Role of Slit2 in Reducing Complications after Surgical Brain Injury

Prativa Sherchan
Role of Slit2 in Reducing Complications after Surgical Brain Injury

by

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A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Physiology

March 2016
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<td>Description</td>
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<tr>
<td>SBI</td>
<td>Surgical Brain Injury</td>
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<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<td>Robo</td>
<td>Roundabout</td>
</tr>
<tr>
<td>srGAP1</td>
<td>Slit-Robo GTPase activating protein 1</td>
</tr>
<tr>
<td>BWC</td>
<td>Brain Water Content</td>
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<td>NS</td>
<td>Neurological Score</td>
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<td>LPO</td>
<td>Lipid Peroxidation</td>
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<td>MPO</td>
<td>Myeloperoxidase</td>
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<td>MDA</td>
<td>Malondialdehyde</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td>AQ4</td>
<td>Aquaporin-4</td>
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<td>COX-2</td>
<td>Cyclooxygenase-2</td>
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<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<td>ZO-1</td>
<td>Zona Occludens-1</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrixmetalloproteinase</td>
</tr>
<tr>
<td>HBO-PC</td>
<td>Hyperbaric oxygen preconditioning</td>
</tr>
<tr>
<td>LRRs</td>
<td>Leucine Rich Repeats</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>BTB</td>
<td>Blood Tumor Barrier</td>
</tr>
<tr>
<td>PMECs</td>
<td>Pulmonary Microvascular Endothelial Cells</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human Umbilical Vein Endothelial Cells</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>FAK</td>
<td>Focal Adhesion Kinase</td>
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<tr>
<td>N-WASP</td>
<td>N-Wiskott Aldrich Syndrome Proteins</td>
</tr>
<tr>
<td>Erk1/2</td>
<td>Extracellular Signal Regulated Kinase-1/2</td>
</tr>
<tr>
<td>CD45</td>
<td>Cluster of Differentiation 45 (CD45)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ICV</td>
<td>Intracerebroventricular Injection</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal Nuclei</td>
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<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
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<tr>
<td>PAK1</td>
<td>p21-Activated Protein Kinase</td>
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<tr>
<td>PBD</td>
<td>p21-Binding Domain</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>vWF</td>
<td>von Willibrand Factor</td>
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ABSTRACT OF THE DISSERTATION

Role of Slit2 in Reducing Complications After Surgical Brain Injury

by

Prativa Sherchan

Doctor of Philosophy, Graduate Program in Physiology
Loma Linda University, March 2016
Dr. John H. Zhang, Chairperson

Surgical brain injury (SBI) is the inadvertent injury to brain tissue at the perisurgical site which occurs due to neurosurgical maneuvers such as incision, retraction, and electrocoagulation that can result in post-operative complications. Blood brain barrier (BBB) disruption and neuroinflammation are major pathophysiological consequences after neurosurgical injury. Blood brain barrier dysfunction leads to increased influx of water and plasma proteins as well as peripheral immune cell infiltration into the brain parenchyma that further potentiates brain edema and worsens post-operative neurological function. Activated resident immune cells and infiltrated peripheral immune cells release inflammatory mediators and promote oxidative stress and cell death which contributes to neuroinflammation and progression of injury.

In this project we have identified Slit2, an extracellular matrix protein expressed endogenously in the brain, as a potential therapeutic target for reducing SBI-induced complications. Our central hypothesis was that Slit2 will increase after SBI as an endogenous protective mechanism, and recombinant Slit2 pretreatment will reduce neuroinflammation and stabilize the BBB via Robo receptor signaling pathway. The dual effects of Slit2— anti-migratory and anti-
permeability—were explored under three aims using the rat SBI model. The first aim evaluated the role of Slit2 in reducing neuroinflammation and BBB permeability after SBI. The second aim investigated the role of Slit2 and its receptor Robo1 in reducing neuroinflammation after SBI. Lastly, the third aim investigated the role of Slit2 and its receptor Robo4 in stabilizing the BBB after SBI.

Our findings showed that endogenous Slit2 increased in the perisurgical site in response to injury and had a protective function after SBI. Exogenous recombinant Slit2 pretreatment attenuated brain edema and neuroinflammation and thereby improved neurological function after SBI. Furthermore, we observed that recombinant Slit2 reduced neuroinflammation after SBI by inhibiting peripheral immune cell infiltration to the injury site possibly through Robo1-srGAP1 pathway mediated Cdc42 inactivation. Lastly, we elucidated that recombinant Slit2 reduced BBB permeability by stabilizing endothelial tight junction after experimental SBI possibly via Robo4-paxillin dependent Rac1 activation.

These observations suggest that Slit2 may be beneficial to reduce neurosurgical injury and improve post-operative outcomes in neurosurgical patients.
CHAPTER ONE

INTRODUCTION TO SURGICAL BRAIN INJURY

Adapted from

Surgical Brain Injury

Surgical brain injury (SBI) is the inadvertent injury to brain tissue at the perisurgical site which occurs due to neurosurgical maneuvers (Jadhav et al., 2007a, Huang et al., 2014). The unique nature of brain tissue poses considerable challenges for neurosurgery. Under the rigid and tough protection of the skull and meninges, the brain is extremely vulnerable to the mechanical insults produced by neurosurgical maneuvers, such as direct incisions, electrocauterization, and retraction. Healthy tissues at the margins of the operative target are inevitably subjected to injury during surgical procedures.

Post-operative complications are frequently encountered after intracranial surgeries, which increase patient morbidity and mortality (Bruder and Ravussin, 1999, Rolston et al., 2014). Major complications are encountered in 13-27% patients after intracranial surgeries (Bruder, 2002). New onset of neurological deficits or worsening of neurological function is reported to be the second most common adverse event encountered after intracranial surgeries with a reported occurrence of up to 20% (Wong et al., 2012). Blood brain barrier (BBB) disruption, hemorrhage and neuroinflammation are major pathophysiological consequences of SBI that can result in post-operative complications such as brain edema and neurological deficits following neurosurgeries (Bruder, 2002, Yamaguchi et al., 2007, Hyong et al., 2008)
Pathophysiology of Surgical Brain Injury

Brain Edema

Post-operative edema has been reported to occur in 2 to 10% cases following neurosurgical procedures in a literature review series that examined the frequency of occurrence of adverse events after cranial tumor resection (Wong et al., 2012). The blood brain barrier comprising the vascular endothelium, pericyte, and astrocytic processes, prevents the leakage of plasma proteins from the vascular bed into the brain tissue (Nag et al., 2009). Vasogenic edema results from the passage of water along with plasma proteins into the brain tissue because of damage to the capillary endothelium and the interendothelial tight junctions of the BBB, whereas cellular swelling of the injured brain cells results in cytotoxic edema (Nag et al., 2011). Both result in increased intracranial pressure, which may lead to further brain injury from cell death or hypoperfusion (Bruder and Ravussin, 1999).

Neuroinflammation

Neuroinflammation plays an important role in the progression of brain edema after neurosurgical injury (Hyong et al., 2008). Previous studies have shown evidence of neuroinflammation characterized by increase in proinflammatory cytokines, proinflammatory enzymes, activation of resident immune cells and infiltration of peripheral leukocytes at the perisurgical site (Hyong et al., 2008, Ayer et al., 2012, Huang et al., 2015). Activated resident immune cells and infiltrated peripheral immune cells release inflammatory mediators and promote oxidative stress and cell death which contributes to
Recent studies show that targeting specific inflammatory insult reduced
perisurgical brain edema and improved outcomes after experimental SBI (Huang
et al., 2015, Sherchan et al., 2015).

**Surgical Brain Injury Rat Model**

Currently, SBI is clinically addressed by nonspecific postoperative care; however, it has become possible to study potential therapies in the preclinical
laboratory setting using the rodent model for SBI. The SBI rat model was
designed to mimic injuries sustained from neurosurgical manipulation of brain
tissue. The rodent brain is exposed through a small cranial window in the right
frontal bone through which partial frontal lobe resection is performed. The
margins of the resection are designated in relation to the bregma, as shown in
Fig. 1.
**Figure 1.** Partial right frontal lobe resection. Two incisions (*dashed lines*) are made leading away from the bregma (*white X*) along the sagittal and coronal planes, 2 mm lateral to sagittal and 1 mm rostral to coronal respectively.
The SBI model provides consistently measureable brain edema using the brain water content in the perisurgical site. Studies have shown that brain edema in the perisurgical site peaked 24 h after inducing SBI, was significantly higher up to day 3 post-injury, and subsided by day 7; SBI was associated with neurobehavior deficits that dissipated by day 7 (Matchett et al., 2006, Jadhav et al., 2007b, Yamaguchi et al., 2007). The SBI rat model allows opportunity to better understand SBI pathophysiology and to test specific SBI targeted therapeutic strategies for possible clinical use in neurosurgical patients. The experimental therapeutics that have been tested in the SBI rodent model is outlined in Table 1 and has been further discussed in this chapter.
Table 1. Experimental rodent studies of therapeutic agents for surgical brain injury.

<table>
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<tr>
<th>References</th>
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<th>Treatment outcomes</th>
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<tr>
<td>Matchett et al., J Neurosci.Methods, 2006</td>
<td>Erythropoietin pretreatment</td>
<td>SBI: ↑brain water content (BWC)</td>
<td>Treatment (Tx): harmful, ↑↑BWC</td>
</tr>
<tr>
<td>Lo et al., Neurosci Lett, 2007</td>
<td>NADPH oxidase KO or apocynin pretreatment</td>
<td>SBI: ↑BWC, ↓neurological scores (NS)</td>
<td>KO: ↑NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tx: no effect</td>
</tr>
<tr>
<td>Jadhav et al., J Neurosurg, 2007</td>
<td>PP1 pretreatment</td>
<td>SBI: ↑VEGF, ↑p-ERK1/2, ↓ZO-1, ↑BWC</td>
<td>Tx: ↓VEGF, ↓p-ERK1/2, ↑ZO-1, ↓BWC</td>
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<tr>
<td>Yamaguchi et al., Neurosurgery, 2007</td>
<td>MMP inhibitor-1 pretreatment</td>
<td>SBI: ↑BWC, ↓NS</td>
<td>Tx: ↓BWC</td>
</tr>
<tr>
<td>Lee et al., Acta Neurochir Suppl, 2008</td>
<td>Simvastatin Pretreatment</td>
<td>SBI: ↑BWC, ↓NS</td>
<td>Tx: no effect</td>
</tr>
<tr>
<td>Lee et al., Acta Neurochir Suppl, 2008</td>
<td>Melatonin pretreatment</td>
<td>SBI: ↑BWC, ↓NS, ↑lipid peroxidation (LPO)</td>
<td>Tx (low dose): ↓BWC, ↑NS, ↓LPO</td>
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<td></td>
<td></td>
<td></td>
<td>Tx (high dose): ↑↑BWC, ↓↓NS, ↑↑LPO</td>
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<tr>
<td>Bravo et al., Brain Res, 2008</td>
<td>L-histidine and thioperaamide post-treatment</td>
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<td>Hyong et al., Brain Res, 2008</td>
<td>Rosiglitazone pretreatment</td>
<td>SBI: ↑BWC, ↓NS, ↑myeloperoxidase (MPO), ↑TNFα, ↑IL-1β</td>
<td>Tx: ↓MPO, ↓TNFα, ↓IL-1β</td>
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<tr>
<td>Di et al., Neurosci Lett, 2008 Hao et al., Brain Res 2009</td>
<td>Aminoguanidine post-treatment</td>
<td>SBI: ↑BWC, ↓NS, ↑TNFα, ↑NF-κβ, ↑malondialdehyde (MDA), ↑glutathione (GSH), ↑aquaporin-4 (AQ-4)</td>
<td>Dx: ↓BWC, ↓MDA, ↑GSH, ↓AQ-4</td>
</tr>
<tr>
<td>JadHAV et al., Stoke, 2009</td>
<td>Hyperbaric oxygen preconditioning</td>
<td>SBI: ↑BWC, ↓NS, ↑cyclooxygenase-2 (COX-2), ↑hypoxia inducible factor-1α (HIF-1α)</td>
<td>Tx: ↓BWC, ↑NS, ↓COX-2, ↓HIF-1α</td>
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Experimental Therapeutics for Surgical Brain Injury

Vascular Endothelial Growth Factors

Vascular endothelial growth factors (VEGFs) are a large family of proteins designated VEGF-A through VEGF-E and are expressed in the choroid plexus and neurons in the normal brain (Merrill and Oldfield, 2005). A well-known growth factor with angiogenic, mitogenic, and permeability-inducing effects, VEGF has also been shown to contribute to SBI-induced brain edema. VEGF-A has a potent hyperpermeability inducing effect on the microvascular endothelium that is mediated through its receptor VEGF receptor-2 (VEGF-2, kdr) (Merrill and Oldfield, 2005). VEGF-2, a transmembrane tyrosine kinase present in the endothelium of the brain vessels, activates MAPK signaling (Kusaka et al., 2004). An upregulation in VEGF has also been reported in models of TBI predominantly due to the infiltrating neutrophils (Chodobski et al., 2003). VEGF administration and overexpression with viral vectors have both been associated with compromised integrity of the BBB in the rodent brain (Proescholdt et al., 1999). A potential mechanism by which VEGF-A might increase BBB permeability involves downregulating the expression of occludin, a tight junction protein; this would disrupt the organization of occludin and ZO-1, another junctional protein, leading to tight junction disassembly (Petty and Lo, 2002). Although VEGF may have potentially reparative actions during later phases after an injury, early inhibition of VEGF or its upstream mediator Src tyrosine kinase have been shown to reduce brain edema in various stroke models (Kusaka et al., 2004, Jadhav et al., 2007a). The expression of VEGF was increased at 24 h after SBI in the ipsilateral frontal region surrounding the surgical resection site (Jadhav et al., 2007b). The Src
family is implicated in VEGF-dependent hyperpermeability and has been shown to be involved in SBI-induced BBB disruption and subsequent brain edema in rodents (Jadhav et al., 2007b). An increase in expression of VEGF and p-ERK 1/2 as well as a corresponding decrease in the tight junction protein ZO-1 were reversed when rats were pretreated with PP1, an Src tyrosine kinase inhibitor, prior to inducing SBI (Jadhav et al., 2007b). In addition, VEGF was shown to play a possible role in the erythropoietin-induced increase in brain edema during the early phase in a SBI rodent model. Erythropoietin administration led to significantly increased brain water content in the perisurgical region at 24 h and was associated with increased expression of VEGF (Matchett et al., 2006). Thus, VEGF-A and its upstream Src tyrosine kinase present potential therapeutic targets for preserving the BBB and reducing brain edema following neurosurgical procedures.

VEGF-B, another member of the VEGF family expressed in the CNS, mediates its effects through the VEGF receptor-1 (VEGF-1, flt-1); VEGF-B is likely responsible for maintaining the BBB in a steady state. Furthermore, when bound to the soluble extracellular portion of VEGFR-1, VEGF is inactive, sequestered, and unable to bind to VEGF receptors. The role of VEGF-B and its receptors in brain edema development following SBI remains to be elucidated (Merrill and Oldfield, 2005).

**Matrix Metalloproteinases**

Matrix metalloproteinases (MMPs), zinc-dependent endopeptidases
involved in tissue remodeling and repair, have been implicated in the SBI-induced destruction of the extracellular matrix proteins of the neurovascular unit. The target substrates of MMPs include collagen IV, fibronectin, and laminin, all of which are critical to maintaining the integrity of the BBB. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) degrade the basal lamina and tight junction proteins of the BBB and promote BBB injury that leads to vasogenic edema during the acute stage in experimental models of brain injury and stroke (Petty and Lo, 2002, Donkin and Vink, 2010). Upregulation of MMPs has been demonstrated following subarachnoid hemorrhage, cerebral ischemia, traumatic brain injury (TBI), and intracerebral hemorrhage, and has been shown to contribute to BBB disruption during the early stage of injury (Sehba et al., 2004, Rosenberg and Yang, 2007, Ding et al., 2009, Wu et al., 2010). Additionally, MMP-9 knockout mice had improved functional outcomes, lowered BBB permeability, and reduced lesion volume after transient focal cerebral ischemia and TBI (Wang et al., 2000, Asahi et al., 2001). Similarly, the role of MMPs in mediating BBB disruption and brain edema after SBI was demonstrated by Yamaguchi and colleagues. An upregulation of MMP-2 and MMP-9 was temporally associated with BBB disruption after SBI in rats. A significant increase in MMP activity, particularly that of MMP-9, compared with pre-surgery levels was detected at days 1–14 after SBI, with highest MMP activities observed at days 1 and 3 coinciding with peak values in brain edema; MMP inhibitor, reversed these effects, preserving the BBB integrity and reducing SBI-induced brain edema as early as 3 h post-injury (Yamaguchi et al., 2007). Evidence of the role of MMPs in the development of
brain edema in the early stages after stroke and in SBI models supports the potential use of MMP inhibition to prevent brain edema following neurosurgical procedures.

**Cyclooxygenase-2**

Various inflammatory mediators have been implicated in BBB disruption and brain edema after stroke and brain injury. The role of cyclooxygenases, enzymes that catalyze the conversion of arachidonic acid to prostaglandins and thromboxanes, in mediating brain edema development has been extensively studied. The upregulation of cyclooxygenase-2 (COX-2), expressed in various cell types including neurons, astrocytes, endothelial cells, macrophages, and microglia in the CNS, has been demonstrated following focal and global cerebral ischemia, neonatal hypoxia–ischemia, and intracerebral hemorrhage (Chu et al., 2004, Donkin and Vink, 2010, Fathali et al., 2010, Cheng et al., 2011). Selective inhibition of COX-2 provided protection after ICH by reducing prostaglandin E2 production, thereby decreasing inflammation, brain edema, and cell death, which translated into improved functional recovery (Chu et al., 2004). Further, the role of COX-2 in mediating ischemic preconditioning-induced protection has been suggested. COX-2 has been shown to mediate ischemic preconditioning in vitro (Kim et al., 2007). Studies from our laboratory have determined COX-2 to be a potential mediator of hyperbaric oxygen preconditioning (HBO-PC) in SBI and global ischemia rodent models; animals preconditioned for 1 h daily for 5 days with HBO prior to inducing injury had significantly improved neurological function.
and brain edema (Jadhav et al., 2009, Cheng et al., 2011). Cyclooxygenase-2 has been shown to play a part in SBI pathophysiology as well. An increase in COX-2 expression was detected 24 h after SBI in mice, and HBO-PC significantly attenuated the increase in COX-2 possibly through suppression of HIF-1, the upstream regulator of COX-2 (Ostrowski et al., 2005). The study showed that HBO-PC increased COX-2 level two-fold in comparison to the fourfold increase by the SBI, which suggested that protection conferred by HBO-PC might have involved brain preconditioning by increasing COX-2 to subinjurious levels. Furthermore, the protective effects of HBO-PC were reversed in the presence of selective COX-2 inhibitor. These studies demonstrate HBO-PC or COX-2 inhibition to be promising therapies in attenuating brain edema following neurosurgical procedures.

**Conclusion**

The SBI rodent model mimics injuries caused by neurosurgical procedures and produces post-operative brain edema. It allows for the study of the cellular signaling pathways and the identification of key molecular targets for neuroprotective pretreatment before neurosurgical intervention. The various edema promoting factors including VEGF, MMPs, and COXs have been revealed to be potential targets for therapy. Similarly, recent studies have explored specific neuroinflammatory therapies to target SBI induced complications.

As clinically applicable therapeutic intervention for SBI is likely to result in significant benefits for patients and healthcare organizations, further preclinical
and clinical studies are necessary to explore the applicability of these targets.
References


CHAPTER TWO
ROLE OF SLIT2 IN CNS PATHOLOGY

Introduction to Slit2-Robo

Slits are evolutionarily conserved extracellular matrix proteins originally identified in the nervous system (Ballard and Hinck, 2012, Yuen and Robinson, 2013). Slit proteins are key regulators in guiding the migration of axons and neuronal precursors during development by preventing abnormal midline crossings (Marillat et al., 2002). The mammalian Slit protein contains an N-terminal signal peptide, four leucine-rich repeats (LRRs), nine epidermal growth factor repeats (EGF), a laminin G domain, and a cysteine rich C terminus (Wong et al., 2002). There are three known isoforms of Slit (Slit1-Slit3) in the vertebrates (Wong et al., 2002, Chaturvedi and Robinson, 2015). Slit1 is predominantly expressed in the developing nervous system but Slit2 and Slit3 are also expressed outside the nervous system including the kidney, lung, heart and immune cells (Wu et al., 2001, Prasad et al., 2007, Chaturvedi and Robinson, 2015). Among the Slit proteins, Slit2 has been most widely studied during pathological states in the nervous system. Slit2 is expressed by neurons in the normal adult brain (Marillat et al., 2002, Guan and Rao, 2003). However, in response to injury Slit2 is expressed by glial cells in addition to the neurons (Hagino et al., 2003), which implicates distinct function of Slit2 in the adult brain after an injury.

Slit2 exerts its function by binding to its receptor Roundabout (Robo) (Guan and Rao, 2003). The Robo family of transmembrane receptors (Robo1-
Robo4) mediates the intracellular signal transduction pathways for the Slit ligands. Robo is a single-pass transmembrane protein which belongs to the immunoglobulin superfamily (Ballard and Hinck, 2012). The extracellular region of Robo contains five immunoglobulin (Ig) domains and three fibronectin type III repeats, a transmembrane domain and four conserved cytoplasmic motifs (CC0-CC3) (Guan and Rao, 2003). The LRR domain of Slit2 directly binds with the Ig domain of Robo to transduce downstream signaling pathways (Ballard and Hinck, 2012). Among the Robo family, the Robo1 receptor is expressed by neurons in the normal brain while glial cells are either negative or faintly positive for Robo1 expression (Mertsch et al., 2008) whereas, Robo4 is expressed by the brain microvascular endothelial cells (Cai et al., 2015). In addition, Robo1 is also highly expressed by non-neuronal cells such as the peripheral immune cells (Wu et al., 2001, Prasad et al., 2007)

**Role of Slit2 During CNS Development**

Multiple factors are involved in guiding the axons projecting from diverse neuronal populations in the brain towards the appropriate direction (Zhang et al., 2012). Among such factors, Slit2-Robo signaling has emerged as a conserved system that guides the path of axons in the developing nervous system across species. Slit proteins in the brain are crucial during development of the forebrain axons since it maintains the proper position, extension and crossing of axons and axonal tracts in the forebrain (Nguyen-Ba-Charvet and Chedotal, 2002). During development the forebrain exhibits complementary pattern of expression of the Slit proteins (1 and 2) and Robo (1 and 2).
projecting axons avoid regions expressing high levels of Slit2 mRNA during development of the axonal tract projections in embryonic mice (Nguyen-Ba-Charvet and Chedotal, 2002). Additionally, Slit mutant mice exhibited abnormal patterns and positioning of axonal tract projections during development (Nguyen-Ba-Charvet and Chedotal, 2002). There is some evidence that in addition to Slit2, the other Slits also aid in the guidance of the developing axons. Slit2 antisense morpholino oligonucleotides that targeted translation of Slit2 mRNA reduced the formation of axonal projections, and simultaneous inhibition of both Slit2 and Slit3 function further reduced the projecting axons (Zhang et al., 2012). Additionally, Robo2 knockdown led to severe loss of the forebrain descending tract fibers (Alajez et al., 2011). Likewise, studies using Slit or Robo knockout mice showed that axons abnormally approached and crossed the midline during development in different regions of the brain (Bagri et al., 2002, Andrews et al., 2006, Lopez-Bendito et al., 2007, Zhang et al., 2012).

Although initially identified as an axonal repellent critical for development of the nervous system, the function of Slit2-Robo has been expanded to regulate disease processes in the CNS, which is discussed further in this chapter.

**Role of Slit2 in CNS Disorders**

**Glioma**

Malignant glioma is the most common primary brain tumor in adults and is associated with high mortality and poor prognosis. (Gu et al., 2015). The invasive nature of glioma increases the risk of its recurrence despite surgical removal and
is the most common indicator for poor prognosis (Gu et al., 2015). The dysregulation of cell motility has been postulated to be involved in promoting glioma cell infiltration which might promote the invasive nature of the tumor (Yiin et al., 2009, Gu et al., 2015).

While the role of Slit-Robo system in axon and neuronal migration is well established, its role in CNS tumors has remained relatively unexplored. Slit2 is normally expressed by neurons and astrocytes in the normal brain and is reported to have a tumor suppressive function (Mertsch et al., 2008, Xu et al., 2010). In human glioma cells Slit2 was expressed at very low levels (Mertsch et al., 2008, Yiin et al., 2009) and more so with malignant progression (Mertsch et al., 2008). However, the percent of tumors that expressed Robo1 increased as the invasiveness of the tumor progressed (Mertsch et al., 2008). Mertsch et al. suggested that hypermethylation of the SLIT2 promoter associated with increasing grade of glioma may be responsible for the lower expression of Slit2 in high grades tumors similar to what has reported in peripheral tumors outside the CNS including colorectal, lung, renal, breast and cervical cancers (Mertsch et al., 2008). There is some supporting evidence that methylation of SLIT2 gene regulates its expression in glioma cells. While normal brain samples lacked any methylation at the SLIT2 promoter region, the CpG island on the SLIT2 promoter was frequently hypermethylated in tumor samples (59%) and in glioma cell lines (71%) with a corresponding loss in the expression of Slit2, which was restored after treatment with demethylating agent 5-aza-2'-deoxycytidine (Dallol et al., 2003). This was associated with reduced expression of Slit2 depending on the
degree of methylation of the promoter. Furthermore, treatment with
demethylating agent restored SLIT2 gene expression in the methylated glioma
cell lines but did not change the expression of SLIT2 in unmethylated glioma cell
lines indicating that the hypermethylation of SLIT2 promoter resulted in reduced
expression of Slit2 in the tumors (Dallol et al., 2003)

Even though the Slit-Robo system has been shown to be important
regulators in the guiding the motility of various cell types, fewer studies have
examined the involvement of Slit2 and Robo in tumor cell migration in the CNS.
Yinn et al. observed that glioma cells treated with recombinant Slit2 or stably
transfected with Slit2 gene exhibited reduced capacity to migrate and invade
compared to controls in vitro (Yiin et al., 2009). In addition, when glioma cells
transfected with Slit2 gene were implanted intracranially, the capacity of the
tumor cells to infiltrate and invade into the brain parenchyma was impaired in
vivo (Yiin et al., 2009). Furthermore, the anti-migratory effect of Slit2 on tumor
cells was dependent on Robo1. When glioma cells were transfected with Robo1
siRNA, the anti-migratory effects of recombinant Slit2 was reversed suggesting
that Slit2 inhibited glioma cell migration in a Robo1 dependent manner (Yiin et
al., 2009). Similar findings were observed by Mertsch et al. in which Slit2 reduced
migration of glioma cell lines that was neutralized by RNAi mediated knockdown
of Robo1 in vitro (Mertsch et al., 2008). These observations suggest that Slit2-
Robo1 has a critical role in glioma cell migration which may be important in
understanding the invasive nature of gliomas. Since glioma cell invasion into the
brain parenchyma is a major contributor to tumor recurrence, a better
understanding of the cues involved in glioma migration and invasion is essential for developing effective treatment strategies against malignant gliomas.

**Traumatic and Surgical Brain Injury**

The role of Slit2 after brain injury has been studied to an even lesser extent. In a cryo-injured traumatic brain injury mouse model Slit2 was found to be highly expressed by the neurons and astrocytes surrounding the lesion (Hagino et al., 2003). Slit2 was the predominant member of the Slit family proteins expressed in surrounding necrotic tissue following cryo-injury, while Slit1 and Slit3 showed much weaker expression (Hagino et al., 2003). Slit2 mRNA was elevated during the early phase of the injury by day 2 and reached a peak at day 7 followed by a decline in expression on day 14 post-injury. The proximal reactive astrocytes surrounding the lesion expressed Slit2 but not the distal astrocytes, microglia or oligodendrocytes (Hagino et al., 2003). Furthermore, Slit2 co-localized with the heparan sulfate proteoglycan, glypican-1, indicating a possible involvement of Slit2-glypican-1 interaction in the recovery after traumatic brain injury. The heparin sulfate chain of glypican-1 binds with high affinity to Slit2 and the authors postulated that such a binding may increase the interaction of Slit2 with Robo during recovery after injury. Likewise, we recently published that Slit2 was increased at the perisurgical site following neurosurgical brain injury in a rat model, and Slit2 was expressed by both neurons and astrocytes (Sherchan et al., 2015). We speculate that Slit2 likely has a protective function after brain injury, since knockdown of endogenous Slit2 using siRNA worsened brain edema after SBI. Additionally, recombinant Slit2 pretreatment improved outcomes after
surgical brain injury by reducing brain edema and neuroinflammation at the perisurgical site (Sherchan et al., 2015).

**Cerebral Ischemia**

The inhibitory effect of Slit2 on peripheral leukocyte migration has been a recent addition to the spectrum of the anti-migratory effects of Slit2 on various cell types (Wong et al., 2002). Slit2 has been shown to inhibit the migration of various cell types including neurons, pericytes, endothelial cells, and peripheral leukocytes (Wong et al., 2001, Wong et al., 2002). Altay et al. explored the role of Slit in reducing cerebral leukocyte migration in rodent models (Altay et al., 2007). Exogenous Slit administration decreased leukocyte adherence by 98% in the cerebral microvessels 4 hours following TNFα exposure (Altay et al., 2007). Similar effects were observed after experimental global cerebral ischemia in which 79% reduction in leukocyte adherence was observed (Altay et al., 2007). Administration of RoboN, the soluble receptor for Slit reversed the inhibitory effect of Slit on leukocyte migration to the cerebral microvessels after global ischemia. Additionally, neutralizing endogenous Slit exacerbated TNFα induced cerebral microvessel leukocyte recruitment suggesting an anti-migratory role of both the endogenous and exogenous Slit following cerebrovascular inflammation (Altay et al., 2007).

**Intracerebral Hemorrhage**

A few studies have explored CNS pathology using Slit2 transgenic
animals. These studies provide evidence that overexpression of Slit2 can be detrimental. Slit2 overexpressing transgenic mice had increased brain microvessel density compared to wild type mice and were more susceptible to collagenase induced ICH (Han and Geng, 2011). The transgenic mice exhibited larger hemorrhagic areas and increased intracerebral hemorrhage volume compared to control mice indicating that the vessels in Slit2 transgenic mice were fragile and susceptible to greater injury given the same degree of insult (Han and Geng, 2011). There was increased brain microvessel permeability in Slit2 transgenic mice demonstrated by Evans blue dye extravasation and Amyloid-β 40 peptides leakage from serum into the brains of Slit2 transgenic mice but not in the control wild type mice (Han and Geng, 2011). Additionally, Slit2 overexpressing transgenic mice had enlarged lateral ventricles with higher ventricular pressure compared to control mice which was associated with abnormal morphology of the choroid plexus characterized by increase in microvessels and enlarged interepithelial and endothelial junctions (Han and Geng, 2011). Likewise, transgenic mice overexpressing human Slit2 exhibited increased BBB permeability and Alzheimer's disease (AD)-like alterations characterized by behavioral changes, hippocampal neuron apoptosis, and amyloid-β (Aβ) protein deposition (Li et al., 2015a).

**Epilepsy**

In a previous study, Fang et al. characterized the role of Slit2 in synaptic plasticity after temporal lobe epilepsy. The study evaluated the expression of Slit2 after temporal lobe epilepsy using temporal neocortex samples from
patients with intractable temporal lobe epilepsy and in experimental animals (Fang et al., 2010). While Slit2 was mainly expressed by neurons in normal human controls, samples taken from patients with intractable temporal lobe epilepsy showed that Slit2 was increased and expressed by both neurons and astrocytes (Fang et al., 2010). Similar findings were observed in the experimental rat model with increased Slit2 expression compared to control rats. Likewise, Slit2 was expressed by neurons in the controls and during the acute and latent phase of epilepsy in a rat model. However, during the chronic phase (30 and 60 days) Slit2 was mainly expressed by astrocytes (Fang et al., 2010). The study by Fang et al. suggests that Slit2 could be an important guidance molecule to direct axonal sprouting towards the correct target during remodeling phase after epilepsy (Fang et al., 2010). Additionally, the authors speculated that decreased expression of Slit2 during acute phase after epilepsy induction with increasing expression in the astrocytes during chronic phase suggests that Slit2 may be involved in astrogliosis after epilepsy. Further, investigation into the role of Slit2 after epilepsy is necessary to delineate its function after epilepsy.

**Role of Slit2 Dependent on Cell Types**

**Endothelial Cells**

Previous study examining the role of Slit2 on the endothelial cells in CNS implicates an endothelial protective function of Slit2. The Robo4 receptor is the endothelial specific receptor for Slit2 that is expressed by human brain microvascular endothelial cells. Previous study used an in vitro model of glioma blood-tumor barrier (BTB) to explore the role of Slit2-Robo4 in maintaining BTB
stability (Cai et al., 2015). The expression of Robo4 was upregulated in human glioma tissues compared to normal brain tissues as well as in the glioma cocultured endothelial cells derived from the BTB model (Cai et al., 2015). Slit2 pretreatment reduced BTB endothelial permeability but this protective effect of Slit2 was not seen in the presence of Robo4 knockdown (Cai et al., 2015). Additionally, Robo4 overexpression reduced BTB permeability by 50.33% which was associated with reduced MMP-9 activity and a corresponding increase in the expression of endothelial tight junction proteins occludin, ZO-1 and claudin-5 (Cai et al., 2015). Whereas, Robo4 knockdown increased the endothelial BTB permeability 1.41 times higher compared to the control group and showed the opposite effect on MMP-9 activity and tight junction protein expression. Furthermore, Src and Erk1/2 were the downstream mediators involved in regulation of the MMP-9 activity by Robo4 (Cai et al., 2015).

However, a dual role of Slit2 has been implicated in the vascular endothelial cells outside of CNS. The endothelial protective function of Slit2-Robo4 was observed following intraocular injections of Slit2 in a retinal vascular hyperpermeability model in mice (Jones et al., 2008). Likewise, Slit2 inhibited Andes virus induced disruption of endothelial adherens junction proteins in human pulmonary microvascular endothelial cells (PMECs) (Gorbunova et al., 2013). However, vascular permeability was increased in Slit2 transgenic mice subjected to laser-induced CNV and in the HUVECs in vitro (Li et al., 2015b). These conflicting results suggest that endogenous Slit2 overexpression and
exogenous Slit2 possibly have diverse functions and mechanisms on the endothelial cells.

Moreover, the endothelial response to Slit2 seems to be dependent on the tissue specific expression of the type of Robo receptors. Studies from systemic pathologies indicate that the expression and function of endothelial Robo4 may be context dependent and can vary depending on the pathology. For instance, Robo4 was found to be upregulated in the endothelial cells in various neoplasm (Cai et al., 2015). The expression of Robo4 was downregulated in virus-infected human PMECs and it predominantly expressed Robo4 but not Robo1 (Gorbunova et al., 2013). However, the anti-permeability effect of Slit2 was not observed in the human umbilical vein endothelial cells (HUVECs) which express similar levels of Robo4 and Robo1 receptors (Gorbunova et al., 2013). These findings suggest that the endothelial response to Slit2 is dependent on the tissue specific endothelial cell expression of the type of Robo receptors.

**Pericytes**

Pericytes are special cell types that provide structural support to the capillaries and interact with endothelial cells by making direct cell-cell contacts and through paracrine signals. Pericytes have been shown to play a critical role during physiological and pathological angiogenesis (Bergers and Song, 2005). During initial stage of angiogenesis, pericytes detach from the endothelial cells which enables endothelial cells to migrate into the surrounding extracellular matrix giving rise to sprouting new vessels (Raza et al., 2010). Slit2 inhibited platelet derived growth factor (PDGF) induced human brain vascular pericyte
motility in an in vitro wound healing assay (Guijarro-Munoz et al., 2012). The pericycle chemorepellent effect of Slit2 was decreased but not abolished by antibodies blocking the Robo1 and Robo4 receptors which suggested potential contribution of other receptors (Guijarro-Munoz et al., 2012). Slit2 also inhibited PDGF-induced lamellipodia formation in the pericytes, features suggestive of a pro-migratory phenotype (Guijarro-Munoz et al., 2012). Additionally, pericytes were found to secrete high levels of Slit2 while negligible amounts of Slit1 and Slit3 were detected. Higher levels of Robo1 and low levels of Robo4 receptors were detected on the surface of the pericytes (Guijarro-Munoz et al., 2012). The authors suggested that Slit2 secreted by the pericytes act in an autocrine manner on the Robo receptors and regulate its function during angiogenesis. Although a dual role of Slit2 has been implicated in angiogenesis dependent on the type of endothelial Robo receptor involved, the regulation of pericyte function also seems to determine the angiogenic regulation by Slit2.

**Regulation of Slit2-Robo in the CNS**

A few upstream regulators have been identified that modulate the expression of Slit-Robo in the CNS. However, detailed studies are lacking and are essential for understanding how Slit-Robo is altered in CNS pathologies and the implications of such changes. Additionally, these upstream regulators could be important targets for potential therapeutic manipulation.

**Transcription Factors Pax6**

Various regulatory genes control the expression of axonal guidance
molecules and thereby regulate the path that axonal fibers travel along. Among few studies that have explored the role of regulatory genes and transcription factors that control expression of Slit and its receptor Robo, the transcription factor Pax6 seems to play a role in regulating axonal pathfinding during brain development by regulating the expression of Slit proteins (Tsuchiya et al., 2009). The expression of Slit1 and Slit2 were downregulated whereas netrin-1 was upregulated in the dorsal thalamus of Pax6 deficient mice lacking the transcription factor Pax6 (Tsuchiya et al., 2009). This was associated with aberrant pattern of axonal projections from the mammillary bodies to the thalamus and midbrain in Pax6 mutant mice (Tsuchiya et al., 2009). The expression of Slit3 did not change and was low in both the wild type and mutant mice. However, the expression patterns of receptor for netrin-1, DCC and Robo1-3 was similar between Pax mutant and wild type embryonic mice (Tsuchiya et al., 2009). Mutations in genes that encode the transcription factor Pax6 may lead to reduced Slit1 and Slit2 expression resulting in abnormal axonal pathfinding.

**MicroRNA 218**

MicroRNAs (miRNAs) may control the migration and invasion of glioma cells by regulating the expression of Slit2 and its receptor. MicroRNAs are short noncoding single-stranded RNAs, ~22 nucleotides in length, that function as oncogenes or tumor suppressors in various types of cancers. MicroRNA 218 (miR-218) in particular was found to be downregulated in glioma cell lines and in human glioma tissues (Gu et al., 2015). Transfection of glioma cells with miR-218 inhibited the migration and invasion of glioma cells through modulation of the
Slit2-Robo1 pathway (Gu et al., 2015). MicroRNA 218 transfection was associated with increased expression of Slit2 but miR-218 downregulated the expression of Robo1 in the glioma cells by targeting the 3’-untranslated region (3’-UTR) of Robo1 (Gu et al., 2015). These findings are similar to previous studies in which miR-218 was shown to inhibit the invasion and progression of gastric and nasopharyngeal cancer cells by downregulating the Slit2-Robo1 pathway (Tie et al., 2010, Alajez et al., 2011).

**Endocannabinoids and Cannabinoid Receptors**

The upstream factors that regulate Slit-Robo signaling, expression and function remains poorly explored. In the developing brain, Slit2 regulates the direction of migrating axons specifically by preventing midline crossing and maintaining the dorsoventral positioning of axons. Endocannabinoids and cannabinoid receptors 1 and 2 have been implicated as upstream regulators of Slit2-Robo1 interaction during development of the forebrain axonal tracts (Alpar et al., 2014).

**Downstream Targets of Slit2-Robo Signaling in the CNS**

Few downstream targets of Slit2-Robo signaling pathways have been identified in the CNS. These studies provide a glimpse into the intracellular mechanism by how Slit2 exerts its anti-migratory function in the CNS.

**Small Rho-GTPase Cdc42**

Various axon guidance cues regulate actin polymerization at the leading
edge of growth cones during cell motility or axon pathfinding by modulating the activity of cytoskeletal regulatory proteins. Cdc42 is a member of the small Rho-GTPases that regulates actin cytoskeletal polymerization during cellular motility. Modulation of Cdc42 activity by various guidance cue molecules has been implicated in directing cell migration (Myers et al., 2012). Yinn et al. showed that Slit2 regulated Cdc42 activity in glioma cells dependent on Robo1 receptor in vitro (Yiin et al., 2009). The expression of Slit2 in glioma cells attenuated Cdc42 activity which was reversed with immunodepletion of Slit2. Likewise, immunodepletion of Slit2 also reversed the effects of Slit2 to inhibit glioma cell migration (Yiin et al., 2009). Additionally, when glioma cells were transfected with Robo1 siRNA the effects of recombinant Slit2 to inhibit cell migration and reduce Cdc42 activity were reversed suggesting that Slit2 inhibited glioma cell migration by attenuating Cdc42 activity dependent on the Robo1 receptor (Yiin et al., 2009).

**Focal Adhesion Kinase**

Previous study showed that Slit2 regulated neuronal motility in a FAK- and Cdc42-dependent manner in vitro (Myers et al., 2012). Positive guidance cues such as BDNF and laminin increased Cdc42 activity and enhanced cellular protrusion, whereas Slit2 decreased Cdc42 activity and lamellipodia formation thereby reducing neuronal migration, which was disrupted with the expression of constitutively active FAK (Myers et al., 2012). This implicates that Slit2 regulates neuronal migration by modulating Cdc42 activity in a FAK-dependent manner. The downstream effectors of Cdc42 such as p21-activated kinase (PAK) and N
Wiskott Aldrich Syndrome Protein (N-WASP) modulate actin polymerization and therefore cell migration (Myers et al., 2012).

**Src and Extracellular Signal-Regulated Kinase 1/2**

Previous study using a glioma BTB model in vitro showed that Slit2 had anti-permeability effect on the endothelial cells derived from the BTB. Robo4 knockdown increased the BTB endothelial permeability by disrupting tight junction proteins which was mediated via the activation of Src-Erk1/2-MMP-9 signaling pathway (Cai et al., 2015). Inhibitors of Src and Erk partially reversed the increase in BTB permeability and MMP-9 activity induced by Robo4 knockdown, which showed that Src and Erk1/2 are downstream mediators involved in endothelial Robo4 activation.

**Research Question, Hypothesis and Research Aims**

Clinical and experimental studies show that adverse events such as brain edema and neurological deficits occur post-operatively following neurosurgical procedures. The mechanisms of influx of blood-borne inflammatory cells into brain parenchyma, which amplifies neuroinflammation and parenchymal damage as well as BBB disruption, which potentiates brain edema after SBI has been explored in this project. Specifically, the role of Slit2, an extracellular matrix protein expressed endogenously in the brain, as well as the effects of recombinant Slit2 as a novel therapeutic strategy to reduce neuroinflammation and BBB disruption after SBI has been studied.
Our central hypothesis was that Slit2 will increase after SBI as an endogenous protective mechanism, and recombinant Slit2 pretreatment will reduce neuroinflammation and stabilize the BBB via Robo receptor signaling pathway in a rat SBI model.

The following three specific aims have been explored in this project.

**Specific Aim 1. Evaluate the Role of Slit2 in Reducing Neuroinflammation and BBB Permeability After SBI**

Our corollary hypothesis was that Slit2 will attenuate neuroinflammation and BBB permeability after SBI, thereby reducing brain edema and neurological deficits. We measured temporal profile of Slit2 expression after SBI and evaluated outcomes after endogenous Slit2 knockdown in the SBI rats. Brain edema, neurological deficits and inflammatory makers were measured with recombinant Slit2 pretreatment.

**Specific Aim 2. Investigate the Role of Slit2 and its Receptor Robo1 in Reducing Neuroinflammation After SBI**

Our corollary hypothesis was that Slit2 will attenuate neuroinflammation after SBI by reducing peripheral leukocyte infiltration by Robo1-srGAP1 mediated Cdc42 inactivation. We measured inflammatory mediators and leukocyte infiltration after SBI and with recombinant Slit2 pretreatment. The effect of antagonizing Robo1 and its downstream effector srGAP1 with recombinant Slit2 pretreatment was also evaluated.
Specific Aim 3. Investigate the Role of Slit2 and its Receptor Robo4 in Stabilizing the BBB After SBI

Our corollary hypothesis was that Slit2 will protect BBB integrity after SBI by stabilizing the BBB junction proteins through Robo4-paxillin mediated Rac1 activation. We measured the expression of BBB junction proteins after SBI and with recombinant Slit2 pretreatment. The effect of antagonizing Robo4 and its downstream effector paxillin with recombinant Slit2 pretreatment was also evaluated.

The schematic representation of specific aims (Fig. 1) illustrates the key players examined in this project.
Figure 1. Schematic representation of specific aims. Surgical brain injury (SBI) leads to peripheral immune cell infiltration at the perisurgical site and disruption of the blood brain barrier (BBB) tight junction (TJ) and adherens junction (AJ) proteins. This potentiates brain edema at the perisurgical site and consequently neurological dysfunction. Aim 1 evaluated the role of endogenous Slit2 after SBI. Next the protective effects of recombinant Slit2 on neuroinflammation and BBB permeability were evaluated. Aim 2 evaluated the mechanism by which Slit2 reduces neuroinflammation after SBI. Aim 3 evaluated the mechanism by which Slit2 decreases BBB permeability after SBI.
References


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CHAPTER 3

RECOMBINANT SLIT2 ATTENUATES NEUROINFLAMMATION AFTER SURGICAL BRAIN INJURY BY INHIBITING PERIPHERAL IMMUNE CELL INFILTRATION VIA ROBO1-SRGAP1 PATHWAY IN A RAT MODEL

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Conflicts of Interest: None.

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Abstract

Background and Purpose: Peripheral immune cell infiltration to the brain tissue at the perisurgical site can promote neuroinflammation after surgical brain injury (SBI). Slit2, an extracellular matrix protein, has been reported to reduce leukocyte migration. This study evaluated the effect of recombinant Slit2 and the role of its receptor roundabout1 (Robo1) and its downstream mediator Slit-Robo GTPase activating protein 1 (srGAP1)-Cdc42 on peripheral immune cell infiltration after SBI in a rat model.

Methods: One hundred and fifty-three adult male Sprague-Dawley rats (280-350 g) were used. Partial resection of right frontal lobe was performed to induce SBI. Slit2 siRNA was administered by intracerebroventricular injection 24 hours before SBI. Recombinant Slit2 was injected intraperitoneally 1 hour before SBI. Recombinant Robo1 used as a decoy receptor was co-administered with recombinant Slit2. srGAP1 siRNA was administered by intracerebroventricular injection 24 hours before SBI. Post-assessments included brain water content measurement, neurological tests, ELISA, western blot, immunohistochemistry, and Cdc42 activity assay.

Results: Endogenous Slit2 was increased after SBI. Robo1 was expressed by peripheral immune cells. Endogenous Slit2 knockdown worsened brain edema after SBI. Recombinant Slit2 administration reduced brain edema, neurological deficits, and pro-inflammatory cytokines after SBI. Recombinant Slit2 reduced peripheral immune cell markers cluster of differentiation 45 (CD45) and myeloperoxidase (MPO), as well as Cdc42 activity in the perisurgical brain.
tissue which was reversed by recombinant Robo1 co-administration and srGAP1 siRNA.

**Conclusions:** Recombinant Slit2 improved outcomes by reducing neuroinflammation after SBI, possibly by decreasing peripheral immune cell infiltration to the perisurgical site through Robo1-srGAP1 mediated inhibition of Cdc42 activity. These results suggest that Slit2 may be beneficial to reduce SBI-induced neuroinflammation.

**Keywords**

Surgical brain injury, Neuroinflammation, Brain edema, Peripheral Immune Cell Infiltration, Slit2, Robo1, srGAP1, Cdc42.

**Highlights**

Peripheral immune cells infiltration increased at the perisurgical site after SBI

Endogenous Slit2 was increased as a protective mechanism after SBI

Recombinant Slit2 attenuated brain edema by reducing neuroinflammation

Recombinant Slit2 reduced neuroinflammation by decreasing immune cell infiltration

Recombinant Slit2 inhibited immune cell migration dependent on Robo1-SrGAP1 pathway
Introduction

Surgical brain injury (SBI) is the inadvertent injury to brain tissue at the perisurgical site which occurs due to neurosurgical maneuvers such as incision, retraction, and electrocoagulation that can aggravate post-operative brain edema and neurological deficits (Jadhav et al., 2007a, Huang et al., 2014). Major complications are encountered in 13-27% patients after intracranial surgeries (Bruder, 2002). Measures to reduce neurosurgical complications are limited (Jadhav et al., 2007a). Furthermore, routine therapy used against SBI including hyperosmolar agents and steroids can have unwanted adverse effects (Li et al., 2014, Xu et al., 2014). Therapeutic strategies that augment endogenous protective mechanisms would be a safe approach to reduce post-operative complications in neurosurgical patients.

Neuroinflammation is a major pathophysiological consequence after SBI (Yamaguchi et al., 2007, Hyong et al., 2008) which contributes to brain edema that can worsen post-operative neurological function (Ayer et al., 2012). Following brain injury, resident immune cells get activated and release cytokines and chemokines that promote migration of peripheral immune cells to injury site in the brain (Wang and Dore, 2007, Rhodes, 2011, Ma et al., 2014). Immune cell infiltration and inflammatory mediators were increased in adjacent brain tissue at the perisurgical site following experimental resection (Jadhav et al., 2007a, Hyong et al., 2008). Infiltrated immune cells release inflammatory mediators that further promote neuroinflammation (Petty and Lo, 2002, Yilmaz et al., 2006, Lo, 2009).
Slit2 is a secreted extracellular matrix protein (Ballard and Hinck, 2012) expressed endogenously in the brain by neurons and astrocytes (Hagino et al., 2003, Prasad et al., 2007). Slit2 was recently identified as an inhibitor of leukocyte chemotaxis (Wu et al., 2001, Ballard and Hinck, 2012) and was shown to be protective in experimental models of systemic inflammation (Kanellis et al., 2004, Tole et al., 2009, London et al., 2010). Slit2 reduced migration of leukocytes to the cortical venules after global cerebral ischemia in mice (Altay et al., 2007). The anti-migratory function of Slit2 is mediated by binding to its receptor roundabout1 (Robo1) (Wong et al., 2002, Ballard and Hinck, 2012).

Furthermore, it has been established that Slit-Robo GTPase Activating Protein 1 (srGAP1) a downstream effector of the receptor Robo1 (Wong et al., 2002) can inhibit Cdc42, which is a critical mediator for cell migration (Bishop and Hall, 2000, Yiin et al., 2009). Robo1 has been shown to be expressed on the surface of peripheral immune cells (Wu et al., 2001, Guan and Rao, 2003, Prasad et al., 2007), which we propose induces the signal transduction pathway that mediates the anti-migratory effect of Slit2 and thereby reduces brain infiltration of peripheral immune cells.

The role of Slit2 after SBI is unknown, and the mechanism by which Slit2 elicits neuroprotection has not been evaluated. The objective of this study was to evaluate the effects of recombinant Slit2 as a novel therapeutic strategy to reduce neuroinflammation after SBI in a rat model.
Materials and Methods

Animals

All procedures were approved by the Institutional Animal Care and Use Committee at Loma Linda University and complied with NIH Guide for the Care and Use of Laboratory Animals. Adult male Sprague Dawley rats (280–350 g) were housed in humidity and temperature controlled environment with 12 hour light/dark cycle. One hundred and fifty-three rats were subjected to either Sham surgery (n=26) or SBI surgery (n=127) by inducing partial resection of the right frontal lobe.

Experimental Design

Experiment 1. The time course expression and localization of endogenous Slit2 and Robo1 was characterized at 24 hours, 72 hours, and day 7 after SBI. Rats (n=24) were divided 4 groups: Sham, SBI 24 hours, SBI 72 hours, and SBI day 7. Brain samples from the residual right frontal lobe were collected for enzyme linked immunosorbent assay (ELISA), western blot and for immunohistochemistry.

Experiment 2. The role of endogenous Slit2 after SBI was evaluated. Rats (n=37) were divided into 4 groups: Sham, SBI, SBI+Slit2 siRNA, SBI+Scramble siRNA. Endogenous Slit2 knockdown was performed by intracerebroventricular (ICV) injection of Slit2 siRNA (Life Technologies, Grand Island, NY, USA) 24 hours before SBI. Brain water content, neurological function and western blot was evaluated at 72 hours after surgery.
Experiment 3. The effect of exogenous recombinant Slit2 pretreatment for SBI was evaluated. Three doses of recombinant Slit2 (1 µg/Kg, 3 µg/Kg, 10 µg/Kg) (R and D Systems, Minneapolis, MN, USA) was tested. Rats (n=74) were divided into 5 groups: Sham, SBI+Vehicle, SBI+Slit2 (1 µg/Kg), SBI+Slit2 (3 µg/Kg), SBI+Slit2 (10 µg/Kg). The dose of recombinant Slit2 (3 µg/Kg) was chosen based on previous publication (Altay et al., 2007). Furthermore, since a dose response effect of Slit2 was previously reported in vitro studies (Prasad et al., 2007), we examined the effects of two additional doses to establish the optimal dose for SBI. Recombinant Slit2 or vehicle normal saline was injected intraperitoneally 1 hour before SBI. Brain water content and neurological function was evaluated at 24 and 72 hours after surgery. Brain samples from the residual right frontal lobe at the perisurgical site were collected at 24 hours for western blot and immunohistochemistry.

Experiment 4. The role of Robo1 and srGAP1 in recombinant Slit2 mediated protection after SBI was investigated. Rats were divided into 6 groups: Sham, SBI+Vehicle, SBI+Slit2 (10 µg/Kg), SBI+Slit2(10 µg/Kg)+Robo1(3 µg/Kg), SBI+Slit2(10 µg/Kg)+srGAP1 siRNA, SBI+Slit2(10 µg/Kg)+scramble siRNA. Rats (n=18) were added for the last 3 groups. Recombinant Robo1 (3 µg/Kg) (R and D Systems, Minneapolis, MN, USA) was co-administered with recombinant Slit2 by intraperitoneal injection 1 hour before SBI. srGAP1 or scramble siRNA (Life Technologies, Grand Island, NY, USA) was given by ICV injection 24 hours before SBI. Neurological function was evaluated at 24 hours after surgery and
brain samples were collected at 24 hours for western blot and Cdc42 activity assay.

**Surgical Brain Injury Model**

Rats were subjected to surgical brain injury as previously described (Jadhav et al., 2007a, Yamaguchi et al., 2007). Briefly, anesthesia was induced by 4% isoflurane in an induction chamber and maintained at 2.5% using a nasal mask. The skin was incised and periosteum was reflected to expose the bregma and frontal bone. A square craniotomy 5x5 mm was made on the right frontal bone with left lower corner towards bregma by using a micro drill. The dura was incised and a partial resection of the right frontal lobe was performed with margins of resection at 2 mm lateral to sagittal suture and 1 mm proximal to coronal suture, with the depth extending to base of skull. Normal saline irrigation and intraoperative packing was done to ensure complete hemostasis after which skin incision was sutured. Sham animals were subjected to right frontal craniotomy but without dural incision or frontal lobe resection. Buprenorphine 0.03 mg/Kg was given by subcutaneous injection for post-operative analgesia. Post-operatively rats were closely observed for complete recovery from anesthesia.

**Intracerebroventricular Injection**

Rats were anesthetized with isoflurane and placed prone on a stereotactic frame. A 10 µL Hamilton syringe (Hamilton Co, Reno, NV, USA) was inserted through a burr hole on the skull into right lateral ventricle using the following
coordinates relative to bregma: 1.0 mm lateral, 1.5 mm posterior and 3.2 mm below the horizontal plane of bregma as previously described (Suzuki et al., 2010, Chen et al., 2013). Slit2 siRNA 500 pmol or srGAP1 siRNA 500 pmol (Life Technologies, Grand Island, NY, USA) each in 2 μL sterile saline was injected at a rate 0.5 μL/min as previously described (Suzuki et al., 2010, Chen et al., 2013). The same volume of scramble siRNA (Life Technologies, Grand Island, NY, USA) was injected as a negative control. The needle was left in situ for additional 10 mins and then slowly withdrawn over 5 mins to prevent leakage. The burr hole was sealed with bone wax and skin incision was sutured. Rats were returned to recovery cages and closely observed for complete recovery from anesthesia.

**Brain Water Content Measurement**

Brain edema was evaluated at 24 and 72 hours after surgery by wet weight/dry weight method as previously described (Jadhav et al., 2007b, Yamaguchi et al., 2007). The brains were quickly removed and dissected into six parts: right frontal, left frontal, right parietal, left parietal, cerebellum and brainstem. The samples were weighed immediately (wet weight) and then placed in an oven at 100°C for 48 hours and weighed again (dry weight). The percent of brain water content in each region was calculated using the formula [(wet weight – dry weight)/wet weight]×100 (Yamaguchi et al., 2007).

**Assessment of Neurological Function**

Neurobehavioral deficits were evaluated by an examiner blinded to the groups by using modified Garcia test and beam balance test at 24 and 72 hours.
after SBI as previously described (Garcia et al., 1995, Yamaguchi et al., 2007). Briefly, Garcia test assessed sensorimotor deficits and included seven tests: spontaneous activity, body proprioception, vibrissae touch, limb symmetry, lateral turning, forepaw outstretching, and climbing. Each test was scored from 0 to 3 with a maximum score of 21. In beam balance test, the rats were allowed to walk on a 90 cm x 2.25 cm beam for 1 min during which distance traveled and time taken to travel the distance was recorded. The score ranged from 0 to 5. Higher scores indicated better function for both the tests.

**Enzyme Linked Immunosorbent Assay (ELISA)**

Slit2 concentration in brain samples were measured using a Slit2-HRP conjugated Elisa kit (MyBiosource, San Diego, CA, USA) as previously described (Doyle et al., 2008, Hu et al., 2014). Sample supernatants from the residual right frontal lobe were incubated in a microtiter plate pre-coated with biotin conjugated antibody specific for rat Slit2. Next, the samples were incubated with avidin conjugated HRP after which TMB substrate was added. The absorbance was measured spectrophotometrically at wavelength of 450 nm using a microplate reader (Biorad, Irvine, CA, USA).

**Western Blotting**

Western blot was performed as previously described (Hasegawa et al., 2011, Chen et al., 2013). Briefly, brain samples were collected after rats were transcardially perfused with phosphate buffered saline (PBS). Samples were homogenized in Ripa lysis buffer (Santa Cruz Biotechnology, Dallas, TX, USA)
for protein extraction (Hasegawa et al., 2011) Supernatants from the residual right frontal lobe samples were collected after centrifugation at 14,000 g at 4°C for 30 mins and protein concentration was determined using a detergent compatibility assay (Biorad, Irvine, CA, USA). Equal amounts of protein (50 µg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes after which the membranes were incubated overnight with the respective primary antibodies: anti-Slit2 (1:200), anti-Robo1 (1:200), anti-myeloperoxidase (MPO) (1:500) (all from Santa Cruz Biotechnology, Dallas, TX, USA), anti-cluster of differentiation 45 (CD45) (1:500), anti-IL-1β (1:1000) and anti-INF-γ (1:1000) (all from Abcam, Cambridge, MA, USA). The same membranes were probed with anti-β Actin (1:2000) (Santa Cruz Biotechnology, Dallas, TX, USA) as loading controls. The membranes were incubated with appropriate secondary antibodies (1:4000) (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour at room temperature. Bands were visualized using ECL Plus Chemiluminescence kit (Amersham Biosciences, Arlington Heights, IL, USA) and quantified using the software Image J (National Institutes of Health, Bethesda, MD, USA). Results are expressed as relative density to β-actin and then normalized to average value of the sham group as previously described (Hasegawa et al., 2011).

**Immunohistochemistry**

Briefly, after PBS perfusion and post-fixation in formalin, brain samples were sectioned into 10-µm-thick slices using a cryostat (CM3050S; Leica Microsystems, Bannockburn, IL, USA). Immunofluorescence staining was
performed as previously described (Hasegawa et al., 2011, Altay et al., 2012). The sections were incubated overnight at 4°C with the following primary antibodies: anti-Slit2 (1:200), anti-Robo1 (1:200), anti-MPO (1:200) (all from Santa Cruz Biotechnology, Dallas, TX, USA), anti-neuronal nuclei (NeuN) (1:500), anti-glia fibrillary acidic protein (GFAP) (1:100) and anti-CD45 (1:150) (all from Abcam, Cambridge, MA, USA). Sections were then incubated with FITC- and Texas Red-conjugated appropriate secondary antibodies (1:100) (Jackson Immuno Research, West Grove, PA, USA) for 2 hours at room temperature and visualized with a fluorescence microscope (Olympus BX51).

**Cdc42 Activity Assay**

A pull down assay was performed using Cdc42 activity assay kit (Cell Biolabs, San Diego, CA, USA) as previously described (Yiin et al., 2009). Briefly, samples were mixed with the p21-activated protein kinase (PAK1)-p21-binding domain (PBD) agarose beads and incubated at 4°C for 1 hour. The beads were then re-suspended in sample buffer and separated by 10% polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was probed with anti-Cdc42 specific antibody provided in the kit to detect the GTP-bound Cdc42.

**Statistical Analysis**

Statistical analysis was performed using the Sigma Plot 10.0 and Sigma Stat version 3.5 (Systat Software, San Jose, CA, USA). Data were presented as mean ± SEM. Statistical differences between groups were analyzed using one-
way ANOVA for multiple comparisons followed by Tukey or Student-Newman-Kuels post hoc tests. P values less than 0.05 was considered statistically significant.

**Results**

All sham-operated rats survived. Nine rats died of the 127 SBI rats. The overall mortality in the SBI group was 7.09%.

*Temporal Expression and Localization of Endogenous Slit2 After SBI*

The expression of Slit2 at the perisurgical site in the brain was measured at 24 hours, 72 hours and 7 days after SBI. The level of endogenous Slit2 was increased at 24 hours following SBI and reached a peak at 72 hours compared to sham (p<0.05 compared to sham, Fig. 1A). The increase in the level of Slit2 was sustained to 7 days after SBI (p<0.05 compared to sham). Double immunofluorescence staining showed that Slit2 was expressed by the neurons and astrocytes (Fig. 1C).
**Figure 1.** Temporal expression and localization of endogenous Slit2 in the brain after SBI. (A) ELISA showed that Slit2 concentration increased at 24 hours, 72 hours and 7 days after SBI with a peak at 72 hours. Data are expressed as mean ± SEM. N=4 to 6 per group. ANOVA, SNK. *p<0.05 compared to Sham, φp<0.05 compared to 24 hours SBI. (B) Histology section showing perisurgical site in the inset used for immunofluorescence staining. (C) Representative microphotographs of immunofluorescence staining showing co-localization of Slit2 (Texas Red/red), with neuronal nuclei (NeuN) or glial fibrillary acidic protein (GFAP) (FITC/green) and DAPI at 24 hours after SBI. Arrows indicate merged cells. Scale bar=50 μm.
Temporal Expression and Localization of Robo1 After SBI

The expression of Robo1 in the brain was measured at 24 hours, 72 hours and 7 days after SBI. Robo1 expression increased at all-time points with a peak at 24 hours after SBI (p<0.05 compared to sham, Fig. 2A). Double immunofluorescence staining of brain samples showed that Robo1 co-localized with the marker for peripheral immune cells, cluster of differentiation 45 (CD45) and with the marker for neutrophils, myeloperoxidase (MPO) at the perisurgical site after SBI (Fig. 2B).
Figure 2. Temporal expression and localization of Robo1 in the brain after SBI. (A) Representative image of western blot and quantitative analysis showed that Robo1 expression increased at 24 hours, 72 hours and 7 days after SBI with a peak increase at 24 hours. Data are expressed as mean ± SEM. N=4/group. ANOVA, SNK. *p<0.05 compared to Sham, φp<0.05 compared to 24 hours SBI. (B) Representative microphotographs of immunofluorescence staining showing co-localization of Robo1 (Texas Red/red) with immune cell markers cluster of differentiation 45 (CD45) or neutrophil marker myeloperoxidase (MPO) (FITC/green) and DAPI at 24 hours after SBI. Arrows indicate merged cells. Scale bar=50 μm
**Endogenous Slit2 Knockdown Worsened Outcomes After SBI**

Western blot showed that Slit2 siRNA effectively suppressed the expression of Slit2 (p<0.05 compared to SBI and scramble siRNA groups, Fig. 3A). SBI rats that received Slit2 siRNA had significantly higher brain water content in the residual right frontal lobe (p<0.05 compared to SBI and scramble siRNA groups, Fig. 3B). Garcia test showed worsened neurological function in the Slit2 siRNA group compared to scramble siRNA (p<0.05 compared to scramble siRNA, Fig. 3C), but there was no significant difference between the groups in beam balance test (Fig. 3D).
Figure 3. Effect of endogenous Slit2 knockdown on Slit2 expression, brain water content and neurological function 72 hours after SBI. (A) Representative image of western blot and quantitative analysis of Slit2 expression after siRNA knockdown showed that Slit2 siRNA but not scramble siRNA significantly reduced the expression of endogenous Slit2 after SBI. (B) Brain water content was significantly increased at the residual right frontal lobe 72 hours after SBI. Slit2 siRNA worsened brain edema compared to SBI and scramble siRNA. RF: right frontal, LF: left frontal, RP: right parietal, LP: left parietal, C: cerebellum, BS: brainstem. (C) Garcia test and (D) Beam balance test showed significantly worse neurological function 72 hours after SBI compared to sham. Slit2 siRNA group performed worse than scramble siRNA group in the Garcia test. Data are expressed as mean ± SEM. N=6/group. ANOVA, SNK. *p<0.05 compared to sham, †p<0.05 compared to SBI, #p<0.05 compared to SBI+scramble siRNA.
Recombinant Slit2 Administration Reduced Brain Edema and Improved Neurological Function 24 and 72 Hours After SBI

Brain water content was significantly increased at the perisurgical site in the residual right frontal lobe at 24 and 72 hours after SBI (p<0.05 compared to sham, Fig. 4A and 4B, respectively). Recombinant Slit2 3 µg/Kg and 10 µg/Kg significantly reduced brain water content at both time points (p<0.05 compared to vehicle). Neurological function evaluated by Garcia test was significantly worse at 24 and 72 hours after SBI (p<0.05 compared to sham, Fig. 4C and 4D, respectively). All three doses of recombinant Slit2 significantly improved Garcia scores at 24 hours (p<0.05 compared to vehicle). However, only recombinant Slit2 10 µg/Kg improved performance in the Garcia test at 72 hours (p<0.05 compared to vehicle). Beam balance test showed significantly worse neurological function after SBI at 24 and 72 hours (p<0.05 compared to sham, Fig. 4E and 4F, respectively). Slit2 10 µg/Kg showed a tendency to improve beam balance scores at 72 hours but was not significant.
Figure 4. Effect of recombinant Slit2 on brain water content and neurological function at 24 and 72 hours after SBI. Brain water content was significantly increased in the residual right frontal lobe at 24 hours (A) and 72 hours (B) after SBI. Recombinant Slit2 3 µg/Kg and 10 µg/Kg significantly reduced brain edema at both time points. RF: right frontal, LF: left frontal, RP: right parietal, LP: left parietal, C: cerebellum, BS: brainstem. Garcia test (C and D) and Beam balance test (E and F) showed significantly worse neurological function at 24 and 72 hours after SBI, respectively. Slit2 10 µg/Kg significantly improved performance in the Garcia test 24 and 72 hours after SBI. Data are expressed as mean ± SEM. ANOVA, Tukey. *p<0.05 compared to Sham, †p<0.05 compared to SBI+Vehicle, ‡p<0.05 compared to SBI+Slit2 10 µg/Kg.
**Recombinant Slit2 Reduced Neuroinflammation and Peripheral Immune Cell Infiltration at the Perisurgical Site 24 Hours After SBI**

The expression of pro-inflammatory cytokines IL-1β and INF-γ in the residual right frontal lobe was quantified by western blot, which showed significant increase after SBI compared to sham (P<0.05 compared to sham, Fig. 5A and 5B, respectively). Recombinant Slit2 10 µg/Kg significantly reduced the expression of both IL-1β and INF-γ (p<0.05 compared to vehicle).

Immunofluorescence staining showed increased CD45 and MPO positive cells at the perisurgical site after SBI compared to sham (Fig. 5C and 5D, respectively). Recombinant Slit2 10 µg/Kg treated group had fewer cells positively stained for CD45 and MPO compared to vehicle group.
Figure 5. Effect of recombinant Slit2 on neuroinflammation and peripheral immune cell infiltration at 24 hours after SBI. Representative western blot image and quantitative analysis showed that IL-1β (A) and INF-γ (B) expression was significantly increased after SBI. Slit2 10 μg/Kg significantly reduced the expression of both pro-inflammatory cytokines. Data are expressed as mean ± SEM. N=6/group. ANOVA, SNK. *p<0.05 compared to Sham, †p<0.05 compared to SBI+Vehicle. (C) Representative microphotographs of immunofluorescence staining showing co-localization of peripheral immune cell marker cluster of differentiation 45 (CD45)-FITC/green with DAPI at the right frontal perisurgical site. Recombinant Slit2 10 μg/Kg group had fewer CD45 positively stained cells. (D) Representative microphotographs of immunofluorescence staining showing co-localization of neutrophil marker myeloperoxidase (MPO)-FITC/green with DAPI at the perisurgical site. Fewer MPO positive cells were visualized in the recombinant Slit2 10 μg/Kg group. Asterix denotes site of injury and scale bar=100 μm for panels C and D.
Recombinant Slit2 Reduced Peripheral Immune Cell Infiltration to the Perisurgical Site Dependent on Robo1 Receptor and its Downstream Mediator srGAP1

Western blot showed that CD45 and MPO expression was significantly increased after SBI (p<0.05 compared to sham, Fig. 6A and 6B, respectively). Recombinant Slit2 10 µg/Kg significantly reduced the expression of both markers (p<0.05 compared to vehicle). Recombinant Robo1 administration abolished the effects of recombinant Slit2 by increasing expression of CD45 and MPO (p<0.05 compared to Slit2) and worsened neurological deficits which was evaluated using Garcia test (p<0.05 compared to Slit2, Fig. 6C). Likewise, srGAP1 siRNA abolished the effects of recombinant Slit2 by increasing the expression of CD45 and MPO (p<0.05 compared to Slit2, Fig 7A and 7B, respectively) and worsened neurological deficits which was evaluated using Garcia test (p<0.05 compared to Slit2, Fig 7C).
Figure 6. Role of Robo1 in Slit2 mediated decrease in peripheral immune cell infiltration. (A) Representative western blot image and quantitative analysis showing peripheral immune cell marker cluster of differentiation (CD45) expression was increased in the right frontal perisurgical site at 24 hours after SBI compared to sham. Slit2 10 μg/Kg significantly reduced the expression of CD45 which was reversed with recombinant Robo1 co-administration. (B) Representative western blot image and quantitative analysis showing increased expression of neutrophil marker myeloperoxidase (MPO) at the perisurgical site after SBI. Slit2 10 μg/Kg significantly reduced the expression of MPO which was reversed with recombinant Robo1 co-administration. (C) Slit2 10 μg/Kg significantly improved neurological function evaluated using Garcia test 24 hours after SBI which was reversed with recombinant Robo1 co-administration. Data are expressed as mean ± SEM, N=6/group. ANOVA, SNK. *p<0.05 compared to Sham, †p<0.05 compared to SBI+Vehicle, &p<0.05 compared to SBI+Slit2 10 μg/Kg.
**Figure 7.** Role of srGAP1 in Slit2 mediated decrease in peripheral immune cell infiltration. (A) Representative western blot image and quantification showing Slit2 10 μg/Kg significantly reduced the expression of peripheral immune cell marker cluster of differentiation 45 (CD45) which was reversed with srGAP1 siRNA but not scramble siRNA. (B) Slit2 10 μg/Kg significantly reduced the expression of neutrophil marker myeloperoxidase (MPO) which was reversed with srGAP1 siRNA but not scramble siRNA. (C) Slit2 10 μg/Kg significantly improved neurological function evaluated using Garcia test 24 hours after SBI which was reversed with srGAP1 siRNA. Data are expressed as mean ± SEM, N=6/group. ANOVA, SNK. †p<0.05 compared to SBI+Vehicle, &p<0.05 compared to SBI+Slit2 10 μg/Kg, @p<0.05 compared to SBI+Slit2+srGAP1 siRNA.
Recombinant Slit2 Reduced Cdc42 Activity After SBI by Robo1-srGAP1 Pathway

Cdc42 activity was significantly increased 24 hours after SBI (p<0.05 compared to sham, Fig. 8A), which was reduced with recombinant Slit2 10 µg/Kg administration (p<0.05 compared to vehicle). Recombinant Robo1 3 µg/Kg partially reversed the reduction in Cdc42 activity by recombinant Slit2 (p=0.097 compared to SBI+Slit2 10 µg/Kg, Fig. 8A), whereas srGAP1 siRNA reversed the reduction in Cdc42 activity by recombinant Slit2 (p<0.05 compared to Slit2, Fig. 8B).
Figure 8. Cdc42 activity assay in the right frontal perisurgical site at 24 hours after SBI. (A) Cdc42 activity was increased 24 hours after SBI which was significantly reduced by recombinant Slit2 10 μg/Kg. Robo1 co-administration partially reversed the effect of Slit2 on Cdc42 activity (p=0.097 compared to SBI+Slit2 10 μg/Kg). (B) srGAP1 siRNA but not scramble siRNA reversed the effect of Slit2 on Cdc42 activity. Data are expressed as mean ± SEM. N=5/group. ANOVA, SNK. *p<0.05 compared to Sham, †p<0.05 compared to SBI+Vehicle, &p<0.05 compared to SBI+Slit2 10 μg/Kg, @p<0.05 compared to SBI+Slit2+srGAP1 siRNA.
Discussion

In this study we focused on the anti-inflammatory function of recombinant Slit2 after SBI, particularly in terms of reducing peripheral immune cell migration to the brain after injury. We observed that endogenous Slit2 was upregulated after SBI, and knockdown of endogenous Slit2 worsened SBI induced brain edema. Robo1, the receptor for Slit2 was expressed by brain infiltrated peripheral immune cells. Administration of recombinant Slit2 prior to inducing SBI reduced brain edema, neurological deficits, neuroinflammation, and peripheral immune cell infiltration at the perisurgical site. Recombinant Slit2 reduced immune cell infiltration and Cdc42 activity after SBI was reversed by recombinant Robo1 co-administration as well as by inhibition of the downstream effector srGAP1. These results suggest that Slit2 may have protective function after SBI, and that recombinant Slit2 may be a beneficial candidate to reduce neuroinflammation.

Among members of the Slit family, Slit2 has important function in the brain especially in regulating migration of neurons and axons in the CNS during development (Marillat et al., 2002). However, its function in the adult brain is still being explored (Hagino et al., 2003). In addition, there are limited studies on the effect of Slit2 on neuroinflammation after brain injuries (Altay et al., 2007). Slit2 was detected at the peri-lesional site 2 days after traumatic brain injury and peaked at 7 days followed by a subsequent decline by day 14 after the injury in mice (Hagino et al., 2003). Consistent with these studies, our results showed that endogenous Slit2 increased at 24 hours, 72 hours and 7 days after SBI with a peak at 72 hours after injury. Slit2 was localized in neurons and astrocytes, which we speculate is the source for increase in endogenous Slit2 after SBI. This
observation complements previous studies that have reported Slit2 was expressed in the neurons and astrocytes (Hagino et al., 2003, Mertsch et al., 2008).

Roundabout 1 (Robo1), the receptor for Slit2 has been shown to mediate the anti-migratory function of Slit2 (Prasad et al., 2007, Yiin et al., 2009). Studies show that Slit2 exerts its repellent function on various cell types by binding to its receptor Robo1 (Guan and Rao, 2003). Robo1 is highly expressed by neutrophils (Tole et al., 2009) and lymphocytes (Wu et al., 2001) and moderately expressed by monocytes (Prasad et al., 2007). We observed that Robo1 expression increased at the perisurgical site after SBI and peaked at 24 hours and started decreasing 72 hours after injury. Robo1 was co-localized with the brain infiltrated peripheral immune cells.

To elucidate the role of endogenous Slit2 after SBI, we performed siRNA knockdown of endogenous Slit2 and evaluated brain edema and neurological function at 72 hours after SBI since endogenous Slit2 levels peaked at this time point. Slit2 knockdown worsened brain edema after SBI. However, there was no significant difference in neurological function after Slit2 siRNA knockdown compared to the SBI group. Given that Garcia test and beam balance test examine the overall gross sensorimotor deficits, it is possible that these tests were not sensitive enough to detect fine sensorimotor deficits which may result from the perisurgical site edema (Krafft et al., 2014, McBride et al., 2015). Additionally, these tests include parameters that evaluate function of the bilateral side and it is likely that the rats compensated for deficits using the uninjured side.
which may have reduced sensitivity of these tests (Krafft et al., 2014).

Nevertheless, our observations suggest that Slit2 is upregulated in response to brain injury possibly as an endogenous protective mechanism after SBI. Endogenous Slit2 may be protective after SBI in several ways. First, Slit2 is a secreted protein (Marillat et al., 2002) that may be secreted into the CSF and thence into blood stream where it may directly bind to Robo1 receptors on peripheral immune cells and thereby eventually slow down migration of these cells to the brain parenchyma. Interestingly, we observed that Robo1 expression started decreasing as the levels of endogenous Slit2 temporally increased after SBI. We speculate that Robo1 expression possibly decreased at 72 hours after SBI as a result of reduced immune cell infiltration by endogenous Slit2. Endogenous Slit2 may be elevated as a potential compensatory mechanism after injury. Second, it is possible that endogenous Slit2 may be increased to compensate for the endothelial barrier breakdown after injury. Slit2 was shown to reduce endothelial barrier permeability through binding to the endothelial specific receptor Robo4 (Jones et al., 2008, Jones et al., 2009). We speculate that Slit2 may be a compensatory mechanism to prevent further breakdown of the blood brain barrier, which could also reduce peripheral immune cell infiltration to the brain parenchyma after SBI. This could likely explain why endogenous Slit2 knockdown worsened brain edema. Lastly, Slit2 may exert direct anti-inflammatory effects by interacting with the Robo4 receptor. Previous study showed that Slit2 reduced pro-inflammatory cytokine and chemokine expression by directly interacting with the endothelial specific Robo4 receptor (Zhao et al.,
Although we did not investigate the protective mechanism of endogenous Slit2 after SBI, our results suggest that Slit2 has a protective function after SBI. Further investigation is needed to explore protective mechanism of endogenous Slit2 and the role of endothelial Robo4 receptor in Slit2 mediated protection.

Given that Slit2 has a protective function after SBI, next we examined whether exogenous recombinant Slit2 administration would have beneficial effects after SBI. Recombinant Slit2 was shown to protect against various systemic inflammatory conditions including renal, peritoneal and lung inflammation in rodents (Kanellis et al., 2004, Tole et al., 2009, Ye et al., 2010). Since neurosurgical procedures are planned events, it allows for pretreatment before injury is inflicted. Three doses of recombinant Slit2 were tested by evaluating outcomes at 24 and 72 hours after injury to establish the most effective dose to reduce SBI. Recombinant Slit2 10 µg/Kg reduced brain edema and improved performance in the Garcia test at both 24 and 72 hours after SBI. We therefore used this dose to complete subsequent experiments.

Inflammatory markers were shown to be elevated at the perisurgical site 24 and 72 hours after SBI (Yamaguchi et al., 2007, Hyong et al., 2008). Resident immune cells get activated due to the primary injury and release chemokines and pro-inflammatory cytokines that induce endothelial cells to upregulate adhesion molecules which promotes peripheral leukocyte trafficking to the injury site (Hickey, 1999, Ransohoff et al., 2003). A number of studies show that leukocytes accumulate in the CNS in response to an injury (Ransohoff et al., 2003, Wang and Dore, 2007, Ma et al., 2014). Infiltrated immune cells and
activated resident microglia release inflammatory mediators such as INF-γ and IL-1β that increases tissue injury and potentiates neuroinflammation (Yilmaz et al., 2006). Slit2 has been shown to have anti-migratory function including inhibition of leukocyte migration (Wu et al., 2001, Tole et al., 2009, London et al., 2010). We observed that recombinant Slit2 reduced expression of pro-inflammatory cytokines and peripheral immune cell markers at the perisurgical site. These results suggest that recombinant Slit2 mediates protection at least in part through reduction of neuroinflammation by decreasing brain infiltration of peripheral immune cells.

To elucidate the anti-migratory mechanism of recombinant Slit2, we explored the role of its receptor Robo1 (Guan and Rao, 2003) which we observed was expressed by the peripheral immune cells. The second LRR domain of Slit2 can directly bind with the first extracellular immunoglobulin (Ig) domain of Robo1 (Wong et al., 2002, Guan and Rao, 2003, Ballard and Hinck, 2012). Robo1 knockdown neutralized the inhibitory effect of Slit2 for cellular chemotaxis in vitro (Prasad et al., 2007, Mertsch et al., 2008). Altay et al. previously showed that recombinant Robo1 acts as a decoy receptor by binding to recombinant Slit2 which thereby makes less Slit2 available to bind to the Robo1 receptors on immune cells to exert its anti-migratory function (Altay et al., 2007). We therefore co-administered recombinant Robo1 along with recombinant Slit2 as a decoy receptor to neutralize the effects of recombinant Slit2. Our results complement the findings of Altay et al. and showed that recombinant Robo1 reversed the protective effect of recombinant Slit2 by increasing brain
infiltrated immune cell markers. This suggests that Slit2 regulates the migration of peripheral immune cells to the brain dependent on Robo1.

Slit-Robo GTPase Activating Protein 1 (srGAP1) is a downstream effector of Robo1 receptor (Wong et al., 2002). The SH3 domain of srGAP1 directly binds to the intracellular CC3 motif of Robo1 receptor (Wong et al., 2001, Guan and Rao, 2003). In the presence of Slit2, there is increased recruitment of srGAP1 to Robo1 which induces the downstream signal transduction pathways. srGAP1 inactivates Cdc42, a small GTPase protein, by converting the active GTP-bound form of Cdc42 to inactive GDP-bound form of Cdc42 (Wong et al., 2001, Yiin et al., 2009). Cdc42 is a critical mediator for cell migration (Bishop and Hall, 2000, Yiin et al., 2009). Activation of Cdc42 leads to filopodial extension at leading edge of migrating cells which enables cells to move forward (Bishop and Hall, 2000, Kumar et al., 2012). Our results showed that srGAP1 siRNA reversed the protective effects of recombinant Slit2 against SBI induced immune cell infiltration. Additionally, we observed that Cdc42 activity was reduced by recombinant Slit2 and this reduction was partially neutralized by recombinant Robo1 co-administration and reversed by srGAP1 siRNA. This suggests that Slit2 activation of the Robo1-srGAP1 pathway inhibits Cdc42 activity which possibly reduced peripheral immune cell infiltration and neuroinflammation after SBI.

In conclusion, our findings indicate that Slit2 contributes to protection against neuroinflammation after SBI. Administration of recombinant Slit2 reduced neuroinflammation after SBI by inhibiting peripheral immune cell infiltration.
possibly through Robo1-srGAP1 pathway mediated Cdc42 inactivation. These observations suggest that Slit2 may be a potential therapeutic option to reduce neurosurgical injury. Modulation of neuroinflammation by targeting peripheral immune cell infiltration may be beneficial to improve post-operative outcomes in neurosurgical patients.
References


CHAPTER 4

RECOMBINANT SLIT2 ATTENUATES BLOOD BRAIN BARRIER PERMEABILITY BY ROBO4 DEPENDENT RAC1 ACTIVATION AFTER SURGICAL BRAIN INJURY IN A RAT MODEL

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Abstract

Background and Purpose: Increase in blood brain barrier (BBB) permeability following neurosurgical procedures can worsen post-operative brain edema and neurological function. The extracellular matrix protein Slit2 has been shown to have vascular anti-permeability effects outside the brain. This study evaluated the effect of recombinant Slit2 and the role of its receptor roundabout4 (Robo4) and its downstream mediator paxillin in preserving the BBB integrity after surgical brain injury (SBI) in a rat model.

Methods: Adult male Sprague-Dawley rats (280-350 g) were subjected to sham or SBI surgery. Surgical brain injury was induced by partial resection of right frontal lobe. Recombinant Slit2 was administered by intraperitoneal injection 1 h before SBI induction. Robo4 siRNA or paxillin siRNA was given by intracerebroventricular injection 24 h before SBI. Evans blue dye extravasation, neurological function, western blot for BBB junction proteins, immunohistochemistry and Rac1 activity were evaluated 24 h after SBI.

Results: Recombinant Slit2 administration reduced Evans blue dye extravasation and stabilized occludin expression after SBI. Robo4 and paxillin was expressed by endothelial cells. The expression of paxillin was reduced after SBI which was normalized with recombinant Slit2 administration. Recombinant Slit2 increased Rac1 activity which was reversed by Robo4 and paxillin siRNA.

Conclusions: Recombinant Slit2 reduced BBB permeability after SBI, possibly by stabilizing the BBB tight junction through Robo4-paxillin mediated Rac1
activation. These findings suggest that Slit2 may be beneficial to stabilize neurosurgical injury to the BBB.

**Keywords**

Surgical brain injury, Blood brain barrier permeability, Slit2, Robo4, Paxillin, Rac1.
Introduction

Brain edema at the perisurgical site is a major pathophysiological consequence following neurosurgical procedures (Bruder and Ravussin, 1999, Bruder, 2002, Rolston et al., 2014). Post-operative edema has been reported to occur in 2 to 10% cases after cranial tumor resection (Wong et al., 2012). Increase in blood brain barrier (BBB) permeability contributes to perisurgical site brain edema following neurosurgical injury in experimental surgical brain injury (SBI) rodent model (Jadhav et al., 2007a, Yamaguchi et al., 2007). Disruption of BBB tight junction and adherens junction proteins increases paracellular permeability leading to the development of subsequent vasogenic edema which can worsen patient neurological outcomes (Nag et al., 2011). Current treatment regimes for post-operative brain edema do not target the specific BBB pathology and have limited use due to unwanted adverse effects (Li et al., 2014, Xu et al., 2014).

Slit2 is a secreted extracellular matrix protein known to regulate the migration of axons and neurons during development (Marillat et al., 2002, Ballard and Hinck, 2012). Recent studies show that Slit2 is increased in the brain following traumatic and surgical brain injury (Hagino et al., 2003, Sherchan et al., 2015), which implicates that Slit2 may have a role to play in the recovery after brain injury in adults (Marillat et al., 2002). Additionally, previous studies have demonstrated that Slit2 reduced endothelial hyperpermeability dependent on the endothelial specific receptor Robo4. In a model of glioma cocultured endothelial cells, Slit2 reduced blood tumor barrier (BTB) permeability which was inhibited with knockdown of the endothelial specific receptor Robo4 (Cai et al., 2015).
Likewise, recombinant Slit2 reduced vascular hyperpermeability in a Robo4 receptor dependent manner in mouse models of lung inflammation and retinopathy (Jones et al., 2008, London et al., 2010). The endothelial stabilizing effect of Robo4 has been shown to be mediated by the downstream effector paxillin, an intracellular adaptor protein (Jones et al., 2009).

The role of Slit2 in regulating BBB permeability after brain injury including SBI has not been explored. Based on these previous studies we propose that recombinant Slit2 will activate Robo4-paxillin signal transduction pathway which will attenuate BBB disruption after SBI in rats.

**Materials and Methods**

**Animals**

All procedures were approved by the Institutional Animal Care and Use Committee at Loma Linda University following the NIH Guide for the Care and Use of Laboratory Animals. Adult male Sprague Dawley rats (280-350 g) were subjected to either SBI or sham surgery. Animals were housed in humidity and temperature controlled environment with a 12 h light/dark cycle and free access to food.

**Experimental Design and Animal Groups**

Experiment 1. The BBB protective effect of recombinant Slit2 was evaluated. Rats were divided into 3 groups: Sham, SBI+Vehicle, SBI+Slit2 (10 µg/Kg). Recombinant Slit2 (R and D Systems, Minneapolis, MN, USA) or vehicle normal saline was injected by intraperitoneal route 1 h before SBI. Neurological
function was evaluated 24 h after surgery and brain samples from residual right frontal lobe were collected for Evans blue dye extravasation assay and to perform Western blot for BBB junction proteins.

Experiment 2. The expression and cellular localization of Robo4 and paxillin after SBI was characterized. Rats were divided into 5 groups: Sham, SBI 6 h, SBI 12 h, SBI 24 h, and SBI 72 h. Brain samples from the residual right frontal lobe were collected for immunohistochemistry and Western blot assay.

Experiment 3. The role of Robo4 and paxillin in recombinant Slit2 mediated protection was evaluated. Rats were divided into 6 groups: Sham, SBI+Vehicle, SBI+Slit2 (10 µg/Kg), SBI+Slit2 (10 µg/Kg)+Robo4 siRNA, SBI+Slit2 (10 µg/Kg)+Paxillin siRNA, SBI+Slit2 (10 µg/Kg)+Scramble siRNA. Recombinant Slit2 or vehicle normal saline was injected intraperitoneally 1 h before SBI. The siRNAs for Robo4, paxillin and scramble siRNA (all from Life Technologies, Grand Island, NY, USA) was injected by intracerebroventricular (ICV) route 24 h before SBI. Neurological function was evaluated at 24 h after surgery and brain samples were collected for Western blot and Rac1 activity assay.

**Surgical Brain Injury Rat Model**

Rats were subjected to surgical brain injury as previously described (Jadhav et al., 2007b, Yamaguchi et al., 2007). Anesthesia was induced with 4% isoflurane and maintained at 2.5% using a nasal mask. A midline skin was made in the scalp and periosteum was reflected to expose the underlying frontal bone. After identifying the bregma, a square craniotomy 5x5 mm was made on the right
frontal bone with left lower corner towards bregma by using a micro drill. The margins of the bone window were made 2 mm lateral to sagittal suture and 1 mm proximal to coronal suture. The underlying dura was incised and a partial resection of the right frontal lobe was performed along margins of the bone window with the depth of resection extending to base of skull. Hemostasis was achieved with normal saline irrigation and intraoperative packing following which the skin incision was sutured. Sham animals were subjected to right frontal craniotomy but without dural incision or frontal lobe resection. Buprenorphine 0.03 mg/Kg was injected subcuatenously for post-operative analgesia. Rats were closely observed post-operatively for complete recovery from anesthesia and then returned back to home cages.

Intracerebroventricular Injection

Rats were anesthetized with isoflurane and placed prone on a stereotactic frame. Intracerebroventricular (ICV) injection into the right lateral ventricle was performed by making a burr hole using the following coordinates relative to bregma: 1.0 mm lateral, 1.5 mm posterior and 3.2 mm below the horizontal plane of bregma as previously described (Suzuki et al., 2010, Chen et al., 2013). A 10 µL Hamilton syringe (Hamilton Co, Reno, NV, USA) was inserted through a burr hole to inject the siRNAs. A total volume of 2 µL either Robo4 siRNA (500 pmol) or paxillin siRNA (500 pmol) (Life Technologies, Grand Island, NY, USA) was injected at a rate 0.5 µL/min as previously described (Suzuki et al., 2010, Chen et al., 2013). The same volume of scramble siRNA (Life Technologies, Grand Island, NY, USA) was injected as a negative control. The needle was left in situ
for additional 10 mins after the completion of injection followed by slow withdrawal over 5 mins to prevent leakage. The burr hole was sealed with bone wax and skin incision was sutured. Rats were closely observed for complete recovery from anesthesia and then returned back to home cages.

**Neurological Evaluation**

The modified Garcia test was performed to evaluate sensorimotor deficits after SBI as previously described (Garcia et al., 1995, Ostrowski et al., 2005, Yamaguchi et al., 2007). Briefly, the Garcia test evaluated six parameters that included: spontaneous activity, symmetry in the movement of all four limbs, forepaw outstrecthing, climbing, body proprioception, and response to vibrissae touch. A total score of 21 was given, and higher scores indicated better performance.

**Evans Blue Dye Extravasation**

Evans blue dye extravasation assay was performed to assess BBB permeability as previously described (Jadhav et al., 2007a, Suzuki et al., 2010). Briefly, Evans blue dye (2%; 5 mL/kg) was given by intraperitoneal injection and allowed to circulate for 4 h after injection. The rats were transcardially perfused with phosphate buffered saline (PBS) after which the brain samples were removed and snap frozen in liquid nitrogen and stored at -80°C until use. The right frontal region was homogenized in PBS (1 mL/300 mg) and then centrifuged at 14,000 rpm for 30 mins after which the supernatant was collected. An equal amount of trichloroacetic acid (50%) was added to 500 μL of the supernatant and
allowed to incubate overnight at 4ºC. The supernatant was centrifuged using the same parameters next day and quantity of extravasated Evans blue dye was measured at 620 nm with a spectrophotometer.

**Western Blot Analysis**

Western blot was performed as previously described (Hasegawa et al., 2011, Huang et al., 2015). Briefly, brain samples were collected after rats were transcardially perfused with phosphate buffered saline (PBS) and then homogenized in Ripa lysis buffer (Santa Cruz Biotechnology, Dallas, TX, USA) for protein extraction. The supernatants from the samples were collected after centrifuging at 14,000 g at 4ºC for 30 mins, and protein concentration in the samples was determined using a detergent compatibility assay (Biorad, Irvine, CA, USA). Equal amounts of protein (50 µg) were loaded on to sodium dodecyl sulfate polyacrylamide gel for electrophoresis and then transferred onto nitrocellulose membranes. The membranes were then incubated overnight at 4ºC with the following primary antibodies: anti-occludin (1:50,000) (Abcam, Cambridge, MA, USA) and anti-VE cadherin (1:200), anti-Robo4 (1:200) and anti-paxillin (1:500) (all from Santa Cruz Biotechnology, Dallas, TX, USA). The same membranes were probed with anti-β Actin (1:2,000) (Santa Cruz Biotechnology, Dallas, TX, USA) as loading controls. This was followed by incubation of the membranes with appropriate secondary antibodies (1:4,000) (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at room temperature. The bands were visualized using ECL Plus Chemiluminescence kit (Amersham Biosciences, Arlington Heights, IL, USA) and quantified using Image J software.
Results were expressed as relative density to β-actin and then normalized to average value of the sham group as previously described (Hasegawa et al., 2011).

**Immunohistochemistry**

Briefly, after PBS perfusion and post-fixation in formalin, brain samples were sectioned into 10-µm-thick slices using a cryostat (CM3050S; Leica Microsystems, Bannockburn, IL, USA). Immunofluorescence staining was performed as previously described (Hasegawa et al., 2011, Altay et al., 2012a). The sections were incubated overnight at 4°C with the following primary antibodies: anti-Robo4 (1:100) or anti-paxillin (1:100) (both from Santa Cruz Biotechnology, Dallas, TX, USA) and co-localized with anti-von willibrand factor (vWF) (1:100) (Santa Cruz Biotechnology, Dallas, TX, USA) or anti-glial fibrillary acidic protein (GFAP) (1:1,000) (Abcam, Cambridge, MA, USA). Sections were then incubated with FITC- and Texas Red-conjugated appropriate secondary antibodies (1:200) (Jackson Immuno Research, West Grove, PA, USA) for 2 h at room temperature and visualized with a fluorescence microscope (Olympus BX51).

**Rac1 Activity Assay**

A pulldown assay was performed using the Rac1 activity assay kit (Cell Biolabs, San Diego, CA, USA) as previously described (Wojciak-Stothard and Ridley, 2002, Raz et al., 2010). Briefly, samples were mixed with PAK1-PBD agarose beads and incubated at 4°C for 1h after which the agarose beads were
separated by centrifugation. The beads were then re-suspended in sample buffer and then subjected to 10% polyacrylamide gel electrophoresis followed by transfer to nitrocellulose membrane. The membrane was then probed with anti-Rac1 specific antibody provided in the kit to detect the GTP-bound Rac1.

**Statistical Analysis**

Statistical analysis was performed using the Sigma Plot 10.0 and Sigma Stat version 3.5 (Systat Software, San Jose, CA, USA). Data were presented as mean ± SEM. Statistical differences between groups were analyzed using one-way ANOVA for multiple comparisons followed by Student-Newman-Kuels or Fisher L SD post hoc tests. P values less than 0.05 was considered statistically significant.

**Results**

*Recombinant Slit2 Administration Attenuated BBB*

**Permeability 24 Hours After SBI**

The permeability of BBB was evaluated using Evans blue dye extravasation assay at 24 h after SBI. Rats subjected to SBI had significantly increased extravasated dye in the perisurgical right frontal region (p<0.05 compared to sham, Fig 1A). Recombinant Slit2 significantly reduced Evans blue dye extravasation at the perisurgical site (p<0.05 compared to vehicle).
Figure 1. Effect of recombinant Slit2 on BBB stability in the right frontal perisurgical site 24 h after SBI. (A) Evans blue dye extravasation assay showed increased leakage of the dye in perisurgical site after SBI which was significantly reduced with recombinant Slit2 10 μg/Kg administration. (B) Representative Western blot and quantitative analysis showed that the expression of occludin was significantly decreased after SBI. Recombinant Slit2 10 μg/Kg significantly increased occludin expression compared to vehicle group. (C) Representative Western blot and quantitative analysis showed that the expression of VE cadherin did not change after SBI. Data are expressed as mean ± SEM. *p<0.05 compared to sham, †p<0.05 compared to vehicle.
Recombinant Slit2 Preserved Tight Junction Protein

Expression 24 Hours After SBI

The BBB junction proteins occludin and VE cadherin in the right frontal region was evaluated using Western blot analysis 24 h after SBI. The expression of occludin was significantly reduced in the perisurgical site 24 h after SBI (p<0.05 compared to sham, Fig. 1B), and recombinant Slit2 significantly increased occludin expression (p<0.05 compared to vehicle). However, the expression of VE cadherin was not significantly different between sham or SBI groups at 24 h (p>0.05, Fig. 1C).

Cellular Localization of Robo4 After SBI

Immunofluorescence staining of brain samples was performed to delineate the cell types that express Robo4 receptor 24 h after SBI. Double immunofluorescence staining showed that Robo4 was expressed by endothelial cells and neurons but not astrocytes in the perisurgical site after SBI (Fig. 2).
Figure 2. Localization of Robo4 in the brain after SBI. Representative microphotographs of immunofluorescence staining showed co-localization of Robo4 (FITC/green) with endothelial marker von Willibrand Factor (vWF) (Texas Red/red) and DAPI in the perisurgical site at 24 h after SBI. The lower panel shows that Robo4 (FITC/green) was expressed by neurons but did not co-localize with the astrocyte marker glial fibrillary acidic protein (GFAP) in the perisurgical site. Scale bar=50 μm.
**Temporal Expression and Cellular Localization of Paxillin**

*After SBI*

The temporal expression of paxillin in the perisurgical site was measured using Western blot analysis at 6h, 12h, 24h and 72h after SBI. The expression of paxillin was not significantly different from sham at 6h, 12h and 24h after SBI (p>0.05 compared to sham, Fig. 3A). However, the expression of paxillin started to decrease at 24 h after SBI and was significantly reduced at 72 h after SBI (p<0.05 compared to sham). Double immunofluorescence staining showed that paxillin co-localized with endothelial cells and astrocytes at the persurgical site 24 h after SBI (Fig. 3B).
Figure 3. Temporal expression and localization of paxillin in the brain after SBI. (A) Representative Western blot and quantitative analysis showed that the expression of paxillin started to decrease 24 h after SBI and showed a significant decline at 72 h after SBI. Data are expressed as mean ± SEM. *p<0.05 compared to Sham, &p<0.05 compared to SBI at 6 h, ‡p<0.05 compared to SBI at 12 h. (B) Representative microphotographs of immunofluorescence staining showing co-localization of paxillin (FITC/green) with endothelial marker von Willibrand Factor (vWF) or astrocyte marker glial fibrillary acidic protein (GFAP) (Texas Red/red) and DAPI in the perisurgical site at 24 h after SBI. Scale bar=50 μm.
Recombinant Slit2 Increased Rac1 Activity After SBI

Dependent on Robo4-Paxillin Pathway

The assay for Rac1 activity was performed at 24 h after SBI. Recombinant Slit2 significantly increased Rac1 activity (p<0.05 compared to vehicle, Fig. 4A) which was reversed with administration of Robo4 siRNA (p<0.05 compared to Slit2) or paxillin siRNA (p<0.05 compared to Slit2) but not with scramble siRNA (p>0.05 compared to Slit2). In addition, paxillin siRNA significantly worsened neurological function compared to Slit2 10 µg/Kg or scramble siRNA which was evaluated using Garcia test 24h after SBI (p<0.05 compared to Slit2 and scramble siRNA, Fig. 4B) whereas, Robo4 siRNA partially worsened neurological function compared to Slit2. Robo4 siRNA but not scramble siRNA effectively suppressed the expression of Robo4 (p<0.05 compared to Slit2 and scramble siRNA, Fig. 4C). Likewise, paxillin siRNA but not scramble siRNA significantly suppressed the expression of paxillin (p<0.05 compared to Slit2 and scramble siRNA, Fig. 4D).
Figure 4. Role of Robo4-paxillin in Slit2 mediated protection after SBI. (A) Rac1 activity assay showed that recombinant Slit2 10 μg/Kg significantly increased Rac1 activity in the right frontal perisurgical site at 24 h after SBI. Robo4 siRNA and Paxillin siRNA reversed this effect but not scramble siRNA. (B) Garcia test showed significantly worse neurological function after SBI. Paxillin siRNA administration along with Slit2 10 μg/Kg significantly worsened neurological function compared to Slit2 10 μg/Kg or scramble siRNA. (C) Representative Western blot image and quantitative analysis showed that the expression of Robo4 was significantly reduced with Robo4 siRNA administration but not with paxillin or scramble siRNA. (D) Representative Western blot image and quantitative analysis showed that paxillin siRNA but not scramble siRNA significantly reduced paxillin expression compared to Slit2 10 μg/Kg. Data are expressed as mean ± SEM. *p<0.05 compared to Sham, †p<0.05 compared to SBI+Vehicle, @p<0.05 compared to SBI+Slit2 10 μg/Kg, #p<0.05 compared to SBI+Slit2+Robo4 siRNA, $p<0.05 compared to SBI+Slit2+paxillin siRNA.
Discussion

This study examined the BBB protective effects of recombinant Slit2 administration in a rat SBI model. Recombinant Slit2 reduced BBB permeability which was associated with preservation of the endothelial tight junction protein. The BBB protective effects of recombinant Slit2 was possibly mediated by stabilization of tight junction through Robo4-paxillin dependent activation of Rac1 after SBI.

Although primarily involved in regulating the migration of developing axons and neurons (Marillat et al., 2002), recent studies have demonstrated that Slit2 can exert its effects on other cells types including endothelial cells and is involved in regulating vascular integrity (Cai et al., 2015). Slit2 was shown to inhibit VEGF-induced endothelial hyperpermeability in vitro and in mouse models of retinal and choroidal vascular disease (Jones et al., 2008). In accordance with previous studies, we observed that recombinant Slit2 administration reduced extravasation of the albumin-bound Evans blue dye into the perisurgical site after SBI suggesting that Slit2 reduced BBB permeability possibly by preserving the endothelial junction integrity.

The BBB integrity is maintained by interendothelial tight junction and adherens junction proteins (Nag et al., 2009). Tight junction proteins such as occludin and claudin-5 maintain barrier function of the BBB, and disruption of tight junction proteins enhances the endothelial barrier permeability after injury to the brain (Altay et al., 2012b). Various factors such as inflammation, oxidative stress and proteases can degrade tight junction proteins after brain injury (Altay et al., 2012b, Krafft et al., 2013). Previous studies show that tight junction
proteins were degraded in the perisurgical site following SBI thereby increasing BBB permeability and brain edema (Yamaguchi et al., 2007). The endothelial protective function of Slit2 has been shown to be associated with stabilization of inter-endothelial junctions in vitro (Gorbunova et al., 2013). In accordance, we observed that recombinant Slit2 administration increased the expression of occludin suggesting that Slit2 regulates the expression of endothelial tight junction proteins after SBI. However, we did not observe any change in the expression of adherens junction protein VE cadherin after SBI with or without Slit2 administration. Furthermore, Slit2 inhibited VEGF induced retinal permeability in Robo4+/+ mice but not in Robo4 null mice suggesting that Slit2-dependent inhibition of VEGF induced permeability was mediated via Robo4 activation (Jones et al., 2008). We therefore investigated the role of Robo4 in Slit2 mediated stabilization of BBB after SBI.

Among the Robo family receptors, Robo4 is known to be the endothelial specific receptor for Slit2 ligand (Huminiecki et al., 2002, Ballard and Hinck, 2012). Robo4 receptor has been demonstrated to be expressed by human brain microvascular endothelial cells (Cai et al., 2015). Consistent with these findings, we observed that Robo4 was expressed by endothelial cells at the perisurgical site in SBI rats. Furthermore, previous study showed that transgenic overexpression of Robo4 by glioma cocultured endothelial cells reduced BTB permeability by preserving tight junction protein expression in vitro (Cai et al., 2015). We speculate that recombinant Slit2 binds to Robo4 receptors on the endothelial cells and regulates the expression of tight junction proteins.
Interestingly, we also found that Robo4 was expressed by neurons but not astrocytes surrounding the perisurgical site. However, the regulation of Robo4 expression after SBI remains to be further explored.

Next, we investigated the role Robo4 and its downstream mediator paxillin in Slit2 mediated BBB stabilization by Rac1 activation. Paxillin is a critical downstream effector of Robo4 in endothelial cells (Jones et al., 2009). The Paxillin Interaction Motif (PIM) in the cytoplasmic tail of Robo4 directly interacts with Lim domain of paxillin (Jones et al., 2009). Binding of Slit2 ligand to Robo4 increases the interaction of Robo4 with paxillin and Arf-GAP complex (Jones et al., 2009). The Arf-GAP complex inactivates Arf6 which is a small GTPase protein that induces the internalization of Rac1 (Palacios et al., 2001, Turner et al., 2001). However, in the presence of Slit2, the interaction between Robo4-paxillin increases and Arf6 gets inactivated thereby restoring Rac1 activity. The small GTPase Rac1 is an important mediator known to stabilize adherens junction and tight junction complexes that maintain endothelial barrier integrity (Wojciak-Stothard and Ridley, 2002, Waschke et al., 2004). We observed that recombinant Slit2 administration increased Rac1 activity after SBI which was reversed with siRNA mediated knockdown of either Robo4 or paxillin. Based on these findings, we propose that Slit2 preserved BBB integrity after SBI possibly by Robo4-paxillin mediated activation of Rac1 which stabilized the tight junction. We observed that paxillin was expressed by endothelial cells and astrocytes surrounding the perisurgical site. Interestingly, we also observed that the expression of paxillin decreased following SBI and was normalized with
recombinant Slit2 administration. Previous study showed that paxillin was localized at the focal adhesions in the cell periphery and Slit2 exposure redistributed paxillin from focal adhesions to the cell surface (Jones et al., 2009). It is likely that recombinant Slit2 redistributed paxillin to the cell surface and restored its expression after SBI-induced injury. However, in depth evaluation of the regulation of paxillin after SBI and with Slit2 remains to be studied.

In conclusion, our findings suggest that recombinant Slit2 reduced BBB permeability by stabilizing endothelial tight junction after experimental SBI possibly via Robo4-paxillin dependent Rac1 activation.
References


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CHAPTER 5
DISCUSSION AND CONCLUSION

Summary of Findings

This project examined the potential role of the extracellular matrix protein, Slit2 and its receptor Roundabout (Robo) in reducing complications following neurosurgical injury. We specifically examined the contribution of Slit2 in reducing neuroinflammation and blood brain barrier (BBB) disruption after surgical brain injury (SBI) using a rat model. The major observations and conclusions obtained from the project have been summarized in this section.

*Endogenous Slit2 has a Protective Function After SBI*

Our findings from Chapter 3 show that endogenous Slit2 has a protective function after SBI. We observed that endogenous Slit2 was upregulated after SBI starting at 24 h upto 7 days after the injury, and knockdown of endogenous Slit2 using siRNA worsened brain edema and neurological function after SBI. In addition, we observed that Slit2 was expressed by neurons and astrocytes after the injury, which we speculate is the source for increase in endogenous levels of Slit2 as a protective response to injury. These findings complement previous study in which Slit2 was shown to be increased after traumatic brain injury (Hagino et al., 2003). Although the function of endogenous Slit2 in adults after brain injury has not been explored, our findings that endogenous Slit2 knockdown worsened outcomes after SBI suggests a protective role of Slit2 following brain injury.
Recombinant Slit2 Reduces SBI Induced Complications

Our findings from Chapter 3 show that recombinant Slit2 pretreatment reduced SBI induced brain edema and neurological deficits in SBI rats. The improved outcomes with recombinant Slit2 pretreatment was associated with reduced neuroinflammation and peripheral leukocyte migration to the perisurgical site after SBI. Post-operative brain edema is one of the major complications encountered in neurosurgical patients (Wong et al., 2012). Neuroinflammation and disruption of the BBB can worsen brain edema and post-operative neurological function (Bruder and Ravussin, 1999, Hyong et al., 2008, Huang et al., 2015), which were both attenuated with recombinant Slit2. Additionally, our results from Chapter 4 show that recombinant Slit2 reduced BBB permeability and stabilized the BBB tight junction at the perisurgical site after SBI. These findings support the therapeutic potential of recombinant Slit2 to reduce neuroinflammation and BBB disruption following brain injury.

Robo Receptor Mediated Signaling Pathways Contribute to the Protective Effects of Slit2

The Robo family of receptors is known to be activated by the Slit2 ligand (Guan and Rao, 2003, Ballard and Hinck, 2012). We evaluated the role of Robo1 and Robo4 receptors in Slit2 mediated protection after SBI. Our findings in Chapter 3 show that Robo1 was expressed by peripheral immune cells that infiltrated at the perisurgical site after SBI. Our results suggest that inactivation of Cdc42 via Robo1-srGPA1 pathway contributed to the inhibition of peripheral immune cell migration by recombinant Slit2. Knockdown of Robo1 or srGAP1
reversed the anti-migratory effect of recombinant Slit2 on the peripheral immune cells. Likewise, our findings in Chapter 4 show that Robo4 was expressed by the endothelial cells. The BBB protective function of recombinant Slit2 was associated with Robo4-paxillin mediated Rac1 activation, and knockdown of Robo4 or paxillin reversed Slit2 mediated Rac1 activation. These findings suggest that Robo1 and Robo4 receptors are primarily involved in mediating the protective effects of Slit2 by modulating the downstream signaling pathways.

**Scientific Contribution**

This project was focused on studying SBI, which is an important clinical problem that has largely been overlooked due to a lack of understanding of the occurrence of the problem. Even though adverse events such as brain edema and post-operative neurological deficits often occur following neurosurgical procedure (Bruder and Ravussin, 1999, Rolston et al., 2014), the pathophysiology of SBI is often not studied. This project explored two major pathophysiological events that occur after SBI. First, the role of peripheral immune cell migration in mediating neuroinflammation after SBI was examined particularly with a focus on Robo1 mediated immune cell migration to the injury site. Second, post-operative brain edema resulting from BBB junction disruption was examined with a focus on restoring BBB stability after SBI.

This project has identified novel molecular mechanisms involved in Slit2 mediated neuroprotection. Slit2 has primarily been studied in the developing nervous system and few studies have explored the role of Slit2 in adult brain (Marillat et al., 2002, Mertsch et al., 2008). Even thought previous studies have
shown that Slit2 reduced injury outside the CNS in animal models of renal and lung inflammation (Kanellis et al., 2004, Ye et al., 2010), the function of Slit2 after brain injury has been relatively unexplored. Slit2 was shown to be increased after experimental traumatic brain injury (Hagino et al., 2003) and exogenous Slit2 was beneficial in a cerebral ischemia rodent model (Altay et al., 2007). However, functional significance of Slit2 after brain injury and the protective mechanism of Slit2 were not determined. Our findings in this project provide evidence that Slit2 has a protective role after brain injury. We have elucidated previously unknown dual function of Slit2 after brain injury, which includes reducing neuroinflammation by inhibiting the migration of peripheral immune cells and antipermeability effects by stabilizing the BBB tight junction. Furthermore, our findings demonstrate that the protective mechanism of Slit2 is mediated via Robo1 receptor expressed by peripheral immune cells and Robo4 receptor expressed by BBB endothelial cells. These novel findings show that exogenous Slit2 administration targets two major pathophysiological events after SBI and thereby augments the body’s endogenous protective response against SBI.

Overall, these findings provide evidence that Slit2 has beneficial role after SBI and is a potentially suitable therapeutic candidate to reduce deleterious consequences after SBI and possibly other brain injuries with similar pathologies.

**Limitations and Future Directions**

The major limitations in this project are discussed in this section.
Role of Other Members of the Slit Protein Remains to be Elucidated

This project primarily focused on exploring the role of Slit2 after SBI even though there are three known isoforms of Slit proteins (Slit1-Slit3) (Guan and Rao, 2003, Hohenester, 2008). Studies show that other Slit members may regulate processes in the CNS too. For instance, Slit1 is predominantly expressed in the developing CNS and Slit3 has been reported to have anti-permeability effects following VEGF induced retinal hyperpermeability (Marillat et al., 2002, Jones et al., 2008). Given these findings, it is likely that Slit1 and Slit3 may be involved during recovery after CNS injury. However, Hagino et al. observed that Slit2 was predominantly expressed surrounding the lesion after traumatic brain injury in mice while the expression of Slit1 and Slit3 was much weaker (Hagino et al., 2003). In accordance, we observed that the expression of Slit2 was increased after SBI. However, we did not evaluate if Slit1 and Slit3 exhibit any changes after SBI. Neither did we explore therapeutic potential of the other Slit members after SBI. A detailed examination of all members of the Slit family after brain injury is warranted.

Alternate Pathways in Slit2 Mediated Protection Need to be Evaluated

This project examined two primary pathways in Slit2 mediated protection after SBI which included the Robo1-srGAP1 pathway regulating neuroinflammation and the Robo4-paxillin pathway maintaining the endothelial barrier stability by recombinant Slit2. However, Slit2 can activate alternate
pathways, which was not explored in this project.

For instance, (1) Downstream effectors such as Abelson kinase (Abl) and Enabled (Ena) can interact with the intracellular motif of Robo and regulate cell migration. Abl antagonizes the repulsive effects of Robo whereas, Ena enhances Robo mediated repulsive signaling pathway (Ballard and Hinck, 2012). (2) We measured Cdc42 activity as the downstream substrate of srGAP1 but other small GTPases including RhoA and Rac1 that regulate cellular actin organization and motility were not evaluated in this project (Wojciak-Stothard and Ridley, 2002, Waschke et al., 2004). (3) Previous studies have shown that Slit2 can inhibit chemokine induced Src kinase activity and Lck kinase activity which have been reported to regulate cell migration (Prasad et al., 2007). (4) The endothelial barrier protective effect of Slit2 may be mediated by modulation of matrixmetalloproteinase (MMP)-9 activity which was not explored (Cai et al., 2015). (5) Lastly, using knockout or transgenic animals would strengthen the findings in our project. It was previously observed that Robo4 null mice had increased severity of lung injury than Robo4+/+ mice when subjected to lung inflammation (London et al., 2010), which proposes a protective function for Slit2-Robo4. However, Slit2 transgenic mice were more susceptible to collagenase induced ICH and had larger hemorrhagic volumes compared to control mice (Han and Geng, 2011). The findings in this study conflict with the protective effects of Slit2 observed in other animal models of CNS injury (Altay et al., 2007, Sherchan et al., 2015). This suggests that endogenous Slit2 overexpression and exogenous Slit2 possibly have diverse functions and mechanisms on the
endothelial cells.

Additionally, the findings in these studies could be attributed to differences in the animal model used, differential mechanism of the endogenous versus exogenous Slit2, or due to differences in the magnitude of exposure to Slit2 in transgenic animals as opposed to exogenous recombinant Slit2 administration. Although the temporal pattern and duration of Slit2 expression in transgenic animals subjected to injury and the mechanism of injury was not explored, these findings necessitate further exploration of the function of Slit2 after brain injury.

**Detailed Exploration of the Robo Receptor Subtypes is Required**

The effect of Slit2 may depend on the type of receptor predominantly expressed following an injury. Furthermore, the expression and function of Robo receptors may be tissue specific and context dependent. For instance, Slit2 reduced permeability in human pulmonary microvascular endothelial cells (PMECs) that predominantly express Robo4 (Gorbunova et al., 2013), but the anti-permeability effect of Slit2 was not observed in the human umbilical vein endothelial cells (HUVECs) which express similar levels of Robo4 and Robo1 receptors (Gorbunova et al., 2013). Therefore, an in depth understanding of the expression of Robo receptors and changes that may occur after SBI is required.

**Upstream Regulators of Slit2 Need to be Explored**

The transcription factor such as Pax6 has been shown to regulate the expression of Slit2 (Tsuchiya et al., 2009). Additionally, changes in SLIT2 gene,
for instance hypermethylation of the gene in systemic tumors and glioma have been observed (Dallol et al., 2003). Our findings showed that endogenous Slit2 levels increased after SBI as a protective response to counteract the consequences of SBI. However, the upstream regulators for Slit2 were not examined. Further exploration into the regulation of endogenous Slit2 is essential to understanding this response.

Conclusion

Our findings suggest that Slit2 has a beneficial role against neuropathological consequences after SBI. The protective effect of Slit2 against SBI was possibly mediated by its anti-migratory function against peripheral leukocytes and by endothelial barrier stabilization dependent on the Robo receptors. The dual protective function of Slit2 makes it a potential therapeutic option to reduce neurosurgical injury and improve post-operative outcomes in neurosurgical patients.
References


