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Key Words. Adult neural stem cell • Nurr1 • Dopamine neurons • Parkinson disease

ABSTRACT

Neural precursor (NP) cells from adult mammalian brains can be isolated, expanded in vitro, and potentially used as cell replacement source material for treatment of intractable brain disorders. Reduced ethical concerns, lack of teratoma formation, and possible ex vivo autologous transplantation are critical advantages to using adult NP donor cells over cells from fetal brain tissue or embryonic stem cells. However, the usage of adult NP cells is limited by the ability to induce specific neurochemical phenotypes in these cells. Here, we demonstrate induction of a dopaminergic phenotype in NP cells isolated from the subventricular zone (SVZ) and white matter of rodent adult brains using overexpression of the nuclear receptor Nurr1 in vitro. Forced expression of Nurr1, a transcriptional factor specific to midbrain dopamine (DA) neuron development, caused in the adult cells an acquisition of the DA neurotransmitter phenotype and sufficient differentiation toward morphologically, phenotypically, and ultrastructurally mature DA neurons. Coexpression of neurogenic factor Mash1 and treatment with neurogenic cytokines brain-derived neurotrophic factor and neurotrophin-3 greatly enhanced Nurr1-induced DA neuron yield. The Nurr1-induced DA neurons demonstrated in vitro presynaptic DA neuronal functionality, releasing DA neurotransmitter in response to depolarization stimuli and specific DA reuptake. Furthermore, Nurr1-engineered adult SVZ NP cells survived, integrated, and differentiated into DA neurons in vivo that can reverse the behavioral deficit in the host striatum of parkinsonian rats. These findings open the possibility for the use of precursor cells from adult brains as a cell source for neuronal replacement treatment of Parkinson disease. STEM CELLS *2007;25: 1252–1262*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Multipotent undifferentiated stem or precursor cells with the capacity for self-renewal play major roles in mammalian development. Most adult tissues harbor tissue-specific precursor cells even after the termination of developmental processes. The brain was long thought to be an exception, until neural precursor (NP) cells were discovered in the subventricular zone (SVZ) of rodent adult forebrain [1–4]. NP cells have since been identified in other regions of the adult brain, such as the hippocampal dentate gyrus [5, 6], midbrain [7], and even the subcortical white matter (WM) [8–10], which is mostly occupied by neuronal fibers and a minor population of oligodendrocyte progenitors.

Parkinson disease (PD) is a neurodegenerative disorder characterized by progressive and specific loss of dopamine (DA)-secreting neurons in the substantia nigra of the midbrain. Given the distinct loss of this single neuronal subtype in a confined brain region, cell-based replacement approaches for PD have garnered much attention over the past decade. Precursor cells from fetal and adult brains can be isolated, maintained in culture in an undifferentiated state, and guided to differentiate into a variety of neurons and glia [11]. The development of these culture techniques offers the opportunity to generate large numbers of specific neurons for cellular replacement strategies. The derivation of DA neurons from stem or precursor cells is of particular interest as a cell-based strategy for PD. DA neurons have only been efficiently derived from NPs of early fetal ventral midbrain [12, 13] and embryonic stem (ES) cells from preimplanted blastocysts of embryos [14, 15]. Clinical use of these cells in PD patients, however, has been hampered by ethical concerns related to destruction of embryos or fetuses, as well as a complication of teratoma formation by undifferentiated ES cells [16]. The usage of NP cells isolated from the adult brain would circumvent these ethical issues and complications. Furthermore, adult NP cell-based autologous transplantation could

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involve isolating precursors from intact brain regions of the patient and ex vivo expansion of these precursor cells.

In this study, we isolated NP cells from the SVZ of the lateral ventricle and subcortical WM of rodent adult brain and examined whether such precursor cells could generate functional DA neurons relevant for cell therapeutic treatment of PD. Historically, NP cells derived from adult brain have primarily differentiated into astroglial cells, and the induction of DA neurons from naïve adult precursor cells has seldom been achieved (reviewed in [17, 18]). We used retrovirus-based transgene expression of Nurr1, a known midbrain DA neuronal phenotype inducer [19, 20], to promote DA neuronal differentiation in adult NP cells. Nurr1-expressing adult precursors efficiently differentiated toward DA cells, demonstrating the functionalities of presynaptic DA neurons in vitro and in vivo by engrafting into the striatum of PD-model rats. These findings suggest that NPs derived from adult brain, in conjunction with *Nurr1* gene manipulation, could be a useful strategy for PD therapy.

MATERIALS AND METHODS

Culture of Neural Precursor Cells Isolated from the SVZ and WM of Adult Rat Brains

Animals were housed and cared for according to NIH guidelines. Brains were freshly obtained in the course of surgical resection from adult male Sprague-Dawley rats (7–8 weeks old; Koatech, Seoul, Korea, http://www.ekoatech.com), washed several times in Ca^{2+}-, Mg^{2+}-free Hanks' balanced salt solution (CMF-HBSS), and sliced anteroposteriorly in a brain matrix (coronal-type; Stoelting, Wooddale, IL, http://www.stoeltingco.com). The SVZ and WM tissues, as indicated in supplemental online Figure 1a, were dissected from the rest of the tissue, free of adjacent cortex and ventricular epithelium. The tissues were mechanically chopped and digested in a solution containing papain (2.5 U/ml papain; Worthington, Freehold, NJ, http://www.worthington-biochem.com), dispase II (1 U/ml; Roche, Indianapolis, http://www.roche.com), and DNase I (2.5 U/ml; Worthington) by incubating at 37°C for 45 minutes on a rocking shaker (model 1012; SHEL LAB, Cornelius, OR, http://www.shellab.com). The pellets were collected by centrifugation at 200*g*, rinsed once with CMF-HBSS, and resuspended in 1 ml of serum-free N2 medium [21] supplemented with basic fibroblast growth factor (bFGF) (20 ng/ml), bFGF+epidermal growth factor (EGF) (20 ng/ml), or bFGF+ platelet-derived growth factor-AA (PDGF) (10 ng/ml; all reagents from R&D Systems Inc., Minneapolis, http://www.rndsystems.com), all of which had the potential to function as mitogens. The dissociated cells were seeded at 5,000 (SVZ) or 10,000 (WM) cells per milliliter in 10-cm culture dishes (3262 ultra low attachment; Corning, NY, http://www.corning.com) and were induced to proliferate by the mitogens, resulting in the formation of floating cell aggregates (neurospheres). Cultures were maintained for 7–14 days at 37°C in a 5% CO_2 incubator. Five ml of medium was added every 3–4 days, and mitogens were added daily. Neurospheres were manually picked under microscopy after 7 (SVZ) or 14 (WM) days in vitro (DIV), transferred into new dishes with freshly prepared medium, and further cultured in identical conditions. For subsequent neurosphere formation (cell passage), cells assembled in the spheres were dissociated into single cells by incubating in CMF-HBSS for 4–5 hours at 37°C followed by mechanical resuspension, seeded in 10-cm culture dishes, and further cultured in the presence of the mitogens. Cell cultures were maintained for an additional >2 months by cell passages at every 7 days up to 10 passages. In other cases, dissociated cells were plated at 25 cells per milliliter in 96-well plates in N2 medium supplemented with the same mitogens and marked wells containing only a single cell per well. Only the wells marked were included in the analysis of the sphere-forming rate, representing percentage of wells in which cell aggregate (sphere, clone) was formed from a single cell at 3–4 days after cell plating. For phenotypic analysis of the neurospheres, 30–50 spheres were collected, immersed in Tissue-Tek (Sakura Finetechnical, Tokyo, http://www.sakuraus.com), and cryosectioned at 35 μm. In other cases, the spheres were dissociated into small clusters by mechanical pipetting and directly plated onto coverslips (12-mm diameter; Carolina Biological Supply Co., Burlington, NC, http://www.carolina.com) precoated with poly-L-ornithine (PLO)/fibronectin (FN) [21]. Immunocytochemical analyses were performed on the cryosectioned spheres or coverslip preparations as described below. In most of the other experiments, neurospheres were dissociated into single cells as described above, and plated at 500 cells per mm^2 onto PLO/FN-coated coverslips in 24-well plates or 6-cm tissue culture dishes (for cell transplantation) in mitogen-supplemented N2. Retroviral transductions were carried out at 50%–60% cell confluence (typically 1 day after plating) as described below. Cell differentiation was induced for 7–14 days by withdrawal of the mitogens in N2 medium supplemented with B27 supplement (1×; Invitrogen, Grand Island, NY, http://www.invitrogen.com) and ascorbic acid (200 μM; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com). In certain experiments, brain-derived neurotrophic factor (BDNF) (20 ng/ml), glial cell-derived neurotrophic factor (GDNF) (20 ng/ml), or neurotrophin-3 (NT3) (20 ng/ml; all from R&D Systems) was added to the medium.

Cells were isolated from rat cortices at embryonic day 14 (E14) and cultured in suspension in N2 supplemented with the mitogens. Neurospheres formed after 3–6 days of cell expansion were dissociated, plated, and differentiated in conditions identical to those of the adult cultures as described above.

Retroviral Construction and Transduction

The retroviral vectors expressing Nurr1 (pNurr1-IRES-LacZ, pNurr1-IRES-green fluorescent protein [GFP], and pNurr1-IRES-Mash1) and Mash1 (pMash1-IRES-LacZ) were constructed as described previously [22]. The retroviral vectors were transfected into 293gpg packaging cells (Lipofectamine; Invitrogen), and supernatant containing viral particles (VSV-G pseudotyped recombinant retrovirus) was harvested 72 hours after incubation. Viral titer was adjusted to 5×10^6 particles per ml. For retroviral transduction, cells on coverslips or 6-cm dishes were incubated with the viral supernatant containing polybrene (1 μg/ml) for 3 hours. After 1–2 days of further cell expansion in the presence of the mitogens described above, cells were induced to differentiate or prepared for cell transplantation. Retroviral infection per se did not affect cell survival, proliferation, or differentiation.

Immunostaining of Cultured Cells and Brain Slices

Cultured cells, cryosectioned brain slices, or neurospheres were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: tyrosine hydroxylase (TH) anti-rabbit (1:250; Pel-Freez, Rogers, AK, http://www.pel-freez.com) or anti-mouse (1:1000; Sigma-Aldrich), neuron-specific class III β-tubulin (TuJ1) anti-mouse (1:500) or anti-rabbit (1:2000; both from Covance, Princeton, NJ, http://www.crpinc.com), anti-rabbit Nurr1 (1:200; Chemicon, Temecula, CA, http://www.chemicon.com), nestin (1:50, provided by Martha Marvin and Ron Mckay, NIH, Bethesda, MD), vesicular monoamine transporter 2 (VMAT2) (1:500; Pel-Freez), anti-mouse GFP (1:400; Roche), Ki67 (1:100; Novocastra Ltd., Newcastle upon Tyne, U.K., http://www.novocastra.co.uk), glial fibrillary acidic protein (GFAP) (1:200; MP Biomedicals, Solon, OH, http://www.mpbio.com), 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) (1:500; Sigma-Aldrich), microtubule-associated protein (MAP2) (1:1,000; Sigma-Aldrich), neuron-specific RNA-binding protein (HuC/D, 1:100; Invitrogen), and anti-rat dopamine transporter (DAT) (1:5,000; Chemicon). For detection of primary antibodies, fluorescently labeled (fluorescein isothiocyanate or Cy3) secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com) were used according to the specifications of the manufacturer. Cells or brain slices were mounted in Vectashield containing 4′,6 diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) and analyzed under an epifluorescent (Nikon, Tokyo, http://www.nikon.com) or confocal (LMS 510; Carl Zeiss, Feldbach, Switzerland, http://www.zeiss.com) microscope.

Immunoelectron Microscopy Analysis

Cells grown on Aclar embedding film (Electro Microscopy Science, Hatfield, PA, http://www.emsdiasum.com) were fixed in 4% paraformaldehyde/0.01% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 (PB), cryoprotected in 30% sucrose in PB, and then incubated with anti-TH antibody (Pel-Freez) 4°C overnight. TH+ cells were visualized with peroxidase reaction with diaminobenzidine (DAB) substrate. TH-stained cells were postfixed with 1% osmium tetroxide in 0.1 M PB for 30 minutes, dehydrated in a graded series of alcohols, embedded in Durcupan ACM (Fluka, Buchs SG, Switzerland, http://www.sigmaaldrich.com) between strips of Aclar film and polymerized at 60°C for 48 hours. Small pieces containing TH+ cells cut and glued onto the plastic block. Ultrathin sections were cut, mounted on formvar-coated single-slot nickel grids and examined on a Hitachi H 7500 electron microscope (Hitachi, Tokyo, http://www.hitachi.com). For specificity of immunoreactivity, a negative control test was carried out. Omission or replacement of the primary antiserum with normal rabbit serum showed an absence of immunoreactivity.

Reverse Transcription-Polymerase Chain Reaction

Total cellular RNA was isolated using Tri Reagent (Molecular Research Center, Inc. Cincinnati, OH, http://www.mrcgene.com) and cDNA was synthesized from 5 μg of total RNA in a 20-μl reaction using the Superscript kit (Invitrogen). Optimal polymerase chain reaction (PCR) conditions for each primer set were determined by various $MgCl_2$ concentrations, annealing temperatures, and cycle numbers to determine a linear amplification range. Information about primer sequences and optimum conditions provided upon request.

Assays for DA Release and Reuptake

DA release was quantified using high-performance liquid chromatography (HPLC) analyses as described previously [23]. Briefly, cells in 24-well plates were incubated in 400 μl/well of freshly prepared N2 (basal release) or N2 supplemented with 56 mM KCl (evoked release) for 30 minutes. The media were collected, stabilized with 0.1 mM EDTA, and analyzed. Samples (100 μl) were injected into a reverse phase μ-Bondapak C18 column (150 × 3.0 mm; Eicom, Tokyo, http://www.eicom-usa.com), and analyzed with an amperometric electrochemical detector (model ECD-300; Eicom). DA levels were calculated using external DA standard (50 nM methyl DOPA) injected immediately before and after each experiment.

Assays for DAT-mediated specific DA uptake were conducted as described previously [23]. Cells were incubated with 50 nM [^{3}H]DA (51 Ci/mmol; Amersham Biosciences, Buckinghamshire, U.K., http://www.amersham.com) with or without 10 μM nomifensine (Sigma-RBI, Natick, MA), a DAT blocker, to determine nonspecific uptake. After incubation for 10 minutes at 37°C, cells were washed with cold PBS and lysed in 0.5 M NaOH. Radioactivity was measured by liquid scintillation counting (MicroBeta TriLux version 4.4; PerkinElmer, Waltham, MA, http://www.perkinelmer.com). Specific DA uptake was calculated by subtracting nonspecific uptake (with nomifensine) from uptake without nomifensine. The level of DA release and uptake per TH+ cell was calculated by dividing each value by the TH+ cell number obtained from cultures conducted in parallel to the DA release and uptake assays.

Electrophysiology

Nurr1-expressing cells were identified by GFP expression under a GFP-filtered microscope in the differentiated cultures for SVZ NP cells transduced with Nurr1-IRES-GFP viruses and subjected to electrophysiological analysis using the whole-cell recording configuration of the conventional "dialyzed" whole-cell patch-clamp technique [22]. Patch electrodes were fabricated from a borosilicate glass capillary (Sutter Instrument Co., San Rafael, CA, http://www.sutter.com) by using a vertical micropipette puller (Narishige, Tokyo, http://www.narishige.co.jp). Resistances of patch electrodes were set to 2–4 M. Cell membrane capacitance and series resistance were compensated for (typically >80%) electronically using a patch-clamp amplifier (Axopatch-200A; Axon Instruments/Molecular Devices Corp., Foster City, CA, http://www.moleculardevices.com). Current protocol generation and data acquisition were performed using pClamp 8.2 software on an IBM computer equipped with an analog-to-digital converter (Digidata 1322A; Axon Instruments). Voltage traces were filtered at 5 KHz by using the four-pole Bessel filter in the clamp amplifier and stored on the computer hard drive for later analysis. All experiments were performed at room temperature (21–24°C). For recording in voltage clamp mode, the patch pipette solution contained (in mM): KCl, 134; $MgCl_2$, 1.2; MgATP, 1; Na_2GTP, 0.1; EGTA, 10; glucose, 14; and HEPES, 10.5 (pH adjusted to 7.2 with KOH). The bath solution contained (in mM): NaCl, 134; KCl, 5; $CaCl_2$, 2.5; $MgCl_2$, 1.2; glucose, 14; and HEPES 10.5 (pH adjusted to 7.4 with NaOH).

In Vivo Transplantation

Generation of 6-hydroxydopamine-lesioned PD model rats and an amphetamine-induced rotation test were performed as described previously [24]. Adult rat SVZ NP cells under bFGF-proliferation were harvested 2 days after Nurr1 transduction and dissociated into single cells in CMF-HBSS as described above. Using a 22-gauge needle, 5 μl of cell suspension (1×10^5 cells per microliter in PBS) per site was deposited at two sites of the striatum (coordinates in anteroposterior, mediolateral, and dorsoventral relative to bregma and dura: (a) 0.07, −0.30, 0.55; (b) −0.10, −0.40, −0.50; incisor bar set at 3.5 mm using a 22-gauge needle. The needle was left in place for 5 minutes following each injection. Control sham-operated rats were injected with a vehicle PBS solution. Rats received daily injections of cyclosporine A (10 mg/kg, i.p.). Amphetamine-induced rotation scores were determined at 2, 4, and 6 weeks post-transplantation. For histological analysis, animals were anesthetized with phenobarbital and perfused transcardially with 4% paraformaldehyde in PBS. Brains were equilibrated with 30% sucrose in PBS and sliced on a freezing microtome (CM 1850; Leica, Wetzlar, Germany, http://www.leica.com). Free-floating brain sections (35 μm thick) were subjected to immunohistochemistry as described above. The total number of TH immunoreactive cells in the graft was estimated as described previously with the Abercrombie correction factor [24].

Cell Enumeration and Statistical Analysis

Cells were counted in microscopic fields randomly chosen across the culture area, using an eyepiece grid at a final magnification of ×200 or ×400. Three to six culture wells were analyzed in each experiment. Data are expressed as mean ± SEM. Statistical comparisons were made by one-way analysis of variance with Tukey post hoc analysis (SPSS 11.0; SPSS, Inc., Chicago, http://www.spss.com) when more than two groups were involved.

RESULTS

Isolation and Characterization of NP Cells Derived from the SVZ and WM of Adult Rat Brain

We have attempted an in vitro expansion of NP cells from the SVZ of the lateral ventricles and subcortical WM of rat adult brains to test whether adult NP cells could provide a renewable source of DA neurons for cell replacement strategy in PD. The culture of SVZ precursor cells was carried out in serum-free N2 medium supplemented with the mitogen bFGF as described previously [25]. Previous efforts have isolated NP cells from subcortical WM by sorting based on early oligodendrocyte progenitor markers CNPase and A2B5 [10]. These protocols are highly complex, however, and difficult to replicate. We tested whether WM NPs could be simply isolated, without sorting, through proliferation in vitro with appropriate mitogens. SVZ cells were induced to proliferate by the mitogenic action of bFGF, and neurospheres appeared within 7 DIV. In contrast, no neurospheres were formed in the bFGF-supplemented WM cultures. Neither supplementation with EGF nor the combination of

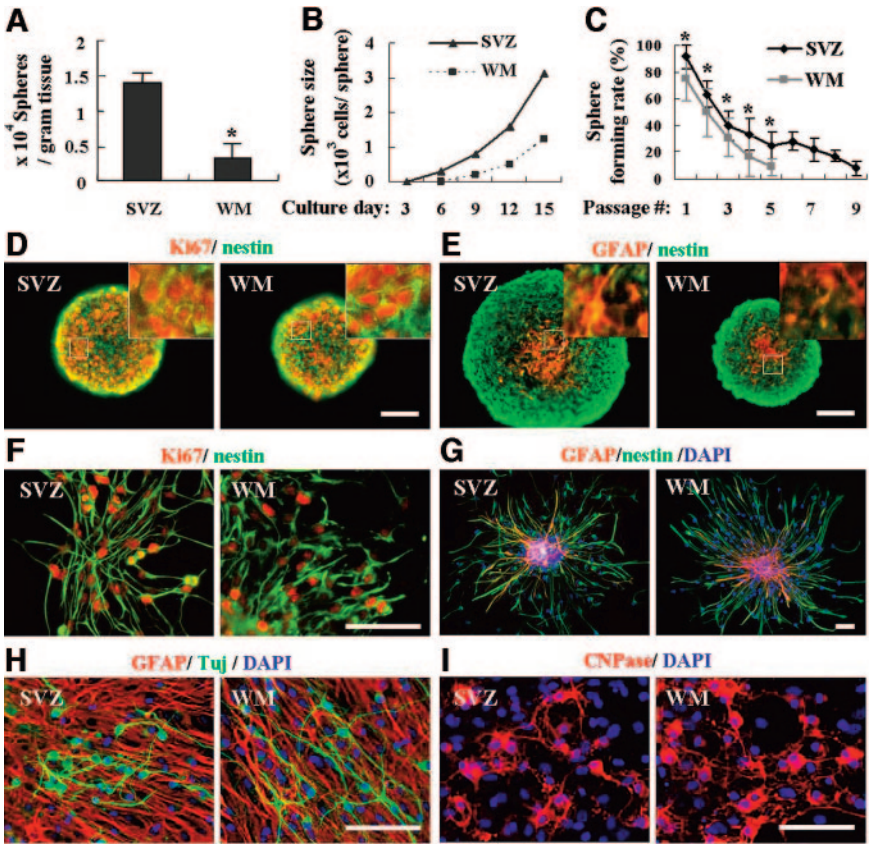

Figure 1. In vitro self-renewal and multipotent properties of neural precursor cells derived from the SVZ and WM of adult rat brain. **(A, B):** Frequency and proliferation capacity of proliferating neural precursor cells were determined by the number of neurospheres per gram of tissue at 14 days in vitro (DIV) **(A)** and neurosphere size (average number of cells in a sphere) over the culture period **(B)**. **(C):** Sphere forming rate. Neurospheres at DIV 12 were dissociated into single cells and seeded at a clonal density (25 cells per milliliter) in 96-well plates. Only the wells containing a single cell were marked for the analysis. The sphere forming rate represents percentage of wells containing spheres compared with total wells marked, at 3–4 days after cell seeding. Similarly, the sphere forming rates for the third through the ninth spheres were evaluated. ∗, Significantly different from WM at $p < .01$. **(D–G):** Cellular phenotypes of SVZ- and WM-derived spheres. Neurospheres, generated after 14 days of in vitro proliferation with mitogens basic fibroblast growth factor (bFGF) (SVZ) and bFGF + platelet-derived growth factor-AA (WM), were cryosectioned **(D, E)**. The phenotypes of the cells assembled in the spheres were determined by the immunocytochemical analyses for Ki67 (proliferation marker), nestin (marker for neural precursor cell), and GFAP (marker for astrocyte or adult-type neural precursor cell). Insets, enlarged images of the boxed areas for nestin+/Ki67+ **(D)** and nestin+/GFAP+ **(E)** cells. The cellular phenotypes of SVZ- and WM-derived spheres were further evaluated by directly plating the spheres onto fibronectin-coated surfaces. After 10 hours of further culture with the mitogens, cellular phenotypes were determined using Ki67/nestin **(F)** and GFAP/nestin **(G)** immunostaining. **(H, I):** Differentiation of the neural precursor cells derived from SVZ and WM. Differentiation of the dissociated cell clusters was induced by withdrawal of the mitogens the day after cell plating. After 7 days of differentiation, phenotypes of the differentiated SVZ and WM precursors were determined by immunocytochemical analyses for GFAP (astrocytic)/TuJ1 (neuronal) **(H)** and CNPase (oligodendrocytic marker) **(I)**. Scale bar = 40 μm. Abbreviations: CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; DAPI, 4′,6 diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; SVZ, subventricular zone; WM, white matter.

bFGF+EGF caused efficient proliferation of WM cells. However, bFGF+PDGF did promote the expansion of WM cells, resulting in conspicuous neurospheres within 7–14 DIV. The minimum periods required for visible sphere formation were 3–4 days in SVZ and 6–8 days in WM cultures. Usually, at 7 (SVZ) and 14 (WM) DIV, the spheres were transferred into fresh dishes using a pipette under the microscope. This procedure was a critical modification that allowed further culturing while keeping cultures free from tissue debris and potential contaminants. No spheres were observed from attempts to culture cells from the cortex or striatum under any of the culture conditions tested (data not shown), confirming that identification and isolation of NP from the SVZ and WM was tissue specific.

At 14 DIV, there was a great difference in the number of spheres per gram of tissue formed in SVZ (14,007.0 ± 1,471.3) and WM cultures (3,202.3 ± 1,985.5; $n = 3$) (Fig. 1A). Furthermore, the average neurosphere size (cells assembled in a sphere) in the SVZ cultures (3,130 cells) was greater than those in the WM cultures (1,220 cells) (Fig. 1B). These findings suggest both the presence of more self-renewable cells and a greater in vitro proliferation capacity in SVZ cells compared with WM cells. When the spheres were dissociated into single cells and seeded at a clonal density (25 cells per milliliter), subsequent (secondary) spheres were generated from 91.2% ± 8.9% and 75.0% ± 16.7% of the dissociated cells in SVZ and WM cultures, respectively (Fig. 1C). Similarly, 63.2% ± 9.5% (SVZ) and 49.6% ± 17.9% (WM) of cells dissociated from the respective secondary spheres gave rise to tertiary spheres. Further reductions were shown in the sphere forming units of fourth to ninth SVZ and WM sphere-forming cultures.

Disregarding the differences in the sphere formation rate and proliferation capacity, the phenotypical properties and in vitro differentiation potentials of the SVZ- and WM-derived spheres were indistinguishable. Immunostaining performed after 14 DIV showed that 99.2% ± 1.0% (SVZ) and 98.5% ±

1.3% (WM) of assembled cells in spheres were positive for nestin, an intermediate filament specific to NP cells. Proliferation cell marker Ki67 was colocalized in 95.0% ± 2.2% and 94.3% ± 1.0% of nestin+ cells in the SVZ and WM cultures, respectively (Fig. 1D, 1F). Interestingly, GFAP, a marker for astrocytic progeny and NP cells in the adult brain, was colocalized in 48.9% ± 3.3% and 51.9% ± 2.6% of nestin+ cells in the SVZ and WM cultures, respectively (Fig. 1E, 1G). Virtually all GFAP+ cells expressed nestin, and the GFAP+/nestin+ cells were always assembled in the center portions of the spheres.

Cell differentiation was induced by dissociating the spheres and plating the cells onto FN-coated plates in the absence of mitogens. After 7 days of differentiation, SVZ cultures consisted of 18.0% ± 1.9% TuJ1+ neurons, 44.2% ± 2.3% GFAP+ astrocytes, and 15.6 ± 1.6% CNPase+ oligodendrocytes. WM cultures consisted of 16.0% ± 2.1% TuJ1+ neurons, 48.1% ± 2.3% GFAP+ astrocytes, and 16.9% ± 1.8% CNPase+ oligodendrocytes (Fig. 1H, 1I). These findings, taken together, suggest that NP cells with self-renewal and multipotent properties from adult brain WM and SVZ can be derived simply but efficiently.

To examine whether proliferative and developmental properties of the adult NP cells can be maintained after long-term cell expansion in vitro, SVZ and WM spheres were further cultured in the presence of the same mitogens for 70 days with 10 passages (as described in Materials and Methods). Total cell numbers increased during the two initial passages of SVZ cultures but leveled off and rather decreased after fourth cell passages (supplemental online Fig. 1d). However, significant increase of total cell numbers was not achieved by the extended period of WM cultures. When cells on each passage were plated and differentiated, more proportions of cells underwent cell apoptosis in the cultures expanded longer in vitro (percentage of cells with apoptotic nuclei in SVZ cultures: 38.4% ± 9.2% in passage 3 [P3] vs. 63.1% ± 6.8% in P6 at differentiation day 7.) Furthermore TuJ1+ neuron yields from SVZ NP cells were gradually decreased over passages (supplemental online Fig. 1f). These findings suggest that further in vitro cell expansion of adult NP cells by a longer period of culture does not offer substantial benefit in the generation of NP cells with self-renewal and multipotent capacities. The following experiments, therefore, were done in unpassaged cultures (neurospheres cultured for 7 days after transferring into freshly prepared dishes), unless otherwise noted.

Dopaminergic Differentiation of SVZ- and WM-Derived NP Cells by *Nurr1* Transgene Expression

Efficient dopaminergic differentiation from adult brain-derived NP cells has been difficult to achieve. Consistently, we found that no differentiated SVZ and WM cells were positive for TH, a key enzyme for DA biosynthesis. Neither treatment of cytokines, such as SHH, FGF8 (early inducers for midbrain DA neuronal development), GDNF, BDNF, and NT3 (differentiation and survival factors for DA neurons), nor alteration of culture conditions, such as cell density or culture period and type (suspension or adhesive culture), was effective in generating TH+ cells (data not shown), suggesting no DA neurogenic potential of naïve adult NP cells.

Genetic manipulation of key transcription factors also has the potential to induce acquisition of DA neurogenic potential in adult NP cells. Nurr1 is an orphan nuclear receptor-type transcription factor with a critical role in midbrain DA neuron development that has been demonstrated in vivo [26–28] and in vitro [19, 20, 22, 29]. We retrovirally induced *Nurr1* transgene expression in SVZ- and WM-derived NP cells using Nurr1-IRES-GFP or Nurr1-IRES-LacZ vector. Efficiency of the retroviral transduction was 46.0% ± 4.9% and 37.9% ± 2.7% of total cells based on the estimation of Nurr1+ and GFP+ cells, respectively, at day 2 postinfection. Of the GFP+ cells, 22.6% ± 2.2% and 24.0% ± 2.3% of cells were positive for TH in the cultures for SVZ- and WM-derived NP cells (Fig. 2A, 2B, arrows), respectively, and TH+ cell numbers were maintained without significant loss up to at least 14 days of in vitro differentiation (data not shown). Subpopulations of TH+ cells expressed DAT, a protein specific to DA homeostasis in pre-

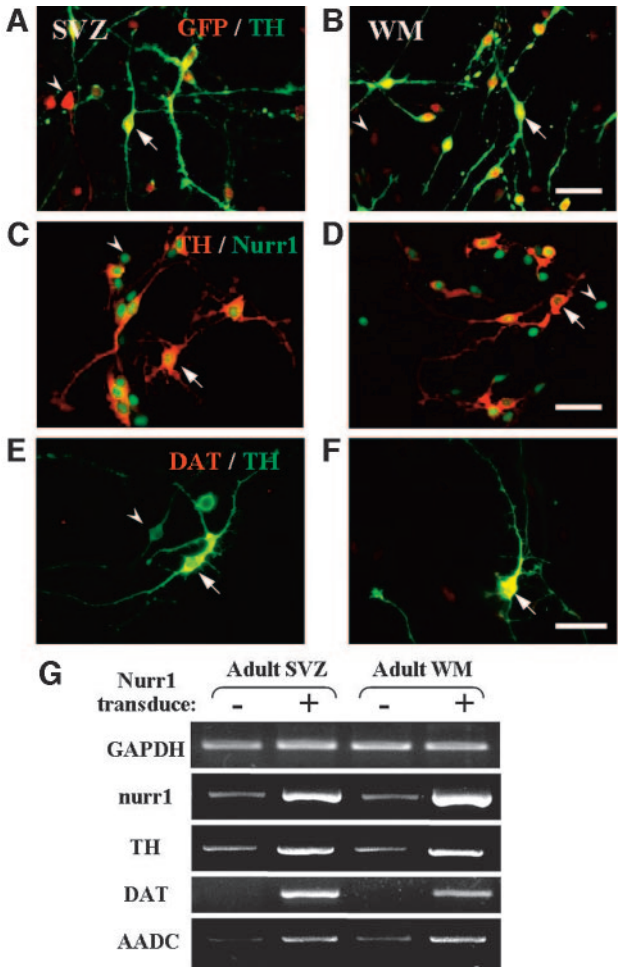

Figure 2. Dopaminergic differentiation from adult SVZ and WM-neural precursor cells by Nurr1 overexpression. Neurospheres derived from adult rat SVZ and WM were dissociated into single cells and cultured on fibronectin-coated coverslips. Cells were transduced with Nurr1-expression viruses (Nurr1-IRES-GFP **[A, B]** or Nurr1-IRES-LacZ), and cell differentiation was induced the following day by withdrawing the mitogens' basic fibroblast growth factor (bFGF) (SVZ) and bFGF+platelet-derived growth factor-AA (WM). Dopaminergic differentiation of the Nurr1-transduced precursor cells was determined at day 7 of in vitro differentiation by reverse transcription-polymerase chain reaction (RT-PCR) **(G)** and immunocytochemical analyses for TH/GFP **(A, B)**, TH/Nurr1 **(C, D)**, and TH/DAT **(E, F)**. Arrows indicate representative TH+ cells expressing GFP **(A, B)**, Nurr1 **(C, D)**, and DAT **(E, F)**, whereas arrowheads indicate GFP+/TH− **(A, B)**, Nurr1+/TH− **(C, D)**, and TH+/DAT− **(E, F)** cells. Scale bar = 20 μm. **(G):** Semiquantitative RT-RCR analyses to examine Nurr1-induced expression of DA neuronal markers TH, DAT, and AADC. Abbreviations: AADC, aromatic acid L-amino acid decarboxylase; DAT, dopamine transporter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; SVZ, subventricular zone; TH, tyrosine hydroxylase; WM, white matter.

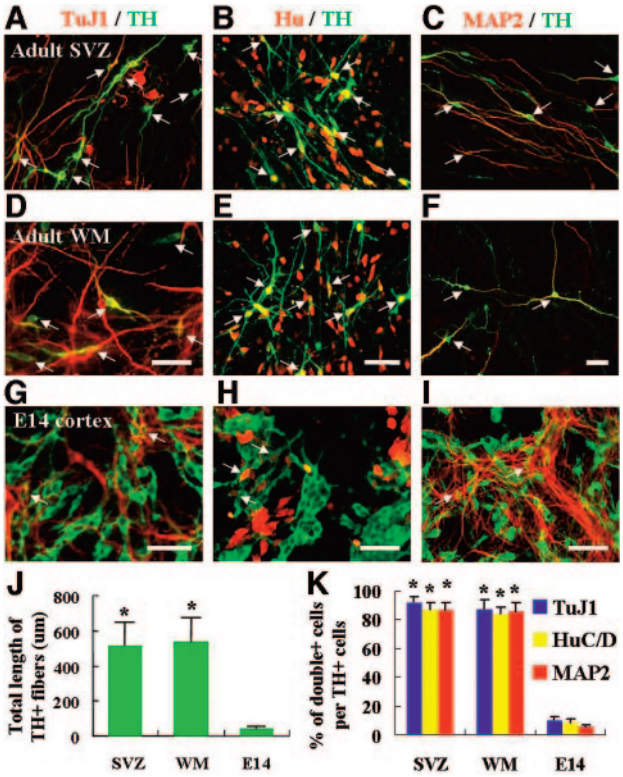

Figure 3. Morphological and phenotypic differentiation of dopamine cells derived from Nurr1-transduced adult neural precursor cells. SVZ- and WM-derived neural precursor cells were cultured, transduced with Nurr1 (Nurr1-IRES-LacZ), and differentiated, as described in the legend of Figure 2. The same procedures were performed with precursor cells isolated from E14 rat cortices. At day 7 of in vitro differentiation, morphologic maturation of TH+ cells and colocalization of the neuronal markers TuJ1, HuC/D, and MAP2 were assessed. Shown in **(A–I)** are the representative images for TH/TuJ1 **(A, D, G)**, TH/HuC/D **(B, E, H)**, and TH/MAP2 **(C, F, I)**. Arrows indicate TH+ cells expressing TuJ1 **(A, D, E)**, HuC/D **(B, E, H)**, and MAP2 **(C, F, I)**. Scale bar = 20 μm. Note that TH+ cells in adult SVZ **(A–C)** and WM **(D–F)** cultures exhibit mature neuronal morphology with multiple TH fibers and that the neuronal markers are colocalized in a major population of TH+ cells derived from the adult cells **(A–F)**. This is in a clear contrast to short or absent processes in TH+ cells and separated localization of TH and neuronal markers in different cells in E14 cortical cultures **(G–I)**. Graphs **(J, K)** depict total length of neurites per TH+ cell and percentage of double-positive cells out of total TH+ cells, respectively. *, Significantly different from E14 cortical cultures at $p < .01$; $n = 20–30$ for each value. Abbreviations: E14, embryonic day 14; MAP2, microtubule-associated protein; SVZ, subventricular zone; TH, tyrosine hydroxylase; WM, white matter.

synaptic DA neurons (Fig. 2E, 2F, arrows); after 7 days of differentiation, 30.4% ± 14.6% (SVZ) and 25.3% ± 12.6% (WM) of TH+ cells were TH+/DAT+. In addition, in semiquantitative reverse transcription-PCR analyses, expression of DA neuron-specific markers TH, DAT, and aromatic acid L-amino acid decarboxylase mRNAs were strikingly enhanced in Nurr1-transduced cultures, compared with the untransduced control (Fig. 2G). These findings suggest a role for Nurr1 in inducing dopaminergic differentiation of adult NP cells.

Morphologic, Phenotypic, and Functional Properties of DA Neurons Derived from Nurr1-Expressing Adult NP Cells

We have previously demonstrated the effect of forced Nurr1 expression in NP cells derived from rat embryonic brains. Nurr1 efficiently induced DA neurotransmitter identity in rat embryonic NP cells, but the Nurr1-induced DA cells were morphologically and functionally immature as presynaptic DA neurons [20, 22, 29]. Consistently, short or absent neurite extensions were observed from the TH+ cells differentiated from Nurr1-transduced precursor cells isolated from rat cortical tissues at E14 (Fig. 3G–3I). By contrast, Nurr1-induced TH+ cells in the differentiated cultures for Nurr1-transduced adult SVZ- and WM-derived NP cells showed mature neuronal morphology with extensive and multiple neurite outgrowths (Fig. 3A–3F). Neurites per TH+ cell at day 7 of differentiation had an average length of 512.7 ± 133.6 μm (SVZ), 531.7 ± 136.2 μm (WM), and 36.1 ± 13.2 μm (E14 cortex) (Fig. 3J; $p < .01$ from E14 cultures; $n = 40$ for each value).

We next tested whether enhanced morphometric maturation was correlated with the expression of mature neuronal or dopaminergic markers. Quantification of the percentage of Nurr1-induced DA cells expressing neuronal markers TuJ1, HuC/D, and MAP2 confirmed our morphometric data on cell differentiation shown in Figure 3. Only a minor proportion (10.5% ± 2.1%) of Nurr1-TH+ cells expressed TuJ1 in the cultures for E14 cortical precursors (Fig. 3G, 3K). In contrast, TH+/TuJ1+ cells were 91.9% ± 4.4% and 87.0% ± 6.5% of total TH+ cells in the cultures for SVZ- and WM-derived precursor cells, respectively (Fig. 3A, 3D, 3K). Similar results were obtained in 87.1% ± 5.2% (SVZ), 84.2% ± 5.7% (WM) of TH+ cells coexpressing HuC/D and 86.2% ± 6.6% (SVZ), 85.3% ± 7.4% (WM) of TH+ cells coexpressing MAP2 specific to mature neuronal cells (Fig. 3B, 3C, 3E, 3F, 3K). In addition, there was a clear contrast in the expression of DAT, which is expressed at later stages of DA neuron development [30]. As shown in Figure 2, DAT protein expression was colocalized in the subpopulations of TH+ cells derived from SVZ and WM NP. In contrast, none of TH+ cells in E14 cultures expressed DAT (data not shown). The generation of synapses is a definite marker for maturity of neuronal cells. Synapsin and synaptophysin, specific for synaptic vesicles, were colocalized in TH+ cells differentiated from Nurr1-transduced SVZ or WM NP cells (Fig. 4A, 4B; data not shown). Immunoelectron microscopic analyses showed that TH-stained axon terminals, which contain neurotransmitter-containing vesicles, have synaptic contacts with distinct postsynaptic densities (Fig. 4C, 4D). Furthermore, ultrastructures were shown for the synapses generated between TH+ dendrites and neighboring axons (Fig. 4E, 4F).

Presynaptic DA neuron function was assessed by measuring DA release in vitro in Nurr1-transduced cultures 10 days after differentiation. HPLC analysis revealed that DA was readily detected in the medium conditioned for 30 minutes in the differentiated SVZ and WM cultures (basal release, SVZ: 10.0 ±1.7 pg per TH+ cell; WM: 8.0 ± 0.4 pg per TH+ cell; $n = 6$ for each value; Fig. 5A, 5B). Elevated potassium was used to determine whether DA release could be evoked by depolarization. DA releases of Nurr1-tranduced SVZ and WM cells were strikingly increased by high potassium treatment. DA levels were 48.1 ± 7.8 pg per TH+ cell (SVZ) and 26.7 ± 0.8 pg per TH+ cell (WM) in the medium supplemented with 56 mM KCl ($n = 6$). In contrast, basal release of DA was undetectable, and DA level in the KCl-supplemented medium was only 0.6 ± 0.1 pg per TH+ cell in Nurr1-transduced E14 cortical cultures. DA was not detected in any untransduced naïve cultures (Fig. 5A). DAT-mediated high affinity reuptake of DA is a critical process in DA metabolism and could be an indicator for functional presynaptic DA neurons. Although reuptake in Nurr1-transduced E14 cells was at the limit of detection (E14: 0.07 ± 0.01 fmol per minute per 1,000 TH+ cells), SVZ and WM cells transduced with Nurr1 exhibited robust DA uptake of 22.75 ± 1.54 and 19.75 ± 0.68 fmol per

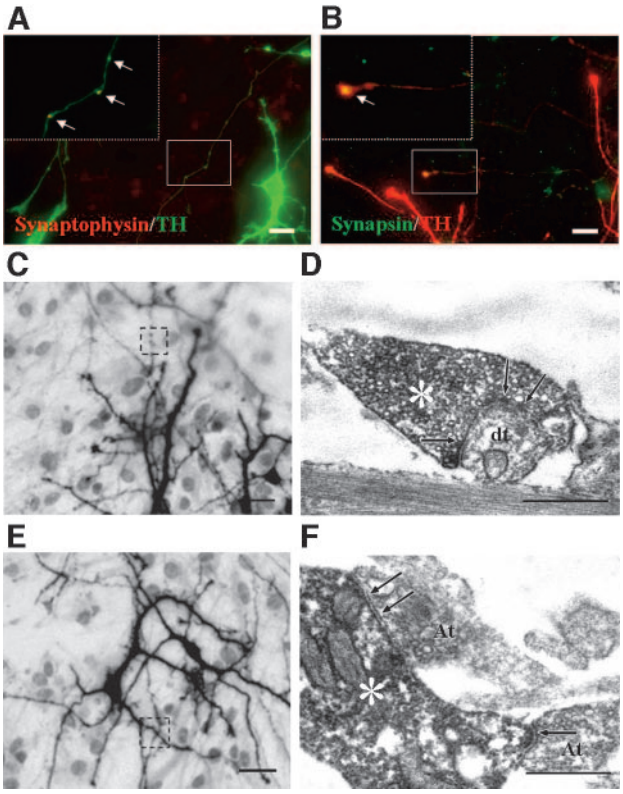

Figure 4. Ultrastructures of synapses generated by Nurr1-SVZ-derived dopamine neurons. **(A, B):** Double immunofluorescent images for TH with the markers for At, synaptophysin **(A)**, and synapsin **(B)** in the differentiated cultures for Nurr1-SVZ cells. Boxed areas are shown magnified in the insets at top left. Note that synaptophysin and synapsin were localized in the boutons on the TH-positive axons. **(C–F):** Light microscopy **(C, E)** and electron microscopy (EM) **(D, F)** for the immunoperoxidase staining of TH-stained SVZ cells. Boxed areas in **(C)** and **(E)** represent the areas for the EM observation in **(D)** and **(F)**, respectively. The image shown in **(D)** demonstrates that a TH-positive At (asterisk) containing round vesicles forms synaptic contact with a TH-negative dt. **(F):** Ultrastructures of synapses are also observed between TH-positive dt (asterisk) with two TH-negative At. Arrows in **(D)** and **(F)** indicate the sites of synaptic contacts. Scale bar = 20 μm **(A–C, E)** and 0.5 μm **(D, F)**. Abbreviations: At, axon terminal; dt, dendrite; TH, tyrosine hydroxylase.

minute per 1,000 TH+ cells, respectively (Fig. 5C). In whole-cell recording on cells differentiated from Nurr1-SVZ NP cells (transduced with Nurr1-IRES-GFP), depolarizing voltage steps elicited both large outward potassium currents and fast inward Na^+ currents. (Fig. 5D), which are hallmark features of differentiated neurons.

Neurogenic Factors Enhance DA Neuron Yields from Nurr1-Expressed Adult NPs

Although most Nurr1-TH+ cells expressed neuronal markers in adult NP cultures, as described above, none of the TH+ cells were positive for glial markers GFAP and CNPase (data not shown). These findings imply that Nurr1-induced TH expression occurs only in neuronal progeny and that DA neuronal yield in the Nurr1-transduced cultures could be further enhanced by neurogenic factor treatments. Treatment with neurogenic cytokines BDNF and NT-3 resulted in two- to threefold increases in TuJ1+ neurons (Fig. 6A, 6D, 6J). Yields of TH+ cells were also strikingly enhanced in the cultures treated with these cytokines (Fig. 6B, 6E, 6J); 5.9% ± 2.0% (untreated), 19.8% ± 4.7% (BDNF), and 15.7% ± 5.4% (NT3) of total cells

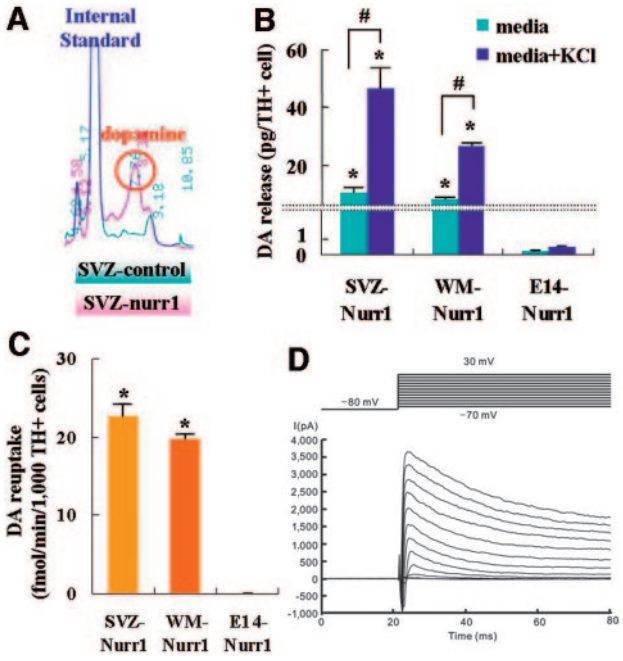

Figure 5. In vitro presynaptic neuronal functions of DA cells differentiated from Nurr1-transduced adult neural precursor (NP) cells. **(A–C):** At day 10 of in vitro differentiation, DA releases and uptakes specific for Nurr1-induced DA cells were obtained by subtracting the respective untransduced control values from those of Nurr1-transduced cultures. Graphs **(B, C)** represent DA release and uptake per unit of Nurr1-induced TH+ cells, calculated by dividing the specific values by TH+ cell numbers in the respective dishes cultured in parallel. Shown in **(A)** is a representative result of high-performance liquid chromatography for DA level in the medium of untransduced control SVZ (blue) and Nurr1-transduced SVZ (red) cultures. Graph **(B)** shows DA levels in the medium (basal release; sky blue) and medium containing 56 mM KCl (evoked release; dark blue). ∗, Significantly different from the respective values of E14 cortical cultures at $p < .01$; $n = 6$ for each value. #, Significantly different from the respective levels of basal DA release at $p < .01$. **(C):** DA transporter-mediated specific DA uptake. ∗, Significantly different from the respective values of E14 cortical cultures at $p < .01$; $n = 10$. **(D):** Electrophysiological properties of differentiated Nurr1-SVZ cells. NP cells derived from SVZ were transduced with Nurr1-IRES-green fluorescent protein (GFP) and differentiated for 7 days. Under GFP-filtered microscopy, Nurr1/GFP-expressing cells were subjected to patch-clamp recording. Shown in graph are large outward K^+ currents and fast inward Na^+ currents elicited by depolarizing voltage steps, which is characteristic feature of mature neuron. Abbreviations: DA, dopamine; E14, embryonic day 14; SVZ, subventricular zone; TH, tyrosine hydroxylase; WM, white matter.

were TH+. Mash1 is a proneural basic helix-loop-helix transcriptional factor that plays an essential role during neuronal specification of precursor cells (reviewed in [31–34]). Forced expression of Mash1 robustly enhanced TuJ1+ neuronal yields from SVZ- and WM-NP cells (Fig. 6G, 6J). Co-expression of Mash1 in Nurr1-transduced cultures by co-infection using the mixture of the viruses expressing Nurr1 (Nurr1-IRES-LacZ) and Mash1 (Mash1-IRES-LacZ) individually resulted in an increased yield of TH+ cells (5.8% ± 2.2% in the SVZ− cultures transduced with Nurr1 alone vs. 21.3% ± 7.2% in the cultures transduced with Nurr1+Mash1). The percentage of TH+ cells out of total cells was further enhanced by co-expression of Nurr1 and Mash1 in the same cells using retroviral transduction with bicistronic Nurr1-IRES-Mash1 vector (37.4% ± 12%; Fig. 6B, 6H, 6J). These findings, taken together, suggest that Nurr1-induced DA differentiation is correlated with neurogenesis of adult NP cells and specifically occurs in the cells of neuronal

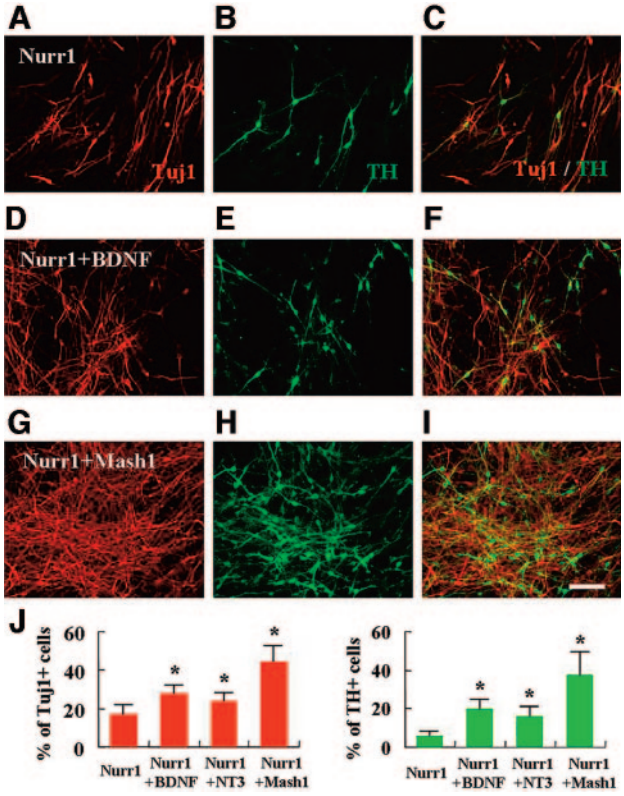

Figure 6. Neurogenic factors enhance TH+ neuronal yields from Nurr1-subventricular zone (SVZ)- and white matter (WM)-derived neural precursor cells. Cultured SVZ and WM precursor cells were transduced with Nurr1 or Nurr1+Mash1 (Nurr1-IRES-Mash1) and differentiated in the absence or presence of neurogenic cytokines BDNF and NT3. The number of TuJ1+ and TH+ cells were determined 7 days after differentiation. Shown in **(A–I)** are representative images for TuJ1+ (left column), TH+ (middle), and merged TuJ1+/TH+ (right) immunocytochemistry on the cells transduced with Nurr1 (upper row), Nurr1+Mash1 (bottom row), and Nurr1+BDNF treatment (middle row). Scale bar = 40 μm. The graph in **(J)** represents the percentage of TuJ1+ and TH+ cells out of total (4′,6 diamidino-2-phenylindole +) cells. Note that co-expression of proneural basic helix-loop-helix Mash1 and BDNF/NT3 treatments greatly enhanced Nurr1-induced DA neuronal yields in the cultures of adult neural precursor cells. ∗, Significantly different from the respective Nurr1-transduced control values at $p < .01$; $n = 20–30$ microscopic fields obtained from 3–5 independent cultures. Abbreviations: BDNF, brain-derived neurotrophic factor; NT3, neurotrophin-3; TH, tyrosine hydroxylase.

lineages. These results also indicate that neurogenic factor treatment and expression might be used for efficient derivation of DA neurons from adult NP cells.

In Vivo Survival, Differentiation, and Function of Nurr1-Transduced SVZ NPs

In vivo analyses were carried out in the hemi-parkinsonian rats grafted with SVZ-NPs transduced with Nurr1 and Nurr1+Mash1. In histological assessment at 6 weeks after transplantation, the numbers of TH+ cells were 412 ± 122 ($n = 4$) and 2,704 ± 368 ($n = 8$) in the animals grafted with Nurr1 and Nurr1+Mash1, respectively. Subpopulations of TH+ cells within the grafts exhibited morphologically mature neuronal shape with extensive neurite arborization, and neuronal markers MAP2 and VMAT2 were localized in the TH+ cell populations (Fig. 7C, 7D). Behavioral assessment in the PD rats grafted was performed using an amphetamine-induced rotation test for 6 weeks after transplantation (Fig. 6G). None of the sham-operated rats (PBS injected; $n = 8$) exhibited a reduction in amphetamine-induced rotation 6 weeks post-transplantation. By contrast, two out of six Nurr1-grafted rats and 9 out of 14 Nurr1+Mash1-grafted rats exhibited >40% reduction in rotation scores. The average rotation scores 6 weeks after transplantation, compared with the pretransplantation values, were 914 ± 129 in the sham controls ($n = 8$) versus 734 ± 259 in the Nurr1-grafted animals ($n = 6$; $p < .05$) and 453 ± 84 in the Nurr1+Mash1-grafted animals ($n = 14$; $p < .01$).

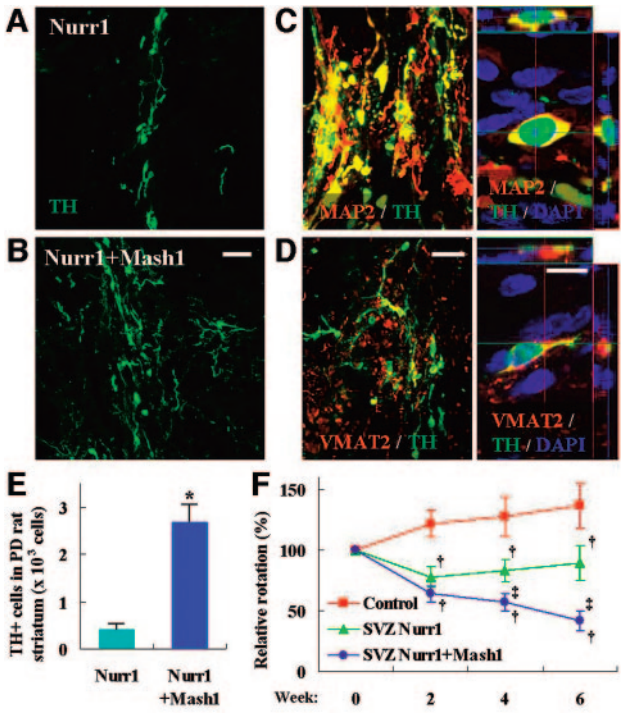

Figure 7. In vivo transplantation study. **(A, B):** Representative confocal images of TH+ cells in the striatal grafts of PD rats transplanted with SVZ-neural precursors (NPs) transduced with Nurr1 **(A)** and Nurr1+Mash1 **(B)** at 6 weeks after transplantation. **(C, D):** Co-expression of TH with mature neuronal (MAP2 **[C]**) and DA neuronal (VMAT2 **[D]**) markers in the grafts derived from Nurr1-SVZ NP cells. Right panels **(C, D)** are enlarged images of TH/MAP2/DAPI and TH/VMAT/DAPI, respectively, with which colocalizations of TH/MAP2 and TH/VMAT2 are further confirmed. Images were obtained by stacking z-series through the thickness of section (35 μm). Scale bars = 20 μm (**[A, B]**; left images in **[C]** and **[D]**) and 5 μm (right images in **[C]** and **[D]**). **(E):** Quantification of viable TH+ cell numbers in the grafts generated by Nurr1- and Nurr1+Mash1-transduced SVZ cells at 6 weeks after transplantation. ∗, Significantly different from Nurr1-grafted at $p < .01$; $n = 4$ (Nurr1) and $n = 8$ (Nurr1+Mash1). **(F):** Rotation score induced by amphetamine for 6 week after transplantation. Significantly different from control (†) and Nurr1-grafted (‡) at $p < .01$; $n = 8$ (shame control, phosphate-buffered saline-injected), $n = 6$ (Nurr1), and $n = 14$ (Nurr1+Mash1). Abbreviations: DAPI, 4′,6 diamidino-2-phenylindole; MAP2, microtubule-associated protein; PD, Parkinson disease; SVZ, subventricular zone; TH, tyrosine hydroxylase; VMAT2, vesicular monoamine transporter 2.

Discussion

In this study, we have demonstrated that NP cells derived from adult rat SVZ and WM can be expanded in vitro and efficiently differentiated toward functional DA neurons, which could potentially be used in basic and translational studies for PD. An adult precursor-based approach has the critical merit of circum-

venting ethical concerns over using embryos or fetuses as a source of precursor cells. Furthermore, an autologous transplantation strategy using adult brain-derived NP cells might work by isolating and expanding adult NP cells in vitro and then reimplanting them into the damaged site of the patient brain. In an autologous grafting approach, the SVZ and WM would be the most likely regions from which NP could be isolated without significant damage to brain function, as these regions are less specific in their physiological functions relative to other NP-containing regions, such as the hippocampus and midbrain substantia nigra, which play critical roles in memory and motor control, respectively.

Nunes et al. [10] demonstrated identification and isolation of multipotent NP cells from adult subcortical WM using oligodendrocyte progenitor marker-based sorting. They claimed that the restriction of oligodendrocyte progenitors into an oligodendroglial phenotype might be due to environmental cues surrounding in vivo adult WM and that purification of these progenitors by sorting is required to remove paracrine influences, thereby causing these cells to acquire the potential to generate neurons and astrocytes. However, here we successfully isolated multipotent NP cells from WM without the purification step, by simply culturing in suspension with the combined mitogen treatment of bFGF+PDGF. Our initial cell maintenance at low cell density and transfer of primitive spheres into fresh dishes (as described in Materials and Methods) might serve to eliminate the paracrine influences removed by oligodendrocyte marker-based sorting [10].

Despite differences in sphere formation rate and proliferation capacity of SVZ and WM NP cells in vitro, the phenotypic properties and general differentiation potentials of these cells were indistinguishable. Over 90% of cells in the neurospheres derived from both brain regions expressed the NP-specific marker nestin. Interestingly, the astroglial marker GFAP was colocalized in approximately 48%–52% of nestin+ cells, and these cells were localized in the center portion of the spheres. Considering the nature of astroglial neural stem cells in the adult SVZ and hippocampal dentate gyrus [17], the nestin+/GFAP+ cells in the center of the spheres might be early stem cells or precursors (B cells [4]) maintaining their original in vivo properties. The nestin+/GFAP− cells on the periphery of the spheres could be either cells that lost their GFAP immunoreactivity during in vitro proliferation or cells directly originating from nestin+/GFAP− transit-amplifying C cells in the SVZ [35, 36] or C-cell-corresponding cells in the WM. However, it is also possible that the nestin+/GFAP+ cells are differentiated progenitor cells with a differentiation phenotype of astrocytic progeny. This concept assumes that cell differentiation could occur preferentially in the center of spheres, where mitogenic signals from the media might not extend through the multilayer cell barrier of the spheres.

Consistent with preferred in vitro astroglial differentiation potentials of adult brain-derived neurospheres [37–39], 40%–50% of the SVZ and WM NP cells differentiated into GFAP+ astrocytes. The preferential astrocytic differentiation of WM-derived NP cells in this study is in contrast to the finding of Nunes et al. [10] that most WM NP cells derived from purified oligodendrocyte progenitors retained differentiation toward oligodendrocytic progeny. Further research is needed to clarify which experimental parameters are responsible for the difference in differentiation potentials between the two approaches.

Efficient DA differentiation of cultured NP cells has been demonstrated to be confined to cells derived from ventral mesencephalon early in embryonic development [13]. To date, no study has shown a high yield of DA neurons from NPs derived from adult brain tissues, including midbrain substantia nigra [7]. A recent article has reported DA neuron differentiation from cultured adult tegmental NP cells using coculture conditions with PA6 stromal cells [37]. However, the DA neuronal yield in the study was extremely low, with only 0.12% of total cells positive for TH. Given the limited success in driving dopaminergic differentiation in naïve NPs using extrinsic cues, we attempted to induce transgene expression of Nurr1, a key transcription factor for DA phenotype expression, in adult NPs. Ectopic Nurr1 expression, combined with neurogenic factor treatments, yielded 30%–40% TH+ cells. This TH+ cell yield is higher than that from precursors derived from rat E12 ventral midbrains (10%–20% [12]) and mouse ES cells (20%–25% [14]). Furthermore DA cells differentiated from the Nurr1-transduced SVZ and WM NPs were not only morphologically, ultrastructurally, and phenotypically mature but also demonstrated robust presynaptic functions of DA release and DAT-mediated specific DA uptake in vitro. These findings collectively suggest the possible large-scale generation of functional DA neurons from Nurr1-engineered adult brain-derived precursor cells.

The Nurr1 effect in adult NP cell cultures is in contrast to that of embryonic brain-derived NP cells demonstrating DA phenotype expression in Nurr1-transduced embryonic NP cells without neuronal differentiation and acquisition of presynaptic DA neuron functionality (Figs. 3, 5) [20, 22, 29, 40, 41]. Experimental culture conditions such as cell density [42], mode of culture (adherent or suspension culture), degree of cell-cell contacts, and ingredients and cytokines in the culture medium can affect neuronal differentiation of cultured NP cells. However, differences in the neuronal differentiation of Nurr1-DA cells derived from adult and embryonic brains are likely to be, rather, intrinsic, because all the possible extrinsic factors were adjusted identically in the cultures of adult and embryonic NP cells: for example, both adult and embryonic NP cells were expanded in the form of floating neurospheres, passaged, Nurr1-transduced, and differentiated with identical procedures and conditions (as described in Materials and Methods). In an effort to understand the molecular basis for the different effects of Nurr1 in adult and embryonic NP cells, we compared expression levels of several differentiation factors, including Bcl-XL, SHH, and Mash1, co-expressions of which in embryonic NP cells cause enhanced neuronal differentiation of Nurr1-DA cells [22]. However, no clear discrepancy was observed in the mRNA expression levels of those factors tested (supplemental online Fig. 2). We further examine the expressions of RhoA (neurite extension [43]) and PC3 (neuronal birth [44]), resulting in no clear differences of their expression levels between the adult and embryonic NP cells. Expression of SCG10, enriched in the growth cones of the developing neurons [45], was rather decreased in the SVZ and WM cultures compared with that of E14 NP cells. Additional studies are required for an understanding of the molecular basis for the difference of the Nurr1 effects between adult and embryonic NP cells.

We further demonstrated that SVZ NPs transduced with Nurr1 or Nurr1+Mash1 survived, differentiated into TH+ DA neurons in the striatum of PD animals for 6 weeks post-transplantation, and functioned to restore the behavioral deficit of PD rats. However, the numbers of viable TH+ cells were only 412 and 2,704 cells out of 1×10^6 cells injected in the animals grafted with Nurr1 and Nurr1+Mash1, respectively: survival rates, calculated by dividing the TH+ cell numbers in vivo by the numbers of TH+ cells counted after 7 days of in vitro differentiation in parallel cultures, were $0.8\% \pm 0.2\%$ in Nurr1 and $1.1\% \pm 0.4\%$ in Nurr1+Mash1 grafts, indicating insufficient in vivo survival or differentiation of these cells. In addition, TH+ cells in the grafts demonstrated a diversity of morphologic differentiation: subpopulations of TH+ cells in the grafts exhibited immature neuronal morphologies with short or

no processes, whereas certain populations of TH+ cells had fully differentiated neuronal shapes and phenotypes with extensive and elongated fibers and co-expression of mature neuronal or DA neuronal markers (Fig. 7A–7D). This is in contrast to the in vitro cultures, where the majority of TH+ cells derived from Nurr1-transuced adult NPs were morphologically, phenotypically, and functionally mature DA neurons (Figs. 3–5). Consistent with our observations in vivo, poor survival and limited neuronal differentiation of adult SVZ cells have previously been reported after ectopic grafting into the adult brain [46]. The poor survival and neuronal differentiation might be attributable to limited senescence of adult NP cells per se, incompatible donor-host interactions, or inadequate cell manipulation during cell dissociation, retroviral transduction, and cell transplantation. Further investigation into ensuring better in vivo survival and differentiation is needed.

In conclusion, our study demonstrates the first example of an efficient derivation of functional DA neurons from adult brain-derived precursor cells using genetic manipulation of dopaminergic and neurogenic genes. The use of genetically manipulated adult NP cells could provide an interesting alternative over the use of fetal midbrains as a DA neuron source and opens the possibility of autologous cell transplantation for PD patients. However, it remains to be determined whether our approach is applicable to human cells and whether the risk of introducing transgenes is manageable in a therapeutic setting.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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