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Mesenchymal Stem Cells Promote Oligodendroglial Differentiation in Hippocampal Slice CulturesFrancisco J. Rivera^{1,3*}, Florian A. Siebzehnruhl^{2*}, Mahesh Kandasamy^{3*}, Sebastien Couillard-Despres³, Massimiliano Caioni³, Anne-Maria Poehler³, Benedikt Berninger^{4,5}, Beatrice Sandner³, Ulrich Bogdahn³, Magdalena Goetz^{4,5}, Ingmar Bluemcke², Norbert Weidner³ and Ludwig Aigner^{1,3}

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Key Words

Mesenchymal stem cells • Neural progenitors • Oligodendrocyte differentiation • Hippocampal slice culture • Multiple Sclerosis

Abstract

We have previously shown that soluble factors derived from mesenchymal stem cells (MSCs) induce oligodendrogenic fate and differentiation in adult rat neural progenitors (NPCs) *in vitro*. Here, we investigated if this pro-oligodendrogenic effect is maintained after cells have been transplanted onto rat hippocampal slice cultures, a CNS-organotypic environment. We first tested whether NPCs, that were pre-differentiated *in vitro* by MSC-derived conditioned medium, would generate oligodendrocytes after transplantation. This approach resulted in the loss of grafted NPCs, suggesting that oligodendroglial

pre-differentiated cells could not integrate in the tissue and therefore did not survive grafting. However, when NPCs together with MSCs were transplanted *in situ* into hippocampal slice cultures, the grafted NPCs survived and the majority of them differentiated into oligodendrocytes. In contrast to the prevalent oligodendroglial differentiation in case of the NPC/MSC co-transplantation, naïve NPCs transplanted in the absence of MSCs differentiated predominantly into astrocytes. In summary, the pro-oligodendrogenic activity of MSCs was maintained only after co-transplantation into hippocampal slice cultures. Therefore, in the otherwise astrogenic milieu, MSCs established an oligodendrogenic niche for transplanted NPCs, and thus, co-transplantation of MSCs with NPCs might provide an attractive approach to re-myelinate the various regions of the diseased CNS.

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Introduction

Oligodendrocytes are the desired cell types for any cell replacement approach in several pathological conditions characterized by myelin loss in the central nervous system (CNS), such as in multiple sclerosis (MS), spinal cord injury (SCI) or multi-system atrophy (MSA). *In vivo*, oligodendroglial progenitors, which are present in the CNS parenchyma, have the capacity to generate oligodendrocytes and re-myelinate axons (for review see [1]). Indeed, in some pathologies including subsets of MS, successful re-myelination is a key event in the course of functional recovery [2]. Conversely, in other subsets of MS and after SCI the occurrence of re-myelination is highly limited [3-5]. In the latter cases, *ex vivo* generation and transplantation of cells with an oligodendrogenic potential constitutes an attractive alternative strategy to promote re-myelination. This might be achieved by the use of neural stem and progenitor cells (NPCs) that can be harvested from the adult CNS and expanded in cell culture [6, 7].

We have recently demonstrated that MSCs and soluble factors secreted by MSCs robustly induced and promoted the oligodendroglial fate and concomitantly inhibited the astroglial fate of adult rat NPCs *in vitro* [8, 9]. Indeed, the co-cultivation of NPCs with MSCs is to our knowledge the most efficient method to differentiate adult NPCs into oligodendrocytes without genetic manipulation [8].

Here, we investigated if the oligodendroglial activity of MSCs on NPCs is maintained after co-transplantation of MSCs/NPCs into a CNS-organotypic environment. Hippocampal slice cultures were used as a target tissue, since i) they are well established and widely used as CNS organotypic cultures containing several well-described nerve fibers [10], and ii) they preserve regions of neurogenic activity and demonstrate neural progenitor proliferation and differentiation [11]. Hence, hippocampal slice cultures served as a host tissue for MSC / NPC transplantation and survival and differentiation of the transplanted NPCs were investigated.

Materials and Methods

Animal subjects

Adult female Fischer 344 rats (Charles River Deutschland GmbH, Germany) weighing 160-180g were used as donors for the isolation of MSCs and NPCs. Nineteen to 21 days postnatal Wistar rats were used as donors for the hippocampal slice cultures. All experiments were carried out in accordance with

the European Communities Council Directive (86/609/EEC) and institutional guidelines. Animals had ad libitum access to food and water throughout the study.

MSC and NPC cultures

NPCs derived from the adult hippocampus and subventricular zone were generated as described [7, 8]. Briefly, five 2 to 4 month-old female Fischer-344 rats (Charles River Deutschland GmbH, Germany) were decapitated. Hippocampi were aseptically removed, transferred to 4°C DPBS (PAN, Germany) with 4.5g/L glucose (Merck, Germany) (DPBS/glu), washed once, transferred to petri dishes and dissociated mechanically. The cell suspension was washed in DPBS/glu and resuspended in PPD-solution containing 0.01% Papain (Worthington Biochemicals, England), 0.1% dispase II (Boehringer, Germany), 0.01% DNase I (Worthington Biochemicals, England) and 12.4 mM MgSO₄ in HBSS (PAN, Germany) without Mg⁺⁺/Ca⁺⁺ (PAA, Germany) and digested for 30 to 40 min at 37°C. The cell suspension was triturated every 10 min. Dissociated cells were collected and resuspended in Neurobasal (NB) medium containing B27 (Gibco BRL, Germany), 2mM L-glutamine and 100U/mL penicillin/ 100 µg/ml streptomycin and washed three times. Finally, the single cell suspension was resuspended in NB medium (Gibco BRL, Germany) supplemented with B27 (Gibco BRL, Germany) (NB/B27), 2 mM L-glutamine (PAN, Germany), 100U/mL penicillin/ 100 µg/ml streptomycin (PAN, Germany), 2 µg/ml heparin (Sigma, Germany), 20 ng/ml bFGF-2 (R&D Systems, Germany) and 20 ng/ml EGF (R&D Systems, Germany). Cultures were maintained at 37°C in an incubator with 5% CO₂. Half of the medium was changed every 7 days. Neurosphere cultures from passage number 2 to 6 were used throughout this study.

MSCs were prepared as described previously [8]. Briefly, bone marrow plugs were harvested from femurs and tibias of 2-4 month-old female Fisher-344 rats (Charles River Deutschland GmbH, Germany). Plugs were mechanically dissociated in α MEM and recovered by centrifugation. Cell pellets were resuspended in α MEM-10% fetal bovine serum (FBS) and seeded at 1x10⁶ cells/cm². After 3 days, media was changed and non-adherent cells were removed. Adherent cells were incubated in fresh α MEM-10% FBS until a confluent layer of cells was achieved. Cells were trypsinized using 0.25% Trypsin (Gibco Cell Culture, Invitrogen GmbH, Germany) and seeded in α MEM-10% FBS at 8,000 cells/cm². After 3-5 days of culture, the resulting monolayer of cells, hereafter named rat bone marrow-derived mesenchymal stem cells (MSCs), was trypsinized and further cultured for experiments or frozen for later use. As demonstrated in our previous work, this cell culture preparation is highly enriched in multipotent MSCs with virtually no hematopoietic contamination [8].

Labeling of cells

NPCs were incubated with 1 µM 5-bromo-2'-deoxyuridine (BrdU, Sigma, Germany) in growth medium for 48 h before transplantation. BrdU-incorporation could be demonstrated in more than 95% of NPCs by immunohistochemistry (data not shown). EGFP-labeled MSCs were used in the hippocampal slice culture transplantation experiments. To this end, we used

an HIV-based lentiviral eGFP vector pseudotyped with VSVG, where eGFP is encoded under the control of the human ubiquitin C promoter [12]. A total of 10^7 MSCs were transduced in 0.5 ml of medium with 10^7 infectious viral particles for 1,5 hrs at 37°C, washed and seeded. Two days later, the eGFP expressing cells are enriched to approximately 90% by FACS sorting using a FACSAria (Becton Dickinson).

NPC pre-differentiation

NPCs were pre-differentiated by the MSCs-derived conditioned medium (MSC-CM). MSC-CM was prepared as described [8]. Briefly, MSCs were plated at 12,000 cells/cm² and incubated in α MEM-10% FBS. After 3 days, the conditioned medium was collected and filtered using a 0.22 μ m-pore filter. BrdU-labeled NPCs were plated on polyornithine (100 μ g/ml) and laminin (5 μ g/ml)-coated dishes and incubated for 2 days with MSC-CM. After this period, cells were treated with 0.25% trypsin (Gibco BRL, Germany) and collected for transplantation (Fig. 1 A). Based on our previous studies, this time frame is sufficient to trigger an oligodendrogenic program in NPCs, since i) the presence of MSC-CM for two to three days was sufficient to increase the expression level of pro-oligodendrogenic transcription factors (olig 1/2) and to decrease expression level of the anti- oligodendrogenic factor Id2 [8], and ii) a two day incubation with MSC-CM was sufficient to induce and promote the expression of the oligodendroglial markers GalC and MBP and to inhibit expression of the astroglial marker GFAP [9].

Organotypic hippocampal slice cultures and cell transplantation

Preparation and maintenance of organotypic hippocampal slice cultures and cell transplantation were performed as described [10]. Briefly, postnatal day 19-21 Wistar rats were decapitated, the brains removed and placed into ice-cold preparation medium. Horizontal 350 μ m sections were prepared on a Leica VT-1000 vibratome (Leica Microsystems, Bensheim, Germany), placed in transwell cell-culture inserts in 6 well plates (Greiner Bioscience, Germany) and cultured as interface cultures [13] in slice culture medium [14]. The slices were cultured in a humidified atmosphere at 35°C. Medium was changed on the first day and every other day thereafter. For stem cell transplantation, a total of 5,000 cells (MSCs, NPCs or a 1:1 mix of both, Fig.1 B) were grafted one day after slice culture preparation into different hippocampal regions (CA region and dentate gyrus) in 0.05 μ l of medium using a Hamilton syringe. Alternatively, 5,000 MSC-CM pre-conditioned (pre-differentiated) BrdU-labeled NPCs were transplanted (Fig. 1 A). After 7 days, slices were fixed in 4 % paraformaldehyde for 24 hours and placed in 30% sucrose.

Immunohistology

Immunostaining and histological analysis was performed with free-floating sections to assess the NPCs differentiation pattern in vivo. To visualize the transplanted BrdU-labeled NPCs, the hippocampal slices were washed 3 times with TBS and incubated for 1h in 50% formamide/2xSSC (0.3M NaCl, 0.03M sodium citrate) at 65°C. Sections were rinsed in 2xSSC, incubated for 30 min in 2M HCl at 37°C and rinsed for 10 min on

0.1M boric acid (pH 8.5). After rinsing in Tris-buffered saline (TBS), sections were blocked in TBS + 3% donkey serum + 0.1% Triton-X100 for 1h. The same solution was used for the antibody solution. Primary antibodies were applied overnight at 4°C. Fluorochrome-conjugated species-specific secondary antibodies were used for immunodetection. The following primary antibodies were used: rat anti-BrdU for grafted NPC 1:500 (AbDSerotec, UK), rabbit anti-GFAP for astroglia 1:1000 (Dako, Denmark A/S, Glostrup, Denmark), mouse anti-Myelin Basic Protein (MBP) for oligodendrocytes 1:750 (SMI-94, Sternberger Monoclonals Inc., U.S.A.), rabbit anti-glutathione S-Transferase placental form (GST-pi) for oligodendrocytes 1:2000 (Biomol, Germany), rabbit anti-green fluorescence protein (GFP) 1:500 for the detection of transplanted GFP-expressing MSCs (Rockland, Gilbertsville, USA), rabbit anti-active caspase-3 1:100 for the detection of cells undergoing apoptosis (Promega, Madison, Wisconsin, USA), rabbit anti-Iba1 1:500 for the detection of microglia (Wako Chemicals GmbH, Neuss, Germany). Secondary antibodies: donkey anti-mouse, rabbit conjugated with Alexa Fluor[®] 488 1:1000 (Molecular Probes, U.S.A.), rhodamine X (RHOX) 1:1000 (Dianova, Germany) or Cy5 1:500 (Dianova, Germany). Specimens were mounted on microscope slides using Prolong Gold Antifade kit (Invitrogen GmbH, Karlsruhe, Germany).

Quantitative Analysis

Transplanted BrdU-labeled NPCs in hippocampal slice cultures were visualized using a Leica microscope (Leica Mikroskopie und Systeme GmbH, Germany) equipped with a Spot[™] digital camera (Diagnostic Instrument Inc, U.S.A.) at 200 x or 400 x magnification. Cell survival was determined as percentage of the total BrdU-positive cells found in respect to the total NPCs transplanted. The fate of BrdU-labeled NPCs was analyzed by double-immunofluorescence staining for BrdU and cell-type specific markers and confocal fluorescence microscopy (Leica TCS-NT). For each condition, 3 to 5 randomly selected observation fields, containing in total 150-400 cells, were analyzed in the z-axis and photographed. Three independent experiments were analyzed.

Statistical Analysis

Statistical analysis was performed for all experiments using non-parametrical one-tailed Mann Whitney t test. Averages are expressed with their standard deviations. Statistical analysis was performed using PRISM 4 software.

Results

The present work investigates the potency of MSC-derived signals to increase the oligodendroglial differentiation capacity of transplanted NPCs. Two different experimental designs were tested: i) NPCs were first pre-conditioned with MSC-derived conditioned medium and then transplanted onto hippocampal slice cultures (Fig. 1A); ii) NPCs were transplanted either alone

Fig. 1. Schematic representation of the transplantation paradigm. (A) Transplantation of pre-differentiated NPCs. NPCs (as neurospheres) were labeled with BrdU under proliferative conditions for 48 hours. Then, these cells were plated on dishes and incubated with MSC-conditioned medium (MSC-CM) for 48 hours. After this period, BrdU-labeled NPCs were transplanted (TX) onto a hippocampal slice. (B) Co-transplantation of MSCs and NPCs. BrdU-labeled NPCs were mixed 1:1 with MSCs or with eGFP-expressing MSC (MIX) and transplanted onto a hippocampal slice. As a control, BrdU-labeled NPCs were transplanted alone (NPCs).

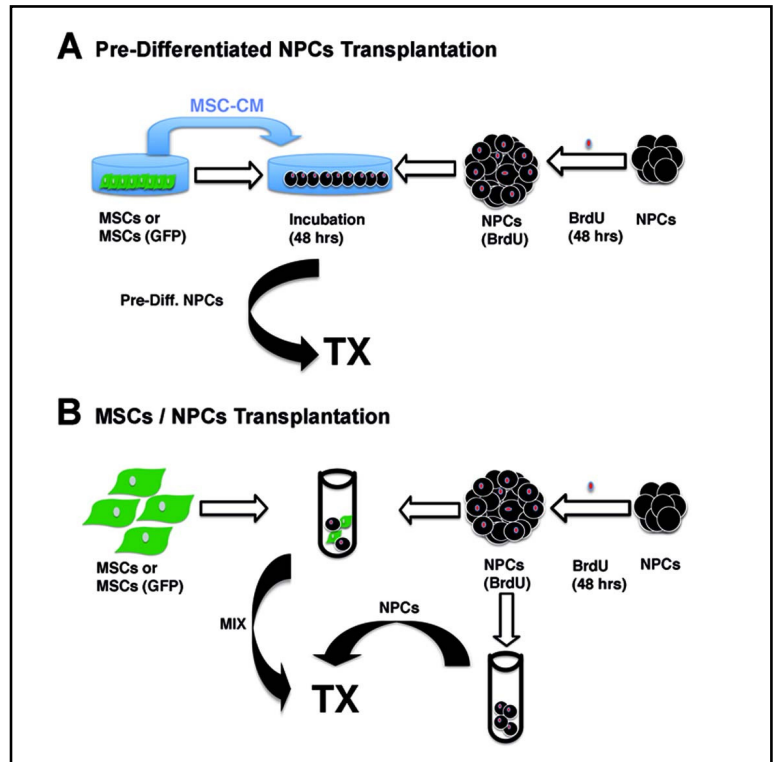


Fig. 2. Presence and survival of transplanted NPCs and MSCs on hippocampal slice cultures. (A) Fluorescence image of GFP-expressing MSCs. MSCs were co-transplanted with NPCs and incubated for 7 days. Note that GFP-expressing MSCs were found in small clusters and in some cases as isolated cells. Scale bar = 50 μ m. (B) Confocal image of transplanted MSC. GFP in green and the microglial marker Iba1 in red. Cell nuclei are illustrated by TOPRO-3 (blue). GFP positive and Iba1 positive cells do not co-localize. Scale bar = 50 μ m. (C, D) Fluorescence images of BrdU-labeled NPCs. NPCs were transplanted alone (C) or co-transplanted together with MSCs (D) onto hippocampal slice cultures and incubated for 7 days. NPCs were detected in both conditions. Upper right panels show an inset of transplanted BrdU-labeled NPCs. Scale bar = 500 μ m. (E) Quantitative analysis of survival of BrdU-labeled NPCs expressed as the number of transplanted cells detected with respect to the total number of transplanted cells. NPCs were transplanted alone (Control) or co-transplanted together with MSCs (+ MSCs). Note that in both conditions more than 40 % of the cells survived after transplantation; there was no significant difference between the two conditions ($p > 0.05$). Graph shows means \pm SD and statistical analysis was performed using non-parametrical one-tailed Mann Whitney t test. (F) Confocal image of transplanted NPCs (BrdU labeling in red) co-stained for active caspase-3 (green). BrdU/active caspase-3 double positive cells were detected, suggesting that at least some of the NPCs undergo cell death by an apoptotic pathway. Scale bar = 50 μ m.

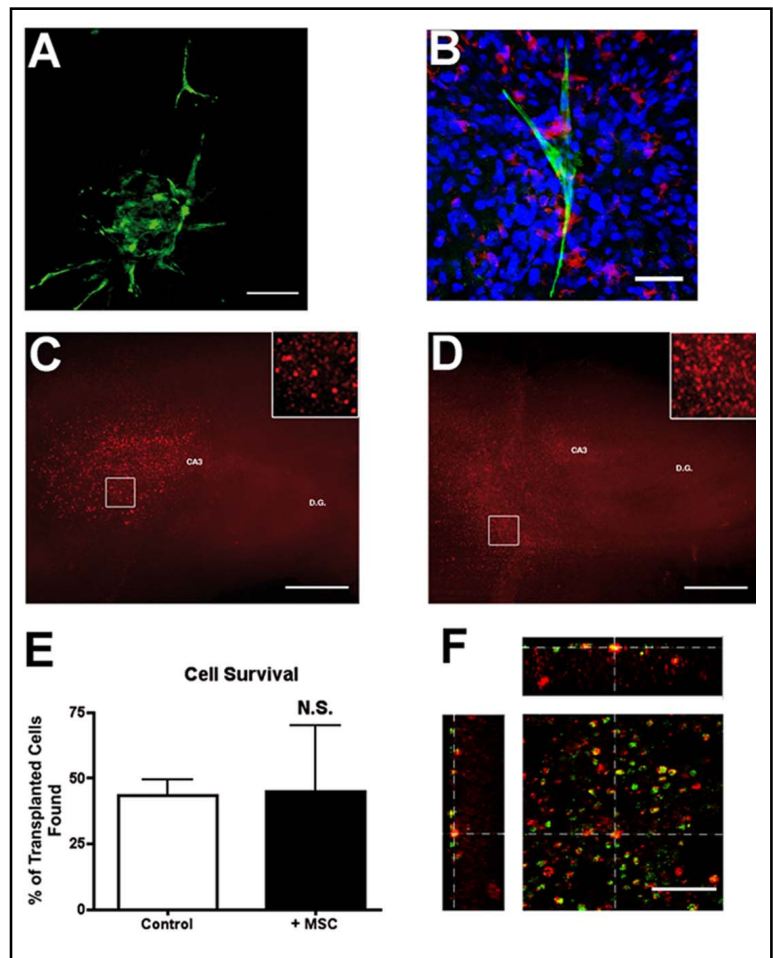
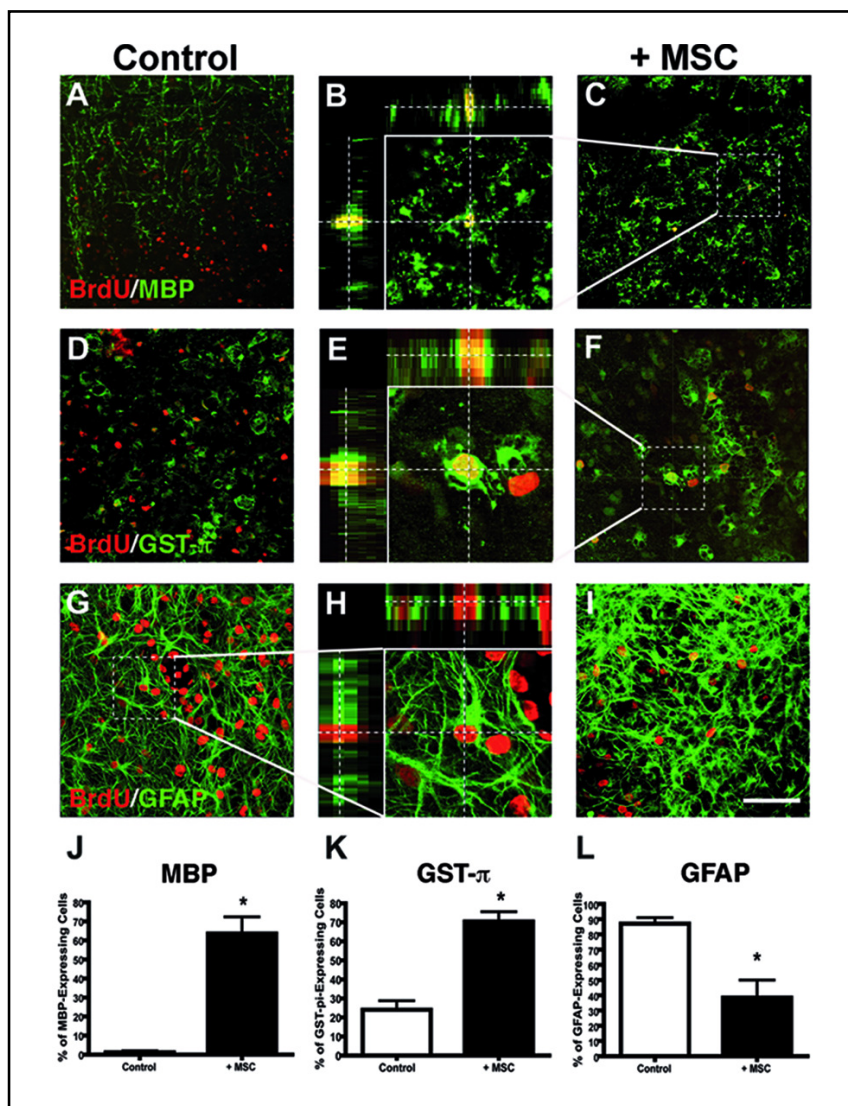


Fig. 3. MSCs promote an oligodendroglial fate of transplanted NPCs on hippocampal slices. (A-I) Confocal images of transplanted NPCs alone (A, D, G, H) or together with MSCs (B, C, E, F, I) in hippocampal slice cultures 7 days after transplantation. To detect the final fate of transplanted NPCs, cultures were processed for immunohistochemistry for BrdU (red, A-I) and for different glial markers: 1) MBP (green, A-C), GST-p (green, D-F) for oligodendrocytes and 2) GFAP (green, G-I) for astrocytes. Middle column (B, E, H) shows a higher magnification image including the Z-axis (C, F and G, respectively). Scale bar = 50 μ m. Note the co-localization of BrdU signal with the respective glial marker. (J-L) Quantitative analysis of the fate of transplanted NPCs. Graphs show the percentage of MBP- (J), GST-p- (K) and GFAP- (L) expressing cells. Note that MSCs enhance the percentage of MBP- and GST-p- expressing cells (oligodendrocytes) as an expense of GFAP- expressing cells (astrocytes). Experiments were done in triplicate. Results are expressed as mean \pm SD and statistical analysis was performed using non-parametrical one-tailed Mann Whitney t test, (*), $p < 0.05$.



or together with MSCs onto hippocampal slice cultures (Fig. 1B).

Limited survival of oligodendroglial pre-differentiated NPCs after transplantation

In our previous study, we demonstrated that a two to three day stimulation of NPCs with MSC-conditioned medium (MSC-CM) was sufficient to trigger an oligodendrogenic program in NPCs [8, 9]. NPCs responded with elevated expression of the oligodendrogenic determinants Olig1, Olig2 and Nkx2.2 and with reduced levels of the anti-oligodendrogenic factor Id2, with induction of GalC and MBP expression and inhibition of GFAP expression [8, 9]. To investigate if NPCs that were pre-differentiated towards the oligodendroglial lineage could survive and integrate into a host CNS environment, we exposed BrdU labeled NPCs to MSC-CM for two days prior to transplantation

on hippocampal slices. Seven days after grafting, we were unable to detect any remaining BrdU-labeled NPCs, suggesting that survival of oligodendroglial pre-differentiated NPCs after transplantation was limited.

MSCs promote oligodendroglial differentiation of NPCs in hippocampal slices

Prior to transplantation, NPCs were labeled with BrdU, which allows the analysis of cell survival and fate. Typically, this procedure labels approximately 95% of exposed NPCs. MSCs were genetically labeled to express eGFP using a lentiviral construct. NPCs were transplanted onto different regions of hippocampal slice cultures either alone or in combination with MSCs. One week later, the slices were analyzed for survival of transplanted cells, as well as for differentiation, in the case of transplanted NPCs.

MSCs were found to be present in the slice 7 days after transplantation indicating that a significant number of MSCs have survived on/in the slice. The cells were not evenly distributed but appeared in clusters suggesting that they did not disperse uniformly in/on the slice (Fig. 2 A). In addition, MSCs were predominantly detected at the slice surface, only very few migrated deeper into the tissue. Due to the lineage relation of MSCs with macrophages/microglia, we analyzed the transplanted MSCs for the presence of the microglia markers Iba1. Confocal analysis revealed that none of the MSCs was found to express Iba1, indicating that MSCs did not differentiate into microglia under this condition (Fig. 2 B).

NPCs, as indicated by the BrdU label, were primarily found in the CA3 region of the hippocampal slice, while some cells were detected also in CA1 and in the dentate gyrus (Fig. 2 C, D). Quantitative analysis of BrdU labeled cells indicated that almost 50% of the transplanted NPCs survived regardless of whether the cells had been transplanted alone or together with MSCs (Fig. 2 E). Active caspase-3 labeling of the BrdU-positive cells suggests that some of the transplanted NPCs underwent apoptosis (Fig. 2 F). Apoptotic and surviving transplanted NPCs co-localized within the same regions, suggesting that there were no regional hot-spots of cell death or survival.

Next, we analyzed the fate of grafted NPCs. Confocal analysis was used to identify and to quantify the percentage of BrdU-positive cells, which co-labeled either with MBP or GST-pi as markers for oligodendrocytes, MBP is an important component of myelin sheath [15] and it has been used as a marker particular for mature oligodendrocytes [16], or with GFAP as marker for astrocytes. When NPCs were transplanted alone, most of the NPCs differentiated into GFAP expressing cells (Fig. 3 L), and only a minor population expressed MBP (Fig. 3 J). In contrast, NPC/MSC co-transplantations generated predominantly cells expressing the oligodendroglial markers MBP and GST-pi (Fig. 3 J and K), while less cells were found to be GFAP-immunoreactive (Fig. 3 L). Neuronal fate of transplanted NPCs was analyzed using the young immature neuronal marker DCX. We could not detect BrdU-labeled cells that co-expressed DCX in any of the transplantation paradigms, suggesting that neuronal differentiation of transplanted NPCs in/on the hippocampal slice was absent. In addition, none of the MSCs (GFP-positive cells) was found to express GFAP, MBP or DCX, indicating that MSCs do not differentiate into astrocytes,

oligodendrocytes or neurons under this condition.

Discussion

The present report substantiates our previous finding that MSCs provide a source of potent factors promoting oligodendroglial fate, differentiation and maturation of adult rodent NPCs [8, 9]. While NPCs alone differentiated predominantly into astrocytes after being transplanted on hippocampal slices, the co-transplantation of NPCs together with MSCs favored oligodendrogenesis of the transplanted NPCs. Thus, MSCs provide a pro-oligodendrogenic microenvironment for transplanted NPCs.

The underlying mechanisms and the molecules involved in the MSC-oligodendrogenic activity are currently unknown. However, we could recently exclude transforming growth factor-beta1, sonic hedgehog, noggin, fibroblast growth factor, vascular endothelial growth factor, epidermal growth factor, ciliary neurotrophic factor, brain derived neurotrophic factor, neurotrophin-3, interleukin-6, thyroid hormone, and the signaling molecules leukotrienes and UDP-glucose as potential candidates for the MSCs-derived oligodendrogenic activity [8, 9]. The identification of the molecular nature of the MSC-derived oligodendrogenic activity could be crucial for the development of molecular therapies. In the meantime, the intraparenchymal or intracerebroventricular infusion of non-fractionated MSC-conditioned medium had been considered as an alternative strategy. However, a recent study demonstrated that MSC-derived conditioned medium had a toxic effect on hippocampal neurons [17], thus confuting the feasibility such an approach.

A cellular approach via the *ex vivo* generation and transplantation of pre-mature oligodendrocytes seems to be a reasonable alternative to a molecular therapy. However, the optimal cell type for such intervention is still a matter of debate. In the present work, NPCs that were pre-differentiated towards oligodendrocytes (pre-mature) failed to survive grafting procedures and/or failed to integrate into the host tissue. This is consistent with previous studies, in which post-mitotic oligodendrocytes as well as oligodendrocyte-precursor cells (OPCs) have been used to promote remyelination in rodent models of focal CNS demyelination. Oligodendrocytes were rather ineffective in remyelination of chemically-induced demyelinated areas [18-20], whereas OPCs provided some cellular repair [20-22]. Similarly, transplantation of adult human OPCs into newborn shiverer mice resulted in

oligodendroglial differentiation and widespread remyelination [23]. Thus, progenitors grafting rather than pre-differentiated or mature oligodendrocytes seem to be better suited for cell transplantation approaches aiming for re-myelination.

MSC transplantation has been recently proposed as a new cell-based therapy for several CNS neurological disorders such as MS. Although the mechanism(s) underlying the therapeutic effects of MSCs are still under investigation, there are evidences that (i) MSCs secrete a number of different cytokines, chemokines and growth factors providing trophic activities [24]. Transplanted MSCs might therefore influence the local host microenvironment and modulate the molecular properties of host tissue. (ii) MSCs modulate and suppress the immuno-response. They inhibit T cell proliferation, induce regulatory T cells, and suppress inflammation by secreting anti-inflammatory molecules [25]. Indeed, there is a growing body of evidence supporting that MSCs protect the CNS from autoimmune demyelination via an immunomodulatory mechanism [25-27]. In addition, (iii) MSCs might regulate the proliferation, apoptosis, migration and differentiation of endogenous stem/progenitor cells [24, 28]. The present report indicates that the MSCs activities

are not restricted to endogenous stem/progenitor cells, since MSCs also had the capacity to define the differentiation fate of transplanted NPCs.

In summary, MSCs provide a regenerative microenvironment for damaged neural tissues by releasing bioactive molecules that can suppress the immuno-response and improve that regeneration potential of endogenous and transplanted progenitors.

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