SPARC MODULATES THE SPINAL CORD NEUROIMMUNE RESPONSE IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

by

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B.Sc. (Hons), Simon Fraser University, Burnaby, British Columbia, Canada 2008

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

February 2014

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Abstract

SPARC (Secreted Protein Acidic and Rich in Cysteine), a secreted glycoprotein, regulates proliferation, migration and differentiation. SPARC is highly expressed in glia and blood vessels during CNS development. SPARC expression is maintained in tissues undergoing rapid turnover and its expression is highly upregulated during injury or disease. SPARC’s modulatory activity in glia and endothelia during injury lead us to investigate the role of SPARC in an animal model of CNS inflammation and demyelination with known BBB dysfunction: Experimental Autoimmune Encephalomyelitis (EAE). We discovered that, in the spinal cord, SPARC is expressed and localized to developing endothelia and radial glia but is down-regulated and retained in specific subpopulations of glia in the adult spinal cord. During the repair response of EAE, CNS glia and endothelia recapitulate their developmental SPARC expression. Furthermore, in the absence of SPARC, EAE onset is delayed even though there is increased blood-brain barrier (BBB) permeability. We provide evidence that SPARC may play a role in neuro-immune and endothelial cross-talk during the repair response following EAE.
Preface

Chapter 3 and 4 are based on work reported in the following:


- Anwar MA, Petit A and Roskams AJ. (To be submitted - 2013) SPARC Modulates the Spinal Cord Neuroimmune Response in Experimental Autoimmune Encephalomyelitis. J Biomed Res (submitted 2013). I was responsible for all of the experiments and analysis. I also wrote the majority of the manuscript.
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List of Abbreviations

AD – ADULT
BBB – BLOOD BRAIN BARRIER
BCSFB – BLOOD CEREBROSPINAL FLUID BARRIER
BFGF – BASIC FIBROBLAST GROWTH FACTOR
BMEC - BRAIN MICROVASCULAR ENDOTHELIAL CELL
CC – CENTRAL CANAL
CD – CLUSTER OF DIFFERENTIATION
CFA – COMPLETE FREUND’S ADJUVANT
CNS – CENTRAL NERVOUS SYSTEM
CSPGS – CHONDROITIN SULPHATE PROTEOGLYCANS
CX3CR1 – CX3C CHEMOKINE RECEPTOR 1
DAPI – 4’, 6-DIAMIDINE-2-PHENYLINDOLE DIHYDROCHLORIDE
EAE – EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS
ECM – EXTRACELLULAR MATRIX
GFAP – GLIAL FIBRILLARY ACIDIC PROTEIN
GFP – GREEN FLUORESCENT PROTEIN
GM – GRAY MATTER
HE – HEMATOXYLIN-EOSIN
HLA – HUMAN LEUKOCYTE ANTIGEN
IBA-1 – IONIZED CALCIUM-BINDING ADAPTER MOLECULE 1
IL – INTERLEUKIN
INF - INTERFERON
MHC – MAJOR HISTOCOMPATIBILITY COMPLEX
MMPS – MATRIX METALLOPROTEINASES
MOG – MYELIN OLIGODENDROCYTE GLYCOPROTEIN
MRI – MAGNETIC RESONANCE IMAGING
MRNA – MESSENGER RIBONUCLEIC ACID
MS – MULTIPLE SCLEROSIS
NSC – NEURAL STEM CELLS
OECs – OLFACTORY ENSHEATHING CELLS
PBS – PHOSPHATE BUFFERED SALINE
PECAM-1 – PLATELET ENDOTHELIAL CELL ADHESION MOLECULE
PFA – PARAFORMALDEHYDE
PCNA – PROLIFERATING CELL NUCLEIC ACID
PCR – POLYMERASE CHAIN REACTION
PDGF – PLATELET-DERIVED GROWTH FACTOR
PNVP – PERINEURAL VASCULAR PLEXUS
ROI – REGION OF INTEREST
SC – SPINAL CORD
SCRG – SPINAL CORD RADIAL GLIA
SPARC – SECRETED PROTEIN ACIDIC AND RICH IN CYSTEINE
SVZ – SUBVENTRICULAR ZONE
NA-FITC – SODIUM FLUORESCEIN ISOTHIOCYANATE
TEER – TRANSENDOTHELIAL ELECTRICAL RESISTANCE
TGF-β1 – TRANSFORMING GROWTH FACTOR BETA-1
VEGF – VASCULAR ENDOTHELIAL GROWTH FACTOR
WM – WHITE MATTER
ZO-1 – ZONULA OCCLUDENS
Acknowledgements

I’d like to acknowledge Dr. Peter Rieckmann, for accepting me as a wide-eye naive graduate student and introducing me to the most complex protein I have ever studied, and my collaborator, Dr. Jacqueline Quandt, for her advice and experimental input for rigor. Sincere thanks goes to the (former) members of the Rieckmann lab and MS group of the UBC Hospital.

I’d like to acknowledge Dr. Jane Roskams for her constant advice and pressure to perform the best possible experiments. I’d also like to thanks all the members of the Roskams lab, those who have completed and those who remain, have all provided me with help and support beyond that which I can thank them for. A special thank you goes to Nicole Janzen for essentially everything, Dr. Samantha Lloyd-Burton and Dr. Audrey Petit for hand-on aid in key experiments and findings.

I’d like to thank all the members of my supervisory committee, Dr. Christian Naus, Dr. Fabio Rossi, and Dr. Wolfram Tetzlaff, for their excellent expertise and wisdom that proved invaluable for my project, and for taking the time out of their busy schedules to meet with me and provide advice at various times during the course of my graduate career. My sincere thanks goes to Dr. Vincent Duronio for his tremendous help and guidance for getting all this done.

I’d like to thank the Canadian Institute for Health Research for providing funds necessary for this project. I’ve learned that mice are royalty and are paid far more than graduate students.

I’d like to thank my father, mother and sister for their love and support. And finally, thank God.
Dedication

I dedicate my work to my loving wife. Thank you for all the love, support and courage.
Chapter 1: Introduction

1.1 Extracellular matrix

The extracellular matrix (ECM) is defined as the extracellular component of connective tissue that provides structural support and regulates intercellular signaling. The ECM modulates cell behavior by sequestering growth factors. Injury or disease can trigger protease activity that can cause the release of these growth factors, allowing for rapid and local growth factor-mediated activation of cellular functions such as repair and regeneration. The ECM of the central nervous system (CNS) is primarily made up of macromolecular glycosaminoglycans, especially chondroitin sulphate proteoglycans (CSPGs) and hyaluronan. The CNS ECM is rich in growth factors, proteases, cytokines and matricellular proteins.

1.1.1 Matricellular proteins

Matricellular proteins are ECM associated proteins that do not serve structural roles in the ECM, instead they bind to matrix proteins, cell surface receptors and other proteins, including proteases and cytokines, and act to modulate ongoing signalling events (Bornstein, 2000, Bornstein, 2002). Matricellular proteins are generally highly expressed during development, but are downregulated in the adult, other than in tissues undergoing morphogenesis, remodelling and repair. Matricellular proteins are vital in processes governing both tissue development and disease pathogenesis. They have been demonstrated to regulate processes such as cellular adhesion, migration and proliferation, all fundamental concepts in CNS development, regeneration and repair. Matricellular proteins can be classified into several different families, including the
secreted protein acidic and rich in cysteine (SPARC) family, the CYR61, CTGF, Nov (CCN) family, the thrombospondin family, and the tenascins (Bornstein, 2000, Clark and Sage, 2008)

1.2 Secreted Protein Acidic and Rich in Cysteine (SPARC)

The SPARC family consists of 9 proteins: SPARC (osteonectin, BM-40) (Brekken and Sage, 2001), hevin (SPARC-like (SPL) 1, SC1, MAST 9, RAGS-1, QR1, ECM 2) (Hambrock et al., 2003), secreted modular calcium binding protein (SMOC) 1 and 2 (SRG) (Vannahme et al., 2002, Vannahme et al., 2003), testicans 1, 2, and 3 (SPARC/osteonectin, CWCV, and Kazal-like domains proteoglycans, SPOCK) (Schnepp et al., 2005, Vannahme et al., 1999, Alliel et al., 1993, Charbonnier et al., 1998a), and follistatin like protein 1 (fstl-1, TSC-36/Flik, follistatin related protein (FRP), TGF-β inducible protein) (Hambrock et al., 2004a). Each SPARC family member contains a characteristic conserved extracellular calcium binding domain with an E-F hand motif, a follistatin-like domain, and an amino terminal acidic domain (Brekken and Sage, 2001, Sullivan and Sage, 2004). The actions of SPARC family matricellular proteins are mediated indirectly, since no specific receptor has been identified for any of the proteins. Instead, they are believed to act primarily through direct binding to other matrix components, growth factors and cytokines. SPARC, the prototypical member of the SPARC family, has a wide variety of biological functions and has been recently implicated in various crucial roles in the CNS, from modulating development to regulating response to injury and disease (Pierani et al., 1995, Pierani et al., 1993, Cifuentes-Diaz et al., 2000, Vincent et al., 2008, Eroglu, 2009, Kucukdereli et al., 2011, Lloyd-Burton and Roskams, 2012, Lloyd-Burton et al., 2013)
1.2.1 Extracellular matrix organization

SPARC binds both fibrillar collagen and collagen type IV, a key component of the basal lamina, thin sheet of fibers that underlies the endothelium which lines the interior surface of blood vessels (Mayer et al., 1991). SPARC-null mice show many abnormal pathologies associated with deficiencies in ECM organization, assembly and composition. SPARC-null mice show abnormal structure and organization of several connective tissues, for example, dermal, heart, adipose, and periodontal ligament tissue were shown to contain less fibrillar collagen (Bradshaw et al., 2003a, Bradshaw et al., 2003b, Bradshaw et al., 2010, Trombetta and Bradshaw, 2010). Collagen fibrils in SPARC-null mice are smaller and have a more uniform diameter than wildtype fibrils (Bradshaw et al., 2003a). Defective basal lamina collagen IV in SPARC-null lens capsule results in early onset cataractogenesis in SPARC-null mice (Yan et al., 2002, Gilmour et al., 1998a).

1.2.2 Matrix metalloproteinase activity

Matrix metalloproteinases (MMPs) are a family of endopeptidases considered to be the primary mediators of ECM degradation and turnover. MMPs play major regulatory roles on cell proliferation, migration, differentiation, angiogenesis, and apoptosis. SPARC has been shown to regulate MMP expression and activity. Exogenous SPARC induced MMP-1, MMP-3, and MMP-9 activity in synovial fibroblasts (Tremble et al., 1993). SPARC increases MT1-MMP and MMP-2 activity in glioma cells and increased MMP-2 activity in breast cancer cells (McClung et al., 2007, Nischt et al., 2001, Gilles et al., 1998). However, SPARC’s regulation of MMPs is tissue environment specific as host SPARC negatively regulates MMP-2 and MMP-9 levels and activity in peritoneal tumors (Said et al., 2007). Regions of SPARC that induced MMP activity
were mapped to the peptides containing the alpha helices of the E-F hand region (Tremble et al., 1993). SPARC’s role in MMP regulation can lead to excessive degradation of matrix components, such as basal lamina collagen IV, blood-brain barrier (BBB) disruption and glial scar formation (Rosell et al., 2008, Anik et al., 2011).

1.2.3 Growth factor and receptor activity
SPARC modulates growth factor signaling, including vascular endothelial growth factor (VEGF) receptor, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β1, by either binding directly to growth factors, as in the case of VEGF, or by binding to growth factor receptors, such as SPARC binding to the TGF-β receptor endoglin, thereby modulating their activity (Rivera et al., 2011, Rivera and Brekken, 2011). SPARC’s regulation of growth factor activity has, for the most part, been shown to occur in the ECM and involve modulation of growth factor receptor activity either through interaction with ligand or with cell-surface receptors.

1.2.4 Adhesion
Adhesion requires three steps: attachment; spreading; and formation of stress fibers and focal adhesions. De-adhesion is characterized by the disassembly of stress fibers and formation of an intermediate state of adherence (Murphy-Ullrich, 2001). Matricellular proteins, unlike most other components of the ECM, are anti-adhesive. SPARC is strongly anti-adhesive towards many cell types to various substrates in vitro, and induces changes in cell shape, leading to cell rounding (Lane and Sage, 1990, Sage et al., 1989). The Ca²⁺-binding domains of SPARC have since been demonstrated to be responsible for this effect on cell-shape (Lane and Sage, 1990,
Murphy-Ullrich et al., 1995). Cells isolated from SPARC-null mice display enhanced spreading and increased numbers of focal adhesion plaques in vitro (Bradshaw et al., 1999).

1.2.5 Migration
The role of SPARC on cellular migration can be specified by two components: an intermediate state of adherence, as previously described, and the regulation of ECM turnover and MMP activity favors migration (DiMilla et al., 1991, Lane and Sage, 1994). Matricellular proteins are highly expressed during development and remodeling, a time frame in which cellular migration is particularly active. Furthermore, the cell types expressing matricellular components under normal conditions are those that are able to move freely through the ECM. In the CNS, highly motile glia such as astrocytes and microglia express significant levels of SPARC (Vincent et al., 2008). Following ischemic and excitotoxic lesion, reactive, hypertrophic microglia rapidly downregulate and release SPARC at the lesion, facilitating microglial motility towards the lesion site (Lloyd-Burton et al., 2013).

1.2.6 Proliferation
SPARC inhibits the proliferation of a wide range of cells, including endothelial cells, mesangial cells, and smooth muscle cells (Funk and Sage, 1991, Funk and Sage, 1993, Pichler et al., 1996). SPARC-null mesangial cells, fibroblasts, and smooth muscle cells proliferate faster than their respective wild-type counterparts (Bradshaw et al., 1999). SPARC-nulls demonstrate enhanced microgliosis in and around the lesion site, following cortical ischemia. Microglia from SPARC-nulls also intrinsically proliferate at a greater rate in vitro - an enhanced effect that can be rescued by the addition of exogenous SPARC (Lloyd-Burton et al., 2013). SPARC may be
acting both intracellularly and extracellularly to influence proliferation. Extracellular SPARC can bind directly to PDGF via its follistatin-like domain and modulate cell cycle progression in certain endothelial cell lines (Funk and Sage, 1991). Intracellular SPARC can translocate to the nucleus and inhibit cell cycle (Yan et al., 2002). Using full-length SPARC as bait the carboxy-terminal domain, which contains two EF-hand high-affinity binding sites, was found to have transcriptional activity. An alternative mechanism of action of intracellular SPARC is suggested by the observation that SPARC is associated with axonemal tubulin in ciliated epithelial cells (Sodek et al., 2002).

1.2.7 Survival

SPARC can promote cell survival and halt apoptosis. SPARC promotes lens epithelial cell survival by binding to β1 integrin resulting in increased integrin-linked kinase (ILK) activity (Weaver et al., 2008). Increased intracellular SPARC expression is associated with increased resistance to the chemotherapy agent imatinib (Fenouille et al., 2010). Decreased SPARC expression in melanoma leads to apoptosis, and is associated with an activation of caspase 3 (Fenouille et al., 2011).

However, in other cases, SPARC can enhance apoptosis. In human colon cancer, breast and pancreatic cancer, SPARC increases apoptosis via the activation of caspase 8 (Rahman et al., 2011, Tang and Tai, 2007). SPARC’s survival activity and regulation of apoptosis appears to be context-dependent and is most likely influenced by other extracellular factors.
1.2.8 SPARC in the developing and adult nervous system

SPARC, identified in the peripheral nervous system (PNS) as a factor produced by Schwann cells, affects neuroblastoma angiogenesis (Chlenski et al., 2002), and can be taken up by retinal ganglion cells in vitro, resulting in the promotion of neurite outgrowth and survival (Bampton et al., 2005). Olfactory ensheathing cells (OECs) share many characteristics with Schwann cells, including SPARC expression and secretion (Au et al., 2007). SPARC is a major component of the neurite outgrowth-promoting activity of OEC-conditioned medium in a dorsal root ganglion assay (Au et al., 2007). SPARC-null mice have increased levels of neuronal activity and show increased levels of anxiety-related behaviors and reduced levels of depression-related behaviors (Campolongo et al., 2012). SPARC also affects cell proliferation in the subgranular zone of the dentate gyrus in the hippocampus, although it does not affect maturation and survival of new neurons (Campolongo et al., 2012).

In the postnatal (P) developing murine CNS, SPARC is highly expressed in subpopulations of radial glia, astrocytes, and microglia at distinct developmental stages. During brain development, SPARC is extensively expressed by radial glia, astrocytes, and developing endothelia (Vincent et al., 2008), which mature postnatally to form the blood-brain barrier (BBB) which tightly regulates the movement of ions, molecules, and cells between the blood and the CNS (Risau and Wolburg, 1990). SPARC’s expression and localization coincides with BBB maturation during brain development (Vincent et al., 2008). In the adult brain, SPARC is expressed by microglia, a subset of astrocytes (specifically in the brainstem), and specialized radial glial derivatives (Müller and Bergmann glia) (Vincent et al., 2008).
SPARC-null mice have a subtle phenotype that is perhaps best revealed by challenge, like abnormal fibroblast migration during dermal wound healing (Basu et al., 2001, Bradshaw et al., 2002) and increased cardiac rupture following myocardial infarction (Schellings et al., 2009). SPARC has the potential to modulate different aspects of CNS repair, including regulating endogenous glial activation to promote neurite outgrowth (Au et al., 2007) and microglial responses following ischemic and excitotoxic lesion in the brain (Lloyd-Burton et al., 2013). Recently, SPARC has also been mechanistically implicated in astrocytic regulation of synaptic stability (Kucukdereli et al., 2011, Jones et al., 2011) and cortical lamination (Gongidi et al., 2004).

1.3 Glial heterogeneity

Virchow first described glia as nerve glue, implying a homogenous population of support cells with a mostly passive role (Somjen, 1988). However, recent studies indicate that glia, such as astrocytes and microglia, are not homogenous but vastly morphologically and functionally diverse and play critical roles in development and disease.

1.3.1 Astrocyte heterogeneity

Astrocytes are the most abundant glia in the CNS and perform many functions, including the biochemical support of nervous tissue and endothelial cells that form the blood–brain barrier (BBB), homeostatic maintenance of the neural microenvironment, and repair/glial scar formation after injury. Most astrocytes express the intermediate filament glial fibrillary acidic protein (GFAP). Following Virchow’s initial description of glia, detailed morphological studies over the subsequent years revealed the first broad category of astrocyte heterogeneity based on location in
the gray versus white matter. Gray matter protoplasmic astrocytes have more irregular processes and few glial filaments (Vaughn and Pease, 1967). Protoplasmic astrocytes extend processes to contact neural synapses, blood vessels or CNS boundaries (Bushong et al., 2003, Bushong et al., 2002, Reichenbach et al., 2010). Fibrous astrocytes are located in the white matter and have characteristic “star-shape” and cylindrical processes. In addition, there are specialized radial astroglia that reside in the CNS, such as Bergmann glia in the cerebellum, and Müller cells in the retina (Metea and Newman, 2006, Parpura and Zorec, 2010). Astrocytes are found throughout the CNS, yet recent evidence shows that astrocytes arise from distinct groups of progenitors in the spinal cord (Zhang and Barres, 2010). Recently, our lab has identified GFAP-expressing radial glial subtype in the adult spinal cord (Petit et al., 2011). These adult radial glia are morphologically reminiscent of the radial glia present during CNS development and may serve similar functions during repair process following CNS injury or disease.

1.3.2 Microglial heterogeneity

Microglia are myeloid cells of the CNS and perform a wide range of roles including immune surveillance, scavenging and phagocytosis, maintenance of the neural microenvironment homeostasis, regulation of cytokines and the protection of the structural and functional integrity of the CNS (Hanisch and Kettenmann, 2007, Ransohoff and Cardona, 2010, Kettenmann et al., 2011, Prinz and Mildner, 2011, Saijo et al., 2011). The ability of microglia to rapidly react to injury or disease has been known as “activation”. However, this term does not adequately reflect the diversity of microglial responses nor the net effect on the CNS (Hanisch and Kettenmann, 2007, Kettenmann et al., 2011). Microglial activation encompasses functional complexity and diverse microglial responses, such as in the induction (or suppression) of genes, leading to
dramatically different CNS impacts (Gordon and Taylor, 2005, Mosser and Edwards, 2008, Murray and Wynn, 2011). We recently showed that SPARC influences the distribution and branching of mature ramified microglia, with significant differences between cortical gray and white matter, and modulates microglial proliferation and migration (Lloyd-Burton et al., 2013).

1.4 Blood-CNS barriers

The CNS microenvironment homeostasis must be maintained within precise ranges to facilitate proper function (Hawkins and Davis, 2005). This is accomplished by two structures: the blood-brain barrier (BBB), formed by specialized endothelial cells stitched together by tight junctions, and the blood-cerebrospinal fluid barrier (BCSFB), formed by choroid plexus epithelial cells.

1.4.1 Blood-brain barrier (BBB)

Tightly apposed CNS endothelial cells stitched closely together by tight junctions form the BBB and selectively restricts the passage of molecules between the CNS and circulation (Lee et al., 2009, Abbott et al., 2006, Martino et al., 2002). Tight junctions (TJs) are transmembrane proteins with extracellular domains that interact with associated submembraneous adapter proteins, such as zonula occludens (ZO) -1, to adhere cells together and provide cytoskeletal support (Engelhardt and Sorokin, 2009). The basement membrane comprised of ECM molecules, such as collagens, fibronectin, proteoglycans, vitronectin, and tenascin, form a complex network between endothelial cells and glial cells in the CNS (Engelhardt and Sorokin, 2009, Persidsky et al., 2006). ECM degradation, which can occur through MMP activity, correlates with increased BBB permeability (Hawkins and Davis, 2005).
Pathological conditions in the CNS are associated with downregulation and mislocalization of TJs, BBB dysfunction and increased permeability, and immune cell extravasation into the CNS parenchyma (Stamatovic et al., 2008).

1.4.1.1 Blood-brain barrier development and maintenance

Unlike other tissues, CNS vascularization is entirely driven by angiogenesis. In rodents, CNS blood vessels are formed starting at embryonic day 9 (E9) by sprouting from a primitive vascular network surrounding the neural tube known as the perineural vascular plexus (PNVP) (Nakao et al., 1988). Under the influence of vascular endothelial derived growth factor (VEGF), angiopoietin-1, and Sonic Hedgehog (Shh) secreted by the neuroepithelium lining the subventricular zone (SVZ) (Nagase et al., 2005), certain PNVP endothelial cells switch their phenotype to a highly invasive and migratory endothelial cell type that initiates blood vessel sprouting into the neural tube. Differentiating endothelial cells are anchored on an early primitive basement membrane formed by extracellular matrix (ECM) proteins including collagen IV, fibronectin, laminin-1 and entactin/nidogen-1 (Flamme et al., 1997, Bader et al., 1998, Virgintino et al., 2007). Also, the rapid association of newly formed microvasculature by pericytes suggests that they may be the first cell type of the neurovascular unit to physically interact with endothelial cells (Virgintino et al., 2007). In addition to pericytes, neighboring undifferentiated neural progenitor cells (NPCs), differentiating NPCs, and radial glia also appear to exercise an influence on the developmental BBB as studies have suggested their ability to induce barrier properties in brain endothelial cells in vitro and in vivo (Daneman et al., 2009, Daneman et al., 2010, Stenman et al., 2008, Weidenfeller et al., 2007). On the other hand, the early stage developing microvasculature remains devoid of astrocytes as such cells only appear
at the end of gestation and early postnatal stages (Zerlin and Goldman, 1997, Senjo et al., 1986). During embryonic development, functional barrier properties are acquired as demonstrated by a continuous increase in tight junction organization (Kniesel et al., 1996, Liebner et al., 2011). This process results in barrier maturation, marked by an increase in transendothelial electrical resistance (TEER) with a simultaneous decrease in permeability to water-soluble compounds such as mannitol, potassium or urea (Preston et al., 1995, Keep et al., 1995).

Although barrier properties are induced during embryonic development, they remain attenuated when compared to the adult BBB. An examination of the multicellular composite that helps maintain the adult BBB reveals that pericytes remain in contact with endothelial cells, sharing a more elaborate basement membrane formed by different ECM components including agrin, laminin, perlecan and SPARC. The developmental CNS parenchyma is replaced by dense meshwork of neurons and glial cells supported by a chondroitin-sulfate proteoglycan-rich matrix (Thorne and Nicholson, 2006). Unlike the early stages of embryonic BBB development when astrocytes are absent, astrocytes play important roles in BBB maturation and maintenance. While the mechanisms driving the further induction and maintenance of the adult BBB are unresolved, several growth factors and signaling molecules have been shown to have effects on the BBB phenotype in vitro. Importantly, the BBB phenotype is dictated by the local microenvironment and is not intrinsic to brain endothelial cells themselves (Stewart and Wiley, 1981); and thus, primary brain microvascular endothelial cells (BMECs) rapidly lose their barrier features in vitro. Thus, it is important to take into account the embryonic and adult neurovascular microenvironments when investigating BBB dynamics.
1.5 Multiple Sclerosis

Multiple Sclerosis (MS) is an inflammatory neurodegenerative disease in which the insulating myelin sheath of brain and spinal cord neurons are damaged by the host immune system resulting in a wide range of signs and symptoms (Compston and Coles, 2008). Patients with MS can have almost every neurological symptom, with autonomic, visual, motor, and sensory impairments being the most common (Compston and Coles, 2008). The specific deficiencies are determined by the locations of the lesions within the CNS. These lesions most commonly affect the white matter in the optic nerve, brain stem, basal ganglia and spinal cord, or white matter tracts close to the lateral ventricles (Compston and Coles, 2008).

1.5.1 Causes of Multiple Sclerosis

Although the exact cause of MS is unknown, it is believed to occur as a result of a combination of genetics and environmental factors such as infectious agents (Compston and Coles, 2008).

1.5.1.1 Genetics

Although MS is not generally considered a hereditary disease, a number of genetic variations have been shown to increase the risk of developing disease (Dyment et al., 2004). Genetic variations in the human leukocyte antigen (HLA) system, a group of genes located on chromosome 6 that serve as the major histocompatibility complex (MHC), are mostly related to increased susceptibility in developing autoimmune disorders, such as MS (Compston and Coles, 2008, Baranzini, 2011). Specifically the HLA-DR15 and HLA-DQ6 alleles of the MHC system have a positive association with increased MS susceptibility (Compston and Coles, 2008). Genome wide association studies have discovered a number of other genes outside of the HLA
locus, such as the genes encoding for the IL-2 receptor and IFN-γ, that increase the probability of MS (Baranzini, 2011, Sospedra and Martin, 2005).

1.5.1.2 Geographical Location

MS is more common in people who reside further from the equator, such as in northern European populations, although there are exceptions (Compston and Coles, 2008, Alonso and Hernan, 2008, Milo and Kahana, 2010). A widely accepted theory is that decreased sunlight exposure due to geographical location results in decreased vitamin D production (Ascherio and Munger, 2007a). Vitamin D is a known modulator of the immune system, and its insufficiency is implicated in increased susceptibility to autoimmune disease (Moran-Auth et al., 2013).

1.5.1.3 Infectious Agents

There are several infectious agent hypotheses proposed as triggers of MS but none have been confirmed. The two most common hypotheses include the prevalence hypothesis, where MS is due to an infectious agent that is more common in regions where MS is common and in most individuals it causes an ongoing infection without symptoms, and the more supported hygiene hypothesis, where exposure to an infectious agent early in life is protective, the disease occurs in response to a late encounter with the same or similar infectious agent (Compston and Coles, 2008, Compston and Coles, 2002, Ascherio and Munger, 2007b, Kurtzke, 1993). The presence of oligoclonal bands in brain and cerebrospinal fluid (CSF) of MS patients and virus mediated demyelinating encephalomyelitis in humans and animals implicate viral agents as potential cause of MS (Gilden, 2005). Candidate viruses include herpes, measles, mumps, rubella, and Epstein-Barr viruses (Compston and Coles, 2008, Ascherio and Munger, 2007b).
1.5.2 Pathophysiology of Multiple Sclerosis

The two main pathological characteristics of MS are demyelination and formation of lesions in the CNS (called plaques), and inflammation (Compston and Coles, 2008). The mechanisms of demyelination and inflammation interact in a complex system that ultimately results in neurodegeneration.

1.5.2.1 Inflammation

The inflammatory process is caused by autoreactive encephalitogenic T cells, which gain entry into the brain through disruptions in the BBB (Figure 1). These T cells recognize myelin as foreign and initiate cell-mediated immune attack on white matter in the optic nerve, brain stem, basal ganglia and spinal cord, or white matter tracts close to the lateral ventricles (Compston and Coles, 2008). The myelin attack initiates the inflammatory processes which triggers other immune cells and the release of soluble factors like cytokines and antibodies. Inflammation further breakdowns the BBB, which results in swelling, microglial/macrophage activation, and further cytokine release through a vicious inflammatory cycle. The soluble immune factors released impair neurotransmission by intact neurons and could enhance the loss of myelin, or they may cause the axon to break down completely (Compston and Coles, 2008).
Activated Th1 cells and Th17 cells are thought to be the main culprits in MS pathogenesis. Interferon (IFN)-γ producing Th1, and Interleukin (IL)-17 producing Th17 lymphocytes are primed by follicular dendritic cells outside the CNS, then cross the BBB and encounter CNS antigen-presenting cells. They produce inflammatory factors and cytokines that damage the myelin and axons. They also activate the resident microglia and produce cytokines that attract further inflammatory cells into the CNS and perpetuate the inflammatory cascade. Antibodies and B cells can also enter the CNS, and plasma cells produce antibodies within the CNS. Antibody mediated damage contributes to the inflammatory demyelination and neurodegeneration.

Figure 1. Summary diagram of some of the key pathological features of MS pathogenesis.
1.5.2.2 Demyelination

MS involves not only the inflammatory destruction of white matter, but also the loss of oligodendrocytes, the glial cells responsible for creating and maintaining the myelin sheath. When the myelin sheath is lost, a neuron can no longer effectively conduct electrical signals. Remyelination takes place in the early phases of MS, but the progressive loss of oligodendrocytes diminishes this repair (Chari, 2007).

1.6 Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is the most commonly used experimental model for the human inflammatory demyelinating disease, multiple sclerosis (MS), in which the interaction between immunopathological and neuropathological mechanisms leads to an approximation of the key pathological features of MS: inflammation, demyelination, blood-brain barrier breakdown, axonal loss and gliosis (Nischt et al., 2001, Tang and Tai, 2007, Ascherio and Munger, 2007a, Compston and Coles, 2002). The processes of anti-inflammation, remyelination, and repair are also initiated in EAE, which, therefore can also serve as a model for these processes. EAE was first described over 75 years ago, originally induced in rabbits using human spinal cord homogenate (McBroom and Sadowski, 1995, Lewis et al., 1995). The technique has since been refined and expanded to many other species: mice, rats, guinea pigs, macaques and rhesus monkeys, providing models of acute monophasic, relapsing–remitting and chronic progressive CNS inflammation (Nakao et al., 1988, Sriram and Steiner, 2005). Disease incidence is higher in susceptible animal strains (Bader et al., 1998, Virgintino et al., 2007).
While there is significant heterogeneity in the susceptibility to the induction, the method of induction and the response to various immunological or neuropharmacological treatments, many elements of MS pathogenesis have been identified, tested or confirmed in EAE. Therefore, EAE is a good model for studying MS mechanisms, even more so than for testing or developing drugs (Nischt et al., 2001). One of the major differences between MS and EAE is that the latter requires an external immunization to induce the disease (Chlenski et al., 2002). In ‘active’ EAE induction, myelin antigen sensitization occurs through the use of an adjuvant, which contains bacterial components that activate pattern recognition receptors of the innate immune system (Yan et al., 2002). In the C57BL/6 mouse, immunization with MOG35-55 peptide in Complete Freund’s Adjuvant (CFA), which is comprised of inactivated Mycobacterium tuberculosis particles in oil emulsion, can induce monophasic or a chronic, progressive form of EAE (Mendel et al., 1995, Raine et al., 1980, Kornek et al., 2000, Gold et al., 2006). This model is characterized by multifocal, confluent areas of mononuclear inflammatory infiltration and demyelination in the spinal cord white matter, and substantial astrocytic, microglial, and macrophage activation in the spinal cord gray matter, without any motor neuron loss (Mendel et al., 1995, Wu et al., 2008). Macrophages and CD4+ T cells are the main cell types in the inflammatory infiltrate (Mendel et al., 1995). In the brain, there is meningitis and perivascular inflammatory cuffing in the cerebellum and hindbrain white matter (Mendel et al., 1995).

There are four key pathological features of EAE: (a) inflammation, which is generally believed to be the main trigger of the events leading to tissue damage; (b) demyelination, where the myelin sheath or the oligodendrocyte cell body is destroyed by the inflammatory process; (c) axonal loss; and (d) astrogliosis. Immune infiltration into the CNS occurs at specific breakdown
points in the BBB, where loss of BBB permeability is a major pathological hallmark that precedes the clinical presentation of symptom (Kermode et al., 1990, Davie et al., 1994, Plumb et al., 2002). In EAE, BBB disruption and inflammatory cell infiltration is unequally distributed in the CNS, with preferential pathology in the spinal cord (Bennett et al., 2010). Immune processes contribute to the initiation and continuation of EAE and studies have indicated that microglia, astrocytes and the infiltrating immune effector cells, such as monocytes and T cells, have distinct roles in the disease pathogenesis (Williams et al., 1994, Trapp et al., 1999, Eng et al.). SPARC appears to mediate the elimination of autoreactive encephalitogenic T cells in EAE (Hara et al., 2011) where SPARC-null mice display a distinct delay in autoimmunity through disruption of immune cell cross-talk in lymphoid tissues (Piconese et al., 2011)

1.7 Hypothesis

I hypothesize during normal state in the spinal cord, SPARC is expressed and maintained in distinct glial subpopulations responsible for sustaining the ECM structure and composition and a proper functional BBB. During EAE, I hypothesize SPARC expression is upregulated by spinal cord glia and endothelia, in a manner similar to development, and is involved in the neuro-immune and endothelial repair responses.
Chapter 2: Materials and Methods

2.1 Animals

Animal procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and the University of British Columbia animal care committee. Mice that are homozygous for the CX3CR1-GFP targeted mutation (B6.129P-Cx3cr1tm1Litt/J), SPARC null mice (B6;129S-Sparctm1Hwe/J), and wild-type mice (B6129SF2/J) were obtained from Jackson Laboratories (Bar Harbor, ME). Equal numbers of male and female mice were used.

2.2 Genotyping

Animals were first genotyped by PCR to identify the SPARC nulls and SPARC wild types using primers MGSPARC-For 5'-GAT GAG GGT GGT CTG GCC CAG CCC TAG ATG CCC CTC AC-3', NEOMYCIN-Rev 5'-GGT GTG CCC AGT CAT AGC CGA ATA GCC TCT CCA CCC AAG-3', and MGSPARC-Rev 5'-CAC CCA CAC AGC TGG GGG TGA TCC AGA TAA GCC AAG-3' followed by PCR for the Cx3cr1-GFP (previously recommended by The Jackson Laboratory) using primers oIMR3945 5'-TTC ACG TTC GGT CTG GTG GG-3' (wildtype), oIMR3946 5'-GGTTCCTAGTGGAGCTAGGG-3' (common to both wild-type and the Cx3cr1-GFP), and oIMR3947 5'-GAT CAC TCT CGG CAT GGA CG-3' (Cx3cr1-GFP only).

2.3 Experimental Autoimmune Encephalomyelitis induction

EAE was performed using a MOG35-55-induced paradigm as previously described (Devaux et al., 1997). Briefly, 6-8 week old female mice were immunized with 100 µg myelin oligodendrocyte glycoprotein (MOG) (35-55) peptide (MEVGWSYPFPAGILVYQGR) in complete Freund's
adjuvant containing 200 µg heat-killed Mycobacterium tuberculosis H37RA by subcutaneous injection over 3 sites on the flank at day 0, with additional intraperitoneal injections of Pertussis toxin (200 ng in sterile saline) on days 0 and 2. Phenotypic assessment was performed daily following the scoring: (0) no signs of disease; (1) limp tail or hindlimb weakness; (2) limp tail and hindlimb weakness; (3) partial hind limb paralysis in one but not both hind limbs; (4) complete hind limb paralysis. Mice were sacrificed at 18–21 days after induction (Score 3–4, peak disease) and tissue prepared as below.

2.4 Tissue processing
Mice were lethally injected with xylazine (Rompun) and ketamine (0.5 mg of xylazine, 5 mg of ketamine, i.p.) and transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA) before the brains were dissected out and cryopreserved with successive 10 and 30% sucrose sinks in PBS for 24 h each. Brains and spinal cords were mounted and frozen in Tissue Tek optimal cutting temperature compound in isopentane on dry ice. Frozen 14 µm sections were collected on Superfrost Plus slides and stored at -20°C.

2.5 Histological examination
Histological examination of spinal cord 14 µm sections was performed as previously described (Okuda et al., 1999). Sections from the cervical, thoracic and lumbar level of the spinal cord were stained with hematoxylin-eosin (HE) and evaluated by in a blind fashion using the following semi-quantitative score: 0, no inflammation; 1, cellular infiltrates only in the perivascular areas and meninges; 2, mild cellular infiltrates in parenchyma (1–10/section); 3,
moderate cellular infiltrates in parenchyma (11–100/section); 4, high cellular infiltrates in parenchyma (>100/section).

2.6 Immunofluorescence

Tissue was permeabilized in 0.1% Triton X-100 (Sigma) for 30 min, washed in PBS, blocked in 4% normal serum for 20 min, and incubated with primary antibodies (see below) in 2% normal serum at 4°C for 16 h. Primary antibodies: goat anti-SPARC (1:125; R&D Systems) (Vincent et al., 2008), rabbit anti-Iba-1 (1:1000; Wako), mouse anti-GFAP directly conjugated to Cy3 (1:1000 Sigma), rat anti-CD31 (PECAM-1) monoclonal antibody (1:500 BD Pharmingen), mouse anti-proliferating cell nucleic acid (PCNA; 1:5000; Sigma), and chicken anti-GFP (1:1000; Aves Labs). Detection used fluorescent secondary antibodies (Alexa Fluor 488 or 594; 1:200; Invitrogen) incubated for 1 h at room temperature, or by peroxidase chromogen reaction using biotinylated secondary antibodies (1:200), and Vectastain ABC kit and Vector VIP kits (Vector Laboratories). Nuclei were counterstained with 0.5 µg/ml 4’,6-diamidine-2-phenylindole dihydrochloride (DAPI; Boehringer Mannheim) and sections were mounted using Vectashield (Vector Laboratories) or ProLong Gold (Invitrogen). For negative controls, primary antibodies were omitted or nonspecific primary antibodies used. Antigen unmasking for PCNA immunofluorescence, was performed by heating sections for 10 min in 10 mM citrate buffer, pH 6.0, before the permeabilization step. If performing antigen unmasking, an anti-GFP antibody must be used to visualize endogenous GFP.
2.7 Image Capture

Images were captured using an Axioplan 2 Imaging epifluorescent microscope (Zeiss) with Zeiss Axiovision software or a Fluoview FV1000 laser scanning confocal microscope. Images were corrected for contrast and brightness using Photoshop CS3 software (Adobe Systems). For quantification, all images were captured with uniform settings, and no post processing was performed.

2.8 SPARC Dynamics in Astrocytes during EAE

The SPARC-expressing GFAP+ cells were identified in combination with DAPI staining in 40,000 µm2 regions of interest (ROI) located in contralateral gray and white matter from the lumbar level spinal cords from wild-type, early EAE, and peak EAE (n = 4 wild-type, n = 3 early and peak EAE). Every sixth slide (minimum of 10 sections) through the length of the lumbar spinal cord was tested to create an accurate sample, and stained by immunofluorescence for SPARC and astrocytes (GFAP). Individual SPARC-expressing GFAP+ cells and GFAP+ only cells (ie. not expressing SPARC) in each ROI were counted using the ImageJ Count feature. Statistical analysis was performed using an unpaired t test with Welch’s correction, which allows for the two groups possibly having unequal variances (accounting for measure of data spread).

2.9 Quantification of microglial morphology

Four serial coronal sections per mouse (n=3) through the brain and spinal cord were prepared for the following analysis. Six z-stacked fields of view at predetermined locations in the brain (cortex and corpus callosum representing the gray and white matter, respectively) and the spinal
cord (gray, white and boundary regions) sections were captured using a 63X objective on an Axioplan 2 Imaging epifluorescent microscope (Zeiss). Z-stacks were subjected to iterative deconvolution using the Zeiss Axiovision deconvolution module, and then flattened using a maximum intensity projection. Three microglia per defined region of brain and spinal cord per serial section were thresholded in ImageJ to create a binary image, with consistent settings used throughout. Process outgrowth was measured using the ImageJ Skeletonize and Histogram features and the pixel value converted to total micrometers. Multiple parameters were analyzed using the ImageJ Analyze Skeleton plug-in. Data were subjected to an unpaired Student’s t test for significance.

2.10 BBB Permeability and quantification

BBB permeability was quantified by Sodium Fluorescein Isothiocyanate (Na-FITC, Sigma; ~400 Da) extravasation using standard techniques (Phares et al., 2006, Saadoun et al., 2009). Briefly, SPARC-null (B6;129S-Sparctm1Hwe/J) and wild-type mice (B6129SF2/J) mice (n=2/group) were anesthetized by intraperitoneal injection with xylazine (Rompun) and ketamine (0.5 mg of xylazine, 5 mg of ketamine, i.p.) and a catheter was implanted into the right internal carotid artery. Injection of Na-FITC, 10 mg in 0.1 mL sterile phosphate buffered saline (PBS) pH 7.4, was perfused through the bilateral carotid arteries at a rate of 1 mL/min (10 mL/kg) by using a syringe pump. In order to avoid elevation of systemic blood pressure, the same amount of blood was withdrawn from the inferior vena cava. The artificial opening of the BBB was performed by similar injection of 20% mannitol into the internal carotid (Suzuki et al., 1985, Ruijter et al., 2003). The brain and spinal cord tissue was harvested and processed as described previously. Four serial coronal brain and spinal cord sections were stained for blood vessels using rat anti-
CD31 (PECAM-1) monoclonal antibody (1:500 BD Pharmingen). Four fields of view at predetermined locations in the brain were captured using a 40X objective on an Axioplan 2 Imaging epifluorescent microscope (Zeiss) and Na-FITC fluorescence mean, maximum and minimum intensity was quantified using ImageJ Measure and Analyze feature. For quantification, all images were captured with uniform settings, and no post processing was performed.
Chapter 3: Results

3.1 SPARC is expressed by radial glia and blood vessels during spinal cord development

Within the early postnatal CNS, endothelial cells collaborate with astrocytes to form the BBB, a specialized structure consisting of basal lamina and tight junctions that prevent the passive diffusion of blood-borne substances into the brain (Risau and Wolburg, 1990). SPARC is extensively expressed in radial glia and endothelia of the embryonic and postnatal mouse brain (Vincent et al., 2008), but little is known about its role in the developing spinal cord. SPARC has also been implicated in regulating basal lamina formation and tight junction expression (Gongidi et al., 2004, Tilling et al., 1998, Baumann et al., 2009, Liddelow et al., 2011, Liddelow et al., 2012). To test if SPARC may play a role in spinal cord development, we used immunofluorescence detection to establish the spatiotemporal expression of SPARC in the mouse spinal cord.

At postnatal day (P) 4/5, SPARC mRNA and protein is highly localized to spinal cord blood vessels (Figure 2A, B). Using immunofluorescence detection of SPARC in P5 spinal cord, most blood vessels co-express both SPARC and cluster of differentiation 31 (CD31), a common endothelial cell surface protein (Figure 2B) A small number of spinal cord CD31+ blood vessels do not co-express SPARC. At P5, Many SPARC-expressing spinal cord endothelia are proliferating (Figure 2E), as indicated by Proliferating Cell Nuclear Antigen (PCNA), a DNA polymerase cofactor expressed during cell division (Leonardi et al., 1992). SPARC+ spinal cord blood vessels also have a maturing BBB as indicated by expression and localization of zonula occludens (ZO)-1 (Figure 2D), a tight junction associated protein that regulates tight
junction assembly and barrier formation (Muller et al., 2005, McNeil et al., 2006).

Subsequently, SPARC is downregulated in adult spinal cord endothelia (Figure 2G). SPARC is also widely expressed in cell bodies, processes and pial endfeet of GFAP+ spinal cord radial glia (SCRG) (Figure 2C). SPARC’s endothelial expression is downregulated as the spinal cord matures and it is expressed by other CNS cells (Figure 2F-G, arrowheads).
Figure 2. SPARC is expressed by radial glia and proliferating endothelia during spinal cord development.

SPARC mRNA and protein is expressed by blood vessels (arrowheads) in (A) postnatal day 4 spinal cord (Allen Brain Atlas) and (B) postnatal day 5 lumbar spinal cord. (C) SPARC (green) is expressed by GFAP+ (red) radial glia, particularly in their processes (arrowheads). (D) SPARC+ (green) blood vessels (bv) co-express ZO-1 (red) (arrowheads). (E) SPARC (green) is highly enriched in mitotic (E) PCNA-expressing endothelia (bv). (F, G) SPARC (green) expression in CD31+ (red) blood vessels is significantly down-regulated in the adult spinal cord. Scale: (B-C) 50 µm (D-E, G) 20 µm (F) 100 µm. Ad: Adult, bv: blood vessel, P5: postnatal day 5, ISH: in situ hybridization.
3.2 SPARC is expressed by subpopulations of GFAP+ glia in the mature spinal cord

Astrocytes are the most abundant and heterogeneous glia in the adult spinal cord and precisely regulate a homeostatic CNS microenvironment (Abbott et al., 2006). BBB maintenance provided by the astrocyte-endothelial and extracellular matrix interactions in the microenvironment, is tightly regulated and necessary for proper barrier function (Engelhardt, 2003). All subtypes of astrocytes express glial fibrillary acidic protein (GFAP) – to different degrees - throughout the mature CNS (Jacque et al., 1978). In the mature spinal cord, SPARC is expressed most highly by GFAP-expressing gray matter protoplasmic astrocytes (Figure 3A, B, 4C) and perivascular astrocytes and their endfeet (Figure 3D, F), with distinct BBB punctuate localization around cross sectioned blood vessels (Figure 3E).

Adult spinal cord progenitors reside in the ependymal and subependymal layers of the central canal (CC) ventricular zone (Alonso, 1999, Horner et al., 2000, Fu et al., 2003). Adult spinal cord progenitors can express GFAP and demonstrate neural stem cell (NSC) characteristics in vivo and in vitro (Martens et al., 2002, Kulbatski et al., 2007a, Sabourin et al., 2009). Subsets of GFAP+ cells (putative progenitors) in the adult CC also express SPARC (Figure 3G). Furthermore, a recently identified adult SCRG framework exists throughout the white matter that may function as potential progenitors situated at the spinal cord-pial interface (Petit et al., 2011). SPARC is expressed in the cell bodies, radial processes, and endfeet of some of GFAP-expressing white matter adult SCRG (Figure 3A, C).
Figure 3. SPARC is expressed by subpopulations of GFAP+ glia in the adult spinal cord.

(A) SPARC (green) is expressed by different GFAP+ glial populations in the mature spinal cord, including (B) gray matter protoplasmic GFAP+ (red) astrocytes, (C) GFAP+ (red) spinal cord radial glia and along their processes (C, arrowhead), and (D) perivascular GFAP+ (red) astrocytes and their (E, F) endfeet (arrowhead). SPARC (green) is also expressed by (G) subependymal central canal GFAP+ (red) putative progenitors. Scale: (A) 200 µm (B, C) 100 µm (D-G) 20 µm. gm: gray matter, wm: white matter, bv: blood vessel, cc: central canal.
3.3 Developmental SPARC expression is recapitulated in the EAE spinal cord

Many CNS pathological conditions are accompanied by BBB dysfunction and loss of barrier permeability. Given SPARC’s distinct spatiotemporal distribution and enrichment in BBB and astrocytes during BBB formation, we decided to test if SPARC becomes redistributed during BBB breakdown and re-formation after CNS lesion. Experimental autoimmune encephalomyelitis (EAE), an animal model of CNS inflammation and demyelination, shows BBB dysfunction, loss of barrier permeability, and altered tight junction structure (Paterson, 1976, Juhler et al., 1984, Juhler et al., 1985, Wekerle et al., 1986, Hawkins et al., 1990, Hawkins et al., 1991, Namer et al., 1992). We therefore stimulated Myelin Oligodendrocyte Glycoprotein (MOG) \textsubscript{35-55} peptide-induced EAE in C57BL/6 female mice and disease progression follows a course with three distinct clinical phases: early, peak and recovery (Figure 4A). Immunofluorescence detection was used to analyze changes in SPARC's expression and localization during EAE progression through early and peak phases.

In response to CNS pathology, astrocytes undergo morphological changes, extend their processes, and proliferate extensively (Stichel and Muller, 1998). Reactive astrocytes play a key role CNS repair include the secretion of signaling molecules, ECM components and formation of a glial “scar” (Stichel and Muller, 1998, Faulkner et al., 2004, Jones et al., 2003a). During early EAE, SPARC is highly expressed by the majority of gray matter protoplasmic GFAP-expressing astrocytes (Figure 5A), white matter GFAP-expressing adult SCRG, particularly in radial processes and pial endfeet (Figure 5B), and by perivascular astrocytes, particularly in their endfeet (Figure 5C). During early EAE, SPARC is particularly enriched in mitotic PCNA-expressing gray matter protoplasmic astrocytes (Figure 4D) and white matter fibrous astrocytes
To test if there is an increase in the numbers of SPARC+ GFAP-expressing glia during EAE progression, we analyzed a series of regions of interest (ROI) (40,000 µm$^2$) in white (Figure 4F, G) and gray (Figure 4I, J) matter. We first counted the total number of GFAP+ glia within a given area (40,000 µm$^2$) of gray and white matter. Subsequently, we counted the numbers of these GFAP-expressing glia that also co-express SPARC (Figure 4I, J). During early phase of EAE, there is a two-fold and three-fold increase in the numbers of SPARC/GFAP-expressing glia in the gray and white matter, respectively (Figure 4H). In peak phase, however, although the numbers of GFAP-expressing glia remain relatively similar, they have mostly all down-regulated their expression of SPARC (Figure 4E, H).

The three-fold increase in the numbers of SPARC+ GFAP-expressing glia in the white matter during early EAE may be in part due to adult SCRG (Figure 4H). Adult SCRG respond to EAE injury by shifting their mitotic and morphological state: they become hypertrophied and proliferative (Petit et al., 2011). During early EAE, SPARC is expressed in cell bodies, radial processes, and pial endfeet of GFAP+ SCRG (Figure 5B).

Peak phase in EAE represents the highest clinical impairment in the mouse (Figure 4A) (Berard et al., 2010). Following peak phase, there is remission of the disease indicated by clinical improvements, suggesting the initiation of potential repair mechanisms during peak phase in EAE (Figure 4A) (Chen et al., 1998). During peak disease, SPARC is down-regulated in GFAP+ glia and expressed by CD31+ blood vessels of the spinal cord (Figure 5F, G). In particular, a high density of SPARC+ blood vessels are present in the dorsal funiculus (Figure
5G arrowhead), an area of significant BBB dysfunction and inflammatory immune cell infiltration into the CNS parenchyma (Kerschensteiner et al., 2004).
Figure 4. Quantification of SPARC-expressing glia during EAE progression in C57BL/6 mice.

(A) MOG\textsubscript{35-55} peptide-induced EAE in C57BL/6 mice follow a clinical course can be broadly divided into three phases: early, peak and recovery. (B) Sample locations of gray and white matter used for SPARC/GFAP expressing cell quantification in normal and early EAE spinal cord. Representative gray and white matter regions (40,000 µm\textsuperscript{2}) with GFAP+/SPARC+ cells. (C-D) SPARC (green) is enriched in mitotic PCNA-expressing (blue) GFAP+ (red) gray matter astrocytes during early EAE. (E) There is a significant increase in the number of GFAP-expressing glia as assayed in representative regions of (F) white and (G) gray matter during EAE disease progression. (H) During early EAE, there is a two-fold and three-fold increase in the
numbers of SPARC+ GFAP-expressing glia in the (I) white and (J) gray matter, respectively (* p<0.05, ** p<0.01, *** p<0.001) Scale: (C, D) 30 µm (F, G, I, and J) 50 µm GM: gray matter, WM: white matter, eEAE: early EAE, pEAE: peak EAE.
Figure 5. SPARC expression shifts from glia to vasculature during EAE progression in C57BL/6 mice.

During early phase, SPARC (green) expression is maintained by GFAP+ glia, including (A, arrowheads) gray matter protoplasmic astrocytes (red), (B) white matter spinal cord radial glia (red), particularly in their processes (B, arrowheads), and (C) perivascular astrocytes (red), particularly in their endfeet (C, arrowheads). During peak phase, SPARC (green) is downregulated by both (D, E) gray and white matter GFAP+ (red) glia and upregulated by (F,G) CD31+ blood vessels (bv). Scale: (A-C, and E) 100 µm (D, G) 150 µm (F) 100 µm. df: dorsal funiculus, gm: gray matter, wm: white matter.
3.4 SPARC is expressed by distinct subpopulations of microglia in the adult mouse central nervous system

Microglia, the resident immune cells of the central nervous system, adopted a mature, ramified morphology and begin to express SPARC following postnatal brain development (Vincent et al., 2008, Lloyd-Burton et al., 2013). Cortical microglia also secrete their SPARC when they become fully activated. In order to test if SPARC may act in a similar manner in microglia in the adult spinal cord, we used immunofluorescence detection in CX3CR1-GFP transgenic mice, where enhanced GFP (EGFP) driven by the Cx3cr1 promoter is expressed by resident microglia (Jung et al., 2000, Nimmerjahn et al., 2005). These mice were crossed with SPARC-null mice to produce offspring that were heterozygous for GFP-CX3CR1 and homozygous SPARC null. In the uninjured adult SPARC-null brain at low magnification, the distribution of GFP-CX3CR1-expressing microglia was altered compared to wildtype (Fig. 6A, B). Following quantification, it became clear that the density of microglial cell bodies is significantly higher in the cortical gray matter, whereas it is significantly lower in the white matter of the SPARC-null brain, compared with controls (Fig. 6C). Furthermore, when visualizing SPARC-null microglia with GFP in the GFPCX3CR1/SPARC-null mouse at high magnification, microglial morphology appeared to be significantly more complex and varied in the SPARC-null. To directly test this, we performed detailed morphological analysis of endogenous GFP distribution in CX3CR1-GFP microglia in the cortex of both SPARC-null and control mice (Fig. 6D–I). Z-stacks of individual microglia, sampled from equivalent sites in the gray and white matter, were deconvolved, flattened (Fig. 6D), skeletonized (Fig. 6E), and analyzed for total process outgrowth and branching (Fig. 6F–I). Microglial processes in both the SPARC-null gray matter and white matter are significantly longer compared with the wild-type reporter mice (Fig. 6J). In addition, microglial processes in
the SPARC-null white matter showed significantly increased branching relative to the wildtype (Fig. 6K).

Although SPARC expression by microglia appears to be universal throughout the brain, especially in the cortical parenchyma (Figure 7A), spinal cord microglia rarely express SPARC. In the normal adult spinal cord gray matter (Figure 7B) and white matter (Figure 7C) resting microglia do not express SPARC. However, a distinct subpopulation of SPARC-expressing spinal cord microglia can be found at the boundary of the gray and white matter (Figure 7E, F).

SPARC regulates mature microglial morphology in the brain with significant differences between the cortical gray and white matter (Lloyd-Burton et al., 2013). In order to test if the absence of SPARC in spinal cord microglia affects morphology, we applied the same analysis to gray matter, white matter, and boundary microglia in the mature spinal cord. Spinal cord gray and white matter microglia, both of which do not express SPARC, show a branching and process architecture similar to SPARC-null cortical gray and white matter microglia (Figure 7G) (Lloyd-Burton et al., 2013). SPARC-expressing boundary microglia, on the other hand, display a distinct morphology that is more similar to cortical gray matter microglia than their neighbors (Figure 7G) (Lloyd-Burton et al., 2013).

Following ischemic and excitotoxic lesions, reactive, hypertropic microglia release SPARC into the lesion (Lloyd-Burton et al., 2013). In order to test if SPARC is up-regulated and released by spinal cord microglia during EAE disease progression, we also used immunofluorescence detection of ionized calcium binding adaptor molecule 1 (Iba-1), a marker for
microglia/macrophages that becomes up-regulated as they respond to pathology (Figure 6D) (Imai et al., 1996). During EAE disease progression, spinal cord microglia, including the SPARC-expressing gray and white matter boundary microglia, do not express SPARC (Figure 7F-H). During peak EAE disease, an accumulation of Iba-1+ immune cells are focused around SPARC-expressing white matter blood vessels, particularly in the area of the dorsal funiculus (Figure 7K).
Figure 6. The absence of SPARC differentially regulates microglial expansion, process length, and branching in cortical gray and white matter in the SPARC-null cerebral cortex.

(A, B) GFP-expressing ramified microglia are evenly distributed, but Iba-1-positive cells (C) are more dense (n=3) in SPARC-nulls than control (+/+) (p<0.01) and significantly less dense in the white matter of the corpus callosum (p<0.05) compared with control mice. (D) Microglial
morphology was analyzed by visualizing 3D deconvolved images of GFP+ microglia in SPARC null/CX3CR1-GFP double transgenic mice and skeletonizing to analyze for process outgrowth and number of primary branch points (E, arrowhead). (F–I) SPARC-null microglia in the gray matter (G; GM) and white matter (I; WM) have increased process outgrowth (J; p<0.05, student’s t test) and, in white matter, have more primary branch points (K; p<0.01) than control microglia.
Figure 7. SPARC is expressed by distinct subpopulations of microglia in the adult mouse central nervous system.

In the normal adult brain, (A) cortical mature ramified CX3CR1-GFP+ microglia (green) express SPARC (red). In the normal adult spinal cord, mature ramified (B) gray and (C) white matter CX3CR1-GFP+ microglia (green) rarely express SPARC (green) except for a specific subset of (E, F) SPARC-expressing (red) CX3CR1-GFP+ microglia (green) (arrowhead) at the boundary between gray and white matter. CX3CR1-GFP+ (green) microglia co-express detectable levels of (D) Iba-1 (red). (G) SPARC-negative gray and white matter spinal cord microglia have increased process outgrowth and branching that is similar to cortical gray and white matter microglia in the SPARC-null cerebral cortex (Figure 6F-I). Boundary SPARC-expressing microglia are morphologically distinct from spinal cord gray and white matter microglia (** p<0.01, *** p<0.001, student’s t test). SPARC (red) was not detected in Iba-1+ (green) gray and white matter spinal cord microglia during (H, I) early and (J) peak phase. (H) Iba-1+ (red) microglia/macrophages can be seen closely associated with SPARC-expressing (green) white matter blood vessels (arrowheads) during peak phase in the dorsal funiculus (df). Scale: (A-D, and F) 20 µm (E) 50 µm (H-K) 50 µm. gm: gray matter, wm: white matter, bd: boundary, df: dorsal funiculus.
3.5 Absence of SPARC delays EAE onset but enhances BBB permeability

Mononuclear leukocyte infiltration into the CNS parenchyma occurs early and persists throughout the acute inflammatory phases, early and peak, during EAE progression and is crucial for the development of neurological symptoms (Charil and Filippi, 2007, Trapp and Nave, 2008, Stadelmann, 2011). In order to ascertain whether loss of SPARC alters immune infiltration and the potential for recovery during EAE early and peak phases, we established EAE disease course in SPARC-null mice and controls. We further used histopathological scoring to quantify changes in neuroimmune pathology by analyzing the numbers of infiltrates into the spinal cord parenchyma. The onset of clinical EAE symptoms in SPARC-null mice is delayed by 3-4 days and fail to reach "peak" scores (3 or above) when compared to wildtype controls (Figure 8A). Perivascular cellular infiltrates are typical signs during EAE disease pathology (Lossinsky et al., 1989, Lyman et al., 1989, Cross et al., 1990). SPARC-nulls display significant infiltrates in the meninges and parenchyma (Figure 9A, arrowheads) when compared to wildtype (Figure 9B, arrowheads). We performed hematoxylin-eosin (HE) staining on multiple spinal cords and employed a histological scoring system to count mean numbers of cellular infiltrates around blood vessels, as previously described (Okuda et al., 1999). In the absence of SPARC, there is a significant increase in the number of perivascular infiltrates that occurs early and persist into peak EAE, scores of (early) 3.00 ± 1.41 and (peak) 2.72 ± 1.16 compared to wildtype (early) 1.88 ± 0.64 and 2.11 ± 0.64 (Figure 9C). Na-FITC extravasation into the brain and spinal cord parenchyma was enhanced in the adult SPARC-null (Figure 8C, E) when compared to wildtype (Figure 8B, D). The normal state SPARC-null CNS displays a dysfunctional BBB, through P20 to adult, as quantified by significantly increased Na-FITC fluorescence intensity in CNS parenchyma (p<0.05) (Figure 8F).
Figure 8. Absence of SPARC delays EAE onset but enhances BBB permeability.

(A) SPARC-null animals show a delayed clinical onset of EAE compared to controls. Wiltype brain (B) and spinal cord (D) display less Na-FITC extravasation into the CNS compared to SPARC-null brain (C) and spinal cord (E). (F) BBB permeability is significantly (p<0.05) enhanced in SPARC-null brain and spinal cord compared to wildtype.
Figure 9. SPARC-null spinal cords display enhanced perivascular and meningeal infiltrates.

(A) Wildtype and (B) SPARC-null spinal cords stained with hematoxylin and eosin display the typical signs of inflammation: immune infiltrate localized throughout the parenchyma and the meninges (arrowheads). (C) Histological scoring of perivascular and meningeal infiltrates show that SPARC-null spinal cords have an overall higher histological scores, (early) 3.00 ± 1.41 and (peak) 2.72 ± 1.16 compared to wildtype (early) 1.88 ± 0.64 and (peak) 2.11 ± 0.64. Scale: (A, B) 150 µm. parenchyma and the meninges (arrowhead).
Chapter 4: Discussion

Using a series of in vivo immunofluorescence analyses to test if SPARC expression changes spatiotemporally in the spinal cord during early postnatal development, and following EAE, we show that (1) SPARC expression in spinal cord astrocytes and microglia is different from the brain; (2) SPARC accumulates in subsets of astrocytes as glial proliferation slows down, and the BBB forms; (3) SPARC spatiotemporal expression changes dramatically in the spinal cord in response to EAE and the (4) absence of SPARC alters how blood vessels and glial cells in the spinal cord respond to EAE.

In the developing brain, SPARC is first detected in early embryonic radial glia and blood vessels (Vincent et al., 2008). In the postnatal brain, its expression is maintained in radial glia as it becomes enriched in glial components of the blood-brain and blood-cerebrospinal fluid barriers (Vincent et al., 2008). During early CNS development, blood vessels are rapidly proliferating (angiogenesis) to form a neurovascular network large enough to support the rapidly expanding CNS (Santer et al., 2005, Johnson et al., 2005). SPARC is enriched in proliferating spinal cord blood vessels during angiogenesis (Figure 2). Angiogenesis is regulated by a number of exogenous factors like vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), matrix metalloproteinases (MMPs) (Santer et al., 2005), the activity of which can be modulated by SPARC. SPARC can be anti-angiogeneic in some cases (Chlenski et al., 2010) and can regulate activity of VEGF, FGF-2 and MMPs (Gigant et al., 1998, Toban et al., 1998, Siche et al., 1998, Bhoopathi et al., 2010). In the developing and early postnatal spinal cord,
SPARC is therefore probably not anti-angiogenic and correlates more highly with expansion of endothelia and establishment of a viable neuro-vascular network and the BBB.

Maturation of the blood-brain barrier (BBB) is the final stage of neurovascular development, and consists of the modification of endothelial tight junction (TJ) assembly and structure, which is regulated by TJ associated proteins (Risau and Wolburg, 1990, Wolburg and Lippoldt, 2002). Zonula occludens (ZO)-1, a TJ associated protein, anchors TJ proteins to the cytoskeleton and regulates signaling pathways that promote BBB formation (Charbonnier et al., 1998b, Decousus et al., 1998). Spinal cord blood vessels largely co-express SPARC and ZO-1 during early postnatal development of the BBB (Figure 2). At P5, SPARC is also expressed by spinal cord radial glia (SCRG) at the pial boundary of the developing spinal cord (Figure 2), and is particularly enriched in their radial processes and pial endfeet. As precursor cells migrate along radial glial scaffolds of the cortex, they induce the maturation of the BBB (Hambrock et al., 2004b). SPARC can also regulate cell adhesion and migration (Brekken and Sage, 2001, Murphy-Ullrich, 2001, Murphy-Ullrich et al., 1995, Bhoopathi et al., 2011) and may be promoting adhesion in SCRGs and perivascular astrocytes, and influencing precursor cell migration along radial glia - both of which, in turn, may impact BBB maturation. SPARC may thus promote BBB formation through its actions with TJ associated protein ZO-1 in vivo and in vitro [Jacquie Quandt, personal communication]. Interestingly, this developmental profile of endothelial SPARC expression appears to be recapitulated in spinal endothelia during different phases of EAE (Figure 5).
In the mature spinal cord, SPARC is primarily restricted to sub-populations of distinct glia (Figure 3, 7). Astrocytes are the most abundant and heterogeneous glial population in the adult spinal cord and, play a central role in combining with endothelial cells to regulate a homeostatic microenvironment for blood-brain barrier maintenance and function (Abbott et al., 2006). SPARC is expressed widely by protoplasmic gray matter astrocytes, which regulate and secrete factors to maintain the CNS microenvironment (Felsch et al., 2004), and is enriched in the end-feet of perivascular astrocytes, which form the BBB with endothelia and produce the ECM of the blood-brain barrier basal lamina (Risau and Wolburg, 1990, Felsch et al., 2004) (Figure 3). Thus, SPARC’s expression in astrocytes positions it to potentially regulate and maintain BBB homeostasis in the spinal cord, and contribute to modulating synaptic activity.

The main progenitors identified in the adult spinal cord reside in the ependymal and subependymal layers of the central canal (CC), and demonstrate neural stem cell (NSC) characteristics in vivo and in vitro (Martens et al., 2002, Sabourin et al., 2009, Kulbatski et al., 2007b). Adult CC progenitors reside alongside the blood-cerebrospinal barrier (BCSFB), a highly specialized structure that, much like the BBB, regulates the transport of substances across from and to the cerebrospinal fluid (CSF) (Mayer et al., 1991). The BCSFB is located at the choroid plexus, which also expresses enriched levels of SPARC (Liddelow et al., 2011, Liddelow et al., 2012), as do adult CC progenitors (Figure 3). A second precursor population in the adult spinal cord - radial glia (SCRG) - are located at the pial boundary, maintain a neural progenitor morphology and expression profile (Petit et al., 2011), and also have enriched levels of SPARC in their radial processes, and pial endfeet (Figure 3). SPARC’s enrichment in both of these progenitors highlights a potential role in modulating SCRG cell adhesion and precursor cell
migration along spinal radial glial scaffolds in the adult spinal cord (Figure 3). In addition, SPARC’s enrichment in the mature BBB and BCSFB positions it once again to participate in proper barrier function.

Microglia, the resident immune cells of the CNS, enter the CNS during embryonic development (Chan et al., 2007) and do not express SPARC during CNS migration but when they reach their final location and mature they adopt a ramified morphology, and become the primary SPARC-expressing cell throughout the brain (Lloyd-Burton et al., 2013). Microglia are heterogenous cells and display some regional phenotypic variation (Gilmour et al., 1998b), with little understanding of functional differences between them. Similarly, during spinal cord development, microglia do not express detectable levels of SPARC, but when they assume a mature ramified morphology, in contrast to the brain, spinal cord microglia still do not express SPARC. Spinal cord microglia that do not express SPARC exhibit similar morphology to SPARC-null cortical microglia (Lloyd-Burton et al., 2013). Using a CX3CR1-GFP mouse that illuminates all microglia throughout the CNS, we were able to locate a small subpopulation of SPARC-expressing spinal cord microglia with a distinctly different morphology that reside at the boundary between the spinal gray and white matter (Figure 7). SPARC modulates microglial structure throughout the CNS and SPARC-expressing microglia may represent a morphological and functional phenotypic variant that may respond differently to activation, within the spinal cord.

Pathological CNS conditions drastically change the CNS microenvironment and are often accompanied by BBB/BCSFB dysfunction (Coisne and Engelhardt, 2011, Wolburg and Paulus,
Leukocytes enter into the CNS parenchyma via regions of BBB breakdown, and facilitate inflammatory damage and demyelination (Kermode et al., 1990, Vercellino et al., 2008). EAE is a complex animal model of CNS inflammation and demyelination where cross-talk between immunopathological and neuropathological mechanisms regulate detrimental inflammation, demyelination, axonal loss and gliosis, and consequent reparative processes (Constantinescu et al., 2011). Interestingly, BBB disruption and the accumulation of inflammatory cellular infiltrates in EAE occurs preferentially in the spinal cord (Bennett et al., 2010).

Astrocytes respond during CNS injury or disease (Marchetti et al., 2013, Skripuletz et al., 2013), by becoming hypertrophic, proliferating and secreting growth factors and cytokines. These, in turn, act as guidance cues for inflammatory cells which interact with microglia, and can stimulate mechanisms for BBB repair (Coussons-Read et al., 1998). Reactive astrocytes modulate the inflammatory response during early phase in EAE, where there is significant inflammation and peripheral immune cell infiltration through a disrupted BBB (Hunzelmann et al., 2001). SPARC is enriched in reactive gray matter protoplasmic astrocytes, and perivascular astrocytes and their endfeet (Figure 5). There is a two-fold increase in the numbers of SPARC-expressing gray matter astrocytes during early phase of disease (Figure 4). This would position it to modulate signaling through VEGF, FGF-2 and MMPs (Krum and Rosenstein, 1998, Madiai et al., 2003, Gottschall and Yu, 1995, Gottschall et al., 1995, Muir et al., 2002) and ECM and alter the CNS microenvironment as EAE progresses (Sweetwyne et al., 2004, Jones et al., 2003b). The highest clinical impairment during EAE is evident during peak phase of the disease, which is followed by remission and clinical improvement (Berard et al., 2010). Recovery from peak disease is associated with growth factor and cytokine secretion from reactive astrocytes,
apoptosis of encephalitogenic T cells in the CNS, and a shift from a pro-inflammatory to a non-pathogenic anti-inflammatory state (Hara et al., 2011, Chen et al., 1998). This process is clearly initiated by neuro-immune signaling at peak disease.

In the white matter, adult SCRG respond to EAE by dividing, and becoming hypertrophied (Petit et al., 2011). SPARC is extensively expressed by GFAP-expressing SCRG, and there is a three-fold increase in the numbers of SPARC-expressing GFAP+ glia during the early phase of disease (Figure 4, 5). Reactive SCRG may thus be providing a biological framework to assist migratory precursors that are activated within the WM in response to disease (Petit et al., 2011, Kulbatski et al., 2007b). SPARC up-regulation in SCRG during early EAE suggests it may contribute to the migration of cells along radial processes, during the WM repair response.

Mice that are homozygous null for SPARC are viable and exhibit mild phenotypes, such as abnormal collagen fibril assembly, cataractogenesis, osteopenia, which are exacerbated following challenge or disease (Brekken and Sage, 2001, Brekken et al., 2003). In EAE, the clinical onset was delayed and the severity of disease was reduced in SPARC-null mice (Figure 8, 9). SPARC-null mice exhibit a significant delay in the development of EAE, due to defective follicular dendritic cell networking, improper lymphoid germinal center formation, and lack of Th17 cell differentiation (Piconese et al., 2011). However, here we observed a greater and earlier accumulation of cellular infiltrates which remain during the peak phase of EAE in SPARC-null spinal cords (Figure 9), which may be due to changes in peripheral activation, or BBB access. To test whether there is a dysfunctional BBB in the absence of SPARC, we performed BBB permeability assay and quantified Na-FITC extravasation into the CNS
parenchyma (Figure 8). Na-FITC extravasation was significantly enhanced in the SPARC-null CNS compared to wildtype (Figure 8). Therefore, SPARC’s role may be vital during BBB development and following EAE may embrace modulating neuro-immune activities both within and beyond the CNS.

Here, we establish that, in the spinal cord, SPARC is expressed and localized to developing endothelia and radial glia but is down-regulated as the spinal cord matures (Figure 10). SPARC expression is retained in specific subpopulations of glia - primarily astrocytes and distinct subpopulations of microglia - in the adult spinal cord. Furthermore, we show that during the repair response of EAE, CNS glia and endothelia recapitulate their developmental SPARC expression, and provide evidence that it may play a role in neuro-immune and endothelial cross-talk during the repair response following EAE.
**Figure 10. Summary of SPARC expression in CNS during development and EAE.**

Summary diagram illustrating SPARC’s spatiotemporal expression in brain and spinal cord from postnatal development into adulthood and during EAE disease progression.
Chapter 5: Future Directions

5.1 Inducible Flp-FRT SPARC-null transgenic mice

The FLP-FRT recombination system, similar to the Cre-lox, can be used to conditionally manipulate gene expression and is becoming more frequently used in mouse-based research. It involves using flippase (FLP) recombinase, derived from the yeast *Saccharomyces cerevisiae* (Sadowski, 1995). FLP recognizes a pair of FLP recombinase target (FRT) sequences that flank a genomic region of interest. A gene of interest with flanking FRT sequences can be activated, repressed, or exchanged for other genes under the control of FLP expression. The activity of the FLP enzyme can be controlled so that it is expressed in a particular cell type or organ and/or triggered by an external stimulus like a chemical signal. Using this technology, the loss of the *SPARC* gene can be studied in a given target cell type of interest (“spatial control”), such as astrocytes, endothelia, or microglia, and can also be studied over time, by using an inducible promoter to trigger the recombination activity at different time points (“temporal control”). This would allow us to determine if distinct glial or endothelial SPARC is responsible for specific functions at particular developmental or disease time points. For instance, we have shown that absence of SPARC delays EAE onset and enhances BBB permeability. However, we cannot be certain whether the altered EAE disease course and BBB parameters in SPARC-null mice is a consequence of the absence of CNS-specific SPARC or disrupted lymph node cytoarchitecture and improper T cell priming (Piconese *et al.*, 2011). Under an inducible FLP-FRT recombination system, we can organize CNS-specific SPARC knockout in different glia or endothelia to determine which components alter EAE disease course and BBB pathophysiology without significant external variables.
5.2  *In vitro* neural stem cell modeling of SPARC’s role at the blood-brain barrier

5.2.1  *In vitro* modeling of the blood-brain barrier

Modeling the BBB *in vitro* can facilitate a variety of studies that are not amenable to *in vivo* investigation. For example, *in vivo* experiments, such as those performed with knockout animals, are largely restricted to evaluating basic phenotype alterations, resulting in a limited understanding of underlying molecular and cellular mechanisms that may govern a physiological process or BBB dysfunction in a disease state. Also, while detailed drug delivery evaluation can only be performed *in vivo*, mining through large combinatorial libraries of small molecule or protein libraries is not compatible with *in vivo* approaches. Finally, *in vivo* investigation of the BBB is mostly performed in animals, with investigation of the human BBB being limited to non-invasive methods such as magnetic resonance imaging (MRI) techniques.

Because of the significant challenges presented by *in vivo* studies, *in vitro* models have been under development and utilized in many diverse scientific studies. One longstanding approach consists of isolating and culturing primary brain microvascular endothelial cells (BMECs). Given the complex intercellular interactions that defines the embryonic and adult neurovasculature, the removal of BMECs from their vital microenvironment and growth in culture leads to loss of BBB phenotype. To date, there has been very limited success in persuading embryonic BMECs to grow *ex vivo* (Mi et al., 2001). On the other hand, adult BMECs have been cultured successfully by many laboratories, but they rapidly lose their *in vivo* phenotype resulting in comparatively poor transendothelial electrical resistance (TEER), higher barrier permeability and decreased transporter expression compared to the same cells *in vivo* (Lyck et al., 2009, Roux and Couraud, 2005, Kniesel and Wolburg, 2000).
In order to improve in vitro BBB properties, various approaches to re-introduce aspects of the in vivo microenvironment have been reported. Astrocyte co-culture systems are the most widely used (Arthur et al., 1987, Dehouck et al., 1990). In this model, BMECs are cultivated with primary astrocytes isolated from newborn rodents. Addition of astrocytes can improve barrier function as measured by increases in TEER, decreases in BBB passive permeability, and enhanced of tight junction complexes as observed by increased tight junction protein levels and proper localization (Dehouck et al., 1990, Rubin et al., 1991, Tao-Cheng et al., 1987, Janzer and Raff, 1987).

Although the BBB properties of co-culture models have improved as a result of the synergistic combination of the various cell types of the neurovascular unit, these models still fail to fully recreate the in vivo BBB phenotype. In addition, implementation of such models is limited by two factors: workflow and scalability. Neurons (embryonic), astrocytes (postnatal), pericytes (adult), and BMECs (adult) are isolated from animals of various ages, resulting in a laborious process of many singular primary cell isolations, and yields from several of these isolations, particularly of BMECs, are quite low.

5.2.2 Stem cell sources for modeling the blood-brain barrier

Stem cells have significant advantages for BBB modeling because of the current challenges with multicellular complexity, scalability, and the inability to culture primary BMECs at different developmental time points, particularly early in embryonic development. Stem cells give rise to all cells in the human body throughout various stages of development and then often reside in
specific locations, or niches, during adulthood. Various populations of stem cells can be isolated during development and from adult tissues, and the properties they possess are dependent on the timing and location of the isolation. Neural stem cells (NSCs) reside in defined neurogenic niches in the CNS, such as the subventricular zone (SVZ) of the lateral ventricle and the spinal cord central canal ependymal zone (SCEZ) (Vincent et al., 2008, Kriegstein and Alvarez-Buylla, 2009, Alvarez-Buylla et al., 2008, Moore, 2006, Riquelme et al., 2008, Shen et al., 2008, Tavazoie et al., 2008). NSCs isolated from the embryonic CNS can differentiate into neurons, astrocytes, and oligodendrocytes (Caldwell et al., 2001, Temple, 1989).

5.2.3 SPARC and NSCs

As mentioned earlier, SPARC can regulate proliferation, migration and differentiation by modulating extracellular matrix (ECM) composition and the accessibility of growth factors (VEGF, bFGF, PDGF, TGF-β1) to their receptors (Brekken and Sage, 2001, Bradshaw et al., 1999, Bradshaw and Sage, 2001, Bradshaw, 2009, Delany et al., 2003, Nie and Sage, 2009, Schiemann et al., 2003, Francki et al., 1999, Francki et al., 2004, Raines et al., 1992, Kupprion et al., 1998, Sage et al., 2003, Motamed et al., 2003). Our lab has shown that SPARC is located in the rodent neurogenic niche in development and adulthood (Vincent et al., 2008). During early postnatal development, SPARC is highly enriched in radial glia in the SVZ and the RMS and its expression is maintained at low levels in the adult SVZ (Vincent et al., 2008). Furthermore, SPARC is expressed by developing radial glia (Figure 2) and adult subependymal putative progenitors (Figure 3) in the spinal cord. SPARC is poised to modulate the CNS niche microenvironment and signaling pathways to influence NSC activity. Investigating SPARC role in an in vitro NSC model of the BBB could lend insights into not only
how SPARC may regulate NSC activity but also how that activity may regulate highly vascularized neurogenic zones, such as the SVZ, and BBB dynamics.
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