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# PSA-NCAM in postnatally generated immature neurons of the olfactory bulb: a crucial role in regulating p75 expression and cell survival

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In the mammalian brain, ongoing neurogenesis via the rostral migratory stream (RMS) maintains neuronal replacement in the olfactory bulb throughout life. Mechanisms that regulate the final number of new neurons in this system include proliferation, migration and apoptosis. Here we show that the polysialylated isoforms of the neural cell adhesion molecule (PSA-NCAM) act as a pro-survival molecule in immature newborn neurons. Confocal microscopic analysis revealed a threefold increase in TUNEL-positive cells in the subventricular zone (SVZ) and the RMS of transgenic animals lacking the gene encoding NCAM (NCAM$^{-/-}$), as compared with wild types. The enhanced apoptotic cell death occurred specifically in the population of mCD24-positive newborn neurons, but not in GFAP-positive astrocytes. Using in vitro cultures of purified SVZ-derived neurons, we demonstrate that the loss or inactivation of PSA on NCAM, as well as the deletion of NCAM, lead to reduced survival in response to neurotrophins including BDNF and NGF. These changes in cell survival are accompanied by an upregulation of p75 neurotrophin receptor expression in vitro as well as in vivo. Furthermore, the negative effects of PSA-NCAM inactivation on cell survival could be prevented by the pharmacological blockade of the p75 receptor-signaling pathway. We propose that PSA-NCAM may promote survival by controlling the expression of the p75 receptor in developing neurons.

**KEY WORDS: Neuronal survival, Neurotrophin, PSA-NCAM, p75 (Ngfr), Olfactory bulb, Neurogenesis**

## INTRODUCTION

One striking feature of postnatal neurogenic zones including the subventricular zone (SVZ) is a strong PSA-NCAM immunoreactivity (Garcia-Verdugo et al., 1998; Rousselot et al., 1995). Polysialic acid (PSA) is a linear homopolymer of α2,8-linked sialic acid that is uniquely attached to the neural cell adhesion molecule (NCAM) in the vertebrate brain (Kiss et al., 2001; Rutishauser and Landmesser, 1996). PSA-NCAM is abundantly expressed in the developing central nervous system (CNS) where its presence is associated with morphogenetic events, such as cell migration, axonal growth and synaptogenesis (Edelman, 1986; Kiss and Rougon, 1997). In the postnatal period, the polysialic content of NCAM tends to decrease (Rutishauser and Landmesser, 1996), but PSA-NCAM does persist in adult brain structures that display a high degree of morphofunctional plasticity (Durbec and Cremer, 2001).

The functional significance of PSA on NCAM at the cell surface of these immature neurons is not completely understood. Genetic deletion of the NCAM molecule (NCAM$^{-/-}$) results in a ~30% decrease in the size of the olfactory bulb (OB), and the overall brain size is reduced by about 10% (Cremer et al., 1994; Tomasiewicz et al., 1993). These defects can be duplicated by the injection of Endo-N, an enzyme that specifically removes the PSA moiety associated with NCAM, suggesting that the observed phenotypical changes in the NCAM$^{-/-}$ animal are primarily related to the absence of the PSA chain itself (Ono et al., 1994). Parallel to the reduction in OB size, an increased number of neuronal precursors are observed in the subventricular zone and rostral migratory stream (SVZ-RMS) of NCAM$^{-/-}$ animals as compared with wild-type (WT) littermates (Chazal et al., 2000; Ono et al., 1994). It has been suggested that this accumulation of neuronal precursors in the SVZ-RMS is the result of impaired chain migration of these cells toward the OB (Hu et al., 1996; Ono et al., 1994).

Although much has been learned about the molecular control of proliferation and migration of SVZ-derived progenitors (Alvarez-Buylla and Garcia-Verdugo, 2002), relatively little is known about factors that control their survival. Sensory inputs (Miwa and Storm, 2005; Rochefort et al., 2002), glutamate (Brazel et al., 2005) and PTEN (Li et al., 2002) have been shown to regulate survival of newly generated neurons. In this study, we explored the possibility that PSA-NCAM is involved in survival of postnatally generated, new neurons by focusing on the SVZ-RMS-OB where PSA-NCAM expression is maintained at high levels throughout life (Garcia-Verdugo et al., 1998). We compared cell death in NCAM$^{-/-}$ mice and WT animals and tested the effect of removing or blocking PSA-NCAM in a culture model. Our results demonstrate that PSA-NCAM promotes neuronal survival by regulating p75 (Ngfr – Mouse Genome Informatics) neurotrophin receptor expression.

## MATERIALS AND METHODS

### Animals and viral injection

Sprague-Dawley rats and C57/B6J mice were housed at the Geneva Medical School facility. All protocols were approved by the local competent authority (Office Cantonale Veterinaire of Geneva). The NCAM-knockout mouse was generated by the partial excision of exons 3 and 4 (Cremer et al., 1994) and maintained in a C57/B6J background (five backcrosses).

Labeling of cells in the SVZ-RMS was performed by means of a lentivector carrying GFP under the regulation of the ubiquitin promoter. Attenuated lentivector particles were produced by transfection of 293T cells according to standard protocols (Klages et al., 2000). WT and NCAM$^{-/-}$ pups at postnatal day 7 (P7) were anesthetized with 2% isofluran (Foren) in

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a 30%:70% mixture of $O_2$:air, and maintained in a stereotaxic frame. One microliter of a suspension containing the lentivector at a concentration of $1\times10^9$ transducing units/ml was stereotactically injected (coordinates from the bregma: 0 mm anterior, 0 mm posterior, 1 mm lateral) with a Hamilton syringe at a depth of 1.5 mm from the surface of the brain. Animals were sacrificed 4 days after the injection and brains processed for immunofluorescence.

### Cell culture and reagents

SVZ-derived cultures were prepared from newborn rats (P0) or P7 mice as previously described (Gascon et al., 2005). Briefly, the SVZ was dissected from coronal slices, dissociated mechanically, trypsinized and purified using percoll gradient centrifugation. Cells were plated onto polyornithine (Sigma, St Louis, MO) -coated cell culture supports and allowed to grow in Neurobasal Medium (Gibco, Paisley, UK) with 2% B27 supplement (Gibco), 2 mM L-glutamine (Gibco) and 1 mM sodium pyruvate (Sigma).

For experiments with neurotrophins, recombinant human NGF and BDNF were purchased from Regeneron Pharmaceuticals (Tarrytown, NY). The inhibitors myriocin, fumonisin B1 and SP600125 were obtained from Biomol (Plymouth Meeting, PA, USA) and K252a from Calbiochem (Merck Biosciences, Germany). To remove PSA from cell surfaces, we used the enzyme Endo-N, purified from phage K1 (Kiss et al., 1994). Endo-N has been shown to rapidly and specifically degrade linear polymers of sialic acid with 2,8-linkage, and with a minimum length of 7-9 residues (Vimr et al., 1984).

### Histology and immunofluorescence

To analyze the SVZ-RMS, P7 mice were sacrificed by decapitation. Brains were removed and fixed overnight in 4% paraformaldehyde (PAF). Brains were then frozen and cut using a cryostat (Leica, Germany). Sagittal slices of 20 μm were plated onto gelatin-coated slides and post-fixed for 30 minutes with 4% PAF. To correlate the extent of the SVZ area with the number of apoptotic cells, adjacent sections were stained by TUNEL labeling (see below) and with Hematoxylin-Eosin (HE) following standard protocols.

For immunolabeling, sections were rinsed three times in PBS and then incubated with primary antibodies in 0.5% BSA (Sigma) 0.3% Triton X-100 in PBS for 48 hours at 4°C. Antibodies used were against GFAP (Dako, Dakopatts, Copenhagen, Denmark) 1/500, mCD24 (generous gift from G. Rougon, IBDML, Marseille, France) 1/200, GFP (Molecular Probes, Invitrogen) 1/2000, doublecortin (Santa Cruz Biotechnology, Santa Cruz, CA) 1/1000, NeuN (Chemicon) 1/500, and p75 (Promega, Madison, WI) 1/1000. After three washes with PBS, Alexa-conjugated secondary antibodies (Molecular Probes) diluted in PBS were applied for 90 minutes at room temperature.

Immunocytochemistry in cultures was performed as previously described (Gascon et al., 2005). Primary antibodies used were: anti-βIII-tubulin (Sigma) 1/500, anti-p75 (Promega) 1/1000, anti-activated caspase 3 (R&D systems, Avingdon, UK) 1/2000, Men B IgM that recognizes α2,8-linked PSA of a chain length greater than 12 residues (Rougon et al., 1986) 1/500, and anti-GABA (Matute and Streit, 1986) 1/2000. For GABA, cells were fixed in 2% PAF, 2% glutaraldehyde in phosphate buffer (pH 7.4) for 30 minutes followed by 1 hour in 4% PAF. Before the incubation with the primary antibody, cells were rinsed three times for 20 minutes each with PBS, and then incubated for 30 minutes in a solution of 0.1 M $NH_4Cl$.

### TUNEL assay

Samples were rinsed with PBS and incubated for 15 minutes with the TUNEL buffer (30 mM Tris, 140 mM sodium cacodylate and 1 mM cobalt chloride). Then, 0.3 U/μl terminal transferase (Roche, Germany) and 6 μM labeled dUTP were applied for 90 minutes at room temperature. The reaction was stopped with 2× SSC (sodium citrate buffer) and the samples washed again with PBS.

For brain slices, 7′-fluorescein dUTP (Roche, Germany) was chosen as a label. The fluorescent labeling enabled quantification of apoptotic cells under a fluorescent microscope (Axiophot, Zeiss, Germany), and evaluation of the colocalization of TUNEL-positive cells with GFAP, mCD24 or p75, under the confocal microscope (LSM 510, Zeiss, Germany).

Unless indicated otherwise, all treatments on cultured cells were performed 5 days after plating and lasted 20 hours. TUNEL cells were revealed using both 7′-fluorescein and 16′-biotin dUTP (Roche, Germany). For biotin detection, cells were incubated either with the ABC Kit (Vector Laboratories, Burlingame, CA) followed by the chromogenic substrate (diaminobencindine, DAB), or with avidin-Texas Red (1/1000) (Vector Laboratories).

### SYBR green assay

Total RNA was extracted using the RNA-Easy Micro Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. 50 ng of total RNA were converted to cDNA using Sensiscript reverse transcriptase (Qiagen). Reverse transcription was performed in a T3 thermocycler (Biometra, Göttingen, Germany) for 1 hour at 37°C. Real-time RT-PCR was carried out in an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR reactions were performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems). Each SYBR Green reaction (10 μl total volume) contained 1 μl of cDNA as template and each primer at 0.3 μM. Controls without template (water) or reverse transcriptase were always negative. The oligonucleotide primers used for β-actin, TrkB, TrkC and p75 have been described elsewhere (Gascon et al., 2005).

### Western blotting

The RMS-OB and cortex were microdissected from P0 or P7 WT and $NCAM^{-/-}$ mice ($n$=5). Samples (tissue or cultured cells) were first homogenized in ice-cold disruption buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40) containing a protease inhibitor mix (Complete Mini Protease Inhibitors, Roche), and then centrifuged for 10 minutes at 10,000×***g*** at 4°C to obtain a total cell lysate. The supernatant was removed and stored at –80°C. 25 μg of protein were electrophoresed and then transferred to a PVDF membrane in 25 mM Tris, 192 mM glycine and 20% methanol. After blocking (for 1 hour at room temperature in PBS containing 5% non-fat dried milk and 0.1% Tween-20), blots were incubated in primary mouse monoclonal antibody anti-α-tubulin 1/8000 (Sigma), rabbit polyclonal anti-p75 1/2000 (Promega), or the rabbit polyclonal anti-phospho Trk (Tyr 490) 1/1000 (Sigma), overnight at 4°C. Membranes were rinsed three times, incubated with the appropriate horseradish peroxidase-linked secondary antibody at 1/5000 (Biorad, Hercules, CA) for 2 hours at room temperature and developed using ECL reagents (Amersham Biosciences, Little Chalfant, UK).

### PSA and p75 quantification

PSA immunolabeling was performed on living cells. Briefly, medium was harvested, cultures were rinsed in PBS and then incubated with a PSA antibody (Ab 735, generous gift from Dr Gerardy-Schahn, Abteilung Zelluläre Chemie, Medizinische Hochschule, Hannover, Germany) for 30 minutes at 4°C. Cells were then fixed with 4% PAF and incubated with the secondary antibody linked to rhodamine. Quantification of PSA surface fluorescence was carried out using confocal microscopy (LSM 510) as previously described (Kiss et al., 1994). Ninety cells at each time point in three different experiments were randomly imaged. Each captured field contained a single cell profile to be evaluated. All the parameters were kept constant for each session of measurements. Fluorescence was scaled between 0 (lowest intensity) and 255 (highest intensity). Levels of PSA were estimated as the proportion of PSA-rich regions (characterized by intensity values between 248 and 255) relative to the total area of the cell (defined as the pixels whose intensity was higher than the background). For p75, after background subtraction, the mean fluorescence intensity was calculated for each cell. These values were grouped into 20 intensity categories and the percentage of cells falling in each category was plotted for the control and the Endo-N group.

### Image acquisition and data analysis

The number of apoptotic cells along the SVZ-RMS and the striatum was counted under a fluorescent microscope. The surface of these regions was estimated with Scion Image software using Hoechst counterstaining on the analyzed sections and HE staining on the next section. More than 10 sections per brain (WT $n$=4; $NCAM^{-/-}$ $n$=4) were used to calculate the density of apoptotic cells. For colocalization of TUNEL with GFAP, mCD24 or p75,

sections were examined under the confocal microscope (LSM 510, Zeiss). For this analysis, TUNEL-positive cells were examined along the *z*-axis and scored as positive if they colocalized completely with the other marker. Any doubtful cell was considered as negative. More than 200 (WT) and 400 (NCAM$^{-/-}$) TUNEL-positive cells were analyzed in six different sections of at least three different brains.

Cultured cells were analyzed under a light microscope (Eclipse TE2000-U, Nikon, Zurich, Switzerland). For quantification of cell death rate, half of the culture medium was replaced by fresh medium containing the appropriate treatment. 20 hours later, dying cells were revealed using TUNEL or caspase 3. Positive cells were counted on 40 random fields (0.064 mm$^2$) in at least two sister cultures and expressed as a percentage of the total cell number. More than 300 cells per condition were considered in each experiment. Results were expressed as mean±s.d. of at least three independent experiments.

For statistical analysis, one-way ANOVA followed by a two-tailed unpaired *t*-test or Holm-Sidak test were performed. Statistical differences were set for $P<0.05$.

## RESULTS

### Increased apoptosis in the SVZ-RMS of NCAM$^{-/-}$ mice

In order to examine the role of PSA-NCAM in neuronal survival in vivo, we compared NCAM$^{-/-}$ brains, lacking both PSA-NCAM and NCAM, with their WT counterparts. In agreement with previous reports (Chazal et al., 2000; Ono et al., 1994), a larger SVZ-RMS and a smaller OB were observed in the NCAM$^{-/-}$ mice (Fig. 1A). Using the TUNEL assay, we observed that dying cells were present in all brain regions but their number was particularly elevated in the SVZ-RMS (Fig. 1B,C). Quantitative analysis revealed a nearly threefold increase in the density of TUNEL-positive cells in the SVZ-RMS of the NCAM$^{-/-}$ animals as compared with WT (Fig. 1B,C). No significant differences were found in the OB or in other brain regions such as the striatum (Fig. 1C), confirming the specificity of the observed increase in the SVZ-RMS system.

We next explored the phenotype of dying cells in the NCAM$^{-/-}$ SVZ-RMS using immunofluorescence. We concentrated on neuroblasts and astrocytes as they represent more than 80% of cells in this area (Garcia-Verdugo et al., 1998). We found that less than 5% of TUNEL profiles colocalized with GFAP (Glial Fibrillary Acidic Protein), a marker of astrocytes. In addition, there was no difference in the percentage of double-labeled cells (TUNEL$^+$ GFAP$^+$) between WT and NCAM$^{-/-}$ mice (5.03±2.8% in WT versus 4.03±1.07% in NCAM$^{-/-}$). We then examined whether TUNEL-positive cells displayed neuronal markers. For this purpose, we used mCD24 (Cd24a), a cell surface protein whose expression largely overlaps with PSA-NCAM (Calaora et al., 1996). We found that many TUNEL-positive nuclei colocalized with mCD24 in the WT animal. However, the proportion of these double-labeled cells was significantly higher in the mutant SVZ-RMS (43.97±8.5% in WT versus 61.05±5.8% in NCAM$^{-/-}$, $P<0.05$). Thus, the absence of NCAM/PSA-NCAM leads to enhanced apoptosis specifically in the population of newly generated neurons.

### Removal or inactivation of the PSA moiety on NCAM modifies the survival of immature SVZ-derived neurons in culture

The increased cell death in the SVZ-RMS of the NCAM$^{-/-}$ mice could be related to a direct effect of the absence of PSA-NCAM on cell survival, or to an indirect effect due to impaired migration and accumulation of newly generated neurons in the RMS (Chazal et al., 2000; Ono et al., 1994). To address this issue, we took advantage of an in vitro model system, where neurons from the postnatal SVZ are purified and developed as olfactory interneurons (Gascon et al., 2005). This model enables the evaluation of cell survival independently of migration. As seen in Fig. 2, neurons in these low-density cultures survive and display a GABAergic phenotype. PSA-NCAM and NCAM were expressed in cells from WT animals, whereas immunostaining for these molecules was completely absent in NCAM$^{-/-}$ cultures (Fig. 2). Under basal conditions, few cells underwent apoptosis by the end of the fourth day in vitro (DIV), as revealed by caspase 3 immunostaining and TUNEL assay (Fig. 3A). TUNEL labeling was significantly increased in WT cultures when the PSA moiety was removed from NCAM by the enzyme Endo-N (PSA$^-$ NCAM$^+$) (Fig. 3B). Enhanced apoptosis was also observed when cells were exposed to the PSA function-blocking antibody m735 (Frosch et al., 1985) (Fig. 3B). Neither Endo-N nor the m735

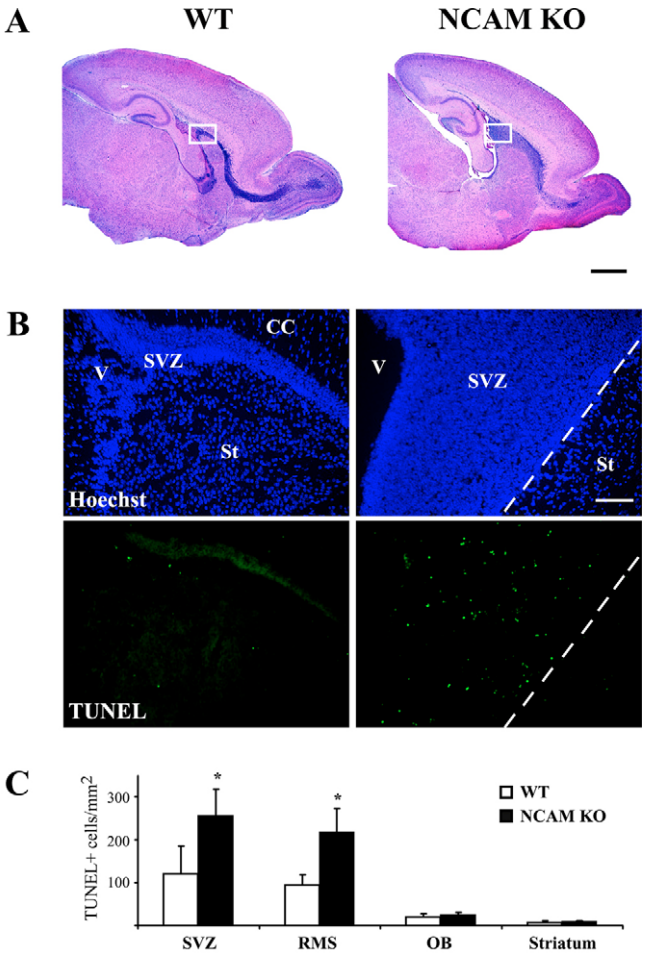


**Fig. 1. Enhanced cell death in the SVZ-RMS of NCAM-deficient animals.** (**A**) Photomicrographs of HE-stained sagittal sections illustrating the enlarged SVZ-RMS in NCAM-deficient (NCAM KO) as compared with WT mice. (**B**) TUNEL assay (lower panels, green fluorescence) reveals that apoptosis is increased in the SVZ-RMS of mice lacking NCAM (right) as compared with WT (left). Hoechst counterstaining on the same sections (upper panels) shows dying cells located in the SVZ-RMS but not in neighboring areas. These pictures correspond to the boxed regions in A. (**C**) Quantification of the density of TUNEL$^+$ cells in WT and NCAM$^{-/-}$ brains demonstrates a significant increase in cell death in the SVZ and RMS of NCAM mutant animals. *, $P<0.05$ (two-tailed unpaired *t*-test). CC, corpus callosum; V, lateral ventricle; St, striatum. The limit between SVZ and St is represented by the dashed line. Scale bar: 1 mm in A; 100 μm in B.

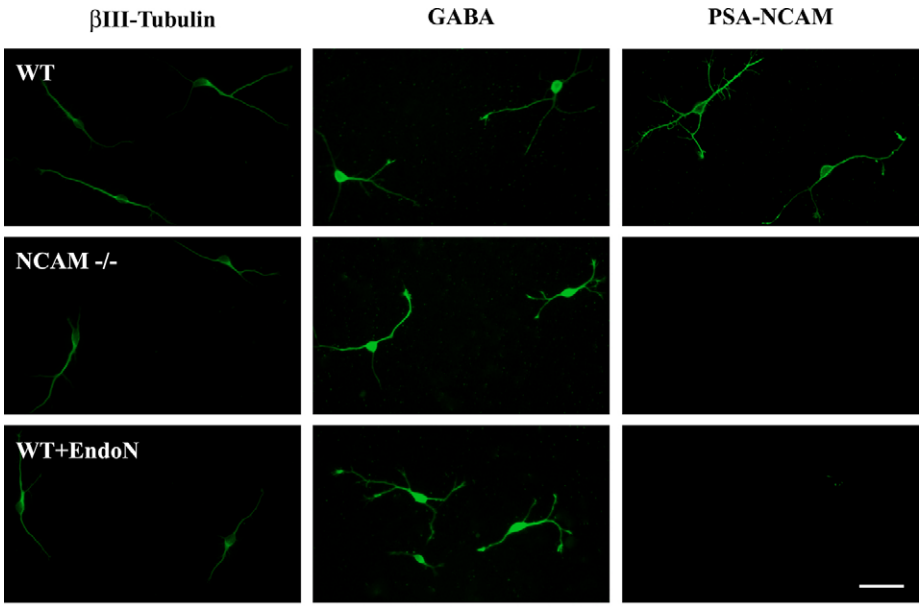


**Fig. 2. Characterization of SVZ-derived cultures from mice.** In culture, SVZ-derived cells prepared from WT mice (top panels) display immunocytochemical markers of immature neurons (strongly positive for βIII-tubulin, GABA and PSA-NCAM). Neither genetic deletion of NCAM (NCAM$^{-/-}$, middle panels) nor the enzymatic removal of PSA (WT+EndoN, bottom panels) affects the morphology or the pattern of markers expressed by these cells. Scale bar: 20 μm.

antibody affected cell survival of neurons isolated from the NCAM$^{-/-}$ mice, arguing against a possible non-specific toxic effect of these treatments (not shown). These data suggests that PSA-NCAM plays a role in the survival of SVZ-derived neurons in culture.

## Immature SVZ-derived neurons respond differently to exogenous BDNF and NGF in the absence of PSA-NCAM

The role of neurotrophins in neuronal survival is well established (Miller and Kaplan, 2001). Accordingly, it has been shown that administration of exogenous neurotrophins in vivo promotes survival of newly generated neurons in the SVZ-RMS-OB (Zigova et al., 1998). To better understand the role of PSA-NCAM in neuronal survival of SVZ-derived neurons, we exposed WT, Endo-N-treated WT and NCAM$^{-/-}$ cells to exogenous BDNF and NGF (Brain Derived Neurotrophic Factor and Nerve Growth Factor, respectively) and assessed the effect of these neurotrophins on cell death rate. As shown in Fig. 3C, BDNF significantly decreased apoptotic cell death in WT cultures and this effect was reduced by Endo-N treatment. BDNF did not reduce cell death, even at high doses, in cultures isolated from NCAM$^{-/-}$ animals (Fig. 3C). The effects of NGF on neuronal survival were very different from those of BDNF. NGF did not modify cell survival in WT cultures; however, it increased cell death in NCAM$^{-/-}$ as well as in Endo-N-treated cultures (Fig. 3C). Thus, PSA seems to modulate the response to neurotrophins in SVZ-derived neurons. It should be emphasized here that the increase in apoptotic rate determined in our short-term experiments led to massive cell loss at longer exposure times. Thus, 3 days of Endo-N treatment, alone or with NGF (50 ng/ml), reduced the number of neurons by 30% or 60%, respectively.

We then took advantage of the fact that, along with maturation, neurons spontaneously downregulate PSA expression (Kiss and Rougon, 1997; Rutishauser and Landmesser, 1996). In agreement with these reports, we observed a time-dependent decrease of PSA-NCAM immunostaining in cultured SVZ-derived neurons. Our quantitative analysis (see Materials and methods) revealed that the intensity of PSA staining decreased nearly fivefold between 3 and 9 DIV (Fig. 4A,B). Consistent with this observation, we found that addition of NGF to 9-DIV cultures (low PSA levels) triggered a significant increase in cell death rate compared with basal conditions (sister cultures treated with the equivalent amount of control medium) (Fig. 4C). By contrast, this effect was not detected in cells challenged with NGF at 3 or 6 DIV (high PSA levels) (Fig. 4C). When the same experiments were repeated with NCAM$^{-/-}$ cells, we found a similar increase in cell death in response to NGF at all time points (increase in cell death, NGF versus control: at 3 DIV, 94.64±29.22%; at 6 DIV, 100.35±36.6%; at 9 DIV, 68.95±36.37%). Thus, the increased sensitivity of WT cells to NGF at mature stages seems to be related to their low PSA content and confirms our observations obtained using Endo-N and the m735 antibody.

Together, these results support the hypothesis that the presence of PSA at the cell surface influences the response of immature SVZ-derived neurons towards neurotrophins and is essential for their proper survival.

## Inhibitors of p75 block the effect of PSA removal on cell survival

Neurotrophins could exert their biological effects through Trk receptors and/or the low-affinity p75 receptor (Miller and Kaplan, 2001). We have previously shown that SVZ-derived neurons in vitro express neurotrophin receptors p75, TrkB and TrkC, but not TrkA (Ngfr, Ntrk2, Ntrk3 and Ntrk1, respectively, Mouse Genome Informatics) (Gascon et al., 2005). Our results, showing that BDNF-induced cell survival was substantially reduced in the absence of PSA and that NGF further increased cell death under these conditions raised the possibility that the presence or absence of PSA might modify cell survival independently of Trk signaling pathways. To test this hypothesis, we used the pan-Trk inhibitor K252a (400 nM), which is known to block signaling through all Trk receptors (Koizumi et al., 1988). Using western blots, we confirmed that K252a effectively blocks phosphorylation of Trk receptors in response to BDNF (Fig. 5A). We also found that in the presence of K252a (Fig. 5B), the number of apoptotic cells was still significantly increased in NCAM$^{-/-}$ cultures as well as after Endo-N treatment, as compared with control WT cultures. Most importantly, in the absence of both Trk signaling and PSA-NCAM at the cell surface, treatment either with BDNF or NGF significantly increased apoptotic cell death (Fig. 5B), indicating that Trk receptors do not mediate this effect.

We also examined whether blocking p75 signaling might rescue cells from death after PSA removal. We inhibited the proapoptotic cascades activated through p75 by exposing cells to fumonisin B1 (10 μM) or myriocin (50 nM). Both molecules are known to block p75-mediated accumulation of the second messenger ceramide by depleting cells of its sphingolipidic precursor (Dobrowsky et al., 1994). Under basal conditions, fumonisin B1 and myriocin did not affect neuronal survival (Fig. 5C). However, cell death induced in the presence of Endo-N was significantly reduced by these inhibitors (Fig. 5C).

The c-Jun-N terminal kinase (JNK; Mapk8 – Mouse Genome Informatics) is another well-established element of the p75-proapoptotic cascade (Casaccia-Bonnefil et al., 1996; Friedman, 2000). Blockade of this enzyme by its selective antagonist SP600125 also reversed the effect of Endo-N (Fig. 5C). These experiments suggest that the p75 signaling pathway plays an essential role in enhanced apoptosis observed after removal or inactivation of PSA-NCAM.

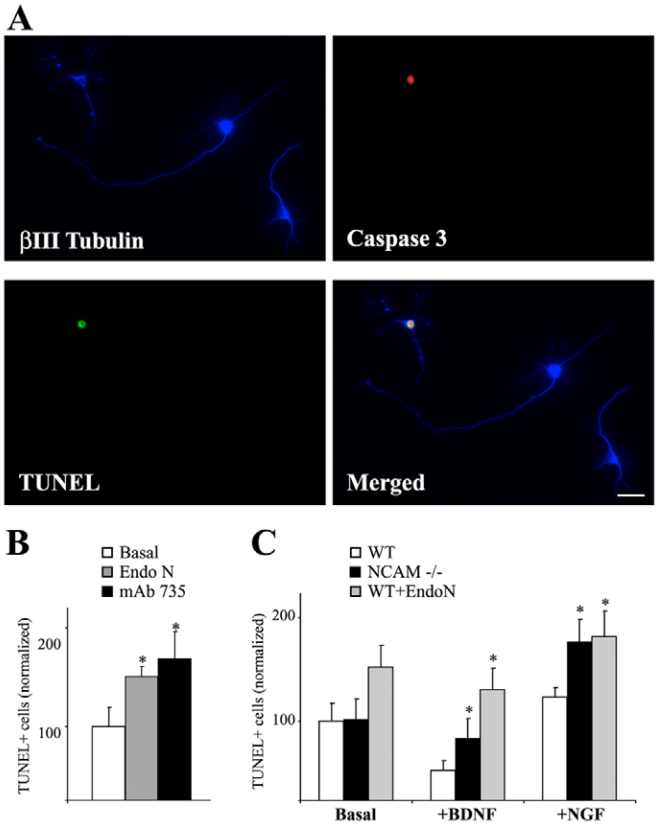


**Fig. 3. Loss of PSA-NCAM reduces survival of cultured SVZ-derived neurons under basal conditions and in response to neurotrophins.** (**A**) Epifluorescence images illustrating apoptotic cells in SVZ-derived cultures co-labeled for activated caspase 3 (red) and TUNEL (green). βIII-tubulin staining (blue) was used to confirm the neuronal phenotype of cultured cells. (**B**) Quantification of cell death rate in the presence or absence of PSA-NCAM. When WT cultures are treated with Endo-N ($PSA^-$ $NCAM^+$) for 20 hours, or with a PSA-blocking antibody (mAb 735), a significant increase in death rate is found. *, $P<0.05$ (ANOVA, Holm-Sidak test). (**C**) Survival of WT cells is promoted by exogenous application of BDNF (100 ng/ml), but not of NGF. By contrast, cells lacking either PSA (WT+EndoN) or PSA and NCAM ($NCAM^{-/-}$) exhibit an enhanced cell death rate in response to NGF (50 ng/ml) and BDNF (100 ng/ml), as compared with WT neurons. *, $P<0.05$ (ANOVA, Holm-Sidak test).

## Increased expression of p75 in the absence of PSA in vitro

Since the above experiments indicated an augmented p75 receptor activation following PSA removal, we explored the possibility that p75 expression was increased under these conditions. First, we assessed the levels of p75 mRNA after Endo-N treatment using real-time RT-PCR. We found that treatment with Endo-N for 20 and 48 hours was sufficient to double the levels of p75 transcripts compared with the vehicle-treated cultures (Fig. 6A). No later time points were considered because of the progressive cell loss in the Endo-N-treated cultures. Importantly, TrkB and TrkC mRNA levels were not modified upon Endo-N administration, arguing against a non-specific effect of Endo-N (Fig. 6A). These findings are consistent with our earlier report demonstrating that the maturation of these cells (and thus the downregulation of PSA) is accompanied by a progressive increase in p75 expression (Gascon et al., 2005).

We next examined whether PSA inactivation triggers an increase in p75 at the protein level. As illustrated in Fig. 6B, treatment with Endo-N for 20 hours resulted in a dramatic augmentation of p75 immunoreactivity compared with vehicle-treated cells. Quantification of p75 intensity using confocal microscopy (see Materials and methods) revealed that Endo-N produced a significant shift in the intensity of p75 immunoreactivity (Fig. 6C), thus confirming our real-time RT-PCR results. Together, these observations suggest that the presence of polysialylated NCAM at the cell surface may closely regulate the expression of p75 receptor.

## Absence of NCAM in vivo leads to an increased expression of p75 in the SVZ-RMS that is accompanied by an early maturation of newborn neurons

We then examined whether p75 expression was also modified in vivo in the absence of PSA-NCAM. Western blot analysis of p75 in RMS-OB or the cerebral cortex from $NCAM^{-/-}$ and WT animals revealed that p75 was downregulated with age in both strains (Fig. 7A), as previously described (Roux and Barker, 2002). More importantly, we found a striking increase in p75 protein levels in $NCAM^{-/-}$ RMS-OB, but not in the cortex, when compared with WT (Fig. 7A). The increase was more prominent in newborn animals, most likely because of the low proportion of cells that expressed p75 at later time points. These results were confirmed by immunofluorescence. In WT animals, we observed a few p75-positive cells scattered along the SVZ-RMS. They displayed a round morphology and occasionally formed clusters (Fig. 7B). In agreement with a previous report (Giuliani et al., 2004), these cells were not associated with doublecortin staining (Fig. 7B), suggesting that they were immature progenitors. In the $NCAM^{-/-}$ mice, p75 labeling was substantially increased in the SVZ-RMS compared with WT animals. Although small round cells similar to those observed in WT animals were found, the majority of p75-positive cells in the $NCAM^{-/-}$ SVZ-RMS were process-bearing cells exhibiting a more complex morphology and colocalized with doublecortin (Fig. 7C).

To further explore the origin and phenotype of this latter cell population in the SVZ-RMS, a lentivector encoding GFP was unilaterally injected into the lateral ventricle of WT and $NCAM^{-/-}$ animals as previously described (Gascon et al., 2006). We used a low number of vector particles in order to transduce a limited number of cells in the SVZ-RMS so as to allow a morphological analysis under the confocal microscope. As illustrated in Fig. 8A, 4 days after vector injection, most cells in the SVZ-RMS of WT animals displayed the typical features of migrating neurons, i.e. an

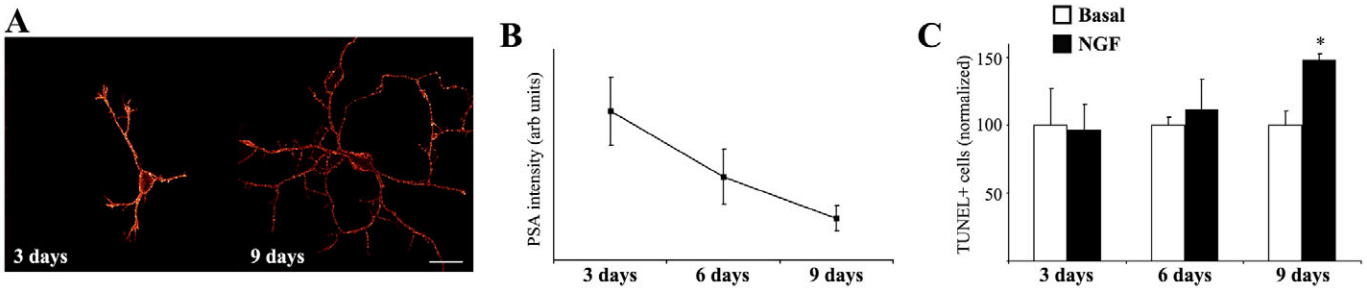


**Fig. 4. Decreased PSA-NCAM expression in SVZ-derived neurons correlates with enhanced cell death in response to exogenous NGF.** (**A**) Confocal photomicrographs of PSA-immunostained neurons demonstrate the downregulation of PSA expression between 3 and 9 DIV. A color scale, where bright regions correspond to PSA-rich areas, was used to better illustrate these differences. (**B**) Surface intensity of PSA immunoreactivity was measured by confocal microscopy and plotted (arbitrary units) against the time in culture. This reveals that the levels of PSA decrease during maturation of neurons in vitro. (**C**) Time-dependent response of SVZ-derived cultures to exogenous NGF. In WT cultures, NGF induces a significant increase in cell death at late stages (9 DIV), but not before (3 and 6 DIV). Results are expressed as the percentage of cell death increase as compared with sister cultures not treated with NGF (basal conditions). *, $P<0.05$, as compared with basal conditions (ANOVA, unpaired *t*-test). Scale bar: 20 μm.

elongated cell body and a leading process oriented towards the OB. In addition, these cells were immunopositive for doublecortin (Fig. 8A). By contrast, in NCAM$^{-/-}$ mice, only a fraction of GFP-expressing cells exhibited this morphology. In agreement with a previous report (Petridis et al., 2004), we found that many of these cells displayed a more complex and branched morphology reminiscent of maturating neurons (Fig. 8A). These cells had a neuronal phenotype as they colocalized with markers such as doublecortin (Fig. 8A). These results raised the possibility that new neurons abnormally maturate in the SVZ-RMS of NCAM$^{-/-}$ animals. To explore this hypothesis, we performed immunostaining with NeuN (Neuna60 – Mouse Genome Informatics), a marker known to detect neurons at more mature stages than doublecortin. A significant number of NeuN$^{+}$ profiles were found in the mutant SVZ-RMS, whereas no labeling was detected in WT animals (Fig. 8B). Interestingly, 4 days after viral injection, most GFP-expressing cells in the NCAM$^{-/-}$ SVZ-RMS were doublecortin$^{+}$, and only a few of them colocalized with NeuN. These data suggest that the lack of PSA-NCAM results in an early neuronal maturation in the SVZ-RMS that is accompanied by increased p75 expression in these cells.

Finally, we tested whether p75 expression is associated with cell death. For that purpose, we examined TUNEL and p75 in the SVZ-RMS of the NCAM$^{-/-}$ mice. We found that a significant proportion of p75$^{+}$ cells displaying a complex morphology (27.1±3.12%, $n$=3) contained TUNEL labeling (Fig. 8C, top panel). Interestingly, most TUNEL$^{+}$ p75$^{-}$ (60.15±9.92%, $n$=3) cells presented high levels of nuclear condensation (Fig. 8C, bottom panels), indicating that they were probably at terminal stages of the apoptotic process and therefore had lost reactivity to most markers. No p75$^{+}$ TUNEL$^{+}$ cells could be found in WT mice, probably reflecting the scarce number of p75$^{+}$ cells present in the wild-type SVZ-RMS. These results confirm our in vitro data demonstrating that p75 is upregulated in the absence of PSA-NCAM. They also demonstrate that increased levels of p75 occur in early maturating cells that undergo apoptosis.

## DISCUSSION

In this study, we have investigated the role of the cell adhesion molecule PSA-NCAM in the survival of newly generated neurons in the postnatal SVZ-RMS-OB. Our data give strong support to the hypothesis that PSA-NCAM is a pro-survival molecule in immature neurons. We demonstrate that deletion of NCAM as well as removal

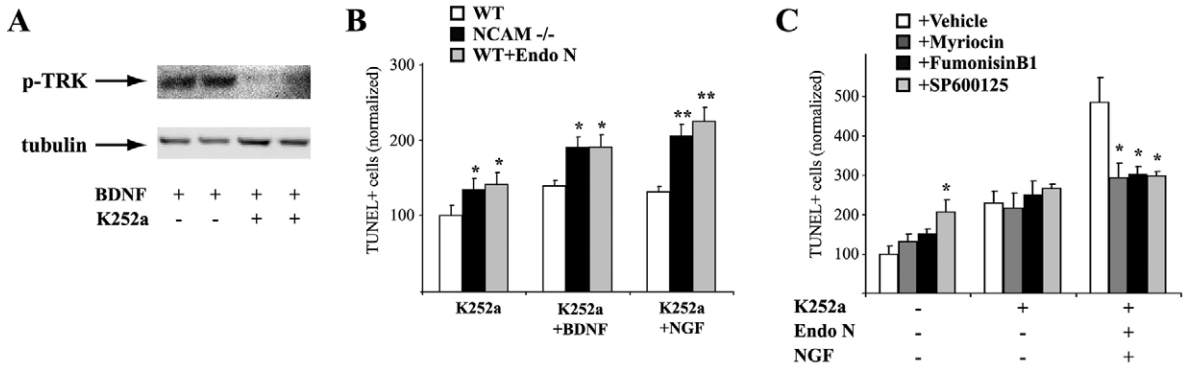


**Fig. 5. Enhanced cell death after PSA removal involves the activation of p75 pathways.** (**A**) K252a effectively blocks phosphorylation of Trk receptors. At 5 DIV, SVZ-derived neurons were exposed to BDNF (100 ng/ml) in the presence or absence of the Trk-inhibitor K252a (400 nM) and the phosphorylation state of Trk receptors was assessed by western blotting. (**B**) The presence of the Trk receptor blocker K252a (400 nM) does not modify the increased cell death observed in response to exogenously applied NGF (50 ng/nl) or BDNF (100 ng/ml) after PSA removal. *, $P<0.05$; **, $P<0.001$ (ANOVA, Holm-Sidak test). (**C**) Blocking p75-dependent pathways by inhibitors of ceramide production (10 μM fumonisin B1 and 50 nM myriocin) or c-Jun N-terminal kinase (SP600125, 20 μM) was able to prevent cell death induced by PSA removal. *, $P<0.05$ (ANOVA, Holm-Sidak test).

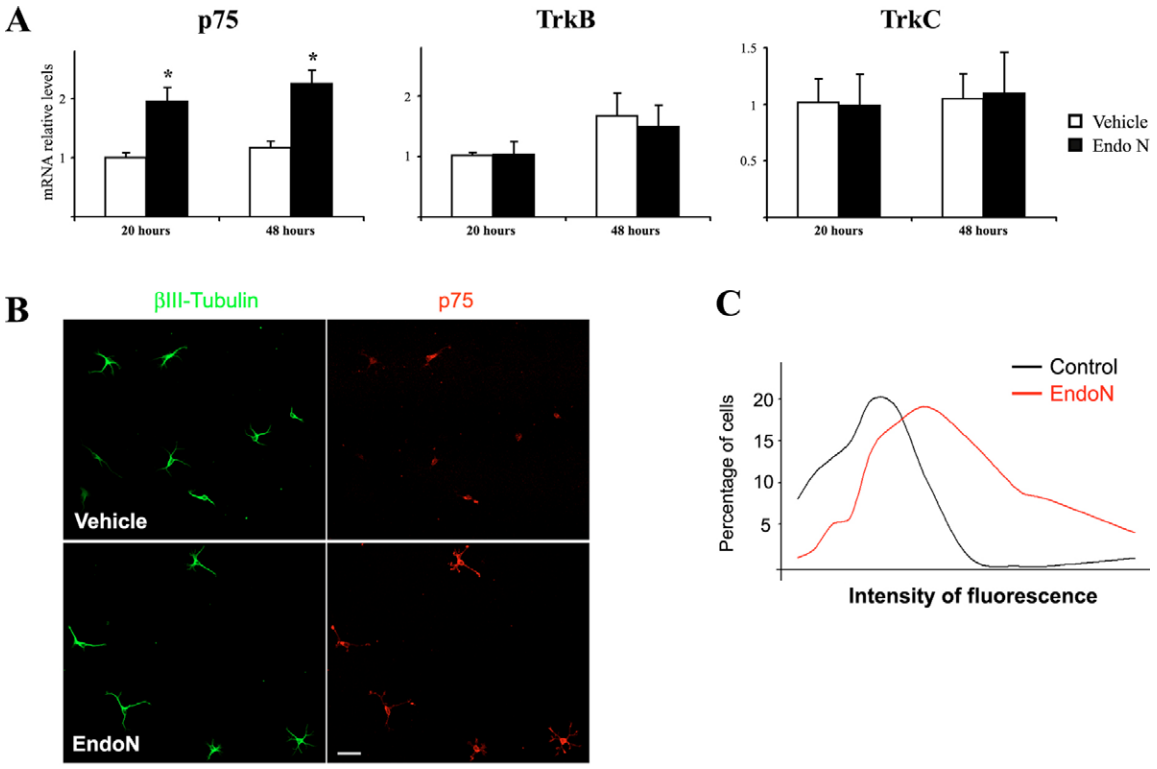


**Fig. 6. Increased expression of p75 after PSA removal.** (**A**) Real-time RT-PCR analysis of p75, TrkB and TrkC mRNA after Endo-N treatment. RNA extracted from SVZ-derived neurons treated either with Endo-N (black bars) or with vehicle (white bars) were used to quantify the expression of p75, TrkB and TrkC in three independent experiments. Compared with vehicle-treated cultures, removal of PSA specifically affected p75 levels. *, $P<0.05$ (two-tailed unpaired *t*-test). (**B**) Confocal photomicrographs of βIII-tubulin (green) and p75 (red) double-labeling in neurons treated with vehicle or with Endo-N demonstrate the upregulation of p75 expression in cells lacking PSA. (**C**) Quantification of p75 immunoreactivity using confocal microscopy. Cells treated with Endo-N for 20 hours showed a significant increase in p75 immunoreactivity compared with controls. Scale bar: 20 μm.

or blocking of the PSA chain on NCAM increase apoptosis of these cells in vivo as well as in vitro. These changes are paralleled by an upregulation of p75 neurotrophin receptor expression. Furthermore, the negative effects of PSA-NCAM inactivation on cell survival could be prevented by the pharmacological blockade of the p75 receptor-signaling pathway. This suggests that regulation of neuroblast survival by PSA-NCAM involves modulation of p75 receptor expression and/or activity. These results reveal a novel mechanism for the control of survival of newly generated neurons in the SVZ-RMS-OB system.

Our data demonstrate that apoptosis is increased nearly threefold in the SVZ and RMS of NCAM$^{-/-}$ animals as compared to WT. This result is novel, as although previous studies explored this possibility, they did not report an increase in cell death in NCAM$^{-/-}$ animals (Ono et al., 1994). The reasons for this discrepancy between the present and earlier reports might be methodological. Thus, to quantify cell death, Ono et al. (Ono et al., 1994) used the pyknotic index, a detection method less sensitive than the TUNEL assay used in our study (Clarke, 1990; McCloskey et al., 1998). The enhanced rate of apoptotic cell death we document here in NCAM$^{-/-}$ animals is cell-specific because it occurred in the population of migrating neuroblasts (PSA$^{+}$ NCAM$^{+}$) but not in GFAP-positive astrocytes (PSA$^{-}$NCAM$^{+}$), supporting the hypothesis that PSA-NCAM and not NCAM is important for cell survival. In addition, we observed increased TUNEL staining only in the SVZ and the RMS of NCAM-deficient brains but not in other brain regions. However, in view of the rapid clearing of apoptotic cells (Ferrer et al., 1990; Savill, 1998) in vivo, we cannot exclude the possibility that enhanced, albeit low levels of apoptosis may occur elsewhere.

The regional and cell-specific occurrence of enhanced TUNEL labeling in the NCAM$^{-/-}$ animal strongly suggests the direct involvement of PSA-NCAM in the adequate survival of newly generated neurons. This hypothesis receives further support from our in vitro experiments: (1) enzymatic removal or antibody blocking of PSA produce a significant increase in TUNEL labeling; (2) in the absence of PSA, levels of apoptosis are higher than in control cultures in response to neurotrophins; and (3) spontaneous downregulation of PSA associated with maturation mimics the effects of genetic or enzymatic elimination of PSA. These results are also consistent with the recent observation that the lack of PSA-NCAM increases cell death of newly generated neurons in cortical cultures (Vutskits et al., 2006). Relevant to the enhanced apoptosis shown in this study in the absence of PSA-NCAM is the observation that the OB as well the whole brain of NCAM$^{-/-}$ animals are significantly smaller than in heterozygous or WT animals (Cremer et al., 1994).

Given the net increase of apoptosis in the SVZ-RMS of NCAM$^{-/-}$ animals, the lack of similarly increased cell death in cultures prepared from NCAM-deficient mice under basal conditions was surprising. This might reflect the existence of compensatory mechanisms in mutant cells that could counteract the effects of the absence of NCAM under basal conditions, but not when cells are challenged in a more complex in vivo environment. Since exogenous application of NGF in culture induced significantly more apoptosis in NCAM$^{-/-}$ cultures than in WT, one possibility is that cells could be exposed to this neurotrophin in the SVZ-RMS in vivo and that this may explain the increased apoptotic cell death. The influence of NGF on SVZ neural precursors has been demonstrated under

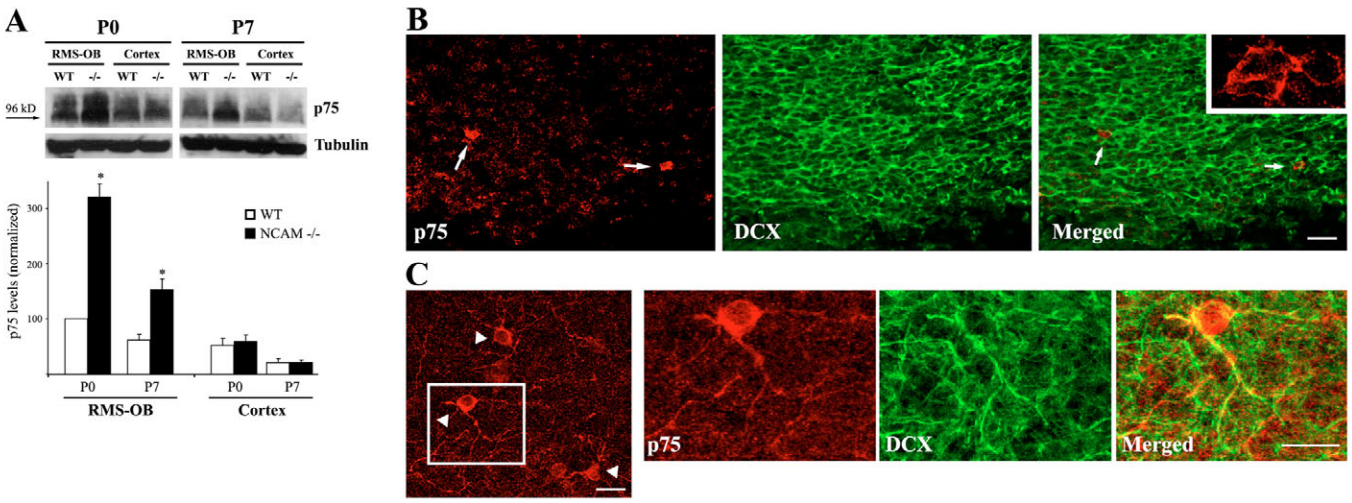


**Fig. 7. p75 upregulation in the SVZ-RMS of NCAM$^{-/-}$ animals is accompanied by the presence of abnormally mature neurons.** (**A**) Western blot analysis of p75 expression in WT and NCAM$^{-/-}$ brain. p75 is expressed at higher levels in the RMS-OB of NCAM mutant mice during early postnatal life. *, $P<0.05$ (two-tailed unpaired *t*-test). (**B**) Confocal image of sagittal brain slices stained for p75 and doublecortin (DCX) demonstrating the presence of small round p75$^{+}$ DCX$^{-}$ cells in the WT SVZ-RMS (arrows). p75$^{+}$ cells occasionally form small clusters (insert). (**C**) Immunolabeling for p75 in NCAM$^{-/-}$ brains revealed an increased number of p75 cells in the SVZ-RMS. These cells often exhibited a branched morphology (arrowheads). A higher magnification image of the area depicted in the left-hand panel illustrates that most of these p75$^{+}$ cells colocalized with the neuronal marker doublecortin. Scale bars: 20 μm.

pathological conditions such as experimental allergic encephalitis (Triaca et al., 2005). It should be noted that in contrast to NCAM$^{-/-}$ cultures, removal of PSA by Endo-N increased apoptosis even under basal in vitro conditions. This raises the possibility that putative compensatory mechanisms might not be operational when PSA is acutely removed, and/or NCAM without PSA may promote cell death at this developmental stage of neuronal precursors. Further studies should clarify these issues.

In previous studies, we found that BDNF signaling through TrkB receptors was impaired in the absence of PSA-NCAM (Muller et al., 2000; Vutskits et al., 2001). The results presented here give support to this observation, because the survival-promoting effect of BDNF was significantly weaker in Endo-N-treated and NCAM$^{-/-}$ cultures than in control preparations. Moreover, our observations point to the involvement of p75 receptor in cell death observed in the absence of PSA-NCAM. In view of the evidence that SVZ-derived neurons in culture express p75, TrkB and TrkC, but not TrkA (Gascon et al., 2005), our finding that NGF significantly increased apoptosis in cells lacking PSA suggests that this effect is mediated through p75. In agreement with these results, pharmacological blockade of p75, but not of Trk receptors, prevented neuronal cell death induced by the removal of PSA. We demonstrate that the inhibition of two well-established pro-apoptotic cascades downstream of p75, ceramide and c-Jun N-terminal kinase (Barrett et al., 1998; Casaccia-Bonnefil et al., 1996; Dobrowsky et al., 1994; Frago et al., 1998; Hirata et al., 2001), completely prevented neuronal cell death induced by the absence of PSA in control as well as NGF-treated cultures. Together, these data raised the possibility that the removal of PSA from NCAM induced an enhanced activation of p75 signaling pathways. In agreement with this idea, we found that both in vivo and in vitro, immature neurons lacking PSA-NCAM express significantly higher levels of p75 than control cells. However, definitive evidence for this hypothesis would require additional experiments involving the use of p75-knockout animals and function-blocking antibodies. We also demonstrated that PSA removal does not affect TrkB or TrkC expression, thus illustrating the specificity of this effect. The current study is, to our knowledge, the first indication of a role of PSA-NCAM in the regulation of p75 receptor expression. Interestingly, p75 expression in Schwann cells is tightly regulated by Egr1 (Zif268) (Nikam et al., 1995), a transcription factor known to be activated downstream of the FGF receptor (Midgley and Khachigian, 2004; Santiago et al., 1999; Wang et al., 1997). Given the fact that NCAM at the cell membrane can interact with and activate FGF receptors (Doherty and Walsh, 1996), this signaling pathway would be an interesting candidate to modulate p75 expression and cell survival. Further studies are required to address this hypothesis.

It has been proposed that p75 receptors function both as a dependence receptor and a death receptor depending on the cellular context, including associated Trk expression, ligand presentation and expression of p75-interacting molecules (Bredesen et al., 2005). In the first case, the expression of p75 creates states of dependence on its ligands and activates death following the withdrawal of neurotrophic factors (Barrett and Bartlett, 1994; Rabizadeh et al., 1993). In the second, p75 may mediate the cellular response to a mismatched neurotrophin (e.g. exposure of a neuron-expressing TrkB and p75 to NGF, which binds TrkA and p75) through its function as a death receptor (Aloyz et al., 1998; Casaccia-Bonnefil et al., 1996; Frade et al., 1996). In both situations, p75-mediated apoptosis may serve to eliminate cells when they experience declining or inappropriate trophic support. One potential implication of our findings is that by limiting p75 expression, PSA-NCAM may protect newborn neurons from being dependent on trophic support before integration into olfactory circuits. This may ensure that enough cells arrive and compete in the OB. Neurons having reached their appropriate place in the granule/glomerular layer, and having established synaptic connections, would be coupled to network activity that is crucial for their long-term survival (Miwa and Storm, 2005; Rochefort et al., 2002). The progressive downregulation of PSA-NCAM and the increase in p75 expression during maturation would contribute to the elimination of non-integrated and/or misplaced cells. This mechanism may become operational precociously in the RMS of the NCAM$^{-/-}$ animal. Our studies

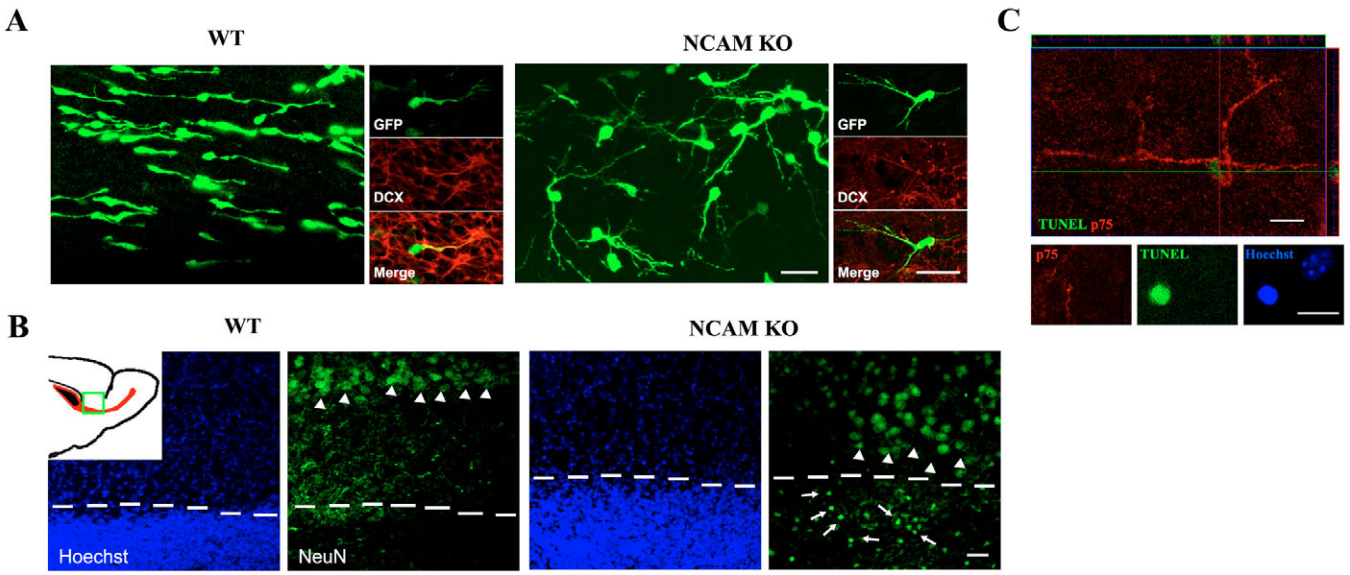


**Fig. 8. Abnormal neuronal maturation in the SVZ-RMS of NCAM$^{-/-}$ animals is associated with apoptosis and p75 expression.** (**A**) Morphological analysis of cells in SVZ-RMS 4 days after a GFP lentiviral vector injection in the lateral ventricles. In WT animals, GFP$^{+}$ cells display a unipolar morphology typical of migrating cells, whereas in NCAM mutant mice many GFP$^{+}$ cells exhibit a complex branched morphology suggestive of maturating neurons. GFP$^{+}$ cells colocalized with the neuronal marker doublecortin (DCX). (**B**) NeuN staining in the SVZ-RMS revealed numerous NeuN-positive nuclei in the NCAM mutant mice (arrows). These nuclei were smaller than those found on the cortex (arrowheads). The dashed line indicates the limit between the SVZ-RMS and the overlying corpus callosum and cortex. (**C**) Colocalization of TUNEL labeling and p75 immunostaining in the NCAM-deficient SVZ-RMS. The upper panel depicts an example of a TUNEL$^{+}$ nucleus that colocalizes with p75, whereas the lower panels illustrate a strongly TUNEL$^{+}$ nucleus that does not colocalize with p75 staining. Note that this latter nucleus is highly condensed, as illustrated by the Hoechst staining. Scale bar: 20 μm in A,B; 10 μm in C.

revealed a large number of disoriented, process-bearing neurons in the RMS of the NCAM$^{-/-}$ animals that were also immunopositive for p75. These results confirm and extend previous reports demonstrating that, in the absence of PSA, chain migration is disrupted (Chazal et al., 2000; Ono et al., 1994) and neuronal precursors start differentiating (Petridis et al., 2004). Whether this precocious differentiation is the cause or the effect of altered migration remains to be determined.

The neuroprotective effect of PSA-NCAM could also be important under pathological conditions. Both PSA and p75 are known to be re-expressed in lesion contexts, such as in mechanical damage, focal ischemia, axotomy, stroke and epileptic seizures (Gage et al., 1989; Giehl et al., 2001; Kokaia et al., 1998; Rende et al., 1993; Roux et al., 1999). Remarkably, blockade or enzymatic disruption of PSA leads to an impaired repair process (Bonfanti et al., 1996; Daniloff et al., 1986) and the NCAM-knockout mice exhibit deficient recovery after cortical lesions (Troncoso et al., 2004). It has been reported that, in a mice model of amyotrophic lateral sclerosis, surviving motoneurons in transgenic animals express PSA-NCAM (Warita et al., 2001), raising the possibility that PSA re-expression protects undamaged and functional neurons from p75-mediated apoptosis. Together, the data presented here provide a rational for the potential application of NCAMs as targets for therapy.

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