

High Efficacy of Clonal Growth and Expansion of Adult Neural Stem Cells

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SUMMARY: Neural stem cells (NSCs) from the adult central nervous system are currently being investigated for their potential use in autologous cell replacement strategies. High expansion rates of NSCs in culture are crucial for the generation of a sufficient amount of cells needed for transplantation. Here, we describe efficient growth of adult NSCs in Neurobasal medium containing B27 supplement under clonal and low-density conditions in the absence of serum or conditioned medium. Expansion of up to 15-fold within 1 week was achieved on low-density NSC cultures derived from the lateral ventricle wall, the hippocampal formation, and the spinal cord of adult rats. A 27% single-cell cloning efficiency in Neurobasal/B27 combination further demonstrates its growth-promoting ability. Multipotency and nontumorigenicity of NSCs were retained despite the high rate of culture expansion. In addition, increased cell survival was obtained when Accutase, instead of trypsin, was used for enzymatic dissociation of NSC cultures. This work provides an important step toward the development of standardized protocols for highly efficient *in vitro* expansion of NSCs from the adult central nervous system to move more closely to the clinical use of NSCs. (*Lab Invest* 2003, 83:949–962).

Cell replacement strategies for the diseased central nervous system (CNS) require a substantial amount of cellular material to be generated *in vitro*. Cells must be nontransformed, well characterized, and able to differentiate into the appropriate cell types. Moreover, they must survive in the diseased CNS and integrate into the neural network after transplantation to functionally improve the clinical outcome of patients. The manifestation of clinical symptoms in neurodegenerative diseases is preceded by the loss of a high number of neurons. In the case of Parkinson's disease, for example, 50% of nigral dopaminergic neurons (approximately 220,000 to 250,000 cells) are lost before clinical symptoms appear (for review see Dunnett and Bjorklund, 1999). Because only 5% to 20% of transplanted dopaminergic neurons survive *in*

vivo (Dunnett and Bjorklund, 1999), the generation of a high number of cells obviously constitutes a first requirement for successful CNS cell therapy.

Different sources for cells, such as dissociated fetal mesencephalic tissue, *in vitro* expanded stem cells derived from blastocysts or embryonic forebrains, and neural stem cells (NSCs) from the adult brain, have been analyzed for their potential use in transplantation experiments (Bain et al, 1995; Bjorklund et al, 2002; Brundin et al, 2000; Brustle and McKay, 1996; Englund et al, 2002; Freed et al, 2001; Gage, 1994, 2000; Svendsen et al, 1998; Widner et al, 1992). In humans with Parkinson's disease, implantation of fetal tissue has resulted in some degree of functional recovery (Brundin et al, 2000; Freed et al, 2001; Piccini et al, 1999). However, the highly limited access to fetal tissue and the ethical concerns surrounding its use have strengthened the search for alternative stem cell sources, in particular those harvested from adult tissues. The isolation of cells bearing NSC properties from the adult mammalian CNS (Arsenijevic et al, 2001; Gage et al, 1995b; Johansson et al, 1999b; Reynolds and Weiss, 1992; Seaberg and van der Kooy, 2002) has opened new avenues, including the exciting prospect of autologous grafting and *ex vivo* gene therapy. As an ideal scenario, a high number of NSCs needs to be generated out of a minimal CNS

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biopsy specimen within a short time. At present, however, there is a lack of efficient protocols for the expansion of adult NSCs, in particular under the low-density or clonal conditions needed for the development of autologous cell and gene therapies for the CNS.

In the adult CNS, NSCs can be isolated both from neurogenic regions, ie, the hippocampus and the ventricle wall, and from non-neurogenic regions including the septum, striatum, optic nerve, neocortex, and spinal cord (Arsenijevic et al, 2001; Johansson et al, 1999b; Palmer et al, 1995, 1999, 2001; Seaberg and van der Kooy, 2002; Shihabuddin et al, 1997; Weiss et al, 1996). In the presence of epidermal growth factor (EGF) and/or basic fibroblast growth factor (bFGF), NSCs grow as monolayer cultures or in 3-dimensional aggregates, so-called neurospheres. A variety of serum-free culture conditions used for the *in vitro* expansion of NSCs have been reported. The combination of Dulbecco's modified essential medium with Ham's F-12 (DMEM/F12) supplemented with either N2 or B27 is widely used for the culture of NSCs (Gage et al, 1995b; Reynolds et al, 1992; Svendsen et al, 1995). These protocols, however, require (i) the initial presence of serum, (ii) preconditioned medium from already established cultures, or (iii) high cell densities, ie, ranging from 100 cells/ μ l up to 20,000 cells/ μ l (Carpenter et al, 1999; Gage et al, 1995a; Geschwind et al, 2001; Johansson et al, 1999b; Palmer et al, 1995, 1999, 2001; Shetty and Turner, 1999; Svendsen et al, 1997; Taupin et al, 2000). Because future clinical applications must be restricted to cells produced under defined conditions, the use of serum or conditioned media is incompatible with the use in humans, and therefore, there is an imperative need for improved cell culture protocols for adult NSCs.

When monolayers of NSCs reach confluency or when neurospheres reach a diameter hindering sufficient central nutrient supply, passaging of cells is required. Current methods use (i) trituration or mechanical dissociation, resulting in mixtures of single cells and small neurospheres, or (ii) enzymatic dissociation using the protease trypsin. The latter is used when single-cell suspensions for clonal growth of NSCs are required, but unfortunately results in significant cell loss (Svendsen et al, 1998).

Here we provide a protocol for increased cell survival and efficient regrowth of dissociated multipotent neurospheres. Moreover, and in contrast to currently used culture conditions for adult NSCs, we describe methods for efficient NSC expansion under clonal and low-density conditions.

Results

Optimization of Neurosphere Culture Conditions

Passaging Methods. In this study, we used NSCs from the adult rat subventricular zone (SVZ), hippocampus (HC), and spinal cord and human neural precursor cells (hNPCs) isolated from an 8-week post-

conception forebrain (Svendsen et al, 1999). NSCs and hNPCs were maintained in suspension cultures in which they grew as aggregates referred to as neurospheres. After a growth phase of typically 1 to 2 weeks, neurospheres became too large to allow for proper nutrient supply in the central part of the cell mass and needed to be dissociated. Hence, the first parameter investigated in this study was the passaging process. Two different dissociation methods are commonly used: (i) enzymatic treatment using trypsin or (ii) sectioning of spheres, also referred to as "chopping." The latter maintains cell-cell interactions and results in faster recovery and higher expansion rates (Svendsen et al, 1998). However, this method is not appropriate when single cells are required (Shetty and Turner, 1998; Shihabuddin et al, 1997; Svendsen, 1995, 1998). Because dissociation of neurospheres using trypsin provokes massive cell death, we tested Accutase, a different enzyme preparation that is proposed to be less aggressive than trypsin (see <http://www.innovativecelltech.com/accutase.html>).

To compare the effects of the two different enzyme preparations, NSCs from passage number 10 and hNPCs were plated at low density (10 cells/ μ l) and allowed to grow for 7 days (Fig. 1a). The resulting neurospheres were then dissociated into single cells using trypsin or Accutase and reseeded. The single-cell suspension was grown for an additional 4 days. At the end of the experiment, the total numbers of neurospheres and cells were determined. One day after enzymatic treatment, a reduced cell number was observed in rodent cultures dissociated with trypsin as compared with Accutase-treated or untreated cultures (Fig. 1b). As opposed to trypsinized cells, Accutase-treated cultures readily formed spheres within 24 hours (Fig. 1b). Compared with untreated control cultures, Accutase dissociation increased by 3-fold the number of spheres by the end of the experiment (Fig. 1, b and f). Starting with equal cell numbers (10^4 cells in 1 ml), the initial cell survival after Accutase treatment was significantly higher (90% to 95%) compared with trypsin (70 to 80%) (data not shown). This enhanced survival was observed in NSC cultures derived from the different CNS regions used. Within 4 days after dissociation, a large proportion of the remaining cells in trypsinized rodent NSC cultures died (Fig. 1c). In contrast, dissociation using Accutase not only increased the initial number of viable cells but also significantly improved the survival of cells. As a consequence, an increase in total cell and sphere numbers was measured in Accutase-treated cultures (Fig. 1, b and d). In the case of fetal hNPCs, initial cell survival and final cell number were higher using Accutase dissociation (Fig. 1e). The number of newly formed hNPC neurospheres was not quantified, because cells formed a monolayer after dissociation (not shown). To exclude the possibility that the effects observed are not specific to the different enzyme compositions, we performed a series of dissociation and passaging experiments using different enzyme concentrations and incubation times. The results consistently demonstrated that at lower concentra-

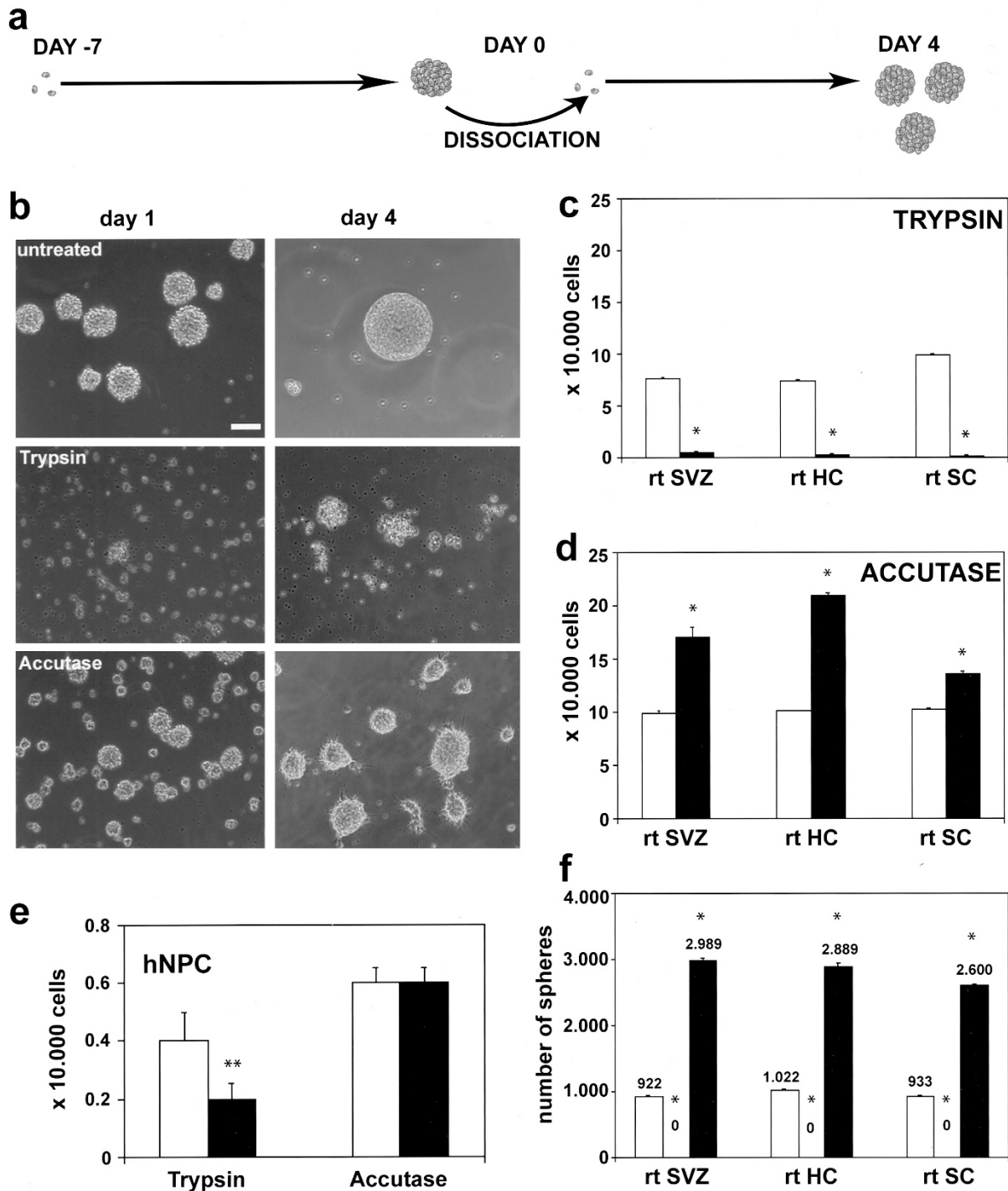


Figure 1.

Comparison of trypsin and Accutase dissociation of neural stem cell (NSC) neurospheres. **a**, Schematic representation of the dissociation paradigm (see “Materials and Methods”). **b**, Phase contrast micrographs of untreated (top), trypsinized (middle), and Accutase-treated (bottom) rat subventricular zone (SVZ) neurosphere cultures on Day 1 (left) and Day 4 (right) after dissociation ($\text{bar} = 100 \mu\text{m}$). **c**, Quantification of the total number of viable cells from adult rat SVZ (rt SVZ), hippocampus (rt HC), and spinal cord (rt SC) immediately after trypsinization (white bars) and 4 days after dissociation (black bars). **d**, Quantification of the total number of viable cells from SVZ, HC, and spinal cord, immediately after Accutase treatment (white bars) and 4 days after dissociation (black bars). **e**, Total number of viable human neural precursor cells (hNPCs) immediately after dissociation (white bars) and 4 days after dissociation (black bars) using trypsin or Accutase. **f**, Number of newly formed neurospheres on post-dissociation Day 4 in untreated cultures (white bars), trypsinized cultures ($n = 0$; gray bars), and Accutase-treated cultures (black bars). Viable cells were determined by trypan blue exclusion test. Data were generated using adult rat NSCs from passage number 10. The data are expressed as average \pm SD from three experiments performed in triplicate. Significance values according to Student's *t* test are * $p < 0.003$ and ** $p < 0.02$.

tions, trypsin is insufficient to generate single-cell suspensions, and at higher concentrations, it results in low survival of cells and in insufficient regrowth of

neurospheres (not shown). In addition, other enzymes such as thermolysin, collagenase II, or the enzyme composition PPD (see “Material and Methods”) were

Table 1. Effect of the Medium Composition on Number of Primary Neurospheres

Number of cells seeded/ml	Number of neurospheres in	
	NB/B27	DMEM/N2
rtSVZ		
10,000	4.7 ± 0.9	1.0 ± 0.0
50,000	20.0 ± 0.8	1.0 ± 0.0
100,000	52.7 ± 4.1	3.3 ± 0.5
rtHC		
10,000	4.3 ± 1.2	1.0 ± 0.0
50,000	39.7 ± 1.2	1.0 ± 0.0
100,000	103.3 ± 4.0	4.0 ± 0.0

Quantification of total number of primary neurospheres from adult rat SVZ grown in Neurobasal medium supplemented with B27 (NB/B27) or DMEM/F12 medium supplemented with N2 (DMEM/N2) at different densities: 10 cells/ μ l, 50 cells/ μ l, and 100 cells/ μ l. The data are expressed as average \pm sd.

insufficient to yield good cell dissociation and survival at the same time (not shown).

Composition of Culture Media. To assess the influence of culture medium compositions on adult rat NSC and hNPC proliferation, neurospheres from passages 3 to 20 were dissociated and seeded as single cells in six different media/supplement combinations at low density (10 cells/ μ l). After a growth cycle of 7 days in the presence of EGF and bFGF, the total cell number in the culture was quantified. We compared cell proliferation rates obtained in DMEM/F12, the most prevalent NSC culture medium, with rates obtained in Neurobasal (NB) medium, a medium previously described for the maintenance of differentiated neurons (Brewer et al, 1994). These media were completed with three different supplements: B27, BIT9500, and N2 (Table 2). N2 was traditionally used for NSC cultures, whereas B27 was described originally for the maintenance of primary neurons in culture (for examples, see Palmer et al, 1995; Svendsen et al, 1995). Finally, BIT9500 was initially used for hematopoietic stem cell cultures (Hogge et al, 1997) and recently applied on adult human NSCs (Palmer et al, 2001).

The different media/supplement combinations significantly influenced cell expansion rates. NSCs readily grew spheres in NB/B27 medium (Fig. 2, a and c), resulting in a high expansion rate of 10- to 15-fold within 7 days (Fig. 2b). In media supplemented with BIT9500, cells were able to re-form spheres; however, the total number of cells did not increase within 7 days, whereas in the other media, the total number of cells decreased within the same period of time (Fig. 2, a to c). As shown in Figure 2, b and c, the number of neurospheres paralleled the total number of cells counted. Human NPCs showed increased expansion rates in NB/B27 and DMEM/F12/BIT9500 (Fig. 2d).

Establishment of Primary Neurospheres. The next set of experiments tested whether the growth-promoting effect of NB/B27 on already-established neurosphere cultures was also applicable on freshly isolated NSCs. After dissociation of adult rat HC and

SVZ, the number of viable cells was estimated and seeded in NB/B27 or DMEM/F12/N2 at three different densities (10 cells/ μ l, 50 cells/ μ l, and 100 cells/ μ l). Single cells began to form small spheres within 5 to 7 days of culture and grew in mass and number during the next several weeks. As presented in Table 1, the highest yields of primary neurospheres were obtained in NB/B27. The higher efficacy of NB/B27 compared with DMEM/F12/N2 was observed at the three cell densities investigated (Table 1).

Primary neurospheres were passaged using Accutase and further expanded in NB/B27. Both SVZ- and HC-derived NSCs yielded a 6000-fold expansion within 21 days (Fig. 3a). In addition, the proliferation rate for both cell types remained constant for at least 3 weeks of culture.

Single Cell Cloning. The future application of adult NSCs in autologous transplantation might require uniform cell material generated by single cell cloning. To achieve that, we plated Accutase-dissociated rat SVZ NSCs derived from established passage number 4 cultures in 96-well plates at a density of 0.5 cells/well by limited dilution. Comparison of the ability of six media/supplement combinations to promote neurosphere formation revealed significant differences. In agreement with the proliferation data on bulk cultures, B27-supplemented NB medium most effectively induced NSC proliferation under clonal conditions. After 6 weeks in culture, approximately 27% of the seeded cells produced new neurospheres (Fig. 3b). Albeit to a lesser extent, media supplemented with BIT9500 were also able to support substantial formation of neuro-

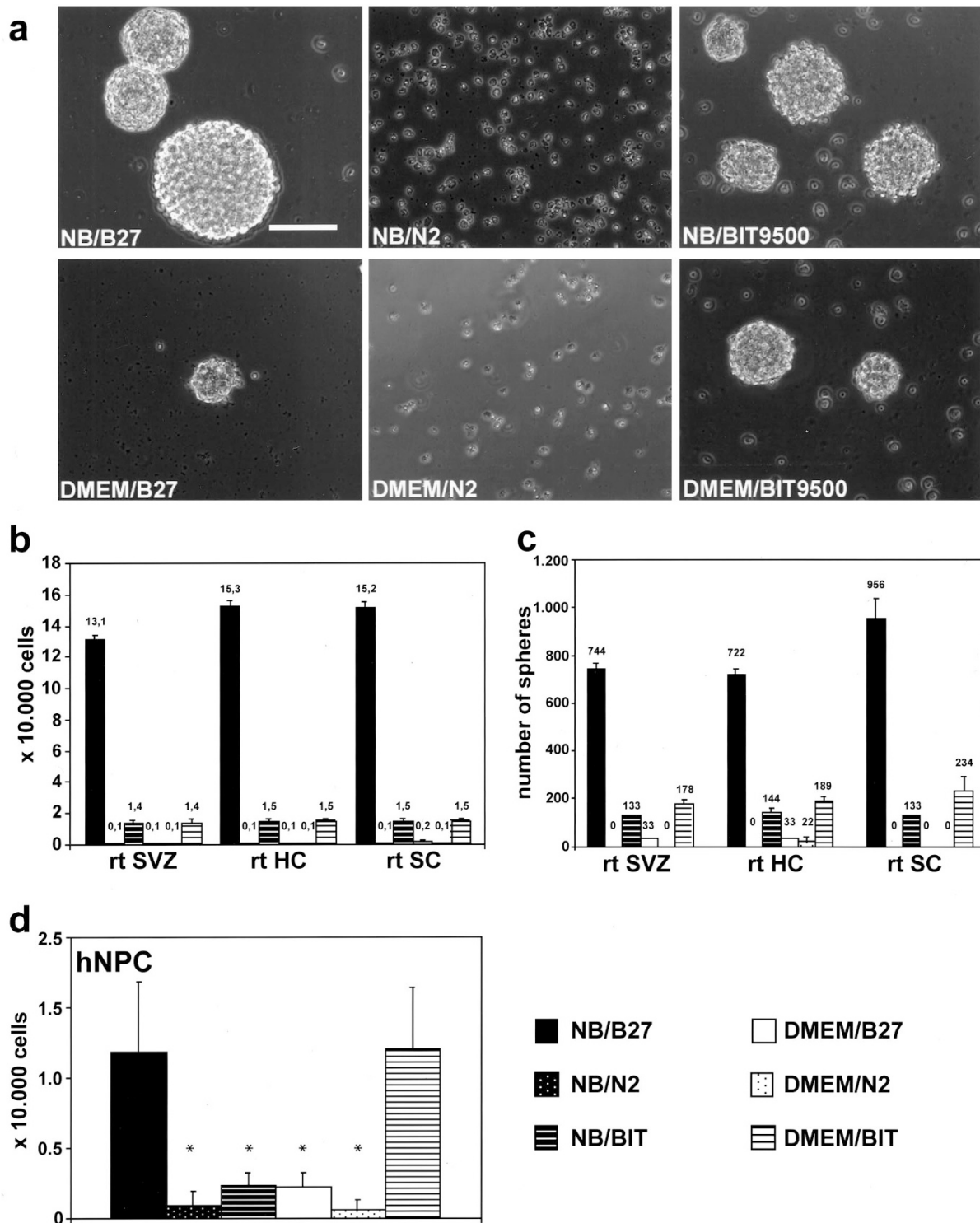
Table 2. Composition of B27, N2, and BIT9500 Supplements

	B27 ^a	N2 ^b	BIT9500 ^c
BSA	+	+	+
Transferrin	+	+	+
Insulin	+	+	+
Progesterone	+	+	—
Putrescine	+	+	—
Sodium selenite	+	+	—
Biotin	+	—	—
L-carnitine	+	—	—
Corticosterone	+	—	—
Ethanolamine	+	—	—
D(+)-galactose	+	—	—
Glutathione (reduced)	+	—	—
Linolenic acid	+	—	—
Linoleic acid	+	—	—
Retinyl acetate	+	—	—
Selenium	+	—	—
T3 (triiodo-L-thyronine)	+	—	—
DL- α -tocopherol (vitamine E)	+	—	—
DL- α -tocopherol acetate	+	—	—
Catalase	+	—	—
Superoxide dismutase	+	—	—

^a See Brewer et al, 1993.

^b Provided by manufacturer (Gibco BRL, Germany).

^c Provided by manufacturer (StemCell Technologies Inc., Canada).



spheres from single cells (4–10%). FACS sorting is a widely used method to isolate single cells. Therefore, we assessed the impact of FACS sorting on rat spinal

cord- and HC-derived NSCs that were dissociated and sorted into 96-well plates at a density of 1 cell/well in NB/B27. As with the limited dilution experiment,

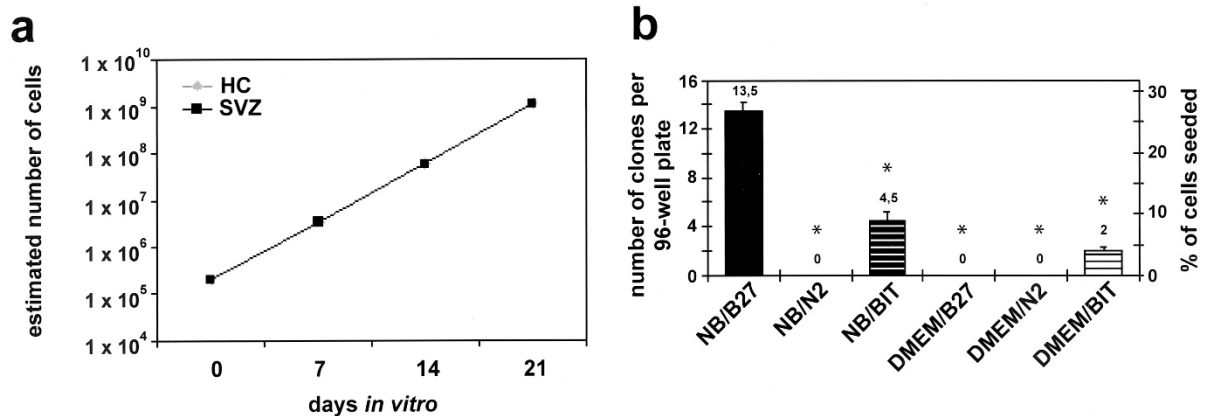


Figure 3.

Cloning efficiency in different media. a, Growth rates of rat SVZ and HC cells in NB/B27 for 21 days in which the total number of cells at each passage has been calculated. Every 7 days adult rat SVZ neurospheres were dissociated and reseeded with a density of 20 cells/ μ l (200,000 cells per flask). b, Quantification of total number of clonally derived neurospheres of rat ventricle wall cells (SVZ) in different media 6 weeks after seeding. Adult rat SVZ neurospheres were dissociated and reseeded in the different media: NB medium supplemented with B27 (black bars), N2 ($n = 0$; black dotted bars), or BIT9500 (black striped bars) and DMEM/F12 medium (DMEM) supplemented with B27 ($n = 0$; white bars), N2 ($n = 0$; white dotted bars), or BIT9500 (white striped bars) by limiting dilution with a density of 0.5 cells/well. All wells were screened for single cells by phase contrast microscopy, and only wells with 1 single cell were considered to be clonal. The data are expressed as average \pm SD from two experiments. Significance values according to Student's *t* test are * $p < 0.0001$ related to cells grown in NB/B27.

25% to 30% of the plated single cells formed neurospheres (not shown).

Differentiation of NSCs: Influence of Medium Composition on NSC Potential

To determine the differentiation status of NSCs under proliferation conditions, clonally derived rat neurospheres from passage 14 after cloning were transferred onto glass coverslips coated with poly-ornithine and laminin and cultured in NB/B27 in the presence of EGF and bFGF. Within a few hours of incubation on the poly-ornithine/laminin matrix, neurospheres became adherent and cells started to migrate out and formed a surrounding monolayer (Fig. 4, a and b). Two days after plating, cells were fixed with 4% paraformaldehyde and processed for immunohistochemistry. Immunodetection of the Ki67 antigen, which is detected in the nucleus during the G1, S, G2, and M phases of the cell cycle, was used to assess proliferation in adherent cultures. As shown in Figure 4, c and d, Ki67 could be detected, suggesting that cultures were still in a proliferative state. In addition, a subpopulation of rat cells was positive for nestin (Fig. 4e), an intermediate filament protein found in noncommitted NSCs (Lendahl et al, 1990), whereas every cell except differentiated young neurons expressed nestin in the hNPC culture (Fig. 4f). To detect neuronal committed cells, we used antibodies directed against β III-tubulin, an early neuronal-specific tubulin isotype (Laferriere and Brown, 1996). In rodent NSC cultures, no β III-tubulin-positive cells were detected under proliferative conditions, whereas in the hNPC cultures, some β III-tubulin-positive cells were observed (Fig. 4f). The detection of glial fibrillary acidic protein (GFAP), an intermediate filament found in astrocytes, was rare in rat NSCs, in hNPC cultures; however, every cell except a few β III-tubulin-positive cells,

expressed GFAP (not shown). Finally, consistent with other reports (Palmer et al, 1997), some rat cells under proliferative conditions were immunoreactive for galactocerebroside (GalC), an oligodendrocytic marker. No GalC-positive cells could be detected in the hNPC cultures used in this study.

In the next set of experiments, we tested clonally derived rat SVZ NSCs grown in NB/B27 for their differentiation potential. NSCs were plated onto poly-ornithine/laminin-coated glass coverslips in NB/B27 without growth factors but in the presence of 1% serum, a standard paradigm used to induce differentiation for NSCs (Arsenijevic et al, 2001; Gage et al, 1995b; Johansson et al, 1999b; Reynolds and Weiss, 1992). Two different SVZ clones were analyzed by immunofluorescence staining for β III-tubulin, GFAP, and GalC. All three markers were expressed in the cultures (Fig. 5, a to c), indicating that the original clones were multipotent and that cells grown in NB/B27 retained multipotency. To test whether the different media/supplement combinations used in the proliferation tests affected neuronal and glial differentiation, NSC bulk cultures derived from rat SVZ and HC were differentiated as described above in the different media/supplement combinations. Cultures were analyzed by immunostaining for β III-tubulin and GFAP. Quantitative analysis revealed that 10% to 33% of cells expressed β III-tubulin, between 5% and 18% of cells were immunoreactive for GFAP, and 0.5% to 1% of cells stained for GalC (Fig. 5d). In HC-derived cultures grown in DMEM medium, there was a tendency toward neuronal versus a glial differentiation; however, this effect was not observed in SVZ-derived cultures. A similar amount of cells survived the differentiation condition in the different media/supplements (not shown). Therefore, selective cell death of one or more cell types is not likely.

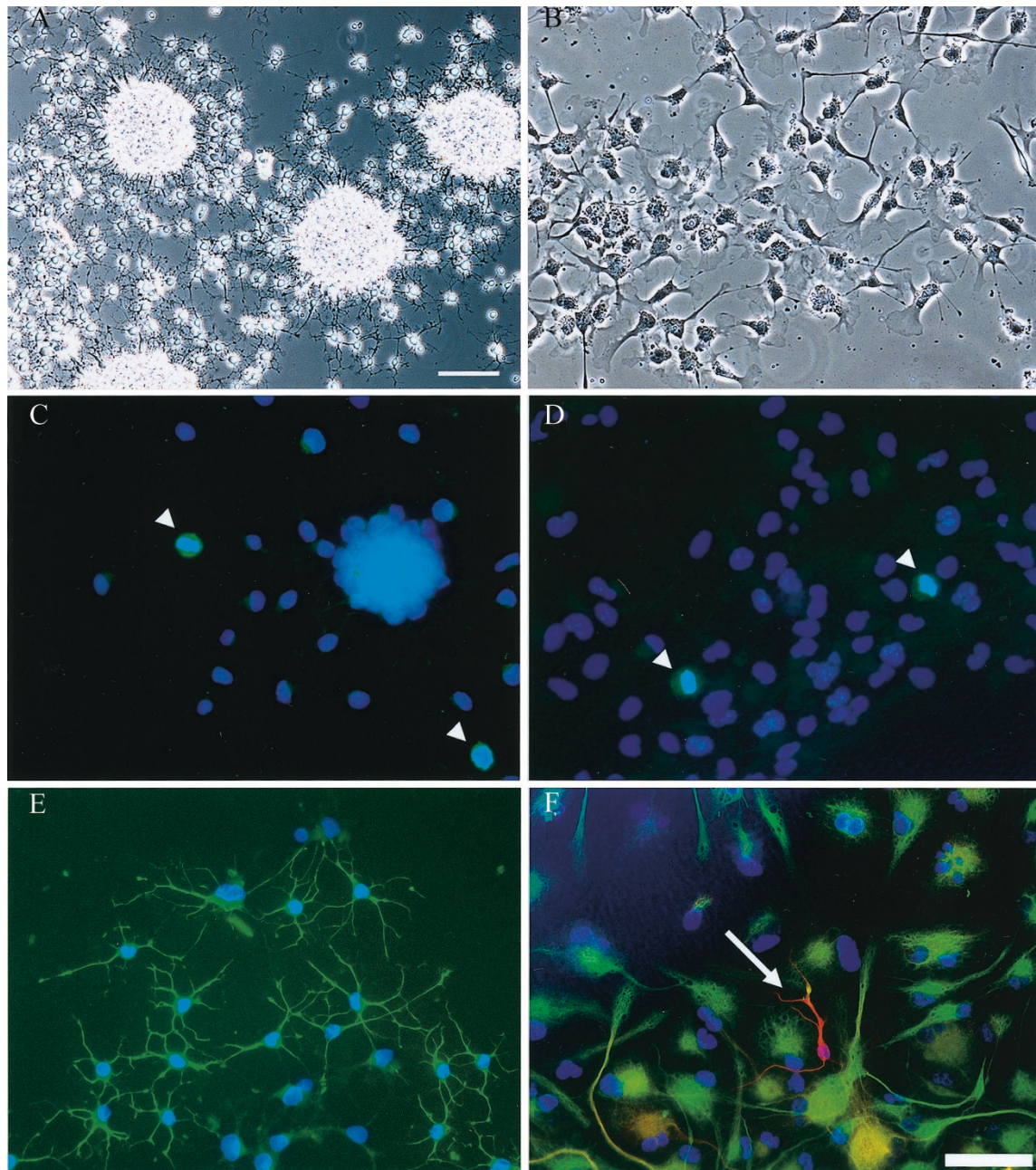


Figure 4.

Immunocharacterization of NSC cultures. Phase contrast micrographs of rat SVZ (a) and hNPC (b) neurospheres plated for 2 days. Immunodetection of the Ki67 antigen (green, *arrowheads*) in rat SVZ (c) and hNPC (d) cultures plated for 2 days. Nuclei were counterstained using DAPI (blue). Immunodetection of nestin (green) in rat SVZ (e) and hNPC (f) cultures plated for 2 days. In hNPC cultures, nestin expression was detected in every cell with the exception of β III-tubulin-positive cells (red, *arrow*) (*bar* = 50 μ m).

Adult Rat NSCs Retain Normal Diploidy and Do Not Transform In Vivo

Risk assessment of tumor formation is crucial for the future clinical application of stem cells. In vitro, proliferating cells frequently escape from growth regulation as a result of genetic abnormalities that manifest in alterations including ploidy and karyotype changes. Adult rat NSCs used in this study possessed consistent morphology and growth characteristics throughout the entire period of analysis. Ploidy was tested by

FACS analysis of propidium iodide-stained DNA from proliferating NSCs from passage number 4 and compared with clonally derived NSCs from passage number 18. Cells from both cultures showed a propidium iodide FACS pattern typical for a diploid culture (Fig. 6a). In addition, the two FACS patterns were almost identical, indicating that chromosomal alterations detected by the propidium iodide FACS method can be excluded up to passage 18. To further investigate the genetic stability of long-term adult rat NSC cultures,

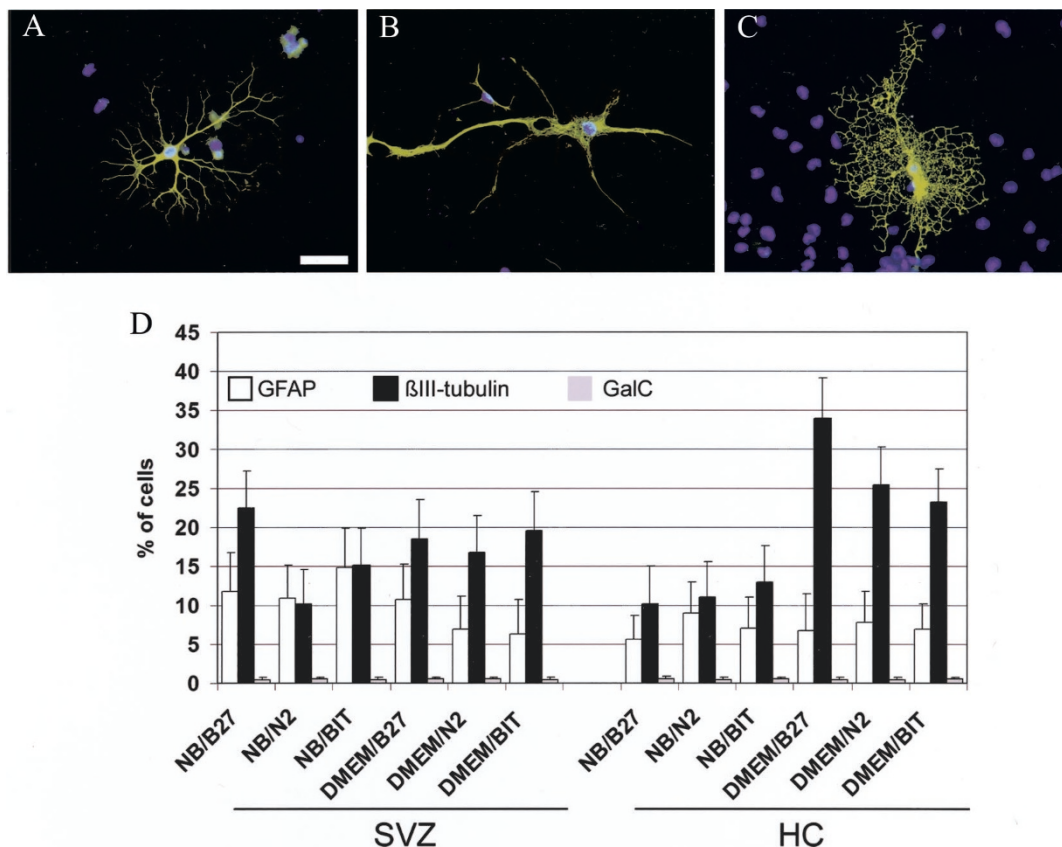


Figure 5.

Lineage potential of cloned rat NSC cultures. Individual clones derived from rat SVZ cultured on poly-ornithine/laminin matrix were differentiated in NB/B27 medium supplemented with 1% FCS for 7 days and immunostained for the presence of neurons with β III-tubulin (a), astrocytes with glial fibrillary acidic protein (GFAP) (b), or oligodendrocytes with galactocerebroside (GalC) (c) (bar = 40 μ m). Cells were cloned at passage number 4 and passaged an additional 14 times after cloning. d, Quantification of the number of GFAP-immunolabeled (white bars), β III-tubulin-immunolabeled (black bars), and GalC-immunolabeled (gray bars) cells detected 7 days after plating in different media/supplement combinations onto poly-ornithine/laminin matrix in the absence of growth factors and in the presence of 1% serum. NSCs were able to produce neuronal and glial cells in all media/supplement combinations used. The data are expressed as average \pm SEM.

karyotype analysis was performed on cells from passage number 18. As shown in Figure 6b, these cells contained 42 chromosomes typical for a female rat. Chromosomes had a normal band pattern without major alterations. The quantitative analysis of chromosomes from 50 metaphases revealed that 80% of metaphases had 42 chromosomes and only 20% had lost either one or two chromosomes. The integrity of the adult rat NSCs used in this study was further tested in vivo after transplantation into the adult rat brain. Here, 500,000 bromodeoxyuridine (BrdU)-prelabeled cells from passage number 18 were transplanted as spheres into the subventricular zone area. As a result, 3 weeks and 2 months later, almost all of the BrdU-positive transplanted cells disappeared from the site of transplantation and migrated out along the rostra-migratory stream into the olfactory bulb. Immunofluorescence analysis for Ki-67 and BrdU revealed that transplanted BrdU-positive cells were immunonegative for Ki-67, indicating that transplanted cells stopped to proliferate in vivo. This demonstrates the nontumorigenic feature of the transplanted rat NSCs used in this study.

Discussion

Standardized protocols for fast in vitro generation of a substantial amount of NSCs are crucial to the success for adult NSCs to provide a reliable source for nervous tissue for autologous transplantation therapies. Here, we describe (i) a protocol for increased cell survival and efficient regrowth of dissociated multipotent rodent and human neurospheres using Accutase instead of trypsin and (ii) high efficacy of NSC growth under clonal and low-density conditions independent of the presence of serum, conditioned medium, or factors like cystatin C.

Passaging of NSCs Using Accutase

NSCs are typically grown as monolayer cultures or in 3-dimensional neurosphere aggregates (Johansson et al, 1999a; Palmer et al, 1995; Reynolds and Weiss, 1992; Svendsen et al, 1998). After reaching a critical cell density or sphere size, or for example, when clonal analysis of NSCs is required, single-cell suspensions are generated by enzymatic treatment. Our compara-

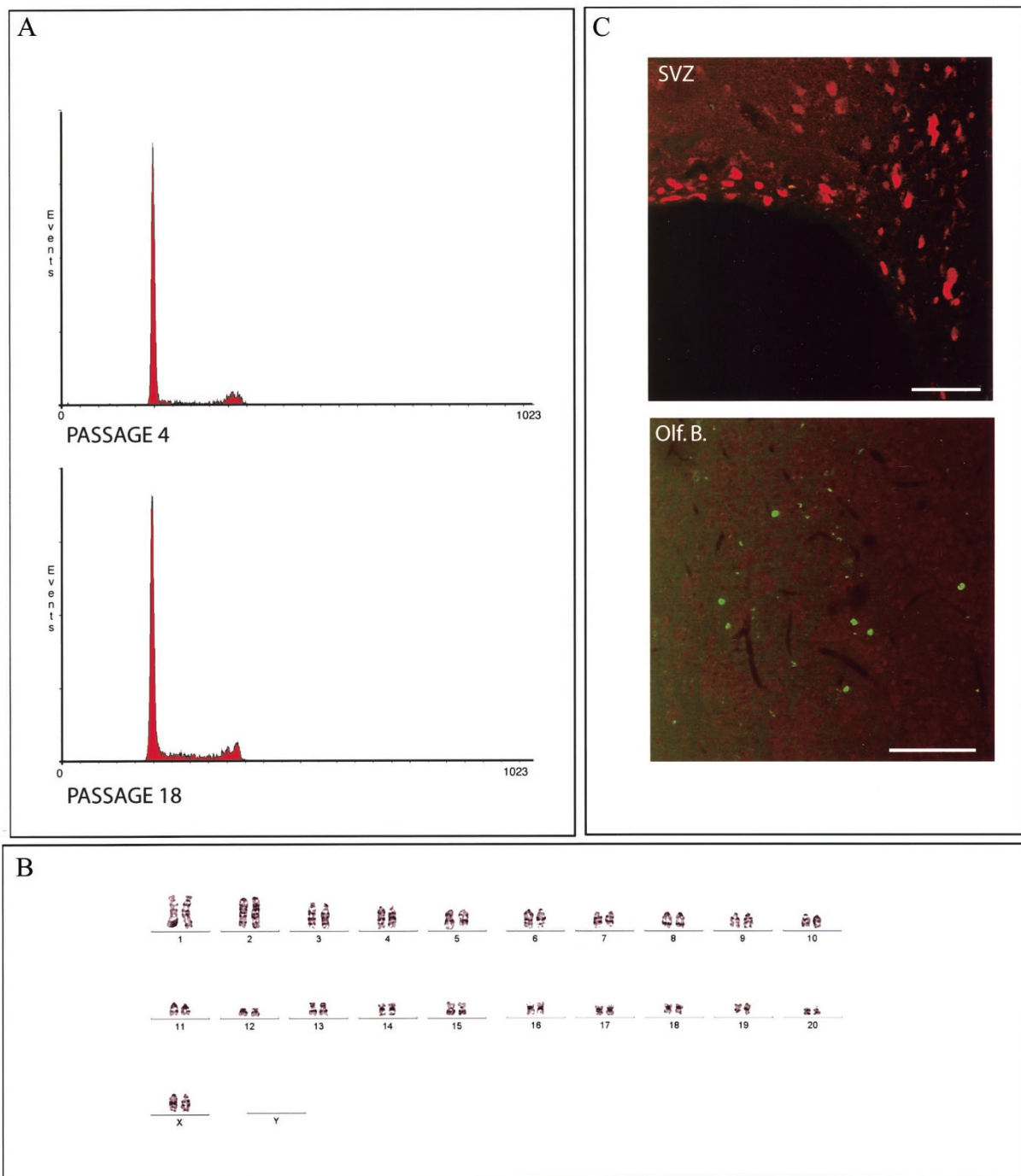


Figure 6.

Chromosomal stability and integrity of adult rat NSCs. Adult rat SVZ-derived NSCs from passages 4 and 18 analyzed for chromosomal content and cellular integrity. a, FACS analysis of propidium iodide-stained DNA from proliferating NSCs from passage number 4 and from clonally derived NSCs from passage number 18. Both cultures show a propidium iodide FACS pattern typical for a diploid culture. b, Karyotype analysis on adult rat SVZ-derived NSCs from passage number 18. c, Immunohistologic double-staining for Ki-67 (red) and BrdU (green) of the SVZ and the olfactory bulb (*Olf. B.*) 2 months after transplantation of BrdU-prelabeled NSCs from passage number 18 into the SVZ. No BrdU-positive transplanted cells are visible in the SVZ area, but Ki-67-positive endogenous proliferating precursor cells are detected in the SVZ. In the olfactory bulb, BrdU-labeled transplanted NSCs accumulate. Note that these cells are negative for Ki-67, indicating that they are not proliferating (*bar* = 50 μ m for SVZ and 100 μ m for *Olf. B.*).

tive analysis using two different enzymatic compositions, trypsin and Accutase, indicated higher acute cell viability and fast recovery of adult NSC spheres after Accutase dissociation. Accutase has been specifically developed for the preparation of cell samples for FACS analysis (<http://sciencepark.mdanderson->

[.org/flow/files/Aggregates.html](http://flow/files/Aggregates.html)). It is a proprietary formulation containing proteolytic and collagenolytic activities and was developed for rapid dissociation with high viability of cells (see <http://www.innovativecelltech.com/accutase.html>). Because the precise formulation of Accutase is not available, we can only spec-

ulate about the nature of this beneficial effect of Accutase. Despite its efficient cell dissociation activity, Accutase is less harmful to NSCs than trypsin, as suggested by the higher cell viability seen immediately after enzymatic treatment. The increase in the total numbers of cells and neurospheres observed 4 days after dissociation also reflects a higher survival of cells after Accutase versus trypsin dissociation. Because the integrity of cell surface markers is crucial for antibody binding and FACS analysis, it is plausible that Accutase does not degrade membrane bound receptors, such as EGF and bFGF receptors, as extensively as trypsin. Alternatively, Accutase might be less destructive to cell adhesion molecules required for neurosphere formation. A deleterious effect by trypsin was also reported after dissociation of primary olfactory receptor neurons and human tumor xenografts (Allalunis-Turner and Siemann, 1986; McEntire and Pixley, 2000). A more gentle and alternative approach to trypsinization for passaging the neurosphere cultures is the "chopping" method, described for human fetal hNPC spheres (Svendsen et al, 1998). However, this method is not applicable when single-cell suspensions are required, eg, for clonal analysis.

Media Supplements Differentially Affect Growth Rates

Current techniques used for the growth of NSCs in neurospheres or as monolayers are based on serum-free DMEM/F12 medium supplemented with N2, B27, or BIT9500 (Gage et al, 1995b; Palmer et al, 2001; Reynolds et al, 1992; Seaberg and van der Kooy, 2002; Svendsen et al, 1995). DMEM/F12 is supportive for the growth of NSCs under high cell density conditions ranging from 20 cells/ μ l to 5×10^4 cells/ μ l (Carpenter et al, 1999; Johansson et al, 1999b; Seaberg and van der Kooy, 2002). It is, however, insufficient for the growth of NSCs under low-density or clonal conditions and requires conditioned medium (Palmer et al, 1999) or additional compounds such as cystatin C (Taupin et al, 2000).

We describe media/supplement combinations that allow for high efficacy of NSC growth under clonal and low-density (10 cells/ μ l) conditions independent of the presence of serum or conditioned medium. hNPCs proliferated best in NB/B27 and DMEM/F12 medium supplemented with BIT9500. Rat NSCs derived from SVZ, HC, or spinal cord showed the highest proliferation rates when grown in NB/B27 medium. Similar results were obtained with NSCs derived from adult mouse neurogenic regions SVZ and HC (not shown). The expansion in rat NSC number in NB/B27 (6000-fold within 21 days) represents a higher expansion rate when compared with previously established NSC culture protocols (Kallós and Behie, 1999; Palmer et al, 2001). In addition, our data showed high efficacy of clonal growth of single NSCs in a serum-free medium, NB/B27. This represents a major advantage for future cell therapy and transplantation experiments. Despite the high expansion rate, in vitro-propagated NSCs did not alter their chromosomal content and did form

tumors 2 months after transplantation, indicating that these cells did not transform.

NB medium was originally described as a supportive medium for primary neuronal cultures (Brewer et al, 1993; Kivell et al, 2000), but so far has not been used for adult NSC cultures. In combination with the supplement B27, it has been described to be supportive for the maintenance of neurons derived from brain regions, including cortex, cerebellum, dentate gyrus, striatum, and substantia nigra (Brewer, 1995). B27 was first reported for the maintenance of embryonic hippocampal neurons and neuronal cell lines (Brewer et al, 1993). In addition to the Bottenstein and Sato N2 formulation (Bottenstein and Sato, 1979) of transferrin, insulin, putrescine, progesterone, and sodium selenate, it includes a range of substances (Table 2 and Brewer et al, 1993). As a supplement to DMEM/F12, B27 was described to promote the growth and expansion of freshly isolated rat embryonic precursor cells and of adult rodent NSCs (Seaberg and van der Kooy, 2002; Svendsen et al, 1995). The use of NB/B27 for cultures of adult NSCs, however, represents a new and highly efficient approach. The BIT9500 supplement, originally used for hematopoietic stem cell cultures (Hogge et al, 1997), has previously been reported to induce the growth of adult human progenitor cells isolated from postmortem tissue (Palmer et al, 2001). It is an efficient supplement for the growth of adult human NSCs; however, B27 in combination with NB was the most potent growth medium for adult rat NSCs.

Neuronal Differentiation of NSCs Grown in NB/B27

The expansion potential of a given cell culture is an important factor of NSC-based transplantation strategies. However, it is also crucial to determine the differentiation potential of the expanded cultures. We induced differentiation of NSC cultures by withdrawal of growth factors and addition of 1% serum, which was sufficient to induce differentiation in the rodent NSC cultures. In contrast, the hNPCs remained refractory to this approach and only a fraction of cells differentiated (not shown). Stronger differentiation stimuli, such as retinoic acid exposition or prolonged differentiation period, may thus be required. Nevertheless, in the subpopulation that underwent differentiation in the hNPCs, we observed some neurons and astrocytes, but we did not detect the expression of GalC, an antigen specific for oligodendrocytes. This is in agreement with previous reports showing that only young human NSC cultures from the embryonic forebrain can generate oligodendrocytes (Murray and Dubois-Dalcq, 1998; Svendsen et al, 1998).

Our observations using different media/supplement combinations during differentiation of adult rat NSCs suggest that all media/supplements used including NB/B27 were able to allow neuronal and glial differentiation. Furthermore, the neuron/glia ratio generated upon differentiation is not massively influenced by the different combinations. Despite the high expansion rate using NB/B27, the results demonstrate that adult

rodent-derived cultures retained multipotency and could generate the three main neural cell types: neuron, astrocyte, and oligodendrocyte. However, the designation of "neuron," based on the detection of β III-tubulin, should be taken with caution. Although the expression of β III-tubulin implies a certain degree of cell maturation toward the neuronal phenotype, other neuronal markers, such as neurofilament proteins and neurotransmitters, and the electrophysiological property of a cell seem more appropriate to qualify a cell as "differentiated neuron."

In conclusion, we demonstrate that the media and supplements currently used for *in vitro* expansion of NSCs promote significantly different rates of proliferation. Optimization of the growth conditions allowed for the expansion of cultures derived from single cells without the use of serum or conditioned medium. It is noteworthy that the different medium compositions did not alter the differentiation potential of adult NSCs.

Materials and Methods

Primary Cell Cultures for Growing Neurospheres of Stem Cells from Adult Hippocampus, Lateral Ventricle Wall, and Spinal Cord

Adult female Fischer-344 rats (3–4 months; Charles River, Germany) were killed, and brains and spinal cords were removed and put in 4° C Dulbecco's phosphate buffered saline (DPBS) (PAN, Germany) with 4.5 gm/L glucose (Merck, Germany) (DPBS/glu). Overlying meninges and blood vessels were removed. HC and ependymal zones, including subependymal and subventricular zones from the lateral wall of the lateral ventricle (SVZ), were aseptically removed. The dissected tissue was transferred to fresh DPBS/glu, washed once, transferred to Petri dishes, and dissociated mechanically. The cell suspension was washed in DPBS/glu to rinse off excess blood and resuspended in PPD solution containing 0.01% papain (Worthington Biochemicals, England), 0.1% dispase II (Boehringer Mannheim, Mannheim, Germany), 0.01% DNase I (Worthington Biochemicals), and 12.4 mM MgSO_4 in HBSS (PAN) without $\text{Mg}^{2+}/\text{Ca}^{2+}$ (PAA, Germany) and digested for 30 to 40 minutes at room temperature. The cell suspension was triturated every 10 minutes. Dissociated cells were collected and resuspended in serum-free DMEM/F12 medium containing 2 mM L-glutamine and 0.1 gm/L penicillin/streptomycin and washed three times with accurate trituration. Finally the single-cell suspension was resuspended in NB medium (Gibco BRL, Germany) supplemented with B27 (Gibco BRL) (NB/B27), 2 mM L-glutamine (PAN), 0.1 gm/L penicillin/streptomycin (PAN), 2 $\mu\text{g}/\text{ml}$ heparin (Sigma, Taufkirchen, Germany), 20 ng/ml bFGF-2 (R&D Systems, Germany), and 20 ng/ml EGF (R&D Systems). Viable cells were counted by trypan blue exclusion assay in a hemocytometer. Cells were seeded in T-25 culture flasks and cultures were maintained at 37° C in an incubator with 5% CO_2 . Single cells began to form spheres within 5 to 7 days of suspension culture and continued to grow

in mass and number during the next weeks. Half of the medium was changed every 7 days. Cells from passage numbers 3 to 20 were used for the experiments. hNPCs were generated and maintained as described (Svendsen et al, 1998).

Passaging of Cells

The culture medium containing floating neurospheres was collected in a 15-ml centrifuge tube and centrifuged at 120 rcf for 5 minutes. The pellet was resuspended in 200 μL of Accutase (Innovative Cell Technologies Inc., distributed by PAA) and triturated approximately 10 times using a pipette. Then, the cell suspension was incubated at 37° C for 10 minutes. Dissociated spheres were again triturated and resuspended in 800 μL of NB/B27 medium. Dissociated cells were centrifuged at 120 rcf for 5 minutes and resuspended in NB/B27 medium. An aliquot was counted by trypan blue exclusion assay in a hemocytometer to determine the amount of viable cells. Cells (10^5) were plated in T75 culture flasks for long-term passaging (10 ml of culture medium per flask) in NB/B27 medium. The cells obtained after Accutase treatment of primary neurospheres proliferated and yielded secondary neurospheres. Secondary neurospheres were passaged 7 to 9 days after plating primary neurosphere cells. Similar to primary cultures and primary neurospheres, single cells obtained after dissociation of secondary neurospheres proliferated and yielded tertiary neurospheres. Rat NSCs used in this study were derived from cultures passaged between 3 and 20 times.

Dissociation Test

Cells (10^4) from either adult rat NSC, passages 3 to 20, or from hNPC cultures per well were seeded in 12-well plates in NB/B27 medium in a volume of 1 ml and were grown for 7 days. At Day 7 the number of neurospheres was determined; the culture medium, containing floating neurospheres, was collected and centrifuged at 120 rcf. The pellet was resuspended in either 200 μL of 0.05% trypsin (597 U/mg; PAN) or Accutase and triturated approximately 10 times using a yellow tip pipette. Cell suspension was incubated at 37° C for 10 minutes. Dissociated spheres were again triturated and resuspended in 800 μL of NB/B27 medium. Dissociated cells were centrifuged at 120 rcf for 5 minutes and resuspended in NB/B27 medium. An aliquot was counted by trypan blue exclusion assay in a hemocytometer to determine the amount of viable and dead cells. Cells were replated in 12-well plates in NB/B27 medium and grown for an additional 4 days. At Day 4 the number of spheres was counted before final Accutase treatment of the cells. Viable cells were counted by trypan blue exclusion.

Proliferation Test

Various media and supplements were used to study the most optimal conditions for adult rat NSC cultures, passages 0 to 20, and for hNPC cultures. NB medium

(Gibco BRL) or DMEM/F12 (1:1) medium (PAN, Germany) were either supplemented with B27 (Gibco BRL), N2 (Gibco BRL), or BIT9500 (StemCell Technologies Inc., Canada). Human low-density lipoproteins (Sigma) were added to the BIT9500-containing media at a final concentration of 40 $\mu\text{g}/\text{mL}$ according to the manufacturer's instruction. All media contained 2 mM L-glutamine, 0.1 gm/L penicillin/streptomycin, 2 $\mu\text{g}/\text{mL}$ heparin (Sigma), 20 ng/mL bFGF (R&D Systems), and 20 ng/mL EGF (R&D Systems). A total of 10^4 cells per well were seeded in 12-well plates in a volume of 1 mL and grown under standard conditions. At Day 7 the grown neurospheres were counted and dissociated by Accutase, and viable cells were counted by trypan blue exclusion assay in a hemocytometer.

Clonal Analysis

Seven-day-old neurospheres of passage number 4 were dissociated by Accutase, and the resulting single-cell suspension was used for clonal analysis. Single cells were transferred to 96-well plates either by limited dilution or by FACS sorting. For limited dilution experiments, single cells were plated at a density of 0.5 cells/well of 96-well plates; for FACS sorting experiments, individual cells were sorted (1 cell/well) in 96-well plates in 200 μL of growth medium. After the first 2 weeks, half of the media was changed; then the media change was performed weekly. After 6 weeks of culture, each well was manually screened for colonies using phase contrast microscopy, and only wells that originally contained one single cell were referred to as clones. Individual clones were used to establish clonal cell lines by dissociating the clonal neurospheres and replating the single cells under the same culture conditions. Cells were grown for an additional 14 passages and then analyzed for their differentiation potential.

Cell Differentiation

To analyze the influence of culture media on the differentiation potential of adult rat NSCs, single-cell suspensions were plated in the above-described combinations of media and supplements and submitted to differentiation-promoting conditions (growth factor withdrawal and 1% FCS) on poly-ornithine (250 $\mu\text{g}/\text{mL}$)- and laminin (5 $\mu\text{g}/\text{mL}$)-coated glass coverslips in 12-well plates at a cell density of 10^5 cells/well and grown for 7 days. In some experiments, neurospheres were plated on coated coverslips and grown for 2 or 7 days. Cells were fixed with phosphate-buffered 4% prewarmed paraformaldehyde (37° C, pH 7.4) (4% w/v paraformaldehyde, 100 mM NaH_2PO_4 , 0.4 mM CaCl_2 , 50 mM sucrose) for 30 minutes and processed for immunohistochemistry.

DNA Staining for FACS Analysis

NSC cultures were dissociated into single cells using Accutase according to the protocol described above. Cells were washed once with PBS, and the pellet was resuspended into 5 mL of ice-cold 70% ethanol and kept overnight at -20° C. The day after, cells were

washed twice with PBS and resuspended into 1 mL of PBS and 1 mL of 0.192 M Na_2HPO_4 , 4 μM citric acid, at pH 7.8. After 5-minute incubation at room temperature, cells were washed and resuspended into 1 mL of PBS containing 20 $\mu\text{g}/\text{mL}$ of propidium iodide and 10 $\mu\text{g}/\text{mL}$ of RNase A. After 30-minute incubation at room temperature, samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Germany). Data were processed with the WinMDI 2.8 software (J. Trotter, USA).

Chromosome Preparation

Cells were resuspended in 5 mL of RPMI/20% FCS (Biochrom AG, Berlin, Germany) and treated with 100 μL Colcemid (10 $\mu\text{g}/\text{mL}$) (Roche, Mannheim, Germany) for 120 minutes at 37° C and centrifuged. The cell pellet was suspended in warm hypotonic solution (75 mM KCl) for 20 minutes at 37° C, centrifuged, and fixed in a 3:1 mixture of methanol and acetic acid. Metaphase spreads were prepared on slides, dried, and Giemsa stained after pretreatment with trypsin.

Transplantation

NSCs from passage number 18 were incubated 48 hours before transplantation with 1 μM BrdU (Sigma), a proliferation marker in the growth medium. Before transplantation, neurospheres were cut into fragments (average size 200 μm) using a McIlwain Tissue Chopper (Mickle Engineering, Gomshall, United Kingdom). Neurospheres were washed twice and resuspended in PBS to yield a final concentration of 1.6 to 1.8×10^5 cells/ μL . The number of NSCs was estimated by dissociating three aliquots (100 μL each) of neurospheres with Accutase. The resulting single-cell suspension was stained with trypan blue (Sigma) and counted. Adult female Fischer-344 rats (3–4 months; Charles River) were deeply anesthetized using a mixture of ketamine (62.5 mg/kg; WDT, Garbsen, Germany), xylazine (3.175 mg/kg; WDT), and acepromazine (0.625 mg/kg; Sanofi-Ceva, Düsseldorf, Germany) in 0.9% sterile NaCl solution. All experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and institutional guidelines. The skin was opened above the skull by a mid-sagittal incision, and the underlying skull was opened above the ventricle wall. A total volume of 3 μL of cell suspension containing 1.8×10^5 cells/ μL was injected stereotactically directly into the area of the subventricular zone of the lateral ventricle through a pulled glass micropipette (200 μm internal diameter) using a Picospritzer II (General Valve, Fairfield, Illinois). At 3 weeks and 2 months after grafting, animals were perfused transcardially with 0.9% NaCl solution followed by 4% paraformaldehyde in PBS. The brains were dissected, immersed overnight in fixative, and transferred to 30% sucrose/100 mM phosphate buffer, pH 7.4, for at least 48 hours. Brains were cut into 40- μm sagittal sections using a sliding microtome. Sections were stored at -20° C in cryoprotectant solution until staining (25% v/v glycerol, 25% v/v ethylene glycol, and 0.05 M phosphate buffer, pH 7.4).

Immunohistochemistry

Cells. After 30 minutes of fixation at room temperature with phosphate-buffered 4% paraformaldehyde, samples were blocked for a minimum of 1 hour in fish skin gelatin buffer [0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 1% BSA, 0.2% Teleostean gelatin (Sigma), 0.1% Triton X-100] at room temperature. The specimens were incubated overnight at 4° C with the primary antibodies at the following dilutions: mouse anti-GalC 1:500 (Chemicon, Temecula, California); rabbit anti-GFAP 1:1000 (Dako, Denmark); rabbit anti-Ki67 1:500 (Novocastra, United Kingdom); rabbit anti-*nestin* 1:200 (Chemicon, USA); mouse anti-*nestin* 1:200 (PharMingen International, San Diego, California); mouse anti- β III-tubulin 1:500 (clone 5G8; Promega, Madison, Wisconsin); mouse anti- β III-tubulin 1:500 (clone TUJ1; Babco, USA). The secondary fluorochrome-conjugated antibodies were diluted 1:500 (donkey anti-mouse or rabbit; Dianova, Germany). All antibody dilutions and washes were performed with the fish skin gelatin buffer. Nuclear counterstaining was performed with 4',6'-diamidino-2-phenylindole dihydrochloride hydrate at 0.25 μ g/ μ l (DAPI; Sigma). After the last wash, the samples were briefly rinsed with PBS and mounted on slides using Prolong (Molecular Probes, The Netherlands). In cases in which antigens were sensitive to detergents (GalC), Triton X-100 was omitted from the fish skin gelatin buffer.

Tissue. To allow for a better penetration of antibodies in areas of high neuronal density, free-floating sections were placed in 1% Triton X-100/TBS (Tris-buffered saline: 0.1 M Tris-HCl, pH 7.4/0.9% NaCl) solution for 15 minutes followed by three consecutive 5-minute washes with TBS. For detection of incorporated BrdU, the sections were subjected to the following procedure: incubation in 0.3 M NaCl/30 mM citrate buffer, pH 7.0/50% (v/v) formamide at 65° C for 2 hours, rinse in 0.3 M NaCl/30 mM citrate buffer, pH 7.0, incubation in 2N HCl at 37° C for 30 minutes, rinse in 0.1 M borate buffer (pH 8.5) for 10 minutes, and rinse in TBS. Sections were blocked in TBS/3% donkey serum/0.1% Triton-X 100 (TBS-DS-TX) for 30 minutes, followed by incubation with primary antibodies in TBS-DS-TX for 48 hours at 4° C. The following primary antibody dilutions were used: rat anti-BrdU 1:500 (Accurate, Westbury, New York) and rabbit anti-Ki-67 1:500 (Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK). The sections were then rinsed in TBS three times for 10 minutes and incubated with secondary antibodies in TBS-DS-TX for 2 hours. The following fluorochrome-conjugated secondary antibodies were used: donkey anti-rat-FITC F(ab)₂ fragment and donkey anti-rabbit-rhodamineX F(ab)₂ fragment (all 2 μ g/ml; Jackson ImmunoResearch, West Grove, Pennsylvania). After several washes in TBS, sections were mounted on gelatin-coated glass slides and cover-slipped using Prolong (Molecular Probes).

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