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ORIGINAL ARTICLE

S100B protein activates a RAGE-dependent autocrine loop in astrocytes: implications for its role in the propagation of reactive gliosis

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Abstract

Extracellular S100B dramatically increases after brain injury. While low S100B levels are neuroprotective, micromolar S100B levels have shown *in vitro* to activate microglia and facilitate neuronal death. In astrocytes, S100B exposure activates nuclear factor kappa B (NF-κB) and induces pro-inflammatory mediators. On microglia and neurons S100B effects are essentially mediated by receptor for advanced glycation end products (RAGE)/NF-κB, but it is not clear if these intracellular cascades are activated by different S100B levels in astrocytes and whether increased extracellular S100B is sufficient to induce reactive gliosis. A better understanding of these pathways is essential for developing successful strategies to preserve the beneficial S100B effects after brain injury. Here, we show that microglia-depleted cultured astrocytes exposed to S100B mimicked several features of reactive gliosis by activating RAGE/Rac-1-Cdc42, RAGE/Erk-Akt or RAGE/NF-κB-dependent pathways. S100B effects include RAGE/Rac1-Cdc42-dependent astroglial hypertrophy and facilitation of migration as well as increased mitosis. S100B exposure improved the astrocytic survival to oxidative stress, an effect that requires Erk/Akt. S100B also activates NF-κB in a dose-dependent manner; increases RAGE proximal promoter transcriptional activity and augmented endogenous RAGE expression. S100B-exposed astrocytes showed a pro-inflammatory phenotype with expression of Toll-like receptor 2 (TLR 2), inducible nitric oxide synthase (iNOS) and interleukin 1-beta (IL-1β), and facilitated neuronal death induced by oxygen-glucose deprivation. *In vivo*, intracerebral infusion of S100B was enough to induce an astroglial reactive phenotype. Together, these findings demonstrate that extracellular S100B in the micromolar level activates different RAGE-dependent pathways that turn astrocytes into a pro-inflammatory and neurodegenerative phenotype.

Keywords: DAMP, inflammation, reactive gliosis.

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S100B is a small EF-hand Ca^{2+} binding protein that has several intracellular actions in astrocytes (reviewed in Donato *et al.* 2009). After brain injury, S100B becomes detectable in cerebrospinal fluid and peripheral blood (Rothermundt *et al.* 2003; Michetti *et al.* 2012). *In vitro* experiments have shown that S100B efflux is increased by

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Abbreviations used: 5-FU, 5-fluorouracyl; BrdU, 5-bromo-2′-deoxyuridine; BSA, bovine serum albumin; CM, conditioned medium; CNS, central nervous system; DAMP, damage-associated molecular pattern; DMEM, Dulbecco's modified Eagle medium; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; IL-1β, interleukin 1-beta; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor kappa B; NO, nitric oxide; OGD, oxygen–glucose deprivation; PRR, pattern recognition receptor; RAGEΔcyto, RAGE lacking the intracellular domain; RAGE, receptor for advanced glycation end products; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4; wtRAGE, wild type RAGE.

oxygen-serum-glucose deprivation or cellular stretching, is independent of *de novo* S100B protein synthesis and does not require an endoplasmic reticulum trafficking (Davey *et al.* 2001; Gerlach *et al.* 2006; Ellis *et al.* 2007). In view of this, it was more recently proposed that S100B behaves as a Damage-Associated Molecular Pattern protein able to activate innate immunity (Sorci *et al.* 2010; Bianchi *et al.* 2011).

The Receptor for Advanced Glycation End products (RAGE) is a Pattern Recognition Receptor involved in the activation of innate immunity and S100B has been shown to interact with RAGE in different cell types (Hofmann *et al.* 1999; Huttunen *et al.* 2000; Villarreal *et al.* 2011). The other prominent members of the pattern recognition receptor family are the Toll-like receptors (TLR). TLR is a family of receptors involved in the recognition of pathogens and Damage-Associated Molecular Patterns. Within central nervous system (CNS) increased TLR expression has been observed and specifically TLR2 and TLR4 have been related to ischemic brain injury (Marsh *et al.* 2009). Evidence from studies developed in non-nervous tissues has shown that RAGE and TLR may cooperate in the innate immunity activation driven by tissue damage (Ibrahim *et al.* 2013).

In astrocytes, recombinant S100B has been shown to induce proliferation (Selinfreund *et al.* 1991), increased inducible nitric oxide synthase (iNOS) expression, nitric oxide release, interleukin 1-beta (IL-1β), interleukin 6 and TNF-α secretion (Hu and Van Eldik 1996, 1999; Hu *et al.* 1996, 1997; Ponath *et al.* 2007). While in microglia S100B has been shown to interact with RAGE to induce the pro-inflammatory mediators and cell migration (Bianchi *et al.* 2007, 2010, 2011), only interleukin 6 and TNF-α secretion was demonstrated to be RAGE-dependent in astrocytes (Ponath *et al.* 2007). It is presently unknown if S100B is able to activate similar intracellular cascades in astrocytes to facilitate the conversion to the reactive phenotype and whether S100B-induced reactive astrocytes are beneficial or detrimental for neuronal survival.

In this study, we used microglia-depleted astrocytic culture exposed to brain-purified S100B and determined that S100B/RAGE, by activating different intracellular pathways, induces profound alterations in astrocytes toward a reactive phenotype *in vitro*, increases RAGE expression, and exacerbated oxygen–glucose deprivation (OGD)-induced neuronal degeneration. *In vivo*, S100B administration in rat brains was sufficient to reproduce several features of injury-induced reactive gliosis.

Materials and methods

Materials

Cell culture reagents were obtained from HyClone, Logan, UT, USA and Invitrogen, Carlsbad, CA, USA. Antibodies were purchased from Millipore Corporation, Bedford, MA, USA [anti-RAGE; anti-actin; anti-MAP2 (microtubule associated protein-2)]; Santa Cruz Biotechnology, Santa Cruz, CA, USA (anti-NF-κB p65 subunit; anti-TLR4); from Sigma, St Louis, MO, USA [anti-S100B; anti-iNOS, anti-vimentin; anti-Bromodeoxiuridine (5-bromo-2′-deoxyuridine, BrdU)], and Dako (anti-Glial Fibrillary Acidic Protein, GFAP). Luciferase reporter plasmid PGL3-basic was purchased from Promega, Madison, WI, USA, green fluorescent protein (GFP) expression plasmid was from Clontech. S100B (Clontech Laboratories, Inc., Mountain View, CA, USA) purified from bovine brain, Bromodeoxyuridine (BrdU), PD98059, LY294002, sulphazalazine (SFZ), lysophosphatidic acid (LPA), 5-fluorouracyl (5-FU), bovine serum albumin free of glycosilated adducts, and other chemicals were obtained from Sigma. Secondary antibodies were from Jackson Immunoresearch. NSC23766 was purchased from Tocris. Nuclear factor kappa B (NF-κB) reporter plasmid was a gift of Dr Humberto Gutierrez (University of Cardiff, UK). Expression plasmids pEBGN and DN-pEBGN-Sp1 were obtained from Dr Gerard Thiel (University of the Saarland Medical Center, Homburg, Germany). wtRAGE and ΔcytoRAGE were kindly provided by Dr Henri Huttunen (University of Helsinki, Finland) and Dr Rosario Donato (University of Perugia, Italy). N17Rac1, wtRac1, N17Cdc42, wtCdc42, and wtRhoA were provided by Dr Gustavo Paratcha and Dr Fernanda Ledda (University of Buenos Aires). Microscopic images were taken in an Olympus IX-81 microscope equipped with a DP71 camera or Olympus FV-1000 confocal microscope (Olympus, Tokyo, Japan).

Astroglial cell culture

Brains from neonatal rat pups were removed; cortices were isolated and dissociated mechanically using scissors and Hank's Balanced Salt Solution (HBSS), followed by trituration using a glass serological pipette till tissue was not longer visible. After decanting tissue, 1 mL of supernatant was transferred to a new centrifuge tube. Extraction was repeated twice. The supernatant was centrifuged and the resulting pellet was washed in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/mL penicillin/streptomycin, and platted in 10 cm diameter plates. After 1 h incubation at 37°C and CO_2 5% to allow the binding of microglia, supernatant containing astrocytes was transferred to poly-L-lysine coated bottles with fresh supplemented DMEM. When the cells reached confluence (8–10 days), they were subjected to shaking at 180 rpm for 24 h at 37°C to detach microglia and oligodendrocytes. Then, cells were washed with pre-warmed complete DMEM, incubated for additional 24 h, trypsinized, and re-seeded for the experimental procedures in either 12 or 24 wells plates that were maintained in 5% CO_2 at 37°C in supplemented DMEM. Cultures obtained with this procedure showed more than 95% astrocytes with positive GFAP staining. Cell culture fixation and immunocytochemistry was done as previously described (Villarreal *et al.* 2011). In cell division studies, BrdU 600 ng/mL was added to the tissue culture medium and cells were fixed 24 h later. S100B purified from bovine brain (Sigma) used for the treatments was dissolved in phosphate-buffered saline to 1 mg/mL, sterile filtered and stored in aliquots at −80°C until use. This value is equivalent to 50 μM of S100B dimmer. Astrocytes were exposed to S100B in serum-free Optimem (Invitrogen, Carlsbad, CA, USA). For loss of function studies, anti-RAGE neutralizing antibody or control immunoglobulin was added to the culture at a concentration of 1.5 μg/mL 1 h before initiating S100B treatment and maintained during 24 h.

Wound healing assay

Astrocytes were cultured until confluence (7–10 days) as stated above. A scratch was made with a sterile micropipette tip to create a cell-free area. Then, medium was changed and different S100B concentrations were added in the presence or absence of anti-RAGE neutralizing antibodies; the chemical blocker of Rac1 NSC 23766 (50 μM) or RhoA activator LPA (1 μM). After 24 or 48 h, astrocytes were fixed and stained with glial markers (anti-GFAP, anti-vimentin, anti-S100B) and Hoechst nuclear counter-staining. Images were captured at different time points (0; 24; 48 h) with the Olympus IX-81 microscope. In a different set of experiments, astrocytes were transfected with one of the expression plasmids (RAGE, RAGEΔϖ cyto, N17-Rac1 (DN-Rac), wtRac, N17-Cdc42 (DN-Cdc42, wtRhoA) and GFP plasmid as transfection control. Typically 1 μg of DNA (0.2 μg GFP plasmid plus 0.8 μg tested plasmid) and 2 μL of Lipofectamine 2000 (Invitrogen) in 100 μL Optimem per well (12 well plates) were used and leaved in contact with astrocytes overnight. The ratio GFP/plasmid was used to ensure the GFP expression in the cells that successfully incorporated the tested plasmid as reported (Alsina *et al.* 2012). Twenty-four hours later, the scratch was performed and the protocol was followed as described above. Cell migration was determined by counting the number of astrocytes invading the scratch with the cell counter plug-in for the NIH ImageJ software (Bethesda, MD, USA). The length of the scratch was used to normalize the values. A total of 10-17 areas along the scratch were randomly selected in each well and the cells were quantified. Experiments were repeated three times with similar results. In another set of experiments, the relative contribution of cell division was determined by adding mitosis inhibitor 5-fluorouracyl (5-FU) to the medium (50 μg/mL).

Primary cortical neurons

The cortical neuronal cultures were prepared from embryonic day (E) 16 Wistar rats according to Goslin *et al.* (1988) with minor modifications described in Villarreal *et al.* (2011). OGD exposure was performed after 7 days *in vitro* (7DIV) by extracting the culture medium and replacing it with glucose and serum-free Neurobasal previously saturated for 20 min with 0.1% O_2, 5% CO_2 and balance N_2. Then, OGD neurons were incubated for 30 min in the hypoxic chamber in the presence of a gas mixture composed of 0.1% O_2, 5% CO_2 and balance N_2. Then, medium was removed and replaced by Neurobasal plus B27. Neurons were incubated during additional 24 h in presence of 50% conditioned medium collected from S100B-exposed astrocytes.

Primers and PCR conditions

RT-PCR was performed as previously described in Ramos *et al.* (2007) using specific primers for TLR2, IL-1β, and actin. Detailed PCR protocols are available from the authors under request. PCR products were run in a 1.5% agarose gel and photographed in a Bio-Rad (Hercules, CA, USA) VersaDoc 4000 imaging system. Each reverse transcriptase-PCR (RT-PCR) experiment was run with negative control reactions that consistently failed to generate a PCR product (data not shown).

Reporter constructs and transcriptional assay

Fragments of the proximal RAGE promoter containing the highly conserved sequences among species (mouse-rat-human) were amplified from human genomic DNA by PCR and cloned into Zero Blunt TOPO system (Invitrogen) and re-cloned into the reporter plasmid pGL3 Basic Luciferase (Promega) where the Luc gene had been replaced by the GFP expression cassette from a pEGFP-N1 plasmid (Clontech). The detailed cloning protocols as well as PCR conditions are available from authors upon request. All sequences were verified by sequencing in the Genome Quebec facilities (McGill University, Montreal, Canada). Astrocytes were transfected with the correspondent reporter plasmid in which the GFP gene was controlled by the cloned fragment of the RAGE promoter. Twenty-four hours later transfected cells were washed; medium was replaced by fresh Optimem with different concentrations of S100B for 24 h. The cells were analyzed for GFP fluorescence immediately after finishing the S100B exposure. The activity of the empty pGL3 reporter and the protein content were used to normalize the GFP intensity obtained with the Optimas 6.2 image analyzer (Media Cybernetics, Bothell, WA, USA). Transfection efficiency controls were routinely performed by cotransfecting a plasmid encoding human glutathione-S-transferase with a nuclear localization signal and counting positive cells per microscopic field.

NF-κB activity reporter assay

Astrocytes were obtained as detailed above and seeded to a density of 150 000 cells per well in 24-well plates. Once astrocytes reached 50% confluence, astrocytes were transfected with the NF-κB reporter plasmid that encodes for the GFP gene downstream of a promoter that has six consensus sites for NF-κB. Twenty hours after transfection, medium was replaced by fresh Optimem added with different S100B concentrations and cells were fixed 24 h later to evaluate the intensity of GFP expression with the Optimas 6.2 image analyzer. A similar transfection protocol was used for dominant negative Sp1 plasmid in loss of function studies.

Immunoblotting

Cultured astrocytes were homogenized in NP-40 lysis buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 10% glycerol], with protease inhibitors cocktail (Sigma), phenylmethylsulfonyl fluoride 1 mM and sodium vanadate 10 mM. Samples with equivalent protein content were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred as described previously (Ramos *et al.* 2007; Villarreal *et al.* 2011). For the separation of triton insoluble (F-actin) and triton-soluble (G-actin) fractions, the protocol described by Baorto *et al.* (1992) was performed followed by the immunoblot detection with monoclonal anti-actin antibodies.

Target	Forward	Reverse
TLR2	GAAAGATGCGCTTCCTGAAC	CGCCTAAGAGCAGGATCAAC
IL-1β	ACAACAAAAAAGCCTCGTGCTG	CCATTGAGGTGGAGAGCTTTCA
actin	CACCACTTTCTACAATGAGC	CGGTCAG0GATCTTCATGAGG

S100B infusion in brain cortex

Adult (250–300 g, 3 months old) male Wistar rats obtained from the Animal Facility of the Pharmacy and Biochemistry School, University of Buenos Aires, were used in this study. Animals were housed in a controlled environment (12/12-h light/dark cycle, controlled humidity and temperature, rat food, and water *ad libitum*. All surgical procedures were performed under anesthesia induced with ketamine-xylazine (90/10 mg/kg). Rats were placed in a stereotaxic apparatus, and a small surface of skull between the coronal suture and the bregma line was exposed. A 1-mm diameter hole was drilled on the skull. Subsequently, 1 μL of 50 μM S100B or bovine serum albumin (BSA) (control protein) was injected into the brain cortex 0.4 mm below the pial surface at a ratio of 0.25 μL/min. After 3 days of recovery time, animals were deeply anaesthetized, fixed by perfusion and brain sections were subjected to the immunohistochemical procedure as described previously (Aviles-Reyes *et al.* 2010). The animal care for this experimental protocol was in accordance with the NIH guidelines for the Care and Use of Laboratory Animals, the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience, the ARRIVE guidelines and it was approved by the CICUAL committee of the School of Medicine, University of Buenos Aires.

Quantitative studies and statistical analysis

Changes in astroglial cell morphology and survival were evaluated by using the NIH ImageJ software on cells observed with phase contrast or immunostained as stated in each figure legend. Each experiment was repeated at least three times. Representative experiments and photographs or gels are presented in the figures. The data were subjected to one-way analysis of variance (ANOVA) and Student–Newman–Keuls post-test unless stated otherwise in the figure legend. The statistical software package used was GraphPad Prism 3.0, (San Diego, CA, USA) and statistical significance was assumed when $p < 0.05$.

Results

S100B exposure modifies astroglial cell morphology in a RAGE-dependent manner

Rat brain astrocytes in culture typically exhibit two distinct phenotypes: a flattened, polygonal morphology, and a filamentous stellated morphology (Chen *et al.* 2005). In the first set of experiments, we determined that both stages, as well as astrocytes with intermediate phenotypes, express RAGE in cytoplasm and projections (Fig. 1a). Since astrocytic density in culture profoundly affects their morphology, we confirmed RAGE expression in higher density culture (Fig. 1b). Having established that these cells express RAGE, we asked if extracellular S100B was able to modify glial cell morphology. For this, we exposed rat brain astrocytes to increasing doses of S100B up to 1 μM for 24 h and we found that the predominant phenotype after S100B treatment was the stellated one (Fig. 2a). Phalloidin staining showed that polygonal astrocytes bearing actin stress fibers were reduced (Fig. 2a) and the pool of triton-soluble actin was increased by S100B exposure (Fig. 2b). Quantitative studies confirmed that the number of polygonal astrocytes was significantly reduced after S100B treatment, while the stellated astrocytes were increased (Fig. 2c). To confirm that S100B-induced stellation was RAGE dependent, we transfected cultured astrocytes with a plasmid expressing a dominant negative RAGE lacking the cytoplasmic domain (RAGEΔcyto, DN-RAGE). The over-expression of RAGEΔcyto reduced the 50 nM S100B-induced stellation, while the wtRAGE over-expression failed to significantly promote it probably indicating a saturation effect (Fig. 2d). The over-expression of RAGEwt or RAGEΔcyto did not significantly alter astroglial stellation induced by 1 μM S100B (Fig. 2d) which may be indicative of a significant effect of endogenous RAGE at that ligand level. To elucidate this, we studied the effect of S100B exposure on stellated astrocytes by blocking endogenous RAGE with neutralizing antibodies. S100B exposure presented a significant effect of increasing the number of secondary branching points in stellated astrocytes (Fig. 2e), while the number of primary projections remained unaffected (data not shown). Figure 2e also shows that RAGE blockage abolished the S100B effect on stellated astrocytes.

Actin breakdown is necessary for astroglial stellation (Baorto *et al.* 1992) and RhoA is a negative regulator of that process (Höltje *et al.* 2005), in the next experiment we asked whether the small RhoGTPases were involved in the S100B/RAGE-induced stellation. Figure 2f shows that the transfection with dominant negative Rac1 (N17-Rac1) or dominant negative Cdc42 (N17-Cdc42) essentially abolished 50 nM and 1 μM S100B-induced stellation; while wtRac1 but not wtRhoA over-expression, potentiated the 1 μM S100B-induced stellation (Fig. 2f). We conclude that S100B induces the astrocytic stellation and increases the complexity of glial cell projections in a RAGE and Rac1/Cdc42 dependent manner.

S100B facilitates astroglial cell migration toward an injured site

Studies in glioma cell lines suggested that, after interaction with its prototypic ligand High-mobility Group Box 1 (HMGB-1), RAGE is able to regulate cell migration by activating Rac1 and Cdc-42 signaling (Taguchi *et al.* 2000; Bassi *et al.* 2008; Hudson *et al.* 2008). These facts, together with our previous findings showing the Rac1 and Cdc42 dependence of S100B/RAGE effects on astrocytic cytoskeleton, led us to ask whether extracellular S100B enhances astrocytes motility. Using scratch wound healing assays, we observed that astrocytes slowly invade the wound (Fig. 3a–c and Figure S1). The initial invasion-related event was the astroglial polarization and the subsequent protrusion of long processes that invaded the wound space (Fig. 3a and b). These processes exhibit a flattened polygonal shape in their distal end, similar to filopodia (Fig. 3a). At later time points, pale GFAP+ cells appeared in the wounded space (Fig. 3c). The invasion of the wound was predominantly leaded by a

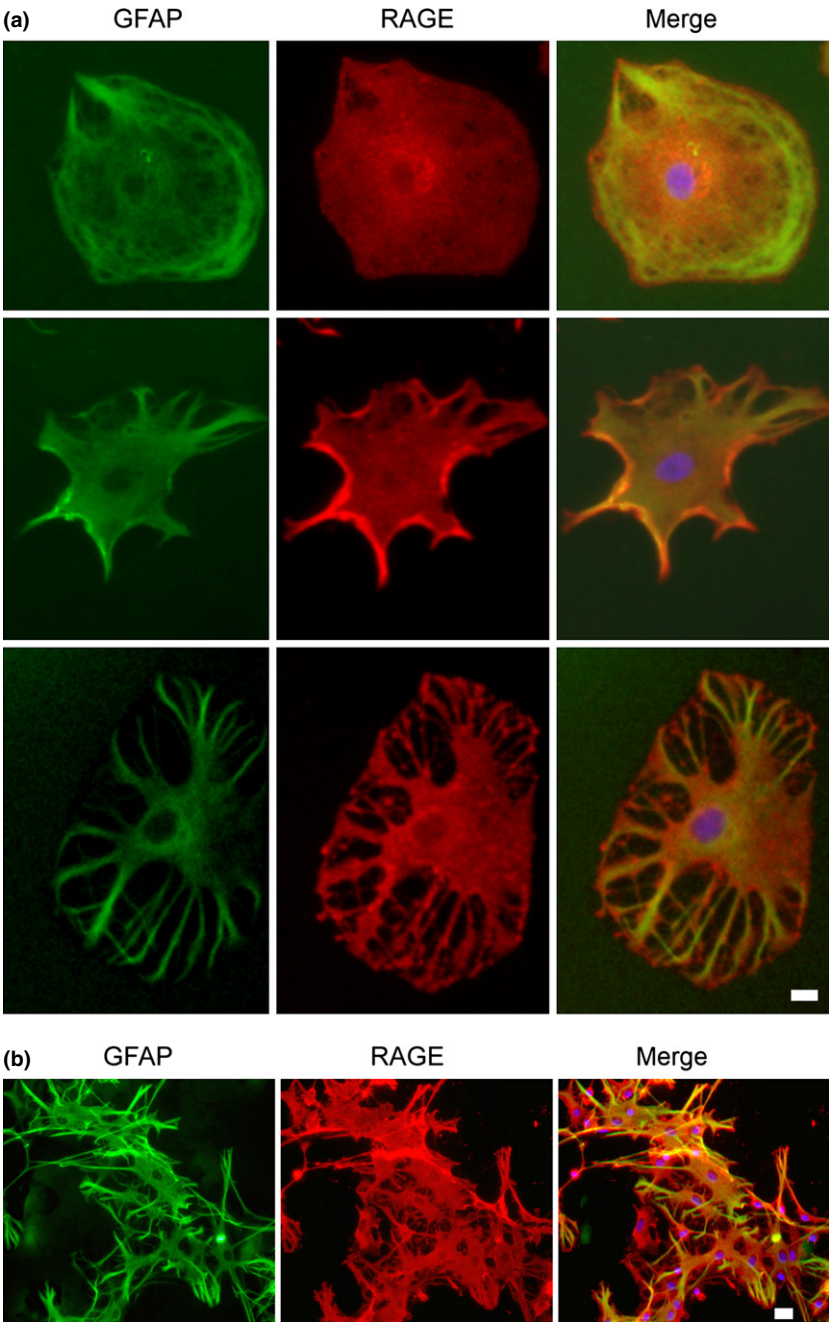
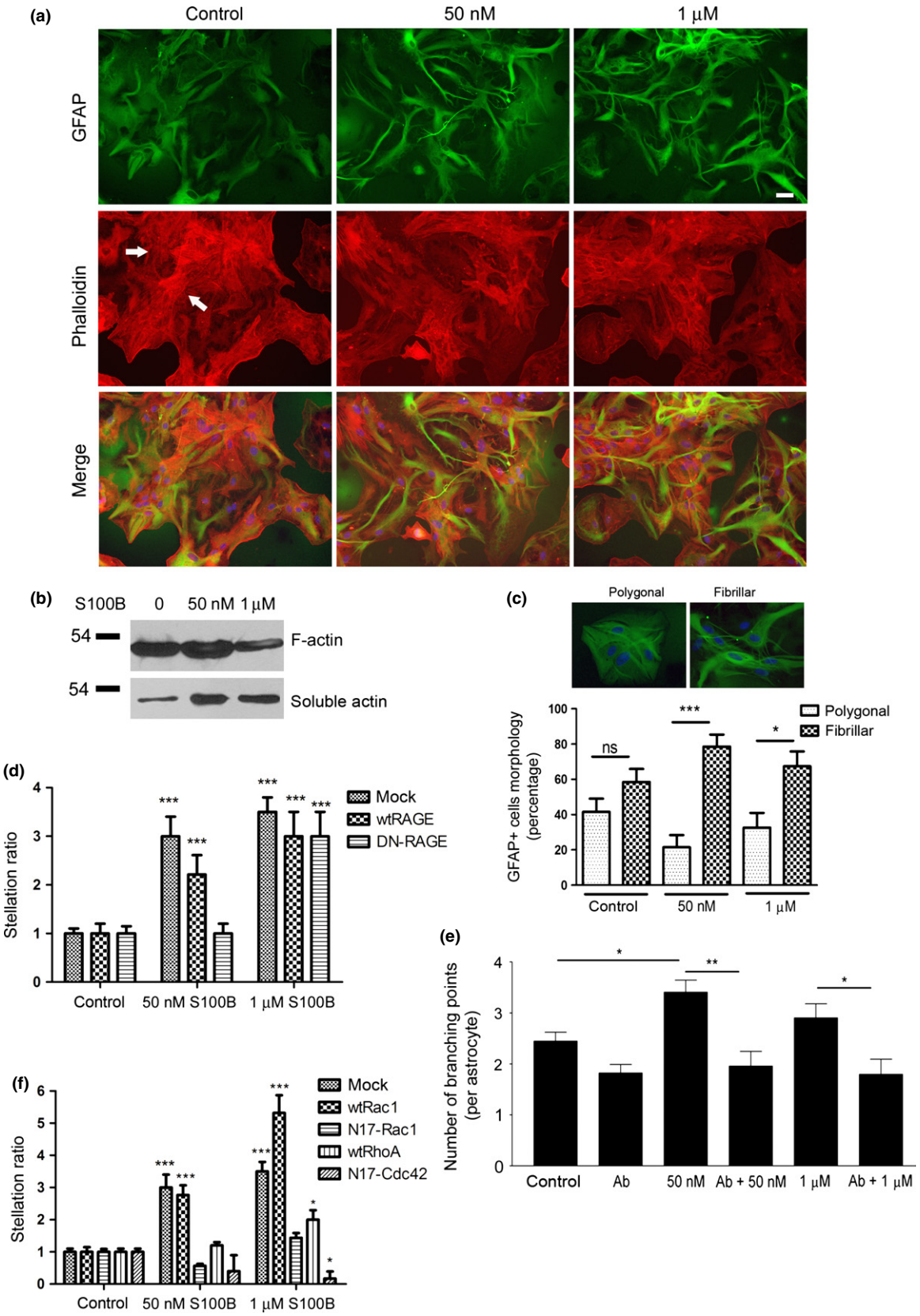


Fig. 1 Rat cortical astrocytes express RAGE. (a) Astroglial different phenotypes showed RAGE expression, bar = 6 μm. (b) Higher density culture with multiple cell-to-cell contacts did not significantly change RAGE, bar = 20 μm.

Fig. 2 S100B induces astrocytic stellation. (a) Representative images of cultured astrocytes exposed to different levels of S100B during 24 h. Arrows show the actin stress fibers. (b) Triton-soluble and insoluble (F) actin pools analyzed by immunoblots. (c) Quantification of filamentous and polygonal astrocytes in Glial Fibrillary Acidic Protein (GFAP)-stained cultures exposed to S100B. (d) Quantitative study showing the stellation ratio of cultured astrocytes transfected with wtRAGE or the dominant negative RAGEΔcyto and exposed to S100B. (e) Quantification of the number of branching points in filamentous (stellated) astrocytes exposed to S100B in presence or absence of RAGE-neutralizing antibodies. (f) Quantification of the stellation ratio of astrocytes transfected with the indicated constructs (wt or dominant negative Rho GTPases) and exposed to S100B as indicated. Results are expressed as mean ± SEM of a representative experiment repeated three times with similar results (*$p < 0.05$, **$p < 0.01$; ***$p < 0.001$ after ANOVA and Student–Newman–Keuls post-test).


(a)
Control
50 nM
1 μM
GFAP
Phalloidin
Merge
(b)
S100B 0 50 nM 1 μM
54
F-actin
54
Soluble actin
(c)
Polygonal
Fibrillar
GFAP+ cells morphology (percentage)
ns
***
*
Polygonal
Fibrillar
Control
50 nM
1 μM
(d)
Stellation ratio
Mock
wtRAGE
DN-RAGE
Control
50 nM S100B
1 μM S100B
(e)
Number of branching points (per astrocyte)
Control
Ab
50 nM
Ab + 50 nM
1 μM
Ab + 1 μM
(f)
Stellation ratio
Mock
wtRac1
N17-Rac1
wtRhoA
N17-Cdc42
Control
50 nM S100B
1 μM S100B

population of vimentin-immunoreactive astrocytes with low GFAP expression (Figure S1) that showed increased BrdU incorporation (data not shown). Low and high concentrations of S100B (50 nM and 1 μM, respectively) facilitated the astrocytic invasion of the wound, and in each case, the enhanced migration was blocked by RAGE neutralizing antibodies (Fig. 3d). Mitosis blockage by 5-FU treatment partially prevented the S100B effect on cell migration (Fig. 3e). Since it has been extensively described that astrocytes require Rac1 and Cdc42 for the initial event of polarization and subsequent migration into the scratch, we then tested if facilitated migration by S100B/RAGE requires Rac1 or Cdc42. As shown in Fig. 3f, astrocytes transfected with the dominant negative N17-Rac1, N17-Cdc42 or wtRhoA showed reduced migration into the scratch. A similar effect was observed with the pharmacological blockage of Rac1 or the RhoA activator LPA (Figure S2). We conclude that S100B facilitates the astroglial invasion of an artificial wound and that effect requires a RAGE/Rac1-Cdc42 dependent pathway.

S100B exposure promotes astroglial mitosis

Earlier studies have reported that extracellular S100B promotes glial cell proliferation (Selinfreund *et al.* 1991). To explore this in more depth, we first asked if the effects of S100B were dose- and RAGE- dependent. Interestingly, nanomolar and low micromolar S100B increased cell number, whereas 10 μM S100B actually reduced the astrocytic cell number, suggesting a toxic effect at this high dose (Fig. 4a). To confirm that the increase in cell number reflected increased cell division, we evaluated the effects of S100B on BrdU incorporation. Figure 4b shows that S100B does promote BrdU incorporation and that this effect was blocked by RAGE neutralizing antibodies. Thus we conclude that S100B increases the astrocytic cell division in a RAGE-dependent manner.

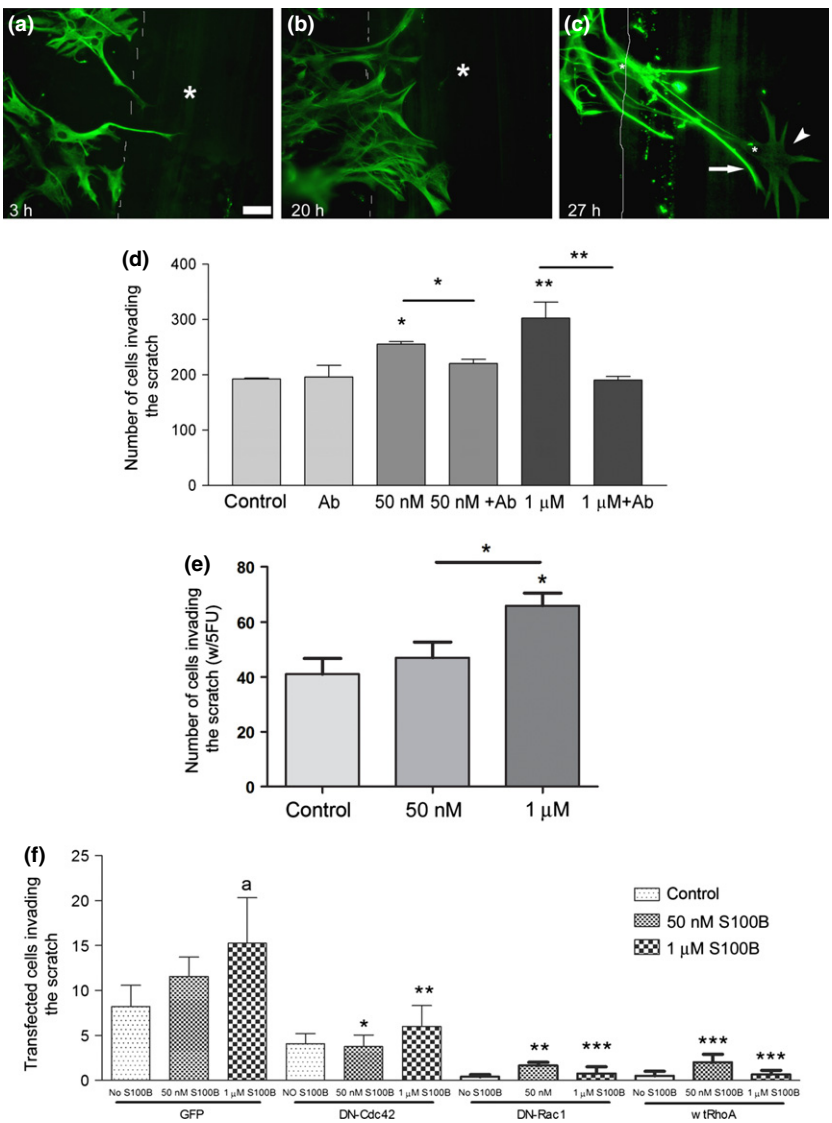


Fig. 3 S100B promotes astroglial invasion to an artificial wound *in vitro*. (a, b, c) Different representative images of astrocytic projections invading the artificial wound performed on low-density astrocytic monolayers wounded with scratches, fixed at the indicated times, and stained with Glial Fibrillary Acidic Protein (GFAP); white dotted line indicates the scratch limit, scratch is identified by the asterisk; arrow head shows an astroglial soma, while arrow shows the glial projection from astrocytes located in the periphery of the wound that are invading the scratch. Bar = 10 μm. (d) Quantitative studies in a similar experiment but using confluent astrocytic monolayers scratch wounded and incubated with S100B in presence of RAGE neutralizing antibodies or control IgG, fixed 24 h later and double stained with GFAP/Hoechst. The analyzed length of the wound was fixed to 2000 μm. (e) A similar experiment using 5-fluorouracyl (5-FU) to block mitosis. (f) Astrocytes were transfected with DN-Rac1 (N17-Rac1), DN-Cdc42 (N17-Cdc42) or wtRhoA, 20 h later the scratch was performed and the number of transfected cells invading the scratch was counted. Values are represented as mean ± SEM and statistical significance of the effects was confirmed using ANOVA and Student-Newman-Keulspost-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. their respective S100B dosis in green fluorescent protein (GFP)-transfected astrocytes; 'a' indicates $p < 0.05$ vs. the no-S100B GFP-transfected cells).

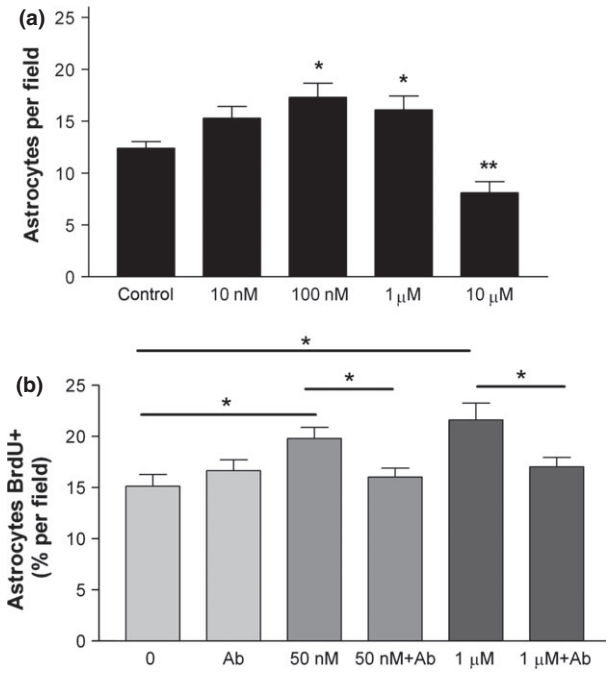


Fig. 4 S100B effects in astrocytic proliferation. (a) Quantitative analysis of cell abundance after 24 h of exposure to the indicated S100B concentrations; values represent the mean ± SEM of cells per 20X microscopic field. (b) Quantitative analysis of 5-bromo-2′-deoxyuridine (BrdU) incorporation after 24 h of incubation with S100B in presence or absence of RAGE-neutralizing antibodies. Values are represented as mean ± SEM (percentage of Glial Fibrillary Acidic Protein (GFAP)+/BrdU+ cells per field) and statistical significance of the effects was confirmed with ANOVA and Student–Newman–Keuls post-test (*$p < 0.05$; **$p < 0.01$).

S100B exposure promotes NF-κB nuclear localization and NF-κB-dependent transcriptional activity

It has been shown that ligand binding to RAGE induces NF-κB activation in different cell types (Liu *et al.* 2005; Rong *et al.* 2005; Villarreal *et al.* 2011). In the next set of experiments, we asked if S100B induces NF-κB activation in cultured astrocytes and we attempted to characterize the effect. For this we examined the nuclear localization of p65 NF-κB subunit in astrocytes exposed to different levels of S100B. Figure 5a shows that 1 μM S100B exposure induced the rapid translocation of the p65 NF-κB subunit to the cell nucleus and this effect is partially blocked by RAGE neutralizing antibodies (Fig. 5a and b). To confirm if the increased nuclear localization of p65 NF-κB subunit correlates with the transcriptional activity, astrocytes were transfected with a reporter plasmid encoding GFP under the control of NF-κB promoter and exposed to three different doses of S100B (50 nM, 100 nM, and 1 μM). Figure 5c and d indeed show a dose-dependent stimulation by S100B. In addition, reporter studies have demonstrated that 50 nM S100B activates NF-κB transcriptional activity, an effect that was not detected by nuclear p65 translocation. This is expected because of the higher sensitivity of the NF-κB reporter plasmid. We conclude that S100B activates NF-κB transcriptional activity in a RAGE- and dose-dependent manner.

S100B exposure improves astrocytic survival to oxidative stress

Reactive astrocytes are known to present improved survival to oxidative stress; we then asked if the S100B exposure increases astrocytic survival to oxidative stress induced by H_2O_2. For this, astrocytes were incubated with 50 nM or 1 μM of S100B for 16 h in serum-free medium, and then exposed to 200 μM H_2O_2 for 2 h. Figure 6a shows that S100B partially protected astrocytes from oxidative stress-induced death. In the next experiments, we explored the signaling pathways involved in this S100B-induced protection. For that purpose, S100B-mediated protection was evaluated in the presence of Erk (PD98059), Akt (LY294002) or NF-κB (BAY 11-7082) chemical blockers. As shown in Figure 6b, Erk inhibition with PD98059 abolished 50 nM and 1 μM S100B-induced protection, while Akt inhibition abolished 50 nM, but not 1 μM S100B-induced protection to oxidative stress (Fig. 6c). Surprisingly, NF-κB pharmacological blockage did not abolish S100B protective effects (Fig. 6d). We conclude that low or high S100B level confer protection to oxidative cellular stress by involving Erk while Akt is required for the 50 nM S100B protective role.

S100B exposure activates proximal RAGE promoter and induces RAGE over-expression in a NF-κB/Sp1-dependent manner

nuclear factor kappa B (NF-κB), Hypoxia-Induced Factor 1 alpha (HIF-1α), and Sp1 transcription factors are able to regulate RAGE expression by acting on different recognition sites along the RAGE promoter (Pichiule *et al.* 2007). It has been also shown that S100B induces Sp1 and NF-κB binding to DNA probes (Liu *et al.* 2005). We next asked if S100B is able to increase RAGE expression, a mechanism that may drive a feed-forward loop to potentiate S100B effects. We observed that S100B exposure increased RAGE expression (Fig. 7a, lanes 1-2-3). Then, we asked if the proximal regions of RAGE promoter were sensitive to S100B exposure. We identified conserved sites in the RAGE promoter among close related mammalian species (rat-mouse-human) and two highly conserved areas were amplified and cloned into GFP-reporter plasmids (Fig. 7b). Plasmid pRAGE-GFP (+14/−501) contained both conserved regions while plasmid pRAGE-GFP (+14/−255) contained only the proximal conserved area (Fig. 7b). Only 1 μM S100B dose significantly increased the transcriptional activity in both RAGE promoter constructs (Fig. 7c). Sp1 and NF-κB consensus sites are present in the RAGE proximal promoter, however NF-κB site is located solely in the pRAGE-GFP (+14/−501)

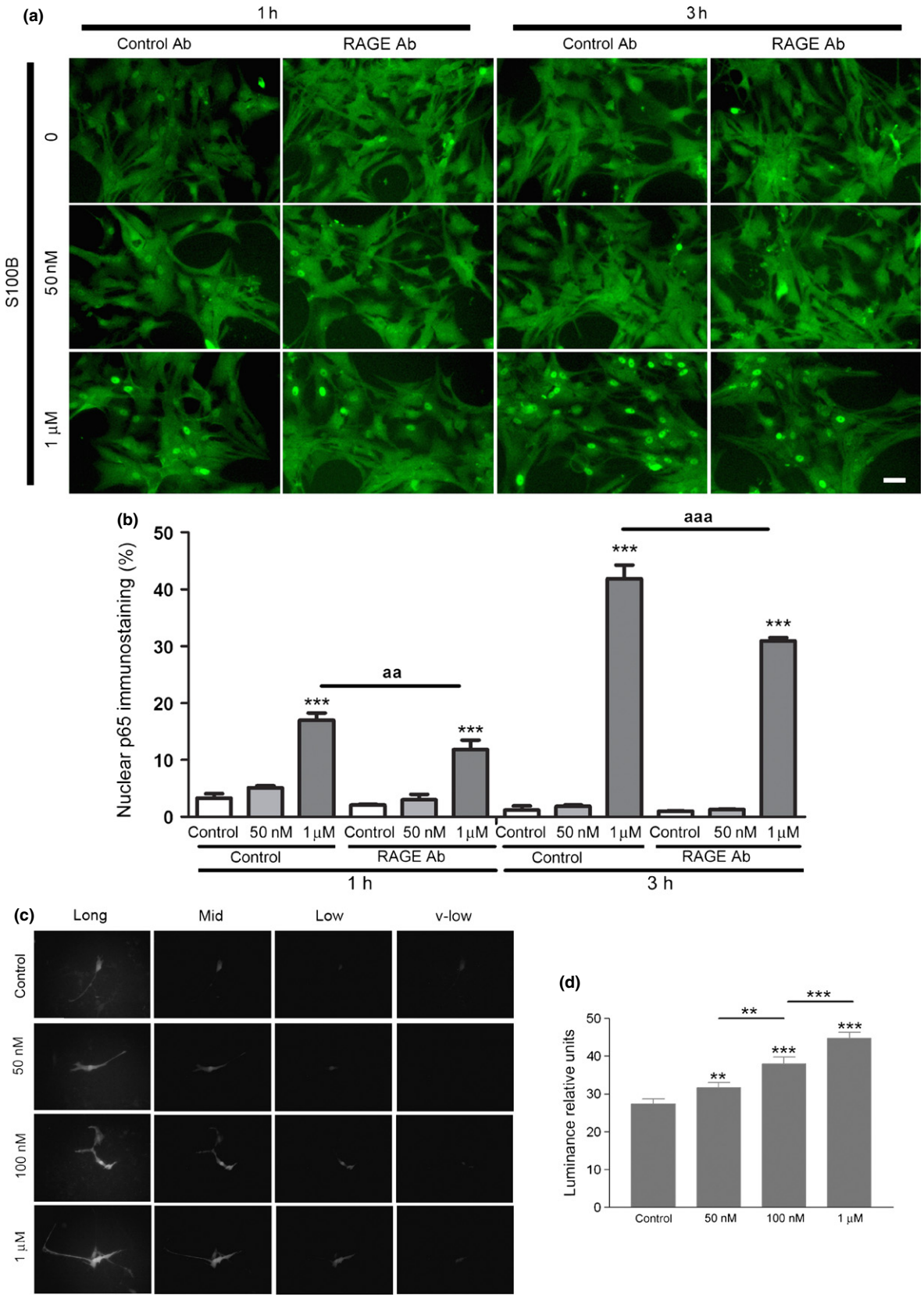

(a)
1 h
3 h
Control Ab
RAGE Ab
Control Ab
RAGE Ab
S100B
0
50 nM
1 μM
(b)
Nuclear p65 immunostaining (%)
50
40
30
20
10
0
aa
aaa
***
***
***
***
Control
50 nM
1 μM
Control
50 nM
1 μM
Control
50 nM
1 μM
Control
50 nM
1 μM
Control
RAGE Ab
Control
RAGE Ab
1 h
3 h
(c)
Long
Mid
Low
v-low
Control
50 nM
100 nM
1 μM
(d)
Luminance relative units
50
40
30
20
10
0
**
***
**
***
***
Control
50 nM
100 nM
1 μM

Fig. 5 Nuclear factor kappa B (NF-κB) transcriptional activity induced by S100B. (a) Nuclear localization of p65 NF-κB subunit induced by S100B exposure at different time points, bar = 25 μm. (b) Astrocytes showing nuclear p65 localization were quantified and results analyzed by ANOVA and Student–Newman–Keuls post-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. the No-S100B treatment; $^{aa}p < 0.05$ and $^{aaa}p < 0.01$ vs. the RAGE neutralizing antibody); after. (c) Astrocytes were transfected with a NF-κB- green fluorescent protein (GFP) reporter plasmid and 20 h later they were exposed to vehicle or different S100B concentrations for 24 h. Images show different exposition times of the photomicrographs to illustrate differences in the activity of the NF-κB reporter (long, mid, low, v-low means long, middle, low, and very low exposures, respectively). (d) Quantification of the NF-κB transcriptional activity induced by S100B expressed as luminance relative units of GFP emission analyzed with the Optimas 6.2 software analyzed by ANOVA and Student-Newman-Keuls post-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

while Sp1-rich areas are present in both constructs (Fig. 7b). When we blocked NF-κB activity using SFZ, the 100 nM S100B-induced RAGE expression was discretely reduced, but higher S100B concentration (1 μM) induced RAGE expression even in presence of SFZ (Fig. 7a, lanes 4-5-6). We also transfected astrocytes with a dominant negative form of Sp1 and Fig. 7d shows that Sp1 blockade partially abolished S100B-induced RAGE expression both at 100 nM and 1 μM. We conclude that S100B induces RAGE expression involving Sp1 and NF-κB.

S100B induces a pro-inflammatory phenotype in astrocytes

It was reported that S100B promotes the secretion of pro-inflammatory mediators (Hu and Van Eldik 1996, 1999; Hu *et al.* 1996, 1997; Ponath *et al.* 2007). To evaluate if astroglial reactivity induced by S100B/RAGE produces a conversion into the pro-inflammatory neurodegenerative profile, we analyzed the expression of TLR2, TLR4, IL-1β, and iNOS in S100B exposed astrocytes. As shown in Fig. 8a, TLR2 mRNA is increased after S100B exposure, while IL-1β dramatically increases following a dose-response profile. Immunoblot studies showed an increase in iNOS and a slight increase in TLR4 expression that did not reach statistical significance (Fig. 8b).

Having found that S100B exposure produces a reactive phenotype in cultured astrocytes we then asked if S100B-exposed astrocytes are able to induce neurodegeneration on stressed neurons. For that purpose, astrocytes were incubated with 50 nM or 1 μM S100B during 24 h, washed and then conditioned medium (CM) was collected during additional 24 h. CM was used to treat primary cortical neurons exposed to control conditions or 30 min to oxygen-glucose deprivation and lactate dehydrogenase levels were assessed. As shown in Fig. 8c, CM obtained from 1 μM but not 50 nM S100B-treated astrocytes significantly reduced cell survival in OGD-exposed neurons. The exposure to CM in control

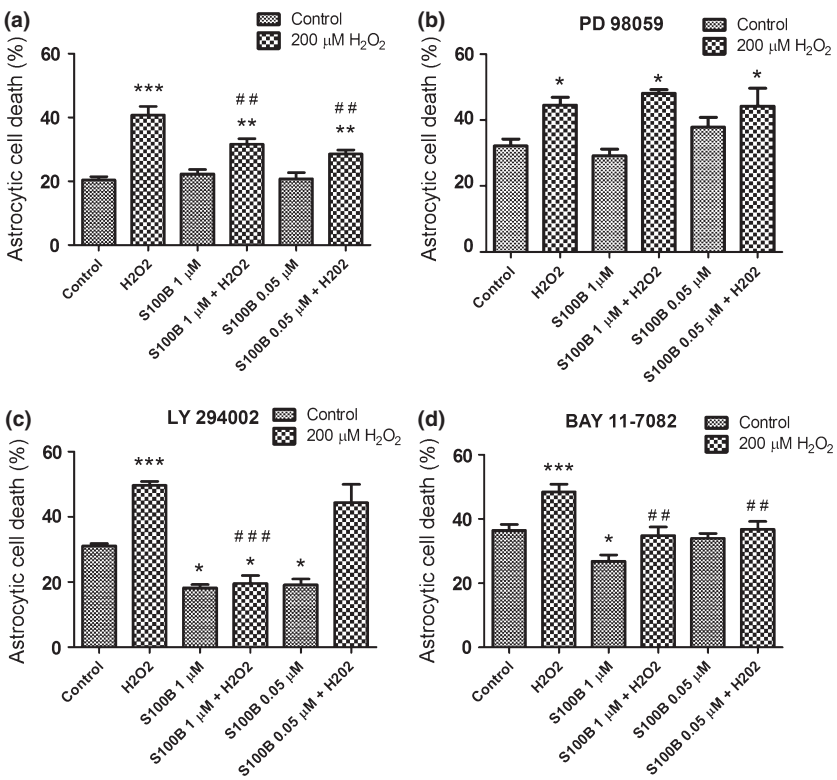


Fig. 6 S100B exposure improves astroglial cell survival to oxidative stress. Astroglial cell degeneration was assessed in cultured astrocytes subjected to 200 μM H_2O_2 exposure in serum-free medium. Astrocytes were pre-incubated with vehicle or S100B in absence (a) or presence of Erk (PD98059) (b), Akt (LY294002) (c) or nuclear factor kappa B (NF-κB) (Bay117082) (d) for 16 h and then exposed to 200 μM H_2O_2 for 2 h, fixed and morphological parameters of cell degeneration were evaluated (pyknotic nucleus, vacuolization, loss of projections). Results were expressed as mean ± SEM percentage of Glial Fibrillary Acidic Protein (GFAP)+ cells in three independent experiments (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. the control astrocytes; ##$p < 0.01$; ###$p < 0.001$ vs. the H_2O_2-treated astrocytes after ANOVA and Student–Newman–Keuls post-test).

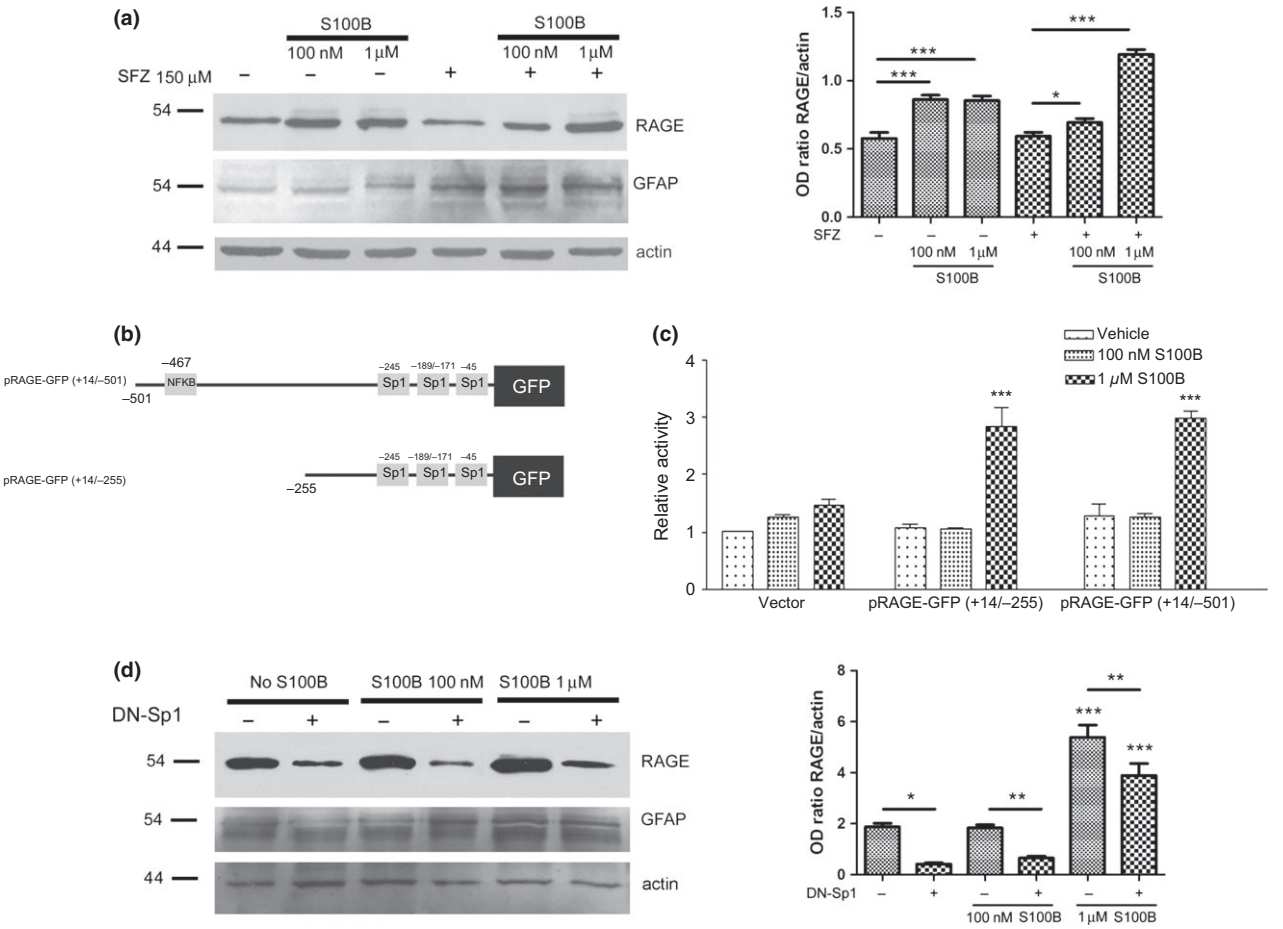


Fig. 7 RAGE promoter transcriptional activity is regulated by S100B. (a) RAGE expression after 24 h of exposure to S100B or to S100B plus 150 μM nuclear factor kappa B (NF-κB) inhibitor sulfazalazine and analyzed by immunoblot; actin was used as a loading control and OD ratios are shown as the intensity of the bands versus the intensity of loading control. (b) Schematic representation of the 5′ proximal RAGE promoter cloned areas with transcription factor consensus sites identified by boxes, numbers referring to the transcription initiation site. (c) Astrocytes were transfected with the indicated RAGE- green fluorescent protein (GFP) reporter construct and GFP expression was quantified by fluorescence intensity. Twenty-four hours after transfection, astrocytes were exposed to 100 nM or 1 μM S100B. Promoter activity was expressed as relative activity of the control (empty) vector as mean ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. vehicle after ANOVA and Student–Newman–Keuls post-test). (d) Astrocytes were transfected with a DN-Sp1 or the empty vector, and after 20 h exposed to S100B for 24 h to evaluate RAGE expression; actin was used as loading control and OD ratios are shown as the intensity of the bands versus the intensity of loading control.

neurons failed to produce significant effects on neuronal survival (data not shown).

In vivo infusion of S100B in rat brain induces reactive gliosis

Our previous results showed that S100B exposure induces a reactive phenotype in cultured astrocytes; we then asked whether this effect is actually possible *in vivo*. For that purpose, 1 μL of 50 μM S100B or BSA as control protein were intracortically infused in the pre-frontal cortex of naïve animals (not exposed to injury) and the astroglial morphology and microglial infiltrate were studied. By 3 days after S100B or BSA infusion, reactive astrocytes were observed surrounding the injection site (Fig. 9a). Astrocytes from the S100B-injected brains showed a larger increase in the soma size and projections length, as well as a more prominent GFAP expression (Fig. 9a and b). Increased number of vimentin-immunoreactive cells was observed in the cortex surrounding injection site and corpus callosum located below the S100B injection site (data not shown). A massive infiltrate of tomato lectin-positive cells was present in the injection site both in BSA or S100B injected hemispheres (Fig. 9a). RAGE expression was detected in glial cells present in the injected cortex and corpus callosum both in BSA and S100B infused brains (Fig. 9c). We conclude that exposure to high S100B level is able to induce morphological features of reactive astrocytes *in vivo*.

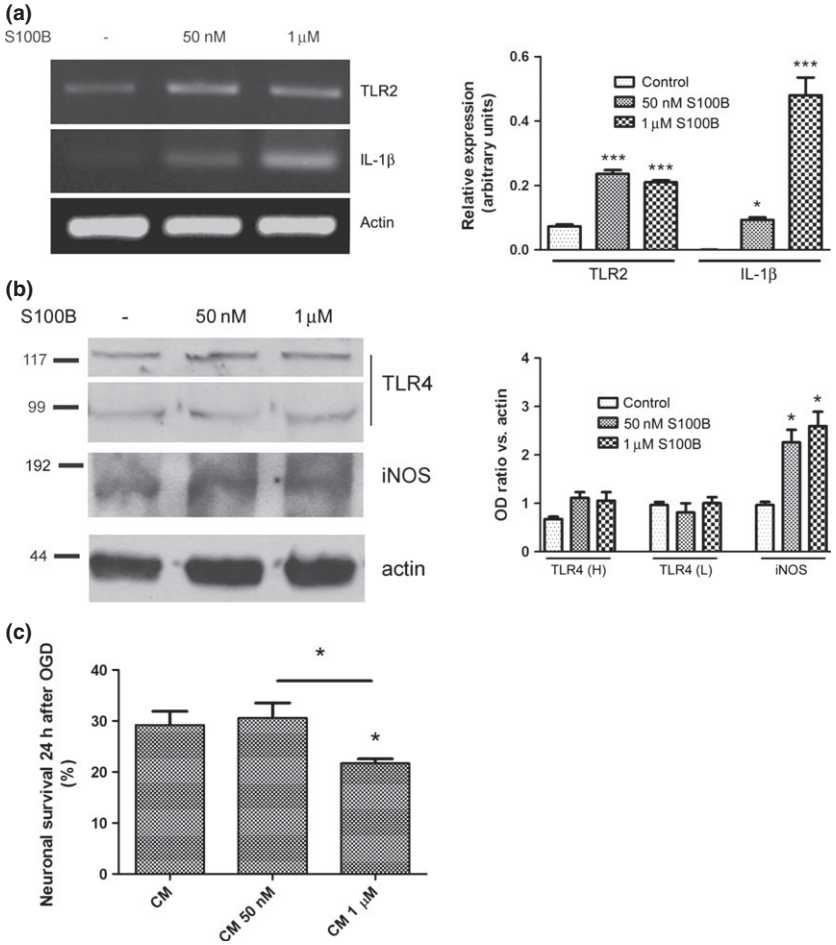


Fig. 8 S100B induces the astroglial polarization to the pro-inflammatory neurodegenerative profile. (a) RT-PCR showing Toll-like receptor 2 (TLR 2) and interleukin 1-beta (IL-1β) expression in astrocytes exposed to the indicated S100B level for 24 h, actin was used as control mRNA and densitometry are shown as the ratio of the target gene versus actin. (b) Immunoblot showing the TLR4 and inducible nitric oxide synthase (iNOS) expression in a similar experiment to that showed in A. TLR4 molecular weights of 120KDa and 95KDa correspond to glycosylated and native protein, respectively. Actin was used as a loading control protein. (c) Primary cortical neurons (7DIV) were exposed to oxygen–glucose deprivation (OGD) for 30 min or control conditions as indicated in methods and then to conditioned medium (CM) collected from astrocytes exposed to S100B concentrations as indicated. Neurons were immunostained with MAP-2, survival was evaluated as the MAP-2 immunoreactive area and referred to 100% (control neurons exposed to CM from astrocytes that did not received S100B). Values are represented as mean ± SEM and statistical significance of the effects was confirmed with ANOVA and Student–Newman–Keuls post-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Discussion

S100B in low level is constitutively secreted from glial cells to the extracellular space where it seems to have a beneficial role facilitating neuronal survival. However, its secretion is largely increased by brain injury (Rothoerl *et al.* 2000; Petzold *et al.* 2003; Mori *et al.* 2008).

RAGE is expressed in cultures of murine astrocytes (Ponath *et al.* 2007) and we here showed that rat cortical astrocytes also express RAGE in cytoplasm and cell membrane. In addition, the different phenotypes (polygonal and fibrillar) present in astrocytic culture express detectable levels of endogenous RAGE.

While *in vivo*, astroglial hypertrophy is a hallmark of reactive gliosis and is evidenced by increased large and complexity of glial cell projections with intermediate filaments over-expression (mainly GFAP), *in vitro* astroglial stellation is usually paralleled to reactive gliosis (Burgos *et al.* 2007; Scarisbrick *et al.* 2012). Similarly to that reported by Hu and Van Eldik (1999), we observed that S100B exposure induces several alterations in astrocytes toward a reactive stellate phenotype. Here, we extend this observation showing that S100B-induced stellation: (i) is RAGE dependent, (ii) requires downstream small Rho GTPases family members Rac1 and Cdc-42 but not RhoA, and (iii) occurs both at nanomolar and micromolar S100B.

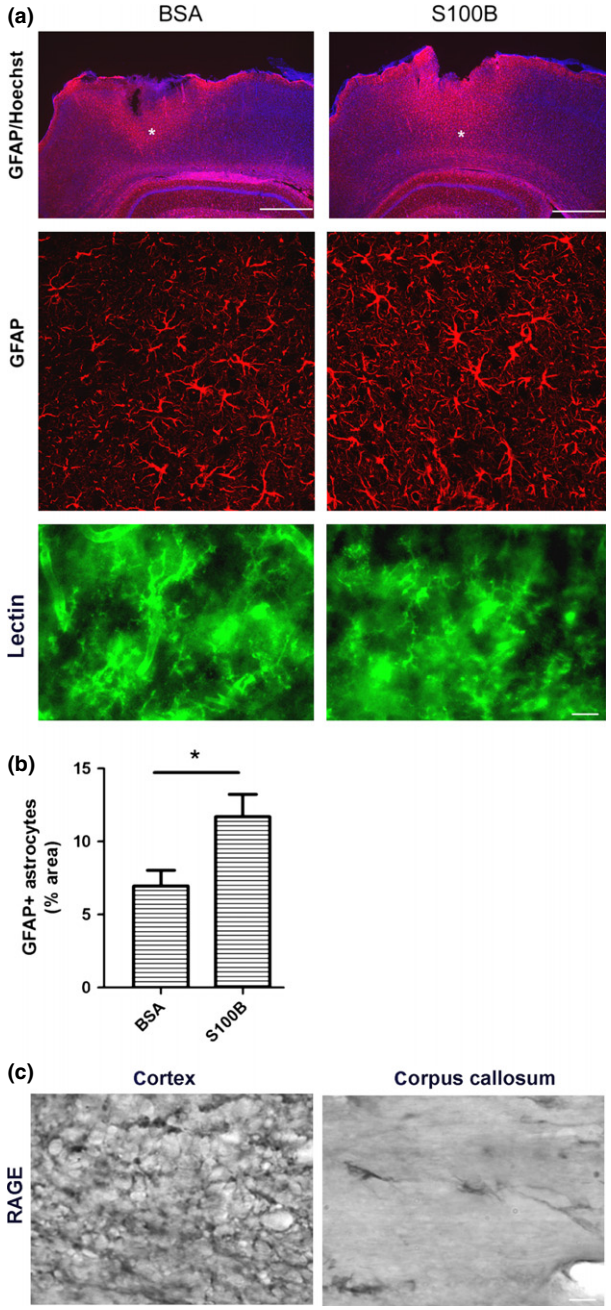


Fig. 9 S100B intracortical infusion in rat brain. (a) Images show the Glial Fibrillary Acidic Protein (GFAP) and tomato lectin staining 3 days after the single S100B or bovine serum albumin (BSA) infusion in the prefrontal cortex. The area used for high magnification images is marked with an asterisk (*). Bar = 1000 μm (upper panel); 30 μm (middle panel); 12 μm (lower panel). (b) Quantitative analysis of the GFAP-immunoreactive area shown as percentage of the field. (c) Images of RAGE immunoreactive glial cells in the cortex surrounding injection site and corpus callosum below the injection site. Bar = 24 μm.

The wtRAGE over-expression did not facilitate S100B induced stellation probably because of a limitation in the downstream signaling. This fact is supported by the increased S100B-induced stellation when we over-express wtRac1. Interestingly, astroglial stellation induced by IL-1β, C3 protein derived from *Clostridium botulinum* ($C3^{bot}$) or cAMP occurs by RhoA inactivation (John *et al.* 2004; Höltje *et al.* 2005; Perez *et al.* 2005) and it has been demonstrated that cytoskeletal glial protrusion depends on Rac1 and Cdc42 (Höltje *et al.* 2005). S100B/RAGE seems to share similar intracellular pathways to induce stellation in astrocytes and the small Rho GTPases appears to be the limiting step for cytoskeletal reorganization in glial cells.

Our experiments also showed that S100B is able to promote the initial steps of astroglial invasion to the artificial wound created by the scratch wound healing assay, an effect that was RAGE dependent and also resulted Rac1 and Cdc42 dependent. Mitosis blockage reduced astroglial invasion of the wound but was unable to fully prevent the S100B effect. Having in mind that the initial event for astroglial invasion of the wound is a Rac1/Cdc42-dependent polarization followed by the extension of large protrusions into the scratch (Etienne-Manneville 2008) and that RhoA is a negative regulator of astroglial migration (Höltje *et al.* 2005), it is not surprising that our data suggest that S100B-facilitated glial invasion requires the same pathway as S100B-induced stellation (RAGE/Cdc42-Rac1). Similarly to what we found in cultured astrocytes, in C6 glioma and microglia, the RAGE-mediated cell migration requires the activity of Rac1 and Cdc-42 (Hudson *et al.* 2008; Bianchi *et al.* 2011). In addition, a population of vimentin-immunoreactive cells migrating into the wound was observed in our experiments stressing the fact that less differentiated astrocytes are the first population to migrate into the artificial wound. A similar population of immature astrocytes with long processes was showed to form the borders of glial scar *in vivo* (Wanner *et al.* 2013).

Reactive astrocytes undergo dramatic changes in the cell morphology but also show increased rate of cell division. In our hands, extracellular S100B induced astroglial cell division and essentially functions as a mitogen by activating a RAGE-dependent pathway. A number of cell lines and primary cultures also showed increased cell division after exposure to S100B, including C6 glioma (Selinfreund *et al.* 1991), cultured astrocytes (Selinfreund *et al.* 1991) and primary mouse myoblasts (Riuzzi *et al.* 2011), however, in the latter cell type these effects seem to be RAGE-independent (Riuzzi *et al.* 2011). Our results clearly showed that S100B mitogenic effect on astrocytes is RAGE-dependent. At higher concentrations (10 μM), we observed that S100B exerts a toxic effect similar to that shown in cultured astrocytes (Hu and Van Eldik 1996), neuroblastoma cell lines (Huttunen *et al.* 2000), osteoblastic cells (Alikhani *et al.* 2007), and primary neurons (Villarreal *et al.* 2011).

Evidence of NF-κB activation after S100B exposure has been observed in neurons (Alexanian and Bamburg 1999; Kögel *et al.* 2004; Villarreal *et al.* 2011), microglia (Adami *et al.* 2004; Liu *et al.* 2005; Bianchi *et al.* 2010) and

astrocytes (Lam *et al.* 2001; Ponath *et al.* 2007). The RAGE dependence of S100B-induced NF-κB activation has been shown in astrocytes by Lam *et al.* (2001) using recombinant S100B in the micromolar level. Consistent with this, we found that 1 μM S100B exposure increases p65 NF-κB subunit nuclear localization in a RAGE-dependent manner. However, by using a NF-κB reporter plasmid, we here extend these observations showing that 50 nM and 1 μM doses of S100B are able to induce NF-κB transcriptional activation.

After brain injury, astrocytes surrounding the injury site are exposed to a very harmful environment and reactive astrocytes are known to have improved survival to these conditions. In concordance with this scenario, our results showed that S100B exposure increased astrocytic survival to oxidative stress conditions requiring Erk and Akt activities.

RAGE expression rises in various forms of brain injury including hypoxia, ischemia, sleep apnea, Alzheimer's, and Parkinson's diseases (Ma *et al.* 2003; Bierhaus *et al.* 2005; Aviles-Reyes *et al.* 2010; Villarreal *et al.* 2011). In cultured astrocytes, we have here observed that S100B exposure activates proximal RAGE promoter and induces RAGE protein expression. Specifically, micromolar S100B activates proximal RAGE promoter, while both nanomolar and micromolar S100B induce endogenous RAGE over-expression. Our *in silico* analysis of the RAGE proximal promoter revealed the presence of consensus binding sites for Sp1 and NF-κB, in agreement with the previous reports using DNA footprinting assays (Li and Schmidt 1997; Li *et al.* 1998). When we blocked NF-κB with sulphazalazine or transfected a dominant negative Sp1, a partial reduction in S100B-induced endogenous RAGE expression was observed. While Sp1 and NF-κB activities are required for the 100 nM S100B effect; higher S100B level seems to partially overcome Sp1 and NF-κB blockage. This may be indicative of upstream transcription factors beyond the proximal promoter and/or epigenetic mechanisms likely to be involved in S100B-induced RAGE over-expression. Pichiule *et al.* (2007) have shown that hypoxia-induced RAGE expression requires the activation of HIF-1α (Pichiule *et al.* 2007), but also functional NF-κB and Sp1 binding sites have been identified in the human RAGE promoter (Li and Schmidt 1997; Lam *et al.* 2001). In fact, we here showed that NF-κB and Sp1 are required for S100B to fully activate RAGE expression. S100B, as other RAGE ligands, induce RAGE expression and thereby promote a feed-forward amplification loop that may be dependent on NF-κB activity.

Astrocytes phenotypically characterized as reactive can be beneficial or detrimental for neuronal survival (Zamanian *et al.* 2012). One of our objectives was to determine if differential S100B concentrations could induce a different type (beneficial or detrimental) of reactive astrocytes. We observed that nanomolar or micromolar S100B levels discretely increased the expression of pro-inflammatory TLR2 and iNOS but micromolar S100B was indeed very effective to increase IL-1β. Functionally, conditioned medium obtained from astrocytes exposed to 1 μM S100B has a detrimental effect on OGD-exposed neurons, thus indicating that 1 μM S100B may induce the reactive astrocytes conversion into the reactive neurodegenerative profile.

In an attempt to mimic S100B release from a focal injury site, we directly administrated S100B in brain cortex and this procedure induced morphological features of reactive glia in the astrocytes surrounding the injected area, thus showing that high extracellular S100B concentration is able to produce a reactive phenotype *in vivo*. Microglia probably acts synergistically with astrocytes in the S100B/RAGE response. RAGE expressing cells were observed in the ipsilateral cortex and corpus callosum and this expression is likely to be induced by the released S100B or by the cannula injury itself.

Taking together our results, we propose that S100B/RAGE pathway participates in the initiation/expansion of reactive gliosis after brain injury by activating a number of intracellular cascades in astrocytes including small RhoGTPases, Erk/Akt, the transcription factor NF-κB and acts synergistically with other pro-inflammatory cellular pathways. In this scenario, S100B/RAGE can promote astroglial hypertrophy, increase local mitosis, facilitate astroglial polarization and migration to the injured site to contribute to the glial scar, and activate RAGE expression, thus configuring a feed-forward loop that may potentiate and expand S100B effects. Micromolar S100B levels turn astrocytes into a pro-inflammatory neurodegenerative phenotype.

Acknowledgments and conflict of interest disclosure

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The authors have no conflict of interest to declare.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Representative images of cultured astrocytes invading the artificial scratch wound at 3 h, 24 h, and 72 h after the scratch.

Figure S2. Representative images of cultured astrocytes invading

the artificial scratch wound by 1 h and 48 h after the scratch. Astrocytes were exposed to the indicated concentrations of S100B in presence of Rac1 blocker (NSC23766), RhoA activator (LPA, lysophosphatidic acid) or vehicle.

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