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DIPLOMOVÁ PRÁCE

Intrinsic differences in stem/progenitor cells and their microenvironment in mice with increased neurogenic potential

Hradec Králové 2008 **Barbora Slabá**

„Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.“

I would like to thank Milos Pekny, MD, DrMedSc, Professor for being my supervisor and for giving me the opportunity to participate on the research in his group at Department of Clinical Neuroscience and Rehabilitation at Sahlgrenska Academy at Göteborg University.

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TABLE OF CONTENTS

1. INTRODUCTION

Throughout most of the twentieth century, neurobiologists believed that the mammalian adult nervous system could not generate new neurons. This belief was based on the absence of neurons with mitotic figures in the brains of adult birds and mammals, in general, and the absence of signs of regenerative neuronal proliferation following brain lesions or trauma in particular. The development of new techniques such as, 3H-thymidine autoradiography (Altman et al., 1965; Altman, 1969) or BrdU labeling, provided evidence that neurogenesis constitutively occurs in specific regions of the adult mammalian brain (Reynolds et al., 1992; Palmer et al., 1995).

The discovery of a population of multipotent, self-renewing cells in the adult CNS (Gage, 2000) and continued research has shown that neural progenitors, precursors and stem cells in the adult central nervous system (CNS) are capable of generating new neurons, astrocytes and oligodendrocytes. These findings bring with them future possibilities for the development of novel neural repair strategies for neurodegenerative diseases and chronic neurological or psychiatric disorders.

2. BACKGROUND

ABBREVIATIONS

bFGF - basic fibroblast growth factor
BL - basal lamina
BrdU - 5´bromodeoxyuridine
BV - blood vessels
CNS - central nervous system
Dapi - 4', 6-diamidino-2-phenylindole
DNAse - deoxyribonuclease
EDTA - ethylene diamine tetraacetic acid
EGF - epidermal growth factor
FBS- fetal bovine serum
FGF-2 fibroblast growth factor–2
GCL - granule cell layer
GFAP - glial fibrillary acidic protein
GV - $GFAP^{-/-}$ $Vim^{-/-}$
HBSS - Hanks Balanced Salts Solution
IF - intermediate filament
LV - lateral ventricle
ML - molecular layer
O4 - monoclonal antibody, oligodendrocyte marker
OB - olfactory bulb
P4 - postnatal day 4
PBS - phosphate buffer saline
PCR - polymerase chain reaction
PORN - poly-L-ornithine
RMS - rostral migratory stream
S100 - calcium binding protein, neuronal marker
SGZ - subgranular zone
SVZ - subventricular zone
Vim - vimentin
WT - wild type

2.1 DEFINING THE PRECURSOR CELL

The term neural precursor cell defines both stem and progenitor cells. These cells can be defined by their functional attributes. Several important characteristics distinguish stem cells from other types of cells:

- *Self-renewal* - the ability to go through numerous cycles of cell division while maintaining an undifferentiated state
- *Multipotency* - the capacity to differentiate into specialized cell types

While stem cells demonstrate unlimited self-renewal and multipotency, precursor cells have more limited self-renewal and are generally unipotent.

The two main types of mammalian stem cells are embryonic stem cells that are found in blastocysts and somatic stem cells, which are found in adult tissues. In the developing embryo stem cells differentiate into all of the specialized embryonic tissues. In contrast, in the adult, stem and progenitor cells act as a repair system for the body, replenishing specialized cells, but also maintain the normal turnover of regenerative organs, such as blood, skin or intestinal tissues.

2.2 CULTURING NEURAL STEM CELLS

Stem cell lines provide valuable research tools for general molecular and cellular neuroscience. Neural cell lines may be defined as clonally derived cells capable of indefinite replication in tissue culture and differentiation into neural cells (Gottlieb, 2002). The standard method of isolating neural stem cells in vitro is to dissect out a region of the fetal or adult brain that has been demonstrated to contain dividing cells in vivo. The tissue is dissociated and then single cells are cultured in a serum-free medium containing mitogens such as epidermal growth factor (EGF) (Reynolds et al., 1992), basic fibroblast growth factor (bFGF) (Gritti et al., 1996) or fibroblast growth factor–2

(FGF-2) (Uchida et al., 2000). Using these mitogens, cells can proliferate in vitro and remain multipotent (Gage, 2000; Carpenter et al., 1999).

Neural stem/progenitor cells can grow either in monolayers on substrate-coated tissue culture plates (Ray et al., 1993; Richard et al., 1992) or as self-adherent complexes of cells, forming clusters known as neurospheres (Reynolds et al., 1992; Carpenter et al., 1999). In both culture systems, these cells retain the capacity to produce the three main mature cell classes of the CNS: neurons, astrocytes and oligodendrocytes.

2.2.1 Neurospheres

Neurospheres continue to proliferate and form new spheres in secondary cultures after their mechanical dissociation and replacement as single cells in the presence of mitogens, demonstrative of self-renewal (Figure 1). When neurospheres are plated on adhesive substrates (poly-L-ornithine-coated coverslips) multipotent cells can migrate out from the neurosphere core and develop the morphological and antigenic properties of astrocytes, oligodendrocytes and neurons, a process called differentiation.

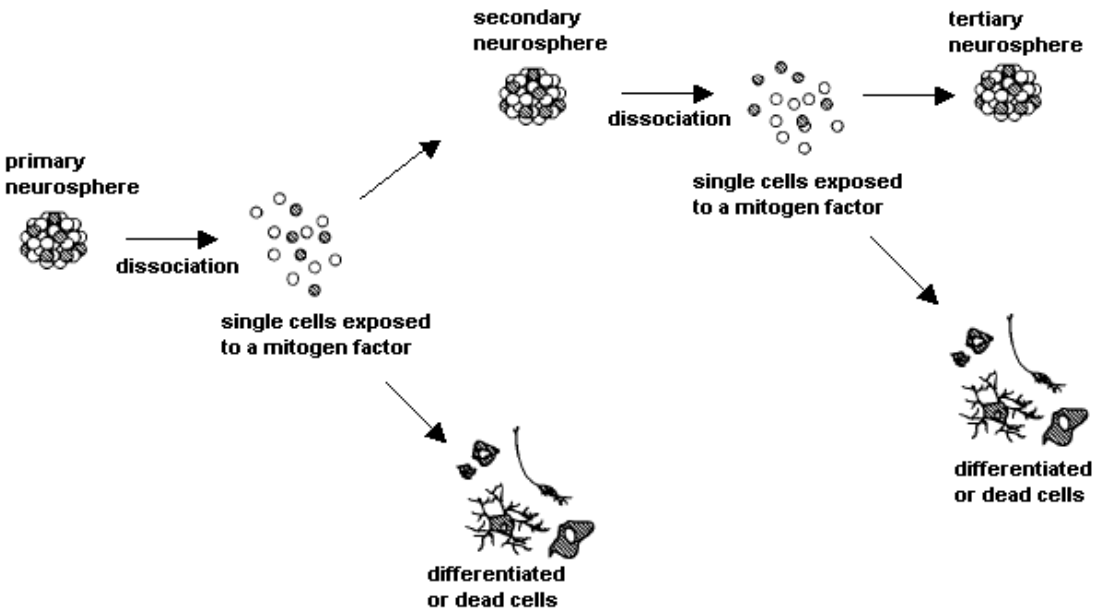

Figure1: Individual primary spheres are dissociated into single cells and replated under clonal conditions in bFGF-containing medium. A subset of cells proliferates and gives rise to secondary clonal spheres. Some cells differentiate and acquire the typical morphology of neuronal or glial cells or die. When this procedure is serially repeated cell proliferation still occurs. This type of experiment demonstrates the existence of pluripotent precursor cells that proliferate in the presence of bFGF and are capable of self-renewal over serial passaging in vitro. Taken and modified from: Gritti A. et al. (1996)

2.3 NEUROGENESIS IN THE ADULT CENTRAL NERVOUS SYSTEM

The process of generating functional neurons from progenitor cells is called neurogenesis. Precursor cells must take a number of steps towards maturation that include proliferation, neuronal fate specification, maturation and functional integration of neuronal progeny into neuronal circuits before they can be defined as newly-formed neurons.

Evidence of adult neurogenesis was first established by the identification of a population of multipotent neural stem cells. [^{3}H]-Thymidine incorporation studies in multiple animal models showed that proliferation persists in specific regions of the adult brain and suggested that these regions might contain immature progenitors of neurons or glia (Ihrie RA et al, 2008).

Two germinal regions or niches within the adult mammalian brain have been shown to contain neural progenitor cells: the subventricular zone (SVZ) along the walls of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus. Newly generated neuronal cells born during adulthood that integrate into neuronal circuits and survive to maturity are stable and may permanently replace nerve cells born during development (Dayer AG et al., 2003).

Neurogenesis may also occur in other areas of the adult mammalian brain, albeit at lower levels, such as the spinal cord, the neocortex, the striatum, the amygdala, and the substantia nigra in certain species, although some of these results have been contradicted by other studies (Palmer et al., 1995; Weiss et al., 1996; Zhao et al., 2003).

2.3.1 The subventricular zone

Proliferation in the SVZ takes place in the medial wall of the lateral ventricle (LV), where stem cells divide to generate transit amplifying cells, which, in turn, give rise to neuroblasts that migrate in the rostral migratory stream (RMS) to their final destination in the olfactory bulb (Figure 2). Several intrinsic factors expressed by stem cells and progenitors control both proliferation rates and the fate of newborn cells. After reaching the core of the olfactory bulb, cells detach from chains, invade the overlaying layers and differentiate into two local interneuron subtypes: granule cells and periglomerular neurons.

2.3.2 The subgranular zone

The second area of adult neurogenesis is the SGZ within the dentate gyrus of the hippocampus. Two populations of SGZ astrocytes, horizontal and radial, can be distinguished by their morphological characteristics. Radial astrocytes have a large cell body with a major radial process that penetrates the granule cell layer (GCL). These radial astrocytes have been identified as the primary SGZ progenitors. Horizontal astrocytes are generally elongated with thin lateral processes intercalated extensively between granule neurons. Both types of SGZ astrocytes first generate intermediate precursors (D cells), which later mature into new granule neurons (Seri et al., 2004) (Figure 3).

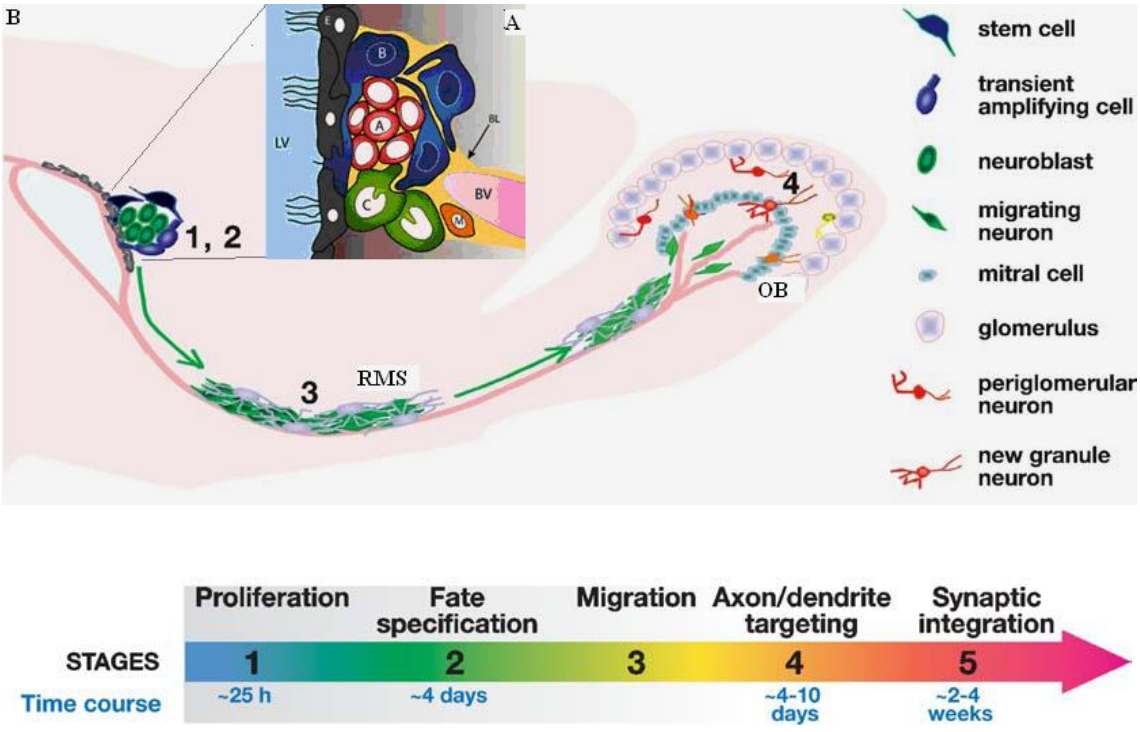

Figure 2:

(A): A detail of the subventricular zone (SVZ) in mouse brain. The SVZ contains slowly dividing astrocyte-like neural stem cells known as type B cells (shown in blue, B). These cells give rise to rapidly proliferating type C cells (C), which in turn give rise to immature neuroblasts, called type A cells (A) that migrate to the olfactory bulb. Adjacent ependymal cells (E) of the lateral ventricle are essential for neuronal fate specification by providing inhibitors of gliogenesis. The cells of the SVZ have extensive contact with the basal lamina (BL) and microglia (M) and also lie near blood vessels (BV).

(B): An illustration of four interneuron developmental stages: proliferation (1), fate specification (2), migration (3) and integration (4). Immature neurons (green) migrate with each other in chains through the rostral migratory (RMS) stream to the olfactory bulb (OB). The migrating neurons are ensheathed by astrocytes (3). Once reaching the bulb, new neurons then migrate radially to the outer cell layers (4).

Taken and modified from: Ming et al., 2005; Ihrie et al., 2008

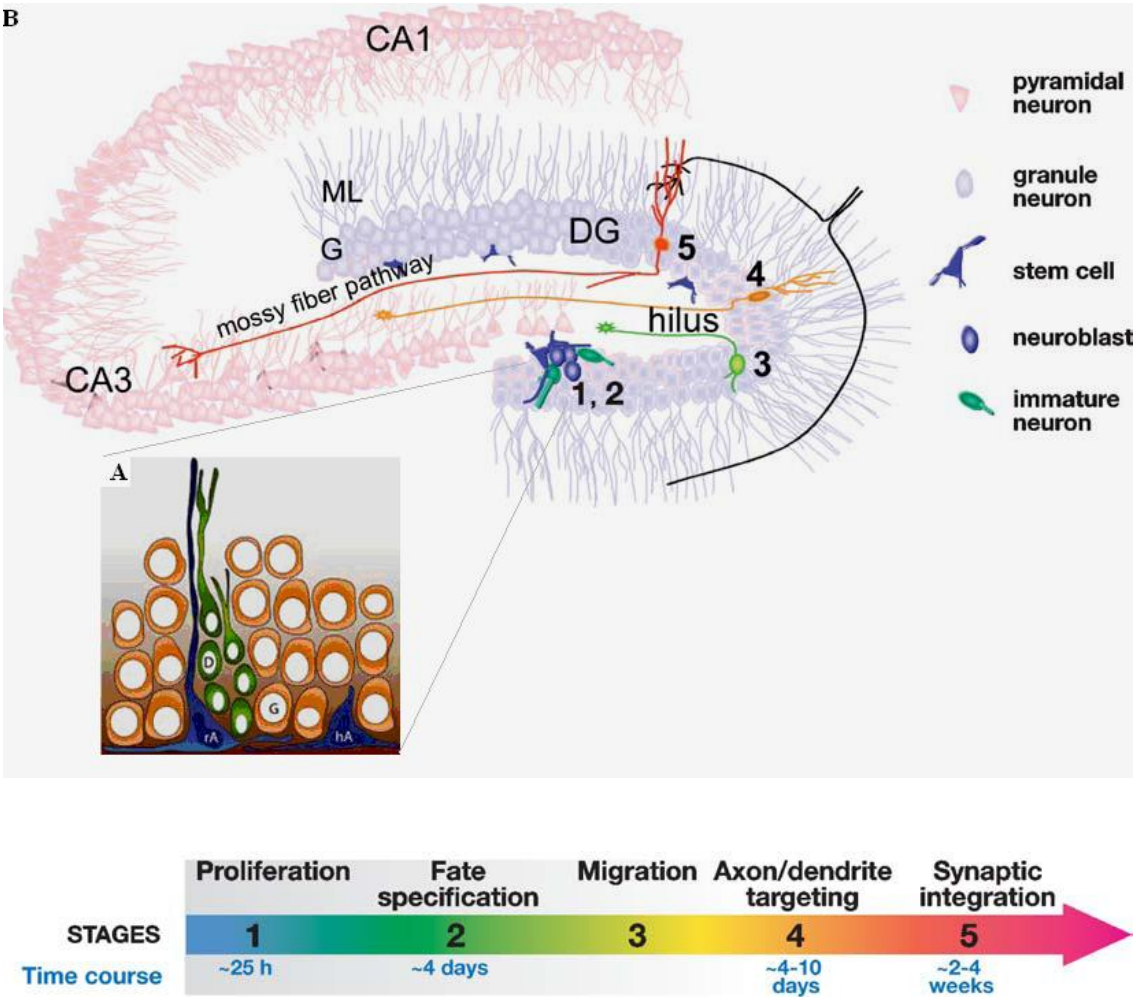

Figure 3:

(A): Model showing the organization of the SGZ. Astrocytes (blue) with their cell bodies located within the subgranular have radial processes (rA) that project through the granular cell layer and short tangential processes (hA) that extend along the border of the granule cell layer and hilus. D cells (green, D) form clusters and as cells mature, they move into the granular cell layer to reside next to older granule neurons (G).

(B): Generation of new granular neurons in the dentate gyrus of the hippocampus. Immature neurons (light green) migrate a short distance into the granule cell layer, G (3). Immature neurons (orange) extend their axonal projections along mossy fiber pathways to the CA3 pyramidal cell layer. They send their dendrites in the opposite direction toward the molecular layer, ML (4). New granule neurons (red) receive inputs from the entorhinal cortex and send outputs to the CA3 and hilus regions (5).

Taken and modified from: Ming et al., 2005; Zhao et al., 2003

2.4 FUNCTION OF NEUROGENESIS DURING ADULTHOOD

The function of the newly generated neuronal cells in the adult brain and the involvement of adult neurogenesis in the physiology and pathology of the CNS is the subject of intense research. Neurogenesis is modulated by a variety of environmental stimulus and pathophysiological conditions. Evidence suggests that newly generated neuronal cells participate in processes like learning and memory, but their contribution to these processes is still largely unknown.

New neuronal cells are also generated after damage at sites of injury and degeneration where they replace some of the lost nerve cell and participate in a regenerative attempt by the CNS (Taupin, 2006).

2.5 THE INFLUENCE OF THE ENVIROMENT/NICHE ON NEUROGENESIS

Multipotent stem cells can be isolated from many non-neurogenic regions, such as the adult spinal cord. Nevertheless active adult neurogenesis occurs only in two specific neurogenic niches: the subventricular zone and the hippocampal subgranular zone. These findings promote the idea that the local environment, in which the neural stem cells are found, may be the crucial for their differentiation potential. The most abundant cells in the CNS are astrocytes, they are known to support proliferation, survival and maturation of developing neurons and neuroblasts that have already committed to neuronal lineage (Song et al., 2002).

Astrocytes may play key roles in the generation of new neurons by providing an environment that is permissive to neurogenesis. An emerging picture of astrocytes suggests their control of the neurogenic niche and show that adult neurogenesis may be controlled by signals provided by regionally specified astrocytes in the adult brain. Astrocytes are involved in neuronal commitment decisions by progenitor cells (Song et al., 2002), direct migration and proliferation of RMS neuroblasts (Manson et al., 2001) and lastly, have been identified as the stem cell of the CNS (Doetsch et al., 1999).

More specifically, in Glial fibrillary acidic protein $^{-/-}$ Vimentin $^{-/-}$ (GFAP$^{-/-}$ Vim$^{-/-}$) and GFAP$^{-/-}$ brains astrocytes are devoid of the main component of the intermediate filament network, GFAP, and vimentin.

Previous work in a transgenic mouse model has shown that deletion of cytoskeletal intermediate filaments GFAP and vimentin creates an astrocytic environment more permissive to transplantation and increased regeneration and highlights the importance of a permissive environment; brain and spinal cord injury experiments in GFAP$^{-/-}$ Vim$^{-/-}$ mice show that there is a more permissive environment for integration of neural transplants in the adult CNS (Kinouchi et al., 2003) as well as improved axonal sprouting (Menet et al., 2003) and there is synaptic regeneration after CNS injury (Wilhelmsson et al., 2004). These outcomes suggest that intermediate filaments depletion in astroglial cells might alter their differentiation state, turning them into cells functionally similar to more immature astrocytes and therefore more supportive of CNS regeneration, which would ultimately allow control of complex processes (Pekny et al., 2007).

3.**AIM**

3.1 HYPOTHESIS

Alterations in intermediate filaments will affect the neurosphere forming capacity and the differentiation potential of GFAP-/-Vim-/- mice when compared to wild-type controls due to alterations in both the stem cell population and the astrocytes within the neurogenic niche.

3.2 OBJECTIVE

The objective of this thesis was to use neurosphere assay in order to determine the primary and secondary neurosphere forming capacity of GFAP-/- vimentin-/- mice compared to wild type controls, to analyze neurosphere growth in terms of size and proliferation and finally to determine the differentiation potential of primary neurospheres.

4. MATERIALS AND METHODS

4.1 MICE

Aged matched postnatal day 4 (P4) mice carrying a null mutation in the GFAP and/or Vimentin gene and wild-type controls were used (Pekny et all 1995, 1999 **). All mice were on a C57Bl6/129Sv/129Ola mixed genetic background. Genotypes were confirmed by PCR.

4.2 STEM/PROGENITOR CELL ISOLATION

P4 mice were decapitated, the brain was dissected out in Leibovitz medium (31415-029, Invitrogen), cut into pieces (1-2 mm) and incubated for 20 min at 37°C in 2 mL of a digestion solution 0,1% trypsin, 0,5mM EDTA (25200-072, Invitrogen) in Hanks Balanced Salts Solution ((HBSS), 14175-053, Invitrogen) for enzymatic dissociation. A trypsin inhibiting solution (0,5% trypsin inhibitor; T6522, Sigma), 0.5% Trypsin Inhibitor and 0,5% DNAse (DN25, Sigma) in HBSS was added and the tissue was mechanically dissociated into a single cell suspension using a 20G needle attached to a 1 mL syringe. The sample was centrifuged at 24°C for 3 min at 1200 rpm. The pellets were washed twice in Neurobasal (21103-049, Invitrogen) and resuspended in Neurosphere media (Table 1).

Neurosphere Media		**Differentiation Media**	
Neurobasal		Neurobasal	
L-glutamine	20 μM/ml	L-glutamine	20 μM/ml
Penicillin	1x	Penicillin	1x
B27	1x	B27	1x
bFGF	20 ηg/ml	Fungizone	0.5ul/10ml
EGF	20 ηg/ml		
Heparin	1 U/ml		
Fungizone	0.5ul/10ml		

Table 1: Media used for neurosphere formation and differentiation.

Neurobasal supplemented with L-glutamine 20 mM/ml, (25030-024, Invitrogen), penicillin, 1x, (15140-163, Invitrogen), B27, 1x, (17504-044, Invitrogen), heparin, 1U/ml, (H1027-50, Sigma), fungizone, 0,5 ml/10ml, (Bristol Meyers Squibb) and

growth factors bFGF, 20ng/ml, (13256-029, Invitrogen) and EGF, 20ng/ml, (02633, stemcell.com) (NBGF).

4.3 PRIMARY NEUROSPHERE PROPAGATION

Single cells were counted using a Burker Chamber and plated at the concentrations of $1x10^5$ cells or $1x10^6$ cells on uncoated 12 well and 24 well plates and cultured in neurosphere media for 7 days at 37°C and 0.5% CO_2.

4.4 SECONDARY NEUROSPHERE PROPAGATION

Primary neurosphere were mechanically dissociated into the single cell suspension using a 20G needle attached to a 1mL syringe. Cells were counted using a Burker chamber and plated at a concentration of 10^5 cells/well in neurosphere media. The cultures were incubated for 7 days at 37°C and 0.5% CO_2.

4.5 NEUROSPHERE COUNTS

After seven days in culture, total numbers of primary and secondary neurospheres per well were counted using an inverted Nikon microscope (TMS, Japan).

4.6 CELL DIFFERENTIATION

10 seven day old neurospheres were plated on glass coverslips coated with Poly-L-ornithine 10mg/ml, (PORN; P3655, Sigma) and laminin, 5mg/ml, (23017-015, Invitrogen) and gently flooded with differentiation media. One day after plating, 1% fetal bovine serum (10106-169, Invitrogen) was added. Cells were fixed in 4% paraformaldehyde in 0,1M phosphate buffer for 20 minutes at room temperature after 5 days of differentiation. Differentiated neurospheres were analyzed on a Nikon eclipse 80i fluorescence microscope and analyzed using Image J software.

4.7 STEM/PROGENITOR CELL PROLITERATION ANALYSIS

Seven day old neurospheres were incubated in 5 μM 5´bromodeoxyuridine (BrdU; DN25, Sigma) in neurosphere media on laminin/PORN coated plates, cultured for 24 hours and fixed in 4% paraformaldehyde in 0.1M phosphate buffer for 20 minutes at room temperature for immunocytochemistry. Neurosphere were photographed with a Leica Confocal and analysed with Image J Software (NIH).

4.8 NEUROSPHERE SIZE MEASUREMENT

Seven day old neurospheres were plated with neurosphere media onto PORN/laminin coated plates and incubated for 30 min at 37°C. Once attached, 25-50 neurospheres were photographed with a Nikon inverted microscope attached to a Leica 290 camera and measured using Image J Software (NIH).

4.9 IMMUNOCYTOCHEMISTRY

BrdU

Cells were incubated in 2M HCl for 10 min at 37°C, washed once in PBS and twice in 0,1 M borate buffer for 5-10 min. Cultures were then incubated in 3% normal goat serum (X0907, Dako), 0.01% Triton X-100 (Sigma) in PBS for 30 min at room temperature. After blocking cells were incubated in anti-mouse BrdU antibody, 1:200, (M0744, Dako) diluted in 0,1 % triton X-100 in PBS overnight at 4°C. Cells were then washed three times in PBS and incubated in goat anti-mouse Alexa 594, 1:500, (A11032, Mol Probes) with 4',6-diamidino-2-phenylindole (Dapi), 1:500, (D8417, Sigma) and Topro, 1:1000 (T3605, Invitrogen)diluted in 0,1 % triton X-100 in PBS with for 1 hr at room temperature, washed three times in PBS and mounted with Moviol (Clariant GmbH) with 2,5% 1,4-diazabicyclo [2.2.2.] octane (DABCO, 33480).

Immunocytochemistry for cell phenotype markers

Differentiated neurospheres were washed three times in PBS, incubated with blocking solution (10% fetal calf serum, 0,5% triton X-100 in PBS) for 1 hr at room temperature incubated with primary antibodies, mouse anti-b-III-tubulin, 1:200, (MMS435P250,

Nordic-Biosite), rabbit anti-S100, 1:100, (Z0311, Dako) mouse anti-O4, 1:100, (MAB345, Chemicon) in PBS overnight at 4°C. Cells were then washed three times in PBS, incubated in goat anti-mouse Alexa 594 or donkey anti-Alexa 488/568, 1:1000, (A11034, Dako) for 1 hr at room temperature, washed three time in PBS and mounted in Moviol with 2,5% 1,4-diazabicyclo [2.2.2.] octane.

5. RESULTS

The neurosphere assay is a suitable tool to analyze proliferation, self-renewal capacity and multipotency of neural stem/progenitor cells.

Increased primary neurosphere formation in GFAP$^{-/-}$Vim$^{-/-}$ and GFAP$^{-/-}$ mice compared to wild type controls

After 7 days in culture the neurosphere forming capacity of genetically modified mice compared to wild-type controls was analysed. Counts of total neurospheres/well in age matched groups of GFAP$^{-/-}$Vim$^{-/-}$ and GFAP$^{-/-}$ mice showed a statistically significant increase in the number of neurospheres in GFAP$^{-/-}$Vim$^{-/-}$ and GFAP$^{-/-}$ mice compared to wild-type controls ($p<0,05$) (Figure 4a).

No difference in secondary neurosphere formation in GFAP$^{-/-}$Vim$^{-/-}$ and GFAP$^{-/-}$ mice compared to wild type controls

Neurospheres were dissociated into single cell suspensions and cultured again under the same conditions for 7 days. In this case, no significant difference was found in secondary neurosphere formation between GFAP$^{-/-}$Vim$^{-/-}$ and GFAP$^{-/-}$ mice and to wild type controls (Figure 4b).

No difference in neurosphere growth in GFAP$^{-/-}$Vim$^{-/-}$ and GFAP$^{-/-}$ mice compared to wild type controls

Diameter measurements of individual primary neurospheres after 7 days of growth showed no significant differences in neurosphere size between GFAP$^{-/-}$Vim$^{-/-}$ and wild type mice (Figure5).

5’ Bromodeoxyuridine (BrdU) was added to primary neurosphere cultures for 24 hours on day 7 in vitro and BrdU incorporation was quantified to determine the number of actively proliferating cells in GFAP$^{-/-}$Vim$^{-/-}$ and wild type neurospheres. There was no significant difference in the percentage of proliferating cells in GFAP$^{-/-}$Vim$^{-/-}$ and wild type animals (Figure6).

Increased neuronal differentiation of GFAP$^{-/-}$Vim$^{-/-}$ neurospheres compared to wild type controls

For verification that neurospheres contain multipotent progenitor cells, several individual neurospheres were plated on laminin/PORN coated glass coverslips in media without growth factors supplemented with 1% fetal bovine serum. Cells immediately adhered to the substrate and were differentiated for 5 days in culture. Mature neural cell lines were evaluated by immunocytochemistry for expression of specific phenotypic markers: b-III-tubulin for neurons, S100 for and O4 for oligodendrocytes.

The number of neurons was assessed by counting beta-III-tubulin positive cells. The results of cell differentiation in vitro show the a significantly higher percentage of neurons generated from GFAP$^{-/-}$Vim$^{-/-}$ animals compared to wild type controls (Figure7).

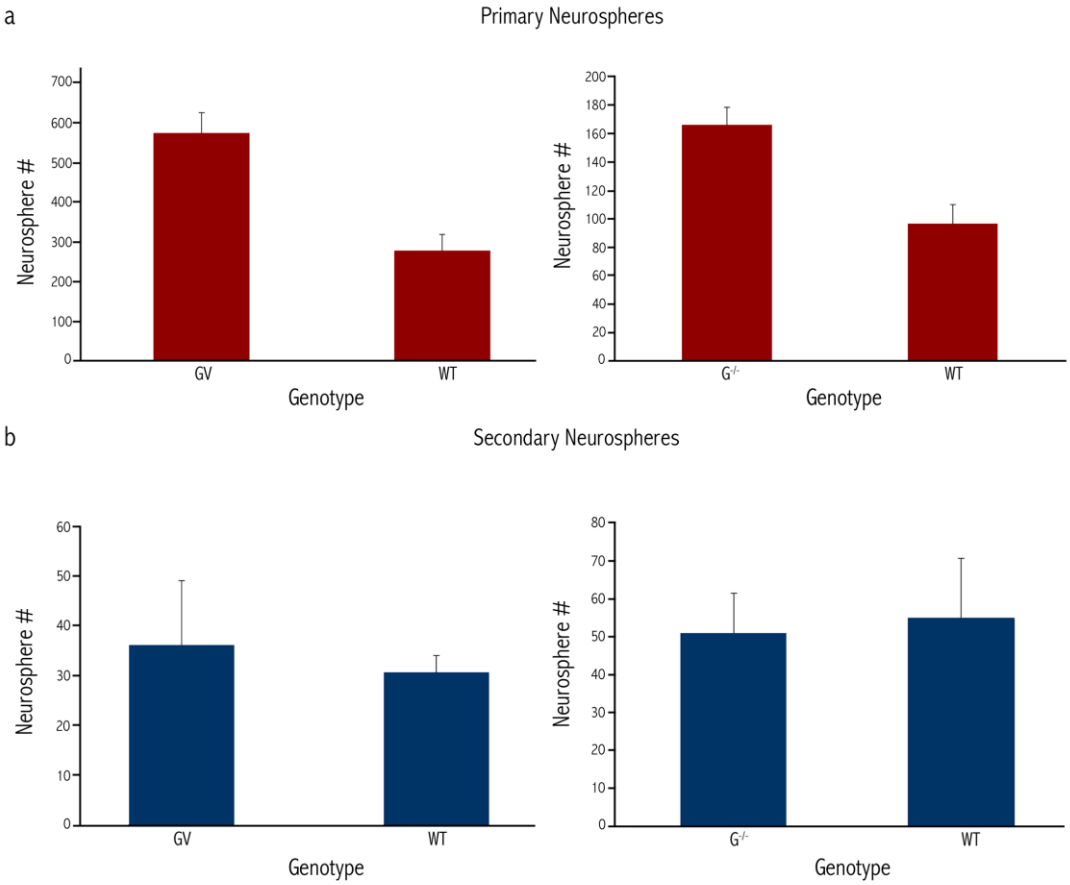

Figure 4: The number of primary neurospheres produced from GFAP$^{-/-}$Vim$^{-/-}$ animals is almost doubled but no difference is seen in secondary neurosphere production.

There is a significant increase in primary neurosphere formation in GFAP$^{-/-}$Vim$^{-/-}$ animals and GFAP$^{-/-}$ single mice when compared to wild type controls (a). No significant difference is found in GFAP$^{-/-}$Vim$^{-/-}$ and GFAP$^{-/-}$ secondary neurosphere formation when compared to WT animals (b). GFAP$^{-/-}$Vim$^{-/-}$ (GV); GFAP$^{-/-}$Vim$^{-/-}$ (G$^{-/-}$); wild type (WT)

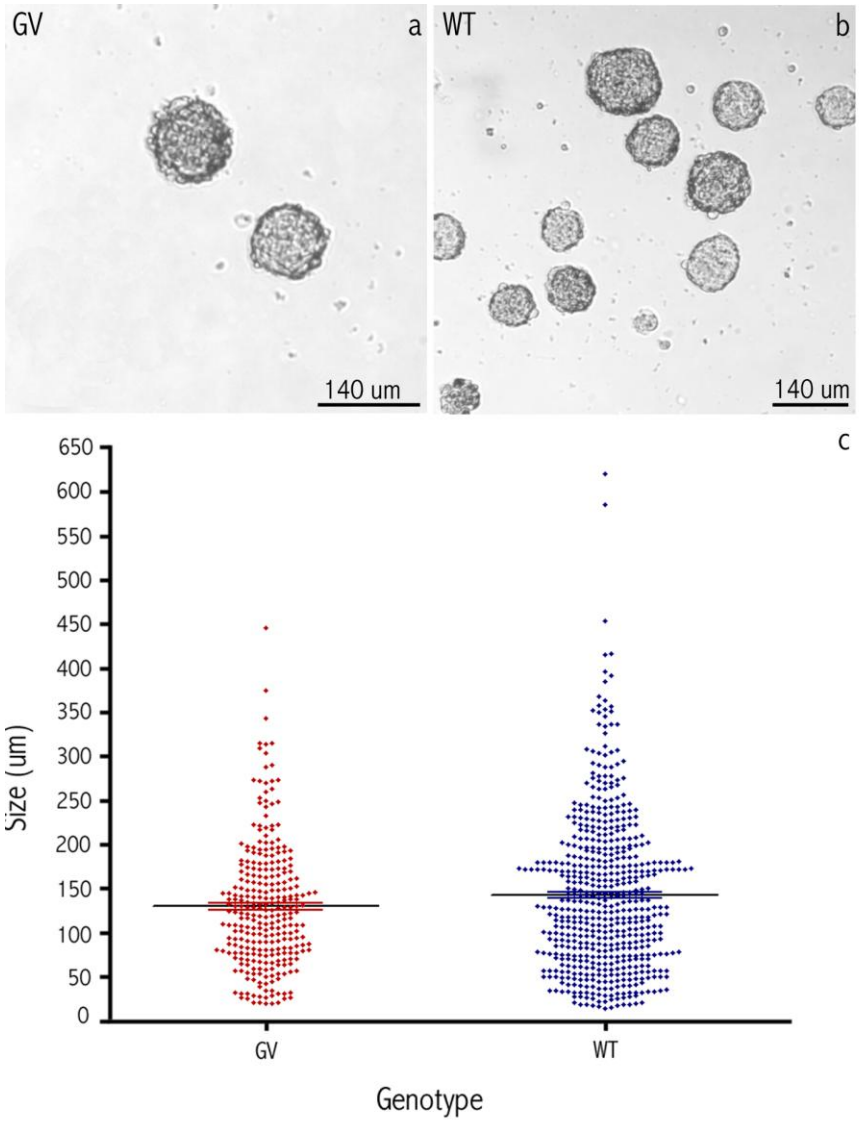

Figure 5: The size of neurospheres was not altered in GFAP$^{-/-}$Vim$^{-/-}$ animals. GFAP$^{-/-}$Vim$^{-/-}$ neurospheres after 7 days in culture (a). WT neurospheres after 7 days in culture (b). The size (diameter of the neurosphere) of GFAP$^{-/-}$Vim$^{-/-}$ neurospheres did not differ significantly from WT controls (c; the mean is shown by the black line). GFAP$^{-/-}$Vim$^{-/-}$ (GV); wild type (WT).

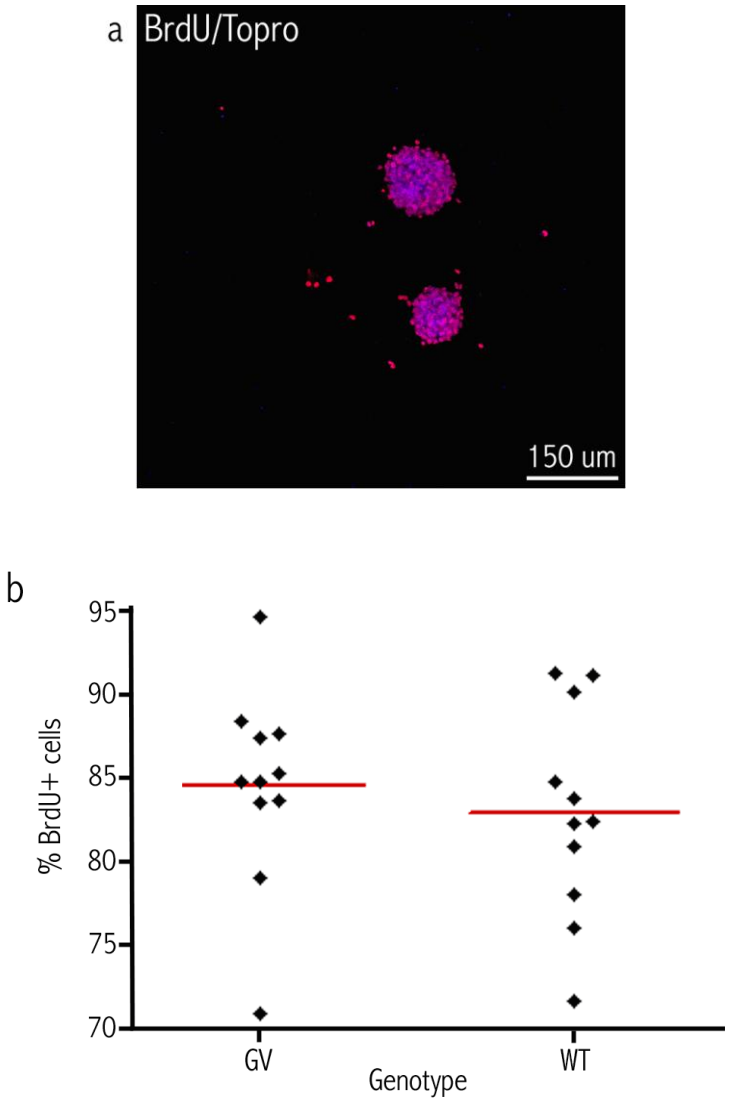

Figure 6: GFAP$^{-/-}$Vim$^{-/-}$ animals did not show any differences in stem/progenitor cell proliferation.

Neurosphere proliferation after 7 days in culture (a). There is no significant difference in the percentage of proliferating cells in GFAP$^{-/-}$Vim$^{-/-}$ and WT animals (b; the mean is shown by the red line). (GFAP$^{-/-}$Vim$^{-/-}$ (GV); wild type (WT); BrdU, red; Topro, blue).

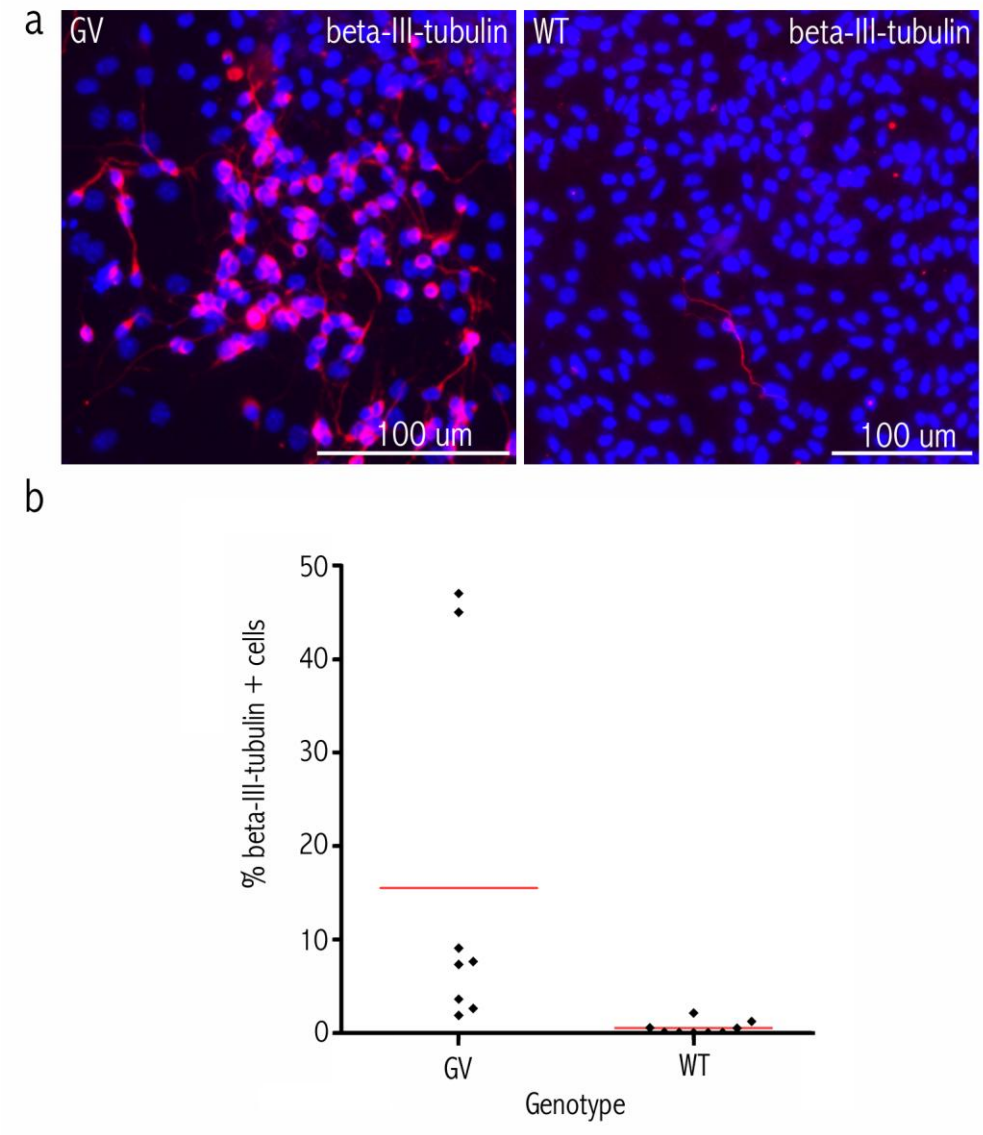

Figure 7: Increased neuronal differentiation from GFAP$^{-/-}$Vim$^{-/-}$ primary neurospheres. Beta-III-tubulin positive cells from GFAP$^{-/-}$Vim$^{-/-}$ and WT primary neurospheres aged 5d in differentiating conditions (a). The percentage of neurons produced is significantly higher after differentiation of primary GFAP$^{-/-}$Vim$^{-/-}$ neurospheres than WT controls (b). GFAP$^{-/-}$Vim$^{-/-}$ (GV); wild type (WT); beta-III tubulin, red; DAPI, blue)

6. DISCUSSION

This study showed that the neurosphere forming capacity of both GFAP$^{-/-}$ Vim$^{-/-}$ and GFAP$^{-/-}$ mice was significantly higher than wild type controls. No differences were seen in their proliferation and size measurements, suggesting that there is no difference in the growth (size-proliferation correlate) between GFAP$^{-/-}$ Vim$^{-/-}$ and GFAP$^{-/-}$ and wild type neurospheres. Moreover, GFAP$^{-/-}$ Vim$^{-/-}$ neurospheres show increased potential for neuronal differentiation.

Doubling of primary neurosphere forming capacity in GFAP$^{-/-}$ Vim$^{-/-}$ and GFAP$^{-/-}$ mice was be due to more permissive environment for stem/progenitors cells

For primary neurosphere cultures, cells were cultured directly from the brain and plated at equal densities. An increased number of neurospheres suggests that GFAP$^{-/-}$ Vim$^{-/-}$ mice have an increased number of progenitor cells. This observation may indicate that modifications in the astrological environment are sufficient to boost the progenitor/stem quotient in GFAP$^{-/-}$ Vim$^{-/-}$ and GFAP$^{-/-}$ brains. The number of secondary neurospheres was not significantly different; single cells from primary neurosphere cultures, plated at equal densities result in equal numbers of stem/progenitor cells able to form secondary neurospheres.

No intrinsic differences in terms of growth between GFAP$^{-/-}$ Vim$^{-/-}$ and wild type neurospheres

The depletion of intermediate filaments in astrocytes does not affect GFAP$^{-/-}$ Vim$^{-/-}$ neurosphere growth. The data presented here shows that there were no differences in proliferation and neurosphere size. However, further investigation, such as real time PCR (qPCR) analysis of gene expression is needed to confirm the assumption that stem/progenitor cells are intrinsically similar.

Increased differentiation of neurons from GFAP$^{-/-}$ Vim$^{-/-}$ neurospheres

The number of neurons was assessed by immunocytochemistry for neuronal specific β-III-tubulin. Under differentiation conditions, GFAP$^{-/-}$ Vim$^{-/-}$ neurospheres generated higher percentages of β-III-tubulin positive neurons than wild type controls. These results suggest that neuronal differentiation of neural progenitor cells was enhanced in GFAP$^{-/-}$ Vim$^{-/-}$ mice. These results are in agreement with a recent study in vivo showing increased neurogenesis of transplanted progenitor cells in the GFAP$^{-/-}$ Vim$^{-/-}$ mouse (Widestrand et al., 2007). Further experiments should define the percent of other differentiated cell types in order to determine if the increase of neurons seen is compensated for by reduced astrocyte or oligodentrocyte differentiation.

7. CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, this work together with findings from previous studies suggests that the absence of intermediate filaments in astrocytes enhances or promotes stem/progenitor cell survival and neuronal differentiation in $GFAP^{-/-}$ $Vim^{-/-}$ mice by modifying the astroglial environment within the neurogenic niche.

FUTURE DIRE CTIONS

- To determine the gene expression profile of neurospheres from $GFAP^{-/-}$ $Vim^{-/-}$and wild type mice
- To determine the primary and secondary neurosphere forming capacity of $Vim^{-/-}$ mice.
- To determine neuronal differentiation from $GFAP^{-/-}$ mice.
- To determine how modifying the environment by using astrocyte-neurosphere co-culture systems influences neurosphere forming capacity and neuronal differentiation in both $GFAP^{-/-}$ $Vim^{-/-}$ and wild type animals.

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ABSTRACT

Neural stem cells (NSCs) are present in the mammalian adult nervous system and are found in two specific regions: the subventricular zone of the forebrain and the subgranular zone in the dentate gyrus of the hippocampus. NSCs undergo fate specification, maturation and functional integration before they can take part in neuronal circuits. Less is known about the mechanisms controlling these processes or the function of the newly born neurons in the adult brain. A large number of molecular and environmental factors can regulate various aspects of proliferation, maturation and differentiation of adult stem cells in adult nervous system. NSCs can give rise to three main types of neural cells: neurons, astrocytes and oligodendrocytes. It is suggested that astrocytes may play critical role in regulating neurogenesis in both the intact adult brain and after injury. Therefore, determining how astrocytes regulate adult neurogenesis at the molecular level is an essential step toward understanding the regulation of adult neurogenesis. To find out how genetic alteration of astrocytic intermediate filament will affect neural stem cells of postnatal mice, the neurosphere assay was performed in order to compare the proliferative and differentiation characteristics of neural stem/progenitor cells in $GFAP^{-/-}Vim^{-/-}$ and wild type mice. The results indicate that the absence of intermediate filaments in astrocytes enhances or promotes stem/progenitor cell survival and neuronal differentiation in $GFAP^{-/-}$ $Vim^{-/-}$ mice by modifying the astroglial environment within the neurogenic niche.

Key words: neural stem/progenitor cell, neurogenesis, neurosphere, GFAP, vimentin, astrocytes

ABSTRAKT

Neurální kmenové buňky (NKB) se vyskytují v dospělém nervovém systému savců, nacházejí se ve dvou specifických oblastech: subventrikulární zóna předního mozku a subgranulární zóna gyrus dentatus v hipokampu. NKB získávají své specifické vlastnosti, vyzrávají a integrují se do funkčních celků, předtím než se mohou funkčně zapojit do nervového systému. Stále víme málo o mechanismech, které kontrolují tyto procesy a o funkci nově vznikajících neuronů v dospělém mozku. Velké množství faktorů na molekulární úrovni a vliv prostředí, to vše se může podílet na regulaci proliferace, vývoje a diferenciace somatických kmenových buněk v dospělém nervovém systému. NKB dávají vznik třem hlavním typům nervových buněk: neuronům, astrocytům a oligodendrocytům. Ukazuje se, že astrocyty mohou hrát klíčovou roli v regulaci neurogeneze: v neporušeném funkčním dospělém mozku i po jeho poškození. Pro odhalení funkce astrocytů v regulaci neurogeneze na molekulární úrovni, je nezbytným krokem nejprve porozumět regulaci neurogeneze v dospělém CNS. Abychom zjistili, jak se projeví odstranění genů intermediálních filament astrocytů (GFAP, vimentin) na počtu NKB, a jak zásah ovlivní vlastnosti neurálních kmenových buněk myší, analyzovali jsme neurosféry neurálních kmenových buněk. Neurosféry sloužili k porovnání charakteristik proliferace a diferenciace neurálních kmenových/progenitorových buněk $GFAP^{-/-}Vim^{-/-}$ myší a myší bez genetické modifikace. Výsledky ukazují, že astrocyty bez intermediálních filament zvyšují nebo podporují přežití neurálních kmenových buněk a diferenciaci neuronů $GFAP^{-/-}Vim^{-/-}$ myší, tím že genetická změna modifikuje astrogliálního prostředí a neurogenní mikroprostředí.

Klíčová slova: neurální kmenové buňky, neurogeneze, neurosféra, GFAP, vimentin, astrocyty