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A novel role for the Receptor for Advanced Glycation End-products in neural progenitor cells derived from adult SubVentricular Zone

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ABSTRACT

The Receptor for Advanced Glycation End-products (RAGE) is a member of the immunoglobulin superfamily of cell surface receptors which interacts with a wide range of ligands, such as High-Mobility Group Box-1 (HMGB-1), S100B, advanced glycation end-products (AGEs). Here we provided evidence for the restricted expression of RAGE in the undifferentiated neural stem/progenitor cells of mouse adult SubVentricular Zone (SVZ) neurogenic region and adult SVZ-derived neurospheres. Additionally, RAGE ligands stimulated both proliferation and neuronal differentiation of SVZ-derived neural progenitor cells (NPC) *in vitro*. NF-κB nuclear translocation occurred upon RAGE activation in SVZ-derived neurospheres and its blockade (by SN-50) or its absence (in $p50^{-/-}$ derived NPC) resulted in the inhibition of the ligand-mediated effects on neuronal differentiation. These novel findings delineate an interesting scenario where the RAGE-NF-κB axis may contribute to regulate adult neural stem/progenitor cell function in physiological and possibly pathological conditions where this axis is upregulated.

Introduction

The Receptor for Advanced Glycation End-products (RAGE) is a multi-ligand receptor of the immunoglobulin superfamily originally described as a cell surface receptor for molecules derived from non-enzymatic glycation and referred to as Advanced Glycation End-products (AGEs) (Neeper et al., 1992). Since then, additional RAGE ligands were identified including amyloid β (Aβ)-peptides and fibrils, constituents of Alzheimer's disease (AD) amyloid plaques, the alarmin HMGB-1 and the calcium-binding proteins S100/calgranulins (Ding and Keller, 2005). Furthermore RAGE was identified as a counter-receptor for the leucocyte β2 integrin Mac-1 (Chavakis et al., 2003). RAGE exists both as membrane-bound and soluble isoforms (sRAGE), with the latter ones acting as decoy receptors that bind circulating ligands (Neeper et al., 1992; Ding and Keller, 2005). Structurally, membrane-bound full length RAGE (FL-RAGE) consists of three extracellular immunoglobulin-like domains (one V- and two C-types), a single transmembrane domain and a short intracellular C-terminal tail, which is essential for intracellular signalling (Huttunen et al., 1999; Dattilo et al., 2007). sRAGE isoforms can be generated by alternative splicing (Hudson et al., 2008; Kalea et al., 2009) or by proteolytic cleavage of FL-RAGE by ADAM10 (Raucci et al., 2008).

In normal adult tissues, RAGE is usually expressed at low levels, except in the lung where it is quite abundant (Hudson et al., 2008; Kalea et al., 2009). Upon ligand accumulation in a tissue, RAGE is commonly upregulated and produces a sustained cell activation through multiple intracellular signalling pathways (Li and Schmidt, 1997; Bierhaus et al., 2001; Schmidt et al., 2001; Ding and Keller, 2005). A vast array of information has been collected on the possible contribution of RAGE activation to several diseases such as diabetes, AD, atherosclerosis, cancer progression and stroke (Schmidt et al., 2001; Arancio et al., 2004; Ding and Keller, 2005; Fang et al., 2010; Takuma et al., 2009). For such reasons, RAGE is viewed as an attractive target for pharmacological intervention in those disorders (Geroldi et al., 2006; Maczurek et al., 2008).

In the adult central nervous system (CNS), RAGE has been shown to be expressed at low levels by neurons, glia, endothelial cells and it has been involved in multiple events including proliferation, neurite outgrowth, migration and apoptosis (Yan et al., 1996, 1997; Huttunen et al., 2000; Lue et al., 2001; Chou et al., 2004; Schmidt et al., 2007; Qin et al., 2008). Among the signalling pathways which lie downstream RAGE engagement are those involving the NF-κB family of transcription factors, whose role in the CNS is widely recognized (Grilli and Memo, 1999). Additionally, NF-κB p50/p65 dimer has been shown to activate an autoregulatory positive feedback loop whereby RAGE itself is upregulated (Li and Schmidt, 1997; Bierhaus et al., 2001). Recently our group provided evidence for the selective expression of NF-κB transcription factors in neurogenic areas of adult mouse brain (Denis-Donini et al., 2005). Moreover, we demonstrated a selective defect in adult neurogenesis in NF-κB p50 knock-out mice (Denis-Donini et al., 2008). Little is known on the possible involvement of RAGE in adult neurogenesis but, interestingly, Manev et al. (2003) reported that the

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receptor is expressed by BrdU-labelled cells in the hippocampal SubGranular Zone (SGZ) after chronic fluoxetine administration, a treatment known to promote neurogenesis (Santarelli et al., 2003). Based on these observations, we decided to investigate the potential role of the RAGE-NF-κB axis in the modulation of adult neurogenesis. We confirmed that *in vivo* RAGE is expressed by the neural stem/progenitor cells in the neurogenic SVZ region of the adult mouse brain. We also characterized *in vitro* the expression of the membrane-bound FL-RAGE isoform in adult mouse neural progenitor cells (NPC). Moreover, we investigated the functional role of the RAGE-NF-κB axis in the proliferation and differentiation of adult NPC.

Results

RAGE is expressed in vivo in the SVZ of adult mice

Immunohistochemical analysis using an N-terminal anti-RAGE antibody confirmed the presence of the receptor in the adult mouse SVZ region (Fig. 1A–I). To further characterize the RAGE-positive cell population we performed double-labelling experiments with GFAP (Glial Fibrillary Acidic Protein), a marker of adult neural stem cells (or type B cells) and of mature astrocytes, and Sox-2 (anti-SRY-related HMG-box gene 2), a transcription factor expressed in the nucleus of undifferentiated stem/progenitor cells (Graham et al., 2003; Brazel et al., 2005). As shown in Fig. 1, a subpopulation of GFAP-positive cells coexpressed RAGE (Fig. 1A–C), while the majority of Sox-2-positive cells were also immunoreactive for RAGE (Fig. 1D–F). Doublecortin (DCX) is a microtubule-associated protein expressed by immature neuroblasts in adult neurogenic areas (Lois and Alvarez-Buylla, 1994). When RAGE/DCX colabelling was performed, no colocalization was observed in the adult SVZ (Fig. 1G–I). To further characterize the nature of the subpopulation of $GFAP^+/RAGE^+$ cells in the adult SVZ, we then decided to performed a triple-labelling experiment with antibodies against RAGE, GFAP and Sox-2. As shown in Fig. 2A, virtually all $RAGE^+/GFAP^+$ cells were also marked by Sox-2 expression suggesting that they may be identified as undifferentiated neural stem/progenitor cells (Brazel et al., 2005).

In order to further analyze the expression of RAGE in $BrdU^+$ proliferating cells, a group of adult CD1 mice were administered a single injection of bromodeoxyuridine (BrdU, 150 mg/kg, intraperitoneally) and sacrificed 24 h later. As shown in Fig. 2B, a triple-labelling experiment with antibodies against RAGE, GFAP and BrdU demonstrated that BrdU immunoreactivity was absent in the $RAGE^+/GFAP^+$ cells, possibly suggesting that this subpopulation could be identified as quiescent neural stem cells (type B cells). Conversely, triple-labelling experiment with antibodies against RAGE, Sox-2 and BrdU (Fig. 2C) showed that several $RAGE^+/Sox\text{-}2^+$ cells were marked by BrdU, possibly suggesting that RAGE is expressed in a

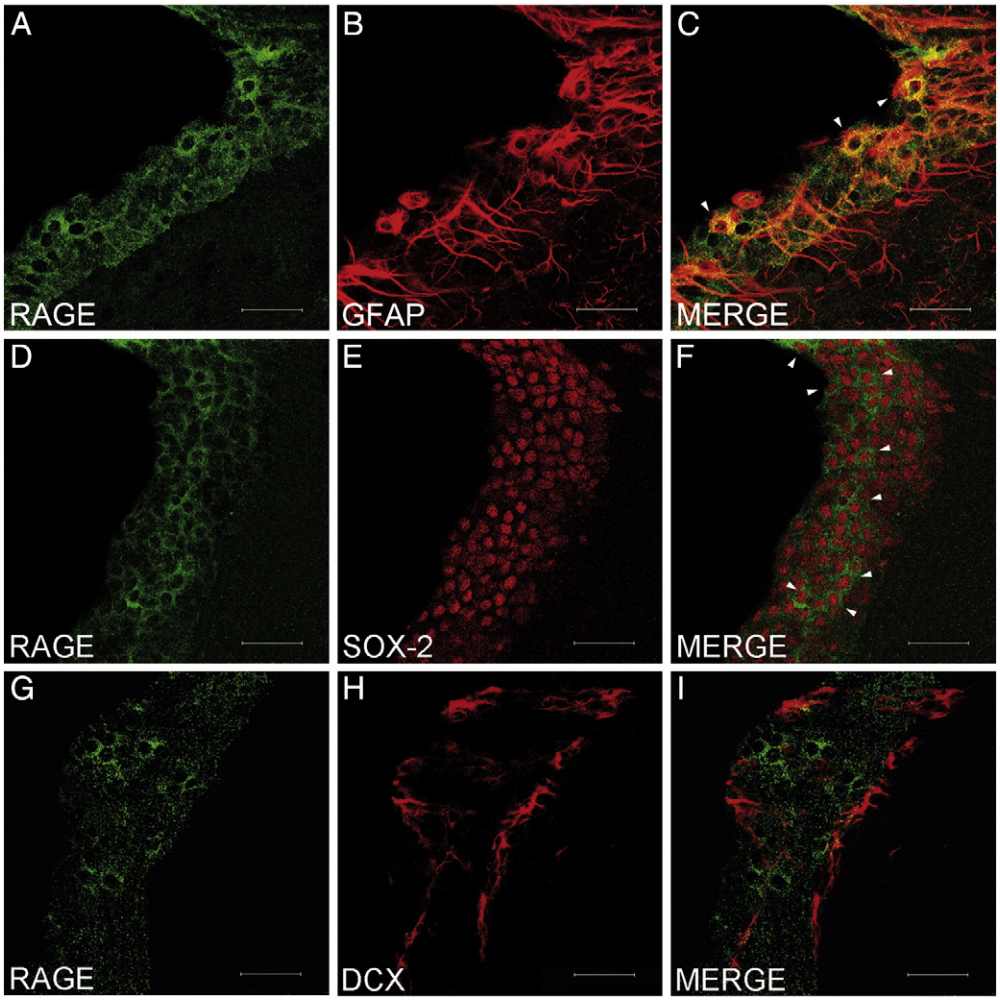

Fig. 1. RAGE is expressed *in vivo* in the neural stem/progenitor cells of the adult SVZ region. (A–I) Representative confocal microscopy images of the adult mouse SVZ region immunostained with antibodies against RAGE (A, D and G, green), GFAP (B, red), a marker of type B cells or mature astrocytes, Sox-2 (E, red), a marker of stem/progenitor cells, or DCX (H, red), a marker of immature neuroblasts. Overlay of the two channels demonstrated that a subpopulation of $GFAP^+$ cells (indicated by arrowheads) coexpressed RAGE (C), the majority of $Sox\text{-}2^+$ cells (indicated by arrowheads) were also immunoreactive for RAGE (F), while no colocalization was observed between RAGE and DCX (I). Magnification = ×800. Scale bars = 37.5 μm.

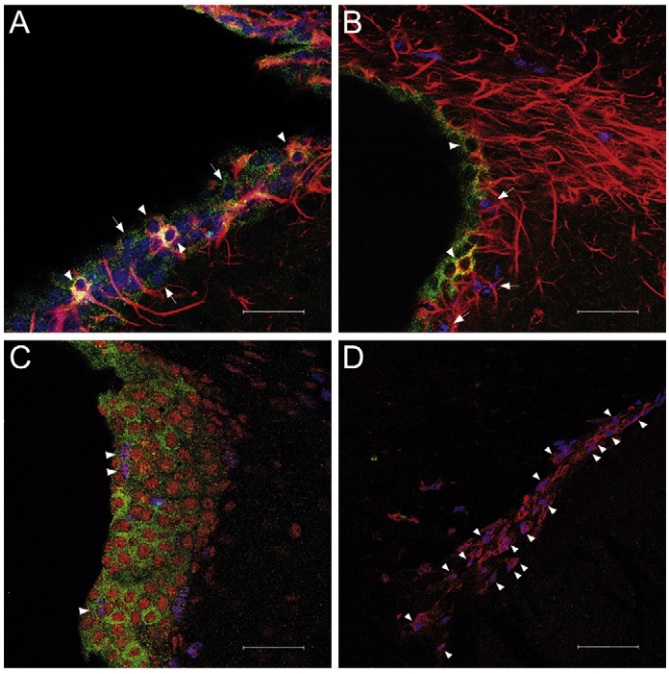

Fig. 2. RAGE is expressed in a subpopulation of quiescent GFAP$^+$/Sox-2$^+$ stem cells and in proliferating Sox-2$^+$ neural progenitors, but not in migrating neuroblasts. (A–C) Representative confocal microscopy images of the adult mouse SVZ region. (A) Immunostaining with antibodies against RAGE (green), GFAP (red) and Sox-2 (blue) demonstrated that RAGE immunoreactivity is detected in GFAP$^+$/Sox-2$^+$ cells (indicated by arrowheads) and GFAP$^-$/Sox-2$^+$ cells (indicated by arrows). (B) Immunostaining with antibodies against RAGE (green), GFAP (red) and BrdU (blue), showed that the receptor is expressed in GFAP$^+$/BrdU$^-$ cells (indicated by arrowheads) but it is absent in GFAP$^+$/BrdU$^+$ cells (indicated by arrows). (C) Immunostaining with antibodies against RAGE (green), Sox-2 (red) and BrdU (blue) demonstrated RAGE expression in a subpopulation of Sox-2$^+$/BrdU$^+$ cells (indicated by arrowheads). (A–C) Magnification = ×800. Scale bars = 37.5 μm. (D) Representative confocal microscopy images of the adult mouse RMS immunostained with antibodies against RAGE (green), DCX (red) and BrdU (blue) showed the absence of RAGE immunoreactivity in BrdU$^+$/DCX$^+$ migrating neuroblasts (indicated by arrowheads). Magnification = ×400. Scale bar = 75 μm.

subpopulation of transit amplifying progenitor cells, such as type C cells. DCX is considered to be a marker of migrating neuroblasts, so we also performed a triple-staining with antibodies against RAGE, BrdU and DCX (Fig. 2D) and we analyzed the rostral migratory stream (RMS), the well-defined route whereby newborn neuroblasts generated in the SVZ reach their final destination, the olfactory bulb (OB). As expected, several BrdU$^+$ cells were seen in the RMS, with the majority of them coexpressing DCX, while RAGE immunoreactivity was totally absent (Fig. 2D).

By Western blot analysis, the presence of the post-translationally modified (54 kDa) FL-RAGE protein was confirmed in SVZ protein extracts by using two distinct commercially available antibodies which recognize different N- and C-terminal epitopes (Fig. 3A). Lung extracts were used as positive control.

RAGE is expressed by undifferentiated nestin and Sox-2 positive cells in SVZ-derived neurospheres

We decided to further investigate the functional significance of RAGE expression in the SVZ taking advantage of neurosphere cultures, a widely utilized *in vitro* model of adult neural progenitor cells (NPC). Western blot analysis confirmed the presence of FL-RAGE protein in SVZ-derived neurosphere extracts by using two antibodies which recognize different N- and C-terminal epitopes (Fig. 3A). Additionally, FL-RAGE expression in NPC was confirmed by RT-PCR analysis (Fig. 3B). Immunocytochemical analysis of non permeabilized SVZ-derived neurospheres by an antibody which recognizes a N-terminal extracellular receptor epitope confirmed that RAGE immunoreactivity localized at the plasma membrane of neurosphere cells (Fig. 3C–N). SVZ-derived neurospheres grown in presence of epidermal growth factor (EGF) and fibroblast growth factor (FGF) are mainly composed of undifferentiated neural progenitor cells expressing markers such as the intermediate neurofilament nestin (Fig. 3D and G) and Sox-2 (Fig. 3J and M). Double-immunolabelling experiments and confocal z-plane analysis confirmed that RAGE-positive cells coexpressed nestin (Fig. 3E and H) and Sox-2 (Fig. 3K and N), suggesting that, in line with the data reported *in vivo*, they are undifferentiated neural progenitor cells.

HMGB-1, S100B and AGE-BSA stimulate neural progenitor cell proliferation via RAGE activation

During this study, we evaluated the effect of 96 h-exposure to three different RAGE ligands on NPC proliferation. Concentrations of HMGB-1 ranging from 1 to 10 ng/ml (40 pM–0.4 nM) significantly increased proliferation with a maximal effect (20.70% ± 2.27) at 1 ng/ml (Fig. 4A; $n = 15$, $p < 0.001$). Similar results were obtained when cells were treated with S100B. Nanomolar concentrations (0.3–30 nM) of the ligand significantly increased proliferation (at least $p < 0.01$) with a maximal effect (20.62% ± 1.18) elicited at 3 nM (Fig. 4B; $n = 12$, $p < 0.001$). Finally, increasing concentrations of Glycated-Bovine Serum Albumin (AGE-BSA), from 25 to 100 μg/ml (371 nM–1.49 μM), induced proliferation in a dose-dependent manner, with a maximal effect (19.41% ± 3.55) at 100 μg/ml (Fig. 4C; $n = 8$, $p < 0.01$). To investigate whether RAGE ligands were involved in the proliferation of NPC or in their survival, a time-course experiment was also undertaken. As shown in Fig. 4D, the growth curve in vehicle-treated NPC suggested a relatively stationary phase up to 48 h followed by an exponential phase up to 96 h. No difference between vehicle and ligand-treated cells could be seen up to 48 h. Compared to vehicle-treated cells, HMGB-1 (1 ng/ml) produced a statistically significant increase at 72 h ($p < 0.01$) and at 96 h ($p < 0.001$), while S100B (3 nM) and AGE-BSA (50 μg/ml) only at 96 h ($p < 0.001$ and $p < 0.01$, respectively). In order to confirm RAGE involvement in the ligand-mediated effects, cells were treated for 96 h with RAGE ligands in absence or presence of 20 μg/ml of a neutralizing anti-RAGE antibody. Effects of 3 ng/ml HMGB-1 (Fig. 4E), 3 nM S100B (Fig. 4F) and 50 μg/ml AGE-BSA (Fig. 4G) were significantly inhibited by the neutralizing antibody ($n = 5$, $p < 0.01$ for HMGB-1 and S100B; $p < 0.001$ for AGE-BSA), confirming that they were indeed receptor-mediated. Interestingly, when applied alone, the neutralizing anti-RAGE antibody did not affect neurosphere proliferation (*data not shown*).

HMGB-1, S100B and AGE-BSA promote neuronal differentiation of neural progenitor cells

Upon removal of EGF and FGF from the medium, neural progenitor cells derived from SVZ neurospheres stop dividing and start to differentiate. By double immunolabelling for markers of mature neurons (Microtubule-Associated Protein 2, MAP-2) and glial cells (GFAP), we could evaluate *in vitro* the differentiation of NPC toward the two lineages. After 22 h in absence of growth factors, neurosphere-derived cells mainly differentiate towards the neuronal lineage, giving rise to 42.17% ± 2.45 of MAP-2$^+$ neurons and 5.87% ± 2.37 of GFAP$^+$ glial cells, over the total number of viable cells (Fig. 5A). Under these experimental conditions, we evaluated the effect of RAGE ligands on NPC differentiation. As shown in Fig. 5B, 1 ng/ml HMGB-1 had a remarkable pro-neurogenic effect, as revealed by double MAP-2/GFAP immunolocalization. With all RAGE ligands, tested at concentrations which are active on proliferation, a significant increase in the number of MAP-2$^+$ neurons was observed in comparison with vehicle-treated cells (% of MAP-2$^+$ neurons over total viable cells: 42.15% ± 2.33; 71.94% ± 3.32; 68.77% ± 2.79; 70.46% ± 2.12 for vehicle, 3 nM S100B,

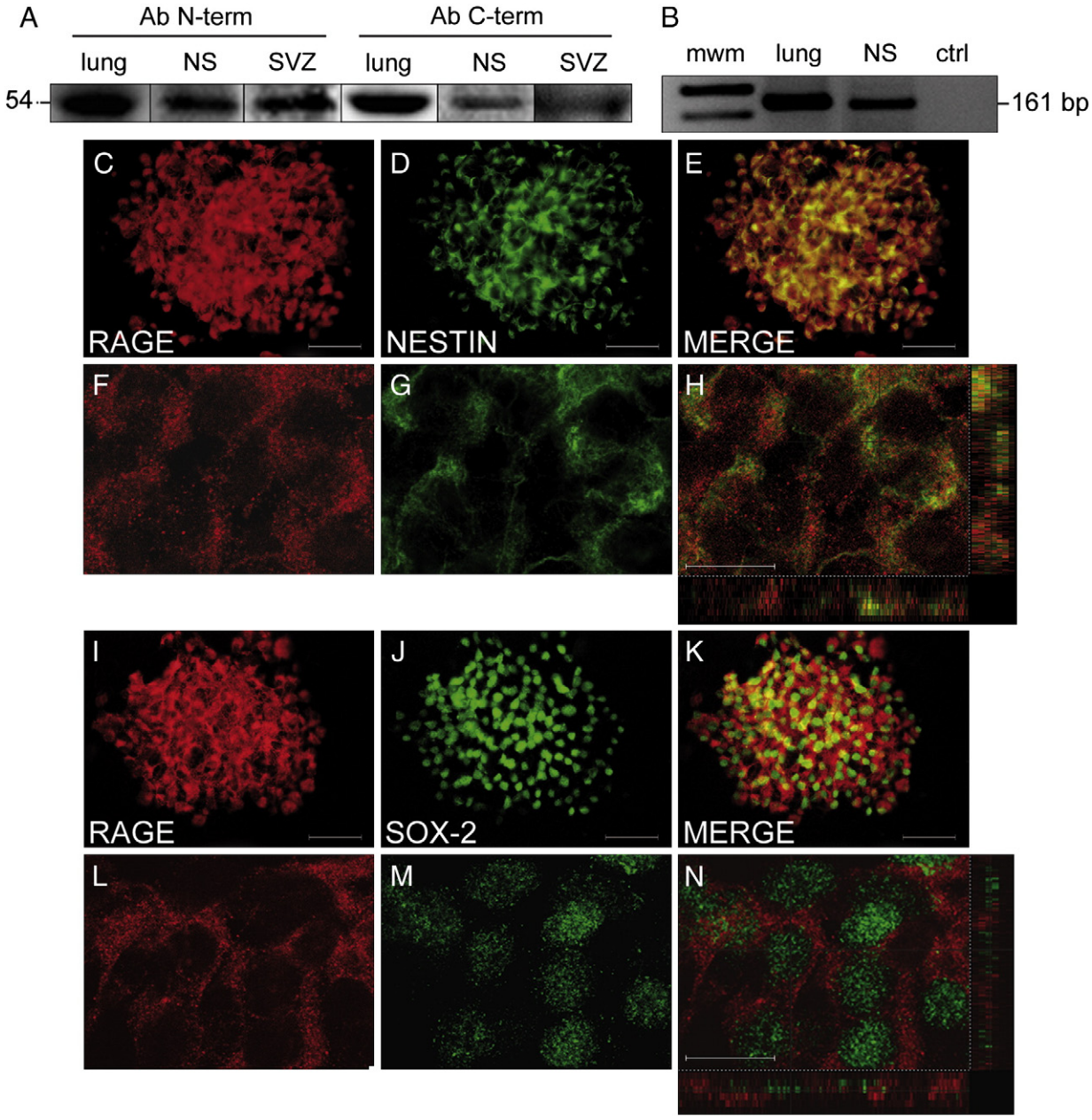

Fig. 3. Expression of FL-RAGE in adult SVZ region and in SVZ-derived neurospheres. (A) Representative immunoblots confirming the presence of the post-translationally modified form (54 kDa) of FL-RAGE in extracts from undifferentiated SVZ-derived neurospheres (NS), SVZ or lung. Two antibodies recognizing different epitopes ("Ab N-term" and "Ab C-term") gave comparable results. (B) RT-PCR analysis of FL-RAGE expression in SVZ-derived neurospheres (NS) and in lung cDNA. A 161 bp PCR product was detected, corresponding to FL-RAGE isoform. PCR reaction without cDNA (ctrl) served as negative control. The 50 bp DNA ladder was used as molecular weight marker (mwm). (C–N) Representative fluorescent microscopy images (C–E and I–K) and confocal z-plane analysis (F–H and L–N) of neurospheres immunolabelled with antibodies against RAGE (C, F, I and L, red), nestin (D and G, green) and Sox-2 (J and M, green). The merged images showed that RAGE immunoreactivity colocalized with nestin (E and H) and Sox-2 (K and N) in SVZ neurosphere cells. (C–E and I–K) Magnification = ×600. Scale bars = 56.25 μm. (F–H and L–N) Scale bars = 11.5 μm.

1 ng/ml HMGB-1 and 50 μg/ml AGE-BSA, respectively. $n = 3$, $p < 0.001$ vs vehicle) (Fig. 5C). Conversely, no effect was elicited by RAGE ligands on the number of newly generated $GFAP^+$ astrocytes, compared to vehicle-treated cells (Fig. 5D; $n = 3$). Within the time frame of our experimental conditions no MBP^+ oligodendrocytes can be observed since oligodendrogenesis requires several days *in vitro* to occur. We therefore investigated the expression of NG2, a marker of oligodendrocyte precursors. We quantified the number of $NG2^+$ positive cells after 22 h treatment with HMGB-1, S100B, AGE-BSA at concentrations that were active on neuronal differentiation and we did not observe any effect compared to vehicle-treated cells (Fig. 5D; $n = 3$).

As shown in Fig. 5E, HMGB-1 effects on neuronal differentiation were receptor-mediated since prevented by pretreating cells with a neutralizing anti-RAGE antibody ($n = 3$, $p < 0.001$). Similar results were obtained by antibody pretreatment on AGE-BSA and S100B-mediated neuronal differentiation (*data not shown*). When applied alone, the neutralizing anti-RAGE antibody did not affect NPC differentiation (Fig. 5E). To investigate whether RAGE activation could affect the survival of NPC or neuroblasts, we evaluated the activity of lactate dehydrogenase (LDH) in the differentiation medium after 22 h in presence of RAGE ligands or vehicle. As shown in Fig. 5F, no difference in the LDH activity was observed in the media of vehicle-

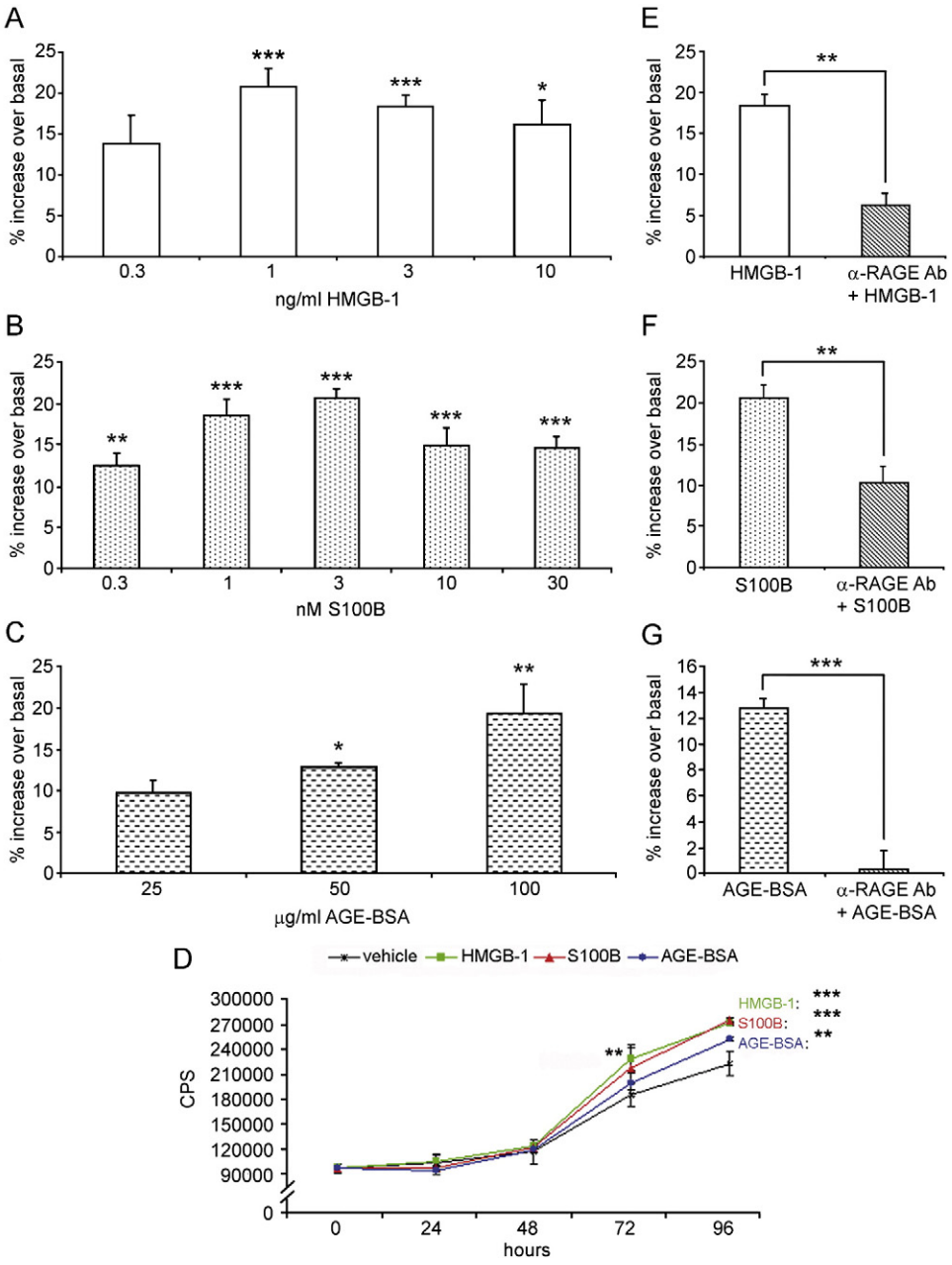

Fig. 4. RAGE activation stimulates the proliferation of adult NPC. (A–C) 96 h treatment with indicated amounts of HMGB-1 (A), S100B (B) and AGE-BSA (C) resulted in statistically significant increase in NPC proliferation. Data, expressed as % increase over basal (vehicle-treated value), represent the mean ± s.e.m. of experiments (n = 15 for HMGB-1; n = 12 for S100B; n = 8 for AGE-BSA), run in triplicates. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ vs vehicle (one-way ANOVA and post-hoc Tukey's test). (D) NPC were treated with 1 ng/ml HMGB-1, 3 nM S100B, 50 µg/ml AGE-BSA or vehicles in a time-course proliferation experiment. Data, expressed as counts *per* second (CPS), represent the mean ± s.e.m. of n = 3 experiments, run in triplicates. $^{**}p<0.01$; $^{***}p<0.001$ vs vehicle (Student's *t*-test). (E–G) 1 h pretreatment with a neutralizing anti-RAGE antibody (α-RAGE Ab, 20 µg/ml) counteracted the proliferative effects of 3 ng/ml HMGB-1 (E), 3 nM S100B (F) and 50 µg/ml AGE-BSA (G) on SVZ-derived NPC. Data, expressed as % increase over basal (vehicle-treated), represent the mean ± s.e.m. of n = 5 experiments, run in triplicates. $^{**}p<0.01$; $^{***}p<0.001$ (Student's *t*-test).

or RAGE ligand-treated cells. Additionally, we analysed the number of apoptotic nuclei in SVZ derived NPC differentiated in presence of vehicle or RAGE ligands. As shown in Fig. 5G, no difference was observed in the percentage of apoptotic nuclei over the total nuclei number in vehicle- versus RAGE ligand-treated cells.

A functional RAGE-NF-κB axis in adult neural progenitor cells

NF-κB p50/p65 dimer-mediated signalling lies downstream RAGE activation (Li and Schmidt, 1997; Bierhaus et al., 2001). Therefore we decided to investigate the presence of a functional RAGE-NF-κB axis in adult neural progenitor cells. *In vivo* double RAGE/p65 immunolocalization experiments confirmed that the majority of RAGE-expressing cells of the adult SVZ costained for cytoplasmic NF-κB p65 (Fig. 6A–C). *In vitro*, NF-κB p65 immunoreactivity was detected in the cytoplasm of undifferentiated neurosphere cells (Fig. 6D) and its nuclear translocation was observed within 30 min after exposure to 10 ng/ml tumor necrosis factor-α (TNF-α), used as a positive control (Fig. 6E). When adult SVZ-derived neurospheres were exposed for 30 min to concentrations of RAGE ligands active on NPC proliferation and differentiation, namely 1 ng/ml HMGB-1 (Fig. 6F), 3 nM S100B (Fig. 6G) and 50 µg/ml AGE-BSA (Fig. 6H), NF-κB p65 nuclear translocation was observed. These data demonstrated that also in adult SVZ-derived NPC RAGE activation is coupled to NF-κB signalling. Furthermore, under differentiating conditions, 1 h-pretreatment of NPC with 10 µg/ml SN-50, a cell-permeable peptide which inhibits NF-κB p50 nuclear translocation

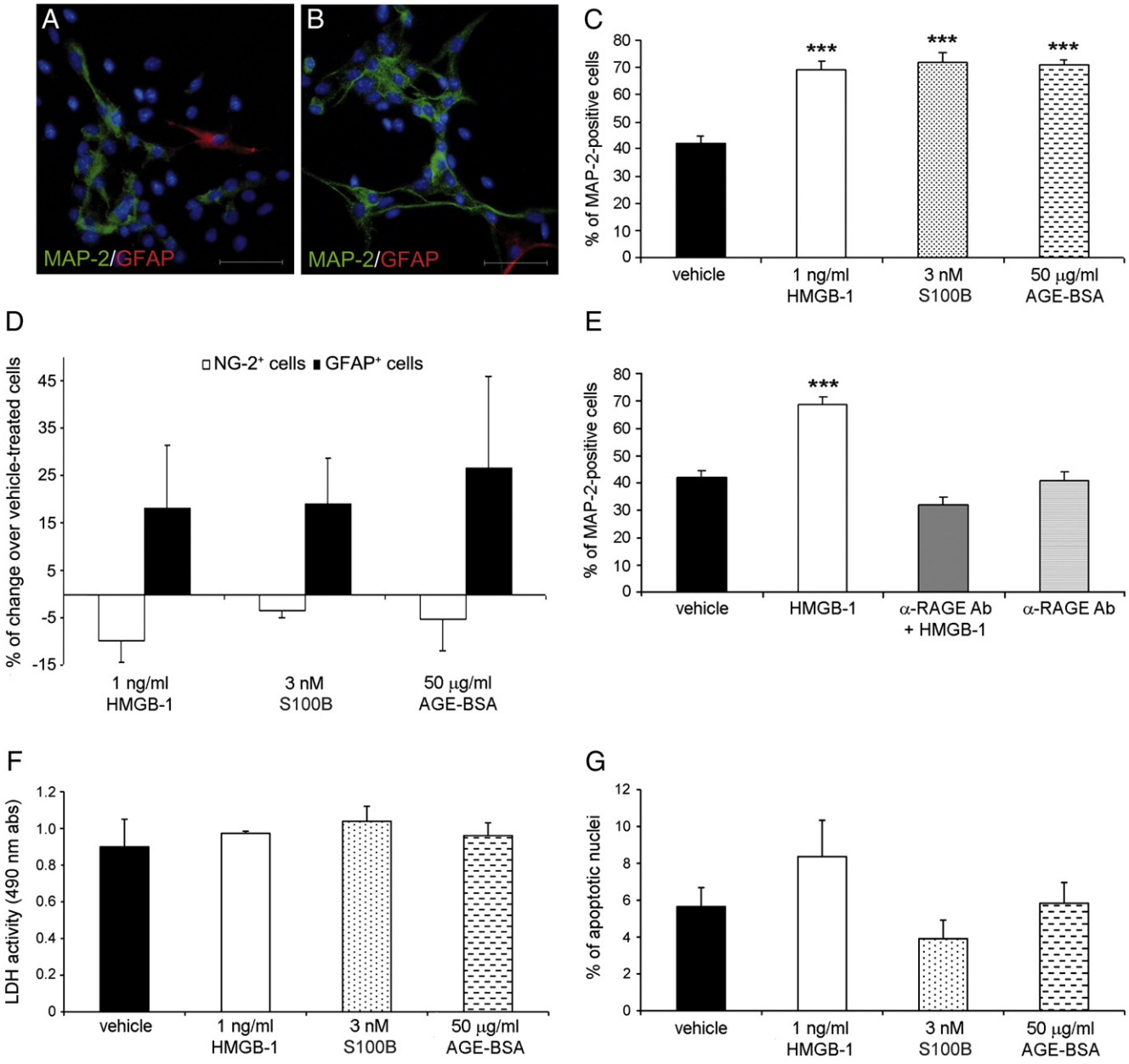

Fig. 5. RAGE activation promotes neuronal differentiation of adult NPC. (A–B) Representative fluorescent microscopy images of MAP-2$^+$ neurons (green) and GFAP$^+$ glial cells (red) derived from NPC after 22 h treatment with vehicle (A) or 1 ng/ml HMGB-1 (B). Nuclei were stained with Hoechst (blue). Magnification = ×1000. Scale bars = 33.75 μm. (C) 22 h treatment with indicated concentrations of RAGE ligands significantly increased the percentage of NPC-derived MAP-2$^+$ neurons compared to vehicle-treated cells. Data are expressed as % of MAP-2-positive cells over total viable cells and represent the mean ± s.e.m. of n = 3 experiments, run in triplicates. ***$p<0.001$ vs vehicle (Student's *t*-test). (D) 22 h treatment with indicated concentrations of RAGE ligands did not significantly change the number of NG-2$^+$ oligodendrocyte precursors (white bars) or GFAP$^+$ glial cells (black bars) compared with vehicle-treated cells. Data are expressed as % of change over vehicle-treated cells and represent the mean ± s.e.m. of n = 3 experiments, run in triplicates. (E) 1 h pretreatment with a neutralizing anti-RAGE antibody (α-RAGE Ab, 20 μg/ml) abolished the effect of 1 ng/ml HMGB-1 on neuronal differentiation of SVZ-derived NPC. When applied alone, the neutralizing anti-RAGE antibody did not affect neuronal differentiation of NPC. Data are expressed as % of MAP-2-positive cells over total viable cells and represent the mean ± s.e.m. of n = 3 experiments, run in triplicates. ***$p<0.001$ vs vehicle (Student's *t*-test). (F) Evaluation of LDH activities in conditioned media of differentiated cell cultures treated for 22 h with vehicle or RAGE ligands. No significant difference in LDH activity was observed in the media of vehicle- or RAGE ligands-treated cells. (G) Evaluation of the percentage of apoptotic nuclei in differentiated cell cultures treated with vehicle or RAGE ligands for 22 h. No significant difference was observed comparing vehicle- or RAGE ligands-treated cells. (F–G) Data represent the mean ± s.e.m. of n = 3 experiments, run in triplicates.

(Lin et al., 1995), completely prevented the increase in the number of MAP-2$^+$ neurons induced by all RAGE ligands (Fig. 6I–M; n = 3, $p<0.001$). As a control, cell culture treatment with 10 μg/ml of SN-50 M inactive peptide (Lin et al., 1995) did not counteract RAGE ligand-mediated effects on NPC neuronal differentiation (Fig. 6M). To confirm the involvement of NF-κB in the effects of RAGE ligands, we utilized wild-type (wt) and NF-κB p50$^{-/-}$ derived SVZ neurospheres. Under basal conditions, no difference in neuronal differentiation of SVZ-derived NPC was observed between the two genotypes (Fig. 6N; n = 3). As expected, in NPC from wt mice RAGE ligands significantly increased the percentage of MAP-2$^+$ neurons over the total viable cells (Fig. 6N; n = 3, $p<0.01$ for 1 ng/ml HMGB-1 and $p<0.001$ for 3 nM S100B and 50 μg/ml AGE-BSA). Conversely, the receptor activation by RAGE ligands produced no effect on neuronal differentiation of NF-κB p50$^{-/-}$-derived SVZ neural progenitor cells (Fig. 6N; n = 3).

Overall, these data confirmed the involvement of NF-κB signalling, and in particular of p50-containing dimers, in the RAGE-mediated effects on differentiation of adult SVZ-derived neural progenitor cells.

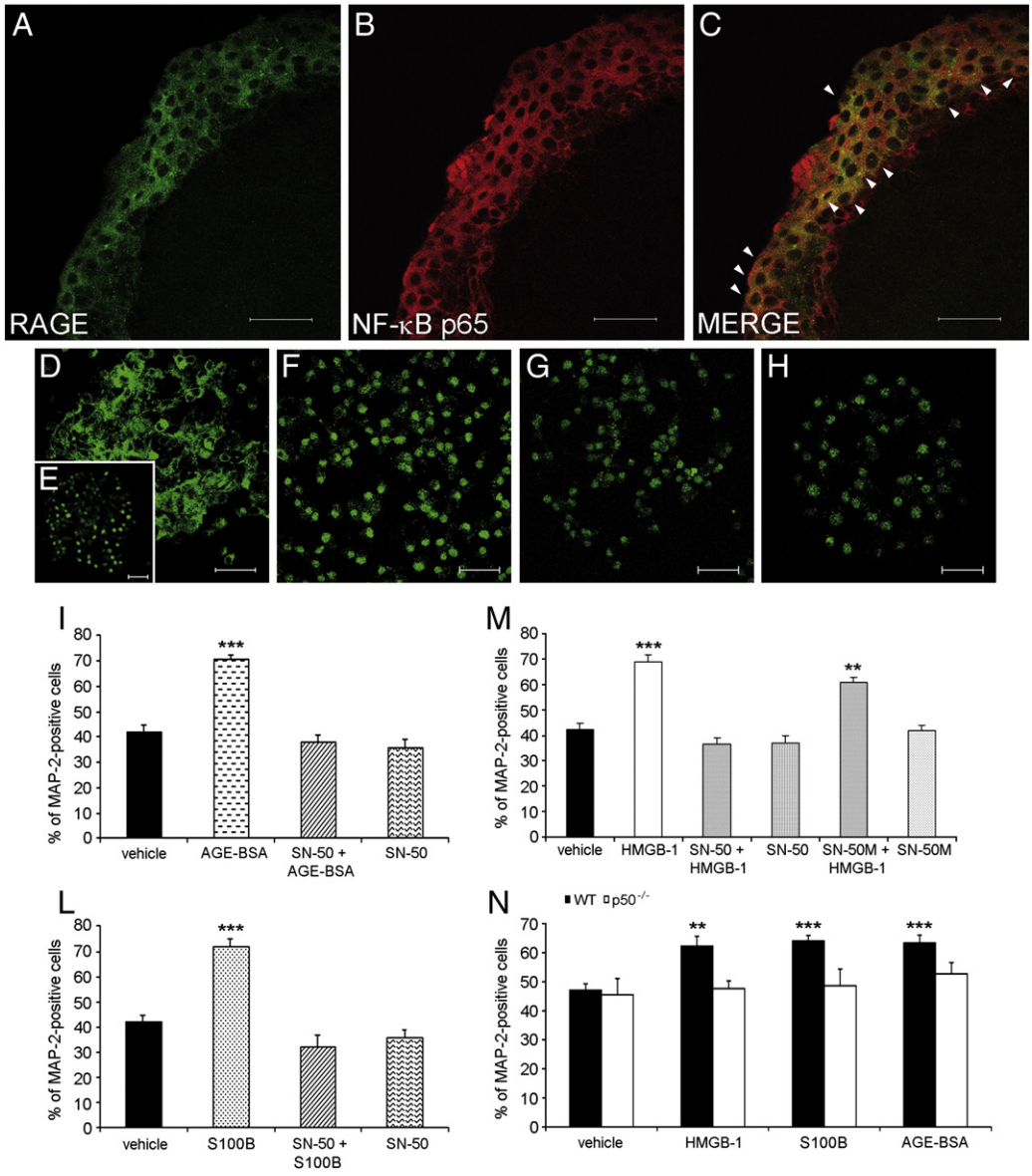

Fig. 6. Functional RAGE-NF-κB axis in adult neural progenitor cells. (A–C) Representative confocal microscopy images of adult mouse SVZ region immunostained with antibodies against RAGE (A, green) and NF-κB p65 (B, red). Overlay of the two channels (C) confirmed RAGE colocalization in p65-expressing cells (indicated by arrowheads). Magnification = ×800. Scale bars = 37.5 μm. (D–H) RAGE ligands induced NF-κB p65 nuclear translocation in undifferentiated SVZ-derived neurospheres. Confocal microscopy images showing cytoplasmatic (D) or nuclear (E–H) NF-κB p65 mmunostaining. Neurospheres were exposed for 30 min to vehicle (D), 10 ng/ml TNF-α (E), or to 1 ng/ml HMGB-1 (F), 3 nM S100B (G), 50 μg/ml AGE-BSA (H). Magnification = ×800. Scale bars = 37.5 μm. (I–M) The inhibition of NF-κB nuclear translocation by 1 h pretreatment with SN-50 peptide (10 μg/ml) completely prevented the increase in MAP-2$^+$ neurons induced by 50 μg/ml AGE-BSA (I), 3 nM S100B (L) and 1 ng/ml HMGB-1 (M). When applied alone, SN-50 peptide did not affect the differentiation of NPC (I–M). SN-50 M (negative control peptide) did not counteract HMGB-1-mediated effect on NPC neuronal differentiation (M). (N) 22 h treatment with 1 ng/ml HMGB-1, 3 nM S100B and 50 μg/ml AGE-BSA or vehicle of wt and NF-κB p50$^{-/-}$ SVZ NPC. Compared to vehicle, RAGE ligands significantly increased the percentage of MAP-2$^+$ neurons from wt but not in p50$^{-/-}$ derived SVZ neural progenitor cells. (I–N) Data are expressed as % of MAP-2-positive cells over total viable cells and represent the mean ± s.e.m. of n = 3 experiments, run in triplicates. $^{**}p<0.01$, $^{***}p<0.001$ vs vehicle (Student's *t*-test).

Discussion

RAGE has been extensively investigated for its potential contribution to chronic disorders such as diabetes, atherosclerosis, cancer and AD, as well as to acute conditions such as cardiac ischemia, stroke and trauma (Arancio et al., 2004; Rong et al., 2005; Muhammad et al., 2008; Fang et al., 2010; Hassid et al., 2009; Riehl et al., 2009; Takuma et al., 2009; Yan et al., 2009). Within the CNS, RAGE is expressed on neurons, glia, endothelial cells and it was demonstrated to be involved in multiple biological events including cell proliferation, neurite outgrowth, migration, apoptosis (Yan et al., 1996, 1997; Huttunen et al., 1999, 2000; Lue et al., 2001; Chou et al., 2004; Schmidt et al., 2007; Qin et al., 2008). Now we propose a novel functional role for RAGE in CNS, namely the modulation of proliferation and neuronal differentiation of adult neural progenitor cells. Here we provided evidence for the expression of RAGE in the adult murine SVZ neurogenic region and in adult SVZ-derived neurospheres. In particular, we demonstrated that *in vivo* RAGE immunoreactivity was present in GFAP$^+$/Sox-2$^+$ neural stem cells, characterized by a low proliferation rate (RAGE$^+$/GFAP$^+$/BrdU$^-$ cells) as reported in quiescent type B cells, and in the majority of transit amplifying Sox-2$^+$/BrdU$^+$ neural progenitor cells (likely corresponding to type C cells) of the adult SVZ. In parallel, RAGE was expressed by nestin$^+$ and Sox-2$^+$ progenitor cells in SVZ derived

neurospheres. On the other hand, we could not detect RAGE immunoreactivity in newborn DCX$^+$ migrating neuroblasts in SVZ and RMS. Since RAGE expression was absent in OB of adult mice and in MAP-2$^+$ mature neurons derived from the differentiation of SVZ neurospheres (*data not shown*), altogether these data support the idea that the receptor expression is restricted to SVZ undifferentiated stem/progenitor cells. Additionally, we demonstrated that RAGE ligands such as HMGB-1, S100B and AGE-BSA stimulated proliferation of NPC *in vitro*. When SVZ-derived NPC were cultured under differentiating conditions, the activation of the receptor significantly increased the number of newly generated MAP-2$^+$ neurons, without any relevant effect on GFAP$^+$ astrocytes or NG2$^+$ oligodendrocyte progenitors. Additionally, no significant differences were observed in the number of apoptotic nuclei and in LDH activity in conditioned media of NPC cells differentiated in presence of RAGE ligands or vehicle, suggesting that RAGE activation did not affect survival of NPC and/or neuroblasts and/or post-mitotic neurons. Both proliferation and neuronal differentiation properties of RAGE ligands appeared to be receptor-mediated since they were inhibited by the pretreatment with a neutralizing anti-RAGE antibody. Neurogenesis is a process resulting from the complex interplay of proliferation, differentiation and cell survival/death events. The available literature data show that in some instances, increased proliferation of SVZ neural stem/progenitor cells can result in reduced neuroblast formation (Kuhn et al., 1997; Jackson et al., 2006). Conversely, several *in vivo* and *in vitro* studies demonstrated that the two events could also proceed in parallel. For example, the dopamine receptor agonists increased both the proliferation and the neuronal differentiation of SVZ progenitors both *in vitro* and *in vivo* (O'Keeffe et al., 2009; Winner et al., 2009).

We also provided evidence for the presence of a functional RAGE-NF-κB axis in adult NPC. NF-κB p65 nuclear translocation occurred upon RAGE activation in SVZ-derived neurospheres and blockade of NF-κB p50 translocation by SN-50 peptide resulted in inhibition of ligand-mediated effects on neuronal differentiation of neural progenitor cells. To further confirm NF-κB involvement, RAGE activation significantly enhanced neuronal differentiation in wt NPC-derived cells, while p50-deficient NPC were unresponsive. Finally, our *in vitro* observations were corroborated by *in vivo* data demonstrating the presence of NF-κB p65 immunoreactivity in RAGE-positive stem/progenitor cells of adult mouse SVZ neurogenic niche.

Our data on a RAGE-NF-κB axis positively modulating SVZ neurogenesis are in line with other results showing that NF-κB signalling is involved in either differentiation and/or proliferation of NPC. Bernardino et al. (2008) demonstrated that the activation of tumor necrosis factor receptor 1 (TNFR1), which in turn activates NF-κB, increased both proliferation and neuronal differentiation of neurospheres derived from neonatal SVZ. Similar results were obtained by Widera et al. (2006) on neurospheres derived from adult SVZ. *In vivo*, Shingo et al. (2001) demonstrated that erythropoietin positively regulates the neuronal differentiation in the adult SVZ through a homeostatic autocrine/paracrine mechanism resulting in NF-κB activation.

Interestingly, available literature data point to more complex activities of the NF-κB signalling in the adult hippocampal neurogenic region. As an example, activation of TNFR1 and interleukin 1 (IL-1) receptors, which are upstream of NF-κB activation, resulted in inhibition of hippocampal neurogenesis (Iosif et al., 2006; Kaneko et al., 2006; Goshen et al., 2008). Rolls et al. (2007) demonstrated both *in vitro* and *in vivo* that Toll-like receptors (TLR) 2 and 4 showed distinct and opposite effects on the adult hippocampal neurogenesis by triggering MyD88 and Protein Kinase C α/β-dependent activation of the NF-κB signalling pathway. Our group recently demonstrated a selective defect in adult hippocampal neurogenesis in NF-κB p50 knock-out mice, possibly occurring at the transition between the maturation stages of newly born neuroblasts/neurons marked by the expression of DCX and Calretinin (Denis-Donini et al., 2008).

These results are not surprising. The neural stem/progenitor cells of the two neurogenic areas likely represent distinct cell populations, endowed with different differentiative potential, which differentially respond to physiological and pathological stimuli. Interestingly, we have recently evaluated if any alteration was present in the SVZ region of NF-κB p50$^{-/-}$ mice. No defect could be observed in the SVZ and in the OB of p50$^{-/-}$ compared to wt mice, revealing a hippocampus-specific influence of this NF-κB family member (Grilli et al., data unpublished).

Additionally, NF-κB is a family of transcription factors whose members can differently combine to form hetero- and homo-dimers of different composition (Grilli et al., 1993). Their regulatory specificity is dependent on their distinct affinities for cofactors and κB consensus elements in target genes and therefore they can elicit multiple, even opposite, functions within the same cell type in the CNS (Leung et al., 2004; Pizzi and Spano, 2006). In this complex context, the involvement of NF-κB in the regulation of adult neurogenesis should also take into account that distinct members of the family may be involved in fine tuning of adult neurogenesis, with dimers of different composition playing different and even opposite role in neural stem/progenitor cells.

The functional significance of the RAGE-NF-κB axis in adult SVZ deserves further investigation but at this stage hypothesis can be made. A typical feature of RAGE in the brain is that it is expressed at relatively low levels in physiological conditions while its expression is strikingly increased in situations characterized by enhanced cellular activation or stress, like inflammation and neurodegeneration associated with several neurological disorders. As an example, RAGE expression is increased in human and experimental stroke (Ma et al., 2003; Zhai et al., 2008). Additionally, after acute brain damage, RAGE ligands such as HMGB-1 and S100B are released by necrotic cells and inflammatory cells (Rothermundt et al., 2003; Goldstein et al., 2006; Kim et al., 2006; Mori et al., 2008; Qiu et al., 2008). Cerebral ischemia is also associated with strong NF-κB activation in neurons, astrocytes, microglia, and infiltrating inflammatory cells (Ridder and Schwaninger, 2009). In turn, NF-κB activation causes the receptor up-regulation through a feed forward signal transduction mechanism, since the presence of NF-κB binding sites in the RAGE promoter region (Li and Schmidt, 1997). According to the general view, in acute brain damage activation of the RAGE-NF-κB axis is deleterious (Mori et al., 2008; Muhammad et al., 2008; Hassid et al., 2009). Recently, Muhammad et al. (2008) provided evidence that in stroke the HMGB-1-RAGE signalling links necrosis with apoptosis and suggested that the receptor may represent a valuable target for anti-inflammatory therapy in cerebral ischemia. Additionally, a potential contribution of AGEs to neurotoxicity and brain damage during ischemic stroke has been suggested (Zimmerman et al., 1995).

The novel finding of a functional RAGE-NF-κB axis in adult neural progenitor/stem cells adds complexity to the scenario of RAGE contribution to brain damage. Indeed there is a substantial evidence supporting enhanced cell proliferation after injury in regions of the adult brain known to harbour neural stem/progenitor cells, and in particular in the SVZ region (Jin et al., 2001; Chen et al., 2003; Romanko et al., 2004; Jin et al., 2006). Additionally, a number of studies indicate that after ischemic and traumatic brain injury the cells of adult SVZ not only have the capacity to replenish their own numbers, but also to replace neurons and glia (Chen et al., 2003). Moreover, intracerebroventricular (icv) injection of S100B following traumatic brain injury resulted in strongly increased hippocampal neurogenesis (Kleindienst et al., 2005). In particular Kleindienst and colleagues showed that icv administration of S100B significantly increased both the number of BrdU-positive cells and the percentage of BrdU$^+$/NeuN$^+$ neurons in the adult hippocampal neurogenic region. They also quantified effects of icv injection of S100B in the SVZ and reported a small but statistically not significant increase in the number of BrdU-positive cells in this region (Kleindienst et al.,

2005). Our *in vitro* data showed that RAGE ligands induced a small increase of NPC proliferation and a dramatic increase of neuronal differentiation in SVZ NPC, suggesting that this latter effect is predominant. Unfortunately, Kleindienst et al. (2005) did not analyse neuronal differentiation in this neurogenic area. Despite this, preliminary data in our laboratory report that the RAGE-NF-κB axis is also functionally active in adult hippocampal NPC (*data not shown*), suggesting that S100B may actually promote neurogenesis in this area via NF-κB.

Overall, the presence of RAGE in the SVZ region of adult mice may suggest a potential role of the multiligand receptor in a pro-neurogenic response to brain injury, possibly via NF-κB-mediated transcription. This hypothesis is in line with recent experimental evidence obtained by our group that the NF-κB family of transcription factors is involved in adult neurogenesis (Denis-Donini et al., 2008). Furthermore, the data presented in this paper suggest the Janus-faced nature of RAGE in situations of brain damage. Indeed, in the area of inflammation and neurodegeneration, RAGE axis activation in inflammatory cells such as macrophages/microglia appears to be detrimental (Muhammad et al., 2008). Conversely, in the SVZ stem/progenitor cells RAGE axis activation in response to damage may potentially contribute to endogenous mechanisms of repair, such as neurogenesis.

RAGE is currently viewed as an attractive target for pharmacological intervention in several neurological disorders including acute brain damage. The mechanisms behind the complex RAGE-mediated responses would imply the need to search for agents that may inhibit RAGE detrimental and maladaptive effects without compromising the potentially adaptive ones like neurogenesis.

Experimental methods

Animals

Adult (4–6 month-old) male CD1 mice, purchased from Charles River Laboratories, and adult (4–6 month-old) NF-κB $p50^{-/-}$ (B6; 129P2-*Nfκb1 tm1 Bal/J*) and wild-type (B6; 129PF2) male mice, obtained from the Jackson Laboratories, were utilized throughout this study. All animals were maintained in high-efficiency particulate air (HEPA)-filtered Thoren units (Thoren Caging System) at the University of Piemonte Orientale animal facility and they were kept in number of 3–4 animals/cage with unlimited access to water and food. Care and handling of animals were performed in accordance with the NIH guidelines and also reviewed and approved by the local IACUC.

Isolation and culture of adult SVZ neurospheres

For neurosphere preparation, the brains from three adult male mice were dissected and the SVZ was isolated under a dissecting microscope. Detailed procedure was previously described (Denis-Donini et al., 2008). Primary (Passage 1, P1) neurospheres were dissociated after 7 days *in vitro* (D.I.V.), whereas P2-P9 neurospheres every 5 D.I.V.. At each passage, cells were plated in 25-cm^2 Falcon cell-culture flask (Becton Dickinson Labware) at a density of 12,000 cells/cm^2 in proliferation medium [Neurobasal-A serum-free medium (Gibco-Invitrogen) containing B27 supplement (Gibco-Invitrogen), 2 mM L-glutamine (Gibco-Invitrogen), 10 ng/ml basic human FGF (Peprotech), 20 ng/ml human EGF (Sigma-Aldrich), 4 μg/ml heparin sodium salt (Sigma-Aldrich) and 100 U/100 μg/ml Penicillin/Streptomycin (Gibco-Invitrogen)]. P3-P9 neurospheres were utilized to study proliferation and differentiation.

Neural progenitor cell proliferation

Dissociated NPC were plated at the density of 10,000 cells/well in LuminNUNC F96 MicroWell (NUNC) in proliferation medium in presence of increasing concentrations of RAGE ligands [0.3–30 nM S100B protein from bovine brain (Calbiochem), 0.3–10 ng/ml human recombinant HMGB-1 (Sigma-Aldrich) and 25–100 μg/ml AGE-BSA (Sigma-Aldrich)] or vehicle (Tris–HCl 50 mM pH 7.8) for 96 h. For neutralization assays, NPC were pretreated for 60 min with 20 μg/ml neutralizing polyclonal anti-RAGE antibody (R&D systems) before RAGE ligand or vehicle addition. The proliferation rate was determined by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), according to manufacturer instructions. All experiments were run in triplicates by using different cell preparations and repeated at least five times. Data are expressed as percent increase over basal conditions (vehicle-treated cells) or as counts *per* second (CPS) and represent the mean value ± s.e.m. To assess NF-κB p65 nuclear translocation, neurospheres were plated at the density of 1000 neurospheres/ml in 24-well plates in proliferation medium and treated for 30 min with vehicle (Tris–HCl 50 mM pH 7.8), 10 ng/ml TNF-α (Peprotech), 1 ng/ml HMGB-1, 3 nM S100B, 50 μg/ml AGE-BSA. Thereafter, neurospheres were harvested on super-frost microscope slides (Menzel-Glaser) by cytospin centrifugation (235 × *g*, 5 min) and fixed with ice-cold 4% paraformaldehyde (PFA) in PBS for 20 min at RT for subsequent immunofluorescent analysis. This analysis was repeated three times by using neurospheres at different passages derived from different cell preparations.

Neural progenitor cell differentiation

For differentiation, P3-P9 neurospheres were dissociated and plated onto laminin-coated (2.5 μg/cm^2) Lab-Tek 8-well permanox chamber slides (NUNC) at a density of 43,750 cells/cm^2 in differentiating medium [Neurobasal-A medium containing B27 supplement, 2 mM L-glutamine and 100 U/100 μg/ml Penicillin/Streptomycin]. Under these experimental conditions, NPC differentiate predominantly into neurons. NPC were treated in presence of indicated concentrations of S100B, HMGB-1 and AGE-BSA or vehicle (Tris–HCl 50 mM, pH 7.8) for 22 h. For neutralization, NPC were pretreated for 60 min with 20 μg/ml neutralizing polyclonal anti-RAGE antibody before RAGE ligand or vehicle addition. For NF-κB p50 inhibition, 10 μg/ml NF-κB SN-50 peptide (Calbiochem) or 10 μg/ml NF-κB SN-50 M peptide (Calbiochem) were added to NPC 60 min before RAGE ligand or vehicle addition. Then cells were washed in PBS and fixed with ice-cold 4% PFA for 20 min at RT for subsequent immunofluorescent analysis. In each experiment, 10 fields/well were counted by using a fluorescent microscope ECLIPSE E600 (NIKON) with a X60 objective. All experiments were run in triplicates by using different cell preparations and repeated at least three times.

Assessment of cell viability

Dissociated NPC were differentiated, as previously described, in presence of indicated concentrations of S100B, HMGB-1 and AGE-BSA or vehicle (Tris–HCl 50 mM, pH 7.8) for 22 h. Media of each samples were collected and LDH activity in the culture supernatants was evaluated by using Cytotoxicity Detection Kit LDH (Roche Applied Science), according to manufacturer instructions. Data are expressed as values of absorbance (490 nm) of the red formazan salt generated in a two-step reaction catalyzed by LDH enzyme. In parallel, in order to evaluate the percentage of apoptotic cells, nuclei of RAGE ligand- or vehicle-treated cells were counterstained with 0.8 ng/ml Hoechst (Sigma-Aldrich) diluted in PBS. The number of apoptotic nuclei was counted in each samples using a fluorescent microscope ECLIPSE E600 (X600). All experiments were run in triplicates by using different cell preparations and repeated at least three times.

Immunocytochemistry

After fixation, neurospheres or differentiated cells were washed three times in PBS and permeabilized in PBS containing 0.48% (vol/

vol) Triton X-100 (Sigma-Aldrich). Permeabilization was omitted for RAGE immunostaining. The primary antibodies against MAP-2 (1:600, rabbit monoclonal; Chemicon), GFAP (1:600, mouse monoclonal; Chemicon), RAGE (1:600, goat polyclonal; Chemicon) and Sox-2 (1:500, rabbit polyclonal; Chemicon) were incubated for 150 min at RT, whereas the primary antibodies against Nestin (1:4000, chicken polyclonal; Neuromics) and p65 (1:500, rabbit polyclonal; Santa Cruz Biotechnology) were incubated overnight at 4 °C in an antibody solution containing 16% (vol/vol) donkey or goat serum (Sigma-Aldrich). Secondary antibodies were as follows: Alexa Fluor 594-conjugated donkey anti-goat (1:1200; Molecular Probes), Alexa Fluor 555-conjugated donkey anti-goat (1:1200; Molecular Probes), Alexa Fluor 488-conjugated donkey anti-rabbit (1:1600; Molecular Probes), Alexa Fluor 594-conjugated donkey anti-mouse (1:1400; Molecular Probes) and Alexa Fluor 488-conjugated goat anti-chicken (1:1400; Molecular Probes). Nuclei were counterstained with 0.8 ng/ml Hoechst diluted in PBS. Slides were coverslipped with Fluorescent Mounting Medium (DakoCytomation) as anti-fading agent. Incubation without primary antibodies served as negative control for immunocytochemistry. Fluorescent microscope or laser scanning confocal microscope (Leica TCS-NT) was used for analysis. Adobe Photoshop CS (Adobe Systems, Inc) was used for digital processing of the images. Only light intensity, brightness, and contrast adjustments were applied to improve information.

RNA isolation and Reverse Transcriptase-PCR analysis

Total mRNA was extracted from SVZ-derived NPC ($0.5–1.5\times10^6$ cells) and lung tissue of adult mice by using the SV Total RNA Isolation System (Promega), according to manufacturer instructions. The cDNA was obtained by using the ImProm-II Reverse Transcription System kit (Promega). The primer sequences used for PCR amplification were as follows: FL-RAGE, sense 5′-CGATGAGGGGCCAGCTGAAG-3′ (exon 9) and antisense 5′-CCTGGCTTTCCGGGGCCTTC-3′ (exon 11). PCR reactions were carried out by using GoTaq Flexi DNA polymerase (Promega) in a final volume of 25 μl containing 56 ng cDNA, 0.4 mM of each primers, 0.2 mM dNTPs, 2.5 mM $MgCl_2$. PCR conditions were: 94 °C for 10 min; 40 cycles, 94 °C for 30 s, 67.6 °C for 30 s, 72 °C for 1 min; final elongation at 72 °C for 10 min. PCR products were run onto 2% agarose gels and bands visualized by staining with ethidium bromide (Sigma-Aldrich).

Protein isolation and Western blot analysis

For protein isolation, tissues from adult mice and SVZ-derived neurospheres were disrupted in hypotonic RIPA buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA pH 8, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS, 1 mM PMSF, 25 mM NaF, 1 mM $NaVO_4$, 1 mM DTT, protease inhibitor mix (Sigma-Aldrich)]. Neurosphere homogenates were incubated on ice for 25 min. To complete the lysis, incubation at −80 °C for 3 min followed by 2 min at 37 °C was repeated three times. Lysates were centrifuged at 15,700 *g* for 10 min at 4 °C and supernatants were collected. For protein isolation from tissues, SVZ and lung were disrupted in hypotonic RIPA buffer by using a tissue homogenizer. Tissue homogenates were incubated on ice for 60 min, centrifuged at $15,700\times g$ for 30 min at 4 °C and the supernatants were recovered. Protein concentration was determined by Bradford assay (Sigma-Aldrich) and equivalent amounts (50 μg) of each sample were separated onto a 10% (wt/vol) Sodium Dodecyl Sulphate (SDS)-PAGE gel and transferred onto nitrocellulose membranes. The membranes were blocked with 5% (wt/vol) BSA in TBS buffer overnight at 4 °C. Immunoblots were carried out for 150 min at RT in an antibody solution containing 3% (wt/vol) BSA in TBS with the following antibodies: anti-RAGE (1:400, goat polyclonal; AB5484, Chemicon; immunogen: synthetic peptide corresponding to aa 42–59 of RAGE; marked as "N-term") and anti-RAGE (1:1200, rabbit polyclonal; R5278, Sigma; immunogen: synthetic peptide corresponding to aa 362-380 of RAGE; marked as "C-term"). After washing, blots were incubated with peroxidase-conjugated goat anti-rabbit (1:10,000; BIO-RAD) or donkey anti-goat (1:10,000; Santa Cruz Biotechnology) antibodies for 60 min at RT and the immunocomplexes were visualised by the Supersignal West Pico Chemiluminescent substrate (Pierce).

Bromodeoxyuridine treatment and immunohistochemistry

Adult male CD1 mice were administered a single injection of bromodeoxyuridine (BrdU; 150 mg/kg body weight, intraperitoneally) and, 24 h later, they were deeply anaesthetized with Avertin (400 mg/kg i.p.) and perfused transcardially with saline, followed by 4% ice-cold PFA in 0.1 M phosphate buffer (PB) pH 7.4. Tissues were prepared as previously described (Denis-Donini et al., 2008). Sections were incubated with primary antibodies anti-p65 (1:300, rabbit polyclonal; Santa Cruz Biotechnology), anti-RAGE (1:600, goat polyclonal; R&D system), anti-GFAP (1:300, mouse monoclonal; Millipore), anti-DCX (1:200, rabbit polyclonal; AbCAM), anti-Sox-2 (1:800, rabbit polyclonal; Chemicon) and anti-BrdU (1:200, rat monoclonal, Novus Biological), overnight at 4 °C. For RAGE immunolabelling, antigen unmasking with Citrate/EDTA, pH 6.2 at 98 °C for 20 min was performed. The next day, after several washes, sections were incubated with Alexa Fluor 555-conjugated goat anti-rabbit secondary antibody (1:300; Molecular Probes), Alexa Fluor 555-conjugated goat anti-mouse secondary antibody (1:300; Molecular Probes), Alexa Fluor 555-conjugated donkey anti-rabbit secondary antibody (1:300; Molecular Probes), Alexa Fluor 633-conjugated goat anti-rabbit secondary antibody (1:300; Molecular Probes), Cy5-conjugated goat anti-rat (1:600; Jackson ImmunoResearch) and biotinylated horse anti-goat secondary antibody (1:400; Vector Laboratory) followed by incubation with Fluorescein Streptavidin (1:100; Vector Laboratory). The sections were then mounted onto slides and coverslipped with Fluorescent Mounting Medium (DakoCytomation) as anti-fading agent. Incubation of tissue without primary antibodies served as negative control for immunohistochemistry. Fluorescent signals were detected using a confocal scanning laser microscope with a X20 objective and X40 PL APO oil objective (1.25 NA), by using an argon laser (exciting at 488 nm) or a helium-neon laser (exciting at 543–633 nm). Adobe Photoshop CS (Adobe Systems, Inc.) was used for digital processing of the images. Only light intensity, brightness, and contrast adjustments were applied to improve information.

Statistical analysis

In all experiments, data are reported as mean ± s.e.m, with at least three replicates per group. Data were analyzed by Student's *t*-test or by one-way ANOVA followed by Tukey's *post hoc* test. Statistical significance level was set for p values less than 0.05.

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