Investigating the role of Fractalkine and S1P receptors on oxidative stress in the CNS

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List of Abbreviations

AD	Alzheimer's disease
ADAM	A disintigrin and metalloprotease
AKT	Small GTPase
ALS	Amyotrophic lateral sclerosis
BBB	Blood brain barrier
BSA	Bovine serum albumin
CAT	Catalase
cAMP	cyclic adenosine monophosphate
CNS	Central Nervous System
CX3CL1	Fractalkine
CX3CR1	Fractalkine receptor
DMSO	Dimethyl sulfoxide
EAE	Experimental autoimmune encephalomyelitis
EDG-1	Endothelial differentiation gene-1
ERK	Extracellular-signal-regulated kinase
FBS	Fetal bovine serum
GOX	Glucose oxidase
GPx	Glutathione peroxidase
GPCR	G protein-coupled receptor
H_2O_2	Hydrogen peroxide
HIV	Human immune-deficiency virus
HO-1	Hemeoxygenase-1
IFNγ	Interferon gamma
IL-6	Interleukin 6
IL-1β	Interleukin 1 beta
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MBP	Myelin basic protein
MOG	Myelin oligodendrocyte glycoprotein
MPP	Matrix metalloprotease
MS	Multiple sclerosis
NFH	Neurofilament-Heavy
NF-κB	Nuclear factor kappa b
NK	Natural Killer
Nrf2	Nuclear factor-like 2
OPC	Oligodendrocyte precursor cell
PD	Parkinson's disease
pFTY720	Phosphorylated FTY720
Pi3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR-MS	Relapsing remitting multiple sclerosis
S1P	Sphingosine 1-phosphate
S1PR	Sphingosine 1-phosphate receptor
SOD1	Superoxide dismutase-1
SphK	Sphingosine kinase
SP-MS	Secondary progressive multiple sclerosis
TNFα	Tumour necrosis factor alpha

Scientific Abstract

The fractalkine ligand (CX3CL1) is a unique chemokine that is synthesised as a transmembrane protein. One of the unique features of fractalkine is its ability to exist as both a membrane tethered adhesion molecule and as a soluble chemotactic ligand via activation of its G protein-coupled receptor CX3CR1. Importantly, the fractalkine ligand is expressed in astrocytes and reported to be involved in regulating the inflammatory process. Here, we examined how fractalkine is regulated and cleaved from human astrocytes. Our studies show, for the first time, that ADAM10 is the main protease responsible for the cleavage of fractalkine from the surface of astrocytes under pro-inflammatory conditions. We also demonstrate the involvement of p38 MAPK and NF-κB in this regulation of sCX3CL1 (**Results, Chapter 3**). Using the S1PR agonist, pFTY720 and recombinant fractalkine (rCX3CL1), we investigated the effects of S1PR and CX3CR1 activation on oxidative stress induced demyelination in organotypic cerebellar slice cultures treated with bolus concentrations of H_2O_2 (0.1-1mM) and low-continuous H_2O_2 (~20μM) generated from glucose oxidase and catalase (GOX-CAT). We report that pFTY720 and fractalkine attenuated both bolus and continuous H_2O_2-induced demyelination (**Results, chapter 4 and 5**). In addition, both bolus H_2O_2 and GOX-CAT treatments caused a significant decrease in vimentin fluorescence in these slice culture models with no change observed in microglial Iba1 fluorescence. Importantly, pre-treatment with pFTY720 partially attenuated bolus H_2O_2-induced decrease in vimentin fluorescence, while significantly attenuating GOX-CAT induced decrease in vimentin fluorescence. Of note, pFTY720 did not reduce GOX-CAT induced levels of the inflammatory cytokines, IL-6 and fractalkine (**Results, chapter 4**). In contrast, we observed that rCX3CL1 attenuated bolus H_2O_2-induced decrease in vimentin fluorescence but had no effect on GOX-CAT induced decrease in vimentin fluorescence. Again we observed that rCX3CL1 did not reduce GOX-CAT induced levels of IL-6 nor did it alter Iba1 fluorescence (**Results, chapter 5**). Overall, this study establishes a new model of oxidative stress induced demyelination through the use of GOX-CAT enzymes and suggests that pFTY720 can attenuate oxidative stress induced demyelination. We also highlight the potential for fractalkine receptor modulation as a new therapeutic avenue for demyelinating diseases.

Lay Abstract

The brain is made up of four major types of brain cells. Nerve cells (neurons) are specialised cells that process and carry 'messages' through electrical signals. Oligodendrocytes are cells which are wrapped around neurons and form a layer of insulation (myelin), which help carry the neuron 'message'. Microglia, are cells which form the brains immune system. Astrocytes help to form and maintain the network on which neurons grow. All brain cells use oxygen to make energy (metabolism) and bi-products of this reaction, such as hydrogen peroxide (H_2O_2), are created for a short period of time. The brain normally clears and detoxifies these bi-products, before they become harmful as they can cause oxidative damage and inflammation. All brain cells have a large number of proteins on their surface called receptors, which are used to communicate with other cells. These receptors are activated by molecules released by other cells and can also be activated by drugs. In this thesis we looked at the drug that is used to treat multiple sclerosis (MS) called Gilenya (pFTY720). This drug can bind specific receptors, which are called sphingosine 1-phosphate receptors (S1PRs) on brain cells and immune cells. T cells are one type of immune cell that are thought to cause damage in the brain during MS. FTY720 is thought to prevent these T cells from entering the brain and causing damage to nerve cells, which can lead to the symptoms of MS. We also looked at a molecule called fractalkine, which is naturally released from neurons and astrocytes. Fractalkine can activate its own receptor called CX3CR1. In many brain diseases such as MS and Alzheimer's disease cells start to die from the build up of toxic products such as H_2O_2. In this thesis we used cultures of astrocytes and brain slices from mice to investigate the drug Gilenya and the natural molecule fractalkine. We showed that they were protective of cells from toxic levels of H_2O_2. This research may provide a better understanding of how we can modulate different brain cell functions to develop new drugs for brain-related illnesses.

Aims

The specific aims of this thesis were to:

- Investigate the expression and release of the chemokine fractalkine from human astrocytes (Results chapter 3)
- Explore the intracellular mechanism of fractalkine synthesis (Results chapter 3)
- Determine the proteases involved in the cleavage of fractalkine from the surface of stimulated human astrocytes (Results chapter 3)
- Study the effects of H_2O_2-induced oxidative stress on myelination in cerebellar slices and establish a new model of oxidative stress induced demyelination through use of the enzymes glucose oxidase (GOX) and catalase (CAT) (Results chapter 4)
- Determine if pFTY720 protects against H_2O_2–induced demyelination in cerebellar slice cultures (Results chapter 4)
- Investigate the potential protective effects of pFTY720 on oxidative stress induced cell death in dissociated astrocyte cultures and astrocytes within organotypic cerebellar slices (Results chapter 4)
- Examine if pFTY720 modulates GOX-CAT induced levels of IL-6 and fractalkine (CX3CL1) (Results chapter 4)
- Explore the potential therapeutic effects of recombinant fractalkine on bolus H_2O_2 and GOX-CAT induced demyelination in cerebellar slice cultures (Results chapter 5)
- Determine if recombinant fractalkine has protective effects on vimentin expression in cerebellar slice cultures (Results chapter 5)
- Investigate if recombinant fractalkine modulates GOX-CAT induced levels of IL-6

Hypothesis

We hypothesise that the chemokine fractalkine can be cleaved from the astrocytic membrane by a member of the ADAMs family of proteases. We propose that bolus H_2O_2 and GOX-CAT induced levels of H_2O_2 will have a deleterious effect on myelination and vimentin in cerebellar slices. We also hypothesise that pFTY720 and fractalkine will have similar protective effects on bolus as well as GOX-CAT induced demyelination.

Value of Research

Fractalkine/CX3CR1 signalling is recognised as a major contributor and regulator of the inflammatory process. By making use of selective compound and pathway inhibitors we show for the first time that ADAM10 proteases combined with P38 MAPK and NF-κB, are important for regulating fractalkine cleavage in astrocytes. These results suggest that ADAM10 is an important regulator of the astrocyte inflammatory response and shows that fractalkine is differentially cleaved in astrocytes and neurons. We also describe for the first time, the use of glucose oxidase and catalase to generate low levels of H_2O_2 continuously, which induces demyelination in organotypic slice cultures, similar to bolus H_2O_2. Importantly, we demonstrate that a drug used in multiple sclerosis pFTY720, which is an S1PR1, 3, 4 and 5 agonist, attenuates demyelination induced by both bolus and continuous H_2O_2. Moreover, we show that the protective effects of pFTY720 are independent of changes in the levels of IL-6. Overall this work suggests that sphingosine 1-phosphate receptors might have utility as drug targets in neurodegenerative illnesses in which oxidative stress and demyelination are implicated. In addition, we suggest fractalkine receptor (CX3CR1) modulation may be a potential new target for protecting against oxidative stress induced demyelination in both inflammatory and non-inflammatory conditions.

Outputs

Manuscripts

- **O'Sullivan SA**, Gasparini F, Mir A.K, Dev K.K, FRACTALKINE SHEDDING IS MEDIATED BY P38 AND THE ADAM10 PROTEASE UNDER PRO-INFLAMMATORY CONDITIONS IN HUMAN ASTROCYTES. Journal of Neuroinflammation, 2016 Aug 22; 13(1):189.
- **O'Sullivan SA.** and Dev K.K, DEMYELINATION INDUCED BY OXIDATIVE STRESS IS REGULATED BY SPHINGOSINE 1-PHOSPHATE RECEPTORS. GLIA. *In revision*
- **O'Sullivan SA.** and Dev K.K, SPHINGOSINE-1-PHOSPHATE RECEPTOR THERAPIES: ADVANCES IN CLINICAL TRIALS FOR CNS-RELATED DISEASES. *Submitted*

Presented Posters

- **O'Sullivan S** and Dev K.K. THE REGULATION OF THE FRACTALKINE LIGAND IN HUMAN ASTROCYTES. Glia conference, Bilbao, Spain (July 2015).
- **O'Sullivan S** and Dev K.K. CX3CL1 SIGNALLING IN HUMAN ASTROCYTES. Frontiers in neurology Ireland conference (Nov 2015)
- **O'Sullivan S**, Healy L.M, Connor TJ, Dev K.K. S1PR–TLR4 SIGNALLING IN ASTROCYTES AND THEIR ROLE IN PRO-INFLAMMATORY CHEMOKINE RELEASE. 9th FENS Forum of Neuroscience in Milan, Italy from 5-9th July 2014
- **O'Sullivan S**, Healy L.M, Connor TJ, Dev K.K. SPHINGOSINE-1-PHOSPHATE–TLR4 SIGNALLING IN ASTROCYTES AND THEIR ROLE IN CHEMOKINE RELEASE. Joint Biochemical society/British Neuroscience society conference on Astrocytes in Health and Neurodegenerative Disease, London, 28-29th April 2014
- **O'Sullivan S**, Healy L.M, Connor TJ, Dev K.K. S1P RECEPTORS REGULATE TLR4-MEDIATED CHEMOKINE RELEASE FROM ASTROCYTES. Neuroscience Ireland conference (Sept 2013)
- **O'Sullivan S**, Healy L.M, Connor TJ, Dev K.K. MODULATION OF S1P RECEPTORS IMPAIRS LPS INDUCED CHEMOKINE SYNTHESIS IN ASTROCYTES**.** Neurology Ireland conference (Nov 2013)
- **O'Sullivan S**, Healy L.M, Connor TJ, Dev K.K. S1P RECEPTOR ACTIVATION ATTENUATES LPS INDUCED CHEMOKINE RELEASE FROM ASTROCYTES Postgraduate research day Trinity College (Nov 2013)

Awards

- All expenses paid IBRO-UNESCO School on computational and Theoretical Neuroscience (Hyderabad India, 2012)
- Biochemical society student travel Bursary (£300) to attend the Astrocytes in Health and Neurodegenerative Disease conference which took place in London from 28-29th April 2014

- Trinity College Dublin Trust Travel Grant (€300) to attend the 9th FENS Forum of Neuroscience in Milan, Italy from 5-9th July 2015

Chapter 1:
Introduction

1. Introduction to fractalkine/CX3CR1 signalling

Fractalkine is a unique chemokine because of its structural and functional properties. Fractalkine signals through CX3CR1, which is a G protein-coupled receptor, permitting chemotactic as well as cell adhesive properties (D'Haese et al., 2010). The constitutive expression of both fractalkine and CX3CR1 in the brain sets it apart from most other chemokine/receptor pairs and gives us a new ligand-receptor duo to consider for discovering the complexities of cell-cell communication in the CNS (Harrison et al., 1998). Although the expression pattern of fractalkine and its receptor between rodent and mammalian cells remains controversial, it is thought the fractalkine ligand is primarily expressed in neurons and to a lesser extent in astrocytes. The fractalkine receptor, CX3CR1 is expressed primarily by microglia (Hatori et al., 2002).

1.1 Fractalkine structure and distribution

Chemokines are classified into four subfamilies, which are organised based on their amino acid sequence. Fractalkine is categorised in the CX3C subfamily (Rostene et al., 2007) and to date it appears that fractalkine only binds one receptor – CX3CR1, which is unusual for chemokines (**Table 1.1**). Murine fractalkine shares 85% and 78% sequence homology with rat and human fractalkine respectively (Harrison et al., 2001) (**Figure 1.1**). The full length fractalkine is larger than most other chemokines at a size of approximately 373 amino acids, compared to the more common size of 70 amino acids (Harrison et al., 2001). Fractalkine can also exist in different forms. Firstly, at approximately 95 kDa, the full length membrane bound molecule contains an N-terminal chemokine domain, a mucin-like stalk region, a single transmembrane hydrophobic region and a short intracellular C-terminal domain (Harrison et al., 2001). This membrane bound form of fractalkine serves as an important adhesion molecule for interactions with CX3CR1 signalling and for firm adhesion of CX3CR1 expressing immune cells (Harrison et al., 2001). The extracellular chemokine domain of the fractalkine ligand can be cleaved by different proteases such as ADAM (a disintigrin and metalloproteinase) 10 and 17 (also known as TNFα converting enzyme or TACE) or cathepsin-S, which is a lysosomal cysteine protease, in a process known as ectodomain shedding (Hundhausen et al., 2003, Garton et al., 2001) which is important for chemotaxis of CX3CR1 expressing cells (**Figure 1.2**).

1.2 Fractalkine and CX3CR1-mediated signalling

Communication between neuronal and glial cells from early development to late adulthood may be an important factor in many CNS pathologies. In the developing brain fractalkine/CX3CR1 signalling has been shown to play an important role in brain modelling (Paolicelli et al., 2011). Studies in CX3CR1$^{-/-}$ mice have shown that these mice have more synapses, a reduced number of circulating microglia in the hippocampus compared to wild type and have delayed maturation of functional glutamate receptors (Hoshiko et al., 2012, Paolicelli et al., 2011). Disruption to CX3CL1/CX3CR1 from conception also causes a differential response to fractalkine treatment in comparison to wild-type animals following inflammatory and neurodegenerative insults (Cardona

et al., 2006, Pabon et al., 2011, Paolicelli et al., 2011). Fractalkine has also been shown to reduce microglial adhesion to poly-L-lysine coated surfaces in in-vitro assays (Maciejewski-Lenoir et al., 1999). Fractalkine signalling in developmental stages may act as a chemoattractant for microglia to enter the brain and perform synaptic pruning, especially in areas of the hippocampus (Paolicelli et al., 2011). Interestingly, fractalkine has an inhibitory effect on neuronal migration possibly through increasing binding and adhesion to the extracellular matrix (Lauro et al., 2006). As well as maintaining homeostasis in the brain, it has also been suggested that fractalkine is one of the first responders to neuronal injury (Tsou et al., 2001). High levels of glutamate have been shown to lead to ADAM17 activation resulting in the cleavage of fractalkine in order to contain the neuronal damage (Tsou et al., 2001). In the adult brain, it is thought that tonic fractalkine signalling keeps microglia in a quiescent state. In vivo studies in aged mice demonstrated decreased levels of fractalkine, which correlated with an increase in CX3CR1 and microglial activation (Lyons et al., 2009). If this decrease in fractalkine in aged mice is comparable to human brains, then a decrease in fractalkine, followed by an increase in microglial activation could play a major role in many neurodegenerative disorders. Studies have also shown that TNFα secreted from LPS activated microglia can be attenuated with the addition of exogenous fractalkine (Mizuno et al., 2003). Additionally, a fractalkine neutralising antibody enhanced LPS induced microglial neurotoxicity, further suggesting that tonic activation of CX3CR1 by fractalkine may serve as an anti-inflammatory signal which helps to maintain CX3CR1 expressing microglia in a quiescent state (Mizuno et al., 2003).

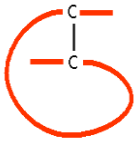
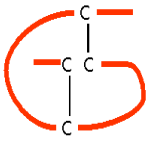
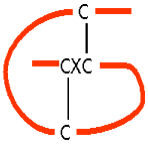
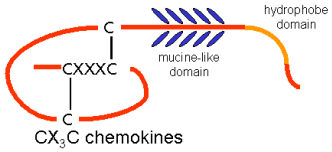
Sub-family	Sub-group	Nomenclature	Common name
CC Chemokines 	Allergenic	CCL1 CCL2 CCL7 CCL8 CCL11 CCL12 CCL13 CCL24 CCL26	I-309 MCP-1 MCP-3 MCP-2 Eotaxin **MCP-5 *MCP-4 Eotaxin-2 *Eotaxin-3
	Inflammatory	CCL3 CCL4 CCL5 CCL6 CCL9 CCL10 CCL18	MIP-1α MIP-1β RANTES **C10 **MIP-1γ **CCL10 *MIP-4
	Hemofiltrate	CCL14 CCL15 CCL16 CCL23	*CC-1 *Leukotactin-1 LEC *MPIF-1
	Developmental	CCL17 CCL22 CCL25	TARC MDC TECK
	Homeostatic	CCL19 CCL20 CCL21	ELC LARC SLC
	Other	CCL27 CCL28	ESkine MEC
CXC Chemokines 	ELR+	CXCL1 CXCL2 CXCL3 CXCL5 CXCL6 CXCL7 CXCL8 CXCL15	GROα GROβ GROγ ENA-78 GCP-2 NAP-2 IL-8 Lungkine
	ELR-	CXCL4 CXCL9 CXCL10 CXCL11 CXCL12 CXCL13 CXCL14 CXCL16	PF-4 MIG IP-10 I-TAC SDF-1 BCA-1 BRAK CXCL16
C Chemokines 	N/A	XCL1 XCL2	Lymphotactin α *Lymphotactin β
CX3C Chemokines 	N/A	CX3CL1	Fractalkine

Table 1.1 Chemokine classifications

Adapted from Savarin-Vuaillat and Ransohoff (2007). *Human only; **mouse only; ELR+/-, glutamic acid-leucine-arginine sequence motif present/absent; N/A, non-applicable.

```
Hu  CX3CL1    MAPISLSWLL  RLATFCHLTV  LLAGQHHGVT  KCNITCSKMT  SKIPVALLIH  YQQNQASCGK  RAIILETRQH  RLFCADPKEQ  WVKDAMQHLD
Mur CX3CL1    MAPSPLAWLL  RLAAFFHLCV  LLPGQHLGMT  KCEIMCDKMT  SRIPVALLIR  YQLNQESCGK  RAIVLETTQH  RRFCADPKEK  WVQDAMKHLD
Rat CX3CL1    MAPSQLAWLL  RLAAFFHLCV  LLAGQHLGMT  KCNITCHKMT  SPIPVTLLIH  YQLNQESCGK  RAIILETRQH  RHFCADPKEK  WVQDAMKHLD

              91                                                                                              180
Hu  CX3CL1    RQAAALTRNG  GTFEKQIGEV  KPRTTPAAGG  MDESVVLEPE  .ATGESSSLE  PTPSSQEAQR  ALGTSPELPT  GVTGSSGTRL  PPTPKAQDGG
Mur CX3CL1    HQAAALTKNG  GKFEKRVDNV  TPGITLATRG  LSPSALTKPE  SATLEDLALE  LTTISQEARQ  TMGTSQEPPA  AVTGSSLSTS  EAQDAGLTAK
Rat CX3CL1    HQTAALTRNG  GKFEKRVDNV  TPRITSATRG  LSPTALAKPE  SATVEDLTLE  PTAISQEARR  PMGTSQEPPA  AVTGSSPSTS  KAQDAGLAAK

              181                                                                                             270
Hu  CX3CL1    PVGTELFRVP  PVSTAATWQS  SAPHQPGPSL  WAEAKTSEAP  STQDPSTQAS  TASSPAPEEN  APSEGQRVWG  QGQSPRPENS  LEREEMGPVP
Mur CX3CL1    PQSIGSFEAA  DIST.TVWPS  PAVYQSGSSS  WAEEKATESP  STTAPSPQVS  TTSPSTPEEN  VGSEGQPPWV  QGQDLSPEKS  LGSEEINPV.
Rat CX3CL1    PQSTGISEVA  AVST.TIWPS  SAVYQSGSSL  WAEEKATESP  PTIALSTQAS  TTSS..PKQN  VGSEGQPPWV  QEQDSTPEKS  PGPEETNPV.

              271                                                                                             360
Hu  CX3CL1    AHTDAFQDWG  PGSMAHVSVV  PVSSEGTPSR  EPVASGSWTP  KAEEPIHATM  DPQRLGVLIT  PVPDAQAATR  RQAVGLLAFL  GLLFCLGVAM
Mur CX3CL1    .HTDNFQERG  PGNTVHPSVA  PISSEETPSP  ELVASGSQAP  KIEEPIHATA  DPQKLSVLIT  PVPDTQAATR  RQAVGLLAFL  GLLFCLGVAM
Rat CX3CL1    .HTDIFQDRG  PGSTVHPSVA  PTSSEKTPSP  ELVASGSQAP  KVEEPIHATA  DPQKLSVFIT  PVPDSQAATR  RQAVGLLAFL  GLLFCLGVAM

              361                                  398
Hu  CX3CL1    FTYQSLQGCP  RKMAGEMAEG  LRYIPRSCGS  NSYVLVPV
Mur CX3CL1    FAYQSLQGCP  RKMAGEMVEG  LRYVPRSCGS  NSYVLVPV
Rat CX3CL1    FAYQSLQGCP  RKMAGEMVEG  LRYVPRSCGS  NSYVLVPV
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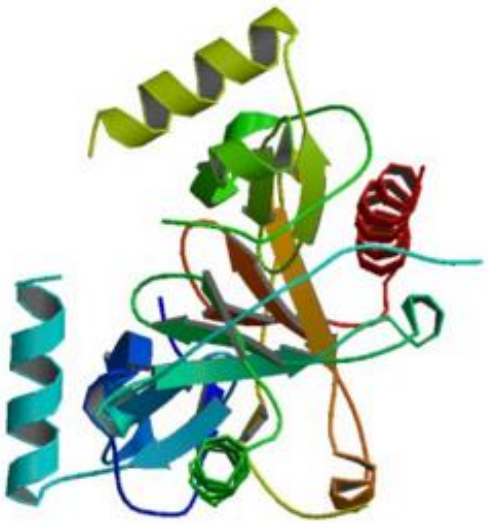


Fractalkine chemokine domain – ribbon diagram

Figure 1.1: Protein sequences of human, rat and mouse fractalkine

The green coloured sequences designate the signal peptide; red sequences are the chemokine domains; blue sequences are the membrane spanning domains; yellow sequences are the C-termini. Any difference in sequences in the coloured domains, between human and mouse or rat, are underlined. Ribbon structure source (Hoover DM, et al., 2000)

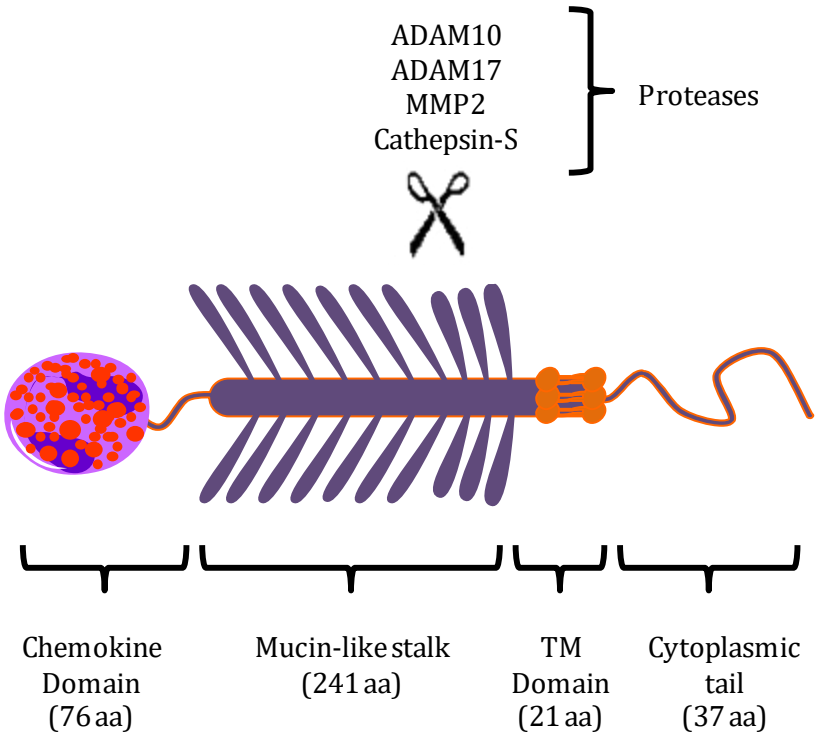


Figure 1.2: Illustration of membrane bound fractalkine

Fractalkine is synthesised as a membrane protein containing a chemokine domain projected out from the cell by a highly glycosylated mucin stalk, a transmembrane domain and an intracellular cytoplasmic tail. Cleavage by metalloproteases occurs at an unknown juxtamembrane site that gives rise to the soluble form of fractalkine.

2. Role of fractalkine signalling in the CNS

2.1. Neurons

CX3CR1 expression on neurons is controversial as many studies have shown the presence or absence of CX3CR1. In support of neuronal CX3CR1 expression, investigations into the direct effects of fractalkine on neurons have shown that signalling through CX3CR1 on neurons activates the ERK1/2 and AKT pathways and inhibit NMDA induced calcium influx (Deiva et al., 2004, Meucci et al., 1998, Meucci et al., 2000). Fractalkine has also been shown to inhibit neuronal cell death (Tong et al., 2000). Neuronal expression of CX3CR1 may also allow fractalkine to signal in an autocrine and paracrine fashion (Hatori et al., 2002). Hippocampal neurons have been shown to express higher levels of fractalkine than neurons in other parts of the brain (Harrison et al., 1998). Although fractalkine is constitutively expressed in neurons in the CNS, its expression can also be upregulated by several stimulators such as IL-1β and TNFα (Maciejewski-Lenoir et al., 1999, Chandrasekar et al., 2003). In general, it is thought that an increase in fractalkine on neurons has an overall protective effect on neurons (Sheridan and Murphy, 2013).

2.2. Glial Cells

To date, there are no studies suggesting the presence of fractalkine or its receptor on oligodendrocytes. In contrast, astrocytes have been shown to express the fractalkine ligand (Hatori et al., 2002). Basal levels of fractalkine expression have been shown to be much lower in comparison to neurons. Many studies agree that microglia express the fractalkine receptor CX3CR1 (Maciejewski-Lenoir et al., 1999). Astrocytes however, do not appear to express the receptor, which is in line with studies demonstrating astrocytes do no not migrate towards fractalkine (Lauro et al., 2006). Astrocytes have been shown to respond to pro-inflammatory cytokines by up-regulating and shedding the fractalkine ligand (Maciejewski-Lenoir et al., 1999). This up-regulation and shedding of fractalkine on astrocytes has been suggested to serve as a microglial attractant to sites of inflammation (Sunnemark et al., 2005). In normal conditions, low levels of soluble fractalkine may therefore be modulating the release of further pro-inflammatory cytokines suggesting an overall anti-inflammatory and neuro-protective effect on the CNS. Chemokines have long been thought of as traffickers of immune cells towards inflammation, however studies show their roles extend into most physiological as well as pathological states (Rollins, 1997).

Fractalkine is not thought to be expressed by microglia, however its unique receptor, CX3CR1, is primarily expressed by microglia in the CNS (Hatori et al., 2002). Studies have shown that this allows neurons to regulate microglial activation and proliferation (Hatori et al., 2002, Limatola and Ransohoff, 2014). Therefore communication between neurons and astrocytes expressing fractalkine and CX3CR1 containing microglia may be an important factor in many CNS diseases (**Figure 1.3**). In vitro studies show that fractalkine decreases LPS-induced MHC class II and CD40 levels as well as IL-1β protein expression in microglia, via Akt and PI3Kinase signalling (Lyons et al., 2009). Microglia also have an important role to play in CNS development as evidence shows that

fractalkine can acts as a chemotropic agent for CX3CR1 expressing microglial cells where they undergo synaptic pruning (Paolicelli et al., 2011). In conjunction, fractalkine has also been shown to reduce microglial adhesion to the extracellular matrix, making it easier for microglial cells to migrate throughout the brain (Maciejewski-Lenoir et al., 1999) (**Figure 1.4**).

3. Fractalkine expression and regulation in the periphery

Fractalkine is also expressed in the periphery and has been detected in heart, kidney, lung and testis tissue (Bazan et al., 1997, D'Haese et al., 2010). Fractalkine is primarily localised to endothelial, epithelial and dendritic cells, in contrast to other chemokines, which are predominantly expressed by immune cells. CX3CR1 expression in the periphery is found to be expressed on immune cells including lymphocytes, monocytes, natural killer (NK) cells, T cells and mastocytes (D'Haese et al., 2010, Imai et al., 1997, Jung et al., 2000, Al-Aoukaty et al., 1998, Cambien et al., 2001, Chapman et al., 2000b, Foussat et al., 2000, Inngjerdingen et al., 2001, Kanazawa et al., 1999, Papadopoulos et al., 2000). Fractalkine expression is normally up-regulated in peripheral tissues during inflammatory conditions. In both in vitro and in vivo studies, fractalkine expression is shown to be increased following stimulation of endothelial cells with pro-inflammatory cytokines such as IL-1, TNFα, IFNγ and LPS (Bazan et al., 1997, Imaizumi et al., 2000). As fractalkine is also a membrane bound chemokine it directly mediates the firm adhesion of leukocytes under physiological flow conditions in a selectin- and integrin-independent manner (Fong et al., 1998, Goda et al., 2000, Haskell et al., 1999). Therefore, fractalkine expression on endothelial cells plays a vital role in leukocyte recruitment from the blood into the tissue interstitium under pro-inflammatory conditions. Consistent with this finding, increased expression of fractalkine on endothelial cells has been found in numerous inflammatory diseases such as glomerulonephritis (Feng et al., 1999, Furuichi et al., 2001), atherosclerosis (Greaves et al., 2001), cutaneous inflammatory disease (Raychaudhuri et al., 2001) and Crohn's disease (Muehlhoefer et al., 2000). In parallel, enhanced expression of CX3CR1 in leukocytes has also been found in these peripheral diseases.

Polarized T-cells can also differentially regulate endothelial fractalkine. The T_{H1} cytokine, IFNγ, has been shown to stimulate fractalkine expression, while IL-4 and IL-13, which are T_{H2} cytokines, have no effect (Fraticelli et al., 2001). Indeed, T_{H2} cytokines can inhibit the increase in fractalkine under pro-inflammatory conditions. In agreement, T_{H1} polarised cells preferentially express CX3CR1 compared to T_{H2} phenotypic cells (Fraticelli et al., 2001). Smooth muscle cells cultured in vitro also express fractalkine in a pro-inflammatory dependent manner, however, unlike endothelial cells, smooth muscle cells appear to require co-application of TNFα and IFNγ (Ludwig et al., 2002). The regulation of fractalkine in this T_{H1} dependent manner is further supported by the increased expression of fractalkine on endothelial cells in TH1 dependent pathologies such as psoriasis, mycobacterium tuberculosis (Fraticelli et al., 2001) and cardiac allograft rejection (Robinson et al., 2000). Alternatively diseases that are thought to be T_{H2} driven responses including atopic

dermatitis, Castelman's disease and reactive lymph node hyperplasia, show no changes in fractalkine expression (Fraticelli et al., 2001). Fractalkine has also been shown to induce chemotaxis in CX3CR1 expressing human dermal microvascular endothelial cells in vitro, suggesting a potential role for fractalkine signalling in angiogenesis (Volin et al., 2001).

Epithelial cells in the bronchi and intestines also express fractalkine (Fujimoto et al., 2001, Muehlhoefer et al., 2000). Enhanced levels of fractalkine, in the bronchial epithelium, has also been documented in patients with inflammatory lung disease as well as increased levels of soluble fractalkine in bronchoalveolar lavage fluid (Fujimoto et al., 2001). In line with these in vivo findings, treatment of cultured bronchial epithelial cells with IL-1 and IFNγ causes an increase in the levels of soluble fractalkine (Fujimoto et al., 2001, Muehlhoefer et al., 2000).

Dendritic cells, which play a vital role in antigen presentation, have shown increased levels of fractalkine when mature. This suggests that fractalkine is involved in the interaction between mature dendritic cells and lymphocytes (Papadopoulos et al., 1999). Enhanced fractalkine expression in dendritic cells of psoriasis tissue (Raychaudhuri et al., 2001) and HIV infected individuals (Foussat et al., 2001) has also been shown. Macrophages are also capable of expressing fractalkine, with T_{H2} cytokines causing an increase in fractalkine expression (Greaves et al., 2001). Notably, increased fractalkine expression in immune cells is evident in rheumatoid arthritis as well increased levels of soluble fractalkine in synovial fluid (Ruth et al., 2001).

Disorder	Therapy	Therapeutic effect	Reference
Ischemic renal failure (mouse)	CX3CR1-blocking antibodies	↓Macrophages & delayed disease progression	(Oh et al., 2008)
Lupus nephritis (mouse)	NH2-terminally truncated fractalkine antagonists	Delayed onset & progression	(Inoue et al., 2005)
CFA-induced monoarthritis (rat)	Anti-CX3CR1 neutralising antibodies	↓Allodynia, ↓hyperalgesia & ↓pain	(Sun et al., 2007)
Chronic intrathecal morphine (rat)	Anti-CX3CR1 neutralising antibodies	Potentiates morphine analgesia, attenuates tolerance, hyperalgesia & allodynia	(Johnston et al., 2004)
Glomerulonephritis (rat)	CX3CR1 antagonism via vMIP-II	↓Leukocyte cheomotaxis & infiltration	(Chen et al., 1998)
Non infectious peritonitis (mouse)	CX3CR1 antagonist	↓Macrophage accummulation	(Dorgham et al., 2009)
Xenograft lung carcinoma (mouse)	Vaccination of 3LL lung carcinoma cells gene-modified with fractalkine	↓Tumour growth	(Guo et al., 2003)
Hepatocellular carcinoma (mouse)	Transfer of a fractalkine eukaryotic expression vector into tumor cells	↓Tumour growth & ↑survival	(Tang et al., 2007)
Peripheral and dorsal horn neuropathy (rat)	Irreversible cathepsin S inhibitor	Anti-nociception, anti-hyperalgesia & reversal of allodynia	(Clark et al., 2007)
Neuropathic pain (mouse)	Intraneural fractalkine injection	↓Neuropathic pain	(Holmes et al., 2008)
Neuroblastoma (mouse)	Fractalkine gene therapy in combination with targeted IL-2	↓Primary tumour growth & ↓liver metastases	(Zeng et al., 2005)
Crohn's disease & RA (human)	E6011 - humanized anti-FKN monoclonal antibody	Phase1/2 - Safe & well tolerated	ClinicalTrials.gov Identifier NCT02039063 & NCT02196558

Table 1.2: Therapeutic approaches involving fractalkine/CX3CR1 signalling. CFA; Complete Freund's adjuvant, vMIP; viral macrophage inflammatory protein, RA; rheumatoid arthritis. Adapted in part from D'Haese et al. (2010).

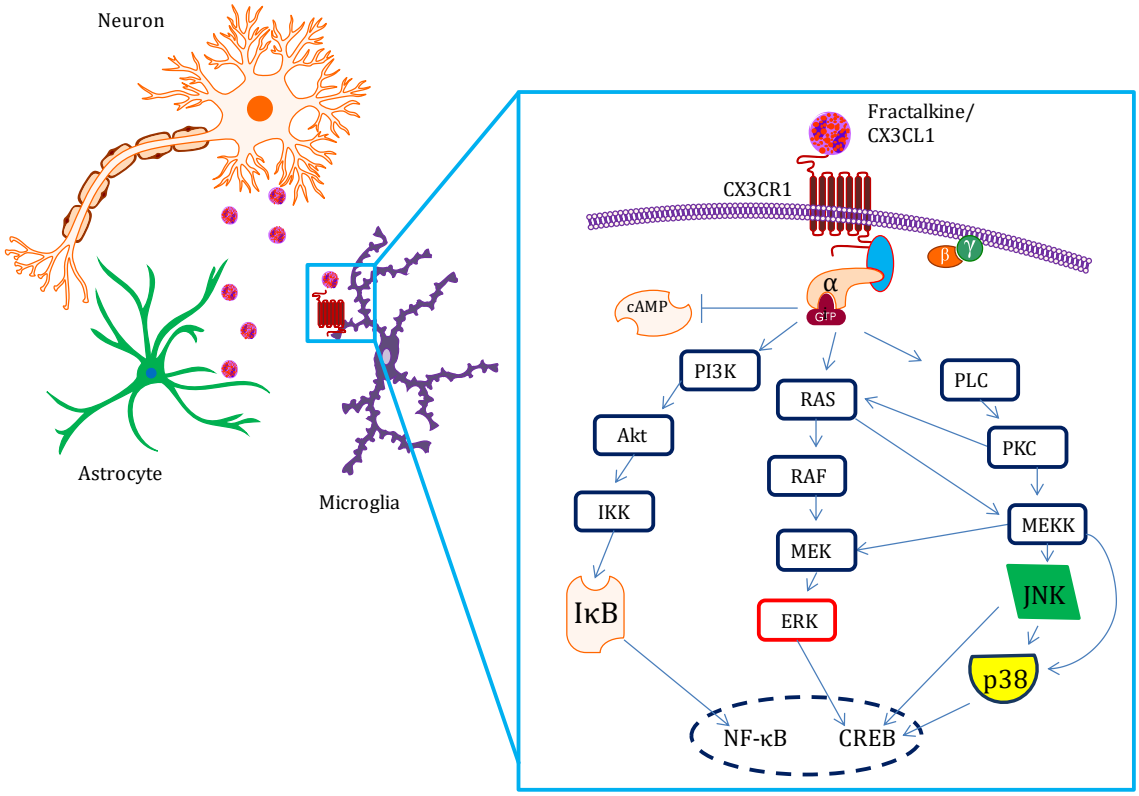


Figure 1.3: CX3CR1 mediated signalling in the CNS

Fractalkine is a G protein-coupled receptor and induces many well known signalling pathways which lead to the activation of NF-κB and CREB. Fractalkine is able to activate numerous signalling cascades which depend on what cell it signals through and whether the ligand is anchored to the membrane or in its soluble form. Pi3K, phosphoinositide 3-kinase; IKK. Inhibitor of kappa B kinase; Akt, serine/threonine-specific protein kinase; RAS, small GTPase; RAF, small GTPase; MEK, mitogen activated protein kinase; ERK, extracellular signal regulated kinase; PLC, phospholipase C; PKC, protein kinase C; JNK, C-Jun N terminal kinase; P38, P38 mitogen activated protein kinase; CREB, cAMP response element-binding protein; NF-κB, nuclear factor kappa B.

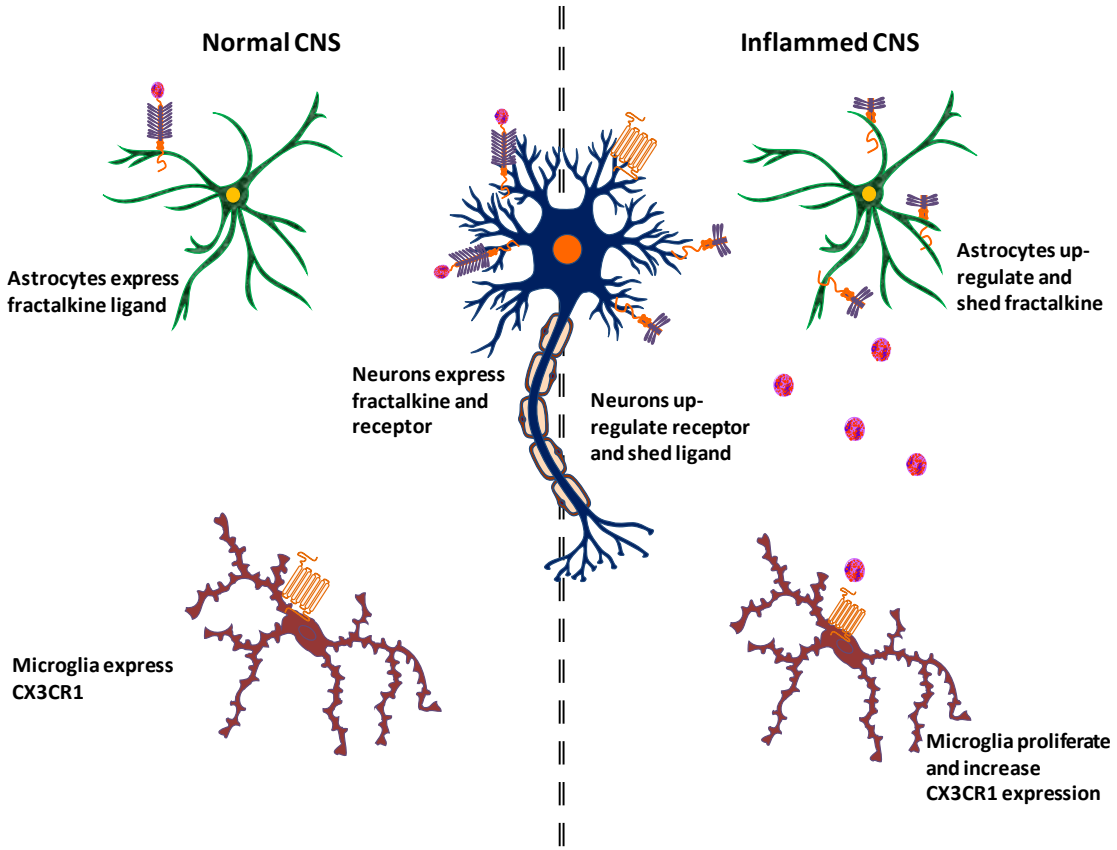


Figure 1.4: Fractalkine and CX3CR1 expression in inflammatory conditions in the CNS

Under normal physiological conditions fractalkine is thought to be expressed on neurons and CX3CR1 expressed on microglia. Astrocytes have little to no detectable levels fractalkine or CX3CR1. Under pathological conditions, fractalkine can be up-regulated on neurons and astrocytes and cleaved by ADAM10 and ADAM17 proteases. Soluble fractalkine activates microglia and creates a chemotactic gradient to attract microglia and other immune cells to the site of damage where they release pro-inflammatory cytokines. Overall, increased fractalkine expression should be protective, and serves to limit inflammation and promote tissue repair.

4. Sphingosine-1-phosphate receptor signalling

Sphingosine-1-phosphate (S1P) is an important signalling sphingolipid. Lipids have important signalling properties and are also implicated in many diverse cellular functions (Toman and Spiegel, 2002). S1P can act as an extracellular ligand for cell surface S1P receptors and intracellularly as a second messenger (Van Brocklyn et al., 1998). The concentration of S1P varies in different body fluids and tissues, and excessive production at inflammatory sites has an impact in various pathological conditions (Brinkmann, 2007). The potential for S1P receptors (S1PRs) as drug targets has been highlighted recently due to a number of studies. FTY720 (Gilenya/Fingolimod) is a structural analogue of sphingosine and targets all known S1P receptor subtypes except S1P2 (Brinkmann, 2007). In-vivo, FTY720 is phosphorylated by sphingosine kinases (SphK) to its active form pFTY720 and it is this form that binds to and modulates S1PRs (Terada et al., 2004).

4.1 S1P distribution and metabolism

S1P is a platelet derived bioactive sphingolipid produced by the metabolism of sphingosine by sphingosine kinase-1 (SphK1) and sphingosine kinase-2 (SphK2) (Pappu et al., 2007). As a result, S1P is found in high concentrations in the plasma and lymph tissue (Pham et al., 2010). High amounts of SphKs along with low levels of S1P phosphatase (converts S1P to sphingosine) and S1P lyase (terminally cleaves the sphingolipid) aids in the production of S1P (**Figure 1.5**). The majority of sphingosine-1-phosphate signalling occurs through the G protein-coupled receptors previously known as the endothelial differentiation gene (EDG) family. These cell surface receptors are expressed ubiquitously across species and together with their diverse cellular functions, sphingosine lipids and their receptors are an important focus of signal transduction research (Toman and Spiegel, 2002, Kono et al., 2008). Of the five known subtypes of S1PRs, (S1PR1-S1PR5), each is approximately 400 amino acids in length. Studies indicate that ceramide, sphingosine and S1P play a very important role in cell survival, growth and differentiation in many cell types including neurons (Terada et al., 2004). Therapeutic strategies based on the interaction and modulation of these receptors is being investigated for a number of different diseases.

4.2 S1P receptor signalling in the CNS

S1P can induce a diverse array of biological responses (Yamagata et al., 2003). Not only is S1P vital for cellular regulation in the adult, it is also critical for the proper development of the cardiovascular and central nervous system during the embryonic stage (Brinkmann, 2007). Four out of the five S1P receptors are found in the CNS, these include S1PR1, 2, 3 and 5, with all four receptors being expressed on neurons, astrocytes, oligodendrocytes and microglia. The level of expression of these receptors can vary depending on the age and growth conditions surrounding the cell. For example, S1PR1 and 3 have been shown to be important for the developing brain and are also up-regulated in the mature brain when exposed to pathological stimuli (Choi and Chun, 2013). In adult cells, S1P can produce cytoskeletal re-arrangements in order to regulate immune

cell trafficking, vascular homeostasis and cell communication in the CNS (Brinkmann, 2007). In the immune system it has been well documented that internalisation of S1PR1 prevents B and T cells from leaving the lymph nodes and entering the lymphatic circulation (Hla and Brinkmann, 2011). This in turn has important ramifications in many disease states where inflammation plays a destructive role.

The active form of FTY720 causes internalisation of the S1PR1 receptor which leads to the reversible inhibition of lymphocyte egress from peripheral lymph nodes. Thus preventing tissue damaging T cells from infiltrating sites of inflammation in the CNS (Groves et al., 2013). As S1P receptors are expressed on all cell types, FTY720 has the capacity to modify diseases on several different levels (Miron et al., 2008b). Although there are multiple S1P receptor subtypes in the brain there is very little known about how they function, either independently or synergistically. Using synthetic analogues of S1P to act as agonists or antagonists is improving our understanding how these receptors work in both healthy and diseased brains (Sim-Selley et al., 2009).

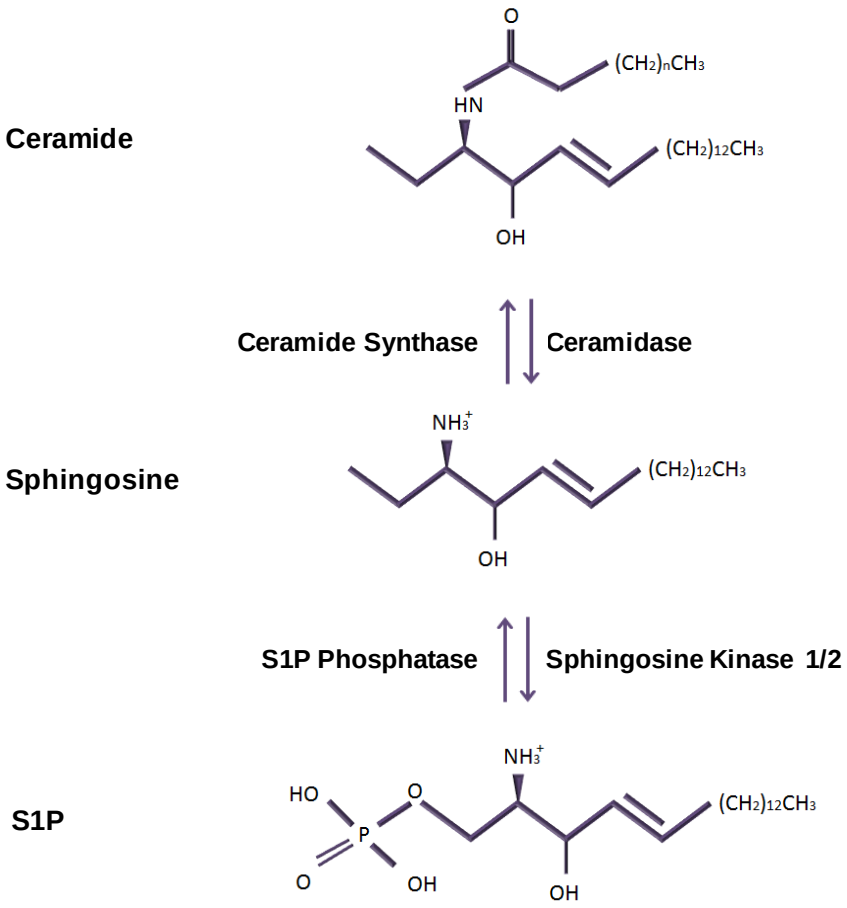


Figure 1.5: Synthesis of Spingosine-1-phosphate

Sphingosine is synthesised from ceramide by ceramidase. In turn, sphingosine is phosphorylated to Sphingosine-1-phosphate (S1P) by one of two sphingosine kinases (Sphingosine kinase-1 and Sphingosine kinase-2).

5. Role of S1P signalling in the CNS

5.1 Neurons

In neurons, *in vitro* experiments reveal that S1P signalling mainly elicits morphological changes related to growth cone formation and neurite extension and retraction (Ishii et al., 2004). As in other cell types, differential expression of S1P receptors can have opposing functions. For example, stimulation of neurons with nerve growth factor (NGF) activates sphingosine kinase 1 (SphK1), which converts sphingosine to sphingosine-1-phosphate. The resulting transactivation of S1P1 promotes neurite extension. Over-expression and application of antisense probes to down-regulate S1P receptors have demonstrated that S1P1 receptors promote neurite extension, while inhibition of this process is caused by signalling of S1P2 and 5 (Toman et al., 2004, MacLennan et al., 2000). In addition, neurons lacking S1P2 have been shown to elicit hyper-excitability (MacLennan et al., 2001). Synaptic activity is another function regulated in part by S1P (Kajimoto et al., 2007, Kanno et al., 2010). The regulation of synaptic activity, along with neuronal viability and neurogenesis, is likely an important factor that relates to psychiatric dysfunctions and memory impairments.

5.2 Glial Cells

Oligodendrocytes are the myelinating cells in the CNS and they also function in development and nervous system repair, in particular remyelination (Snaidero and Simons, 2014). Depending on the maturation state of the myelinating oligodendrocytes, S1P receptor expression will be altered. In mature oligodendrocytes S1P5 is more highly expressed than any other S1P receptor (Kim et al., 2011). S1P5 has been shown to be crucial for survival of mature cells. As a result of S1P5 being expressed at such high levels in mature myelinating oligodendrocytes; it was thought that S1P5 should have a key role in myelination. However, S1P5 null mice develop myelin normally and there were no obvious differences in myelination (Jaillard et al., 2005). Interestingly, animals genetically altered to lack the S1P1 receptor specifically on oligodendrocytes show a decrease in myelin proteins, have a thinner myelin sheath and are more vulnerable to chemically induced demyelination models (Kim et al., 2011). This clearly indicates that S1P1 may have a substantial role in the formation of healthy myelin in the brain.

Astrocytes express S1P3, S1P1 and low levels of S1P2 (Groves et al., 2013). Expression of S1P5 is undetectable under basal conditions but can be up-regulated when exposed to growth factors in culture (Rao et al., 2004). Activated astrocytes up-regulate S1P1 and 3 together suggesting a synergistic or opposing functions of these signalling cascades, in response to pathogens (Choi and Chun, 2013). So far, it has been shown that S1P1 and 3 can manipulate astrogliosis in different disease models including MS and Sandhoffs disease (Choi and Chun, 2013, Wu et al., 2008). However, there are functional differences in that astrogliosis can be either neurotoxic or neuroprotective (Choi and Chun, 2013).

Known as the resident macrophages of the CNS, microglia are the main form of active immune defence in the CNS. As a result of the different activation states of microglia, S1P receptor

expression is very dynamic. For example in activated microglia, S1P1 and 3 are down-regulated but S1P2 is up-regulated (Choi and Chun, 2013). This tells us that S1P receptor expression is extremely important for regulating cellular responses and that the same S1P receptors can elicit different effects in different cell types. Sphingosine lipids have been proposed to regulate pro-inflammatory cytokines such as TNFα, IL-1β and nitric oxide (NO). S1P1 deletion has been shown to reduce microglial activation in experimental autoimmune encephalomyelitis (EAE) spinal cords, however the precise function of these receptors on microglia remains to be established. (**Figures 1.6 and 1.7**)

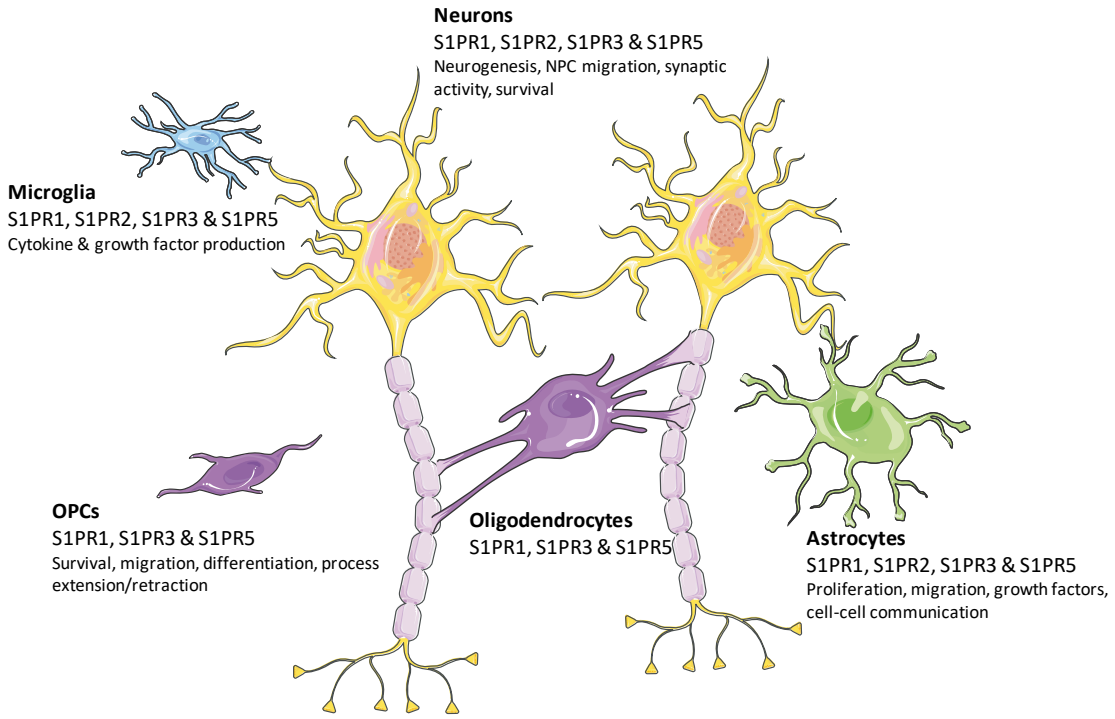


Figure 1.6: S1P receptor expression in the CNS

Distribution pattern and summary of S1P receptor function on cells of the CNS. NPC, neuronal precursor cell; OPCs, oligodendrocyte precursor cell.

6. S1P signalling and distribution in the periphery

S1P receptors have specific physiological roles within the cardiovascular, immune and nervous systems, maintaining vascular tone and barrier integrity and also in the trafficking of lymphocytes (Brinkmann, 2007). S1P receptors also contribute to the pathophysiology of autoimmunity, inflammation and cancer (Brinkmann, 2007, LaMontagne et al., 2006). Consequently, the family of S1P receptors is of high therapeutic value. S1P has a blood concentration of approximately 200-900nM (Murata et al., 2000). S1P is produced by platelets, mast cells and other non-haematopoietic cells such as endothelial cells (Ancellin et al., 2002, English et al., 2000, Prieschl et al., 1999). During inflammatory conditions S1P production is increased through pro-inflammatory cytokine mediated activation of SphK, which in turn converts the pro-apoptotic sphingosine into anti-apoptotic S1P (Chalfant and Spiegel, 2005). S1P also regulates cell trafficking and migration by acting as either a chemoattractant or through modulation of permeability barriers (Brinkmann, 2007). Immune cells such as B and T cells also express S1P1 receptors with lower levels of S1P4. However, some subsets express lower levels of S1P1/4 and higher levels of S1P3 (Sawicka et al., 2005, Wang et al., 2004). Indeed, studies have shown that S1P1 is able to control thymocyte and lymphocyte egress from the thymus and lymphoid tissue into the blood (Matloubian et al., 2004). Dendritic cells in the skin have also been shown to up regulate S1P1 and 3 and migrate into the lymph nodes following activation by antigens (Czeloth et al., 2005). Mast cells, which express S1PR1 and S1PR2 and eosinophils, which express S1PR1 and S1PR2 and S1PR3 are central players in asthma with S1P receptors thought to affect processes of degranulation and chemotaxis. Furthermore, S1P levels in asthmatic patients are raised significantly following an allergen challenge (Chalfant and Spiegel, 2005, Jolly et al., 2004).

Endothelial cells and smooth muscle cells predominantly express S1PR1, 2 and 3 and varying levels depending on vessel type and tissue source. Evidence suggests that S1P can modulate heart rate, blood pressure and endothelial integrity. S1PR1 has also been implicated in blood vessel formation whereby S1P induces tube formation and can also promote de novo angiogenesis, which is critical for tumor progression (Karliner, 2004, Waeber et al., 2004). This widespread expression of S1P receptors throughout the periphery and CNS can therefore lead to side effects of S1P receptor modulation. Common side effects of FTY720 reportedly include fatigue, respiratory tract infections, headaches and gastrointestinal disturbance. Less frequent but more serious side effects can include bradycardia, viral infections, macular edema and atrioventricular block (Ward et al., 2014a).

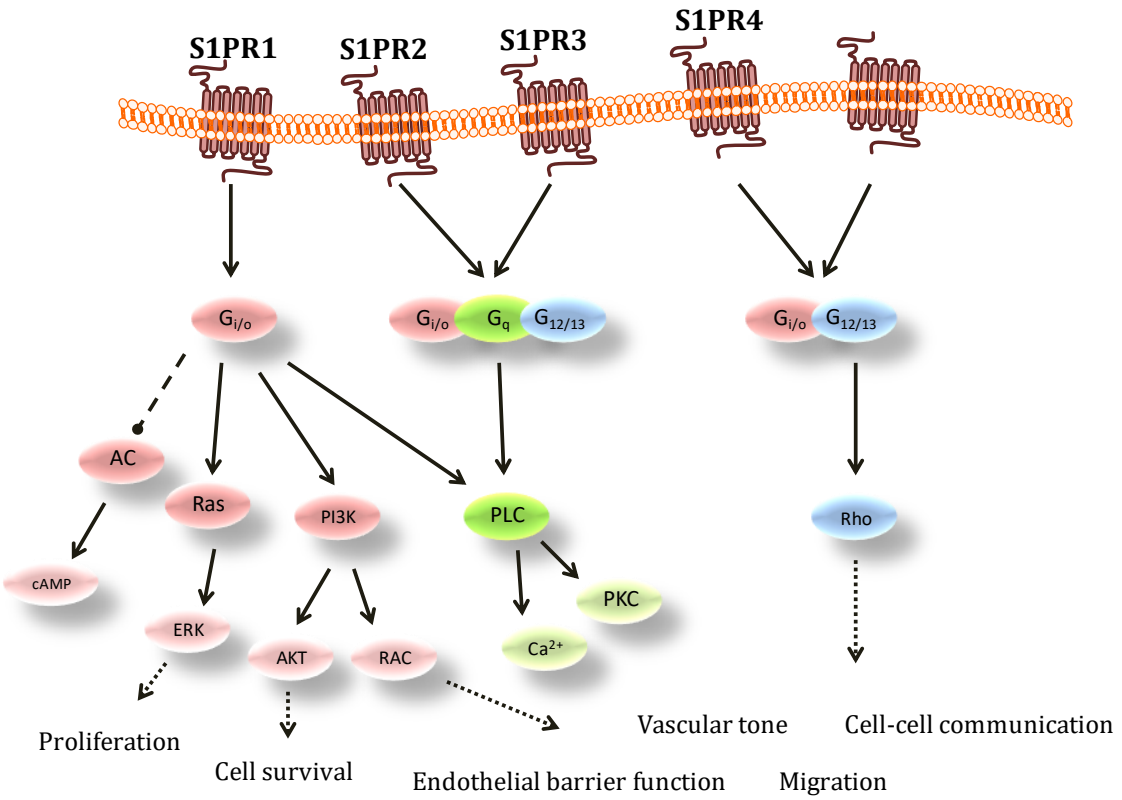


Figure 1.7: S1P receptor signalling pathways

Upon activation, S1P receptors couple to a specific G protein which results in the activation and/or inhibition of downstream second messengers. AC, adenyl cyclase; Ras, small GTPase; Pi3K, phosphoinositol 3-kinase; PLC, phospholipase C.

7. Oxidative stress in the CNS

Oxidative stress is a pathological condition defined as an imbalance between production and removal of reactive oxygen species (ROS). ROS is a term used to collectively describe free radicals and other reactive molecules that have been derived from oxygen (O_2) (Hancock et al., 2001). These oxygen radicals are important by-products of mitochondrial respiration and other enzymatic activities such as oxidation of metals. ROS have a role in many cellular signalling events and can act as both intracellular and extracellular messengers (Hancock et al., 2001, Veal et al., 2007). The electron structure of atomic oxygen makes it susceptible to radical formation, thus, through the reduction of oxygen, many types of ROS can be formed. These include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide (NO) (Hancock et al., 2001, Sies, 2014).

Cells of the CNS are exposed to low concentrations of ROS under normal physiological conditions (Veal et al., 2007). Through the action of enzymatic antioxidants (e.g. superoxide dismutase (SOD) and catalase) and non enzymatic molecules (e.g. glutathione and vitamins C and E), these potentially toxic molecules are neutralised (Satoh and Lipton, 2007) (**Table 1.2**). In particular, SOD catalyses the conversion of superoxide anions into H_2O_2 and O_2. Within the peroxisome, catalase is then able to convert H_2O_2 into water (H_2O) and O_2 (McCord and Fridovich, 1968). The cellular response to oxidative stress will depend on the strength and duration of the exposure. Importantly, Foxo transcription factors are known to play a vital part in regulating cell cycle, metabolism, apoptosis and oxidative stress response (Burhans and Heintz, 2009). The Foxo3a transcription factor is normally held in an inactive state in the cytoplasm, however, during oxidative stress Foxo3a moves to the nucleus and initiates transcription of genes, such as SOD, for oxidant metabolism (Burhans and Heintz, 2009, Kops et al., 2002). It has been suggested that small increases in H_2O_2 can cause cells to re-enter the cell cycle whereas higher, sustained levels of H_2O_2, lead to cell cycle arrest and apoptosis (Choi et al., 2005, Park et al., 2006). Besides endogenous sources, the unstable cellular environment caused by oxidative stress can be generated by UV rays, alcohol, nutrient deficiency, toxins and trauma (**Table 1.3**). ROS can damage DNA, an early step in carcinogenesis and damage other biomolecules, which can lead to atherosclerosis, arthritis, inflammation, diabetes, aging, neurodegenerative diseases and other disorders (**Table 1.4)** (Valko et al., 2007).

The brain and central nervous system are particularly susceptible to damage caused by oxidative stress for several reasons. Brain tissue is composed of nearly 60% lipids and lipids, such as myelin, are particularly sensitive to oxidation (Aikens and Dix, 1991, Chang et al., 2009). Furthermore, the brain has the highest rate of oxygen (O_2) consumption and neurochemical reactions, such as dopamine oxidation, generate high levels of ROS and reactive nitrogen species (RNS) (Chiurchiu and Maccarrone, 2011). There is also a natural increase in the deposition of metal ions with aging, which catalyse the production of ROS and RNS (Zecca et al., 2001, Ward et al., 2014b). However in neurodegenerative diseases, iron accumulation is often greater, than levels in healthy aging (Ward et al., 2014b). ROS are constantly generated by activated microglia and infiltrating macrophages as

part of the inflammatory response and have been implicated in neuronal damage in the neurodegenerative process (Chiurchiu, 2014). Mitochondrial dysfunction in cells of the CNS, are also a likely cause of altered oxidative metabolism, which leads to lipid and DNA oxidation. In addition, ROS have been shown to regulate protease activity such as MMPs and produced direct and indirect damage to the blood brain barrier (BBB) (Leppert et al., 1995, Lehner et al., 2011). Free radicals, derived from ROS, are also known to activate transcription factors such as nuclear factor kappa B (NF-κB), which is involved in the transcription of many pro-inflammatory cytokines and adhesion molecules commonly associated with neurodegenerative diseases **(Figure 1.8)** (Leppert et al., 1995). Examination of brain tissue from patients with neurodegenerative disorders has revealed that, along with increased levels of ROS, there are decreased antioxidants such as SOD, catalase and glutathione (Langemann et al., 1992, Chiurchiu et al., 2016).

ROS production	
Endogenous sources	**Exogenous sources**
Autoxidation of small molecules e.g. thiols, catecholamines and flavins – superoxide is formed by the dioxygen reduction of these molecules	Anti-neoplastic drugs Reduce oxygen to superoxide, hydroxyl radical and hydrogen peroxide
Enzymes e.g. xanthine oxide reductase and aldehyde oxidase – produce superoxide during catalytic cycle	Electromagnetic radiation i.e. x-rays, gamma rays
Mitochondrial electron transport chain Superoxide generated from oxygen metabolism. Radical production from complex I & III	Particle radiation i.e. electrons, protons, deuterons and neutrons
Endoplasmic reticulum and nuclear membrane Both membranes contain cytochromes P450 and b5 which act as oxidisers and reducing agents	Environmental factors e.g. ozone, pesticides, tobacco smoke, solvents, anaesthetics, hydrocarbons.
Peroxisomes Contains oxidase enzymes that produce hydrogen peroxide	Environmental pollution
Plasma membrane Free radicals generated extracellularly cross the plasma membrane which causes lipid/protein oxidation	

Table 1.3: Endogenous and exogenous sources of reactive oxygen species

(Uttara et al., 2009, Jones, 2002, Gandhi and Abramov, 2012)

Antioxidant systems	
Endogenous sources	**Exogenous sources**
Superoxide dismutase (SOD) Catalyses the reduction of superoxide into hydrogen peroxide	Vitamins C & E: high reducing potential, inhibit formation of free radical chain reaction and first barrier to peroxidation of fatty acids
Glutathione system Converts hydrogen peroxide to water and oxygen, reduces peroxides, acts on low concentrations of H_2O_2	Metals, Copper & Selenium: Co-factor for glutathione and synergistic effects with vit. E
Catalase Converts hydrogen peroxide to water and oxygen, acts on high concentrations of H_2O_2	Coenzyme Q10 Transfers electrons from complex I&II to complex III

Table 1.4: Endogenous and exogenous antioxidant systems

(Uttara et al., 2009, Jones, 2002, Gandhi and Abramov, 2012)

8. Role of fractalkine and S1P in neurological conditions

The role of glial cells in brain pathology has become more recognised in recent years. Neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) predominantly involve neuronal death and inflammation in distinct brain areas. Areas of degenerating neurons are known to be populated with glial cells such as microglia and astrocytes (Gao et al., 2003). Traditionally these cells were thought to clear debris but recent research has shown that upon activation, microglia and astrocytes can promote neurodegeneration through the production of cytotoxic molecules such as oxidative stress molecules, cytokines and proteases (Gao et al., 2003). Glial cells are now more accepted as being key neuromodulators for the developing brain as well as in adulthood and therefore must possess several mechanisms of communication with neurons as well as other brain cells.

8.1 Amyotrophic lateral sclerosis

ALS is a neurodegenerative disease that affects both upper and lower motor neurons (Rosen, 1993, Bury et al., 2015, DeJesus-Hernandez et al., 2011). The disease progresses rapidly, resulting in fatality within 3-5 years of diagnosis (Robberecht and Philips, 2013). 10% of ALS cases are inherited while 90% are sporadic. Of the familial cases the most common mutation is in the C9orf72 gene, while the autosomal dominant mutation in the Cu/Zn-SOD (SOD-1) has been the most commonly used model for ALS (Acevedo-Arozena et al., 2011). Mutations in the SOD-1 gene would implicate ROS in disease pathogenesis (Ghadge et al., 1997). Indicators of excess protein oxidation were also found to be increased in patients with sporadic ALS (McLean et al., 2014). Excitotoxicity and dysfunctional axonal transport have been suggested as a consequence of oxidative stress (Carter et al., 2009). Studies in models of mutant SOD-1 in mice have found irregular activation of Nox2 leading to superoxide radical formation and an increase in pro-inflammatory cytokines (Harraz et al., 2008).

Few studies have been conducted in relation to S1P signalling and ALS pathogenesis. To date, there is only one *in vitro* study linking the possible role of S1PR signalling in ALS. L-BMMA, an amino acid capable of triggering neurodegenerative diseases such as ALS, was used on a neuroblastoma cell line. It was found that S1P was protective against L-BMAA induced necrosis (Munoz-Saez et al., 2015). Although there has been no *in vivo* studies examining the role of S1PR signalling or effect of S1PR drugs in ALS pathology, clinical trials are currently underway to investigate the therapeutic value of FTY720 (Fingolimod/Gilenya) in this disease. In addition, CX3CR1 has been shown to be a disease-modifying gene in amyotrophic lateral sclerosis (ALS) (Lopez-Lopez et al., 2014). The rate of disease progression and patient survival time has been associated with one or two copies of the CX3CR1 V249I allele. Patients with 249I/I or 249V/I genotypes had shorter survival times and faster disease progression in comparison to patients with V249V genotypes. However, no association was found between these two variants and risk of developing ALS (Lopez-Lopez et al.,

2014). While this is the strongest genetic link to ALS survival, these results also show the importance of the immune system in ALS pathogenesis.

8.2 Alzheimer's disease

Alzheimer's disease is the most common age related neurodegenerative disorder and the most common disease associated with dementia (Asle-Rousta et al., 2013). This progressive, degenerative disease is characterised by the loss of specific neural populations. The hallmark of AD is deposition of extracellular amyloid beta (Aβ) plaques and a build up of the toxic intracellular tau protein (Asle-Rousta et al., 2013). The involvement of oxidative stress in the disease pathogenesis of AD is supported by the significant amount of lipid peroxidation discovered in AD patient's brains. (Di Bona et al., 2010). Aβ plaques also promote the generation of ROS, causing glial activation and migration, thus triggering localised areas of inflammation. Oxidative stress also decreases neurogensis, limiting the brains neuroregenerative capacity (Zhu et al., 2004, von Bernhardi and Eugenin, 2012, Butterfield et al., 2006).

The main abnormalities that have been documented in AD brains, involving S1P, include a decrease in the total phospholipid and sulfide contents with increases in ceramide and cholesterol (Asle-Rousta et al., 2013). Ceramide, which is a precursor to sphingosine-1-phosphate, can have negative effects on vital cellular processes such as inhibiting glycolysis, inducing oxidative stress and stabilising BACE1 (the Amyloid precursor protein cleaving enzyme) (Asle-Rousta et al., 2013). Despite there being no difference in S1P1 expression in post-mortem Alzheimer's disease tissue (Brana et al., 2014) animal models of AD have been used to investigate the potential neuroprotective and neuro-restorative effects of S1PR modulation. In one such model, AD was mimicked by injecting Aβ into the frontal cortex of rats. Daily FTY720 treatment decreased the Aβ induced activation of caspase-3, reduced hippocampal neuronal loss and improved spatial learning and memory in these rats (Asle-Rousta et al., 2013). In a second study, using the same AD model, daily FTY720 treatment restored the Aβ induced passive avoidance memory impairment to a comparable level as the standard AD drug Memantine (Hemmati et al., 2013). A third *in vivo* study performed aimed to investigate the role of the central S1PR1 in the ability of FTY720 to ameliorate the effects of Aβ in these animal models. These studies conclude that the protective effects of FTY720 against experimental AD are likely mediated via S1PR modulation in CNS resident cells (Asle-Rousta et al., 2014). In addition to the possibility of altered S1PR signalling playing a role in AD pathogenesis, aberrant fractalkine signalling has also been suggested to be present in AD pathology. Post-mortem analyses of AD brains have often reported high levels of microglial activation surrounding Aβ plaques (Mandrekar-Colucci and Landreth, 2010). As neuronal fractalkine decreases naturally with age, this may be contributing to the increased activation of microglial cells (Lyons et al., 2009). Studies in transgenic mouse models of AD, have also suggested that microglial $CX3CR1^{-/-}$ mice have decreased levels of neuronal loss (Fuhrmann et al., 2010). CX3CR1 knockdown in rats injected with $A\beta_{1\text{-}40}$ fibrils has also been shown to rescue LTP expression and spatial memory impairment (Wu et al., 2013).

8.3 Parkinson's disease

Parkinson's disease is characterised by muscle rigidity, bradykinesia, tremor and loss of balance (Chiurchiu et al., 2016). The neuropathology of PD involves degeneration of neurons in the substantia nigra and depletion of the neurotransmitter dopamine and its metabolites. One of the hallmarks of PD is the formation of cytoplasmic lewy bodies containing α-synuclein (Chiurchiu et al., 2016). Although the exact cause of PD is still unknown, post-mortem evidence from PD patients brains suggests a dominant role in mitochondrial dysfunction as well as pro-oxidant toxins from the environment leading to oxidative stress (Blesa et al., 2015, Pamphlett, 2014). Post-mortem studies also show reduced levels of glutathione and increased levels of its precursor glutathione disulfide (GSSG) (Gu et al., 2015).

S1P and CX3CR1 signalling has also been implicated in PD. In an *in vitro* MPP+ (1-methyl-4-phenylpyridinium) model of Parkinson's disease, sphingosine kinases have been implicated as having a key role on neuronal viability (Pyszko and Strosznajder, 2014b). Reduced activity of SphK1 and 2 is observed in neuronal dopaminergic neurons when treated with MPP+, where the addition of exogenous S1P exerts a significant neuroprotective effect (Pyszko and Strosznajder, 2014b). In an MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of Parkinson's disease it has been documented that $CX3CR1^{-/-}$ mice had significantly more dopaminergic cell death in the substantia nigra compared to wild type (Cardona et al., 2006). In contrast fractalkine was reportedly neuroprotective in a 6-hydroxydopamine rat model of PD. This neuroprotection was possibly due to a reduction in microglial activation correlating with protection of dopaminergic neurons (Pabon et al., 2011).

8.4 Multiple sclerosis

Multiple sclerosis (MS) is seen as a chronic inflammatory and progressive degenerative disorder characterised by phases of demyelination and axonal loss with BBB damage, which allows infiltration of peripheral immune cells (Broux et al., 2013). Continuous activation of microglia, leading to excess ROS generation, is also known to occur in MS and is thought to have a direct effect on demyelination (Bo et al., 1994, Karahalil et al., 2015). Free radicals have also been shown to upregulate many genes such as TNFα, iNOS and VCAM-1, involved in human MS and the mouse model of MS, experimental autoimmune encephalomyelitis (EAE) (Winyard and Blake, 1997). Weakened antioxidant defences as well as increased levels of markers of oxidative stress have been found in MS patient samples (Karg et al., 1999, Langemann et al., 1992, Karlik et al., 2015).

The role of S1P/S1P receptors has been well documented in recent years due to the positive effects of FTY720 (also known as Fingolimod/Gilenya) in MS patients. Since 2010, FDA approval of Gilenya/Fingolimod made FTY720 the first orally available effective treatment for relapsing remitting Multiple sclerosis (RR-MS). FTY720 is an analogue of S1P and exerts its actions on MS through sequestering lymphocytes in the peripheral lymph nodes, the down-regulation of

inflammatory genes and vascular adhesion molecules, promotes BBB integrity and reduces S1P lyase, all of which result in an increase in the concentration of S1P in the CNS. This effect may lead to further activation of S1P receptors and an up-regulation of S1P1 and 5 with the concomitant down regulation of S1P3 and 4 (Portaccio, 2011, Foster et al., 2009). It has also been shown that there is no astrocytic expression of S1P1 and 5 in active grey matter lesions in MS, while in inactive lesions it was reported that the receptor expression was returned to normal (Brana et al., 2014). In a mouse model of EAE, FTY720 potently inhibits disease toxicity. However, this effect is non-existent in mice with astrocytes selectively lacking the S1P1 receptor subtype (Cohen and Chun, 2011). Interestingly, the effects of FTY720 in the cuprizone model, which is a non T-cell mediated model of demyelination, attenuated oligodendrocyte and neuronal injury but failed to promote re-myelination (Kim et al., 2011). Due to the efficacy of FTY720 in both models of demyelination, this suggests a vital role of sphingolipid signalling in myelination. Other compounds selective to S1P1 and 5 receptors have also demonstrated comparable efficacy to FTY720 in EAE mouse models (Jin et al., 2014, Ren et al., 2012, Komiya et al., 2013). Although sphingolipids are key components of CNS tissue, S1P receptor expression remains to be fully characterised in normal and diseased conditions.

Studies suggest that Fractalkine signalling may also have a role to play in autoimmune disorders such as MS. In the EAE model for MS it was found that there was a build up of microglia expressing CX3CR1 in active brain lesions and sites of inflammation (Sunnemark et al., 2005). It was also found that there was an accumulation of CX3CR1 mRNA in periplaque regions as well as active and inactive demyelinating lesions, indicating a large number of infiltrating microglia. Increased levels of astrocytic fractalkine have also been observed at sites of inflammation, while no change was observed in neurons. From this we can propose that astrocytes have a role to play in the recruitment of microglia to damaged areas of the brain through the up-regulation of the fractalkine ligand (Sunnemark et al., 2005). $CX3CR1^{-/-}$ mice also reported to have higher levels of inflammatory cytokines, such as TNFα, IFNγ and IL-17 with lower levels of anti-inflammatory cytokines such as IL-10 (Garcia et al., 2013). From these murine studies we can imply that fractalkine/CX3CR1 signalling may be crucially important in the regulation of immune responses and in autoimmune disorders such as MS. Interestingly, genetic studies have revealed that there is a lower incidence of the CX3CR1 I249/T280 haplotype in secondary progressive MS patients compared to patients with primary progressive. This could be indicative of a positive effect of the I249 allele when paired with the T280 allele (Stojkovic et al., 2012). These polymorphisms are located on the transmembrane domain region of the CX3CR1 protein and it is thought that they alter the binding affinity of fractalkine in addition to altering the expression levels of CX3CR1 (Stojkovic et al., 2012). While it has been shown that these polymorphisms are not a determining factor in disease onset, the effects of these variants may be related to the functional properties of CX3CR1 following fractalkine binding, be it the membrane bound or soluble form.

Fractalkine has been shown to chemo-attract natural killer (NK) cells to active lesion sites in the brains of EAE mice (Huang et al., 2006, Imai et al., 1997, Yoneda et al., 2000) It has also been shown that S1P signalling, through S1PR1 and S1PR5, are necessary to mediate NK cell egress from the lymph nodes, which then allow NK cells to respond to migrating cues such as fractalkine (Schwab and Cyster, 2007). FTY720 is known to increase the sensitivity of lymphocytes to certain chemokine signals (Johnson et al., 2010). For example, NK cells are also known to express CXCR4 which is the receptor for CXCL12. CXCR4/CXCL12 signalling is known to promote NK cell migration (Campbell et al., 2001). Despite the fact that both fractalkine and CXCL12 both cause NK migration, treatment with FTY720 in vitro can reduce the migration response of NK cells to fractalkine (Johnson et al., 2011). In contrast, FTY720 can enhance the migratory effect of NK cells to the CXCL12 chemokine (Johnson et al., 2011). This reduction in fractalkine mediated NK cell migration following FTY720 treatment suggests possible crosstalk between S1P and fractalkine receptors.

8.5 Rett syndrome

Rett syndrome is a neuro-developmental disorder usually caused by mutations in the MeCP2 gene (Amir et al., 1999). A mouse model of this disease has since been developed in which MeCP2-deficient mice (*Mecp2*$^{-/y}$) mimic the human Rett syndrome with decreased levels of brain derived neurotrophic factor (BDNF) (Guy et al., 2001, Deogracias et al., 2012). In-vivo studies on MeCP2 deficient mice, also show signs of oxidative brain damage (De Filippis et al., 2015). This symptom supports evidence of higher levels of oxidative stress markers found in the circulation of Rett syndrome patients (De Filippis et al., 2015). This is thought to stem from abnormal mitochondrial function as high levels of H_2O_2 in the brains of Rett syndrome mice (MeCP2-308 heterozygous female mice) appears to be caused by a dysfunction in complex II in the mitochondria (De Filippis et al., 2015).

The use of S1PR modulators may represent a novel therapeutic strategy for the treatment of Rett syndrome. The S1PR agonist FTY720 has been shown to increase levels of BDNF in the MeCP2$^{-/y}$ mice which correlated with increased locomotive activity. The authors suggest that in addition to the beneficial effects of FTY720 on lymphocyte egress, FTY720 may also be contributing to accelerated neuronal repair (Deogracias et al., 2012). MeCP2$^{-/y}$ mice with a CX3CR1$^{-/-}$ in microglia, has also been shown to reduce hippocampal neuronal soma size back to control levels while also decreasing microglia process complexity and increasing microglial insulin-like growth factor release (Horiuchi et al., 2016). CX3CR1$^{-/-}$ is also thought to significantly improve motor performance, respiration, body weight and lifespan of MeCP2$^{-/y}$ mice (Horiuchi et al., 2016)

8.6 Lysosomal storage disorders

Lysosomes are cellular organelles containing enzymes capable of recycling of cellular waste and debris. Lysosomes are highly susceptible to oxidative stress in the aging brain (Bahr and Bendiske, 2002), with evidence suggesting that mitochondrial dysfunction plays an important role in disease

pathogenesis (Vazquez et al., 2012). Lysosomal strorage disorders (LSDs) are a result of a deficiency of a particular enzyme in the lysosome, required for the metabolism of large molecules into smaller subunits, which the cell can utilize. These diseases include Niemann-Pick disease (NPC) (Speak et al., 2014), Sandhoff disease (Wu et al., 2008) and Krabbes disease (Contreras et al., 2010), where altered levels of sphingosine or related molecules have been shown to occur.

Krabbes disease is a heritable LSD, where there is a deficiency in the enzyme galactosylceramide beta galactosidase (GALC) that breaks down and prevents accumulation of the toxic metabolite galactosylsphingosine (psychosine) (O'Sullivan and Dev, 2015). The concentration of psychosine, which accumulates mainly in the CNS, is found to be at least 10 times higher in cerebral tissue effected by Krabbe's disease than normal (Vanier and Svennerholm, 1975) (Contreras et al., 2010). Addition of pyschosine to astrocytes and oligodendrocytes causes cell death as well as demyelination in cerebellar organotypic slice cultures (O'Sullivan and Dev, 2015, Voccoli et al., 2014). Notably, these detrimental effects of pyschosine are attenuated by treatment with pFTY720 (O'Sullivan and Dev, 2015). Sandhoff disease is another neurological LSD in which the catabolic enzymes, β-hexosaminidase A and B, required to degrade neuronal membrane components are deficient (Wu et al., 2008). This results in a toxic build up of lipid metabolites eventually leading to neuronal cell death. In a mouse model of Sandhoff disease the deletion of SphK1 or S1P3 led to a milder disease course, with a reduction in the proliferation of glial cells (Wu et al., 2008). No changes in the levels of fractalkine were detected in a mouse model of Sandhoff disease (Tsuji et al., 2005). In addition, no studies on the involvement of fractalkine have been reported for Krabbes disease.

8.7 Psychiatric disorders

Schizophrenia is a psychiatric disorder that occurs in approximately 1% of the population, with typical symptom onset occurring in adolescence or early adulthood (Hardingham and Do, 2016). Human and animal studies show that irregular firing of fast-spiking paravalbumin-positive inter-neurons (PVI's) together with abnormalities in myelination, may account for the onset of schizophrenic symptoms (Lewis et al., 2012, Mighdoll et al., 2015). Rodent studies propose that in developing brains, reduced glutathione levels along with oxidative stress are sufficient to induce schizophrenic like symptoms (Steullet et al., 2010). Schizophrenia 'risk genes' further emphasise how oxidative stress may be involved in schizophrenia, such as Dystrobrevin binding protein 1 (DTNBP1), proline dehydrogenase 1 (PRODH), neuregulin 1 (NRG1), D-amino acid oxidase activator (DAOA) and disrupted in schizophrenia 1 (DISC1) (Hardingham and Do, 2016). DTNBP1 has an antioxidant role in the CNS and has been shown to be degraded in the presence of oxidative stress (Yap et al., 2014). DAOA has also been associated with oxidative stress, mitochondrial dysfunction and white matter deficits (Filiou et al., 2012). Furthermore, in human studies, evidence of oxidative stress in the blood and cerebrospinal fluid (CSF), has been shown in post-mortem tissue of schizophrenic patients (Yao and Keshavan, 2011, Do et al., 2009). Decreased levels of

glutathione in the cortex are also thought to be associated with more severe negative symptoms (e.g social withdrawal) in schizophrenic patients (Matsuzawa and Hashimoto, 2011).

Animal models of anxiety like behaviour show a higher level of S1P in serum samples, which was reported to cause neurodegeneration (Jang et al., 2011). Notably, elevated levels of S1P were reported in the serum as well as various brain regions in rats after immobilization and electric shock stressors (Jang et al., 2011). These elevated levels of S1P, particularly in the amygdale, may give rise to stress induced anxiety (Jang et al., 2011). These studies suggest a link between psychiatric and neurological disorders associated with sphingolipid metabolism (Muhle et al., 2013). Sphingomyelin and ceramide levels are also altered in post-mortem brain tissue in schizophrenic patients (Kucharska-Mazur et al., 2014). In a particular study, the plasma levels of S1P were found to be a predictor for the risk of psychotic episodes, especially in schizophrenia subjects (Kucharska-Mazur et al., 2014). Recently, fractalkine has also been associated with schizophrenia as being an important biomarker (Stuart and Baune, 2014).

9. Concluding remarks

Oxidative stress is known to contribute to many diseases within the CNS. Fractalkine/CX3CR1 and S1P/S1PR signalling systems are an important communication link between neurons and glial cells in order to maintain appropriate homeostatic processes. Furthermore, Fractalkine and S1P seem to take part in several physiological and pathological processes through the recruitment of immune cells to the site of inflammation and regulating oxidative stress. The importance of CX3CR1 and S1PR modulation in several neurological and inflammatory disorders is already well recognised, however, the full therapeutic extent of fractalkine and S1P involvement in disease processes remains to be elucidated. Therefore, the family of S1PRs as well as CX3CR1 appear worthy of continued study and may provide significant therapeutic opportunities.

Disease	Affected areas	Connection to oxidative stress	Reference
Macular degeneration	Eyes (retina)	Reactive oxygen species (ROS) and intermediates	(Beatty et al., 2000)
Diabetes	Multiple organ	ROS, SOD, catalase and glutathione enzyme dysfunction	(Maritim et al., 2003)
Chronic fatigue	Multiple organ	Inflammation, C-reactive protein (CRP), ROS	(Maritim et al., 2003)
Autoimmune disorders	Multiple organs, joints, immune system	Inflammation and ROS	(Scofield et al., 2005)
Asthma	Lungs	Inflammation and ROS (H_2O_2)	(Cho and Moon, 2010)
Neurodegenerative diseases (Alzheimer's, Parkinson's, ALS)	Central and peripheral nervous system	Inflammation and ROS	(Uttara et al., 2009)
Arthritis	Joints	Free radicals	(Ziskoven et al., 2010)
Nephritis	Kidney	ROS and glutathione dysfunction	(Blackburn et al., 2011)
Melanoma	Skin	DNA damage, lipid peroxidation and ROS	(Sander et al., 2003)
Myocardial infarction	Heart	ROS	(Di Filippo et al., 2006)

Table 1.5 Diseases associated with oxidative stress

Adapted from Rahal et al. (2014).

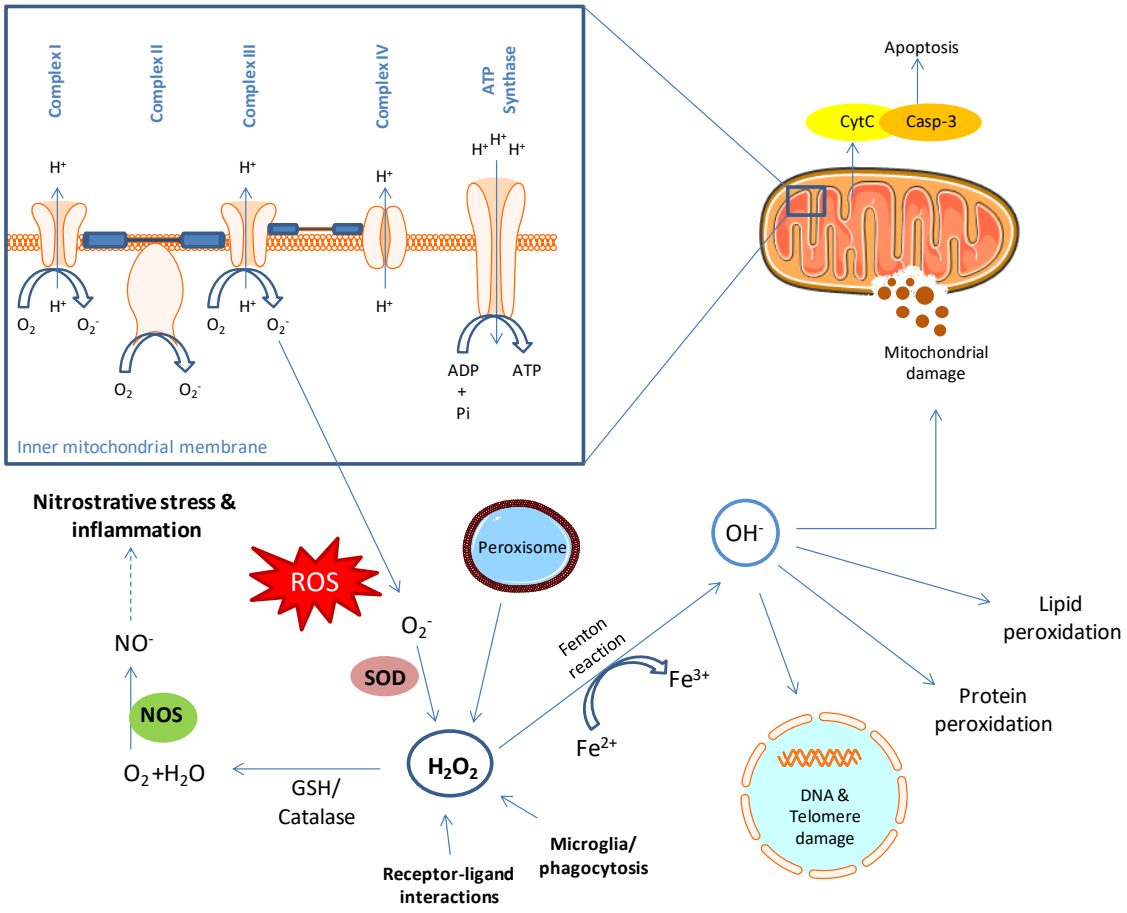


Figure 1.8: H_2O_2 generation and pathways of oxidative stress

Schematic diagram of main sources of oxidative stress and its link to inflammation, lipid and protein peroxidation, mitochondrial damage and cell death

Chapter 2:
Materials and Methods

1. Materials

1.1 Compounds and treatments

All compounds used are summarised and can be seen in Table 2.1. All inhibitors used TACE/ADAM10 inhibitor (BMS-561392), specific TACE inhibitor (BMS-566394), specific ADAM10 inhibitor (TOCRIS; GI 254023X), MMP inhibitor (Millipore; 444289), inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) (TOCRIS; 2559) and the p38 MAPK inhibitor -VX702 (TOCRIS; 3916) were prepared as 10mM stock solutions dissolved in dimethyl sulfoxide (DMSO, Sigma; D8418). pFTY720 used in all experiments was the pure active (S)-enantiomer of pFTY720 (2-amino-2-(2-(4-octylphemyl)ethyl)propane-1,3-dioll) prepared as 10mM stock solutions dissolved in 90% DMSO and 20mM HCL. Lipopolysaccharide (LPS) was prepared as 1mg/ml stock in H_2O (Sigma, L4391). Amplex Red used was obtained from biosciences (A12222). The cytokines used were IL-1β (R&D Systems, PHC0815), TNFα (R&D Systems, 210-TA) and Interferon γ (IFNγ; GIBCO, PHC4031). Hydrogen peroxide (H_2O_2; Sigma, 216763) was prepared fresh for every experiment by diluting appropriately in serum free media. Glucose oxidase solution (GOX; G0543) and catalase solution (CAT; C3155) were both sourced from Sigma. GOX was supplied at a concentration of 200U/mg, where 1 unit of enzyme activity oxidises 1μM of D-glucose/min to H_2O_2. GOX was used at a fixed concentration of 0.1 units/ml (i.e. 1:100,000 dilution) throughout all experiments. CAT was supplied at a concentration of 30,000U/mg, where 1 unit of enzyme activity decomposes 1μM of H_2O_2/min to H_2O. CAT was used at varying concentrations from 0.03 units/ml (i.e. 1:1,000,000 dilution) to 300 units/ml (i.e. 1:100 dilution) as indicated in the figure legends and is stable for up to 24 hrs (**Figure 2.1**).

1.2 Antibodies

Table 2.2 highlights all the antibodies used in this study. Primary antibodies used were: myelin basic protein (MBP; Abcam, ab40390), CX3CL1 (ebioscience; 14-7986), Vimentin (Santa-Cruz; sc-373717), Neurofilament-H (Abcam; ab5539), myelin oligodendrocyte glycoprotein (MOG, Millipore; MAB5680) and Ionised calcium binding adapter molecule-1 (Iba1, WAKO; 019-19741). Secondary antibodies used were: Anti-mouse 549 (Jackson ImmunoResearch; 115-506-068), Anti-rabbit 488 (Alexa; A11008), Anti-chicken 633 (Alexa; A21103). Human primers used were; CX3CL1 (Life Technologies; Hs00171086_M1), GAPDH (Life Technologies; Hs02758991_g1) and HPRT1 (Life Technologies; Hs01003267_M1)

1.3 Stains

Dyes and chemicals used for cellular staining were: Nuclear stain Hoechst 34580 (Invitrogen, H21486) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT (Invitrogen, M6494).

Compound	Company details	Use
ADAM17 inhibitor	Novartis BMS-566394	ADAM17 inhibitor, Cell culture
ADAM10 inhibitor	Tocris 3995	ADAM10 inhibitor, Cell culture
ADAM17/ADAM10 dual inhibitor	Novartis BMS-561392	ADAM17/10 inhibitor, Cell culture
MMP inhibitor	Millipore 444289	Broad spectrum MMP inhibitor, Cell culture
VX-702	Tocris 3916	P38 MAPK inhibitor, Cell culture
IKKβ/TPCA-1	Tocris 2559	NF-κB inhibitor, Cell culture
pFTY720	Novartis	S1P receptor agonist, Cell/slice culture
Recombinant fractalkine	R&D Systems 458-MF	CX3CR1 agonist, Slice culture
Amplex red	biosciences A12222	Quantification of H_2O_2, Slice culture supernatant
IL-1β	R&D Systems PHC0815	Cytokine, Cell culture
TNFα	R&D Systems 210-TA	Cytokine, Cell culture
IFNγ	GIBCO PHC4031	Cytokine, Cell culture
H_2O_2	Sigma 216763	Oxidative stress inducer, Cell/slice culture
GOX-CAT	Sigma G0543, C3155	Oxidative stress inducer, Cell/slice culture

Table 2.1 List of compounds used

Primary Antibodies						
Antibody	**Type**	**Host**	**Supplier**	**Cat number**	**Dilution**	**Application**
Vimentin	Monoclonal	Mouse	Santa Cruz	Sc-373717	1/500	IHC
MBP	Polyclonal	Rabbit	Abcam	Ab40390	1/1000	IHC
NFH	Polyclonal	Chicken	Millipore	AB5539	1/1000	IHC
MOG	Monoclonal	Mouse	Millipore	MAB5680	1/1000	IHC
Iba1	Polyclonal	Rabbit	Wako	019-19741	1/1000	IHC
Fractalkine	Polyclonal	Rabbit	eBioscience	14-7986	1/200	ICC

Secondary Antibodies					
Antibody	**Host**	**Supplier**	**Cat number**	**Dilution**	**Application**
Anti-rabbit Alexa 488	Goat	Invitrogen	A11008	1/1000	IHC
Anti-mouse Dylight 549	Donkey	Jackson Immuno-research	115-506-068	1/1000	IHC
Anti-chicken Alexa 633	Goat	Invitrogen	A21103	1/1000	IHC

Table 2.2 List of primary antibodies used

2. Cell Culture

2.1 Aseptic technique

All *in vitro* cell culture procedures were carried out under strict aseptic technique. All instruments used were sterilised before use by autoclaving and spraying with 70% ethanol before being placed in the laminar flow hood (FASTER). A specific cell culture laboratory coat was worn at all times and gloved hands were sprayed with 70% ethanol before entering the hood or cell culture incubator to ensure sterility. When not in use the interior of the laminar flow hood including all equipment were exposed to a germicidal UV lamp which emits ultraviolet radiation at 253.7nm to ensure sterility before use. All tissue culture preparations took place under the hood.

2.2 Human astrocyte culture

Human astrocytes from fetal brains were purchased from ScienCell Research Laboratory, USA (1800, Lot No. 9063 and 11065) as we have described previously (Healy et al., 2013, Elain et al., 2014, Rutkowska et al., 2015, O'Sullivan and Dev, 2015). Human astrocytes were cultured at 37°C and 5% CO_2 in a humidified incubator and grown in human astrocyte media (ScienCell; 1801) or DMEM/F12 (Fisher; 10770245) supplemented with 1% astrocyte growth supplement (ScienCell; 1852), 10% fetal bovine serum (FBS, Sigma; F7524), and 1% Penicillin/Streptomycin (pen/strep, Sigma; P4333) in T75 culture flasks (Corning) unless otherwise indicated in figure legends. The cells were grown for 14 days until 90% confluent then re-plated in six or 24 well-plates and used when 80% confluent.

2.3 Murine astrocyte cell culture

Mixed glia cell cultures were prepared from cortical tissue of either male or female one day old wild type C57BL/6 mice. Animals were decapitated, skull removed and the cortical tissue was detached from both hemispheres with forceps. The cortical tissue was cross-chopped with a scalpel, placed in pre-warmed Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12, Fisher, 10770245) supplemented with 10% Fetal Bovine serum (FBS, Labtech; FB-1090) and 1% penicillin/streptomycin (pen/strep, Sigma, P4333) and gently triturated until a clear solution was obtained. The resulting solution was passed through a cell strainer (40µm). The tubes were centrifuged at 1,200 x g for three minutes at room temperature and the cell pellet was re-suspended in media (DMEM/F12, 1% pen/strep, 10% FBS) and plated into separate T75 flasks. Cells were cultured at 37°C and 5% CO_2 in a humidified incubator for 14 days at which point microglial cells were separated from mouse astrocytes by placing them on a rotating shaker for two hours at 37°C. The supernatant, with detached microglia, was removed and purified astrocytes were trypsinised and plated accordingly.

2.4 Mouse organotypic cerebellar slice culture

Cerebellar slice cultures were prepared as described previously (O'Sullivan and Dev, 2015, Pritchard et al., 2014, Sheridan and Dev, 2012, O'Sullivan et al., 2016) using brain tissue isolated

from both male and female postnatal day 10 C57BL/6 mice, according to the protocol reported earlier (Sheridan and Dev, 2012). Mice were decapitated; cerebellar tissue was removed from the skull and separated from hindbrain with spatulas on ice. The cerebellum was cut into 400µm parasagittal slices using a McIlwain tissue chopper. Slices were separated in to individual slices under a dissection microscope. Four slices were grown on each cell culture insert (Millicell, PICMORG50). Slices were cultured in media containing 50% Opti-MEM (Invitrogen, 11058021), 25% Hanks' buffered salt solution (HBSS, Invitrogen, 14025-050), 25% heat inactivated horse serum (Biosera, HO-290) supplemented with 2mM Glutamax (Invitrogen, 35050-038), 28mM D-Glucose (Sigma, G8769), 1% pen/strep, 10mM HEPES (Sigma, H3784) for 12-14 days in vitro. Slices cultures were grown at 35.5˚C and 5% CO_2 in a humidified incubator. Prior to treatments, slices were starved in serum free media for four hours prior to all treatments (**Figure 2.2**).

3. Molecular Biology

3.1 Quantitative Polymerase Chain Reaction (Q-PCR)

For mRNA expression of CX3CL1, human astrocytes were grown in six well plates until 80% confluent. Prior to stimulation, cells were starved in serum free media for three hours. The cells were then pre-treated with either VX702 (p38 inhibitor) or an IKKβ inhibitor in serum free media for 30 min after which IL-1β, TNFα or IFNγ were added and incubated for 3 hr or 18 hr. After treatment, the media was removed and cells were washed twice with phosphate buffered saline (PBS) in preparation for Q-PCR analysis. RNA extraction was performed using either Qiagen or Macherey-Nagel methods as described by manufacturers. Using Qiagen kits, the cell pellets were suspended in RLT lysis buffer (Qiagen Hilden, Germany) and then frozen at -80ºC. RNA was isolated using RNeasy mini kit (Qiagen, 74104). To eliminate genomic DNA, an on-column DNase digestion was carried out with the RNase-free DNase set (Qiagen 79254). After reverse transcription of mRNA (10 minutes at 25ºC; 120 minutes at 37°C; 5 seconds at 85ºC) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814), RT-PCR was performed with the 7900HT Fast Real-Time PCR System (Applied Biosystems, 4329001) according to the Standard Thermal Cycler Protocol (2 minutes at 50ºC; 10 minutes at 95ºC; 40 cycles of 15 seconds at 95ºC and one minute at 60ºC). The threshold was set manually for all samples. The analysis was performed with the SDS 2.3 software. For the Macherey-Nagel method (Macherey-Nagel; 740955), cells were suspended in RA1 buffer with 1% beta-mercaptoethanol and frozen at -80ºC. To eliminate genomic DNA, an on-column DNase digestion was carried out with the RNase-free DNase. After reverse transcription of mRNA (10 minutes at 25ºC; 120 minutes at 37°C; 5 seconds at 85ºC) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814), RT-PCR was performed using Step One Plus real time PCR system (Applied Biosystems) according to the Standard Thermal Cycler Protocol (2 minutes at 50ºC; 10 minutes at 95ºC; 40 cycles of 15 seconds at 95ºC and one minute at 60ºC). TaqMan Gene Expression Assays using TaqMan probes of human CX3CL1 (Applied Biosystems; Hs00171086_M1) and HPRT (Applied Biosystems; Hs01003267_M1) (all FAM dye labelled) were used. The relative expression of CX3CL1 to the reference gene HPRT

was determined. Each condition was run in triplicates and the experiments were repeated independently three times unless otherwise indicated in the figure legends.

4. Biochemistry

4.1 Amplex Red assay

The production of H_2O_2 in cell culture media was measured through the Amplex Red® assay. In the presence of H_2O_2, horseradish peroxidise (HRP) catalyses the conversion of amplex red (10-acetyl-3,7-dihydroxyphenoxazine) to the fluorescent resorufin. Production of H_2O_2 is proportional to the conversion of amplex red to resorufin. Thus the amount of resorufin is considered a quantitative indicator of the amount of H_2O_2 produced. A 16-point standard curve was generated for H_2O_2 (Sigma, 216763), which was diluted in serum free slice media. Freshly prepared standards (100µl) were added to a black 96 well plate (Greiner; 655076) in duplicate. The assay is sensitive to 10pM H_2O_2. Samples were added to the plate in triplicate. The amplex red mix was prepared by adding amplex red (50mM) to 100units/ml of HRP and PBS, vortexed quickly and 100µl was added to each well. The plate was read in a fluorescent plate reader (Molecular devices; SpectraMax Gemini X5) heated to 37°C, shaken for 30secs and read at excitation 550nm and emission 585nm, cutoff 570nm (**Figure 2.3**).

4.2 Enzyme Linked Immunosorbent Assay (ELISA)

Cytokine levels in cell culture supernatant were measured with R&D systems ELISA kits according to the manufacturer's instructions. Mouse IL-6 with a range of 15.60 - 1,000 pg/ml (DY406), mouse CX3CL1/fractalkine with a range of 0.391 - 25 ng/ml (DY472) and human CX3CL1/fractalkine with a range of 0.63ng/ml-20ng/ml (DY365). Briefly, 96 well ELISA plates (Thermo Scientific; 95029780) were coated overnight at room temperature with capture antibodies diluted in PBS. The plates were washed three times with wash buffer (0.05% Tween 20 (Sigma; P7949), PBS, pH 7.4) and then blocked for one hour at room temperature with the appropriate reagent diluent. The plates were then washed three times with wash buffer and any remaining buffer was removed from the wells by inverting and blotting plate on tissue paper. A standard curve was prepared using serial dilutions of the recombinant protein diluted in the appropriate reagent diluents. The samples and standards were then incubated in the antibody coated ELISA plate for two hours at room temperature. The plate was then washed three times with wash buffer and detection antibody (diluted in reagent diluent) was added to each well for two hours. Following three more washes, Streptavidin-HRP diluted in reagent diluent was added to each well and incubated for 20 minutes at room temperature, protected from light. Three more washes were carried out and the wells were incubated with substrate solution (R&D Systems; DY999) for 20-40 minutes at room temperature protected from light. The colour reaction was stopped with the addition of 1M H_2SO_4. To determine the optical density of each well absorbance was read immediately using a plate reader at 450nm and 570nm (Labsystem Multiskan). 570nm readings were subtracted from the 450nm readings to correct for optical imperfections in the plate. The standard curve was calculated by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and a best

fit line was generated to find the slope. All samples were averaged, measured in the linear range of the curve and calculated using the slope to convert the absorbance values to pg/ml.

4.3 MTT assay

Human or mouse astrocytes were plated in 96-well plates and cultured for at least 24 hours until 70% confluent. The cells were starved for four hours and then treated for 18 or 24 hours with H_2O_2 with or without pFTY720 at various concentrations indicated in the figure legends. After the treatments, media with the compounds was removed and replaced with 100µl of fresh serum free media supplemented with 10µl of 12mM MTT (Invitrogen, M6494). The cells were incubated with MTT for three hours at 37°C. Next 75µl of media was removed and 50µl of DMSO was added per well for 10 minutes and incubated at 37°C. The samples were mixed and absorbance was read at 540nm.

4.4 Immunocytochemistry

Human astrocytes were plated on glass cover slips in six well plates (VWR) and cultured for at least 24 hours until 80% confluent. The cells were then washed with PBS and fixed with 4% formaldehyde solution (Sigma, F1635) for 10 minutes at room temperature. Permeabilisation and nonspecific binding was reduced by incubating cells for 1 hour at room temperature in blocking buffer (PBS supplemented with 1% bovine serum albumin (BSA) and 0.1% Triton-X 100). For all antibody incubation and wash steps, diluted blocking buffer (PBS supplemented with 0.5% BSA and 0.01% Triton-X 100) was used. The cells were incubated overnight at 4°C with rabbit anti-CX3CL1, which binds the N-terminal chemokine domain, (1:500, eBioscience; 14-7986), washed three times, and incubated with anti-rabbit secondary antibody conjugated to Alex488 (1:1000; Invitrogen; A11008) for 1 hr at room temperature. After washing three times in PBS supplemented with 0.01% Triton-X 100, the cells were then incubated with Hoechst nuclear stain (diluted 1:10,000 in PBS)(Invitrogen; H21486) for 10 min and washed twice again. The coverslips were the mounted on glass slides in antifade reagent (life technologies, S36936) and the edges sealed with varnish. Samples were stored at 4°C in the dark until imaged. The cells were visualized with a confocal microscope (Leica SP8).

4.5 Immunohistochemistry

Immunohistochemistry for organotypic slices was performed by washing the slices twice in PBS. The slices were then fixed by incubation in 4% formaldehyde solution (Sigma, F1635) for 10 mins. The slices were washed twice in PBS and incubated overnight in blocking buffer (PBS supplemented with 10% BSA and 0.5% Triton-X 100). For all antibody dilutions PBS supplemented with 2% BSA and 0.1% Triton-X 100 was used. Slices were incubated for 24 hours at 4˚C in primary antibody, washed three times with PBS supplemented with 0.01% Triton-X 100 and incubated overnight at 4ºC in secondary antibody. The slices were washed again three times and mounted on glass cover slides in antifade reagent (life technologies, S36936) and the edges sealed with varnish. Samples were stored at 4°C in the dark until imaged. Slices were visualised with a Leica SP8

confocal microscope at 10X magnifications. Four slices per condition were grown and six to eight images per slice were taken to cover the whole slice, thereby limiting bias and variation between conditions. Values in graphs show the mean fluorescent intensity of about 130 measurements with standard error of the mean. Confocal images were captured as 12 bit.lif files of 1024 x 1024 pixel resolution. Image acquisition settings were kept constant across treatments. Image analysis was conducted using imageJ software (https://imagej.nih.gov/ij/). Confocal images were split into single channels and a minimum threshold was adjusted to remove background staining/noise. Arbitrary fluorescence values were averaged across the four slices per condition to give a single value for the total fluorescent intensity of that sample.

5. Statistical analysis

All statistical analysis was performed using GraphPad Prism 6. Independent student t-tests were performed where only two groups were compared. In experiments where three or more groups were compared an ordinary one way analysis of variance (ANOVA) was performed and was followed by post-hoc tests. Tukey's post-hoc test was used for experiments where all columns were compared to each other. Detailed data analysis methods are provided in the methods section, figure legends and results sections.

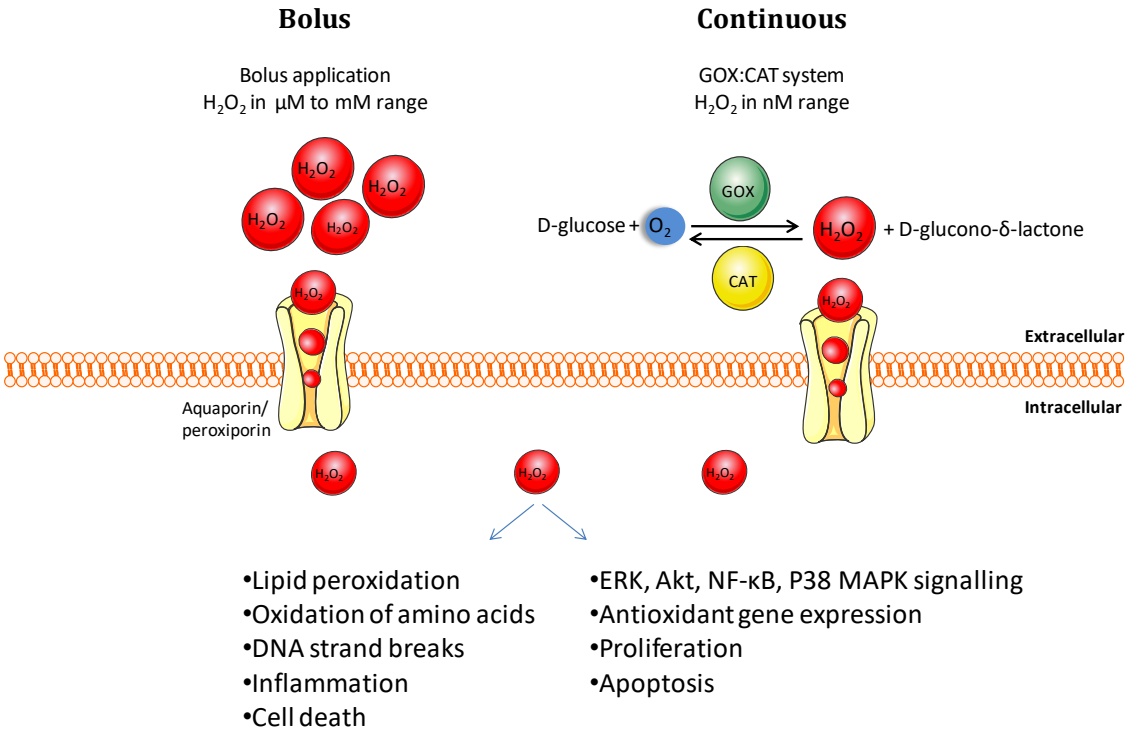


Figure 2.1: Schematic depicting bolus versus continuous application of H_2O_2 to cell culture

Concentration dependent effects of H_2O_2 and signalling pathways are differentially regulated by various concentrations of H_2O_2. H_2O_2; hydrogen peroxide, GOX; glucose oxidase, CAT; catalase, O_2; oxygen.

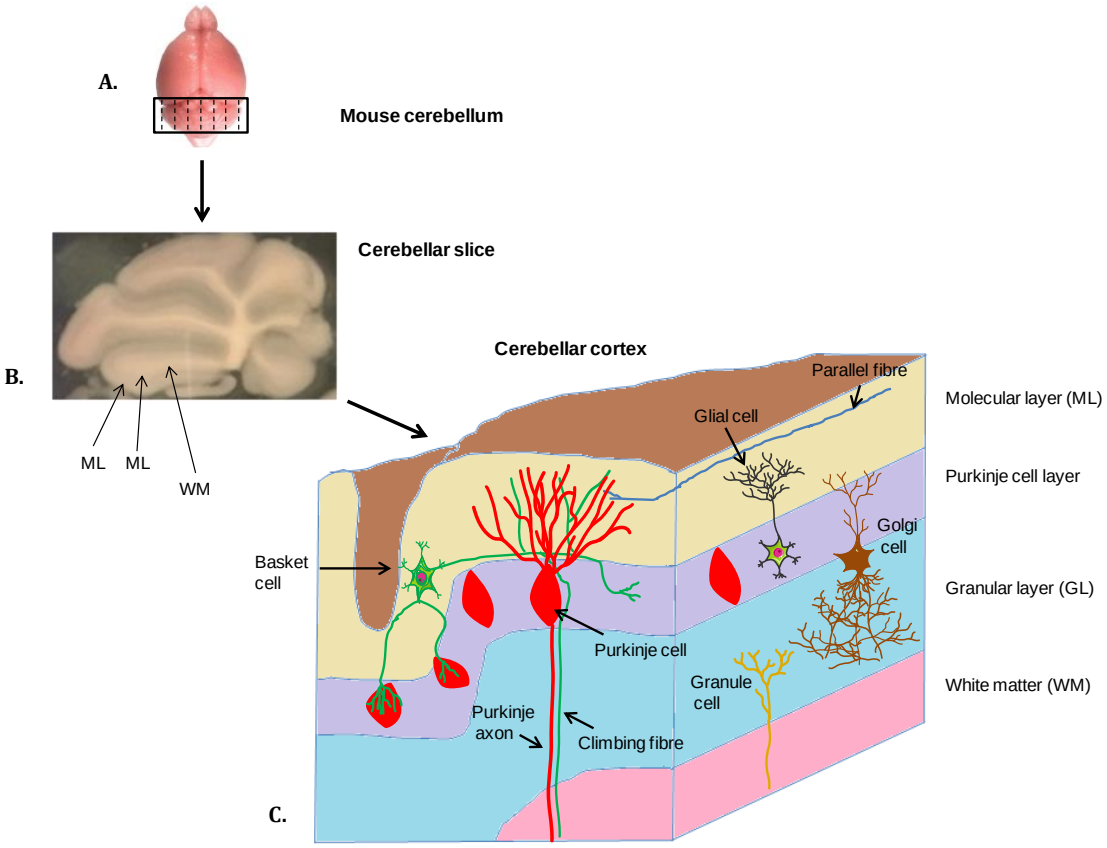


Figure 2.2: Molecular structure of cerebellum

A. Mouse brain with lines indicating parasagittal sectioning of cerebellum. B. Representative image of cerebellar slice post dissection. C. Illustration showing architecture of the cerebellar cortex highlighting the relative locations of purkinje cells and surrounding glia.

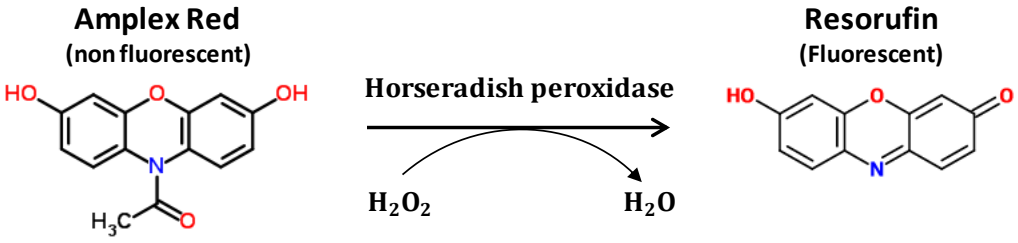


Figure 2.3: Conversion of amplex red to resorufin

HRP catalyses the conversion of amplex red to resorufin in the presence of H_2O_2. Lower level of detection is 10pM H_2O_2.

Chapter 3:

Fractalkine shedding is mediated by p38 and the ADAM10 protease under pro-inflammatory conditions in human astrocytes

Chapter aims

The specific aims of this study were to:

- Investigate fractalkine expression in human astrocytes
- Identify the proteases responsible for the cleavage of the ligand from the cell surface
- Explore fractalkine signalling in human astrocytes
- Determine intracellular signalling pathways involved in the regulation of fractalkine

Abstract

The fractalkine ligand (CX3CL1) is expressed in astrocytes and reported to be neuro-protective. When cleaved from the membrane, soluble fractalkine (sCX3CL1) activates the receptor CX3CR1, which is expressed in neurons and microglia. The membrane bound form of fractalkine additionally acts as an adhesion molecule for microglia and infiltrating white blood cells. Here, the mechanisms involved in the up-regulation and cleavage of fractalkine from human astrocytes was investigated. A combination of ADAM17 (TACE) and ADAM10 protease inhibitors were found to attenuate IL-1β, TNFα and IFNγ induced sCX3CL1 levels in astrocytes. A specific ADAM10 (but not ADAM17) inhibitor also attenuated these effects, suggesting ADAM10 proteases induce release of sCX3CL1 from stimulated human astrocytes. A p38 MAPK inhibitor also attenuated the levels of sCX3CL1 upon treatment with IL-1β, TNFα or IFNγ. In addition, an IKKβ inhibitor significantly reduced the levels of sCX3CL1 induced by IL-1β or TNFα in a concentration dependent manner, suggesting a role for the NF-κB pathway. These findings are important for understanding the role of fractalkine in healthy and stimulated astrocytes and may benefit our understanding of this pathway in neuro-inflammatory and neurodegenerative diseases.

1. Introduction

The chemokine fractalkine is expressed in the CNS at relatively high levels in comparison to most other cytokines (Hulshof et al., 2003). Fractalkine is a transmembrane protein with a chemokine domain attached to a mucin like stalk (Bazan et al., 1997). Although somewhat controversial, over the years it has been shown that fractalkine is expressed primarily on neurons and astrocytes, with its receptor, CX3CR1, expressed on microglia and neurons (Hatori et al., 2002, Hughes et al., 2002, Hulshof et al., 2003, Lauro et al., 2006, Maciejewski-Lenoir et al., 1999, Mizuno et al., 2003, Nishiyori et al., 1998). Therefore, neurons and astrocytes can signal to neurons and microglia expressing CX3CR1. Fractalkine can exist in two different forms, with the membrane bound form important for adhesion (Haskell et al., 1999). Fractalkine can also be cleaved by ADAM10, ADAM17 or cathepsin S, depending on the cell type and the microenvironment (Clark et al., 2009, Bourd-Boittin et al., 2009). This cleavage from the cell surface of neurons or astrocytes mainly represents a response to insult or injury. Within the CNS, fractalkine has potential to be both neuroprotective and toxic (Cook et al., 2010, Catalano et al., 2013, Koyama et al., 2013, Milligan et al., 2008, Mizuno et al., 2003). It is not completely understood the exact roles for either form of fractalkine within the CNS but in general because of the high levels in the adult brain, fractalkine is thought to help maintain a homeostatic environment as well as help regulate the response to CNS insults (Hughes et al., 2002, Lauro et al., 2006, Sunnemark et al., 2005). Studies also suggest that tonic signalling of the fractalkine ligand has an anti-inflammatory effect by somewhat controlling microglial activation states. Therefore inhibition of this tonic signalling may support inflammatory processes (Zujovic et al., 2000).

Transmembrane proteins, such as fractalkine, can be proteolytically cleaved at the juxtamembrane region which results in the detachment of their extracellular region (ectodomain) in a process known as ectodomain shedding (Montes de Oca, 2010). This shedding process can release proteins such as cytokines and growth factors from their membrane bound form, or alternatively, it can down-regulate receptors from the cells surface (Montes de Oca, 2010). Proteases such as the matrix metalloprotease (MMP) family shed several cell surface substrates (Fu et al., 2013, Kim et al., 2014). One such family of metalloproteases that mediate these events are known as ADAMs (a disintigrin and metalloprotease), of which the TNFα converting enzyme (TACE/ADAM17) and ADAM10 are most studied (Moss et al., 2007, Garton et al., 2001). Recent findings suggest that altered functions of ADAM10 could contribute to neurodegenerative disease processes such as Alzheimer's disease and encephalopathies (Allinson et al., 2003, Checler and Vincent, 2002). Much work has been done on ADAMs in the periphery, as they are highly abundant in many cells types (Bourd-Boittin et al., 2009). This shedding process can be activated by cytokines and growth factors via mitogen activated protein kinase (MAPK) pathways (Bandyopadhyay et al., 2006, Diaz-Rodriguez et al., 2002, Sheng et al., 2009). For example, it is thought the p38 MAPK is involved in the constitutive shedding of fractalkine (Jones et al., 2013).

Many studies performed on peripheral and neuronal cells implicate ADAM10 in the constitutive shedding of the CX3CL1 ligand from various cell types, while ADAM17 is the main protease regulating inducible cleavage of this ligand (Hundhausen et al., 2003, Cook et al., 2010). While a low level of CX3CL1 expression in astrocytes has been reported (Hatori et al., 2002), little information currently exists regarding its regulation, constitutive shedding or inducible cleavage in this cell type. Here, we present new data examining cytokine-induced regulation of CX3CL1 ligand in human astrocytes.

2. Results

2.1 Fractalkine release from human astrocytes is stimulated by pro-inflammatory cytokines

Fractalkine is expressed in the CNS at relatively high levels compared to other chemokines (Harrison et al., 1998). Fractalkine is a transmembrane protein and is expressed in astrocytes at low levels, but can be up-regulated in response to inflammation (Sunnemark et al., 2005). When cleaved from the membrane, soluble fractalkine (sCX3CL1) activates the receptor CX3CR1, which is expressed primarily in microglia. The membrane bound form of fractalkine/CX3CL1 additionally acts as an adhesion molecule for microglia and infiltrating white blood cells. As a result of the low expression of fractalkine on astrocytes, its functional expression has not been well characterised to date. Here, we first measured the levels of fractalkine constitutively released from human astrocytes. The data showed low levels of sCX3CL1 with no significant change in the levels of sCX3CL1 in astrocyte conditioned media between 6 and 36 hr suggesting minimal surface expression and subsequent cleavage of fractalkine under basal conditions (**Figure 3.1A**), in agreement with previous observations (Maciejewski-Lenoir et al., 1999, Hatori et al., 2002). In contrast, the treatment of human astrocytes with IL-1β induced a concentration- and time-dependent increase in the levels of sCX3CL1 (**Figure 3.1B**). Specifically, human astrocytes treated with IL-1β for 6 hr showed no significant increase in the levels of sCX3CL1, whereas 18 hr treatment with IL-1β at 1pg/ml (220.5 +/- 17.5), 10pg/ml (711.8 +/- 12.8), and 100pg/ml (911.5 +/- 10.0) significantly increased levels of sCX3CL1 (**Figure 3.1B**). Immunocytochemistry also showed the treatment of human astrocytes with IL-1β (100pg/ml), TNFα (10ng/ml) or IFNγ (10ng/ml) increased the levels of CX3CL1 in human astrocytes, using an antibody directed against the chemokine N-terminal domain of CX3CL1 (eBioscience; 14-7986) (**Figure 3.1C**).

2.2 IL-1β, TNFα and IFNγ increases levels of fractalkine mRNA

In human astrocytes pro-inflammatory cytokines have been shown to up-regulate the mRNA expression of IL-6, IL-8, T_{H2} cytokines CXCL1, CXCL2, CCL20 as well as BACE1 and the amyloid precursor protein (Elain et al., 2014, Zhao et al., 2011). To determine whether IL-1β, TNFα and IFNγ alter the levels of fractalkine by increasing protein synthesis, we examined their effect on fractalkine mRNA using qPCR. Treatment of human astrocytes with IL-1β (100pg/ml, **Figure 3.2B**), TNFα (10ng/ml, **Figure 3.2C**) and IFNγ (10ng/ml, **Figure 3.2D**), for 3 hr, significantly increased the levels of fractalkine mRNA in all three cases, by 20-fold or more. These effects appeared transient in so far as cytokine treatment for 18 hr increased levels of fractalkine mRNA but not to the same extent as 3 hr. In particular, IL-1β treatment significantly increased levels of fractalkine mRNA but only by approximately 2-fold (**Figure 2E**) and treatment for 18 hr with TNFα increased the levels of fractalkine mRNA by approximately 5-fold compared to control (**Figure 3.2F**). Overall, these results show that the cytokines IL-1β, TNFα and IFNγ increase the expression of fractalkine, likely by promoting mRNA and protein synthesis.

2.3 Matrix metalloprotease inhibitors attenuate IL-1β, TNFα and IFNγ induced levels of CX3CL1 in human astrocytes

Matrix metalloproteases (MMPs) are a family of proteases that are capable collectively of degrading proteins in the extracellular matrix (ECM). MMPs are also involved in the cleavage of cell surface receptors and ligands such as cytokines and chemokines (Van Lint and Libert, 2007). Thus, MMPs can play important roles in regulating cellular processes such as cell death and inflammation (Van Lint and Libert, 2007). Notably, however, the ability of MMPs to cleave the fractalkine ligand is less well studied in glial cells, particularly in astrocytes. To determine whether MMPs play a role in the release of the fractalkine ligand from human astrocytes, the soluble levels of fractalkine were measured in astrocyte conditioned media using ELISA. Human astrocytes were pre-treated with a pan MMP inhibitor, RS-130830, (1μM; IC_{50}) for 30 mins prior to 18h treatment with the pro-inflammatory stimuli; IL-1β (100pg/ml), TNFα (10ng/ml) or IFNγ (10ng/ml). Treatment with IL-1β (1,328.0 +/- 253.8) (**Figure 3.3A**), TNFα (2,185.0 +/- 113.3) (**Figure 3.3B**) and IFNγ (1,687 +/- 181.2) (**Figure 3.3C**) increased the levels of soluble fractalkine after 18 h, compared to control (###p<0.001 n=3; One way ANOVA and Tukey's post-hoc test). Importantly, pre-treatment with a pan MMP inhibitor, RS-130830, attenuated the fractalkine induced increase by IL-1β (666.8 +/- 46.8; **p<0.01), TNFα (1,207.0 +/- 108.4; ***p<0.001) and IFNγ (821.0 +/- 110.9; *p<0.05) respectively, compared to matched treated group (One way ANOVA and Tukey's post hoc test) (**Figure 3.3A-C**). Notably, IL-1β, TNFα, IFNγ or the MMP inhibitor, RS-130830 had no effect on cell viability (*Data not shown*).These results suggest that human astrocytes shed the fractalkine chemokine in response to IL-1β, TNFα and IFNγ and that pre-treatment a pan MMP inhibitor significantly reduces this response.

2.4 ADAM10 inhibitors reduce pro-inflammatory cytokine mediated increase of CX3CL1 in human astrocytes

ADAM10 and ADAM17 proteases have also been implicated in the cleavage of fractalkine from the cell surface of ECV-304 cells and fibroblasts (Hundhausen et al., 2003, Jones et al., 2013), with ADAM10 involved in constitutive shedding of the ligand. To our knowledge it is unknown whether the same proteases are used to cleave fractalkine on astrocytes. We again noted low levels of sCX3CL1 in conditioned media taken from human astrocytes, suggesting low levels of expression in these cells under control conditions and which also suggests the amount of constitutive shedding from human astrocytes is low. Next, to investigate whether ADAM10 and/or ADAM17 proteases are involved in the inducible shedding of fractalkine from human astrocytes under stimulated conditions, these cells were pre-treated for 30 min with the dual ADAM10/ADAM17 (BMS-561392) inhibitor, the selective ADAM17 inhibitor (BMS-566394) or the selective ADAM10 inhibitor (TOCRIS; GI 254023X) before treatment with the pro-inflammatory recombinant cytokines, IL-1β (100pg/ml), TNFα (10ng/ml) or IFNγ (10ng/ml). As expected, IL-1β (84.6 +/- 8.7, **Figure 3.4A**), TNFα (400.6 +/- 63.06, **Figure 3.4B**) and IFNγ (163.5 +/- 49.05, **Figure 3.4C**) significantly increased the levels of sCX3CL1 (###p<0.001 compared to control). Importantly, pre-treatment with the ADAM10/ADAM17 dual inhibitor significantly decreased the levels of sCX3CL1 induced by

IL-1β (663.7 +/- 39.5, **Figure 3.4A**), TNFα (600.3 +/- 30.2, **Figure 3.4B**) and IFNγ (847.8 +/- 146.6, **Figure 3.4C**) (***p<0.001, **p<0.01 compared to matched treated group). In contrast, the selective inhibitor for ADAM17 did not alter the levels of sCX3CL1 induced by IL-1β (973.5 +/- 64.3, **Figure 3.4D**), TNFα (944.2 +/- 39.0, **Figure 3.4E**) and IFNγ (1776.0 +/- 129.3, **Figure 3.4F**). However, the specific ADAM10 inhibitor also attenuated the IL-1β (84.5 +/- 8.7 vs. 32.0 +/- 5.0, **Figure 3.4G**), TNFα (401.0 +/- 63.1 vs. 115 +/- 36.0, **Figure 3.4H**) and IFNγ (163.5 +/- 49.1 vs. 47.9 +/- 8.6, **Figure 3.4I**) (**p<0.01, *p<0.05 compared to matched treated group). Taken together, this data suggests ADAM10 plays a central role in the shedding of fractalkine from astrocytes. During our experiments, we noted variations in the absolute levels of CX3CL1, between experiments. These can be linked to the different batches of human astrocytes used and the age/passage number the cells were used at. Importantly, however, while the absolute levels of CX3CL1 differed between experiments, the effects of the cytokines IL-1β, TNFα and IFNγ were similar as were the effects of the inhibitors used. To convince ourselves further of a role for ADAM10 in the inducible increase in levels of CX3CL1, we replicated experiments examining the effects of ADAM10 inhibitor by co-stimulating astrocytes with TNFα and IFNγ (1ng each). In this experiment we still found that the ADAM10 inhibitor significantly attenuated TNFα/IFNγ-induced levels of fractalkine (19,948 +/- 2,357 vs. 5,208 +/- 1,569) **Supplemental Figure 1** (***p<0.001, compared to matched treated group).

2.5 Inhibition of the p38 MAP Kinase attenuates IL-1β, TNFα and IFNγ induced levels of soluble CX3CL1

Several studies have shown that p38 MAP kinase is required for phosphorylation and activation of ADAM10 and ADAM17 in peripheral cells (Jones et al., 2013, Bandyopadhyay et al., 2006). To investigate if IL-1β, TNFα and IFNγ promote cleavage of fractalkine via a p38 MAPK-dependent pathway, we tested if the p38 MAPK inhibitor, VX-702 (TOCRIS; 3916), attenuated the increase of sCX3CL1 levels induced by these pro-inflammatory cytokines. Human astrocytes were serum starved for 3 hrs prior to pre-treatment with the p38 inhibitor, VX-702 (1μM for 30 min), and then incubated with IL-1β (100pg/ml), TNFα (10ng/ml) or IFNγ (10ng/ml) for 18 hrs. In line with previous data (Ralay Ranaivo and Wainwright, 2010) (Sheng et al., 2009), the p38 MAPK inhibitor attenuated IL-1β (2552 +/- 91.18 vs. 1254 +/- 36.46) (**Figure 3.5A**), TNFα (1475 +/- 91.7 vs. 969.7 +/- 49.07) (**Figure 3.5B**) and IFNγ (2033 +/- 187.6 vs. 1118 +/- 231.6) (**Figure 3.5C**) induced levels of sCX3CL1 (***p<0.001, *p<0.05, compared to matched treated group). This data may be likely explained by a required phosphorylation, preceding an ADAM10-mediated shedding of fractalkine from human astrocytes.

2.6 IKKβ inhibitor attenuates pro-inflammatory cytokine induced protein levels of CX3CL1 in human astrocytes

The transcription factor NF-κB regulates the production of several pro-inflammatory cytokines (Li and Verma, 2002). In order to further investigate the mechanism by which pro-inflammatory cytokines increase fractalkine levels in human astrocytes, effects of the NF-κB pathway inhibitor

IKKβ (TPCA-1) were examined on the protein levels of sCX3CL1 using ELISA. Human astrocytes were pre-treated for 30 min with the IKKβ inhibitor (TPCA-1, 3μM, 1μM, 0.3μM, 0.1μM and 0.03μM), prior to the addition of IL-1β (100pg/ml) or TNFα (10ng/ml) for 18 hr. As demonstrated previously, IL-1β and TNFα increased the levels of sCX3CL1, which were significantly attenuated by the IKKβ inhibitor (TPCA-1, using 1μM, 628.3 +/- 138.5 vs. 47.7 +/- 14.9 for IL-1β; 748.0 +/- 120.0 vs. 116.4 +/- 31.7 for TNFα) (**Figure 3.6B**) (***$p<0.001$ and **$p<0.01$ *$p<0.05$ compared to matched treated group). From this data, we conclude that the pro-inflammatory cytokines IL-1β and TNFα increase the levels of sCX3CL1 by promoting NF-κB-dependent synthesis which results in subsequent enhanced shedding of fractalkine via an ADAM10 dependent mechanism.

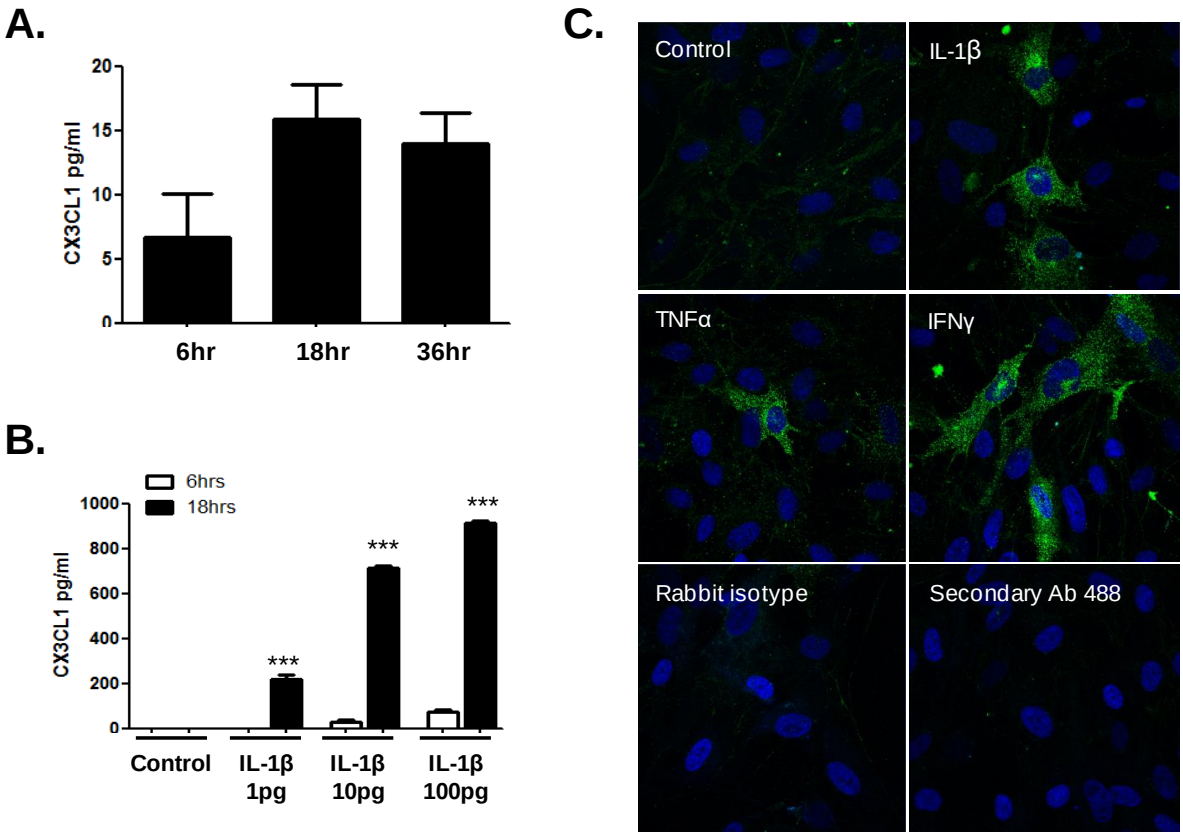


Figure 3.1: Soluble fractalkine (sCX3CL1) release from human astrocytes

Human astrocytes were **(A)** serum starved for 6 hr, 18 hr, and 36 hr to measure constitutive release of sCX3CL1. **(B)** Cells were treated with IL-1β (1pg/ml, 10pg/ml and 100pg/ml) for 6 or 18 hr to measure induced release of sCX3CL1. **(C)** Images show immuno-staining of up-regulated CX3CL1 on human astrocytes when treated with IL-1β (100pg/ml), TNFα (10ng/ml) and IFNγ (10ng/ml) for 18 hr. **(B)** and **(C)** groups were serum starved 3 hr before treatments. Quantification of CX3CL1 ELISA revealed **(A)** no significant increase in CX3CL1 release between 6 and 36 hr. **(B)** CX3CL1 release was significantly higher after 18 hr treatments compared to 6 hr. All graphs expressed as mean+/- SEM; n=4, each condition done in duplicate. One way ANOVA and Tukey's post-hoc test; ***p<0.001 compared to control.

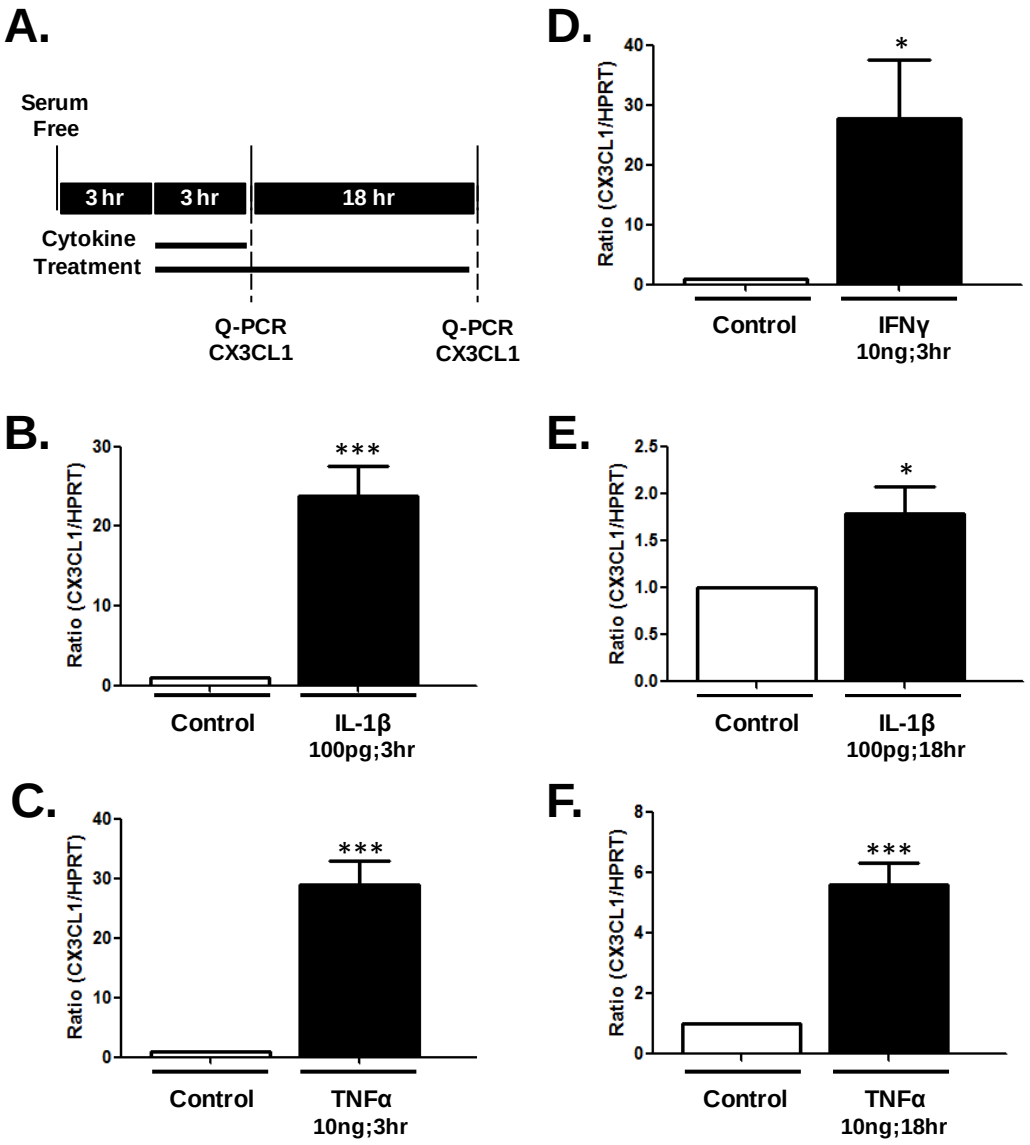


Figure 3.2: IL-1β, TNFα and IFNγ induce fractalkine mRNA synthesis in human astrocytes

Human astrocytes were serum starved for 3 hr prior to stimulation with **(B+E)** IL-1β (100pg/ml), **(C+F)** TNFα (10ng/ml) or **(D)** IFNγ (10ng/ml) for either 3 or 18 hr. All graphs show significant increase in CX3CL1 mRNA levels after 3 and 18 hr. Data presented as mean +/- SEM, n=4-8, unpaired t-test, *P<0.05, ***p<0.001 vs. corresponding control.

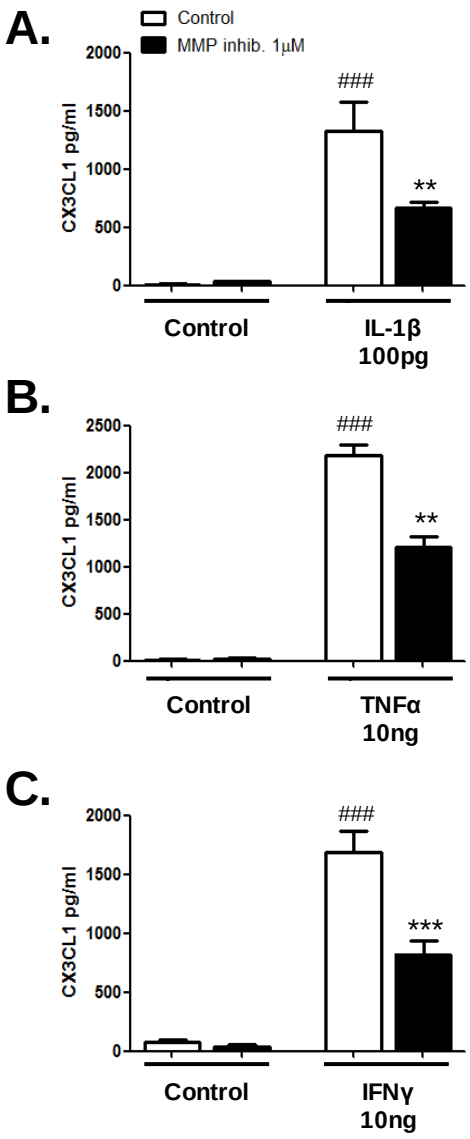


Figure 3.3: Inhibition of matrix metalloproteases attenuates sCX3CL1 release from human astrocytes

Human astrocytes were pre-treated with a pan MMP inhibitor (MMP inhib.) Marimastat (1μM) for 30 min. Cells were then stimulated with either **(A)** IL-1β (100pg/ml), **(B)** TNFα (10ng/ml) or **(C)** IFNγ (10ng/ml) for 18 hr. All groups were serum starved 3 hr before treatments. Quantification of CX3CL1 ELISA revealed a significant decrease in CX3CL1 when cells were pre-treated with Marimastat for all treatment groups. ###p<0.001 Compared to own control; ***p<0.001 and **p<0.05 compared to matched treated group. All values expressed as averages +/- SEM; n=3-5, each condition done in duplicate. (One way ANOVA and Tukey's post-hoc test).

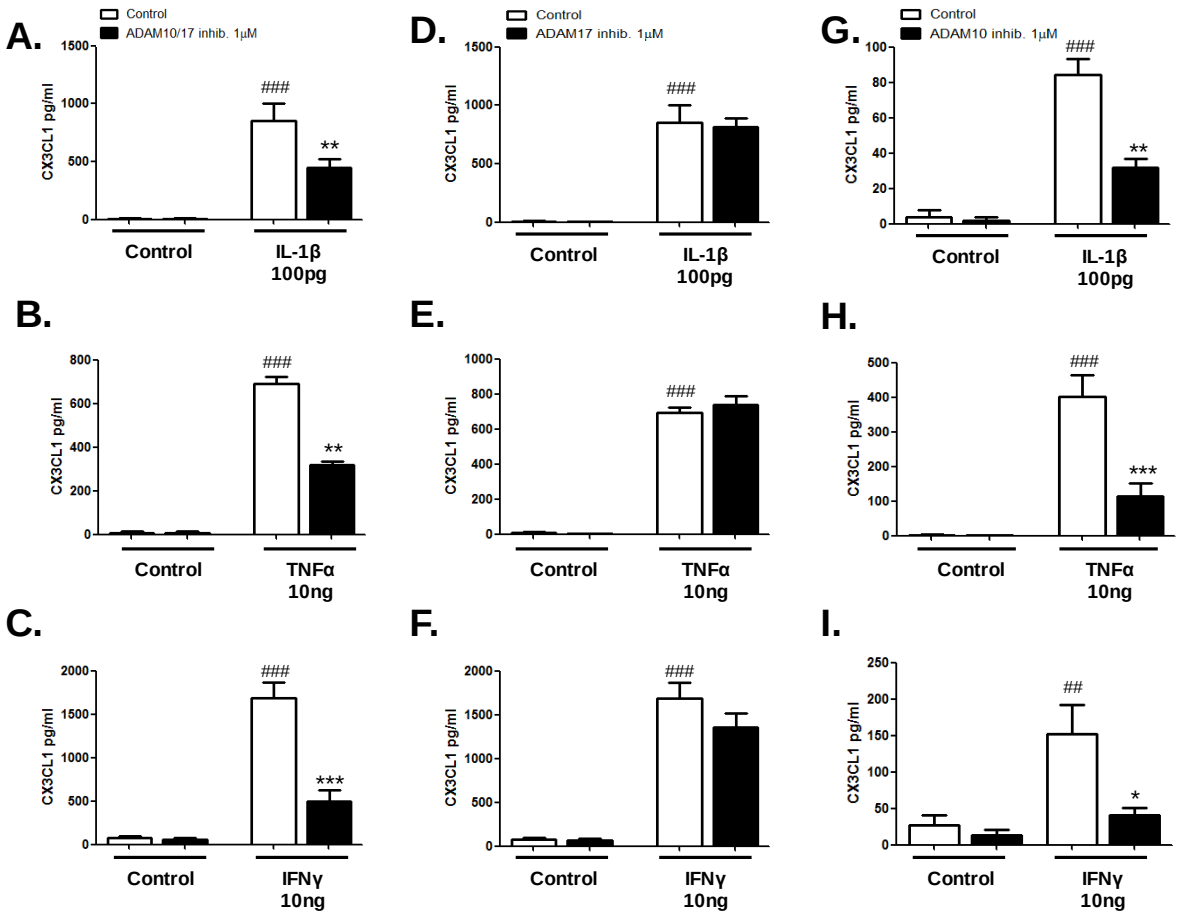


Figure 3.4: ADAM10 inhibitor attenuates IL-1β, TNFα and IFNγ induced sCX3CL1 release

Human astrocytes were serum starved for 3 hr and pre-treated with a combination of ADAM10/17 inhibitor (BMS-561392; 1μM), specific TACE inhibitor (BMS-566394; 1μM), or specific ADAM10 inhibitor (GI 254023X; 1μM) for 30 min. Cells were then stimulated with either IL-1β (100pg/ml) **(A+D+G)**, TNFα (10ng/ml) **(B+E+H)** or IFNγ (10ng/ml) **(C+F+I)** for 18 hr. Quantification of CX3CL1 ELISA revealed a significant decrease in sCX3CL1 when pre-treated with ADAM10/17 inhibitor and specific ADAM10 inhibitor. No significant difference was seen with the specific ADAM17 inhibitor. ###p<0.001 Compared to own control; *p<0.05 **p<0.01 and ***p<0.001 compared to matched treated group. Values expressed as averages +/- SEM; n=4-8, each condition done in duplicate. (One way ANOVA and Tukey's post-hoc test).

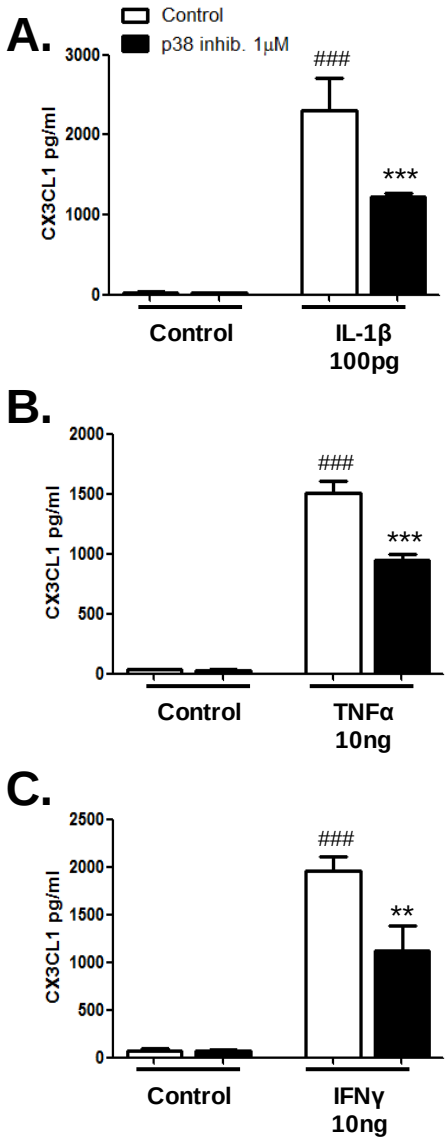


Figure 3.5: The p38 inhibitor (VX-702) reduces IL-1β, TNFα and IFNγ induced sCX3CL1 release

Human astrocytes were serum starved for 3 hr and pre-treated with a the P38 inhibitor, VX-702 (1μM) for 30 min. Cells were then stimulated with either **(A)** IL-1β (100pg/ml), **(B)** TNFα (10ng/ml) or **(C)** IFNγ (10ng/ml) for 18 hr. Quantification of CX3CL1 ELISA revealed a significant decrease in sCX3CL1 when pre-treated with VX-702 for all treatment groups. ###p<0.001 Compared to own control; ***p<0.001 and **p<0.01 compared to matched treated group. Values expressed as averages +/- SEM; n=3, each condition done in duplicate. (One way ANOVA and Tukey's post-hoc test).

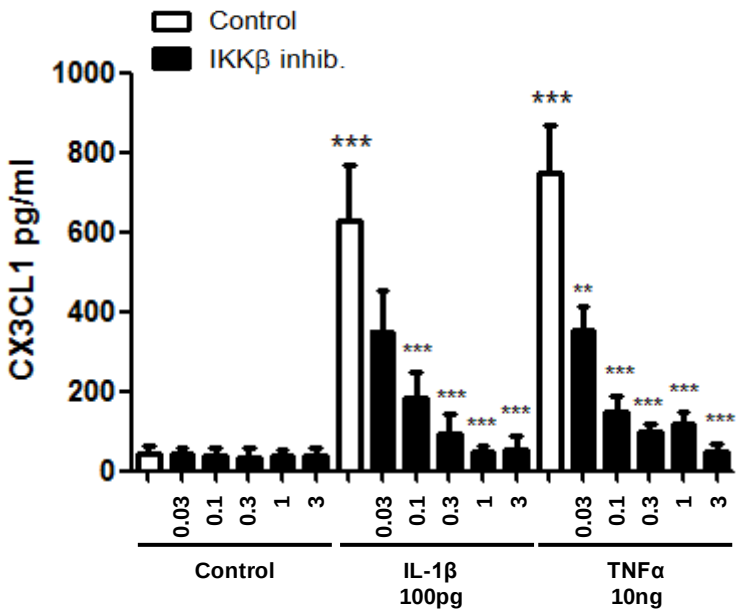
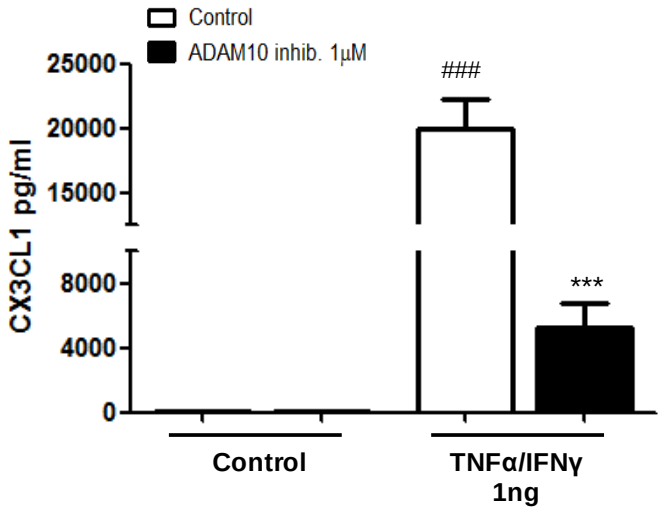


Figure 3.6: IKKβ inhibitor attenuates sCX3CL1 release

Human astrocytes were serum starved for 3 hr and pre-treated with the NF-κB inhibitor, TPCA-1 (IKKβ inhib; 3μM, 1μM, 0.3μM, 0.1μM and 0.03μM) for 30 min. Cells were then stimulated with either IL-1β (100pg/ml) or TNFα (10ng/ml) for 18 hr. Quantification of CX3CL1 ELISA revealed a concentration dependent decrease in sCX3CL1 release for both IL-1β and TNFα treated groups. ###p<0.001 Compared to own control; **p<0.01 and ***p<0.001 compared to matched treated group. Values expressed as averages +/- SEM; n=3 each condition done in duplicate. (One way ANOVA and Tukey's post-hoc test).



Supplemental Figure 1. Soluble fractalkine levels are attenuated with ADAM10 inhibition following co-stimulation with TNFα and IFNγ. Astrocytes were pre-treated with the ADAM10 inhibitor (GI 254023X; 1µM), for 30 min. Cells were then co-stimulated with TNFα (1ng/ml) and IFNγ (1ng/ml) for 18 hr. Quantification of CX3CL1 ELISA revealed a significant decrease in soluble fractalkine when pre-treated with ADAM10 inhibitor. ###$p<0.001$ compared to own control and ***$p<0.001$ compared to matched treated group (One way ANOVA and Tukey's post-hoc test). Values expressed as averages +/- SEM; n=4, duplicates.

3. Discussion

3.1 Summary of Findings

In the current study, we investigated fractalkine (CX3CL1) expression and regulation in human astrocytes. We showed firstly that fractalkine is expressed in human astrocytes and is constitutively shed at low levels. On stimulation with pro-inflammatory cytokines such as IL-1β, TNFα and IFNγ this low level of sCX3CL1 is significantly up-regulated. We noted that a broad spectrum MMP inhibitor attenuated this increase in sCX3CL1. Using a dual inhibitor of ADAM10/ADAM17 and selective ADAM10 and ADAM17 inhibitors, we showed further that ADAM10 is the main protease responsible for the cytokine-mediated inducible shedding of fractalkine. We also found these cytokines stimulated the mRNA levels of fractalkine, suggesting a mechanism involving increased mRNA and protein synthesis. Using a p38 MAPK inhibitor, we demonstrated that the pro-inflammatory cytokine-mediated increase in sCX3CL1 likely involved a phosphorylation event mediated by p38 MAPK. An inhibitor of IKKβ also attenuated the increase in sCX3CL1 induced by the pro-inflammatory cytokines IL-1β and TNFα, suggesting involvement of the NF-κB pathway. Taken together, the data indicates that pro-inflammatory cytokines regulate fractalkine expression on human astrocytes and that ADAM10 is the main protease involved in the liberation of sCX3CL1 from human astrocytes.

3.2 ADAM10 regulates cytokine-mediated levels of soluble fractalkine in astrocytes

Both ADAM10 and ADAM17 have been implicated in the development of inflammatory diseases such as Alzheimer's disease (Manzine et al., 2013, Lammich et al., 1999, Allinson et al., 2003). Studies have also shown that ADAM10 expression is widespread in the CNS compared to a more restricted ADAM17 expression (Karkkainen et al., 2000). A more ubiquitous expression of ADAM10 would suggest this enzyme may be responsible for the constitutive basal processing of ligands such as fractalkine (Karkkainen et al., 2000, Lammich et al., 1999). In contrast, a low expression of ADAM17 appears to coincide with its proposed involvement in inducible proteolytic processing. For example, previous studies show that pro-inflammatory cytokines, such as CXCL12, can regulate cleavage of the fractalkine ligand from neurons (Cook et al., 2010). The treatment of neurons with CXCL12 stimulates the expression of ADAM17 causing an increase in sCX3CL1 (Cook et al., 2010). Studies also show that ADAM10 is responsible for constitutive neuronal fractalkine cleavage (Hundhausen et al., 2003). In contrast, our studies on human astrocytes would suggest that ADAM10 is the main protease responsible for the cytokine-mediated proteolytic processing of sCX3CL1. This finding raises the possibility that differential regulation of ADAM10 and ADAM17 may be cell type dependent and provide a mechanism for regulating different cellular responses to the same stimuli.

3.3 Involvement of p38 MAPK in the regulation of ADAM10

Similar to studies investigating the release of fractalkine from neurons, we show here that the shedding of fractalkine occurs both constitutively and in response to inflammatory stimuli in

astrocytes (Hundhausen et al., 2003, Jones et al., 2013). We find, in particular, that ADAM10 likely regulates pro-inflammatory cytokine-induced levels of fractalkine in astrocytes. Notably, several other ligands are shed by ADAM10, such that cleavage by this protease is likely controlled by multiple regulatory mechanisms (Seals and Courtneidge, 2003). The cytosolic tail of ADAM10, as well as other ADAM proteases, contains many interaction motifs for signalling and adapter proteins, which could conceivably regulate protease activity (Seals and Courtneidge, 2003). Indeed, studies have shown that downstream signalling molecules such as p38 interact with the cytoplasmic domain of ADAM10 and regulate ADAM-dependent ligand shedding (Diaz-Rodriguez et al., 2002, Fan et al., 2003, Jones et al., 2013). Popular thought is that constitutive shedding is governed by p38 (Seals and Courtneidge, 2003, Black et al., 2003). In neurons, it appears that p38 regulates the constitutive shedding of fractalkine via ADAM10 (Bandyopadhyay et al., 2006, Diaz-Rodriguez et al., 2002). Here we show that, in astrocytes, inhibition of p38 caused a significant decrease in the levels of sCX3CL1 induced by treatment with pro-inflammatory cytokines. It is therefore reasonable to suggest that, in astrocytes, p38 via ADAM10 may enhance cleavage of fractalkine and/or that a p38 signalling pathway is required for increased production of this ligand.

3.4 Functions of astrocyte derived fractalkine

Fractalkine signalling is crucial for proper development during brain maturation (Paolicelli et al., 2011), synaptic plasticity (Bertollini et al., 2006, Rogers et al., 2011), neuroprotection (Pabon et al., 2011, Limatola et al., 2005) and neurotoxicity (Cardona et al., 2006) (Sheridan and Murphy, 2013). More specifically, sCX3CL1 has the capability of enhancing microglial response to CNS damage thereby promoting a pro-inflammatory environment (Cardona et al., 2006). However, membrane tethered fractalkine can also serve as an adhesion molecule thereby anchoring cells to the extracellular matrix and preventing them from migrating (Lauro et al., 2006). In a MOG induced EAE rat model of MS, neuronal fractalkine was reported unchanged and remained at control levels (Sunnemark et al., 2005). However, at sites of inflammation and surrounding active lesions there is an increase in astrocyte associated fractalkine along with increased expression of CX3CR1 on microglia (Sunnemark et al., 2005). A possible role for astrocyte derived fractalkine may be to enhance CX3CR1 signalling on microglia and at the same time promote levels of neuronal fractalkine as an attachment factor. This dual role of fractalkine within the CNS may thus serve to protect surrounding tissue from damaged neurons while also mobilizing microglia and astrocytes to eliminate the damaged tissue.

3.5 Studies using fractalkine as a drug target – lessons learned so far

The genetic manipulation of fractalkine signalling has led to contradictory findings in mouse models of peritonitis regarding its role in immune defence (Jung et al., 2000, Ishida et al., 2008). In particular studies have shown that monocyte extravasation is not affected in CX3CR1 deficient mice, suggesting that CX3CR1 signalling is not necessary for invading monocytes (Jung et al., 2000). In contrast CX3CR1 mediated signalling is demonstrated to be essential for macrophages in mounting defence against bacterial infection (Ishida et al., 2008). As the fractalkine-CX3CR1

interaction appears exclusive, in contrast to other chemokines, this specificity has been considered as worthy of exploitation for drug development. Previous studies involving either fractalkine or CX3CR1 deficient mice have, however, lead to contradictory findings, where fractalkine is suggested as neuroprotective or neurotoxic. Studies, for example, on middle cerebral artery occlusion (MCAO) in fractalkine deficient mice exhibited smaller infarct lesions and had lower mortality rates (Soriano et al., 2002). However, administration of exogenous fractalkine, to fractalkine deficient mice, post ischemia, resulted in neurotoxicity and increased infarct size (Soriano et al., 2002). Notably, these neurotoxic effects of fractalkine were not observed in CX3CR1 deficient mice (Soriano et al., 2002). Studies, in contrast, also show that fractalkine administered to wild type rats, just prior to MCAO, elicits long lasting neuroprotective effects (Cipriani et al., 2011). In addition to this, a narrow therapeutic concentration range for fractalkine appears to exist, where low concentrations are neuroprotective (15-70pM) with higher levels (>150pM) becoming toxic (Cipriani et al., 2011). Therefore, from these studies its seems that the concentration of fractalkine, its administration pre or post insult, as well as activity of the endogenous fractalkine signalling pathway, determine the efficacy of fractalkine in diseased models.

3.6 Concluding Remarks

Here, we find that fractalkine shedding from astrocytes is mediated by pro-inflammatory cytokines via an ADAM10-dependent pathway and that p38 as well as NF-κB signalling is required. This study indicates further a complex role for fractalkine in both neuronal and glial cells during inflammatory events and suggests regulation of this signalling pathway for the purposes of drug development may require targeting in a cell-specific, temporal and spatial manner.

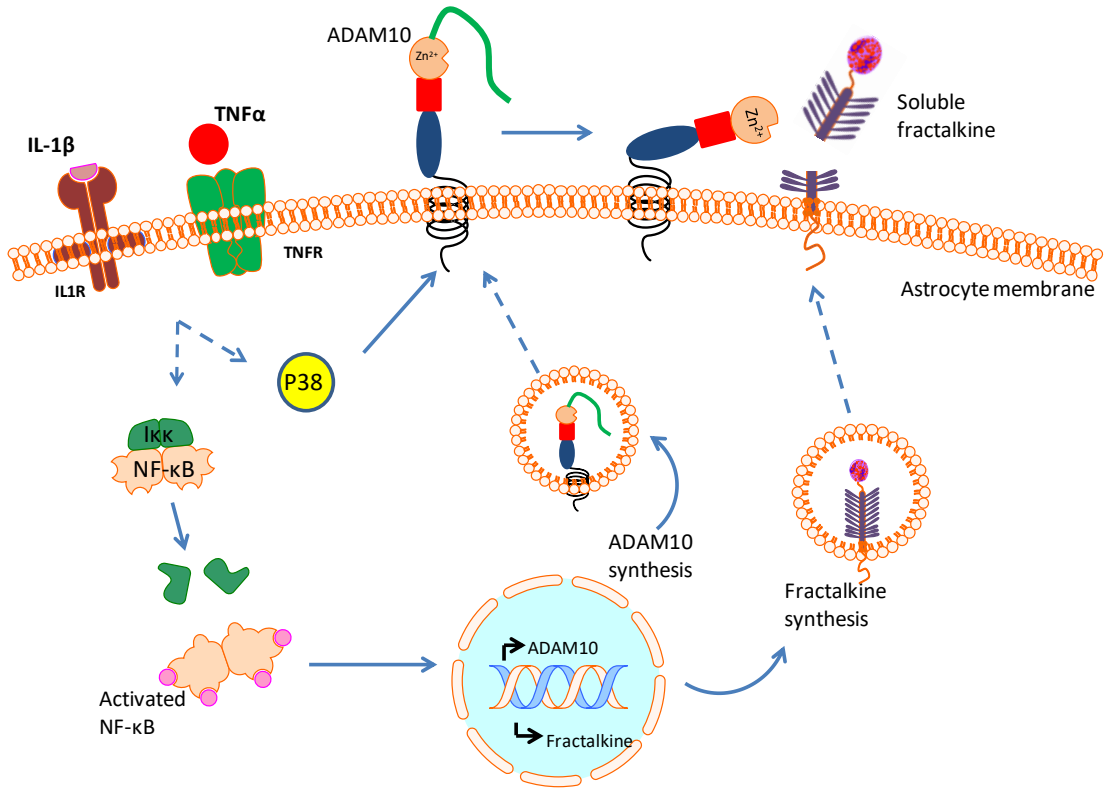


Figure 3.7: Overview of the possible mechanisms of cytokine mediated increases in fractalkine release from astrocytes

IL-1β and/or TNFα signal through NF-κB which causes *de-novo* synthesis of fractalkine. It is then transported to the cell surface where it can be cleaved by ADAM10. P38 MAPK may be phosphorylated simultaneously, which interacts with the cytoplasmic tail of ADAM10 causing it to switch from its pro- to active form.

Chapter 4:

Demyelination induced by oxidative stress is regulated by sphingosine 1-phosphate receptors

Chapter aims

The specific aims of this chapter were to:

- Investigate the effects of bolus H_2O_2 on myelination and astrocyte reactivity in cerebellar slice cultures
- Investigate the effects of low, sustained levels of H_2O_2 generated by glucose oxidase and catalase enzymes on myelination and astrocyte reactivity in cerebellar slice cultures
- Compare the effects of H_2O_2 on astrocytes in dissociated cell culture versus cerebellar slice culture
- Determine whether the S1P receptor agonist, pFTY720, exerts protective effects on H_2O_2 toxicity in cerebellar slices
- Investigate the effects of H_2O_2 on levels of Iba1 in microglia
- To examine the levels of select pro-inflammatory cytokines in cerebellar slices treated with H_2O_2

Abstract

Oxidative stress is a pathological condition defined as an imbalance between production and removal of reactive oxygen species (ROS). This process can cause structural cell damage, disrupt DNA repair and induce mitochondrial dysfunction, all of which are implicated in neurodegeneration. Many *in vitro* studies have used direct bolus application of H_2O_2 to investigate the role of oxidative stress in cell culture. In the current study, using mouse organotypic cerebellar slice cultures, the effects of H_2O_2 induced oxidative stress on myelination state were examined, using bolus concentrations of H_2O_2 (0.1-1mM) and low-continuous H_2O_2 (~20µM) generated from glucose oxidase and catalase (GOX-CAT). Using these models, the potential therapeutic effects of pFTY720, an oral therapy use in relapsing-remitting multiple sclerosis, were also examined. Here, bolus treatment of H_2O_2 (0.5mM) was found to induce demyelination and, for the first time, it was demonstrated that low-continuous H_2O_2 (GOX-CAT) exposure also induces demyelination in these slices. Both bolus H_2O_2 and GOX-CAT treatments caused a significant decrease in vimentin expression in these slice culture models as well as increased cell death in isolated astrocyte cultures. Importantly, pre-treatment with pFTY720 significantly attenuated both bolus H_2O_2 and GOX-CAT induced demyelination and significantly attenuated the GOX-CAT induced decrease in vimentin in cerebellar slices, without altering levels of the pro-inflammatory cytokine IL-6. Taken together, this data establishes a novel GOX-CAT model of demyelination and demonstrates that pFTY720 can act independently of inflammatory cytokines such as IL-6, to attenuate decreases in vimentin as well as demyelination induced by oxidative stress.

1. Introduction

Reactive oxygen species (ROS), under physiological conditions, are involved in many biological events including protection from pathogens, oxidative modifications and regulation of apoptosis (Veal et al., 2007). The prototypical ROS, hydrogen peroxide (H_2O_2), is found at intracellular concentrations of approximately 10nM (Sies, 2014), of which the major cellular source is NADPH oxidases or complex III in the mitochondria (Boveris et al., 1972). H_2O_2 is under strict regulatory control by peroxiredoxins, glutathione peroxidises as well as catalase. H_2O_2 is readily transported across cell membranes by aquaporins (peroxiporins), similar to H_2O (Bienert et al., 2006). This reactive molecule acts as a second messenger, for example, regulating insulin signalling (May and de Haen, 1979, Alim et al., 2014), growth factor signalling (Bae et al., 1997, Lo and Cruz, 1995) and activation of Akt and MAPK pathways (Ushio-Fukai et al., 1999), as well as regulating transcription factors such as Nrf2 (Kobayashi et al., 2009), CREB (Ozgen et al., 2009), NF-κB (Schreck et al., 1991) and NOTCH (Marinho et al., 2014). H_2O_2 can also regulate kinase activity and phosphorylation, likely by inactivating protein phosphatases (Sies, 2014). In mammalian cells, H_2O_2 is known to regulate cell proliferation (Ma et al., 2015), differentiation (Ho et al., 2013) and inflammation (Tschopp and Schroder, 2010).

Oligodendrocytes make up roughly 5-10% of the total glial population in the brain (Barateiro et al., 2015) and have the highest rate of metabolic activity within the CNS (Perfeito et al., 2007). High concentrations of iron, polyunsaturated fatty acids combined with low levels of glutathione make oligodendrocytes an ideal target for oxidative stress (Gruber et al., 2015). In oligodendrocytes, H_2O_2 is normally removed efficiently from surroundings with an increased ability to detoxify H_2O_2 as the cells mature (Dringen et al., 2005). In mature oligodendrocytes, uncontrolled levels of H_2O_2 can induce cell death, in part, by the phosphorylation of ERK and a decrease in p38 MAPK signalling (Lee and Won, 2014). Increases in ROS production have also been shown to affect the expression of genes expressing myelin in oligodendrocytes (Gruber et al., 2015). Some *in vitro* studies demonstrate that non-lethal oxidative stress can also induce a reversible shortening of oligodendrocyte cell processes (Fernandez-Gamba et al., 2012). These studies highlight the susceptibility of oligodendrocytes to oxidative stress, which contributes to many diseases including amyotrophic lateral sclerosis (ALS) (Carri et al., 2015), Alzheimer's disease (AD) (Milton, 2004, Wang et al., 2014b), multiple sclerosis (MS) (Lee et al., 2012a, Aydin et al., 2014) and even schizophrenia (Filiou et al., 2012, Do et al., 2015).

Sphingosine 1-phosphate receptors (S1PR) are a family of G-protein coupled receptors composed of subtypes S1PR1-5 (Dev et al., 2008). These receptors are the drug targets of the first oral therapy used in relapsing-remitting multiple sclerosis, namely fingolimod, of which the principle active molecule is the phosphorylated version of FTY720 (pFTY720) (Dev et al., 2008). S1PRs are expressed in neurons and glia and have been shown to regulate a range of cellular functions, including neuronal survival, astrocyte migration, microglia activation state and oligodendrocyte-

mediated myelination (Brinkmann et al., 2002, Dev et al., 2008). We have demonstrated previously that S1PR agonists, such as pFTY720, attenuate demyelination induced by a range of toxins and inflammatory insults, such as LPC (Sheridan and Dev 2012), psychosine (O'Sullivan and Dev, 2015, O'Sullivan et al., 2016) and MOG-reactive splenocytes (Pritchard et al., 2014).

Here, we aimed to develop a new model of H_2O_2-induced demyelination through the use of organotypic cerebellar slice cultures. Our objective was to assess the effects of FTY720 on myelin-related proteins within a multi-cellular environment that maintains physiological cell-cell connections. In this study, we firstly induced demyelination in organotypic cerebellar slice cultures by bolus application of H_2O_2 and then, for the first time, developed a new model of H_2O_2-based demyelination using a glucose oxidase and catalase system (GOX-CAT system). We also describe, herein, the protective effects of pFTY720 in both of these demyelinating models.

2. Results

2.1 Application of bolus H_2O_2 to cerebellar slices causes demyelination

The oxidant H_2O_2 can cause cellular damage through the production of free radicals (Halliwell, 1992). To study the effects of H_2O_2 on myelination state, organotypic cerebellar slices were prepared from postnatal day 10, C57BL/6J mice and cultured for 12-14 days *in vitro*. On day 12 the slices were treated with 0.1mM, 0.5mM or 1mM of bolus H_2O_2 for 18hrs (**Figure 4.1A**). The slices were then subjected to immunohistochemical analysis using confocal microscopy to quantify the fluorescence levels of myelin basic protein (MBP; as a myelin marker) and neurofilament-H (NFH, as a neuronal marker) (**Figure 4.1D**). The results show that MBP fluorescence is significantly decreased after 0.5mM H_2O_2 (Control; 52.9 RFU +/- 3.7 RFU vs. 0.5mM H_2O_2; 31.5 RFU +/- 4.6 RFU) and 1mM H_2O_2 (22.4 RFU +/- 3.0 RFU), with 0.1mM H_2O_2 having no significant effect on the levels of MBP (46.3 RFU +/- 8.4 RFU) (***p<0.001, **p<0.01 Student t-test) (**Figure 4.1B**). We also found a significant decrease in the levels of NFH fluorescence at a high concentration of 1mM H_2O_2 (Control; 49.3 RFU +/- 6.4 RFU vs. 1mM H_2O_2; 27.8 RFU +/- 2.9 RFU; *p=0.015) (**Figure 4.1C**). In contrast, treatment with 0.5mM (36.9 RFU +/- 4.4 RFU; p=0.153) and 0.1mM (45.4 RFU +/- 6.3 RFU) *p<0.05, n=5, student t-test. H_2O_2 did not cause a significant decrease in NFH fluorescence (**Figure 4.1C**). This data shows that H_2O_2 can induce demyelination, without significant damage to neurons, in a concentration-dependent manner.

2.2 pFTY720 attenuates H_2O_2 induced demyelination in cerebellar slice cultures

Fingolimod (FTY720) is the first oral therapy for the demyelinating disease, multiple sclerosis (MS) and has shown clinical efficacy in preventing relapses in these patients (Kappos et al., 2006). High levels of oxidative stress have been documented in MS as well as several other neurodegenerative diseases (Karlik et al., 2015, Wang et al., 2014b, Blesa et al., 2015). Thus, to determine whether fingolimod protects from the loss of myelin induced by oxidative stress, we incubated cerebellar slices with 0.5mM bolus H_2O_2 for 18hrs, to induce demyelination without the loss of NFH expression. Slices were treated with H_2O_2 in the presence or absence of the phosphorylated version of fingolimod, namely, pFTY720. As demonstrated above, a concentration of 0.5mM H_2O_2 did not significantly alter the levels of NFH fluorescence (82.9% +/- 9.6%, Student t-test, n=4) (**Figure 4.2A and C**). In contrast, bolus 0.5mM H_2O_2 treatment induced demyelination in cerebellar slices, as determined by MBP fluorescence (66.1% +/- 5.6%; ###p<0.001, student's t-test), which was significantly attenuated by pre-treatment with 1nM FTY720 (101.9% +/- 8.6%; *p<0.05, one-way ANOVA and Tukey's post-hoc test, n=4) (**Figure 4.2A and B**). These results demonstrate that pFTY720 can prevent the deleterious effects of H_2O_2 on myelination state.

2.3 Effects of pFTY720 on astrocytes in cerebellar slices treated with bolus H_2O_2

In vitro studies suggest that cortical astrocytes play a protective role in the defence of neurons against oxidative stress (Desagher et al., 1996). To demonstrate H_2O_2-mediated astrocyte cell death and possible protection by pFTY720, we examined these effects in isolated cell cultures. The treatment of dissociated human astrocytes with H_2O_2, induced a concentration dependent decrease

in cell viability (**Figure 4.3A**) (Control 100% vs. 0.7mM H_2O_2 28.6% +/- 0.6%; ***p<0.001), which was not significantly reversed by pFTY720 (**Figure 4.3B**) (34.8% +/- 8.1%). In hippocampal organotypic slice cultures and in dissociated hippocampal astrocytes and neurons, astrocytes have been shown to be the most sensitive to H_2O_2 toxicity (Feeney et al., 2008). Thus, we also investigated the effects of H_2O_2 on astrocytes in cerebellar slice cultures. A modest decrease in vimentin fluorescence was evident when cerebellar slices were treated with 0.1mM H_2O_2, however this is not significantly different from the control (Control; 100% vs. 0.1mM H_2O_2; 82.9% +/- 16.9%) (**Figure 4.3C**). At higher H_2O_2 concentrations of 0.5mM (57.5% +/- 13.3%; *p<0.05) and 1mM (36.6% +/- 1.9% **p<0.01) we observed a significant decrease in vimentin fluorescence, n=5; student t-test (**Figure 4.3C**). We next examined the effects of pFTY720 on 0.5mM H_2O_2 induced reduction in the levels of vimentin, in cerebellar slice cultures and observed that pre-treatment with 1nM pFTY720 partly reduced the H_2O_2-mediated decrease in vimentin fluorescence (87.7% +/- 11.2%) compared to 0.5mM H_2O_2 treatment alone (63.9% +/- 9.7%). We note, however these effects were not statistically significant, n=4 (**Figure 4.3D**).

2.4 GOX-CAT induced demyelination in cerebellar slice cultures

The generation of H_2O_2 using the enzymatic system of glucose oxidase (GOX) and catalase (CAT) allows for low, continuous production of H_2O_2 in cell culture for up to 24hrs leading to a state of oxidative stress (Mueller et al., 2009). In contrast to bolus H_2O_2 treatments, the GOX-CAT model of oxidative stress is seen as more physiologically relevant with regards to the amount of time that H_2O_2 is present in the media and its concentration. Differences in intracellular signalling pathways and cellular responses have been observed in response to bolus and continuous low levels of H_2O_2 (Pathipati et al., 2013, Millonig et al., 2012). Using this GOX-CAT system, generation of H_2O_2 can be controlled by maintaining glucose oxidase constant at a dilution of 1:100,000 and varying the dilution of CAT. Using GOX (1:100,000 dilution) and CAT (1:500,000 dilution) we quantitatively determined the amount of H_2O_2 present in the media after 18hrs by means of amplex red. A control containing the same GOX-CAT dilution in the absence of organotypic slices was found to have a concentration of approximately 20µM H_2O_2 (20.0 µM +/- 1.4 µM) (**Figure 4.4B**). However, in the presence of four organotypic cerebellar slices, the levels of H_2O_2 were found to be approximately 350nM (345.0 µM +/- 47.5 µM) (**Figure 4.4B**). We therefore next examined the effect of increasing CAT dilutions (i.e. rising H_2O_2 concentration) on the expression of MBP and NFH in cerebellar slices media. Our results showed a concentration dependent decrease in MBP fluorescence after 18hrs treatment with GOX-CAT, where a significant decrease in MBP fluorescence was seen as the CAT dilution increases (**Figure 4.4C (i)**) (control 44.53 RFU +/- 3.6 RFU; 1:50,000 CAT 31.7 RFU +/- 3.5 RFU; 1:100,000 CAT 29.27 RFU +/- 4.3 RFU; 1:500,000 CAT 25.4 RFU +/- 4.3 RFU; 1:1,000,000 CAT 20.2 RFU +/- 6.8 RFU; *p<0.05, **p<0.01, n=4, student t-test). In contrast to observed changes in levels of MBP, we observed no concentration dependent effect of GOX-CAT treatment on NFH fluorescence (except at a CAT dilution of 1:1,000,000; *p<0.05, student t-test) (**Figure 4.4C (ii)**). Overall the data is suggestive of the idea that oligodendrocytes exhibit a higher degree of vulnerability to the effects of low continuous H_2O_2 production in comparison to neurons.

2.5 pFTY720 prevents GOX-CAT induced demyelination in cerebellar slice cultures

As demonstrated above, we showed that the bolus H_2O_2-induced decrease in MBP expression is attenuated by pre-treatment with pFTY720. To further this finding, we next investigated the effects of pFTY720 on demyelination induced by GOX-CAT treatment. As before, GOX-CAT treatment (using a 1:500,000 CAT dilution) significantly reduced MBP fluorescence (70.8% +/- 4.6%, student t-test; $^{###}$p<0.001, n=6) compared to untreated control (**Figure 4.5A and B**), without decreasing the expression of NFH **(Figure 4.5C)**. Importantly, pre-treatment with 1nM pFTY720 significantly attenuated this GOX-CAT-induced decrease in MBP fluorescence (101.7% +/- 8.9%, one way ANOVA with Tukey's post-hoc test **p<0.01, n=6) (**Figure 4.5B**), without significantly changing levels of NFH. When examining the expression levels of MOG, similar results were found; in particular that GOX-CAT treatment induced a significant reduction in the level of MOG (100% v.s 66.15% +/- 8.4%, student t-test; $^{##}$p<0.01, n=5), which was significantly attenuated by pre-treatment with pFTY720 (66.1% +/- 8.4% vs. 130.9% +/- 22.47%, One-way ANOVA; *p<0.05, n=5) (**Figure 4.6**). Taken together, this data strongly suggests that pFTY720 can limit demyelination caused by low levels of continuous H_2O_2 treatment (as generated by GOX-CAT treatment), as well as by higher concentrations of acute H_2O_2 (during bolus treatments).

2.6 pFTY720 attenuates GOX-CAT induced vimentin decrease in cerebellar slices

Cells that consume oxygen continuously generate H_2O_2, which is thought to be formed at the highest quantities than compared to other peroxides (Dringen et al., 2005). Astrocytes are known to play a role in the detoxification of H_2O_2, with catalase thought to be the main hydrogen peroxidase (Desagher et al., 1996). To date, many studies investigating the effects of bolus H_2O_2 on glial cells have been conducted using dissociated glial cell cultures (Ito et al., 2015), although only a limited number have used GOX-CAT. Notably, no studies have yet been reported for astrocytes, therefore we next examined the effects of low- continuous H_2O_2 on the viability of astrocytes in both isolated astrocyte cultures and organotypic cerebellar slice cultures. In isolated astrocyte cultures treatment with GOX-CAT caused a concentration-dependent decrease in cell viability (Control 100% vs. CAT 1:50,000 52.8% +/- 5.1%, ***p<0.001) (**Figure 4.7A**), which was not rescued by pFTY720 (55.9% +/- 5.7%) (**Figure 4.7B**); similar to data shown for bolus H_2O_2 (**Figure 4.3A and B**). Using organotypic cerebellar slice cultures, however, we observed a significant concentration dependent decrease in vimentin fluorescence at a CAT dilution of 1:1,000,000 and 1:500,000 (Control; 100% vs. 1:500,000 CAT; 60.8% +/- 11.8%; *p=0.05) and 1:1,000,000 CAT (53.2% +/- 15.4%, *p<0.05, n=4) (**Figure 4.7C**), that was significantly attenuated with 1nM FTY720 treatment (66.9% +/- 5.9% vs. 90.4% +/- 8.1%, one way ANOVA with Tukey's post-hoc test *p<0.05, n=6) (**Figure 4.7D**). Overall, this data suggests that pFTY720 may be protective of astrocyte cell loss in slice cultures at low concentrations of H_2O_2 (as generated when using GOX-CAT), but not in isolated cell cultures or at higher concentrations of H_2O_2 (when using bolus H_2O_2 treatments).

2.7 pFTY720 attenuates GOX-CAT induced demyelination independent of changes in microglial Iba1 and pro-inflammatory cytokines

We have previously reported that pFTY720 protects against LPC- (Sheridan and Dev, 2012), psychosine- (O'Sullivan and Dev, 2015) and splenocyte-induced (Pritchard et al., 2014) demyelination. In these studies, pFTY720 appears to attenuate LPC-induced levels of pro-inflammatory cytokines, such as LIX, MIP1α, MIP3α, which may in part explain its protective effect on demyelination induced by this toxin (Sheridan and Dev, 2012). In contrast, previous studies in our lab have shown that psychosine-induced demyelination and protection by pFTY720 is not associated with altered levels of pro-inflammatory cytokines such as IL-6, TNFα or IL-1β, and occurs independently of microglia cell response. These studies suggest that pFTY720 may therefore also have the ability to provide protection independent of the inflammatory response (O'Sullivan and Dev, 2015). Here, we further investigated whether pFTY720 attenuates H_2O_2-induced demyelination by dampening inflammatory response. Using organotypic cerebellar slices we first examined the effects of GOX-CAT in the presence or absence of pFTY720 on the expression levels of the pro-inflammatory marker IL-6. Treatment with bolus H_2O_2 showed a modest increase in levels of IL-6, which were not significant (**Figure 4.8A**), while GOX-CAT treatment caused a bi-phasic concentration-dependent increase in the levels of IL-6, which was significant at CAT dilutions of 1:1,000 (Control 43.9 +/- 6.3 vs. 383.6 +/- 66.33; $^{\#\#\#}p<0.001$), 1:500,000 (232.2 +/- 76.7; $^{\#}p=0.05$) and 1:1,000,000 (586.0 +/- 88.1; $^{\#\#\#}P<0.001$) student t-test (**Figure 4.8A and B**). In our hands, however, H_2O_2 did not increase the levels of IL-6 in either isolated mouse or human astrocytes, using either bolus H_2O_2 or GOX-CAT derived H_2O_2 (**Figure 4.8C**). Moreover, the effects of bolus H_2O_2 or GOX-CAT treatments on levels of IL-6 in organotypic cerebellar slices were not significantly altered by pFTY720 **(Figure 4.8A).** Similar results were also found for another pro-inflammatory chemokine, namely, fractalkine (Control 84.8 +/- 16.4 vs. 389.3 +/- 45.8; ***p<0.001) (**Figure 4.8D**). This is similar to our previous studies demonstrating the effects of pFTY720 and psychosine on the levels of IL6 (O'Sullivan and Dev, 2015). A study using isolated mouse microglia cultures has reported these cells release IL-6 at low concentrations of continuous H_2O_2 (GOX 1:100,000:CAT 1:5,000) but not in response to higher levels (1:20,000 and 1:80,000 CAT dilution) with cell death reported at 1:80,000 CAT dilution (Pathipati et al., 2013). Thus we next examined the effects of GOX-CAT and pFTY720 on levels of the microglia marker, Ionized calcium binding adapter molecule 1 (Iba1), although we note that Iba1 is not a specific marker of altered microglia reactivity. In these experiments, no significant changes in expression of Iba1 were observed with any of the treatments used (**Figure 4.8E**). Overall, therefore, these results are in agreement with our previous data (O'Sullivan and Dev, 2015), suggesting that pFTY720 can rescue demyelination independent of changes in the pro-inflammatory response.

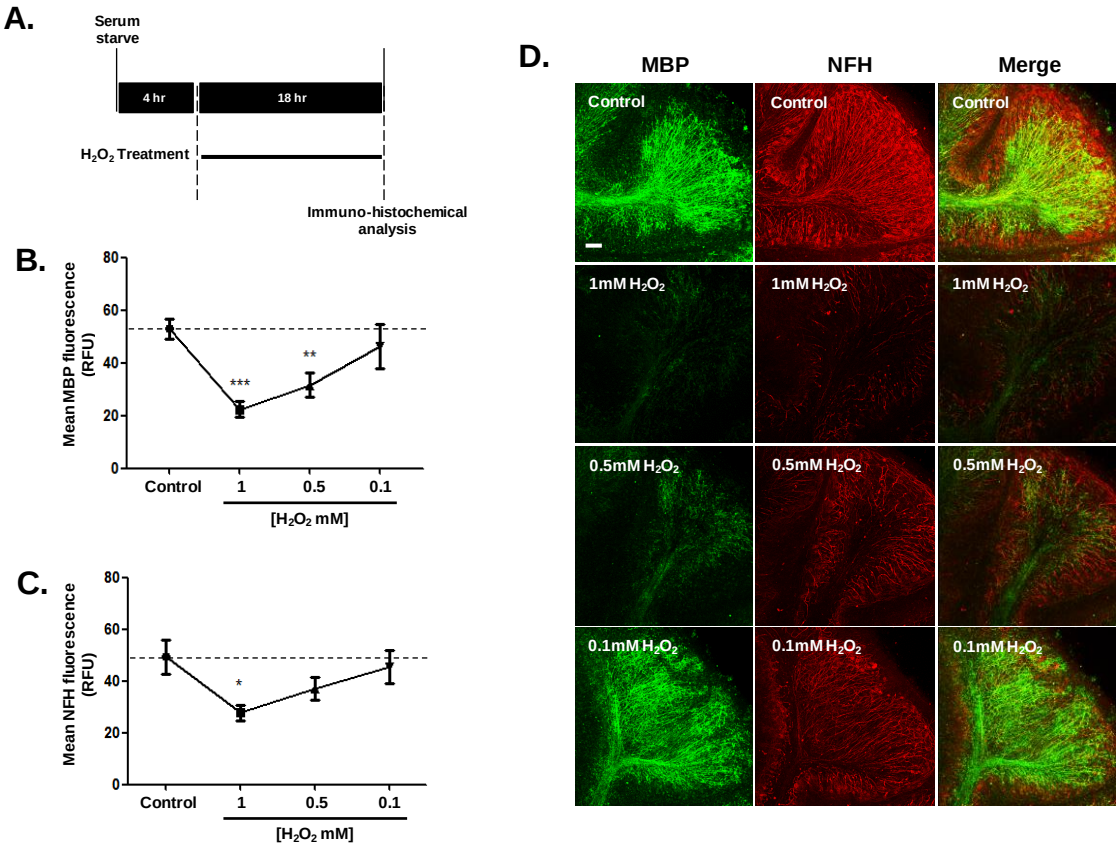


Figure 4.1: Bolus H_2O_2 induces demyelination in cerebellar slice cultures

After 12-14 DIV, P10 cerebellar slices were serum starved for 4 hrs prior to addition of H_2O_2 (0.1mM, 0.5mM and 1mM) for 18 hrs. **(A)** Experimental timeline shown. **(B)** Shows a significant decrease in MBP fluorescence at 1mM and 0.5mM H_2O_2. **(C)** Graph shows a significant decrease in NFH fluorescence at 1mM H_2O_2 only. Slices were analysed using confocal microscopy. **(D)** Images are representative of 5 separate experiments with images taken at 10x magnification. Scale bar, 100µm. Graphs expressed as means +/- SEM, t-test; ***p<0.001, **p<0.01 and *p<0.05.

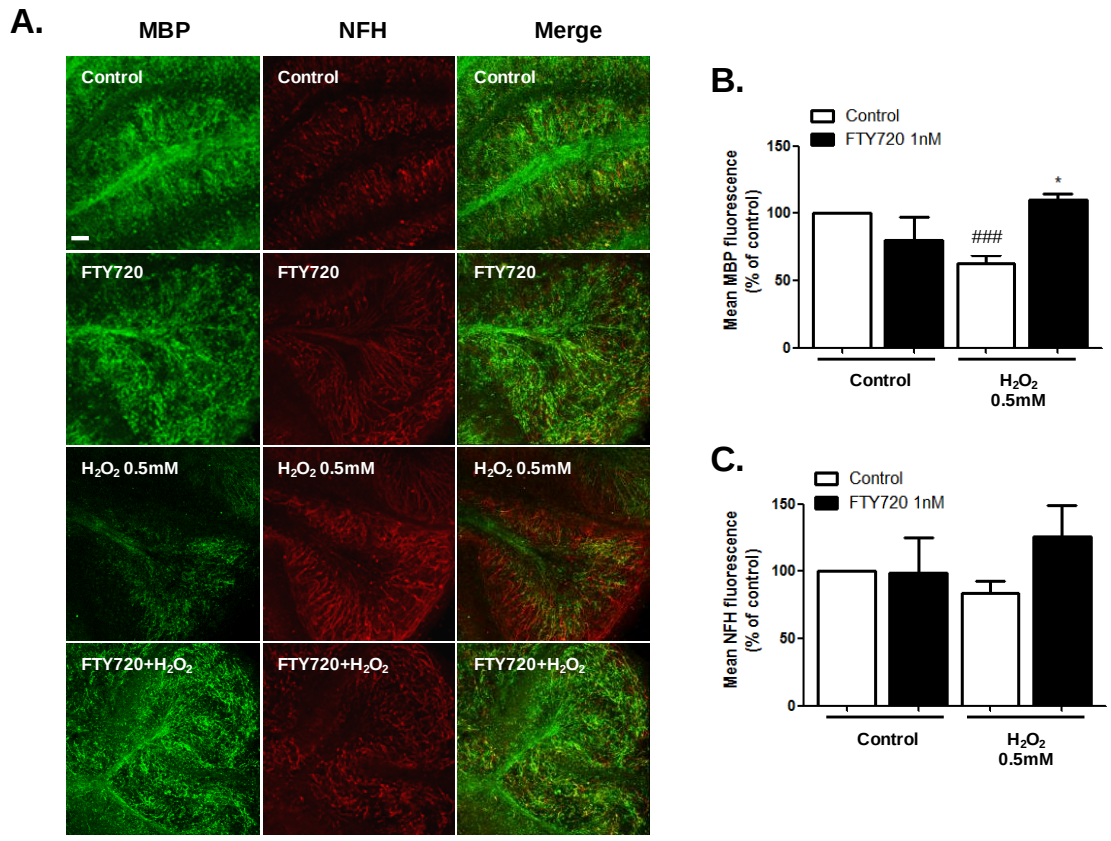


Figure 4.2: pFTY720 attenuates H_2O_2 induced demyelination in cerebellar slice cultures

Organotypic cerebellar slices were serum starved for 4 hrs prior to pre-treatment with pFTY720 (1nM) for 1 hr. This was followed by the addition of bolus H_2O_2 (0.5mM) for 18 hrs. **(A)** Images are representative of 4 separate experiments. Confocal images taken at 10x magnification. Scale bar, 100µm. **(B)** Graph show a significant decrease in MBP fluorescence which is attenuated by pFTY720. **(C)** NFH shows no significant decrease with GOX-CAT treatment as determined by student t-test. All data expressed as mean +/- SEM, t-test; $^{###}p<0.001$ compared to own control, One way ANOVA; $*p<0.05$ and Tukey's post-hoc test compared to matched treated group, n=4.

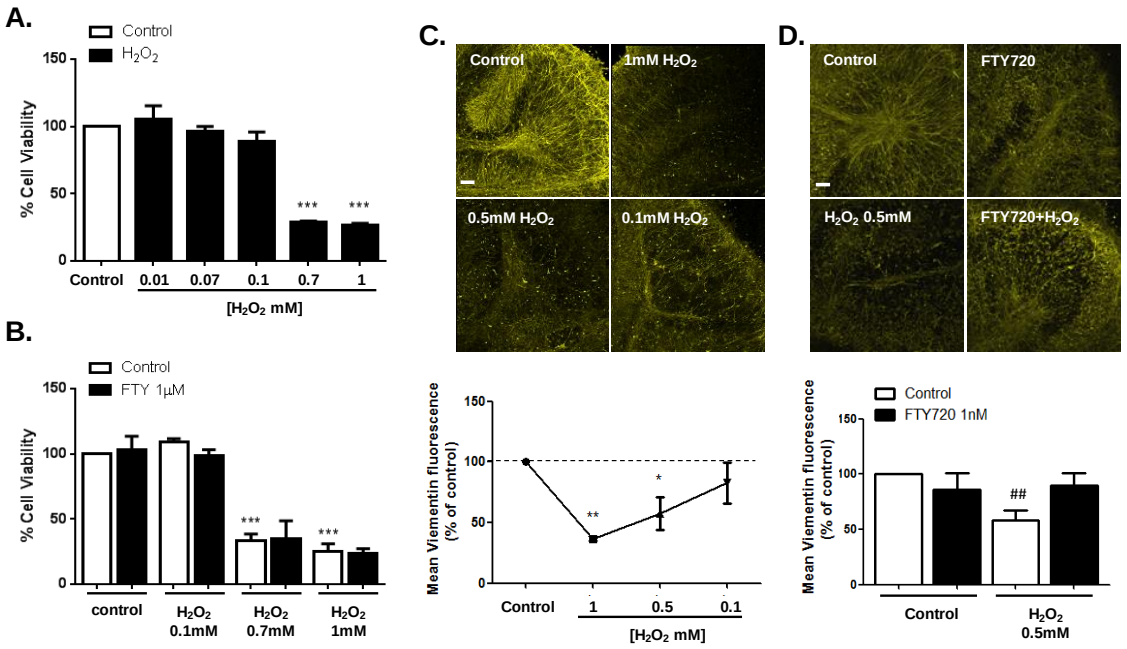


Figure 4.3: Bolus H_2O_2 induced astrocyte cell death is not attenuated with pFTY720

(A) MTT assay of human astrocytes treated with various concentrations of H_2O_2 as indicated on graph. Significant decrease in cell viability at 0.7mM. **(B)** Human astrocytes pre-treated with pFTY720 (1μM) does not attenuate astrocyte cell death induced by H_2O_2. **(C)** Organotypic cerebellar slices treated with H_2O_2 (0.1mM, 0.5mM and 1mM) for 18 hrs. Graph shows a significant decrease in vimentin fluorescence at 0.5mM compared to control. **(D)** Graph shows a significant decrease in vimentin fluorescence when treated with 0.5mM H_2O_2, which is not attenuated with pFTY720 (1nM) treatment as determined by one-way ANOVA. Graphs expressed as mean +/- SEM, t-test; ***p<0.001 **p<0.01 and *p<0.05. Images are representative of 4 - 5 separate experiments. Confocal images taken at 10x magnification. Scale bar, 100μm.

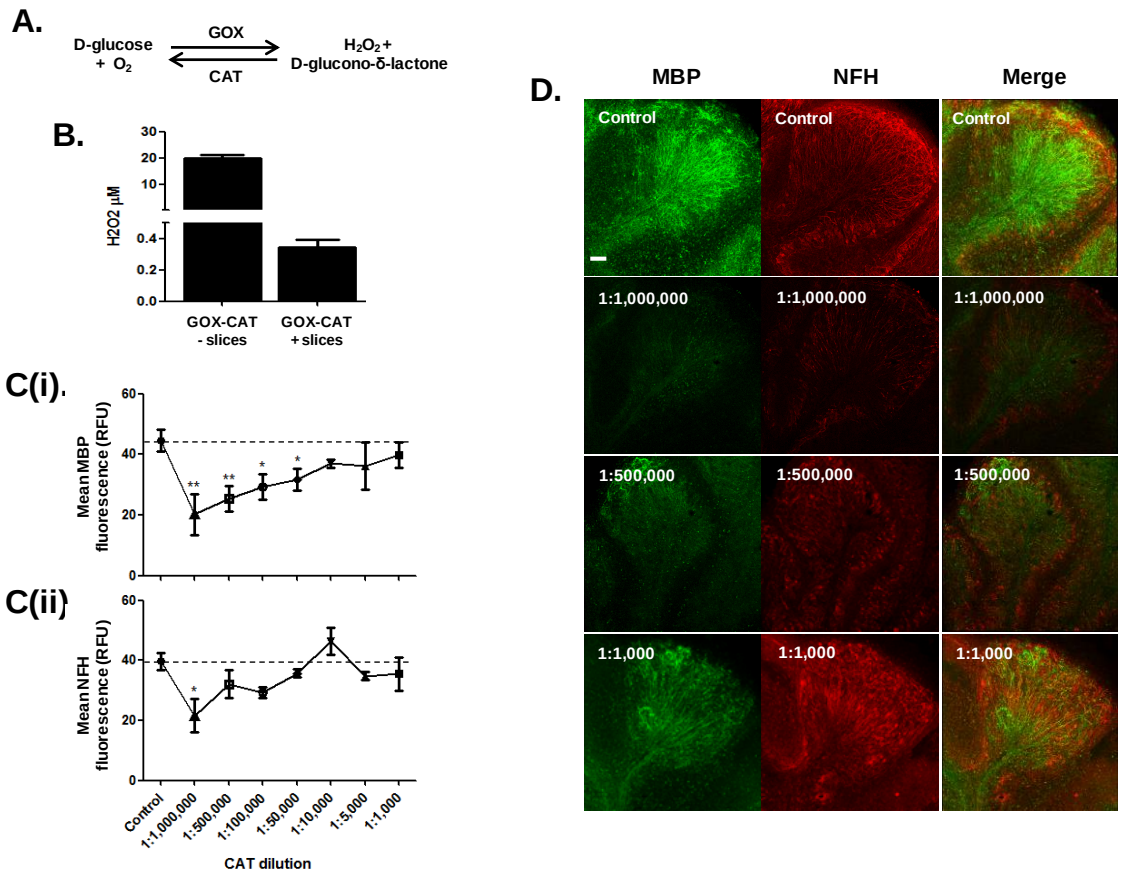


Figure 4.4: GOX-CAT induced demyelination in cerebellar slice cultures

(A) Schematic illustrating the formation of H_2O_2 in a GOX-CAT system. **(B)** GOX (1:100,000 dilution) and CAT (1:500,000 dilution) mediated H_2O_2 was measured in serum free slice media using amplex red and measured after 18hrs. Graph shows quantitative H_2O_2 measurement where approx. 20µM H_2O_2 is generated in serum free slice media compared to approx. 350nM when cultured with four cerebellar slices. All experiments carried out contained 4 slices per insert; n=3. **(C)** Organotypic cerebellar slices were serum starved for 4 hrs prior to addition of GOX and CAT. GOX (1:100,000 dilution) was maintained constant while the CAT dilution was varied as indicated on graph. As the CAT concentration decreases, a decrease in myelination is evident as measured by MBP fluorescence. **(D)** Images are representative of 3-5 separate experiments. Confocal images taken at 10x magnification. Scale bar, 100µm. **(C)** Graph shows a significant decrease in MBP fluorescence at CAT dilutions of 1:1,000,000, 1:500,000, 1:100,000 and 1:50,000 and a significant decrease in NFH fluorescence at a CAT dilution of 1:1,000,000. Graphs are expressed as the mean +/- SEM, t-test; **p<0.01 *p<0.05.

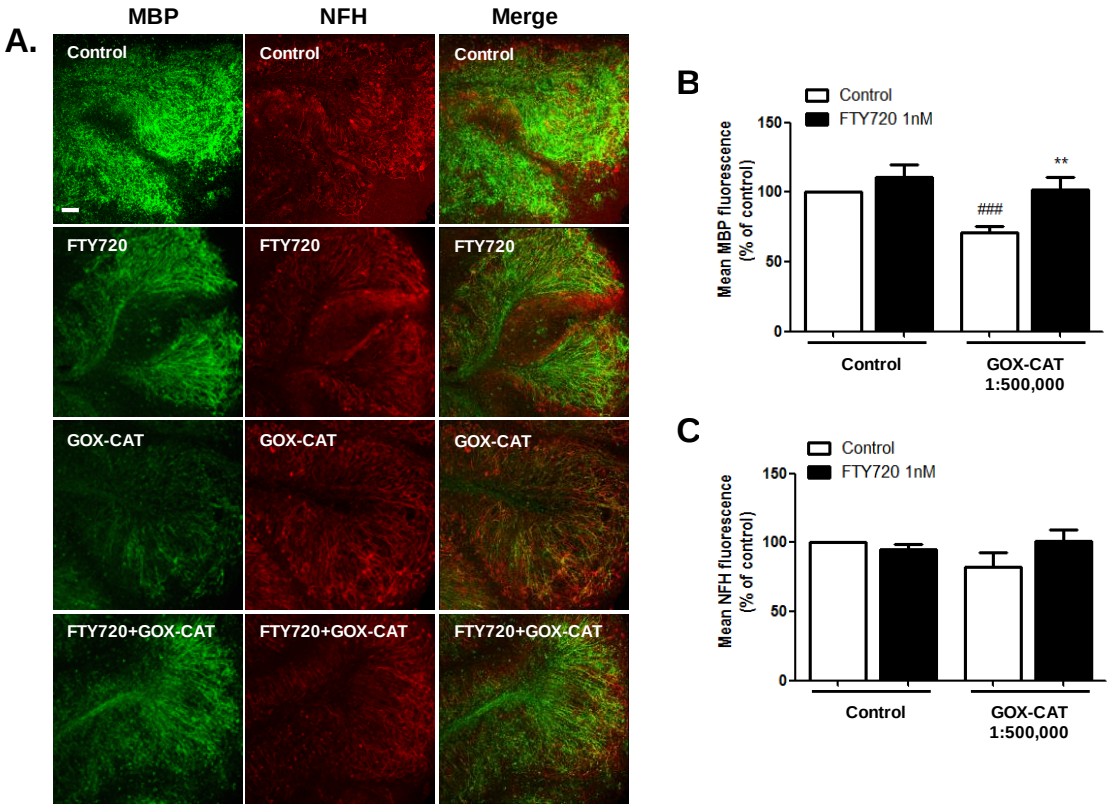


Figure 4.5: pFTY720 prevents GOX-CAT induced demyelination in cerebellar slice cultures

Organotypic cerebellar slices were serum starved for 4 hrs prior to pre-treatment with pFTY720 (1nM) for 1 hr. GOX-CAT enzymes for production of H_2O_2 were then added for 18hrs. GOX was used at a dilution of 1:100,000 and CAT was used at 1:500,000. **(A)** Confocal images are representative of 3-6 separate experiments. Images taken at 10x magnification. Scale bar, 100µm. **(B)** Graph shows a significant decrease in MBP fluorescence when treated with GOX-CAT. This effect is rescued with pFTY720 pre-treatment. **(C)** NFH shows no significant decrease with GOX-CAT treatment as determined by t-test. Graphs show means +/- SEM, t-test; $^{###}p<0.001$ and one way ANOVA with Tukey's post-hoc test $^{*}p<0.05$.

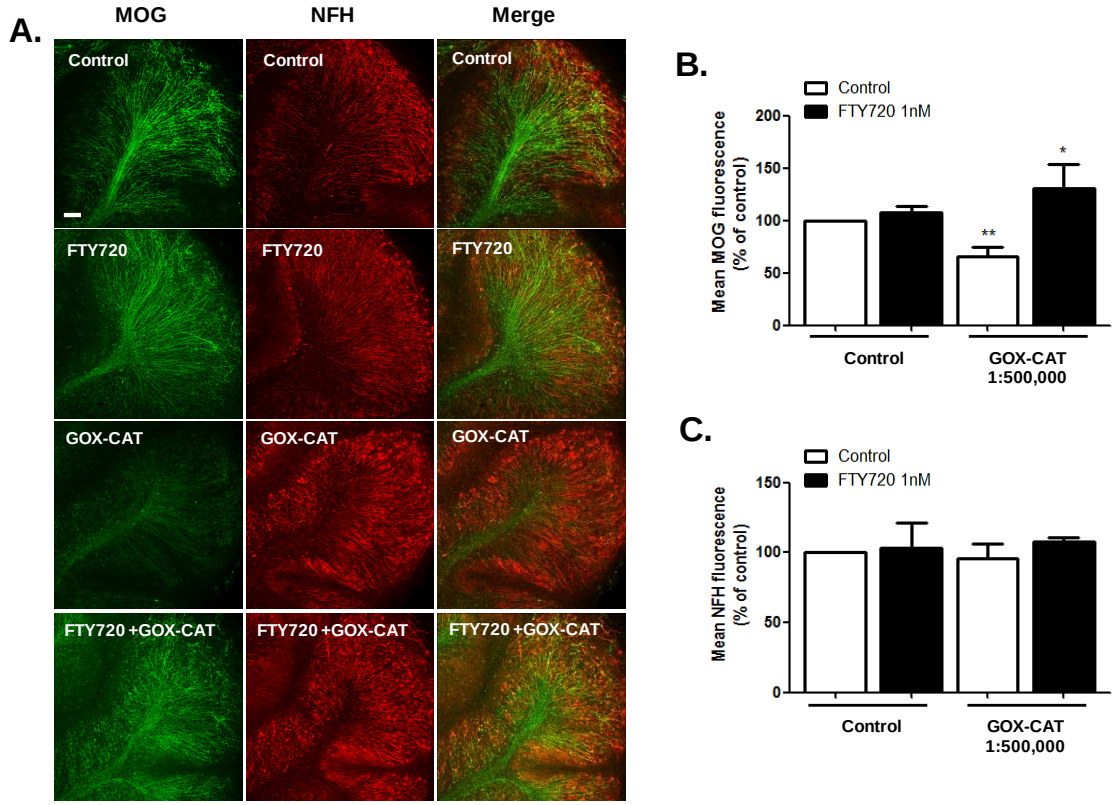


Figure 4.6: GOX-CAT induced reduction in MOG expression is attenuated with pFTY720

Organotypic cerebellar slices were serum starved for 4 hrs prior to pre-treatment with pFTY720 (1nM) for 1hr. GOX-CAT enzymes for production of H_2O_2 were then added for 18hrs. GOX was used at a dilution of 1:100,000 and CAT was used at 1:500,000. **(A)** Confocal images are representative of 5 separate experiments. Images taken at 10x magnification. Scale bar, 100µm. **(B)** Graph shows a significant decrease in MOG fluorescence when treated with GOX-CAT. This effect is rescued with pFTY720 pre-treatment. **(C)** NFH shows no significant decrease with GOX-CAT treatment as determined by t-test. Graphs show means +/- SEM, t-test; $^{\#\#}p<0.01$ and one way ANOVA with Tukey's post-hoc test $^{*}p<0.05$.

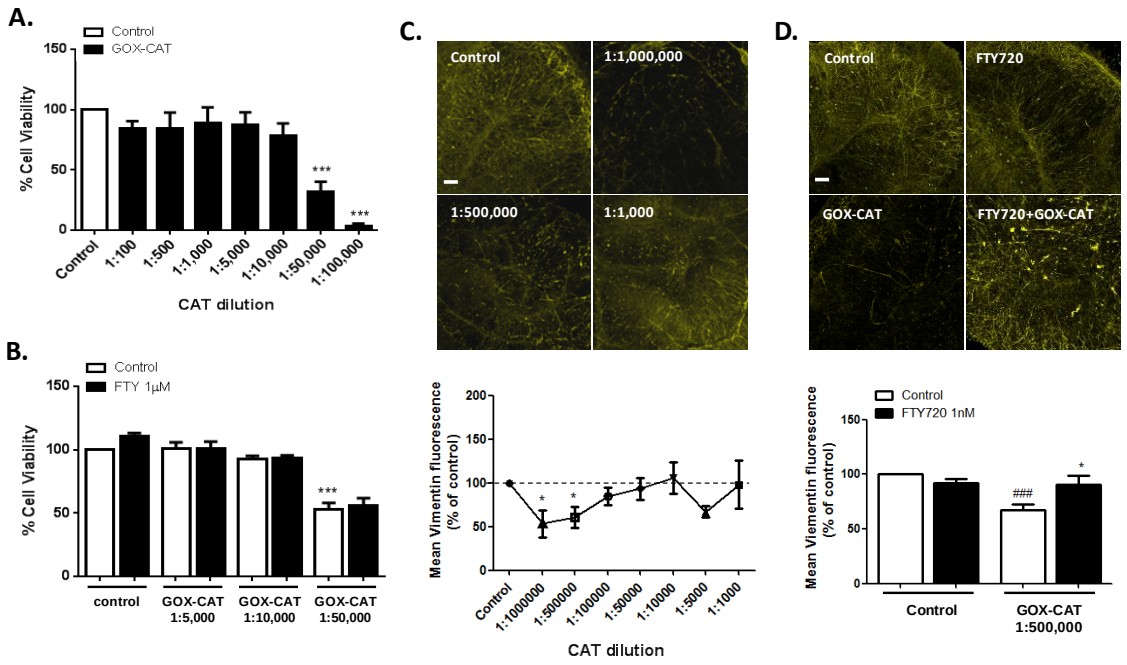


Figure 4.7: GOX-CAT induced decrease in vimentin is attenuated by pFTY720 in cerebellar slices

(A) MTT assay of human astrocytes treated with GOX 1:100,000 with CAT dilution indicated on graph. Significant decrease in cell viability at 1:50,000 CAT. **(B)** Human astrocytes pre-treated with pFTY720 (1μM) does not attenuate astrocyte cell death induced by GOX-CAT. **(C)** Organotypic cerebellar slices treated with GOX 1:100,000 dilution while the CAT concentration was varied as indicated on graph. Upon addition of GOX-CAT, an decrease in vimentin fluorescence is observed. Graph shows a significant decrease in vimentin fluorescence at CAT dilutions of 1:1,000,000 and 1:500,000 compared to control. **(D)** Shows vimentin fluorescence is significantly decreased when treated with GOX-CAT and this effect is attenuated by 1hr pre-treatment with pFTY720 (1nM). Graphs expressed as mean +/- SEM, t-test; ###p<0.001 and one way ANOVA with Tukey's post-hoc test *p<0.05. **(A and B)** Images are representative of 3 - 5 separate experiments. Confocal images taken at 10x magnification. Scale bar, 100μm.

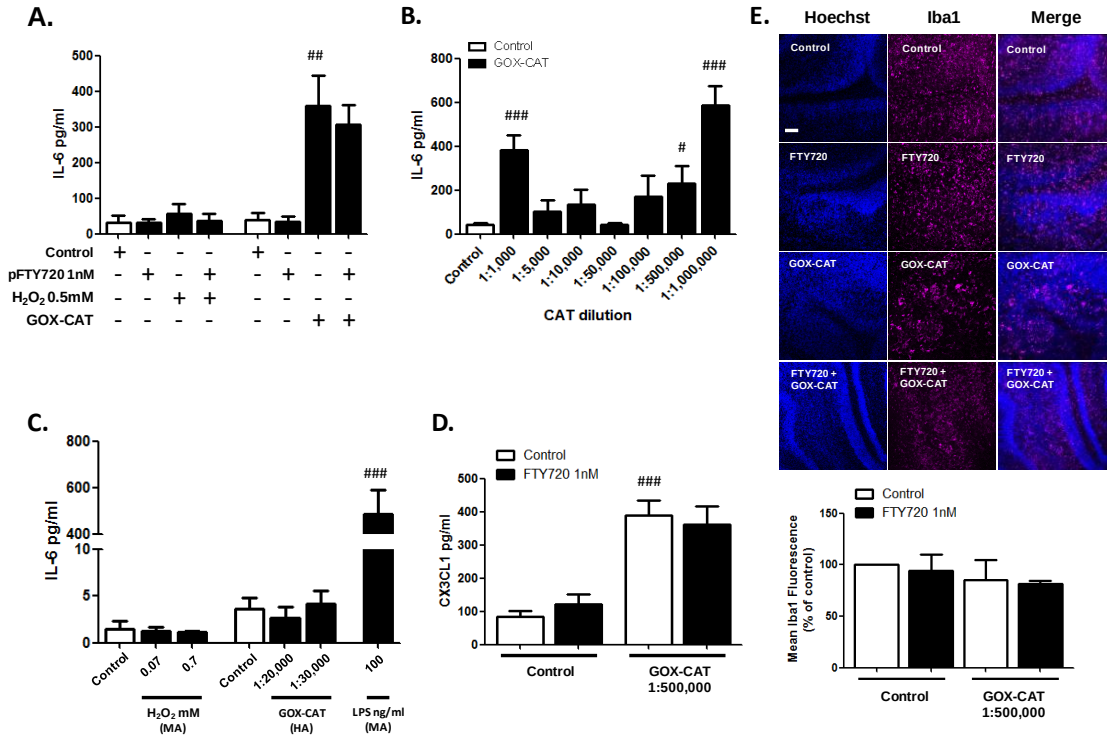


Figure 4.8: pFTY720 attenuates GOX-CAT induced demyelination independent of changes in microglial Iba1 and pro-inflammatory cytokines

Organotypic cerebellar slices were pre-treated with pFTY720 for 1 hr prior to addition of 0.5mM H_2O_2 or GOX-CAT (1:100,000-1:500,000) enzymes. **(A)** Graph indicates IL-6 levels, as measured by ELISA, from cerebellar slices treated with 0.5mM H_2O_2 and GOX-CAT (1:100,000-1:500,000). Significant increases in IL-6 are seen with GOX-CAT treatment which is not attenuated by 1nM pFTY720. **(B)** GOX-CAT (GOX 1:100,000 and CAT dilution indicated on graph) caused a bi-phasic concentration-dependent increase in the levels of IL-6. **(C)** Mouse (MA) and human astrocytes (HA) were serum starved for three hrs prior to treatment with H_2O_2 and GOX-CAT respectively for 18hrs. Graph shows mouse astrocytes treated with 0.07 and 0.7mM bolus H_2O_2 and human astrocytes treated with GOX (1:100,000) and CAT (1:20,000 or 1:30:000). Mouse astrocytes treated with LPS (100ng/ml) used as positive control. **(D)** Shows pFTY720 (1nM) does not attenuate soluble fractalkine (sCX3CL1) levels from slices treated with GOX-CAT (1:100,000-1:500,000). **(E)** Shows no change in microglial Iba1 fluorescence with GOX-CAT treatments. Confocal images taken at 10x magnification. Scale bar, 100µm. All graphs expressed as mean +/- SEM, t-test; $^{###}p<0.001$, $^{##}p<0.01$, $^{#}p<0.05$ and one way ANOVA with Tukey's post-hoc test; n=3-5.

3. Discussion

3.1 Summary of findings

The excessive generation of ROS, such as H_2O_2, is thought to be commonly associated with neurodegenerative conditions, for example, Alzheimer's disease (Milton, 2004, Wang et al., 2014b), multiple sclerosis (MS) (Lee et al., 2012a, Aydin et al., 2014) and post-ischemic injuries (Gruber et al., 2015). This accumulation of H_2O_2 leads to free radicals and in the case of iron-rich oligodendrocytes, causes extensive cell damage (Gruber et al., 2015). In this study we investigated the effects of short acting, bolus concentrations of H_2O_2 on myelination in cerebellar organotypic slice cultures, and for the first time also report the effects of low-continuous concentrations of H_2O_2 liberated by a GOX-CAT system. We demonstrate that GOX-CAT generates H_2O_2 at nanomolar concentrations and that both bolus H_2O_2 and GOX-CAT treatment induces demyelination, as determined by a decrease in MBP and/or MOG expression. Importantly, pFTY720 significantly attenuated this damage in both cases. In organotypic cerebellar slice cultures, both bolus H_2O_2 and GOX-CAT also decreased the expression of vimentin, where pFTY720 attenuated or to a large extent reduced these effects. In isolated astrocyte cultures, pFTY720 was unable to rescue decreased astrocyte cell viability induced by treatment with either bolus H_2O_2 or GOX-CAT. We also observed that treatment of cerebellar slices with GOX-CAT, while not causing changes in expression of the microglia marker Iba1, caused a bi-phasic concentration-dependent increase in the levels of IL-6. Notably, pFTY720 did not change the levels of Iba1 expression, nor did it attenuate the levels of IL-6 induced by GOX-CAT treatment. Together these results suggest that the GOX-CAT system can induce demyelination in organotypic slice cultures and shows that pFTY720 attenuates H_2O_2 induced demyelination. The findings also suggest these protective effects of pFTY720 may be independent of changes of Iba1 and the pro-inflammatory cytokine IL-6, but may involve limiting a decrease in the expression of the astrocyte marker vimentin.

3.2 Using the GOX-CAT system to induce demyelination

To induce oxidative stress *in vitro*, the amount of radicals in a system can be increased by direct addition of H_2O_2 or by inhibiting antioxidant defences, for example, using inhibitors of catalase (CAT) (Gille and Joenje, 1992). High concentrations of H_2O_2 added directly to the media, tends to result in a short term exposure of the initial high concentration (Gille and Joenje, 1992). This bolus H_2O_2 treatment has a short half-life, with estimations ranging from only 4-10 mins exposure in cell culture conditions (Dringen and Hamprecht, 1997, Mueller et al., 2009). The rate of H_2O_2 degradation also depends on the cell type, density and antioxidant capabilities (Gille and Joenje, 1992). In cell culture experiments, bolus concentrations of H_2O_2 ranging from 50μM to 1mM have been used extensively to study the harmful effects of H_2O_2. However, these high concentrations are in contrast to physiological levels, which have been reported to range from 10nM to 10μM (Mueller et al., 2009). In an attempt to establish a more physiologically relevant means of studying oxidative stress, reports have used a glucose oxidase-catalase (GOX-CAT) system. In this system the levels of H_2O_2 are governed by a ratio of the two enzymes GOX and CAT (Mueller et al., 2009), which can generate near constant levels of H_2O_2 for up to 24hrs (Sobotta et al., 2013). In our study, we utilised

the GOX-CAT system to develop a new model of H_2O_2-induced demyelination. We found that the enzymatic mixture of GOX and CAT in serum free slice media generated approximately 20μM H_2O_2 when there was no other source of potential peroxidases and, as expected, in the presence of cerebellar slices the levels of H_2O_2 were reduced to approximately 300-400nM. Importantly, our results show that the addition of GOX-CAT to cerebellar slices induced a loss of the key components of myelin, namely MBP and MOG, which was attenuated by treatment with pFTY720.

3.3 H_2O_2 induced astrocyte cell toxicity

In neurons, pFTY720 protects against damage from bolus H_2O_2 *in vitro*, through the blockade of astrocyte derived nitric oxide (Colombo et al., 2014) as well as directly decreasing levels of phosphorylated Foxo3a, a transcription factor involved in oxidative stress mediated neuronal cell death (Safarian et al., 2014). In vitro studies on endothelial and granulosa cells have also shown that activation of S1PRs attenuates bolus H_2O_2-induced apoptosis (Moriue et al., 2008, Nakahara et al., 2012). In contrast, the potential therapeutic effects of S1PR modulation, via pFTY720, on astrocyte cell death mediated by H_2O_2-induced oxidative stress, have thus far not been reported. Our data shows that dissociated astrocyte cultures undergo significant cell death when treated with bolus H_2O_2 or GOX-CAT as well as in organotypic slices, where both treatments decrease the expression levels of vimentin. While pFTY720 was unable to rescue bolus H_2O_2 or GOX-CAT induced astrocyte cell death in dissociated cell cultures, in the organotypic slice culture model, pFTY720 limited the decrease in vimentin caused by these treatments. These results may be likely explained by the toxic effects of H_2O_2 and the protective effects of pFTY720 being dependent on toxin concentration (i.e. high bolus H_2O_2 concentrations *versus* low continuous GOX-CAT generated H_2O_2) as well as cellular density (i.e. dissociated single cell cultures *versus* organotypic slice culture cellular networks). In agreement, we have shown previously that pFTY720 protects against psychosine induced astrocyte cell death in a manner that is dependent on psychosine concentration as well as astrocyte cell density (O'Sullivan and Dev, 2015).

3.4 pFTY720 attenuates demyelination in a cytokine dependent and independent manner

Studies have shown that pFTY720 attenuates increased levels of pro-inflammatory cytokines, such as IL-6, IL-1β and IL-17, in experimental autoimmune encepthalomyelitis (EAE) (Choi et al., 2011). In organotypic cerebellar slices, we show that induction of demyelination with MOG-reactive splenocytes can be attenuated via pFTY720, which is associated with a reduction in IFNγ and IL-6 cytokines (Pritchard et al., 2014). In addition, others and we observe that pFTY720 protects against demyelination induced by LPC, whereby enhanced levels of cytokines are reduced via S1PR modulation (Sheridan and Dev, 2012, Miron et al., 2010, Miron et al., 2008a, Miron et al., 2011). Of interest, demyelination induced by LPC appears to involve activated microglia, increased ROS, and raised levels of IL-6, TNFα and IL-1β (di Penta et al., 2013). In addition, it has been reported that pFTY720 can attenuate levels of cytokines in isolated microglial cell cultures stimulated with LPS (Noda et al., 2013). More recently, we have demonstrated that demyelination induced by

psychosine is attenuated by S1PR modulation in a manner that is independent of pro-inflammatory cytokines (O'Sullivan and Dev, 2015, O'Sullivan et al., 2016). Here, we examined further the inflammatory response associated with demyelination, and show that treatment of cerebellar slices with GOX-CAT induced an increase in the levels of IL-6 and fractalkine, without altering expression levels of the microglial marker Iba1, although we note that Iba1 is not necessarily a marker of microglia activation state. Notably, in these slice culture experiments, neither the levels of IL-6, fractalkine or Iba1 were altered by pFTY720. Given that we have shown previously that astrocytes are also capable of releasing pro-inflammatory cytokines such as IL-6 (Giralt et al., 2013, Elain et al., 2014, Pathipati et al., 2013), we also examined the effects of H_2O_2 on the levels of IL-6 in isolated astrocytes, with an aim to then examine the effects of pFTY720. In our hands, however, H_2O_2 did not increase the levels of IL-6 in either isolated mouse or human astrocytes, using either bolus H_2O_2 or GOX-CAT derived H_2O_2..

We also note that different concentrations of pFTY720 were used for dissociated cell culture experiments with astrocytes (1μM) and organotypic cerebellar slice cultures (1nM). Our lab has found in previous experiments with cerebellar slices, that lower concentrations of pFTY720 are effective at preventing demyelination and in promoting remyelination (O'Sullivan and Dev, 2015, Pritchard et al., 2014, Sheridan and Dev, 2012). This concentration of pFTY720 is also more therapeutically relevant, i.e. a dose that would reach the CNS in human MS patients as levels of FTY720 in rodent CSF are reportedly in the sub-nanomolar range (Miron et al., 2008b). Concentrations of pFTY720 used in dissociated cell culture experiments can range from 10nM-10μM (Miron et al., 2008b). Higher concentrations of pFTY720 may be used in order to study acute responses of pFTY720 and to show rapid internalisation of S1P receptors (Healy et al., 2013). In our experiments on astrocyte cell viability with pFTY720, we used both 1nM (Data not shown) and 1μM.

In conclusion, these results suggest that the GOX-CAT model may be useful in the studying the role of oxidative stress on myelination as well as examine the effects of novel therapies. They also suggest that cell toxicity and protection by pFTY720 are dependent on the cell densities used as well as the cell culture models. Most importantly, these findings suggest that differing demyelinating agents might regulate levels of cytokines by specific pathway(s), not all of which are sensitive to modulation by S1PRs, and that S1PRs may have capacity to rescue from demyelination in both inflammatory and non-inflammatory models.

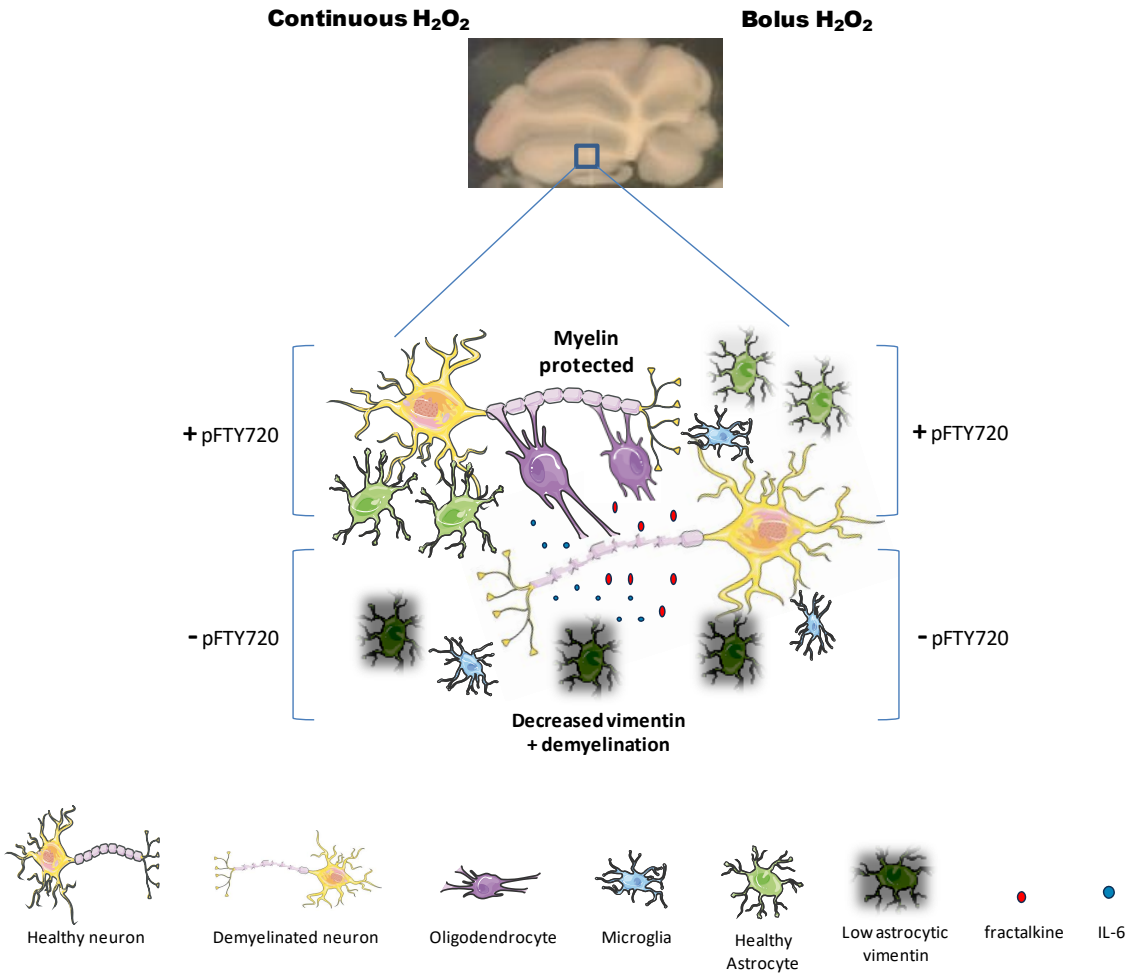


Figure 4.9: Demyelination induced by oxidative stress is regulated by sphingosine 1-phosphate receptors

pFTY720 attenuated demyelination and limited the decrease in vimentin fluorescence caused by low-continuous (GOX-CAT) and bolus H_2O_2. pFTY720 did not alter levels of IL-6 or fractalkine. Iba1 fluorescence levels remained unchanged with treatments.

Chapter 5:

The chemokine fractalkine (CX_3CL1) attenuates H_2O_2 induced demyelination in cerebellar slices

Chapter aims

The specific aims of this chapter were to:

- Investigate the effects of recombinant fractalkine (rCX3CL1) on bolus H_2O_2 and GOX-CAT-induced demyelination
- Explore any protective effects of rCX3CL1 on astrocytes in cerebellar slices under oxidative stress conditions
- To investigate if the protective of rCX3CL1 on oxidative stress induced demyelination was due to a decrease in the levels of IL-6 or changes to Iba1 expression in microglial cells within cerebellar slices

Abstract

Fractalkine/CX3CR1 signalling has been implicated in many neurodegenerative and neurological diseases of the central nervous system (CNS). This signalling pathway plays a important role in regulating reactive oxygen species (ROS), as well as itself being altered in conditions of oxidative stress. Here we investigated the effects of recombinant fractalkine (rCX3CL1) in models of H_2O_2-induced demyelination and astrocyte toxicity, within organotypic cerebellar slice cultures. We show here, for the first time, that rCX3CL1 significantly attenuated bolus H_2O_2 induced demyelination as measured by expression of MBP and also attenuated reduced vimentin expression. Using a GOX-CAT system to continuously generate low levels of H_2O_2 and induce demyelination, we observed similar protective effects of rCX3CL1 on MBP and MOG fluorescence, although in this model, the decrease in vimentin expression was not altered. Interestingly, we observed that bolus H_2O_2, induced demyelination was not associated with increased levels of IL-6. In contrast, GOX-CAT treatment increased the levels of IL-6, without any significant change in microglial Iba1 fluorescence. In all these cases, pre-treatment with rCX3CL1 neither altered levels of IL-6 or Iba1. Taken together this data highlights the protective effects of fractalkine signalling in oxidative stress related demyelination, in inflammatory as well as non-inflammatory conditions.

1. Introduction

Fractalkine and its receptor, CX3CR1, are constitutively expressed in the central nervous system. This is in contrast to most other chemokines, whose expression in the brain is only detected during inflammation (Harrison et al., 1998, Nishiyori et al., 1998, Pan et al., 1997). One of the unique features of fractalkine is its ability to exist as both a membrane tethered adhesion molecule and as a soluble chemotactic ligand (Harrison et al., 2001). In naive rodent brains, CX3CR1 has been localised to microglia (Harrison et al., 1998, Hughes et al., 2002) and shown to be expressed intracellularly by neurons (Hughes et al., 2002). Fractalkine (CX3CL1) is shown to be localised to neurons (Harrison et al., 1998, Hughes et al., 2002) and astrocytes (Hughes et al., 2002). It is thought that fractalkine expression in the brain is an important mediator of neuronal-glial communication. It is also thought to play a homeostatic role in normal physiological functions with the ability to regulate immune responses during times of injury (Tsou et al., 2001, Boddeke et al., 1999).

Several studies provide evidence of neurotoxic insults increasing the expression of fractalkine on neurons and astrocytes as well as increased microglial migration (Cardona et al., 2006, Cho et al., 2011, Garcia et al., 2013). In the rodent EAE model for MS an increase in astrocytic levels of fractalkine was observed at sites of inflammation, while neuronal fractalkine remained unchanged. Microglial CX3CR1 expression has also been reported to be increased around active demyelinating lesions (Sunnemark et al., 2005). Further experiments suggest that fractalkine plays a direct neuroprotective role in the regulation of inflammation under oxidative and ischemic conditions (Chen et al., 2013, Zujovic et al., 2001). Fractalkine has been shown to promote human monocyte survival through a reduction in intracellular ROS (White et al., 2014). However, studies also show that ROS such as H_2O_2 can enhance the expression of fractalkine and other adhesion molecules on endothelial cells, which may contribute to vascular injury (Chen et al., 2011). Thus, evidence suggests that fractalkine can be neuroprotective or neurotoxic depending on the cellular environment (Wu et al., 2015, Soriano et al., 2002, Cipriani et al., 2011).

Ischemic brain injury has been associated with the generation of excess ROS, which can lead to oxidative stress and in turn, neuronal damage (Radak et al., 2014). Increases in ROS production have also been shown to affect myelin producing oligodendrocytes. High concentrations of iron, polyunsaturated fatty acids combined with low levels of glutathione, make oligodendrocytes an ideal target for oxidative stress (Gruber et al., 2015). Decreasing the levels of ROS surrounding these oligodendrocytes has been shown to increase myelin production with a concurrent reduction in the anti-oxidative response elements Nrf2 and hemeoxygenase-1 (HO-1) (Gruber et al., 2015, Fernandez-Gamba et al., 2012). Importantly, neuronal fractalkine has been shown to directly activate the Nrf2 pathway in microglia (Lastres-Becker et al., 2014). Given the susceptibility of neuronal and glial cells to oxidative stress and the potential protective effects of fractalkine/CX3CR1 signalling, we investigated the effects of fractalkine, on H_2O_2 induced

demyelination, astrocytic vimentin expression, microglial Iba1 expression and levels of the pro-inflammatory cytokine IL-6, in organotypic cerebellar slice cultures.

2. Results

2.1 Fractalkine (CX3CL1) prevents bolus H_2O_2 induced demyelination in cerebellar slices

Oxidative stress is thought to play a role in aging as well as many neurodegenerative diseases (Beal, 2005). Fractalkine and CX3CR1 expression has been shown to be altered in demyelinating lesions associated with EAE (Sunnemark et al., 2005, Zhu et al., 2013). To determine whether the soluble fractalkine ligand (sCX3CL1) is protective of myelin, in an environment of oxidative stress, we pre-treated cerebellar slices for 1 hr with recombinant fractalkine (rCX3CL1; R&D systems; cat#458-MF) prior to addition of 0.5mM bolus H_2O_2. Our results show, after 18hrs of bolus H_2O_2 treatment there is a significant decrease in MBP fluorescence (59.6% +/-2.5%), which was significantly attenuated in groups pre-treated with rCX3CL1 (94.5% +/- 10.3%), (student t-test; $^{###}p<0.001$, one way ANOVA and Tukey's post-hoc test; $^{*}p<0.05$) (**Figure 5.1B(i)**). We also note a small decrease in NFH fluorescence in comparison to the control; however this was not found to be significant (81.7% +/- 14.6%) (**Figure 5.1B(ii)**). This data shows that rCX3CL1 may have protective effects on myelin state in the cerebellum, in an environment of oxidative stress.

2.2 Fractalkine (CX3CL1) prevents bolus H_2O_2 induced astrocyte cell death in cerebellar slice cultures

Fractalkine has been shown to have anti-apoptotic effects on human monocytes through a reduction in intracellular oxidative stress (White et al., 2014). In certain diseases, the up-regulation of fractalkine has been shown to help prevent microgliosis, through the activation of the Nrf2 transcription factor and up-regulation of heme-oxygenase 1 proteins (Lastres-Becker et al., 2014). Therefore, in addition to the positive effects of rCX3CL1 on myelination in an organotypic slice model of oxidative stress, we next examined the effect of 0.5mM bolus H_2O_2 on astrocytes. Vimentin fluorescence was significantly decreased through the addition of bolus H_2O_2 (74.2% +/- 4.0%) (**Figure 5.2A and B**). Importantly, pre-treatment with rCX3CL1 (10ng/ml), significantly prevented this loss in vimentin fluorescence (127.7% +/- 12.6%) (Student t-test; $^{##}p<0.01$ and one way ANOVA and Tukey's post-hoc test, $^{**}p<0.01$) (**Figure 5.2B**). These results show that rCX3CL1 displays a significant protective effect on astrocytes in cerebellar tissue when exposed to large concentrations of H_2O_2.

2.3 GOX-CAT induced demyelination is prevented by Fractalkine (CX3CL1) treatment

Administration of large bolus doses of H_2O_2 is now being challenged as an inaccurate model of oxidative stress, as it involves non-physiological H_2O_2 concentrations (Sobotta et al., 2013). Bolus H_2O_2 has also been shown to be metabolised within minutes in cell culture (Mueller, 2000). Alternatively, the glucose oxidase-catalase (GOX-CAT) system is thought to produce physiologically appropriate concentrations of H_2O_2, over a physiologically appropriate time (Mueller et al., 2009).

Therefore, differing biological outcomes may result from either large bolus or low continuous H_2O_2 delivery. Given we find that rCX3CL1 protects myelin and astrocytes from the effects of a large bolus dose of H_2O_2 (**Figure 5.1 and 5.2**), we next investigated if rCX3CL1 would have similar protective effects when using the GOX-CAT model. Cerebellar slices were treated with 100ng/ml, 10ng/ml or 1ng/ml of rCX3CL1 for 1 hr prior to treatment with GOX-CAT (GOX dilution 1:100,000, CAT dilution 1:500,000) for 18hrs. This GOX-CAT treatment caused significant demyelination (70.8 +/- 4.6%, student t-test, ###p<0.001; n=4) (**Figure 5.3B-D**), which was attenuated by pre-treatment for 1 hr with rCX3CL1 (100ng/ml) (105.4% +/- 9.0%, one way ANOVA and Tukey's post-hoc test, **p<0.01; n=4) (**Figure 5.3B**). These effects of rCX3CL1 were concentration-dependent, where pre-treatment with 10ng/ml (94.9% +/- 4.7%) and 1ng/ml of rCX3CL1 (95.8% +/- 7.8%) did not have a significant protective effect (**Figure 5.3C and D**). In the conditions tested, we observed no significant difference in NFH fluorescence (**Figure 5.3E-G**)**.** There were also no significant effects of rCX3CL1 treatment alone on MBP fluorescence, at any of the three concentrations tested, (100ng/ml, 105.8 +/- 9.2%; 10ng/ml, 127.3% +/- 27.9%; 1ng/ml, 112.0% +/- 18.3%) and NFH fluorescence (100ng/ml, 107.1% +/-12.7%; 10ng/ml, 112.2% +/- 11.2%; 1ng/ml, 98.9% +/- 13.9%) (**Figure 5.3E-G**).

2.4 GOX-CAT induced reduction in MOG is attenuated by fractalkine

In vitro studies on oxidative stress, have led to mixed reports as to the protective (White et al., 2014) or toxic effects (Chen et al., 2011, Xuan et al., 2012) of fractalkine. Like many other cytokines, fractalkine has been shown to be either anti-inflammatory (Zujovic et al., 2000) or neurotoxic (Mattison et al., 2013) depending on the circumstances. It has been suggested that the timing and concentration of fractalkine administration is of importance in determining the response of neurons and glia to potential neurodegeneration (Sheridan and Murphy, 2013). Given we find that soluble fractalkine attenuates GOX-CAT induced decrease in MBP (Figure 3), to further convince ourselves that GOX-CAT treatment is having a deleterious effect on myelin and that recombinant fractalkine (rCX3CL1) exerts a protective effect, we investigated the effects of GOX-CAT and rCX3CL1 on levels of MOG. We report, GOX-CAT treatment caused a significant decrease in MOG fluorescence compared to control (100% vs. 62.7% +/- 7.4%). Pre-treatment with rCX3CL1 (100ng/ml) for 1 hr, produced significant protective effects on MOG levels (110.7% +/- 11.9%), t-test; ###p<0.001, and one way ANOVA *p<0.05; n=5 (**Figure 5.4**). As before, we show that neither rCX3CL1 nor GOX-CAT significantly reduce NFH fluorescence. These finding suggest fractalkine can significantly attenuate the toxic effects of low-continuous H_2O_2 on myelin proteins.

2.5 Fractalkine does not protect astrocytes from low continuous H_2O_2 exposure

Many *in-vitro* studies on the effects of oxidative stress on astrocytes have used large bolus H_2O_2 concentrations in order to measure biological effects (Ito et al., 2015, Kim and Kwon, 2013, Dringen and Hamprecht, 1997). In this study, we have used the GOX-CAT system to generate low continuous

concentrations of H_2O_2 in order to induce demyelination in cerebellar slice cultures. In this model, fluorescence levels of the astrocyte marker vimentin, decreased significantly when exposed to GOX-CAT (GOX dilution 1:100,000, CAT dilution 1:500,000) for 18hrs (66.9% +/- 5.9%) (**Figure 5.5B and C**). Pre-treatment with rCX3CL1 for 1 hr at 100ng/ml (70.4% +/- 11.8%) (**Figure 5.5B**) and 10ng/ml (69.6% +/- 7.4%) (**Figure 5.5C**) (One-way ANOVA and Tukey's post-hoc test, n=4) were unable to significantly attenuate this effect of GOX-CAT treatment on astrocytes. This data shows that even though rCX3CL1 is able to significantly attenuate the bolus H_2O_2 induced decrease in vimentin fluorescence (see **Figure 5.2**); rCX3CL1 is unable to counteract the toxic effects of low continuous H_2O_2. These results may be explained by astrocytes displaying a higher vulnerability to continuous low-level concentrations of H_2O_2, in comparison to transient large bolus concentrations.

2.6 Protective effects of recombinant fractalkine on myelination is independent of IL-6 levels

H_2O_2 is not thought to be a potent inducer of pro- or anti-inflammatory cytokines (Pathipati et al., 2013). In vitro stimulation of primary microglial cells with bolus H_2O_2 does not cause significant IL-6 release. This is in contrast to GOX-CAT treatment, whereby a 2-fold increase in levels of IL-6 are seen in comparison to controls (Pathipati et al., 2013). Similarly, in these set of experiments, cerebellar slices were treated with 0.5mM bolus H_2O_2, which did not cause a significant increase in IL-6 levels (Control 16.6pg/ml +/- 8.7pg/ml vs. 0.5mM H_2O_2 73.8pg/ml +/- 34.4pg/ml, t-test; p=0.182). In contrast, when treated with low-continuous H_2O_2 (GOX-CAT) a significant increase in IL-6 is detected (control 47.8pg/ml +/- 8.6 pg/ml vs. 152.5 pg/ml +/- 23.9 pg/ml) Student t-test, $^{\#}p<0.05$; n=3-4 (**Figure 5.6A**). To investigate whether or not this increase in IL-6 was due to increased microglial fluorescence, which could infer microglial proliferation/activation, cerebellar slices were pre-treated for 1 hr with rCX3CL1 (100ng/ml) prior to addition of GOX-CAT and stained for Iba1. No significant difference in fluorescence between treatment groups was observed. A small increase in Iba1 fluorescence is noted in the rCX3CL1 treated control group, however this is not significant (**Figure 5.6B**).

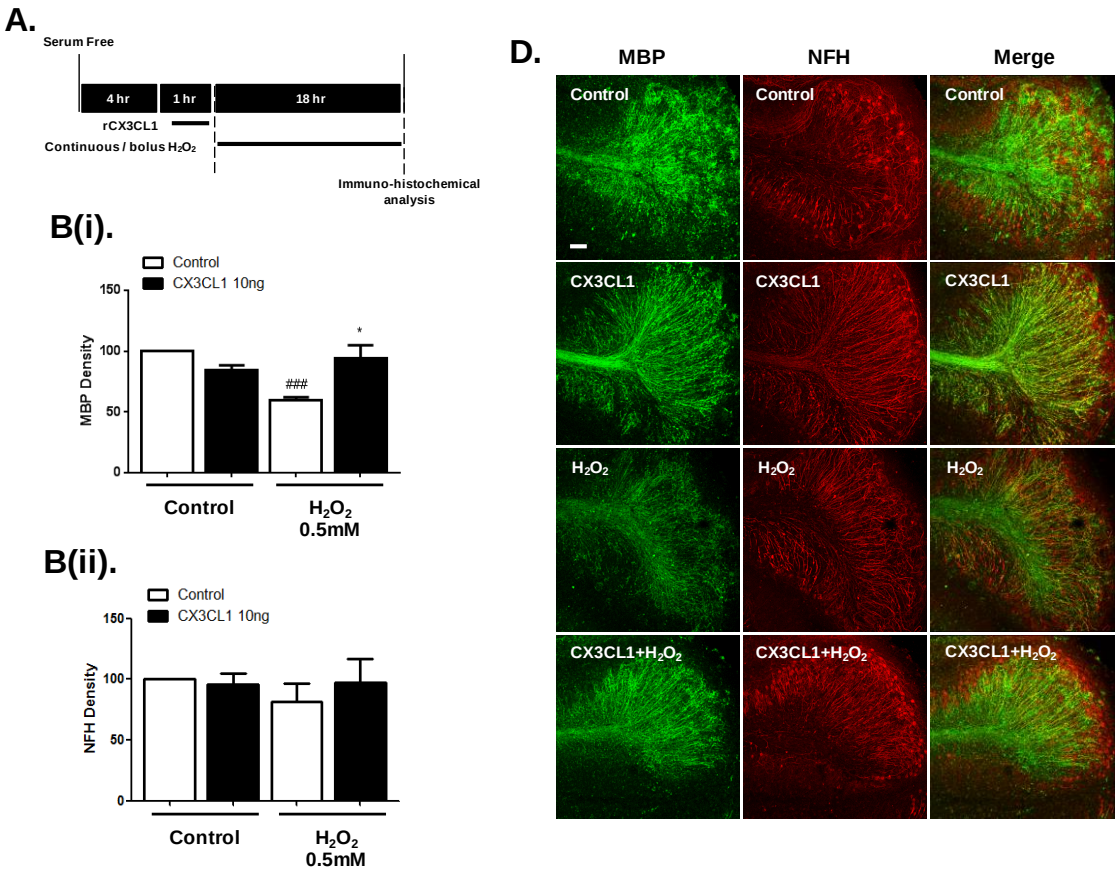


Figure 5.1: Fractalkine prevents bolus H_2O_2 induced demyelination in cerebellar slice cultures

(A) Experimental timeline shown. **(Bi)** Organotypic cerebellar slices were pre-treated with fractalkine (CX3CL1; 10ng) for 1 hr prior to addition of bolus H_2O_2 (0.5mM) for 18hrs. Graph shows a significant decrease in MBP fluorescence after H_2O_2 treatment .This effect is rescued with CX3CL1 treatment. **(Bii)** NFH shows no significant decrease with bolus H_2O_2 treatment compared to control. **(D)** Images are representative of 3 separate experiments. Confocal images taken at 10x magnification. Scale bar, 100 µm. Data expressed as mean +/- SEM, t-test; ###p<0.001, one-way ANOVA and Tukey's post-hoc test, *p<0.05.

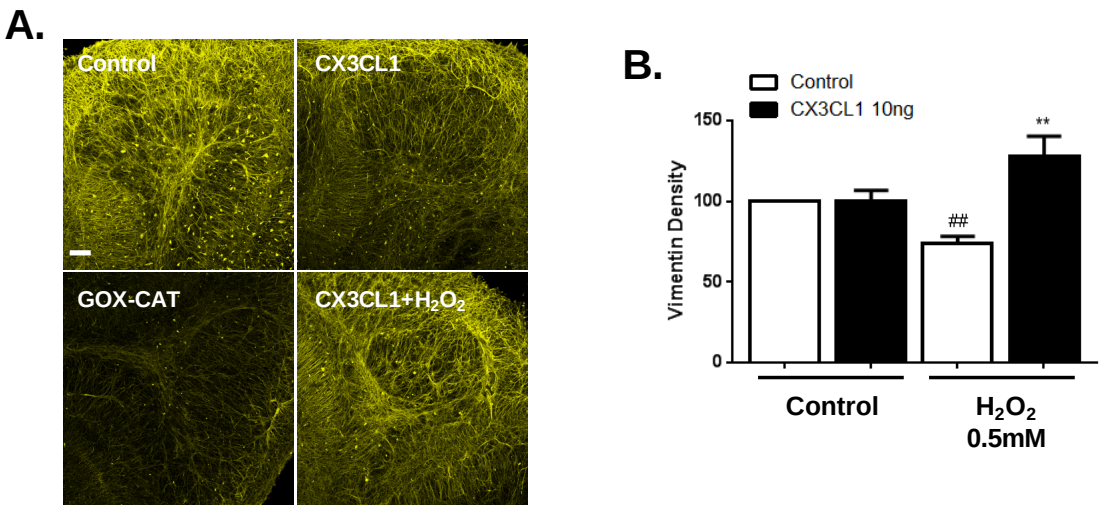


Figure 5.2: Fractalkine prevents bolus H_2O_2 induced astrocyte cell death in cerebellar slice cultures

Organotypic cerebellar slices were pre-treated with fractalkine (CX3CL1; 10ng/ml) for 1 hr prior to addition of bolus H_2O_2 (0.5mM) for 18hrs. **(A)** Images are representative of 3 separate experiments. Confocal images taken at 10x magnification. Scale bar, 100 µm. **(B)** Graph shows a significant decrease in vimentin fluorescence when treated with bolus H_2O_2. This effect is rescued when pre-treated with fractalkine. Data expressed as mean +/- SEM, student t-test; $^{\#\#}p<0.01$ and one way ANOVA and Tukey's post-hoc test, $^{**}p<0.01$.

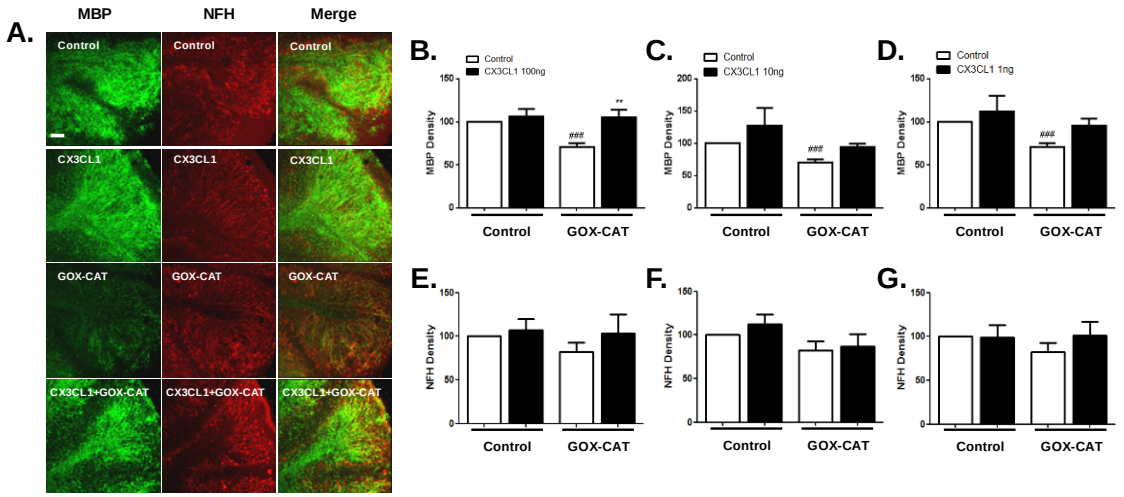


Figure 5.3: Fractalkine prevents oxidative stress induced demyelination in cerebellar slice cultures

Organotypic cerebellar slices were pre-treated with fractalkine (CX3CL1; 100ng/ml, 10ng/ml or 1ng/ml) for 1 hr, prior to treatment with glucose oxidase (GOX) and catalase (CAT). Concentrations were maintained constant at 1:100,000 and 1:500,000 respectively in order to generate low levels of H_2O_2 continuously. **(A)** Images are representative of fractalkine (CX3CL1) at 100ng/ml treatment group. Confocal images taken at 10x magnification. Scale bar, 100 µm. **(B)** Graph shows a significant decrease in MBP fluorescence with GOX-CAT treatment. This effect is rescued with fractalkine treatment at 100ng/ml but not at **(C)** 10ng/ml or **(D)** 1ng/ml. NFH shows no significant decrease in fluorescence with GOX-CAT treatment **(E-G)**. Graphs expressed as mean +/- SEM, n=4-6. Student t-test; ###p<0.001 and one-way ANOVA **p<0.01.

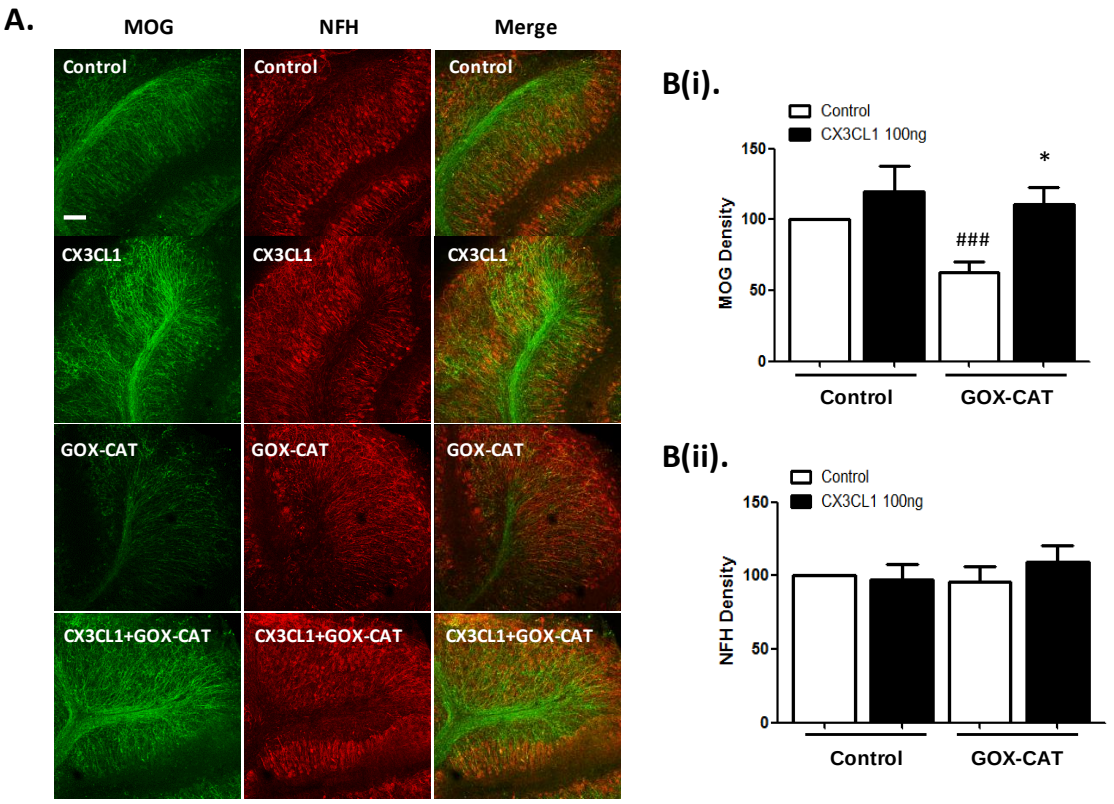


Figure 5.4: GOX-CAT induced reduction in MOG is attenuated by fractalkine

Organotypic cerebellar slices were pre-treated with fractalkine (CX3CL1; 100ng/ml) for 1 hr prior to addition of glucose oxidase (GOX; 1:100,000) and catalase (CAT; 1:500,000). **(A)** Images are representative of 5 separate experiments. Scale bar, 100µm. Confocal images taken at 10x magnification. **(Bi)** Graph shows a significant decrease in MOG fluorescence with GOX-CAT treatment. This effect is rescued with fractalkine treatment at 100ng/ml. **(Bii)** NFH shows no significant decrease in fluorescence with GOX-CAT treatment. Graphs expressed as mean +/- SEM, t-test; ###p<0.001, and one way ANOVA *p<0.05; n=5.

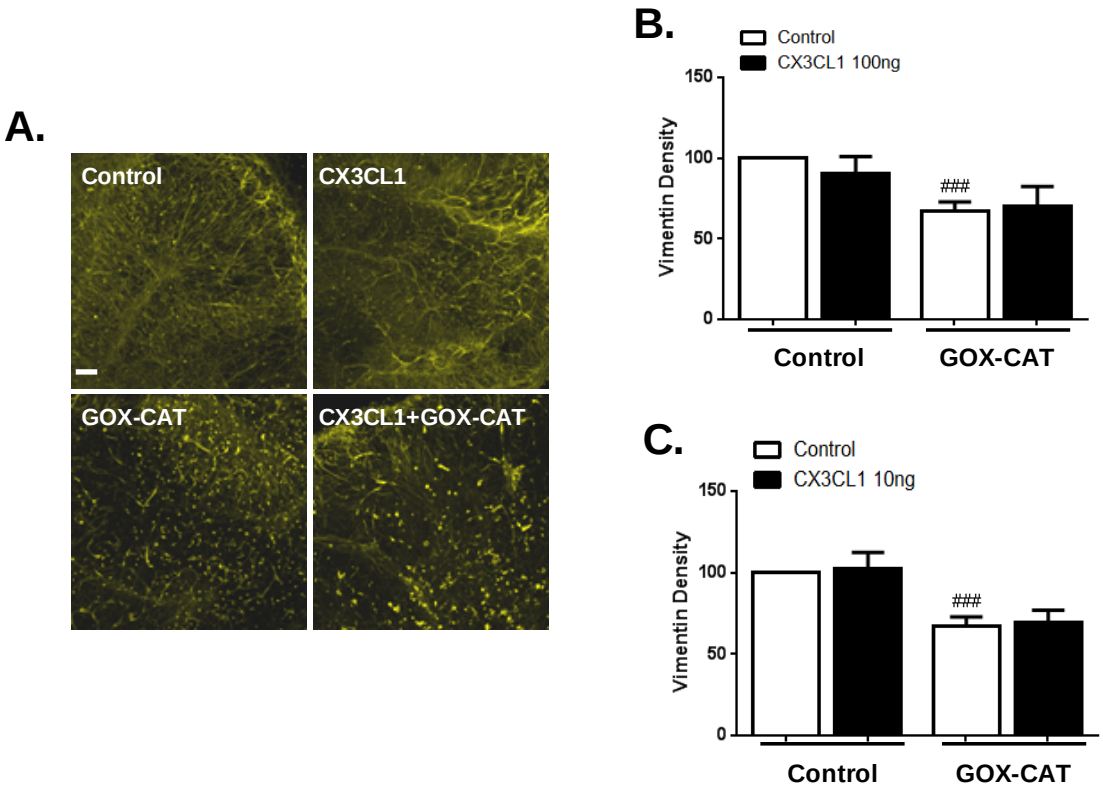


Figure 5.5: Fractalkine does not protect astrocytes from low continuous H_2O_2 exposure

Cerebellar slices were pre-treated with fractalkine (CX3CL1; 100ng/ml or 10ng/ml) for 1 hr prior to addition of glucose oxidase (GOX; 1:100,000) and catalase (CAT; 1:500,000) in order to generate low levels of H_2O_2 continuously **(A)** Images are representative of 4-6 separate experiments. Confocal images taken at 10x magnification. **(B and C)** Graph shows a significant decrease in Vimentin fluorescence when treated with GOX-CAT. Fractalkine treatment does not prevent this astrocyte cell death. Data expressed as mean +/- SEM, student t-test; $^{###}p<0.001$ and one way ANOVA with Tukey's post-hoc test.

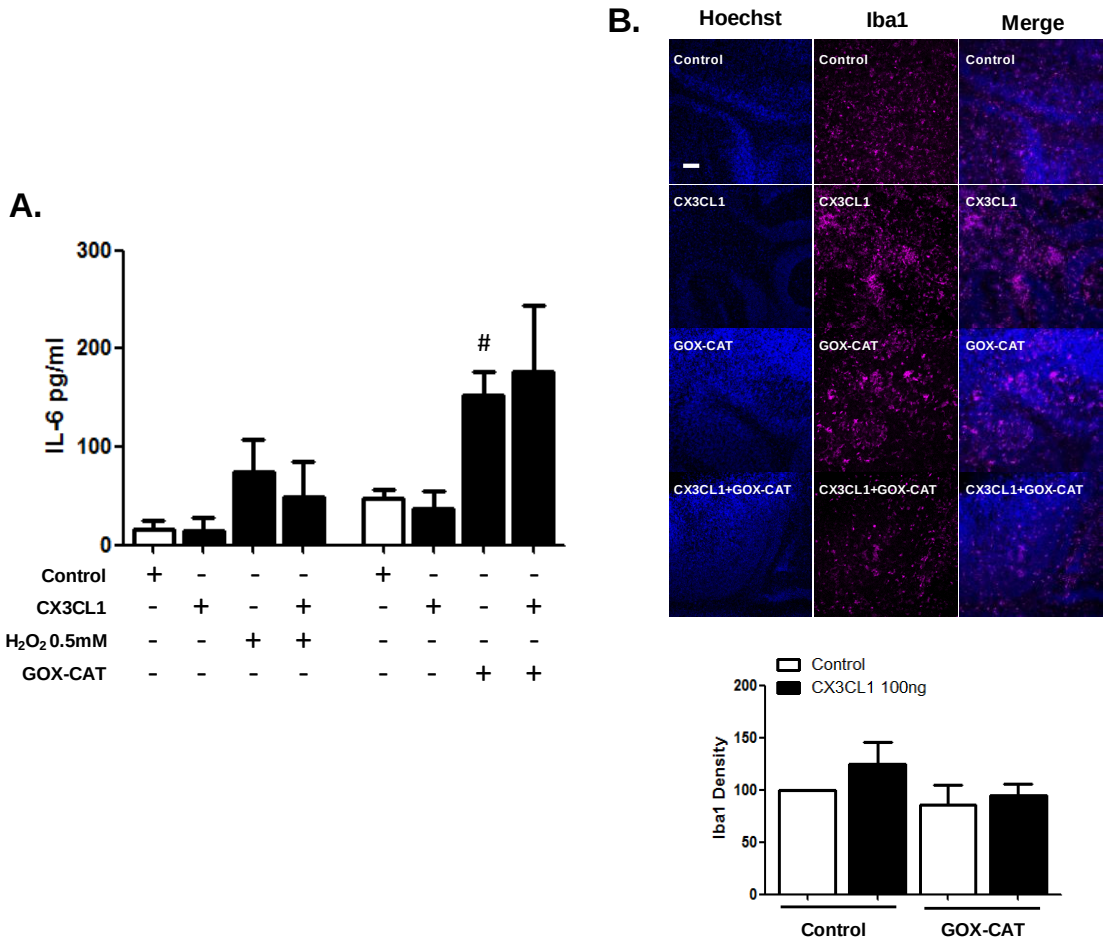


Figure 5.6: Protective effects of fractalkine on myelination are independent of IL-6 levels

(A) Graph indicates IL-6 release from cerebellar slices pre-treated with fractalkine (CX3CL1; 100ng/ml or 10ng/ml) for 1 hr followed by 0.5mM H_2O_2 or GOX-CAT (1:100,000-1:500,000). Cerebellar slices were then processed for immunohistochemistry and media used for ELISA. A significant increase in IL-6 is seen with GOX-CAT treatment, which is not attenuated by CX3CL1. **(B)** Shows no significant change in microglial Iba1 expression between treatments. Confocal images taken at 10x magnification. Scale bar, 100µm. Graphs expressed as mean +/- SEM, t-test, $^{\#}p<0.05$; n=3.

3. Discussion

High concentrations of H_2O_2 can cause oxidation of proteins and lipids resulting in cellular damage (Barbouti et al., 2002). To date, most studies have focused on bolus H_2O_2 treatments to study the toxic effects of H_2O_2 on isolated cells. Addition of GOX-CAT enzymes allows for the generation of low, continuous levels of H_2O_2 to be generated in vitro, which may better mimic the sustained rise in oxidative stress levels seen in neurological disorders (Denes et al., 2008, Wang et al., 2014a). Given that oxidative damage is associated with the initiation and progression of many neurological diseases, it is important to identify new cellular targets for preventing oxidative injury. Fractalkine is a chemokine, which can regulate neuronal and glial cell responses to oxidative stress (Zujovic et al., 2001, Lastres-Becker et al., 2014). Here, we investigated the effects of recombinant fractalkine (rCX3CL1) in cerebellar slices treated with bolus H_2O_2 or GOX-CAT mediated H_2O_2. Addition of fractalkine to cerebellar slices, prior to H_2O_2 induced oxidative stress, significantly attenuated the loss of myelin associated with H_2O_2 treatments. Unlike slices treated with bolus H_2O_2, GOX-CAT treatment showed a significant increase in levels of IL-6. These levels were not altered by rCX3CL1 treatment. The increase in IL-6 was independent of changes in microglial Iba1 fluorescent intensity. Turning our attention to astrocytes, fractalkine significantly attenuated bolus H_2O_2-induced decrease in vimentin fluorescence but had little-to-no protective effect over GOX-CAT treated slices. Overall these studies suggest that fractalkine may protect astrocytes against high levels of H_2O_2 insults but may be limited in its protective effects against low-sustained levels of H_2O_2. In addition fractalkine may regulate myelination state in both inflammatory and non-inflammatory conditions.

3.1 Protective effects of fractalkine on myelination

The involvement of fractalkine in regulating the oxidative stress response has been documented (Lastres-Becker et al., 2014, White et al., 2014). Administration of fractalkine prior to ischemic insult in wild type rodents produces neuroprotective effects, with smaller infarct sizes and a reduced mortality rate reported (Cipriani et al., 2011). In contrast, studies with $CX3CR1^{-/-}$ and $CX3CL1^{-/-}$ rodents following ischemic injury had a better outcome in comparison to their wild type counterparts, which suggests that fractalkine contributes to neurotoxicity (Cipriani et al., 2011, Soriano et al., 2002). This apparent contradiction highlights the complex signalling pathways evoked by fractalkine, suggesting it can be protective or toxic depending on the period of release and the microenvironment (Re and Przedborski, 2006). In our studies, we demonstrated H_2O_2 induced demyelination, as reflected by a decrease in the levels of MBP and MOG fluorescence, are attenuated by pre-treatment with fractalkine, prior to H_2O_2 insult. It should be noted that in our study we used the fractalkine chemokine domain and not full-length fractalkine, which includes the mucin stalk. Addition of recombinant fractalkine (rCX3CL1) prior to H_2O_2 insult may be an important factor for the protective effects observed. Contrasting effects of chemokine blockade during the initiation or progression phase of inflammation has been documented (Bruhl et al., 2004, Sheridan and Murphy, 2013). Therefore, rCX3CL1 may pre-condition microglia, increasing the efficiency of the antioxidative stress response (Iga et al., 2007).

3.2 Contrasting responses of astrocytes to bolus and GOX-CAT generated H_2O_2

Studies have suggested that different sub-populations of astrocytes in the brain can vary considerably in sensitivity to bolus H_2O_2. For example hippocampal astrocytes are sensitive to concentrations as low as 50μM, whereas cortical and cerebellar astrocytes are resistant to 500μM H_2O_2 (Desagher et al., 1996, Feeney et al., 2008, Ferrero-Gutierrez et al., 2008). Astrocytes may also vary in their sensitivity towards the method of H_2O_2 delivery where different or opposing biological outcomes may be elicited by cells in response to bolus or GOX-CAT H_2O_2 (Sobotta et al., 2013). Using organotypic cerebellar slice cultures, decreased fluorescent intensity of vimentin, an intermediate filament protein in astrocytes, is caused by both bolus and GOX-CAT generated H_2O_2. In contrast to GOX-CAT generated H_2O_2, rCX3CL1 significantly attenuated bolus H_2O_2 induced decrease in vimentin fluorescence. The protective effects of rCX3CL1 on bolus H_2O_2 insult may be due to the short half-life of H_2O_2 in cell culture (Dringen and Hamprecht, 1997, Mueller et al., 2009). The slow dissociation rate of fractalkine from its receptor also suggests that fractalkine signalling may outlast the effects of bolus H_2O_2 and thus contribute to its protective effects. Similarly, the lack of protective effects observed in GOX-CAT treated slices may be due to the half-life of rCX3CL1 (in comparison to GOX-CAT), which for chemokines is generally thought to be short (Beal, 2005, Lambeir et al., 2001, Greer et al., 1994). In addition, persistent H_2O_2 levels due to GOX-CAT activity may outlast any protective effects of rCX3CL1 due to the short signalling duration and half life of most chemokines. However, the exact half-life of fractalkine in cell culture is unknown. Thus, rCX3CL1 may be protective of astrocytes in conditions of short-lasting bolus H_2O_2 treatments, but not with GOX-CAT generated H_2O_2.

3.3 Protective effects of fractalkine are independent of IL-6

In primary dissociated microglial cultures, low levels of sustained H_2O_2 have been shown to cause activation of microglia, which promotes a pro-inflammatory response followed by an anti-inflammatory phenotype several hours later (Pathipati et al., 2013). This suggests that a pro-inflammatory, neurotoxic phase may precede a second anti-inflammatory and a neuroprotective phase. Given the important role IL-6 plays in inflammation and the differential levels of IL-6 seen in cerebellar slices treated with bolus versus continuous H2O2 we wanted to further investigate whether CX3CR1 modulation was regulating these levels of IL-6 in cerebellar slice cultures. In this study, we observed a significant increase in levels of IL-6 from GOX-CAT treated cerebellar slices, which were not attenuated, nor exacerbated, by pre-treatment with rCX3CL1. Notably, no significant increase in IL-6 was observed in bolus H_2O_2 treated groups. Increased levels of IL-6 from GOX-CAT may stem from an initial immune mediated response elicited by microglia or perhaps from microglia-astrocyte mediated cross-talk (Catalano et al., 2013). However, the presence of IL-6 in the media would suggest that rCX3CL1 is not decreasing levels of inflammation generated from GOX-CAT mediated oxidative stress. On the contrary, the presence of IL-6 may be an indicator of the regenerative process (Becker et al., 2004). Pro-inflammatory responses of IL-6 are mediated

through 'trans-signalling', whereby a specific protein, namely, gp130, present on cell surfaces responds only to a complex of IL-6 and soluble IL-6 receptor (sIL-6R) (Drucker et al., 2010). sIL-6R is generated through ADAM metalloprotease mediated cleavage (Chalaris et al., 2007). Regenerative or anti-inflammatory activities of IL-6 are mediated by 'classic signalling'. Here, IL-6 targets the few cell types which express membrane bound IL-6R, mainly hepatocytes, lymphocytes, neutrophils, macrophages and neurons, which leads to the activation of JAK/STAT pathways and the activation of target genes within the nucleus (Drucker et al., 2010, Scheller et al., 2011). Thus, traditionally, high levels of IL-6 are associated with harmful effects of inflammation, however, IL-6 may also have an anti-inflammatory effect, which helps regenerate tissue damage (Scheller et al., 2011).

3.4 Effects of GOX-CAT on microglial Iba1 expression

Iba1 is a calcium binding protein that is specifically expressed in microglia in the CNS (Ito et al., 2001). Studies show that Iba1 can play an important role in regulating microglial activation and enhanced Iba1 expression has been associated with microglial activation in the ischemic and oxidative stressed rodent brain (Ito et al., 2001, Matsumoto et al., 2008, Gerecke et al., 2013). However, in our study, no increase in Iba1 fluorescence was observed between treatments, which would suggest that microglia were not in an activated state. A caveat to this conclusion, however, is that we cannot rule out microglial activation based on these observations alone due to the complexity of the microglial phenotype. Therefore in order to characterise fully the microglial response, alternative markers of activation, such as arginase-1, or changes in microglial morphology should be considered.

In summary, we demonstrate that rCX3CL1 protects astrocytes against bolus H_2O_2 induced decrease in vimentin fluorescence, however these protective effects are not observed with GOX-CAT treated groups. We also show in our study that rCX3CL1 may protect against demyelination caused by pathological levels of H_2O_2 during oxidative stress conditions. Similar to pFTY720, rCX3CL1 exerts its protective effects independently of IL-6 levels.

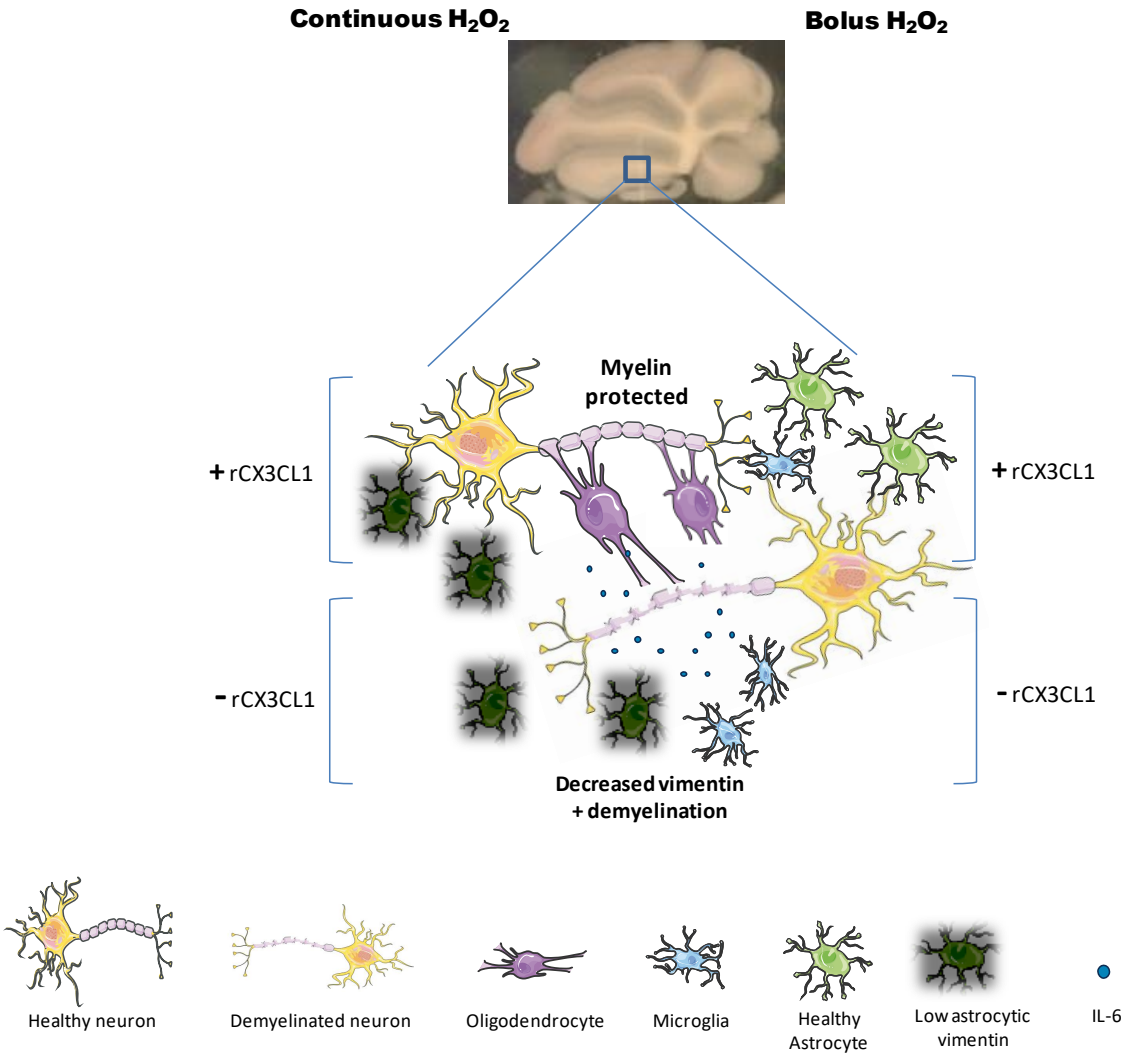


Figure 5.7: The chemokine fractalkine attenuates H_2O_2 induced demyelination in cerebellar slices

Fractalkine attenuated demyelination caused by low-continuous (GOX-CAT) and bolus H_2O_2. Fractalkine did not attenuate GOX-CAT induced decrease in vimentin fluorescence but did rescue bolus induced decrease in vimentin. Fractalkine did not alter levels of IL-6. Iba1 fluorescence levels remained unchanged with treatments.

Chapter 6:

Discussion

6.1. Study overview

The S1P/S1PR and fractalkine/CX3CR1 signalling systems serve as important communication links between neurons and glial cells (Paolicelli et al., 2011, Brinkmann, 2007). S1P and fractalkine signalling are known to participate in inflammatory and oxidative stress conditions through the recruitment of immune cells to the site of inflammation and up-regulation of anti-oxidant genes (Noda et al., 2011, Colombo et al., 2014, Lauro et al., 2015, Aarthi et al., 2011). Both of these signalling pathways have been associated with a wide range of pathological conditions (Sheridan and Murphy, 2013, Aarthi et al., 2011). The vital role of S1PR signalling in neurological and inflammatory disorders is already well recognised with regulatory approval of the S1PR modulator, FTY720, for the treatment of relapsing remitting MS (Brinkmann et al., 2010). Fractalkine/CX3CR1 signalling has received a great deal of attention since its discovery in the late 90's, presenting us with a novel neurotherapeutic target for such conditions as Alzheimer's, stroke and MS (Desforges et al., 2012). Here, we examined the effects of S1PR modulation and CX3CR1 activation in H_2O_2 induced demyelination. Furthermore we aimed to develop and validate a new H_2O_2 model of oxidative stress induced demyelination, in order to test the efficacy of pFTY720 and to put forth the fractalkine receptor (CX3CR1) as a new therapeutic target for demyelinating conditions.

Studies to date on the fractalkine ligand have mainly focused on neurons (Lauro et al., 2015), therefore we initially investigated fractalkine expression and regulation in human astrocytes. Here we confirmed that fractalkine is expressed in human astrocytes and is constitutively shed at low levels. On stimulation with pro-inflammatory cytokines such as IL-1β, TNFα and IFNγ, this low level of soluble fractalkine (sCX3CL1) was significantly up-regulated at both the protein and mRNA level. In addition, we demonstrated that the pro-inflammatory cytokine-mediated increase in sCX3CL1 likely involved a phosphorylation event mediated by p38 MAPK and the NF-κB pathway. Importantly, ADAM10 was found to be the main protease responsible for the cytokine-mediated shedding of sCX3CL1 from astrocytes (**Chapter 3 results**). As oxidative stress is commonly associated with inflammation in diseases of the CNS (Li et al., 2013), the effects of short acting, bolus H_2O_2 and low-continuous concentrations of H_2O_2 liberated by a GOX-CAT system on myelination was investigated in organotypic cerebellar slice cultures. We demonstrated both bolus H_2O_2 and GOX-CAT induced demyelination was significantly attenuated by pFTY720. Both of these H_2O_2 models, decreased the expression of vimentin, where pFTY720 attenuated or to a large extent reduced these effects. From these results, the protective effects of pFTY720 may be independent of changes in Iba1 and the pro-inflammatory cytokine IL-6 (**Chapter 4 results**). Finally, fractalkine/CX3CR1 signalling may be a potential target for therapeutic manipulation in diseases where oxidative stress contributes to demyelination. Fractalkine has previously been shown to regulate neuronal and glial cell responses to oxidative stress (Zujovic et al., 2001, Lastres-Becker et al., 2014). Focusing on our H_2O_2 models, the effects of recombinant fractalkine (rCX3CL1) on demyelination were investigated. We demonstrate that addition of fractalkine to cerebellar slices, prior to H_2O_2 induced oxidative stress, significantly attenuates the loss of myelin associated with

toxic levels of H_2O_2. Furthermore, unlike GOX-CAT, fractalkine significantly attenuated bolus H_2O_2 induced decrease in vimentin fluorescence. Similar to our previous results, we show that the protective effects of rCX3CL1 are independent of changes in microglial Iba1 expression as well as levels of IL-6 (**Chapter 5 results**).

6.2 Glial cells regulate oxidative stress and neuro-inflammation

6.2.1 Astrocytes

Astrocytes play an important role in defending the brain against oxidative stress such as H_2O_2 (Dringen and Hamprecht, 1997), utilising both glutathione peroxidise and catalase to counteract H_2O_2 toxicity (Dringen et al., 2005). Astrogliosis (reactive astrocytes), are often used as hallmarks of pathological diseases in the CNS (Muller et al., 2011). Reactive astrocytes have also been associated with increases in the cytoplasmic intermediate filaments, GFAP and vimentin (Kamphuis et al., 2015)**,** which are thought to play an important role in the astrocytic response to oxidative stress (de Pablo et al., 2013, Salminen et al., 2011). In vitro, rodent neurons appear to be more susceptible to oxidative stress damage than astrocytes and this is thought to be a result of neurons containing much lower levels of glutathione than astrocytes (Sagara et al., 1993, Noble et al., 1994, Desagher et al., 1996). It is therefore thought that glial cells and in particular, astrocytes, are more resistant to oxidative stress when compared to neurons. However, studies on hippocampal slices suggest astrocytes may be more sensitive to H_2O_2 toxicity than neighbouring neuronal and glial cells (Feeney et al., 2008). In agreement with this study, we demonstrate that H_2O_2 caused a significant decrease in vimentin fluorescence (but not in neuronal NFH fluorescence) in cerebellar slices, which could suggest a decrease in astrocyte reactivity or perhaps increased cell death. This highlights further, the idea that astrocytes in different brain regions may be differentially susceptible to H_2O_2 induced toxicity.

6.2.2 Microglia

Activation of microglia in neuroinflammatory conditions are known to be an important source of ROS, which can help to eliminate pathogens as well as regulate pro-inflammatory genes in an autocrine manner (Innamorato et al., 2009). Uncontrolled production of ROS through improper functioning of the Nrf2 pathway may cause microglia to remain in an activated state and exacerbate the inflammatory response (Innamorato et al., 2009). ROS producing microglia are considered key initiators of the demyelination process, with peroxynitrite production important for the initiation of phagocytosis (Ladeby et al., 2005). Here, we detected no increase in microglial Iba1 fluorescence after GOX-CAT induced H_2O_2 treatment, which may suggest microglia were not in an activated state and/or were not proliferating. In some cases damaged neurons can produce fractalkine and FGF-2, which suppresses microglial activation and promotes an anti-inflammatory and anti-oxidant microglial phenotype (Mizuno, 2014). Our results showed an increase in fractalkine upon GOX-CAT treatment, which may be released from neurons and altering microglial activation state.

6.2.3 Oligodendrocytes

The myelin sheath is responsible for rapid and efficient conduction of nerve signalling and also for maintaining and preserving axonal integrity (Barateiro et al., 2015). Oligodendrocytes have the highest rate of metabolic activity within the CNS (Perfeito et al., 2007). High concentrations of iron, polyunsaturated fatty acids combined with low levels of glutathione make oligodendrocytes an ideal target for oxidative stress (Gruber et al., 2015). During peak myelinating periods, the elevated production of myelin by oligodendrocytes may make these cells vulnerable to damage, resulting in the production of less myelin or myelin of a poor quality, thus contributing to demyelinating diseases. (Barateiro et al., 2015). Oligodendrocytes use a large amount of oxygen and ATP in order to maintain myelin production, which in turn can lead to the production of excess H_2O_2 if not removed efficiently (Thorburne and Juurlink, 1996). Large intracellular stores of iron, required for myelin production, can interact with H_2O_2 causing increased levels of hydroxyl radicals through the fenton reaction. Hydroxyl radicals are known to cause lipid peroxidation, impair protein functions and promote membrane damage (Brito et al., 2008). This is in agreement with our studies where H_2O_2 caused a significant decrease in myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG). MBP is one of the main constituents of myelin in the CNS and amounts to 30% of the total protein in myelin (Perfeito et al., 2007). In oligodendrocytes, excess H_2O_2 can be efficiently removed, with the ability to detoxify H_2O_2 improving as the cells mature (Dringen et al., 2005). Increases in ROS production have also been shown to affect genes expressing myelin in oligodendrocytes. Decreasing the levels of ROS surrounding these oligodendrocytes has been shown to increase myelin production with a concurrent reduction of the Nrf2 transcription factor and hemeoxygenase-1 (HO-1) protein (Gruber et al., 2015). As fractalkine is also known to regulate the Nrf2 pathway in microglia (Lastres-Becker et al., 2014) the protective effects we see from rCX3CL1, upon H_2O_2 induced demyelination may be attributed in part to fractalkine mediated upregulation of the Nrf2 transcription factor and its associated antioxidant proteins.

6.3 Induction of H_2O_2-mediated oxidative stress in cell culture

Previous studies have shown that organotypic cerebellar slice cultures are a more physiologically accurate way to study the complex multicellular organisation of the brain in comparison to isolated cell cultures (Birgbauer et al., 2004, Sheridan and Dev, 2012). Many studies on oxidative stress have been carried out on dissociated cell cultures in relation to cell viability and the unique adaptive responses of different cell types (Desagher et al., 1996, Dringen and Hamprecht, 1997, Ito et al., 2015). This thesis looks at the effects of oxidative stress on organotypic slice cultures and how cells are affected within a multi-cellular structure that has retained its original cytoarchitecture. Here, we focused on two different means of increasing the radical load through the addition of H_2O_2. Addition of a high concentration of diluted H_2O_2 directly to the media, results in short term exposure of the initial high concentration, to cells (Gille and Joenje, 1992). The concentration of H_2O_2 diminishes rapidly depending on the cell type, density and antioxidant capabilities (Gille and Joenje, 1992). H_2O_2 easily penetrates the cell membrane where it is detoxified by catalase. Studies suggest that in the presence of high bolus concentrations of H_2O_2, cell sensitivity is inversely

correlated with cellular catalase activity, whereas glutathione peroxidise may act as the dominant antioxidant against lower H_2O_2 concentrations (Engstrom et al., 1990). The deleterious effects of H_2O_2, such as lipid peroxidation and DNA damage, are thought to be caused indirectly through its ability to generate the radical hydroxide (OH^-) (Gille and Joenje, 1992). Addition of high bolus doses of H_2O_2 has been shown to cause cellular damage in a fast and transient manner. Bolus concentrations of H_2O_2 between 50 and 500μM are regularly used in cell culture experiments to study the harmful effects of H_2O_2. These high concentrations are in contrast to physiological concentrations which have been reported to range from 10nM to 10μM (Mueller et al., 2009). Bolus H_2O_2 also has a very short half life, with estimations ranging from 4-10 mins in dissociated cell culture conditions (Dringen and Hamprecht, 1997, Mueller et al., 2009). For chronic, continuous exposure, H_2O_2 can be generated enzymatically by glucose oxidase (GOX) and catalase (CAT). As it remains unclear to what extent bolus H_2O_2 treatment mimics the physiological response of cells, this GOX-CAT system is a novel approach to studying the effects of H_2O_2 in organotypic slice culture.

6.4 S1P receptors as targets for oxidative stress

Much evidence shows that oxidative stress can modulate S1P metabolism, which can ultimately affect the sphingolipid signalling balance between levels of pro-apoptotic ceramide and sphingosine and the pro-survival sphingosine 1-phosphate (S1P) (Pyne and Pyne, 2010, Neubauer and Pitson, 2013). There is evidence to suggest that oxidative stress causes apoptosis through sphingomyelinase mediated production of ceramide (Barth et al., 2012, Hernandez et al., 2000). High concentrations of ROS degrades SphK1 thereby reducing its activity (Maceyka et al., 2007, Pchejetski et al., 2007). Importantly, mild levels of oxidative stress have been shown to activate SphK1, thereby shifting sphingolipid metabolism in favour of the pro-survival S1P (Van Brocklyn and Williams, 2012). Reports also suggest that exogenous S1P can actively reduce levels of intracellular ROS and attenuate apoptosis in dissociated cells (Pyszko and Strosznajder, 2014a). Support for the anti-apoptotic effects of exogenous S1P have also been shown in PC12 neurons subjected to serum starvation and cortical neurons exposed to amyloid beta (Aβ) (Edsall et al., 1997). Interestingly, it appears the pro-survival effects of S1P depend critically on its transient generation. If S1P is prevented from being broken down, for example with a deficit in S1P-lyase, S1P initiates apoptosis (van Echten-Deckert et al., 2014, Hagen et al., 2009). Notably, S1P derived from SphK2 has been shown to have pro-apoptotic effects (Hagen et al., 2009). However, apoptotic effects of SphK2 may be more complicated as evidence suggests SphK2 induced apoptosis can be either direct or receptor mediated (Liu et al., 2003). Oxidative stress induced apoptosis has been reported to be mediated though activation of the JNK signalling pathway. Oxidative stress induced activation of JNK causes the inhibition of the anti-apoptotic protein Bcl-2 and causes the upregulation of pro-apoptotic proteins such as Bax and Bak (Sinha et al., 2013). Together with the knowledge that exogenous S1P inhibits JNK (Lee et al., 2012b) and cytochrome-c release from mitochondria, S1PRs are possible targets for regulating oxidative stress induced apoptosis. Alternative evidence also suggests that (**Figure 6.1**).

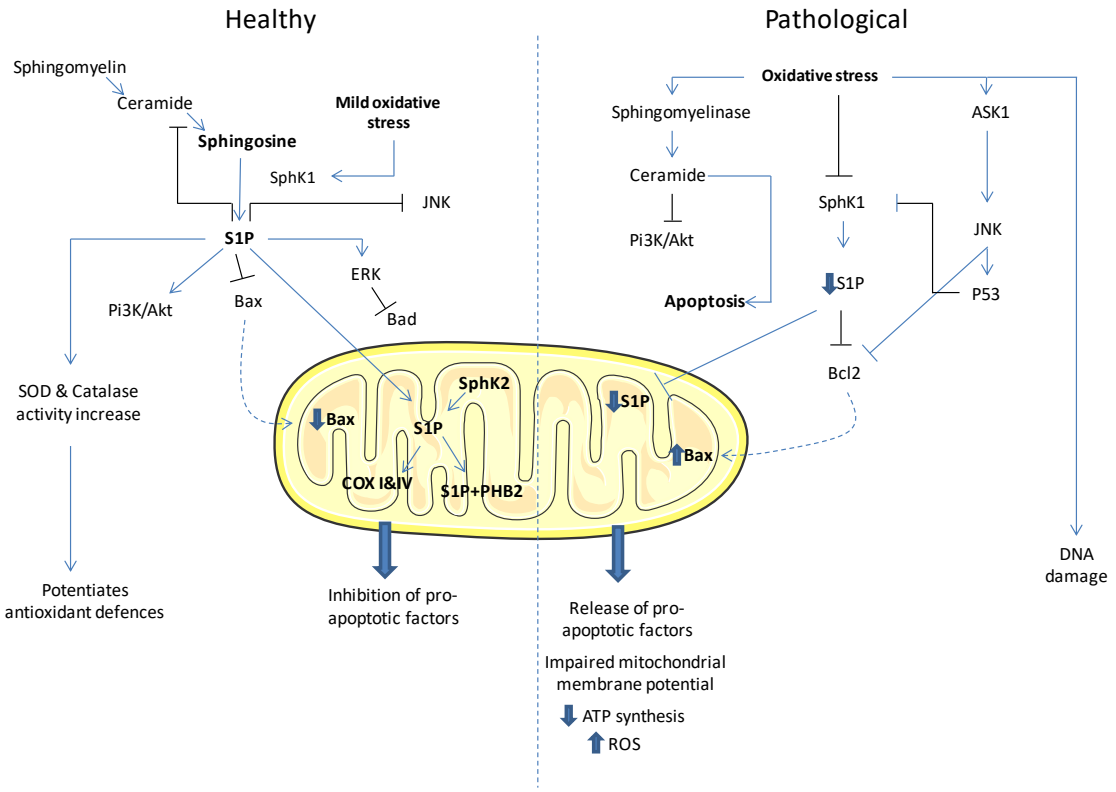


Figure 6.1: Sphingolipid signalling homeostasis

Simplified schematic of non receptor mediated S1P formation and signalling events, which can influence mitochondrial function in normal and oxidative stress conditions. ASK1, apoptosis signal-regulating kinase 1; CytC, cytochrome C; S1P, sphingosine 1-phosphate; SphK, sphingosine kinase; SOD, superoxide dismutase

6.5 Fractalkine structure and functional significance

In this thesis, we used the chemokine domain of fractalkine and not full-length fractalkine, which includes the mucin stalk. Even though there is no known signalling domain within this glycosylated mucin stalk, which led to the earlier conclusion that the mucin stalk functioned only as a spacer (Mizoue et al., 2001), more recent reports suggest that full-length fractalkine elicits a more potent anti-apoptotic effect (White et al., 2014). It is possible that the presence of the mucin stalk region allows for a more stable receptor-ligand conformation resulting in more potent signalling. The only other reported membrane spanning chemokine to contain a mucin-stalk, which tethers a chemokine domain, is CXCL16 (Matloubian et al., 2000). CXCL16 has been shown to exert differential effects depending on whether or not the stalk is present (Petit et al., 2008). Therefore, examination of the effects of full-length fractalkine on demyelination and vimentin expression may be of further interest.

The functional importance of the glycosylated stalk is unknown as it has not been shown to contribute to fractalkine/CX3CR1 signalling interactions (Harrison et al., 2001). The fractalkine chemokine domain, similar to that of other chemokines, (Hoover et al., 2000, Mizoue et al., 1999) has been shown to be globular in structure while the mucin domain projects as a long stalk (Fong et al., 2000). This data suggests that the role of the stalk region may be to extend the chemokine domain out from the cell towards the CX3CR1 expressing cells (Fong et al., 2000, Ostuni et al., 2014). Fractalkine has a relatively slow dissociation rate from CX3CR1, which may contribute to its adhesion properties (Fong et al., 2002, Haskell et al., 2000). As a signalling molecule, the chemokine domain alone (as well as varying lengths of the attached mucin stalk) has been shown to induce changes in levels of intracellular calcium, ERK and Akt signalling as well as mechanisms involved in migration (Bazan et al., 1997, Deiva et al., 2004, Limatola et al., 2005). It has also been proposed that soluble fractalkine does not activate integrins (Haskell et al., 1999). Thus, it is possible that membrane bound fractalkine activation of CX3CR1 mediates cellular adhesion, in the absence of G protein activation. In contrast, the cleaved molecule activates G protein-mediated signalling (Felouzis et al., 2016). The higher affinity of fractalkine for CX3CR1, in comparison to most other chemokine/receptor pairs, may also contribute to the protective effects of fractalkine (Combadiere et al., 1998, Fong et al., 2000, Haskell et al., 2000, Imai et al., 1997).

6.6 Fractalkine shedding by the ADAMs family

Metalloproteases, such as ADAM10 and ADAM17, are known to enzymatically cleave fractalkine from its membrane tethered form (Garton et al., 2001, Tsou et al., 2001, Chapman et al., 2000a). The ability to regulate soluble and membrane bound fractalkine may also be cell type specific as our studies revealed ADAM10 as the primary metalloprotease responsible for inducible cleavage of fractalkine from human astrocytes. This is in contrast to neuronal studies where ADAM17 is shown to be the main protease responsible for inducible cleavage of fractalkine (Cook et al., 2010). These findings add to the complexity of the regulation of fractalkine signalling in the CNS. Hence, astrocyte

derived soluble fractalkine may serve a different function to neuronal derived soluble fractalkine, which may serve to regulate individual cellular responses to the environment. From our studies on human astrocytes we report that P38 MAPK is involved in the regulation of fractalkine shedding, as P38 inhibition significantly attenuates levels of soluble fractalkine. In general, the family of p38 MAPKs play an important role in cytokine production and the cellular stress response (Baan et al., 2006, Mittelstadt et al., 2005). To date,, four isoforms of p38 MAPK have been identified, namely p38α, β, γ and δ (Baan et al., 2006, Freshney et al., 1994). This family of kinases are activated in response to many cellular stress responses including osmotic shock, growth factors, UV light, LPS and inflammatory cytokines (Freshney et al., 1994, Han et al., 1994, Lee et al., 1994, Raingeaud et al., 1995, Rouse et al., 1994). Activation of p38 MAPKs can be caused by MKK3 (MAPK kinase-3) and MKK6 (MAPK kinase-6) induced phosphorylation. Many transcription factors such as NF-κB are known to be direct targets of p38 leading to p38 MAPK manipulation of gene transcription (Baan et al., 2006, Mittelstadt et al., 2005). p38 MAPKs can also play a major role in apoptosis and cytoskeletal reorganisation. P38 MAPKs have been implicated in sepsis, ischemic heart disease, arthritis, HIV and Alzheimer's disease (Kelkar et al., 2005, Lee and Dominguez, 2005). Previous studies have linked P38 signalling to the fractalkine shedding process (Garton et al., 2001, Fan et al., 2003). P38 has also been suggested to regulate activity of ADAM17 through interaction with the cytoplasmic domain (Killock and Ivetic, 2010). Therefore, it is possible that P38 MAPK may interact with the cytoplasmic tail of ADAM10, causing activation or enhancing its enzymatic abilities in human astrocytes.

ADAM10 has more than 40 substrates that belong to three different classes of membrane anchored proteins (Endres and Fahrenholz, 2010). The amyloid precursor protein is probably the most well known of these substrates. ADAM10 cleaves proteins that are involved in migration (N-cadherin) (Kohutek et al., 2009), proliferation (CXCL16) (Gough et al., 2004), cell signalling (Notch) (Tian et al., 2008) and immune system regulation (low affinity immunoglobulin E receptor) (Lemieux et al., 2007). Most of the effects produced from ADAM10's cleavage activities can be associated with the substrates N-terminal ectodomains, which are released into the extracellular space. However, studies have shown that the intracellular domains of the substrates can also elicit effects. For example, following cleavage of the Notch receptor, its C-terminus acts a transcription factor and translocates to the nucleus (Schroeter et al., 1998, Bozkulak and Weinmaster, 2009). This implies that shedding of the fractalkine ligand from astrocytes may not only elicit effects on CX3CR1 expressing cells, but that the remaining C-terminus may modulate functions within the astrocyte itself. As fractalkine has been shown to regulate its own production through its G protein-coupled receptor in peripheral smooth muscle cells (Chandrasekar et al., 2003), one possibility is that this auto-regulation may be mediated through its cytoplasmic tail. If this intracellular signalling cascade is somehow disrupted in pathological conditions, this could lead to the dysregulation of the fractalkine ligand and aberrant inflammatory signals.

6.7 Expression pattern of fractalkine and CX3CR1 in the CNS

Even though we did not characterise the full expression pattern of fractalkine and its receptor, many reports provide evidence of their expression throughout the CNS. The fractalkine ligand has been shown to be primarily expressed on neurons (Harrison et al., 1998, Hughes et al., 2002) and astrocytes (Hughes et al., 2002). Studies have reported CX3CR1 expression on neurons, microglia and astrocytes in the rodent brain (Harrison et al., 1998, Jiang et al., 1998, Maciejewski-Lenoir et al., 1999, Meucci et al., 1998). However, later studies suggest CX3CR1 protein is not expressed on astrocytes (Hughes et al., 2002). Interestingly, in contrast to microglia, astrocytes were shown to have no migratory responses to fractalkine (Lauro et al., 2006). However, fractalkine has been shown to stimulate calcium mobilisation within rat astrocytes (Maciejewski-Lenoir et al., 1999). CX3CR1 protein has been shown to be present in healthy adult human astrocytes (Hulshof et al., 2003). It is noteworthy however, that in vitro studies on human fetal astrocytes showed no CX3CR1 mRNA expression (Hatori et al., 2002). This is in line with our findings where no CX3CR1 mRNA was detected in control or cytokine treated human fetal astrocytes (data not shown). Several groups have demonstrated that rodent neurons express CX3CR1 (Lauro et al., 2006, Meucci et al., 2000, Hughes et al., 2002). CX3CR1 mRNA and moderate CX3CR1 protein immune-reactivity has also been shown to be expressed in adult human neurons (Hatori et al., 2002, Hulshof et al., 2003). Only one study has been found with regards to the expression on oligodendrocytes in which they report the CX3CR1 was not expressed on cells with “typical oligodendrocyte morphology” (Hughes et al., 2002).

6.8 Protective effects of pFTY720 in models of demyelination

Previously, our lab has demonstrated the demyelinating effects of LPC (Sheridan and Dev, 2012) and MOG reactive splenocytes (Pritchard et al., 2014). Both of these demyelinating agents have an inflammatory component associated with their pathology (**Figure 6.2**). The protective effects of pFTY720 have been partly attributed to its ability to attenuate pro-inflammatory cytokine release. More recently we have also demonstrated the demyelinating capabilities of psychosine (O'Sullivan and Dev, 2015). Psychosine is a toxic metabolite that builds up in the brain which is caused by a mutation in the lysosomal enzyme galactosylceramidase (GALC) (O'Sullivan and Dev, 2015). Cerebellar slices treated with psychosine however, do not cause the release of the pro-inflammatory cytokines IL-1β, TNFα and IL-6. Nevertheless, pFTY720 has been shown to attenuate demyelination and the neuronal damage associated with this toxic metabolite, through the pleiotropic effects of pFTY720 on neuronal and glial S1PRs (O'Sullivan and Dev, 2015) (**Figure 6.1**). In the present set of experiments we demonstrate that high bolus H_2O_2 concentrations and low-continuous H_2O_2 can also lead to demyelination in organotypic cerebellar slice cultures. Similar to psychosine, pFTY720 attenuates H_2O_2 induced demyelination independently of the presence of IL-6 and CX3CL1 pro-inflammatory cytokines. The exact protective mechanism of S1PR modulation on myelination remains unknown. However, studies show following LPC induced demyelination, pFTY720 increased the number and promoted process extension of oligodendrocyte progenitor cells (OPC’s), with the remyelination phase of pFTY720 thought to be mediated through S1PR3 and

S1PR5 (Miron et al., 2010). FTY720 has also been shown to increase the number of phagocytosing microglia, while promoting the secretion of microglial and astrocytic growth factors and attenuating pro-inflammatory cytokines (Noda et al., 2013). Functional antagonism of S1PR1 has been shown to play a vital role in FTY720 mediated efficacy in EAE (Choi et al., 2011). FTY720 has also been shown to exert direct protection of neurons by increasing growth factors such as BDNF (Doi et al., 2013, Fukumoto et al., 2014).

6.9 Neuroprotective effects of membrane tethered and soluble fractalkine equilibrium

The effects of CX3CR1 modulation in myelination has not been looked at thus far. We demonstrate that addition of recombinant fractalkine in cerebellar slices attenuates H_2O_2 induced demyelination. We observe that the protective effects of fractalkine may be independent of pro-inflammatory cytokines (**Figure 6.2**). The protective effects of fractalkine on myelination in cerebellar slices may however be partly attributed to the regulation of CX3CR1 on microglia. Studies have shown that exogenous fractalkine reduces microglial mediated neurotoxicity, by suppressing microglial activation in a rodent model of Parkinson's disease (Pabon et al., 2011). Fractalkine also modulates microglial mediated neurotoxicity through the activation of the Nrf2 transcription factor and the up-regulation of antioxidant proteins (Lastres-Becker et al., 2014). Fractalkine secreted from damaged or dying neurons promotes microglial phagocytosis without inducing nitric oxide (NO) or pro-inflammatory cytokines in order to maintain healthy neuronal connections, while simultaneously up-regulating the antioxidant enzyme hemeoxygenase-1 (HO-1) (Noda et al., 2011). Direct neuroprotective effects have also been attributed to the equilibrium between membrane tethered and soluble fractalkine (Harrison et al., 1998), therefore regulating the cleavage of fractalkine may have important physiological impacts on cell survival. In the rodent model for MS, MOG-induced experimental autoimmune encephalomyelitis (EAE) shows an accumulation of CX3CR1 expressing microglia in and around active demyelinating lesions. Interestingly, neuronal mRNA levels are reportedly unchanged whereas an increase in astrocytic fractalkine was reported to be attracting microglia to the sites of inflammation (Sunnemark et al., 2005). Even though mRNA levels of fractalkine were measured and not the protein levels, it is possible that astrocytic fractalkine may have been up-regulated and shed from the cell surface in order to promote microglial chemotaxis, which would coincide with the up-regulation of CX3CR1 on microglia. This cell specific regulation of fractalkine may be possible due to the different ADAM proteases that are used for cleaving neuronal (ADAM17) versus astrocyte fractalkine (ADAM10). It is therefore possible that fractalkine/CX3CR1 signalling may be dysregulated in EAE/MS as evidenced from the studies mentioned. Fractalkine receptor antagonism at a specific stage of the disease may be a viable therapeutic target. Furthermore, therapeutic effects of Tecfidera (dimethyl fumerate/BG-12), the marketed drug for relapsing remitting MS (RR-MS), is thought to modulate the Nrf2 pathway, thus strengthening the therapeutic potential of fractalkine/CX3CR1 mediated Nrf2 modulation in diseases such as MS.

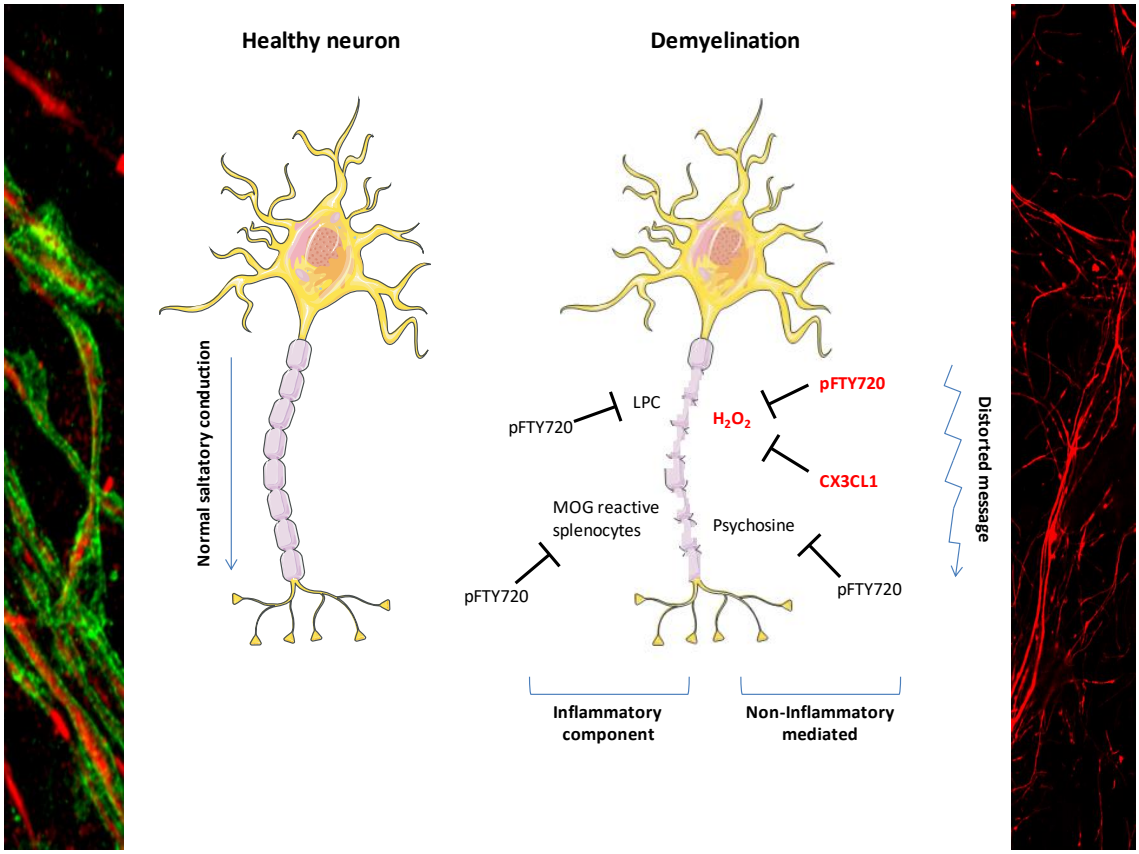


Figure 6.2: Overview of demyelinating agents and the protective effects of pFTY720 and fractalkine

pFTY720 attenuates LPC-, and MOG reactive splenocyte-induced demyelination in part through a reduction in pro-inflammatory cytokines. pFTY720 and fractalkine also attenuated H_2O_2- and psychosine-induced demyelination independently of pro-inflammatory cytokines. Confocal image left hand side shows axons (NFH; red) surrounded by myelin (MBP; green), control conditions; 63x magnification. Confocal image on right hand side shows demyelinated axons (NFH; red), bolus H_2O_2 treated; 40x magnification.

6.10 Therapeutic potential of fractalkine/CX3CR1 modulation

The role of fractalkine in several CNS disorders has already been recognised (Arli et al., 2013, Liu et al., 2014, Stuart and Baune, 2014, Lopez-Lopez et al., 2014). Attempts are being made to study the possible effects of fractalkine inhibition as well as CX3CR1 agonists and antagonists in the CNS through siRNA and knockout studies in rodents (Mattison et al., 2013, Bertollini et al., 2006, Cardona et al., 2006, Denes et al., 2008, Lesnik et al., 2003). Studies published, show the potential for fractalkine/CX3CR1 signalling as a major target for treatment of CNS disorders. However, no clinical trials have been initiated thus far. One of the fundamental reasons as to the difficulty in developing chemokine based therapies is due to the many regulatory functions that chemokines possess. Problems and unexpected results may arise when moving from rodent to human studies, as all studies to date on the therapeutic effects of fractalkine have been reported in animals. Human and mouse chemokine families have been shown to exhibit differences in gene clusters and therefore a similar species difference may also be true with fractalkine, which needs to be taken into consideration when translating animal findings to human experiments (Zlotnik et al., 2006, Nomiyama et al., 1999). Experiments using blocking antibodies or peptides may also be more advantageous than genetic knockout experiments as this would allow for more controlled timing of agonist/antagonistic effects. This is particularly important for chemokine modulation as reports suggest chemokines can be both beneficial and harmful depending on the timing and concentration at which they were administered (Bruhl et al., 2004, Cipriani et al., 2011).

Chemokines actively participate in the development of inflammatory and oxidative stress related diseases, with modulation of their receptors attracting considerable attention from pharmaceutical companies (Mladic et al., 2015). There is particular interest on the development of antagonist molecules for CX3CR1, which may prove useful in peripheral inflammatory diseases (Milligan et al., 2004). CX3CR1 agonists should also be investigated, as fractalkine has been shown to control microglial activation (Lyons et al., 2009) and neuronal survival (Limatola et al., 2005). However, while peripheral fractalkine may play the more conventional inflammatory chemokine role, its role in the CNS may also include more homeostatic functions. Thus, particular care will have to be taken in relation to fractalkine and its effects on the CNS. CX3CR1 signalling has also been reported to promote atherosclerosis (Cybulsky and Hegele, 2003), therefore potential therapeutic applications of fractalkine/CX3CR1 modulation may need to be carefully titrated in order to avoid an increased risk in cardiovascular disease. Further studies on other chemokines which may be able to bind CX3CR1 will also have to be investigated. Fractalkine is thought to bind specifically CX3CR1 and because CX3CR1 is not thought to bind any other ligand, the modulation of fractalkine/CX3CR1 signalling may have promising therapeutic applications. However, one study has suggested a possible alternate ligand, namely CCL26, which may have an affinity for CX3CR1 (Nakayama et al., 2010).

6.11 Limitations and future directions

The current study demonstrated the protective effects of S1PR and CX3CR1 modulation over H_2O_2 induced demyelination in an *in vitro* setting as well as highlighted a major difference in the protective effects of rCX3CL1 on vimentin depending on the method of H_2O_2 delivery. Some of the limitations we observed over the course of our studies are worthy of mention. Firstly, the expression levels of fractalkine and its receptor CX3CR1 have previously been characterised in the rodent brain (Hughes et al., 2002). However, pre- and post-oxidative stress levels of fractalkine/CX3CR1 expression in cells of the mouse cerebellum have not been characterised in our study. In this regard, fractalkine and CX3CR1 expression levels could be characterised at embryonic, newborn and adult stages in naive and mice subjected to oxidative stressed in order to fully characterise the role of fractalkine signalling in oxidative stressed brains. There are also no reported studies on the expression of fractalkine or CX3CR1 on oligodendrocytes. Therefore, clarification on the expression pattern of fractalkine/CX3CR1 is of high importance to fully understand its role in myelination. A more comprehensive analysis on cytokine levels through the use of multiplex arrays may also help us to understand the effects of oxidative stress on cytokine levels, which in turn could lead to new drug targets.

In this thesis we have used fluorescent intensity of myelin markers such as MBP and MOG as an indicator of myelin state. However, these proteins are also present in non-myelinating oligodendrocytes and cell debris and consequently may provide an overestimation of myelin. Additionally, this technique does not address the thickness or compaction of the myelin sheath. Considering this, the use of electron microscopy to corroborate our findings would be warranted. Electrical conduction studies could also be carried out in order to test the functional quality of the restored myelin. Examining the half-life of fractalkine in cell culture would also aid in our understanding of the efficacy of fractalkine in experimental conditions. Further insight on the functional role of astrocyte-derived fractalkine and how important it is that ADAM10 is the selective protease involved would also prove beneficial. The target cell type with which FTY720 and CX3CL1 are exerting their therapeutic effects could also be addressed through slice culture experiments with tissue specific knock-out mice or through siRNA knock-down of specific S1P receptors as well as cell specific knock-down of CX3CR1.

6.12 Closing remarks

Oxidative stress is a common feature in many neurodegenerative illnesses. The neuroprotective effects of pFTY720, across a wide range of neurological diseases, are supported by evidence from *in vivo* studies (Guy et al., 2001, Hemmati et al., 2013, Gao et al., 2012). In addition, the efficacy of FTY720 (Fingolimod) in patients with multiple sclerosis seems now well accepted, promoting the use of this drug in additional clinical trials for CNS illnesses, where safety and tolerability studies are being conducted (Clinicaltrials.gov identifier: ALS, NCT01786174; Stroke, NCT02002390; Schizophrenia, NCT01779700; Rett syndrome, NCT02061137; Glioblastoma, NCT02490930). Drugs with a similar method of action to FTY720 also show promising therapeutic effects in peripheral

diseases. For example Ozanimod (RPC1063), which is an S1PR1 agonist is currently undergoing clinical trials for patients with ulcerative colitis (NCT02531126) as well as RR-MS (NCT02576717). In the current study, we demonstrated that the sphingosine 1-phosphate receptor agonist, pFTY720, attenuated demyelination as well as attenuated the reduction in astrocytic vimentin, induced by both bolus and continuous H_2O_2. Moreover, we show that the protective effects of pFTY720 are independent of changes in the levels of IL-6. This data further supports the use of pFTY720 as a potential therapy for a wide range of neurological disorders where oxidative stress is a major contributing factor.

Centrally acting therapies which protect from demyelination or enhance remyelination following a demyelinating episode are highly sought after. Fractalkine and its receptor CX3CR1 are expressed differentially on cells of the CNS, however, the role of fractalkine on myelination state has not been investigated thus far. Here, we demonstrate that recombinant fractalkine (rCX3CL1) has protective effects on H_2O_2-induced demyelination. Furthermore, we show rCX3CL1 may also have protective effects on astrocytes under oxidative stress conditions. In closing, we suggest that this work raises the exciting possibility that CX3CR1 modulation may provide neuroprotection in neurological disorders associated with oxidative stress. Due to the specificity of fractalkine for CX3CR1, development of agonists/antagonists may be a promising therapeutic tool in modulating CNS diseases.

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