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The Signalling and Function of EBI2 in the Central Nervous System



This thesis submitted to University of Dublin, Trinity College

for the degree of

Doctor of Philosophy

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(14th October 2014)

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Table of common abbreviations

Abbreviation	Definition	Abbreviation	Definition
7α25HC	7α, 25-dihydroxycholesterol	**HPRT1**	Hypoxanthine-guanine phosphoribosyltransferase
Ab	Antibody	**HSD3B7**	Cholest-5-ene-3β,7α-diol 3β-dehydrogenase
AD	Alzheimer disease	**IFN**	Interferon
APC	Antigen presenting cell	**IL**	Interleukin
BBB	Blood brain barrier	**KO**	Knock-out
BCR	B cell receptor	**LPC**	Lysophosphatidylcholine
BDNF	Brain derived neurotropic factor	**LPS**	Lipopolysaccharide
BSA	Bovine serum albumin	**LXR**	Liver X receptor
CCL/CXCL/...	Chemokine ligand	**MAP**	Mitogen-activated protein
CCR/CXCR/...	Chemokine receptors	**MBP**	Myelin basic protein
CH25H	Cholesterol-25-hydroxylase	**MOG**	Myelin oligodendrocyte glycoprotein
CNS	Central nervous system	**MRC**	Marginal reticular cell
CHO	Chinese hamster ovary cell	**MS**	Multiple sclerosis
CI	Cell index	**MTT**	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
CSF	Cerebrospinal fluid	**NFAT**	Nuclear factor of activated T cells
CYP7B1	Hydroxycholesterol 7-alpha-hydroxylase	**NFκB**	Nuclear factor kappa B
DC	Dendritic cell	**NK**	Natural killer cell
DMSO	Dimethyl sulfoxide	**NO**	Nitric oxide
EAE	Experimental autoimmune encephalomyelitis	**OPC**	Oligodendrocyte progenitor cell
EBI2	Epstein-Barr virus-induced gene 2	**PD**	Parkinson's disease
EBV	Epstein-Barr virus	**PBS**	Phosphate buffered saline
ECIS	Electric Cell-Substrate Impedance Sensing (ECIS)	**PBMC**	Peripheral blood mononuclear cell
ERK	Extracellular-signal –regulated kinase	**PLP**	Proteolipid protein
FACS	Fluorescence-activated cell sorter	**Ptx**	Pertussis toxin
FBS	Fetal bovine serum	**ROS**	Reactive oxygen species
FDC	Follicular dendritic cell	**S1P**	Sphingosine-1-phosphate
FGF	Fibroblast growth factor	**S1PR1**	S1P receptor 1
GC	Germinal centre	**TBS**	Tris-buffered saline
GFAP	Glial fibrillary acidic protein	**TLR**	Toll-like receptor
GPCR	G-protein coupled receptor	**TNFα**	Tumor necrosis factor alpha
HC	Dihydroxycholesterol	**WT**	Wild type

Scientific abstract

The G protein-coupled receptor EBI2 (Epstein-Barr virus-induced gene 2) is activated by 7α, 25-dihydroxycholesterol (7α25HC) and plays a role in T cell-dependent antibody response and B cell migration. Aberrant EBI2 signalling is implicated in a range of autoimmune disorders however, its role in the CNS remains unknown. Here we characterize the functional role of EBI2 in the brain using primary human and mouse astrocytes, organotypic cerebellar slice cultures and EBI2 knockout animals. We find that human and mouse astrocytes express EBI2 and the enzymes necessary for synthesis and degradation of 7α25HC. In astrocytes, EBI2 activation stimulates ERK phosphorylation, Ca^{2+} signalling and induces cellular migration (**Results 1**), in addition to attenuating LPS and IL17/TNFα-mediated release of IL6 and TNFα in human and mouse astrocytes. Activation of EBI2 also inhibits IL17/TNFα-induced signalling and NFκB translocation to the nucleus. Importantly, analysis of mRNA levels of EBI2 and its signalling pathway in mouse astrocytes treated with LPS showed that these cells respond to immune challenge in part by regulating the expression pattern of EBI2 and oxysterol related genes. The data also showed that astrocytes stimulated with LPS can induce macrophage migration and that EBI2 can regulate this cellular crosstalk (**Results 2**). In addition to playing important roles in astrocyte biology and immune system regulation in the CNS, the data showed that EBI2 signalling is involved in myelin development and that inhibition of EBI2 signalling induces a delay in myelin development. We also show that EBI2 signalling is protective in LPC-induced demyelination in organotypic cerebellar slice cultures and that the protection is, at least partially, mediated via inhibition of pro-inflammatory cytokine release (**Results 3**). These results, for the first time, demonstrate a role for EBI2 in astrocyte function and myelin development and protection against demyelinating insults and suggest that modulation of this receptor may be beneficial in neuroinflammatory and neurodegenerative disorders.

Lay abstract

The four major brain-specific cell types in the brain are: (i) neurons, which transmit and process information via electrical signals, (ii) oligodendrocytes, which insulate neuronal axons with fatty sheets called myelin to ensure fast and complete electrical signal transduction, (iii) microglia, which provide defence against potential infections in the brain and, (iv) astrocytes, that serve many important roles such as immune defence, cleaning up debris, ensuring proper neuronal functioning and also communication with other cells. These cells, and indeed all living cells, have a wide range of proteins called receptors on their surface, which are activated by signalling molecules. EBI2 is one such receptor that plays very important roles in the biology of immune cells, where it regulates antibody production to fight infections. In this thesis, we investigated whether EBI2 is also present on the cell surface of brain cells, namely astrocytes. We asked whether EBI2 receptor is involved in regulation of astrocyte physiology and fighting of infections in the brain, and also if it can regulate oligodendrocyte myelination of neurons. Our research has shown for the first time that EBI2 is present on the surface of astrocytes and that it does play a role in immune function in the brain. When EBI2 is in contact with its signalling molecule (called oxysterol 7α25HC) it causes the astrocytes to migrate and also stops these cells from releasing harmful inflammatory signals. The EBI2 receptor is also capable of sensing infections and responding to them by releasing molecules that attract immune cells into the CNS, to help fight infection. In addition, we found that the EBI2 receptor is necessary for appropriate and timely myelination of neurons and that activation of the EBI2 receptor can protect the cells from chemically induced loss of myelin. These findings are important because they inform us about the role of the EBI2 receptor in the brain, and about how astrocytes and oligodendrocytes are modulated by this receptor. These findings also allow us to develop new ways to control brain cell function and limit pro-inflammatory processes. We believe this work is important for development of new drugs that might be beneficial in treating diseases such as multiple sclerosis or Alzheimer's disease.

Aims and hypothesis

The aims of the study are as follows:

- To investigate EBI2 expression in the cells of the central nervous system, specifically astrocytes **(Results 1)**.
- To identify whether astrocytes express enzymes necessary for the EBI2 ligand, oxysterol 7α25HC, synthesis and degradation **(Results 1)**.
- To explore EBI2 signalling pathways in astrocytes **(Results 1)**.
- To study whether EBI2 agonism induces astrocyte migration **(Results 1)**.
- To examine if EBI2 inhibits pro-inflammatory cytokine release **(Results 2)**.
- To examine whether activation of EBI2 attenuates pro-inflammatory signalling in astrocytes **(Results 2)**.
- To investigate whether EBI2 and 7α25HC related enzymes are regulated in response to immune challenge **(Results 2)**.
- To explore the role of EBI2 in astrocytes in cellular cross-talk **(Results 2)**.
- To study the role of EBI2 signalling in myelin development *in vivo* and *in vitro* using wild type and EBI2 knock-out mice **(Results 3)**.
- To identify the role of EBI2 signalling in myelin development **(Results 3)**.
- To investigate whether EBI2 protects from chemically-induced demyelination **(Results 3)**.
- To examine the role of EBI2 in pro-inflammatory cytokine signalling during chemically-induced demyelination **(Results 3)**.

We hypothesise that EBI2 is expressed in astrocytes and that it regulates astrocyte signalling and function. Specifically, we hypothesise that astrocytes express EBI2 and enzymes necessary for 7α25HC synthesis and degradation, EBI2 agonism with 7α25HC activates downstream signalling pathways, EBI2 stimulates migration of astrocytes, EBI2 alters cytokine/chemokine levels in astrocytes, EBI2 and 7α25HC-related enzymes are regulated in astrocytes upon immune challenge, and that EBI2 in astrocytes is involved in inter cellular communication. We also hypothesise that EBI2 is involved in myelin development, that EBI2 activation has protective effects on LPC-induced demyelination in cerebellar slices and that it might exhibit its protective effects via inhibition of pro-inflammatory cytokine release in slices.

Value of research

EBI2 is a fairly recently deorphanised receptor that has attracted much interest over the last 4-5 years. EBI2 is mainly expressed in the lymphoid tissue and the cells of the immune system such as B cells. For these reasons its function and expression in the immune system have been the main focus of research. Astrocytes, among other functions, also serve immune functions in the CNS. They present antigens to peripheral immune cells and release cytokines and chemokines that activate and drive immune cell influx into the CNS. Even though astrocytes are necessary for a proper CNS function and physiology, they have also been implicated in a range of neurodegenerative diseases with an immune component such as multiple sclerosis, Alzheimer's disease or Parkinson's disease. Moreover, cholesterol is a major component of myelin and the EBI2 agonists, oxysterols, are cholesterol derivatives involved in its homeostasis. It is therefore warranted to investigate the expression and function of EBI2 in the CNS and its involvement in astrocyte biology and myelination. Therapies that control astrocyte activation may prove useful in managing neuroinflammatory or neurodegenerative diseases and EBI2 might be a valuable tool via which inflammatory signalling and myelination can be controlled. This study explored the expression and function of EBI2 in human and mouse astrocytes and its role in myelination. The data showed that EBI2 is expressed in astrocytes and that it plays important roles in the cell physiology. Activation of EBI2 induced intracellular signalling and cellular migration and dampened pro-inflammatory signalling. Importantly, this study showed that astrocytes respond to immune challenge by regulating EBI2 and oxysterol expression. The data also suggests that astrocytes communicate with the cells of the immune system, such as macrophages, via the EBI2 signalling system (the receptor and 7α25HC synthesising enzymes). Importantly, the data showed that EBI2 is involved in myelin development and can protect from LPC-induced demyelination most likely via inhibition of pro-inflammatory cytokine signalling. Taken together, these studies support the possibility that EBI2 in astrocytes regulates inflammatory responses and astrocyte migration. These results provide initial evidence for the use of EBI2 as a novel drug target to altering astrocyte function in neuroinflammatory and neurodegenerative illnesses and warrant further *in vitro* and *in vivo* research into the role of EBI2 in the central nervous system biology.

Outputs

Papers

Rutkowska A., Sailer A.W. and Dev K.K. EBI2 regulates myelination state and protects from LPC-induced demyelination in organotypic cerebellar slice cultures. *In preparation.*

Preuss I., **Rutkowska A.**, Dev K.K., and Sailer A.W. Signalling of the oxysterol / EBI2 system in immune cells. *In preparation.*

Rutkowska A., Sailer A.W. and Dev K.K. EBI2 regulates levels of IL6 and TNFα in astrocytes and their signalling with macrophages. *EMBO Reports, to be submitted.*

Rutkowska A., Preuss I., Gessier F., Sailer A.W. and Dev K.K. EBI2 regulates intracellular signalling and migration in human astrocytes. *Glia,* 10.1002/glia.22757.

Rutkowska, A., Elain, G., Jeanneau, K., Healy, L.M., Mir, A.K., Dev, K.K. S1P receptors regulate IL17-induced levels of IL6 in human astrocytes. *Glia, in preparation.*

Elain, G., Jeanneau, K., **Rutkowska, A.**, Mir, A.K., Dev. K.K. The selective anti IL17A monoclonal antibody Secukinumab (AIN457) attenuates IL17A-induced levels of IL6 in human astrocytes. *Glia*, 2014; 62(5):1098-1136.

Healy, L.M., Sheridan, G.K., Pritchard, A.J., **Rutkowska, A.**, Mullershausen, F., Dev, K.K. Pathway specific modulation of S1P1 receptor signalling in rat and human astrocytes. *British Journal of Pharmacology*, 2013; 169:1114-1129.

Doyle, F., Rohde, D., **Rutkowska, A.**, Morgan, K., Cousins, G., McGee, H. Systematic review and meta-analysis of the impact of depression on subsequent smoking cessation in patients with coronary heart disease: 1990-2013. *Psychosomatic Medicine*, 2014; 76:44-57.

Posters

Rutkowska, A., Preuss, I., Sailer, A.W., Dev, K.K. EBI2 regulates intracellular signalling and migration in human astrocytes. 9th FENS Forum of Neuroscience, Milan, Italy, July 5-9 2014.

Rutkowska, A., Sailer, A.W., Dev, K.K. Oxysterol-EBI2 signalling in astrocytes and their role in demyelination. Astrocytes in Health and Neurodegenerative disease, a joint Biochemical Society/British Neuroscience Association Focused Meeting, London, April 28-29 2014.

Rutkowska, A., Sailer, A.W., Dev, K.K. EBI2 directs astrocyte migration and protects from demyelination. Poster presented at 3rd Annual Meeting Frontiers in Neurology Ireland, Dublin, Ireland, 2013.

Preuss, I., Voshol H., Abraham Y., Lasbennes MC., Laurent S., **Rutkowska A.**, Baumgarten B., Zhang J., Gessier F., Dev K.K., Seuwen K., Sailer A.W. Signalling of the oxysterol/EBI2 system in immune cells. Poster presented at 13th International Conference on Bioactive Lipids in Cancer, Inflammation, and Related Diseases, Puerto Rico, 2013.

Elain, G., Jeanneau, K., **Rutkowska, A.**, Dev, K.K., Mir, A.K. IL17A and TNFα regulate levels of chemokines and cytokines in human astrocytes via MAP kinase and NFκB signalling pathways. Poster presented at European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS), Copenhagen, Denmark, 2013.

Rutkowska, A., Dev, K.K., Sailer, A.W. Oxysterols stimulate astrocyte migration via the EBI2 receptor. Poster presented at the 5th School of Medicine Postgraduate Research Day, TCD, Dublin, 2012.

Rutkowska, A., Dev, K.K., Sailer, A.W. EBI2 receptor directs human astrocyte migration. Poster presented at the NSI Conference, RCSI, Dublin, 2012.

Rutkowska, A., Dev, K.K., Mosbacher, J., Deymier, C., & Muehlemann, A. The role of S1P receptors in LPS-induced cellular stress in microglia. Poster presented at the NSI Conference, Maynooth, 2011.

Awards

Best poster award for a poster presented at the 3rd Annual Meeting Frontiers in Neurology Ireland, Dublin, Ireland, 2013. Rutkowska, A., Sailer, A.W., Dev, K.K. EBI2 directs astrocyte migration and protects from demyelination.

Biochemical Society Student Travel Bursary (£300) to attend Astrocytes in Health and Neurodegenerative disease conference taking place in London on April 28-29 2014.

Trinity College Dublin Trust Travel Grant (€300) to attend 9th FENS Forum of Neuroscience, Milan, Italy, July 5-9 2014.

Chapter 1: Introduction

Overview

Epstein-Barr virus (EBV)-induced gene 2 (EBI2), also known as GPR183, is a G protein-coupled receptor (GPCR) which is highly expressed in peripheral blood mononuclear cells (PBMCs) **(Birkenbach et al., 1993, Benned-Jensen et al., 2012)**. EBI2 regulates B cell positioning within the lymphoid tissue and is crucial for launching appropriate T cell-dependent antibody response **(Gatto et al., 2009, Gatto et al., 2011, Kelly et al., 2011, Pereira et al., 2010)**. In addition to being crucial for launching correct humoral response, EBI2 is also involved in inflammatory responses **(Gatto et al., 2011, Gatto et al., 2009, Pereira et al., 2009)**. EBI2 is activated by oxysterols and signals through the pertussis toxin (PTx)-sensitive heterotrimeric G proteins of the Gi/o family leading to a decrease in cyclic adenosine monophosphate (cAMP) production, calcium mobilization and stimulation of the extracellular-signal-regulated kinase (ERK) pathway **(Hannedouche et al., 2011, Liu et al., 2011, Benned-Jensen et al., 2013, Benned-Jensen et al., 2012)**.

Oxysterol 7α, 25-dihydroxycholesterol (7α25HC) and closely related compounds have been identified as high affinity agonists for EBI2 **(Liu et al., 2011, Hannedouche et al., 2011)**. Oxysterols are oxygenated derivatives of cholesterol and play important roles in metabolism of vitamins and lipids, cholesterol trafficking, activation of nuclear hormone receptors, regulation of inflammatory signalling pathways, and many more **(Schroepfer, 2000, Leoni and Caccia, 2011, Russell, 2000)**. Oxysterols have chemotactic properties inducing cell migration *in vitro* and *in vivo* **(Liu et al., 2011, Hannedouche et al., 2011)**. Moreover, oxysterols have immunomodulatory properties having both, pro- and anti-inflammatory effects depending on the oxysterol and cell type **(Koarai et al., 2012, Aye et al., 2012, Diczfalusy et al., 2009, Rosklint et al., 2002, Lemaire-Ewing et al., 2009, Moog et al., 1988)**.

While the role of EBI2 in the immune system is the main focus of research its expression and function in the cells of the central nervous system (CNS) have not been investigated. Astrocytes are one of the four resident brain cells that serve a range of physiological functions in the CNS. Among other roles, they are involved in CNS immune defence. They communicate with cells of the immune system via signalling molecules (for example by cytokines and chemokines) and cell-cell interactions (for example by antigen presentation) **(Kimelberg and Nedergaard, 2010, Ransom et al., 2003)**. Astrocytes also isolate inflamed or injured areas by forming glial scars. They express a wide range of receptors and release various signalling molecules allowing them to be involved in both, normal and pathophysiological processes in the CNS **(Kimelberg and Nedergaard, 2010, Ransom et al., 2003)**. Importantly, these glial cells have been implicated in many neurodegenerative diseases such as multiple sclerosis (MS), Parkinson's disease (PD) and Alzheimer's disease (AD) **(Lin et al., 1993, Knott et al., 2002, Sidoryk-Wegrzynowicz et al., 2010, Kimelberg, 2010, Sriram, 2011)**.

A quarter of the total body cholesterol is located in the brain of in oligodendrocytes, neurons and astrocytes **(Leoni and Caccia, 2011)**. Cholesterol is the main building block of myelin and is crucial for correct and timely myelin formation **(Nelissen et al., 2011)**. Astrocytes participate in myelination during development by upregulating cholesterol synthesis **(Pfrieger and Ungerer, 2011)**. Oxysterols are also involved in cholesterol homeostasis and turnover and have been shown to play important roles in myelination **(Nelissen et al., 2011)**. In addition to being involved in normal myelin development, oxysterols can have detrimental effects on myelin and oligodendrocytes **(Makoukji et al., 2011, Trousson et al., 2009)**. Abnormal levels of oxysterols have been found in a number of

neurodegenerative diseases such as Alzheimer's disease or multiple sclerosis **(Lutjohann et al., 2000, Leoni et al., 2002)**. Upregulated levels of oxysterols have been detected in the brains of EAE animals and MS patients **(Diestel et al., 2003)**. In spastic paraplegia gene 5 (SPG5) mutations in CYP7B1 gene lead to neuropathy of upper motor neurons as well as periventricular and subcortical white matter lesions **(Schule and Schols, 2011, Biancheri et al., 2009)**.

Here, the expression and role of EBI2 in the CNS were investigated. First, expression of EBI2 and 7α25HC synthesising enzymes were measured, then intracellular signalling and effects of EBI2 activation on human and mouse astrocyte migration were investigated. To further demonstrate the role of EBI2 in astrocytes, we examined their function in regulating the levels of pro-inflammatory cytokines and the NFκB signalling pathway. In addition, we report that EBI2 may be involved in sensing invading pathogens in CNS and respond to them by regulating the receptor and oxysterol related enzymes. We also demonstrate how EBI2-signalling in astrocytes may control their cellular communication with macrophages. Lastly, the effect of EBI2 activation on myelin development was examined *in vitro*, using organotypic cerebellar slices, and *in vivo*, in wild type (WT) and EBI2 knock-out (KO) animals.

1 EBI2 Receptor

1.1 Structure and expression of EBI2

EBV-induced gene 2 (EBI2, also known as GPR183) is a G protein-coupled receptor (GPCR) which belongs to the largest and most studied family of GPCRs called rhodopsin-like receptors (also known as family A) **(Birkenbach et al., 1993)**. EBI2 is located in chromosome 13 in region q32.3 very close to GPR18 which is its closest homologue **(Rosenkilde et al., 2006)**. EBI2 also has some homology with GPR17, cysteinyl leukotriene receptors and purinergic receptors (e.g. PSY2, PSY6) **(Norregaard et al., 2011)**. Among the most prominent characteristics of EBI2 structure is the lack of a signal peptide. More interestingly, the sequence encoding human EBI2 has two methionine codons in the N-terminus, and it is currently unclear which one is responsible for initiation of translation. The murine sequences lack the first methionine but they do not seem to have any functional consequences in terms of expression **(Rosenkilde et al., 2006, Birkenbach et al., 1993, Norregaard et al., 2011)**. Figure 1.1 shows aligned human, rat and mouse sequences of EBI2 and Figure 1.2 shows EBI2 phylogenetic tree.

EBI2 is expressed at the cell surface mainly in lymphoid tissue (spleen and lymph nodes) and peripheral blood mononuclear cells (PBMC) mainly on B and T cells **(Rosenkilde et al., 2006)**. EBI2 is upregulated during B cell maturation and is expressed in recirculating B cells in the spleen, lymph node and bone marrow **(Pereira et al., 2009)**. EBI2 expression is highest in naïve B cells and after interaction with T helper cells, and it is almost undetectable in germinal centre (GC) B cells **(Rosenkilde et al., 2006, Norregaard et al., 2011)**. EBI2 expression has also been found in granulocytes, dendritic cells, macrophages, monocytes, neutrophils, human platelets, tonsils and pancreas **(Birkenbach et al., 1993, Pereira et al., 2009, Amisten et al., 2008, Chan et al., 2010b, Hannedouche et al., 2011, Liu et al., 2011, Norregaard et al., 2011)**. Figure 1.3 illustrates EBI2 expression in different tissue and cells (adapted from **Rosenkilde et al., 2006**).

1.2 Signalling of EBI2

EBI2 is coupled exclusively to the G_i protein and when activated signals via two mitogen-activated protein (MAP) kinases, p38 and extracellular-signal-regulated kinase (ERK), as well as calcium and serum response element (SRE) transcription factor in a pertussis toxin (Ptx)-sensitive manner. Notably, it does not signal via nuclear factor of activated T cells (NFAT) or nuclear factor kappa B (NFκB) **(Liu et al., 2011, Rosenkilde et al., 2006, Benned-Jensen et al., 2011, Benned-Jensen et al., 2013, Hannedouche et al., 2011, Daugvilaite et al., 2014)**. Figure 1.4 illustrates EBI2 signalling pathways. EBI2 is activated by several oxysterols and among them, 7α, 25-dihydroxycholesterol (7α25HC) has the highest affinity and is the most potent endogenous agonist for EBI2 with affinity ranging from 2 +/- 2 nM **(Hannedouche et al., 2011, Liu et al., 2011)**. Other related oxysterols activate EBI2 with lower potency ranging from 5 +/- 4 nM for 7α27HC, 121 +/- 54 nM for 7β25HC, 310 +/- 261 nM for 7βHC, 1453 +/- 1163 nM for 7αHC, 3032 +/- 2645 nM for 25HC, to above 10,000 nM for cholesterol **(Hannedouche et al., 2011)**. In addition to agonists, there are currently two characterised EBI2 competitive antagonists, NIBR189 and GSK682753A **(Benned-Jensen et al., 2013, Gessier et al., 2014)**. Figure 1.5 shows the chemical structure of EBI2 agonists and antagonists.

Downstream activation of EBI2 with 7α25HC induces a wide range of functional responses such as cell migration or calcium mobilization **(Hannedouche et al., 2011, Liu et al., 2011)**. 7α25HC chemoattractant properties display a bell shaped activity curve where higher concentrations reduce

chemoattractant properties, indicating a ligand-induced receptor desensitization, most likely due to receptor internalization **(Liu et al., 2011, Hannedouche et al., 2011)**. In fact, 7α25HC induces EBI2 internalization after as little as 10 minutes of treatment with 7α25HC **(Hannedouche et al., 2011, Liu et al., 2011, Yi et al., 2012)**. Other related oxysterols such as 7α27HC, 7β25HC, 7αHC and 25HC also induce EBI2 expressing cell migration although with much lower potency than 7α25HC **(Hannedouche et al., 2011)**.

1.3 Oxysterols

Oxysterols are oxygenated derivatives of cholesterol, which are involved in metabolism of vitamins and lipids, cholesterol homeostasis and trafficking, bile acid synthesis, apoptosis, necrosis, inflammation, immunosuppression, and many others **(Otaegui-Arrazola et al., 2010, Bjorkhem and Diczfalusy, 2002, Schroepfer, 2000, Leoni and Caccia, 2011, Russell, 2000)**. Oxysterols, other than 7α25HC, are also agonists for nuclear hormone receptors such as liver x receptors (LXR) **(Spann and Glass, 2013)**. Agonism of LXR has effects on gene expression in brain cells such as astrocytes, for instance, by inducing synthesis of ATP-binding cassette (ABC) transporters **(Whitney et al., 2002).** Oxysterols are formed via several pathways such as autoxidation, action of monooxygenase or by enzymatic or non-enzymatic lipid peroxidation **(Bjorkhem and Diczfalusy, 2002)**. Oxysterols formed by oxidation in the ring have been found to have cytotoxic effects while oxysterols formed via enzymatic reaction tend to have a range of beneficial biological functions **(Otaegui-Arrazola et al., 2010)**. 7α25HC is synthesised from cholesterol through enzymatic action of cholesterol-25-hydroxylase (CH25H) followed by a cytochrome P450 hydroxycholesterol 7-alpha-hydroxylase (CYP7B1) and is degraded by cholest-5-ene-3β,7α-diol 3β-dehydrogenase (HSD3B7) **(Yi et al., 2012)**. Figure 1.5 shows the synthetic pathways of 7α25HC.

Oxysterols can easily cross lipophilic membranes and have very short half-lives in the brain compared to cholesterol, which has a half-life of about five years. Oxysterol concentrations are very low ranging from 0.01-0.1 μM in serum and plasma to less than 0.005-0.46 μg/g in tissue (e.g. rat liver) **(Schroepfer, 2000, Bjorkhem and Diczfalusy, 2002)**. Adult rodent's brain has 10–20 mg/g of cholesterol and 20 μg/g of 24SHC wet weight **(Wang et al., 2014)**. Of interest, 24SHC, 25HC and 27HC and their synthetic enzymes CYP46A1, CH25H and CYP27A have all been found in the brain and specifically in oligodendrocytes **(Shackleford et al., 2013)**. A quarter of the total body cholesterol is located in the brain in myelin sheath and plasma membranes of oligodendrocytes, astrocytes and neurons. Cholesterol is synthesised *de novo* in the brain as it cannot gain access to the brain from the circulation due to the blood-brain barrier (BBB) **(Leoni and Caccia, 2011, Bjorkhem and Meaney, 2004).** Cholesterol metabolite 24SHC is a brain-specific oxysterol which can easily cross BBB and is excreted from the brain to the periphery to maintain steady levels of cholesterol in the CNS **(Bjorkhem and Meaney, 2004)**. It has been shown that brain is the only source of 24SHC in the human body and levels of circulating 24SHC have been proposed as biomarkers for brain cholesterol homeostasis and neurodegenerative diseases such as multiple sclerosis (MS) and Alzheimer's (AD) **(Bjorkhem and Meaney, 2004, Leoni et al., 2002, Lutjohann et al., 2000)**. Oxysterols are transported bound to apolipoprotein E (apoE), mainly produced by astrocytes, and are one of the most bioactive components of oxidized low density lipoprotein (oxLDL) **(Bjorkhem and Meaney, 2004, Shentu et al., 2012).** Oxysterols also have been shown to play important roles in astrocyte biology. For instance, they inhibit astrogliosis and a correlation has been found between cholesterol synthesis in the CNS and reactive astrocyte proliferation **(Bochelen et al., 1995)**.

1.4 EBI2 function in the immune system

EBI2 plays very important roles in the immune system by being involved in the correct positioning of activated B cells in the lymphoid organs and launching appropriate antibody response **(Gatto et al., 2013, Kelly et al., 2011)**. In brief, chemokine receptor type 5 (CXCR5) guides naïve B cells to the follicles in the lymphoid tissue. During normal humoral immune response, shortly after B cell receptor (BCR) engagement, activated B cells upregulate EBI2 which transiently guides them to the outer regions of the follicle, possibly to encounter more antigens entering from circulation **(Kelly et al., 2011)**. This effect is counteracted by strong signals from chemokine receptor type 7 (CCR7) (also known as EBI1) which is upregulate about 6 to 10 hours after BCR engagement. CCR7 directs B cells to the B zone - T zone (B-T) boundary where they interact with cognate T helper cells **(Kelly et al., 2011, Pereira et al., 2010, Pereira et al., 2009, Gatto et al., 2009, Gatto et al., 2011)**. CXCR5 and EBI2 continue to signal contributing to equal distribution of B cells along the B-T boundary ensuring correct B cell to T cell interactions **(Kelly et al., 2011)**. Subsequently, following CCR7 downregulation and cluster of differentiation 40 (CD40) engagement, EBI2 function takes over and directs cells away from the B-T boundary and towards the outer- and inter- areas of the follicle **(Kelly et al., 2011)**. From there, some cells move to the extra follicular areas in CXCR4- and sphingosine-1-phosphate (S1P1)-dependent manner, differentiate into plasmablasts and mount a rapid antibody response. Some cells however, move to the follicle centre, differentiate into GC B cells and undergo antibody affinity maturation, necessary for a long-term antibody response **(Kelly et al., 2011, Pereira et al., 2010, Pereira et al., 2009, Gatto et al., 2009, Gatto et al., 2011)**. The signals that are involved in directing B cells to the GC and stimulating them to leave the follicle to mount a short-term antibody response are still unclear. Figure 1.6 illustrates the sequence of events described above.

EBI2 deficiency does not affect normal GC response, follicular organization, lymphocyte numbers or B cell migration to the B-T boundary that occurs within hours of BCR stimulation **(Pereira et al., 2009, Gatto et al., 2009)**. However, in the subsequent stages of the humoral response EBI2 deficient B cells fail to move to the outer- and inter-follicular regions and instead favour the follicle centre **(Gatto et al., 2009, Pereira et al., 2009)**. In addition, EBI2 deficiency results in a decreased early phase T cell dependent antibody defence showing that EBI2 is crucial for appropriate humoral response before the involvement of high-affinity GC plasma cells occurs **(Gatto et al., 2009)**. On the other hand, EBI2 overexpression or suppression of its downregulation after T cell interaction results in a diminished GC response and increased B cell migration to the outer- and inter-follicular regions followed by greater plasmablast formation and increased concentration of antibodies in the early phases of T cell dependent antibody response **(Gatto et al., 2009, Pereira et al., 2009)**.

In CH25H (cholesterol-25-hydroxylase, a 7α25HC synthesising enzyme) deficient mice, activated B cells fail to migrate to the outer- and inter-follicular regions and favour B-T boundary and central areas of the follicle **(Hannedouche et al., 2011)**. These CH25H-deficient mice also show a three-fold decrease in the extent of their antibody response. On the other hand, saturating the microenvironment with 7α25HC or inhibition of CYP7B1 (hydroxycholesterol 7-alpha-hydroxylase, the second 7α25HC synthesising enzyme) results in equal distribution of B cells in the lymphoid tissue suggesting that 7α25HC gradient is crucial for appropriate localization of B cells in the lymphoid tissue **(Hannedouche et al., 2011, Liu et al., 2011)**. Thus, to ensure correct functioning of the humoral antibody response the gradient of 7α25HC has to be tightly controlled. Since B cells do not themselves express the 7α25HC synthesising (CH25H and CYP7B1) nor the degrading enzyme (HSD3B7) they depend on other cells to regulate the gradient. Indeed, it has been shown that

lymphoid stromal upregulate the levels of 7α25HC via CH25H, CYP7B1 enzymes and follicular dendritic cells downregulate the levels via HSD3B7 enzyme **(Yi et al., 2012)**.

EBI2 is involved in various stages of the humoral response exerting a wide range of effects **(Kelly et al., 2011)**. As Gatto and colleagues **(2009)** suggested, regulation of EBI2 expression may be a powerful tool to control B cell localization within secondary lymphoid tissue. This may be achieved via the B cell lymphoma 6 (BCL-6) transcriptional repressor and the non-canonical NF-κB pathway which have been found to regulate EBI2 expression **(Gatto et al., 2009)**. It has been shown that BCR-triggered NF-κB activation transiently induces upregulation of EBI2 in naïve B cells and that BCL-6 triggers downregulation of EBI2 in GC B cells **(Shaffer et al., 2000, Glynne et al., 2000, Gatto et al., 2009)**. EBI2 expression has also been found to be regulated by cytokines such as interleukin 4 (IL4), IL6 and IL10 **(Shaffer et al., 2000, Lam et al., 2008, Gatto et al., 2009)**.

EBI2 expression and function have so far only been investigated with regards to the cells of the immune system and the receptor's expression and function in the central nervous system remains currently unknown. However, studies suggest that dysregulated expression of EBI2 or of its related oxysterols have been implicated in a number of CNS diseases such as Alzheimer's disease or multiple sclerosis **(Papassotiropoulos et al., 2000, Bjorkhem, 2006, Fukumoto et al., 2002, Koldamova et al., 2003, Lutjohann et al., 2000, Vaya and Schipper, 2007, Vega et al., 2003)**. It is therefore warranted to investigate the role of EBI2 in the CNS as well as the immune system. EBI2 involvement in disease states is discussed in detail in section four of this chapter.



Figure 1.1 Human, mouse, and rat EBI2 sequences. Alignment of human, mouse, and rat EBI2 sequences **(http://blast.ncbi.nlm.nih.gov/BlastAlign.cgi)**. Amino acid residues that are identical in human EBI2 are indicated by dashes; differences are shown. The transmembrane domains are underlined **(www.uniprot.org, www.ensemble.org)**.

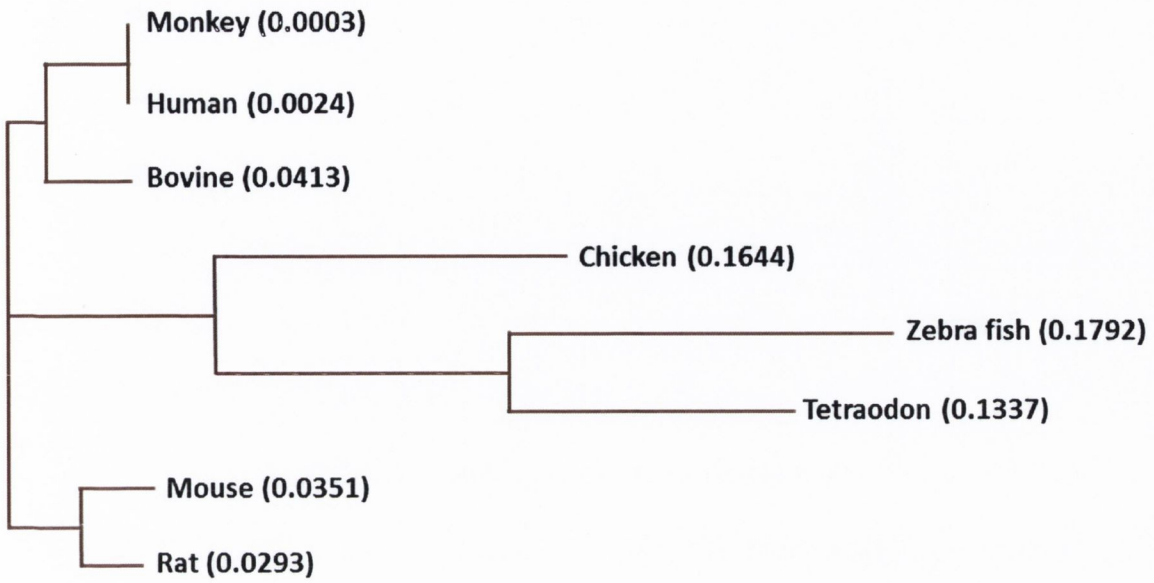


Figure 1.2 EBI2 phylogenetic tree.

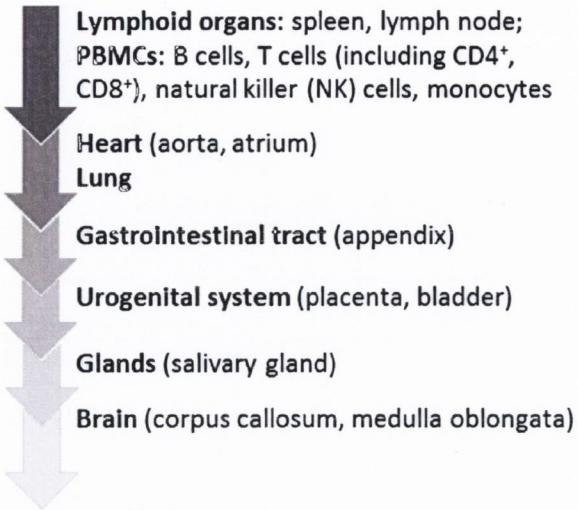


Figure 1.3 EBI2 expression in cells and tissue. Brackets indicate tissue with highest expression (adapted from **Rosenkilde et al., 2006**).

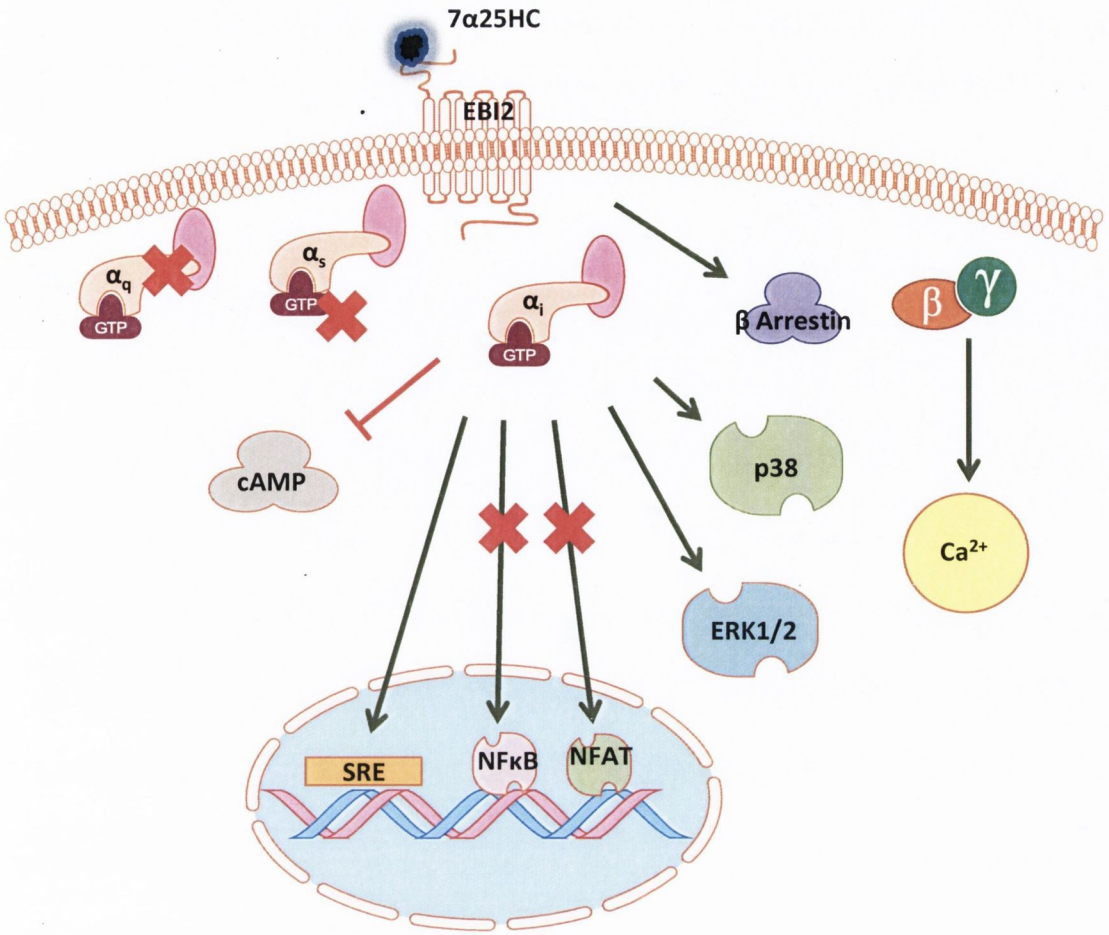


Figure 1.4 EBI2 signalling pathways. EBI2 signals through G_i protein in a Ptx-sensitive manner leading to inhibition of cAMP. EBI2 activates SRE (but not NFκB or NFAT), ERK, p38, β arrestin (independently of G protein) and Ca^{2+} signalling (via βγ subunits) **(Liu et al., 2011, Rosenkilde et al., 2006, Benned-Jensen et al., 2011, Benned-Jensen et al., 2013, Hannedouche et al., 2011, Daugvilaite et al., 2014)**.

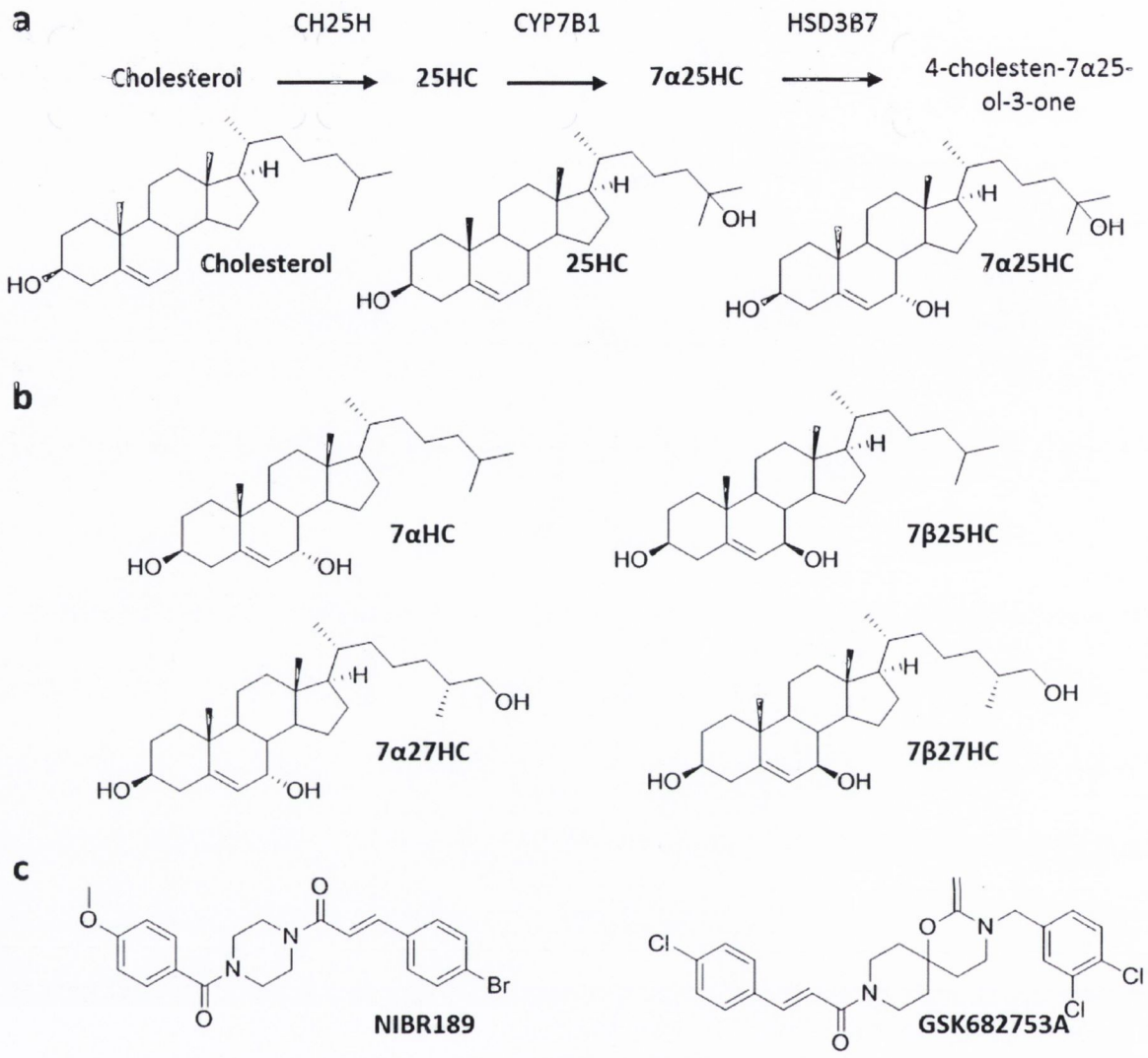


Figure 1.5 Chemical structures and synthetic pathways of **(a)** cholesterol, 25HC and 7α25HC and **(b)** other EBI2 activating oxysterols. **(c)** Chemical structure of EBI2 antagonists, NIBR189 and GSK682753A.

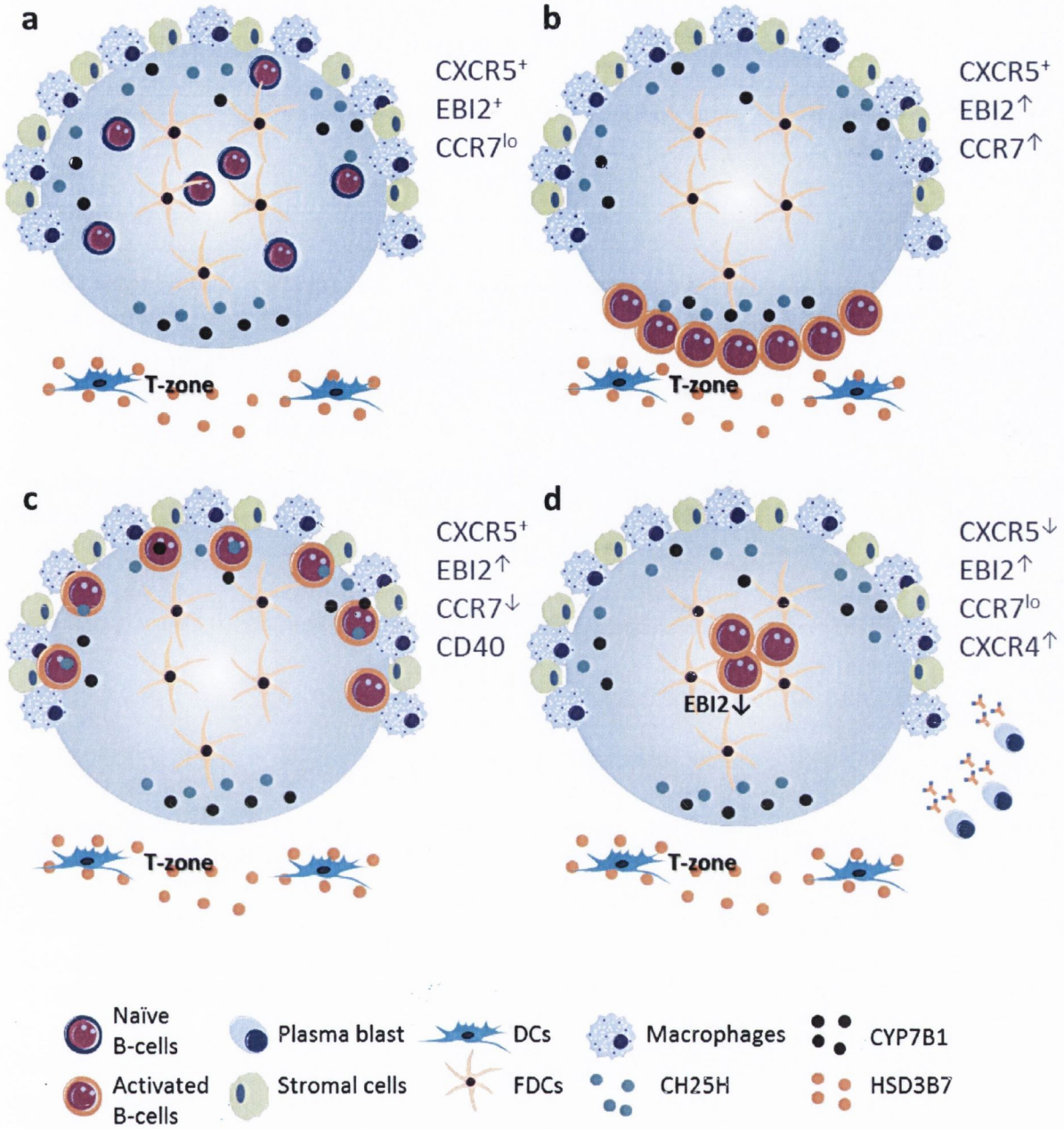


Figure 1.6 Function of EBI2 in the immune system. (a) Naïve B cells enter follicles to look for antigens and/or deliver opsonized antigen. If no antigen is encountered they leave the follicle in S1P dependent manner. (b) B cells encounter an antigen and become activated. Shortly after BCR engagement they upregulate EBI2 and migrate towards outer regions of the follicle where stromal cells produce 7α25HC. Between 6 to 10 hours after BCR engagement CCR7 is upregulated and EBI2 chemoattraction is counteracted. The cells begin to migrate to the B-T zone to interact with cognate T cells. Dendritic cells are the main downregulators of 7α25HC keeping 7α25HC levels low in the T-zone (c) Within two to four days after interaction with T cells and after CD40 engagement, B cells downregulate CCR7 and in EBI2 dependent manner move away from the B-T zone and enter the inter- and outer- follicular areas. (d) Some of the activated B cells sharply downregulate EBI2 and move to the follicle centre to form GCs. Others, leave the follicles in CCR4 dependent manner, become plasma blasts and launch a short-term antibody response (adapted from **Pereira et al., 2010, Pereira et al., 2009, Gatto et al., 2009, Gatto et al., 2011, Yi et al., 2012, Kelly et al., 2011**). Abbreviations: FDC - follicular dendritic cell; DCs – dendritic cells

2 Astrocytes

2.1 Astrocyte origins and types

Astrocytes are one of the three well known types of resident CNS glial cells alongside microglia and oligodendrocytes (and not withstanding others such as glial antigen 2 (NG2) positive glia). Astrocytes are homeostatic cells of the CNS characterised by a star-shaped morphology and spreading numerous extended processes that surround neurons and blood vessels **(Wang and Bordey, 2008, Kimelberg, 2010, Butt et al., 2002)**. They are non-excitable and are identified by intermediate filaments called glial fibrillary acidic proteins (GFAP) **(Wang and Bordey, 2008, Kimelberg, 2010)**. Of importance, it has to be noted that not all astrocytes have a star-shaped morphology or express GFAP **(Kimelberg, 2004)**.

Astrocytes originate from neural progentitors that give rise to both, macroglia (astrocytes and oligodendrocytes) and neurons **(Verkhratsky and Butt, 2013)**. The neural progenitors differentiate into radial glial cells in the ventricular zone and can give rise to astrocytes, neurons and some oligodendrocytes. Most oligodendrocytes, however, are generated in distinct areas of the CNS from specific glial precursors. Astrocytes, on the other hand, originate from both, radial glial cells and glial precursors **(Verkhratsky and Butt, 2013)**.

Astrocytes are an exceptionally varied cell type and many subtypes that distinguish one type from the other can be named based on their function, cellular presence, morphology, gene expression, physiological properties and response to stimuli **(Verkhratsky and Butt, 2013)**. One classification divides these cells into type 1 and type 2 astrocytes. Type 1 astrocytes are defined as fibroblast-type cells that originate early in development and secrete growth factors that, in turn, can stimulate the growth of type 2 astrocytes **(Raff et al., 1983, Armstrong et al., 1990).** Type 2 astrocytes can differentiate into O-2A progenitor cells and give rise to oligodendrocytes **(Raff et al., 1983, Armstrong et al., 1990)**. Other classification divides astrocytes into protoplasmic astrocytes, found in the grey matter of the brain and the spinal cord, and fibrous astrocytes, found in the white matter in the brain, the spinal cord, optic nerve and retina **(Verkhratsky and Butt, 2013)**. These are the most numerous subtypes of astrocytes, however, there are other types which include radial glia and other radial glia-type cells such as Müller glia in the retina and Bergmann glia in the cerebellum **(Verkhratsky and Butt, 2013)**. In addition, there are other, much less abundant subtypes which include: perivascular and marginal astrocytes, velate astrocytes of the cerebellum and olfactory bulb, interlaminar and polarised astrocytes found in the primate cortex, and varicose projection astrocytes present only in human brains **(Verkhratsky and Butt, 2013)**. Other subtypes include tanacytes found in the periventricular organs, hypothalamus and the spinal cord, pituicytes in the pituitary gland, and retinal and ventrical cells such as ependymocytes, choroid plexus cells and retinal pigment epithelial cells **(Verkhratsky and Butt, 2013)**.

2.2 Astrocyte functions

Astrocytes are a very abundant cell type in the brain and serve a range of physiological functions. For instance, they control neuronal maturation, form and maintain synapses, uptake neurotransmitters, release antioxidants and are responsible for extracellular ion buffering **(Kimelberg and Nedergaard, 2010, Wang and Bordey, 2008, Ransom et al., 2003, Kimelberg, 2010)**. Astrocytes promote myelination and speed up myelin wrapping around axons and formation of compact myelin during myelin development **(Watkins et al., 2008, Sorensen et al., 2008)**. Studies have shown that GFAP

expression is necessary for correct myelination and blood-brain barrier formation **(Liedtke et al., 1996)**. In addition to supporting neurons, astrocytes also play a role in maintenance of the BBB and communication with the immune system via antigen presentation and cytokine/chemokine production **(Kimelberg and Nedergaard, 2010, Wang and Bordey, 2008, Ransom et al., 2003, Kimelberg, 2010)**. They also regulate synaptic transmission by processing and integrating neuronal activity and coordinating calcium waves, suggesting astrocyte involvement in intercellular communication **(Kimelberg and Nedergaard, 2010, Wang and Bordey, 2008, Ransom et al., 2003, Kimelberg, 2010)**. Table 1.1 lists various types of astrocyte functions.

Astrocytes also express a wide range of receptors such as G protein coupled receptors (GPCRs), ionotropic receptors as well as receptors for growth factors, chemokines, cytokines, steroids, toll-like receptors (TLRs), and, themselves, release neurotransmitters, growth factors and other signalling molecules allowing them to be involved in both normal and pathophysiological processes taking place in the CNS **(Kimelberg and Nedergaard, 2010, Wang and Bordey, 2008, Ransom et al., 2003, Kimelberg, 2010)**. Figure 1.7 illustrates some of the large repertoire of proteins expressed by astrocytes.

During CNS damage or injury astrocytes facilitate healing of the injured tissue, form glial scars to limit the extent of damage and release pro-inflammatory cytokines and chemokines leading to activation of microglia and influx of leukocytes into the CNS. Astrocytes have also been found to present myelin antigens to T cells in MS **(Fontana et al., 1984)**. Although others have shown that only microglia, and not astrocytes, present myelin basic protein (MBP) antigens to T cells and astrocytes are involved in suppression of T cell proliferation and release of anti-inflammatory cytokines but not in phagocytosis **(Matsumoto et al., 1992)**.

2.3 Similarities and differences between rodent and human astrocytes

Part of our knowledge about astrocyte biology and function comes from *in vitro* studies that use mouse or rat primary cells. While human and rodent brain cells share many similarities there are also differences between species that need to be taken into account when conducting research **(Oberheim et al., 2009)**. For instance, both rodent and human astrocytes respond to metabotropic agonists by intracellular Ca^{2+} changes. On the other hand, human astrocytes are more varied in morphology and are approximately 2.6-fold larger than rodent astrocytes **(Oberheim et al., 2009)**. Murine and human astrocytes also express different receptors. For instance, mouse astrocytes express a wide range of toll-like receptors (TLRs) while human astrocytes preferentially express TLR3 and do not express TLR4 receptors **(Farina et al., 2005)**. These differences in cell biology may account for the fact that many compounds fail when tested in humans in spite of excellent therapeutic effects in animals. Table 1.2 lists the similarities and differences between rodent and human astrocytes **(Oberheim et al., 2009).**

2.4 Astrocyte involvement in disease

Because astrocytes are key for proper brain functioning they have also been implicated in many neurodegenerative diseases such as multiple sclerosis (MS), Parkinson's disease (PD) and Alzheimer's disease (AD) **(Sidoryk-Wegrzynowicz et al., 2010, Sriram, 2011)**. Their increased activity exacerbates the inflammatory response driving the progression of these diseases. Reactive astrocytes are the most abundant cell type in MS plaque and have been found in non-inflamed MS and experimental autoimmune encephalomyelitis (EAE) lesions without the presence of other

inflammatory infiltrates **(D'Amelio et al., 1990, Morcos et al., 2003, Smith and Sommer, 1992)**. Figure 1.8 illustrates how astrocytes exacerbate the pathophysiology of multiple sclerosis. Astrocytes also contribute to neurodegeneration in Parkinson's disease via release of toxic molecules such as 1-Methyl-4-phenylpyridinium (MPP^+) that is taken up by dopaminergic neurons and causes neurotoxicity **(Rappold and Tieu, 2010)**. In Alzheimer's disease, β-amyloid plaques activate astrocytes, which in turn secrete pro-inflammatory cytokines that exacerbate the neuronal injury taking place during Alzheimer's disease **(Johnstone et al., 1999)**.

There is a bi-directional communication between astrocytes and peripheral immune cells via cytokine/chemokine system and other signalling molecules. At the site of injury, astrocytes release cytokines and chemokines to activate resident immune cells and induce influx of leukocytes from the periphery **(Miljkovic et al., 2011, Miljkovic et al., 2007)**. They stimulate interferon gamma (IFN-γ) and IL17 production by T helper 1 (Th1) and Th17 cells in multiple sclerosis plaques and respond to the stimulation with these cytokines by increasing expression of inducible nitric oxide (iNOS), NO and other pro-inflammatory mediators such as IL6 implicated in multiple sclerosis pathogenesis **(Miljkovic et al., 2011, Bo et al., 1994, Miljkovic et al., 2007)**. Increased levels of IL6 enhance its own production in a positive feedback loop and also drive IL17 synthesis and release **(Ma et al., 2010, Meares et al., 2012, McGeachy et al., 2007)**. Some studies have suggested that astrocytes also drive the development of EAE by phagocytosing myelin and presenting myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP) antigens to $CD4^+$ T cells. However, the role of astrocytes and their phagocytosing capabilities remain a topic of debate in the field **(Tan et al., 1998, Kort et al., 2006, Lee et al., 1990, Fontana et al., 1984)**.

Increased numbers of astrocytes in inflamed areas of the CNS do not necessarily indicate that astrocytes deteriorate the disease states. Astrocyte activities within inflamed brain also have protective functions via secretion of neurotropic factors and antioxidants, scavenging reactive oxygen species (ROS), elimination of free radicals and toxic molecules, and sealing the inflamed area from the healthy tissue **(Lin et al., 1993, Knott et al., 2002, Sidoryk-Wegrzynowicz et al., 2010)**. Astrocytes are also capable of inducing apoptosis in T cells thus limiting the extent of inflammation **(Hara et al., 2011)**. However, it might be beneficial to moderate astrocyte activity when the degree of inflammation is detrimental to healthy tissue or in autoimmune diseases such as multiple sclerosis where astrocytes drive activation and recruitment of peripheral immune cells that attack myelin.

Function	Details
Support to neurons	K^+ buffering
	Uptake of glutamate, gamma-aminobutyric acid (GABA), glycine and taurine
	Involvement in neurogenesis
	Control of neuronal maturation
	Production of lactate and glycogen for neurons
	Secretion of antioxidants
	Synapse formation and plasticity
	Regulation of synaptic transmission via peri-synaptic processes (ensheathment of groups of synapses to limit interaction between groups of neurons)
	Processing and integration of neuronal activity
	Promotion of myelination
Other	Involvement in angiogenesis
	BBB formation and maintenance
	Control of cerebral blood flow and vascular tone
	H_2O, O_2, CO_2, NO transport via Aquaporin 4
	Control of extracellular pH
	Production of intra- and inter-cellular Ca^{2+} waves
	Gliotransmission (e.g. adenosine triphosphate (ATP), glutamate, d-serine, polyunsaturated fatty acids)
	Promotion of oligodendrocyte wrapping around axons
	A plethora of immune functions (described in text)

Table 1.1 Astrocyte functions **(Wang and Bordey, 2008, Kimelberg, 2010, Barres, 2008, Kimelberg and Nedergaard, 2010)**.

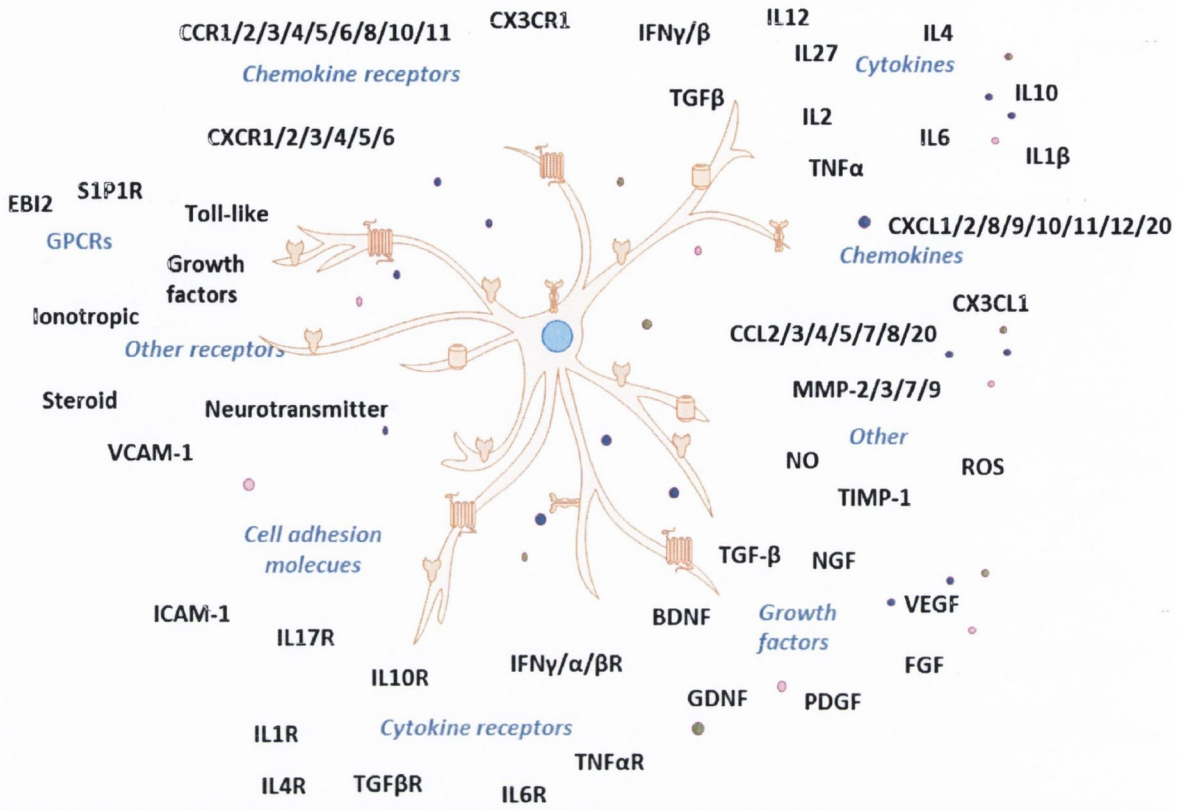


Figure 1.7 Receptors and ligands expressed in astrocytes (Sidoryk-Wegrzynowicz et al., 2010, Wang and Bordey, 2008, Dorf et al., 2000, Flynn et al., 2003, Tada et al., 1994, Rosenman et al., 1995, Larochelle et al., 2011, Gao and Ji, 2010, Milligan et al., 2008, Meeuwsen et al., 2003). Abbreviations: CCR/CXCR/CX3CR – CC/CXC/CX3C chemokine receptors; CCL/CXCL/CX3CL - CC/CXC/CX3C chemokine ligand; VCAM-1/ICAM-1 - vascular cell/intercellular adhesion molecule-1; NGF – nerve growth factors; VEGF - vascular endothelial growth factor; BDNF – brain derived neurotropic factor; GDNF - glial derived neurotropic factor; PDGF - platelet-derived growth factor; FGF - fibroblast growth factor; TIMP-1 - tissue inhibitor of metalloproteinase -1; MMP - Matrix metalloproteinase.

	Rodent Astrocytes	Human Astrocytes
Types of astrocytes	- Protoplasmic - Fibrous	- Protoplasmic (grey matter) - Fibrous (white matter) - Varicose projection astrocyte (humans only) - Interlaminar (primates only)
Dimensions	Protoplasmic astrocyte: - diameter 56.0 +/- 2.0 μm - processes diameter 2.2 +/- 0.13 μm - processes length 37.2 +/- 2.0 μm	Protoplasmic astrocyte: - diameter 142.6+/-5.8 μm (2.6 fold larger) - processes diameter 2.9 +/- 0.18 μm - processes length 97.9 +/-5.2 μm (2.6 fold longer)
Complexity	Protoplasmic and fibrous astrocytes, less complex, organized in domains with little overlap, equally spaced out. - Domain diameter 85.6 +/- 2.7 μm - Average area of domain overlap 11.8 +/- 2.2 μm^2 - Overlapping processes length 4.2 +/- 0.7 μm	Protoplasmic astrocytes are most abundant in rodent and mammalian brains but are more complex in human. Fibrous astrocytes in humans are not organized in domains but cell bodies are equally spaced out. - Domain diameter 183.2 +/- 6.1 μm - Average area of domain overlap 204.7 ± 44.1 μm^2 - Overlapping processes length 53.0 +/- 10.7 μm
Number of synapses	- 1,397 million synapses/mm^3 in rodents - 20-120,000 synapses within a single domain	- 1,100 million synapses /mm^3 in humans - 270,000 to 2 million synapses within a single domain
Number of processes	3.75 +/- 0.115	37.5 +/- 5.17 (10-fold more)
Functional differences	Slower Ca^{2+} waves (8.6 +/- 0.6 μm/s) Patches of endfeet around vasculature	Faster Ca^{2+} waves (43.4 +/- 4.7 μm/s) Endfeet completely encompass the vessel
TLR receptor expression	Express TLR2, TLR4, TLR5, TLR9	Express TLR2 and TLR3

Table 1.2 Differences between human and rodent astrocytes (adapted from **Oberheim et al., 2009, Esen et al., 2004, DeFelipe et al., 2002, Farina et al., 2005, Bsibsi et al., 2002, Bowman et al., 2003**).

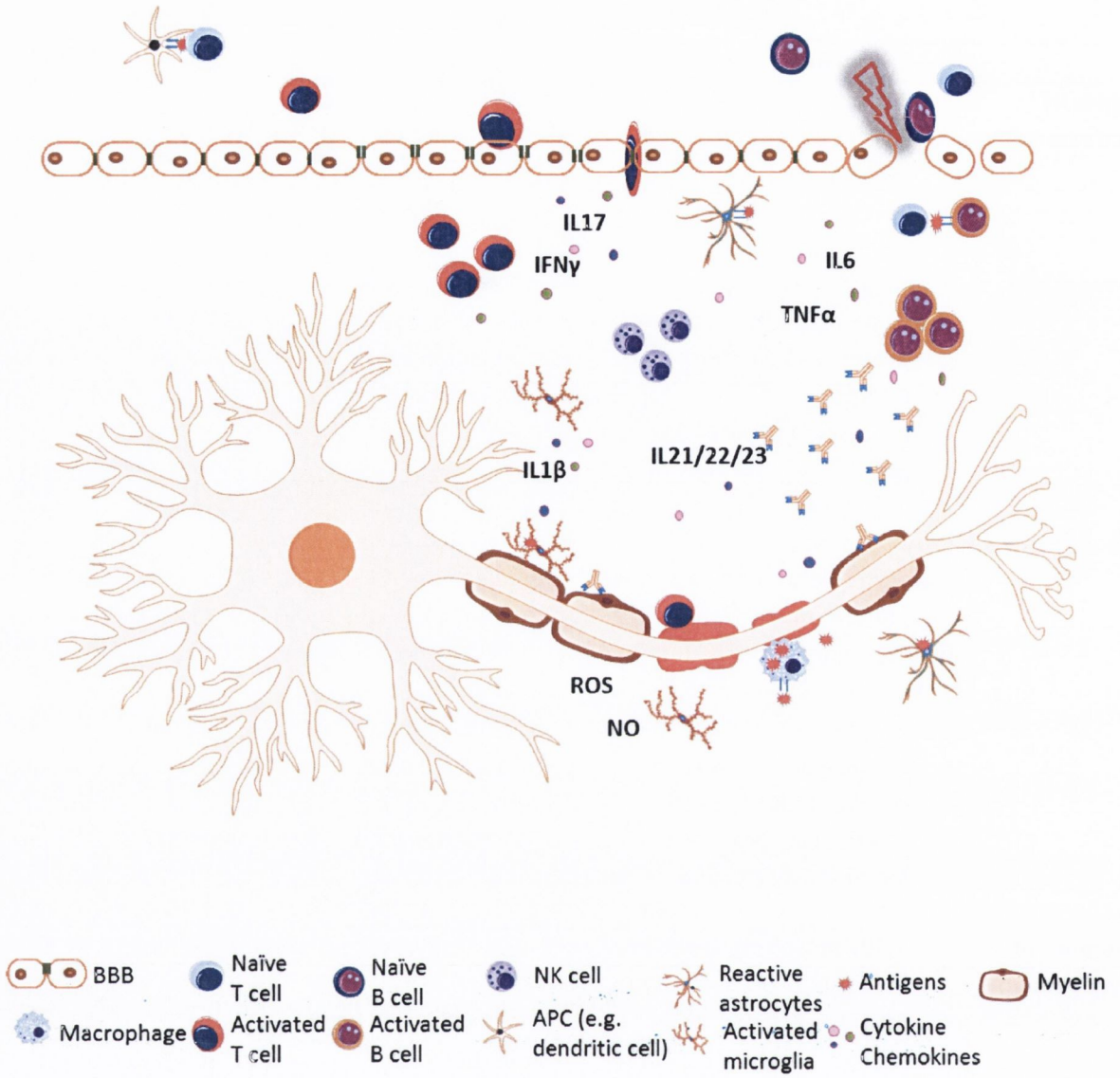


Figure 1.8 Cellular processes involved in multiple sclerosis. Leukocytes gain access to the CNS either by being pre-activated in the periphery or via damaged BBB. After crossing the BBB, T cells are reactivated in the CNS or are presented antigen and initiate Th1 type response leading to inflammation and destruction of myelin driven by cytokines. B cells contribute to inflammation and myelin destruction by production of cytokines, antigen presentation to T cells and, most of all, by production of antibodies that directly destroy myelin. Microglia/macrophages are present in MS lesions to clean cell debris, prepare the microenvironment for repair and facilitate restructuring of damaged neural circuits. They also contribute to inflammation by secreting ROS, NO, cytokines/chemokines and antigen presentation. Astrocytes respond to CNS damage by migrating to the site of injury where they release pro-inflammatory cytokines and chemokines leading to activation of microglia and influx of leukocytes. They also phagocytose debris to facilitate healing of the injured tissue and form glial scar limiting the extent of damage. They also serve as antigen presenting cells further contributing to the pro-inflammatory response **(Krumbholz et al., 2012, Larochelle et al., 2011, Keegan and Noseworthy, 2002, Neumann et al., 2009)**.

3 Oligodendrocytes

3.1 Oligodendrocyte morphology and function

As for oligodendrocytes, their main function is to form myelin, an insulating layer of lipids wrapped around neuronal axons, to ensure a fast, complete and uninterrupted conduction of electrical signals on long distances **(Snaidero and Simons, 2014)**. In addition to signal conduction, oligodendrocytes have been found to serve other functions such as axonal maintenance and regulation of axonal development and size **(Baumann and Pham-Dinh, 2001)**. Mature oligodendrocytes have many extended processes that wrap around axons of several neurons, where one axon has myelin sheets formed by several oligodendrocytes **(Snaidero and Simons, 2014, Bunge, 1968)**. One oligodendrocyte can myelinate as many as 30 axons **(Butt and Ransom, 1989)**.

Oligodendrocytes have several characteristic features that distinguish them from other glial cells such astrocytes. For instance, they are smaller in size, have greater cytoplasm and nucleus density, they do not have intermediate filaments or glycogen in the cytoplasm but, in contrast, have many microtubules in the processes **(Baumann and Pham-Dinh, 2001)**. Within their own lineage oligodendrocytes also vary in morphology and thus different classifications have been proposed. One classification, originally proposed by Del Rio Hortega, is based on the size and shape of the cell body, area where they are most numerous, thickness of myelin they form and the diameter of axon they myelinate **(Baumann and Pham-Dinh, 2001, Verkhratsky and Butt, 2013)**. Four types of oligodendrocytes have been distinguished based on the oligodendrocyte shape, size and axonal diameter. Type I/II oligodendrocytes have small cell bodies and form short and thin myelin around large number of small diameter axons. Type III/IV oligodendrocytes form thick and long myelin along fewer axons with largest diameter **(Butt et al., 1995, Baumann and Pham-Dinh, 2001)**. According to a second classification based on the cytoplasm density, there are light, medium and dark oligodendrocytes where the darkest oligodendrocytes are the most mature ones **(Mori and Leblond, 1970)**. In addition, there are other non-myelinating oligodendrocytes, NG2-glia and satellite oligodendrocytes **(Verkhratsky and Butt, 2013)**.The NG2-glia are characterised by expression of NG2 chondroitin sulphate proteoglycan (CSPG4) and the platelet-derived growth factor α (PDGFRα), and they do not express the A2B5 marker for oligodendrocyte progenitor cells (OPCs) or 29,39-cyclic nucleotide-39-phosphohydrolase (CNPase) for myelinating oligodendrocytes **(Verkhratsky and Butt, 2013, Richardson et al., 2011, Nishiyama et al., 2009)**. The NG2-glia, common in the white and grey matter, are a source of oligodendrocyte progenitor cells (OPCs) in mature CNS but also have been found to form synapses with neurons and react to neurotransmitters released in the synapses **(Verkhratsky and Butt, 2013, Richardson et al., 2011, Kang et al., 2010)**. The satellite oligodendrocytes of grey matter express CNPase, A2B5 and PDFRβ but do not express NG2 or PDGFRα **(Takasaki et al., 2010, Szuchet et al., 2011)**. Their function is currently unknown **(Verkhratsky and Butt, 2013)**.

3.2 Myelin development and maintenance

Oligodendrocyte precursors originate from the epithelial cells in the ventricular zone **(Baumann and Pham-Dinh, 2001, Gallo and Deneen, 2014)**. In humans, myelination begins at the foetal stage in the spinal cord, peaks at one year of life and is complete by 20 years. In mouse, myelination starts after birth, also the in spinal cord, and is complete by 45-60 post-natal days **(Baumann and Pham-Dinh, 2001)**. In mammals, oligodendrocytes mature during early post-natal period. At this time they are highly motile and after they have positioned themselves along mature axons they lose the ability to

migrate and proliferate and begin to mature **(Baumann and Pham-Dinh, 2001)**. Oligodendrocytes undergo several stages of development before they become fully mature myelin forming cells. Initially, they are migratory and mitotic precursors, then they develop into OPCs and finally become post-mitotic myelinating cells **(Baumann and Pham-Dinh, 2001)**. Different stages of oligodendrocyte development are characterised by expression of various cell surface and intracellular antigens that appear or disappear at precisely orchestrated developmental stages. Figure 1.9 shows expression of oligodendrocyte and myelin markers during differentiation and maturation (adapted from **Baumann and Pham-Dinh, 2001, Rettig and Old, 1989, Hardy and Reynolds, 1993, Pfeiffer et al., 1993, Arnett et al., 2004, Nishiyama et al., 2009, Schumacher et al., 2012, Verkhratsky and Butt, 2013**).

Various hormones and several growth factors regulate oligodendrocyte maturation, differentiation and survival. The most important ones include PDGF, basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), neurotrophin-3 (NT-3), glial growth factor (GGF), ciliary neurotophic factor (CNTF) and transforming growth factor β (TGFβ), **(Baumann and Pham-Dinh, 2001, Verkhratsky and Butt, 2013)**. OPC differentiation, migration and proliferation are regulated by neurons and astrocytes via signalling molecules such as leukemia inhibitory factor (LIF), IL6, IL11, IL2, TNFα, IFNγ, IL1β, CXCL1 and CXCL12 which either inhibit or stimulate the OPCs **(Hardy and Reynolds, 1993, Baumann and Pham-Dinh, 2001, Richardson et al., 2000, Verkhratsky and Butt, 2013)**. Regulation of numbers of mature oligodendrocytes during development is dependent on activation of programmed cell death **(Hardy and Reynolds, 1993, Baumann and Pham-Dinh, 2001, Richardson et al., 2000)**.

3.3 Myelin-specific and other proteins expressed in oligodendrocytes

Myelin is built in 70% from lipids such as cholesterol, phospholipids (e.g. galactocerebroside (GalC)) and glycolipids, and in 30% from other proteins, which are described in more detail in Table 1.3 (adapted from **Baumann and Pham-Dinh, 2001, Verkhratsky and Butt, 2013**). In addition, markers of oligodendrocyte maturation include nestin, isoform DM-20 of proteolipid protein (PLP/DM-20), PDGFRα, polysialylated neural cell adhesion molecule (PSA-NCAM), ganglisides GD3, A2B5, neuron-glial antigen 2 (NG2) and O4. Moreover, specific oligodendrocyte proteins include GalC and RIP antigen, while the main myelin-specific proteins are CNPase, PLP, myelin basic protein (MBP), myelin-associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG) **(Baumann and Pham-Dinh, 2001)**. Other minor myelin-specific proteins are myelin/oligodendrocyte specific protein (MOSP), myelin-associated/oligodendrocyte specific protein (MOBP), oligodendrocyte-myelin glycoprotein (OMgp), NOGO, P2, transferrin and others **(Verkhratsky and Butt, 2013)**. CNPase is the first expressed myelin-specific protein which is promptly followed by expression of MBP along with PLP and MAG. Expression of these proteins occurs just before myelination begins **(Baumann and Pham-Dinh, 2001)**. The last protein to be expressed is MOG, which is only present in mature myelin sheets. More details regarding the function and expression of these myelin-specific proteins are discussed in Table 1.3.

Similar to astrocytes, oligodendrocytes express a wide range of receptors including voltage-operated ion channels (potassium, sodium, calcium, chloride and acid-sensing), glutamate receptors (both ionotropic and metabotropic), purinergic receptros (P1, P2X, P2Y), neurotransmitter receptors such as gamma-aminobutyric acid (GABA), glycine, acetylcholine and dopamine receptors **(Verkhratsky and Butt, 2013)**. In addition, they also express other receptors such as cannabinoid receptors, opioid receptors, bradykinin receptors and adrenaline receptors **(Verkhratsky and Butt, 2013)**. As was

described above, oligodendrocyte proliferation, migration and differentiation are regulated, among other signals, via growth factors and cytokine/chemokine signals. Accordingly, oligodendrocytes express receptors for these signalling molecules **(Verkhratsky and Butt, 2013)**.

3.4 Diseases of oligodendrocytes

Leukodystrophies are genetic diseases of myelin that affect humans and can be a result of dysmyelination (defective structure of myelin), hypomyelination (decreased myelination) or demyelination (loss of normal myelin) **(Baumann and Pham-Dinh, 2001)**. They are characterised by failure of myelin to form properly during development in early life, for instance in Pelizaeus-Merzbacher, or as a result of metabollic dysfunction such as occur in Krabbe, Alexander or Canavan diseases. Damage to oligodendrocytes leading to loss of myelin also occurs in disorders called leukoencephalopathies. Leukoencephalopathies are a group of diseases where demyelination is a result of factors such as ischemia, mitochondrial or neuronal dysfunction **(Baumann and Pham-Dinh, 2001)**.

Oligodendrocytes and myelin can also be damaged as a result of bacterial, viral or protozoal infection and factors such as toxins, CNS trauma, neurodegeneration, autoimmunity, neuroinflammation, radiation, stroke, diabetes or hypertension **(Merrill and Scolding, 1999)**. Oligodendroglia cell death has also been shown to be triggered by immune mediators and factors such as cells (T cells, macrophages and microglia), cytokines (FGF, NGF, IFNγ, IL2, TNFα) or various brain affecting diseases (e.g. CNS human immunodeficiency virus). Indeed, the list of agents that are harmful to oligodendrocytes is long and includes free radicals, calcium and toxins such as lysophosphatidylcholine (LPC), psychosine or ceramide (reviewed in **Merrill and Scolding, 1999**). There are several *in vivo* animal models of the above diseases that provide insight into the mechanisms mediating myelin damage. For instance, cytokine knock-out mouse models allow to study the effects of pro-inflammatory cytokines on myelin development and damage **(Merrill and Scolding, 1999)**. Other genetic mouse models of myelin diseases include the jimpy, rumpshaker, shiverer, quaking or twitcher mice characterised based on their phenotypes and myelin deficits **(Baumann and Pham-Dinh, 2001)**. The jimpy and rumpshaker mice both have mutations in the PLP protein. The jimpy mutants have reduced populations of oligodendrocytes by 50% and deficits in myelin sheath axon coverage and compaction. They are also characterised by tremor, seizures and death at postnatal week four **(Duncan et al., 1989, Knapp et al., 1986, Baumann and Pham-Dinh, 2001)**. The rumpshaker mutants, on the other hand, do not die prematurely, have somewhat higher numbers of oligodendrocytes than the WTs but exhibit hypomyelination and astrocytosis **(Griffiths et al., 1990, Fanarraga et al., 1992, Baumann and Pham-Dinh, 2001)**. The shiverer mutants lack MBP protein and as a result they have a reduced lifespan of four to five months and exhibit tremor and seizures **(Baumann and Pham-Dinh, 2001)**. In these mice there is an apparent lack of compacted myelin, hypomyelination and absence of the major dense line **(Privat et al., 1979, Mikoshiba et al., 1981, Baumann and Pham-Dinh, 2001)**. The twitcher mutant is a model of Krabbe disease, a human globoid cell leukodystrophy, where the lipid metabolising enzyme, galactosylceramidase, has been mutated **(Suzuki, 1983)**. In these mice effects of mutation start to appear at P20-22 when incoordination, weakness and tremors start to appear and ends with their death at postnatal month three **(Suzuki, 1983)**. There is also marked loss of axons, astrogliosis and demyelination throughout the CNS **(Suzuki, 1983)**.

There are also several induced models of demyelination mediated by immune activation, viral infection or toxic agents **(Baumann and Pham-Dinh, 2001)**. The examples include the autoimmune encephalomyelitis (EAE) model of multiple sclerosis, A59 mouse hepatitis virus model, and toxin-mediated cuprizone, ethidium bromide and LPC models **(Merrill and Scolding, 1999, Baumann and Pham-Dinh, 2001)**. The EAE model, for instance, is the most commonly used animal model of multiple sclerosis. Importantly, there are many subtypes of the EAE model, although they do not represent the full MS pathology, given its heterogeneity **(Mix et al., 2008, Mix et al., 2010)**. The toxin-induced models induce dysfunction in mitochondrial DNA leading to necrosis **(Baumann and Pham-Dinh, 2001)**. In the cuprizone model there is a widespread loss of oligodendrocytes however, the axons remain intact **(Ludwin, 1978)**. Recovery, although not complete, of myelin begins within a week of return to the normal diet **(Ludwin, 1978)**. Similarly, in the LPC model, injection of LPC, an activator of phospholipase A2, into specific areas in the brain induces local demyelination followed by remyelination within one month **(Nathoo et al., 2014)**.

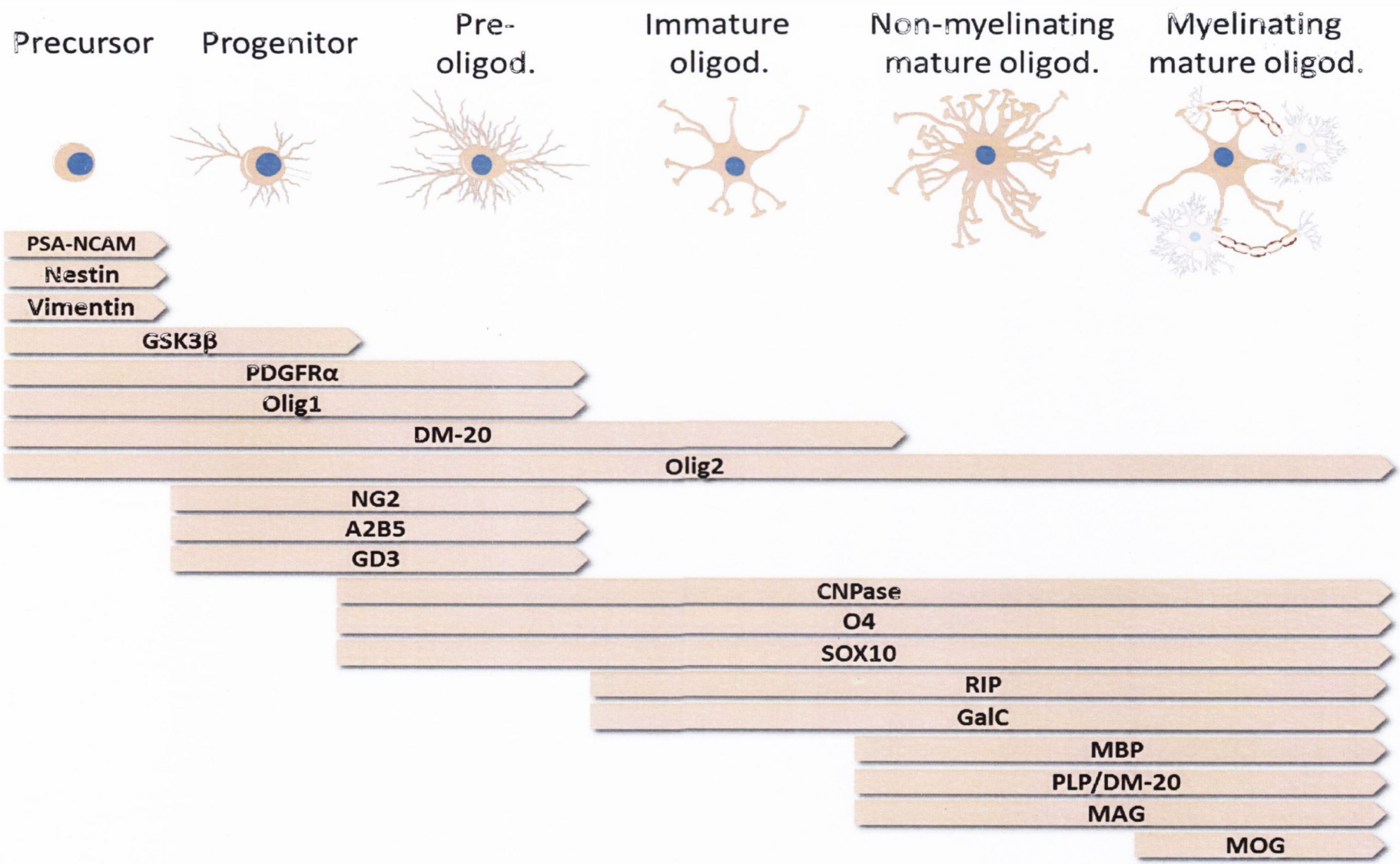


Figure 1.9 Oligodendrocyte markers expressed during development (adapted from **Baumann and Pham-Dinh, 2001, Rettig and Old, 1989, Hardy and Reynolds, 1993, Pfeiffer et al., 1993, Arnett et al., 2004, Nishiyama et al., 2009, Schumacher et al., 2012, Verkhratsky and Butt, 2013**). Olig1/2 (oligodendrocyte transcription factor 1/2).

Myelin-specific proteins	Function and expression
PLP	Constitutes 50% of all myelin proteins Human and rodent sequences are 100% identical Important for stabilisation of membrane junction which occurs after compaction Has four transmembrane domains, two outside and three intra-cytoplasmic domains Expressed at the intra-periodic and major dense line Also expressed in PNS in Schwann cells and transcripts also present in macrophages and lymphocytes
MBP	Constitutes 30% of all myelin proteins Earliest expression found in the spinal cord in embryonic day 14 mice Characterised by many isoforms and various masses Isoforms 20.2 and 21.5 kDa in humans are expressed during myelin formation and are found in chronic MS lesions where they correlate with remyelination Important for myelin compaction
CNPase	Constitutes 4% of all myelin proteins Earliest expression detected in mouse spinal cord at embryonic stages Also expressed in photoreceptors Is not present in compact myelin but localises in paranodal loops and cytoplasm of non-compacted myelin Expressed on plasma membrane Transcripts also present in lymphocytes
MAG	Constitutes 1% of all myelin proteins Has a membrane-spanning domain and an extracellular part with five segments Regulates fyn tyrosine kinase, which is important for myelination Expressed in the periaxonal part of myelin Gene knock-out results in delayed myelin compaction Involved in interactions between oligodendrocytes and neurons Inhibits axonal regeneration Also present in Schwann cells
MOG	Constitutes <0.1% of all myelin proteins Expressed only in mammals Present on the outside layer of compact myelin and in the plasma membrane of oligodendrocytes Consists of only one transmembrane domain Coincides with final stages of myelin maturation Can induce T-cell mediated inflammation and antibody-mediated demyelination in EAE

Table 1.3 Myelin-specific proteins and their function (adapted from **Baumann and Pham-Dinh, 2001**).

4 EBI2 and oxysterols in disease

4.1 EBI2 in disease

Dysregulated expression of EBI2 has been found in melanoma metastases and some neoplasmic and autoimmune diseases such as lymphocytic leukaemia or systemic lupus erythematosus **(Aalto et al., 2001, Ye et al., 2003, Qin et al., 2010)**. EBI2 expression was also shown to be increased in chronic rhinosinusitis with nasal polyps **(Hulse et al., 2013)**. Type 1 Diabetes (T1D) is a macrophage-associated autoimmune disease which has been linked to differential expression of interferon regulatory factor 7 (IRF7) driven inflammatory network (IDIN) genes **(Heinig et al., 2010)**. EBI2 regulates these genes and knock-down of EBI2 in rat macrophages results in increased expression of IRF7 - a master regulator of the type 1 interferon response. Suggesting that EBI2 is a negative regulator of the innate immune response in macrophages **(Heinig et al., 2010)**. There is some evidence suggesting that EBI2 might play an important role in EBV-associated diseases **(Norregaard et al., 2011)**. For instance, EBI2 is overexpressed in EBV positive post-transplant lymphoproliferative disorder (PTLD) samples as opposed to EBV negative samples **(Craig et al., 2007)**. In addition, Epstein-Barr virus (EBV) infection of Burkitt's lymphoma cells *in vitro* results in an induction of nine genes including EBI2 which expression increases over 200-fold **(Birkenbach et al., 1993)**. The exact role of EBI2 in EBV-associated diseases remains however to be elucidated **(Norregaard et al., 2011)**.

4.2 Hereditary spastic paraplegias type 5

Various oxysterols have been implicated in a wide range of peripheral and CNS diseases such as atherosclerosis **(Zieden et al., 1999, Harik-Khan and Holmes, 1990)**, inflammatory bowel disease **(Biasi et al., 2009)**, osteoporosis **(Liu et al., 2005)**, Alzheimer's disease **(Papassotiropoulos et al., 2000, Bjorkhem, 2006, Fukumoto et al., 2002, Koldamova et al., 2003, Lutjohann et al., 2000, Vaya and Schipper, 2007, Vega et al., 2003)**, Parkinson's disease **(Bosco et al., 2006, Rantham Prabhakara et al., 2008)**, multiple sclerosis **(Teunissen et al., 2007, Leoni et al., 2004, Diestel et al., 2003, Leoni et al., 2002, Leoni et al., 2005, Teunissen et al., 2003)**, age-related macular degeneration **(Ong et al., 2003, Rodriguez et al., 2004)** and hereditary spastic paraplegia type 5 (SPG5) **(Schule et al., 2010)**. Table 1.4 summarises the diseases where EBI2 or oxysterols have been implicated. A particularly interesting link between EBI2 signalling and disease is seen in patients with spastic paraplegia gene 5 (SPG5), primarily due to support from genetic studies. In particular, mutations in CYP7B1 gene have been identified as the cause of SPG5. This rare autosomal recessive neurodegenerative disorder belongs to a group of diseases called hereditary spastic paraplegias, and is characterised by neuropathy of upper motor neurons, optic atrophy as well as periventricular and subcortical white matter lesions as shown on magnetic resonance imaging **(Biancheri et al., 2009, Schule and Schols, 2011)**. The mutation in CYP7B1 gene leads to decline in a cytochrome P450 enzyme, CYP7B1, which in turn results in increased levels of oxysterol 27HC in cerebrospinal fluid (CSF) **(Roos et al., 2013)**.

4.3 Alzheimer's disease

Increased levels of 27HC in CSF have also been found in patients with Alzheimer's disease **(Leoni et al., 2004)**. Alzheimer's disease is a progressive debilitating neurodegenerative disease characterised by aggregation of β-amyloid plaques in the brain and extensive loss of neurons **(Vejux and Lizard, 2009)**. Increased levels of 27HC in Alzheimer's disease may be explained by the loss of metabolically active neurons leading to a decrease in CYP7B1 needed to metabolise 27HC **(Meaney et al., 2007)**. However, there is a possibility that 27HC gains access to the brain from the circulation via a damaged

blood-brain barrier, which is a hallmark of many neurodegenerative diseases **(Blennow et al., 1990, Bjorkhem et al., 2009)**. In addition, there is a genetic predisposition to Alzheimer's disease in patients carrying the Swedish amyloid precursor protein 670/671 mutation **(Shafaati et al., 2011)**. It has been shown that these patients have marked accumulation of 27HC which might accelerate neurodegeneration **(Shafaati et al., 2011)**. Studies have found altered levels of 24SHC and 27HC in the brain samples of Alzheimer's disease patients that may contribute to the formation of β-amyloid plaques **(Bjorkhem et al., 2006, Lutjohann et al., 2000)**. Plasma ratio of 24SHC to cholesterol might therefore be a marker for Alzheimer's disease **(Papassotiropoulos et al., 2000)**. Statins inhibit cholesterol accumulation, decrease 24SHC plasma levels and also limit β-amyloid formation **(Vega et al., 2003)**. *In vitro* studies suggest that reduction of cholesterol inhibits β-amyloid formation in hippocampal neurons while *in vivo* studies showed that increased cholesterol uptake in mice leads to greater amyloid deposits **(Simons et al., 1998, Refolo et al., 2000)**. In addition, in membranes with low cholesterol content there is evidence of less β-amyloid being formed **(Simons et al., 1998, Puglielli et al., 2003)**.

There is some contradicting evidence regarding the role of oxysterol-induced ATP-binding cassette transporter one (ABCA1) levels. Some studies suggest that in mouse and human neuroblastoma cells oxysterol-induced ABCA1 leads to a rise in secreted β-amyloid **(Fukumoto et al., 2002)**. On the other hand, 22HC has been shown to increase ABCA1 levels but decrease β-amyloid secretion in primary rat neurons, astrocytes and microglia **(Koldamova et al., 2003, Sun et al., 2003)**. More importantly, 24SHC and 27HC have been shown to inhibit β-amyloid synthesis and amyloid precursor protein (APP) release in primary neuronal cultures **(Brown et al., 2004)**. In addition, CD36 receptor, which is involved in β-amyloid uptake by microglia and is upregulated by oxysterols, has been suggested to be involved in Alzheimer's disease by enhancing β-amyloid build-up **(Leonarduzzi et al., 2008, Koenigsknecht and Landreth, 2004)**.

4.4 Multiple sclerosis

Multiple sclerosis is an autoimmune, neurodegenerative disease of the CNS of unknown origin, which is characterised by inflammation, demyelination, neuronal loss and occurrence of sclerotic plaques **(Compston and Coles, 2002)**. It has been suggested that during progression of multiple sclerosis when myelin sheaths are being damaged, large quantities of reactive oxygen species (ROS) are being released which, in turn, lead to oxidation and breakdown of more myelin. Since the myelin sheath is made up primarily of cholesterol, when broken down, it may act as a source of oxysterols that might have cytotoxic effects on brain cells **(Leoni et al., 2005, Diestel et al., 2003)**. In support of this, abnormal levels of oxysterols have been detected in the brains and cerebrospinal fluid of EAE animals and multiple sclerosis patients **(Diestel et al., 2003, Leoni et al., 2005)**. Some studies suggest that certain oxysterols exacerbate the pathology of multiple sclerosis and development of EAE **(Teunissen et al., 2007, Teunissen et al., 2003, Islam et al., 2012, Quintana et al., 2008)**. Others have found that genes such as CH25H are upregulated in microglia during demyelinating and remyelinating stages of EAE to support myelination **(Olah et al., 2011)**. Also, a study showed that CH25H deficient mice develop an exacerbated course of EAE **(Reboldi et al., 2014)**.

The brain is the only source of 24SHC in the human body and therefore levels of circulating 24SHC have been proposed as biomarkers for brain cholesterol homeostasis and neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, Parkinson's disease or Huntington disease **(Bjorkhem and Meaney, 2004, Leoni et al., 2002, Lutjohann et al., 2000, Leoni and Caccia, 2011)**.

Plasma levels of 24SHC is proportional to the number of metabolically active neurons and levels of 24SHC in the circulation correlate with BBB dysfunction **(Leoni and Caccia, 2011)**. In addition, high incidence of Epstein-Barr virus (EBV) infection has been found in multiple sclerosis patients although it is not certain whether the MS patients are more susceptible to the infection or whether EBV infection exacerbates the diease **(Cocuzza et al., 2014, Kvistad et al., 2014, Torring et al., 2014)**.

4.5 Parkinson's disease

Parkinson's disease is an advancing neurodegenerative disorder characterised by a marked loss of dopaminergic neurons and presence of abnormal aggregates of proteins called Lewy bodies **(Vejux and Lizard, 2009)**. Apoptosis, mitochondrial dysfunction and oxidative stress have been suggested as causes of this disease **(Rantham Prabhakara et al., 2008)**. Studies have found a link between 27HC, increased levels of α-synuclein and cell apoptosis in Parkinson's disease **(Rantham Prabhakara et al., 2008)**. In addition, oxysterol levels in cerebral cortices of patients with presence of Lewy bodies where found to be much higher than in matched controls suggesting that increased levels of oxysterols found in Lewy bodies may lead to greater α-synuclein fibrilization and progression of Parkinson's disease **(Bosco et al., 2006)**.

4.6 Age-related macular degeneration

Age related macular degeneration is characterised by deposits of macular drusen that build up in the Bruch's membrane of the eye. They are mainly made up of glycolipids, proteins, and cellular debris. Age related macular degeneration leads to a loss of central vision in aged population **(Parmeggiani et al., 2013)**. Cholesterol and oxysterols have been found in primate retina and it has been suggested that during age-related macular degeneration disease development cholesterol is being oxidised which might lead to the formation of oxysterols that, in turn, accumulate and lead to cytotoxic events, which contribute to the development of the disease **(Moreira et al., 2009, Rodriguez et al., 2004)**.

Disease	Target	References
Melanoma metastases	EBI2	**(Qin et al., 2010)**
Neoplasmic diseases (e.g. lymphocytic leukaemia)	EBI2	**(Aalto et al., 2001)**
Autoimmune diseases (e.g. systemic lupus erythematosus)	EBI2	**(Ye et al., 2003)**
Chronic rhinosinusitis with nasal polyps	EBI2	**(Hulse et al., 2013)**
Type 1 Diabetes (T1D)	EBI2	**(Heinig et al., 2010)**
EBV infection (e.g. MS, PTLD)	EBI2	**(Norregaard et al., 2011)**
Atherosclerosis	7βHC, 26HC	**(Zieden et al., 1999, Harik-Khan and Holmes, 1990)**
Inflammatory bowel disease	7-K, 7αHC, 7βHC, α-EPOX and β-EPOX	**(Biasi et al., 2009)**
Osteoporosis	Cholestane-3β,5α,6β-triol	**(Liu et al., 2005)**
Alzheimer's disease	24SHC; 22(R)HC; 25HC; 27HC; CYP7B1	**(Papassotiropoulos et al., 2000, Bjorkhem, 2006, Fukumoto et al., 2002, Koldamova et al., 2003, Lutjohann et al., 2000, Vaya and Schipper, 2007, Vega et al., 2003) (Meaney et al., 2007, Yau et al., 2003)**
Parkinson's disease	24SHC; 27HC	**(Bosco et al., 2006, Rantham Prabhakara et al., 2008)**
Multiple sclerosis EAE	7-ketocholesterol; 24SHC; 27HC; CH25H	**(Teunissen et al., 2007, Leoni et al., 2004, Diestel et al., 2003, Leoni et al., 2002, Leoni et al., 2005, Teunissen et al., 2003, Kvistad et al., 2014, Cocuzza et al., 2014, Torring et al., 2014) (Olah et al., 2011)**
Age-related macular degeneration	7-ketocholesterol; 24SHC	**(Ong et al., 2003, Rodriguez et al., 2004)**
Hereditary spastic paraplegia type 5 (SPG5)	CYP7B1; 27HC	**(Biancheri et al., 2009, Schule and Schols, 2011) (Schule et al., 2010)**
Huntington disease	24SHC	**(Bjorkhem and Meaney, 2004, Leoni et al., 2002, Lutjohann et al., 2000, Leoni and Caccia, 2011)**

Table 1.4 EBI2 and oxysterols implicated in diseases (adapted from **Vejux and Lizard, 2009**).

5 Closing statement

In summary, evidence suggests that EBI2 and oxysterols are important players not only in the immune system but also in the central nervous system. Proper functioning of the EBI2/oxysterol system seems crucial for healthy physiology and prevention of the onset or progression of several neurodegenerative diseases. Therefore, knowledge about EBI2 expression and function in the CNS, as well as the immune system, might be beneficial in managing neurological and neurodegenerative disease with inflammatory component. In this thesis we have attempted to determine the role of EBI2 in astrocytes **(Results 1)**, signalling between astrocytes and macrophages **(Results 2)** and finally EBI2 role in oligodendrocytes and myelination **(Results 3)**.

Chapter 2: Materials and methods

1 Materials

1.1 Compounds

EBI2 antagonist (NIBR189) **(Gessier et al., 2014)** and 7α25HC were prepared as 10 mM stock solutions dissolved in 90% dimethyl sulfoxide (DMSO, Sigma). All experiments used the pure active (S)-enantiomer of *p*FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl] propane-1,3-diol) prepared as a 10 mM stock solution in 90% DMSO and 20 mM HCl. Cholesterol was prepared as 10 mM solution in 90% ethanol and glutamate was prepared as a 100 mM stock solution (Sigma, G1251). Lipopolysaccharide (LPS) was prepared as 1 mg/ml stock in H_2O (Sigma, L4391). The final concentration of vehicles (DMSO or ethanol) was below 0.01% and therefore, the additional vehicle control was not included in the experiments. Lysolecithin from egg yolk (L-α-Lysophosphatidylcholine, LPC, Sigma, L4129) was prepared as 25 mg/ml stock in serum-free media. The cytokines used were interleukin 17A (IL17A) (R&D Systems, 317-ILB) and tumor necrosis factor α (TNFα) (R&D Systems, 210-TA).

1.2 Antibodies

Table 2.1 shows all antibodies used in the study. Primary rabbit polyclonal antibodies were: anti-glial fibrillary acidic protein (GFAP, Abcam, ab7260), anti-myelin basic protein (MBP, Abcam, ab40390), anti-Iba1 (Wako, 019-19741), anti-actin (Pierce, PA1-16889) and anti-nuclear factor κB (NFκB) p65 (Santa-Cruz, sc-109). Primary monoclonal anti-human EBI2 antibodies were generated by genetic immunization of mice **(Costagliola et al., 2000)** and made available through Euroscreeen S.A. Belgium. Two hybridoma subclones 57C9B51C9 (batch ACE20691) and 607B1C31C1 (batch ACE25368), known as 57C and 607B respectively, were used. Primary rat monoclonal antibodies were: anti-CD31 (BD, 553370), anti-CD11b FITC conjugated (BD, 553310) and anti-F4/80 (eBiosciences, 14-4801-81). Mouse monoclonal antibodies used were: anti-ERK 1/2 (p44/p42) (Millipore, 05-1152), anti-MBP (Covance, SMI 94), anti-olig1 (Millipore, MAB5540), anti-CNPase (Millipore, MAB326R), anti-MOG (Millipore, MAB5680), anti-CD68 (Serotec, MCA341R) and anti-163 (Serotec, MCA342R). The other primary antibodies used in this study were: rabbit monoclonal antibody anti-phospho-ERK 1/2 (Millipore, 05-797R) and chicken polyclonal anti-neurofilament heavy chain (Millipore, AB5539).

Secondary goat antibodies used for immunocytochemistry were: anti-mouse 488 (Invitrogen Alexa, A11029), anti-mouse 633 (Invitrogen Alexa, A21052), anti-rabbit 488 (Invitrogen Alexa, A11008), anti-rabbit 633 (Invitrogen Alexa, A21070), anti-chicken 633 (Invitrogen Alexa, A21103), anti-rat 488 (Invitrogen Alexa, A-11006), anti-rat 633 (Invitrogen Alexa, A21094). Human secondary mouse antibody used was anti-human 488 (Invitrogen Alexa, A10631). Other secondary Jackson ImmunoResearch donkey antibodies used were anti-chicken 549 (703-505-155) and anti-mouse 549 (715-505-020). Secondary antibodies used for Western blotting (WB) and conjugated to horse-radish peroxide (HRP) were: donkey anti-rabbit IgG (GE Healthcare, NA934), goat anti-mouse (Sigma, A8924).

Antibodies used for fluorescence-activated cell sorting (FACS) analysis were: Alexa Fluor 647 anti-hEBI2 (57C 9B5 1C9) mIgG2a antibody (anti-hEBI2-57, Novartis, Switzerland), Alexa Fluor 647 anti-hEBI2 (607B 1C3 1C1) mIgG2b (anti-hEBI2-607, Novartis, Switzerland), Alexa Fluor 647 mIgG2a isotype control or Alexa Fluor (BioLegend, MOPC-173) and Alexa Fluor 647 mIgG2b isotype control (BioLegend, MPC-11).

1.3 Stains

Dyes and chemicals used for cellular staining were: nuclear stain Hoechst 34580 (Invitrogen, H21486), Calcein AM (Invitrogen, C3100MP), Fluo-4 (Invitrogen, F-14127) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) (Invitrogen, M6494).

2 Animal husbandry

The EBI2 double (-/-) knock-out (KO) mouse strain (mixed genetic background C57BL/6 x C129) were purchased from Deltagen **(Hannedouche et al., 2011)**. All mice, the KO and wild type (WT) littermates were housed in filter-top cages under specified pathogen-free conditions, and a standard diet and water were provided *ad libitum*. All animal procedures were approved by the institutional ethics committee (Novartis Pharma, Basel, Switzerland and Trinity College Dublin, Ireland). For myelin development experiments, WT and EBI2 KO animals were sacrificed at indicated time points, whole brains were removed from skulls and snap frozen in liquid nitrogen. Heads of embryonic day 14 (E14) animals were detached and frozen in liquid nitrogen with the skin and remaining tissue.

3 Cell Culture

3.1 Human astrocyte culture

All cell cultures were grown at 37°C and 5% CO_2 in a humidified incubator. Human astrocytes from a fetal brain (cerebral cortex), male, at 21 weeks gestation were purchased from ScienCell Research Laboratory (1800, Lot No. 9063), as we have described previously **(Healy et al., 2013, Elain et al., 2014)**. Cells were grown in human astrocyte media (ScienCell, 1801) supplemented with 1% astrocyte growth supplement (ScienCell, 1852), 10% fetal bovine serum (FBS, Labtech, FB-1090) and 1% penicillin/streptomycin (pen/strep, ScienCell, 0503) in T75 culture flasks unless otherwise indicated in figure legends. The cells were grown until 90% confluent at which point they were plated into cell culture plates. After re-plating the cells were grown to and used when 80% confluent.

3.2 Murine astrocyte culture

Mixed glia cell cultures were prepared, as we have described previously **(Healy et al., 2013)**, from cortical tissue of zero to one day old wild type C57BL/6 or EBI2 KO mice or Wistar rats. In brief, animals were decapitated, skull removed and the revealed cortical tissue was detached from both hemispheres with forceps. The cortical tissue was cross-chopped with a scalpel, placed in pre-warmed 50% Dulbecco's Modified Eagle's Medium (DMEM) and 50% F12 (DMEM/F12, Fisher, SH30023.01) supplemented with 1% pen/strep (Sigma, P4333) and 10% FBS and then gently triturated until a clear solution was obtained. The resulting solution was passed through a cell strainer (40 µM). The tubes were then centrifuged at 3,220 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 in separate T25 flasks. The cells were incubated for two hours to allow the cells to adhere and then were flooded with further 8 ml of media. The cells were cultured for 12 days at 37°C in a humified incubator with 5% CO_2. Media was exchanged the day after preparation and then every three days. After 12 days in culture microglial cells were separated from 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 trypsynized and plated accordingly.

3.3 Culture of mouse organotypic slices

Organotypic cerebellar slice cultures are an established *in vitro* assay for studying myelin development and maintenance. They represent a closer to *in vivo* microenvironment having all the cellular components and structure found *in vivo*. They are accessible to biochemical manipulation and analysis. Various biochemical methods have been used to induce demyelination in slice cultures such as lysolecithin (LPC), lipopolysaccharide (LPS), cuprizone or MOG reactive T cells **(Notterpek et al., 1993, Sheridan and Dev, 2011, di Penta et al., 2013, Pritchard et al., 2014, Sun et al., 2012)**. Cerebellar slice cultures were prepared, using brain tissue isolated from postnatal day 10 WT C57BL/6 mice or EBI2 KO mice according to the protocol reported earlier **(Sheridan and Dev, 2011, Pritchard et al., 2014)**.

Briefly, mice were decapitated, cerebellar tissue was removed from the skull, cooled down in cold Opti-MEM (Invitrogen, 11058021), and separated from hinbrain with spatulas on ice. Cerebellum was cut into 400 µm parasagittal slices using a McIlwain tissue chopper. Slices were cooled in Opti-MEM on ice and later separated into individual slices under dissection microscope. Four to six slices were grown on each cell culture insert (Millicell, PICMORG50). Slices were cultured using an interface method with 1 ml of medium per 35 mm well. Slices were grown in 50% Opti-MEM, 25% Hanks' buffered salt solution (HBSS, Invitrogen, 14025-050), 25% heat-inactivated horse serum (Bio-Sera, HO-290) supplemented with 2 mM Glutamax (Invitrogen, 35050-038), 28 mM D-glucose (Sigma, G8769), 1% pen/strep, 10 mM HEPES (Sigma, H3784) for 12-14 days *in vitro* unless otherwise indicated in figure legends. Slice cultures were grown at 35.5°C and 5% CO_2 in a humidified incubator. Prior to treatment, slices were starved in serum free media for four hours and then transferred to fresh serum-free medium containing LPC (0.4 mg/ml) (Sigma, L-4129) and incubated overnight for 18 hours, with or without 7α25HC (1 µM) and/or NIBR189 (NIBR189, 1 µM). Following 18 hours LPC, 7α25HC and/or NIBR189 treatment, slices were transferred to medium containing 7α25HC (1 µM) and NIBR189 (1 µM) alone for further 30 hours (48 hours in total). For myelin development experiments, slices were grown in 7α25HC (1 µM) or NIBR189 (1 µM) or control media immediately after preparation and for seven, 14 or 20 days *in vitro* (DIV). Fresh compounds were added every other day when media was changed. For myelin development in WT versus EBI2 KO animals, slices were grown in complete media without compounds for indicated time. To measure the extent of neuronal demyelination, immunocytochemistry was performed.

3.4 Heterologous cell line culture

Chinese hamster ovary cells stably expressing human EBI2 (CHO-hEBI2) were made by cloning human EBI2 gene from HL-60 cDNA (GenBank accession number NP_004942) **(Hannedouche et al., 2011)**. Human EBI2 was inserted into the pEFIN3 vector at the EcoR1 and Xbal sites in the multiple cloning site and plasmids expressing human EBI2 were stably transfected in CHO-K1 expressing the apo-aequorin and Gqi5 **(Hannedouche et al., 2011)**. The CHO-hEBI2 and CHO-A21 control cell lines were grown in DMEM/F12, 10% FBS, 0.25 mg/ml zeocin (Invivogen, Ant-zn-1p), 5 µg puromycin (Sigma, P8833) and 1% pen/strep. CHO-hEBI2 cell media was supplemented with 0.4 mg G418 (Sigma, G5013). The cells were grown in T75 flasks until confluent at which point they were plated into eight-well imaging plates (Ibidi, 161107) or used for FACS analysis. Human monocyte (U937), human B cells (RS11846) and mouse macrophages (RAW264.7) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with Glutamax, 10% FBS, 1% sodium pyruvate (Invitrogen, 11360-070), 1% MEM non-essential amino acids (NEA, Invitrogen, 11140), 0.1% 2-Mercaptoethanol (Sigma, M7154), 1% pen/strep in T75 culture flasks.

4 Molecular Biology

4.1 Preparation of mRNA

For IL6 mRNA expression analysis human astrocytes were cultured in 24 well plates for 48 hours until 80% confluent. Prior to stimulation, cells were starved in serum-free media for four hours. The cells were then pre-treated with the compounds in serum free media for one hour after which IL17 (50 ng/ml) and TNF (10 ng/ml) (TNFα/IL17) were added and the cells incubated for 18 hours at 37°C. After treatment, the cells were washed twice with phosphate buffered saline (PBS), scraped in RA1 lysis buffer (Machery-Nagel, 740961) supplemented with 10% β-mercapthoethanol and frozen at -80°C. For mRNA expression of EBI2, CH25H, CYP7B1 and HSD3B7 human and mouse astrocytes were grown in T75 flasks until 80% confluent, washed twice with PBS, trypsynized (Invitrogen, 25300-096) and centrifuged for 10 minutes at 3,220 x g. Media was removed and the cells were resuspended in RA1 lysis buffer and frozen at -80°C. The U937 and RS11846 cells were centrifuged for 10 minutes at 3,220 x g and resuspended in RA1 lysis buffer and frozen at -80°C. For EBI2, CH25H, CYP7B1 and HSD3B7 mRNA expression after LPS treatment, mouse astrocytes were grown until 90% confluent, washed with PBS and starved for four hours. The cells were then treated with LPS (100 ng/ml) at indicated time points. After treatments, media was removed and frozen for use in migration experiments and the cells were washed twice with PBS, scraped in RA1 lysis buffer and frozen at -80°C. RNA was isolated using the RNeasy Mini Kit (Qiagen, 74104). To eliminate genomic DNA contamination an on-column DNase digestion was carried out with the RNase-Free DNase Set (Qiagen, 79254).

4.2 Real time quantitative polymerase chain reaction (RT-qPCR)

After reverse transcription of mRNA (10 minutes at 25°C; 120 minutes at 37°C; five seconds at 85°C) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814), RT-qPCR was performed with the 7900HT Fast Real-Time PCR System (Applied Biosystems, 4329001) according to the standard thermal cycler protocol (two minutes at 50°C; 10 minutes at 95°C; 40 cycles of 15 seconds at 95°C and one minute at 60°C). TaqMan Gene Expression Assays using TaqMan (Applied Biosystems) probes of human EBI2 (Hs00953886-m1), mouse EBI2 (Mm02620906-s1), human CH25H (Hs02379634-s1), mouse CH25H (Mm00515486-s1), human CYP7B1 (Hs00191385-m1), mouse CYP7B1 (Mm00505894-g1), human HSD3B7 (Hs00986913-g1), mouse HSD3B7 (Mm01545399), human IL6 (Hs00985639-m1), and the house keeping gene human HPRT1 (Hs02800695-m1) and mouse HPRT1 (Mm01545399-m1) all FAM dye-labeled were used. Experiments were run in 384-well plates (Greiner, 784076). Each condition was run in quadruplicates, with each quadruplicate RNA sample run in duplicates and the experiments were repeated three to four times. The threshold was set manually for all samples. The analysis was performed with the SDS 2.3 software. The relative expression of EBI2, CH25H, CYP7B1, HSD3B7 and IL6 to the reference gene (HPRT1) was determined.

5 Biochemistry

5.1 Proteome profiler antibody arrays

Human and rat astrocytes were grown in 24-well plates and starved for four hours in serum free media before treatments with LPS (100 ng/ml) with or without 7α25HC (1 μM) for four hours at 37°C. The supernatants were analysed with Human Cytokine Array Panel A (ARY005 R&D Systems) and Rat Cytokine Array Panel A (ARY008, R&D Systems) according to manufacturer's protocol, similar to our previous studies **(Sheridan and Dev, 2011)**. In brief, the detection antibody cocktail was reconstituted with 100 μl of dH_2O and 25x wash buffer was diluted to 1x with dH_2O. Four pre-coated

membranes were removed from protective sheets using flat-tip tweezers and placed in Array Buffer 6 and incubated for one hour at room temperature to block non-specific binding. While membranes were being blocked, the samples were prepared by mixing 500 µl of sample with 500 µl of Array Buffer 4 and adjusted with Array Buffer 5 or 6 (depending on the rat versus human kit) to the final volume of 1.5 ml followed by addition of Detection Antibody Cocktail and incubated for one hour at room temperature. After blocking, Array Buffer 6 was removed and the sample cocktail prepared earlier was added to each membrane and incubated overnight at 4°C on a rocker. Next day, the membranes were removed from the dish and placed in petri dishes containing 20 ml of the wash buffer. The membranes were washed twice by 10 minutes on a rocker at room temperature. Subsequently, streptavidin-HRP was diluted accordingly in Array Buffer 5 or 6 and pipetted into the four-well dish containing the membranes and incubated for one hour at room temperature on a rocker. The membranes were then washed three times as described above and exposed to a chemiluminescent reagent for five to 10 minutes (Thermo Scientific, SuperSignal West Pico Substrate, 34077/87). The membranes were then covered with a plastic wrap taking care to remove all bubbles and placed in an X-ray film cassette. The film was exposed for one and two minutes and developed with Agfa X-ray machine. The films were then scanned and densitometry of cytokine spots was analysed with ImageJ software (U.S. National Institutes of Health, www.imagej.nih.gov/ij/).

5.2 Homogeneous time resolved fluorescence (HTRF)

Human astrocytes were grown in 24 well plates and cultured for 48 hours until 80% confluent. Prior to stimulation cells were starved in serum free media for four hours. The cells were pre-treated with 7α25HC (1 µM) for one hour after which TNFα/IL17 were added and the cells incubated overnight at 37°C in 5% CO_2. Levels of IL6 in the supernatant were measured with homogeneous time resolved fluorescence (HTRF) human IL6 kit (Cisbio, 62IL6PEB) according to the manufacturer's instructions and as we have described previously **(Elain et al., 2014)**. Briefly, 8 µl of the supernatants were transferred into a 384 well plate and mixed with 8 µl of a pre-mixed IL6 detection antibody cocktail containing 4 µl of anti hIL6-XL665 and 4 µl of anti hIL6-K. The mixture was incubated for three hours at room temperature in the dark after which absorbance was read at 450 nm with Envision plate reader (Perkin Elmer, 2104). A standard curve was prepared using serial dilutions of IL6 calibrator diluted in the supplied dilute (50 mM PBS, pH 7, 0.2 % bovine serum albumin (BSA, Santa Cruz, 9048-46-8), 0.02 % NaN_3). All points of the standard curve were performed in triplicates and the samples were run in quadruplicates. The standard curve was calculated by plotting the standards against the absorbance values and levels of IL6 were measured in ng/ml.

5.3 The enzyme-linked immunosorbent assays (ELISAs)

All cells were starved in serum free media before treatments. Human astrocytes were treated with a combined treatment of IL17 (50 ng/ml) and TNFα (10 ng/ml) (TNFα/IL17) with or without 7α25HC (1 µM) overnight. Primary mouse astrocytes were treated with LPS (100 ng/ml) overnight with or without 7α25HC (1 µM), the media was collected and frozen at -20°C. Mouse cerebellar slices were treated as described above and media was collected following 18 hour LPC treatment with or without the compounds treatment and frozen at -20°C. Levels of cytokines in the supernatant were measured with the following RnD Systems ELISA kits: human IL6 (DY206), mouse IL6 (DY406), mouse TNFα (DY410) and mouse IL1β (DY401) according to the manufacturer's instructions, and similar to our previous reports **(Elain et al., 2014)**. 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 1% BSA. The plates were then washed three times with wash buffer and any remaining buffer was removed from the wells by aspiration. A standard curve was prepared using serial dilutions of the recombinant protein diluted in their reagent diluents: 1% BSA in PBS or for mouse IL1β 0.1% BSA, 0.05% Tween-20 in tris-buffered saline (TBS, Fisher, 10785341). The samples and standards were incubated in the antibody coated ELISA plate for two hours at room temperature. The plate was then washed three times with wash buffer and 50 µl of the detection antibody (diluted in their respective reagent diluents) was added to all wells. Following three further washes, 50 µl of streptavidin-HRP diluted in reagent diluents was added to each well and incubated for 20 minutes at room temperature, protected from light. Three further washes were carried out and the wells were incubated with 50 µl of substrate solution (RnD Systems, DY999) for 20-40 minutes at room temperature, protected from light. The colour reaction was stopped by the addition of 50 µl 1 M H_2SO_4 and absorbance was read immediately using a plate reader at 450 nm (Labsystem Multiskan). The standard curve was calculated by plotting the standards against the absorbance values and the cytokine levels were measured in pg/ml.

5.4 Western blot analysis

For ERK phosphorylation experiments, human astrocytes were grown in six well plates until 80% confluent. Before treatment, the cells were starved in serum free media for four hours after which they were pre-treated with or without the EBI2 antagonist (NIBR189) at indicated concentrations for 30 minutes. The compound 7α25HC (at indicated concentrations) was then added for one to 10 minutes. After indicated time the cells were washed twice with ice cold PBS, scraped in PTxE lysis buffer (1% Triton-x (Sigma, T9284), 1 mM ethylenediaminetetraacetic acid (EDTA, Sigma, E6758), PBS pH 7.4) and frozen at -20°C. Mice whole brains were quickly dissected and frozen in liquid nitrogen and then kept at -80°C until used. The brains were homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo, 89900) using Qiagen Tissue lyser (85220) with metal beads for 30 seconds three to four cycles. The protein content was then analysed with a bicinchoninic acid assay (BCA, Thermo Scientific, 23225). The samples were then equalised to 30 µg/well of protein with H_2O.

For all Western blotting the samples were mixed with the sample buffer (Bio-Rad, 161-0737 supplemented with 10% β-mercaptoethanol, Sigma, M6250) and incubated for 10 minutes at 75°C. The protein samples were then separated in 10% SDS-polyacrylamide gels and transferred onto a polyvinylidene membrane (Immobilon P, Millipore, IPVH00010). The membrane was blocked for one hour at room temperature, incubated with primary antibody overnight at 4°C, washed four times five minutes and then incubated with secondary antibody for one hour at room temperature. Blocking and antibody incubation steps were performed in PBS supplemented with 0.1% Tween-20 (PBS-T) and 5% non-fat milk and wash steps using PBS-T. After further four times five minutes wash, the membrane was incubated with chemiluminescent HRP substrate (Immobilon Western, Millipore, MDR-100-030Q) and read using Image Reader LAS-3000 (Fujifilm). Densitometry measurement of band intensity was used for quantification using ImageJ software. Bar graphs show mean relative density of the target protein to the reference protein.

6 Cellular staining studies

6.1 Fluorescence-activated cell sorting (FACS)

FACS experiments were performed by Dr. Inga Preuss in Novartis Pharmaceuticals, Basel, Switzerland. CHO-A21 and CHO-hEBI2 cell lines were plated in a Costar V bottom 96-well plate at 150,000 cells per well and stained with anti-hEBI2 antibodies 57C and 607B or isotype control diluted in FACS buffer (2% FBS, 2 mM EDTA in PBS). After incubation on ice for 30 minutes the labelled cells were washed with FACS buffer and fixed with fixation buffer (1% formaldehyde, Sigma, 47608). EBI2 cell surface expression was measured by flow cytometry using CyFlow Space with a RobbyWell plate reader (Partec). FlowJo software was used to analyse data.

6.2 Immunocytochemistry

Immunocytochemistry of human astrocytes was performed using cells plated in eight well plates (Ibidi, 161107) and cultured for 24 hours until 70% confluent. For NFκB studies the cells were starved for four hours in serum free media and then pre-treated with 7α25HC (1 μM) for one hour followed by addition of IL17 (50 ng/ml) and TNFα (10 ng/ml). The cells were incubated with the compounds and/or IL17/TNFα mix overnight at 37°C. For EBI2 internalisation studies U937 cells were washed once with PBS and incubated with 7α25HC (10 μM) or NIBR189 (10 μM) for one hour at 37°C. The U937 cells where than attached to cover slides using Thermo Scientific Cytospin 4 Cytocentrifuge (A78300003). All cells were washed with PBS and fixed with fixation/permeabilisation kit (BD Cytofix/Cytoperm, 554714) for 20 minutes on ice. Nonspecific binding was reduced by incubating cells for one hour at room temperature in TwB buffer (PBS supplemented with 0.1% Tween-20, 1% BSA). For all antibody incubation and wash steps, TwB* (PBS supplemented with 0.05% Tween-20 and 0.5% BSA) were used. The cells were incubated overnight at 4°C with the primary antibodies, washed twice and incubated with secondary antibodies for one hour. The cells were then washed twice, incubated with Hoechst in PBS for 10 minutes and washed twice again and stored in PBS at 4°C in the dark until imaged. The cells were visualized with a Zeiss LSM 700 confocal microscope. For quantification of co-localizing NFκB pixels, the co-localisation add-in to the Zeiss software was used. The software automatically, during image acquisition, calculates pixels emitting signals in two channels, Hoechst (nucleus) and Alexa 488 (NFκB) in these sets of experiments. On average, 10 images were taken per well and treatments were run in duplicates. The graphs show the mean number of co-localising pixels in 8-12 images per condition.

Immunocytochemistry for organotypic slices was performed by firstly washing the slices twice in PBS. The slices were then fixed and permeabilised by incubation in 4% paraformaldehyde (PFA) for 10 minutes on ice followed by incubation in ice-cold 100% methanol for five minutes. The slices were then washed twice five minutes in PBS and incubated overnight in the TxBN buffer (PBS supplemented with 0.5% Triton-X100, 10% BSA, 1% normal goat serum (NGS, Abcam, ab7481)) to reduce nonspecific binding. For all antibody incubation and wash steps TxB* (PBS supplemented with 0.1% Triton-X and 2% BSA) was used. The slices were incubated overnight at room temperature with the primary antibodies, washed twice and incubated overnight with secondary antibodies. The slices were then washed twice and mounted on glass cover slides with Tissue-Tek OCT Compound (Sakura, 4583). The slices were visualized with a Zeiss LSM 700 confocal microscope at various magnifications indicated in figure legends. Image stacks were initially used to analyse all layers of the slice and showed that optimal staining was confined to the central layers; images were thus taken from central layers within the tissue and then mean fluorescence intensity of MBP staining in the

central white matter tracts was analysed using ImageJ software. On average, four to six slices per condition were grown, six to eight images per slice were taken and four regions per image measured. Values in graphs show mean MBP fluorescence intensity of about 140 measurements with standard error of the mean (SEM).

6.3 MTT assay

The MTT assay is widely used as an indirect measurement of cell viability and proliferation **(Mosmann, 1983, Gerlier and Thomasset, 1986, Cai et al., 2014, Yuan et al., 2014)**. In this assay, yellow MTT is enzymatically reduced by metabolically active cells to purple formazan, which can then be solubilised and its levels measured spectrophotometrically. Human astrocytes were plated in 96-well plates and cultured for 24 hours until 70% confluent. The cells were starved for four hours and then treated overnight with 7α25HC and NIBR189 at increasing concentrations, pFTY at 0.0.1 µM and 10% FBS was used as a positive control. After the treatments, media with compounds was removed and replaced with 100 µl of fresh media supplemented with 10 µl of 12 mM MTT (Invitrogen, M6494). The cells were incubated with MTT for four hours at 37°C. Next, 85 µl of media was removed and 50 µl of DMSO per well was added for 10 minutes and incubated at 37°C. The samples were then mixed and absorbance read at 540 nm. Bar graphs show mean and SEM of the values obtained from the reader.

7 Functional Studies

7.1 Calcium signalling

Human astrocytes were plated on glass bottom, 35 mm FluoroDishes (World Precision Instruments) and grown until 80% confluent. Cells were prepared according to the protocol described earlier **(Healy et al., 2013)**. Briefly, cells were washed once with 1 ml of 37°C HBSS supplemented with 20 mM HEPES buffer and 5.5 mM glucose. Cells were incubated with 2 µM Fluo-4 AM (Invitrogen, F-14201) in supplemented HBSS (pre-warmed to 37°C) for 20 minutes in a cell culture incubator (37°C and 5% CO_2). After incubation with Fluor-4 AM, the cells were washed once with supplemented HBSS (pre-warmed to 20°C) for 20 minutes at room temperature and protected from light. After 20 minutes, the wash buffer was removed and replaced with 1 ml of fresh supplemented HBSS. The cells were treated with 7α25HC (1 µM), pFTY720 (1 µM) and cholesterol (1 µM) followed by glutamate (30 µM). Changes in calcium concentration were recorded with a Zeiss LSM 510 confocal microscope using one frame per second scanning speed. The baseline was recorded for 30 seconds after which the test compound was added followed by glutamate treatment after 180 seconds. The recording lasted for 240 seconds in total. Data analysis was performed with ImageJ software. The mean fluorescence was measured in the stacks and z-axis profile was plotted. The bar graphs show mean image fluorescence taken from the first 30 frames, which represent the baseline, compared to the following 30 to 90 frames taken during treatments.

7.2 xCELLigence impedance assay

Impedance measurements of human astrocytes were conducted using E-plate 96 (Roche Applied Biosciences) and recorded with the Real-Time Cell Analyzer (RTCA) Cardio Instrument (Roche Applied Biosciences). After plating, the cells were allowed to reach 80% confluence and then starved for four hours followed by 30 minutes pre-treatment with the 7α25HC (1 µM) and then treatment with IL17 (50 ng/ml) and TNFα (10 ng/ml). The impedance measurements were taken every 15 minutes. All conditions were run in quadruplicates. A unit-less impedance value called cell index (CI) was given to represent the changes in electrical impedance detected during measurement. The CI value is

calculated automatically by the machine's software and represents a ratio of impedance measured at a single time point divided by the baseline impedance measured at the start of the experiment.

7.3 Migration and wound healing assays

Wound healing and migration studies were conducted at 37°C and 5% CO_2 in a humidified incubator using either the Electric Cell-substrate Impedance Sensing (ECIS) platform (Applied BioPhysiscs) or the xCELLigence directed migration platform (Roche Applied Biosciences). Figure 2.1 shows the experimental setup with both assays. Human astrocyte wound healing and migration were measured using the ECIS platform **(Chan et al., 2010a, Wu et al., 2010, Jiang et al., 2008, Waters et al., 2006)**. The cells were plated on L-cystein (10 mM) pre-coated 8-well plates with 10 electrodes per well (8W10E, Applied BioPhysiscs). The attachment and growth of the cells was monitored for 24 hours using ECIS platform. Before treatment, the cells were starved for four hours in serum free media, then pre-treated with or without the EBI2 antagonist NIBR189 (0.25 μM) for 30 minutes. The compounds, 7α25HC (at indicated concentrations) and pFTY720 (0.01 μM) were then added and the cells were subjected to a high current to create a wound (20 seconds, 2500 μA, 64000 Hz). Impedance was measured at 16,000 Hz every 20 seconds for 24 hours. All conditions were run in duplicates. Wound healing and migration traces are normalised impedance values measured at 16,000 Hz. Graphs show normalized mean area under curve with SEM. To determine whether application of the high current resulted in formation of 250 nm in diameter wound spots, cells were died with Calcein, AM (Invitrogen, C1430) before and after the current was applied and after 24 hours. The cells were imaged using Zeiss Axio Imager.M1 fluorescent microscope with Axio Cam MRc5 and images were captured at five times magnification.

Directed migration of mouse macrophages (RAW264.7), WT and EBI2 KO mouse astrocytes was conducted using the xCELLigence platform with 16-well CIM-plates. The bottom chamber of the plate was filled with serum free media supplemented with increasing concentrations of 7α25HC with or without EBI2 antagonist NIBR189. For macrophage (RAW264.7) migration the bottom chamber was filled with either astrocyte conditioned media or serum free media supplemented with 7α25HC (1 μM) with or without EBI2 antagonist NIBR189 (10 μM). The plates were inserted into the humidified incubator with the impedance reader to allow for the plate and media to equilibrate to the same temperature and CO_2 level. The baseline readout was then performed and cells plated onto the top chamber of the plate and left for 20 minutes on the bench top to allow the cells to attach. The cell migration was recorded immediately after the plate was re-inserted into the reader and recorded for 4 hours. In the xCELLigence assay CI index is calculated automatically similarly to the impedance measurements described above. The platform's software also calculates the slope which can be used to evaluate migration. The slope describes the steepness, incline, gradient, and changing rate of a CI curve within the given time period. The linear fitting of the defined CI curve is calculated according to the following formula: CI=intercept + slope * time.

8 Statistical analysis

All statistical analysis was performed using GraphPad Prism 5. Independent student t-tests were run 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. Dunnett's post-hoc test was performed when the treatment groups were compared to a control group. Bonferroni's post-hoc test was run for selected pairs of columns in experiments where only certain groups were compared. 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.

Primary antibodies						
Antibody	**Type**	**Host**	**Manufacturer**	**Cat #**	**Dilution**	**Use**
GFAP	Polyclonal	Rabbit	Abcam	Ab7260	1/1000	ICC
MBP	Polyclonal	Rabbit	Abcam	Ab40390	1/1000	ICC
Anti-NF H	Polyclonal	Chicken	Millipore	AB5539	1/1000	ICC
Anti- NFκB p65	Polyclonal	Rabbit	Santa Cruz	Sc-109	1/50	ICC
Anti-EBI2 (57C)	Monoclonal	Human	Novartis	N/A	1/100	ICC
Anti-EBI2 (607B)	Monoclonal	Human	Novartis	N/A	1/100	ICC
Anti-CD11b FITC conjugated	Monoclonal	Rat	BD	553310	1/250	ICC
Anti-Iba1	Polyclonal	Rabbit	Wako	019-19741	1/250	ICC
Anti-F4/80	Monoclonal	Rat	eBiosciences	14-4801-81	1/1000	ICC
Aniti-CD31	Monoclonal	Rat	BD	553370	1/250	ICC
Anti-pERK 1/2	Monoclonal	Rabbit	Millipore	05-797R	1/3000	WB
Anti-ERK 1/2	Monoclonal	Mouse	Millipore	05-1152	1/3000	WB
Anti-CD68	Monoclonal	Mouse	Serotec	MCA341R	1/250	ICC
Anti-CD163	Monoclonal	Mouse	Serotec	MCA342R	1/250	ICC
Anti-MOG	Monoclonal	Mouse	Millipore	MAB5680	1/1000	WB
Anti-MBP	Monoclonal	Mouse	Covance	SMI 94	1/1000	WB
Anti Actin	Polyclonal	Rabbit	Pierce	PA1-16889	1/1000	WB
Anti-Olig1	Monoclonal	Mouse	Millipore	05-1152	1/1000	WB
Anti-CNPase	Monoclonal	Mouse	Millipore	MAB326R	1/1000	WB
hEBI2 mIgG2a Alexa 647	N/S	Mouse	Novartis	57C 9B5 1C9	1/100	FACS
hEBI2 mIgG2b Alexa 647	N/S	Mouse	Novartis	607B 1C3 1C1	1/100	FACS
Alexa 647 mIgG2a isotype control	N/S	Mouse	BioLegend	MOPC-173	1/100-1000	FACS
Alexa 647 mIgG2b isotype control	N/S	Mouse	BioLegend	MPC-11	1/100-1000	FACS
Secondary antibodies						
Antibody	**Type**	**Host**	**Manufacturer**	**Cat #**	**Dilution**	**Use**
Anti-mouse Alexa 488	N/S	Goat	Invitrogen	A11029	1/500	ICC
Anti-mouse Alexa 633	N/S	Goat	Invitrogen	A21052	1/500	ICC
Anti-rabbit Alexa 488	N/S	Goat	Invitrogen	A11008	1/500	ICC
Anti-rabbit Alexa 633	N/S	Goat	Invitrogen	A21070	1/500	ICC
Anti-human Alexa 488	N/S	Mouse	Invitrogen	A10631	1/500	ICC
Anti-chicken 549	N/S	Donkey	Jackson Immuno Research	703-505-155	1/500	ICC
Anti-mouse 549	N/S	Donkey	Jackson Immuno Research	715-505-020	1/500	ICC
Anti-chicken Alexa 633	N/S	Goat	Invitrogen	A21103	1/500	ICC
Anti-rat Alexa 488	N/S	Goat	Invitrogen	A-11006	1/500	ICC
Anti-rat Alexa 633	N/S	Goat	Invitrogen	A21094	1/500	ICC
Anti-rabbit HRP	N/S	Donkey	GE Healthcare	NA934	1/5000	WB
Anti-mouse HRP	N/S	Goat	Sigma	A8924	1/5000	WB

Table 2.1 List of primary and secondary antibodies. N/S = not specified

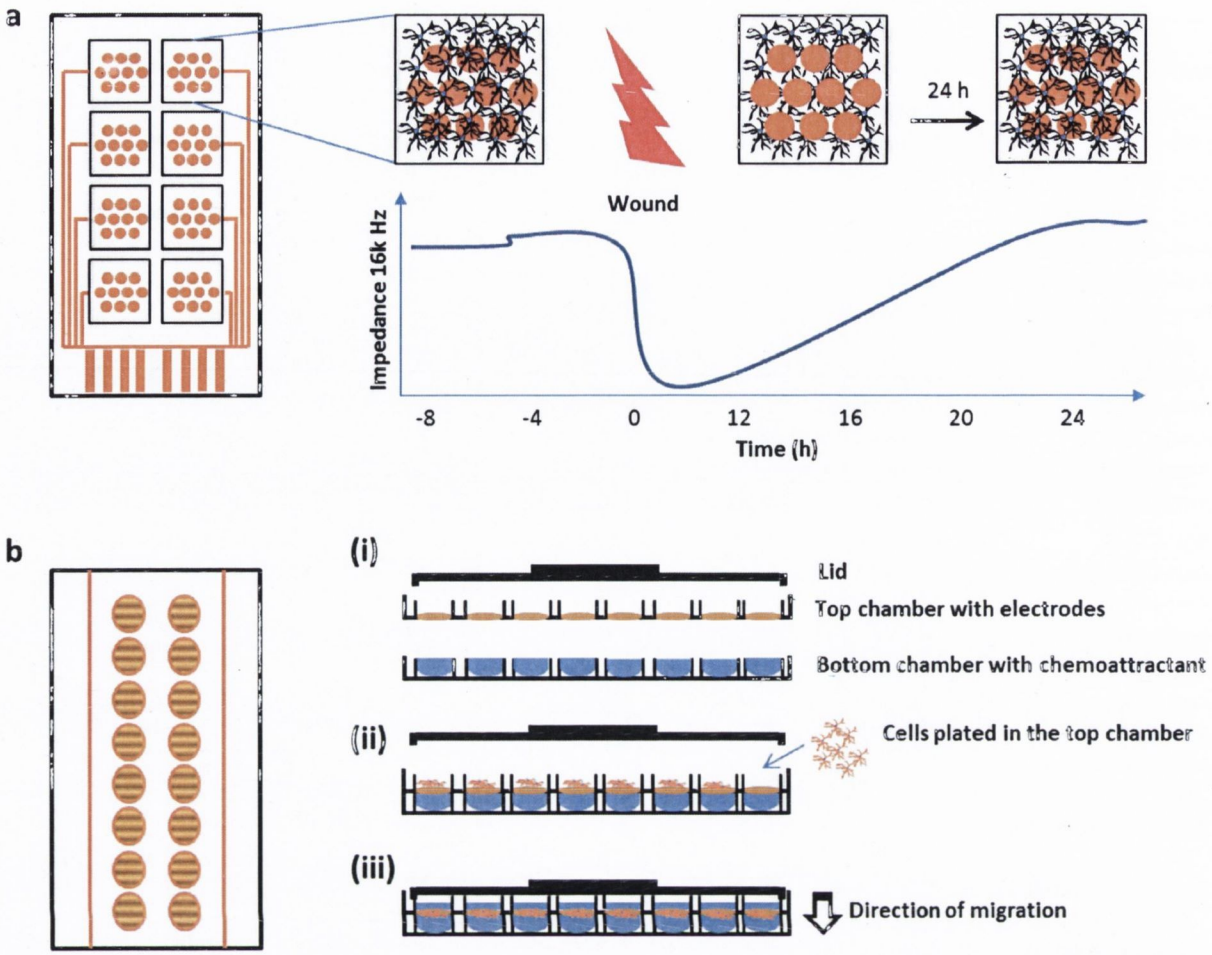


Figure 2.1 Experimental setup using ECIS and xCELLigence platforms. (a) Experimental setup with ECIS platform. Top view of the plate shows eight wells with 10 electrodes each. A close-up shows a monolayer of astrocytes growing on top of the electrodes. The number of cells, spread and strength of adhesion corresponds to the impedance values shown in the graph below. When a high electrical current is applied, only the cells growing directly on top of the electrodes are electorporated and within 10-15 minutes detach from the electrodes. This can be observed in the graph by impedance values dropping to those of an open electrode. Following the application of the wound, the healthy cells around the electrodes begin to heal the wound growing over the empty electrodes resulting in the gradual increase in impedance values as seen on the graph. **(b)** Experimental setup with xCELLigence migration platform. Top view of the plate shows 16 wells with numerous electrodes placed at the bottom of each well. Panels on the right show a side view of the plate. First, the bottom chamber of the plate is filled with media with or without compounds **(i)**. Then, the top chamber with electrodes is installed on top of the bottom chamber and cells are plated in the top chamber **(ii)**. There are pores between the electrodes in the top chamber allowing the cells to migrate through from the top chamber to the bottom towards the direction of the chemoattractant placed in the bottom chamber **(iii)**. Impedance is measured on the inside of the bottom chamber only after the cells have migrated to the other side.

Chapter 3: EBI2 regulates intracellular signalling and migration in astrocytes

Chapter aims

The study aims are to:

- investigate EBI2 expression in human astrocytes
- identify if human astrocytes express enzymes necessary for EBI2 ligand synthesis and/or degradation
- explore EBI2 signalling in human astrocytes
- study whether EBI2 agonism induces human astrocyte migration

Abstract

The G protein-coupled receptor EBI2 (Epstein-Barr virus-induced gene 2) is activated by 7α, 25-dihydroxycholesterol (7α25HC) and plays a role in T cell-dependant antibody response and B cell migration. Aberrant EBI2 signalling is implicated in a range of autoimmune disorders however its role in the CNS remains unknown. Here we characterize the functional role of EBI2 in glia cells using primary human astrocytes and EBI2 knock-out animals. We find human and mouse astrocytes express EBI2 and the enzymes necessary for synthesis and degradation of 7α25HC. In astrocytes, EBI2 activation stimulates ERK phosphorylation, Ca^{2+} signalling and induces cellular migration. These results, for the first time, demonstrate a role for EBI2 in astrocyte function and suggest that modulation of this receptor may be beneficial in neuroinflammatory disorders.

1 Introduction

The G protein-coupled receptor (GPCR), EBI2 (Epstein-Barr virus (EBV)-induced gene 2, also known as GPR183), is highly expressed in immune cells and is activated by oxysterols **(Hannedouche et al., 2011, Birkenbach et al., 1993, Liu et al., 2011)**. EBI2 signals through the pertussis toxin (PTx)-sensitive heterotrimeric G proteins of the Gi/o family leading to a decrease in cAMP production, calcium mobilization and stimulation of the extracellular-signal-regulated kinase (ERK) pathway **(Gatto et al., 2011, Benned-Jensen et al., 2013)**. The 7α, 25-dihydroxycholesterol (7α25HC) and closely related compounds have been identified as high affinity agonists for EBI2 **(Liu et al., 2011, Hannedouche et al., 2011)**. Oxysterols are oxygenated derivatives of cholesterol, which play roles in metabolism of vitamins and lipids, cholesterol trafficking, activation of nuclear hormone receptors, regulation of inflammatory signalling pathways, and many others **(Schroepfer, 2000, Leoni and Caccia, 2011)**. The synthesis of 7α25HC from cholesterol occurs via enzymatic action of cholesterol-25-hydroxylase (CH25H) and hydroxycholesterol 7-alpha-hydroxylase (CYP7B1) **(Russell, 2000)**. Degradation of 7α25HC is accomplished by the enzyme HSD3B7, which moves the double bond from ring B to ring A and oxidizes the hydroxyl group at position three to a ketone **(Russell, 2000)**. Oxysterols act as chemoattractants leading to directed cell migration *in vitro* and *in vivo* **(Liu et al., 2011, Hannedouche et al., 2011)**. It has also been demonstrated that the appropriate positioning of activated B cells within the lymphoid follicles is dependent on a precise gradient of 7α25HC **(Yi et al., 2012)**. In addition to being crucial for launching appropriate T cell-dependant humoral response, EBI2 is also involved in inflammatory responses **(Gatto et al., 2011, Gatto et al., 2009, Pereira et al., 2009)**. For example, upon immune challenge *in vitro* with lipopolysaccharide (LPS), human B cells, macrophages, dendritic cells, natural killer (NK) cells and neutrophils simultaneously upregulate expression of EBI2 and the enzymes necessary for 7α25HC synthesis, such as CYP7B1 and CH25H **(Park and Scott, 2010, Diczfalusy et al., 2009)**. Moreover, oxysterols have been implicated in having both, pro- and anti-inflammatory effects depending on the oxysterol and cell type. For instance, the precursor to 7α25HC, 25HC, is a pro-inflammatory oxysterol, which enhances release of pro-inflammatory cytokines from placental throphoblasts, airway epithelial cells, monocytic THP-1 cells and macrophages **(Koarai et al., 2012, Aye et al., 2012, Diczfalusy et al., 2009, Rosklint et al., 2002, Lemaire-Ewing et al., 2009)**. On the other hand, 7β25HC, was found to have immunosuppressive effects in lymphocytes **(Moog et al., 1988)**.

While the role of EBI2 in the immune system is well studied, its role in the cells of the CNS, in particular astrocytes, has not been investigated. Astrocytes are a very abundant cell type in the brain and they serve a range of physiological functions. For instance, they control neuronal maturation, form and maintain synapses, uptake neurotransmitters, release antioxidants and are responsible for extracellular ion buffering **(Kimelberg, 2010, Ransom et al., 2003)**. In addition to supporting neurons, astrocytes also play a role in formation and maintenance of the blood-brain barrier (BBB) and communication with the immune system via cytokine/chemokines and other signalling molecules **(Kimelberg, 2010, Ransom et al., 2003)**. These cells process and integrate neuronal activity and coordinate calcium waves indicative of intercellular communication **(Kimelberg, 2010, Ransom et al., 2003)**. Astrocytes also form glial scars, express a wide range of receptors and release neurotransmitters and other signalling molecules allowing them to be involved in both normal and pathophysiological processes in the central nervous system (CNS) **(Kimelberg, 2010, Ransom et al., 2003)**. Importantly, these glial cells have been implicated in many neurodegenerative diseases such

as multiple sclerosis (MS), Parkinson's disease (PD) and Alzheimer's disease (AD) **(Sidoryk-Wegrzynowicz et al., 2010, Sriram, 2011)**. Specifically to MS, reactive astrocytes are the most abundant cell type in MS plaques and have been found in lesions without the presence of other inflammatory infiltrates **(Morcos et al., 2003)**. Astrocytes are thought to drive the development of experimental autoimmune encephalomyelitis (EAE) by phagocytosis of myelin and presenting myelin basic protein (MBP), myelin oligodendrocyte oligoprotein (MOG) and proteolipid protein (PLP) antigens to $CD4^+$ T cells, however, their role in phagocytosis has been debated in the field **(Fontana et al., 1984, Tan et al., 1998, Kort et al., 2006)**.

Notably, a quarter of the total body cholesterol is located in the brain mainly in oligodendrocytes, to form myelin, and neurons and astrocytes **(Leoni and Caccia, 2011)**. Of interest, abnormal levels of oxysterols have been detected in the brains of EAE animals and MS patients **(Diestel et al., 2003)**. Some studies suggest that certain oxysterols exacerbate the pathology of MS and development of EAE **(Teunissen et al., 2007, Teunissen et al., 2003, Quintana et al., 2008)**, while others have found that genes such as CH25H are upregulated in glia during demyelinating and remyelinating stages of EAE to support myelination **(Olah et al., 2011)**. Another study has shown that in CH25H knock-out animals, which cannot synthesise 25HC, mice display aggravated progression of EAE **(Reboldi et al., 2014)**. Thus, to examine the role of EBI2 signalling in the CNS, here, the expression, signalling and function of EBI2 in human astrocytes were demonstrated. In addition, the effect of EBI2 activation on astrocyte migration was also examined in wild type (WT) and EBI2 knock-out (KO) animals.

2 Results

2.1 EBI2 is expressed in human and mouse astrocytes

EBI2 expression and function in the immune system has been shown to be crucial for appropriate T cell-dependant antibody response and correct B cell migration and localisation in B cell follicles **(Pereira et al., 2009, Gatto et al., 2009, Gatto et al., 2011)**. In contrast, information on the functional role of this receptor in the CNS still remains limited. Here, the expression and function of EBI2 in glial cells, specifically astrocytes were examined. First, the two human EBI2 monoclonal antibodies, and isotype controls, were tested in control CHO cells (A21) and CHO cells stably expressing the human EBI2 (CHO-hEBI2) **(Hannedouche et al., 2011)** using fluorescence activated cell sorting (FACS). While the isotype control antibodies showed no staining in either control or hEBI2-expressing CHO cells, both EBI2 monoclonal antibodies (57C and 607B) stained the CHO-hEBI2 cell line (Figure 3.1b), but not control CHO cells (Figure 3.1a), demonstrating antibody selectivity. In addition, immunocytochemistry using the 57C and 607B EBI2 antibodies confirmed expression of human EBI2 in the CHO-hEBI2 cell line and showed cell surface and intracellular expression of EBI2 (Figure 3.1c). Moreover, pure human astrocyte cultures were stained for EBI2 and counter-stained with GFAP (Figure 3.1d). The confocal images showed expression of EBI2 on human astrocytes, which appeared similar in localisation to the CHO-hEBI2 cells. Next, the mRNA expression levels of EBI2 in human and mouse astrocytes and human monocytes (U937) were investigated. Cultured mouse astrocytes expressed EBI2 mRNA at approximately 34.1% levels (+/- 10.2%) and human astrocytes at 7.1% (+/- 3.5%) of that found in human monocytes (U937) (Figure 3.1e). Taken together, the data demonstrated that EBI2 is expressed in astrocytes at both, mRNA and protein levels.

2.2 Human and mouse astrocytes express enzymes necessary for 7α25HC synthesis and degradation

It has been shown that 7α25HC gradients are tightly controlled within the secondary lymphoid tissue in order to ensure appropriate B cell trafficking during humoral response **(Yi et al., 2012)**. This gradient is maintained via enzymes necessary for 7α25HC formation (CH25H, CYP7B1) and degradation (HSD3B7) (Figure 3.2a). To determine whether human astrocytes express enzymes responsible for 7α25HC synthesis and degradation, the mRNA levels of CH25H (Figure 3.2b), CYP7B1 (Figure 3.2c) and HSD3B7 (Figure 3.2d) were measured. Mouse astrocytes were found to express all three enzymes, which was in contrast to human astrocytes which did not express CH25H mRNA and relatively much smaller amounts of CYP7B1 (8.1% +/- 5.3%) and HSD3B7 (22.4% +/- 12.5%) (Figure 3.2b-d). Human B cells (RS11846) did not express any of the enzymes needed for 7α25HC synthesis or degradation while human monocytes (U937) expressed only HSD3B7 mRNA and, relatively to human astrocytes, expressed it in low amounts (26.4% +/- 39.1%) (Figure 3.2b-d). These studies indicated that astrocytes express both EBI2 and enzymes necessary for 7α25HC synthesis and degradation, although species differences (at least *in vitro*) may occur.

2.3 EBI2 activation induces pERK 1/2 signalling in human astrocytes

EBI2 has been suggested previously to be coupled to the ERK 1/2 signalling pathway **(Benned-Jensen et al., 2013)**. To determine if activation of EBI2 with its endogenous agonist, 7α25HC, also leads to ERK phosphorylation in human astrocytes, the levels of phosphorylated ERK were examined. Time course studies showed that the EBI2 agonist 7α25HC induced a robust and statistically significant increase in levels of pERK after 1 minute treatment (510.4% +/- 107.9% 1 μM, ***$p<0.001$, one-way

ANOVA and Dunnett's post-hoc test) and remained for up to 10 minutes (*data not shown*). Moreover, the 7α25HC-mediated increase in levels of pERK were concentration dependent (Figure 3.3a) and inhibited by the EBI2 antagonist (NIBR189) (48.2% +/- 36.3% 10 μM, one-way ANOVA and Dunnett's post-hoc test, p>0.05) (Figure 3.3b). These results demonstrate that, in human astrocytes, EBI2 signals via the pERK 1/2 pathway and that ERK phosphorylation is mediated by agonist activation of the receptor and subsequently blocked by the receptor antagonism.

2.4 Agonism of EBI2 induces Ca^{2+} signalling in human astrocytes

Previous studies showed that activation of EBI2 with 7α25HC induces Ca^{2+} signalling in CHO-hEBI2 cells **(Hannedouche et al., 2011)**. Here, the effects of the EBI2 receptor agonist 7α25HC on Ca^{2+} signalling in pure human astrocyte cultures were further demonstrated (Figure 3.4). Stimulation of human astrocytes with 7α25HC (1 μM) caused a statistically significant, transient increase in the levels of Ca^{2+} (153.8% +/- 68.7%, **p<0.01, one-way ANOVA and Dunnett's post-test) (Figure 3.4b-d). In line with previous studies, cholesterol did not activate EBI2 and did not alter intracellular Ca^{2+} levels (76.5% +/- 5.5%, p>0.05, one-way ANOVA and Dunnett's post-test) (Figure 3.4b-d) **(Hannedouche et al., 2011)**. The data also confirmed previous findings that stimulation of astrocytes with the sphingosine-1-phosphate receptor (S1PR) agonist, pFTY720, caused a significant increase in the levels of Ca^{2+} in astrocytes (302.9% +/- 208.6%, ***p<0.001, one-way ANOVA and Dunnett's post-test) **(Mullershausen et al., 2007)**. Notably, the effects of 7α25HC on increased Ca^{2+} levels were approximately 50% of that observed with pFTY720. These differences may be explained by pan-S1PR activation by pFTY720 **(Mullershausen et al., 2007)** versus a more selective EBI2 activation by 7α25HC.

2.5 EBI2 regulates wound healing/migration in human astrocytes

Studies have suggested that 7α25HC is a chemoattractant to EBI2 expressing immune cells and that it guides B cell migration and correct positioning in the B cell follicles via EBI2 **(Hannedouche et al., 2011, Liu et al., 2011)**. To investigate whether 7α25HC induces migration (coordinated movement of cells from one place to another as a result of receptor activation by chemoattractant molecules) in astrocytes, the effects of 7α25HC on human astrocyte migration and wound healing capabilities was measured using ECIS wound healing assay (Figure 3.5a). Application of a high current (i.e. 20 seconds, 2500 μA, 64000 Hz) resulted in the formation of approximately 250 nm in diameter 'wound-spots' directly on top of the electrodes (Figure 3.5b). Moreover, astrocytes were able to re-populate these wound-spots 24 hours after application of the high current (Figure 3.5b). Neither the agonism nor the antagonism of EBI2 impacted the cell viability and proliferation of human astrocytes (110.9% +/- 6.2% 7α25HC, 92.6% +/- 18.8% NIBR189, p>0.05, one-way ANOVA and Dunnett's post-hoc test). In contrast to the treatment with 10% FBS, which resulted in significant increase in cell viability and numbers, as determined by MTT assay (154.4% +/- 14.2%, **p<0.01, one-way ANOVA and Dunnett's post-hoc test) (Figure 3.5c). Treatment of human astrocytes with increasing concentrations of 7α25HC demonstrated that the compound induced wound healing and migration of human astrocytes in a concentration-dependent manner (106.2% +/- 8.6% at 0.06 μM versus control) (Figure 3.5d and e). To further confirm whether the effects of 7α25HC on astrocyte wound healing and migration were dependant on EBI2 activation, the cells were pre-treated with EBI2 antagonist, NIBR189 (0.25 μM), for 30 minutes followed by 7α25HC at indicated concentrations. Antagonism of EBI2 by NIBR189 inhibited the 7α25HC-induced human astrocyte wound healing and migration (47.1% +/- 15.9%, ***p<0.001, one-way ANOVA and Bonferroni's post-hoc test) (Figure

3.5f and g). Notably, the effects of 7α25HC on astrocyte migration where similar to that observed for the S1PR agonist pFTY720 as previously reported (Figure 3.5f) **(Mullershausen et al., 2007)**.

2.6 7α25HC does not induce migration in EBI2 knock-out astrocytes

To further confirm that the effects of 7α25HC on astrocyte migration are EBI2 dependant, astrocytes from wild type (WT) and EBI2 knock-out (EBI2 KO) mice were prepared. Astrocyte migration in response to 7α25HC was then measured with xCELLigence directed migration assay (Figure 3.6). Similar to human astrocytes WT mouse astrocytes migrated towards 7α25HC in a concentration-dependant manner (163.4% +/- 13.8%) (Figure 3.6b, c). In contrast, EBI2 KO astrocytes did not migrate towards 7α25HC confirming EBI2-dependant effects of 7α25HC on astrocyte migration (Figure 3.6b). In addition, the antagonism of EBI2 in WT mouse astrocytes blocked 7α25HC-induced cell migration (103.3% +/- 36.9%, **p<0.01, unpaired t-test) (Figure 3.6c). Taken together, these genetic and pharmacological studies demonstrate that that 7α25HC induces astrocyte migration via EBI2 and that these effects can be blocked with EBI2 antagonism.

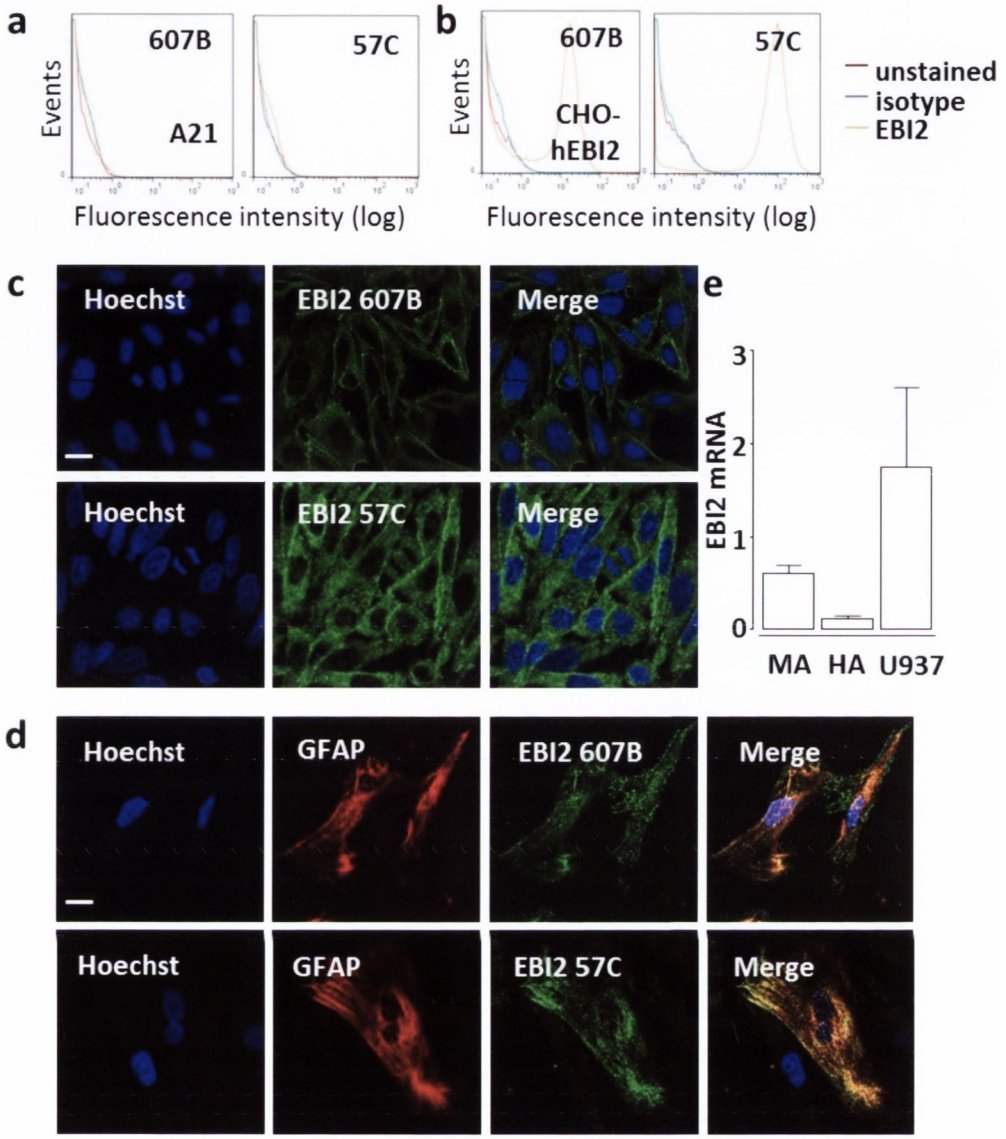


Figure 3.1 EBI2 is expressed in human and mouse astrocytes. FACS analysis of anti-human EBI2 monoclonal antibodies (607B and 57C) in control CHO cells (A21) (a) and in CHO cells stably expressing the human EBI2 (CHO-hEBI2) (b) showed staining only in the CHO-hEBI2 cell line, but not in the control CHO cells (A21), demonstrating antibody selectivity. (c) Expression of EBI2 in CHO cells stably expressing hEBI2 as determined with anti-human EBI2 monoclonal antibodies (607B and 57C). Cells were also dyed with Hoechst stain as a nuclear marker. (d) Expression of EBI2 in human astrocytes as determined with anti-human EBI2 monoclonal antibodies (607B and 57C). Cells were also immunostained with anti-GFAP polyclonal antibody as an astrocyte marker and Hoechst stain as a nuclear marker. Confocal images were captured at 40x magnification (n=4). Scale bar, 10 μm. (e) EBI2 mRNA expression in mouse astrocytes (MA), human astrocytes (HA) and human monocytes (U937) as measured using TaqMan Gene Expression Assays (Applied Biosystems). Data is expressed as average values +/- SEM, relative to internal standard HPRT1 (n=3-5).

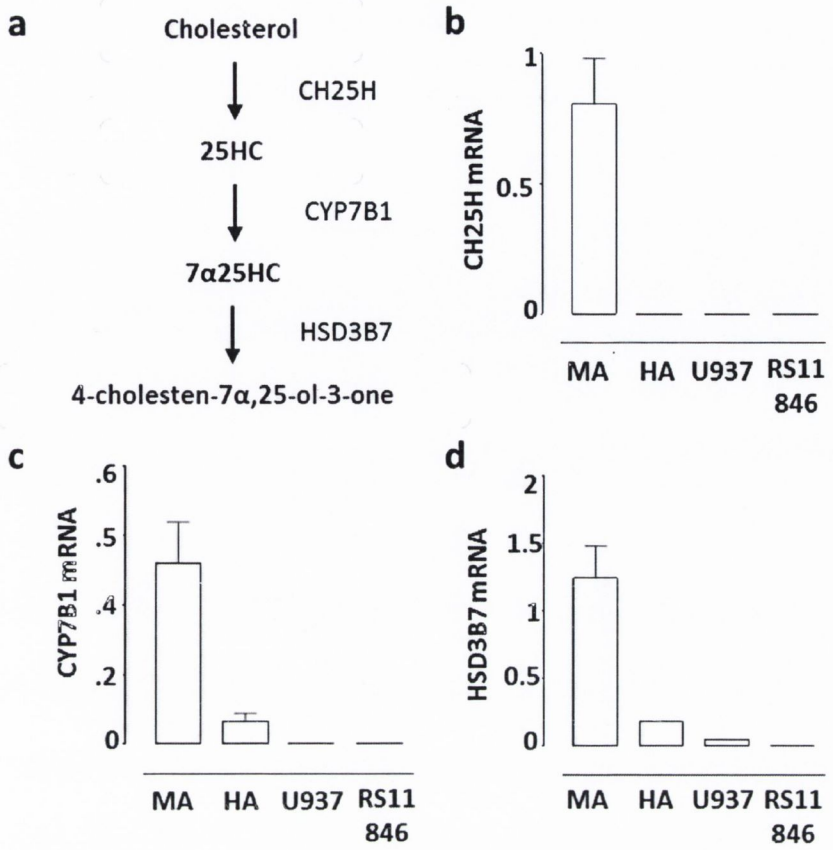


Figure 3.2 Human and mouse astrocytes express enzymes necessary for 7α25HC synthesis and degradation. **(a)** Schematic diagram showing enzymatic pathway of 7α25HC. mRNA expression of CH25H **(b)**, CYP7B1 **(c)** and HSD3B7 **(d)** in mouse astrocytes (MA), human astrocytes (HA), human monocytes (U937) and human B cells (RS11846). Data is expressed as averages +/- SEM, relative to internal standard HPRT1 (n=3-5).

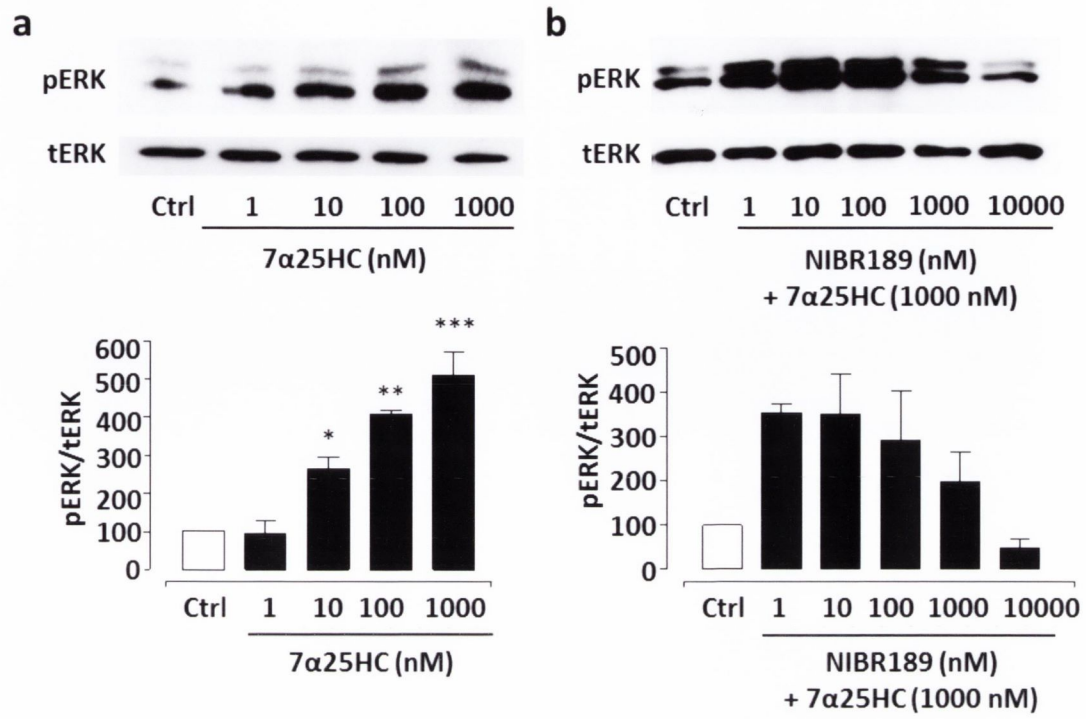


Figure 3.3 EBI2 activation induces ERK signalling in human astrocytes (a) A dose response curve shows that 1 minute treatment with increasing concentrations of 7α25HC induces ERK phosphorylation in a concentration-dependent manner. (b) A dose-response curve of the EBI2 antagonist NIBR189 (10 μM) shows that increasing concentrations of NIBR189 inhibit 7α25HC (1 μM) induced pERK1/2 phosphorylation although statistical significance was not reached. Corresponding bar graphs show densitometric analysis of the Western blot bands. Values are shown as pERK/tERK, where tERK is the total ERK present and pERK is the level of phosphorylated ERK1/2. Data presented as mean +/- SEM (n=3-5), one-way ANOVA and Dunnett's multiple comparison post-tests, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. corresponding control.

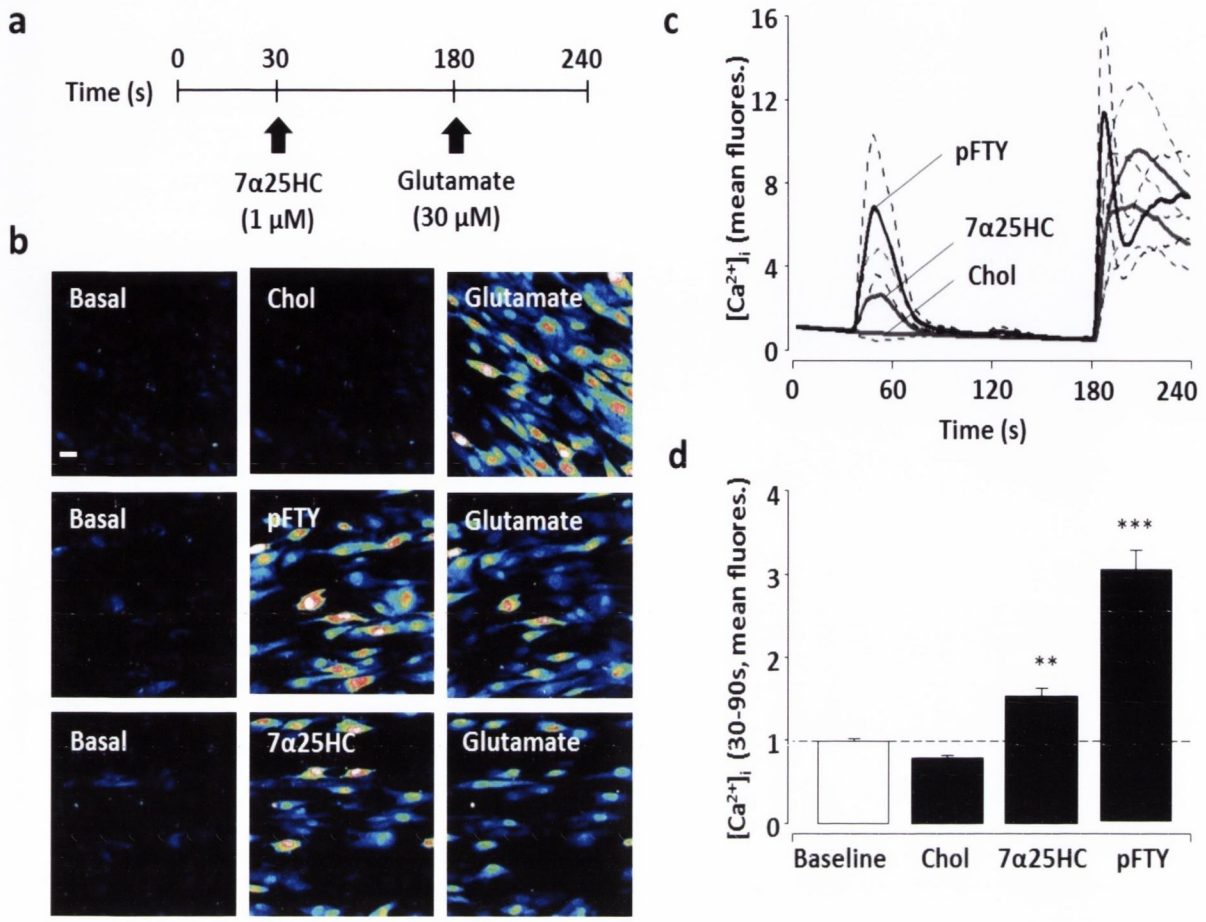


Figure 3.4 EBI2 activation induces Ca^{2+} signalling in human astrocytes (a) Schematic diagram illustrating experimental design. **(b)** Representative images taken from time-lapse series, showing basal levels of calcium, after addition of cholesterol (chol) (1 μM), pFTY720 (1 μM) or 7α25HC (1 μM) and after stimulation with glutamate (30 μM). Confocal images were captured at 40x magnification. Scale bar, 20 μm. **(c)** Representative, traces show changes in Ca^{2+} levels over time. Each trace is average +/- SEM of 6-9 independent observations. Solid lines represent averages and dotted lines represent SEM. **(d)** Corresponding bar graphs show that 7α25HC causes increase in Ca^{2+} levels (30-90 seconds). Data is expressed as mean +/- SEM (chol n=6; 7α25HC n=9; pFTY n=6), One-way ANOVA and Dunnett's post-test, ** $p<0.01$, *** $p<0.001$ vs. baseline.

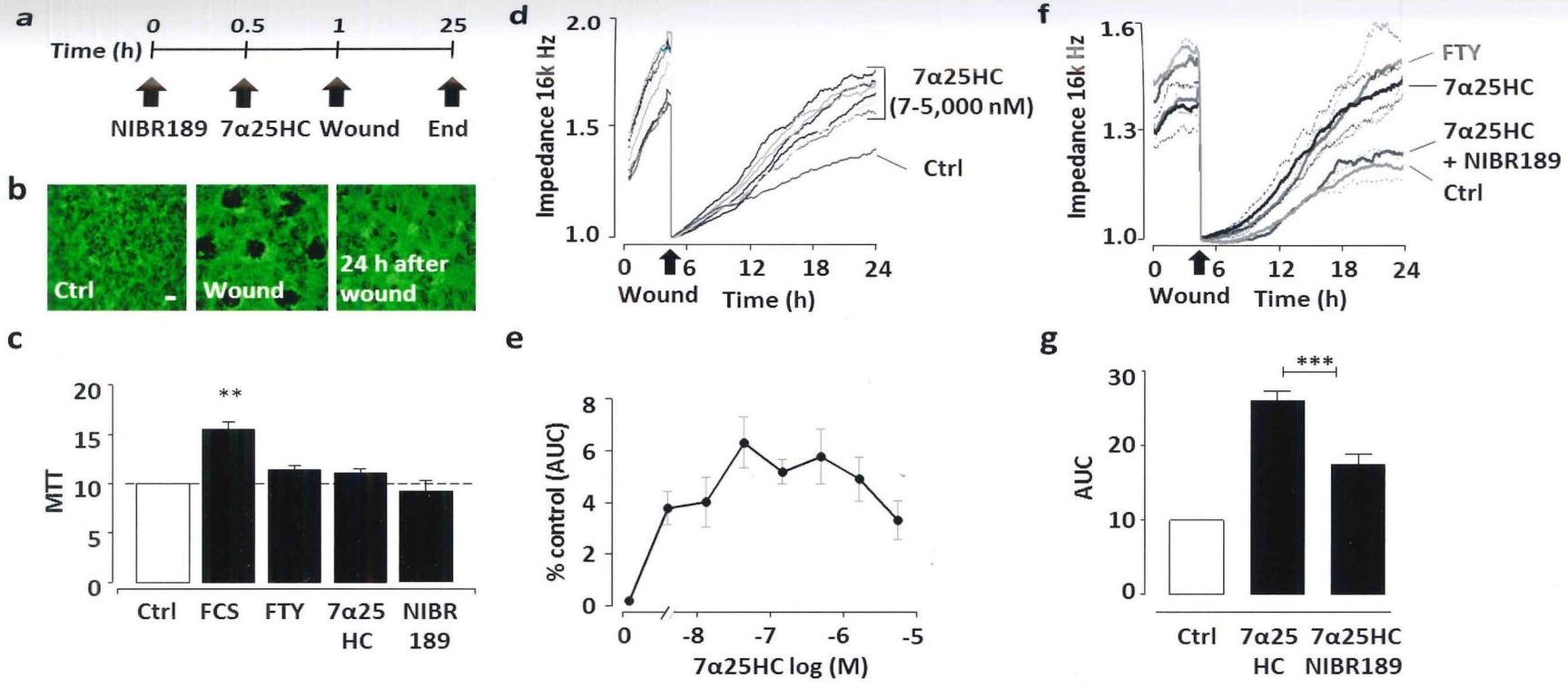


Figure 3.5 7α25HC induces human astrocyte wound healing/migration. **(a)** Diagram illustrating experimental design with human astrocytes using ECIS. **(b)** Images showing cell detachment from electrodes after application of high current (wound). Fluorescent images taken at 5x magnification. Scale bar, 200 μm. **(c)** MTT assay showing effects of FCS (10%), pFTY720 (FTY, 0.01 μM), 7α25HC (1 μM) and NIBR189 (0.25 μM) on astrocyte viability and proliferation after 24 hour treatment. Data presented as mean +/- SEM (n=3), one-way ANOVA and Dunnett's post-test, ** $p<0.01$ vs. corresponding control. **(d)** Representative traces show wound healing/cell migration in response to increasing concentrations of 7α25HC over a 24 hour time period. **(e)** Corresponding graph shows a concentration-dependent effect of 7α25HC at a 24 hour time point. Data presented as normalized AUC +/- SEM (n=3). **(f)** Representative traces show inhibition of 7α25HC (0.01 μM) induced wound healing/migration with EBI2 antagonist (NIBR189, 0.25 μM) over a 24 hour time period. Each trace is an average +/- SEM of two independent observations. Solid lines represent averages and dotted lines represent SEM. **(g)** Corresponding bar graph shows NIBR189 (0.25 μM) mediated inhibition of 7α25HC (0.01 μM) at 24 hour time point. All traces were normalized to 1.0 at time of wound. Data presented as AUC (with a further subtraction of 90 units) +/- SEM (n=3), Bonferroni's post-test, ***$p<0.001$ vs. corresponding control.

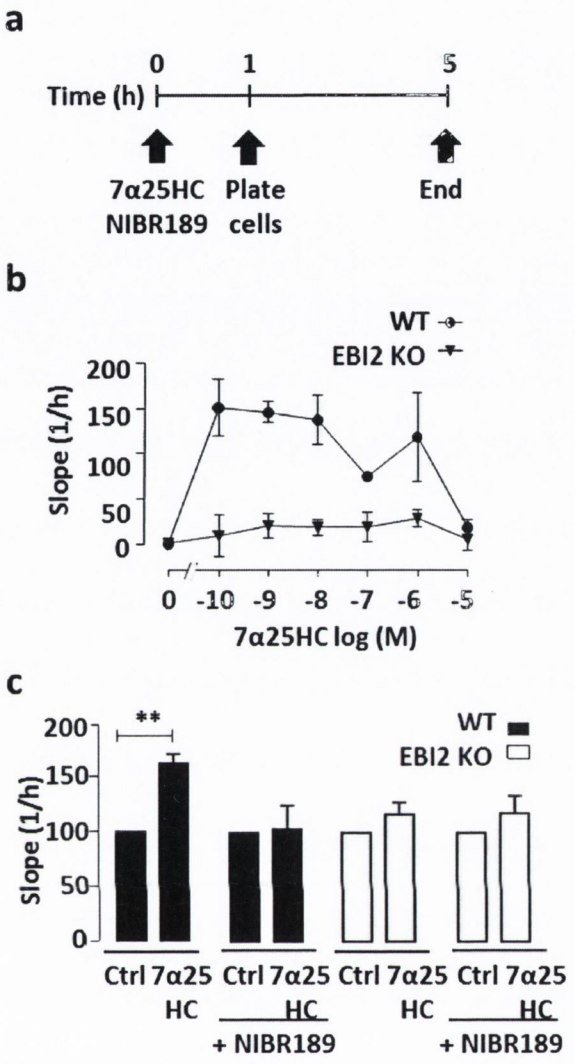


Figure 3.6 7α25HC does not induce migration in EBI2 knock-out astrocytes. **(a)** Schematic diagram illustrating experimental design with mouse astrocyte using xCELLigence. **(b)** Effect of 7α25HC on migration of mouse astrocytes prepared from wild type (WT) and EBI2 knock-out (EBI2 KO) animals. Similar to human astrocytes **(Figure 3.5)** 7α25HC induces a concentration-dependant migration of WT mouse astrocytes. In contrast, 7α25HC does not induce migration of EBI2 KO astrocytes confirming receptor mediated mechanism. Data presented as mean +/- SEM (n=3). **(c)** Bar graph shows inhibition of 7α25HC (0.01 µM) induced migration with EBI2 antagonist (NIBR189, 10 µM) in WT mouse astrocytes over a 4 hour time period. No change in migration is observed in EBI2 KO astrocytes. Data presented as mean +/- SEM (n=3), student t-test ** $p<0.01$ vs. corresponding control.

3 Discussion

Studies have shown that EBI2 regulates immune cell function such as B cell trafficking, differentiation and T cell dependant antibody response **(Gatto et al., 2009, Hannedouche et al., 2011)**. In contrast to knowledge of EBI2 function in the immune system, little is known about its expression and function in the CNS. Here, EBI2 expression and function in astrocytes was investigated by examining intracellular signalling and cell migration. The data showed that astrocytes express EBI2 and enzymes needed for 7α25HC synthesis and degradation. *In vitro* stimulation of EBI2 with 7α25HC caused an increase in the levels of pERK and enhanced intracellular Ca^{2+} signalling. Importantly, 7α25HC promoted wound healing/migration of human astrocytes, which was inhibited by an EBI2 antagonist and did not occur in astrocytes prepared from EBI2 KO mice.

3.1 Astrocytes are involved in normal and pathophysiological processes in the CNS

Astrocytes serve a wide range of functions including control of BBB, regulation of synaptic transmission, release of growth factors and signalling molecules, which allows them to be involved in regulation of cell migration, cellular communication and inflammatory responses **(Kimelberg, 2010)**. During CNS damage or injury, astrocytes release pro-inflammatory cytokines and chemokines leading to activation of microglia and influx of leukocytes **(Kimelberg, 2010)**. These cells also facilitate healing of the injured tissue by form glial scar limiting the extent of damage **(Kimelberg, 2010)**. Astrocytes have also been suggested to also serve as antigen presenting cells playing a further contributing role to the pro-inflammatory response, although this function of astrocytes has been debated in the field **(Fontana et al., 1984)**. In addition to being involved in a range of physiological brain functions, astrocytes have also been implicated in pathophysiological events taking place during many neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease or Parkinson's disease **(Sidoryk-Wegrzynowicz et al., 2010)**. Their increased activity has been shown to exacerbate the inflammatory response driving the diseases progress. Therapies that control astrocyte activation may therefore prove useful in managing autoimmune and neurodegenerative diseases.

3.2 Astrocytes express EBI2 and 7α25HC synthesising and degrading enzymes

Studies have reported that peripheral blood mononuclear cells (PBMCs) express EBI2, however these cells do not themselves regulate the gradient levels of 7α25HC in the lymphoid tissue. Instead, it has recently been shown that gradient levels of 7α25HC in the lymphoid tissue can be controlled by lymphoid stromal cells via the enzymes CH25H, CYP7B1 and HSD3B7 **(Yi et al., 2012)**. The current study showed that mouse and human astrocytes expressed EBI2 as well as the CH25H, CYP7B1 and HSD3B7 enzymes that synthesise and degrade 7α25HC, respectively. The conversion of 25HC by CYP7B1 may be a mechanism used by astrocytes to limit inflammation in the CNS. The current data also indicated that astrocytes express HSD3B7 responsible for degradation of oxysterols. It is possible that expression of this enzyme by astrocytes within inflammatory lesions may also lead to the attenuation of pro-inflammatory signals. Astrocytes present at the site of inflammation may express HSD3B7 to degrade oxysterols and attenuate the pro-inflammatory signals and limit the influx of resident and peripheral immune cells. We noted, however, differential expression of the three enzymes, CH25H, CYP7B1 and HSD3B7, in human versus mouse astrocytes, which indicates a species difference, at least *in vitro*. How these differences relate to *in vivo* situation and their importance when using animal models to investigate EBI2 function relating to the human setting requires further study, particularly in the context of investigating EBI2 drugs in animal models of

disease. Nevertheless, the current data suggests that astrocytes express EBI2 as well as enzymes that synthesise and degrade 7α25HC.

3.3 EBI2 induces migration/wound healing in astrocytes

Previous studies have also shown that activation of EBI2 with 7α25HC induced migration of EBI2 expressing immune cells **(Hannedouche et al., 2011, Liu et al., 2011)**. The current data showed that 7α25HC also promoted astrocyte migration/wound healing in a concentration-dependant manner. Notably, higher concentrations resulted in diminished migration, which may be indicative of receptor desensitisation. Moreover, this astrocyte migratory response was inhibited with an EBI2 antagonist and absent in astrocytes prepared from EBI2 KO animals. These genetic and pharmacological studies support the idea that 7α25HC-induced migration of human astrocytes was EBI2 dependent. Interestingly, increased levels of various oxysterols including 7α25HC have been found in different diseases especially those with an inflammatory component **(Diestel et al., 2003, Olah et al., 2011, Leoni and Caccia, 2011)**. Taken together, these studies support the possibility that astrocytes during CNS injury respond to various cytokines, chemokines and possibly oxysterols such as 7α25HC to regulate inflammatory response. These results provide initial evidence for the use of EBI2 as a novel drug target to altering astrocyte function in neuroinflammatory and neurodegenerative illness.

Chapter 4: EBI2 regulates levels of IL6 and TNFα in astrocytes and their signalling with macrophages

Chapter aims

The study aims are to:

- investigate the role of EBI2 in induction and inhibition of pro-inflammatory cytokines in mouse and human astrocytes
- identify EBI2-regulated intracellular signalling pathways involved in regulation of pro-inflammatory signals
- study whether EBI2 and 7α25HC related enzymes are modified by pro-inflammatory signals
- explore whether the EBI2/oxysterol system in astrocytes is involved in regulation of cellular cross-talk

Abstract

EBI2 is a G protein-coupled receptor activated by oxysterol 7α, 25-dihydroxycholesterol (7α25HC) and regulates T cell-dependant antibody response and B cell migration. In addition to immune cell function, we find that EBI2 is expressed in astrocytes and that its activation induces astrocyte migration and intracellular signalling. Here, the role of EBI2 in regulating astrocyte immune function was investigated. While LPS did not induce cytokine release in human astrocytes, we found IL17/TNFα significantly increased levels of IL6 in these cells. Importantly, the EBI2 agonist, 7α25HC, attenuated IL17/TNFα-mediated increase of IL6 and also inhibited the nuclear translocation of NFκB. In contrast to human astrocytes, LPS increased levels of IL6 and TNFα in rodent astrocytes, which were again inhibited by 7α25HC. Notably, LPS treatment of rodent astrocytes also altered mRNA levels of EBI2 and oxysterol related genes suggesting that the EBI2 signalling pathway is sensitive to LPS-mediated immune challenge. Significantly, we also found astrocyte conditioned media induced macrophage migration which was inhibited with the EBI2 antagonist NIBR189. These results demonstrate a role for EBI2 in the release of pro-inflammatory cytokines from astrocytes and in their communication with innate immune cells such as macrophages.

1 Introduction

Oxysterols are oxygenated derivatives of cholesterol that regulate lipid, cholesterol, hormonal and inflammatory signalling pathways **(Russell, 2000)**. The G protein-coupled receptor, EBI2 (Epstein Barr virus-induced gene 2, GPR183), is activated by the oxysterol, 7α, 25-dihydroxycholesterol (7α25HC) and highly expressed in peripheral blood mononuclear cells (PBMCs) **(Hannedouche et al., 2011, Birkenbach et al., 1993, Liu et al., 2011)**. EBI2 regulates B cell positioning within the lymphoid tissue and is crucial for launching appropriate T cell-dependant antibody response **(Gatto et al., 2011, Gatto et al., 2009, Pereira et al., 2009)**. The most potent EBI2 agonist 7α25HC, is synthesised from cholesterol through enzymatic action of cholesterol-25-hydroxylase (CH25H) followed by cytochrome P450 hydroxycholesterol 7-alpha-hydroxylase (CYP7B1) and is degraded by cholest-5-ene-3β,7α-diol 3β-dehydrogenase (HSD3B7) **(Yi et al., 2012, Russell, 2000)**. Upon immune challenge *in vitro* with lipopolysaccharide (LPS), human B cells, macrophages, dendritic cells, natural killer (NK) cells and neutrophils simultaneously upregulate expression of EBI2 and the CYP7B1 and CH25H enzymes, while decreasing the degrading enzyme HSD3B7, thus apparently promoting EBI2 signalling **(Park and Scott, 2010, Diczfalusy et al., 2009, Preuss et al., 2014a)**. Moreover, increased levels of 25HC (the precursor to 7α25HC) occurs in the blood of humans and mice after injection of LPS **(Bauman et al., 2009, Diczfalusy et al., 2009)**. In addition, *in vitro* treatment of viruses with 25HC inhibits the activity of human immunodeficiency virus, herpes simplex virus 1 and Ebola virus **(Liu et al., 2013, Spann and Glass, 2013)**. Indeed, it has recently been shown that macrophages deficient in CH25H enzyme produce greater amounts of IL1β and CH25H KO mice exhibit exacerbated course of EAE **(Reboldi et al., 2014)**. The induction of oxysterols such as 25HC in response to TLR4 stimulation or type I/II interferons might thus be a host strategy to fight such infections **(Park and Scott, 2010, Diczfalusy et al., 2009, Spann and Glass, 2013, Blanc et al., 2013)**.

In addition to playing important roles in the immune system, we have shown EBI2 is expressed in the cells of the central nervous system (CNS), namely astrocytes, and this receptor regulates astrocyte signalling, as well as astrocyte cell migration **(Rutkowska et al., 2014)**. Astrocytes serve a range of physiological and pathophysiological functions in the CNS. They control neuronal maturation, maintenance of synapses and uptake of neurotransmitters **(Wang and Bordey, 2008, Ransom et al., 2003, Kimelberg, 2010)**. These cells also secrete neurotropic factors and antioxidants, scavenge reactive oxygen species, free radicals and toxins and can seal off an inflamed area by forming glial scars **(Lin et al., 1993, Knott et al., 2002, Sidoryk-Wegrzynowicz et al., 2010)**. In addition, astrocytes bi-directionally communicate with immune cells via signalling molecules (for example by cytokines and chemokines) and cell-cell interactions. For instance, astrocytes are capable of inducing apoptosis in T cells, thus limiting the extent of inflammation **(Hara et al., 2011)**. In contrast, however, astrocytes contribute to the progression of disease in experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis, by phagocytising myelin and presenting myelin antigens to $CD4^+$ T cells **(Tan et al., 1998, Kort et al., 2006, Lee et al., 1990, Fontana et al., 1984)**. These cells also release cytokines and chemokines at the site of injury to activate immune cells and induce their influx from the periphery. Astrocytes also stimulate IFNγ and IL17 production by Th1 and Th17 cells and respond to stimulation with these cytokines by increasing expression of inducible nitric oxide synthase, nitric oxide and other pro-inflammatory mediators such as IL6 **(Miljkovic et al., 2011, Bo et al., 1994, Miljkovic et al., 2007)**. We have shown that treatment of astrocytes with IL17/TNFα induces levels of IL6, via the NF-κB pathway **(Elain et al, 2014)**. Moreover, this increased

level of IL6, in turn, can enhance its own production and also that of IL17 **(Ma et al., 2010, Meares et al., 2012, McGeachy et al., 2007)**.

A quarter of the total body cholesterol is located in the brain in myelin sheets and plasma membranes of astrocytes and neurons **(Leoni and Caccia, 2011, Bjorkhem and Meaney, 2004)**. Cholesterol and oxysterols inhibit astrogliosis, where the rate of cholesterol synthesis in the brain has been correlated with reactive astrocyte proliferation **(Bochelen et al., 1995)**. Oxysterols have both, pro- and anti-inflammatory effects depending on the oxysterol and the cell type in question. Increased levels of 24SHC occur in the cerebrospinal fluid of patients with neurological diseases **(Leoni et al., 2004)**. Oxysterols upregulate expression of adhesion molecules (ICAM-1, VCAM-1), growth factors (TGFβ1), cytokines (IL8) and chemokines (monocyte chemotactic protein 1, MCP1) involving ERK and NFκB signalling pathways **(Leonarduzzi et al., 2005, Leonarduzzi et al., 2001, Lemaire et al., 1998, Otaegui-Arrazola et al., 2010)**. *In vitro*, 25HC enhances the release of pro-inflammatory cytokines from placental throphoblasts, airway epithelial cells, monocytic THP-1 cells and macrophages **(Koarai et al., 2012, Aye et al., 2012, Diczfalusy et al., 2009, Rosklint et al., 2002, Lemaire-Ewing et al., 2009)**. On the other hand, oxidised low density lipoprotein and several oxysterols (7βHC, 24SHC, 25HC and 27HC) inhibit TNFα and IL1β mRNA and protein levels in macrophages via NFκB and AP-1 transcription factors **(Ohlsson et al., 1996, Englund et al., 2001)**. In addition, 7β25HC, has immunosuppressive effects in lymphocytes **(Moog et al., 1988)** where EBI2 is a negative regulator of IFNγ response and cytokine levels **(Chiang et al., 2013)**.

To further demonstrate the role of EBI2 in astrocytes, here, we examined the role of EBI2 in human and rodent astrocytes on regulating the levels of pro-inflammatory cytokines and the NFκB signalling pathway. In addition, we report how EBI2 signalling in astrocytes may control their cellular communication with macrophages.

2 Results

2.1 LPS does not induce cytokine release in human astrocytes

Oxysterols exert both, pro- and anti-inflammatory effects depending on the type of oxysterol and cell type **(Aye et al., 2012, Koarai et al., 2012, Fowler et al., 2003, Kim et al., 2006)**. Thus, we first examined the effects of EBI2 agonist 7α25HC (1 μM) or TLR4 agonist LPS (100 ng/ml) treatment of human astrocytes on the levels of a panel of cytokines, by qualitative measurements using cytokine arrays (Figure 4.1). Overall, neither 7α25HC nor LPS induced cytokine release in human astrocytes, with the exception of IL6, which showed a moderate increase compared to non-treated control after LPS treatment (Figure 4.1a and b). Given others and we have also shown that human astrocytes release IL6 after treatment with IL17/TNFα, but not LPS **(Farina et al., 2005, Hartupee et al., 2009, Tarassishin et al., 2014, Elain et al., 2014)**, we next tested the effects of IL17/TNFα on cytokine levels in human astrocytes by quantitative ELISA. Treatment of human astrocytes with 7α25HC (1 μM) or LPS (100 ng/ml) showed no effect on IL6 release ($p>0.05$, n=3, one-way ANOVA and Dunnett's post-test) (Figure 4.1c). Treatment with TNFα (10 ng/ml) or IL17 (50 ng/ml) alone induced a moderate, although not statistically significant, increase in IL6 levels ($p>0.05$, n=3, one-way ANOVA and Dunnett's post-test) (Figure 4.1c). However, the combined treatment of TNFα/IL17 increased IL6 levels by 13-fold, compared to control, (***$p<0.001$, n=3, one-way ANOVA and Dunnett's post-test) (Figure 4.1c). These data corroborate recent findings that human astrocytes do not respond to LPS and that simultaneous activation of IL17 and TNFα receptors in human astrocytes induces an increase in levels of IL6 **(Elain et al., 2014, Tarassishin et al., 2014)**.

2.2 7α25HC attenuates IL17/TNFα-induced levels of IL6 and NFκB translocation to the nucleus in human astrocytes

Oxysterols inhibit inflammatory responses in LPS stimulated microglia **(Kim et al., 2006)**. To investigate whether 7α25HC has similar inhibitory effects on IL6, human astrocytes were pre-treated with or without the EBI2 agonist 7α25HC (1 μM), then stimulated with IL17/TNFα (50/10 ng/ml) and both the mRNA and protein levels of IL6 were measured (Figure 4.2a). The pre-treatment of human astrocytes with 7α25HC (1 μM) significantly attenuated IL17/TNFα-induced protein levels of IL6 (85.4%+/-9.8%, *$p<0.05$, n=3, one-way ANOVA and Bonferroni's post-test) (Figure 4.2b) and mRNA levels of IL6 (53.3%+/-19.3%, **$p<0.01$, n=3, one-way ANOVA and Bonferroni's post-test) (Figure 4.2c), compared to IL17/TNFα treatment alone. These results show that EBI2 activation inhibits IL17/TNFα-induced release of IL6 in human astrocytes. To determine whether 7α25HC-mediated IL6 inhibition involves the NFκB pathway, human astrocytes were treated with IL17/TNFα (50/10 ng/ml) with or without 7α25HC (1 μM) and stained for NFκB and counterstained with Hoechst nuclei dye (Figure 4.2d). Treatment with IL17/TNFα induced translocation of NFκB to the nucleus (218.9%+/-88.9%), in agreement with our previous study **(Elain et al., 2014)**, which was significantly inhibited by 7α25HC (47.8%+/-25.6%, *$p<0.05$, n=4, one-way ANOVA and Bonferroni's post-test) (Figure 4.2e), compared to control. In addition, 7α25HC alone had a moderate inhibitory effect on translocation of NFκB to the nucleus (65.1%+/-25.4%, $p>0.05$, n=4, one-way ANOVA and Bonferroni post-hoc test) possibly indicating tonic activity of EBI2, although this effect was not statistically significant compared to control (Figure 4.2d and e). Taken together, these data suggest that EBI2 activation does not alter NFκB signalling pathway under control conditions and that 7α25HC may exert its immune modulatory effects on IL6 levels via inhibition of the NFκB pathway.

2.3 EBI2 agonism inhibits IL17/TNFα signalling in human astrocytes

To further investigate the role of EBI2 on IL17/TNFα signalling, the effects of 7α25HC on cell impedance was measured using xCELLigence. The inhibitory effects of the EBI2 agonist, 7α25HC (1 μM), on IL17/TNFα-induced changes in cell impedance were investigated (Figure 4.3). Treatment of human astrocytes with IL17/TNFα (295.3%+/-40.6%, ***p<0.001, n=7-12, one-way ANOVA and Bonferroni's post-test) led to a significant increase in impedance cell index (CI) values approximately 20 minutes following treatment, compared to control (Figure 4.3a and c). Notably, treatment with 7α25HC (90.3%+/-13.9%) did not cause a significant change in impedance CI values, compared to control (p>0.05, n=7-12, one-way ANOVA and Bonferroni's post-test) (Figure 4.3a and c). Importantly, pre-treatment with 7α25HC significantly attenuated (78.3%+/-12.9%, ***p<0.001, n=7-12, one-way ANOVA and Bonferroni's post-test) the IL17/TNFα-induced increase in impedance CI values, compared to IL17/TNFα treatment (Figure 4.3b and c). These results further demonstrated that human astrocytes respond to IL17/TNFα and that pre-treatment with 7α25HC significantly inhibits this response.

2.4 7α25HC attenuates LPS-induced levels of IL6 and TNFα in rodent astrocytes

Previous studies have indicated that human astrocytes, as opposed to their rodent counterparts, do not express TLR4 receptors **(Farina et al., 2005)**. In agreement we found LPS (100 ng/ml) did not induce cytokine levels in human astrocytes (Figure 4.1) and therefore, next investigated the effects of LPS and 7α25HC in both rat and mouse astrocytes. Firstly, rat astrocytes were used to demonstrate that LPS increased the levels of several cytokines, whereas 7α25HC treatment showed no effect on cytokine levels (Figure 4.4a). Importantly, pre-treatment of these rat astrocytes with 7α25HC attenuated LPS-induced cytokine release as qualitatively measured by cytokine arrays (Figure 4.4a). Next we used mouse astrocytes to demonstrate if EBI2 had the ability to dampen inflammatory response mediated by LPS. In agreement with both human (Figure 4.2) and rat astrocyte (Figure 4.4a) data, in mouse astrocytes, 7α25HC treatment attenuated the LPS-mediated release of IL6 (90.1%+/-0.4%, *p<0.05, n=3, one-way ANOVA and Bonferroni's post-test) (Figure 4.4b and c) and TNFα (75.3%+/-11.4%, ***p<0.001, n=3, one-way ANOVA and Bonferroni's post-test) (Figure 4.4b and d), compared to control. Taken together, the data showed that EBI2 has an ability to dampen pro-inflammatory signalling induced via different immune receptor signalling pathways such as TLR4 and TNFαR/IL17R.

2.5 LPS induces changes in mRNA levels of EBI2 and 7α25HC related enzymes in mouse astrocytes

We and others have previously demonstrated that LPS alters molecules of the EBI2 signalling pathway, namely EBI2 and the 7α25HC synthesising and degrading enzymes (Figure 4.5a) **(Park and Scott, 2010, Diczfalusy et al., 2009, Preuss et al., 2014a, Lattin et al., 2008, Schroder et al., 2012)**. In accordance with these studies, here we found that LPS induced a rapid and lasting suppression of EBI2 mRNA expression in mouse astrocytes (Figure 4.5b). In addition, LPS induced expression of both 7α25HC synthesising enzymes namely CH25H and CYP7B1. CH25H mRNA peaked after four to six hours (631.5%+/-224.8% at four hours and 606.3%+/-335.1% at six hours compared to control, ***p<0.001, *p<0.05, n=3, one-way ANOVA and Dunnett's post-test) (Figure 4.5c) and CYP7B1 mRNA expression slowly and gradually increased over 24 hour period (635.8%+/-436.8% at 24 hours, compared to control, ***p<0.001, n=3, one-way ANOVA and Dunnett's post-test) (Figure 4.5d). The mRNA levels of the 7α25HC degrading enzyme HSD3B7 remained constant for the first eight hours of LPS treatment and increased after 24 hours (150.3%+/-6.6%, compared to control, *p<0.05, n=3, one-way ANOVA and Dunnett's post-test) (Figure 4.5e). These results demonstrate that molecules of

the EBI2 signalling pathway in astrocytes are sensitive to immune challenges (such as LPS) and support the idea that EBI2 may be involved in sensing infection in both immune and central nervous systems.

2.6 Mouse astrocyte conditioned media induces macrophage (RAW264.7) migration

We have shown that macrophages express enzymes necessary for oxysterol synthesis and suggest they release oxysterols to induce migration of other cells **(Preuss et al., 2014a)**. These effects on migration were partially inhibited with EBI2 antagonist (NIBR189) indicating EBI2 mediated signalling **(Preuss et al., 2014a)**. Here, the effects of 7α25HC and media taken from LPS stimulated mouse astrocytes on migration of macrophage (RAW264.7) cell line was examined using xCELLigence migration assay chamber system (Figure 4.6a). The direct application of exogenous 7α25HC (0.01 μM for 4 hours) to the lower chamber induced a significant increase in the migration of macrophages, compared to control (242.5%+/-81.3%, ***$p<0.001$, n=5, one-way ANOVA and Dunnett's post-test) (Figure 4.6b). Significantly, we also observed that media collected from astrocytes stimulated with LPS induced macrophage migration in a time-dependent manner in contrast to non-treated astrocytes (192.2%+/-50.2% at 6 hours, 211.0%+/-66.5% at 8 hours, compared to control, *$p<0.05$, n=3 and 4, one-way ANOVA and Dunnett's post-test) (Figure 4.6b). Notably, media from astrocytes treated with LPS for 2, 4 and 24 hours did not induce macrophage migration, indicating a temporal response and also indicating that LPS itself was not having a direct effect on macrophage migration in these set of experiments (Figure 4.6b). To investigate whether the observed migratory effects were EBI2 dependant, the astrocyte-conditioned media was supplemented with EBI2 antagonist (NIBR189, 10 μM). In agreement with previous studies **(Preuss et al., 2014a)**, the antagonism of EBI2 inhibited the effects of exogenous 7α25HC on macrophage migration (109.5%+/-57.9%, *$p<0.05$, n=3, one-way ANOVA and Bonferroni's post-test). Similar to previous studies **(Preuss et al., 2014a)**, conditioned media taken from astrocytes stimulated with LPS for 6 and 8 hours and supplemented with EBI2 antagonist (NIBR189) attenuated previously observed macrophage migration however, statistical significance was not reached ($p>0.05$, n=3, one-way ANOVA and Bonferroni's post-test) suggesting partial EBI2 involvement in regulation of macrophage migration (Figure 4.6c). Overall, therefore, the data suggests that astrocytes stimulated with LPS can induce macrophage migration and, moreover, that EBI2 can regulate this cellular crosstalk.

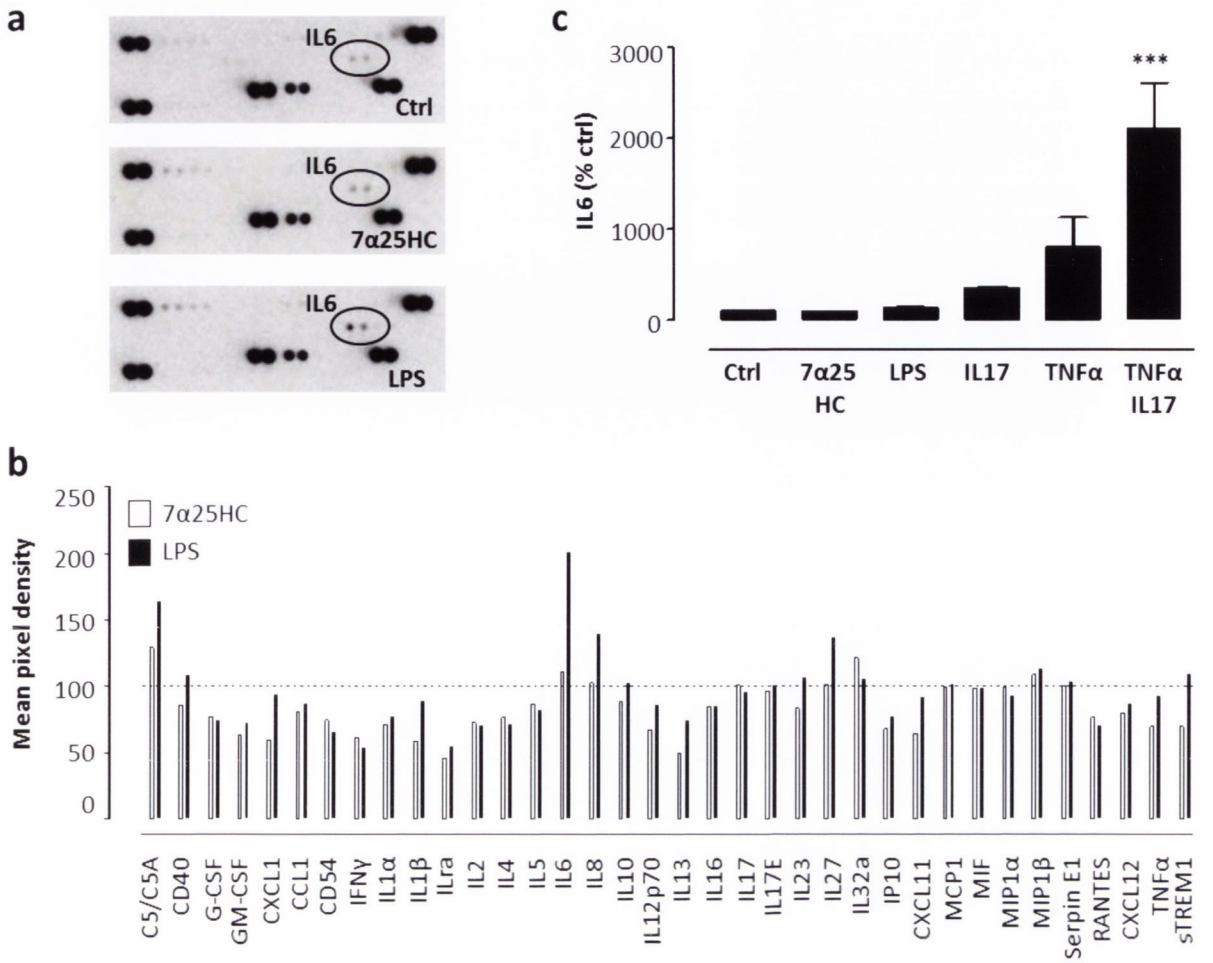


Figure 4.1 LPS does not induce release of cytokines and chemokines in human astrocytes (a) Qualitative multiple cytokine array data shows that neither LPS (100 ng/ml, 4 hours) nor 7α25HC (1 μM, 4 hours) treatment of human astrocytes induces release of cytokines **(b)** graphical densitometry of the cytokine panels shown in **(a)**. **(c)** Quantitative ELISA data shows that combined treatment with IL17/TNF (50/10 ng/ml, 18-24 hours) induces a 13-fold increase in IL6 levels in human astrocytes, whereas IL17 (50 ng/ml) and TNFα (10 ng/ml) alone showed only moderate effects. LPS (100 ng/ml) and 7α25HC (1 μM) did not induce IL6 release in mouse astrocytes. Data presented as mean +/- SEM, n=3, one-way ANOVA and Dunnett's post-hoc test, ***$p<0.001$ vs. control.

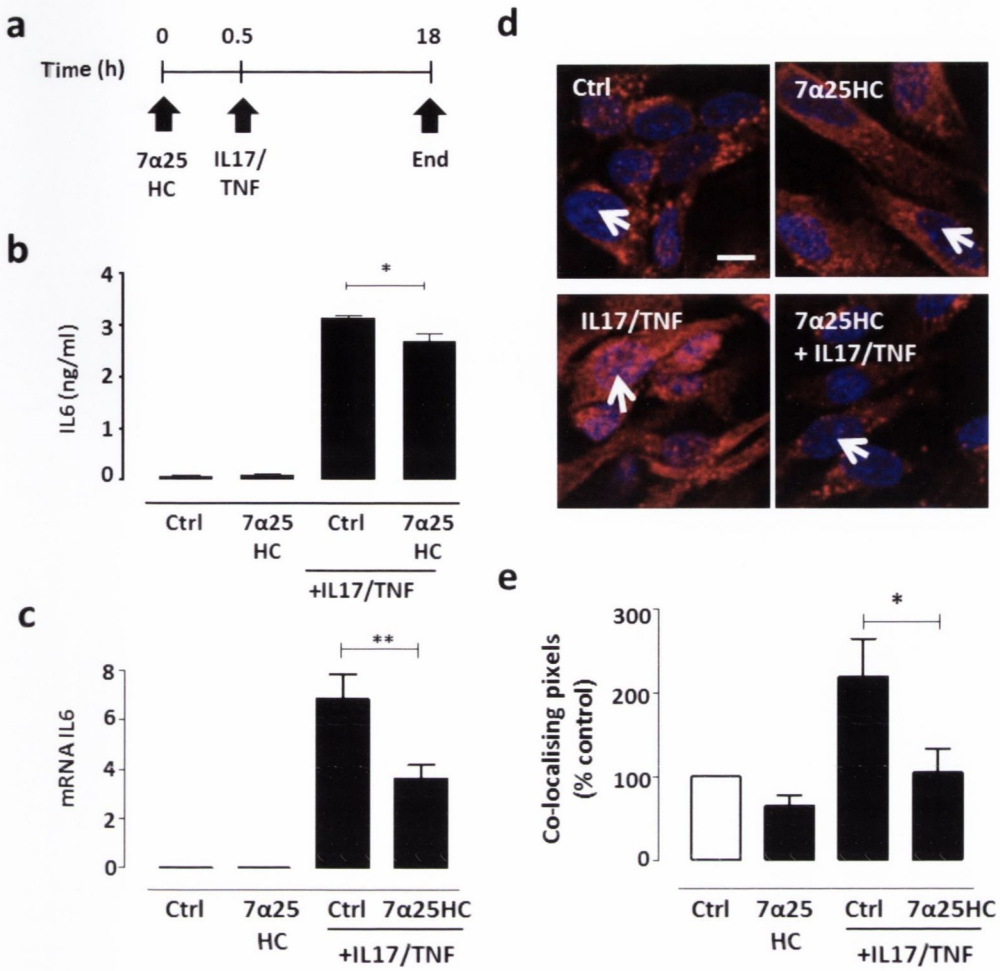


Figure 4.2 7α25HC attenuates IL17/TNFα-induced levels of IL6 and NFκB nuclear translocation in human astrocytes (a) Schematic diagram illustrating experimental design. (b) Pre-treatment with 7α25HC (1 μM) attenuates IL17/TNF (50/10 ng/ml) mediated increase in the levels of IL6 protein and (c) mRNA levels in human astrocytes. Data shown is a representation of three separate experiments (n=3). Data presented as mean +/- SEM, one-way ANOVA and Bonferroni post-test, *p<0.05, **p<0.01 vs. corresponding control. (d) Representative confocal images show NFκB (red) and nuclei (Hoechst, blue) immunostaining. (e) Bar graph shows 30 minutes pre-treatment with 7α25HC (1 μM) inhibited NFκB translocation to the nucleus induced by IL17/TNF (50/10 ng/ml, 18-20 hours) in human astrocytes. The number of Hoechst (blue) and NFκB (red) co-localising pixels were quantified by Zen 2011 software. Data presented as mean +/- SEM (n=4), one-way ANOVA and Bonferroni's post-test, *p<0.05, vs. corresponding control. Images taken at 40x magnification. Scale bar, 10 μM.

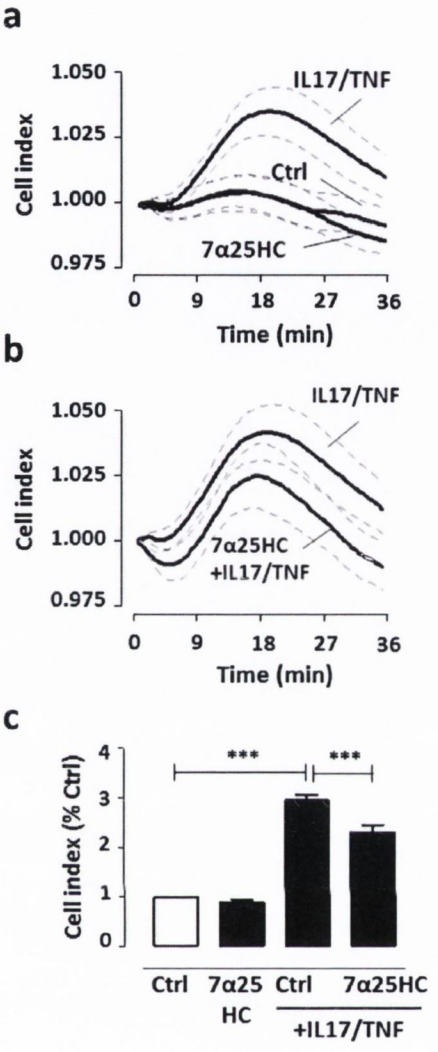


Figure 4.3 7α25HC inhibits IL17/TNFα-induced signalling in human astrocytes (a) Representative traces show effects of IL17/TNF (50/10 ng/ml) and 7α25HC (1 μM) on impedance cell index values over a time period of 36 minutes in human astrocytes. Corresponding bar graph illustrates treatment with IL17/TNFα (50/10 ng/ml) causes significant increase in cell impedance, whereas treatment with 7α25HC (1 μM) does not induce such changes compared to control. **(b)** Representative traces showing 30 minutes pre-treatment with 7α25HC (1 μM) inhibits IL17/TNFα (50/10 ng/ml) induced increase in impedance cell index values over a time period of 36 minutes in human astrocytes. **(c)** Bar graph illustrates pre-treatment with 7α25HC (1 μM) significantly reduces IL17/TNFα (50/10 ng/ml) induced increase in impedance cell index value. For graphical data, normalized cell index was calculated by subtracting the maximum response from the minimum response for each condition. Graphical data is presented as average (solid line) +/- SEM (dotted line) (n=7-12, observations), one-way ANOVA and Bonferroni's post-test, ***$p<0.001$ vs. corresponding control.

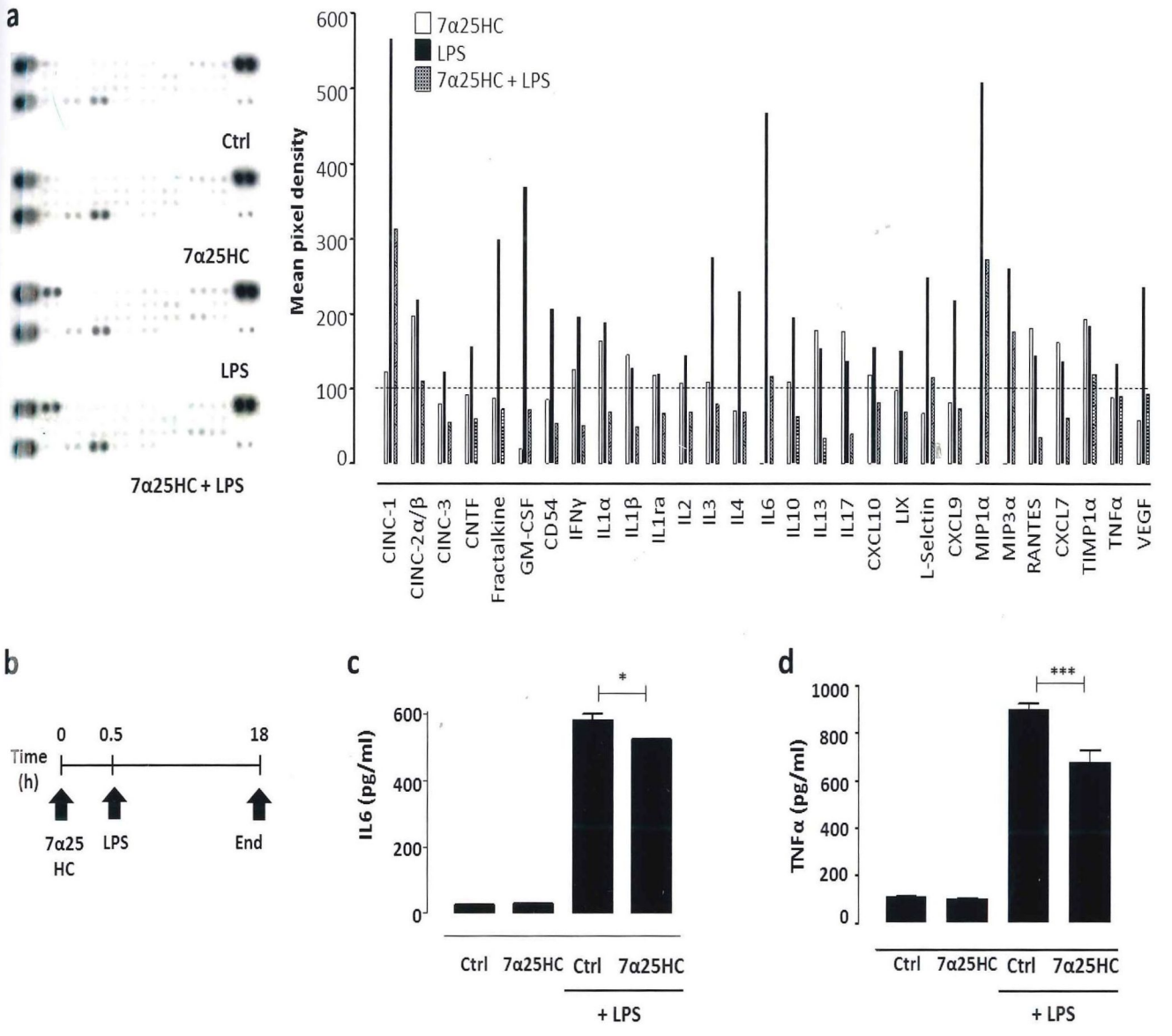


Figure 4.4 7α25HC attenuates LPS-induced levels of IL6 and TNFα in rodent astrocytes (a) Qualitative multiple cytokine array data shows that 7α25HC (1 μM, 4 hours) does not increase levels of cytokines in rat astrocytes, whereas LPS (100 ng/ml, 4 hours) induces an increase in cytokines. A 30 minute pre-treatment with 7α25HC dampens LPS (100 ng/ml, 4 hours) induced increase of cytokines/chemokines in rat astrocytes (*left panel,* shows cytokine arrays; *right panel,* shows graphical densitometry). **(b)** Schematic diagram illustrating experimental design using mouse astrocytes. Treatment of mouse astrocytes with 7α25HC (1 μM) attenuates LPS (100 ng/ml) mediated increase in the levels of **(c)** IL6 and **(d)** TNFα protein levels. Data shown is a representation of three separate experiments (n=3). Data presented as mean +/- SEM, one-way ANOVA and Bonferroni's post-test, *$p<0.05$, ***$p<0.001$ vs. corresponding control.

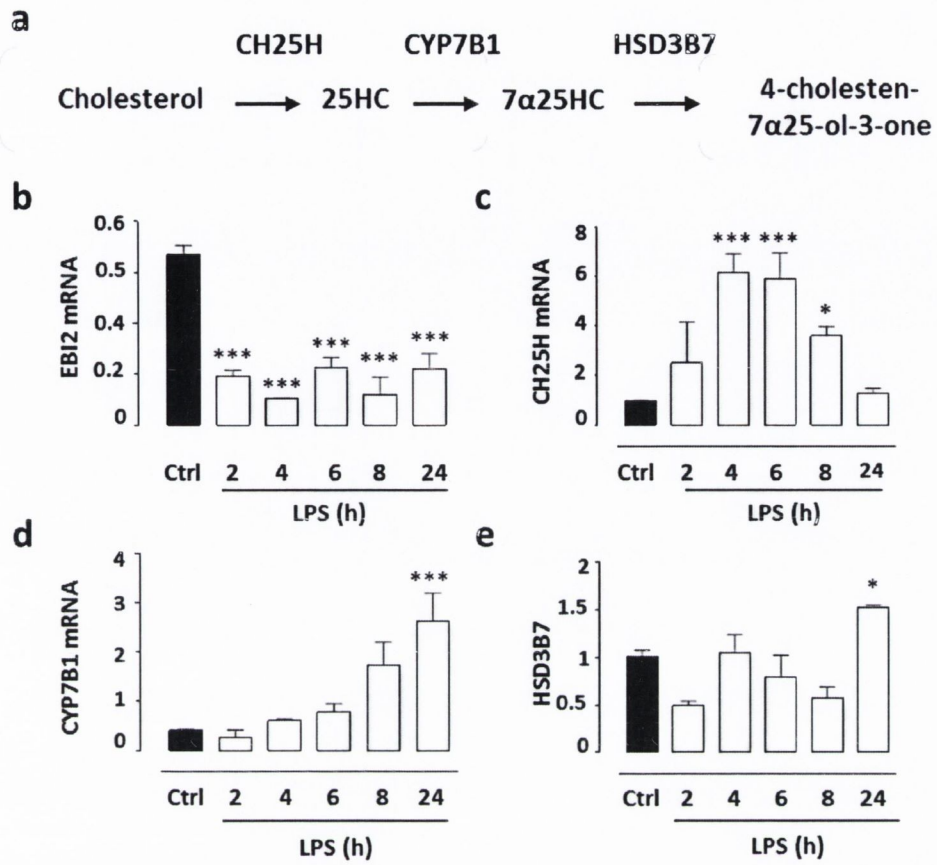


Figure 4.5 LPS induces changes in mRNA levels of EBI2 and 7α25HC synthesizing and degrading enzymes in mouse astrocytes (a) Synthetic and degrading pathway of 7α25HC. **(b)** Treatment of mouse astrocytes with LPS (100 ng/ml) induces a rapid and lasting suppression of EBI2 mRNA expression. **(c)** LPS induces CH25H mRNA expression in mouse astrocytes that peaks after four to six hours and declines thereafter. **(d)** LPS induces a slow and gradual increase in CYP7B1 mRNA expression in mouse astrocytes. **(e)** LPS induces increase in HSD3B7 mRNA expression after 24 hour treatment. Data presented as mean +/- SEM, n=3, one-way ANOVA and Dunnett's post-test, *p<0.05, ***p<0.001 vs. corresponding control.

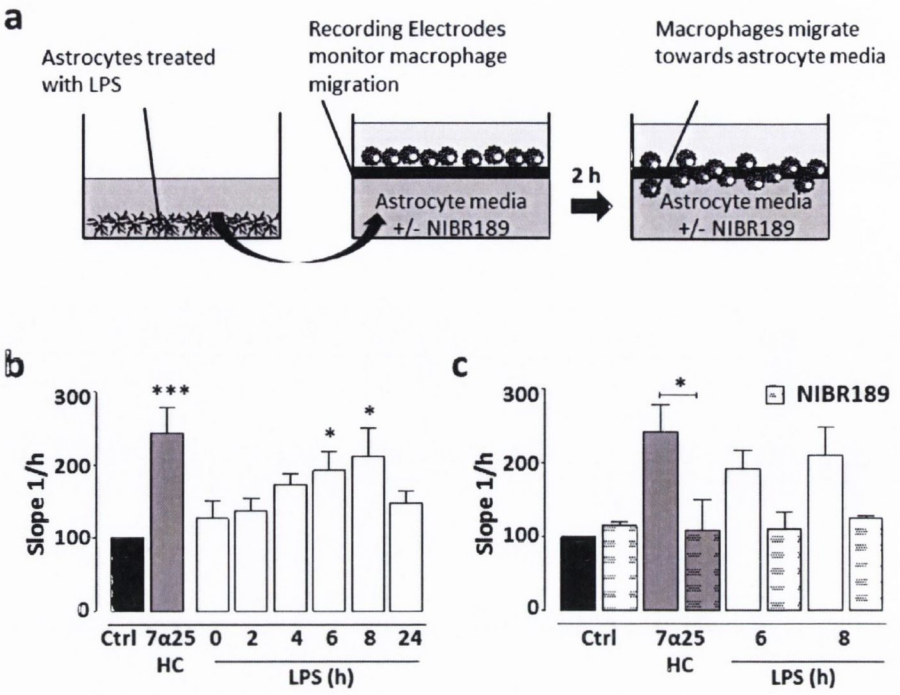


Figure 4.6 Mouse astrocyte conditioned media induces macrophage (RAW264.7) migration. (a) Schematic of experimental setup. Transwell system where macrophages (RAW264.7) were plated on the recording electrodes (upper chamber) and mouse astrocyte conditioned media with or without 7α25HC or NIBR189 was added to the lower chamber. The migration of macrophages from the upper chamber to the lower chamber was measured by the recording electrodes four hours after addition of astrocyte media or 7α25HC to the bottom chamber. **(b)** Treatment with 7α25HC (0.01 μM) induced a significant increase in macrophage migration compared to control. Moreover, supernatant from mouse astrocytes treated with LPS (100 ng/ml) for zero to 24 hours was collected and added to the lower chamber in the xCELLigence migration assay. Astrocyte media collected after six and eight hours of LPS (100 ng/ml) treatment induced macrophage (RAW264.7) migration while media from non-treated astrocytes or treated with LPS for zero, two, four and 24 hours did not induce macrophage (RAW264.7) migration indicating an indirect LPS effect of astrocyte media on macrophage migration. Data presented as mean +/- SEM, n=3-5, one-way ANOVAs with Dunnett's post hoc tests, *$p<0.05$, ***$p<0.001$ vs. corresponding control. **(c)** Both 7α25HC (0.01 μM) and astrocyte conditioned media obtained from six and eight hour LPS-treated mouse astrocytes induced macrophage migration. However, addition of the EBI2 antagonist (NIBR189, 10 μM) to 7α25HC or the LPS-treated mouse astrocyte media attenuated astrocyte conditioned media effects on macrophage migration however, significant effects were only observed for the effects exerted by the exogenously added 7α25HC indicating EBI2 mediated effects. Data presented as mean +/- SEM n=3, one-way ANOVAs with Bonferroni's post-tests, *$p<0.05$, vs. corresponding control.

3 Discussion

Astrocytes are one of the four glial cells in the CNS that serve a multitude of roles including CNS immune defence, regulation of blood-brain barrier, synaptic transmission and cellular communication by means of release of various growth factors and signalling molecules **(Kimelberg, 2010)**. Astrocytes are not, however, only involved in regulation of the normal physiological brain functions. They are implicated as having detrimental effects and driving the disease progress in many autoimmune and neurodegenerative diseases. Astrocyte increased activity exacerbates inflammatory responses in multiple sclerosis, Parkinson's disease and Alzheimer's disease **(Sidoryk-Wegrzynowicz et al., 2010)**. Therapies that control astrocyte activation may therefore prove useful in managing these disorders. In the current study, EBI2 signalling and function in human and rodent astrocytes were investigated by examining their role in regulating the levels of cytokines in astrocytes and demonstrating the role of EBI2 in cellular communication between astrocytes and macrophages. We found that 7α25HC attenuated the TNFα/IL17-induced increase in levels of IL6 in human astrocytes, where NFκB was likely involved. EBI2 also inhibited LPS-induced increase in levels of IL6 and TNFα in rodent astrocytes. We also demonstrated that mouse astrocytes regulated mRNA levels of EBI2 and both the 7α25HC synthesising and degrading enzymes in response to a challenge with LPS. Importantly, conditioned media obtained from LPS-stimulated astrocytes induced macrophage (RAW264.7) migration that was partially attenuated with the EBI2 antagonist NIBR189. Taken together, the data indicates that EBI2 regulates the levels of pro-inflammatory cytokines in astrocytes, that molecules in the EBI2 signalling pathway are sensitive to pro-inflammatory signals, and that EBI2 plays a role in communication between astrocytes and macrophages.

3.1 EBI2 inhibits pro-inflammatory cytokine release and NFκB signalling in astrocytes

There is a bi-directional communication between astrocytes and peripheral immune cells via signalling molecules such as cytokines, chemokines, growth factors and others. At the site of injury, astrocytes release cytokines and chemokines to activate resident immune cells and induce influx of leukocytes from periphery **(Kimelberg, 2010)**. On the other hand, leukocytes can influence astrocytes by secreting cytokines such as IL17, TNFα and IL1β **(Larochelle et al., 2011, Bo et al., 1994)**, which induce astrocytes to release pro-inflammatory signals such as IL6. In turn, the release of molecules such as IL6 can augment positive feedback loop that drives IL6 and IL17 signalling **(Ma et al., 2010, Meares et al., 2012, McGeachy et al., 2007)**. Previous research has shown that mice deficient in CH25H enzyme cannot synthesise oxysterol 25HC and show aggravated course of EAE **(Reboldi et al., 2014)**. In the same study, CH25H deficient mouse macrophages released greater amounts of IL1β after LPS stimulation than their wild type counterparts, suggesting that 25HC has anti-inflammatory properties **(Reboldi et al., 2014)**. In the current data, we have shown that pre-treatment of human astrocytes with 7α25HC inhibited both mRNA and protein levels of IL6 in TNFα/IL17-treated human astrocytes and, furthermore, that 7α25HC also reduced the protein levels of IL6 and TNFα in LPS-challenged rat and mouse astrocytes. In the current study, we also showed that treatment of human astrocytes with 7α25HC alone did not induce pro-inflammatory cytokine release and did not activate NFκB signalling, however, it attenuated the nuclear translocation of NFκB mediated by TNFα/IL17 treatment. In addition to regulating anti-inflammatory effects via NFκB, both ERK1/2 and Ca^{2+} signalling have been implicated in oxysterol-induced cytokine regulation **(Aye et al., 2012, Lemaire-Ewing et al., 2009, Dugas et al., 2010)**. It may be relevant therefore, that we have previously shown activation of EBI2 with 7α25HC also induces ERK1/2 and Ca^{2+} signalling

(Rutkowska et al., 2014). Thus, in astrocytes, we suggest that EBI2 regulation of NFκB, ERK1/2 and Ca^{2+} signalling may play a role in altering cytokine signalling induced by TNFα/IL17 and LPS.

3.2 EBI2 regulates inter-cellular communication between astrocytes and macrophages

In addition to the regulation of cytokine levels, our data also suggests that the EBI2 signalling pathway (namely the receptor and 7α25HC metabolic enzymes) expressed in astrocytes respond to immune challenge (for example LPS stimulation). Similar findings were reported for primary human monocyte-derived macrophages with the exception that in the current study primary mouse astrocytes significantly down-regulated mRNA expression of EBI2 as opposed to transient increase of EBI2 transcripts in human macrophages **(Preuss et al., 2014a)**. Such species differences in human and rodent astrocytes toward responsiveness to immune-challenge has been reported previously **(Tarassishin et al., 2014)** and was corroborated by our data, showing mouse astrocytes, but not human astrocytes respond to LPS by downregulating EBI2 transcripts. More specifically, we found that the 7α25HC synthetic enzymes CH25H and CYP7B1 showed increased expression in astrocytes treated with LPS, while the degrading enzyme HSD3B7 showed modest changes at later time-points. We propose, therefore that LPS could transiently increase levels of 7α25HC at four to eight hours after challenge, which would return to baseline 24 hours later. The potential that LPS could increase the levels of 7α25HC, although this would require direct measurements of the levels of 7α25HC, may induce EBI2 desensitisation, internalisation and subsequent degradation in astrocytes, potentially forming a feedback mechanism, in agreement with our findings that LPS decreases levels of EBI2 expression. More importantly, an increase in 7α25HC may drive the migration of immune cells into the CNS, which is in sync with our data showing that astrocytes challenged with LPS can promote migration of macrophages and that EBI2 plays a role in this process. Thus, regulation of the EBI2 signalling pathway (both the receptor and the enzymes that control levels of the ligand) in astrocytes might therefore be involved in the influx of peripheral blood cells into the CNS during inflammation. Taken together, these findings suggest a potential role for EBI2 in equipping astrocytes with the ability to directly sense and respond to pathogenic attack.

We have discussed recently the potential roles of the EBI2 signalling pathway molecules in disease and their potential uses as drug targets for EBV infection and EBV-mediated diseases as well as type-1-diabetes, multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus **(Daugvilaite et al., 2014)**. A more direct link between EBI2 signalling pathway molecules and brain cell function, however, comes from the findings that CYP7B1 mutations result in hereditary spastic paraplegia type 5 (SPG5) **(Schule et al., 2010, Di Fabio et al., 2014)**. Notably, in this illness, deficits in axonal trafficking, altered myelination state and neurodegeneration have been observed, suggesting a dysfunction in EBI2 signalling may induce brain pathophysiology as well as aberrant immune cell function. Whether mutations in CYP7B1 result in reduced levels of 7α25HC and subsequent reduced EBI2 activation in patients with SPG5 and whether astrocytes play a specific role in this disease remains, however, to be clarified. It has recently been shown that animals deficient in the other 7α25HC synthesising enzyme, namely CH25H, exhibit worsened course of EAE and that CH25H KO macrophages produce increased levels of IL1β in response to LPS **(Reboldi et al., 2014)**. Here, we show that the EBI2 signalling pathway in astrocytes equips these cells with a mechanism to respond to immune-challenge, to regulate pro-inflammatory response of astrocytes, and to communicate with macrophages.

Chapter 5: EBI2 regulates myelin development and inhibits LPC-induced demyelination

Chapter aims

The study aims are to:

- investigate the role of EBI2 in myelin development in wild type and EBI2 knock-out animals
- explore oligodendrocyte markers expression in wild type and EBI2 knock-out mice brains during myelin development
- study whether EBI2 signalling is involved in myelin development and maintenance in organotypic cerebellar slices
- identify the role of EBI2 in LPC-induced demyelination in slice cultures
- investigate the effects of 7α25HC on LPC-induced pro-inflammatory cytokine release in slices

Abstract

The G protein-coupled receptor EBI2 (Epstein-Barr virus-induced gene 2) is activated by 7α, 25-dihydroxycholesterol (7α25HC) and plays a role in T cell-dependant antibody response and B cell migration. Aberrant EBI2 signalling is implicated in a range of autoimmune disorders however its role in the CNS remains poorly understood. Here we characterize the role of EBI2 in myelination under normal and pathophysiological conditions using organotypic cerebellar slice cultures and EBI2 knock-out (KO) animals. We find that MBP expression in brains taken from EBI2 KO mice is delayed compared to those taken from wild type (WT) mice. In agreement with these *in vivo* findings, we show that antagonism of EBI2 reduces MBP expression *in vitro*. Importantly, we demonstrate that EBI2 activation attenuates lysolecithin-induced demyelination in mouse organotypic slice cultures. Moreover, EBI2 activation also inhibits lysolecithin-mediated release of pro-inflammatory cytokines such as IL6 and IL1β in cerebellar slices. These results, for the first time, display a role for EBI2 in myelin development and protection from demyelination under pathophysiological conditions and suggest that modulation of this receptor may be beneficial in neuroinflammatory and demyelinating disorders.

1 Introduction

A quarter of the total body cholesterol is located in the brain of which the majority is in oligodendrocytes, neurons and astrocytes **(Leoni and Caccia, 2011)**. During myelin development cholesterol synthesis in the CNS is upregulated in neurons, astrocytes and oligodendrocytes. When myelination is complete the synthesis of cholesterol is reduced and supplied to neurons mainly by astrocytes **(Pfrieger and Ungerer, 2011)**. Cholesterol is a crucial component of myelin and deficiency or lack of cholesterol staggers the rate of myelin formation and results in motor symptoms, as has been shown in genetically modified mice in which myelinating oligodendrocytes cannot synthesise cholesterol **(Saher et al., 2005)**.

Oxysterols, oxygenated derivatives of cholesterol, are involved in cholesterol homeostasis and turnover and have been shown to play important roles in myelination **(Nelissen et al., 2011)**. The oxysterol 24SHC is a brain specific cholesterol metabolite that is thought to be synthesised exclusively in the brain and secreted into circulation via the blood-brain barrier **(Bjorkhem and Meaney, 2004)**. In addition to 24SHC other oxysterols such as 25HC and 27HC are synthesised in the brain via enzymatic action of cholesterol-25-hydroxylase (CH25H) and cholesterol 27-hydroxylase (CYP27A1) **(Russell, 2000)**. Studies have also shown that peripheral application of LPS induces mRNA and protein levels of CH25H in mouse brains and leads to a significant increase in the levels of 25HC protein in the brain **(Bauman et al., 2009, Waltl et al., 2013)**. In addition to being involved in normal myelin development, oxysterols have also been shown to have detrimental effects on myelin. For instance, they inhibit myelin gene expression in the peripheral nervous system via liver x receptor (LXR) α and β mediated signalling **(Makoukji et al., 2011)**. Oxysterols have also been shown to have adverse effects on oligodendrocyte viability *in vitro*. Indeed, treatment of an oligodendrocyte cell line 158N with 25HC or 22SHC induced cell death and morphological changes independently of LXR signalling **(Trousson et al., 2009)**. On the other hand, mass spectroscopy analysis of oxysterols shows the presence of oxysterol biosynthetic enzymes and oxysterols in oligodendrocytes indicating that oxysterols may signal in an autocrine/paracrine manner in oligodendrocytes **(Trousson et al., 2009)**. In addition, oxysterols enhance expression and activity of phospholipase A2 leading to a protective effect in oligodendrocytes **(Trousson et al., 2009)**. These studies show that oxysterols may play dual role in oligodendrocyte/CNS biology having either detrimental or supportive functions **(Trousson et al., 2009)**.

Abnormal levels of oxysterols have been found in a number of neurodegenerative diseases such as Alzheimer's disease, multiple sclerosis or cerebrotendinous xanthomatosis **(Lutjohann et al., 2000, Leoni et al., 2002, Panzenboeck et al., 2007)**. Upregulated levels of oxysterols have been detected in the brains of experimental autoimmune encephalomyelitis (EAE) animals and multiple sclerosis (MS) patients **(Diestel et al., 2003)**. Some studies suggest that certain oxysterols exacerbate the pathology of MS and development of EAE **(Teunissen et al., 2007, Teunissen et al., 2003, Quintana et al., 2008)**, while others have found that genes such as CH25H are upregulated in microglia during demyelinating and remyelinating stages of EAE to support myelination **(Olah et al., 2011)**. It has also been shown that deficiency in CH25H in mice results in exaggerated course of EAE and also leads to overproduction of IL1β in CH25H KO macrophages suggesting anti-inflammatory properties of 25HC **(Reboldi et al., 2014)**. Of interest, mutations in the CYP7B1 gene, which encodes for the enzyme that converts 25HC into a 7α25HC, have been identified as the cause of spastic paraplegia gene 5 (SPG5). This neurodegenerative disorder belongs to a group of diseases called hereditary spastic paraplegias,

and is characterised by neuropathy of upper motor neurons as well as periventricular and subcortical white matter lesions **(Schule and Schols, 2011, Biancheri et al., 2009)**.

Inflammation in the CNS and the presence of pro-inflammatory cytokines such as TNFα/β and IL1β have been shown to induce demyelination *in vivo* **(Redford et al., 1995, Ferrari et al., 2004)**. Direct injection of TNFα/β into the sciatic nerve induced inflammation, axonal damage and demyelination **(Redford et al., 1995)**. In another study chronic IL1β expression induced inflammation, breakdown of the blood-brain barrier (BBB), activation of astrocytes and microglia and pronounced demyelination without the loss of neurons **(Ferrari et al., 2004)**. In *in vitro* studies, organotypic cerebellar slices are commonly used to examine development of myelin as well as processes regulating demyelination and remyelination **(Birgbauer et al., 2004, Sheridan and Dev, 2011, Miron et al., 2010)**. Inflammation and presence of pro-inflammatory cytokines and oxidative stress have been shown to induce demyelination in organotypic slice cultures **(di Penta et al., 2013, Sheridan and Dev, 2011)**. Studies have shown that lysophosphatidylcholine (LPC) and lipopolysaccharide (LPS) induce increase in IL1β, IL6, TNFα, CXCL5 (LIX) and CCL3 (MIP1α) in organotypic slices and lead to oligodendrocyte and axonal damage and demyelination **(di Penta et al., 2013, Sheridan and Dev, 2011)**. Previous studies have suggested that inhibition of pro-inflammatory chemokine/cytokine levels in cerebellar slices might be a mechanism via which LPC-induced demyelination is restricted **(Sheridan and Dev, 2011)**. Treatment of cerebellar slices with FTY720, an S1P receptor agonist that has immunomodulatory properties, attenuated LPC-induced demyelination and pro-inflammatory chemokine release **(Sheridan and Dev, 2011)**. Similar mechanisms were observed for cuprizone-induced demyelination in corpus callosum where treatment with FTY720 protected from demyelination via inhibition of pro-inflammatory cytokine release such as IL1β **(Kim et al., 2011)**. Others have also shown that inhibition of TNFα signalling attenuated demyelination in organotypic slices indicating a direct role of pro-inflammatory cytokines in mediating myelin damage **(di Penta et al., 2013)**.

The main signalling events involved in oligodendrocyte generation, differentiation and myelination include sonic hedgehog, Notch signalling, growth factors such as PDGF and FGF, and transcription factors such as Sox10 **(Verkhratsky and Butt, 2013)**. Other pathways crucial for the myelin gene expression include the Wnt signalling pathway that has been shown to be differentially regulated by oxysterols in the central and peripheral nervous system (PNS) **(Shackleford et al., 2013)**. It has been shown that oxysterols inhibit Wnt transcripts in Schwann cells and activate them in oligodendrocytes **(Shackleford et al., 2013)**. Oxysterol 25HC also has differential effects on myelin genes (myelin protein zero (MPZ) and peripheral myelin protein-22 (PMP22)) expression. Namely, it inhibited MPZ and PMP22 in Schwann cells but not in the oligodendrocyte cell line **(Shackleford et al., 2013)**. Other major signalling pathways involved in oligodendrocyte differentiation and myelination are Fyn kinase, phosphatidylinositol 3' -kinase (PI3K) - Akt (PI3K-Akt), mitogen-activated protein kinase (MAPK), cyclic AMP response element-binding (CREB) and Janus kinase - STAT (JAK/STAT) signalling **(Verkhratsky and Butt, 2013)**. Some studies have also shown that the extracellular signal-regulated kinase (ERK1/2) signalling promotes normal myelin development as well remyelination after injury and persistent ERK1/2 signalling increases myelin thickness after injury **(Ishii et al., 2012, Fyffe-Maricich et al., 2011, Xiao et al., 2012)**. Other studies have shown that increase in ERK1/2 signalling induced by rolipram (phosphodiesterase 4 inhibitor which prevents breakdown of cAMP) promotes oligodendrocyte maturation and enhances remyelination after LPC- and cuprizone-induced demyelination in mice and cerebellar slices **(Sun et al., 2012)**.

Since oligodendrocytes use cholesterol as a building block for myelin and oxysterols are involved in cholesterol homeostasis and turnover, it is possible that EBI2, which is activated by oxysterols, may play an important role in myelination **(Nelissen et al., 2011)**. Thus, to examine the role of EBI2 signalling in myelination under normal and pathophysiological conditions, the effect of EBI2 activation on myelination state was examined *in vitro* using organotypic cerebellar slices and *in vivo* in wild type (WT) and EBI2 knock-out (KO) animals.

2 Results

2.1 Organotypic cerebellar slices maintain host tissue cytoarchitecture and physiology

Previous studies have shown that cerebellar organotypic slice cultures can be used to study the complex multi-cellular organisation of the brain and are more physiologically accurate than isolated single cell type cultures **(Lossi et al., 2009, Sheridan and Dev, 2011, Birgbauer et al., 2004)**. These slices can be maintained in culture for several weeks and have been shown to be a suitable *in vitro* assay for studying myelination **(Dupont et al., 2006, Takacs and Metzger, 2002, Notterpek et al., 1993, Birgbauer et al., 2004)**. Here, the cytoarchitecture of cerebellar tissue in organotypic slices prepared from postnatal day 10 (P10) mice and cultured for 14 days *in vitro* (14 DIV) was investigated with immunocytochemistry. The data showed that CNS resident cells including neurons (Figure 5.1b), astrocytes (Figure 5.1a), microglia (Figure 5.1d) and oligodendrocytes (Figure 5.1b) were present in the slices after 14 DIV. Peripheral immune cells such as macrophages, monocytes, dendritic cells and lymphocytes (Figure 5.1c, e and f) were also present in the slices. This data suggests, perhaps, that CNS-immune system interactions that occur *in vivo* may have potential to take place in these slice cultures. In addition, we find that microvascular morphology is also preserved as shown by alignment of CD31 (endothelial cells, Figure 5.1a), with GFAP (astrocytes, Figure 5.1a) and F4/80 (macrophages, Figure 5.1c); suggestive that GFAP and F4/80 positive cells envelop the CD31 cells in the brain as found in the blood-brain barrier. Most importantly, the data showed that myelination, which is mainly observed in the central white matter at P10, continues to develop in this *in vitro* model for at least 14 DIV **(Birgbauer et al., 2004)** making it a model for studying myelination processes under normal and pathophysiological conditions (Figure 5.1b).

2.2 EBI2 deficiency results in transient delay in MBP expression

Since oligodendrocytes use cholesterol as a building block for myelin and oxysterols are involved in cholesterol homeostasis and turnover, it is possible that activation of EBI2 by oxysterols plays an important role in myelination. In agreement with this idea, studies have shown that activation of liver x receptors by oxysterols indeed regulates cholesterol homeostasis in oligodendrocytes **(Nelissen et al., 2011)**. Thus, to investigate whether EBI2 also has a role in regulation of myelination state, cerebellar slices were prepared from P10 EBI2 KO and WT mice and cultured for 1-14 DIV (Figure 5.2). The extent of myelination was measured with confocal microscopy quantifying MBP staining. The data showed less MBP staining in cerebellar slices prepared from EBI2 KO mice compared to WT mice in the first one to two DIV (75.1% +/- 20.6% at one DIV and 70.6% +/- 21.3% at two DIV) and reaching WT levels after four to five DIV (95.1% +/- 26.2%) (**$p<0.01$, ***$p<0.001$, five to six animals per group, independent student t-test) (Figure 5.2a and b). To further study the effects of EBI2 deficiency on the observed lower levels of MBP expression, whole mouse brain lysates of WT and EBI2 KO animals were prepared and MBP protein expression was measured with Western blots (Figure 5.2c-e). The analysis showed that MBP expression in brains from EBI2 KO mice is deficient at P14 compared to MBP expression in brains from WT mice (54.9% +/- 22.9%, ***$p<0.001$, three animals per time point, independent student t-test) (Figure 5.2c and d). In agreement with organotypic slice data, MBP expression *in vivo* was deficient only at earlier stages of development and reached WT levels at P30 (80.5% +/- 19.5%, $p>0.05$, three animals per time point, independent student t-test) (Figure 5.2c and e). The data suggest that EBI2 signalling is involved in myelin development at least during earlier stages of myelination and warrants further investigations into the role of EBI2 and oxysterols in myelination.

2.3 Olig1, CNPase and MOG are not differentially expressed in EBI2 KO and WT mice

Various proteins are differentially expressed during oligodendrocyte differentiation, maturation, myelin formation and in mature myelin. Markers such as Olig1 is expressed in oligodendrocyte progenitor cells (OPC) during differentiation into myelinating oligodendrocytes **(Paes de Faria et al., 2014)**. In contrast, 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase) is expressed very early in developing oligodendrocytes. Moreover, myelin oligodendrocyte glycoprotein (MOG) is present in mature oligodendrocytes and involved in myelin maintenance **(Baumann and Pham-Dinh, 2001, Birgbauer et al., 2004)**. These markers can thus be used to monitor myelin at varying stages of development and maturation. To further investigate the effects of EBI2 deficiency on myelin formation, the expression of these and other oligodendrocyte markers were examined in brain lysates prepared from WT and EBI2 KO mice by Western blotting. In contrast to MBP expression, the markers Olig1 **(Figure 5.3a and b)**, CNPase **(Figure 5.3a and c)** and MOG **(Figure 5.3a and d)** were not differentially expressed in WT and EBI2 KO mice brains. These data are suggestive of the idea that EBI2 regulates the expression of MBP, and these effects are not dependent on the expression of other oligodendrocyte markers.

2.4 Downregulation or antagonism of EBI2 signalling staggers myelination

Myelin sheets are made up to a great extent from cholesterol and oxysterols are involved in cholesterol homeostasis **(Russell, 2000, Nelissen et al., 2011)**. Cholesterol deficiency in myelinating oligodendrocytes can result in disrupted and delayed myelination showing that cholesterol is a crucial component of myelin. This lack of cholesterol can slow the rate of myelin formation and results in motor deficiency in mutant mice where myelinating oligodendrocytes cannot synthesise cholesterol **(Saher et al., 2005)**. To investigate the effects of long-term EBI2 agonism and antagonism on myelination, organotypic cerebellar slices were prepared from 10 day old mice and cultured with or without the EBI2 agonist (7α25HC, 1 μM) or antagonist (NIBR189, 1 μM) for 9, 14 or 20 days *in vitro* (DIV) **(Figure 5.4)**. Neither agonism nor antagonism of EBI2 for 9 DIV (7α25HC 106.4% +/- 24.5%, NIBR189 97.38% +/- 21.1%, $p>0.05$, n=6, one-way ANOVA and Dunnett's post-test) nor 14 DIV (7α25HC 97.3% +/- 20.5%, NIBR189 103.0% +/- 30.0%, $p>0.05$, n=4, one-way ANOVA and Dunnett's post-test) induced changes in myelination compared to non-treated control as measured by MBP staining and immunocytochemistry **(Figure 5.4a and b)**. However, long-term 20 DIV incubation of slices with the compounds induced a significant delay in MBP expression compared to non-treated controls (7α25HC 74.5% +/- 8.8%, NIBR189 77.0% +/- 7.1%, $^{**}p<0.01$, $^{***}p<0.001$, n=4, one-way ANOVA and Dunnett's post-test) **(Figure 5.4c)**. Interestingly, both agonism and antagonism of EBI2 induced similar inhibitory effect on MBP expression. To explain these apparently contradictory findings we suggest that the EBI2 agonist 7α25HC is likely inducing receptor internalisation and thus functional antagonistic effect as we have described for sphingosine 1-phosphate receptors (S1PR) **(Mullershausen et al., 2009, Healy et al., 2013)**. In agreement, we and others have observed that 7α25HC induces EBI2 receptor internalization in CHO-hEBI2 stable cells and in the U937 monocyte/macrophage cell line **(Figure 5.4d)** **(Hannedouche et al., 2011, Liu et al., 2011, Yi et al., 2012, Preuss et al., 2014b,** *in preparation*). Taken together, the data showed that EBI2/oxysterol signalling may regulate myelination state.

2.5 7α25HC attenuates LPC-induced demyelination in mouse cerebellar slices

Studies have suggested that EBI2 and oxysterols are implicated in the pathogenesis of various autoimmune diseases **(Ye et al., 2003, Heinig et al., 2010)**. It has also been found that CH25H is up-regulated during demyelination as well remyelination in animal models of multiple sclerosis, EAE, to

promote myelination **(Olah et al., 2011)**. In the current study, the effect of 7α25HC on LPC-induced demyelination was examined, similar to that we have described previously for the S1PR agonist, pFTY720 (Figure 5.5) **(Sheridan and Dev, 2011)**. The effects of LPC and 7α25HC on myelination in slices prepared from WT mice (Figure 5.5a and c) and EBI2 KO mice (Figure 5.5b and d) were investigated. In contrast to previous experiments the slices were cultured for 14 DIV in complete media without compounds allowing them to myelinate fully before short-term treatments with LPC (18 hours) and the compounds (48 hours). Short-term treatment of fully myelinated WT slices with 7α25HC or NIBR189 alone had no significant effects on myelin state compared to control, as determined by MBP immunostaining (Figure 5.5a and b). Importantly, 7α25HC (99.6% +/- 35.1%) significantly protected from LPC-induced (55.4% +/- 19.1%) demyelination in WT slices (**p<0.01, n=8, one-way ANOVA and Tukey's post-test) (Figure 5.5c). Furthermore, the pre-treatment of slice cultures with EBI2 antagonist, NIBR189, reversed the protective effects of 7α25HC (44.1% +/- 17.2%), indicating a receptor dependent mechanism (**p<0.01, n=4, one-way ANOVA and Tukey's post-test) (Figure 5.5c). To further confirm that the observed effects are EBI2 dependent, slices from EBI2 KO mice were treated same as above and immunostained for MBP (Figure 5.5b). The data showed that treatment with 7α25HC does not protect from LPC-induced demyelination (49.8% +/- 12.7%) in EBI2 KO mice confirming EBI2 mediated effects (**p<0.01, n=4, one-way ANOVA and Tukey's post-test) (Figure 5.5d). Taken together, the data suggests that oxysterols as well as EBI2 signalling are involved in preserving myelination state under conditions of demyelination.

2.6 EBI2 attenuates LPC-induced IL6 and IL1β release in mouse cerebellar slices

Studies have shown that oxysterols may exert anti-inflammatory effects inhibiting pro-inflammatory cytokine release **(Englund et al., 2001, Ohlsson et al., 1996, Moog et al., 1988)**. For instance, it has been shown that mice deficient in the CH25H enzyme, which cannot synthesise oxysterol 25HC, show intensified EAE disease progress **(Reboldi et al., 2014)**. CH25H deficient mouse macrophages also released greater amounts of IL1β after LPS stimulation than their wild type counterparts, indicating that 25HC has anti-inflammatory properties **(Reboldi et al., 2014)**. It has also been shown that pro-inflammatory cytokines can induce demyelination **(Redford et al., 1995, Ferrari et al., 2004)**. To investigate whether LPC-induced demyelination was accompanied by increase in pro-inflammatory cytokines and whether EBI2 activation had an effect on cytokine release in these slices, media collected after 18 hour treatment with LPC (0.4 μg/ml) with or without EBI2 agonist (7α25HC, 1μM) or antagonist (NIBR189, 1 μM) was analysed with IL6 and IL1β ELISAs (Figure 5.6a). Treatment with LPC alone significantly induced levels of IL6 (239.5% +/- 98.2%) (Figure 5.6b) and IL1β (244.8% +/- 62.2%) (Figure 5.6c) in cerebellar slices after 18 hour (**p<0.01, ***p<0.001, n=3, one-way ANOVA and Bonferroni post-test). Treatment with 7α25HC (98.2% +/- 3.1% for IL6, 89.4% +/- 6.2% for IL1β) and NIBR189 (87.1% +/- 9.9% for IL6, 94.9% +/- 6.7% for IL1β) alone had no effect on IL6 and IL1β release from organotypic slices (Figure 5.6b and c) (p>0.05, n=3, one-way ANOVA and Bonferroni post-test). However, pre-treatment of slices with 7α25HC attenuated the LPC-induced levels of IL6 (115.9% +/- 46.1%) (Figure 5.6b) and IL1β (99.1% +/- 20.7%) (Figure 5.6c) (*p<0.05, **p<0.01, n=3, one-way ANOVA and Bonferroni post-test). Moreover, demonstrating EBI2 dependent mechanism, NIBR189 blocked the anti-inflammatory effects of 7α25HC on LPC-induced levels of IL6 (91.1% +/- 6.3%) (Figure 5.6b) and IL1β (73.1% +/- 19.8%) (Figure 5.6c) (p>0.05 versus LPC, n=3, one-way ANOVA and Bonferroni post-test) and supported the earlier findings that EBI2 attenuates pro-inflammatory cytokine release **(Rutkowska et al., 2014)**. In summary, the data shows that EBI2 and oxysterols are involved in regulation of normal myelination as well as protection

during inflammatory processes possibly via inhibition of pro-inflammatory cytokine levels and other mechanisms.

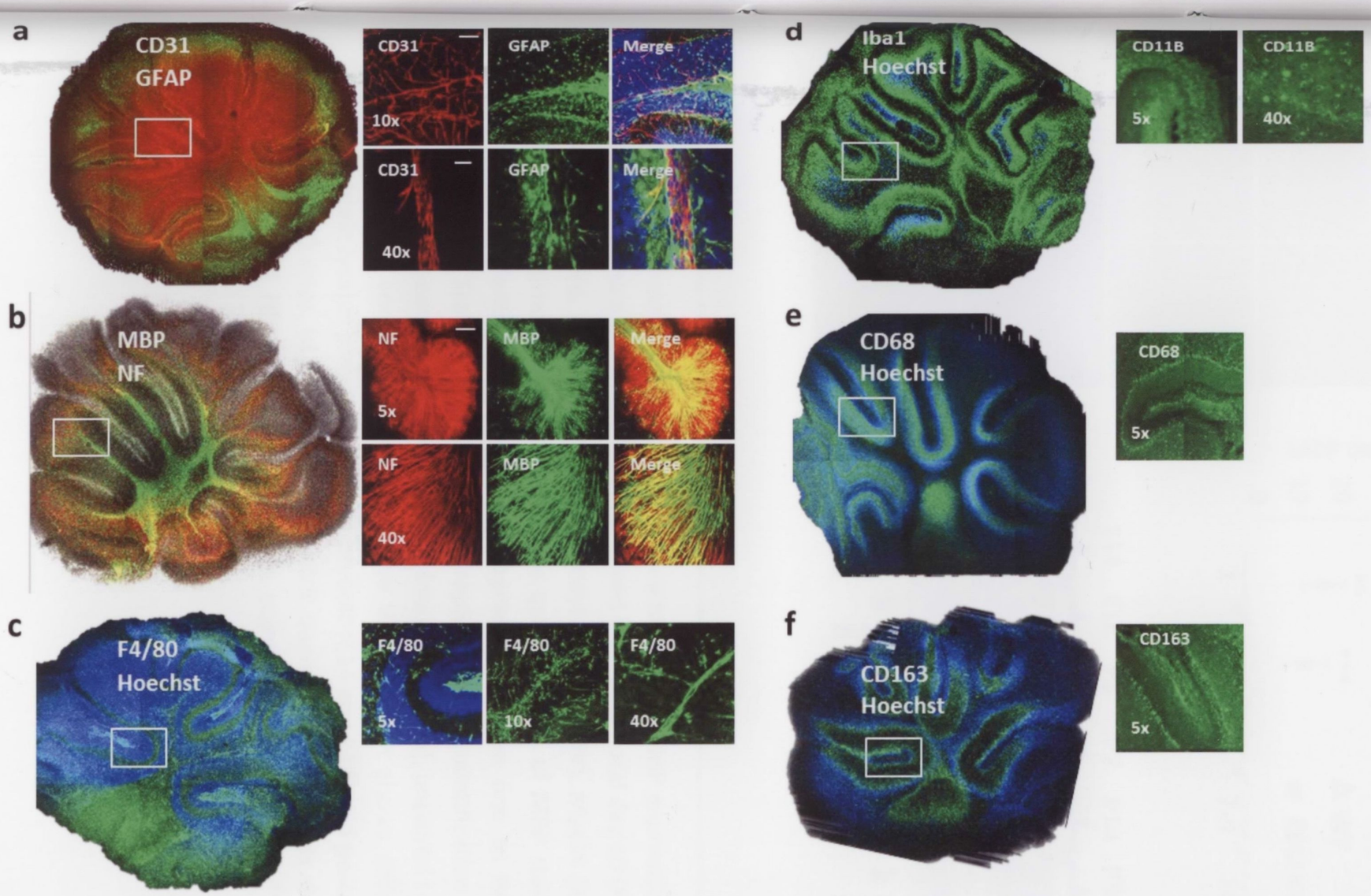


Figure 5.1 Organotypic cerebellar slices maintain host tissue cytoarchitecture and physiology. Confocal images show preserved cytoarchitecture in organotypic slice cultures prepared from P10 mice and cultured for 14 DIV. **(a)** Preserved blood vessel structure shown with CD31/PECAM-1 (red), endothelial cell marker. Images also show astrocyte (GFAP, green) staining throughout the entire slice as well as complete ensheathment of blood vessels with astrocytic end feet (higher magnification images). **(b)** Images showing a complete axon (NF, red) myelination with oligodendrocytes (MBP, green). **(c)** Presence of immune system cells shown here with anti-F4/80 antibody, a marker of mature macrophages and monocytes (green). Macrophages and monocytes allign along the blood vessels as can be seen in higher magnification (10x and 40x) images forming the blood brain barriere together with endothelial cells and astrocytes. **(d)** Widespread microglia presence shown with anti-Iba1 and anti-CD11b antibodies. **(e and f)** Macrophages, monocytes, lymphocytes, fibroblasts and dendritic cells are also present in the slices. White rectangles in the tiled images show a representative area of images taken at higher magnification. Images taken at 10x (scale bar 100 µM), 40x (scale bar 50 µM) and 5x (scale bar 200 µM) magnification.

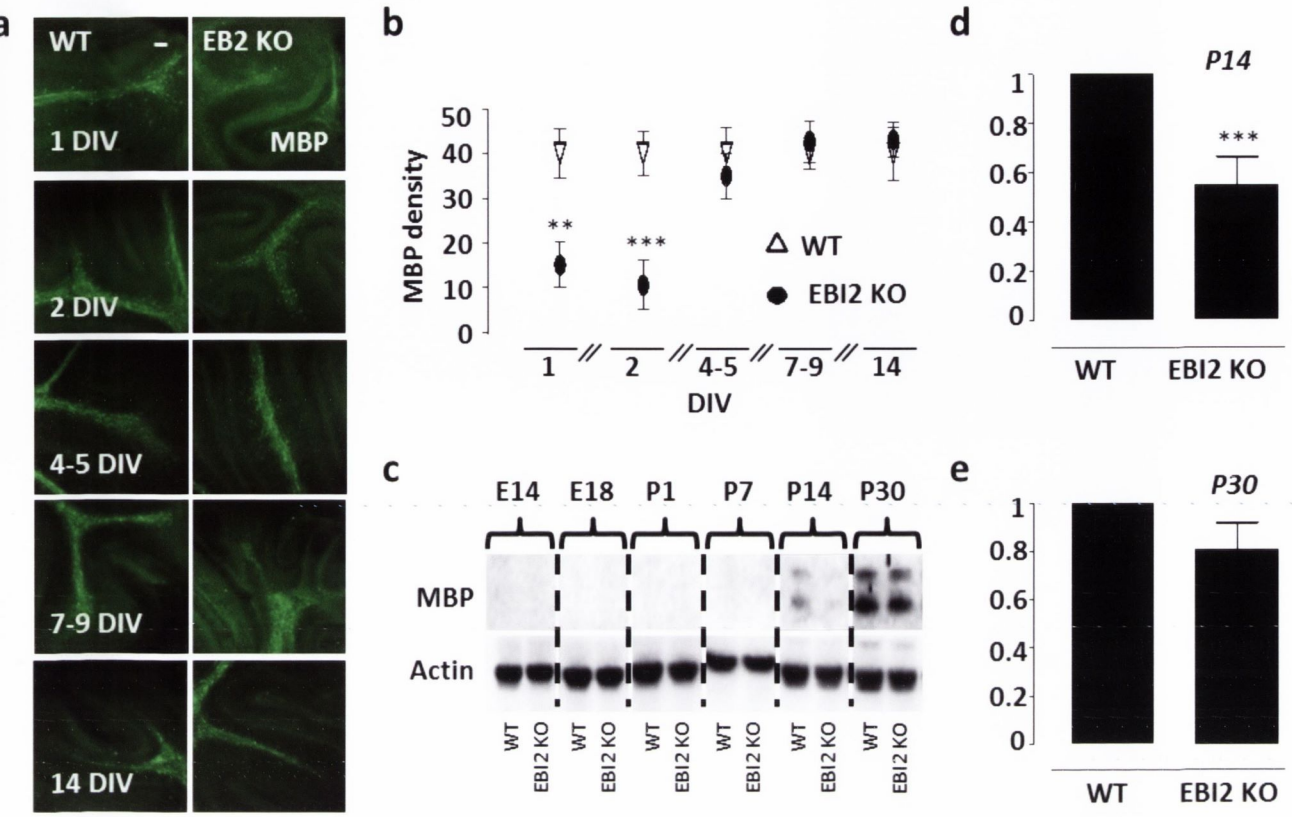


Figure 5.2 EBI2 deficiency results in transient delay in MBP expression. (a) Representative confocal images show mice cerebellar slices prepared from post natal day 10 (P10) mice and cultured for one, two, four to five, seven to nine and 14 days in vitro (DIV). Myelin (MBP, green) staining is shown. Myelin development was determined by quantification of MBP staining density. Analysis showed significant differences in myelin development between one to two DIV. Images taken at 10x magnification. Scale bar, 100 µM. (b) Graph showing quantification of images shown in (a). Data presented as mean +/- SEM (five to six animals per group), unpaired t-test, **p<0.01, ***p<0.001 vs. corresponding control. (c) Representative Western blot shows differential expression of MBP in whole brain lysates prepared from embryonic (E14 and E18) and postnatal (P1, P7, P14 and P30) WT and EBI2 KO mice. (d and e) Densitometric quantification of blots shown in (c). Data presented as mean +/- SEM (three animals per time point), unpaired t-test, *** p<0.001 vs. corresponding control.

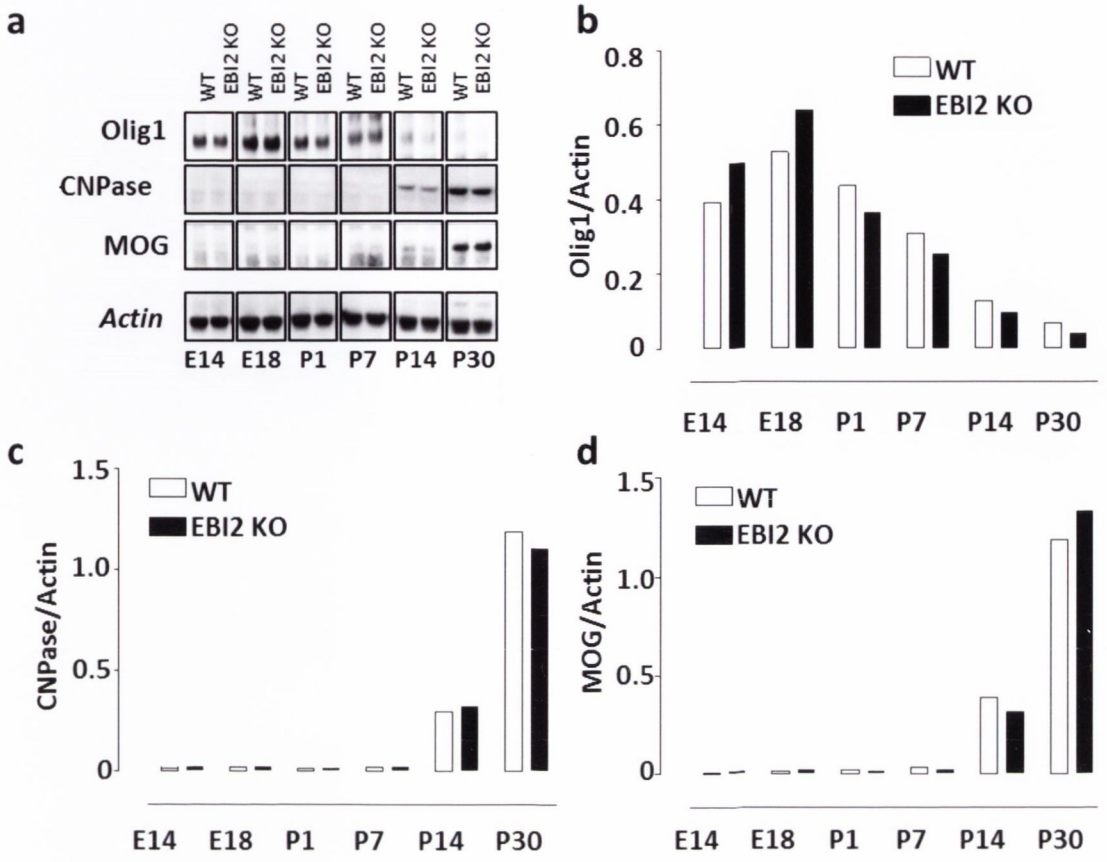


Figure 5.3 Olig1, CNPase and MOG are not differentially expressed in WT and EBI2 KO mice. (a) Representative Western blots show quntitative differences in oligodendrocyte marker expression in whole brain lysates prepared from embryonic (E14 and E18) and postnatal (P1, P7, P14 and P30) mouse brains. Densitometric quantification of blots shown in (a) indicating that Olig1 (b) CNPase (c) and MOG (d) expression is not different in WT and EBI2 KO animals. Data presented as single measurement, three animals per time point.

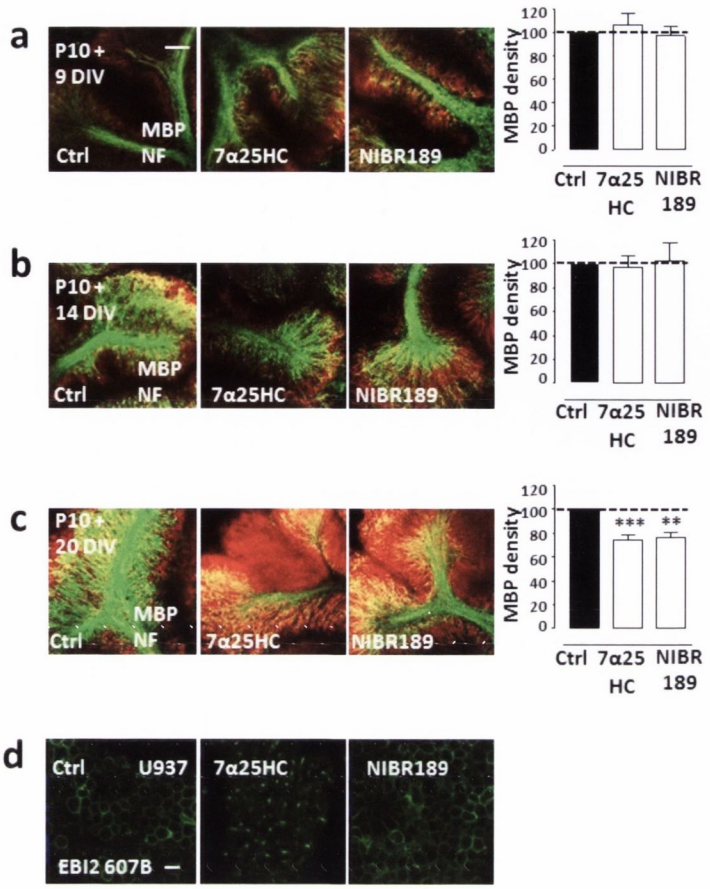


Figure 5.4 Downregulation or antagonism of EBI2 signalling staggers myelination. Representative confocal images show MBP expression in WT cerebellar slices prepared from P10 mice and cultured for (a) 9 DIV (b) 14 DIV and (c) 20 DIV with or without 7α25HC (1 μM) and NIBR189 (1 μM). Corresponding bar graphs shows quantification of MBP staining. There are no differences between non-treated and treated slices at 9 DIV (a) and 14 DIV (b). However, treatment of slices with 7α25HC or NIBR189 for 20 DIV (c) inhibits normal myelin development in WT cerebellar slices as measured by MBP staining. Data presented as mean +/- SEM (n=4 and 6), One-way ANOVA and Dunnett's post-test, ** $p>0.01$, *** $p<0.001$ vs. corresponding control. Images taken at 10x magnification. Scale bar 100 μm (d) Treatment of human monocytes (U937) with 7α25HC (10 μM) for 45 minutes induces EBI2 internalisation. NIBR189 (10 μM) does not induce receptor internalisation. Images taken at 40x magnification. Scale bar 20 μM.

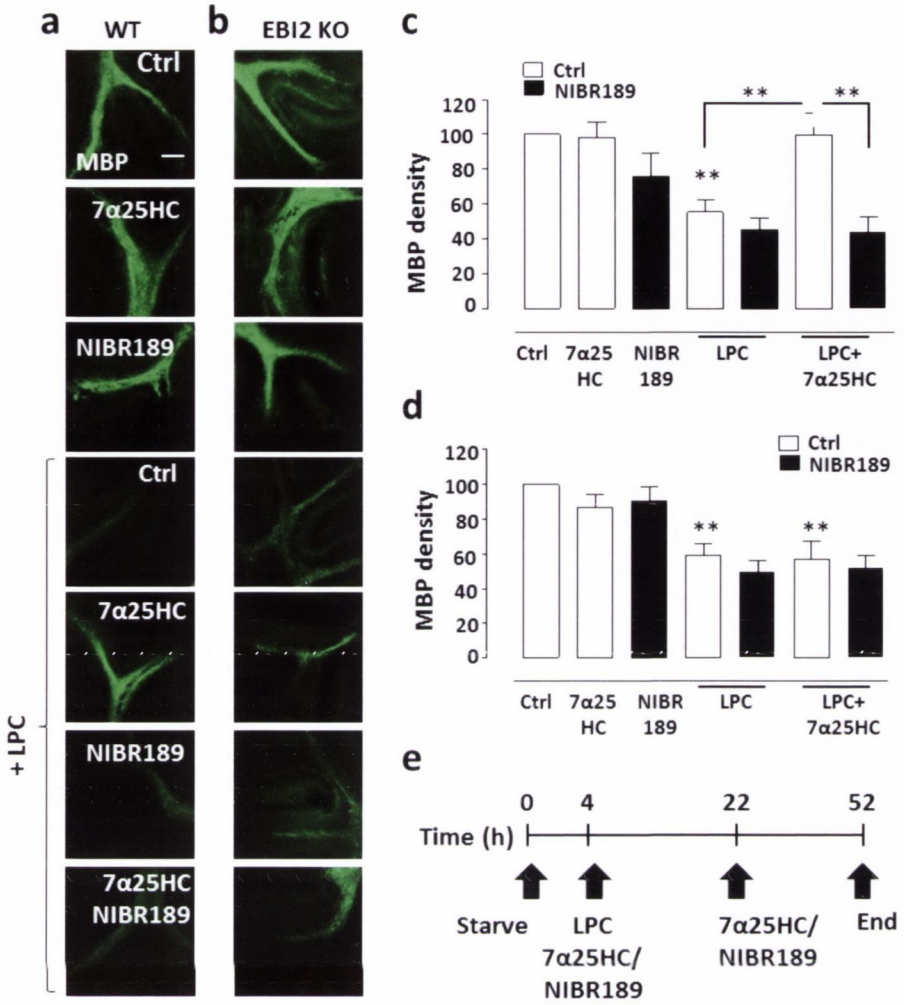


Figure 5.5 7α25HC protects from LPC-induced demyelination. Representative confocal images show WT (a) and EBI2 KO (b) mice cerebellar slices treated with 7α25HC (1 μM) and/or NIBR189 (1 μM) +/- LPC (0.4 mg/ml). Anti-MBP (green) immunostaining is shown. Images taken at 10x magnification. Scale bar, 100 μM. (c) A corresponding bar graph shows quantification of myelin staining in WT slices shown in (a). 7α25HC significantly protects from LPC-induced demyelination and this protective effect is inhibited with the EBI2 antagonist NIBR189 indicating EBI2 mediated protection from demyelination. Data presented as mean +/- SEM (n=4 & 8), one-way ANOVA and Tukey's multiple comparison post-test, ** $p<0.01$ vs corresponding control. (d) A bar graph shows that 7α25HC does not protect from LPC-induced demyelination in slices prepared from EBI2 KO animals confirming EBI2 dependent mechanism shown in (a and c). Data presented as mean +/- SEM (n=4), one-way ANOVA and Tukey's multiple comparison post-test, **$p<0.01$ vs control. (e) Schematic diagram illustrating experimental design.

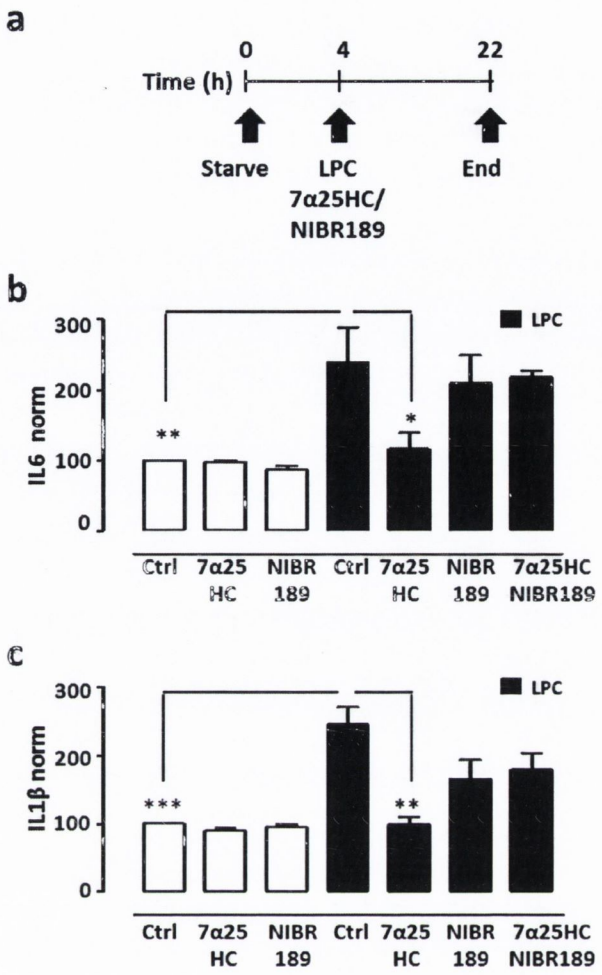


Figure 5.6 7α25HC attenuates LPC-induced levels of IL1β and IL6 in cerebellar slices. (a) Schematic diagram illustrating experimental design. Treatment of slices with LPC (0.4 mg/ml) induces an increase in IL6 **(b)** and IL1β **(c)** protein release. 7α25HC (1 μM) attenuates LPC (0.4 mg/ml) induced levels of IL6 **(b)** and IL1β **(c)**. Co-treatment with EBI2 antagonist (NIBR189, 1 μM) blocks the 7α25HC mediated effects on the levels of IL6 **(b)** and IL1β **(c)** indicating EBI2-dependant regulation of cytokine release. Treatment with 7α25HC and NIIBR189 alone did not alter IL6 **(b)** and IL1β **(c)** release. Data presented as mean +/- SEM (n=3), one-way ANOVA and Bonferroni's post-test, *p<0.05, **p<0.01, *** p<0.001 vs. corresponding control.

3 Discussion

EBI2 is a G protein-coupled receptor that plays an important role in orchestrating T cell-dependent antibody response in lymphoid tissue **(Gatto et al., 2009, Kelly et al., 2011)**. The oxysterol 7α25HC is the most potent known endogenous agonist for EBI2 **(Hannedouche et al., 2011, Liu et al., 2011)**. Oxysterols are derivatives of cholesterol which, among other functions, also act as immune regulators **(Russell, 2000)**. The majority of body's cholesterol is located in the brain where it serves as a building block for myelin sheets of oligodendrocytes **(Bjorkhem and Meaney, 2004)**. Aberrant oxysterol signalling has been reported in certain diseases of the CNS with immune and demyelinating component **(Leoni and Caccia, 2011, Leoni, 2009)**. For instance, CH25H is an enzyme involved in 7α25HC synthesis which has been found to be upregulated in microglia during demyelinating and remyelinating phases of EAE most likely to enhance myelination **(Olah et al., 2011)**. Mutation in another enzyme needed for 7α25HC synthesis, namely CYP7B1, is the cause of a neurodegenerative disorder called spastic paraplegia type 5 (SPG5), which is characterised by white matter lesions **(Schule and Schols, 2011, Biancheri et al., 2009)**. The role of EBI2 in CNS immune defence and its potential involvement in myelination have not been studied so far. Here, the effects of EBI2 on normal myelin development and demyelination under pathophysiological conditions were investigated. It was demonstrated that myelin in EBI2 KO mice develops slower than in WT mice. In agreement with the knock-out studies, pharmacological studies showed that long-term inhibition of EBI2 signalling either via persistent antagonism or receptor internalization and downregulation staggers myelin development. In addition to regulation of myelin development under normal conditions, the data showed that activation of EBI2 protects from LPC-induced demyelination and that the protective effects might be mediated via inhibition of pro-inflammatory cytokine release.

3.1 EBI2 is involved in myelin development

Studies have shown the importance of high cholesterol synthesis and homeostasis for proper myelin development **(Saher et al., 2005)**. Abnormalities in cholesterol or oxysterol levels have also been linked to defective myelination **(Saher et al., 2005, Trousson et al., 2009, Makoukji et al., 2011)**. In the current study, investigations of the expression of myelin proteins in WT and EBI2 KO mice at various stages of development showed that MBP expression in the brains of EBI2 KO mice is delayed compared to WT mice. Notably, a similar pattern of MBP expression was observed *in vivo* and *in vitro*. MBP expression was reduced in cerebellar slices prepared from P10 EBI2 KO mice grown *in vitro* for 1-14 days compared to slices prepared from WT mice. The data showed an initial delay of MBP expression and thereafter an eventual MBP recovery in EBI2 KO animals. MBP is thought to be involved in myelin compaction during oligodendrocyte maturation **(Campagnoni and Macklin, 1988)**. In shiverer mice, where the MBP gene has been mutated, formation of the major dense line in compact myelin is affected **(Privat et al., 1979, Roach et al., 1985)**. It is therefore possible that EBI2 signalling is involved in later stages of myelin development when myelin is being compacted. At this stage of myelination an asymmetry in lipid composition during myelin compaction is being formed **(Baumann and Pham-Dinh, 2001)**. The glycolipids are positioned in the outer surface in the intraperiodic line and negatively charged phospholipids are located on the inner part **(Baumann and Pham-Dinh, 2001)**. Given that we find EBI2 regulates MBP levels, this receptor might play an important role in the process of compaction.

Interestingly, we have not observed differences in Olig1, CNPase or MOG protein expression between WT and EBI2 KO mouse brains. These and other markers are expressed at various stages of

oligodendrocyte development to guide the maturation of myelin. Olig1, for instance, is a transcription factor that is important for differentiation of OPCs into myelinating oligodendrocytes **(Paes de Faria et al., 2014)**. The enzyme CNPase is one of the first myelin-specific proteins expressed in developing oligodendrocytes. In addition, MBP expression is detected 2-3 days after CNPase, just before myelin formation begins. Lastly, MOG is a mature myelin protein that is expressed at late stages of myelin development and is a marker of fully differentiated mature oligodendrocytes **(Baumann and Pham-Dinh, 2001)**. The lack of differences in the expression of these proteins, except MBP, in WT and EBI2 KO animals indicates that EBI2 signalling is not involved at all stages of myelin development. This may also suggest that EBI2 may only be involved in regulation of fine-tuning of myelin content and compaction.

3.2 Inhibition of EBI2 signalling leads to delay in MBP expression

The data from pharmacological in *vitro* studies confirmed the findings from the *in vivo* and *in vitro* genetic knock-out experiments. Both, long-term EBI2 antagonism and agonism induced a delay in MBP expression. The EBI2 agonist, 7α25HC, has been shown to induce receptor internalization in CHO cells stably expressing EBI2 and in the U937 monocyte/macrophage cell line (**Hannedouche et al., 2011, Liu et al., 2011, Yi et al., 2012, Preuss et al., 2014b,** *in preparation*). It is therefore possible that long-term EBI2 agonism led to receptor internalisation and its subsequent downregulation on the cell membrane inducing a functional antagonist effect as has previously been described for sphingosine 1-phosphate receptors **(Mullershausen et al., 2009, Healy et al., 2013)**. The effects of the EBI2 compounds on MBP expression was observed after as long as 20 DIV and was not present at earlier time points. The differences in myelination might not have been observed at earlier stages due to the fact that EBI2 signalling was present up to day 10 of postnatal development (P10) at which stage the slices were prepared and maintained in media supplemented with the EBI2 compounds. Thus, it is conceivable that the MBP expression continued for some time after the signal was interrupted pharmacologically with EBI2 compounds resulting in the slowing down of MBP expression.

3.3 EBI2 protects from LPC-induced demyelination via inhibition of pro-inflammatory cytokine release

Lastly, the current study demonstrated that activation of EBI2 can attenuate LPC-induced demyelination in WT mouse cerebellar organotypic slice cultures, but not in those prepared from EBI2 KO mice. It also showed that a reduction in the levels of pro-inflammatory cytokines may play a role in these protective effects. However, other signalling events instigated by activation of EBI2 in various CNS and immune cells, may have been responsible for attenuation of LPC-induced demyelination as well as the delay in MBP expression in EBI2 KO animals *in vivo*. These mechanisms, however, were not investigated in this study. Moreover, the EBI2 antagonist inhibited the effects of 7α25HC on LPC-induced demyelination in organotypic mice cerebellar slice cultures. These protective effects of 7α25HC were lost in slices prepared from EBI2 KO animals. Overall, these experiments provide genetic and pharmacological support for an EBI2-mediated process. Of interest, the effects of 7α25HC on attenuating LPC-induced demyelination in organotypic mice cerebellar slice cultures resembled the effects of the S1P1R modulator pFTY720 in our previous studies **(Sheridan and Dev, 2011)**.

To our knowledge, there are currently no studies investigating the expression or function of EBI2 in neurons, oligodendrocytes or microglia. We acknowledge that the observed effects of oxysterols and

EBI2 signalling on myelination may have been a result of indirect effects exerted by neurons, oligodendrocytes, microglia or astrocytes. Soluble factors released by neurons or glial cells as well as proteins expressed on the surface of these cells might have exerted an indirect effect on both, normal myelin development and protection from demyelination. It is important to note that this study did not investigate the signalling events involved in myelin development or protection from LPC-induced demyelination in any of the above cell types. Future studies should investigate the mechanisms of action of EBI2 mediated signalling on myelination mediated by neurons, oligodendrocytes, microglia and astrocytes.

Given the data presented in the current study, examining the effects of EBI2 agonists and antagonists in animal models of MS is now warranted. In addition, the use of EBI2-null animals in models such as EAE, are also important to determine the value of this receptor as a drug target in such illnesses. It is equally interesting to note that mutations in CYP7B1 have been identified in patients with an autosomal recessive form of hereditary spastic paraplegia called SPG5 (for a recent review see **(Schule et al., 2010)**). Several groups reported white-matter MRI abnormalities indicative of possible demyelination in cases of SPG5 **(Biancheri et al., 2009, Criscuolo et al., 2009, Goizet et al., 2009)**. These results suggest further that reduced levels of EBI2-activating oxysterols may alter myelination state. Moreover, these genetic findings support the idea that activation of EBI2 could be important for therapeutic intervention in selected patients suffering from SPG5. In closing, we suggest this work and further *in vivo* studies will support the use of EBI2 as a novel drug target for treatment of MS and possibly other neuroinflammatory and neurodegenerative diseases.

Chapter 6: Discussion

6.1 Study overview

Investigations into the function of EBI2 receptor have mainly focused on the immune system and its resident cells. It is now well established that EBI2 regulates immune cell function such as B cell trafficking, differentiation and T cell dependant antibody response **(Gatto et al., 2009, Hannedouche et al., 2011)**. EBI2 expression and function have not, however, been investigated in the cells of the CNS that very often act as the first line of immune defence in the brain and thus might express similar receptors as the immune cells. Therefore, the current study set out to examine the expression and function of EBI2 in astrocytes and in regulation of neuroinflammatory and demyelinating processes in the CNS. Intracellular signalling, cell migration, cytokine release, cellular communication, myelin development and protection from demyelination were investigated. The data showed that astrocytes express EBI2 and the enzymes needed for 7α25HC synthesis and degradation. Activation of EBI2 with 7α25HC, *in vitro*, induced phosphorylation of ERK, enhanced intracellular Ca^{2+} signalling and, importantly, promoted human and mouse astrocytes motility, which was inhibited by an EBI2 antagonist and did not occur in astrocytes prepared from EBI2 KO mice **(Results 1)**. Stimulation of EBI2 with 7α25HC also attenuated pro-inflammatory signalling in human and mouse astrocytes. Furthermore, the pre-treatment of human and mouse astrocytes with 7α25HC inhibited TNFα/IL17-induced synthesis and release of IL6 from human astrocytes and LPS-induced release of IL6 and TNFα from mouse astrocytes. In addition, the data showed that EBI2 signalling in astrocytes is sensitive to pro-inflammatory signals and that EBI2 plays a role in communication between astrocytes and macrophages **(Results 2)**. Lastly, it was shown that EBI2 and oxysterols play a role in normal myelin development and that EBI2 may be protective in chemically-induced demyelination **(Results 3)**. Figure 6.4 summarises the multimodal role of EBI2 in glial and immune cells.

6.2 The role of astrocytes in CNS physiology

The complexity and variety of astrocyte function is truly remarkable. Alongside the long known and well-established functions such as ion buffering, uptake of neurotransmitters, metabolic and antioxidant support, additional and more complex roles of astrocytes are now emerging. Astrocytes have been found to control neuronal maturation, synapse formation and plasticity. Strikingly, astrocytes have also been shown to be involved in synaptic transmission by processing and integrating neuronal activity via peri-synaptic processes **(Wang and Bordey, 2008, Kimelberg, 2010, Barres, 2008, Kimelberg and Nedergaard, 2010)**. In addition to the above functions, these glial cells also serve astrocyte-specific roles such as involvement in angiogenesis, BBB formation and maintenance, gliotransmission, regulation of oligodendrocyte maturation and wrapping around axons, communication via Ca^{2+} waves and release of various signalling molecules **(Wang and Bordey, 2008, Kimelberg, 2010, Barres, 2008, Kimelberg and Nedergaard, 2010)**. These cells also participate in immune defence against invading pathogens and neuroinflammatory events occurring due to stroke or neurodegeneration. In these instances, they serve as immune mediators by releasing inflammatory cytokines, chemokines and other molecules, and possibly phagocytosing debris **(Wang and Bordey, 2008, Kimelberg, 2010, Barres, 2008, Kimelberg and Nedergaard, 2010)**. Astrocytes also form glial scars to seal off intact tissue and limit the extent of inflammation, scavenge the microenvironment and prepare it for repair, stimulate oligodendrocyte maturation, repair synapses and damaged BBB. They can also modulate immune cells by reducing their presence via induction of apoptosis or activate them by presenting antigens and releasing pro-inflammatory cytokines and chemokines **(Larochelle et al., 2011, Neumann et al., 2009, Kimelberg, 2010, Sidoryk-Wegrzynowicz**

et al., 2010). As a result of such a varied functionality, astrocytes are involved in regulation of the majority of processes taking place in the CNS both, normal and pathophysiological. Their reactivity during neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease or Parkinson's disease has been shown to exacerbate inflammation and deteriorate the disease state **(Sidoryk-Wegrzynowicz et al., 2010)**. New therapies that regulate astrocyte activation may therefore prove useful in managing autoimmune, demyelinating and neurodegenerative diseases.

6.3 Expression of EBI2 and 7α25HC synthesising and degrading enzymes in astrocyte biology

Studies have reported that immune cells such as macrophages, monocytes, B and T cells express EBI2 but do not themselves regulate the levels of 7α25HC in the secondary lymphoid tissue **(Yi et al., 2012)**. Instead, it has been shown that gradient of 7α25HC is tightly regulated by lymphoid stromal cells and follicular dendritic cells that up- or down-regulate the synthesising enzymes (CH25H and CYP7B1) and the degrading enzyme (HSD3B7) in order to keep 7α25HC at precise concentrations **(Yi et al., 2012)**. Here, we showed that mouse and human astrocytes expressed EBI2 as well as the CH25H, CYP7B1 and HSD3B7 enzymes, although in varying degrees **(Results 1)**. We suggest, therefore, that astrocytes may regulate the levels of 25HC via the enzyme CH25H and the levels of 7α25HC via the enzyme CYP7B1, and thus limit or enhance inflammatory responses in the CNS. Figure 6.1 illustrates the expression of EBI2 and 7α25HC synthesising enzymes and signalling cascades activated by EBI2 in astrocytes. In this putative model, increased levels of 25HC, which has been shown to have pro-inflammatory properties, may be upregulated initially to enhance inflammatory signals within the inflamed tissue. With time, astrocytes may change the response to a more anti-inflammatory profile via synthesis of 7α25HC. Similarly, delayed upregulation of HSD3B7 (the degrading enzyme) may be used by astrocytes to limit immune cell migration into the CNS after the initial pro-inflammatory signals. It has to be noted, however, that we found mouse astrocytes expressed higher levels of EBI2 and the synthesising enzymes than their human counterparts **(Results 1)**. Reasons and functional roles for this species difference observed *in vitro* requires further investigations and remains unclear. Thus, whether these differences are observed *in vivo* and the possible implications that may arise need to be further explored. Such species differences in the expression of EBI2 signalling molecules would be important to gain further understanding of the developmental importance of EBI2 as well as for testing drugs that target this pathway in animal models of diseases.

6.4 The role of EBI2 in astrocyte migration

Previous studies showed that the EBI2 agonist, oxysterol 7α25HC, induces migration of EBI2 expressing cells, such as B cells **(Hannedouche et al., 2011, Liu et al., 2011)**. Here, we showed that 7α25HC also induces astrocyte migration in a concentration-dependant manner **(Results 1)**. Notably, we found that astrocyte migration was inhibited with the EBI2 antagonist and did not occur in mouse astrocytes prepared from EBI2 KO animals showing that 7α25HC-induced migration was EBI2-dependant **(Results 1)**. It is of interest that upregulated levels of oxysterols have been found in neurodegenerative disease where injured or apoptotic neurons and oligodendrocytes are the source of cholesterol and oxysterols **(van de Kraats et al., 2013)**. Figure 6.1 shows the effects of EBI2 activation on astrocyte migration. Thus, although further studies are required, perhaps astrocytes may sense the increased levels of oxysterols via their surface expression of EBI2 and migrate towards the area of injury and inflammation. At this stage, these EBI2-stimulated astrocytes may then propagate or inhibit the inflammatory response dependent upon the temporal and spatial cues. Taken together, these studies support the possibility that astrocytes migrate towards areas of

inflammation in the CNS in response to various chemokines and possibly oxysterols such as 7α25HC. The EBI2/oxysterol signalling may therefore be another tool used by astrocytes in immune defence in the central nervous system.

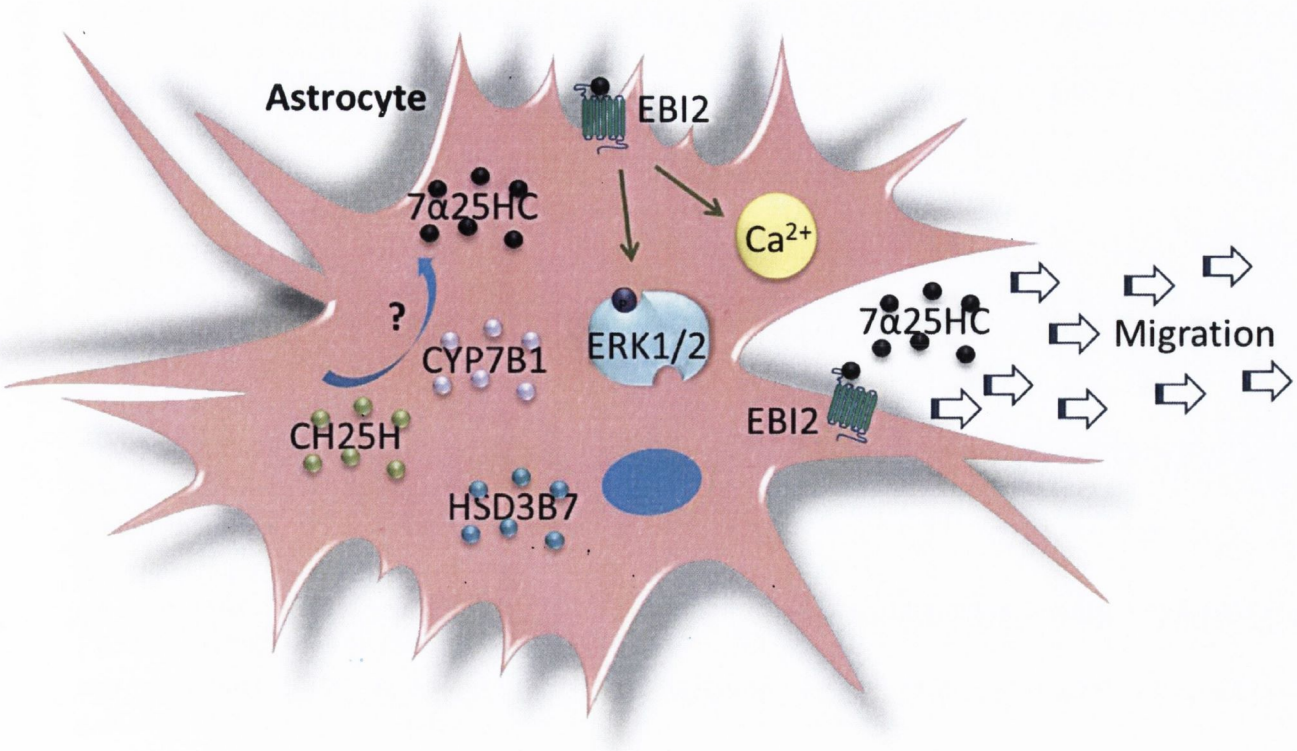


Figure 6.1 EBI2 regulates intracellular signalling and migration in astrocytes. Astrocytes express EBI2 and enzymes for 7α25HC synthesis and degradation. Activation of EBI2 with 7α25HC in astrocytes activates pERK 1/2 and Ca^{2+} signalling and induces astrocyte migration.

6.5 Regulatory role of EBI2 and oxysterols in inflammation

There is some contradictory data regarding regulation of inflammatory signalling pathway by oxysterols. For instance, the precursor to the EBI2 agonist, 25HC, has been shown to both, induce and inhibit the release of pro-inflammatory cytokines in various cell types, for example in macrophages or placental throphoblasts **(Ohlsson et al., 1996, Aye et al., 2012, Englund et al., 2001)**. Recently, however, it has been shown that CH25H deficient macrophages release increased amounts of IL1β as a result of their inability to produce 25HC suggesting anti-inflammatory properties of this oxysterol **(Reboldi et al., 2014)**. There is a possibility that certain oxysterols have pro- and others anti-inflammatory properties and that these effects vary between cell types. Our data, here, showed that 7α25HC inhibited LPS-induced IL6 and TNFα levels in mouse astrocytes and mRNA and protein levels of IL6 in TNFα/IL17-stimulated human astrocytes **(Results 2)**. In contrast to previous research showing that 25HC induces NFκB activation, we found 7α25HC did not induce NFκB translocation to the nucleus **(Results 2)** indicating that agonism of EBI2 with 7α25HC does not activate this "classical" pro-inflammatory signalling pathway **(Aye et al., 2012)**. Moreover, we found that treatment of the cells with 7α25HC inhibited the nuclear translocation of NFκB mediated by TNFα/IL17 treatment, suggesting a possible signalling pathway in EBI2-regulated pro-inflammatory cytokine release **(Results 2)**. Figure 6.2 illustrates the EBI2 modulated inflammatory pathways. Two additional pathways have been implicated in oxysterol-regulated cytokine release: ERK1/2 and Ca^{2+} signalling **(Aye et al., 2012, Lemaire-Ewing et al., 2009, Dugas et al., 2010)**. Supporting this suggestion, our data showed that in human astrocytes 7α25HC induces pERK and Ca^{2+} signalling **(Results 1)**. Activation of both, or either, of these pathways by EBI2 might thus be involved in regulation of cytokine release by astrocytes. More research investigating the exact pathways regulating cytokine release in astrocytes is now needed to address this question.

6.6 The role of EBI2 in inflammatory response in astrocytes

Astrocytes communicate with each other and other brain-specific cells but also send and receive information from the peripheral immune cells they come in contact with. Astrocytes utilise cytokines and chemokines to activate and attract immune cells to the sites of injury or inflammation. They have also been suggested to scavenge the pathophysiological environment to clear debris and look for antigens they can then present to the immune cells **(Kimelberg, 2010)**. Thus, astrocytes have been suggested to play roles as antigen presenting cells (APC), as well as microglia, in the CNS, although this function of astrocytes has been debated in the field **(Fontana et al., 1984)**. Notably, astrocytes, also receive and respond to the signals sent by peripheral immune cells by releasing pro-inflammatory cytokines such as IL6 or TNFα **(Miljkovic et al., 2011, Bo et al., 1994, Miljkovic et al., 2007)**. In our studies, we did not examine whether EBI2 regulates APC responses of astrocytes; we believe these studies would be worthy of consideration. In our study, we did however show that, in addition to the regulation of cytokine levels, the EBI2/oxysterol signalling (the receptor and 7α25HC metabolic enzymes) is regulated in astrocytes in response to immune challenge such as LPS **(Results 2)**. Figure 6.2 shows the EBI2 response to pro-inflammatory stimuli. These results indicate a possibility that EBI2 and its signalling pathway might be an additional tool at the disposal of astrocytes by which they may be able to directly sense and respond to pathogenic attack in the CNS.

6.7 The role of EBI2 in astrocyte-mediated cellular cross-talk

Previous research has shown that stimulation of primary human macrophages with LPS leads to a rapid upregulation of CH25H and CYP7B1 enzymes **(Preuss et al., 2014a)**. Supernatants harvested from these LPS-treated macrophages were used to stimulate macrophage migration and were

partially inhibited by the EBI2 antagonist NIBR189 **(Preuss et al., 2014a)**. The data showed that LPS-induced mRNA levels of CH25H and CYP7B1 enzymes resulted in a sufficient synthesis and release of 7α25HC to stimulate cell motility. In our current study, we found that LPS also induced CH25H and CYP7B1 in mouse astrocytes and that LPS-treated astrocyte media induced macrophage migration **(Results 2)**. These findings suggest that LPS-induced increase in 7α25HC synthesising enzymes may lead to 7α25HC release in sufficient amounts to induce cell migration. Our studies also showed that, in addition to LPS regulating the levels of the 7α25HC synthesising enzymes, LPS treatment also suppressed EBI2 mRNA levels in mouse astrocytes **(Results 2)**. Figure 6.2 illustrates the proposed mechanism of action of EBI2 regulated cellular cross-talk. Based on these studies, we propose that an increase in 7α25HC may drive the migration of immune cells into the CNS, which is a hallmark of many neuroinflammatory diseases such as multiple sclerosis or Alzheimer's disease. We suggest that regulation of EBI2 signalling molecules, in astrocytes, might therefore be a mechanism by which these cells sense and/or regulate the influx of peripheral cells into the CNS during inflammation. We acknowledge, however, that further experiments directly measuring the effects of inflammatory agents, such as LPS, on the levels of 7α25HC released from astrocytes would be required to support these claims.

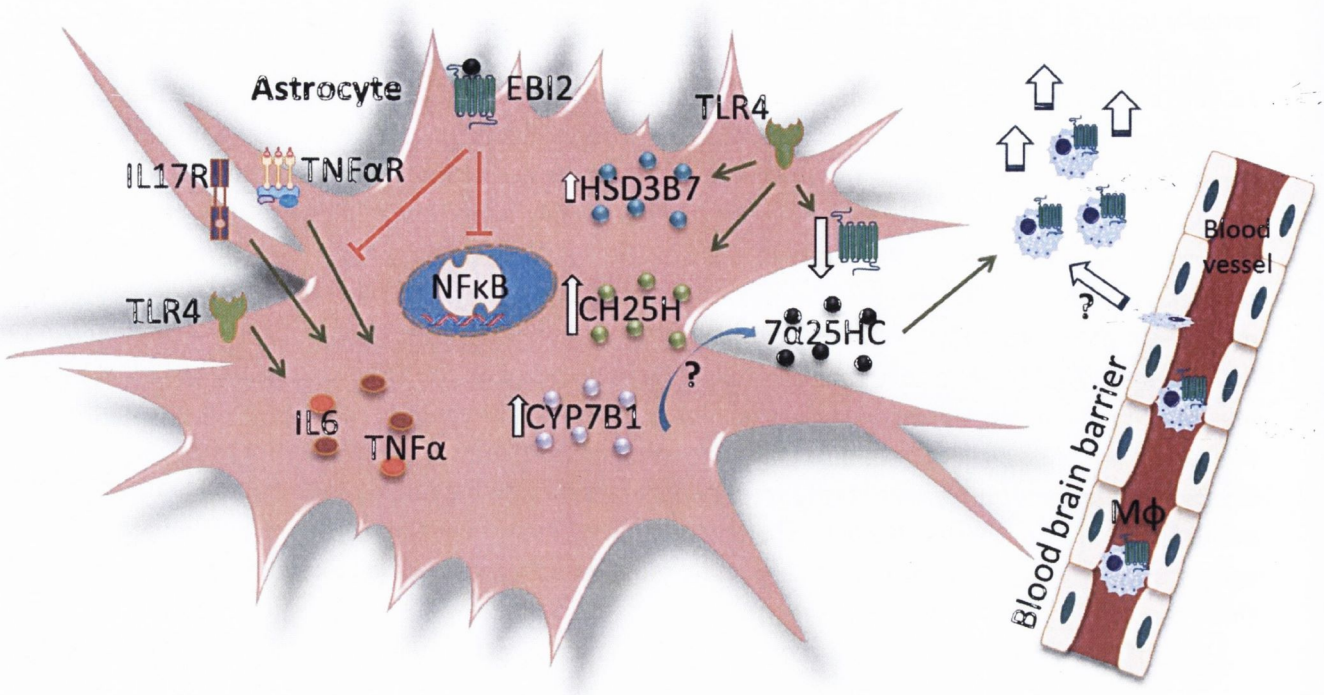


Figure 6.2 EBI2 regulates levels of IL6 and TNFα in astrocytes and their signalling with macrophages. Combined TNFα/IL17 signalling induces IL6 release in human astrocytes, which can be attenuated with 7α25HC pre-treatment. Activation of EBI2 with 7α25HC in human astrocytes inhibits NFκB nuclear translocation. Stimulation of mouse astrocytes with LPS induces IL6 and TNFα release. Pre-treatment with 7α25HC attenuates LPS-induced effects. Astrocytes respond to LPS challenge by modulating the mRNA levels of EBI2 and 7α25HC synthesising and degrading enzymes. Astrocytes conditioned media from LPS treated mouse astrocytes induces macrophage migration.

6.8 EBI2 signalling in myelin development

Since cholesterol is the main component of myelin and its breakdown products, oxysterols, are agonists for EBI2, we hypothesised that EBI2 plays a role in myelination **(Saher et al., 2005)**. In our current study the involvement of EBI2 signalling in myelin development was therefore investigated. Here, for the first time, we showed that EBI2 is involved in normal myelin development **(Results 3)**. We found that MBP was expressed with delay in 14 day old EBI2 KO animals as compared to WT animals and these effects were normalised at 30 days. Similar effects were observed *in vitro* where slices prepared from 10 day old EBI2 KO animals showed delayed MBP expression at early culture timepoints of 1-2 DIV, which again normalised at 4-5 DIV. Importantly, complete myelin development in EBI2 KO animals was not prevented but only delayed and did not result in any overt changes in myelination, at least as observed in the current study. Further support that EBI2 signalling is required for MBP expression came from our studies that showed that treatment of slices with EBI2 compounds limited MBP expression in a time dependent manner **(Results 3)**. We suggest that downregulation of EBI2 *in vivo* and in cerebellar slices may result in delayed myelin development. Figure 6.3 highlights the role of EBI2 in myelin development. Of importance, these effects of EBI2 inhibition were restricted to MBP protein expression and did not alter expression for earlier oligodendrocyte markers Olig1 or CNPase or the mature myelin marker MOG. Interestingly, MBP is one of the major proteins found in myelin and expressed in myelinating oligodendrocytes, and is also involved in myelin formation and compaction **(Baumann and Pham-Dinh, 2001, Campagnoni and Macklin, 1988)**. It is therefore possible that EBI2/oxysterol signalling is needed for timely myelin maturation and is not involved in the earlier stages of OPC migration or differentiation. Nor does EBI2/oxysterol signalling, from our data, seem to interfere with myelin maturation. For example, MOG is a myelin-specific protein that is only expressed in mature myelin forming oligodendrocytes and we did not find it to be altered in EBI2 KO animals. It is therefore possible that EBI2 signalling is not involved in early stages of oligodendrocyte recruitment and differentiation or in later stages of myelin maturation and maintenance. The data suggests that EBI2 may be involved during myelin sheet development and compaction and that a lack of EBI2 signalling may delay these processes.

6.9 The role of EBI2 in protection from chemically induced demyelination

Finally, we showed that EBI2 is not only involved in normal myelin development but its modulation can protect from chemically induced demyelination. Short-term treatment of slices prepared from WT mice with the EBI2 agonist prevented LPC-induced demyelination as well attenuated pro-inflammatory cytokine release. These effects were not observed in slices prepared from EBI2 KO animals, suggesting an EBI2 receptor dependent mechanism **(Results 3)**. Previous studies have shown that pro-inflammatory cytokines such as IL1β or TNFα can induce demyelination **(Ferrari et al., 2004, Redford et al., 1995)**. In the current study, the data showed that 7α25HC could inhibit cytokine release. We also showed that 7α25HC could inhibit the pro-inflammatory intracellular signal propagation of NFκB induced by LPS, TNFα/IL17 and LPC **(Results 2 and 3)**. It is therefore possible that the protective effects of 7α25HC on demyelination are partially mediated via inhibition of pro-inflammatory cytokine release. Figure 6.3 summarises the EBI2 mediated effects on cytokine release in slices. Taken these demyelinating studies together with the myelination studies above, we suggest a model whereby EBI2 signalling might be important for myelin development under normal conditions and where EBI2 signalling is important for attenuating demyelination.

Since there are currently no studies investigating the expression or function of EBI2 in the cells of the CNS other than astrocytes **(Rutkowska et al., 2014)** and this study did not investigate the signalling

events involved in myelin development or protection from LPC-induced demyelination in any of the above cell types. It is impossible to conclude whether the EBI2-induced effects were mediated via neurons, microglia, oligodendrocytes, astrocytes or all of these cells. Therefore, future studies should investigate the mechanisms of action of EBI2 signalling on myelination mediated by neurons, oligodendrocytes, microglia and astrocytes in order to answer the question of how EBI2 signalling affects myelination.

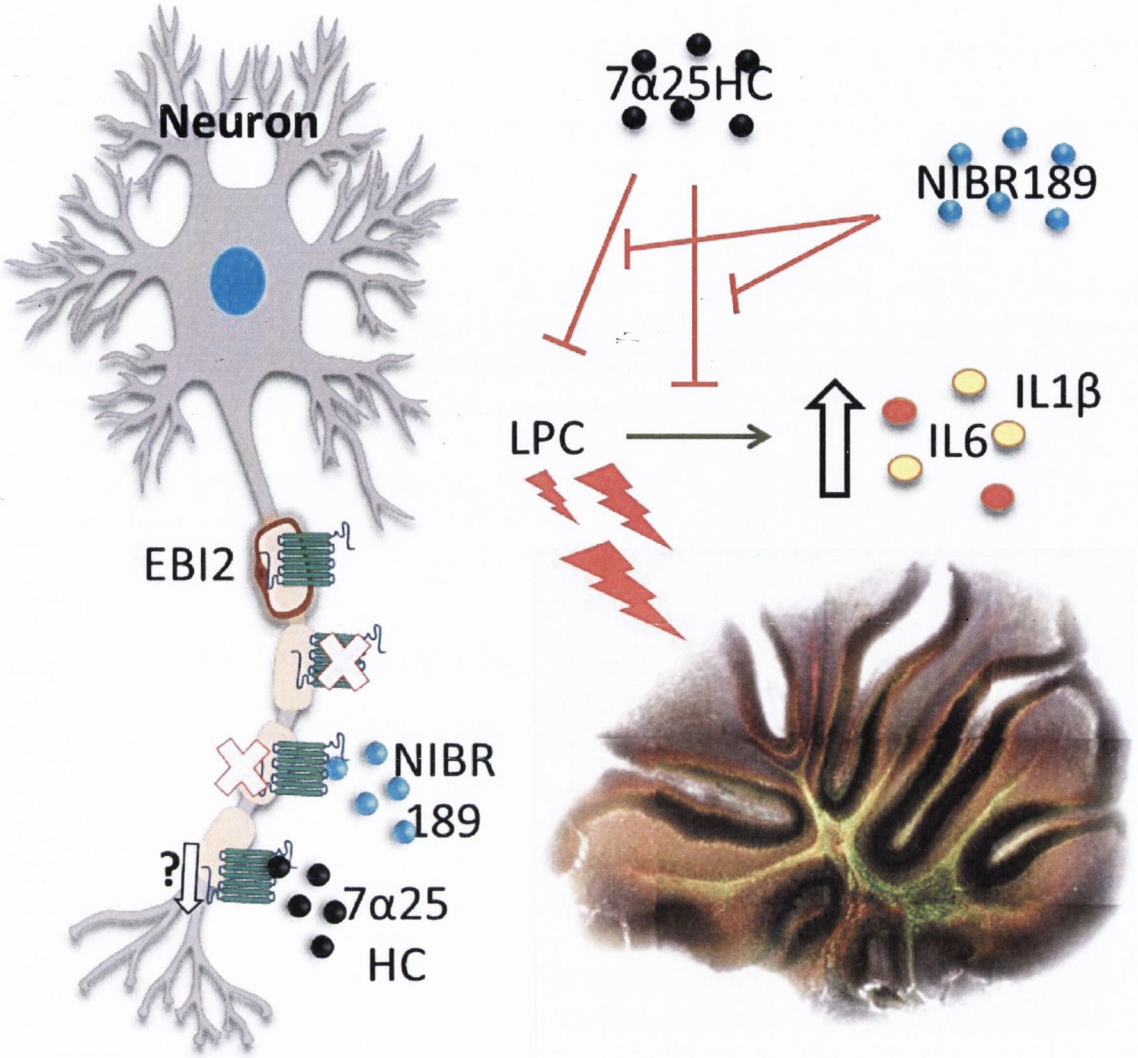


Figure 6.3 EBI2 regulates myelin development and inhibits LPC-induced demyelination. Expression of MBP is delayed in EBI2 KO animals and cerebellar slices prepared from EBI2 KO animals. Expression of Olig1, CNPase and MOG is not affected in EBI2 KO animals. Downregulation or antagonism of EBI2 signalling during development delays myelination as measured by MBP expression. EBI2 protects from LPC-induced demyelination in organotypic cerebellar slices prepared from WT but not EBI2 animals. Treatment with 7α25HC attenuates IL6 and IL1β release in LPC-stimulated slices.

6.10 Limitations and future directions

While the current study provided considerable insight into the basic biology, signalling and function of EBI2 in the CNS there are certain limitations that should be addressed. Firstly, the role of EBI2 signalling in disease context and what implications, if any, does aberrant EBI2 signalling have on disease development, progression or dissolution have not been explored in our study. In this regard, animal models of diseases such as EAE for multiple sclerosis, shiverer mice for MBP deficiency or cuprizone model for demyelinating diseases could be used to answer some of these questions. In addition, EBI2 role in virus mediated models of multiple sclerosis and inflammatory demyelinating disease may also be explored due to the fact that EBI2 is sharply upregulated after Epstein-Barr virus infection and is a negative regulator of IFNγ response **(Daugvilaite et al., 2014, Baumann and Pham-Dinh, 2001)**. Secondly, the current study did not explore the differences in receptor and enzyme expression between rodent and human cells and the possible implications that may arise from it. The translation of results from animal studies and their applicability to human disease might therefore be questioned and require study in greater detail. Other questions such as whether the observed anti-inflammatory effects *in vitro* are sufficient to make a difference *in vivo* also form part of work that represents future directions.

One of the challenges encountered in the study was quantification of oxysterol levels in cells and media. While the expression of the mRNA levels of synthesising and degrading enzyme was possible, we were not able to measure the oxysterol levels in astrocytes in various treatment conditions. Oxysterols, due to their unstable nature and short half-life, are difficult to measure. As such, we could therefore only infer that the changes in enzymes mRNA levels translated into changes in the levels of oxysterols being made by astrocytes. Other challenges encountered during the course of this study included the lack of good anti-mouse EBI2 antibodies for the purpose of conducting EBI2 expression level and trafficking studies. In addition, the mechanisms of LPC-mediated demyelination are not currently very well understood which, we acknowledge renders incomplete the data investigating the role of EBI2 in LPC-induced demyelination. Moreover, the lack of knowledge of pathways involved in myelination and exact role of MBP and other myelin proteins in the process of myelination has been a challenge for the field in general **(Baumann and Pham-Dinh, 2001)**. Taking these factors into account, we propose that future studies exploring the role of EBI2 in animal models of neuroinflammatory and demyelinating diseases of the CNS are warranted.

6.11 Closing remarks

Neurodegenerative diseases such as multiple sclerosis or Alzheimer's disease have a neuroinflammatory component to them. They are the most common diseases of the central nervous system and have devastating effects on patients' lifespan and quality of life. There is currently no cure for these disorders. There are a few disease modifying drugs such as Gilenya, marketed for relapsing-remitting multiple sclerosis, but the cure for MS and other neurodegenerative diseases is still to be discovered. Intriguingly, it is the immune system rather than the CNS that is targeted with the current therapies. There is an unmet need in finding centrally acting therapies that enhance remyelination after a demyelinating episode or protect from demyelination during inflammation in the CNS. EBI2 is mainly expressed in the cells of the immune system such as B and T cells and lymphoid tissue, therefore its role has been mainly studied with regards to the immune system and neglected in the CNS **(Pereira et al., 2010, Daugvilaite et al., 2014)**. Here, the effects of EBI2 on the cells of the CNS and on myelination state and demyelination have been investigated. Resident CNS cells such as astrocytes and microglia are the first line of defence against invading pathogens and

therefore express many of the same receptors and may behave in a similar fashion as the cells of the immune system such as macrophages or monocytes **(Miljkovic et al., 2011, Tan et al., 1998, Kort et al., 2006, Lee et al., 1990, Miljkovic et al., 2007)**. We have therefore investigated the role of EBI2 in the CNS looking at EBI2 function in astrocytes, neuroinflammatory signalling and (de)myelination. Figure 6.4 Summarises the findings in the current study. The data showed that EBI2 and oxysterols are involved in several functions such as migration, cytokine release, inflammatory signalling and myelination. It is therefore possible that the EBI2-oxysterol signalling may be a part of the larger immunomodulatory system involved in astrocyte-mediated immune defence and CNS repair.

Given the data presented in the current study, further investigation of the effects of EBI2 agonists and antagonists in animal models of neuroinflammatory disease are now warranted. In closing, we suggest this work and further *in vivo* studies will support the use of EBI2 as a novel drug target for treatment of neuroinflammatory and neurodegenerative diseases.

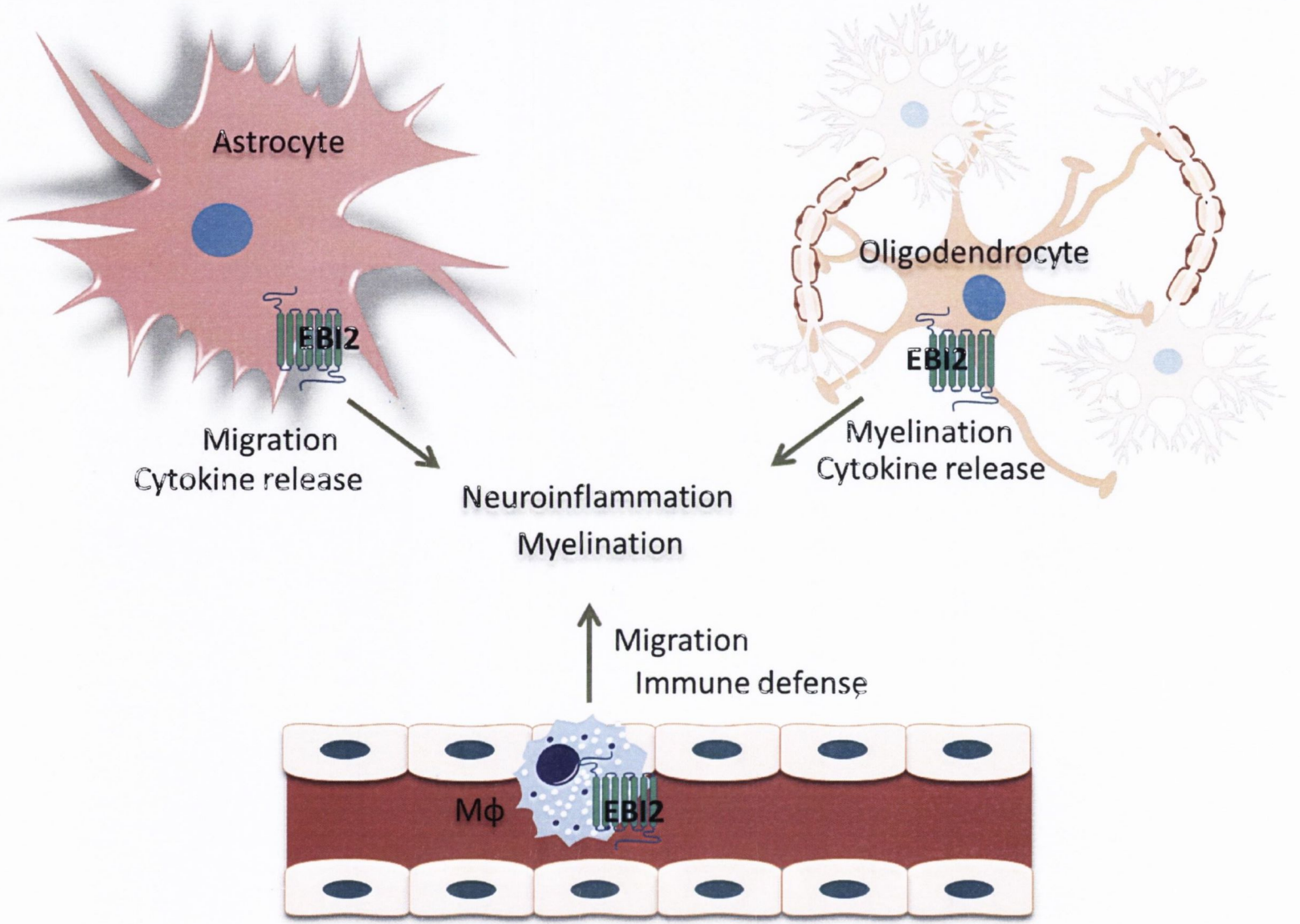


Figure 6.4 Multimodal roles of EBI2 in immune and glial cells. EBI2 serves various roles in both, the central and the immune systems. The major central effects include migration and anti-inflammatory properties. The main immune system roles include chemotaxis of EBI2 expressing cells and modulation of immune defence.

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