Molecular Neurobiology (2019) 56:4395–4413
https://doi.org/10.1007/s12035-018-1367-3

Astrocyte-Like Cells Differentiated from Dental Pulp Stem Cells Protect Dopaminergic Neurons Against 6-Hydroxydopamine Toxicity

Kavina Ganapathy[1,2] · Indrani Datta[1] · Ramesh Bhonde[2,3]

Received: 23 August 2018 / Accepted: 25 September 2018 / Published online: 16 October 2018

Abstract

Dental pulp stem cells (DPSCs) are promising for use in neurodegenerative-diseases because of their neural crest origin. While neuronal differentiation of DPSCs has been shown, their plasticity towards astrocyte-like cells remains to be studied. We aimed to examine differentiation potential of DPSCs to astrocytes and their consequent neuroprotective role towards dopaminergic (DA) neurons under 6-hydroxydopamine (6-OHDA) toxicity. Induction of DPSCs to astrocytes with differentiation factors showed definitive increase in astrocyte-specific markers glial fibrillary acidic protein (GFAP), and excitatory amino acid transporter 2 along with glial calcium-binding protein S100β through FACS and immunofluorescence assays. RT-PCR and ELISA showed significant increase in BDNF and GDNF expression and secretion in astrocyte-differentiated DPSCs over naïve DPSCs. Neuroprotective role of these cells on DA neurons under 6-OHDA stress was evaluated by both contact and non-contact methods. FACS analysis of PKH26-stained SH-SY5Y homogenous cells in contact method and of TH immunopositive cells in primary midbrain culture in non-contact method both indicated higher survival of DA neurons in astrocyte-differentiated DPSCs over naïve DPSCs. Recovery of β-tubulin III and TH immunopositive cells was reduced in the presence of TrkB inhibitor, suggesting a key neuroprotective role of BDNF secretion by DPSCs. When nitric oxide (NO) release was inhibited by L-NAME in primary midbrain culture, BDNF release in co-culture under 6-OHDA stress reduced further in naïve DPSCs than in astrocyte-differentiated DPSCs, suggesting that BDNF release in naïve DPSCs is primarily regulated by paracrine signaling while for differentiated DPSCs, it is equally through autocrine and paracrine signaling with NO being the mediator. In conclusion, we suggest that DPSCs exposed to glial commitment cues exhibit substantial differentiation towards astrocyte-like cells with better neuroprotective activity against 6-OHDA toxicity than naïve DPSCs.

Keywords Neuroprotection · Astrocyte differentiation of dental pulp stem cells · Brain-derived neurotrophic factor · Nitric oxide · Dopaminergic neurons · 6-Hydroxydopamine

Introduction

Astrocytes are the most abundant glial cell type in the central nervous system and are critical for the protection of neurons in the adult brain. Among its numerous functions, the astrocyte is known to maintain an optimal milieu for the survival and functioning of neuronal cells through antioxidant protection, glutamate clearance, release of neurotrophic factors and cytokines, and maintenance of ionic balance [1–3]. An insult to the central nervous system (CNS) in response to an injury or a disease may result in cellular, molecular, and functional changes in astrocytes, leading to a state known as "reactive astrogliosis" [4, 5]. Studies have suggested that the transient or permanent impairment of these astroglial cells during brain damage leads to neuronal death [2, 6]. SOD1 mutation in astrocytes has been reported in familial amyotrophic lateral sclerosis (fALS) contributing to the selective death of motor

Kavina Ganapathy and Indrani Datta contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12035-018-1367-3) contains supplementary material, which is available to authorized users.

✉ Indrani Datta
indranidatta.nimhans@gmail.com; indrani@nimhans.kar.nic.in

[1] Department of Biophysics, National Institute of Mental Health and Neurosciences, P.B. No – 2900, Hosur Road, Bengaluru, Karnataka 560029, India

[2] School of Regenerative Medicine, Manipal University, Bengaluru, Karnataka 560065, India

[3] Dr. D. Y. Patil Vidyapeeth, Pimpri, Pune, Maharashtra 411018, India

neurons [7, 8]; similarly, studies have shown that genes (DJ1, PINK1, iPLA$_2$, Parkin, LRRK2) known to have a contributory role in the development of PD have key roles in astrocyte function [9–16]. These reports highlight the impairment of niche cells of specialized neurons in the disease state. Whether this endogenous niche cell function can be mimicked by tissue derived stem cells is therefore the next question.

To understand if tissue-derived stem cells could be a potential way to mimic the endogenous niche cell function, we carried out our study with dental pulp stem cells (DPSCs). Among the adult tissue-derived stem cells, DPSCs are of prime interest for use in nervous system diseases because of their part cranial neural crest origin [17, 18]. Tooth organogenesis studies indicate the contribution of embryonic ectomesenchymal, ectodermal epithelial, and peripheral nerve-associated glial origin of DPSCs [17, 19–21], making them an attractive source for cell replacement of the nervous system. DPSCs are non-tumorogenic and share immunomodulatory properties, mesenchymal surface marker profile, and tri-lineage differentiation potential with BM-MSCs. Unlike BM, extracted teeth are discarded tissue and hence DPSCs inherently have fewer ethical concerns. Our earlier study has shown the capability of DPSCs to respond to developmental midbrain cues for differentiation to dopaminergic neurons [22] in vitro. Their capability for differentiation to neuronal cells in vivo following transplantation in rodent brain in instances of brain injury and neurodegenerative diseases has also been reported [23–25]. However, their plasticity towards non-neuronal supportive cells of the CNS is yet to be reported. Given the crucial importance of replacing impaired endogenous niche cells of the CNS in neurodegenerative diseases with healthy exogenous supportive cells, it is essential to evaluate the capability of DPSCs to differentiate to functional astrocyte cells. Therefore, the aim of our present study was to evaluate the differentiation potential of DPSCs to astrocytes and validate their neuroprotective role towards dopaminergic neurons under 6-hydroxydopamine (6-OHDA) toxicity in comparison with naïve DPSCs. This will clearly indicate whether naïve DPSCs or astrocyte-like differentiated DPSCs are the better cell of choice as exogenous supportive cells in Parkinson's disease.

Materials and Methods

Schematic representation of experimental design is provided in Fig. 1.

Culture and Characterization of Dental Pulp Stem Cells

Third molar teeth were collected from patients aging 18–40 years, undergoing extractions for various medical conditions at the Department of Dentistry, Manipal Hospital, Bangalore, India (approved by the Institutional Ethical Committee, Manipal Hospital, Bangalore). The isolation procedure for each tooth was performed as per our previous report [26]. The pulp tissue post extraction was minced into smaller pieces and further subjected to an enzymatic dissociation in 2 mg/ml of collagenase (Sigma-Aldrich, St. Louis) for 1 h at 37 °C in a humified chamber. Isolated cells were plated in Knock-out Dulbecco's modified Eagle's medium (KO-DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS), 5 mM L-glutamine, and 50 U/ml pencillin-streptomycin (all purchased from Invitrogen) and incubated at 37 °C with 5% CO_2 and > 80% humidity to avoid evaporation. Growth medium was replaced twice a week and then cells were characterized and evaluated for multipotency by inducing DPSCs to osteogenic and adipogenic lineages. Cells were passaged either at the time of the experimental assay or at confluence (85%). The expression of mesenchymal markers was analyzed using direct immune phenotyping as described by Datta et al. (2011) [27]. In brief, cells were detached by enzyme 0.25% Trypsin-EDTA (Sigma), washed using phosphate buffer saline (PBS; Invitrogen) and fixed in 2% paraformaldehyde (PFA) at 4 °C for 30 min. Cells were labeled with specific cell surface epitopes like CD73-Fluorescein isothiocyanate (FITC), CD90-FITC, CD105-FITC, CD80-FITC, and HLADR-FITC along with their respective isotype controls (all antibodies obtained from BD Pharmingen). Flow cytometry analysis was performed on FACS Verse (BD Biosciences). Cells were identified by light scatter for 10,000 gated events and analyzed using BD FACSuite software. Next, multipotency was assessed through osteogenic and adipogenic differentiation. DPSCs were subjected to osteogenic and adipogenic differentiation media for 21 days as described in Majumdar et al. [22]. After 21 days of induction, calcium mineralization was assessed by Von Kossa staining in the plate containing osteogenic medium and the presence of lipid droplets was assessed by Oil Red O staining in the plate containing adipogenic medium. Further validation was done by analyzing messenger RNA (mRNA) expression of osteocyte and adipocyte markers, osteocalcin (OCN), Runx2, peroxisome proliferator-activated receptor (PPAR)γ, and fatty acid-binding protein 4 (FABP4), respectively.

Astroglial Induction of DPSCs

DPSCs were plated at the density of 1×10^5 per 35-mm dish. After 24 h, cells were primed with pre-differentiation medium, containing 2 mM glutamine, 1× N2 supplement (Invitrogen), 20 ng/ml human epidermal growth factor (hEGF), and 20 ng/ml human basic fibroblast growth factor (bFGF; Immuno Tools) in DMEM basal media for 72 h as described by Bahat-Stroomza et al. (2009) [28] with few modifications.

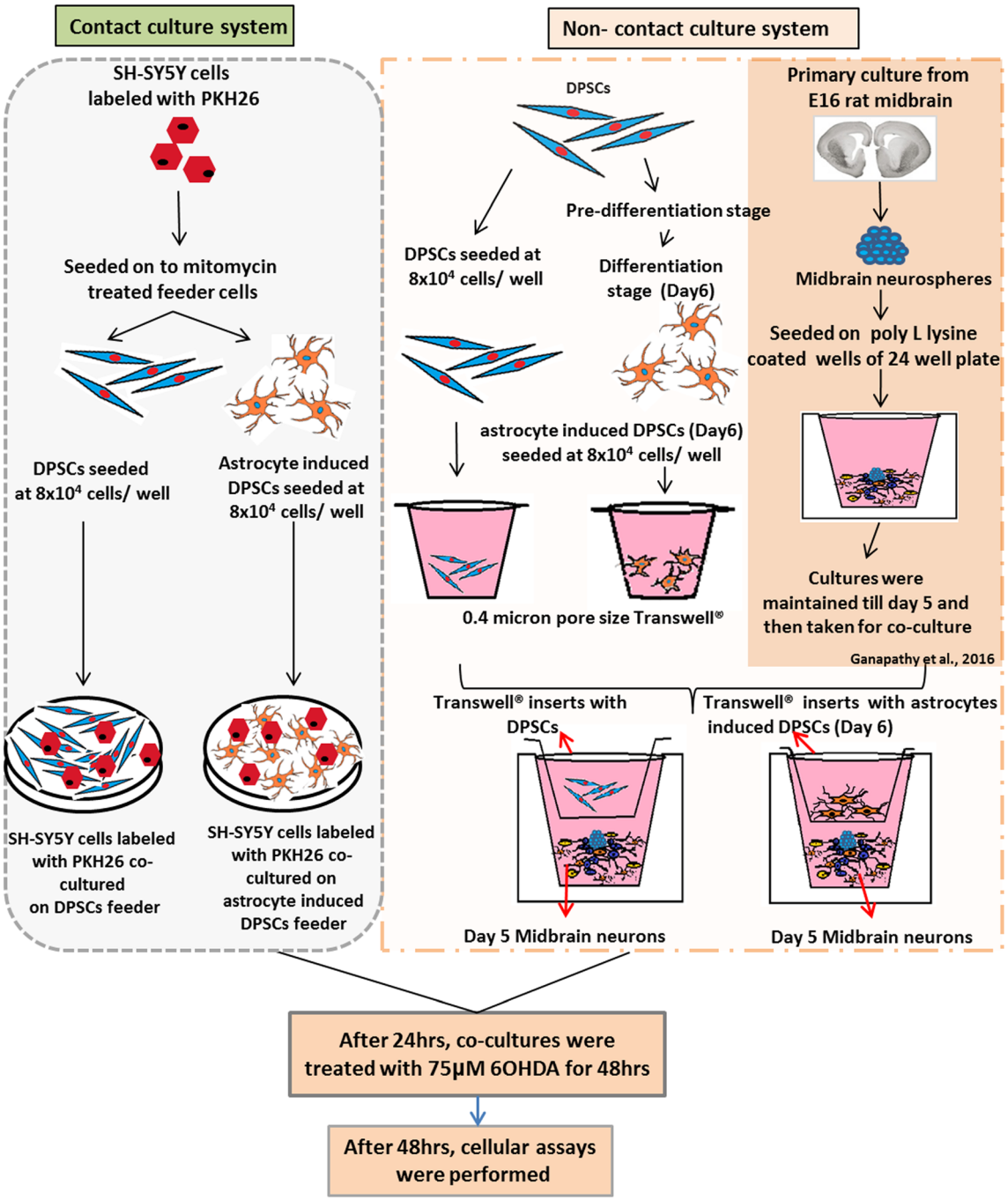

Fig. 1 Co-culture model of DPSCs, astrocyte-induced DPSCs with SH-SY5Y and primary midbrain culture under 6-OHDA stress. Two sources of dopaminergic cells were used in the two types of co-culture system: (1) contact co-culture where homogenous secondary cell line (SH-SY5Y) were used and (2) non-contact co-culture where day 5 primary midbrain culture obtained from rat E16 midbrain by following the procedure described in [31] were used. First, DPSCs were induced to CNS supportive cells (astrocytes) in vitro, and then the DPSCs and the differentiated counterparts were co-cultured in contact and non-contact systems. In contact system, PKH26-labeled SH-SY5Y cells were seeded on the mitomycin-treated DPSCs and astrocyte-differentiated DPSCs. In non-contact system, DPSCs and astrocyte-differentiated DPSCs were seeded on trans-well which was then placed in the well containing midbrain primary neurons. After 24 h, both the contact and non-contact systems were treated with 75 µM 6-OHDA for 48 h and evaluated for several cellular events

After 72 h, cells were induced with DMEM/F12 medium containing 50 ng/ml human neuregulin1-β1, 20 ng/mL bFGF, 5 ng/ml platelet-derived growth factor (PDGF) (all factors from Immuno Tools), and 1 mM dibutyryl cyclic AMP (db-cAMP) (Sigma). Medium was replaced for every 72 h. Differentiation day points in 72 h intervals, i.e., day 3, day 6, and day 9, were further taken for characterization experiments.

FACS Analysis for Astroglial and Neuronal Markers

For intracellular staining for astrocyte-specific markers, cells were stained using indirect method. Wherein, DPSCs and differentiated DPSCs at different day points day 3, day 6 and day 9 were fixed with 4% PFA, permeabilized with 0.1% Triton X-100 for 15 min at room temperature. Then, cells were blocked using 3% BSA for 1 h at RT followed by washes with 0.1% sodium azide. Cells were incubated with primary antibodies such as glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100β), excitatory amino acid transporter 2 (EAAT2), achaete-scute homolog 1 (ASCL1), and human natural killer-1 (HNK1) for 1 h at 4 °C followed by washes. Similar staining steps were used for the evaluation of fluorescent-labeled immunopositive cell population in primary midbrain culture co-cultured with DPSCs and differentiated DPSCs and the primary antibodies used were TH, β-tubulin III, and GFAP. Then, cells were incubated with secondary antibody conjugated with FITC. Cells were analyzed using FACS Caliber or FACS verse (BD Biosciences). Cells were identified by light scatter for 10,000 gated events and analyzed using the software CellQuestPro or FACS Suite software. Percentage immunopositive population was plotted as mean ± SEM for $n = 3$.

Immunocytochemistry

DPSCs and differentiated DPSCs were analyzed for astrocyte-specific markers fixed with 4% paraformaldehyde for 20 min at 4 °C. Permeabilization of cells was performed with 0.1% Triton X-100 for 15 min followed by blocking with 3% BSA for 1 h at room temperature. Cells were then washed with PBST (PBS with 0.05% Tween 20). These cells were incubated with primary antibodies such as GFAP (Chemicon), S100β (Abcam), and EAAT2 (Sigma) at 1:100 dilution at 4 °C overnight. Secondary antibody conjugated with FITC (anti-mouse IgG-FITC and anti-rabbit IgG-FITC at 1:500 dilutions) was added to the cells at room temperature for 45 min. PBS washes were repeated and then counterstained with a nuclear stain, 4′,6′-diamino-2-phenylindole dihydrochloride (DAPI; Sigma) for 15 min at 1:10,000 dilution for 1 min. Cover slips were mounted on DABCO mounting medium and visualized under a Nikon fluorescent microscope.

Gene Expression

RNA was isolated from DPSCs and astrocyte-induced DPSCs using TRIzol-LS Reagent (Invitrogen). Real-time PCR was performed as described earlier [29]. Briefly, complementary DNA (cDNA) was synthesized from 1 μg of total RNA using Superscript III reverse transcriptase (Invitrogen). The primers used for the RT-PCR experiments were as follows: ASCL1 forward—5′-AAGCAAGTCAAGCGACAGCG-3′, reverse—5′-GTCGTTGGAGTAGTTGGGG-3′; GFAP forward—5′-TAGCTACATCGAGAAGGTCC-3′, reverse—5′-AAGAACTGGATCTCCTCCTC-3′; BDNF forward—5′-TCATACTTTGGTTGCATGAAGGC-3′, reverse—5′ GCCGAACTTTCTGGTCCT-3′; GDNF forward—5′-GATT GCGAACTCTTGCCCCT-3′, reverse—5′-GAGC CGCTGCAGTACCTAAA-3′; NGF forward—5′-AGGG AGCAGCTTTCTATCCTG-3′, reverse—5′-GGCA GTGTCAAGGGAATGC-3′; VEGF forward—5′-TTCA TGGATGTCTATCAGCG-3′, reverse—5′-CATC TCTCCTATGTGCTGGC-3′; HGF forward—5′-CATC AAAGCCCTTGTCGGGAT-3′, reverse—5′-ACGA ACACAGCTATCGGGGTA-3′. Real time was performed using KAPA SYBR FAST qPCR kit master mix (Kappa Biosystems) as per the manufacturer's instructions. SDS.v1.4 software was used to analyze the data. The fold change in expression level of each marker was calculated using the formula $2^{-\Delta\Delta C_T}$, where C_T is the cycle threshold difference. DPSCs and trans-differentiated cell gene expression were normalized with respect to 18S. The results are presented as relative fold expression (mean ± SEM) for $n = 3$ in duplicates.

Neurotrophic Factor Release Assay

Cell supernatant was collected from a seeding density of 100,000 cells of control and differentiated DPSCs at day 3, day 6, and day 9 of astroglial induction and BDNF and GDNF was measured using enzyme-linked immunosorbent assay (ELISA). The concentration obtained was represented as nanogram per milliliter or picogram per milliliter for 100,000 cells. The co-culture experiments of naïve and differentiated DPSCs were seeded as 80,000 cells along with primary midbrain culture obtained from three differentiated neurospheres and the data is represented as picogram per milliliter. Extracellular release of BDNF (Ray Biotech) and GDNF (Elabscience) was measured according to the manufacturer's instructions. The absorbance was recorded at 450 nm using a microplate reader (Infinite®M200, TECAN).

Co-culture of SH-SY5Y Cells with DPSCs and Differentiated DPSCs in Contact Culture System

The human neuroblastoma cell line (SH-SY5Y) was a kind gift from Dr. Asok Mukhopadhyay, National Institute of Immunology (NII). These cell lines were maintained in Dulbecco's modified Eagle's medium/F12 (DMEM:F12) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Himedia), 1× glutamax (Invitrogen), and 100 μg/ml pencillin/streptomycin incubated at 37 °C and 5% CO_2. These cells were plated on mitomycin C (10 μg/ml)-treated monolayer cultures of naïve DPSCs and day 6 differentiated DPSCs at a density of 8×10^4 cells/well.

6-Hydroxydopamine Treatment

Ten millimolar stock of 6-hydroxydopamine (6-OHDA) (Sigma) solution containing 0.01% (*W/V*) ascorbic acid was prepared in buffer according to the manufacturer's instructions. LC_{50} concentration, i.e., 75 μM obtained from our previous study [30], was used for co-culture experiments and varying concentrations from 10 to 150 μM were used to assess the cell survival of DPSCs and differentiated DPSCs in monolayer culture.

Cell Survival Assay

MTT assay was performed to assess the cell survival of DPSCs and differentiated DPSCs under varying concentrations of neurotoxin 6-OHDA. In brief, control and treated cells of both DPSCs and differentiated DPSCs were incubated with 1 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) at 37 °C and 5% CO_2 for 2 h. MTT medium was aspirated and violet formazan crystals were dissolved using dimethyl sulphoxide (DMSO). Absorbance was measured at 560 nM using a microplate spectrophotometer (TECAN infinite M200). The results are presented as mean ± SEM from $n = 5$.

Cell survival was also estimated through FACS analysis using PKH26 labeling for SH-SY5Y cells. SH-SY5Y cells were fluorescently labeled using the lipophilic, cell tracking red dye, PKH26 (Sigma), as per the manufacturer's protocol. 1×10^7 cells were re-suspended in a freshly prepared PKH26 (2 μM) with diluent C for 5 min at RT. The reaction was stopped with FBS followed by washes with phosphate-buffered saline (PBS). Labeled cells were re-suspended in complete SH-SY5Y cell growth medium before seeding. Only live cells retain the PKH26 labeling. PKH26-labeled SH-SY5Y cells were seeded at density of 5×10^4cells on a mitomycin C (10 μg/ml)-treated monolayer cultures of naïve DPSCs and day 6 differentiated DPSCs at a density of 8×10^4cells/well. Co-cultures were then treated with 75 μM 6-OHDA for 48 h. Cells from control and treated groups were then harvested with 0.25% Trypsin-EDTA (Sigma) and the pellet was fixed with ice-cold 2% paraformaldehyde (PFA). The fluorescently labeled SH-SY5Y cells from all the experimental parameters were measured using BD FACS Verse (BD Biosciences). Cells were identified by light scatter for 10,000 gated events and analyzed using the FACS Suite Software (BD Biosciences).

Isolation and Culture of Primary Midbrain Neuronal Culture

Sequential culture technique as described in our previous study [31] was followed to obtain primary midbrain culture. In brief, female Sprague Dawley mother rat was euthanized at day 16 according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CUPCSEA). Midbrain was isolated, meninges were removed, and the tissue was subjected to enzymatic dissociation using accutase for 10 min at 37 °C. Dissociated cells were neutralized with serum containing medium and seeded in hanging drop method [31] using proliferative medium containing neurobasal media supplemented with 1% FBS, 1× N2 supplement, 2 mM glutamax, and 100 μg/ml penstrep (all from Invitrogen) and incubated at 37 °C with 5% CO_2. At day 2, spheres were gently pooled into a nonadherent dish and allowed to grow until day 5 in proliferative medium. Neurospheres at day 5 were seeded on poly-L-lysine (PLL)-coated plates with differentiation medium consisting of neurobasal medium with 1× N2 supplement, 30 mM glucose (Sigma), 100 μM ascorbic acid (Sigma), 2 mM glutamax, 100 μg/ml penstrep, and 20 ng/ml basic fibroblast growth factor (bFGF) (Immuno Tools) and incubated at 37 °C with 5% CO_2. Medium was changed for every 2 days until day 5 and then cells were taken for co-culture experiments.

Non-contact Co-culture System

DPSCs and differentiated DPSCs were seeded at density of 8×10^4 cells/trans-well (0.4 mm porous, Corning) and incubated at 37 °C in 5% CO_2. After 24 h, trans-well containing DPSCs and differentiated DPSCs was inserted into culture wells of day 5 primary midbrain neurons respectively (as shown in the Fig. 1). Co-cultures were treated with freshly prepared 6-OHDA at 75 μM and incubated for 48 h at 37 °C in 5% CO_2. After 48 h, primary midbrain culture was taken for cellular assays such as FACS analysis and ELISA.

Addition of Inhibitors

To assess the neuroprotective effect through BDNF secreted by DPSCs and differentiated DPSCS on primary midbrain neurons, ANA12 a TrkB inhibitor was used to block the BDNF receptor in the primary midbrain culture [29]. Day 5 primary midbrain culture was incubated with 1 μM ANA12 in growth medium for 2 h at 37 °C and 5% CO_2. Next, cultures were washed with PBS and taken for trans-well co-culture system with DPSCs and differentiated DPSCs treated with 75 μM 6-OHDA as mentioned in above methodology. Further, midbrain primary culture was immunostained for TH, β-tubulin III, and GFAP and analyzed by FACS as described earlier.

For nitric oxide inhibition, DPSCs, differentiated DPSCs, and primary midbrain cultures were separately treated with NO inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME) at concentration of 100 μM for 2 h at 37 °C and 5% CO_2. Further, the experiments for BDNF release and nitrite estimation were performed under control and 6-OHDA treatment.

Nitrite Estimation

As described earlier by Prasanna et al. [32], Griess reagent was used to measure nitrite levels as an indicator for NO. Supernatant was collected from midbrain neuronal cultures, DPSCs, and differentiated DPSCs separately and also co-cultured together under control and 6-OHDA-treated conditions. This was transferred into 96-well flat-bottom microtiter plates. One hundred microliter of 1× Griess reagent was added to 100 μl of supernatant in triplicates and incubated at RT for 15 min. Reading was taken using a spectrophotometer ELISA plate reader (TECAN Infinite M200) at a wavelength of 550 nm and optical density was plotted against the standard curve of sodium nitrate $NaNO_2$; the results are presented as mean ± SEM from $n = 5$ in duplicates.

Results

Characterization of Human DPSCs for Mesenchymal Properties

Human DPSCs were sub-cultured and characterized for mesenchymal stromal cell-specific surface antigens by flow cytometry. DPSCs were immunopositive for CD90 (94.48%), CD73 (87.85%), and CD105 (93.03%) and negligible expression of hematopoetic marker CD34 (1.13%) and MHC class II surface receptor HLADR (2.3%) was observed (Fig. 2a). Further, the differentiation potential of DPSCs towards osteogenic and adipogenic lineages was assessed. The induced cells were positive for Von Kossa and Oil Red O staining representing osteogenic and adipogenic differentiation respectively (Fig. 2b). Control DPSCs without the induction factors were negative for both the stains (Fig. 2b). RT-PCR analysis showed expression of early osteogenic transcription factor Runx2 in control DPSCs but the terminal osteocyte marker osteocalcin was expressed only upon induction (Fig. 2c). Control DPSCs also showed basal expression of early adipocyte marker PPARγ2 and it increased in the differentiated state. Low expression of terminal adipocyte marker fatty acid-binding protein 4 (FABP4) was observed in control DPSCs and this too increased upon adipogenic induction (Fig. 2c).

Morphological, Gene Expression, and Immunophenotypical Changes of DPSCs upon Astroglial Induction

Control cells without differentiation factors showed flattened fibroblast-like morphology (Fig. 3(A)). Wherein, induced cells showed stellated morphology from day 3 onwards. Day 6 exhibited more complex processes which remained intact even at day 9 (Fig. 3(A)). Comparative and quantitative evaluation of early and late astrocyte-specific markers by flow cytometry analysis showed the quantitative increase in immunopositive population of S100β at day 3 (48%), day 6 (44%), and day 9 (67%) compared to control (14%). Glial fibrillary protein GFAP showed higher expression than control (35.6%) and remained similar within the differentiation day points (45%, 59%, and 53% for days 3, 6, and 9 respectively). Glutamate aspartate transporter EAAT2 expression showed increase upon astroglial induction from 11% (control) to 44% (day 9) as show in Fig. 3(B). The neural progenitor fate-specific marker Ascl1 and neural crest marker HNK1 expressed in control DPSCs decreased upon astroglial induction showing the shift from neural progenitor state to astrocyte-differentiated state. FACS histograms of each marker with respect to differentiation day points are represented in supplementary figure 1. Gene expression of control DPSCs showed higher level of Ascl1 than days 3 and 9 of induction [Fig. 3(C(a))]. Similarly, expression of GFAP showed marked increase at all induction day points with respect to control, confirming the transition of DPSCs to astrocytes [Fig. 3(C(b))]. Astrocyte-specific protein expression was further confirmed by immunocytochemistry. Qualitative representation of day 6 induced cells for astrocyte-specific proteins showed GFAP and EAAT2 along with glial calcium-binding protein S100β expression in stellated cells compared to control cells (Fig. 3(D)). Analysis of mesenchymal stromal surface antigens in differentiated cells showed less expression of CD73 (14.79%), CD90 (14.5%), and CD105 (22.52%) and negligible expression of HLADR (1.02%). In addition, analysis of co-stimulatory marker CD80 which is responsible for immune response was nil (0.96%). However, early neuronal marker nestin (45%) showed moderate expression in astrocyte-induced DPSCs (Fig. 4).

Neurotrophic Factors of Differentiated DPSCs

Neural crest-derived DPSCs are known to produce neurotrophic factors. So, next, we determined whether the astrocyte-like cells obtained from DPSCs secrete glial-derived factors and retain the secretion of NTFs. At day 3 of induction, gene expression and extracellular release assay of GDNF and BDNF showed remarkable increase with respect to control ($P < 0.05$). Although there was decreased gene expression of BDNF seen at day 6 and day 9, BDNF release remained comparable with control ($P > 0.5$) [Fig. 5(A(a), B)]. A significant enhancement of neurotrophic factors, VEGF, and HGF was noticed at all day points ($P < 0.05$) [Fig. 5(d, e)] and NGF was downregulated with respect to control [Fig. 5(c)].

Effect of 6-OHDA Stress on Naïve and Differentiated DPSCs

Endogenous astrocytes are known to play an important role in neuroprotection. Hence, the neuroprotective role of

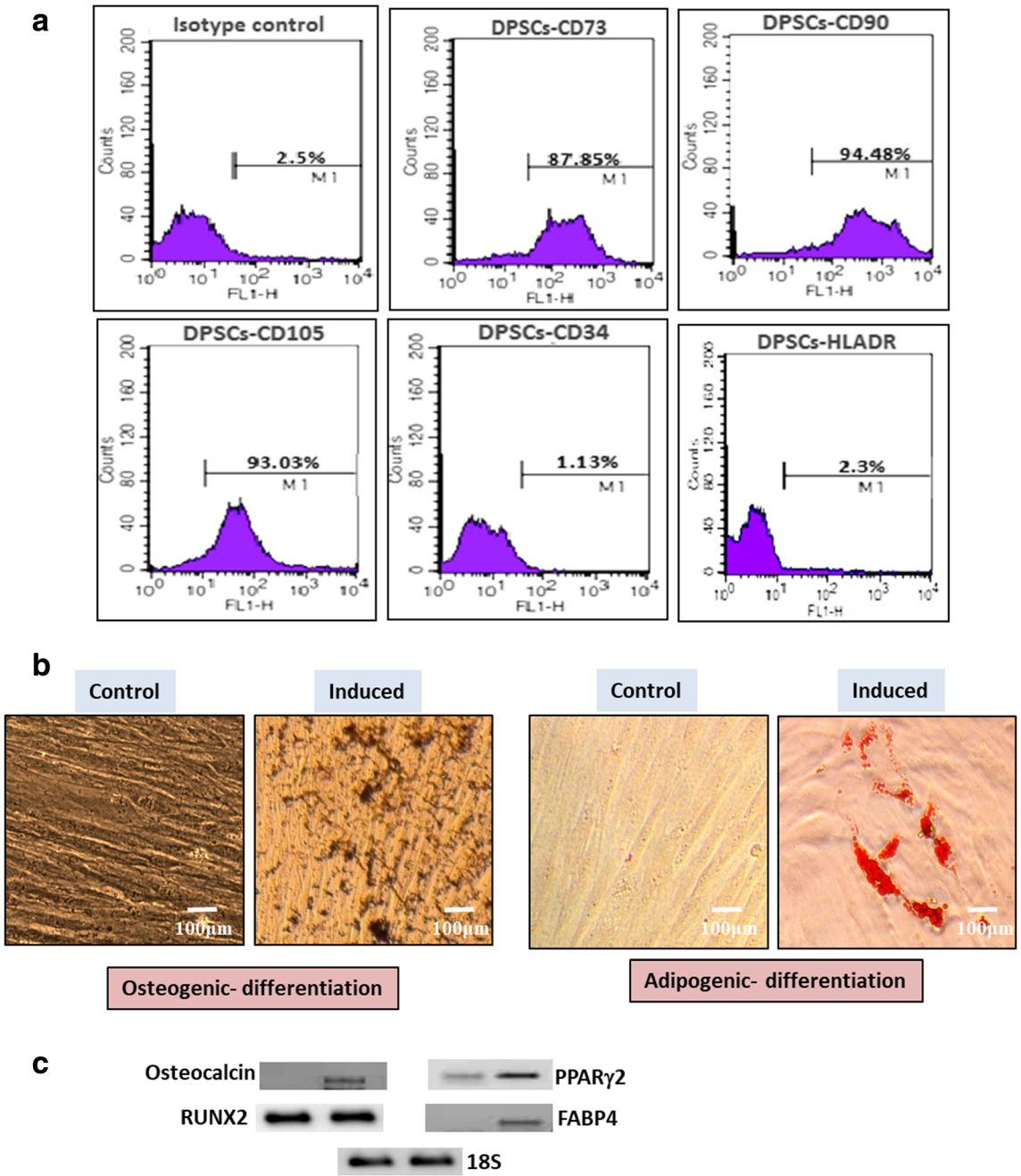

Fig. 2 Characterization of DPSCs for mesenchymal stromal markers and multipotency. **a** Representative FACS histogram of DPSCs immunolabeled with CD73, CD90, CD105, CD34, and HLADR. **b** DPSCs induced for both osteogenic and adipogenic lineages displayed calcification in osteogenic induced cells (Von Kossa stain) and oil droplets (Oil O red stain) in adipogenic induced cells compared to control. **c** RT-PCR revealed that the gene expression pattern of osteogenic markers osteocalcin and RUNX2 and adipogenic markers FABP4 and PPARγ2 in control and astrocyte-induced DPSCs

naïve and differentiated DPSCs on survival of SH-SY5Y cells under 6-OHDA stress was assessed through contact co-culture system. First, the effect of 6-OHDA on naïve and differentiated DPSCs was assessed using MTT assay. DPSCs at constant seeding density under varying concentrations of 6-OHDA at 48 h showed reduced survival with respect to control from 10 μM 6-OHDA onwards ($P < 0.005$). On contrary, differentiated DPSCs at 40 μM and 75 μM 6-OHDA treatments showed increase in cell survival ($P < 0.05$; Fig. 6a, b). Upon higher concentration of 6-OHDA treatment, the cell survival showed a mild decrease ($P > 0.05$). This evidence supports our previous study of endogenous astrocytes showing gliosis during 6-OHDA stress [29].

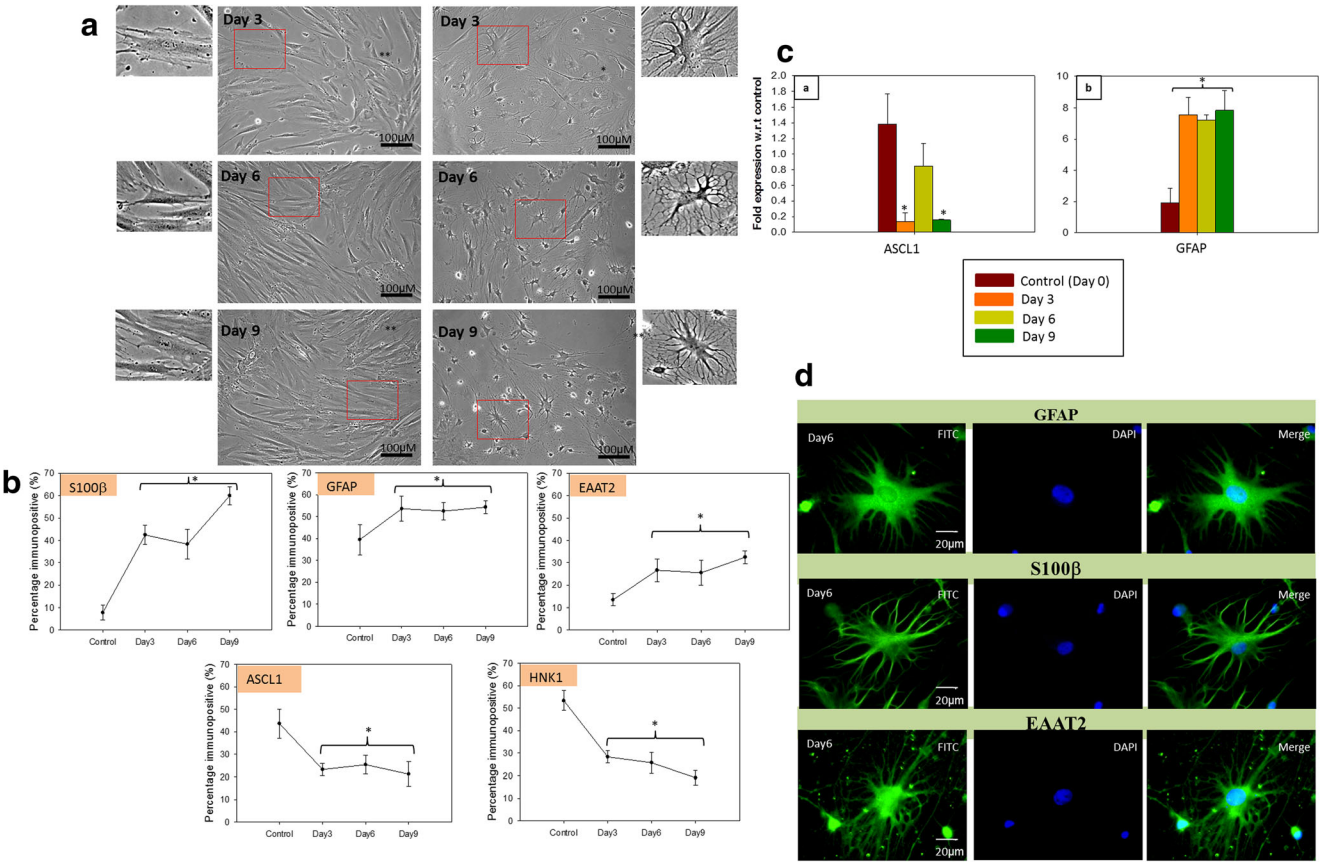

Fig. 3 Characterization of astrocyte-induced DPSCs. (A) Representative bright field images of differentiation at different day points showing the morphological changes. Higher magnification of the red inset is shown for all day points. (B) Graphical representation of FACS analysis for DPSCs at different day points of astrocyte induction for astrocyte-specific markers GFAP and EAAT2 along with glial calcium-binding protein S100β, neural progenitor fate-specific marker ASCL1, and neural crest marker HNK1. (C) Relative fold expression in early neuronal transcriptional factor ASCL1 (a) and terminal astrocyte marker GFAP (b). The data for (B, C) are presented as mean ± SEM for $n = 3$. Significant difference between control and differentiated DPSCs is shown by $^*P < 0.05$. (D) Representative immunofluorescence images of differentiated DPSCs for GFAP, S100β, and EAAT2. Cell nuclei were stained with DAPI

Effect of Naïve DPSCs and Differentiated DPSCs on Survival of 6-OHDA-Treated SH-SY5Y Cells in Contact Co-culture System

At feeder density of 8×10^4 cells/well (optimum density of feeder cells was obtained from the previous study involving endogenous astrocytes), naïve and differentiated DPSCs were co-cultured with PKH26-stained SH-SY5Y cells (5×10^4 cells/well). After treating with 75 μM 6-OHDA for 48 h, PKH26-positive cells were analyzed using flow cytometry (Fig. 7). PKH26-stained SH-SY5Y cells showed mild decrease in the presence of DPSCs and differentiated DPSCs as feeder cells under control conditions ($P > 0.05$). 6-OHDA treatment, further, showed significant decrease in SH-SY5Y cells both in the absence ($P < 0.005$) and in the presence of feeders ($P < 0.05$; Fig. 7). Nonetheless, SH-SY5Y co-cultured on naïve DPSCs (55.66 ± 2.72%) and differentiated DPSCs showed significant higher survival rate (65 ± 2.88%) in comparison with SH-SY5Y cells treated in the absence of feeder (45.66 ± 2.96%).

Effect of Naïve and Differentiated DPSCs on Survival of Different Cell Populations in Primary Midbrain Culture Under 6-OHDA Stress

Non-contact Co-culture System The survival of β-tubulin III, GFAP, and TH immunopositive populations in primary midbrain cells under stress and co-cultured with DPSCs and differentiated DPSCs was assessed in non-contact culture system (Fig. 8). As shown in Fig. 8a, under 6-OHDA stress, total neuronal population labeled with β tubulin III showed a significant decrease in primary midbrain culture. In the presence of DPSCs and differentiated DPSCs in the trans-well under 6-OHDA stress, a significant increase in β-tubulin III immunopositive population ($P < 0.05$) was observed; however, it was still significantly lesser than the yield of β-tubulin III immunopositive cells under control conditions in the presence of DPSCs and differentiated DPSCs ($P < 0.05$). A considerable increase in GFAP immunopositive population was detected in primary midbrain culture under 6-OHDA stress ($P < 0.05$) that further decreased in the presence of DPSCs and differentiated DPSCs in

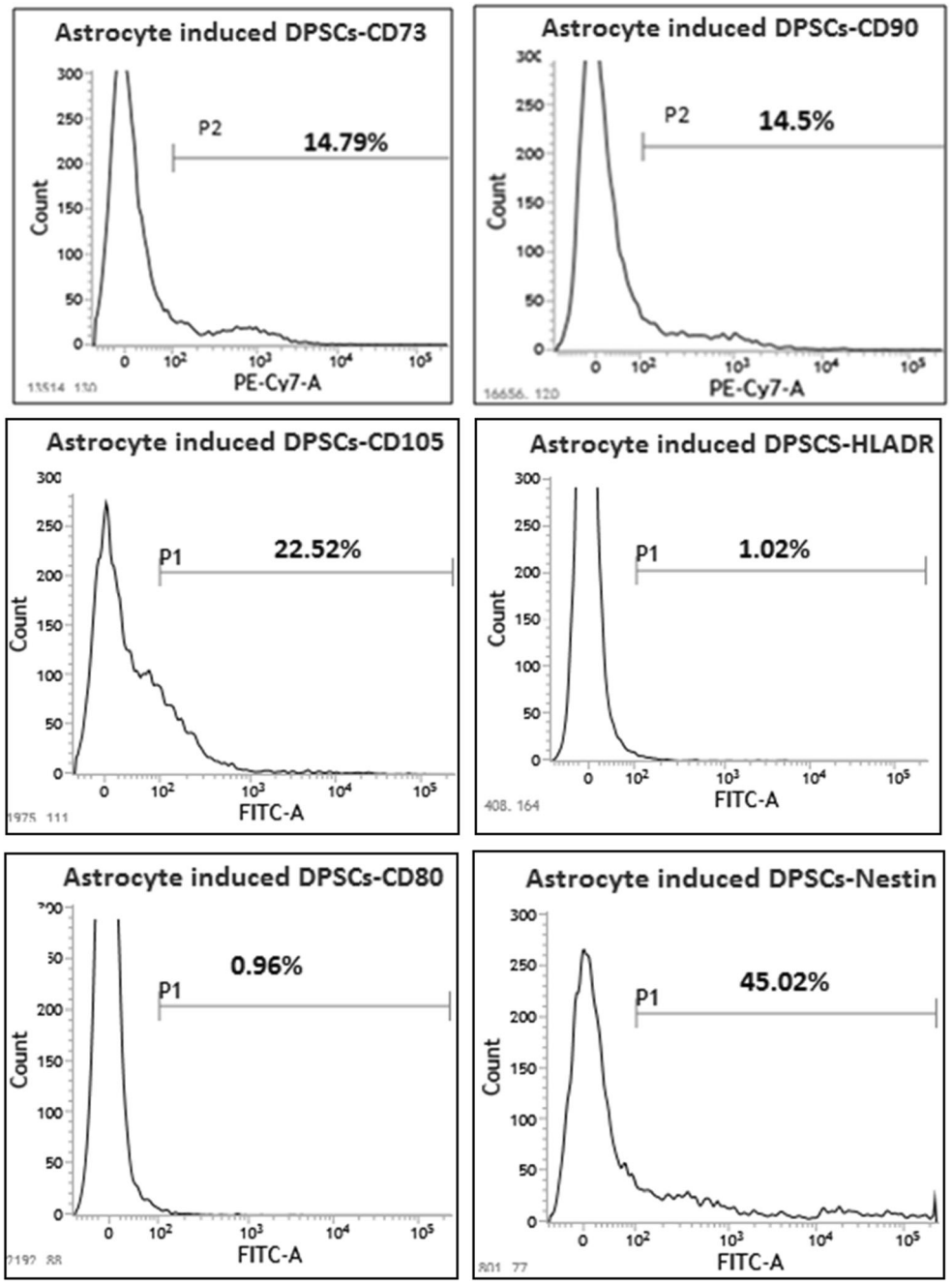

Fig. 4 Representative FACS histogram plots of MSC-specific surface markers CD73, CD90, and CD105; HLADR; early neural marker nestin; and co-stimulatory marker CD80 in astrocyte-differentiated DPSCs. The percentage is presented as mean ± SEM for $n = 3$

trans-well ($P < 0.05$). FACS analysis was also performed for TH immunopositive cells as represented in Fig. 8c. The presence of DPSCs and differentiated DPSCs in the trans-well inserts brought about a significant increase in TH immunopositive cells in comparison with 6-OHDA-treated primary midbrain cultures without DPSCs in inserts ($P < 0.05$). A comparable yield of TH immunopositive population was detected in the primary midbrain culture in the presence of DPSCs and differentiated DPSCs under control ($P = 0.273$). Further, between control and 6-OHDA stress, a significant difference in TH immunopositive cells was observed in the presence of naïve and differentiated DPSCs ($P < 0.05$). The TH immunopositive cells in primary midbrain culture under 6-OHDA stress was higher under the influence of differentiated DPSCs in comparison with naïve DPSCs ($P < 0.05$).

Effect of BDNF Inhibitor on Dopaminergic Neuron Survival Under 6-OHDA Stress in the Presence of Naïve and Differentiated DPSCs

To detect the protective role of BDNF secreted by naïve and differentiated DPSCs on the survival of dopaminergic neuronal

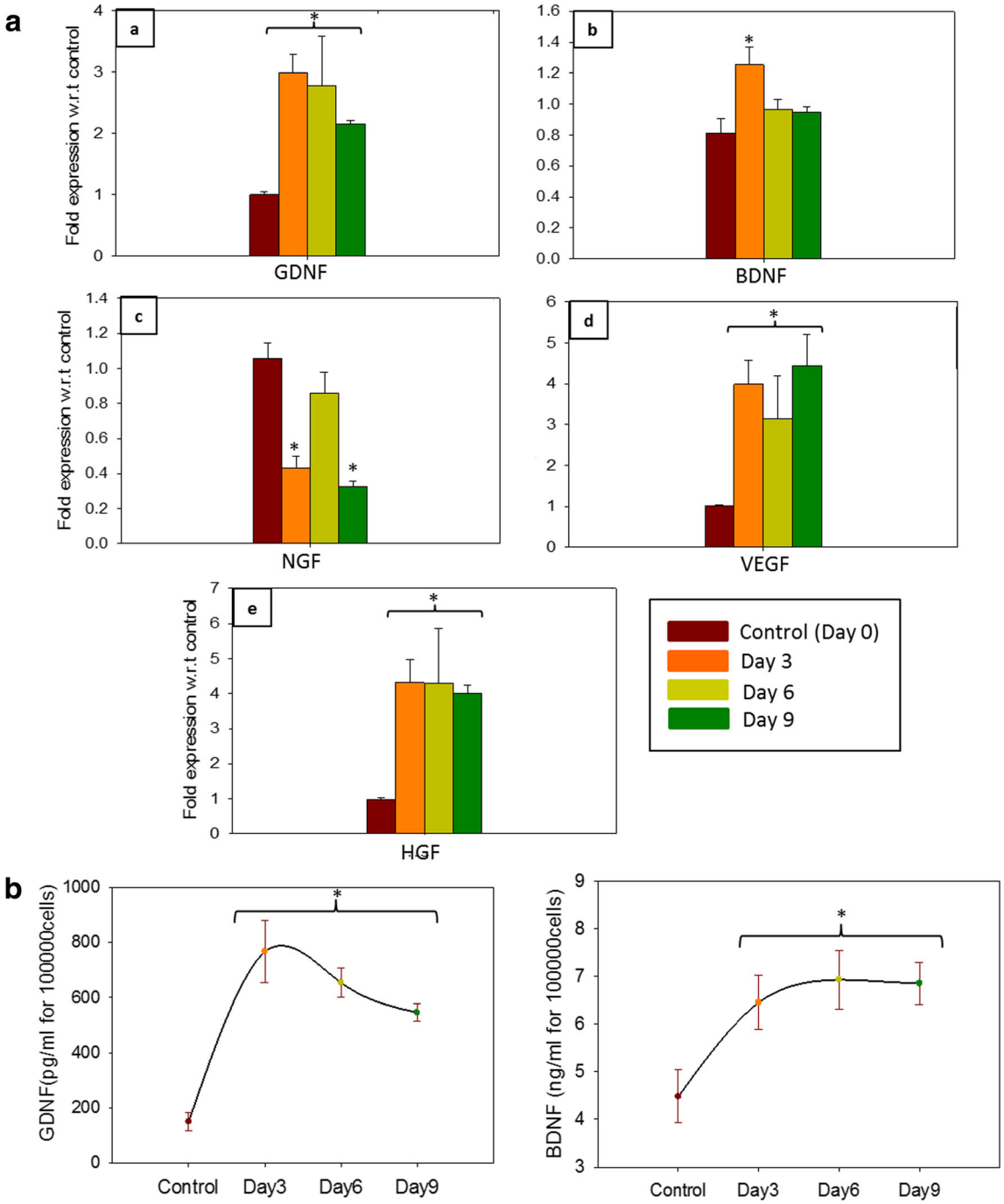

Fig. 5 Gene expression and NTF release in DPSCs and differentiated DPSCs. (A) Relative fold expression change in NTF markers GDNF (a), BDNF (b), NGF (c), VEGF (d), and HGF (e) in DPSCs at different day points of astrocyte induction; mean ± SEM for $n = 3$. Significant difference between control (day 0) and differentiated DPSCs of days 3, 6, and 9 is denoted by $^*P < 0.05$. (B) Graphical representation of BDNF and GDNF release in control and differentiated DPSCs. The data is presented as mean ± SEM for $n = 5$. The significant differences are denoted by $^*P < 0.05$

population in the primary midbrain culture under 6-OHDA stress; inhibitor for BDNF receptor was used. Primary midbrain cultures were incubated with BDNF receptor (TrkB) blocker ANA12 and then exposed to 6-OHDA stress with midbrain astrocytes in the trans-well inserts and the GFAP, β-tubulin III, and TH immunopositive populations were determined through FACS analysis. As represented in Fig. 9, a distinct decrease in β-tubulin III and TH immunopositive populations was noted in the presence of ANA12 in comparison with the primary midbrain culture under 6-OHDA stress in the presence of naïve and differentiated DPSCs ($P < 0.05$). The β-tubulin III and TH immunopositive populations in the presence of ANA12 was

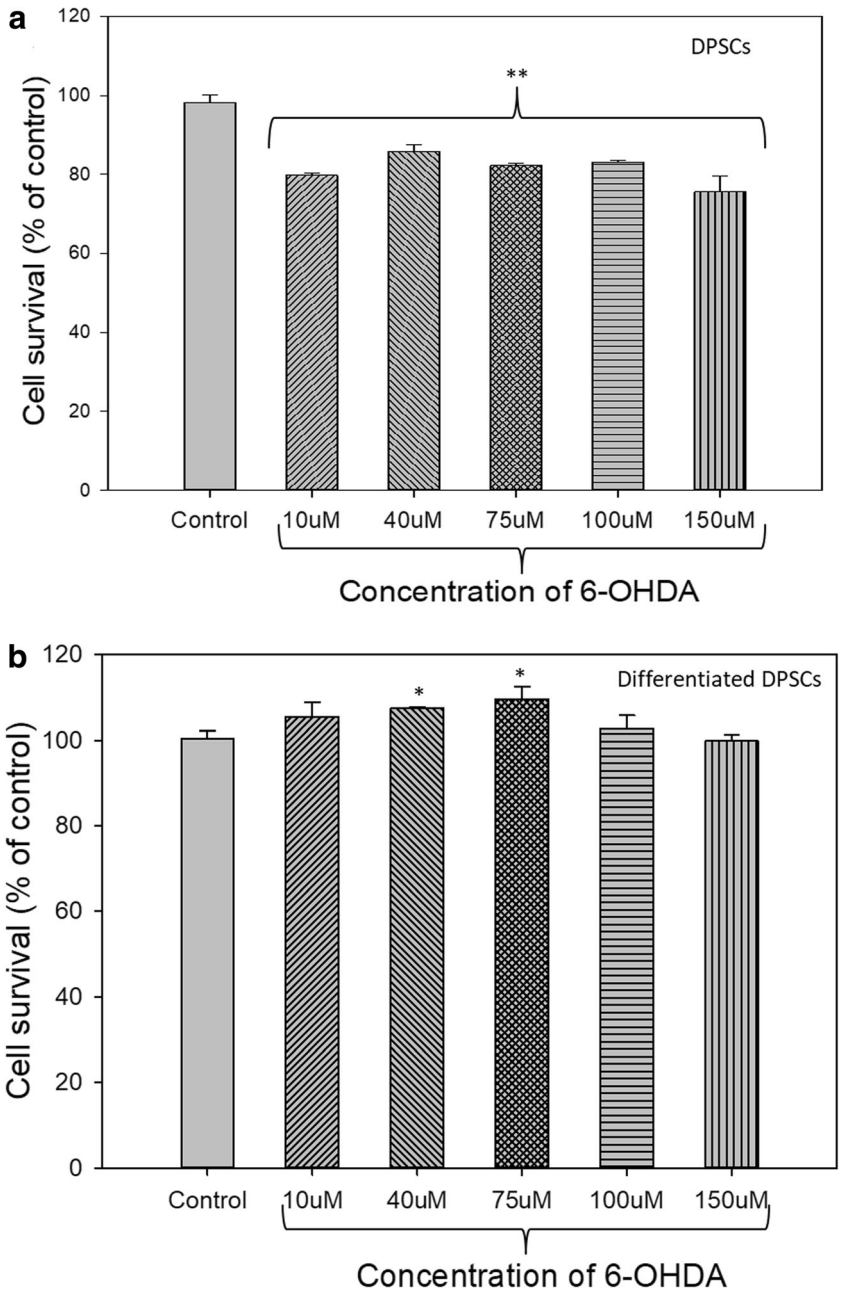

Fig. 6 Cell survival of DPSCs and differentiated DPSCs. Effect of 6-OHDA at different concentrations after 48 h was measured by MTT assay in DPSCs (**a**) and astrocyte-differentiated DPSCs (**b**). The percentage of cell survival rate is presented as mean ± SEM for $n = 5$. The significant differences are denoted by $^{*}P < 0.05$

comparable to the cells under 6-OHDA stress without the DPSCs ($P > 0.05$). A mild decrease in GFAP immunopositive population was observed in the presence of ANA12 in comparison with the cells under 6-OHDA stress without DPSCs ($P > 0.05$). Thus, BDNF receptor blocker distinctively impaired the protective effect of naïve and differentiated DPSCs on the β-tubulin III and TH populations of midbrain neuronal cells under 6-OHDA stress.

Comparison of BDNF-Mediated Neuroprotection Between DPSCs and Differentiated DPSCs

BDNF is a crucial NTF for the survival and function of dopaminergic neurons. Under control conditions, BDNF release from the co-culture of primary midbrain culture with differentiated DPSCs was higher than the BDNF release from the co-culture with naïve DPSCs ($P < 0.05$; Fig. 10a). Furthermore, upon 6-OHDA treatment, an increase in BDNF release was observed in the co-cultures of primary midbrain culture with naïve and differentiated DPSCs with respect to their controls ($P < 0.05$). BDNF release by the co-culture of differentiated DPSCs with primary midbrain culture was significantly higher than that by the co-culture of naïve DPSCs with primary midbrain culture ($P < 0.05$).

In our previous publication [29], a NO-dependent release of BDNF from the endogenous supportive cells was observed under 6-OHDA stress. Here too, upon inhibition of nitric

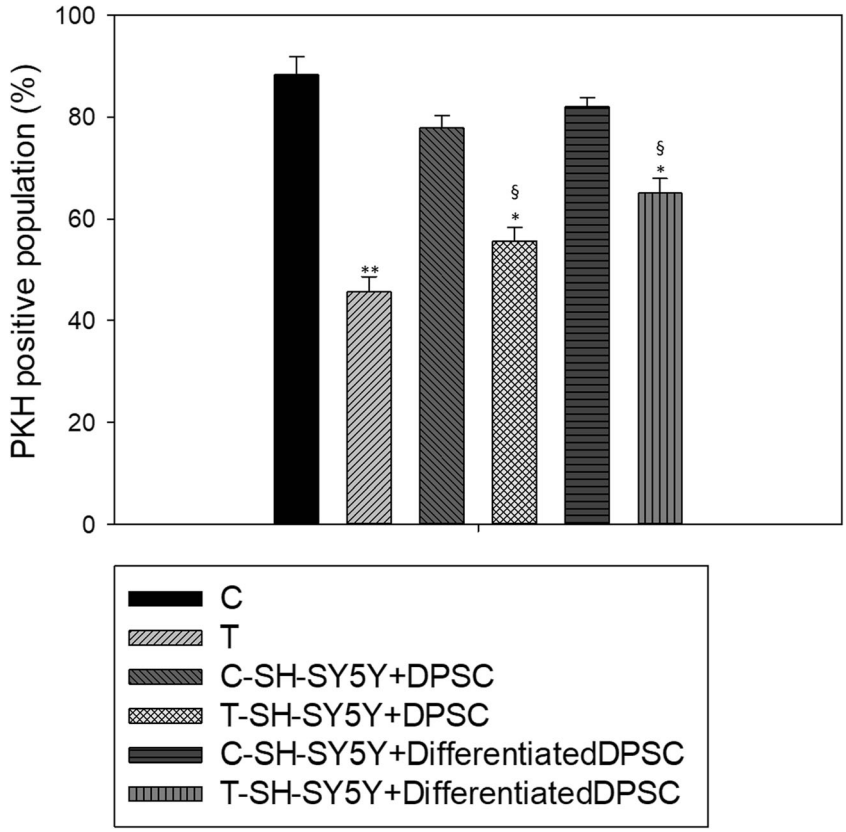

Fig. 7 Effect of 6-OHDA on the cell survival of SH-SY5Y cells in contact system. Graphical representation of FACS analysis of PKH26-positive SH-SY5Y cells co-cultured with DPSCs and astrocyte-differentiated DPSCs under 6-OHDA stress. The PKH26-positive population of SH-SY5Y cells under control (C) and 6-OHDA treated (T) without the presence of DPSCs was also detected. Data is presented as mean ± SEM for $n = 3$. The significant difference of 6-OHDA-treated groups w.r.t its respective controls is denoted by $^{*}P < 0.05$ and the distinct difference between 6-OHDA-treated SH-SY5Y cells without any feeder (T) and 6-OHDA-treated SH-SY5Y cells in the presence of DPSCs and differentiated DPSCs are denoted by $^{\S}P < 0.05$

oxide by treating the primary midbrain culture alone with L-NAME, a decrease in extracellular BDNF was detected in the co-culture of naïve and differentiated DPSCs with primary midbrain culture under 6-OHDA treatment ($P < 0.05$). Interestingly, a difference in response between naïve and differentiated DPSCs was observed during the presence of L-NAME for the total co-culture (i.e., naïve or differentiated DPSCs + primary midbrain culture). In this experimental set-up, the co-culture of primary midbrain culture along with naïve DPSCs did not show additional attenuation of BDNF release whereas the co-culture of differentiated DPSCs with primary midbrain culture showed a further reduction in BDNF secretion ($P < 0.05$; Fig. 10b, c). This suggests that NO release from the cells of primary midbrain culture is crucial for inducing BDNF release from naïve DPSCs under 6-OHDA stress. On contrary, the BDNF secretion from differentiated DPSCs is regulated by NO release from both the cells of primary midbrain culture and the differentiated DPSCs.

Nitrite Release upon 6-OHDA Stress on Primary Midbrain Cultures Co-cultured with Naïve and Differentiated DPSCs

The NO release by naïve and differentiated DPSCs co-cultured with primary midbrain culture was also measured under 6-OHDA stress (Fig. 11). 6-OHDA-treated co-cultures of naïve and differentiated DPSCs with primary midbrain culture showed a distinctly higher release of nitrite in comparison with their respective controls ($P < 0.05$). For the DPSC co-cultured with primary midbrain culture, the presence of L-NAME blocking the nitric oxide release from only the primary midbrain culture was comparable to the nitrite release from the co-culture where the total cell population was blocked by L-NAME ($P > 0.05$). On the other hand, the nitrite release by the differentiated DPSCs co-cultured with primary midbrain culture, the presence of L-NAME blocking the nitric oxide release from only the primary midbrain culture was significantly higher than the nitrite release from the co-culture where the total cell population was blocked by L-NAME ($P < 0.05$). The nitric oxide inhibition of the total cell population in co-cultures was comparable between naïve and differentiated DPSCs ($P > 0.05$). This suggests that the nitrite release in the co-culture system for differentiated DPSCs and primary midbrain culture was contributed by both the cultures. Thus, the release of nitrite by the differentiated DPSCs mimicked that of the endogenous supportive cells, the astrocytes (as mentioned in our previous publication [29]).

Discussion

The regeneration of specialized neurons lost in neurodegenerative diseases such as PD using stem cells has been a primary

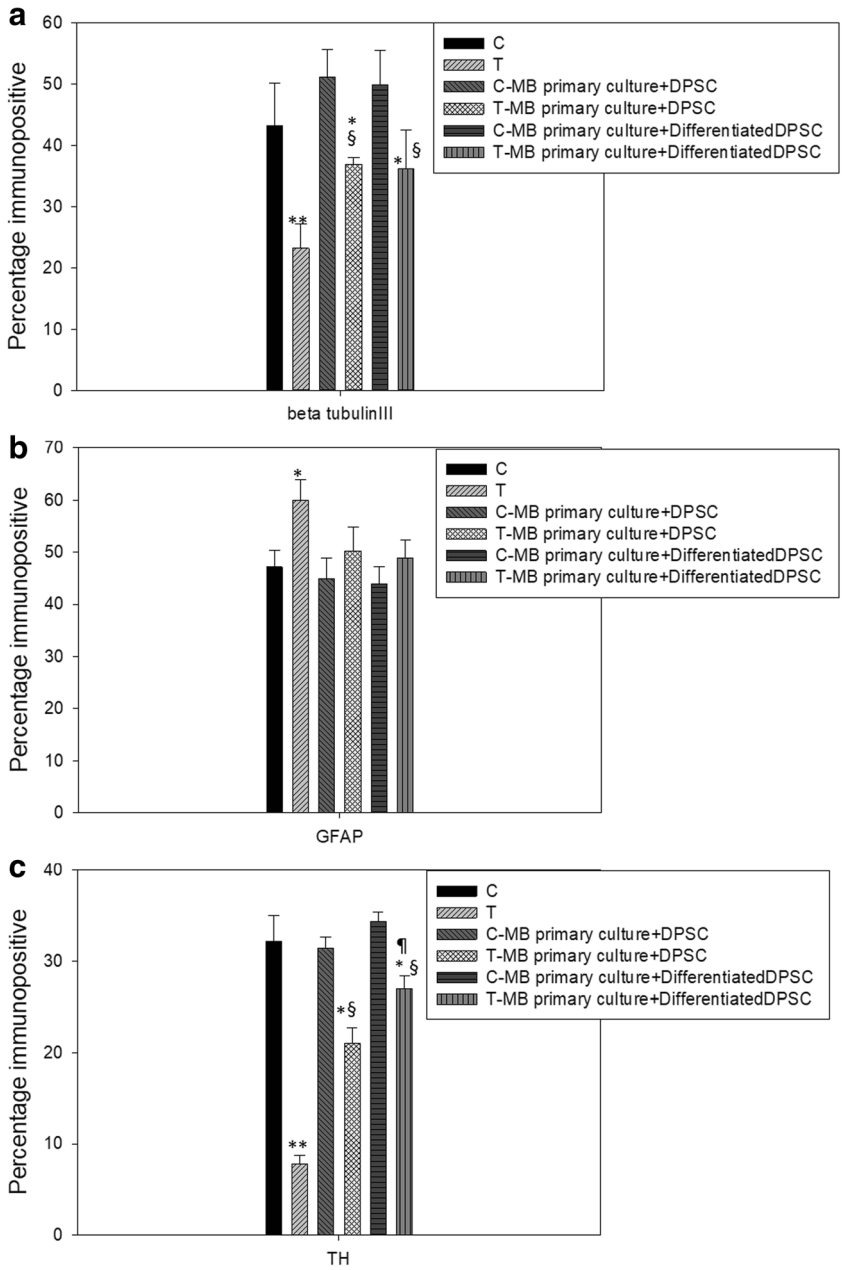

Fig. 8 Effect of 6-OHDA on the survival of primary midbrain cells in non-contact system. Graphical representation of FACS analysis of **a** β-tubulin III (total neuronal cells), **b** GFAP (astrocytes), and **c** TH (dopaminergic neurons) populations surviving in the primary midbrain cells co-cultured with DPSCs and astrocyte-differentiated DPSCs under 6-OHDA treatment. These immunopositive populations of these markers under control (C) and 6-OHDA treated (T) without the presence of DPSCs were also measured. The data is presented as mean ± SEM for $n = 3$. The significant difference of 6-OHDA-treated groups w.r.t its respective controls is denoted by $^{*}P < 0.05$ and the distinct difference between 6-OHDA-treated primary midbrain culture without any feeder (T) and 6-OHDA primary midbrain cells in the presence of DPSCs and differentiated DPSCs are denoted by $^{\S}P < 0.05$. Significant difference in the yield of these populations in primary midbrain cell under 6-OHDA stress between the presence of differentiated DPSCs and the presence of naïve DPSCs is represented as $^{¶}P < 0.05$

focus area for the past decade. An increasing body of work however suggests that a healthy endogenous niche is of crucial importance in addressing the vulnerability of specialized neurons in the CNS during neurodegenerative conditions. PD is characterized by the selective loss of dopaminergic (DA) neurons in the substantia nigra (SN) of the midbrain region and not in the ventral-tegmental area and other catecholaminergic cell group areas [33–37]. Postmortem studies of PD patient brains and in vivo PD models highlight that a lesser density of astrocytes may be a primary and crucial factor for the preferential loss of DA neurons in the substantia nigra in comparison with other catecholaminergic cell group areas of the brain [38]. Therefore, in the present study, we aim to assess the neuroprotective potential of naïve DPSCs and astrocyte-like differentiated DPSCs for dopaminergic neurons under 6-OHDA stress. We also studied the nitric oxide (NO) and BDNF crosstalk in mediating the survival, yield, and function of these DA neurons.

In line with earlier studies, we too observed that DPSCs shared a common mesenchymal surface marker profile and differentiation potential with MSCs derived from the bone marrow. While a few studies [26, 39–41] have reported the differentiation of DPSCs to mature neuronal and dopaminergic cell types, there are no reports yet on the differentiation of DPSCs to astrocytes. First, the cells were subjected to a pre-differentiation medium by exposure to EGF, bFGF, and N2

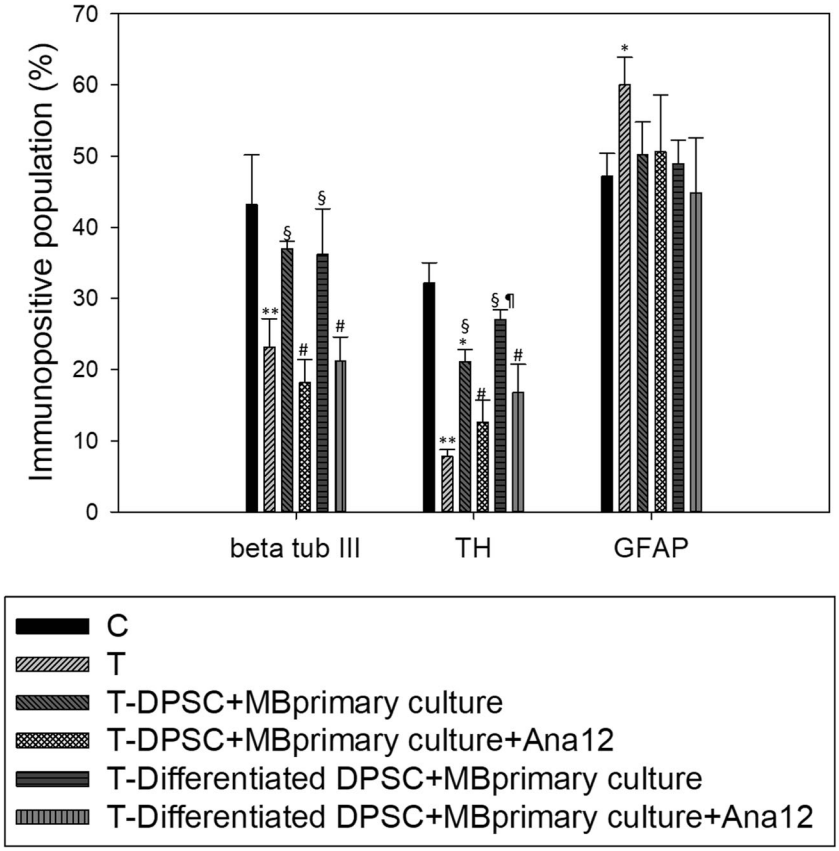

Fig. 9 Effect of BDNF in the survival of primary midbrain neurons of the non-contact co-culture system under 6-OHDA stress. Graphical representation of FACS analysis of β-tubulin III (total neuronal cells), GFAP (astrocytes), and TH, (dopaminergic neurons) populations in the primary midbrain cells co-cultured with DPSC and astrocyte-differentiated DPSCs under 6-OHDA stress with or without TrkB inhibitor ANA12. These immunopositive populations of these markers under control (C) and 6-OHDA treated (T) without the presence of DPSCs were also detected. The data is presented as mean ± SEM for $n = 3$. The significant difference of 6-OHDA-treated groups w.r.t its respective controls is denoted by $^{*}P < 0.05$ and the distinct difference between 6-OHDA-treated primary midbrain culture without any feeder (T) and 6-OHDA primary midbrain cells in the presence of DPSCs and differentiated DPSCs is denoted by $^{\S}P < 0.05$. Significant difference in the yield of these populations in primary midbrain culture under 6-OHDA stress between the presence of differentiated DPSCs and the presence of naïve DPSCs is represented as $^{¶}P < 0.05$

supplement. Stimulation of the epidermal growth factor receptor (EGFR) can initiate numerous effects on CNS cells in vitro including neuronal survival and differentiation, astrocyte proliferation, and the proliferation of multipotent progenitors [42–44]. Moreover, a pioneer study by Kornblum et al. [45] using EGFR-null mice showed that the lack of EGF receptor induced abnormal astrocyte development and neuronal death. Eventually, several other studies established the significance of EGF receptor stimulation for astrocyte commitment from neural progenitors [46–50].

This pre-differentiation step was followed by addition of differentiation factors neuregulin, PDGF, and db-cAMP. Neuregulins (NRGs) comprise a large family of EGF-like signaling molecules involved in cell-cell communication during development and disease [51–54]. In astrocyte development, NRGs have been found to facilitate the action of TGFα, for glial maturation and glia-neuron interaction [55–61]. PDGF and db-cAMP are supportive factors required for the maintenance of mature astrocytes and neuron-glia communication [62–64]. Similar factors were used earlier for generation of astrocytes from pluripotent stem cells and bone marrow mesenchymal stem cells [28, 65–68].

Induction of DPSCs to astrocytes with differentiation factors showed changes in cell morphology at different day points of induction. A simple stellated morphology was seen on day 3 of induction, which enhanced and exhibited complex astrocyte morphology at day 6 and day 9. FACS analysis showed a definitive increase in astrocyte-specific markers GFAP and EAAT2 along with glial calcium-binding protein S100β from day 3 onwards. Though GFAP can be expressed by ependymal cells of the brain, the expressions of S100β and EAAT2 are specific for mature astroglial cells [69–71]. S100β is a protein that gets localized in the cytoplasm and nucleus and is expressed primarily by a subtype of mature astrocytes that sheathe blood vessels [72]. EAAT2 is a glutamate transporter primarily expressed by some mature astrocytes [73]. In addition, the MSC-specific markers CD73, CD90, and CD105 and the neural crest marker HNK1, as

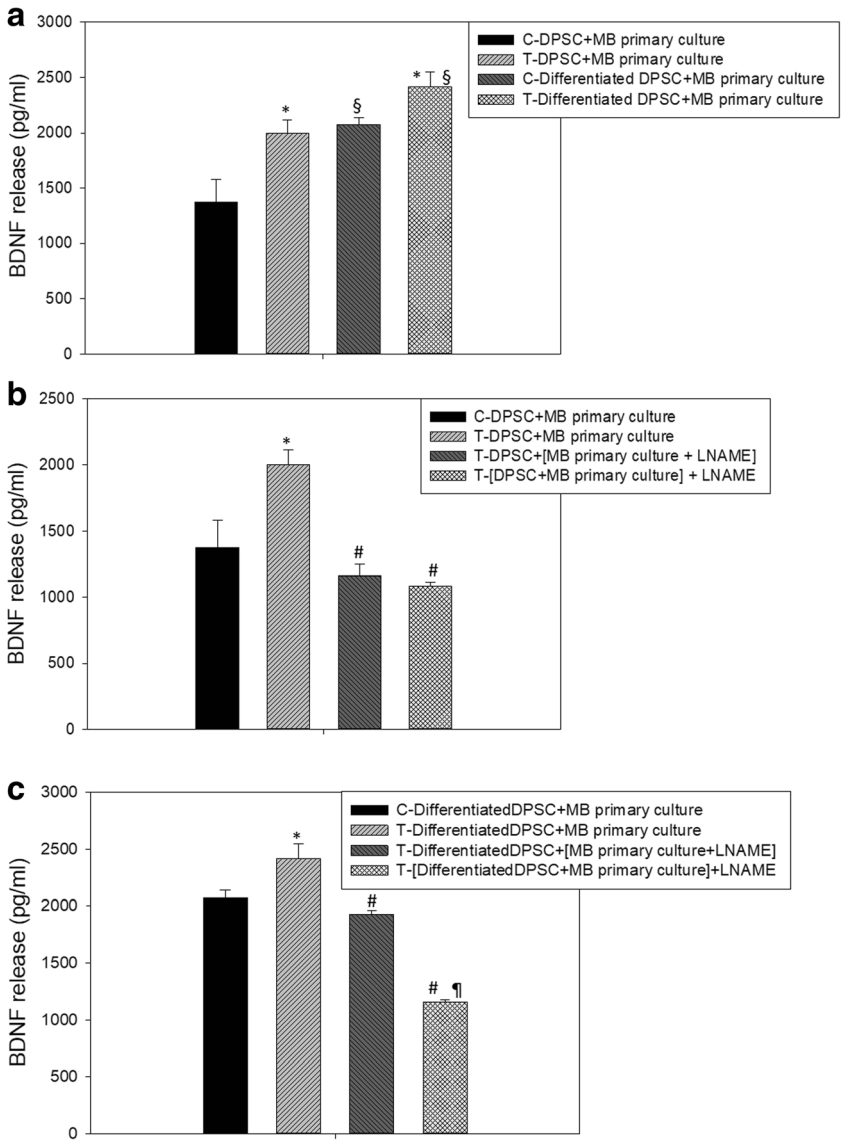

Fig. 10 BDNF release in the non-contact co-culture system of both DPSCs and astrocyte-differentiated DPSCs with or without inhibiting NO. **a** Represents BDNF concentration from co-cultures of primary midbrain culture with DPSCs and astrocyte-differentiated DPSCs under 6-OHDA stress. Control and 6-OHDA treatment are shown as (C) and (T). Data is presented as mean ± SEM for $n = 5$. Significant differences between control and 6-OHDA treated for each group is represented as $^{*}P < 0.05$ and distinct differences between DPSCs as feeder to differentiated as feeder are denoted by $^{\S}P < 0.05$. **b** Represents BDNF concentration from co-cultures of primary midbrain culture with DPSCs under 6-OHDA stress with and without NO inhibitor L-NAME. **c** Represents BDNF concentration from co-cultures of primary midbrain culture with astrocyte-differentiated DPSCs under 6-OHDA stress with and without NO inhibitor L-NAME. Data in **b**, **c** is presented as mean ± SEM for $n = 5$. Significant difference between control and 6-OHDA treated is shown by $^{*}P < 0.05$ and the difference between 6-OHDA treated and L-NAME added to the cultures in different combinations is denoted by $^{\#}P < 0.05$

well as the early neural commitment marker ASCL1, were significantly downregulated in astrocyte-differentiated DPSCs. The astrocyte-differentiated population comprised > 65% cells expressing astrocyte-associated markers and almost 45% of cells expressing nestin-positive neural progenitor pool. Probably, the DPSCs which do not express astrocyte-specific markers are in the early neural progenitor stage. MSCs are also uniquely characterized by their immunomodulatory property [74, 75] and it is thus essential to assess the expression of HLADR and co-stimulatory markers in astrocyte-induced DPSCs in vitro. The expression of co-stimulatory molecule CD80, necessary for T cell activation [76, 77], was found absent in astrocyte-induced cells ensuring no activation of T cell or immune response. Taken together, this evidence suggests that DPSCs can be successfully differentiated to astrocytes in vitro without losing their immunomodulatory property.

It is known that DPSCs possess the innate ability to secrete neurotrophic factors that supports the survival, axon growth, function, and attachment of neurons [78–80]. Similarly, endogenous astrocytes are also known to provide soluble trophic factors to promote and maintain neural function in the CNS niche [81–84]. In the present study, NTF expression and their secretion in astrocyte-induced DPSCs showed significant increase for BDNF, GDNF, VEGF, and HGF, but not for NGF, over naïve DPSCs. These findings are in line with those of earlier studies showing NTF expression and also non-NGF-type NTF expression in astrocytes [85].

Significantly higher levels of BDNF and GDNF were released from astrocyte-differentiated DPSCs in comparison with naïve DPSCs. In addition, the increase in proliferation of astrocyte-differentiated DPSCs when treated with varying concentrations of 6-OHDA, as assessed by MTT assays, is in line with several earlier studies reporting astrogliosis during

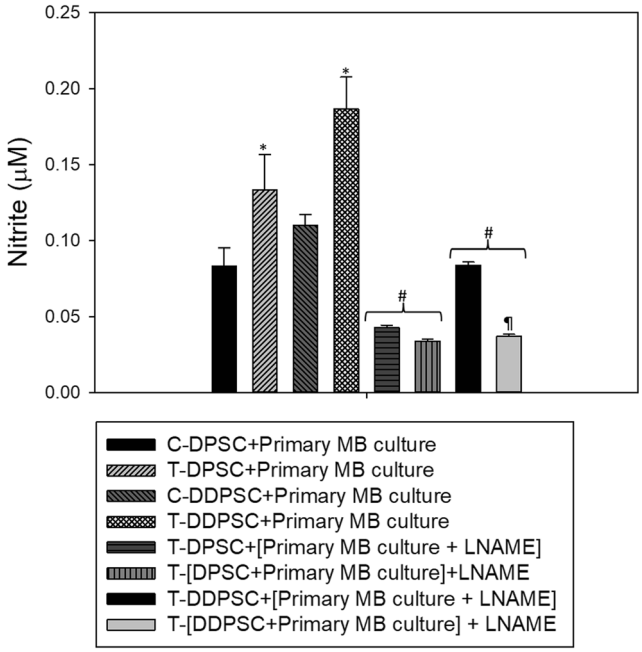

Fig. 11 NO release in the non-contact co-culture system of DPSCs and astrocyte-differentiated DPSCs with and without L-NAME. Graphical representation of nitrite concentration measured using Griess reagent from co-cultures of primary midbrain culture with DPSCs and astrocyte-differentiated DPSCs under 6-OHDA stress. L-NAME was either incubated with primary midbrain culture or incubated with the total co-culture (as indicated in the figure). Data is presented as mean ± SEM for $n = 5$. Significant difference between control and 6-OHDA treated is shown by $^{*}P < 0.05$ and the difference between 6-OHDA treated and L-NAME added to the cultures in different combinations is denoted by $^{\#}P < 0.05$. $^{¶}P < 0.05$ was used for significant difference between L-NAME incubated with primary midbrain culture alone and L-NAME added to total co-culture

injury [86, 87]. This also aligns with our observation of increase in GFAP-positive cells in the midbrain primary culture under 6-OHDA treatment in our earlier publication [29]. This increase in astrocytes was observed only in neuron-glia mixed cultures and not in purified astrocyte cultures. In the present study, too, a similar observation was noted in the astrocyte-differentiated DPSCs, as they are a mixed population of astrocytes and neural progenitors. This evidence of the ability of astrocyte-differentiated DPSCs to proliferate and secrete NTFs, in comparison with naïve DPSCs, may be expected to help neuronal survival.

The effect of astrocyte-induced DPSCs on survival of neurons under 6-OHDA stress in comparison with naive DPSCs was evaluated by both contact and non-contact methods. Homogenous dopaminergic-like SH-SY5Y cells were used in the contact method to assess the effect of naïve DPSCs and astrocyte-differentiated DPSCs on an individual neuronal population. FACS analysis of PKH26-stained SH-SY5Y cells showed higher SH-SY5Y cell survival when co-cultured with astrocyte-differentiated DPSCs in comparison with naive DPSCs. Further, the evaluation of the neuroprotective role in primary midbrain neurons using non-contact method demonstrated the increased yield of TH immunopositive cells and mature neuronal cells immunopositive for β-tubulin III in primary midbrain culture co-cultured with astrocyte-differentiated DPSCs in comparison with naive DPSCs, accompanied by a reduction in endogenous astrogliosis of the primary midbrain culture. Overall, the data highlight the significant ability of astrocyte-differentiated DPSCs to protect neurons with or without contact.

The primary role of astrocytes in the CNS is secretion of neurotrophic factors that are crucial for the survival of neuronal cells. We had also stated in our earlier publication [29] that the density of region-specific astrocytes is crucial for the survival of mature and dopaminergic neurons and that BDNF played a major role in combating cell death under 6-OHDA toxicity. The secretion of NTFs such as BDNF and GDNF was found in higher level in astrocyte-differentiated DPSCs than in naïve DPSCs. BDNF is reported to be an essential NTF for the survival of dopaminergic neurons [88–92]. A significant release of BDNF by astrocyte-differentiated DPSCs in both control and treated co-cultures in comparison with naïve DPSCs was noted under 6-OHDA stress. Also, the recovery of β-tubulin III and TH immunopositive yields observed in the presence of naïve DPSCs and differentiated DPSCs was significantly reduced in the presence of BDNF inhibitor Ana12, suggesting a key neuroprotective role rendered by the BDNF secretion of the DPSCs (both naïve and differentiated).

According to earlier studies, nitric oxide (NO) is an important mediator of glia-glia and neuron-glia crosstalk during injury/stress and can initiate the release of glio-transmitters and cytokines [93–97]. In our earlier study [29], we reported a similar event where NO-induced BDNF release played a crucial role in bringing about survival of TH-positive neurons. Further, the endogenous release of NTFs from midbrain astrocytes was regulated by both autocrine and paracrine communication. When nitric oxide release was inhibited by L-NAME in primary midbrain culture, the BDNF release in co-culture under 6-OHDA stress was reduced more in naïve DPSCs than in astrocyte-differentiated DPSCs, suggesting that the BDNF release in naïve DPSCs is primarily regulated by paracrine signaling, whereas for differentiated DPSCs it is equally through autocrine and paracrine signaling with NO as the mediator. The corresponding nitrite levels measured in the presence of L-NAME showed that the nitrite release for the co-culture with the primary midbrain culture with naïve DPSCs is primarily from the midbrain cells, while in the co-culture with astrocyte-differentiated DPSCs the nitrite release is equally from both midbrain cells and differentiated DPSCs. When NO was inhibited in total culture of co-culture system, BDNF release was reduced in both naïve DPSCs and astrocyte-differentiated DPSCs. This suggests that the differentiated DPSCs have a similar autocrine signal loop like the endogenous astrocytes for releasing BDNF, while both naïve and differentiated DPSCs respond in a paracrine process

mediated by NO to release BDNF during 6-OHDA stress on primary midbrain culture. Also, irrespective of 6-OHDA stress and NO level, BDNF release is higher in astrocyte-differentiated DPSC in comparison with naïve DPSCs, suggesting the presence of both paracrine and autocrine loops in the former case.

Conclusion

In sum, our data demonstrate that DPSCs exposed to glial commitment cues show a substantial differentiation towards astrocyte-like cells and that these cells attain functional maturity with respect to their interaction with midbrain neuronal cells to bring about neuroprotection. The data further suggest that astrocyte-differentiated DPSCs may be appropriate candidates for further evaluation as stem cell therapy-based treatments in animal models of neurological diseases and injury involving modification of midbrain microenvironment. In conclusion, based on the evidence of the study, DPSCs may be evaluated as a preferred option for mimicking the role and function of endogenous astrocytes in neurodegenerative disease.

Contract grant sponsor "Innovative Young Biotechnologist Award" received by ID from the Department of Biotechnology (DBT), Government of India, New Delhi (contract grant no. BT/07/IYBA/2013-7)

References

1. Barreto GE, Gonzalez J, Torres Y, Morales L (2011) Astrocytic-neuronal crosstalk: implications for neuroprotection from brain injury. Neurosci Res 71(2):107–113
2. Hamby ME, Sofroniew MV (2010) Reactive astrocytes as therapeutic targets for CNS disorders. Neurotherapeutics 7(4):494–506
3. Volterra A, Meldolesi J (2005) Astrocytes, from brain glue to communication elements: the revolution continues. Nat Rev Neurosci 6(8):626–640
4. Buffo A, Rite I, Tripathi P, Lepier A, Colak D, Horn AP, Mori T, Götz M (2008) Origin and progeny of reactive gliosis: a source of multipotent cells in the injured brain. Proc Natl Acad Sci 105(9):3581–3586
5. Ridet JL, Malhotra SK, Privat A, Gage FH (1997) Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci 20(12):570–577
6. Rappold PM, Tieu K (2010) Astrocytes and therapeutics for Parkinson's disease. Neurotherapeutics 7(4):413–423
7. Nagai M, Re DB, Nagata T, Chalazonitis A, Jessell TM, Wichterle H, Przedborski S (2007) Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. Nat Neurosci 10(5):615–622
8. Pehar M, Beeson G, Beeson CC, Johnson JA, Vargas MR (2014) Mitochondria-targeted catalase reverts the neurotoxicity of hSOD1G93AAstrocytes without extending the survival of ALS-linked mutant hSOD1 mice. PLoS One 9(7):e103438
9. Booth HDE, Hirst WD, Wade-Martins R (2017) The role of astrocyte dysfunction in Parkinson's disease pathogenesis. Trends Neurosci 40(6):358–370
10. Choi I, Choi DJ, Yang H, Woo JH, Chang MY, Kim JY, Sun W, Park SM et al (2016) PINK1 expression increases during brain development and stem cell differentiation, and affects the development of GFAP-positive astrocytes. Mol Brain 9:5
11. Henry AG, Aghamohammadzadeh S, Samaroo H, Chen Y, Mou K, Needle E, Hirst WD (2015) Pathogenic LRRK2 mutations, through increased kinase activity, produce enlarged lysosomes with reduced degradative capacity and increase ATP13A2 expression. Hum Mol Genet 24(21):6013–6028
12. Kim KS, Kim JS, Park JY, Suh YH, Jou I, Joe EH, Park SM (2013) DJ-1 associates with lipid rafts by palmitoylation and regulates lipid rafts-dependent endocytosis in astrocytes. Hum Mol Genet 22(23):4805–4817
13. Ledesma MD, Galvan C, Hellias B, Dotti C, Jensen PH (2002) Astrocytic but not neuronal increased expression and redistribution of parkin during unfolded protein stress. J Neurochem 83(6):1431–1440
14. Lev N, Barhum Y, Ben-Zur T, Melamed E, Steiner I, Offen D (2013) Knocking out DJ-1 attenuates astrocytes neuroprotection against 6-hydroxydopamine toxicity. J Mol Neurosci 50(3):542–550
15. Miklossy J, Arai T, Guo JP, Klegeris A, Yu S, McGeer EG, McGeer PL (2006) LRRK2 expression in normal and pathologic human brain and in human cell lines. J Neuropathol Exp Neurol 65(10):953–963
16. Yun HM, Choi DY, Oh KW, Hong JT (2015) PRDX6 exacerbates dopaminergic neurodegeneration in a MPTP mouse model of Parkinson's disease. Mol Neurobiol 52(1):422–431
17. Janebodin K, Horst OV, Ieronimakis N, Balasundaram G, Reesukumal K, Pratumvinit B, Reyes M (2011) Isolation and characterization of neural crest-derived stem cells from dental pulp of neonatal mice. PLoS One 6(11):e27526
18. Janebodin K, Reyes M (2012) Neural crest-derived dental pulp stem cells function as ectomesenchyme to support salivary gland tissue formation. Dentistry S13:001. https://doi.org/10.4172/2161-1122S13-001
19. Chai Y, Jiang X, Ito Y, Bringas P Jr, Han J, Rowitch DH, Soriano P, McMahon AP et al (2000) Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development 127:1671–1679
20. Kaukua N, Shahidi MK, Konstantinidou C, Dyachuk V, Kaucka M, Furlan A, An Z, Wang L et al (2014) Glial origin of mesenchymal stem cells in a tooth model system. Nature 513(7519):551–554
21. Thesleff I, Tummers M. (2009) Tooth organogenesis and regeneration. In: StemBook from: https://www.ncbi.nlm.nih.gov/books/NBK27071/. https://doi.org/10.3824/stembook.1.37.1
22. Majumdar D, Kanafi M, Bhonde R, Gupta P, Datta I (2016) Differential neuronal plasticity of dental pulp stem cells from exfoliated deciduous and permanent teeth towards dopaminergic neurons. J Cell Physiol 231:2048–2063
23. Jung J, Kim JW, Moon HJ, Hong JY, Hyun JK (2016) Characterization of neurogenic potential of dental pulp stem cells cultured in xeno/serum-free condition: in vitro and in vivo assessment. Stem Cells Int 2016:6921097
24. Leong WK, Henshall TL, Arthur A, Kremer KL, Lewis MD, Helps SC, Field J, Hamilton-Bruce MA et al (2012) Human adult dental pulp stem cells enhance poststroke functional recovery through non-neural replacement mechanisms. Stem Cells Transl Med 1(3):177–187
25. Young FI, Telezhkin V, Youde SJ, Langley MS, Stack M, Kemp PJ, Song B (2016) Clonal heterogeneity in the neuronal and glial differentiation of dental pulp stem/progenitor cells. Stem Cells Int 1290561

26. Kanafi M, Majumdar D, Bhonde R, Gupta P, Datta I (2014) Midbrain cues dictate differentiation of human dental pulp stem cells towards functional dopaminergic neurons. J Cell Physiol 229(10):1369–1377
27. Datta I, Mishra S, Mohanty L, Pulikkot S, Joshi PG (2011) Neuronal plasticity of human Wharton's jelly mesenchymal stromal cells to the dopaminergic cell type compared with human bone marrow mesenchymal stromal cells. Cytotherapy 13(8):918–932
28. Bahat-Stroomza M, Barhum Y, Levy YS, Karpov O, Bulvik S, Melamed E, Offen D (2009) Induction of adult human bone marrow mesenchymal stromal cells into functional astrocyte-like cells: potential for restorative treatment in Parkinson's disease. J Mol Neurosci 39(1–2):199–210
29. Datta I, Ganapathy K, Razdan R, Bhonde R (2018) Location and number of astrocytes determine dopaminergic neuron survival and function under 6-OHDA stress mediated through differential BDNF release. Mol Neurobiol 55(7):5505–5525
30. Ganapathy K, Datta I, Sowmithra S, Joshi P, Bhonde R (2016a) Influence of 6-hydroxydopamine toxicity on α-synuclein phosphorylation, resting vesicle expression, and vesicular dopamine release. J Cell Biochem 9999:1–18
31. Ganapathy K, Sowmithra S, Bhonde R, Datta I (2016b) By changing dimensionality, sequential culturing of midbrain cells, rather than two-dimensional culture, generates a neuron-glia ratio closer to in vivo adult midbrain. Cells Tissues Organs 201:445–463
32. Prasanna SJ, Saha B, Nandi D (2007) Involvement of oxidative and nitrosative stress in modulation of gene expression and functional responses by IFNγ. Int Immunol 19(7):867–879
33. Fearnley JM, Lees AJ (1991) Ageing and Parkinson's disease: substantia nigra regional selectivity. Brain 114(Pt. 5):2283–2301
34. German DC, Manaye KF, Sonsalla PK, Brooks BA (1992) Midbrain dopaminergic cell loss in Parkinson's disease and MPTP-induced parkinsonism: sparing of calbindin-D28k-containing cells. Ann N Y Acad Sci 648:42–62
35. Hirsch EC, Graybiel AM, Agid Y (1988) Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. Nature 334:345–348
36. Hirsch EC, Graybiel AM, Agid Y (1989) Selective vulnerability of pigmented dopaminergic neurons in Parkinson's disease. Acta Neurol Scand Suppl 126:19–22
37. Kordower JH, Olanow CW, Dodiya HB, Chu Y, Beach TG, Adler CH, Halliday GM, Bartus RT (2013) Disease duration and the integrity of the nigrostriatal system in Parkinson's disease. Brain 136: 2419–2431
38. Damier P, Hirsch EC, Javoy-Agid F, Zhang P, Agid Y (1993) Protective role of glutathione peroxidase against neuronal death in Parkinson's disease. Neuroscience 52:1–6
39. Arthur A, Rychkov G, Shi S, Koblar SA, Gronthos S (2008) Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem Cells 26: 1787–1795
40. Karaöz E, Demircan PC, Saglam O, Aksoy A, Kaymaz F, Duruksu G (2011) Human dental pulp stem cells demonstrate better neural and epithelial stem cell properties than bone marrow-derived mesenchymal stem cells. Histochem Cell Biol 136:455–473
41. Kiraly M, Porcsalmy B, Pataki A, Kadar K, Jelitai M, Molnar B, Hermann P, Gera I et al (2009) Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. Neurochem Int 55:323–332
42. Farkas LM, Krieglstein K (2002) Heparin-binding epidermal growth factor-like growth factor (HB-EGF) regulates survival of midbrain dopaminergic neurons. J Neural Transm (Vienna) 109(3):267–277
43. Galvez-Contreras AY, Quiñones-Hinojosa A, Gonzalez-Perez O (2013) The role of EGFR and ErbB family related proteins in the oligodendrocyte specification in germinal niches of the adult mammalian brain. Front Cell Neurosci 7:258
44. Kornblum HI, Zurcher SD, Werb Z, Derynck R, Seroogy KB (1999) Multiple trophic actions of heparin-binding epidermal growth factor (HB-EGF) in the central nervous system. Eur J Neurosci 11:3236–3246
45. Kornblum HI, Hussain R, Wiesen J, Miettinen P, Zurcher SD, Chow K, Derynck R, Werb Z (1998) Abnormal astrocyte development and neuronal death in mice lacking the epidermal growth factor receptor. J Neurosci Res 53(6):697–717
46. Angenieux B, Schorderet DF, Arsenijevic Y (2006) Epidermal growth factor is a neuronal differentiation factor for retinal stem cells in vitro. Stem Cells 24(3):696–706
47. Burrows RC, Wancio D, Levitt P, Lillien L (1997) Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. Neuron 19(2):251–267
48. Kilpatrick TJ, Bartlett PF (1995) Cloned multipotential precursors from the mouse cerebrum require FGF-2, whereas glial restricted precursors are stimulated with either FGF-2 or EGF. J Neurosci 15(5):3653–3661
49. Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH (1997) Epidermal growth factor and fibroblast growth Factor-2 have different effects on neural progenitors in the adult rat brain. J Neurosci 17(15):5820–5829
50. Shen YM, Meltzer H, Saljooque F, Sang U (2001) Stimulation of the epidermal growth factor receptor induces glial-specific protein expression in the human DAOY neuroectodermal cell line. Dev Neurosci 23(1):84–90
51. Agarwal A, Zhang M, Trembak-Duff I, Unterbarnscheidt T, Radyushkin K, Dibaj P, de Souza DM, Boretius S et al (2014) Dysregulated expression of neuregulin-1 by cortical pyramidal neurons disrupts synaptic plasticity. Cell Rep 8(4):1130–1145
52. Britsch S (2007) The neuregulin-I/ErbB signaling system in development and disease. Springer Science & Business Media 190:1–65
53. Mei L, Nave KA (2014) Neuregulin-ERBB signaling in the nervous system and neuropsychiatric diseases. Neuron 83(1):27–49
54. Ting AK, Chen Y, Wen L, Yin DM, Shen C, Tao Y, Liu X, Xiong WC et al (2011) Neuregulin 1 promotes excitatory synapse development and function in GABAergic interneurons. J Neurosci 31(1):15–25
55. Ma YJ, Berg-von der Emde K, Rage F, Wetsel WC, Ojeda SR (1997) Hypothalamic astrocytes respond to transforming growth factor-alpha with the secretion of neuroactive substances that stimulate the release of luteinizing hormone-releasing hormone. Endocrinology 138(1):19–25
56. Moore CS, Abdullah SL, Brown A, Arulpragasam A, Crocker SJ (2011) How factors secreted from astrocytes impact myelin repair. J Neurosci Res 89(1):13–21
57. Ojeda SR, Ma YJ (1999) Glial-neuronal interactions in the neuroendocrine control of mammalian puberty: facilitatory effects of gonadal steroids. J Neurobiol 40:528–540
58. Parker MW, Chen Y, Hallenbeck JM, Ford BD (2002) Neuregulin expression after focal stroke in the rat. Neurosci Lett 334(3):169–172
59. Schmid RS, McGrath B, Berechid BE, Boyles B, Marchionni M, Šestan N, Anton ES (2003) Neuregulin 1–erbB2 signaling is required for the establishment of radial glia and their transformation into astrocytes in cerebral cortex. Proc Natl Acad Sci 100(7):4251–4256
60. Thompson RJ, Roberts B, Alexander CL, Williams SK, Barnett SC (2000) Comparison of neuregulin-1 expression in olfactory ensheathing cells, Schwann cells and astrocytes. J Neurosci Res 61(2):172–185
61. Tokita Y, Keino H, Matsui F, Aono S, Ishiguro H, Higashiyama S, Oohira A (2001) Regulation of neuregulin expression in the injured rat brain and cultured astrocytes. J Neurosci 21(4):1257–1264

62. Paco S, Hummel M, Plá V, Sumoy L, Aguado F (2016) Cyclic AMP signaling restricts activation and promotes maturation and antioxidant defenses in astrocytes. BMC Genomics 17:304
63. Schlag BD, Vondrasek JR, Munir M, Kalandadze A, Zelenaia OA, Rothstein JD, Robinson MB (1998) Regulation of the glial Na+-dependent glutamate transporters by cyclic AMP analogs and neurons. Mol Pharmacol 53:355–369
64. Yoshida H, Imaizumi T, Tanji K, Sakaki H, Metoki N, Hatakeyama M, Yamashita K, Ishikawa A et al (2005) Platelet-activating factor enhances the expression of nerve growth factor in normal human astrocytes under hypoxia. Mol Brain Res 133(1):95–101
65. Lee G, Kim H, Elkabetz Y, Al Shamy G, Panagiotakos G, Barberi T, Tabar V, Studer L (2007) Isolation and directed differentiation of neural crest stem cells derived from human embryonic stem cells. Nat Biotechnol 25(12):1468–1475
66. Rao M, Malik N (2013) Neural differentiation from pluripotent stem cells. InStem Cells Handbook. Springer, New York, pp. 149–160
67. Schwartz PH, Brick DJ, Stover AE, Loring JF, Müller FJ (2008) Differentiation of neural lineage cells from human pluripotent stem cells. Methods 45(2):142–158
68. Zelenaia O, Schlag BD, Gochenauer GE, Ganel R, Song W, Beesley JS, Grinspan JB, Rothstein JD et al (2000) Epidermal growth factor receptor agonists increase expression of glutamate transporter GLT-1 in astrocytes through pathways dependent on phosphatidylinositol 3-kinase and transcription factor NF-κB. Mol Pharmacol 57(4):667–678
69. DeSilva TM, Borenstein NS, Volpe JJ, Kinney HC, Rosenberg PA (2012) Expression of EAAT2 in neurons and protoplasmic astrocytes during human cortical development. J Comp Neurol 520(17): 3912–3932
70. Jungblut M, Tiveron MC, Barral S, Abrahamsen B, Knöbel S, Pennartz S, Schmitz J, Perraut M et al (2012) Isolation and characterization of living primary astroglial cells using the new GLAST-specific monoclonal antibody ACSA-1. Glia 60(6):894–907
71. Markiewicz I, Lukomska B (2006) The role of astrocytes in the physiology and pathology of the central nervous system. Acta Neurobiol Exp (Wars) 66(4):343–358
72. Wang DD, Bordey A (2008) The astrocyte odyssey. Prog Neurobiol 86(4):342–367
73. Ayers-Ringler JR, Jia YF, Qiu YY, Choi DS (2016) Role of astrocytic glutamate transporter in alcohol use disorder. World J Psychiatry 6(1):31–42
74. Angoulvant D, Clerc A, Benchalal S, Galambrun C, Farre A, Bertrand Y, Eljaafari A (2004) Human mesenchymal stem cells suppress induction of cytotoxic response to alloantigens. Biorheology 41:469–476
75. Fibbe WE, Noort WA (2003) Mesenchymal stem cells and hematopoietic stem cells transplantation. Ann N Y Acad Sci 996:235–244
76. Akira S, Takeda K, Kaisho T (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2(8): 675–680
77. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, Gilliet M, Ho S et al (2002) Human epithelial cells trigger dendritic cell–mediated allergic inflammation by producing TSLP. Nat Immunol 3(7):673–680
78. Arthur A, Shi S, Zannettino AC, Fujii N, Gronthos S, Koblar SA (2009) Implanted adult human dental pulp stem cells induce endogenous axon guidance. Stem Cells 27(9):2229–2237
79. Mead B, Logan A, Berry M, Leadbeater W, Scheven BA (2014) Paracrine-mediated neuroprotection and neuritogenesis of axotomised retinal ganglion cells by human dental pulp stem cells: comparison with human bone marrow and adipose-derived mesenchymal stem cells. PLoS One 9(10):e109305
80. Young F, Sloan A, Song B (2013) Dental pulp stem cells and their potential roles in central nervous system regeneration and repair. J Neurosci Res 91(11):1383–1393
81. Kimelberg HK, Nedergaard M (2010) Functions of astrocytes and their potential as thera- peutic targets. Neurotherapeutics 7(4):338–353
82. Parpura V, Grubisic V, Verkhratsky A (2011) Ca(2+) sources for the exocytotic release of glutamate from astrocytes. Biochim Biophys Acta 1813(5):984–991
83. Ramaswamy S, Kordower JH (2009) Are growth factors the answer? Parkinsonism Relat Disord 15(Suppl 3):S176–S180
84. Yasuda T, Mochizuki H (2010) Use of growth factors for the treatment of Parkinson's disease. Expert Rev Neurother 10(6):915–924
85. Yoshida K, Toya S (1997) Neurotrophic activity in cytokine-activated astrocytes. Keio J Med 46(2):55–60
86. Hausmann R, Riess R, Fieguth A, Betz P (2000) Immunohistochemical investigations on the course of astroglial GFAP expression following human brain injury. Int J Legal Med 113(2):70–75
87. Wachter B, Schürger S, Rolinger J, von Ameln-Mayerhofer A, Berg D, Wagner HJ, Kueppers E (2010) Effect of 6-hydroxydopamine (6-OHDA) on proliferation of glial cells in the rat cortex and striatum: evidence for de-differentiation of resident astrocytes. Cell Tissue Res 342(2):147–160
88. Baquet ZC, Bickford PC, Jones KR (2005) Brain-derived neurotrophic factor is required for the establishment of the proper number of dopaminergic neurons in the substantia nigra pars compacta. J Neurosci 25(26):6251–6259
89. Baydyuk M, Xu B (2014) BDNF signaling and survival of striatal neurons. Front Cell Neurosci 8(254):1–10
90. Feng L, Wang CY, Jiang H, Oho C, Mizuno K, Dugich-Djordjevic M, Lu B (1999) Differential effects of GDNF and BDNF on cultured ventral mesencephalic neurons. Brain Res Mol Brain Res 66:62–70
91. Hyman C, Hofer M, Barde YA, Juhasz M, Yancopoulos GD, Squinto SP, Lindsay RM (1991) BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature 350: 230–232
92. Murer MG, Yan Q, Raisman-Vozari R (2001) Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. Prog Neurobiol 63:71–124
93. Bal-Price A, Moneer Z, Brown GC (2002) Nitric oxide induces rapid, calcium-dependent release of vesicular glutamate and ATP from cultured rat astrocytes. Glia 40:312–323
94. Buskila Y, Amitai Y (2010) Astrocytic iNOS-dependent enhancement of synaptic release in mouse neocortex. J Neurophysiol 103: 1322–1328
95. Ikeda H, Murase K (2004) Glial nitric oxide-mediated long-term presynaptic facilitation revealed by optical imaging in rat spinal dorsal horn. J Neurosci 24:9888–9896
96. Mehta B, Begum G, Joshi NB, Joshi PG (2008) Nitric oxide–mediated modulation of synaptic activity by astrocytic P2Y receptors. J Gen Physiol 132(3):339–349
97. Xie AX, Petravicz J, McCarthy KD (2015) Molecular approaches for manipulating astrocytic signaling in vivo. Front Cell Neurosci 9:144