Investigating the molecular, metabolic, and psychological effects of minocycline



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Abstract

An immune component has been suggested to underlie the pathophysiology of psychiatric diseases, and is thought to involve pro-inflammatory cytokines. Thus, there is the potential for drugs with anti-inflammatory properties to act as therapeutic agents for psychiatric illnesses. Minocycline has shown promise in pre-clinical animal models and early phase clinical trials for a variety of psychiatric disorders. Previous studies on minocycline have also shown its ability to suppress microglia activity and reduce inflammatory cytokine levels, and its behavioural effects on depressive-like behaviour in animals and humans. However, the underlying mechanisms that lead to minocycline's psychotropic effects are not clear.

This dissertation investigated the proposal that the anti-inflammatory mechanisms of minocycline underlie its psychotropic effects, as well as explored the pathways that mediate inflammation-induced behavioural changes. To that end, four studies were conducted. The first study looked at inflammation-induced modulation of GluN2 subunit expression and downstream signalling, effects which were rescued by prior treatment with minocycline (Chapter Two). The second study explored the effects of inflammation and minocycline administration on plasma and brain metabolites in mice (Chapter Three). The third study and fourth study investigated the psychological effects (Chapter Four) and biochemical effects (Chapter 5) of an acute dose of minocycline in healthy volunteers. It was revealed that minocycline administration decreased fear misclassification, increased contextual learning, decreased levels of the inflammatory marker CRP, and increased serum lipoprotein.

Overall, minocycline was able to ameliorate inflammation-induced changes, suggesting that anti-inflammatory mechanisms are linked to glutamatergic signalling and synaptic transmission which lead to downstream psychotropic effects. However, metabolomic analysis also revealed changes in lipid metabolism and energy-related metabolites that could potentially underlie a separate mechanism for minocycline's actions. Nevertheless, given the association between metabolites, and inflammation, it is possible that these pathways interact.

Declaration

This dissertation is the result of my own work and includes nothing that is the outcome of work done in collaboration except as specified in the text.

I state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Oxford or any other University of similar institution.

In accordance with the guidelines, this thesis does not exceed 50,000 words, and it contains less than 150 figures.

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List of Abbreviations

1D – One dimensional

2D – Two dimensional

5-HIAA - 5-hydroxyindoleacetic acid

5HTR – Serotonin receptor

5HTT – Serotonin transport

ADP – Adenosine di-phosphate

AMPAR – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ANOVA – Analysis of variance

APP – Acute phase proteins

APR – Acute phase response

Arc – Activity-regulated cytoskeleton-associated protein

ASM - Acidic sphingomyelinase

ATP – Adenosine tri-phosphate

AWERB - Animal Welfare and Ethical Review Body

BBB – Blood Brain Barrier

BCAA – Branched chain amino acids

BDI – Becks Depression Inventory

BDNF - Brain-Derived-Neurotrophic-Factor

BFS – Befindlichkeits scale

BMI – Body mass index

CA – Cornu Ammonis

CamKII – Calcium/calmodulin-dependent protein kinase II

CANTAB - Cambridge Neuropsychological Test Automated Battery

CGI – Clinical Glocal Impression questionnaire

CNS – Central Nervous System

COSY – Correlation spectroscopy

COX – Cyclooxygenase

CPMG – Carr-Purcell-Meiboom-Gill

CPT-IP – Continuous Performance Test – Identical Pairs

CRP – C reactive protein

CSF – Cerebrospinal fluid

CUREC – Central University Research Ethics Committee

CUS – Chronic unpredictable stress

CVO – Circumventricular organs

D_2O – Deuterium Oxide

DAMP - Damage-associated molecular patterns

DG – Dentate gyrus

dH_2O – Deionized water

DSM V – Diagnostics and Statistical Manual of Mental Disorders, 5th Edition

EAAT – Excitatory Amino Acid Transport

ECAT – Emotional Categorization Task

EDTA – Ethylenediaminetetraacetic acid

eEF2 – Eukaryotic elongation factor 2

ELISA – Enzyme-linked immunosorbent assays

EMEM – Emotional Recognition Memory Task

EPQ – Eysenck Personality Questionnaire

EREC – Emotional Recall Task

ERK – Extracellular signal-regulated kinases

ETB – Emotional test battery

FC – Frontal Cortex

FERT – Facial Expression Recognition Task

FST – Forced swim test

GABA – γ-Aminobutyric acid

GluN2 – NMDAR subunit 2

GPCR – G-Protein Coupled Receptor

GS – Glutamine synthetase

HAM-D – Hamilton Depression Rating Scale

Hc – Hippocampus

HCl – Hydrochloric acid

HDL – High density lipoprotein

HMDB – Human metabolome database

HPA – Hypothalamic-pituitary-adrenal

ICAD – Inflammatory cytokine-associated depression

IDO – Indoleamine 2,3-dioxygenase

IFNγ – Interferon-γ

IL-1β – Interleukin-1β

IL-1r – Interleukin-1 receptor

IL-6 – Interleukin-6

IL-10 – Interleukin-10

i.p. – Intraperitoneal

ISH – *in situ* Hybridization

KA – Kynurenic acid

KAT – Kynurenine aminotransferase

KOH – Potassium hydroxide

KMO – Kynurenine 3-monooxygenase

LAT1 – Large neutral amino acid Transport

LDL – Low density lipoprotein

LPS – Lipopolysaccharide

LTP – Long term potentiation

LVLDL – Large very low density lipoprotein

MADRS – Montgomery-Asberg Depression Rating Scale

MAP – Mitogen activated protein

MAO – Monoamine oxidase

MDD – Major Depressive Disorder

MHC – Major Histocompatibility Complex

mRNA – messenger RNA

MRS – Magnetive resonance spectroscopy

MS – Mass spectrometry

mTOR – Mammalian target of rapamycin

NAA – N-Acetyl aspartate

NART – National Adult Reading Test

NF H_2O – Nuclease-free water

NLRP3 – Nod-like receptor pyrin domain-containing 3

NMDAR – N-methyl D-aspartate receptor

NMR – Nuclear magnetic resonance spectroscopy

NO – Nitrous oxide

NOESY – Nuclear Overhauser Effect Spectroscopy

NOS – Nitric oxide synthase

OPLS-DA – Orthogonal partial least squares discriminant analysis

P2X7R – Purinergic type 2X7 receptor

PAMP – Pathogen-associated molecular patterns

PANAS – Positive and Negative Affective Scale

PBS – Phosphate buffered saline

PCA – Principal component analysis

PFC – Pre-frontal cortex

PRR – Pattern recognition receptors

PUFA – Poly-unsaturated Fatty Acid

QC – Quality control

QUIN – Quinolinic acid

qPCR – quantitative polymerase chain reaction

RS – Retrosplenial cortex

RSD – Spectral relative standard deviation

SAA – Serum amyloid A

SAM – S-adenosyl methionine

SAP – Spontaneous activity-driven potentiation

SART – Sustained Attention to Response Test

SDS – Sodium Dodecyl Sulphate

S.E.M. – Standard error mean

siRNA – small interfering RNA

SNRI – selective noradrenaline reuptake inhibitors

SSC – Saline sodium citrate

SSRI – Selective serotonin reuptake inhibitor

STAI – Stait-Trait Anxiety Index

TBARs – thiobarbituric acid reactive substances

TC – Technical control

TdT – Terminal Deoxynucleotidly Transfer

TEA – Triethanolamine

TFRC – Transferrin receptor

TLR4 – Toll-like receptor

TNFα – Tumour necrosis factor α

TrkB – Tropomyosin receptor kinase B

TSP – 3-trimethylsilylpropanoic acid

TSPO – Translocator protein

TST – Tail suspension test

VIP – Variable importance in projection

VAS – Bond-Lader visual analogue scale

VLDL – Very low density lipoprotein

Chapter 1: Introduction

1.1 The immune system and the central nervous system

While the immune system and the central nervous system (CNS) have very different functions, the distinct elements that make up these systems have been shown to perform similar functions, and to interact with each other, influencing downstream behavioural changes.

1.1.1 Glia: Immune-like cells in the brain

Glial cells consist mainly of astrocytes, microglia and oligodendrocytes. They are non-neuronal cells in the brain that were once thought to function solely as the "glue" between neurons, but have since been discovered to perform important support functions (Jäkel & Dimou, 2017). In particular, microglia and astrocytes have been shown to perform functions linked to immunomodulatory effects, such as antigen presentation and the release of cytokines and chemokines (Constantinescu et al., 2005; J. A. Smith, Das, Ray, & Banik, 2012).

Microglia are viewed as the brain's resident immune-like cells. They perform similar functions to macrophages, including immune surveillance of the brain and phagocytosis (Kreutzberg, 1996; Ousman & Kubes, 2012). Activated microglia have been shown to release potentially neurotoxic substances such as nitrous oxide (NO), glutamate, and reactive oxygen species, and have been proposed to be a key player driving neuroinflammation and excitotoxicity (Kreutzberg, 1996; Takeuchi, 2013). Given its functions, enzymes expressed in microglia include inflammatory markers such as nitric oxide synthase (NOS), and kynurenine 3-monooxygenase (KMO), which catalyses the conversion of kynurenine to quinolinic acid (QUIN), a product of the kynurenine pathway (Guillemin et al., 2001; Sierra et al., 2014).

Astrocytes are involved in synaptic transmission and the maintenance of homeostasis in the brain (Bélanger & Magistretti, 2009). They also play a key role in the recycling of glutamate by expressing glutamate transporters, which are involved in the uptake of glutamate into

astrocytes from the synaptic cleft, and glutamine synthetase (GS), that converts glutamate to the less harmful glutamine (Rose, Verkhratsky, & Parpura, 2013). Astrocytes also express kynurenine aminotransferase (KAT), which catalyses the conversion of kynurenine to kynurenic acid (KA), a product of the kynurenine pathway (Guillemin et al., 2001).

Aberrant glial function has been reported in psychiatric diseases (Jo, Zhang, Emrich, & Dietrich, 2015; Rajkowska & Stockmeier, 2013). Patients with mood disorders have been observed to have decreased glial numbers in the pre-frontal cortex and limbic system in a post-mortem study (Ongur, Drevets, & Price, 1998; Torres-Platas, Nagy, Wakid, Turecki, & Mechawar, 2016). In a mouse chronic unpredictable stress model, glial ablation resulted in depressive-like behaviours such as anhedonia, and anxiety (Mounira Banasr & Duman, 2008). Increased microglia activation was also observed in multiple brain regions in post-mortem studies of depressed suicides (Steiner et al., 2008; Torres-Platas, Cruceanu, Chen, Turecki, & Mechawar, 2014). As immune functions within the CNS contribute to behavioural changes, this suggests that the immune system may also be involved.

1.1.2 Inflammatory mechanisms: the acute phase response

The acute phase response (APR) is part of the innate immune system against threats such as bacterial infection and stress. It involves the altered production of acute phase proteins (APP) by the liver. APPs can be classified as positive or negative, based on whether their production is increased or decreased respectively. Two major APPs in humans are C reactive protein (CRP) and serum amyloid A (SAA), whose levels are increased more than ten-fold during the APR, making them ideal markers of inflammation (Cray, Zaias, & Altman, 2009).

The APR is induced by the actions of pro-inflammatory cytokines, and thus, the peak increase of APPs is after that of pro-inflammatory cytokines. APP levels also remain raised for around 48 hours, and have a more prolonged effect than pro-inflammatory cytokines (Gruys, Toussaint, Niewold, & Koopmans, 2005). The APR has been shown to be robustly induced by the inflammatory agent lipopolysaccharide (LPS) in animal models (Chiba et al., 2011; Wait et al.,

2005). The APR has also been linked to altered brain function, as an APR has been observed in rodent chronic stress models, and in patients with schizophrenia, depression, and mania (Maes et al., 1997). Therefore, communication pathways must exist between the brain and the peripheral immune system.

1.1.3 Immune-brain interactions

The CNS was once thought to be an immune privileged system due to the lack of an immune response against implanted tissue grafts in the CNS parenchyma. The traditional view was that the blood brain barrier (BBB) restricted immune cell entry to the brain, and the lack of a lymphatic system in the CNS meant that antigens in the CNS were considered invisible to the systemic immune system. (Galea, Bechmann, & Perry, 2007)

However, increasing evidence has shown that there are interactions, and that the efferent and afferent arms of neuro-immune interactions are more complex than previously thought. Recent research has challenged the view that the CNS lacks a lymphatic system, and studies using detectable tracers have shown that CNS interstitial fluid and cerebrospinal fluid (CSF) drain into cervical lymph nodes through intra-mural perivascular pathways and lymphatic vessels in the dura mater surrounding the brain, respectively (Engelhardt, Vajkoczy, & Weller, 2017). The CNS has also been shown to mount an immune response through its resident immune cell, the microglia, and activate mechanisms that recruit peripheral immune cells, such as T-cells, to the CNS (Prinz & Priller, 2017). In addition, the inflammatory reflex, where vagus nerve activation suppresses peripheral cytokine synthesis through splenic T-cells, is another potential route for a bi-directional communication between the brain and the immune system (Pavlov & Tracey, 2017).

The brain can influence the immune system via the hypothalamic-pituitary-adrenal (HPA) axis, through release of glucocorticoids that suppress the function of various immune cells, the autonomic nervous system, and through small molecule compounds such as catecholamines and neuropeptides (Bellavance & Rivest, 2014; Procaccini, Pucino, De Rosa,

Marone, & Matarese, 2014). However various pathways also exist through which the immune system can modulate behaviour. One key example is infection-mediated sickness behaviour, mainly classified by decreased mobility that leads to depressed mood, and cognitive impairments, through inflammatory cytokines binding to their cognate receptors on paraganglia surrounding the vagus nerve (Maier & Watkins, 1998). Through such pathways, peripheral immune changes are able to induce an immune response in the CNS without direct access to it. Circumventricular organs, such as the pineal gland and median eminence, provide another access point. They are located around the third and fourth ventricles, and defined as so-called "leaky" structures in the brain that have extensive vasculature and lack a normal BBB, allowing for the exchange of substances between the CNS and peripheral blood flow (Duvernoy & Risold, 2007; Quan, 2008).

What is clear is that significant neuropathology and behavioural deficits can result from a compromised peripheral immune system. Thus, while traditional models of psychiatric disorders have focused on the dysregulation of various neurotransmitters, particularly the monoaminergic and glutamatergic systems, there are suggestions that a dysfunctional immune system may underlie the neuropathophysiology of several psychiatric illnesses (Lotrich, 2015; Muller & Schwarz, 2007).

1.2 Inflammation in the pathology of psychiatric diseases

Many studies support the idea that there is an immune component in the pathophysiology of psychiatric diseases. For example, some anti-psychotics and antidepressants have been shown to have anti-inflammatory properties (Obuchowicz, Bielecka, Paul-Samojedny, Pudelko, & Kowalski, 2014; Sugino, Futamura, Mitsumoto, Maeda, & Marunaka, 2009). Chronic inflammatory diseases also have a high co-morbidity with psychiatric diseases such as depression (Walker, Kavelaars, Heijnen, & Dantzer, 2014). Parallels between neuroinfectious diseases and neurological/psychiatric disorders provide a hint of the mechanisms involved.

1.2.1 Neuroinfectious Diseases

Neuroinfectious diseases result in lasting neural and behavioural changes, such as depression, decreased cognition, and reduced motor functions, long after the infection has been cleared, suggesting that the damage was not caused by the pathogen but was immune-mediated. For example, patients who have recovered from bacterial meningitis may present with cognitive deficits or dementia (Grindborg, Naucler, Sjolin, & Glimaker, 2015), and patients recovering from tuberculous meningitis frequently display behavioural abnormalities and psychiatric disorders (Cherian & Thomas, 2011). The immune response to the pathogen involves alteration of the blood brain barrier, allowing the entry of T-cells, activation of microglia and astrocytes, and the production and release of signalling molecules such as cytokines, nitrogen species, and oxidants, that contribute to neurotoxicity (Klein, Garber, & Howard, 2017). A marker of microglial activation, the translocator protein, has also been shown to be more abundant in patients with schizophrenia (Bloomfield et al., 2016). Indeed, the Major Histocompatibility Complex (MHC) has been linked with this disorder in a recent genome-wide association study (Mukherjee et al., 2014). Furthermore, the activation of the complement system in response to pathogens has been suggested to promote synapse remodelling and elimination, which may underlie neuronal damage (Hong et al., 2016).

Therefore, it is likely that similar events occur in the pathology of psychiatric diseases i.e. aberrant microglia activation, and reactivation of developmental pathways regulating synaptic plasticity lead to abnormal neuronal function which eventually manifest as anomalous behaviours. In neuroinfectious diseases, these responses are brought about by recognition of pattern recognition receptors (PRRs) on microglia recognizing pathogen-associated molecular patterns (PAMPs) (Klein et al., 2017), a mechanism mirrored in the response to psychological stress mediated by the inflammasome.

1.2.2 The inflammasome

The inflammasome, an intracellular multiprotein complex, contains PRRs that enable them to detect pathogens, but are also able to respond to other danger signals aside from PAMPs (Kaufmann et al., 2017).

Several types of inflammasomes have been characterized on the basis of their protein components, but the Nod-like receptor pyrin domain-containing 3 (NLRP3) inflammasome has gained particular interest. The NLRP3 inflammasome has a cytosolic PRR and is activated by a broad array of damage-associated molecular patterns (DAMPs). This includes signals like elevated levels of fatty acids and glucose that could link inflammation to systemic diseases (Iwata, Ota, & Duman, 2013). Thus, the NLRP3 inflammasome functions as an adaptor that translates potentially harmful signals to an immune response. For example, it has been shown to be activated by psychological stress, providing the link between stress, inflammation and depression. Iwata et al. have conducted a series of experiments with a rodent chronic unpredictable stress (CUS) model of depression that demonstrates how this pathway works. Stress results in a release of adenosine tri-phosphate (ATP) that activates the Purinergic type 2X7 receptor (P2X7R) receptor which in turns results in the formation and activation of the NLRP3 inflammasome, leading to downstream depressive behaviour. These behaviours are ameliorated with a P2X7R antagonist and Nlrp3-null mice, thereby supporting the role of these components in the development of stress-mediated depression (Iwata et al., 2016).

The NLRP3 inflammasome contains a caspase-1 that can be activated to cleave pro-interleukin-1β (IL-1β) to the active IL-1β form, stimulating downstream immune processes (Lechtenberg, Mace, & Riedl, 2014). This release of IL-1β is thought to be the underlying factor driving the progression of pathology in psychiatric diseases like depression. Given that cytokines coordinate many of the immune responses involved in physiology and pathology, it stands to reason that cytokines play a key role in neuroinflammation and the translation of peripheral inflammation to pathology in the CNS.

1.2.3 The role of cytokines

Pro-inflammatory cytokines, such as IL-1β, Interleukin-6 (IL-6), Tumour Necrosis Factor α (TNFα), and Interferon γ (IFNγ), have been proposed to mediate the pathophysiology of psychiatric disorders (Al-Hakeim, Al-Rammahi, & Al-Dujaili, 2015; Paul-Samojedny et al., 2011; Rothwell, Allan, & Toulmond, 1997). These proteins, mainly produced by immune cells, facilitate cell-to-cell communication in the innate inflammatory response, and their elevation in the blood have been associated with neurodegeneration (Allan & Rothwell, 2001), schizophrenia (Di Nicola et al., 2013; Petrikis et al., 2015) and mood disorders (Hillhouse & Porter, 2015). Further evidence for pro-inflammatory signals mediating brain dysfunction come from studies reporting that depressive symptoms with suicide ideation are one of the side effects of cytokine therapy for Hepatitis C and cancer (Raison et al., 2010; Schaefer et al., 2004). Furthermore, inducing inflammation with endotoxins such as lipopolysaccharide (LPS), that increase levels of pro-inflammatory cytokines, results in 'depressive-like' behaviours in animals (Frenois et al., 2007), and increased anxiety and depressed mood and decreased performance in memory tasks in healthy volunteers (Reichenberg et al., 2001).

These cytokines are rapidly induced in response to tissue injury, infection, or inflammation (Allan & Rothwell, 2001), but have also been found to play important physiological roles in unchallenged-tissue. For example, cytokines have been proposed to play important roles in cognitive functions such as learning and memory. Baune et al. demonstrated that mice deficient in TNF and its receptors performed significantly worse in behavioural tasks assessing cognition such as the novel object test (Baune et al., 2008). Likewise, Avital et al. discovered that IL-1 and its receptor are needed for hippocampal-dependent memory tasks. The IL-1 receptor type I (IL-1R1) knock-out mice were significantly worse than control mice in the spatial water maze task (Avital et al., 2003). This suggests that cytokines perform dual functions, with basal levels necessary for synaptic plasticity and neurogenesis, but under- or over-expression leading

to impairment. Given their inverted U-shape function, the levels of cytokines are therefore tightly regulated. This is best demonstrated by IL-1β.

Interleukin-1β

IL-1β is regarded as the key cytokine linking inflammation to psychiatric diseases, and there are multiple layers of control over its levels. The interleukin-1 family consists of IL-1α, IL-1β, and the IL-1 receptor antagonist (IL-1ra). These cytokines bind to two receptors – IL-1 receptor type I and type II (IL-1R1 and IL-1R2 respectively). IL-1α and IL-1β have high sequence homology and mainly differ in terms of location. IL-1α is based in cells, whereas IL-1β is secreted. On the other hand, IL-1ra binds with high affinity to IL-1R1 and is an endogenous antagonist of IL-1β. Furthermore, IL-1β binds with high affinity to IL-1R2, but IL-1R2 is a decoy receptor and does not result in any further downstream signalling. Finally, IL-1β is produced in an inactive form, and has to be cleaved by caspase-1 for activation. (McAfoose & Baune, 2009).

While basal levels of IL-1β has been shown to be important for synaptic function and long term potentiation (LTP), pathophysiological levels of IL-1β impair LTP and result in detrimental effects on learning and hippocampal-dependent memory (Goshen et al., 2007; Khairova, Machado-Vieira, Du, & Manji, 2009). With regards to psychiatric diseases, a combination of stress and IL-1β has been shown to decrease neurogenesis in the adult hippocampus, a phenomenon linked to anhedonia (Goshen et al., 2008). IL-1β also plays a key role in inflammasome-mediated neuroinflammation, where activation of the inflammasome leads to the formation of mature, active IL-1β. IL-1β is said to be the most potent of the pro-inflammatory cytokines in the brain (Maier & Watkins, 1998) and blocking IL-1β dampens much of sickness behaviour.

However, it is not entirely clear how increased levels of cytokines in the CNS lead to downstream behavioural deficits. Links between neurotransmitter pathways and the immune system suggest an answer. Changes in glutamate, the most abundant neurotransmitter in the brain, has been proposed in several psychiatric disorders, and it has been suggested that

cytokines can directly modulate glutamatergic neurotransmission through the N-Methyl-D-Aspartate receptor (NMDAR) (B. Viviani et al., 2003).

1.3 Glutaminergic signalling in psychiatric diseases

Glutamate is the main excitatory neurotransmitter in the brain, and glutaminergic signalling is involved in many key functions in the brain and implicated in many neurodegenerative and psychiatric diseases. A broad range of receptors are activated by glutamate – this includes NMDA receptors, AMPA receptors, kainate receptors, and metabotropic glutamate receptors.

1.3.1 The N-methyl D-aspartate Receptor

1.3.1.1 NMDAR Structure and Function

The NMDARs are a class of ionotropic glutamate receptors widely expressed in the brain. They are composed of heterotetramers -two glycine-binding GluN1 subunits and two glutamate-binding GluN2 subunits (GluN2A, GluN2B, GluN2C and GluN2D) (M. L. Blanke & Van Dongen, 2009). This composition allows for NMDAR variability at different stages of development or in different regions of the brain, where different combinations of subunit variants can form NMDARs with different properties. Recent research has also discovered a new class of NR subunits – GluN3 - that can replace GluN2 in the tetramer, further increasing NMDAR variability. In the adult brain, the majority of NMDARs are a combination of GluN1 with GluN2A and/or GluN2B (Papadia & Hardingham, 2007).

NMDAR subunits have three main components – an N-terminal extracellular domain, a transmembrane channel pore, and a C-terminal intracellular tail (Monaghan & Jane, 2009). The extracellular domain contains the binding sites for NMDAR agonists, such as glutamate and NMDA, and co-agonists, such as glycine and D-serine (Leeson & Iversen, 1994). The transmembrane channel is permeable to ions such as sodium and calcium upon activation, and is otherwise blocked by a magnesium ion (Nowak, Bregestovski, Ascher, Herbet, & Prochiantz,

1984). The intracellular tail recruits signaling proteins downstream of NMDAR activation, including kinases, such as post synaptic density (PSD95), and calcium-dependent signaling molecules, such as nitric oxide synthase (NOS) (Sheng, 2001). The NMDAR has been found to be upstream of signalling molecules such as extracellular signal-regulated kinase 1 and 2 (ERK1/2), and calcium/calmodulin-dependent protein kinase II (CamKII) that are involved in many key signalling pathways (Cao, Ren, Zhu, Zhao, & Zhang, 2012; Chiba et al., 2011). NMDAR activation is tightly regulated. For example, the binding of both an agonist and a co-agonist is required. Also, the magnesium ion block in the transmembrane pore has to be removed before the channel is permeable to ions (Evans, Francis, & Watkins, 1977). This requires the membrane to be partially depolarized by sodium influx through AMPA receptors (M.L. Blanke & VanDongen, 2009). The high level of regulation suggests that NMDARs regulate important functions.

At physiological levels, NMDARs play important roles in neurodevelopment (Burnashev & Szepetowski, 2015), synaptic plasticity (Hunt & Castillo, 2012), learning (Morris, Anderson, Lynch, & Baudry, 1986), and memory (Riedel, 2003). Conversely, dysregulation of NMDARs is associated with some neuropsychiatric disorders, with hypoactivity linked to cognitive impairment (Umbricht et al., 2000), and overstimulation linked to excitotoxicity and neurodegeneration (Zhou, Ding, Chen, Yun, & Wang, 2013). This has led to the inverted-U curve hypothesis of NMDAR function (Lipton & Nakanishi, 1999), where too little or too much activation of NMDARs could lead to neuronal cell death.

1.3.1.2 NMDARs in psychiatric diseases

The potential of NMDAR neuromodulators as therapeutic interventions in psychiatric diseases supports the theory that glutaminergic transmission is dysregulated in psychiatric diseases. For example, NMDAR co-agonists (glycine, D-serine, D-alanine) have been shown to have therapeutic efficacy when used as adjunctive therapies in treating the negative symptoms of schizophrenia (Balu & Coyle, 2015; Tsai, Yang, Chang, & Chong, 2006). NMDAR antagonists, such as nitrous oxide, lanicemine and ketamine, have shown promise in pre-clinical models and

early-phase clinical trials of depression, and have been hailed as a new class of antidepressants (Berman et al., 2000; Downey et al., 2016; Zorumski, Nagele, Mennerick, & Conway, 2015). In particular, a large body of research has been conducted on ketamine, suggesting a possible mechanism by which NMDAR dysregulation links to psychiatric diseases.

The case for ketamine

Ketamine is a non-competitive NMDAR antagonist, discovered to induce rapid and long-lasting antidepressant effects at a single sub-anesthetic dose (Berman et al., 2000). Key elements mediating the antidepressant effects of ketamine have been elucidated. Ketamine blockade of NMDARs has been shown to lead to downstream de-suppression of Brain-Derived-Neurotrophic-Factor (BDNF) translation (Autry et al., 2011), which together with its receptor, Tropomyosin receptor kinase B (TrkB), has been positively correlated with antidepressant efficacy, possibly through their roles in synaptogenesis and neurogenesis (R. S. Duman & Monteggia, 2006; Saarelainen et al., 2003). BDNF has also been shown to activate the mammalian target of rapamycin (mTOR) signalling pathway (Nosyreva et al., 2013), which is involved in protein synthesis and increased excitatory neurotransmission (Miller et al., 2014). Finally, ketamine administration also activated growth factor signaling proteins, increased levels of synaptic proteins and AMPAR subunits, and increased dendritic spine densities (N. Li et al., 2010).

While the role of NMDARs in psychiatric diseases is not yet fully understood, with conflicting theories (active inhibition versus disinhibition) and evidence (both antagonists and co-agonists are able to act as antidepressants) (Chan, Matthews, & Burnet, 2016), it is clear that NMDARs are important in the pathophysiology of psychiatric diseases. However, what factors lead to dysregulation of NMDARs? Pathways linking inflammation and NMDARs suggest that peripheral inflammation may be involved.

1.3.1.3 Links between NMDARs and inflammation

Several mechanisms link inflammation to NMDARs. One of the more established mechanisms is through the kynurenine pathway. However, there is also evidence supporting that IL-1β can play a direct role on NMDAR modulation.

Kynurenine Pathway

The kynurenine pathway (Fig 1.1) is involved in the catabolism of tryptophan in a series of enzyme-mediated reactions (Schwarcz & Pellicciari, 2002). Therefore, the products of the pathway are dependent on the activity and types of enzymes available, as different enzymes are expressed in different cell types and modulated by different signals.

Tryptophan, an essential amino acid, can be processed in two main pathways to form either serotonin or kynurenine. The enzyme indoleamine-2,3-dioxygenase (IDO) metabolizes tryptophan to form kynurenine, and kynurenine can in turn be further metabolized by KMO and KAT to form QUIN and KA respectively (Guillemin et al., 2001; Hillhouse & Porter, 2015).

The products of the kynurenine pathway are of particular interest as they both bind to the NMDAR glycine site, but have contrasting effects. QUIN is produced by microglia and is a NMDAR agonist, while KA is produced by astrocytes and is a NMDAR antagonist (Guillemin et al., 2001). The kynurenine pathway is linked to inflammation, as pro-inflammatory cytokines have been shown to induce IDO, therefore shuttling tryptophan away from serotonin synthesis, and resulting in the depletion of serotonin (O'Connor et al., 2009). Therefore, peripheral inflammation and high levels of pro-inflammatory cytokines would result in the increased levels of kynurenine and its downstream products QUIN and KA, and modulation of NMDAR activity. What is unclear is how it is determined which kynurenine metabolite is formed. The Kynurenine pathway also fails to take into account the possible direct role of IL-1β on NMDARs.

IL-1R1-mediated Pathway

A second possible mechanism by which inflammation affects NMDARs is through the pro-inflammatory cytokine IL-1β. Viviani et al. have shown that administration of IL-1β to

primary hippocampal neurons resulted in an increase of NMDA-induced intracellular calcium and increased phosphorylation of the NMDA receptor subunits NR2A/NR2B. This effect was attenuated by addition of IL-1 receptor antagonist (IL-1ra) (B. Viviani et al., 2003; Viviani et al., 2006). The same group showed that IL-1β affected NMDAR phosphorylation through the Src family of kinases. Gardoni et al. built on this research by showing that the IL-1 receptor, IL-1 receptor accessory protein and NR2B co-immunoprecipitate from the same sub-cellular location (Gardoni et al., 2011). Therefore, it is possible that upon activation by IL-1β, IL-1R1 forms a signalling complex that includes the IL-1R accessory protein which initiates a signalling cascade that activates the Src family of kinases that in turn phosphorylate and hyperactivate NR2B (Fig 1.2).

Hence, peripheral inflammation and increased pro-inflammatory cytokine levels could result in increased cytokine levels in the CNS through vagus nerve activation, uptake through the BBB and diffusion through leaky CVOs. This in turn results in the formation of a IL-1R1 signalling complex that activates signalling pathways to phosphorylate and hyperactivate NR2B.

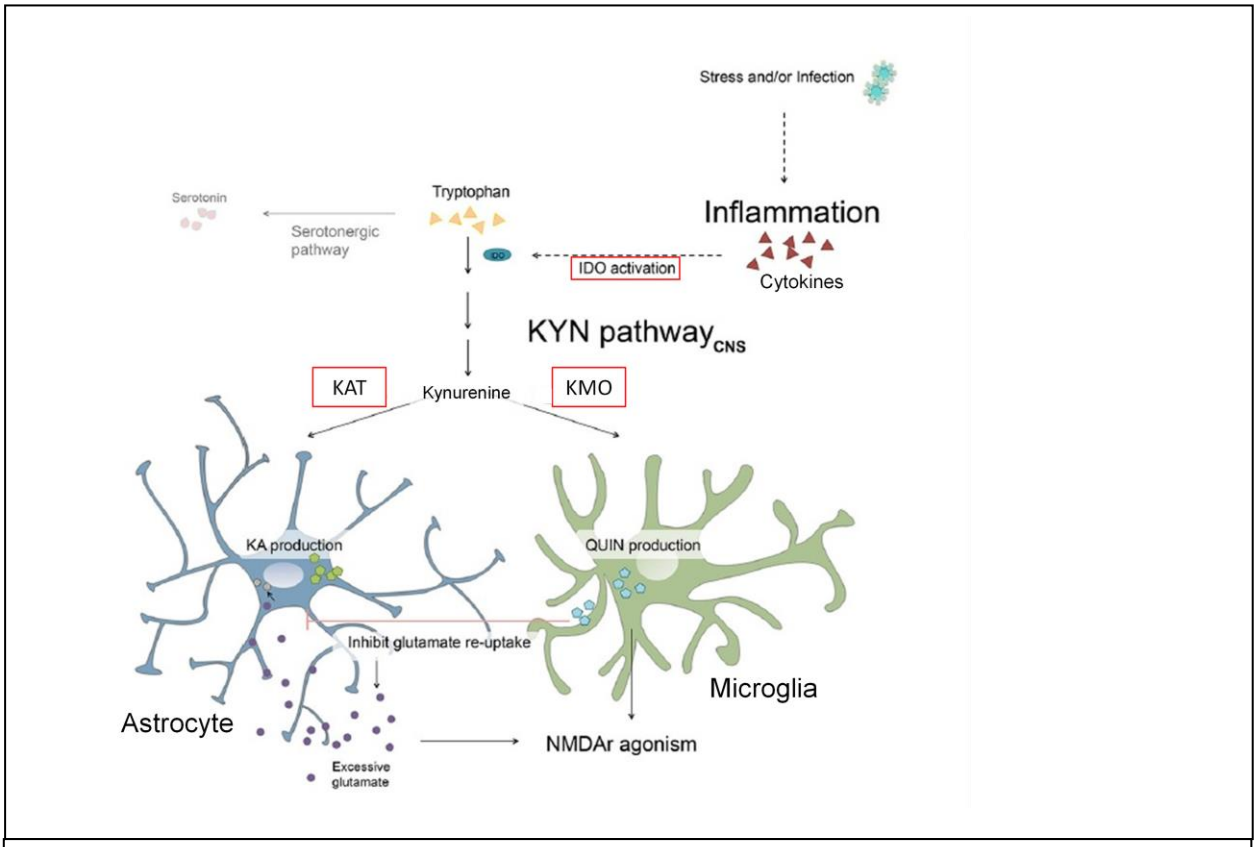


Fig 1.1 The kynurenine pathway (adapted from Jo et al., 2015) showing the metabolism of tryptophan to kynurenine and its neuroactive metabolites with the enzymes catalysing key reactions highlighted in red.

IDO: Indoleamine 2,3-dioxygenase; KAT: Kynurenine Aminotransferase; KMO: Kynuremine mono-oxygenase; KA: Kynurenic Acid; QUIN: Quinolinic acid

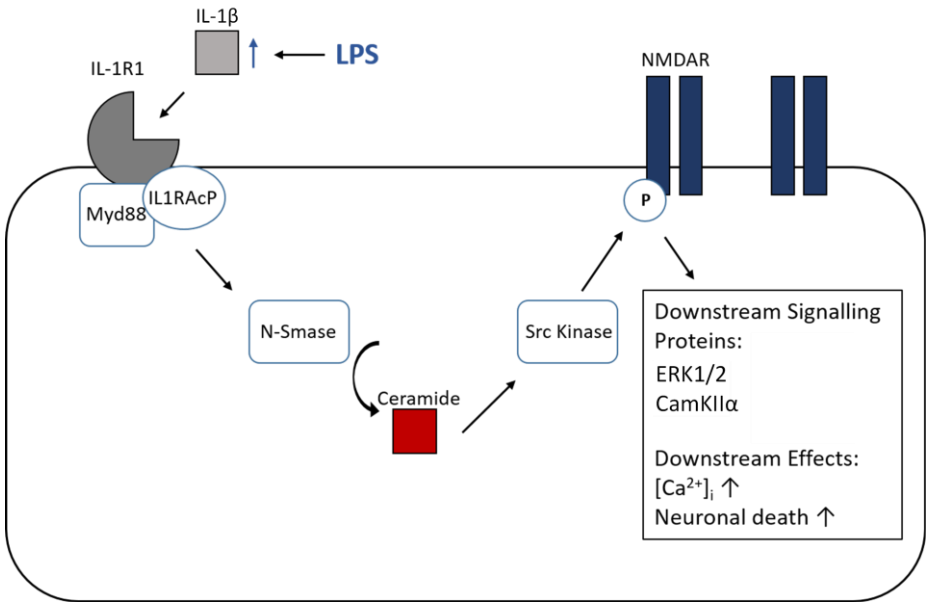


Fig 1.2 The IL-1R1 pathway showing how increased IL-1β increased NMDAR activity through Src kinase phosphorylation of GluN2B

IL-1R1: Interleukin-1 receptor 1; IL1RAcP: IL-1R accessory protein; Myd88: Myeloid differentiation primary response 88; N-smase: Neutral sphingomyelinase

1.4 Metabolic changes in psychiatric diseases

The metabolome is a collection of all the small molecule metabolites in a sample at a given point of time. Metabolites include amino acids, lipids, carbohydrates and nucleic acids, and are the products of biochemical processes catalysed by enzymes. They are highly variable, and are able to reflect the influence of both genetic background and environmental factors (for example, diet and stress) (Larrieu et al., 2017; Thévenot, Roux, Xu, Ezan, & Junot, 2015). Therefore, different phenotypes (for example, tissue type, biofluids, disease state) have different metabolic profiles. Key metabolic changes that are distinct between phenotypes can be identified through metabolomics - the study of the metabolome combined with multivariate statistical analysis. Multivariate analysis identifies components, made up of combinations of metabolites, which can be used to build predictive models that are able to predict which phenotype an unknown sample belongs to. This is made possible by advances in metabolomics-

based technologies, such as nuclear magnetic resonance (NMR), mass spectrometry (MS), and magnetic resonance spectroscopy (MRS), which are fast, reproducible, sensitive, and minimally invasive techniques able to measure all the metabolites in a tissue simultaneously.

All in all, metabolomics has the potential to identify altered metabolic pathways, and biomarkers for disease subtypes and severity, as well as treatment response (Sethi & Brietzke, 2015). In particular, untargeted metabolomics has been used as an unbiased method to generate new hypotheses without restrictions from prior knowledge of specific targets (Johnson, Ivanisevic, & Siuzdak, 2016).

Given the multi-factorial and heterogeneous nature of psychiatric disorders, it is not surprising that dysregulation of metabolic pathways could contribute to disease pathology. Changes in metabolite levels have been identified in various psychiatric disorders that provide hints to the dysregulated systems. For example, metabolite changes in schizophrenia suggest that energy metabolism dysfunction and the involvement of oxidative stress may play a role in disease pathology (Fukushima et al., 2014; Orešič et al., 2011). Another study observed increased levels of ornithine, and decreased levels of arginine, glutamine, histidine, and phosphatidylcholine species in patients with schizophrenia that have also been associated with schizophrenia risk genes, highlighting genotype-phenotype interactions (He et al., 2012). Likewise, similar metabolic perturbations have been identified in depression. Kawamura et al. have proposed that decreased levels of plasma phosphoethanolamine (PEA) in MDD patients could be a possible candidate as a biomarker for MDD severity (Kawamura et al., 2018). Further metabolic changes suggest a shift towards fatty acid and amino acid catabolism in depression from gluconeogenesis, with depressed patients having decreased levels of free fatty acid and acyl carnitine, a fatty acid carrier (X. Liu et al., 2015). This is supported by lower levels of plasma or urine glucose, lactate and pyruvate, and increased levels of Kreb cycle metabolites that suggest an imbalance between gluconeogenesis and glycolysis (X. Liu et al., 2015; Nedic Erjavec et al., 2018; Zheng et al., 2013). Changes in levels of metabolites related to neurotransmitters,

such as glutamate, tryptophan and tyrosine, also provide links to the monoaminergic and glutamatergic systems (Kaddurah-Daouk et al., 2012; X. Liu et al., 2015).

1.4.1 Glutamate and glutamine

As the main excitatory neurotransmitter in the brain, glutamate is associated with many important physiological functions, and its availability is tightly controlled in the glutamate-glutamine cycle.

Glutamine serves as both a precursor for the synthesis of glutamate, as well as the product for glutamate metabolism. Glutamate is released by neurons, where it is taken up by excitatory amino acid transporters (EAAT) into astrocytes. Apart from EAATs, astrocytes also express glutamine synthetase (GS), an enzyme that converts glutamate into glutamine. Glutamine is released back into the extracellular space, and taken up into nerve terminals through transporters. Glutamine is then metabolized by glutaminase to form glutamate, where it can be used for glutamatergic signalling or in other metabolic pathways (Hertz, 2013).

Therefore, apart from glutamate levels, glutamine levels and the ratio of glutamine over glutamate have also been proposed as biomarkers for different phenotypes. Several studies have associated mood disorders with increased brain glutamate levels. Hashimoto et al. showed increased glutamate levels in the pre-frontal cortex of post-mortem brain tissue of patients with depression and bipolar disorder (Hashimoto et al., 2016a). This was supported by MRS imaging studies, where patients with MDD were shown to have increased glutamate in the occipital cortex (Sanacora et al., 2004). However, other studies have also shown otherwise, where changes in the brain were not seen between depressed patients and healthy controls for glutamate levels alone, but a significant reduction was observed in depressed patients when both glutamine and glutamate (Glx) levels were considered (Danilo Arnone, Mumuni, Jauhar, Condon, & Cavanagh, 2015). Finally, Hashimoto et al. (2016) have shown that patients with MDD had an increased glutamine over glutamate ratio in the CSF when compared to healthy controls,

while no such differences were seen for individual glutamate and glutamine levels (Hashimoto et al., 2016a).

In general, glutamine production is seen as protective, as excess glutamate levels is strongly linked to excitotoxicity. This is supported by a study showing that inhibition of GS enhanced the inflammatory response. Palmieri et al. suggested that glutamine plays a role in suppressing inflammation, and observed an increase in pro-inflammatory markers when GS was inhibited in LPS-stimulated microglia (Palmieri et al., 2017).

Finally, apart from glutamine, glutamate also has links to the Krebs cycle and γ-aminobutyric acid (GABA). Other than glutamine, glutamate can also be produced from α-ketoglutarate, one of the intermediate metabolites in the Krebs cycle, in a reaction catalysed by glutamate dehydrogenase (GDH). Glutamate can also be converted to the main inhibitory neurotransmitter GABA through glutamic acid decarboxylase (GAD) (Hertz, 2013).

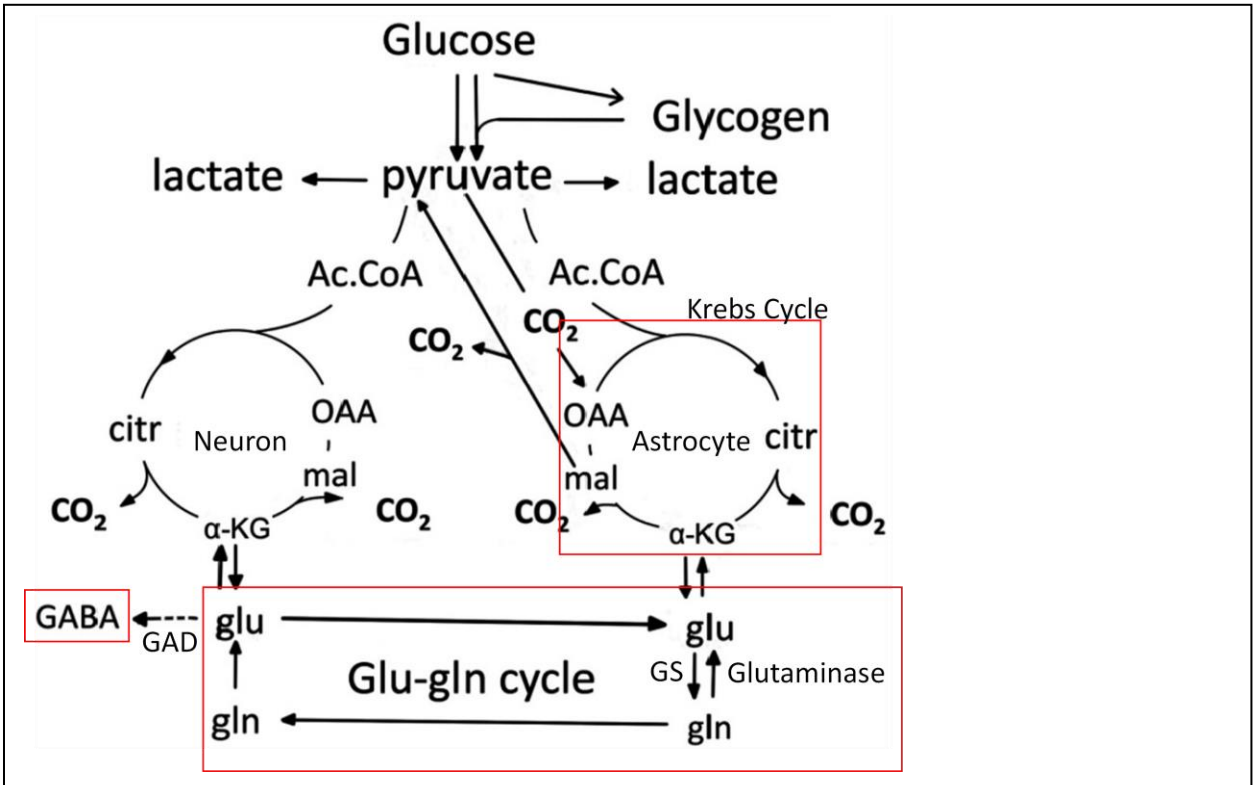


Fig 1.3 The glutamate-glutamine cycle (Adapted from Hertz, 2013) and links between glutamate, Kreb cycle metabolites, and GABA

Glu: Glutamate; Gln: Glutamine; GS: Glutamine Synthetase; GABA: γ-aminobutyric acid; GAD: Glutamic acid decarboxylase; α-KG: α-Ketoglutarate

1.4.2 Branched chain amino acids

The branched chain amino acids (BCAA) include isoleucine, leucine, and valine. They are essential amino acids that can only be obtained from diet. BCAAs are metabolized in a series of reactions to form products that can enter the Krebs cycle (S. Zhang, Zeng, Ren, Mao, & Qiao, 2017). However, BCAAs have also been implicated in glucose and fatty acid metabolism. BCAA supplementation have been proposed to shift nutrient utilization from carbohydrates to lipids, thus increasing fatty acid metabolism (Nishimura, Masaki, Arakawa, Seike, & Yoshimatsu, 2010). However, BCAA deficiency has also been linked to increased lipolysis, suggesting that too much or too little BCAA would dysregulate lipid metabolism (Y. Cheng et al., 2010). In terms of glucose metabolism, BCAAs have been observed to increase glucose consumption and utilization through the upregulation of glucose transporters (Nishitani, Takehana, Fujitani, & Sonaka, 2005).

Finally, BCAAs are also suggested to influence behaviour through affecting the availability of neurotransmitter-related amino acids, such as tryptophan and tyrosine. The transport of BCAAs to the brain involves the large neutral amino acid transport 1 (LAT1), which also transports tryptophan and tyrosine (Pardridge & Choi, 1986). Thus, increased levels of BCAA could reduce the availability of tryptophan and tyrosine, which are precursors of the neurotransmitters serotonin and dopamine respectively. This is supported by studies showing that increased BCAA levels was linked to behavioural impairment (Coppola et al., 2013).

1.4.3 Lipids

Most studies investigating the pathophysiological characteristics of diseases focus on protein targets, such as enzymes and receptors that may be abnormally expressed/activated. However, lipids perform important physiological roles, and provide support for protein function. For example, lipids are the main components of cell membranes, and also regulate signalling through membranes (Schneider et al., 2017). Therefore, changes in lipid levels also suggest mechanisms underlying disease pathology, and provide potential therapeutic targets. The major

lipid species more commonly associated with psychiatric diseases include phospholipids, cholesterol and sphingolipids.

Phospholipids and fatty acids

Phospholipids are the main component of all cell membranes, and are comprised of fatty acids, including the omega-3 and omega-6 poly-unsaturated fatty acids (PUFA). The composition of phospholipids influences membrane properties by affecting the structure and function of membrane-bound proteins, thus affecting cell signalling (Salem, Litman, Kim, & Gawrisch, 2001). Free fatty acids are also able to influence signalling through their metabolic products, such as prostaglandins and thrombaxanes, and influence gene expression by activating transcription factors (Horrocks & Farooqui, 2004; Khan & Vanden Heuvel, 2003; Serhan & Petasis, 2011).

Phospholipids and fatty acids have been implicated in both the pathology and treatment of psychiatric diseases. Decreased brain levels of the omega-3 PUFA, docosahexaenoic acid (DHA), or a decreased omega-3 over omega-6 PUFA ratio, have been observed in patients with depression and anxiety (Assies et al., 2010; Lotrich, Sears, & McNamara, 2013; Verly-Miguel et al., 2015). Pre-clinical animal models suggest that DHA may regulate serotoninergic and dopaminergic signalling. Diet-induced decreases in brain DHA correlated with decreases in serotonin levels and dopamine receptor D2 concentration (Davis et al., 2010; McNamara et al., 2009). Reduction in brain DHA content also increased serum corticosterone levels in a rodent stress model, and reduced brain-derived neurotrophic factor (BDNF) expression (Levant et al., 2008). Increased DHA content also decreased depressive-like behaviours, measured by decreased immobility time in the FST and TST (Ferraz et al., 2011; Huang, Yang, Chiu, Pariante, & Su, 2008; Jiang, Liang, & Shi, 2012). These are similar results to those induced by antidepressant treatment in pre-clinical depression models, highlighting the antidepressant effects of DHA. This is also supported by studies in humans, where adjunctive treatment with DHA improved depression and mania scores in bipolar patients (Clayton et al., 2009).

Cholesterol and lipoproteins

Cholesterol is a component of membranes, where it influences membrane fluidity and hence, affects membrane proteins and cell signalling. Hence, in the brain, cholesterol plays important roles in neurotransmission and synaptic plasticity (Parekh, Smeeth, Milner, & Thuret, 2017). As cholesterol is unable to cross the BBB, brain cholesterol is mainly recycled or synthesized locally (Bjorkhem & Meaney, 2004). Thus the transport of cholesterol, through the binding of apolipoproteins to form lipoproteins, is of key importance to the regulation of cholesterol levels. Furthermore, although the link between peripheral and central levels of cholesterol is unclear, Lutjohann et al. have shown that both dietary cholesterol supplementation and cholesterol-lowering statins can affect central cholesterol levels (Lutjohann et al., 2004).

Cholesterol are carried in circulation by three main types of lipoproteins – very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). While LDL is generally associated with inflammation and atherosclerosis (Gotto, 2011), HDL is proposed to have anti-inflammatory properties (Barter et al., 2004).

Serum levels of cholesterol have been associated with psychiatric diseases. Patients with MDD have been shown to have decreased total serum cholesterol compared to healthy controls (M. Horsten, S. P. Wamala, A. Vingerhoets, & K. Orth-Gomer, 1997; Rabe-Jablonska & Poprawska, 2000). In terms of the different types of cholesterol, patients with MDD have also been observed to have decreased levels of HDL, increased levels of LDL, and an increase in the ratio of LDL over HDL (Gupta, Petkar, Jadhav, & Dubey, 2013; Myriam Horsten, Sarah P. Wamala, Ad Vingerhoets, & Kristina Orth-Gomer, 1997; Parekh et al., 2017). However, other studies have also shown the opposite effect where increased HDL was correlated with increased depression, and that circulating LDL levels were inversely associated with depression (Olusi & Fido, 1996). In terms of mechanisms underlying its effects on the pathophysiology of psychiatric diseases, cholesterol has been proposed to influence serotonergic transmission (Scanlon, Williams, &

Schloss, 2001; Sun, Yang, Mao, Jia, & Zhang, 2015), and inflammation (Kurano et al., 2011; Lin et al., 2003). Finally, therapeutic interventions for mood disorders have been shown to increase serum cholesterol levels (Kurt et al., 2007), suggesting a role of cholesterol metabolism in treating psychiatric diseases.

Sphingolipids and ceramides

Sphingolipids are also important components of membranes. They contain a hydrophilic head group and a hydrophobic ceramide component, which enables interaction with cholesterol to form lipid rafts (membrane structures rich in GPCRs) in membranes, thus influencing the biophysical properties of membranes (Schneider et al., 2017; Simons & Ikonen, 1997). Sphingomyelin, a type of sphingolipid, can be hydrolysed by acidic sphingomyelinase (ASM) to form ceramides. This reaction has been shown to be triggered by bacterial infection and stressful stimuli, such as UV light (Charruyer et al., 2005; Grassmé et al., 1997; Grassmé et al., 2001). Ceramide-enriched membranes have a receptor-trapping effect, thus modulating the distribution of receptors on the membrane and facilitating receptor communication with downstream signalling molecules, promoting signal transduction (Hering, Lin, & Sheng, 2003).

Pre-clinical animal models suggest that sphingomyelin and ceramide levels may play a role in psychiatric diseases. Overexpression of ASM in a mouse model resulted in increased ceramide levels and depressive-like behaviour (Gulbins et al., 2013). In the hippocampus, this increase in ceramide resulted in reduced levels of neurogenesis, neuronal maturation, and neuronal survival, suggesting a mechanism by which ceramides could influence neuronal function (Gulbins et al., 2013). On the other hand, knocking out ASM in mice reduced ceramide levels, and resulted in reduced anxiety and depressive-like behaviours (Gulbins et al., 2013). Chronic unpredictable stress in rats also resulted in increased ceramide levels and decreased sphingomyelin levels in the hippocampus and PFC, and increased serum corticosterone levels (Oliveira et al., 2016). Administration of antidepressant drugs reversed the effects of stress on

mice overexpressing ASM, but not in ASM knock out mice, indicating antidepressants may function through ASM inhibition (Gulbins et al., 2013).

In humans, elevated ceramide levels were found in white matter of patients with bipolar disorder, and ASM activity was shown to be increased in peripheral blood mononuclear cells of MDD patients (Kornhuber et al., 2005; Schwarz et al., 2008).

1.5 Depression

Major depressive disorder (MDD) is a common psychiatric disorder estimated to affect 350million people worldwide. It is predicted to be the leading cause of disease burden by 2030, and its economic burden is estimated to cost slightly over 200 billion dollars in the United States alone (Greenberg, Fournier, Sisitsky, Pike, & Kessler, 2015).

MDD is classified by persistent sadness and a loss of interest in activities one usually enjoys for at least two weeks. Symptoms include a loss of energy, changes in sleep patterns, changes in appetite, reduced concentration, feelings of worthlessness and/or guilt, and suicide ideation (Uher, Payne Jennifer, Pavlova, & Perlis Roy, 2013). The classical antidepressants target the monoamine system and are known as selective serotonin reuptake inhibitors (SSRIs) or selective noradrenaline reuptake inhibitors (SNRIs) (Charney, 1998). Thus, the mechanism of most antidepressants is to potentiate serotonergic and noradrenergic transmission. However, such treatments are only truly effective in slightly over 30% of depressed patients (Joffe, Levitt, & Sokolov, 1996), underscoring the heterogeneity of the pathophysiology of MDD. The possibility that there could be different subtypes of MDD, based on the dysregulation of different systems but resulting in converging behaviour changes, highlights the need for antidepressants that target different pathways from the typical antidepressants.

1.5.1 Traditional models of depression – The monoaminergic and glutamatergic hypotheses

The models of depression have traditionally focused on the monoaminergic and glutamatergic pathways.

Monoaminergic

The monoamine neurotransmitters (serotonin, noradrenaline, and dopamine) are most commonly linked to depression. Classical antidepressants have been shown to increase availability of serotonin in the synaptic cleft (Hillhouse & Porter, 2015), leading to the serotonin hypothesis of depression, where diminished activity of serotoninergic transmission is believed to play a causal role in the pathophysiology of depression (Cowen, 2008; Hirschfeld, 2000). Support for this hypothesis include biochemical evidence – brain tryptophan, a precursor of serotonin synthesis, depletion resulted in a relapse of depressive symptoms in recovered depressed patients (K. A. Smith, Fairburn, & Cowen, 1997) – and genetic evidence where genetic polymorphisms in the serotonin transporter (5HTT) gene have been associated with increased vulnerability towards depression (Caspi et al., 2003). The serotonin receptor is also implicated - the binding density of serotonin receptor 1A ($5HT_{1A}R$), an auto-receptor that functions as a serotonin sensor, is increased in depressed patients, and knocking out the $5HT_{1A}R$ with small interference RNA (siRNA) in mice resulted in increased serotonin release, and antidepressant effects in behavioural tasks (Ferres-Coy et al., 2013).

It is clear that monoaminergic transmission is strongly associated with the pathophysiology of depression, with brain serotonin abnormalities seen in depressed patients. However, the monoamine hypothesis of depression has been criticised as being insufficient to explain several aspects of depression. Firstly, not all serotonin potentiating drugs have clinical efficacy as antidepressants (Meltzer, Bastani, Jayathilake, & Maes, 1997). Secondly, abnormalities in monoaminergic transmission are still present in patients who have recovered from depression, indicating that monoamines may not be directly correlated to depressive

symptoms (Cowen, 2008), and may be a marker of vulnerability towards depression instead. For example, in tryptophan deletion studies, unlike recovered depressed patients, healthy volunteers do not display depressive symptoms as a result of a transient decrease in serotonin levels (Ruhe, Mason, & Schene, 2007). Therefore, the monoamine hypothesis by itself is too simplistic, and other systems are also dysregulated in depression.

Glutamatergic

Given the importance of glutamate as the main excitatory neurotransmitter in the brain, glutamatergic transmission is involved in many key areas of the brain linked to depression (amygdala, hippocampus, pre-frontal cortex) (Sanacora, Treccani, & Popoli, 2012), and it is unlikely that classical antidepressants would not alter glutamatergic signalling. In fact, the success of Ketamine, a noncompetitive NMDAR antagonist, suggests that depression is a disease of glutamatergic hyperfunction. However, this theory is limited by the lack of evidence showing that increased glutamate availability leads to depressive symptoms. Nevertheless, apart from the evidence of glutamatergic neuromodulators acting as a new class of antidepressants (see section 1.3.1.2), clinical evidence also exists supporting the role of glutamatergic dysregulation in the pathophysiology of depression.

Abnormalities in glutamate and glutamine levels have been observed in patients with depression. In plasma, elevated glutamate levels and a decreased glutamine over glutamate ratio have been shown in several studies, an effect that was ameliorated by antidepressant treatment (Altamura et al., 1993; Küçükibrahimoğlu et al., 2009). Frye et al. showed reduced glutamate levels in the CSF of patients with depression or bipolar disorder (Frye, Tsai, Huggins, Coyle, & Post, 2007). Proton MRS studies have also linked glutamate levels to mood, where patients with depressive episodes have decreased glutamate levels in the frontal cortex and cingulate regions (Auer et al., 2000; Michael et al., 2003). While the exact nature of the glutamatergic changes is not well understood and dependent on many factors (brain region,

stage of illness progression, medication), the above studies suggest that glutamate clearance and metabolism may be dysregulated in MDD.

Apart from glutamate levels, studies looking at glutamate receptors and associated downstream signalling proteins link glutamatergic signalling to other aspects of MDD. Genetic polymorphisms of GRIN2B, the gene encoding the GluN2B subunit of the NMDAR, is thought to be a predictive marker for treatment-resistant depression (TRD) in patients (C. Zhang et al., 2014). Patients with depression have also been found to have increased NMDAR activation (R. Trullas & P. Skolnick, 1990). Antidepressant administration increased brain derived neurotrophic factor (BDNF) levels through a NMDAR-mediated signalling pathway.

Glutamatergic and monoaminergic signalling are also linked through the kynurenine pathway (See section 1.3.1.3), where shifting tryptophan metabolism towards the kynurenine pathway results in decreased availability of serotonin, and increased availability of the NMDAR modulators QUIN and KA.

Finally, one particularly interesting aspect of the glutamatergic hypothesis of depression is the convergence between glutamatergic signalling and neuroinflammation. Glutamate clearance and metabolism is tightly linked to the function of glial, as the transporters, excitatory amino acid transport (EAAT), and enzymes, glutamine synthase, involved are found on astrocytes. In fact, the enzymes involved in breaking down kynurenine to KA and QUIN are found on astrocytes and microglia. This has led to proposals that dysregulation of the immune system underlies and integrates both the monoaminergic and glutamatergic systems.

1.5.2 Inflammation model of depression

There is strong support for an immune component in MDD, mediated through pro-inflammatory cytokines. An activated immune system has been observed in patients with MDD, with increased numbers of circulating lymphocytes, increased levels of APP in serum, and increased plasma levels of pro-inflammatory cytokines (Dowlati et al., 2010; Maes et al., 1993). More tellingly, exogenous administration of cytokines or substances that increase inflammatory

cytokine levels (endotoxins, vaccines) in humans and animals can induce depressive-like symptoms (see section 1.2.3). Pro-inflammatory cytokines are key in the inflammation model of depression as they link inflammation to the dysregulation of other systems. These cytokines have been found to activate indoleamine-2,3-dioxygenase (IDO), the enzyme that converts tryptophan to kynurenine (see section 1.3.1.3), thus contributing to decreased serotonin availability.

The role of cytokines is further supported by genetic evidence. Polymorphisms in several cytokine genes that modulate expression of the cytokines have been linked to increased vulnerability towards depression (Barnes, Mondelli, & Pariante, 2016). Inflammation-related genes have also been found to be differentially transcribed (Shelton et al., 2010).

Aside from cytokines, patients with depression have been found to have abnormal glia volume and function (see section 1.1.1). In particular, patients with depression have been observed to have reduced astrocyte numbers in key areas such as the PFC and limbic system (Johnston-Wilson et al., 2000; Rajkowska & Stockmeier, 2013). This impairment of astrocyte function in the PFC has been associated with anhedonia, anxiety and helplessness behaviour in a rodent model of chronic unpredictable stress (M. Banasr et al., 2010). Human post-mortem studies have also shown reduced oligodendrocyte numbers in the PFC and amygdala (Hamidi, Drevets, & Price, 2004; Uranova, Vostrikov, Orlovskaya, & Rachmanova, 2004). On the other hand, higher microglial activation has been observed in patients with MDD (Steiner et al., 2008).

This imbalance in astrocyte and microglial function is important in several ways. Firstly, given that the distribution of the enzymes involved in the metabolism of kynurenine to KA and QUIN are localized to the astrocyte and microglia respectively, patients with MDD have higher levels of QUIN, and lower levels of the neuroprotective KA, in the CSF (Hillhouse & Porter, 2015). QUIN acts as an agonist of the NMDAR, and is also able to stimulate glutamate release from neurons (Tavares et al., 2002). Secondly, impaired astrocyte function would result in impaired glutamate clearance, as enzymes that transport and metabolize glutamate are localized to

astrocytes. Taken together, this imbalance would result in potentiated glutamatergic transmission.

Through pathways like the kynurenine pathway and the inflammasome, the immune system is able to link to the other dysregulated systems involved in the pathophysiology of MDD. Increased levels of pro-inflammatory cytokines results in decreased synthesis and higher turnover of monoamines, and increased release and reduced clearance of glutamate. This results in the hypo-serotonergic and hyper-glutamatergic phenotype observed in MDD. Furthermore, through the IL-1R and associated signalling proteins, IL-1β is able to influence NMDARs directly (B. Viviani et al., 2003). Finally, the immune system has also been shown to modulate the HPA axis and the stress response by modulating glucocorticoid receptor resistance (Van Bogaert et al., 2011).

This begs the question of how pro-inflammatory cytokine levels are raised in patients with MDD. Many factors can contribute to this elevation, including childhood trauma, chronic stress, diet and obesity, the gut microbiome composition, cellular damage, infections, and vaccines (Lotrich, 2015). Thus, through DAMP-recognizing PRRs, the immune system provides the link between environmental factors and downstream mechanisms of MDD (Iwata et al., 2013).

However, not every individual with elevated cytokine levels develops MDD. Thus, there are different subtypes of MDD, including an inflammatory cytokine-associated depression (ICAD), brought about by a variety of factors (environmental, genetic) that confer vulnerability to certain individuals. For example, ICAD is more strongly associated with symptomatic melancholic features, and pre-existing elevations of pro-inflammatory cytokines, which serves as a good marker for susceptibility to ICAD (Lotrich, 2015). Unfortunately, this marker is specific to ICAD, and the variable success of classical antidepressants, and the long latency period before improvements in clinical symptoms are observed highlights the need for efficacy markers to measure early effects of MDD and treatment.

1.5.3 Neuropsychological model of antidepressant drug action

It is well established that patients suffering from MDD have a negative affective bias. That is, when presented with positive and negative information, they are more likely to remember the negative information, or perceive situations to be more negative than they actually are (Beck, Rush, Shaw, & Emery, 1979). This is said to play a role in the maintenance of mood disorders.

The neuropsychological model of antidepressant drug action proposes that antidepressants function by shifting the processing of emotional information towards a more positive valence (Harmer, Hill, Taylor, Cowen, & Goodwin, 2003). This effect has been shown to occur early after drug administration (within a few hours compared to the few weeks needed for mood changes), and has been robustly shown after just a single clinical dose (Harmer et al., 2003; Harmer, O'Sullivan, et al., 2009). The theory is that this shift towards increased salience of positive information gradually results in improved mood as the patient starts interpreting information and situations in a more positive way. This provides a possible explanation to the delayed action of antidepressant drugs.

Most current antidepressant drugs potentiate monoaminergic transmission, however the series of events that translate increased availability of serotonin to improved mood is not well understood. It appears that monoaminergic pathways do not regulate mood directly, as SSRIs do not lead to mood improvements in healthy volunteers (Cowen, 2008). Studies showing that lowering serotonin levels can produce changes in emotional perception and susceptibility to MDD provide a hint that negative biases in emotional processing could be involved. Thus, the neuropsychological model provides an explanation for how serotonin transmission could translate to mood changes.

1.5.3.1 The emotional test battery (ETB)

Evidence supporting the neuropsychological model of antidepressant action comes from a series of behavioural tasks designed to detect changes in emotional processing, collectively termed the emotional test battery (ETB). These tasks are sensitive to the various components of emotional processing, including visual perception, attention, and memory.

The ETB comprises of the Facial Expression Recognition Task (FERT), the Emotional Categorization Task (ECAT), the Attentional Dot-Probe task, the Emotional Recall Task (EREC), and the Emotional Recognition Memory Task (EMEM). These tasks assess the perception, interpretation, and memory of facial expressions and self-referential personality characteristic words, as well as biases in attention to positive or negative stimuli. These tasks are described in detail in Section 4.2.4.

The ETB has been robustly shown to be sensitive to the negative affective bias in patients in depression. A variety of antidepressants, including SSRIs and NSRIs, have also been shown to reduce these negative biases early on in treatment (Harmer, O'Sullivan, et al., 2009). Interestingly, the ETB has also demonstrated emotional bias changes in healthy volunteers after antidepressant changes (D. Arnone, Horder, Cowen, & Harmer, 2009; Browning, Reid, Cowen, Goodwin, & Harmer, 2007; Harmer et al., 2003). Therefore, the ETB has the potential to act as a tool to assess antidepressant properties of novel drug targets with an acute dose, after a short period of time, and in healthy volunteers. The use of healthy volunteers allows for the study of drug effects without any confounding changes in mood.

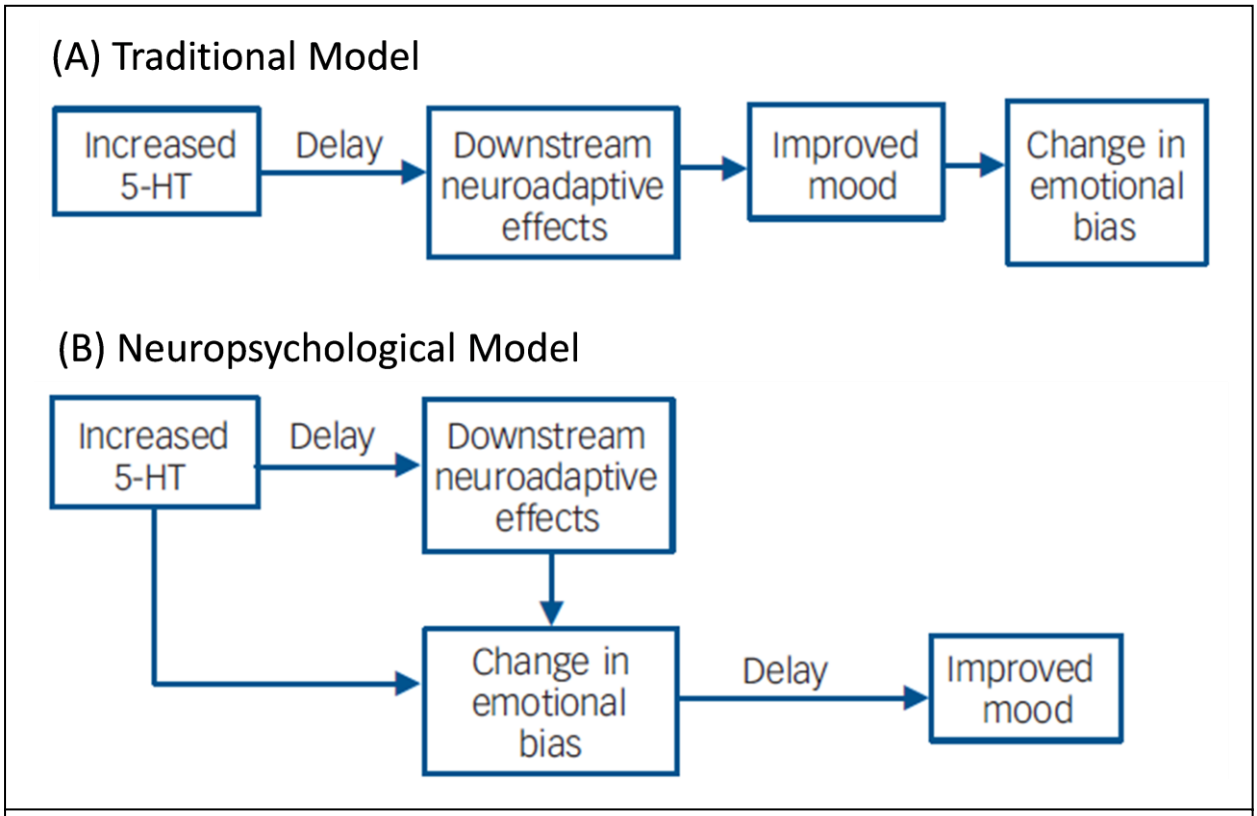


Fig 1.4 Neuropsychological model of antidepressant drug action adapted from Harmer et al. 2009 (A) Traditional model of antidepressant action where delayed improved mood results in a change in emotional bias, as a result of downstream neuroadaptive effects (B) Neuropsychological model of antidepressant action where mood improvements are mainly mediated by early changes in emotional bias, with some contribution from downstream neuroadaptive effects.

1.6 Minocycline

Given the strong evidence supporting an activated immune component in psychiatric diseases, drugs with anti-inflammatory properties present a potential new class of therapeutics that target different mechanisms from classical treatments. One such example is minocycline, a broad-spectrum tetracycline-derivative antibiotic (Pae, Marks, Han, & Patkar, 2008) that has shown promise in both pre-clinical animal models and early phase clinical trials for psychiatric diseases.

1.6.1 Biochemical structure and function

Minocycline is a member of a class of antibiotics derived from tetracycline. Tetracycline antibiotics target both gram-positive and gram-negative bacteria by binding to the bacterial 30S ribosomal subunit and inhibiting protein synthesis. However, alternate uses of tetracycline antibiotics was suggested when it was discovered that tetracyclines had biological effects separate from their anti-microbial properties (Garrido-Mesa, Zarzuelo, & Galvez, 2013). While most tetracyclines have been found to have non-microbial effects, in particular anti-inflammatory and anti-apoptotic properties, minocycline has stood out as the most promising candidate for CNS diseases.

Minocycline shares a core structure of four six-membered rings with tetracycline (Noble, Garwood, & Hanger, 2014). However, modifications at three sites improved the pharmacokinetic profile of minocycline, increasing its absorption, half-life, penetrance of tissue, and bioavailability. Minocycline is also suitable for chronic use as it is well tolerated in humans and has a good safety record (Goulden, Glass, & Cunliffe, 1996). As the most lipid soluble of the tetracycline derivatives, minocycline is able to pass through the BBB and has good penetrance into the CNS (Barza, Brown, Shanks, Gamble, & Weinstein, 1975), making it a promising therapeutic for psychiatric diseases. It has also been shown to be the most neuroprotective tetracycline derivative.

1.6.2 Minocycline as a potential therapeutic in CNS diseases

Minocycline studies have shown therapeutic efficacy in a broad range of CNS disorders, including neurodegenerative disorders, brain injuries, and psychiatric diseases. For this section, we will be focusing on the evidence of minocycline as a potential therapeutic for psychiatric diseases, and some of the proposed mechanisms underlying its therapeutic efficacy.

1.6.2.1 Evidence from pre-clinical models

Minocycline has shown efficacy in behavioural tasks modelling psychiatric diseases. O'Connor et al. have shown that there is a significant LPS x minocycline interaction effect in a mouse LPS-induced depression model. LPS administration increased immobility time in mice in the forced swim test (FST) and tail suspension test (TST), two established tasks modelling depressive-like behaviour, while treatment with minocycline normalized immobility time back to control duration (O'Connor et al., 2009). At the biochemical level, LPS significantly increased expression of IL-1β, IFN-γ, and IDO, as well as the ratio of plasma and brain kynurenine levels over tryptophan levels. Pre-treatment with minocycline was able to significantly decrease the LPS-mediated increases, suggesting that the antidepressant effect of minocycline was mediated by its anti-inflammatory properties and its effects on the kynurenine pathway (O'Connor et al., 2009). This was supported by measurements of brain levels of serotonin, and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), that were similar between the mice treated with LPS and mice treated with both LPS and minocycline, implying that serotonin levels are not responsible for the antidepressant effects in this model. Therefore, LPS-induced depressive behaviour may model the ICAD subtype, and be important for the development of treatment-resistant depression.

On the other hand, Monte et al. have shown the therapeutic potential of minocycline in a ketamine-induced model of schizophrenia. A series of behavioural tasks modelling the positive (locomotor activity and pre-pulse inhibition of startle), negative (social interaction) and cognitive (Y maze) symptoms were used. In all the behavioural tasks, minocycline was able to prevent and

reverse the ketamine-induced changes in outcomes in a manner similar to the established anti-psychotic risperidone (Monte et al., 2013). At the biochemical level, minocycline was able to prevent the ketamine-induced decrease in glutathione, a marker for oxidative stress, and the ketamine-induced increases in nitrile levels and thiobarbituric acid reactive substances (TBARs), a marker for lipid peroxidation, in brain regions associated with schizophrenia (PFC, Hc, striatum) (Monte et al., 2013). This study suggests that minocycline might have therapeutic efficacy in ameliorating all three types of symptoms in patients with schizophrenia through correcting oxidative and nitrergic imbalances. Similar behavioural changes were observed in two other models of schizophrenia induced by the NMDAR antagonists MK-801 and phencyclidine (Fujita et al., 2008; Yechiel Levkovitz, Levi, Braw, & Cohen, 2007).

1.6.2.2 Evidence from early phase clinical trials

Given that minocycline shows promise in pre-clinical animal models, the next question would be to determine if these results translate to the clinic. Several early phase clinical trials suggest that minocycline has potential as an adjunctive or complementary treatment to current medications.

Minocycline has shown antidepressant properties as an adjunctive treatment in randomized controlled clinical trials (RCT) for several subtypes of depression, including MDD (Dean et al., 2017), bipolar depression (Savitz et al., 2018), psychotic depression (Miyaoka et al., 2012), and treatment resistant depression (TRD) (Husain et al., 2017). The primary outcome for these RCTs include questionnaires measuring mood changes, specifically the Hamilton Depression Rating Scale (HAMD) and the Montgomery-Asberg Depression Rating Scale (MADRS). While Dean et al. did not observe significant reductions in the MADRS scores in the minocycline group compared to placebo in MDD patients, significant improvements in secondary measures, such as the clinical global improvements scale (CGI), and questionnaires involving functioning and quality of life, suggest that minocycline has clinical efficacy (Dean et al., 2017). Conversely, adjunctive minocycline resulted in significant decreases in HAMD scores

in TRD and psychotic depression, two forms of depression associated with poor response to antidepressants. This suggests that minocycline may be more efficacious in subtypes of depression with non-monoaminergic-based pathology. Results from Savitz et al. suggest inflammation may be involved. In this trial, an adjunctive combination of aspirin and minocycline significantly improved response rates in bipolar depression, while minocycline alone had no significant effect. However, measurements of inflammatory cytokines revealed that patients with a higher baseline level of IL-6 responded better to minocycline treatment, suggesting that therapeutics with anti-inflammatory properties would be more effective in patients with inflammation-based pathology. (Savitz et al., 2018)

Apart from improvements in depressive symptoms, Miyaoka et al. also showed that adjunctive minocycline significantly improved psychotic symptoms in psychotic depression (Miyaoka et al., 2012). The use of minocycline as an adjunctive treatment for schizophrenia has been studied in several RCTs and assessed in a meta-analysis (Xiang et al., 2017). While minocycline has been found to significantly improve the negative symptoms of schizophrenia, its effects on cognitive and positive symptoms are less conclusive. While Levkovitz et al. showed that minocycline had beneficial effects on cognitive functioning, especially executive functions related to the frontal lobe, Liu et al. found that minocycline had minimal effect on cognition with only a small improvement in attention compared to placebo-controlled groups (F. Liu et al., 2014). However, preliminary findings from the recently concluded BeneMin trial showed that 12 months of adjunctive minocycline treatment had no effect on total negative symptoms, avolition-apathy, expressive deficits or depression in schizophrenia patients (Krynicki et al., 2018; Lisiecka et al., 2015). Nevertheless, one point of note is that inflammation markers were low in both minocycline and placebo groups, further suggesting that the efficacy of minocycline is linked to a subset of patients with inflammation-based pathology. All in all, minocycline has shown clinical efficacy in patients with schizophrenia with minimal adverse reactions, and is a safe and effective adjunctive treatment.

1.6.2.3 Suggested mechanisms

This section will cover the proposed mechanisms underlying the therapeutic efficacy of minocycline in CNS diseases.

Firstly, it is well established that minocycline has anti-inflammatory properties. Minocycline has been shown to inhibit microglia activation (Fan et al., 2007), with studies showing that minocycline specifically inhibits polarization towards the pro-inflammatory state of microglia (Kobayashi et al., 2013). Minocycline also regulates the levels of pro-inflammatory agents, possibly by inhibiting or decreasing the expression of the enzymes that are involved in their synthesis or activation. For example, minocycline inhibits the interleukin converting enzyme (ICE), which cleaves pro-IL-1β to active IL-1β, as well as inducible nitric oxide synthase (iNOS), which catalyzes the synthesis of nitric oxide from L-arginine (Henry et al., 2008; Yrjänheikki, Keinänen, Pellikka, Hökfelt, & Koistinaho, 1998). This is supported by studies showing that minocycline administration attenuated mRNA expression of inflammatory genes (IL-6, IL-1β, MHCII, TLR2) in brain regions (Henry et al., 2008; Nikodemova, Watters, Jackson, Yang, & Duncan, 2007). Finally, minocycline has been shown to be an inhibitor of IDO, a key enzyme in the kynurenine pathway that links inflammation to neurotransmitter systems (O'Connor et al., 2009).

Apart from its anti-inflammatory properties, evidence from models of neurotoxicity and brain injury has highlighted the neuroprotective potential of minocycline. Minocycline has been found to promote neurogenesis in neurotoxicity models (Giri et al., 2018). Minocycline also has anti-oxidant properties, and can act as a free radical scavenger to attenuate ROS-mediated neuronal and axonal destruction (Kraus et al., 2005). In addition, minocycline is able to attenuate glutamate excitotoxicity. Several studies suggest that minocycline is able to chelate calcium ions, preventing hyperglutamatergic activation (Antonenko, Rokitskaya, Cooper, & Krasnikov, 2010). However, other studies propose that minocycline may modulate signalling pathways downstream of glutamate receptors. For example, Pi et al. have shown that minocycline inhibits

glutamate-induced p38 activation, and was involved in the disinhibition of the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway (Pi et al., 2004).

1.6.3 The paradox of minocycline in schizophrenia and depression models

Given the multi-factorial nature of most psychiatric disorders, a broad-spectrum therapeutic like minocycline has great potential in addressing the complex interactions between multiple dysregulated systems. However, this raises the question of how minocycline is able to have therapeutic effects in both schizophrenia and depression, two disorders with seemingly opposite pathophysiology. As inflammation has been proposed to activate NMDARs, depression, with its hyper-activated glutamatergic transmission (Muller & Schwarz, 2007; R. Trullas & P. Skolnick, 1990), fits in with current literature, and ketamine that blocks NMDARs is being investigated as a treatment for refractory depression (Lara, Bisol, & Munari, 2013). Conversely, schizophrenia has been hypothesized to be an illness of NMDAR hypofunction (Gunduz-Bruce, 2009). However, minocycline has been shown to improve cognitive impairment in schizophrenia patients, a phenomenon associated with increased activation of NMDARs. Therefore, minocycline appears to decrease NMDAR activation in depression but increase it in schizophrenia.

Our understanding of how minocycline affects NMDARs is further complicated by the fact that while current literature agrees that inflammation has an effect on NMDARs, evidence on the exact effect is mixed. Depending on the samples used –hippocampal slices *in vitro*, primary cell cultures from different regions of the CNS, and hippocampal tissue *in vivo*– and how inflammation was induced, researchers have found different effects on the levels or activity of different subunits of NMDARs (Ma et al., 2014; Riazi et al., 2015; B. Viviani et al., 2003). Thus, there is a gap in knowledge on exactly how peripheral inflammation affects NMDARs in the hippocampus, and whether anti-inflammatory treatments can attenuate these effects.

1.7 Summary and aims of this thesis

This thesis aims to investigate the mechanisms underlying the psychotropic function of minocycline in psychiatric diseases, which we believe is facilitated by its anti-inflammatory properties. The introductory chapter (Chapter One) has discussed the immune component in psychiatric diseases (with the specific example of MDD), how it could link the various dysregulated endocrine and neurotransmitter systems (glutamatergic and monoaminergic) involved, and the potential of minocycline as a complementary treatment. Current literature has proposed several mechanisms underlying the effects of minocycline in CNS diseases, however only a few studies have looked at psychiatric diseases, and only at a specific disease (MDD or schizophrenia). Therefore, there are gaps in knowledge that have yet to be explored, and we aim to explore the mechanism of minocycline in four ways – modulation of NMDARs and downstream signalling (Chapter Two), metabolomics effects in animals (Chapter Three), psychological effects in humans (Chapter Four), and biochemical effects in humans (Chapter Five).

Chapter Two, the first experimental chapter, will look at the direct effects of peripheral inflammation (induced by LPS) on central NMDARs, and whether minocycline will ameliorate these effects. Currently, a large body of research has focused on minocycline's effects on NMDARs through the kynurenine pathway and thus, many studies have looked at the effects of IDO and the kynurenine metabolites. However, given that IL-1β could directly modulate NMDARs through the IL-1R1 receptor as well, we hypothesize that the increase in peripheral pro-inflammatory cytokines induced by LPS administration would result in central NMDAR changes, and affect downstream signalling proteins, leading to changes in neuronal activity. A second hypothesis is that minocycline would ameliorate some of the LPS-induced effects.

Chapter Three will explore the effects of LPS-induced inflammation and minocycline on both plasma and brain metabolites. Given the links between inflammation and metabolism, the metabolome could provide hints to the pathways dysregulated during inflammation, and

whether minocycline functions by ameliorating the LPS-mediated effects. As minocycline performs a variety of functions, it is also possible that minocycline's therapeutic efficacy is unrelated to its anti-inflammatory properties. Comparing the minocycline-treated group to control will reveal changes to the metabolome. Two dosing regimens of minocycline were studied – an acute dose and a repeated study.

Chapter Four will look at the psychological effects of an acute dose of minocycline in healthy volunteers. Currently, while the therapeutic efficacy of minocycline as an adjunctive treatment has been studied in RCTs, no one has looked at its effects on emotional and cognitive processing. Based on the neuropsychological model of antidepressants, we hypothesize that minocycline would reduce negative biases in healthy volunteers. A secondary outcome involves investigating the effects of minocycline on cognitive tasks, as minocycline has been shown to be efficacious in improving cognitive symptoms in schizophrenia RCTs.

Chapter Five is the last experimental chapter, and will look at biochemical measures from the minocycline study in Chapter Four. Given its anti-inflammatory properties, we hypothesize that minocycline would reduce levels of inflammatory markers. ^{1}H NMR will also be used to study the effects of an acute dose of minocycline on the serum metabolome. Based on results from our acute dose mouse study, we hypothesize that minocycline would affect lipoprotein levels.

Table 1.1 Summary of studies conducted including details of aims, design, and methods

CD1 Mice			**Humans (Healthy Volunteers)**	
Molecular Study:	Metabolomic Study:		Psychological Study:	Biochemical Measures:
Repeated doses	Single dose	Repeated doses	Single dose	Single dose
Study Aims: Investigating the effects of inflammation and/or minocycline on: 1. Inflammatory markers 2. NMDAR subunit expression 3. Downstream signalling proteins 4. Arc expression	Study Aims: Investigating the effects of a single dose of minocycline on: 1. Plasma metabolites 2. Hippocampal metabolites 3. Frontal cortex metabolites	Study Aims: Investigating the effects of inflammation and/or minocycline on: 1. Plasma metabolites 2. Hippocampal metabolites 3. Frontal cortex metabolites	Study Aims: Investigating the effects of a single dose of minocycline on: 1. State (e.g. mood) 2. Emotional processing 3. Cognitive processing	Study Aims: Investigating the effects of a single dose of minocycline on: 1. Cortisol 2. Inflammatory cytokines 3. Serum metabolites including lipoproteins
Design: 2x2 factorial 7 days treatment of minocycline (50mg/kg) or saline. On the 7th day, treatment with LPS (<1mg/kg) or saline. Samples collected 24 hours after last dose. All treatments administered through intraperitoneal injection. Samples: 3 batches, N = ~64	Design: Single intraperitoneal injection of minocycline (50mg/kg) or saline. Samples collected 2 hours after dose. Samples: 1 batch, N = ~19	Design: 2x2 factorial 7 days treatment of minocycline (50mg/kg) or saline. On the 7th day, treatment with LPS (<1mg/kg) or saline. Samples collected 24 hours after last dose. All treatments administered through intraperitoneal injection. Samples: 3 batches, N = ~60	Design: Randomized, between-subjects, double-blind, placebo-controlled study. Recruited 40 healthy controls. 2 capsules of minocycline (total dose 200mg/kg) or placebo administered to participants. Psychological tasks conducted 2 hours post-dose.	Design: Randomized, between-subjects, double-blind, placebo-controlled study. Recruited 40 healthy controls. 2 capsules of minocycline (total dose 200mg/kg) or placebo administered to participants. Plasma/serum collected before drug intake, and 4 hours post-dose.
Method: Inflammatory markers measured by ELISA and qPCR. NMDAR subunit and Arc expression measured by ISHH. Signalling proteins measured by western blot.	Method: Metabolite changes detected by ^{1}H NMR, spectra processing, and multivariate analysis (OPLS-DA)	Method: Metabolite changes detected by ^{1}H NMR, spectra processing, and multivariate analysis (OPLS-DA)	Method: State changes assessed by self-reported questionnaires. Emotional processing assessed by the ETB. Cognitive processing assessed by tasks measuring contextual cueing, priming, and the working memory	Method: Inflammatory markers measured by ELISA. Metabolite changes detected by ^{1}H NMR, spectra processing, and multivariate analysis (OPLS-DA)

Chapter 2: The molecular effects of inflammation and minocycline on inflammatory markers and NMDAR signalling

2.1 Introduction

2.1.1 Lipopolysaccharide model of neuroinflammation

The association between inflammation and the pathophysiology of psychiatric disorders has been shown in both human studies and pre-clinical animal models. One of the most established animal models is the post-inflammatory depression paradigm where endotoxin administration leads to depressive-like behaviours in rodents. Dunn et al. have shown that administration of the pro-inflammatory cytokine interleukin-1β (IL-1β), and the bacterial endotoxin lipopolysaccharide (LPS) leads to increased immobility in the Porsolt forced swim test (FST) and the tail suspension test (TST), which are two measures of depressive-like behaviour or despair (Dunn & Swiergiel, 2005). Likewise, Jain et al. showed that LPS administration increased immobility in the FST, an outcome that was reversed by treatment with antidepressants (Jain, Kulkarni, & Singh, 2001). However, in both studies, behavioural tasks were conducted two hours post-LPS dose, at a time where sickness behaviour is at its peak, and the results of the FST and TST could not be dissociated from reduced locomotor activity shown by reduced crossings and rearings in the open field test (OFT) (Dunn & Swiergiel, 2005).

To avoid the confounding effects of LPS-induced sickness behaviour, Frenois et al. conducted behavioural tasks at two time-points; 6 and 24 hours post-LPS administration. While sickness behaviour was still prevalent at 6 hours post-LPS, after 24 hours, motor activity and food intake was normalized to control levels. However, depressive-like behaviours defined by increased immobility in the FST and decreased sucrose consumption, a measure of anhedonia, were still present (Frenois et al., 2007). Therefore, this implies that behavioural changes

observed 24 hours post-LPS dose are attributed to LPS-induced depressive behaviour, and largely dissociated from LPS-induced sickness behaviour.

This is supported at the molecular level by changes in inflammatory markers. Pro-inflammatory plasma cytokine levels are shown to peak 2 to 4 hours after LPS administration, and normalize to control levels 24 hours post-LPS. However, changes in pro-inflammatory cytokine levels can still be detected in the brain 24 hours after the LPS administration (Custodio et al., 2013). Other markers shown to have a delayed increase include brain nitrite levels (Custodio et al., 2013), and major acute phase proteins (APP) in the liver such as serum amyloid A (SAA) (Gruys et al., 2005).

2.1.2 Inflammation and NMDARs

Inflammation has been proposed to influence behaviour through several mechanisms, including serotonergic neurotransmission and glucocorticoid resistance (inability of the stress hormone, corticosterone, to suppress the inflammatory response). However, given the importance of glutamatergic signalling in functions affected by psychiatric illnesses, the effects of inflammation on glutamatergic-related mechanisms cannot be ignored.

One key way inflammation affects glutamatergic signalling is through modulating indoleamine 2,3-dioxygenase (IDO) activity and the kynurenine pathway. O'Connor et al. have shown that LPS-activated IDO increased brain pro-inflammatory cytokine expression, increased plasma kynurenine levels, and resulted in depressive-like behaviour. The importance of kynurenine was further supported by a study showing that the administration of kynurenine increased immobility in the FST and TST (O'Connor et al., 2009). Kynurenine produces metabolites in the brain that act on glutamate receptors (Hillhouse & Porter, 2015).

However, the kynurenine pathway is not the only way for inflammation to affect glutamatergic signalling. Viviani et al. have also shown that IL-1β administration increased N-methyl D Aspartate Receptor (NMDAR) activity, and resulted in increased intracellular calcium

levels, and increased neuronal cell death, which links inflammation to excitotoxicity. This association between IL-1β and NMDARs was shown to be mediated by the interleukin-1 receptor (IL1R) and the Src family of kinases (B. Viviani et al., 2003), thus elucidating a distinct mechanism apart from the kynurenine pathway.

2.1.3 NMDAR-associated downstream signalling proteins

The activation of NMDAR stimulates a cascade of downstream signalling proteins. Of particular interest are the extracellular signal-regulated kinases 1 and 2 (ERK1/2), and the calcium/calmodulin-dependent protein kinase IIα (CamKIIα). ERK1/2 and CamKIIα are signal transduction proteins that are activated by phosphorylation on key residues after NMDAR activation, and in turn phosphorylate target signalling proteins to modulate their activity. ERK1/2 is a part of the mitogen activated protein (MAP) kinase family, and has been shown to phosphorylate serine and threonine residues on transcription factors, cytoskeleton proteins and other protein kinases (Hetman & Gozdz, 2004). It is suggested to have anti-excitotoxic effects, and is involved in functions such as cell proliferation and survival, and neuronal differentiation and plasticity. Thus, increased levels/activity of ERK is proposed to be neuroprotective (Karmarkar, Bottum, Krager, & Tischkau, 2011).

CamKIIα has been shown to exhibit GluN2 subunit preference, and associates more strongly with GluN2B than GluN2A (Chiba et al., 2011). CamKIIα activity is modulated by binding to the cytoplasmic tail of NMDAR subunits, and has been associated with neuronal functions such as long term potentiation (LTP) and synaptic plasticity. Therefore, increased levels/activity of CamKIIα has been shown to increase LTP and spontaneous activity-driven potentiation (SAP) (Barria & Malinow, 2005).

2.1.4 Arc

The activity-regulated cytoskeleton-associated protein (Arc) has been shown to be important for LTP and synaptic function. As such, Arc mRNA has been shown to be induced by synaptic activity, and increased Arc expression is a marker for synaptic function (Bramham et al., 2010). Arc expression has also been linked to activation of NMDARs and downstream associated signalling proteins, such as ERK (Waltereit et al., 2001). Therefore, Arc expression can be said to be a functional marker of NMDAR activity.

2.1.5 Aims

In this study, the inflammation mouse paradigm described by O'Connor et al. (2009) and Frenois et al. (2007) was chosen to model the increase in pro-inflammatory cytokine levels that is seen in some patients with a psychiatric disorder in the absence of overt sickness. A non-sepsis-inducing dose of LPS (0.83 mg/kg) was chosen, similar to that used in the above two studies. The current study also used the same species of mice (CD1), and conducted experiments 24 hours post-injection when LPS-induced depressive behaviour are prevalent, but LPS-induced sickness behaviour is returning to baseline.

The current study first established that the chosen dose of LPS induced inflammatory effects in line with current literature. Based on previous studies, peripheral inflammatory cytokine levels are hypothesized to have returned to baseline, while brain inflammatory cytokine levels are still elevated. The effect of minocycline on these inflammatory parameters was then investigated. Given that minocycline has been shown to have anti-inflammatory properties, minocycline administration was hypothesized to ameliorate the LPS-induced changes.

The next section investigated the effects of LPS on NMDARs, and whether minocycline ameliorated these effects. As IL-1β has been shown to affect NMDAR activity, it was

hypothesized that NMDAR expression levels would be modulated by LPS, and that minocycline would ameliorate these changes.

Finally, the effect of LPS and minocycline on downstream NMDAR-associated signalling proteins, as well as a functional marker Arc, was investigated. It was hypothesized that changes in the levels or activities of these proteins would mirror that of the changes in NMDAR levels or activity.

2.2 Methods

2.2.1 Drugs

Lipopolysaccharide (LPS) from *Escherichia coli*, and Minocycline Hydrochloride (Minocycline) were used (all from Sigma Aldrich).

2.2.2 Animals

The experiments were performed on CD-1 mice (male, adult 6-8 weeks). The mice were housed 5 to a cage under standard environmental conditions and had access to food and water *ad libitum*. All animal procedures were carried out in accordance with UK Home Office Animals (Scientific Procedures) Act (1986) and associated Home Office guidelines. The local Animal Welfare and Ethical Review Body (AWERB) at the University of Oxford approved the procedures specific to this study.

3 cohorts of animals were treated for the various experiments. Samples from cohorts 1 and 2 were used for *in situ* hybridization (ISH) experiments, samples from cohorts 2 and 3 were used for western blotting experiments, and samples from cohorts 3 were used for quantitative polymerase chain reaction (qPCR) experiments. Plasma samples from this study and the next chapter were used for corticosterone measurements.

For each cohort, CD-1 mice were randomly divided equally into 4 groups (N = 20 or 24) in a 2x2 factorial arrangement, and treated once daily for 7 days with minocycline (50mg/kg) or saline. On the seventh day, mice were also treated with LPS (0.83 mg/kg) or saline (Fig 2.2.1). All treatments were administered through intraperitoneal (i.p.) injections.

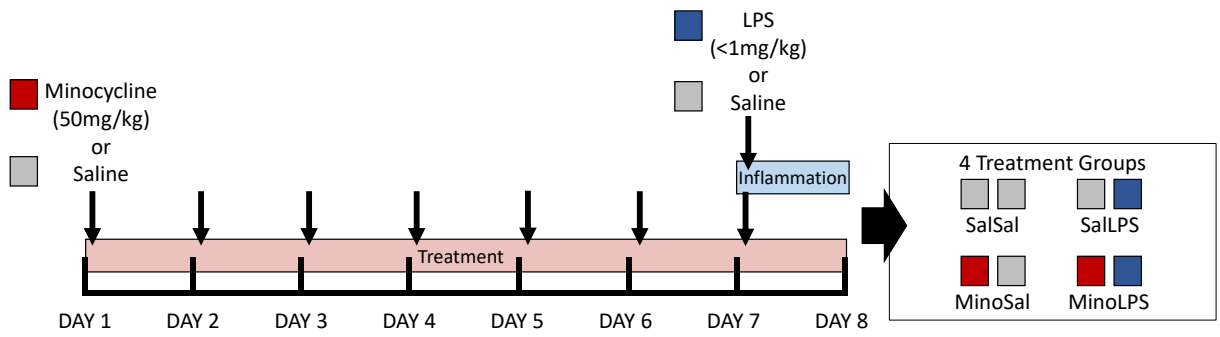


Fig 2.2.1 Illustration of treatment regime of repeated dosage of minocycline and a single injection of LPS in CD1 mice

2.2.3 Tissue processing

The hippocampal and frontal cortex tissue were weighed with a weighing balance. To obtain protein lysates for western blotting, RIPA buffer (Sigma) containing proteinase inhibitor (Roche) was added to frontal cortex and hippocampal tissues. Tissue were then lysed with a Tissuelyser LT (Qiagen), and centrifuged at 17,000 xg for 5 min. Aliquots of the supernatant were then stored at -80°C.

2.2.4 *In situ* hybridization

Coronal and sagittal sections (14 µm) containing the frontal cortex and hippocampus were cut with a cryostat and collected on slides (2 sections per slide). Slides then underwent pre-hybridization treatment: Sequentially, slides were fixed with 4% paraformaldehyde, washed in PBS, and treated in TEA buffer (0.1M Triethanolamine hydrochloride, 0.9% Sodium Chloride, 0.25% Acetic Anhydride), increasing concentrations of ethanol, chloroform and decreasing concentrations of ethanol. All solutions and apparatus were pre-treated with diethylpyrocarbonate.

A mixture of oligonucleotide probe, 5X buffer, ^{35}S Radioisotope and Terminal Deoxynucleotidly Transferase Buffer (TdT) (Promega) was prepared for labelling of oligonucleotide probes with radioactivity. This mixture was then incubated at 37°C for 30 min. After purification with Nick Columns containing Sephadex G-50 DNA grade, radioactivity of the labelled probes was counted with the scintillation counter. The amount of probe needed to obtain a concentration of $1x10^6$ counts per min/section was then calculated and prepared, and 75 µL of the solution was added to each section. Slides were incubated overnight at 37 °C with Saline Sodium Citrate (SSC) buffer to maintain humidity.

The slides were then washed (thrice at 55 °C for 20 min followed by twice at room temperature for an hour) in SSC, rinsed in distilled water, and left to dry. Slides were then arranged in a cassette and exposed to film. Radioactivity count of the Cornu Ammonis 1 (CA1), Cornu Ammonis 3 (CA3) and dentate gyrus (DG) regions in the hippocampus were then measured with a Northern Light R95 white trans-illuminator (Imaging Research Inc).

2.2.5 Western blotting

Total protein lysates were normalized against Bradford assay. 5 µg of each sample was loaded into each lane of 4-20% Mini-Protean TGX Gels (Biorad) and separated with SDS-PAGE in 1X Running Buffer (25 mM Tris base, 250 mM Glycine, 0.1% SDS, dH_2O). Proteins were then transferred to a PVDF membrane overnight using electroblotting in 1X Transfer Buffer (25 mM Tris base, 192 mM Glycine, 20% Methanol, dH_2O). The membrane blots were reactivated in methanol and incubated for 40 min at room temperature in Blocking Buffer (0.1% PBS-Tween20 with 5% nonfat Milk). Primary and secondary antibodies were prepared in 0.1% PBST with 2% nonfat milk and added to blots sequentially for 1 hour and 40 min respectively. Immunoreactivity was visualized with enhanced chemiluminescence (Amersham ECL Prime) and exposure to Hyperfilm (Amersham) at various time points. Densitometric analysis was done to

quantify the changes in protein levels (AlphaImager3400) using film exposures below the saturation point. β-Actin was used as an internal control to normalize the optical density values in each sample.

Antibodies used in western blot include anti-CamKIIα (1:40000, Abcam), anti-pTyr286-CamKIIα (1:1000, Abcam), anti-Erk1/2 (1:2000 , Millipore), anti-p-Erk (1:500 , Millipore), and anti-β-Actin (1:1000000, Sigma). Horse-radish peroxidase-conjugated goat anti-rabbit (1:10000, Biorad) and goat anti-mouse (1:5000, Biorad) were used as secondary antibodies.

2.2.6 Enzyme-linked immunosorbent assays (ELISAs)

Plasma corticosterone levels were detected using enzyme-linked immunosorbent assays (ELISA) according to manufacturer's instructions (Enzo). Samples were diluted 1:10 (v:v).

2.2.7 RNA extraction and reverse transcription

Brain and liver tissue samples were homogenized in TRI-reagent (Sigma), at the manufacturer-recommended concentration of 1 ml per 50-100 mg of tissue, with a Tissuelyser LT (Qiagen). After centrifugation of the tissue lysate at 17,000 xg for 5 min, the supernatant was transferred to a fresh tube. Total RNA was extracted from the supernatant through a series of steps involving the addition of chloroform (1:5 v/v of TRI-reagent), and isopropanol (1:2 v/v of TRI-reagent). After centrifugation at 17,000 xg for 10 min at 4 °C, the resulting pellet was washed in 70% ethanol, and left to dry, before resuspension in around 30 µL of nuclease-free water (NF H_2O). Total RNA yield was determined by absorbance at 260 nm using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). RNA was diluted to a total concentration of 2000 ng in 8 µl of NF H_2O, and treated with DNase (for each sample: 2 µL of RNase-free DNase (Promega), incubated at 20 min at 37 °C, followed by 5 min at 75 °C). RNA was reverse transcribed to cDNA as follows: A reverse transcription (RT) reaction mix (1 µL of 10X RT buffer,

0.4 µL 25 mM dNTP mix, 1 µL 10X random primers, 0.5 µL RNase inhibitor, 1 µL reverse transcriptase, 1.1 µL NF H_2O) was added to 5 µL of each sample for a final concentration of 100 ng/µL. This mixture was then incubated for 1 hour at 37 °C. All reagents were obtained from Thermo Fisher Scientific.

2.2.8 Quantitative polymerase chain reaction (qPCR)

qPCR was performed using a QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific) in 384 well format. Each sample was run in triplicate as a 12 µL reaction consisting of 1 µL forward primer, 1 µL reverse primer, 6 µL SYBR Green (Thermo Fisher Scientific), and 4 µL of sample (3 ng/µL). Relative gene expression of SAA2, IL-10 and TNFα were explored in the liver, while IL-1β, IL-6, TNFα, IDO, COX2, and TLR4 expression were investigated in the brain. B2M was used as the housekeeping gene for liver samples, and TFRC was used as the housekeeping gene for brain samples.

2.2.9 Statistical Analyses

Statistical analyses were performed using SPSS v20. For experiments with 2x2 factorial arrangements, the effects of treatments were analysed using two-way ANOVA with Bonferroni correction. Results are expressed as mean +/- S.E.M. and biological significance was determined using appropriate post hoc tests (pairwise comparisons).

2.3 Results

2.3.1 Validation of LPS-induced inflammation model

2.3.1.1 Corticosterone

Corticosterone levels have been shown to be positively correlated with inflammation. This is supported by our results where a significant LPS main effect was observed ($F_{1,119} = 19.591$, $p < 0.001$). Pairwise comparisons show that this effect was driven by differences between the LPS-treated group (SalLPS) and control (SalSal) (+391.214%, p = 0.001), and the group treated with both minocycline and LPS (MinoLPS) and minocycline alone (MinoSal) (+279.704%, p = 0.003). Groups were not normally distributed, and the assumption of homogeneity of variances was violated, however, as group sizes were large (>=30) and similar between treatment groups (30-32), 2-way ANOVA was still conducted.

As an extra measure, a non-parametric test analysing differences in distribution between treatment groups was also conducted. Results obtained were similar to that of the parametric tests, where groups were found have significant differences in distribution (Kruskal Wallis; Test statistic (3) = 23.161, $p < 0.001$), driven by differences between SalLPS and SalSal ($p < 0.05$) and MinoLPS and MinoSal (p = 0.001).

Results obtained support that LPS treatment has induced inflammation, but does not show that minocycline ameliorates the LPS-induced increase in corticosterone levels.

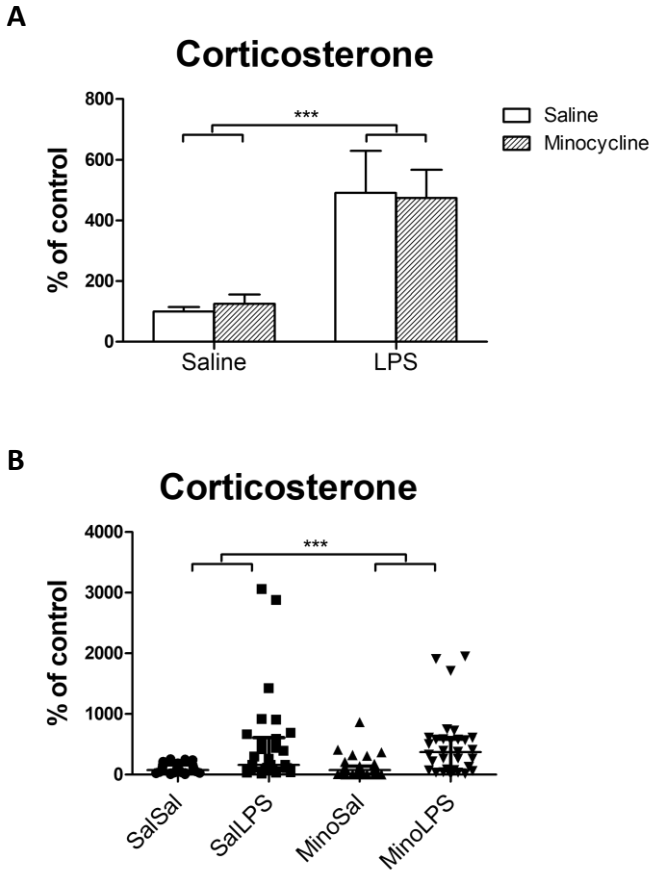


Fig 2.3.1 Plasma corticosterone levels were increased in LPS-treated groups. Results are expressed as mean with SEM (A) and as a scatter plot (B)

2.3.1.2 Inflammatory markers

The effects of LPS and minocycline were explored in a range of inflammatory markers in the liver and the brain. Liver markers were chosen due to the association between inflammation and the acute phase response (APR).

Liver

There was a significant LPS main effect ($F_{1,19}$ = 53.241, p < 0.001), and a significant minocycline main effect ($F_{1,19}$ = 4.736, p = 0.042) for serum amyloid A2 (SAA2), a major positive protein in the APR. Pairwise comparisons revealed that LPS increased SAA2 levels with (MinoLPS vs MinoSal: +815.1%, p < 0.001) or without (SalLPS vs SalSal: +4235.0%, p < 0.001) prior treatment with minocycline. There was also a significant difference between the SalLPS and MinoLPS group, where mice treated with both minocycline and LPS had significantly higher levels of SAA2 mRNA than mice treated with LPS alone (MinoLPS vs SalLPS: +55.81%, p = 0.022). Given that variance was not equal between treatment groups, a non-parametric test was also conducted. Results obtained were similar to that of the parametric tests, where groups were found to have significant differences in distribution (Kruskal Wallis; Test statistic (3) = 18.572, p < 0.001), driven by differences between SalLPS and SalSal (p = 0.015) and MinoLPS and MinoSal (p = 0.030).

No differences were found between treatment groups for TNFα and interleukin-10 (IL-10), indicating that LPS-mediated cytokine changes in the liver had returned to baseline.

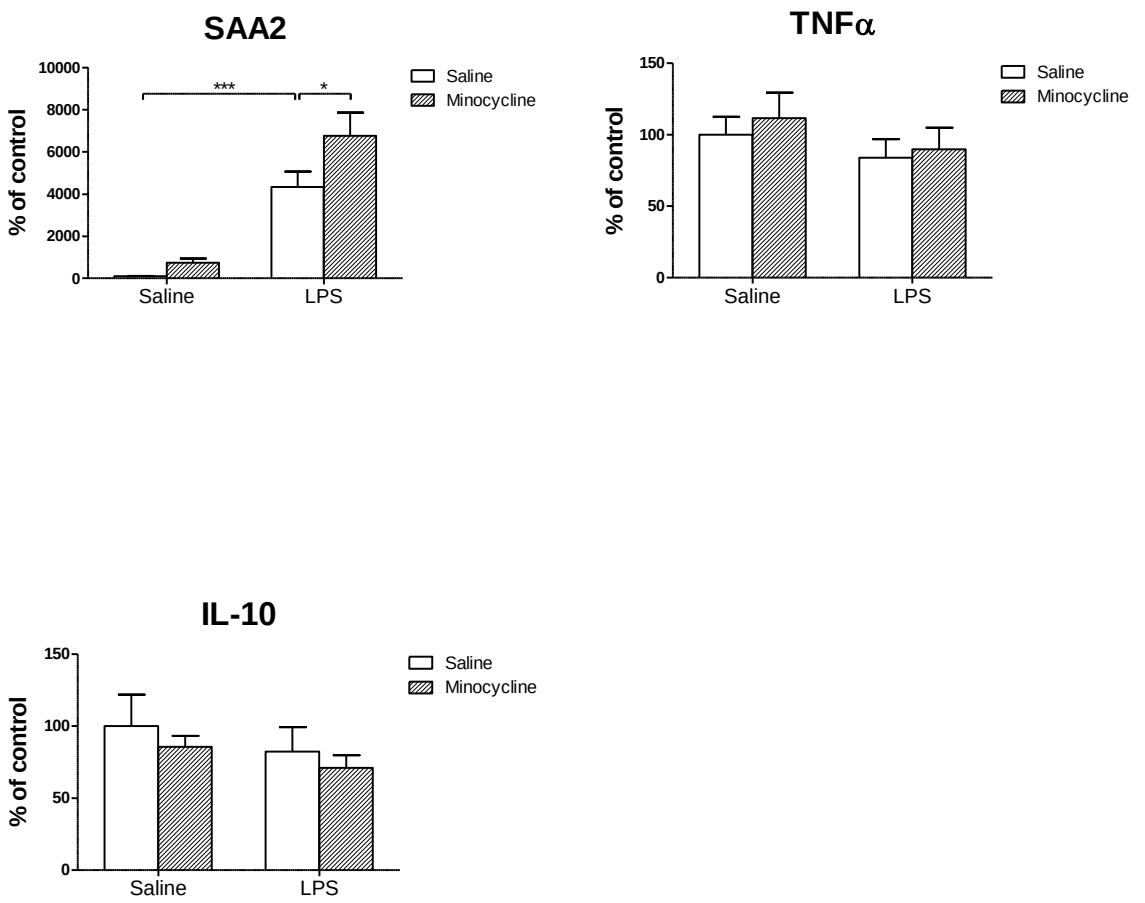


Fig 2.3.2 Liver mRNA expression of inflammatory markers 24 hours post-treatment. SAA2 levels were significantly increased as a result of LPS treatment.

SAA: Serum Amyloid A2; TNFα: Tumour Necrosis Factor α; IL-10: Interleukin-10

Brain

Cytokines

LPS administration had different effects on IL-1β and IL-6 in the hippocampus (Hc) and frontal cortex (FC). For the Hc, there was no significant differences in IL-1β expression between groups 24 hours post-treatment. However, there was a trend of an interaction, LPS*minocycline interaction on IL-6 expression ($F_{1,17} = 3.401$, $p = 0.083$), driven by differences between SalSal and SalLPS (+116.1%, $p < 0.05$), and trend differences between SalLPS and MinoLPS (-44.66%, $p = 0.075$).

For the FC, there was a significant LPS main effect ($F_{1,15}$ = 10.427, p = 0.006), minocycline main effect ($F_{1,15}$ = 6.376, p = 0.023), and LPS*minocycline interaction effect ($F_{1,15}$ = 4.887, p = 0.043) on IL-1β expression. Pairwise comparisons revealed that this was driven by differences between SalSal and SalLPS (+177.5%, p = 0.002), and SalLPS and MinoLPS (-55.68%, p = 0.004). For IL-6, there was a trend of a LPS main effect ($F_{1,18}$ = 3.460, p = 0.079).

Similar effects were obtained for both brain regions for TNFα. A significant LPS main effect was observed for both the Hc (F1,18 = 12.857, p = 0.002) and FC (F1,18 = 10.801, p = 0.004). As Hc samples in the treatment groups were not of equal variance, an additional independent samples T-test was conducted between the LPS-treated animals and non-LPS treated animals. TNFα expression was still significantly different between the two groups (p = 0.002) when equal variances were not assumed. For the FC samples, the LPS main effect was driven mainly by differences between the SalSal and SalLPS treatment group (SalLPS +351.2%, p = 0.010). There was also a trend for a decrease in TNFα expression when LPS-treated animals were treated with minocycline beforehand (MinoLPS -44.08%, p = 0.084).

Pro-inflammatory enzymes and receptors

The expression of enzymes associated with pro-inflammatory mechanisms (IDO, COX2) and the LPS receptor TLR4 was also investigated as additional inflammatory markers. IDO was also of particular interest, as it has been shown to be inhibited by minocycline.

Once again, different effects were observed in the 2 brain regions. In the Hc, there was a main effect of minocycline treatment on IDO expression (F1,18 = 12.759, p = 0.002). Pairwise comparisons showed that minocycline reduced IDO expression with or without LPS treatment (MinoSal vs SalSal -43.1%, p = 0.010; MinoLPS vs SalLPS -35.6%). There was also a trend for a LPS main effect on COX2 expression (F1,18 = 3.021, p = 0.099). Finally, there was a main effect of minocycline treatment (F1,18 = 8.803, p = 0.008), and a trend for a LPS main effect (F1,18 = 4.228, p = 0.055) for TLR4 expression. Pairwise comparisons revealed that this was mainly driven

by differences between the MinoSal and SalSal groups (MinoSal -25.6%, $p = 0.045$). There was also a trending difference between the MinoLPS and SalLPS groups (MinoLPS -20.7%, $p = 0.057$).

On the other hand, no changes were observed for IDO and COX2 expression in the FC, but there was a trend for a LPS main effect ($F_{1,18} = 3.444$, $p = 0.080$) for TLR4 expression.

All in all, our results show that the effects of minocycline and LPS are still detectable 24 hours post-dose. In the liver, inflammatory cytokine expression has returned to baseline, but the changes resulting from the APR can still be observed. Also, a peripherally-administered dose of LPS resulted in changes in the brain, although after 24 hours, some of the inflammation response has returned to baseline. Likewise, peripherally-administered doses of minocycline also resulted in detectable changes in the brain.

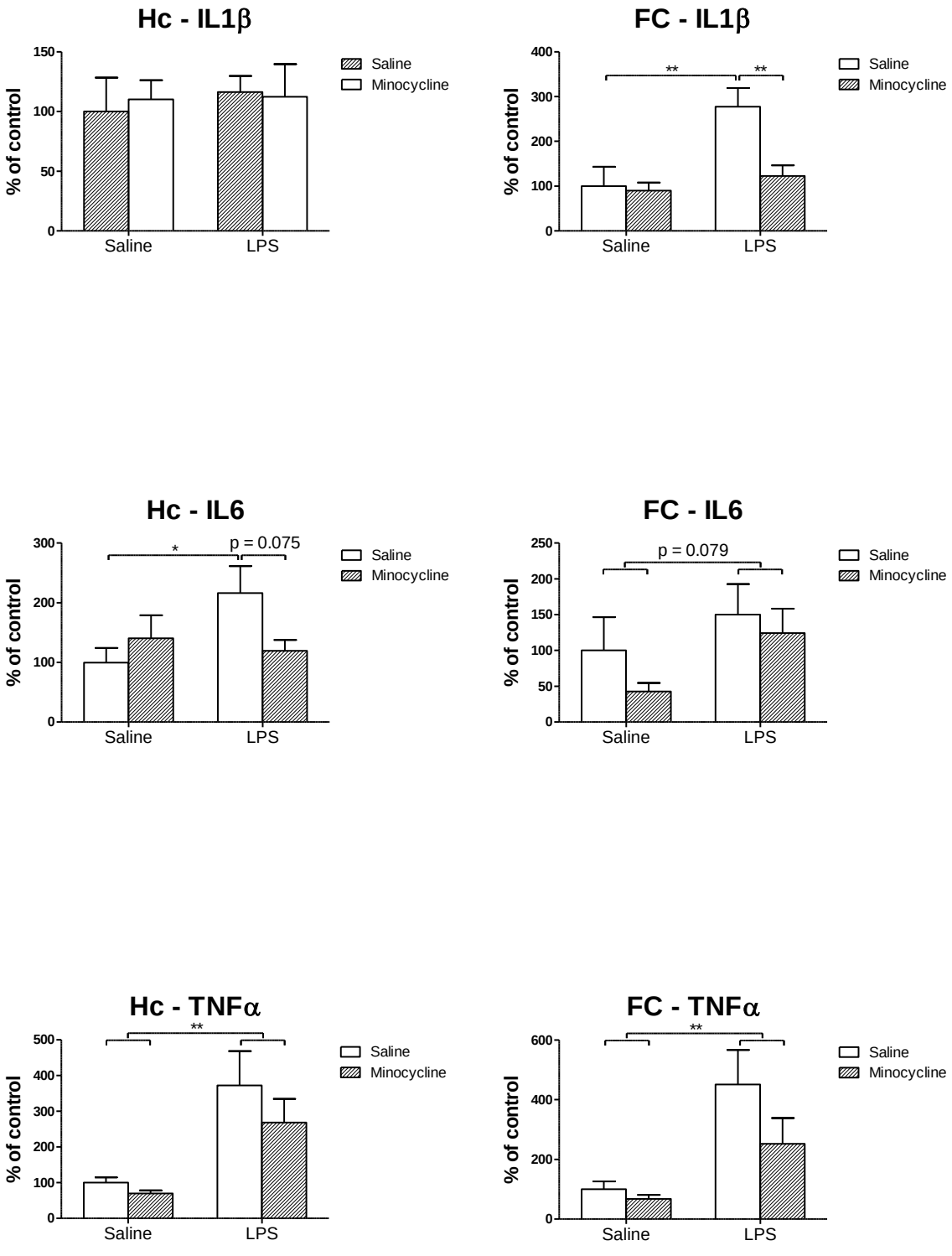


Fig 2.3.3 Brain mRNA expression of pro-inflammatory cytokines were significantly increased by LPS 24 hours post-treatment. Minocycline ameliorated the LPS-induced increase in IL-1β in the FC.

Hc: Hippocampus, FC: Frontal Cortex; IL-1β: Interleukin-1β; IL-6: Interleukin-6

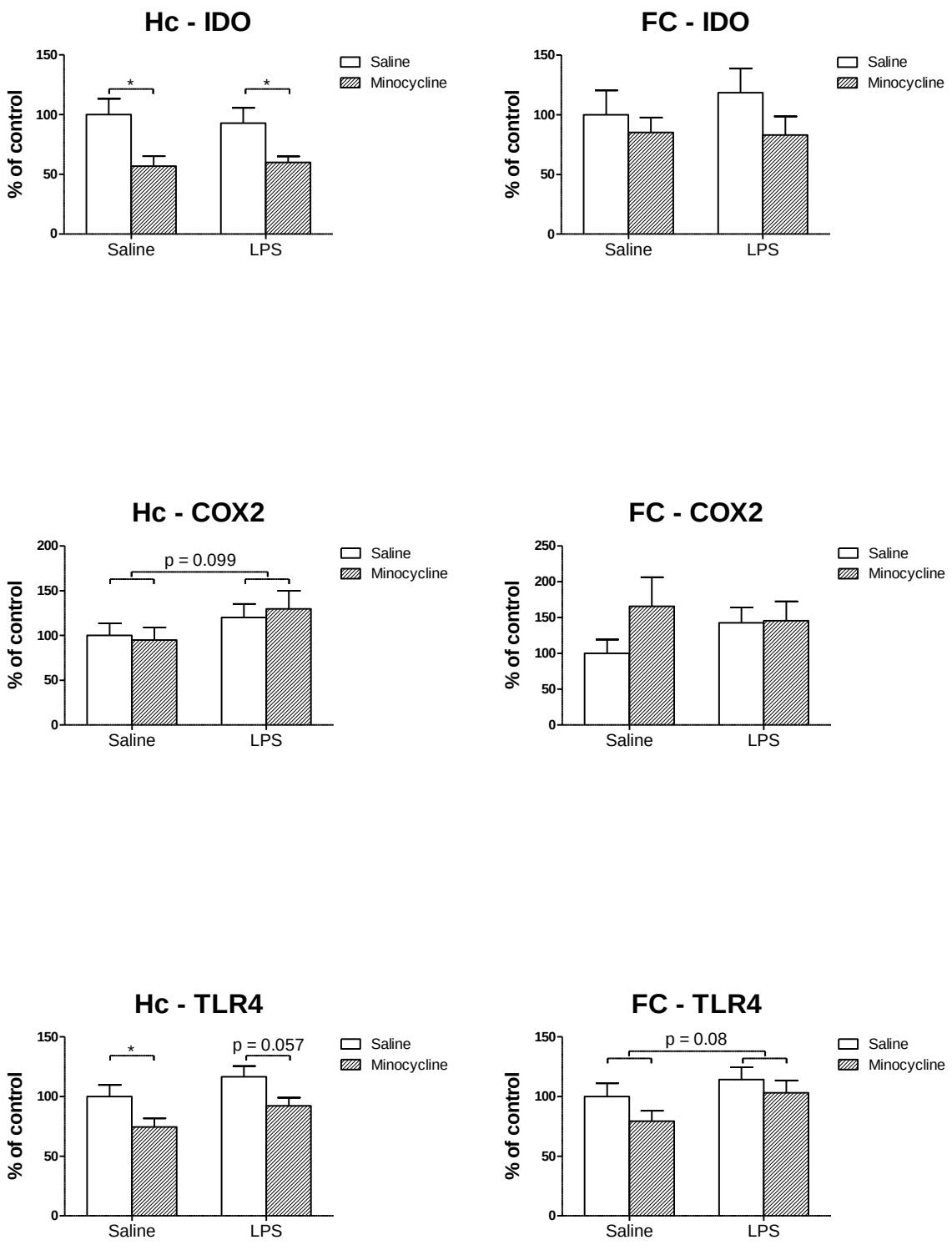


Fig 2.3.4 Brain mRNA expression of pro-inflammatory markers.

IDO: Indoleamine 2,3-dioxygenase; COX2: Cyclooxygenase 2; TLR4: Toll-like receptor 4

2.3.2 Expression of central glutamate receptor genes

2.3.2.1 NMDARs

The next part of the study investigated the links between inflammation and the different GluN2 subunits. Sub-region-specific analysis revealed that most changes occurred in the CA1 region of the hippocampus.

There was a significant minocycline main effect ($F_{1,34} = 7.548$, $p = 0.010$), and a significant LPS*minocycline interaction effect ($F_{1,34} = 6.920$, $p = 0.013$) for the GluN2B subunit in the CA1 region of the hippocampus. Pairwise comparisons revealed that a single peripherally-administered low dose of LPS reduced GluN2B expression compared to control (SalLPS -15.48%, $p = 0.016$). However, minocycline administration increased GluN2B mRNA in LPS-treated animals (MinoLPS +23.31%, $p < 0.001$). A similar result was obtained for GluN2A expression where a significant LPS*minocycline interaction ($F_{1,33} = 12.452$, $p = 0.001$) was observed. A significant LPS main effect ($F_{1,33} = 6.411$, $p = 0.016$) was also observed. As tests of normality was violated for some treatment groups, an additional non-parametric test was conducted. A significant difference was found for the distribution between treatment groups (Kruskal Wallis, $p = 0.002$), with pairwise comparisons revealing that this was driven by differences between the SalSal and SalLPS group (SalLPS -25.15%, $p = 0.003$), and the MinoLPS and SalLPS group (MinoLPS +21.79%, $p = 0.009$).

A significant minocycline effect ($F_{1,34} = 5.790$, $p = 0.022$) was also observed for the GluN2B subunit in the dentate gyrus (DG). This was mainly driven by the decrease in GluN2B expression in the SalLPS group compared to the MinoLPS group (SalLPS -16.87%, $p = 0.036$).

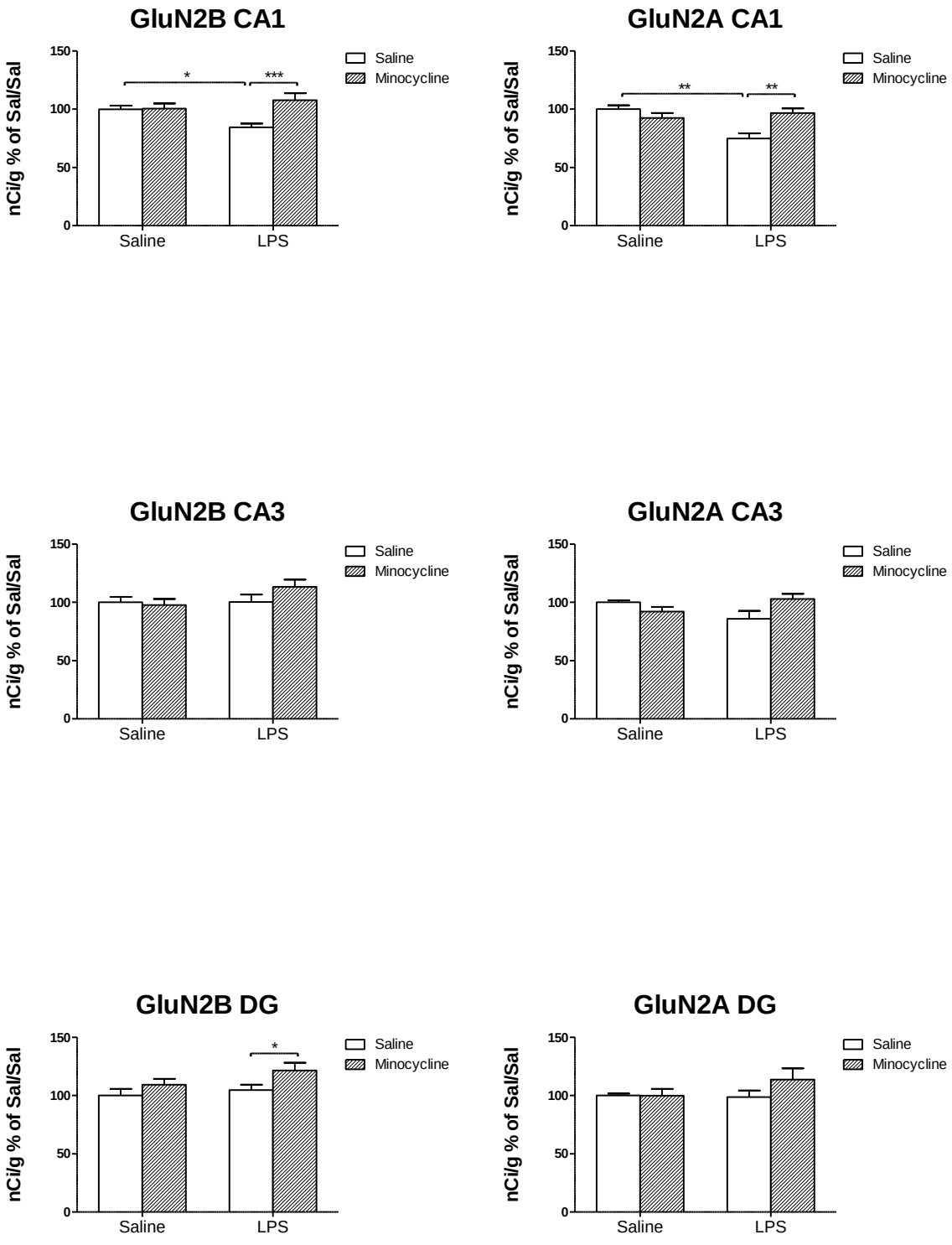


Fig 2.3.5 Sub-region specific expression of the GluN2 subunit in the hippocampus. The LPS-induced decrease in GluN2B and GluN2A expression in the CA1 region was ameliorated by minocycline treatment.

CA1: Cornu Ammonis 1; CA3: Cornu Ammonis 3; DG: Dentate Gyrus

2.3.3 NMDAR-associated signalling protein activity and levels

2.3.3.1 Signalling proteins (CamKIIα and ERK1/2)

The previous section showed that GluN2 expression was decreased by peripherally administered LPS-induced inflammation, and that this decrease was not observed in animals treated with both minocycline and LPS. The next step was then to investigate the functional implications of this change. In this section, the level (expressed as the ratio of total protein levels over the levels of the loading control β-Actin) and the activity (expressed as the ratio of phosphorylated protein levels over total protein levels) of 2 NMDAR-associated signalling proteins were investigated.

ERK1/2

There was a significant LPS main effect ($F_{1,39} = 5.554$, $p = 0.024$) and a significant LPS*minocycline interaction ($F_{1,39} = 5.784$, $p = 0.021$) on total ERK levels. Pairwise comparison revealed that this was mainly driven by the increase in ERK levels as a result of LPS administration compared to control (SalLPS +16.083%, $p = 0.002$). However, there was also a trending increase in ERK levels as a result of minocycline administration (MinoSal +9.301%, $p = 0.062$). As the assumption of equal variance between groups was violated, a non-parametric analysis was also conducted. It was determined that there a significant difference in distribution across the treatment groups (Kruskal Wallis, $p = 0.022$), driven by differences between the SalSal and SalLPS groups ($p = 0.013$ adjusted). There was no difference in the activity of ERK1/2 between treatment groups.

CamKIIα

There were no significant main effects or interactions on CamKIIα levels and activity. However, a trending LPS*minocycline interaction effect ($F_{1,38} = 3.864$, $p = 0.057$) was observed for CamKIIα activity, driven by a decrease in activity as a result of minocycline administration

compared to control (MinoSal -33.768%, p = 0.058). This decrease was ameliorated in animals treated with both minocycline and LPS (MinoLPS +46.132%, p = 0.078). As the assumption of equal variance between groups was violated, a non-parametric analysis was also conducted. Results determined that there was no significant difference in distribution between treatment groups (Kruskal Wallis, p > 0.05).

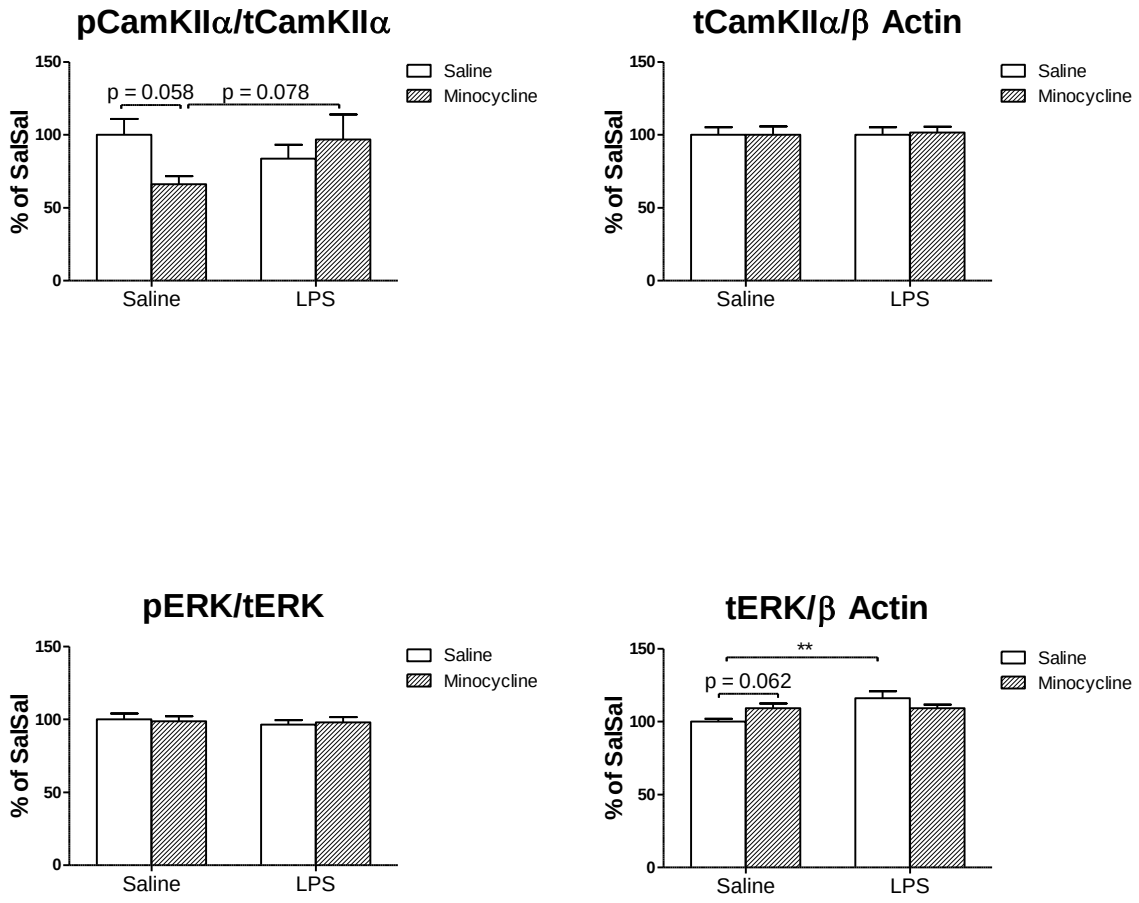


Fig 2.3.6 Protein expression levels of total and phosphorylated CamKIIα and ERK in hippocampal lysates.

pCamKIIα/tCamKIIα: ratio of phosphorylated CamKIIα over total CamKIIα; tCamKIIα/β Actin: ratio of total CamKIIα over levels of the internal control β Actin; pERK/tERK: ratio of phosphorylated ERK over total ERK; tERK/β Actin: ratio of total ERK over levels of the internal control β Actin.

2.3.3.2 Variation between cohorts

Large variations were observed between cohorts for the measurements of the activity and levels of signalling proteins (Fig 2.3.7). Initial changes observed in the first cohort were not observed when the experiment was replicated in the second cohort. In the case of CamKIIα activity, some results had the opposite effect when the experiment was replicated. For example, CamKIIα activity increased compared to control versus the decrease observed in the first cohort for the MinoLPS group. Thus, it was difficult to draw meaningful conclusions from the results on signalling proteins.

Nevertheless, the results of this study hint that peripherally-administered inflammation and minocycline administration modulate NMDAR-associated signalling proteins, although the exact effect is difficult to elucidate.

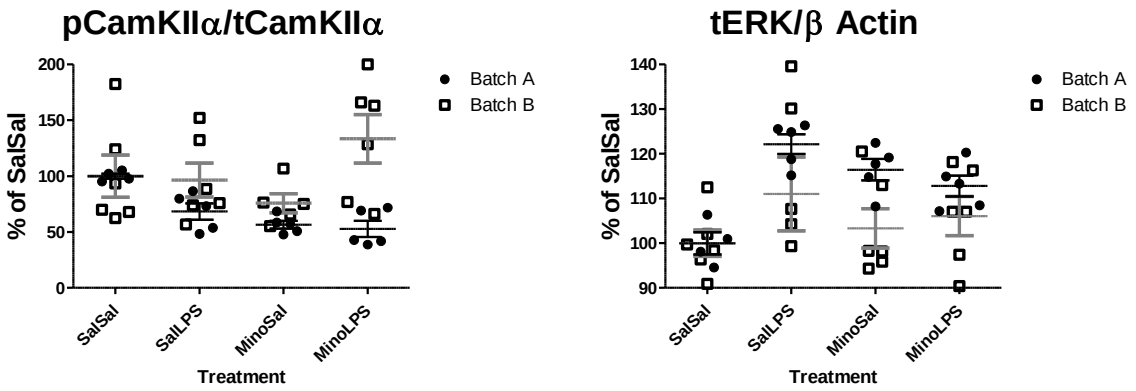


Fig 2.3.7 Representative scatter-plots showing variation between cohorts grouped by treatment

2.3.4 Arc gene expression

The Arc protein has been shown to be important for LTP, and Arc expression has been shown to be a marker for neuronal activity. Thus, Arc expression was studied to show functional changes downstream of NMDAR expression changes.

Two-way ANOVA revealed that Arc expression mirrors that of the GluN2 subunit. A significant LPS*minocycline interaction effect ($F_{1,32} = 50.177$, $p < 0.001$) was observed in the CA1 region of the hippocampus. Pairwise comparisons showed that this was driven by a decrease in Arc expression in mice treated with LPS alone compared to control (SalLPS -13.73, $p < 0.001$), and an increase in Arc expression in the MinoLPS group compared to the SalLPS group (SalLPS -20.631%, $p < 0.001$).

However, minocycline administration also reduced Arc expression in the CA1 region of the hippocampus. There was a significant decrease in Arc expression when mice treated with minocycline alone were compared with control (MinoSal -13.34%, $p < 0.001$). There was also a significant decrease in Arc expression when mice treated with minocycline alone were compared with mice treated with both minocycline and LPS (MinoSal -20.24%, $p < 0.001$). Thus the increase in Arc expression was only observed when mice previously treated with minocycline were stimulated with LPS.

Arc expression was also altered by LPS and minocycline administration in the FC. A significant minocycline main effect ($F_{1,13} = 6.383$, $p = 0.025$), and LPS*minocycline interaction effect ($F_{1,13} = 5.210$, $p = 0.040$) was observed. Pairwise comparisons revealed that this was mainly driven by differences between the SalSal and SalLPS groups (SalLPS -23.82%, $p = 0.048$), and between the SalLPS and MinoLPS groups (MinoLPS +47.46%, $p = 0.006$). A similar result was obtained for a sub-region of the FC, the retrosplenial cortex (RS). A significant LPS*minocycline interaction effect ($F_{1,13} = 6.519$, $p = 0.024$) was also observed, driven by trending differences

between the SalSal and SalLPS group (SalLPS -16.73%, p = 0.051), and trending differences between the SalLPS and MinoLPS group (MinoLPS +17.40%, p = 0.085).

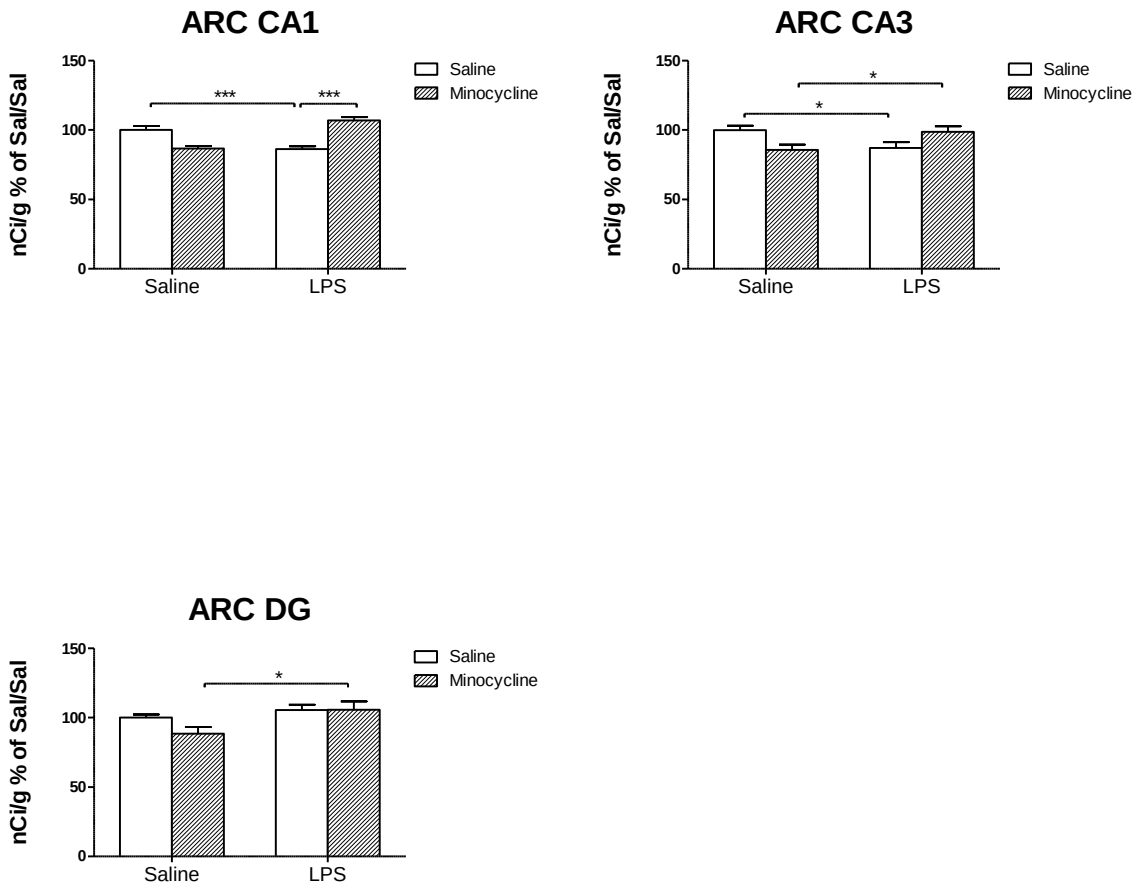


Fig 2.3.8 Sub-region specific expression of the Arc in the hippocampus. The LPS-induced decrease in Arc expression in the CA1 region was ameliorated by minocycline treatment.

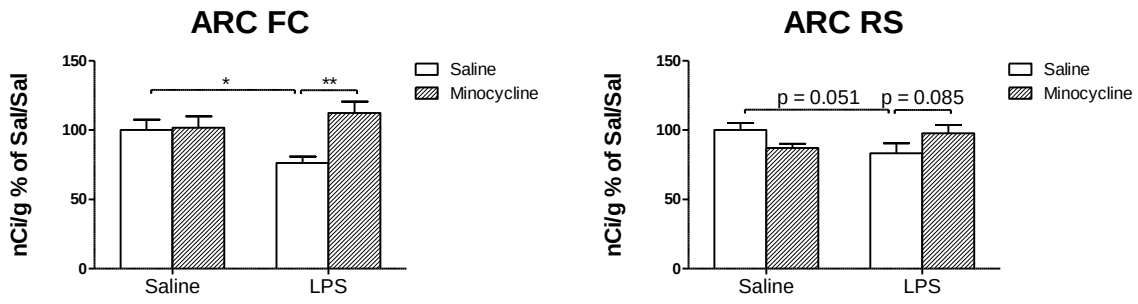


Fig 2.3.9 The LPS-induced decrease in Arc expression in the FC was ameliorated by minocycline treatment. Similar trending differences were found in the RS sub-region.

Arc: Activity-regulated cytoskeleton associated protein; RS: Retrosplenial Cortex

2.4 Discussion

Summary of results

The results of this study showed that LPS administration increased levels of inflammatory markers in the periphery (SAA2 mRNA and plasma corticosterone), and in the brain (inflammatory cytokines and TLR4 mRNA). LPS administration also decreased the expression of the GluN2 subunits in the CA1 region of the hippocampus. However, this decrease was prevented when animals were treated with minocycline. Similar effects were observed for Arc expression in the CA1 region of the hippocampus, where the LPS-induced decrease was ameliorated by prior treatment with minocycline. An unexpected result to note was the increased liver SAA2 expression following minocycline administration.

LPS effects 24 hours post-dose: Insights and comparisons with current literature

The cytokine hypothesis of depression proposes that there is an inflammatory component, indicated by increased levels of pro-inflammatory cytokines in the circulation of patients (Maes, 2008; Wohleb, Franklin, Iwata, & Duman, 2016). This has brought about induced-inflammation animal models of depression, such as through administration of LPS. However, one of the challenges of this model is to dissociate between LPS-induced sickness behaviour and LPS-induced depressive behaviour. Frenois et al. (2007) has shown that 24 hours post-LPS dose is a time-point where sickness behaviour has normalized to control, while depressive behaviours are still prevalent.

The results obtained in this study validate the LPS model of depression at the molecular level. The lack of difference in liver TNFα expression between treatment groups suggests that LPS-induced increases in pro-inflammatory cytokine levels in the periphery have normalized after 24 hours. However, significant increases in the expression of pro-inflammatory cytokine levels in the brain can still be observed. Inflammatory markers, such as TLR4, are also suggested to still be elevated in the brain after 24 hours.

Although peripheral inflammatory cytokine levels were normalised 24 hours after LPS, liver SAA2 expression remained significantly increased in LPS-treated animals compared to control. This is in line with current literature, as the increase in pro-inflammatory cytokines is thought to induce the APR, and the expression of major APR proteins like SAA2 have been shown to be elevated up to 48 hours post-LPS (Cray et al., 2009; Gruys et al., 2005).

While O'Connor et al. (2009) stated that pro-inflammatory cytokine upregulation was ubiquitous in discrete brain regions, the current study suggested that LPS had different effects on different brain regions, with differential expression of inflammatory markers in the frontal cortex and hippocampus.

Inflammation and NMDARs

The prevailing theory underlying the mechanism of how inflammation modulates glutamatergic signalling is through the catabolism of tryptophan to form neuro-active compounds that act on NMDARs (Hillhouse & Porter, 2015). However, current research on the kynurenine pathway and its effects on NMDARS are limited to the formation of the NMDAR antagonist KA and the NMDAR agonist QUIN, and to behavioural effects where kynurenine administration has been shown to induce depressive-like behaviour. Thus, they do not investigate actual effects on NMDARs and downstream signalling proteins.

A separate mechanism was proposed by Viviani et al. (2003) where IL-1β modulated NMDAR activity through binding to the IL-1 receptor (IL-1R) and forming a signalling complex leading to phosphorylation of NMDARs at the GluN2 subunit. Thus, they have shown that inflammation increased the activity of NMDARs through phosphorylation of GluN2B at the tyrosine-1472 residue. They also demonstrated some downstream molecular effects, namely an increase in intracellular calcium concentration and increased neuronal cell death. However, there is still a gap in our knowledge of the downstream effect on NMDAR-associated signalling proteins.

The current study attempted to address that by investigating GluN2 subunit expression in the hippocampus, as well as the activity and levels of downstream signalling proteins, and a functional marker Arc expression. In contrast to the results obtained by Viviani et al. (2003) where only activity changed, the current data show an alteration in the expression of the GluN2 subunits, where LPS treatment decreased both the GluN2A and GluN2B subunits. However, this is not surprising given the methodological differences. The Viviani et al. study was conducted in primary cultures of hippocampal neurons which were only exposed to IL-1β for several minutes, and NMDA was also added as a NMDAR agonist. The present study, however, was an *in vivo* investigation of the effects of peripherally-administered inflammation on hippocampal tissue after 24 hours. It is possible that any initial change in phosphorylation would not be detected after 24 hours, and that expression changes may occur instead.

Also, given that decreased expression of mRNA levels does not necessarily translate to decreased protein levels, the functional effects of NMDAR modulation was explored. We observed a significant increase in ERK levels and a significant decrease in Arc expression, indicating that the decrease in GluN2 subunits had functional effects. The parallel decreases in the expression of the GluN2 subunits and Arc mRNAs suggest that LPS administration resulted in NMDAR-mediated decreases in synaptic activity.

As a corresponding change was not observed for IL-1β expression in the hippocampus, it is possible that the NMDAR and Arc changes were not mediated by IL-1β, and occurred through a separate mechanism from that which was proposed by Viviani and colleagues. It is also possible that changes in IL-1β expression may have returned to control levels after 24 hours. Nevertheless, this study has shown that LPS-induced inflammation decreased NMDAR and Arc expression. As Arc has been shown to be important for synaptic plasticity, LTP, and the consolidation of implicit and explicit memory (Bramham et al., 2010; Shepherd & Bear, 2011), impaired Arc function could be a possible mechanism underlying the inflammatory mechanisms in psychiatric diseases.

The effects of repeated minocycline administration 24 hours post-LPS

Minocycline administration was shown to increase liver SAA2 expression. This was in contrast to our hypothesis that minocycline would ameliorate LPS-induced effects on inflammatory markers. Nevertheless, SAA2 is a major APP with immunomodulatory effects, and while it is more commonly associated with pro-inflammatory actions (Ebert et al., 2015), SAA2 has also been proposed to have immunosuppressive properties (Ye & Sun, 2015), and it is possible that the increase in SAA2 expression is a compensatory mechanism to suppress inflammation.

Minocycline administration also significantly decreased IDO expression in the hippocampus. This shows that under the current study's dosing regimen, peripheral administration of minocycline has central effects, and supports the action of minocycline as an inhibitor of IDO (O'Connor et al., 2009).

With regards to glutamate transmission, prior treatment with minocycline prevented the LPS-induced decrease of GluN2 subunit expression in the CA1 region of the hippocampus. This effect was also mirrored by Arc expression in the same sub-region, and suggests that minocycline rescued the LPS-induced impairment of NMDAR function and thus restored synaptic activity. However, similar results were not obtained for ERK, a key indicator of NMDAR function. Thus, it is possible that ERK changes, while relevant to LPS-induced inflammation, may not explain minocycline's activity, and both it and LPS are influencing NMDAR and ARC expression separately.

Signalling proteins: Variation

The effect of inflammation on signalling proteins is inconclusive due to large variations between the cohorts of animals used in each batch of replicate experiments. Another confounder may be that phosphorylation of signalling proteins is transient, and may have returned to baseline before 24 hours. This is conceivable given that phosphorylation of proteins

is a rapid post-translational process, as previously demonstrated (B. Viviani et al., 2003), whereas alterations in gene expression are more gradual over time.

Limitations of study

This study has several limitations. Firstly, having a larger sample size would have increased the power of the investigation. As the current study involved several techniques with different tissue processing methods, some experiments were only conducted with a single cohort. Furthermore, signalling protein changes were very variable, and a larger sample size may have helped with forming more conclusive results.

Due to time constraints, the current study focused mainly on the hippocampus. However, results showed that LPS treatment had a significant increase on IL-1β expression in the frontal cortex, a change that was ameliorated by minocycline treatment. ISH results of Arc expression also showed that LPS and minocycline administration resulted in similar effects in the frontal cortex and the hippocampus. It would have been interesting to investigate if NMDAR changes in the frontal cortex would be similar to that observed in the hippocampus, and if NMDAR expression changes would match that of IL-1β expression.

2.5 Conclusion

This study has shown that a peripherally-administered dose of LPS has both peripheral and central effects after 24 hours, some of which are ameliorated by treatment with minocycline. Previous studies have proposed mechanisms through which inflammation can influence NMDAR signalling, and increase depressive-like behaviours. The current study has built on these experiments by showing that LPS treatment induced decreases in the expression of GluN2 subunits and Arc. This suggests that inflammation could lead to the behavioural changes observed through reduced synaptic plasticity. Prior treatment of minocycline prevented the LPS-induced elevation of brain inflammatory markers, and the reduced GluN2 and Arc expression. These results therefore present a mechanism for how the anti-inflammatory effects of minocycline may underlie its psychotropic effects. However, minocycline also induced main effects that were not directly linked to preventing LPS-induced changes, such as the increase in liver SAA2 expression, that implicate non-anti-inflammatory effects.

This will be explored further in the next chapter, which investigated the effects of LPS and/or minocycline on plasma and brain metabolites, where metabolite changes could discern mechanisms underlying the psychotropic properties of minocycline that may not be linked to its anti-inflammatory effects.

Chapter 3: The effects of inflammation and minocycline on plasma and brain metabolites

3.1 Introduction

3.1.1 Background

The psychotropic effects of minocycline have been studied in pre-clinical animal models and neuronal cultures, which show that its anti-inflammatory properties are key to its central actions. O'Connor et al. have linked the anti-inflammatory effects of minocycline to the metabolism of tryptophan through the kynurenine pathway (O'Connor et al., 2009). While Chapter 2 focused on the effects of minocycline administration on central glutamate receptors and downstream cell signalling in a mouse inflammation model, this chapter will take a more systems-wide approach, and look at the effects of minocycline on plasma and brain metabolites using the same model.

Studies on the metabolome have proven useful in identifying metabolic profiles – changes in specific metabolites – that are characteristic of a phenotype. For instance, Larrieu et al. demonstrated the metabolic profiles that were linked to social ranking in a mouse chronic social defeat stress model, and susceptibility to depressed behaviour (Larrieu et al., 2017).

While there are not many studies in current literature exploring the effects of inflammation on the metabolome, Dai et al. have explored inter-individual variation in response to LPS administration with metabolomics techniques in a rat model. They found that inter-individual susceptibility to LPS was mainly associated with lipid metabolism, where different species of lysophosphatidylcholines were found in the survival and non-survival groups, and sphingosine was unique to the survival group. Other metabolites increased as a result of LPS administration were related to energy metabolism, such as S-adenosyl methionine (SAM), and components of the Krebs cycle, including glucose, and citrate, linking LPS treatment to oxidative

stress (Dai et al., 2016). These studies suggest the importance of metabolites in separating between phenotypes and revealing the perturbations of pathways that could explain downstream behaviour/pathology.

3.1.2 Aims and hypotheses

The current study investigated the metabolic effects of minocycline in mice under two treatment regimes, a single dose, and repeated administration. The single dose study is a hypothesis-generating study to identify early metabolite changes that could explain the psychotropic effects of minocycline. For the repeated administration study, the first objective was to study the effects of inflammation, stimulated by LPS administration, on both brain and plasma metabolites. The second objective was to study the effects of repeated administration of minocycline on brain and plasma metabolites, and also to determine if prior repeated dosage of minocycline would ameliorate the effects of inflammation.

Although the current study is not the first to study the effects of LPS on metabolites, its objective and protocols differ from that of Dai and colleagues. The objective of the latter study was to investigate inter-individual variation in response to LPS with survival as the primary outcome, and so a high dose of LPS (10mg/kg) was used which was lethal to two-thirds of the population within 72 hours post-treatment. On the other hand, the mouse inflammation model used here involved a low dose of LPS (0.83mg/kg) to model increased levels of pro-inflammatory markers, without sickness behaviour, as seen in patients with psychiatric diseases. The previous work was also limited to examining plasma metabolite changes, while both plasma and brain metabolites were investigated here. Nevertheless, based on the results of Dai and colleagues, the hypothesis was that a low-grade inflammation would also affect plasma metabolites related to lipid and energy metabolism. Given the effects of peripherally-administered inflammation on central glutamate receptors (Chapter 2), it was also hypothesized that intra-peritoneal

administration of LPS would result in observable differences in brain metabolites, possibly related to glutamate.

To our knowledge, this current study is the first to look at the effects of minocycline on plasma and brain metabolites. As such, an untargeted hypothesis-generating metabolomics analysis was conducted to identify global metabolite differences. Nevertheless, other studies have used metabolomics analysis to study the effects of drugs with antidepressant properties on metabolites. Their results also suggest changes in metabolites related to lipid and energy metabolism. For example, Weckmann et al. have shown that ketamine administration reduced the ratio of adenosine triphosphate (ATP) over adenosine diphosphate (ADP) (Weckmann et al., 2017). Finally, given the anti-inflammatory properties of minocycline, it was hypothesized that minocycline would ameliorate some of the inflammatory effects of LPS.

3.2 Methods

3.2.1 Drugs

Lipopolysaccharide (LPS) from Escherichia coli, and Minocycline Hydrochloride (Minocycline) were used (all from Sigma Aldrich).

3.2.2 Animals

The experiments were performed on CD-1 mice (male, adult 6-8weeks). The mice were housed 5 to a cage under standard environmental conditions and had access to food and water *ad libitum*. All animal procedures were carried out in accordance with UK Home Office Animals (Scientific Procedures) Act (1986) and associated Home Office guidelines. The local Animal Welfare and Ethical Review Body (AWERB) at the University of Oxford approved the procedures specific to this study.

Acute Dose:

Mice were randomly divided into two groups of 10 each (N=20), and were administered minocycline or saline via an intra-peritoneal injection (Fig 3.2.1A). Two hours after, mice were humanely culled.

Repeated Dose:

Mice were treated in batches of 20 (N=5/treatment group) in a 2x2 factorial arrangement (Fig 3.2.1B). Four batches were combined to get 20/treatment group, however one of the batches was excluded at the analysis stage for a total of 15/treatment group (N=60), as it was determined that lack of availability of dry ice for immediate freezing of samples introduced additional variability. Mice were injected (intraperitoneal) with minocycline or saline for seven days, once a day. On the 7th day, mice were also injected with LPS or saline. After 24 hours, mice were humanely culled.

3.2.3 Tissue and plasma collection

Frontal cortex, hippocampus, and trunk blood was collected. Trunk blood was collected in a tube containing ethylenediaminetetraacetic acid (EDTA), and spun at 17,000 xg for 10 min. The supernatant (plasma) was then transferred to a separate tube. All samples were frozen at -80 °C until extraction.

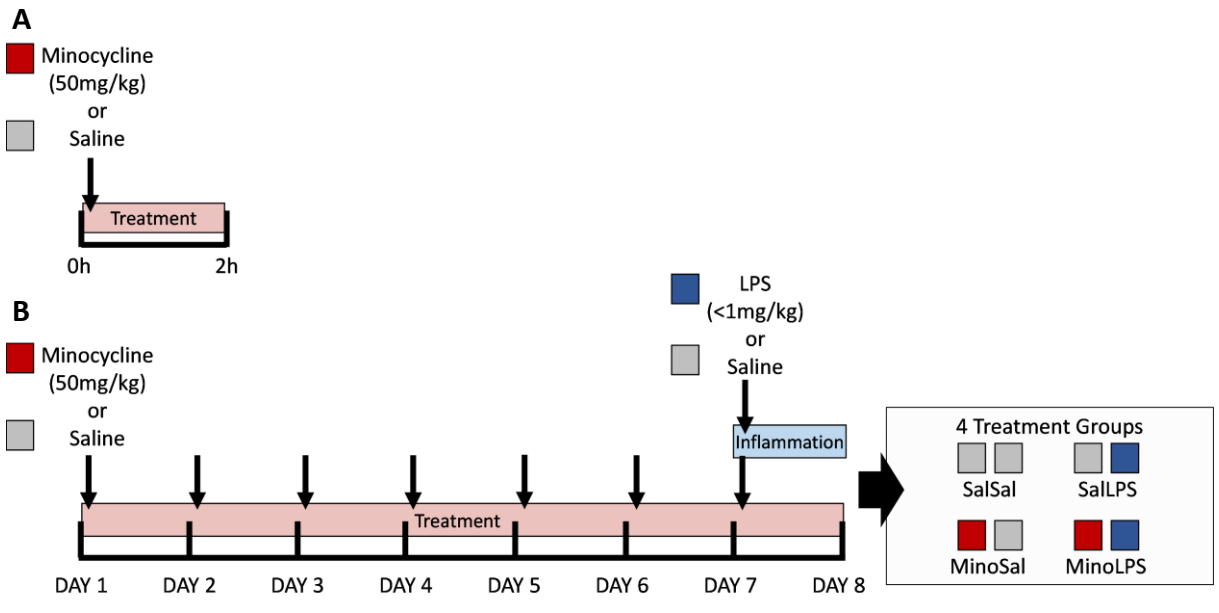


Fig 3.2.1 Illustration of treatment regime of (A) acute dose of minocycline and (B) repeated dosage of minocycline and a single injection of LPS in CD1 mice

3.2.4 Aqueous-phase metabolite extraction

Brain tissue metabolite extraction protocol was adapted from (Viant, 2007). Briefly, brain tissue metabolites were extracted in a solution of 0.1 N HCl/Methanol (200% v/w), 0.02 N HCl (1000% v/w) and 3 M Perchloric Acid (250% v/w) with a glass mortar and pestle. Samples were then centrifuged at 3500 xg for 15 min at 4 °C, and supernatants containing aqueous-phase metabolites were transferred to a new tube. Pellets containing lipid-phase metabolites were stored at -80 °C for further extraction.

The supernatants were then neutralized in 10 M KOH (92.2% v/w) and cold 0.5 M phosphate buffer (276% v/w). pH was adjusted to 7 with pH test strips (Sigma). The neutralized solution

was then left on ice for 15 min, and centrifuged for an additional 15 min at 3500 xg at 4 °C. Supernatants were then transferred to a fresh tube and lyophilized in a freeze-vac (Savant™ Speedvac™ concentrator, ThermoFisher Scientific, UK). Samples in the form of lyophilized powder were then stored at -80 °C until further analysis.

3.2.5 ^{1}H NMR

Plasma samples: Plasma samples were defrosted on ice and centrifuged at 17,000 xg for 5 min at 4 °C. An equal volume of plasma was aliquoted (100 µL) into a fresh tube, and diluted to 600 µL in a 75 mM phosphate buffer (5:1 disodium phosphate Na_2HPO_4, monosodium phosphate NaH_2PO_4 in 100% D_2O, pH 7.4). The volume of plasma used was limited by the volume of plasma collected, and the volume chosen was the maximum volume available for 90% of samples.

Brain tissue samples: Lyophilized samples were resuspended in 600 µL of phosphate buffer (0.2M Na_2HPO4, 0.043 NaH_2PO4, in 100% D_2O with 0.05 wt % 3-trimethylsilylpropanoic acid (TSP)).

Samples were then transferred to 5 mm Borosilicate Glass NMR tubes (Norrell). ^{1}H NMR spectra were acquired using a 700-MHz Bruker AVII spectrometer operating at 16.4 T equipped with a ^{1}H (^{13}C/^{15}N) TCI cryoprobe. Sample temperature was stable at 310 K. ^{1}H NMR spectra were acquired using a one dimensional (1D) Nuclear Overhauser Effect Spectroscopy (NOESY) pre-saturation scheme for attenuation of the water resonance with a 2 s presaturation.

<u>Plasma Samples:</u>

An additional sequence, the spin-echo Carr-Purcell-Meiboom-Gill (CPMG) sequence, was used for plasma samples to suppress broad signals arising from large molecular weight plasma components with a τ interval of 400 µs, 80 loops, 32 data collections, an acquisition time of 1.5 s, a relaxation delay of 2 s, and a fixed receiver gain. CPMG spectra provide a measurement of small molecular weight metabolites and mobile side chains of lipoproteins in the plasma sample and were used for all further analysis of plasma samples.

The NMR spectrometer was operated by a research collaborator, Dr Fay Probert, from the department of Pharmacology.

3.2.6 Processing and analysis

NMR

Processing: Processing methods were adapted from published paramters (Jurynczyk et al., 2017). NMR spectra were imported into MestreNova (Mestrelab Research, Spain) and each spectrum was then processed manually with phase 0 (PH0) correction, baseline correction (Bernstein polynomial fit, order = 3), and referencing to an added standard (TSP referenced to δ0) for brain tissue, and an internal standard (Lactate referenced to δ1.33) for plasma. The individual spectra were then stacked, and binned (sum method, width of each integral region = 0.02ppm). Binning refers to a function where the whole spectrum is divided into bins of equal width, and all the peaks in each bin is integrated to obtained a value representing the area of all the peaks in a bin. Binned values were then exported as a spreadsheet (.xlsx) for further analysis. Inter-individual variation was reduced by total area normalization (each bin normalized to a ratio of the individual with the lowest total area over total area of the individual) to account for any dilution error. Brain tissue samples were also further normalized to TSP (each bin normalized to a ratio of the individual with the lowest TSP area over TSP area of the individual). Finally, noise areas were removed by two methods. First, spectral relative standard deviation (RSD) values were calculated for each bin, and bins with RSD greater than 100 were removed (Parsons, Ekman, Collette, & Viant, 2009). Second, the addition of the mean and standard deviation was calculated for each bin, and the average taken for a noise region. Bins with a value lower than that of the noise region would be removed. Broadly, areas that were removed include the water peak, regions before 0.7ppm, regions after 9.38ppm, noise region 5.0 to 6.0ppm, and contamination EDTA peaks for plasma samples.

Preliminary exploratory analysis: The normalized values were then imported into SIMCA (Umetrics, Sweden). Outliers were identified with principal component analysis (PCA) scores plots with pareto scaling. PCA is an unsupervised multi-variate analysis method that attempts to explain as much variation as possible in as few components (van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006). Pareto scaling removes the bias given to large peaks without inflating the noise, thus allowing the detection of changes in small and medium-sized peaks. Supervised multi-variate analysis was conducted using orthogonal partial least squares discriminant analysis (OPLS-DA), which attempts to find a linear relationship between a predictor matrix (spectrometric bin values) and a response matrix (treatment groups) (Triba et al., 2015).

Model building and validation: OPLS-DA models were built in R 3.3.2 (R Core Team, 2016) using the ROPLS package (Thévenot et al., 2015) and an in-house R script using 10-fold cross validation. The total number of samples were divided into 10 groups, with nine groups used to build the model (training set) and the last group used to test the model (testing set). For each iteration of the script, each of the 10 groups was used as the testing set once, thus 10 models were built for each iteration. The 10-fold cross validation was then repeated for a total of 100 iterations, and the robustness of the ensemble of models determined by looking at mean accuracy on the test set (i.e. how accurate the model is at predicting which group a sample in the test set belongs to). Other parameters include the average specificity of the models, sensitivity of the models, average Q^2 values, R^2X values, and R^2Y values. The Q^2 measures the internal predictive ability of the model (the accuracy of the model on the training set) and is used to optimise the model, while the R^2 measures the goodness of fit of the model (Triba et al., 2015), with R^2X and R^2Y measuring the fraction of the variation explained by the model of the X and Y variables respectively.

To validate the genuine OPLS-DA models built, the same cross-validation process was used with the classes of the data randomly permuted; samples were randomly assigned to treatment groups. This ensemble of OPLS-DA models represents the null distribution; the accuracy which can be achieved purely by chance, using random data. If the true OPLS-DA models perform significantly better than the permutation test, then the discrimination observed cannot have occurred by chance and thus the results are significant. Results of the comparisons between the ensemble of OPLS-DA models and the permutation tests are summarised in Appendix 3.1. For significant, predictive models, the variable importance in projection (VIP) scores were looked at to identify the key bins that were important for building the model. Bins with a high VIP score are bins that are significantly different between the treatment groups.

Metabolite identification and direction of change: Metabolites were assigned to peaks in bins with high VIP scores through a combination of literature values (Govindaraju, Young, & Maudsley, 2000; Misra & Bajpai, 2009), reference to the human metabolome database (HMDB) (Wishart et al., 2013), and confirmed with two-dimensional (2D) correlation spectroscopy (COSY). COSY provides confirmation that peaks occupying different positions on the spectrum belong to the same metabolite through cross-peaks that show a correlation between signals. The direction of change between groups was also determined by comparing means in SPSS (independent samples T-test for acute study, 2-way ANOVA for repeated dose study).

The flow of events from treatment to metabolite identification is summarised below (Fig 3.2.2).

Pathway analysis: Pathway analysis was conducted with the pathway analysis function in MetaboAnalyst 4.0 (Chong et al., 2018) using the hypergeometric test for over representation analysis, and the relative-betweeness centrality test for pathway topology analysis.

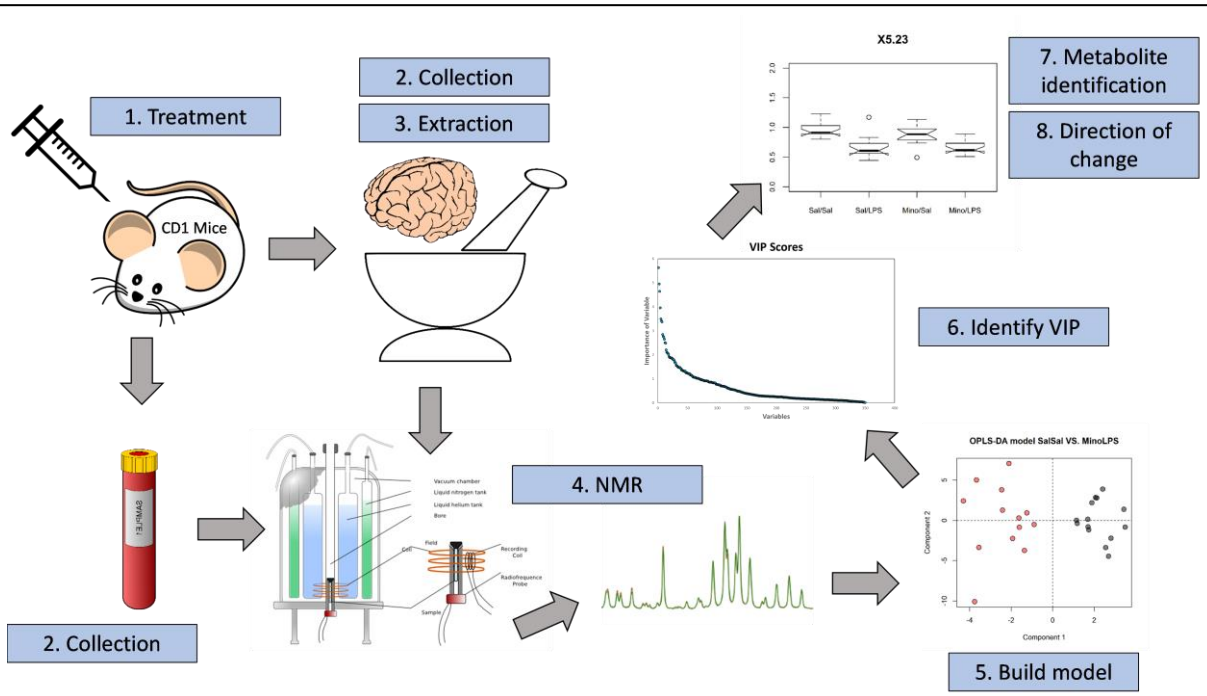


Fig 3.2.2 Summary of the flow of events from treatment of mice to metabolite identification numbered in ascending order

VIP: Variable importance in projection

3.3 Results

3.3.1 Validation of methods

3.3.1.1 Representative spectra

One-dimensional (1D) ^{1}H NMR Spectra obtained were of sufficient quality for the identification of metabolites in both aqueous phase brain tissue extracts and plasma samples (Fig 3.3.1). This was determined by peak width at half-height of the TSP (brain) and lactate doublet (plasma) peaks being less than 1.2 Hz, and visual inspection of the alignment of spectra after stacking. As the plasma samples did not undergo extraction, broad peaks representing mobile fatty acids in lipoproteins were identified. EDTA, a contaminant used as an anti-coagulant in plasma collection, was also identified in the spectra and the relevant peaks were removed before further analysis.

3.3.1.2 Variability – NMR spectrometer and batch

To build robust predictive models differentiating between treatment groups, other forms of variability that could potentially be greater than, and thus mask, inter-treatment group variability were checked for through a combination of PCA scores plots and spectral relative standard deviation (RSD) values.

To minimise spectrometer variation, brain tissue samples from the same study were ran in a single session. Technical controls (TC), aliquots of the same sample, were ran at intervals throughout the session. The PCA scores plot of all the brain tissue samples from the repeated dose study (Fig 3.3.2A) show tight clustering of the TCs, indicating low spectrometer variability. The clustering of TCs are also a validation of spectra processing methods.

For the repeated dose study, to obtain sufficient numbers in each treatment group, four batches of 20 animals (5/treatment group) were combined. This introduced an additional source of variation – inter-batch variation. Fig 3.3.2A revealed that brain region contributed the largest variation in all the samples, with clear separation of the two brain regions – hippocampus (Hc) and frontal cortex (FC). The PCA scores plot of experimental samples of both brain regions

coloured by batch (Fig 3.3.2B) showed that Batch 4 clustered separately from the other 3 batches, and was thus removed before further analysis. This was expected as brain tissue from Batch 4 was not immediately frozen after collection unlike the other three batches.

The other three batches formed a single cluster and suggested inter-batch variation was not significant in these cases. This was supported by the spectrum-wide RSD values of the different datasets (Table 3.3.1). Median RSD values have been proposed to be a useful measure for characterizing both technical and inter-individual biological variation (Parsons et al., 2009). As expected, the median RSD was lowest for TCs at 5.34%. Median RSD values for brain tissue extracts and plasma were similar to that seen in literature, with median RSD values higher in biofluids (plasma). There was no significant increase in variation between a single batch (Batch 2) compared to a dataset combining three batches (All) for plasma, Hc and FC samples. Furthermore, batch controls (BC) and quality controls (QCs) were also ran to check for variation between different batches of mice for treatment and metabolite extraction respectively. Briefly, for BCs, additional samples of cortex were collected for each treatment batch, and for QCs, samples were collected from a single mouse and divided into portions that were extracted with each batch. BCs and QCs did not cluster according to batch. Therefore, it was concluded that the different batches in the repeated dose study could be combined into a single dataset for further analysis.

3.3.1.3 Weight of animals as a result of treatment

For the repeated dose study, another area of concern was that any inter-group differences in metabolites could be a result of animals feeling unwell and eating less, rather than a change in metabolic pathways as a result of treatment. Therefore, two batches of animals were weighed before (Day 1) and after (Day 8) treatment (Fig 3.3.4). 2-way ANOVA showed no significant LPS effect, minocycline effect, or LPS*minocycline interaction on weight change (%) after treatment (Fig 3.3.4). Therefore, the conclusion was that metabolite changes between the groups are the result of treatment effects.

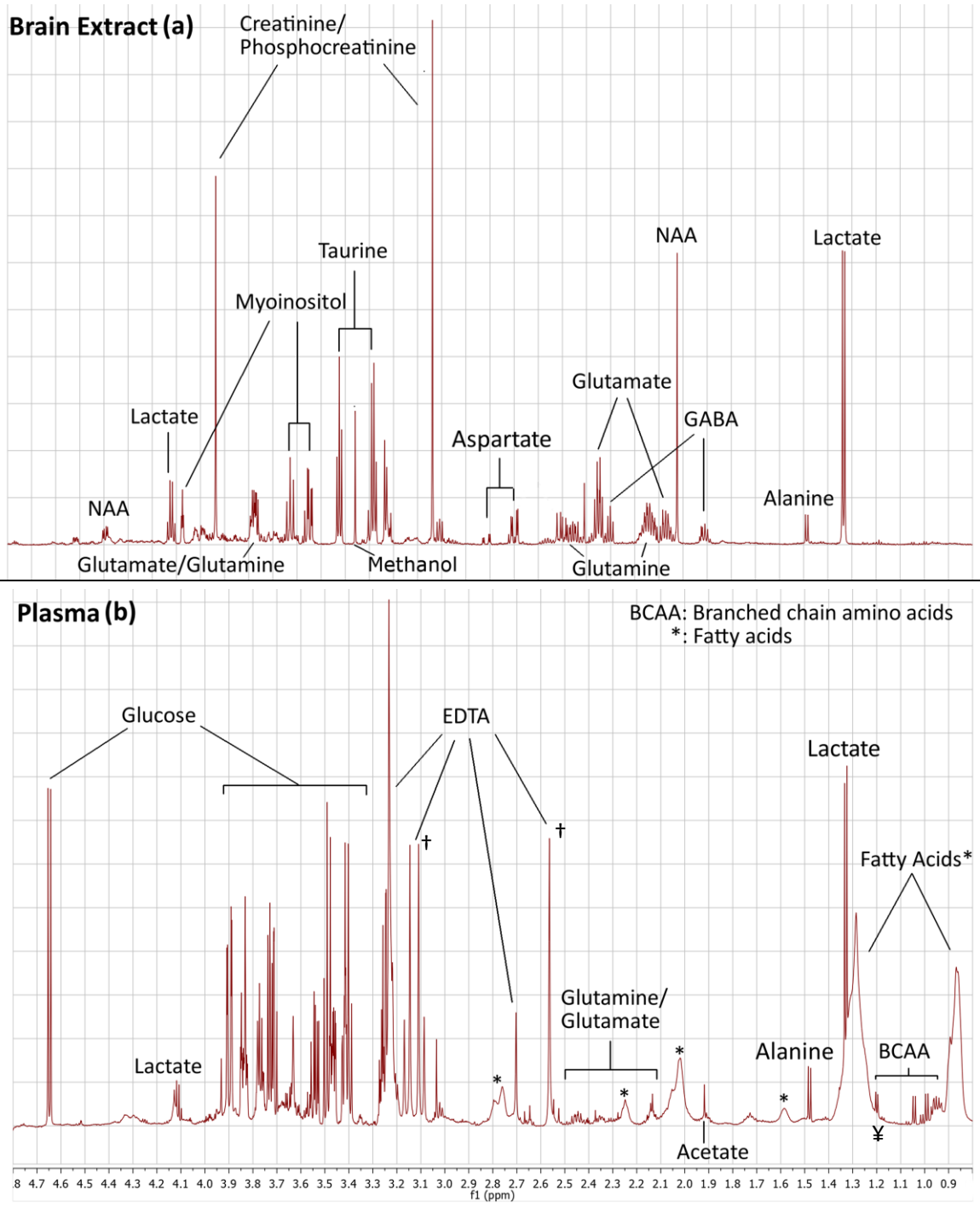


Fig 3.3.1 Representative ^{1}H NMR spectra of (A) aqueous phase brain tissue extracts and (B) plasma with labelled common metabolites.

BCAA: Branched chain amino acids, including ¥ β-hydroxybutyrate;
EDTA: Ethylenediaminetetraacetic acid (both free EDTA and † Mg^{2+} bound EDTA); GABA: γ-Aminobutyric acid; NAA: N-Acetyl aspartate

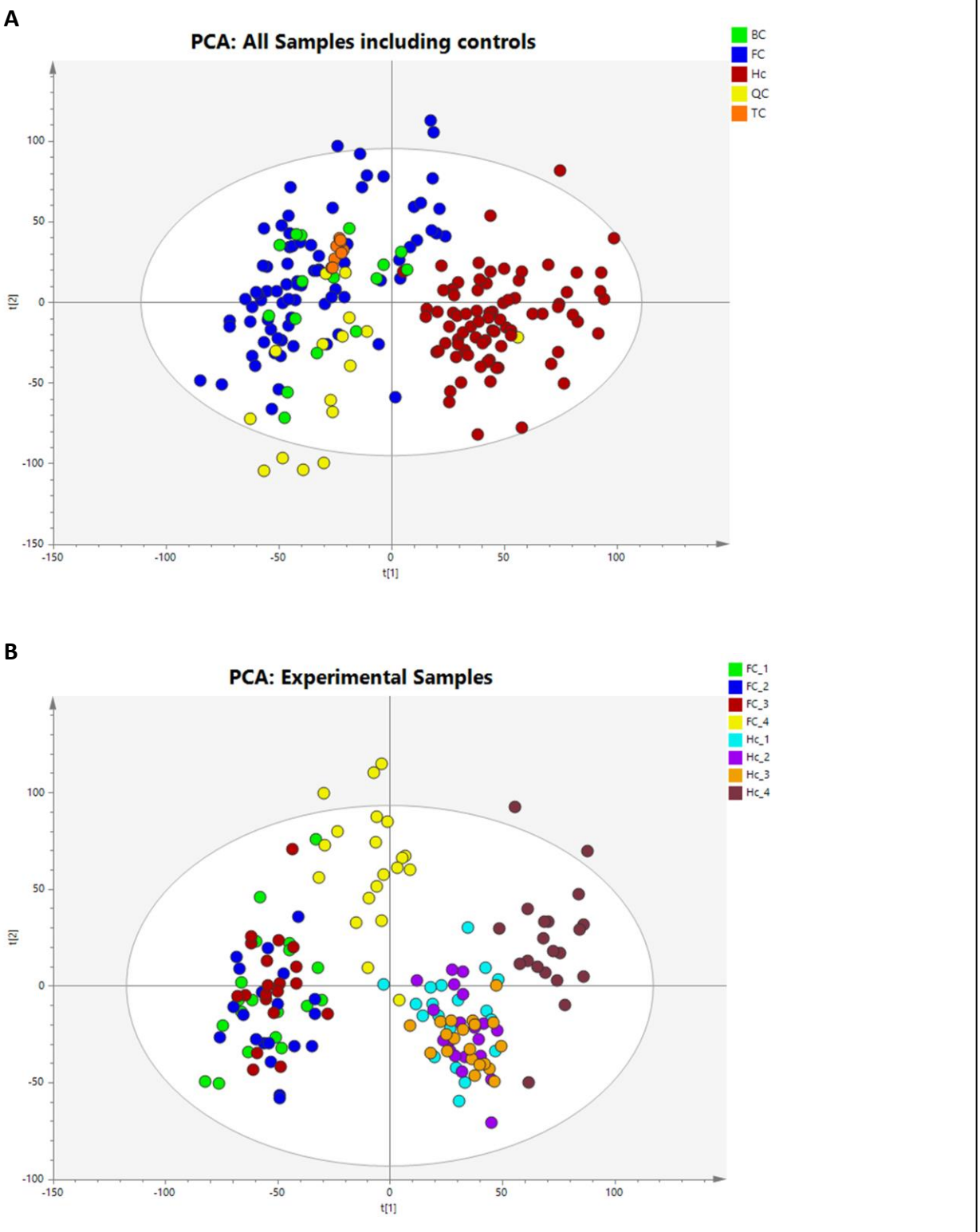


Fig 3.3.2 PCA scores plot of (A) all brain tissue samples [No.of components = 19; Q^2(cum) = 0.635; R^2X(cum) = 0.87], and (B) experimental brain tissue samples from the repeated dose study [No.of components = 16; Q^2(cum) = 0.621; R^2X(cum) = 0.859].

BC: Batch controls; QC: Quality controls; TC: Technical controls

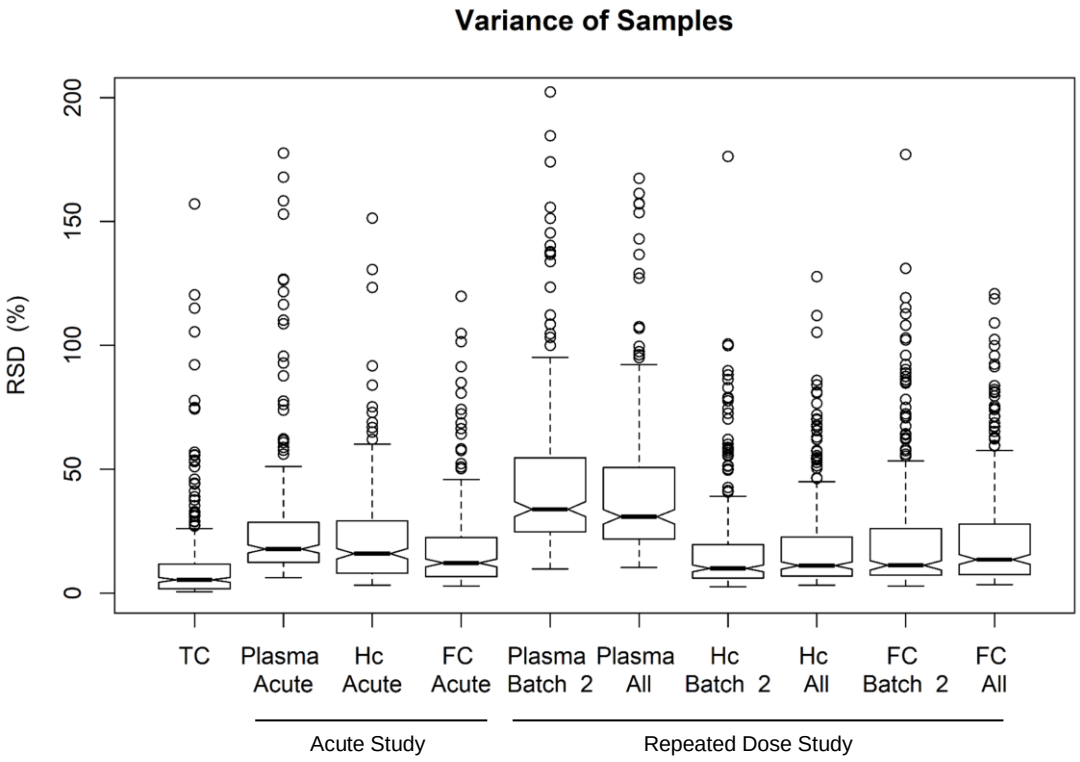


Fig 3.3.3 Spectrum wide RSD values of different datasets limited to 200% RSD

Table 3.3.1 Summary of spectrum wide RSD values of different datasets

	Technical Controls (TC)	Acute Dose Study			Repeated Dose Study					
		Plasma	Hc	FC	Plasma		Hc		FC	
					Batch 2	All	Batch 2	All	Batch 2	All
Min	0.5582	6.21	3.164	2.893	9.689	10.26	2.546	3.196	2.818	3.443
1st Quartile	1.7425	12.31	7.968	6.67	24.724	21.81	6.108	6.906	7.159	7.507
Median	5.3414	17.79	15.883	12.119	33.834	30.79	9.959	11.095	11.178	13.525
Mean	12.355	37.31	25.561	21.335	93.159	55.33	18.588	19.404	23.093	22.928
3rd Quartile	11.6467	28.55	28.922	22.468	54.369	50.7	19.544	22.485	25.99	27.632
Max	157.1159	961.88	601.687	483.372	9555.778	1078.09	176.188	127.673	177.045	120.853

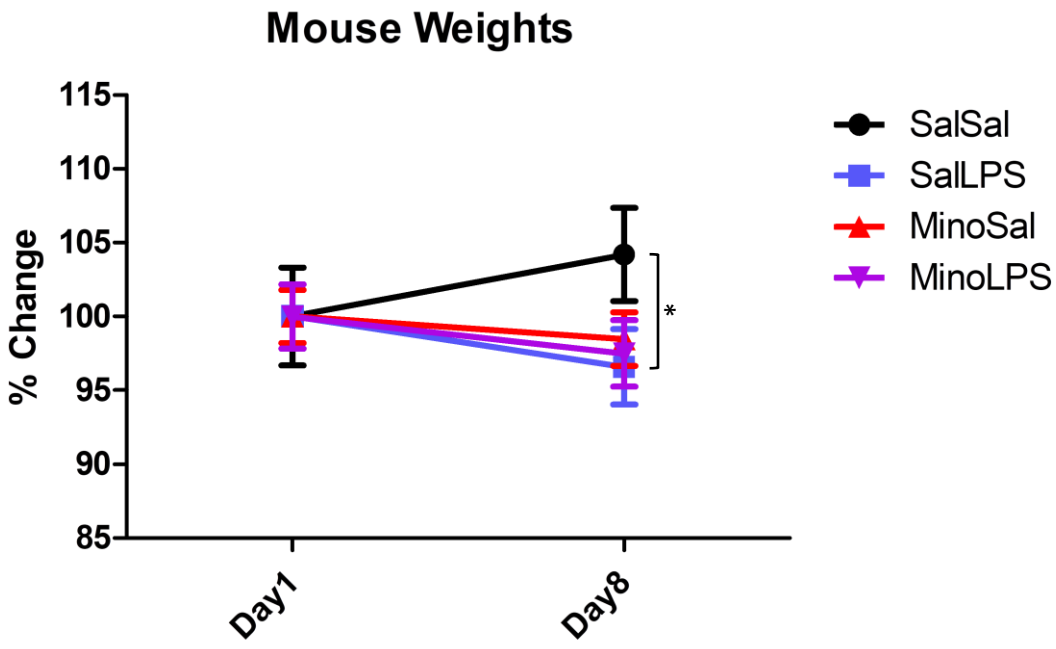


Fig 3.3.4 Weight change of mice (%) before and after treatment displayed as mean per treatment group.

3.3.2 Acute Minocycline Dose Study

3.3.2.1 Plasma metabolites

Orthogonal partial least squares discriminant analysis (OPLS-DA) built an ensemble of significant discriminatory models between the plasma metabolites of minocycline-treated and saline-treated groups with clear clustering of the two groups (Fig 3.3.5). These models were significantly better than chance, and were significantly more accurate (+32%, $p < 0.001$) at predicting which group a sample in the independent test set belonged to compared to the randomly permuted model ensemble (Fig 3.3.6). The Q^2 (0.556 compared to -0.5333, $p < 0.001$), sensitivity (+25.7%, $p < 0.001$), and specificity (+28.6%, $p < 0.001$) of the models were also significantly better than that of the permutation test. This suggests that a two hour treatment of minocycline resulted in identifiable changes in plasma metabolites compared to control.

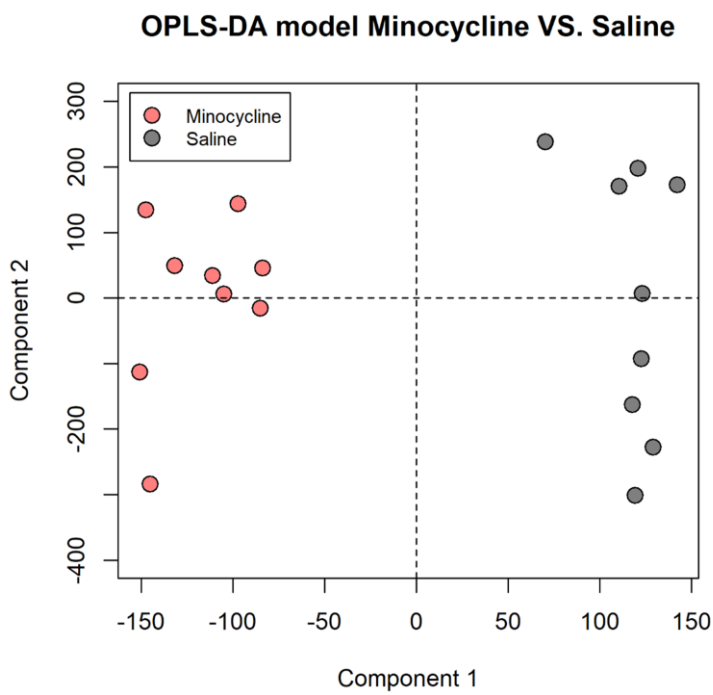


Fig 3.3.5 Plasma: Representative OPLS-DA model based on treatment (Minocycline/Saline). [Predictive component = 1; Orthogonal component = 5; $Q^2Y = 0.769$; $R^2X = 0.911$; $R^2Y = 0.965$]

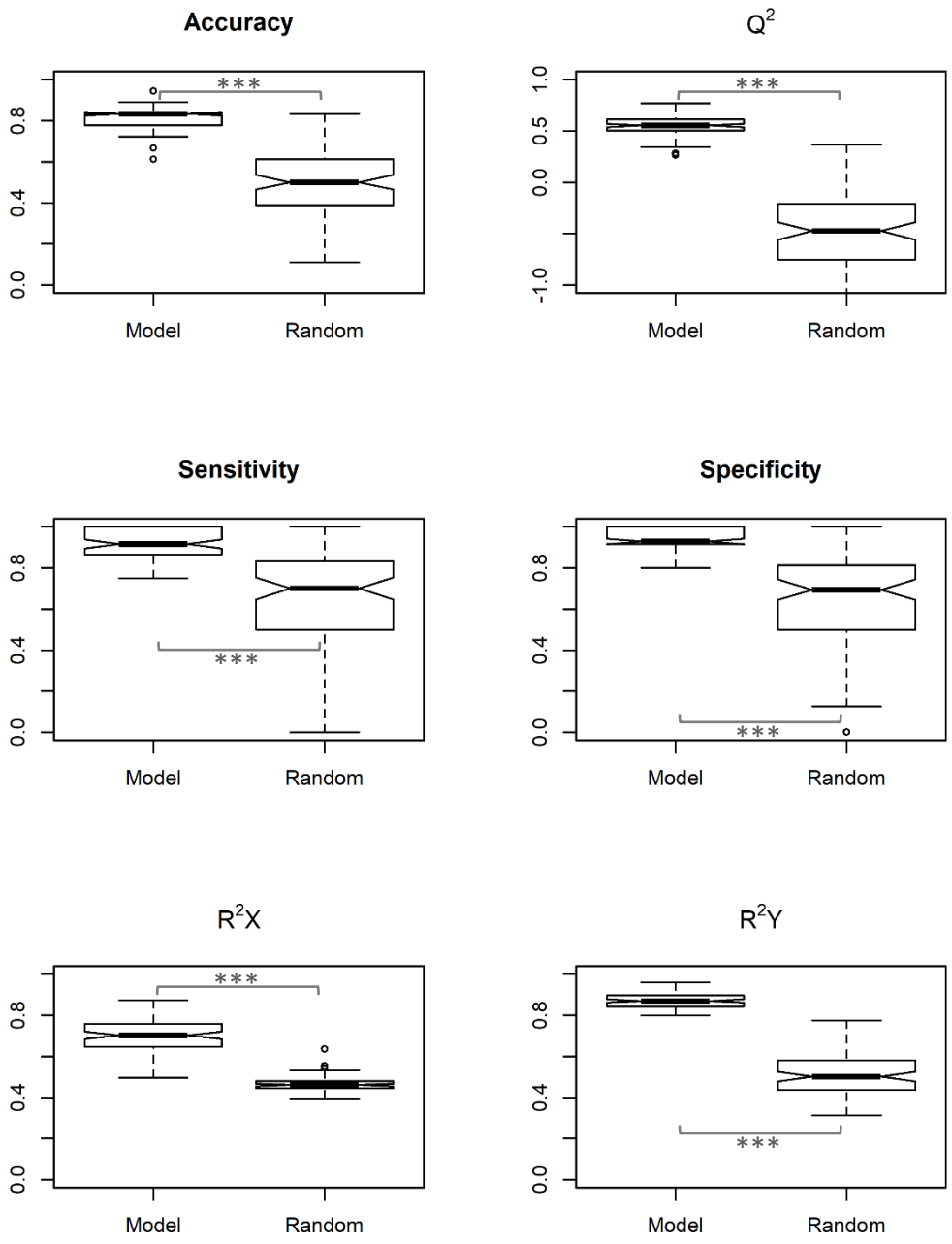


Fig 3.3.6 Comparisons between the Minocycline/Saline models (Model) and randomly permutated models (Random). On all counts (Accuracy, Q^2, Sensitivity, Specificity, R^2X, and R^2Y), the Minocycline/Saline models were significantly better (independent samples T-test). ***$p < 0.001$

Table 3.3.2 Plasma metabolites important for building the OPLS-DA models (VIP>2) and their direction of change

Metabolite	Minocycline vs Saline
$-CH_3$ lipoprotein	↓*
$(-CH_2-)_n$ lipoprotein	↓**
Creatine	↑**
Taurine	↑*
Glucose	↑*

Comparisons made based on mean bin integrals; *p < 0.05; **p < 0.01

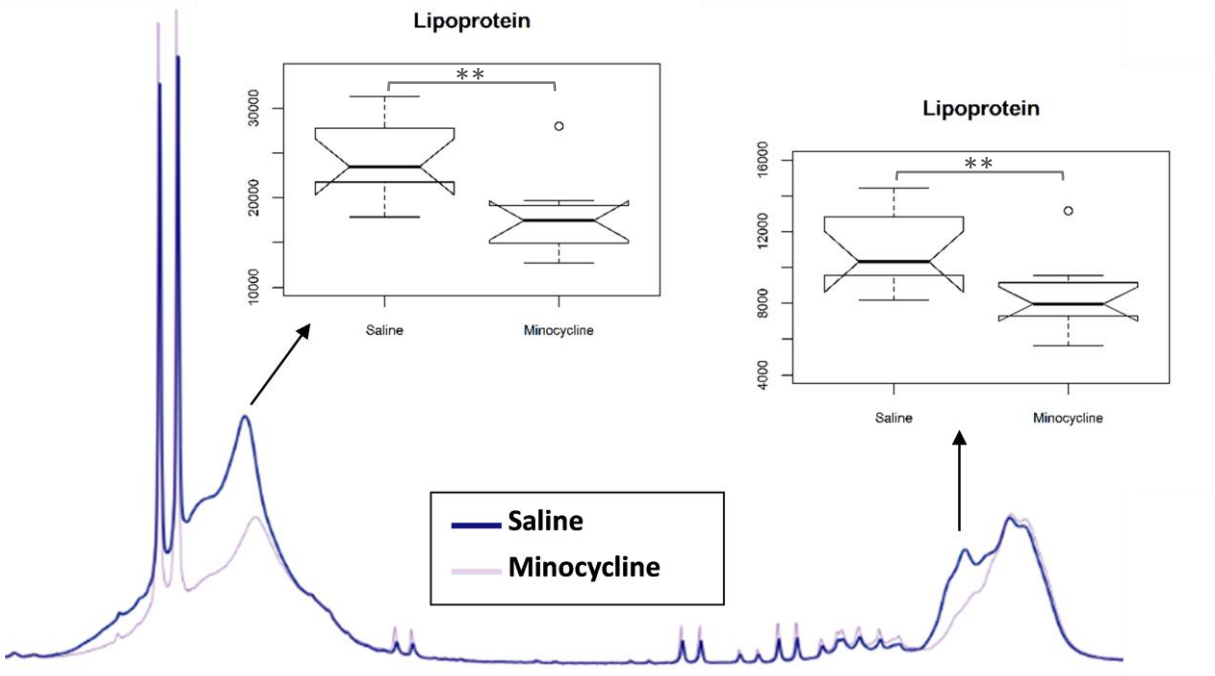


Fig 3.3.7 Representative NMR peaks showing treatment differences on plasma lipoproteins.

Boxplot values based on mean bin integrals; **p < 0.01

The key components contributing to the models were identified based on their VIP scores and summarized in Table 3.3.2. Minocycline treatment resulted in decreased levels of lipoproteins (Fig 3.3.7) and increased levels of creatine, taurine and glucose.

3.3.2.2 Aqueous brain metabolites

Orthogonal partial least squares discriminant analysis (OPLS-DA) analysis built models between the aqueous phase metabolites of minocycline-treated and saline-treated groups for both Hc tissue (Fig 3.3.8A) and FC tissue (Fig 3.3.8B). However, the FC models were not significantly more accurate than randomly permutated models, implying that a two hour treatment of minocycline did not result in identifiable changes in aqueous phase FC metabolites compared to control.

On the other hand, the Hc models were significantly better than chance, although predictive accuracy was low (+10.2%, $p < 0.001$). This indicates that changes in the Hc metabolic profile as a result of minocycline administration were quite subtle.

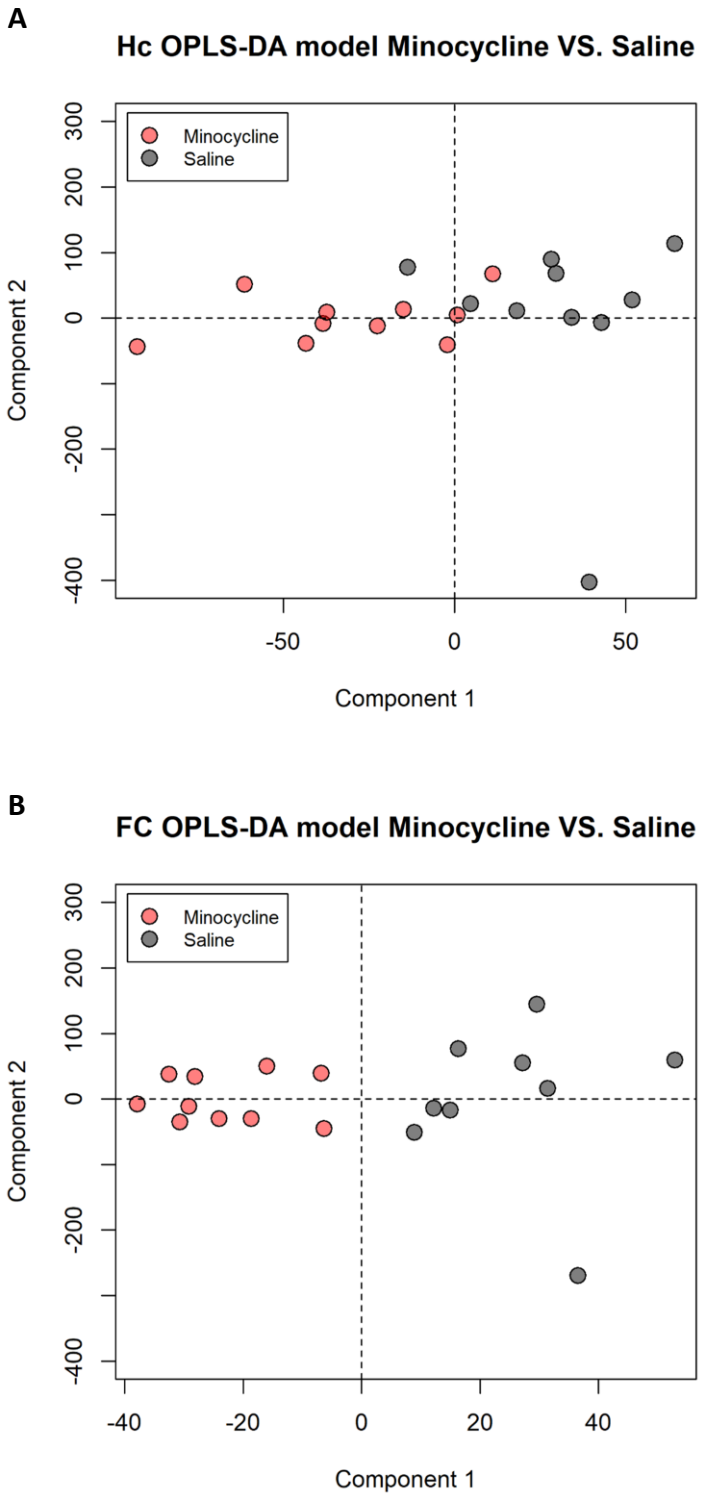


Fig 3.3.8 Representative OPLS-DA models based on treatment (Minocycline/Saline) for (A) Hippocampus (Hc) [Predictive component = 1; Orthogonal component = 1; Q^2Y = -0.271; R^2X = 0.439; R^2Y = 0.571], and (B) Frontal Cortex (FC) [Predictive component = 1; Orthogonal component = 1; Q^2Y = -0.27; R^2X = 0.302; R^2Y = 0.81]

3.3.3 Repeated Minocycline Dose Study

Given the 2x2 factorial study design, there are two fixed factors in this study, namely minocycline treatment (minocycline or saline) and LPS treatment (LPS or saline). This resulted in 4 treatment groups in this study – control animals treated with saline for both treatments (SalSal), animals treated with Saline then LPS (SalLPS), animals treated with minocycline without LPS (MinoSal) and animals treated with both minocycline and LPS (MinoLPS).

3.3.3.1 Plasma metabolites

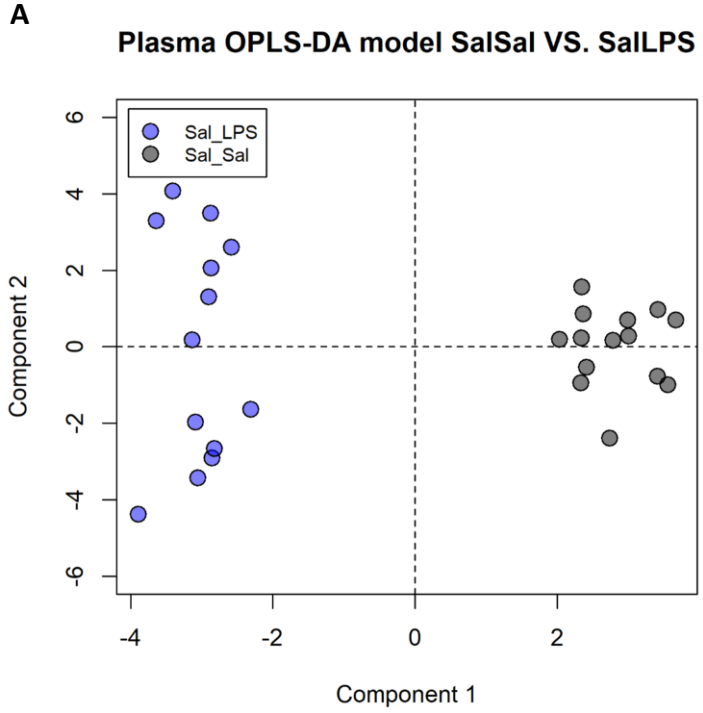
3.3.3.1.1 The effect of inflammation on plasma metabolites

To study the effect of inflammation on plasma metabolites, comparisons were made between animals treated with and without the inflammatory agent lipopolysaccharide (LPS). Thus, two OPLS-DA analyses were undertaken - metabolite changes between SalSal and SalLPS, and metabolite changes between MinoSal and MinoLPS.

In both comparisons, OPLS-DA analysis built models that were significantly better than chance, and visually there was clear clustering of the two treatment groups (Fig 3.3.9). When compared to randomly permutated models, the OPLS-DA models between SalSal and SalLPS (+49%, $p < 0.001$, Fig 3.3.10), and between MinoSal and MinoLPS (+34.9%, $p < 0.001$, Fig 3.3.11) were also significantly more accurate at predicting which group a sample belonged to. Likewise, in other measures such as the Q^2 (SalSal/SalLPS: 0.86 compared to -0.31, MinoSal/MinoLPS: 0.479 compared to -0.322, both $p < 0.001$), sensitivity (SalSal/SalLPS: +41%, MinoSal/MinoLPS: +32.6%, both $p < 0.001$), and specificity (SalSal/SalLPS: +37%, MinoSal/MinoLPS: +30.3%, both $p < 0.001$), the OPLS-DA models were significantly better than the randomly permutated models. This suggests that a single dose of LPS resulted in changes in plasma metabolites that were still identifiable after 24 hours.

The key components contributing to the models were identified based on their VIP scores and summarized in Table 3.3.3. LPS treatment resulted in decreased levels of lipoproteins (Fig 3.3.12) and glucose (Fig 3.3.13), and increased levels of creatine, and isoleucine (Fig 3.3.12).

A



B

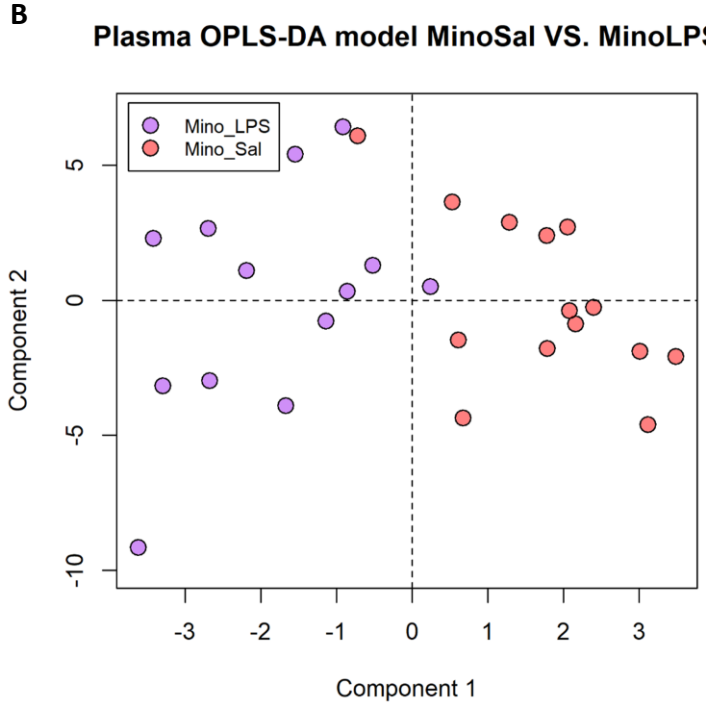


Fig 3.3.9 Plasma: Representative OPLS-DA models based on inflammation (LPS) for (A) control (SalSal) and LPS-treated animals (SalLPS) [Predictive component = 1; Orthogonal component = 4; Q^2Y = 0.905; R^2X = 0.891; R^2Y = 0.975], and (B) minocycline-treated (MinoSal) animals and animals treated with both LPS and minocycline (MinoLPS) [Predictive component = 1; Orthogonal component = 2; Q^2Y = 0.491; R^2X = 0.764; R^2Y = 0.712]

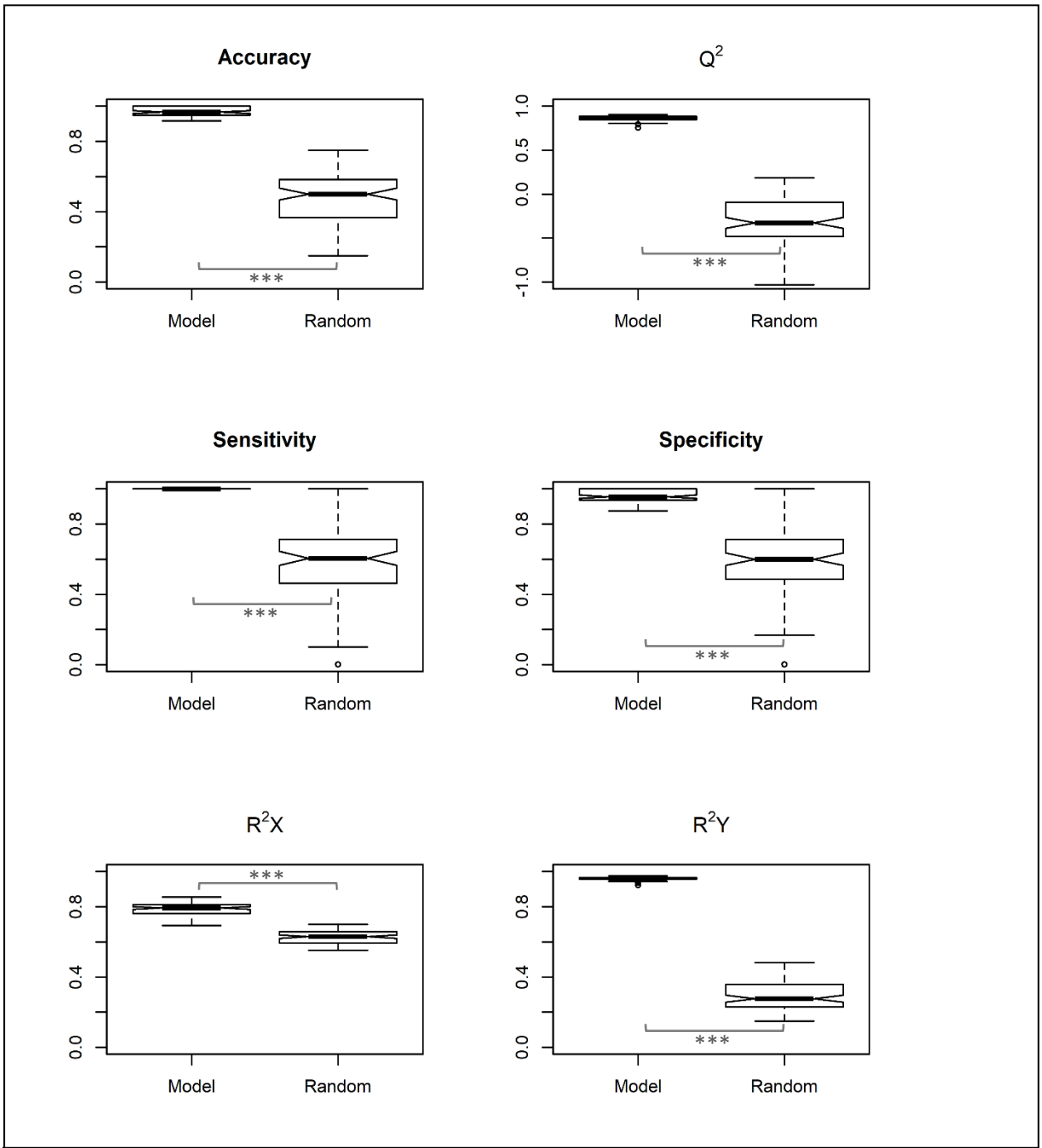


Fig 3.3.10 Plasma: Comparisons between the SalSal/SalLPS models (Model) and randomly permutated models (Random). On all counts (Accuracy, Q^2, Sensitivity, Specificity, R^2X, and R^2Y), the SalSal/SalLPS models were significantly better. ***$p < 0.001$

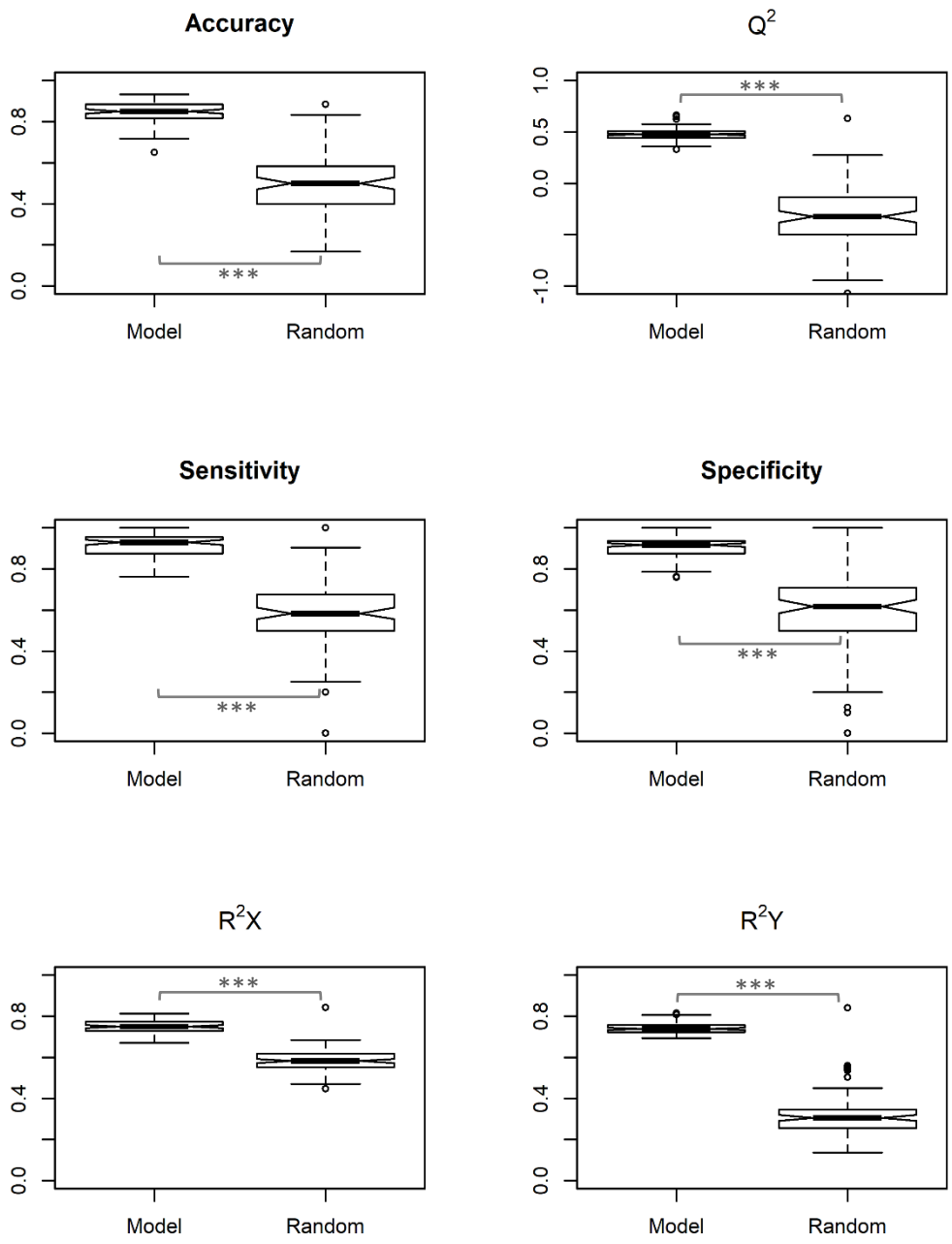


Fig 3.3.11 Plasma: Comparisons between the MinoSal/MinoLPS models (Model) and randomly permutated models (Random). On all counts (Accuracy, Q^2, Sensitivity, Specificity, R^2X, and R^2Y), the MinoSal/MinoLPS models were significantly better. ***$p < 0.001$

Table 3.3.3 Plasma metabolites important for building the OPLS-DA models (VIP>2) and their direction of change

Key plasma metabolites	Direction of change			
	SalLPS vs SalSal	MinoLPS vs MinoSal	MinoSal vs SalSal	MinoLPS vs SalLPS
-CH_3 lipoprotein HDL	↓**		↓*	
-CH_3 lipoprotein VLDL	↓*	↓(trend)		
Isoleucine	↑***	↑*		↓*
Isoleucine/Leucine	↑***	↑(trend)		↓(trend)
$(-CH_2-)_n$ lipoprotein	↓**	↓*		
α-glucose	↓***	↓**		
β-glucose	↓***	↓**		
Creatine	↑*	↑***		

Comparisons made based on mean bin integrals; *p < 0.05; **p < 0.01; ***p < 0.001

HDL: High density lipoprotein; VLDL: Very low density lipoprotein

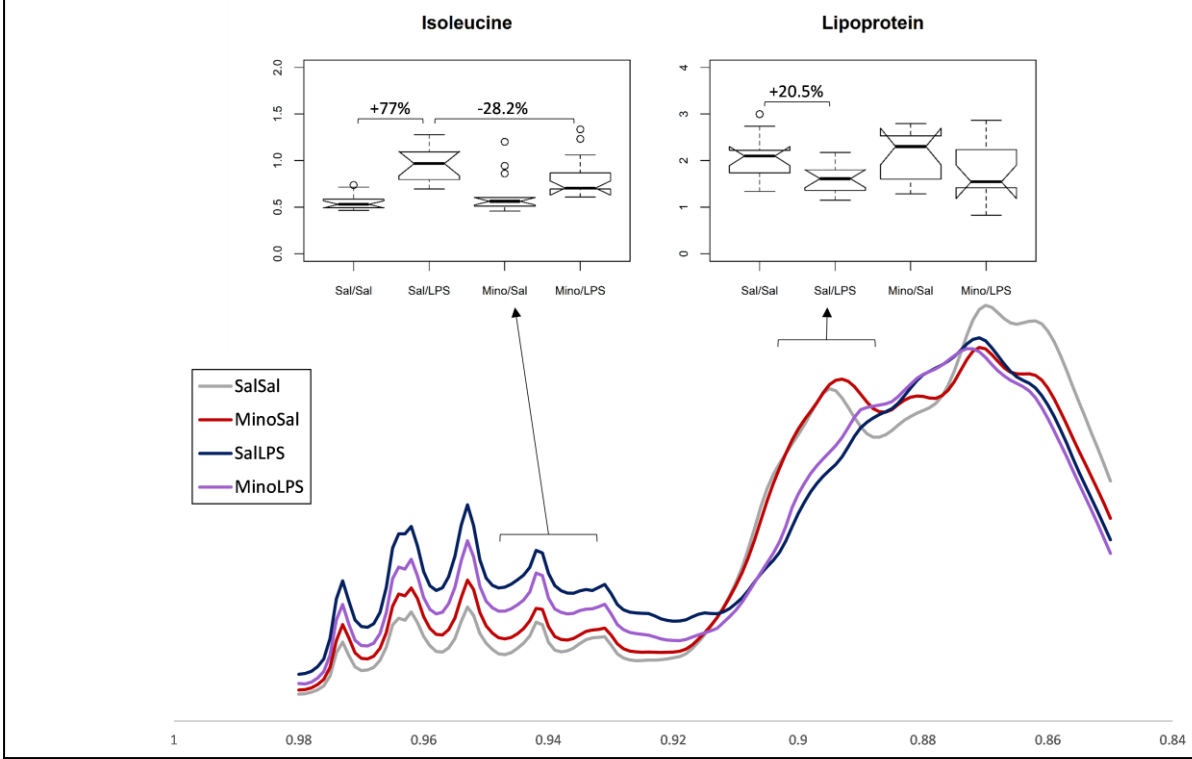


Fig 3.3.12 Representative NMR peaks showing treatment differences on plasma lipoproteins and isoleucine; Boxplot values based on mean bin integrals

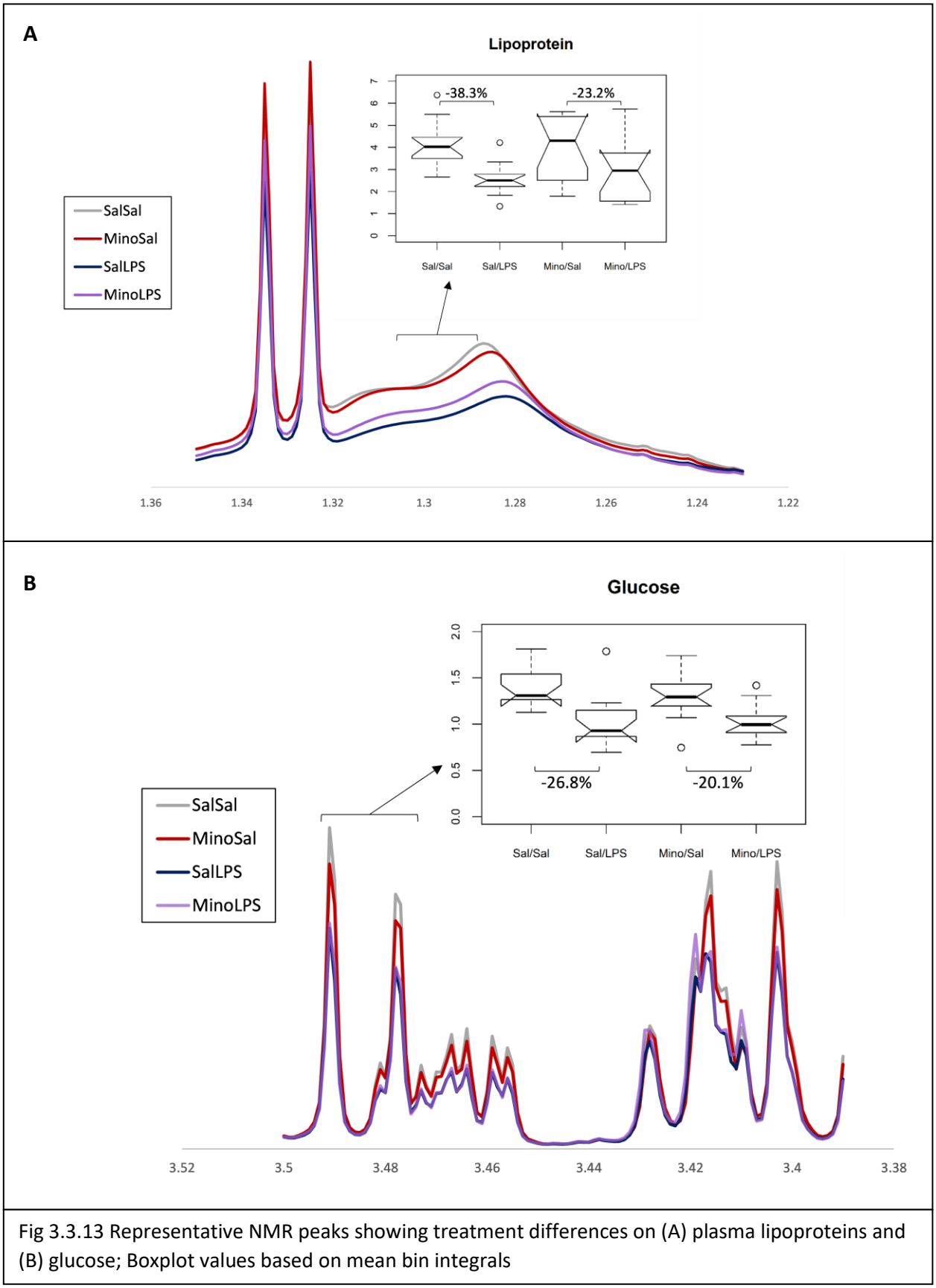


Fig 3.3.13 Representative NMR peaks showing treatment differences on (A) plasma lipoproteins and (B) glucose; Boxplot values based on mean bin integrals

3.3.3.1.2 The effect of minocycline on plasma metabolites

To study the effect of minocycline on plasma metabolites, comparisons were made between animals treated with and without minocycline. Thus, two OPLS-DA analyses were undertaken - metabolite changes between SalSal and MinoSal, and metabolite changes between SalLPS and MinoLPS.

In both comparisons, OPLS-DA analysis built models (Fig 3.3.14A and B) that were significantly better than chance, although predictive accuracy was low (SalSal with MinoSal – Model: 58.45%, Random: 49.25%; SalLPS with MinoLPS - Model: 60.12%, Random: 51.67%, $p < 0.001$). While the predictive accuracy of the models was lower than for LPS treatment, the fact that the models perform significantly better than random chance confirms that significant differences in metabolite concentrations are present, as a result of minocycline treatment, which the multivariate analysis is able to detect. This confirms that a seven day repeated dose treatment of minocycline resulted in subtle, but detectable, changes in plasma metabolites up to 24 hours following administration of the final dose of minocycline.

Furthermore, several significant changes in metabolites identified by the inflammation models were found following minocycline administration (Table 3.3.3). Minocycline significantly decreased plasma lipoproteins in the non-LPS treated animals (Fig 3.3.15) and significantly ameliorated the increase in plasma isoleucine levels brought about by LPS treatment (Fig 3.3.15).

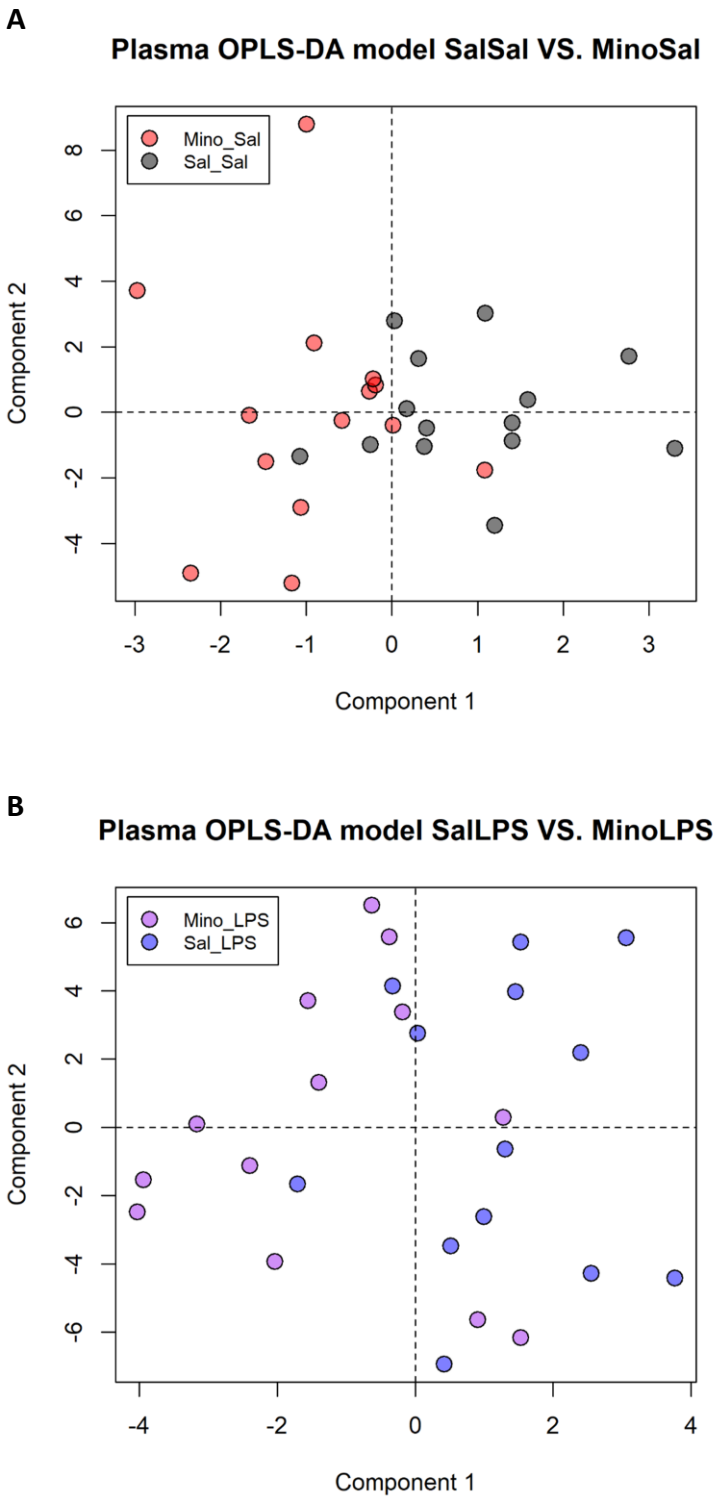


Fig 3.3.14 Plasma: Representative OPLS-DA model based on minocycline for (A) control (SalSal) and minocycline-treated animals (MinoSal) [Predictive component = 1; Orthogonal component = 1; Q^2Y = 0.152; R^2X = 0.397; R^2Y = 0.425], and (B) LPS-treated (SalLPS) animals and animals treated with both LPS and minocycline (MinoLPS) [Predictive component = 1; Orthogonal component = 1; Q^2Y = -0.122; R^2X = 0.551; R^2Y = 0.365]

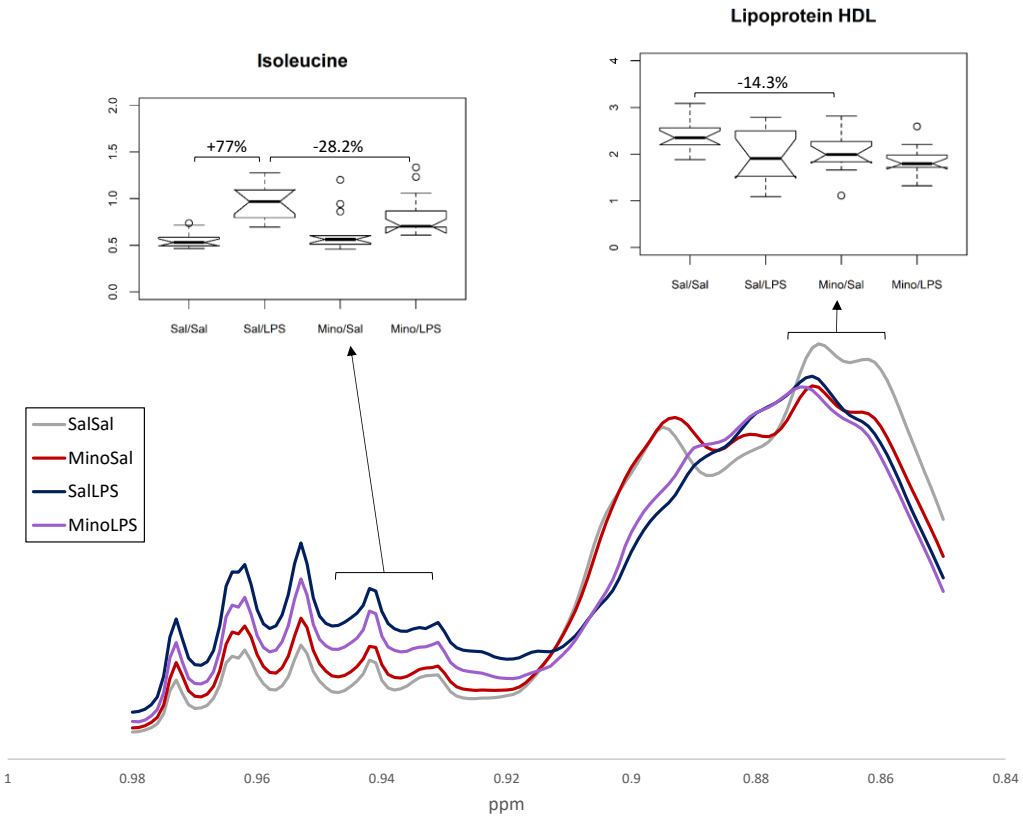


Fig 3.3.15 Representative NMR peaks showing treatment differences on plasma lipoproteins and isoleucine; Boxplot values based on mean bin integrals

3.3.3.2 Aqueous brain metabolites

3.3.3.2.1 The effect of inflammation (LPS) on aqueous brain metabolites

Similar to plasma metabolites, the same approach was used to study the effect of inflammation on aqueous brain metabolites; two OPLS-DA analyses were conducted between animals treated with and without the inflammatory agent lipopolysaccharide (LPS) – SalSal compared with SalLPS, and MinoSal compared with MinoLPS.

SalSal and SalLPS Models

In both Hc and FC tissue, OPLS-DA analysis built models that were significantly better than chance, and visually there was clear clustering of the two treatment groups (Fig 3.3.16). When compared to randomly permutated models (Fig 3.3.17 and Fig 3.3.18), the ensemble of OPLS-DA models between SalSal and SalLPS were also significantly more accurate (Hc: +16.4%, FC: +34.2%, both $p < 0.001$) at predicting which group a sample belonged to. Likewise, in other measures such as the Q^2 (Hc: 0.225 compared to -0.343, FC: 0.537 compared to -0.315, both $p < 0.001$), sensitivity (Hc: +18.7%, FC: +33.0%, both $p < 0.001$), and specificity (Hc: +16.9%, FC: +29.8%, both $p < 0.001$), the OPLS-DA models were significantly better than the randomly permutated models.

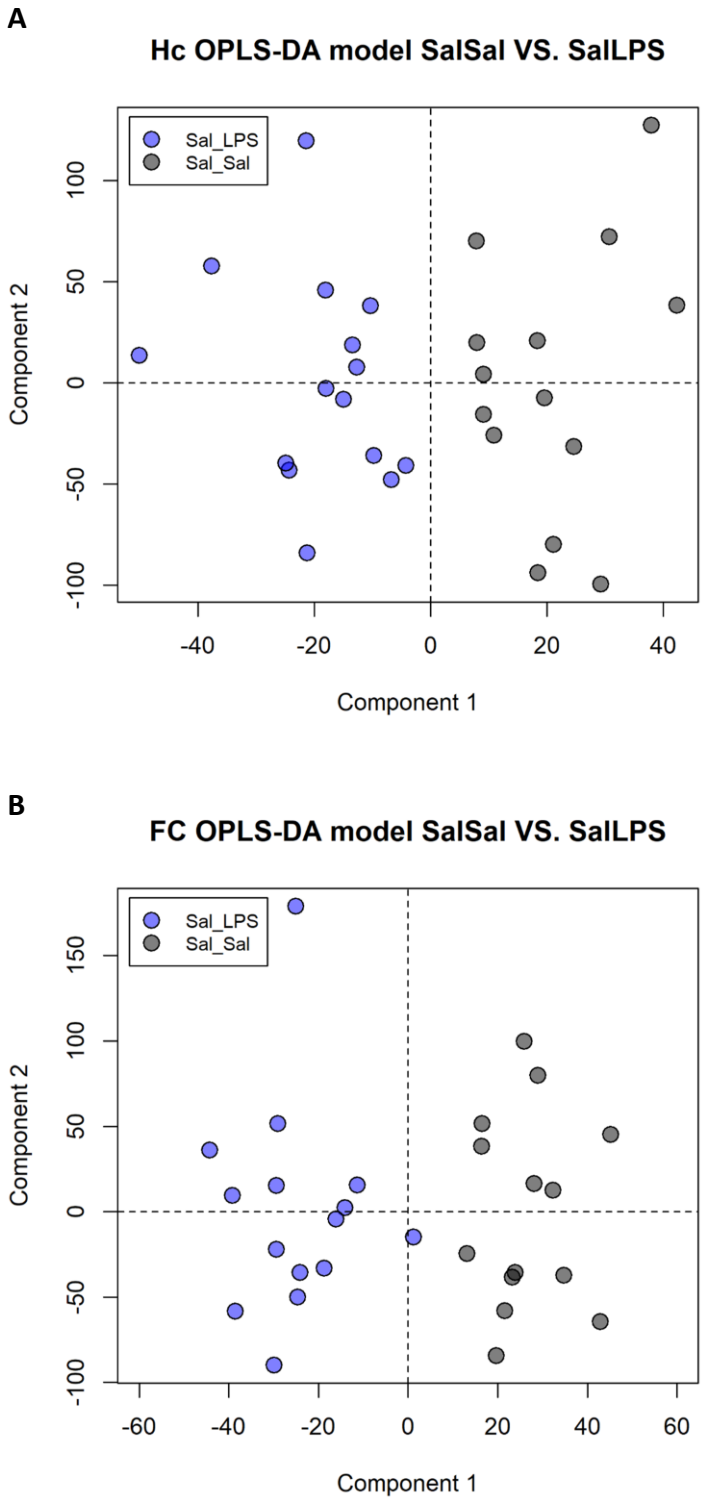


Fig 3.3.16 Representative OPLS-DA models between the SalSal and SalLPS groups for (A) aqueous phase hippocampus (Hc) metabolites [Predictive component = 1; Orthogonal component = 1; Q^2Y = 0.079; R^2X = 0.241; R^2Y = 0.757], and (B) aqueous phase frontal cortex (FC) metabolites [Predictive component = 1; Orthogonal component = 2; Q^2Y = 0.492; R^2X = 0.458; R^2Y = 0.859]

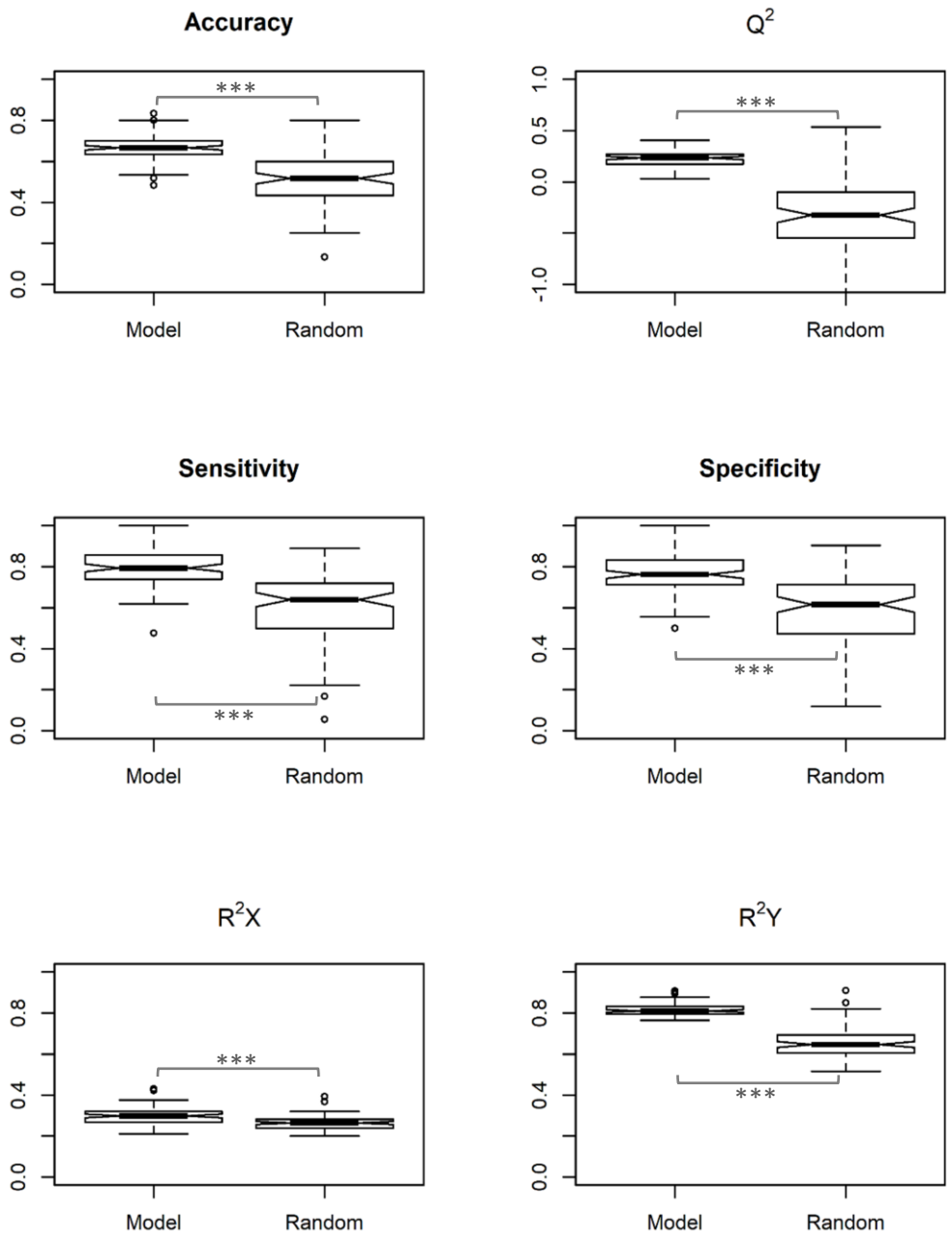


Fig 3.3.17 Hippocampus (Hc): Comparisons between the SalSal/SalLPS models (Model) and randomly permutated models (Random). On all counts (Accuracy, Q^2, Sensitivity, Specificity, R^2X, and R^2Y), the SalSal/SalLPS models were significantly better. ***$p < 0.001$

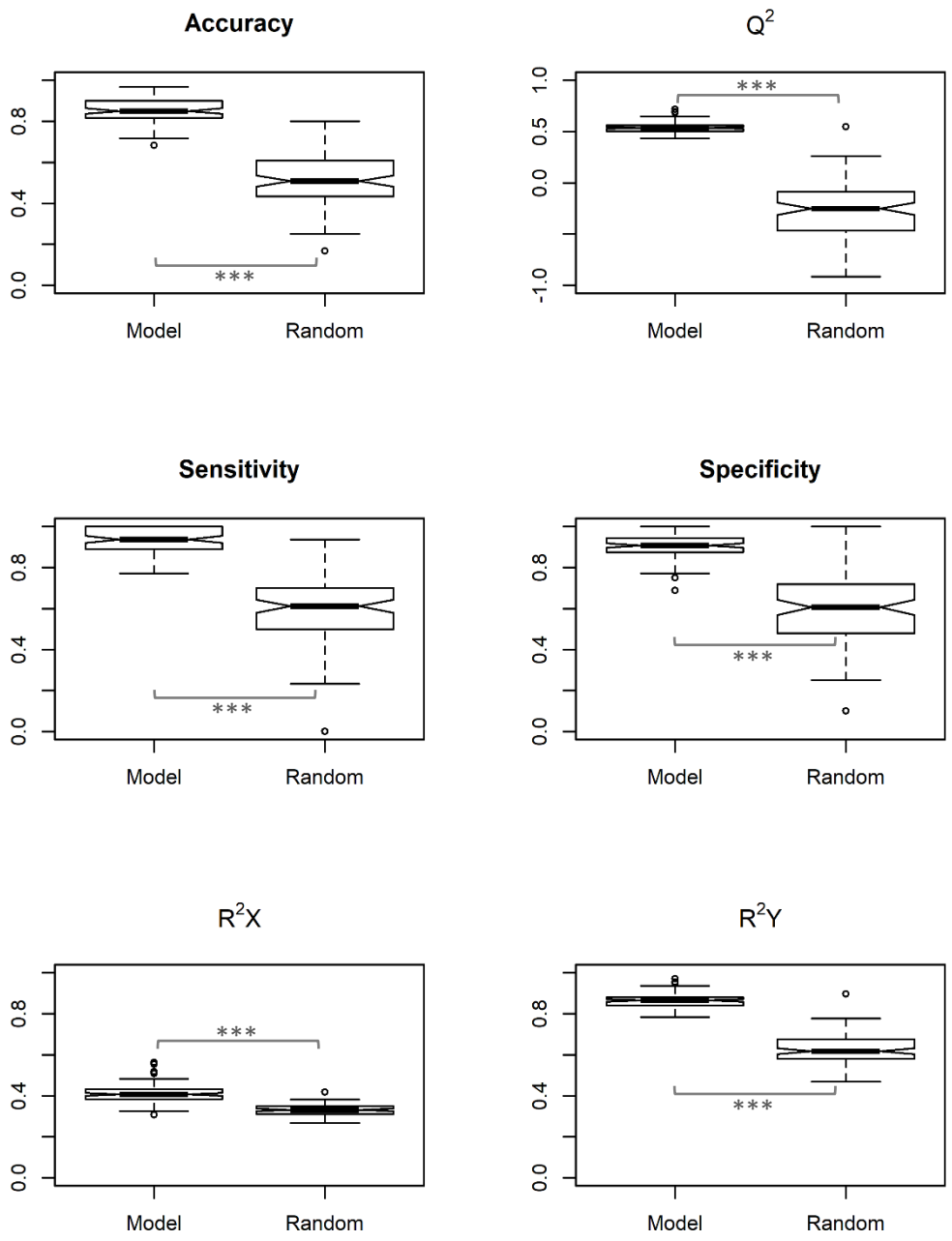


Fig 3.3.18 Frontal Cortex (FC): Comparisons between the SalSal/SalLPS models (Model) and randomly permutated models (Random). On all counts (Accuracy, Q^2, Sensitivity, Specificity, R^2X, and R^2Y), the SalSal/SalLPS models were significantly better. ***$p < 0.001$

MinoSal and MinoLPS Models

In Hc tissue, OPLS-DA analysis built models that were not significantly better than chance (Fig 3.3.19A). This implies that in aqueous phase hippocampal metabolites, a single dose of LPS did not result in sufficient identifiable changes after 24 hours when animals were also treated with minocycline. Thus, this suggests that minocycline is ameliorating the effects of LPS in the Hc.

In FC tissue, OPLS-DA analysis built models that were significantly better than chance (Fig 3.3.19B). When compared to randomly permutated models (Fig 3.3.20), the OPLS-DA models between MinoSal and MinoLPS were also significantly more accurate (+13.2%, $p<0.001$) at predicting which group a sample belonged to. Likewise, in other measures such as the Q^2 (0.0304 compared to -0.355, $p<0.001$), sensitivity (+17.8%, $p<0.001$), and specificity (+12.4%, $p<0.001$), the OPLS-DA models were significantly better than the randomly permutated models. However, the mean improvements from the ensemble of MinoSal/MinoLPS models were not of as large a magnitude as that of the SalSal/SalLPS models.

Key metabolites identified

Nevertheless, significant changes were still observed between the SalSal and SalLPS groups and the MinoSal and MinoLPS groups in both brain regions. The key components contributing to the models were identified based on their VIP scores and summarized in Table 3.3.4. In both brain regions, LPS treatment resulted in a significant increase in glutamine levels in both control (SalSal vs. SalLPS), and minocycline treated animals (MinoSal vs. MinoLPS) (Fig 3.3.21 and Fig 3.3.22). This was surprising for the MinoSal/MinoLPS analysis in the Hc, as the models built were not significantly better than the permutation test. This suggests that the change in glutamine levels was subtle and not sufficient to build models that were able to accurately distinguish between treatment groups.

For the SalSal/SalLPS analysis, a significant increase in N-acetyl aspartate (NAA) was also observed in both brain regions. However, more changes were observed in the FC alone. In the FC, LPS treatment also resulted in a significant decrease in alanine, glutamate, serine and histidine/phenylalanine, with a significant increase in glycerol levels.

Likewise, for the MinoSal/MinoLPS analysis, more changes were observed in the FC than the Hc. In the FC, LPS treatment also significantly increased NAA levels and significantly decreased serine levels.

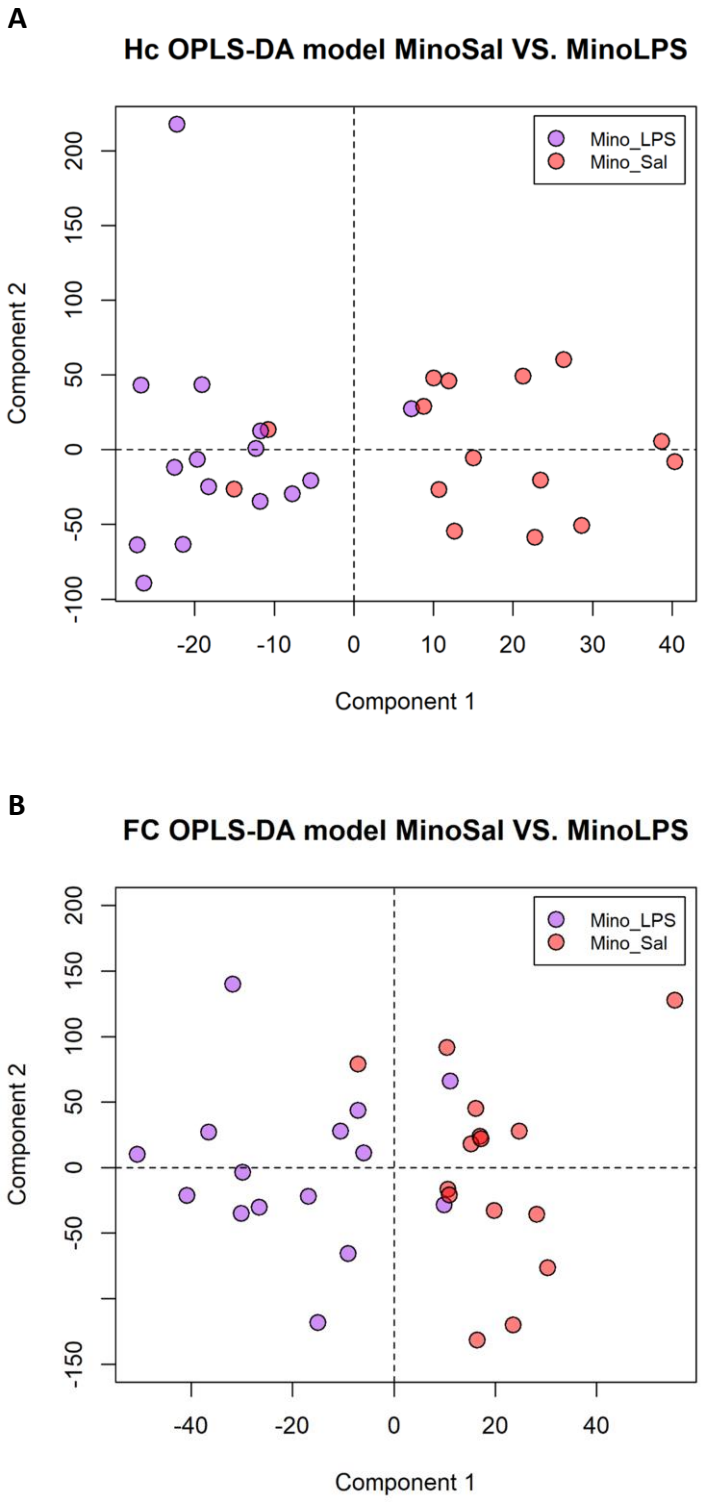


Fig 3.3.19 Representative OPLS-DA models between the MinoSal and MinoLPS groups for (A) aqueous phase hippocampus (Hc) metabolites [Predictive component = 1; Orthogonal component = 1; Q^2Y = -0.158; R^2X = 0.242; R^2Y = 0.636], and (B) aqueous phase frontal cortex (FC) metabolites [Predictive component = 1; Orthogonal component = 1; Q^2Y = 0.082; R^2X = 0.309; R^2Y = 0.615]

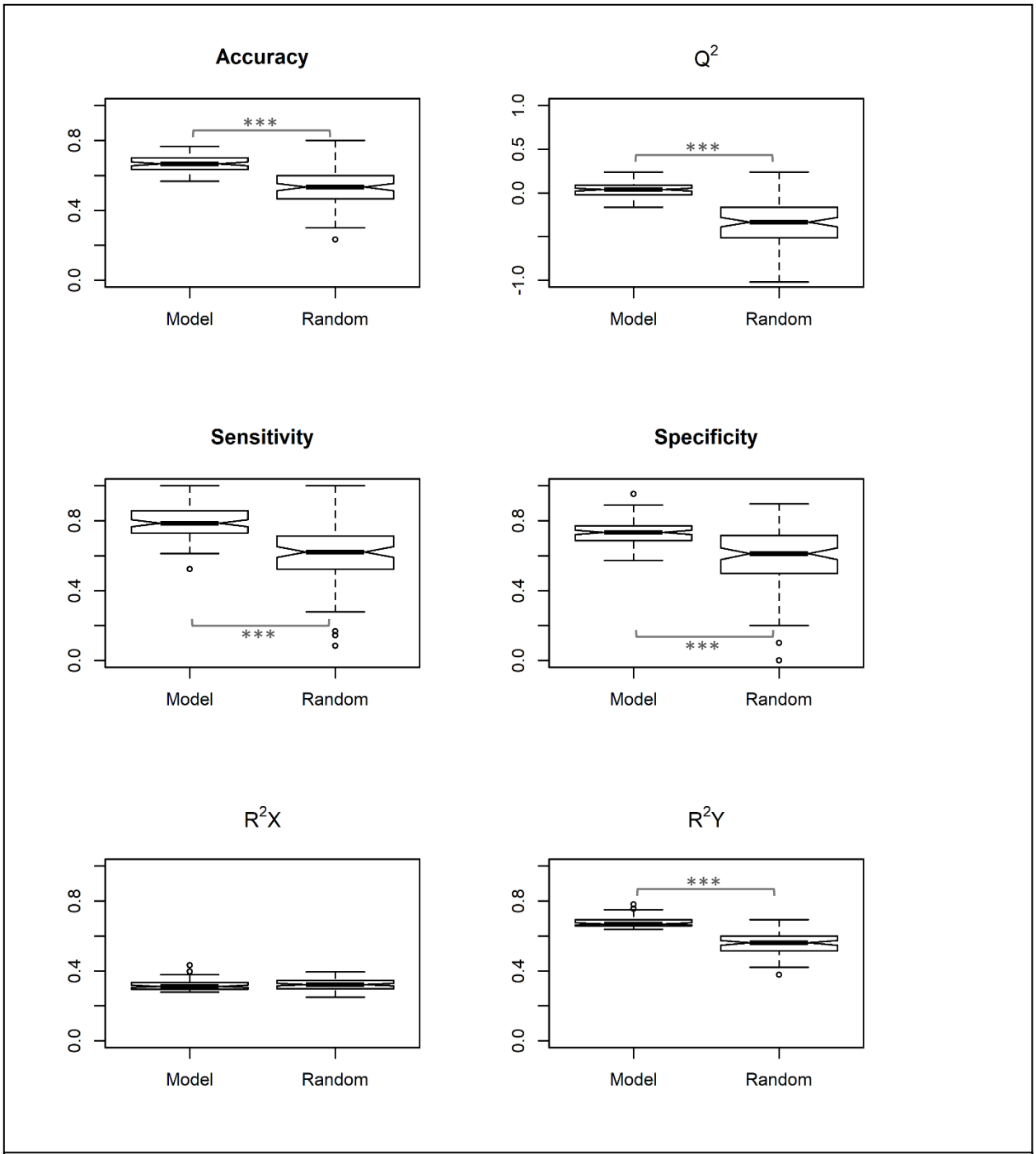


Fig 3.3.20 Frontal Cortex (FC): Comparisons between the MinoSal/MinoLPS models (Model) and randomly permutated models (Random). On most counts (Accuracy, Q^2, Sensitivity, Specificity, R^2X, and R^2Y), the MinoSal/MinoLPS models were significantly better. ***$p < 0.001$

Table 3.3.4 Aqueous brain metabolites important for building the OPLS-DA models (VIP>2) and their direction of change

Metabolites	Hippocampus		Frontal Cortex	
	SalLPS vs SalSal	MinoLPS vs MinoSal	SalLPS vs SalSal	MinoLPS vs MinoSal
Alanine			↓**	
Glutamate			↓*	
Glutamine	↑**	↑*	↑***	↑**
N-acetylaspartate	↑**		↑***	↑**
Glycerol			↑**	
Serine			↓***	↓***
Histidine/Phenylalanine			↓**	

Comparisons made based on mean bin integrals; *p < 0.05; **p < 0.01; ***p < 0.001

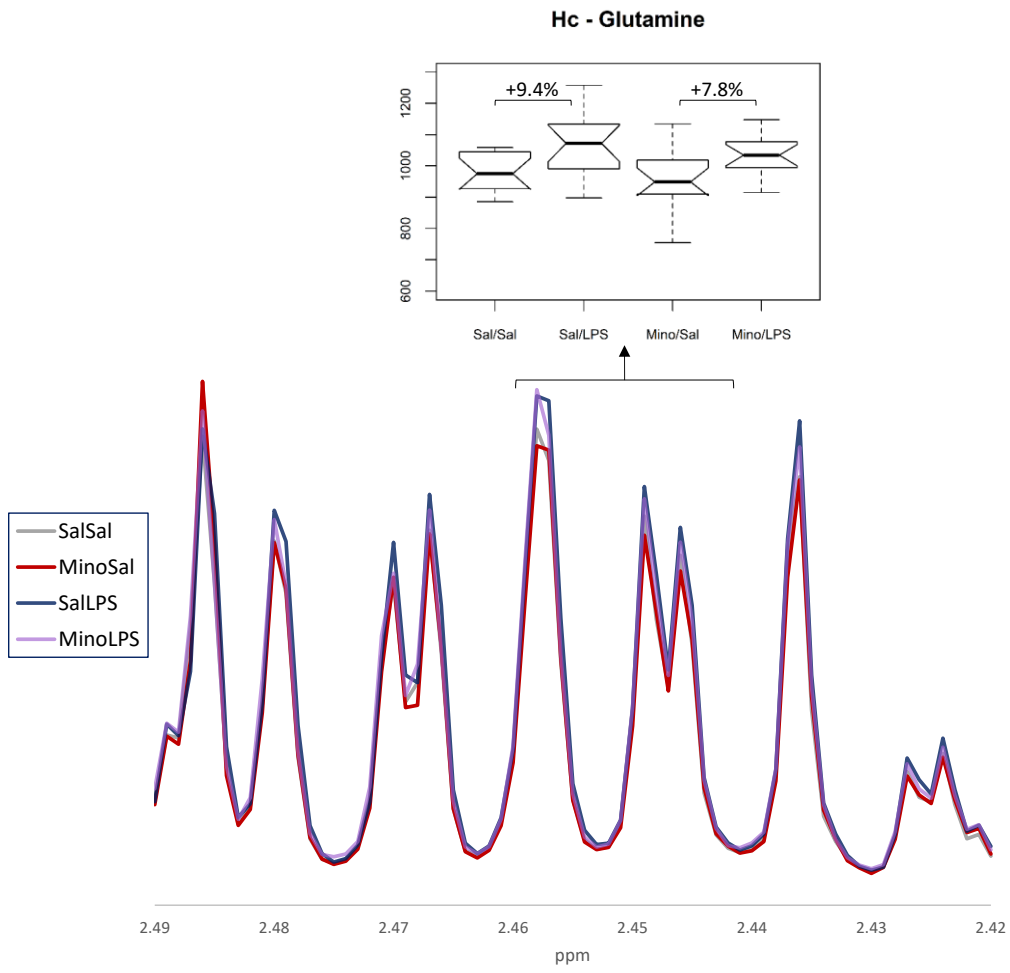


Fig 3.3.21 Representative NMR peaks showing treatment differences on brain glutamine levels in the Hippocampus (Hc); Boxplot values based on mean bin integrals

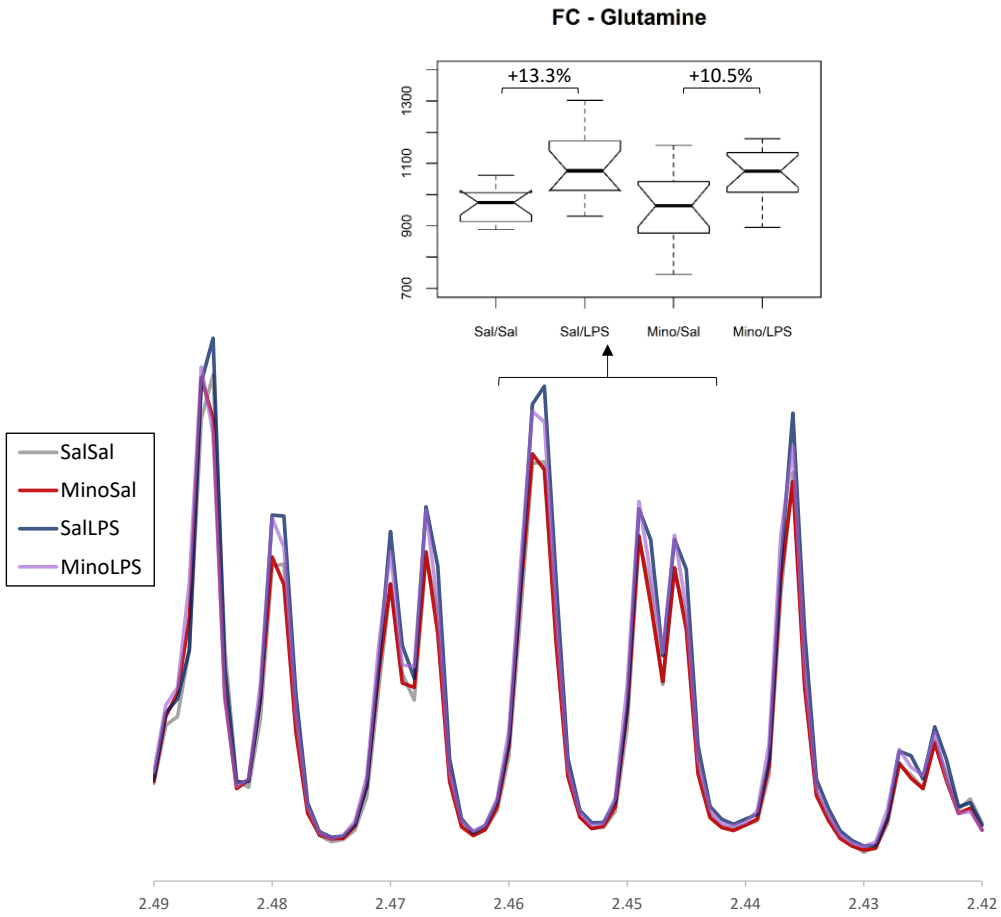
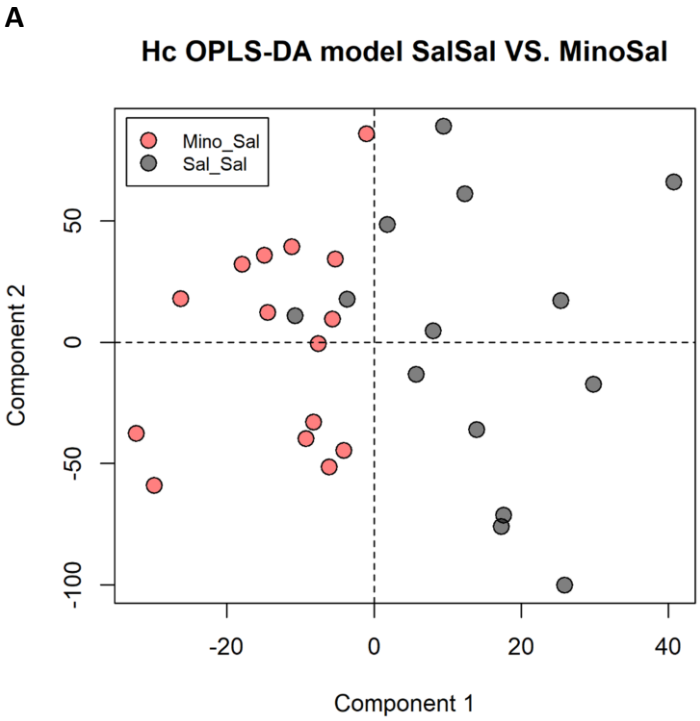


Fig 3.3.22 Representative NMR peaks showing treatment differences on brain glutamine levels in the Frontal Cortex (FC); Boxplot values based on mean bin integrals

3.3.3.2.2 The effect of minocycline on aqueous brain metabolites

To study the effect of minocycline on aqueous-phase brain metabolites, comparisons were made between animals treated with and without minocycline in the Hc and FC. Two OPLS-DA analyses were conducted between animals treated with and without minocycline – SalSal compared with MinoSal, and SalLPS compared with MinoLPS.

For both brain regions and in both comparisons, OPLS-DA analysis built models that were not significantly better than chance (Fig 3.3.23 and Fig 3.3.24), implying that a seven day repeated dose treatment of minocycline did not result in sufficient identifiable changes in aqueous phase brain metabolites after 24 hours.



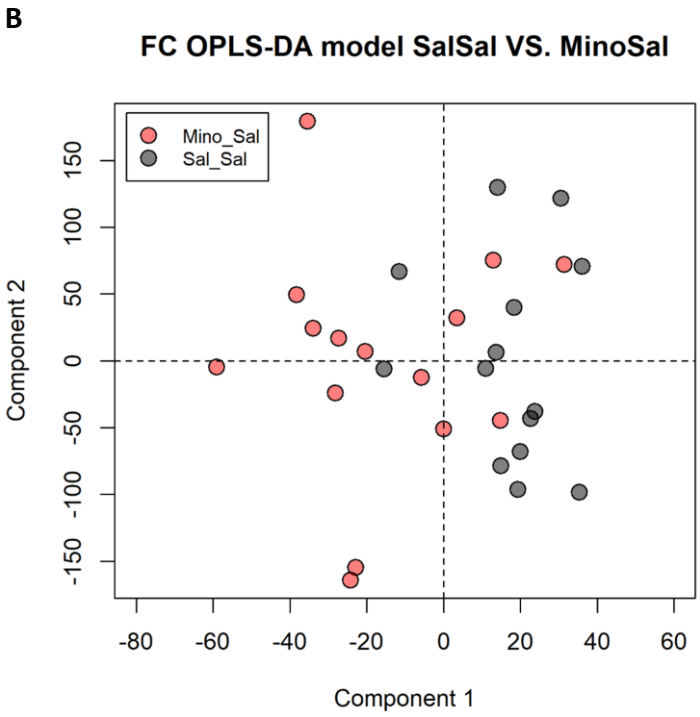


Fig 3.3.23 Representative OPLS-DA models between the SalSal and MinoSal groups for (A) aqueous phase hippocampus (Hc) metabolites [Predictive component = 1; Orthogonal component = 1; Q^2Y = -0.572; R^2X = 0.211; R^2Y = 0.579], and (B) aqueous phase frontal cortex (FC) metabolites [Predictive component = 1; Orthogonal component = 1; Q^2Y = -0.351; R^2X = 0.392; R^2Y = 0.402]

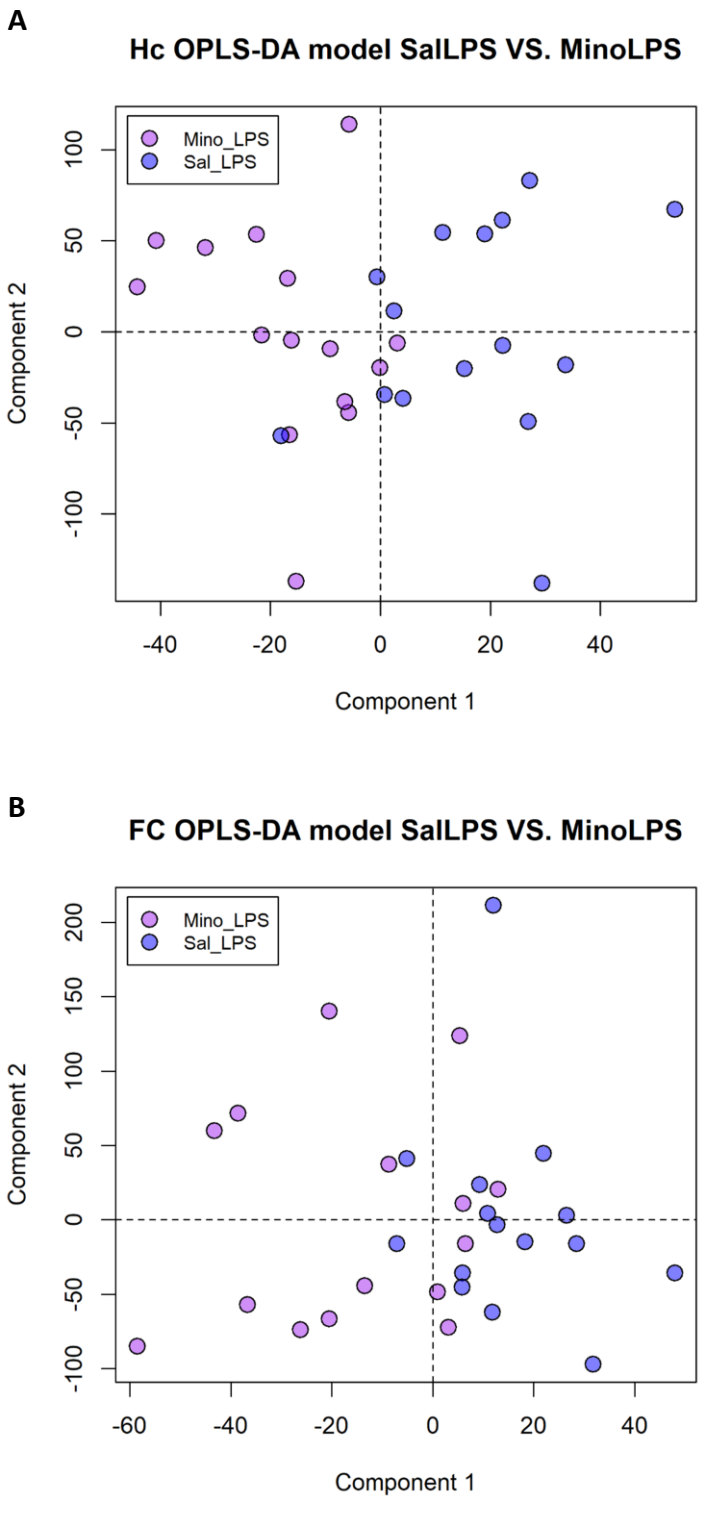


Fig 3.3.24 Representative OPLS-DA models between the SalLPS and MinoLPS groups for (A) aqueous phase hippocampus (Hc) metabolites [Predictive component = 1; Orthogonal component = 1; Q^2Y = -0.299; R^2X = 0.265; R^2Y = 0.545], and (B) aqueous phase frontal cortex (FC) metabolites [Predictive component = 1; Orthogonal component = 1; Q^2Y = -0.299; R^2X = 0.324; R^2Y = 0.429]

3.3.3.3 Pathway analysis

Pathway analysis was conducted for metabolites that changed as a result of inflammation (Table 3.3.5). This revealed that LPS administration, in particular, affected the alanine, aspartate and glutamine metabolism pathway, as well as the D-glutamine and D-glutamate metabolism pathway (Fig 3.3.25). Pathway analysis was not conducted for minocycline-induced metabolite changes, as there were too few metabolite changes for a meaningful analysis.

Table 3.3.5 Pathway analysis of metabolite changes resulting from LPS administration

	Total	Expected	Hits	Raw p	-LOG(p)	Holm adjust	FDR	Impact
Alanine, aspartate and glutamate metabolism	24	0.15243	4	7.56E-06	11.793	0.00062	0.00062	0.40928
Aminoacyl-tRNA biosynthesis	69	0.43825	5	2.57E-05	10.57	0.0020803	0.001053	0.12903
D-Glutamine and D-glutamate metabolism	5	0.031757	2	0.0003553	7.9425	0.028424	0.0097115	1
Nitrogen metabolism	9	0.057163	2	0.0012623	6.6748	0.09972	0.025877	0
Arginine and proline metabolism	44	0.27946	3	0.0020611	6.1845	0.16077	0.033802	0.10545
Glycine, serine and threonine metabolism	31	0.19689	2	0.015159	4.1891	1	0.20718	0.23848
Cyanoamino acid metabolism	6	0.038109	1	0.037574	3.2814	1	0.44015	0
Methane metabolism	9	0.057163	1	0.055886	2.8844	1	0.57283	0.4
Valine, leucine and isoleucine biosynthesis	11	0.069866	1	0.067921	2.6894	1	0.61884	0.33333
Histidine metabolism	15	0.095272	1	0.091584	2.3905	1	0.68271	0
Selenoamino acid metabolism	15	0.095272	1	0.091584	2.3905	1	0.68271	0
Glycerolipid metabolism	18	0.11433	1	0.10898	2.2166	1	0.68794	0
Starch and sucrose metabolism	19	0.12068	1	0.11471	2.1653	1	0.68794	0.03958
Sphingolipid metabolism	21	0.13338	1	0.12608	2.0709	1	0.68794	0
Butanoate metabolism	22	0.13973	1	0.13171	2.0271	1	0.68794	0
Galactose metabolism	26	0.16514	1	0.15393	1.8713	1	0.68794	0.03644
Glutathione metabolism	26	0.16514	1	0.15393	1.8713	1	0.68794	0.05534
Porphyrin and chlorophyll metabolism	27	0.17149	1	0.1594	1.8363	1	0.68794	0
Cysteine and methionine metabolism	27	0.17149	1	0.1594	1.8363	1	0.68794	0.02533
Valine, leucine and isoleucine degradation	38	0.24135	1	0.21757	1.5252	1	0.86772	0
Fatty acid metabolism	39	0.24771	1	0.22268	1.502	1	0.86772	0.00134
Pyrimidine metabolism	41	0.26041	1	0.2328	1.4576	1	0.86772	0
Purine metabolism	68	0.4319	1	0.35846	1.0259	1	1	0

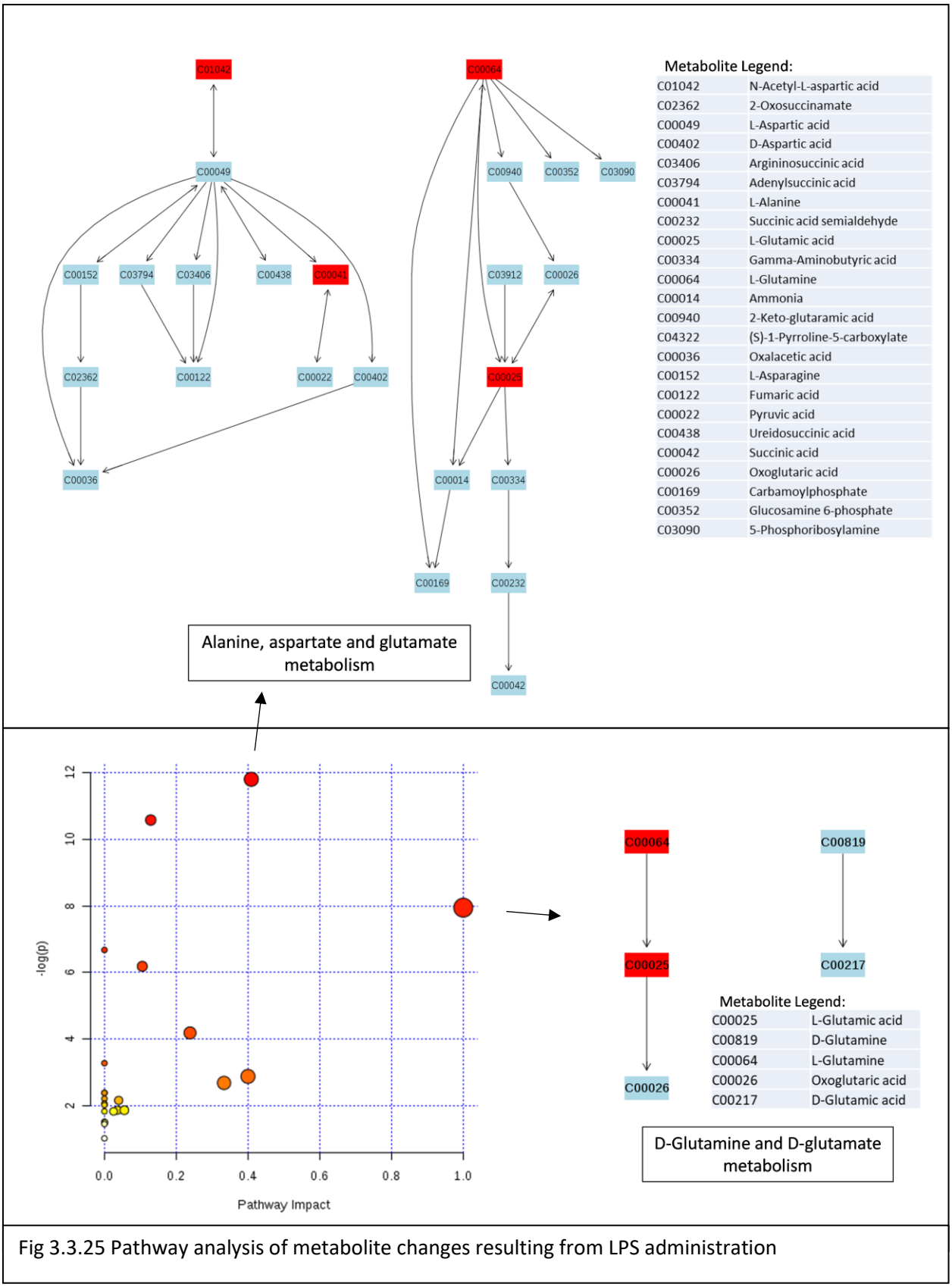


Fig 3.3.25 Pathway analysis of metabolite changes resulting from LPS administration

3.4 Discussion

Summary of results

A summary of the significant models built and key metabolite changes identified is shown in Table 3.4.1.

Acute study

A single dose of minocycline resulted in plasma metabolite changes after two hours including decreased levels of lipoproteins, and increased levels of creatine, glucose, and taurine compared to saline. An ensemble of models differentiating between the minocycline and saline treatment groups was also built for hippocampal metabolites, but predictive accuracy of the model was low, indicating metabolite changes following minocycline administration in the brain are subtle.

Repeated administration study

Changes in plasma and aqueous phase brain metabolites were prevalent 24 hours after a single LPS dose. Plasma metabolite changes mainly involved components of nutrient utilization (lipids and glucose metabolism), and mitochondrial energetics (creatine). Aqueous phase brain metabolite changes involved amino acids linked to neurotransmission.

While minimal effects were observed following minocycline treatment in the brain, repeated minocycline administration resulted in marked decreases in lipoprotein and isoleucine levels in plasma. Differences between the SalSal and SalLPS models and the MinoSal and MinoLPS models also suggest the subtle effects of minocycline on metabolites.

Table 3.4.1 Summary of results for both the acute dose and repeated administration studies.

	Treatment		Plasma	Hippocampus	Frontal Cortex
Acute Study	Minocycline vs Saline		✓	✓ (Low magnitude)	
	Metabolite changes:		↓ lipoproteins ↑ creatine ↑ taurine ↑ glucose	↓ lactate ↑ histidine/phenylalanine	
Repeated Dose Study	LPS	SalSal vs SalLPS	✓	✓	✓
		Metabolite changes	↓ lipoproteins ↓ glucose ↑ (iso)leucine ↑ creatine	↑ glutamine ↑ NAA	↑ glutamine ↑ NAA ↑ glycerol ↓ glutamate ↓ serine ↓ histidine/phenylalanine ↓ alanine
		MinoSal vs MinoLPS	✓		✓ (Low magnitude)
		Metabolite changes	↓ lipoproteins ↓ glucose ↑ (iso)leucine ↑ creatine	↑ glutamine	↑ glutamine ↑ NAA ↓ serine
	Minocycline	SalSal vs MinoSal	✓ (Low magnitude)		
		Metabolite changes	↓ lipoproteins		
		SalLPS vs MinoLPS	✓ (Low magnitude)		
		Metabolite changes	↓ (iso)leucine		

Ticks represent models that were significantly better than chance. Low magnitude refers to <10% increase in predictive accuracy between OPLS-DA models and random permutation models

NAA – N Acetyl Aspartate

Effects of inflammation (LPS) on metabolites: SalSal compared with SalLPS models

This study has revealed some insights on the effects of low-grade inflammation after twenty-four hours when the initial acute phase (innate) immune response was expected to have normalised. Thus, at 24 hours post-LPS injection, inflammatory cytokine levels have been shown to have returned to baseline in plasma, and behavioural changes associated with sickness behaviour, such as reduced locomotor activity, have also subsided (Custodio et al., 2013). However, elevated inflammatory cytokine levels and expression are still observed in the brain, along with neuroinflammatory mechanisms such as increased immune cell recruitment and activation (Cazareth, Guyon, Heurteaux, Chabry, & Petit-Paitel, 2014; Custodio et al., 2013).

In this context, this study has shown that the metabolite profile is significantly perturbed as a result of a peripherally-administered low dose of LPS in plasma, Hc, and Fc and these perturbations persist for up to 24 hours. This suggests that the plasma metabolite changes are downstream of altered plasma cytokines and persist when cytokine changes have normalized. This suggests a link to the acute phase response (APR), which can still be detected 24 hours post-LPS dose in the periphery (Gruys et al., 2005), while brain metabolite changes are linked to inflammatory cytokine levels.

It is also interesting to note that LPS administration appears to have different effects on the hippocampus and frontal cortex, suggesting that distinct brain regions have different sensitivities to inflammation. Results appear to suggest that the frontal cortex is more susceptible to LPS, with more metabolite changes detected in the FC models.

Significance of metabolite changes

Lipoproteins (Plasma):

There is a strong association between inflammation, the APR and lipoproteins. The APR is induced by pro-inflammatory cytokines (Gruys et al., 2005), and causes metabolic changes due to the breakdown of serum proteins and synthesis of acute phase proteins (APP). One of the major APPs synthesized is serum amyloid A (SAA), which is an apolipoprotein involved in cholesterol transport. SAA has been shown to increase dramatically after LPS administration (Chapter 2), and its functions are linked to reduction in high density lipoprotein (HDL) levels (Jahangiri, 2010) which is consistent with the decrease in lipoprotein resonances observed in this study. Another APP upregulated by inflammation is endothelial lipase that facilitates phospholipid hydrolysis (Badellino, Wolfe, Reilly, & Rader, 2008). Therefore, the reduction in plasma lipoprotein levels observed in the present study is in line with current literature that links lipoproteins to inflammation through the APR. While SAA has been shown to be immunological active, such as by activating the inflammasome cascade, some studies have also suggested that

SAA has anti-inflammatory properties that inhibit inflammatory processes (Tilg, Dinarello, & Mier, 1997; Ye & Sun, 2015). Nevertheless, SAA and other APPs have immunomodulatory effects linked to their effects on lipid-based systems, such as cholesterol transport and phospholipid hydrolysis.

Energy metabolism (Plasma):

The other key plasma metabolites (glucose, isoleucine, creatine) altered by LPS administration are involved in nutrient utilization and mitochondrial energetics. Alterations in glucose metabolism and insulin resistance have been linked to LPS. Raetzsch et al. have shown that a single dose of LPS decreased glucose utilization and production (Raetzsch et al., 2009), keeping with the decreased glucose levels observed in the current study. However, in their study, the decrease in plasma glucose levels was normalized to control levels 24 hours post-dose, while the current study shows that levels are still significantly decreased. It is possible that this discrepancy is due to method differences, as Raetzsch et al. used a handheld glucose meter. This study suggests that the effects of LPS on glucose metabolism may last longer than originally presumed and that metabolomics analysis of plasma may provide a more sensitive measure of ongoing inflammation.

Both isoleucine and creatine levels were shown to be decreased 24 hours after LPS administration. Isoleucine is part of the branched chain amino acids (BCAA), that have been linked to many nutrient utilization functions, including enhancing lipolysis and glucose consumption (S. Zhang et al., 2017). Creatine, along with phosphocreatine, are essential for ATP regeneration, where phosphocreatine provides a phosphate to ADP to form ATP (Guimarães-Ferreira, 2014).

Glutamine and glutamate (Brain):

Glutamate is the main excitatory neurotransmitter in the brain, and as such, plays a key role in cell signalling. Its availability is tightly controlled by a glutamate-glutamine shuttle, where

glutamate is synthesized from glutamine by glutaminase, while glutamine synthetase metabolizes glutamate to glutamine in astrocytes (Hertz, 2013). Dysregulation in glutamatergic signalling has been strongly linked to psychiatric diseases. Given that inflammation results in changes in glutamatergic signalling, inflammation-mediated glutamatergic perturbations could underlie the pathophysiology of psychiatric diseases. Hashimoto et al. have shown that the ratio of glutamine over glutamate in CSF is increased in patients with depression, and correlated to severity of depression (Hashimoto et al., 2016b). Thus, this is consistent with the current study, where LPS administration resulted in increased levels of glutamine in both brain regions, along with reduced levels of glutamate in the FC.

Other amino acids linked to neurotransmitters (Brain):

For aqueous phase brain metabolites, LPS administration also altered levels of amino acids linked to neurotransmitters. Serine metabolism has been linked to lipids as it is an input for ceramide synthesis (Gao et al., 2018). Serine is also an essential co-agonist of NMDARs, providing another link between LPS-induced inflammation and glutamatergic signalling (Chapter 2) (Mothet et al., 2000). N-acetylaspartate (NAA) has been linked to myelin and oligodendrocytes, proposed to function as a reservoir for glutamate, and is also linked to glutamatergic signalling together with N-acetyl-aspartyl-glutamate (NAAG) (Chakraborty, Mekala, Yahya, Wu, & Ledeen, 2001; Clark et al., 2006; Kołodziejczyk, Hamilton, Wade, Káradóttir, & Attwell, 2009). Finally, phenylalanine is converted to tyrosine, which is a precursor for serotonin synthesis, a neurotransmitter implicated in many psychiatric diseases (Strasser, Sperner-Unterweger, Fuchs, & Gostner, 2017).

Effects of minocycline on metabolites

Single versus repeated dose minocycline study

A single dose of minocycline resulted in sufficient changes in plasma metabolites to build a significantly predictive model. However, repeated administration of minocycline (once a day for seven days) resulted in only a weakly predictive model, suggesting that, while significant differences in metabolite concentrations were identified in the MinoSal compared to the SalSal treatment groups, the magnitude of these changes 24 hours post administration is less than the acute dose (measured 2 hours after administration). This was a counter-intuitive result as greater changes were expected after the repeated administration of minocycline. However, it is possible that compensatory mechanisms could have developed to adapt to the initial changes brought about by minocycline. A second explanation is that samples were collected at different time-points post-treatment in both studies. Samples were collected two hours post-treatment in the acute dose study, while samples were collected 24 hours after the last dose of minocycline in the repeated dose study. Thus, given the transient nature of metabolite changes, the lack of a significantly predictive model does not necessarily imply no effects, but possibly that the observed alterations could have returned to baseline levels at the time-point we sampled. Finally, given that minocycline administration has been shown to change the gut microbiome (Wong et al., 2016), another possibility is that a single dose and repeated administrations of minocycline could have different effects on the microbiome, accounting for the different metabolic effects observed between the two studies.

Significance of metabolite changes

Lipoproteins, creatine and glucose levels are also changed after minocycline administration:

Plasma lipoprotein levels were decreased after both a single and repeated dose of minocycline. Interestingly, this effect is similar to what is seen after a single dose of LPS. This is also counter-intuitive to what was expected, as the original hypothesis was that minocycline,

given its anti-inflammatory effects, would ameliorate the effects of LPS. However, it is possible that 24 hours post-LPS administration, compensatory anti-inflammatory mechanisms may have been activated to bring changes back to baseline. This was shown by studies on the effects of LPS plasma inflammatory cytokines and glucose levels, where peak changes occur within eight hours post-LPS dose, and slowly return to baseline levels thereafter (Raetzsch et al., 2009). Therefore, it is possible that the anti-inflammatory effects of minocycline on plasma lipoproteins are similar to that induced by anti-inflammatory compensatory mechanisms activated in response to LPS administration. This is further supported by studies showing that the APR has immuno-suppressive effects and acts as a check to pro-inflammatory mechanisms stimulated by infection (N. Cheng, He, Tian, Ye, & Ye, 2008; Ye & Sun, 2015). This explanation could also apply to the increased plasma creatine levels observed after minocycline, which were similar to those seen 24 hours post-LPS administration.

However, what is interesting is that glucose levels are increased after minocycline administration, compared to the decrease seen after LPS administration. This suggests that the mechanisms that lead to glucose changes are separate from that of the post-inflammatory mechanisms linked to lipoprotein and creatine changes.

Taurine

Plasma taurine levels were increased two hours post-minocycline administration. Taurine has been shown to have neuroprotective effects, and can safeguard against glutamate-mediated excitotoxicity in the brain (Ripps & Shen, 2012). Taurine, along with creatine, glutamine and NAA, have also been implicated in increasing resilience in a chronic stress model where increased availability of energy-related metabolites are proposed to increase ability to cope with higher energy demands (Larrieu et al., 2017). This suggests a potential mechanism of minocycline separate from its anti-inflammatory effects.

Comparisons with the metabolomics profile of other antidepressants

The metabolite profile observed after a single dose of minocycline is in line with that of other antidepressants, where changes in lipoproteins and energy-related metabolites were also observed.

The role of energy metabolism in psychotropic therapeutics is demonstrated by the example of ketamine. A proteome and metabolome analysis of ketamine administration on mouse hippocampi revealed that ketamine administration reduced the adenosine triphosphate (ATP) over adenosine diphosphate (ADP) ratio. This ratio is correlated with depressive-like behaviour in mice, where a reduced ratio was positively correlated with decreased floating time in the forced swim test (Weckmann et al., 2017). Minocycline could have a similar mechanism, where altered levels of creatine, a source of high energy phosphate for the formation of ATP, could imply alterations in the ratio of ATP over ADP.

Furthermore, metabolic studies of chronic (four weeks) administration of antidepressants in mice showed lipidomic changes, in particular, that of decreased levels of phosphatidylcholine species, and increased levels of lysophospholipid species (Lee et al., 2009). It is noteworthy that greater differences in lipids were observed in the frontal cortex, compared to other brain regions like the hippocampus and striatum (Lee et al., 2009), similar to the results we observe for a single dose of LPS.

Minocycline as an anti-inflammatory guard against LPS

One of the main aims of this study is to determine if minocycline's anti-inflammatory properties underlie its psychotropic effects. To study that, a mouse inflammation model was used to test if prior repeated doses of minocycline ameliorated the effects of LPS. Given that the models built between the MinoLPS and SalLPS treatment groups were not significantly more accurate than that of chance, it was concluded that metabolite changes between the two groups were subtle. Nevertheless, out of all the metabolites identified to have been changed by LPS

administration, the increase in isoleucine levels was shown to be ameliorated by prior treatment with minocycline. As one of the BCAA, isoleucine has been identified to have immunomodulation functions. High levels of isoleucine have been linked to increased vulnerability to excitotoxicity, and alterations in microglial reactivity. De Simone et al. have shown that high levels of BCAA reduced expression of pro-inflammatory agents in microglia, but also increased levels of reactive oxygen species, and decreased levels of neuroprotective agents. Finally, high levels of BCAA were also shown to alter immunological responses after LPS administration (De Simone et al., 2013).

One possible explanation for the difficulty in detecting changes in metabolite levels between the MinoLPS and SalLPS treatment groups is that metabolite changes may be intermediate between control levels and SalLPS levels, thus the actual effect size may be small. However, differences between the SalSal and SalLPS models and the MinoSal and MinoLPS models suggest that minocycline is having an effect on metabolites.

Limitations of study and future work

One of the limitations of our study, particularly for the acute study, is that sample size was small. Statistical power was sufficient to detect changes in metabolite levels. However, given that samples have to be divided into a training set (to build the model) and testing set (to test the model), greater sample numbers could reveal more information, particularly when metabolite changes are subtle.

A second limitation is that we were unable to conduct parallel measurements of inflammatory markers in plasma and brain tissue to study the effects of cytokines on metabolites. This was due to technical limitations, as insufficient amounts of plasma samples remained after NMR analysis for further use, and all of the brain tissue samples were processed for NMR analysis. Nevertheless, previous studies have shown that 24 hours post-LPS treatment, mRNA levels of inflammatory cytokines are elevated in the brain (Chapter 2).

A third limitation is that the NMR provides limited information on lipid-phase metabolites. In plasma samples, lipids are detected as broad peaks which contain a range of lipid species. Thus, from our current NMR data alone, we are unable to identify the specific lipid species that are affected by LPS and minocycline. For the brain samples, the perchloric acid extraction protocol removes all lipid-phase metabolites and proteins from the sample, thus we are only able to analyse aqueous-phase metabolites. Given that lipoproteins were affected by a single dose of minocycline, repeating this study with a mass spectrometer might reveal more information about the acute and chronic effects of minocycline on lipids.

A fourth limitation is that we only investigated the effects of minocycline at one time-point post-treatment per study – two hours for the acute study, and 24 hours for the repeated administration study. The 24 hour time-point was chosen as that is the time-point in an established mouse inflammation model where depressive behaviour is observed that is not attributed to sickness behaviour. However, the time-points chosen might not be the optimal time-point to observe metabolite changes, and a time-course metabolomics study might provide valuable information on the effects of inflammation and/or minocycline.

Finally, the dosing regimen chosen was based on established inflammation models showing LPS-induced depressive behaviour, and the ability of minocycline to ameliorate those effects (Frenois et al., 2007; O'Connor et al., 2009). However, to confirm the antidepressant effects of minocycline, and biological relevance of the metabolite changes identified, it would be useful to repeat the experiment and include some behavioural tests.

3.5 Conclusion

In conclusion, peripherally-induced inflammation resulted in changes in both plasma metabolites and aqueous-phase brain metabolites. Key plasma metabolite changes were involved in energy metabolism, including lipoproteins, glucose, creatine, and isoleucine. Key polar brain metabolite changes include amino acids linked to neurotransmission such as glutamine, serine, and NAA. A single dose of minocycline also resulted in changes in similar plasma metabolites involved in energy metabolism, in this case lipoproteins, glucose, creatine, and taurine. While repeated doses of minocycline did not result in OPLS-DA models with significantly better predictive accuracy of large magnitude, minocycline does have an effect on LPS-treated samples. Minocycline ameliorated the LPS-mediated increase in isoleucine, and differences in the SalSal compared with SalLPS and MinoSal compared with MinoLPS models suggest that minocycline's effect on LPS-treated samples may be present but not sufficiently large to build a significantly predictive model.

Changes in lipoproteins and energy-related metabolites like glucose and creatine are in line with that seen in previous studies of inflammation and antidepressants. This study has also identified taurine and isoleucine as possible metabolites linked to minocycline action.

While animal models have revealed information about the effects of minocycline on central signalling and metabolites, animal models are limited in information and relevance compared to human studies. Therefore, the next two chapters will look at the psychological (Chapter 4) and biochemical (Chapter 5) effects of minocycline in healthy volunteers.

Chapter 4: The effect of acute minocycline administration on emotional processing and cognition in healthy volunteers

4.1 Introduction

4.1.1 Background

The use of minocycline as a therapeutic for psychiatric diseases has been explored in several early phase clinical trials, particularly for depression and schizophrenia. However, these clinical trials usually involved the use of minocycline as an adjunct to the medication the patients had already been prescribed. The main outcome for trials in depressed patients were mood questionnaires, such as Montgomery-Asberg Depression Rating Scale (MADRS) or the Hamilton-Depression Rating Scale (HAM-D), and quality of life questionnaires, where minocycline was shown to improve HAM-D scores in patients with treatment resistant depression (Husain et al., 2017), and Clinical Global Impression (CGI) and quality of life scores (Dean et al., 2017). Thus, these clinical trials were longitudinal studies involving repeated doses of minocycline, usually over a period of several months.

Clinical trials are very useful in clinical research, allowing the assessment of the efficaciousness of a given drug in treating a particular disease. However, they lack the methods necessary to understand the neurobiological and psychological mechanisms underlying the action of different drug agents. Recent developments in experimental medicine have allowed for the investigation of the effects of different antidepressants and other drugs by using more sensitive measures of emotional processing and cognition. These measures can be used in a wide range of populations and have been shown to be sensitive to the effects of antidepressant drugs within hours of administration, therefore providing insight into their mechanisms of action. For example, administration of a single dose of citalopram, a common selective serotonin reuptake inhibitor (SSRI), has been shown to increase attentional bias to positive words

compared to placebo in healthy volunteers (Browning et al., 2007). Similarly, administration of acute reboxetine (a norepinephrine reuptake inhibitor) improved recognition of happy facial expressions (Harmer et al., 2003). These studies support the cognitive neuropsychological model of antidepressant drug action (Harmer, Goodwin, & Cowen, 2009), which suggests that antidepressants act early to increase the processing of positive versus negative stimuli, therefore targeting the negative bias that is commonly seen in depression. As demonstrated by Tranter et al., these changes are thought to contribute to later improvements in mood. In this study, increased accuracy to detect happy faces within the first 2 weeks of treatment was predictive of later improvements in depression scores (Tranter et al., 2009).

Investigating the acute effects of pharmacological agents has the advantage of measuring the influence of a drug without any confounding changes in symptoms. Therefore, the current study investigated the early effects of a single administration of minocycline on psychological processes, to provide insight into the psychological pathways influenced by this drug. An extensive literature search suggests that this is the first study measuring the acute effects of a single dose of minocycline on emotional and cognitive processing. To explore emotional processing, the emotional test battery (ETB), a series of computer-based tasks shown to be sensitive to early changes effected by psychotropic agents, was used. Previous studies have shown that the ETB captures the shift towards a positive valence bias caused by a single dose of an antidepressant in healthy volunteers (D. Arnone et al., 2009; Browning et al., 2007; Harmer et al., 2003).

The ETB has shown to be useful not only in proposing a possible mechanism of action for antidepressants, such as SSRIs, but also to identify other compounds with potential antidepressant properties. These agents target different systems, and have mechanisms that are distinct from that of the SSRIs. For example, a single dose of memantine, an anti-NMDA antagonist that targets the glutamatergic system, was shown to reduce false alarm responses for negative-valence words in an emotional memory task (Pringle et al., 2012). Thus, the use of

the ETB has demonstrated that the antidepressant action of different, pharmacologically unrelated drugs involves changes in the processing of emotional information.

Given that minocycline has also shown to improve the negative and cognitive symptoms in early-phase schizophrenia trials (Y. Levkovitz et al., 2010; F. Liu et al., 2014), it would be interesting to explore if a single dose of minocycline could affect the processing of cognitive information. As previous work in animals have shown that minocycline ameliorated inflammation-induced changes in the hippocampus (Chapter 2), 2 hippocampal-dependent cognitive functions were used in the current investigation. These were the implicit functions of contextual cueing, and implicit memory. The N-back task for working memory was also chosen to explore the effects of minocycline in the pre-frontal cortex.

4.1.2 Aims and hypotheses

The current study explored the early effects of a single dose of minocycline on emotional and cognitive processing in a population of healthy volunteers. In accordance with previous studies, healthy volunteers were recruited in order to study the psychological effects of minocycline without complications from any psychiatric diseases or ongoing/prior psychotropic medication.

The primary outcome of this study was the effect of minocycline on emotional processing measured through the ETB. Based on previous similarly-designed studies, the hypothesis was that minocycline would increase processing of positive information through the increased recognition of positive-valence facial expressions or words. It was also possible that minocycline could decrease the processing of negative information through reduced recognition of negative-valence facial expressions or words. The secondary outcome of this study was the effect of minocycline on cognitive processing measured through the contextual cueing task, negative priming task, and the N-back task. This tested the hypothesis that minocycline would improve performance in cognitive tasks, based on studies outlined above.

4.2 Methods

4.2.1 Participants and Recruitment

A previous study found that acute antidepressant administration reduced accuracy to detect fearful faces in healthy volunteers, with an effect size of 1.09. Informed by these data, an *a priori* sample size calculation for the current between-subjects design yielded n=19 as the minimum sample size required to detect changes in accuracy (difference between 2 independent means: two tailed, alpha = 0.05, effect size = 1.09, power = 0.9) (Harmer, Shelley, Cowen, & Goodwin, 2004).

40 healthy adults (ages 18 to 55 inclusive) were recruited using web and poster advertisements. Participants who were outside the age limit, had a current or past history of any psychiatric disorder, had any medical contra-indication, were taking medication that would interfere with minocycline or cause any contra-indications, had known hypersensitivity to tetracyclines or to any of the excipients in the minocycline or placebo capsules, were currently pregnant or breastfeeding, were lactose intolerant, smoked more than 5 cigarettes a day, had a body mass index (BMI) outside the range of 18-30 inclusive, or were dyslexic were excluded. A washout period was also enforced for participants who had previously participated in studies involving the use of medication (3 months), brain stimulation (1 month), or who had recently used psychotropic drugs (3 months). To avoid learning effects, participants who had taken part in studies involving the same battery of emotional processing tasks were also excluded. Finally, to ensure that female participants would not be unduly influenced by hormonal changes, testing sessions during the pre-menstrual period was avoided.

4.2.2 Study design

Ethical approval for this study was obtained from the Central University Research Ethics Committee (CUREC). This study is a double blind, randomized, placebo-controlled study.

Participants were reimbursed £60 along with reasonable transport costs for their time. The study methodology is outlined in Fig 4.2.1.

Screening

During the screening visit, participants were given a chance to ask questions about the study before giving written consent. Participants were then assessed for mental and physical health through examination of their medical history, and the Structured Clinical Interview for DSM V respectively. Physical measurements (sitting and standing blood pressure and heart rate, height, and weight) were taken. Participants were then asked to do 3 questionnaires – the National Adult Reading Test (NART) (Nelson & Wilson, 1991) to assess verbal IQ, the trait version of the STAI (STAI-T) (Spielberger, Gorsuch, & Lushene, 1970), and the Eysenck Personality Questionnaire (EPQ) (Eysenck & Eysenck, 1975). STAI-T measures trait anxiety, and the EPQ measures four aspects of personality - neuroticism, extraversion, social desirability, and psychoticism. The screening visit usually lasted for an hour.

Testing

Participants that passed the screening visit were then invited back for the testing visit. The testing visit was conducted in the morning, and participants were advised to eat breakfast at least half an hour before the visit. Participants were asked to complete the first round of questionnaires, followed by collection of blood samples, and drug/placebo administration. Participants were then given 2 hours to rest before a second round of questionnaires. Participants then performed a series of computer-based psychological tasks measuring emotional and cognitive processing. The testing visit ends off with a third round of questionnaires and a second collection of blood samples.

Participants were asked to refrain from consuming alcohol 12 hours before the testing visit, to refrain from heavy exercise on the day of the visit, and to refrain from smoking during the duration of the testing visit.

4.2.3 Subjective ratings - Questionnaires

Participants were asked to complete the same questionnaires at three time-points on testing day. These comprised of the state version of the STAI (STAI-S) (Spielberger et al., 1970), the positive and negative affective scale (PANAS) (Crawford & Henry, 2004), the Befindlichkeits scale (BFS) (von Zerssen, Strian, & Schwartz, 1974), and the Bond-Lader visual analogue scale (VAS) (Bond & Lader, 1974). These questionnaires were used to identify subjective changes in state and mood. The Becks Depression Inventory (BDI) (Beck, Ward, Mendelson, Mock, & Erbaugh, 1961) was used to check that both groups were similar at baseline and only completed once at the start of the testing visit.

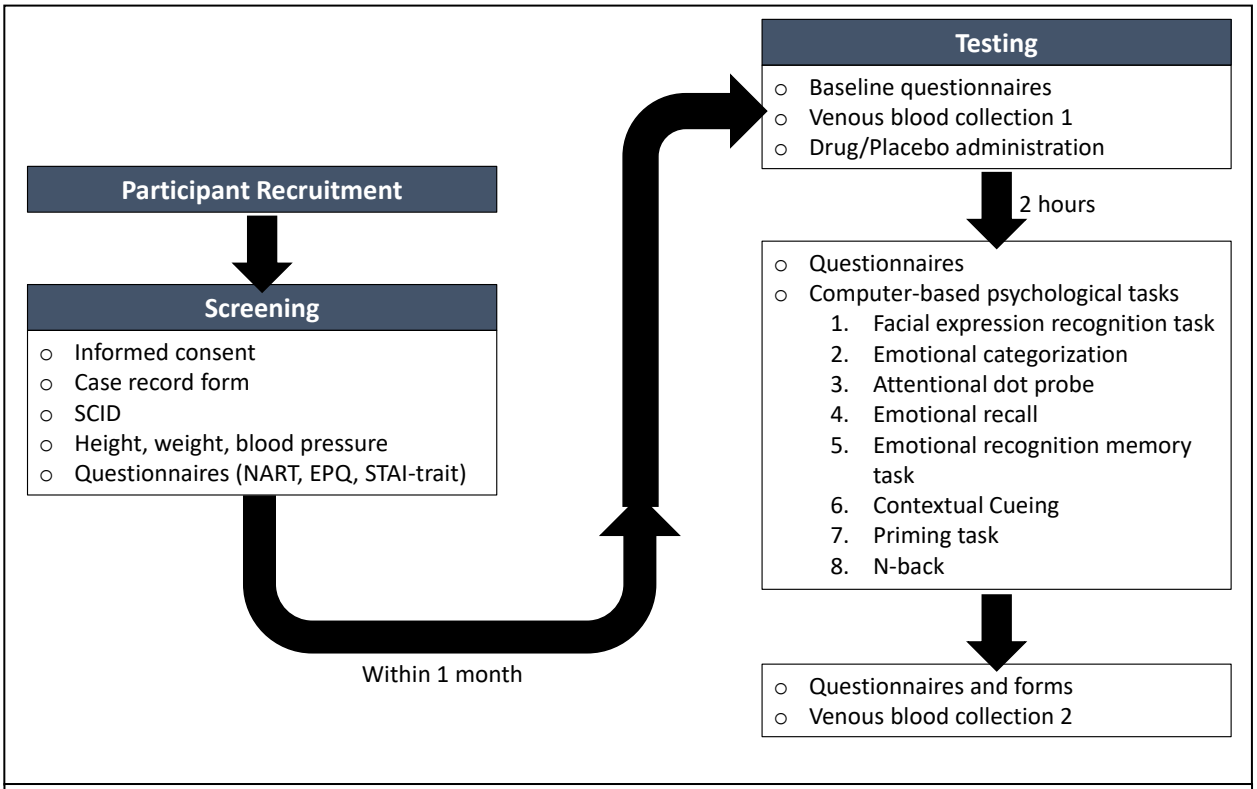


Fig 4.2.1 Outline of study methodology

4.2.4 Psychological tasks – ETB

Participants were given the same tests in the order as described below.

Facial Expression Recognition Task (FERT)

The FERT measured a participant's ability to differentiate between 6 basic emotions (anger, disgust, fear, sadness, surprise, and happiness) and neutral, presented as images of facial expressions. The images were taken from the Pictures of Affect Series (Ekman & Friesen, 1976) and shown at varying intensities in 10% gradations ranging from 0% (neutral) to 100% (full emotion). A total of 250 (10 individuals contributing a total of 4 examples of each emotion at each intensity, plus a neutral expression for each individual) randomized images were presented on a computer monitor for 500 ms per image. Participants were given a break at the midpoint of the task, and were asked to respond as quickly and as accurately as possible.

Analysis of the data involved looking at accuracy scores, reaction time, and misclassifications. Accuracy scores comprised of total accuracy (total number of correct responses for each emotion), as well as accuracy scores at 20% intensity intervals (correct responses at each emotional intensity averaged across 20% intensity bins). Misclassifications are defined as the number of responses when the participant chose a facial expression when it was actually an expression of a different category. A signal detection analysis was also performed to assess discriminability (d'), a measure of sensitivity, and response bias (β), a measure of conservativeness. Grier calculated these measures using the equations $d' = 0.5 + ((y-x)(1+y-x)/4y(1-x))$ and $\beta = (y(1-y)-x(1-x))/(y(1-y)+x(1-x))$ where x = probability of false alarms (no.of false alarms/total number of distractors) and y = accuracy (no.of hits/no.of targets). (Grier, 1971)

For all analyses, emotions were defined as the 6 emotions (anger, disgust, fear, happy, sad, surprise) and neutral was considered as baseline and not included in the analyses.

Emotional Categorization Task (ECAT)

Participants were presented with 60 personality characteristic words on a computer screen for 500 ms. The words (Anderson, 1968) were selected to be positive or negative in nature (30 each),

and were matched for length, frequency and meaningfulness. Participants were asked to think of the words as though overhearing someone describing them with that characteristic, and their task was to determine if they would like or dislike to be described in that manner.

Analysis of the data involved looking at the number of classifications, and response times for correct identification.

Attentional Dot-Probe Task

This task measured a participant's attentional bias towards positive or negative emotional stimuli.

Participants were shown a display where a pair of faces would appear (one on the top half of the screen and the other at the bottom half), followed by 2 dots that were either in vertical or horizontal orientation in the location of one of the faces. Participants were asked to respond by pressing a key corresponding to the orientation of the dots. There were three possible emotional pairings of the faces shown – 1. Neutral-neutral 2. Happy-neutral 3. Fearful-neutral. If participants had an attentional bias towards negative stimuli, they would respond faster when the dots appear in the location previously occupied by a fearful face than when the dots appeared in the other location. The emotional faces (happy and fearful) appeared in both locations at equal frequency.

The faces shown were photographs of 20 individuals with different facial expressions taken from the JACFEE/JACNeuF sets of facial expressions (Matsumoto & Ekman, 1988).

The time at which the faces were presented were also variable – 100 ms (subliminal) and 1000 ms (supraliminal).

Data was analysed by calculating attentional vigilance scores for each participant from mean reaction times. Namely, comparing between congruent trials, where probes appears in the same location as the emotional face, and incongruent trials, where probes appeared in the opposite position. Positive scores would imply an attentional vigilance towards the emotional stimuli.

Emotional Recall Task (EREC)

Participants were given 2 min to write down as many characteristic words as they could remember from the ECAT. This was a surprise recall task. Emotional biases in memory was measured as the relative recall of positive versus negative words.

Emotional Recognition Memory Task (EMEM)

Participants were presented with a series of personality characteristic words on a computer screen. Half of the words presented were positive and negative words previously seen by participants in the ECAT, while the other half were novel distracter words. Participants were asked to determine if they had previously seen the word (familiar) in the ECAT or not (novel). This task measured emotional biases in memory, and data analysis involved looking at the accuracy and response time of the recall, and the valence of the recall (i.e. whether positive information was remembered better).

4.2.5 Psychological tasks – Cognitive tasks

Participants were given a short break in between the emotional and cognitive processing tasks. The cognitive tasks were also done in the order as described below.

Contextual Cueing

This task, adapted from Chun & Jiang (1998), measured the learning of spatial contextual information (Marvin M. Chun, 2000; M. M. Chun & Jiang, 1998). Participants were presented with a display of an array of 11 distracter "L"s with a single target "T" on a computer screen. Participants were then asked to locate the T and press a key indicating if it was tilted to the left or to the right. In each trial, a central fixation dot is shown for 500 ms, followed by the display array for 700 ms or until participant response. There are 48 trials in a block, with the display array a repeat of a previously presented array in half the trials, and novel display arrays for the remaining half. Altogether, participants are asked to complete a practice block (where the "T" is

in a different colour from the distracters) and 10 actual blocks where participants received feedback at the end of each block (percentage accuracy and time taken).

Data was analysed by calculating difference scores from the reaction time and accuracy for repeated arrays versus reaction time and accuracy for novel arrays. Reaction times greater than 2000 ms were not recorded.

Priming task

This task measured implicit memory, where prior exposure to a stimulus influences responses to the next stimulus (Klinge et al., 2018). Participants were asked to locate an "X" that would appear at one out of 4 possible locations on the screen, and press a corresponding location key on the keyboard. A distracter "O" would also be shown at a second location. Each trial starts with the presentation of a fixation cross for around 1500 ms, followed by a priming display. Participants will then be presented with a second shorter fixation cross for around 400 ms, and finally a probe display. Participants will have to respond to both the priming and probe display, and both displays remain on screen until a response is recorded.

The locations of the "X" and "O" within the two displays in a trial result in 4 conditions – 2 experimental conditions (repetition priming and negative priming), and 2 control conditions (imbalance and control). There are a total of 192 trials (48 trials per condition), presented to participants in 3 blocks with a 15 second break in between blocks.

Data was analysed by calculating difference scores from reaction times from the 2 experimental conditions compared to the control condition. Only trials with correct responses to the prime display were included in the analysis.

N-Back task

The N-back task measured working memory (Harvey et al., 2005). Participants were shown a series of letters, and asked to respond by clicking either the key "same" or "different" depending on the task condition. This task had four conditions. In the 0-back condition, participants were asked to select "same" when the letter "X" appeared, and different for all other letters. In the

1-back condition, participants were asked to compare each letter with the one before and select "same" if the current letter was the same as the one before. In the 2-back condition, participants were asked to compare between the current letter and the letter shown two places ago. Finally, in the 3-back condition, participants were asked to compare between the current letter and the letter shown three places ago. Each trial starts with a screen stating the condition, followed by a central fixation cross. The end of the trial is indicated by the appearance of a central fixation cross.

Data analysis for this task involved looking at reaction time and accuracy.

4.2.6 Analyses

Demographic data (except gender) and baseline characteristics were analysed with independent sample t-tests. All other measures and tasks were analysed with split plot ANOVA where group (minocycline or placebo) was the between-subjects factor, and time, valence and emotions were the within-subject factors. Equality of variance was tested with Mauchly's test of sphericity, and when this assumption was not met, the Greenhouse-Geisser corrected value was reported instead. All analyses were done using SPSS and Graphpad Prism.

The effects of gender were also considered with gender as an additional between-subjects factor. A gender effect was reported if there was a significant gender*group*measure interaction effect.

4.3 Results

4.3.1 Demographic data and baseline trait characteristics

There were no significant differences between treatment groups in demographic data such as gender, age, BMI, and NART scores, a measure for verbal IQ (Table 4.3.1).

The 2 groups were also well matched at baseline for depression scores (BDI), trait anxiety scores (STAI-T), and personality trait scores (EPQ). Analysis was done with independent samples T-test, unless assumptions were violated, in which case Mann-Whitney U test was reported instead. In addition, no significant group*gender interactions were observed for any of the baseline measures when analysed with 2-way ANOVA (Appendix 4.1).

Table 4.3.1 Demographics and baseline data of participants are similar between treatment groups.

	Minocycline Group	**Placebo Group**	**Significance (*p* value)**
Gender	10M/10F	10M/10F	1.0
Age (Years)	27.50 (4.56)	27.50 (6.89)	0.464
BMI	23.84 (3.06)	23.31 (1.79)	0.797
NART	115.95 (6.63)	114.62 (4.43)	0.459
STAI (Trait)	31.55 (5.83)	31.90 (7.28)	0.849
BDI	1.30 (2.32)	2.10 (3.74)	0.673
EPQ: Neuroticism	4.15 (2.92)	5.40 (3.97)	0.264
EPQ: Psychoticism	2.65 (1.81)	3.05 (2.50)	0.711
EPQ: Extraversion	14.00 (4.22)	14.30 (4.11)	0.821
EPQ: Lie	9.75 (3.77)	11.60 (3.72)	0.126

Values represent the mean with standard deviation in parentheses.

BMI: Body mass index; NART: National Adult Reading Test; STAI: State-trait Anxiety Index; BDI: Beck Depression Inventory; EPQ: Eysenck Personality Questionnaire

4.3.2 Subjective state changes – questionnaires

Subjective state changes were measured at 3 time points – before drug/placebo administration, 2 hours after drug administration (post-treatment 1), and after the psychological testing session (approximately 4 hours after drug administration, post-treatment 2). State changes measured include affect (PANAS), mood and energy (BFS), and the Bond-Lader visual analogue scale (VAS). These were analysed with repeated measures ANOVA.

The results of the subjective state changes are summarized in Table 4.3.2, expressed as mean of the treatment group with standard deviation in parenthesis. There were no significant main effect of group or interaction between group and time points (STAI-S and BFS-Total), or interaction between group, time points, and valence (PANAS, BFS energy and mood, VAS) (all p values > 0.1).

For PANAS, valence was defined as positive or negative. For BFS, valence was defined as energy or mood. For the VAS, the 16 different items were combined to form sub-scales of calmness, contentedness, and vigilance ranging from 0 to 100, with 0 representing maximum calmness, contentedness, and vigilance.

Therefore, at baseline (pre-treatment), there were no significant differences in mood or state between the treatment groups. Also, a single dose of minocycline resulted in no significant difference in mood or state after 2 hours (post-treatment 1) and 4 hours (post-treatment 2) (Table 4.3.2). There were also no significant gender*group*time-point interactions for all measures.

Table 4.3.2 Self-reported state changes assessed at three time-points were similar between treatment groups.

	Minocycline Group			**Placebo Group**		
	Pre-treatment	Post-treatment 1	Post-treatment 2	Pre-treatment	Post-treatment 1	Post-treatment 2
STAI (State)	28.8 (5.56)	26.8 (6.11)	31.2 (7.16)	27.35 (6.81)	24.8 (5.76)	28.35 (8.63)
PANAS: Positive	32.15 (6.78)	32.8 (8.10)	31.60 (8.34)	33.45 (6.88)	37.55 (5.42)	35.25 (5.83)
PANAS: Negative	12.45 (3.99)	11.00 (2.27)	12.35 (5.14)	12.15 (3.10)	10.95 (1.73)	11.60 (2.26)
BFS: Energy	2.50 (3.93)	3.20 (5.77)	5.85 (6.39)	2.20 (3.62)	2.05 (2.86)	4.60 (5.48)
BFS: Mood	8.25 (10.99)	7.40 (12.00)	10.40 (13.49)	6.00 (5.33)	5.10 (5.19)	7.15 (11.63)
BFS: Total	10.750 (14.54)	10.60 (16.88)	16.25 (18.79)	8.20 (8.29)	7.15 (6.87)	11.75 (15.82)
VAS: Calmness	24.70 (16.63)	25.30 (15.64)	28.35 (18.03)	22.23 (17.02)	24.85 (17.33)	21.60 (16.89)
VAS: Contentedness	20.17 (14.81)	19.68 (12.73)	21.04 (14.97)	18.62 (15.14)	16.81 (11.79)	17.86 (14.06)
VAS: Vigilance	22.35 (13.41)	29.87 (18.13)	33.01 (18.19)	24.93 (14.13)	24.74 (14.68)	31.73 (16.46)

Values represent the mean with standard deviation in parentheses.

STAI: State-trait Anxiety Index; PANAS: Positive and Negative Affective Scale; BFS: Befindlichkeits Scale; VAS: Bond-Lader Visual Analogue Scale

4.3.3 Side effects and efficacy of blinding

Participants were asked to rate how well they were feeling at the end of the study on a scale of 0 to 100 with 0 indicating feeling poorly and 100 indicating feeling well. There was no significant difference in wellbeing between the minocycline group and the placebo group (81.50 ± 14.83 vs 88.30 ± 12.44, $p > 0.05$). A majority of participants also reported feeling no, or minimal, side effects at the end of the study, with the most common side effects (headaches, dizziness and blurred vision) attributed to the computer tasks and the taking of blood samples. No major adverse side effects were reported.

Participants were also asked to choose which group they believed they were in, and their choices were independent of their actual groups ($\chi^2(2, n = 40) = 2.31$, $p > 0.05$). This suggests that blinding was effective in this study.

Table 4.3.3 Participant-reported side-effects at the end of the study.

Side Effects	**Score Frequency**				**Linked to Drug?**
	0	1	2	3	
No side effects	20				NA
Headache		5	1		1 – Yes
Dizziness/Lightheaded		7	3	1	2 – Yes
Blurred Vision		3			1- Yes
Loss of appetite				1	1- Yes
Sore Mouth			1		No
Itching		2			No
Coughing		3			No
Difficulty swallowing		1			No
Numbness in hands and feet		4			No
Stomach discomfort				1	Yes
Skin rashes		1			No

4.3.4 Effects of minocycline on emotional processing

4.3.4.1 FERT

Misclassifications

There was a significant group*emotion interaction for percentage misclassifications (F(3.715, 137.446) = 2.531, p = 0.047). Pairwise comparisons revealed that this was driven by fearful facial expressions, where participants in the minocycline group made significantly fewer fear misclassifications (-42.524%, p = 0.006) (Fig 4.3.2A). Percentage misclassification was taken by calculating total misclassification per participant and dividing misclassifications in each emotion category by the total.

Total Accuracy and Emotional Intensity

There was no significant interaction between emotion and group for total accuracy. However, given that the task included different levels of emotion intensities, which are known to influence the ability to accurately detect a facial expression, an exploratory analysis was conducted to investigate drug effects on different intensity levels (0-20% to 80-100%). There was a significant group*emotion interaction at the 70-80% intensity bin (F(5, 185) = 3.963, p = 0.002) (Fig 4.3.1). Pairwise comparisons revealed that this was driven by the sad and surprise expressions, where participants in the minocycline group were significantly more accurate at recognizing facial expressions of sadness and surprise (Sad: +19.737%, p = 0.002; Surprise: +13.651%, p = 0.036).

This was supported by results looking at the effect of treatment group on the accuracy scores across emotional intensities of individual emotions. There was a significant group*intensity interaction for surprise facial expressions (F(4, 144) = 2.793, p = 0.028), where minocycline administration increased the accuracy of recognizing surprise at the 70-80% bin (+13.446%, p = 0.037). There was also a significant group main effect for sad expressions, where

minocycline increased the accuracy of recognizing sad facial expressions at the 70-80% bin (+8.608%, F(1, 36) = 4.423, p = 0.043).

Reaction Time

There was no significant effect of minocycline on the speed at which participants correctly identified facial expressions in this task (Fig 4.3.2B).

Signal detection theory: Response bias and sensitivity index

A signal detection analysis was conducted by calculating response bias and sensitivity index. Response bias determines the likelihood ratio of participants choosing a particular emotion, and a higher value is associated with a more conservative response. There was a significant group*emotion interaction for the response bias (F(3.726, 137.874) = 3.015, p = 0.023). Pairwise comparisons revealed that participants in the minocycline group were more conservative when picking fearful expressions compared to the placebo group (+19.069%, p = 0.008) (Fig 4.3.3A).

However, there was no difference in the sensitivity index between the 2 treatment groups for all 6 emotions (Fig 4.3.3B).

There were no significant gender interactions for all measures.

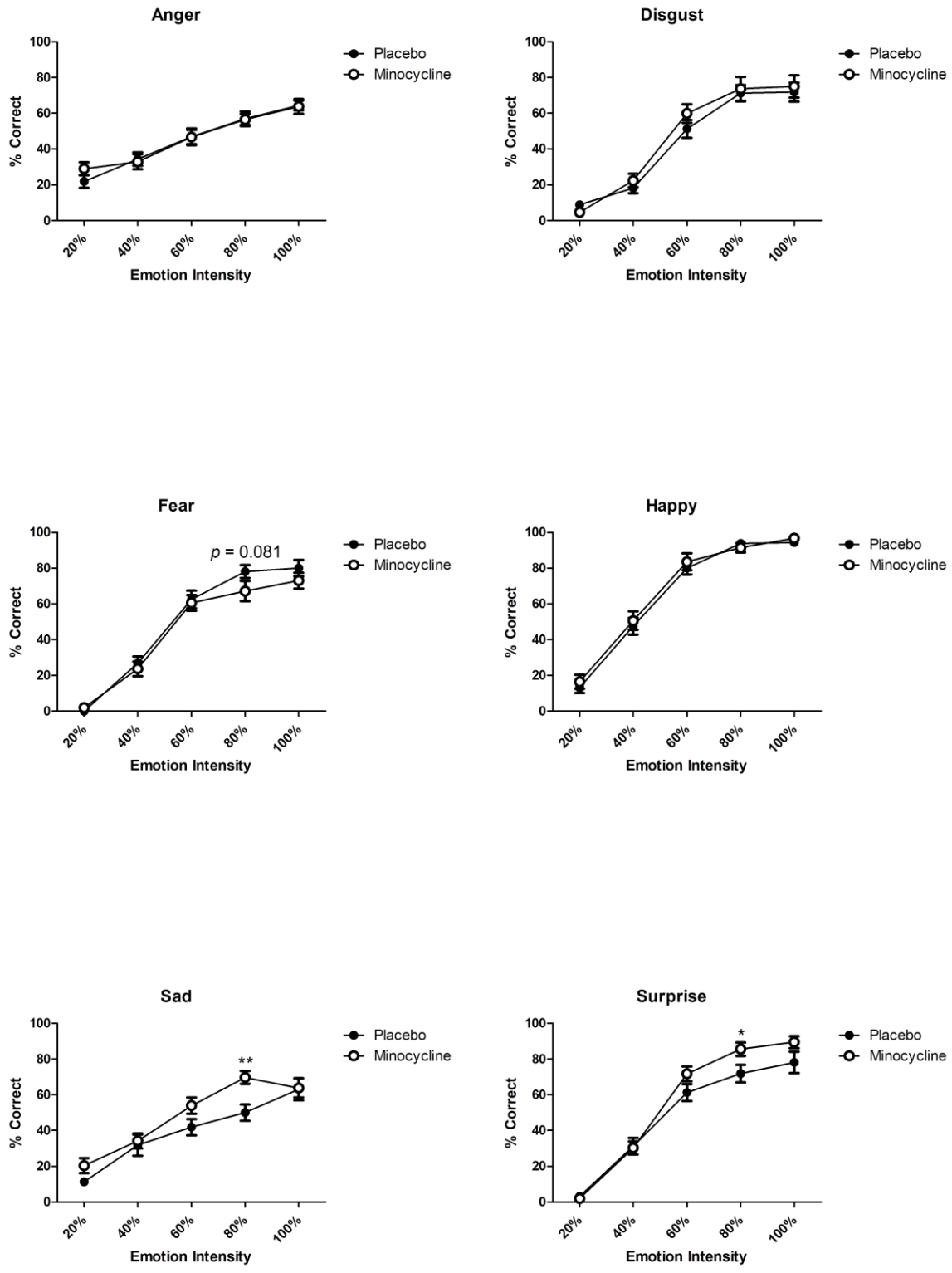


Fig 4.3.1 FERT: Facial expression recognition over different intensity levels in 20% blocks separated by treatment group. Values are expressed as means ± standard error mean (SEM). Minocycline increased recognition of the sad and surprise facial expressions at the 70-80% intensity bin.

*p < 0.05; **p < 0.01

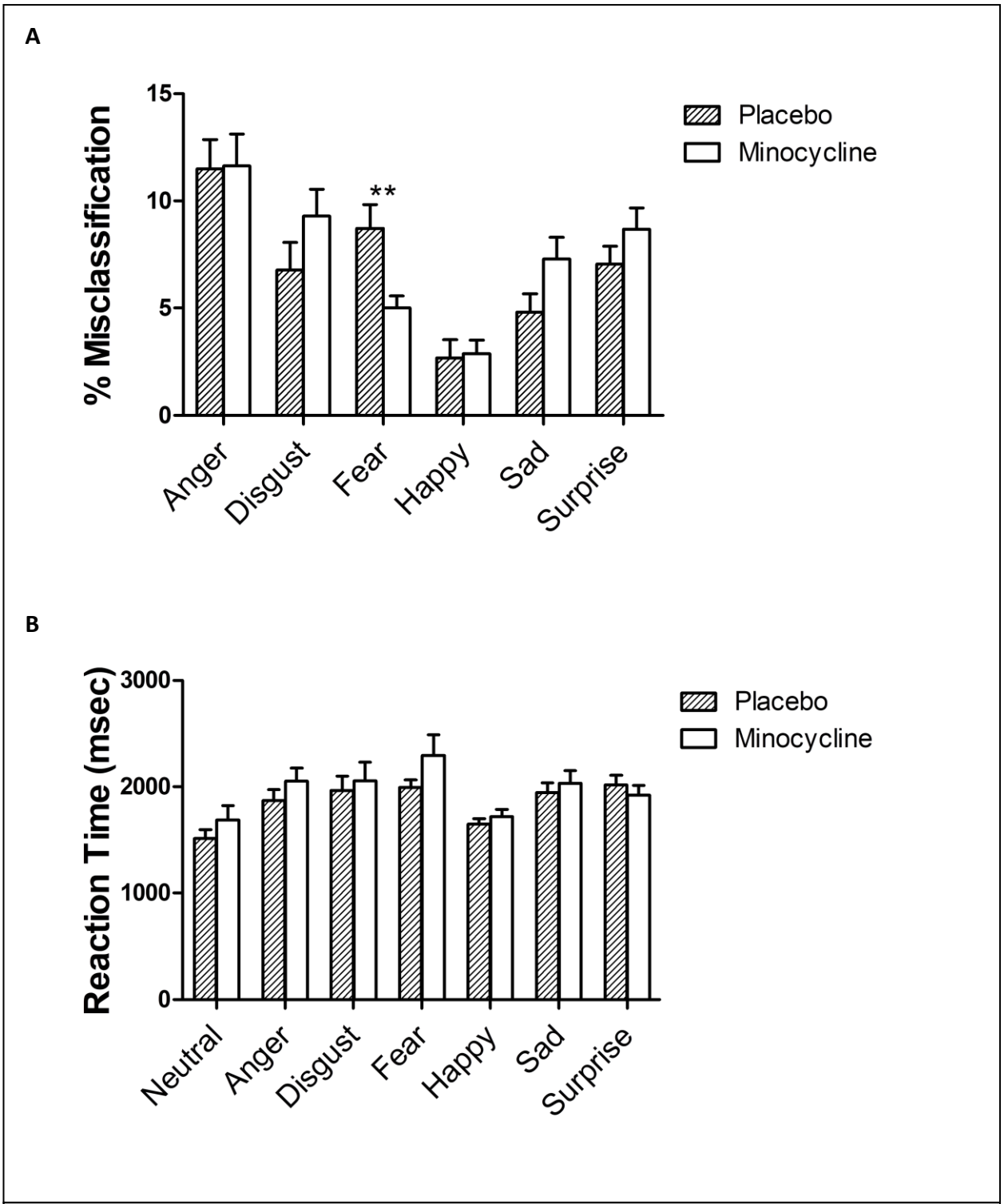


Fig 4.3.2 FERT: Effects of minocycline on (A) misclassification (expressed as % of total misclassification) and (B) reaction time in msec. Minocycline decreased misclassification of fearful expressions. No difference was seen in reaction times between minocycline and placebo.

**$p < 0.01$

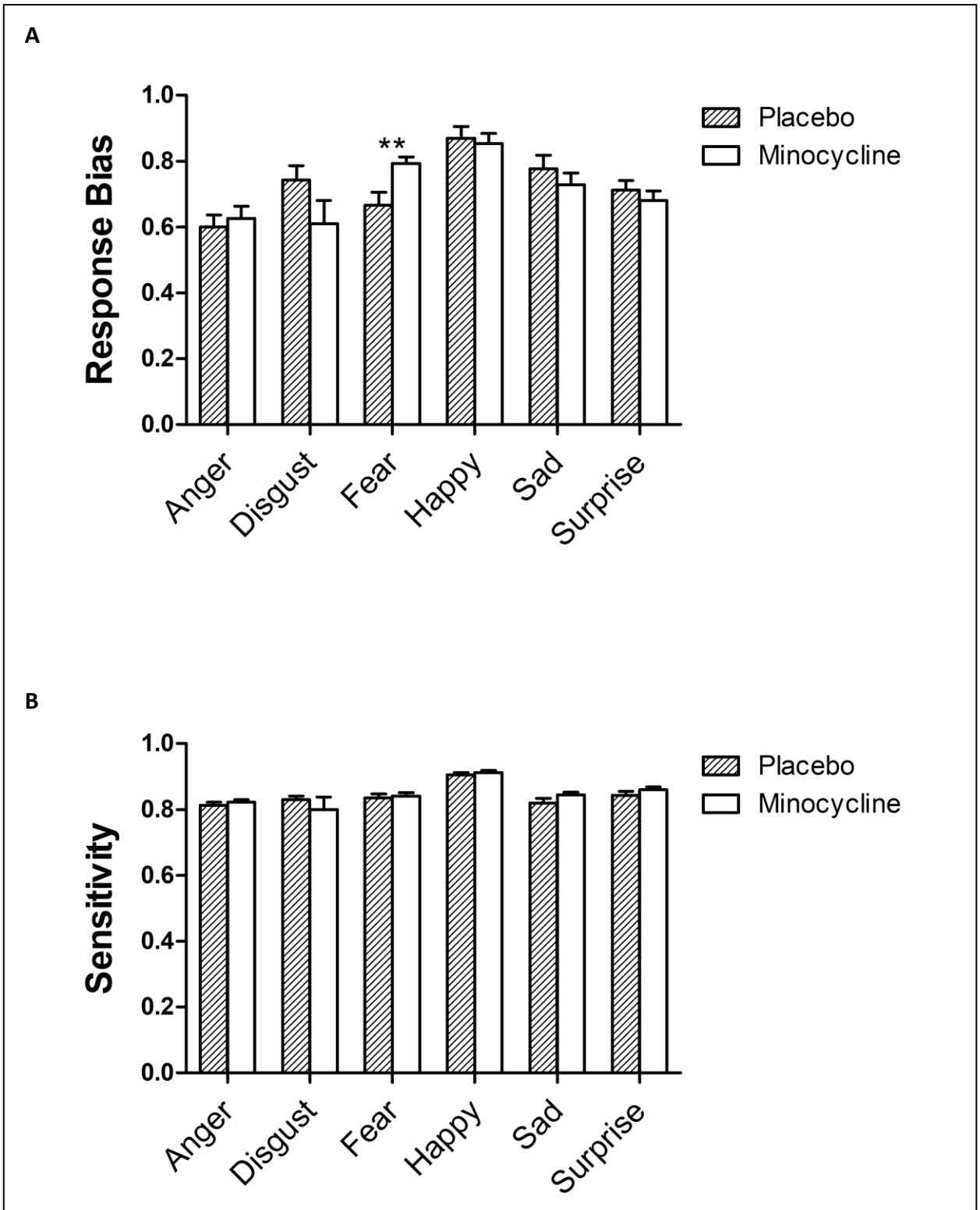


Fig 4.3.3 FERT: Minocycline increased the (A) response bias score for fearful expressions, implying increased conservativeness when choosing fearful faces, but had no effect on (B) sensitivity.

**$p < 0.01$

4.3.4.2 ECAT

Accuracy

There was no significant valence*group interaction for correct responses. The percentage of positive and negative correct responses out of total correct responses for each participant was also calculated to account for variations in total correct responses. There was also no significant valence*group interaction for percentages (Fig 4.3.4A).

However, there was a significant valence*group*gender interaction for both raw numbers ($F(1, 34) = 4.789$, $p = 0.036$) and percentages ($F(1, 34) = 4.571$, $p = 0.040$). For percentages, there was a significant difference between treatment groups in the male group, where participants in the minocycline group had decreased correct responses for positive-valence words (-2.077%, $p = 0.016$), and increased correct responses for negative-valence words by the same magnitude.

Reaction Time

There was no significant valence*group interaction for both raw reaction time (in msec) and percentages of positive and negative responses out of total reaction time (Fig 4.3.4B). There was also no significant valence*group*gender interaction for both measures.

Given that there was a significant decrease in correct responses to positive-valence words in the male group, reaction time in the male group was looked at. For males, an exploratory pairwise comparison showed a significant increase in reaction time for both positive- and negative-valence words in the minocycline group (Positive: +23.211%, $p = 0.008$; Negative: +18.6%, $p = 0.022$). Nevertheless, for both genders, participants responded slower to negative-valence words, and no such group differences were seen for percentages, indicating that the difference is due to inter-individual variation in total response time, rather than a skew in valence.

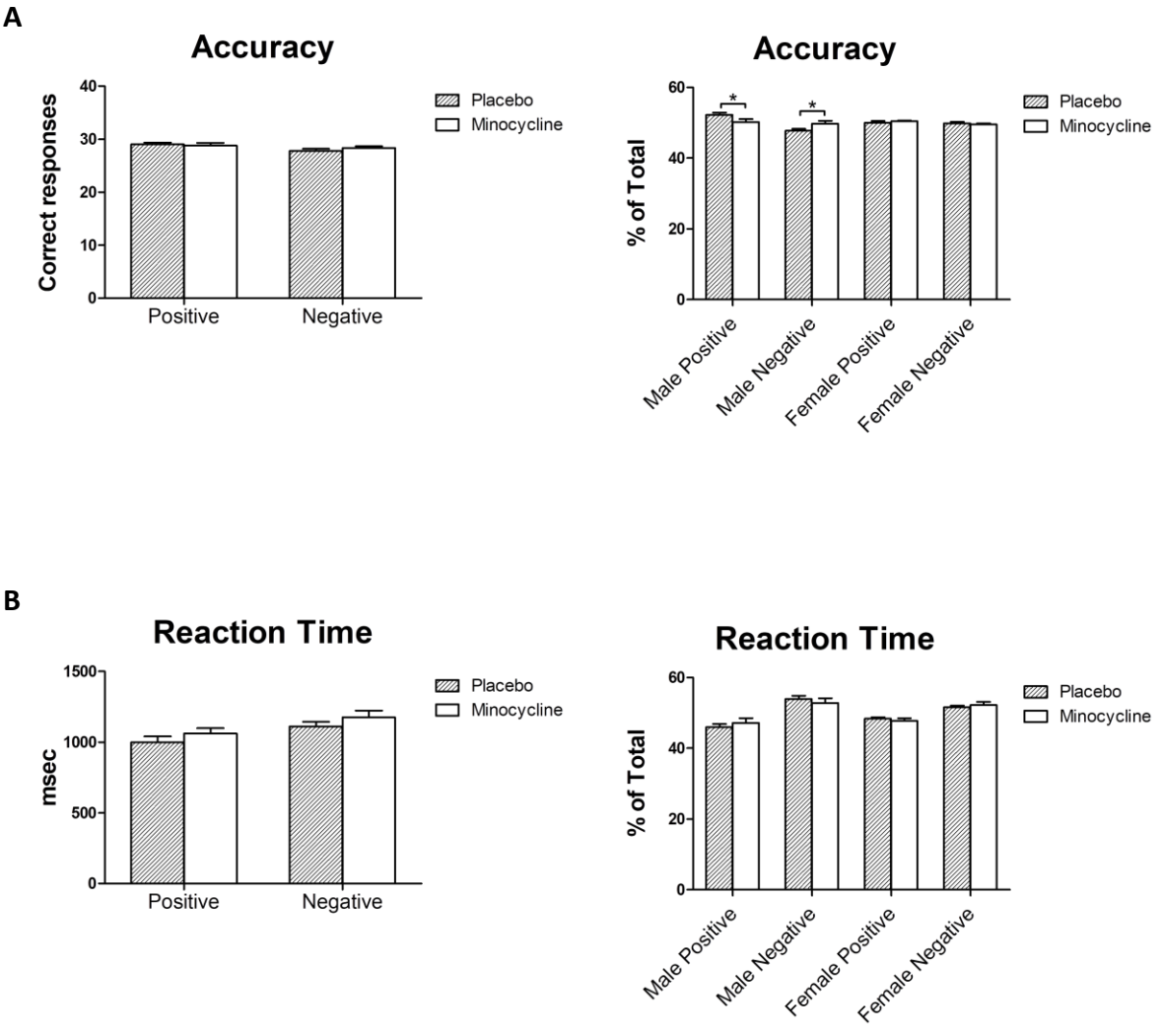


Fig 4.3.4 ECAT: (A) Accuracy - Minocycline had no effect on number of correct responses, but decreased the accuracy of positive valence words in males. (B) There was no difference in reaction time between the minocycline and placebo groups. *p < 0.05

4.3.4.3 Attentional Dot Probe

Accuracy

Both groups performed well on this task with no significant differences in accuracy between treatment groups (Placebo: 94.35%; Minocycline: 93.30%, p = 0.605). There was no significant group*congruence*valence interaction, or gender*group*congruence*valence interaction.

Vigilance Scores

There was no significant group*mask*valence interaction, no mask*group or valence*group interaction, and no gender*group*mask*valence interaction for vigilance scores.

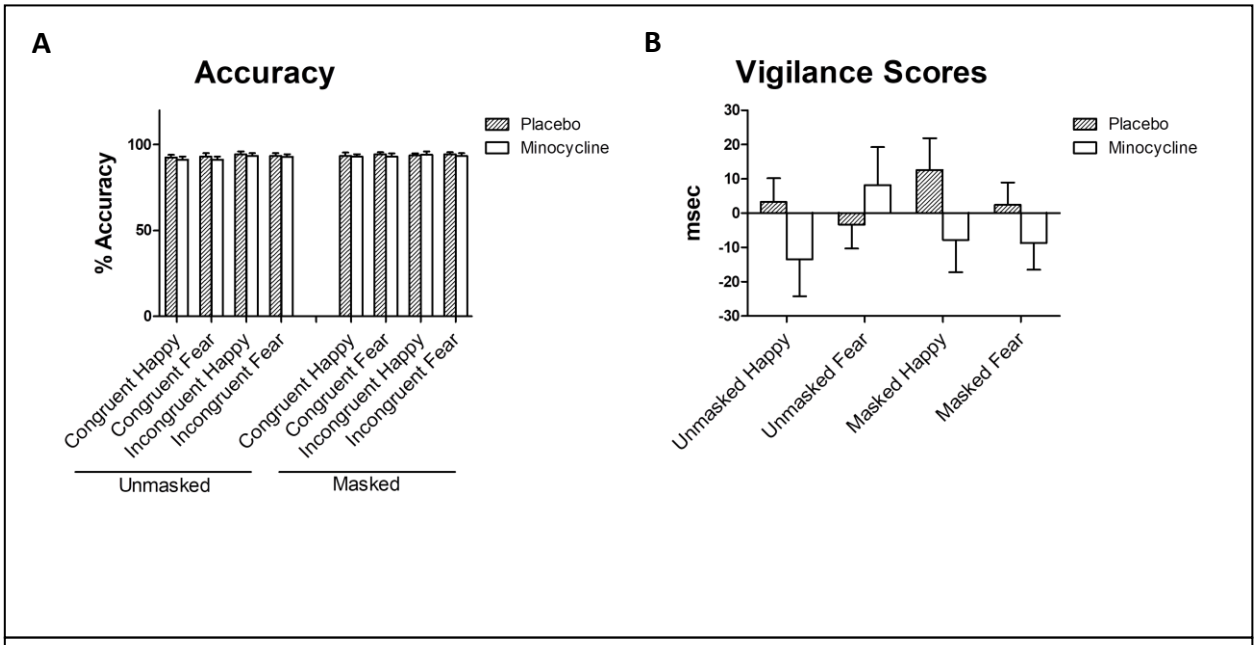


Fig 4.3.5 Attentional Dot Probe task: (A) Accuracy was similar between minocycline and placebo treatment groups. (B) Minocycline decreased mean vigilance scores for the masked condition compared to placebo, implying decreased attention towards emotional stimuli.

4.3.4.4 EREC

There were no significant differences between treatment groups in terms of the percentage of positive/negative correctly recalled words, and positive/negative false alarms (Fig 4.3.6A and B). A positive bias (Positive Total) was calculated by subtracting the total number of negative words from the positive words and dividing that by the total number of words irrespective of whether the words were correctly recalled or false alarms. A positive skew was also calculated by subtracting the ratio of correctly recalled negative words over total negative words from the ratio of correctly recalled positive words over total positive words. In both cases, there was no significant difference between treatment groups (Fig 4.3.6C). There were also no gender effects on all measures.

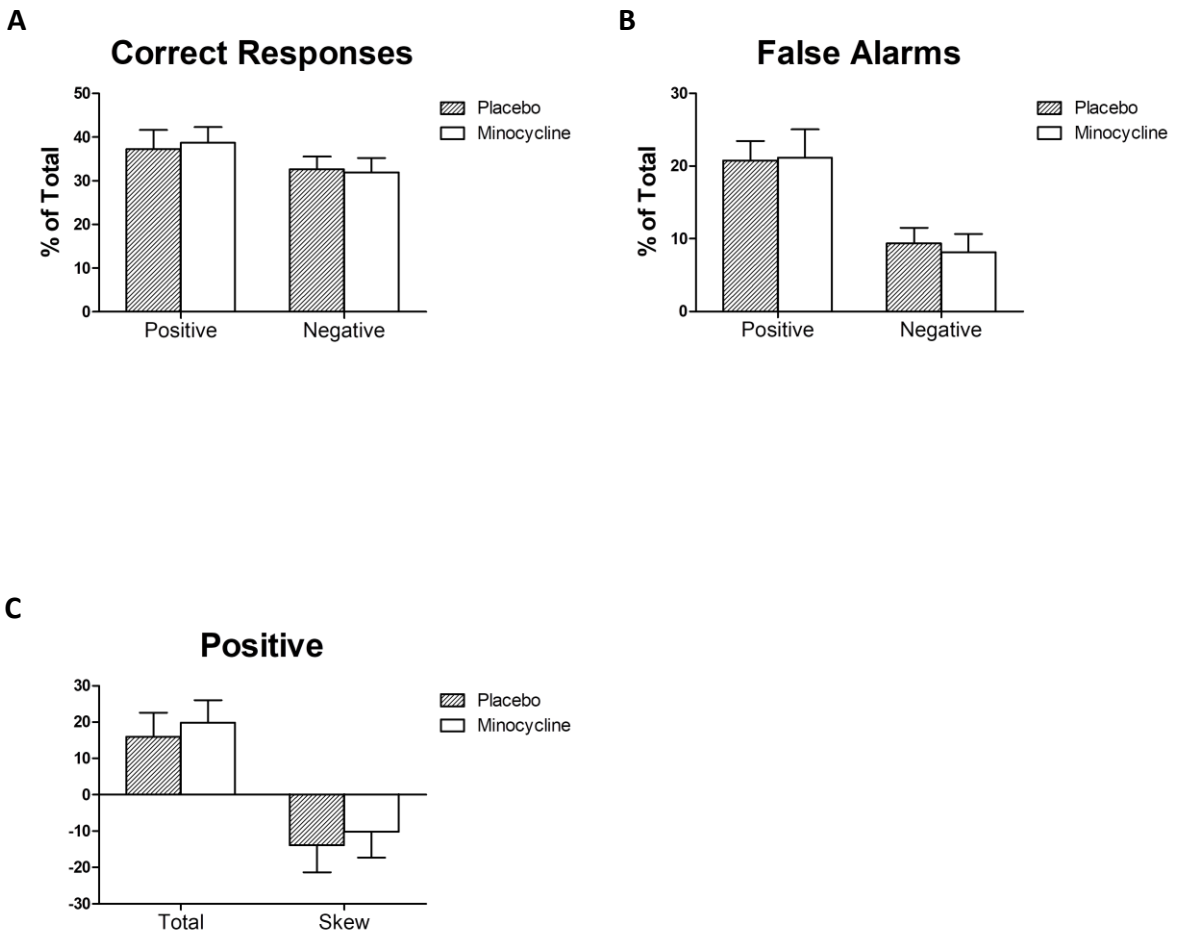


Fig 4.3.6 EREC: There was no difference between the minocycline and placebo treatment groups for (A) correct responses and (B) false alarms, both separated by valence (expressed as a percentage of total responses), and (C) positive bias.

4.5.4.5 EMEM

There were no significant differences between treatment groups in all the outcomes for this task, namely the number of correct responses, the number of correct rejections, the number of false alarms, and reaction times for correct responses (Fig 4.3.7). There were also no gender effects on all measures.

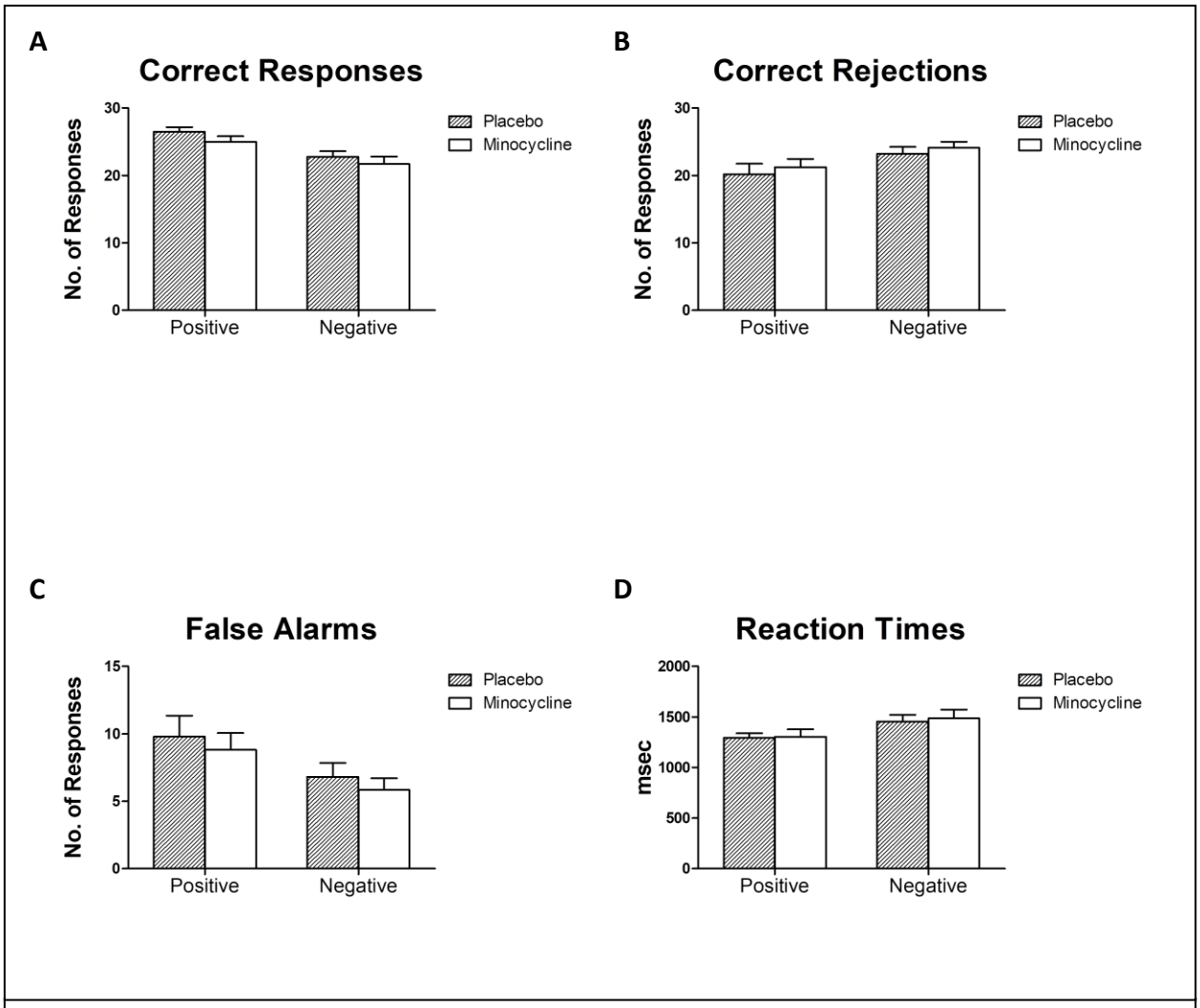


Fig 4.3.7 EMEM: There was no difference between the minocycline and placebo treatment groups for (A) correct responses, (B) correct rejections, (C) false alarms, and (D) reaction times.

4.3.5 Effects of minocycline on cognitive tasks

4.3.5.1 Contextual cueing

Accuracy

Difference scores were computed for accuracy by subtracting novel values from repeated values, therefore positive scores indicated learning. There was a main group effect for accuracy difference scores averaged across all 10 blocks ($F(1, 36) = 5.917$, $p = 0.020$), with minocycline administration increasing accuracy difference scores (Fig 4.3.8).

Scores from the first 5 blocks (blocks 1 to 5) and the last 5 blocks (blocks 6 to 10) were combined respectively, to form 2 phases. There was no group*phase interaction, however, an exploratory pairwise comparison was conducted to see if there were any difference between phases. Pairwise comparisons show that the increase in accuracy difference scores for the minocycline group was driven by the first five blocks (+0.028, $p = 0.026$). There was also no gender*group*phase interaction.

Reaction Time

Difference scores were computed for reaction time by subtracting repeated values from novel values. There was no main group effect for reaction time difference scores when scores were averaged across all 10 blocks (Fig 4.3.8). There was also no group*phase interaction, and no gender*group*phase interaction.

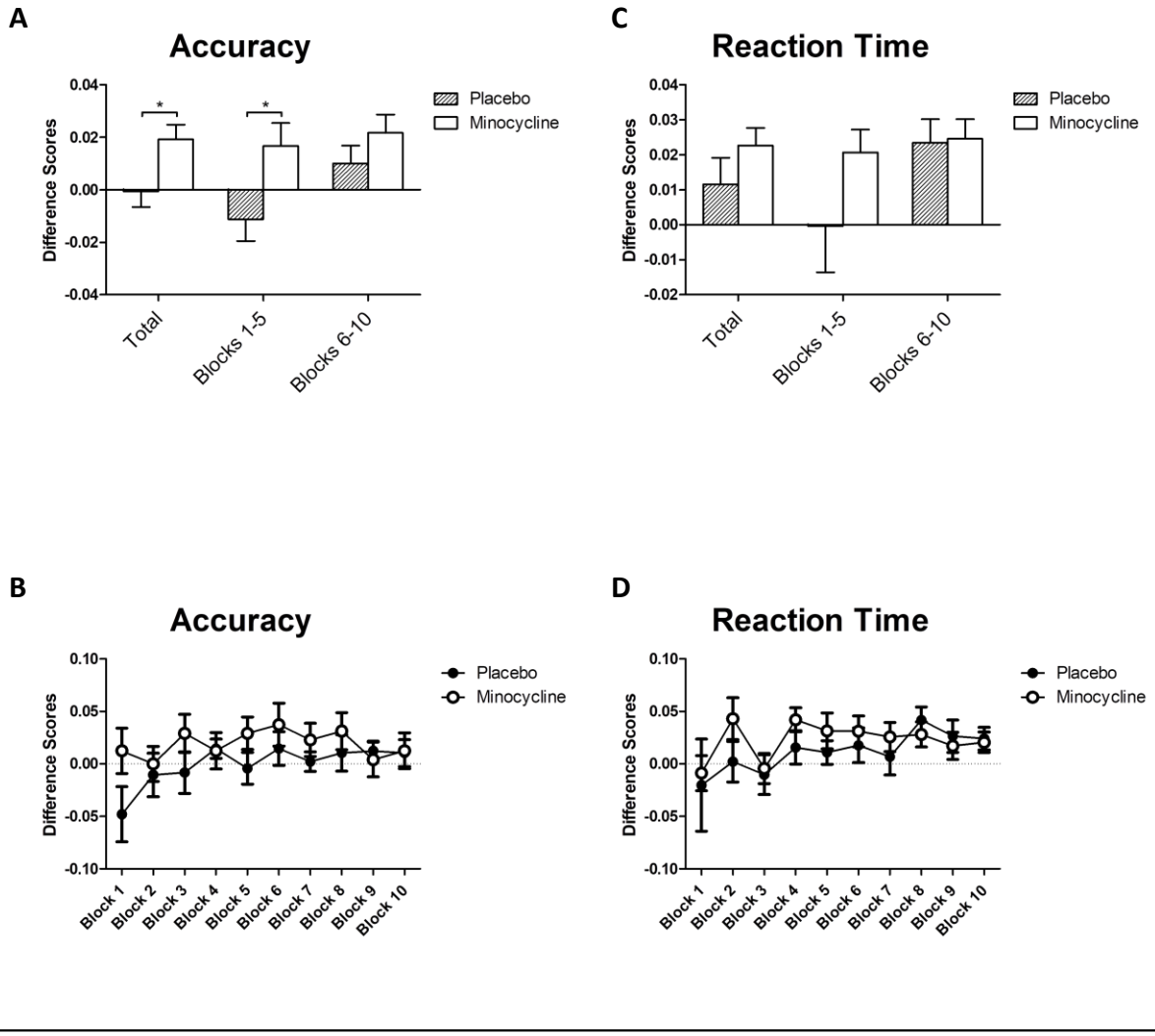


Fig 4.3.8 Contextual Cueing task: Minocycline increased (A and B) accuracy difference scores across all ten blocks and across the first five blocks of the task. Reaction times (C and D) were similar between treatment groups. *p < 0.05

4.3.5.2 Priming task

Accuracy

Accuracy for priming effects was computed as taking the accuracy for the repetition condition and negative condition as a percentage of the neutral control condition (Fig 4.3.9A). There was no main group effect, and no significant group*condition interaction. There were also no gender effects, with no significant gender*group*condition interaction.

Reaction Time

Reaction time for priming effects was computed for repetition priming and negative priming (Fig 4.3.9B). For repetition priming, the median reaction time of the repetition condition was subtracted from the median reaction time of the control condition. The negative priming effect was calculated by subtracting the median reaction time of the control condition from the median reaction time of the negative condition. There was no main group effect, and no significant group*condition interaction. There were also no gender effects.

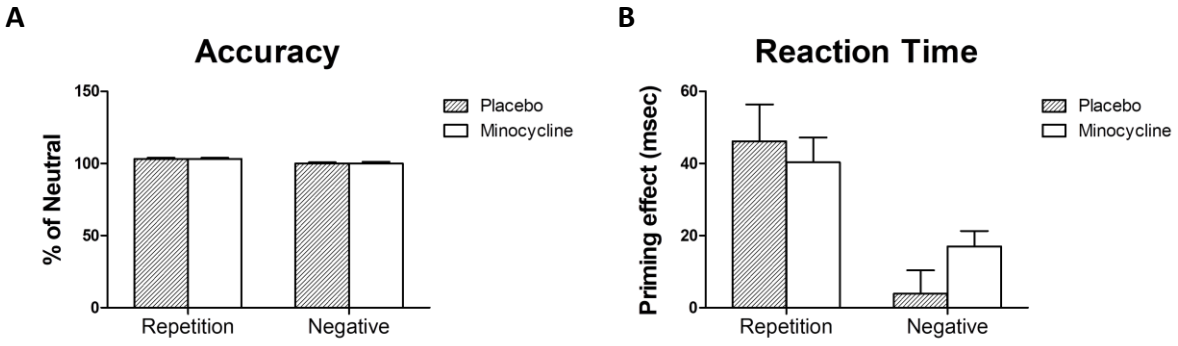


Fig 4.3.9 Priming task: There was no difference between the minocycline and placebo treatment groups for (A) accuracy and (B) reaction time.

4.3.5.3 N-back

Accuracy

Overall performance on this task was high with total accuracy (Fig 4.3.10A) for all four conditions similar between the two treatment groups (Placebo: 85.41%; Minocycline: 83.46%, p = 0.069). When broken down into the different conditions, there was no main effect of group, no significant group*condition interaction, and no gender*group*condition interaction.

Reaction Time

Reaction time (RT) was computed by subtracting the mean reaction time for the control condition (0-back) from the mean reaction time for the other 3 conditions (Fig 4.3.10B). There was no main effect of group, no significant interaction between group*condition, and no gender*group*condition interaction.

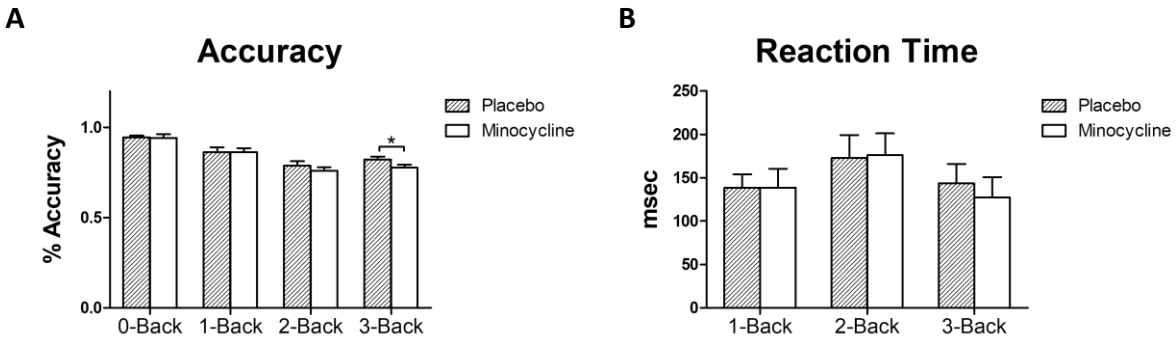


Fig 4.3.10 N-Back task: There was no difference between the minocycline and placebo treatment groups for (A) accuracy and (B) reaction time.

4.4 Discussion

Aims of Study and Hypotheses

This study investigated the effects of a single dose of minocycline on emotional and cognitive processing. Emotional processing was explored through a series of tasks measuring responses to positive- and negative-valence emotions. Given the antidepressant effects of minocycline in clinical trials, it was hypothesized that minocycline would respond in a similar manner to other antidepressants and increase emotional processing of positive information, such as by increasing recognition of happy facial expressions, or by better remembering positive-valence words.

Summary of results

However, the results of the current study show that minocycline did not increase the processing of positive information. Nevertheless, minocycline does have an effect on emotional processing. Decreased misclassification of fearful expressions in the minocycline group suggests that minocycline may function by reducing the processing of negative information. This is supported by a higher response bias score, a measure of conservativeness in signal detection theory, where participants in the minocycline group were more conservative when picking fearful expressions. Minocycline administration also increased accuracy for both sadness and surprise at the 70-80% intensity bin.

Minocycline administration resulted in a 2 percent decrease in the correct identification of positive-valence self-referential words. However, a lack of difference between treatment groups in the raw numbers of responses and other emotional memory tasks suggest that minocycline does not increase the processing of negative information.

In terms of cognitive tasks, minocycline increased total accuracy difference scores (averaged over all 10 blocks), driven by scores in the 1st phase (averaged over 1st 5 blocks) for the contextual cueing task, indicating that minocycline improves implicit learning.

Neuropsychological model of depression and negative biases

Based on the neuropsychological theory of depression, antidepressant effect can be measured by amelioration of the negative bias observed in patients with depression. However, one of the potential criticisms of studying the negative bias in healthy volunteers is that healthy volunteers do not actually have a negative bias at baseline. Thus, one challenge at the analysis stage is how to define improvements in negative bias or other antidepressant effects in healthy volunteers. Other studies investigating emotional processing changes in healthy volunteers provide some suggestions.

Similarly-designed studies from the Harmer group suggest that an increase in the processing of positive information, shown for example by an increase in the accuracy of detecting happy facial expressions, is characteristic of antidepressant action in healthy volunteers (Harmer et al., 2003). However, other acute studies also show that changes in attentional vigilance to emotional expressions, and dampened responses to stressful social stimuli are possible outcomes (Attwood et al., 2017; Wardle, Bershad, & de Wit, 2016). Thus, a reduced perception of negative-valence emotions, such as fear or anger, has also been observed in healthy volunteers after acute administration of a psychotropic manipulation.

Study results: Explanations and theories

This study was based on other similar single-dose studies using the same battery of tasks in healthy volunteers. However, those studies mainly involved known antidepressants drugs such as SSRIs. As such, the results obtained from those studies may only be relevant to mechanisms brought about by SSRI administration. The differences in results obtained from this study suggests that the effects of minocycline may have a different mechanism than that of SSRIs, but that emotional processing changes may still be involved.

Emotional Processing: Anxiety and fear

Other studies with similar outcomes, but using different tasks from the ETB, provide hints of other possible mechanisms. Wardle et al. showed that naltrexone, a non-specific opioid antagonist, slowed identification of sad and fearful expressions (Wardle et al., 2016). This effect was attributed to its inhibition of kappa-opioid receptors, whose antagonism is linked to antidepressant and anxiolytic effects. Buprenorphine, a partial mu-opioid receptor agonist and kappa-opioid antagonist, has also been shown to reduce fear perception (Ipser et al., 2013). Given that minocycline was observed to have similar effects on fear processing in the current study, it is possible that minocycline may have effects on opioid receptors as well. While there are few pre-clinical studies investigating the effect of minocycline on opioid receptors, minocycline has been shown to have analgesic effects (Mika et al., 2014).

Studies investigating emotional processing changes in anxiety suggest that minocycline may have an anxiolytic effect. Anxiety has been shown to be associated with impairment in the recognition of emotional expressions, and a hyper-vigilance to threat (Attwood et al., 2017). The current study has shown that minocycline increased the recognition of sad and surprise facial expressions, and also reduced fear misclassification, thus reducing fear perception. Parenthetically, an exploratory pairwise comparison showed that minocycline also significantly reduced attentional vigilance to emotional expressions for both valences (happy and fearful) in the masked condition (-15.748msec, $p = 0.029$) of the attentional dot probe task, although there was no significant group*mask*valence interaction. Nevertheless, this suggests a decrease in vigilance that may help to dampen the hyper-vigilant response to threats.

Cognitive Processing: Implicit learning

Minocycline has previously been shown to have subtle effects on improving cognitive deficits in patients with schizophrenia. Liu et al. have shown that minocycline administration improved attention/vigilance scores in the Continuous Performance Test – Identical Pairs (CPT-

IP) task, but not in other cognitive domains (F. Liu et al., 2014). Levkovitz et al. associated minocycline administration with improvements in executive functions (working memory, cognitive flexibility, and planning) assessed by the Cambridge Neuropsychological Test Automated Battery (CANTAB) (Y. Levkovitz et al., 2010). Both of these studies were chronic administration studies, 16 weeks and 6 months respectively, and it is interesting that greater cognitive improvements were seen in the longer study, associating improvements with duration of treatment. Indeed, Levkovitz et al. observed a significant time*treatment interaction for improvements. However, improvements in cognitive performance have also been shown in an acute minocycline study in healthy volunteers. Sofuoglu et al. showed that minocycline administration (5 days) resulted in faster reaction times in a Sustained Attention to Response Test (SART), and linked this result to changes in glutamate and dopamine neurotransmission (Sofuoglu, Mooney, Kosten, Waters, & Hashimoto, 2011).

As the current study only involved a single dose of minocycline and implicit tasks have been suggested to be more sensitive than explicit tasks (Pringle, Browning, Parsons, Cowen, & Harmer, 2013), tasks measuring implicit learning were chosen. A second reason was also because implicit learning has been linked to hippocampal function (Degonda et al., 2005), and data from our animal studies showed that minocycline ameliorated inflammation-induced effects on NMDAR signalling in the CA1 region of the hippocampus (Chapter 2). The sensitivity of these tasks was demonstrated by Klinge et al., where improvements in the repetition priming condition resulting from a single dose of methylphenidate was captured by the priming task (Klinge et al., 2018). The current study showed that a single dose of minocycline improved contextual learning, particularly in the early phase of the task. There is the possibility that participants in the minocycline treatment group were simply better at the task, however, performance between the 2 groups were similar in other cognitive tasks, and both groups also achieved similar scores on the NART.

Visual contextual cues provide important information about the environment and situation, giving meaning and influencing perception of what is observed (Marvin M. Chun, 2000). Dysfunction in contextual learning has been associated with schizophrenia and substance abuse disorders (Baez et al., 2013; Tipps, Raybuck, Buck, & Lattal, 2015). While the contextual cueing task focuses on spatial context cues, there are other types of contextual information which would be relevant to emotional processing, such as cognitive and social contexts, and it would be interesting to investigate if minocycline also improves contextual learning in those areas. Contextual learning experiments in animal models commonly involve conditioning experiments and fear extinction, and have provided evidence for hippocampal modulation of fear responses in the amygdala. These hippocampal-amygdala interactions have been implicated in anxiety, and provide another potential mechanism for minocycline's effects (Maren, Phan, & Liberzon, 2013).

NMDARs and behavioural changes

Given that minocycline has been shown to affect NMDARs and downstream signalling in Chapter 2, it is possible that glutamatergic signalling changes underlie minocycline's effects on emotional processing and implicit learning. For example, Pringle et al. also showed that a single dose of the NMDAR antagonist memantine reduced false alarm responses for negative-valence words in the EMEM (Pringle et al., 2012). NMDAR modulators, such as antagonists (ketamine), and partial agonists (GLYX13) have also been shown to have antidepressant effects (Chan et al., 2016). Furthermore, hippocampal NMDARs have been associated with anxiolytic effects in anxiety tests in rodents (Barkus et al., 2010). Finally, D-cycloserine, an NMDAR partial agonist, has been shown to both improve implicit learning in patients with Alzheimer's (Schwartz, Hashtroudi, Herting, Schwartz, & Deutsch, 1996), as well as shift healthy participants' response strategy to be slower and more accurate (Günthner et al., 2016).

Gender Effects

The current study shows that psychological effects in the ECAT were driven by changes in males. There was no group*gender interaction in the rest of the measures, indicating that the effect of minocycline was similar in both genders. It is noteworthy, however, that an exploratory pair-wise comparison revealed that there was also a stronger effect in males in the contextual cueing task. Pairwise comparisons reveal that there was a significant increase in accuracy difference scores for the males in the minocycline group (+0.047, p = 0.009). (Appendix 4.2).

Previous studies have shown that minocycline has a gender effect. Li and McCullough showed that minocycline was ineffective in reducing ischemic damage in female mice in an experimental stroke model (J. Li & McCullough, 2009). With regards to human studies, Amiri-Nikpour et al. have shown that adjunctive minocycline administration resulted in minimal clinical improvements on ischemic stroke recovery in females compared to males (Amiri-Nikpour, Nazarbaghi, Hamdi-Holasou, & Rezaei, 2015). One possibility is that minocycline effects are more subtle in females, and higher powered studies are needed to see effects. It is also possible that minocycline may be less sensitive in females, and higher dosages should be prescribed in females. This could be a result of differences in the endocrine system between genders. As participants were given the same dose of minocycline, it is also possible that the pharmacokinetics of minocycline were affected by size differences between genders. Nevertheless, this highlights a limitation in animal models, where most studies are conducted with mice of the same gender to minimize variation. This would result in the loss of information if gender effects were present.

Limitations

This study has several limitations. First, given that minocycline was not designed as a psychotropic drug, unlike antidepressants like SSRIs, psychological effects might be more subtle. A greater number of participants recruited would have resulted in greater statistical power to

detect more subtle effects, particularly after a single dose. A more high-powered study might also reveal significant effects in females as well as males.

Second, as study participants were a healthy volunteer population, the focus of this study was more of investigating the mechanisms underlying the downstream behavioural effects of minocycline. Thus, the clinical relevance of this study is limited. Ideally, a patient population should also be included for comparison to determine if a single dose of minocycline actually ameliorates the negative bias seen in patients with depression.

Third, the aims of this study was mainly mechanistic, involving the study of early effects of a single dose that may provide an explanation for how minocycline could function as a complementary drug for schizophrenia and depression. However, actual use in the clinic would probably involve chronic usage of minocycline. Hence, it might have been useful to conduct a chronic longitudinal study with the same battery of emotional processing tasks. This would provide more information on the possible link between early changes in emotional processing and downstream mood or behaviour changes.

4.5 Conclusion

In conclusion, a single dose of minocycline is sufficient to induce detectable psychological changes 2 hours after drug administration. Reductions in fear misclassification suggest that minocycline affects emotional processing in a way that implies an anxiolytic action, a similar mechanism to the decreased processing of threatening stimuli induced by anxiolytic drugs. Improvements in accuracy difference scores in the contextual cueing task also suggest that minocycline improves implicit learning. All in all, this study showed that minocycline has psychotropic properties, supporting its use as an adjuvant therapeutic for psychiatric disorders, and suggesting that the mechanism underlying its antidepressant effects could be early changes in emotional processing.

The next chapter will look at the biological effects of minocycline on inflammatory markers and metabolites. As minocycline's anti-inflammatory properties have been proposed to be key to its psychotropic properties, biological changes may provide a further clue of the mechanisms that lead to such psychological changes.

Chapter 5: The effects of an acute dose of minocycline in healthy volunteers on inflammatory markers and serum metabolites

5.1 Introduction

5.1.1 Background

Minocycline has been shown to have psychotropic effects in human studies, particularly in early-phase clinical trials for treatment-resistant depression, schizophrenia, and addiction, which used subjective mood ratings, and computer-based psychological tasks. In Chapter 4, it was shown that a single dose of minocycline resulted in changes in emotional processing and implicit learning. Given the current hypothesis that the psychotropic effects of minocycline are based on its anti-inflammatory properties, it would be expected that a single dose of minocycline will also result in changes in inflammatory markers, but what is not clear are the associated metabolic changes that are likely to result from an altered inflammatory response.

The anti-inflammatory effects of minocycline have been widely demonstrated in pre-clinical animal models and cell cultures, where decreases in pro-inflammatory cytokine levels, and inhibition of inflammation-related enzymes such as caspase-1, nitric oxide synthase (NOS), and indoleamine-2,3-dioxygenase (IDO) have been reported (M. Chen et al., 2000; O'Connor et al., 2009). However, the biochemical/biological effects of minocycline are less well studied in humans. Sofuoglu et al. have shown that a short period of daily minocycline treatment (3-5 days) resulted in decreased plasma cortisol levels in healthy volunteers, although this result is complicated by the addition of dextroamphetamine in the study design (Sofuoglu et al., 2011). Another study observed a one point drop in C reactive protein (CRP) levels after 12 weeks of minocycline administration compared to placebo, though the change was not statistically robust (Husain et al., 2017). In a study involving 6 weeks of minocycline treatment, Savitz et al. showed a positive correlation between reductions in depression scores and decreases in serum IL-6

levels, attributing the antidepressant effects of minocycline to its anti-inflammatory properties (Savitz et al., 2018). In both studies, higher levels of inflammatory markers at baseline were correlated with increased efficacy of minocycline as a therapeutic, measured by larger decreases in established depression ratings.

Earlier research has suggested close interactions between inflammation and the metabolome (Fitzpatrick & Young, 2013; Jurynczyk et al., 2017). Metabolomics enables the understanding of systemic changes underlying a particular phenotype, and biofluid metabolomics in humans have been robustly shown to build predictive models separating patients with neurological or psychiatric diseases from healthy populations (Kawamura et al., 2018). This highlights the hypothesis-generating utility of metabolomics; disease-specific metabolic profiles can act as diagnostic and prognostic biomarkers.

However, apart from identifying potential biomarkers from predictive disease models, metabolomics can also be helpful in furthering our understanding of how therapeutics work. The biochemical actions of metabolites have important functions such as acting as co-substrates, regulating post-translational modifications, acting as messengers for signal initiation and transduction, being involved in the transport of proteins, and indirectly influencing their environment through homeostatic regulation (Johnson et al., 2016). Thus, the metabolic differences resulting from drug administration could reveal pathways that are important for the mechanism of the drug. In particular, untargeted metabolomics is an unbiased tool for identifying metabolite changes without *a priori* assumptions that may lead to novel and unexpected discoveries. In the current study, proton NMR (^{1}H NMR) was used to provide an unbiased approach, enabling the measurement of a broad range of metabolites simultaneously, with a single measurement.

5.1.2 Aims and hypotheses

The aim of this study was to explore the biochemical effects of a single dose of minocycline on plasma inflammatory markers and serum metabolites in a healthy population.

As highlighted in Chapter 4, healthy volunteers were studied so that the effects of minocycline alone were not confounded by the complications from any psychiatric disorders or ongoing/prior psychotropic medication.

To our knowledge, this is the first study that has investigated the anti-inflammatory effects of a single dose of minocycline on plasma inflammatory markers in humans. Given the findings of previous studies (O'Connor et al., 2009; Tikka, Fiebich, Goldsteins, Keinanen, & Koistinaho, 2001), it was hypothesized that minocycline administration would reduce inflammatory markers (CRP, inflammatory cytokines) compared to placebo. However, from the previous studies on mice (Chapter 2), we hypothesized that minocycline and placebo administration would have similar effects on plasma cortisol levels.

The current study is also the first to investigate the effects of a single dose of minocycline on serum metabolites. An untargeted hypothesis-generating metabolomics analysis to identify global metabolite differences between the two treatment groups was applied. In addition, based on the mice studies on acute minocycline administration (Chapter 3), a targeted metabolomics analysis looking specifically at the metabolites that changed in the mouse model was also applied. In view of the rodent data, it was hypothesized that a single dose of minocycline would be sufficient to observe detectable changes in serum metabolites, in particular lipoproteins, creatine, taurine, and glucose.

5.2 Methods

5.2.1 Participants and Recruitment

See Section 4.2.1

5.2.2 Blood Collection and Processing

Blood was collected from participants twice - once in the morning before minocycline/placebo administration (time-point 1), and once at the end of the study (time-point 2). Each time, approximately 4 mL of serum were collected in serum vacutainer tubes, and 6 mL of plasma were collected in lithium-heparin-containing vacutainer tubes. The tubes were inverted eight times, and stored upright at room temperature for 20 min (plasma tubes) and 30 min (serum tubes). Tubes were centrifuged at 1300 xg for 10 min. Plasma and serum samples were then aliquoted into labelled 2 mL Eppendorf tubes® and stored upright in a -80 °C freezer.

5.2.3 Cortisol and inflammatory markers Measurements

Plasma cortisol and inflammatory markers were measured with enzyme-linked immunosorbent assays (ELISAs) for human cortisol, C-reactive protein (CRP), Interleukin-6 (IL-6), Interleukin-1β (IL-1β), and Tumour Necrosis Factor α (TNFα) according to manufacturer's instructions. ELISA kits for cortisol, CRP, IL-6 and IL-1β were bought from Abcam, and the ELISA kit for TNFα was bought from RnD systems. Samples were diluted 1:5000 for CRP, and used neat for the rest of the measurements.

5.2.4 ^{1}H-NMR

Serum samples were defrosted on ice and centrifuged at 17,000 xg for 5 min at 4 °C. An equal volume (150 µL) of serum were aliquoted into a fresh tube and diluted to 600 µL with 74 mM sodium phosphate buffer D_2O (pH 7.4) (see section 3.2.5). Volume of serum used was

determined as previously published (Jurynczyk et al., 2017). All NMR spectra were acquired using a 700-MHz Bruker AVII spectrometer operating at 16.4 T equipped with a ^{1}H ($^{13}C/^{15}N$) TCI cryoprobe. Sample temperature was stable at 310 K. ^{1}H NMR spectra were acquired using a 1D NOESY presaturation scheme for attenuation of the water resonance with a 2 s presaturation. A spin-echo Carr-Purcell-Meiboom-Gill (CPMG) sequence with a τ interval of 400 µs, 80 loops, 32 data collections, an acquisition time of 1.5 s, a relaxation delay of 2 s, and a fixed receiver gain was used to suppress broad signals arising from large molecular weight plasma components. CPMG spectra provide a measurement of small molecular weight metabolites and mobile side chains of lipoproteins in the plasma sample and were used for all further analysis. 750 µL of serum from consenting participants (N = 19 x 2 time-points) was also sent to numares AG (Regensburg, Germany) for further analysis of the lipoprotein populations and composition using the AXINON® lipoFIT® analysis platform.

The NMR spectrometer was operated by a research collaborator, Dr Fay Probert, from the department of Pharmacology.

5.2.5 Processing and analysis

ELISAs

Statistical Analysis: Raw values were divided by values of time-point 1 to compute the change between time-points, where time-point 1 values were expressed as 1, and time-point 2 values were expressed as a ratio of time-point 1. This was done to normalize the baseline inter-individual variation between participants. The adjusted values were then analysed with split plot analyses of variance where group (minocycline or placebo) was the between-subjects factor, and time was the within-subject factor. Equality of variance was tested with Mauchly's test of sphericity, and when this assumption was not met, the Greenhouse-Geisser corrected value was reported instead. All analyses were done using SPSS and Graphpad Prism.

NMR

Processing: Processing methods were adapted from published parameters (Jurynczyk et al., 2017). NMR spectra were imported into MestreNova (Mestrelab Research, Spain) and each spectrum was then processed manually with phase 0 (PH0) correction, baseline correction (Bernstein polynomial fit, order = 3), and referencing to an internal standard (Lactate referenced to δ1.33) similar to section 3.2.8. The individual spectrums were stacked, and binned (sum method, width of each integral region = 0.02 ppm). Binning refers to a function where the whole spectrum is divided into bins of equal width, and all the peaks in each bin is integrated to obtained a value representing the area of all the peaks in a bin. Binned values were then exported as a spreadsheet (.xlsx) for further analysis. The water peak and noise areas were also removed (see section 3.2.8), with a final number of 175 bins included for further analysis.
As there were two time-points for each participant, the percentage change between time-points for each bin was calculated by taking the ratio of time-point 2 divided by time-point 1 and multiplying the ratio by 100.

Preliminary exploratory analysis: The original bin values and ratios were then imported into SIMCA (Umetrics, Sweden). Principal component analysis (PCA) scores plot were used to identify outliers as well as inter-individual variation. Pareto scaling was used for original values, whereas UV scaling was used for the percentage ratios. Supervised multi-variate analysis was conducted with orthogonal partial least squares discriminant analysis (OPLS-DA), where samples were separated by treatment.

Model building and validation: OPLS-DA models were built in R 3.3.2 (R Core Team, 2016) using the ROPLS package (Thévenot et al., 2015) and a 10-fold cross validation method (see section

3.2.8). A total of 100 iterations was conducted and mean values of accuracy, specificity, sensitivity, Q^2, R^2X, and R^2Y of the models were calculated and presented as boxplots.

Validation of the models was similar to that of section 3.2.8 where the genuine OPLS-DA models were compared with models generated by randomly permuting the class assignments to see if the genuine models performed significantly better (determined by the accuracy, specificity, and sensitivity). For significant, predictive models, the variable importance in projection (VIP) scores were calculated to identify the key bins that were important for the discrimination between classes. Key bins were identified by sorting all bins in descending order of VIP score and plotting a graph of bins (x axis) against VIP scores (y axis). Bins which result in the largest decrease in accuracy when removed from the model (steepest gradient in the VIP plot) are the most important discriminatory variables, hence the inflection point in the graph (the point at which the graph first plateaus) is taken as the threshold VIP value; all bins with VIP scores above this value are considered significant for driving the discriminatory model.

Metabolite identification and direction of change: Metabolites were assigned to peaks in bins with high VIP scores through a combination of literature values (Misra & Bajpai, 2009), reference to the human metabolome database (HMDB) (Wishart et al., 2013), and confirmed with two-dimensional (2D) correlation spectroscopy (COSY). The direction of change between groups was also determined by comparing means in SPSS (independent samples T-test). When assumptions for parametric testing were not met, the non-parametric test Mann-Whitney was used instead.

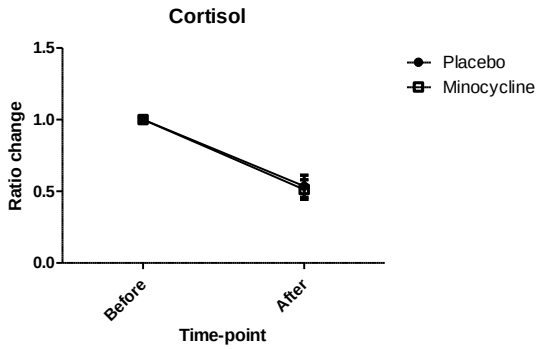
5.3 Results

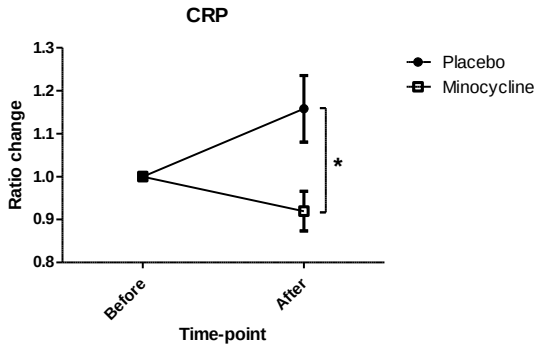
5.3.1 Cortisol and inflammatory markers measurements

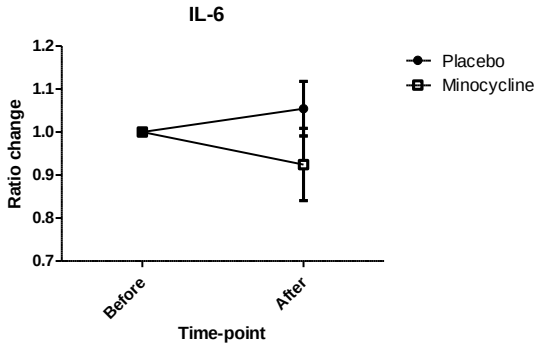
Minocycline has been shown to be anti-inflammatory in animal models, and it is this property that is proposed to drive its psychotropic effects. Therefore, the anti-inflammatory effect of a single dose of minocycline was determined by measuring plasma inflammatory markers before and after drug administration. This includes CRP, IL-6, IL-1β, and TNFα. Cortisol levels were also measured as the glucocorticoid system has also been implicated in psychiatric diseases, and has been shown to have interaction effects with the immune system.

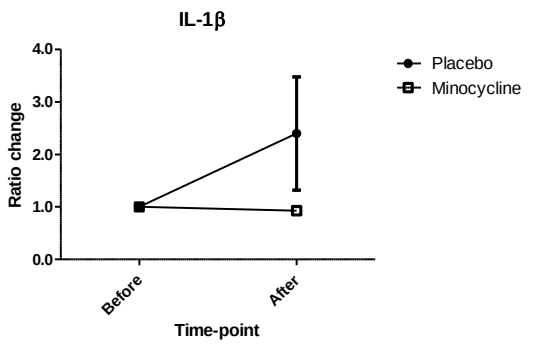
Quantitative measurements with ELISAs revealed that there was significant time-point*group interaction for CRP ($F_{1,35}$ = 6.788, p = 0.014). CRP levels were significantly lower in the minocycline group compared to the placebo group (Fig 5.3.1) when taken as a ratio of time-point 2 divided by time-point 1 (-23.8%, p = 0.014). No significant difference between treatment groups was found for the inflammatory cytokines. However, that was not unexpected as our participants are healthy volunteers and baseline levels of inflammatory cytokines are low to undetectable even with high sensitivity kits. For example, IL-1β levels were detectable in only 35% of participants.

Cortisol levels showed a decrease at the second measurement (Fig 5.3.1), which is in line with literature, as wakening cortisol levels are at its highest. However, there was no significant difference in cortisol levels between treatment groups, indicating that cortisol levels do not play a role in the psychotropic effects of minocycline.









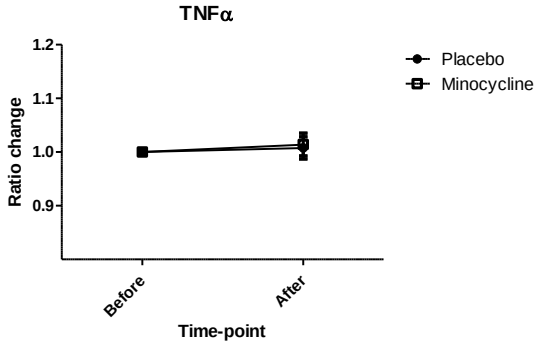


Fig 5.3.1 Plasma cortisol and inflammatory cytokines levels before and after placebo/minocycline administration expressed as a ratio of before. *$p < 0.05$

5.3.2 Serum metabolomics

5.3.2.1 Variance of the data

Compared to animal models, human metabolomics data show greater inter-individual variation due to environmental influences such as body mass index (BMI) and diet. Therefore, the effect of minocycline on serum metabolites was determined by measuring metabolite levels before and after minocycline/placebo administration, where each individual acts as their own control. Similar to the mice metabolomics studies, inter-treatment group variability was checked for through a combination of principal component analysis (PCA) scores plots and spectral relative standard deviation (RSD) values (Parsons et al., 2009).

To minimise spectrometer variation, all serum samples from both time-points were ran in a single session. Technical controls (TC), aliquots of the same sample, were ran at intervals through the session, and PCA scores show tight clustering of the TCs, indicating low spectrometer variability across time (Fig 5.3.2A). This was supported by spectrum-wide RSD values of the TCs (Table 5.3.1), where the TC median RSD value was lowest at 6.35%.

As participants were fasted between time-points, it was expected that there would be large differences between time-points, and samples would cluster by time-points. However, PCA scores of all samples from both time-points show that inter-individual differences were greater than that of time-point differences (Fig 5.3.2A). This was supported by median RSD values, where samples from both time-points, and time-point 2 alone had the highest values of 21.06% and 20.52% respectively. Therefore, the percentage ratio of time-point 2 over time-point 1 was used for further analysis. The percentage ratio PCA scores plot (Fig 5.3.2B) showed some natural clustering between treatment groups, and lower variation compared to all samples (Table 5.3.1). The PCA scores plot also showed some outliers that were removed before model building.

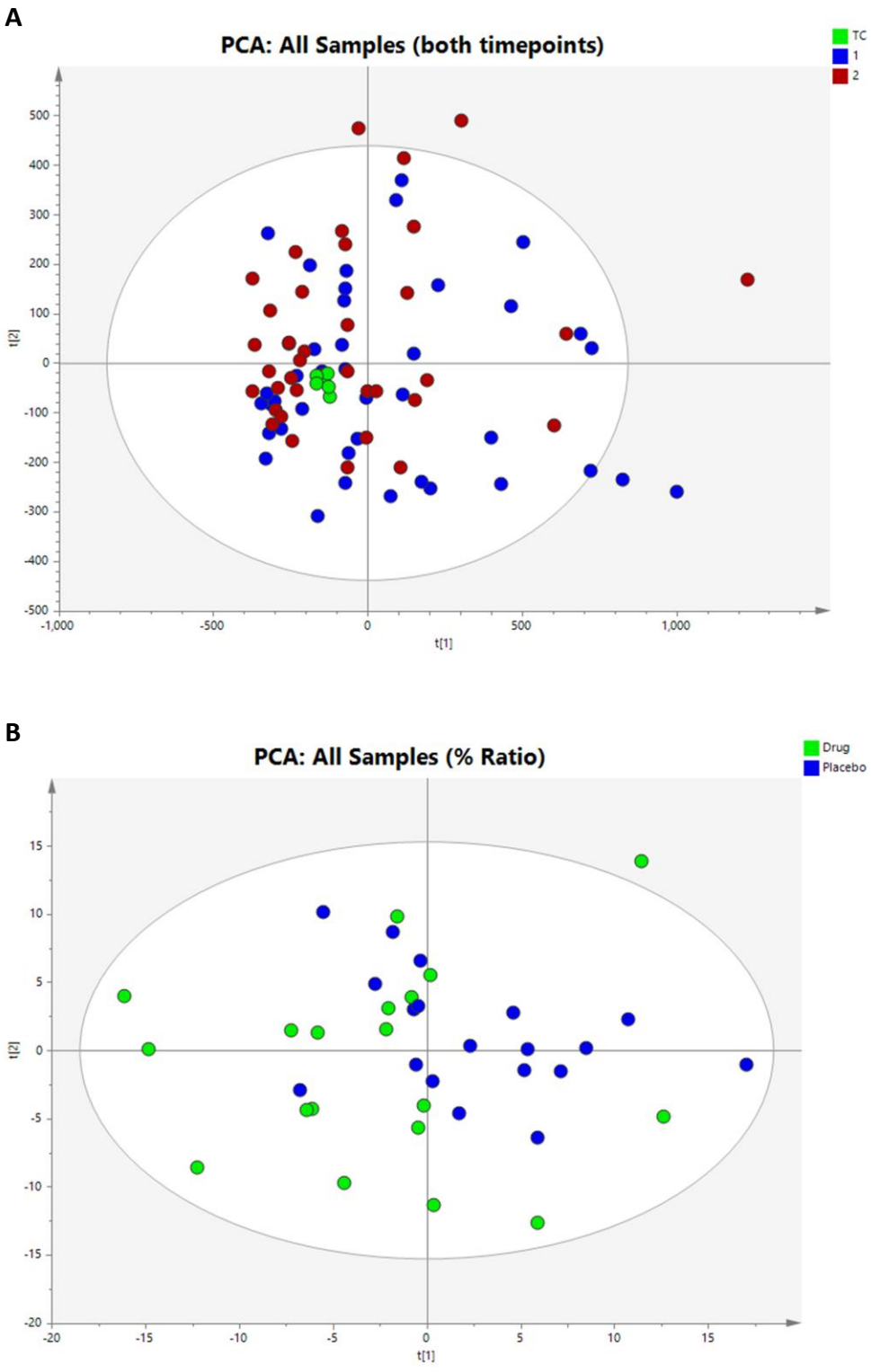


Fig 5.3.2 PCA scores plot of (A) all samples including technical controls (TC) coloured by time-point (before drug administration 1; after drug administration 2) [No.of components = 5; Q^2(cum) = 0.861; R^2X(cum) = 0.907], and (B) all experimental samples (without TCs) expressed as the percentage ratio of time-point 2/time-point 1 coloured by treatment [No.of components = 7; Q^2(cum) = 0.521; R^2X(cum) = 0.766].

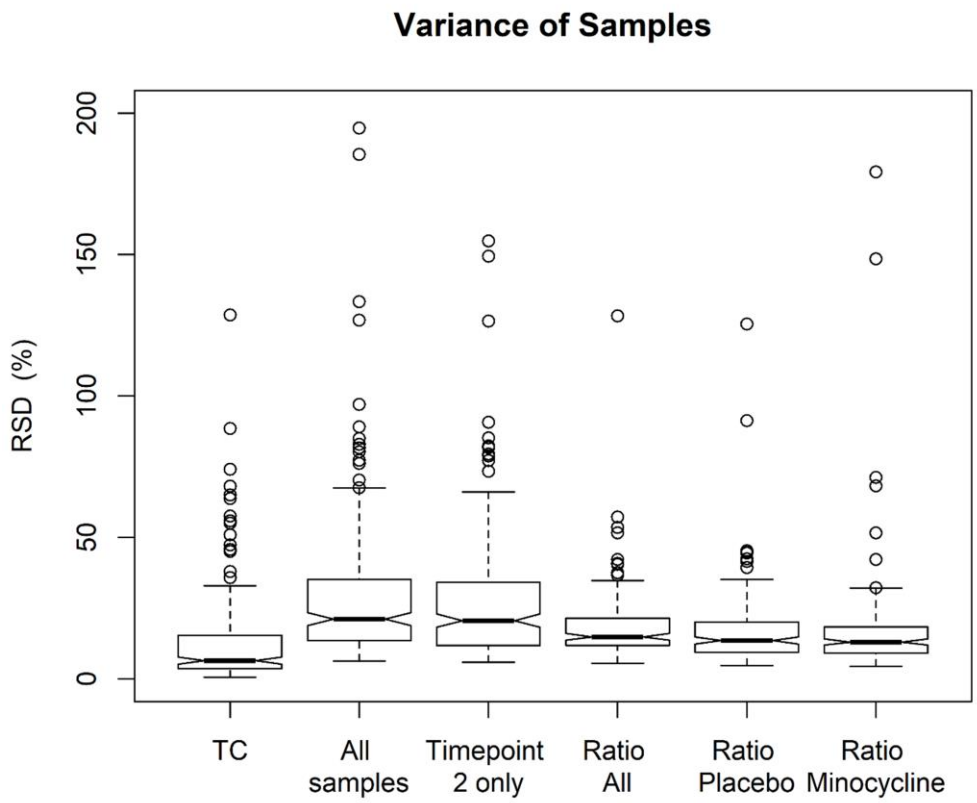


Fig 5.3.3 Spectrum wide RSD values of different datasets limited to 200% RSD.

Table 5.3.1 Summary of spectrum wide RSD values of different datasets

	Technical Controls (TC)	Area of Peaks (Bins)		Ratio (Time-point 2/Time-point 1)		
		All Samples	Time-point 2 alone	All Samples	Placebo group	Drug group
Min	0.5863	6.326	5.813	5.496	4.717	4.343
1st Quartile	3.4782	13.474	11.804	11.754	9.379	9.001
Median	6.3536	21.057	20.517	14.818	13.473	12.979
Mean	14.0552	28.89	28.678	25.715	18.038	54.156
3rd Quartile	15.3345	35.025	34.119	21.438	20.022	18.275
Max	365.2692	194.678	382.887	1140.73	254.686	6577.363

5.3.2.2 The effect of minocycline on serum metabolites

Untargeted OPLS-DA analysis

Untargeted orthogonal partial least squares discriminant analysis (OPLS-DA) analysis built a significant model between the serum metabolites of minocycline-treated and placebo-treated groups with clear separation of the two groups (Fig 5.3.4). This ensemble of models was significantly more accurate (+23.2%, $p < 0.001$) at predicting which group a sample belonged to compared to the randomly permuted model ensemble (Fig 5.3.5). The Q^2 (0.2107 compared to -0.3944, $p < 0.001$), sensitivity (+26.2%, $p < 0.001$), and specificity (+22.3%, $p < 0.001$) of the models were also significantly better than that of randomly permutated models (Appendix 5.1). This suggests that a single dose of minocycline resulted in identifiable changes in serum metabolites compared to control.

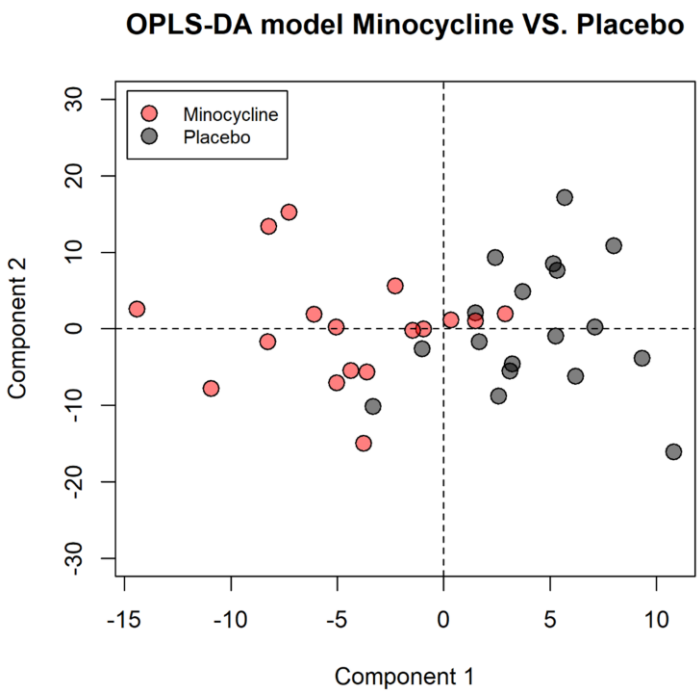


Fig 5.3.4 Untargeted representative OPLS-DA model of serum metabolites based on treatment (Minocycline/Placebo). [Predictive component = 1; Orthogonal component = 1; $Q^2Y = 0.248$; $R^2X = 0.417$; $R^2Y = 0.561$]

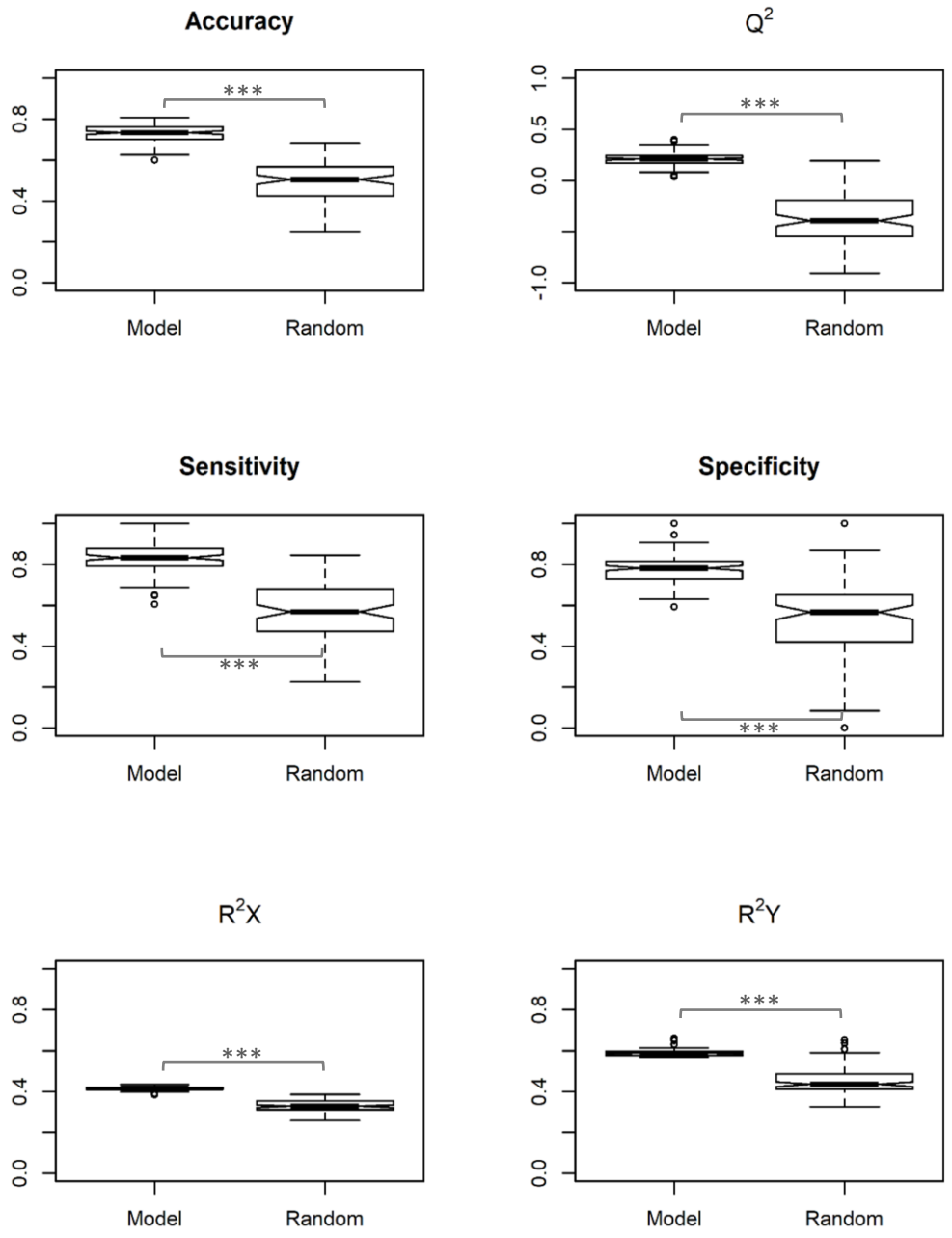


Fig 5.3.5 Comparisons between the Minocycline/Placebo models (Model) and randomly permutated models (Random). On all counts (Accuracy, Q^2, Sensitivity, Specificity, R^2X, and R^2Y), the Minocycline/Placebo models were significantly better. ***$p < 0.001$

Key metabolite changes

The key components contributing to the models were identified based on their VIP scores and summarized in Table 5.3.2. Minocycline treatment resulted in a reduction in decreased levels of lipoproteins (Fig 5.3.6 and 5.3.7) compared to the placebo group in the untargeted analyses.

Targeted analysis with variables from mice acute dose study

In chapter 3, an acute dose of minocycline for two hours resulted in decreased levels of lipoproteins and increased levels of creatine, taurine, and glucose (Table 3.3.2) in mice. Therefore, a second analysis was conducted targeting these four metabolites. A univariate analysis was conducted with the bins associated for each metabolite with the results summarized in Table 5.3.2. Unlike the mice acute dose study, there was no significant difference in the levels of creatine, taurine, and glucose between treatment groups. However, lipoprotein levels were significantly different between treatment groups, although the direction of change was reversed compared to the mice acute dose study.

Table 5.3.2 Serum metabolites important for building the Minocycline/Placebo model and their direction of change. Significance determined by independent samples T-test; ***p < 0.001

Metabolite	Minocycline vs Placebo
$-CH_3$ mobile lipoprotein	↑***
$(-CH_2-)_n$ mobile lipoprotein	↑***
-CH mobile lipoprotein	↑***
Creatine	N.S.
Glucose	N.S.
Taurine	N.S.

Comparisons made based on bin integrals

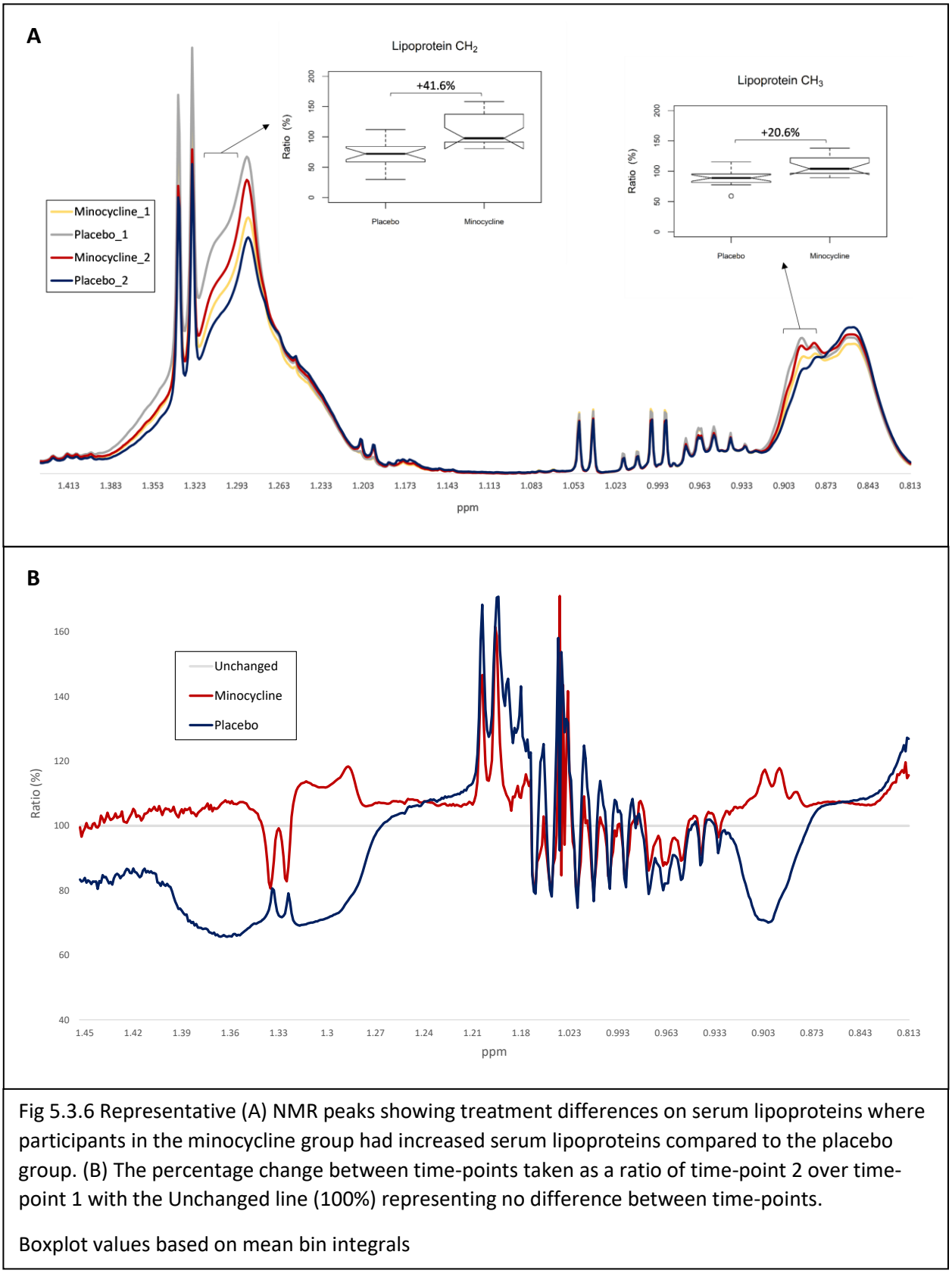


Fig 5.3.6 Representative (A) NMR peaks showing treatment differences on serum lipoproteins where participants in the minocycline group had increased serum lipoproteins compared to the placebo group. (B) The percentage change between time-points taken as a ratio of time-point 2 over time-point 1 with the Unchanged line (100%) representing no difference between time-points.

Boxplot values based on mean bin integrals

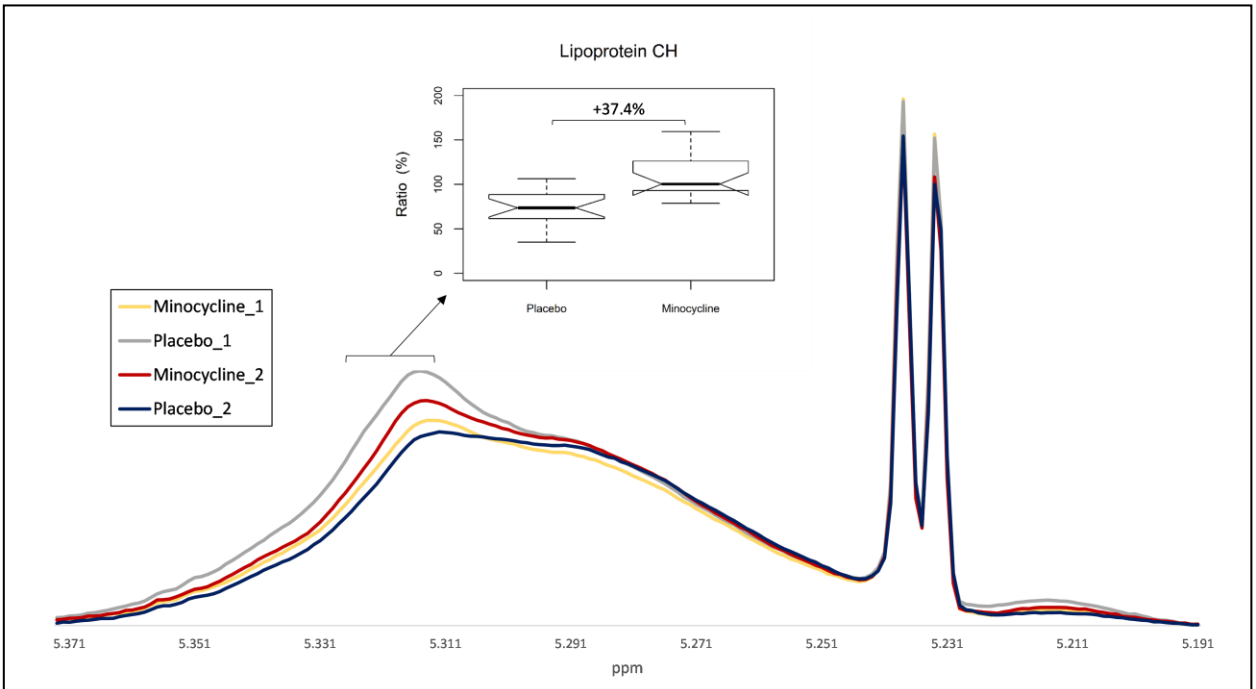


Fig 5.3.7 Representative NMR peaks showing treatment differences on serum lipoproteins where participants in the minocycline group had increased serum lipoproteins compared to the placebo group; Boxplot values based on mean bin integrals

Total Sum Differences

The detectable serum metabolite differences between the minocycline and placebo treatment groups is further supported by differences in the sum area of the spectra before (time-point 1) and after (time-point 2) minocycline or placebo administration. The percentage difference between the time-points was calculated by subtracting the sum of the area of the peaks at time-point 1 from the sum of the area of the peaks at time-point 2 and dividing the difference by the sum of time-point 1. There was a significant difference in the percentage difference of the sum area of the spectra between treatment groups with the placebo group having a decrease in sum area compared to the placebo group (Placebo: -9.186%; Minocycline: +0.634%, $p < 0.01$).

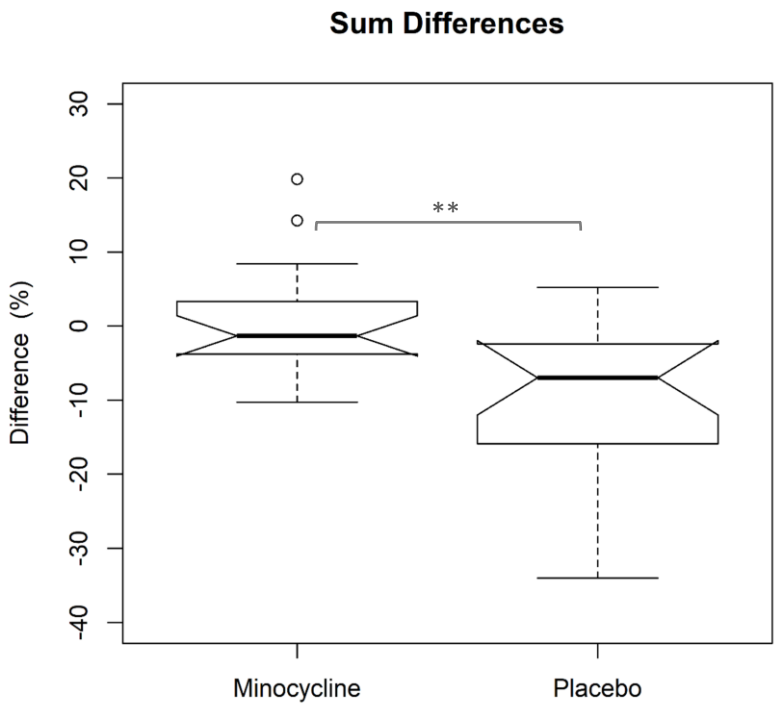


Fig 5.3.8 Percentage difference of the sum of the spectra separated by treatment groups, calculated by subtracting the sum of the area of the peaks at time-point 1 from the sum of the area of the peaks at time-point 2 and dividing the difference by the sum of time-point 1. **p < 0.01

5.3.2.3 Lipoprotein population and composition

The expanded analysis by numares AG revealed more detailed results on lipoprotein populations and compositions. They measured concentrations and diameters of the different classes of cholesterol particles (VLDL, LDL, and HDL), as well as the cholesterol concentration in each class. Further information was revealed by measuring the particle and cholesterol concentration of sub-classes, where each class is further divided into small and large particles. Finally total serum levels of cholesterol and triglycerides were also measured. The full results obtained are summarised in Appendix 5.2.

Minocycline administration resulted in increased concentration of large VLDL (L-VLDL) particles, diameter of VLDL particles, cholesterol concentration of VLDL particles, cholesterol concentration in small particles of LDL, and concentration of triglycerides in serum compared to placebo (Table 5.3.2). When significance was adjusted for multiple testing (Bonferroni), there was a significant increase in the cholesterol concentration in small particles of LDL, and a trending increase in the mean diameter of VLDL particles in the minocycline group compared to placebo.

Table 5.3.3 Summary of significant lipoprotein differences between minocycline and placebo expressed as the % ratio of time-point 2 over time-point 1

Metabolite	Minocycline (N = 11)		Placebo (N = 7)		Sig	Adj Sig
	Mean	SD	Mean	SD		
LVLDL-p	137.77	45.75	60.37	52.58	0.0216	0.5391
VLDL-s	104.01	3.75	95.39	6.39	0.0031	0.0781
VLDL-c	104.32	10.66	90.76	14.61	0.0422	1.0555
LDL.C-c	118.75	17.41	80.45	12.67	0.0002	0.0043
Triglycerides	103.62	25.71	63.47	21.84	0.0043	0.1072

LVLDL-p: Concentration of large VLDL particles; VLDL-s: Mean diameter of VLDL particles; VLDL-c: Cholesterol concentration in VLDL class; LDL.C-c: Cholesterol concentration in LDL subclass small particles; Sig: Significance (p value); Adj Sig: Significance adjusted for multiple testing with Bonferroni

5.4 Discussion

Summary of results

A single dose of minocycline resulted in several detectable biochemical changes in serum compared to placebo approximately four hours after drug administration. Firstly, the anti-inflammatory effect of minocycline was shown by the reduction in CRP levels in the minocycline group compared to the placebo group. Secondly, percentage changes between time-points in lipoprotein peaks in ^{1}H NMR spectra suggest that minocycline has an effect on lipid metabolism in humans.

Inflammatory Markers

Most studies of minocycline in humans with neuropsychiatric disorders have been early phase clinical trials involving minocycline as an adjunctive treatment for several months. Thus, the biochemical effects of a single dose of minocycline has not yet been shown. In this study, a single dose of minocycline had anti-inflammatory effects on CRP levels. While the levels of inflammatory cytokines (IL1β, IL6 and TNFα) were not significantly different between treatment groups, this was not unexpected as our participants are healthy volunteers with low levels of the measured cytokines. However, given that inflammation has been shown to contribute to the pathophysiology of psychiatric diseases, and that patients with depression have been shown to have increased serum and CSF levels of inflammatory cytokines, the anti-inflammatory effect of minocycline on cytokines might be more prevalent in those populations.

Cortisol was also measured as stress and the inflammation response are linked, and therapeutics such as antidepressants have been shown to reduce levels of cortisol. This has also been demonstrated in mouse inflammation models where corticosterone, the main glucocorticoid hormone in rodents, is increased with the administration of the inflammatory agent lipopolysaccharide (Hasko, Elenkov, Kvetan, & Vizi, 1995). However, the present measurements show that a single dose of minocycline does not affect cortisol levels. This is

supported by the mouse studies in section 2.3.1, where minocycline did not significantly ameliorate the increase in corticosterone resulting from LPS administration. These data, therefore, suggest that unlike other antidepressants (Manthey et al., 2011), the glucocorticoid system may not be involved in the psychotropic effect of minocycline.

Serum Metabolomics

Mobile fatty acid side-chains of lipoproteins are detected in the ^{1}H CPMG NMR spectra as broad peaks with different sections of the spectra representing hydrogen nuclei from the -CH_3, -CH_2- and CH moieties in fatty acids. As such, it is difficult to specifically identify which lipoprotein species has changed. The expanded analysis by numares AG revealed that minocycline administration mainly affected VLDL and LDL particles, an unexpected result given that it is HDL that is most often linked to anti-inflammatory effects. However, APP have been shown to interact with lipoproteins (Chiba et al., 2011). While SAA was not measured in this study, CRP levels were observed to have changed as a result of minocycline administration. CRP has been linked to LDL, and has been shown to bind to oxidized LDL (Chang, Binder, Torzewski, & Witztum, 2002). Studies on the interactions between CRP and LDL are generally related to atherosclerosis, however, and thus are limited to a context where both have increased levels. Therefore, it is unclear what effect decreased CRP levels would have on LDL particle and cholesterol concentration, and whether the metabolite changes observed are linked to the anti-inflammatory effects of minocycline.

Mouse versus Human metabolome comparison

In Chapter 2, a similar acute study was conducted in mice, where mice were treated with minocycline or saline for two hours. In that study, minocycline treatment resulted in decreased levels of lipoproteins, namely the -CH_3 and -CH_2- moieties, and increased levels of creatine, taurine, and glucose. While lipoprotein levels were changed as a result of minocycline

administration in both humans and mice, the direction of change was opposite between the two species. From a comparison between mouse and human plasma, Cruickshank-Quinn et al. showed that 57.3% of plasma metabolites in mice were also detected in humans. Using a mass spectrometer, they detected 2478 compounds common in both species, of which 2208 were lipid species, and 270 were aqueous-phase metabolites (Cruickshank-Quinn et al., 2017). Given the wide range of lipid species possibly present in the broad lipid peaks detected by the NMR, it is possible that different lipid species were driving the peaks altered in the mice and human models. That said, it is also important to note that the main classes of lipids associated with psychiatric diseases (ceramides, sphingolipids and phospholipids) were still common between the two species.

Another explanation could be the protocol differences between the two experiments. For the human study, two time-points (before and after drug administration) were measured, and the decrease in lipoproteins in the placebo group was a result of the participants being fasted in between time-points. For the mouse study, only one time-point (after drug administration) was measured, and mice were not fasted at all. Thus, the differences observed could be a result of minocycline having different effects on lipid metabolic pathways in differing environments (fasting versus *ad libitum* access to food). A second difference in protocol is that mice were only exposed to minocycline for two hours before sample collection, whereas the second time-point of blood collection would have been around four hours after minocycline administration for human participants. Therefore, this difference could also be a result of a time-course change in the actions of minocycline.

Finally, given that the mouse study only involved male mice, a univariate analysis with group as the between-subjects variable was conducted where the important variable bins were split by gender. However, the direction of change of human serum metabolite levels as a result of minocycline administration was the same in both genders (Appendix 5.3).

Minocycline and lipids: Theories

Given the associations between lipids and psychiatric diseases, it is possible that minocycline's effects on lipid metabolism underlies its psychotropic effects. Certain lipid species and the enzymes that modulate their levels have been implicated in the pathology of depression, in particular ceramide levels resulting from acidic sphingomyelinase (ASM) activity (Gulbins et al., 2013). This is supported by studies showing that some antidepressants may, in part, achieve their effects by acting on sphingolipids and phospholipids (Lee et al., 2009). Lee et al. have shown that four weeks of treatment of antidepressants, such as Maprotiline and Paroxetine, resulted in decreased levels of phosphatidylcholine, increased levels of lysophosphatidylcholine, decreased levels of sphingomyelins, and increased levels of ceramides in the mouse frontal cortex. Therefore, it is possible that minocycline might act as a modulator of enzymes involved in lipid metabolism.

A second possibility is that minocycline may be involved in modulating energy metabolism, especially if minocycline activity is affected by fasting. Results from animal studies show that short-term fasting affects the expression of enzymes involved in lipid metabolism. For example, Saneyasu et al. have shown that four hours after fasting, adipose triglyceride lipase mRNA levels were significantly increased, while that of lipoprotein lipase were significantly decreased in white adipose tissue (Saneyasu, Shiragaki, Nakanishi, Kamisoyama, & Honda, 2013). However, it is important to note that while diet is controlled in animal studies, we are unable to control what participants eat before the start of the study, and there is a possibility that changes in metabolite levels could be a result of variations in diet. Nevertheless, this was controlled for as much as possible by starting the study at the same time for each participant, as far as possible, and instructing participants to eat breakfast at least an hour before the start of the study. Thus, the second time-point of blood collection will at least be five hours post-prandial for all participants. Furthermore, given the clear differences in multiple lipid peaks between treatment groups, it is unlikely that the differences observed are a result of diet

differences. Therefore, it is possible that minocycline modulates the activity of lipid-metabolizing enzymes that increase the breakdown and release of certain lipid species for energy as a result of fasting.

Limitations of study and future work

There are some limitations to the present study. First, while the NMR is a robust, quick, and inexpensive, it is not as sensitive as the mass spectrometer, especially when detecting lipids. Lipids appear on the ^{1}H NMR spectrum as broad peaks, and it is difficult to decipher which lipid species exactly is affected by treatment. Given that lipids are suggested to play an important role in the mechanisms of minocycline, it may be useful to conduct a lipidomic analysis with a mass spectrometer to identify the specific class of lipids involved. This may also help to explain the different results obtained for the mice and human studies as it is possible that different species of lipids were changed in both studies.

Second, we have identified that lipoproteins are affected by the administration of minocycline, however we have yet to determine the biological relevance of this result. Thus, it is important to determine the specific pathways that are perturbed that leads to this increase/lack of decrease of lipoproteins in human serum. Ideally, it would also be informative to integrate this information with data from other systems, such as the genome or the proteome. For example, if a specific lipid species is elevated, genomic analysis may show reduced copies of an enzyme involved in breaking it down.

Third, given the design of the study, it would not have been feasible to collect blood from participants more than twice. Therefore, we only have metabolomics data from one time-point (four hours post minocycline treatment). It is possible that we may have missed other metabolite changes more prevalent at different time-points (for example, two hours post treatment, or 24 hours post treatment). Thus, this metabolomics study could be expanded by exploring serum metabolites at more time-points post-treatment.

5.5 Conclusion

In conclusion, a single dose of minocycline was sufficient to cause detectable biochemical changes approximately four hours after drug administration. Reductions in CRP levels compared to the placebo group show the anti-inflammatory effects of minocycline, and suggest that the anti-inflammatory effects of minocycline could underlie its psychotropic properties (Chapter 4). Increases in lipoprotein levels in the minocycline group compared to the placebo group suggest that lipid metabolism could be involved in the mechanism of minocycline. This is in contrast to what was previously seen in the preceding mouse study, which highlights the importance of conducting human studies – given that not all animal work is translational. Nevertheless, it is interesting that the same class of metabolites (lipids) was affected in both mice and humans. This begs the question of whether lipid perturbation could underlie the psychotropic effects of minocycline, and whether this is linked or separate from its anti-inflammatory properties.

Chapter 6: General discussion and conclusion

6.1 Summary of results

Systemically-administered minocycline had peripheral and central effects in both animals and humans.

For CD1 mice, LPS increased peripheral and central inflammatory markers, and decreased hippocampal GluN2 subunit expression, leading to decreased downstream Arc expression. Prior treatment with minocycline was able to prevent the LPS-induced changes in some markers, and in GluN2 and Arc expression. LPS administration also altered plasma metabolites related to nutrient utilization (lipoproteins, glucose, isoleucine, creatine), and brain metabolites linked to neurotransmitters, such as glutamine, glutamate and serine. Repeated administration of minocycline ameliorated the LPS-induced increase in plasma isoleucine only, whereas, an acute dose of minocycline also had effects on plasma metabolites linked to nutrient utilization, such as lipoproteins.

In humans, an acute dose of minocycline resulted in psychological changes in emotional processing (decreased fear misclassification, increased accuracy of sadness and surprise), cognitive processing (contextual learning), and biochemical measures such as reduced CRP levels, and increased serum lipoproteins.

6.2 LPS and the anti-inflammatory effects of minocycline

It was hypothesized that the anti-inflammatory effects of minocycline underlie its psychotropic properties in psychiatric disorders. Therefore, the ability of minocycline to ameliorate inflammation-induced changes was investigated in an established mouse LPS model. Previous studies have shown that LPS administration induced depressive-like behaviour, such as increased immobility in the FST and TST (Dunn & Swiergiel, 2005). O'Connor et al. have also

shown that prior treatment of minocycline prevented the LPS-induced behaviour changes (O'Connor et al., 2009).

This dissertation first validated the effects of the LPS dosing regimen (i.p. injection, non-sepsis-inducing <1mg/kg dose, samples collected 24 hours post-dose). The results obtained (Fig 6.1) are in-line with previous studies showing induced APR and increased brain pro-inflammatory cytokine expression that led to depressive-like behaviour (i.e. increased immobility in the FST and TST) (Frenois et al., 2007; O'Connor et al., 2009). The next step was then to evaluate the anti-inflammatory effects of minocycline. Finally, LPS-induced molecular and metabolite changes, and the influence of minocycline on those effects were investigated to identify a possible anti-inflammatory mechanism that might explain minocycline's antidepressant effects.

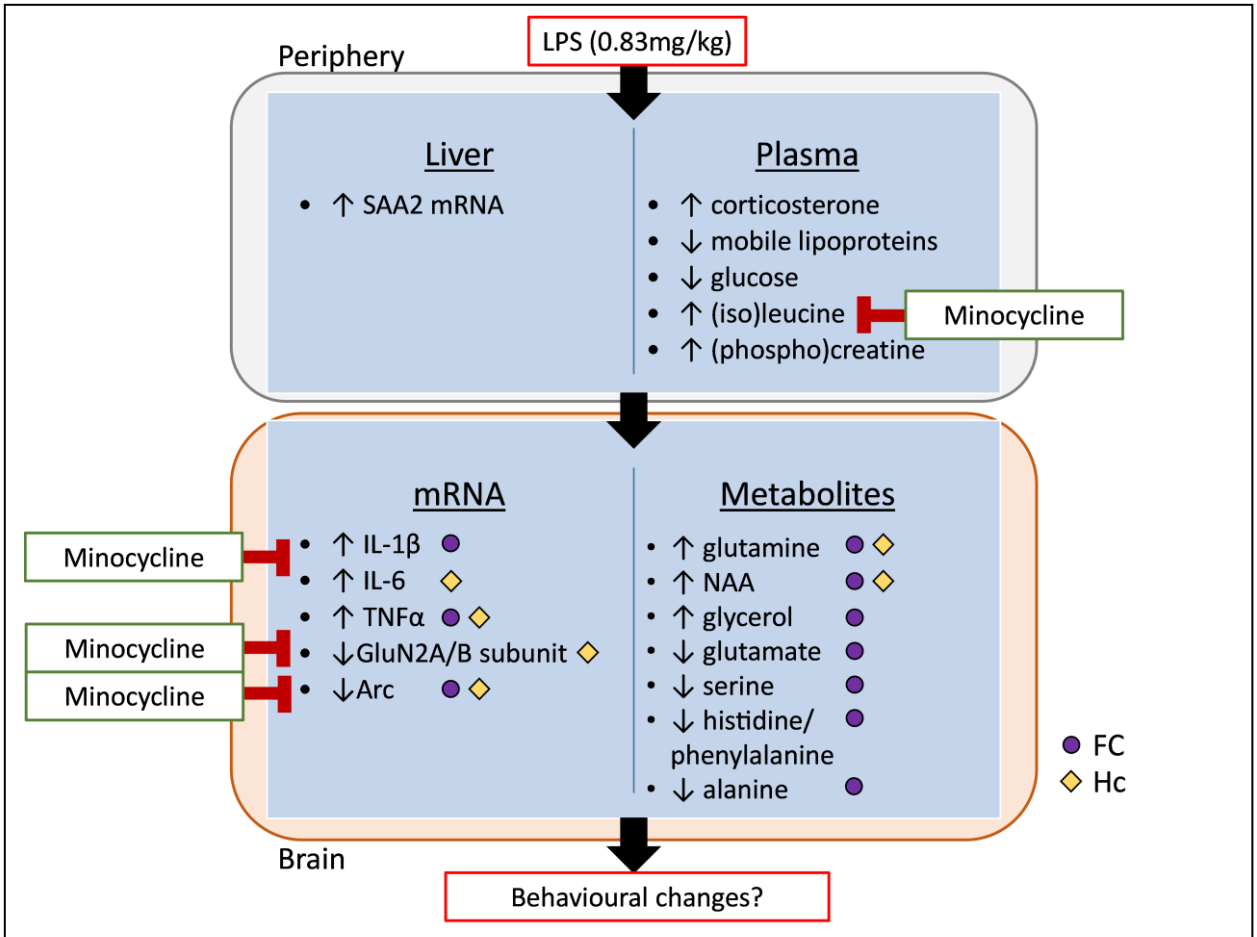


Fig 6.1 Summary of effects of LPS dosing regimen in a CD1 mouse inflammation model. Effects that were ameliorated by prior treatment with minocycline are highlighted. FC expression of GluN2A/B was not investigated.

Anti-inflammatory effects of minocycline

The anti-inflammatory effects of minocycline have been demonstrated in both mice and humans in the current study (Fig 6.1 and 6.2). In mice, prior administration of minocycline reduced the LPS-induced increase in IL-1β and IL-6 in the brain. Minocycline also reduced the expression of pro-inflammatory enzymes/receptors IDO and TLR4 in the hippocampus. Finally, in humans, a single dose of minocycline was sufficient to reduce CRP levels after four hours. Therefore, it is possible that downstream functional and behavioural changes observed in mice and humans are a result of minocycline's anti-inflammatory effects.

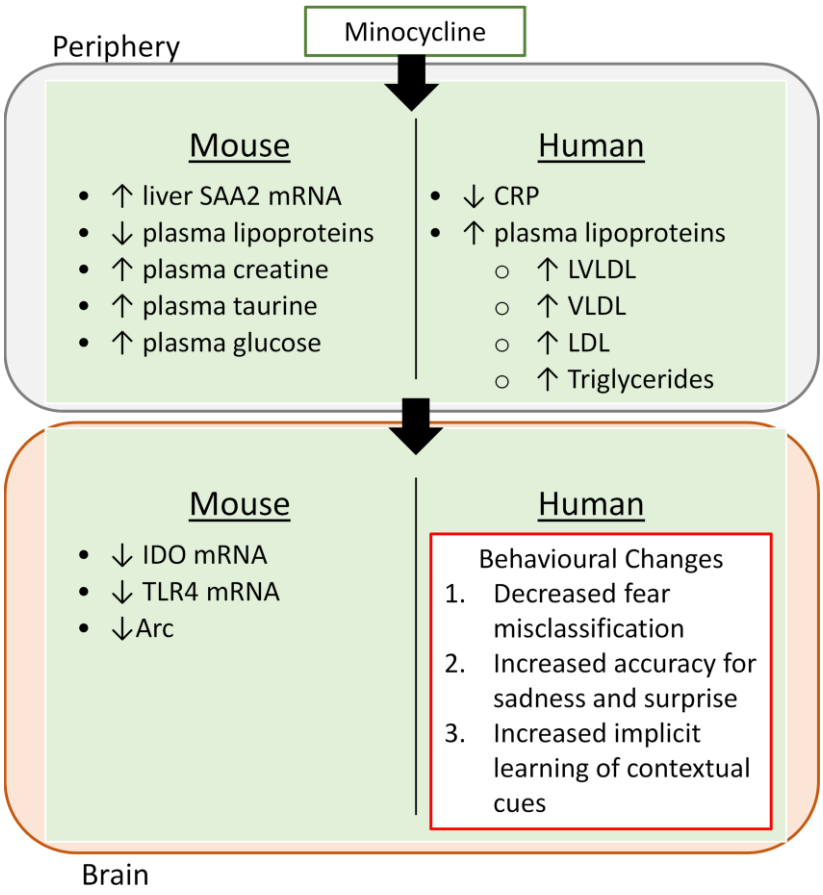


Fig 6.2 Summary of effects of minocycline administration in both CD1 mice and humans

All mouse brain changes occurred in the Hc

Inflammation and glutamatergic signalling

Previous studies suggested that glutamatergic signalling could be involved in mediating the inflammatory effects of minocycline. Viviani et al. showed that IL-1β induced the phosphorylation of GluN2B, increasing its activity and leading to increased intracellular calcium concentration and neuronal death downstream (B. Viviani et al., 2003). Thus, this suggests that inflammation could result in NMDAR-mediated excitotoxicity. Another study by O'Connor et al. suggested that inflammation influences NMDARs through the neuroactive metabolites QUIN and KA, an agonist and antagonist of NMDARs respectively, produced in the kynurenine pathway (O'Connor et al., 2009).

The effect of inflammation on NMDARs was supported by the results of the current study showing that LPS administration reduced GluN2 subunit expression in the CA1 region of the hippocampus. While the current study did not measure changes in GluN2 protein levels or activity, a downstream decrease in Arc expression in the same brain region implied functional changes that resulted from the decrease in GluN2 expression. Alterations in glutamatergic signalling is also supported by metabolite changes in the brain. LPS administration resulted in increased glutamine and NAA levels, and decreased glutamate and serine levels. Similar changes in glutamine/glutamate levels have been observed in patients with depression (Hashimoto et al., 2016a; Levine et al., 2000), suggesting dysregulation in the glutamate-glutamine cycle. It has also been proposed that NAA may function as a reservoir for glutamate (Clark et al., 2006). Furthermore, D-serine functions as a co-agonist of NMDAR, and it is synthesised in the brain from L-serine by serine racemase (Wolosker, Blackshaw, & Snyder, 1999). Therefore the observed decrease in L-serine levels in the current study may reduce D-serine availability and so would impair NMDAR signalling. While the results of the current study may suggest glutamatergic hypofunction, it would be premature to draw conclusions as the effects observed may be a result of compensatory mechanisms. It is possible that inflammation may result in increased NMDAR activity, shown by Viviani et al. (2003) in hippocampal cultures, and the

decreased expression of GluN2 subunits and availability of NMDAR agonists acts to compensate for increased NMDAR activity. While changes in ERK1/2 protein levels were observed, this result was inconclusive due to the large variation between cohorts. Thus, it is not clear which signalling proteins mediate the NMDAR-induced changes in Arc expression, although some studies have proposed that ERK signalling regulates Arc expression. Nevertheless, it is clear that inflammation dysregulates glutamatergic signalling, and the current study has shown that a possible downstream functional effect is decreased Arc expression, implicating decreased synaptic function.

Prior treatment with minocycline ameliorated the LPS-induced decrease in GluN2 subunit and Arc expression. Thus, it is possible that anti-inflammatory effects of minocycline on glutamatergic signalling and synaptic function underlie its psychotropic effects. Synaptic plasticity has been shown to be disrupted in psychiatric disorders such as depression, which is marked by reduced FC and Hc volume, and the loss of synapses, which has been demonstrated in the post mortem PFC of depressed patients (Ronald S. Duman, Aghajanian, Sanacora, & Krystal, 2016). Altered levels of growth or neurotrophic factors, and neurotransmitters also disrupt signalling pathways that lead to the synthesis of synaptic proteins. Elevated pro-inflammatory cytokine levels are also thought to activate pathways leading to loss of synapses. Finally, antidepressants, such as fluoxetine, have been observed to have subtle augmenting effects on synaptic plasticity through BDNF.

As Arc has been shown to be a critical regulator of synaptic function (Shepherd & Bear, 2011), it is a promising target for minocycline. To provide further support for the anti-inflammatory effects of minocycline underlying its psychotropic effects, Arc expression was only shown to be increased with both minocycline and LPS administration. Arc expression was shown to be decreased when just minocycline was administered. Finally, Arc expression in the FC has also been shown to be suppressed in chronic stress models of depression, lending further support to Arc changes underlying minocycline's psychotropic effects (Y. Li et al., 2015).

BCAA

Apart from glutamatergic signalling, LPS administration also resulted in increased isoleucine levels, an effect that was also ameliorated by minocycline. As elevated levels of BCAA have been linked to increased neurotoxicity and increased microglial reactivity (De Simone et al., 2013), it is possible that part of minocycline's anti-inflammatory action involves ameliorating the potentially excitotoxic actions of increased BCAA levels.

However, minocycline also has effects that may be separate from its anti-inflammatory effects. The next section will explore the non-anti-inflammatory effects of minocycline that may also underlie its psychotropic effects.

6.3 Non-anti-inflammatory mechanisms of minocycline

In chapter 2, minocycline was shown to increase liver SAA2 expression, which is counter-intuitive to its anti-inflammatory effects. The acute dose study of minocycline in chapter 3 also showed that minocycline administration alone altered levels of plasma metabolites. Of particular note is the effect of minocycline on plasma lipoproteins. This effect of minocycline on lipoproteins was also observed in healthy volunteers. Apart from its effects on CRP, a single dose of minocycline also resulted in increased levels of lipoproteins, providing another possible explanation for the behavioural effects observed.

SAA2 and Lipoproteins

One of the most consistent change, resulting from minocycline administration, is that of plasma lipoproteins. Lipoprotein levels were altered in both humans and mice within four hours after a single dose of minocycline. While it is difficult to determine the exact lipid species altered by minocycline administration from ^{1}H NMR results, the extended lipid peak analysis by numares AG revealed that plasma levels of LVLDL, VLDL, LDL, and triglycerides were increased in

participants in the minocycline group, suggesting alterations in cholesterol metabolism. In mice, lipoproteins levels were reduced as a result of minocycline administration.

In chapter 3, it was suggested that plasma metabolite changes were linked to the APR. In mice, liver SAA2 expression was observed to increase as a result of minocycline administration. While SAA2 is one of the major proteins of the APR, and thus, linked to the inflammatory response, increased levels suggests a non-anti-inflammatory mechanism of minocycline. Logically, minocycline would be expected to dampen the APR and reduce SAA2 expression. Given that SAA2 is an apolipoprotein, it is possible that the APR contributes to the lipid effects observed. For one, SAA binds to HDL, and is transported in plasma as a component of HDL (Coetzee et al., 1986). While this is generally linked to a reduction in HDL's anti-inflammatory properties and atherosclerosis, Sato et al. have also shown that SAA-HDL has higher antioxidant properties (Sato et al., 2016). Furthermore, there are links between SAA and lipid metabolism where SAA was shown to increase the expression of genes related to lipolysis and decrease the expression of genes related to lipogenesis, which could explain the decrease in lipoprotein levels observed (C. H. Chen et al., 2008; Faty, Ferre, & Commans, 2012).

In humans, minocycline administration resulted in increased levels and/or cholesterol concentration of serum LVLDL, VLDL, LDL and triglycerides. However, it is important to note that this is the result of an acute dose of minocycline, and chronic administration of minocycline in clinical trials have not reported weight gain as an adverse drug effect. While increased levels of LVLDL, VLDL, and LDL may not seem beneficial, it is interesting to note that depression is associated with decreased serum LDL levels (Parekh et al., 2017; Rabe-Jablonska & Poprawska, 2000). Therefore, more studies are required to understand the interaction between lipoprotein metabolism and minocycline, and should include the comparisons between acute and repeated administrations of the drug. Overall, however, the results of this present investigation in both mice and humans, unequivocally demonstrated that minocycline has a lipid modulating component with its biological effects.

Lipid changes have been linked to the pathology of psychiatric disorders, and brain lipid levels have been observed to be altered by chronic administration of antidepressants (Gulbins et al., 2013; Lee et al., 2009). Furthermore, lipids play important roles in membranes and cell signalling, and changes in lipid components may affect synaptic function in the brain (Schneider et al., 2017). The changes observed were peripheral, but peripheral lipids have been shown to alter the levels and composition of lipids in the brain (Lutjohann et al., 2004). Therefore, it is possible that the effects of minocycline on the modulation of lipid metabolism may underlie its psychotropic function. However, minocycline administration alone also resulted in changes in other metabolite levels.

Nutrient utilization and bioenergetics

Minocycline administration alone resulted in increased levels of plasma creatine, taurine, and glucose in mice, which suggested that minocycline may have improved nutrient utilization and bioenergetics in the animals. In humans, minocycline prevented the decrease in lipoprotein levels observed in the placebo group. Given that participants were not allowed to eat during the course of the study, it is expected that metabolite levels would fall by the second blood collection. Thus, the lipoprotein changes observed in the section above could also be linked to increased nutrient availability – the higher levels of serum LDL observed in participants in the minocycline group carries nutrients such as cholesterol and lipids to the rest of the body from the liver. This suggests a role for minocycline in increasing the availability of nutrients during short-term starvation.

Deficiency in nutrients such as omega-3 fatty acids, and minerals have been shown in patients with psychiatric disorders. Furthermore, amino acids are precursors to neurotransmitters essential for proper brain function, and supplements containing them have been discovered to reduce symptoms. Thus, changes in nutritional availability could also underlie the psychotropic effects of minocycline. (Rao, Asha, Ramesh, & Rao, 2008)

6.4 Interactions between anti-inflammatory and lipidomics effects

The previous sections have looked at the evidence for anti-inflammatory and non-anti-inflammatory mechanisms underlying the psychotropic effects of minocycline. This section will explore the possibility that these mechanisms are linked.

For one, CRP levels have been shown to be inversely correlated with LDL levels (Sammalkorpi, Valtonen, & Maury, 1990), thus the decrease in CRP levels could be associated with the increase in LDL levels observed in healthy volunteers. Furthermore, despite the strong association between SAA and the pro-inflammatory response, other studies have shown that SAA also triggers anti-inflammatory mechanisms, and may have a homeostatic role balancing pro- and anti-inflammatory responses instead (Tilg et al., 1997; Ye & Sun, 2015). Therefore, it is possible that increased SAA expression may be a compensatory response to the pro-inflammatory effects induced by LPS administration. This could also explain the similar effects on plasma lipoproteins, creatine, and taurine observed after a single dose of minocycline, and LPS administration. Another point worth noting is the proposed difference in functions between plasma SAA levels, which are largely contributed by hepatic SAA induced by the APR, and SAA produced by inflammatory tissue. Plasma SAA bind to HDL and are involved in lipid metabolism, while tissue SAA bind to inflammatory receptors and promote pro-inflammatory responses (Ye & Sun, 2015). Therefore, it is possible that the effects of SAA2 and lipid metabolism induced by minocycline could also be linked to its anti-inflammatory effects in response to stimulation by an inflammatory agent.

There are also links between lipids and inflammation, best shown through the example of atherosclerosis (van Diepen, Berbee, Havekes, & Rensen, 2013). It is telling therefore that there is a high incidence of comorbidity between atherosclerosis and depression. Increased inflammation and lipid levels have traditionally been thought to be positively correlated. For example, LPS administration in rodents has been found to induce a hyperlipidemic response, especially through the increase in plasma levels of triglycerides and total cholesterol (Wahby,

Yacout, Kandeel, & Awad, 2015). However, the links between inflammation and lipids are not that clear. LPS administration reduced plasma cholesterol levels in humans, including a decrease in LDL (Hudgins et al., 2003). In chronic inflammatory disorders, such as rheumatoid arthritis, patients have also been found to have reduced plasma cholesterol levels, including decreased cholesterol concentration in both HDL and LDL (Lazarevic et al., 1992). Anti-inflammatory treatments, such as treatments targeting pro-inflammatory cytokines, have also been shown to have mixed effects on lipids. Therefore, although lipids and inflammatory markers interact, further work is needed to decipher the effects of their interactions. Nevertheless, given the importance of lipids, particularly membrane-derived lipids, in synaptic function, it is possible that both lipids and anti-inflammatory mechanisms have converging effects on synaptic transmission.

Finally, Viviani et al. have shown that a lipid species, ceramide, is involved in the IL-1R-mediated increase in GluN2B phosphorylation (B. Viviani et al., 2003). All in all, given the pleiotropic effects of minocycline, it is possible that the results obtained in this dissertation may have been triggered simultaneously by different mechanisms. Nevertheless, links between anti-inflammatory effects, lipids, and the APR suggest that they could all be part of the same pathway.

6.5 Limitations and future work

This dissertation was a mechanistic study investigating the downstream processes brought about by the anti-inflammatory effects of minocycline. As such, in mice, the focus was at the molecular level, looking at possible molecular systems disrupted by inflammation and rescued by minocycline. While this dissertation has established signalling and potential functional changes brought about by LPS and minocycline, it is not clear whether these changes translate to downstream effects on behaviour. Based on previous studies, whose methodology this study has followed (Frenois et al., 2007; O'Connor et al., 2009), it is clear that LPS resulted

in depressive-like behaviours which were rescued by prior treatment with minocycline. The assumption, therefore, is that the molecular and metabolite changes observed in the current investigation would lead to the same downstream behavioural changes observed in previous studies. This was supported by results from the human study, showing that minocycline had psychotropic effects in healthy volunteers. Nevertheless, behavioural studies showing the antidepressant effects of minocycline as a result of the molecular changes identified may prove useful to provide a molecular-behavioural link.

One interesting result identified from this dissertation is the effect minocycline has on lipoproteins. As this dissertation conducted an untargeted hypothesis-generating metabolomics analysis with ^{1}H NMR, global changes in metabolites were investigated rather than any specific class. However, given that minocycline resulted in changes in lipoproteins in both mice and humans, a lipidomics analysis with mass spectrometry would expand on the results obtained from this dissertation. In particular, it would be useful to determine if minocycline results in similar lipid changes as other antidepressants (Lee et al., 2009).

Furthermore, the current study chose the time-points of 2 hours (acute study) and 24 hours (repeated dose study) post-dose to match that of inflammation mouse model in the molecular study, as well as the single dose human study. However, given the transient nature of metabolite levels and metabolic changes, conducting a metabolomics analysis on more time-points would provide a more complete picture of the metabolomic changes brought about by minocycline.

Also, this dissertation was interested in looking at the effect of increased pro-inflammatory cytokines levels on downstream pathways without overt sickness behaviour, as seen in patients with depression, thus a non-sepsis-inducing dose of LPS was used. However, an acute dose of LPS may result in compensatory mechanisms different from that seen in patients with psychiatric disorders, where there is a chronic elevation of inflammatory markers. Therefore, another way to expand knowledge in the field of inflammation in psychiatric

disorders and the potential of therapeutics with anti-inflammatory properties would be to use a chronic inflammation mouse model instead.

In addition, this dissertation analysed results from the different techniques employed separately. However, the analysis can be further expanded by conducting correlation tests. For example, one way of exploring the links between the anti-inflammatory effects of minocycline and downstream psychological effects could be to measure the correlation coefficient between CRP levels and fear misclassification.

Finally, this focus of this dissertation was on the anti-inflammatory effects of minocycline, and non-anti-inflammatory effects of minocycline observed were still within the context of an inflammation model. To truly decipher other non-anti-inflammatory effects, it would be useful to use a different animal model – perhaps one involving learned helplessness or a chronic stress model. The neuroprotective effects of minocycline has also been shown in brain injury models, although this is less relevant to the pathophysiology of psychiatric disorders.

6.6 Conclusion

All in all, this dissertation has shown evidence that supports the theory that the anti-inflammatory mechanisms of minocycline underlie its psychotropic effects. In a rodent inflammation model, prior treatment with minocycline rescued LPS-induced effects on NMDAR signalling, and downstream decrease in Arc expression. In healthy volunteers, minocycline administration reduced inflammatory markers, shown by decreased levels of CRP, which could underlie behavioural changes in emotional processing (reduced fear misclassification) and cognition (improved contextual learning). Metabolomic analysis also revealed changes in lipid metabolism and energy-related metabolites that could potentially underlie a separate mechanism for minocycline's actions. However, the links between metabolites, inflammation, and downstream psychotropic effects suggest that these pathways interact.

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Appendix

Appendix 3.1 Summary of results comparing the ensemble of OPLS-DA models with the ensemble of randomly permutated models

Acute Dose Study:

Acute Study		Accuracy		Sensitivity		Specificity		Q2		R2Y		R2X	
		Model	Rand	Model	Rand	Model	Rand	Model	Rand	Model	Rand	Model	Rand
Plasma	Summary	80.9%	48.9%	91.5%	65.8%	94.2%	65.6%	0.555	-0.533	0.872	0.510	0.701	0.465
	T test	<0.001 ***		<0.001		<0.001		<0.001		<0.001		<0.001	
	K.S. Test	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
Hc	Summary	60.8%	50.6%	82.1%	66.3%	78.5%	67.9%	0.0285	-0.2565	0.631	0.633	0.458	0.436
	T test	<0.001 ***		<0.001		<0.001		<0.001		>0.05		<0.001	
	K.S. Test	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
FC	Summary	48.5%	50.6%	66.5%	65.7%	66.3%	66.6%	0.0086	-0.234	0.844	0.792	0.344	0.344
	T test	>0.05		>0.05		>0.05		<0.001***		<0.001		>0.05	
	K.S. Test	>0.05		>0.05		>0.05		<0.001		<0.001		<0.001	

Repeated Dose Study:

SalSal/SalLPS		Accuracy		Sensitivity		Specificity		Q2		R2Y		R2X	
		Model	Rand	Model	Rand	Model	Rand	Model	Rand	Model	Rand	Model	Rand
Plasma	Summary	97%	48%	100%	59%	97%	60%	0.86	-0.31	0.960	0.293	0.787	0.627
	T test	<0.001 ***		<0.001		<0.001		<0.001		<0.001		<0.001	
	K.S. Test	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
Hc	Summary	67.1%	50.7%	78.9%	60.2%	76.5%	59.6%	0.225	-0.343	0.816	0.653	0.297	0.262
	T test	<0.001 ***		<0.001		<0.001		<0.001		<0.001		<0.001	
	K.S. Test	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
FC	Summary	85.3%	51.1%	92.9%	59.9%	90.2%	60.4%	0.537	-0.315	0.864	0.622	0.411	0.330
	T test	<0.001 ***		<0.001		<0.001		<0.001		<0.001		<0.001	
	K.S. Test	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	

MinoSal/ MinoLPS		Accuracy		Sensitivity		Specificity		Q2		R2Y		R2X	
		Model	Rand	Model	Rand	Model	Rand	Model	Rand	Model	Rand	Model	Rand
Plasma	Summary	84.5%	49.6%	91.6%	59%	90.5%	60.2%	0.479	-0.322	0.743	0.314	0.749	0.585
	T test	<0.001 ***		<0.001		<0.001		<0.001		<0.001		<0.001	
	K.S. Test	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
Hc	Summary	51.1%	50.2%	61.1%	57.9%	59.8%	54.8%	-0.362	-0.421	0.671	0.642	0.248	0.240
	T test	0.525		0.109		<0.05 *		<0.001 ***		<0.001		<0.001	
	K.S. Test	0.999		0.111		0.174		<0.001 ***		<0.001		<0.001	
FC	Summary	65.6%	52.3%	78.7%	61.0%	72.8%	60.5%	0.0304	-0.355	0.676	0.559	0.317	0.319
	T test	<0.001 ***		<0.001		<0.001		<0.001		<0.001		>0.05	
	K.S. Test	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	

SalSal/MinoSal		Accuracy		Sensitivity		Specificity		Q2		R2Y		R2X	
		Model	Rand	Model	Rand	Model	Rand	Model	Rand	Model	Rand	Model	Rand
Plasma	Summary	58.5%	49.3%	69.8%	57.9%	68.6%	58.1%	-0.0483	-0.374	0.426	0.314	0.471	0.518
	T test	<0.001 ***		<0.001		<0.001		<0.001		<0.001		<0.001	
	K.S. Test	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
Hc	Summary	41.4%	49.0%	48.3%	56.2%	49.8%	58.1%	-0.786	-0.44	0.638	0.658	0.214	0.240
	T test	<0.001 ***		<0.001		<0.001		<0.001		<0.001		<0.001	
	K.S. Test	<0.001		<0.01		<0.001		<0.001		<0.001		<0.001	
FC	Summary	38.5%	49.4%	47.8%	59.5%	45.5%	56.8%	-0.535	-0.305	0.473	0.575	0.391	0.384
	T test	<0.001 ***		<0.001		<0.001		<0.001		<0.001		<0.001	
	K.S. Test	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	

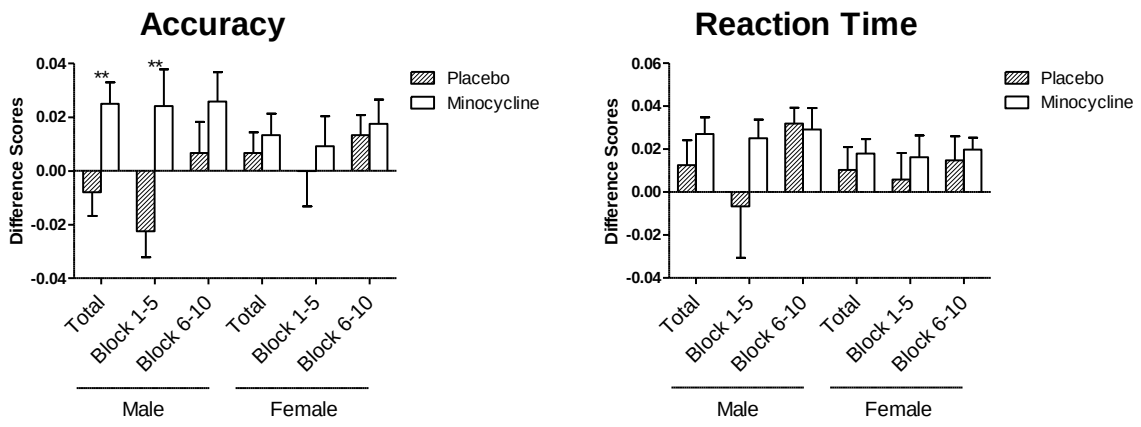
SalLPS/MinoLPS		Accuracy		Sensitivity		Specificity		Q2		R2Y		R2X	
		Model	Rand	Model	Rand	Model	Rand	Model	Rand	Model	Rand	Model	Rand
Plasma	Summary	60.1%	51.7%	73.6%	62.4%	71.6%	63.3%	-0.0059	-0.309	0.392	0.323	0.566	0.532
	T test	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
	K.S. Test	<0.001		<0.001		<0.01 **		<0.001		<0.001		<0.001	
Hc	Summary	45.6%	49.1%	53.3%	55.0%	53.2%	55.5%	-0.459	-0.472	0.589	0.601	0.269	0.261
	T test	<0.01 **		>0.05		>0.05		>0.05		<0.001		<0.001	
	K.S. Test	>0.05		>0.05		>0.05		<0.001		<0.001		<0.001	
FC	Summary	35.9%	49.8%	41.9%	54.8%	43.0%	57.0%	-0.592	-0.416	0.487	0.585	0.314	0.309
	T test	<0.001 ***		<0.001		<0.001		<0.001		<0.001		<0.05 *	
	K.S. Test	<0.001		<0.001		<0.001		<0.001		<0.001		<0.01 **	

	Minocycline Group		Placebo Group		Significance (P value)
	Male	Female	Male	Female	Group*Gender
Gender	10	10	10	10	1.0
Age (Years)	26.3 (4.47)	28.7 (4.55)	24.7 (2.71)	30.3 (8.68)	0.368
BMI	24.18 (3.29)	23.49 (2.95)	22.95 (0.82)	23.66 (2.42)	0.391
NART	115.22 (5.60)	116.69 (7.76)	115.03 (4.08)	114.20 (4.93)	0.532
STAI (Trait)	32.9 (6.71)	30.2 (4.76)	34.2 (8.87)	29.6 (4.62)	0.645
BDI	1.9 (2.96)	0.7 (1.33)	2.6 (3.53)	1.6 (4.06)	0.920
EPQ: N	3.6 (3.10)	4.7 (2.79)	6.8 (3.79)	4.0 (3.80)	0.078
EPQ: P	2.9 (1.97)	2.4 (1.71)	4.1 (2.73)	2.0 (1.83)	0.235
EPQ: E	14.2 (4.80)	13.8 (3.79)	13.2 (4.83)	15.4 (3.10)	0.334
EPQ: L	10.0 (4.97)	9.5 (2.27)	10.2 (3.22)	13.0 (3.80)	0.167

A4.1 Summary of baseline measures separated by treatment group and gender. 2-way ANOVA analysis revealed no significant gender*group interaction effects. Values represent the mean with standard deviation in parentheses.

BMI: Body mass index; NART: National Adult Reading Test; STAI: State-trait Anxiety Index; BDI: Beck Depression Inventory; EPQ: Eysenck Personality Questionnaire

Appendix 4.2 Contextual cueing task separated by gender



A4.2 The increase in accuracy difference scores as a result of minocycline administration was mainly driven by males (Males: +0.047, p = 0.009).

Appendix 5.1 Summary of results comparing the ensemble of OPLS-DA models with the ensemble of randomly permutated models

Minocycline Study		Accuracy		Sensitivity		Specificity		Q2		R2Y		R2X	
		Model	Rand	Model	Rand	Model	Rand	Model	Rand	Model	Rand	Model	Rand
Serum	Summary	72.8%	49.6%	83.3%	57.1%	77.0%	54.7%	0.211	-0.394	0.591	0.452	0.413	0.330
	T test	<0.001 ***		<0.001		<0.001		<0.001		<0.001		<0.001	
	K.S. Test	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	

Appendix 5.2 Expanded lipoprotein analysis by numares AG

Metabolite	Minocycline (N = 11)		Placebo (N = 7)		Sig	Adj Sig
	Mean	SD	Mean	SD		
LVLDL-p	137.77	45.75	60.37	52.58	0.0216	0.5391
LDL-p	101.81	9.38	105.77	19.32	0.5802	14.5050
LLDL-p	100.42	9.99	97.68	19.61	0.7094	17.7341
HDL-p	106.76	9.47	104.15	10.80	0.6051	15.1275
LHDL-p	115.31	16.27	109.29	9.85	0.4341	10.8520
SHDL-p	104.15	10.25	101.88	11.67	0.6765	16.9118
VLDL-s	104.01	3.75	95.39	6.39	0.0031	0.0781
LDL-s	99.81	0.94	99.22	1.28	0.2875	7.1879
HDL-s	101.51	0.90	100.59	1.34	0.1099	2.7479
VLDL-c	104.32	10.66	90.76	14.61	0.0422	1.0555
IDL-c	105.90	9.51	113.18	15.52	0.2471	6.1765
LDL-c	106.02	9.63	111.36	18.60	0.4672	11.6793
LDL.A-c	99.06	8.66	112.92	23.30	0.1028	2.5701
LDL.C-c	118.75	17.41	80.45	12.67	0.0002	0.0043
HDL.A-c	107.84	9.37	113.71	26.64	0.5254	13.1356
HDL.C-c	111.68	25.87	100.17	27.32	0.3912	9.7797
Total-Cholesterol	105.60	8.82	104.08	14.52	0.7913	19.7825
LDL-Cholesterol	103.76	11.41	119.29	28.92	0.1418	3.5442
HDL-Cholesterol	112.86	11.48	114.59	12.07	0.7696	19.2394
Triglycerides	103.62	25.71	63.47	21.84	0.0043	0.1072
Glucose	90.05	15.56	94.77	12.49	0.5101	12.7518
Alanine	69.00	11.66	75.23	11.31	0.2804	7.0090
Valine	84.98	10.96	79.81	2.79	0.2442	6.1048
Leucine	76.26	17.82	73.69	8.32	0.7456	18.6405
Isoleucine	75.66	18.93	71.72	12.80	0.6354	15.8860

A5.2 Expanded lipoprotein analysis by numares AG. Significant results (independent samples T-test) are highlighted in red. Adj Sig refers to significance when Bonferroni correction for multiple testing is applied.

Metabolite Legend:

LVLDL-p	Concentration of large VLDL particles	nmol/l
LDL-p	Concentration of LDL particles	nmol/l
LLDL-p	Concentration of large LDL particles	nmol/l
SLDL-p	Concentration of small LDL particles	nmol/l

HDL-p	Concentration of HDL particles	nmol/l
LHDL-p	Concentration of large HDL particles	nmol/l
SHDL-p	Concentration of small HDL particles	nmol/l
VLDL-s	Mean diameter of VLDL particles	nm
LDL-s	Mean diameter of LDL particles	nm
HDL-s	Mean diameter of HDL particles	nm
VLDL-c	Cholesterol concentration in VLDL class	mg/dl
IDL-c	Cholesterol concentration in IDL class	mg/dl
LDL-c	Cholesterol concentration in LDL class	mg/dl
LDL.A-c	Cholesterol concentration in LDL subclass A (large particles)	mg/dl
LDL.B-c	Cholesterol concentration in LDL subclass B (medium-sized particles)	mg/dl
LDL.C-c	Cholesterol concentration in LDL subclass C (small particles)	mg/dl
HDL.A-c	Cholesterol concentration in HDL subclass A (large particles)	mg/dl
HDL.B-c	Cholesterol concentration in HDL subclass B (medium-sized particles)	mg/dl
HDL.C-c	Cholesterol concentration in HDL subclass C (small particles)	mg/dl
Total-Cholesterol	Concentration of total cholesterol in serum	mg/dl
LDL-Cholesterol	Concentration of LDL cholesterol in serum	mg/dl
HDL-Cholesterol	Concentration of HDL cholesterol in serum	mg/dl
Triglycerides	Concentration of total triglycerides in serum	mg/dl
Lactate	Concentration of lactate in serum	mg/dl
Glucose	Concentration of glucose in serum	mg/dl
Alanine	Concentration of alanine in serum	µmol/l
Valine	Concentration of valine in serum	µmol/l
Leucine	Concentration of leucine in serum	µmol/l
Isoleucine	Concentration of isoleucine in serum	µmol/l

Appendix 5.3 Serum metabolite changes split by gender

	Bins	X0.893	X0.913	X1.293	X1.313	X1.393	X1.413	X2.233	X2.253
Male	**Placebo**	84.44782	67.45458	74.54227	66.76194	66.50452	79.40014	63.59062	67.60787
	Drug	113.2987	120.5195	119.087	120.2479	112.0232	108.5527	122.9408	111.3128
Female	**Placebo**	92.65038	76.93054	84.90505	75.4248	74.07475	86.16755	73.92416	76.83582
	Drug	105.5216	106.5189	106.7959	105.9738	97.9779	101.3452	110.5081	97.88814

A5.3 Mean values of % ratio (time-point 2/time-point 1) of representative lipoprotein bins split by gender and treatment group.

Metabolite changes were similar between both genders (i.e. lipoprotein levels were higher in the minocycline treatment group for both genders)