

MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

NCAM expression induces neurogenesis *in vivo*Camille Boutin,¹ Brigitte Schmitz,² Harold Cremer^{1,*} and Simone Diestel^{1,*,†}¹Institut de Biologie du Développement de Marseille-Luminy, UMR 6216, CNRS/Université de la Méditerranée, Campus de Luminy-case 907, 13288 Marseille cedex 9, France²Institute of Animal Sciences, Department of Biochemistry, University of Bonn, Katzenburgweg 9a, 53115 Bonn, Germany**Keywords:** heterophilic NCAM interactions, *in vivo* electroporation of postnatal mice, olfactory neurogenesis, PSA-NCAM

Abstract

Neural cell adhesion molecule (NCAM) plays an important role during neural development and in the adult brain, whereby most functions of NCAM have been ascribed to its unique polysialic acid (PSA) modification. Recently we presented evidence suggesting that expression of NCAM *in vivo* interferes with the maintenance of forebrain neuronal stem cells. We here aimed at investigating the fate of cells generated from NCAM-overexpressing stem cells in postnatal mouse brain and at elucidating the functional domains of NCAM mediating this effect. We show that ectopic expression of the NCAM140 isoform in radial glia and type C cells induces an increase in cell proliferation and consequently the presence of additional neuronal type A cells in the rostral migratory stream. A mutant NCAM protein comprising only fibronectin type III repeats and immunoglobulin-like domain 5 was sufficient to induce this effect. Furthermore, we show that the neurogenic effect is independent of PSA, as transgenic NCAM is not polysialylated in radial glia and type C cells. These results suggest that heterophilic interactions of NCAM with other components of the cell membrane must be involved.

Introduction

Neural cell adhesion molecule (NCAM) is widely expressed in the brain and has been implicated in a variety of processes. NCAM can interact in *cis* and *trans* homophilically via the immunoglobulin-like (Ig) domains 1–3. Furthermore, some heterophilic binding partners have been identified. Additionally, NCAM can activate several signal transduction pathways initiated by homophilic NCAM–NCAM binding or interaction with heterophilic ligands in the extracellular region [i.e. glial cell line-derived neurotrophic factor (GDNF), prion protein, fibroblast growth factor receptor (FGFR); reviewed in Hinsby *et al.*, 2004]. NCAM carries a unique modification in its extracellular domain, polysialic acid (PSA). PSA is mainly expressed during development, reaches its maximum expression perinatally and is then drastically downregulated. In later developmental stages the PSA-modification remains only in regions of the brain that maintain neurogenesis, including the subventricular zone (SVZ), the granule cell layer of the hippocampus, particular regions of the hypothalamus and in regions undergoing structural plasticity (reviewed in Rutishauser, 2008; Bonfanti & Theodosis, 2009). PSA has been suggested to regulate NCAM function based on the observation that mice lacking the two specific PSA transferases, thus being PSA-deficient but NCAM-

positive, develop a more severe phenotype than animals lacking both NCAM and PSA (Cremer *et al.*, 1994; Weinhold *et al.*, 2005; Angata *et al.*, 2007). Although considerable effort has been made to unravel the molecular mechanisms of NCAM actions, our current understanding concerning this point is still rather fragmentary.

Several studies suggested a role of NCAM as an inducer of neuronal differentiation. However, the results obtained in there were highly controversial (Amoureux *et al.*, 2000; Shin *et al.*, 2002; Kim *et al.*, 2005; Röckle *et al.*, 2008; Burgess *et al.*, 2008). This could be attributed to the fact that most of the studies were based on *in vitro* assays and that different cell populations were investigated as, for example, hippocampal precursor cells (Amoureux *et al.*, 2000; Shin *et al.*, 2002; Kim *et al.*, 2005; Burgess *et al.*, 2008) or SVZ-derived neuroblasts (Röckle *et al.*, 2008).

In this study, we investigated *in vivo* the effect of NCAM on neurogenesis in the anterior mouse forebrain. In the postnatal periventricular region, radial glia cells localized in the ventricular zone (VZ) lining the lateral ventricle are stem cells that generate type A cells. These migrate to the olfactory bulb (OB) where they differentiate into interneurons (Merkle *et al.*, 2004). This accessible neurogenic system can easily be visualized and genetically manipulated via electroporation (Boutin *et al.*, 2008), thus representing a powerful model to study the molecular mechanism underlying neurogenesis. Here we present gain-of-function experiments showing that ectopic NCAM expression in neural stem cells favours neurogenesis. Furthermore, we demonstrate that this effect is independent of NCAMs polysialylation.

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Materials and methods

Antibodies

Hybridoma cells producing monoclonal antibody 123C3 against human NCAM were provided by R. Michalides (Amsterdam, the Netherlands), monoclonal mouse anti-L1 antibody (UJ 127.11) was provided by P. Altevogt (Heidelberg, Germany; Ebeling *et al.*, 1996). Monoclonal mouse anti-PSA antibodies were kind gifts of G. Rougon, Marseille, France (MenB) and R. Gerardy-Schahn (735), University of Hannover, Hannover, Germany. Monoclonal mouse RC2 antibody was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA); monoclonal mouse anti-Ki67 and MASH1 antibodies were from BD Pharmingen (Erembodegem, Belgium). Polyclonal guinea pig anti-doublecortin (Dcx) antibody was purchased from Millipore (Molsheim, France). Secondary antibodies conjugated to Cy3, Cy2 or horseradish peroxidase were purchased from Dianova (Hamburg, Germany); Alexa-Fluor-633- or Alexa-Fluor-488-conjugated secondary antibodies were from Molecular Probes (Eugene, OR, USA).

Plasmids

pCX-MCS2 and pCX-EGFP-N1 plasmids have been described by Morin *et al.* (2007), expression plasmid for human NCAM140 by Diestel *et al.* (2007). L1 cDNA was subcloned into the pCX-MCS2 plasmid. Human sialyltransferase (St8Sia II; Stx) was a kind gift of H. Hildebrandt (University of Hannover) and subcloned into pCDNA3 plasmid. Plasmids used for *in vivo* electroporation were prepared using EndoFree Plasmid Kits (Qiagen, Hilden, Germany) and resuspended in phosphate-buffered saline (PBS; 5 µg/µL final concentration).

Construction of human NCAMΔCT (hNCAMΔCT) and hNCAMΔNCT constructs

The hNCAMΔCT construct was created using pCX-MCS2-NCAM140wt (further termed hNCAM140) construct as template (described in Diestel *et al.*, 2007) by polymerase chain reaction (PCR) using the following primers: sense primer: 5'-CCC AAG CTT GGG ATG CTG CAA ACT AAG GAT CTC ATC TGG-3' and antisense primer: 5'-CGG GAT CCC GTC AGG TGA TGT CCA CAA CCA G-3' (introducing a stop codon directly after the transmembrane domain). The PCR product was then introduced into pCX-MCS2.

For construction of the hNCAMΔNCT construct, the above-described hNCAMΔCT construct was used as template. Overlap extension PCR was carried out to delete Ig 1–Ig 4 domains between the signal peptide and Ig 5 domain. The first PCR was carried out with the following primers: sense primer 1: 5'-CCA AAT CTG GCG GAG CCG AAA TCT GG-3' (annealing in pCX-MCS2 plasmid) and antisense primer 2: 5'-CTG GGA GTC CTG CAC CTG CAG AGA AAC TGC AGT TCC CAG-3' (deleting Ig 1–Ig 4 domains). For the second PCR, sense primer 3: 5'-CT CTG CAG GTG CAG GAC TCC CAG TCC ATG TAC CTT-3' (also deleting Ig 1–Ig 4 domains) and antisense primer 4: 5'-CGG GAT CCC GTC AGG TGA TGT CCA CAA CCA CCA G-3' were used. A third PCR was carried out using PCR products 1 and 2 as templates, with the following primers to generate the complete hNCAMΔNCT construct: sense primer 1: 5'-CCA AAT CTG GCG GAG CCG AAA TCT GG-3' and antisense primer 4: 5'-CGG GAT CCC GTC AGG TGA TGT CCA CAA CCA CCA G-3'. All constructs were verified by direct DNA sequencing (MWG Biotech, Ebersbach, Germany). The final PCR product was also introduced into pCX-MCS2.

Postnatal electroporation procedure

Animals were treated and experiments were carried out according to guidelines approved by the French ethical committee and the European Communities Council Directive (24 November 1986, 86/609/EEC). The electroporation procedure was carried out as described in Boutin *et al.* (2008). Briefly, P1 mice of CD1 strain (Charles River, Lyon, France) were anaesthetized by hypothermia (4 min). Mice were fixed to a support and plasmid DNA was injected into the lateral ventricle (5 µg/µL, 2 µL per injection). Co-electroporation was carried out with cDNAs in a ratio of 3 : 1 [DNA of interest : green fluorescent protein (GFP)]. This ratio results in a successful co-electroporation rate of 85.4% (Boutin *et al.*, 2008). Successfully injected animals were subjected to five electrical pulses (100 V, 50 ms, separated by 950-ms intervals) using the CUY21 edit device (Nepagene, Chiba, Japan) and 10-mm tweezer electrodes (CUY650P10; Nepagene). Reanimated animals were returned to the mother.

NCAM-deficient mice have been described previously (Cremer *et al.*, 1994) and were treated like CD1 mice.

Immunohistochemistry

For histological analysis, pups were deeply anaesthetized with an overdose of ketamin/xylazin (100 mg and 10 mg/kg body weight, respectively). Perfusion was performed intracardially with a solution of 2% paraformaldehyde in PBS. The brains were dissected out and immersed overnight in the same fixative at 4°C. Coronal sections were cut at 50 µm using a microtome (Microm, Walldorf, Germany). Immunohistochemistry was carried out on floating vibratome sections. Sections were first blocked with 5% horse serum in PBS (blocking solution) for 1 h at room temperature. For Ki67 and MASH1 staining, cells were permeabilized with 0.5% Triton X-100 during the blocking step. Respective first antibodies were incubated overnight at 4°C (123C3 4.5 µg/mL, MenB 1 : 200, RC2 1 : 100, 735 2 µg/mL, Ki67 1 : 200, MASH1 1 : 100, Dcx 1 : 200) in blocking solution. After three washing steps with blocking solution, incubation with labelled secondary antibodies followed for 1 h. Before mounting, cell nuclei were stained with Hoechst 33258 (Sigma, Lyon, France). Images were taken either using a fluorescence microscope (Axioplan2, ApoTome system; Zeiss, Germany) with a Plan Apochromat 20 ×/0.75 objective or a laser confocal scanning microscope with a Plan Apochromat 63 ×/1.40 oil immersion objective (LSM510; Zeiss, Germany).

Cell culture, transfection and immunoblot analysis

For analysis of *in vitro* PSA-modification of NCAM, rat B35 neuroblastoma cells were used (Schubert *et al.*, 1974; Diestel *et al.*, 2007). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin, and plated on 0.01% poly-L-lysine-coated plastic dishes. Transient transfections were carried out using Lipofectamine Plus™ reagent (Invitrogen, Karlsruhe, Germany). For co-transfection of hNCAM140 or hNCAMΔNCT together with St8Sia II, a ratio of 1 : 1 of both cDNAs was used. After transfection the cells were cultured for 1 day followed by differentiation using 1 mM dbcAMP in OptiMEM (Invitrogen) and further incubation for 24 h. Cells were lysed in RIPA buffer and protein concentration was determined using the Bicinchoninic acid assay (Sigma). Proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked in blocking solution [2 h, 5% milk powder

in Tris-buffered saline (TBS)/0.1% Tween-20] prior to incubation with the first antibodies in 5% bovine serum albumin in TBS/0.1% Tween-20 (overnight, 4°C). Membranes were washed and incubated with peroxidase-conjugated secondary antibodies for 1 h in blocking solution. Proteins were detected by enhanced chemiluminescence (Perbio Science, Bonn, Germany).

Statistical analysis and quantification of radial glia cells and type A cells

Statistical analysis was carried out with one-way ANOVA followed by the Bonferroni *post hoc* test for multiple comparisons. *P*-values equal to or < 0.05 were considered statistically significant (**P* < 0.05, ***P* < 0.01, ****P* < 0.001). Data are presented as mean ± SEM. SPSS software (Munich, Germany) was used to analyse the data for statistical significance. For most experiments (if not differently indicated) data from at least three animals were analysed.

For an approximate quantification of radial glia cells compared with non-radial glia cells in the periventricular zone the unique morphology of radial glia cells was taken as a basis. They make contact with the ventricle lumen and send a long, radial process to the brain surface. These cells were defined as radial glia cells. All other cells observed in the SVZ were counted and classified as non-radial glia cells. Errors in classification that might have occurred by this way of counting would occur in control and NCAM-electroporated brains, and have therefore statistically not been taken into account. For this experiment, at least five mice with a minimum of six slices have been analysed per condition.

For quantification of neural precursors 4 days post-electroporation (dpe) GFP-positive cells in the SVZ of each slice were counted, the mean was calculated and – because of the heterogeneity of the electroporation efficiency – set to 100%. This kind of calculation therefore does not take into account the different cell numbers of GFP-positive cells in the SVZ after NCAM or control electroporation (see Fig. 2). Cells in the rostral migratory stream (RMS) and OB were also counted and matched to 100% GFP-positive cells in the SVZ.

Results

We have recently described that expression of human NCAM140 (hNCAM140) in postnatal radial glia cells induces a loss of this cell type and the generation of type A cells (Boutin *et al.*, 2008). To further analyse this phenotype and to understand if this effect on radial glia cells is specific for NCAM or can also be induced by overexpression of other cell adhesion molecules, co-electroporation of GFP with either empty plasmid (control), hNCAM140 or L1 – a related cell adhesion molecule – into P1 mouse brain was carried out. Radial glia cells have a unique morphology that allows them to be unequivocally identified with their cell body lining the ventricles and long processes projecting to the pial surface. At 2 dpe in control brains these cells represented 30% of all GFP-positive cells observed in the periventricular region (corresponding to a ratio of non-radial glia to radial glia of 3.33 to 1, *n* = 34 slices; Fig. 1A and B). Expression of hNCAM140 induced a reduction of radial glia to 15% of all GFP-positive cells (which corresponds to a ratio of non-radial glia to radial glia of 6.4 to 1, *P* < 0.001, *n* = 32 slices, one-way ANOVA) and the appearance of tangentially oriented cells in the SVZ. In contrast, the proportion of radial glia cells was not affected when L1 was expressed (*P* = 0.184, one-way ANOVA; Fig. 1A and B). These results demonstrate that the observed effect on radial glia cells is specific for NCAM rather than a general property shared by adhesion molecules.

In light of these results we investigated the endogenous expression of NCAM and PSA (because of its importance for NCAM function) in this cell type postnatally. Expression of PSA-NCAM has been described in radial glia cells in embryonic stages of rat brain (Li & Grumet, 2007). Co-staining of PSA and RC2, a marker for radial glia cells (Misson *et al.*, 1988), was carried out in sections of non-electroporated control brains. Expression of PSA could be observed in the SVZ and in a small cell population directly at the ventricular wall of the lateral ventricle. However, these cells did not exhibit radial glia cell morphology and no co-expression of PSA with RC2 could be detected (Fig. 2). Co-staining of endogenous NCAM and RC2 revealed a broad expression of NCAM in the VZ/SVZ. However, in the VZ it did not co-localize with RC2, and NCAM-positive cells at the wall of the lateral ventricle did not exhibit radial glia morphology (Fig. 2). Altogether, these results show that neither NCAM nor PSA is endogenously expressed in radial glia cells at postnatal stages.

Ectopic expression of NCAM has consequences on radial glia cells, which according to the current model turn postnatally into type B cells, which represent the neural stem cells of the adult SVZ (reviewed in Merkle & Alvarez-Buylla, 2006). Thus, we investigated the consequences of NCAM overexpression on other cell populations occurring in this neurogenic system.

Type C cells represent a highly proliferative intermediate progenitor population that has been well described in the adult (Doetsch *et al.*, 1999). These cells are characterized by the expression of the basic helix-loop-helix transcription factor MASH1 in the adult (Parras *et al.*, 2004) and in postnatal stages (own unpublished observation). To analyse the fate of electroporated cells, the number of GFP-positive type C cells was quantified at 2 dpe. In the control situation we found considerable amounts of MASH1-expressing C cells ($24.0 \pm 2.2\%$, *n* = 26; Fig. 3A and B). Expression of hNCAM140 led to a significant increase of MASH1-positive cells in this compartment ($36.4 \pm 2.3\%$, *P* = 0.004, *n* = 21, one-way ANOVA; Fig. 3A and B), indicating that increased amounts of radial glia may enter the neurogenic pathway.

In the adult it has been shown that type C cells give rise to migrating type A cells expressing Dcx (Nacher *et al.*, 2001) and PSA-NCAM (Doetsch *et al.*, 1997). In agreement, we found that in postnatal stages large amounts of GFP-positive type A cells travel through the RMS into the OB at 4 dpe (Boutin *et al.*, 2008). Quantification of the number of GFP-positive cells in the SVZ, RMS and OB revealed that significantly more cells were present in the RMS and OB when hNCAM140 was expressed compared with control expression (RMS: *P* = 0.021, *n* = 13; OB: *P* = 0.001, *n* = 15, one-way ANOVA; Fig. 3D). The identity of these cells as migrating type A cells was verified by Dcx staining (Fig. 3C). This calculation is based on the fact that the electroporation efficiency is identical for hNCAM140 and control plasmid. Taken together, these data show that cell proliferation after NCAM electroporation passes via type C cells and that the neuronal lineage is favoured as a consequence of NCAM gain-of-function.

We observed earlier that the total number of GFP-positive cells was increased by approximately 30% after ectopic NCAM expression (Boutin *et al.*, 2008). Therefore, we investigated next the cell proliferation in the periventricular zone. Wild-type P1 mouse brains were co-electroporated with GFP and hNCAM140 or a control plasmid, and staining with the proliferation marker Ki67 was performed at 2 dpe. We found that in control conditions at this time point $29.6 \pm 2.8\%$ of electroporated cells expressed Ki67 (*n* = 21; Fig. 4A and B). When hNCAM140 was expressed, this number increased significantly to $49.2 \pm 3.2\%$, (*P* = 0.001, *n* = 23, one-way ANOVA; Fig. 4A and B). Altogether, these results strongly suggest that

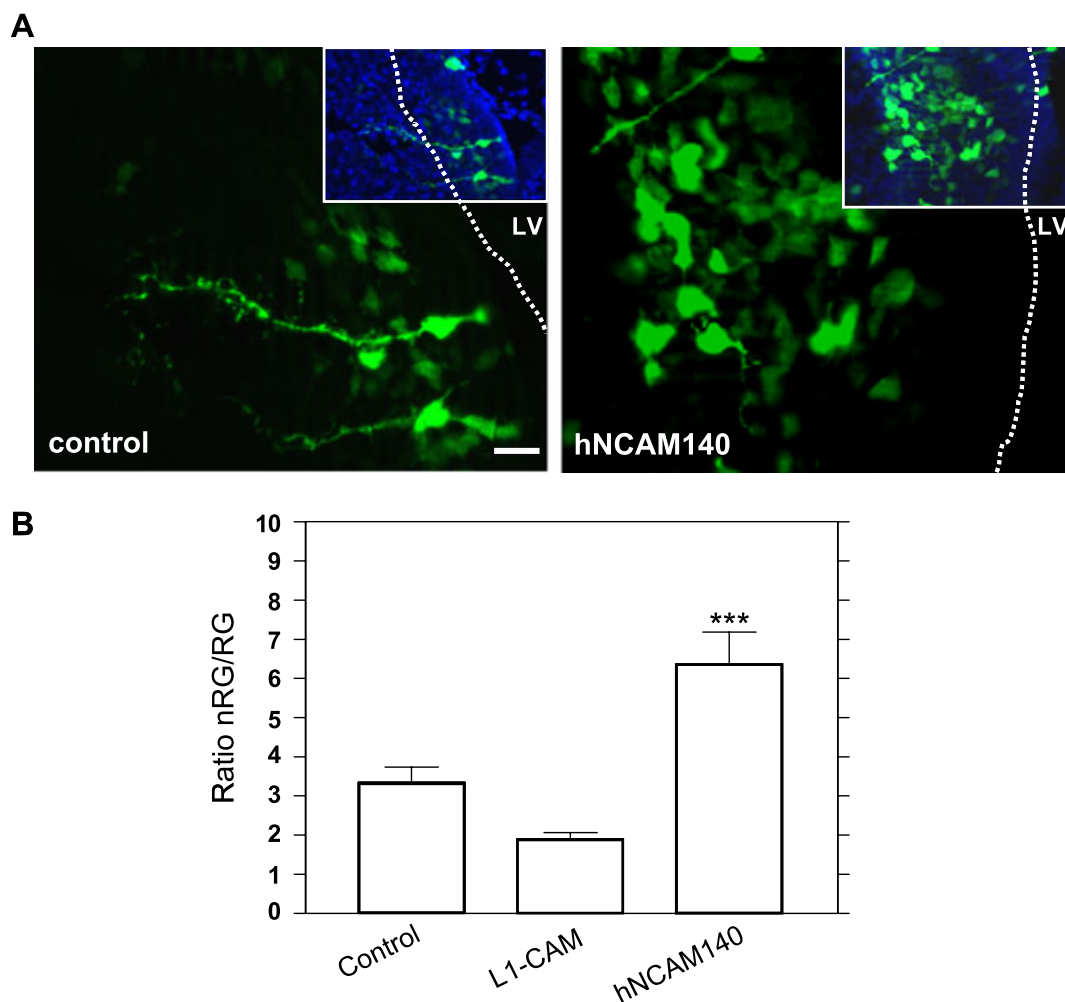


FIG. 1. HNCAM140 interferes with radial glia cell maintenance. (A) Wild-type mice were co-electroporated with GFP and an empty expression plasmid (= control), or with GFP and hNCAM140, respectively. Mice were analysed 2 dpe. Inserts show the overlay of GFP fluorescence and Hoechst 33258 staining. The hNCAM140 electroporated cells show more tangentially oriented cells and less cells with radial glia morphology. Scale bar: 20 μ m. (B) Quantification of the ratio of radial glia cells (RG) to other cell types (nRG, non-radial glia cells) in the SVZ after electroporation of control, L1 or hNCAM140 together with a GFP expression plasmid (2 dpe). Cells with radial glia and all cells with other morphology in the SVZ were counted, and the ratio between both cell numbers was calculated. For quantification, at least five animals have been analysed with a minimum of six slices per animal investigated. Data are given in mean $\pm$ SEM, *** P < 0.001. LV, lateral ventricle; NCAM, neural cell adhesion molecule.

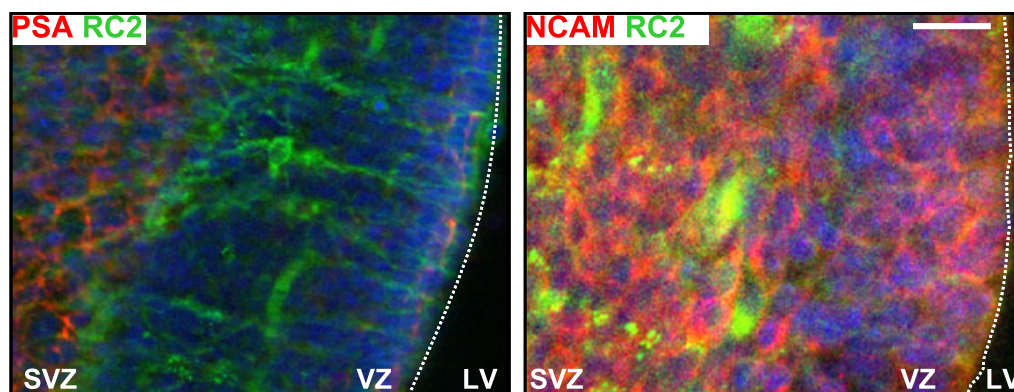


FIG. 2. Polysialic acid (PSA) and neural cell adhesion molecule (NCAM) are not present in radial glia cells of postnatal mice. Radial glia cells of P0 wild-type mouse brains in the subventricular zone (SVZ) were identified by staining with antibody RC2 (green); PSA was detected with antibody 735 (left, red). Endogenous NCAM was detected with mouse NCAM-specific antibody H28 (right, red). Nuclei were counterstained with Hoechst 33258; scale bar: 10 μ m. LV, lateral ventricle; VZ, ventricular zone.

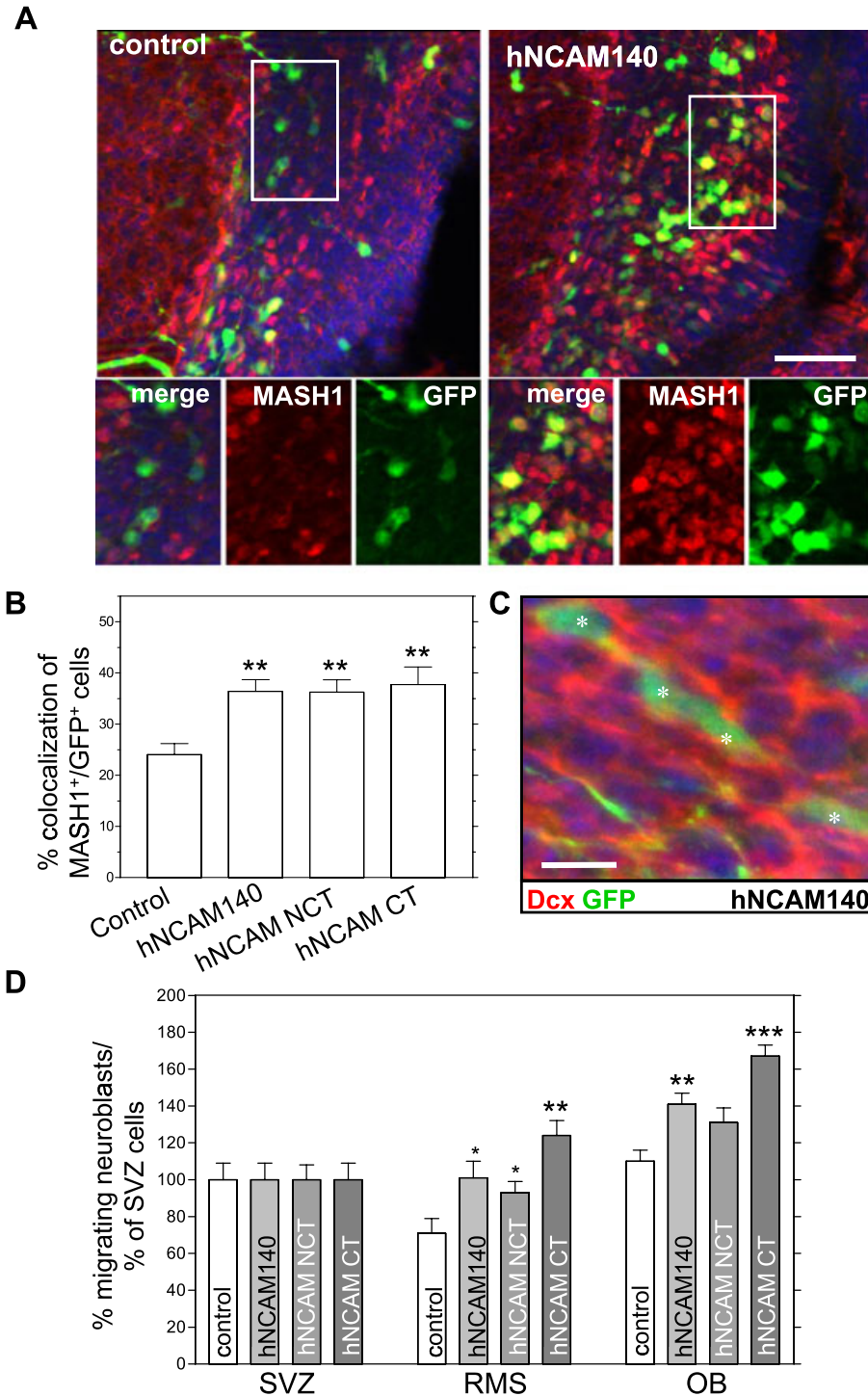


FIG. 3. Neural cell adhesion molecule (NCAM) enhances olfactory neurogenesis. (A) Wild-type mice were co-electroporated with control/green fluorescent protein (GFP) or hNCAM140/GFP plasmids. Type C cells were immunostained 2 dpe with antibody MASH1 (red) and Hoechst 33258 (blue); scale bar: 50 μ m. (B) Quantification of cells double-positive for GFP and MASH1 in the subventricular zone (SVZ) at 2 dpe reveals significantly more hNCAM140/GFP-positive type C cells than control/GFP-expressing type C cells. Similar results were obtained for the NCAM fragments hNCAM Δ NCT and hNCAM Δ CT (see Fig. 5 for a description of these isoforms). For quantification, at least three animals were analysed per condition with at least seven slices per animal. Data are given in mean $\pm$ SEM, ** P < 0.01. (C) Wild-type mice were co-electroporated with hNCAM140/GFP plasmids. Migrating neuroblasts were immunostained 4 dpe with a Dcx-specific antibody (red) and Hoechst 33258 (blue); scale bar: 5 μ m. An image section from the rostral migratory stream (RMS) is shown. (D) Wild-type mice were co-electroporated with hNCAM140/GFP, hNCAM Δ NCT/GFP, hNCAM Δ CT/GFP or with control/GFP. Cells that remained in the SVZ 4 dpe were counted and the mean cell number from all slices analysed was set to 100%. The number of cells in the RMS and olfactory bulb (OB), respectively, were matched to the 100% of SVZ cells. Note that more cells were detected in the RMS and OB of either NCAM isoform expressing brains compared with control brains. For quantification, at least five animals were analysed per condition with at least six slices per animal. Data are given in mean $\pm$ SEM, * P < 0.05, ** P < 0.01, *** P < 0.001.

NCAM expression positively influences proliferation of cells of the neurogenic lineage in the SVZ.

To further analyse the underlying mechanism of the effect of NCAM on postnatal neurogenesis, different NCAM constructs were generated (Fig. 5A). In the first construct (termed hNCAM Δ NCT) Ig domains 1–4 and the carboxyterminal region were deleted, leaving only domains relevant for efficient polysialylation (Close *et al.*, 2003), but lacking all domains that are necessary for homophilic and most heterophilic extracellular interactions. This protein is also unable to activate NCAM-dependent signal transduction pathways. The second construct (hNCAM Δ CT) misses the cytoplasmic tail, thereby generating a protein that is able to interact with all physiological binding partners in its extracellular region but can not activate NCAM-dependent signal transduction pathways. The correct and efficient polysialylation of the hNCAM Δ NCT construct was verified by expression in B35 neuroblastoma cells in the presence of S8Sia II (Fig. 5B), one of the two enzymes that mediate polysialylation of NCAM (for review, see

Rutishauser, 2008). Additionally, PSA-modification of the hNCAM Δ NCT construct was verified in type A cells by immunohistochemistry *in vivo*. These cells express in the RMS high amounts of PSA-NCAM (Rousselot *et al.*, 1995; Chazal *et al.*, 2000). hNCAM Δ NCT was co-electroporated with GFP into postnatal day 1 (P1) brains of heterozygous (NCAM^{+/−}) or NCAM-deficient (NCAM^{−/−}) mice, and PSA expression was analysed at 5 dpe. Expression of hNCAM Δ NCT induced a detectable increase in PSA expression in transgenic cells compared with the already PSA-positive RMS environment (Fig. 5C). When the construct was expressed in NCAM- and, therefore, PSA-deficient mice (Cremer *et al.*, 1994), only GFP-positive cells in the RMS showed efficient polysialylation as already demonstrated for hNCAM140 (Boutin *et al.*, 2008). Altogether, these results demonstrate that hNCAM140 and hNCAM Δ NCT become correctly and efficiently polysialylated *in vitro* and *in vivo*.

We compared the consequence of expression of the two modified protein variants hNCAM Δ CT and hNCAM Δ NCT with the effect of

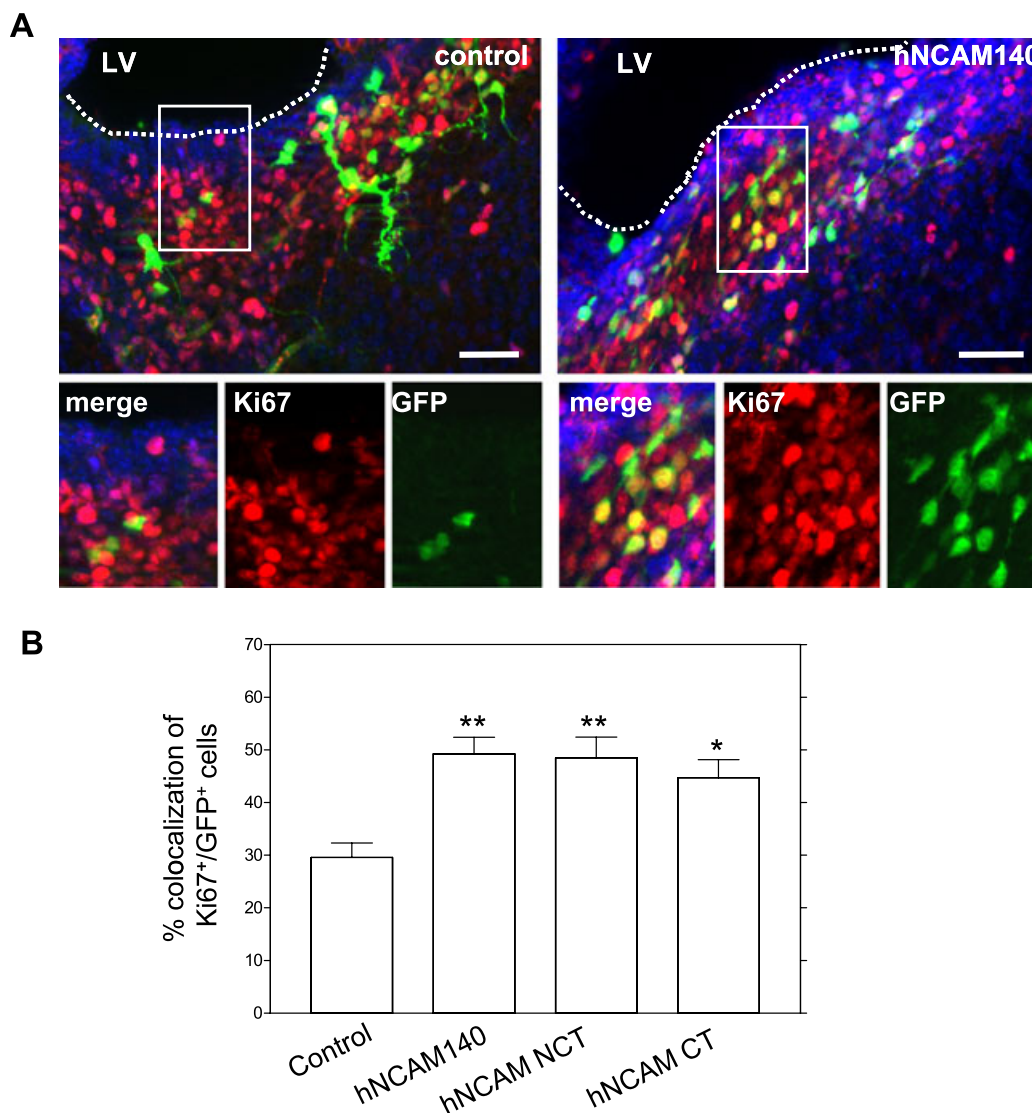


FIG. 4. Neural cell adhesion molecule (NCAM) expression increases proliferation in the SVZ. (A) Cells were electroporated with control/green fluorescent protein (GFP) or hNCAM140/GFP plasmids. Proliferating cells (2 dpe) were stained with antibody Ki67 (red) and cell nuclei with Hoechst 33258 (blue). Scale bar: 50 μ m. (B) Quantification of Ki67- and GFP-double-positive cells (2 dpe) in the SVZ reveals a significantly higher proliferation rate of hNCAM140/GFP-, hNCAM Δ NCT/GFP- and hNCAM Δ CT/GFP-expressing cells than control/GFP cells. For quantification, at least three animals were analysed with at least seven slices per animal. Data are given in mean $\pm$ SEM, * P < 0.05, ** P < 0.01.

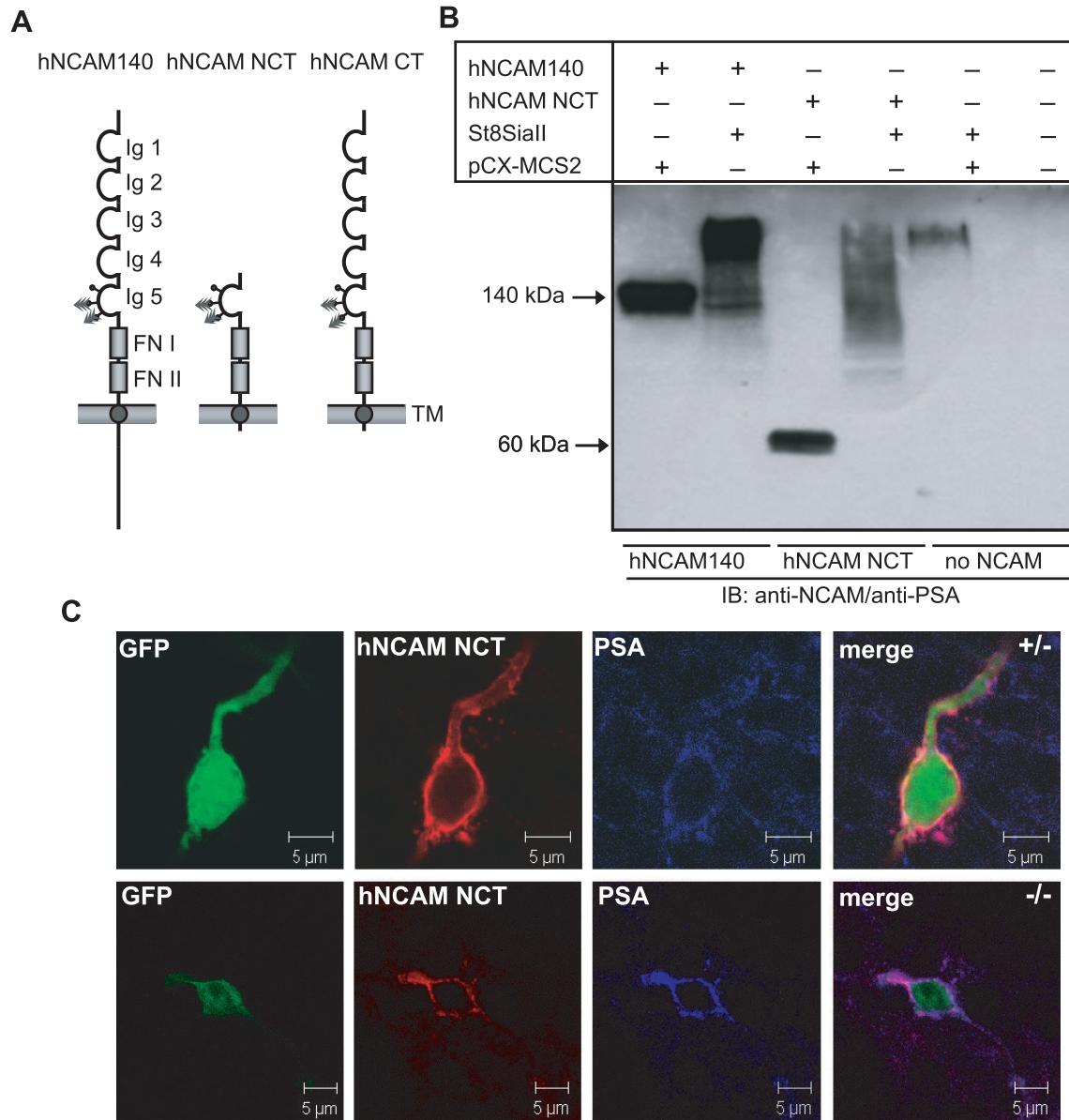


FIG. 5. hNCAM140 and hNCAM Δ NCT are polysialylated *in vitro* and *in vivo*. (A) Human NCAM140wt was used as a template to generate the deletion mutants. FN, fibronectin type III domain; Ig, immunoglobulin-like domain; TM, transmembrane domain; arrowheads indicate the polysialylation sites. The hNCAM Δ NCT construct misses Ig 1–4 domains and the cytoplasmic tail. In hNCAM Δ CT only the cytoplasmic tail was deleted. (B) B35 neuroblastoma cells were transfected with hNCAM140 or hNCAM Δ NCT together with an empty expression plasmid (pCX-MCS2) or with a plasmid encoding sialyltransferase (St8Sia II). hNCAM140 and hNCAM Δ NCT were detected with the human neural cell adhesion molecule (NCAM)-specific antibody 123C3, and polysialic acid (PSA) with the PSA-specific antibody 735. The molecular weight shift in the St8Sia II-transfected lanes shows efficient polysialylation of hNCAM140 and hNCAM Δ NCT. The signal in lane 5 represents polysialylation of endogenous NCAM. (C) Heterozygous (NCAM $^{+/-}$) or NCAM-deficient (NCAM $^{-/-}$) mice were co-electroporated with green fluorescent protein (GFP) and hNCAM Δ NCT. Immunohistochemistry was carried out at 5 dpe. Cells in the RMS were labelled with the PSA-specific antibody Men B (blue) or human NCAM-specific antibody 123C3 (red). hNCAM Δ NCT is specifically expressed in NCAM $^{-/-}$ and NCAM $^{+/-}$ mice in electroporated cells. PSA expression was increased in NCAM $^{+/-}$ mice in electroporated cells compared with surrounding cells, and specifically detected in electroporated cells in NCAM $^{-/-}$ mice.

full-length hNCAM140 concerning their effects on neurogenesis. We found that neither deficiency of the cytoplasmic domain nor additional deletion of major parts of the extracellular domains induced a loss of the neurogenic potential of NCAM (Figs 3B and D, and 4). First, the number of MASH1-positive type C cells was increased in comparison to the control, but to the same level after full-length NCAM expression (Fig. 3B). Second, the number of A cells in the RMS and the OB was augmented at a level comparable to that induced by hNCAM140 (Fig. 3D). In addition, proliferation as measured by Ki67 expression

was increased to the same level as by hNCAM140 (Fig. 4B). Thus, at all investigated levels, the two constructs were indistinguishable from hNCAM140 in their ability to potentiate neurogenesis.

The isoforms have in common their ability to become PSA-modified. Therefore, we investigated the possibility that the observed effect on neurogenesis is due to this modification and not to a function of the NCAM backbone itself. We analysed the polysialylation status of overexpressed NCAM in radial glia and type C cells at postnatal stages. hNCAM140 was co-electroporated with GFP into P1 mouse

brains, and analysis of radial glia cells was carried out at 1 dpe. This early time point was chosen because NCAM-electroporated radial glia cells had not yet turned into their progeny at this stage compared with later stages at which the other experiments have been performed (2 dpe, 4 dpe, 5 dpe). Electroporated radial glia cells were identified by expression of GFP and their specific morphology. We found that radial glia cells that showed strong expression of transgenic NCAM did not stain for PSA (Fig. 6A). The same was true for type C cells after 2 dpe, as demonstrated by the absence of colocalization of PSA and MASH1 in GFP-positive cells (Fig. 6B). Altogether, these results strongly indicate that the positive effect of NCAM on neurogenesis in the periventricular region can be attributed to the NCAM protein itself rather than its PSA moiety. Furthermore, the finding that even the minimum fragment represented by hNCAM Δ NCT can mediate the effect demonstrates that it is independent of: (i) homophilic NCAM interactions; and (ii) NCAM-mediated signal transduction.

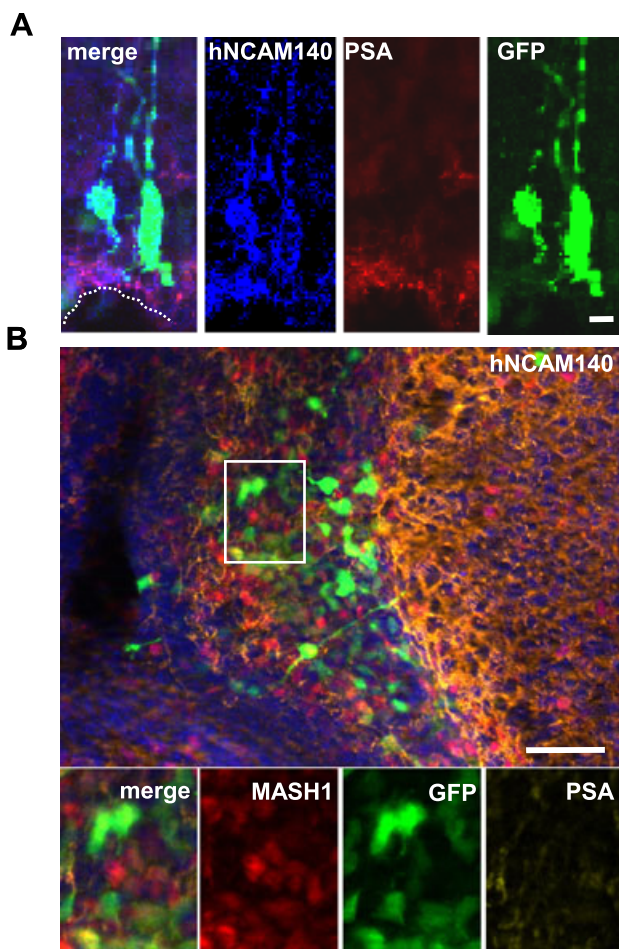


FIG. 6. Electroporated neural cell adhesion molecule (NCAM) is not polysialic acid (PSA)-modified in radial glia cells and type C cells. (A) Wild-type mice were electroporated with hNCAM140/green fluorescent protein (GFP) plasmids and analysed at 1 dpe. Electroporated NCAM was labelled using the human NCAM-specific antibody 123C3 (blue), PSA with the PSA-specific antibody Men B (red). None of the electroporated radial glia cells in the SVZ show PSA expression; scale bar: 5 μ m. (B) After co-electroporation with hNCAM140/GFP plasmids, type C cells were identified by labelling with antibody MASH1, PSA was detected with MenB antibody. No PSA expression was detectable in MASH1-positive type C cells. Scale bar: 50 μ m. Data are given in mean $\pm$ SEM. For quantification, at least three animals were analysed per condition with at least seven slices per animal.

Discussion

In this study we took advantage of an *in vivo* electroporation method to evaluate the effect of NCAM expression on olfactory neurogenesis of postnatal mouse brain. We found that overexpressing this protein in radial glia cells influences their maintenance and increases the generation of migrating A cells via transit amplifying type C cells.

Postnatal radial glia cells are able to generate different cell types, like oligodendrocytes, ependymal cells, astrocytes and OB interneurons (Merkle *et al.*, 2004). Our results indicate that premature NCAM expression in radial glia cells influences their maintenance and increases their potential to generate migrating A cells via transit amplifying type C cells. This is in accordance with previous reports that described NCAM as an inducer of neurogenesis *in vitro* (Amoureux *et al.*, 2000; Shin *et al.*, 2002; Kim *et al.*, 2005). Interestingly, we observed increased proliferation of cells in the periventricular region before the start of migration through the RMS to give rise to interneurons of the OB (Boutin *et al.*, 2008; and data not shown).

With the immunohistochemical analysis used we cannot clearly distinguish which cell type(s) exhibit accelerated proliferation within the SVZ. However, we observed more transit amplifying type C cells in all experiments. Therefore, these and/or earlier generated cells in the periventricular zone are probably induced by NCAM to proliferate.

It is also possible that ectopic NCAM might not increase cell proliferation itself but that it might rather influence cell survival. This would also lead to more type C cells and therefore conclusively to more proliferating cells. This would be in accordance with two studies describing a positive effect of NCAM on cell survival (Gascon *et al.*, 2007; Röckle *et al.*, 2008). The first study demonstrated reduced cell survival of A cells in NCAM-deficient animals. This effect could also be induced by enzymatic removal of PSA in wild-type mice. Therefore, the survival of A cells depended on the PSA-modification of NCAM. The latter study (Röckle *et al.*, 2008) showed a role of non PSA-modified NCAM on survival of type A cells *in vitro*. As an underlying mechanism an increased NCAM *trans* interaction has been proposed (Röckle *et al.*, 2008). Because our results demonstrate that the effect is not mediated by PSA and our hNCAM Δ NCT construct is not capable of homophilic *trans* interactions, these mechanisms seem not to play a role under the *in vivo* conditions described here. Moreover, both studies describe effects on A cells, we here describe a gain-of-function effect of NCAM on earlier generated cells. Therefore, an increase in proliferation is more likely than an increase in cell survival.

The discrepancy between our results and other studies showing that NCAM reduces proliferation while increasing differentiation (Amoureux *et al.*, 2000; Shin *et al.*, 2002; Kim *et al.*, 2005) might be explained by the fact that we here present an effect of NCAM on cell proliferation *in vivo*, whereas earlier reports described the effect on neural progenitor cells with more restricted potency *in vitro*. Furthermore, NCAM affects clearly already radial glia cells, which disappear after NCAM expression. Therefore, the proliferation induced by NCAM seems to be associated with a differentiation shift of radial glia cells towards the neuronal lineage.

To elucidate how NCAM mediates these effects we created different constructs, differing in their ability to bind homo- and heterophilically or to mediate signal transduction, but all of them capable to become PSA-modified. The observation that all three isoforms used here mediated the same neurogenic effect indicates that neither homophilic binding to other NCAM molecules in *cis* and/or *trans*, nor NCAM-dependent signal transduction plays a role. Moreover, interactions with many extracellular binding partners of NCAM as, for example,

heparansulphate, chondroitin sulphate proteoglycans or cell surface oligosaccharides could be excluded.

Removal of PSA has been shown to shift neuronal precursor cells towards a more differentiated neuronal phenotype in the SVZ (Röckle *et al.*, 2008) and in the hippocampus (Burgess *et al.*, 2008). We were interested if this differentiation-inducing function is also active in earlier populations of the OB lineage, namely in radial glia and type C cells. Both cell types do not express PSA endogenously (reviewed in Doetsch, 2003; and own observation). The PSA expression that we observed directly at the ventricular wall of the lateral ventricle may result from PSA-expressing immature ependymal cells, which have been detected as early as P0 (Tramontin *et al.*, 2003). Furthermore, we observed neither in radial glia cells nor in type C cells a PSA-modification of electroporated NCAM, indicating that the responsible enzymes ST8Sia II and ST8Sia IV are most likely not expressed in these cell types and that the neurogenic activity of NCAM is independent of its PSA-modification.

Therefore, there remains only one possibility how NCAM can act on neurogenesis in the periventricular region. Heterophilic interactions in the part of NCAMs extracellular domain still possible for the hNCAM Δ NCT construct (Fig. 5A) may be responsible for enhanced neurogenesis. Such type of interactions with other proteins might be favoured as the NCAM protein does not carry the PSA-modification that could sterically inhibit them (for review, see Rutishauser, 2008). Heterophilic interactions that can still take place include FGFR, ATP and the prion protein (reviewed in Hinsby *et al.*, 2004).

The FGFR is activated upon dimerization by ligand binding. The mechanism of FGFR activation by NCAM has not been clarified completely, but it has been proposed that NCAM (present in *cis* dimers) at the cell surface is associated with one FGFR molecule. NCAM *trans* interactions may then induce dimerization of the FGFR and lead to FGFR-dependent signal transduction. The FGFR seems to be a good candidate because: (i) it has been shown to be active in the SVZ (Palmer *et al.*, 1995; Kuhn *et al.*, 1997); and (ii) an *in vitro* study showed that NCAM could induce a neuronal phenotype only in the presence of basic FGF. Ig domains (1–3) alone could not mimic this effect and therefore not function as a neuronal inducer (Amoureux *et al.*, 2000). This study supports our results that the neurogenic effect of NCAM is located within the domains present in the hNCAM Δ NCT construct.

The second known interaction partner for the extracellular part without Ig-like domain 1–4 is the prion protein whose binding site is located in the Ig 5, fibronectin type III domain 1 and/or fibronectin type III domain 2 (Schmitt-Ulms *et al.*, 2001; Santucci *et al.*, 2005). However, the function of the prion protein has never been investigated in radial glia cells. Therefore, according to current knowledge, the FGFR and maybe ATP, which has been suggested to regulate FGFR binding to NCAM together with not yet identified interaction partners of NCAM, remain the most likely candidates to mediate the observed effect.

Altogether, the results presented here point to a positive role of NCAM in neurogenesis in the periventricular region. Postnatal neuronal stem cells hold high promise for brain repair following brain trauma or neurodegenerative diseases. Therefore, expression of NCAM in stem cells might be one of many factors useful for therapeutic approaches in the future.

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Abbreviations

Dcx, doublecortin; dpe, days post-electroporation; FGFR, fibroblast growth factor receptor; GFP, green fluorescent protein; Ig, immunoglobulin-like domain; NCAM, neural cell adhesion molecule; OB, olfactory bulb; P, postnatal day; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PSA, polysialic acid; RMS, rostral migratory stream; ST8Sia, sialyltransferase; SVZ, subventricular zone; TBS, Tris-buffered saline; VZ, ventricular zone.

References

- Amoureux, M.C., Cunningham, B.A., Edelman, G.M. & Crossin, K.L. (2000) N-CAM binding inhibits the proliferation of hippocampal progenitor cells and promotes their differentiation to a neuronal phenotype. *J. Neurosci.*, **20**, 3631–3640.
- Angata, K., Huckaby, V., Ranscht, B., Terskikh, A., Marth, J.D. & Fukuda, M. (2007) Polysialic acid-directed migration and differentiation of neural precursors are essential for mouse brain development. *Mol. Cell. Biol.*, **27**, 6659–6668.
- Bonfanti, L. & Theodosis, D.T. (2009) Polysialic acid and activity-dependent synapse remodeling. *Cell Adh. Migr.*, **23**, 43–50.
- Boutin, C., Diestel, S., Desoeuvre, A., Tiveron, M.C. & Cremer, H. (2008) Efficient electroporation of the postnatal forebrain. *PLoS ONE*, **3**, e1883.
- Burgess, A., Wainwright, S.R., Shihabuddin, L.S., Rutishauser, U., Seki, T. & Aubert, I. (2008) Polysialic acid regulates the clustering, migration and neuronal differentiation of progenitor cells in the adult hippocampus. *Dev. Neurobiol.*, **68**, 1580–1590.
- Chazal, G., Durbec, P., Jankovski, A., Rougon, G. & Cremer, H. (2000) Consequences of neural cell adhesion molecule deficiency on cell migration in the rostral migratory stream of the mouse. *J. Neurosci.*, **20**, 1446–1457.
- Close, B.E., Mendiratta, S.S., Geiger, K.M., Broom, L.J., Ho, L.L. & Colley, K.J. (2003) The minimal structural domains required for neural cell adhesion molecule polysialylation by PST/ST8Sia IV and STX/ST8Sia II. *J. Biol. Chem.*, **278**, 30796–30805.
- Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kraemer, P., Scheff, S., Barthels, D., Rajewski, K. & Wille, W. (1994) Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. *Nature*, **367**, 455–459.
- Diestel, S., Schaefer, D., Cremer, H. & Schmitz, B. (2007) NCAM is ubiquitinated, endocytosed and recycled in neurons. *J. Cell Sci.*, **120**, 4035–4049.
- Doetsch, F. (2003) The glial identity of neural stem cells. *Nat. Neurosci.*, **6**, 1127–1134.
- Doetsch, F., García-Verdugo, J.M. & Alvarez-Buylla, A. (1997) Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J. Neurosci.*, **17**, 5046–5061.
- Doetsch, F., Caillé, I., Lim, D.A., García-Verdugo, J.M. & Alvarez-Buylla, A. (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell*, **97**, 703–716.
- Ebeling, O., Duczmal, A., Aigner, S., Geiger, C., Schöllhammer, S., Kemshead, J.T., Möller, P., Schwartz-Albiez, R. & Altevogt, P. (1996) L1 adhesion molecule on human lymphocytes and monocytes: expression and involvement in binding to alpha v beta 3 integrin. *Eur. J. Immunol.*, **26**, 2508–2516.
- Gascon, E., Vutsits, L., Jenny, B., Durbec, P. & Kiss, J.Z. (2007) PSA-NCAM in postnatally generated immature neurons of the olfactory bulb: a crucial role in regulating p75 expression and cell survival. *Development*, **134**, 1181–1190.
- Hinsby, A.M., Berezin, V. & Bock, E. (2004) Molecular mechanism of NCAM function. *Front. Biosci.*, **9**, 2227–2244.
- Kim, J.H., Lee, J.H., Park, J.Y., Park, C.H., Yun, C.O., Lee, S.H. & Son, H. (2005) Retrovirally transduced NCAM140 facilitates neuronal fate choice of hippocampal progenitor cells. *J. Neurochem.*, **94**, 417–424.
- Kuhn, H.G., Winkler, J., Kempermann, G., Thal, L.J. & Gage, F.H. (1997) Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult brain. *J. Neurosci.*, **17**, 5820–5829.
- Li, H. & Grumet, M. (2007) BMP and LIF signaling coordinately regulate lineage restriction of radial glia in the developing forebrain. *Glia*, **55**, 24–35.
- Merkle, F.T. & Alvarez-Buylla, A. (2006) Neural stem cells in mammalian development. *Curr. Opin. Cell Biol.*, **18**, 704–709.
- Merkle, F.T., Tramontin, A.D., García-Verdugo, J.M. & Alvarez-Buylla, A. (2004) Radial glia give rise to adult neural stem cells in the subventricular zone. *Proc. Natl Acad. Sci. USA*, **101**, 17528–17532.

- Misson, J.P., Edwards, M.A., Yamamoto, M. & Caviness, V.S. Jr (1988) Identification of radial glia cells within the developing murine central nervous system: studies based upon a new immunohistochemical marker. *Brain Res. Dev. Brain Res.*, **44**, 95–108.
- Morin, X., Jaouen, F. & Durbec, P. (2007) Control of planar divisions by the Gprotein regulator LGN maintains progenitors in the chick neuroepithelium. *Nat. Neurosci.*, **10**, 1440–1448.
- Nacher, J., Crespo, C. & McEwen, B.S. (2001) Doublecortin expression in the adult rat telencephalon. *Eur. J. Neurosci.*, **14**, 629–644.
- Palmer, T.D., Ray, J. & Gage, F.H. (1995) FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. *Mol. Cell. Neurosci.*, **6**, 474–486.
- Parras, C.M., Galli, R., Britz, O., Soares, S., Galichet, C., Battiste, J., Johnson, J.E., Nakafuku, M., Vescovi, A. & Guillemot, F. (2004) Mash1 specifies neurons and oligodendrocytes in the postnatal brain. *EMBO J.*, **23**, 4495–4505.
- Röckle, I., Seidenfaden, R., Weinhold, B., Mühlenhoff, M., Gerardy-Schahn, R. & Hildebrandt, H. (2008) Polysialic acid controls NCAM-induced differentiation of neuronal precursors into calretinin-positive olfactory bulb interneurons. *Dev. Neurobiol.*, **68**, 1170–1184.
- Rousselot, P., Lois, C. & Alvarez-Buylla, A. (1995) Embryonic (PSA) NCAM reveals chains of migrating neuroblasts between the lateral ventricle and the olfactory bulb of adult mice. *J. Comp. Neurol.*, **351**, 51–61.
- Rutishauser, U. (2008) Polysialic acid in the plasticity of the developing and adult vertebrate nervous system. *Nat. Rev. Neurosci.*, **9**, 26–35.
- Santuccione, A., Sytnyk, V., Leshchyns'ka, I. & Schachner, M. (2005) Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59fyn and to enhance neurite outgrowth. *J. Cell Biol.*, **169**, 341–354.
- Schmitt-Ulms, G., Legname, G., Baldwin, M.A., Ball, H.L., Bradon, N., Bosque, P.J., Crossin, K.L., Edelman, G.M., DeArmond, S.J., Cohen, F.E. & Prusiner, S.B. (2001) Binding of neural cell adhesion molecules (N-CAMs) to the cellular prion protein. *J. Mol. Biol.*, **314**, 1209–1225.
- Schubert, D., Heinemann, S., Carlisle, W., Tarikas, H., Kimes, B., Patrick, J., Steinbach, J.H., Culp, W. & Brandt, B.L. (1974) Clonal cell lines from the rat central nervous system. *Nature*, **249**, 224–227.
- Shin, M.H., Lee, E.G., Lee, Y.S. & Son, H. (2002) Neural cell adhesion molecule promotes the differentiation of hippocampal precursor cells to a neuronal lineage, especially to a glutamatergic neural cell type. *Exp. Mol. Med.*, **34**, 401–410.
- Tramontin, A.D., García-Verdugo, J.M., Lim, D.A. & Alvarez-Buylla, A. (2003) Postnatal development of radial glia and the ventricular zone (VZ): a continuum of the neural stem cell compartment. *Cereb. Cortex*, **13**, 580–587.
- Weinhold, B., Seidenfaden, R., Röckle, I., Mühlenhoff, M., Schertzinger, F., Conzelmann, S., Marth, J.D., Gerardy-Schahn, R. & Hildebrandt, H. (2005) Genetic ablation of polysialic acid causes severe neurodevelopmental defects rescued by deletion of the neural cell adhesion molecule. *J. Biol. Chem.*, **280**, 42971–42977.