IL-1β-mediated changes in cerebral perfusion and neural activity in a rat model of neuroinflammation and excitotoxicity

A thesis submitted to the University of Manchester for the degree of
Ph.D. Neuroscience
in the Faculty of Life Sciences

2013

NATASHA J. BRAY
# Contents

1. Introduction ........................................................................................................... 14
   1.1 Neuroinflammation ......................................................................................... 14
   1.2 Interleukin-1 .................................................................................................. 15
      1.2.1 IL-1β signalling ...................................................................................... 16
      1.2.2 IL-1β and neurodegeneration .................................................................. 17
      1.2.3 Glutamate and excitotoxicity .................................................................... 20
   1.3 IL-1β and excitotoxicity .................................................................................... 22
   1.4 The AMPA+IL-1β model ................................................................................. 24
      1.4.1 Hypotheses for AMPA+IL-1β-induced cortical cell death ................. 26
      1.4.2 The vascular hypothesis ......................................................................... 28
   1.5 Optical imaging of the haemodynamic response ........................................... 29
   1.6 The haemodynamic response ............................................................................ 31
   1.7 Optical imaging spectroscopy ........................................................................... 33
   1.8 The rat barrel cortex ....................................................................................... 36
   1.9 Extracellular potentials in the sensory cortex ............................................... 40
   1.10 Aims and Objectives ...................................................................................... 42

2. Materials and Methods ......................................................................................... 45
   2.1 Procedure ........................................................................................................ 45
      2.1.1 Animals .................................................................................................... 45
      2.1.2 Surgical preparation ............................................................................... 46
      2.1.3 Optical imaging spectroscopy and hardware .................................... 48
      2.1.3.1 Whisker stimulus ............................................................................ 49
      2.1.4 Striatal injection ..................................................................................... 50
      2.1.5 Tissue processing, immunohistochemistry and histology ............... 51
      2.1.6 Electrode recordings .............................................................................. 53
   2.2 Analysis ............................................................................................................ 55
5.3 Methodological considerations ................................................................. 143
  5.3.1 AMPA+IL-1β Model .............................................................................. 143
  5.3.2 Isoflurane anaesthesia ........................................................................ 144
  5.3.3 Optical imaging spectroscopy ............................................................... 144
  5.3.4 Tissue oxygenation and electrode recordings ...................................... 145
5.4 Future directions ....................................................................................... 147
6. References .................................................................................................. 154

Word Count: 38,325 with references, introductory material and lists; 29,557 without.
ii) List of figures

Figure 1-1 Structure of the blood brain barrier ............................................................. 18
Figure 1-2 Absorption spectra of oxy- and deoxy-haemoglobin ....................................... 34
Figure 1-3 Example OIS whisker stimulation response .................................................... 35
Figure 1-4 The rat whisker lemniscal pathway .............................................................. 37
Figure 2-1 Creation and application of automated binomial mask .................................... 57
Figure 2-2 Vessel depth and diameter analysis .................................................................. 59
Figure 2-3 MUA analysis ................................................................................................. 61
Figure 2-4 Gamma and beta LFP analysis ........................................................................ 62
Figure 2-5 Analysis sites in the brain ................................................................................ 64
Figure 3-1 Maximum area time courses .......................................................................... 69
Figure 3-2 HbO response time course .............................................................................. 70
Figure 3-3 Physiological parameters in time course study ............................................... 72
Figure 3-4 Time course of neutrophil recruitment ........................................................... 75
Figure 3-5 Time course of IgG extravasation and oedema ................................................ 76
Figure 3-6 Maximum spatial areas ..................................................................................... 79
Figure 3-7 HbT responses ................................................................................................. 80
Figure 3-8 HbO responses ................................................................................................. 82
Figure 3-9 HbO latencies and profiles ............................................................................... 84
Figure 3-10 HbR early and late responses ......................................................................... 86
Figure 3-11 Vessel depths ................................................................................................. 88
Figure 3-12 Vessel diameters ........................................................................................... 89
Figure 3-13 MCA depth and HbO response correlation .................................................... 90
Figure 3-14 Physiological parameters comparing groups ................................................... 92
Figure 3-15 IgG and oedema are IL-1β-dependent ............................................................ 94
Figure 3-16 SJC-positive neutrophils in scortex, striatum and piriform ............................. 96
Figure 3-17 Example neutrophil photomicrographs ....................................................... 97
Figure 3-18 Correlations of OIS and histological markers ................................................... 99
Figure 4-1 Baseline oxygenation and time-to-maximum baseline measurement ............... 109
Figure 4-2 Illustration of progression to negative oxygenation responses ....................... 110
Figure 4-3 Absolute stimulus-induced change in oxygenation ......................................... 111
Figure 4-4 Correlation of baseline and absolute change oxygenation values ................... 112
Figure 4-5 Changes in oxygenation response percentages ............................................... 113
Figure 4-6 Gamma and beta peak activity ...................................................................... 116
Figure 4-7 Correlation of gamma and beta peak .............................................................. 117
Figure 4-8 Stimulus-evoked and spontaneous MUA ....................................................... 119
Figure 4-9 Correlation of gamma and beta with MUA ...................................................... 121
Figure 4-10 Coupling of gamma and beta activity with oxygenation response ............... 122
Figure 4-11 Physiological parameters from electrode study ........................................... 125
Figure 4-12 Oedema and IgG in electrode studies ......................................................... 127
Figure 4-13 Cresyl violet interhemispheric paling ......................................................... 129
Figure 4-14 Example photomicrographs of cresyl violet tissue ................................................. 130
Figure 4-15 Correlation of striatal and cortical paling with IgG ............................................. 131
Figure 5-1 Schemative diagram showing possible mechanisms. ............................................. 142
iii) List of Abbreviations

AA – arachidonic acid

AMPA - amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid

AMPAR - amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid receptor

ANOVA – analysis of variance

APOX – action potential/oxygenation

ATP – adenosine triphosphate

BBB – blood brain barrier

BOLD – blood oxygen level-dependent

BSA – bovine serum albumin

bpm – beats per minute

Ca\textsuperscript{2+} - calcium ion

CBF – cerebral blood flow

CCD – charge-coupled device

CNS – central nervous system

DAC – digital-analog-converter

DAMP – damage-associated molecular pattern

dH\textsubscript{2}O – distilled water

DPX – distyrene plasticiser and xylene

EAAT – excitatory amino acid transporter

ECG - electrocardiogram

EEG - electroencephalogram

ET-1 – endothelin-1

expCO\textsubscript{2} – expired carbon dioxide

fMRI – functional magnetic resonance imaging

GABA – gamma-aminobutyric acid

GluR – glutamate receptor

Glu - glutamate

GUI – graphical user interface
H₂O - water
HbO – oxyhaemoglobin
HbR - deoxyhaemoglobin
HbT – total haemoglobin
IFN-γ – interferon-gamma
IgG – immunoglobulin G
i.p. - intraperitoneal
i.st. - intrastriatal
ICAM-1 – intercellular adhesion molecule-1
IL - interleukin
IL-1α – interleukin-1 alpha
IL-1β – interleukin-1 beta
IL-1Ra – interleukin-1 receptor antagonist
IL-1R1 – interleukin-1 receptor
IL-1RAcP – interleukin-1 receptor accessory protein
JAM – junction adhesion molecule
K⁺ - potassium ion
KGA – kidney-type glutaminase
LFP – local field potential
LGIC – ligand-gated ion channel
M - mean
MAP – mitogen activated pathway
MCA – middle cerebral artery
Mg²⁺ - magnesium ion
MK-801 - [5R,10S]-[+]-5-methyl-10,11- dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine
MMP – matrix metalloproteinase
mRNA – messenger ribonucleic acid
MUA – multi-unit activity
Na⁺ - sodium ion
NIH – National Institute of Health
NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA – N-methyl-D-aspartate
NO – nitric oxide
NREM – non-rapid eye movement
NVU – neurovascular unit
O₂ – oxygen
OI – optical imaging
OIS – optical imaging spectroscopy
OCT – optical coherence tomography
PAMP – pathogen-associated molecular pattern
P2X₇ – P2X purinoceptor 7
PC – personal computer
PCI – peripheral component interconnect
PBS – phosphate buffered saline
PGE₂ – prostaglandin E₂
POM – posterior medial nucleus of the thalamus
rCBF – regional cerebral blood flow
S-AMPA – (S)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
S₁ – primary somatosensory cortex
S₂ – secondary somatosensory cortex
SD – standard deviation
SEM – standard error of the mean
sEPSC – spontaneous excitatory post-synaptic current
sIPSC – spontaneous inhibitory post-synaptic current
SpO₂ – blood oxygen saturation
T – temperature
TBI – traumatic brain injury
TNF-α – tumour necrosis factor alpha
VCAM-1 – vascular adhesion molecule-1

VPM – ventral posterior medial nucleus of the thalamus

VPMdm – dorsomedial section of the ventral posterior medial nucleus of the thalamus
iv) Declaration
No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

v) Copyright statement

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trade marks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://documents.manchester.ac.uk/DocuInfo.aspx?DocID=487), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.manchester.ac.uk/library/aboutus/regulations) and in The University’s policy on Presentation of Theses.
vi) Acknowledgements

I would like the opportunity to thank all the people that have helped me over the past three years.

First of all I would like to thank Dr Ingo Schiessl for his supervision and guidance over the past three years. His everlasting calm, kindness, patience, and - dare I say it - efficient, German manner all make him a fantastic supervisor and I’m really grateful for everything he’s taught me (not just about MATLAB!).

Secondly, I’d like to thank Prof Stuart Allan for his undying optimism and enthusiasm with this project, and for always making time in his hectic schedule to chat. I’d also like to thank my advisor Dr Rasmus Petersen for his insightful and helpful advice, and Dr Mark Dickinson for the technical know-how.

Big thanks go to all the colleagues and friends I’ve made in the past three years: Fiona for putting up with me, all of the Gigg and Montemurro labs for the motivational chats, Zoltan, Mike and Kyle for their programming wizardry and all of the undergraduate and Master’s students that have graced the Schiessl lab.

Thanks to all of the Allan, Lawrence, Brough, Pintaux, Boutin, Rothwell, Luckman, Lucas, Canal, Gardiner, Petersen and Grieve lab members that have inspired me along the way (past and present); to the FLS Postgrad Society; to the Supersonicós; and the Brain Bank bloggers.

Special thanks go to Simon for always listening; Amy and Gaurav for keeping me smiling most of the time and the C’house gang for when I needed a break. Thanks to my family, especially my brother and my parents for not laughing too hard when I told them I wanted to do a PhD, and for their continuing fervent interest in my research and whatever else I do.

Finally, I thank the BBSRC for funding me through this project.
vii) Abstract

The University of Manchester
Natasha J. Bray
Submitted 28.9.13; Final Submission 26.11.13
Doctor of Philosophy, Neuroscience

IL-1β-mediated changes in cerebral perfusion and neural activity in a rat model of neuroinflammation and excitotoxicity

Neuroinflammation is a major driver of secondary brain cell death after ischaemic stroke, seizure activity and traumatic brain injury. In a model of excitotoxic neuroinflammation, striatal injection of a toxic dose of AMPA causes cell death in the striatum after 24 hours. Co-injection of AMPA with the pro-inflammatory cytokine interleukin-1β (IL-1β) leads to additional cortical cell death. Injected alone, IL-1β leads to little or no cell death.

It is hypothesised that IL-1β may exacerbate cell death by interfering with blood flow coupling. In the first study, two-dimensional optical imaging spectroscopy was used to measure early changes in the haemodynamic response in the anaesthetised rat barrel cortex before and for 6 hours after injection of vehicle, AMPA, IL-1β, or AMPA+IL-1β. After injection of IL-1β, with or without AMPA, the oxygenated blood flow response to mechanical whisker stimulation approximately halved over the course of 6h.

In the second study, to determine whether the IL-1β-dependent changes in blood flow response are reflected by altered cellular activity, local field potentials, multi-unit activity and local tissue oxygenation responses to whisker stimulation were recorded simultaneously from the active barrel before and up to 6h after injection. A similar reduction in the size of the oxygenation response was seen again in the IL-1β- and AMPA+IL-1β-treated groups. Importantly, the level of gamma frequency oscillations at stimulus onset decreased within the first hours after injection of AMPA+IL-1β or IL-1β, suggesting a disruption of the fast-spiking interneuron network in the barrel cortex.

These findings, along with histological observations of IL-1β-dependent markers of neuroinflammation, suggest that IL-1β may exacerbate AMPA-induced excitotoxicity by potentiating seizure activity and decoupling the neurovascular response in the cortex.
1 Introduction

1.1 Neuroinflammation

The word ‘inflammation’ comes from the Latin ‘inflammare’: ‘to set on fire’. The body’s normal acute inflammatory response to infection or injury consists of five cardinal signs: tumor (swelling), rubor (increased perfusion), dolor (pain), calor (heat) and function laesa (loss of function), although these symptoms may in some cases present without actual infection or injury (Ryan and Majno, 1977). Neuroinflammation, while by no means identical to the classic inflammation seen in the periphery, comprises a number of dynamic stages and especially involves the resident immune cells microglia and astrocytes, as well as immune cells from the periphery (Danton and Dietrich, 2003).

The objective and, perhaps, selective advantages for the brain to have its own immune response are much the same as that in the periphery: namely, damage limitation and repair (Ryan and Majno, 1977). However, chronic and unresolved, or excessive acute neuroinflammation contribute to central nervous injury in a number of disorders. Moreover, the prevalence of systemic inflammatory disease, such as atherosclerosis, urinary tract infections, or obesity, has been associated with a higher risk of brain diseases and can worsen outcome after acute brain injury (Smeeth et al., 2004, Murray et al., 2013, Emsley et al., 2003). Acute, excessive neuroinflammation is especially relevant in situations such as after stroke (Danton and Dietrich, 2003), traumatic brain injury (TBI) (Lu et al., 2005) and epileptic seizures (Choi and Koh, 2008, Fabene et al., 2008, van Vliet et al., 2007).
1.2 Interleukin-1

Chemical mediators involved in the inflammatory response to acute brain injury include chemokines, prostaglandins and cytokines (Danton and Dietrich, 2003, Murray et al., 2013). Cytokines are polypeptide messengers with multiple targets and downstream effects, most with both pro- and anti-inflammatory actions, though distinguishing between pro- and anti- may not be so easy in the brain. Some cytokines – including IL-1, interferon-alpha (IFN-α) and tumour necrosis factor-alpha (TNF-α) are most notably instrumental in driving and responding to neuroinflammation (Allan and Rothwell, 2001). IL-1 has been extensively studied over recent years due to its critical role in stroke and other neurodegenerative diseases (Allan and Rothwell, 2001, Allan et al., 2005).

The IL-1 family consists of three main ligands: the two agonists IL-1α and β (March et al., 1985), and the endogenous IL-1 receptor antagonist, IL-1Ra (Hannum et al., 1990). This thesis will focus particularly on IL-1β and its actions in the brain.

IL-1β is primarily produced by endothelial cells, astrocytes, microglia and macrophages (Dinarello, 2002). Synthesis of IL-1β is a two-step process. In the first step, pathogen- or damage- associated molecular patterns (PAMPs or DAMPs) bind to Toll-like receptors on the cell surface, triggering the expression of the 31kDa precursor protein pro-IL-1β (Giri et al., 1985) via the action of the transcription factor NF-κB (Netea et al., 2008). Secondly, a second ‘damage’ stimulus, for instance ATP released from dying cells, binds to P2X7 purinoceptors (Le Feuvre et al., 2002, Ferrari et al., 1997), which in turn activate the inflammasome. The inflammasome is a molecular scaffold upon which pro-caspase-1 is cleaved to caspase-1 (Martinon et al., 2002), which then cleaves pro-IL-1β to the mature 17kDa...
active form (Thornberry et al., 1992, Giri et al., 1985). IL-1β is not processed through the Golgi apparatus and the mechanism through which cytosolically cleaved IL-1β leaves the cells is yet unknown (Brough and Rothwell, 2007).

1.2.1 IL-1β signalling

Once outside the cell, IL-1β has multiple effects on various cell types throughout the brain in both health and disease. It exerts its effects through the IL-1 type I receptor (IL-1RI) (Sims et al., 1988), which must be bound to the IL-1RI accessory protein (IL-1RAcP) after ligand binding to allow signal transduction into the targeted cell (Greenfeder et al., 1995). Another receptor, the IL-1 type II receptor, does not feature an intracellular IL-1RAcP domain so it thought to be a decoy receptor, sequestering excess IL-1α and β (Colotta et al., 1993).

IL-1RI is expressed by and found on the surface of neurons, microglia, astrocytes, ependymal cells, pericytes and endothelial cells of the cerebrovasculature (Ericsson et al., 1995), as well as on circulating neutrophils (Brandolini et al., 1997). When injected directly into the brain, IL-1β has been found to rapidly diffuse via volume transmission along white matter tracts and blood vessels (Konsman et al., 2000). The primary effect of IL-1 signal transduction is the expression and mRNA-stabilisation of pro-inflammatory mediators (Rothwell and Luheshi, 2000, Thornton et al., 2010).

In a healthy brain, IL-1β is important for a number of different processes, including non-rapid eye movement sleep (NREM; (Obal et al., 1990), adaptive sickness behaviour via the hypothalamus (Dinarello, 1984), and long term potentiation (LTP; (Viviani et al., 2003). The latter effect is attributed to IL-1β
binding to IL-1RI, which leads to an intracellular cascade involving ceramide-dependent upregulation of Src tyrosine kinase activity. This cascade results in the phosphorylation and potentiation of NMDA glutamate receptors (Viviani et al., 2003). This upregulation of NMDA receptor activity aids in normal LTP processing but also may render the cell more vulnerable to the excitotoxic effects of glutamate, described later.

IL-1β is a pro-inflammatory cytokine; while it has some beneficial effects on repair, neovascularisation and formation of the astrocytic scar (Giulian et al., 1988), its acute effects are generally thought to be detrimental to the brain (Allan and Rothwell, 2001, Allan et al., 2005).

1.2.2 IL-1β and neurodegeneration

IL-1β is not harmful per se to neuronal cultures (Strijbos and Rothwell, 1995) and neurotoxic effects have only been reported in neonatal rodent models (Cai et al., 2004). However, it has deleterious effects in neurodegenerative disorders via myriad mechanisms (Allan et al., 2005). Acute effects of IL-1β are considered to mediate neurodegeneration in epilepsy (Choi and Koh, 2008), stroke (McColl et al., 2008, Allan et al., 2005) and brain injury (Hutchinson et al., 2007). In contrast, IL-1Ra, the endogenous antagonist, has been shown to be neuroprotective in models of ischaemic stroke (Betz et al., 1995, Relton and Rothwell, 1992, Loddick and Rothwell, 1996, Girard et al., 2012), neonatal hypoxia (Girard et al., 2012), subarachnoid haemorrhage (Greenhalgh et al., 2012) and spinal cord injury (Zong et al., 2012).
One of the main sites of IL-1β’s deleterious actions in neurodegeneration is on the blood-brain barrier (BBB). The brain’s internal environment requires a delicate and different balance of ions to that of the circulation and so is vulnerable to many substances found in the blood (Hawkins and Davis, 2005). The BBB, the interface between the intraluminal blood and the brain, protects the brain from pathogens and losing ion balance by strictly limiting the passage of materials and cells into the brain parenchyma. The anatomical substrate of the BBB, called the neurovascular unit (Figure 1-1), includes the endothelial cells of the blood vessel, pericytes, the extracellular matrix, neurons and a basal laminal membrane which is continuous with astrocytic end-feet (Hawkins and Davis, 2005). Tight junctions exist between the endothelial cells of the cerebral vasculature (Kniesel and Wolburg, 2000) and these are adaptable by means of up-or down-regulating transcription of different intercellular and intracellular adhesion molecules. Intercellular molecules, including claudins, occludins and junction adhesion molecules (JAMs) are scaffolded to intracellular actin-binding proteins such as zonula occludins, 7H6 and cingulin (Hawkins and Davis, 2005). Altogether, the structure of the NVU usually provides a secure, protective interface between the blood supply and the brain tissue.

Figure 1-1 Structure of the blood brain barrier
Structure of the blood-brain barrier, comprising different types closely surrounding the blood vessel.
However, IL-1β is thought to alter endothelial cell function via mitogen-activated protein (MAP) kinase and NF-κB pathway upregulation (Thornton et al., 2010), stimulating the expression of adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) (Konsman et al., 2007). Adhesion molecules anchor circulating leukocytes, primarily neutrophils, in the lumen of the blood vessel and help them pass trans- or paracellularly into the brain parenchyma at the site of injury (McColl et al., 2008). Once there, extravasated neutrophils may release decondensed DNA and protease tangles known as neutrophil-extracellular traps or NETs which are detrimental to neuronal survival (Allen et al., 2012). Leukocytes also degranulate, releasing more cytokines and matrix metalloproteinase-9 (MMP-9), which breaks down the extracellular matrix and disrupts the structure and function of the neurovascular unit, weakening the BBB and leading to oedema (Gidday et al., 2005).

IL-1β production and release from microglia, astrocytes and neurons leads to several other effects. It elicits a fever response via the thermoregulators in the central hypothalamus, (Rothwell and Luheshi, 2000) which can dangerously overheat the brain (Suehiro et al., 1999). IL-1β also increases the astrocytic production of arachidonic acid (AA) (Stella et al., 1997), which increases release and reduces re-uptake of glutamate (Vazquez et al., 1994). IL-1β is also involved in the regulating the nitric oxide synthase production of nitric oxide, which also has positive effects on the extracellular concentration of glutamate (Vidwans and Hewett, 2004). For these reasons and more discussed later, IL-1β is considered to have adverse effects on excitotoxic injury, the focus of this thesis project.
1.2.3 Glutamate and excitotoxicity

Glutamate is an excitatory amino acid which is released from the vast majority (~80%) of all synapses in the brain (Meldrum, 2000). It acts via several families of glutamate receptors, namely the metabotropic receptors (mGluRs), and ligand-gated ion channels (LGICs) including: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) and kainate (Doble, 1999). Over-activation of glutamate receptors leads to excitotoxicity via mass influx of Ca$^{2+}$ ions (Olney, 1969, Beal, 1992).

AMPA receptors (AMPARs) are cation channels but are generally less permeable than NMDA channels, especially to Ca$^{2+}$ ions (Dingledine et al., 1999). AMPARs lacking a GluR2 subunit are, however, much more calcium-permeable, leading some to believe that this particular subset of receptors are responsible for AMPA-mediated Ca$^{2+}$-excitotoxicity (Noh et al., 2005).

NMDA receptors are ionotropic receptors; however their effects are slower than the other two families of ionotropic channels. NMDA receptors are non-specific cation channels, which, when activated, allow the free down-gradient movement of K$^+$, Na$^+$ and importantly, Ca$^{2+}$ to the other side of the membrane (Dingledine et al., 1999). NMDA receptors can be blocked by intracellular Mg$^{2+}$ ions while the cell is polarised, but upon depolarisation the magnesium ‘unplugs’ the receptor, allowing further depolarisation (Mayer et al., 1984). In this way, NMDA receptor activation is indicative of strong excitatory transmission. Excitotoxicity can occur via these receptors, but usually in an exacerbative fashion, as cells need to depolarise first for magnesium to unstop the channel. Once open, activated NMDA channels can lead to a huge influx of Ca$^{2+}$ ions (Olney, 1969), leading to excitotoxicity.
Excitotoxicity has been implicated as the possible initial trigger or cause of progression in many neurological diseases, including stroke, traumatic brain injury, Alzheimer’s disease, Parkinson’s disease, multiple sclerosis and epilepsy (Beal, 1992, Doble, 1999, Pitt et al., 2003, Gressens et al., 2005). Evidence for the involvement of excitotoxicity in these diseases arises in some part from the experimental efficacy of glutamatergic antagonists against the progression of these conditions (Gressens et al., 2005, Lees and Leong, 2001).

The term excitotoxicity collectively describes what happens in the cell after over-activation of glutamatergic receptors. It often occurs when there is not enough oxygen and glucose to metabolise and produce sufficient levels of ATP to maintain transmembrane potential (Doyle et al., 2008). Neurons require a high amount of energy to maintain this potential, but when energy levels falter, the active transport ion pumps that usually provide the membrane potential fail and cells depolarise. After a stroke or epileptic seizure, this occurs on a much larger scale (Hossmann, 1996), leading to efflux of K+ and influx of Na+. These events invoke action potentials down the neuron’s axon (Caplan, 2000) resulting in further propagation of the mass release of glutamate.

Since the concentration of glutamate inside a neuron is comparatively high relative to the extracellular space, injured or necrotic neurons that spill their contents dangerously increase the extracellular concentration of glutamate from approximately 0.6μmol/L to as much as 5μmoL/L (Mark et al., 2001). Lowered energy stores inhibit astrocytes’ ability to remove released glutamate from the synapse via excitatory amino acid transporters (EAATs) (Gomi et al., 2000), allowing the extracellular concentration of glutamate to continue rising.
There are two different mechanisms of cell death in excitotoxicity: necrosis, or delayed apoptosis, depending on whether the over-activation of glutamate receptors is severe or mild/moderate, respectively (Ankarcrona et al., 1995, Bonfoco et al., 1995). In severe cases of excitotoxicity, ATPases needed to lower the intracellular concentration of Ca^{2+} no longer work due to a lack of energy, so the levels of Ca^{2+} rise from 0.1-1μM to approximately 100 times normal concentration (Beal, 1992). Ca^{2+}-dependent lipases, proteases and DNAses become activated and the cell’s organelles are enzymatically broken down (Doyle et al., 2008). Mitochondria collapse and dendritic and nuclear swelling occurs, ultimately followed by lysis as the cell dies from necrosis.

Delayed apoptosis, on the other hand, occurs when the cell temporarily recovers mitochondrial potential and neuronal energy charge within 3-12h after initial glutamate exposure, before undergoing cell-programmed apoptosis around 24h later (Ankarcrona et al., 1995, Bonfoco et al., 1995). The temporal dichotomy between early necrosis and delayed apoptosis is similar to that seen after stroke, where neurons in the vulnerable surrounding tissue tend to die from apoptosis some time after the core injury, which mainly undergoes rapid necrosis (Siesjo, 1992). The same difference between cell death mechanisms is also seen after TBI (Raghupathi, 2004) and in epilepsy (Henshall, 2007).

1.3 IL-1β and excitotoxicity

IL-1β’s role in neurodegeneration has often been attributed to its actions during excitotoxicity. IL-1β mRNA is upregulated in microglia following excitotoxic challenge with NMDA (Pearson et al., 1999) and the expression of IL-1β can be
alleviated by NMDA antagonism following ischaemic challenge (Jander et al., 2000), suggesting that IL-1β is reversibly dependent on excitotoxic activity. Upregulated expression of IL-1β is induced after status epilepticus (De Simoni et al., 2000) or exposure to kainate (Vezzani et al., 1999). IL-1β may exert its pro-excitotoxic effects through increased microglial activation (Hailer et al., 2005), by potentiating NMDA receptors via Src kinase activity (Viviani et al., 2003), or by priming cells before the insult (Aden et al., 2010).

IL-1β has been shown to prime cells to become more vulnerable to excitotoxic attack both in slice cultures (Bernardino et al., 2008) and in vivo (Vezzani et al., 1999), perhaps partially through its upregulation of neuronal kidney-type glutaminase (KGA) which raises the concentration of glutamate released from dying neurons (Ye et al., 2013).

The antagonistic actions of IL-1Ra, on the other hand, have repeatedly been shown to be neuroprotective in models of excitotoxicity (Relton and Rothwell, 1992, Lawrence et al., 1998) and seizure behaviour (Vezzani et al., 2000). More recently, it was reported that the lack of an isoform of the IL-1RacP protein, IL-1RacPb, decreased long-term survival after kainate excitotoxicity (Gosselin et al., 2013).

The exact mechanisms behind IL-1β’s enhancement of excitotoxic damage remain largely unknown. However, in order to better treat patients suffering from excitotoxicity, a driver of cell death in many neurological disorders, it is important to understand IL-1β’s actions.
1.4 The AMPA+IL-1β model

There have been several models of IL-1β-exacerbated excitotoxicity studied in the past, though most involve the injection of excitotoxic compounds – for instance NMDA, kainic acid or (S)-α-amino-3-hydroxy-5-4-isoxazolepropionic acid (S-AMPA, referred to henceforth as AMPA) directly into the brain, followed by application of IL-1β, which alone has very little, if not no, neurotoxic effect per se.

One study pertaining to this concept found that, even though striatally-injected IL-1Ra reduces the amount of NMDA- or AMPA-induced excitotoxic damage in the striatum, striatal co-injection of IL-1β with AMPA, but not with MGlut (an agonist specific for NMDA receptors) leads to not only excitotoxic damage in the striatum, but additional extensive damage in the cortex. While the initial striatal damage is mediated via striatal AMPA receptors, the NMDA receptor antagonist MK-801 applied directly to the cortex inhibited the AMPA+IL-1β-induced cortical damage, suggesting the cortical damage in mediated by NMDA receptor transmission.

Since the extensive cortical damage is particular only to the co-injection of AMPA+IL-1β, it has been used as a marker to study the exacerbation of excitotoxicity by inflammation. Furthermore, AMPA+IL-1β injected together leads to more severe seizure behaviour, making the AMPA+IL-1β a model of epilepsy-like disorders, as well as a tool with which to examine acute effects of IL-1β in the brain. In fact the model has even been likened to the pattern seen in stroke injury, where a vulnerable ‘penumbra’ tissue surrounding the core, dead zone may be fatally influenced by inflammation.
Interestingly, there are no ‘straightforward’ excitatory circuits leading from the striatum to the area of cortex damaged by AMPA+IL-1β challenge (Cotman et al., 1987, Nieoullon and Kerkerian-Le Goff, 1992), though there may be synaptic pathways via the piriform cortex (described more later) or thalamocortical circuits involved. IL-1β injected into the contralateral striatum after ipsilateral striatum application of AMPA exacerbates ipsilateral cortical damage, suggesting IL-1β exerts its actions by influencing neural pathways, rather than by diffusing through the brain (Lawrence et al., 1998).

IL-1β may act anterogradely upon these pathways, although evidence from synaptosomes suggests if this is the case, IL-1β would most likely potentiate glutamatergic transmission postsynaptically (described more later), rather than presynaptically (Allan et al., 1998). While retrograde transport of IL-1β to the cortex is theoretically possible, the timescale of damage seen in this model (<24h) deem this mode of action unlikely (Lawrence et al., 1998). The AMPA-alone damage is thought to occur in the striatum about 4-6h after injection, while the cell death that occurs in the cortex when AMPA+IL-1β are co-injected is delayed by approximately 8h (Patel et al., 2006).

As such, the AMPA+IL-1β model presents an opportunity to examine IL-1β-specific effects in excitotoxic challenge; in particular, within the cortex. Changes that occur within the cortex in the AMPA+IL-1β animals and not in the AMPA alone group may be attributable to IL-1β’s synergistic effects. In particular, since the model is acute, it is especially pertinent to study the early events that underlie the exacerbation of neuronal death seen in the AMPA+IL-1β group.
1.4.1 Hypotheses for AMPA+IL-1β-induced cortical cell death

While the acute mechanisms for the IL-1β-mediated exacerbations remain unclear, there have been several hypotheses regarding IL-1β’s actions derived from this model.

It has been suggested that IL-1β mediates cell death in the cortex by increasing seizure activity. Cortical cell death in kainic acid and AMPA-induced excitotoxicity models has previously been correlated to the amount of seizure activity, and anti-convulsants such as diazepam prevent the characteristic cell death (Benari et al., 1979, Patel et al., 2006, Lees and Leong, 2001). Blockade of NMDA glutamate receptors in the cortex prevents the cortical cell death usually seen after striatal application of AMPA+IL-1β (Lawrence et al., 1998). Interestingly, direct application of IL-1β onto rat hippocampal neurons potentiates NMDA currents but decreases synaptic transmission (Yang et al., 2005). In spinal cord experiments, IL-1β has been shown to increase the frequency and amplitude of spontaneous excitatory post-synaptic currents (sEPSCs), while decreasing the same aspects of spontaneous inhibitory post-synaptic currents (sIPSCs), by enhancing AMPAR activity and suppressing GABA and glycine currents (Kawasaki et al., 2008).

The AMPA+IL-1β has been described as an ‘all-or nothing’ model, in that the co-injection of AMPA+IL-1β either leads to the additional, marked cell death in a large area of the cortex, or does not exhibit much damage besides in the striatum and the piriform cortex. The piriform cortex has been especially implicated in this model, as AMPA+IL-1β-induced cell death, and in some cases the injection of AMPA alone, will lead to cell death in this area. Cortical damage tends to be worse the more ventrolaterally (that is, the closer to the piriform cortex) AMPA+IL-1β is
injected (Grundy et al., 2002, Patel et al., 2006). This area has often been implicated in the progression and generalisation of seizures to the rest of the hemisphere’s cortex (Loescher and Ebert, 1996). Moreover, the piriform cortex serves as a ‘synaptic gateway’ to the cortex from the striatum, since there are no direct striatum-cortical projections (Cotman et al., 1987). One hypothesis for the cortical damage seen in the AMPA+IL-1β model, therefore, is that seizure activity in the piriform cortex is exacerbated by IL-1β in the piriform cortex, leading to extensive seizures spreading dorsally to the rest of the cortex. This may explain how the cortex is affected by IL-1β, despite the fact that when AMPA is injected into the striatum and IL-1β into the cortex, cortical death does not occur (Lawrence et al., 1998).

IL-1β’s known disruptive effects on the BBB via recruitment of neutrophils (Shaftel et al., 2007, Anthony et al., 1997), has been shown to cause oedema (Anthony et al., 1997), which in turn exacerbates seizure activity (Fabene et al., 2008, van Vliet et al., 2007). Albumin, a serum plasma protein usually not found in the brain parenchyma, but which permeates through the leaky BBB, increases susceptibility to and lowers the threshold for seizures (Frigerio et al., 2012). Oedema and the presence of leukocytes in blood vessels have been also hypothesised, though not tested experimentally, to mechanically impair blood flow in this model, in a similar way to that seen after reperfusion in MCAo.

Another hypothesis arising from the AMPA+IL-1β model is the possibility that the IL-1β-dependent acute fever response – evidenced by an increase in heart rate and temperature – worsens neuronal injury (Allan et al., 2000). Elevated brain temperature can exacerbate seizure activity (Suehiro et al., 1999) and glutamate excitotoxicity during ischaemia (Campos et al., 2012). However, if AMPA is
injected striatally and IL-1β administered to the ventricles, the cortical damage does not occur, so it is unlikely that body temperature is the main driver of the extrastriatal damage caused by striatal AMPA+IL-1β (Grundy et al., 1999).

1.4.2 The vascular hypothesis

Functional decoupling of neuronal activity from the normal, reactive increase in blood supply is another action through which IL-1β has been hypothesised to contribute its effects in the AMPA+IL-1β model.

Decoupling of the haemodynamic response is a logically plausible effect of IL-1β in the brain. Seizure activity, which is exacerbated by IL-1β and inflammation (Choi and Koh, 2008, Fabene et al., 2008, Viviani et al., 2003, Degos et al., 2013), has been shown to lead to a decoupling of the haemodynamic response (Meldrum and Nilsson, 1976, Bahar et al., 2006, Fox et al., 1988, Geneslaw et al., 2011, Hirose et al., 2009). By increasing seizure activity and impairing the replenishing hyperaemia, IL-1β could mediate a metabolic imbalance in cortical cells that could be lethal or render them more susceptible to excitotoxicity.

The neurovascular unit that controls the dilation of blood vessels in response to neural activity is made up of astrocytes, endothelial cells, pericytes and neurons, all of which are affected in various ways by IL-1β (John et al., 2005, Giulian et al., 1988, Hawkins and Davis, 2005, McColl et al., 2008, Thornton et al., 2010). In particular, there is evidence that cerebral IL-1β induces astrocytic end-feet swelling and astrogliosis (Giulian et al., 1988), which could theoretically impede cerebral blood flow responses by compressing capillary walls.
In order to test whether IL-1β affects the functional coupling of blood flow and neural activity, it would be pertinent to use an imaging method that fits particular experimental requirements. The method (or methods) must be relatively fast, but repeatable, as cell death in the AMPA+IL-1β model occurs in the striatum at 4-6h after injection. It also must have high spatial and temporal resolution, to measure fast changes in regional blood flow, and it should be possible to record functional changes prior to injection, to act as a baseline functional response. In this project, two main methods have been used to study the effects of IL-1β on the haemodynamic response and neural activity during excitotoxicity. Firstly, optical imaging spectroscopy (OIS), which uses different wavelengths of light to examine the various components of the haemodynamic response, and secondly, a miniaturised Clark-type tissue oxygenation probe coupled with an electrode for measuring electrical potentials.

1.5 Optical imaging of the haemodynamic response

Many techniques have been engineered with the aim of imaging the brain; in particular, the areas of increased activity in response to certain sensory, motor or cognitive demands. While single- and multi-cell recordings offer the best method of investigating the function of single cortical cells, it is often useful to study the response of a group of these cells. Groups of neurons in the brain that are clustered together often share a function or respond in a similar way to the same stimulus (Mountcastle, 1957, Hubel and Wiesel, 1965).

Optical imaging is an advantageous method of imaging blood flow on the scale of groups of neurons (Grinvald et al., 2001). It is an in vivo method of
obtaining recordings with especially high spatial and temporal resolutions; 50µm and milliseconds, respectively. Secondly, when used on anesthetised rat, it allows repeated measurements from the same animal over extended periods of time as the skull and dura can remain intact over the brain. Unlike a number of other techniques used in imaging neuroinflammation, optical imaging does not require ionising radiation, isotopes or fluorescent gene markers, reducing possible confounds in its technology (Wunder et al., 2009).

Optical imaging uses a very sensitive charge-coupled camera (CCD) camera to capture light reflected back from the brain, to create an image of the illuminated area of cortex. Some of the changes that are detectable by optical imaging techniques are very small, around 0.1%, so the equipment must be extremely accurate and must not fluctuate for response recordings to be reliable. Changes in the amount of light that is reflected back to the camera depend on how much light is absorbed by the tissue and the blood – or rather, its various components within it, described in the next section.

When using optical imaging, it is important to aim for the best signal-to-noise ratio possible, as the changes being detected are very small and vulnerable to physiologic effects such as heartbeat and respiration, as well as general artefacts (Grinvald et al., 2001, Mayhew et al., 1996). A lot of care must be taken in data processing and analysis to avoid over-manipulating the data, while still achieving clear results specific to the experiment conditions (Schiessl et al., 2008).

The main limitation with optical imaging using visible wavelengths of light is that presently it can only be used to image either flat or more convex surfaces of the brain, about 1mm below the surface (Yousef et al., 1999), not including the sulci, or
folds in the cortex. The other limitation, specifically in imaging animals larger than mice, is that the skull must be carefully thinned to about 100µm before light can sufficiently penetrate to and be reflected back from the cortex (Frostig et al., 1994). The procedure of thinning the skull (usually with a drill) also carries the risk of causing heat damage to the cortical area being imaged.

1.6 The haemodynamic response

The brain’s blood supply is extremely precious for the maintenance of neuronal function. Despite being only consisting 2% of the body’s mass, the brain demands 20% of the total oxygen and energy demand, which is of course delivered via the bloodstream. It is estimated that the brain uses 3.5mL O₂ per 100g tissue per minute (Siesjo, 1978, Ames, 2000). Neurons create most of this demand: 80% of the blood demand of the brain is a result of neuronal metabolism requirements, a lot of which is used in transmitting the action potential down one neuron and synapsing onto the next (Rolfe and Brown, 1997). When neurons are active, their demand for oxygen and glucose increases to allow for metabolism to generate ATP stores (Leniger-Follert and Hossmann, 1979).

Following neuronal metabolism, the blood vessels supplying the active area vasodilate, increasing the regional cerebral blood flow (rCBF) (Fox et al., 1986). The typical haemodynamic response seen following somatosensory activation, for instance, through tactile stimulation, comprises an initial ‘dip’ of oxygenation as cells metabolise glucose and oxygen stores. This is accompanied by a concurrent increase in the concentration of deoxyhaemoglobin, and followed by a large increase in oxygenation, peaking at 3-4 seconds after the start of stimulation, termed
functional hyperaemia, or (collectively with the ‘initial dip’) the haemodynamic response. Cellular activity has been shown, under normal physiological conditions, to be tightly associated with the resulting haemodynamic response in a system called neurovascular coupling (Berwick et al., 2005b). There are several mechanisms through which blood supply is coupled to neural activity.

One proposed process involves neuronal products of the activation and $\text{Ca}^{2+}$ ion influx, including nitric oxide (NO), which diffuse out of the cell and act on smooth muscle cells, causing them to dilate (Stefanovic et al., 2007). An alternative hypothesis is that on binding or taking up glutamate from the synapse, increased intracellular levels of astrocytic $\text{Ca}^{2+}$ results in the activation of a cascade producing prostaglandin E$_2$. This may then exit the cell via the astrocyte’s end-feet, directly onto smooth muscle cells, inducing vasodilation (Rossi, 2006).

Another theory is that pericytes - the vascular smooth muscle cells surrounding the cerebral blood vessels - constrict in response to mediators such as ATP and noradrenaline, whereas their processes grow, allowing vasodilation, when substances such as glutamate are administered (Peppiatt et al., 2006). Lastly, it is possible that the vessel’s smooth muscle cells are directly innervated by neurons in the active region itself. It is most likely that neurovascular coupling is a result of a mixture of all of these proposed mechanisms. Interestingly, and perhaps luckily for those wanted to study the control of blood flow in the brain, the haemodynamic response is an overcompensatory reaction to neural activity, both spatially and temporally, although one possible explanation for the spatial ‘overspill’, particularly within the somatosensory cortex, could be that the extra blood is directed to surrounding cortices to support predicted neural responses in these areas (Berwick et al., 2008).
One of the important ways in which the specific elements haemodynamic response are being understood is through optical imaging spectroscopy (OIS), which uses optical imaging to dissect various components of the functional hyperaemia to stimulation.

1.7 Optical imaging spectroscopy

Optical imaging spectroscopy (OIS) involves using a number of different filtered wavelengths of light to illuminate the cortex in order to obtain signals relating to concentration changes of imaged chromophores. By using a modified version of the Beer Lambert law, it is possible to pinpoint various points on the wavelength spectrum which coincide with the maximum absorption of a certain substance, for instance, oxy-haemoglobin (HbO) (Berwick et al., 2005b). Figure 1-2 illustrates the absorption spectra of oxy- and deoxy-haemoglobin. Using OIS of the cortex vasculature, it is possible to identify changes weighted towards levels of oxyhaemoglobin (HbO) by using a 577nm wavelength filter; a filter for 560nm for the deoxy-haemoglobin (HbR) and a filter for either of the isobestic points (in this case, around 550nm) for the total concentration of haemoglobin (HbT) (Berwick et al., 2005b).
Figure 1-2 Absorption spectra of oxy- and deoxy-haemoglobin
Diagram showing part of the absorption spectra of HbO (red) and HbR (blue). Also shown are the position of the wavelength filters used in optical imaging spectroscopy. Changes in reflectance imaged using Filter 1 (577±5nm) are associated with changes in HbO, while Filter 2 (560±5nm) can be used to image differences in levels of HbR. Filter 3 (550±5nm) can be used to image changes in HbT. This data was calculated with the assumptions that the concentration and saturation of haemoglobin are 104µM and 50% respectively (Prahl, 1999).

The typical haemodynamic response as measured using OIS consists of several components, each of which shows different temporal characteristics (Figure 1-3). The first is the measure of total haemoglobin (HbT), the increase in the volume of blood in the vessels, which occurs around 3s after the onset of the stimulus. The second, after a similar delay, is the HbO content of the supplying blood vessels. Thirdly, OIS can detect the changes in the concentration of deoxy-haemoglobin (HbR). A time course of HbR concentration during stimulation usually shows what is referred to as the ‘deoxy-dip’ or ‘initial dip’, followed by a relative decrease, indicating the net increase in HbT. It has been heavily debated as to what the initial
dip may be attributed (Hu and Yacoub, 2012), although it is generally thought to be due to the activity-dependent deoxygenation of haemoglobin in the blood before an increase in blood flow has become established (Schiessl et al., 2008, Mayhew et al., 2000, Berwick et al., 2005b, Berwick et al., 2008).

![Figure 1-3 Example OIS whisker stimulation response](image)

**Figure 1-3 Example OIS whisker stimulation response**
Characteristic frame time courses showing the oxygenated haemoglobin (HbO), total haemoglobin (HbT) and deoxygenated haemoglobin (HbR) in response to 4s whisker stimulation. Maximum response frames are indicated by arrows. Darker pixels are representative of a lower concentration of the blood component. While HbO and HbT exhibit an increase around 3s after stimulus onset, HbR shows a small initial increase, followed by a relative decrease as the deoxygenated haemoglobin is ‘washed away’. Figure from the Schiessl lab.

OIS provides a relevant, high-resolution and fast method for measuring the haemodynamics upon cortical activation. To test whether IL-1β modulates the neurovascular response in the brain during excitotoxic challenge, it is important to utilise a well-researched paradigm of such functional coupling. The rat barrel cortex is a very well-researched system and incidentally is situated inside the area of cortex typically injured by the striatal co-injection of AMPA+IL-1β, close to branches of the middle cerebral artery (Lawrence et al., 1998, Paxinos and Watson, 2006).
1.8 The rat barrel cortex

The barrel cortex in rodents is one area of brain research to which optical imaging can – and has been – applied successfully (Berwick et al., 2005b, Jones et al., 2005, Mayhew et al., 2000). The somatosensory barrel cortex is a somatotopic representation of the mystacial pad, which holds the whiskers or vibrissae. When a whisker is deflected, mechanically-gated Merkel, Ruffini and Lanceolate sensory receptors are activated and transmit action potentials along the maxillary nerve – the second branch of the trigeminal nerve - to the ipsilateral brainstem, where they synapse in the trigeminal nucleus’ ‘barrelette’ representation of the whisker (Veinante and Deschenes, 1999, Petersen, 2007). Here, the inputs from the whisker split into three pathways: the (main) lemniscal, paralemniscal and extralemniscal. The lemniscal pathway extends from the principal nucleus of the trigeminal nucleus across the midline to the dorsomedial section of the ventral posterior medial nucleus of the contralateral thalamus (VPMdm), where again groups of neurons are arranged somatotopically, this time in so-called ‘barreloids’ (Brecht and Sakmann, 2002).

From here, glutamatergic neurons project to layer IV of the somatosensory cortex, congregating together to form separate barrels of densely packed spiny, stellate neurons (Woolsey and Vanderlo, 1970), as well as weakly innervating upper layer VI. Some neural communication overlaps between barrels, presumably to integrate multi-whisker inputs (Petersen, 2007). The majority of sensory input to the barrel arrives from the lemniscal pathway. The paralemniscal pathway leaves the trigeminal nucleus from the rostral interpolar nucleus instead, synapsing in the posterior medial nucleus of the thalamus (POM) and projecting to layers I and Va of the barrel cortex. This pathway is thought to be involved in active exploration and is not considered to be as active in anaesthetised animals (Furuta et al., 2006).
extrastriatal pathway goes via the interpolar nucleus and the ventrolateral section of the VPM, before sending inputs to the septa between barrels and the secondary somatosensory cortex (S2)

**Figure 1-4 The rat whisker lemniscal pathway**

A. The lemniscal pathway from the whisker matrix via the brainstem and thalamus to the barrel cortex, particularly layers IV, and partially VI. B. A tangential brain section showing the somatotopic barrel cortex map, stained with cytochrome C oxidase. C. Cortical layers of the barrel cortex I-VI, with barrels indicated by asterisks. Picture from a review (Schubert et al., 2007).

Thanks to the wealth of previous research carried out on the barrel cortex, the normal neural and coupled haemodynamic responses to whisker stimulation are well-characterised and mapped (Berwick et al., 2005b, Mayhew et al., 2000, Berwick et al., 2008). It is a good model of the somatosensory cortex in general because the barrels are so clearly defined and obtaining a typical response from a whisker deflection is relatively replicable and easy to do, even when the animal is under anaesthesia (Berwick et al., 2005b, Berwick et al., 2008). Each barrel is approximately 400-500µm wide so the resolution that OIS provides is ideal for imaging this system.
The rat barrel cortex lends itself particularly well to the study of cerebral blood flow. Blood vessels in the barrel cortex are arranged in a particularly conserved fashion, where the vessel branches from the MCA are directed towards cerebral vascular units, closely coupled with the barrels themselves (Cox et al., 1993). From the surface of the brain, vessels descend, arborizing most in layers III/IV and least in layers I and V, resulting in a ‘spindle’-form vascular arrangement (Patel, 1983). The laminae themselves therefore tend to receive layer-weighted shares of the cerebral blood flow, though this may be due to the various levels of activity (Goense et al., 2012). Unlike non-cortical sites, the barrel cortex generally exhibits linear coupling between the neural activity and the amplitude of the blood flow response (Devonshire et al., 2012). By intensifying stimulation of the whisker, the haemodynamic response is proportionately increased (Mayhew et al., 2000).

The origins of neural activity in the rodent barrel cortex are also well-researched, as it is considered representative of most sensory cortices (Buzsaki et al., 2012). If we are to understand possible interactions between IL-1β, glutamate excitotoxicity, and cerebral blood flow, we need to obtain a picture of the functioning neurons themselves and the relationship with the blood flow responses to their stimulation. The neurovascular coupling relationship upheld within the barrel cortex in response to single whisker stimulation has, again, been extensively studied in the past using BOLD fMRI (Devonshire et al., 2012, Goense et al., 2012), slit spectroscopy (Mayhew et al., 2000), laser Doppler (Hewson-Stoate et al., 2005), 2-D OIS (similar to that used here in this thesis) (Berwick et al., 2008, Devor et al., 2003), electrode recordings (Einevoll et al., 2007), and by combining electrode and imaging techniques (Berwick et al., 2005a, Berwick et al., 2008).
While OIS may be lauded for its measurement of haemodynamics within the system, simultaneous electrode recordings are one of the only ways of determining whether or not neurovascular coupling is ongoing. The present study uses mechanical whisker stimulation to evoke a neural and haemodynamic response in the barrel cortex. Various techniques have been used previously to determine whether the relationship between the neural and haemodynamic responses in the rat somatosensory cortices to increasing stimulus intensity (frequency or amplitude) of stimulation is linear (Martindale et al., 2003, Ngai et al., 1999), non-linear (Devor et al., 2003, Sheth et al., 2004), or, as it may appear, linear at mid-range intensities (Hewson-Stoate et al., 2005). Since the current study utilises such a ‘mid-range’ frequency of whisker stimulation (8 Hz), we would expect a linear relationship between the extent of the neural and haemodynamic responses.

The APOX electrode is a Clark-type oxygen tension probe and platinum action potential microsensor (Thompson et al., 2003). It records electrical activity and tissue oxygen partial pressure simultaneously around the electrode tip, giving a measure of the haemodynamic integrity and cellular function (Thompson et al., 2003). Using this polarographic oxygen sensor, the typical biphasic signal is seen: the initial dip in oxygenation, followed by a positive overshoot (Thompson et al., 2003, Li and Freeman, 2007, Li and Freeman, 2011). As described previously, this typical profile represents the initial increased oxygen consumption by active cells, followed by the functional hyperaemia replenishing the active area with fresh blood flow (Malonek and Grinvald, 1996, Mayhew et al., 2000). Previous work using this instrument, predominantly by Freeman and colleagues, have examined neurometabolic coupling in the anaesthetised cat’s visual system (Li and Freeman, 2007, Li and Freeman, 2011) and have also found a largely linear relationship.
between the stimulus-evoked neural activity and corresponding haemodynamic response (Li and Freeman, 2007). Because of the initial dip’s relatively small amplitude (more closely representing the spatial scale of cellular activity), the change in oxygenation can be recorded from up to a few hundred micrometres around the tip of the electrode (Thompson et al., 2005), the same volume from which extracellular potentials can be received by the electrode (Legatt et al., 1980, Katzner et al., 2009), meaning that simultaneous oxygen concentration and indirect measures of neural metabolism are derived from the same volume of active tissue (Thompson et al., 2005). On the other hand, the positive overshoot of oxygenation elicited after the initial dip can spread over 1-2mm (Thompson et al., 2005), so positive changes may be picked up by the tissue oxygen sensor further away from the active group of neurons.

1.9 Extracellular potentials in the sensory cortex

Extracellular potentials recorded by a microelectrode placed in the resting or stimulated sensory cortex may be split into two major components: local field potentials (LFPs), which are low frequency (0-100 Hz) and multi-unit activity (MUA), comprising high frequency (>300 Hz) activity (Logothetis, 2002). LFPs are generally thought to result from synchronised dendro-somatic inputs into the system. They are representative of the sum current of the slower oscillations produced by the cells within a relatively large volume of cortex from which the electrode records, usually around 0.5-3mm (Mitzdorf, 1987, Juergens et al., 1999). On the other hand, MUA reflects the total current elicited by all the spiking action potentials, or synaptic outputs, near (within a 50-350µm radius) to the tip of the electrode (Legatt
et al., 1980). In previous studies of the barrel cortex, whisker stimulation-evoked extracellular potentials have been shown to peak and decrease rapidly after stimulus onset (Devor et al., 2003), followed by a slower activation (Moore and Nelson, 1998), thought to be representative of the slower-acting NMDA-receptor activation (Armstrong-James et al., 1993).

Pre-synaptically-derived LFP oscillations have long been thought to be responsible for the lion’s share of the cortex’s energy demand (Attwell and Laughlin, 2001). This conclusion arises from the estimate that around 80-90% of the glucose metabolised within the cortex is owing to glutamatergic communication (Sibson et al., 1998) and ATP-dependent glutamate/glutamine recycling by astrocytes (Magistretti and Pellerin, 1999, Pellerin and Magistretti, 2004).

To visualise LFPs, broadband signal from the cortex can be low-pass filtered usually between 0-150 Hz. The low-frequency oscillatory signal from the cortex can then be subsequently divided into frequency bands, including delta (0-4Hz), theta (4-8 Hz), alpha (8-13 Hz), beta (13-24 Hz) and gamma (24-100 Hz), which are each associated with increasing LFP amplitudes and different arousal states. Delta and theta waves are believed to reflect sleep, while alpha activity is associated with relaxed wakefulness. In contrast, beta and gamma oscillations are attributed to thalamocortical loops associated with more intense mental activity, for instance the processing of sensory inputs. These rhythms are known to be influenced by the ascending and basal forebrain arousal networks (Steriade and Hobson, 1976, Steriade et al., 1993).

The LFPs included in the gamma range depend on tightly synchronised inhibitory interneuron networks. Inhibitory interneurons make up around 15% of the
cells in the cortex (Galarreta and Hestrin, 1999). These fast-spiking GABAergic neurons oscillate rhythmically and reciprocally and, in doing so, create windows of inhibition for excitatory inputs carrying sensory information to assemble coherently (Jones and Barth, 1997).

MUA in layer IV of the somatosensory cortex is thought to be dependent on pyramidal cell spiking activity. These spikes, each lasting 0.4-1 ms (Logothetis, 2002), carry information on aspects of the stimulus presented to the whisker, including the frequency, direction and amplitude of deflections (Khatri et al., 2004, Bale and Petersen, 2009).

The LFP signal tends to be more tightly coupled with the haemodynamic response than MUA, even though MUA and LFP activity usually correlate with each other (Kikuchi et al., 2010, Magri et al., 2012, Bruyns-Haylett et al., 2013, Haslinger et al., 2006). In particular, beta (Stevenson et al., 2011) and gamma frequencies have been shown to be more strongly coupled with the haemodynamic response (Niessing et al., 2005, Logothetis, 2002, Stevenson et al., 2011), with some evidence suggesting that the power and ratio of gamma and beta oscillations in the cortex may predict the amplitude and latency of the BOLD response (Magri et al., 2012).

1.10 Aims and Objectives

The aim of this thesis was to elucidate the early mechanisms through which IL-1β exacerbates AMPA-induced excitotoxicity. The AMPA+IL-1β model comprises a strong seizure activity, as well as elements of oedema, fever and neutrophil recruitment, all known to have deleterious consequences in the CNS. One hypothesis of IL-1β’s actions is that it may upregulate seizure activity while
impairing the haemodynamic response, the functional supply of oxygenated blood to active regions of the cortex. Together with the AMPA-induced excitotoxic challenge, neurovascular decoupling could manifest as a symptom of neuronal dysfunction, or it may worsen the injury by making the cortex more vulnerable to the metabolic disruption incurred by seizure activity. Importantly, since AMPA+IL-1β-induced cell death takes a matter of hours to occur, one of the main objectives of this thesis was to identify the early changes in neural activity and the haemodynamic response, before significant cortical damage was sustained.

To meet these aims, two methods were used: optical imaging spectroscopy and extracellular electrode recordings with simultaneous tissue oxygen sensing. Combined with histological methods quantifying the infiltration of neutrophils and disruption of the BBB, these methods were used to examine the haemodynamic response, as well as LFP and MUA responses to whisker stimulation within the first hours after the striatal injection, before any significant cell death occurred.
2 Materials and Methods

This chapter will describe the materials and methods used for both results chapters detailed in this thesis. The first results chapter comprises two studies. Firstly, a time-course characterisation of early OIS and histological effects in anaesthetised rats injected with striatal AMPA+IL-1β and maintained under anaesthesia for a further 2, 4, 6, or 8h. Subsequently, haemodynamic responses and histological parameters were compared between groups of anaesthetised rats injected with vehicle, AMPA, IL-1β or AMPA+IL-1β.

The second results chapter investigates changes in cellular activity (LFP and MUA) and local tissue oxygenation in the stimulated whisker barrel in anaesthetised rats before and up to 6 h after striatal injection of vehicle, AMPA, IL-1β or AMPA+IL-1β.

2.1 Procedure

2.1.1 Animals

All animal procedures were carried out in accordance with the UK Animal (Scientific Procedures) Act 1986. Male Lister Hooded rats (Charles River, UK) weighing 250g-400g were housed in cages of up to 5, at 21-23°C and 60±5% humidity, with a 12h light/dark cycle and with access to standard rat chow and water ad libitum. Previously, the AMPA+IL-1β model has used male Sprague Dawley rats, but a pilot study indicated that the co-injection of IL-1β with AMPA into Lister Hooded rats does still lead to a poorer outcome (as assessed by seizure severity and rate of mortality) than injection of AMPA alone. Since the majority of optical
imaging studies have been carried out in outbred strains such as Lister Hooded, these rats were chosen for the purposes of this thesis.

### 2.1.2 Surgical preparation

Anaesthesia was induced with a 4ml/kg i.p. injection of 25% Hypnorm (0.315mg/mL fentanyl, 10mg/mL fluanisone; Roche UK) and 25% Hypnovel (1mg/mL midazolam; Vetapharm Ltd. UK) in sterile water (Braun, UK). This method of anaesthesia induction wears away after approximately 20 min, allowing for the surgical preparation needed for artificial ventilation. Once the pedal reflex was lost, the animal’s scalp and throat were shaved and the animal kept on a warming plate throughout tracheotomy surgery. Iodine surgical scrub (Betadine, Seton Healthcare Group, UK) was used to disinfect the skin over the trachea and 0.2mL xylocaine (AstraZeneca) injected subcutaneously along the line of incision. The animals were then tracheotomised using a 2.0 trachea tube (Vygon UK, Ltd.) before being placed on a stereotaxic frame (Kopf Instruments, USA) and another 0.1mL xylocaine administered into each ear before inserting ear bars.

Animals were artificially ventilated with 1% isoflurane (Abbott, Berkshire) in room air using a Zoovent Jetsys ventilator (Universal Lung Ventilators Ltd. UK), and endtidal CO$_2$ was monitored using a Capnogard ETCO2 (Novametrix Medical Systems Inc., USA) and the breathing rate occasionally minimally adapted to maintain an endtidal CO$_2$ of between 25-39mmHg for the duration of the experiment to ensure adequate ventilation and to prevent hypercapnia, which may affect baseline perfusion as well as the haemodynamic response. The animal’s core temperature was monitored using a rectal probe and maintained at no less than 37°C by a
homeothermic heat mat (Harvard Apparatus). A Tiger Pulse Veterinary Oximeter (Thames Medical) with a rectal probe was used to measure core tissue oxygenation to ensure the animal’s blood was well-oxygenated throughout the experiment. Electrocardiogram (ECG) recording electrodes were attached either side of the animal’s chest and a ground electrode was placed on the hind leg. The ECG signal was amplified using a D150 amplifier (Digitimer Ltd., UK) and visualised with a counter (TA200, IVO GmbH & Co., KG). ECG and core temperature readings were recorded every 20 min before the start of each imaging or electrode experiment to measure febrile responses elicited by striatal injection of IL-1β. While small mammals are not able to maintain their body temperature under anaesthesia (Malkinson et al., 1988), the heat mat feedback mechanism did not allow the animal’s core temperature to fall below 37.6°C, so increases in body temperature (and heart rate) served as a marker of the fever response often seen after central injection of IL-1β (Busbridge et al., 1989).

Once the animal was maintained on anaesthesia and no corneal reflex could be elicited, 0.1mL xylocaine was injected along the dorsal midline of the scalp. This line was then incised with a scalpel and the cranium exposed using a surgical scraper tool. A hole in the cranium for the intrastriatal injection was drilled using a dental drill with a 0.8mm drill bit (Mesinger, Germany) at co-ordinates 2.7mm ipsilateral (the animal’s left) to and 0.5mm anterior to Bregma (Paxinos and Watson, 2006). For electrode recording experiments, a shallow dimple was drilled in the cranium at 4mm contralateral (animal’s right) and 4mm posterior relative to Bregma, into which a flat-point screw was inserted to act as a ground electrode.

For OIS, a rectangular area of bone measuring ~4x4mm over the somatosensory cortex was thinned slowly and uniformly to translucency (100µm)
using a dental drill, intermittently cooled using chilled (4°C) 0.9% saline (Braun) to prevent heat damage. Any bleeding vessels in the cranium were stopped with bone wax (Ethicon, UK) and a well was made around the imaging area using dental cement (Kemdent, UK). The well was filled with warmed (37°C) 0.9% saline, reducing the effect of specularities on the surface of the bone, and sealed with a 1.2mm diameter glass coverslip (Scientific Laboratory Supplies Ltd., UK) using Vaseline (Unilever) to prevent evaporation.

2.1.3 Optical imaging spectroscopy and hardware

OIS was used to measure the functional haemodynamic response to whisker stimulation before and every 20 min after striatal injection of AMPA, IL-1β, AMPA+IL-1β or vehicle (see Striatal injection below).

A charge-coupled device (CCD) 1M30 Pantera camera (Dalsa Imaging) was positioned normal to the imaged surface, which was illuminated with light from a halogen projection lamp (6958 24V 250W G6.35, Phillips) that passed through four wavelength filters (550±10nm, 560±10nm, 577±10nm and 700±20nm) transmitted via a fibreglass cable. Images obtained using the 700nm wavelength filter, associated with changes in tissue scattering, were not utilised in these studies.

Filters were alternated at 32Hz by a Lambda DG-4 filter changer (Sutter Instruments) to obtain an 8Hz frame-rate for each wavelength. The Lambda DG-4 was powered by an uninterrupted CPX200 Powerflex power supply (TTi) using 20V and 10A. Image acquisition, stimulus generation and filter alternation were synchronised and mediated using a BNC 2090 (National Instruments) connected to a
PCI data acquisition card (PCI-MIO-16E-4, National Instruments) all controlled by MATLAB (MathWorks Inc., USA).

A BNC 2090 connector block (National Instruments) generated three output channels: to the stimulus generator; to the Lambda DG-4 (allowing synchronisation between the stimulus and the filter changer) and to the PC (IBM) via a multi input-output card (National Instruments). A PC frame grabber card with a CameraLink port and a parallel port connected to the filter changer enabled synchronisation between the CCD camera and the filter changer to ensure that the filter and frame rates were identical. The BNC 2090 also collected data from the electrode (described in 2.1.6) and feedback data from the stimulus generation.

A region of interest (ROI) of the barrel cortex was selected using a custom-made general user interface (GUI) created on MATLAB. Immediately after an imaging experiment, the quality of the experiment was checked using a custom-made program displaying: the spatial activation of the barrel; the mean ROI grey level throughout the experiment; the average trial for each wavelength filter and the power spectrum of the collected signal. This meant that experiments affected by, for instance, filter skips or bubbles/bleeds occurring within the ROI could be repeated.

### 2.1.3.1 Whisker stimulus

Mechanical stimulation of the E1 whisker was achieved using a piezoelectric ceramic actuator (Picma) with an 8Hz square wave from an alternating current amplifier (E-650.00 LVPZT; Physik Instrumente GmbH & Co. KG). This frequency and waveform was chosen from previous work done in the lab as giving the largest reliable haemodynamic response (unpublished results). One imaging experiment
consisted of 30 trials, each 13s long, comprising 1s pre-stimulus, 4s stimulation, followed by 8s recovery. After the baseline imaging and subsequent injection of vehicle/AMPA/IL-1β/AMPA+IL-1β, imaging experiments or electrode recordings were carried out every 20 min up to 2, 4, 6 or 8h depending on the end point of the study.

2.1.4 Striatal injection

After baseline OIS or electrode recordings, animals received direct striatal injection of a 1µL solution of either vehicle, 5nM S-AMPA (Tocris Cookson, UK) in vehicle, 50ng/µL human recombinant IL-1β (hrIL-1-β; New Brunswick Scientific Co., Inc.) in vehicle, or AMPA+IL-1β combined (5nM and 50ng/µL respectively). Human recombinant IL-1β has always been used within the AMPA+IL-1β model, and evidence suggests IL-1β highly conserved (Bird et al., 2002). Solutions also contained a small amount of mistral blue in order visualise the solution during injection and the site of injection after the experiment. All solutions were made using the vehicle: 0.5% sterile endotoxin-free bovine serum albumin (BSA) in PBS. All injected solutions were mixed on the day of injection from stock aliquots stored at -80°C and were kept on ice until use.

Injections were carried out using a pulled glass microneedle (Sigma-Aldrich, P0549) with the pressure from a 5mL syringe at an approximate rate of 0.5µL/min. Glass microneedle injections lead to less collateral damage and inflammation around the needle tract than Hamilton syringe injections (McCluskey et al., 2008). Injected solutions for animals were not randomised or blinded, but injections were varied in order where possible to avoid effects of age or weight between groups. The injection
site was 0.7mm anterior, 2.7mm lateral to Bregma, and 5.5mm ventral to the dura, which was nicked with a 24 gauge needle just before injection. After injection, the microneedle was kept in place for 5 min - to prevent reflux of the solution - before removal and recommencement of imaging/electrode recording.

2.1.5 Tissue processing, immunohistochemistry and histology

After the final imaging experiment or electrode recording, animals were overdosed with anaesthetic (5% isoflurane and i.p. injection of 2mL 20% sodium pentobarbitone (Pentoject; AnimalCare Ltd.) and perfused transcardially with 4°C 0.9% saline nitrate and then 4% paraformaldehyde for 10 min. Brains were fixed overnight in 4% paraformaldehyde and post-fixed in 30% sucrose/PBS for >24 h. Coronal slices 30µm thick were cut in series 360µm apart on a sledge microtome and stored at -20°C in cryoprotectant (6.6g/L disodium hydrogen orthophosphate and 0.79g/L sodium dihydrogen orthophosphate in 30% ethylene glocol, 20% glycerol in dH₂O) until needed for immunohistochemical analysis.

The Nissl stain cresyl violet, while appropriate for demarcating areas of cell death at longer time points (~24h), did not leave a visible outline of damaged tissue at this early time point, however, some automated analyses were used on cresyl violet stained tissue described below. SJC-positive neutrophils and IgG immunostaining were found to be the best markers for early inflammation in this model.
2.1.5.1 Immunohistochemistry

Immunostaining of immunoglobulin G (IgG) and neutrophils was performed on free-floating sections. To identify neutrophils, sections were washed for 10 min three times with PBS, incubated with 0.3% hydrogen peroxide (Sigma) for 10 min and washed in PBS, again for 10 min three times. The sections were then incubated in 2% normal goat serum (Vector) in primary diluent (0.3% Triton in PBS) for 1h before being incubated overnight at 4°C with the primary anti-SJC antibody (raised in rabbit; kindly gifted by Dr Daniel Anthony and Sandra Campbell, University of Oxford, UK) at a dilution of 1:300 in primary diluent. The next day the slices were washed 3 times with PBS and then incubated with a biotinylated anti-rabbit secondary antibody (1:500; Vector) in primary diluent for 2h. An ABC kit (Vectastain) mix of components A and B in PBS (17.5µL + 17.5µL in 13mL respectively) was prepared 30 min before use. After washing in PBS as before, slices were incubated for 1h in the ABC mix, before developing using 0.01% diaminobenzidine (DAB; Sigma) and 0.005% hydrogen peroxide in dH₂O. Samples were finally washed in PBS before mounting onto slides and coverslipping using DPX mounting medium (Sigma-Aldrich). For IgG immunostaining the same steps were carried out as with the SJC, except no primary antibody step was required and a biotinylated anti-rat IgG antibody (made in goat; Vector) diluted 1:500 in primary diluent was used instead of the secondary antibody.

2.1.5.2 Histology

Cresyl violet stain was performed on slices from brains taken from electrophysiology experiments. Sections were mounted on gelatinised slides before incubating in ascending concentrations of ethanol (50, 70, 90, 100%) for 2 min each,
before placing in Ultraclear (Mallinckrodt Baker, UK) for 9 min and subsequent
immersions in descending concentrations of ethanol (100, 90, 70, 50%). Slides were
then stained in 2% cresyl violet solution for 2.5 min and cleared for 3 min in
ascending concentrations of ethanol (50, 70, 90%, 90% with acetic acid, 100%)
before being placed in Ultraclear for 4 min. Slides were coverslipped with DPX and
left to dry overnight in a fume cupboard before analysis.

2.1.6 Electrode recordings

In the third study, Lister Hooded rats were used to investigate the function-
associated neural activity within layer IV of the barrel cortex before and after
injection of vehicle, AMPA, IL-1β, or AMPA+IL-1β. The E1 barrel was located
using three repeats of the OIS experiments described earlier.

The APOX electrode (Unisense) allows for simultaneous recording of local
tissue oxygenation and electrical field potentials from the same volume. Before
insertion into the cortex, the electrode switched on, left to stabilise for >30 minutes
and was calibrated as per the manufacturer’s instructions before each insertion.
Firstly, the electrode tip was placed in dH₂O that had been vigorously bubbled with
room air for 5 min at room temperature to obtain an atmospheric ‘Sₐₙ’ reading. Then
the electrode tip was placed in an anoxic solution of 0.1M sodium hydroxide and
0.1M sodium ascorbate (Sigma-Aldrich) to obtain a zero measurement, ‘S₀’, before
rinsing well with dH₂O. The values of ‘Sₐₙ’ and ‘S₀’ were then placed in an equation
with the constant ‘a’ to calculate the micromolar concentration of oxygen (C, in
µmolO₂/L) from the current reading (S) in pA. The constant ‘a’ in this case,
dependent on the salinity and temperature of the calibrating water, was 283.9µmolO$_2$/L (from Unisense Manual table).

\[ C = a \times (S - S_0)/(S_{at} - S_0) \]

After calibration, a small craniotomy was performed over the E1 barrel and the dura nicked using a 24 gauge needle. The APOX probe was lowered 600µm into layer IV/V at a rate of approximately 5µm/s into the active barrel, normal to the surface of the cortex using a custom-built microdrive (UD-800B Microdrive Controller). The position of the probe was then adjusted on a micrometre scale to find a consistent neural response to manual mechanical stimulation of the E1 whisker. As a control, the ipsilateral whiskers were mechanically stimulated manually to ensure that the signal only came from contralateral whisker stimulation and not as a result of artefacts derived from whisker motion. The animal was shielded from electrical noise from the piezoelectric actuator using aluminium foil, while environmental 50Hz noise was removed from the electrode signal using a Humbug 50/60Hz Noise Eliminator (Quest Scientific). The probe’s tissue oxygenation signal was grounded on the headstage and connected to a 2-channel PA-2000 picoammeter (Unisense), which displays the tissue oxygenation while the signal was also transferred to the BNC 2090 (National Instruments, UK).

The electrical signal from the APOX probe was grounded to the headstage, which in turn was grounded to the flat-tipped head-screw in the contralateral hemisphere cranium, the ear bars and foil shield. The broadband electrical signal from the electrode was transferred through the Humbug to the BNC 2090, where it was bandpass-filtered into two channels. The first contained the lower frequencies (5-300Hz) associated with LFP activity as seen in EEG recordings. The other
channel carried higher frequencies associated with MUA (300-3000Hz). The tissue oxygenation data from the PA-2000 was carried in a third channel. All channels were recorded at a sampling rate of 20kHz and were acquired using the BNC 2090 and collected onto the PC using a Data Acquisition System controlled by MATLAB.

2.2 Analysis

2.2.1 Imaging analysis

All data was processed and analysed using custom-written programs on MATLAB. Automated programs were used where possible to prevent experimenter bias.

Images obtained from OIS experiments were separated into three stacks per experiment, one for each of the three visible wavelengths used to illuminate the cortex - namely 550, 560 and 577nm.

The experiments were averaged over their 30 trials and processed using a modified Beer-Lambert equation which utilises path-length scaling algorithm (PLSA) to obtain three new stacks which more accurately represented changes in the concentrations of HbO, HbR and HbT by taking into account the effect of the varying wavelengths on the light’s ability to penetrate and be reflected from tissue back into the camera (Berwick et al., 2005b).

The area supplied by the haemodynamic response, called the ‘activated area’ was defined using an automated, custom-made program which selected pixels that changed beyond the threshold of >1.5 SD or >2.5 SD of the mean value of the first-frame ROI over the course of the experiment. The program then processes selected
pixels to improve the signal-to-noise and a convex polygon surrounding this activated area was constructed (see Figure 2-1A). In cases where the program mistakenly selected large ‘activated’ vessels instead of the barrel, the ROI was reduced manually to exclude the vessel.

To measure the amplitude of the HbT, HbO and HbR components of the functional haemodynamic response within the active barrel, an automated binomial mask was generated for each animal’s pre-injection imaging experiments using the same ‘activated area’ polygon produced using a 1.5 SD threshold as detailed above. The activated area pixels contained within the mask had a value of 1, while surrounding pixels had a value of 0. In the few cases where there was a shift in the camera’s position relative to the imaged area, the mask’s co-ordinates were aligned pixel-by-pixel by eye using vessels as landmarks so that the same area was selected throughout all of the animal’s OIS experiments.

HbT, HbO and HbR image stacks from each imaging experiment were multiplied by the binomial mask to isolate the activated area’s pixels (Figure 2-1B). These pixels were then averaged for each frame of the stack and plotted to visualise the average time-course of the HbT, HbO and HbR components of the haemodynamic response. The peak amplitude of the HbT and HbO responses, relative to prestimulus, were found for each imaging experiment before and after striatal injection (Figure 2-1C). For HbR responses, the peak value during stimulation (early increase) and the lowest value after the peak value (late decrease) were found instead.
2.2.1.1 Vessel cross-section analysis

The middle cerebral artery (MCA) passes through the imaged area and usually through or very near the imaged E1 barrel. To test the hypothesis that oedema or changes in intracranial or intraluminal pressure could be affecting arterial vessel, two measures of the MCA’s diameter was taken: from the x-y perspective, called the ‘diameter’ (as seen from the surface of the cranium) and from a x-z perspective, called the ‘depth’ (that is, an estimate of the ventral vessel thickness, using pixel grayscale values from HbO images). A straight line crossing the MCA either within, or as near as possible to the activated area mask (detailed above) was manually selected in the pre-injection baseline experiment HbO stack. As endtidal CO$_2$ and SpO$_2$ were maintained at a constant level, and the image used to compare vessel measurements was always taken from before whisker stimulation, this
measure was used to represent the vessel’s HbO content at a resting level. For comparisons, and to ensure any changes seen were not a result of, for example, total cerebral blood volume, the same was done for a dural vessel within the active area.

The maximum difference between the pixel values of the pale parenchymal tissue and of the dark vessel was measured by a custom-made automated program for images at the beginning of each imaging experiment. The maximum difference in pixel value between the parenchyma and vessel was found for each imaging experiment before and after intrastriatal injection. These differences reflect the ‘depth’ or ‘content’ of the vessels (Figure 2-2). The same cross-sections were thresholded using another automated program to find the diameter of the vessel. This was the distance in pixels between the two co-ordinates at the palest common pixel value intersecting the cross-section when normalised to 0 as shown in Figure 2-2.

2.2.2 APOX probe recordings analysis

In the (final) study, electrode and oxygenation recordings of the response to whisker stimulation before and after injection of vehicle/AMPA/IL-1β/AMPA+IL-1β were collected in three channels: tissue oxygenation, LFP signal (5-300 Hz) and MUA (300-3000Hz). For each channel, the signal was analysed only for animals that exhibited a discernible response to the stimulus before the striatal injection to allow for comparisons with recordings taken after injection. Group N numbers are therefore reported in each results section.
**Figure 2-2 Vessel depth and diameter analysis**

A, Example cross-section (red dashed line) over a branch of the supplying MCA. Black scale bar: 0.5mm; arrow points rostral. B, The pixel values of the vessel part of the cross-section, showing the two parameters used in this study: the depth (red) and diameter (blue) of the vessel. The same cross-section was used for all following experiments for that animal.

### 2.2.2.1 Tissue oxygenation analysis

Animals were excluded from the analysis of tissue oxygenation recordings if no ‘classic’ haemodynamic response (with a small initial dip and subsequent overshoot starting before the end of the stimulus) was recorded before intrastriatal injection. Therefore, groups numbers were as follows: vehicle, $N = 5$; AMPA, $N = 4$; IL-1β, $N = 5$; AMPA+IL-1β, $N = 5$.

Firstly, the $\mu$mol/L concentration of oxygen was calculated using the picoampere reading from the APOX electrode/PA-2000 in the calibration equation as described earlier. The concentration of oxygen was averaged over the first second of each experiment (that is, before the stimulus was applied) before and after striatal injection to find how the oxygenation reading changed over the 6 h after injection.

Secondly, the mean $O_2$ measurement value at the end of the 4s stimulus (4-5s) was normalised to the pre-stimulus oxygenation measurement. These resulting
values - the pre-stimulus and the ‘peak’ oxygenation - were found for all recordings, before and after striatal injection.

Lastly, since using converted concentrations of oxygen did not demonstrate any differences between groups, the ‘peak’ reading was expressed as a percentage of the pre-stimulus measurement, as done in previous studies using the APOX probe (Li and Freeman, 2007). These percentages were subtracted from the pre-injection percentage to visualise the changes in the size of the oxygenation response after injection.

2.2.2.2 Analysis of electrical field potentials

2.2.2.2.1 Analysis of multi-unit activity

MUA was analysed by thresholding the raw 300-3000Hz channel data at 3SD of the amplitude above the mean of the recording. A matrix was made to represent the incidence of the resulting ‘spikes’ in time within each of the 30 trials per experiment. Habituation to the stimulus occurred after 100-400ms in all animals. Spikes were binned into 100ms compartments, which were averaged over the 30 trials. The value of the maximum spike rate at the onset of the stimulus, minus the average spike rate within the last 1.5s (16x100ms bins) of the stimulus period was normalised to the average spike rate after stimulus offset, or ‘(Peak – Plateau)/Spontaneous’ as shown in Figure 2-3 below. The spontaneous spike rate was also measured. Calculated values of the peak and spontaneous spike rates were normalised to the pre-injection values for comparison of recordings before and after striatal injection.
Figure 2-3 MUA analysis
Illustration showing an example MUA spike density histogram indicating the analysed MUA parameters. At the stimulus (grey box) onset, there is a peak in MUA, followed by a reduction that either returns to pre-stimulus levels, or stays at an elevated plateau, quantified in this study by averaging activity in the last 1.5s of the stimulus and removed.

2.2.2.2 Analysis of local field potentials

LFP recordings (5-300Hz) were separated into EEG spectrum frequency bands (theta [5-8Hz] alpha [8-13Hz], beta [13-24Hz] and gamma [40-100Hz]) using Fourier spectrogram analysis of the raw recording. Spectrograms were created using the built-in function on MATLAB, with a 200ms time window and 100ms overlap. Gamma (40-100Hz) and beta (13-24Hz) frequency bands were found to best reflect the oxygenation response, as previously reported (Magri et al., 2012), so these frequency bands were selected for further analysis.

A log spectrogram was made for each electrode recording and the time window values averaged over the 30 trials (Figure 2-4A). From these new spectrograms, gamma and beta bands were averaged over frequencies 40-100Hz and 13-24Hz respectively to visualise a mean response to stimulation for each band (Figure 2-4B and C). The amplitude of the peak of gamma and beta power at the
onset of the stimulus (at 1s), minus the average gamma and beta power in the last 2.5s of the stimulus was found for each recording. Peak amplitudes were then normalised to express the pre-injection value as 100%, then logged to base 10, since the spectrograms were generated in log space.

Figure 2-4 Gamma and beta LFP analysis
A, Example $10^6 \log(\text{power})$ spectrogram (with window width 0.5s, 0.25s overlap and in 0.2Hz intervals) showing averaged over 30 trials with 1s pre-stimulus, 4s whisker stimulation (black bar) followed by 8s recovery. Gamma (40-100Hz) and beta (13-24Hz) frequency bands are indicated. Note the marked increase in all frequencies at the onset of the stimulus at 1s. B, Gamma and C, beta frequency bands averaged to show activity through the average trial. Again, note the increase in activity at the onset of the stimulation (grey box), which reduces to pre-stimulus levels by ~1s into the stimulus.
2.2.3 Analysis of histology and immunohistochemistry

Slides carrying brain sections were blinded until after analysis had been carried out to prevent experimenter bias. All sections used for immunohistochemistry and histology were the same thickness (30µm) and had undergone the same tissue processing methods.

To assess breakdown of the BBB as reflected by IgG extravasation, the ratio between the mean pixel values of the contralateral and ipsilateral hemispheres of each section immunostained with IgG was found by drawing an outline around each hemisphere and measuring using ImageJ software (National Institute of Health). Since DAB staining is not stoichiometric (van der Loos, 2008) and so can’t be compared between animals, this method was preferred because the contralateral hemisphere served as a control for each individual. Any effect of oedema (for example, ipsilaterally in the AMPA+IL-1β group) on the level of IgG immunostaining was diminished by averaging over the area of the whole hemisphere in each section.

The area of each ipsilateral hemisphere was measured by free-hand selecting each hemisphere of at least three sections per animal using ImageJ, which provided an area measurement. The average ratio of the ipsilateral:contralateral area for each animal was calculated to assess any oedema.

SJC-positive neutrophils in 0.56x0.42mm areas were counted manually from photomicrographs of the cortex, piriform cortex and striatum of three sections per brain around the injection site at 10x magnification using ImageJ (shown in Figure 2-5).
To analyse tissue stained with cresyl violet, 10x magnification photomicrographs were taken of the striatum and cortex (as above) on each hemisphere, from at least three coronal sections surrounding the injection site per brain. From these micrographs, the difference between the mean pixel value of the ipsilateral and contralateral striatum and layer IV/V of the somatosensory cortex (the layer from which recordings were taken) was found and compared between injection groups.

2.3 Statistics

Statistical analyses were carried out using MATLAB or GraphPad (Prism). Amplitudes of HbT, HbO and HbR responses and electrode recording results were average into hourly time bins, statistically tested (described in next paragraph), and then normalised to pre-injection recordings (100%), to minimise between-subject variation for easier presentation in graphs. All statistical significance indicated on graphs was assessed according to non-normalised data. Net changes (used for presentation purposes) in the % oxygenation response amplitude, heart rate and core
temperature measurements were found by subtracting the pre-injection measure from subsequent values after binning into hourly average values. Correlations between parameters were quantified using Pearson’s r, except in the case of the normalised log gamma and beta activity, where Spearman’s r for non-parametric data was used.

OIS analysis of the HbT, HbO and HbR responses, MCA/dural vessel depth/diameters, oxygenation, MUA, heart rate and core temperature were compared over time and between groups using two-way repeated-measures ANOVA with Bonferroni post-hoc tests, and with repeated-measures one-way ANOVA with Bonferroni correction for multiple comparisons with the pre-injection measures to further evaluate differences within groups at different time points. In the majority of cases, results of ANOVA are reported from analyses carried out on raw data, represented by the letter ‘r’ in brackets; however, in a few cases, normalised values, excluding the ‘Pre’-injection value (to which the data has been normalised), have been analysed instead – these results are reported with the phrase ‘n-Pre’ in brackets. SJC-positive neutrophils, IgG immunostaining and oedema were compared between groups using one-way ANOVA and Student’s t-tests. Statistical significance was taken at the 5% level.
3 Results 1

This chapter reports the results of the first two separate studies: a characterisation of injury over time after striatal AMPA+IL-1β injection, and a comparison of four injection conditions, namely vehicle, AMPA, IL-1β and AMPA+IL-1β. In both studies, imaging, histological data and physiological data (heart rate and temperature) are reported.

3.1 Time series

Animals were prepared for OIS and a baseline haemodynamic response to whisker stimulation recorded using the three wavelengths (550±10nm, 560±10nm, 577±10nm) before striatal injection of AMPA+IL-1β as described in Chapter 2. Optical imaging experiments consisting of 30 trials were carried out every 20 min for 2 (N = 4), 4 (N = 4), 6 (N = 3), or 8h (N = 3), following which animals were perfused transcardially with saline nitrate and PFA for histological analysis.

This initial study was conducted to determine evaluate changes in the haemodynamics and histology (if any) happened at early time points (prior to cell death at ~8h) in response to AMPA+IL-1β injection. It was also important that a marker of early inflammation could be identified for further comparison with other test groups in subsequent experiments. All 14 animals in this study survived until the experiment end point.
3.1.1 Optical imaging spectroscopy

3.1.1.1 Active area analysis

Automated masks demarcating the active barrel were made for each animal, multiplied with image stacks representing the HbO and the resulting pixels averaged to assess the HbO response over the course of the experiment (2-8h).

For the purposes of this time-course characterisation study, the HbO response was investigated (rather than the HbT and HbR) since changes in the HbO signify more about the functionality of the imaged area. In addition, during pilot studies it was noted that changes in the HbO response were more pronounced than other components of the haemodynamic response.

Pilot studies within the lab (H. Buggey, unpublished results) had previously indicated that injection of AMPA+IL-1β may trigger a shrinking of the area supplied by the haemodynamic response to stimulation. Therefore, the activated area was measured for each imaging experiment before and after striatal injection of AMPA+IL-1β. The areas were normalised to the pre-injection imaging experiment for each individual animal so that changes relative to the pre-injection could be compared between animals.

In Figure 3-1A, a threshold of 1.5 SD above the mean pixel value has been used to define the activated area, while in Figure 3-1B a higher threshold of 2.5 SD is applied instead. When the active area is defined by a lower threshold (1.5 SD) the size of the area supplied by the haemodynamic response is not significantly affected ($F(r) = 0.8952, p > 0.05$; one-way ANOVA; Figure 3-1A). However, when the higher threshold (2.5 SD) is applied instead, the averaged area appeared well-maintained until after 6h, when there was a sharp decline (Figure 3-1B). One-way
ANOVA identified a significant effect of the injection \( (F(r) = 2.449, \ p < 0.05) \), however, the reduction seen at 6-8h was not found to be significantly different to the pre-injection area measure, presumably because of the low group number at the stage \( (N=3) \).

Looking at the individual maximum areas, it became apparent that it is not a case of the activated area becoming smaller spatially; rather, fewer pixels within the area reach above the threshold and so are not included by the automated program. Therefore, in order to elucidate the nature of any injection-specific changes it proved more useful to look at the amplitude of the various components of the haemodynamic response.

3.1.1.2 Amplitude of HbO response

In Figure 3-2, the amplitude of the HbO response was seen to decrease significantly from the pre-injection baseline after injection of AMPA+IL-1β after 1h \( (F(r) = 4.579, \ p < 0.001; \) one-way ANOVA). While animals culled at the earlier time points (<6h, \( N = 8 \)) did not exhibit an obvious slow decline in the HbO response over the hours after injection of AMPA+IL-1β, there was consistently a ‘drop’ in the amplitude of the response of approximately 40% of the baseline between the pre-injection and first post-injection imaging experiment (at 5 min) in the majority of the animals (12 out of 14).
Figure 3-1 Maximum area time courses
The maximum thresholded activated area of the HbO response, before (‘Pre’) and after injection of 5nM AMPA and 50ng IL-1β, A, using a 1.5 SD threshold, and B, using a 2.5 SD threshold. Fourteen animals were used and culled at 2 (N = 4), 4 (N = 4), 6 (N = 3) or 8 (N = 3) time points after injection, represented by dotted lines. Group mean ± SEM shown.
Figure 3-2 HbO response time course
The maximum amplitude of the HbO response to E1 whisker stimulation before (‘Pre’) and after striatal injection of 5nM AMPA and 50ng IL-1β. Each point represents the group’s average across 2-4 experiments, each consisting of 30 trials; group mean ± SEM. Animals were culled at 2h ($N = 4$), 4h ($N = 4$), 6h ($N = 3$) or 8h ($N = 3$) indicated by dotted lines. Asterisks indicate significant difference between group mean and pre-injection response (100%) in one-way ANOVA on raw data; *, $p < 0.05$; ** $p < 0.01$.

In most animals, a slower more consistent decline was also seen in the hours following the injection, with less variation between animals as time progressed. A linear regression analysis ($R^2 = 0.3804$) reveals that the slope deviates significantly from zero ($F(n-Pre) = 47.89, p < 0.0001$).

3.1.2 Physiological parameters
The net change in heart rate of the animals injected with AMPA+IL-1β increases significantly over time ($F(r) = 8.787, p < 0.001$; $F(n) = 11.27, p < 0.001$; one-way ANOVA; Figure 3-3A). The average increase in beats per minute (bpm)
became significantly higher than before the injection after 3h in a 1-way ANOVA between time points ($t(3h \text{ vs. Pre}, r) = 5.219, p < 0.001$) and peaked at approximately 4h ($t(4h \text{ vs. Pre}, r) = 5.730, p < 0.001$, Bonferroni), with significant differences maintained until the 7h end point. Of the animals maintained for longer than 2h after injection of AMPA+IL-1β, only one animal (in the 6h group) did not show a marked increase in heart rate.

According to one-way ANOVA, the average temperature increased above the pre-injection baseline ($F(r) = 3.937, p < 0.001; F(n) = 4.207, p < 0.001$), to a peak at 4h ($t(4h \text{ vs. Pre}, r) = 3.889, p < 0.01$), after which it began to decrease (Figure 3-3B). Between 4-6h there was a marked increase in variability in the temperature response, with one animal showing no change. Hence, due to low group numbers, data was not significant after 5h.

The increase in temperature was less obvious and more variable than the increase in heart rate, however, the normalised measurements of both parameters correlate significantly ($r = 0.7143, p < 0.0001$), as seen in Figure 3-3C. The association seems to be limited by the increase in heart rate, which plateaus at approximately 180bpm despite continued increases in temperature beyond 0.5°C.
Figure 3-3 Physiological parameters in time course study
Net average changes in the A, heart rate and B, core temperature (ΔT) before (‘Pre’) and after striatal injection of AMPA+IL-1β. Dotted lines represent end points for animals euthanized at different time points (2h, 4h, 6h, 8h; N = 4, 4, 3, 3 respectively). Mean ± SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.0001 in Bonferroni post hoc vs. pre-injection, using raw values. C Normalised measures of Δ heart rate and Δ temperature (ΔT) correlate significantly.
3.1.3 Histology

SJC-positive neutrophils and immunoglobulin G (IgG) were both found to be suitable markers for assessing the neuroinflammation in response to AMPA+IL-1β administration. Brains taken from animals imaged for 2/4/6/8h after injection of AMPA+IL-1β were analysed for these two markers and oedema to establish a time-course of neuroinflammatory changes after striatal injection of AMPA+IL-1β.

The number of SJC-positive neutrophils counted in the cortex increased over time in the three analysed areas: the somatosensory cortex, striatum and piriform cortex (Figure 3-4). According to one-way ANOVA between the time points, there was a significant increase in the number of ipsilateral cortical neutrophils over time ($F = 10.98, p < 0.01$), but no effect on contralateral neutrophils ($F = 1.793, p > 0.05$). There were significant differences in the number of ipsilateral neutrophils in the cortex between brains taken at 2h vs. 6h ($t = 4.503, p < 0.01$), 2h vs. 8h ($t = 6.753, p < 0.001$) and 4h vs. 8h ($t = 6.575, p < 0.001$). There was also a significant increase in the number of neutrophils in the ipsilateral piriform cortex ($F = 21.32, p < 0.001$), though again not in the contralateral piriform cortex ($F = 1.793, p > 0.05$). There was a significant difference found between ipsilateral neutrophil counts in the piriform between 2h and 6h ($t = 5.771, p < 0.001$), 2 and 8h ($t = 7.195, p < 0.001$) and 4 and 8h ($t = 4.885, p < 0.01$). In the same way, ipsilateral striatal neutrophils increased over time ($F = 21.32, p < 0.001$), whereas contralateral striatal neutrophils did not ($F = 0.9074, p > 0.05$). Significant increases in neutrophil counts in the ipsilateral striatum were found between 2 and 6h ($t = 5.074, p < 0.01$), 2 and 8h ($t = 5.024, p < 0.01$), 4 and 6h ($t = 4.855, p < 0.01$) and 4 and 8h ($t = 4.804, p < 0.01$).

More SJC-positive neutrophils were present in the injected hemisphere vs. contralateral hemisphere at both 6h (cortex, $t = 4.150, p < 0.05$; striatum, $t = 4.870, p$
< 0.01) and 8h (cortex, \( t = 6.279, p < 0.001 \); striatum, \( t = 4.877, p < 0.01 \)) after injection of AMPA+IL-1β. More neutrophils were counted in the ipsilaterial piriform cortex than in the contralateral piriform after 8h (\( t = 6.279, p < 0.001 \)). Interestingly there was an increase in the number of contralateral neutrophils in the piriform cortex at 6 and 8h; however, this was not found to be statistically significant compared to 2h, presumably because there were just 3 animals for each of the later time points.

Breakdown of the BBB, measured using IgG was evident in brains at 6h after injection (\( F = 26.99, p < 0.0001 \); one way ANOVA). According to Bonferroni multiple comparison tests, there was no significant increase in the amount of ipsilateral staining between 2h and 4h, however there was between 2h and 6h (\( t = 7.620, p <0.001 \)), 2h and 8h (\( t = 7.076, p < 0.001 \)), 4h and 6h (\( t = 4.962, p < 0.01 \)), and 4h and 8h (\( t = 4.418, p < 0.01 \)). Compared to the contralateral hemisphere, the ipsilateral BBB breakdown was significantly worse at 4h (\( t = 3.487, p < 0.05 \)), 6h (\( t = 12.88, p < 0.01 \)) and 8h (\( t = 41.09, p < 0.001 \); Figure 3-5). IgG extravasation was evident in the whole striatum and also seemed to emanate from large radial vessels in the cortex, before becoming almost homogenous over the entire hemisphere after 8h.

There was no significant difference found between groups in the amount of tissue oedema as determined by ipsilateral:contralateral volume ratios, though the two later time points (6/8h) comprised low N numbers (3 each).
Figure 3-4 Time course of neutrophil recruitment

A-C Counts of contralateral (C) and ipsilateral (I) SJC-positive neutrophils at different time points after striatal injection of AMPA+IL-1β: 2h (N = 4), 4h (N = 4), 6h (N = 3) and 8h (N = 3). SJC-positive neutrophils within a 0.56mm x 0.42mm 10x mag. rectangle in the A, somatosensory cortex, B, striatum and C the piriform cortex. Each point signifies the average of counts from at least three sections per animal. Mean ±SEM shown; *, p < 0.05; **, p < 0.01; ***, p < 0.001 in paired t-test between hemispheres and one-way ANOVA Bonferroni multiple comparisons indicated between time points. D-F Example 10x micrographs of the ipsilateral D, somatosensory cortex, E, striatum and F, piriform cortex. Scale bar: 100μm.
Figure 3-5 Time course of IgG extravasation and oedema

IgG extravasation at 2, 4, 6 and 8h after striatal injection of AMPA+IL-1β. A. Average pixel value ratio between ipsilateral and contralateral hemispheres. Each point represents an average of at least three sections per animal. Mean ± SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001 in one-sample t-test vs. 0 and one-way ANOVA Bonferroni multiple comparisons between groups indicated by bars. B-D. Example sections from brains of animals culled at A, 2h, B, 4h, C, 6h and D, 8h after injection. Note the spread into the cortex at 6h (E) and then the ipsilateral hemisphere’s homogenous spread of IgG at 8h (F). Scale bar: 2.5mm.
3.2 Six-hour experiments

Following the characterisation of the time course of inflammatory markers and the HbO response after striatal injection of AMPA+IL-1β, the 6h time point was chosen for the second study comparing groups injected striatally with 1μL solution of vehicle, 5nM AMPA, 50ng IL-1β or AMPA+IL-1β combined ($N = 5$ each throughout this section). As in the previous study, imaging data, histology results and physiological parameters (heart rate and temperature) are reported. One animal injected with AMPA+IL-1β died only 2.5h after injection and so has been omitted from all analyses and one more animal was consequently used to replace it, ensuring $N = 5$ in all groups for all analyses.

3.2.1 Imaging results

OIS was used to measure the HbO, HbT and HbR components of the haemodynamic response to whisker stimulation before and after striatal injection of the four solutions. The following results section will firstly describe results from analysis of the size of the activated area obtained from HbO image stacks. Then the response amplitude of each component (HbT, HbO, HbR) will be examined, with a particular emphasis on the HbO response as the most representative indicator of haemodynamic function.

3.2.1.1 Activated area analysis

The activated area analysis, as done in the previous study, was repeated using a 1.5 SD threshold on HbO stacks to verify any changes in the spatial extent of the haemodynamic response after striatal injections. The baseline average areas for each
group were found to be not significantly different from each other ($F = 0.3134, p > 0.05$).

Figure 3-6 shows that, compared to the vehicle condition, the activated area is not significantly affected by any of the three test injections over time ($p > 0.05$, two-way repeated measures ANOVA, shown in graph), although significant effects of time and subjects were found ($F(time, r) = 3.979, p < 0.01$); $F(sub, r) = 17.51, p < 0.001$; two-way repeated measures ANOVA). Further, none of the groups showed any significant effect of injection over time using one-way ANOVA ($p > 0.05$).

However, it noted when calculating the areas that the polygon created around the thresholded area, although often the same total size, again contained fewer dilated discs representing >1.5 SD ‘activation’, just as in the previous study. This may explain the apparent ‘shrinking’ of the area of activation in the IL-1β group. Therefore, it seems more likely that the intensity or amplitude, rather than the spatial extent, of the haemodynamic response is affected. Therefore the next sections will describe changes in the amplitude of the haemodynamic response.
Changes in the maximum activated area (defined by a 1.5 SD threshold) over 6h following injection of vehicle, 5nM AMPA, 50ng IL-1β or 5nM AMPA + 50ng IL-1β. Each point represents the average across the group, with each animal’s average comprising at least two imaging experiments consisting of 30 trials each, normalised to the baseline. No significant differences were found using two-way repeated measures ANOVA or one-way ANOVA for each group.

3.2.1.2 HbT response

Binomial masks delineating the active barrel from the pre-injection experiment were multiplied with image stacks representing changes in HbT, resulting in an averaged pixel value HbT response time course for each imaging experiment. The maximum peak of HbT was found for each imaging experiment before and after striatal injection. Raw value baseline amplitudes did not vary significantly between groups ($F = 1.076, p > 0.05$).

Intrastriatal injection of vehicle had no significant effect on the amplitude of the HbT response over time. Injection of IL-1β however, led to a significant decrease
in the HbT response over time compared to the vehicle group at 6h \((F(\text{IL-1}\beta, r) = 3.737, p < 0.01)\).

### Figure 3-7 HbT responses
Amplitudes of the HbT response to whisker stimulation pre- and up to 6h post-injection of vehicle, AMPA, IL-1\(\beta\), or AMPA+IL-1\(\beta\). Points represent average of groups where 2-4 imaging experiments (each consisting of 30 trials) has been binned per animal. Mean + SEM; *, \(p < 0.05\); **, \(p < 0.01\); repeated two-way ANOVA Bonferroni post-tests vs. vehicle using normalised values excluding the ‘Pre’ measurement.

A two-way repeated measures ANOVA comparing normalised values after the injection (that is, without the ‘Pre’ value) found a significant effect of the injected solution on the size of the HbT response \((F(n-Pre) = 3.670, p < 0.05); \text{Figure 3-7})\). Compared to vehicle, the AMPA group exhibited a smaller HbT response at 5h \((t(n-Pre) = 2.982, p < 0.05)\), and 6h \((t(n-Pre) = 2.972, p < 0.05)\), while the IL-1\(\beta\) group showed a significantly lower response than the vehicle group at 5h \((t(n-Pre) = 2.884, p < 0.05)\) and 6h \((t(n-Pre) = 3.866, p < 0.01)\). The AMPA+IL-1\(\beta\) group’s HbT response was significantly lower than vehicle at 6h \((t(n-Pre) = 3.249, p < 0.05)\).
While the size of the HbT response is a useful measure of functional perfusion, further analysis was carried out on the two separated components of the haemodynamic response, HbR and particularly HbO, in an effort to understand any changes in demand and functional supply of oxygenated blood to the active area of the cortex.

3.2.1.3 HbO response

The amplitude of the HbO response to mechanical stimulation of the E1 whisker was found for each imaging experiment before and after striatal injection. The raw value size of the baseline HbO responses (before normalising) did not differ significantly between groups according to one-way ANOVA ($F = 0.5317, p > 0.05$).

Two-way ANOVA on raw data identified a significant effect of an injection ($F(r) = 3.371, p < 0.05$) and time ($F(r) = 3.372, p < 0.01$) on the size of the HbO response. The size of the HbO response significantly decreased over time after injection of IL-1β or AMPA+IL-1β when compared to the vehicle (Figure 3-8). Bonferroni post-hoc tests indicate that, compared to vehicle, the size of the HbO response was significantly smaller at 6h in the IL-1β group ($t(r) = 3.391, p < 0.01$), and AMPA+IL-β group, ($t(r) = 3.151, p < 0.05$). Both the IL-1β and AMPA+IL-1β groups suffered a 50% reduction in the size of the HbO response 6h after injection.
Figure 3-8 HbO responses
Graph showing the change in the amplitude of the HbO response before (‘Pre’) and up to 6h after striatal injection of vehicle, AMPA, IL-1β or AMPA+IL-1β (N = 5 each). Mean ± SEM shown; *, p < 0.05; **, p < 0.01, according to two-way ANOVA conducted on data before normalising, with Bonferroni tests vs. vehicle group. Symbols xx, p < 0.01; xxx, p < 0.001 indicate significant differences according to one-way ANOVA comparing groups’ time points against pre-injection, again using data before normalisation.

As one animal in the vehicle group showed a large increase in the amplitude of the HbO response and so may have contributed to an exaggeration of differences between the vehicle and test groups, one-way ANOVA comparing different time points were performed (Figure 3-8). Neither the vehicle, AMPA of IL-1β groups showed any significant deviation from the size of the pre-injection HbO response (vehicle, F(r) = 1.217; AMPA, F(r) = 1.117; IL-1β, F(r) = 2.636; p > 0.05), although the IL-1β group did reach near significance (p = 0.0516).

However, in the group injected with AMPA+IL-1β, there was a significant decline in the HbO response. The AMPA+IL-1β group’s HbO response significantly
decreased relative to pre-injection baseline measurements (100%) at 5h \( t(r) = 4.568, p < 0.01 \) and 6h after injection \( t(r) = 5.510, p < 0.001 \); Figure 3-8).

One animal in the AMPA group did have an exceptionally large pre-injection baseline HbO response, meaning that normalised post-injection responses may have been weighted lower than if the pre-injection response had been a more ‘usual’ size. Despite this, there is still no significant decrease in the AMPA group’s HbO response over time.

In sum, statistical analyses (two-way ANOVA vs. vehicle and one-way ANOVA vs. pre-injection) identified significant decreases in the IL-1β and AMPA+IL-1β groups’ HbO responses, while the AMPA and vehicle groups consistently show no statistical differences from baseline over time.

The latency of the HbO responses was investigated to see whether the neurovascular coupling had become slower to act despite the same assumed metabolic demand for oxygenated blood (Figure 3-9). There were no differences in the latency to peak over the hours after injection in any of the groups at any time point \( p > 0.05 \), using either two-way ANOVA or one-way ANOVA on raw binned data), indicating that it is the amplitude, rather than the time to peak, of the HbO response that changes in the IL-1β and AMPA+IL-1β conditions.
Figure 3-9 HbO latencies and profiles
Latency of the peak of the HbO response before and after striatal injections (left). Below, averaged hourly HbO response profiles pre- and after injection of vehicle, AMPA, IL-1β or AMPA+IL-1β (N = 5 each). The profile of the HbO ‘overshoot’ is similar in all groups before injection and the time-to-peak stays consistent in all groups yet progressively flattens over time in the IL-1β and AMPA+IL-1β groups.
3.2.1.4  

**HbR components**

Two elements of the HbR response were studied: the initial transient increase in HbR concentration (known as the ‘deoxy dip’ or ‘early increase’) and the subsequent decrease that occurs as a result of a relative increase in HbT and HbO, referred to here as the ‘late decrease’ HbR response.

Changes in the concentration of HbR were calculated for all imaged responses using the same binomial mask used on the HbT and HbO stacks. A two-way ANOVA of raw data identified a significant effect of time ($F_{(r, time)} = 2.596, p < 0.05$) and Bonferroni post hoc tests demonstrated that the IL-1β group exhibited a smaller HbR late decrease than vehicle at 6h ($t_{(r)} = 2.939, p < 0.01$; Figure 3-10A).

However, because the average vehicle response appeared to increase, one-way ANOVA were performed to test for significance between the pre-injection amplitude and the group averages at hourly time points, again using the data before normalisation. Using this method, there was no difference over time in any of the groups of the size of the late HbR response ($p > 0.05$).

As the late HbR response is largely thought to reflect the overall increase in HbT in the supplied region, the smaller, initial increase in HbR, was also considered (Figure 3-10B). This parameter is associated with the increased metabolism of cells at the beginning of stimulation (Mayhew et al., 2000).

The raw values of the initial ‘deoxy dip’ pre-injection did not show significant variation between groups ($p > 0.05$), although a significant effect of time was found ($F_{(r)} = 2.263, p < 0.05$). There were no differences between groups in the amplitude of the ‘deoxy dip’, and none of the groups exhibited any significant
deviation from the baseline at any of the hourly time points as determined using one-way ANOVA.

Figure 3-10 HbR early and late responses
Analysis of the two HbR response elements. A, The size of the late decrease in the relative concentration of HbR reduced over time in IL-1β and AMPA+IL-1β-injected animals. Mean ± SEM; **, *p* < 0.05; two-way repeated measures ANOVA Bonferroni post-tests using raw data. B, The size of the ‘deoxy dip’ did not change significantly over time in any of the four conditions.
3.2.2 Vessel volume analysis

Branches of the MCA inside or closest to the active barrel (as found using OIS) were analysed by selecting a 2-D pixel value cross-section over the vessel HbO stack and observing changes in the value of the darkest pixel (the ‘depth’ of the vessel) and the diameter of the vessel.

Raw values of the maximum ‘depth’ of the MCA did not vary significantly between groups before injection ($F(r) = 2.057$, $p > 0.05$). Two-way repeated measures of the raw data indicated a significant effect of an interaction ($F(r) = 1.730$, $p > 0.05$) and time ($F(r) = 7.213$, $p < 0.001$).

To identify changes within groups, one-way ANOVA was used on each group’s raw data to compare the pre- and subsequent post-injection MCA ‘depths’. No change was detected in the depth of the MCA over time in animals injected with vehicle. A non-significant, decreasing trend was seen in the groups injected with AMPA or IL-1β separately, whereas the MCA in the AMPA+IL-1β group became about 40% ‘lighter’ than before injection at between 2 and 6h ($2h, t(r) = 3.831, p < 0.05$; $3h, t(r) = 4.529, p < 0.01$; $4h, t(r) = 4.011, p < 0.05$; $5h, t(r) = 4.438, p < 0.01$; $6h, t(r) = 5.889, p < 0.001$; Bonferroni post-tests; Figure 3-11A).

Dural vessels were used as a comparison, since these vessels are not involved in the haemodynamic response. There was no significant effect of injection found for dural vessel depths over time according to two-way ANOVA ($p > 0.05$; Figure 3-11B). One-way ANOVA of each group vs. pre-injection values, on the other hand, showed reduction in the depth of the dural vessel in the AMPA+IL-1β group at 2-6h after injection ($F(r) = 5.605$, $p < 0.001$; $2h, t(r) = 4.200, p < 0.01$; $3h, t(r) = 4.677, p < 0.01$; $4h, t(r) = 4.469, p < 0.01$; $5h, t(r) = 3.911, p < 0.05$; $6h, t(r) = 3.495, p <$
In addition, injection of IL-1β was also found to diminish the dural vessel ‘depth’ (F(r) = 2.943, p < 0.05), though Bonferroni post hoc tests revealed no specific time points demonstrating this effect.

![A. MCA depth](image)

![B. Dural vessel depth](image)

**Figure 3-11 Vessel depths**

Diameters of the A, MCA and B, dural vessel, before and after striatal injections. Mean ± SEM; *, p < 0.05, **, p < 0.01; ***, p < 0.001 in one-way ANOVA, Bonferroni post hoc vs. pre-injection using data before normalising.
Diameters of the same vessels were also determined (Figure 3-12). In both cases - the MCA and the dural vessel - the diameter of the vessel did not significantly alter from the pre-injection baseline over the course of the experiment, according to repeated measures two- and one-way ANOVA ($p > 0.05$), although a significant, but weak, interaction effect was found for the MCA diameters ($F(r) = 1.728, p < 0.01$).

Figure 3-12 Vessel diameters
Analysis of MCA and dural vessel diameters before and after striatal injections. A. MCA and B, dural vessel cross-sectional diameters. No differences were found over time or between groups using one- or two-way ANOVA.
The normalised measures of the depth of the MCA correlated significantly with the normalised values of the HbO response over the 6h in the AMPA+IL-1β group ($r = 0.6644, p < 0.0001$), but not in any of the other three groups.

![Figure 3-13 MCA depth and HbO response correlation](image)

Correlation between the normalised values for the HbO response amplitude and the depth of the MCA in animals injected with AMPA+IL-1β.

### 3.2.3 Physiological parameters

ECG heart rate measurements were taken before and at 20 min time points after injection of vehicle/AMPA/IL-1β/AMPA+IL-1β (Figure 3-14A). A two-way ANOVA comparing all the groups over the 6h after injection found a significant effect of injection ($F(r) = 3.550, p < 0.05$), with groups injected with IL-1β or AMPA+IL-1β exhibiting a significant increase in heart rate relative to vehicle, and the AMPA group not showing any noticeable change. IL-1β-injected animals had a higher heart rate than vehicle animals at 3-6h (3h, $t(r) = 3.576, p < 0.01$; 4h, $t(r) = 4.079, p < 0.001$; 5h, $t(r) = 3.335, p < 0.01$; 6h, $t(r) = 3.840, p < 0.01$), while injection of AMPA+IL-1β together caused a significant increase in heart rate compared with
vehicle from 3-5h (3h, \( t(r) = 3.682, p < 0.01 \); 4h, \( t(r) = 3.564, p < 0.01 \); 5h, \( t(r) = 2.820, p < 0.05 \)). One way ANOVA comparing groups’ time points with pre-injection heart rates found similar effects in IL-1β \( (F(r) = 13.35, p < 0.0001) \) and AMPA+IL-1β \( (F(r) = 10.56, p < 0.0001) \) groups.

According to repeated measures two-way ANOVA, there was no significant effect of injection on core temperature, perhaps because the measured changes were relatively small, although significant effects of time \( (F(r) = 6.943, p < 0.01) \) and interaction \( (F(r) = 1.860, p < 0.05) \) were found. Bonferroni post hoc test indicated that animals injected with IL-1β were significantly warmer than vehicle-injected animals at 4h \( (t(r) = 3.122, p < 0.05; \) Figure 3-14B). One-way ANOVA on raw temperature data, on the other hand, found an effect of injection of IL-1β \( (F(r) = 3.200, p < 0.05) \) – though not at specific time points – and AMPA+IL-1β \( (F(r) = 6.258, p < 0.001) \), specifically at 3 and 4h after injection \( (3h, t(r) = 3.824, p < 0.05; \) 4h, \( t(r) = 4.895, p < 0.01 \)). As found in the previous study, normalised (net) changes in heart rate and temperature correlate significantly \( (r = 0.6901, p < 0.0001; \) Figure 3-14C).
Figure 3-14 Physiological parameters comparing groups
Net changes in A, heart rate and B, temperature (T) in imaged animals. Mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; two-way repeated-measures ANOVA Bonferroni post-tests on data before normalising. x, $p < 0.05$; xx, $p < 0.01$ according to one-way r.m. ANOVA vs. pre-injection baseline (non-normalised). C, Measurements of net increases in heart rate and temperature correlate significantly.
3.2.4 Histology

3.2.4.1 IgG and oedema

Brains injected with IL-1β, with or without AMPA, showed similar levels of BBB breakdown at 6h, while injection of vehicle or AMPA barely seemed to affect BBB permeability. According to one-way ANOVA vs. vehicle, the ratio of ipsilateral:contralateral IgG immunostaining was significantly higher in brains injected with IL-1β or AMPA+IL-1β (IL-1β, t = 8.210, p < 0.001; AMPA+IL-1β, t = 8.335, p < 0.001; Bonferroni multiple comparison tests; Figure 3-15A). AMPA-injected brains showed no more ipsilateral IgG immunostaining than the vehicle vehicle group, though there was more immunostaining in the ipsilateral hemisphere than in the contralateral hemisphere in all groups, probably due to the insertion of the microneedle (vehicle, t = 6.349, p < 0.01; AMPA, t = 2.894, p = 0.05; IL-1β, t = 9.310, p = 0.001; AMPA+IL-1β, t = 12.09, p = 0.001; one-sample t-tests).

According to one-way ANOVA, there was no significant difference in the amount of oedema as defined by the difference in volume between the ipsilateral and contralateral hemispheres. Student’s t-test did however find that the AMPA+IL-1β group showed a higher ipsilateral:contralateral volume ratio than animals injected with vehicle (t = 2.534, p = 0.0350; Figure 3-15B).
Figure 3-15 IgG and oedema are IL-1β-dependent
A Ipsilateral IgG immunostaining, normalised to contralateral immunostaining, 6h after injection of vehicle/AMPA/IL-1β/AMPA+IL-1β (N = 5 each). Each point represents the average of >3 slices for one animal. Mean ± SEM; ***, p < 0.001, one-way ANOVA Bonferroni. B, The ipsilateral:contralateral volume ratio in brain slices from animals 6h after injection of vehicle/AMPA/IL-1β/AMPA+IL-1β. Mean and SEM; *, p < 0.05, Student’s t-test. C-F, Representative slices showing IgG immunostaining after injection of C, vehicle, D, AMPA, E, IL-1β and F AMPA+IL-1β. Slices taken from approximately Bregma -0.4mm. Scale bar: 2.5mm.

3.2.4.2 Neutrophils

One-way ANOVA with Bonferroni multiple comparisons tests was used to evaluate the counts of neutrophils. In general, more neutrophils were present in IL-β
or AMPA+IL-1β-injected brain regions in than in vehicle- or AMPA-injected animals. IL-1β-injected animals had more neutrophils present in the injected hemisphere’s striatum ($t = 7.043$), piriform ($t = 7.547$) and somatosensory cortex ($t = 10.85$; all $p < 0.001$; Figure 3-16, A-C). The AMPA+IL-1β group showed more neutrophil recruitment in the ipsilateral piriform ($t = 7.333$) and somatosensory cortices compared to the contralateral side ($t = 8.294$; both $p < 0.001$). However, there was no difference between hemispheres in the number of striatal neutrophils in this group ($t = 1.481$, $p > 0.05$). Striatal infusion of AMPA or vehicle had no effect on the number of ipsi vs. contralateral neutrophils.

Compared to vehicle, there were more neutrophils in the ipsilateral piriform ($t = 10.55$), cortex ($t = 11.22$), and striatum ($t = 7.033$) in IL-1β animals (all $p < 0.001$). AMPA+IL-1β injection led to more ipsilateral neutrophils in the piriform ($t = 8.794$) and somatosensory cortex ($t = 8.451$, both $p < 0.001$) than vehicle injection, but interestingly no significant difference was found in the number of striatal neutrophils ($t = 1.618$, $p > 0.05$; Figure 3-16B).
Figure 3-16 SJC-positive neutrophils in scortex, striatum and piriform
SJC-positive neutrophil analysis in the contralateral (C) and ipsilateral (I) A, somatosensory cortex, B, striatum and C, piriform cortex. Each point represents the average count in a 1x1.2mm rectangle of 3 slices around the site of injection for each animal. Mean ± SEM; ***, p < 0.001, one-way ANOVA Bonferroni multiple comparison tests.
Figure 3-17 Example neutrophil photomicrographs
Representative 10x magnification photomicrographs of SJC-positive neutrophils in the ipsilateral A, cortex; B, striatum; and D, piriform cortex. Scale bar: 100μm.
3.2.5 Correlation analyses

Correlation analyses were performed to detect associations between the following parameters for each animal in all groups ($N = 5$ each): AUC of net changes in heart rate and temperature; ipsilateral oedema; ipsilateral IgG; number of ipsilateral cortical neutrophils; normalised values of the MCA ‘depth’ at 6h post-injection; and normalised values of the amplitude of the HbO response at 6h post-injection.

The reduction of the HbO response correlated positively with oedema ($r = -0.6789, p < 0.01$), IgG ($r = -0.4552, p < 0.05$), and cortical neutrophils ($r = -0.5502, p < 0.05$). The measure of the MCA depth negatively correlated significantly with both BBB breakdown ($r = -0.5330, p < 0.05$) and the AUC of the net change in heart rate ($r = -0.4840, p < 0.05$). BBB breakdown and oedema were positively associated ($r = 0.5457, p < 0.05$), and the level of IgG immunostaining and the number of cortical neutrophils showed tight correlation ($r = 0.8053, p < 0.0001$).
Figure 3-18 Correlations of OIS and histological markers
Correlations between A, Normalised HbO response at 6h and ipsilateral IgG; B, HbO response at 6h and oedema; C, HbO response at 6h and number of cortical neutrophils; D, MCA ‘depth’ and IgG; E, Normalised MCA ‘depth’ and the AUC net increase in heart rate; F, Oedema and ipsilateral IgG and G, IgG and number of cortical neutrophils. Each point represents one animal; line of best fit shown.
3.3 Discussion

3.3.1 Summary of findings

In this chapter, OIS was used to identify and compare changes in the various components of the barrel cortex’s haemodynamic response to whisker stimulation in rats injected striatally with vehicle, AMPA, IL-1β or AMPA+IL-1β.

The first set of experiments aimed to define a time course of the early haemodynamic, physiological and histological changes in the most severe group, AMPA+IL-1β together. The markers eventually chosen – IgG immunostaining and SJC-positive neutrophils – were both elevated between 4 and 6h after injection (in agreement with previous studies; (Anthony et al., 1997, Blamire et al., 2000), so a 6h time point was chosen for the main study reported in this chapter. These initial experiments also served to optimise the imaging data analysis.

The main comparison study found that the size of the haemodynamic response, in particular the HbO component, was hindered by the injection of IL-1β with or without AMPA (using either one- or two-way repeated measures ANOVA). It is important to note that, while the normalised size of the positive HbO overshoot approximately halved, the barrels’ haemodynamic responses to whisker stimulation were not delayed temporally or particularly affected spatially by IL-1β or co-injection of AMPA+IL-1β. Conversely, the HbO response remained statistically unchanged in both the vehicle and AMPA groups.

Overall, however, it may be concluded that striatal IL-1β, with or without AMPA, leads to an impedance of the functional haemodynamic response to stimulation in the barrel cortex. Interestingly, the reduction in the size of the HbO response at 6h correlated significantly with the levels of ipsilateral IgG, oedema and
cortical neutrophils, despite significant oedema only occurring in the AMPA+IL-1β group.

The size of the HbT response fell significantly over time in only the IL-1β condition at 6h when assessing the raw data, though when normalised values were tested, HbT responses were found to become smaller over time in groups excepting vehicle. Within the AMPA group, there was one animal that had an extremely large pre-injection haemodynamic response, so the small decline in haemodynamic response seen in this group may be, at least in part, attributed to this. However, the HbT responses of animals in the IL-1β and AMPA+IL-1β showed significant reductions towards the end of the experiment when compared to the first experiment after injection.

Moreover, when the late HbR decrease was teased apart from the overall HbT response and examined separately, a reduction in the amplitude of the HbR response was seen in the IL-1β group. This may be because the net HbR ‘late decrease’ response is largely indicative of the larger influx changes in HbT, replenishing the levels of HbO and washing away the waste products of oxidative metabolism, including HbR itself.

The initial increase in HbR at the onset of the stimulus, thought to be indicative of the metabolism of oxygen by active neurons (Hu and Yacoub, 2012), did not change in any of the groups, suggesting that the metabolic demand for oxygenated blood was not significantly affected in the imaged barrels. However, the ‘deoxy dip’ is incompletely understood and is comparatively small, so would require averaging over many more trials than were used here to infer solid conclusions.
The results of the vessel analysis suggest that the MCA of animals injected with AMPA+IL-1β carried less HbO over time, as signified by a reduction in the ‘depth’ of the vessel from 2h after injection. The diameter of these arteries did not, however, change significantly, while dural vessels in all groups stayed the same, both in measurements of diameter (x-y) and depth (x-z). This may imply that the MCA vessels in the AMPA+IL-1β may have been undergoing pressure in the x-z axis, though not along the x-y axis, perhaps due to swelling or oedema of the inflamed brain.

The histology data overall confirms that injection of IL-1β leads to recruitment of neutrophils and extravasation of IgG between 4 and 6h. Perhaps unsurprisingly, IgG levels and the number of neutrophils and amount of oedema correlate, although oedema and cortical neutrophils were not significantly associated.

3.3.2 Interpretation of results

The imaging data taken together suggest that striatal injection of IL-1β hampers the neurovascular response to stimulation of the barrel cortex, at least to a depth reached by the optical imaging (up to a maximum of approximately 1mm). IL-1β has been reported to have diverse and modulatory effects on CBF, ranging from vasodilation via prostaglandins and nitric oxide (Monroy et al., 2001, Osuka et al., 1997) to vasospasm and net reduction in CBF (Maher et al., 2003, Seto et al., 2006). Administration of IL-1β has also been shown to reduce blood flow in the infarct after MCAo (Parry-Jones et al., 2008) and endogenous levels of IL-1β are found to correlate with vasospasm after subarachnoid haemorrhage (Fassbender et al., 2001).
However, the effects of IL-1β on the functional neurovascular response to sensory stimulation (rather than the net change in CBF) have not, to our knowledge, previously been studied. It may be that a disruption to the function of the cortex, resulting in an uncoupling of the neurovascular response may contribute to IL-1β’s exacerbation of AMPA-induced excitotoxicity. This hypothesis is supported by the association between the decrease in the HbO response at 6h and the markers of BBB breakdown, neutrophils and oedema in the injected hemisphere.

No cell death was found in preliminary studies using markers for apoptosis. However, we may safely assume that despite the reduction in the haemodynamic response, there would have been no cortical cell death in the IL-1β condition; several previous studies have injected higher doses into rat striatum and seen similar levels of IgG extravasation and neutrophils, but no cell death (Anthony et al., 1997, Blamire et al., 2000). It remains to be seen what the defining factor is that triggers the demise of eventual cortical neurons in the AMPA+IL-1β group but not in the IL-1β cohort.

Early (4-6h) presence of IgG and oedema has been reported after injection of IL-1β before, with BBB permeability resolving after 24h (Blamire et al., 2000). It is well-documented that transmigrated neutrophils release, amongst other inflammatory chemicals, matrix metalloproteinases that further disrupt the integrity of the BBB (Gidday et al., 2005). Leukocyte depletion with anti-PMN serum or radiation before injection of IL-1β has previously been shown to prevent breakdown of the BBB (Anthony et al., 1997, Blamire et al., 2000). Mice lacking the IL-1R1 receptor do not show the same leukocyte recruitment in response to IL-1β, suggesting the extravasation of neutrophils is also IL-R1-dependent (Ching et al., 2005). In this study, however, it was intriguing to find that there was no significant increase in the
number of striatal neutrophils at 6h in the AMPA+IL-1β group of the second study, even though the cortical neutrophil count and level of IgG immunostaining matched that of the IL-1β group. The reason behind this is not clear, though it’s possible that the IL-1β-dependent recruitment of neutrophils to the striatum was diminished if endothelial cell production of adhesion molecules was impaired by the co-injection of AMPA.

One of the hypotheses behind the AMPA+IL-1β induced cortical pattern of cell death is that cerebral oedema may cause a physical obstruction of the feeding surface arteries, compressing them against the inside of the dura, which is relatively inflexible. This hypothesis would be largely supported by the vessel data here: while the dural vessel diameter and content did not change significantly in any of the groups, and the diameter across the MCA as seen from the surface of the brain did not alter either, it seemed as though the artery was compressed normal to the surface of the skull. Further support for this hypothesis comes from the negative correlation between cerebral oedema and the MCA content; it may be that the more the brain had swelled, the less HbO the MCA could carry. An alternative explanation for this result in the AMPA+IL-1β could be vasoconstriction, though no other groups showed a similar pattern. This may suggest that any active vasoconstriction may be as a result of synergistic effects of IL-1β and AMPA, or via indirect modulatory actions on vasoactive mechanisms.

The depth in the MCA was also negatively associated with increases in heart rate, indicating that systemic changes in blood flow and pressure may play a role in this model. However, it is unclear why the IL-1β would not elicit a similar drop in MCA content if this is the case. Hypotension and elevated heart rate are both typical of septic shock, and i.v. interleukin-1 has previously been shown to led to similar
symptoms in rabbits (Dinarello et al., 1989). Moreover, AMPAR activity is known to be involved in the baroreceptor reflex in the nucleus tractus solitarii. While glutamate acting here usually promotes sympathetic hypertension, AMPA-induced excitotoxic death of these neurons may impair the baroreceptive response and lead to hypotension (Li et al., 2012).

Increases in heart rate and core temperature acted as indirect markers for the bioactivity of the IL-1β. The pro-inflammatory cytokine is known to elicit a febrile response in the brain via IL-1RI receptors in the hypothalamus (Busbridge et al., 1989). The increases in these physiological parameters occur as early as 1-2h after injection of IL-1β with or without AMPA and correlate strongly, though there appears to be an upper limit on the change in heart rate (about +180 bpm), at which point this association plateaus despite further increases in core temperature.

In addition, the fever response seen did not (negatively) correlate significantly with the size of the HbO response, which may suggest these factors are dissociated from one another. Despite the injection of IL-1Ra into the lateral hypothalamus protecting against the characteristic cortical cell death (Allan et al., 2000), the increase in temperature in the AMPA+IL-1β group has been found to be dissociated from cortical damage in this model (Grundy et al., 1999).

### 3.3.3 Further work

The purpose of this study was to use optical imaging spectroscopy to explore the haemodynamic response in the context of excitotoxicity and neuroinflammation. The deterioration of the haemodynamic response seen in the IL-1β and AMPA+IL-1β raises more questions. Does the drop in the blood flow response reflect a lower
demand for oxygen due to diminished cellular activity? Or, alternatively, does the reduction in blood flow impoverish the active cells in the barrel?

The study within the next chapter will aim to address these questions. By measuring cellular activity and tissue oxygenation responses to whisker stimulation simultaneously, it may be possible to further characterise the AMPA+IL-1β model in order to elucidate the role of IL-1β in exacerbating AMPA-induced excitotoxicity.
4 Results 2

This chapter describes the results of the experiments carried out using the APOX probe, as well as physiological parameters and the histology performed on the brains after electrode recordings. This study aimed to elucidate whether the IL-1β-dependent haemodynamic depression seen in the previous chapter using OIS is a symptom, or a preceding factor associated with changes in neural activity, as measured in MUA and LFPs.

4.1 Tissue oxygenation

Local tissue oxygenation recordings were taken from layer IV/V of the active whisker barrel during whisker stimulation before and after striatal injection using the APOX probe. In all animals, when the probe was inserted, an extremely low oxygenation reading was seen, which then rose gradually, sometimes to a plateau over a number of hours. It is speculated that the initial low recordings reflect an artefact of the probe exerting pressure on the surrounding tissue, and vice versa.

Figure 4-1A shows the change in the (unstimulated) tissue oxygenation readings in the different groups. All animals were included in this analysis - as it did not require a response to stimulation - so the groups were as follows: vehicle, \( N = 6 \); AMPA, \( N = 4 \); IL-1β, \( N = 9 \); and AMPA+IL-1β, \( N = 7 \). The \([O_2]\) before injection did not differ significantly between groups (\( F = 0.7283, p = 0.5460 \)). There was no effect of the injection on the baseline tissue concentrations of oxygen (\( F = 1.589, p = 0.2253 \)), but there was a significant effect of time on the groups (\( F = 10.92, p < 0.0001 \)) as all groups increased over time. As there appears to be a strong global
effect over time in baseline tissue oxygenation recordings, it is difficult to comment on any effects of injection. However, it appears that the \([O_2]\) in animals injected with AMPA+IL-Lβ rose faster to a peak at about 3h, returning by 6h to a similar level to the other groups, which show a very steady increase to a plateau oxygenation level. However, there were no significant differences in the baseline \([O_2]\) measurements between any of the groups at any time points, and no difference between groups’ time-to-maximum baseline \([O_2]\) \((F = 1.667, p = 0.2032;\) one-way ANOVA; Figure 4-1B).

Another observation made was that the profile of the oxygenation response to whisker stimulation changed over time; instead of an initial dip followed by a classic overshoot of oxygen, ‘negative’ responses were increasingly seen over time (Figure 4-2). This occurred in all the groups at a similar rate.
Figure 4-1 Baseline oxygenation and time-to-maximum baseline measurement

A, Average resting tissue concentration of oxygen over time before (‘Pre’) and for 6h after injection of vehicle (N = 6), AMPA (N = 4), IL-1β (N = 9) or AMPA+IL-1β (N = 7). No significant difference was found between the groups at any time point. Mean ± SEM shown.

B, The time to maximum measurement of unstimulated tissue [O₂] in each group. There was no significant difference found between groups.
Example of the change seen in the profile of the oxygenation response. After the
animal was injected with vehicle, these recordings (A-F, 1-6h respectively) were
made approximately each hour after injection of AMPA+IL-1β. The grey box in
each case represents the 4s whisker stimulation, and each example has been averaged
over 30 trials. Over time, the peaks of the oxygenation overshoot (seen especially in
A at 6-7s) becomes smaller than the initial dip, and it takes longer to return to the
pre-stimulus level. Note that the size of the dip does not grow significantly in
amplitude, rather, the latency of its resolution increases.

The stimulus-elicited changes in [O₂] (whether positive or negative) were
determined and compared between groups to establish whether there was an effect of
the injection on the ‘height’ of the response. Only animals that showed a ‘classic’
positive response (with a positive overshoot at offset of the stimulus) previous to
injection were used in this comparison to remove any animals in which the
oxygenation response might already be impaired. Within the animals included (N =
5, 4, 5 and 5 for vehicle, AMPA, IL-1β and AMPA+IL-1β respectively), there was
no significant difference between groups’ average raw pre-injection responses (F =
0.5444, \( p > 0.05 \); Figure 4-3) and no effect of injection on the decline of the size of the oxygenation response to whisker stimulation (\( F = 0.03344, \ p > 0.05 \)). However, for all groups there was a significant effect of time on the micromolar \([O_2]\) response, which declined markedly by 6h to approximately -100% of the pre-baseline response (\( F = 7.669, \ p < 0.0001 \)).

![Stimulated \( \Delta O_2 \) (\( \mu \text{mol} \))](image)

**Figure 4-3 Absolute stimulus-induced change in oxygenation**
The average size of the oxygenation response, determined by subtracting the pre-stimulus baseline from the mean oxygenation just for the 2.5s after stimulation. Responses were recorded before and after injection of vehicle, AMPA, IL-1β or AMPA+IL-1β (\( N = 5, 4, 5 \) and 5 respectively). Mean ± SEM shown.

The size of the oxygenation response correlated significantly with the corresponding resting concentration of oxygen in all groups (vehicle, \( r = -0.4301, \ p < 0.05 \); AMPA, \( r = -0.5337, \ p < 0.01 \); IL-1β, \( r = -0.4461, \ p < 0.05 \); AMPA+IL-1β, \( r = -0.5824, \ p < 0.01 \)). In other words, the size of the oxygenation response decreased or became more negative as the resting concentration increased Figure 4-4.
Because there was a global effect over time on the tissue oxygenation response, the absolute values of oxygenation were not used to further compare between groups. Instead, the percentage change in oxygenation in response to whisker stimulation before and after injection was calculated. Before injection, there was no significant difference between the groups’ average percentage change in oxygenation ($F(Pre, r) = 2.988, p > 0.05$). Two-way ANOVA performed on non-normalised data did not reveal any effect of injection on the oxygenation responses over time (Figure 4-5), though differences were identified used within-group analyses, described below.

According to one-way repeated measures (within group) ANOVA on non-normalised data, IL-1β caused a decline in the ‘height’ of the oxygenation response
(F(r) = 3.562, p < 0.05), specifically at 2h (t(r) = 3.507, p < 0.05), 3h (t(r) = 3.660, p < 0.05), 4h (t(r) = 3.426, p < 0.05) and at 6h (t(r) = 3.480, p < 0.05) compared to the pre-injection response. In the same way, repeated measures one-way ANOVA comparing groups to the pre-injection measurement identifies a significant reduction in the AMPA+IL-1β group’s responses almost immediately after injection, from 1-6h (1h, t(r) = 3.948, p < 0.01; 2h, t(r) = 4.019, p < 0.01; 3h, t(r) = 5.931, p < 0.001; 4h, t(r) = 5.768, p < 0.001; 5h, t(r) = 4.833, p < 0.001; 6h, t(r) = 4.958, p < 0.001); Bonferroni’s multiple comparison tests; Figure 4-5). In contrast, no decrease was found in the two other groups over time (vehicle, F(r) = 2.488; AMPA, F(r) = 1.283; p > 0.05; one-way repeated measures ANOVA). While the two statistical analyses paint slightly different pictures, the extent of the normalised reduction of the neurovascular response in IL-1β and AMPA+IL-1β groups was similar (-50%).

**Figure 4-5 Changes in oxygenation response percentages**
The change in the size of the oxygenation response (% baseline concentration) to whisker stimulation. The graph shows that animals injected with IL-1β with or without AMPA showed more negative oxygenation responses that AMPA or vehicle groups. Mean ± SE; *, p < 0.05; **, p < 0.01; one-way repeated measures ANOVA.
It should be noted that although all animals’ responses tended towards the negative BOLD response more frequently over the 6h, those injected with AMPA+IL-1β or IL-1β alone showed a markedly accelerated progression to the negative BOLD profile than the other groups.

4.2 Local field potentials

Gamma- and beta-frequency oscillations were quantified using spectrogram analysis described in the Methods. Animals were excluded from analysis if there was no positive peak in gamma and beta activity at the onset of the stimulus in experiments before injection to allow for comparison with post-injection results, resulting in groups as follows: vehicle, $N = 4$; AMPA, $N = 2$, IL-1β, $N = 5$; AMPA+IL-1β, $N = 5$.

Two-way ANOVA detected an effect of time on both gamma and beta activity peaks (gamma, $F(r) = 16.09, p < 0.001$; beta, $F = 28.75, p < 0.001$) and an interaction effect for the size of the gamma peaks ($F(r) = 1.963, p < 0.05$). One-way ANOVA were therefore used to probe the changes within groups.

AMPA+IL-1β animals showed a rapid decline in gamma activity after injection ($F(r) = 18.42, p < 0.0001$; Figure 4-6A), with significantly impaired gamma activity after only 1h for the duration of the experiment (1h, $t(r) = 5.851$; 2h, $t(r) = 7.693$; 3h, $t(r) = 7.825$; 4h, $t(r) = 7.928$; 5h, $t(r) = 8.248$; 6h, $t(r) = 8.595$; all $p < 0.0001$. Similarly, IL-1β-injected animals showed a decline in gamma activity, ($F(r) = 14.22, p < 0.0001$), reaching significance after 2h (2h, $t(r) = 4.346, p < 0.01$; 3h, $t(r) = 5.705$; 4h, $t(r) = 6.151$; 5h, $t(r) = 7.163$; 6h, $t(r) = 7.417, p < 0.001$). In contrast, the vehicle group only showed a gradual and non-significant decline in gamma activity over time (vehicle, $F(r) = 1.501$, $p > 0.05$), and since the AMPA
group only comprised two animals, no valid statistical analysis could be used for this group.

Two way ANOVA found a significant effect of time on beta activity \( (F(r) = 24.59, p < 0.001) \). However, due to large (though non-significant) variation in the size of the beta activity peaks before injection, one-way ANOVA were used to analyse changes in beta activity in each of the groups. Beta peaks became smaller in IL-1β and AMPA+IL-1β groups at time points at 3 and 6h (IL-1β: 3h, \( t(r) = 4.548, p < 0.01; 4h, t(r) = 4.844, p < 0.01; 5h, t(r) = 5.146, p < 0.001; 6h, t(r) = 5.829, p < 0.001; \) AMPA+IL-1β, 3h, \( t(r) = 2.018; 4h, t(r) = 5.824; 5h, t(r) = 5.618; 6h, t(r) = 5.535; \) all \( p < 0.001 \), Figure 4-6B). Although the AMPA data points at 4-6h seem to be similarly different to vehicle in the beta analysis, there were only two animals in this group and so no statistical comparison could be made. The vehicle also showed a statistical decline in beta activity over time \( (F(r) = 2.964, p < 0.05) \), though Bonferroni post hoc tests did not reveal any specific time point at which beta activity was significantly lower than the pre-injection levels, so may be considered a general effect over time.
Figure 4-6 Gamma and beta peak activity

A Graph showing the averaged net (i.e. ‘Pre’ normalised to 0) changes in the log amplitude of the A, gamma and B, beta peaks at the onset of the stimulus in groups before (‘Pre’) and after striatal injection. Mean ± SEM; **, p < 0.01; ***, p < 0.001; repeated measures one-way ANOVA Bonferroni post-tests vs. Pre, using raw data.
The size of the net change in the amplitude of beta and gamma peaks (as shown in Figure 4-6) correlated significantly in all groups (vehicle, \( r = 0.5387, p < 0.01 \); AMPA, \( r = 0.9058, p < 0.001 \); IL-1\( \beta \), \( r = 0.6513, p < 0.001 \); AMPA+IL-1\( \beta \), \( r = 0.6095, p < 0.001 \); Figure 4-7).

![Figure 4-7 Correlation of gamma and beta peak](image)

Correlation between the net change in log amplitudes of peaks in gamma and beta frequency oscillations at the onset of the stimulus. Lines of best fit shown.

### 4.3 Multi-unit activity

MUA (300-3000Hz) in response to whisker stimulation was recorded before and up to 6h after striatal injection of vehicle, AMPA, IL-1\( \beta \) or AMPA+IL-1\( \beta \). Animals were excluded from MUA analysis if they did not exhibit a peak in MUA at stimulus onset in the trials before striatal injection to ensure only activity from stimulated barrels was included, leaving the final group \( N \)-numbers as: vehicle, \( N = 4 \); AMPA, \( N = 3 \); IL-1\( \beta \), \( N = 6 \); AMPA+IL-1\( \beta \), \( N = 4 \).
The raw values of the peak MUA response calculated from pre-injection experiments did not significantly differ between groups, though a significant effect of time was identified \( F(r) = 5.095, p < 0.001 \). Repeated measures one-way ANOVA comparing the pre-injection trials with subsequent measures of the MUA peak after the injection found that only the vehicle group showed a significant decrease, between 4 and 6h \( F(r) = 4.793, p < 0.01; 4h, t(r) = 4.091; 5h, t(r) = 4.123; 6h, t(r) = 3.841; \) all \( p < 0.05 \); no other groups deviated significantly from the baseline (Figure 4-8A). Interestingly, two of the four animals in the AMPA+IL-1β group exhibited a large increase in the level of stimulus-evoked MUA immediately after injection, though the other two did not.

The level of ‘spontaneous’ or resting activity – that is, MUA outside whisker stimulation, recorded before and after injection - was also investigated (Figure 4-8B). The raw values of the spontaneous MUA before injection did not differ significantly between groups \( F(r) = 0.7368, p > 0.05 \). There was no significant difference between the groups’ resting MUA over time, according to two-way ANOVA \( p > 0.05 \). One-way ANOVA showed that there was an effect of vehicle between 4-5h \( F(r) = 3.453; 4h, t(r) = 3.601; 5h, t(r) = 3.591; \) all \( p < 0.05 \) and AMPA+IL-1β \( F(r) = 2.677, p < 0.05 \) on the level of spontaneous MUA, though no particular time point was indicated by Bonferroni multiple comparisons for AMPA+IL-1β differences.
Figure 4-8 Stimulus-evoked and spontaneous MUA

A, Graph showing net change in the amplitude of the peak in MUA at onset of the stimulus. B, Graph showing the level of spontaneous MUA outside stimulation before and after striatal injection. Mean + SEM shown; *, p < 0.05; repeated measures one-way ANOVA vs. pre-injection, Bonferroni multiple comparisons.
4.4 Correlation analyses

Oxygenation, MUA and LFP data were tested for significant correlations to investigate associations between neural activity and the oxygenation response. For each comparison, the data from animals used in the previous results were normalised to give percentage changes relative to the pre-injection measurement, but only normalised data from after the injection were compared, averaged as before into hourly time points.

Normalised changes in MUA at the onset of the stimulus correlated with the size of gamma activity peaks in AMPA ($r = 0.7133, p < 0.01$), IL-1β ($r = 0.8532, p < 0.0001$) and AMPA+IL-1β groups ($r = 0.8555, p < 0.0001$), though not in the vehicle group (Figure 4-9A). Likewise, beta activity correlated significantly with MUA in the AMPA ($r = 0.7692, p = 0.0034$) and AMPA+IL-1β ($r = 0.8425, p < 0.0001$) groups (Figure 4-9B).

Interestingly, the size of the peak in gamma oscillations only correlated with the % change in oxygenation in the vehicle group ($r = 0.4070, p < 0.05$; Figure 4-10A). No significant correlation was found between beta activity and the percentage oxygenation responses (Figure 4-10B), though a trend was seen in the vehicle group ($r = 0.3765, p > 0.05$, n.s.).
Correlation between the log changes in A, gamma and B, beta frequency activity at the onset of the stimulus, and the change in the amount of MUA. All groups that showed a significant correlation are indicated with a line of best fit.
Figure 4-10 Coupling of gamma and beta activity with oxygenation response

Scatter graph showing A, gamma and B, beta peak amplitude and the change in the size of the oxygenation response (relative to pre-injection). Line of best fit shown where significant correlation occurred.
4.5 Physiological parameters

As in the other studies, heart rate and temperature were monitored throughout the animal experiments (vehicle, $N = 7$; AMPA, $N = 5$; IL-1β, $N = 9$; AMPA+IL-1β, $N = 7$).

A two-way ANOVA found the difference in heart rate between the vehicle group and IL-1β-injected animals at 2-6h (2h, $t(r) = 6.747$; 3h, $t(r) = 6.633$; 4h, $t(r) = 6.248$; 5h, $t(r) = 5.127$; 6h $t(r) = 5.160$; all $p < 0.001$). Similarly, the heart rate of animals injected with AMPA+IL-1β was significantly higher than those injected with vehicle between 2-6h ($t(r) = 3.501$, $p < 0.01$; 4h, $t(r) = 4.281$; 4h, $t(r) = 4.748$; 5h, $t(r) = 4.377$; 6h, $t(r) = 4.219$; $p < 0.001$; Figure 4-11A). Repeated measures one-way ANOVA within groups revealed mirrored the same indication of large increases in heart rate in the two groups injected with IL-1β.

Two-way ANOVA revealed a significant effect of the injection ($F(r) = 3.033$, $p < 0.001$), time ($F(r) = 3.145$, $p < 0.001$) and an interaction ($F(r) = 3.309$). The elevation in core temperature was most obvious among the IL-1β animals, with IL-1β-injected animals becoming significantly warmer than the control group between 3 and 4h after injection (3h, $t(r) = 3.044$, $p < 0.05$; 4h, $t(r) = 3.447$, $p < 0.01$). Although the AMPA+IL-1β-injected animals did become warmer than previous to the injection, the two-way ANOVA Bonferroni post hoc tests did not detect a significant difference compared to vehicle (Figure 4-11B). One-way ANOVA detected effect of the injection of IL-1β at 3-4h ($F(r) = 6.068$, $p < 0.001$; 3h, $t(r) = 3.044$, $p < 0.05$; 4h, $t(r) = 3.447$, $p < 0.01$); and in the AMPA ($F(r) = 2.912$, $p < 0.05$) and AMPA+IL-1β ($F(r) = 2.795$, $p < 0.05$) groups, though Bonferroni multiple comparison tests did not identify significantly deviated values at particular time points for these groups.
As seen previously, the net change in heart rate and core temperature correlated significantly ($r = 0.7682, p < 0.0001$), indicating that these changes are related to a fever response elicited by the injection of IL-1β with or without AMPA, rather than an artefact of the maintenance of anaesthesia (Figure 4-11C).
Physiological parameters from electrode study

Net changes in A, heart rate and B, temperature (ΔT) in animals used for electrode experiments. Mean ± SEM shown; *, p < 0.05; **, p < 0.01; ***, p < 0.001; repeated measures one-way ANOVA Bonferroni multiple comparisons. C, Net changes in heart rate and temperature correlate significantly.
4.6 Histology

Brains taken after electrophysiological experiments were analysed for IgG immunostaining, ipsilateral vs. contralateral volume ratios, and cresyl violet optical density. Brains were excluded from these analyses if the APOX probe was inserted more than once into the brain prior to striatal injection to limit the possible confound of probe insertion on IgG leakage and ipsilateral hemisphere volume (leaving vehicle, \( N = 5 \); AMPA, \( N = 4 \); IL-1β, \( N = 5 \); AMPA+IL-1β, \( N = 5 \)).

Significantly more IgG was found in the ipsilateral versus the contralateral hemisphere in AMPA (\( t = 3.962, p < 0.05 \)), IL-1β (\( t = 6.551, p < 0.01 \)) and AMPA+IL-1β groups (\( t = 9.626, p < 0.001 \)), but not in the vehicle group (\( t = 2.259, p > 0.05 \)). Insertion of the probe probably would have contributed to elevated IgG extravasation in the ipsilateral hemisphere, so test groups’ IgG immunostaining levels were compared with vehicle in one-way ANOVA (\( F = 11.70, p < 0.001 \); Figure 4-12A). As in previous studies, more ipsilateral IgG immunostaining was present in brains injected with IL-1β and AMPA+IL-1β compared to the vehicle group (IL-1β, \( t = 4.920, p < 0.01 \); AMPA+IL-1β, \( t = 4.522, p < 0.001 \)), while there was no significant difference between the AMPA and vehicle groups (\( t = 1.048, p > 0.05 \)).

In addition, injection of AMPA+IL-1β led to significantly more ipsilateral oedema than the AMPA group (\( t = 1.773, p < 0.05 \); one-way ANOVA; Figure 4-12B). No other group showed significant ipsilateral oedema compared to other groups in this one-way ANOVA (\( F = 5.406, p < 0.05 \)). According to Student’s t-test, however, the AMPA+IL-1β group did show significantly more oedema than vehicle (\( t = 2.631, p < 0.05 \)).
Cresyl violet dye, known to stain Nissl bodies in neurons, was used to assess any early indications of neuronal ER stress, oedema or cell death. The difference between the ipsilateral and contralateral hemispheres’ striatum and somatosensory cortex were measured and compared between injection groups. Even though through the microscope, there were no obviously perceptible differences between the hemispheres, there were some effects of injection on the optical density difference.
between the injected and contralateral hemispheres in the striatum and layer IV/V of the somatosensory cortex.

Layer IV/V of the ipsilateral cortex was found to be significantly less optically dense than the contralateral hemisphere in all groups (vehicle, \( t = 3.456, p < 0.05 \); AMPA, \( t = 4.396, p < 0.05 \); IL-1β, \( t = 4.4775, p = 0.01 \); AMPA+IL-1β, \( t = 7.984, p = 0.001 \); one-sample t-tests), presumably due to the insertion of the probe. One-way ANOVA (\( F = 3.286, p < 0.05 \)) revealed a significant difference between the cortical ipsi:contra optical density shift between animals injected with vehicle and those injected with IL-1β (\( t = 3.002, p < 0.05 \)). According to Student’s t-test, the difference between the ipsilateral and contralateral cortex was significantly greater in animals injected both with IL-1β (\( t = 2.609, p < 0.05 \)) and AMPA+IL-1β (\( t = 3.361, p < 0.01 \)). In other words, the interhemispheric optical density difference was bigger in the cortex of animals injected with IL-1β, with or without AMPA, than vehicle.

No significant difference was found between the ipsilateral and contralateral striatum of animals injected with vehicle (\( t = 1.452, p > 0.05 \)) or IL-1β (\( t = 2.339, p > 0.05 \); Student’s t-tests). In contrast, the mean optical density of the ipsilateral striatum in animals injected with AMPA or AMPA+IL-1β was significantly lower than the contralateral hemisphere (IL-1β, \( t = 6.611, p < 0.01 \); AMPA+IL-1β, \( t = 2.910, p < 0.05 \); Student’s t-test). One-way-ANOVA indicates an effect of groups on the extent of the interhemispheric difference in striatal optical density (\( F = 4.857, p < 0.05 \)), with a significant difference between vehicle and AMPA+IL-1β groups (\( t = 2.996, p < 0.05 \)). Unpaired t-tests identify a significant difference in the interhemisphere optical density between AMPA-and AMPA+IL-1β-injected animals compared to vehicle (AMPA, \( t = 4.874, p < 0.001 \); AMPA+IL-1β, \( t = 2.402, p < 0.05 \)).
Figure 4-13 Cresyl violet interhemispheric paling
Difference in optical density between the ipsilateral and contralateral A, somatosensory cortex and B, striatum; higher values indicate that the ipsilateral hemisphere had a lower optical density than the contralateral hemisphere. Mean ± SEM shown; *, p < 0.05; **, p < 0.01; ***, p < 0.001 in unpaired t-tests vs. vehicle.

Interestingly, the IgG score for each brain correlated significantly with the ipsi:contra difference in optical density within the cortex (r = 0.7627, p = 0.0001; Figure 4-15A), but not for the striatum (r = 0.05983, p < 0.05; Figure 4-15B).
Figure 4-14 Example photomicrographs of cresyl violet tissue
Example photomicrographs of the contralateral and ipsilateral hemispheres of the same section of cresyl violet-stained A, somatosensory cortex and B, striatum taken at 10x magnification.
Figure 4-15 Correlation of striatal and cortical paling with IgG
The difference between ipsi- and contra-lateral A, cortex and B, striatum compared with the level of ipsilateral IgG for each brain. A paling of the ipsilateral cortex is associated with higher levels of IgG, but there is no significant correlation between striatal paling and IgG.

There were no significant associations between net changes in heart rate or core temperature and the paling of the ipsilateral cortical tissue ($\Delta$HR, $r = 0.3358$, $p > 0.05$; $\Delta$T, $r = 0.3627$, $p > 0.05$).
4.7 Discussion

4.7.1 Summary of findings

In this set of experiments, an APOX probe was used to measure stimulus-elicited changes in tissue oxygenation and neural activity simultaneously from layer IV/V of the active barrel, before and up to 6h after injection of vehicle, AMPA, IL-1β or AMPA+IL-1β.

When using the supposed absolute concentrations derived from the calibration equation supplied by the APOX manufacturer, there was no difference between groups in the unstimulated concentration of oxygen in the cortex, or in the size of changes in the concentration of oxygen during stimulation. All groups exhibited negative responses towards the end of the 6h. However, when the oxygenation was expressed in terms of percentage change from the pre-stimulus concentration (Li and Freeman, 2007), we could more easily compare changes between groups. Using this method, a fall in the size of the oxygenation response was found in both the IL-1β and AMPA+IL-1β groups, while the vehicle and AMPA groups showed little reduction in the size of the percentage change oxygenation response compared to pre-injection.

While this result does not explain why negative responses were increasingly seen over time, it does imply that even the vehicle and AMPA groups’ negative responses did not represent a deterioration of the oxygenation response as it did in the IL-1β-injected animals. This finding may be related to the (non-significant) trend observed, where the unstimulated oxygenation reading in IL-1β-injected animals rose to a peak and then started to decline, unlike the measures for the vehicle and AMPA groups, which kept rising consistently. In future, it would be a good idea to
calibrate the instrument after removal from the cortex to check whether the shift in
the signal within the brain was due to a gradual rise of gases within the electrode
during the experiment. If this were the case, a linear slope calibration timescale
could be constructed and the data processed to take this into account.

Neural activity responses were recorded in this study to examine the extent of
neurovascular coupling. The peaks of gamma and beta frequency oscillations at the
stimulus onset were found to give the best SNR measure of cortical cell activity in
the vehicle group. The AMPA+IL-1β and IL-1β groups both showed a diminishing
of the gamma frequency component about 2h after injection compared to the pre-
injection baseline. Compared to the vehicle group, peaks of beta activity were also
found to be significantly smaller in the IL-1β- and AMPA+IL-1β-injected animals
towards the end of the experiment (4-6h). Unfortunately the AMPA group in these
analyses was too small (N = 2) to allow valid comparisons with regard to these
parameters.

The MUA analysis detected a significant decline in the postsynaptic activity
in the vehicle group at 4-6h. One possible explanation for this decline in the control
group could be that the majority of the vehicle experiments were carried out before
the other groups, when still ensuring the set-up would work. These experiments
would have taken longer to set-up than when the instruments became more familiar,
so the maintenance of the animal under longer durations of anaesthesia may have
had a negative effect on the animal’s cortical activity. A (non-significant) ‘dip’ in the
amount of resting MUA was detected in the AMPA+IL-1β group between 1-3h after
injection, but after this point the level of spontaneous MUA returned back to normal
pre-injection levels. One possible hypothesis for this phasic pattern is discussed in
the next subsection.
Positive correlative associations were found between normalised stimulus-evoked gamma activity and corresponding MUA peaks in both the IL-1β and AMPA+IL-1β groups, as well as between beta activity and MUA in AMPA and AMPA+IL-1β groups. The level of gamma activity is widely thought to be associated with the strength of the BOLD response (Magri et al., 2012), and indeed, the vehicle group showed a significant correlation between these parameters. Beta oscillations, on the other hand, showed no significant association with the size of the oxygenation response in any of the groups.

As in the previous results chapter, the striatal injection of IL-1β (with or without AMPA) mediated a significant rise in heart rate (with similar, though less significant effects on temperature seen), as well BBB breakdown. The AMPA+IL-1β group also exhibited significantly more oedema than the vehicle group. Interestingly, although the time points used here were too early for cresyl violet stain to clearly delineate areas of any cell death, there were differences found between hemispheres in the striatum and cortex in various groups. AMPA, with or without co-injection of IL-1β, led to a paling of the ipsilateral striatum, while IL-1β, with or without AMPA, led to a paling of the ipsilateral cortex. While cell death in the striatum is known to occur as a result of AMPA-induced excitotoxicity, we know that in this model, IL-1β alone does not lead to cell death. Also, from closer examination of the morphology of cells in the cortex, it was clear that while striatal cells exposed to AMPA (with or without Il-1β) looked damaged, the cortical cells in animals injected with IL-1β (with or without AMPA) did not look ‘unwell’ – rather, there was just more space between them. A strong correlation was found between the levels of IgG and the amount of ipsilateral paling in the cortex, but not in the striatum. This evidence suggests that increased extracellular fluid resulting from IL-1β-dependent BBB
leakiness could be particular to the vulnerable cortex. Moreover, since the AMPA-induced striatal cell death is well-characterised, we may speculate that the paling in the striatum even at this early time point is related to the demise of neurons in this area after injection of AMPA or AMPA+IL-1β.

4.7.2 Interpretation of results

The size of the oxygenation response decreased and even became negative, in all groups. Negative BOLD responses have been associated with deceased gamma-band activity (Magri et al., 2012), although it may have been a reflection of low levels of beta activity as well (Magri et al., 2012). Lower beta activity has been shown to affect the latency of the positive overshoot typically seen in a ‘normal’ haemodynamic response, so it could be that the change in the oxygenation profile may not be a reduction of the overshoot, but rather a delay, such that the next trial started before the oxygen deficit was compensated for.

The level of multi-unit activity, both spontaneous and stimulus-evoked, was consistent in the other groups, but decreased dramatically early on (1-3h) in half the animals injected with AMPA+IL-1β (though the group average was not significantly affected). This time course is consistent with a pattern of early necrosis/late apoptosis (Choi, 1987, Ankarcrona et al., 1995, Bonfoco et al., 1995), where cells that suffer an excitotoxic dose of intracellular Ca$^{2+}$ either die rapidly from loss of membrane potential and subsequent necrosis, or regain membrane potential after ~3h, and so still can produce an ‘output’ but die approximately 24h later from programmed apoptosis. This order of events fits that observed in this model; cortical cells do not die until around 24h after injection. It may be that the cortex in the AMPA+IL-1β group suffers at the early time point from the excitotoxicity more than
that of the AMPA group. The fact that only two of the four AMPA+IL-1β-injected animals exhibited this may also help signify why the cortical damage seen in the AMPA+IL-1β model tends to be ‘all-or-nothing’, though more animals would have to be tested to see whether changes in MUA (or indeed any of the measures described here) correlate with cell death at a later time point.

Crucially, the size of the gamma activity peak correlated significantly with the percentage change in the oxygenation response in the vehicle group, but not in any of the other groups. A linear relationship between these two parameters has previously been reported (Magri et al., 2012, Niessing et al., 2005), so it may be argued that in the test conditions, the lack of correlation indicates a loss of coupling between neural activity and the corresponding neurovascular response. In particular, in Figure 4-10A, we can see that there are four ‘clusters’ corresponding to the four groups. While the coupling between gamma activity and the oxygenation response remains intact within the vehicle group (demonstrated by the correlation), tight neurovascular coupling is lost in the other three test groups. While the AMPA group shows a slight reduction in the oxygenation response, there does not seem to be a great change in gamma activity seen. The IL-1β points show largely a loss of the oxygenation response, and little reduction of gamma activity. On the other hand, the AMPA+IL-1β are spread to show not only a reduction of the oxygenation response, but also a dramatic decrease in the relative size of the stimulus-evoked gamma activity peak.

Gamma oscillations are mediated by the alternating firing of inhibitory interneurons and excitatory pyramidal cells carrying sensory information. The inhibition provided by the GABAergic interneurons making up 20% of the barrel creates phasic windows for sensory inputs (Whittington et al., 1995, Cardin et al.,
A reduction in the amount of gamma activity may either reflect a loss of the tight synchronicity between the inhibitory interneurons, or overall diminished inhibitory:excitatory ratio. It could be argued, therefore, that the dip in gamma activity and spontaneous MUA in the AMPA+IL-1β group represented disinhibition of inputs to cortical layer IV, perhaps related to the incidence of seizure activity. Looking at the stimulus-evoked MUA data, it is difficult to comment on whether the reduction of gamma activity in the AMPA+IL-1β group is reflected in the MUA activity, though half animals injected with AMPA+IL-1β exhibited a large increase in the size of the MUA peak in the first hour after injection.

4.7.3 Limitations of the current study

The APOX probe that was used measures around 30μm at the tip, and so perhaps exerts more pressure on the surrounding tissue than other, smaller electrodes might and this may have affected the oxygenation readings. This may also help explain why all groups exhibited a systematic increase in the concentration of oxygen after probe placement, and why the responses to stimulation were better expressed as percentage changes in oxygenation.

Another issue arising from the oxygenation data was the decrease in the size of the oxygenation response, with most animals eventually demonstrating a negative response to whisker stimulation. This occurred in almost all animals, though there was no correlation found between the length of time under anaesthesia preceding probe insertion and the decline of the oxygenation response. In the optical imaging we see no such effect on the size of the oxygenation response, so it is possible that the change is instead due to removal of the cranium over the barrel cortex before probe insertion.
This study would benefit from using more animals, especially in the AMPA group, which for many analyses only provided data from two animals. It would be interesting to see whether increased N-numbers would decrease variability to reveal a significant increase in the level of MUA immediately after injection in the AMPA and AMPA+IL-1β groups.

The next chapter will aim to combine and comment on these two results chapters, recommend further work and draw overall conclusions amounting from this thesis.
5 Discussion

5.1 Summary of key findings

In the first chapter, OIS was utilised to identify an early (<6h) IL-1β-dependent abatement of the barrel cortex’s hemodynamic response to whisker stimulation. A febrile reaction, and increased BBB breakdown and neutrophil infiltration paralleled this finding, with the latter correlating with the net decline in HbO response amplitude at 6h after injection.

The second results chapter demonstrated the IL-1β-mediated reduction of the functional neurovascular response using an APOX probe, which simultaneously records oxygen levels and electrical potentials. The AMPA+IL-1β group exhibited lower levels of gamma frequency activity at the onset of the stimulus than the other groups at 2h. Most interestingly, while the vehicle group showed a linear correlation between the size of the peak in gamma activity and the size of the oxygenation response, the other three test groups did not. In addition, less stimulus-evoked gamma and beta activity was found in the IL-1β group and AMPA+IL-1β groups after injection, while there were no notable seizure-related changes (which may have been expected) seen in MUA, or ‘output’ of the barrel cortex system. Levels of IgG matched the IL-1β-dependent pattern seen in the previous study and correlated with cortical paling using cresyl violet.

5.2 Interpretations and biological hypotheses

The AMPA+IL-1β model has been used to create several hypotheses concerning the actions of IL-1β in worsening glutamatergic excitotoxicity. However, the data presented in this thesis point towards an alteration in cellular activity, concurrent (though not necessarily
mechanistically associated) with an IL-1β-dependent diminishing of the haemodynamic response.

The finding that gamma oscillations decreased in the IL-1β and AMPA+IL-1β groups is intriguing. Previous work on the AMPA+IL-1β model has shown that activation of cortical NMDA receptors is required for IL-1β to exacerbate AMPA-induced excitotoxicity (Lawrence et al., 1998). NMDA antagonists have been repeatedly shown to upregulate gamma oscillation activity (Hakami et al., 2009, Anver et al., 2011, McNally et al., 2011), while NMDA receptor agonists are commonly pro-convulsants (Frenk et al., 1986, Piredda and Gale, 1986). Gamma oscillations are dependent on tight synchronicity between inhibitory interneurons (Traub et al., 1997), and NMDA antagonists are known to alter gamma oscillations in models of psychosis (Carlen et al., 2012). It is therefore conceivable that a reduction in gamma activity suggests disinhibition or overexcitation, perhaps via NMDA receptors.

NMDA receptors are highly calcium permeable (Lu et al., 1996) and their activity is potentiated by IL-1β (Viviani et al., 2003). Some research shows that neutrophils and oedema exacerbate and lead to seizure progression (Fabene et al., 2008, van Vliet et al., 2007), in this case further worsening the scenario. While the initial insult may not be enough to lead to cell necrosis in the cortex, excitotoxic damage may trigger cell-programmed apoptosis (Ankarcrona et al., 1995, Bonfoco et al., 1995), which would become evident about 24h after injection (Lawrence et al., 1998). Combined with the reduced oxygenation response to activity - a mechanism which may be important for even small fluctuations in cellular activity (Bruyns-Haylett et al., 2013) - these vulnerable cortical cells may be further impaired in successfully regaining their mitochondrial potential and homeostatic balance (Beal, 1992, Carriedo et al., 1998). In addition, if blood flow mechanisms are impaired by IL-1β, waste
products, reactive oxygen species (ROS) and excess glutamate may not be ‘washed’ away from the vulnerable region sufficiently for the cells to recover.

**Figure 5-1 Schematic diagram showing possible mechanisms.** IL-1β potentiates NMDA-receptor activity in the cortex, leading to a reduction of GABAergic gamma activity. Since the haemodynamic response is tightly coupled with gamma oscillations, neurovascular coupling is lost. This is not lethal to cortical cells in itself, but with added metabolic demand elicited by AMPA on the system (perhaps through thalamocortical circuits) through seizure activity, which is in itself aggravated by IL-1β through BBB breakdown and oedema, the strain breaks a threshold and leads to cell death in the cortex.

In sum, the evidence demonstrated within this thesis point towards a seizure hypothesis of IL-1β-exacerbated AMPA excitotoxicity. The co-injection of IL-1β may potentiate NMDA receptors in the piriform, and subsequently the rest of the cortex (Viviani et al., 2003), which would explain the reduction of gamma oscillations in layer IV/V and the protective properties of cortically administered NMDA receptor antagonists in this model (Lawrence et al., 1998). A decoupling of the neural activity and the neurovascular response, demonstrated using both OIS and the APOX probe, may render the cortex less able to re-establish normal metabolism and mitochondrial balance, even though the haemodynamic
effects and impairment of stimulus-evoked gamma activity elicited by IL-1β alone are not enough to be detrimental to neurons *per se*.

Crucially, these effects were all observed in this model at very early time points - too early to be a reflectance of actual cell death - as indicated by the fact that MUA barely changed over time. By focusing on the mechanisms described above, it may be possible to intervene to prevent IL-1β’s deleterious effects on excitotoxic injury.

### 5.3 Methodological considerations

#### 5.3.1 AMPA+IL-1β Model

The AMPA+IL-1β model serves as a valuable paradigm with which to study IL-1β effects on neuronal death in order to determine its underlying mechanisms in neurodegeneration. While AMPA injected striatally causes local cell death at 24h, co-injection with IL-1β leads to extensive cortical damage as well as similar detriment to the striatum (Lawrence et al., 1998). We know that IL-1β does not cause cell death even when injected striatally, although there have been previous reports of reversible and non-lethal meningitis, oedema, recruitment of neutrophils and BBB breakdown (Anthony et al., 1997, Blamire et al., 2000). IL-1β alone is not toxic to pure neuronal cultures (Strijbos et al., 1993), but has been widely reported to exacerbate damage in several neurological disease contexts (Allan and Rothwell, 2001, Allan et al., 2005, Betz et al., 1995, Greenhalgh et al., 2012, Parry-Jones et al., 2008).

While using the AMPA+IL-1β model is valuable to study the isolated role of IL-1β in the excitotoxic context, studies that focus on the first crucial hours, before significant cell
death, are much less often reported than experiments that use cell death as an end point (Patel et al., 2006, Grundy et al., 2002, Grundy et al., 1999, Lawrence et al., 1998). Conversely, it was not possible to detect clear cell death in these studies, so the possibility that the haemodynamic response decreased due to cell death may be eliminated.

5.3.2 Isoflurane anaesthesia

Isoflurane is a known systemic vasodilator (Schwinn et al., 1990), though its positive effects on local cerebral blood flow occur generally only at concentrations greater than used here (Maekawa et al., 1986). It also has purported negative effects on seizure activity (Isaeva, 2008) and has been shown to prevent AMPA-induced excitotoxicity in the rat cortex (Kimbro et al., 2000), though nonetheless some studies have found it to be pro-convulsant (Veronesi et al., 2008). Isoflurane does have a dose-dependent negative influence on the level of gamma oscillations (Hudetz et al., 2011) and high doses worsen cell death in a model of traumatic brain injury (Hertle et al., 2012). However, the relatively low dose used for these studies (1%) did not need to be changed and so was a constant variable throughout all experiments.

A possible future control experiment would be to maintain rats under anaesthesia for a full 24 h after injection of AMPA+IL-1β, or the other test solutions, to ensure that cell death does still occur in the cortex despite the potentially dampening effect of isoflurane on seizure activity.

5.3.3 Optical imaging spectroscopy

The optical imaging technique used in this thesis has been used to study functional haemodynamic coupling within the barrel cortex for many years (Grinvald et al., 2001,
Berwick et al., 2005b), due to its high spatial and temporal resolution, and the fact that it is relatively non-invasive. Drawbacks to OIS include limits on depth to which visible wavelengths of light can penetrate the cortex and the relatively small area of imaged tissue, compared with, for instance, fMRI. However, in the case of this thesis, which focused on assessing the well-characterised neurovascular response to whisker stimulation with high spatial and temporal resolution, the method pertained very well to the experimental requirements.

It would have been interesting if it were possible to reliably investigate the data from the fourth wavelength used in the optical imaging set-up (>700nm). Imaging of the intrinsic optical signal (IOS) may have indicated potential differences between groups in the AMPA+IL-1β; however there are several reasons why this could not validly be used. Firstly, we know that significant oedema occurs in the model, which may alter the osmotic/light scattering properties of imaged neurons and surrounding tissue. Secondly, and most importantly, the number of trials required to average for a satisfactorily representative response to the whisker stimulation would hardly be possible considering the hourly time scale of the actions of AMPA+IL-1β.

5.3.4 Tissue oxygenation and electrode recordings

The APOX probe couples local oxygenation measurement with electrical field potential recordings. We found that using the APOX probe to measure absolute concentrations did not give easily comparable results, perhaps due to pressure artefacts from surrounding tissue. However, percentage changes in the concentration of oxygen for each recorded response gave a more robust picture of the fluctuations in oxygenation within the active barrel.
Importantly, we were able to replicate the optical imaging results – the IL-1β-dependent decrease in the size of the oxygenation response to whisker stimulation - using the APOX electrode. The AMPA group showed some (insignificant) decline, but the vehicle group served as a healthy control and demonstrated coupling between the gamma oscillations and the relative size of the oxygenation response, which has been reported before (Niessing et al., 2005), despite the invasive nature of this method. The three test groups, in contrast, appeared to lose this coupling of neural activity and oxygenation response.

One aspect that was not completely understood, however, was the incidence of negative oxygenation responses to whisker stimulation. In the optical imaging set-up, positive responses were seen even at 8h after injection, whereas all groups in the electrode study exhibited negative responses to stimulation towards the end of the experiment. It may be that, in opening the cranium for probe insertion, pressure required for a positive response is lost. Compared to OIS, the insertion of an electrode is obviously more invasive and this may explain the differences observed between methods. Negative oxygenation responses have been reported, and attributed to lower levels of neural activity (Niessing et al., 2005, Magri et al., 2012) and are considered more common in deeper cortical layers (Boorman et al., 2010, Goense et al., 2012). One point to bear in mind concerning the electrode recordings is that there may be layer-specific responses to IL-1β. The IL-1β reduction of the HbO response that we see in the optical imaging combines the signal from the surface to deeper layers, and the blood flow to layer IV/V descends from the imaged surface. It is possible that the negative responses seen in the APOX results from layer IV do not represent all the layers of the cortex, or that optical imaging is merely weighted by layer IV/V in its view of the cortex from the surface.

The electrode recording analysis focused mainly on higher frequency oscillations, partly due to the accidental omission of low-frequency (<5Hz) oscillations from the electrode
signal. In later animals, the lower frequencies were recorded yet no patterns were discerned using these frequencies. It would have been beneficial to see whether reductions in gamma and beta frequency bands are accompanied with increases within other frequency spectra, for instance seizure-associated delta (Sitnikova and van Luijtelaar, 2009), especially since seizures constitute a large aspect of the AMPA+IL-1β model.

5.4 Future directions

It would be useful to know a) whether the changes seen continue or recover over time and b) whether reductions in functional blood flow could predict the eventual cell death in the cortex at 24h. In particular, it would be valuable to be able to estimate the total duration or size of the decrease in the haemodynamic response required to irreversibly damage neurons in the cortex.

In the same way, it would be interesting to see whether early changes in measures of LFP and MUA components correlate with future cell death seen in the AMPA+IL-1β group. Later time points (8-24h) comparing markers of apoptosis and necrosis after injection of AMPA+IL-1β would be valuable in defining the exact nature of the cortical death, especially when maintained under anaesthesia so that it may be compared more readily with data from recovered animals.

Exactly how IL-1β mediates the loss of the HbO response remains to be elucidated. The mechanisms underlying the neurovascular response are numerous and as yet, still incompletely understood, especially with regard to pathophysiological conditions (Girouard and Iadecola, 2006). The four mechanisms outlined in the introductory chapter (neuronal products, astrocyte signalling, pericyte communication and direct innervation) may all be
modulated by IL-1β. Moreover, the previously mentioned effects of IL-1β on the resting vasculature may also contribute to the phenomenon seen in these studies (Seto et al., 2006).

From the previous experiments carried out using this model, however, we may assume that the reduction of the HbO response is not lethal to cortical cells in itself. Rather, there may be a decoupling of the neurovascular response which renders the cortex vulnerable to excitotoxicity (which in turn may be propagated to the cortex by the actions of IL-1β). For this reason, it is recommended that further experiments are carried out to find out if and when the haemodynamic response recovers from the disruption caused by IL-1β. For example, animals could be kept under anaesthesia until the acute effects of IL-1β on the BBB breakdown have subsided at 24h. Measures of the BBB permeability and oedema at the 24h time point (for instance using horseradish peroxidase) would help to determine whether disruption of the BBB is directly associated with the haemodynamic effects.

As it is known that NMDA receptors mediate the cortical cell death observed after injection of AMPA+IL-1β (Lawrence et al., 1998), it would follow logically to test the NMDA antagonist MK-801 in the cortex to see whether it prevents the loss of the haemodynamic response or stimulus-evoked gamma activity, or indeed whether any protection against neurovascular decoupling correlates with cortical neuroprotection. This could be measured both in terms of the possible protection of gamma oscillations and the haemodynamic response to whisker stimulation. This experiment would help to ascertain whether IL-1β’s diminishing of the haemodynamic response or gamma activity are associated with the same mechanisms through which it exacerbates AMPA excitotoxicity.

It would also be helpful to conduct experiments where IL-1β is directly administered to the cortex to see whether IL-1β mediates its effects on the haemodynamic response via actions particularly in the striatum or by, for example, diffusing into the cortex. Direct
application of IL-1Ra to the cortex protects against striatal AMPA+IL-1β-induced cortical cell death (unpublished observations). It would be worth testing whether striatal or cortical IL-1Ra rescues the neurovascular response, as this would determine where IL-1β exerts its actions. Comparison of site-specific effects of antagonism with IL-1Ra on the cortical LFP and MUA responses to stimulation might similarly help show where IL-1β acts to worsen excitotoxicity.

Further work could be carried out on elucidating whether the loss of the haemodynamic response and the reduction of gamma activity arise via the same IL-1β mechanisms, or whether the two effects are independent from one another. Artificially impeding the haemodynamic response, for instance, by inducing hypercapnia, has been shown to alter the barrel cortex’s neural response to whisker stimulation, tending towards cortical arousal (Jones et al., 2005). Conversely, applying the GABA(A) agonist muscimol directly to the cortex greatly impedes intracortical processing and results in an impaired haemodynamic response, though does not completely abolish it (presumably due to the remaining thalamocortical input’s contribution to overall energy demands) (Harris et al., 2010). It is possible therefore that IL-1β may affect cortical gamma activity, or the oxygenation response, but that one aspect then induces the other via another mechanism. To answer this question, each aspect could be ‘rescued’ – for instance, blood flow to the brain could be ‘boosted’ by inhalation of nitrous oxide; or gamma activity induced using locally administered NMDA antagonists – and the effect on the alternate aspect measured to check for reciprocal relationships between the two. The drug 7-nitroindazole reportedly prevents nitric-oxide-induced neurovascular coupling but without affecting somatosensory-evoked potentials (Burke and Buhrle, 2006), and so could serve as a useful comparison to mimic the decoupling seen after injection of IL-1β, to determine whether the impairment of the
haemodynamic response, combined with striatal injection of AMPA, leads to eventual cortical cell demise.

Another useful addition to the paradigm set up within this thesis would be the additional measurement of arterial blood gases and blood pressure. These parameters have been well-documented in the haemodynamic literature as possible mediators of enhanced/diminished neurovascular coupling (Sumiyoshi et al., 2012, Duong, 2007, Jones et al., 2005), partly because they are strong indicators of sympathetic/parasympathetic balance (Ramaekers et al., 2002) and partly because they have obvious impacts on the amount of available oxygen for oxidative metabolism (Jones et al., 2005). Although a rectal tissue oximeter was used throughout these experiments, the oxygen saturation of animals that demonstrated a febrile response (to IL-1β) was not easily monitored as the instrument did not function if the heart rate was faster than 350bpm. Therefore, to ensure that injection of IL-1β with or without AMPA does not induce altered blood gas concentrations that may impact both on cortical activity and the HbO/oxygenation response, it would be essential to check this in future experiments. Checking blood gas concentrations would also help rule out a possible change in total cerebral blood volume response - and help isolate purely the HbO element of the haemodynamic response - in the results that have described here through the OIS experiments.

The evidence from the first results chapter concerning the possible compression of arteries on the surface of the cortex makes the need to measure blood pressure more compelling. Thus, in future experiments, arterial blood pressure and intracranial pressure should both be measured, especially after injection of AMPA+IL-1β, where the MCA’s x-z diameter appeared to shrink drastically in less than 2h after injection. Laser Doppler flowmetry would also be a useful tool to determine whether arterial flow on the surface of the cortex is impeded, either by arterial compression by oedema or swollen astrocyte end-feet, or
even by possible obstruction by intraluminal neutrophils. It would also help establish, or rule out, whether the haemodynamic response is smaller independently of resting perfusion, or whether the cortex is ‘over-perfused’ and neural activity fails to trigger the usual neurovascular response, as hypothesised following similar results seen after application of i.v. cocaine (Berwick et al., 2005a). Calibrating the APOX probe at the end of the experiment and producing a slope calibration curve may help determine absolute [O2] more accurately, which would help in establishing the extent of resting oxygenation as well. In a multiple linear regression study of the physiological parameters affecting neurovascular coupling, heart rate within a normal physiological range was identified as having the most significant positive effect on the response seen with fMRI (Sumiyoshi et al., 2012). However, it could be posited that a pathological increase of heart rate, such as that seen after injection of IL-1β with or without AMPA, may be detrimental to neurovascular coupling instead. Beta-blockers or similar drugs could be administered after injection to see whether the haemodynamic response, and/or the cortex may be saved after injection of AMPA+IL-1β.

In addition, it would be useful to examine the LFP and MUA data more closely, as there may be more telling changes in the latencies and profiles of MUA and LFP responses to whisker stimulation. Recordings of single-cell or multi-arrays of cortical activity from different layers of the barrel cortex would also help elucidate the differential modulations of neural activity that are mediated by the striatal injection of AMPA and/or IL-1β. Studying fluctuations in the amount of low frequency oscillations might help detect seizure activity, as might recording MUA from a larger cohort of animals.

Future intervention studies would be of particular interest, considering the role of both IL-1β and excitotoxicity in a number of neurological diseases. IL-1β antagonism with IL-1Ra may not act to rescue the effects seen here. However, early uncoupling of the neurovascular response, decreased gamma and increased seizure activity may represent new targets for
preventing IL-1β-worsened excitotoxic injury. These findings may therefore be especially relevant to the study of disorders comprising both neuroinflammatory and excitotoxic elements. Interestingly, studies combining the use of EEG and near-infra red spectroscopy (NIRS) on patients with epilepsy have observed a decrease in cerebral oxygenation during seizure activity, and this has been associated with continuation of the seizure (Buchheim et al., 2004, Adelson et al., 1999).

This thesis is, to my knowledge, the first to examine IL-1β-mediated effects on the functional haemodynamic response. The two methods used to quantify this effect showed similar IL-1β-induced reduction of the oxygenation response to whisker stimulation and IL-1β-dependent decreases in stimulus-evoked gamma oscillations, without significant effects on MUA. Importantly, the changes seen in these studies were observed very soon - as early as 1h - after injection, providing a picture of the very early functional changes brought about by acute neuroinflammation and excitotoxicity.
6. References


1 type-II receptor - a decoy target for IL-1 that is regulated by IL-4. *Science*, 261, 472-475.


HAKAMI, T., JONES, N. C., TOLMACHEVA, E. A., GAUDIAS, J., CHAUMONT, J., SALZBERG, M., O'BRIEN, T. J. & PINAULT, D. 2009. NMDA receptor hypofunction leads to generalized and persistent aberrant gamma oscillations


MOUNTCASTLE, V. B. 1957. Modality and topographic properties of single neurons of cat’s somatic sensory cortex. 20, 408-434.


