Neurochem Res (2006) 31:215–225
DOI 10.1007/s11064-005-9021-7

ORIGINAL ARTICLE

The Polysialylated Neural Cell Adhesion Molecule Promotes Neurogenesis in vitro

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Accepted: 29 November 2005 / Published online: 30 March 2006

Abstract A characteristic feature of neurogenic sites in the postnatal brain is the expression of the polysialylated forms of the neural cell adhesion molecule (PSA-NCAM). To investigate the role of PSA-NCAM in generation of neuronal populations, we developed an in vitro model where neurogenesis occurs in primary cortical cultures following serum withdrawal. We show that removal or inactivation of the PSA tail of NCAM in these cultures leads to a significant decrease in the number of newly generated neurons. Similarly, cultures prepared from NCAM knock-out mice exhibit a significantly reduced neurogenesis. Pulse-chase experiments using the proliferation marker BrdU reveal that the lack of PSA does not affect the mitotic rate of neural progenitors but rather, it reduces the early survival of newly generated neurons. These results suggest that, in addition to its role in the migration of neuronal progenitors, PSA-NCAM is required for the adequate survival of these cells.

Keywords Neuronal survival · Postnatal neurogenesis · PSA-NCAM · Proliferation

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Introduction

One strategy by which plasticity is maintained in the postnatal central nervous system (CNS) is the generation and integration of new neurons into functional circuits [1]. It is now well established that neurons are continuously generated in the rostral part of the adult subventricular zone adjacent to the lateral ventricle from where immature neuroblasts migrate toward the olfactory bulb and integrate into existing neural circuits [2]. Neurogenesis also occurs in the postnatal dentate gyrus of the hippocampus and recent observations indicate that this phenomenon might have a role in learning and memory [3]. Newly generated neurons from the subventricular zone were shown to be recruited into the neocortex of adult primates [4] and a potential for neurogenesis within the neocortex itself was also suggested [5–7], though this phenomenon is still debated [8]. In addition to the well-defined neurogenic zones of the postnatal brain, progenitors with neurogenic potential were also isolated from the adult cerebral cortex [9]. In line with these results, generation of new neurons was observed in confluent astroglial cultures of the postnatal rat cerebral cortex [10–11].

A characteristic feature of neurogenic sites is the expression of the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) at the surface of immature neuroblasts [9, 12–13]. Polysialic acid is a linear homopolymer of alpha 2,8-linked sialic acid that is uniquely attached to NCAM in the vertebrate nervous system [14–15]. The expression of PSA-NCAM at the cell surface appears to be important for morphogenetic events in both the developing and adult CNS, including migration, axonal regeneration and neuronal survival [15–16]. However, the biological importance of PSA-NCAM in neurogenesis remains to be elucidated.

To address this issue, we developed and characterized an in vitro model where neurogenesis occurs in confluent primary cortical cultures from newborn rat and mice following serum withdrawal. We show in this manuscript that removal or blockade of PSA leads to a substantial decrease in neurogenesis. Similarly, generation of neurons is impaired in cultures prepared from the NCAM knockout mice, lacking both NCAM and PSA-NCAM. Pulse-chase experiments with the proliferation marker BrdU revealed that the absence of PSA does not affect the mitotic activity of progenitors but rather the early survival of newly generated neurons.

Experimental procedures

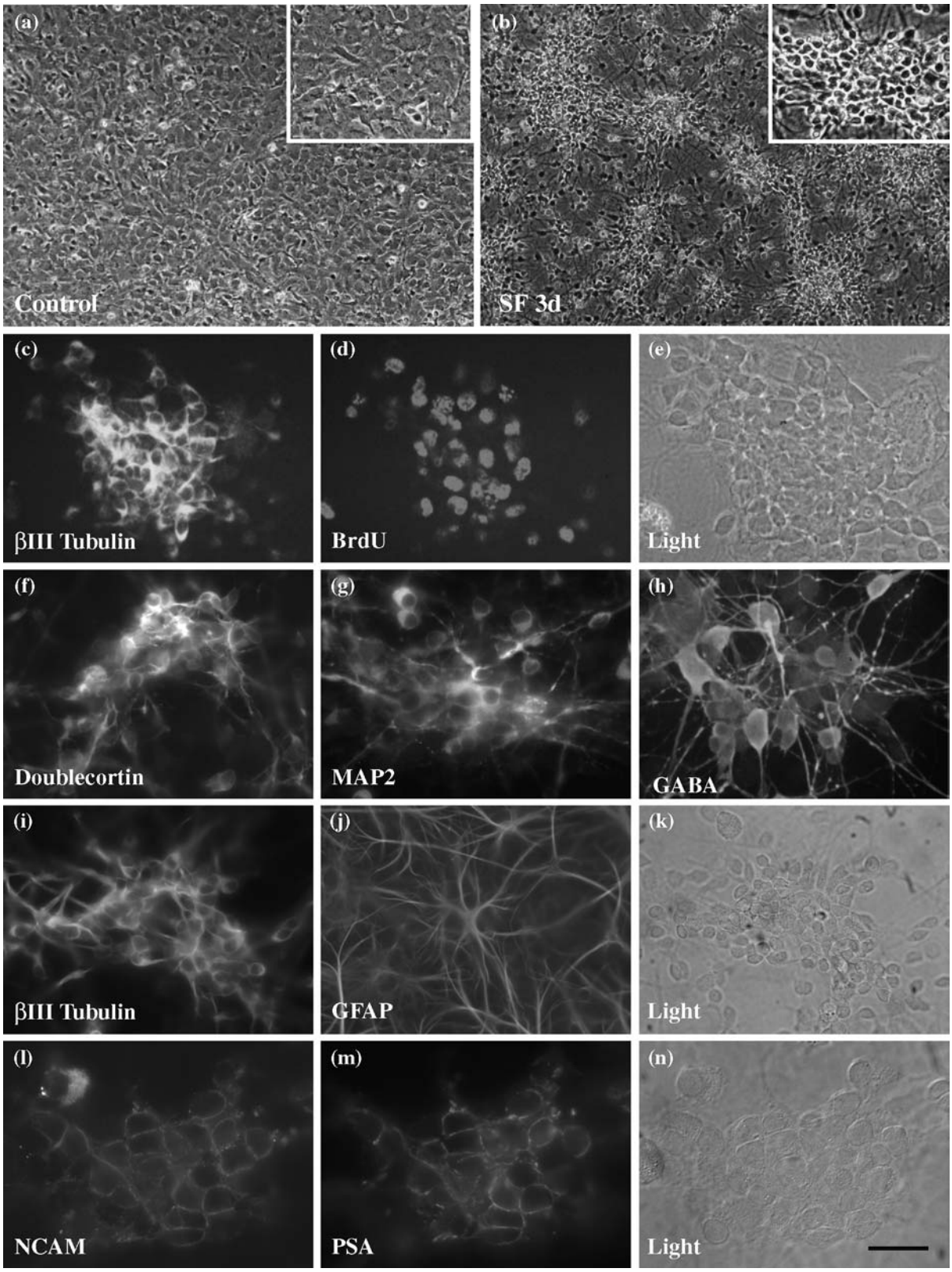
Cell cultures

Primary mixed cultures of neuronal and glial cells were prepared from the cerebral cortex of postnatal 1-day-old Sprague–Dawley rats, as previously described [17]. Cells were plated on polylysine-coated (Sigma, Saint Louis, MO, USA) coverslips in petri dishes (35 × 10 mm, Falcon, Plymouth, UK) with a seeding density of 7×10^5 cells per cover slip and grown in Dulbecco's modified Eagle's medium (DMEM), 10% foetal calf serum (FCS). When cultures became confluent at the seventh day in vitro (DIV), the medium was changed to serum-free medium (DMEM Gibco, 15 μg/ml insulin, 20 μg/ml transferrin, 20 nM progesterone, 100 mM putrescine and 30 nM sodium selenite). Under these conditions cultures remained viable for more than 2 weeks. Cortical cultures from mice where NCAM was genetically deleted [18] as well as from their wild type (WT) counterparts (C57Bl6 strains) were prepared and handled similarly as cultures from rats with the exception that the initial seeding density was 4×10^6 cells per petri dish.

To remove PSA from cell surfaces, the enzyme Endoneuraminidase N (Endo N) purified from phage K1 [19] was used. Endo N was shown to degrade rapidly and specifically linear polymers of sialic acid with 2,8-linkeage, with a minimum length of 7–9 residues [20]. Cells were treated with Endo N (0.5 U/ml) in serum-free medium from 1 h to 96 h according to experimental protocols. In order to inactivate PSA, we used a mouse IgG antibody (Ab) (m735; 2 μg/ml), kindly provided by Rita Gerardy–Schahn, that binds to PSA with eight or more residues [21]. To evaluate cell proliferation, bromodeoxyuridine (BrdU, Sigma, Saint Louis, MO, USA) was added to cultures in the presence of serum-free medium for 1 h to 72 h, according to experimental protocols. In order to study neuronal survival following the BrdU pulse, we used the antimitotics AraC (2.5 μM; Sigma, Saint Louis, MO, USA) and aphidicolin (50 μM, Calbiochem Laboratories, La Jolla, CA, USA) for up to 8 h.

Immunocytochemistry, antibodies and cell counts

Cultures were fixed in cold (4°C) 4% paraformaldehyde in 0.1 M phosphate buffer for 40 min, and then washed several times in phosphate buffered saline (PBS) solution. Primary Abs directed against cell surface antigens (PSA, NCAM, O4, A2B5) were diluted in PBS/0.5% BSA solution, while Abs against intracellular antigens were diluted in PBS/0.5% BSA/0.3% Triton X-100 solution. Cultures were incubated with primary Abs at room temperature for 2 h or at 4°C overnight. Bound Abs were revealed with rhodamine-conjugated sheep anti-mouse IgG (dilution 1:40; Boehringer Mannheim Biochemicals, Rotkreuz, Switzerland) or fluorescein-conjugated sheep anti-rabbit IgG (dilution 1:80; Boehringer Mannheim Biochemicals, Rotkreuz, Switzerland) secondary Abs (diluted in PBS/0.5% BSA solution). The following primary Abs were used: (1) a rabbit polyclonal Ab (Dakopatts, Copenhagen, Denmark) to GFAP was used (1:400 dilution) to identify astrocytes; (2) a mouse monoclonal Ab directed against β-tubulin isotype III (1:400 dilution; Sigma, Saint Louis, Missouri, USA) as well as the mouse monoclonal anti-MAP2 (1:100 dilution; Sigma, Saint Louis, Missouri, USA); a goat polyclonal Ab against Doublecortin (1:1000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and a mouse monoclonal Ab against NeuN (1:300 dilution; Chemicon, Temecula, CA, USA) were used to identify neurons; (3) the monoclonal antibody anti-GABA 3D5 (1:2000 dilution; kindly provided by P. Streit, Zurich, Switzerland) was used to detect GABA immunopositive neurons; (4) a mouse monoclonal A2B5 Ab hybridoma supernatant (ATCC, Rockville, MD, USA; 1:5 dilution; ref 22) was used to label O-2A progenitor cells; (5) O4 monoclonal Ab (hybridoma supernatant; 1:5 dilution; ref 22) was used to identify oligodendrocytes; (6) monoclonal Abs, Men B IgM (1:500 dilution) that recognizes specifically alpha 2–8-linked PSA with chain length superior to 12 residues [23] and m735 IgG [21] were used to detect PSA; (7) the rabbit antiserum directed against the NCAM protein core was a site-directed pAb recognizing the seven NH_2-terminal residues of NCAM (1:600 dilution) [24]; (8) BrdU incorporation into cells was detected with a mouse monoclonal Ab (1:50 dilution, Boehringer Mannheim, Germany) or with a rat monoclonal Ab against BrdU (1:200 dilution; Harlan Sera Lab). Cultures were examined by either a fluorescence microscope (Axiophot; Zeiss, Oberlochen, Germany) or a confocal microscope (Bio-Rad MRC-600 laser scanning confocal imaging system). Cells


(a)
Control
(b)
SF 3d
(c)
βIII Tubulin
(d)
BrdU
(e)
Light
(f)
Doublecortin
(g)
MAP2
(h)
GABA
(i)
βIII Tubulin
(j)
GFAP
(k)
Light
(l)
NCAM
(m)
PSA
(n)
Light

◀

Fig. 1 Immunocytochemical characterization of neurogenesis in cortical cultures. (**a**) Phase contrast images show culture at the stage of confluence (control) and (**b**) another preparation 3 days following serum withdrawal (SF3d). Note the formation of cellular clusters on the top of the glial monolayer in the presence of serum-free medium (for higher magnifications see inserts). Cells inside the clusters were positively stained with the neuron specific marker β III tubulin (**c**) and incorporated the proliferation marker BrdU (**d**). (**e**) Phase contrast photograph corresponding to (**c**) and (**d**). The neuronal fate of newly generated cells was further confirmed by immunostaining with neuron specific markers such as doublecortin (**f**), MAP2 (**g**) and GABA (**h**). While virtually all cells inside the clusters expressed β III tubulin (**i**), these cells did not express the astrocytic marker GFAP (**j**). (**k**) Phase contrast photograph corresponding to (**i**) and (**j**). Most cells in clusters exhibit NCAM (**l**) and PSA (**m**) at the cell surface. (**n**) Phase contrast photograph corresponding to (**l**) and (**m**). Scale bars: (a–b): 120 μm; (c–g): 50 μm; (h): 40 μm; (i–k): 50 μm; (l–n): 40 μm

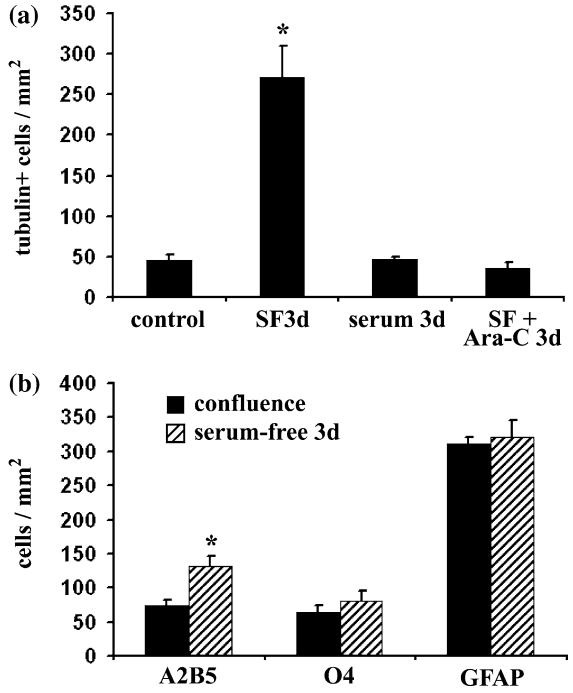


Fig. 2 Removal of serum promotes neurogenesis in confluent cortical cultures. (**a**) Cultures were maintained in serum-complemented medium until they became confluent (7 days), further cultured in the absence of serum for 3 days, fixed and immunostained for the neuronal marker β tubulin III. In controls, a small number of neurons were present in culture. Three days following serum withdrawal (SF3d) the number of neurons was substantially increased. In contrast, the number of neurons did not increase in the presence of serum (serum 3d) or an antimitotic (SF+Ara-C 3d). (**b**) Serum withdrawal for three days also induced a small but significant increase in the number of A2B5 immunopositive, presumably O-2A progenitor cells. On the contrary, the number of O4 positive oligodendrocytes and GFAP positive astrocytes was not affected by serum removal. Results are mean ± SEM, n=3 independent experiments for each time-point and each treatment expressed. Differences between groups were first determined by ANOVA and then the paired t-test was performed where t was corrected for multiple comparisons against the control (confluence) group. $^*P < 0.05$ was considered as significant

were counted with the help of a square grid, placed into the ocular of the microscope. On each coverslip 35 samples were randomly taken and then samples were pooled. Data are expressed as either the number of cells per mm^2 or the percentage of the initial cell number, according to the experimental setup. Differences between groups were first discriminated by ANOVA and then the paired t-test was performed where t was corrected for multiple comparisons against the control (serum-free) using the Bonferroni correction. $^*P < 0.05$ compared to controls was considered significant in each experiment.

Results

Removal of serum promotes neurogenesis in cortical cultures from newborn rats

Primary cortical cultures from newborn rat cerebral cortex developed a confluent monolayer in the presence of serum after 7 DIV as previously described [25]. At this stage, a small number of immature neurons as well as some oligodendrocytes—type 2 astrocyte (O2-A) precursors could be observed on the top of the astroglial monolayer. We observed that removal of serum from the culture medium at the stage of confluence induced the formation of clusters of small, round-shaped cells with dense perykaria as early as 24 h after changing the medium (Fig. 1a–b). The number of these cells increased progressively (Fig. 1b) until 7 days after serum withdrawal (the longest time-point examined). Growing clusters flattened as cells begin to migrate from the centre towards the periphery and grow neurite-like processes. When the proliferation marker bromodeoxyuridine (BrdU) was administered into the culture medium,

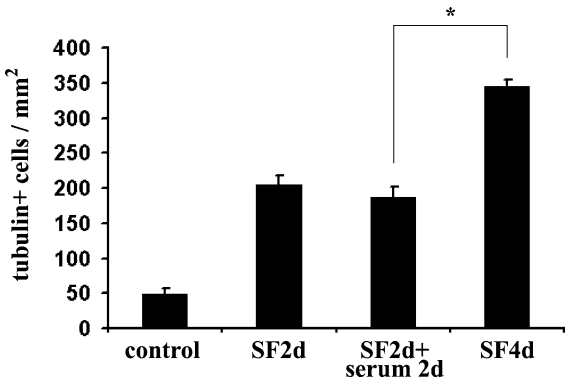


Fig. 3 Readministration of serum into the culture medium stops neurogenesis. Compared to control cultures, kept in the presence of serum-free medium for 4 days (SF4d), neurogenesis was significantly reduced if serum was readministered for 2 days after a 2 day serum-free period (SF2d+serum 2d). $^*P < 0.05$

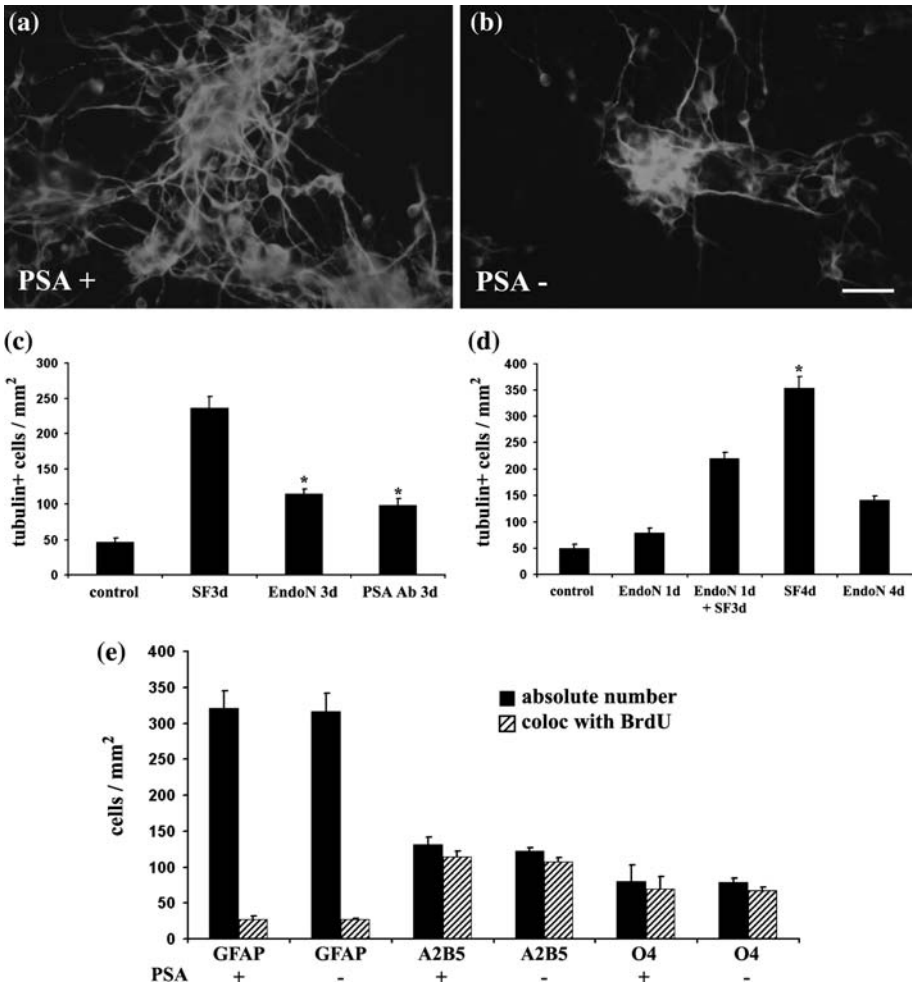


Fig. 4 Neurogenesis is reduced after removal or inactivation of PSA. After reaching confluence (control), cells were further cultured in the presence of Endo N or anti PSA Ab (PSA Ab) for 3 days, fixed and immunostained for β tubulin III. The number of immunostained neurons was substantially decreased in the presence of Endo N (**b**) compared with controls (**a**). (**c**) Quantitative analysis of the effect of Endo N (EndoN 3d) and anti-PSA Ab (PSA Ab 3d) on neurogenesis. (**d**) The effect of Endo N treatment is reversible. When cultures are exposed to the enzyme for 24 h (EndoN 1d) and then kept in serum-free medium for an additional 3 days (EndoN 1d + SF3d), the number of neurons was significantly higher compared with groups where Endo N was present during the whole experimental period (EndoN 4d). (**e**) Endo N treatment affected neither survival nor proliferation of GFAP+ astrocytes, A2B5+ O-2A precursor or O4+ oligodendrocytes compared to control serum-free conditions during a 3-day-long experimental period. Black columns show the absolute number of cells, according to the indicated immunostaining per mm^2, while dashed columns stand for the number of these cells showing colocalization with the proliferation marker BrdU. In these experiments, BrdU was added to the culture medium for the last 48 h. Results are mean ± SEM, n=3 independent experiments for each time-point and each treatment expressed. Differences between groups were first discriminated by ANOVA and then the paired t-test was performed where t was corrected for multiple comparisons against the control (serum-free) group. $^*P < 0.05$ was considered as significant. Scale bars: (a–b): 50 μm

cells in clusters incorporated BrdU (Fig. 1c–e) indicating ongoing cell proliferation. Accordingly, when the antimitotics Ara-C or aphidicolin were added to the culture medium, no formation of cell clusters was observed (Fig. 2a).

The phenotypes of newly generated cells were identified immunocytochemically. The large majority of cells within clusters expressed the neuron specific marker β-tubulin III (Fig. 1c–e). These cells also expressed other established markers for neurons, such as doublecortin, MAP2, NeuN as well as GABA (Fig. 1f–h). Moreover, we observed that at later time points these cells developed processes and immunostained for synapsin (not shown). In contrast, we did not observe cells within the clusters that were positive for the astrocytic marker GFAP (Fig. 1i–k) or the oligodendrocyte marker O4. Cells in clusters were immunostained with antibodies against PSA and NCAM (Fig. 1 l–n), which is consistent with the notion that immature neurons in vivo as well as in vitro, express the polysialylated isoforms of NCAM [15]. Subsequent cell counts indicated that in addition to the generation of new neurons (Fig. 2a), removal of serum also resulted in a small but significant

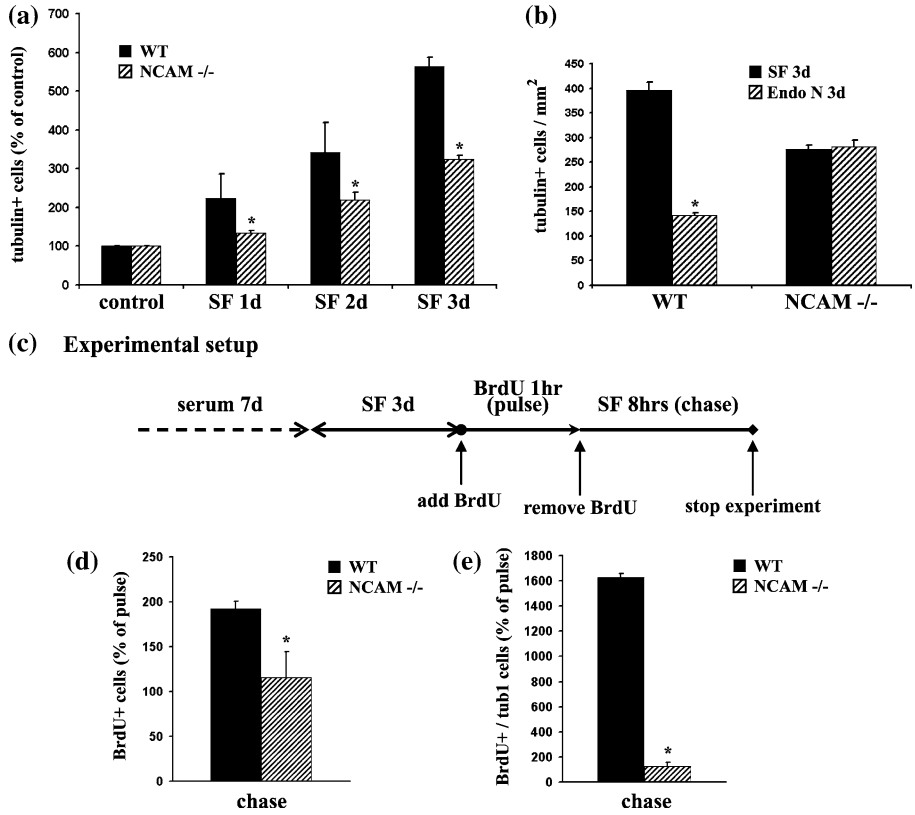


Fig. 5 Decreased neurogenesis in cultures from the NCAM−/− mice. After confluence of cultures (control), serum-complemented medium was withdrawn and cultures from WT and NCAM−/− animals were kept in serum-free (SF) medium for 1–3 days with or without Endo N. The number of neurons was assessed after tubulin immunostaining. (**a**) The rate of neurogenesis is significantly slower in the NCAM−/− compared with the WT mouse. Values are expressed as percentage of control value at the stage of confluence in both strains (confluence=100%; absolute number of tubulin+ cells was 38±9/mm^2 in the WT and 79±15/mm^2 in the NCAM−/−). (**b**) A 3-day Endo N treatment does not affect neurogenesis in NCAM−/− cultures. (**c**) Evaluation of neurogenesis by pulse-chase experiments using BrdU. Following the 3 day period in the presence of serum-free medium, BrdU was added to the culture medium for 1 h (pulse). BrdU was then washed out and cultures were kept in serum-free medium for an additional 8 h before fixation (chase). The number of BrdU positive cells at the end of the pulse (''pulse''=100%=210±20/mm^2 in WT and 190±30/mm^2 in NCAM−/−) was determined for both WT and NCAM−/− cultures. The number of BrdU+ (**d**) as well as BrdU+/tubulin+ cells, (**e**) determined at the end of the chase was expressed as percentage of ''pulse'' in each strain. Results are mean ± SEM, n=3 independent experiments for each time-point and each treatment in both strains. The paired t-test was performed between values obtained in WT and NCAM−/− strains at each time point (except in A, where correlation between serum-free and Endo N treated groups was examined) and $^*P < 0.05$ was considered as significant

increase in the number of A2B5+ O-2A progenitor cells (Fig. 2b), but these cells were homogenously distributed all over the astrocytic monolayer without forming clusters. No increase in the number of GFAP+ astrocytes and O4+ oligodendrocytes was observed (Fig. 2b).

If cultures were maintained in the presence of serum after reaching confluence, we did not observe cluster formation (Fig. 3). The inhibitory effect of serum was further confirmed in a series of experiments where the serum withdrawal-induced progenitor proliferation was stopped by readministration of serum into the culture medium (Fig. 3).

Collectively, these results demonstrate that primary cortical cultures from newborn rat retain a robust neurogenic potential that could be revealed by removal of serum from the culture medium.

Neurogenesis is reduced after removal or inactivation of PSA

To investigate the role of PSA-NCAM in neurogenesis, two complementary approaches were taken. First, at the time of serum withdrawal, cultures were exposed to the enzyme Endo N that selectively removes PSA from NCAM [20]. Consistent with previous reports [17], 2 h of Endo N treatment was sufficient to completely remove PSA immunoreactivity from all neuronal surfaces without any visible effect on NCAM immunoreactivity on neurons or glial cells. However, to prevent rapid re-expression of PSA after removal of the enzyme [17], Endo N was maintained in the culture medium during the whole study period. We observed that in the presence of Endo N, the number of newly

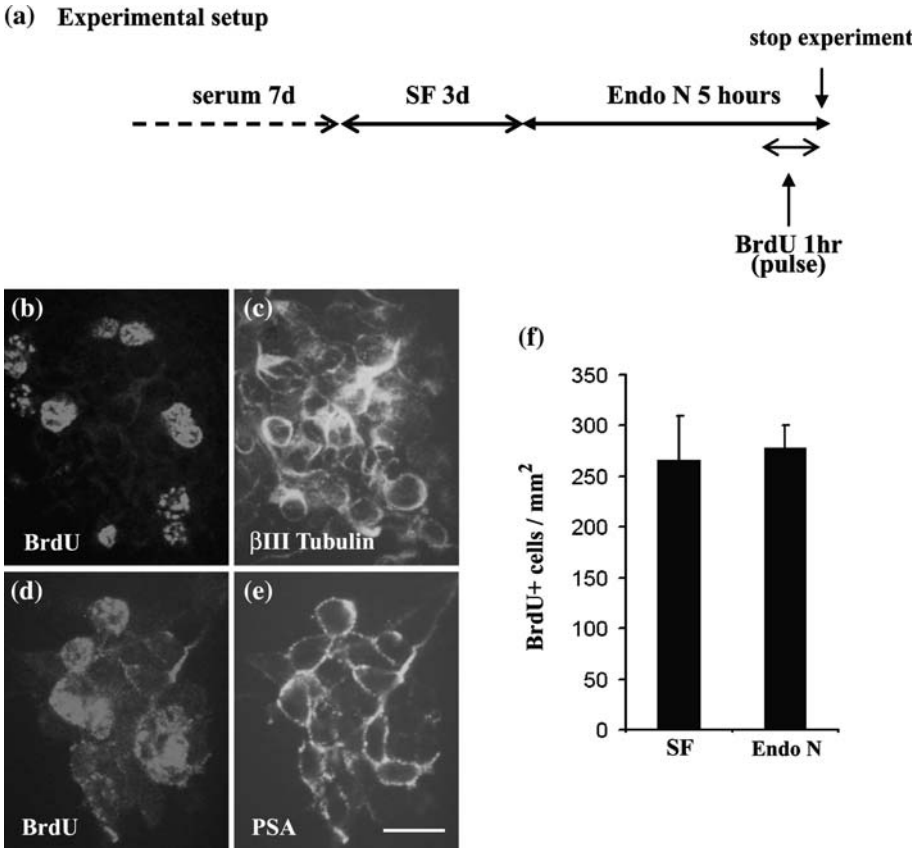


Fig. 6 Removal of PSA from NCAM does not affect the mitotic rate of neuronal precursors. (**a**) Experimental setup. After reaching confluence, cultures were kept in serum-free medium for 3 days and then Endo N was added to the culture medium. Following 4 h of Endo N treatment, cultures were exposed for 1 h to BrdU and then fixed. Double immunolabelling shows that a 1-hour-long BrdU pulse results in the incorporation of this proliferation marker (**b**) into β III tubulin+ cells (**c**). The majority of cells inside the clusters incorporating BrdU during the pulse (**d**) also express PSA-NCAM at the cell surface (**e**). (**f**) The number of cells incorporating BrdU during the pulse period did not differ between control and Endo N treated groups. Values are expressed as the total number of BrdU+ cells/mm^2. Results are mean±SEM, n=3 independent experiments. The paired t-test was performed and $^*P < 0.05$ was considered as significant. Scale bars: (b–e): 35 μm

generated neurons was significantly reduced (>50%) in cultures (Fig. 4a–c). This effect was reversible, as removal of the enzyme, and consequently the re-expression of PSA at the cell surface, increased the number of neurons compared to cultures where Endo N was continuously present during the study period (Fig. 4d). In contrast to neurons, Endo N treatment affected neither the number nor the proliferation rate of other non-neuronal cell types in our cultures (Fig. 4e).

As a second approach to establish a role for PSA-NCAM in neurogenesis, a monoclonal IgG Ab (m735) directed against PSA was included in the culture medium at the time of serum withdrawal and the number of neurons was determined 3 days later. The specificity of this antibody has been characterized extensively in previous studies [21]. Using immunocytochemistry, we verified that after this 72 h of culture period the Ab remained bound to the cell surface, indicating that a single administration of this Ab (2 μg/ml) was sufficient to block the PSA groups permanently. Figure 4c shows that similar to the effect of for Endo N treatment, exposure of cultures to the PSA Ab, but not to preimmune IgG, resulted in a reduced number of neurons compared to control groups.

Neurogenesis in cultures from NCAM knockout mice

To further evaluate the role of PSA in cortical neurogenesis in vitro, we took advantage of the NCAM knockout (NCAM−/−) mouse lacking both NCAM and PSA. The phenomenon of serum withdrawal-induced neurogenesis also occurred in primary cortical cultures of newborn mice prepared from WT as well as from the NCAM−/− animals. When Endo N was administered to these cultures, neurogenesis was found to be significantly reduced in the WT but not in the NCAM−/− cultures (Fig. 5b). The lack of Endo N-induced changes in NCAM−/− cultures further argues against a potential toxic effect of the enzyme.

Comparison of the rate of neurogenesis between the WT and NCAM−/− cultures revealed that the increase in the

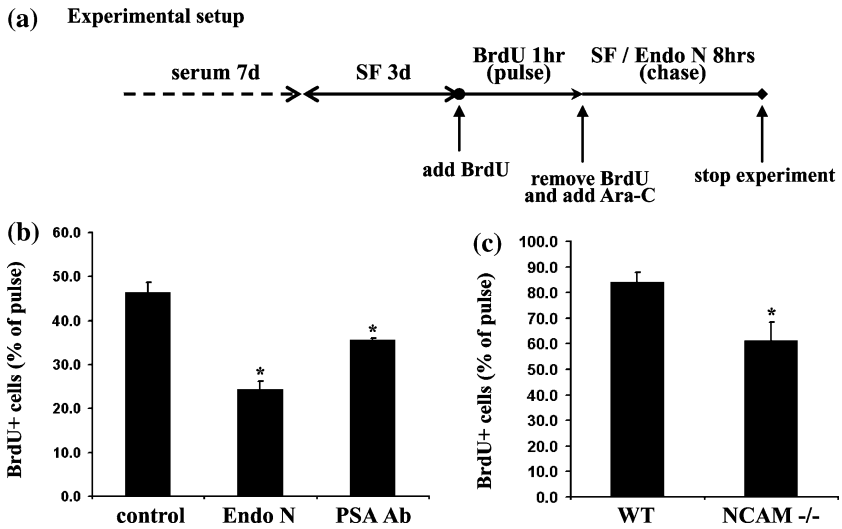


Fig. 7 Short-term survival of newly generated neurons is reduced in the absence of PSA-NCAM or NCAM. (**a**) Experimental setup. After reaching confluence, cultures were kept in serum-free medium for 3 days that was followed by 1-hour BrdU pulse. BrdU containing medium was then removed and cultures from rat or mice (WT and NCAM−/−) were kept in serum-free medium in the presence of an antimitotic for an additional 8 h. Under these conditions, survival of BrdU+ cells at the end of the chase was significantly decreased in the presence of Endo N or the anti-PSA Ab in cortical cultures from rats (**b**) as well as in cultures prepared from NCAM−/− animals (**c**) compared with control groups. Values are expressed as percentage of pulse (i.e. the number of BrdU+ cells at the end of the pulse) in each strain (rat, mouse WT, mouse NCAM−/−). Results are mean ± SEM, n=3 independent experiments for each treatment expressed. In B, differences between groups were first discriminated by ANOVA and then the paired t-test was performed where t was corrected for multiple comparisons against the control (serum-free) group. In (**c**), the paired t-test was performed between WT and NCAM−/− groups. In both cases, $^*P < 0.05$ was considered as significant

number of immature tubulin positive neurons after serum removal is significantly slower in cultures prepared from the NCAM−/− mice (Fig. 5a). To confirm these observations we also conducted pulse-chase experiments using the cell proliferation marker BrdU. In preliminary experiments, using colocalization of BrdU with cell cycle specific markers, we determined that after 1 h of BrdU pulse more than 90% of BrdU+ cells were also stained with an antibody against cyclin A (not shown). Hence these data confirm that 1 h of BrdU pulse can detect practically all cells that pass through the S-phase during this period and that by the end of the pulse, we do not deal with BrdU+ postmitotic (G0) cells. Colocalization of BrdU+ cells with cell specific markers following the 1-hour-long pulse labelling revealed that the majority of BrdU+ cells (>75%) were localized to the cell clusters of β tubulin III positive neuronal cells (Fig. 6b–c). Some GFAP+ astroglia and O4+ oligodendrocytes also showed colocalization with BrdU but the distribution of these cells did not show any colocalization with the clusters of newly generated neurons.

Following these preliminary observations, we performed a series of pulse-chase experiments with BrdU to further evaluate the rate of neurogenesis in the WT and the NCAM−/− mice (for experimental protocol see Fig. 5c). When BrdU was added to the culture medium for 1 h (pulse) at the end of the 3 days period in serum-free medium, a small number of β tubulin III positive cells incorporated this proliferation marker in both the WT and the NCAM−/− strains. BrdU was then washed out and cultures were left in serum-free medium for an additional 8 h and then cultures were fixed (chase). As seen in Fig. 5d, the number of BrdU+ cells increased in both groups during the 8-hour-long chase period, but this was significantly higher in cultures prepared from the WT animals. This difference was even more important when the number of β tubulin III—BrdU double labelled cells was compared between cultures from the two strains (Fig. 5e). In cultures from the WT animals the number of BrdU positive neurons was increased from $2\pm1/mm^2$ (pulse) to $33\pm6/mm^2$ (chase), while in the NCAM−/− mice $3\pm2/mm^2$ such double labelled cells were found following the pulse and $4\pm2/mm^2$ following the chase period.

Altogether, these data demonstrate a role for PSA-NCAM in generating new neurons. Given that the decreased neurogenesis observed in the NCAM−/− compared to the WT mouse is reproduced by removal or blocking PSA, our results also suggests that this effect is rather related to the presence of the PSA chain than the NCAM protein core itself.

Removal of PSA from NCAM does not affect the proliferation rate of neuronal precursors

The phenomenon of neurogenesis involves the mitotic cycle as well as the survival and differentiation of neuronal progenitors. To investigate whether the presence of PSA at

the cell surface is involved in the proliferation of precursors, cultures were treated with Endo N for 4 h following serum withdrawal and then BrdU was added to the culture medium for 1 h (see experimental protocol in Fig. 6a). As described above, a short BrdU pulse (1 h) was sufficient to obtain a small number of BrdU+ cells inside the clusters (Fig. 6b–c). The majority of these BrdU-labelled cells expressed PSA-NCAM at the cell surface (Fig. 6d–e). Endo N treatment did not affect the proliferation rate, as revealed by the 1-hour-long BrdU chase, neither in the rat (Fig. 6f) nor in the WT mouse (not shown) cultures suggesting that the mitotic cycle per se in our cultures did not depend on the presence of PSA-NCAM.

Short-term survival of newly generated neurons is reduced in the absence of PSA

In vivo, a high percentage of newly generated neurons undergo early apoptosis in the proliferative zones of the prenatal as well as postnatal brain [26]. The presence of PSA at the cell surface could play a role in this process. We thus tested the hypothesis that removal of PSA decreases early survival of newly generated cells. For this purpose, cortical cultures from the newborn rat were kept for 3 days following serum withdrawal, and then BrdU was added to the culture medium for 1 h (pulse) as described above (for experimental protocol see Fig. 7a). Following the pulse labelling, BrdU was removed and cultures were kept for an additional 8 h in serum-free medium in the presence or the absence of Endo N. To differentiate between the proliferation and survival of the pulsed labelled BrdU+ cells during the 8-hour-long chase period, we also included the antimitotics Ara-C or aphidicolin into the culture medium. We found that, under these experimental conditions, more than 50% of BrdU+ cells disappeared by the end of the 8 h of chase even in the presence of PSA at the cell surface. However, compared to these control groups, Endo N treatment resulted in a further and significant increase in cells loss during the chase period (Fig. 7b). Similar results were obtained using the anti-PSA Ab (m735) (Fig. 7b). Survival of newly generated cells during the chase period was also significantly decreased in cultures prepared from the NCAM−/− compared to WT mice (Fig. 7c).

Discussion

The functional significance of PSA-NCAM at the surface of neuronal progenitors is a pertinent question. PSA-NCAM is expressed in immature neuroblasts in neurogenic zones of the embryonic as well as the postnatal brain [9, 12–13] and this adhesion molecule has been implicated in the migration of these cells [27]. Our data suggest that PSA-NCAM might take part in the trophic regulation of immature neurons. Using an in vitro model of neurogenesis, we demonstrate that removal of the polysialic tail of NCAM by Endo N decreases the number of newly generated neurons. Similar results were obtained when PSA was blocked by a specific antibody and in cultures prepared from the NCAM−/− mice. Using pulse-chase labelling of neuronal progenitors with the proliferation marker BrdU, we could differentiate between two distinct although closely related events of neurogenesis, namely the mitotic activity per se and the early survival of newly generated neurons. We show that the lack of PSA-NCAM or NCAM does not influence mitotic activity, but rather it increases early cell death of newly generated immature neurons.

In these studies we used an in vitro model where neurogenesis occurred in primary cultures of the microdissected cerebral neocortex of newborn rat and mouse. At confluence, these preparations consist essentially of an astrocytic monolayer with a few postmitotic neurons and oligodendrocyte progenitors on the top of it. In agreement with previous observations [10], we found that the astrocytic monolayer can generate large populations of immature neurons following removal of serum from the culture medium. This was demonstrated by immunocolocalization of the cell proliferation marker BrdU with neuron specific markers, such as tubulin-β-III, MAP2, doublecortin and NeuN. In the present study, we did not investigate the cells of origin of newly generated neurons. The microdissection of cortical tissues was performed in such a way that we could exclude cells from the neurogenic matrix of the subventricular zone. Thus cells with neurogenic potential are likely to be derived from the cortex. This hypothesis is in agreement with recent evidence suggesting that the cerebral cortex and the subcortical white matter contains multipotential progenitor cells or stem cells until the end of the second postnatal week [11, 28]. Accumulating evidence suggest that GFAP positive astrocytes, and radial glia cells have attributes of stem cells and could give rise to multipotential neurospheres and neurons under in vivo as well as in vitro conditions [9, 11, 29, 30]. Whether cells exhibiting neurogenic potential in our cultures belong to the multipotential astrocytic stem cell population or rather they correspond to committed neural progenitors remains to be determined.

We provide three lines of evidences for a role PSA-NCAM in neurogenesis in this model. First, Endo N—that specifically cleaves PSA without affecting the protein core of the NCAM molecule—causes a significant (>50%) decrease in the number of newly generated neurons compared to control groups. Endo N does not seem to exert a non-specific or toxic effect, as exposure to this enzyme does not influence the proliferation of other, non-neuronal cell types in our cultures. Also, the effect of Endo N on neurogenesis

can be reversed by the removal of the enzyme from the culture medium and, importantly, neurogenesis is not influenced by Endo N in cultures prepared from the NCAM knock-out mice. Second, when cultures were grown in the presence of a monoclonal anti-PSA Ab, generation of new neurons was also significantly (>50%) reduced. Third, neurogenesis is substantially decreased in cultures prepared from the NCAM−/− animals. Collectively, these results suggest that the PSA moiety or PSA-NCAM rather than the NCAM protein core plays a role in neurogenesis.

Generation of neuronal populations could be regulated by modifying the mitotic activity of progenitors or by promoting the survival of neuroblasts. Using a series of pulse-chase labelling with the proliferation marker BrdU, we could differentiate between these two processes. Our experiments suggest that PSA-NCAM is not involved in the mitotic activity of cortical progenitors in these cultures. These observations are in agreement with previous reports, showing that proliferation of neuronal progenitors in the postnatal subventricular zone is not affected in the NCAM−/− animals or in animals treated with Endo N [27]. It should be mentioned that removal of PSA from tumour cells was shown to inhibit proliferation [31]. Moreover, it has been proposed that homophilic NCAM to NCAM interactions inhibit cell proliferation in primary cultures of rat astrocytes as well as in populations of isolated hippocampal progenitor cells [32–33]. Differences between these and our observations could be due to either the use of different cell types in these studies or to differences in experimental protocols to evaluate cell proliferation.

Our observations suggest that PSA-NCAM is implicated in the early survival of newly generated neurons. In these experiments, following the 1-hour-long BrdU pulse, cells were kept in the presence of an antimitotic, either Ara-C or aphidicolin. This experimental setup allowed avoiding further proliferation of labelled precursors during the pulse period and thus permitted us to address directly the effect of PSA-NCAM on cell survival. In control cultures, following an 8-hour-long chase period in the presence of an antimitotic, we found about 50% decrease in the number of pulse-labelled BrdU positive cells. Compared to these controls, removal or blockade of PSA induced a further and significant decrease in the number of detectable BrdU positive cells following the chase. Although we cannot formally exclude that the observed high rate of cell death following the chase even in control cultures is due in part to blockade of some cells in the S-phase, the occurrence of such an important loss of newly generated neurons is well established in vivo [26]. These data are in agreement with previous studies demonstrating that interfering with PSA-NCAM affects the survival of neurons in dissociated [34] and organotypic cultures [35], as well as in vivo in neurogenic areas of the postnatal brain (36).

The mechanisms by which the removal or blockade of PSA interferes with neurogenesis remain to be elucidated. As PSA has a high negative charge density, its presence confers to NCAM a large hydrated volume that was hypothesised to attenuate cell to cell contacts [37]. According to this scenario, the absence of PSA at the cell surface would favour NCAM to NCAM interactions that, in turn, could induce apoptosis of young neurons [38]. The PSA chain may directly interfere with NCAM-mediated signalling such as the activation of the Ras/MAP kinase pathways [39] or the FGF receptor-mediated signalling [40–41]. An alternative possibility would be that PSA-NCAM, at the surface of newly generated neurons, may modulate growth factor responsiveness of these cells [16, 34–35, 41–43]. PSA-NCAM could interfere directly with BDNF/TrkB [34, 42] or other growth factor signalling [35, 43] that are implicated in neurogenesis. Clearly, further studies are needed to answer these questions.

Acknowledgements We wish to thank to Sylvie Chliate (technician, Department of Neuroscience, University of Geneva Medical School, Switzerland) for technical assistance. This work was supported by Swiss National Foundation Grants (Bern, Switzerland) 3100A0-104059/1 and by the European Community Grant Promemoria No. 512012-2005 to Jozsef Z. Kiss.

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