Published in final edited form as:

Exp Neurol. 2014 March ; 253: 72–81. doi:10.1016/j.expneurol.2013.11.022.

Ribosomal S6 kinase regulates ischemia-induced progenitor cell proliferation in the adult mouse hippocampus

Kate Karelina^a, Diego Alzate-Correa^b, and Karl Obrietan^{a,*}

^aDepartment of Neuroscience, The Ohio State University, Columbus OH 43210

^bDivision of Pharmacology, The Ohio State University, Columbus OH 43210

Abstract

Ischemia-induced progenitor cell proliferation is a prominent example of the adult mammalian brain's ability to regenerate injured tissue resulting from pathophysiological processes. In order to better understand and exploit the cell signaling mechanisms that regulate ischemia-induced proliferation, we examined the role of the p42/44 mitogen-activated protein kinase (MAPK) cascade effector ribosomal S6 kinase (RSK) in this process. Here, using the endothelin-1 ischemia model in wild type mice, we show that the activated form of RSK is expressed in the progenitor cells of the subgranular zone (SGZ) after intrahippocampal cerebral ischemia. Further, RSK inhibition significantly reduces ischemia-induced SGZ progenitor cell proliferation. Using the neurosphere assay, we also show that both SGZ- and subventricular zone (SVZ)-derived adult neural stem cells (NSC) exhibit a significant reduction in proliferation in the presence of RSK and MAPK inhibitors. Taken together, these data reveal RSK as a regulator of ischemia-induced progenitor cell proliferation, and as such, suggest potential therapeutic value may be gained by specifically targeting the regulation of RSK in the progenitor cell population of the SGZ.

Keywords

RSK; endothelin-1; ischemia; adult progenitor cell proliferation

Introduction

The adult mammalian brain, once believed to be highly limited in its ability to self-repair, is now understood to be capable of a substantial degree of regeneration in the wake of traumatic brain injury (TBI). A key locus of regeneration is the neurogenic niche located in the subgranular zone of the dentate gyrus (SGZ). Within the SGZ, radial glia-like progenitor cells generate neural precursor cells which express markers of immature neurons, migrate

© 2013 Elsevier Inc. All rights reserved.

*Corresponding author: Karl Obrietan, Department of Neuroscience, The Ohio State University, Graves Hall, Rm 4030, 333 W. 10th Ave Columbus OH 43210, Phone 1 (614) 292-4420, Fax 1 (614) 688-8742, obrietan.1@osu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors have declared that no competing interests exist.

into the granule cell layer (GCL) of the dentate gyrus, mature, and integrate into the hippocampal network (Emsley et al., 2005; Kempermann et al., 2004; van Praag et al., 2002). In response to injury, such as transient cerebral ischemia, there is a rapid increase in progenitor cell proliferation within the SGZ. Although only a small percent of SGZ newborn cells survive the inflammatory milieu of the ischemic brain (Arvidsson et al., 2002; Parent et al., 2002), a restorative role of ischemia-induced progenitor cell proliferation has been noted (Arvidsson et al., 2002; Emsley et al., 2005; Nakatomi et al., 2002). This ability to replenish lost cells after injury is all the more impressive given the pathophysiological environment resulting from cell death, excitotoxicity, oxidative stress and neuroinflammation that bathe the injured tissue and form physical barriers to neuronal regeneration (Brouns and De Deyn, 2009; Candelario-Jalil, 2009; Deierborg et al., 2009). As ongoing research yields further insight into the functional consequences of neural progenitor cell proliferation, there is an increasing need to identify the underlying signaling events that regulate these processes.

In line with this idea, we have focused on understanding the role of the p42/44 mitogen-activated protein kinase (MAPK) cascade in TBI-induced progenitor proliferation. Interest in this pathway is based in part on work showing that MAPK signaling is tightly coupled to an array of growth factors, cytokines, transmitters and free radicals that are released following TBI (Sawe et al., 2008). Further, MAPK signaling is rapidly activated by cerebral ischemia (Gu et al., 2001; Wu et al., 2000) and regulates both cytoplasmic and nuclear targets to promote cell growth, differentiation and apoptosis (Pan et al., 2012; Zhang and Liu, 2002). Regulation of these processes occurs via both direct ERK phosphorylation of transcription factors (Zhang and Liu, 2002) and also via phosphorylation of downstream effector kinases such as the mitogen and stress-activated kinase (MSKs) and ribosomal S6 kinases (RSKs) (Choi et al., 2012; Hauge and Frödin, 2006; Karelina et al., 2012; Yves et al., 2012), which are the focus of the current study.

The RSK family of kinases consists of 4 isoforms (RSK1-4: here collectively referred to as RSK), which show a good degree of redundancy with respect to expression patterns, mechanisms of activation, and substrate specificity (Dümmeler et al., 2005; Frödin and Gammeltoft, 1999). ERK activation of RSK occurs via phosphorylation at the C-terminus kinase domain (CTKD). The CTKD then phosphorylates the RSK N-terminus kinase domain which leads to PDK1-dependent (3-phosphoinositide-dependent protein kinase-1) activation of RSK (Cargnello and Roux, 2011; Frödin and Gammeltoft, 1999; Pearce et al., 2010). RSK targets an array of proteins within the cytoplasm, and is also translocated to the nucleus where it modulates transcription factor activation (Chen et al., 1992; Zhao et al., 1996; Hauge and Frödin, 2006). Of particular relevance to the data presented here, RSKs are directly involved in the regulation of cell cycle progression and cell survival (Clark et al., 2005; Nebreda and Gavin, 1999; Shimamura et al., 2000; Smith et al., 2005) via phosphorylation of key substrates including glycogen synthase kinase-3, cyclin D1, the cyclin-dependent kinase inhibitor p27^{kip1}, and Bad (Eisinger-Mathason et al., 2008; Fujita et al., 2003; Shimamura et al., 2000; Sutherland et al., 1993). Hence, the rapid activation of ERK during cerebral ischemia coupled with its role in activity-dependent cell proliferation makes RSK ideally positioned to regulate ischemia-induced progenitor cell proliferation.

In this study, we employed a model of transient cerebral ischemia induced via endothelin-1 (ET-1) infusion. ET-1 is a 21 amino acid peptide that has been identified as a potent vasoconstrictor (Yanagisawa et al., 1988). ET-1 is not directly neurotoxic (Nikolov et al., 1993) but induces decreased regional cerebral blood flow and brain infarction following direct intrahippocampal infusion (Faraji et al., 2011; Máteffyová et al., 2006). We report that a single intrahippocampal infusion of ET-1 induces RSK activation and progenitor cell proliferation in the SGZ. Moreover, ischemia-induced progenitor cell proliferation is substantially reduced via pharmacological inhibition of RSK. Using the neurosphere cell culture approach, we further show that the effects of RSK inhibition on neural stem cell (NSC) proliferation are cell-autonomous. Together, these data reveal a central role for RSK in the regulation of activity-dependent progenitor cell proliferation.

Materials and Methods

Animals

Adult C57/Bl6 mice (5–8 weeks old) were maintained on a 12:12 light/dark cycle in a temperature- and humidity-controlled vivarium. All mice were allowed *ad libitum* access to food and water. The study was conducted in accordance with Ohio State University guidelines for the care and use of animals and under protocols approved by the Institutional Animal Care and Use Committee.

Pharmacological agents

Endothelin-1 (Sigma Aldrich) is a potent vasoconstrictor that induces a transient decrease in regional cerebral blood flow and brain infarction (Faraji et al., 2011; Hughes et al., 2003). The following MAPK/RSK inhibitors were used: U0126 (Mek inhibitor; EMD Millipore), SL0101 (RSK inhibitor; Toronto Research Chemicals, Inc, # S560000), and BI-D1870 (RSK inhibitor; Enzo Life Sciences).

Stereotaxic surgery and infusion

Mice (5–8 per group/mixed gender) were anesthetized with a single intraperitoneal (i.p.) injection of ketamine (95.2 mg/kg) and xylazine (30.8 mg/kg). Fur was then removed from the scalp and protective ointment was applied to the eyes. Mice were then placed in the stereotaxic apparatus (Cartesian Research, Inc) and the unilateral coordinates (AP –2.06 mm; ML 1.30 mm; DV –2.00 mm) were used to place the tip of a 5 µL Hamilton syringe above the top blade of the hippocampal dentate gyrus (Figure 1A). Mice were infused with 1 µL of either BI-D1870 (5 mM) or the vehicle dimethyl sulfoxide (DMSO; Sigma Aldrich). Thirty minutes following DMSO or BI-D1870 infusion, mice received a 0.5 µL infusion of ET-1 (1 µg/µL) or the vehicle saline. All agents were infused at a rate of 1 µL/min. All mice were returned to their home cages and monitored for a period of 48 hours.

BrdU injections and tissue processing

In order to label newly generated cells, 5-bromo-2'-deoxyuridine (50mg/kg in saline, Sigma Aldrich) was injected (i.p.) two times as previously reported: 4 and 2 hours before sacrifice (Choi et al., 2008). Mice were sacrificed via transcardial perfusion 48 hours following ET-1 infusion. Mice were perfused with cold saline followed by 4% paraformaldehyde. Brains

were post-fixed in 4% paraformaldehyde and cryoprotected with 30% sucrose. Coronal sections (40 μ m) through the hippocampus were prepared using a freezing microtome.

Cresyl violet staining

Sections were mounted on gelatin-coated slides, dehydrated in graded alcohol solutions and incubated in 0.3% cresyl violet solution. Sections were then destained with 0.1% glacial acetic acid in 95% ethanol, cleared in xylene and mounted with Permount.

Fluoro-Jade B histology

Cell degeneration was assayed using Fluoro-Jade B labeling (FJB; Millipore). Tissue was immersed in 95% and 70% ethanol and washed in water. Sections were then incubated in 0.06% potassium permanganate for 10 min, washed and incubated in 0.001% FJB in 0.1% acetic acid. Finally, tissue was immersed in xylene and cover-slipped with DPX.

Immunohistochemistry

For immunohistochemistry, sections were washed and incubated in 0.3% hydrogen peroxide in 20% methanol for 20 minutes. Following washes in phosphate buffered saline with 0.1% triton-x (PBS-T), sections were blocked with 10% normal goat serum, then incubated overnight at 4° with rabbit anti-phospho p90 RSK (pRSK, 1:250; Thr359/Ser363, Cell Signaling) or rabbit anti-Ki67 (1:2000; Vector Labs). Following PBS-T washes, sections were then incubated for 2 hours in biotinylated goat anti-rabbit secondary antibody (1:500; Vector Labs). Finally, sections were processed using the ABC staining method (Vector Labs) and visualized with nickel-intensified DAB (Vector Labs).

For immunofluorescence, sections were washed in PBS-T and blocked with 10% normal horse or goat serum followed by overnight incubation at 4° with the following antibodies: rabbit anti-pRSK (1:100), mouse anti-pERK (1:300, Cell Signaling), goat anti-SOX2 (1:500, Santa Cruz), mouse anti-nestin (1:250, Millipore), mouse anti-GFAP (1:500), goat anti-doublecortin (1: 1000, Santa Cruz), or rat anti-BrdU (1:200; Accurate Chemical & Scientific Corp). Following PBS-T washes, sections were then incubated with AlexaFluor secondary antibodies conjugated with Alexa 488, Alexa 594 or Alexa 633 (each 1:500; Invitrogen). Fluorescence images were captured using a Zeiss 510 Meta confocal microscope. Where relevant, DraQ5 (1:5000) was used as a nuclear counterstain.

Cell quantitation

To quantitate BrdU and Ki67 expression in the SGZ, photomicrographs were captured at a 10 $\times$ magnification. Cells were counted unilaterally (ipsilateral to the infusion) in 3 dorsal hippocampal sections (defined as Bregma -1.70 through Bregma -2.06) spaced 120 μ m apart and summed. The mean $\pm$ SEM from 5–8 mice per group is presented. The SGZ was defined as the approximately 50 μ m-wide band between the hilus and GCL.

Fluoro-Jade B quantitation was performed using ImageJ software (Schneider et al., 2012) to quantify percentage of the area over threshold. The threshold level for Fluoro-Jade B positive cells over background was defined once and maintained constant for all image comparisons. Hippocampal subfields were individually analyzed by creating a boxed 'region

of interest' (ROI) around the GCL, hilus, and CA1. Once an ROI was created for a specific subfield, the same parameters of that ROI were kept constant for all images.

To quantitate pRSK expression in SGZ stem cells, first, stem cells were identified based on co-expression of both SOX2 and Nestin. The total number of SOX2/Nestin-positive stem cells was then quantified in the ipsilateral SGZ of 3 dorsal hippocampal sections. The percent of those stem cells that also co-labeled for pRSK was then determined for each mouse, and a mean was obtained for each group. All quantitation analyses were performed by an individual blinded to experimental conditions.

Neurosphere Culture

Neurosphere cultures were established using published methods (Pacey et al., 2006). SGZ and SVZ cells were microdissected from adult mice (5 weeks old) and digested with trypsin for 90 minutes at 37°C. The cells were then treated with a trypsin inhibitor (Roche), mechanically triturated, and filtered through a sterile cell strainer (40µm; BD Biosciences). The cells were plated in suspension at a density of 15 cells/µL in serum free medium (SFM) containing 30% glucose, 1M HEPES buffer (Sigma), Progesterone (Sigma), B-27 supplement (Invitrogen), insulin-transferrin-sodium selenite supplement (Roche), heparin (Sigma), fibroblast growth factor-basic (Sigma; bFGF) and epidermal growth factor (Sigma; EGF). Neurospheres were passaged every 7–9 days.

Following the second passage, the inhibitors BI-D1870 (0.1 µM, 1 µM or 10 µM), SL0101 (1 µM, 10 µM or 100 µM), U0126 (0.1 µM, 1 µM or 10 µM) or the vehicle DMSO were added to the single cell suspension in SFM. RSK inhibitor doses were selected based on protein kinase assays which indicate the most effective doses for RSK inhibition. Importantly, these assays revealed that BID1870 inhibits RSK with a higher specificity compared to SL0101 and inhibits activity of all four RSK isoforms by as much as 98% without significantly affecting other protein kinases (Bain et al., 2007; Sapkota et al., 2007). The doses for U0126 were selected based on previously published doses (Fujita et al., 2008; Hawes et al., 2006; Torroglosa et al., 2007). The final concentration of DMSO per each well was 0.01%. All inhibitors and vehicle were supplemented every 2–3 days. Cell proliferation was quantified as previously published (Alagappan et al., 2009; Regad et al., 2009) after 7 days *in vitro* based on total number of neurospheres and neurosphere size distribution (50–100, 100–150, 150–200, 200–250 µm in diameter). Neurospheres smaller than 50 µm in diameter were excluded from analysis. Stem cell self-renewal was assessed by quantifying the number of tertiary neurospheres generated relative to secondary neurospheres ($3^{\circ}/2^{\circ}$). All analyses were performed by an individual blinded to experimental conditions. A total of 3 mice were used for each experiment, and each experiment was replicated at least twice.

A subset of neurospheres were grown as a monolayer on poly-D-lysine (Sigma) coated glass coverslips (18 mm round) for immunocytochemical processing as described below.

Immunocytochemistry

For immunofluorescent labeling, neurospheres that were adhered to glass coverslips were fixed in 4% paraformaldehyde, washed with PBS-T, treated for 10 minutes in 0.3 M glycine

and blocked with 10% normal goat serum. Neurospheres were then incubated overnight at 4° with rabbit anti-Ki67 (1:200) or mouse anti-nestin (1:200). Following PBS-T washes, sections were incubated with AlexaFluor 488 (1:500), counterstained with the nuclear marker DraQ5 (1:5,000; Biostatus Limited), washed, and coverslipped. Fluorescence images were captured using a Zeiss 510 Meta confocal microscope.

Neurosphere cell viability assay

After seven days in culture, intact neurospheres (~200 µm in diameter) were treated with DMSO, BI-D1870 (10 µM), SL0101 (100 µM) or U0126 (10 µM) for 24 hours. Neurospheres were then double stained with CellTracker Green CMFDA (5 µM; Molecular Probes) for detection of living cells and propidium iodide (PI: 25 µM; Sigma Aldrich) for detection of dead cells. Both viability dyes were added to the neurosphere culture, followed by a 30-minute incubation at 37° C. Neurospheres were then washed with SFM, fixed in 4% paraformaldehyde and coverslipped. Spacers made from cover glass (Fisher Scientific) were placed under the coverslip in order to maintain the 3-dimensional integrity of the neurospheres. Images were captured as a z-stack using a Zeiss 510 Meta confocal microscope. Cell death was quantified as number of dead cells (PI-positive) per 1000 mm³. All analyses were performed by an individual blinded to experimental conditions.

Statistical Analysis

Immunohistochemistry, NSC proliferation and NSC survival data comparisons were made using a one-way ANOVA followed by a posthoc Tukey analysis. Data were considered significant for p-values < 0.05.

Results

Intrahippocampal ischemia induction via endothelin-1 infusion

Transient focal cerebral ischemia was induced by a unilateral intrahippocampal infusion of ET-1 (Figure 1A–B) and tissue was collected 2 or 8 days post-injury (DPI). Representative Fluoro-Jade B labeling data from the 2-day time point revealed dead/degenerating cells predominately in the CA1, and to a lesser extent in the GCL and hilus regions of the hippocampus (Figure 1C). In order to determine whether ischemia increases RSK activation, hippocampal tissue was immunolabeled with an antibody against the phosphorylated form of RSK (pRSK). Specifically, the pRSK antibody detects RSK1 (and to a limited extent, RSK3) phosphorylated at threonine 359 and serine 363: two events associated with RSK1 activation. Indeed, Frödin et al (1999) showed that phosphorylation of serine 363 is a critical step for the initiation of RSK activity. Using immunohistochemically labeling, we found that ET-1 mediated triggered marked RSK phosphorylation (here also referred to as activation) within the GCL as well as in the SGZ (Figure 1D) of the dentate gyrus.

In order to determine which SGZ cell populations exhibited increased RSK activity, we conducted a triple-label immunofluorescent labeling experiment in which pRSK was labeled along with SOX2 (a marker for multipotent stem cells (Ellis et al., 2004)) and nestin (a marker of neural stem cells). Under both basal conditions and at 2-days post-ET-1 infusion, pRSK expression was detected in SOX2/nestin-positive stem cells of the SGZ (Figure 2A).

Notably, quantitative analysis of SOX2/nestin-positive stem cells revealed that ET1-evoked ischemia produced a significantly greater percentage of pRSK-positive stem cells than saline infusion (Figure 2B). Quantitation of the total number of SOX2/Nestin-positive stem cells further revealed that the number of stem/progenitor cells did not differ between conditions (Figure 2C). Interestingly, a parallel analysis of pRSK expression at the 8 day post-DPI time point did not detect marked RSK activity in any of the noted cell populations (data not shown). Together, these data indicate that cerebral ischemia evokes a transient increase in RSK activation within the SGZ progenitor population.

Lastly, to extend this analysis to additional cell populations, we performed triple-label immunofluorescence assays in which pRSK was labeled along with GFAP (glial fibrillary acidic protein) and doublecortin (a marker of immature neurons) following ET1-evoked ischemia (Supplemental Figure 1). Low-level pRSK expression was detected in doublecortin-positive cells (Supplemental Figure 1A and 1C), whereas negligible pRSK labeling was detected in GFAP-positive astrocytes located in the GCL (Supplemental Figure 1).

RSK regulates ischemia-induced progenitor cell proliferation

The activation of RSK in neural progenitor cells of the SGZ raised the possibility that it contributes to ischemia-induced cell proliferation. To examine the role of RSK in this process, mice received an intrahippocampal infusion of the RSK inhibitor BI-D1870 or the vehicle (DMSO) 30 minutes before ET-1 or saline infusion, and animals were sacrificed 2 or 8 days later. BI-D1870 is a small molecule inhibitor that potently disrupts RSK activity and does not affect upstream kinases or other AGC family members, including PKA and PKC activity (Bain et al., 2007; Sapkota et al., 2007). To assess mitotic activity, mice were injected with BrdU (a thymidine analog that is taken up by cells during the S phase of the cell cycle) 4 and 2 hours before sacrifice. At the 2 DPI time point, DMSO/ET-1 infusion led to a significant increase in BrdU-positive cells in the SGZ compared to control DMSO/SAL (Figure 3A). However, inhibition of RSK before the onset of the ischemic event (BI-D1870/ET-1) significantly reduced mitotic labeling. Interestingly, infusion of BI-D1870 without ischemia (BI-D1870/SAL) did not significantly reduce progenitor cell proliferation, relative to vehicle-infused tissue. To complement the BrdU labeling, 2 DPI tissue was also processed for Ki67, a mitotic marker that is expressed at all phases of the active cell cycle (Supplemental Figure 2). Consistent with the results using BrdU labeling, quantitative analysis of Ki67 revealed a significant increase in proliferating progenitor cells following ischemia (DMSO/SAL vs DMSO/ET-1) as well as a significant reduction of ischemia-induced Ki67 labeling in those animals that were infused with BI-D1870 prior to ET-1. Again, BI-D1870 infusion without ischemia (BI-D1870/SAL) did not significantly affect progenitor cell proliferation. Of note, both male and female mice were used in these studies. In order to address the potential sex-specific effects, we ran a 2-factor ANOVA (the factors were gender and treatment (BI-D1870 vs DMSO) to determine whether gender differences contribute to post-ischemic progenitor cell proliferation in our model. Our analysis revealed no significant effects of gender ($p = 0.725$) nor a gender/treatment interaction ($p = 0.540$). Taken together, these data indicate that RSK functions as a regulator of activity-dependent, but not basal, progenitor cell proliferation in the SGZ.

Given the profound effect of RSK inhibition on acute proliferation (i.e., the 2-day time point), we conducted a similar experiment using a time point that more closely reflects peak proliferative activity following injury, 8 DPI (Takagi et al., 1999; Zhang et al., 2001). Similar to the results obtained at 2 DPI, mitotic activity at 8 DPI was also significantly reduced by RSK inhibition, compared to DMSO (Figure 3B). Here it is worth restating that, for this experiment, RSK was inhibited at the onset of the ischemic insult, and that pRSK analysis revealed that RSK activity was not detected at the 8 day post-ET-1 infusion time point. Together, these data indicate that the transient ET-1-evoked bout of RSK activity contributes to a long-lasting proliferative program (out to at least 8 days post-injury).

RSK regulates neural stem cell proliferation and self renewal *in vitro*

In order to examine whether RSK regulates progenitor cell proliferation in a cell-autonomous manner, adult NSCs were derived from the SGZ and cultured at a density of 15 cells/ μ L. Single cell suspensions were treated with BI-D1870 or DMSO and allowed to form neurospheres over 7 days. Neurospheres expressed Ki67 and nestin, confirming that they consist of actively cycling NSCs (Figure 4A). To analyze the proliferative capacity of the cultured NSCs in the presence of the RSK inhibitor, we counted the number of neurospheres present in each well and measured their diameter. Relative to DMSO-treated wells, BI-D1870 treatment dose-dependently reduced *in vitro* NSC proliferation (Figure 4B). Indeed, the two higher doses of BI-D1870 (1 μ M and 10 μ M) completely eliminated neurosphere formation, while the low dose (0.1 μ M) treatment did not affect neurosphere formation.

Given the profound effect of RSK inhibition on SGZ-derived NSC proliferation, we conducted additional experiments to determine whether this regulatory mechanism exists in another neurogenic niche located in the SVZ (Figure 5A–B). To that end, adult NSCs were derived from the SVZ and again cultured at a density of 15 cells/ μ L. Single cell suspensions were treated in the same manner as above with BI-D1870 or DMSO. Analysis of the number of neurospheres that formed after 7 days of treatment was consistent with SGZ-derived neurosphere data. BI-D1870 treatment dose-dependently reduced *in vitro* progenitor/stem cell proliferation relative to DMSO treatment. To confirm the specificity of RSK inhibition, we treated an additional set of SVZ-derived cells with another small-molecule RSK inhibitor, SL0101 (Smith et al., 2005). A dose-dependent effect of SL0101 was also observed whereby the low dose (1 μ M) increased the number of small (50–100 μ m) neurospheres, suggesting a reduced proliferative capacity of SL0101-treated cells. Of note, neurosphere formation occurs as a combination of clonal cell division (i.e. one NSC produces one neurosphere) as well as aggregate formation of small neurospheres (Singec et al., 2006). As such, the presence of a larger number of small neurospheres after 7 days *in vitro* likely reflects reduced responsiveness to growth factors, decreased self-renewal, and ultimately reduced proliferation (Pastrana et al., 2011). The highest dose of SL0101 tested (100 μ M) nearly completely eliminated neurosphere formation. Finally, as a way of testing the specificity of the effects of the RSK inhibitors, we examined whether these effects could be replicated by the disruption of MAPK pathway activity. To this end, cells were treated with the MEK-inhibitor U0126. A dose-dependent effect of U0126 was observed that paralleled the outcome of RSK inhibition via BI-D1870 (Figure 5A–B). These data provide

mechanistic support for the RSK inhibitor data, and further, suggest that MAPK signaling functions via the RSK pathway to regulate NSC proliferation *in vitro*.

To determine whether RSK regulates self-renewal, we examined the effect of RSK inhibition (via BI-D1870 and SL0101) or MAPK inhibition (via U0126) on neurosphere formation (Supplemental Figure 3). Given that the highest doses tested for each inhibitor resulted in potent disruption of proliferation, only the two lower doses were tested for each inhibitor. In addition, only SVZ-derived neurospheres were used for this analysis because hippocampus-derived neurospheres have been shown to have very limited capacity for self-renewal across multiple passages (Bonaguidi et al., 2008). Our results indicate that DMSO-treated wells demonstrated a normal capacity for self-renewal, with the number of neurospheres increasing by 58.6% from the secondary to tertiary passage. In contrast, RSK inhibition via BI-D1870 reduced neurosphere self-renewal to 33.0% (0.1 μ M) and 24.3% (1.0 μ M). Similarly, SL0101 treatment reduced self-renewal capacity to 37.8% (1.0 μ M) and 42.8% (10.0 μ M). U0126 treatment resulted in a modest reduction in self-renewal; 49.2% (0.1 μ M) and 49.1% (1.0 μ M). Taken together, these data indicate that MAPK/RSK signaling contributes to both the proliferation and self-renewal of cultured neural stem cells.

MAPK and RSK inhibition reduces neural stem cell survival *in vitro*

The reduction in neurosphere numbers resulting from RSK inhibition likely reflects an impairment of both NSC proliferative capacity and cell survival. To specifically test whether MAPK and RSK inhibition reduces NSC survival, intact SVZ-derived neurospheres (~200 μ m in diameter) were treated with DMSO, BI-D1870 (10 μ M), SL0101 (100 μ M), or U0126 (10 μ M) for 24 hours. These doses were selected based on the most effective inhibition of NSC proliferation obtained from the dose-response experiments (Figure 5). To estimate cell viability, neurospheres were double labeled with cell tracker green, a marker of intact/live cells, and propidium iodide, a dye that penetrates only necrotic cells with damaged membranes. Each of the inhibitors significantly increased the number of dead cells, suggesting that MAPK and RSK signaling affects *in vitro* NSC proliferative capacity at least in part by regulation of cell survival (Figure 6). Consistent with the data from the SVZ, treatment of SGZ-derived neurospheres with BI-D1870 (10 μ M) led to a marked increase in cell death. In fact, RSK inhibition via BI-D1870-evoked cell death in SGZ-derived neurospheres was far greater than the level of cell death observed in SVZ-derived neurospheres. One possible explanation is that unlike the pure stem cell culture derived from the SVZ, the SGZ consists of both stem cells and progenitor cells and is thus a mixed culture (Bonaguidi et al., 2008; Seaberg and van der Kooy, 2002). Together our data suggest that RSK inhibition of progenitor cells may result in a greater rate of cell death, however current limitations in distinguishing stem cells from progenitor cells make it difficult to directly test this hypothesis.

Discussion

The neurogenic niches of the adult brain contain progenitor cells that continue to produce neurons, astroglia and oligodendrocytes throughout life (Alvarez-Buylla and Lim, 2004). Under pathophysiological conditions, this phenomenon is believed to function as a means of

regenerating injured CNS tissue (Nakatomi et al., 2002). A key goal of this study was to identify the cell signaling events that contribute to this process following transient cerebral ischemia. In this study, we present data supporting the hypothesis that RSK regulates ischemia-induced progenitor cell proliferation. Our experiments reveal a marked increase in ischemia-induced phosphorylated RSK expression in SOX2/nestin-positive NSCs of the SGZ. This expression pattern, coupled with evidence of substantial progenitor cell proliferation after ischemia, led to the investigation of a regulatory role for RSK in this process. Indeed, inhibition of RSK via intrahippocampal infusion of BI-D1870 prior to ET-1 induction of ischemia resulted in a marked decrease in progenitor cell proliferation as determined by Ki67 and BrdU labeling.

Rapid and sustained activation of ERK within neurogenic regions plays a critical role in TBI-induced stimulation of progenitor cell proliferation (Li Y et al., 2010; Sung et al., 2007; Tian et al., 2009). A simple, mechanistic, description of this process would likely start with extracellular factors (i.e. mitogens) produced during an ischemic event driving the activation of ERK, which in turn would regulate an array of cytoplasmic and nuclear targets that promote proliferation, growth, and differentiation (Zhang and Liu, 2002). The data presented here indicate that the effects of ERK on NSC proliferation are mediated in part by RSK. These findings are consistent with *in vitro* data suggesting that RSK activation is necessary for ERK-mediated cell proliferation (Gayer et al., 2010; Godeny and Sayeski, 2006). Moreover, inhibition of RSK has been shown to suppress proliferation in breast (Smith et al., 2005), prostate (Clark et al., 2005), and colon (Park and Cho, 2012) cancer cell lines. Of note, in our study, RSK inhibition did not affect progenitor cell proliferation under control (non-ischemic) conditions. This is consistent with a role for the MAPK cascade as an activity-dependent signaling pathway. Indeed, we and others have reported that U0126 infusion blocks inducible but not basal rates of progenitor cell proliferation (Choi et al., 2008; Fournier et al., 2012; Pourié et al., 2006). These data suggest that progenitor cell proliferation is tightly regulated by intracellular signal transduction in response to extracellular factors produced by injured tissue.

Post-ischemic progenitor cell proliferation is a highly complex process driven by multiple inter-related factors that modulate cell cycle progression, growth, migration, differentiation and survival (Wiltout et al., 2007). Consistent with this, transient focal cerebral ischemia induces a cascade of pathophysiological events that involve glial and neuronal production of growth factors, cytokines, reactive oxygen species, neurotransmitters, and hormones (Brouns and De Deyn, 2009; Candelario-Jalil, 2009). These factors interact and converge to influence the rate of progenitor cell proliferation (Jin-qiao et al., 2009; Li J et al., 2010; Luo et al., 2007; Merino et al., 2011; Schabitz et al., 2007; Wang et al., 2010), thus making it difficult to identify cell-autonomous regulation of this process *in vivo*. However, by isolating adult-derived NSCs and culturing them using a neurosphere assay, we were able to determine a central role of RSK as a cell-autonomous regulator of NSC proliferation. Indeed, NSCs cultured in the presence of RSK inhibitors show reduced capacity for forming neurospheres. Of note, NSCs were cultured in serum free media containing EGF and bFGF; both growth factors initiate the Ras-MEK-ERK cascade leading to activation of RSK. Thus,

our *in vitro* data are consistent with a role for RSK as an inducible regulator of progenitor cell proliferation.

It is well known that the number of proliferating progenitor cells far exceeds the number of cells that successfully mature and incorporate into the existing hippocampal network (Kuhn et al., 2005). In fact, as many as 80% of TBI-induced newborn cells do not survive past the immature neuronal lineage-restricted state (Arvidsson et al., 2002). Importantly, under pathophysiological conditions (i.e. neurodegenerative disease) newborn cell survival is regulated in large part by the MAPK signaling cascade (Miloso et al., 2008). More specifically, RSK activation has been shown to increase cell survival via regulation of key substrates including the pro-apoptotic Bad (Bonni et al., 1999), the death-associated protein kinase (Anjum et al., 2005), and the CCAAT/enhancer binding protein β (Buck et al., 2001). Our data confirm a role for both MEK and RSK in cell survival; however, it is important to note that neurospheres are heterogeneous populations made up of stem cells, progenitor cells and a few spontaneously differentiating cells (Jensen and Parmar, 2006; Parmar et al., 2003). Thus, additional work will be required to determine the cell type(s) that is vulnerable to cell death following MEK or RSK inhibition in neurospheres.

Conclusions

Taken together, these data reveal RSK as a critical component of the cellular signaling cascade that regulates inducible progenitor cell proliferation. The findings presented here advance our understanding of the complex set of events that must take place for the successful production of new cells in a pathophysiological brain. Ultimately, a clear understanding of the cellular/molecular mechanisms that regulate this process may reveal novel therapeutic targets and strategies for the alleviation of CNS tissue injury in the event of TBI such as ischemia or status epilepticus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Yujia (Jennie) Liu for technical assistance. This work was supported by The American Heart Association Postdoctoral Fellowship number 11POST7410015; and the National Institutes of Health Grant numbers: NS066345, NS06740 and MH062335.

Abbreviations

ERK	Extracellular signal regulated kinases 1 and 2
ET-1	endothelin-1
GCL	granule cell layer
MAPK	mitogen-activated protein kinase
NSC	neural stem cell

RSK	ribosomal S6 kinase
SGZ	subgranular zone
SVZ	subventricular zone

References

- Alagappan D, Lazzarino DA, Felling RJ, Balan M, Kotenko SV, Levison SW. Brain injury expands the numbers of neural stem cells and progenitors in the SVZ by enhancing their responsiveness to EGF. *ASN neuro* 1. 2009
- Alvarez-Buylla A, Lim DA. For the long run: maintaining germinal niches in the adult brain. *Neuron*. 2004; 41:683–686. [PubMed: 15003168]
- Anjum R, Roux PP, Ballif BA, Gygi SP, Blenis J. The tumor suppressor DAP kinase is a target of RSK-mediated survival signaling. *Curr Biol*. 2005; 15:1762–1767. [PubMed: 16213824]
- Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med*. 2002; 8:963–970. [PubMed: 12161747]
- Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, Mclauchlan H, Klevernic I, Arthur JSC, Alessi DR, Cohen P. The selectivity of protein kinase inhibitors: a further update. *Biochem J*. 2007; 408:297–315. [PubMed: 17850214]
- Bonaguidi MA, Peng CY, McGuire T, Falciglia G, Gobeske KT, Czeisler C, Kessler JA. Noggin expands neural stem cells in the adult hippocampus. *The Journal of Neuroscience*. 2008; 28:9194–9204. [PubMed: 18784300]
- Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and-independent mechanisms. *Science*. 1999; 286:1358–1362. [PubMed: 10558990]
- Brouns R, De Deyn PP. The complexity of neurobiological processes in acute ischemic stroke. *Clin Neurol Neurosur*. 2009; 111:483–495.
- Buck M, Poli V, Hunter T, Chojkier M. C/EBP β phosphorylation by RSK creates a functional XEXD caspase inhibitory box critical for cell survival. *Mol Cell*. 2001; 8:807–816. [PubMed: 11684016]
- Candelario-Jalil E. Injury and repair mechanisms in ischemic stroke: considerations for the development of novel neurotherapeutics. *Curr Opin Invest Dr*. 2009; 10:644–654.
- Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiology and Molecular Biology Reviews*. 2011; 75:50–83. [PubMed: 21372320]
- Chen R, Sarnecki C, Blenis J. Nuclear localization and regulation of erk-and rsk-encoded protein kinases. *Molecular and cellular biology*. 1992; 12:915–927. [PubMed: 1545823]
- Choi YS, Cho HY, Hoyt KR, Naegel JR, Obrietan K. IGF-1 receptor-mediated ERK/MAPK signaling couples status epilepticus to progenitor cell proliferation in the subgranular layer of the dentate gyrus. *Glia*. 2008; 56:791–800. [PubMed: 18338791]
- Choi YS, Karelina K, Alzate-Correa D, Hoyt KR, Impey S, Arthur JSC, Obrietan K. Mitogen- and stress-activated kinases regulate progenitor cell proliferation and neuron development in the adult dentate gyrus. *J Neurochem*. 2012; 123:676–688. [PubMed: 23020821]
- Clark D, Errington T, Smith J, Frierson H, Weber M, Lannigan D. The serine/threonine protein kinase, p90 ribosomal S6 kinase, is an important regulator of prostate cancer cell proliferation. *Cancer Res*. 2005; 65:3108–3116. [PubMed: 15833840]
- Deierborg T, Staflin K, Pesic J, Roybon L, Brundin P, Lundberg C. Absence of striatal newborn neurons with mature phenotype following defined striatal and cortical excitotoxic brain injuries. *Exp Neurol*. 2009; 219:363–367. [PubMed: 19427853]
- Dümler BA, Hauge C, Silber J, Yntema HG, Kruse LS, Kofoed B, Hemmings BA, Alessi DR, Frödin M. Functional characterization of human RSK4, a new 90-kDa ribosomal S6 kinase, reveals constitutive activation in most cell types. *J Biol Chem*. 2005; 280:13304–13314. [PubMed: 15632195]

- Eisinger-Mathason TS, Andrade J, Groehler AL, Clark DE, Muratore-Schroeder TL, Pasic L, Smith JA, Shabanowitz J, Hunt DF, Macara IG, Lannigan DA. Codependent functions of RSK2 and the apoptosis-promoting factor TIA-1 in stress granule assembly and cell survival. *Mol Cell*. 2008; 31:722–736. [PubMed: 18775331]
- Ellis P, Fagan BM, Magness ST, Hutton S, Taranova O, Hayashi S, McMahon A, Rao M, Pevny L. SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult. *Dev Neurosci*. 2004; 26:148–165. [PubMed: 15711057]
- Emsley JG, Mitchell BD, Kempermann G, Macklis JD. Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. *Prog Neurobiol*. 2005; 75:321–341. [PubMed: 15913880]
- Faraji J, Metz GA, Sutherland RJ. Stress after hippocampal stroke enhances spatial performance in rats. *Physiol Behav*. 2011; 102:389–399. [PubMed: 21130791]
- Fournier NM, Lee B, Banasr M, Elsayed M, Duman RS. Vascular endothelial growth factor regulates adult hippocampal cell proliferation through MEK/ERK-and PI3K/Akt-dependent signaling. *Neuropharmacology*. 2012; 63:642–652. [PubMed: 22580375]
- Frödin M, Gammeltoft S. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol Cell Endocrinol*. 1999; 151:65–77. [PubMed: 10411321]
- Fujita N, Sato S, Tsuruo T. Phosphorylation of p27Kip1 at threonine 198 by p90 ribosomal protein S6 kinases promotes its binding to 14-3-3 and cytoplasmic localization. *J Biol Chem*. 2003; 278:49254–49260. [PubMed: 14504289]
- Fujita Y, Hiroshima M, Sanbe A, Yamauchi J, Murase S, Tanoue A. ETOH inhibits embryonic neural stem/precursor cell proliferation via PLD signaling. *Biochemical and biophysical research communications*. 2008; 370:16–173. [PubMed: 18339308]
- Gayer CP, Craig DH, Flanagan TL, Reed TD, Cress DE, Basson MD. ERK regulates strain-induced migration and proliferation from different subcellular locations. *J Cell Biochem*. 2010; 109:711–725. [PubMed: 20069571]
- Godeny MD, Sayeski PP. ERK1/2 regulates ANG II-dependent cell proliferation via cytoplasmic activation of RSK2 and nuclear activation of elk1. *Am J Physiol Cell Physiol*. 2006; 291:C1308–C1317. [PubMed: 16723511]
- Gu Z, Jiang Q, Zhang G. Extracellular signal-regulated kinase 1/2 activation in hippocampus after cerebral ischemia may not interfere with postischemic cell death. *Brain Res*. 2001; 901:79–84. [PubMed: 11368953]
- Hauge C, Frödin M. RSK and MSK in MAP kinase signalling. *J Cell Sci*. 2006; 119:3021–3023. [PubMed: 16868029]
- Hawes JJ, Narasimhaiah R, Picciotto MR. Galanin and galanin-like peptide modulate neurite outgrowth via protein kinase C-mediated activation of extracellular signal-related kinase. *European Journal of Neuroscience*. 2006; 23:2937–2946. [PubMed: 16819983]
- Hughes PM, Anthony DC, Ruddin M, Botham MS, Rankine EL, Sablone M, Baumann D, Mir AK, Perry VH. Focal lesions in the rat central nervous system induced by endothelin-1. *J Neuropathol Exp Neurol*. 2003; 62:1276–1286. [PubMed: 14692703]
- Jensen JB, Parmar M. Strengths and limitations of the neurosphere culture system. *Mol Neurobiol*. 2006; 34:153–161. [PubMed: 17308349]
- Jin-qiao S, Bin S, Wen-hao Z, Yi Y. Basic fibroblast growth factor stimulates the proliferation and differentiation of neural stem cells in neonatal rats after ischemic brain injury. *Brain Dev*. 2009; 31:331–340. [PubMed: 18657919]
- Karelina K, Hansen KF, Choi YS, DeVries AC, Arthur JSC, Obrietan K. MSK1 regulates environmental enrichment-induced hippocampal plasticity and cognitive enhancement. *Learn Memory*. 2012; 19:550–560.
- Kempermann G, Wiskott L, Gage FH. Functional significance of adult neurogenesis. *Curr Opin Neurobiol*. 2004; 14:186–191. [PubMed: 15082323]
- Kuhn HG, Biebl M, Wilhelm D, Li M, Friedlander RM, Winkler J. Increased generation of granule cells in adult Bcl-2-overexpressing mice: a role for cell death during continued hippocampal neurogenesis. *Eur J Neurosci*. 2005; 22:1907–1915. [PubMed: 16262630]

- Li J, Siegel M, Yuan M, Zeng Z, Finnucan L, Persky R, Hurn PD, McCullough LD. Estrogen enhances neurogenesis and behavioral recovery after stroke. *J Cerebr Blood F Met.* 2010; 31:413–425.
- Li Y, Peng Z, Xiao B, Houser CR. Activation of ERK by spontaneous seizures in neural progenitors of the dentate gyrus in a mouse model of epilepsy. *Exp Neurol.* 2010; 224:133–145. [PubMed: 20226181]
- Luo CX, Zhu XJ, Zhou QG, Wang B, Wang W, Cai HH, Sun YJ, Hu M, Jiang J, Hua Y. Reduced neuronal nitric oxide synthase is involved in ischemia-induced hippocampal neurogenesis by up-regulating inducible nitric oxide synthase expression. *J Neurochem.* 2007; 103:1872–1882. [PubMed: 17854382]
- Mátéffyová A, Otáhal J, Tsenov G, Mareš P, Kubová H. Intrahippocampal injection of endothelin-1 in immature rats results in neuronal death, development of epilepsy and behavioral abnormalities later in life. *Eur J Neurosci.* 2006; 24:351–360. [PubMed: 16836644]
- Merino JJ, Gutiérrez-Fernández M, Rodríguez-Frutos B, Álvarez-Grech J, Alcalde ME, Vallejo-Cremades MT, Díez-Tejedor E. CXCR4/SDF-1 α -chemokine regulates neurogenesis and/or angiogenesis within the vascular niche of ischemic rats; however, does SDF-1 α play a role in repair? *Int J Stroke.* 2011; 6:466–467. [PubMed: 21951413]
- Miloso M, Scuteri A, Foudah D, Tredici G. MAPKs as mediators of cell fate determination: an approach to neurodegenerative diseases. *Curr Med Chem.* 2008; 15:538–548. [PubMed: 18336268]
- Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell.* 2002; 110:429–441. [PubMed: 12202033]
- Nebreda AR, Gavin AC. Cell survival demands some Rsk. *Science.* 1999; 286:1309–1310. [PubMed: 10610536]
- Nikolov R, Rami A, Kriegstein J. Endothelin-1 exacerbates focal cerebral ischemia without exerting neurotoxic action in vitro. *Eur J Pharm- Environ.* 1993; 248:205–208.
- Pacey L, Stead S, Gleave J, Tomczyk K, Doering L. Neural stem cell culture: neurosphere generation, microscopical analysis and cryopreservation. *Nat Protoc.* 2006; 215:1–14.
- Pan YW, Zou J, Wang W, Sakagami H, Garelick MG, Abel G, Kuo CT, Storm DR, Xia Z. Inducible and conditional deletion of extracellular signal-regulated kinase 5 disrupts adult hippocampal neurogenesis. *J Biol Chem.* 2012; 287:23306–23317. [PubMed: 22645146]
- Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann Neurol.* 2002; 52:802–813. [PubMed: 12447935]
- Park YS, Cho NJ. EGFR and PKC are involved in the activation of ERK1/2 and p90 RSK and the subsequent proliferation of SNU-407 colon cancer cells by muscarinic acetylcholine receptors. *Mol Cell Biochem.* 2012; 370:191–198. [PubMed: 22865467]
- Parmar M, Sjöberg A, Björklund A, Kokaia Z. Phenotypic and molecular identity of cells in the adult subventricular zone: in vivo and after expansion in vitro. *Mol Cell Neurosci.* 2003; 24:741–752. [PubMed: 14664822]
- Pastrana E, Silva-Vargas V, Doetsch F. Eyes wide open: a critical review of sphere-formation as an assay for stem cells. *Cell Stem Cell.* 2011; 8:486–498. [PubMed: 21549325]
- Paxinos, G.; Franklin, KBJ. The mouse brain in stereotaxic coordinates. Academic Press; London: 2001.
- Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. *Nature Reviews Molecular Cell Biology.* 2010; 11:9–22.
- Pourié G, Blaise S, Trabalon M, Nédélec E, Guéant JL, Daval JL. Mild, non-lesioning transient hypoxia in the newborn rat induces delayed brain neurogenesis associated with improved memory scores. *Neuroscience.* 2006; 140:1369–1379. [PubMed: 16650606]
- Regad T, Bellodi C, Nicotera P, Salomoni P. The tumor suppressor Pml regulates cell fate in the developing neocortex. *Nature neuroscience.* 2009; 12:132–140.
- Sapkota GP, Cummings L, Newell FS, Armstrong C, Bain J, Frodin M, Grauert M, Hoffmann M, Schnapp G, Steegmaier M. BI-D1870 is a specific inhibitor of the p90 RSK (ribosomal S6 kinase) isoforms in vitro and in vivo. *Biochem J.* 2007; 401:29–38. [PubMed: 17040210]

- Sawe N, Steinberg G, Zhao H. Dual roles of the MAPK/ERK1/2 cell signaling pathway after stroke. *J Neurosci Res.* 2008; 86:1659–1669. [PubMed: 18189318]
- Schabitz WR, Steigleder T, Cooper-Kuhn CM, Schwab S, Sommer C, Schneider A, Kuhn HG. Intravenous brain-derived neurotrophic factor enhances poststroke sensorimotor recovery and stimulates neurogenesis. *Stroke.* 2007; 38:2165–2172. [PubMed: 17510456]
- Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nature methods.* 2012; 9:671–675. [PubMed: 22930834]
- Seaberg RM, van der Kooy D. Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. *The Journal of Neuroscience.* 2002; 22:1784–1793. [PubMed: 11880507]
- Shimamura A, Ballif BA, Richards SA, Blenis J. Rsk1 mediates a MEK-MAP kinase cell survival signal. *Curr Biol.* 2000; 10:127–135. [PubMed: 10679322]
- Singec I, Knoth R, Meyer RP, Maciaczyk J, Volk B, Nikkhah G, Frotscher M, Snyder EY. Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. *Nat Methods.* 2006; 3:801–806. [PubMed: 16990812]
- Smith JA, Poteet-Smith CE, Xu Y, Errington TM, Hecht SM, Lannigan DA. Identification of the first specific inhibitor of p90 ribosomal S6 kinase (RSK) reveals an unexpected role for RSK in cancer cell proliferation. *Cancer Res.* 2005; 65:1027–1034. [PubMed: 15705904]
- Sung SM, Jung DS, Kwon CH, Park JY, Kang SK, Kim YK. Hypoxia/reoxygenation stimulates proliferation through PKC-dependent activation of ERK and Akt in mouse neural progenitor cells. *Neurochem Res.* 2007; 32:1932–1939. [PubMed: 17562163]
- Sutherland C, Leighton IA, Cohen P. Inactivation of glycogen synthase kinase-3 beta by phosphorylation: new kinase connections in insulin and growth-factor signalling. *Biochem J.* 1993; 296:15–19. [PubMed: 8250835]
- Takagi Y, Nozaki K, Takahashi J, Yodoi J, Ishikawa M, Hashimoto N. Proliferation of neuronal precursor cells in the dentate gyrus is accelerated after transient forebrain ischemia in mice. *Brain research.* 1999; 831:283–287. [PubMed: 10412007]
- Tian HP, Huang BS, Zhao J, Hu XH, Guo J, Li LX. Non-receptor tyrosine kinase Src is required for ischemia-stimulated neuronal cell proliferation via Raf/ERK/CREB activation in the dentate gyrus. *BMC Neurosci.* 2009; 10:139. [PubMed: 19943942]
- Torroglosa A, Murillo-Carretero M, Romero-Grimaldi C, Matarredona ER, Campos-Caro A, Estrada C. Nitric Oxide Decreases Subventricular Zone Stem Cell Proliferation by Inhibition of Epidermal Growth Factor Receptor and Phosphoinositide-3-Kinase/Akt Pathway. *Stem Cells.* 2007; 25:88–97. [PubMed: 16960136]
- van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. Functional neurogenesis in the adult hippocampus. *Nature.* 2002; 415:1030–1034. [PubMed: 11875571]
- Wang S, Zhang Z, Guo Y, Sui Y, Sun Y. Involvement of serotonin neurotransmission in hippocampal neurogenesis and behavioral responses in a rat model of post-stroke depression. *Pharmacol Biochem Behav.* 2010; 95:129–137. [PubMed: 20045434]
- Wiltout C, Lang B, Yan Y, Dempsey RJ, Vemuganti R. Repairing brain after stroke: a review on post-ischemic neurogenesis. *Neurochem Int.* 2007; 50:1028–1041. [PubMed: 17531349]
- Wu DC, Ye W, Che XM, Yang GY. Activation of mitogen-activated protein kinases after permanent cerebral artery occlusion in mouse brain. *J Cerebr Blood F Met.* 2000; 20:1320–1330.
- Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.* 1988; 332:411–415. [PubMed: 2451132]
- Yves R, Xiaocui Z, Philippe PR. Regulation and function of the RSK family of protein kinases. *Biochem J.* 2012; 441:553–569. [PubMed: 22187936]
- Zhang R, Zhang Z, Zhang L, Chopp M. Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. *Neuroscience.* 2001; 105:33–41. [PubMed: 11483298]
- Zhang W, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res.* 2002; 12:9–18. [PubMed: 11942415]

Zhao Y, Bjørbæk C, Møller DE. Regulation and interaction of pp90^{rsk} isoforms with mitogen-activated protein kinases. *Journal of Biological Chemistry*. 1996; 271:29773–29779. [PubMed: 8939914]

Highlights

- Cerebral ischemia induces progenitor cell proliferation in the dentate gyrus SGZ.
- This study examined the role of the ERK effector kinase RSK in this process.
- Cerebral ischemia induced pRSK expression in SGZ neural stem cells.
- RSK inhibition reduced ischemia-induced progenitor cell proliferation.
- Both MAPK and RSK inhibition reduced neurosphere proliferation.

Figure 1. Intrahippocampal endothelin-1 infusions induce cell degeneration and RSK activation

A) Schematic of stereotaxic infusion site (adapted with permission from (Paxinos and Franklin, 2001)). Endothelin-1 (ET-1) or saline (SAL) were infused into the hippocampus and tissue collected 48 hours later. B) Representative cresyl violet-stained section showing the needle track into the hippocampus; scale bar = 400 μ m. C) Fluoro-Jade B labeling revealed that ET-1 infusion triggered cell degeneration in the CA1 hippocampal subfield; limited cell death was detected in the hilus and GCL. Cell death was not detected in the saline-infused control. Scale bar = 200 μ m. D) Quantitative analysis of Fluoro-Jade B-labeled degenerating cells in the CA1, GCL and hilus (see *Materials and Methods* for details on quantitative analysis method). E) Immunohistochemical labeling revealed ET-1-induced phosphorylation of RSK in the GCL and SGZ; upper panel scale bar = 100 μ m, lower panel scale bar = 50 μ m.

Figure 2. Ischemia-induced activation of RSK in SGZ neural stem cells

A) Representative images show phosphorylated RSK expression in SOX2/Nestin-positive neural stem cells of the SGZ following saline infusion or ET-1-induced ischemia. The boxed region in the line drawing represents the SGZ region shown in the immunofluorescence image (adapted with permission from (Paxinos and Franklin, 2001)). Scale bar in panel A = 20 μ m. B) Quantitative analysis revealed an increase in the percent of pRSK-positive stem cells following ET-1 ischemia. C) The total number of SOX2/Nestin-positive stem cells per slice was not affected by ET1. An asterisk (*) denotes a statistically significant difference, $p < 0.05$.

Figure 3. RSK inhibition reduces ischemia-induced progenitor cell proliferation

BrdU labeling experiments revealed that RSK inhibition via BI-D1870 infusion 30 minutes before ET-1 infusion reduced ischemia-induced progenitor cell proliferation in the SGZ. A) BrdU-positive expression is significantly increased at 2 days post-ET-1 treatment in DMSO, but not BI-D1870 treated SGZ. B) At a later time point (8 days post-injury), the number of BrdU-positive proliferating cells was still reduced by BI-D1870 treatment. Scale bar = 200 μ m. Data are presented as mean $\pm$ SEM. An asterisk (*) denotes a statistically significant difference from the DMSO group, $p < 0.05$.

Figure 4. BI-D1870 inhibits SGZ neurosphere proliferation

Adult-derived stem/progenitor cells were isolated from the SGZ (the region that was isolated is highlighted in red in the line drawing; adapted with permission from (Paxinos and Franklin, 2001)). Cells were grown in a neurosphere culture containing EGF and bFGF. A) Neurospheres were confirmed to consist of actively proliferating stem cells via immunofluorescent detection of nestin and Ki67 expression; scale bar = 50 μm . B) Treatment of the neurospheres with BI-D1870 inhibited stem/progenitor cell proliferation in a dose-dependent manner; scale bar = 300 μm . Data are presented as mean $\pm$ SEM. An asterisk (*) denotes a statistically significant difference from the DMSO group, $p < 0.05$.

Figure 5. MAPK/RSK inhibition attenuates SVZ progenitor/stem cell proliferation

Adult-derived stem/progenitor cells were isolated from the SVZ (the region that was isolated is highlighted in red in the line drawing; adapted with permission from (Paxinos and Franklin, 2001)). Cells were grown in a neurosphere culture containing EGF and bFGF. Compared to neurospheres treated with DMSO, the RSK inhibitors BI-D1870 and SL0101, or the Mek inhibitor U0126 resulted in a dose-dependent reduction of progenitor/stem cell proliferation. A) Representative images of SVZ-derived neurospheres in each treatment condition. B) Quantitative analysis of the number and diameter of neurospheres per well. Data are presented as mean $\pm$ SEM. An asterisk (*) denotes a statistically significant difference from the DMSO group, $p < 0.05$.

Figure 6. MAPK/RSK inhibition reduces survival of proliferating progenitor/stem cells
 SGZ- and SVZ-derived neurospheres were treated with DMSO, BI-D1870, SL0101 or U0126 for 24 hours and stained with the cell viability dyes propidium iodide (red) and cell tracker (green), (the region that was isolated is highlighted in red in the line drawings; adapted with permission from (Paxinos and Franklin, 2001)). Representative orthogonal views through confocal z-stacks indicate reduced cell survival following treatment with the MAPK and RSK inhibitors. A) SGZ-derived neurospheres treated with 10 μM BI-D1870 have significantly more dead cells relative to DMSO treatment. B) SVZ-derived neurospheres treated with 10 μM BI-D1870, 100 μM SL0101, or 10 μM U0126 all show a significant increase in cell death relative to DMSO treatment. Data are presented as mean $\pm$ SEM. An asterisk (*) denotes a statistically significant difference from the DMSO group, $p < 0.05$.