Human Adult White Matter Progenitor Cells Are Multipotent Neuroprogenitors Similar to Adult Hippocampal Progenitors

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ABSTRACT

Adult neural progenitor cells (aNPC) are a potential autologous cell source for cell replacement in neurologic diseases or for cell-based gene therapy of neurometabolic diseases. Easy accessibility, long-term expandability, and detailed characterization of neural progenitor cell (NPC) properties are important requisites for their future translational/clinical applications. aNPC can be isolated from different regions of the adult human brain, including the accessible subcortical white matter (aNPCWM), but systematic studies comparing long-term expanded aNPCWM with aNPC from neurogenic brain regions are not available. Freshly isolated cells from subcortical white matter and hippocampus expressed oligodendrocyte progenitor cell markers such as A2B5, neuron-glial antigen 2 (NG2), and oligodendrocyte transcription factor 2 (OLIG2) but at a small fraction of cells, 1 of 694 cells from white matter and 1 of 1,331 hippocampal cells, was able to generate neurospheres. Studies comparing subcortical aNPCWM with their hippocampal counterparts showed that both NPC types expressed mainly markers of glial origin such as NG2, A2B5, and OLIG2, and the NSC/NPC marker Nestin, but no pericyte markers. Both NPC types were able to produce neurons, astrocytes, and oligodendrocytes in amounts comparable to fetal NSC. Whole transcriptome analyses confirmed the strong similarity of aNPCWM to aNPChip. Our data show that aNPCWM are multipotent NPC with long-term expandability similar to NPC from hippocampus, making them a more easily accessible source for possible autologous NPC-based treatment strategies.

INTRODUCTION

Neural progenitor cells (NPC) isolated from the adult human brain have been the focus of increased attention during the last years because of their potentials in autologous cell replacement for neurologic diseases such as Parkinson’s disease, stroke, or in cell-based gene therapy of neurometabolic diseases. Human adult NPC (aNPC) can be isolated from known neurogenic regions such as the hippocampus [1–3] as well as from nonneurogenic regions such as subcortical white matter [4, 5] or cortex [6, 7]. aNPC were mainly expanded as neurospheres, which contain neuroprogenitors but also a certain number of differentiated neurons and glial cells [8]. Neurospheres are believed to act as stem cell niches. A niche provides both conceptual and physical depth to the stem cell and has anatomical as well as functional dimension that enables stem cells to reproduce and self-renew [9]. Neurospheres are thus creating a microenvironment harboring different cell types that support themselves mechanically and biochemically by sending instructive and neurotrophic signals [10].

In contrast to fetal neural stem cells (fNSC), data on human aNPC are very limited and systematic characterization are lacking, particularly concerning comparative studies of NPC from different brain regions [11]. Human aNPC were successfully isolated and cultivated from white matter and hippocampal tissue. These cells are only expandable for several passages. It is not yet known whether human aNPC from known neurogenic regions as the hippocampus behave differently compared with NPC from subcortical white matter. The white matter cells would be much more interesting for autologous cell-based treatment approaches because the isolation is less harmful for the patients. A recent study by Knoth and colleagues showed for the first time that human hippocampal neurogenesis is very
similar in vivo to the well-described murine neurogenesis [12]. However, it is not entirely known which cell type from hippocam-
pal tissue is the source of hippocampal aNPC (aNPC<sub>hip</sub>) and how these behave in vitro compared with white matter NPC (aNPC<sub>WM</sub>) [1, 3, 13, 14].

In this study, we systematically compare neurosphere cultures derived from human adult neural tissue isolated from two different brain regions, the subcortical white matter and the hippocampus, asking whether aNPC<sub>WM</sub> behave similarly to aNPC<sub>hip</sub>, thus making these more easily accessible cells a primary cell source for potential autologous NPC-based therapeutic approaches.

## MATERIALS AND METHODS

### Isolation and Propagation of Multipotent NPC

Adult human hippocampal (hip) and subcortical white matter (wm) tissue was obtained from routine epilepsy surgery procedures following informed consent of the subjects. All procedures were in accordance with the Helsinki convention and approved by the Ethical Committee of the University of Dresden (47032006). All subjects underwent high-resolution magnetic resonance imaging, excluding tumors, and were screened for the presence of infectious disease. In all cases the neuropathological examinations did not reveal evidences for tumor formation. The patient groups used for generation of the various adult NPC (aNPC) cultures did not show significant differences with respect to age ($F$ value, 0.099; $p = .960$; one-way analysis of variance [ANOVA]), gender ($p = .820$, Fisher’s exact test), histology/diagnosis ($p = .153$, Fisher’s exact test), or antiepileptic medication ($p = .708$, Fisher’s exact test). Demographic details of subjects, including diagnosis and antiepileptic drug therapy, are given in Table 1.

Figure 1A shows the experimental design and time scale. Tissue was taken and stored in ice-cold Dulbecco’s phosphate-buffered saline containing 10% glucose and 1% penicillin/streptomycin for transportation (maximum, 1 hour). Tissue samples were cut into small pieces, incubated in trypsin (2.5 mg/ml; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) for 20 minutes at room temperature, incubated in DNase (40 μg/ml; Sigma-Aldrich) for 15 minutes at 37°C, and homogenized to a quasi-single-cell suspension by gentle triturating. Cells were plated onto poly-α-lysine-coated chamber slides, kept for 4 hours under expansion conditions, and processed for immunofluorescence stainings.

For expansion of neurospheres, cells were added to 25-cm<sup>2</sup> flasks in knockout Dulbecco’s modified Eagle’s medium (Gibco BRL, Life Technologies, Tulsa, OK, http://www.invitrogen.com), supplemented with 10% serum replacement, 2% B-27 supplement, 1% Glutamax, 1% penicillin/streptomycin (all from Gibco), and 20 ng/ml of both epidermal growth factor (EGF) and fibroblast growth factor-2 (both from Sigma-Aldrich) at 5% CO<sub>2</sub>, 92% N<sub>2</sub>, and 3% O<sub>2</sub> using an incubator equipped with an O<sub>2</sub>-sensitive electrode system (Heraeus; Thermo Scientific, Bremen, Germany, http://www.thermoscientific.com). Neurospheres were cultured, as described previously [1]. Human fetal neural stem cells (NSC) were generated, as described previously [16].

Sphere-forming assays were done as introduced by Lefkovits [17] and recently described in detail [18]. Primary cells were plated at reducing densities in 96-well plates with 200 μl per well in the above-described expansion medium. Cell suspensions were diluted by serial 1:2 dilutions, and cells were plated at 10,000–20,000 cells per well. After 7 days in vitro, the fraction of wells negative for neurosphere formation was quantified. These data were then log transformed and plotted against plating density. A linear regression was performed and a straight line passing through zero fitted to the data (including the 95% confidence intervals [95% CIs]) using the GraphPad Prism 5 software. The intercept of log (37%) gave the frequency of cells capable of proliferating to form a neurosphere (see [17] for the theory underlying this procedure).

Mesenchymal stem cells were cultured as described by [19, 20], and adult human pericytes as reported by [21].

### Differentiation

Neurosphere cultures of aNPC were plated onto poly-α-lysine coated slides in expansion medium without mitogens. Induction of differentiation of fetal NSC was initiated by plating them onto poly-α-ornithine and fibronectin-coated dishes in Neurobasal medium (Gibco) containing 2% B-27 supplement, 1% Glutamax, 1% penicillin/streptomycin, 10 μM forskolin (Sigma-Aldrich), and 100 μM AMP (Sigma-Aldrich). Cells were differentiated for 14 days before immunocytochemical analysis.

### Immunocytochemistry

Cell cultures were fixed with Accustain (Sigma-Aldrich) for 30 seconds, washed with phosphate-buffered saline (PBS), and blocked for 2 hours with PBS containing 0.2% Triton X-100 and 3% donkey serum. Cultures were incubated with primary antibodies overnight at 4°C, followed by secondary fluorescence-conjugated antibodies for 1 hour at room temperature. Cell nuclei were counterstained with Hoechst 33342 (Invitrogen, Grand Island, NY, http://www.invitrogen.com). The following primary antibodies were used: mouse anti-A2B5 1:250, mouse anti-GaIC 1:750, mouse anti-Nestin 1:500, mouse anti-neuron-glia antigen 2 (NG2) 1:200, rabbit anti-EgFr 1:100, rabbit anti-Olig2 1:500, mouse anti-NeuN 1:200 (all from Millipore, Schwalbach, Germany, http://www.millipore.com); rabbit anti-NG2 1:200, rabbit anti-CD133 1:500, rabbit anti-platelet-derived growth factor receptor α (PDGFrα) 1:500, chicken anti-αlglial fibrillary acidic protein (GFAP) 1:1,000, rabbit anti-GLAST 1:500, rabbit anti-Pax6 1:100 (all from Abcam, Cambridge, U.K., http://www.abcam.com); mouse anti-Map2ab 1:400, rat anti-CD5 1:200 (both from BD Biosciences, Bedford, MA, http://www.bdbiosciences.com); mouse anti-Tuj1 1:500, rabbit anti-β-Tubulin III 1:2,000 (both from Covance, Richmond, CA, http://www.covance.com); mouse anti-Sox2 1:500 (R&D Systems, Minneapolis, MN, http://www.rndsystems.com); mouse anti-fibronectin 1:500, mouse anti-O4 1:500, mouse anti-Vimentin 1:1,000 (Sigma-Aldrich); goat anti-

DCCX 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com); rabbit S100β 1:1,500 (Swant, Bellinzona, Switzerland, http://www.swant.com); mouse anti-CC1 1:50 (Merck, Darmstadt, Germany, http://www.merck.com); and rabbit anti-Ki67 1:500 (Novocastra Ltd., Newcastle upon Tyne, U.K., http://www.novocastra.co.uk). The secondary antibodies conjugated to Alexa 488, 555, 594, and 647 1:500 were obtained from Invitrogen (Carlsbad, CA, http://www.invitrogen.com). The following primary antibodies were used: mouse anti-A2B5 1:250, mouse anti-GaIC 1:750, mouse anti-Nestin 1:500, mouse anti-neuron-glia antigen 2 (NG2) 1:200, rabbit anti-EgFr 1:100, rabbit anti-Olig2 1:500, mouse anti-NeuN 1:200 (all from Millipore, Schwalbach, Germany, http://www.millipore.com); rabbit anti-NG2 1:200, rabbit anti-CD133 1:500, rabbit anti-platelet-derived growth factor receptor α (PDGFrα) 1:500, chicken anti-αlglial fibrillary acidic protein (GFAP) 1:1,000, rabbit anti-GLAST 1:500, rabbit anti-Pax6 1:100 (all from Abcam, Cambridge, U.K., http://www.abcam.com); mouse anti-Map2ab 1:400, rat anti-CD5 1:200 (both from BD Biosciences, Bedford, MA, http://www.bdbiosciences.com); mouse anti-Tuj1 1:500, rabbit anti-β-Tubulin III 1:2,000 (both from Covance, Richmond, CA, http://www.covance.com); mouse anti-Sox2 1:500 (R&D Systems, Minneapolis, MN, http://www.rndsystems.com); mouse anti-fibronectin 1:500, mouse anti-O4 1:500, mouse anti-Vimentin 1:1,000 (Sigma-Aldrich); goat anti-

DCCX 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com); rabbit S100β 1:1,500 (Swant, Bellinzona, Switzerland, http://www.swant.com); mouse anti-CC1 1:50 (Merck, Darmstadt, Germany, http://www.merck.com); and rabbit anti-Ki67 1:500 (Novocastra Ltd., Newcastle upon Tyne, U.K., http://www.novocastra.co.uk). The secondary antibodies conjugated to Alexa 488, 555, 594, and 647 1:500 were obtained from Invitrogen, and those conjugated to Rhodamine Red-X and Dylight 649 1:200 were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, http://www.jacksonimmuno.com). Images were captured using the Leica DM IRE2 (Leica, Heerbrugg, Switzerland, http://www.leica.com).
The number of cells immunoreactive for a given marker was determined by counting the number of positive cells in relation to the number of Hoechst-stained nuclei. In a typical experiment, 500–1,000 cells were counted within randomly selected fields per well. Statistical comparisons were made using the ANOVA model and post hoc test with Bonferroni adjustment, as appropriate. All data are presented as mean ± SEM.

**Table 1. Donor characteristics**

| No. | Age (years) | Gender | Donor tissue type | Diagnosis/indication/surgery | Antiepileptic medication |
|-----|-------------|--------|-------------------|-----------------------------|--------------------------|
| 1   | 34          | Female | White matter      | Ganglioglioma WHO I°         | OXC 1,200 mg             |
| 2   | 56          | Female | White matter      | AHS/anterior temporal lobectomy + AHE | LEV 5,500 mg, OXC 300 mg, CBZ 450 mg |
| 3   | 27          | Female | White matter      | Complex malformation with FCD 1+2 | OXC 1,200 mg             |
| 4   | 46          | Female | White matter      | FCD 2                        | OXC 2,850 mg             |
| 5   | 38          | Male   | White matter      | Angioma                      | LEV 1,500 mg             |
| 6   | 49          | Female | White matter      | AHS                         | LTG 500 mg               |
| 7   | 7           | Female | White matter      | FCD 2                        | OXC 720 mg, TPM 200 mg, LTG 200 mg |
| 8   | 53          | Male   | White matter      | Astrogliosis, residues from brain bleeding | LTG 300 mg             |
| 9   | 33          | Male   | White matter      | Cavernoma                    | LEV 2,000 mg             |
| 10  | 61          | Female | White matter      | Cavernoma                    | TPM 300 mg, PHT 225 mg, OXC 1,200 mg |
| 11  | 55          | Female | White matter, hippocampus | FCD 1+2/anterior temporal lobectomy + AHE | LTG 400 mg             |
| 12  | 29          | Male   | White matter, hippocampus | FCD 1b                      | OXC 2,100 mg, LCM 400 mg |
| 13  | 35          | Male   | White matter      | AHS                         | TPM 150 mg               |
| 14  | 42          | Male   | White matter      | Cavernoma                    | LEV 2,000 mg             |
| 15  | 52          | Male   | White matter, hippocampus | FCD 1+2, AHS                | LCM 300 mg, LEV 2,000 mg, TPM 300 mg |
| 16  | 25          | Male   | Hippocampus       | AHS                         | LTG 300 mg               |
| 17  | 39          | Female | Hippocampus       | AHS/anterior temporal lobectomy + AHE | LTG 200 mg, OXC 1,800 mg, CLB 10 mg |
| 18  | 23          | Male   | Hippocampus       | FCD 2, AHS                  | LEV 3,000 mg             |
| 19  | 52          | Male   | Hippocampus       | AHS                         | LCM 350 mg, ZON 300 mg   |
| 20  | 49          | Male   | Hippocampus       | AHS, FCD 1+2               | LCM 500 mg, LEV 1,500 mg |
| 21  | 32          | Male   | Hippocampus       | AHS, FCD 2                 | LTG 350 mg               |
| 22  | 50          | Male   | Hippocampus       | AHS, FCD 1                 | LCM 300 mg, LEV 3,500 mg |
| 23  | 44          | Male   | Hippocampus       | Cavernoma                   | LTG 300 mg               |
| 24  | 20          | Female | Hippocampus       | AHS, FCD 1                 | LEV 3,000 mg             |
| 25  | 34          | Male   | Hippocampus       | AHS, FCD 1+2               | LTG 500 mg               |
| 26  | 59          | Male   | Hippocampus       | AHS, FCD 1+2               | LTG 400 mg               |
| 27  | 56          | Female | Hippocampus       | AHS, FCD 1+2               | LEV 3,500 mg             |

Abbreviations: AHE, amygdaohippocampactomy; AHS, Ammon’s horn sclerosis; CBZ, carbamazepine; CLB, clobazam; FCD, focal cortical dysplasia classified according to [15]; LCM, lacosamide; LEV, levetiracetam; LTG, lamotrigine; OXC, oxcarbazepine; PHT, phenytoin; TPM, topiramate; ZON, zonisamide; WHO, World Health Organization.

**Cell Counting and Statistics**

The number of cells immunoreactive for a given marker was determined by counting the number of positive cells in relation to the number of Hoechst-stained nuclei. In a typical experiment, 500–1,000 cells were counted within randomly selected fields per well. Statistical comparisons were made using the ANOVA model and post hoc test with Bonferroni adjustment, as appropriate. All data are presented as mean ± SEM.

**RNA Extraction and Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis**

For gene expression analysis, a small piece of the sample was removed before starting the tissue preparation and immersed into RNAlater (Ambion, Austin, TX, http://www.ambion.com) solution. Total tissue RNA was extracted using the RNeasy Lipid Tissue mini kit (Qiagen, Hilden, Germany, http://www.qiagen.com), according to the manufacturer’s protocol. Total cellular RNA was extracted from human neural stem and progenitor cells during expansion using RNeasy total RNA purification kit, followed by treatment with RNase-free DNase (Qiagen). Quantitative real-time one-step reverse transcription-polymerase chain reaction (RT-PCR) was carried out using the LightCycler System from Stratagene (La Jolla, CA, http://www.stratagene.com) and the QuantiTect SYBR Green polymerase chain reaction (PCR) kit from Qiagen. PCR conditions were as follows: 25 minutes at 50°C, 15 minutes at 95°C; 45 cycles of 15 seconds at 94°C, 20 seconds at 55°C, 30 seconds at 72°C, 1 minute at 95°C; and 1 cycle of 30 seconds at 55°C. To demonstrate product specificity, melting curves of the RT-PCR products were acquired and an agarose gel electrophoresis was performed to confirm the product length. Primer sequences and lengths are available from the authors upon request.

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Figure 1. Isolation of multipotent human aNPC from white matter and hippocampal tissue. (A): Schematic representation of experimental design and time course. (B): Representative stainings of freshly isolated cells from white matter tissue. Nuclei were counterstained with Hoechst dye. Scale bars = 100 μm. (C): Quantitative analysis of marker protein expression in freshly isolated cells from white matter or hippocampal tissue. No cells with marker expression were detected for CD133 (Prominin1), SOX2, PAX6, GALC, O4, GFAP, S100b, glutamate aspartate transporter, polysialylated neural cell adhesion protein Vimentin, and fibronectin. *p < .05 when compared with white matter tissue (two-sided t test). (D): Quantitative transcription profile of the tissue samples. qRT-PCR analyses were performed for marker genes of neural stem cells (SOX2, MSI1, NESTIN, MKI67), glial lineage (CSPG4, OLIG2, PDGFRα, GALC, GFAP), and neuronal lineage-restricted progenitors (SOX1, PAX6, TUBB3). Results are mean values ± SEM from at least three independent experiments. *p < .05 when compared with white matter tissue (two-sided t test). Limiting dilution assay for primary neurospheres derived from white matter (E) and hippocampus (F). Data are shown as mean ± SEM (n = 3–5), and dotted lines represent the 95% confidence interval (95% CI). The intercept of log (37% negative wells) gave the neurosphere-forming frequency. Insets show representative photographs of respective primary neurospheres. Note the statistical significant difference in neurosphere-forming capacity between the two tissues. Scale bars = 100 μm. Abbreviations: CI, confidence interval; EGFR, epidermal growth factor receptor; GALC, galactocerebroside; GFAP, glial fibrillary acidic protein; HMBS, hydroxymethylbilane synthase; NG2, neuron-glial antigen 2 (chondroitin sulfate proteoglycan 4); OLIG2, oligodendrocyte transcription factor 2; PDGFRα, platelet-derived growth factor receptor; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.
Figure 2. Expansion of multipotent human adult neural progenitor cells (aNPC) from white matter and hippocampal tissue. Human aNPC from white matter and hippocampus were cultured in suspension (neurosphere) culture for up to 6 weeks and stained for various marker proteins. aNPC were compared with human fetal neural stem cell (NSC) as a multipotent NSC control. (A): Representative selection of stainings for typical marker proteins in aNPCWM, aNPCHIP, and fNSC. Nuclei were counterstained with Hoechst dye. Scale bars = 100 μm. (B–D): Quantitative analysis of marker proteins. Cells were stained against NSC and proliferation markers (CD133, SOX2, Nestin, KI67), markers for oligodendroglial progenitor cells (A2B5, NG2, OLIG2, PDGFRα), and differentiated cells (β-Tubulin III, MAP2ab, GALC, GFAP). One-way analysis of variance revealed statistical significant differences for CD133 (F value: 12.00; p = .008), SOX2 (F value: 59.31; p < .001), Nestin (F value: 17.16; p < .001), KI67 (F value: 15.85; p = .002), OLIG2 (F value: 6.46; p = .032), and β-Tubulin III (F value: 7.33; p = .011). Results are mean values ± SEM from at least three independent experiments. *, p < .05; **, p < .01; ###, p < .001 when compared with aNPCWM (post hoc Bonferroni adjusted t test). #, p < .05; ##, p < .01; ###, p < .001 when compared with aNPCHIP (post hoc Bonferroni adjusted t test). (E): aNPC do not express the pericyte marker SMA. Depicted are representative images of aNPC, MSC, and adult human brain-derived pericytes. Magnification ×200. Abbreviations: aNPCWM, hippocampal adult neural progenitor cell; aNPCHIP, white matter adult neural progenitor cell; fNSC, fetal neural stem cell; GALC, galactocerebroside; GFAP, glial fibrillary acidic protein; MAP2, mitogen-activated protein 2; MSC, mesenchymal stem cell; NG2, neuron-glial antigen 2; OLIG2, oligodendrocyte transcription factor 2; PDGFR, platelet-derived growth factor receptor; TubIII, β-Tubulin III.
request. The results are expressed relative to the housekeeping gene HMBS (hydroxymethylbilane synthase).

**Gene Expression Microarray Analysis**

For the gene expression microarray analysis, we used the Affymetrix U133A chips containing 22,215 probe sets representing at least 12,905 individual genes. The whole procedure was performed following the manufacturer’s standard protocol (Affymetrix, Santa Clara, CA, http://www.affymetrix.com). For the data processing, normalization was calculated with the GC content-corrected Robust Multiblue Analysis algorithm [22]. Data post-processing and graphics were performed with in-house developed functions in MATLAB. Hierarchical clustering of genes and samples was performed with one minus correlation metric and the nonweighted average distance (UPGMA, also known as group average) linkage method. The gene ontology terms were taken from the AmiGO Gene Ontology database [23].

**Electrophysiology**

Cells were investigated for membrane currents between days 20 and 30 of differentiation using the standard whole-cell patch-clamp technique with an EPC-9 amplifier and PulseFit software (HEKA, Lambrecht, Germany, http://www.heka.com), as described previously [16]. In brief, the external bath solution contained (in mM) the following: 142 NaCl, 1 CaCl₂, 8 KCl, 6 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4; 320 mOsm). Electrodes had resistances of 2–4 MΩ when filled with the internal solution containing (in mM): 153 KCl, 1 MgCl₂, 10 HEPES, 5 EGTA, and 2 MgATP (pH 7.3; 320 mOsm). Whole-cell currents were low-pass filtered at 1–5 kHz, digitized at 10 kHz, and analyzed with PulseFit (HEKA) and GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, http://www.graphpad.com). Membrane potentials were measured in current-clamp mode. Numerical data of all experiments were expressed as mean ± SEM and statistical significance using Student’s t test (two-sided, unpaired).

**RESULTS**

**Freshly Isolated aNPC<sub>WM</sub> Show Similar Characteristics as aNPC<sub>CHIP</sub>**

To assess differences of aNPC<sub>WM</sub> and aNPC<sub>CHIP</sub> within the isolates, we analyzed the cells directly after isolation by immunocytochemistry. As shown in Figure 1B and 1C, approximately 20% of freshly isolated cells from both tissues were positive for oligodendrocyte progenitor cell markers A2B5, NG2, and oligodendrocyte transcription factor 2 (OLIG2). EGF receptor (EGFR) and PDGFRα were less frequently expressed with differences between white matter and hippocampal cell isolates only for EGFR+ cells. Single-labeled images for better visualization of the coexpression are provided in supplemental online Figure 1. The number of NG2+ cells derived from hippocampal tissue (25% ± 5%) was significantly elevated compared with white matter tissue (18% ± 5%; p = .039, two-sided t test). Double immunostainings revealed in general only very few NG2+ OLIG2+ cells (2.6%–4.2%) and EGFR+/PDGFRα+ cells (0.7%–1.0%), but 12.8%–17.2% A2B5+/OLIG2+ cells and 4.9%–9.8% A2B5+/NG2+ cells with no differences between white matter and hippocampal cell isolates (supplemental online Fig. 2). Quantitative RT-PCR (qRT-PCR) analyses of known NSC genes as well as for genes encoding for the glial and neuronal lineage did not show any difference between hippocampus or white matter origin except—as expected—for the oligodendrocyte marker gene galactocerebroside (GALC) with higher values in white matter tissue (Fig. 1D). We found high levels for genes of the glial lineage, which was consistent with the findings in the immunostainings. Data also revealed high levels of the NSC marker SOX2 and the early neuroectodermal markers NESTIN and MS11 (encoding for Musashi1). Within the neural lineage we found low levels of the proneuronal gene SOX2 but elevated levels of the neuronal gene TUBB3.

Quantitative sphere-forming assays as introduced by Lefkowitz [17] were carried out to determine the number of isolable aNPC (Fig. 1E). This assay showed that 1 of 694 cells (95% CI: 528–1,000) within the white matter cell suspension had the ability to form a sphere, whereas only 1 of 1,331 cells (95% CI: 1,052–1,816) within the hippocampal cell suspension was able to form a sphere (significant difference with p = .046).

**aNPC<sub>WM</sub> and aNPC<sub>CHIP</sub> Depict Very Similar Marker Protein Expression Patterns**

After initial characterization of freshly isolated aNPC, we cultured aNPC from both tissues as neurosphere suspensions (aNPC<sub>WM</sub> and aNPC<sub>CHIP</sub>). Human fNSC cultured as neurosphere suspensions (n = 5) were used to compare aNPC with multipotent tissue-specific NSC. After 4 weeks in vitro, suspension cultures formed neurospheres so that these could be analyzed on protein and gene expression levels (Figs. 2, 3). We first analyzed protein expression of established stem cell, oligodendrocyte progenitor, and differentiation markers. Fetal NSC were much easier to propagate, showing significantly more KI67+ cells compared with both aNPC types. Interestingly, CD133 and SOX2 protein could only be detected in INSC but not in aNPC cultures. Next, we analyzed the oligodendrocyte progenitor markers A2B5, NG2, OLIG2, and PDGFRα [24, 25]. We observed more NG2+ cells in both aNPC types compared with fNSC, whereas no differences were found in A2B5 and OLIG2 expression. Relevant amounts of PDGFRα+ cells were only detected in fetal NSC. In neurosphere cultures, we did not find any expression of phenotypic markers currently used to identify pericytes, such as regulator of G protein-signaling 5 selectively expressed in pericytes in brain capillaries [26] and α-smooth muscle actin [21, 27] (Fig. 2E).

Concerning spontaneous differentiation within the cell systems, we were able to identify significantly more immature β-Tubulin III+ cells in both aNPC cultures compared with fNSC, whereas a number of GFAP+ astrocytes were similar. Mitogen-activated protein 2 (MAP2) and GALC were not detected in any culture. Notably, aNPC<sub>WM</sub> and aNPC<sub>CHIP</sub> did not significantly differ in their marker protein expression pattern (Fig. 2).

**aNPC<sub>WM</sub> and aNPC<sub>CHIP</sub> Depict Very Similar Transcriptome Expression Profiles**

We comparatively analyzed the whole-genome gene expression profile of aNPC from both tissues to evaluate their differences within the entire transcriptome. The global gene expression heat map indicates a close transcriptomics fingerprint of both aNPC types (Fig. 3A). Accordingly, both hierarchical clustering analyses and pairwise scatter plots displayed that aNPC<sub>WM</sub> closely resembled aNPC<sub>CHIP</sub>, but both aNPC clearly differed from fNSC (Fig. 3B, 3C). The transcriptome of aNPC<sub>WM</sub> and aNPC<sub>CHIP</sub> differed only in 232 genes (details are given in Table 2). Of note, both aNPC types showed similar expression levels of NSC markers, such as OLIG2,
SOX2, and MASH1/ASCL1. The transcriptomes of aNPCWM and fNSC differed significantly, as previously reported for aNPCHPI [13], but both cell types overexpressed other genes commonly associated with a neural stem/progenitor cell fate, such as Jagged 1 (JAG1) [28], SOX2 [29], SOX4 [30], Nestin (NES) [31], OLIG2 [32], and the G protein-coupled receptor 56 (GPR56) [33]. The NSC marker Musashi1 (MS1 [34]) was also highly expressed in aNPC and fNSC.
Table 2. The 232 genes differentially expressed in white matter adult neural progenitor cells versus hippocampal adult neural progenitor cells

| Category                  | Genes                                                                 |
|---------------------------|-----------------------------------------------------------------------|
| Signaling (7.86%)         | BST2, SFRP1, SOX9, STC1, GAD1, GRIA2, WNT5A, CXCL6, GPR56, WISP1, SALL1, ABAT, GABBR2, PLP1, FZD2, KCNQ3, GREM1, GDP15 |
| Extracellular matrix (18.34%) | SPARC1, THBS1, IGFBP7, COL6A3, TGFβ1, LUM, EFEMP1, DNM, NICD1, SFRP1, COL1A1, COL1A2, PCOLCE, SERpine1, LTBP1, FN1, FN1, LOXL1, NID2, FLRT2, PTPRZ1, CHL1, LTBP2, CLEC3B, WNT5A, COL2A1, COL1A1, CTGF, COL6A2, TFAP2C, OPC1, SPON2, CPZ, LAMBI, COL6A1, COL5A1, BGN, COL5A1, LAMA2, CD248, BCAN, COL5A2 |
| Cell membrane (26.64%)    | DSP, SDCl, IFITM2, EMP1, COL6A2, TGFβ1, MYOF, SFRP1, SVIL, SERpine1, CAV1, GPRCSA, CD4, GPRCSB, SLAC4A4, MAPT, FZD6, EDNRB, PROM1, RGS4, CHL1, PTGER4, VAMP5, SPPN, GAD1, GRIA2, PLAU, BAI3, PCDH17, WNT7A, GPR56, OXTR, OMG, TGFβR2, MCAM, CTGF, COL6A2, GMP6B, GMP6A, TSPAN4, GMP6A, SMAPG, CXL12, CD24, GABBR2, FZD2, COL1A3, CXC4, COL6A1, NCAM1, ADAM12, BGN, IFITM1, ITGA8, KCNQ3, LAMA2, STEAP3, FLVCR2, THY1, GPR124, NLG4X |
| Cytoskeleton (3.06%)      | DSP, SVIL, HIP1, TRIM9, MICAL2, PPP2R2B, MLPH                         |
| Receptor activity (14.41%) | ENG, EFERMP1, SFRP1, SD4, LTBP1, ANPEP, LOXL2, GPPRSA, CD4, GPRCSB, SEMA3C, FZD6, EDNRB, PTPRZ1, PTGER4, PDGFR, GRIA2, BAI3, GPR56, OXTR, KDEL3R, TGFβ2R, GABBR2, FZD2, TYRO3, CXC4R, PTTP4, ITGA8, PAQR6, COLEC12, NTRK2, GPR124, NLG4X |
| Metabolic processes (35.81%) | DSP, OX8, THBS1, SDC1, STAT6, EFEMP1, SRGN, DCM, SFRP1, NNMT, COL1A1, IGF2, CYP1B1, CTSG, PCOLCE, CRMP1, PAX9A, IL8, DAP2, CAV1, CYBA, PAPP1, CAV1, GATM, CD4, LOXL1, GCH1, NNAT, PROM2, COX7A1, AOX1, GAD1, HIP1, PLAU, WNT5A, LHX2, RET1, SALL1, NXX2-2, ASS1, CITED1, RBMS1, MEIS2, PTGIS, PTF, TGFβR2, CGF, PLAG1, ABAT, CTNFND22, CD24, TRIM9, GLT252D2, FAP, IGBP53, PLP1, CPZ, PDE4B, TYRO3, HOPX, CJC4R, MICAL2, COL5A1, TRIM22, PTPRD, PAM1, ADAM12, CFH, BGN, UBB2, FUT9, COL5A1, LOX, HEY1, OSE, FKB2111, 3T66GA05, IMA1, NTRK2 |
| Developmental processes (50.66%) | DSP, OX8, THBS1, IGFBP7, DAB2, SDC1, EMP1, STAT6, COL6A3, TGFβ1, KRT18, BST2, LUM, ENG, SRGN, DCM, SFRP1, SD4, NNMT, COL1A1, COL1A2, IGF2, CYP1B1, PCOLCE, CRMP1, SVIL, SERpine1, FN1, IL8, ANPEP, SOX9, LOXL2, PAPP52, CAV1, GATM, MFAP2, GFAP, CD4, SEMA3C, MAPT, FZD6, NNT, EDNRB, PROM1, PTPRZ1, CXC1, STC1, PTGER4, VAMP5, FABP7, CLEC3B, HIP1, SCRG1, PLAU, SRPX2, TAGLN, MWS1, WNT5A, FOX1, LHX2, FOX1D1, GDF1, GPR56, OXTR, SALL1, NXX2-2, ASS1, OMG, CITED1, MEIS2, CCN2, TGFβ2R, COL1A1, MCAM, CTGF, NDRG4, GMP6B, ID4, ECM1, GMP6A, PLEKHB1, CTNDN2, SIOOB, CXL12, CD24, PHDL2A, PLP |
| Cell adhesion (20.09%)    | DSP, THBS1, IGFPG7, COL6A3, TGFβ1, ENG, NICD1, CYP1B1, SOX9, LOXL2, TPbg6, CD4, NID2, FLRT2, CHL1, SVPN, NCAN, SRPX2, PCHD17, GPR56, WISP1, OMG, ISLR, COL1A1A, MCAM, CTGF, COL6A2, SPON2, CTNDN2, CXL12, CD24, PCHD1A, COL1A3, TYRO3, LAMBI, COL6A1, COL5A1, NCAM1, PTPRD, ADAM12, ITGA8, COL1A3, LAMA2, BCAN, THTHY1, NLG4X |
| Apoptotic processes (5.24%) | DSP, KRT18, SRGN, MAT, HIP1, CITED1, TGFβ2R, PHDL2A, CXC4R, PPP2R2B, STEAP3, GREM1 |
| Others (16.16%)           | OPTN, TSPAN7, KIFSC, RASSF2, IGFPG7, ARHGAAP29, PHACTR2, IFIL6, PLLP, TFF3, COL9A3, TRIL, PMPD2, FAM107A, MXRAS, CARD10, SYNRG1, CLEC11A, KIAA1199, WSCD1, ATRPDE02, RUFY3, C17orf91, TM4SF1, OLFLM1, LNX, BAALC, KLDHC8A, TMEM45A, COPZ2, CACNA2D3, C7orf58, STTNN4, GLTBD2, C1orf61, RNC |

qRT-PCR analyses of known NSC markers as well as for genes encoding for the glial and neuronal lineage confirmed the genome-wide profile analyses (Fig. 4). Even though not found on protein level, we could detect significant levels of CD133 and high levels of SOX2 expression in aNPCWm and aNPChip as well as fNSC. aNPC expressed significantly less PAX6 compared with fNSC (Fig. 4). Glial and oligodendrocyte precursor cell marker expression confirmed the results seen by the immunostainings (Figs. 2, 4).

aNPCWm Showed Multitropotential Differentiation and Functional Capacity Similar to aNPChip

Long-term expanded NPC (up to 12 weeks) were able to differentiate into the three major cell types of the central nervous system: neurons, oligodendrocytes, and astrocytes. For comparison of the differentiation potential of aNPCWm and aNPChip, the spontaneous differentiation potential was investigated by removal of mitogens. After 14 days of differentiation, we detected in aNPChip cultures 11% ± 2% β-Tubulin III immature neurons, 7% ± 4% mature MAP2ab+ neurons, 15% ± 7% GALC+ oligodendrocytes, and 10% ± 3% GFAP+ astrocytes (Fig. 5B). Same results were obtained for the hippocampal aNPCWm cultures. For fNSC cultures, we detected significantly more MAP2ab+ neurons and less GALC+ oligodendrocytes compared with both aNPC types. We did not observe coexpression of markers for different neural cell lineages. To prove that neurons acquired typical signs of neuronal functions, we performed patch-clamp analyses of neurons derived from aNPC, demonstrating large sodium inward currents of approximately 1 nA (mean maximum of all investigated cells was 317 pA; Fig. 5C, 5D). Almost half of the aNPC (6 of 13; 46%) were able to give rise to action potentials (Fig. 5E). The majority of NPC-derived neuronal cells were not fully mature, showing sodium current peak amplitudes below 200 pA and therefore not able to fire action potentials.

DISCUSSION

We report a detailed characterization of human aNPC cultures derived from white matter tissue showing that long-term expanded white matter aNPC are nearly identical to aNPC isolated from the hippocampus, the standard neurogenic brain region for aNPC isolation. Because tissue samples from the subcortical white matter tissue are relatively easy to obtain by neurosurgical intervention and the risk for the patients is much lower compared with surgeries on neurogenic brain regions such as the subventricular zone (SVZ) and hippocampus, aNPC generated from subcortical white matter have important potentials in translational medicine toward clinical applications. Although there are some studies...
dealing with either white matter or hippocampal derived human aNPC [1, 4–7, 14, 35–37], comparative studies of aNPCWM with aNPC derived from neurogenic regions are lacking. This comparison is of pivotal importance, because aNPCWM are generated from a non-neurogenic region, and the isolation efficacy and their multipotent stem cell performance remain enigmatic. In the present study, we did not detect relevant differences of proliferation, stem cell performance, and transcriptome profile between NPC derived from subcortical white matter and those from the neurogenic hippocampus, except an even higher number of isolable NPC within the white matter.

Approximately 20% of freshly isolated white matter and hippocampal cells were positive on the protein level for known OPC markers such as A2B5, NG2, and OLIG2 [24, 38], but we did not detect cells positive for the NSC markers CD133 (Prominin1), NESTIN, SOX2, or PAX6. qRT-PCR studies largely confirm these results, but with a major discrepancy in SOX2 expression, which was only observed on mRNA level in contrast to the protein level. The EGF receptor (EGFR) protein was expressed in 18% of white matter and 7% of hippocampal cells. Although this is not surprising for white matter-derived cells, taking into account that most of the cycling progenitor cells in the human adult brain are NG2+ and OLIG2+ [39], this was not expected for aNPCWM with its known neurogenic niche [12, 40]. Independent of this marker expression pattern, only a small fraction of 1 cell of 700–1,300 cells is able to generate neurospheres, showing—in agreement with the EGFR expression data—a higher neurosphere-forming capacity of the white matter tissue compared with hippocampus using EGF as a mitogen. This amount of neurosphere-forming cells is comparable to non-neurogenic periventricular brain regions of mice [18, 41, 42] but, interestingly, even higher compared with murine hippocampal tissue [41].

Expanded aNPC from both brain regions showed similar expression patterns of marker proteins for NPC, OPC, and differentiated neural cell markers and were positive for known oligodendrocyte progenitor cell markers such as A2B5, PDGF, NG2, and OLIG2 [24, 38]. This marker expression pattern is expected for aNPCWM but not for aNPCWM. The reason for the similar marker expression pattern of both aNPC might be that the NG2+ cells grow faster than the original aNPCWM so that the culture conditions might favor NG2+/OLIG2+ cells. However, a recent paper showed that the acquisition of NG2/OLIG2 expression is

**Figure 4.** Quantitative reverse transcription-polymerase chain reaction characterization of multipotent human adult neural progenitor cells (aNPC) from white and hippocampal tissue. Gene expression analysis of marker genes for neural stem/progenitor cells (A), glial lineage-restricted cells (B), and neuronal restricted progenitor cells (C) in aNPC and fNSC during expansion using quantitative reverse transcription-polymerase chain reaction. One-way analysis of variance revealed statistical significant differences for SOX2 (F value: 6.6; p = .015), MSI1 (F value: 11.0; p = .004), MKI67 (F value: 20.4; p < .001), PDGFRa (F value: 35.4; p < .001), PAX6 (F value: 6.6; p = .025), and TUBB3 (F value: 5.3; p = .040), but not for CD133 (F value: 0.2; p = .805), NESTIN (F value: 3.3; p = .086), CSPG4 (F value: 1.6; p = .248), OLIG2 (F value: 0.9; p = .437), and TUBB3 (F value: 3.4; p = .091), and SOX1 (F value: 152; p value < .0001). Results are mean values ± SEM from at least three independent experiments. *, p < .05; **, p < .01; ###, p < .001 when compared with aNPCWM (post hoc Bonferroni adjusted t test). Abbreviations: aNPCWM, white matter adult neural progenitor cell; aNPCWM, white matter adult neural progenitor cell; fNSC, fetal neural stem cell; GALC, galactocerebroside; GFAP, glial fibrillary acidic protein; HMBS, hydroxymethylbilane synthase; MSI1, Musashi1; OLIG2, oligodendrocyte transcription factor 2; PDGFR, platelet-derived growth factor receptor.
a typical phenomenon of aNPC culture, suggesting this cell population as the common adult NPC in vitro phenotype [43]. This pattern is clearly different from fNSC lacking expression of NG2 but being SOX2+.

Most studies with human aNPC from white matter [4], the SVZ [3, 44], or the hippocampus [1, 3, 13, 14] only reported short-term expandability and few population doublings. There is yet only one report showing long-term expandability of hippocampal and temporal cortex NPC [45]. These NPC expressed OPC and glial markers such as A2B5, NG2, and GFAP and could be differentiated into neurons also after long-term expansion, thus confirming our results in this work. However, the study did not provide quantification of neuronal differentiation after long-term expansion nor transcriptome analysis, thereby not excluding that these cell cultures were contaminated with the recently identified brain pericytes (perivascular mesenchymal cells) [21, 27]. We extended these data to more easily accessible white matter-derived aNPC by showing their long-term expandability and neuronal differentiation potential with almost identical properties compared with those of aNPCHIP isolated from a neurogenic region. As brain regionalization occurs in early embryonic development, the differentiation capacity of fetal and adult NPC isolated from different brain regions is hard to compare [18, 46]. The rather high gliogenic potential of these adult human NPC is typical for mammalian...
adult NPC cultures [1, 47]. Both neurosphere cultures were not contaminated with perivascular mesenchymal stem cells (Fig. 2E). The comparative transcriptome data of both aNPC types confirm the similarity of both neuroprogenitor cell types.

Our data show that the majority of aNPC-derived nerve cells were not fully mature. This was, however, expected using this short-term differentiation protocol, but the readout of functional neuronal differentiation only served us to characterize these cells as NPC with essential neuronal differentiation capacity.

Of note, the NPC mentioned in this work were all isolated from patients suffering from treatment-resistant epilepsy. Thus, we cannot exclude that these NPC do not fully resemble healthy adult human NPC. It is known that seizures induce neurogenesis in vivo [48]. Therefore, aNPC from healthy donors might even be less apparent, possibly explaining the increased numbers of colony-forming cells from human compared with mice hippocampal tissue [41]. However, a recent study reports that treatment with the antiepileptic drug valproate blocks seizure-induced neurogenesis [49]. Thus, it remains unknown whether human NPC derived from epileptic surgery reflect healthy or diseased NPC.

**CONCLUSION**

In conclusion, we were able to successfully derive and long-term expand NPC from human adult white matter tissue with multipotent neural differentiation capacity. We describe that the morphological and functional properties of these cells are very similar to those from aNPC<sub>up</sub> generated from an established neurogenic niche of the adult brain. We furthermore provide here-with for the first time a detailed transcriptome analysis of human aNPC<sub>up</sub>, showing an almost identical transcriptome as aNPC<sub>up</sub> serving as a framework for standardized comparative gene expression analysis of human brain-derived NPC with other stem cell populations or differentiated tissues. It is unclear from our data whether both aNPC types acquire similar properties by long-term cell culturing in defined conditions, but the very similar pattern of NPC markers in fresh cell isolates suggests a similar NPC configuration in both brain regions. However, for translational/clinical aspects, the displayed similarities of the more easily accessible white matter aNPC with original neurogenic niche aNPCs is of great advantage, making aNPC from subcortical white matter a primary cell source for future autologous NPC-based therapeutic approaches.

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**AUTHOR CONTRIBUTIONS**

X.L.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; A.H.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript; F.W., M.J.A.-B., S.H.-E., M.K., and J.S.: collection and assembly of data, data analysis and interpretation, manuscript writing; H.R.S.: provision of study material, manuscript writing, financial support, administrative support; A.S.: conception and design, data analysis and interpretation, manuscript writing, financial support, administrative support.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.
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