

# An Aminopropyl Carbazole Derivative Induces Neurogenesis by Increasing Final Cell Division in Neural Stem Cells

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## Abstract

P7C3 and its derivatives, 1-(3,6-dibromo-9*H*-carbazol-9-yl)-3-(*p*-tolylamino)propan-2-ol (1) and *N*-(3-(3,6-dibromo-9*H*-carbazol-9-yl)-2-hydroxypropyl)-*N*-(3-methoxyphenyl)-4-methylbenzenesulfonamide (2), were previously reported to increase neurogenesis in rat neural stem cells (NSCs). Although P7C3 is known to increase neurogenesis by protecting newborn neurons, it is not known whether its derivatives also have protective effects to increase neurogenesis. In the current study, we examined how 1 induces neurogenesis. The treatment of 1 in NSCs increased numbers of cells in the absence of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2), while not affecting those in the presence of growth factors. Compound 1 did not induce astrocytogenesis during NSC differentiation. 5-Bromo-2'-deoxyuridine (BrdU) pulsing experiments showed that 1 significantly enhanced BrdU-positive neurons. Taken together, our data suggest that 1 promotes neurogenesis by the induction of final cell division during NSC differentiation.

**Key Words:** Neurodegenerative diseases, Neural stem cells, Neurogenesis, Final cell division, Aminopropyl carbazoles

## INTRODUCTION

Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, and amyotrophic lateral sclerosis (ALS) are characterized by progressive loss of neurons (Bossy-Wetzel *et al.*, 2004). The number of AD patients in the United States in 2012 was approximately 5.4 million, while that of PD patients in 2010 was roughly 630,000 (Kowal *et al.*, 2013). However, currently there is no pharmacological agent that cures neurodegenerative diseases.

Neural stem cells (NSCs) can self-renew and possess the ability to differentiate into neurons, astrocytes, and oligodendrocytes (Gage, 2000). NSCs are present not only in the developing central nervous system (CNS) but also in the adult CNS (Gage, 2000). NSCs in the adult CNS are known to produce new neurons throughout the subventricular zone and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Taupin and Gage, 2002). Thus, small molecules that can modulate the promotion of neurogenesis from endogenous

NSCs may be considered as novel drug candidates for the treatment of these diseases.

Small molecules that increase neurogenesis from NSCs have recently been reported, however, the underlying mechanisms behind neuron generation have not yet been fully identified. A natural product, forskolin (FSK) was found to increase neuronal differentiation of NSCs (Lairson *et al.*, 2013). FSK enhanced neurogenesis by stimulating adenylyl cyclase and resulted in an increase in the concentration of intracellular cyclic adenosine monophosphate. In addition, NSCs were synergistically differentiated into neurons upon treatment with FSK in combination with retinoic acid. Another synthetic molecule, neuropathiazol, has been reported to increase neuronal differentiation by inducing the mRNA expression of *neuroD*, while suppressing astrocytogenesis in NSCs (Warashina *et al.*, 2006). Sertraline, an antidepressant drug, was found to increase neurogenesis through the glucocorticoid receptor in neural progenitor cells (NPCs) (Anacker *et al.*, 2011; Peng *et al.*, 2012). Sodium butyrate, a histone deacetylase inhibitor, was also shown to promote neurogenesis (Kim *et al.*, 2009a).

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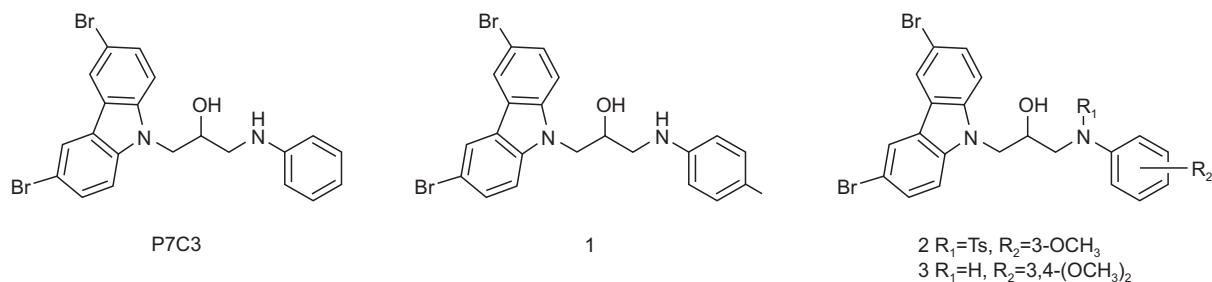
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**Fig. 1.** Chemical structures of P7C3 and 1-3.

Sodium butyrate treatment resulted in the sprouting of dendrites, an increase in the number of synapses, and up-regulation of *neuroD* expression (Fischer *et al.*, 2007). We have also recently reported that a phytochemical, kuwanon V, induced neurogenesis not only in the absence of but also in the presence of mitogens such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) (Kong *et al.*, 2015).

Recently, a synthetic molecule, an aminopropyl carbazole, termed P7C3, was reported to increase neurogenesis in the mouse SGZ of the dentate gyrus, and improved the cognition of aged rats (Pieper *et al.*, 2010). In addition, P7C3 derivatives had effects on PD and ALS in animal models (De Jesus-Cortes *et al.*, 2012; Tesla *et al.*, 2012). It has been reported that P7C3 exerted its proneurogenic activity by protecting newborn neurons from apoptosis but not by promoting the proliferation of mice hippocampal NPCs (MacMillan *et al.*, 2011). The neuroprotection exerted by P7C3 is through the targeting of nicotinamide phosphoribosyltransferase (NAMPT) to produce nicotinamide adenine dinucleotide (NAD) (Wang *et al.*, 2014). In a rodent model of blast-mediated traumatic brain injury (TBI), P7C3 treatment preserved axonal integrity after injury, before neuronal cell death occurred (Yin *et al.*, 2014).

We have previously synthesized and identified analogues of P7C3 that enhance *in vitro* neurogenesis (Yoon *et al.*, 2013). Treatment of NSCs derived from the cortex of embryonic day (E) 14 rats with 5.0  $\mu$ M of three derivatives (Fig. 1), 1-(3,6-dibromo-9H-carbazol-9-yl)-3-(p-tolylamino)propan-2-ol (1), N-(3-(3,6-dibromo-9H-carbazol-9-yl)-2-hydroxypropyl)-N-(3-methoxyphenyl)-4-methylbenzenesulfonamide (2), and 1-(3,6-dibromo-9H-carbazol-9-yl)-3-((3,4-dimethoxyphenyl)amino)propan-2-ol (3), significantly increased the percentage of TuJ1 (neuronal marker)-positive cells, compared to the vehicle-treated control group (Yoon *et al.*, 2013). In the previous study, the mode of action of 2 was investigated, and it was reported that 2 boosts the survival of NSCs in the absence of EGF and FGF2, while inhibiting the proliferation of NSCs in the presence of EGF and FGF2 (Yoon *et al.*, 2013). In the current study, we investigate the neurogenic mechanisms of 1 and found that 1 increased final cell division during differentiation of NSCs to enhance neurogenesis.

## MATERIALS AND METHODS

### Cell culture

NSCs were cultured as previously described (Kim *et al.*, 2013). Briefly, NSCs isolated from the cortex of E14 Sprague-Dawley rat embryos were dissociated, seeded onto tissue cul-

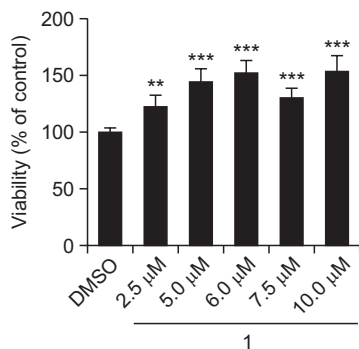
ture flasks (200,000 cells/ml), and expanded as neurospheres for 7 days in Dulbecco's modified Eagle's medium/F12 supplemented with 1% (v/v) PSA, 2% (v/v) B27 (all from Gibco, Grand Island, NY, USA), 20 ng/ml EGF and 20 ng/ml FGF2 (both from Chemicon, Temecula, CA, USA). The medium was replaced every 2 days. Neurospheres were dispersed into a single-cell suspension with accutase (Chemicon), and dissociated cells were seeded onto tissue culture plates pre-coated sequentially with 0.01% poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) and 10  $\mu$ g/ml laminin (Invitrogen, Grand Island, NY, USA). The cells were maintained at 37°C in 5% CO<sub>2</sub>.

### Cell viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay. NSCs were plated onto 48-well plates (Corning, Corning, NY, USA) at a density of  $3 \times 10^4$  cells/well and treated with different concentrations of 1 or 0.1% dimethyl sulfoxide (DMSO; Sigma-Aldrich) as a vehicle for 2 days. The cells were incubated with MTT solution (1 mg/ml) for 2 hours at 37°C. Formazan crystals that formed in the NSCs were solubilized with 20% sodium dodecyl sulfate (Amresco, Solon, OH, USA) in 50% aqueous N,N-dimethyl-formamide (Sigma-Aldrich). Ly-sates were transferred to 96-well plates (Corning) and the absorbance was measured at 550 nm using the Synergy H1 Hybrid Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA).

### Immunocytochemistry and cell counting

Immunocytochemical examination was performed as previously described (Kim *et al.*, 2007). Cell cultures were fixed with 4% paraformaldehyde (PFA; USB Products, Cleveland, OH, USA) for 30 minutes and washed with phosphate-buffered saline (PBS). Fixed cells were blocked with 5% normal goat serum (Millipore, Temecula, CA, USA) and 0.2% Triton X-100 (Amresco) in PBS for 30 minutes and incubated with primary antibodies against cleaved caspase-3 (rabbit monoclonal antibody, 1:400; Cell Signaling), glial fibrillary acidic protein (GFAP, rabbit polyclonal antibody, 1:1000; Dako, Denmark), and  $\beta$ III Tubulin (TuJ1, mouse monoclonal antibody, 1:1000; Sigma-Aldrich) for 1 hour. After rinsing with PBS, the cells were incubated for 30 minutes with secondary antibodies conjugated to Cy3 (goat anti-rabbit immunoglobulin G [IgG], 1:1000; Jackson ImmunoResearch, West Grove, PA, USA) or Alexa Fluor 488 (goat anti-mouse IgG, 1:1000; Invitrogen) followed by 5 minutes in 4',6-diamidino-2-phenylindole (DAPI, 1:10,000 in PBS; Sigma-Aldrich) to stain the nuclei. Images were obtained using an inverse fluorescence microscope (DMIL; Leica, Wetzlar, Hesse, Germany). Cleaved cas-



**Fig. 2.** 1 increases cell viability during NSC differentiation. NSCs were treated with various concentrations (2.5, 5.0, 6.0, 7.5, and 10.0 μM) of 1 or DMSO for 2 days in the absence of EGF and FGF2. Cell viability was assessed by an MTT assay. The values are presented as the mean ± S.D. (n=7, \*\*p<0.01 and \*\*\*p<0.001). Statistical analysis was performed using Student's *t*-test.

pase-3-, GFAP-, TuJ1-, and DAPI-positive cells were counted and normalized to total DAPI-positive cell numbers. The value of the 1-treated group was divided by that of the DMSO-treated group to yield fold changes.

#### BrdU assay

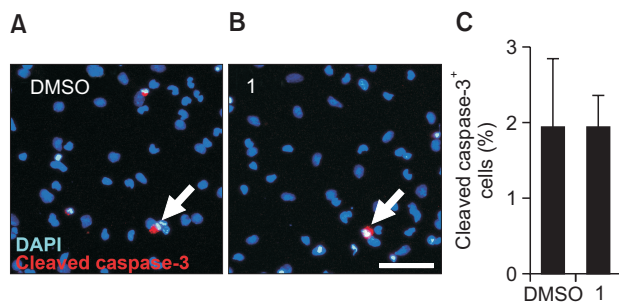
One day after plating, NSCs were treated with 5.0 μM compound 1 or DMSO for 4 days, and 5-bromo-2'-deoxyuridine (10.0 μM, BrdU; Sigma-Aldrich) was added to the media either during 0-12 hours or during 12-24 hours. The cells were fixed with 4% PFA for 30 minutes and then fixed with 70% ice-cold MeOH (Merck, Darmstadt, Hesse, Germany) for 10 minutes at room temperature. The cells were exposed to 2 M HCl (Sigma-Aldrich) for 20 minutes at 37°C and then incubated with 0.1 M sodium borate (Sigma-Aldrich) for 10 minutes at room temperature. Cells were washed with PBS containing 0.2% Triton X-100 and incubated with 5% normal goat serum and 0.2% Triton X-100 in PBS for 1 hour, followed by an overnight incubation at 4°C with an anti-BrdU antibody (mouse monoclonal antibody, 1:2000; Sigma-Aldrich). After rinsing, the cells were incubated with a Cy3-conjugated secondary antibody for 1 hour, followed by DAPI for 5 minutes. The images were acquired with an inverse fluorescence microscope. BrdU-positive cells were counted and normalized to the total cell number.

#### Neurosphere growth rate

Neurosphere growth was measured as previously described (Gamm *et al.*, 2008; Kim *et al.*, 2009b). Briefly, individual spheres (approximately 100-200 μm in diameter) were transferred to single wells of 96-well plates containing 200 μl growth media with 1 or DMSO (n≥4, for each treatment). The diameter of the spheres was measured daily using a lens-mounted microscope (Leica). The volume of the neurospheres was calculated by the equation  $V=4/3\pi r^3$ , where  $r=1/2$  diameter. Neurosphere volume on each day (day 0-3) was divided by the volume at day 0 and then multiplied by 100 to obtain the percentage increase in sphere volume.

#### Neurosphere formation assay

NSCs expanded as neurospheres in the presence of EGF and FGF2 for 1 week were dissociated and plated as single



**Fig. 3.** 1 has no effect on cell survival during NSC differentiation. NSCs differentiated for 4 days in the presence of 1 (5.0 μM) or DMSO were immunostained with an anti-cleaved caspase-3 antibody. (A, B) Representative immunofluorescence images for apoptotic cells (red, cleaved caspase-3-positive) and nuclei (blue, DAPI-positive). Scale bar, 50 μm. (C) Quantification of cleaved caspase-3-positive apoptotic cells. The values are presented as the mean ± S.D. (n=3). Statistical analysis was performed using Student's *t*-test.

cells onto uncoated 48-well plates at clonal density (1 cell/μl) in 250 μl media in each well (Coles-Takabe *et al.*, 2008). The cells were grown as floating cell aggregates (neurospheres) for 7 days in the presence of mitogens added with 5.0 μM compound 1 or DMSO. The number of neurospheres was counted from 3 independent fields photographed using the JULI-Digital Bio (NanoEnTek, Seoul, Korea).

#### Statistical analysis

Data were expressed as the mean ± standard error of the mean (S.E.M.) or the mean ± standard deviation (S.D.), and statistical significance was determined using Student's *t*-test (\*p<0.05, \*\*p<0.01, and \*\*\*p<0.001).

## RESULTS

### Increased cell viability by compound 1 during NSC differentiation

NSCs proliferate in the presence of EGF and FGF2, and differentiate into neurons or glia in the absence of mitogens. It was previously reported that 5.0 μM of 1 increased neurogenesis compared to DMSO-treated control during differentiation (Yoon *et al.*, 2013). To investigate the mode of action of 1, we first tested whether 1 affected the cell viability in the absence of growth factors. NSCs were isolated from the cortex of E14 rat embryos, dispersed into a single cell suspension, and expanded in tissue culture flasks as neurospheres for 6 days in the presence of EGF and FGF2. Neurospheres were dissociated into single cells, seeded onto 48-well plates, and treated with various concentrations (2.5, 5.0, 6.0, 7.5, and 10.0 μM) of 1 or DMSO as a vehicle for 2 days in the absence of mitogens. Subsequently, cell viability was assessed by an MTT assay. Treatment with 1 significantly increased the cell viability by 22.79, 44.44, 52.00, 30.64, and 53.63% at 2.5, 5.0, 6.0, 7.5, and 10.0 μM, respectively, compared with the DMSO-treated control (Fig. 2). These data suggest that 1 enhances cell viability/numbers during NSC differentiation.

**Facilitation of final cell division by compound 1 during NSC differentiation**

To test whether the increased cell viability/number by 1 in the absence of EGF and FGF2 was due to increased cell survival, we examined the levels of cleaved caspase-3, an apoptosis marker, by immunocytochemistry. NSCs were treated with 5.0 μM compound 1 or DMSO for 4 days in the absence of growth factors, and apoptotic cells were immunostained with an anti-cleaved caspase-3 antibody. However, there was no significant difference in the number of cleaved caspase-3-positive cells between the 1- and DMSO-treated groups (Fig. 3). These data suggest that 1 does not affect cell survival during differentiation.

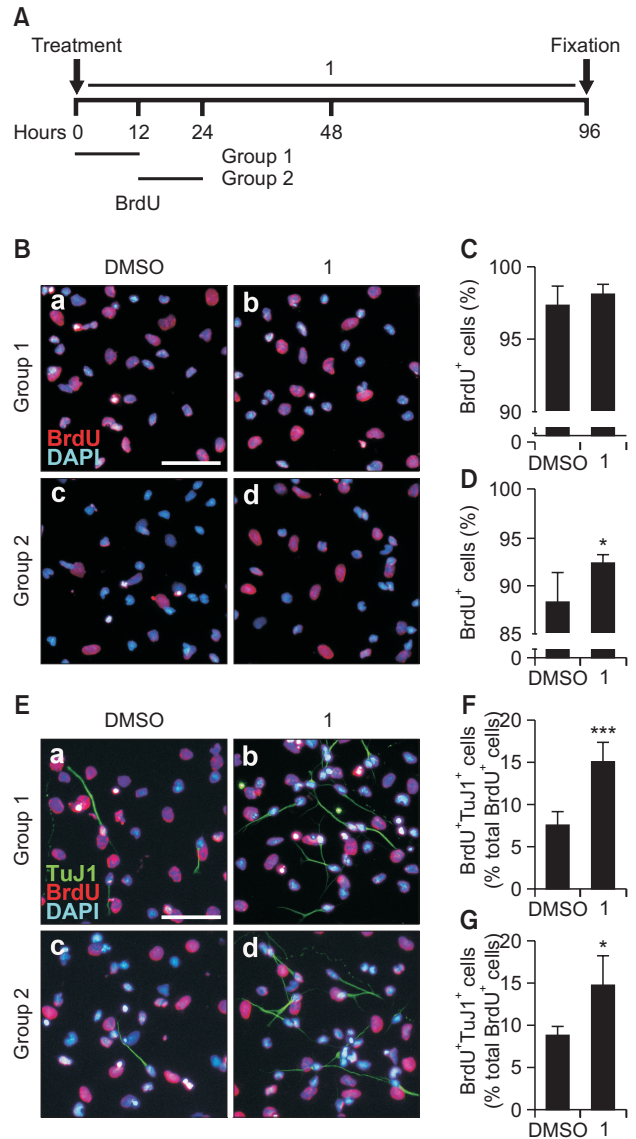
We then performed BrdU pulsing experiments to investigate whether the increased cell viability/number was a result of the stimulated proliferation during NSC differentiation. When BrdU, a thymidine analog that incorporates into the DNA of proliferating cells in the S-phase, was pulsed during the first 12 hours of differentiation, the number of BrdU-positive cells in the 1-treated group was not significantly different from the DMSO-treated control (Fig. 4B[a,b], 4C). However, when BrdU was added during the next 12 hours of differentiation, the number of BrdU-positive cells in the 1-treated group was significantly increased compared with that of cells treated with DMSO (Fig. 4B[c,d], 4D). Moreover, 1 markedly increased the number of BrdU/TuJ1 double-positive cells compared with the DMSO control when BrdU was added for both the first 12 hours and the next 12 hours of differentiation (Fig. 4E-4G). These data suggest that 1 promotes final cell division to generate neurons during NSC differentiation.

**Compound 1 does not affect NSC proliferation in the presence of mitogens**

We next examined whether 1 affected NSC proliferation in the presence of EGF and FGF2. Firstly, we performed a neurosphere growth assay. The sizes of the neurospheres treated with 5.0 μM compound 1 or DMSO in the presence of growth factors were measured daily for 4 days (day 0-3). As shown in Fig. 5A, the sizes of the neurospheres became bigger in a time-dependent manner. To evaluate the effect of 1 on NSC proliferation, we measured the diameters of neurospheres and calculated the volumes of spheres using the equation  $V=4/3\pi r^3$ , where  $r=1/2$  diameter. Neurosphere volume on each day was divided by the volume on day 0 and then multiplied by 100 to obtain the percentage increase in sphere volume. However, there was no significant difference in the volume increase of the neurospheres between the two groups (Fig. 5B). Furthermore, when we dissociated, plated NSCs at clonal density (1 cell/μl), and cultured for 7 days in the presence of mitogens with 5.0 μM compound 1 or DMSO to determine neurosphere formation ability, we observed no significant difference between the two groups (Fig. 5C, 5D). Taken together, these data suggest that 1 does not change the proliferation or neurosphere formation ability of NSCs in the presence of growth factors.

**Induction of neurogenesis but no significant changes in astroglial differentiation by compound 1 during NSC differentiation**

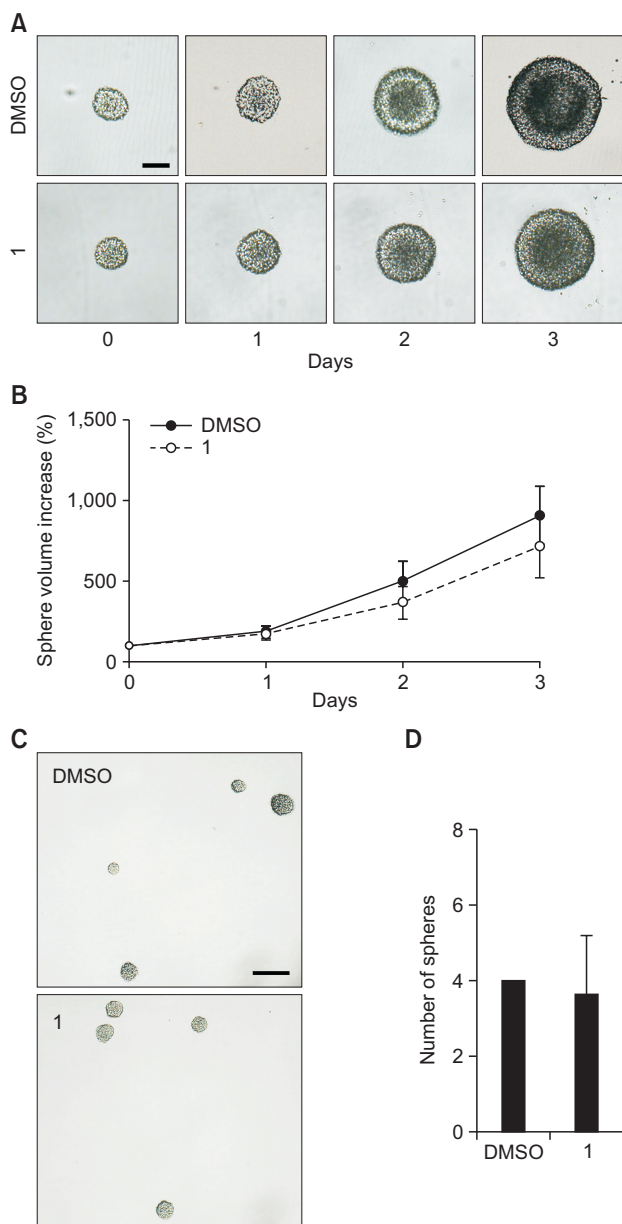
In order to investigate whether 1 has effects on the astroglial differentiation of NSCs, NSCs were treated with either 5.0 μM compound 1 or DMSO for 4 days in the absence of EGF and FGF2. Astroglia and neurons were detected using



**Fig. 4.** 1 promotes final cell division during NSC differentiation. (A) NSCs were treated with 5.0 μM compound 1 or DMSO for 4 days in the absence of growth factors, and BrdU was added either during 0-12 hours (group 1) or 12-24 hours (group 2). After fixation, cells were immunostained with an anti-BrdU antibody. (B) Representative immunofluorescence images of BrdU-positive cells (red) and nuclei (blue). Scale bar, 50 μm. (C, D) Quantification of BrdU-positive cells in group 1 (C) and group 2 (D). (E) Representative immunofluorescence images of BrdU-positive cells (red), TuJ1-positive cells (green), and nuclei (blue). Scale bar, 50 μm. (F, G) Quantification of BrdU/TuJ1 double-positive cells in group 1 (F) and group 2 (G). The values are presented as the mean ± S.D. (n=4, \*p<0.05 and \*\*\*p<0.001). Statistical analysis of all data was performed using Student's t-test.

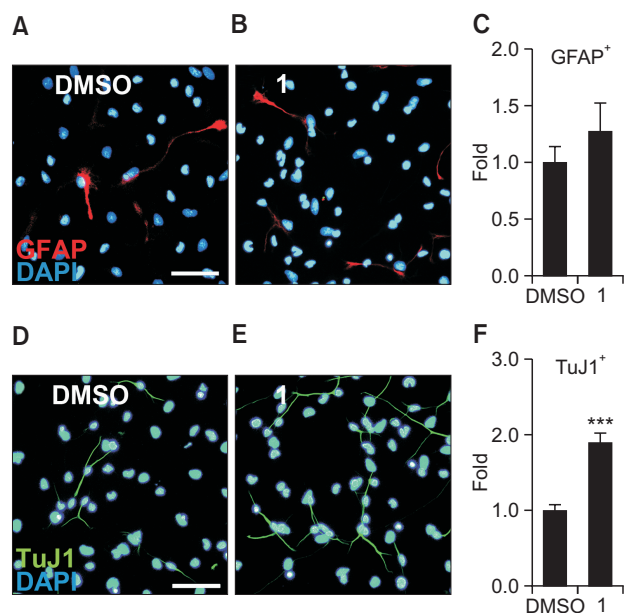
antibodies against GFAP and TuJ1, respectively. Immunocytochemistry results showed that there was no significant difference in the number of GFAP-expressing astrocytes between the 1-treated group and the DMSO-treated control group (Fig. 6A-6C). Conversely, we confirmed the neurogenic effects of 1 that had been reported in our previous study (Yoon *et al.*,





**Fig. 5.** 1 does not affect NSC proliferation in the presence of mitogens. (A) Individual neurospheres were treated with either 5.0  $\mu$ M compound 1 or DMSO for 4 days in the presence of EGF and FGF2. Digital images of neurospheres on day 0-3 are shown. Scale bar, 100  $\mu$ m. (B) Volumes of neurospheres were calculated using the measured diameters. The values are the mean  $\pm$  S.D. ( $n \geq 4$ , for each treatment). (C) Passaged NSCs were dissociated, plated as single cells at clonal density (1 cell/ $\mu$ l), and grown as neurospheres for 7 days in the presence of growth factors added with 5.0  $\mu$ M compound 1 or DMSO. Scale bar, 100  $\mu$ m. (D) The number of spheres was counted from 3 independent randomly chosen fields. The values are the mean  $\pm$  S.D. ( $n=3$ ). Statistical analysis of all data was performed using Student's *t*-test.

2013). As shown in Fig. 6D-6F, 1 significantly increased the percentage of neurons by 1.90-fold, compared to the DMSO-treated control. These data suggest that 1 promotes neurogenesis but does not affect astroglial differentiation in NSCs.



**Fig. 6.** Compound 1 induces neurogenesis while not affecting astrocytogenesis. NSCs treated with 5.0  $\mu$ M compound 1 or DMSO for 4 days during differentiation were immunostained with a TuJ1 or anti-GFAP antibody. (A, B) Representative immunofluorescence images for astrocytes (red, GFAP-positive) and nuclei (blue). Scale bar, 50  $\mu$ m. (C) Quantification of GFAP-positive astrocytes. (D, E) Representative immunofluorescence images for neurons (green, TuJ1-positive) and nuclei (blue). Scale bar, 50  $\mu$ m. (F) Quantification of TuJ1-positive neurons. The values are presented as the mean  $\pm$  S.E.M. ( $n=3$ , \*\*\* $p < 0.001$ ). Statistical analysis of all data was performed using Student's *t*-test.

## DISCUSSION

NSCs can self-renew and differentiate into neurons, astrocytes, and oligodendrocytes (Gage, 2000). Since neurodegenerative diseases are characterized by a loss of neurons (Bossy-Wetzel *et al.*, 2004), several studies have attempted to identify small molecules that increase the neuronal differentiation of NSCs to facilitate regeneration (Kim and Jin, 2012).

Recently, P7C3 has been discovered to increase neurogenesis (Pieper *et al.*, 2010) and compound 1, a derivative of P7C3, has been identified as a neurogenesis enhancer in our previous study (Yoon *et al.*, 2013). P7C3 functions as a neuroprotective compound by activating NAMPT and increasing NAD levels in cells (Wang *et al.*, 2014). P7C3 and its derivative, P7C3-S243 not only protect neurons from death but also preserve axonal integrity, learning and memory, and motor coordination in a rodent TBI model (Yin *et al.*, 2014). In the present study, we investigated whether a P7C3 derivative, compound 1, functions in a similar manner to P7C3 during the neurogenesis of NSCs.

When NSCs were treated with 1 or DMSO in the absence of growth factors, 1 significantly increased not only neuronal differentiation but also overall cell viability compared with the DMSO control. Due to the fact that P7C3 and its derivatives including compound 2 are known to protect newly generated neurons from death and block axonal degeneration (Pieper *et al.*, 2010; Yoon *et al.*, 2013; Yin *et al.*, 2014), we first hypothesized that 1 would also enhance cell survival like other

aminopropyl carbazoles. However, treatment with 1 did not change the number of cleaved caspase-3-positive cells compared with the DMSO-treated control, suggesting that 1 may act differently to P7C3.

Interestingly, it was shown that 1 promoted cell division during NSC differentiation. When BrdU was pulsed during 12-24 hours of differentiation, 1 significantly increased the number of proliferating cells compared with the DMSO-treated control. Moreover, 1 considerably increased the number of BrdU/TuJ1 double-positive cells within 24 hours of differentiation. During neurogenesis, neural progenitor cells undergo symmetric or asymmetric divisions (Noctor *et al.*, 2004). A time-lapse and immunocytochemistry study revealed that just before neurogenesis NSCs do not transform to neurons but undergo final cell division (Kim *et al.*, 2007). Thus, data obtained in the current study suggest that compound 1 may facilitate final cell division to produce daughter neurons during NSC differentiation. Similar to compound 1, kuwanon V, a phenolic compound isolated from the *Morus bombycis* bark, increased neurogenesis and final cell division in early NSC differentiation (Kong *et al.*, 2015). Insulin-like growth factor-1 is also known to induce neurogenesis by stimulating cell division of granule cell precursors (Lin and Bulleit, 1997).

Although the detailed mechanisms involved in increased neurogenesis by aminopropyl carbazole derivatives should be investigated in further studies, our results provide another underlying proneurogenic mechanism of aminopropyl carbazole derivatives that is different from those reported with P7C3 and its other derivatives (Yoon *et al.*, 2013; Wang *et al.*, 2014; Yin *et al.*, 2014). P7C3 was found to increase neurogenesis by increasing survival of newborn neurons but not by promoting proliferation of neural precursor cells, and 2 was also shown to enhance neurogenesis by boosting cell survival during NSC differentiation (MacMillan *et al.*, 2011; Yoon *et al.*, 2013). In contrast, 1 did not show protective effects but increased neurogenesis by the facilitation of final cell division during NSC differentiation. Identification of P7C3 derivatives or variants may provide not only neuroprotective but also neuroproducing agents for neurodegenerative diseases.

## ACKNOWLEDGMENTS

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