UNIVERSITY OF CALIFORNIA, SAN DIEGO

Activation of the mTOR Pathway in Astrocytes After Spinal Cord Ischemia

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

in

Molecular Pathology

by

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2008

The dissertation of Simone Codeluppi is approved, and it is acceptable in quality and form for publication on microfilm:

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University of California, San Diego

2008

DEDICATION

To Greta and Saiane Marie-Emilie.

EPIGRAPH

"I have been impressed by the urgency of doing.
Knowing is not enough; we must apply.
Being willing is not enough; we must do."
—Leonardo Da Vinci

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ACKNOWLEDGEMENTS

I would like to acknowledge Professor Elena Pasquale for her support as advisor during my graduate career. I would also like to acknowledge my committee members Steven Gonias, Darwin Berg, Daniel Donoghue and Barbara Ranscht for their expert advices and encouraging comments as I progressed towards the dissertation. I would like to acknowledge Mike Hefferan and Martin Marsala of the University of California, San Diego as collaborators in the animal studies included in this dissertation.

Thanks to Professor Gianluigi Rossi because without his support this adventure would have not taken place.

Thanks Fatima for your help, support and coffees all along the way; you are a great friend! I would also like to acknowledge all the members of the Pasquale lab especially Caroline and Morgan for their help in some of the projects that are not included in the dissertation and for the help in making the lab a happy and noisy workplace.

Many thanks to all my friends at the Burnham Institute for Medical Research for their help but also for being great friends during these years.

Thanks Josh, Joaquim, Ryan, James, Mari, Martin, Leonardo to drag me out from the lab and for being always there.

My friends back home, Alberto, Simone, Nicola, Rossano and Silvia for always being close even if so far away. Thanks for taking care of me every time I am back home!

Thanks Maria, Gigi, Matteo, Cristiano, Gianluca and Lucia for all the support, it meant a lot!

I would like to thank my family; Vittorio, Teresa, Greta and Saiane for believing in me and remind me everyday what is important in life.

Last but definitively not least I would like to thank Camilla for her constant support even through the hardest storms at work and in life. I would not have made it without you!

Chapter 2 is being submitted for publication as The Rheb-mTOR pathway is upregulated in reactive astrocytes of the injured spinal cord by Codeluppi S., Svensson CI., Hefferan MP., Silldorff MD., Marsala M. and Pasquale EB. The dissertation author is the primary investigator on this paper.

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PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

Activation of the mTOR Pathway in Astrocytes After Spinal Cord Ischemia

by

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Doctor of Philosophy in Molecular Pathology
University of California San Diego, 2008
Professor Steven L. Gonias, Chair
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Spinal cord injury is characterized by cell death, macrophage infiltration, and formation of a glial scar. Functional recovery from this severe damage is dependent on the regeneration and growth of the axons of the surviving neurons beyond the lesion site and on the formation of the proper synaptic connections between the axons and their targets. However, the regeneration process is affected by inhibitory molecules expressed by the astrocytes of the glial scar and derived from the rupture of the myelin sheats. A better environment for spinal cord regeneration can be created by regulating glial scar formation and by blocking the effects of the myelin derived inhibitors. In the adult central nervous system, epidermal growth factor (EGF) regulates astrocyte activation and scar formation. Inhibition of EGF receptor signaling showed beneficial effects and promoted functional recovery and locomotion in an animal model of contusion-induced spinal cord injury.

We showed that EGF receptor activates the mammalian target of rapamycin (mTOR) pathway in primary cultures of adult spinal cord astrocytes. EGF receptor activation causes Akt mediated phosphorylation and downregulation of the mTOR pathway inhibitor Tuberin. Since Tuberin is a GTPase-activating protein that regulates the activity of the small GTPase Rheb, EGF treatment increases Rheb and mTOR activity. Furthermore, in primary cultures of spinal cord astrocytes, mTOR

regulates proliferation and EGF-induced migration. We also detected increased activation of the EGF receptor and the mTOR pathway in hypertrophic astrocytes in the spinal cord after ischemia-induced injury.

These in vitro and in vivo findings suggest that EGF-induced astrocyte hypertrophy is regulated by the mTOR pathway. Inhibition of mTOR activity with rapamycin may reduce astrocyte hypertrophy as well as scar formation and prove beneficial for axonal regeneration and functional recovery after spinal cord injury.

Chapter 1

Introduction

1.1 Spinal Cord Injury

Spinal cord trauma often has severely debilitating effects and poor clinical prognosis. Depending on the location of the lesion site the trauma may cause loss of muscle movement, sensation, bladder, bowel, and breathing control. The lesioned nerves are capable of only limited sprouting, which provides little functional recovery. Due to this lack of regenerative ability, the functional impairments associated with spinal cord injury will affect the victims for their lifetime. Even though no treatment currently exists, the advances in understanding the mechanisms that prevent regeneration have provided new hope for spinal cord repair.

For many years it was thought that injured CNS axons were not capable of spontaneous regeneration, unlike those of the peripheral nervous system that can regenerate after injury [1]. This idea changed when a series of grafting experiments showed that CNS axons in a transected spinal cord elongated when tissue from the peripheral nervous system was grafted into the lesion [2]. This revolutionary experiment suggested that lesions in the CNS cause the formation of an inhibitory environment that prevents axon regrowth. The best characterized environmental inhibitors of axon regeneration are associated with the CNS myelin and the glial scar.

Myelin components released upon injury to the CNS inhibit axon regrowth. Three major inhibitors have been extensively characterized: Nogo-A, Myelin-Associated Glycoprotein, and Oligodendrocyte-Myelin Glycoprotein.

Nogo-A is a transmembrane protein expressed by oligodendrocytes and some neuronal cell types. It is localized in both the inner and outer loop of myelin and on the surface of oligodendrocytes. The Myelin-Associated Glycoprotein is a member of the immunoglobulin superfamily expressed on the surface of oligodendrocytes and in the periaxonal membrane in the internodal segments of the myelin sheats. The Oligodendrocyte-Myelin Glycoprotein is a GPI-anchored protein highly expressed in many types of neurons and immature oligodendrocytes, and it is enriched in the region of the nodes of Ranvier. All three inhibitors bind to the same receptor complex, which is localized on growing axons and is formed by the Nogo receptor, the neurotrophin binding protein p75ntr, and the signal transducing subunit LINGO-1. Ligand-Nogo receptor complex formation results in activation of the small GTPase RhoA and inhibition of axon growth. The Rho family of small GTPases play an important role in transducing extracellular signals to the actin cytoskeleton, and activation of RhoA leads to actin depolymerization, growth cone collapse, and inhibition of neurite growth [3].

Another well characterized inhibitor of axon regeneration is the glial scar, which is formed in the CNS after injury [4, 5]. Many types of spinal cord trauma such as compression, contusion, and ischemia all lead to the same final outcome of cell death. This occurs in both the neuronal and the glial cell populations and results in the formation of a lesion cavity [6]. In addition, an inflammatory response is triggered by the injury. Resident microglia become activated and secrete pro-inflammatory cytokines that recruit immune cells from the circulation. Phagocytic immune cells have a dual role, they can reduce the inhibitory effects of myelin inhibitors and promote axonal sprouting but also increase spinal cord damage through secretion of neurotoxic factors and production of reactive oxygen species [7, 8, 9]. The degenerative effects of inflammation on the tissue surrounding the injury are reduced by the formation of a glial scar, which insulates and fills the lesion cavity. Noteworthy, the amount of cell division associated with gliosis is small and confined to the area near the lesion core [10]. In lesions where the meninges are not damaged, the scar is composed primarily of hypertrophic astrocytes that express high amounts of intermediate filament proteins, such as glial fibrillary acidic protein (GFAP), vimentin, and nestin

[11, 12, 13]. However, in lesions where the meninges are damaged the astrocytes are mixed with invading connective tissue cells [14, 15]. The majority of the hypertrophic astrocytes migrate towards the site of injury, surround the non-regenerating nerves, and form a scar that in the long run becomes an impenetrable barrier. Even though reactive astrocytes express many inhibitors of axon growth such as tenascins, semaphorins, and ephrins, the inhibitory effects of the scar are mainly mediated by the chondroitin sulfate proteoglycans, which are highly upregulated in hypertrophic astrocytes [16, 17, 18, 19, 20, 21]. These proteoglycans consist of a core protein that is covalently linked to one or more glycosaminoglycans and are negatively charged due to sulfation of the sugar chains. The molecular mechanisms that regulate the effect of proteoglycans on axon regeneration are not well characterized but may involve signaling through neuronal receptors and regulation of axonal cytoskeleton and membranes. Furthermore, proteoglycans can also interact with constituents of the extracellular matrix that normally promote growth and inhibit their activities [22]. Even though the glial scar has a negative effect on axon regeneration, it is beneficial for the stabilization of the CNS after injury. Components of the scar repair the blood-brain barrier, control of the inflammatory response and reduce cellular degeneration [10]. Indeed, glial scarring is important for the survival of the animal but its inhibitory effects on axon regeneration also cause the loss of long term functional recovery.

A possible strategy to improve the regeneration potential of the injured spinal cord is to block the myelin inhibitory factors and regulate glial scar formation. Blocking the interaction between myelin-derived inhibitors and their receptor using antibodies and peptides has been shown to cause significant axonal regeneration and functional recovery in injured animals [3]. However, regulating the extent of glial scar formation presents a challenge because in the initial period after spinal cord injury astrocytes have a positive effect on survival. A therapy that inhibits astrocyte hypertrophy and scar formation will have to maintain the beneficial effects of the astrocytes but block the later stages of scar formation when the reactive gliosis is no longer beneficial. The aim of my thesis is to understand the molecular processes of glial scar formation in order to identify molecular targets that can be used to develop treatments to regulate

reactive gliosis, and therefore potentially improve axon regeneration after spinal cord injury.

1.2 Role of EGF Receptor and mTOR is Astrocytes Physiology

In the CNS, astrocytes regulate neuronal activity and also connect the different cell types of the CNS into a functional network. Indeed, astrocytes form GAP junctions not only with other astrocytes, but also with oligodendrocytes, ependimal cells, and endothelial cells [23]. Astrocytes become reactive after CNS injuries such as ischemia and trauma. They become hypertrophic, migratory, and the size and complexity of their processes increase. The hallmark of astrocyte activation is the upregulation of intermediate filament proteins such as GFAP, vimentin, and nestin [11, 12, 13].

Astrocytes activation is regulated by the epidermal growth factor (EGF) receptor [24]. During development EGF receptor signaling is important for the differentiation of astrocytes, but EGF receptors are then downregulated in mature astrocytes of the adult mammalian CNS [25]. After injury, EGF receptor expression is rapidly upregulated in astrocytes [26, 27]. Comparison of the transcriptional profiles of astrocytes treated with EGF or left untreated using gene microarrays has shown that the majority of the differentially expressed genes could be grouped in three main categories: genes that regulate extracellular matrix organization, genes that regulate cell migration, and cytokines and cytokine receptors [25]. These changes in gene expression suggest that the EGF receptor regulates cell migration and tissue remodeling after neural injury. Furthermore, inhibition of the EGF receptor in activated astrocytes has been shown to promote functional recovery, locomotion, and nerve regeneration in a contusion model of spinal cord injury. Even though activation of the EGF receptor is known to cause a reactive phenotype in astrocytes, the downstream pathways that are involved have not been fully characterized.

The EGF receptor, also known as ErbB1, is a member of the tyrosine kinase receptor superfamily. Ligand binding to receptor tyrosine kinases induces receptor dimerization, upregulation of the kinase activity, and phosphorylation of tyrosines in the both the receptor and its downstream targets [28]. Phosphorylated tyrosines in receptor tyrosine kinases can function as docking sites for proteins containing Src homology 2 (SH2) or Phosphotyrosine-binding (PTB) domains. Upon binding

to phosphorylated residues, the phosphotyrosine-binding proteins can recruit downstream effectors and activate the Ras-Erk pathway, the phosphoinositide-3 kinase (PI3 kinase)-Akt pathway, and Rho, which mediate cell proliferation, survival, migration, and invasion.

The mammalian target of rapamycin (mTOR) is one of the proteins that can be regulated by Erk and Akt (Fig. 1.1). mTOR is a serine/threonine kinase that belongs to the phosphoinositide-3-kinase-related kinase family and has a central role in the integration of growth factor and nutrient signaling [29]. Studies on patients with Tuberous Sclerosis have linked mTOR to the regulation of astrocyte physiology. Tuberous Sclerosis is characterized by benign tumors, called hamartomas, that develop in the brain, lung, kidney and skin [30]. An high percentage of patients with Tuberous Sclerosis have mutations in the TSC2 gene, which encodes Tuberin. Tuberin forms a tumor suppressor heterodimer with the protein Hamartin and inhibits mTOR activity. Indeed, hamartomas isolated from patients with Tuberous Sclerosis have high levels of mTOR activity [31]. The role of Hamartin is to stabilize Tuberin and protect it from ubiquitination and degradation [32]. In the CNS, hamartomas are formed by hypertrophic astrocytes [33]. Knockout of upstream regulators of the mTOR pathway such as Tuberin, Hamartin, or the protein phosphatase and tensin homolog (PTEN) that dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate counteract PI3-Kinase activity reducing Akt activation, has shown that astrocyte proliferation and cell size are regulated by the mTOR pathway and that an abnormal activation of mTOR induces astrocyte hypertrophy and increases proliferation [34, 35, 36].

Growth factors signaling can activate mTOR through Erk and Akt-mediated phosphorylation and downregulation of Tuberin (Fig. 1.1) [32]. Tuberin is a GTPase-activating protein that inhibits the GTPase Rheb [37]. Phosphorylation by Akt inhibits Tuberin, resulting in increased Rheb activity. GTP-bound Rheb binds the immunophilin FKBP38 thus preventing its inhibitory interaction with mTOR [38]. Downstream of mTOR are the 4EBP1 protein and the p70 ribosomal S6 kinase, which are involved in the regulation of mRNA translation initiation and ribosome biogenesis [39].

mTOR can form two functionally distinct signaling complexes (Fig. 1.1). The mTOR complex 1 (mTORC1) is characterized by the presence of the protein Raptor and is sensitivity to the macrolide antibiotic rapamycin [29]. Rapamycin binds the FK506-binding protein FKBP12. The rapamycin-FKBP12 complex binds mTOR on a region exposed only when mTOR is bound to Raptor, resulting in inhibition of mTOR activity [40]. mTORC1 is known to regulate protein translation, ribosome biogenesis, autophagy and adaptation to hypoxia [39]. The mTOR complex 2 (mTORC2) is characterized by the presence of the protein Rictor and is not sensitive to rapamycin [29]. mTORC2 regulates the actin cytoskeleton through the PKC and Rho pathways [41]. Furthermore, mTORC2 activates Akt through phosphorylation of S473 in the Akt hydrophobic motif [42]. Phosphorylation at S473 together with the PDK1 dependent phosphorylation of T308 in the catalytic domain are responsible for activation of Akt downstream of growth factors [42]. These phosphorylations require Akt plasma membrane localization, which occurs by binding to PI(3,4,5)P3, a phospholipid generated by PI3 kinase.

The positive effects of EGF receptor inhibition on functional recovery after spinal cord injury supports the notion that the EGF receptor plays an important role in glial scar formation and the creation of the inhibitory environment that blocks axon regeneration. An aspect of the work presented here is to determine whether the effects of EGF receptor signaling are mediated by the mTOR pathway in activated astrocytes. We also sought to determine if the EGF receptor-mTOR pathway is active in reactive astrocytes after spinal cord injury and if mTOR can be used as drug target to regulate glial scar formation and improve axonal regeneration and recovery after spinal cord injury.

1.3 Spinal Cord Ischemia

To study the role of the EGF receptor-mTOR pathway in astrocyte activation and glial scar formation in the spinal cord, we used a model of ischemia-induced injury [43]. A balloon catheter is introduced into the left femoral artery of an adult rat and guided into the descending thoracic aorta where it is inflated at the level of the subclavian artery (Fig. 1.2). Systemic hypotension is maintained during blood flow occlusion for 10 min. The lumbar region of the spinal cord is the area primarily affected by ischemia in this model. The cervical and upper thoracic regions of the spinal cord continue to receive blood throughout the procedure via the vertebral and segmental radicular arteries arising from the descending aorta. The ischemia causes a selective loss of small inhibitory interneurons resulting in a spastic paraplegia of the animal [43]. Interneuron death causes the formation of a cavity that is invaded by infiltrating macrophages and isolated from the surrounding healthy tissue by reactive astrocytes. The meninges are intact and the injury site is not invaded by meningeal fibroblasts. Therefore, using this model of spinal cord injury reactive astrocytes can be evaluated without considering secondary effects due to the presence of infiltrating fibroblasts. Three weeks after injury, a glial scar characterized by strong GFAP immunoreactivity is visible in the region where the interneurons were located. Therefore, we selected this time point for our analysis.

1.4 Primary Cultures of Astrocytes

Many animal models have been developed to study the different types of brain injury and the associated reactive gliosis but these in vivo systems are not suitable for the study of the biochemical mechanisms that regulate astrocyte activation. Tissue culture offers a good opportunity for the molecular dissection of the pathways that regulate reactive gliosis. However, most studies on cultured astrocytes have used cells prepared from newborn animals. This is in contrast with the in vivo animal models of CNS injury, which utilize adult animals. The ideal tissue culture model would allow direct comparison between normal adult astrocytes and reactive adult astrocytes. We developed such a model system by using primary cultures of astrocytes from the adult rat spinal cord. We were able to obtain cultures with a proportion of astrocytes greater than 95% (measured by counting the number of GFAP positive cells) (Fig. 1.3A). When grown in media with serum the cells in culture have the characteristics of reactive astrocytes and express high levels of intermediate filament proteins (Fig 1.3). Using this culture system we have been able to study how the EGF receptor and the mTOR pathway regulate astrocyte activation.

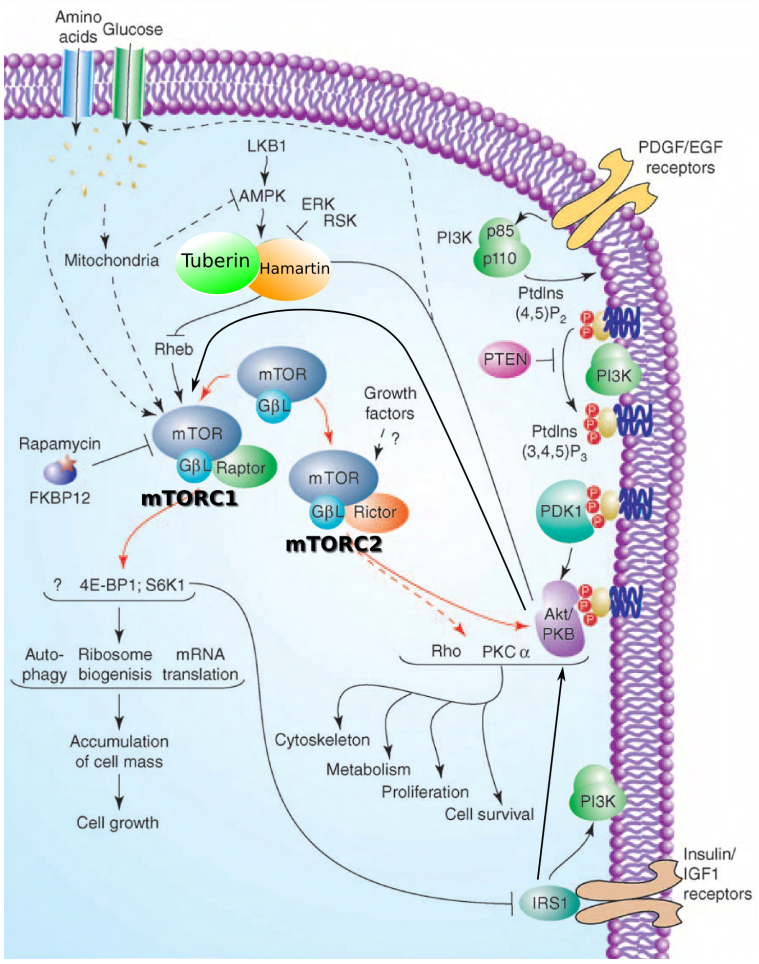


Figure 1.1: **PI3 kinase-Akt and mTOR signaling pathways.** The raptor-mTOR complex 1 (mTORC1) is rapamycin sensitive and regulates cell growth through S6 kinase (S6K1) and 4E-BP1. mTORC1 integrates the signals from nutrients and growth factors through the upstream regulators tuberin, hamartin and Rheb. The rictor-mTOR complex 2 (mTORC2) is rapamycin insensitive and regulates Akt, PKC, Rho and Rac to control cell survival, metabolism and the cytoskeleton. How mTORC2 is regulated is unknown. Dashed lines indicate interactions that are not direct. (Modified from Sarbassov et al. 2005).

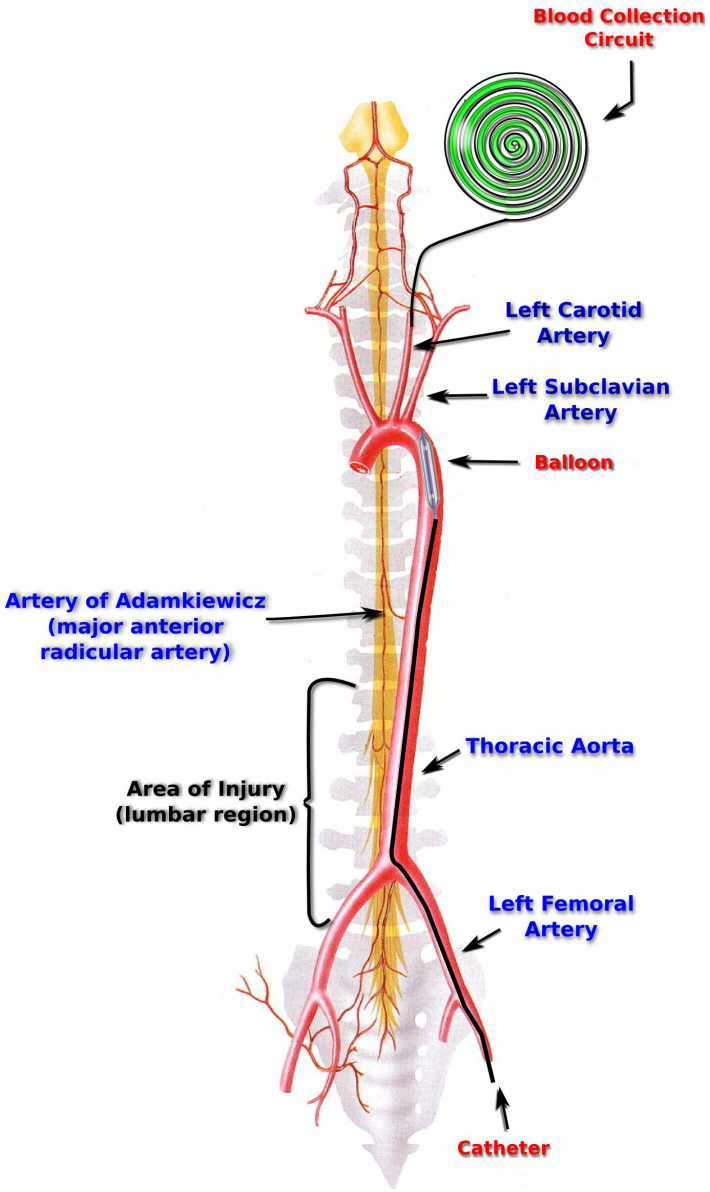


Figure 1.2: **Model of ischemia-induced spinal cord injury.** A balloon catheter is introduced into the left femoral artery of an adult rat and guided into the descending thoracic aorta where it is inflated at the level of the subclavian artery causing ischemia.

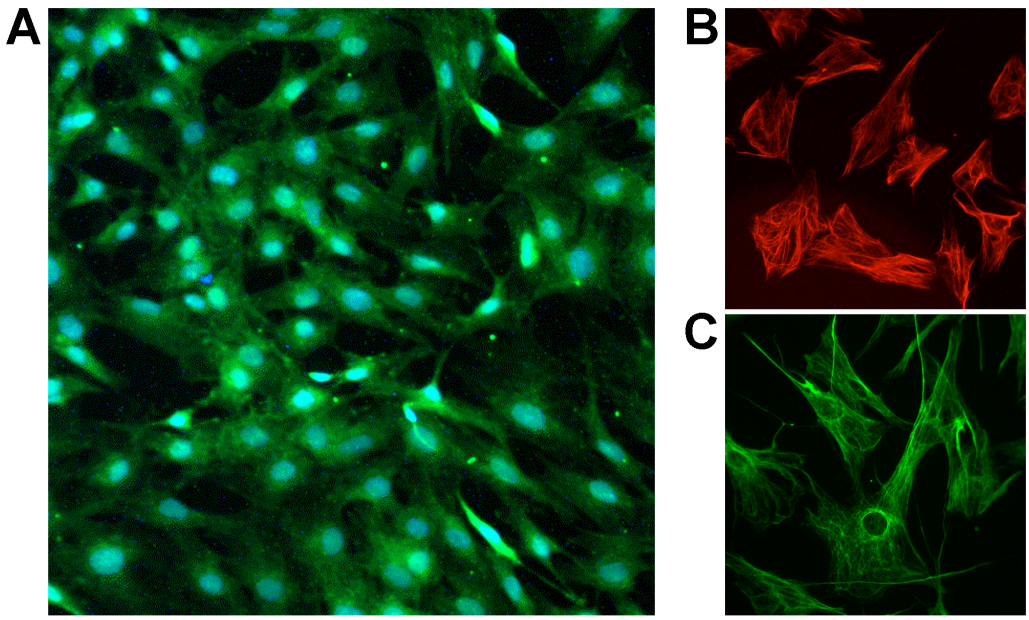


Figure 1.3: **Purity of the cultures from adult spinal cord astrocytes.** Astrocyte cultures were prepared from spinal cords of adult male rats. After removal of the meninges, the tissue was chemically and mechanically dissociated. The cells were plated on poly-L-lysine coated plates. After 10 days dead neurons, olygodendrocytes, and microglia were removed by shaking the cultures. The cultures contain greater than 95% GFAP-positive cells (A, green). The astrocytes maintained in serum-containing media, have the phenotypical hallmark of reactive astrocytes, expressing high levels of intermediated filament proteins such as GFAP **(A)**, vimentin **(B, red)** and nestin **(C, green)**. Nuclei are labelled in blue with DAPI.

Chapter 2

The Rheb-mTOR pathway is upregulated in reactive astrocytes of the injured spinal cord

2.1 Abstract

Astrocytes in the central nervous system respond to tissue damage by becoming reactive. They migrate and undergo hypertrophy, and form a glial scar that inhibits axon regeneration. Therefore, limiting astrocyte responses represents a potential therapeutic strategy to improve functional recovery. It was recently shown that the epidermal growth factor (EGF) receptor is upregulated in astrocytes after injury and promotes their transformation into reactive astrocytes. Furthermore, EGF receptor inhibitors were shown to enhance axon regeneration in the injured optic nerve and to promote structural and functional recovery after spinal cord injury. However, the signaling pathways involved were not elucidated. Here we show that in cultures of adult spinal cord astrocytes EGF activates the mTOR pathway, a key regulator of astrocyte physiology. This occurs through Akt-mediated phosphorylation of the GTPase-activating protein Tuberin, which inhibits the ability of Tuberin to inactivate the small GTPase Rheb. Indeed, we found that Rheb is required for EGF-dependent mTOR activation in spinal cord astrocytes, whereas the Ras-MAP kinase pathway does not appear to be involved. Moreover, astrocyte growth and EGF-dependent

chemoattraction were inhibited by the mTOR-selective drug rapamycin.

We also detected elevated levels of activated EGF receptor and mTOR signaling in reactive astrocytes in vivo in an ischemic model of spinal cord injury. Interestingly, increased Rheb expression likely contributes to mTOR activation in the injured spinal cord. These results suggest that rapamycin could be used to harness astrocyte responses in the damaged nervous system, thus promoting the development of an environment more permissive to axon regeneration.

2.2 Introduction

Injuries to the central nervous system not only cause damage to neuronal circuits but also induce dramatic changes in the glial cells [44, 45, 46, 47]. Following spinal cord injury, astrocytes upregulate expression of intermediate filament proteins such as nestin, vimentin and glial fibrillary acidic protein as well as proteoglycans and other molecules that are inhibitory to axon growth. Although some proliferation occurs following injury, the most dramatic astrocyte responses involve migration towards the site of injury and hypertrophy. Over time, reactive astrocytes deposit extracellular matrix molecules and form a glial scar. The glial scar plays an important role in the repair process because it contributes to the re-establishment of homeostasis by insulating the injury site and restoring the integrity of the blood-brain barrier [10, 46]. However, the glial scar also represents a physical and biochemical barrier that greatly contributes to the failure of damaged axons to regenerate and re-establish functional connections, thus hindering functional recovery [45, 10, 46].

Growth factors such as EGF and TGFα have been implicated in the transformation of quiescent astrocytes into reactive astrocytes, and it has been recently reported that EGF receptor expression and activation are upregulated in astrocytes after injuries to the central nervous system [48, 49, 24, 50]. However, the signaling pathways stimulated by EGF that lead to astrocytes activation have not been fully characterized. The serine/threonine kinase mTOR (mammalian target of rapamycin) is a key regulator of cell size and proliferation downstream of growth factor receptors, besides its role in mediating cell responses to nutrients [29, 51, 28]. Various mTOR upstream regulators have been reported to play an important role in astrocytes. For example, inactivation of a negative regulator of the pathway, the tumor suppressor Pten, promotes astrocyte hyperthropy and proliferation, which can lead to the formation of benign glial tumors called hamartomas as well as malignant glial tumors [34, 52, 53]. Inactivation of another tumor suppressor, Tuberin, also leads to glial cell hypertrophy and the formation of glial hamartomas [53]. Tuberin is a GTPase-activating protein for Rheb, a Ras family GTPase that activates mTOR by disrupting its inhibitory interaction with the FK506-binding protein FKBP38 [38]. Akt, Tuberin and Rheb activate mTOR when it is part of a protein complex called mTORC1 (mTOR

complex 1), which is exquisitely sensitive to the drug rapamycin [54, 40, 29]. Interestingly, rapamycin acts by promoting the inhibitory interaction of mTOR with another FK506-binding protein, FKBP12. mTOR also exists as part of a second protein complex, called mTORC2, which is regulated differently and is insensitive to acute inhibition by rapamycin. Our data suggest that a pathway involving mTORC1 plays an important role in spinal cord astrocytes downstream of the EGF receptor.

2.3 Materials and Methods

Antibodies and other reagents.

Antibodies against the phosphorylated EGF receptor (Tyr845), p70 S6 kinase, phosphorylated p70 S6 kinase (Thr389),
Erk1/Erk2, phosphorylated Erk1/Erk2 (Thr202/Tyr204), Akt, phosphorylated Akt (Ser473), phosphorylated Tuberin (Thr1462) and phosphorylated S6 (Ser235/Ser236) were from Cell Signaling (Danvers, MA); the anti-Rheb antibody for immunofluorescence microscopy was from Cell Signaling (Danvers, MA) and the anti-Rheb antibody for immunoblotting was from AbNova (Taipei City, Taiwan); antibodies against the EGF receptor, Tuberin and vimentin were from Santa Cruz Biotechnology (Santa Cruz, CA); the anti-glial fibrillary acidic protein (GFAP) antibody was from Millipore (Billerica, MA); and the anti-paxillin antibody was from BD Transduction Laboratories (Franklin Lakes, NJ). Laminin was from BD Transduction Laboratories (Franklin Lakes, NJ); MTT, EGF, DMSO and phosphatase inhibitor cocktails 1 and 2 were from Sigma (Saint Louis, MI); rapamycin was from LC Laboratories (Woburn, MA) and PD98059, LY294002 and FTI-277 were from Calbiochem (La Jolla, CA). Horseradish peroxidase-conjugated secondary antibodies for immunoblotting were from GE Healthcare (Piscataway, NJ) and Alexa Fluorconjugated secondary antibodies for immunofluorescence were from Invitrogen (Carlsbad, CA). Rhodamine-phalloidin was from Invitrogen.

Astrocyte cultures.

Astrocyte cultures were prepared from spinal cords of adult male Sprague-Dawley rats (P65 to P70, weighing 300-350 grams) using a method previously described [55] with some modifications. The spinal cords were ejected from the vertebral column using a saline-filled syringe. The tissue was chemically dissociated by 0.5% Trypsin-EDTA for 10 min followed by mechanically trituration in Dulbeccos modified eagles medium (DMEM) (Gibco, Grand Island, NY, USA). After centrifugation at 1,200 rpm for 5 min, the cells were suspended in DMEM containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin and plated in a flask coated with poly-

L-lysine (Sigma). The cultures were maintained in a humidified atmosphere of 95% air/5% CO2 at 37C for 10 days while changing medium at days 4 and 7. Approximately on day 10 and 11, oligodendrocytes and microglial cells growing on top of the confluent astocyte layer were removed by shaking at 200 rpm for 2 hours at 37C and replacing the medium. The next day, the cells were trypsinized and replated in 6-well plates (40,000 cells/well). The cultures, which routinely contained greater than 95% glial fibrillary acidic protein-positive cells, were used for experiments when confluent (typically within 4-6 days).

For EGF stimulation, confluent astrocyte cultures were starved for 24 hours in 0.1% FBS-containing medium and then stimulated with 100 ng/ml EGF or vehicle for 15 min. In some experiments the cultures were pretreated for 30 min with 100 nM rapamycin, 25 μM PD98059, 50 μM LY294002 or vehicle only as a control. For farnesyltransferase inhibition, the cultures were incubated for 24 hours with 10 μM FTI-277. For the MTT growth assays and the Akt phosphorylation time course shown in Fig. 3, astrocytes were initially sparse (3,000 cells/well in a 96-well plate), and EGF and rapamycin were added every day and the culture medium was replaced every third day.

MTT growth assay.

To measure cell growth, the culture medium was replaced with 100 μl of 5 mg/ml MTT in serum-free medium. After 4 hours the supernatant was aspirated from the wells without disturbing the formazan precipitate, the formazan crystals were dissolved in 100% DMSO and the absorbance was measured at 570 nm with a microplate reader.

Immunoblotting.

Astrocyte cultures were lysed in 0.5% Triton-X100, 3% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA containing protease inhibitors, NaF and phosphatase inhibitor cocktails 1 and 2. Samples were analyzed by SDS-PAGE followed by immunoblotting. Membranes were incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and SuperSignal chemilumi-

nescence reagents (Thermo Scientific Rockford, IL). Membranes were stripped using the ReBlot Western Blot Recycling Kit (Millipore, Billerica, MA) before reprobing with a different antibody.

Transwell migration assay.

Astrocyte cultures were trypsinized and incubated in suspension in serum-free medium with or without 100 nM rapamycin for 15 min. Fifty thousand cells were added to the top compartment of each Transwell (8 μm filter pore diameter, Corning Acton, MA) precoated with 10 μg/ml laminin and containing serum free medium in both upper and lower compartments. To stimulate chemotactic migration, 20 ng/ml EGF were added in the lower compartment. For rapamycin treatment, 100 nM rapamycin was present in the upper and lower compartments. The astrocytes were allowed to migrate for 4 hours and then the filters were washed with PBS and the cells from the top surface of the filters were removed with a cotton swab. The filters were then fixed with 4% formaldehyde and cell nuclei were stained with DAPI (Invitrogen, Carlsbad, CA). The nuclei of cells that had migrated to the bottom surface of the filter were photographed under a fluorescence microscope (6 fields/filter using a 10X objective) and counted with ImageJ (NIH Bethesda, MA). The average number of cells in 3 replicate wells was determined for each condition in each of 3 independent experiments.

Lentivirus packaging and infection.

For lentivirus packaging, 6 x 10^6 293FT cells (Invitrogen, Carlsbad, CA) were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with a mixture of 6 μg pLKO.1-Rheb shRNA plasmid or control pLKO.1-GFP shRNA (OpenBiosystems, Huntsville, AL), 2 μg envelope-encoding pMD2.G plasmid and 2 μg of the packaging vector psPAX2 plasmid (Addgene, Cambridge, MA). The culture supernatant containing the packaged virus was harvested 48 hours after transfection and titered using C6 rat glioma cells. For viral infection, confluent plates of astrocytes were incubated for 24 hours with 200 μl virus (2 x10^5 transducing units/ml) and 6 μg/ml polybrene as transduction enhancer. After infection, the astrocytes were grown in

medium containing 0.5 μg/ml puromycin, which was replaced every third day, in order to eliminate non-infected cells. After 10 days, the astrocytes were grown to confluency in puromycin-free medium.

Real-time PCR.

All the reagents used were from Applied Biosystems (Foster City, CA). The TaqMan Probes and primers for Rheb (assay ID Rn00566263-m1) were "assay-on-demand" gene expression products. Cultured astrocytes were washed with PBS and total RNA was extracted using RNA-STAT-60 and reversed transcribed using random hexanucleotide primers. TaqMan PCR amplification reactions were carried out in 25 μl containing 50 ng of cDNA in MicroAmp Optical Plates with MicroAmp Optical Caps using the TaqMan Universal Master Mix. Incubations at 50 C for 2 min and at 95 C for 10 min were carried out to activate the AmpliTaq polymerase, followed by 40 cycles at 95 C for 15 s and 60 C for 1 min. Hypoxanthine-guanine phosphoribosyltransferase was used as a loading control for each sample and the standard curve method was used for quantification [56].

Spinal cord ischemia.

Spinal cord ischemia was induced in anesthesized male Sprague Dawley rats according to a previously described protocol [57, 43]. This protocol involves inflating a balloon catheter placed in the descending thoracic aorta at the level of the left subclavian artery to occlude aortic blood flow for 10 min while also maintaining systemic hypotension. After 3 weeks, rats were anesthetized with pentobarbital and phenytoin and transcardially perfused with 200 ml of heparinized saline solution followed by 250 ml of 4% formaldehyde in PBS in order to obtain spinal cord tissue for analysis. All experiments were performed according to protocols approved by the Institutional Animal Care Committee of the University of California, San Diego.

Immunolabeling.

Spinal cords were dissected and postfixed in 4% formaldehyde in PBS overnight at 4C and then cryoprotected in 30% sucrose PBS and frozen. Transverse spinal cord sections (30 m in thickness) were stored in PBS. Sections were permeabilized with PBS, 0.2% Triton-X100 for 10 min, blocked for 1 hour in PBS 5% goat serum, 0.2% Triton-X100 and then incubated overnight with primary antibodies in PBS, 0.2% Triton-X100, 5% goat serum followed by an 1 hour incubation with Alexa Fluorconjugated secondary antibodies (1:250) in the same solution. Cultured astrocytes were fixed in 4% formaldehyde in PBS for 15 min, permeabilized in PHEM buffer (120 mM Pipes, 50 mM Hepes, 20 mM EGTA, 8 mM Magnesium) with 0.1% Triton-X100 for 5 min, blocked for 1 hour in PHEM buffer with 5% goat serum, incubated for 1 hour with primary antibodies in PHEM buffer with 5% goat serum and incubated with Alexa Fluorconjugated secondary antibodies (1:300) in PHEM buffer with 5% goat serum. Images were captured using a multiphoton laser point scanning confocal microscopy system (Radiance 2100/AGR-3Q, Bio-Rad Laboratories, Hercules, CA) equipped with Lasersharp 2000 software (Bio-Rad Laboratories, Hercules, CA).

2.4 Results

EGF activates the mTORC1 pathway in cultured spinal cord astrocytes

To examine EGF-dependent signaling pathways that may induce changes in astrocytes following injury, we used cultured astrocytes isolated from adult rat spinal cord. Immunoblotting and immunocytochemistry experiments verified EGF receptor expression and increased receptor tyrosine phosphorylated in response to EGF stimulation (Fig. 1A and Suppl. Fig. 1). To determine whether the mTORC1 pathway was activated following EGF stimulation, we used antibodies that detect phosphorylation of p70 S6 kinase on threonine 389, a site well known to be selectively phosphorylated by mTORC1 and widely used to monitor mTORC1 activation [40, 51]. EGF stimulation substantially increased the levels of phosphorylated pS6 kinase, indicating robust activation of mTORC1 in spinal cord astrocytes (Fig. 1A).

Studies with other cell types suggest that several signaling connections may lead to mTORC1 activation following EGF stimulation [29, 51, 28]. For example, the Erk1/Erk2 MAP kinases and their downstream target p90 Rsk can phosphorylate Tuberin and inhibit its activity, leading to downstream Rheb and mTORC1 activation [40, 58]. EGF treatment promoted phosphorylation and activation of Erk1/Erk2 in cultured astrocytes (Fig. 1B), as expected because the Ras-MAP kinase pathway is a major pathway known to be activated by the EGF receptor [59]. However, treatment with an inhibitor of the Erk pathway, PD98059, did not significantly reduce S6 kinase phosphorylation (Fig. 1B). Thus, activation of mTOR in spinal cord astrocytes stimulated with EGF does not depend on Erk1/Erk2 activation.

EGF stimulation also caused phosphorylation of Akt at serine 473 (Fig. 1C), which is one of the sites involved in Akt activation [40, 29]. Akt in turn can activate mTORC1 by phosphorylating Tuberin at serine 939 and threonine 1462, leading to its inactivation [58]. Tuberin T1462 phosphorylation was indeed increased following EGF stimulation and the PI3 kinase inhibitor, LY294002, abolished not only Akt phosphorylation but also Tuberin phosphorylation, suggesting that Tuberin phosphorylation occurs downstream of PI3 kinase and Akt (Fig. 1C). Although LY294002

can also target mTOR [54], inhibition of mTORC1 with rapamycin did not reduce Akt phosphorylation (Fig. 1D). Rather, we observed an upregulation of Akt phosphorylation similar to that previously described in several other cell types treated with rapamycin [40, 53]. However, rapamycin abolished S6 kinase phosphorylation, confirming that this phosphorylation depended on mTORC1 because rapamycin is a very selective mTOR inhibitor [54, 29]. Taken together, these data suggest that EGF activates mTORC1 in spinal cord astrocytes through Akt-mediated Tuberin inactivation. However, Akt has also been reported to activate mTOR through different signaling mechanisms that are independent of Tuberin and Rheb [40, 29, 28].

To assess the involvement of Rheb in EGF-dependent mTORC1 activation, we used the farnesyltransferase inhibitor FTI-277 because Rheb cannot activate mTORC1 if its membrane localization is impaired by the absence of the carboxy-terminal farnesyl group [60]. Treatment of cultured astrocytes with FTI-277 significantly inhibited the EGF-dependent increase in S6 kinase phosphorylation (Fig. 2A), suggesting that Rheb participates in mTORC1 activation. We also knocked down Rheb mRNA and protein expression by using an shRNA delivered by lentiviral infection (Fig. 2B,C). Rheb knockdown inhibited S6 kinase phosphorylation both in the absence and in the presence of EGF (Fig. 2D), indicating that Rheb is necessary for mTORC1 activation by EGF and possibly other stimuli in astrocytes from the adult spinal cord.

The mTORC1 inhibitor rapamycin negatively affects the growth and EGF-dependent migration of spinal cord astrocytes.

Because rapamycin has been shown to inhibit the growth of many cell types, indicating a critical role of mTORC1 in cell proliferation [29, 51, 28], we examined the effects of EGF and rapamycin on the growth of spinal cord astrocytes over a period of several days by using the MTT cell viability assay. Stimulation with EGF in the absence of serum was not sufficient to promote the growth of astrocytes from adult spinal cord (Fig. 3A). On the other hand, astrocyte growth induced by serum was almost completely inhibited by rapamycin, concomitant with inhibition of S6 kinase phosphorylation (Fig. 3B,C). We obtained similar results by measuring cell numbers (data not shown), indicating that the astrocytes are proliferating in the course of the

assay and not simply increasing in size. Phosphorylation of S6 kinase also decreased in the absence of rapamycin, once the cells became confluent and stopped growing (days 4 to 6). Concomitant with the decrease in S6 kinase phosphorylation induced by either confluency or rapamycin, we again observed an increase in Akt phosphorylation on S473 (Fig. 3C). Elevated Akt phosphorylation in rapamycin-treated cells was accompanied by an increase in the levels of the scaffolding protein insulin receptor substrate-2 (IRS-2), which is an upstream regulator of Akt (Fig. 3D). The increase in phosphorylated Akt suggests that survival signals may be upregulated [61] and therefore that the effects of rapamycin probably do not involve increased cell death. Indeed, we did not detect cleaved caspase 3 in the rapamycin-treated astrocytes while low levels were detectable in the untreated, confluent astrocytes (Fig. 3D).

Astrocytes are known to migrate towards sites of spinal cord injury [10]. Immunolabeling for filamentous actin and paxillin, a component of focal adhesions, showed that the cultured astrocytes were well spread and had many actin stress fibers and focal adhesions (Fig. 4A), consistent with a high adhesion to the substrate and a low level of cell motility. EGF treatment for 15 min decreased cell spreading, focal adhesions and stress fibers while causing the formation of long processes, as previously described and consistent with the ability of EGF to promote astrocyte motility [62]. Rapamycin treatment prevented the EGF-dependent morphological changes in spinal cord astrocytes (Fig. 4A) as well as the chemotactic effects of EGF in Transwell migration assays (Fig. 4B), suggesting a role for mTORC1 in the regulation of spinal cord astrocyte migration towards EGF. Akt phosphorylation on S473 was increased in cultured spinal cord astrocytes treated with rapamycin for 4 hours, corresponding to the duration of the Transwell migration assay (Fig. 4C). These results implicate mTORC1 activated downstream of Tuberin and Rheb in the regulation of astrocyte migration and indicate that rapamycin could be used to inhibit not only astrocyte growth but also chemotactic migration.

The mTOR pathway is activated in the rat spinal cord after ischemic injury.

To determine whether activation of mTORC1 also occurs in reactive astrocytes in vivo, we used a model where spinal cord injury is induced by transient occlusion of the descending thoracic aorta combined with systemic hypothension, leading to transient lumbar spinal cord ischemia [57, 43]. We examined spinal cord frozen sections by immunohistochemistry 3 weeks after reperfusion, while control spinal cords were obtained from rats that underwent a sham operation. We detected prominent upregulation of markers of activated astrocytes such as vimentin, glial fibrillary acidic protein, the glial glutamate transporter GLT-1 and the NG2 condroitin sulfate proteoglycan in the white matter of the injured spinal cord (Fig. 5A and data not shown). Furthermore, we observed increased labeling with a phosphospecific antibody that recognizes the activated EGF receptor and possible also activated ErbB2 (Fig. 5B), similar to the increase in activated EGF receptor previously reported in a spinal cord contusion model and other central nervous system injury models [48, 24, 62]. The substantial colocalization of phosphorylated EGF receptor with the glial glutamate transporter GLT-1 and the partial colocalization with the glial intermediate filament protein vimentin (Fig. 5B), which is similar to the colocalization with vimentin observed in cultured astrocytes (Suppl. Fig. 1), suggest that the activated EGF receptor is present in reactive astrocytes and may regulate their responses to the ischemic injury.

To determine whether the mTOR pathway is activated in reactive spinal cord astrocytes in vivo, we immunolabeled spinal cord sections for phosphorylated ribosomal protein S6, which is a substrate of S6 kinase, because the antibodies to phosphorylated S6 kinase did not work well for immunohistochemistry. Prominently elevated levels of phosphorylated S6 ribosomal protein were detected in the white matter of injured spinal cords compared to controls (Fig. 6A). The substantial colocalization with vimentin staining (Fig. 6A), which is reminiscent of that observed in cultured astrocytes (Suppl. Fig. 1), confirmed upregulation of S6 phosphorylation in reactive astrocytes. We then examined whether Rheb was present in the injured spinal cord and may therefore play an in vivo role in regulating mTORC1 activation down-

stream of the activated EGF receptor in reactive astrocytes, as suggested by our in vitro results. We detected Rheb immunoreactivity in control spinal cords, predominantly in the gray matter (Fig. 6B). Interestingly, Rheb expression was prominently upregulated in the white matter of the injured spinal cord and showed extensive colocalization with vimentin (Fig. 6B). Given that Rheb overexpression typically results in higher levels of activated Rheb [63], this suggests an additional Rheb-dependent mechanism besides Tuberin inhibition downstream of the EGF receptor that may contribute to in vivo activation of mTORC1 in reactive astrocytes of the injured spinal cord.

2.5 Discussion

In this study we show that activation of the EGF receptor in astrocytes triggers a downstream signaling pathway that involves Akt, Tuberin, Rheb and mTOR. This pathway was elucidated using cultured astrocytes isolated from the adult spinal cord and appears to also be functional in reactive astrocytes in vivo, in a well characterized model of spinal cord injury in which transient ischemia causes neuronal loss, paraplegia and reactive gliosis [57, 43]. Immunohistochemistry showed that in reactive astrocytes of the injured spinal cord there was an increase not only in the levels of activated EGF receptor but also in the expression of the mTORC1 activator Rheb. Thus, upregulation of Rheb expression may be another mechanism contributing to mTORC1 activation *in vivo* [63]. It will be interesting to determine whether increased Rheb expression is a common feature of nervous system injuries characterized by reactive gliosis.

Adult spinal cord astrocytes treated with rapamycin did not grow, confirming that astrocytes require mTORC1 activity in order to grow in culture, as has been shown for many other cell types [29, 51, 28]. However, EGF was not sufficient to stimulate astrocyte growth in the absence of other serum components, suggesting that additional signaling pathways besides activation of mTORC1 are important for growth regulation in these cells. On the other hand, EGF was sufficient to induce changes in the actin cytoskeleton and to stimulate chemotactic migration. These effects of EGF appear to also be regulated by mTORC1 because they were acutely inhibited by rapamycin, which due to its unusual mechanism of action is an absolutely specific mTORC1 inhibitor [54]. A role for mTORC1 in cell migration has been reported in only a few cell types, such as fibroblasts and trophoblast cells, where growth factor-induced migration can be inhibited by a short exposure to rapamycin similarly to what we observed in astrocytes [64, 65, 66]. In contrast, mTOR has a widespread role in the regulation of cytoskeletal remodeling and cell migration when it is part of the mTORC2 complex [67, 68]. Although mTOR in mTORC2 is insensitive to rapamycin, prolonged exposure to rapamycin can deplete the levels of mTOR available to form mTORC2 complexes [69]. However, such indirect inhibition of mTORC2 is not likely to be responsible for the effects of rapamycin on EGF-dependent changes

in cytoskeletal organization and migration in adult spinal cord astrocytes due to the relatively short time course of rapamycin exposure in our migration experiments. Furthermore, rapamycin did not decrease Akt phosphorylation at S473, which is the site phosphorylated by mTORC2 [42].

On the contrary, treatment of the astrocytes with rapamycin rapidly and persistently increased Akt phosphorylation at S473. Previous studies have shown that rapamycin can promote Akt phosphorylation in some cell types through inhibition of a negative feedback loop involving S6 kinase-mediated phosphorylation and degradation of insulin receptor substrates (IRS) [40, 70, 53]. By increasing IRS levels, rapamycin inhibition of mTORC1 activity can increase PI3 kinase and, therefore, Akt activation. A similar mechanism appears to accounts for the inverse regulation of S6 kinase and Akt phosphorylation that we observed in adult spinal cord astrocytes because we observed increased levels of IRS-2 protein in rapamycin-treated astrocytes.

The rapamycin-dependent increase in Akt phosphorylation may play a role in the inhibitory effects of rapamycin on astrocyte migration. The roles of Akt in cell survival and metabolism are well known, while its role in cell migration is less characterized [61]. Interestingly, recent studies have shown that Akt1 and Akt2 can have different effects on migration depending on the cell type [71]. For example, activation of Akt1 but not Akt2 decreases EGF-dependent breast epithelial cell migration by inhibiting Erk kinases [72] and hyperactivation of Akt1 in breast cancer cells inhibits motility and invasion through a pathway that involves Tuberin phosphorylation and degradation, leading to decreased Rho activation [62]. Furthermore, activation of Akt2 but not Akt1 has been shown to decrease fibroblast migration, likely by inhibiting Rac and Pak1 [73] while phosphorylation and inhibition of GSK-3 by Akt family members can have negative effects on cell migration [74]. Downregulation of S6 kinase activity may also play a role in the inhibitory action of rapamycin on the cytoskeletal and chemotactic effects of EGF. Besides its role in the regulation of protein synthesis, S6 kinase has been shown to associate with the actin cytoskeleton and has been implicated in the regulation of cell morphology and migration [64, 65].

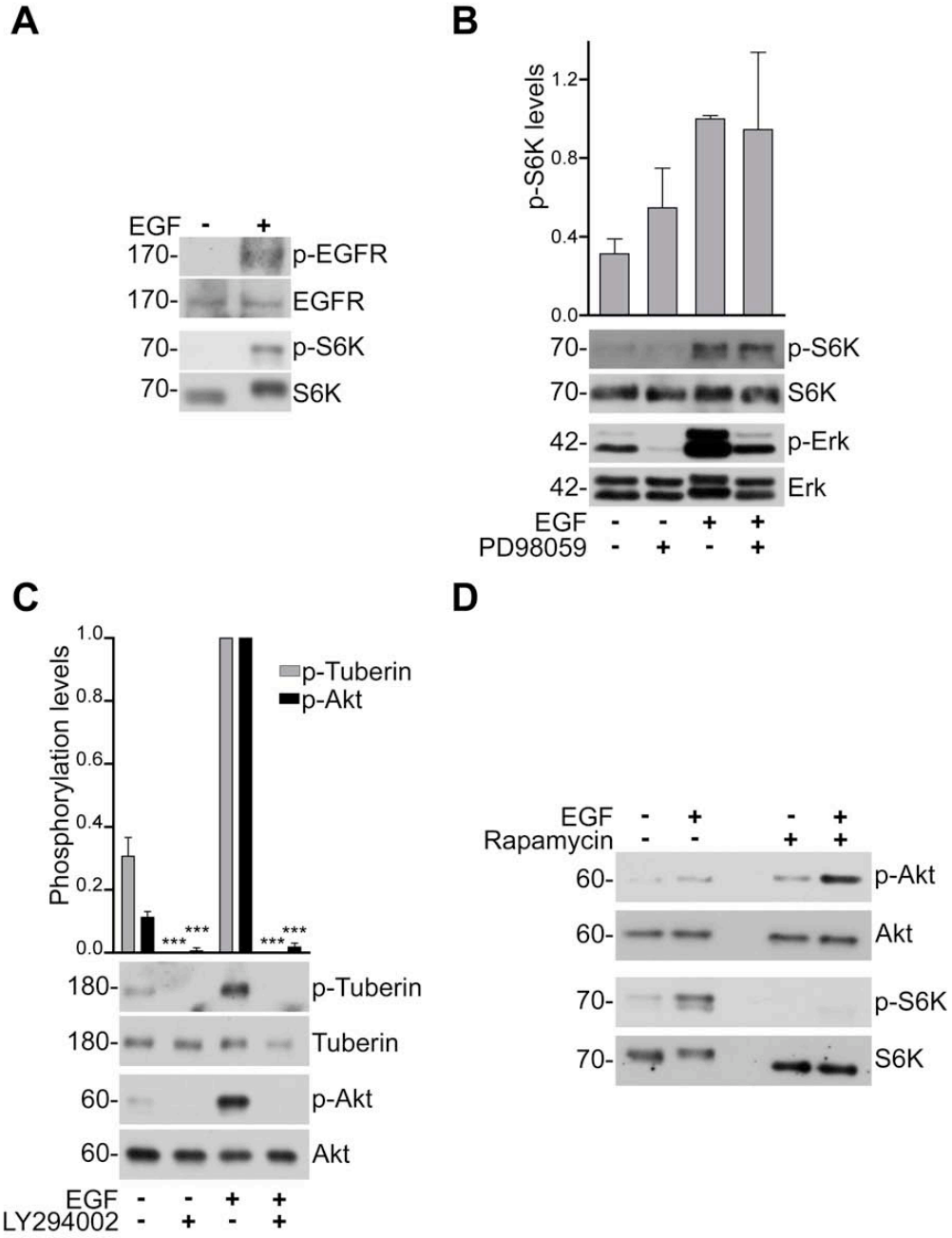
An important implication of our findings is that blocking the mTORC1 pathway with rapamycin, which is already in use as an immunosuppressive and antipro-

liferative drug [54, 29], could be beneficial in the treatment of spinal cord injury to reduce astrocyte proliferation and migration and promote functional recovery. It will also be interesting to examine whether prolonged rapamycin treatment can downregulate expression of proteins responsible for astrocyte hypertrophy and reactive gliosis, whose expression may depend on EGF receptor and mTORC1 activity [75, 34, 49, 25, 24, 76, 36]. Moreover, the increased Akt activation seen after rapamycin treatment should enhance astrocyte survival [61], which could be beneficial in conjunction with inhibiting excessive astrocyte growth and motility. While moderate astrocyte gliosis would be desirable to reduce tissue damage and neuronal cell death following injury [10], excessive gliosis may be limited with the use of rapamycin.

Several studies with animal models have shown that a brief administration of rapamycin near the time of ischemic injury to the central nervous system does not have neuroprotective effects [77, 78, 79]. In contrast, a single dose of rapamycin administered 4 hours after traumatic brain injury in mice has been shown to reduce inflammatory processes and neuronal loss and to improve functional recovery [80]. However, the effects of prolonged rapamycin treatment on astrocyte activation and glial scar formation have not been investigated. It is encouraging that in vitro rapamycin inhibits astrocyte growth and migration (as we show here), reduces the size of hypertrophic astrocytes in which mTOR is hyperactivated [36], and promotes neurite outgrowth [81]. Nevertheless, it will be important to examine the effects of rapamycin in vivo due to the complexities of the mTOR pathways and the existence of feedback mechanisms that may affect the outcome of prolonged rapamycin treatment in the damaged nervous system [29, 51, 28]. In clinical trials for the treatment of tuberous sclerosis-associated astrocytomas and Pten-deficient glioblastomas, rapamycin has already shown some promising anti-tumor activity [82, 83]. The EGF receptor inhibitors PD168393 and AG1478 have been shown to promote axon regeneration and functional recovery in pre-clinical studies using animal models of spinal cord and optic nerve injury through inhibitory effects on reactive astrocytes [48, 25] as well as by decreasing neuronal responses to inhibitors of axon outgrowth [84]. These inhibitors can also target another member of the EGF receptor family, ErbB2, which is also expressed in reactive astrocytes where it may function by dimerizing

with ligand-bound EGF receptor [85, 49, 86]. Our studies suggest that rapamycin may be useful as an alternative or in combination with EGF receptor inhibitors [87] to reduce glial scar formation and improve axon regeneration after spinal cord injury and possibly other injuries to the central nervous system.

Figure 2.1: **EGF stimulation of astrocytes from adult spinal cord activates the mTOR pathway**. **A**, EGF promotes phosphorylation of the EGF receptor and S6 kinase. Astrocytes were stimulated for 15 min with EGF (+) or vehicle (-) and lysates were probed by immunoblotting with antibodies to phosphorylated EGF receptor (p-EGFR) and phosphorylated S6 kinase (p-S6K) and reprobed with antibodies to the EGF receptor and S6 kinase. **B**, The Erk MAP kinase pathway is not involved in EGF-dependent S6 kinase phosphorylation. Astrocytes treated with the PD98059 inhibitor (+) or vehicle (-) were stimulated with EGF and lysates were probed by immunoblotting with antibodies to phosphorylated S6 kinase (p-S6K) or phosphorylated Erk1 and Erk2 MAP kinases (p-Erk) and reprobed with antibodies detecting the non-phosphorylated forms of the proteins. The levels of phosphorylated S6 kinase determined from the optical density of the bands were normalized to total S6 kinase protein levels. The histogram shows average levels of phosphorylated S6 kinase relative to the level after EGF treatment. The error bars represent the standard error (SE) from 3 independent experiments. PD98059 did not cause significant differences in S6 phosphorylation as determined by one-way ANOVA and Bonferronis post-hoc test. **C**, The PI3 kinase-Akt pathway mediates EGF-depenent Tuberin phosphorylation. Astrocytes incubated with the LY294002 PI3 kinase inhibitor (+) or vehicle (-) were stimulated with EGF and lysates were probed by immunoblotting with the indicated antibodies. The levels of phosphorylated proteins were quantified as described in B for S6 kinase. The histogram shows average levels of phosphorylated Tuberin and phosphorylated Akt relative to the levels after EGF treatment. The error bars represent the SE from 3 independent experiments. ***$p<0.001$ for the comparison between untreated and LY294002 treated by one-way ANOVA and Bonferronis post-hoc test. **D**, The mTORC1 selective inhibitor rapamycin blocks S6 kinase phosphorylation but not Akt phosphorylation. Astrocytes incubated with rapamycin (+) or vehicle (-) were stimulated with EGF and the lysates were probed by immunoblotting with the indicated antibodies.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D
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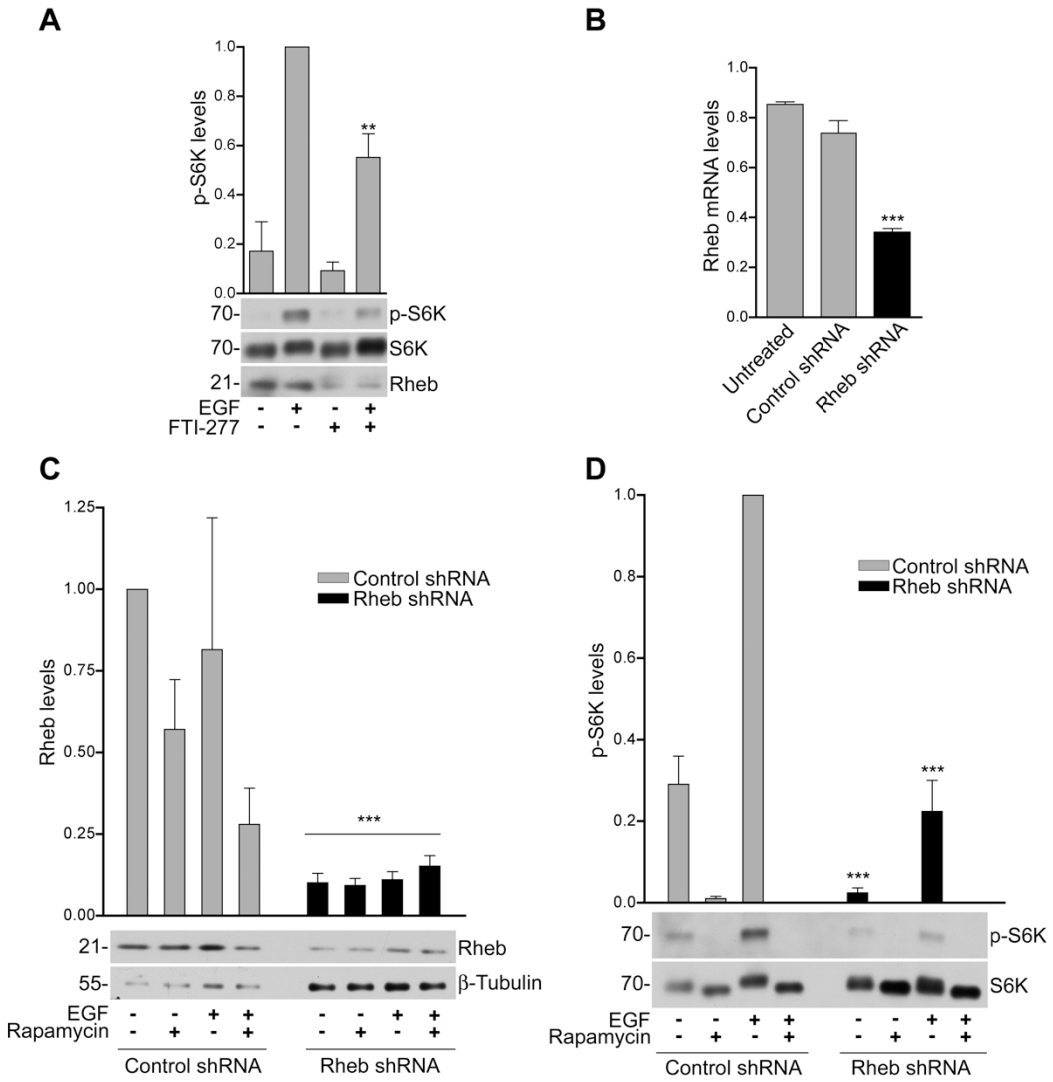
Rapamycin - - + +
60- p-Akt
60- Akt
70- p-S6K
70- S6K

Figure 2.2: **Rheb is required for EGF-dependent S6 kinase phosphorylation in spinal cord astrocytes. A**, The FTI-277 farnesyltransferase inhibitor decreases phosphorylation of S6 kinase. Astrocytes incubated with FTI-277 were stimulated with EGF and lysates were probed by immunoblotting with antibodies to phosphorylated S6 kinase (p-S6K) and Rheb, and reprobed with antibodies to S6 kinase. Note that non-farnesylated Rheb has a different molecular weight, so Rheb immunoreactivity appears weaker because the protein is no longer concentrated in a single band. The levels of phosphorylated S6 kinase determined from the optical density of the bands were normalized to total S6 kinase protein levels. The histogram shows average levels of phosphorylated S6 kinase relative to the level after EGF treatment. The error bars represent the SE from 3 independent experiments, each with duplicate measurements. **$p<0.01$ for the comparison between untreated and FTI-277 treated by one-way ANOVA and Bonferronis post-hoc test. **B, C**, Lentiviral delivery of Rheb shRNA decreases Rheb mRNA and protein levels in spinal cord astrocytes. Astrocytes were infected with a lentivirus encoding Rheb shRNA or a control (GFP) shRNA. In B, Rheb mRNA was quantified by quantitative real-time PCR and the histogram shows the averages ± SE from 3 independent experiments, each with duplicate measurements. ***$p<0.001$ for the comparison with cells treated control shRNA by one-way ANOVA and Bonferronis post-hoc test. In C, astrocytes were incubated with rapamycin (+) or vehicle (-) and stimulated with EGF. The levels of Rheb protein were determined from the optical density of the bands and normalized to the levels of β-tubulin in the lysates. The histogram shows average levels of Rheb relative to the level in unstimulated control cells. The error bars represent the SE from 4 independent experiments, each with duplicate measurements. ***$p<0.001$ for the comparison of control shRNA- versus Rheb shRNA-treated samples by two-way ANOVA. **D**, Rheb knockdown decreases S6 kinase phosphorylation. Astrocytes infected with lentivirus encoding Rheb shRNA or a control shRNA were incubated with rapamycin (+) or vehicle (-) and stimulated with EGF. The histogram shows the average levels of phosphorylated S6 kinase normalized to total S6 kinase and relative to the level in EGF stimulated control astrocytes. The error bars represent the SE from 4 independent experiments, each with duplicate samples. ***$p<0.001$ for the comparison of control shRNA versus Rheb shRNA by one-way ANOVA and Bonferronis post-hoc test.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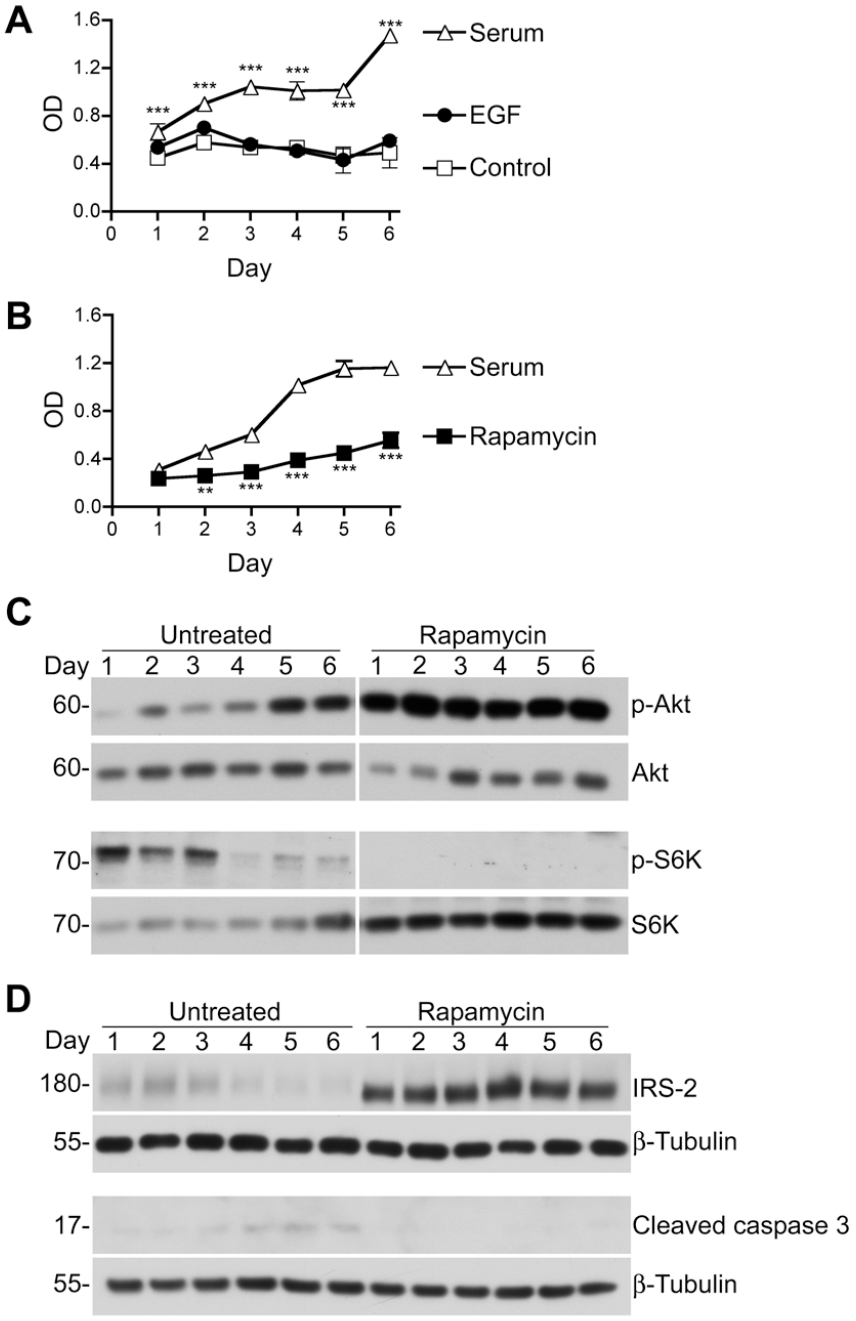
Rapamycin - + - + - + - +
Control shRNA
Rheb shRNA

Figure 2.3: **mTORC1 is required for the growth of cultured spinal cord astrocytes. A**, Fetal bovine serum promotes the growth of astrocytes. Astrocytes were grown for the indicated number of days and their growth was quantified using the MTT assay. The graph shows averages ± SE from 3 independent experiments, each with triplicate measurements. ***$p<0.001$ for the comparison with control-treated by two-way ANOVA and Bonferronis post-hoc test. **B**, Rapamycin inhibits astrocyte growth. The graph shows averages ± SE from 3 independent experiments, each with triplicate measurements. ***$p<0.001$ for the comparison between serum and rapamycin treated by two-way ANOVA and Bonferronis post-hoc test. **C**, Confluency and rapamycin inhibit S6 kinase phosphorylation and enhance Akt phosphorylation. Lysates from untreated and rapamycin treated cells were probed by immunoblotting with the indicated antibodies. All the lanes are from the same gel, and a lane between untreated and rapamycin treated samples was digitally removed. **D**, Rapamycin treatment increases the levels of IRS-2 and does not increase the levels of cleaved caspase 3. Lysates from untreated and rapamycin treated cells were probed by immunoblotting with the indicated antibodies.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55-
β-Tubulin

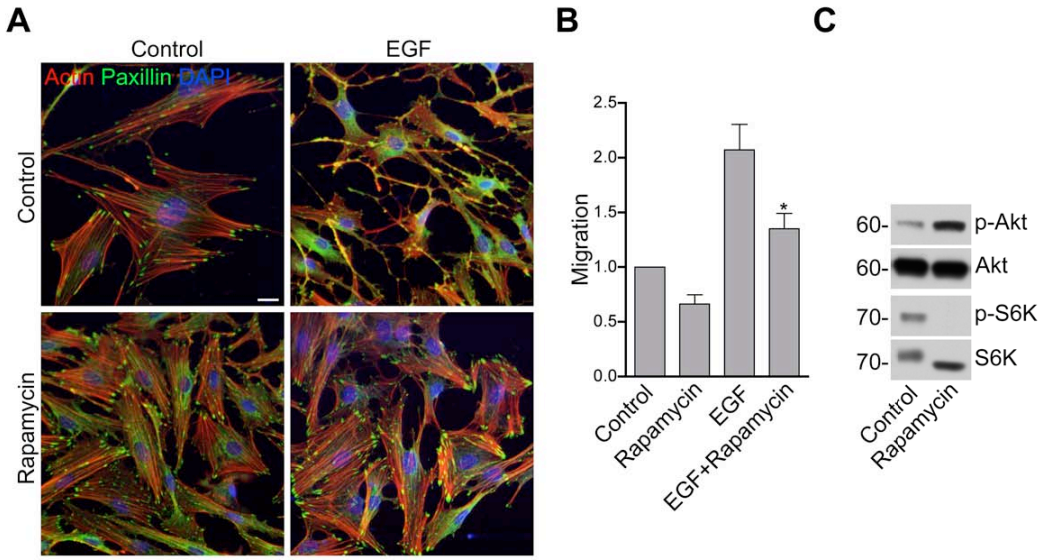

Figure 2.4: **mTORC1 promotes EGF-dependent cytoskeletal reorganization and migration of spinal cord astrocytes. A**, EGF induces morphological changes in astrocytes. Astrocytes treated with rapamycin were stimulated for 15 min with EGF, fixed and stained for paxillin (green) and F-actin (red). Scale bar 20 μm. **B**, Rapamycin inhibits astrocyte chemotactic migration towards EGF. Astrocytes were seeded on Transwell filters coated with laminin and allowed to migrate through the filters towards EGF for 4 hours. The histogram shows the average number of cells that migrated to the lower side of the filters in the different conditions relative to control. Error bars indicate the SE from 3 independent experiments, each with triplicate samples. **C**, Lysates from astrocytes treated for 4 hours with rapamycin and untreated controls were probed by immunoblotting with antibodies to phosphorylated Akt (p-Akt) and phosphorylated S6 kinase (p-S6 kinase) and reprobed with antibodies to Akt and S6 kinase.

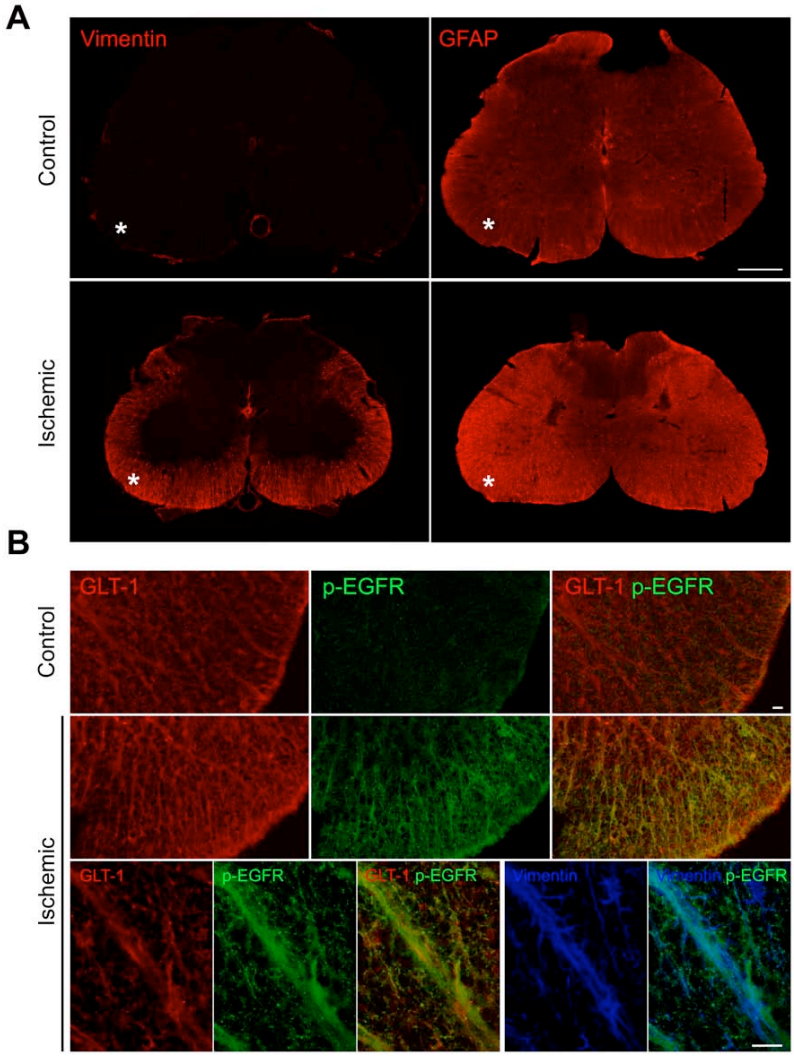

Figure 2.5: **The EGF receptor is activated in vivo in the injured spinal cord.** **A**, The increased vimentin and glial fibrillary acidic protein (GFAP) immunoreactivity reveals the presence of reactive astrocytes in the white matter of the lumbar spinal cord (asterisks) following an ischemic injury. Scale bar = 500 μm. **B**, Immunolabeling for phosphorylated EGF receptor (green) is increased in the white matter of the spinal cord after ischemia and shows substantial colocalization with the astrocytic glutamate transporter GLT-1 (red) as well as partial colocalization with the cytoskeletal protein vimentin (blue) in triple-labeled sections. Scale bars = 20 μm.

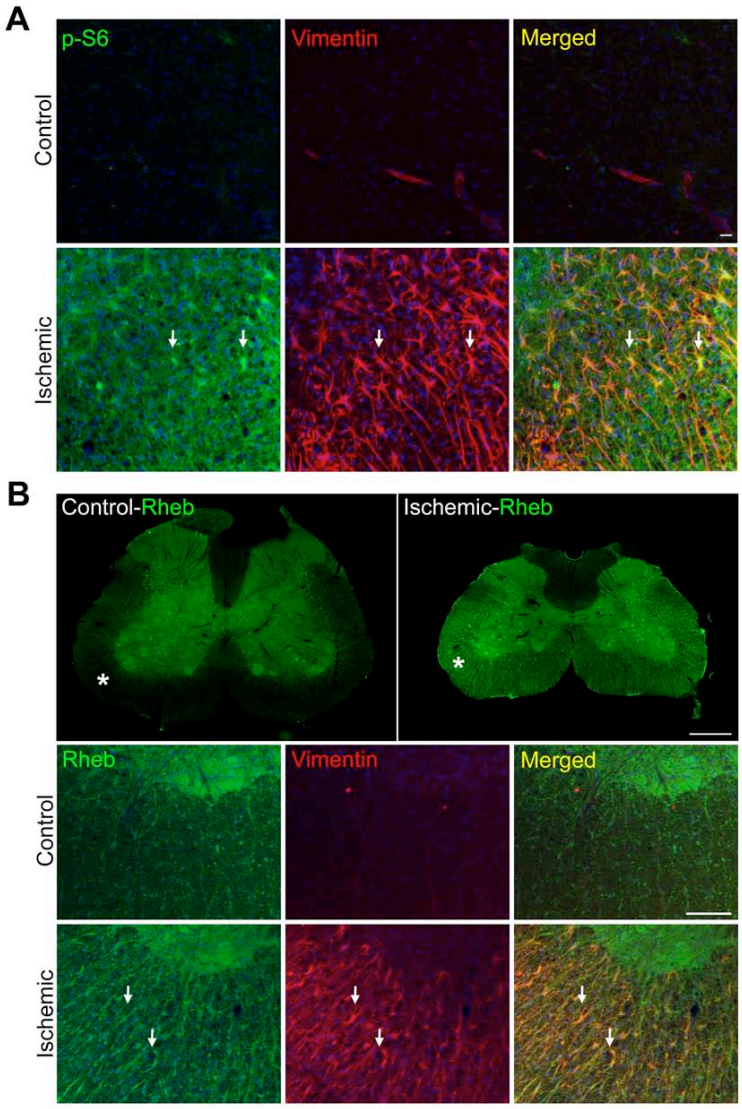

Figure 2.6: **Rheb is upregulated and mTORC1 is activated in vivo in the injured spinal cord. A**, Immunolabeling for phosphorylated S6 ribosomal protein (green) is dramatically upregulated in the vimentin-positive astrocytes (red) within the white matter of injured spinal cord. Scale bar = 20 μm. **B**, Immunolabeling for Rheb (green) is also dramatically upregulated in the vimentin-positive astrocytes (red) in the white matter of the injured spinal cord after ischemia (asterisks). Scale bars = 500 μm (top) and 20 μm (bottom).

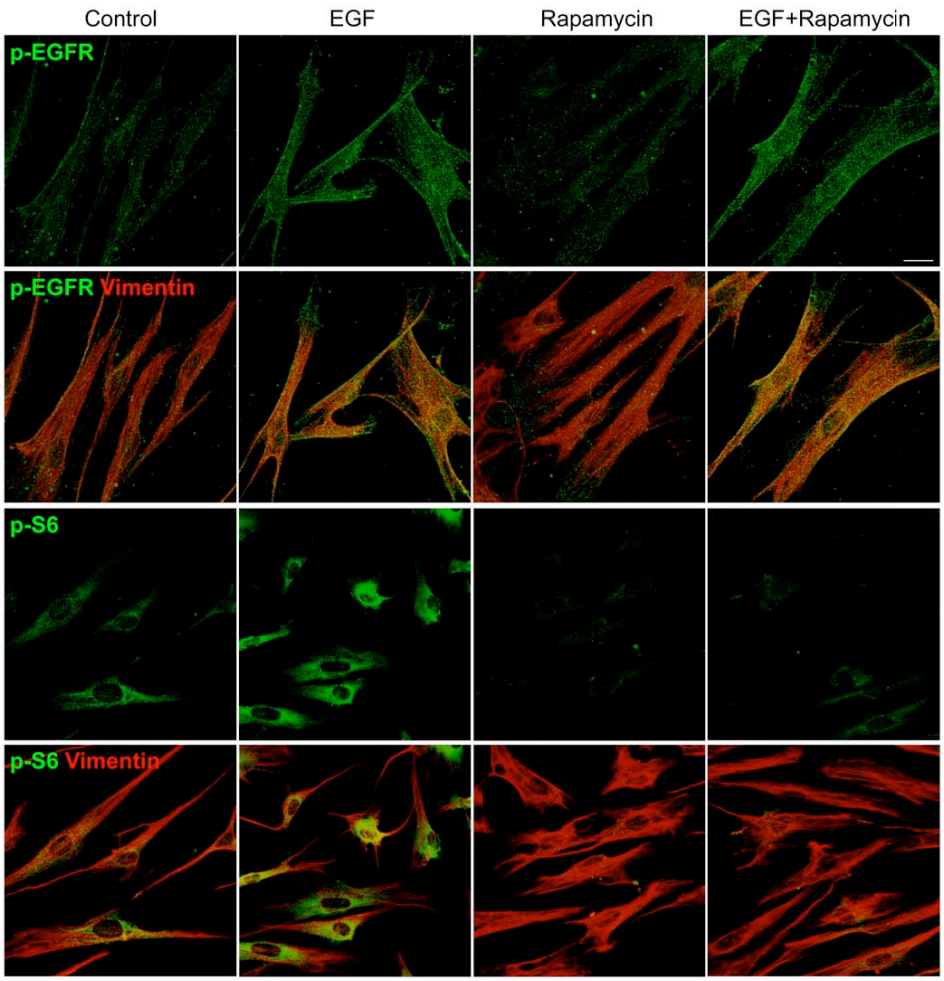

Figure 2.7: **EGF stimulation increases EGF receptor phosphorylation and phosphorylation of the mTORC1 downstream target S6 ribosomal protein.** Astrocytes were treated with EGF for 15 min in the presence or in the absence of rapamycin and double-labeled for phosphorylated EGF receptor (green) and vimentin (red) or for phosphorylated S6 ribosomal protein (green) and vimentin (red). Rapamycin decreases the levels of phosphorylated S6 without affecting EGF receptor phosphorylation, as expected. Scale bar = 20 μm.

Chapter 3

Discussion

Using primary cultures of adult astrocytes, we characterized a pathway where the EGF receptor activates mTORC1 through regulation of the Akt-Tuberin-Rheb cascade. Interestingly, the Rheb related protein RhebL1 is expressed at low level in primary cultures of astrocytes (Fig. 3.1) and is not involved in the regulation of mTORC1 downstream of EGF (Fig. 2.2C,D-3.1). We found that the EGF receptor-mTORC1 pathway is activated in vivo in the hypertrophic astrocytes of the ischemic spinal cord. Furthermore, we also observed a substantial increase in Rheb expression, suggesting that regulation of Rheb protein levels after ischemia may be a new mechanism to regulate mTORC1 activity. Rheb activates mTORC1 by sequestering the inhibitory protein FKBP38 [38].

Our experiments show that the EGF receptor regulates astrocyte proliferation and migration through mTORC1. The role of the mTORC1 downstream of the EGF receptor in the regulation of astrocyte proliferation has been determined using the macrolide antibiotic rapamycin. Rapamycin binds the immunophilin FKBP12 and the complex is then able to interact with mTORC1. Interestingly, the rapamycin binding site in FKBP12 is exposed only when mTOR is part of mTORC1. In mTORC2 the

binding site is masked and the complex is insensitive to rapamycin [40].

Although astrocyte growth requires mTORC1 activity, EGF is not sufficient to induce astrocyte growth in cultures maintained in low serum, suggesting that other growth factors are involved in the regulation of astrocyte growth. Treatment with rapamycin inhibits astrocyte growth without inducing apoptotic cell death (Fig. 2.3A,D-3.2). Furthermore, Akt activity and IRS-2 protein levels are increased after incubation with rapamycin, indicating an activation of survival pathways (Fig. 2.3D). The observed inhibition of growth together with the lack of apoptosis and increased activation of survival pathways suggest that the major effect of rapamycin is to inhibit astrocyte proliferation rather than promoting cell death.

The chemoattractive effect of EGF on astrocyte migration was investigated using a Transwell migration assay. EGF treatment causes disassembly of focal adhesions and depolymerization of actin stress fibers in astrocytes (Fig. 2.4). The chemoattractive effect of EGF on astrocytes is regulated by mTORC1 and is sensitive to rapamycin. A role of mTORC1 in cell migration as been previously reported only in few cell types, such as fibroblasts and trophoblast cells, and for the first time here on astrocytes [64, 65, 66].

Even though rapamycin is selective for mTORC1, long term treatment with rapamycin can deplete the amount of free mTOR in some cell types, inhibiting the formation of mTORC2 and thus possibly cell migration [39, 69]. To determine if the effect of rapamycin effect on astrocyte migration is the result of secondary mTORC2 inhibition, we measured mTORC2 activity using a phospho-specific antibody recognizing the Akt phosphorylation site that is targeted by mTORC2. Akt activity in astrocytes is not decreased after long term incubation with rapamycin, confirming that the chemoattractive effect of EGF is mediated by mTORC1 (Fig. 2.4C-2.3C).

It will be interesting to identify the pathways that regulate rapamycin inhibition of cell migration. Even thought our experiments showed that the mTORC1 pathway regulates astrocyte migration towards EGF, how mTORC1 affects cell migration in astrocytes is not known (Fig. 3.3). Results obtained using fibroblasts have shown that the mTORC1 downstream effector, S6 kinase, can directly affect cell migration [64]. Reduced S6 kinase activity as consequence of mTORC1 inhibiton can affect cell

migration by blocking depolymerization of actin stress fibers [64]. The increase in Akt phosphorylation caused by rapamycin treatment in the astrocytes may also play a role in inhibition of cell migration. Akt activation after rapamycin treatment can be the result of a negative feedback loop that modulates the growth factor receptor-mTORC1 signaling cascade and is regulated by the IRS-2 [70]. IRS family proteins activate the PI3 kinase-Akt pathway downstream of growth factor receptors, and this activation correlates with IRS protein levels. IRS phosphorylation by S6 kinase promotes the degradation of IRS causing a decrease in PI3 kinase-Akt signaling [70]. In our experiments we have shown that long term treatment with rapamycin inhibits S6 kinase and results in increased IRS-2 protein levels and Akt phosphorylation in cultured astrocytes, where IRS-2 is the main IRS protein expressed (Fig. 2.3D-3.4).

Akt is a family of serine/threonine kinases with three members: Akt1, Akt2, and Akt3 [88]. The different Akt proteins can promote or inhibit cell motility and migration through regulation of the actin cytoskeleton [72, 62, 73]. Glycogen synthase kinase-3 is an Akt substrate that can regulates cell motility by affecting focal adhesion dynamics. Downregulation of glycogen synthase kinase-3 through phosphorylation by Akt reduces cell motility through inhibition of focal adhesion kinase and the small GTPase Rac [74]. Akt1 can also inhibit migration through Tuberin or the Erk pathway. Akt1 phosphorylation promotes the degradation of Tuberin and, through an unknown mechanism, this decreases Rho activation and thus cell motility [89, 72, 90]. Furthermore, Akt2 has been shown to decrease migration through inhibition of the small GTPase Rac and its effector p21-activated protein kinase [73].

Indirect evidence that inhibition of the mTORC1 pathway can be beneficial in the treatment of CNS injury comes from studies in which EGF receptor inhibitors where used in spinal cord injury models. Inhibition of EGF receptor signaling has been shown to improve functional recovery by affecting reactive astrocytes, possibly at least in part through regulation of mTORC1 activity. However, the mTOR pathway is also regulated by other growth factors and the use of rapamycin in the treatment of spinal cord injury may have a broader effects by blocking more pathways. Experiments on neurons have also shown that EGF receptor inhibitors can also affect neurons by blocking the inhibitory effect of the proteoglicans expressed by astrocytes

on axon regeneration [84]. Whether mTOR may also contribute to the effects of EGF receptor signaling in neurons remains to be determined. In conclusion, EGF receptor inhibitors and rapamycin may provide complementary approaches to regulate glial scar formation and improve axon regeneration and functional recovery after spinal cord injury.

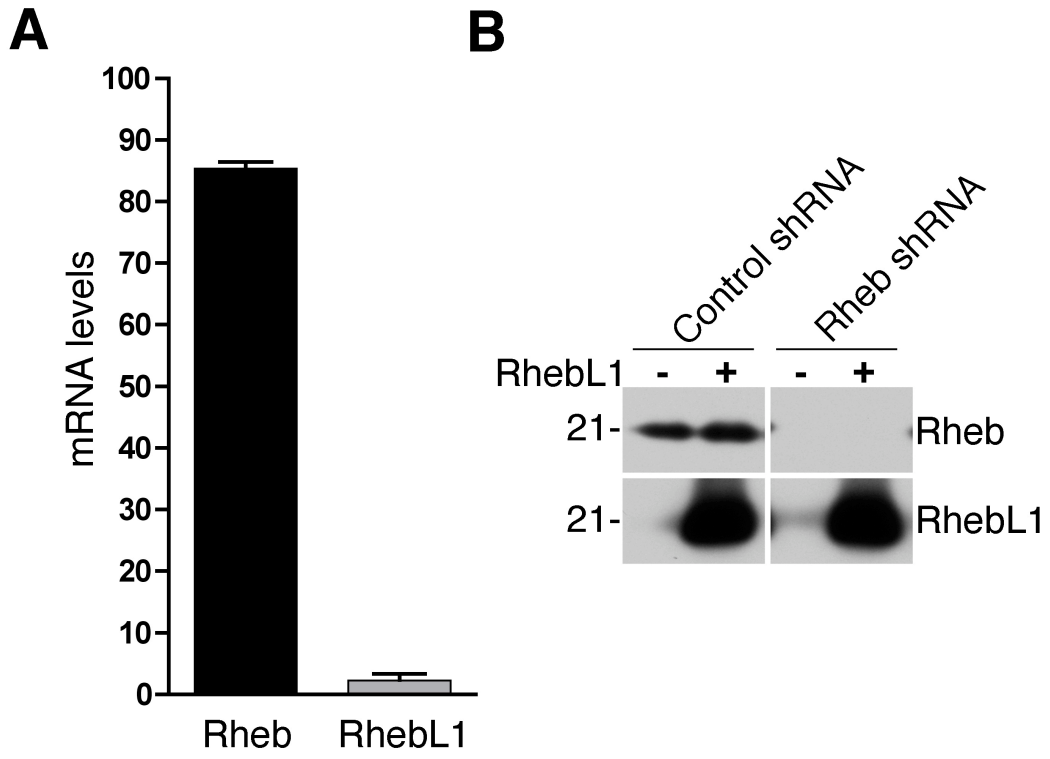

Figure 3.1: **Rheb-like1 is is poorly expressed in cultures of adult spinal cord astrocytes and is not affected by Rheb specific shRNA. (A)** Quantitative real-time PCR (qPCR) measurement of Rheb-like 1 (RhebL1) and Rheb mRNA levels in astrocytes shows very low expression levels. **(B)** Stable clones of C6 astrocytoma cells lines transfected with a RhebL1 construct or empty vector control were infected with infected with a virus encoding Rheb shRNA of control shRNA. Lysates were probed by immunoblotting with the indicated antibodies. Rheb shRNA does not affect RhebL1 expression.

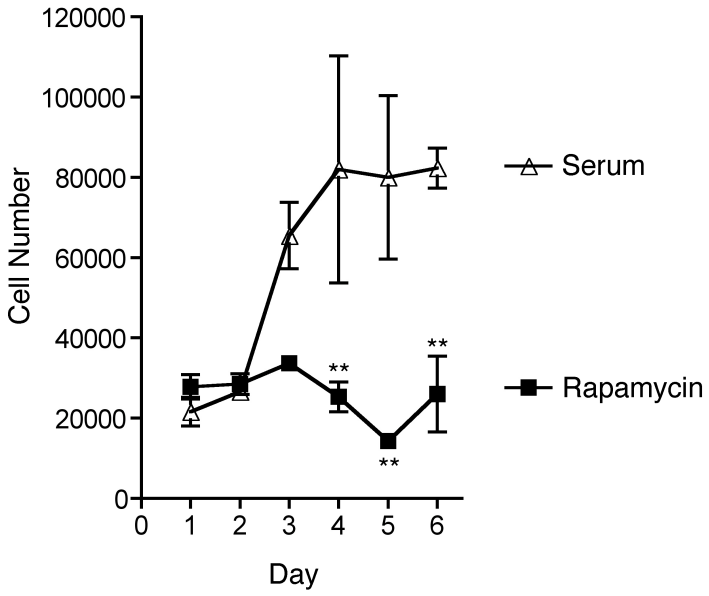

Figure 3.2: Rapamycin inhibits astrocyte growth induced by fetal bovine serum. The graph shows averages ±SE of triplicate measurements from one experiments, which is representative of three experiments that were carried out. **$p<0.01$ for the comparison between serum and rapamycin treated by two-way ANOVA and Bonferronis post-hoc test.

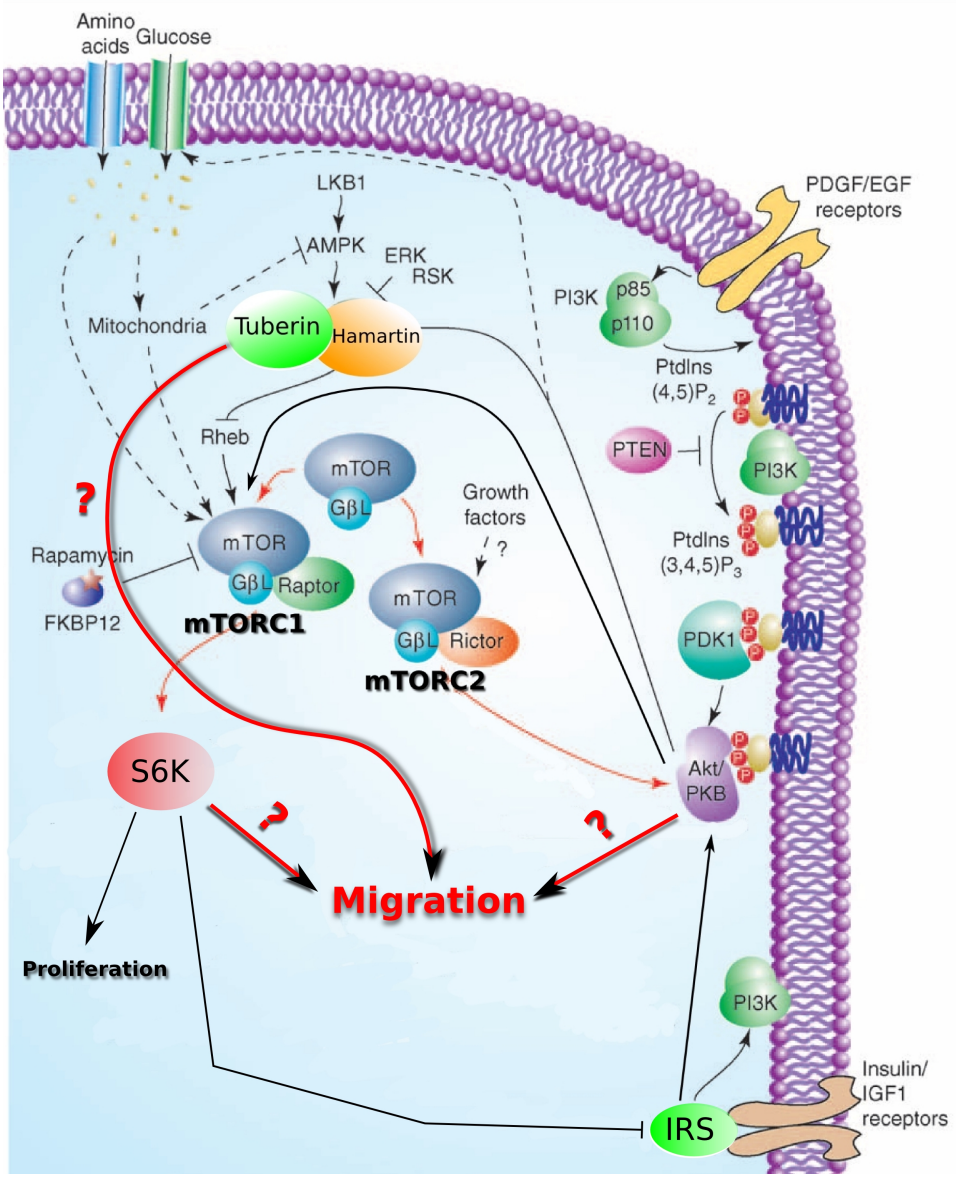

Figure 3.3: **EGF receptor-mTOR signaling pathway in adult spinal cord astrocytes.** mTORC1 is activated downstream of the EGF receptor. The activation is mediated by the Akt-Tuberin-Rheb cascade in astrocytes. mTORC1 regulates astrocyte proliferation and migration towards EGF and the treatment with the mTORC1 specific inhibitor rapamycin blocks the chemoattractive effect of EGF. The effects of rapamycin on cell migration may result from a role of S6 kinase on the actin cytoskeleton. Akt and Tuberin may also be involved in the regulation of the actin cytoskeleton. The question marks represent the regulatory points that may affect astrocyte migration and that will need further investigation.

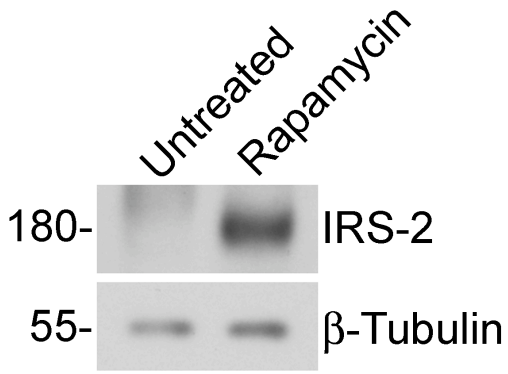

Figure 3.4: **Rapamycin treatment for 4 hours increases the levels of IRS-2.** Lysates from untreated and rapamycin treated cells were probed by immunoblotting with the indicated antibodies.

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