpr3, these findings suggest that rat Fpr3 is unlikely to be mediating the inhibitory effect of fMLF at the ten micromolar concentration.

Taken together, the lack of an overall inhibitory effect of fMLF on ACTH release in the present studies, at concentrations known to stimulate Fpr1 in leukocytes, reaffirms the existing evidence that Fpr1 is not expressed within the pituitary, or involved in the regulation of ACTH secretion (John *et al.* 2007). The prototypical Fpr1 ligand does not mediate effects via mouse Fpr3 at supra-micromolar concentrations (Hartt *et al.* 1999; Liang *et al.* 2000), therefore, the inhibition of ACTH release at such concentrations is

likely to be mediated via a lower affinity receptor such as Fpr2, whose transcripts have been found within the mouse and rat pituitary (John *et al.* 2007).

5.4.2 WKYMVm

The data indicate that WKYMVm has pharmacological effects on the release of ACTH from primary dispersed anterior pituitary cells. At all concentrations tested, WKYMVm was associated with significantly upregulated basal ACTH release versus the untreated control. The effect of WKYMVm on ACTH release was similar both in the presence and absence of the secretagogue CRH. At micromolar WKYMVm, there was a slight decrease in ACTH release, even when co-incubated with CRH. To the author's knowledge, this is the first report of WKYMVm-mediated modulation of ACTH release from dispersed anterior pituitary cells.

WKYMVm is a synthetic hexapeptide originally identified from a peptide library. It is different from WKYMVM, structurally, because of the *dextro*-methionine about a C-terminal amide, and functionally with respect to its affinity for FPR/Fpr subtypes, particularly in leukocytes. Human neutrophils express FPR1 and FPR2/ALX, whereas monocytes express FPR1, FPR2/ALX, and FPR3 (Ye *et al.* 2009). Literature concerning the relative affinity of WKYMVm is largely based on its effects via human FPR2/ALX in neutrophils with a pEC₅₀ of 10.12 (Migeotte *et al.* 2005), and via human FPR3 in monocytes, where it acts with a pEC₅₀ of 8.52 (Christophe *et al.* 2001). However, WKYMVm also has some effects via FPR1 with a pEC₅₀ of 7.60 (Le *et al.* 1999).

Initial characterisation of the effects of WKYMVm on mouse neutrophils, suggested the agonist upregulated lytic granule release via Fpr1 with a pEC₅₀ of 8.82 (He *et al.* 2000), with activity at Fpr2 or Fpr3 with a pEC₅₀ of 7.48 (Boxio *et al.* 2005). The relative affinities of WKYMVm with the major mouse Fpr subtypes have since been updated to Fpr1 > Fpr2 >>> Fpr3, with respective pEC₅₀ values of 10.1, 9.3, and approximately 6 (He *et al.* 2013a). In terms of mechanistic details, the mobilisation of Ca²⁺ appears to be important in eliciting the pro-exocytotic effects of WKYMVm. The incubation of micromolar WKYMVm with HL-60 cells overexpressing Fpr2, for instance, readily permits the detection of Ca²⁺ with the Fluo3 tracer versus vehicle-treated cells (Prat *et al.* 2006). An alternative approach to demonstrating the role of Ca²⁺ involves its chelation with the compound BAPTA (1,2-bis(o-aminophenoxy)ethane-

N,N,N',N'-tetraacetic acid), and then indexing the exocytosis of lytic granules by a β -hexosaminidase assay. Using this approach in the rat basophilic leukaemia cell line (RBL-2H3) expressing human FPR2/ALX, resulted in a blockade of the pro-exocytotic effect of WKYMVm (Gomperts 1990; Bae *et al.* 2003b).

In the context of the present pro-exocytotic effects of WKYMVm on ACTH release, there are several strands of evidence from the literature on signalling cascades downstream of Fpr/FPR that corroborate these findings. There is evidence for the role of Ca^{2+} mobilisation within the corticotrophs (Gomperts 1990) and also the phosphorylation of extracellular regulated kinase (ERK) (Sullivan *et al.* 2005), with each of these associated with an increased secretion of ACTH (Leong 1988). The incubation of WKYMVm in wildtype macrophages has been found to increase the concentration of phosphorylated ERK (Kang *et al.* 2005). Importantly, the phosphorylation of ERK has been shown to be significantly reduced in leukocytes obtained from Fpr2/3^{-/-} mice versus wildtypes (Dufton *et al.* 2010), but it was not possible to conclude whether Fpr2 or Fpr3 has a greater contribution to this effect. This also applies to the experiments of Boxio and coworkers (2005), who showed the pro-exocytotic effect of WKYMVm on neutrophil granule release is halved by the human FPR2/ALX antagonist C2 (WPLTHTLRHTIW, 10^{-5} mol.l⁻¹), but could not stipulate whether this was Fpr2 or Fpr3-mediated. Given the findings of a pro-exocytotic effect of WKYMVm, here on ACTH secretion, and elsewhere on lytic granule release via human FPR2/ALX, along with the loss of effects following blockade or knockout of Fpr2 and Fpr3, it seems plausible to suggest that the WKYMVm-stimulated release of ACTH is mediated via Fpr2 or Fpr3, or perhaps both, since these are expressed within mouse and rat pituitary, where Fpr1 is not (John *et al.* 2007).

5.4.3 F2L

These data show F2L—an acetylated 21 amino acid oligopeptide derived from the amino terminal end of the intracellular haem-binding protein (Migeotte *et al.* 2005)—upregulates ACTH release from dispersed rat anterior pituitary cells when co-incubated with CRH, versus the untreated control cells. To the author's knowledge, this is the first report of F2L-mediated stimulation of ACTH release from dispersed anterior pituitary cells. The vehicle treatment of 2.5% DMSO, however, also had a stimulatory effect on ACTH release, which was roughly equal to the effect of CRH treatment. F2L was inhibitory, however,

when used at a low concentration ($0.5 \times 10^{-7} - 2 \times 10^{-7} \text{ mol.l}^{-1}$) versus the vehicle group. This inhibitory effect of F2L versus vehicle was reduced by higher concentrations of F2L ($4 \times 10^{-7} - 8 \times 10^{-7} \text{ mol.l}^{-1}$) and reversed when co-incubated with CRH at the top concentration of F2L ($8 \times 10^{-7} \text{ mol.l}^{-1}$).

The stimulatory effect of F2L on ACTH secretion observed here might be explained in the context of existing pharmacological data. Early experiments with F2L identified it as the first endogenous ligand with both potent and selective chemotactic actions via human FPR3 with a $\text{pK}_d/\text{pEC}_{50}$ of 8.2, and only weak activity at FPR2/ALX with a $\text{pK}_d/\text{pEC}_{50}$ of 6.3, and none via FPR1 (Migeotte *et al.* 2005). High concentrations (above 10 micromolar) of the Fpr2-/FPR2/ALX-selective antagonist WRW_4 have been shown to also antagonise FPR3 (Bae *et al.* 2004). Blockade of FPR3, the high affinity receptor for F2L, with WRW_4 has been shown to inhibit completely the F2L-induced influx of Ca^{2+} and its activation of $\text{NF}\kappa\text{B}$ (Shin *et al.* 2006). WRW_4 is also a selective antagonist of mouse Fpr2 (Önnheim *et al.* 2008) and rat Fpr2 (Braun *et al.* 2011), and have broadly similar pIC_{50} values, 6.6 and 6.9 respectively. Taken together, the present data do not refute the observation that F2L is a pure agonist of mouse Fpr2 in the nanomolar range with a pEC_{50} of 6.70, from a study that confirmed F2L has negligible binding at mouse Fpr1 or Fpr3 (Gao *et al.* 2007). Thus, the pEC_{50} of F2L via mouse Fpr2 informed the choice of the central concentration in the present study ($10^{-6.7} = 2 \times 10^{-7} \text{ mol.l}^{-1}$), as there are no published data on the effects of F2L via the rat Fpr subtypes.

The molecular mediators known to be involved in CRH-stimulated ACTH secretion are concordant with those invoked downstream of Fpr/FPR subtypes following activation by F2L. These include, for instance, the increased cytosolic concentration of Ca^{2+} , cAMP, pERK1/2, and pMAPK, each of which is invoked by F2L via human FPR3 (Kang *et al.* 2005; Migeotte *et al.* 2005), via mouse Fpr2 (Gao *et al.* 2007), as well as by CRH via CRH-R1 in corticotrophs. The mechanisms induced by F2L acting via human FPR3, and via mouse Fpr2, are therefore consistent with secretagogues of ACTH such as CRH, and might therefore explain the observed stimulation of ACTH secretion by F2L.

5.4.4 15-*epi*-LXA₄

Unlike other leukotrienes and prostaglandins, the lipoxins are a class of eicosanoid that downregulate leukocyte recruitment and are anti-inflammatory (Gewirtz *et al.* 1999). Lipoxin A₄ (LXA₄) is a metabolite

derived from a double lipoxygenation of arachidonic acid (Serhan & Chiang 2008). In addition to its potent anti-inflammatory effects, LXA₄ has also been shown to inhibit ACTH secretion, ostensibly via rat Fpr2 or Fpr3 (John *et al.* 2007). The present results show that the 15-epimeric isomer of LXA₄, 15-epi-LXA₄ suppressed both basal and CRH-stimulated ACTH release. To the author's knowledge, this is the first time that 15-epi-LXA₄ has been shown to inhibit ACTH release from dispersed anterior pituitary cells from rats.

LXA₄ and 15-epi-LXA₄ are each biosynthesised from arachidonic acid derivatives by 5-, 12-, or 15-lipoxygenase (LOX), except that the synthesis of 15-epi-LXA₄ also requires the acetylation of cyclooxygenase 2 (COX-2) by aspirin (Clària & Serhan 1995; Gewirtz *et al.* 1999). Although the lipoxins have not been directly measured within the pituitary, there is evidence of expression of these biosynthetic enzymes (Cover *et al.* 2001), and the major precursors 5-, 12-, and 15-hydroxyeicosatetraenoic acid are detectable within the tissue (Pilote *et al.* 1982). Functional evidence for the endogenous role of the LOX enzymes on pituitary ACTH release comes from studies that inhibit the enzymes or test the effect of their metabolites. Incubation of dispersed rat pituitary cells with the lipoxygenase inhibitor AA861 resulted in a reduction in secretagogue-stimulated ACTH release (Cowell *et al.* 1991), whereas the LXA₄ precursor 15(S)-hydroxyeicosatetraenoic acid had a stimulatory effect on ACTH release (Won & Orth 1994).

Previous work from this laboratory has shown that low, sub-micromolar concentrations of LXA₄ inhibited ACTH release from rat pituitary segments in forskolin-stimulated, but not basal conditions (John *et al.* 2007). This inhibitory effect of LXA₄ was comparable to the effect of the synthetic GC, dexamethasone. The anti-inflammatory effects of 15-epi-LXA₄ have also been shown to be similar in potency to dexamethasone (Takano *et al.* 1997), and occur along with an upregulation of human FPR2/ALX (Hashimoto *et al.* 2007). Aspirin-triggered lipoxin, 15-epi-LXA₄ is ten times more potent an agonist for human FPR2/ALX with a pEC₅₀ of 10.10 (Maddox *et al.* 1997; Chiang *et al.* 2000) compared with a pEC₅₀ of 9.10 for LXA₄ (Maddox *et al.* 1997; Chiang *et al.* 2000), which is also less stable⁶ (Serhan *et al.* 1995). In mice, the anti-inflammatory effects of these lipoxins are mediated via Fpr3/Alx, and have equal affinity with a pK_d of 8.82 (Takano *et al.* 1997), and their affinity for rat Fpr2l/Alx is similar with a pK_d

⁶ Due to 15-hydroxy prostaglandin dehydrogenase activity.

of 8.30 (Chiang *et al.* 2003). The similar affinities of the lipoxins for the mouse and rat Fpr subtypes is perhaps explained by the 84% sequence homology of these rodent receptors, compared with 74% homology to the higher affinity human FPR2/ALX (Ye *et al.* 2009).

5.4.5 ANXA1_{Ac2-26}

Here, ANXA1_{Ac2-26} inhibited ACTH release at the micromolar level but only when co-incubated with WRW₄. This effect was reversed when ANXA1_{Ac2-26} was co-incubated with both WRW₄ and CRH, and lost altogether at $3 \times 10^{-5} \text{ mol.l}^{-1}$ ANXA1_{Ac2-26}. This suggests that ANXA1_{Ac2-26} is not stimulatory when a WRW₄-sensitive receptor is blocked, ostensibly the rat Fpr2, and so could exert inhibitory effects via Fpr3 or Fpr1. This could mean that the inhibitory effect of ANXA1_{Ac2-26} on pituitary ACTH release is conditional on Fpr2 blockade.

This finding is partly consistent with previous studies from this lab which demonstrated a role for ANXA1 as a mediator of the negative feedback effects of glucocorticoids on hormone release (Taylor *et al.* 1993; John *et al.* 2002; 2007). Evidence was also found for ANXA1 binding sites on the endocrine cells of the anterior pituitary, and thus gave credence to the hypothesised paracrine/juxtacrine mode of ANXA1 action (Christian *et al.* 1997). Walther and coworkers (2000) identified human FPR1 as the first receptor target of ANXA1 action on leukocytes. When the FPR2/ALX and FPR3 subtypes were also identified in human tissues, their functional relationship with ANXA1 seemed to be more significant than FPR1 (Perretti *et al.* 2002; Gavins *et al.* 2003; Ernst *et al.* 2004b; Hayhoe *et al.* 2006).

Taken together, the present observations do not refute those made previously of ANXA1 or ANXA1_{Ac2-26}-mediated inhibition on secretagogue-stimulated ACTH release. Given that the CO₂ regulator failed during this experiment however, the current data should be interpreted with caution until the experiment can be repeated.

5.4.6 Biological significance and scope of findings

The present data militate against a role for the rat equivalent of mouse Fpr1 and human FPR1, as a target for ANXA1 action within the anterior pituitary gland. fMLF had no effect on basal or CRH-stimulated ACTH release at the sub-micromolar concentrations at which rat Fpr1 is selectively bound (Marasco *et al.* 1983). This is in accord with previous data from this lab, where the inhibitory effects of ANXA1_{Ac2-26}

were unaffected by either the genetic knockout of Fpr1, or pharmacological blockade of Fpr1 with the human FPR1/mouse Fpr1 antagonist Boc1 used at $50 \mu\text{mol.l}^{-1}$ (John *et al.* 2007). This suggests that the rat Fpr1 is functionally, as well as structurally, equivalent to mouse Fpr1 and human FPR1.

Further support for this is evident in that while ANXA1 suppressed ACTH release (Taylor *et al.* 1993; John *et al.* 2007) it did not bind to FPR1 in human systems (Perretti *et al.* 2002) and FPR1/Fpr1 is not expressed within the anterior pituitary gland from humans (Becker *et al.* 1998) mice, or rats (John *et al.* 2007). As for other Fpr subtypes, this group found evidence of Fpr2 and Fpr3 expression within the rat pituitary, and the mouse pituitary also contained the subtypes Fpr2, Fpr3, Fpr-rs6, and Fpr-rs7 (John *et al.* 2007). Rat Fpr2l/Alx has been described as the orthologue of human FPR2/ALX (Chiang *et al.* 2003), which mediates the effects of LXA₄, 15-epi-LXA₄, ANXA1, and ANXA1_{Ac2-26} (Takano *et al.* 1997; Hartt *et al.* 1999; Wang & Ye 2002). The observation that the aspirin-triggered lipoxin, 15-epi-LXA₄, when used at $10^{-8} \text{ mol.l}^{-1}$ was able to inhibit the CRH-stimulated ACTH release is consistent with its affinity for mouse Fpr3/Alx (Takano *et al.* 1997) and rat Fpr2l/Alx (Chiang *et al.* 2003), and with the inhibition elicited by the less potent LXA₄ used at $2 \mu\text{mol.l}^{-1}$ (John *et al.* 2007).

The stimulated release of ACTH by CRH is dependent on an intact signalling mechanism downstream of CRH-R1 receptors on corticotrophs. First, production of cAMP activates protein kinase A, and this triggers an influx of Ca^{2+} via L-type Ca^{2+} channels which, results in membrane fusion of secretory vesicles and exocytosis of their ACTH (Aguilera *et al.* 1983; Taylor *et al.* 1993). Previous data have shown that the inhibition of ACTH release mediated by ANXA1 does not interfere with Ca^{2+} influx, and might instead involve a mechanism downstream of an FPR/Fpr subtype (Taylor *et al.* 1993; John *et al.* 2002). Indeed, activation of the FPR/Fpr subtypes coupled with heterotrimeric G_i proteins have resulted in reduced CRH-stimulated ACTH release in the corticotrophic AtT20 cell line (Aguilera *et al.* 1983; Luini & Matteis 1990); an effect that can be blocked by pertussis toxin (Le *et al.* 2002). The rest of the signalling pathway downstream of ANXA1/Fpr activation is unclear, but some evidence has shown that ANXA1 induces actin polymerisation beneath the cell membrane, blocking the exocytosis of lytic granules and hormones from leukocytes and corticotrophs, respectively (Castellino *et al.* 1992; Maderna *et al.* 2002; McArthur *et al.* 2009).

In the context of previous literature, the evidence above suggests that the effects of 15-*epi*-LXA₄, ANXA1_{Ac2-26}, WKYMVm, and F2L on the secretion of ACTH from the anterior pituitary might be mediated via rat Fpr2 or Fpr3. The Fpr2 and Fpr3 in mice and rats have 84% nucleotide sequence homology, and all share functional properties and structural homology with human FPR2/ALX and FPR3. However, there are conflicting reports in the literature as to whether Fpr2 or Fpr3 in these rodents is the functional orthologue of human FPR2/ALX, FPR3, or both (Hartt *et al.* 1999). This is partly because both mouse receptors are responsive to LXA₄ although Fpr2 requires G-alpha subunit 16 coupling (Vaughn *et al.* 2002) but Fpr3 has an ANRV inserted after Leu141 in transmembrane domain 4 which readily permits LXA₄ binding (Takano *et al.* 1997; Gao *et al.* 1998). Dufton and coworkers (2010) found that in the mouse air pouch model, Fpr2/3 ligands such as LXA₄ and ANXA1_{Ac2-26} reduced granulocyte migration whereas serum amyloid A (SAA) increased granulocyte recruitment, despite each having similar affinity for Fpr2. All of these effects were absent in Fpr2/3^{-/-} mice, suggesting that Fpr2 or Fpr3 mediate the opposing effects of these Fpr ligands *in vivo*. Therefore, it is possible that two separate Fpr subtypes exist to produce opposing effects on pituitary hormone secretion, with Fpr2 mediating a positive stimulatory effect and Fpr3 producing an inhibitory effect. Another possibility that is under ongoing research, is that these Fpr subtypes possess bidirectional functions that are ligand-dependent (Bae *et al.* 2003b; Brancaleone *et al.* 2011; Bena *et al.* 2012).

The vehicles for some of the drugs were perhaps used unnecessarily. The manufacturer's data sheets for the drugs mostly suggested the use of a diluent such as glacial acetic acid, DMSO, or ethanol. It may have been cogent to perform concentration-effect curves with some of these vehicles, particularly in the case of DMSO (for F2L), as this appeared to have a potent stimulatory effect on pituitary ACTH release. This is consistent with the finding that DMSO has been associated with stimulated ACTH release *in vivo* (Ni *et al.* 1995). Further experiments should either use an alternative diluent, or use a lower proportion of DMSO (1% total volume as per John *et al.* (John *et al.* 2007), rather than the 2.5% used here). There is no reason to believe that this effect results from a ligand-receptor interaction however, and the simplest explanation is likely to be non-specific cell death. Incidentally, the trypan blue exclusion test for cell death was only performed to count cells and as a check for their viability prior to the incubation with drugs. It would have been worthwhile checking cell viability both before and after the incubation of drugs.

The dispersed anterior pituitary cell culture was generated from a pool of multiple glands into a monolayer of cells. It is assumed that the process of filtration through nylon mesh and the dispersal facilitated by trypsin did not affect the proportion of each pituitary cell subtype. There is no reason to believe, for instance, that any fewer folliculostellate cells were in the monolayer than would be found within the native gland. It has been demonstrated, however, that the communication between the various cell subtypes depends to a great extent on the interaction of these cells within three-dimensional networks (Lafont *et al.* 2010). In this regard, and for further work, aggregate culture of dispersed anterior pituitary cells, or pituitary segments, would be favoured over the static incubation of dispersed cells used here.

5.4.7 Conclusion

These functional data have demonstrated that Fpr/FPR ligands are able to modulate the release of ACTH from dispersed anterior pituitary cells, with the following rank of efficacy: WKYMVm >>> 15-epi-LXA₄ > F2L >> fMLF > ANXA1_{Ac2-26}. The secretion of ACTH from dispersed rat anterior pituitary cells was stimulated by the mouse Fpr2-selective ligands, F2L (human FPR3-selective) and WKYMVm (human FPR2/ALX-selective), but inhibited by agonists with similar affinity for human FPR2/ALX and the rat Fpr2l/Alx, namely, ANXA1_{Ac2-26} and 15-epi-LXA₄. Given the slight inhibition of ACTH release induced by fMLF and WKYMVm at high concentrations associated with a loss of their Fpr1/Fpr2-selectivity, it seems plausible to suggest that these inhibitory effects might also be mediated by the rat Fpr2l/Alx.

**Chapter Six: Study of the effects of a formyl
peptide receptor ligand on pituitary and
adrenal function during experimental
endotoxaemia**

6.1 Introduction

Intraperitoneal injection of lipopolysaccharide (LPS) is a commonly used experimental model of sepsis-related endotoxaemia, and has been shown to induce changes in both the structure and function of the adrenal cortex, and there is some evidence that formyl peptide receptor (Fpr/FPR) subtypes and their ligands might be important in this regard. In wildtype mice, for instance, intraperitoneal administration of LPS leads to a reduction in adrenocortical sterol vacuolation and refractory responses to adrenocorticotrophic hormone 1-24 (ACTH₁₋₂₄) in dispersed adrenal cells (Buss 2010). This effect was limited however, in Fpr2/3^{-/-} mice, suggesting a role of Fpr2 and/or Fpr3 in the adrenal response to LPS (Buss 2010). Inflammogenic stressors such as LPS, have been shown to upregulate the expression of Fpr/FPR subtypes, particularly Fpr2/FPR2/ALX (Iribarren *et al.* 2003). In the preceding chapters, we have seen evidence that the Fpr2-/FPR2/ALX-selective antagonist Trp-Arg-Trp-Trp-Trp (WRW₄) was able to limit the inhibition of corticosterone secretion elicited by the bacterial Fpr/FPR ligand formyl-methionyl-leucyl-phenylalanine (fMLF) (see section 4.3.2). WRW₄ has a pIC₅₀ for FPR2/Fpr2 in human, mouse and rat cells of between 6.6 and 6.9 (Bae *et al.* 2004; Önnheim *et al.* 2008; Braun *et al.* 2011). Based on the above evidence however, it is not clear whether pharmacological blockade of the rat Fpr2 *in vivo* will modulate the response to LPS, in terms of the structure and function of the adrenal cortex.

6.2 Methodology

Based on the above evidence, it was hypothesised that: 1) the inflammogenic stressor LPS would reduce sterol vacuolation of the adrenal cortex within 24 hours; and 2) the Fpr2-/FPR2/ALX-selective antagonist, namely, WRW₄, would inhibit this effect. In order to test this hypothesis, groups of male Wistar rats were injected with either saline, or LPS (750 µg.kg⁻¹ i.p.) and then decapitated either immediately, or after 2, 4, or 24 hours, respectively (four groups), to collect the following tissues: brain, pituitary, thymus, and spleen (n = 4), and adrenal (n = 1) for histological analysis; and trunk blood for the assay of plasma corticosterone and ACTH concentrations (n = 4). The animals were injected and killed in a time course staggered over two weekdays to ensure the time of day for the injections and blood collection for each group was the same (12.30 pm), plus or minus thirty minutes. The remaining adrenals (n = 3) were dispersed in Earle's balanced salt solution (EBSS) and collagenase and aliquotted into 96-well plates prior to centrifugation, then equilibrated at 37 °C at 95% O₂/5% CO₂ for an hour. The cells were then incubated for 4 hours of contact with fresh EBSS containing graded concentrations of ACTH₁₋₂₄.

The above experiments were able to determine the approximate time at which LPS had maximal effects on adrenocortical sensitivity to ACTH₁₋₂₄ and sterol vacuolation. To determine whether the Fpr2-/FPR2/ALX-selective antagonist WRW₄ was able to inhibit these effects, male Wistar rats (n = 6) received intraperitoneal injections of either saline, LPS (750 µg.kg⁻¹), WRW₄ (250 µg.kg⁻¹), or LPS and WRW₄. After 4 hours, tissues were collected as above, except the dispersed adrenal experiments and the determination of plasma ACTH were not performed for this part of the study owing to time constraints.

6.3 Results

6.3.1 The effect of an intraperitoneal injection of LPS on the concentration of ACTH in plasma obtained from groups of male Wistar rats after 2, 4, or 24 hours

Figure 6.1 shows the concentration of ACTH in plasma obtained from groups of male Wistar rats injected intraperitoneally with either saline, or LPS ($750 \mu\text{g} \cdot \text{kg}^{-1}$ i.p.), and then decapitated immediately, or after 2, 4, or 24 hours, respectively.

The concentration of ACTH was significantly increased in plasma obtained from rats after 2 hours ($*** = P < .001$), and 4 hours ($** = P < .01$) following the administration of LPS, versus the saline control group. After 24 hours, the plasma ACTH concentration in rats injected with LPS was no different from the saline control group ($\text{ns} = P > .05$).

The highest concentration of ACTH observed was in plasma obtained from rats after 2 hours following LPS administration, and was significantly higher than from the four-hour group ($*** = P < .001$) and the twenty-four-hour group ($*** = P < .001$). Although the plasma ACTH concentration after 4 hours was lower than in the two-hour group ($*** = P < .001$ vs. LPS 2 h), it was still significantly greater than in the twenty-four-hour ($* = P < .05$) and saline control ($* = P < .05$) groups.

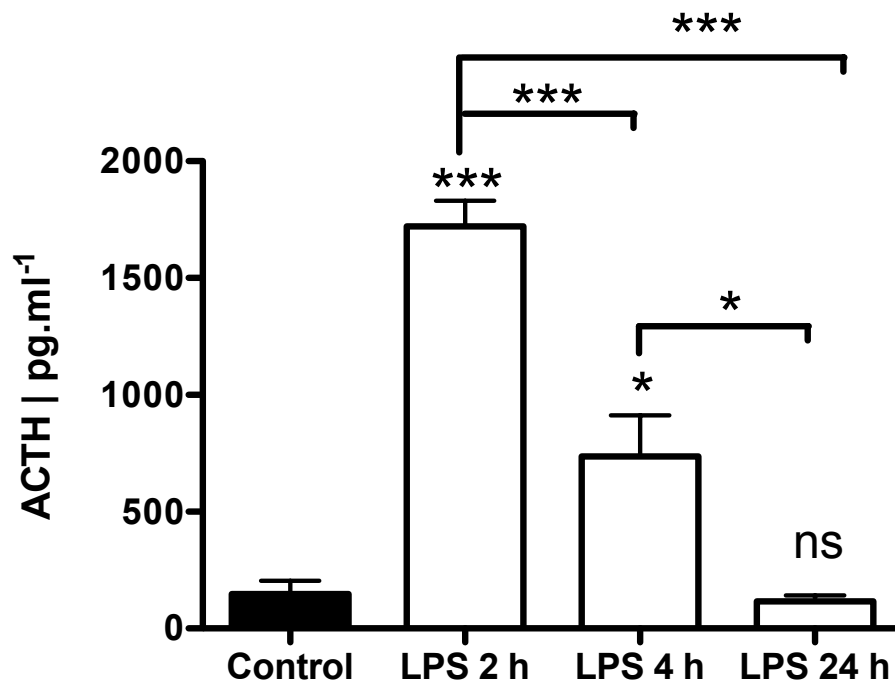


Figure 6.1: Effect of an intraperitoneal injection of lipopolysaccharide (LPS) on the concentration of adrenocorticotrophic hormone (ACTH) in plasma obtained from groups of male Wistar rats after 2, 4, or 24 h.

Male Wistar rats ($n = 4$) received an intraperitoneal (i.p.) injection of either saline (control), or LPS ($750 \mu\text{g.kg}^{-1}$ i.p.), and then decapitated immediately, or after 2, 4, or 24 h, respectively. Trunk blood was collected into tubes coated with ethylenediaminetetraacetate (EDTA) and centrifuged for 10 min at 3,000 rcf. Plasma samples were aliquotted, snap frozen in liquid nitrogen, and then stored at -80°C until the ACTH concentration was determined by immuno-radiometric assay (IRMA) at two sites on the ACTH epitope. Data are arithmetic mean $\pm$ the standard error of mean of $n = 4$ animals, compared by one-way ANOVA with Bonferroni tests for multiple comparisons: *** = $P < .001$, ** = $P < .01$, * = $P < .05$, ns = not significant, vs. control or as indicated by significance bars.

6.3.2 The effect of an intraperitoneal injection of LPS on the concentration of corticosterone in plasma obtained from groups of male Wistar rats after 2, 4, or 24 hours

Figure 6.2 shows the concentration of corticosterone in plasma obtained from groups of male Wistar rats injected intraperitoneally with either saline, or LPS ($750 \mu\text{g.kg}^{-1}$ i.p.), and then decapitated immediately, or after 2, 4, or 24 hours, respectively.

The concentration of corticosterone was not significantly different in plasma obtained from rats after two hours, four hours, or twenty-four hours following the administration of LPS, versus the saline control group ($\text{ns} = P > .05$). Despite this, there appears to be a downward trend from the highest plasma corticosterone concentration observed in rats two hours following LPS administration, towards the levels in the four-hour and twenty-four-hour groups.

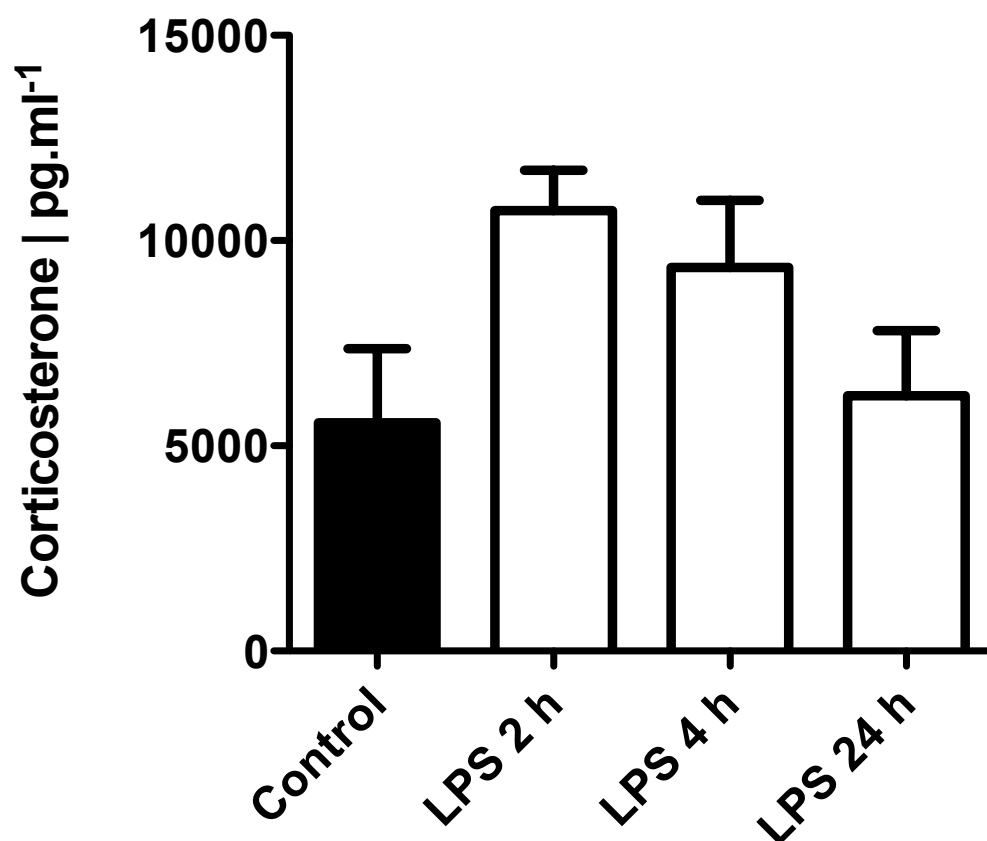


Figure 6.2: Effect of an intraperitoneal injection of lipopolysaccharide (LPS) on the concentration of corticosterone in plasma obtained from groups of male Wistar rats after 2, 4, or 24 h.

Male Wistar rats ($n = 4$) received an intraperitoneal (i.p.) injection of either saline (control), or LPS ($750 \mu\text{g.kg}^{-1}$ i.p.), and then decapitated immediately, or after 2, 4, or 24 h, respectively. Trunk blood was collected into tubes coated with ethylenediaminetetraacetate (EDTA) and centrifuged for 10 min at 3,000 rcf. Plasma samples were aliquotted, snap frozen in liquid nitrogen, and then stored at -80°C until the corticosterone concentration was determined by enzyme immunoassay (EIA). Data are arithmetic mean $\pm$ the standard error of mean of $n = 4$ animals, compared by one-way ANOVA with Bonferroni tests for multiple comparisons.

6.3.3 The effect of an intraperitoneal injection of LPS on the concentration of corticosterone released in response to graded concentrations of ACTH₁₋₂₄ in dispersed adrenal cells obtained from groups of male Wistar rats after 2, 4, or 24 hours

The following results are from experiments performed in order to explore the effects of LPS treatments on the capacity of the adrenal gland to secrete corticosterone when challenged with ACTH.

Figure 6.3 shows the effects of graded concentrations (10^{-11} – 10^{-7}) of ACTH₁₋₂₄ on the release of corticosterone from dispersed adrenal cells obtained from male Wistar rats injected intraperitoneally with either saline, or LPS (750 $\mu\text{g.kg}^{-1}$ i.p.), and then decapitated immediately, or after 2, 4, or 24 hours, respectively.

Two-way ANOVA revealed there were significant main effects of ACTH₁₋₂₄ concentration ($F_{(4, 35)} = 9.293$ (> 1), *** = $P < .001$) and time point ($F_{(3, 35)} = 39.840$, *** = $P < .001$). There was also a significant interaction of these main effects ($F_{(12, 35)} = 4.826$, *** = $P < .001$) on the release of corticosterone.

ACTH₁₋₂₄ caused significant, concentration-dependent increases in corticosterone release from dispersed adrenal cells taken from the saline control group (* = $P < .05$ vs. drug-free control, $\text{pEC}_{50} = 8.02 \pm .78$). By contrast, the corticosterone secretory response to ACTH₁₋₂₄ of adrenal cells from LPS-injected rats was refractory in all groups (ns = $P > .05$ vs. drug-free control). Thus, while basal corticosterone release was higher in dispersed adrenals following LPS administration in the two-hour group (*** = $P < .001$), the four-hour group (*** = $P < .001$), and the twenty-four-hour group (** = $P < .01$), versus the drug-free control from the saline group, these cells were unresponsive to ACTH₁₋₂₄ (ns = $P > .05$ vs. drug-free control). Corticosterone release from adrenal cells of rats injected 24 hours previously with LPS was higher than the saline control group in basal conditions, but was unresponsive to ACTH₁₋₂₄ (10^{-11} – 10^{-6} mol.l^{-1} , ns = $P > .05$ vs. drug-free control).

The *post hoc* tests revealed the peripheral LPS administration resulted in a loss of ACTH₁₋₂₄ concentration-related increases in corticosterone release. The dispersed adrenals collected at 2 hours following LPS administration had a significantly higher corticosterone release versus the saline-injected controls (** = $P < .01$, LPS 2 h vs. saline) at all concentrations of ACTH₁₋₂₄ except for 10^{-7} mol.l^{-1} . The response to ACTH₁₋₂₄ in dispersed adrenals from the four-hour group was broadly in line with the control

response but significantly inverse to the control adrenals at the highest (10^{-7} mol.l⁻¹) and lowest (10^{-11} mol.l⁻¹) concentrations of ACTH₁₋₂₄ (* = P < .05, *** = P < .001, LPS 4 h vs. saline). The response to ACTH₁₋₂₄ in dispersed adrenals 24 hours following LPS administration was significantly lower than the saline control group, but only at the highest concentration of ACTH₁₋₂₄ (10^{-7} mol.l⁻¹, * = P < .05, LPS 24 h vs. saline). Between the different time points for collecting adrenals following LPS administration, the corticosterone release in response to ACTH₁₋₂₄ in the four-hour LPS group was significantly lower than in the two-hour LPS group in the central part curve (10^{-10} mol.l⁻¹ – 10^{-8} mol.l⁻¹ ACTH₁₋₂₄, && = P < .001, LPS 2 h vs. LPS 4 h). The corticosterone release in response to ACTH₁₋₂₄ was also significantly lower in the twenty-four hour LPS group, than the two-hour LPS group, at each concentration of ACTH₁₋₂₄ (** = P ≤ .01) except for 10^{-7} mol.l⁻¹ (ns = P > .05).

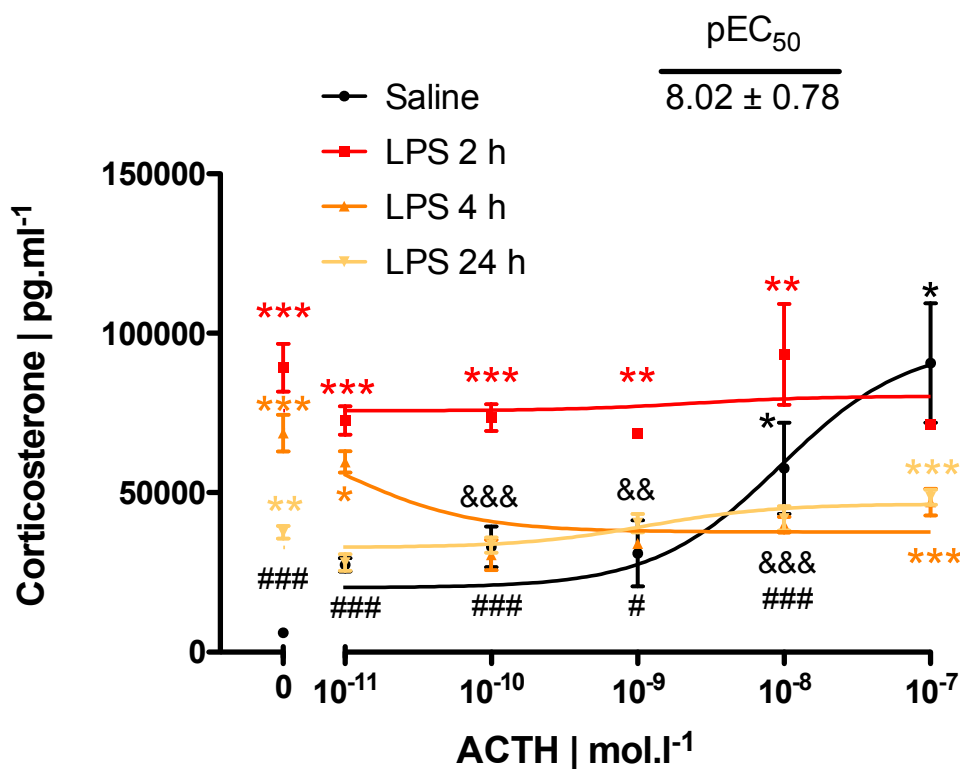


Figure 6.3: Effect of an intraperitoneal injection of lipopolysaccharide (LPS) on the concentration of corticosterone released in response to graded concentrations of adrenocorticotrophic hormone 1-24 (ACTH₁₋₂₄) in dispersed adrenal cells obtained from groups of male Wistar rats after 2, 4, or 24 h.

Male Wistar rats ($n = 4$) received an intraperitoneal (i.p.) injection of either saline (control), or LPS ($750 \mu\text{g.kg}^{-1}$ i.p.), and then decapitated immediately, or after 2, 4, or 24 h, respectively. Adrenal glands were excised from male Wistar rats, and dispersed enzymatically with collagenase in Earle's balanced salt solution (EBSS), centrifuged, and equilibrated at 37°C in 95% $\text{O}_2/5\%$ CO_2 for 1 h. Cells were then incubated for 4 h with or without (basal) ACTH₁₋₂₄ ($10^{-11} - 10^{-7}$) in EBSS. Data are arithmetic mean $\pm$ the standard error of mean of $n = 6$ wells, compared by one-way ANOVA with Dunnett's *post hoc* tests: * = $P < .05$, ** = $P < .01$ vs. control; and by two-way ANOVA with Bonferroni tests for multiple comparisons: && = $P < .01$, &&& = $P < .001$ 2 h vs. 4 h; # = $P < .05$, ## = $P < .01$, ### = $P < .001$ 2 h vs. 24 h.

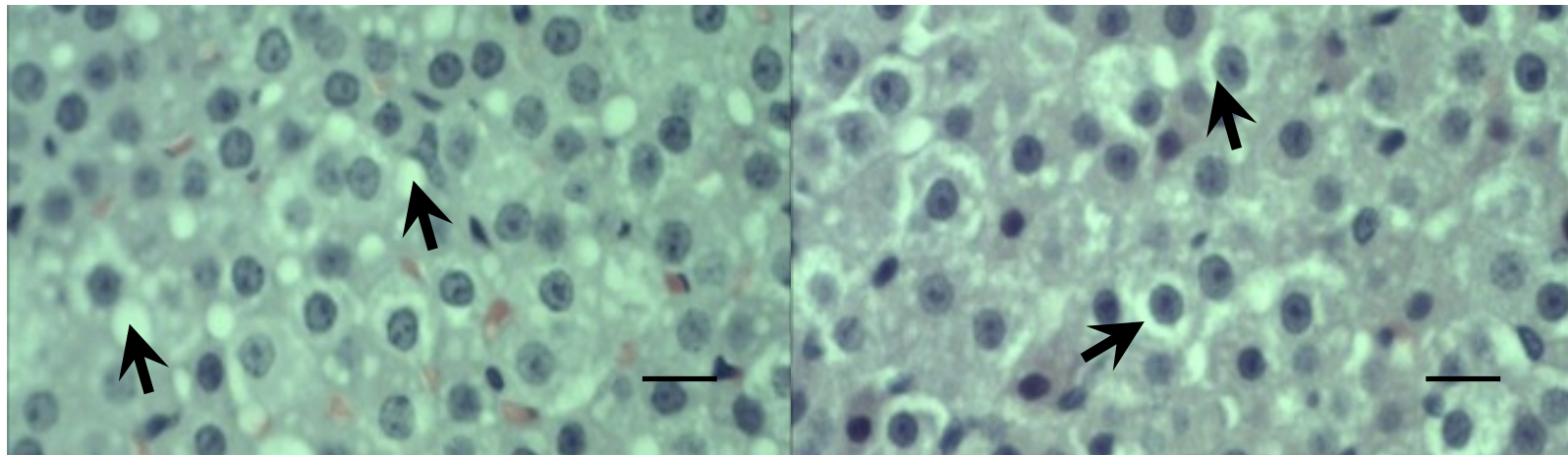
6.3.4 The effect of an intraperitoneal injection of LPS on the morphology of the zona fasciculata within adrenal glands obtained from groups of male Wistar rats after 2, 4, or 24 hours

Figure 6.4 shows the morphology of the zona fasciculata within adrenal glands obtained from groups of male Wistar rats injected intraperitoneally with either saline, or LPS ($750 \mu\text{g.kg}^{-1}$ i.p.), and decapitated either immediately, or after 2, 4, or 24 hours, respectively.

The adrenal cortex is largely made up of endocrine steroid secreting cells. A modified haematoxylin and eosin (H&E) staining protocol allowed histological examination of the adrenocortical cells and sterol content. Unesterified cholesterol is typically localised to the cell membranes, and cholesteryl esters within the cytosol. Although the H&E technique does not differentiate between these sterol compartments, the presence of sterol vacuolations within the cortex are detectable.

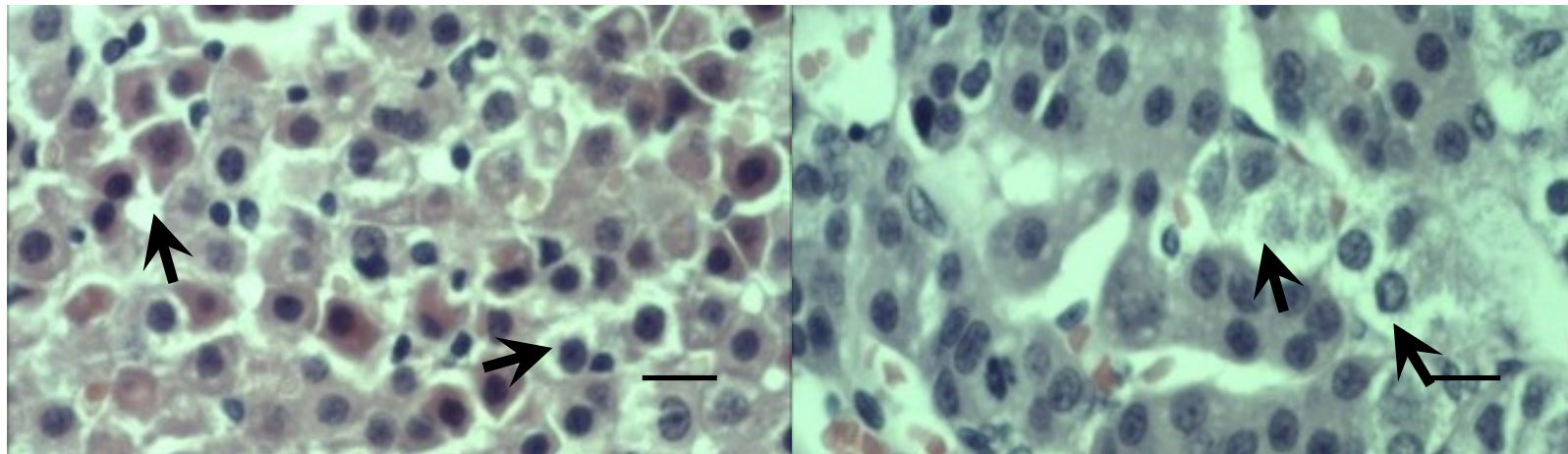
The control section in Figure 6.4 (a) shows a normal arrangement of confluent adrenocortical cells with large, haematoxylin stained (dark blue) nuclei, and sterol vacuolations. The effect of the intraperitoneal injection of LPS is apparent in sections from the two-hour group in (b) with fewer sterol vacuolations and more interstitial space, versus the controls. In sections from the four-hour group (Figure 6.4 c), the interstitium is characterised by islands of cells and fewer sterol vacuolations than the control, along with more eosinophilic cytoplasm than in the two-hour group. After 24 hours (Figure 6.4 d), the interstitial space is markedly increased and although the presence of vacuolated cells remains evident, there are fewer eosinophilic cytoplasm than in the four-hour group.

In contrast to these changes within the adrenal cortex, there were no obvious histological differences in the sections of the hypothalamus, pituitary, spleen, or thymus between each group in this study (data not shown).



(a) Control – Adrenal Z.f.

(b) LPS 2 h – Adrenal Z.f.



(c) LPS 4 h – Adrenal Z.f.

(d) LPS 24 h – Adrenal Z.f.

Figure 6.4: Effect of an intraperitoneal injection of lipopolysaccharide (LPS) on the morphology of the zona fasciculata within adrenal glands obtained from groups of male Wistar rats after 2, 4, or 24 h.

Male Wistar rats ($n = 4$) received an intraperitoneal (i.p.) injection of either saline (control), or LPS ($750 \mu\text{g} \cdot \text{kg}^{-1}$ i.p.), and then decapitated immediately, or after 2, 4, or 24 h, respectively. Transverse sections ($4 \mu\text{m}$) of adrenal glands were stained with Gill's No 1 haematoxylin (75 s), rinsed in tap water (10 min) and counterstained (7 min) with Eosin Y solution (5% w/v dH_2O) acidified with acetate ($50 \mu\text{l}/200 \text{ ml}$). Sections were then dehydrated in 75% and 100% ethanol, each for 30 s, twice, before fixing in xylene for 1 min and mounting coverslips with DPX. The zona fasciculata was imaged by light microscopy (40x). The cytoplasm is stained pale blue/pink, nuclei are dark blue, and red blood cells are orange. Arrows denote sterol vacuolations.

6.3.5 The effect of an intraperitoneal injection of LPS and/or WRW₄ on the concentration of corticosterone in plasma obtained from groups of male Wistar rats after 4 hours

The following results are from experiments performed in order to examine the effects of treatment of rats with the Fpr2-/FPR2/ALX-selective antagonist WRW₄ on the adrenocortical responses to LPS.

Figure 6.5 shows the effect of an intraperitoneal injection of LPS (750 µg.kg⁻¹ i.p.), WRW₄ (250 µg.kg⁻¹ i.p.), or LPS and WRW₄ combined, versus saline, on the concentration of corticosterone in the plasma of male Wistar rats after 4 hours.

WRW₄ alone had no effect on basal corticosterone release (ns = $P > .05$ vs. control). The administration of LPS caused a significant increase in plasma corticosterone concentration within 4 hours ($* = P < .05$ vs. control). The co-administration of LPS and WRW₄ also resulted in a significant increase in plasma corticosterone concentration ($* = P < .05$ vs. control), but this was not different from the effect of LPS alone (ns = $P > .05$).

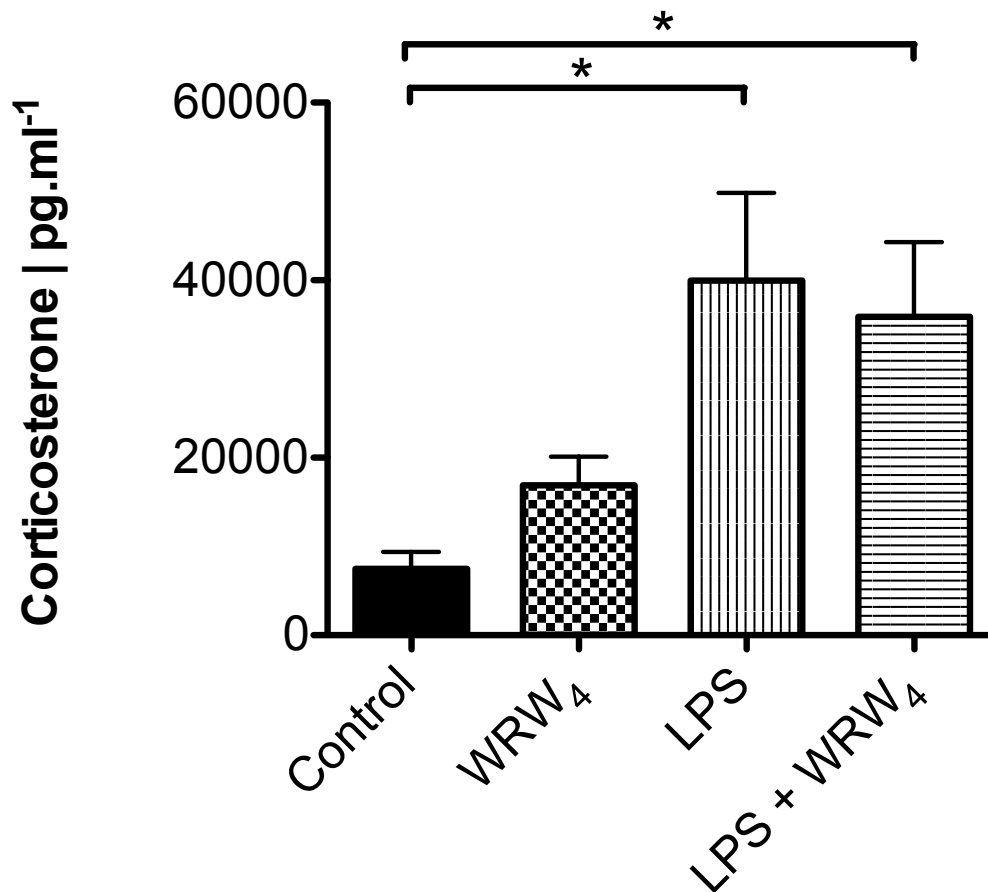


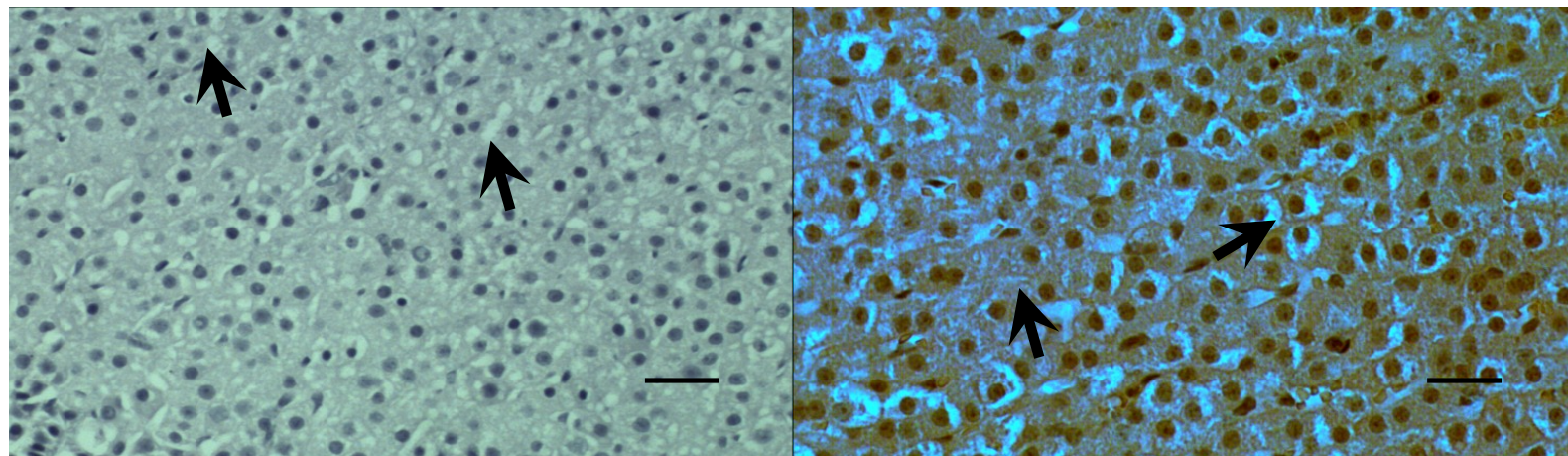
Figure 6.5: Effect of intraperitoneal injections of lipopolysaccharide (LPS) and/or Trp-Arg-Trp-Trp-Trp-Trp (WRW₄) on the concentration of corticosterone in plasma obtained male Wistar rats after 4 h.

Male Wistar rats ($n = 6$) received intraperitoneal injections of either saline (control), LPS ($750 \mu\text{g.kg}^{-1}$), the formyl peptide receptor 2 (FPR2/ALX)-selective antagonist WRW₄ ($250 \mu\text{g.kg}^{-1}$), or LPS and WRW₄, and were then decapitated after 4 h. Trunk blood was collected into tubes coated with ethylenediaminetetraacetate (EDTA) and centrifuged for 10 min at 3,000 rcf. Plasma samples were aliquotted, snap frozen in liquid nitrogen, and then stored at -80°C until the corticosterone concentration was determined by enzyme immunoassay (EIA). Data are arithmetic mean $\pm$ the standard error of mean of $n = 6$ animals, compared by one-way ANOVA with Bonferroni's correction for multiple comparisons: * = $P < .05$ vs. control.

6.3.6 The effect of an intraperitoneal injection of LPS and/or WRW₄ on the morphology of the zona fasciculata within adrenal glands obtained from groups of male Wistar rats after 4 hours

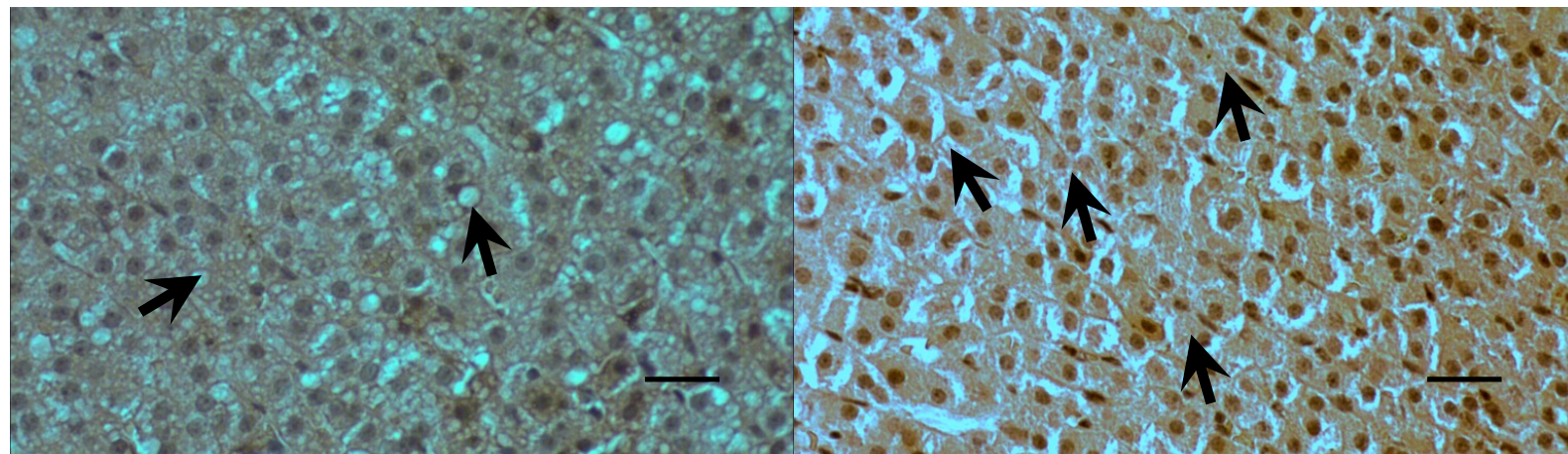
Figure 6.6 shows the effect of an intraperitoneal injection of LPS (750 $\mu\text{g.kg}^{-1}$ i.p.), WRW₄ (250 $\mu\text{g.kg}^{-1}$), or LPS and WRW₄ combined, versus saline, on the morphology of the zona fasciculata within the adrenal glands of male Wistar rats after 4 hours.

The control section in Figure 6.6 (a) shows a typical arrangement of confluent adrenocortical cells with large, nuclei stained dark blue with haematoxylin, and lipid globules within the interstitium. The effect of the intraperitoneal injection of LPS is evident after 4 hours in (Figure 6.6 b) with observations of reduced sterol vacuolations and more interstitial space compared to the control group. In sections from the WRW₄ group (Figure 6.6), the confluency of the adrenocortical cells remains largely unchanged, with an interstitial space and sterol vacuolations that compare with the control group. The effect of the combined administration of LPS and WRW₄ is evident in Figure 6.6 (d), with more vacuolated cells and a loss of the lipid globules versus the WRW₄ and control groups; however, these changes are broadly similar to those seen in the LPS group.



(a) Control – Adrenal Z.f.

(b) LPS 4 h – Adrenal Z.f.



(c) WRW4 4 h – Adrenal Z.f.

(d) LPS + WRW4 4 h – Adrenal Z.f.

Figure 6.6: Effect of an intraperitoneal injection of lipopolysaccharide (LPS) and/or Trp-Arg-Trp-Trp-Trp-Trp (WRW₄) on the morphology of the zona fasciculata within adrenal glands obtained from groups of male Wistar rats after 4 h.

Male Wistar rats ($n = 6$) received an intraperitoneal (i.p.) injection of either saline (control), LPS ($750 \mu\text{g.kg}^{-1}$ i.p.), WRW₄ ($250 \mu\text{g.kg}^{-1}$), or LPS and WRW₄, then decapitated after 4 h. Transverse sections ($4 \mu\text{m}$) of adrenal glands were stained with Gill's No 1 haematoxylin (75 s), rinsed in tap water (10 min) and counterstained (7 min) with Eosin Y solution (5% w/v dH_2O) acidified with acetate ($50 \mu\text{l}/200 \text{ ml}$). Sections were then dehydrated in 75% and 100% ethanol, each for 30 s, twice, before fixing in xylene for 1 min and mounting coverslips with DPX. The zona fasciculata was imaged by light microscopy (40x). The cytoplasm is stained pale blue/pink, nuclei are dark blue, and red blood cells are orange. Arrows denote sterol vacuolations.

6.4 Discussion

This study has provided evidence for the following main findings. First, LPS increases the plasma concentration of ACTH within 2 hours of administration. Second, LPS induces a time-dependent decrease in sterol vacuolation within the zona fasciculata that is maximal within 4 hours of administration. Third, the GC-secretory function of adrenal cells *in vitro* was elevated in basal conditions, but became maximally refractory to graded, physiological concentrations of ACTH₁₋₂₄ within 4 hours of LPS administration *in vivo*. Fourth, intraperitoneal injections of the Fpr2-/FPR2/ALX-selective antagonist WRW₄ had no effect on basal or the LPS-induced increase in the plasma concentration of corticosterone within 4 hours of administration.

To the author's knowledge, this is the first reported use of the Fpr2-/FPR2/ALX-selective antagonist, WRW₄ (250 µg.kg⁻¹ i.p.) to determine the contribution of Fpr2 on pituitary and adrenal stress hormone release in male Wistar rats during experimental endotoxaemia induced by LPS (750 µg.kg⁻¹ i.p.). This work is important because of the need to find safe, efficacious alternatives to corticosteroids during sepsis.

Previous research has assessed the effects of experimental endotoxaemia induced by intraperitoneal injection of LPS on pituitary and adrenal function in mice and rats. Studies of dispersed adrenals obtained from wildtype and Fpr2/3^{-/-} mice have also been performed to assess the contribution of Fpr2 and/or Fpr3 in the regulation of GC secretion. Buss and colleagues (2010) found evidence of increased adrenocortical infiltration of leukocytes (eosinophils, neutrophils, and monocytes) and upregulated transcripts for Fpr1, Fpr2, and Fpr3, and sterol vacuolation within 4 hours of intraperitoneal injection of LPS in wildtype mice. In contrast, the LPS-induced sterol vacuolation was less pronounced in the Fpr2/3^{-/-} mice, suggesting Fpr2 and/or Fpr3 could be involved in the adrenal response to experimental endotoxaemia (Buss 2010). In the present study design, instead of using genetic knockout of Fpr2 and/or Fpr3 in mice, the Fpr2-/FPR2/ALX-selective antagonist WRW₄ was used to pharmacologically block Fpr2 in rats. The present study aimed to advance the field by assessing the role of Fpr2 on pituitary and adrenal stress hormone function during sepsis-related endotoxaemia in male Wistar rats, through pharmacological blockade with the Fpr2-/FPR2/ALX-selective antagonist WRW₄. This was intended to develop a model

that could be translated into a clinical therapy in the form of a drug, as opposed to the germ line knockout mouse models.

Previous research is concordant with the finding that within 4 hours of *in vivo* administration, LPS induced maximal effects on adrenocortical cells *in vitro*, including sterol vacuolation and elevated basal GC secretion whilst the response to graded, physiological concentrations of ACTH₁₋₂₄ was refractory. The stimulatory effect of LPS on adrenal corticosterone secretion has been well documented (Lenczowski *et al.* 1997; Grinevich *et al.* 2001; Campisi *et al.* 2003; Zimomra *et al.* 2011). Previous research has also found that peripheral injection of LPS in mice *in vivo* resulted in adrenocortical cells that were refractory to ACTH₁₋₂₄ when dispersed and cultured *in vitro* (Buss 2010; Mohn *et al.* 2011). Repeated LPS injections result in further uncoupling of the corticosterone secretory response to ACTH₁₋₂₄, despite increases in adrenal mass and expression of steroidogenic enzymes (Grinevich *et al.* 2001). Although the mechanisms behind adrenals being refractory to ACTH₁₋₂₄ following LPS require further investigation, it has been shown that LPS-stimulated mouse macrophages produce a factor that inhibits the action of ACTH₁₋₂₄ on rabbit adrenal cells *in vitro* (Mathison *et al.* 1983; 1984; Ehrhart-Bornstein *et al.* 1998). Also, mice knocked-out for scavenger receptor B-I (SR-BI)—an HDL receptor for the uptake of cholesterol ester—lack the upregulated corticosterone secretion in response to LPS, suggesting *de novo* synthesis of corticosterone from cholesterol/pregnenelone precursors contributes to the LPS-induced hypersecretion of corticosterone (Cai *et al.* 2008). In addition, macrophage and lymphocytes native to the adrenal cortex have been shown to be involved in the infiltration of eosinophils induced by LPS (Bozza *et al.* 1994).

The lack of an effect of WRW₄ alone on the plasma corticosterone concentration is not surprising. WRW₄ is a full, Fpr2-/FPR2/ALX-selective antagonist and as such will not elicit an effect on its own. Given the evidence that LPS has been shown to upregulate adrenal Fpr2 expression, and evidence that Fpr2 mediates anti-inflammatory signalling mechanisms induced by endogenous ligands such as ANXA1 and LXA₄ (Dufton *et al.* 2010), then it was thought that the blockade of Fpr2 might limit some of the effects of LPS on adrenal morphology and function. Given the intraperitoneal route of administration, and the 250 µg.kg⁻¹ dose of WRW₄, it is conceivable that the blood concentration of WRW₄ was not sufficient to block Fpr2 signalling within the adrenal cortex.

6.4.1 Biological significance and scope of findings

Overall, the finding that intraperitoneal administration of LPS in a rat model of sepsis-related endotoxaemia reduced both the secretion of ACTH₁₋₂₄-stimulated corticosterone and the presence of sterol vacuolations within the adrenal cortex has implications for our understanding of pituitary and adrenal function. As these effects were maximal after 4 hours, this implies that this time period could be investigated as a window for Fpr/FPR ligands that preserve the adrenal GC secretory response to ACTH. Although the dose and route of administration of WRW₄ may not have been sufficient to block Fpr2 in this study, this does not rule out the potential importance of ligands that target Fpr2 in sepsis-related endotoxaemia.

Intravenous (i.v.) administration of WRW₄ was considered but was rejected in favour of the i.p. route which is less stressful for the animals. Colleagues have used WRW₄ at 2.2 µg.kg⁻¹ i.v. when observing leukocyte migration during experimental endotoxaemia in mice (Hughes 2012). Despite the present study using a considerably higher dose of 250 µg.kg⁻¹ WRW₄ i.p. in rats, it is possible that the intraperitoneal route of administration resulted in an insufficient blood concentration of WRW₄ to have any effect on LPS-induced changes in adrenal morphology or function. Given that mice have an approximate blood volume of 58.5 ml.kg⁻¹ body mass (Wolfensohn & Lloyd 2003), the maximum blood concentration of a 2.2 µg.kg⁻¹ dose of WRW₄ given intravenously to a 25 g mouse with 1.5 ml of blood would therefore be 3.3 mmol.l⁻¹. Similarly, in rats, blood volume is approximately 64 ml.kg⁻¹ body mass, so the maximum blood concentration of a 250 µg.kg⁻¹ dose of WRW₄ given to a 250 g rat with 16 ml blood would be 3.5 µmol.l⁻¹. These concentrations are above the IC₅₀ of 0.13 µmol.l⁻¹ for WRW₄ via Fpr2/FPR2/ALX Fpr2 (Önnheim *et al.* 2008; Braun *et al.* 2011). Other researchers investigating the role of Fpr/FPR in alopecia, found that WRW₄ was able to block the anti-alpecia effects of the Fpr1 agonist fMLF and the Fpr2 agonist MMK-1, even though it was administered intraperitoneally (Tsuruki *et al.* 2007). The dose of WRW₄ however, was given over several days and was 120 times higher (30 mg.kg⁻¹ i.p.) than the single dose in the present study (250 µg.kg⁻¹ i.p.). It seems likely that the dose in the present study was insufficient to account for the intraperitoneal route of administration.

6.4.2 Conclusion

The finding that this rat model of LPS-induced, sepsis-related endotoxaemia led to an increased plasma concentration of ACTH within 2 hours, and refractory GC secretory responses to ACTH₁₋₂₄ within 4 hours, is consistent with previous research in mice (Buss 2010). The apparent lack of an effect of the Fpr2-/FPR2/ALX-selective antagonist WRW₄ however, does not refute the previous findings that LPS led to reductions in both the secretion of ACTH₁₋₂₄-stimulated corticosterone and the presence of sterol vacuolations within the adrenal cortex was limited by the removal of Fpr2 and/or Fpr3 in Fpr2/3^{-/-} mice. The present work has advanced the field by providing evidence for the disruptive effects of LPS on adrenocortical structure and function occur within 2 hours, and are maximal within 4 hours in rats, thus confirming previous findings in mice. These findings improve our understanding of the temporal nature of changes in pituitary and adrenal function during sepsis-related endotoxaemia.

Chapter Seven: **Summary**

1. Microscopic evaluation of immunostained tissue sections from LPS-stimulated Fpr2/3^{-/-} mice revealed nuclear GFP immunoreactivity within endocrine cells of the anterior pituitary gland, the adrenocortical cells of the adrenal glands, and the thymus, but not in the hypothalamus or the median blade of the dentate gyrus. The nuclear GFP signal was visible after 2 hours, but was absent at 6 hours following LPS injection.
2. Anterior pituitary gland sections from LPS-stimulated Fpr2/3^{-/-} mice had nuclear GFP immunoreactivity in cells with cytoplasmic ACTH, PRL, and GH immunoreactivity, but not the S100b-positive folliculostellate cells.
3. Human anterior pituitary gland sections from MS donors exhibited nuclear immunoreactivity for FPR3 that was colocalised to cells with cytoplasmic ACTH immunoreactivity but not the S100b-positive folliculostellate cells.
4. Although the human pituitary gland also contained FPR2/ALX and FPR1 immunoreactivity, the automated image analysis indicated these subtypes were not significantly colocalised with either ACTH or S100b immunoreactivity.
5. Dispersed anterior pituitary cells from rats had a reduced CRH-stimulated release of ACTH when incubated with micromolar fMLF, micromolar WKYMVm, and 15-epi-LXA₄.
6. The Fpr1-/FPR1-selective agonist fMLF generally did not affect basal or CRH-stimulated ACTH release except at the high, 10 micromolar concentration, where CRH-stimulated ACTH release was inhibited.
7. The Fpr2-/FPR2/ALX-selective agonist WKYMVm stimulated the basal release of ACTH, but generally did not affect CRH-stimulated ACTH release except at a high micromolar concentration of WKYMVm where this was inhibited, and the nanomolar concentration where it was enhanced.
8. The Fpr2-/FPR3-selective agonist F2L stimulated basal release of ACTH at all concentrations, but only affected CRH-stimulated ACTH release at the highest 800 nanomolar concentration where it was enhanced.
9. The Fpr3/Alx-/FPR2/ALX-selective agonist 15-epi-LXA₄ stimulated basal ACTH release at low concentrations, but was reversed at concentrations of or above 10 nanomolar where it also potently inhibited CRH-stimulated ACTH release.

10. The non-selective Fpr/FPR agonist ANXA1_{Ac2-26} did not affect basal or CRH-stimulated ACTH release except at the 3 micromolar concentration where it enhanced it. Basal release of ACTH was inhibited by ANXA1_{Ac2-26} at concentrations of 3 micromolar or below, but only in the presence of the Fpr2-/FPR2/ALX-selective antagonist WRW₄ in an experiment where CRH failed to stimulate ACTH release.
11. The approximate efficacy of the tested Fpr/FPR agonists for their modulation of basal or CRH-stimulated ACTH release can be summarised as: WKYVMm >>> 15-epi-LXA₄ > F2L >> fMLF > ANXA1_{Ac2-26}.
12. Basal ACTH release was most potently modulated by the stimulatory effect of the Fpr2-/FPR2/ALX-selective agonist WKYVMm.
13. CRH-stimulated ACTH release was most potently modulated by the inhibitory effect of the Fpr3/Alx-/FPR2/ALX-selective agonist 15-epi-LXA₄.
14. Dispersed adrenal cells from male Wistar rats had reduced ACTH₁₋₂₄-stimulated corticosterone release when incubated with fMLF, ANXA1_{Ac2-26}, and 15-epi-LXA₄.
15. The Fpr1-selective agonist fMLF elicited the most powerful inhibition of corticosterone release irrespective of the presence of ACTH₁₋₂₄, but was partly inhibited by the Fpr2-selective antagonist WRW₄.
16. The Fpr2-/FPR2/ALX-selective agonist WKYVMm partly suppressed basal corticosterone release, and was unaffected by ACTH₁₋₂₄ or WRW₄.
17. The Fpr2-/FPR3-selective agonist F2L stimulated basal corticosterone release at a low concentration, but enhanced ACTH₁₋₂₄-stimulated corticosterone in the presence of the Fpr2-/FPR2/ALX-selective antagonist WRW₄.
18. The Fpr3/Alx-/FPR2/ALX-selective agonist 15-epi-LXA₄ suppressed both basal and ACTH₁₋₂₄-stimulated corticosterone release, but was unaffected by WRW₄.
19. The non-selective Fpr/FPR agonist ANXA1_{Ac2-26} suppressed both basal and ACTH₁₋₂₄-stimulated corticosterone release.
20. The approximate efficacy of the tested Fpr/FPR agonists for inhibition of basal and ACTH₁₋₂₄-stimulated corticosterone release after 4 hours of contact can be summarised as: fMLF >>> WKYVMm > ANXA1_{Ac2-26} > 15-epi-LXA₄ >> F2L.

21. Despite being an Fpr1-/FPR1-selective agonist at low concentrations, the effects of fMLF were probably mediated via Fpr2 or Fpr3, especially since fMLF was the only agonist to be inhibited by WRW₄.
22. The basal, *in vitro* release of corticosterone from dispersed adrenal cells obtained from rats intraperitoneally injected with LPS four hours earlier, was higher than from rats injected with saline.
23. At all time points following LPS administration, the release of corticosterone from dispersed adrenal cells was refractory to ACTH₁₋₂₄, compared to the ACTH₁₋₂₄ concentration-dependent increase in corticosterone release by adrenal cells from the saline control group.

Chapter Eight: **General Discussion**

8.1 Main findings

To the author's knowledge, this is the first time that antibodies targeting the human FPR subtypes have been used to detect their distribution within the human anterior pituitary gland. Secondly, the factorial study design for the functional investigations meant that co-incubation of dispersed anterior pituitary or adrenal cells with stress hormone secretagogues, and Fpr/FPR agonists and antagonists allowed their interactive effects to be detected. Thirdly, to the author's knowledge, this is the first time that F2L, WKYMVm, and 15-epi-LXA₄ have been used in both dispersed anterior pituitary, and adrenal cell systems. This combination of immunohistochemical and pharmacological approaches, together with novel aspects in the design of the studies, has resulted in the following main findings discussed in turn below.

8.1.1 The anterior pituitary gland from organisms exposed to inflammogenic stressors exhibits colocalisation of ACTH with FPR3 in MS-diseased humans, and ACTH, GH, and PRL with GFP as a proxy for Fpr2 and Fpr3 in LPS-exposed mice

Intraperitoneal injection of LPS into Fpr2/3^{-/-} mice with a GFP transgene in place of Fpr2 and Fpr3 resulted in immunofluorescence for GFP that was colocalised with ACTH, GH, and PRL, but not with S100b. Within the anterior pituitary of deceased humans with MS, FPR3 was colocalised with ACTH immunofluorescence within corticotrophs. The present findings are concordant with those from previous research. Although folliculostellate cells express ANXA1 (Christian *et al.* 1997), they do not possess ANXA1-binding sites, putatively an Fpr/FPR subtype, either within tissue sections (Traverso *et al.* 1999) or in the folliculostellate-like TtT/GF cell line (Inoue *et al.* 1992; McArthur *et al.* 2009). The long thread-like projections of folliculostellate cells do however, show immunogold localisation of ANXA1 at sites in contact with endocrine cells such as corticotrophs (Traverso *et al.* 1999). The distribution of GFP observed here in the Fpr2/3^{-/-} mouse is consistent with the expression of Fpr2 and Fpr3 transcripts within the anterior pituitary glands of C57BL/6 mice and Sprague-Dawley rats (John *et al.* 2007). The present data from the Fpr2/3^{-/-} mouse anterior pituitary was partly corroborated by immunostaining of FPR subtypes within the human anterior pituitary that found significant colocalisation of ACTH with FPR3, but not with FPR1 or FPR2/ALX, and S100b fluorescence was singular in both species. Not all FPR3 was colocalised with ACTH however, and further experiments with anti-GH antisera could confirm whether

the wide distribution of FPR3 is colocalised with GH within human somatotrophic cells. If so, this would be reflective of the widespread colocalisation of GFP and GH within the Fpr2/3^{-/-} mouse pituitary observed here, and consistent with somatotrophs being the dominant endocrine subpopulation within the anterior pituitary of these species (Philip *et al.* 2001; Ganong 2005). The sparse fluorescence for FPR1 is consistent with FPR1-positive leukocyte infiltration observed in brains from MS patients (Müller-Ladner *et al.* 1996). Unfortunately, non-diseased control tissues were not available at the time of the experiments. In summary, the anterior pituitary gland exhibits ACTH colocalisation with FPR3 in MS-diseased humans, and with GFP as a proxy of Fpr2 and Fpr3 in mice exposed to LPS but not in vehicle controls. Taken together, this suggests these subtypes could be targets for Fpr/FPR ligands such as ANXA1 in the control of ACTH secretion, particularly during the inflammogenic stress of MS or endotoxaemia.

8.1.2 Fpr/FPR ligands modulate the secretagogue-induced secretion of pituitary and adrenal stress hormones from dispersed glands derived from rats in vitro

Within four hours, a range of chemically diverse Fpr/FPR ligands exhibited a hierarchy of efficacy in terms of their modulation of basal and/or secretagogue-induced secretion of stress hormones from dispersed cells of both the rat anterior pituitary gland: WKYMVm >>> 15-epi-LXA₄ > F2L >> fMLF > ANXA1_{Ac2-26}; and adrenal glands: fMLF >>> WKYMVm > ANXA1_{Ac2-26} > 15-epi-LXA₄ >> F2L.

The modulatory effects of Fpr/FPR ligands on the CRH-stimulated release of ACTH from the dispersed anterior pituitary gland are plausible given what is known of the signal transduction events downstream of their receptors. Upon CRH binding, CRH-R1 recruits G_s to trigger adenylate cyclase to produce cAMP and activate PKA. The actions of PKA triggers an influx of Ca²⁺ via L-type Ca²⁺ channels which, results in membrane fusion of secretory vesicles and exocytosis of ACTH (Aguilera *et al.* 1983; Taylor *et al.* 1993). Previous data have however shown that the ANXA1-mediated inhibition of ACTH release does not interfere with Ca²⁺ influx, but involves a different mechanism, ostensibly downstream of an Fpr/FPR subtype (Taylor *et al.* 1993; John *et al.* 2002). Indeed, activation of the Fpr/FPR subtypes coupled with heterotrimeric G_i proteins reduced the release of CRH-stimulated ACTH from the corticotrophic AtT20 cell line (Aguilera *et al.* 1983; Luini & Matteis 1990), and this can be blocked by pertussis toxin (Le *et al.* 2002). The other events downstream of ANXA1-Fpr activation are unclear, but some evidence suggests that ANXA1-activated FPR2/ALX leads to actin polymerisation and G_{rho}/G_{rac}/G_{rac}-mediated inhibition of

exocytosis of ACTH from corticotrophs (McArthur *et al.* 2009) and lytic granules from leukocytes (Castellino *et al.* 1992; Maderna *et al.* 2002). Consistent with John and coworkers (2007) the present data militate against a role for the rat equivalent of mouse Fpr1 and human FPR1, as a target for ANXA1 action in the anterior pituitary gland. The Fpr1-/FPR1-selective agonist fMLF had no effect on basal or CRH-stimulated ACTH release at the sub-micromolar concentrations at which these subtypes are selectively bound (Prossnitz *et al.* 1991; He *et al.* 2000) and elicit exocytosis of lytic granules (Schiffmann *et al.* 1975a; Marasco *et al.* 1984). This is consistent with previous observations of inhibitory effects of ANXA1_{Ac2-26} that were unaffected by either the genetic knockout of Fpr1, or pharmacological blockade of Fpr1 with the human FPR1/mouse Fpr1 antagonist Boc1 (John *et al.* 2007). Further support for this is evident in that while ANXA1 suppressed ACTH release (Taylor *et al.* 1993; John *et al.* 2007), the Fpr1/FPR1 subtypes are not expressed in human (Becker *et al.* 1998) or rodent anterior pituitary tissue (John *et al.* 2007). As for the other Fpr subtypes, this laboratory previously found evidence of Fpr2 and Fpr3 expression within the rat pituitary, and the mouse pituitary also contained Fpr-rs6, Fpr-rs7 (John *et al.* 2007). The Fpr2l/Alx subtype in the rat (Chiang *et al.* 2003) and Fpr3/Alx in the mouse (Takano *et al.* 1997) have been described as the orthologues of human FPR2/ALX which mediate the effects of LXA₄, 15-epi-LXA₄, ANXA1, and ANXA1_{Ac2-26} (Takano *et al.* 1997; Hartt *et al.* 1999; Wang & Ye 2002). The present finding that the aspirin-triggered lipoxin, 15-epi-LXA₄ at 10⁻⁸ mol.l⁻¹, inhibited CRH-stimulated ACTH release is consistent with signalling via rat Fpr2l/Alx (Chiang *et al.* 2003), and the inhibition elicited by the less potent LXA₄ used previously at 2 x 10⁻⁶ mol.l⁻¹ (John *et al.* 2007). Conversely, the agonist WKYMVm had a stimulatory effect on ACTH release that is consistent with its pro-exocytotic effects on leukocytes at Fpr2-/FPR2/ALX-selective concentrations (Kim *et al.* 2006). Taken together, this might suggest that Fpr2 mediates stimulatory and Fpr2l/Fpr3 mediates inhibitory effects of Fpr/FPR ligands on the secretion of ACTH, at least from dispersed anterior pituitary glands from rats and over a period of four hours.

The inhibitory effects of Fpr/FPR ligands on the ACTH₁₋₂₄-stimulated release of corticosterone are plausible given what is known about the signalling mechanisms downstream of their receptors. Upon binding of ACTH, MC₂R recruits G_{sα} and adenylate cyclase hydrolyses ATP, producing cyclic AMP. This activates PKA to phosphorylate cAMP response element binding (CREB) protein that then binds to cAMP response elements (CRE) to upregulate the transcription of genes for steroidogenic enzymes

including, P450scc in the rate-limiting conversion of pregnenolone from cholesterol (Richman *et al.* 1973; Simpson & Waterman 1988; Stocco *et al.* 2005; Manna *et al.* 2009). Conversely, upon binding of fMLF, Fpr1 recruits $G_{i\alpha}$ and G_{β} which activate PLC- β and in turn, PKC, which is then able to phosphorylate NF κ B (Naccache *et al.* 1979). Prior to its phosphorylation, the NF κ B heterodimer (p65 (Rel A) and p50) is kept in the cytosol by association with inhibitory factor I κ B. Once phosphorylated, NF κ B is free to enter the nucleus and bind to response elements including CRE, thereby enhancing the transcription of various genes (McKay & Cidlowski 1999). It is plausible to hypothesise, therefore, that the inhibition of corticosterone release induced by fMLF might somehow be achieved by the competition of NF κ B with CREB for its DNA response element downstream of Fpr1 and MC₂R activation, respectively, thereby limiting the ACTH₁₋₂₄-induced expression of steroidogenic enzymes. This hypothesis requires further investigation, but given the bacterial origins of fMLF, this could have implications for bacterial sepsis.

Intriguingly, the Fpr/FPR ligands in the dispersed anterior pituitary gland and adrenal gland experiments exhibited non-classical concentration-effect curves. Although the scope of the present studies was unable to obtain an explanation for this observation, it can be interpreted in the context of several strands of the existing literature. Firstly, the apparently opposing effects of ligands supposedly selective for a given Fpr/FPR subtype could be due to ligand-specific binding pockets. Bena and coworkers (2012) constructed chimeric FPRs to investigate this possibility, and found specific binding pockets for ANXA1_{Ac2-26}, LXA₄ and serum amyloid A on human FPR2/ALX, which mediates both anti-inflammatory and pro-inflammatory effects in polymorphonuclear leukocytes (Chiang *et al.* 2006a). Alternatively, given the high structural homology between the Fpr/FPR subtypes (Ye *et al.* 2009), ligand activity might involve recruitment of multiple Fpr/FPR subtypes or signal mechanisms. The amino acid residues Asp-106 and Arg-201 to Arg-205 in the mouse Fpr1, Fpr2, and Fpr3/Alx subtypes, for instance, are conserved in human FPR1 and FPR2/ALX, whereas FPR3 contains FLILH in this position and other substitutions in the third cytoplasmic loop that binds G proteins (Alvarez *et al.* 1996). This means that mice, and ostensibly rats, lack a direct structural homologue of FPR3 but instead have 3 genes closely related to FPR1 and FPR2/ALX (Gao *et al.* 1998), whereas Fpr-rs3, Fpr-rs4, and Fpr-rs5 have multiple substitutions in residues that are thought to be key to the ligand-specificity of Fpr1, Fpr2, and Fpr3/Alx (Mills *et al.* 2000).

The decision to incubate Fpr/FPR agonists and antagonists with endogenous secretagogues in dispersed anterior pituitary and adrenal cells derived from rats rather than mouse was novel, and necessary to gain an adequate yield of cells to be incubated with a range of ligand concentrations. This came at the expense of limited availability of genetic and immunological tools for the rat Fpr subtypes, as well as disrupting the paracrine communication networks within the pituitary (Baes *et al.* 1987; Denef *et al.* 1989) and possibly the adrenal glands (Haidan 1998; Ehrhart-Bornstein & Bornstein 2008).

8.1.3 Within two hours of experimental endotoxaemia, adrenocortical cells become refractory to ACTH-induced secretion of corticosterone in vitro with a loss of sterol vacuolation that is limited by the knockout of Fpr2 and Fpr3 genetically in mice, but not by pharmacological antagonism in rats

Within two hours, experimental endotoxaemia induced by intraperitoneal injection of LPS (750 µg.kg⁻¹ i.p.) into rats, increased the secretion of ACTH *in vivo*, and of corticosterone from dispersed rat adrenal glands *in vitro*, which remained refractory to graded concentrations of ACTH₁₋₂₄ for up to twenty-four hours. The morphology of the adrenal glands exhibited an LPS-induced decrease in sterol vacuolation within two hours in wildtype mice but was limited in Fpr2/3^{-/-} mice, with a similar effect observed in the rat adrenal gland within two hours that was maximal by four hours, but unaffected by the Fpr2-/FPR2/ALX-selective antagonist WRW₄.

The LPS-induced decrease in adrenal sterol vacuolation that is evident within 4 hours in wildtype mice, but limited by the genetic deletion of mouse Fpr2 and Fpr3 is consistent with previous research (Buss 2010). Following intraperitoneal injection, LPS enters the blood from the peritoneal cavity primarily via the hepatic portal vein (haematogenous route) or via the lymphatic system (lymphogenous route) (Romanovsky *et al.* 2000). From here, LPS is able to enter the well perfused adrenal cortex and exert a well-documented stimulation of adrenal GC secretion (Lenczowski *et al.* 1997; Grinevich *et al.* 2001; Campisi *et al.* 2003; Zimomra *et al.* 2011). The present observation that LPS *in vivo* precedes refractory GC secretion in response to graded concentrations of ACTH in adrenocortical cells *in vitro*, is supported by previous research which also found evidence of downregulated MC₂R expression (Liu *et al.* 2011). Although LPS initially stimulates the production and release of GCs in a mechanism involving COX-2 activation (Vakharia & Hinson 2004; Zacharowski *et al.* 2006), following this, the GC secretory response

to further LPS or ACTH becomes downregulated along with the expression of steroidogenic enzymes and MC₂R (Liu *et al.* 2011). Since there is very little *de novo* synthesis of cholesterol, the cholesteryl ester within the adrenal cortex largely comes from LDL and HDL bound to SR-BI, in an uptake process that is critical for adrenocortical steroidogenesis (Temel *et al.* 1997; Prigent *et al.* 2004). Sepsis-related experimental endotoxaemia is associated with adrenocortical depletion of sterol vacuolation (Marik 2002; Buss 2010; Peng & Du 2010) that coincides with hypocortisolemia (Cai *et al.* 2008), an aberrant SR-BI pathway, and adrenocortical apoptosis (Kanczkowski *et al.* 2013).

WRW₄ is an antagonist selective for Fpr2/FPR2/ALX in cells from mice (Önnheim *et al.* 2008), rats (Braun *et al.* 2011), and humans (Bae *et al.* 2004). Although the intraperitoneal injection of WRW₄ (250 µg.kg⁻¹), a dose intended to pharmacologically knockout the rat Fpr2, failed to significantly affect the plasma concentration of ACTH₁₋₂₄ or corticosterone, or adrenal sterol vacuolation within 4 hours, the fact that genetic deletion of Fpr2 and Fpr3 limited the LPS-induced decrease in adrenal sterol vacuolation in mice has implications for the management of sepsis-related endotoxaemia.

8.2 Future directions and implications

Advances in imaging techniques and mathematical modelling of endocrine tissues have allowed research to obtain spatial and functional data simultaneously. One such technique is two-photon imaging of pituitary cells in organised networks and how these impinge on endocrine function. Transgenic animals are typically used to visualise individual endocrine lineages, such as PRL-DsRed for lactotrophs (He *et al.* 2011), and GH-eGFP for somatotrophs (Lafont *et al.* 2010), and so on. The technique can be applied to visualise the pituitary *in vivo* via cranial window and intravital microscopy or *in vitro* on pituitary slices (Lafont *et al.* 2010). The method creates three-dimensional reconstructions of the pituitary gland to allow optical planes to be selected and cell positions to be identified precisely. The tissue is subjected to a calcium indicator and a laser pulsed via an array of mirrors yielding 64 beamlets for rapid multi-photon imaging of a large tissue volume (100 μm x 100 μm x 40 μm) (see Hodson *et al.* 2010). These experiments could be combined with the administration of LPS conjugated with a fluorochrome such as fluorescein isothiocyanate (FITC) or rhodamine, to monitor the effects of LPS on Ca^{2+} -induced hormone secretion in *Fpr2/3^{-/-}* mice *in vivo* (Feng *et al.* 2011; Vagaja *et al.* 2013). In summary, this technique marries spatial information of pituitary cell networks with functional measurements of Ca^{2+} -induced hormone exocytosis, and has also been applied to other endocrine cell types such as the pancreas (see Schaeffer *et al.* 2011). In addition, the two-photon microscopy technique could be applied to human stem cells that have undergone self-organisation into a three-dimensional culture of interacting pituitary cells akin to that of an intact pituitary gland (Suga *et al.* 2011). Further research could combine these advances in three-dimensional cultures of stem cell-derived human pituitary glands with two-photon microscopy, to allow functional and spatial information to be obtained simultaneously, and on tissues that are directly relevant to the human family of FPR subtypes.

With regard to the adrenal gland, further work should seek to understand adrenal insufficiency in conditions such as sepsis-related endotoxaemia and Addison's disease, as these can lead to septic shock or Addisonian crises which are fatal if poorly managed (Marik 2002). Corticosteroid therapies have undesirable side effects and the dosing regimen requires careful management to meet large variations in physiological demand. Recent advances in methods for obtaining, culturing, and transplanting adrenocortical stem cells could be used to investigate whether adrenal insufficiency, can be prevented by

targeting FPR subtypes with drugs, or treated by regeneration of the adrenal cortex with stem cells. Studies of the latter have been found to improve the survival rates of mice under stress following bilateral adrenalectomy (Zupekan & Dunn 2011). Adrenocortical stem cells can now be obtained by deformability-activated cell sorting (DACS) of progenitor cells which exhibit less clustering and steroidogenic capacity than differentiated cells, avoiding the use of lipophilic dyes such as Nile Red that are incompatible with clinical transplantation (Hur *et al.* 2012). Further work in this area is important because whilst corticosteroids can reverse septic shock, they are scarcely sufficient to reverse the underlying adrenal insufficiency in up to 65% of patients with sepsis (Briegel *et al.* 1996; Marik 2002; Marik & Zaloga 2003).

8.3 Conclusion

These studies have contributed to a growing body of knowledge on the role of formyl peptide receptors in pituitary and adrenal function, and have provided evidence in support of the hypothesis. Within the pituitary gland, ACTH was colocalised with FPR3 in MS-diseased humans and with GFP in Fpr2/3^{-/-} mice which retained more adrenocortical sterols following experimental endotoxaemia than wildtypes. The secretion of pituitary ACTH was generally stimulated by mouse Fpr2-selective, and inhibited by mouse Fpr3/Alx- or rat Fpr2l/Alx-selective agonists within four hours. The role of Fpr/FPR subtypes in adrenal corticosterone secretion remains complicated by the observation that the Fpr1-/FPR1-selective fMLF was the only agonist whose inhibitory effect was prevented by the Fpr2-/FPR2/ALX-selective antagonist WRW₄ within four hours. Experimental endotoxaemia in rats induced adrenal insufficiency in terms of reduced adrenocortical sterol vacuolation and corticosteroid secretion in response to ACTH *in vitro* that was maximal within four hours. Taken together, these findings provide evidence in support of a modulatory role of formyl peptide receptor subtypes, particularly rodent Fpr2, Fpr2l/Alx, Fpr3/Alx and human FPR3, in terms of pituitary and adrenal stress hormone secretion following inflammogenic stressors and endogenous secretagogues. The findings could have implications for understanding the dissociation of pituitary and adrenal function in inflammogenic diseases such as sepsis-related endotoxaemia.

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