Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2011

doi: 10.1111/j.1471-4159.2011.07207.x

S100B alters neuronal survival and dendrite extension via RAGE-mediated NF-κB signaling

Alejandro Villarreal,* Rolando X. Aviles Reyes,* Maria Florencia Angelo,* Analia G. Reines† and Alberto Javier Ramos*

*Laboratorio de Neuropatología Molecular, Instituto de Biología Celular y Neurociencia "Prof. E. De Robertis", Facultad de Medicina, Universidad de Buenos Aires, Ciudad de Buenos Aires, Argentina

†ININFA-CONICET and Cátedra de Farmacología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Ciudad de Buenos Aires, Argentina

Abstract

S100B is a soluble protein secreted by astrocytes that exerts pro-survival or pro-apoptotic effects depending on the concentration reached in the extracellular millieu. The S100B receptor termed RAGE (for receptor for advanced end glycation products) is highly expressed in the developing brain but is undetectable in normal adult brain. In this study, we show that RAGE expression is induced in cortical neurons of the ischemic penumbra. Increased RAGE expression was also observed in primary cortical neurons exposed to excitotoxic glutamate (EG). S100B exerts effects on survival pathways and neurite extension when the cortical neurons have been previously exposed to EG and these S100B effects were prevented by anti-RAGE blocking antibodies. Furthermore, nuclear factor kappa B (NF-κB) is activated by S100B in a dose- and RAGE-dependent manner and neuronal death induced by NF-κB inhibition was prevented by S100B that restored NF-κB activation levels. Together, these findings suggest that excitotoxic damage can induce RAGE expression in neurons from ischemic penumbra and demonstrate that cortical neurons respond to S100B through engagement of RAGE followed by activation of NF-κB signaling. In addition, basal NF-κB activity in neurons is crucial to modulate the extent of pro-survival or pro-death S100B effects.

Keywords: apoptosis, glia, ischemia, neuronal death.

J. Neurochem. (2011) 10.1111/j.1471-4159.2011.07207.x

S100B is a small EF-hand Ca^{2+}-binding protein abundant in the cytoplasm of brain astrocytes (Kligman and Hilt 1988). S100B has several intracellular actions, including regulation of Ca^{2+} homeostasis (Xiong *et al.* 2000), control of microtubule stability (Sorci *et al.* 2000) and modulation of mitosis (Donato 2003). S100B can be secreted from glial cells to the extracellular space by a poorly defined mechanism that is insensitive to the endoplasmic reticulum-Golgi trafficking inhibitor brefeldin (Davey *et al.* 2001) yet is sensitive to $5HT_{1A}$ agonists (Azmitia and Whitaker-Azmitia 1991; Ahlemeyer *et al.* 2000; Ramos *et al.* 2004), to fluoxetine (Manev *et al.* 2001; Tramontina *et al.* 2008), and to severe metabolic stress induced by oxygen-glucose-serum deprivation (Gerlach *et al.* 2006).

Brain trauma induces S100B release from astrocytes and increased S100B levels are found in CSF and peripheral blood in a number of pathological states and neurodegenerative diseases, such as ischemic stroke, traumatic brain injury, and Alzheimer's disease (Van Eldik and Griffin 1994; Rothermundt *et al.* 2003; Braga *et al.* 2006).

S100B has dose-dependent effects on neurons. Nanomolar levels of S100B protect neurons from stress-induced apoptosis (Ahlemeyer *et al.* 2000; Kögel *et al.* 2004), stimulate neurite outgrowth and MAP-2 expression (Reeves *et al.* 1994; Nishi *et al.* 1997, 2000) and modulate long-term neuronal plasticity (Nishiyama *et al.* 2002). However, exposure to micromolar S100B concentrations increases β-amyloid neurotoxicity (Businaro *et al.* 2006) and causes neuronal apoptosis (Hu *et al.* 1997).

Received August 16, 2010; revised manuscript received December 31, 2010; accepted January 18, 2011.

Address correspondence and reprint requests to Dr Alberto Javier Ramos, Laboratorio de Neuropatología Molecular, Instituto de Biología Celular y Neurociencia "Prof. E. De Robertis", Facultad de Medicina, Universidad de Buenos Aires, Calle Paraguay 2155 3er piso, (1121) Ciudad de Buenos Aires, Argentina. E-mail: jramos@fmed.uba.ar

Abbreviations used: CD, cortical devascularization; DIV, days *in vitro*; DPL, days post-lesion; GFAP, glial fibrillary acidic protein; NF-κB, nuclear factor kappa B; PBS, phosphate-buffered saline; RAGE, receptor for advanced glycation end products; SFZ, sulfasalazine.

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily and can be bound by several ligands, including S100B (Hofmann *et al.* 1999; Rong *et al.* 2005; Ramasamy *et al.* 2009). The pro-survival effects of S100B in neurons appear to be due to RAGE-mediated nuclear factor kappa B (NF-κB) activation which, in turn, induces transcription of Bcl-2 and other anti-apoptotic genes (Alexanian and Bamburg 1999; Huttunen *et al.* 2000; Kögel *et al.* 2004). In contrast, the toxic effect of micromolar S100B exposure apparently relies on production of reactive oxygen species, cytochrome C release from mitochondria and caspases activation (Vincent *et al.* 2007).

Little is known about the role of the S100B/RAGE axis in brain ischemia. Penumbral neurons are exposed to large quantities of S100B, released from reactive astrocytes, and neurons expressing RAGE have been detected after experimental and human brain ischemia (Qiu *et al.* 2008; Zhai *et al.* 2008; Hassid *et al.* 2009). The present study was designed to investigate whether extracellular S100B affects neuronal outcome in conditions that mimic the ischemic penumbra. We demonstrate that RAGE expression is selectively induced in neurons from ischemic penumbra and show that RAGE expression is induced in primary cortical neurons exposed to glutamate excitotoxicity. This enables increased sensitivity to S100B-induced survival and death. We then demonstrate that S100B-induced survival effects are RAGE- and NF-κB-dependent and show that basal NF-κB activity determines the prevalence of pro-death or pro-survival S100B effects in neurons.

Experimental procedures

Reagents

Cell culture reagents were obtained from HyClone (Logan, UT, USA) and Invitrogen (Carlsbad, CA, USA). Antibodies were purchased from Chemicon (Temecula, CA, USA; anti RAGE cat# MAB5328; anti-NeuN cat# MAB377); from Santa Cruz Biotechnology (Santa Cruz, CA, USA; anti- NF-κB p65 cat# sc-372, anti-Bcl-XL cat# sc-634; anti- αIκB cat# sc-371) and Cell Signaling Technologies (Beverly, MA, USA; anti-phospho p65 (Ser 536) cat# 3036). The horseradish peroxidase-conjugated secondary antibodies, biotinilated antibodies, extravidin complex, glutamate, S100B purified from bovine brain and other chemicals were obtained from Sigma (St Louis, MO, USA). Secondary fluorescent antibodies were from Jackson Immunoresearch (West Grove, PA, USA). The enhanced chemiluminescence used in the detection of immunoreactive bands was purchased from Pierce (Rockford, IL, USA).

Cortical devascularization

Adult male Wistar rats (250–300 g) 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, free access to standard laboratory rat food and water) under the permanent supervision of a professional technician. All surgical procedures were performed under gas anesthesia induced with sevofluorane (4% vol/vol). Animals were subjected to a unilateral cortical devascularization as previously described (Herrera and Robertson 1989; Figueiredo *et al.* 1993; Angelo *et al.* 2009). All efforts were made to reduce the suffering and the number of animals used. 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.

Fixation

After various recovery times [3, 7 or 14 days post-lesion (DPL)], animals were deeply anaesthetized with ketamine/xylazine (90/10 mg/kg, i.p.) and were perfused through the left ventricle as described (Aviles-Reyes *et al.* 2010). Brains were cryoprotected, snap frozen and coronal 25–50 μm thick brain sections were cut using a cryostat. Free floating sections were kept in a cryoprotective solution (30% glycerol, 20% ethylene glycol in 0.05 M phosphate buffer) at −20°C until use.

Immunohistochemistry

Brain sections of animals belonging to the different experimental groups were simultaneously processed in the free floating state (Angelo *et al.* 2009; Aviles-Reyes *et al.* 2010). Controls for the immunohistochemistry procedure were routinely performed by omitting the primary antibody. Photographs were taken in a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany) equipped with an Olympus Q-Color 5 camera or in an Olympus IX-81 inverted microscope equipped with a DP71 camera (Olympus, Tokyo, Japan).

Primary neuronal culture

The cortical neuronal cultures were prepared from embryonic day (E) 16 Wistar rats according to Goslin *et al.* (1988) with minor modifications. Briefly, cortical neurons were dissociated by treatment with 0.25% trypsin followed by trituration through a Pasteur pipette. Cells plated at a density of 1×10^5 or 7×10^3 cells/cm^2 on poly-D-lysine-coated plastic dishes or glass coverslips, respectively, and maintained for up to 10 days in NeuroBasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen) and 0.5 mM glutamine. Induction of excitotoxicity in neurons was performed after 7 days *in vitro* (7 DIV) by extracting 50% of culture medium and adding glutamate (final concentration 300 μM) or control buffer. After 5 or 10 min, the medium was completely removed, cells were washed and a mixture of 50% conditioned medium and fresh medium containing B27 was added. Neurons were incubated during additional 20 h before lysing them in NP-40 buffer as shown below. For immunocytochemistry, neurons were washed with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde plus 4% sucrose in PBS pH 7.2 for 15 min at 18–25°C. Cells were then washed three times with cold PBS and permeabilized with 0.1% Triton X-100. The procedure was then followed as stated for tissue sections. After finishing the immunofluorescence protocol, slides containing primary cortical neurons were rinsed with PBS and

incubated with a solution of Hoechst 33342 (2 μg/mL in PBS), in a dark chamber, for 5 min at 18–25°C. After further rinses in PBS, the coverslips were mounted on gelatin-coated slides, using a solution of glycerol 30% in PBS

Exposure to S100B

S100B purified from bovine brain (Sigma) was dissolved in PBS to 1 mg/mL, sterile filtered and stored in aliquots at −80°C. This value is equivalent to 50 μM of S100B dimer. Neuronal cultures were exposed to S100B in neurobasal-B27 medium. Alternatively, neurons were exposed for 24 h to S100B plus sulfazalazine 2.5 μM to perform the NF-κB loss of function experiments. The RAGE blockage experiments were done by exposing neurons to anti-RAGE blocking antibody (MAB 5328; Chemicon) that recognizes RAGE extracellular domain (Fiuza *et al.* 2003; Chou *et al.* 2004). Neurons were exposed to 1.5 μg anti-RAGE or unrelated mouse IgG for 2 h and then exposed to different concentrations of S100B.

Immunoblotting

Neurons 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. Cortical areas of animals subjected to cortical devascularization (CD) were homogenized in modified RIPA lysis buffer for tissues (150 mM NaCl; 50 mM Tris; 1 mM EDTA; 1% Triton X-100; 1% sodium deoxycholic acid; 0.1% sodium dodecyl sulfate and protease inhibitors). Proteins were determined using bovine serum albumin (BSA) as standard using the BCA assay (Pierce). Samples with equivalent protein content were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and proteins were transferred onto nitrocellulose membrane. The membrane was blocked in Tris-buffered saline/Tween [10 mM Tris (pH 8.0), 150 mM NaCl, 0.2% Tween 20] added with 5% (w/v) dried skim milk powder. The primary and secondary antibody incubations were carried out in the blocking solution. Immunoreactive bands were detected using the enhanced chemiluminescence solution (Pierce) and AGFA films.

Quantitative studies

Morphometrical studies to analyze dendrite length per neuron were performed on microscopy images taken from cell culture labeled slides with the NeuronJ plugin for the NIH ImageJ software (Meijering *et al.* 2004). Using this software, we analyzed 20–30 neurons per field, 8–10 fields (20× primary magnification), per experimental condition in each experiment and the total dendrite length of MAP-2+ projections was measured. Experiments were repeated 3–4 times with similar results. Neuronal death was evaluated in Hoechst stained sections by quantifying the percentage of neurons showing apoptotic cell bodies or pycnotic nuclei. Nuclear p65 localization was determined in p65/Nf-200 KDa or p65/NeuN stained sections. Immunoreactive bands in western blot studies were analyzed with the NIH ImageJ software and intensity of bands related to the loading control. Statistical analysis was performed by one-way ANOVA and Student–Newman–Keuls post-test. Significance was set at $p < 0.05$. Data were presented as mean ± SEM unless stated different.

Results

RAGE expression is induced in cortical neurons after brain ischemia

RAGE expression is prominent during CNS development, but RAGE protein is undetectable in the normal adult brain. As previous reports have shown that brain hypoxia or ischemia can induce RAGE expression, we set out to identify RAGE-expressing cells in brains subjected to CD-induced ischemia. Figure S1 shows that the ischemic lesion induced by CD affected the cortical layers I–IV. The necrotic core was noted by the absence of individual cells and the penumbra displayed pyknotic nuclei and profuse reactive gliosis. Figure 1(a) and (b) shows that RAGE was expressed in cells with neuronal morphology in the ischemic penumbra, while contralateral hemisphere only showed a very weak RAGE staining in blood vessels endothelium (Fig. 1f). The morphology of RAGE expressing cells were of two main classes: pyramidal-like neurons with a dense cytoplasmic RAGE staining and non-distinguishable nucleus (Fig. 1c) and rounded-shaped neurons with a membrane-like staining and negative nucleus (Fig. 1d). Both types of RAGE+ neurons were present in the ischemic penumbra surrounding the ischemic core (see schematic representation in Fig. 1e). Figure 1g shows that RAGE+ cells co-stain with neuron-specific enolase but not with glial fibrillary acidic protein (GFAP) (Figure S2). In addition, 200 KDa-neurofilament (Nf-200 KDa) immunostaining was observed on RAGE+ neuronal projections (Figure S2). Therefore, we conclude that RAGE+ cells are neurons.

RAGE+ neurons showed pyknotic as well as normal Hoechst-stained nucleus (Fig. 1g). These RAGE+ cells were prominent at 3 DPL, 7 DPL but only isolated neurons retained RAGE staining by 14 DPL (data not shown). Immunoblot analyses demonstrated that RAGE expression increased from 3 DPL to 7 DPL in the ipsilateral hemisphere (Ipsi) becoming undetectable after 14 DPL (Fig. 2). Interestingly, RAGE expression reached the maximal level before the apparent peak of reactive gliosis detected by the over-expression of astroglial markers GFAP and Vim that have a maximal abundance at 7 DPL and 7–14 DPL, respectively.

Glutamate excitotoxicity or S100B exposure induce RAGE expression in cortical primary neurons

In our next set of experiments, we studied the mechanisms responsible for the specific localization of RAGE in the ischemic penumbra neurons. For that purpose, we prepared primary cortical neurons (7 DIV) and exposed them to an excitotoxic stress that mimics the conditions of ischemic penumbra. Figure 3a shows that 5 or 10 min exposure to 300 μM glutamate increased RAGE expression in cortical neurons and increased the level of Sp1 transcription factor in

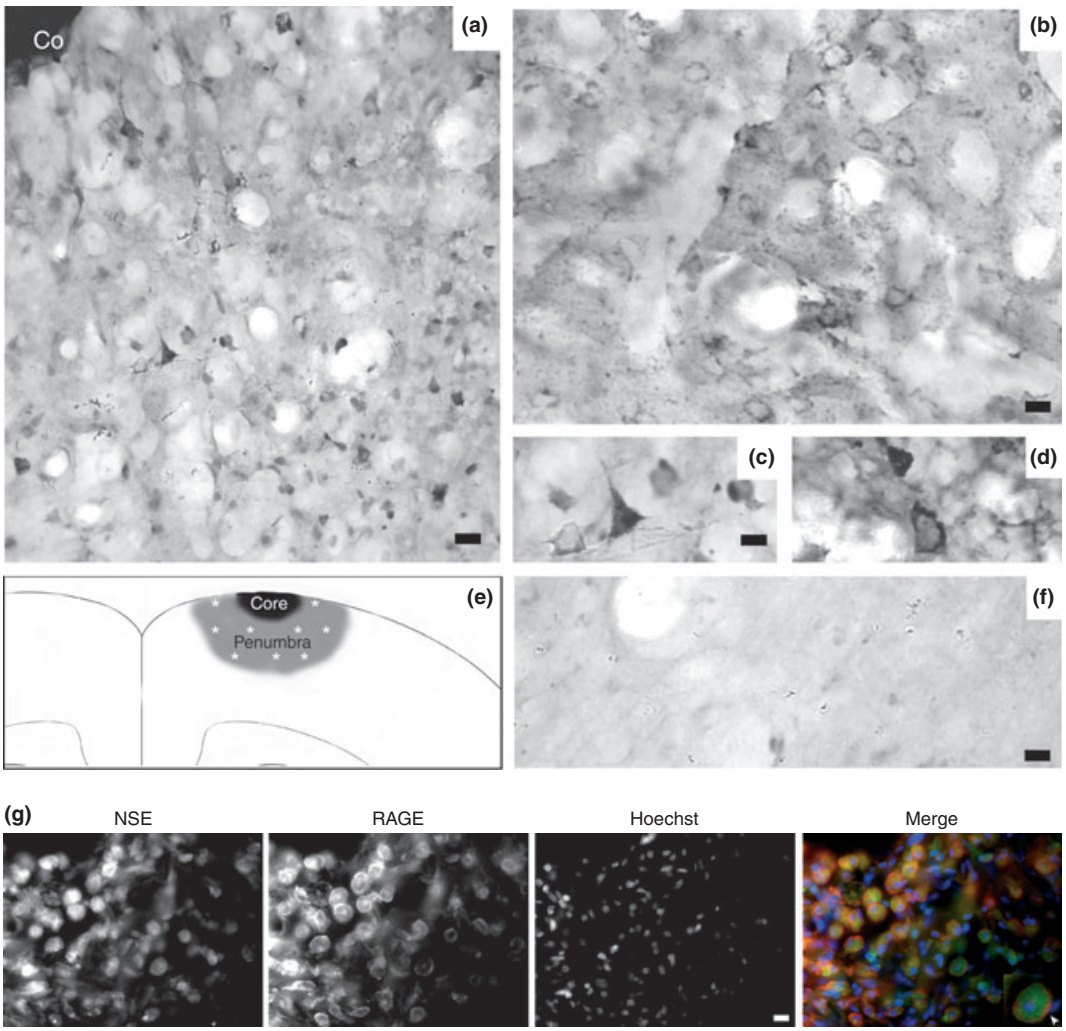

Fig. 1 RAGE expression in neurons from the ischemic penumbra analyzed 3 days post-lesion (3 DPL). (a) Low magnification photograph showing the relative localization of RAGE+ neurons to the ischemic core (Co), bar = 10 μm. (b–d) Larger magnification details of the different morphology of RAGE+ neurons, bar = 20 μm (b) and 25 μm (c, d). (e) Schematic representation of the relative localization of ischemic lesion core (Co) and the RAGE+ neurons (*). (f) Contralateral hemisphere showing absence of RAGE+ neurons. (g) Double immunostaining of the ischemic lesion showing neuron-specific enolase (NSE) (green)/RAGE (red) co-expression, bar = 10 μm, the inset shows a larger detail of a RAGE+/NSE+ neuron.

these cells. Sp1 has been previously demonstrated to be involved in the control of RAGE expression (Li and Schmidt 1997; Li *et al.* 1998). Sp1-dependent transcription is efficiently stimulated by hypo-osmolar exposure (Ramos *et al.* 2007) and Fig. 3b shows that neurons exposed to hypo-osmolar medium (50% osm, 160 mOsm/kg) also increased RAGE expression. RAGE ligands are known to enhance RAGE expression and in our next experiments we asked if S100B was able to induce RAGE expression in cortical neurons. As shown in Fig. 3c, control primary cortical neurons treated with different S100B doses showed increased RAGE expression only at the highest S100B dose (7.5 μM S100B). S100B did not significantly increase RAGE expression in glutamate-exposed neurons (Fig. 3c).

S100B effects on neurons exposed to glutamate-induced excitotoxicity

We next asked if the response of neurons to S100B was altered by glutamate exposure. Although micromolar S100B had a significative effect affecting survival of control primary neurons, the previous exposure to an excitotoxic pulse of glutamate potentiated S100B pro-survival or pro-death effects at nanomolar and micromolar concentrations, respectively (Fig. 4a). We then asked if the S100B effects observed in glutamate-exposed cells were actually RAGE-dependent, using a RAGE-neutralizing antibody. Figure 4b shows that 50 nM S100B increased cell survival, while RAGE blockage restored cell death in glutamate-exposed neurons. RAGE blockage also reduced the killing induced by high concen-

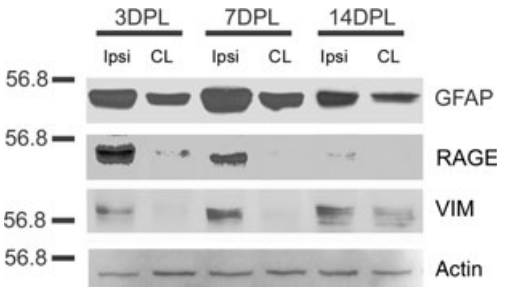

Fig. 2 Time–course of RAGE expression in brain cortical extracts from animals subjected to ischemia. Animals were killed at 3, 7 or 14 days post-ischemic lesion (DPL). Ipsilateral hemisphere (Ipsi) and contralateral hemisphere (CL) were dissected and soluble cortical brain extracts were analyzed by immunoblot, actin was used as internal loading control.

tration of S100B (Fig. 4b). Surprisingly, RAGE blockade reduced neuronal survival in control neurons, even in the absence of S100B (Fig. 4b). Together, these data indicate that after excitotoxicity, S100B effects are RAGE-dependent and that RAGE mediates survival signaling in control neurons.

S100B can also induce neurite outgrowth and in our next experiments, we asked whether the neurite extension ability of S100B was modified by excitotoxicity and if it was RAGE dependent. In control neurons, MAP-2+ neurite outgrowth was modestly stimulated by 1 μM S100B and this was abolished by exposure to the RAGE-blocking antibody. Exposure to excitotoxicity increased neurite extension in the surviving neurons exposed to 50 nM and 1 μM S100B and again, RAGE blockage abolished these effects (Fig. 5). These data indicate that neurite extension by S100B is a RAGE-dependent effect and is potentiated by the previous excitotoxic stress.

S100B exposure induces NF-κB activation in primary cortical neurons

NF-κB activation is a hallmark of RAGE engagement (Hofmann *et al.* 1999; Huttunen *et al.* 1999; Rong *et al.* 2005; Ramasamy *et al.* 2009) and we therefore asked whether S100B activates NF-κB and if the activation was RAGE-dependent. Primary cortical neurons were exposed to S100B for 3 h in presence or absence of RAGE-neutralizing antibodies. As shown in Fig. 6a, p65 subunit nuclear localization was increased in S100B-treated neurons, an effect reduced by RAGE-blocking antibodies.

The transcriptional activity of p65 (relA), the most abundant NF-κB subunit in neurons, is increased by phosphorylation of the transactivation domain region at Ser536 (Chen and Greene 2004; Ho *et al.* 2005; Ridder and Schwaninger 2009). In next experiments, we analyzed a number of molecules involved in S100B-induced NF-κB activation and studied p65 phosphorylation. Figure 6b shows that S100B exposure induced a dose-dependent degradation

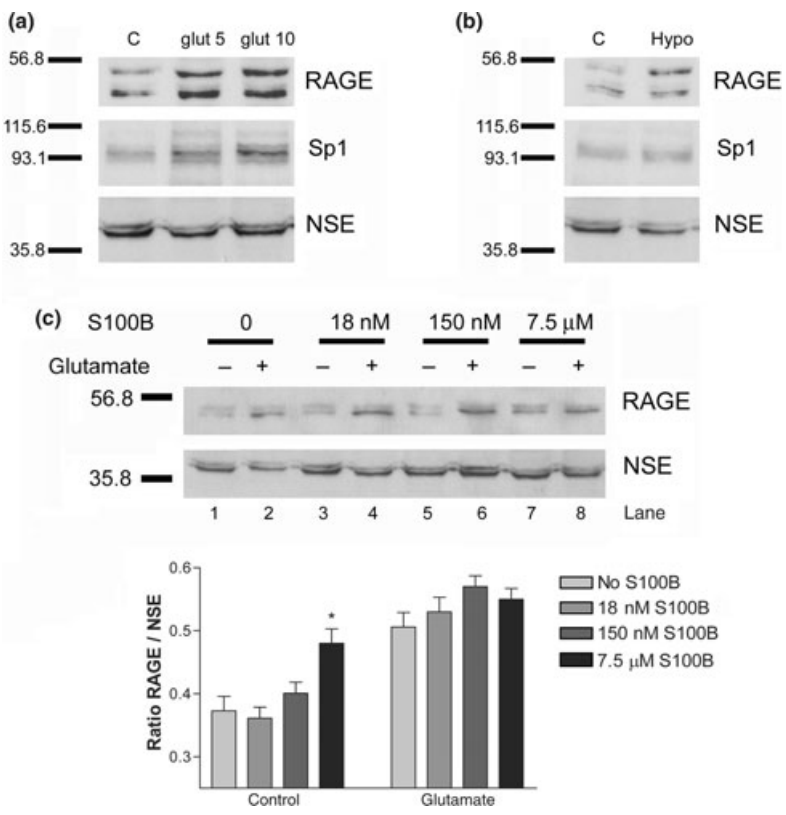

Fig. 3 Induction of RAGE expression by stressful stimuli. (a) RAGE expression in primary cortical neurons induced by a glutamate pulse of 300 μM during 5 or 10 min (glut 5 or glut 10, respectively) and analyzed 24 h later. (b) RAGE expression induced by hypo-osmolarity (50% Osm, 160 mOsm/Kg) during 20 h. (c) RAGE expression induced by 24 h exposure to S100B. Neuron-specific enolase (NSE) was used as loading control. The graph shows the quantification of RAGE/NSE ratio ($n = 3$). Data were analyzed by one-way ANOVA and Student–Newman–Keuls post-test (*$p < 0.05$). Both in a, b and c experiments RAGE run as a double band, in a, b bands were separated using a 15% sodium dodecyl sulfate–PAGE gel, whereas in c a 12% gel was used.

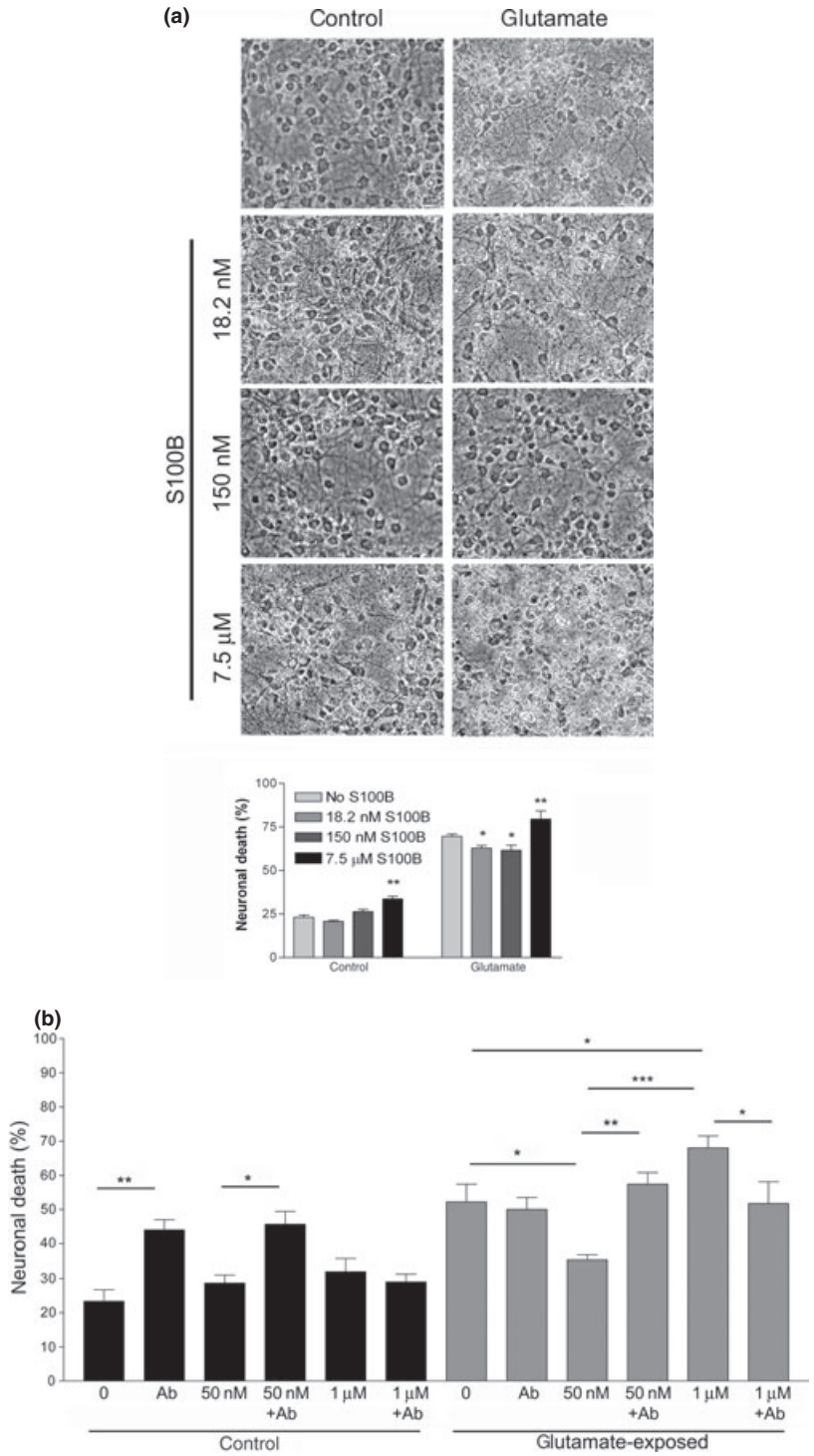

Fig. 4 Neuronal survival is modified by S100B. (a) Primary cortical neurons were exposed to vehicle or to glutamate 300 μM for 10 min and 24 h later to different S100B concentrations as shown. Representative pictures of microscopic fields obtained with phase contrast and the quantification of neuronal death with statistical comparisons made against the respective (no-S100B) control are shown. (b) Quantification of neuronal death after a similar experiment but including a RAGE-blocking antibody (Ab) and only two S100B doses. The number of dying neurons was determined by nuclear morphology with Hoechst staining and expressed as percent of dying neurons per field. Data were analyzed by one-way ANOVA and Student–Newman–Keuls post-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

of IκB in control neurons and previous exposure to glutamate rendered a trend to further degradation of IκB. The analysis of Ser536 showed that phosphorylation at this residue of p65 was increased by S100B in a dose-dependent manner and that previous exposure to glutamate significantly enhanced phosphorylation at this site (Fig. 6b). Hypo-osmolar exposure also increases Ser536 phosphorylation level and enhances IκB degradation in primary cortical neurons (Fig. 6c). We conclude that S100B activates NF-κB in a dose- and RAGE-dependent manner and previous glutamate exposure potentiates S100B-induced NF-κB activation by increasing phosphorylation of p65 subunit.

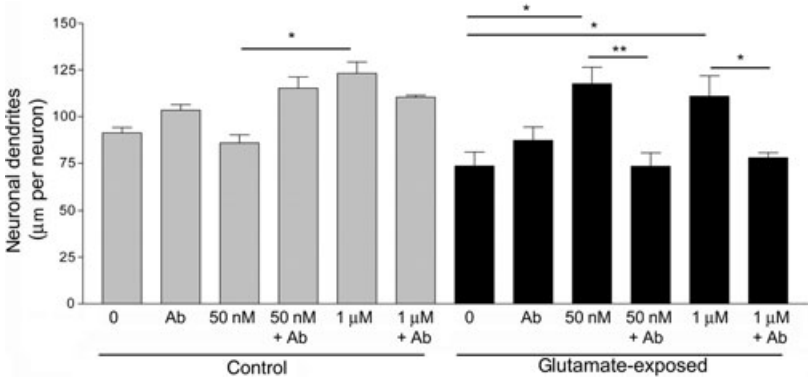

Fig. 5 Dendrite extension induced by S100B. Quantification of the MAP-2+ neurite projections in low-density primary cortical neuronal culture exposed to the glutamate pulse and S100B in presence or absence of RAGE-blocking antibodies and immunostained for MAP-2. Data were analyzed by one-way ANOVA and Student–Newman–Keuls post-test (*$p < 0.05$; **$p < 0.01$).

Effects of NF-κB blockage on primary cortical neurons exposed to glutamate and S100B

Constitutive NF-κB activity is present in cortical neurons and it is required for neuronal survival *in vitro* (Bhakar *et al.* 2002). As S100B induces NF-κB activation, we asked if S100B could be able to prevent the neuronal death induced by the chemical blockage of NF-κB with sulfasalazine (SFZ), a drug that prevents IκB degradation. For that purpose, we first determined that 2.5 µM SFZ was the lowest SFZ dose that reduced neuronal survival in dose-response studies (data not shown). Then, we treated control or glutamate-exposed primary cortical neurons with 2.5 µM SFZ in presence or absence of S100B. Figure 7(a) and (b) show that SFZ treatment drastically reduced neuronal survival; however, when neurons were co-incubated with SFZ and S100B, neuronal survival was restored to control values. Previous glutamate exposure improved the survival of SFZ-exposed neurons, probably because of activation of NF-κB signaling by glutamate (Fig. 7a and b).

In our final experiments, we asked if S100B treatment was able to restore the NF-κB activity in presence of NF-κB blocker SFZ. Figure 7c shows that SFZ treatment reduced NF-κB activation as shown by reduced IκB degradation, reduced p65Ser536 phosphorylation and slight p65 accumulation in the cytoplasmic compartment. However, the reduction in NF-κB activation by SFZ was prevented by the co-incubation with 1 µM S100B, as shown by the increase in p65Ser536 phosphorylation, reduction of cytoplasmic p65 accumulation and partial restoration of IκB degradation (Fig. 7c). As RAGE expression is induced by NF-κB, we then asked if the short 3 h treatment with SFZ was able to modify RAGE abundance in these cortical neurons. As shown in Fig. 7c, RAGE expression was not significantly changed by 3 h exposure to SFZ treatment, either in presence or absence of S100B. We conclude that S100B prevents NF-κB blockage-induced death by partially restoring NF-κB activity.

Discussion

RAGE expression is induced in human and experimental ischemia (Ma *et al.* 2003; Pichiule *et al.* 2007; Qiu *et al.* 2008; Zhai *et al.* 2008; Hassid *et al.* 2009). Here, we show that RAGE-expressing neurons are present specifically in the ischemic penumbra where a profuse reactive gliosis with increased S100B expression has been demonstrated (Ramos *et al.* 2004). Although it was believed that RAGE is expressed only in dying neurons (Ma *et al.* 2003), by analyzing the nuclear morphology we found that RAGE expression was present in both healthy and dying neurons.

RAGE expression seems to be controlled by a number of transcription factors, including Sp1; NF-κB and hypoxia induced factor 1 alpha (HIF-1α) (Li and Schmidt 1997; Li *et al.* 1998; Tanaka *et al.* 2000; Pichiule *et al.* 2007). In cortical neurons, we observed that glutamate excitotoxicity-induced RAGE expression and increased Sp1 abundance. Kögel *et al.* (2004) showed that NMDA agonists are able to increase RAGE mRNA and thus we assume that glutamate is probably acting through this receptor to induce RAGE expression. Brain edema, a common consequence and clinical complication of acute ischemia can be modeled *in vitro* by hypo-osmolar exposure of cell cultures and this paradigm demonstrated to increase Sp1-dependent transcription (Ramos *et al.* 2007; Kommaddi *et al.* 2011) and RAGE expression (Fig. 3b). We confirmed that S100B, behaving like a classical RAGE ligand, stimulates RAGE expression in cortical primary neurons but found that S100B was unable to further increase RAGE expression in glutamate-exposed neurons. Together these data show that stressful stimuli capable of activating Sp1 and/or NF-κB dependent transcription increase RAGE expression in cortical neurons.

Nanomolar S100B levels protect neurons from excitotoxicity when they are pre-incubated with S100B (Ahlemeyer *et al.* 2000; Kögel *et al.* 2004) or are exposed to S100B immediately after oxygen-glucose-deprivation (Pichiule *et al.* 2007). In an attempt to reproduce ischemic penumbra environment where S100B is massively released after ischemia, we added S100B to the culture medium and showed that it rescues neurons from the glutamate-induced neuronal death even when is applied 24 h after the primary excitotoxic stimulus. However, we observed that micromolar S100B levels have a detrimental effect on glutamate-exposed neurons. This result has an interesting correlation

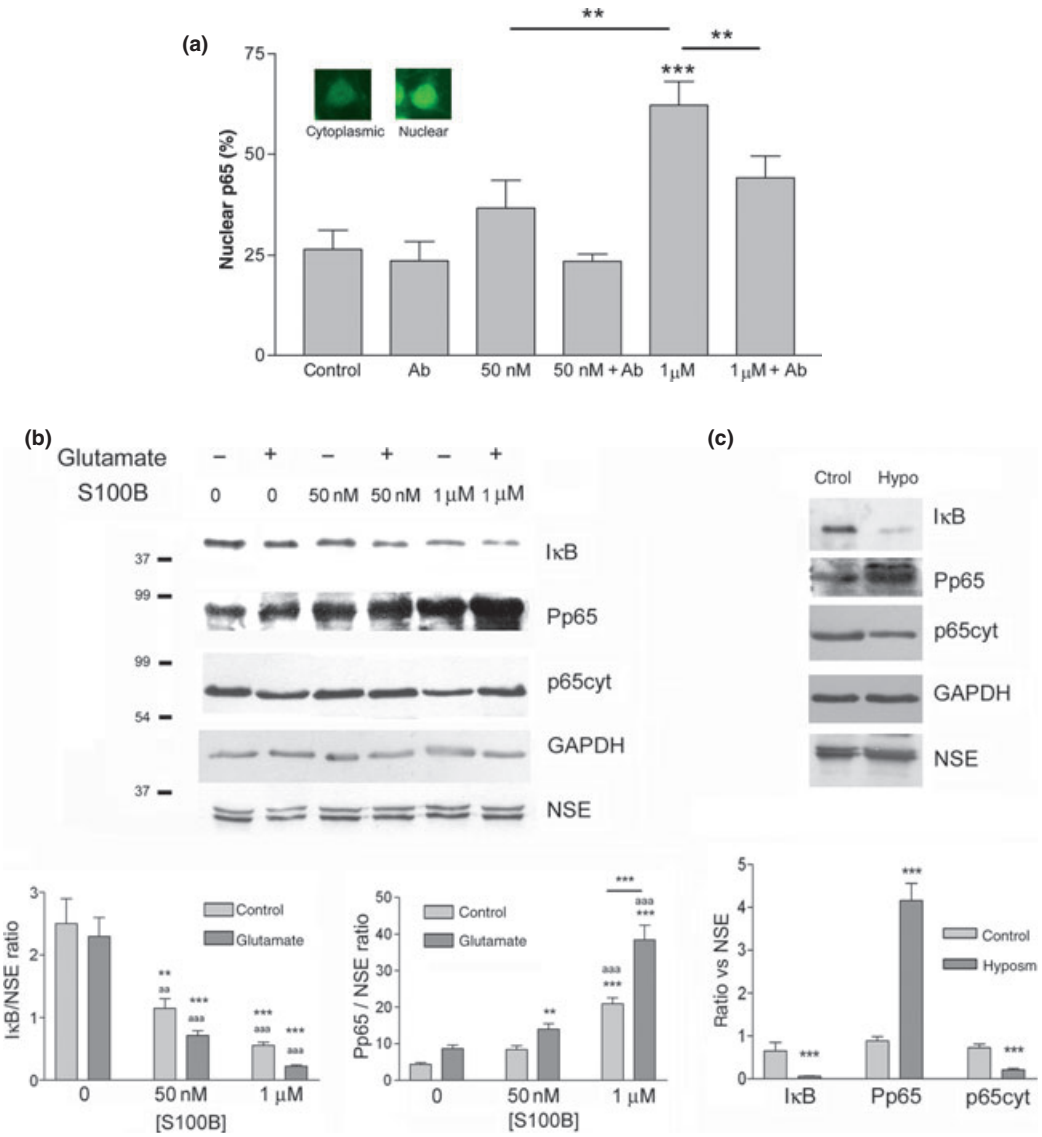

Fig. 6 NF-κB activation after S100B exposure. (a) Primary cortical neurons were exposed to S100B for 3 h, double labeled for p65/NeuN and the relative number of neurons with nuclear p65 was determined with the ImageJ Cell Counter plugin. (b) Neurons were exposed to control medium or to glutamate 300 μM for 10 min and 24 h later different S100B concentrations were added for additional 3 h. Neuron-specific enolase (NSE) and GAPDH were used as loading controls. The graphs show the densitometry of the immunoblots analyzed by one way ANOVA followed by Student–Newman–Keuls post-test. The significance versus the respective control (non-glutamate exposed neurons) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) or versus the glutamate-exposed neurons ($^{a}p < 0.05$; $^{aa}p < 0.01$; $^{aaa}p < 0.001$) is indicated. (c) The effect of 20 h exposure to hypo-osmolarity in NF-κB activation with densitometry is shown as a ratio of the band intensities versus NSE loading control (***$p < 0.001$).

with studies showing that S100B over-expression precedes delayed infarct expansion into the penumbra (Matsui *et al.* 2002) and that inhibition of S100B over-production by astrocytes may provide neuroprotection (Asano *et al.* 2005). Using RAGE-neutralizing antibodies, we observed that RAGE blockage in neurons exposed to excitotoxicity abolishes the nanomolar S100B pro-survival effect and prevents neuronal death induced by micromolar S100B, thus supporting the hypothesis that S100B differential effects on neuronal survival are RAGE-dependent in these conditions. RAGE-dependence of S100B pro-death effects after excitotoxicity correlates with reports showing that knockout mice lacking of RAGE or the RAGE blockade induce a reduction in the brain infarct size (Muhammad *et al.* 2008).

Extracellular S100B increases neurite outgrowth. Indeed, S100B was originally described as a neurite extension factor (Kligman and Marshak 1985; Zhou *et al.* 1995). S100B has

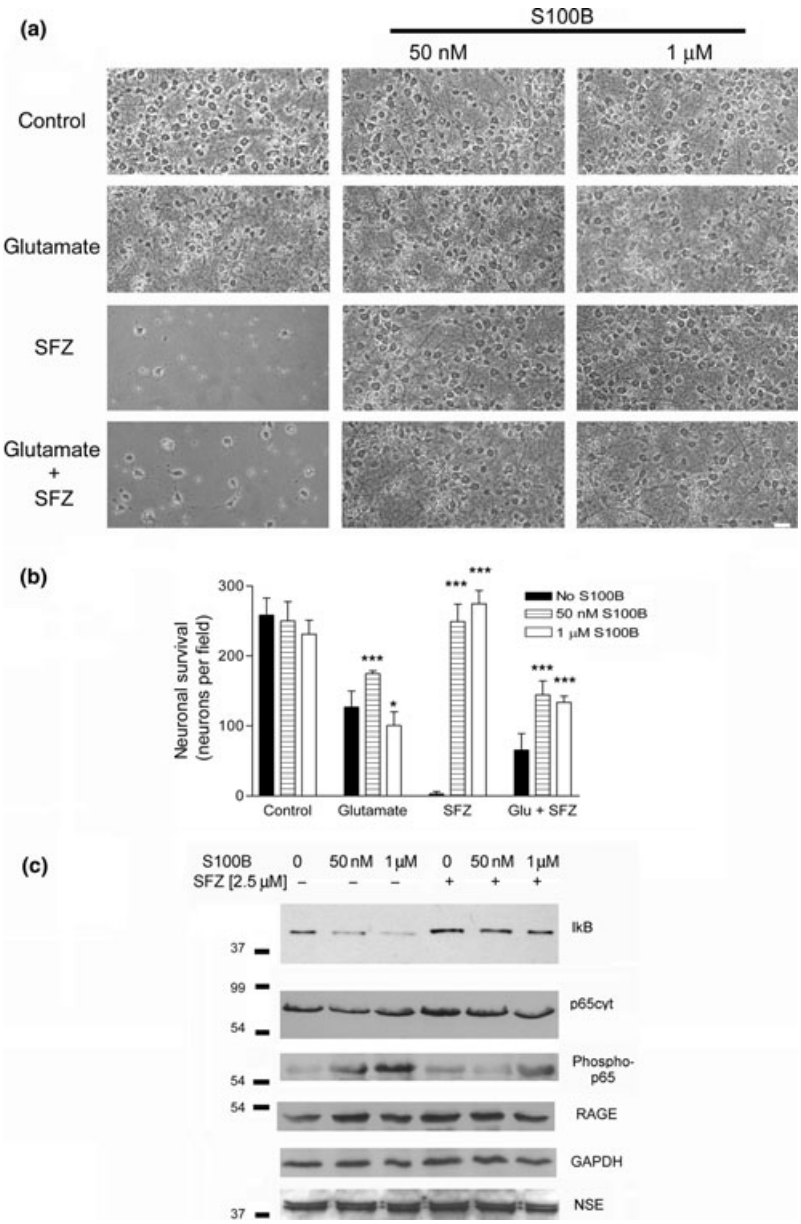

Fig. 7 NF-κB blockade effects in cortical neurons. (a) Primary cortical neurons exposed to a glutamate pulse of 300 μM for 10 min or vehicle were exposed 24 h later to S100B for additional 24 h in presence or absence of 2.5 μM NF-κB chemical inhibitor sulfazalazine (SFZ). Representative photographs of microscopic fields obtained in an inverted microscope with phase contrast are shown. (b) Quantification of neuronal survival after the experiment shown in panel (a) with the number of surviving healthy neurons determined by Hoechst nuclear staining. Data were analyzed by one-way ANOVA and Student–Newman–Keuls post-test (*$p < 0.05$; ***$p < 0.001$ versus the respective control without S100B). (c) Following the same paradigm as in panel (a) but exposing 3 h to S100B with or without SFZ, cells were lysed and cytoplasmic extracts subjected to immunoblot to verify the effect of S100B in restoring the NF-κB activity. Neuron-specific enolase (NSE) and GAPDH were used as loading controls.

shown to reverse colchicine-induced collapse of microtubules (Brewton *et al.* 2001), to promote dendrite extension (Nishi *et al.* 1997, 2000) and to regulate neurite outgrowth in a neuroblastoma cell line (Huttunen *et al.* 2000). We determined that S100B stimulates dendrite outgrowth in the surviving excitotoxicity-exposed neurons in a RAGE-dependent manner. RAGE knockdown in primary cerebellar granule neurons also inhibits neurite outgrowth in absence of S100B, but probably by blocking some additional autocrine RAGE signaling (Wang *et al.* 2008).

The downstream signaling after RAGE activation by S100B in different cell types, including astrocytes, microglia and neuroblastoma cell lines, is mostly attributed to NF-κB activation (Donato 2007; Donato *et al.* 2009). In primary hippocampal neurons, Kögel *et al.* (2004) have described the activation of a p65/cREL atypical NF-κB complex when neurons were pre-incubated with S100B and then exposed to NMDA. Considering that excitotoxicity activates NF-κB, we used a different paradigm where neurons were exposed to the excitotoxic pulse, and 24 h later incubated with S100B to determine NF-κB activation. Our results indicate that S100B activates NF-κB in a RAGE-dependent manner and that previous exposure to glutamate excitotoxicity significantly increases p65 phosphorylation that is known to augment its transcriptional activity (Perkins 2006). Interestingly, a prominent increase in Ser536 phosphorylated p65 has been demonstrated in the neurons of the ischemic penumbra (Ridder and Schwaninger 2009).

To further establish the NF-κB dependence of S100B effects, we used the NF-κB inhibitor sulfasalazine (SFZ) that reduced neuronal survival because basal NF-κB activity is essential for neuronal survival *in vitro* (Bhakar *et al.* 2002). As expected, neuronal survival in presence of SFZ was improved by S100B and this correlated with reduced IκB, reduced cytoplasmic p65 and partial recovery of Ser536 phosphorylation, all indicative of active NF-κB. Interestingly, SFZ has prevented the detrimental effects of micromolar S100B on neurons exposed to glutamate, probably by limiting the over activation of NF-κB pathway. It has been previously described that S100B pro-survival effects in chicken forebrain neurons and neuroblastoma cells are NF-κB-dependent (Alexanian and Bamburg 1999; Huttunen *et al.* 2000). We now propose that both the pro-survival and pro-death S100B effects are mediated by NF-κB. The basal NF-κB activity in neurons and the S100B-induced level of NF-κB activity, probably determined by phosphorylation of p65 transactivation domain, emerge as the main determinants for the prevalence of pro-survival or pro-death S100B effects. The p65 phosphorylation is a key control point for NF-κB transcriptional activity and over-activation of NF-κB in neurons contributes to cell death in ischemia (Zhang *et al.* 2005; Ridder and Schwaninger 2009).

It is important to note that *in vivo*, RAGE is probably interacting not only with S100B released form reactive astrocytes, but also with other ligands released by the neighboring necrotic core. Other RAGE ligands such as high mobility group box 1 (also known as amphoterin or HMG-1) released by the necrotic neurons from ischemic core can act to recruit brain macrophages and promote inflammation (Muhammad *et al.* 2008; Qiu *et al.* 2008) and high mobility group box 1 blockade-ameliorated brain damage (Liu *et al.* 2007; Kim *et al.* 2008; Muhammad *et al.* 2008).

In conclusion, we have shown that RAGE expression and basal NF-κB activity in neurons determine the beneficial or detrimental effects mediated by S100B on neuronal survival and dendrite extension. *In vivo* in ischemic penumbra, the convergence of different RAGE ligands are probably contributing to the final response in target cells and defining neuronal survival or death.

Acknowledgements

This work was supported by grants CONICET PIP1728 (AJR), ANPCYT (grant PICT 2008-1590 to AJR and PICT 2005-34397 to AGR) and IBRO Return Home Fellowship (AJR). RXAR, MFA and AV are fellows from CONICET (Argentina). AJR and AGR are researchers from CONICET (Argentina). We thank Ms Andrea Pecile for her help with the animal care; Dr G. Paratcha, Dr F. Ledda and Dr P. Barker for helpful discussion and revision of the manuscript; Dr P. Power and Dr G. Gutkind for the initial purification of S100B used in exploratory experiments.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Photocomposition showing the focal isquemia induced by cortical devascularization (CD).

Figure S2. Double immunostaining of CD lesion after 7 DPL showing GFAP/RAGE and Nf-200 KDa/RAGE, note that astrocytes GFAP+ do not express RAGE, whereas RAGE+ neurons present projections Nf-200 KDa+ bar = 10 μm.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

References

Ahlemeyer B., Holger B., Irina S., Christine S. and Josef K. (2000) S-100B protects neurons against glutamate- and staurosporine-induced damage and is involved in the antiaptotic action of the 5HT1A- receptor agonist, Bay x 3702. *Brain Res.* **858**, 121–128.

Alexanian A. R. and Bamburg J. R. (1999) Neuronal survival activity of s100beta is enhanced by calcineurin inhibitors and requires activation of NF-kappaB. *FASEB J.* **13**, 1611–1620.

Angelo M. F., Aviles-Reyes R. X., Villarreal A., Barker P., Reines A. G. and Ramos A. J. (2009) p75(NTR) Expression is induced in isolated neurons of the penumbra after ischemia by cortical devascularization. *J. Neurosci. Res.* **87**, 1892–1903.

Asano T., Mori T., Shimoda T., Shinagawa R., Satoh S., Yada N., Katsumata S., Matsuda S., Kagamiishi Y. and Tateishi N. (2005) Arundic acid (ONO-2505) amiliorates delayed ischemic brain damage by preventing astrocytic overproduccion of S100B. *Curr. Drug Targets CNS Neurol. Disord.* **4**, 127–142.

Aviles-Reyes R. X., Angelo M. F., Villarreal A., Rios H., Lazarowski A. and Ramos A. J. (2010) Intermittent hypoxia during sleep induces reactive gliosis and limited neuronal death in rats: implications for sleep apnea. *J. Neurochem.* **112**, 854–869.

Azmitia E. C. and Whitaker-Azmitia P. M. (1991) Awakening the sleeping giant: anatomy and plasticity of the brain serotonergic System. *J. Clin. Psychiatry* **52**, 4–16.

Bhakar A. L., Tannis L. L., Zeindler C., Russo M. P., Jobin C., Park D. S., MacPherson S. and Barker P. A. (2002) Constitutive nuclear factor-kappa B activity is required for central neuron survival. *J. Neurosci.* **22**, 8466–8475.

Braga C. W., Martinez D., Wofchuk S., Portela L. V. and Souza D. O. (2006) S100B and NSE serum levels in obstructive sleep apnea syndrome. *Sleep Med.* **7**, 431–435.

Brewton L. S., Haddad L. and Azmitia E. C. (2001) Colchicine-induced cytoskeletal collapse and apoptosis in N-18 neuroblastoma cultures is rapidly reversed by applied S-100beta. *Brain Res.* **912**, 9–16.

Businaro R., Leone S., Fabrizi C., Sorci G., Donato R., Lauro G. M. and Fumagalli L. (2006) S100B protects LAN-5 neuroblastoma cells against Abeta amyloid-induced neurotoxicity via RAGE engagement at low doses but increases Abeta amyloid neurotoxicity at high doses. *J. Neurosci. Res.* **83**, 897–906.

Chen L. F. and Greene W. C. (2004) Shaping the nuclear action of NF-kappaB. *Nat. Rev. Mol. Cell Biol.* **5**, 392–401.

Chou D. K., Zhang J., Smith F. I., McCaffery P. and Jungalwala F. B. (2004) Developmental expression of receptor for advanced glycation end products (RAGE), amphoterin and sulfoglucuronyl

(HNK-1) carbohydrate in mouse cerebellum and their role in neurite outgrowth and cell migration. *J. Neurochem.* **90**, 1389–1401.

Davey G. E., Murmann P. and Heizmann C. W. (2001) Intracellular Ca^{2+} and Zn^{2+} levels regulate the alternative cell density-dependent secretion of S100B in human glioblastoma cells. *J. Biol. Chem.* **276**, 30819–30826.

Donato R. (2003) Intracellular and extracellular roles of S100 proteins. *Microsci. Res. Tech.* **60**, 540–551.

Donato R. (2007) RAGE: a single receptor for several ligands and different cellular responses: the case of certain S100 proteins. *Curr. Mol. Med.* **7**, 711–724.

Donato R., Sorci G., Riuzzi F., Arcuri C., Bianchi R., Brozzi F., Tubaro C. and Giambanco I. (2009) S100B's double life: intracellular regulator and extracellular signal. *Biochim. Biophys. Acta* **1793**, 1008–1022.

Figueiredo B. C., Piccardo P., Maysinger D., Clarke P. B. and Cuello A. C. (1993) Effects of acidic fibroblast growth factor on cholinergic neurons of nucleus basalis magnocellularis and in a spatial memory task following cortical devascularization. *Neuroscience* **56**, 955–963.

Fiuza C., Bustin M., Talwar S., Tropea M., Gerstenberger E., Shelhamer J. H. and Suffredini A. F. (2003) Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. *Blood* **101**, 2652–2660.

Gerlach R., Demel G., König H.-G., Gross U., Prehn J. H. M., Raabe A., Seifert V. and Kögel D. (2006) Active secretion of S100B from astrocytes during metabolic stress. *Neuroscience* **141**, 1697–1701.

Goslin K., Schreyer D. J., Skene J. H. and Banker G. (1988) Development of neuronal polarity: GAP-43 distinguishes axonal from dendritic growth cones. *Nature* **336**, 672–674.

Hassid B. G., Nair M. N., Ducruet A. F., Otten M. L., Komotar R. J., Pinsky D. J., Schmidt A. M., Yan S. F. and Connolly E. S. (2009) Neuronal RAGE expression modulates severity of injury following transient focal cerebral ischemia. *J. Clin. Neurosci.* **16**, 302–306.

Herrera D. G. and Robertson H. A. (1989) Unilateral induction of c-fos protein in cortex following cortical devascularization. *Brain Res.* **503**, 205–213.

Ho W. C., Dickson K. M. and Barker P. A. (2005) Nuclear factor-kappaB induced by doxorubicin is deficient in phosphorylation and acetylation and represses nuclear factor-kappaB dependent transcription in cancer cells. *Cancer Res.* **65**, 4273–4281.

Hofmann M. A., Drury S., Fu C. *et al.* (1999) RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell* **97**, 889–901.

Hu J., Ferreira A. and Van Eldik L. J. (1997) S100beta induces neuronal cell death through nitric oxide release from astrocytes. *J. Neurochem.* **69**, 2294–2301.

Huttunen H. J., Fages C. and Rauvala H. (1999) Receptor for advanced glycation end products (RAGE)-mediated neurite outgrowth and activation of NF-kappaB require the cytoplasmic domain of the receptor but different downstream signaling pathways. *J. Biol. Chem.* **274**, 19919–19924.

Huttunen H. J., Kuja-Panula J., Sorci G., Agneletti A. L., Donato R. and Rauvala H. (2000) Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation. *J. Biol. Chem.* **275**, 40096–40105.

Kim J. B., Lim C. M., Yu Y. M. and Lee J. K. (2008) Induction and subcellular localization of high-mobility group box-1 (HMGB1) in the postischemic rat brain. *J. Neurosci. Res.* **86**, 1125–1131.

Kligman D. and Hilt D. C. (1988) The S100 protein family. *Trends Biochem. Sci.* **13**, 437–443.

Kligman D. and Marshak D. R. (1985) Purification and characterization of a neurite extension factor from bovine brain. *Proc. Natl. Acad. Sci. U S A* **82**, 7136–7139.

Kögel D., Peters M., König H. G., Hashemi S. M., Bui N. T., Arolt V., Rothermundt M. and Prehn J. H. (2004) S100B potently activates p65/c-Rel transcriptional complexes in hippocampal neurons. Clinical implicances for the role of S100B in excitotoxic brain injury. *Neuroscience* **127**, 913–920.

Kommaddi R. P., Dickson K. M. and Barker P. A. (2011) Stress-induced expression of the p75 neurotrophin receptor is regulated by O-GlcNAcylation of the Sp1 transcription factor. *J. Neurochem.* **116**, 396–405.

Li J. and Schmidt A. M. (1997) Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products. *J. Biol. Chem.* **272**, 16498–16506.

Li J., Qu X. and Schmidt A. M. (1998) Sp1-binding elements in the promoter of RAGE are essential for amphoterin-mediated gene expression in cultured neuroblastoma cells. *J. Biol. Chem.* **273**, 30870–30878.

Liu K., Mori S., Takahashi H. K. *et al.* (2007) Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. *FASEB J.* **21**, 3904–3916.

Ma L., Carter R. J., Morton A. J. and Nicholson L. F. (2003) RAGE is expressed in pyramidal cells of the hippocampus following moderate hypoxic–ischemic brain injury in rats. *Brain Res.* **966**, 167–174.

Manev R., Uz T. and Manev H. (2001) Fluoxetine increases the content of neurotrophic protein S100beta in the rat hippocampus. *Eur. J. Pharmacol.* **420**, R1–2.

Matsui T., Mori T., Tateishi N., Kagamiishi N., Satoh S., Katsube N., Morikawa E., Morimoto T., Ikuta F. and Asano T. (2002) Astrocytic activation and delayed infartct expansion after permanent focal ischemia in rats. Part I: enhanced astrocytic synthesis of S100B in the periinfarct area precedes delayed infarct expansion. *J. Cereb. Flow Metab.* **22**, 711–722.

Meijering E., Jacob M., Sarria J., Steiner P., Hirling H. and Unser M. (2004) Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytometry Part A* **58**, 167–176.

Muhammad S., Barakat W., Stoyanov S. *et al.* (2008) The HMGB1 receptor RAGE mediates ischemic brain damage. *J. Neurosci.* **28**, 12023–12031.

Nishi M., Kawata M. and Azmitia E. C. (1997) S100beta promotes the extension of microtubule associated protein2 (MAP2)-immunoreactive neurites retracted after colchicines treatment in rat spinal cord culture. *Neurosci. Lett.* **229**, 212–214.

Nishi M., Kawata M. and Azmitia E. C. (2000) Trophic interactions between brain-derived neurotrophic factor and s100beta on cultured serotonergic neurons. *Brain Res.* **868**, 113–118.

Nishiyama H., Knopfel T., Endo S. and Itohara S. (2002) Glial protein S100B modulates long-term neuronal synaptic plasticity. *Proc. Natl. Acad. Sci. U S A* **99**, 4037–4042.

Perkins N. D. (2006) Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. *Oncogene* **25**, 6717–6730.

Pichiule P., Chavez J. C., Schmidt A. M. and Vannucci S. J. (2007) Hypoxia-inducible factor-1 mediates neuronal expression of the receptor for advanced glycation end products following hypoxia/ischemia. *J. Biol. Chem.* **282**, 36330–36340.

Qiu J., Nishimura M., Wang Y., Sims J. R., Qiu S., Savitz S. I., Salomone S. and Moskowitz M. A. (2008) Early release of HMGB-1 from neurons after the onset of brain ischemia. *J. Cereb. Blood Flow Metab.* **28**, 927–938.

Ramasamy R., Yan S. F. and Schmidt A. M. (2009) RAGE: therapeutic target and biomarker of the inflammatory response-the evidence mounts. *J. Leukoc. Biol.* **86**, 505–512.

Ramos A. J., Rubio M. D., Defagot C., Hischberg L., Villar M. J. and Brusco A. (2004) The 5HT1a receptor agonist, 8-OH-DPAT, protects neurons and reduces astroglial reaction after ischemic damage caused by cortical devascularization. *Brain Res.* **1030**, 201–220.

Ramos A., Ho W. C., Forte S., Dickson K., Boutilier J., Favell K. and Barker P. A. (2007) Hyposmolarity induces p75ntr expression in central nervous system neurons by activating sp1-dependent transcription. *J. Neurosci.* **27**, 1498–1506.

Reeves R. H., Yao J., Crowley M. R., Buck S., Zhang X., Yarowsky P., Gearhart J. D. and Hilt D. C. (1994) Astrocytosis and axonal proliferation in the hippocampus of S100b transgenic mice. *Proc. Natl. Acad. Sci. U S A* **91**, 5359–5363.

Ridder D. A. and Schwaninger M. (2009) NF-kappaB signaling in cerebral ischemia. *Neuroscience* **158**, 995–1006.

Rong L. L., Gooch C. and Szaboles M. (2005) RAGE: a journey from the complications of diabetes to disorders of the nervous system striking a fine balance between injury and repair. *Restor. Neurol. Neurosci.* **23**, 355–365.

Rothermundt M., Peters M., Prehn J. H. and Arolt V. (2003) S100B in brain damage and neurodegeneration. *Microsci. Res. Tech.* **60**, 614–632.

Sorci G., Agneletti A. L. and Donato R. (2000) Effects of S100A1 and S100B on microtubule stability. An in vitro study using triton-cytoskeletons from astrocyte and myoblast cell lines. *Neuroscience* **99**, 773–783.

Tanaka N., Yonekura H., Yamagishi S., Fujimori H., Yamamoto Y. and Yamamoto H. (2000) The receptor for advanced glycation end products is induced by the glycation products themselves and tumor necrosis factor-alpha through nuclear factor-kappa B, and by 17beta-estradiol through Sp-1 in human vascular endothelial cells. *J. Biol. Chem.* **275**, 25781–25790.

Tramontina A. C., Tramontina F., Bobermin L. D., Zanotto C., Souza D. F., Leite M. C., Nardin P., Gottfried C. and Gonçalves C. A. (2008) Secretion of S100B, an astrocyte-derived neurotrophic protein, is stimulated by fluoxetine via a mechanism independent of serotonin. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **32**, 1580–1583.

Van Eldik L. J. and Griffin W. S. (1994) S100 beta expression in Alzheimer's disease: relation to neuropathology in brain regions. *Biochim. Biophys. Acta* **1223**, 398–403.

Vincent A. M., Perrone L., Sullivan K. A., Backus C., Sastry A. M., Lastoskie C. and Feldman E. L. (2007) Receptor for advanced glycation end products activation injures primary sensory neurons via oxidative stress. *Endocrinology* **148**, 548–558.

Wang L., Li S. and Jungalwala F. B. (2008) Receptor for advanced glycation end products (RAGE) mediates neuronal differentiation and neurite outgrowth. *J. Neurosci. Res.* **86**, 1254–1266.

Xiong Z., O'Hanlon D., Becker L. E., Roder J., MacDonald J. F. and Marks A. (2000) Enhanced calcium transients in glial cells in neonatal cerebellar cultures derived from S100B null mice. *Exp. Cell Res.* **257**, 281–289.

Zhai D. X., Kong Q. F., Xu W. S. *et al.* (2008) RAGE expression is up-regulated in human cerebral ischemia and pMCAO rats. *Neurosci. Lett.* **445**, 117–121.

Zhang W., Potrovita I., Tarabin V., Herrmann O., Beer V., Weih F., Schneider A. and Schwaninger M. (2005) Neuronal activation of NF-kappaB contributes to cell death in cerebral ischemia. *J. Cereb. Blood Flow Metab.* **25**, 30–40.

Zhou F. C., Azmitia E. C. and Bledsoe S. (1995) Rapid serotonergic fiber sprouting in response to ibotenic acid lesion in the striatum and hippocampus. *Dev. Brain Res.* **84**, 89–98.