THE STUDY OF GLIAL SCAR FORMATION AFTER BRAIN ISCHEMIA USING IN VITRO STRATEGIES

For the degree of Master in Science (M.Sc.)

INTERNATIONAL MASTER PROGRAM IN BIOMEDICAL SCIENCES

(IMBS)

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Cohort 2013-2015

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Abstract

Reactive gliosis is a generic response to Central Nervous System (CNS) injury mediated by astrocytes and microglia. Following ischemic damage to the CNS parenchyma, the injured area becomes surrounded by a dense astroglial cell layer known as glial scar. Glial scar formation has been recognized for many decades as a major impediment for neuronal reconnection and a serious obstacle for functional recovery. However, more recent studies have shown that scar limits the area of damage, preventing the diffusion of blood-derived activated immune cells into the CNS that could cause a generalized proinflammatory-neurodegenerative response.

In spite that it has been morphologically recognized for many years since Ramon y Cajal times, to study the biochemical signaling cascades involved in glial scar formation has been difficult mostly because of the *in vivo* nature of the process.

In this context, we studied here the mechanisms of glial scar assembly/disassembly *in vitro* to identify potential pharmacological targets for therapeutic interventions. To achieve this goal we will use the classical 2-Dimensional (2D) astroglial cultures, but we will also develop 3-dimensional (3D) astroglial cultures by using nanotube matrixes to attempt to better reproduce the *in vivo* situation. The results of this thesis showed that meningeal macrophages or ischemia-activated macrophages induce astroglial retraction and formation of scar-like structures in vitro. Scar-forming astrocytes over-express GFAP, S100B and TLR2-4. Using the NF-κB antagonist BAY-11-7082 we demonstrated that scar formation and its density is partially NF-κB dependent. Finally, in 3D astroglial culture grown on hydromatrix nanotubes, we showed that DAMPs can induce astroglial polarization but not the formation of the glial scar in vitro. We conclude that TLR/ NF-κB pathway is probably implicated in the glial scar formation or stabilization and that DAMPs and macrophages are necessary for the formation of glial scars in vitro.

Acknowledgements

First of all I would like to express my thanks and gratitude to Dr. Alberto Javier Ramos for giving me the opportunity to do my Master´s Thesis work in his lab. He has been an excellent mentor and motivator, and he is always full of good advice. He treats his lab members like his close friends and keeps the lab atmosphere lively with his great sense of humour. I have had good friends and good mentors, but for the first time I have a good friend and mentor in Javi. He will be one of the sources of inspiration for my future career.

Thank you to Dr. Roland Mertelsmann for Co-Directing my Master´s Thesis and for creating a wonderful program, the IMBS!

I would like to thank Dr. Veronica Murta for being a wonderful teacher. I have learned a lot of things from her. I hope I will put in to practice at least some of them! Without her guidance I would not have made it.

For my every day survival in the lab, I want to thank Gerardo Rosciszewski, Alicia Rossi, Vanesa Cadena, Jeronimo Lukin and Jeronimo Auzmendi. They have been wonderful colleagues and Amigos!

I would like to thank to the IBCN members for helping me with equipment, the tissue culture room and microscopy facilities.

I would like to thank María Esquiroz and Yvonne Weigand for their help and support during the last two years.

Last, Big thanks to my family and friends for their unconditional love and support.

Abbreviations

AFA: Astrocyte free areas

ATP: Adenosine triphosphate

BBB: Blood brain barrier

CBF: Cerebral blood flow

CSPGs: Chondroitin sulfate proteoglycans

DAMP: Damage-associated molecular pattern

DMEM: Dulbecco´s Modified Eagle Medium

ECM: Extracellular matrix

GFAP: Glial fibrillary acidic protein

GFP: Green fluorescent protein

HBSS: Hank's balanced Salt Solution

HMGB-1: High Mobility Group Box-1

IκB: Inhibitor kappaB

IKK: Inhibitor kappaB kinase

IL-1: Interleukin-1

iNOS: Inducible nitric oxide synthase

LPS: Lipopolysaccharide

NF-κB: Nuclear Factor kappaB

NG2: Neural/glial antigen 2 (NG2 chondroitin sulfate proteoglycan)

NO: Nitric oxide

PAMP: Pathogen-associated molecular pattern

S100B: S100 calcium-binding protein B

TLR: Toll like receptors

TLR2: Toll like receptor 2

TLR4: Toll like receptor 4

TNF-α: Tumor necrosis factor alpha

INTRODUCTION

Brain ischemia and its clinical presentation in Stroke:

Brain remains as one of the organs we are still unable to provide a solid connection between the function of individual cellular players and the function of the organ itself. A critically important area of neuroscience research today is to understand the pathophysiology of stroke, one of the most common neurological diseases. Cerebrovascular diseases (CVDs), which account for 55% of all neurological diseases, are the leading cause of permanent disability, cognitive and motor disorders and dementia.

World Health Organization (WHO) produced a detailed report in 2008 showing the worldwide incidence of neurological disorders (WHO, 2008). In that report it was shown that only in 2005, chronic diseases accounted for 35 million (60%) of the 58 million deaths that occurred worldwide. Heart disease was responsible for 11.7 million of these deaths and stroke, the second most common cause of death, caused 5.8 million. Forty percent of stroke deaths occurred in people <70 years of age. More recently, it was calculated that globally, approximately 15 million new acute stroke events occur every year, and approximately 55 million people have had a stroke at some point in the past. Two thirds of these individuals live in low- and middle-income countries. Every two seconds, someone across the globe suffers a symptomatic stroke (Pandian et al., 2007). The risk of cerebrovascular disease is disproportionately higher in low to middle income countries where, in addition, the stroke patients' care is difficult to reach to these regions. During the last two decades a major transformation in the stroke field took place with the emergence of evidence-based approaches to stroke prevention, acute stroke management, and stroke recovery. The current challenge lies in implementing these interventions, particularly in regions with high incidence of stroke and limited healthcare resources, as the emerging countries.

Well-organized stroke services and emergency transport services are lacking, many treatments are unaffordable, and socio- cultural factors may influence access to medical care for many stroke victims (Lindsay et al., 2014). By 2050, it is anticipated that 80% of stroke events will occur in people living in low and middle income regions. Stroke should be a priority on the health and basic research agenda in developing countries. The experience of high-income nations clearly shows that a significant improvement in the outcome of stroke patients can be achieved with sustained interventions. We should consider that

reaching a 2% reduction per annum in stroke mortality due to better management, would result in 6.4 million fewer deaths from stroke (Pandian et al., 2007).

Unfortunately there are only a few options to treat stroke patients. The immediate goal of acute ischemic stroke therapy is to save the ischemic penumbra through recanalization of the occluded cerebral blood vessel. This is currently attempted through thrombolytics, which are pharmacological agents that can break up a clot blocking the blood flow. To date, the only approved thrombolytic for treatment of acute ischemic stroke is the recombinant tissue plasminogen activator (Alteplase®, r-tPA). However, r-tPA has enormous limitations due to small time window and high costs, as well as concerns regarding adverse bleeding risk due to the hemorrhagic conversion of the stroke (Balami et al., 2013). Other than thrombolytic drugs, there are not yet good treatments that minimize brain tissue loss and dysfunction after stroke. Over 1000 drug trials for stroke have now failed (O'Collins et al., 2006). In most of these trials, neurons were exclusively targeted, for instance using drugs that blocked neuronal glutamate receptors (Appendix 1). Yet the extent to which glial are killed in stroke and the consequences of this cell loss for the surrounding neuronal networks has received relatively little attention. Also there is a crucial need to more fundamentally understand why the Central Nervous System (CNS) is so much more vulnerable to ischemia than non-CNS tissues.

Excitotoxicity is certainly a central element of ischemic damage that is unique to the CNS and it was the main reason that aimed the basic and clinical research on strategies to antagonize the glutamate excitotoxicity after stroke during the last 20 years. However, the massive failure of these strategies in the clinical field (see appendix 1) makes necessary to deeply re-analyze the mechanisms underlying this differential sensitivity of CNS to ischemia. Going to the most straightforward hypothesis, it could be considered that neurons have exactly the same intrinsic vulnerability to ischemia as any other cell type and that the greater brain vulnerability to ischemia might simply rely on the lower redundancy and repair ability of the brain compared with liver or kidney. Although it is simple, this hypothesis does not consider the complexity of brain networks, the task division and crosstalk among different CNS cell types that allows brain function and information processing. The neurovascular unit hypothesis presented in 2006 by Del Zoppo (Del Zoppo, 2006) has been a main addition to understand brain response to ischemia. Neurovascular unit consists of

microvessels (endothelial cells-basal lamina matrix-astrocyte end-feet and pericytes), astrocytes, neurons and their axons, and other supporting cells (e.g., microglia and oligodendroglia) that present different functions but also show a clear division of metabolic activities and functional crosstalk (Figure 1). This *unit* provides a framework for bi-directional communication between neurons and their supply microvessels with the participation of the intervening astrocytes. The neurovascular unit accounts both for cellular intercommunication and nutrient perfusion at the same time (Del Zoppo et al., 2006). Virtually every aspect of brain development and function involves a neuron-glial partnership; therefore the answer to every important question about brain disease will also involve glia. In the context of the neurovascular unit, a better strategy would be to understand how glial pathology contributes to neuronal dysfunction and vice versa (Lobsiger and Cleveland, 2007).

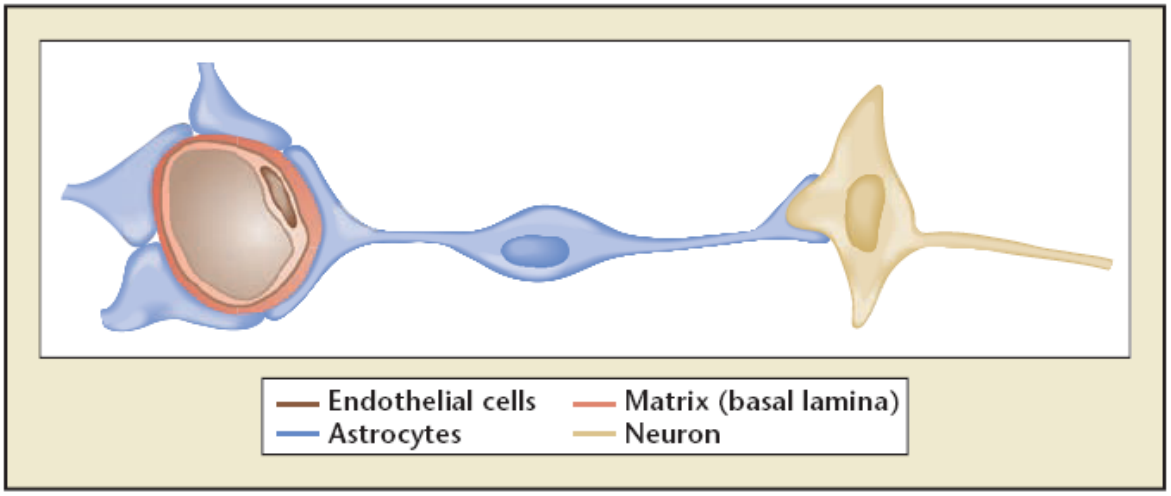

Figure 1. **The neurovascular unit.** A simplified depiction of the relationship between neuron and axon location and a supply microvessel. The astrocyte end-feet form the abluminal portion of capillaries and small microvessels via their attachment to the basal lamina matrix, and astrocyte arborizations contact neurons (Del Zoppo et al., 2010).

Ischemic core and penumbra:

The concept of the ischemic penumbra was initially proposed by Astrup in 1981 and represented an important milestone for understanding the temporal and spatial evolution

of focal ischemic brain injury. Acute stroke causes an irreversibly damaged ischemic core and a potentially salvageable surrounding tissue named *penumbra*, a reversibly injured brain tissue around ischemic core; which is the pharmacological target for acute ischemic stroke treatment (Astrup et al. 1981).

The penumbra concept suggests that different areas within the ischemic region evolve into irreversible brain injury over time and that this evolution is most critically linked to the severity of decline in cerebral blood flow (CBF). The ischemic penumbra was initially defined as a region of reduced CBF with absent spontaneous or induced electrical potentials that still maintained ionic homeostasis and transmembrane electrical potentials. This narrow definition has evolved, and 2 subsequent definitions should be focused upon. Hossmann (1999) characterized the ischemic penumbra as "*a region of constrained blood supply in which energy metabolism is preserved*". Ginsberg and Pulsinelli (1994), proposed that energy metabolism might be intermittently compromised within the ischemic penumbra. If the ischemic penumbra is characterized by these approaches, then a reduction of CBF to levels between a lower threshold of 10–15 mL/100 g/min and an upper threshold of approximately 25 mL/100 g/min is likely to identify penumbral tissue. The ischemic core of infracted tissue has a CBF value below the lower threshold. The upper threshold for CBF decline approximates that level associated with the development of neurologic deficits and is substantially less severe than the CBF decline associated with loss of electrical activity, as proposed by the original ischemic penumbra concept. The penumbra had a CBF rate below normal, but it was higher than the focal zone (Ischemic core). This reduced CBF caused cessation of electrical signals but not loss of ion homeostasis. Thus, the penumbral cells are still viable but their metabolism is reduced because the CBF was lower than normal (25% to 50%), exhibiting acidosis, edema and decreased protein synthesis. It is argued that electric failure and membrane failure occurred at different CBF, and that this was the underlying reason for the two zones (Figure 2).

It was also argued that the necrosis that characterizes the ischemic core slowly invades the area of the penumbra, recruiting more and more cells that will eventually die by apoptosis due to delayed neuronal death. Any potential intervention in focal ischemic stroke would have to focus on preventing the spread of the necrotic zone into the penumbra, since

these are the cells at risk after a stroke. Hence, the important goal in stroke treatment is to save the penumbra as much and as early as possible.

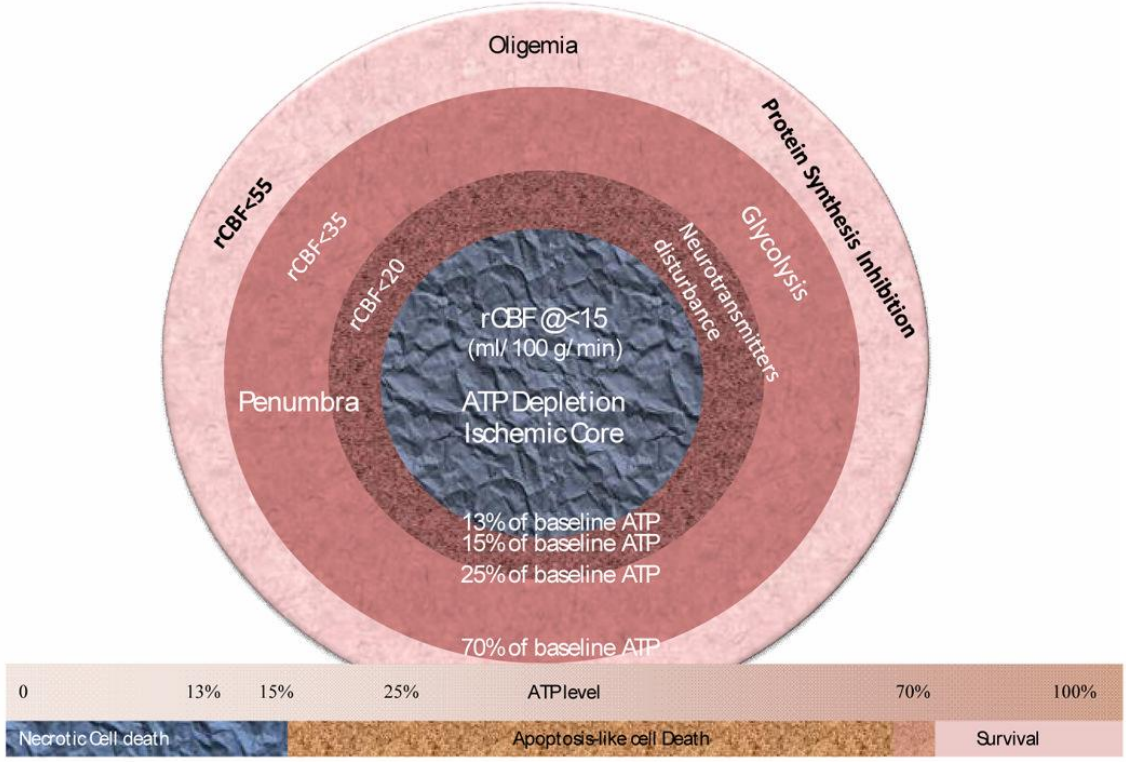

Figure 2. **Correlation between energy thresholds and blood flow thresholds.** The form and pathway of cell death closely are closely associated with energy state levels. Blood flow reduction causes specific metabolic disturbances at certain blood flow thresholds. The ischemic core has depleted ATP level whilst the penumbra has gradient reduction of ATP level between normal tissue and ischemic core (Liu et al., 2010).

Glial cells in the CNS:

Oligodendrocytes:

Oligodendrocytes are the myelinating glia in the CNS and wrap their membranes around axons to form myelin. In addition to providing insulation and trophic support to neuronal axons, oligodendrocytes are active participants in nervous system function, sculpting the structural and electrical properties of axons by controlling their diameter, as well as the spacing and clustering of ion channels at nodes and paranodes. The molecular mechanisms that enable oligodendrocytes to recognize, ensheath, and wrap axons are not completely understood (Chan et al., 2004). Oligodendrocytes are also lost in brain trauma and spinal cord injury either directly or as an indirect result of axon injury, degeneration and

inflammation. Axonal signals, not yet identified, are required for the differentiation of Oligodendrocyte Precursor cells (OPCs) into oligodendrocytes and for the survival of oligodendrocytes (Ben Barres 2008). More recently it has been shown that oligodendrocytes crosstalk not only with the axons that wrap with myelin, but also interact with microglia and astrocytes (Peferoen et al., 2014).

Microglia:

Microglia are the professional immune system cells that are resident in the CNS and constitute about 10% of CNS glia (Hanisch and Kettenmann, 2007; Soulet and Rivest, 2008). Normally blood-derived macrophages are situated in the perivascular space, whereas microglia is located within the brain parenchyma. Like perivascular macrophages of the brain, microglia is derived from uncommitted myeloid progenitor cells but they invade the brain neonatally (Santambrogio et al., 2001). Although microglia displays at least some phagocytic ability, they do not appear to have the strong professional phagocytic ability exhibited by activated macrophages (Ben Barres 2008). In addition to affecting synaptic activity, microglia plays an important role during CNS development in mediating the selective elimination of inappropriate synaptic connections during the formation of mature neural circuits.

Microglia express Toll-Like Receptors (TLR) and NOD-like receptors (NLR), allowing them to detect Pathogen-Associated Molecular Patterns (PAMPs) and the molecular signatures of injury named Damage-Associated Molecular Patterns (DAMPs). The PAMP/DAMP-TLR interaction usually leads to the transcription of NF-κB dependent proinflammatory cytokine genes. Local (Suzuki et al., 2011) and systemic (Püntener et al., 2012) infections, neurodegenerative conditions (Browne et al., 2013), and sterile injury (Wang 2010) have been reported to activate microglia. Once activated, microglia retracts their ramifications and acquires an amoeboid morphology, becoming indistinguishable from activated macrophages. Resident microglia and peripheral macrophages play pivotal roles in the post-ischemic inflammation. As both cell types derive from primitive myeloid cells, they express many shared markers (CD11b, F4/80, Iba-1) (Ginhoux et al., 2010), complicating the study of microglial activity by immunohistochemistry. Like macrophages, microglia can have

either an M1, classically activated phenotype, “classically activated via toll-like receptors or interferon-γ”, or an M2, alternatively activated phenotype, “alternatively activated by interleukin 4 or interleukin 13” (Perry et al., 2010)(Figure 3). M1 microglia are considered to be proinflammatory and secrete TNF-α, iNOS, and CCL2. They express CD80, CD86, and MHCII on their cell surface, possessing the capability to present antigens to T cells (Starossom et al., 2012). They express IL-23, giving implications that microglia T-cell crosstalk may occur. Microglia also produces IL-1β and IL-18 through activation of the inflammasome (Ransohoff and Brown 2012).

M2 microglia are interpreted to be healing cells that have a role in neuroprotection and repair after injury showing arginase activity and upregulation of neurotrophic factors (Kawanokuchi et al., 2008)(Figure 3). Because of this M1 or M2 polarization, microglia has the potential to contribute to the inflammatory secondary injury or to facilitate neuroprotection in CNS disease and injury. Microglia also shows an extensive crosstalk with astrocytes.

Astrocytes release signals such as CSF-1 and ATP that can signal to microglia, whereas microglia release signals such as TNF-α that can signal to astrocytes and modulate their response to injury. Microgliosis and reactive astrocytosis generally occur together, but it is not clear whether there is a causal connection and, if so, in which direction. Nor is there agreement on whether lessening either type of gliosis will be helpful or harmful for neuronal survival after CNS injury (Ben Barres 2008).

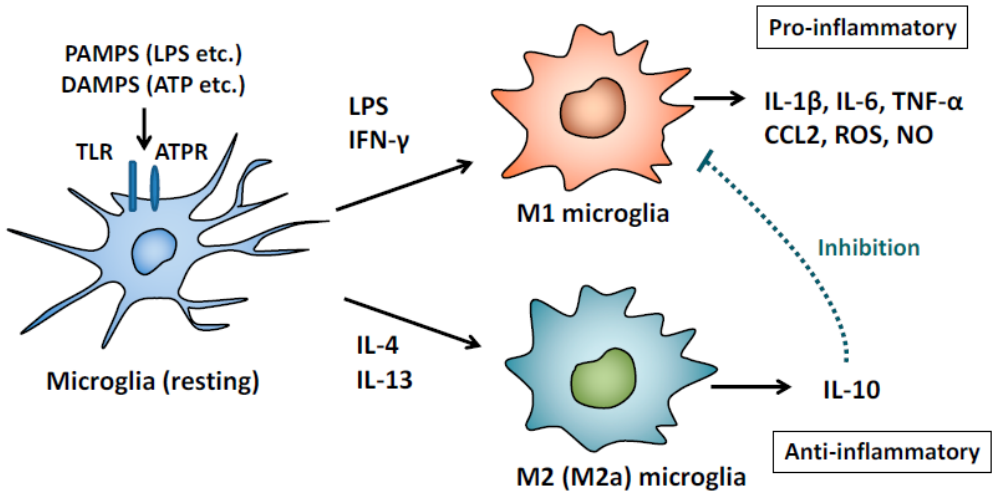

Figure 3. **Microglia polarization is characterised by distinct phenotypes** (Nakagawa Y, Chiba K 2014).

Astrocytes:

Astrocytes constitute nearly half of the cells in the human brain. Classical functions of astrocytes giving physical and metabolic support to neurons were described several decades ago, but more recent findings have unveiled a plethora of astroglial functions in healthy and injured CNS (Figure 4). Astrocytes fall into at least two main classes distinguished by morphology, antigenic phenotype, and location. Accordingly, it is speculated that they most likely differ in some of their main functions; however these differences are far from being demonstrated in vivo. Protoplasmic astrocytes are found throughout all gray matter and exhibit morphology of several stem branches that give rise to many finely branching processes in a uniform globoid distribution. On the other hand, fibrous astrocytes are found throughout all white matter and exhibit a classical morphology with many long fiber-like processes intermingled among myelinated axons (Ramon y Cajal 1909).

In gray matter, a single astrocyte extends thousands of fine membranous processes that ensheath synapses and microvasculature (Araque et al., 1999; Ventura and Harris, 1999; Bushong et al., 2002). Beyond their protoplasmic or fibrous morphologically defined phenotypes, gene profiles indicate a surprising amount of regional astrocyte heterogeneity that has been underestimated (Zhang and Barres, 2010).

Astrocytes have a main role in neuronal development, and thus it is a common view in the field that those mechanisms are re-called during the regenerative processes occurring in the long term after focal CNS injury. During CNS development, molecular boundaries formed by astrocytes take part in guiding the migration of developing axons and certain neuroblasts (Powell and Gelle 1999). In latter steps, astrocytes are essential for the formation and functional activation of developing synapses by releasing molecular signals such as thrombospondin (Ben Barres, 2008, Christopherson et al., 2005, Ullian et al., 2001). Astrocytes secrete a protein, not yet identified, that induces postsynaptic glutamate (AMPA) responsiveness of neurons (Christopherson et al., 2005). Astrocytes appear also to influence developmental synaptic pruning by releasing signals that induce expression of complement C1q in synapses and thereby tag them for elimination by microglia (Ben Barres 2008, Stevens et al., 2007). Interestingly, several evolutionarily conserved phagocytic pathways

were found to be highly enriched in astrocytes including the Draper/Megf10 and Mertk/integrin alpha (v) beta5 pathways, suggesting that mammalian astrocytes have the potential of being professional phagocytes, which leads to an interesting possibility that astrocytes are actively mediating synapse elimination by phagocytosis using these pathways during development, normal adulthood, or after injury (Chung et al., 2013).

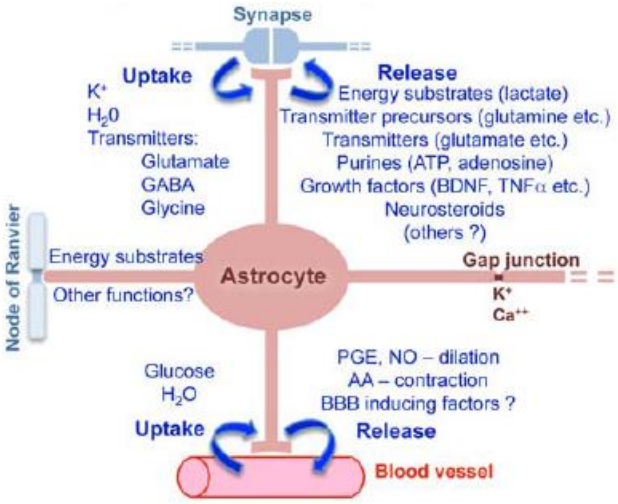

Figure 4. **Astrocyte functions in healthy CNS** (Sofroniew and Vinters, 2010)

The blood brain barrier (BBB) is a diffusion barrier that impedes the influx into brain parenchyma of certain molecules on the basis of polarity and size (Abbott et al., 2006, Ballabh et al., 2004). The principal cellular constituents of the BBB are cerebral capillary endothelial cells that form tight junctions and are surrounded by a basal lamina, perivascular pericytes, and astrocyte endfeet. Studies suggest a role for astrocytes in regulating BBB properties of cerebral endothelial *in vivo* in adult mice through specific signaling mechanisms on astrocyte endfeet that when disrupted cause BBB leaks (Araya et al., 2008).

Astrocytes make extensive contacts and have multiple bidirectional interactions with blood vessels, including regulation of local CNS blood flow. Recent findings show that astrocytes produce and release various molecular mediators, such prostaglandins (PGE), nitric oxide (NO), and arachidonic acid (AA), that can increase or decrease CNS blood vessel diameter and blood flow in a coordinated manner based on the neuronal activity and local environmental conditions (Gordon et al., 2007; Iadecola and Nedergaard, 2007; Koehler RC et al., 2009).

Astrocyte processes are rich in the aquaporin 4 (AQP4) water channel and in transporters for the uptake of K^+ (Seifert et al., 2006, Simard et al., 2004, Zador et al., 2009). AQP4 water channels are densely clustered along astrocyte processes that contact blood vessels and play a critical role in regulating fluid homeostasis in healthy CNS and play roles in both vasogenic and cytotoxic edema. Astrocyte processes envelop essentially all synapses (Brown and Ransom 2007; Peters et al., 1991) and exert essential functions in maintaining the fluid, ion, pH, and transmitter homeostasis of the synaptic interstitial fluid in a manner that is critical for healthy synaptic transmission. However, these astrocytic end-feet surrounding microvasculature are also responsible for a further reduction in blood perfusion in areas surrounding ischemic core when cell swelling and cytotoxic edema occurs (Nedergaard and Dirnagl, 2005).

Beyond their role in the synaptic maturation, stabilization and pruning commented above, astrocyte processes at synapses also play essential roles in transmitter homeostasis by expressing high levels of transporters for neurotransmitters such as glutamate, GABA, and glycine that serve to clear the neurotransmitters from the synaptic space (Figure 4) (Sattler et al., 2006).

Bidirectional communication occurs between neurons and astrocytes. This means that synapses do not consist of just pre- and postsynaptic neuronal elements, but also have an astrocytic projection that envelops the synapse. This close spatial relationship has led to the hypothesis of the *tripartite synapse*, to acknowledge the astrocytes contribution (Figure 5). The synaptic localization of astrocytes means they are ideally placed to monitor and respond to synaptic activity. Astrocytes play direct roles in synaptic transmission through the regulated release of synaptically active molecules including glutamate, purines (i.e. ATP and adenosine), GABA, and D-serine (Halassa MM et al., 2007, Nedergaard Met al., 2003, Perea G et al., 2009, Shigetomi E et al., 2008). The release of such gliotransmitters occurs in response to changes in neuronal synaptic activity, involves astrocyte excitability as reflected by increases in astrocyte intracellular calcium , and can alter neuronal excitability (Halassa et al., 2007, Nedergaard et al., 2003, Perea et al., 2009, Shigetomi et al., 2008). In spite of being a prevalent view during the last 15 years, the concept of a regulated, vesicular release of gliotransmitters has been recently seriously questioned. Fujita and colleagues (2014) demonstrated that the transgenic mice expressing dnSNARE in astrocytes used to

demonstrate the *in vivo* role of the vesicular release of gliotransmitters present leaky neuronal transgene expression that could led to a misinterpretation of the results (Fujita et al., 2014; Sloan and Barres, 2014).

Astrocytes have the potential to exert long-term influences on synaptic function through the release of growth factors and related molecules. Astrocytes are also sources of neuroactive steroids (neurosteroids), including estradiol, progesterone and various intermediaries and metabolites that can have synaptic effects, particularly at GABA receptors (Garcia-Segura and Melcangi, 2006).

Astrocytes, which have processes that on the one hand contact blood vessels and on the other hand contact neuronal perikarya, axons (at nodes of Ranvier), and synapses, are well positioned to take up glucose from blood vessels and furnish energy metabolites to different neural elements in gray and white matter (Figure 4). Astrocytes are the principal storage sites of glycogen granules in the CNS and the greatest accumulation of astrocytic glycogen occurs in areas of high synaptic density (Peters et al., 1991, Phelps 1972). Compelling evidence now demonstrates that astrocytic glycogen utilization can sustain neuronal activity during hypoglycemia and during periods of high neuronal activity (Brown and Ransom 2007, Suh et al., 2007).

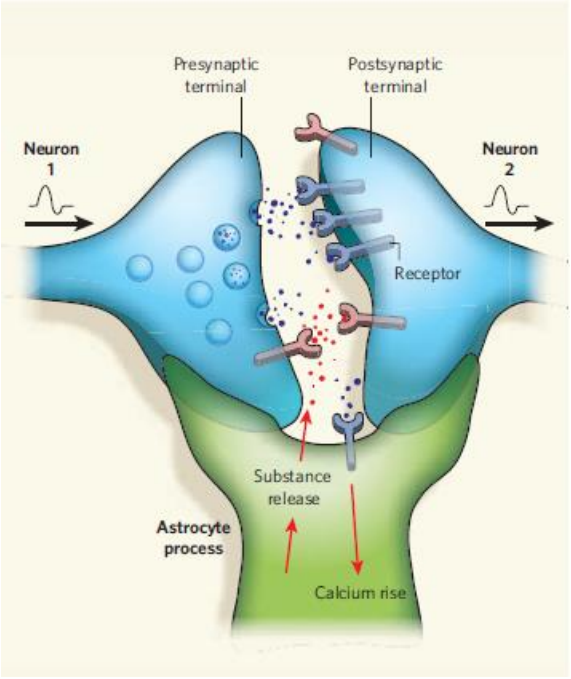

Figure 5. **A tripartite synapse.** Astrocytes express many of the same receptors as neurons. When neurotransmitters are released from the presynaptic terminal of a neuron, astrocytic receptors are thought to be activated, leading to a rise in calcium ions in the astrocyte and the release of various active substances, such as ATP, which act back on neurons to either inhibit or enhance neuronal activity. However this evidence obtained *in vitro* remains to be clarified in vivo (Sloan and Barres, 2014) Astrocytes also release proteins, which control synapse formation, regulate presynaptic function and modulate the response of the postsynaptic neuron to neurotransmitters (Allen and Barres, 2009).

Reactive Gliosis and glial scar formation is a generic response to brain injury:

Astrocytes respond to all forms of insults by a generic process commonly referred to as reactive astrogliosis. Reactive astrogliosis is not simply an all-or-none phenomenon, but rather a constitutive, graded, multi-stage, and evolutionary conserved defensive astroglial reaction ranging from subtle alterations in gene expression to scar formation (Sofroniew, 2009). Although the increasing severity of reactive astrogliosis transition seamlessly along a continuum, it is convenient for purposes of description and classification to recognize three broad categories as originally proposed by Sofroniew in 2009:

Mild to moderate reactive astrogliosis. In mild or moderate reactive astrogliosis there is variable up regulation of genes, as well as hypertrophy of cell body and processes that occurs within the domains of individual astrocytes (Wilhelmsson et al., 2006) without substantive intermingling or overlap of processes of neighbouring astrocytes or loss of individual domains, and with increased GFAP expression (Figure 6b). Because there is little reorganization of tissue architecture, if the triggering injury is resolved, then mild or moderate reactive astrogliosis exhibits the potential for resolution in which the astrocytes return to an appearance similar to that in healthy tissue (Sofroniew 2009).

Severe diffuse reactive astrogliosis. In severe diffuse reactive astrogliosis there is pronounced up regulation of expression of GFAP and other genes, together with hypertrophy of cell body and processes, as well as astrocyte proliferation, resulting in considerable extension of processes beyond the previous domains of individual astrocytes. As a result, there is substantive intermingling and overlapping of neighbouring astrocyte processes with disruption of individual astrocyte domains (Figure 6c). These changes can result in long-lasting reorganization of tissue architecture that can extend diffusely over substantive areas. Severe diffuse reactive astrogliosis is generally found in areas surrounding severe focal lesions, infections or areas responding to chronic neurodegenerative injury.

Severe reactive astrogliosis with compact glial scar formation. Severe reactive astrogliosis with compact glial scar formation includes pronounced up regulation of GFAP and other genes, and pronounced hypertrophy of cell bodies and processes. In addition, groups of reactive astrocytes exhibit overlapping of reactive astrocyte processes, loss of individual

astrocyte domains, evidence of substantive astrocyte proliferation, and the clear formation of dense, narrow, and compact glial scars (Figure 6d). Recent experimental evidence indicates that these astrocyte scars act as neuroprotective barriers to inflammatory cells, and that they form in particular along borders to severe tissue damage necrosis, infection or autoimmune-triggered inflammatory infiltration (Wanner et al., 2013). An important feature of these glial scars is the deposition of a dense chondroitin sulphate proteoglycans (CSPG) extracellular matrix that inhibits axonal growth. Triggering insults include penetrating trauma, severe contusive trauma, invasive infections or abscess formation, neoplasm, chronic neurodegeneration or systemically triggered inflammatory challenges. Glial scar formation is associated with substantive tissue reorganization and structural changes that are long lasting and persist long after the triggering insult may have resolved.

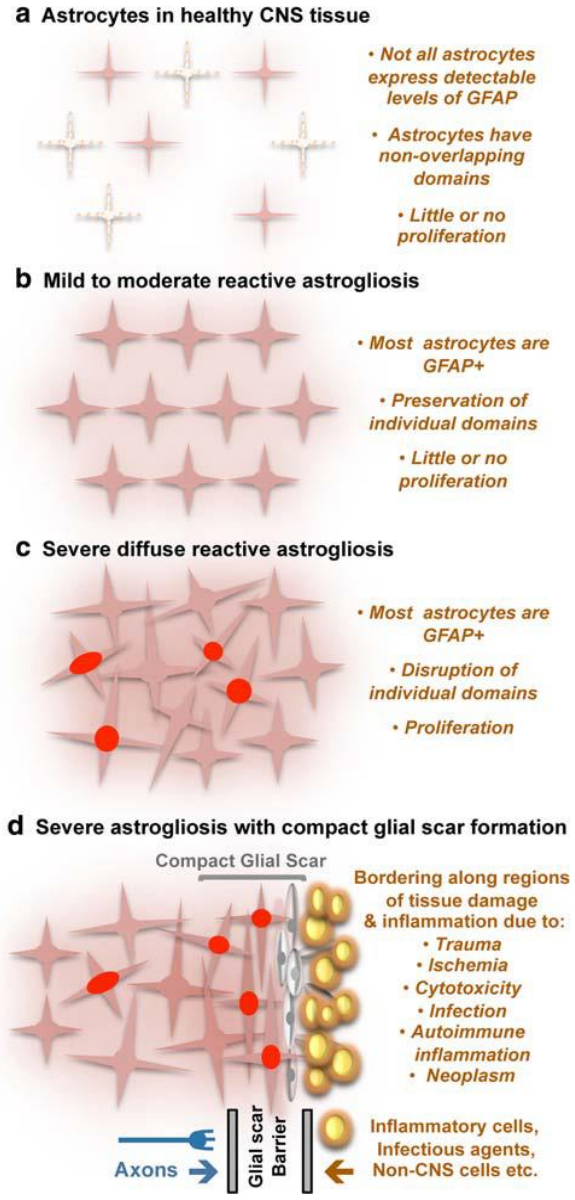

Figure 6. **Schematic representations that summarize different levels of reactive astrogliosis.** a) Astrocytes in healthy CNS tissue. b) Mild to moderate reactive astrogliosis comprises variable changes in molecular expression and functional activity together with variable degrees of cellular hypertrophy. c) Severe diffuse reactive astrogliosis includes changes in molecular expression, functional activity and cellular hypertrophy, as well newly proliferated astrocytes (with red nuclei in figure). d) Severe reactive astrogliosis with compact glial scar formation occurs along borders to areas of overt tissue damage and inflammation, and includes newly proliferated astrocytes (with red nuclei in figure) and other cell types (gray in figure) such as fibromeningeal cells, blood derived macrophages, NG2 OPCs, as well as deposition of dense extracellular matrix. In the compact glial scar, astrocytes have densely overlapping processes (from Sofroniew and Vinters, 2010).

Reactive astrogliosis and glial scar formation play essential roles in regulating CNS inflammation. In response to different kinds of stimulation, reactive astrocytes express and secrete different molecules with either pro- or anti-inflammatory potential (Eddleston et al., 1993, John et al., 2003), and reactive astrocytes can exert both pro- and anti-inflammatory effects on microglia/macrophages (Farinas et al., 2007, Min et al., 2006). A large body of experimental studies indicates both pro- and anti-inflammatory regulatory functions in vivo are regulated by specific molecular signalling in a context-dependent manner that is pathways (Burda and Sofroniew 2014). Reactive astrocytes exert different activities at different times after insults, or in different geographical locations in relation to lesions, as determined by context-specific signalling mechanisms. For example, reactive astrocytes may exert pro-inflammatory roles at early times after insults and in the centre or immediate vicinity of lesions, but exert anti-inflammatory roles at later times and at the borders between lesions and healthy tissue.

Apart from the hallmark features of hypertrophy of astrocyte cellular processes and upregulation of intermediate filament proteins, in particular the upregulation of glial fibrillary acidic protein (GFAP), reactive astrogliosis and glial scar formation are also accompanied with increased production of CSPGs, inducible NO synthase (iNOS), and calcium-binding protein S100B, etc. (Table 1). These factors are considered as the functional markers of astrocyte reactivity whose levels are upregulated following CNS injuries (Sofroniew 2009, Escartin et al., 2008, Kang et al., 2011, Eddleston et al., 1993).

Many different types of intercellular signalling molecules are able to trigger reactive astrogliosis, including: i) Large polypeptide growth factors and cytokines such as IL6, LIF, CNTF, TNF-α, INFc, TGF-β, FGF2, etc. secreted by glia or blood derived macrophages; ii) Mediators of innate immunity such as PAMPs and DAMPS by activating Pattern Recognition Receptors like RAGE and TLR (Villarreal et al., 2014); iii) Neurotransmitters such as glutamate and noradrenalin; iv) Purines such as ATP; v) Reactive oxygen species (ROS) including nitric oxide (NO); vi) Hypoxia and glucose deprivation; vii) Products associated with neurodegeneration such as β –amyloid or with systemic metabolic toxicity such as $NH4^+$ (reviewed in Sofroniew, 2009; Burda and Sofroniew 2014).

Evidence suggests that pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 may act as the initial triggers of reactive astrocytes in the acute phase of spinal cord injury (Kordek et al., 1996, Lin et al 2006). However, accumulating evidence is showing that DAMP gradient originated by focal CNS lesions can be sufficient to induce a graded reactive gliosis as seen *in vivo* (Villarreal et al., 2014; Burda and Sofroniew, 2014). At later stages, other mediators including autocrine molecules like S100B are able to maintain astrocytes reactivity (Vitellaro-Zuccarello et al 2008, Buffo et al., 2010; Villarreal et al., 2014). Interestingly, reactive astrocytes can release the majority of these triggering molecules themselves, which in turn activate more distal astrocytes, microglia and other cell types. It is not completely understood how activated microglia and leukocytes play a modulatory role in astrogliosis and it remains to be elucidated the complex crosstalk among these cell types after focal CNS injury.

STAT3 signalling has been implicated as a key mediator of astrocytic scar formation after SCI (Herrmann et al., 2008, Okada et al 2006). STAT3 conditional knockout mice failed to form a typical glial scar after SCI and that led to a widespread lesion and poor recovery of function. Lack of STAT3 activation particularly resulted in the inability of astrocytes to migrate to the lesion site and contain the injured area. This resulted in exacerbated influx of inflammatory cells at the site of SCI (Herrmann et al., 2008, Okada S et al 2006; Wanner et al., 2013). This evidence emphasized the impact of STAT3 activation in astrocytes and the importance of reactive astrogliosis and glial scar formation in restraining leukocyte infiltration and reducing the initial tissue damage after SCI. Role of NF-κB in glial scar formation has not been studied, but there are several studies showing the activation of NF-κB pathway in astrocytes following SCI (Wanner et al., 2013), brain ischemia (Villarreal et al., 2011, 2014), sleep apnea (Aviles Reyes et al., 2010). Moreover, blockage of the NF-κB pathway or upstream RAGE pattern recognition receptor showed to reduce reactive gliosis *in vivo* in a model of sleep apnea (Angelo et al., 2014).

Like RAGE, TLR are also pattern recognition receptors that are critical components of the innate immune system activated after ischemic injury in the CNS. TLR activation induce the nuclear translocation of the transcription factor nuclear factor-kappa B (NF-κB), leading to the subsequent expression of several proinflammatory genes (reviewed in Hanke and Kielian, 2011).

Nuclear transcription factor-κB (NF-κB), which can be activated by stimuli associated with damage and in turn can promote expression of pro-inflammatory cytokines, is thought to play a central role in most inflammatory responses. When the CNS is damaged, the resident cell types that activate NF-κB mount a response have not been fully defined, but are likely to include astrocytes; microglia and blood derived infiltrating cells.

Table 1. **Different functional categories of genes and molecules modulated by reactive astrocytes in vivo or in vitro.** ↑↓ arrows indicate upregulation or downregulation. Note that some families of molecules (e.g. cytokines) simultaneously can have some members that are ↑ regulated and some that are ↓ regulated. In addition, some individual molecules (e.g. glutathione) can be either ↑ or ↓ regulated by different triggers of astrogliosis or at different times after insults (Sofroniew MV 2009).

Functional categories	Molecular changes in reactive astrocytes
Structural	GFAP ↑; Vimentin ↑; Nestin ↑
Transcriptional regulators	NF-κB ↑; STAT3 ↑; cAMP ↑; Olig2 ↑; mTor ↑; SOX9 ↑; etc.
Extracellular matrix and cell–cell interactions	Chondroitin sulfate proteoglycans ↑↓; collagens ↑↓; laminins ↑; integrins ↑; Cadherins ↑↓; ephrins ↑; metalloproteases ↑
Inflammatory cell regulators	Cytokines and growth factors ↑↓; glutathione↑↓
Fluid and ion homeostasis	AQP4 ↑↓; Na/K transporters ↑↓
Extracellular transmitter clearance	Glutamate transporter ↑↓
Vascular regulators	PGE ↑↓; NO ↑↓
Purines and receptors	ATP ↑↓; P2Y ↑↓
Oxidative stress and protection	NO ↑↓; NOS ↑; SOD↑↓ ; glutathion ↑↓
Gap junction proteins	Cx43 ↑↓
Energy provision	Lactate ↑↓
Synapse formation and remodeling	Thrombospondin ↑↓; complement C1q ↑↓

Increasing evidence shows glial scar as a highly complex system of interacting cell types where, dynamic polarization of different populations of cells at distinct times after injury orchestrates the formation of a structurally layered scar. Reactive astrocytes, activated microglia/macrophages, NG2+ oligodendrocyte progenitors and PDGFβ expressing fibroblasts/stromal cells are the major cell types (Figure 7). Breakdown of the blood–brain barrier (BBB) and leakage of blood and serum elements into the CNS parenchyma is considered a key event in glial scar formation. In agreement with this scenario, several molecules derived from the blood or produced via inflammation have been envisioned as potential triggers of scar formation. Perhaps equally as important, leukocyte extravasation and accumulation of inflammatory cells in the lesion core have a main role in the positioning of the glial scar (Figure 7). Recent work using 3D-imaging combined with genetic labeling of microglia and infiltrating monocyte derived macrophages (CX3CR1-GFP) has revealed that the density of inflammatory cells increases by many folds in the lesion core (Ertürk et al., 2011). Indeed, activated macrophages/microglia markedly increase expression of matrix metalloproteases (MMPs) after injury, and this contributes to vascular permeability and accumulation of more inflammatory cells within the lesion. While these pools of activated macrophages/microglia are thought to be important for lesion debridement, they also drive secondary injury through inflammatory processes that facilitate reactive astroglia conversion into the proinflammatory-neurodegenerative phenotype (Zamanian et al., 2012).

Penetrating injuries that disrupt the meninges are associated with meningeal fibroblast infiltration in the lesion core. However, in a recent study, Soderblom et al. (2013) used genetic fate mapping to show that collagen-1α1/PDGFRβ/CD13 positive perivascular fibroblasts proliferate even in response to contusive injury where the meninges are largely intact, and form a major cellular component of the fibrotic scar. The fibrotic core of the glial scar appears in its mature form by 2 weeks post-injury (Göritz et al., 2011; Soderblom et al., 2013). Adding to the considerable complexity of the lesion environment the core surrounded by the scar also show several different cell types that undergo migration and proliferation when their usual niche is disturbed due to trauma. For example, injury disrupts progenitor niches. After damage, a large and heterogeneous population of immature cells expressing markers associated with progenitors (including nestin, vimentin, and NG2) invade the lesion core (Busch et al., 2010; Lytle et al., 2006; Zai and Wrathall, 2005). *In vivo* imaging

has demonstrated that NG2 oligodendrocyte progenitors rapidly extend processes and migrate toward CNS lesions in a role that seems not to be directly connected with the generation of new myelinating oligodendrocytes (Hughes et al., 2013). This migration is preceded by microglia temporally, and it is thought that dynamic polarization of different populations of cells at distinct times after injury orchestrates the formation of a structurally layered scar (Hughes et al., 2013). Ultimately the glial scar serves as a vital structure that preserves intact circuit elements from damage by intense inflammation at the lesion core.

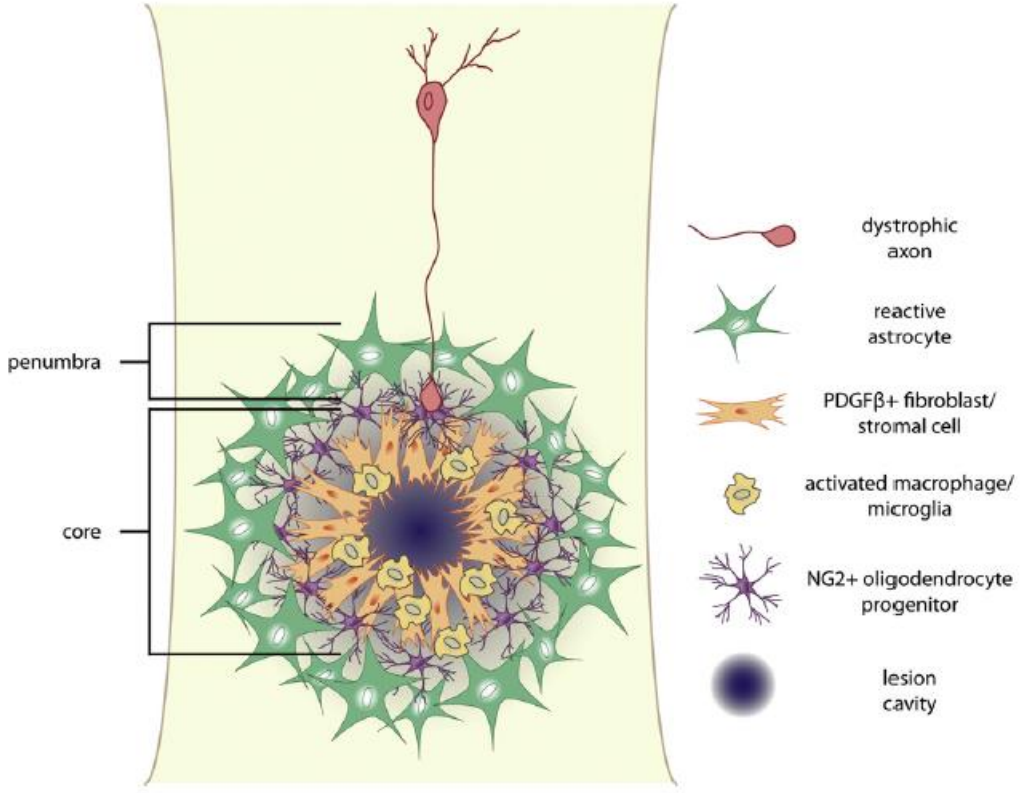

Figure 7**. Anatomy of a contusive spinal cord lesion to show the cellular components of the core and surrounding scar.** As in brain ischemia, spinal cord lesions have two distinct components—the lesion penumbra composed of hypertrophic astrocytes, and the lesion core composed of NG2+ oligodendrocyte precursor cells, PDGFRβ+ fibroblasts, and macrophages/microglia. Note the layered architecture of the glial scar (Cregg et al., 2014).

Glial Scar formation as physiological and bio-molecular barrier for axonal regrowth:

Most of the studies on glial scar formation, its advantages, and its effects on regeneration failure are studies performed in spinal cord injury models due to its importance for the functional recovery failure in humans. Axons attempting to cross the glial scars are trapped and become dystrophic in a process that was described by Ramon y

Cajal almost a century ago (Ramón y Cajal, 1928), but still today not completely understood. Phenotypical transformation of scar-forming astrocytes includes a dramatic change in the star-like unpolarized morphology towards a polarized phenotype that presents an intermingled network of astroglial processes around the lesion. In addition, some reports evidenced a rapid migration of astrocytes away from the inflammatory epicentre that is speculated to collaborate in the initiation of the astrocytic scar at the lesion penumbra (Cregg et al., 2014). After forming the mesh-like layer of entangled filamentous processes, scar-forming astrocytes begin synthesizing and depositing chondroitin sulphate proteoglycans (CSPGs) into the extracellular matrix within 24h after injury. High concentrations of CSPGs persist throughout the lesion for months, which acts as a physical and molecular barrier that entraps dystrophic axons and severely limits their ability to regenerate (Davies et al., 1997).

Dystrophic growth cones were first described as "*sterile clubs*" by Ramón y Cajal, who also noted their persistence in a stable and quiescent state (Ramón y Cajal, 1928). For many years, these unusually shaped end bulbs were considered to be sterile, and therefore incapable of extending a growth cone. More recent research has indicated that axons with dystrophic endings do not lose their ability to regenerate, and that they can in fact return to active growth states (Houle et al., 2006; Kwon et al., 2002; Li and Raisman, 1995). Live imaging of growth cones that stall and become dystrophic in a gradient of CSPGs has revealed that—despite their abnormal appearance and lack of forward movement—dystrophic endings can be extremely dynamic at least for a few days *in vitro* (Tom et al., 2004). Disorganized microtubules underlie the formation of the dystrophic growth cone (Ertürk et al., 2007).

After injury a large population of fibroblasts takes up residence in the lesion core, forming a fibrotic scar replete with extracellular fibronectin, collagen, and laminin (Shearer and Fawcett, 2001). Within the lesion, meningeal fibroblasts have an active role segregating from astrocytes via Ephrin-B2/EphB2 bidirectional signaling (Bundeson et al., 2003), and are thought to contribute to the inhibitory properties of the scar by expression of repulsive axon guidance molecules (Pasterkamp et al., 1999) and also by promoting astrocytic reactivity (Wanner et al., 2008).

At least a subset of invading progenitors, NG2-OPC glia, seems to act as a highly attractive substrate for dystrophic axons, helping to stabilize them within the hostile lesion environment (Busch et al., 2010; McTigue et al., 2006). It is not completely understood how NG2 cells interact with entrapped axons and which is the final effect for the constrain of axonal growth (Bergles et al., 2000; Chittajallu et al., 2004; Lin et al., 2005).

More recent experiments with *in vivo* imaging of bone marrow chimeric animals have led to the surprising finding that activated macrophages, and not microglia, are responsible for prolonged dieback of injured axons after injury (Horn et al., 2008). *In vitro* experiments indicate that macrophages initiate dieback through matrix metalloprotease activity and direct physical interaction with injured axons (Horn et al., 2008).

Having in mind the enormous importance of the axonal growth inhibition by components of the glial scar for the human health, the study of glial scar formation and the strategies for overcoming axonal growth collapse in glial scars are a main objective of modern neuroscience.

Technical and physiological problems to study glial scar formation:

One of the main reasons for the lack of complete understanding about the pathways involved in the glial scar formation and subsequent axonal failure to regrowth is the *in vivo* nature of the phenomenon. Traumatic brain injury, brain ischemia and spinal cord injury share the main sequential steps in the development of the lesion. Indeed, these three different pathological states share the core/penumbra distribution and the glial scar formation that prevents axons from regrowing.

The traditional in vitro 2-dimensional culture has been used to study many features of reactive gliosis and glial scar formation. However, these systems suffer from number of limitations, such as prominent signs of cellular stress, undesired baseline activation, or the loss of many *in vivo* morphological and physiological features of astrocytes. On the other hand, cells cultured in 3D matrixes exhibit features that are closer to the complex *in vivo* conditions (Vinci et al., 2012). The 3D culture models have proven to be more realistic for translating the study findings for *in vivo* applications. One important contribution for the

"*closer-to-in vivo*" behavior of cells when grown as 3D cultures is the matrices and scaffolds that are used for obtaining such cultures. The CNS is a very unique tissue, as compared to other organs in the body, as it has very unusual extracellular matrix (ECM) structures, as well as many other unique characteristics, including its soft physical properties. Natural brain tissue, which has an elastic modulus of around 500 Pa, is very soft compared to other tissues, such as muscle, which possesses an elastic modulus of around 10^4 Pa (Leipzig et al., 2009). These differences in mechanical properties, structure, and composition of ECM have a profound effect on cell function. Whereas cells in most other organs bear loads, and therefore experience mechanical stimuli, cells in the CNS do not. Another major difference is the composition of the ECM of the CNS. Many ECM components, such as fibronectin and collagen, which are plentiful in other organs, are virtually absent in the CNS, whereas there is an abundance of different types of proteoglycans, localized to intercellular spaces between neurons and glial cells (Bandtlow et al., 2000).

Many different types of hydrogel systems have been evaluated for CNS applications in order to attempt to mimic the biochemical and mechanical properties of the CNS. These include both naturally derived hydrogels, including collagen, hyaluronic acid (HA), agarose, alginate, chitosan, methyl cellulose (MC), xyloglucan, Matrigel, fibrin, PolySia-based hydrogels, peptide hydrogels, as well as synthetic hydrogels, such as polyacrylamide and polyethylene glycol (PEG) hydrogels (Nisbet et al., 2008, Straley et al., 2010). Synthetic hydrogels have some notable advantages over natural hydrogels, namely adjustable mechanical properties, as well as easy control of scaffold structure and chemical composition. So, 3D systems seem to be a good alternative. As explained above, most of the studies in the glial scar formation are *in vivo* studies in spinal cord injury. Few studies were done in ischemic brain injury models *in vivo*. On the other hand, signaling pathways involved in glial scar formation have been studied in classical 2-D astroglial culture (Wanner et al., 2008, 2013). To our knowledge, there are not reports studying glial scar formation and reactive gliosis in the more realistic 3D culture model which is one of the objectives of this thesis.

OBJECTIVES AND HYPOTHESIS

General aim:

To contribute to the study of the intracellular pathways involved in glial scar formation after ischemia to evidence potential pharmacological targets for future therapeutic intervention.

Hypothesis:

The presence of blood-infiltrating cells and the gradient of DAMPs released from ischemic core are able to induce the glial scar formation by reactive astrocytes. These mechanisms could probably involve NF-κB activity.

Specific aims:

Specific Aim I: **To evaluate glial scar formation *in vitro* using meningeal cells and astroglial 2D co-cultures.** Mixed primary glial cell cultures including astrocytes, microglia and oligodendrocytes obtained from Wistar rat pups were grown in the classical 2D culture plates and incubated with meningeal cells obtained from rat pups. Glial cell clustering and morphology was analyzed by live image time-lapse microscopy and immunocytochemistry.

Specific Aim II: **To evaluate glial scar formation *in vitro* using activated macrophages obtained from ischemic explants and naive astroglia.** Mixed primary glial cell cultures including astrocytes, microglia and oligodendrocytes obtained from Wistar rat pups were grown in the classical 2D culture plates and incubated with explants obtained from wild type or GFP transgenic rats subjected to ischemia. Glial scar formation in vitro and sensitivity to NF-κB blockage was tested.

Specific aim III**: To evaluate glial cell viability, morphology and reactivity in 3D collagen or 3D nanofiber scaffolds**. Mixed primary glial cell cultures including astrocytes, microglia and oligodendrocytes obtained from Wistar rat pups were grown in the 3D matrixes and glial cell viability and morphology was analyzed by live image time-lapse microscopy and immunocytochemistry with glial markers.

Specific Aim IV: **Reproduction of ischemic penumbra and glial scar formation by controlled release of DAMPs into the matrix**. One of the major hypotheses in glial cell biology after focal brain injury is that the gradient of DAMPs or other morphogens is able to induce the glial scar formation that surrounds the ischemic core and basically overlaps with the penumbra (Villarreal et al., 2014; Burda and Sofroniew, 2014). In this objective we performed focal injections of DAMPs or necrotic cell lysates in the 3D matrix with the mixed glia to induce a gradient of activation resembling the proposed gradient that occurs in ischemic penumbra *in vivo*.

MATERIALS AND METHODS

Materials. Medium and reagents: Hank's balanced Salt Solution (HBSS; Invitrogen-1644011), Dulbecco's Modified Eagle Medium high glucose with glutamine (DMEM; Invitrogen-119965092), Ham´s F-12 Nutrient mixture (F-12; Invitrogen-1584933), 1:1 of DMEM and F-12 (DMEM/F-12), penicillin/streptomycin (Sigma-P4333), poly-L-lysine (poly-L; Sigma-P4707), 5- Fluorouracil (5-FU; Sigma-F6627), 0.05% trypsin, 0.25% trypsin, 0.2% (Sigma-T6567), collagenase(Sigma-C5894), Fetal Bovine Serum (FBS; Natocor), Xylazine (Pharmavet), Ketamine (Richmond), BAY-11-7082 (Sigma), paraformaldehyde (Sigma-P6148), Sucrose (Anedra-7118), Triton X-100, normal Equine Serum (Natocor). Primary antibodies: anti-GFAP (DAKO-Z0334), anti S100B (Sigma-S2532), anti TLR-2 and anti-TLR4 (Santa Cruz- sc-16237 and sc-30002). Secondary antibodies: Alexa 594 (Red; Jackson-111-585-144), Alexa 488 (Green; Jackson-111-545-144). Cellular staining: Tomato Lectin - Fluorescein isothiocyanate (Tomato Lectin-FITC; Sigma-L0401). Nuclear staining: 4^{I}, 6-Diamidino-2-phenylindole (DAPI; Sigma-D9542). 3D matrices: Collagen Solution, Type 1 from rat tail (Collagen matrix; Sigma-C3867), Hydromatrix (Sigma-A6982). Reagents used in 3D cultures: High Mobility Group Protein (HMGB-1; Sigma-H4652).

Animals. Wistar strain rats were used from the animal facility of Faculty of Medicine of the University of Buenos Aires. All rats were housed in a 12 h light/dark cycle in a specific pathogen free facility with controlled temperature and humidity and were allowed free access to food and water. All experiments were conducted according to protocols approved by the Comité Institucional para el Cuidado y Uso de Animales de Laboratario (CICUAL), from the faculty of Medicine of the University of Buenos Aires. These protocols were developed according to the standards for care and use of experimental animals from the National Institutes of Health of the United States of America (NIH), and the recommendations of the Society for Neuroscience (SfN).

Astroglial cell culture. Primary cultures were obtained from neonatal rat pups (P3-P5). Brains were removed, meninges were set aside, and cortices were dissected and dissociated

mechanically using scissors and a serological pipette in HBSS. When tissue was no longer visible, and after decanting for 1-2 minutes, the supernatant was transferred to a new centrifuge tube. This extraction was repeated twice. Following a 5 min centrifugation at 1000 rpm, pellet was washed in DMEM supplemented with 10% FBS, 100 µg/ml penicillin/streptomycin (DMEM complete) and platted in poly-L-lysine coated culture flasks. When the cells reached a state of confluence (8-10 days), flasks were closed tightly and wrapped in a ziploc sprayed with alcohol. Approximately 7ml of fresh medium was added to the flasks , making sure the lid remained dry during agitation. Flasks were agitated at 180 rpm for 24 h, and the medium was removed and replaced by fresh complete medium. This procedure was repeated twice. After 48 h of agitation, 1 µl of mitotic inhibitor 5-FU was added per 8ml of fresh medium, and the flasks were further incubated for 24h. After incubation the medium with 5-FU was removed, and the cells were washed with 5ml of fresh complete medium to completely get rid of 5-FU. Finally, 10ml of fresh complete medium was added and cells were let to recover for 48 h. Cells were trypsinized (0.05%) for 5minutes, and re-seeded in either 12 or 24 poly-L coated wells plates. For the experimental procedures, they were maintained in 5% CO_2 at 37°C in complete DMEM. After 10-12 days in vitro (DIV) astrocytes reached confluence.

Isolation of meningeal cells. Cortical meninges from neonatal rat pups (P3-P5) were collected and placed in HBSS. For tissue dissociation, enzymatic digestion was performed with 0.25% trypsin, 0.2% collagenase, in HBSS for 30 min at 37^{O}C. Mechanical disaggregation was performed sequentially with a P1000 and a P200 tip in the presence of 25U of DNase I. Washed cells were collected by 5 min centrifugation at 1000 rpm. The cell suspension from 5 meninges was grown to confluence in DMEM/F12, 10% fetal bovine serum (FBS), 100 µg/ml penicillin/streptomycin in a 3.5 cm diameter plate, previously coated with poly-L-lysine for 7-10 days.

Astrocyte meningeal co-cultures. Co-cultures were prepared by adding a suspension of 15,000–30,000 meningeal cells to each well of confluent astrocytes prepared in a 24 well

plate. Co-cultures were incubated in DMEM/F12, 10% FBS, 100 µg/ml penicillin/streptomycin for 2–3 days.

Cortical devascularization. Adult male Wistar rats (300-350 g) were obtained from the Animal Facility of the Pharmacy and Biochemistry School, University of Buenos Aires and transgenic rat strain [Wistar-TgN (CAG-GFP)184ys] (Mothe et al., 2005) was obtained from Dr. Fernando Pitossi (FIL, Buenos Aires, Argentina). Animals were housed in a controlled environment (12/12-h light/dark cycle, controlled humidity and temperature, free access to standard laboratory rat food and water) under the permanent supervision of a professional veterinarian. For all surgical procedures animals were anaesthetized with ketamine/xylazine (90/10 mg/kg i.p., (Intraperitoneally)). Animals were subjected to a unilateral cortical devascularization (CD) as previously described (Herrera and Robertson 1989; Villarreal et al., 2011) making every effort to reduce the suffering and the number of animals used. Protocols were approved by the animal care committee CICUAL (Facultad de Medicina, UBA).

Dissociated cell culture and explants from adult ischemic tissue. After recovery time of 3 days post lesion (DPL), animals were deeply anaesthetized with ketamine/xylazine (90/10 mg/kg i.p.) and sacrificed by decapitation. Brains were rapidly removed and placed in Dulbecco's Modified Eagle Medium (DMEM) under sterile conditions. Meninges where then carefully removed and a very small region of the cortex containing the ischemic area (typically 2 mm x 2 mm x 2 mm) was dissected and placed in fresh DMEM. A similar fragment from an equivalent cortical area in the contralateral hemisphere was removed to be processed as control. Immediately after dissection, ischemic tissue was cut into four slices of similar size with a sterile blade and immediately each slice was plated in poly-L-lysine coated dishes. Co-culture experiments of tissue explants on primary astrocytes were done essentially in the same way but explants were seeded on confluent astrocytes monolayer obtained from neonatal pups as described previously.

Cell culture fixation and immunocytochemistry. Cells were washed with phosphate-buffered saline (PBS) 0.1M, and incubated in fixating solution (4% paraformaldehyde plus 4% sucrose in PBS, pH 7.2) for 15 minutes at room temperature. After washing three times with PBS, cell permeabilization was achieved by using Triton X-100 in PBS (0.1%) for 15 min. After washing with PBS, cells were incubated for 30 minutes in blocking solution (containing normal Equine Serum 5% in PBS). Cells were then incubated overnight with primary antibodies diluted in blocking solution at 4°C. After washing 3 times with PBS, cells were incubated with secondary antibodies in blocking solution for 4 hours at room temperature. Cells were then washed 3 times with PBS, and were incubated overnight with Tomato Lectin at 4°C. Finally the cells were washed once with PBS, and incubated for half an hour with DAPI at room temperature for staining the nuclei of the cells. Primary antibodies used: anti-GFAP (1/3000), anti S100B (1/1000), anti TLR-2 (1/1000) and anti-TLR4 (1/1000). Fluorescence immunocytochemistry was performed using secondary antibodies conjugated to Alexa 488 (green), Alexa 594 (red), and Tomato Lectin-FITC (1/1000) and DAPI 1µg/ml.

Cell encapsulation in semisolid matrices. Collagen and Hydromatrix 3D matrices were tested for astrocytic viability and survival. While collagen matrix was not successful, Hydromatrix allowed cell survival for at least 45 days. Hydromatrix was used according to manufacturer's specification. Briefly, a 2x Working Solution was prepared by adding 1 volume of 10mg/ml Hydromatrix Stock Solution, 2 volumes of sterile 20% sucrose solution, and 1 volume of water. Cells were centrifuged and resuspended in DMEM containing 10% sucrose (w/v) (to protect cells from the low pH during gelification) at twice the final desired cell concentration. The 2x Working Solution was added to the 2x cell/sucrose mixture. Then, 80µL of the mixture were added to each well of a 96-well plate. The gel formation was initiated by adding 1–2 volumes of medium to the side of each well. The gelification was usually completed after 1 h at 37°C. Tissue culture medium was added on the top of the gel and changed every two days.

Injection of DAMP. When cells in the 3D matrix reached confluence, DAMPS were injected in to the matrix with the pipette tip at a point near to the wall of the each well. In control

wells, PBS was injected. DAMPS used: Astrocyte lysate (5µl/well) and HMGB-1 (5 µl/well). Astrocyte lysate is prepared by lysis of astrocytes by osmotic shock, followed by centrifugation for 5min at 1000rpm. The supernatant solution is used as astrocyte lysate.

3D cultures immunocytochemistry. Cells were fixed with 30 minutes of incubation with 4% paraformaldehyde plus 4% sucrose in PBS pH 7.2 at room temperature. After washing with PBS, 6 times over 15 minutes, cell permeabilization was achieved by using Triton X-100 in PBS (0.1%) for 30 min. After washing with PBS double times to the normal protocol cells were incubated in blocking solution containing normal Equine Serum (5%) in PBS for 1 hour. Cells were then incubated 24h with primary antibodies diluted in blocking solution. After washing 5 times with PBS over 15 minutes, cells were incubated with secondary antibodies overnight in blocking solution.

Photomicrographs. Pictures were taken with an Olympus IX-81 microscope equipped with a DP71 cooled camera.

Quantitative studies. Total area occupied by Astrocyte free area (AFA) was quantified with NIH Image J and statistically analyzed using Graphpad Prism 5.0.

RESULTS

Primary meningeal cells induce retraction of astrocytes in culture:

To overcome some of the problems associated with the study of the different molecules and cellular pathways involved in glial scar formation *in vivo*, we used meningeal cells to induce astroglial scar like structures in confluent astrocyte cultures *in vitro* as was reported by Wanner and colleagues (Wanner et. al; 2013).

For that purpose, astrocytes were cultured to confluence and meningeal cells extracted from 3-5 days old rat pups were separately cultured for 15 days. Following this period, meningeal cells were added to the confluent astrocyte cultures (as described in Materials and Methods), and the co-culture was followed by time lapse microscopy under phase contrast. After 3 days, the co-cultures were fixed and immunocytochemistry was performed. Astrocytes were identified by glial fibrillary acidic protein (GFAP) immunostaining, while meningeal cells were labeled with fluorescent tomato lectin.

Time lapse studies over 3 days revealed that, starting on day 1; astrocytes were gradually retracting away from central meningeal cell, forming circular areas that we named astrocyte free areas (AFA). Over time, some of the AFA coalesced to form larger AFA. We calculated the total surface area of these AFA per well at different meningeal cell concentrations: 15,000 or 30,000 meningeal cells/well. We observed that doubling the number of meningeal cells did not produce a significant change in the surface covered by the AFA (Figure 8).

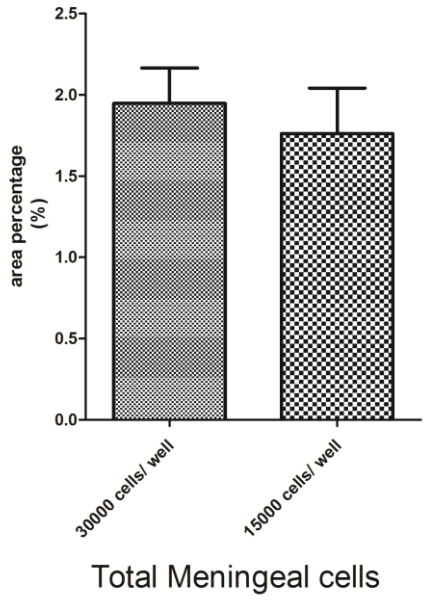

Figure 8. **Total surface area of AFA per well at 2 different meningeal cell concentrations.** Doubling the number of meningeal cells (30,000 meningeal cells per well), did not produce a significant increase in AFA compared to the AFA in the wells that contained 15,000 meningeal cells.

In order to study the morphology of the astrocytes corralling the meningeal cells, we performed immunocytochemistry on the co-cultures after 3 days of incubation with meningeal cells. The images revealed the classical corral-like structure of these AFA with isolated tomato lectin positive cells located in the AFA. Astrocytes were forming a circular structure of GFAP positive filaments with intermingled projections forming a scar-like structure (Figure 9). In addition, astrocytes showed an intense fibrilar GFAP staining and stellated morphology that is classically considered as the consequence of reactive gliosis *in vitro*. In the same images, weak tomato lectin labeling was observed on GFAP+ astrocytes, probably due to their reactive gliosis state induced by the presence of meningeal cells and/or the retraction occurring in neighbor areas of the culture.

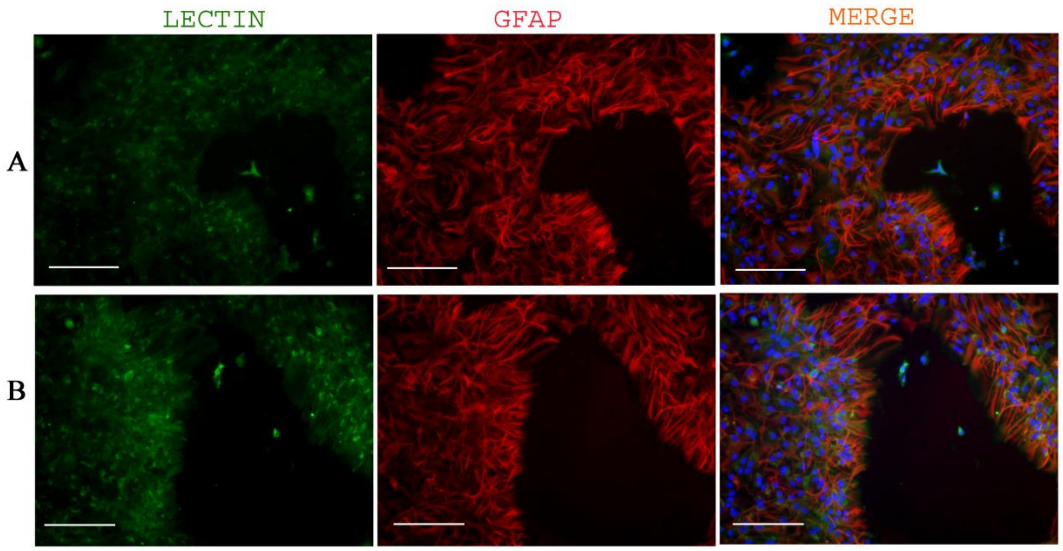

Figure 9. **Meningeal cells, astrocyte free areas (AFA) and reactive gliosis in primary astrocytic culture.** Meningeal cells are strongly tomato lectin positive and do not express GFAP. They are present in AFAs induced in confluent astrocyte monolayers (A, B). GFAP staining in red, Tomato Lectin in green, and nuclei stained with DAPI in blue. Scale bar: 200 µm.

As evidenced by DAPI nuclear staining, there is a very low cellular density in the AFA, with almost no cells showing GFAP reactivity. On the other hand, GFAP positive areas have a very high cellular density and a healthy status without showing images of degenerating cells or pyknotic nuclei (Figure 10).

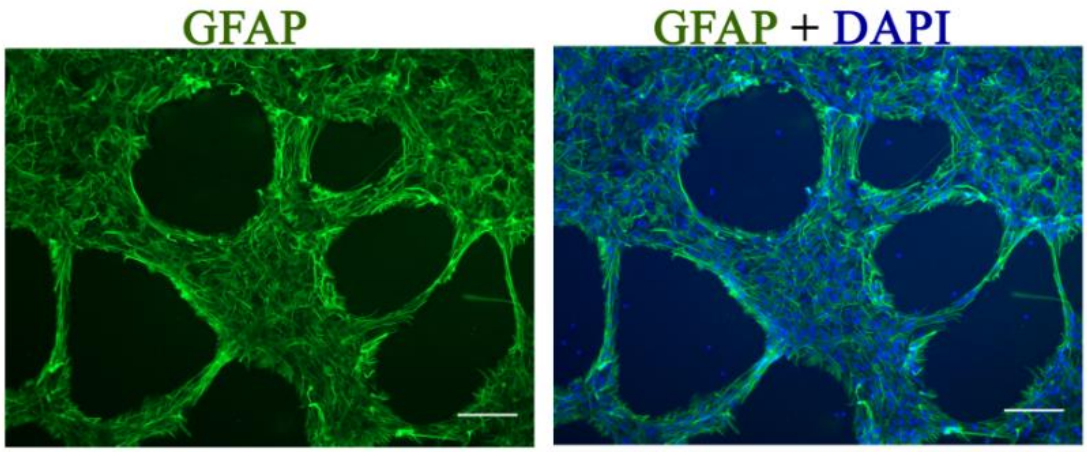

Figure 10. **Astroglial corral-like like structures.** Image on the left showing the astroglial corral-like structures. Image on the right shows DAPI nuclear staining to evidence the cell distribution. GFAP staining is shown in green and DAPI in blue. Scale bar: 200 µm.

Barrier-forming astrocytes resemble glial scar forming astrocytes seen *in vivo* after a focal ischemic or traumatic brain injury:

After closer observation of the astrocytes forming the astroglial scar like structures *in vitro,* we decided to compare them with the astrocytes forming astroglial scar *in vivo*. Following traumatic or ischemic injury to the brain, astrocytes become activated and surround the ischemic core forming a glial scar. The activated astrocytes forming *in vivo* glial scars are different to the normal astrocytes in their morphology and protein expression profiles (Amat et al., 1996).

To evaluate if these *in vitro* astrocytes surrounding the AFAs induced by meningeal cells were similar to those observed *in vivo*, we performed immunocytochemical studies and a detailed observation of the astrocytes surrounding the AFAs. We observed that AFA were surrounded by a layer of high density, clustered, packaged overlapping and intermingled astrocytes (Figure 11). The astrocytes limiting the AFAs surrounding the meningeal cells showed cellular hypertrophy and had elongated cellular processes with extensive overlapping with the adjacent astrocytes perpendicular to the direction of their migration, particularly with the astrocytes on either side (Figure 11). Higher magnification images

showed that GFAP filaments were intermingled showing a wall-like structure similar to that observed *in vivo* during glial scar formation (Figure 12).

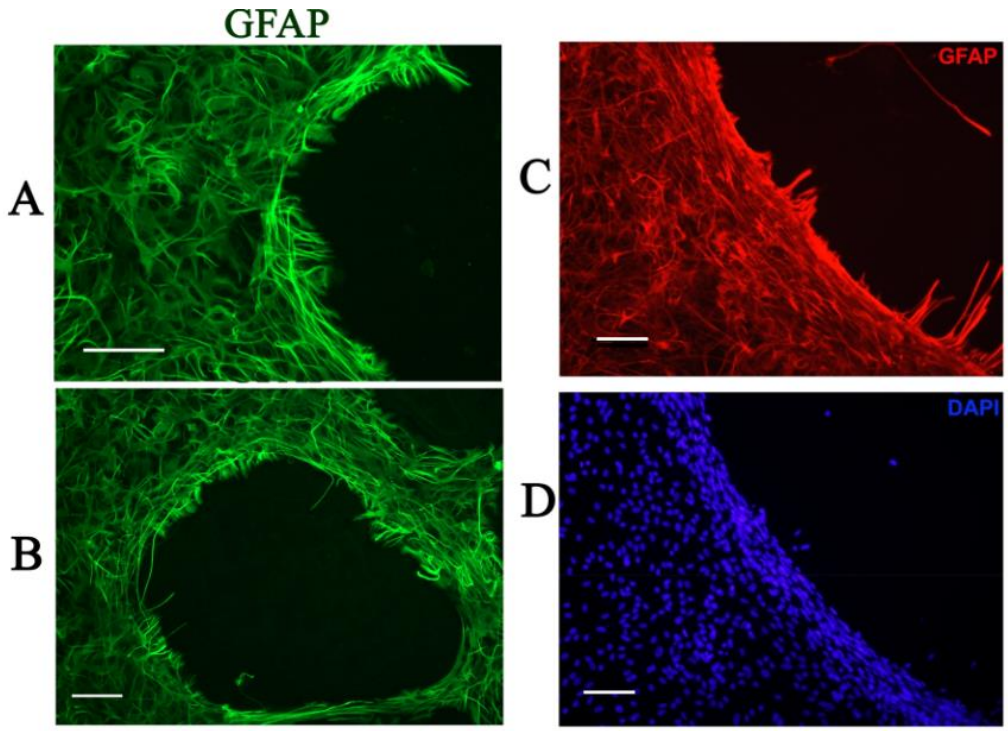

Figure 11**. Morphology and density of astroglial scar like structure.** Astrocytes forming the borders of astroglial scar like structure are hypertrophied, and have highly elongated processes, and great overlapping. The processes run over the scar border. A,B: higher and lower magnification respectively; C: Image shows clustered and highly overlapping astrocytes at the border and confluent astrocytes, deep from border; D: Image shows DAPI nuclear staining allows visualization of astrocyte clustering, higher density of nuclei (astrocytes) at the scar like structure compared to the nuclei (astrocytes) distant from AFA. GFAP staining in green (A,B), red (C) and DAPI in blue. Scale bar: 200 µm.

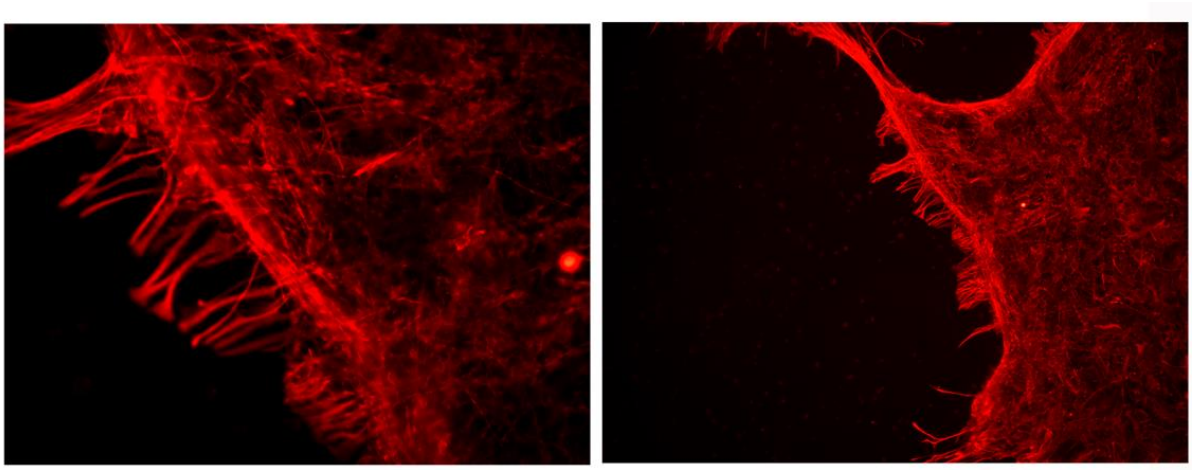

Figure 12**. Morphology and density of astroglial scar-like structure.** High magnification images showing the detailed organization of the glial scar-like structures in the borders of the AFA. Note that astrocytic retraction is not absolute and long projections persist into the AFA.

Figure 12, also shows that astrocytes located more distant from the central meningeal cells were confluent with high degree of overlapping, and presented cellular hypertrophy resembling reactive glia distal to focal CNS injury. We conclude that the astrocytes forming astroglial scar like structure *in-vitro* had similarities with glial scar *in vivo*.

Scar forming astrocytes have differential expression of TLR, GFAP and S100B:

Taking into consideration the fact that scar forming astrocytes have shown a more activated phenotype, we decided to evaluate if there was heterogeneity in the expression of some proteins related to astrocyte activation as the distance from the meningeal cells increased. For that purpose, we performed immunocytochemical studies with specific antibodies to TLR2 and TLR4, two pattern recognition receptors related to innate immunity responses in the CNS. This study revealed that astrocytes forming the scar-like structures *in vitro* showed increased expression of TLR2 (Figure 13) and TLR4 (Figure 14). The TLR2 and TLR4 protein expression of astrocytes was inversely proportional to the distance from the meningeal cells: as the distance of astrocytes from the central meningeal cells increases the protein expression decreases.

S100B is an astroglial protein that can behave as a DAMP activating pattern recognition receptors and inducing reactive gliosis *in vitro* and *in vivo* (Villarreal et al., 2014). In addition, it has been shown that astrocytes increase S100B expression and S100B controlled release when they are reactive *in vitro* and *in vivo* (Ramos et al., 2004; Gerlach et al., 2006). In agreement with the reactive state that we observed in scar-forming astrocytes, images show these astrocytes also express increased S100B protein (Figure 14).

We conclude that, even though all the astrocytes are activated or reactive, the astrocytes forming the scar-like barrier had distinctive morphological and probably different functional characteristics.

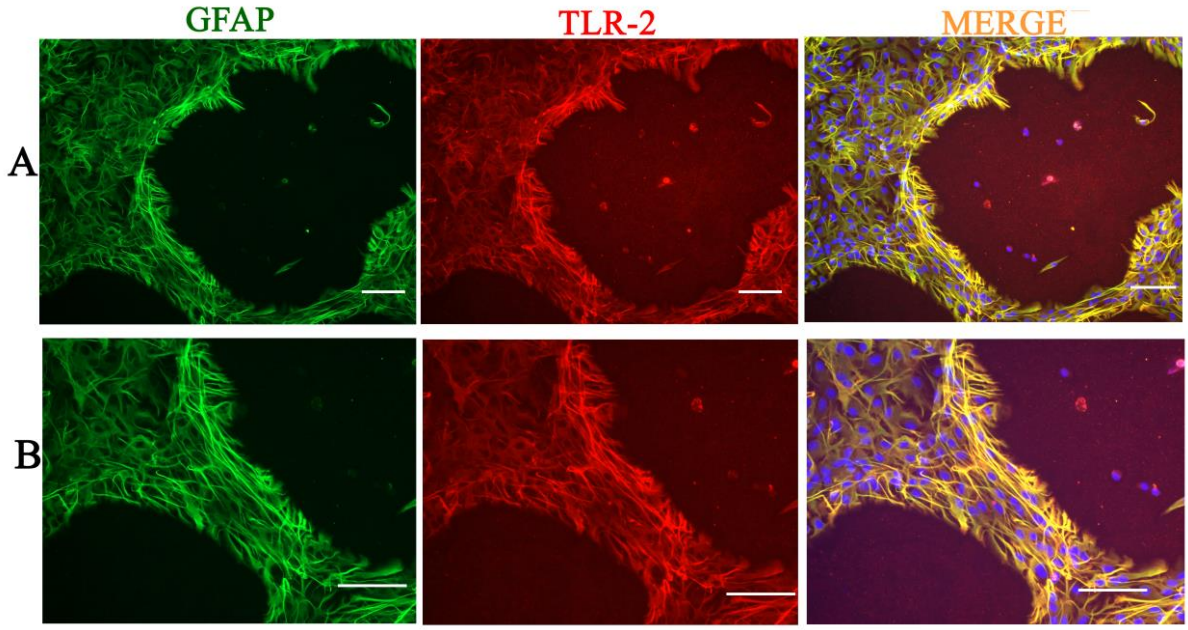

Figure 13. **Expression of different proteins associated with astrocyte activation**. Astrocytes forming the astroglial scar like structure showed increased GFAP and TLR2 expression. The expression decreases with increasing distance from the menigeal cells. (A,B, lower and higher maginfication respectively). GFAP staining in green and TLR2 in red, and nuclei in blue with DAPI. Scale bar: 200 µm.

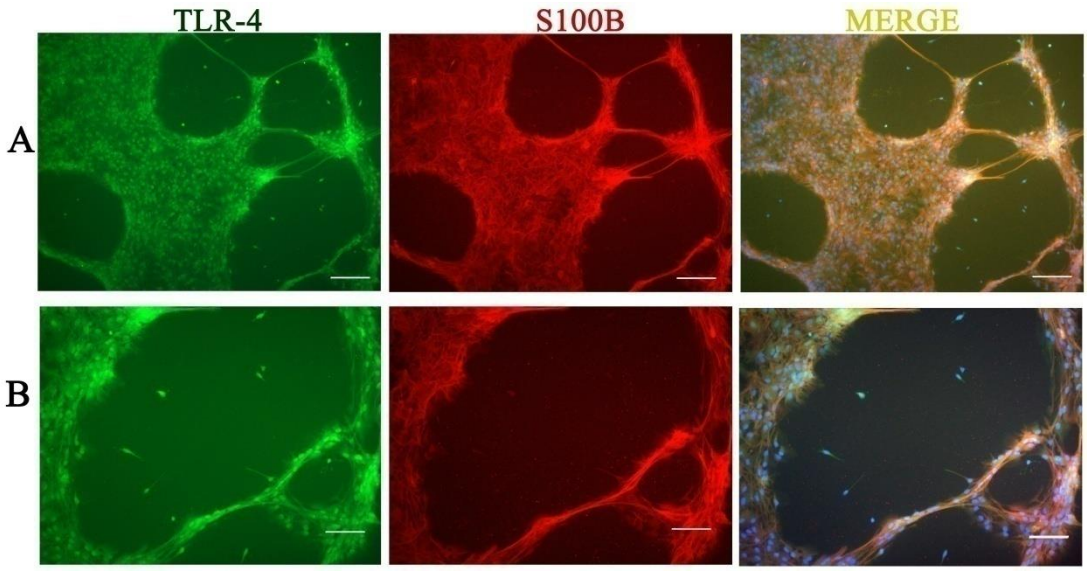

Figure 14. **Differential protein expression by the astrocytes.** Astrocytes at the border of AFA are highly elongated with high expression of TLR4 and S100B, while the astrocytes that are distant from the meningeal cells, TLR4 stained, had lower expression of TLR4 and S100B and were confluent. (A,B lower and higher magnification respectively). TLR4 staining in green and S100B in red. Scale bar: 200 µm.

Biochemical pathways involved in glial scar formation *in vitro*:

The initial active displacement of astrocytes from the areas where meningeal cells are located, and the subsequent change in astroglial cell polarization from the stellated shape to a wall-like structure with intermingled projections are intriguing proccesses. Having in mind the differential expression of TLR2 and TLR4 receptors in the scar-forming astrocytes, we evaluated if the TLR downstream effector NF-κB could be involved in the cellular pathways leading to scar formation *in vitro*.

For that purpose, we used the cortical devascularization model of brain ischemia (Ramos et al., 2004; Villarreal et al., 2011, 2014) in GFP-expressing rats ([Wistar-TgN(CAG-GFP)184ys]) (Mothe et al., 2005), and 3 days after the ischemic injury we obtained ischemic tissue explants from brain cortex. These cortical ischemic tissue explants were cultured alone or co-cultured over confluent astrocytic cultures and followed by live imaging time-lapse microscopy.

Ischemic cortical explants, but not explants obtained from the contralateral hemispheres, released a large number of cells morphologically identified as macrophages activated by the ischemic injury (Figure 15).

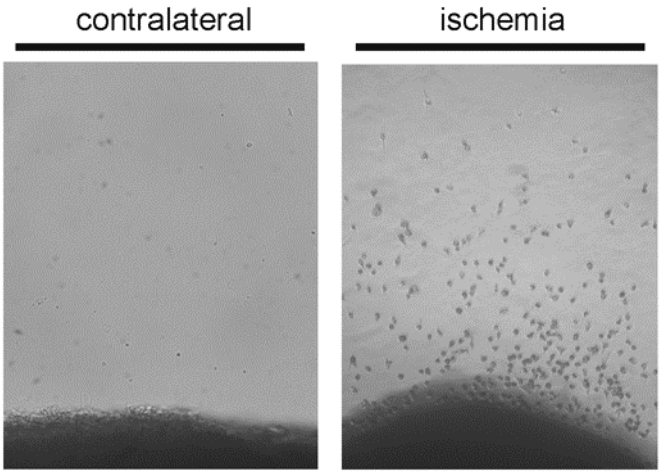

Figure 15: Low magnification images showing the escape of activated macrophages from ischemic tissue explants that are absent in similar explants of the contralateral (control) hemisphere. Images were taken after 1 day in vitro.

When the ischemic explants were co-cultured with astrocytes, we observed that astrocytes retracted from the areas occupied by macrophages resembling the effects observed in meningeal cells-astrocytes co-cultures (Figure 16).

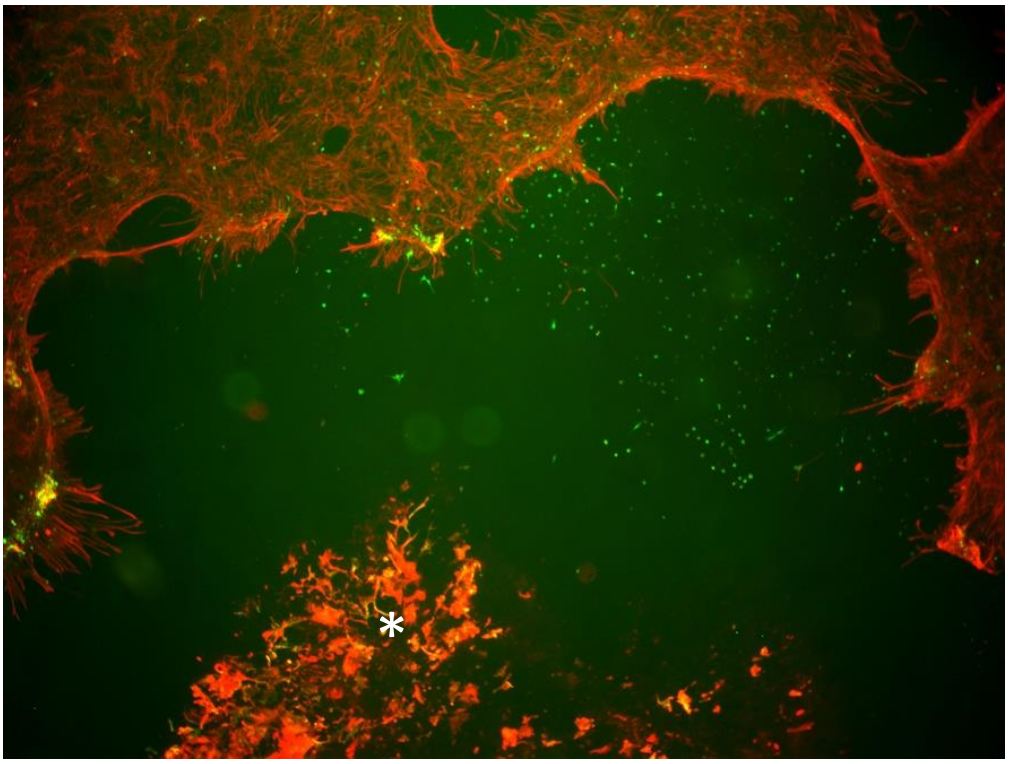

Figure 16**: Ischemic explants cultured over astrocytes.** Low magnification images showing the escape of activated GFP+ (green) macrophages from ischemic tissue explants that induce the scar-like structures in the confluent astrocytic monolayer. The place where ischemic explant was placed is indicated by the asterisk (*). Images were taken after 5 days *in vitro,* and explants were from ischemic animals of 3 days of recovery time.

In order to study the role of NF-κB in the formation of glial scar *in vitro*, we performed a loss of function experiment by repeating the same paradigm, but pre-incubating astrocytes during 1 h with the NF-κB inhibitor BAY-11-7082 to block NF-κB activation in the astrocytes that will receive the explants.

As shown in Figure 17, NF-κB blockage in the astrocytic monolayer induced a reduced retraction of the astrocytic monolayer. We conclude that macrophages activated by ischemia in vivo are highly mobile and induce astroglial retraction. The astroglial retraction from the area occupied by macrophages, as the initial step for glial scar formation, requires NF-κB activity in astrocytes.

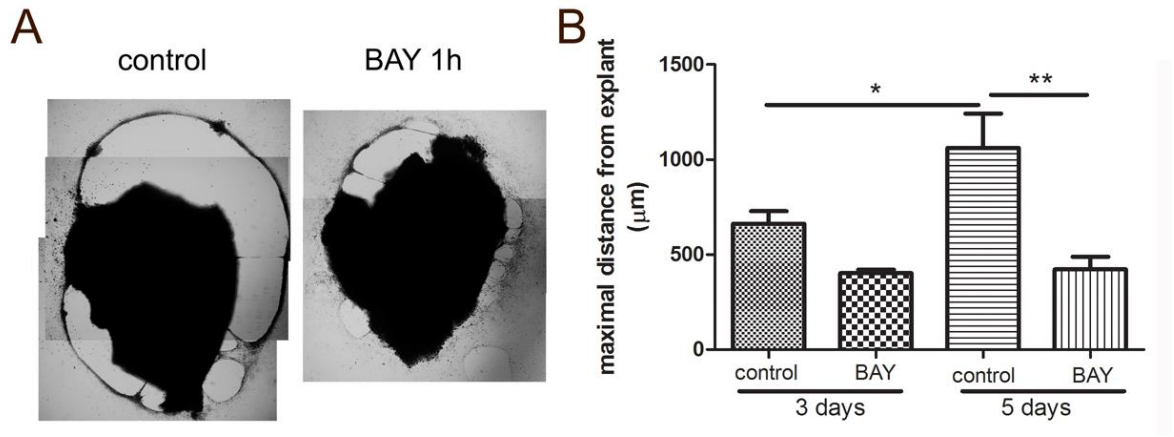

Figure 17: A: **Scar formation requires NF-κB.** Ischemic cortical brain explants cultured on a confluent astrocytic monolayer showed the reduced distance reached by the astroglial scar. B: Quantitative analysis of the maximal distance at which the glial scar is formed after 3 and 5 days in vitro. Statistical significance was tested by one way ANOVA and Student Newman Keuls post-test.

Astrocytes growing in 3D cultures are also sensitive to DAMPs gradients, but do not form scars:

After culturing the astrocytes to confluence in 3D hydrogel matrices during 16 days, we injected recombinant HMGB-1 as a DAMP or soluble astrocyte lysate in the gel. The purpose of this experiment was to test the astroglial response to a focal DAMP release that would mimic the situation of the ischemic core. In the ischemic core *in vivo*, neuronal necrotic cell death induce a focal source of DAMP that reach the astrocytes and probably

contributes to glial scar formation. Using the nanotube Hydromatrix matrices, astrocytes grow in a more realistic 3D environment, and survive for several weeks with excellent viability (Figures 18, 19). After injecting the DAMPs, cultures were followed by time lapse microscopy during 3 days and then they were fixed. Immunocytochemistry revealed that astrocytes growing in 3D culture were sensitive to DAMPs and responded with reactive gliosis and increased GFAP expression. However, we could not find scar-like structures assembled by astrocytes (Figure 20). In another set of experiments, we used mixed glia (microglia plus astrocytes) and no scar formation was observed. We conclude that a focal release of DAMPs is not sufficient to induce glial scar formation by astrocytes.

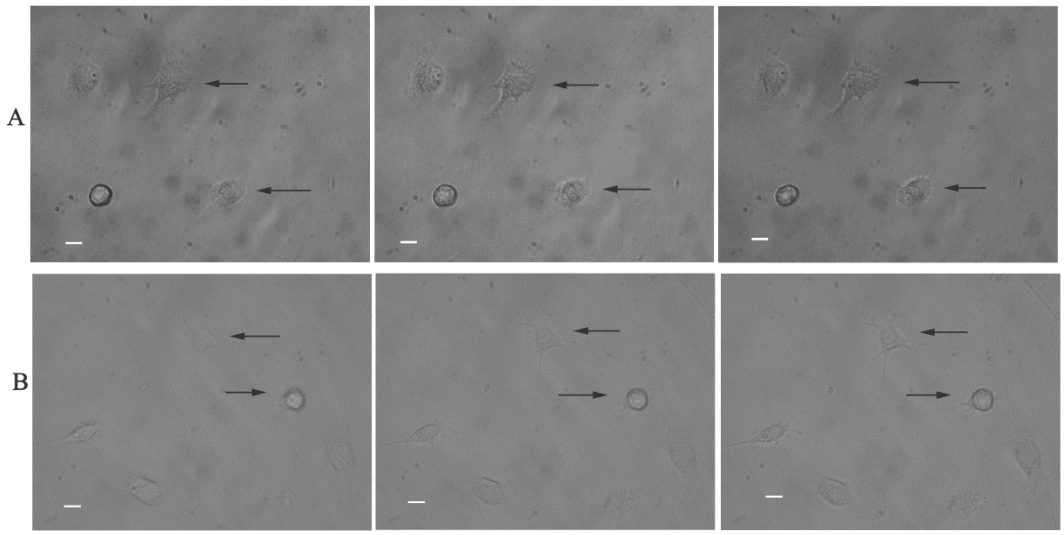

Figure 18. **Three dimensional growth of astrocytes in hydromatrices.** The astrocytes cultures in Hydromatrices showed morphology with growth in three dimensions as shown by arrows, similar to the *in vivo* astrocytes, and they are also cultured for longer times compared to 2D (or astrocytes have longer life span in 3D cultures compared to the 2D cultures). Scale bar: 50 µm.

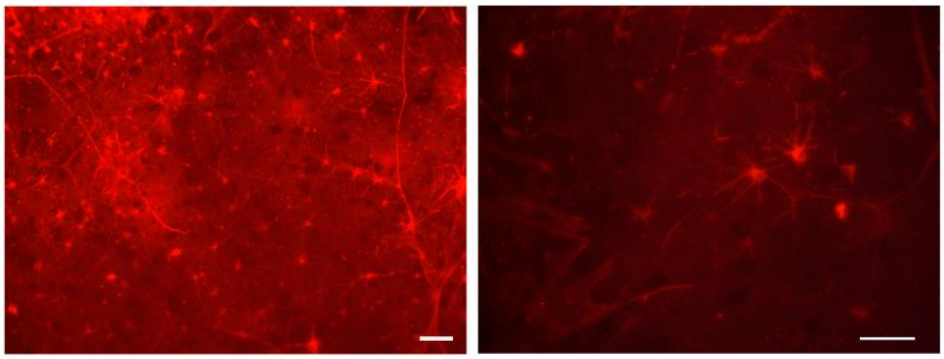

Figure 19. **Morphology and distribution of astrocytes in 3D hydromatrices.** Astrocytes in 3D have fine philopodia and round cell body compared to the 2D monolayer astrocytes. Reaching the confluence is not possible for astrocytes growing in 3D matrixes. The astrocytes in 3D are maintaining their territory compared to the astrocytes in 2D, where astrocytes have intense overlapping after reaching confluence. GFAP staining in red. Scale bar: 200 µm

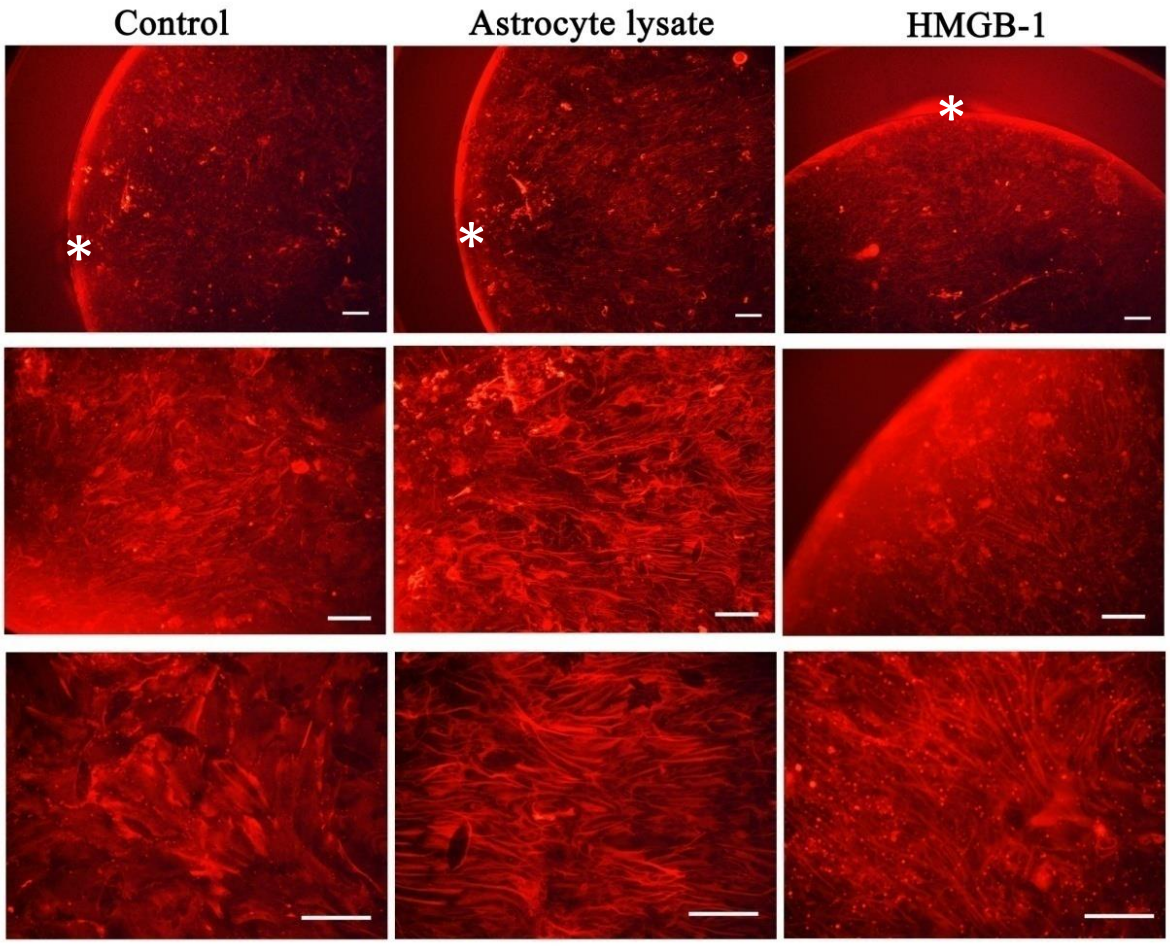

Figure 20. **Sensitivity of 3D astrocytes to recombinant pure DAMP (HMGB-1), or mixed DAMPs (soluble glial lysate obtained by osmotic shock).** Reactive gliosis is induced by the DAMP injection but no glial-scar structures were observed. The astrocytes were immunostained by GFAP and the pictures shows increasing magnification from top to bottom. GFAP staining in red. Site of injection is shown by an asterisk (*). Scale bar: 200 µm.

DISCUSSION AND CONCLUSIONS

We initially reproduced the technique detailed by Wanner and colleages (2013) to induce in vitro glial scar formation in the confluent astrocytes culture by the addition of primary meningeal macrophages. In spite of the fact that we used two meningeal cell concentrations (30,000 meningeal cells per well and 15,000 meningeal cells per well), we noticed that there is no significant increase in the total AFA even though we doubled the meningeal cells per well. This is an intriguing result that is probably reflecting that, after certain number of meningeal cells, there will be no further increase in total AFA per well as the astrocyte enzymes involved in astroglial migration away from the macrophages will get saturated. Alternatively, it might be also possible that, in spite of having higher level of soluble mediators secreted by macrophages, there might not be further free migration of the astrocytes because of the crowding of the astrocytic monolayer.

For glial scar formation, it is critical to understand the molecular machinery governing astrocyte polarity and recruitment to the injury site (as the case of NG2 glial cells) or the migration away from the ischemic core (astrocytes). The Rho family of small GTPases, mediate the convergence of both extracellular and intracellular signals onto the cytoskeleton, resulting in morphological plasticity (Abbracchio et al., 1997, Chen et al., 2005). The small RhoGTPase Cdc42 has emerged as a key regulator of polarization, influencing directional migration in cultured fibroblasts and astrocytes. After interaction with its prototypic ligand High-Mobility Group Box 1 (HMGB-1), RAGE regulates cell migration by activating Rac1 and Cdc-42 signaling (Taguchi et al. 2000; Bassi et al. 2008; Hudson et al. 2008).

The laboratory of my director also showed that changes in astroglial cytoskeleton leading to the stellated reactive astroglial phenotype and the migration towards cell free areas *in vitro* share the RAGE/Cdc42-Rac1 pathway (Villarreal et al., 2014). The initial event in astroglial migration was the astroglial polarization towards the injury site and the subsequent protrusion of long processes. These processes exhibit a flattened polygonal shape in their distal end, similar to philopodia and very similar to those observed in our present results in scar-forming astrocytes. However, most of the literature on astroglial cell migration was obtained by the analysis of scratch assays. While scratch assays induce

astroglial migration towards a cell-free area, a process that is facilitated *in vitro* due to loss of contact inhibition, migration away from macrophages or from the ischemic core involves the override of cell contact inhibition and probably even detaching from the culture surface. Even though this is an extremely stressful situation for cells in culture, we could not observe images of pyknotic nuclei or degenerating cells. Based on the available information and our present observations, we speculate that small RhoGTPases Cdc42 and Rac1 would be crucial for astrocyte polarization required for glial scar formation.

Most of the glial scar studies are done in spinal cord injury (SCI) models. After SCI crush, the reactive astrocytes that are more distant (> 1mm) from the large central lesion core, exhibited a clear and pronounced upregulation of GFAP and some somatic hypertrophy. Nevertheless, these reactive astroglia retained both their basically stellate appearance with GFAP and their basic bushy appearance. In contrast, astroglia in scar borders immediately adjacent to central lesion core, exhibited distinctive elongated morphologies with overlapping and interacting processes. With increasing distance from lesion borders, reactive astroglia transitioned in a clearly graded fashion to morphologies more similar to those seen in healthy tissue. In our *in vitro* experiments we demonstrated this striking differences between glial scar forming astrocytes (astrocytes forming the border of AFA), and the the astrocytes that are distant from lesion (AFA) as seen *in vivo* glial scar. We noticed the formation of well-organized glial scar with highly elongated, overlapping and interacting process. The astrocytes distant from the area occupied by macrophages retained the normal stellate morphology being confluent with less overlapping. We also noticed that the astrocyte forming the border of AFA have projections towards the center of the AFA, which might be because of the incomplete retraction of astrocytes forming the scar. However, we can also interpret that, as the border seems to be well formed and organized, these processes have the role of sensing the molecular environment in the AFA *in vitro* or in the lesion core *in vivo*.

Toll-like receptors (TLR) are one of several classes of pattern-recognition receptors (PRRs), which are involved in the activation of the innate immune system and the subsequent orchestration of the adaptive immune response (Kaisho and Akira, 2006; Medzhitov and Janeway, 2000). Upon recognition of conserved molecular motifs expressed by PAMPs or DAMPs, TLRs initiate a cascade of intracellular events involving the Nuclear

Factor kappa B (NF-κB)-dependent production and release of cytokines and chemokines (Hirschfeld et al., 1999; Medzhitov et al., 1997). DAMPs can bind and trigger TLR activation and consequently contribute to sterile inflammation. Some DAMPs are confined to the intracellular space under physiological conditions, and are released into the extracellular space following injury- or disease-induced cellular damage and death. These DAMPs include HMGB-1 (Park et al., 2004), heat shock proteins (HSPs) (Asea et al., 2002; Ohashi et al., 2000), microRNA (Lehmann et al., 2012), mitochondrial RNA and DNA (Kariko et al., 2005; Zhang et al., 2010) and histones (Huang et al., 2011).

HMGB-1 is a DNA binding protein that regulates gene transcription. In pathological conditions, HMGB-1 is passively released by damaged and necrotic cells or actively released by activated inflammatory cells (Scaffidi et al., 2002). It is increasingly clear that TLRs do in fact play a role in ischemic damage, especially TLR2 and TLR4 have been shown to play a role in cerebral ischemic damage (Marsh et al., 2009). Following ligand binding, TLRs form homodimers (Bovijn et al., 2012) or heterodimers (Hajjar et al., 2001; Ozinsky et al., 2000) and initiate signaling through either the myeloid differentiation 88 (MyD88)-dependent or MyD88-independent pathways (Akira and Takeda, 2004). Injury to the CNS induces an increase in the expression of TLRs and their downstream effectors (Kigerl et al., 2007), leading to the generation of inflammatory mediators such as TNF α, IL1, IL6, and iNOS, all known to be associated with stroke damage (Marsh et al., 2009).

As previous studies show increasing importance of TLR in ischemic injury, we decided to test the expression of TLR by the scar forming astrocytes. We tested the TLR expression in the model of glial scar formation by the primary meningeal cells (Wanner et al., 2013). We noticed the increased expression of TLR2 and TLR4 in scar forming astrocytes, while the remaining reactive astrocytes presented lower expression. Based on our results we can only speculate on the causes of such differential expression. While scar-forming astrocytes are exposed to high levels of DAMPs *in vivo* and it is known that DAMP can initiate a feed-forward loop to increase TLR and RAGE expression (Villarreal et al., 2014; Pelham et al., 2014); this could not be the case *in vitro*. However, it is plausible that macrophages secrete acetylated HMGB-1 (Liesz et al., 2015) that could exert such feed-forward autocrine loop by activating the NF-κB responsive elements in TLR promoters. An alternative explanation is

that scar-forming astrocytes are the more reactive phenotype and thus it is expected that they present higher expression levels of proinflammatory mediators like TLR.

In the central nervous system (CNS), the transcription factor nuclear factor NF-κB is a key regulator of inflammation and secondary injury processes**.** In CNS trauma, the first phase of damage, dependent on mechanical destruction of the nervous tissue, is followed by a secondary phase caused by severe local disturbance of the blood supply and a massive release of pro-inflammatory mediators and neurotoxins from invading and resident cells (Schwab et al., 2002). A prominent source of inflammatory mediators is astroglia, which, in response to injury, undergoes a profound activation known as reactive astrogliosis (Ridet et al., 1997). Even though reactive astrocytes release factors essential for neuronal survival and wound healing (Faulkner et al., 2004), they are also responsible for the production of molecules detrimental to functional recovery and for creating an environment unfavorable to nerve regeneration (Menet et al., 2003).

Many of the processes occurring in reactive astrocytes as a consequence of CNS damage are regulated by NF-κB (O'Neill et al., 1997). After brain injury, NF-κB is highly activated (Bethea et al., 1998, Beni et al., 2004) and the expression of NF-κB–dependent genes is up regulated (Bethea et al., 1999, Siren et al., 2001), indicating a critical function of this factor in CNS pathophysiology. NF-κB is an important transcription factor downstream in the TLR2, TLR4 and RAGE-mediated signaling pathways which respond to a variety of stimuli (Toshchakov et al., 2002).

In unstimulated cells, NF-κB proteins are sequestered in the cytoplasm by IκB inhibitory proteins. Various immune stimuli induce the IκB kinase (IKK) to phosphorylate IκBs, triggering their ubiquitination and degradation. Released NF-κB dimers are further activated through various posttranslational modifications and translocate to the nucleus where they bind to specific DNA sequences and promote transcription of target genes. The transcription factor NF-κB is a key regulator of hundreds of genes involved in cell survival and inflammation (Hayden et al., 2008).

Having in mind that TLR2 and TLR4 were overexpressed in scar-forming astrocytes *in vitro*, we hypothesised that NF-κB may play crucial role in glial scar formation and

maintenance. Following our hypothesis we tested the effect of NF-κB on glial scar formation induced by ischemia-activated macrophages. By using ischemic cortical brain explants co-cultured over a confluent astrocytic monolayer, we observed a dense compact glial scar formation after 3-5 days *in vitro*. When astrocytes receiving the ischemic explants with activated macrophages were pre-treated with the NF-κB blocker BAY 11-7082 for 1 h, we observed a reduced migration of astrocytes away from the ischemic tissue. Furthermore, we also observed that the thickness of the glial scar was less compact when NF-κB activation was blunted. Hence, we conclude that NF-κB plays a crucial role in the astroglial response that leads to the glial scar formation induced by ischemia-activated macrophages.

Immunohistochemical studies from the cortical ischemic injury of cortex showed, astrocytes with STAT3 and NF-κB activation as soon as 2 to 4 hours post-lesion, preceding cell hypertrophy and GFAP up regulation, and being maintained in the long-term formed glial scar (Acarin et al., 2000). From previous studies (Wanner et al., 2013) and from the group of my director, we know that both NF-κB and STAT3 pathways play important roles in cell migration, immune cell activation inflammation and astroglial hypertrophy. The present results blocking NF-κB, and those obtained by Wanner and colleages (2013) blocking STAT3 in vitro and in vivo, show that blockage of these pathways does not prevent the formation of the glial scar but reduces its quality. In fact, we observed that the cell density of the scar and the distance from the macrophages is reduced after NF-κB blockage and STAT3 knock-out mice show increased invasion of blood derived leukocytes after SCI (Wanner et al., 2013). So far, we consider it is probable that the NF-κB and STAT3 pathways act in parallel, and probably synergistically to assembly or maintaining the scar.

Cells cultured in 3D matrixes exhibit features that are closer to the complex *in vivo* conditions (Vinci et al., 2012). In conventional 2D conditions, the extracellular matrix components, cell-to-cell and cell to-matrix interaction that are important for differentiation, proliferation and cellular functions *in vivo* are lost (Mazzoleni et al., 2009). The "*closer-to-in vivo*" behavior of cells when grown as 3D cultures is mainly because of the matrices and scaffolds that are used for obtaining such cultures. These matrices and scaffolds mimic the native extracellular matrix by porosity, fibrous, permeability and mechanical stability. The micro architecture enhances the biophysical and biochemical interaction of the adhered

cells to be better expressed *in vitro*. The 3D matrix provides a biologically active environment for the cells to proliferate, differentiate and secreted cell specific extracellular matrix. The choice of such matrices and scaffolds is based on cell type and the nature of the study. Also, the pore size, ligand density and stiffness can be varied easily, and thus making it easy to change the structural properties of the gel (Baker et al., 2009, 2011; Harjanto and Zaman, 2011). Thus, 3D culture models have proven to be more realistic for translating the study findings for *in vivo* applications.

The cell culture dimension from conventional 2D monolayer to a 3D setting leads to down regulation of astrocyte activation markers, indicating that astrocytes in 3D are less reactive than their 2D counterparts, thus resembling the physiological *in vivo* situation and providing the first 'ground state' system in which triggers of astrogliosis can be investigated (East et al., 2009). As the movement of cells into or within biomaterial scaffolds is a prerequisite for the studies on glial scar formation. In addition, as the hydrogels we are using for the 3D experiments are polypeptide polymers, using 3D matrices it would be more helpful in studying whether the astroglial migration through the matrices is proteolytic (mesenchymal) migration with the involvement of matrix metalloproteases (MMP´s) or non-proteolytic (amoeboid) migration.

Flow-cytometry data shows cells in 3D matrix had more cells in G0/G1 phase and fewer cells in S phase (Ravi et al 2015). In our experiments, astrocytes cultured in the Hydromatrix, reached the confluence slowly compared to the 2D counterparts and also hydromatrix allowed cell survival for at least 45 days without reseeding the cells. This might be attributed to the property of hydrogels to keep cells hydrated and nourished for a longer time along with providing attachment and development support. Thus, cells grown in matrices instead of as monolayers, are able to survive for extended time and at slower proliferation rates which is pretty much closer to the *in vivo* scenario.

The key morphological differences that we observed are that cells in 2D had larger cell area and flattened morphology with wider philopodia compared to astrocytes in 3D matrix. Astrocytes in 3D have fine filopodia and are generally rounder and had a smaller cell area. Also, even when the astrocytes reach the confluence in 3D are maintaining or well

within their territory compared to the astrocytes in 2D where astrocytes have some overlapping after reaching confluence.

After the treatment with DAMPs (astrocyte lysate or recombinant HMGB-1), astrocytes appeared considerably more ramified compared to control cultures at the same time points. As we have injected the DAMPs at one border of the well, we noticed the graded response of the astrocytes to the treatment. The astrocytes that are close to the site of injection had high GFAP expression, they were hypertrophied with long and elongated process stretching in to the surrounding astrocytes territories compared to the astrocytes that are distant from the site of injection, where astrocytes have little elongations. Astrocytes showed signs of migration with small cell free area and alignment of the elongated cellular process away from the site injection that is towards their direction of migration, especially in the wells where astrocyte lysate was injected. The astrocytes treated both with the pure DAMP, HMGB-1, or the DAMP mixture (astroglial cell lysate) showed the morphological features of reactive and reoriented astrocytes with highly polarized phenotype and highly elongated processes, except that there is no clear scar formation with interacting and overlapping filaments perpendicular to the direction of their migration. The astroglial polarization and reoriented cytoskeleton, but lacking of glial scar formation can be interpreted in different ways. It is plausible that technical variations, including the requirement of longer incubation times could be required for 3D cultures compared with 2D or the quality of the DAMP gradient in the matrix compared with the native gradient *in vivo*, could account for the lack of glial scar formation *in vitro* in 3D hydromatrixes. On the other hand, when we compare the *in vivo* glial scar formation, the successful scar formed in 2D macrophages-astroglial co-culture and the dense scars obtained in 2D ischemic explants-astroglial co-culture, it is clear that macrophages and cytokines derived from them are missing in 3D cultures. Thus, these results stress the fact that activated macrophages and DAMPs are probably required to form a glial scar that resemble those found *in vivo* after ischemia or SCI.

In summary, our results show that intracellular pathways and molecules involved in glial scar formation can be studied *in vitro* using different approaches in 2D or 3D platforms. Based in our findings, we conclude that NF-κB is required for the glial scar formation and this transcription factor probably lies downstream of the TLR2 and TLR4 innate immunity

receptors that are overexpressed by scar-forming astrocytes. Finally, we were unable to produce glial scars when we used a focal release of recombinant HMGB-1 (a pure synthetic DAMP) or soluble cell lysate (mixed DAMPs) probably due to the absence of macrophages and/or cytokines produced by these cells.

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APPENDIX 1

Glutamate-based clinical trials in ischemic injury (O'Collins et al., 2006).

Name	First Trial[a]	Mechanism	Level of Protection (%)[b]	N[c]	Experimental Model[d]									STAIR Quality[e]
					Focal			Global			Culture			
					+	O	−	+	O	−	+	O	−	
Excitotoxicity														
ACEA 1021 (licostinel)	1997	NMDA glycine site antagonist	37	25	19	6	0	0	3	0	2	0	0	7
ARL 15896(AR-A15896AR)	1999	NMDA antagonist	39	15	10	8	0	8	8	0	2	0	0	10
Baclofen	2001	GABA-B agonist	—	0	1	1	0	3	2	0	0	0	0	1
BIII 890 CL	2001	Sodium channel block	27	6	6	0	0	0	0	0	1	0	0	4
BMS-204352	1998	Potassium channel opener	14	9	7	1	0	0	0	0	0	0	0	5
CGS 19755 (selfotel)	1995	NMDA antagonist	47	2	4	1	1	4	1	0	2	1	0	7
Clomethiazole (CMZ, Zendra)	1996	GABA agonist	42	7	8	2	0	3	1	0	0	0	0	9
CNS1102 (Cerestat, aptiganel)	1994	NMDA ion channel blocker	51	11	11	2	0	0	0	0	0	0	0	6
CP101.606-27	1999	NMDA ion channel blocker	61	3	3	0	0	0	0	0	0	0	0	8
Dextrorphan	1994	NMDA ion channel blocker	50	17	13	6	0	6	0	0	3	0	0	7
Eliprodil (SL 82.0715)	1994	NMDA polyamine antagonist Sigma ligand	48	4	6	0	0	3	2	0	1	0	0	6
Diazepam (valium)	2000	Benzodiazepine	—	0	0	1	0	12	1	0	1	0	0	1
Fosphenytoin	1995	Sodium channel blocker, Glutamate release inhibitor.	—	0	0	0	0	1	0	0	0	0	0	0
Gavestinel (GV150526A)	1999	NMDA glycine antagonist	60	18	8	6	0	0	0	1	1	0	0	3
Glycine	1996	NMDA antagonist	—	0	0	0	0	0	1	0	2	0	1	0
Lifarizine (RS-87476)	1995	Sodium/calcium channel blocker	46	8	5	4	0	3	0	0	0	0	0	5
Lubeluzole	1994	Sodium/calcium channel blocker NOS inhibitor	23	19	13	8	0	3	2	0	4	2	0	6
Magnesium sulphate	1993	NMDA ion channel blocker. Calcium antagonist	35	10	11	0	0	17	9	1	1	1	0	7
Nalmefene	1998	Opiod antagonist	—	0	0	0	0	1	0	0	0	0	0	0
Naloxone	1981	Opiod antagonist	29	7	8	7	0	5	7	2	2	0	0	8
Nicergoline	1985	α2 adrenoceptor agonist Enhances glutamate uptake	—	0	1	0	0	8	3	0	0	0	0	2
NPS 1506	1998	NMDA ion channel blocker	—	0	6	2	0	0	0	0	1	0	0	4
NS1209/SPD 502	1999	Gluamate antagonist	44	2	2	0	0	0	0	0	0	1	0	3
Remacemide	1994	NMDA ion channel blocker Sodium channel blocker	49	1	1	0	0	0	0	0	1	0	0	2
S-1746	2001	NMDA glycine/AMPA antagonist	—	0	0	0	0	0	0	0	0	0	0	0
Sipatrigine (BW619C89)	1995	Sodium channel antagonist Glutamate release inhibitor	41	37	40	4	0	1	0	0	1	0	0	8
YM872	1999	AMPA antagonist	27	32	22	8	0	0	0	0	1	0	0	7
YM90K	1997	AMPA antagonist	31	23	19	6	0	5	0	0	0	1	0	8
ZK200775 (MPQX)	1997	AMPA antagonist	19	21	12	9	0	1	0	0	0	1	0	6
Antiinflammatory														
Dexamethasone	1971	Glucocorticoid, antiinflammatory	19	11	7	8	1	5	6	6	1	0	0	8
Enlimomab (anti–ICAM-1 antibody)	1996	Leukocyte migration and adhesion inhibitor	14	9	6	7	1	0	0	0	0	0	0	8
FK506 (pacrolimus)	2004	Immunosuppressant	31	72	52	27	0	20	12	0	12	6	0	9
Fludrocortisone	1999	Mineralocorticoid	—	0	0	0	0	0	0	0	0	0	0	0
Hu23F2G (LeukArrest)	1999	Leukocyte adhesion inhibitor	57	1	1	0	0	0	0	0	0	0	0	1
Indomethacin	2001	Cyclooxygenase inhibitor	23	2	3	2	0	11	10	0	2	2	0	4
LDP-01 (Anti–β-2-integrin antibody)	1999	Leukocyte adhesion and migration inhibitor	—	0	0	0	0	0	0	0	0	0	0	0
Neutrophil inhibitory factor (rNIF, UK-279.276)	2000	Neutrophil inhibitor	31	12	8	4	0	0	0	0	0	0	0	4
Paracetemol (acetaminophen)	1987	Analgesic/antipyretic COX inhibitor	8	1	0	1	0	0	0	0	0	0	0	1
Ganglioside GM1	1984	Metabolism, growth	4	1	6	4	0	9	2	0	0	0	0	8
Insulin	1993	Lowers glucose	16	5	4	1	2	8	1	0	0	0	0	8
Xanthinol nicotinate (Sadamin)	1977	Vitamin B(3): metabolic enhancer	—	0	0	0	0	1	1	0	0	0	0	0
Antioxidant														
Ebselen (Harmokisane)	1998	Free radical scavenger	27	9	10	6	0	0	0	0	2	0	1	7
MCI-186 (Edaravone)	2001	Free radical scavenger	−24	8	7	5	0	4	1	1	0	0	0	7
Nicaraven (N,N′-propylenedinicotinamide)	2001	Free radical scavenger	17	4	2	2	0	0	0	0	0	0	0	3
NXY-059	2001	Free radical scavenger	43	27	24	5	0	0	0	0	0	0	0	10
Tirilazad (U74006F)	1994	Free radical scavenger	26	16	11	8	0	6	5	0	4	2	0	8
Antiapoptotic/regeneration														
Basic fibroblast growth factor (trafermin. Fiblast)	1998	Growth factor	29	35	22	19	0	5	2	0	14	3	0	10
Erythropoietin	2002	Antiapoptosis, oxygen delivery	39	9	11	2	0	12	15	0	11	11	0	9
PS519/MLN519	2000	Proteasome inhibitor	32	14	11	3	0	0	0	0	0	0	0	5

Name	First Trial[a]	Mechanism	Level of Protection (%)[b]	N[c]	Experimental Model[d]									STAIR Quality[e]
					Focal			Global			Culture			
					+	O	−	+	O	−	+	O	−	
Calcium/adrenergic modulators/ antihypertensives														
Atenol (Tenormin)	1988	Beta blocker	—	0	0	0	0	0	0	0	0	0	0	0
Candesartan cilexetil (TCV-116, Blopress, CV-11974)	1999	AT1 receptor antagonist Anti-hypertensive	34	5	4	1	0	0	1	0	0	0	0	3
Cyclandelate	1966	Vasodilator (calcium modula-tor)	—	0	0	0	0	0	0	0	0	0	0	0
DP-b99 (DP-BAPA)	2000	Calcium chelator	—	0	0	0	0	0	0	0	0	0	0	0
Flunarizine	1990	Calcium channel blocker	−6	3	4	1	1	24	7	1	3	0	0	6
Nicardipine	1988	Calcium antagonist	11	6	8	10	0	12	15	0	1	2	0	7
Nicergoline	1985	Alpha2 adrenoceptor agonist Enhances glutamate uptake	—	0	1	0	0	8	3	0	0	0	0	2
Nimodipine	1984	Calcium channel blocker	26	37	24	28	0	11	10	0	1	2	0	9
Papaverine	1976	Calcium channel blocker	−3	1	0	1	0	0	0	0	0	0	0	2
Propranolol	1988	Beta-adrenergic blockade, Membrane stabilization	34	4	3	8	0	0	0	0	0	0	0	6
PY 108-068	1986	Calcium antagonist	—	0	2	0	0	0	0	0	0	0	0	5
S-0139 (SB-737004)	1999	Endothelin antagonist	36	4	3	1	0	0	0	0	0	0	0	2
Vinpocetine (ethyl apovincami-nate)	1986	Calcium inhibitor, Vasodila-tor, Sodium blocker.	42	1	1	0	0	9	0	0	0	0	0	
Thrombolytic														
Abciximab (reopro, c7E3 Fab)	1998	Antiplatelet: glycoprotein in-hibitor	27	2	1	1	0	0	0	0	0	0	0	3
Aminophylline	1976	Phosphodiesterase inhibitor	—	0	0	0	0	0	0	0	1	0	0	0
Anerod	1983	Fibrinogen depleting	21	4	4	1	0	0	0	0	0	0	0	4
Argatroban	1986	Anticoagulant	11	4	3	3	0	1	1	0	0	0	0	5
Aspirin	1995	Antiplatelet	31	19	9	13	0	0	2	0	10	0	0	8
Batroxobin (defibrase, DF-521)	1995	Fibrinogen depleting	—	0	4	0	0	1	0	0	1	0	0	1
Certoparin	2000	Anticoagulant	—	0	0	0	0	0	0	0	0	0	0	0
Dalteparin	2000	Anticoagulant	—	0	0	0	0	0	0	0	0	0	0	0
Defibrotide (polydeoxyribo-nucleotide)	1989	Antiplatelet: glycoprotein in-hibitor	—	0	0	0	0	1	0	0	0	0	0	0
Desmoteplase (DSPA)	2002	Antithrombotic	—	0	0	0	0	0	0	0	0	0	0	0
Enoxaparin	2003	Antithrombotic	25	25	12	13	0	0	0	0	0	0	0	6
Eptifibatide (cromafiban; Inte-grilin)	2003	Antiplatelet: glycoprotein in-hibitor	—	0	0	0	0	0	0	0	0	0	0	0
Heparin	1979	Anticoagulant	32	17	10	10	3	2	0	0	1	0	0	8
Nadroparin	1995	Antithrombotic	—	0	0	0	0	0	0	0	0	0	0	0
Org 10172 (danaparoid, Orga-ran)	1997	Antithrombotic	—	0	0	0	0	0	0	0	0	0	0	0
Pentoxifylline	1981	Improve capillary flow	—	0	0	1	0	3	0	0	0	0	0	2
Propentofylline (HWA 285)	1992	Phosphodiesterase inhibitor	37	7	9	2	0	7	2	0	2	1	0	6
Prosatacyclin	1984	Antiplatelet: eicosanoid Vaso-dilator	−6	1	1	1	0	9	1	0	1	0	0	5
Prourokinase	1998	Antithrombotic	55	12	12	0	0	0	0	0	0	0	0	3
RPR 109891	1998	Antiplatelet glycoprotein in-hibitor	—	0	0	0	0	0	0	0	0	0	0	
n-PA/tPA (alteplase)	1988	Antithrombotic	4	86	52	38	11	0	1	0	1	0	0	9
Streptokinase	1963	Thrombolytic	−525	6	1	4	5	0	0	0	0	0	0	6
Tinzaparin	1998	Anticoagulant	—	0	0	0	0	0	0	0	0	0	0	0
Tirofiban (MK-383, aggrastat)	2001	Antiplatelet: glycoprotein in-hibitor	—	0	0	0	0	0	0	0	0	0	0	0
TNK (tenecteplase)	2000	Thrombolytic agent	35	2	2	0	0	0	0	0	0	0	0	4
Triflusal (2-acetoxy-4-trifluoromethylbenzonic acid)	2001	Arachidonic acid metabolism inhibitor (antiplatelet)	—	0	1	2	0	0	0	0	0	0	0	2
Urokinase	1976	Thrombolytic	53	12	13	1	0	0	0	0	0	0	0	7
Nootropic/stimulant														
Amphetamines	2003	Stimulant	−3	1	1	2	0	2	1	0	0	0	0	7
Cerebrolysin	2001	Nootropic	—	0	1	1	0	6	1	0	1	0	0	4
Citicoline (CDP choline)	1987	Membrane precursor, antioxi-dant Vascular insufficiency Immunostimulatory Nootropic	25	13	4	9	0	8	1	0	0	0	0	8
EGB-761 (Gingko biloba ex-tract)	1995	MAO inhibitor Antiplatelet. Antioxidant Reduces leukocyte activation Increases cerebral blood flow	25	15	13	3	0	4	0	0	10	1	0	7
Hydergine	1978	Nootropic, antioxidant.	—	0	0	0	0	2	0	0	0	0	0	0
Nafronyl oxalate (naftidrofuryl)	1978	Serotonin antagonist	38	5	6	2	0	2	0	0	1	0	0	7
Piracetam	1988	AMPA (NA+) modulator	39	5	4	1	0	6	2	0	0	1	0	4
Semax	1997	Derivative of ACTH-4-10	—	0	0	0	0	4	0	0	1	0	0	0

Name	First Trial[a]	Mechanism	Level of Protection (%)[b]	N[c]	Experimental Model[d]									STAIR Quality[e]
					Focal			Global			Culture			
					+	O	−	+	O	−	+	O	−	
Fluid regulation														
Glycerol	1972	Hyperosmolar agent	—	0	0	2	0	1	2	0	0	0	0	0
Dextran	1969	Hemodilution	34	7	4	5	1	0	0	1	0	0	0	7
Hydroxyethyl starch pentastarch	1980	Hemodilution	23	3	4	3	1	0	0	0	0	0	0	8
Mannitol	1978	Hyperosmotic agent. Reduces edema and ICP	34	19	10	15	1	8	5	1	0	0	0	8
Oxygen delivery[f]														
Diaspirin cross-linked hemoglobin	1998	Oxygen delivery Free radical scavenger	48	5	5	1	0	0	0	0	0	0	0	5
Oxygengated flurocarbon nutrient emulsion (OFNE)	2001	Oxygen delivery	94	1	2	0	0	3	0	0	0	0	0	5
Hyperbaric oxygen treatment	1966	Oxygen delivery	24	17	13	5	2	7	4	0	0	0	0	7
Other														
Caffeinol	2002	Stimulant, depressant, diuretic Adenosine receptor modulator	51	10	8	2	0	0	0	0	0	0	0	4
Corticotrophin	1987	GABA receptor modulator Pituitary hormone	—	0	0	0	0	0	0	0	0	0	0	0
Glyceryl trinitrate (nitroglycerin, GTN)	1999	NO donor	—	0	1	0	0	0	0	0	1	0	1	0
Hypothermia	1998	Reduce reducing cerebral oxygen demand (CMRO2), Metabolic and synaptic transmission inhibitor.	46	92	94	28	0	77	28	0	13	3	1	10
ONO-2506	2003	Astrocyte modulating agent Anenuates extracellular monamine	25	8	5	3	0	0	0	0	0	0	0	5
Radix salviae miltiorrhizae	2000	Antioxidant Partial endothelin-1 inhibitor Increases VIP	—	0	1	1	0	1	0	0	1	0	0	1
Repinotan (BAY × 3072)	2000	Serotonin agonist	56	2	2	0	0	2	0	0	7	0	0	2
Simvastatin	2001	HMGCoA reductase inhibitor Antioxidant	30	20	11	1	0	3	1	0	1	0	0	7
TAK-218	2001	Dopamine suppressor Sodium channel modulator	10	1	0	1	0	0	0	0	1	0	0	1
Tinofedrine (D 8955, Novocebrin)	1978	Blood flow, increased metabolism	—	0	0	0	0	0	0	0	0	0	0	0
Trazodone (Desyrel)	1986	Serotonin reuptake inhibitor	—	0	0	0	0	0	0	0	0	0	0	0

[a]First trial = the year in which the drug was first reported to have been given to acute stroke patients.
[b]Level of protection = average neuroprotection from infarct volume changes in focal ischemia studies.
[c]N = number of studies from which the level of neuroprotection has been calculated
[d]+ = number of experimental contrasts with a significant improvement in outcome in the treatment group vs control; 0 = number of experimental contrasts showing no significant in outcome in the treatment group vs control; − = Number of experimental contrasts showing a worse outcome in the treatment group vs control.
[e]STAIR quality = number of STAIR criteria met by the drug (maximum 10: see Table 3).
[f]Analyzed with other group because of low numbers.