RESEARCH ARTICLE

Cell Autonomous and Noncell-Autonomous Role of NF-κB p50 in Astrocyte-Mediated Fate Specification of Adult Neural Progenitor Cells

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In previous work, we demonstrated that NF-κB p50 acts as crucial regulator of adult hippocampal neural progenitor cells (ahNPC). Indeed, NF-κB p50 knockout (KO) mice are characterized by remarkably reduced hippocampal neurogenesis. As a follow up to that work, herein we show that when cultured *in vitro*, ahNPC from wild type (WT) and p50KO mice are not significantly different in their neurogenic potential. This observation prompted us to investigate cell-autonomous and noncell-autonomous consequences of p50 absence on neuronal fate specification of ahNPC. In particular, we focused our attention on astrocytes, known to provide soluble proneurogenic signals, and investigated the influence of WT and p50KO astrocyte conditioned media (ACM) on WT and p50KO ahNPC differentiation. Interestingly, while WT ACM promoted both neuronal and astroglial differentiations, p50KO ACM only supported astroglial differentiation of WT ahNPC. By using a LC–MS/MS approach, we identified some proteins, which are significantly upregulated in p50KO compared with WT astrocytes. Among them, lipocalin-2 (LCN-2) was recognized as a novel astroglial-derived signal regulating neuronal fate specification of ahNPC. Interestingly, LCN-2 proneurogenic effect was greatly reduced in p50KO NPC, where LCN-2 receptor gene expression appeared downregulated. In addition to that, we demonstrated p50KO NPC unresponsiveness to both neuronal and astroglial fate specification signals from WT and p50KO ACM, and we identified a reduced expression of α2δ1, a thrombospondin-1 receptor, as another phenotypic change occurring in ahNPC in the absence of p50. Altogether, our data suggest that dysregulated NPC-astrocyte communication may contribute to a reduced hippocampal neurogenesis in p50KO mice *in vivo*.

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Introduction

Adult neurogenesis occurs in restricted areas of postnatal and adult mammalian brain, namely the subventricular zone (SVZ) in the lateral wall of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus (Bond et al., 2015; Kempermann et al., 2015; Ming and Song, 2005). Recent studies also suggested an additional neurogenic region in the adult hypothalamus (Kokoeva et al., 2005).

Adult hippocampal neurogenesis (ahNG) has attracted great research interest due to its potential involvement in critical functions including cognition, mood and emotional behavior, stress response (Aimone et al., 2010, 2014; Eisch and Petrik, 2012; Lacar et al., 2014). Although complex, the molecular mechanisms that modulate neurogenesis deserve investigation since this may turn into a better understanding of ahNG in brain homeostasis and in neuropsychiatric/neurodegenerative disorders where this process is profoundly deregulated (Apple et al., 2016; Winner et al., 2011).

Within the CNS, NF-κB pathway activation has been involved in a wide range of functions both under

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physiological and pathological conditions (Kaltschmidt and Kaltschmidt, 2009; Mattson and Meffert, 2006; Oikawa et al., 2012). A few years ago our group demonstrated that NF-κB proteins are expressed at considerable levels in areas of active neurogenesis in the postnatal and adult mouse brain (Denis-Donini et al., 2005). Since then, a vast array of information has been collected on the complex involvement of NF-κB proteins in different aspects of postnatal neurogenesis. In particular, several extracellular signals and membrane receptors have been identified as being able to affect neural stem cells/neural progenitor cells (NSC/NPC) and their progeny via NF-κB activation (Meneghini et al., 2010, 2013; Rolls et al., 2007; Wada et al., 2006; Widera et al., 2008; Zhang et al., 2012).

Since the initial observation of NF-κB presence in adult neurogenic areas, our group further explored the role of these regulatory proteins in adult neurogenesis with a specific focus on the NF-κB1 (p50) subunit (Bortolotto et al., 2014; Grilli and Meneghini, 2012). By taking advantage of p50KO mice (Sha et al., 1995) we demonstrated that absence of p50 can deeply affect the *in vitro* response of adult hippocampal NPC (ahNPC) to several endogenous signals (Meneghini et al., 2013) and to proneurogenic drugs (Valente et al., 2012). *In vivo*, we also proved that p50KO mice display a dramatic reduction in adult hippocampal neurogenesis, which correlated with a selective defect in hippocampal-dependent short-term memory (Denis-Donini et al., 2008). Interestingly, *in vivo* and *in vitro*, the proliferation rate of hippocampal NSC/NPC in p50KO mice appeared to be similar to that of WT mice. Moreover, the apoptotic rate in the hippocampal region was not increased in mutant mice compared with their WT counterpart. A detailed phenotypic characterization of newly generated hippocampal cells in p50KO mice suggested that the absence of the NF-κB p50 subunit may trigger a rather selective defect in late maturation of newly generated neurons (Denis-Donini et al., 2008).

Homeostasis of adult neurogenesis requires permissive and instructive signals for aNSC/NPC. Several elegant studies have investigated the mechanisms through which local environment in the neurogenic niche may control fate specification of aNSC/NPC. Among others, a pivotal work demonstrated that astroglial cells can instruct stem cells to adopt a neuronal fate (Song et al., 2002). Since the well documented role of NF-κB in neuronal but also in non-neuronal cells (Brambilla et al., 2009; Lian et al., 2015), we recently decided to further dissect the role of NF-κB p50 in the cross-talk between adult NPC and astrocytes. Exposure of WT and p50KO hippocampal NPC to conditioned media from WT and p50KO astrocytes was utilized as a strategy to study the potential influence of astroglia on aNPC fate specification and the cell autonomous or noncell-autonomous role played by NF-κB p50 signaling in that context.

Materials and Methods

Animals

Wild type (WT; C57BL/6; The Jackson Laboratories) and NF-κB $p50^{-/-}$ (p50KO; C57BL/6 $Nfkb1^{tm1Bal}$/J; The Jackson Laboratories) mice were housed under light- and temperature-controlled conditions in high-efficiency particulate air (HEPA)-filtered Thoren units (Thoren Caging Systems) at the University of Piemonte Orientale animal facility. Mice were kept 3–4/cage with *ad libitum* access to water and food. Animal care and handling were performed in accordance with European Community Directive and approved by the local IACUC (Institutional Animal Care and Use Committees).

Isolation and Culture of WT and p50KO Adult Mouse Hippocampal NPC

For preparing NPC primary cultures from hippocampi, three adult (3–4 months old) male WT and p50KO mice were sacrificed and cell suspension was prepared. Briefly, the brains were removed, and hippocampi were isolated and collected in ice-cold PIPES buffer pH 7.4 containing 20 mM PIPES, 25 mM glucose, 0.5 M KCl, 0.12 M NaCl (Sigma-Aldrich), and 100 U/100 μg/ml penicillin/streptomycin solution (Life Technologies). After 5 minutes at 110*g* centrifugation, the tissue was digested for 40 minutes at 37°C using the Papain Dissociation System (Worthington DBA). Cell suspension was plated onto 25 cm^2 cell-culture flask (Thermo-Fisher Scientific) and cultured as floating neurospheres through subsequent passages (Valente et al., 2012). Primary (Passage 1, P1) neurospheres were dissociated after 7–10 days *in vitro* (DIV), whereas P2–P30 neurospheres every 5 DIV. At each passage cells were plated in T25 flask at a density of 12,000 cells/cm^2 in growing medium: Neurobasal-A medium, supplemented with B27 supplement, 2 mM L-glutamine (Life Technologies), human Epidermal Growth Factor (hEGF, 20 ng/ml; Peprotech), basic Fibroblast Growth Factor (bFGF, 10 ng/ml; Peprotech) and heparin sodium salt (0.0004%, Sigma-Aldrich).

Adult Hippocampal NPC Differentiation

NPC (P5–P30) were used for differentiation experiments. Briefly, neurospheres were dissociated into single cells and plated onto laminin-coated Lab-Tek 8-well permanox chamber slides (Nunc) at a density of 47,000 cells/cm^2 in NPC differentiation medium (NDM, Neurobasal-A medium supplemented with B27, 2 mM L-glutamine and 100 U/100 μg/ml penicillin/streptomycin). NPC were treated in the presence of hippocampal astrocyte conditioned media (ACM) derived from WT or NF-κB p50KO glial cultures, or in the presence of NDM (referred to as standard, STD medium) for 24 hours. WT NPC were treated in the presence of 0.01–1 μg/ml recombinant mouse lipocalin-2 (LCN-2, Cell Signaling), 2 μg/ml purified human thrombospondin-1 (Amsbio), or corresponding vehicle for 24 hours. For receptor for advanced glycated end-products (RAGE) inhibition, 60 minutes before ACM or NDM treatment, 20 μg/ml neutralizing polyclonal anti-RAGE antibody (α-RAGE Ab; R&D System) was added to NPC.

Primary Astrocyte Cultures

Primary mixed glial cultures were prepared from hippocampus of neonatal (P1–2) C57BL/6 WT and p50KO mice and grown in DMEM high glucose, 10% FBS, 15 mM HEPES, 2 mM L-glutamine, and 100 U/100 μg/ml penicillin/streptomycin at a density of 78,000 cells/cm^2. When cells reached confluence (around 10–12 DIV), proliferation of nonastrocytic cells was blocked by 10 μM cytosine arabinoside (Sigma-Aldrich) for 96 hours. Then cultures were switched to NDM. Phenotypic characterization of cultures was performed by immunocytochemistry with antibodies against GFAP (mouse monoclonal, 1:600, Millipore) and CD11b (rat monoclonal; 1:150, Millipore). Contaminating microglial cells below 3% of total cells, with the remaining cells being GFAP$^+$. ACM was collected after 48 hours in the presence of NDM. Briefly, ACM was centrifuged for 10 minutes at 16,000*g* to eliminate cells and debris and passed through 0.2-μm filter before use. ACM was used diluted 1:2 in fresh NDM.

Immunolocalization Studies in Differentiated Adult Hippocampal NPC

NPC were treated in presence of WT or p50KO ACM or STD medium for 24 hours. Phenotypic characterization of NPC-derived cells was carried out by immunolocalization for MAP-2 (rabbit polyclonal, 1:600; Millipore), GFAP (mouse monoclonal, 1:600; Millipore), nestin (chicken monoclonal, 1:1,500; Neuromics), NG-2 (rabbit polyclonal, 1:500; Millipore). Secondary antibodies were as follows: Alexa Fluor 555-conjugated goat antirabbit (1:1,400; Molecular Probes); Alexa Fluor 488-conjugated goat antichicken antibody (1:1,400; Molecular Probes); Alexa Fluor 488-conjugated goat antirabbit (1:1,400; Molecular Probes); and Alexa Fluor 488-conjugated goat antimouse (1:1,600; Molecular Probes). Nuclei were counterstained with 0.8 ng/ml Hoechst (Thermo Fisher scientific) diluted in PBS. In each experiment, 5 fields/well (corresponding to about 150–200 cells/well) were counted with a 60× objective by a Leica DMIRB inverted fluorescence microscope. Cells positive for each marker were counted and their percentage over total viable cells was calculated. All experiments were run in triplicates using different cell preparations and repeated at least three times.

LC–MS/MS Analysis and Protein Quantification

WT and p50KO ACM were subjected to TCA precipitation. Briefly, TCA was added to a final 12% (wt/vol) concentration and kept on ice for 2 hours, followed by centrifugation (16,000*g*, 10 minutes). The supernatant was carefully removed, and 1 ml of tetrahydrofuran (precooled in ice) was added to each pellet and vortexing was carried out until the pellet dissolved completely. Proteins were then reduced with dithiothreitol (DTT), alkylated by iodoacetamide, and digested with trypsin at 37°C O/N. Data acquisition was performed with a micro-LC Eksigent Technologies (Dublin) system, with as stationary phase a Halo Fused C18 column (0.5 mm × 100 mm, 2.7 μm; Eksigent Technologies Dublin), interfaced with a Triple-TOF 5600+ system (AB Sciex, Concord, Canada) equipped with a DuoSpray Ion source. The mobile phase was a mixture of 0.1% (vol/vol) formic acid in water (A) and 0.1% (vol/vol) formic acid in acetonitrile (B), eluting at a flow-rate of 15.0 ml/minutes at an increasing concentration of solvent B from 2% to 40% in 30 minutes. An initial data-dependent acquisition (DDA) analysis was performed on the p50KO and WT ACM samples to generate the SWATH-MS spectral library: the mass spectrometer analysis was performed using a mass range of 100–1,500 Da (TOF scan with an accumulation time of 0.25 second), followed by a MS/MS product ion scan from 200 to 1,250 Da (accumulation time of 5.0 ms) with the abundance threshold set at 30 cps (35 candidate ions can be monitored during every cycle). The samples were then subjected to cyclic data indipendent analysis (DIA) of the mass spectra, using a 25-Da window: the mass spectrometer was operated such that a 50-ms survey scan (TOF-MS) was performed, and subsequent MS/MS experiments were performed on all precursors. These MS/MS experiments were performed in a cyclic manner using an accumulation time of 40 ms per 25-Da swath (36 swaths total) for a total cycle time of 1.5408 seconds (Geromanos et al., 2009; Gillet et al., 2012; Venable et al., 2004). The ions were fragmented for each MS/MS experiment in the collision cell using the rolling collision energy. The MS data were acquired with Analyst TF 1.7 (AB SCIEX, Concord, Canada). Three replicates for each sample were subjected to the DIA. The mass spectrometry files were searched using Protein Pilot (AB SCIEX, Concord, Canada) and Mascot (Matrix Science Inc., Boston) with the following parameters: cysteine alkylation, digestion by trypsin, no special factors and false discovery rate at 1% for Protein Pilot. For Mascot, the following parameters were used: the digestion enzyme selected was trypsin, with three missed cleavages, a search tolerance of 0.4 Da was specified for the peptide mass tolerance, and 0.4 Da for the MS/MS tolerance. The charges of the peptides to search for were set to 2+, 3+, and 4+, and the search was set on monoisotopic mass and the following modifications were specified for the search: carbamidomethyl cysteins as fixed modification and oxidized methionine as variable modification. The search was conducted using the UniProt Swiss-Prot database containing mouse proteins (version 25.03.2015, containing 41,741 sequence entries). Dual filtering criteria for protein identification were employed by combining FDR test from target-decoy database search with a cutoff *P*-value of 0.05, and protein/peptide confidence above 95% probability, with a minimum of two unique peptides per protein. The label-free quantification was performed with Skyline (MacCoss Lab Software, University of Washington) by importing the SWATH-MS runs. The library of the identified proteins used for the processing of SWATH data was generated by combining the results of the database search performed with Protein Pilot and Mascot (Manfredi et al., 2016). The quantification was performed by integrating the extracted ion chromatogram of all the unique ions for a given peptide and by using MSstats, a Skyline external tool for statistical analysis (Choi et al., 2014).

Western Blot Analysis

Protein extracts from adult NPC cultures were obtained by lysis in 3× (vol/vol) of ice-cold 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, 1% (wt/vol) SDS, 10 mM NaF, 1 mM $NaVO_4$, 1 mM DTT, protease inhibitor mix (Sigma-Aldrich)] for 30 minutes on ice. To complete the lysis, incubation at −80°C for 3 minutes

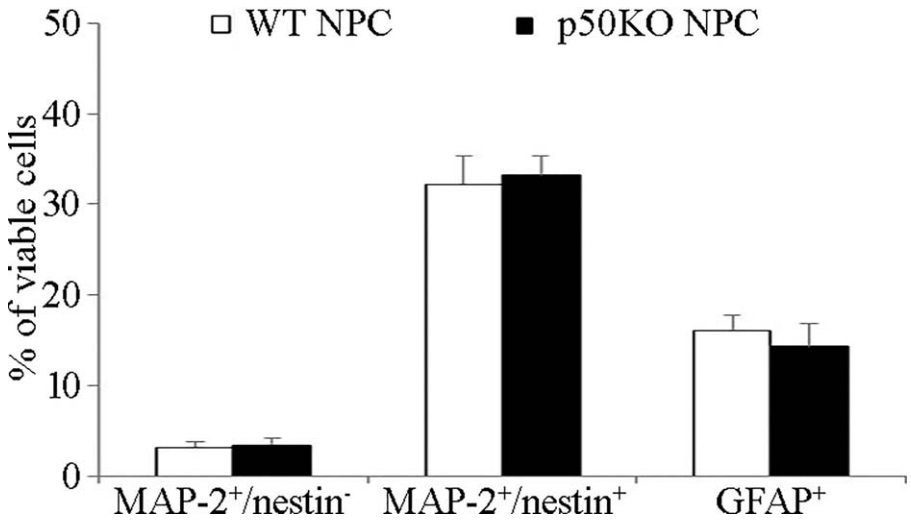

FIGURE 1: Adult hippocampal NPC derived from WT and p50KO mice show similar *in vitro* neuronal and glial differentiation rates. Under differentiating conditions, WT and p50KO ahNPC spontaneously give rise to similar percentages of MAP-2$^+$/nestin$^-$ neurons, MAP-2$^+$/nestin$^+$ neuroblasts, and GFAP$^+$ astrocytes. Data are expressed as mean % ± SD of n = 4 experiments, run in triplicates, and analyzed by Student's t-test.

followed by 2 minutes at 37°C was repeated three times. All lysates were centrifuged at 16,100*g* for 10 minutes at 4°C and supernatants were collected. Protein concentration was determined by Bradford assay (Sigma-Aldrich), and equivalent protein amounts (25 μg) were separated by SDS-PAGE gel (6%) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% BSA in TBS (Sigma-Aldrich) buffer for 60 minutes at RT. Immunoblots were carried out overnight in an antibody solution containing 3% (wt/vol) BSA in TBS-tween 0.1% with the primary antibody against the $\alpha 2\delta$ subunit of dihydropyridine/thrombospondin-1 (TSP-1) receptor (1:500; Sigma-Aldrich). After washing, blots were incubated with peroxidase-conjugated goat antimouse antibody (1:10,000; BIO-RAD) for 60 minutes at RT and immunocomplexes were visualized by the Supersignal West Pico Chemiluminescent substrate (Pierce). Densitometric analysis was performed using the Image Lab software system (Bio-Rad Laboratories), and each band was normalized to β-actin signal (mouse monoclonal, 1:1,000; Sigma-Aldrich).

Quantitative Reverse Transcriptase PCR (qRT-PCR)

RNA was extracted and cDNA generated using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Penzberg, Germany). qRT-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and the CFX384 Real-Time PCR Detection System (Bio-Rad).

The following qRT-PCR primer pairs were used (KiCqStart® SYBR® Green Primers Predesigned): for LCN-2 receptor (LCN-2R/24p3R), 5′-CATTATGGCTCTTCGGTTTC-3′ (forward), 5′-TAGA AATCGCCAGTCCTTAG-3′ (reverse); for β-actin, 5′-GATGTAT GAAGGCTTTGGTC-3′ (forward), 5′-TGTGCACTTTTATTGGT CTC −3′ (reverse). LCN-2R/24p3R expression levels were normalized against β-actin as housekeeping gene, and their relative ratio was calculated.

Statistical Analysis

All experiments were run in triplicates using different cell preparations and repeated at least three times. Data were calculated as mean ± SD and analyzed using Student's t-test when only two independent groups were compared, or by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test when three or more groups were compared. Statistical significance level was set for P values <0.05.

Results

In Vitro WT and p50KO ahNPC Do Not Show Differences in Differentiation and Survival Rates

Multipotent nestin$^+$, sox2$^+$, and GFAP$^-$ NPC isolated from adult mouse hippocampi can be maintained for several passages in an undifferentiated proliferative state (Cuccurazzu et al., 2013). When grown onto laminin-coated chamber slides, upon removal of growth factors and exposure to a serum-free defined medium (STD medium), NPC stop dividing and spontaneously differentiate toward both neuronal and glial lineages. As previously published, by double immunolabeling for markers of neuronal cells (MAP-2) and undifferentiated progenitors (nestin), the appearance of newly generated MAP-2$^+$/nestin$^-$ neurons and MAP-2$^+$/nestin$^+$ neuroblasts can be evaluated and quantified (Meneghini et al., 2013). Under these experimental conditions, both WT and p50KO derived NPC gave rise to the same percentage of neurons and neuroblasts (mean % ± SD of MAP-2$^+$/nestin$^-$ cells: 3.1 ± 0.6 and 3.3 ± 0.8 for WT and p50KO NPC; MAP-2$^+$/nestin$^+$ cells: 32.2 ± 3.2 and 33.1 ± 2.2 for WT and p50KO NPC; Fig. 1). When exposed to STD medium in the absence of growth factors, ahNPC spontaneously differentiate also toward the astrocytic lineage (Meneghini et al., 2013).

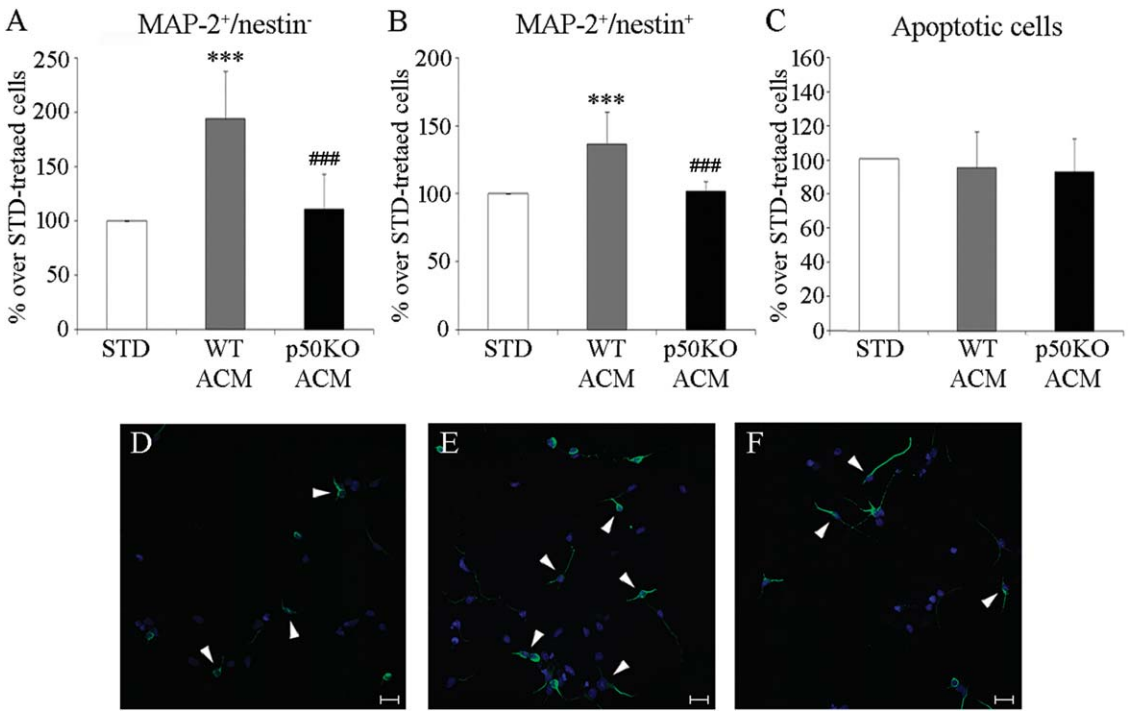

FIGURE 2: WT, but not p50KO ACM, promote neuronal differentiation of WT ahNPC cultures. Under differentiating conditions, 24 hours treatment of WT hippocampal NPC with WT ACM significantly increased the percentage of MAP-2$^+$/nestin$^-$ neurons (A) and MAP-2$^+$/nestin$^+$ neuroblasts (B) compared with standard differentiation medium (STD). Conversely, p50KO ACM was devoid of any proneurogenic effect. (C) No significant difference was observed in the apoptotic rate of cultures exposed to STD medium, WT, and p50KO ACM. Representative confocal microscopy images of MAP-2$^+$ cells (green, white arrowheads) generated from ahNPC after 24 hours in the presence of STD medium (D), WT ACM (E), and p50KO ACM (F). Nuclei were counterstained with Hoechst (blue). Magnification = 400×. Scale bar = 20 μm. Data were calculated as mean values ± SD of *n* = 3 experiments, run in triplicates and analyzed by one-way ANOVA followed by Tukey's *post hoc* test. ****P* < 0.001 *versus* STD; ###*P* < 0.001 *versus* WT ACM. Data are expressed as percentage of increase over STD-treated cells.

The percentage of astrocytes (GFAP$^+$ cells) generated *in vitro* by WT and p50KO NPC was not different (mean % ± SD of GFAP$^+$ cells: 16 ± 1.7 and 14.2 ± 2.6 for WT and p50KO NPC; Fig. 1). Finally, no significant difference in the basal apoptotic rate could be observed between the two genotypes (11.8 ± 6.5% and 12.4 ± 5.9% for WT and p50KO NPC, respectively).

Effect of WT- and p50KO-Derived ACM on Neuronal Differentiation of WT ahNPC Cultures

We investigated the possibility that noncell-autonomous effects were contributing to remarkably reduced hippocampal neurogenesis in p50KO mice (Denis-Donini et al., 2008). Since soluble factors released by astrocytes have been shown to modulate differentiation of NPC (Song et al., 2002), we evaluated their effect on the differentiation rate of ahNPC toward neuronal and nonneuronal lineages. Primary mixed glial cultures were prepared from hippocampi of neonatal (P1–2) WT and p50KO mice. When cells reached confluence, proliferation of nonastrocytic cells was blocked by 10 μM cytosine arabinoside so to obtain an astrocytic-enriched cell population with less than 3% of contaminating microglia. ACM was added to WT ahNPC cultures. Under these experimental conditions, we tested the effect of WT and p50KO ACM, in comparison with standard differentiation (STD) medium, on NPC neuronal differentiation. After 24 hours, a significant increase in the percentage of *in vitro* generated neurons and neuroblasts was observed in WT cells exposed to WT-derived ACM, compared with STD medium condition (percentage increase over STD medium-treated cells: +93.8 ± 43.3 for MAP-2$^+$/nestin$^-$ cells, $P < 0.001$; +36.6 ± 23.3 for MAP-2$^+$/nestin$^+$ cells, $P < 0.001$; Fig. 2A,B). Under the same *in vitro* experimental conditions, no significant difference was observed between WT NPC treated with STD medium or p50KO ACM on both neuron and neuroblast subpopulations (Fig. 2A,B). To investigate whether ACM could affect cell survival in addition to neuronal differentiation, we analyzed the apoptotic rate of ahNPC and their progeny. No difference in the percentage of apoptotic cells was observed in the different media conditions (Fig. 2C).

Both WT- and p50KO-Derived ACM Significantly Promote Astroglial Differentiation of WT ahNPC

We then tested the effect of ACM on astroglial differentiation of ahNPC. We observed a significant increase in the

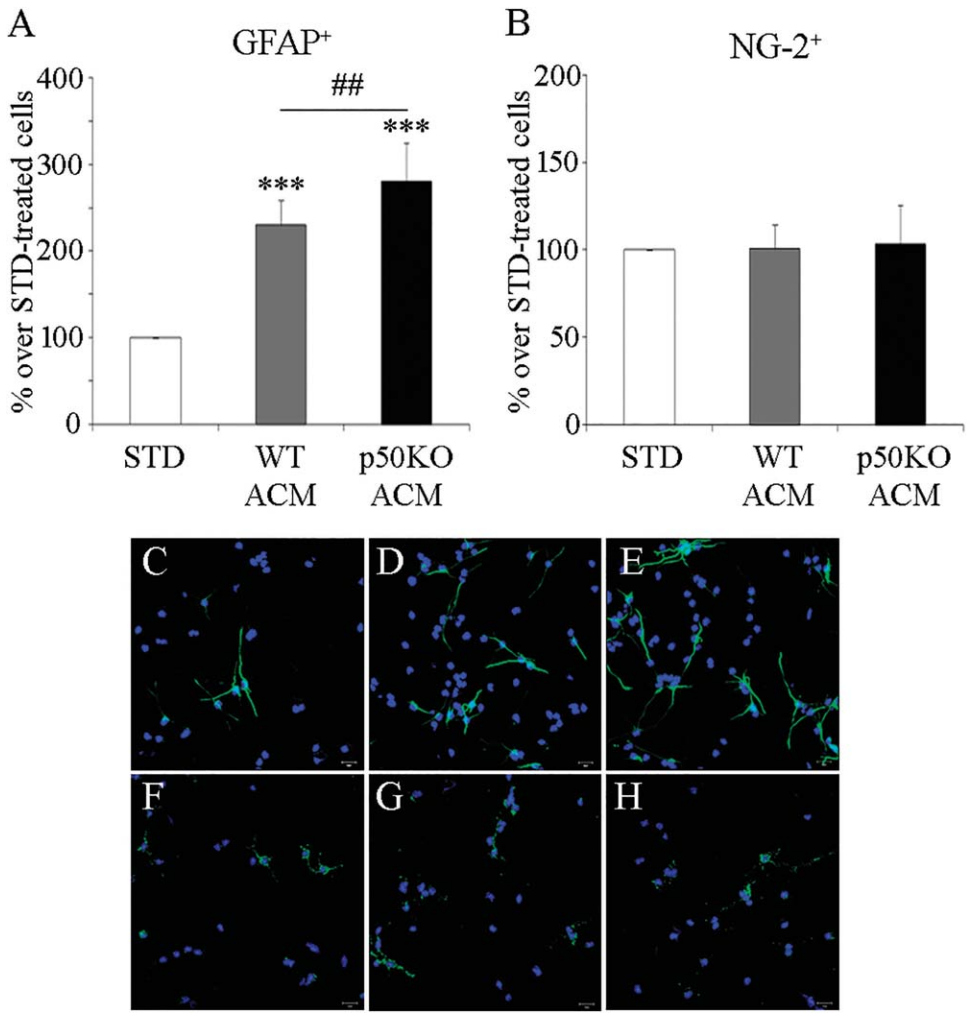

FIGURE 3: Both WT and p50KO ACM promote astroglial differentiation of WT ahNPC. (A) 24 hours treatment of WT ahNPC with WT and p50KO ACM significantly increased the percentage of GFAP$^+$ astroglial cells, compared with STD medium. p50KO ACM was more effective on astrogliogenesis than WT ACM. (B) No significant differences were observed in the percentage of NG-2$^+$ cells upon exposure to different media conditions. Representative confocal microscopy images of ahNPC cultures differentiated in the presence of STD (C and F), WT ACM (D and G), and p50KO ACM (E and H) and immunolabelled for GFAP (C–E) and NG-2 (F–H). Nuclei were counterstained with Hoechst (blue). Magnification = 400×. Scale bar = 20 μm. Data were calculated as mean values ± SD of $n = 3$ experiments, run in triplicates and analyzed by one-way ANOVA followed by Tukey's *post hoc* test. ***$P < 0.001$ *versus* STD; $^{\#\#}P < 0.01$ *versus* WT ACM. Data are expressed as percentage of increase over STD-treated cells.

percentage of GFAP$^+$ cells being generated *in vitro* when WT NPC were exposed to both WT and p50KO ACM (percentage increase over STD medium: +130.2 ± 28.1 and +180 ± 44.8 for WT and p50KO ACM, respectively; $P < 0.001$; Fig. 3A). Actually, in our experimental setting, p50KO ACM was more effective than WT ACM in promoting astrogliogenesis (Tukey's *post hoc*, $P < 0.01$; Fig. 3A). A subpopulation of NG-2$^+$ cells is also present both in proliferating and differentiating conditions in our cellular model. NG-2$^+$ cells, also known as polydendrocytes, are commonly regarded as oligodendrocyte precursor cells (Nishiyama et al., 2009) but also, although controversial, as multipotential progenitors (Kondo and Raff, 2000). Interestingly, under differentiating conditions, the percentage of NG-2$^+$ cells was not different in the presence of STD, WT ACM, and p50KO ACM (Fig. 3B).

p50KO NPC Are Unresponsive to Neuronal and Astroglial Differentiation Signals from Both WT and p50KO Astrocytes

We then tested the effect of WT and p50KO ACM on p50KO NPC differentiation toward neuronal and nonneuronal lineages. Surprisingly, neither WT nor p50KO ACM promoted neuronal differentiation of KO NPC, and their effect was indistinguishable from that of STD medium (Fig. 4A,B). In addition, unlike WT NPC, KO NPC did not even respond to the astrogliogenic effects of WT and KO ACM (Fig. 4C). Similarly to what observed in WT NPC, we could

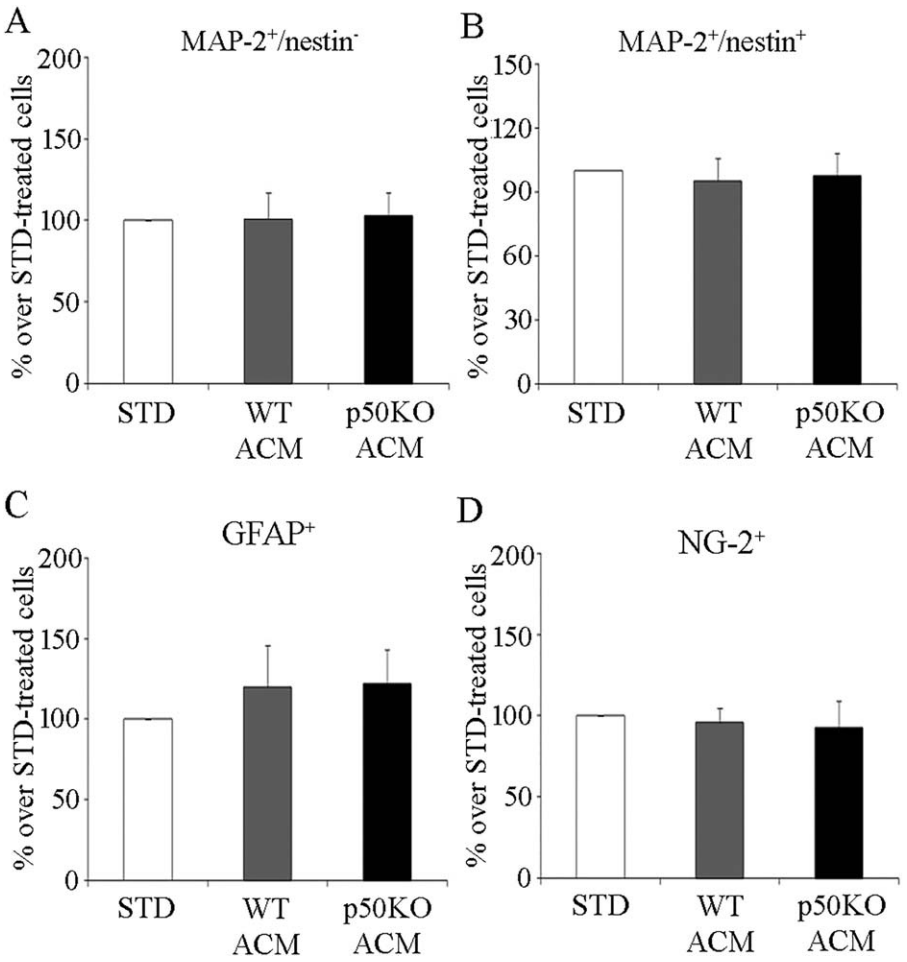

FIGURE 4: p50KO ahNPC are unresponsive to neuronal and astroglial differentiation signals from both WT and p50KO astrocytes. Under differentiating conditions, 24 hours treatment of p50KO hippocampal NPC with WT and p50KO ACM did not affect the percentage of MAP-2$^+$/nestin$^-$ neurons (A) and MAP-2$^+$/nestin$^+$ neuroblasts (B) compared with STD condition. No significant difference was observed in the percentage of GFAP$^+$ cells (C) and NG-2$^+$ cells (D) in the presence of STD medium, WT, and p50KO ACM. Data are expressed as mean values ± SD of *n* = 3 experiments, run in triplicates. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test.

not observe any difference in the percentage of NG-2$^+$ cells when p50KO NPC were exposed to different media conditions (Fig. 4D). Finally, also in p50KO cultures, apoptotic rates were not different in the presence of the different media conditions (data not shown).

RAGE Is Not Involved in the Proneurogenic Activity of ACM on WT ahNPC Differentiation

RAGE is functionally expressed by ahNPC (Meneghini et al., 2010), and its activation by astrocytes released molecules, like HMGB-1 and S100B, promotes neuronal differentiation of ahNPC (Meneghini et al., 2013). In order to understand if the activation of RAGE could mediate the proneurogenic activity of WT ACM on WT ahNPC, we pretreated cultures with a neutralizing anti-RAGE antibody (α-RAGE Ab, 20 μg/ml). α-RAGE Ab was not able to block the increase in MAP-2$^+$ cells induced by WT ACM (percentage increase over STD medium: +70.07 ± 24 for WT ACM, $P < 0.001$; +50.6 ± 27.8 for WT ACM + α-RAGE Ab, $P < 0.01$; Fig. 5A).

p50KO NPC Are Defective in Their Response to TSP-1 and Display Downregulated α2δ1 Expression

TSP-1 is an astrocyte-derived proneurogenic factor (Lu and Kipnis, 2010). Recently, our group demonstrated that α2δ1, a TSP-1 receptor (Eroglu et al., 2009), is functionally expressed by ahNPC (Valente et al., 2012). Recombinant human TSP-1 (2 μg/ml) promoted an increase in the percentage of newly formed neurons in WT NPC cultures, when compared with vehicle (percentage increase over vehicle: +71.7 ± 29.5 in TSP-1-treated cells; $P < 0.001$; Fig. 5B). Conversely, TSP-1 treatment was ineffective in p50KO NPC (Fig. 5B). Then, we evaluated α2δ1 expression levels in WT and p50KO ahNPC by western blot analysis. Interestingly, the α2δ1 subunit appeared strongly downregulated in p50KO compared with WT NPC (−59.5%; $P < 0.05$, Fig. 5C).

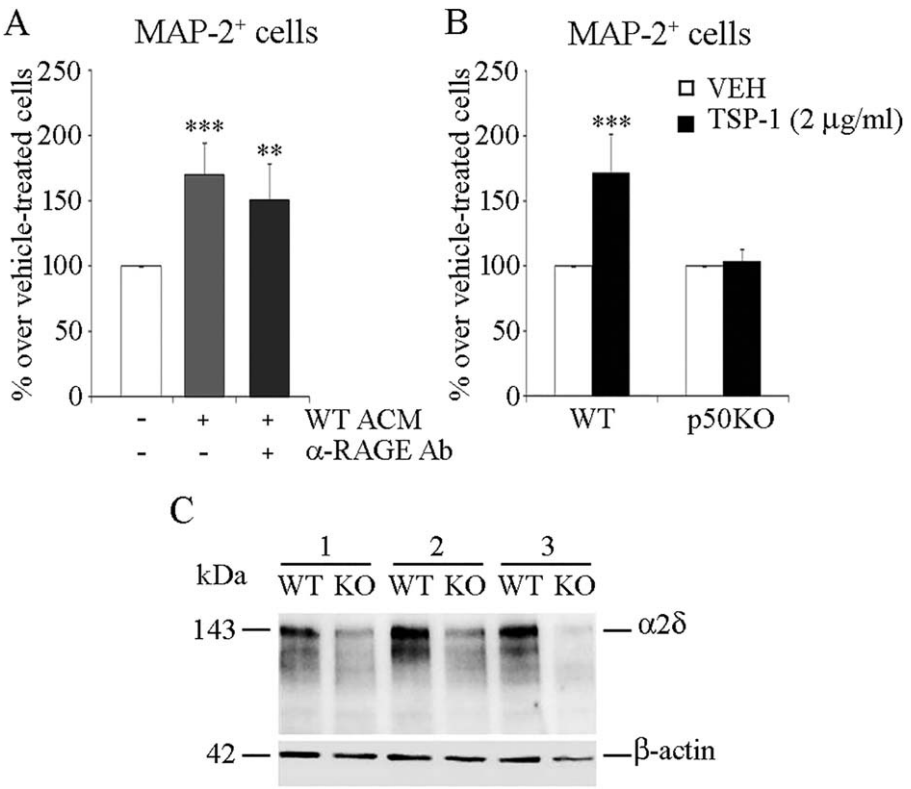

FIGURE 5: p50KO NPC are defective in their proneurogenic response to thrombospondin-1 and display downregulated expression of α2δ1. (A) RAGE activation does not mediate the proneurogenic effects of WT ACM. Pretreatment with a neutralizing anti-RAGE antibody (20 μg/ml) does not counteract the proneurogenic effects of WT ACM on WT ahNPC, as assessed by MAP-2$^+$ counting. (B) Under differentiating conditions, 24 hours treatment of WT NPC with human recombinant TSP-1 (2 μg/ml) significantly increased the percentage of MAP-2$^+$/nestin$^-$ cells, compared with vehicle. No effect was observed in TSP-1-treated p50KO NPC. Data have been calculated as mean values ± SD of *n* = 4 experiments, run in triplicates. ***$P < 0.001$ *versus* vehicle (Tukey's *post hoc* test). Data are expressed as percentage of increase over vehicle-treated cells. (C) Representative immunoblot analysis of α2δ1 subunit and β-actin expression levels in extracts of undifferentiated WT and p50KO ahNPC. Three (1–3) different WT and p50KO cell preparations were collected at different passages and analyzed.

Proteomic Analysis of Soluble Factors Released by WT and p50KO ACM

In order to identify proteins which are secreted by WT and p50KO astrocytes, we analyzed ACM samples using a proteomic approach based on a hybrid system that combines the triple quadrupole technology with a time of flight mass spectrometer platform (Andrews et al., 2011). The analysis was performed on peptides produced by tryptic digestion of ACM proteins. Each sample was injected separately into the HPCL columns in a data dependent acquisition mode in order to build the spectra library of proteins identified from the runs. The protein spectra were then subjected to SWATH (Sequential Window Acquisition of Theoretical mass spectra) analysis, that allows complete evaluation of fragmented ions from detectable peptides in biological samples. Through this approach we were able to identify and quantify, as listed in Table 1, three proteins, which were differentially expressed in WT and p50KO ACM, with *P* values below 0.05. Specifically, the following proteins appeared upregulated in p50KO compared with WT ACM: neutrophil gelatinase-associated lipocalin-2 (NGAL/LCN-2), C–C motif chemochine-2 (CCL2), and H-2 class I histocompatibility antigen, K–K alpha chain (HA1K). Fold-change increase in each identified protein in p50KO ACM *versus* WT ACM is reported in Table 1.

Lipocalin-2 Is an Astrocyte-Derived Proneurogenic Factor Whose Activity Is Strongly Reduced in p50KO NPC

Lipocalin-2 (LCN-2) is 24 kDa iron-related protein whose modulatory role for diverse cell phenotypes in the CNS has recently attracted interest (Jha et al., 2015). Since no data are currently available on the role of LCN-2 on adult neurogenesis we focused our attention on this upregulated protein as a potential contributor to the absence of proneurogenic effects by p50KO ACM. We tested recombinant mouse LCN-2 (0.01–1 μg/ml) initially on differentiating WT ahNPC. To our surprise LCN-2 promoted, in a concentration-dependent manner, neuronal differentiation of WT NPC, as assessed by counting the percentages of MAP-2$^+$/nestin$^-$ cells generated in presence of recombinant protein or vehicle (one-way

TABLE 1: Differentially expressed proteins in WT and p50KO ACM as identified by LC–MS/MS

Protein name	Accession number (UniProt Swiss-Prot)	Coverage (%)	*P*-value	Fold change (KO)
H-2 class I histocompatibility antigen, K–K alpha chain	sp|P04223|HA1K_MOUSE	11.1	0.0081	3.90
C–C motif chemokine 2 (monocyte chemoattractant protein 1)	sp|P10148|CCL2_MOUSE	22.3	0.0010	3.35
Neutrophil gelatinase-associated lipocalin (NGAL) (lipocalin-2)	sp|P11672|NGAL_MOUSE	31.5	0.0013	2.75

Protein coverage percentage and fold-change expressed as KO/WT ratio are provided for each identified protein.

ANOVA; $P < 0.001$, Fig. 6A). Maximal proneurogenic effect was elicited in presence of 0.1 μg/ml LCN-2 (percentage increase over vehicle-treated cells: +86.78 ± 29.18, $P < 0.001$; Fig. 6A). When tested on p50KO NPC LCN-2 was ineffective up to 0.3 μg/ml and only 1 μg/ml of protein significantly increased the percentage of newly generated neurons (percentage of increase over vehicle-treated cells: +70.27 ± 31.1, $P < 0.001$; Fig. 6A). Under the same concentration range, LCN-2 had no effect on cell survival in both genotypes (Fig. 6B). We then evaluated mRNA levels for LCN-2 receptor (24p3R) and *β*-actin in WT and p50KO NPC by qRT-PCR. qRT-PCR analysis showed that LCN-2R/24p3R gene expression is markedly reduced in p50KO compared with WT cells (percentage reduction: −64.4 ± 2.4, Student's *t*-test: $^{**}P < 0.01$; Fig. 6C).

Discussion

In recent years, experimental data on the involvement of NF-κB proteins in the regulation of adult neurogenesis have been generated (as reviewed in Bortolotto et al., 2014; Grilli and Meneghini, 2012). A few years ago, our group proved that p50KO mice display a dramatic reduction in adult hippocampal neurogenesis and, in parallel, a selective defect in hippocampal-dependent short-term memory. A detailed phenotypic characterization of newly generated hippocampal cells strongly suggested that lack of p50 is associated with defects in the late maturation of newly generated neurons, in absence of alterations in survival and proliferation rates of ahNPC (Denis-Donini et al., 2008). As a follow up to that work, herein we show that when cultured *in vitro*, ahNPC from WT and p50KO mice are not significantly different in their neurogenic potential. By using double immunocytochemistry we could distinguish two distinct MAP-2^+ cell populations, which are generated *in vitro* by ahNPC: MAP-2^+/nestin$^+$ cells, which may be indicative of NPC commitment toward the neuronal lineage and MAP-2^+/nestin$^-$ cells, which may better reflect neuronal maturation. Under basal conditions we could not observe significant differences in the two populations between WT and p50KO NPC cultures. Altogether we

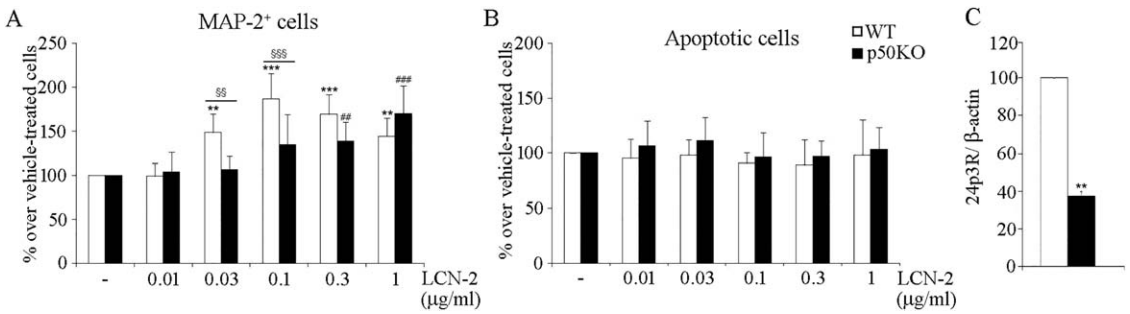

FIGURE 6: Lipocalin-2 is a novel astrocyte-derived proneurogenic signal whose activity is reduced in p50KO NPC. (A) 24 hours treatment with mouse LCN-2 (0.01–1 μg/ml) promoted neuronal differentiation of WT ahNPC in a concentration-dependent manner. Under the same experimental conditions, p50KO NPC responsiveness to LCN-2 is significantly reduced. (B) LCN-2 has no effect on the survival rate of WT and p50KO NPC and their progeny. Data were calculated as mean values ± SD of $n = 3$ experiments, run in triplicates, and analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* WT vehicle; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *versus* p50KO vehicle; $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ in WT *versus* P50KO NPC. Data are expressed as percentage of increase over vehicle-treated cells. (C) QRT-PCR evaluation of LCN-2R/24p3R expression levels in WT and p50KO NPC. LCN-2R expression levels were normalized against *β*-actin as housekeeping gene, and their relative ratio was calculated. LCN-2R expression was downregulated in p50KO NPC. $^{**}P < 0.01$ *versus* WT NPC, Student's *t*-test.

concluded that absence of NF-κB p50 did not correlate *in vitro* with alteration in NPC commitment to neuronal lineage or with maturation of newly generated neurons, as observed *in vivo* (Denis-Donini et al., 2008).

Since the well documented role of NF-κB in nonneuronal cells (Brambilla et al., 2009; Lian et al., 2015), we dissected the role of p50 in ahNPC and astrocytes. Exposure of WT and p50KO hippocampal NPC to conditioned media from WT and p50KO astrocytes was utilized as a strategy to study the influence of astroglia-generated soluble factors on NPC fate specification and the cell-autonomous or noncell-autonomous role played by p50 signaling in that context. Initially we tested the effects of WT and p50KO ACM on WT ahNPC. As previously shown (Song et al., 2002), also in our hands WT ACM promoted neuronal differentiation of ahNPC. Additionally, WT primary astrocytes produced soluble factors that promoted astrogliogenesis of ahNPC, again in line with published reports (Barkho et al., 2006; Chang et al., 2003). Interestingly, when ahNPC were exposed to p50KO ACM we could not observe proneurogenic effects. Conversely, p50KO ACM-derived astrogliogenic signals appeared intact and even enhanced, compared with WT ACM. Of note, no differences in the survival rate of NPC and/or their progeny could be observed after exposure to WT or p50KO ACM.

One interpretation for the lack of proneurogenic effects elicited by p50KO ACM is that absence of the NF-κB subunit could directly affect the secretory profile of astrocytes. It could be hypothesized that, in absence of NF-κB p50, primary astrocytes either become defective in the production of proneurogenic molecule(s) or, alternatively, produce antineurogenic molecule(s). In such context direct and indirect effects of p50 absence could be envisioned. p50 absence may indeed affect transcription of genes encoding for astrocyte-secreted molecules. This could occur as a consequence of the fact that target gene(s) transcription is repressed by p50 homodimers (in that case the gene would become overexpressed) or because, in absence of p50, a profound rearrangement occurs in the available pool of NF-κB transcriptional dimeric complexes, which may in turn result in activation and/or repression of target gene(s). Of course, we cannot even exclude that p50 absence may affect astrocyte secretory profile also by altering the expression of genes encoding proteins which may influence the activity of secreted proteins via posttranscriptional modifications, i.e., kinases, phosphatases, and proteases. Although unlikely, p50 absence may even affect astrocyte secretory pathways or transcription of proteins directly participating in secretory mechanisms. Of course, we do not disregard the possibility that NF-κB p50 absence may change, in addition to their secretory profile, other phenotypic features of astrocytes, including receptor expression and intracellular signaling pathways, but we did not experimentally address this.

We also tested the effect of WT and p50KO ACM on p50KO NPC differentiation. Surprisingly, neither WT nor p50KO ACM promoted neuronal and astroglial differentiation of p50KO NPC over basal conditions, suggesting that in absence of p50 cell autonomous changes may also occur and affect responsiveness of ahNPC to astrocyte-derived proneurogenic and astrogliogenic signals.

In search for phenotypic changes in p50KO NPC which may explain their reduced responsiveness to astrocyte-derived proneurogenic signals, we took into consideration the possibility that these cells may be deficient in membrane receptors for astrocyte-derived proneurogenic molecules. RAGE is expressed by adult NPC where it mediates the proneurogenic effects of several molecules like HMGB-1 and S100B, which are secreted by astrocytes (Meneghini et al., 2010, 2013). Receptor neutralization had no effect on WT ACM proneurogenic effects, suggesting that RAGE activation was not involved in ACM-mediated neuronal differentiation of ahNPC. TSP-1 is also an established astrocyte-derived proneurogenic factor (Lu and Kipnis, 2010). Our group demonstrated that α2δ1, a TSP-1 receptor (Eroglu et al., 2009), is functionally expressed by ahNPC (Valente et al., 2012). Interestingly, α2δ1 expression levels were significantly reduced in p50KO ahNPC, compared with their WT counterpart. In line with this observation, TSP-1 promoted an increase in the percentage of newly formed neurons in WT, but not in p50KO, NPC cultures. Overall these data confirmed that, in absence of p50, cell-autonomous defects may reduce ahNPC responsiveness to astrocyte-derived proneurogenic signals, and in particular to TSP-1, via downregulation of its receptor.

We also actively searched for proteins which may be differentially secreted by astrocytes in the absence of p50 and whose expression could correlate with the lack of proneurogenic effects of KO ACM. LC-MS/MS allowed us to identify proteins differentially expressed in WT and p50KO ACM. More specifically, three molecules were upregulated in p50KO compared with WT ACM, namely C–C motif chemochine-2 (CCL2), neutrophil gelatinase-associated/lipocalin-2 (NGAL/LCN-2), H-2 class I histocompatibility antigen, K–K alpha chain (HA1K). Interestingly, the absence of NF-κB p50 is commonly linked to inflammation in periphery and in brain (Bernal et al., 2014; Rolova et al., 2014) and both CCL-2 and LCN-2 share an established role in inflammation. Moreover, among them, CCL-2 and LCN-2 are both encoded by NF-κB target genes and NF-κB signaling activation lies downstream their receptor activation (Bu et al., 2006; Kohda et al., 2014; Ueda et al., 1994). We hypothesized that one of these proteins may indeed exert

anti-neurogenic activities on NPC and we focused our attention on LCN-2.

LCN-2 is a 24 kDa iron-related protein whose modulatory role in the CNS has recently attracted interest (Jha et al., 2015), although its function is not completely understood (Ferreira et al., 2015). LCN-2 is secreted by brain astrocytes and regarded as an autocrine promoter of their classical proinflammatory activation (Bi et al., 2013; Jang et al., 2013). Since no information was available on the role of LCN-2 on adult neurogenesis, we concentrated our attention on this protein, whose expression is increased by about threefold in p50KO compared with WT ACM. To our surprise, LCN-2 promoted, in a concentration-dependent manner, neuronal differentiation of WT NPC. In contrast with previous observations that LCN-2 can promote apoptosis of neurons and astrocytes (Lee et al., 2012), in our cellular model and within the tested concentration range the protein had no negative effect on the survival of ahNPC and their progeny. Under the same experimental conditions, LCN-2 had no or little effect on neuronal differentiation of p50KO NPC, except at the highest concentration. Altogether, these data suggest, for the first time, that LCN-2 is an astroglial-derived signal which promotes neuronal fate specification of ahNPC and that p50KO NPC are less responsive to this protein. Such reduced sensitivity correlates with the downregulation of LCN-2R/24p3R mRNA levels in p50KO ahNPC. Although it has been demonstrated that LCN-2R is constitutively expressed at high levels in brain, including the hippocampus (Chia et al., 2015), to our knowledge, this is the first demonstration that this receptor is expressed in aNPC. Interestingly, NF-κB signaling activation has been shown to occur downstream of LCN-2/24p3R activation (Dizin et al., 2013).

At this stage of knowledge, we have not yet identified a culprit for reduced proneurogenic effects of p50KO ACM on WT NPC. As far as CCL-2, literature data suggested both proneurogenic (Liu et al., 2007) and antineurogenic effects (Lee et al., 2013) of this chemokine. In our cellular model also CCL-2 exerted proneurogenic activity (data not shown), suggesting that upregulated expression of this chemokine cannot justify reduced proneurogenic effects of p50KO ACM on WT NPC. H-2 class I histocompatibility antigen, K–K alpha chain (HA1K) was also identified by LC–MS/MS in astrocyte-conditioned media and appeared upregulated in p50KO ACM. Of course, at this stage, we cannot exclude that its presence in media may represent an artefact, but since exosomes bearing MHC class I molecules have been demonstrated in other cell types (Zitvogel et al., 1998), in the future, we would like to explore the possibility that extracellular exosomes/microvesicles of astrocytic origin could contribute, with their cargo, to ACM composition and, potentially, to astrocyte-NPC communication. The proteins that we identified in ACM by LC–MS/MS are likely to represent a small fraction of all astrocyte-secreted proteins, so we may have missed other contributors to the reduced proneurogenic potential of p50KO ACM. Among NF-κB target genes that are expressed in astrocytes and whose product is secreted, there are indeed many additional inflammatory and anti-inflammatory cytokines and chemokines (for example, IL-1, IL-6, IL-8, IL-10, IL-12, IL-23, and TNF) (Choi et al., 2014), as well as growth factors like BDNF (Marini et al, 2004). The role of BDNF in neurogenesis is well established, but preliminary experiments in our laboratory did not reveal significant differences in its expression levels or its maturation process between WT and p50KO astrocytes (data not shown).

An additional explanation for reduced proneurogenic effects of p50KO ACM on WT NPC could be that p50KO ACM components may negatively affect expression or functionality of receptor(s) for astrocyte-derived proneurogenic molecules on WT NPC. Since this event occurs in 24 hours, internalization/downregulation or posttranslational modifications of receptors may potentially take place in WT NPC when exposed to p50KO ACM.

In our experimental setting p50KO NPC were unresponsive not only to proneurogenic but also to astrogliogenic astrocyte-derived signals. In the future, we may also attempt identifying the nature of astrocyte-secreted signals that promote astrogliogenesis and of the complex cell-autonomous changes that take place in NPC in the absence of p50. We cannot exclude that the inability of p50KO NPC to respond to both proneurogenic and astrogliogenic signals may also depend on p50-requirement downstream of ACM treatment.

Altogether we propose that reduced hippocampal neurogenesis in p50KO mice *in vivo* could be ascribed to complex defects in the cross-talk between astrocytes and ahNPC. Of course, we cannot exclude the contribution of other cell phenotypes to a reduced hippocampal neurogenesis in p50KO mice and in particular of microglial cells, which are well known to affect NPC fate specification (Sierra et al., 2014). Our experimental work demonstrates that complex cell autonomous and noncell-autonomous changes can affect NPC fate specification in the absence of NF-κB p50. We also show, for the first time, that LCN-2 is an astroglial-derived signal, which promotes neuronal fate specification of ahNPC and whose activity, in parallel with a downregulation of LCN-2R, is strongly reduced in p50KO NPC. Altogether, these data add further complexity to a growing body of data suggesting the relevance of astrocytes and NF-κB signaling in the modulation of adult neurogenesis.

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Conflict of Interest

None declared.

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