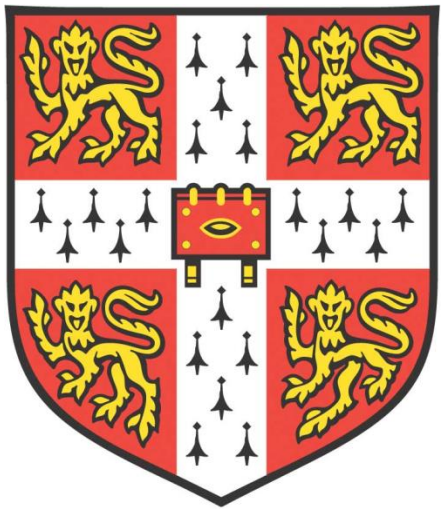
THE ROLE OF TOLL-LIKE RECEPTORS IN PARKINSON'S DISEASE



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DECLARATION

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the relevant Degree Committee.

ABSTRACT

Inflammation in the brain has been strongly implicated in the development and progression of Parkinson's disease (PD). Neuroinflammation in PD is thought to occur in response to aberrant α-synuclein aggregates, and may be mediated by Toll-like receptors (TLRs). TLRs are pattern recognition receptors with the ability to detect damage-associated molecular patterns. The activation of TLRs, potentially occurring through the detection of aggregated α-synuclein, triggers the release of pro-inflammatory cytokines. This may result in chronic inflammation, which produces an environment toxic to neurons and thus causes neurodegeneration such as that seen in the substantia nigra of the PD brain. I hypothesise that the blockade of TLRs will result in the slowing of pathological progression in an animal model, and the blockade of an α-synuclein inflammatory response in human PBMCs.

Chapter 1 describes the clinical and pathological characteristics of PD and some of the suggested mechanisms behind the progression of pathology, focusing on neuroinflammation. Additionally, it introduces TLRs and summarises the current literature surrounding their connection to PD, in particular that of TLRs 2 and 4.

Chapter 2 describes the reproduction of the Kuan et al., (2019) α-synuclein animal model, including behavioural and neuropathological features. To test the effect of TLR blockade on the development of pathology the drug candesartan was used. This is a licensed drug used in the treatment of hypertension through its action as an AT_1 receptor blocker, but has also been identified as decreasing the expression of TLRs 2 and 4. This work demonstrated a protective effect of TLR blockade on the development of cholinergic neurodegeneration and olfactory deficits, both features of early human PD.

Chapter 3 investigates the mechanism behind the protective effects of candesartan seen in chapter 2. Candesartan showed only small trends towards decreasing the expression and protein levels of TLRs 2 and 4. Neuroinflammation was not identified within the brain, and thus candesartan did not appear to have any effects on this inflammation. Peripheral inflammation was identified at a 2-month timepoint, and candesartan decreased this inflammation, providing a potential protective mechanism.

Chapter 4 describes the optimisation of an *in vitro* assay to investigate the inflammatory response of human peripheral immune cells to different forms of α-synuclein. This work showed that human peripheral immune cells show a pro-inflammatory response to both monomeric and oligomeric α-synuclein, and that this is blocked using candesartan. This response was shown to be TLR4- but not TLR2-dependent.

Chapter 5 summarises the main conclusions of my thesis. Candesartan is having a protective effect on the development of neurodegeneration within the α-synuclein animal model, however the mechanisms behind this are unclear. This protective effect could arise through the suppression of peripheral inflammation, through the inhibition of autophagy through decreasing the expression of TLRs on neurons, or through the blockade of AT_1 receptors. Further evidence supporting an anti-inflammatory mechanism arises from the decrease in α-synuclein-mediated inflammation in *in vitro* human peripheral immune cells on candesartan treatment. This thesis finishes with suggestions for further work to provide more insight into the mechanisms behind the protective action of candesartan, and to provide further rationale for the use of candesartan in a clinical trial.

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Chapter 1.

INTRODUCTION

DECLARATION

Chapter 1.4 **"Toll-like receptors"** is a modified excerpt of the published review article **"Toll-like receptors and their therapeutic potential in Parkinson's disease and α-synucleinopathies".** This article was published in Brain, Behaviour, and Immunity: **https://doi.org/10.1016/j.bbi.2019.06.042** under Green open access with a 12-month embargo which has now lifted. The accepted manuscript is freely available to view within Apollo – the University of Cambridge repository at:

https://www.repository.cam.ac.uk/items/2abbd448-b6b5-4aa5-a22c-22279f75ecd9

The author contributions are as follows: I, Catherine Kennedy (née Horne), and Dr Antonina Kouli are joint first authors and equally contributed to the writing of the text. The figures were produced by Dr Antonina Kouli. Critical feedback was provided by Dr Caroline Williams-Gray in addition to the editing of the final manuscript.

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1.1 What is Parkinson's disease?

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease. It was first described by James Parkinson in 1817. The current estimate for PD prevalence in the UK puts number of cases at around 145,000 according to Parkinson's UK. The prevalence of PD is growing (Ou et al., 2021), and indeed is predicted to grow at an even faster rate within the next 20 years (Dorsey et al., 2018). This growth is driven by its being an age-related disease and the continuing improvements in medicine resulting in increased longevity and consequently in a growing ageing population. This has made Parkinson's a key area for research to improve quality of life for millions of people.

1.1.1 Clinical characteristics

There are four key motor characteristics used in the diagnosis of PD: bradykinesia, tremor at rest, rigidity, and postural instability. Bradykinesia is the slowness of movement; this results in a slowness in performing daily living activities. Tremor at rest is a very common feature of PD, though not universal. It is often seen at disease onset as described in a study by Hughes et al., (1993) where 68% of 100 patients had a resting tremor at disease onset. It was also reported in this study that 75% of patients experienced tremor at some point during disease duration. Rigidity is manifested through increased resistance to the passive movement of limbs. Rigidity in certain areas can lead to postural deformities, for example axial rigidity can result in spinal deformities such as camptocormia (Protopsaltis et al., 2016). Postural instability is the loss of postural reflexes leading to poor balance and falls. (Reviewed in Palakurthi and Burugupally, 2019).

PD is generally diagnosed when motor symptoms develop. However, it is thought that the disease begins many years prior to this with prodromal non-motor symptoms. Olfactory dysfunction (or hyposmia) is very common in PD, with up to 90% of 81 patients in one study showing deficits, though interestingly it was found that over 70% of these PD patients were unaware of their having this symptom prior to olfactory testing (Doty et al., 1988). Idiopathic olfactory dysfunction is linked to an increase in the probability of developing PD (Haehner et

al., 2007; Ponsen et al., 2004). Autonomic disturbances, including gastrointestinal function, are also a common non-motor feature, with constipation occurring as a prodromal feature in some cases. A systematic review of constipation and PD carried out by Adams-Carr et al., (2016) has shown that people with constipation are at a greater risk of developing PD than those without. Furthermore, Camacho et al., (2021) have shown in a long-term incident cohort that the severity of constipation at diagnosis is predictive of faster progression to dementia in PD. Sleep disturbance is a common non-motor symptom of PD, and the sleep disorder RBD (REM sleep behavioural disorder) is another prodromal feature of PD and other synucleinopathies (including multiple system atrophy – MSA – and Dementia with Lewy Bodies – DLB). In a cohort of patients diagnosed with RBD (89 patients) 30% developed a synucleinopathy after 3 years, and by 7.5 years this was at 66% (Postuma et al., 2015). Within 12 years of diagnosis over 70% will develop a form of parkinsonism or dementia (Roguski et al., 2020). In PD, the prevalence of RBD is nearly 50% (Zhang et al., 2017).

As mentioned above, many non-motor symptoms are thought to precede the development of motor symptoms. Some of these symptoms, including gastrointestinal dysfunction, are found outside of the central nervous system – that is to say peripherally. It has been hypothesised that PD is initiated peripherally and then spreads to the brain where the further development of disease leads to the characteristic motor symptoms (Reviewed by Klingelhoefer and Reichmann, 2015). Clinical symptom progression consists of the development in severity of motor symptoms; these continue to worsen for the duration of the disease. Postural instability is a major disease milestone with regards to this having a large impact on quality of life; this occurs around 4 years into the disease course (Evans et al., 2011). In addition, other non-motor related symptoms develop. Pain is a common development in people with PD and is seen in over 70% of patients according to a study carried out by Valkovic et al (2015). Cognitive dysfunction can be a feature of early PD but worsens over disease course and can evolve into dementia later in disease progression; dementia is considered to be a symptom of late-stage PD. In an incident population-representative PD cohort 46% had developed dementia by 10 years from diagnosis (Williams-Gray et al., 2013), and another study reported 83% of surviving patients had dementia after 20 years from PD diagnosis (Hely et al., 2008). Symptoms of psychosis, including hallucinations, are a further feature of late-stage PD. Visual hallucinations are reported in around 30% of patients (Fénelon, 2008). These symptoms are often linked to severe cognitive deficits and dementia.

1.1.2 Pathological characteristics

There are two main pathological characteristics of PD. These are the degeneration of dopaminergic neurons in the substantia nigra (SN), and the presence of α-synuclein aggregates (Lewy bodies) within the brain.

The death of SNpc neurons and the loss of the corresponding neuron terminals in the striatum results in decreased dopamine levels in the nigrostriatal system and subsequent motor deficits. It is thought that motor symptoms occur when neuron loss in the SNpc reaches around 50% (Marsden, 1990; Ross et al., 2004), though this differs depending on study, for example Fearnley and Lees estimated only a 30% loss at the point of symptom development (Fearnley and Lees, 1991). The exact percentage loss is difficult to calculate with high accuracy but it is clear that there is already substantial loss by the time of motor-symptom development. It is thought that the striatal dopaminergic neuron terminals could be the initiation point of degeneration, and that the subsequent loss of dopaminergic neurons in the SN could result from the 'dying-back' of the striatal neurite projections (Bernheimer et al., 1973; Tagliaferro and Burke, 2016). This theory is supported by work in the MPTP monkey model where it was shown that the degeneration of dopamine axon terminals in the striatum preceded the loss of dopaminergic cell bodies in the nigra (Herkenham et al., 1991). The loss of striatal dopaminergic markers at motor symptom onset is also thought to be substantial, with estimates taken from post-mortem studies and radioligand imaging analysis coming to around 50-70% loss (Lee et al., 2000; Scherman et al., 1989). The progression of motor symptoms in PD patients occurs due to the continued degeneration of these dopaminergic neurons and striatal fibres, in addition to other neurotransmitter networks, such as the cholinergic system which is implicated in gait and balance dysfunction (Karachi et al., 2010; Rochester et al., 2012). These lead to further loss of the ability to control and coordinate movement. The amount of degeneration is dependent on the duration of disease (Fearnley and Lees, 1991) and correlates with motor deficit severity, however there is variability in this (Asenbaum et al., 1997).

The presence of α-synuclein aggregated to form Lewy bodies is the second key feature of the Parkinson's brain. The aggregation of proteins within the brain is a characteristic of multiple neurodegenerative diseases including Parkinson's and Alzheimer's. Though the specific function of the protein involved and the location of deposition vary between diseases, this commonality suggests that the protein aggregation and dysfunction in protein handling and clearance mechanisms is in some way toxic to neurons. A-synuclein is a 140 amino acid long

protein coded for by the SNCA gene. It is made up of 3 regions: an amino terminus which confers the ability to form α-helices on binding to membranes; a central hydrophobic region which is key to the β-sheet forming potential; and a carboxyl terminus which is unstructured (Stefanis, 2012). It localises to pre-synaptic terminals (George, 2002) and has the ability to bind to membranes; these abilities suggest a potential role in pre-synaptic transmitter release (Cabin et al., 2002; Murphy et al., 2000). A-synuclein was first linked to PD through the discovery of a mutation in the SNCA gene being linked to familial PD (Polymeropoulos et al., 1997). Further alterations to the gene including duplication and triplication have also been found in familial Parkinson's cases (Miller et al., 2004; Singleton et al., 2003). Further study showed that a gene dosage effect was present: a duplication of the gene resulted in late-onset PD whereas a triplication resulted in early-onset PD and dementia (Fuchs et al., 2007). This could indicate that the amount of α-synuclein in the brain is key. Following on from this work α-synuclein was found to be the main component of Lewy bodies (Spillantini et al., 1997). These initial studies have led to the generally accepted theory that aggregated α-synuclein is the toxic species in PD, and that through as yet undetermined mechanisms its aggregation results in the death of dopaminergic neurons in the SN.

1.1.3 Disease progression

Pathological progression is thought to occur via the spread of α-synuclein through the brain along specific anatomical circuits as hypothesised by Braak et al., (2003) in a theory known as Braak's hypothesis. In this study post-mortem PD brains were used to assess the progression of pathology through the brain over time. The first α-synuclein pathology was identified in the dorsal IX/X motor nucleus (the glossopharyngeal/vagal nerve nucleus respectively) or the olfactory bulb; this is identified as Braak stage 1. Pathology continues to spread to other areas of the brain in stage 2, and it isn't until stage 3 that pathology reaches the substantia nigra leading to motor deficits. In the final 3 stages pathology spreads to the temporal cortex followed by the neocortex, this alongside associated cholinergic deficits may underlie the cognitive impairment and dementia seen in late-stage PD. This theory points towards a peripheral first insult, resulting in α-synuclein aggregation which then spreads through unknown mechanisms into the central nervous system through the olfactory bulb or through the vagal nerve from the enteric nervous system of the gastrointestinal tract. This would explain the presence of hyposmia and gastrointestinal dysfunction prior to the development of motor symptoms and the diagnosis of PD. These two areas are particularly vulnerable to

environmental pathogenic exposure, and as such, a dual-hit hypothesis has been suggested (Hawkes et al., 2007). In this theory a neurotropic pathogen enters the brain through two routes: nasally, with anterograde transport from the olfactory system into the temporal lobe; and gastric, with transsynaptic transmission to the vagus nerve from the gut and retrograde transport into the midbrain. Alternatively, a single-hit hypothesis has been suggested whereby Parkinson's either arises in a 'body-first' situation – α-synuclein pathology initiates in the peripheral nervous system before spreading to the brain through the vagal nerve – or a 'brain-first' situation – α-synuclein pathology initiates in the olfactory system before secondary spreading to other brain regions (Borghammer et al., 2022). Both of these hypotheses explain the presence of non-motor symptoms prior to the motor symptoms. Further evidence for pathology arising outside of the brain comes from the presence of α-synuclein aggregates in the enteric nervous system (Braak et al., 2007, 2006; Del Tredici et al., 2010; Kupsky et al., 1987; Wakabayashi et al., 1988). These are seen mainly in post-mortem studies, but also in gut biopsies of relatively early PD patients (diagnosis <5 years) (Lebouvier et al., 2008) and in gut biopsies prior to the onset of PD motor symptoms (Hilton et al., 2014; Shannon et al., 2012; Stokholm et al., 2016). Further evidence arises from the discovery that people who have had a vagotomy (the cutting of one or more branches of the vagal nerve; carried out as a treatment for peptic ulcers) have their risk of developing PD reduced by approximately 40% when study results are combined (Liu et al., 2017; Svensson et al., 2015a, 2015b). In addition, recent work investigating a model of prodromal Parkinson's disease – produced through the injection of adeno-associated viruses containing mutated α-synuclein gene (A53T) into the vagus nerve – found that phosphorylated α-synuclein was deposited in the vagal Schwann cells. This resulted in vagus nerve dysfunction, a neuroinflammatory response, and peripheral autonomic dysfunction – in the form of gastrointestinal deficits – which preceded motor deficits and central pathology (Cheng et al., 2023).

The mechanism of propagation of α-synuclein throughout the brain, as suggested by Braak, is a controversial one; there are many differing theories on how this could take place. One hypothesis is that α-synuclein has the ability to act in a prion-like manner. Prions are infectious particles composed solely of a modified protein. Transmission occurs through converting the normal protein into the modified protein through posttranslational misfolding into β-sheet-rich structures; the original misfolded protein acts as a template for this conversion into pathogenic protein (Prusiner, 1998). Supporting evidence for α-synuclein acting in this manner is provided by various studies. The analysis of post-mortem brains of PD patients who

received grafts of human embryonic mesencephalic tissue showed that when patient death occurred 18 months after the transplant, the dopaminergic phenotype of the graft appeared normal, however 11-16 years post-transplant the grafts showed evidence of Lewy body-like inclusions (Kordower et al., 2008, 1995; Li et al., 2008; Lindvall et al., 1994). This transmission of pathology could be occurring through α-synuclein acting in a prion-like manner, resulting in the pathogenic form of α-synuclein present in the healthy grafts (Olanow and Prusiner, 2009) Further evidence for this mechanism arises from animal and cellular models of PD. These show similar transmission of pathology into healthy grafts (Desplats et al., 2009; Hansen et al., 2011; Kordower et al., 2011), and also that a PD model can be produced through the injection of α-synuclein pre-formed fibrils (PFFs) which act as a seed to trigger the formation of endogenous α-synuclein into Lewy-like inclusions (Luk et al., 2012, 2009). This supporting evidence does not necessarily provide clear support for the prion hypothesis, and could be argued simply to support a non-cell autonomous neurodegenerative process. Other hypotheses exist to support this, including the role of glial cells in the pathological spread of α-synuclein (Xia et al., 2019), and selective vulnerability of specific neuronal populations (Surmeier et al., 2017).

There is not yet a treatment to delay or halt the progression of PD. Only symptomatic treatments are available, which act to stimulate the dopaminergic system. Dopaminergic drugs are effective at alleviating motor symptoms, however this diminishes as the disease progresses due to the underlying neurodegeneration (Schapira, 2009). No disease-modifying drugs currently exist. In order to successfully modify the disease course, a clearer understanding of the mechanisms which drive disease initiation and development is needed.

1.2 What causes Parkinson's disease?

As discussed above, the pathology of PD involves the spread of aggregated α-synuclein through the brain, this is thought to result in the death of dopaminergic neurons. The cause of the initial aggregation of α-synuclein and of its toxicity is unknown. Some theories for the causes of PD do not even involve aggregated α-synuclein, which may be a by-product of other failed cellular mechanisms which are actually responsible for the neurodegeneration. Furthermore, there are some genetic forms of PD which lack Lewy bodies at post-mortem such as *Parkin*-associated PD where Lewy bodies are seen in only a minority of patients, however this is known as a very different form of PD (Doherty and Hardy, 2013). Determining the cause

of PD and its progression could provide a solution to preventing, halting, or slowing the progression of the disease.

1.2.1 Neurodegenerative mechanisms

1.2.1.1 Oxidative stress and mitochondrial deficits

Mitochondrial deficits have long been thought to play a role in the development of PD. Initial work on this arose from the discovery that the toxin MPTP caused parkinsonian symptoms in those accidentally exposed to it (Burns et al., 1985; Langston et al., 1983); this was found to occur through its selective inhibition of mitochondrial complex I (Nicklas et al., 1987). This resulted in the death of dopaminergic neurons in the substantia nigra and subsequent parkinsonism. Oxidative damage has been seen in post-mortem idiopathic Parkinson's brains (Reviewed by Greenamyre et al., 2001). The deficit in mitochondrial complex I results in the release of reactive oxygen species (ROS) and it is these which are thought to be toxic to the dopaminergic neurons and result in their degeneration. The susceptibility of the dopaminergic degeneration may arise from these neurons being particularly vulnerable to oxidative stress. This is due to their already producing high levels of ROS through the metabolism of dopamine (Hermida-Ameijeiras et al., 2004). Oxidative stress as a cause of PD does not immediately involve α-synuclein, however studies have shown that oxidative damage can cause the nitration of α-synuclein thus increasing its propensity to misfold and aggregate. This potentially implicates oxidative stress as the primary event resulting in the onset of pathogenesis (Giasson et al., 2000). Further evidence for a role of oxidative stress and mitochondrial deficits in the development of PD arises from studies of gene mutations in familial forms of PD. Multiple gene mutations which result in mitochondrial dysfunction have been linked to Parkinson's including mutations in Parkin (Palacino et al., 2004), PINK1 (Gautier et al., 2008), and DJ-1 (Krebiehl et al., 2010). However, it has also been suggested that oxidative stress and the evidence for this within Parkinson's patients could be a secondary effect of the death of neurons, or of α-synuclein aggregation, and not specifically involved in the aetiology of PD. Evidence for this arises from work showing that the aggregation of α-synuclein in mitochondria resulted in mitochondrial deficits through decreased complex I activity (Devi et al., 2008; Hsu et al., 2000; Liu et al., 2009).

1.2.1.2 Altered proteolysis

A build-up of aggregated α-synuclein could occur due to impaired processes of proteolysis. There are two main systems through which damaged/dysfunctional proteins are degraded in cells: the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway. A-synuclein can be broken down by both of these systems (Cuervo et al., 2004; Liu et al., 2003; Webb et al., 2003), and a failure of these systems has been identified in human PD (Alvarez-Erviti et al., 2010; Anglade et al., 1997; Chu et al., 2009; Dehay et al., 2010; McNaught and Jenner, 2001; McNaught et al., 2003; Murphy et al., 2015; Ullrich et al., 2010) and in Parkinson's models (Bai-Yun Zeng et al., 2006a, 2006b). Dysfunction of the UPS/autophagy-lysosome pathway is thought to play a role in the spread of neuropathology through aiding α-synuclein propagation between cells (Poehler et al., 2014). This has been shown through the increased exocytosis of α-synuclein when autophagy is inhibited and the subsequent decreased survival of neighbouring neurons (Alvarez-Erviti et al., 2011; Danzer et al., 2012; Lee et al., 2013). A further failure of the autophagic/lysosomal pathway is seen in those with GBA mutations; mutations in this gene are the most common genetic risk factor for PD. This gene codes for the lysosomal enzyme glucocerebrosidase; mutations can result in the loss of function of this enzyme and can result in dysfunction in the autophagic/lysosomal pathway (Smith and Schapira, 2022). Hence the failure of these protein degradation systems may result in the accumulation of the presumed toxic species of α-synuclein in addition to the propagation of α-synuclein through the brain, and thus may be a key feature of neurodegeneration in PD. Alternatively, it has been shown that α-synuclein overexpression can inhibit autophagy (Tanik et al., 2013; Winslow et al., 2010; Yan et al., 2014); accordingly the altered proteolysis seen in PD could be a secondary effect of the development of the pathology rather than involved in the aetiology.

1.2.1.3 Neuroinflammation

Neuroinflammation has been strongly implicated in both the development and progression of PD. This thesis will focus on the role of inflammation in PD, and in particular on the role that Toll-like receptors might play in the inflammatory response. Hence these topics will now be discussed in detail below.

1.3 Neuroinflammation in Parkinson's disease

The function of the inflammatory response in the brain is to protect and restore neurons and glia within the CNS, particularly neurons, as these cells lack the ability to regenerate (Ransohoff, 2016). However, in addition to neuroprotective functions – such as phagocytosis and the subsequent destruction of misfolded proteins – neuroinflammation can also be detrimental. Neuroinflammation involves the activation of immune cells resident to the central nervous system such as microglia, the infiltration of peripheral immune cells, a resulting increase in the release of pro-inflammatory cytokines/chemokines, and the production of reactive oxygen species (ROS). Chronic neuroinflammation has been shown to occur in many neurodegenerative diseases including Alzheimer's disease and Parkinson's disease, and may accelerate disease development and progression (Frank-Cannon et al., 2009).

1.3.1 Evidence that immune activation plays an aetiological role in PD

There has been some debate over whether brain inflammation and immune activation are a secondary effect of the neurodegenerative process or a primary aetiological driver.

1.3.1.1 Genetics

Mutations in a number of immune system-related genes are known to increase susceptibility to developing PD. These genes include those in the HLA region (Saiki et al., 2010; International Parkinson Disease Genomics Consortium et al., 2011) and those in LRRK2 (Russo et al., 2014) in addition to other immune-related genetic susceptibilities found through pathway analysis of GWAS data (Holmans et al., 2013). HLA (human leucocyte antigen) is region of the genome encoding the major histocompatibility complex which plays a critical role in the presentation of antigens and hence is essential for an effective targeted immune response (Saiki et al., 2010). LRRK2 (leucine-rich repeat kinase 2) mutations are implicated in multiple immune-related disorders including inflammatory bowel disease (IBD) and tuberculosis. They are also known to result in an autosomal-dominant familial PD, and are found in approximately 1% of sporadic PD cases (Simón-Sánchez et al., 2009; Zimprich et al., 2004). How LRRK2 mutations cause PD is not well understood. LRRK2 is highly expressed in many immune cells, and an increase in this expression has been seen in response to inflammatory stimuli such as LPS and IFN-γ (Gardet et al., 2010; Hakimi et al., 2011; Moehle et al., 2012; Reviewed by Wallings and Tansey, 2019). It has been suggested that mutated

forms of LRRK2 may push microglia (immune cells found in the brain) towards an activated pro-inflammatory phenotype in addition to regulating their migration and phagocytic properties (Russo et al., 2014).

1.3.1.2 Epidemiological studies

Further evidence arises from epidemiological studies which have shown a reduced risk of developing PD in people regularly taking nonsteroidal anti-inflammatory drugs (NSAIDs) (Gagne and Power, 2010) and studies showing a reduced PD incidence of a third in patients who have taken immunosuppressant drugs (Racette et al., 2018). Furthermore, studies have shown an increased risk of PD associated with autoimmune disorders. A GWAS study identified 17 loci which overlap between PD and autoimmune disorders including type I diabetes and Crohn's disease (Witoelar et al., 2017). In one study, patients with autoimmune diseases – including multiple sclerosis and Hashimoto's disease – were shown to have a 33% higher risk of developing PD (Li et al., 2012). In addition, there is growing evidence for a link between IBD, a disease involving severe intestinal inflammation, and PD. Individuals with IBD are at a greater risk of developing PD than the general population (Zhu et al., 2021).

1.3.1.3 Animal models

In addition, multiple animal studies have implicated inflammation as an initiating factor in PD. PD models can be produced specifically through the induction of inflammation, such as the LPS model. Central injection of LPS (bacterial endotoxin) into the nigra causes specific dopaminergic degeneration (Castaño et al., 1998); this has been shown to occur through the induction of microglial activation and the subsequent production of pro-inflammatory cytokines including TNFα and nitric oxide species (Herrera et al., 2000; Lu et al., 2000). Peripheral injection of LPS, such as intraperitoneal injection, also results in the death of nigral dopaminergic neurons specifically. This effect is thought to be due to the induction of peripheral inflammation resulting in the translocation of peripheral immune cells to the site of inflammation and the production of cytokines which are able to cross the blood-brain barrier and induce neuroinflammation and subsequent neurodegeneration (Qin et al., 2007). In further work it was found that the overexpression of TNF-α in the substantia nigra results in the activation of microglia, the recruitment of peripheral immune cells to the CNS, and the death of dopaminergic neurons and associated motor symptoms (De Lella Ezcurra et al., 2010). These models provide compelling evidence for the role of inflammation in the aetiology of Parkinson's. In this work the induction of inflammation is sufficient for the specific death of

dopaminergic neurons and no direct toxic insult is required. Double-hit models also exist where an α-synuclein-overexpressing animal is also given an inflammatory stimulus such as LPS. This has been shown to potentiate the toxicity in the model, where this dual hit resulted in persistent neuroinflammation (only early acute inflammation was seen in WT animals), progressive degeneration of dopaminergic pathways, and the formation of Lewy-like pathology (Gao et al., 2011). Neuroinflammation can also be seen in toxin models of PD, such as the MPTP model , the 6-OHDA model, or the rotenone model (Członkowska et al., 1996; Purisai et al., 2007; Srivastava et al., 2012). While inflammation is not the direct cause of degeneration in these models, it does exacerbate the effects of the toxin. Protective effects have been seen in these toxin-induced models through manipulation of the immune system – including the knockout of mature T-lymphocytes (Brochard et al., 2009); the knockout of MHCII (Martin et al., 2016); the knockout of TNF-α (Sriram et al., 2002); and the use of immunosuppressant drugs (Khan et al., 2013; McCoy et al., 2006; Mogi et al., 2000; Purisai et al., 2007; Świątkiewicz et al., 2013). The knockout of key components of the immune system has also been seen to show a protective effect in α-synuclein overexpressing animal models, for example the knockout of MHCII was shown by Harms et al., (2013) to prevent the α-synuclein-induced inflammation and dopaminergic degeneration.

1.3.2 The neuroinflammatory hypothesis of PD

The link between neuroinflammation and neurodegeneration is thought to arise through the release of proinflammatory factors by glial cells which have a detrimental effect on neuron survival. Neuron death also leads to further microglial activation. This forms a self-perpetuating cycle of degeneration whereby neuronal death causes microglial activation, resulting in the increased production of pro-inflammatory cytokines & chemokines, and increased oxidative stress, which in turn lead to further neuron loss (Gao and Hong, 2008). (**Figure 1.1**). This hypothesis has been expanded to include the role of the peripheral immune system in this cycle of degeneration. There is evidence in animal models for the activation of peripheral immune cells by antigens which leak into the periphery through cervical lymph nodes (Benner et al., 2008). In addition, peripheral immune cells can be activated by α-synuclein aggregates external to the brain, such as those in the gut (Stolzenberg et al., 2017). These activated peripheral immune cells may then traffic back into the CNS to further activate microglia or to have toxic effects directly on neurons (Sommer et al., 2018). Post-mortem

studies have shown the presence of T-cells in Parkinson's brains, providing further evidence for this hypothesis (Brochard et al., 2009).

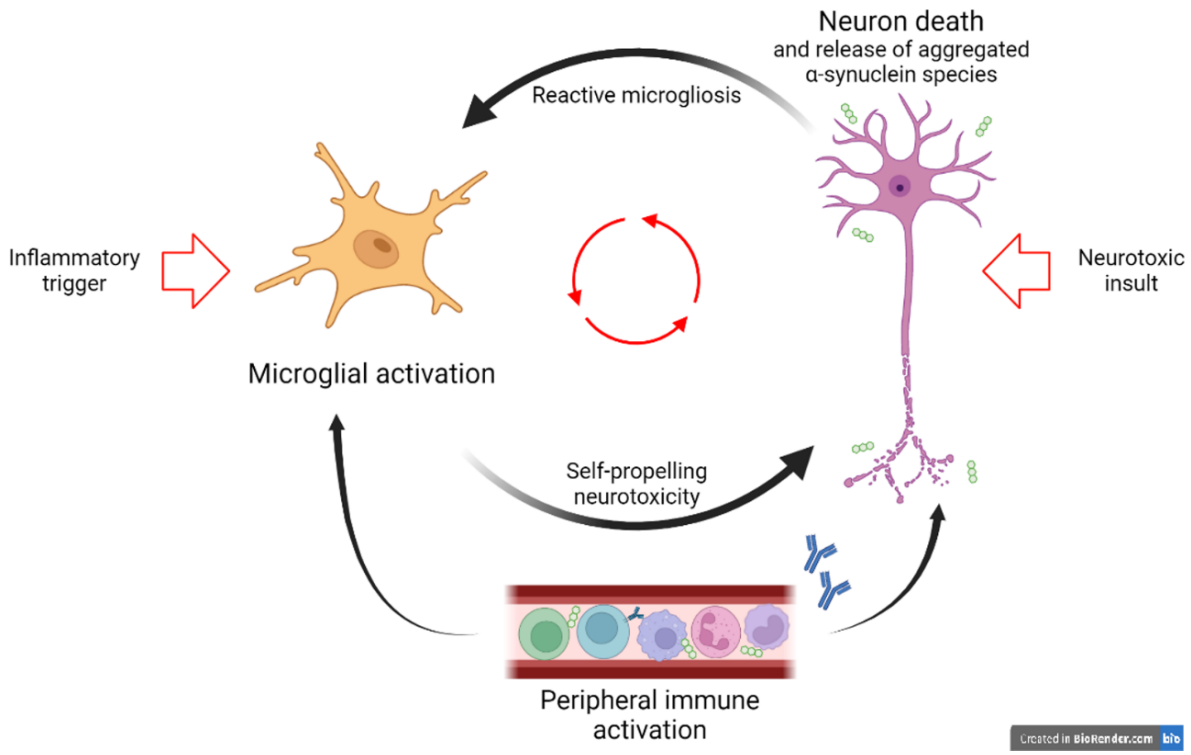


Figure 1.1 Self-perpetuating cycle of degeneration. *Neuronal death causes the release of antigens such as pathological α-synuclein aggregates. These cause immune activation, resulting in increased production of pro-inflammatory cytokines & chemokines, and increased oxidative stress, which in turn leads to further neuron loss. Antigens, such as aggregated α-synuclein, are released from neurons and can leak into the periphery and activate peripheral immune cells. These may also be involved in this cycle through their trafficking back into the CNS to further activate microglia or to have toxic effects directly on neurons.*

1.3.3 Microglia

Microglia are the resident immune cells of the brain. They are found throughout the brain but are particularly concentrated at certain regions, including the hippocampus and substantia nigra (Lawson et al., 1990). Microglia are generally categorised as existing in either a surveying state – originally termed the resting state – or an activated state. Historically this dual categorisation has been strict, however new approaches are moving towards a continuum of activation with 'activated' and 'surveying' states forming the extremes of the spectrum (Mosser and Edwards, 2008). Microglia in the surveying state are described as having ramified

morphology with many extending processes to allow them to constantly survey the local environment for changing conditions (Nimmerjahn et al., 2005). Microglia in the activated state are described as having amoeboid morphology where the cell has a large cell body and few cell processes. The gene expression profiles also differ dramatically between these two states. The activated state is associated with pro-inflammatory functions including the release of pro-inflammatory cytokines, such as TNF-α and IL-1β, in addition to other inflammatory phenotypic features, such as the upregulation of inducible nitric oxide synthase and the release of reactive oxygen species (Le et al., 2001). The surveying state is associated with immunoregulatory functions, the dampening of the immune system, and tissue repair through the production of anti-inflammatory cytokines (Reviewed by Colton, 2009).

1.3.3.1 Microglia in Parkinson's disease

Activated microglia were first observed in the substantia nigra of patients with PD at post-mortem by McGeer and colleagues in 1988 (McGeer et al., 1988). Subsequent studies reported an increase in the levels of pro-inflammatory cytokines in the striatum of PD brains compared to healthy controls; these included TNF-α, IL-1β, and IL-6 as well as other proteins related to microglial activation (Knott et al., 2000; Mogi et al., 1994a, 1994b). In agreement with these post-mortem studies, positron emission tomography (PET) neuroimaging using the tracer [^{11}C]-PK11195 has demonstrated increased microglial activation in the brains of PD patients compared to age-matched healthy individuals, and this appears to start early on in the disease course (Gerhard et al., 2006; Ouchi et al., 2005). Furthermore, recent studies using advanced techniques, such as single-nuclei RNA sequencing, have also shown an increase in activated microglia in the nigra of PD patients and that these cells are enriched in cytokine secretion (Smajić et al., 2022).

Microglia have the ability to be activated by multiple extracellular signals, including those from pathogens, dead or dying cells, or foreign material. The activation of microglia in PD has been hypothesised to occur in response to oligomeric α-synuclein. The mechanism by which aggregated α-synuclein activates microglia is still not fully established but a key theory involves α-synuclein working as a damage-associated molecular pattern (DAMP) to activate Toll-like receptors (Beraud et al., 2011).

1.3.4 Peripheral immune system

In addition to inflammation occurring in the Parkinson's brain, it also occurs in the periphery. The overactivation and dysregulation of both the innate and adaptive immune responses results in the overproduction and release of pro-inflammatory cytokines. These then contribute to the further activation and recruitment of immune cells, resulting in chronic inflammation and an environment which is toxic to cells, including dopaminergic neurons. Mean levels of inflammatory cytokines are elevated in the serum in people with Parkinson's compared to age-matched controls, and a pro-inflammatory cytokine response at diagnosis is associated with a more rapid disease progression over time (Brodacki et al., 2008; Qin et al., 2016; Reale et al., 2009; Williams-Gray et al., 2016). In addition to changes to inflammatory cytokines, alterations in peripheral immune cell subsets also occur in people with PD. An increase in classical (inflammatory) monocytes and monocyte activation markers – including TLR2 and 4 – was shown in people with Parkinson's as compared to healthy controls, and this was most pronounced in cases at a higher risk of dementia (Wijeyekoon et al., 2020). Differential T-call subsets have also been identified in PD, with increased effector/memory T-cells and regulatory T-cell dysfunction being associated with disease severity (Saunders et al., 2012) and decreased CD8+ T-cell replicative senescence and an increase in CD8+ cell activation markers in PD (Williams-Gray et al., 2018). A-synuclein may play a key role in the activation of the peripheral immune system. A-synuclein specific T cells have been identified and are present at higher frequency in PD cases than controls; α-synuclein peptides act as antigens to trigger helper and cytotoxic T-cell responses (Sulzer et al., 2017). A-synuclein-specific antibodies, which may act to propagate inflammation, are increased in people with PD; this is particularly seen in early PD (Scott et al., 2018). A-synuclein is also able to activate monocytes as shown through work with the immortalised monocytic cell line THP-1 (Klegeris et al., 2008) and in cultured patient monocytes/PBMCs and other monocytic/microglial cell lines (Codolo et al., 2013; Grozdanov et al., 2019; Liu et al., 2022; Nissen et al., 2019; White et al., 2018).

1.4 Toll-like receptors

A specific aspect of the immune system which has been gaining traction as playing an important role in PD is the involvement of Toll-like receptors (TLRs).These receptors have been implicated in mediating the inflammatory response of microglia and peripheral monocytes

to pathological α-synuclein, in addition to having important roles in the transmission of α-synuclein, and in the altered proteolysis of α-synuclein.

1.4.1 Function and localisation

TLRs are a family of transmembrane pattern recognition receptor (PRR) proteins key to the activation of the innate immune response. Their primary role is in the detection of foreign microbial and viral molecules which they achieve through their recognition of pathogen-associated molecular patterns (PAMPs). TLRs also have the ability to recognise damage-associated molecular patterns (DAMPs) which are found on molecules endogenous to the host (Reviewed in Bianchi, 2007). On their recognition of either PAMPs or DAMPs, TLRs induce a signalling cascade resulting in the activation of an immune response to clear the pathogen from the host or to resolve the damage. TLRs are involved in the activation of both the innate immune system – consisting of non-specific responses such as the initiation of pro-inflammatory cytokine production and phagocytosis – and in a less direct manner the adaptive immune system – consisting of a response specific to the pathogen mediated by T and B lymphocytes, through their upregulation of co-stimulatory molecules required for robust T-cell responses (Hoebe and Beutler, 2004).

The TLR family is made up of 10 functional TLRs in humans. Each of these receptors has the ability to recognise distinct molecular patterns, allowing a wide range of patterns to be recognised and an appropriate immune response to be initiated (Hasan et al., 2005). TLRs are type I transmembrane proteins formed of an extracellular leucine-rich repeat (LRR) domain responsible for specific pattern recognition, a transmembrane region, and a cytoplasmic TIR (Toll/interleukin-1 receptor) domain which transduces the signal to activate the correct downstream signalling pathway (Reviewed by Kawai and Akira, 2011). The human TLR family can be split into two groups based on where the receptors are located in the cell: TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are found in the plasma membrane, while TLR3, TLR7, TLR8, and TLR9 are found in intracellular compartments (see **Figure 1.2**). This differential localisation occurs due to the specialisation of the different members of the TLR family to recognise specific pathogens.

TLRs normally function in homodimers, with the exception of TLR2 which forms heterodimers with either TLR1 or TLR6, as well as homodimers (Jin et al., 2007; Kang et al., 2009). Some TLRs work in combination with additional molecules or co-receptors that enable

more sensitive ligand recognition; notably TLR4 requires both MD2 (Shimazu et al., 1999) and CD14 (Wright et al., 1990) to recognise and be activated by bacterial lipopolysaccharides (LPS).

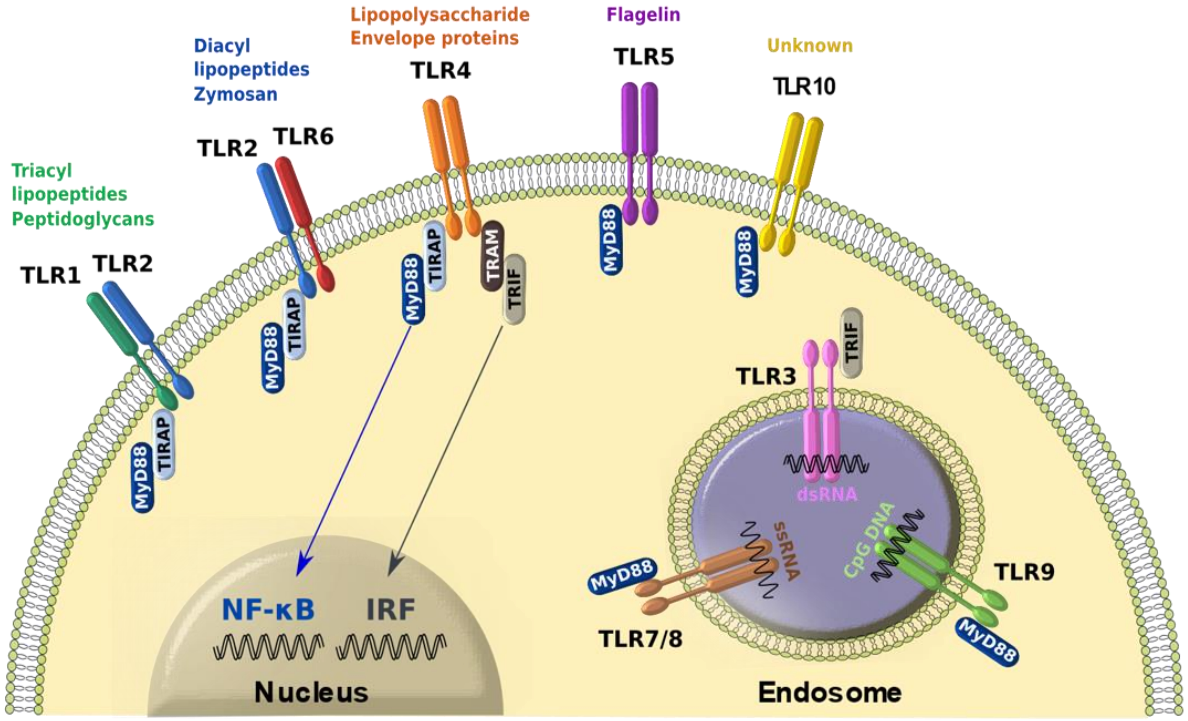


Figure 1.2 Cellular localisation and signal transduction of human Toll-like receptors (TLRs). *TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are located on the plasma membrane while TLR3, TLR7, TLR8 and TLR9 are found intracellularly on endosomal or lysosomal membranes. Most TLRs are believed to function in homodimers, though TLR2 forms heterodimers with either TLR1 or TLR6. All TLRs except for TLR3 signal through a MyD88 (myeloid differentiation 88)- dependent downstream pathway. Signalling via TLR2 and TLR4 requires an additional intracellular adapter protein TIRAP (TIR domain-containing adapter protein), which in turn recruits the central adapter MyD88. This triggers a downstream signalling cascade resulting in the nuclear translocation of the transcription factor NF-κB and the expression of pro-inflammatory cytokines such as TNFα, IL-1β and IL6. There is also a MyD88-independent pathway which involves TLR3 and TLR4. Activation of TLR3 engages the adapter protein TRIF (TIR-domain-containing adapter-inducing interferon-β), while TLR4 signalling requires both TRAM (TRIF-related adapter molecule) and TRIF. This results in the downstream activation of the IRF transcription factors (interferon-regulatory factor) which regulate the expression of interferon type 1 genes. Abbreviations: ssRNA: single stranded RNA, dsRNA: double stranded RNA, CpG DNA: cytosine-guanine rich DNA. Image and legend reproduced from review co-authored by myself (Kouli, Horne et al., 2019).*

On PAMP or DAMP recognition by the extracellular LRR domain, the signal is transduced intracellularly, and the correct adaptor proteins (each with a TIR domain) are recruited to and activated by the cytoplasmic TIR domain of the TLR to trigger an appropriate immune response (Akira et al., 2006). There are multiple adaptor proteins which can be activated on pattern recognition by the TLR, and the combination of these proteins determines the specific downstream pathway. MyD88 (myeloid differentiation 88) is one such adaptor protein; it can be activated by all TLRs, with the exception of TLR3, and as such is known as the central adaptor protein (Deguine and Barton, 2014). MyD88 recruitment results in the downstream activation of NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) and mitogen-activated protein kinases (MAPKs), leading to the activation of the NLRP3 inflammasome and the expression of multiple genes involved in the immune response. These include genes responsible for the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Kawai and Akira, 2011). NFκB can also be activated in a MyD88-independent pathway involving the TLR3 and TLR4 adaptor protein TRIF (TIR-domain-containing adaptor-inducing interferon-β). (**Figure 1.2**). (Yamamoto et al., 2003). Most signalling pathways resulting from TLR activation can be classified as either MyD88-dependent or TRIF-dependent. There are several other molecules and adaptor proteins involved in the TLR signalling pathways which have been extensively reviewed elsewhere (Drouin-Ouellet and Cicchetti, 2012).

In the central nervous system TLRs are expressed in microglia, astrocytes, and neurons. Human and mouse microglia express all members of the TLR family, and their expression varies depending on the stimulation state of the cell and the TLR type (Bsibsi et al., 2002; Olson and Miller, 2004). The most highly expressed microglial TLRs are TLRs 1-4, with TLR2 having the highest expression (Bsibsi et al., 2002). A healthy individual shows low TLR expression on astrocytes, with upregulation on activation, though at a slower rate than that seen in microglia (Trudler et al., 2010). Microglia and astrocytes have distinct functional responses on exposure to TLR ligands, such as differential pro-inflammatory cytokine induction (Jack et al., 2005). Both human and mouse neurons have been shown to express various TLRs, however the expression levels show high variation dependent on the specific TLR (Barajon et al., 2009; Lafon et al., 2006; Ma et al., 2006; Tang et al., 2007; Zhou et al., 2009). In the periphery, TLR expression is highest in tissues which are in close contact with the environment, namely the lungs and the gastrointestinal tract, as well as in peripheral blood mononuclear cells and the spleen (Zarember and Godowski, 2002). Several cell types, both immune and non-immune,

have been found to express TLRs, including dendritic cells, macrophages, B and T lymphocytes (both cytotoxic and helper T lymphocytes, although expression is lower in the latter), natural killer (NK) cells, as well as epithelial, endothelial and fibroblast cells (Fitzner et al., 2008; McClure and Massari, 2014; Muzio et al., 2000; Yao et al., 2015). Expression levels are dependent on the cell type, the specific member of the TLR family, and the stimulation state of the cell (Hornung et al., 2002).

The activation of microglia through TLR engagement by PAMPs results in an inflammatory response induced to clear pathogens, but it can also be induced by endogenous signals (DAMPs) such as heat shock proteins, uric acid, HMGB-1 (high mobility group box chromosomal protein 1), and aggregated, modified or misfolded proteins (Beraud et al., 2011).

1.4.2 Toll-like receptors in Parkinson's disease

Microglial activation in α-synucleinopathies is thought to be triggered by α-synuclein aggregation. The mechanism by which aggregated α-synuclein activates microglia is still being established, but a key theory involves α-synuclein working as a DAMP to activate Toll-like receptors (Beraud et al., 2011). Initial experiments using the BV2 microglial cell line demonstrated that treatment with α-synuclein led to altered expression of several TLRs, including upregulation of TLR1, TLR2, TLR3, and the adapter MyD88, and an increase in expression and release of pro-inflammatory molecules (Beraud et al., 2011). This is similarly seen in other microglial and monocytic cell lines, in addition to in human peripheral blood mononuclear cells (PBMCs) (Bearoff et al., 2023; Codolo et al., 2013; da Rocha Sobrinho et al., 2021). In transgenic animal models, such as Thy1-α-synuclein mice which overexpress wild-type human α-synuclein, there is an increase in TLR1, TLR2, TLR4, and TLR8 mRNA expression in brain regions showing microglial activation (Watson et al., 2012). This upregulation of TLRs is widely seen across a number of different PD models, but the level and timecourse of upregulation depend on the specific model (McCabe et al., 2017). It is worth noting that TLRs have also been shown to be activated in PD models which do not show α-synuclein pathology, such as the MPTP model (Drouin-Ouellet et al., 2011; Panaro et al., 2008; Ros-Bernal et al., 2011). Activation of TLRs in this and similar toxin models may be induced by DAMPs related to oxidative stress, such as cell debris or molecules released by damaged or dying neurons including heat shock proteins, uric acid, and others (Bianchi, 2007).

Of particular note, TLR2 and TLR4 have been consistently reported to be upregulated in many α-synuclein-overexpressing or toxin-induced animal models (Drouin-Ouellet et al., 2015; Kim et al., 2013; Noelker et al., 2013; Watson et al., 2012), and accumulating evidence from human studies further implicates these receptors in the pathogenesis of PD.

1.4.2.1 Toll-like receptor 2

In human PD, upregulation of TLR2 has been observed in post-mortem brains. (Summarised in **Table 1.1**). Specifically, transcriptomic analysis revealed increased TLR2 expression in the substantia nigra, accompanied by an increase in the expression of genes involved in the TLR2 signalling pathway including CD14, IRAK2, and NF-κB (Kim et al., 2013). An increase in TLR2 protein levels was also observed in the caudate/putamem of post-mortem PD brains (Drouin-Ouellet et al., 2015). Furthermore, TLR2 was found to co-localise with amoeboid microglia, suggesting upregulation of TLR2 expression by activated microglia in PD brains (Doorn et al., 2014; Kim et al., 2013; Ping et al., 2018). TLR2 has also been reported to be expressed on neurons in human PD brains, and the level of neuronal TLR2 expression was strongly correlated with disease stage and disease duration (Dzamko et al., 2017). However, analysis of publicly available transcriptomic data from the cortex of control post-mortem brains has indicated that TLR2 is almost exclusively expressed on microglia (Hughes et al., 2019), hence neuronal TLR2 expression may be disease specific. TLR2 upregulation, as well as NF-κB activation, have also been identified in peripheral immune blood cells of PD patients (da Rocha Sobrinho et al., 2021; Drouin-Ouellet et al., 2015; Ping et al., 2018; Wijeyekoon et al., 2020).

Table 1.1 Summary of studies evaluating TLR2 in PD patients. *(Modified from Tables in Kouli, Horne et al., 2019).*

References	Study design	Key observation
Dzamko et al., 2017; Drouin-Ouellet et al., 2015	Human PD and control postmortem brains	TLR2 level is increased in the striatum and anterior cingulate cortex of PD brains.
Ping et al., 2018; Dzamko et al., 2017; Doorn et al., 2014; Kim et al., 2013	Human PD and control postmortem brains	Increased co-localization of TLR2 with activated microglia in PD. Neuronal TLR2 correlates with disease burden and duration.
Ping et al., 2018; Drouin-Ouellet et al., 2015; Shin et al., 2015; da Rocha Sobrinho et al., 2021	Human PD and control PBMCs	TLR2, TLR4, and NF-κB are upregulated in PD PBMCs.
Wijeyekoon et al., 2020	Human PD and control PBMCs and serum	Higher percentage of TLR2+ monocytes in PD patients

PD: Parkinson's disease; PBMCs: peripheral blood mononuclear cells

There is also a growing body of evidence from *in vitro* and *in vivo* studies in animal models implicating TLR2 in the pathogenesis and progression of PD. (Summarised in **Table 1.2**). Endogenous α-synuclein binds to TLR2 to trigger microglial activation, and it has been suggested that this occurs in a conformation-dependent manner, with the oligomeric form, but not monomeric or dimeric forms, acting as a TLR2 agonist (Kim et al., 2013). This was further verified with the use of microglia from TLR2-deficient mice. Kim showed diminished microglial activation indicated by a complete loss of cytokine and chemokine upregulation on treatment with endogenous α-synuclein released by human α-synuclein overexpressing SHSY5Y cells, compared to microglia from wild-type mice. The same group later demonstrated that endogenous α-synuclein released from neurons induced TLR2-mediated microglial activation and this appeared to be neurotoxic. This neurotoxicity was entirely abolished by TLR2 gene depletion (Kim et al., 2016). Similarly, Daniele et al (2015) showed that an oligomeric form (with higher-ordered structure) of α-synuclein activates microglia, and additionally reported that this is mediated through a TLR1/2 heterodimer, resulting in the nuclear translocation of the p65 NF-κB subunit. However, work by others has contradicted that it is only the oligomeric form of α-synuclein which can activate TLRs. Gustot et al., (2015) reported that α-synuclein fibrils, not oligomers or monomers, cause the activation of the NF-κB pathway through TLR2 activation *in vitro*. Another study in primary human monocytes suggested that both monomeric and fibrillary α-synuclein instigated a TLR2-mediated inflammatory response, though α-synuclein oligomers were not used in this work (Codolo et

al., 2013). A combination of different sized fibrils in the form of PFFs were shown to activate primary mouse microglia and the BV2 microglial cell line, and this was prevented through the use of a TLR2-blocking agent (Dutta et al., 2021). It is possible that these discrepancies result partly from a lack of consistency in the definition and size of oligomers and fibrils across different studies. Although the precise aggregate size which is most pathologically relevant in this context remains uncertain, it seems clear that at least one, if not several species of the protein, can act as DAMPs (Roberts and Brown, 2015). Studies in models of other diseases have similarly suggested that other misfolded proteins can also act as DAMPs, for instance Liu et al (2012) identified TLR2 as the receptor to which amyloid-β binds and initiates microglial inflammatory responses.

Activation of neuronal TLR2 has been suggested to result in increased intracellular α-synuclein protein. This was seen in work by Chung et al., (2022) showing an increase in TLR2 activation and a concomitant increase in neuronal α-synuclein on exposure to activated microglia. This was also seen in studies by Dzamko et al., (2017) on the SHSY5Y cell line and iPSC-derived (induced pluripotent stem cell) human neural progenitor cells using the TLR2 agonist PAM3CSK4. It is interesting to note that the use of rapamycin – an inducer of autophagy – ameliorated this TLR2-induced increase in neuronal α-synuclein, potentially implicating autophagy in this pathway. This is further confirmed in a recent study where the activation of neuronal TLR2 impaired the autophagy/lysosomal pathway and potentiated α-synuclein pathology; a small molecule TLR2 inhibitor was seen to ameliorate this (Chedid et al., 2022). Building on their earlier work, Kim et al., (2015) proposed that TLR2 activation can lead to an AKT/mTOR-mediated inhibition of autophagy resulting in accumulation of α-synuclein within neurons. In this study, $TLR2^{-/-}$ mice had reduced neuronal α-synuclein accumulation, reduced microgliosis and astrogliosis, and showed improved motor behavioural deficits compared to control mice (Kim et al., 2015). A recent study by Kim et al., (2021) provided further evidence for this showing that stimulation of TLR2 increased α-synuclein fibril uptake in neurons, and delayed the degradation of the α-synuclein within the neurons. Additionally, they showed a change in morphology of microglia on TLR2 activation, confirming previous work on the activation of microglia in response to α-synuclein through TLR2 activation.

TLR2 has also been implicated in the MPTP toxin model of PD, as shown by its upregulation on MPTP treatment, and subsequent upregulation of proinflammatory cytokines

(Chao et al., 2016; Drouin-Ouellet et al., 2011; Jang et al., 2017; Koo et al., 2017; Sun et al., 2021). In α-synuclein overexpressing mice, immunization with anti-TLR2 antibodies resulted in reduced α-synuclein accumulation, reduced microgliosis and pro-inflammatory cytokine secretion, and a reduced memory deficit as shown using behavioural tests (Kim et al., 2018; La Vitola et al., 2018). TLR2 was shown to be activated in the vagal Schwann cells of a model overexpressing mutated α-synuclein (AAV-A53T injected into the vagus nerve); silencing TLR2 expression attenuated α-synuclein-induced GI dysfunction and vagus nerve demyelination and axonal loss. This model shows a role for TLR2 in a prodromal model of PD (Cheng et al., 2023). Furthermore, the use of a TLR2 neutralising antibody in an *in vitro* set of experiments revealed additional roles for TLR2 in the neuron-to-neuron and neuron-to-astrocyte transfer of α-synuclein. Specifically, after TLR2 inhibition the neuron-to-neuron transfer of α-synuclein was markedly reduced. A similar reduction in α-synuclein transfer was observed in a co-culture of SHSY5Y neurons with primary human astrocytes (Kim et al., 2018).

Table 1.2 Summary of studies evaluating TLR2 in in vitro and in vivo PD models. *(Modified from Tables in Kouli, Horne et al., 2019).*

References	Study design	Key observation
Beraud et al., 2011	α-Synuclein-treated BV2-microglia	Upregulation of microglial TLR2.
Daniele et al., 2015; Kim et al., 2013	α-Synuclein-treated WT & $TLR2^{-/-}$ mouse microglia/TLR2 antagonists	TLR2-mediated microglial activation; inhibition of TLR1/2 ameliorates inflammation.
Gustot et al., 2015	THP1 cells treated with α-synuclein and anti-TLR2 antibodies	Fibrillar α-synuclein activates TLR2.
Codolo et al., 2013	Human monocytes treated with anti-TLR2 antibodies prior to α-synuclein	Both fibrillar and monomeric α-synuclein activate TLR2.
Dzamko et al., 2017	SHSY5Y and IPSC-derived neural cells treated with a TLR2 agonist.	Neuronal TLR2 activation leads to increase α-synuclein.
Chung et al., 2022	SHSY5Y cells co-cultured with LPS-activated microglia and treated with TLR2 inhibitor	Inhibition of TLR2 mitigates α-synuclein aggregation and improves autophagic flux.
Chedid et al., 2022; Kim et al., 2015	SHSY5Y cells treated with TLR2 agonist and autophagy inducer or inhibitor	Neuronal TLR2 regulates autophagy.
Kim et al., 2021	SHSY5Y cells treated with TLR2 stimulator; upregulation of TLR2 in a synucleinopathy mouse model	TLR2 stimulation accelerates α-synuclein uptake, delays its degradation *in vitro*, increases Lewy-like inclusions, and activates microglia *in vivo*.
Kim et al., 2016	WT & $TLR2^{-/-}$ mice	TLR2-mediated microglial activation is neurotoxic.
Kim et al., 2015	$A53T^{+}$ $TLR2^{+/+}$ and $A53T^{+}TLR2^{-/-}$ mice	TLR2 ablation reduces neuronal α-synuclein accumulation and improves motor function.
Dutta et al., 2021	PFF-seeded $A53T^{+/+}$ and $A53T^{\Delta TLR2}$ mice treated with a TLR2 inhibitor.	TLR2 inhibition reduces inflammation and α-synuclein spreading, and protects neurons.
Cheng et al., 2023	AAV-A53T injected into vagal nerve of mice; TLR2 silencing	TLR2 activated in Schwann cells; TLR2 silencing reduced inflammation and pathology.
Chao et al., 2016; Drouin-Ouellet et al., 2011; Sun et al., 2021	MPTP mice	MPTP treatment upregulates TLR2.
Kim et al., 2018	Anti-TLR2 immunisation of α-synuclein overexpressing mice	Anti-TLR2 immunization alleviates neuroinflammation, neurodegeneration and behavioural deficits.
La Vitola et al., 2018	Anti-TLR2 immunisation of WT mice	Anti-TLR2 pre-treatment ameliorates α-synuclein-induced memory deficits.

PD: Parkinson's disease; WT: wild-type; SHSY5Y: human neuroblastoma cell line; BV2: mouse microglia cell line; THP1 cells: human monocyte cell line

1.4.2.2 Toll-like receptor 4

In human studies, analysis of transcriptomic data from post-mortem control brains reveals ubiquitous expression of TLR4 and the adapter MyD88 throughout the brain, with their expression being higher in the substantia nigra and the putamen (Hughes et al., 2019). Additional studies comparing PD with control brains showed increased TLR4 protein levels in PD cases, specifically in the substantia nigra, the frontal cortex, the amygdala, and the caudate/putamen (Drouin-Ouellet et al., 2015; Kouli et al., 2020; Shin et al., 2015). This increase was also observed in peripheral immune cells in PD patients (Drouin-Ouellet et al., 2015; Ping et al., 2018; Shin et al., 2015) and one study also showed that the increased TLR4 is most prominent in PD cases with a higher risk of dementia (Wijeyekoon et al., 2020). Additional evidence supporting a role of TLR4 in human PD is provided from genetic studies and the discovery that a polymorphism in the TLR4 gene is linked with the risk of PD in a Chinese Han population (Zhao et al., 2015). (Summarised in **Table 1.3**).

Table 1.3 Summary of studies evaluating TLR4 in PD patients. *(Modified from Tables in Kouli, Horne et al., 2019).*

References	Study design	Key observation
Drouin-Ouellet et al., 2015; Hughes et al., 2019; Kouli et al., 2020; Shin et al., 2015	Human PD and control postmortem brains	TLR4 protein levels are increased in the substantia nigra, caudate/putamen, frontal cortex, and amygdala of PD brains.
Drouin-Ouellet et al., 2015; Ping et al., 2018; Shin et al., 2015	Human PD and control PBMCs	TLR2, TLR4, and NF-κB are upregulated in PD PBMCs.
Perez-Pardo et al., 2019	Human PD and control intestinal mucosa biopsies	TLR4 and pro-inflammatory cytokine expression is upregulated in the intestinal mucosa of PD patients compared to controls.
Zhao et al., 2015	PD and healthy Chinese Han population	TLR4 polymorphisms are linked to PD.
Wijeyekoon et al., 2020	Human PD and control PBMCs and serum	Higher percentage of TLR4+ monocytes in PD patients, significant increase in TLR4+ monocytes in higher dementia risk group

PD: Parkinson's disease; PBMCs: peripheral blood mononuclear cells

TLR4 has also been implicated through *in vitro* and *in vivo* work in animal models of PD, though some studies support a neuroprotective role while others argue that it acts in a detrimental manner. (Summarised in **Table 1.4**).

Stefanova et al., (2011) showed that TLR4 ablation leads to impaired microglial phagocytosis of α-synuclein in an *in vitro* model using BV2 microglia. In the same study, TLR4 deficiency was found to increase the dopaminergic neuron loss in the SN of an α-synuclein overexpressing mouse model, in addition to exacerbating the motor problems seen in the model. This neuronal death was proposed to occur due to the lack of clearing of α-synuclein by microglial phagocytosis, implicating TLR4 in this process (Stefanova et al., 2011). Further implicating TLR4 in neuroprotection, work by Mariucci et al., (2018) demonstrated that overexpression of α-synuclein mRNA occurs in $TLR4^{-/-}$ mice in several brain areas, though the causes of this have not been elucidated. One suggestion is that TLR4 plays an important role in the clearance of α-synuclein through the autophagy/lysosomal pathway. Venezia et al., (2021) showed that TLR4 deficiency facilitated the spread of α-synuclein, and that this is associated with a deficit in autophagy-lysosomal activity. Further evidence comes from work specifically showing that neuron-released α-synuclein activates microglia, which then engulf the α-synuclein and clear it through selective autophagy. It was shown that this process is dependent on microglial TLR4 due to its role in upregulating the autophagy receptor p62, a receptor key to degradation processes in autophagy (Choi et al., 2020).

Conversely, Tu et al., (2021) showed that microglial autophagic impairment occurred on the activation of TLR4 by α-synuclein, though through a different pathway than that investigated by Choi. In addition, Lee et al., (2019) showed that the activation of TLR4 resulted in the suppression of autophagy-related genes in microglia, however it is worth noting that the TLR4 activation was carried out using LPS and not α-synuclein which may result in the different downstream effects. In this way, TLR4 may be acting in a detrimental manner. This has consistently been seen in toxin-induced models of PD. TLR4 has been implicated in the MPTP model of PD, with MPTP treatment resulting in TLR4 upregulation, and inhibition of TLR4, either by gene knockout or with siRNA silencing, ameliorating MPTP induced deficits. For instance, $TLR4^{-/-}$ mice appeared to be less vulnerable to the toxin as they showed less dopaminergic cell death, in addition to fewer activated nigral microglia (Conte et al., 2017; Mariucci et al., 2018; Noelker et al., 2013; Zhao et al., 2016). Campolo et al., (2019) added to this data by showing that the absence of TLR4 also leads to inhibition of inflammasome activation and an improvement in MPTP-induced motor impairments. Zhou et al., (2016) using an *in vitro* TLR4 siRNA silencing approach found a reduction in microglial activation after MPTP treatment and decreased NF-κB activation. Upregulation of TLR4 was observed in an additional toxin model induced by 6-OHDA; in this model TLR3 expression was also

upregulated (McCabe et al., 2017). In a rotenone-induced model, motor deficits and dopaminergic cell loss were ameliorated in TLR4$^{-/-}$ animals (Perez-Pardo et al., 2019). The paraquat toxin-induced production of proinflammatory cytokines by BV2 microglia was reduced on TLR4 knockdown (Huang et al., 2020).

Recent work suggests that not only TLR2 but also TLR4 is necessary for the recognition of α-synuclein and initiation of inflammatory responses. Upon α-synuclein stimulation, TLR4-deficient microglia produced significantly less TNF-α and reactive oxygen species compared to wild-type microglia (Fellner et al., 2013). In agreement with this, Shao et al., (2018) recently showed that either pharmacological inhibition or gene depletion of TLR4 in microglia *in vitro* attenuates α-synuclein-induced TNF-α secretion. Further work showed TLR4 to be involved in mediating the astrocyte response to α-synuclein as well, resulting in upregulation of pro-inflammatory cytokines through activation of the NF-κB pathway (Rannikko et al., 2015). This data suggests that TLR4 is responsible for detecting α-synuclein as a DAMP and initiating an immune response. Work by Hughes et al., (2019) revealed that physiological levels of soluble oligomeric α-synuclein (but not monomers or fibrils) could induce TLR4-mediated cytokine production in microglia *in vitro*, with a 10- to 100-fold reduction in TNF-α production induced by α-synuclein in TLR4$^{-/-}$ microglia compared to wild-type. The reduction in the inflammatory response to α-synuclein in TLR2$^{-/-}$ microglia compared to wild type was much more modest, suggesting that the TLR4 pathway is the primary mechanism underlying the microglial response to oligomeric α-synuclein at physiological concentrations.

Finally, recent evidence implicates TLR4 activation in the gastrointestinal tract with PD pathogenesis. It has been widely proposed that α-synuclein pathology in PD may be initiated in the periphery, specifically in the intestinal and/or olfactory mucosa (Hawkes et al., 2007), and α-synuclein aggregates are present in human colonic mucosa even in very early disease (Malek et al., 2014). In further support of early gut involvement in PD, patients have been found to have an altered microbiome in the intestine (Keshavarzian et al., 2015; Scheperjans et al., 2015). Furthermore, studies in germ-free α-synuclein-overexpressing mice have demonstrated that colonisation of the gut with microbiota from PD patients exacerbates disease severity, compared with colonisation with microbiota from healthy human donors (Sampson et al., 2016). As such, the microbiome itself may play a role in driving disease progression by triggering an abnormal inflammatory response. TLR4 is primarily activated by

lipopolysaccharides found on the surface of Gram-negative bacteria (Poltorak et al., 1998) and is therefore uniquely poised to sense and be activated by dysbiotic gut microbiota. In line with this theory, Perez-Pardo et al., (2019) have demonstrated increased expression of TLR4 mRNA in human intestinal mucosa biopsy samples from PD patients compared to controls, with a concomitant increase in the expression of pro-inflammatory cytokines (IL-1β, IFN-γ) and chemokines (CCL2, CCL5) in the sigmoid mucosa. In a second set of experiments using the rotenone mouse model of PD, the same authors showed that $TLR4^{-/-}$ mice were protected to some extent from the toxic effects of rotenone both in the gut and the brain, and their motor deficit was significantly less severe compared to wild-type rotenone-treated mice. However, in a different study it was found that the knockout of MyD88 had no effect on the MPTP treatment-induced degeneration of the dopaminergic nigrostriatal pathway (Drouin-Ouellet et al., 2011) despite the increased survival of myenteric dopaminergic neurons seen in the same MPTP-treated $MyD88^{-/-}$ mice (Côté et al., 2011). In another animal study, transplantation of faecal microbiota from wild-type to MPTP mice led to reduced activation of the TLR4 pathway, dopaminergic neuron rescue, and improved motor function (Sun et al., 2018).

In summary, there is evidence to suggest that TLR4 may have both neuroprotective and detrimental roles in PD, although this may depend on the model system, or on the timepoint in the disease process. Specifically, in an acute setting or in early disease, TLR4 activation by α-synuclein may promote microglial phagocytosis to facilitate intracellular degradation of aggregated α-synuclein. Conversely in the setting of chronic inflammation, in either the gut or brain, TLR4 may play a critical role in escalating the inflammatory response (Fellner et al., 2013; Lee et al., 2008).

Table 1.4 Summary of studies evaluating TLR2 in in vitro and in vivo PD models. *(Modified from Tables in Kouli, Horne et al., 2019).*

References	Study design	Key observation
Shao et al., 2018b; Fellner et al., 2013	α-Synuclein-treated WT or TLR4$^{-/-}$ mouse microglia	α-Synuclein triggers microglial activation through TLR4.
Rannikko et al., 2015	α-Synuclein-treated WT or TLR4$^{-/-}$ mouse astrocytes	α-Synuclein triggers astrocytic activation through TLR4.
Stefanova et al., 2011	BV2 microglia with anti-TLR4 antibody	TLR4 inhibition impedes microglial α-synuclein phagocytosis.
Zhou et al., 2016	TLR4 knockdown in MPTP-treated BV2 microglia	TLR4 silencing reduces MPTP-induced microglial activation.
Chung et al., 2022	SHSY5Y cells co-cultured with LPS-activated microglia treated with TLR4 inhibitor	Inhibition of TLR4 mitigates α-synuclein aggregation and improves autophagic flux activity.
Tu et al., 2021	Primary microglia from WT and TLR4$^{-/-}$ mice stimulated with human α-synuclein	A-synuclein-induced microglial autophagy inhibition is TLR4-dependent.
Mariucci et al., 2018	WT & TLR4$^{-/-}$ mice	α-Synuclein expression is upregulated in the brain of TLR4$^{-/-}$ mice.
Venezia et al., 2021	WT & TLR4$^{-/-}$ mice with stereotaxic injection of PFFs	TLR4 deficiency facilitates spreading of α-synuclein, reduces lysosomal function of microglia, and accelerates neurodegeneration.
Mariucci et al., 2018; Conte et al., 2017; Zhao et al., 2016; Noelker et al., 2013; Shao et al., 2019	WT & TLR4$^{-/-}$ MPTP mice	MPTP treatment upregulates TLR4. TLR4 ablation rescues MPTP-induced neuroinflammation, neurotoxicity and motor deficits.
Campolo et al., 2019	WT & TLR4$^{-/-}$ MPTP mice	TLR4 ablation blocks inflammasome activation.
McCabe et al., 2017	WT & 6-OHDA treated rats	On 6-OHDA treatment, striatal TLR4 expression is increased.
Perez-Pardo et al., 2018	WT & TLR4$^{-/-}$ rotenone-treated mice	TLR4 knockout partially rescues dopaminergic cell loss in the substantia nigra and ameliorates the motor deficits.
Huang et al., 2020	BV2 and TLR4$^{-/-}$ microglia treated with paraquat	TLR4 knockout reduced paraquat-induced production of pro-inflammatory cytokines.
Choi et al., 2020	Mice injected with AAV9-human α-synuclein	Neuronal α-synuclein activates microglia which engulf α-synuclein in a TLR4-dependent process.

PD: Parkinson's disease; WT: wild-type; SHSY5Y: human neuroblastoma cell line; BV2: mouse microglia cell line; THP1 cells: human monocyte cell line

1.4.3 Potential therapeutic strategies

Immunomodulatory drugs already exist for a multitude of conditions, however so far none are regularly prescribed to patients with PD. Currently clinical trials of immunomodulatory agents are underway in PD – for example the AZA-PD trial (Greenland et al., 2020) – but are using broad spectrum agents with the intention of gaining proof of concept. This means that off-target immunosuppressive effects may be problematic. For increased efficiency and tolerability, agents focusing on specific immune pathways have been suggested. Establishing the drug targets requires further exploration of the fundamental processes underlying the role of inflammation in Parkinson's progression. There is strong evidence for a role of Toll-like receptors in Parkinson's disease, as discussed above, and as such these may provide a specific therapeutic target.

1.4.3.1 Candesartan cilexetil

A potential candidate of interest is Candesartan cilexetil. This is the prodrug of candesartan, and is rapidly converted to candesartan during the process of gastrointestinal absorption. Candesartan specifically and potently inhibits angiotensin II type I (AT_1) receptors. AT_1 receptors are found in vascular smooth muscle, in addition to main organs and glands including the heart and the brain. Angiotensin II works through its binding to AT_1 receptors, and through this action has effects including vasoconstriction and increased sodium retention. As a result of its antagonism of AT_1 receptors and the subsequent downstream effects, candesartan is prescribed for the treatment of hypertension and causes a dose-dependent decrease in diastolic blood pressure in response to angiotensin II (Gleiter et al., 2004). Candesartan is well tolerated and has limited side effects.

Candesartan has recently been shown to suppress TLR expression, specifically TLR2 and TLR4 (Dasu et al., 2009). This was demonstrated both *in vitro* in primary human monocytes and *in vivo* in mice peritoneal macrophages. In this study it was shown that candesartan treatment reduced the expression of TLR2 and TLR4, inhibited the secretion of pro-inflammatory mediators including TNF-α, IL-1β, and IL-6, and was protective against challenge by LPS. Qie et al., (2020) similarly showed a protective effect against LPS challenge whereby candesartan reduced the BV2 microglial activation phenotype, including a reduction in pro-inflammatory cytokines. This was shown to occur in a TLR4-dependent manner. Another angiotensin II receptor blocker (telmisartan) also induced a reduction in TLR2

expression in LPS-stimulated BV2 microglia (Elkahloun et al., 2019). A different study using human renal tubular epithelial cells found that candesartan reversed the LPS-induced upregulation of TLR4 expression and inhibited downstream inflammatory effects. Interestingly, this was not altered by knockdown of AT_1 receptors, thus suggesting that the anti-inflammatory effects of candesartan seen in this study could be through a novel pathway independent of AT_1 receptors (Zhao et al., 2013). Furthermore, it has been shown that candesartan can act to rescue microglial phenotype and function after *in vitro* stimulation with α-synuclein, providing direct evidence for a potential therapeutic role in preventing the α-synuclein-induced inflammation in PD (Daniele et al., 2015). Additionally, candesartan can reduce TLR 2 and 4 in the brain *in vivo*. A study investigating the use of candesartan to treat experimentally-induced ischemic stroke in mice showed that orally-dosed candesartan reduced the insult-induced rise in TLR2 and TLR4 expression in the brain and the subsequent rise in pro-inflammatory factors (Barakat et al., 2014). This study is not directly applicable to PD, but does show that candesartan appears to be able to cross the blood-brain barrier and to result in anti-inflammatory effects in the brain. This study also shows that candesartan is having this effect independent of changes in blood pressure as a dose of candesartan was chosen specifically to be under this threshold; the study focuses solely on the effect of candesartan on the TLR signalling cascade. Candesartan has been similarly shown to have a neuroprotective effect alongside its anti-inflammatory action in response to α-synucleinopathy. (Rodriguez-Perez et al., 2018). This was carried out using an α-synuclein-overexpressing animal model. Rats injected with an AAV9 virus expressing α-synuclein were dosed daily with candesartan or telmisartan or vehicle. Animals treated with candesartan or telmisartan showed less dopaminergic cell loss in the substantia nigra, reduced microglial activation, and reduced release of pro-inflammatory factors than the untreated AAV9-injected animals. The role of TLRs was not investigated in this work however, so the mechanistic basis of the reduction in neurodegeneration and neuroinflammation was not clear.

Candesartan has been considered as a potential candidate for the treatment of neurodegenerative diseases, such as Alzheimer's, though so far these studies have focused on its role as an anti-hypertensive and have not investigated inflammation. A double-blind randomized placebo-controlled clinical trial has been carried out to assess the safety of treatment with candesartan for one year in non-hypertensive Alzheimer's patients. The effects of treatment were assessed using biomarkers and clinical measures of Alzheimer's disease severity. Candesartan was found to be safe for treatment over this time, and crucially did not

result in hypotension. It was also found that candesartan treatment was associated with reduced amyloid accumulation (as assessed using CSF biomarkers), with an increase in functional network connectivity in subcortical networks, and with improved executive function. There was no proof of target engagement in this trial. (Hajjar et al., 2022; Okafor et al., 2021). A recent randomized double-blind placebo-controlled clinical trial (parallel phase II) using candesartan to treat cognitive impairment in 14 people with PD did not show positive effects on cognitive outcome over 28 weeks (the main trial outcome), or on motor symptoms (Kulisevsky et al., 2023). The authors discussed that this trial may not have been long enough to show clinical effect. Candesartan was chosen for this trial due to its function as an AT_1 receptor blocker through which it has a protective effect on oxidative stress and inflammation, however the effects of candesartan treatment on these factors were not explored, and so no conclusions can be based on this.

Many of these studies provide evidence to support further investigation of candesartan as a candidate treatment for PD. However the mechanism of action and whether this is through its TLR-blocking actions needs to be determined.

1.5 Summary and hypothesis

Parkinson's disease is a common neurodegenerative disorder. Patients experience characteristic motor symptoms in addition to other non-motor features including sleep disorders and gastrointestinal dysfunction; the disease commonly results in the eventual development of dementia. Patients experience a substantial decrease in quality of life as the disease progresses. There is currently no treatment to slow the progression of PD and existing therapies work only to alleviate symptoms. A deeper understanding of the mechanisms underlying the disease may be key to the development of treatments.

There is strong evidence for neuroinflammation playing a crucial role in the development and progression of PD. This evidence arises from animal models, *in vitro* work, and post-mortem and neuroimaging studies in humans. Further evidence implicates TLRs in mediating this inflammation through their action as pattern recognition receptors. TLRs have the ability to recognise damage-associated molecular patterns such as aggregated α-synuclein. TLR activation results in the release of proinflammatory factors which may go on to activate other immune cells and eventually to create an environment detrimental to the survival of neurons.

I hypothesise that Toll-like receptors play an important role in the development and progression of PD through mediating the response of microglia and peripheral monocytes to the binding of aberrant forms of α-synuclein, leading to neuroinflammation and exacerbation of neurodegeneration. I propose that TLR blockade will have a protective effect on the development of pathology in an α-synuclein animal model (***Chapter 2***) through its suppression of inflammation (***Chapter 3***) in addition to reducing the inflammatory response of patient-derived immune cells stimulated with α-synuclein (***Chapter 4***). This has important implications for potential new therapeutic routes for the treatment of Parkinson's disease.

1.6 Objectives

1. To reproduce the α-synuclein PFF model developed by Kuan et al., (2019) and to use this model to determine the effects of the inhibition of TLRs 2 and 4 on the progression of pathology. TLR inhibition will be carried out using candesartan, a licensed drug for the treatment of hypertension repurposed in this study.
2. To determine whether any protective action of candesartan seen in the model is occurring through the suppression of TLR expression and subsequent suppression of inflammation.
3. To optimise a protocol to be used in the investigation of the α-synuclein-induced inflammatory response, and for testing whether candesartan and other TLR-blockers may be protective against this inflammation.

Chapter 2.

INVESTIGATING HOW THE USE OF CANDESARTAN AFFECTS THE PROGRESSION OF AN ANIMAL MODEL OF A-SYNUCLEINOPATHY

DECLARATION

The preparation of α-synuclein pre-formed fibrils and their intravenous injection into the rats was carried out by Dr Wei-Li Kuan. Drug dosing was carried out by myself, Sarah Fox, and staff at The Mira Building animal facility (University of Cambridge Biomedical Services). Blood samples were taken by staff at The Mira Building. Animal culling and tissue collection was carried out with the assistance of Dr Wei-Li Kuan and Pam Tyers.

Immunohistochemistry slide scanning was carried out by the Histopathology and ISH core facility at CRUK Cambridge.

2.1 Aims of Chapter

Though many studies have investigated the mechanistic basis of PD using animal models, few of these models accurately recapitulate the early phase of PD. The PFF:RVG9R method of transporting aggregated α-synuclein from the periphery into the brain results in a slowly progressing degenerative model of PD featuring behavioural deficits representative of early/prodromal PD. The nature of this model also allows the exploration of how a drug intervention may slow the progression of pathology in the model, and allows for the testing of drug efficacy over a long period of time in addition to the testing of drug tolerability.

In this chapter I aimed to reproduce the α-synuclein PFF model developed by Kuan et al., and use this model to determine the effect of candesartan, a putative TLR2 and TLR4 antagonist, on the progression of pathology and of behavioural deficits.

2.1.1 Objectives

1. To confirm that PFF:RVG9R peripheral injections lead to α-synuclein aggregation in the brain.
2. To determine the effect of candesartan treatment on behavioural deficits in the PFF:RVG9R model.
3. To determine the effect of candesartan on neuronal loss in the PFF:RVG9R model.

A further objective of the experiment was to investigate whether candesartan has an effect on neurodegeneration, however this will be described in ***Chapter 3***.

2.2 Introduction

2.2.1 Animal models of Parkinson's disease

The key clinical and pathological characteristics of PD, as discussed in ***Chapter 1***, need to be recapitulated by an animal model to allow mechanistic study of disease pathogenesis. Animal models of Parkinson's disease are useful in researching organisms as a whole. They are a helpful way to monitor the progression of disease pathology long-term, and allow the use of modifying therapies whereby the effects of this intervention can be monitored in a whole system and measured over time. In spite of the usefulness of animal models, the vast majority of drugs successful in slowing the progression of pathology in an animal model eventually fail when transitioned to a clinical trial. This failure is in part due to the lack of success in recapitulating features of the human disease in animals. This is obviously impossible to do fully, however an improvement in similarity of models to disease is needed for higher success rates in the transfer of drugs to clinical trial. The accuracy with which an animal model follows the pathophysiology of the human disease is key to the applicability of the results of work using the model. Many animal models of PD exist; these replicate PD to different extents and emphasise different features of PD.

2.2.1.1 Toxin models

Toxin models were used as the first models of PD, discovered through the observation that the contamination of an illicit drug with MPTP resulted in parkinsonian symptoms in users (Langston et al., 1983). MPTP is one of the most popular toxin models having been found to produce a reliable dopaminergic lesion in mice and monkeys (Visanji and Brotchie, 2005), and showing reproducible behavioural deficits including akinesia and rigidity (Sedelis et al., 2001). On administration through either IV injection or infusion (Jackson-Lewis and Przedborski, 2007), MPTP crosses the blood-brain barrier and is converted to MPP^+ by monoamine oxidase B. MPP^+ is transported into dopaminergic neurons through the DAT transporters. MPP^+ acts in a toxic manner through blocking mitochondrial complex I activity (Nicklas et al., 1987). Another popular model is the 6-hydroxydopamine (6-OHDA) model produced through the injection of the toxin directly into the nigrostriatal system, commonly into the substantia nigra,

the medial forebrain bundle, or the caudate-putamen complex (Deumens et al., 2002). This neurotoxin is transported into dopaminergic neurons and results in the degeneration of nerve terminals. Its toxicity occurs through its inhibition of mitochondrial complexes I and IV (Deumens et al., 2002; Glinka et al., 1997). Other neurotoxins used in the production of PD models include rotenone and paraquat. Both of these toxins similarly work to induce oxidative stress in dopaminergic neurons though mitochondrial-related mechanisms (Betarbet et al., 2000; Fei et al., 2008). These models are effective at replicating the vast loss of dopaminergic neurons in the substantia nigra as is seen in the human disease and the resulting motor deficits. However, these models do not produce the characteristic Lewy bodies seen in human PD and thus appear to reproduce the pathological end-point of PD – i.e. the death of dopaminergic neurons – but not the processes involved to get there.

2.2.1.2 A-synuclein overexpression models

The α-synuclein overexpressing animal model is now frequently utilised. Overexpression is induced either by transgenics – commonly through α-synuclein overexpression under the Thy1 promotor; most common in mice – or through a viral vector – generally using the stereotactic injection of lentiviruses or adeno-associated viruses into the substantia nigra; most common in rats (Chesselet, 2008). These models were produced after the discovery that some familial forms of PD show a duplication or triplication mutation in the SNCA gene coding for α-synuclein. (Miller et al., 2004; Singleton et al., 2003). Fuchs et al., (2007) showed that a gene dosage effect was present, in that a duplication of the gene resulted in late-onset PD, whereas a triplication resulted in early-onset and dementia. This indicated that the level of overexpression of α-synuclein in the brain is key to the development of the disease. Different iterations of these overexpression models show different effects, these include synucleinopathy, neurodegeneration, and motor deficits (Decressac et al., 2012; Kirik et al., 2003, 2002; Lo Bianco et al., 2002). These models are effective in regards to these pathologies, however, they are based on the rare familial forms of PD not the more common idiopathic PD. Consequently, it is difficult to know how relevant these models are due to α-synuclein being expressed at supra-physiological levels. These models are exposed to α-synuclein at a much higher level than would be seen in idiopathic Parkinson's where there are differing reports of whether α-synuclein gene expression is elevated or not (Gründemann et al., 2008; Kingsbury et al., 2004; Neystat et al., 1999; Su et al., 2017; Tan et al., 2005). This not only fails to recapitulate the brain environment experienced by people with idiopathic PD, but

also results in excessive neuroinflammation which may mask other more subtle neuroinflammatory effects seen in the idiopathic PD brain.

2.2.1.3 Pre-formed fibril models

A-synuclein pre-formed fibrils (PFFs) are artificially-produced aggregated α-synuclein species. PFFs are able to act as a template for the recruitment of soluble endogenous α-synuclein into insoluble Lewy body-like inclusions (Volpicelli-Daley et al., 2011). This model was first developed by Volpicelli-Daley in mouse primary neuron cultures. They showed that PFFs were able to act as a template for the recruitment of soluble endogenous α-synuclein into insoluble Lewy body-like inclusions. This model was revolutionary as it uses endogenous levels of α-synuclein, and so produces an environment where physiological levels of α-synuclein are aggregating to form pathological inclusions. The toxicity of these injections of α-synuclein PFFs does not arise from elevated levels of α-synuclein, but instead from the PFF-induced recruitment of endogenous α-synuclein into intracellular aggregates. This was proven through the use of α-syn$^{-/-}$ neurons whereby the addition of α-synuclein pre-formed fibrils did not induce neuron death (Volpicelli-Daley et al., 2011). This model has been extended to the production of animal models, including mice, rats, and primates (Abdelmotilib et al., 2017; Duffy et al., 2018; Luk et al., 2012; Paumier et al., 2015; Shimozawa et al., 2017).

The use of PFFs in the production of animal models generally requires injection into the striatum; this then causes the development of Lewy-like pathology in areas which innervate the striatum including, crucially, the substantia nigra. However, one big limitation of this is that the location of these inclusions is injection site-specific. In addition, it has been suggested that PD begins in the periphery (in the gut or olfactory bulb) and then spreads to the brain where degeneration of dopaminergic neurons leads to subsequent motor symptoms (Hawkes et al., 2007). This means that models created by injection directly into the brain may be less relevant to the human disease. Furthermore, injection into the brain can cause site-specific trauma from the needle resulting in effects, such as inflammation, that cannot be entirely separated from this trauma.

2.2.1.4 A novel PFF model

To circumvent the limitations with injecting directly into the brain, as discussed above, a novel model was developed by Kuan et al., (2019), which is produced through the injection of α-synuclein PFFs into the tail vein of Sprague Dawley rats. PFFs on their own have

been shown to have the ability to cross the blood-brain barrier, however not in sufficient volumes to trigger inclusions or neurodegeneration. Kuan therefore utilised the pre-existing transport system of conjugation to the rabies glycoprotein RVG9R which had previously been utilised to transport RNA into the brain (Kuan et al., 2011; Kumar et al., 2007). This ligand is thought to work through binding a neural-specific acetylcholine receptor which results in a transient membrane inversion, thus allowing the passage of the ligand and its cargo into the brain (Zeller et al., 2015). Kuan showed that this RVG ligand is able to reversibly complex to PFFs, and that they are transvascularly delivered to the brain without affecting their conformation. This model therefore provides an effective periphery to central nervous system transport of α-synuclein PFFs. This replicates processes that are thought to occur in human PD, though in this model the α-synuclein enters the brain through the transport in blood and crossing of the blood-brain barrier, not through neuronal connections with the gut or olfactory bulb, thus making it a less effective model than one incorporating these conditions. Kuan then went on to show that once in the brain, and over a 6 month time period, the PFFs induced site-specific and time-dependent α-synucleinopathy, behavioural deficits associated with early PD – including olfactory deficits (Kuan et al., 2019) and deficits in fine motor ability (Kuan et al., 2022) – and neurodegeneration of both dopaminergic neurons in the substantia nigra and cholinergic neurons in the vagus nerve nucleus. Hence this was shown to be an effective model and potentially better replicates the early stages of human PD as pathology develops relatively slowly with subtle effects even after 6 months.

2.2.2 Candesartan

Candesartan is a licensed drug used in the treatment of hypertension through its actions as an angiotensin II type I receptor blocker. It has recently been shown to act as a blocker of Toll-like receptors 2 and 4 (TLR2, TLR4) *in vitro* and *in vivo*, though the mechanism of this has not been determined (Dasu et al., 2009). Given its ability to act as a TLR blocker and hence potentially reduce neuroinflammation candesartan has been suggested as a candidate for further exploration as a disease modifying treatment for PD. The role of TLRs in PD has been discussed in ***Chapter 1.4***, and will be further discussed in ***Chapter 3***.

2.3 Methods

2.3.1 Study design

Adult male Sprague Dawley rats (aged 14 weeks at start of experiments, N=72) were used in this study. Baseline behavioural tests were carried out and a blood sample obtained from each of the rats. The rats were split into 3 groups: a control group injected with saline only (N=12), a candesartan-treated pre-formed fibril-injected (PFF-injected) group (N=30), and an untreated PFF-injected group (N=30). All of these groups were given an injection at Day 0, with the candesartan treatment being started at Day -1. The PFF injection was a one-off intravenous injection of pre-formed fibrils conjugated to a RVG9R peptide which allowed the transvascular delivery of the fibrils to the brain. This has been shown to lead to a 20% loss of dopaminergic neurons in the substantia nigra and a 20% loss of cholinergic neurons in the vagus nerve nucleus at 6 months, as well as aggregation of α-synuclein in both of these brain regions (Kuan et al., 2019). Behavioural tests and blood sampling were repeated at 2, 4, and 6 month timepoints post-injection. A subset of rats were culled for post-mortem analysis at each 2 month timepoint: 3 from the control group, and 6 from each PFF-injected groups at the 2 and 4 month timepoints. The remaining 6 from the control group and 18 from each PFF-injected group were culled at the final 6 month timepoint. (**Figure 2.1**). At 2 months some animals were chosen for culling if they refused to voluntarily take the drug or control treatment (thus making daily administration more difficult), at 4 months the animals were chosen for culling on a random cage-by-cage basis.

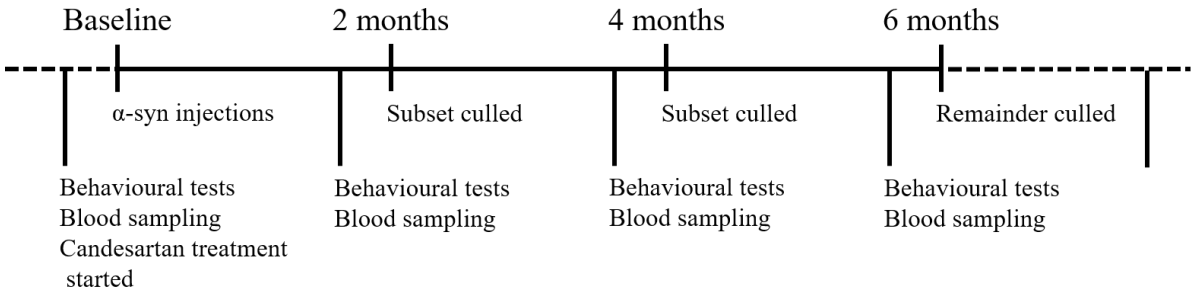


Figure 2.1 Timeline of study design.

A parallel experiment in a larger group of control (non-PFF injected) rats was carried out at a later timepoint to establish whether the effects of the drug were disease model-specific. Adult male Sprague Dawley rats (aged 8 weeks at start of experiments, N=30) were used in this parallel study. Baseline behavioural tests were carried out and a blood sample obtained from each of the rats. The rats were split into 2 groups: a candesartan-treated group (N=16) and an untreated group (N=14). Candesartan treatment was started at Day -1, and a saline injection mimicking the PFF injection of the parallel study was carried out at Day 0. Behavioural tests and blood sampling were repeated at 2, 4, and 6 months post-injection.

2.3.2 Animals, PFF-injection, and candesartan treatment

The rats were housed (3 per cage in the initial experiment and 2 per cage in the second control experiment) in standard laboratory conditions: 12-hour light and dark cycle, 22˚C room temperature, 55% relative humidity, food and water *ad libitum*. When required, food restriction was carried out prior to behavioural tests. All food was removed at 16:00 PM the day prior to the tests, and the tests were carried out between 08:00 AM and 13:00 PM with food being replaced by 14:00 PM. Animals were anaesthetised using isoflurane via inhalation prior to injection of α-synuclein pre-formed fibrils (see below for preparation) into the tail veins of the rats in the initial experiment, and saline was injected in the same way into the rats in the second parallel experiment. This injection was carried out by a researcher who was licensed and competent in this technique (Dr William Kuan), not by me. Candesartan treatment was delivered to the rats in the treated groups on a daily basis at 10 mg/kg/day. The dose was selected based on previous studies using Sprague Dawley rats. In these studies doses between 1 mg/kg/day and 10 mg/kg/day were used, which were well-tolerated, and for up to 40 weeks no severe adverse effects were seen (Fu et al., 2011; Hashikawa-Hobara et al., 2012; Jin et al., 2009; Otsuka et al., 2004). The rats were dosed orally using syringes placed into their mouths, and the candesartan was made palatable to the rats through crushing and mixing with 6ml chocolate spread. Untreated rats were fed with chocolate spread only at the same volume.

2.3.3 Preparation of α-synuclein pre-formed fibrils

Human WT full-length α-synuclein pre-formed fibrils were prepared as previously described in the literature (Volpicelli-Daley et al., 2014). A-synuclein monomers (rPeptide) were resuspended in 10 mM Tris-HCl, 50 mM NaCl, pH 7.6 at a concentration of 5 mg/ml. A 37˚C thermomixer was used to shake them for 7 days at 1000 r.p.m., they were then aliquoted

and transferred to a -80˚C freezer. Prior to use, the PFFs were thawed and diluted to 0.1 μg/μl and sonicated using a Soniprep 150 Plus ultrasonic disintegrator (MSE) with 60 pulses at 10% power with a pause every 10 pulses to allow the solution to cool.

2.3.4 Animal culling and brain preparation

Animals were culled through overdose of carbon dioxide followed by decapitation. The brains were removed and dissected into each hemisphere. The left hemisphere was fixed immediately in 4% PFA for 24 hours and then transferred to 30% sucrose solution with sodium azide. The fixed brains were stored in the sucrose solution at 4˚C until sectioning. The right hemisphere was dissected into 5 brain regions, namely the olfactory bulb, the brainstem, the cerebellum, the cortex, and the midbrain, and then flash frozen. The dissected brain regions were stored at -80˚C until use. If on removal the left hemisphere was damaged then the right hemisphere was fixed instead and the damaged left hemisphere dissected. The PFA-fixed brains were cut into 40 μm sections in the sagittal plane using a microtome. 12 series of consecutive sections were obtained and stored as free-floating sections in TBS-azide at 4˚C until use in immunohistochemistry.

2.3.5 Blood samples

Blood was obtained through bleeding the animals from the saphenous vein. This was collected using uncoated tubes. The blood was allowed to clot at room temperature for 15 minutes before being centrifuged for 15 minutes at 2000 rpm. The serum was then collected and stored at -80˚C until use. More details will be provided in the next chapter.

2.3.6 Immunoprecipitation

Immunoprecipitation was carried out to isolate α-synuclein from the frozen rat brains. Flash-frozen rat cortex was homogenised in 1% Triton X-100 buffer supplemented with Complete protease inhibitors, and a BCA Protein Assay Kit (Thermo Fisher *#23227*) was used to quantify protein concentration. Lysate containing 1 mg protein in a final volume of 500 ul was spun in an ultracentrifuge at 120000 g and 4˚C for 60 min (Beckman Optima Max-XP). The supernatant was collected (triton-soluble fraction). 500 ul Triton X-100 buffer supplemented with Complete protease inhibitors was added to the pellet and a needle used to redistribute the pellet, this formed the triton-insoluble fraction which was then used in the immunoprecipitation. A 5% input was removed from the sample and NuPAGE LDS sample

buffer added before being stored at -20˚C until use in immunoblot. To the remaining 95% of the sample the IP antibody against α-synuclein (Abcam *ab212184*; 1:100) was added. Samples were then incubated at 4˚C overnight on a rotating wheel. Dynabeads™ (Thermofisher Protein G immunoprecipitation kit *#10007D*) were prepared and added to the samples, before a further incubation for 30 min at 4˚C followed by incubation for 10 min at room temperature. The Dynamag-2 magnet was used to isolate the beads; these were washed 3 times in buffer before being eluted using NuPAGE LDS sample buffer. The eluted sample was stored at -20˚C with the Dynabeads™ until use in the immunoblot.

2.3.6.1 Immunoblot

Immunoblotting was carried out to establish the presence of aggregated α-synuclein in the rat brains. IP samples were defrosted on ice before being put onto the Dynamag-2 magnet to isolate the beads while the eluted sample was removed. NuPAGE reducing agent was added to both the Input samples and the IP samples before they were denatured at 70˚C for 10 minutes. All IP samples were run on one 4-12% polyacrylamide gel; all input samples were run on a second 4-12% polyacrylamide gel. Transfer onto a polyvinylidene fluoride membrane was carried out for 90 min at 30 V. Both membranes were then fixed for 1 hour in 4% PFA according to the known practise when blotting for α-synuclein (Lee & Kamitani, 2011). Each membrane was blocked with 5% milk-TBST and then incubated overnight at 4˚C with a second anti-α-synuclein antibody (*BD610787*; 1:1000). The membrane was washed and then incubated with the appropriate HRP-conjugated secondary antibody for 1 hour (Sigma *A9917*; 1:20,000). Immunoreactivity was visualised with chemiluminescence.

2.3.7 Behavioural tests

2.3.7.1 The buried food test

The buried food test was carried out to assess the ability of the rats to discriminate odours. Animals were familiarised with the food pellets (Kelloggs Coco pops) and fasted overnight before this test was carried out. Rats were individually placed in a cage where a single pellet had been buried in the wood shavings lining the bottom of the cage. A timer was started when the animal was placed in the cage and stopped either when the pellet had been found or when 15 minutes had passed. This test was carried out once per 2 month timepoint and the position of the pellet within the cage was changed at each of these timepoints. This test was carried out only in the first study with the PFF-injected animals.

2.3.7.2 The staircase test

The staircase test was carried out to assess the fine motor ability of the rats in the form of reaching and grasping ability. This test was carried out immediately following the odour discrimination test. Animals were food-deprived overnight before the tests. The staircase was made up of 7 steps, with 2 food pellets (Kelloggs Coco pops) placed into wells on the right and left of the step (making 28 pellets total). Rats were individually placed into the staircase box and left for 30 minutes. (**Figure 2.2**). After the test interval, animals were removed from the staircase and the uneaten pellets were counted. This test was carried out once per 2 month timepoint. Animals were discounted from the final analysis if they failed to eat any pellets at all 4 of the study timepoints (baseline, 2 months, 4 months, and 6 months).

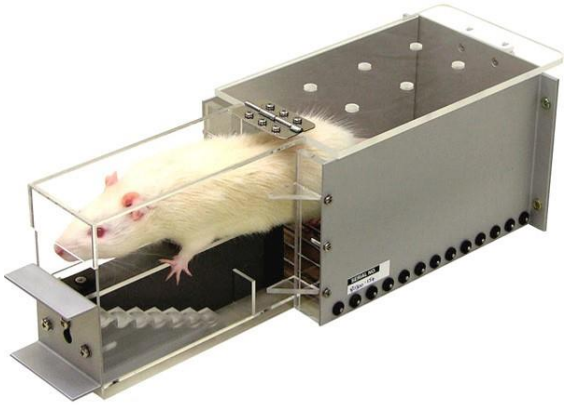


Figure 2.2 The staircase test. *Equipment used in the staircase test with demonstration of how rat uses the equipment.*

2.3.8 Immunohistochemistry

2.3.8.1 Dopaminergic and cholinergic

A 1:12 series of post-mortem brain sections was immunostained for either tyrosine hydroxylase or choline acetyltransferase to stain dopaminergic or cholinergic neurons respectively. Sections were first quenched with 10% methanol and 10% hydrogen peroxide before being blocked in TBS with 0.3% Triton X-100 and 5% serum for 1 hour. Sections were then incubated overnight at 4˚C with primary antibodies diluted in TBS with 3% Triton X-100 and 1% serum, as listed in **Table 2.1**, followed by a 3 hour incubation with biotinylated secondary antibody, and 1 hour 30 minute incubation with avidin-biotin complex (Vector; *#PK-6100*). Antigen visualisation was carried out using 3,3'-Diaminobenzidine (DAB

solution; Vector; *#SK-4100*). DAB solution was left on the tissue from 1 minute to 4 minutes, dependent on tissue colour change. Sections were mounted on coverslips and dehydrated in sequential transfers in 70% EtOH, 90% EtOH, and 10 0% EtOH, followed by immersion in 100% xylene for 30 minutes. Finally, they were mounted with DPX mounting medium (Thermo Scienific).

Table 2.1 Antibodies used for immunohistochemistry on brain sections.

Marker	Species	Dilution	Manufacturer/Catalogue No.
Tyrosine hydroxylase (TH)	Mouse monoclonal	1:500	Millipore Sigma *#MAB318*
Choline acetyltransferase (ChAT)	Goat polyclonal	1:500	Millipore Sigma *AB144P*

Quantification of tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT) staining was carried out in a similar fashion. To ensure non-bias the animal code and group number was blinded. Using a Leica DM6000 microscope with a fixed digital camera, and using the Stereo Investigator 9.10.3 software (MBF Bioscience), the boundaries of the regions of interest (ROI) were drawn. The ROI for TH staining was the substantia nigra, and the ROI for ChAT staining was the hypoglossal nerve nucleus and the vagus nerve nucleus. The identification of these regions was carried out with the assistance of the Paxinos rat brain atlas (5^{th} edition). Neurons were counted using unbiased stereology.

2.3.8.2 A-synuclein

A 1:12 series of post-mortem brain setions were immunostained for a selection of α-synuclein antibodies. Sections were first mounted on SuperFrost Plus Adhesion slides (Epredia #10149870) and left to dry at 37˚C for 4 hours. Antigen retrieval was carried out using sodium citrate buffer for 20 minutes in the microwave. Sections were then quenched with 20% methanol and 3% H_2O_2 for 30 minutes before being blocked in TBS with 0.3% Triton X-100 and 5% serum for 1 hour. Sections were then incubated overnight at 4˚C with primary antibodies diluted in TBS with 0.3% Triton X-100 and 1% serum, as listed in **Table 2.2**, followed by a 2 hour incubation with biotinylated secondary antibody, and 55 minute incubation with avidin-biotin complex (Vector; *#PK-6100*). Antigen visualisation was carried out using 3,3'-Diaminobenzidine (DAB solution; Vector; *#SK-4100*). DAB solution was left on the tissue from 1 minute to 4 minutes, dependent on antibody used. Counterstaining was

carried out using haematoxylin. Sections were dehydrated in sequential transfers in 70% EtOH, 90% EtOH, and 100% EtOH, followed by immersion in 100% xylene for 30 minutes. Finally, they were mounted with DPX mounting medium (Thermo Scienific).

Table 2.2 Antibodies used for α-synuclein immunohistochemistry on brain sections

Marker	Species	Dilution	Manufacturer/Catalogue No.
A-synuclein-42	Mouse monoclonal	1:500	BD Biosciences *#610787*
A-synuclein-211	Mouse monoclonal	1:500	Invitrogen *MA5-12272*
A-synuclein 2A7	Mouse monoclonal	1:500	Antibodies.com *A85290*

2.3.9 Immunofluorescence

A 1:12 series of post-mortem brain sections was immunostained for tyrosine hydroxylase to identify dopaminergic striatal fibres. Sections were blocked in TBS with 0.3% Triton X-100 and 5% serum for 1 hour before being incubated overnight at 4˚C with primary antibodies diluted in TBS with 3% Triton X-100 and 1% serum. Sections were then incubated for 3 hours with DAPI and anti-rabbit-568 at room temperature in the dark. Sections were mounted and then coverslipped using Fluorsave™ reagent (Millipore Sigma).

Quantification of TH striatal immunofluorescence was carried out using the Leica DMI8 microscope. An area of interest was drawn around the striatum and the tile scanning function was used to form a single image of the entire striatum. These images were analysed using ImageJ and a mean greyscale was calculated for each region; all sub-sections containing striatum were used to produce a mean striatal intensity through the whole striatum.

2.3.10 Statistical analysis

Data analysis and graph generation was carried out using GraphPad Prism 8.3. Data normality was assessed using the Shapiro-Wilk test. Pairwise comparisons were carried out between untreated and candesartan-treated PFF or control groups using an unpaired T-test or a Mann-Whitney test (for parametric or non-parametric data respectively). For data comparing >2 groups a one-way ANOVA was carried out followed by Tukey's posthoc test or a Kruskal-Wallis test followed by Dunn's posthoc analysis or a (for parametric or non-parametric data respectively).

2.4 Results

2.4.1 Study design

The model used in this study was developed and established by Dr Wei-Li Kuan. My study focus was to compare disease development and progression in a PFF-injected group treated with the drug candesartan, (which has been shown to block Toll-like receptors 2 and 4), with a PFF-injected group not treated with candesartan. I included a small saline-injected control group in the original study design but limited this to only 6 animals, given that this model was already established and I aimed to reduce the total number of animals used (in accordance with the 3 Rs of animal research). This small control group was included to ensure that the degeneration had occurred as expected, and also because a new behavioural test was carried out which wasn't used in the original paper so controls were required to compare to a non-disease state.

2.4.2 What effect does the injection of α-synuclein PFFs have on endogenous aggregation of α-synuclein within the rat brain?

The original model produced by Kuan et al., (2019) showed aggregation of α-synuclein in the brains of rats injected with PFFs conjugated to an RVG9R peptide, but not in the brains of rats injected with unconjugated PFFs or those injected with saline only. We used the conjugated PFF:RVG9R and saline only to inject our animals – no unconjugated PFFs were used in this study. For simplicity, from here on in our PFF:RVG9R-injected animals will be referred to as PFF-injected or PFF-only. To confirm that α-synuclein aggregation in the brain was occurring as expected in my study, sequential ultracentrifugation was carried out to isolate brain homogenate containing only triton-insoluble α-synuclein, followed by immunoprecipitation to confirm the presence of high molecular weight species in the brain. This was only carried out in the cortex due to a lack of sufficient amounts of other brain tissue which is required in large amounts for this experiment. The immunoprecipitation showed the presence of these high molecular weight species in the cortex of PFF-injected animals. High molecular weight species were also unexpectedly seen in cortical samples from the control

animals. (**Figure 2.3a**). Due to this unforeseen result, it was clear that an error had occurred in the experimental set-up and it seemed likely that the control animals were accidentally injected with α-synuclein PFFs. For this reason, I decided in conjunction with my supervisors to exclude the original controls and conduct a repeat study using control animals. This second study also allowed me to test the effect of candesartan in non-PFF rats to determine whether any effect of the drug on behaviour and pathology was specific to the PFF-model. (**Figure 2.4**). Though every effort was made to directly replicate the first study in the PFF-injected animals in order to allow for comparison, due to some specific unavoidable differences – namely the age of the rats, the number of rats housed per cage, and the timing of the post-mortem work carried out – it was decided that this second control study would be carried out as a parallel study and data analysis would not involve direct comparison between the 2 studies.

Immunoprecipitation was carried out in the new control groups, as in the first study, to confirm the absence of high molecular weight α-synuclein species in the new controls. No high molecular weight species were seen in either of the new control groups (candesartan-treated or untreated), but were present in the PFF-injected animals, confirming the validity of control versus PFF status of the experimental groups. (**Figure 2.3b**).

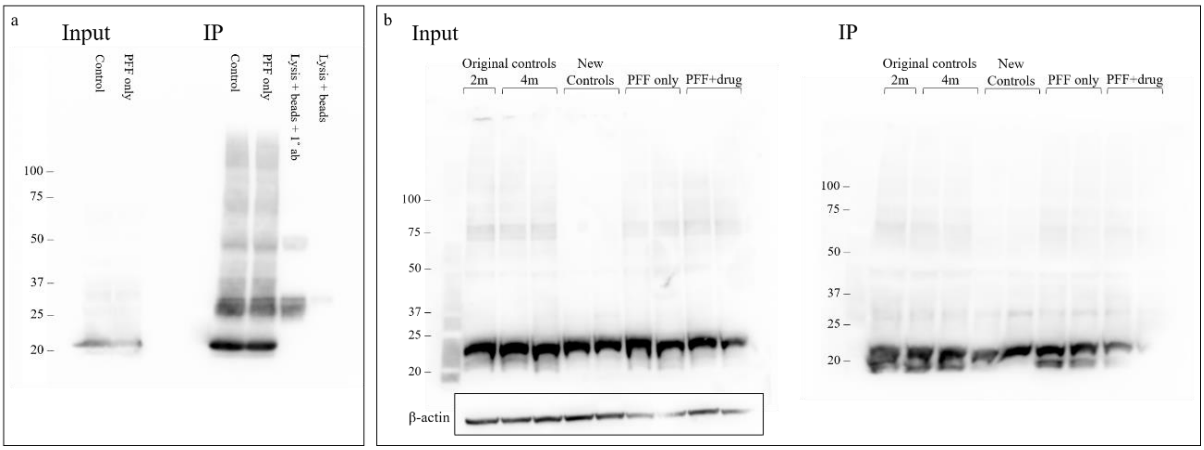


Figure 2.3 Model characterisation. *Immunoprecipitation on homogenised rat cortex in* ***(a)*** *original study with comparison between original control animal and PFF-injected animal, and* ***(b)*** *original and parallel control study with comparison between original control animals, and untreated/drug-treated PFF-injected/new control animals. Loading controls can be seen in the 5% input.*

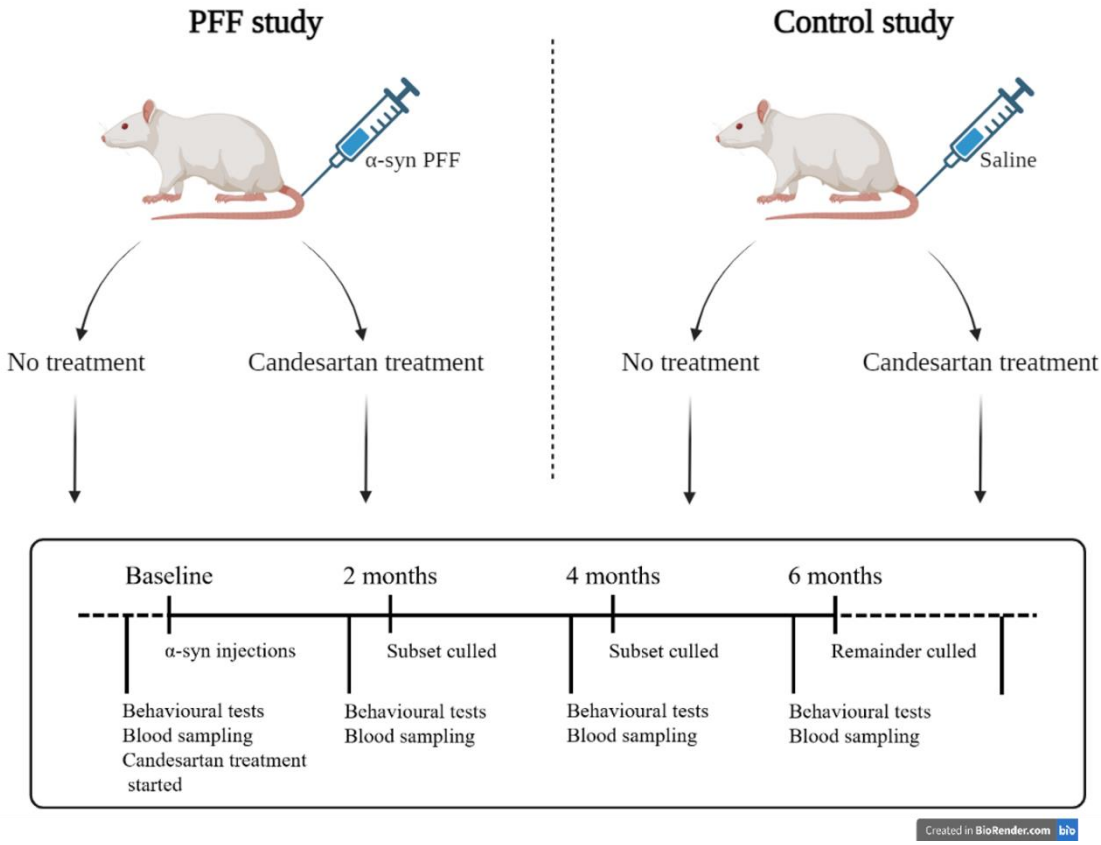


Figure 2.4 PFF and control studies were carried out in parallel. *Diagram showing study design of parallel PFF and control studies, and the timeline of these studies.*

Further work was carried out to determine whether the α-synuclein could be seen in key pathological brain areas in this model, i.e. the brainstem and substantia nigra, using immunohistochemistry techniques. This was carried out in 4 PFF-injected animals and 4 saline injected control animals (controls from the parallel study) using multiple different α-synuclein antibodies to ensure that any changes would not be missed through the use of an ineffective antibody. Suprisingly, there were no clear differences in levels of α-synuclein staining in the brainstem of PFF-injected and control saline-only injected rats (controls from the parallel study). This was consistently seen on staining brainstems using the α-synuclein-42 (**Figure 2.5**) and α-synuclein-2A7 (**Figure 2.6**) antibodies, however no staining at all was seen in either animal group using the α-synuclein-211 antibody (**Figure 2.7**). Positive staining was identified using the α-synuclein-2A7 and α-synuclein-42 antibodies, however this was difficult to interpret fully in regards to differences between PFF-injected and saline-injected control rats due to high non-specific background staining. No positive staining was seen in the substantia

nigra using either the α-synuclein-42 or α-synuclein-2A7 antibodies, though high levels of non-specific background staining was seen. (**Figure 2.8**).

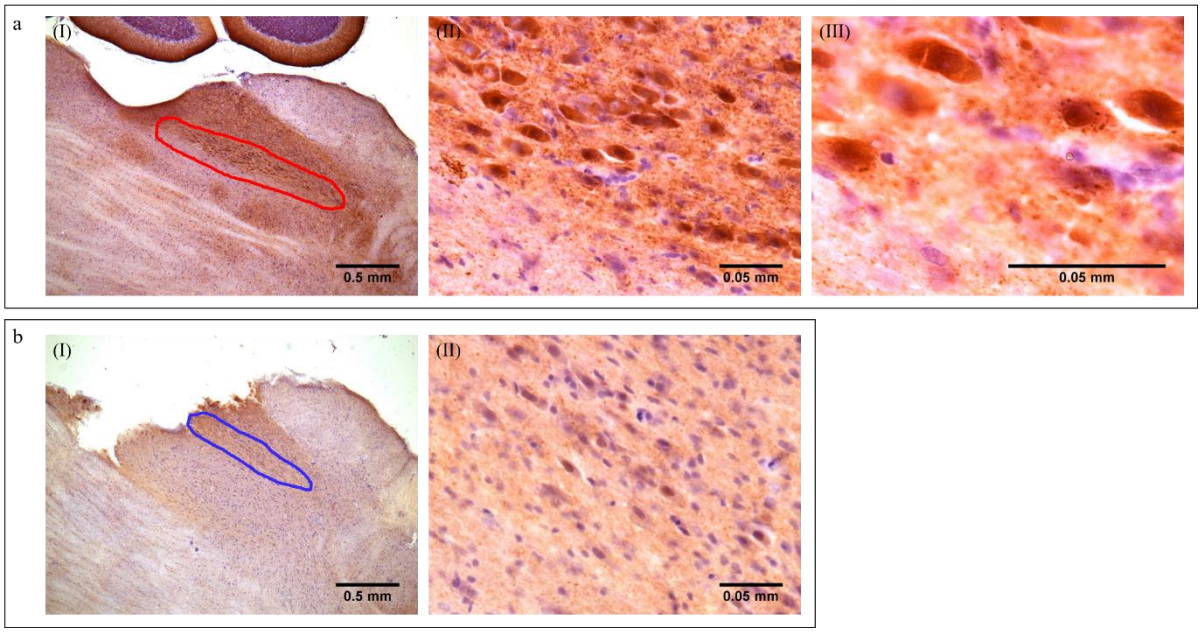


Figure 2.5 α-synuclein-42 staining in the vagus nerve nucleus. *Representative images of α-synuclein staining in the vagus nerve nucleus (outlined) in* ***(a)*** *control (red outline) and* ***(b)*** *PFF-only (blue outline) rats at* ***(I)*** *4X,* ***(II)*** *40X, and* ***(III)*** *100X magnification.*

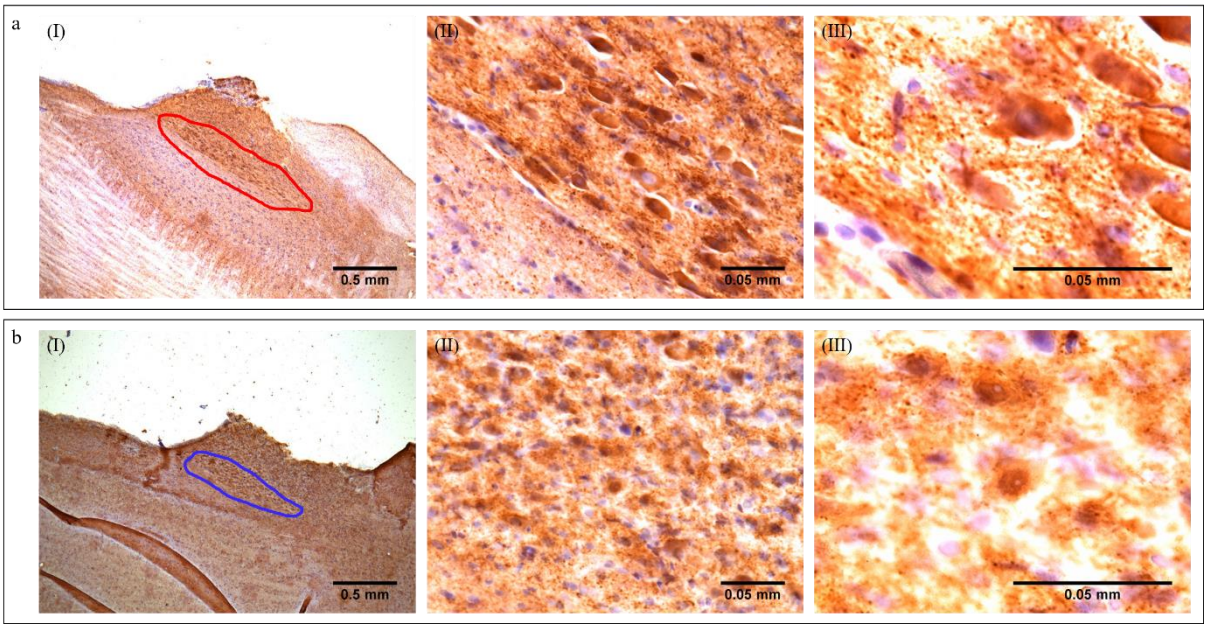


Figure 2.6 α-synuclein-2A7 staining in the vagus nerve nucleus. *Representative images of α-synuclein staining in the vagus nerve nucleus (outlined) in* ***(a)*** *control (red outline) and* ***(b)*** *PFF-only (blue outline) rats at* ***(I)*** *4X,* ***(II)*** *40X, and* ***(III)*** *100X magnification.*

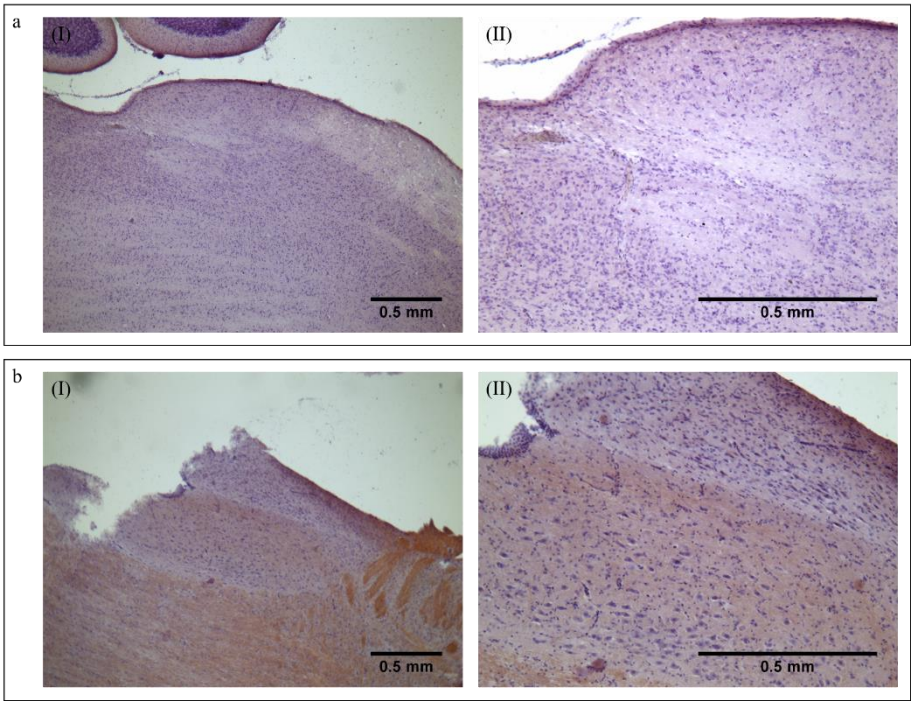


Figure 2.7 α-synuclein-211 staining in the vagus nerve nucleus. *Representative images of α-synuclein staining in the vagus nerve nucleus in* ***(a)*** *control and* ***(b)*** *PFF-only rats at* ***(I)*** *4X and* ***(II)*** *10X magnification.*

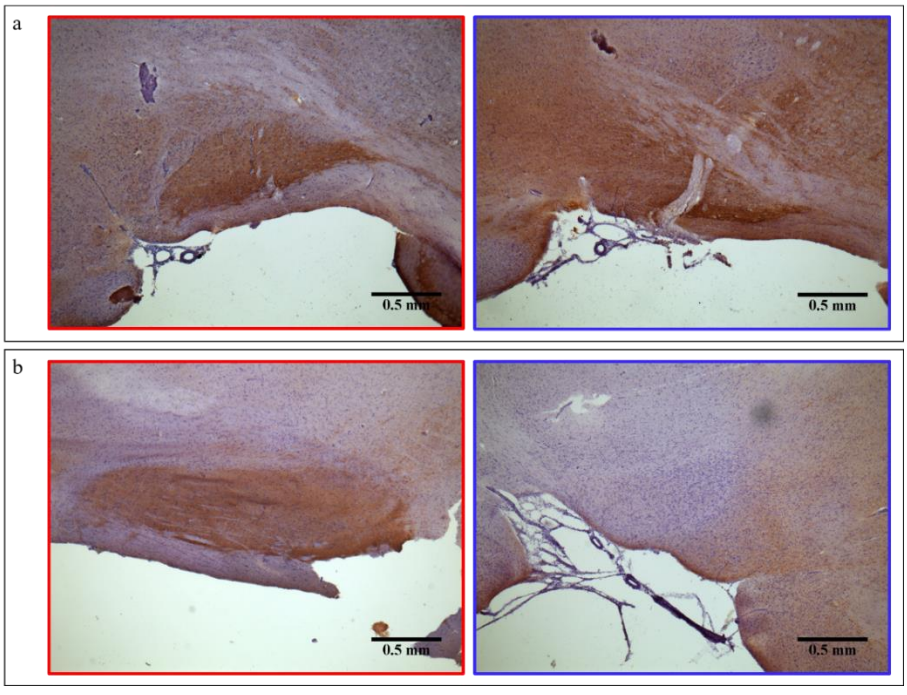


Figure 2.8 α-synuclein staining in the substantia nigra. *Representative images of* ***(a)*** *α-synuclein 2A7 and* ***(b)*** *α-synuclein -42 staining in the substantia nigra in control (left; red-outline) and PFF-only (right; blue-outline) rats at 4X magnification.*

2.4.3 Does candesartan treatment affect the development of olfactory deficits?

Behavioural tests were carried out to assess hyposmia, a clinical feature of PD. The behavioural test chosen was the buried food test whereby animals are tested on their ability to use olfactory cues to forage for a food pellet after overnight fasting. Previously published work using this animal model (Kuan et al., 2019) showed significantly increased retrieval latency – indicative of olfactory deficits – in the PFF-injected animals as compared to the controls. In the current study, there was a trend towards an increase in mean retrieval latency in the untreated PFF-injected animals which was not seen to the same extent in the candesartan-treated group, though this did not reach statistical significance (Friedman test; p=0.4612). (**Figure 2.9a**). Comparison between treatment groups at each individual timepoint did not show any significant differences between the untreated and drug-treated olfactory ability. (Multiple Mann-Whitney tests adjusted for False Discovery Rate). (**Figure 2.9b**). There appeared to be a greater proportion of animals that failed to find the pellet (as shown by a retrieval latency of 900 seconds which was the cut-off) in the untreated PFF-injected group as compared to the candesartan-treated group, though this does not reach significance (Fisher's exact test p=0.0877). (**Figure 2.9b**). This test was not repeated in the second control study due to time constraints.

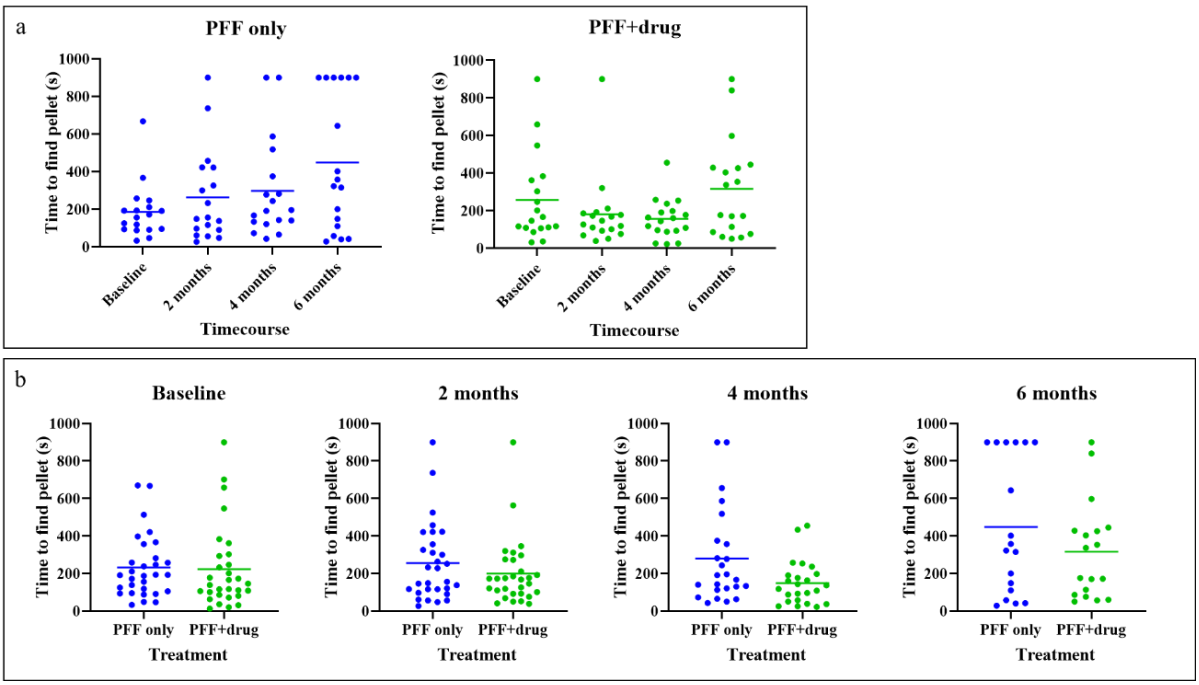


Figure 2.9 Timecourse of olfactory deficits. (a) *Retrieval latency over time in the treated and untreated group. Only animals surviving to the end of the study used, to allow for analysis over time. N =18 per treatment group per timepoint. Friedman test; p=0.4612.* ***(b)*** *Retrieval latency in candesartan treated versus untreated PFF-injected animals at each measurement timepoint. Baseline N=30 per group; 2m N=30 per group; 4m N=24 per group; 6m N=18 per group. Multiple Mann-Whitney tests adjusted for False Discovery Rate; Fisher's exact test comparing number of successes to number of failures; p=0.0877.*

2.4.4 Does candesartan treatment protect animals against motor deficits?

Previous work using this model used common motor behavioural tests such as the ladder climbing test and adjusted step test, however these tests did not show significant behavioural deficits (Kuan et al., 2019). I therefore used a theoretically more sensitive test to determine whether fine motor deficits developed in the PFF model over 6 months, and whether this was altered by candesartan treatment. The staircase test was used to determine fine motor ability in the form of reaching and grasping ability. Initial results showed that from the 2 month timepoint there was a significant difference in motor ability between the drug-treated PFF-injected animals compared to the untreated PFF-injected animals with the drug-treated animals having better motor ability (Mann-Whitney test; $p<0.05$). This significantly better motor ability was sustained at the 4 and 6 month timepoints (Mann-Whitney test; $p<0.01$). These differences between drug-treated and untreated were not seen in the parallel control study. (**Figure 2.10a-**

b). In addition, there was a trend towards decreased motor ability in the untreated PFF-injected group which was not seen in any other treatment groups. (**Figure 2.10c**). However, I also found that many of those animals unable to retrieve any pellets were heavier than the others. When tested it was found that weight significantly inversely correlated with motor ability in both the PFF-injected and saline-injected studies (simple linear regression; PFF $p=0.0014$, Control $p=0.0363$) (**Figure 2.11a**). The untreated PFF-injected animals weighed significantly more than the candesartan-treated animals (Unpaired T-test; $p<0.0001$) (**Figure 2.11b**). This occurred due to the weight-loss effects of candesartan (Müller-Fielitz et al., 2011). Due to these results, an ANCOVA was carried out to assess whether adding in weight as a covariance would remove the significance seen in the initial analysis. Significance did not withstand the ANCOVA; this indicates that the beneficial effect of candesartan on motor ability might be mediated through its effect on weight rather than a protective effect on the development of pathology in the disease model.

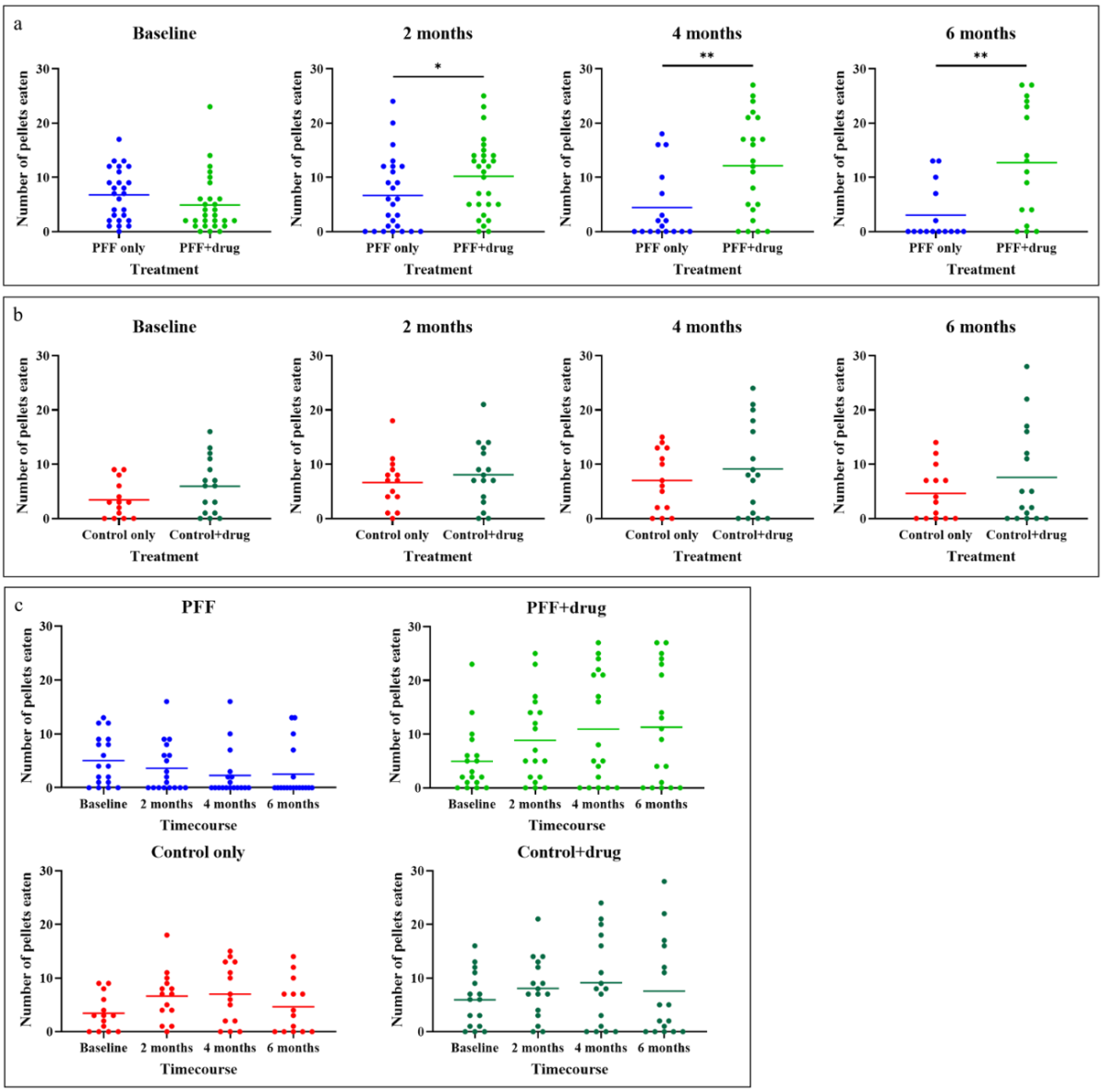


Figure 2.10 Timecourse of motor deficits. *Motor ability as determined by number of pellets eaten in untreated vs drug-treated* ***(a)*** *PFF-injected animals and* ***(b)*** *saline injected control animals at each measured timepoint. Mann-Whitney test; p*<0.05; p**<0.01.* ***(c)*** *Timecourse of motor ability in each individual treatment group.*

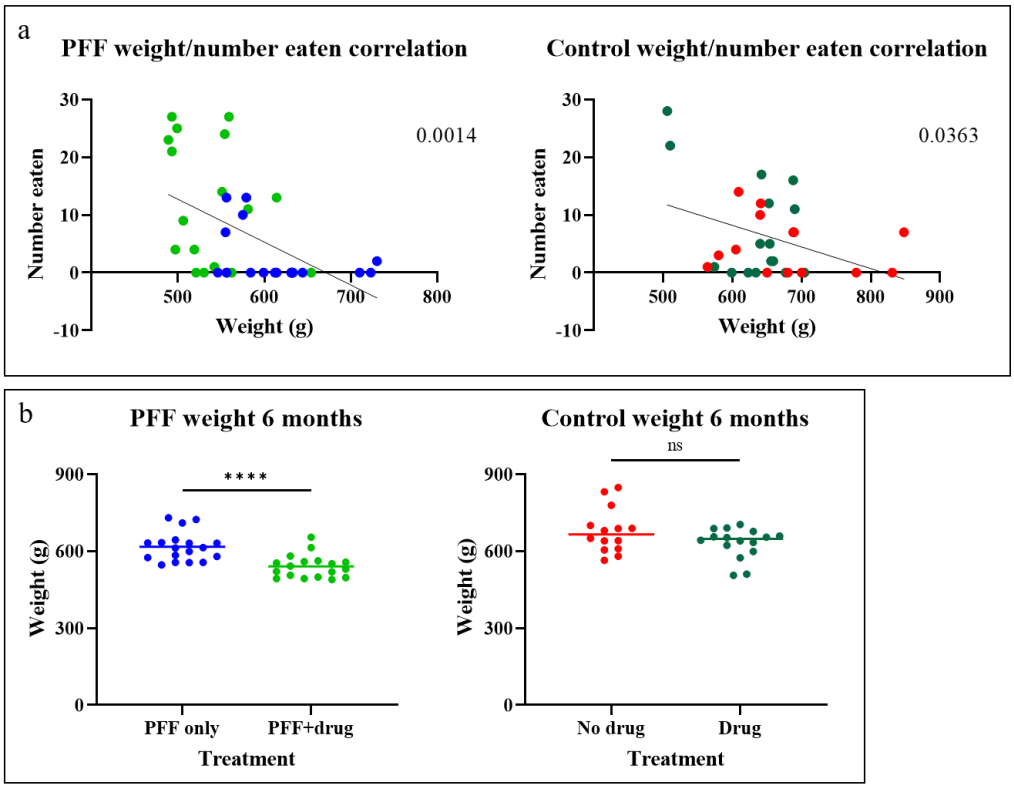


Figure 2.11 Weight as a confounding variable. (a) *Correlation between animal weight and number of pellets eaten in PFF-injected animals (left)(p=0.0014) and control saline-injected animals (right)(p=0.0363). Simple linear regression.* ***(b)*** *Comparison between animal weights in untreated and drug treated PFF-injected (left) and saline injected control (right) animals. Unpaired T-test; p****<0.0001. Mann-Whitney test.*

2.4.5 Does treatment with candesartan affect the survival of cholinergic neurons?

The vagus nerve nucleus is an easily identifiable region of interest within the brainstem containing cholinergic neurons which degenerates early in PD. According to Braak's hypothesis, Parkinson's pathology begins in the brainstem (Braak et al., 2003), and as such the vagus nerve nucleus was chosen as a key region to examine for evidence of neurodegeneration. This analysis was carried out in the majority of animals, though not all due to the lack of useable tissue in a small number of animals. The untreated PFF-injected rats showed significant degeneration of cholinergic neurons in the vagus nerve nucleus over 6 months post PFF-injection (One-way ANOVA followed by Tukey's posthoc test; $p<0.05$). The candesartan-treated PFF-injected rats did not show degeneration over the same period of time, indicative of a protective effect of candesartan. (**Figure 2.12a**). Due to differences in study design it is not

known whether degeneration over time was also seen in either of the saline-injected control groups (control animals were only culled at the 6 month timepoint). Candesartan treatment protected cholinergic neurons in this brain region in the PFF-injected rats as evidenced by a higher density of cholinergic neurons in the candesartan-treated PFF-injected rats at 6 months as compared to the untreated PFF-injected rats (Mann-Whitney test; $p<0.01$). No such difference was observed between the untreated and candesartan-treated saline-injected rats. (**Figure 2.12b-c**). The degeneration of cholinergic neurons was not found to correlate to behavioural tests (Simple linear regression $p=0.8584$). (**Figure 2.12d**). This suggests a disease-state specific protective effect. The degeneration of cholinergic neurons in the PFF-injected rats (One-way ANOVA followed by Tukey's posthoc test; $p<0.05$) and the protective effect of candesartan treatment on cholinergic neuron survival (Unpaired T-test; $p<0.01$) was similarly seen in the hypoglossal nerve nucleus. (**Figure 2.13**). This region has not been established as being pathologically relevant in human PD but is located directly adjacent to the vagus nerve nucleus.

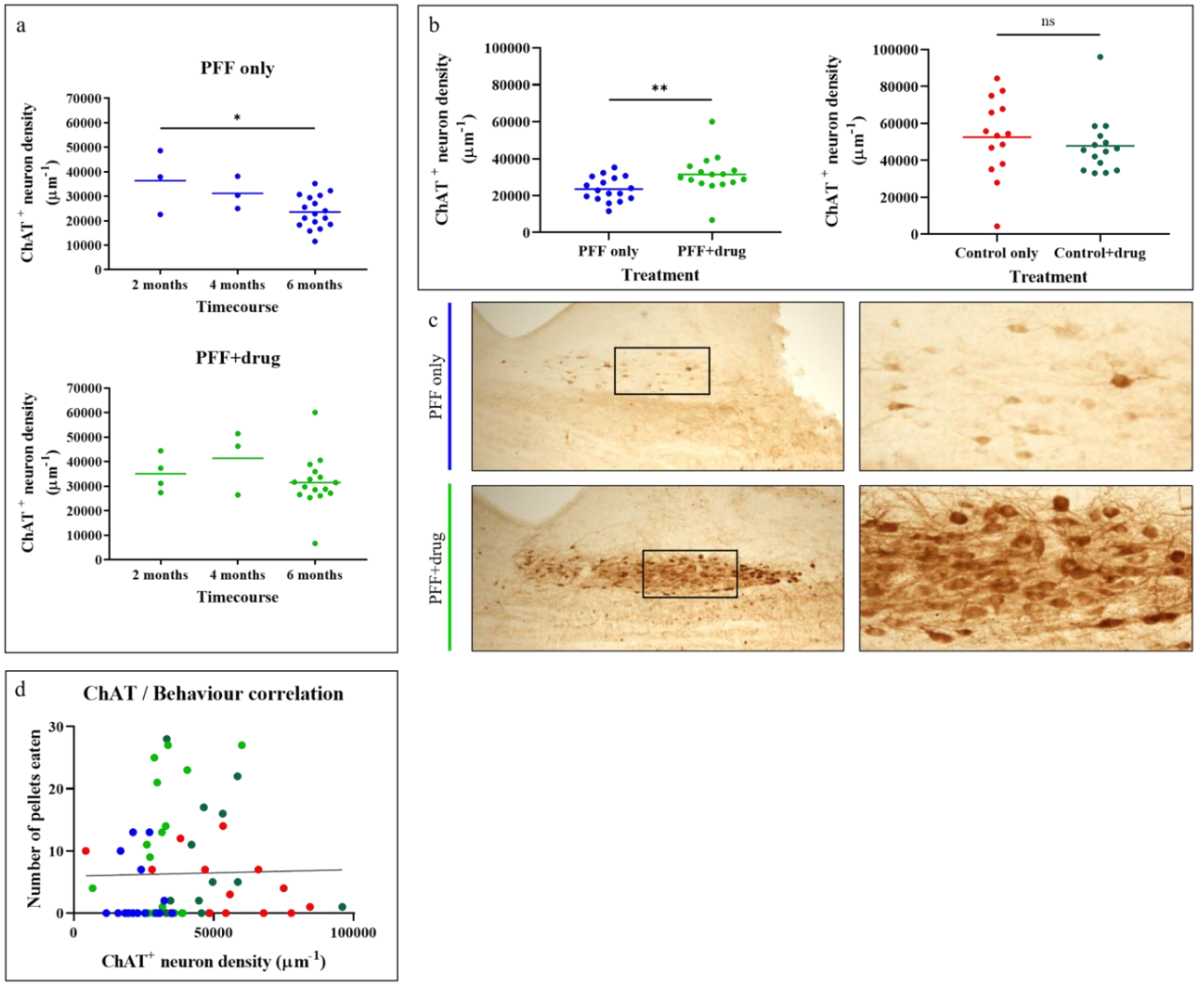


Figure 2.12 Candesartan treatment has a protective effect on cholinergic neuron survival in the vagus nerve nucleus. (a) *Timecourse of cholinergic neuron ($ChAT^+$) density in the vagus nerve nucleus of untreated (top; n=3 at 2m, 3 at 4m, 17 at 6m) and drug-treated (bottom; n=4 at 2m, 3 at 4m, 16 at 6m) PFF-injected animals. One-way ANOVA followed by Tukey's posthoc test; p*<0.05.* ***(b)*** *Cholinergic neuron density in the vagus nerve nucleus of untreated and drug-treated PFF-injected animals (left; n=17 and 16 respectively) and saline injected control animals (right; n=14 and 16 respectively). Mann-Whitney test; p**<0.01.* ***(c)*** *Representative images of the vagus nerve nucleus stained with anti-ChAT antibody in an untreated PFF-injected animal (top) and a drug-treated animal (bottom).* ***(d)*** *Correlation between cholinergic neuron density and number of pellets eaten indicative of motor ability. Simple linear regression.*

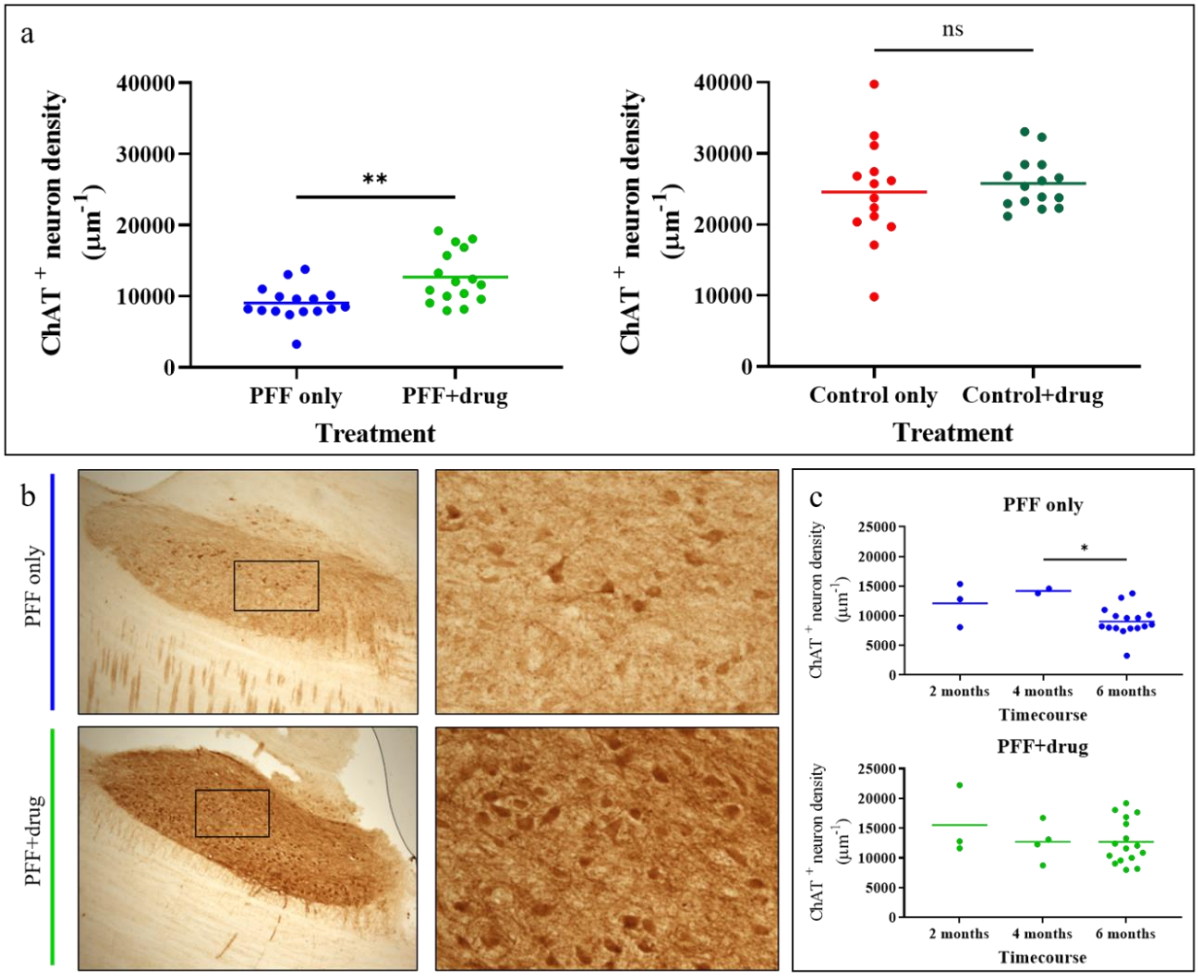


Figure 2.13 Candesartan treatment has a protective effect on the survival of cholinergic neurons in the hypoglossal nucleus. ***(a)*** *Cholinergic neuron density in the hypoglossal nucleus of untreated and drug-treated PFF-injected animals (left; n=16 for each group) and saline-injected control animals (right; n=14 and 15 respectively). Unpaired T-test; p**<0.01.* ***(b)*** *Representative images of the hypoglossal nerve nucleus stained with anti-ChAT antibody in an untreated (top) and drug-treated (bottom) PFF-injected animal.* ***(c)*** *Timecourse of cholinergic (ChAT$^+$) density in the hypoglossal nerve nucleus of untreated (drug; n=3 at 2m, 2 at 4m, 16 at 6m) and drug-treated (bottom; n=3 at 2m, 4 at 4m, 16 at 6m) PFF-injected animals. One-way ANOVA followed by Tukey's posthoc test; p*<0.05.*

2.4.6 Does candesartan treatment have an effect on dopaminergic degeneration in the substantia nigra?

The substantia nigra is a key pathological site in the Parkinson's brain, making it an important region to identify the degeneration of dopaminergic neurons. This degeneration was identified in the Kuan et al., (2019) paper, however it was harder to characterise in my study due to the lack of controls to directly compare to. Surprisingly, no degeneration of the

dopaminergic neurons in the substantia nigra was seen over time, and the average number of neurons remained relatively constant regardless of timepoint or treatment group. (**Figure 2.14a**). Though no significant differences were seen between treatment groups, within these groups there was a large spread of neuron number. (**Figure 2.14b-c**). I hypothesised that degeneration was only occurring in specific animals. To test this, relationships between dopaminergic neuron number and other pathological and behavioural measures were explored. No correlations were found between the number of dopaminergic neurons and either motor deficits, olfactory dysfunction, or cholinergic neuron density. (**Figure 2.14d**). This demonstrates that the number of dopaminergic neurons is not indicative of whether other pathology is progressing in the model.

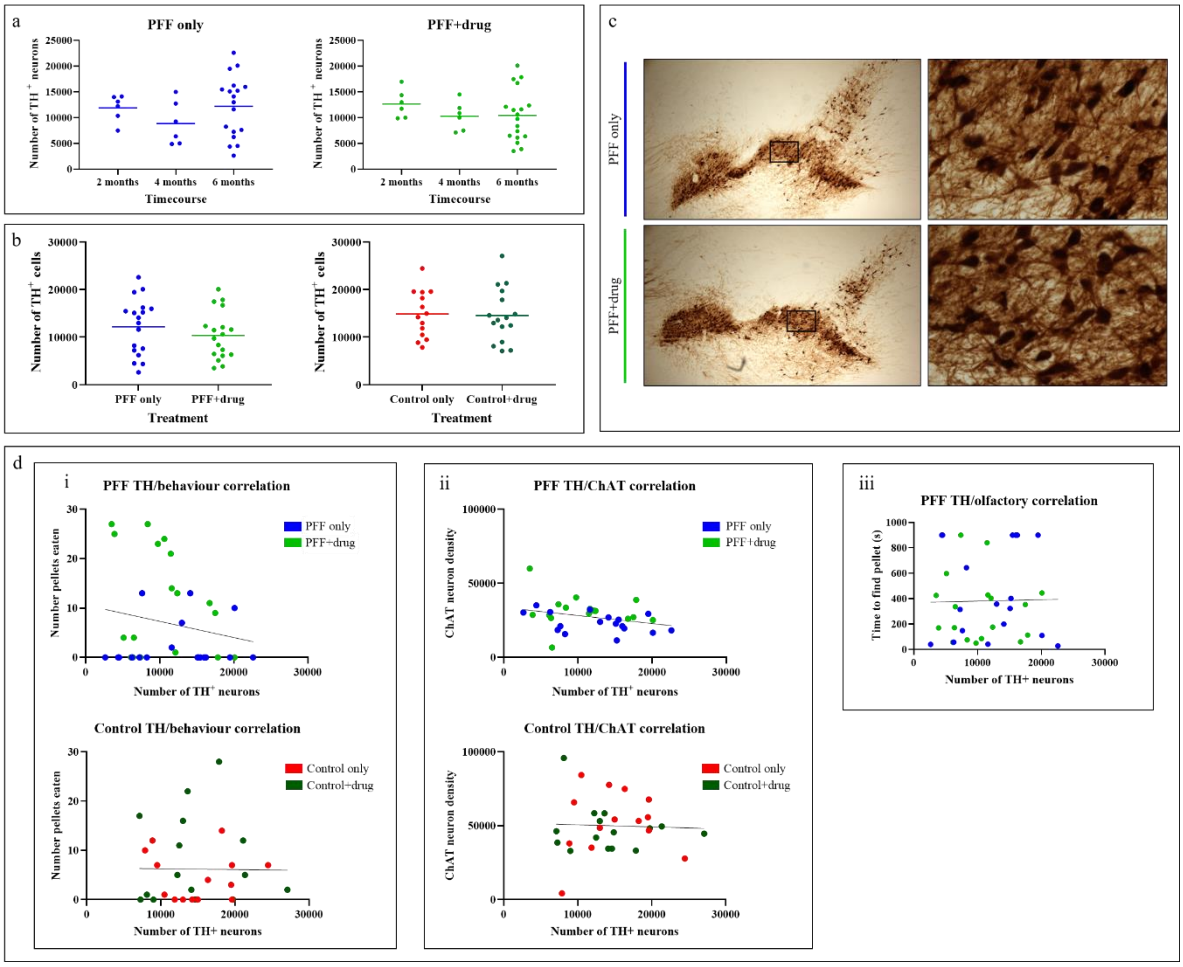


Figure 2.14 Dopaminergic degeneration in the substantia nigra is not occurring in this work despite degeneration being seen in the original Kuan model. ***(a)*** *Timecourse of number of dopaminergic (TH^+) neurons in the substantia nigra of untreated animals (left; n=6 at 2m, 6 at 4m, 18 at 6m) and drug-treated animals (right; n=6 at 2m, 6 at 4m, 18 at 6m).* ***(b)*** *Number of dopaminergic neurons in the substantia nigra of untreated and drug-treated PFF-injected animals (left; n=18 per group) and saline-injected control animals (right; n=14 and 16 respectively).* ***(c)*** *Representative images of substantia nigra stained with anti-TH antibody in an untreated (top) and drug-treated (bottom) animal.* ***(d)*** *Correlation between* ***(i)*** *number of dopaminergic neurons and motor ability,* ***(ii)*** *cholinergic neuron density, or* ***(iii)*** *olfactory ability. Simple linear regression; p>0.05 for all.*

Determining fibre density in the striatum is another way to assess dysfunction in dopaminergic tracts, and may be a better reflection of early dopaminergic dysfunction. Immunofluorescence staining of TH-positive nerve fibres was carried out in the striatum. No significant differences were seen between untreated and candesartan-treated animals in the PFF-injected group which is where the differences were expected. Surprisingly a significant

difference was observed between the untreated and candesartan treated control animals with the drug-treated animals showing higher levels of TH^+ fibres in the striatum. (**Figure 2.15a**). There was no correlation between striatal density and TH neuron number, so not supporting the theory that the lack of dopaminergic neuron degeneration is due to the degeneration being yet to spread along the dopaminergic tracts. (**Figure 2.15b**). There was also no correlation between striatal fibre density and motor deficits, providing further evidence that the motor effects were not due to a dopaminergic deficit in the PFF-injected animals. (**Figure 2.15c**).

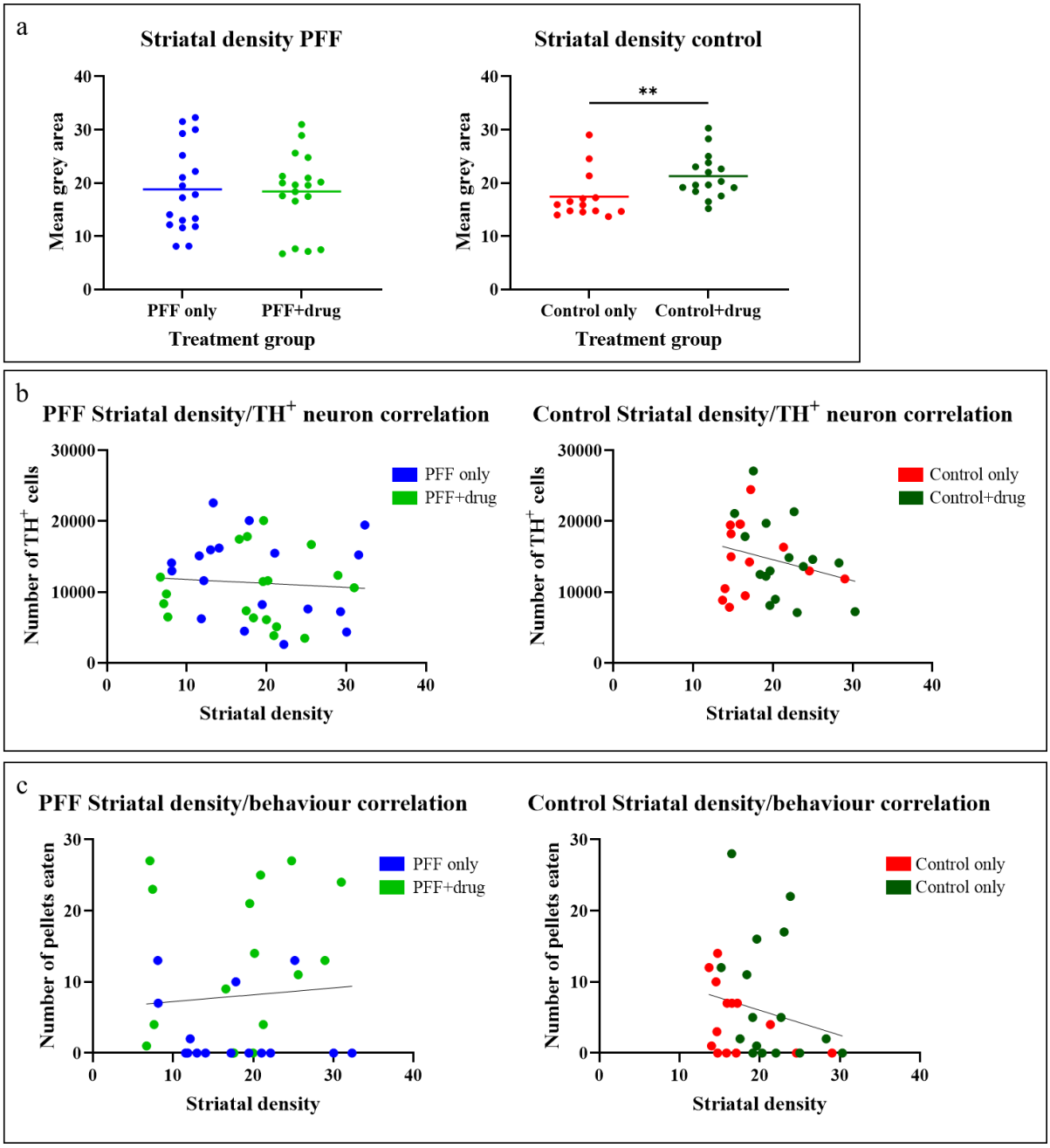


Figure 2.15 No effect of candesartan on striatal dopaminergic fibre density. (a) *Striatal fibre density (as determined by mean grey area of IF images) of untreated and drug-treated PFF-injected animals (left) and saline injected control animals (right) Mann-Whitney test; p**<0.01. Correlation between striatal density and* ***(b)*** *number of dopaminergic (TH^+) neurons or* ***(c)*** *number of pellets eaten in staircase behavioural test in PFF-injected (left) or control (right). Simple linear regression; p>0.05 in all. PFF only N=18; PFF+drug N=18; Control only N=14; Control+drug N=16.*

2.5 Discussion

2.5.1 Summary

In this chapter I sought to identify how long-term treatment with candesartan affected the development of pathology in a slow-progressing model of α-synucleinopathy. The results suggest that candesartan has a protective effect on the survival of cholinergic neurons in both the vagus nerve nucleus (an area seen to degenerate in human PD) and the hypoglossal nucleus (not seen to degenerate in human PD but found directly adjacent to the vagus nerve nucleus). A protective effect is also seen on motor ability, however this effect could be due to weight differences rather than an improvement in dexterity. Furthermore, a trend towards a candesartan-mediated protective effect on olfactory ability was seen but did not reach significance. There was a lack of dopaminergic degeneration in the substantia nigra, possibly due to the fact that this model represented an early (prodromal) disease stage and so the effect of candesartan on dopaminergic pathology could not be adequately tested. However overall, these results show a trend towards a protective effect of candesartan in specific (but not all) areas of disease progression in this model, particularly on the progression of cholinergic degeneration in the brainstem.

The protective effects of candesartan demonstrated in this study using a peripherally-initiated animal model of early PD provide a novel angle on the TLR-blockage as a protective agent hypothesis. The model used is subtle, similarly to the early stages of PD, and has meant that the protective effects of candesartan treatment, though present, are also subtle. The most important result from this study is the protective effect of candesartan on the survival of cholinergic neurons in the vagus nerve nucleus. According to Braak's hypothesis neuronal Lewy bodies first appear in the dorsal motor vagal nucleus in the brainstem before spreading to other brain regions including the substantia nigra in the midbrain. I have not been successful in quantifying α-synuclein levels in specific brain regions, however I have identified specific areas of neurodegeneration. The degeneration of cholinergic neurons in the vagus nerve nucleus of the brainstem, according to Braak's hypothesis, could be the first indication of pathology in the rat α-synucleinopathy model. In this way, this model forms a good

representation of early PD before neurodegeneration has spread elsewhere in the brain, such as to the substantia nigra. This model also provides compelling evidence for PD to begin peripherally, as the peripheral injection of α-synuclein PFFs results in the initiation of brain degeneration in the same brain region as suggested by Braak.

Nevertheless, although the results from this work alone are not sufficient for the immediate transferral to the human disease through the repurposing of candesartan into a clinical trial, there is sufficient data for making candesartan a clear target for further study.

2.5.2 Lack of dopaminergic degeneration

The most obvious limitation with my study is that although the rats developed cholinergic degeneration as expected, they did not develop dopaminergic degeneration in the substantia nigra. Alongside α-synuclein inclusions, dopaminergic degeneration is the most important aspect of a model of PD; these are the two main pathophysiological characteristics of the disease. There are several possible reasons for the lack of development of a dopaminergic deficit in the model which are discussed below.

2.5.2.1 Slow development of neurodegeneration

As mentioned above, this model is a slowly developing model making it a good model for PD which is a slowly progressing disease. According to Braak's hypothesis, Parkinson's pathology begins in the dorsal motor nucleus of the vagus before spreading to other regions of the brain (Braak et al., 2003). This may explain why neurodegeneration is seen in the brainstem in this study, but not in the substantia nigra – i.e. pathology is yet to spread to the nigra. Dopaminergic degeneration was observed in the Kuan et al.,(2019) study after 6 months, and this continues to develop further post 6 months as was shown in an extended study (Kuan et al., 2022). There are possible sex differences in progression rates in PD, and it is possible that the males rats used in this study showed a slower rate of progression than the females in the Kuan study (Cerri et al., 2019; Iwaki et al., 2021). If left for longer than 6 months, dopaminergic pathology may have developed in the nigra in my study. This feature of the model used in this study may actually be a positive. As discussed in ***Chapter 1.1.2***, at the point of diagnosis – generally occurring on the development of motor features – neuron loss in the SNpc is at around 50% (Marsden, 1990; Ross et al., 2004). PD is thought to initiate many years prior to diagnosis, and thus the model used in this study – where α-synuclein pathology is present and

degeneration seen in the vagus nerve nucleus but little to no degeneration in the SN – may be a good representation of the earliest disease processes.

2.5.2.2 Model mechanism

As described earlier, the mechanism through which this model is produced utilizes the rabies virus glycoprotein RVG9R peptide to transport the conjugated PFFs transvascularly into the brain. This peptide works through its binding specifically to neural acetylcholine receptors; these receptors are found on cholinergic neurons. As a result, the PFFs may be entering cholinergic neurons through this mechanism, inducing α-synuclein aggregation, and resulting in the cholinergic degeneration. Acetylcholine receptors are not found on dopaminergic neurons. As a result, the RVG9R-conjugated PFFs will not be directly entering the dopaminergic neurons after injection, however they may spread through the brain from their origins in the cholinergic neurons. The underlying mechisms for this spread are a key field of interest in Parkinson's research and theories include their being spread through anatomical circuits (Braak et al., 2003), through α-synuclein acting in a prion-like manner (Olanow and Prusiner, 2009), or through transfer via the actions of glial cells (Xia et al., 2019). The lack of dopaminergic degeneration in my work may be a result of the failure of the PFFs, or the PFF-induced aggregated α-synuclein, spreading to other areas of the brain. The spread of RVG-conjugated α-synuclein and of RVG-conjugated GFP wa shown to occur in work by Kuan et al., (2019). In this work it was identified in the cortex, hippocampus, thalamus, and nigra. However, as discussed, there are sufficient differences between the work carried out in this chapter and the work carried out by Kuan that it cannot be assumed that this spread of α-synuclein from cholinergic neurons in the brainstem to other neuron types and other areas of the brain is occurring in the animals used in my work. If the spread of α-synuclein has failed then this could explain why degeneration is only seen in cholinergic neurons.

2.5.2.3 Sex differences

The main difference between the study carried out here and the original study carried out by Kuan et al., (2019) was the sex difference between the animals. The original study used female animals whereas we used male animals. Male rats were used in keeping with the majority of animal studies due to concerns about hormonal changes having a neuroprotective effect (Liu and Dluzen, 2007). PD is known to be more prevalent in males as opposed to females with a ratio of approximately 2:1 (Wooten et al., 2004). There is also data showing that the immune response in PD patients may differ between males and females, which – as

suggested by our overall hypothesis – could alter the development of the disease in different sexes (Nissen et al., 2019). It has been suggested that the decrease in Parkinson's prevalence in females might occur due to the protective effect of oestrogen on dopaminergic neuron survival. Evidence for this arises from toxin-induced Parkinsonism where females seem to experience a protective effect on dopaminergic degeneration in response to exposure to various environmental toxins. This is seen in both animal models (including rotenone and paraquat neurotoxin models) and in human epidemiological studies (investigating the link between PD risk and occupation where men and woman hold equivalent occupations) (Adamson et al., 2022; Liu and Dluzen, 2007). However, though evidence strongly supports differences in propensity to develop Parkinson's between males and females, this doesn't help to explain the differences seen between the Kuan study and our study. Based on the above evidence, it would be expected that the male animals used in our study would experience higher levels of dopaminergic degeneration due to a lack of oestrogen, however this is not the case. Consequently, this does not explain the lack of degeneration seen in this study.

2.5.2.4 Weight differences

The method used for dosing the animals was crushing candesartan tablets and mixing them with chocolate spread. The control for this was chocolate spread on its own. Dosing was carried out in this way as a preliminary study showed that when the drug was crushed and mixed with water the rats would not drink the water and so were not getting the correct dose of candesartan. The method of dosing with chocolate spread was very effective in terms of ease of dosing as the rats were very keen to eat it. However, this was ultimately detrimental to the study as after 6 months of eating 0.6 ml of chocolate spread every day the rats gained weight. This was particularly problematic as the weight was not gained equally between the treatment groups – candesartan is known to cause weight loss and it is likely that this counteracted the weight gain from the chocolate spread in these treatment groups (Müller-Fielitz et al., 2011). This created a confounding effect in the motor behaviour test as weight correlated negatively with motor ability. The differences between the candesartan-treated and untreated PFF-injected groups in terms of motor ability no longer reached significance when weight was added as a covariate. Differences in weight and fat content could also be relevant to the lack of dopaminergic degeneration in this study, when compared to Kuan's study.

A link between a high fat diet or increased weight and dopaminergic degeneration has been suggested, however available evidence indicates that high fat/weight is associated with

increased dopaminergic degeneration, in contrast to our findings. The phosphatase PP2A is one of the only phosphatases that dephosphorylates tyrosine hydroxylase (the enzyme which converts L-tyrosine to L-Dopa – the first step in the synthesis of dopamine in dopaminergic neurons) (Nagatsu, 1995). Through its dephosphorylation of TH, PP2A acts to reduce TH activity (Zhang et al., 2007). PP2A shows increased expression after exposure to a high fat diet (Jun et al., 2008), hence the high fat diet in our study might be expected to result in increased expression of PP2A, and the subsequent dephosphorylation and decrease in activity of TH. Further evidence for this arises from **Supplementary Figure 2.1** where a western blot was carried out to determine the levels of TH in the midbrain. This was carried out in a selection of animals from this study, and a selection of animals from the published Kuan et al., (2019) study. The blot showed that the TH in the animals used in this study had undergone post-translational modifications – potentially the dephosphorylation discussed above – which have resulted in bands of a lower molecular weight than that seen in the animals from the Kuan et al., (2019) study which did not receive a high fat diet. This could indicate a potential increase in PP2A. This does not explain the lack of degeneration seen over time in our study, as it would be expected that TH levels would be lower in my study compared to the Kuan et al., (2019) study due to the effect of a high fat diet-induced rise in PP2A. However, it could be the case that this high fat diet rapidly effects the downregulation of TH meaning that by the 2 month timepoint TH is already downregulated. Consequently, it might not have been possible to detect further dopaminergic neurodegeneration over time in my study. Unfortunately, due to a lack of any baseline post-mortem rat brains I was unable to confirm this. This may also explain the lack of significant difference in dopaminergic neuron number between the candesartan-treated and untreated animals, as even though a difference in weight was seen, the amount of fat quantity in their diet is the same.

Finally, weight may have an effect on dopaminergic neurodegeneration due to differences in insulin signalling. Insulin is thought to act in a protective manner towards neuron survival. This has been shown to occur in various types of neurons and against various toxic insults (Ribeiro et al., 2014; Sun et al., 2010). Evidence points to this protective effect also occurring in dopaminergic neurons in an insulin dose-dependent manner through reduction of reactive oxygen species, nitric oxide release, and calcium ion influx (Pang et al., 2016; Ramalingam and Kim, 2016). Evidence for the neuroprotective effect further occurs through the link between type-2 diabetes-mediated insulin resistance and the development of PD (Reviewed in Athauda and Foltynie, 2016). Could it be possible that a temporary rise in insulin

on the addition of daily chocolate spread to the diets of the animals used in this study could be having a protective effect on dopaminergic neuron survival in the substantia nigra, and so negating any detrimental effects of PFF-injection? Though possible, this is unlikely. A high fat diet is strongly associated with insulin resistance (Hancock et al., 2008). Various studies suggest this would make a subject more prone to developing PD (Hu et al., 2007; Xu et al., 2011), the progression of disease would be faster (Cereda et al., 2012; Kotagal et al., 2013), and the dopaminergic neuron loss would be greater (Choi et al., 2005; Morris et al., 2011, 2010). This is not what is seen in our study (with a high fat diet) compared to the Kuan study (with a normal diet). In addition, insulin is a very transient hormone, so to measure levels of insulin and assess how this may be linked to the survival of dopaminergic neurons in our model would require more than the post-mortem tissue we have available, so making this unfeasible to test.

2.5.3 Lack of α-synuclein pathology

In work carried out by Kuan et al., (2022) using this RVG9R:PFF model, α-synuclein pathology was suggested to be diffuse throughout the brain. They did not carry out immunohistochemistry identifying any aggregates in this paper, and instead demonstrated the presence of high molecular weight α-synuclein using immunoprecipitation. Immunohistochemistry was used in the earlier Kuan et al., 2019 paper and showed α-synuclein aggregates through pFTAA and Ser-129 co-staining – though this is not conclusive evidence of α-synuclein aggregates as pFTAA recognises any oligomeric and fibrillar proteins and is not specific to α-synuclein. I similarly found high molecular weight α-synuclein species in the cortex of my PFF-injected rats using immunoprecipitation. However, when using immunohistochemistry for α-synuclein, there was no clear evidence of α-synuclein aggregates and no differences in α-synuclein staining in the brainstem or substantia nigra between the PFF-injected and saline-injected control animals.The lack of any α-synuclein staining in the substantia nigra in the PFF-injected animals could explain the lack of dopaminergic neurodegeneration seen in the PFF-injected animals, and therefore why I couldn’t show a protective effect of candesartan on dopaminergic neurodegeneration (as there is no deficit which candesartan could act upon). The lack of differences in α-synuclein staining between the PFF-injected and control animals in the brainstem counfounds the assumption that the cholinergic degeneration seen in the PFF-injected animals was due to α-synuclein pathology.

However, the α-synuclein staining in the control animals was unexpectantly high, hence non-specific staining may have affected the interpretability of the results.

A potential explanation for these unexpected results could be that the PFF-injected animal brain sections are 2 years older than the control animal brain sections. Though these were stored appropriately, it could be that the age of the sections has resulted in their degradation and so are less able to be effectively stained. The staining level in different animals may also have been influenced by anatomical differences in the exact area of the brain being compared as it is not possible to select sections through the brain which cut through the region of interest in exactly the same way in two different animals. This could result in the section from the control animal featuring a more central section through the vagus nerve nucleus whereas the section from the PFF animal could only be catching the edge of the nucleus. To test this co-staining would have to be carried out with an anti-ChAT antibody. The use of further antibodies identifying phosphorylated α-synuclein may also have been useful to look for pathological α-synuclein aggregates. Unfortunately attempts to do this using an anti-Serine-129 antibody did not produce any useable images, and there was not sufficient time to replicate this work. It is also a possibility that neurotoxicity in this model is being driven by small oligomeric α-synuclein species rather than larger aggregated species. These are not detectable using standard immunohistochemistry staining, and instead would require a much higher sensitivity imaging process such as that used by Emin et al., (2022). This work uses super resolution imaging techniques to image oligomeric α-synuclein, however homogenised or soaked brain tissue samples are required for this and as a result it is not possible to image these species in precise brain regions.

These explanations require further work to establish the true cause of the lack of α-synuclein staining seen in this model. Due to these results, I am unable to conclusively state that the cholinergic degeneration seen in the animals used in this study is due to α-synuclein.

2.5.4 Lack of olfactory deficits

The original study carried out by Kuan et al., (2019) showed olfactory deficits in PFF-injected animals after 6 months which were not seen in the control animals. This was less significant in our study, though a trend towards a deficit in the untreated PFF-animals was seen. As mentioned above, the sex of the animals used in this study was different to those used in the Kuan study. Sex may also underlie this difference in olfactory ability. In humans, women

are known to show higher olfactory sensitivity than men, and similarly this is thought to be the case in most mammals, including rodents (Kass et al., 2017; Sorokowski et al., 2019). In human PD there are sex-dependent differences in the development of non-motor symptoms including hyposmia. One study looking at 951 patients found that a significantly higher percentage of female PD patients reported loss of taste/smell than male PD patients (Martinez-Martin et al., 2012). Hence tests of olfaction may be less sensitive in male rats because of inherent differences in olfactory ability, or pathological differences in the male versus female disease model. The potential differences in olfaction in the male and female α-synuclein rats would require further work in order to be clarified, possibly requiring a more sensitive olfactory test.

Weight gain could also have affected the rats' ability to perform in this behavioural test. The paradigm of this test requires starvation overnight to ensure that animals are keen to find food. Dosing with chocolate spread was carried out immediately prior to starvation (as this was the time when dosing was carried out every day). It is possible that due to the high fat content nature of the chocolate spread, starvation was not as effective, resulting in the animals being less motivated to find the pellet than they would otherwise have been. It also may hold that the allure of the Coco pop food pellet was less to these animals as they were accustomed to the smell and taste of chocolate spread. Furthermore, it is also possible that the weight of the animals made them less inclined or less generally able to move, which again would disincentivise them to retrieve the pellet.

2.5.5 Parallel studies

A further limitation with all of these experiments is that it was not possible to directly compare data from the PFF-injected animals with the saline-injected control animals because of subtle differences in study design and timing – the animals were a slightly different age, were housed in a different manner (fewer rats per cage), and the timings of the post-mortem work carried out were different. In retrospect, incorporation of larger treated and untreated saline control arms in the original study design would have been useful to allow confirmation of whether degeneration has occurred in brain regions. Though this has been shown through timecourse comparison in the PFF groups, it would have also been beneficial to directly compare the numbers/density of neurons in non-PFF animals

2.5.6 The use of candesartan

In addition to its role as an AT2-receptor antagonist, which underlies its use as a treatment for hypertension, candesartan has been shown to inhibit TLRs 2 and 4 in human monocytes and *in vivo* in mice (Dasu et al., 2009). The relevance of its action as a TLR2/4 inhibitor is particularly key as these are the two TLRs most implicated in PD. Different researchers argue for one of either TLR2 or TLR4 to be most relevant to the development and/or progression of PD. An advantage of using candesartan in this project is that the reagent inhibits both TLRs 2 and 4, allowing me to look at the combined effect of inhibition. Furthermore, candesartan is a licensed drug for use in humans thus allowing the direct and rapid translation of this work to the human disease if the study findings were clearly positive, and the potential repurposing of this drug in a clinical trial in PD patients.

In my study, candesartan has successfully protected the survival of the cholinergic neurons in both the vagus nerve nucleus and the hypoglossal nucleus of PFF-injected animals. This appears to be disease-state specific due to the lack of any significant differences between the cholinergic neuron density in untreated and candesartan-treated saline-injected control animals. This specificity of the effect to the disease-state implies an α-synuclein-dependent mechanism. The mechanism of action of candesartan will be investigated in ***Chapter 3***. If our hypothesis is correct, then the mechanism is through candesartan decreasing the expression levels and/or the activation state of TLRs 2 and 4, resulting in an attenuated pro-inflammatory response to aggregated α-synuclein by microglia, and so not producing the neurotoxic cellular environment resulting from chronic inflammation. The relevance of the protection of cholinergic neurons within the vagus nerve nucleus is strong as this is thought to be one of the first regions to degenerate within the human PD brain according to Braak's hypothesis. The lack of a significant effect of candesartan in other brain regions is not necessarily conclusive to candesartan not having a protective effect, because this was likely due to the failure of the model to develop pathology in some key regions. For example, the model failed to result in neurodegeneration in the substantia nigra, and as a result candesartan had no significant effect on the number of dopaminergic neurons within this area. However, this does not exclude the possibility that had this degeneration occurred, candesartan would have had a protective effect on the survival of these neurons. Unfortunately this would require a second study to be carried out where dopaminergic degeneration occurred, and this was not within the scope of this PhD to carry out. As such, my data indicates that candesartan may be a useful protective agent

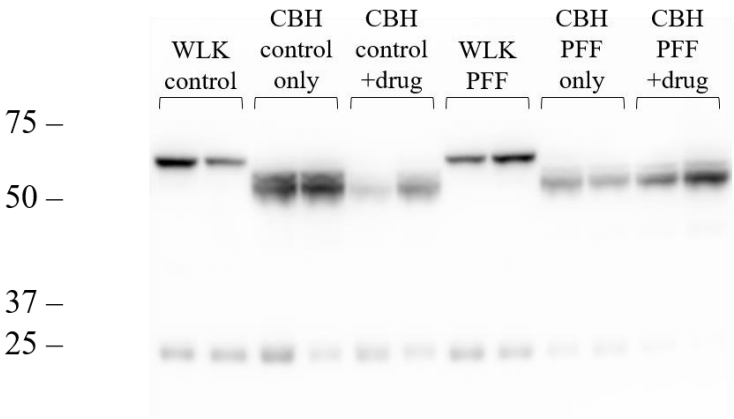
against the development of α-synucleinopathy but more work is required to confirm its impact on dopaminergic degeneration.

In terms of practicality/feasibility, candesartan was a good candidate for use in this study. It was easily obtainable from a pharmacy due to its already being a licensed drug. The production of the drug-chocolate spread mixture for dosing was easy to carry out, though fairly time-consuming as this had to be carried out on a daily basis for the treatment of a large number of animals. The dosing of the animals was very simple as the chocolate spread was very appealing to the rats and the vast majority consumed it without even having to be removed from the cage – that is to say, no gavage was needed, and each rat took less than a minute to dose. This was particularly advantageous as due to there being a large number of rats needing dosing on a daily basis for 6 months, having to gavage or even inject animals in this manner for this length of time would simply not have been feasible, and would potentially have been traumatic to the animals. In addition, the tolerability of this drug was clearly seen, as treatment was carried out for a large number of animals (46 drug-treated animals total across the two parallel studies) over 6 months with no adverse effects seen in any animal.

Though a clear protective effect of candesartan treatment was seen in the increased survival of cholinergic neurons in the vagus nerve nucleus as compared to the untreated animals, this difference was not extensive and did not encompass all animals. The rats used in this study are Sprague Dawleys which are an outbred strain, making them non-genetically identical. This will result in differential effects both of the development of pathology in the model, and in the effect of the drug on the development of pathology. This has potentially resulted in the range of densities of cholinergic neurons: the genetically different rats responding diversely to candesartan treatment. This range of response will also be present if applied to humans, to a much greater extent – pathology will be much more heterogeneous in humans and in addition they may have multiple comorbidity conditions, experience different environmental factors, and be taking other medication. The key question arising from this study is whether the protective effect of candesartan on cholinergic neuron survival provides sufficient evidence to support testing this agent in the human disease. It could be argued that the limits of the protective ability of candesartan on any other aspects of the model means that further work is required to support the repurposing of this drug in a clinical trial.

A further point of interest is whether candesartan dosed orally is actually successfully reaching the brain to carry out its inhibitory effects. This was proven using liquid chromatography mass spectrometry (LCMS) and will be discussed in ***Chapter 3.4.1***.

Another important consideration regarding candesartan use is that its effects are not limited to the inhibition of TLRs 2 and 4, and indeed its licensed function in the treatment of hypertension is through its inhibition of angiotensin II type 1 receptors. This inhibition of angiotensin has already been suggested as a method through which candesartan could be effective in the treatment of PD. For example, Rodriguez-Perez et al., (2018) published a study soon after I began my project, involving similar work with candesartan and other angiotensin type 1 receptor antagonists in an α-synuclein overexpression model produced through viral vector injection directly in the brain. Candesartan showed a protective effect on the development of pathology in this model in terms of protection against dopaminergic degeneration in the substantia nigra, against a loss of dopaminergic striatal terminals, and against a decline in motor ability – as determined through behavioural tests including the rotarod test. This work suggests that candesartan might be having a protective effect in our model through blocking angiotensin type 1 receptors rather than TLRs. In the next chapter, I will investigate potential mechanisms by which candesartan is exerting a protective effect on cholinergic neurodegeneration and behaviour.



Supplementary Figure 2.1 TH protein in my study shows post-translational modification not seen in Kuan model. *Western blot for TH protein using samples taken from the original Kuan study and from my own study.*

Chapter 3.

INVESTIGATING HOW CANDESARTAN IS HAVING A PROTECTIVE EFFECT ON THE PROGRESSION OF THE A-SYNUCLEINOPATHY RAT MODEL

DECLARATION

The single ion recording (SIR) method used in the liquid chromatography mass spectrometry (LCMS) experiments was produced by Dr Stephen Thompson (Department of Clinical Neurosciences, University of Cambridge). The samples were run through by me with the assistance of Dr Thompson.

Immunohistochemistry slide scanning was carried out by the Histopathology and ISH core facility at CRUK Cambridge.

3.1 Aims of chapter

The PFF rat model produced by Kuan et al., (2019) is novel in its recapitulation of early features of PD, and in its modelling the slow rate of progression of disease. This makes it ideal for the exploration of drug intervention and how this affects the early stages of disease development – which is highly relevant in terms of future therapies to delay disease onset – as presented in ***Chapter 2***. An unexplored feature of this model thus far is inflammation. *In vitro* work and many animal studies have demonstrated an inflammatory response to α-synuclein. The presence of aggregated α-synuclein in the brain of the PFF-injected animals has been demonstrated in the previous chapter and in the original publication by Kuan et al., (2019), however it has not previously been investigated whether this correlates with an increase in inflammation.

In the previous chapter it was found that daily candesartan treatment had a protective effect on the development of pathology in the PFF rat model of PD; the aim of the work presented in this chapter was to investigate the mechanistic basis of this effect, and in particular to determine whether candesartan suppresses inflammation in the rat brain via a TLR-dependent mechanism.

3.1.1 Objectives

1. To confirm that candesartan is crossing the blood-brain barrier, and for how long it is detectable within the brain.
2. To determine whether the peripheral injection of α-synuclein PFFs results in inflammation in the brain and/or periphery.
3. To determine the effect of candesartan treatment on levels of TLRs 2 and 4, and on inflammation in the α-synuclein PFF rat model.

3.2 Introduction

3.2.1 Neuroinflammation in Parkinson's disease

As discussed in ***Chapter 1***, neuroinflammation is thought to play an important role in the progression of Parkinson's disease. The immune response is initially beneficial for injured tissue through promoting the clearance of cell debris and the secretion of neurotrophic factors to enhance neuron survival (Gao and Hong, 2008; Kordower, 2003; Trapp et al., 2007). However, a persistent and uncontrolled inflammatory response will result in chronic inflammation and the extended release of pro-inflammatory cytokines, reactive oxygen species, and nitric oxide. These are neurotoxic and further exacerbate neuron damage (Gao and Hong, 2008). The proposed role of inflammation in the progression of PD provides a potential avenue for therapeutic intervention. I hypothesised that the protective effect of candesartan seen in ***Chapter 2*** was occurring through blocking the chronic α-synuclein-induced inflammation, specifically through TLRs. Candesartan is a candidate for drug repurposing and has been proposed to act via TLRs 2 and 4, although its mechanism of action as an anti-hypertensive is through AT_1 receptor blockade (as discussed in ***Chapter 1.4.3.1***).

3.2.2 Investigating inflammation in α-synuclein PFF model

As discussed in ***Chapter 1.3.1.3***, inflammation has been seen in various animal models of PD and it has been suggested that it plays a role in the development of pathology within these models, however the specific link between this inflammation and α-synuclein is not clear. It is thought that α-synuclein activates the immune system; this has been shown through *in vitro* work in addition to in α-synuclein overexpressing models. In these latter models high levels of inflammation are seen in response to α-synuclein, however the proportion of this inflammation due to the supraphysiological levels of α-synuclein is hard to determine. The PFF model works through PFFs acting as a template for the recruitment of soluble endogenous α-synuclein into insoluble Lewy body-like inclusions (Volpicelli-Daley et al., 2011). For this reason, the PFF model provides a better model to study inflammation in response to endogenous α-synuclein levels, as would be experienced in the human idiopathic Parkinson's

brain. The utilisation of the PFF model in understanding neuroinflammation in PD has been reviewed by Stoll and Sortwell., (2022).

The presence of inflammation in PFF models of PD is less well established than in more acute models, perhaps indicating that the inflammatory response to α-synuclein isn't as high when only endogenous levels of α-synuclein are present. An acute inflammatory reaction directly to the injected PFFs has been reported; this occurs in the brain at 1-3 days post-injection, prior to the induction of any phosphorylated α-synuclein inclusions (Harms et al., 2017; Karampetsou et al., 2017). It is possible that this early inflammation occurs due to surgical injections causing mild trauma to the site of injection, and not as a reaction to the foreign PFFs. The relevance of this early inflammation is unknown – it is possible that this initial inflammation immediately fades and plays no role in the further development of inflammation, alternatively, it may play a key role in model development and early inclusion formation. A potential mechanism may involve the priming of immune cells. Previous work has shown that pre-exposure of microglia to α-synuclein, or a different pro-inflammatory stimulant such as LPS, prior to a secondary stimulus such as a TLR agonist (Roodveldt et al., 2013), an endogenous factor such as neuromelanin (Wilms et al., 2003), a proinflammatory factor (Facci et al., 2014), an endotoxin (Purisai et al., 2007), or exposure to a pro-inflammatory stimulant in a pre-existing disease model (Cunningham et al., 2005; McColl et al., 2007) results in a higher pro-inflammatory response on secondary exposure. During the establishment of centrally-injected PFF animal models more chronic inflammation has also been identified. This includes an upregulation of MHCII in response to α-synuclein inclusions (Duffy et al., 2018; Harms et al., 2017; Thomsen et al., 2021) – which is not seen in PBS-injected or monomer-injected animals – and an increase in $Iba1^+$ cells showing morphology indicative of microglial activation (Duffy et al., 2018).

3.2.3 Determining inflammation within the animal brain

Inflammation within the post-mortem animal brain can be determined by quantifying cell activation markers, peripheral immune cell infiltration, or measuring downstream products of immune cell activation, such as pro-inflammatory cytokine levels. (Summarised in **Figure 3.1**).

3.2.3.1 Morphological changes

Microglial activation can be determined through morphological changes to the cell in addition to alterations in the expression of cell surface markers. An activated microglial cell shows amoeboid morphology with a large cell body and few cell processes – in contrast, microglia in the surveying state show ramified morphology with a small cell body and many extended processes to allow their surveying of the environment (Nimmerjahn et al., 2005). These morphological changes can be identified using simple light microscopy after staining with Iba1.

3.2.3.2 Marker expression

Iba1 (ionised calcium binding adapter molecule 1) is a specific marker for microglia/macrophages (Imai et al., 1996). It is a pan-microglial marker expressed in both ramified and activated microglia, though on activation expression of Iba1 increases (Ito et al., 1998). As such, Iba1 may be used as a marker of inflammation, but the presence of $Iba1^+$ microglia alone is not sufficient for identifying inflammation – this should be used alongside morphological changes of the $Iba1^+$ cells, differences in intensity of staining, or microgliosis (an increased number of microglia at the area of inflammatory insult). Other markers have been suggested as being specific for microglial activation; ED1 (or its equivalent in humans – CD68) is frequently cited as being one such marker. ED1 is specific to phagocytic microglia; it is expressed in the lysosomal membranes of phagocytes and this expression increases during phagocytic activity (Bauer et al., 1994; Damoiseaux et al., 1994). However this does not make it entirely specific to activated microglia – ED1 is upregulated on microglial activation but is still expressed along the spectrum of non-activated microglia (Jurga et al., 2020). MHCII is a further marker that is expressed in all microglia, but significantly upregulated in activated microglia (Jurga et al., 2020). MHCII mediates the adaptive immune response involving the activation of T-cells via antigen presentation by MHCII (Harms et al., 2013). Neither of these markers is specific for activated microglia, however the level of their expression may be indicative of the activation state of the cell.

Another feature of inflammation is the infiltration of peripheral immune cells into the brain. An example of this is the movement of peripheral macrophages and monocytes to a site of insult. Within the brain, peripheral macrophages/monocytes are usually restricted to perivascular locations, however when an inflammatory insult occurs these cells can move towards the insult to areas lacking perivascular spaces. In rats these cells express Iba1 and

MHCII, similarly to microglia, but can be distinguished through their expression of CD163 (Harms et al., 2017; Polfliet et al., 2006). As such, the expression of CD163 on cells within the rat brain and away from vasculature can be assumed to be infiltrating macrophages/monocytes and thus be indicative of an inflammatory insult.

3.2.3.3 Downstream pro-inflammatory factors

A further method of determining inflammation in the brain may be through the identification of downstream pro-inflammatory factors. The end result of inflammatory pathways is the activation of pro-inflammatory cytokines and chemokines, the upregulation of nitric oxide synthase, and the release of reactive oxygen species. These systems work so as to coordinate the activation and recruitment of immune cells and to regulate the immune response as a whole. This is with the eventual aim of controlling and eradicating the invading pathogen or intrinsic damage. As such, the detection of these pro-inflammatory factors within the brain is indicative of inflammation.

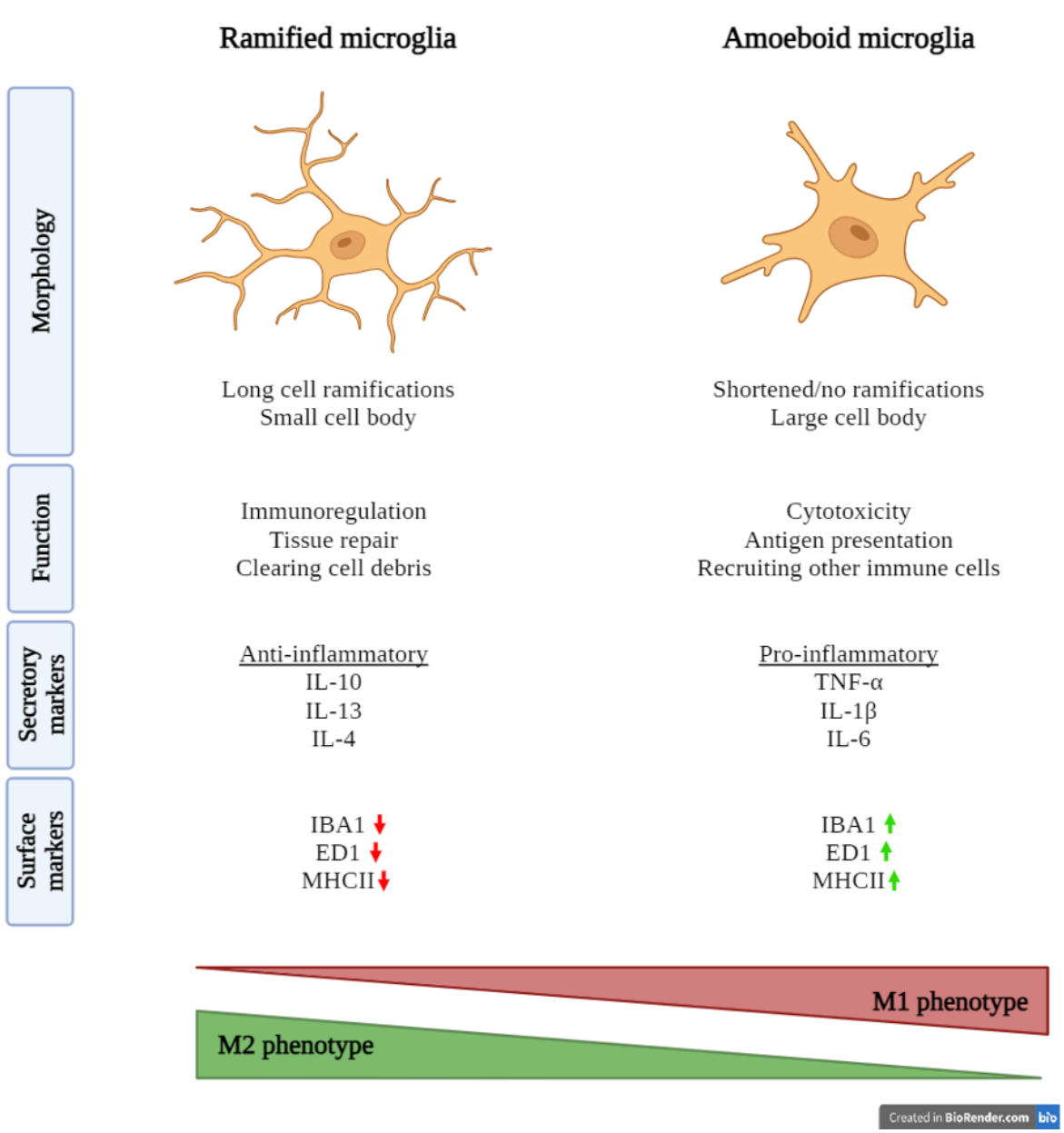


Figure 3.1 Microglial activation spectrum. *Spectrum between ramified and amoeboid microglia including morphology, function, secretory markers, and surface markers. M1 and M2 phenotypes form a continuum; M1 describe classically activated pro-inflammatory microglia whereas M2 describe alternately activated anti-inflammatory microglia.*

3.3 Methods

3.3.1 Study design

The overall study design is as described in ***Chapter 2.3.1***.

3.3.2 Liquid chromatography mass spectrometry

Liquid chromatography mass spectrometry (LCMS) was carried out to assess whether candesartan had successfully reached the brain. Six Sprague Dawley rats were obtained and housed, 3 per cage, in standard laboratory conditions: 12-hour light and dark cycle, 22˚C room temperature, 55% relative humidity, food and water ad libitum. Each rat was given one dose of candesartan (10 mg/kg/day; mixed with chocolate spread; *Amias*) at either 1 hour, 3 hours, 8 hours, 24 hours, or 30 hours pre-culling, the sixth rat was not dosed. All rats were culled via overdose of CO_2. A cannula was inserted into the heart to obtain 2 ml of blood and then a major vein was cut. The rat was then decapitated and the brain removed; simultaneously the liver and spleen were also removed. These were all flash frozen and stored at -80˚C. All tissues were homogenised the following day in the organic solvent acetonitrile at a ratio of 9 MeCN: 1 H_2O using a mechanical homogeniser. After homogenising, samples were spun at 15000 g and 4˚C for 20 minutes, the supernatant was removed and put through a 0.2 μm filter. A positive control was created by dissolving pure candesartan in MeCN, this was used to develop an SIR (single ion recording) method; this was carried out by Dr Stephen Thompson (Department of Clinical Neurosciences, University of Cambridge). All samples, including the positive control, were then run through the LCMS machine by me.

3.3.3 qPCR

qPCR was carried out to measure the expression levels of TLRs 2 and 4 in addition to the pro-inflammatory factors TNF-α, IL-1β, and IL-6. Brainstem flash frozen immediately prior to death was removed from the freezer, weighed, and the correct volume of QIAzol lysis reagent (Qiagen; *#79306*) added before being vortexed until no visible tissue could be seen. RNA was isolated using the RNeasy Plus Universal Mini kit (Qiagen; *73404*). RNA

concentration and chloroform contamination was determined using a nanodrop. RNA to cDNA conversion was carried out according to instructions in SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit (Thermo Fisher Scientific *#11752050*). Samples (5 µl cDNA) were loaded in triplicate into a MicroAmp™ Optical 384 Well reaction plate (Thermo Fisher Scientific *#4309849*) using an automatic liquid pipetting robot (Eppendorf *epMotion® 5070*). The reaction mixture was formed of 10 µl TaqMan™ Genexpression Master Mix (Thermo Fisher Scientific *4369510*), 1 µl TaqMan primer (see **Table 3.1**) and 4 µl UltraPure™ DNase free H_2O (Thermo Fisher Scientific *#10977035*). This formed a total volume of 20 µl in each well. For each primer a No Template Control (NTC) was also loaded onto the plate as a negative control; this consisted of the TaqMan™ mastermix, a TaqMan primer, and DNase free H_2O. The qPCR was carried out on the QuantStudio™ 12K Real-Time PCR System. The loaded plate was incubated for 2 min at 50˚C and 10 min at 95˚C, followed by 40 cycles of 10 sec at 95˚C and 1 min at 60˚C. To compare between the candesartan treatment groups the C_T was normalised both to GAPDH, and to the average of the untreated group – i.e. the control+drug were normalised to the control only, and the PFF+drug were normalised to the PFF only. This gave both the control and PFF untreated groups normalised to 1, and the fold change of the candesartan-treated groups compared to the corresponding value.

***Table 3.1** TaqMan primers used for qPCR.*

Gene	TaqMan Assay ID	Reference sequence
TLR2	Rn02133647_s1	NM_198769.2
TLR4	Rn00569848_m1	NM_019178.1
TNF-α	Rn99999017_m1	NM_012675.3
IL-1β	Rn00580432_m1	NM_031512.2
IL-6	Rn01410330_m1	NM_012589.2
AT-1	Rn02758772_s1	NM_030985.4
GAPDH	Rn01775763_g1	NM_017008.4

3.3.4 Western blot

Western blots to quantify protein levels of TLRs and their downstream factors were carried out on animal midbrain and brainstem. Flash frozen brain regions were individually homogenised in RIPA lysis buffer with Complete protease inhibitors (Merck *#11873580001*), and a BCA Protein Assay Kit (Thermo Fisher *#23227*) was used to quantify protein concentration. Proteins (60 µg) were separated by electrophoresis on a 4-12% polyacrylamide gel (Invitrogen *NP0336BOX*), after which they were transferred to a polyvinylidene fluoride membrane (Cytiva *#10600023*) for 90 min at a constant voltage of 30 V. Effective transfer was checked using ponceau staining. Each membrane was blocked with either 5% milk-TBST or

5% BSA and then incubated overnight at 4˚C with the specific primary antibody, as listed in **Table 3.2**. The membrane was washed and then incubated with the appropriate HRP-conjugated secondary antibody for 1 hour. Immunoreactivity was visualised with chemiluminescence (Cytiva *GERPN2232*).

Table 3.2 Antibodies used for western blotting.

Gene	Species	Dilution	Manufacturer/Catalogue No.
TLR2	Rabbit monoclonal	1:1000	Abcam *ab209217*
TLR4	Rabbit polyclonal	1:1000	Abcam *ab13867*
MyD88	Rabbit polyclonal	1:1000	Abcam *ab2064*
TNF-α	Rabbit polyclonal	1:1000	Abcam *ab6671*
TNF-α	Rabbit monoclonal	1:1000	Abcam *ab205587*
NFκB	Rabbit monoclonal	1:1000	Cell signalling Technology *#3033*
B-actin HRP-conjugated	Mouse monoclonal	1:5000	Santa Cruz Biotech *sc-47778HRP*

3.3.5 Immunohistochemistry

Immunohistochemistry of various microglial and monocytic markers was carried out on a 1:12 series of post-mortem brain sections. Sections were first quenched with 10% methanol and 10% hydrogen peroxide before being blocked in TBS with 0.3% Triton X-100 and 5% serum for 1 hour. Sections were then incubated overnight at 4˚C with primary antibodies diluted in TBS with 3% Triton X-100 and 1% serum, as listed in **Table 3.3**, followed by a 3 hour incubation with the relevant biotinylated secondary antibody (Invitrogen *#A10042*; Vector Laboratories *#BA2001*), and a 30 minute incubation with avidin-biotin complex (Vector *#PK-6100*). Antigen visualisation was carried out using 3,3'-Diaminobenzidine (DAB solution; Vector *#SK-4100*). DAB solution was left on the tissue from 1 minute to 4 minutes, dependent on tissue colour change. Sections were mounted on coverslips and dehydrated in sequential transfers in 70% EtOH, 90% EtOH, and 100% EtOH followed by immersion in 100% xylene for 30 minutes and then mounted with DPX mounting medium (Thermo Scienific).

Table 3.3 Antibodies used for immunohistochemistry on brain sections

Gene	Species	Dilution	Manufacturer/Catalogue No.
Iba1	Rabbit polyclonal	1:500	Wako Chemicals *#019-19741*
MHCII	Mouse monoclonal	1:500	BD Biosciences *#554926*
ED1	Mouse monoclonal	1:500	BioRad *MCA341GA*
CD163	Mouse monoclonal	1:500	BioRad *MCA342GA*

Quantification of Iba1 staining was carried out using 2 different methods. The slides were scanned at 20X magnification at CRUK Cambridge along with ChAT-stained slides and TH-stained slides from the same animals. Iba1 and ChAT/TH staining had been carried out on adjacent 1:12 sections. Regions of interest on Iba1 stained sections were drawn through comparison with the adjacent ChAT/TH-stained section where the region of interest, i.e. the hypoglossal and vagus nerve nuclei, the substantia nigra, and the striatum could clearly be seen. (**Figure 3.2**).

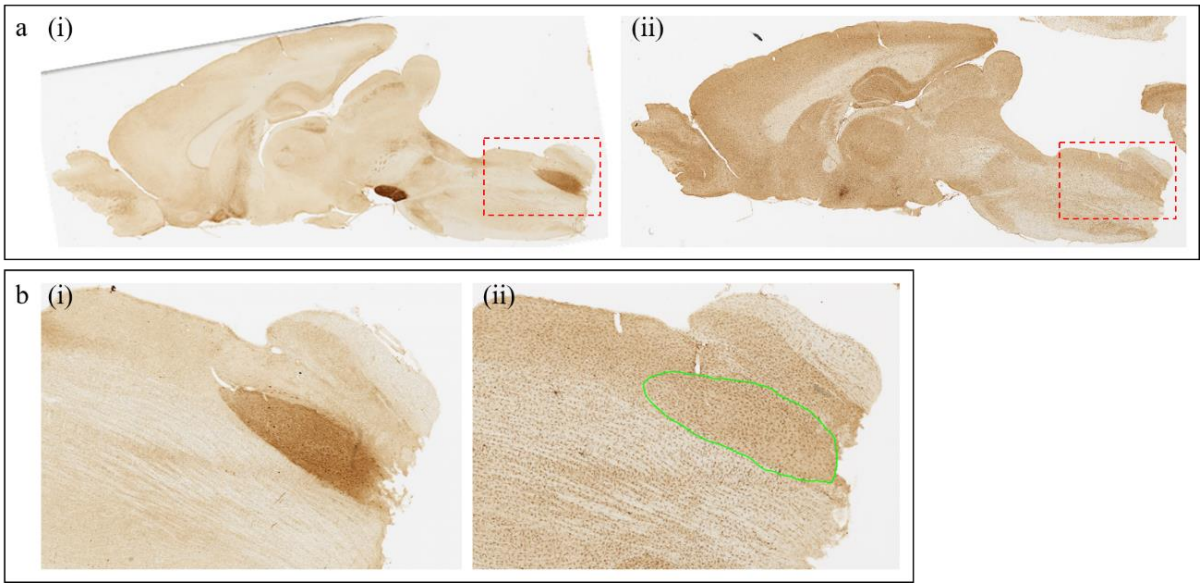


Figure 3.2 Scanned slides for IHC quantification of Iba1 staining. (a) (i) *ChAT stained slide with clear hypoglossal and vagus nerve nuclei shown in red box;* ***(ii)*** *Iba1 stained slide with region corresponding to* ***(i)*** *shown in red box.* ***(b)*** *Higher magnification of* ***(i)*** *ChAT staining;* ***(ii)*** *region of interest shown in green clearly corresponding to ChAT-positive staining in (i).*

The first method used Aperio ImageScope software, specifically the Positive Pixel Count v9 algorithm which can be optimised to quantify brown-coloured staining (DAB staining). The algorithm works by analysing each pixel and allocating them into 4 colour

intensity ranges: background (blue), weak positive (yellow), medium positive (orange), and strong positive (red). The parameters were optimised so as to identify microglia as 'strong positive'. (**Figure 3.3**). The software produces outputs of number of strong positive pixels and intensity of strong positive pixels which were determined as the measure of microglial area and intensity. The ROIs showed a large variation in area and so to account for this the output is reported as number of strong positive pixels divided by the ROI area in mm^2. The parameters were kept the same across all of the samples. This method was problematic as background staining would be identified as a 'strong positive pixel' resulting in inaccurate detection of the specific staining of microglia (see comparison between **Figure 3.3b & c**). This method also only gave an output in terms of positive pixels, so not allowing a specific quantification of number of microglia.

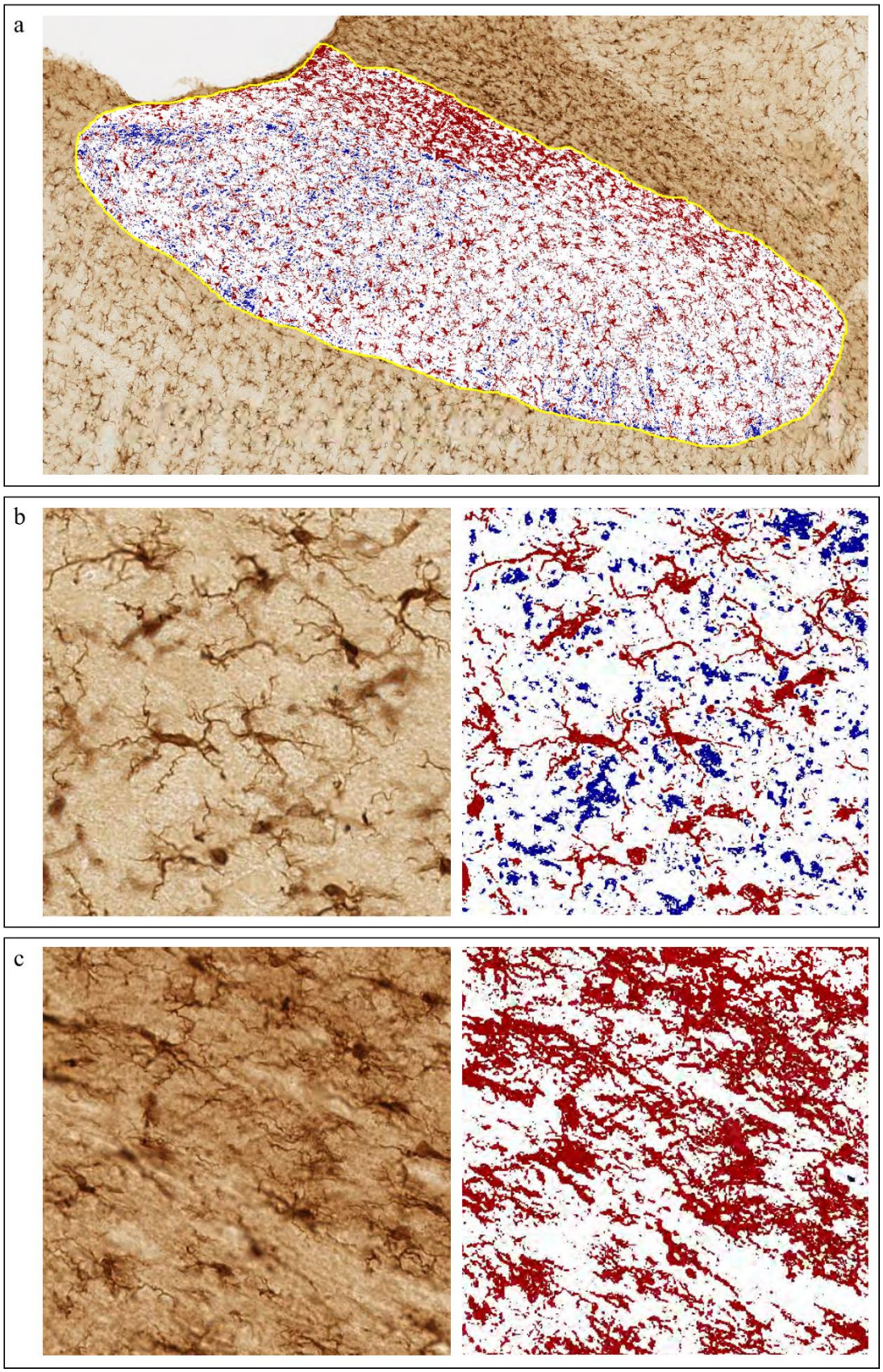


Figure 3.3 Imagescope software analysis. (a) *ROI with optimised parameters applied; microglia identified in red.* ***(b)*** *Low background staining and* ***(c)*** *high background staining with corresponding overlay mask.*

The second method used ImageJ software; this method allowed the counting of microglia through thresholding methods where only particles over a specified diameter were selected. 0.5 mm^2 square ROIs in each brain region were extracted for analysis. Briefly, the colour and size threshold were optimised for each box to select the cell bodies alone, an overlay mask was created (seen in red **Figure 3.4a**) and particle analysis was carried out to count the number of microglia in addition to the area of the selected cell bodies; this was averaged over the number of boxes per brain region. The parameters had to be adjusted for each box due to differing levels of background staining. To ensure non-bias when altering parameters the animal code and group number was blinded. This method was validated through the manual counting of a select number of boxes (N=17), which was shown to positively correlate with the automated count (Simple linear regression R^2=0.6615; black line). (**Figure 3.4**). I decided that this was the optimal method and my reported results are based on this.

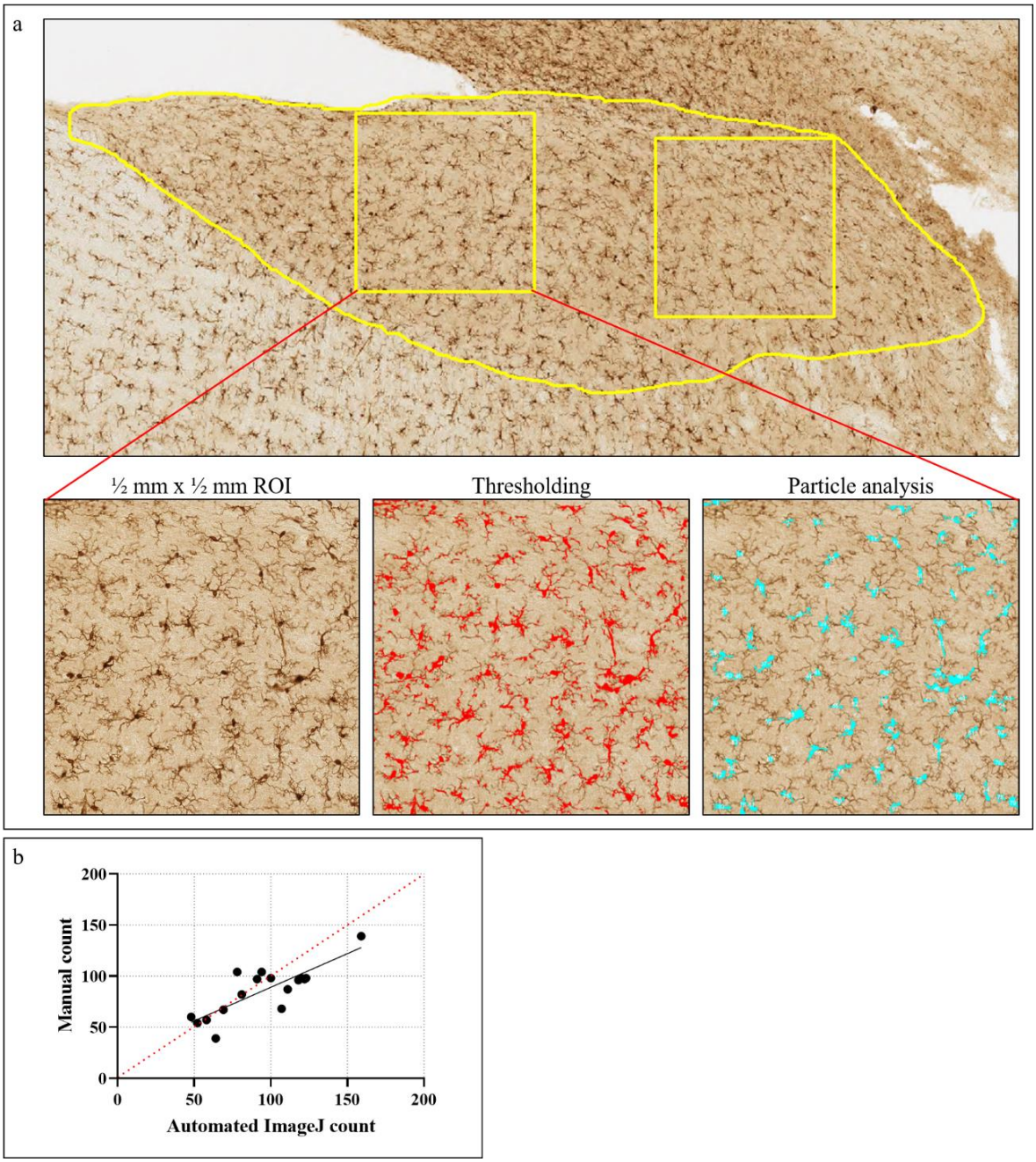


Figure 3.4 ImageJ software analysis. (a) *Example of step-wise image processing to produce microglial cell count and area quantification.* ***(b)*** *Comparison of automated and manual microglial cell count shows a positive correlation as determined through a simple linear regression (black solid line).* R^2*=0.6615.*

3.3.6 Meso Scale Discovery assay

Meso Scale Discovery (MSD) assays were carried out on animal serum to assess levels of pro-inflammatory cytokine expression. MSD assays use proprietary technology integrating electrochemiluminescence and multiarray techniques to detect multiple proteins from one sample. The technology utilised allows for much higher sensitivity than a regular ELISA

(enzyme-linked immunosorbent assay). A blood sample was taken from each animal at baseline; this was then repeated every 2 months until the end of the study. The blood was allowed to clot at room temperature before being spun for 15 minutes at 2000 g. The serum was removed and stored at -80˚C prior to later batch analysis. Mesoscale discovery V-PLEX Proinflammatory Panel 2 kits (MSD *K15059D*) were used to analyse the serum for the pro-inflammatory cytokines IFN-γ, IL-1β, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-13, and TNF-α following the instructions in the kit. Briefly, the plates were blocked for 1 hour using the blocking agent H provided, the plates were then washed before samples and calibrators were added, these were then incubated for 2 hours with shaking. The plates were incubated with the detection antibodies for 2 hours before, finally, read buffer was applied to the washed plates and the plates immediately read using the MSD plate reader. Results were analysed using the MSD Discovery software.

3.3.7 Statistical analysis

Data analysis and graph generation was carried out using GraphPad Prism 8.3. Data normality was assessed using the Shapiro-Wilk test. Pairwise comparisons were carried out between untreated and candesartan-treated PFF or control groups using a Mann-Whitney test. For repeated measures analysis of groups over time a Mixed-effect analysis was carried out followed by Šídák's multiple comparisons test.

3.4 Results

3.4.1 Does candesartan cross the blood brain barrier?

The premise of my study design requires that candesartan is getting into the brain to have its hypothesised action on the TLRs on neuroimmune cells. Liquid chromatography mass spectrometry (LCMS) was carried out on the brains, livers, and spleens of 6 animals each dosed with candesartan at a different timepoint prior to culling (1 hour, 3 hours, 8 hours, 24 hours, and 30 hours pre-culling). A clear peak at the correct molecular weight indicating the presence of candesartan in the brain, liver, and spleen was present in all of the animals dosed 24 hours or less prior to culling. No significant peaks above background noise were seen in the untreated negative control animal, or in the animal dosed 30 hours prior to culling. The highest peak seen at 1.91 does not have the correct molecular weight to be candesartan, and does not correspond to the clear peak seen in the pure candesartan positive control sample. (**Figure 3.5**). This experiment shows that candesartan is able to cross the blood-brain barrier of the rats within an hour of their dosing, and is still present in the brain at 24 hours post-dosing, but has been metabolised by 30 hours post-dosing. Similar peaks were also observed in the liver (see **Figure 3.6**).

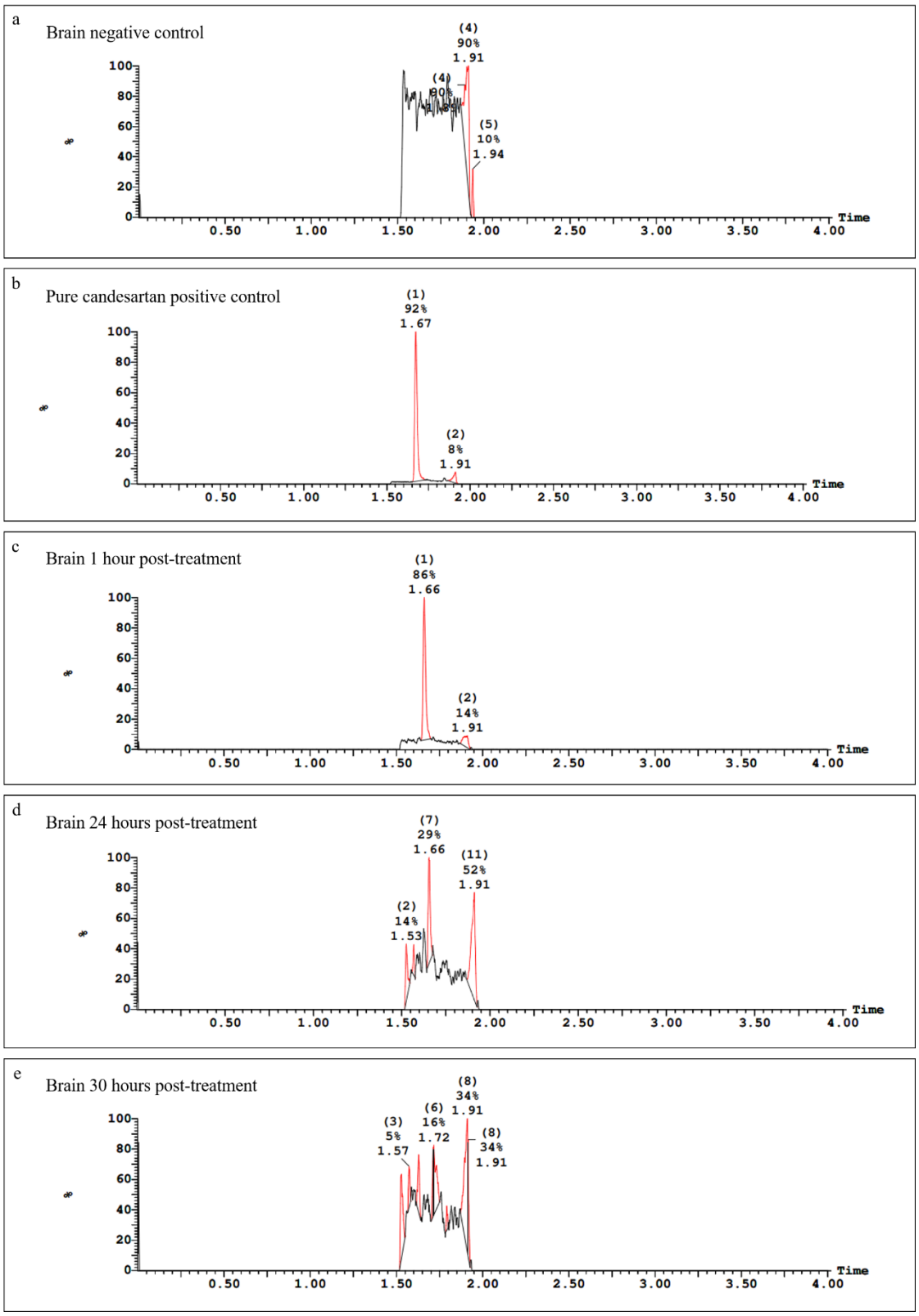


Figure 3.5 Candesartan is able to enter the brain, and persists for 24 hours. *LCMS plots of* ***(a)*** *homogenised brain from untreated rat;* ***(b)*** *pure candesartan suspended in HPLC-grade acetonitrile; and homogenised brain from rat dosed with candesartan* ***(c)*** *1 hour,* ***(d)*** *24 hours, and* ***(e)*** *30 hours prior to culling.*

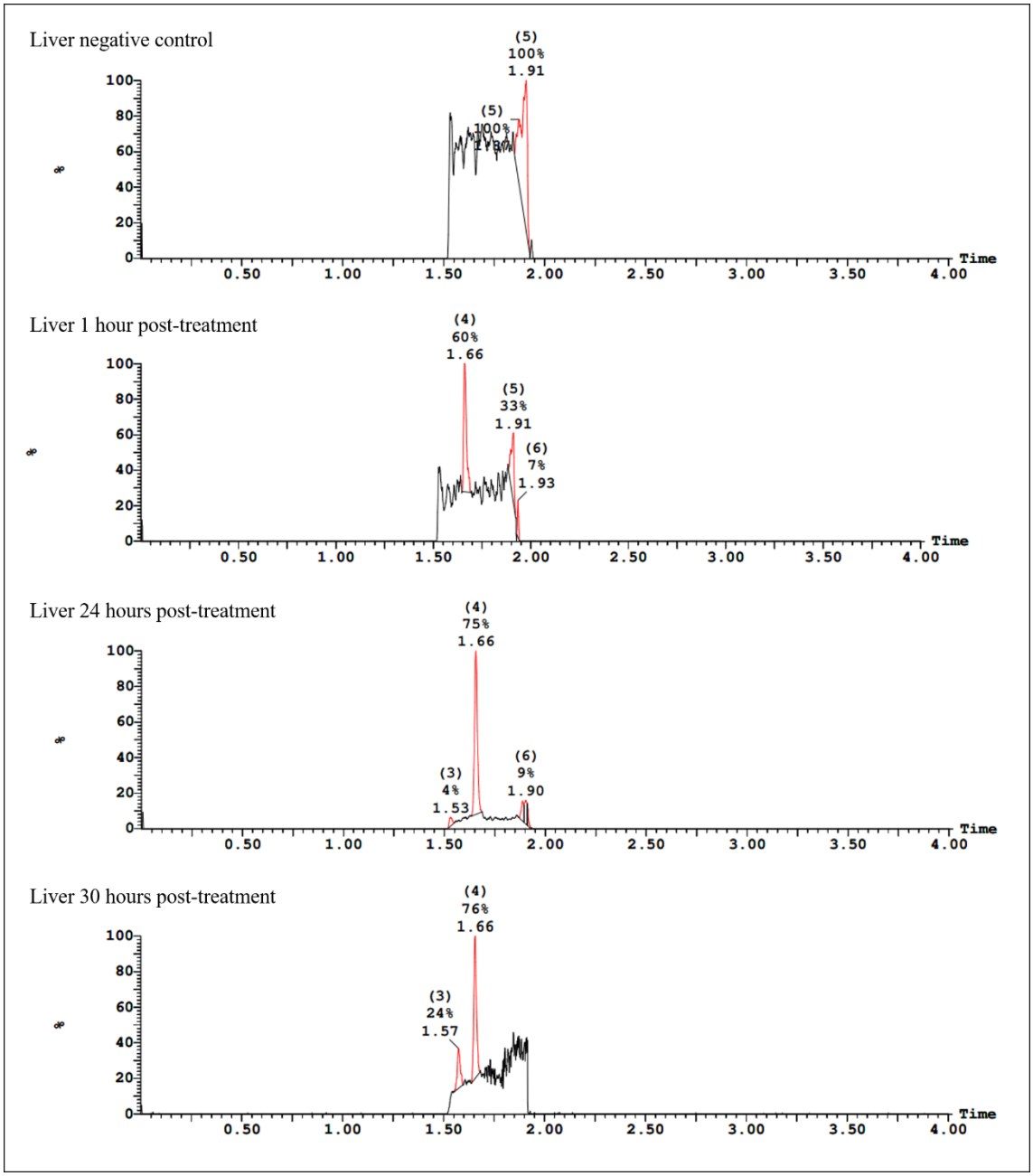


Figure 3.6 Candesartan persists in the liver. *LCMS plots of (top to bottom) homogenised liver from untreated rat; and from rat dosed with candesartan 1 hour, 24 hours, and 30 hours prior to culling.*

3.4.2 Is candesartan successfully reducing the expression of TLR2/4?

The hypothesis underlying this study is that candesartan will have a protective effect on the development of pathology in the α-synuclein animal model through its blocking of TLRs, and subsequent pro-inflammatory effects. To test whether candesartan was having this effect, I determined the levels of TLR expression, and TLR protein levels in the animal brains. qPCR of TLRs 2 and 4 showed no fold-change in the expression of either TLR2 or TLR4 in rat brainstem on candesartan treatment compared to untreated; all normalised to GAPDH and to either the untreated control or untreated PFF groups. (**Figure 3.7**)

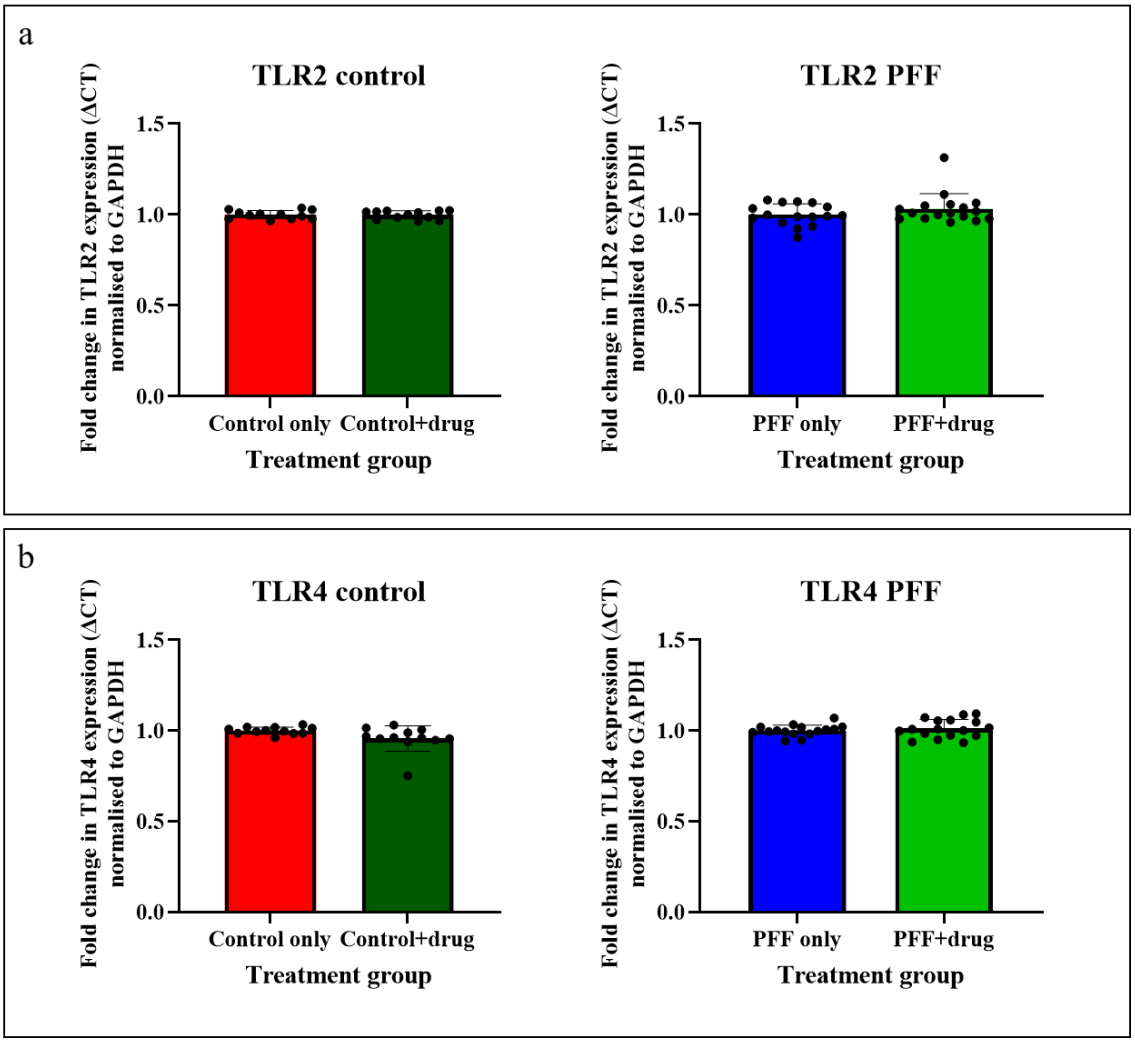


Figure 3.7 Candesartan treatment does not have an effect on the expression of TLRs in the rat brainstem. *Comparison between the fold change in* ***(a)*** *TLR2 and* ***(b)*** *TLR4 mRNA levels of drug-treated compared to untreated control (left) and PFF-injected (right) animals. Normalised to GAPDH.*

Western blots using tissue from the midbrain showed a trend towards decreased TLR protein levels in both drug-treated PFF-injected animals and drug-treated control animals compared to untreated PFF-injected and control animals respectively. These trends did not reach significance for either PFF-injected or control animals. (**Figure 3.8**). (Mann-Whitney test; **Figure 3.8b** control p=0.1049, PFF p=0.1381; **Figure 3.8c** control p=0.2786, PFF p=0.1017). There also appears to be a trend towards increased TLR protein levels in the PFF-injected animals compared to the controls, though with the caveat that these cannot be directly compared due to their being conducted as parallel studies at different times.

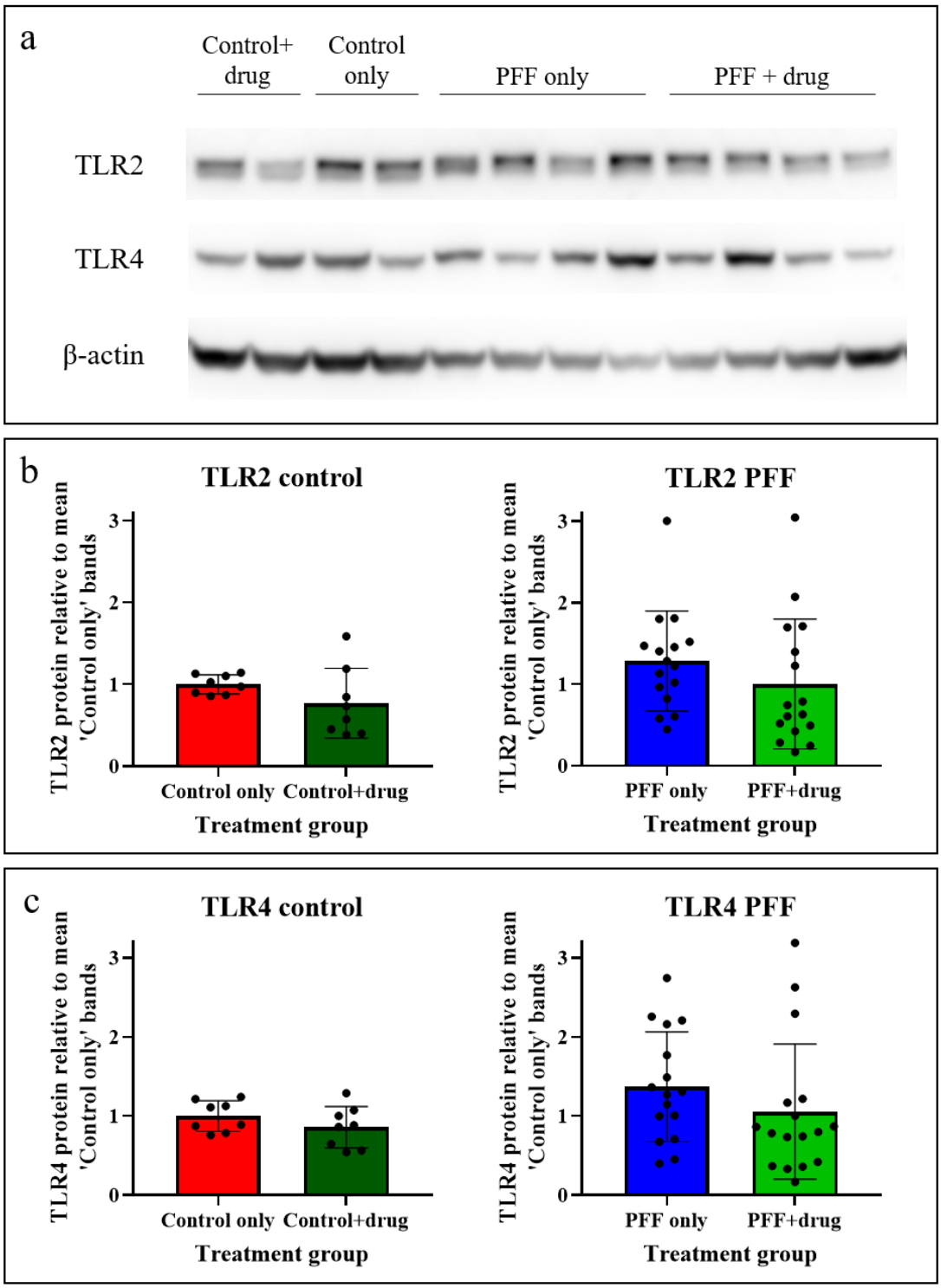


Figure 3.8 Candesartan treatment results in a trend towards decreased protein levels of TLRs 2 and 4 in the rat midbrain. (a) *Representative western blot of TLR2 and TLR4 with β-actin as a loading control; quantification of* ***(b)*** *TLR2; and* ***(c)*** *TLR4 protein levels of untreated and drug-treated saline-injected control (left) and PFF-injected (right) animals. Bands normalised to β-actin and then to the mean of 'control only' bands to allow comparison between different blots. Standard deviation shown in error bars. Mann-Whitney test; p>0.05 for all comparisons).*

In the brainstem, TLR2 was significantly decreased in the candesartan-treated control animals (Mann-Whitney test; $p<0.05$), however this was not seen in the brainstem of PFF-injected animals. (**Figure 3.9**). No significant changes in TLR4 levels were observed between treated and untreated groups (Mann-Whitney test; $p>0.05$ for all other pairwise comparisons).

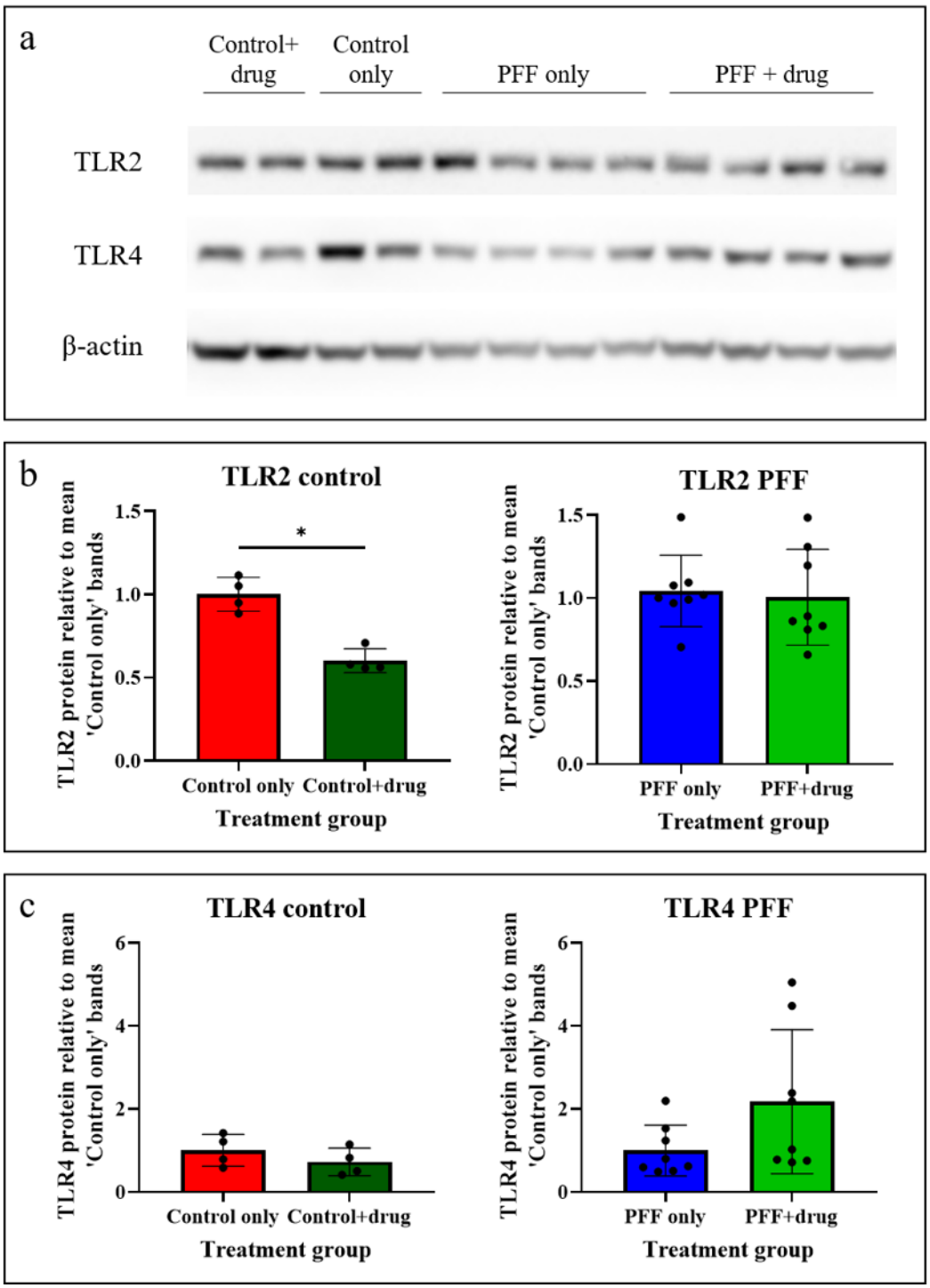


Figure 3.9 Candesartan treatment results in a trend towards decreased protein levels of TLR2 but not of TLR4 in the rat brainstem. (a) *Representative western blot of TLR2 and TLR4 with β-actin as a loading control; quantification of* ***(b)*** *TLR2; and* ***(c)*** *TLR4 protein levels of untreated and drug-treated saline-injected control (left) and PFF-injected (right) animals. Mann-Whitney test; p*<0.05. Bands normalised to β-actin and then to the mean of 'control only' bands to allow comparison between different blots. Standard deviation shown through error bars. Mann-Whitney test; p>0.05 for all comparisons).*

Further work to identify whether candesartan was blocking TLRs 2 and 4 used western blots of MyD88, the universal adapter protein of TLRs, in animal midbrain. The antibody used claimed to show bands at 35 kDa, however, though these bands were present in the blots, they were not able to be quantified due to the non-specific bands surrounding the correct MW. Instead the bands at 70 kDa were used for quantification as they were assumed to be MyD88 dimers (Loiarro et al., 2005). A trend towards decreased MyD88 was seen in the candesartan-treated compared to untreated control animals, however this did not reach significance (Mann-Whitney test). No such trend was seen in the PFF-injected animals. (**Figure 3.10**).

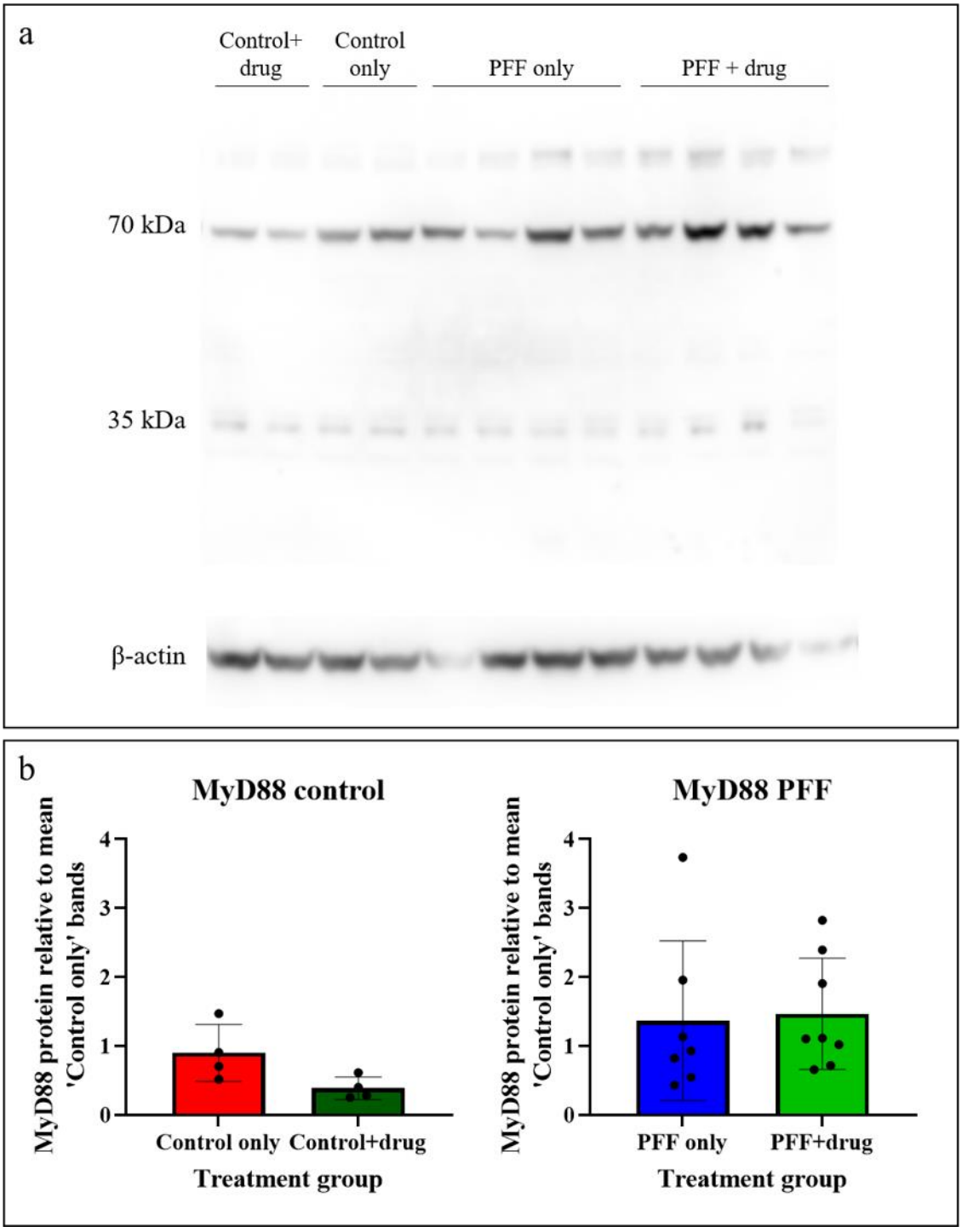


Figure 3.10 Candesartan treatment does not alter protein levels of MyD88. (a) *Representative western blot of MyD88 with β-actin as a loading control;* ***(b)*** *quantification of MyD88 protein levels of untreated and drug-treated saline-injected control (left) and PFF-injected (right) animals. Bands normalised to β-actin and then to the mean of 'control only' bands to allow comparison between different blots. Mann-Whitney test; p>0.05 for all comparisons).*

The primary function of candesartan is as an AT_1 blocker, however studies have also shown a decrease in $AT1_1$ expression on candesartan treatment (Rodriguez-Perez et al., 2018). Therefore the expression of AT_1 in the animal brain was determined using qPCR. No fold change was seen in the expression of AT_1 on candesartan treatment compared to untreated; all normalised to GAPDH and to either the untreated control or untreated PFF groups. (**Figure 3.11**).

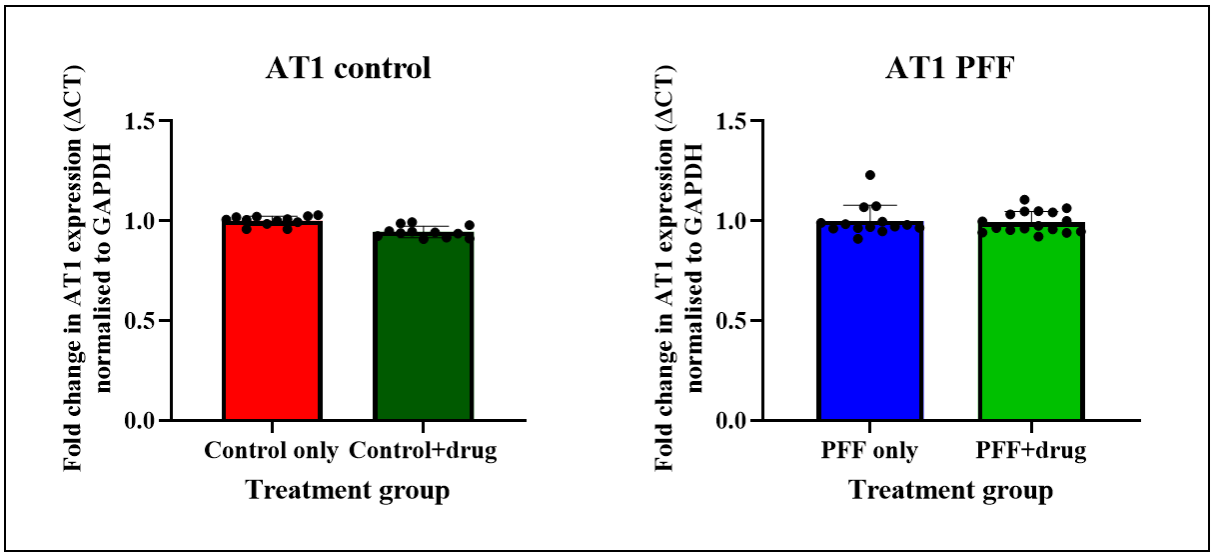


Figure 3.11 Candesartan treatment does not alter AT_1 expression. *Comparison between the fold change in AT_1 mRNA levels of drug-treated compared to untreated control (left) and PFF-injected (right) animals. Normalised to GAPDH.*

3.4.3 Does candesartan treatment impact microglial activation?

I looked at the effect of candesartan treatment on microglial activation using Iba1 immunohistochemistry. This was carried out in 3 areas of interest – the vagus and hypoglossal nuclei (grouped together for analysis), the substantia nigra, and the striatum. At 6 months, Candesartan was shown to have no effect on microglial activation in either PFF-injected animals or saline-injected control animals as determined by the number and area of the microglia. This was the case in the vagus/hypoglossal nerve nuclei (**Figure 3.12**), the substantia nigra (**Figure 3.13**), and the striatum (**Figure 3.14**). In addition, although direct statistical comparison between control and PFF groups was not made due to the fact that the studies were conducted separately (as described in ***Chapter 2.4.2***), the shape and size of the microglia look very similar between the controls and the PFF-injected animals. It is possible therefore that the

lack of response to candesartan is due to a lack of activation of microglia as produced by this α-synuclein model.

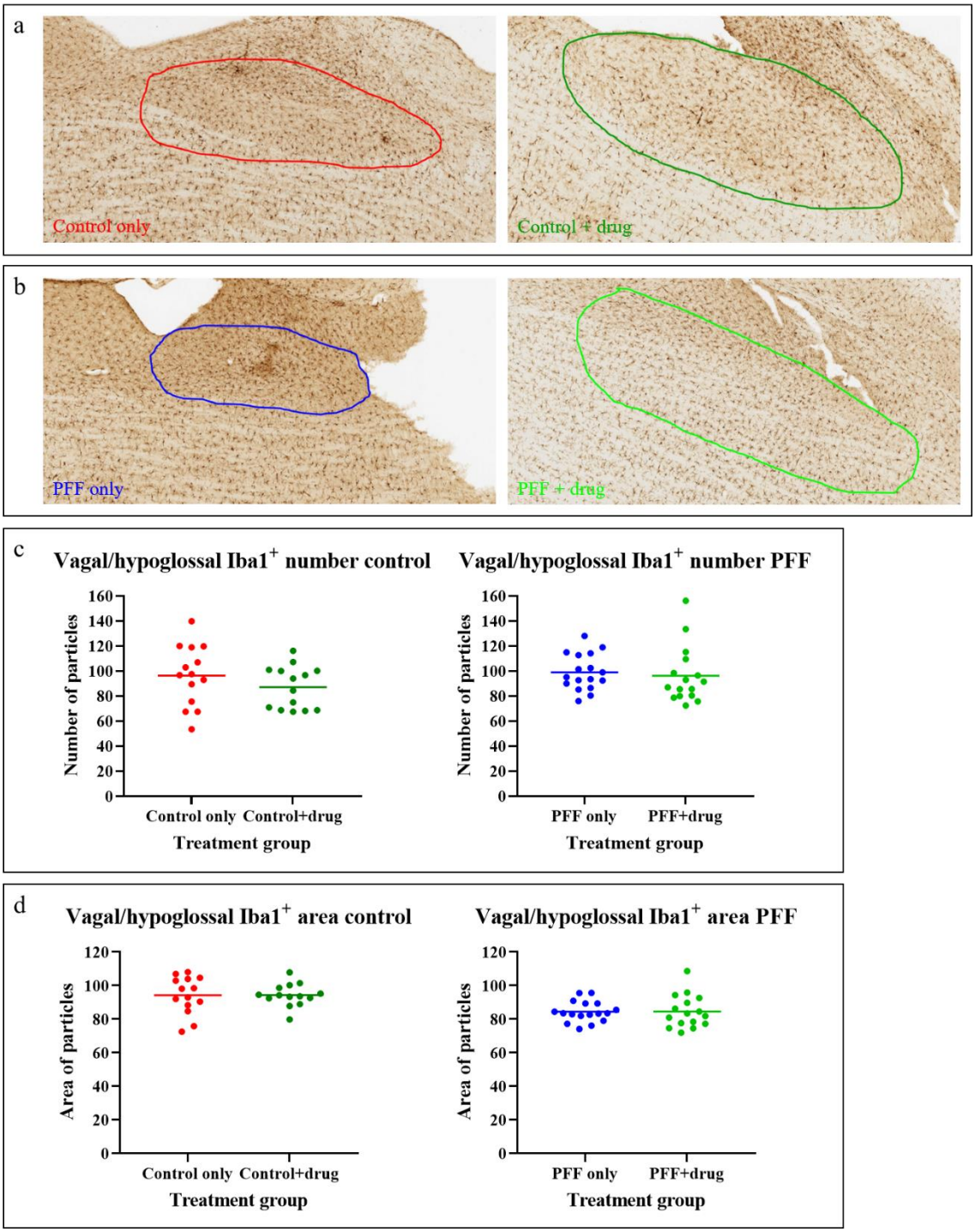


Figure 3.12 Candesartan does not have any effect on microglial number or morphology in the hypoglossal and vagal nerve nuclei. *Representative images of Iba1 staining in the outlined vagal nerve/hypoglossal nuclei in* ***(a)*** *control animals and* ***(b)*** *PFF-injected animals. Quantification of* ***(c)*** *number of Iba1$^+$ cells and* ***(d)*** *area of Iba1$^+$ cells in control (left) and PFF-injected (right) animals. Quantification carried out on 0.5 mm^2 boxes placed within the region of interest.*

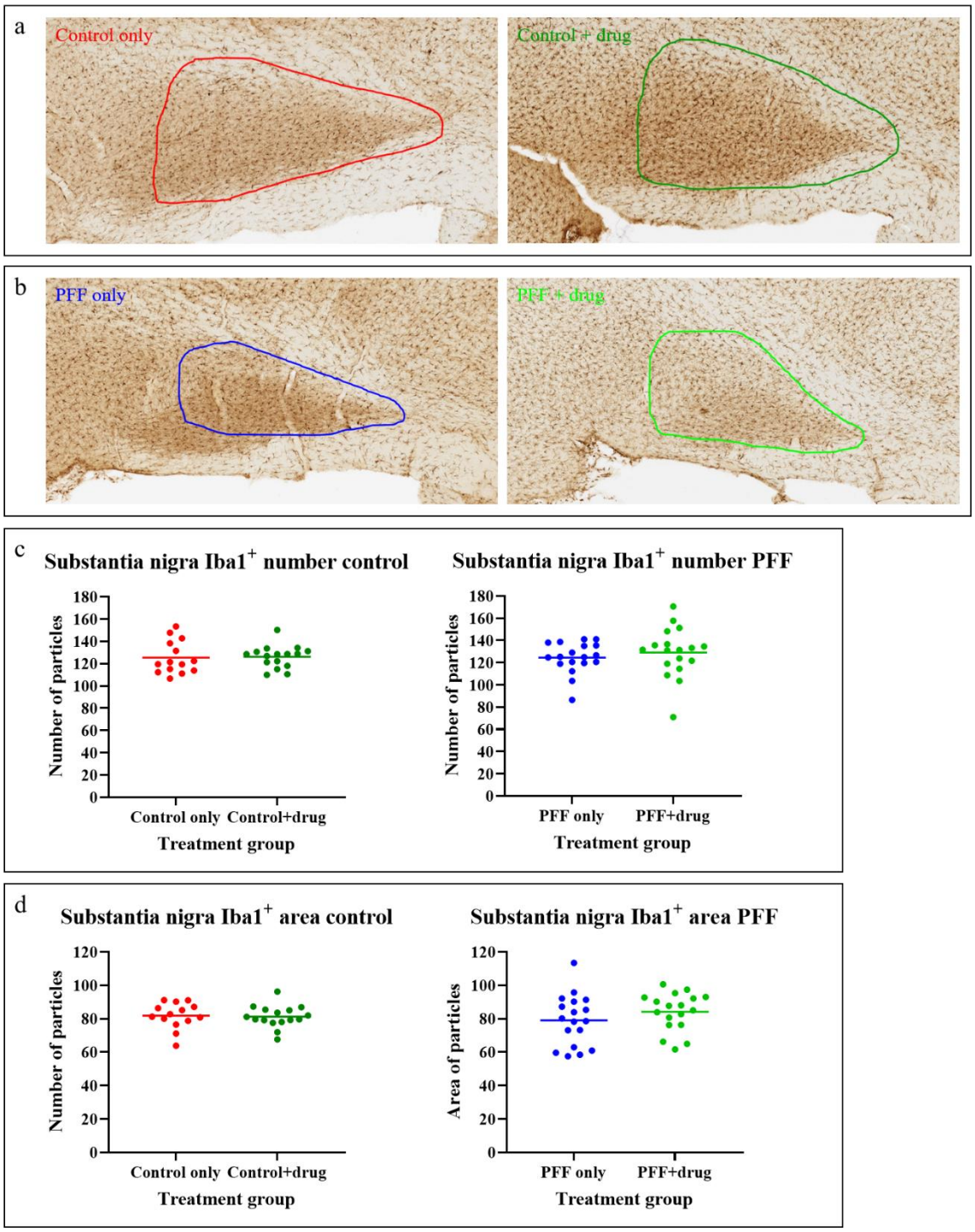


Figure 3.13 Candesartan does not have any effect on microglial number or morphology in the substantia nigra. *Representative images of Iba1 staining in the outlined substantia nigra in* ***(a)*** *control animals and* ***(b)*** *PFF-injected animals. Quantification of* ***(c)*** *number of Iba1$^+$ cells and* ***(d)*** *area of Iba1$^+$ cells in control (left) and PFF-injected (right) animals. Quantification carried out on 0.5 mm^2 boxes placed within the region of interest.*

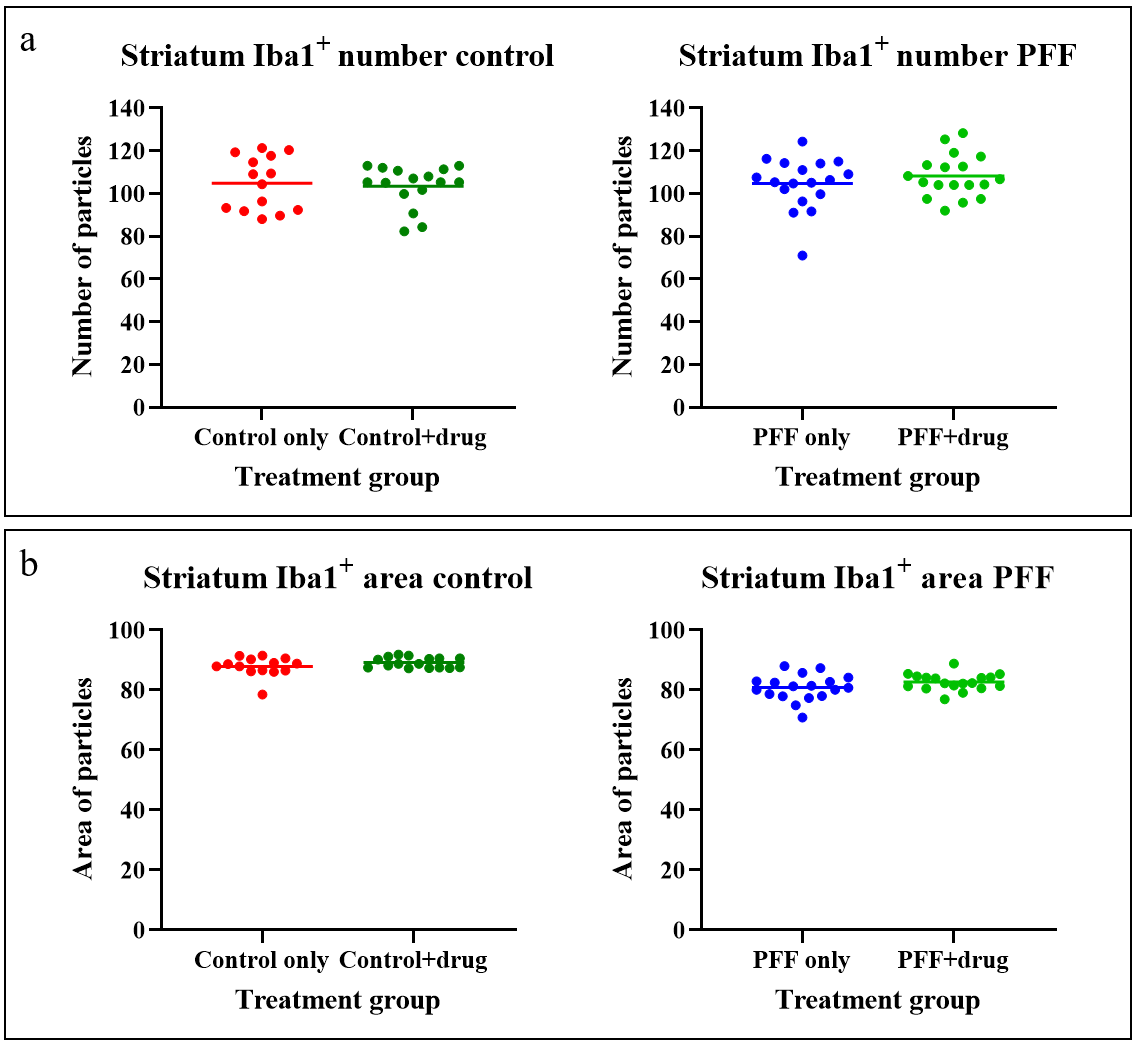


Figure 3.14 Candesartan does not have any effect on microglial number or morphology in the striatum. *Quantification of* ***(a)*** *number of Iba1$^+$ cells and* ***(b)*** *area of Iba1$^+$cells in control (left) and PFF-injected (right) animals. Quantification carried out on 0.5 mm^2 boxes placed within the region of interest.*

I used a selection of other antibodies aimed specifically at markers of microglial activation. MHCII is expressed on all microglia; the antibody used here has been seen in various studies (including Harms et al., 2017 and Rodriguez-Perez et al., 2018) to identify activated microglia due to their expressing higher levels of MHCII. There were no apparent differences between treatment groups at 6 months, and indeed a very low number of cells throughout the brain expressing MHCII – as detected by this antibody – at all. (**Figure 3.15a-b**) An animal showing a clear inflammatory insult in the neck of the cerebellum was used as a positive control. (**Figure 3.15c**) Through comparison with this, it can be seen that the pro-inflammatory effects of the PFF-injection are negligible, and thus treatment with candesartan

has no clear effect on the activation of microglia. (**Figure 3.15**). Staining of MHCII was also carried out at the 2 and 4 month timepoints; no differences were seen between any treatment groups. (**Figure 3.16**). In addition, MHC staining was compared between the PFF animals 4 months post PFF injection, and the control animals 6 months post saline injection in order to allow comparison of animals at a similar age (PFF-injected animals were 14 weeks old at the point of injection whereas the saline injected animals were 8 weeks old). This was done because age may have an effect on neuroinflammation. No differences were seen between the 4-month PFF animals and the 6-month control animals indicating that inflammation is not occurring in this model. (**Figure 3.17**). Due to very low levels of staining, the IHC was not quantified, however further investigation was carried out through western blots to quantify levels of MHCII. Western blots also did not show any significant differences in levels of MHCII between treatment group in either the brainstem or the midbrain (Mann-Whitney test; $p>0.05$). (**Figure 3.18**).

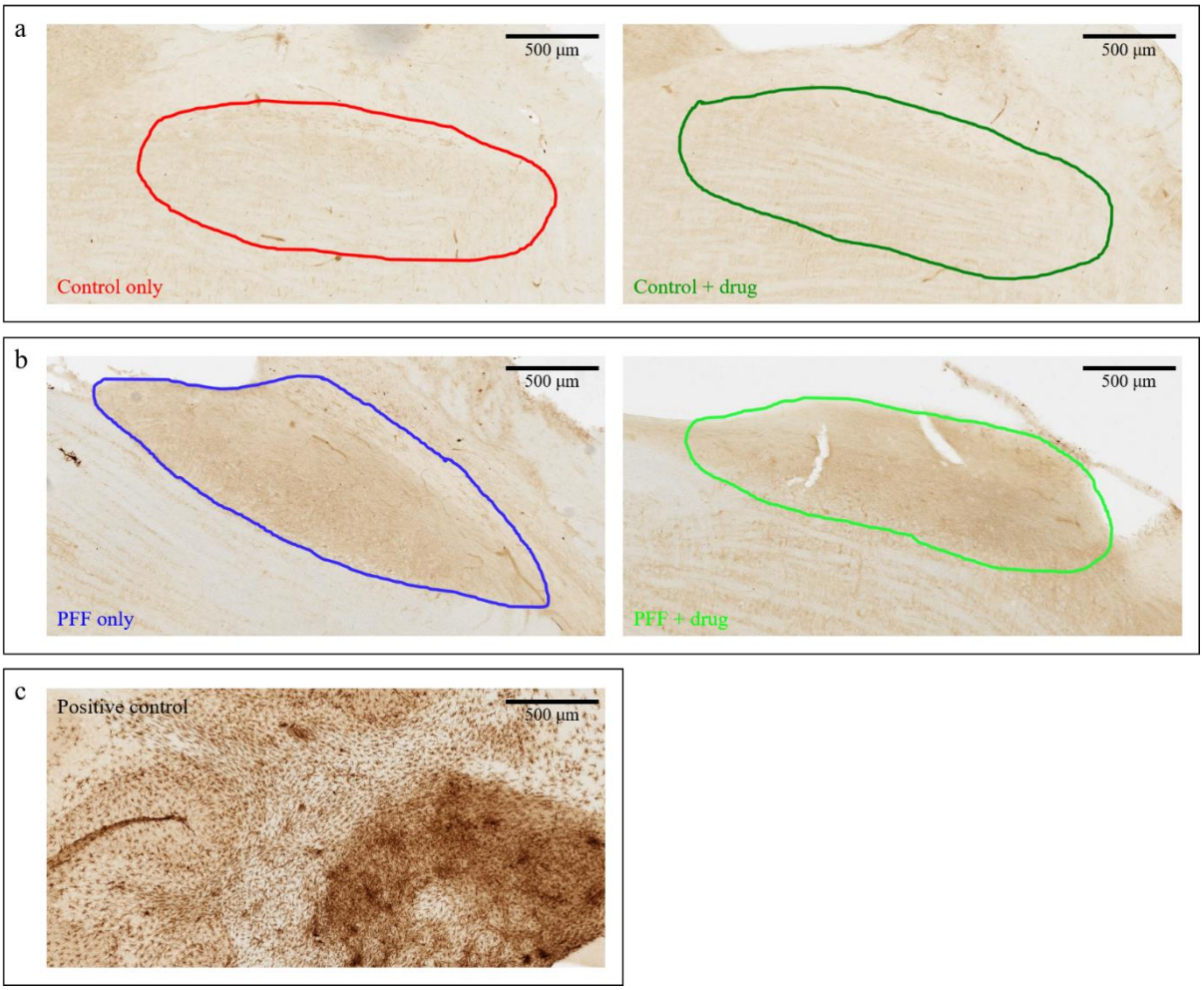


Figure 3.15 Candesartan has no effect on MHCII expression at 6 months. *Representative images of MHCII staining in the vagal nerve/hypoglossal nuclei in* ***(a)*** *control animals,* ***(b)*** *PFF-injected animals, and* ***(c)*** *an animal experiencing an inflammatory insult in the cerebellum. All images are at same magnification and have similar levels of background staining.*

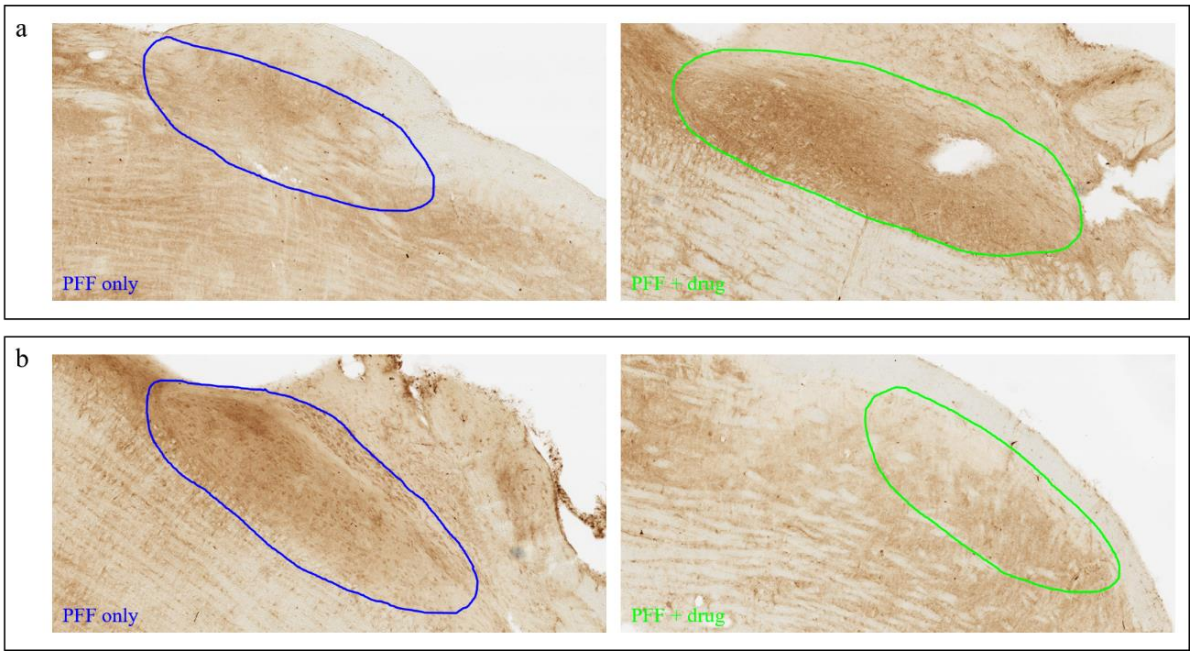


Figure 3.16 Candesartan has no effect on MHCII expression at different timepoints. *MHCII staining in untreated (left; blue) and candesartan-treated (right; green) hypoglossal/vagal nerve nuclei at* ***(a)*** *2 month and* ***(b)*** *4 month timepoints.*

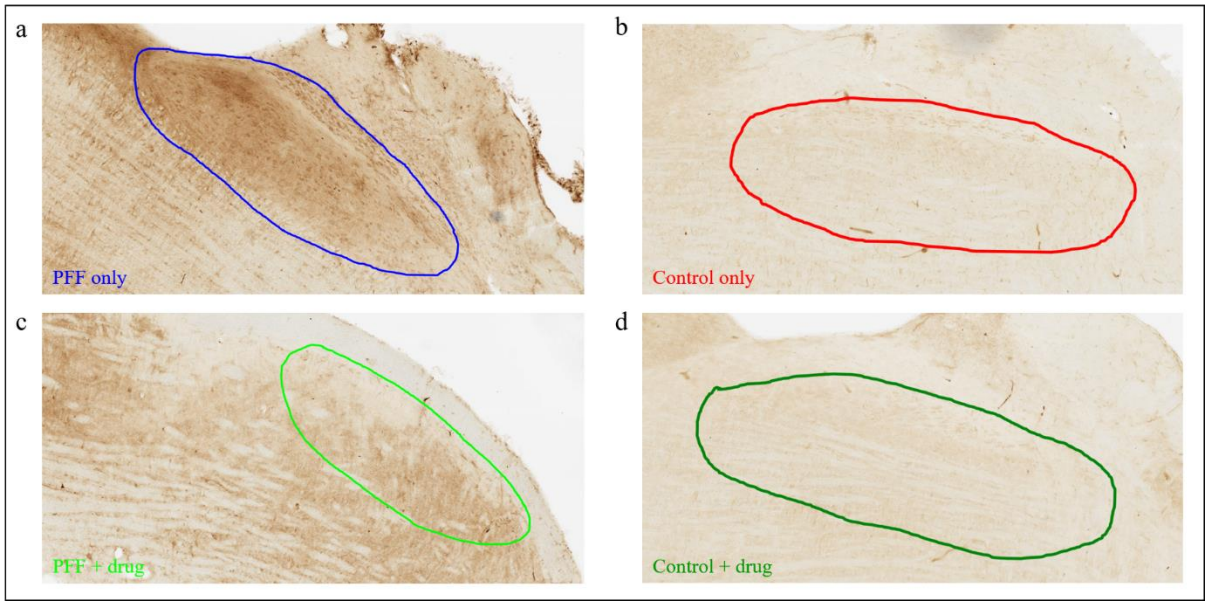


Figure 3.17 No differences in inflammation (as assessed using MHCII) are seen between PFF-injected and saline-injected control animals when at comparable ages. *MHC staining in* ***(a)*** *untreated or* ***(c)*** *candesartan-treated PFF-injected and* ***(b)*** *untreated or* ***(d)*** *candesartan-treated control saline-injected animals at a similar age. PFF-injected animals at 30 weeks old; saline-injected control animals at 32 weeks old.*

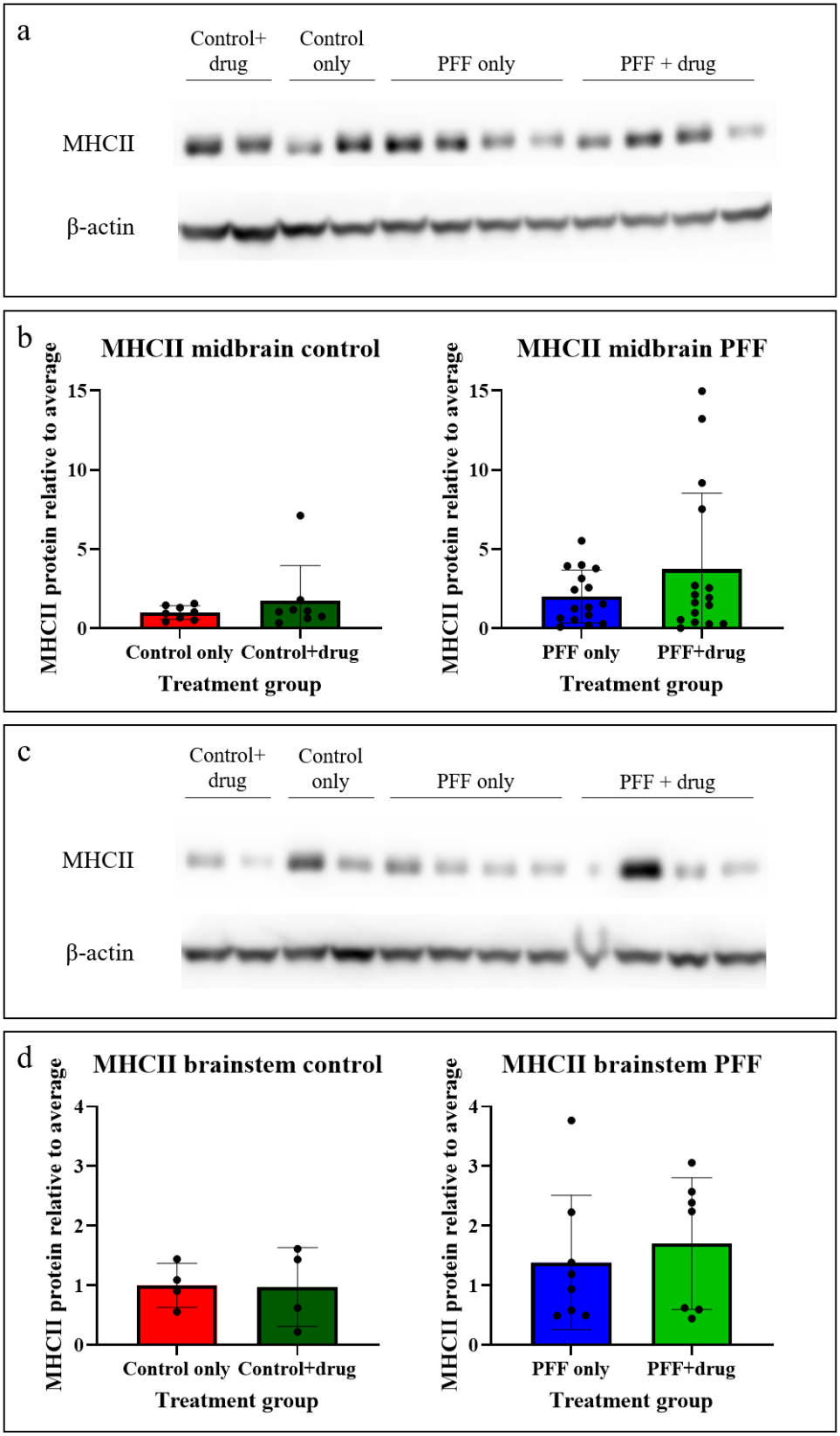


Figure 3.18 Candesartan is not associated with changes in MHCII protein levels. *Representative western blots of MHCII and quantification of blots in the* ***(a-b)*** *midbrain and* ***(c-d)*** *brainstem of control and PFF-injected animals. The well in (c) – well 9 in PFF+drug – which can be seen to have run poorly was removed from all analysis. Mann-Whitney test; p>0.05.*

A further marker of microglial activation is ED1, expressed by amoeboid microglia. This staining did not show any apparent differences between treatment groups on visual inspection. However, it was very difficult to tell whether the staining was successful. Even in the animal with an inflammatory insult in the cerebellum which is being used as a positive control there was very little obvious staining. (**Figure 3.19**). Only when viewed at the highest magnification was there some noticeable staining in the positive control, but this was not seen at all in any of the other animals (data not shown). Finally, as a further indicator of inflammation (though not specifically of microglial activation), a marker of infiltrating monocytes, CD163, was used. This is indicative of whether peripheral cells are becoming activated and infiltrating the brain. There were no apparent differences on visual inspection of stained sections between treatment groups at 6 months. (**Figure 3.20**).

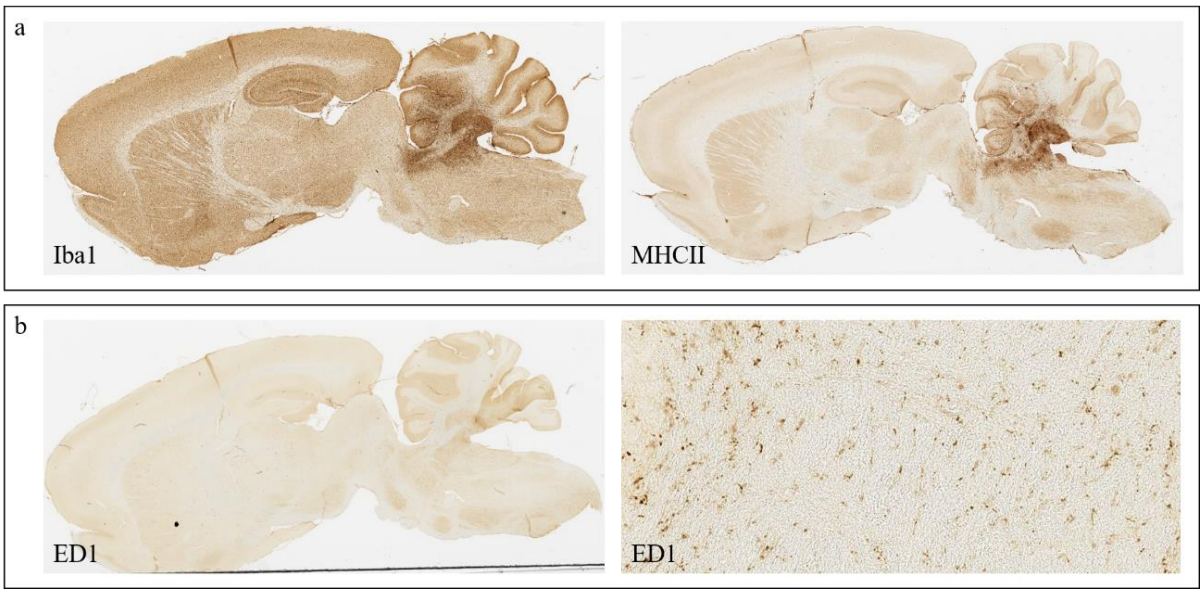


Figure 3.19 ED1 staining is difficult to identify in an animal with an inflammatory insult in the cerebellum being used as a positive control. (a) *Iba1 staining and MHCII staining in the whole brain clearly showing an increase in expression at the neck of the cerebellum.* ***(b)*** *ED1 staining is not clear anywhere in the brain, including in the neck of the cerebellum where inflammation is occurring. At 20X magnification (right) staining can be seen as small dots at the area of inflammation, however this is not seen elsewhere in the brain.*

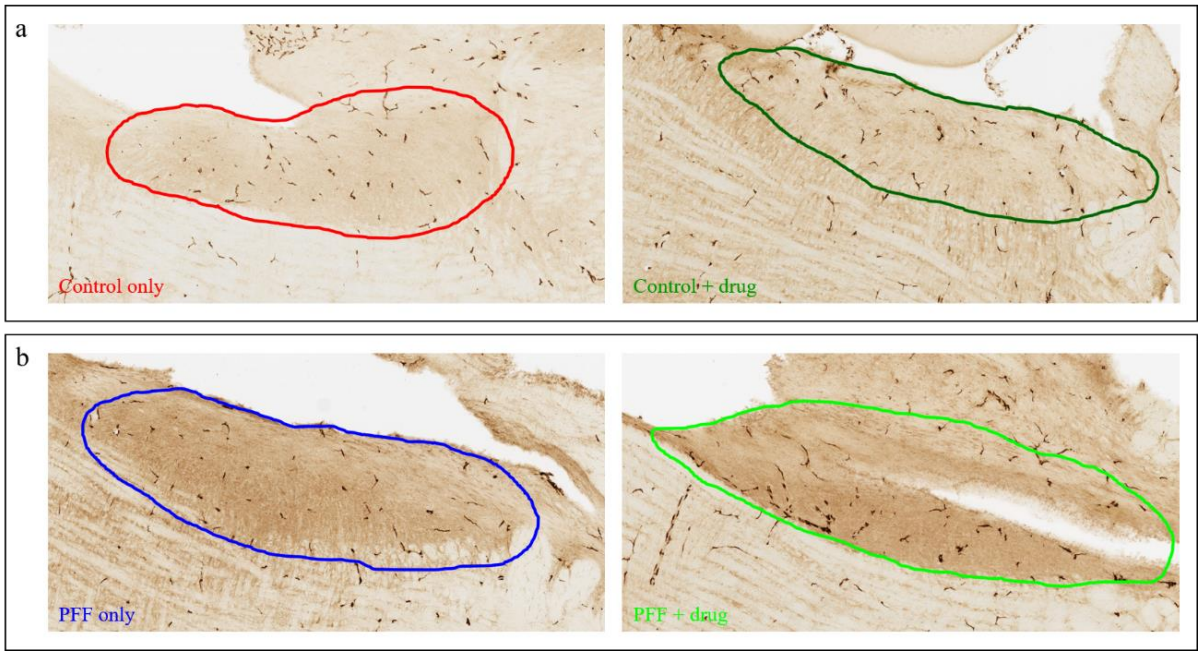


Figure 3.20 CD163 is not differentially expressed in different treatment groups. *CD163 staining in untreated (left) and candesartan-treated (right)* ***(a)*** *PFF-injected and* ***(b)*** *saline-injected control animals. Magnification 20X.*

3.4.4 Is candesartan treatment associated with changes in downstream inflammatory markers?

Activation of TLRs was investigated in the brainstem – the area where neurodegeneration was seen in this model – through measuring the expression of downstream inflammatory molecules. TNFα, IL-1β, and IL-6 expression levels were determined using qPCR. No differences were seen in either the control or PFF-injected groups on candesartan treatment. (**Figure 3.21**).

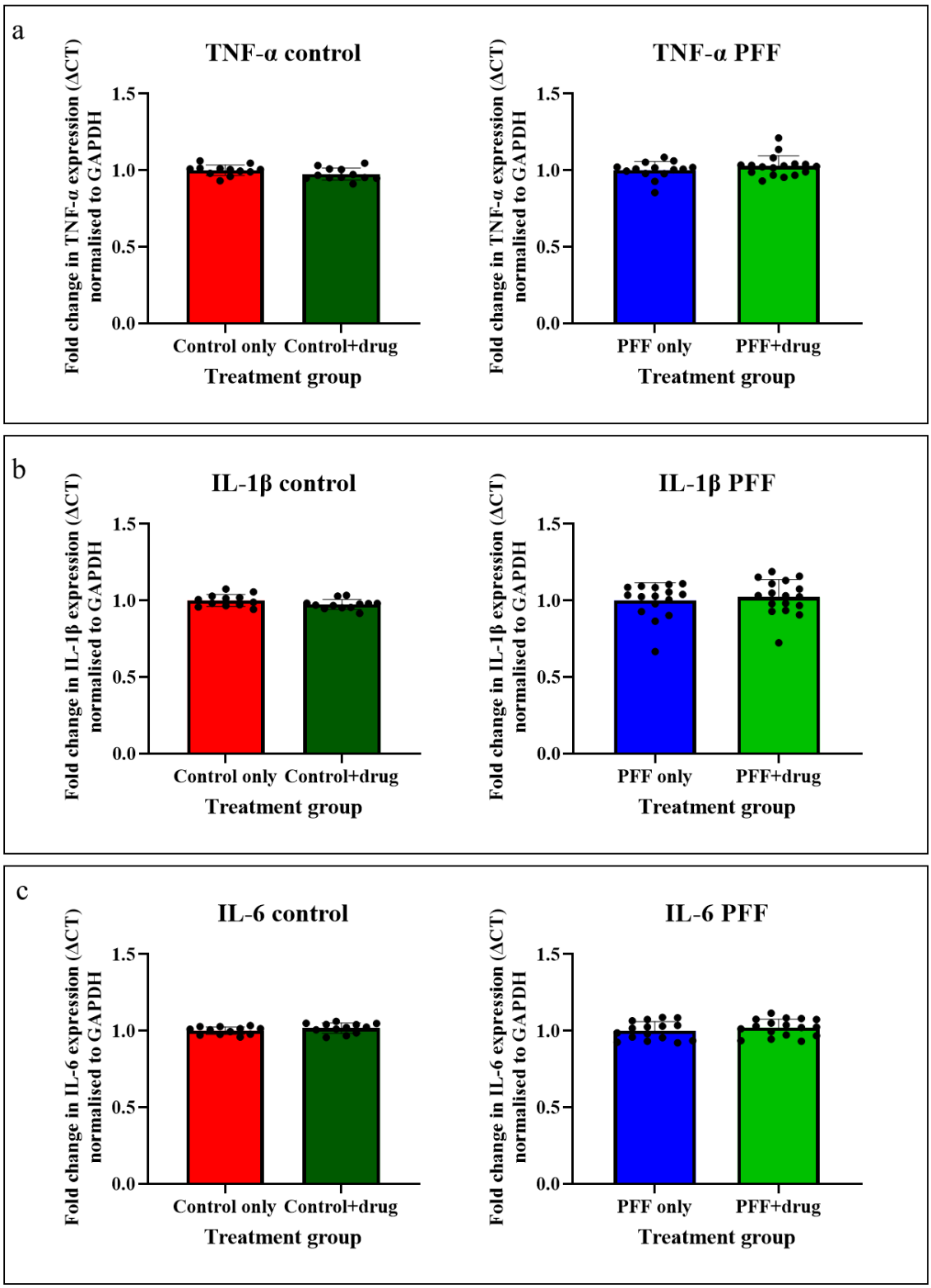


Figure 3.21 Candesartan treatment is not associated with alterations in the expression of downstream pro-inflammatory cytokines in the brainstem. *Expression of* ***(a)*** *TNF-α,* ***(b)*** *IL-1β, and* ***(c)*** *IL-6 in untreated and candesartan-treated control (left) and PFF-injected (right) animals. Normalised to GAPDH. Untreated normalized to 1 and drug-treated displayed as fold change of the untreated animals in each respective group.*

I also attempted to measure the downstream inflammatory protein levels of TNF-α (**Figure 3.22**) and NF-κB (**Figure 3.23**) using western blotting techniques, but none of the antibodies were successful at identifying a band at the correct MW so protein expression levels of these factors were not determined.

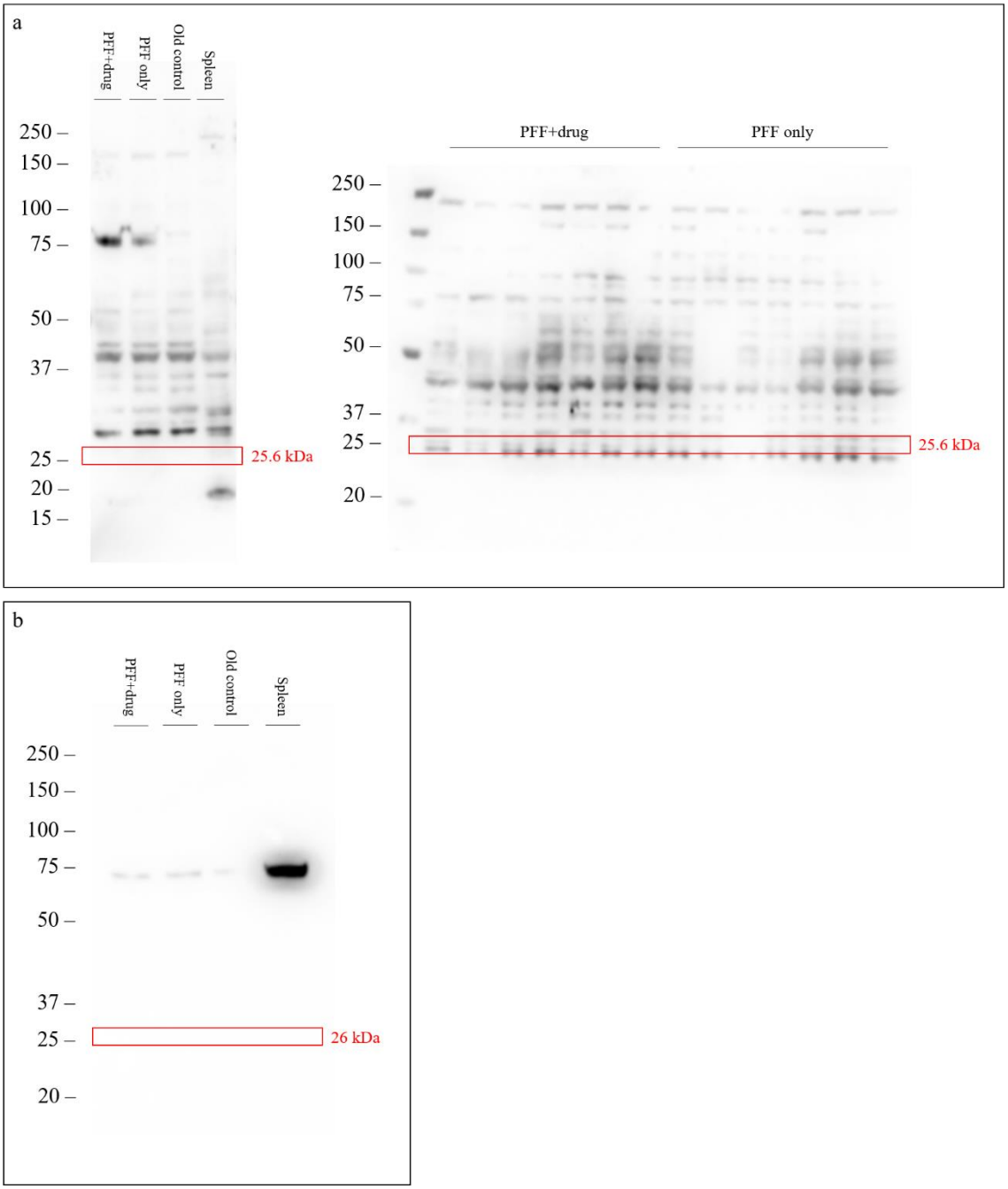


Figure 3.22 Western blots of TNF-α were unsuccessful at identifying a band at the correct molecular weight for TNF-α (26 kDa). *Example western blots using* ***(a)*** *Abcam ab6671 or* ***(b)*** *Abcam ab205587.*

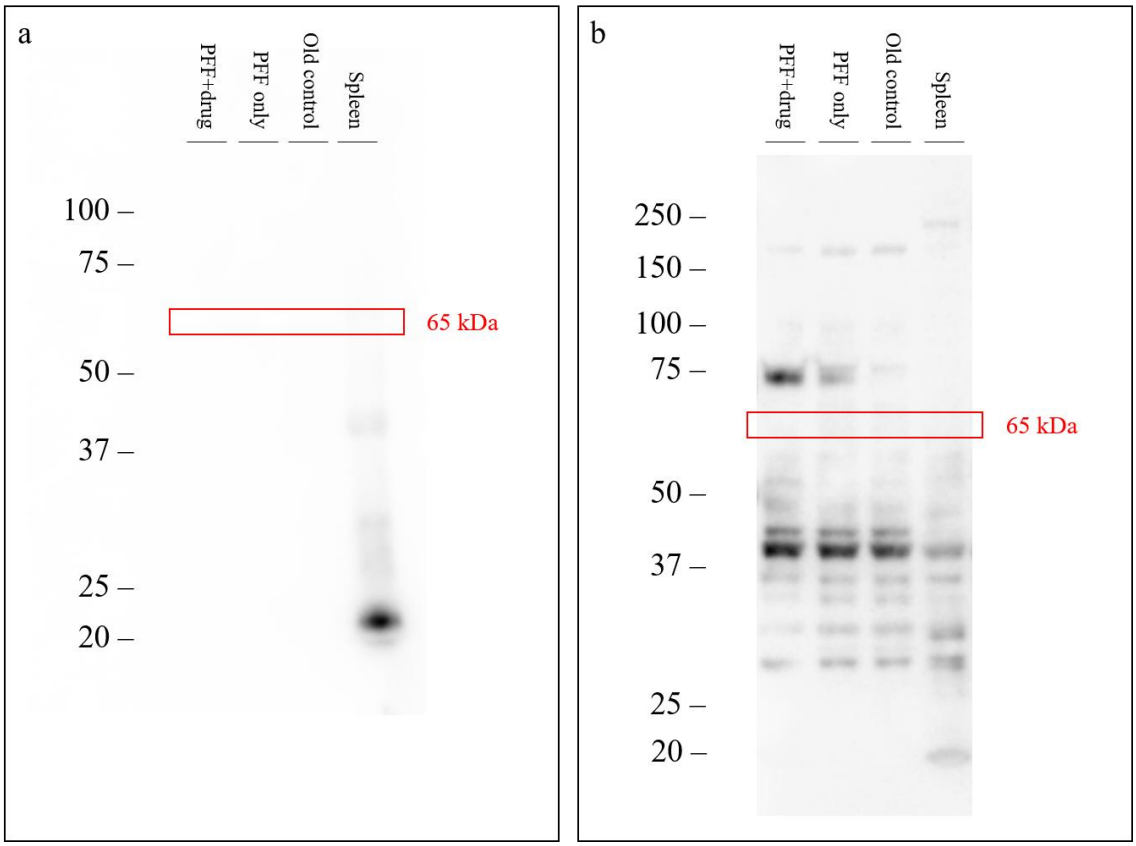


Figure 3.23 Western blots were unsuccessful at identifying band at the correct molecular weight for NF-κB (65 kDa). *Western blot using NFκB Cell Signalling Technology #3033 antibody blocked and diluted in* ***(a)*** *5% milk or* ***(b)*** *5% BSA. Spleen used as positive control.*

3.4.5 Does candesartan treatment influence serum peripheral immune factors?

Peripheral inflammatory markers were measured using an MSD assay. This was carried out on animal serum taken from all animals at baseline and then each 2 month timepoint. The majority of the cytokines in the multiplex assay were below the limit of detection or had such low values that the mean coefficient of variation (CVs) were too high and so deemed unacceptable for use. The three cytokines with acceptable CVs and so used for investigation were TNF-α, IL-10, and KC/GRO (a chemoattractant for monocytes and neutrophils known as CXCL1 in humans). These three assays had mean CVs of below 40% - 30.8%, 32.2%, and 3.7% respectively. These cytokines also contained many samples below the limit of detection – TNF-α PFF 31.6%, control 26.3%; IL-10 PFF 57.4%, control 7.9%; KC/GRO PFF 5.1%, control 0%. For analysis, these samples below the limit of detection were allocated a value between 0 and the lower limit of detection, as is standard practice.

Investigating changes in cytokines over time does not show any significant differences in the levels of TNF-α or IL-10 over time. (**Figure 3.24a-b**). In addition, the levels of IL-10 at the 2 and 6 month timepoints were all below the level of detection, possibly indicating an issue with this cytokine assay. However, KC/GRO shows a clear peak at the 2 month timepoint in the PFF-injected animals which is not seen in the control animals which show a significant decrease in KC/GRO over time; this could be indicative of an early peripheral immune response in the PFF-injected animals. (**Figure 3.24c**). (Mixed-effects analysis followed by Šídák's multiple comparisons test; $p<0.05$; $p<0.01$; $p<0.0001$). Comparison between treatment groups at different timepoints showed significant decreases in KC/GRO on candesartan treatment at 2 months, indicating that candesartan may be attenuating the early peripheral immune response. Candesartan is also seen to significantly decrease KC/GRO in the control study at the 6 month timepoint (**Figure 3.24c**). (Mixed-effects analysis followed by Šídák's multiple comparisons test; $p<0.05$). No other significant differences were seen between treated and untreated groups.

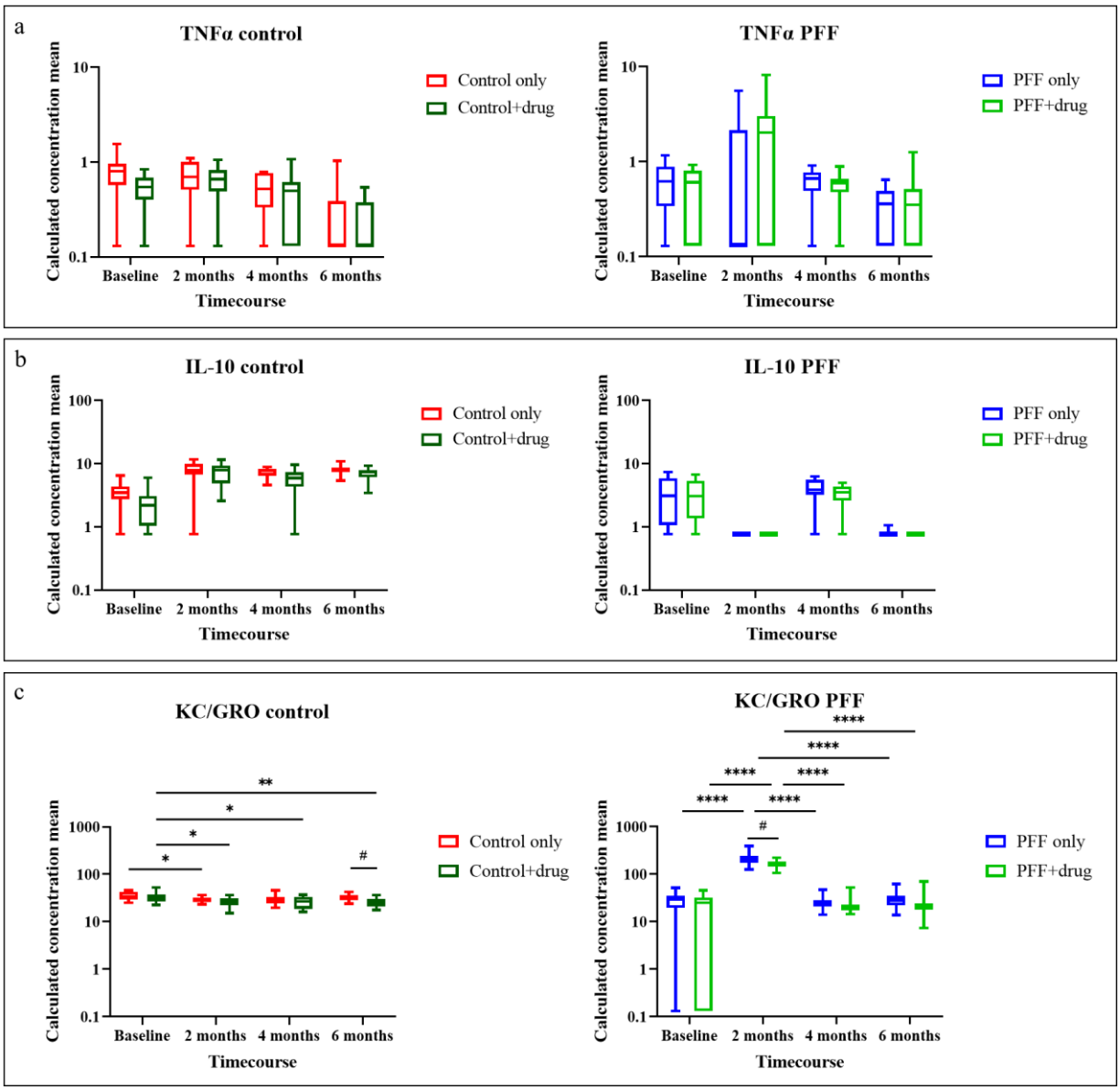


Figure 3.24 Peripheral serum cytokine levels over time in candesartan treated versus untreated animals. *Calculated concentration mean of* ***(a)*** *TNF-α,* ***(b)*** *IL-10, and* ***(c)*** *KC/GRO in candesartan-treated and untreated control (left) and PFF-injected (right) animals. Mixed-effects analysis followed by Šídák's multiple comparisons test; p*<0.05; p**<0.01; p****<0.0001. Mixed-effects analysis followed by Šídák's multiple comparisons test; $p^{\#}$<0.05.*

3.5 Discussion

3.5.1 Summary

Neuroinflammation has been implicated in the development and progression of Parkinson's disease. The hypothesis being investigated in this thesis is whether this neuroinflammation is mediated though the binding of aggregated α-synuclein to Toll-like receptors found on microglia and innate immune cells, leading to an exacerbation of neurodegenerative pathology. To test this, the drug candesartan, a putative TLR2 and 4 blocker, was used in an α-synuclein animal model. The use and effects of candesartan treatment on behaviour and neurodegeneration in this animal model is described in the previous chapter.

In this chapter I sought to identify whether the mechanism through which candesartan was having a protective effect on the development of cholinergic degeneration was through the inhibition of TLR activation, and subsequent neuroinflammation. The results show that candesartan is present in the brain, and thus potentially able to cross the blood brain barrier. However my data do not clearly show a significant impact of candesartan on the levels of TLR expression or protein levels in the brains of PFF-injected animals – though a significant decrease in TLR2 protein levels in the brainstem of candesartan-treated compared to untreated control animals was seen. There also does not appear to be any effect of candesartan on downstream neuroinflammation as shown by a lack of microglial activation, and by the lack of detectible changes to the expression of downstream inflammatory cytokines in the brain including TNF-α and IL-1β. Finally, the PFF model appears to show early activation of the peripheral immune system as seen through a significant increase in the chemokine CXCL1 (referred to as KC/GRO in the MSD assay). This is attenuated on candesartan treatment, indicating that candesartan could be having a protective effect through reducing peripheral inflammation.

These results suggest that candesartan is having a minimal effect on TLR activation and inflammation in the brain of this α-synuclein PFF animal model. However, the data indicate that very limited inflammation is occurring within the PFF-injected animals' brains, thus

making it difficult to measure any impact of candesartan on brain inflammatory processes. Whilst it is possible that early reduction of peripheral inflammation in candesartan treated PFF animals could have some impact on reducing neurotoxicity, it seems likely that the action of candesartan to attenuate degeneration of cholinergic neurons in the brainstem is occurring through mechanisms other than anti-inflammatory ones.

3.5.2 Lack of neuroinflammation

The main limitation of this study was that significant brain inflammation was not induced by the peripheral injection of the α-synuclein PFFs, making it difficult to evaluate any anti-inflammatory effect of candesartan. This lack of inflammation was shown through comparison of PFF-injected and saline-injected control animals at the same experimental timepoint and through a comparison of PFF-injected and saline-injecte animals at the same age but different experimental timepoint (required due to the two parallel studies having animals at slighty different ages at the start of experimentation).

Inflammation is found in centrally-induced PFF models – including an upregulation of MHCII (Duffy et al., 2018; Harms et al., 2017; Thomsen et al., 2021) and an increase in activated $Iba1^{+}$ microglia (Duffy et al., 2018). The markers of inflammation reported in centrally-induced PFF models were investigated here in the Kuan et al., (2019) model but neither a change in microglial morphology nor an increase in MHCII expression was seen at any of the recorded timepoints. The peripheral site of injection and the conjugation of the PFFs to RVG9R (the rabies glycoprotein allowing the passage of the PFFs across the blood-brain) are the two main differences between the model used in this study and other models showing PFF-induced inflammation.

As described in ***Chapter 2.2.1.4***, one of the advantages of the Kuan et al., (2019) model is that the injection occurs peripherally. This decreases the probability that surgical injection-specific inflammation will be seen in the brain, potentially removing a source of immune cell priming in the brain. The PFFs still enter the brain, but through less invasive mechanisms. If true that PFFs are responsible for priming immune cells, as discussed in the chapter introduction, it is unclear why this would not occur in the model used here unless the RVG9R conjugation prevented the immunogenicity of the PFFs without affecting their ability to induce endogenous α-synuclein aggregation. In the original study using RVG9R, Kumar et al., (2007) showed that repeated administration of RVG9R did not induce inflammatory

cytokines or antibodies against the peptide. This negates the possibility of the RVG9R itself resulting in the initial activation or priming of the immune system in the model used here. Other peripherally-induced models, such as that produced through the injection of PFFs into the duodenum (Challis et al., 2020), have shown local inflammation induced early post-injection. Unfortunately they have not investigated whether this inflammation persists post the initial injection insult, or whether there is inflammation in the brain in addition to the α-synuclein pathology which spreads to the brains of these mice when aged (Challis et al., 2020). Investigation into this would be interesting to determine whether later-onset neuroinflammation, independent to the initial acute response to a foreign substance (i.e. the PFFs), occurs without CNS injection.

The immunogenic α-synuclein species in PD has not been fully established. It is thought that pro-inflammatory effects arise from the aggregation of the endogenous α-synuclein into soluble oligomeric species followed by arrangement into larger fibrils and ultimately Lewy body-like inclusions (Cremades et al., 2012). Multiple studies have shown the formation of these inclusions on CNS-injection of PFFs. Some have suggested fibrils to have the greatest pro-inflammatory effect (Grozdanov et al., 2019; Hoffmann et al., 2016). Conversely, others suggest that the oligomeric form has the greatest pro-inflammatory effect and is therefore the most toxic (Emin et al., 2022). In the animal model used in my work, aggregation into high molecular weight (HMW) species has been identified in ***Chapter 2.4.2*** using immunoprecipitation and immunoblotting techniques. However, the specific presence of Lewy body-like pathology was not established in this work despite attempts made to replicate that seen in Kuan et al., (2019). This unfortunately made it impossible to determine specifically where α-synuclein pathology was occurring and as a result to determine whether there was any colocalisation with activated immune cells. Antibodies claiming to distinguish different α-synuclein species have been produced, however their specificity is doubted by many in the field. As a result, it is very difficult to identify forms of α-synuclein other than large insoluble fibrils, and so the presence of oligomeric species within the animal model used here remain elusive. Investigation into the immunogenic species of α-synuclein will be carried out in ***Chapter 4***.

It has been suggested that inflammation in human PD occurs very early in the disease course (Iannaccone et al., 2013; Kim et al., 2022; Ouchi et al., 2005). This could similarly be occurring in this rat model of PD, however the earliest timepoint at which data was gathered –

including blood for peripheral inflammation determination and post-mortem brains for central inflammation determination – was at 2 months post PFF injection. No neuroinflammation is occurring at this earliest timepoint, however in the periphery there is a significant increase in the chemokine KC/GRO (CXCL1). CXCL1 is a chemoattractant for multiple immune cells including monocytes, T-cells, and neutrophils (Serdar et al., 2020). This could be indicative of the peripheral immune system being activated by PFFs, either as the injected PFFs themselves (though it is unlikely that these will still be present in the animal at 2 months post-injection), or in response to the aggregation of endogenous α-synuclein. Monocytes can be activated directly by α-synuclein, and α-synuclein may then be presented to T-cells and B-cells resulting in their activation and further peripheral inflammation. It is possible that the KC/GRO response seen at 2 months is the tail-end or the initiation of a more intense peripheral immune activation which has not been detected at earlier timepoints through lack of data, or has already faded by the 4 month timepoint. Peripheral inflammation can have neurotoxic effects, however these commonly occur through the induction and exacerbation of central inflammation, leading to a neurotoxic environment (Filipov, 2019) which is not seen in my work. Candesartan is having an attenuating effect on this peripheral inflammation at 2 months, and so however peripheral inflammation is having a neurotoxic effect, the action of candesartan in reducing this may be a contributory factor to the neuroprotective effects observed in ***Chapter 2***. One limitation of the MSD assays was that different timepoints were carried out on different plates and so inter-plate variability may contribute to differences seen between timepoints. This could be rectified through the use of a positive control within each plate, however unfortunately this was not carried out within the assays completed here.

Future investigation into whether there is an early acute inflammatory effect specifically to the injected PFFs could be carried out. It would be interesting to observe whether there is an initial inflammatory response to the foreign substance of PFFs, as if not this could aid the discussion of whether this priming of the immune system is crucial to the development of inflammation further along the disease progression. It would also be interesting to determine whether there is an inflammatory response within these first 2 months leading to the induction of KC/GRO, and whether in my study I have simply missed a larger peripheral response due to not collecting data at the correct timepoint. This work warrants further exploration into peripheral inflammation, and could involve peripheral immunophenotyping studies in this model.

3.5.3 Alternative mechanism of action of candesartan

Candesartan is a licensed drug for the treatment of hypertension. It achieves this through its action as an angiotensin II receptor blocker (AT_1 blocker). Candesartan binds specifically to the AT_1 receptor and inhibits the binding of angiotensin II through a non-competitive mechanism. It has been suggested that through this blockade candesartan may be able to have an effect on the progression of Parkinson's disease. Some work has shown that candesartan inhibits the expression of AT_1 receptors (Rodriguez-Perez et al., 2018), however this is not universal and others have shown no effect on mRNA expression of AT_1 (Nishimura et al., 2000). My work similarly showed no differences in mRNA expression of AT_1 receptors on candesartan treatment. LCMS was carried out to ensure that the candesartan was reaching the brain and clear peaks corresponding to candesartan were seen in the brain for up to 24 hours (**Figure 3.5**). One consideration in this particular experiment is that the animal brains were not perfused and for that reason there would still have been blood in the cerebral blood vessels. As a result, it cannot be claimed for certain that any candesartan detected in the homogenised animal brains is from candesartan in the brain parenchyma rather than from residual blood in these cerebral blood vessels. This could explain why AT_1 mRNA levels are not decreased. Further indicators of AT_1 blockade were not assessed. Though likely that candesartan is carrying out its blockade of AT_1, as supported by a wealth of evidence in other studies, it has not been specifically proven in my study.

Angiotensin II binds to angiotensin II type 1 receptors (AT_1), and through this has proinflammatory actions (Suzuki et al., 2003). AT_1 receptors are expressed in the substantia nigra and the striatum (Allen et al., 1992). Angiotensin II acts to stimulate NADPH oxidase to produce reactive oxygen species (ROS) (Griendling and Ushio-Fukai, 2000). Dysregulation of ROS results in oxidative stress, the production of toxic species including 4-hydroxynonenal (HNE), the oxidation of proteins, lipids, and DNA, and an environment generally toxic to cells (Jenner, 2003; Rodriguez-Pallares et al., 2008). It is thought that oxidative stress may play a role in Parkinson's disease whereby it results in an environment toxic to dopaminergic neurons, resulting in their death and the development of PD. Though oxidative stress is mainly linked to mitochondrial dysfunction, it may also be associated with dysfunction in the renin-angiotensin system. An increase in AT_1 expression has been seen in response to α-synuclein as seen in the AAV9-α-synuclein animal model (Rodriguez-Perez et al., 2018), however this has not been identified in human PD. AT_1 serum autoantibodies (which act as agonists to AT_1 receptors) were seen to be increased in a group of people with PD compared to controls

(Labandeira et al., 2022). Therefore, the action of candesartan in blocking AT_1 receptors and reducing oxidative stress may explain how candesartan is having a protective effect on the survival of $ChAT^+$ neurons in ***Chapter 2.4.5*** despite not altering levels of inflammation. A study into how candesartan and other AT_1 blockers affected the progression of pathology in an α-synuclein overexpression animal model showed that the blockage of AT_1 had a protective effect on the development of pathology (Rodriguez-Perez et al., 2018). This work looked into the anti-inflammatory action of candesartan – showing a decrease in MHC II-positive microglia and in pro-inflammatory cytokine expression in treated vs untreated α-synuclein overexpressing animals – however this was not investigated in regards to the involvement of TLRs. Furthermore, it was shown that the AT_1 blockers decreased the oxidative stress induced by α-synuclein overexpression.

3.5.3.1 Alternative TLR-mediated mechanism

In addition to their presence on immune cells, TLRs are also found on neurons (Barajon et al., 2009; Lafon et al., 2006; Ma et al., 2006; Tang et al., 2007; Zhou et al., 2009). In these cells the activation of TLRs results in different downstream effects; one such effect is in the process of autophagic flux. As discussed in ***Chapter 1.2.1.2***, dysfunction of the autophagy-lysosome pathway is thought to aid α-synuclein propagation between cells through resulting in the increased exocytosis of α-synuclein (Alvarez-Erviti et al., 2011; Danzer et al., 2012; Lee et al., 2013; Poehler et al., 2014). Multiple studies have shown that the activation of neuronal TLRs results in the inhibition of autophagy. The activation of neuronal TLR2 (Chedid et al., 2022; Kim et al., 2021) and TLR4 (Chung et al., 2022) has been shown *in vitro* in SH-SY5Y neurons. When co-cultured with LPS-activated microglia (Chung et al., 2022), treated with a TLR2 agonist (Chedid et al., 2022), or with α-synuclein fibrils (Kim et al., 2021) an increase in the autophagic flux marker p62 was observed, indicative of a dampening of flux (Liu et al., 2016). The use of TLR2/4 inhibitors ameliorated this accumulation of p62 in addition to the build-up of phosphorylated α-synuclein, demonstrating a role for activated TLRs in the inhibition of autophagy. This inhibition of autophagy could also explain the increase in neuron-to-neuron transmission of α-synuclein as seen in work by Kim et al., (2015). Alterations to autophagic flux were similarly seen in microglia in a PFF-injected mouse brain. However, in this work TLR4 was crucial for autophago-lysosomal activity to occur and TLR4 deficient mice showed deficits in this activity (Venezia et al., 2017). These studies show that the suppression of TLRs can have effects independent of inflammation. It is possible that the protective effect of candesartan described in ***Chapter 2*** is occurring through its action in

suppressing the expression of neuronal TLRs, leading to increased autophagic flux. This would result in a decrease in α-synuclein accumulation, a decrease in the exocytosis of α-synuclein, and therefore a decrease in the pathological spread of α-synuclein through the brain. If true, this could explain the protective effect of candesartan treatment on the survival of the $ChAT^+$ neurons. In my study, levels of TLRs 2 and 4 have only been quantified through western blotting. This does not allow the determination of cell-specific expression, and may be weighted towards microglial TLR expression due to their expressing higher levels of TLRs than neurons. This could be achieved through immunohistochemistry, however this was not possible in this study due to a failure to find any convincing antibodies staining specifically for TLR expression.

The work described in this chapter has conclusively shown that inflammation is not present within the brain in this animal model, and indicates that candesartan is having a minimal effect on brain inflammation, though whether this is due to the lack of PFF-induced neuroinflammation is unclear. Further work is needed to determine the protective mechanism by which candesartan is slowing the progression of disease pathology as shown in ***Chapter 2***.

Chapter 4.

Investigating whether blocking TLRs alters α-synuclein-induced inflammation in peripheral immune cells of people with Parkinson's disease

DECLARATION

Chapter 4.5.4 **"TLR blockers"** contains some text modified from the published review article **"Toll-like receptors and their therapeutic potential in Parkinson's disease and α-synucleinopathies".** This article was published in Brain, Behaviour, and Immunity: **https://doi.org/10.1016/j.bbi.2019.06.042** under Green open access with a 12-month embargo which has now lifted. The accepted manuscript is freely available to view within Apollo – the University of Cambridge repository at:

https://www.repository.cam.ac.uk/items/2abbd448-b6b5-4aa5-a22c-22279f75ecd9

The author contributions are as follows: I, Catherine Kennedy (née Horne), and Dr Antonina Kouli are joint first authors and equally contributed to the writing of the text. The figures were produced by Dr Antonina Kouli. Critical feedback was provided by Dr Caroline Williams-Gray in addition to the editing of the final manuscript.

The extraction of peripheral blood mononuclear cells (PBMCs) from whole blood was carried out by a combination of myself, Dr Antonina Kouli, Dr Jonathan Holbrook, and Lakmini Kahanawita. The blood was obtained from participants (both patients and controls) attending the Cambridge Parkinson's Disease Research Clinic at the John Van Geest Centre for Brain Repair in Cambridge. Participants had provided written consent for donation of their blood samples and use in this research (REC number 03/303). The clinic is run by Dr Caroline Williams-Gray.

The production of α-synuclein pre-formed fibrils was carried out by Dr Wei-Li Kuan.

Endotoxin testing was carried out by the Lonza™ Rapid Endotest service.

Super-resolution microscopy and analysis of the images taken was carried out by Dr Evgenia Lobanova.

4.1 Aims of chapter

Given that I was not able to demonstrate a clear mechanism by which candesartan was having a neuroprotective effect in the α-synuclein PFF animals, a key aim of this chapter was to determine whether candesartan blocks the inflammatory response to α-synuclein *in vitro*, using human peripheral immune cells.

Candesartan is reported to reduce the expression of both TLR2 and TLR4, and both receptors have been implicated in mediating the inflammatory response of immune cells to α-synuclein in model systems. However, a key question is whether the response in human PD is predominantly driven by the activation of TLR2 or TLR4. This has important implications for future therapeutic strategies targeting TLRs in PD. Though TLR-blockers have been used in various *in vivo* and *in vitro* studies relevant to PD (as reviewed in ***Chapter 1.4***) there is very limited work investigating how they impact on immune responses using human cells, and in particular no studies have specifically investigated the effect of TLR4-specific blockers on human immune cell responses. In this chapter I aimed to optimise a protocol to study the contribution of both TLRs 2 and 4 to the α-synuclein-induced pro-inflammatory response of immune cells in PD, and to collect pilot data to inform future studies using cells from larger cohorts of PD patients and controls.

4.1.1 Objectives

1. To determine whether α-synuclein produces an inflammatory response in human PBMCs, and which species of α-synuclein produces the most robust response.
2. To investigate whether candesartan is able to block the inflammatory response to α-synuclein in ex vivo human cells
3. To investigate whether blockers specifically targeting TLR2, TLR4, or both receptors, are able to block the inflammatory response to α-synuclein.
4. To investigate the optimal experimental conditions for measuring the α-synuclein induced inflammatory response in human immune cells, and the effects of TLR blockade.

4.2 Introduction

In this chapter, work is being transitioned from animal models to models using human cells. Animal models are useful in researching organisms as a whole and allow the effects of an intervention to be monitored in a whole system, however they are limited in the extent to which they replicate the human disease. For this reason, in this chapter human peripheral blood mononuclear cells (PBMCs) were used to investigate TLR responses.

4.2.1 Monocytes

The use of PBMCs in this chapter is in part due to the growing interest in the role of the peripheral immune system in PD. PBMCs include monocyte cells; these are key cells of the innate immune system. There are three main subgroups of monocytes: classical, intermediate, and non-classical. Classical monocytes are enriched in the blood of people with PD compared to healthy controls (Grozdanov et al., 2014; Wijeyekoon et al., 2020). This has been associated with an increase in TREM2 which is thought to be involved in TLR stimulation, in TLRs 2 and 4 expression (Drouin-Ouellet et al., 2015; Wijeyekoon et al., 2020), and in LRRK2 levels which is thought to contribute to monocyte dysregulation (Bliederhaeuser et al., 2016). Further weight to monocytes playing a role in PD is provided by evidence for their having a distinct gene expression pattern in early stage PD compared to controls. Genes involved in immune activation including TNF-α and MyD88 (the TLR central adapter protein) were found to be differentially expressed (Schlachetzki et al., 2018). This increase in monocyte sub-populations and further dysregulation of monocytes and their expression profiles could result in an increased inflammatory response and could play an important role in the pathology of PD. In addition, monocytes are the predominant cell type expressing TLRs in the peripheral immune system; they express all TLRs with TLRs 1, 2, and 4 at the highest levels (Hornung et al., 2002). This makes them particularly useful in the study of PD where the mostly highly implicated TLRs are TLRs 2 and 4.

The use of PBMCs in this study is also in part due to their forming an easily accessible human cell model. The immune cells of potentially highest interest in PD are microglia,

however these cells are difficult to obtain from humans to study *in vitro*. The only methods of observing microglia *in situ* are through neuroimaging techniques or through post-mortem studies. Neuroimaging allows observation of microglia at different stages of PD, and sequential neuroimaging allows the monitoring of changes in microglial activation over time. (Gerhard et al., 2006; Ouchi et al., 2005). However, these studies do not allow the investigation of individual microglia, or provide methods of studying mechanisms behind microglial activation. Post-mortem work allows the investigation of individual microglia, and some investigation into mechanisms of action. However, in these works microglia are static, and are often capturing late-stage PD. *In vitro* methods of investigating human microglia can involve extracting 1˚ human microglia, or through the use of iPSCs (induced pluripotent stem cells) produced from patient cells. These *in vitro* methods are very useful, however they can be complicated. Peripheral monocytes have some similarities with microglia, though they are ontogenetically distinct. These two cell types are indistinguishable in regards to morphology, can both form inflammatory macrophages, and express many of the same factors – such as Iba1. (Baufeld et al., 2018). I am utilising the similarities between these cell types so as to use monocytes as an easily accessible cell model of microglia. There are differences between the cell types, but it is being assumed that the main TLR-mediated inflammatory responses are similar, and may give us some insight into how microglia may respond to α-synuclein. Another method of modelling microglial responses is through the use of microglial cell lines such as the BV2 mouse microglia cell line. These have been used extensively by others, and, though useful, do not fulfil the remit of this chapter aiming to move towards the human disease.

For the experiments in this chapter, I aimed to stimulate the TLR pathway in human PBMCs/monocytes and used TNF-α as the key read-out due to its production by monocytes downstream of the TLR/NF-κB activation pathway. The role of TNF-α has been particularly implicated in the aetiology and progression of Parkinson's. A study of patients with inflammatory bowel disease (IBD; a disease associated with increased risk of the development of PD) found that those IBD patients who had received anti-TNF therapy showed reduced PD incidence (Peter et al., 2018). In addition, anti-TNF treatments in animal models of PD have shown attenuation of the loss of dopaminergic neurons (Barnum et al., 2014; Harms et al., 2011; McCoy et al., 2008, 2006). From these studies it can be seen that the levels of cytokines, including TNF-α, in patients is important. This TNF-α production may occur due to the activation of TLRs as this cytokine is downstream of their NF-κB activation pathway. The peripheral activation of TLRs 2 and 4 has been shown by Wijeyekoon et al., (2020) and Drouin-

Ouellet et al., (2015). Further research is required to determine how the inhibition of these TLRs may affect the release of TNF-α in human peripheral immune cells, and how this may differ in people with PD or healthy controls.

4.2.2 Toxic species of α-synuclein

Pathogenic forms of α-synuclein are thought to play a key role in activating innate immune cells via TLR 2 and 4, as reviewed in ***Chapter 1.4***, but there is some uncertainty regarding which form is the most inflammatory.

The specific species of aggregated α-synuclein which exerts the highest pro-inflammatory effect is unknown. Recent work by Emin et al., (2022) showed that small soluble α-synuclein aggregates resulted in a higher pro-inflammatory response in BV2 microglia than larger aggregates. One major criticism of many studies is the lack of characterisation of aggregates. This study used a density centrifugation method to separate a heterogeneous mixture of lab-generated aggregate species, and then characterised the species using high resolution microscopy, providing an accurate characterisation of exactly what was being added to the cultured BV2 cells. Additionally, in this study soluble α-synuclein aggregates from soaked PD and healthy control brains were characterized. These aggregates were extracted utilising a technique produced by Hong et al., (2018) whereby post-mortem brain is soaked to extract the soluble oligomeric species. PD brains had a higher proportion of small aggregates compared to the controls, and these small aggregates were more inflammatory to BV2 microglia than the larger aggregates extracted from control brains. Hughes et al., (2019) also showed that oligomeric α-synuclein treatment resulted in higher pro-inflammatory effects than fibrils in the BV2 microglial cell line. These oligomers were produced using simple incubation with shaking methods. In contradictory work, also using BV2 microglia, Hoffmann et al., (2016) showed that fibrillar α-synuclein had an increased pro-inflammatory effect on these microglia compared to monomeric or oligomeric species. The production of oligomers in this study was carried out using incubation with 4-hydroxy-2-nonenal (HNE). HNE induces oligomerisation through covalent modification of α-synuclein, and these oligomers differ in conformational properties to fibrils (Bae et al., 2013). Furthermore, a comprehensive study by Grozdanov et al., (2019) likewise showed that fibrils induce increased inflammation compared to other α-synuclein species. This study used a series of well-categorised recombinant α-synuclein species including multiple types of oligomeric species (both small homogenous and larger heterogeneous species), fibrillar species (fibrils, ribbons, and those produced at differing

pHs), and mutated monomeric species. These were used to stimulate healthy control peripheral blood monocytes and BV2 microglia.

The pro-inflammatory nature of α-synuclein has thus been well-established, however further work is required to determine the most pro-inflammatory species.

4.2.3 TLR blockers

Candesartan has been repurposed as a TLR blocker through its reduction of the expression of both TLRs 2 and 4 (Dasu et al., 2009). The blockade of the α-synuclein-induced inflammatory response by candesartan has been investigated by Daniele et al., (2015), however this was carried out in primary mouse microglia. The effectiveness of candesartan at blocking the response of human immune cells to α-synuclein has not been established. This work also used the TLR2 specific blocker CU-CPT22. CU-CPT22 is a small molecule inhibitor, which acts to inhibit the TLR1/TLR2 heterodimer through interaction with the extracellular domain of the TLR (Cheng et al., 2012). Through this they showed that the α-synuclein-induced inflammatory response was dependent on TLR2 signalling. However, TLR4 was not investigated in this work, and so although a TLR2-dependent response is seen, the contribution of TLR4 is unknown. Others, including Hughes et al., (2019) have shown that the inflammatory response of microglia (in this case the BV2 mouse microglial cell line) is TLR4-dependent. This work was carried out using TAK-242, another small molecule inhibitor. It works to suppress TLR4 activation via its action on the intracellular domains of the TLR - specifically the TIR domain – through which it inhibits signal conduction (Ii et al., 2006; Kawamoto et al., 2008). These two studies, due to their only investigating either TLR2 or TLR4, have not fully established which TLR is the most essential to the α-synuclein-induced inflammatory response, and neither have observed this in humans. The use of candesartan in this chapter will allow the inhibition of expression of both TLRs 2 and 4. The use of CU-CPT22 and TAK-242 will allow the determination of which TLR blockade is most effective at attenuating an α-synuclein-induced response.

4.3 Methods

4.3.1 PBMC extraction

Venous blood was collected from participants (both patients and controls) attending the Cambridge Parkinson's Disease Research Clinic at the John Van Geest Centre for Brain Repair in Cambridge. Participants had provided written consent for donation of their blood samples and use in this research (REC number 03/303). Blood was collected in $EDTA^{+}$ tubes to prevent clotting. The blood was mixed with sterile PBS 1:1 and added to SepMate^{TM} tubes containing Ficoll. These were then spun at 120000 g for 10 min to separate the PBMCs. The PBMCs were washed in PBS and spun twice before being resuspended in 10 ml PBS and counted. The cells were then centrifuged and resuspended in 90 % FBS/10% DMSO before being quickly transferred to the -80˚C freezer initially, and then to long-term storage in liquid nitrogen. Cells were extracted either by myself or by a research associate within the lab group.

4.3.2 PBMC thawing

Cryovials were thawed and immediately diluted in warmed RPMI media supplemented with foetal bovine serum and pen-strep. The cells were then spun at 400 g for 5 min, the supernatant discarded, and the cells resuspended in warm culture medium. This process was carried out very quickly to ensure that the cells were left in toxic DMSO for as little time as possible. The cells were counted before being centrifuged again and resuspended to make a solution of 1 million cells/ml.

4.3.3 Monocyte separation

Monocyte separation was carried out using a kit from Miltenyi Biotec (*Cat. No. 130-050-201*). The kit protocol was used. Briefly, after thawing, cells were counted and spun at 300 g and 4˚C for 10 min before being resuspended in MACS buffer. The correct volume of CD14 beads according to PBMC number (20 µl per 10^7 cells) was added and the solution incubated for 15 min at 4˚C. The column was placed into the magnetic field of a MACS separator and washed before the cell suspension was added. Unlabelled cells passed through the column and

the column was washed to ensure only labelled cells remained. The unlabelled cells were discarded. The column was removed from the magnetic field and the magnetically labelled cells eluted using a plunger. The cells were then counted before use in the assay.

4.3.4 Stimulation assays

PBMCs or monocytes were plated in flat-bottomed 96-well plates with 100,000 cells per well and left to rest for 2 hours in an incubator at 5% CO_2 and 37˚C.

4.3.4.1 Stimulation experiments

The cells were stimulated with either LPS (10 ng/ml), α-synuclein monomers (concentration dependent on oligomeric concentration in specific assay; rPeptide), α-synuclein oligomers (concentration between 0.5 μM and 5 μM and optimised as described later; see below for method of production), or α-synuclein PFFs (4 μM; see below for method of production). These were then left for 24 hours, 48 hours, or 72 hours in a 37˚C incubator. The supernatant was removed into a v-bottomed 96-well plate. This was spun at 300g for 5 minutes to pellet the cells. The supernatant was then removed, aliquotted, and frozen at -80˚C for future ELISA experiments.

4.3.4.2 TLR blockade experiments

After the cells were plated and left to rest for 2 hours, either Candesartan (concentration between 5 μM and 30 μM and optimised as later described; ThermoFisher *Cat. No. 466190010*), TAK-242 (1 μM; Tocris Biosciences *Cat. No. 6587*), or Cu-CUPT22 (concentration between 5 μM and 30 μM and optimised as later described; Tocris Biosciences *Cat. No. 4884*) was added, or the cells were left untreated. All drugs were resuspended in DMSO according to the manufacturer's instructions. The cells were treated with blocking agents alone (or left untreated) for 24 hours before the addition of stimulants. The stimulation and supernatant collection were carried out as described above.

4.3.5 Preparation of α-synuclein pre-formed fibrils

A-synuclein pre-formed fibrils were prepared as previously described in the literature (Volpicelli-Daley et al., 2014). A-synuclein monomers (rPeptide) were resuspended in 10 mM Tris-HCl, 50 mM NaCl, pH 7.6 at a concentration of 5 mg/ml. A 37˚C thermomixer was used to shake them for 7 days at 1000 r.p.m., they were then aliquoted and transferred to a -80˚C

freezer. Prior to use the PFFs were thawed and diluted to 0.1 μg/μl and sonicated using a Soniprep 150 Plus ultrasonic disintegrator (MSE) with 60 pulses at 10% power with a pause every 10 pulses to allow the solution to cool.

4.3.6 Preparation of α-synuclein oligomers

A-synuclein oligomers were prepared using a technique developed and characterised by the Klenerman group (Department of Chemistry, University of Cambridge). A-synuclein monomers (rPeptide) were resuspended in PBS at a concentration of 70 μM. A 37˚C thermomixer was used to shake them for 6 hours at 200 r.p.m., they were then either kept at 4˚C for up to 24 hours before use or were aliquoted and transferred to a -80˚C freezer. For use in stimulation assays, the oligomers were thawed (if frozen) and diluted to the required concentration directly in the well containing the media-PBMC solution.

4.3.7 Endotoxin testing

The α-synuclein monomers obtained from rPeptide were tested for endotoxin using the Lonza™ Rapid Endotest service. This service used a Kinetic-QCL™ LAL Assay. Briefly, the chromogenic substrate (consisting of the LAL reagent – Limulus amebocyte lysate; reacts to bacterial endotoxin and LPS – and a chromogenic reagent) is added to the test sample. This will turn yellow after an amount of time inversely proportional to the amount of endotoxin in the sample. The colour change is monitored using an incubating absorbance plate reader measuring absorbance at 405 nm. The amount of endotoxin can be measured through comparison to a standard curve. (Methods obtained from the Lonza webpage for the Kinetic-QCL™ Kinetic Chromogenic LAL Assay; all aspects of the testing process were carried out by Lonza and not by me).

4.3.8 Super-resolution microscopy

All super resolution microscopy was carried out by Dr Evgenia Lobanova. For full methods see Emin et al., (2022). Briefly, the anti-α-synuclein capture antibody (MJFR-14-6-4-2; Abcam; *ab227047*) diluted to 10 nM was added to the prepared coverslip and incubated for 10 min. The coverslip was washed and then the samples incubated for an hour. The detection antibody (MJFR-14-6-4-2-647; Abcam; *ab216309*) was incubated for up to 30 min. The coverslip was washed and then imaged. STORM microscopy was used for super-resolution microscopy; 8000 frames of 15 ms each.

4.3.9 Enzyme-linked immunosorbent assays (ELISA)

The levels of TNF-α and IL-1β in cell culture supernatants were quantified using ELISAs. Two different kits were used due the failure of a kit partway through experimental process, these were the Invitrogen kit *Cat No. CHC1753* (TNF-α) or *CHC1213* (IL-1β) and the R&D Systems kit *Cat No. DY210* (TNF-α) or *DY201* (IL-1β). Optical density was measured at 450 nm using the FLUOstar Omega plate reader. Each standard and sample were measured in 2 adjacent replicates and an average value calculated. The measured optical density was plotted against the known concentration of the standards to generate a 4 parameter curve logistic curve fit using the *MARS* data analysis software.

4.3.9.1 ELISA kit failure

Having successfully used the Invitrogen kit for many experiments, as indicated above, over several months, it stopped worked partway through the optimisation of the oligomer concentration gradients. The nature of the failure was unknown. Despite many repeats using new kits from the same supplier, and direct comparison of old and new standards, the standard curves were not developing. A subset of images of these plates is included in **Figure 4.1**. After multiple plate failures, wastage of samples, and communication with the manufacturer it was decided to try a new manufacturer – R&D systems.

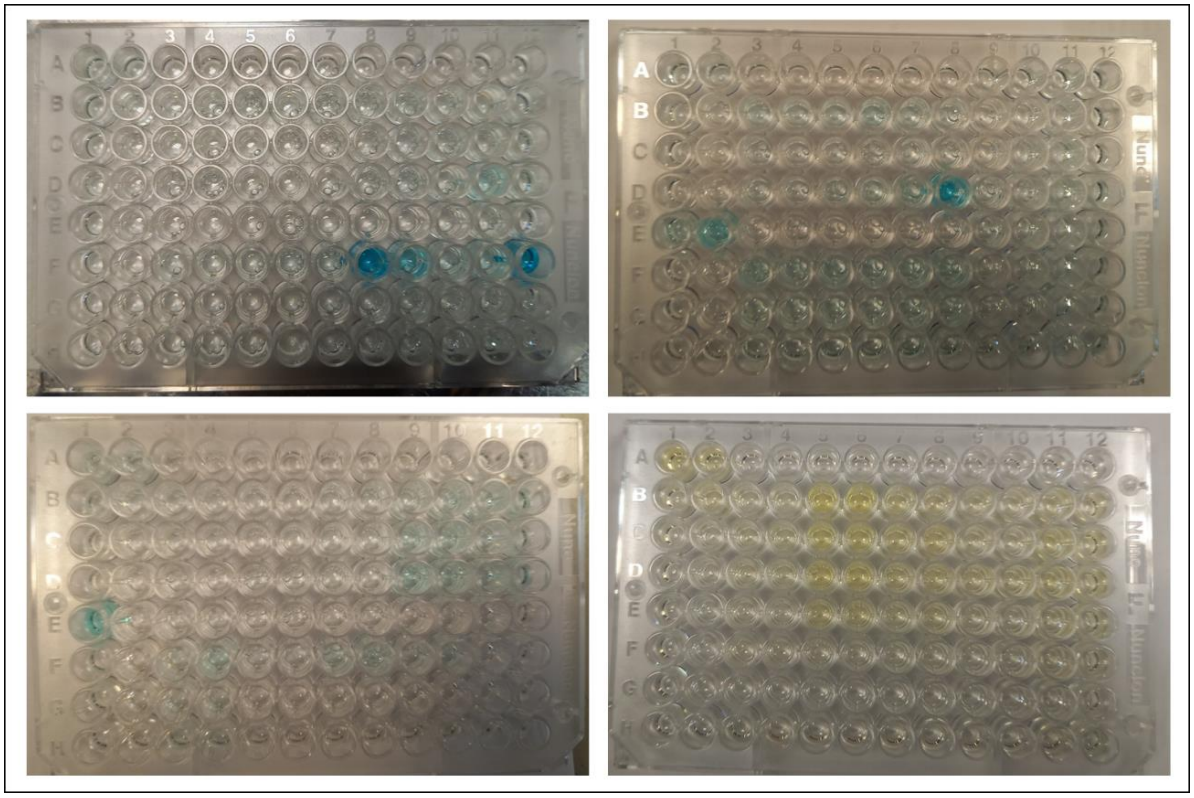


Figure 4.1 Failed ELISA plates using the Invitrogen kit. *Standard curve in duplicate is in the first two columns on the left of each plate, remainder of wells are samples in duplicate. The plate on the bottom right was photographed after adding 2M sulphuric acid to stop the colour change reaction, hence the yellow colour, the other plates were photographed before the addition of the sulphuric acid.*

4.3.9.2 Dilutions in ELISA

The most effective dilution of supernatant to use in the assay was dependent on the kit used and on the individual being tested. When using the Invitrogen kit a dilution of 1:8 was used. This dilution was recommended by the manufacturer and had been tested by others in the lab prior to the work carried out here. On the transition from the Invitrogen kit to the new kit from R&D systems, it was found that a 1:8 dilution caused plate saturation (**Figure 4.2aI**). Samples were diluted to 1:16 and also resulted in complete plate saturation (**Figure 4.2aII**). A concentration gradient was then carried out from 1:32 down to 1:128 (**Figure 4.2aIII**). These dilutions resulted in measurements of TNF-α well-within the standard curve. In later experiments, with various individual's cells, the results were below the level of detection. It was decided that the dilution gradient needed repeating to ensure that results were not being missed through samples being over-diluted. This was repeated first with two subjects' cells and surprisingly it was found that TNF-α produced in response to all stimulants was well below the

top of the standard curve but still not at an acceptable level even in response to LPS which would be expected to result in a much higher response (**Figure 4.2bI**). This was then repeated with more samples to ensure that this wasn't solely the response of one outlying sample. In all 4 of these samples the response was substantially below the top of the standard curve even at a 1:8 dilution (**Figure 4.2bII**). These experiments showed unexpected results where the monomers/oligomers barely showed a response above the unstimulated, and even the LPS did not have as high a response as had been seen before. For this reason, a final dilution gradient was carried out with new batches of monomers, oligomers, and LPS. This showed a response similar to that seen before, and even with these high responses they remained sufficiently below the standard curve at a 1:8 dilution (**Figure 4.2bIII**). It was decided to use the 1:8 dilution from this point forward as there was the concern that, though the responses were well below the standard curve, diluting even less may result in the complete saturation of some individuals due to the wide range of individual responses.

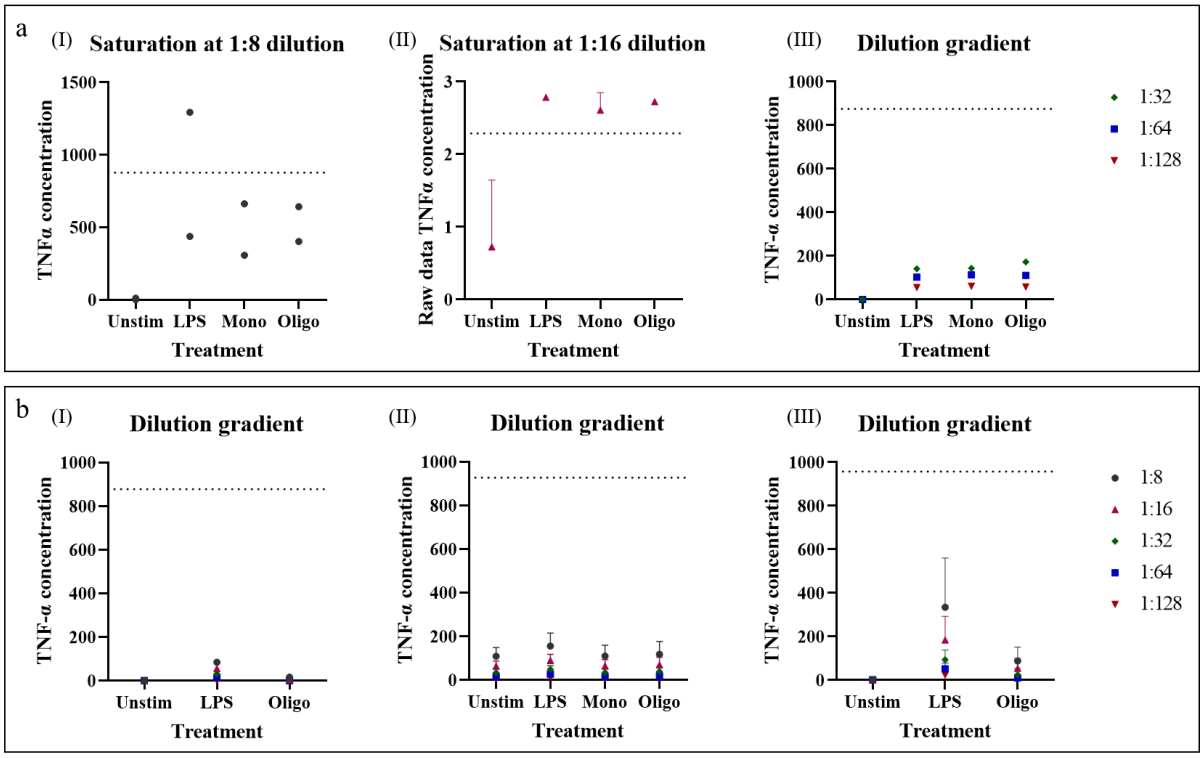


Figure 4.2 Dilution gradients of various subject's cells. (a) *Plate saturation at* ***(I)*** *1:8 dilution (N=2);* ***(II)*** *1;16 dilution (N=2);* ***(III)*** *a partial dilution gradient of 1:32-1:128 (N=4).* ***(b)*** *Full dilution gradient of 1:8-1:128.* ***(I)*** *N=2;* ***(II)*** *N=4;* ***(III)*** *N=4. Dotted line represents top of standard curve for each assay.*

4.4 Results

4.4.1 Monocytes show a high basal level of inflammation

I originally planned to use MACS separated monocytes in my stimulation experiments. However my preliminary experiments showed a high level of basal inflammation in unstimulated cultured monocytes, which might mask any subtle stimulatory effects induced by α-synuclein. The reason for this basal inflammation was not clear so work was carried out to establish whether this was occurring due to stress induced by the cell thawing process, or due to the monocyte cell separation process. A comparison between both fresh and frozen/thawed monocytes and PBMCs was carried out (**Figure 4.3a-b**). These cells were all taken from the same individual and processed at the same time. Fresh monocytes showed increased levels of basal TNF-α production compared to fresh PBMCs, potentially indicating that the cell separation process induces cell stress and cytokine production. The frozen/thawed monocytes showed the highest basal levels of TNF-α (**Figure 4.3c**). This indicates that the thawing process followed by the cell separation process stresses the cells despite resting periods. In contrast, basal TNF-α production in PBMCs was low, and did not differ when the PBMCs had been frozen and thawed before culture compared to fresh cells. The responses to stimulation with LPS, α-synuclein, and PFFs was also compared between fresh and frozen/thawed monocytes and PBMCs and showed a TNF-α response in all cell types. The response of the monocytes to α-synuclein stimulation does not appear to exceed the unstimulated response, whereas it does in whole PBMCs. From these experiments, I decided to use whole PBMCs for further stimulation experiments. Frozen PBMCs were used given that it was not practical to obtain fresh blood from patients every time an assay was carried out, and these preliminary experiments did not suggest an inferior response in frozen/thawed PBMCs.

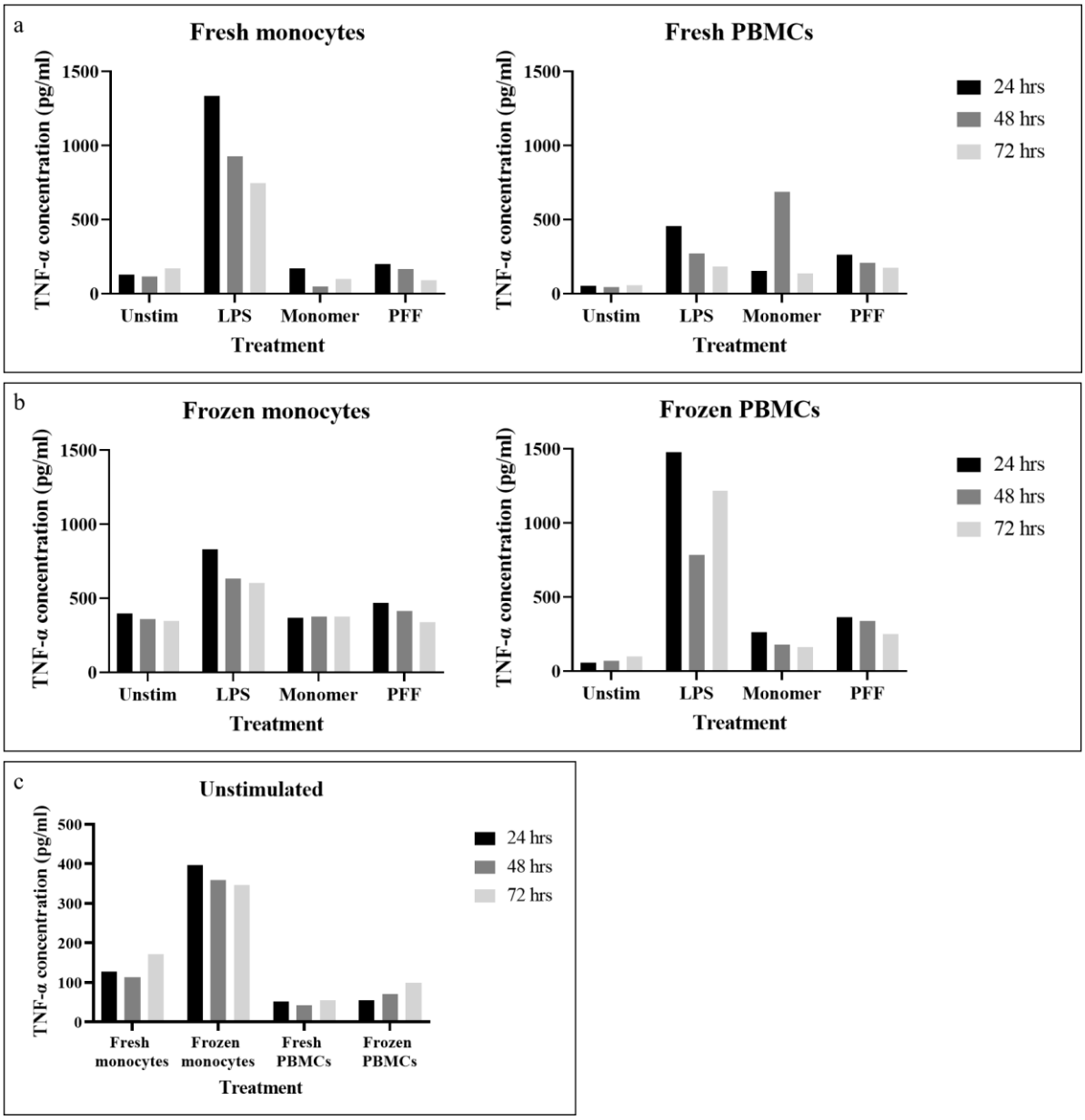


Figure 4.3 TNF-α concentration in supernatant of fresh or frozen cells in response to stimulation. ***(a)*** *Fresh and* ***(b)*** *frozen monocyte (left) and PBMC (right) response to stimulation with LPS and different forms of α-synuclein after having been stimulated for 24 hours, 48 hours, or 72 hours.* ***(c)*** *Conflated unstimulated cell comparison. (N=1). Average CV=3.80%.*

4.4.2 The α-synuclein used is endotoxin-free

A sample of the α-synuclein being used in this study in addition to a sample of media was sent off to an external company (*Lonza*) for high sensitivity endotoxin testing. The samples being endotoxin free is very important as previous work from our group has shown that even very low levels of endotoxin (1.6 EU/ml) induced a similar pro-inflammatory effect to α-

synuclein (White et al., 2018). Measurements carried out by *Lonza* showed that levels in both the monomeric α-synuclein and media alone contained <0.05 EU/ml endotoxin (the lower level of detection of their assay). The monomeric α-synuclein was the same product used to create the further aggregated species of α-synuclein (both oligomers and PFFs). This was carried out under sterile conditions – i.e. using sterile equipment and in an extraction hood – so as to avoid endotoxin contamination. Due to the unfeasibility of sending off a sample every time a new batch was made, it was assumed that the aggregated products would have similar levels of endotoxin to the monomeric species tested.

4.4.3 PBMCs are responsive to monomeric and oligomeric α-synuclein

I investigated the response of PBMCs to different forms of α-synuclein compared to LPS as a positive control, and compared different durations of stimulation. The main species of interest in this study were oligomers due to a prior hypothesis by our collaborator Dr Derya Emin in Professor David Klenerman's lab where she showed that oligomers are the most inflammatory species (Emin et al., 2022). For this reason oligomers were used at different concentrations in these stimulation assays and resulted in a dose-dependent TNF-α response. Oligomer concentration was measured as the starting concentration of monomers in the preparation of the oligomers. At the highest concentration the oligomers resulted in an inflammatory response comparable to LPS. However even at the high concentration of 5 μM (concentration measured as starting concentration of monomers in preparation) the PFFs did not cause a significant inflammatory response. Surprisingly, the monomeric α-synuclein (4 μM) also caused an inflammatory response comparable in magnitude to LPS and oligomers. (**Figure 4.4a**). Different durations of stimulation were assessed to determine the optimal timecourse of α-synuclein-induced inflammation. A robust inflammatory response to α-synuclein was seen at 24 hours post-stimulation, this was maintained at 48 hours, but had started to decline after 72 hours. (**Figure 4.4a**). 24 hours was chosen for use in further experiments due to observing an optimal α-synuclein response, and due to it being the most practical of the timepoints. This experiment was carried out using samples from both PD and controls to investigate whether there were any differences in the cells presumed to have had prior exposure to pathological forms of α-synuclein (i.e. those from people with PD) and cells presumed not to have had this exposure (i.e. those from controls), and the data is presented stratified according to group in **Figure 4.4b**. No significant differences were seen between patient and control groups (Two-way ANOVA followed by Bonferroni's multiple comparison

test; p>0.99), though there was a consistent trend towards a decreased response in patients with each stimulant and at each timepoint. (**Figure 4.4b**).

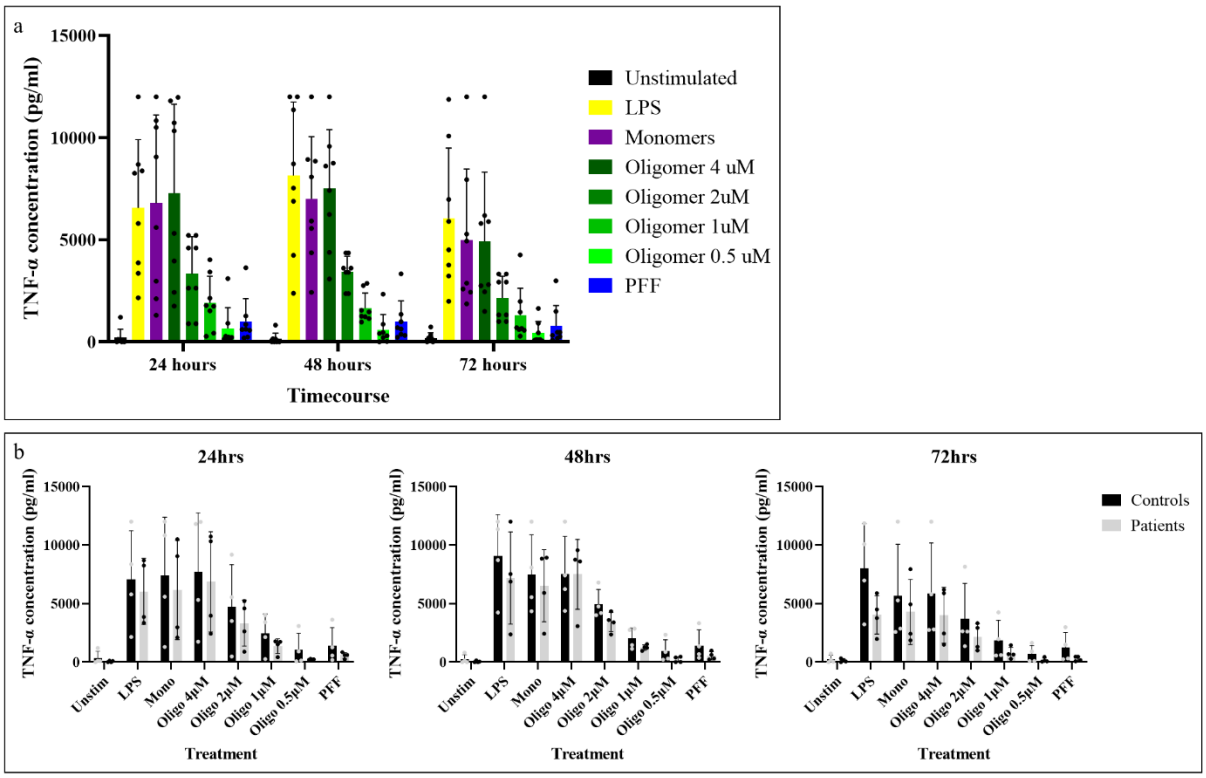


Figure 4.4 TNF-α concentration in supernatant of PBMCs in response to stimulation. (a) *PBMC response to stimulation with LPS and different forms of α-synuclein after having been stimulated for 24 hours, 48 hours, or 72 hours. (N=8)* ***(b)*** *Stratification of (a) into PD (N=4) and control (N=4) responses at 24, 48, and 72 hours of stimulation. Average CV=3.73%.*

The high response of monomeric α-synuclein was surprising though not entirely unprecedented as an inflammatory response to monomers has been seen by Nissen et al., (2019) and White et al., (2018), however several previous studies carrying out similar work (using cultured PBMCs/monocytes/BV2 microglial cell line/THP-1 monocytic cell line) show a monomer-induced response similar to baseline unstimulated levels (Bearoff et al., 2023; Codolo et al., 2013; Grozdanov et al., 2019; Hughes et al., 2019; Pike et al., 2021; Trudler et al., 2021). One hypothesis was that the monomers were aggregating in the culture medium to form oligomers which were more inflammatory. To investigate this, I sent the supernatant from unstimulated cells, from monomer-stimulated cells (at 24 hours and 48 hours of stimulation), and from PFF-stimulated cells to our collaborator Dr Evgenia Lobanova in Professor David Klenerman's lab for single molecule imaging using super resolution microscopy (N=1; 4

images were analysed from each sample) (**Figure 4.5**). This work (carried out and images produced by Dr Lobanova) showed that the culture medium from PBMCs stimulated with monomer for 24 hours showed an increased proportion, and an increased total number, of larger aggregates (length >150 nm) than the supernatant from unstimulated cells (**Figure 4.5b**). Furthermore, when the culture is prolonged for an extra 24 hours (48 hours total) there is an even higher proportion, and total number, of larger aggregates in the medium. As expected, there is a much higher fraction of these larger aggregates in PFFs which have been deliberately aggregated. (**Figure 4.5b**). These results suggest that monomers are aggregating in solution, and this could explain why the inflammatory reaction of cells to this incubation with supposed monomers is higher than expected and comparable to incubation with oligomers. On the basis of these experiments, the optimal form of α-synuclein for future experiments is oligomeric α-synuclein and not PFFs. Monomeric α-synuclein will still be used but not as a negative control as was originally intended. The optimal timecourse is 24 hours as the inflammatory response is at a high level and has not begun to decline.

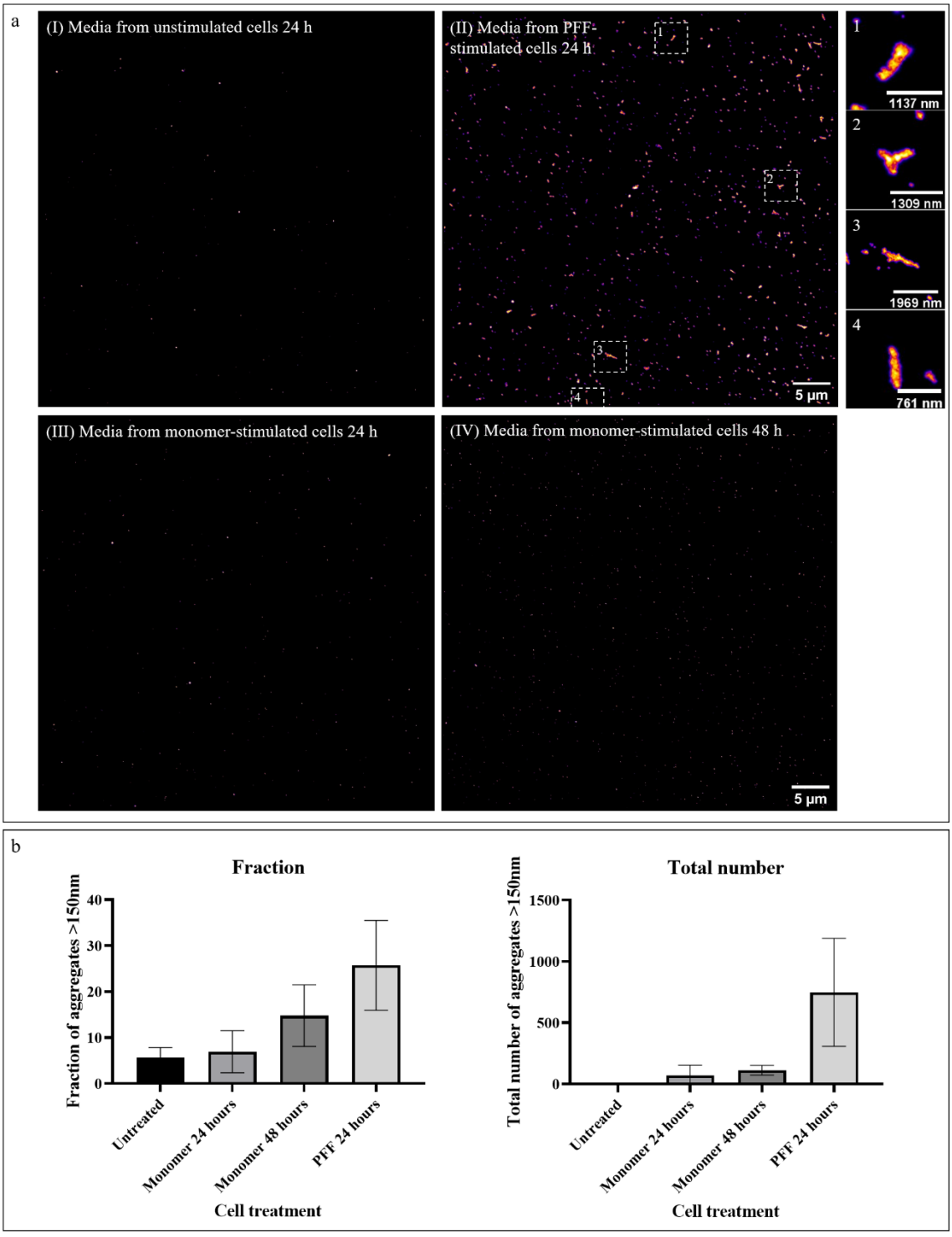


Figure 4.5 Super resolution microscopy and SiMPull of α-synuclein aggregates in supernatant from stimulated cells. (a) *Super resolution microscopy images of α-synuclein aggregates in supernatant from* ***(I)*** *unstimulated cells;* ***(II)*** *cells stimulated with PFFs for 24 hours;* ***(III)****; cells stimulated with monomer for 24 hours;* ***(IV)*** *cells stimulated with monomer for 48 hours.* ***(b)*** *Quantification of (left) the percentage of total aggregates with length >150nm and (right) the total number of aggregates with length >150nm. N=1; 4 images were analysed for each sample.*

4.4.4 PBMCs respond to oligomeric α-synuclein in a dose-dependent manner

Further experiments were carried out to establish a dose-response relationship for oligomeric α-synuclein and to confirm whether this was consistent across subjects in a larger group. All participant PBMCs responded in a dose-dependent manner, though some had a much stronger response than others. Both patients and controls responded in a similar manner. The optimal concentration was determined to be 4 μM of oligomers; at this concentration all participant cells produced an inflammatory response. This was seen for both TNF-α (**Figure 4.6a**) and IL-1β (**Figure 4.6b**). These experiments showed a consistently robust TNF-α response, however the IL-1β response was less robust and less consistent, and even at a concentration of 5 μM the response was not high. For this reason, it was decided to focus on the TNF-α inflammatory response in the following experiments.

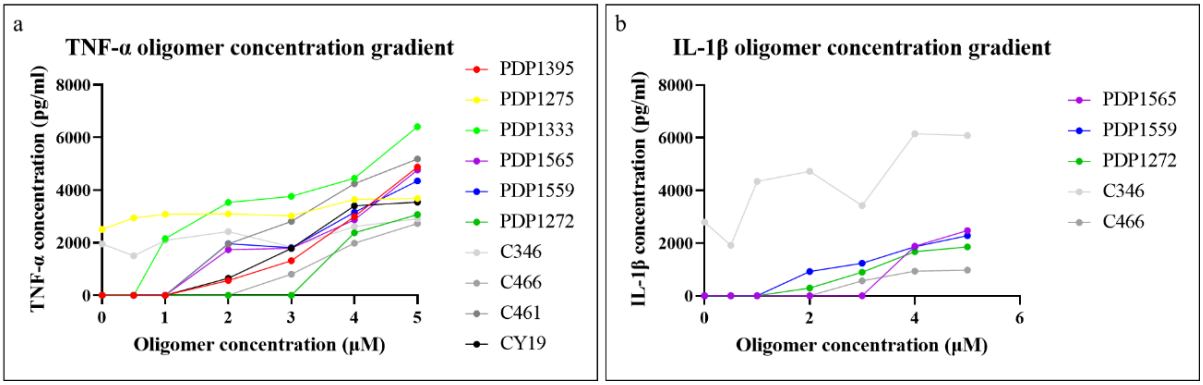


Figure 4.6 Inflammatory response on stimulation with oligomer concentration gradient. (a) *TNF-α. (PD are labelled PDPxxxx, N=6; control are labelled Cxxx, N=4).* ***(b)*** *IL-1β (PD N=3; control N=2). Average TNF-α CV=2.83%; IL-1β CV=2.15%.*

4.4.5 The inflammatory response is TLR4 but not TLR2- dependent

Treatment of PBMCs with the specific TLR4 blocker TAK-242 for 24 hours prior to stimulation resulted in a significant decrease in TNFα release on cell stimulation with LPS, α-synuclein monomers, and α-synuclein oligomers (N=4 participant samples; two-way ANOVA followed by Tukey's posthoc test; $p<0.001$). (**Figure 4.7a**). The concentration of TAK-242 used (1 μM) was chosen based on work by Hughes et al., (2019). In a second experiment (N=3 participant samples), I compared the PBMC response to stimulation following treatment for 24 hours with the TLR2-specific blocker Cu-cpt22, and with candesartan which, as discussed in

Chapter 2.2.2, has been reported to decreases the expression of both TLRs 2 and 4. Both TAK-242 and Candesartan fully abrogated the TNF-α release in response to cell stimulation with LPS (Two-way ANOVA followed by Tukey's posthoc test; p<0.0001). Cu-cpt22 did not have a significant effect on LPS-induced TNF-α release. (**Figure 4.7b**). The monomers and oligomers used in this experiment did not have the expected effect of inducing TNF-α release, so it was not possible to assess the effects of the drug treatments on α-synuclein-induced inflammation in this particular experiment.

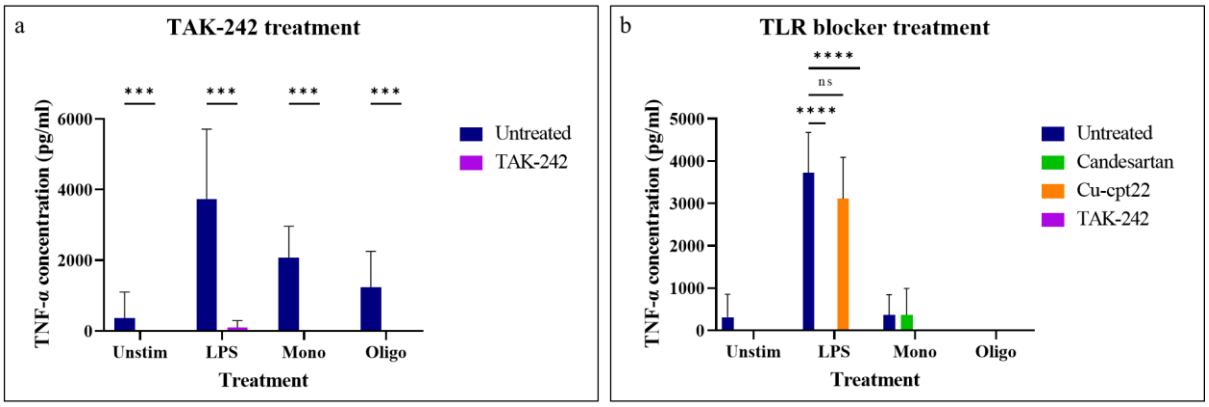


Figure 4.7 Effect of TLR blocking treatment on TNF-α release from stimulated cells. (a) *Untreated and TLR4-blocker TAK-242-treated (N=4; Average CV=6.13%) and* ***(b)*** *untreated, TAK-242, TLR2/4-blocker candesartan, and TLR2-blocker Cu-cpt22-treated (N=3; Average CV=3.55%)) PBMC's response to LPS and different forms of α-synuclein. Two-way ANOVA followed by Tukey's posthoc test; p***<0.001, p****<0.0001.*

The above experiment (**Figure 4.7b**) was repeated again with a new batch of monomers and oligomers which triggered an immune response. TAK-242 consistently supressed the immune response to LPS, and to monomeric and oligomeric α-synuclein. (**Figure 4.8a**). A candesartan concentration gradient was carried out to assess the lowest concentration at which it was effective at blocking the inflammatory response. This was carried out from both an assay optimisation standpoint, and also so that concentrations used here could be translated into use in a drug repurposing clinical trial at a later date. The gradient was chosen according to concentrations used successfully in the literature. Multiple studies used candesartan at 1 μM (Dasu et al., 2009; Qie et al., 2020), however a preliminary experiment using this concentration did not result in any inflammatory effects (data not shown). Daniele et al., (2015) used candesartan up to 30 μM and so a similar range was used here. A clear dose-dependent inhibitory effect on TNF-α was seen. TNF-α levels were significantly decreased from those

released from untreated LPS-stimulated cells when treated with a concentration of 10 μM candesartan, and this response was fully abrogated at a candesartan dose of 20 μM.(Two-way ANOVA followed by Tukey's post-hoc test; $p<0.05$; $p<0.001$). (**Figure 4.8b**). Further work was carried out to assess whether the failure of action of Cu-cpt22 was due to it being used at a suboptimal concentration – the original concentration of 10 μM having been used by Daniele et al., (2015) on primary microglia. A concentration gradient revealed that treatment with Cu-cpt22 across a range of concentrations did not result in a decrease in TNF-α release on cell stimulation with either LPS or α-synuclein. (**Figure 4.8c**). The LPS -stimulated Cu-cpt22-treated cells produced increased TNF-α compared to LPS stimulated but untreated cells, this difference reached significance at a concentration of 10 μM Cu-cpt22 ($p>0.001$) but there did not appear to be a dose-dependent effect above this concentration. Though LPS is thought to have some stimulatory action on TLR2 in addition to its main receptor, TLR4, in these experiments it was clear that the TLR2 blocker Cu-cpt22 did not block the TNF-α response to LPS and may in fact have caused a compensatory upregulation of TLR4 to make up for the loss of TLR2, resulting in increased TNF-α release.

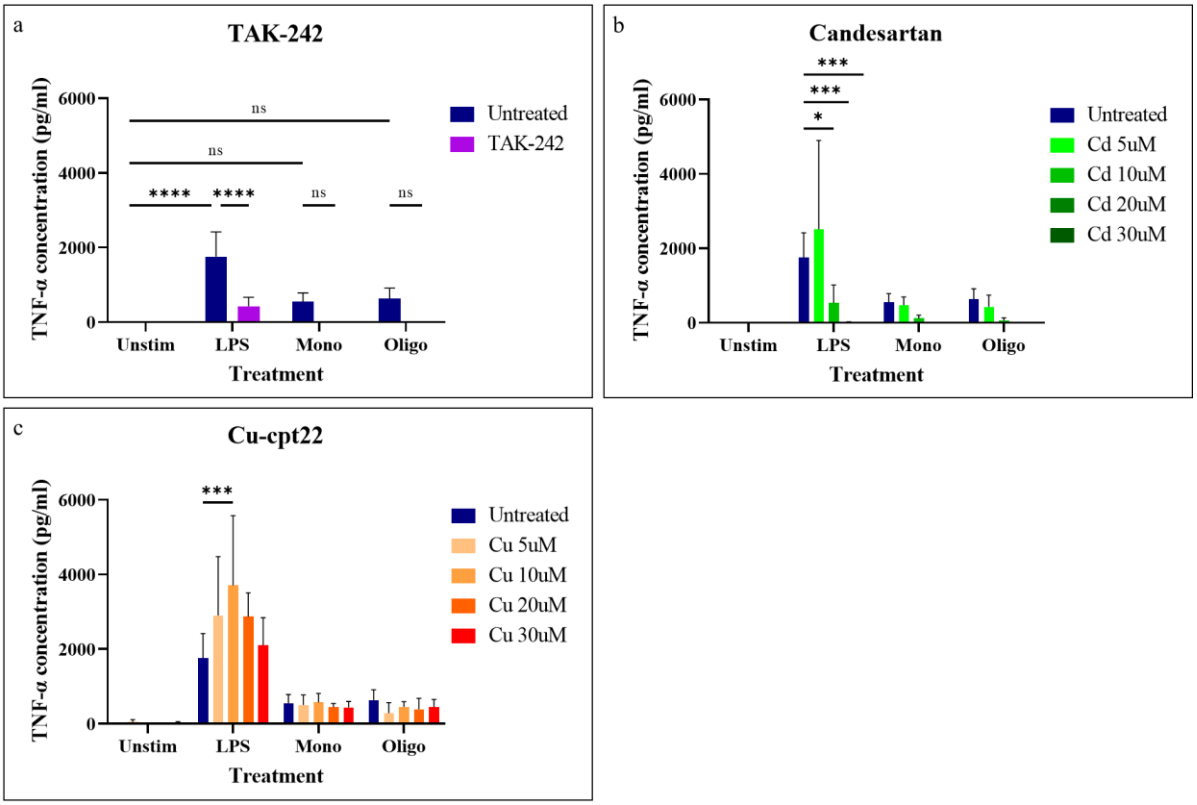


Figure 4.8 Effect of TLR blocking treatment on TNF-α release from stimulated cells. (a) *Comparison between untreated and TAK-242-treated stimulated PBMCs. (N=4).* ***(b)*** *Comparison between untreated and a concentration gradient of candesartan-treated stimulated PBMCs. (N=4).* ***(c)*** *Comparison between untreated and a concentration gradient of Cu-cpt22-treated stimulated PBMCs. (N=4). Two-way ANOVA followed by Tukey's posthoc test; p<0.05, p***<0.001, p****<0.0001. Average CV=2.87%.*

4.5 Discussion

4.5.1 Summary

In this chapter I sought to identify whether human peripheral immune cells responded to different forms of α-synuclein, and whether blocking TLRs would inhibit inflammatory responses to α-synuclein in human immune cells. This question has obvious relevance to Parkinson's irrespective of whether the cells are derived from patients or controls given that α-synuclein is the key pathogenic protein in PD. However, I had also hoped to extend this work into a larger study comparing the responses of PBMCs from a cohort of PD patients versus age-matched healthy controls. Due to a variety of unavoidable circumstances, including kit failures, Covid and Brexit-related reagent supply issues, and a large variance in individual response to stimulants, I was not able to progress to a patient versus control study within the time available for my PhD. However I have made a number of valuable observations which will inform future work. These include:

1. High basal levels of inflammation occur in human monocyte preparations and may confound the response to exogenous stimulation – hence PBMCs may be a better choice of cell type for this work.
2. Using frozen/thawed PBMCs does not significantly affect the inflammatory (TNF-α) response to stimulation.
3. Careful preparation of solutions allows the α-synuclein response to not be confounded by endotoxin, and so unexpected results can be trusted to be legitimate.
4. Oligomers are the optimal stimulant, and show consistent robust response at a concentration of 4 μM.
5. The optimal timecourse for stimulation is 24 hours.
6. The inflammatory response to LPS and to α-synuclein monomers and oligomers is TLR4-dependent.
7. Candesartan effectively blocks the inflammatory response to α-synuclein, this is seen in human immune cells despite the difficulty in showing this occurring in the α-synuclein animal model in ***Chapter 3***.

This work was carried out on PBMCs from individuals with PD, as well as controls. The results suggest that PBMCs from both controls and PD patients respond in a pro-inflammatory fashion to stimulation with both monomeric and oligomeric α-synuclein as shown through their increased production of TNF-α. Whilst the work was underpowered to compare PD versus control responses, this preliminary work suggests that the observed peripheral inflammation measured in PD cohorts might relate to extrinsic factors (e.g. the presence of pathogenic α-synuclein) rather than inherent differences in the function of innate immune cells in the disease state.

In summary, these results show a positive impact of both candesartan and TAK242 on controlling the α-synuclein induced inflammation in human peripheral immune cells. This work has optimised conditions for stimulating and blocking TLR pathways in these cells. As a result, this paves the way for a larger project investigating TLR-dependent innate immune responses to α-synuclein in a large cohort of PD and controls.

4.5.2 Assay failures

4.5.2.1 Kit failure

The initial work carried out in this chapter was using a Thermofisher ELISA kit, however partway through experimentation this kit stopped working – specifically the standards consistently failed to develop. Though initially it was thought that there was a problem with the standards themselves, repetition of the experiment multiple times using a new kit sent by Thermofisher (with a different lot number) continually produced no or very faint standards despite the protocol being followed to the letter, and being carried out using the same reagents and equipment that had previously worked well. This problem was not resolved despite discussion with the manufacturer. It was decided that an aspect within the kit had been changed (unconfirmed by the manufacturer). This was most likely the capture antibody as in part of the troubleshooting the standards and detection antibodies from previous working kits were used and still failed to result in standard development, unfortunately there was no remaining capture antibody from a previous kit to test. Alternatively this could be due to a change in the plate being used – to the best of my knowledge the supplier of these was consistent, however it was noted that between the working plates and the failed plates a new box of plates had been opened. It is possible that a change to the plate resulted in a failure of the capture antibody binding, and so resulting in a failure of all standards – even those which had worked before.

This problem was eventually surmounted through the use of an ELISA kit from a different manufacturer – R&D systems. This unfortunately resulted in a number of experiments being aborted, wastage of samples and reagents, and the repetition of optimisation steps due to having to use a new kit, and consequently to large delays to the project.

4.5.2.2 Variability

In addition to the failure of kits, there was very large variability between the responses observed using different subject's cells. A degree of variability was expected, however the extent to which this occurred was not foreseen. This made it difficult to determine the optimal dilution to use to allow for the detection of very low levels of TNF-α, and the prevention of the saturation of very high levels of TNF-α. A dilution of 1:8 was settled on, however it cannot be guaranteed that in any further work saturation will not occur with some particularly reactive subject cells. Other studies, including Nissen et al., (2019) and White et al., (2018) have similarly seen large variability in the inflammatory responses of human PBMCs. Grozdanov et al., (2019) appeared to show less variability in levels of cytokine production in response to different forms of α-synuclein, this could be due to the presentation of data as fold increase over LPS. This may have been carried out to remove the large inter-subject variability and could be considered in any future work continuing my project.

The variability seen by myself, White, and Nissen may result from the variability in the ages of individuals included in the studies. White et al., (2018) used a cohort comprised of early stage PD patients with an age range of 55-80 years old. The Nissen et al., (2019) PD cohort comprised an age range of 43-79 years old. My own work also used a large range of ages (44-85 years old) as, due to the preliminary nature of the work carried out here, a specific cohort had not been chosen. Ageing is known to result in immunosenescence leading to immune deficiency, a decline in the immune response, and increased susceptibility to infection (Castle, 2000). This has been investigated using whole PBMCs and monocytes derived from elderly people. An impaired immune response of peripheral monocytes in aged subjects is seen by Bruunsgaard et al., (1999), Delpedro et al., (1998), and Nyugen et al., (2010) where a deficit in the induction of pro-inflammatory cytokines is seen in response to LPS and other TLR agonists. This impaired immune response in elderly subjects was similarly seen by Metcalf et al., (2015) in whole PBMCs. Contradictorily, others have shown an increased production of cytokines from aged compared to young subjects. (Fagiolo et al., 1993; Pinke et al., 2013; Roubenoff et al., 1998). Though a conclusive result has not been found, it is clear that ageing

affects the immune reaction of cells, and that this results in differing levels of cytokine production. This could explain the large variation in TNF-α seen in my subjects on stimulation, and emphasises the importance of a carefully selected and age-matched cohort in future work.

The disease duration of people with PD may also have an effect on the inflammatory response of cells. This is controlled for in the White et al., (2018) paper (with a mean disease duration of 4.3 years), however in the Nissen et al., (2019) cohort the disease duration ranges from 3-13 years. In my work there is also a spread in disease duration (0.21-5.84 years). Studies are indicative of alterations in immune function as disease progresses. Mixed data is published on whether correlations between the production of pro-inflammatory cytokines and Hoehn-Yahr stage/UPDRS III score are positive or negative (Hasegawa et al., 2000; Reale et al., 2009). However, progression through H&Y or UPDRS III does not occur at the same rate in all people with PD and so is not directly linked to disease duration. Work by Devos et al., (2013) looked specifically at disease duration and found a negative correlation with colon mRNA levels of pro-inflammatory cytokines. This evidently does not directly correspond to the behaviour of PBMCs, however does provide more evidence that peripheral inflammation is altered in PD, and may be dependent specifically on disease duration. Further indications of alterations in immune function through disease duration arise from studies identifying an increase in auto-antibodies to α-synuclein in early PD which declines as the disease progresses (Scott et al., 2018). Differences in the immune response, and specifically in the PBMC response, correlating to disease duration are less conclusive than changes seen with age. However, there is evidence that this altered immune response may occur, and as a result disease duration is a further factor that should be considered when selecting a cohort for future work.

A further factor which it is becoming increasingly clear must be controlled for is sex. Sex differences in both innate and adaptive immune response are well established, with females mounting stronger immune responses than males. (Klein and Flanagan, 2016). These immune differences can also be seen in a disease state. Nissen et al., (2019) found that immune responses within PD were altered dependent on sex, whereby some differences in immune function observed between controls and PD were seen only in males.

There are many other factors which may result in high variability in the response of PBMCs to stimulation. These include the health of the subject when the blood was taken, the vaccination status of the subject, any comorbidities, and the method of freezing down and then thawing the PBMCs – this can cause cell stress. These factors should all be taken into account

in an effort to control variability. However, even controlling for all of these factors, heterogeneity in human cell responses should be expected and many intrinsic factors will contribute to the response of PBMCs. The aim with this work is to explore whether in spite of the heterogeneity in human cell responses that when comparing between factors such as patient groups and cell treatment groups there is sufficient signal to rise above any of the noise from the heterogeneity.

4.5.2.3 Failure in oligomer production

Throughout the period of experimentation there were many issues with the assays failing to produce consistent responses to monomers/oligomers. Initial work showed a consistently high inflammatory response to both monomers and oligomers, this occurred in all individuals tested, though a large variability in the degree of response was seen – as discussed above. The oligomers used in this work were produced in accordance with the protocol developed by the Klenerman group (Department of Chemistry, University of Cambridge; collaborators of the Williams-Gray group). This was an updated protocol of that used by Hughes et al., (2019). It is a very simple protocol and initially produced consistent results between multiple separate monomer aggregations. Unfortunately, this consistency did not continue and stimulation experiments started to produce either an attenuated response, or no response at all. Stimulation experiments were repeated with PBMCs from the same individual/batch and I found that subjects who had previously produced a strong response to oligomers now produced little/no response to the new batch of oligomers, even though all other experimental conditions were identical (**Supplementary Figure 4.1**). This showed that these later failures were oligomer dependent.

The production of oligomers, though carried out in a regulated fashion, does not produce exactly the same combination of sizes of oligomers every time due to the random nature of aggregation. This could result in one batch producing more pro-inflammatory species than another. This may explain some of the differences in response to oligomers between experiments. A further reason for differences may be due to freezing/thawing of the oligomers. Based on initial variability in oligomer response it was decided to produce a large batch of oligomers and freeze them in aliquots so that the differences in oligomer size between batches could be controlled for. However, it was found that the inflammatory response to oligomers was attenuated if not entirely abrogated after the freeze/thaw process (**Figure 4.7b**). When the oligomers did not undergo freezing/thawing the PBMC inflammatory response to them was

retained (**Figure 4.8**). Future studies should carefully consider how to control for differences in oligomer species between batches.

4.5.3 The inflammatory response to monomers versus oligomers

There is some controversy over α-synuclein monomer-induced PBMC/monocyte inflammatory responses with much of the literature suggesting that oligomers are the more inflammatory species. Conflicting studies arise around this topic. Papers such as White et al., (2018) and Nissen et al., (2019) show an inflammatory response to monomers from PBMCs or monocytes, similar to that seen with aggregated α-synuclein. Conversely, (Bearoff et al., 2023; Codolo et al., 2013; Grozdanov et al., 2019; Hughes et al., 2019; Pike et al., 2021; Trudler et al., 2021) show no, or limited, inflammatory response to monomer treatment as compared to aggregated α-synuclein treatment. These studies differ on the type of cells stimulated, the method of production of aggregated α-synuclein, and the chosen concentration of monomers used to stimulate. The inflammatory effect of monomers seen in this chapter was thought to be due to the high concentration of monomer chosen (4 μM). This concentration was chosen to correspond to the optimal oligomer concentration – 4 μM as according to the concentration gradient seen in **Figure 4.6**. It could be speculated that the high inflammatory response seen in response to this monomer concentration could be due to the sheer amount of protein being added to the culture medium. However, Pike et al., (2021) used concentrations of monomer up to 20 μM and did not see any inflammatory effects – though this was in 1˚ human microglia, and they were only interested in NLRP3 inflammasome-mediated IL-1β secretion – no other cytokine production was monitored. As shown in **Figure 4.5**, a different effect of using such a high concentration of monomers is that this makes them more prone to aggregation into higher molecular weight species. Though this has not occurred extensively in our culture medium, it can be seen that a proportion of monomers have formed oligomeric species >150 nm and that this proportion increases further when left in culture for an extra 24 hours. It is possible that these aggregated species within the culture medium of the monomer-stimulated cells are what is actually responsible for the inflammatory effects seen. This is very difficult to control for; other studies have used techniques such as filtration of monomer solutions before addition to cultures (Codolo et al., 2013; Pike et al., 2021), and vigorous trituration (to remove clumps) (Bearoff et al., 2023) to ensure that no high weight molecular species were found to contaminate the monomeric solution. This should be considered for future work using this assay. A further consideration is whether to control for the starting concentration of monomers,

or whether to control for the number of molecules at the end of the aggregation process. Within this chapter the former control was used – so the concentration of oligomers specified as being used was actually the concentration of the monomers which had been aggregated. This means that when monomers and oligomers at a corresponding concentration are added to the cultured cells, the total number of oligomers present is fewer than the total number of monomers present – as the oligomers are formed from, multiple monomers. This could explain why the oligomers, though suggested to be the more immunogenic species, produce a very similar response to the presumed less immunogenic but more abundant monomeric species.

4.5.4 TLR blockers

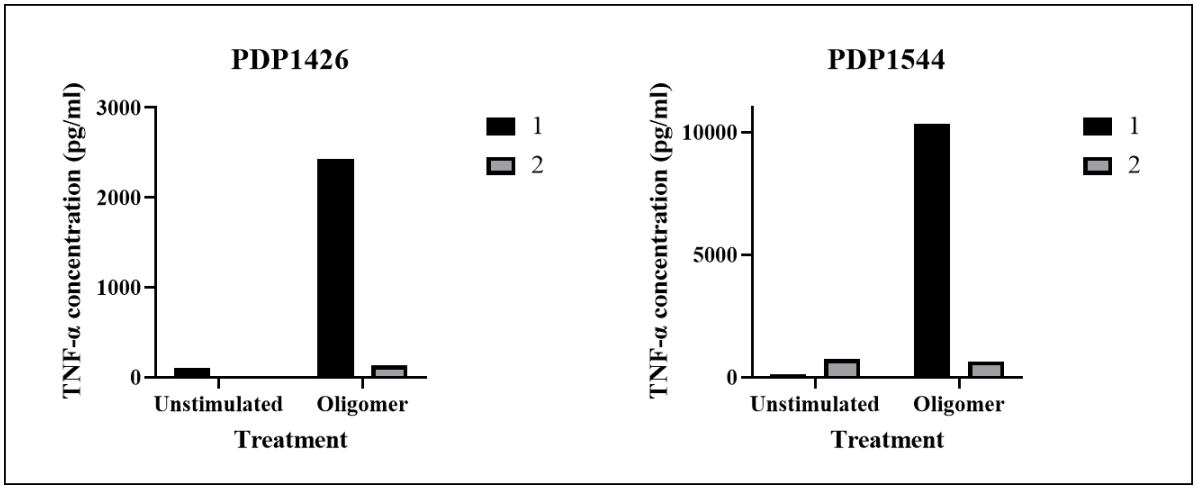
The use of candesartan abrogated the inflammatory response of the cultured immune cells in response to both LPS (as a positive control) and α-synuclein species. This occurred partially when at a candesartan dose of 10 μM, and fully when at 20 μM and above. The method through which candesartan achieved this is not conclusive due to candesartan having varied effects. Candesartan works to block AT_1 receptors in its role as an anti-hypertensive; AT_1 receptors can enhance the activation of NF-κB (Skultetyova et al., 2007) and thus the blockage of these AT_1 receptors by candesartan may block this NF-κB activation and the subsequent production of pro-inflammatory cytokines. It is possible that candesartan is having anti-inflammatory effects in this manner in this assay, however this would not explain the blockage of a response to LPS. LPS is known to exert its inflammatory effects through binding to and activating TLR4, resulting in the activation of downstream pro-inflammatory factors. In this way candesartan appears to be having an anti-inflammatory effect – at least in the LPS-stimulated cells – through reducing the expression of TLR4 (and potentially also TLR2) as shown in work by Dasu et al., (2009). Further work is required to determine whether candesartan's action in these α-synuclein stimulation experiments is through reduction of TLR4 gene expression. A simple suggested experiment to provide supporting evidence for this could be the use of flow cytometry to measure TLR expression on stimulated PBMCs with and without candesartan treatment. Regardless of mechanism, candesartan is having anti-inflammatory effects. This could be conducive to its having a neuroprotective effect, as was seen in ***Chapter 2*** (though through an undetermined mechanism). The tolerability of long-term treatment with candesartan is well characterised through its use as an anti-hypertensive. Therefore its anti-inflammatory properties to stimulation with α-synuclein combined with its

already being a licensed drug make it an ideal candidate for further study and a potential clinical trial.

Similarly to candesartan, TAK-242 abrogated the inflammatory response of the cultured immune cells to both LPS and α-synuclein species. This was expected for LPS, as it is a TLR4 ligand, but shows that TLR4 is also essential for the α-synuclein-mediated inflammatory response, and so provides further evidence for α-synuclein species acting as a DAMP to activate TLR4. TAK-242 has been used in a clinical trial for the treatment of sepsis (Rice et al., 2010). Though unsuccessful in its primary outcomes, this trial demonstrated that TAK-242 is well tolerated – with the rate of adverse events being only slightly higher in those treated with TAK-242 than placebo, and the most frequent adverse event being methemoglobinemia. However, this study only administered TAK-242 for a short period of time – a 30 minute loading dose followed by a 96 hour continuous infusion – for a very acute disease. The treatment of PD – a slowly progressing rather than acute disease – would require long-term treatment with TAK-242 which has not been assessed in any human trials. For this reason, candesartan makes a more promising candidate for use in treating PD in humans. However, TAK-242 is useful in helping to determine the mechanism of the protective effect of candesartan.

The blockade of TLR4 by TAK-242 clearly prevented α-synuclein-induced inflammation, however TLR2 has also been suggested to recognise α-synuclein as a DAMP. Candesartan has been shown to decrease the expression of both TLRs 2 and 4 (Dasu et al., 2009), so it is possible that the anti-inflammatory effects seen on candesartan treatment could be due to a combination of decreased TLR2 and 4 expression. The TLR2-specific blocker Cu-cpt22 was chosen to test this. Cu-cpt22 failed to suppress α-synuclein-induced inflammation in this assay, this indicates that the α-synuclein may be acting specifically via TLR4 to activate the PBMC immune response. However, further work is required to justify this claim as no positive control was used in this study to confirm that Cu-cpt22 was blocking TLR2 – though this has been established in the literature by Cheng et al., (2012), and has been used by Daniele et al., (2015) against α-synuclein-induced TLR2 activation. LPS predominantly activates TLR4, so explaining why the suggested blockage of TLR2 by Cu-cpt22 does not significantly affect the inflammation resulting from this. A TLR2 agonist, such as Pam-3-csk could be used in tandem with Cu-cpt22 to prove that Cu-cpt22 is indeed blocking TLR2 activation.

This work is demonstrating a potential therapeutic mechanism whereby treatment with a TLR blocker decreases the α-synuclein-induced inflammatory response of human immune cells, which could have a protective effect on the progression of PD. Further work is required to demonstrate the applicability of this in humans; this will be discussed in ***Chapter 5***.



Supplementary Figure 4.1 Effect of frozen oligomers on oligomer-stimulated TNF-α production*. Experiment 1 used a batch of oligomers produced and used within 24 hours (full data from this experiment can be seen in Figure 4). Experiment 2 uses batches of oligomers produced and then frozen prior to use in an assay (full data for PDP 1426 can be seen in Figure 2bI; full data for PDP1544 can be seen in Figure 2bII; PDP1426 and PDP1544 were stimulated with different batches of oligomers, both were frozen before use).*

Chapter 5.

DISCUSSION

5.1 Introduction

In this thesis I explored the theory that TLRs play a key role in the progression of Parkinson's disease. I tested this by using TLR-blockers in an α-synuclein animal model, as well as in cultured human peripheral immune cells, to assess their effect on the inflammatory response of these systems. I aimed to address the key hypotheses that:

- TLRs play an important role in the development and progression of PD through mediating the pro-inflammatory response of microglia and peripheral monocytes to the disease state.
- TLR blockade will have a protective effect on the development of pathology in an α-synuclein animal model through the suppression of inflammation.
- TLR blockade will reduce the inflammatory response of human peripheral immune cells to α-synuclein.

5.2 Key findings

The key findings from this thesis are summarised as follows:

- **Candesartan shows a protective effect on the development of cholinergic neurodegeneration in an α-synuclein animal model** ***(Chapter 2)***. An animal model of PD was produced through the injection of α-synuclein PFFs into the tail vein of rats, replicating the model produced and published by Kuan et al., (2019). Animals were dosed daily with candesartan to test how blocking TLRs may alter disease progression. A protective effect was seen in terms of reduced cholinergic degeneration in the vagus nerve nucleus in candesartan-treated as opposed to untreated PFF-injected animals. No differences in cholinergic neuron density were seen between candesartan-treated and untreated control animals, suggesting a disease-specific effect. The area of the brainstem where cholinergic degeneration occurred is a region where the earliest PD pathology is thought to occur in the human disease (Braak et al., 2003), though this α-synuclein pathology was not demonstrated in this area in my work. This could implicate

the blockage of TLRs as a key therapeutic target in early PD. A small protective effect of candesartan was also seen in terms of olfactory deficits (another early feature of human PD) with the untreated animals showing a trend towards a higher proportion of failure in the behavioural test compared to the treated animals. Furthermore, a protective effect of candesartan treatment was seen on the development of motor dysfunction, however this may be occurring due to confounding weight effects. No effect of candesartan was seen on dopaminergic degeneration, however this may have been due to this being an early stage model, and therefore it had not yet developed α-synuclein pathology in the dopaminergic nigrostriatal system.

- **The mechanism by which candesartan is exerting a neuroprotective effect is unclear** ***(Chapter 3)***. The α-synuclein PFF animal model does not result in measurable neuroinflammation, and so candesartan does not appear to be exerting the observed protective effect through reducing brain inflammation. It also did not reduce the expression of AT_1 receptors, but it was not determined whether blockage of these receptors was occurring. Though established as decreasing the expression of TLRs 2 and 4 by Dasu et al., (2009), in my work, candesartan treatment showed only a small trend towards a decrease in the expression and in the protein levels of TLRs 2 and 4 in the rat brain. The expression of downstream factors in the brain, including the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, did not show a decrease on candesartan treatment. Similarly, the majority of peripheral cytokines, including TNF-α, showed no changes on candesartan-treatment, and in fact many consistently remained below the limit of detection of the MSD assay regardless of treatment group. KC/GRO (CXCL1) was the exception to this, showing significant upregulation at 2 months. This upregulation was attenuated by candesartan treatment, indicative of a potential protective effect. Finally, no differences were seen in microglial activation on candesartan treatment as shown through assessing microglial morphological changes, microgliosis, and MHCII expression. There does not appear to be any induction of neuroinflammation in the animal model when measured at 6 months, however this may be due to a failure to assess brain inflammation at an earlier timepoint, particularly given that peripheral inflammation – in the form of upregulation of KC/GRO – was identified at 2 months.
- **A-synuclein species are pro-inflammatory to human peripheral immune cells, and this inflammation appears to be TLR4-dependent** ***(Chapter 4)***. Different forms of

α-synuclein, including oligomers and monomers, resulted in a pro-inflammatory TNF-α response in peripheral immune cells from both people with PD and healthy controls. Optimal experimental timecourses and concentrations of α-synuclein were established to lay the ground for future work to explore further whether there are differences in inflammatory responses between controls and PD. Three different TLR blockers were used to distinguish whether TLR 2 or 4 is the most important in mediating the α-synuclein-induced inflammatory response, and also to assess whether the licensed drug candesartan – used in the previous chapters in an animal model – was capable of suppressing this inflammation. Optimal concentrations were established for these TLR blockers to allow this work to be extended to a large cohort of controls and PD. This work showed suppression of α-synuclein-induced inflammation by higher concentrations of candesartan, and by the specific TLR4-blocker TAK-242. The TLR2-blocker Cu-cpt22 did not suppress this inflammation.

5.3 General discussion

Interest in the role of TLRs in PD, and indeed TLRs in neurodegenerative diseases in general, has grown over the past 15 years. Many studies have provided evidence to support a role for TLRs in Parkinson's disease, particularly TLRs 2 and 4 –see **Table 1.1, Table 1.2, Table 1.3, Table 1.4** – however the use of a TLR blocker to treat PD in a clinical trial has yet to be carried out. In this thesis I aimed to gain pre-clinical evidence to support the use of the licensed drug candesartan in a clinical trial, by investigating whether candesartan could attenuate α-synuclein related pathology in an animal model. There is disagreement in the literature over whether TLR2 or TLR4 is the most important in relaying the inflammatory response to α-synuclein, but candesartan has been reported to decrease the expression of both TLRs 2 and 4 (Dasu et al., 2009), thus providing a way to target both TLRs with one drug. Candesartan is an ideal candidate for repurposing in a clinical trial due to it already being a licensed drug, and its well-established chronic use to treat hypertension, with minimal adverse effects.

My work in ***Chapter 2*** shows that candesartan is having a protective effect against the development of neurodegeneration within the animal model, providing evidence for a positive effect if used therapeutically. Due to the model not developing as expected, the main pathological deficits seen were cholinergic degeneration in the hypoglossal and vagal nerve

nuclei. These areas are the first areas which degenerate within the human PD brain (Braak et al., 2003). In keeping with this, the animals exhibited olfactory deficits, which represent one of the first features of human PD, occurring over 20 years prior to the development of motor symptoms according to some studies (Fereshtehnejad et al., 2019). As such the model produced by Kuan et al., (2019) and replicated within my thesis could be described as a prodromal model of PD whereby neurodegeneration has begun in specific tracts of the brain but is yet to spread to the dopaminergic tracts, and the beginnings of behavioural deficits are being seen but these are yet to extend to motor deficits. Further study by Kuan et al., (2022) showed that when left for up to 18 months post PFF-injection degeneration of TH neurons occurred to an even greater extent. This suggests that if my animals were left to progress for longer than 6 months then pathology may have been seen within the dopaminergic system. Prodromal animal models of PD are not common (Taguchi et al., 2020). Many models do display some features seen in prodromal PD – such as olfactory dysfunction and GI dysfunction. However, these frequently occur alongside largescale neurodegeneration (Höglinger et al., 2015; Prediger et al., 2006; Rodrigues et al., 2014; Tian et al., 2008), or are induced through non-physiological means such as acute environmental toxin exposure without Lewy pathology or injection directly into the olfactory bulb or GI tract (Anderson et al., 2007; Drolet et al., 2009; Kim et al., 2019; Rey et al., 2016). As a result, these models are not particularly accurate models of idiopathic PD, and furthermore they frequently develop too quickly to allow long-term drug intervention to be assessed. The model that I utilised therefore provides a novel tool for investigating the initial stages of α-synuclein induced pathology, including prodromal features, and provides a solid basis for my work investigating the effect of TLR suppression, using candesartan treatment, on pathology development. Furthermore, there is increased interest in clinical trials being carried out in prodromal PD, such as in people with genetic mutations which predispose them to developing PD, or people experiencing REM sleep behaviour disorder (RBD) who have a high chance of going on to develop PD (Roguski et al., (2020) estimate there to be around 70% of RBD patients who go on to develop PD). My work is particularly relevant for these prodromal trials as candesartan is having a protective effect on prodromal symptoms in the α-synuclein animal model. However, in this model candesartan was given prior to the induction of disease which is not possible to achieve in humans as even during the prodromal phase pathology has already started.

Many studies investigating the role of TLRs in mediating neurodegenerative pathology use TLR-knockout models (see **Table 1.2** and **Table 1.4** for specific studies).

Though effective as a proof of concept for TLR-related protective mechanisms, TLR knockout is clearly not a viable solution for use in humans. Other studies have investigated the blockage of TLRs using anti-TLR antibodies (Codolo et al., 2013; Gustot et al., 2015; Kim et al., 2018; La Vitola et al., 2018; Stefanova et al., 2011), or TLR inhibitors (Chung et al., 2022; Daniele et al., 2015; Dutta et al., 2021). None of these therapies have been used in clinical trials to date. Antibody therapies have been suggested in PD but the focus so far has been on anti-α-synuclein antibodies. These have shown some progress in animal models and early clinical development (Schneeberger et al., 2016), but there are risks associated with such therapies, exemplified by those associated with the lecanemab and aducanumab Alzheimer's trials (Mahase, 2022; Walsh et al., 2021). Kim et al., (2018) suggest that antibody therapies aimed at neuroinflammation should also be explored. However more readily available and less experimental treatments may be a more successful way forward. TLR inhibitors such as Cu-cpt22 (Daniele et al., 2015), C29, CLI-095 (Chung et al., 2022), wtTIDM (Dutta et al., 2021), and TAK-242 (Hughes et al., 2019) have all been explored in relation to *in vitro* cell cultures and PD animal models. C29 (TLR2 small molecule inhibitor), CLI-095 (TLR4 small molecule inhibitor), and wtTIDM (peptide blocking TLR2-interaction domain of MyD88) have only been used in a limited number of studies relating to PD animal or *in vitro* models. Though suggested to be protective against α-synuclein-induced inflammation in these PD models, they have only contributed to mechanistic knowledge and following on from this the authors have not suggested their therapeutic use. TAK-242 (TLR4 small molecule inhibitor) has shown neuroprotective effects in animal models for conditions including amyotrophic lateral sclerosis (ALS) and traumatic brain injury (TBI) through its blockade of TLR4 and subsequent reduction in neuroinflammation. (Fellner et al., 2017; Feng et al., 2017). Hughes et al., (2019) also showed this may be relevant to PD with reduction in α-synuclein-induced oxidative stress, neuronal cell death, and inflammation on TAK-242 treatment of BV2 microglial cells. TAK-242 has previously been used in a Phase I randomised, double-blind, placebo-controlled clinical trial for the treatment of sepsis which, although negative for its primary outcome, demonstrated the safety and short-term tolerability of this compound (Rice et al., 2010). Although these studies were in disorders other than α-synucleinopathies, they indicate that TAK-242 could have beneficial effects in PD through the blocking of TLR4 and subsequent decrease in the neuroinflammatory effects of α-synuclein. However, there is little research on the safety of this compound for long-term treatment which would be essential for its use in the treatment of PD. CU-CPT22 (TLR2 small molecule inhibitor) has been shown to reduce α-synuclein-induced TNF-α production in primary microglia (Daniele et al., 2015). A protective effect against

pathological α-synuclein accumulation and motor and sensory dysfunction was similarly seen in an MPTP animal model. CU-CPT22 has been used in a type 2 diabetes mellitus mouse model and suppressed the expression of pro-inflammatory cytokines – including TNF-α, IL-1β, and IL-6. Though not directly relevant to PD, this work does show that CU-CPT22 can be tolerated and has anti-inflammatory effects *in vivo* (Tian et al., 2021). No work has been carried out investigating the safety and tolerability of CU-CPT22 in humans.

My work described in this thesis has provided further evidence for a potential protective role of TAK-242 in α-synuclein-induced inflammation and for the first time demonstrated that this protective effect occurs in human immune cells. This effect was not seen for CU-CPT22, in contrast to the literature, though further work needs to be done to ensure that TLR2 was successfully blocked in my study. However, the main focus of this thesis in terms of TLR blockers was candesartan. This drug has a number of benefits over blockers such as TAK-242 and CU-CPT22. Candesartan is a licensed drug and used in the long term treatment of hypertension meaning that its safety and tolerability over a long timescale has already been assessed. My work has shown that candesartan has protective effects against the development of Parkinson's-like pathology in an animal model. I could not clearly demonstrate the mechanism through which this occurred but candesartan has multiple potential mechanisms of action. Firstly, the blockage of AT_1 receptors (Rodriguez-Perez et al., 2018). Secondly, the inhibition of autophagy through decreasing the expression of TLRs on neurons (Chedid et al., 2022; Chung et al., 2022; Kim et al., 2021). Thirdly, anti-inflammatory effects, as demonstrated in human peripheral immune cells stimulated with α-synuclein. These effects may be occurring through decreasing the expression of TLRs 2 and 4. Though not proven in my animal study, there is abundant evidence from the literature that inflammation plays a role in the development and progression of PD-like pathology in animal models and in the risk for, and progression of human PD. Hence the anti-inflammatory effect of candesartan may be relevant to its neuroprotective action.

5.4 Future directions

5.4.1 Further exploration of candesartan

The work I have carried out using the Kuan et al., (2019) animal model has shown promising results in terms of demonstrating that the licensed drug candesartan can slow the

development of neurodegeneration in a model of prodromal/early PD. My work provides additional support for repurposing candesartan in a clinical trial in PD, however further work is need to clarify the mechanism of action, and to determine whether candesartan also impacts the progression of more established disease.

In this study, candesartan was used in a model of PD specifically focussing on the development and prodromal stages of PD, including cholinergic neuron degeneration in the brainstem and olfactory deficits. This study therefore provides evidence suggesting candesartan might be beneficial to modify the course of prodromal or early PD. However, due to this model not displaying some key features of established PD – namely the presence of pathological α-synuclein in key PD-related brain areas, degeneration of dopaminergic neurons in the substantia nigra, the degeneration of dopaminergic fibres in the striatum, and motor deficits – it is unknown whether candesartan also protects against these features which develop later in disease (according to Braak et al., 2003). Further work should be carried out to investigate the effect of candesartan treatment on these key features. A more acute animal model of Parkinson's could be used, such as a double hit α-synuclein overexpressing model injected with LPS (Gao et al., 2011). This model produces substantial dopaminergic degeneration in the striatum and substantia nigra over a period of 5 months post injection, in addition to aggregated α-synuclein inclusions and neuroinflammation. Though not the best model in regards to emulating idiopathic PD because of the non-physiological levels of α-synuclein in addition to the injection of a foreign inflammatory agent which clearly does not occur in human PD, this model would allow determination of the effects of candesartan on dopaminergic degeneration and on inflammation. In addition, similarly to the Kuan model, this model develops over a relatively long time period (5 months) so allowing the assessment of relatively long-term drug intervention, though evidently not a time period as long as that over which human PD develops. This would build on the data from my work focusing on early cholinergic degeneration in the Kuan et al., (2019) model, and will provide further rationale for the use of candesartan in a clinical trial.

The investigation of the protective effects of candesartan on the development of pathology in a model of PD was carried out using a protocol where treatment was started prior to the establishment of pathology in the model. This is a commonly used approach, done so as to maximise the effect of the drug on pathology, however this is clearly not possible to achieve when treating the human disease. The pathology of PD is thought to begin many years prior to

the development of motor symptoms and diagnosis of disease. Even in people with prodromal features of PD such as hyposmia and RBD, the pathological process has already started. Therefore, any follow-on studies to the α-synuclein animal work carried out in this thesis should carefully consider ways in which it would be possible also to test the effect of candesartan treatment on the progression of pathology when pathology has already been established. This could be carried out using the Kuan et al., (2019) model where candesartan treatment could be started at the 6 month timepoint where pathology is known to have developed, and these animals could then be studied for a further 12-months as a follow up study by Kuan et al., (2022) showed the further development of pathology up to 18 months post PFF injection.

Though my work has suggested a protective effect of candesartan treatment, the mechanism through which candesartan is exerting this effect has not been successfully established. My original hypothesis was that candesartan would act as a TLR blocker, and that this blockage of inflammation would result in protective effects on pathology. Unfortunately, neuroinflammation was not detectable within the animal model used in my studies, and as result I could not measure the impact of candesartan on inflammation – as shown in ***Chapter 3***. Furthermore, the expected inhibition of expression of AT_1 receptors on candesartan use was not seen, and so the action of candesartan was not determined within this work. Mechanism of action is important to establish before use in a clinical trial so as to allow proof of target engagement and the monitoring of relevant mechanistic biomarkers. Further work to establish whether candesartan is exerting an anti-inflammatory effect *in vivo* could include looking at an earlier timepoint in the PFF model, or looking at a timepoint in between those used in my work; this could expand on the KC/GRO upregulation seen in the PFF model at 2 months. Alternatively, an LPS double-hit model could be used due to LPS being well-established as resulting in inflammation. These works should carefully assess the peripheral immune response in addition to the central immune response so as to build on the candesartan-induced reduction in KC/GRO seen in my work. Moreover, TLRs are expressed on innate immune cells in the periphery as well as in the brain; the peripheral levels of TLRs have not been determined in my work and may be playing an important role.

As discussed in ***Chapter 3.5.3***, these protective effects may arise from candesartan's action on the renin-angiotensin system and its potential regulation of oxidative and inflammatory stresses through this mechanism. Further work is needed to assess the effect of

candesartan on the renin-angiotensin system in my work. Though the expression of AT_1 was measured, this did not show any differences in AT_1 mRNA levels on candesartan treatment. Techniques such as quantitative autoradiography may be used as a more accurate alternative to assess the ability of angiotensin II to bind to the AT_1 receptors on candesartan treatment (as carried out in work including Armando et al., (2001) and Nishimura et al., (2000)).

Alternatively, candesartan may still be working through neuronal TLRs, however instead of regulating inflammation it may be having an effect on the autophagy-lysosome system to remove aggregated α-synuclein. The assessment of this in an animal model would require a method of measuring TLR expression specifically on different cell types – potentially though immunofluorescence double-labelling with a neuronal marker and an anti-TLR antibody. In addition, post-mortem work could be carried out to assess the levels of key readouts of autophagy in neurons such as p62 expression. Finally, TLRs expressed by innate peripheral immune cells may also play a role in the development of pathology; these could be assessed using flow cytometry techniques.

5.4.2 Human PBMC studies

The work carried out in ***Chapter 4*** has optimised a protocol for further studies on human PBMC inflammatory responses to α-synuclein. This work was planned with the ultimate goal of carrying out a much larger study comparing the responses of PBMCs from a cohort of PD patients versus age-matched healthy controls. Unfortunately, due to time restraints, this was not possible to achieve within the timeframe of my PhD, however this will be an important future experiment. This work could utilise existing PBMC samples from the NET-PDD (Neuroinflammation and Tau aggregation in Parkinson's Disease Dementia) study carried out by Dr Antonina Kouli and Dr Caroline Williams-Gray. This study recruited newly-diagnosed patients – within 2 years of diagnosis – stratified into two prognostic groups according to their risk of developing dementia during disease progression, and age-matched controls. As the primary goal of the study was to monitor neuroinflammation longitudinally, the cohort was recruited with strict exclusion criteria in regards to any confounding inflammation – i.e. no chronic inflammatory or autoimmune disorders and no use of anti-inflammatory or immune modulating medications. This makes the samples ideal for use in this study to determine the inflammatory reaction of PBMCs to α-synuclein.

Stored PBMCs from participants from each of the aforementioned stratified prognostic groups in addition to age and sex-matched healthy controls could be used. These could be cultured, stimulated, and treated as optimised in ***Chapter 4***. This would allow an investigation of whether the TLR-mediated inflammatory response is related to clinical heterogeneity and the progression of disease. It would also allow further investigation into whether the activation of peripheral immune cells observed in patient-derived PBMCs (Drouin-Ouellet et al., 2015; Grozdanov et al., 2014; Wijeyekoon et al., 2020) is due to inherent differences in the function of immune cells in the disease state, or whether it is due to extrinsic factors such as exposure to pathogenic α-synuclein aggregates when in the disease state. This work could then be extended to see how immune cells interact within an environment more physiologically similar to that which would be experienced within the PD brain. Work by Emin et al., (2022) has utilised a technique produced by Hong et al., (2018) whereby post-mortem brain is soaked to extract the soluble oligomeric species. Emin showed that soaked PD brains contained a larger proportion of smaller α-synuclein aggregates than control brain, and that these aggregates produced a greater inflammatory response when added to cultured BV2 microglia. The use of soluble extracts from soaked PD or control brains to stimulate PBMCs from PD or control individuals would allow further analysis of whether the immune response of PBMCs is inherently different when the immune cells are derived from people with PD, or whether the extrinsic environment (i.e. the nature of α-synuclein species from the soaked diseased brain) is a greater factor in the differing levels of inflammation seen in people with PD. These studies using soaked brain could also allow further analysis of whether TLR blockers, in particular candesartan, are sufficient to control an immune response triggered by pathological α-synuclein species.

The work completed in ***Chapter 4*** was carried out using whole PBMCs due to my early work demonstrating that cultured monocytes show high basal levels of inflammation, potentially due to stress from the combination of the freeze/thaw and monocyte isolation processes. It was also thought that PBMCs may more accurately demonstrate physiological conditions whereby interactions between different types of immune cells are occurring. Further work could be carried out to determine which immune cell type primarily underlies the PBMC response to α-synuclein shown in my work. This could be achieved using post-culture flow cytometry to investigate specific immune cell activation markers, and could be further extended to intracellular flow to measure the cytokine production – particularly focussing on TNF-α – from these different cell types. Flow cytometry could also be carried out to assess TLR levels

on different cell types, and to investigate whether the candesartan treatment is decreasing the TLR expression, or whether it is having its anti-inflammatory effects through different mechanisms.

The question of whether inflammation is key to the neurodegeneration seen in the PD brain is a contentious one. The *in vitro* assays used in ***Chapter 4*** could be extended to explore the link between neuroinflammation and neurodegeneration. The treatment of cultured neurons – in the form of patient iPSC-derived neurons, a neuron cell line such as SH-SY5Y, or rat primary neurons – with the supernatant from the cultured and α-synuclein stimulated human PBMCs used in this work could be carried out. This would provide an insight into how the inflammatory factors released from α-synuclein-stimulated cells affect the survival and health of neurons. This could then be extended to investigate the protective effects of TLR blockers and whether their action on immune cells is sufficient to decrease the hypothesised toxicity on neurons. Furthermore, TLR blockers could be used specifically on cultured neurons to investigate the suggestion previously discussed that the protective action of candesartan in the animal model is due to candesartan abrogating the neuronal TLR-induced inhibition of autophagy. This work would contribute to an understanding of the protective effect seen on cholinergic neurons in the animal model despite a lack of anti-inflammatory effects.

These further projects would extend the work I have carried out and provide further evidence to support the use of TLR blockers, such as candesartan, in a clinical trial for PD. Ultimately this may lead to a new therapeutic strategy through which the progression of PD may be slowed, with implications for improving the quality of life of many around the world experiencing PD.

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