Published in final edited form as:

Neuroscience. 2006 June 30; 140(2): 377–388. doi:10.1016/j.neuroscience.2006.02.064.

N-CADHERIN MEDIATES NITRIC OXIDE-INDUCED NEUROGENESIS IN YOUNG AND RETIRED BREEDER NEUROSPHERES

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Abstract

Neurogenesis may contribute to functional recovery after neural injury. Nitric oxide donors such as DETA-NONOate promote functional recovery after stroke. However, the mechanisms underlying functional improvement have not been ascertained. We therefore investigated the effects of DETA-NONOate on neural progenitor/stem cell neurospheres derived from the subventricular zone from young and retired breeder rat brain. Subventricular zone cells were dissociated from normal young adult male Wistar rats (2–3 months old) and retired breeder rats (14 months old), treated with or without DETA-NONOate. Subventricular zone neurosphere formation, proliferation, telomerase activity, and Neurogenin 1 mRNA expression were significantly decreased and glial fibrillary acidic protein expression was significantly increased in subventricular zone neurospheres from retired breeder rats compared with young rats. Treatment of neurospheres with DETA-NONOate significantly decreased neurosphere formation and telomerase activity, and promoted neuronal differentiation and neurite outgrowth concomitantly with increased N-cadherin and β -catenin mRNA expression in both young and old neurospheres. DETA-NONOate selectively increased Neurogenin 1 and decreased glial fibrillary acidic protein mRNA expression in retired breeder neurospheres. N-cadherin significantly increased Neurogenin 1 mRNA expression in young and old neurospheres. Anti-N-cadherin reversed DETA-NONOate-induced neurosphere adhesion, neuronal differentiation, neurite outgrowth, and β -catenin mRNA expression. Our data indicate that age has a potent effect on the characteristics of subventricular zone neurospheres; neurospheres from young rats show significantly higher formation, proliferation and telomerase activity than older neurospheres. In contrast, older neurospheres exhibit significantly increased glial differentiation than young neurospheres. DETA-NONOate promotes neuronal differentiation and neurite outgrowth in both young and older neurospheres. The molecular mechanisms associated with the DETA-NONOate modulation of neurospheres from young and older animals as well age dependent effects of neurospheres appear to be controlled by N-cadherin and β -catenin gene expression, which subsequently regulates the neuronal differentiating factor Neurogenin expression in both young and old neural progenitor cells.

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Keywords

neuronal differentiation; β -catenin; Neurogenin1; Ngn1; nitric oxide donor; neurite outgrowth

Neural precursor cells contribute to adult neurogenesis and to the limited attempt of brain repair after injury. Induction of self-renewal or differentiation of neural progenitor cells depends on the interaction of intrinsic and environmental cues. Age is an intrinsic factor, which may influence neurogenesis. However, neurogenesis, which contributes to the ability of the adult brain to function normally and adapt to disease, declines with advancing age (Cameron and McKay, 1999; Kuhn et al., 1996). In addition, accelerated glial reactivity in aged rats also correlates with reduced functional recovery after stroke (Badan et al., 2003a). Glial reactivity increases with age, which coincides with the accumulation of beta-amyloid peptide (Abeta) (Badan et al., 2003b). Abeta can evoke reactive gliosis in response to neurodegeneration (Nichols, 1999), whereas, the nitric oxide (NO)/cGMP pathway opposes the effects of Abeta in the regulation of vasoconstriction (Paris et al., 1999). As a pharmacologic neuro-restorative approach, treatment of stroke in rats with an NO donor, DETA-NONOate, promotes neurogenesis and significantly improves functional outcome after stroke in young adult rats (Zhang et al., 2001). These observations prompted our investigation whether DETA-NONOate could promote neurogenesis and modify gliogenesis in young adult and aged brain.

Cellular adhesion plays a key role in a number of developmental events, including proliferation, cell fate, morphogenesis, neurite outgrowth and synaptogenesis. N-cadherin, important for cell-to-cell interaction and adhesion in the development of the CNS, promotes neuronal survival and promotes synaptic plasticity (Suzuki et al., 2004). β -Catenin, which links cadherins and the actin cytoskeleton, is a modulator of cadherin-based adhesion and regulates synaptic structure and neuronal differentiation (Otero et al., 2004; Yu and Malenka, 2003). Over-expression of β -catenin increases axonal length and complexity (Yu and Malenka, 2004). In addition, the proneural basic helix-loop-helix (bHLH) transcription factor neurogenin 1 (Ngn1) promotes neuronal differentiation and suppresses formation of astrocytes (Sauvageot and Stiles, 2002). NO regulates synaptic remodeling and neuronal differentiation in the adult mammal CNS (Bicker, 2001; Ciani et al., 2004; Seidel and Bicker, 2000; Sunico et al., 2005; Truman et al., 1996). We, therefore, hypothesize, DETA-NONOate upregulates the N-cadherin/ β -catenin pathway and Ngn1 expression and thereby promotes neurogenesis in both young and older rats.

In this study, subventricular zone (SVZ) neurosphere cultures with or without DETA-NONOate were employed to test neurosphere formation, proliferation, telomerase activity, and to elucidate the mechanisms by which DETA-NONOate regulates neurogenesis and associated gene expression in young adult and retired breeder rats.

EXPERIMENTAL PROCEDURES

SVZ neurosphere culture

Adult and retired breeder male Wistar rats were purchased from Charles River (Wilmington, MA, USA). SVZ cells were dissociated from normal young adult male Wistar rats (2–3 months) and retired breeder rats (14 months old), as previously reported (Morshead et al., 1994). Mechanically dissociated SVZ cells were plated at 3×10^4 cells/ml in DMEM-F-12 medium containing 20 ng/ml epidermal growth factor (EGF, R&D System, Minneapolis, MN, USA) and 20 ng/ml basic fibroblast growth factor (bFGF, R&D System). DMEM-F-12 medium contains DMEM-F-12 (R&D System), L-glutamine (2 mM), glucose (0.6%) and B27. Primary neurospheres were cultured for 7 days, after which they were collected and passaged by

mechanical dissociation, resuspended in proliferation medium, and plated at 2×10^4 cells/ml in each well of a 24 well plate (Corning, Corning, NY, USA).

The SVZ neurosphere experimental groups

Table 1 shows the experimental groups employed in the present study. Neurospheres from passages 6–8 were cultured under different conditions (Table 1) for 7 days. The floating neurosphere formation number and size were measured at 7 days DIV in groups 1–12 under a microscope. The attachment of neurospheres to the flask was not evaluated.

Real time-PCR

The experimental groups are composed of groups 1–12 identified above. The neurospheres were collected at 7 days DIV for real time PCR assay. Cells were harvested and total RNA was isolated from treated cells with TRIzol (Invitrogen, Carlsbad, CA, USA), following a standard protocol (Livak and Schmittgen, 2001). Quantitative PCR was performed using the SYBR Green real time PCR method. Quantitative RT-PCR was performed on an ABI 7000 PCR instrument (Applied Biosystems, Foster City, CA, USA) using three-stage program parameters provided by the manufacturer, as follows; 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that a single DNA sequence was amplified during PCR. Each sample was tested in triplicate, and samples were obtained from three independent experiments that were used for analysis of relative gene expression data using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The following primers for real-time PCR were designed using Primer Express software (ABI) (see Table 2).

Separate groups of SVZ neurospheres from groups 1–6 were cultured for 3 days to measure cell viability, and cultured for 24 h to measure cell proliferation (bromodeoxyuridine (BrdU) enzyme-linked immunosorbent assay (ELISA)) and telomerase activity. Additional groups of SVZ neurospheres from groups 1–6 were cultured in differentiation medium for 7 days for measurements of cell differentiation (β -tubulin III (TUJ1) and glial fibrillary acidic protein (GFAP) immunostaining) and TUJ1 positive cell neurite outgrowth, respectively.

Viability of the SVZ neurosphere measurement (Chen et al., 2005)

Neurospheres from passages 6–8 were mechanically dissociated to single-cells and were plated at 2.0×10^4 cells/ml onto laminin-coated wells of a 24 well plate (Corning), and treated with conditions 1–6 identified above (see Table 1) for 3 days ($n=4$ /group). SVZ neurosphere cells were identified using fluorescence microscopy according to the procedure provided with the live-dead viability-cytotoxicity kit (Molecular Probes, Eugene, OR, USA). A mixture of calcein acetoxymethyl ester (AM) and ethidium homodimer was added to cover and incubate the SVZ neurosphere cells for 20 min at 37 °C. Live cells (possessing intracellular esterase activity) convert calcein AM to calcein. Calcein is retained in the cell and produces green fluorescence when excited. Dead cells (with their compromised plasma membranes) allow the entrance of ethidium homodimer, which undergoes a 40-fold increase in red fluorescence after binding to nucleic acids. Therefore, the nuclei of the dead cells appear red under the fluorescence microscope. The data are presented as a percentage of dead cells to live cells.

BrdU ELISA assay for SVZ neurosphere cell proliferation (Gasparri et al., 2004)

To evaluate DNA synthesis, the SVZ neurosphere cells from groups 1–6, identified above, were plated at 2×10^4 cells/ml in each well of a 96 well plate (Corning) and cultured for 24 h ($n=4$ /group). BrdU (50 μ M) was added to the SVZ neurosphere culture medium 12 h before fixation, after which samples were spun down and analyzed using a BrdU ELISA for detection

of BrdU incorporated into cellular DNA (BrdU labeling and detection kit III, Roche, Indianapolis, IN, USA) following the manufacturer's standard protocol. The BrdU incorporation was then quantified by colorimetric immunoassay at 450 nm by an automated microplate reader. The absorbance background signal measured was subtracted from all the values reported.

Telomerase activity assay (Chen et al., 2005)

To determine telomerase activity, the Telo TAGGG telomerase PCR ELISA kit (Cat:1854666, Roche) was used. Cells (2×10^5) from neurospheres were cultured for 24 h ($n=4$ /group), collected, and lysed. The experimental groups consisted of groups 1–6 identified above. The supernatant was used for PCR with the following profile; 25 °C 25 min, 94 °C 5 min (94 °C 30 s, 50 °C 30 s, 72 °C 1.5 min) for 30 cycles, 72 °C 10 min, 4 °C hold. The resulting PCR product was denatured and used for ELISA assay. Five microliters of the PCR product was added to a microtiter plate, and incubated at 37 °C for 2 h while being shaken at 300 r.p.m. Anti-digoxigenin-peroxidase was added, and incubated at 25 °C at 300 r.p.m. for 30 min. Then 100 μ l of substrate was added, and incubated at 25 °C at 300 r.p.m. for 20 min. Stop solution was added and the plate was immediately read at 450 nm with reference wavelength of 690 nm.

SVZ neurosphere cell type immunohistochemistry staining and quantification

Neurospheres from passages 6–8 were mechanically dissociated to single-cells, and were plated ($2 \times 10^4/\text{cm}^2$) onto laminin-coated glass coverslips in serum-free differentiation medium (without heparin, bFGF and EGF). The experimental groups consisted of groups 1–9 identified above ($n=4$ /group). Incubation was terminated at 7 days after plating and immunostaining for TUJ1 (a marker of immature neuronal phenotype) and GFAP (a marker of astroglial phenotype), was performed for evaluation of SVZ neurosphere cell differentiation). Immunofluorescent staining for cultured cells was performed, as previously described (Chen et al., 2003). SVZ neurosphere cells were fixed with 4% paraformaldehyde. Primary antibodies used were: mouse anti-TUJ1 (1:1000; Novus Biologicals Inc., Littleton, CO, USA) and rabbit anti-GFAP (1:200; Dako). DAPI was used as a nuclear counterstain. Control experiments consisted of staining as outlined above, but omitted the primary antibodies, as previously described (Li et al., 1998). Positive cells were counted in five randomly selected microscopic fields per slide under a 20 $\times$ objective. The percentages of reactive cells within the total number of DAPI positive cells are presented.

SVZ neuronal cells neurite outgrowth

To identify neurite outgrowth and the axonal arbors of TUJ1 positive cells, photomicrographs were captured using a 20 $\times$ objective (Olympus BX40) via the MCID computer imaging analysis system. A hundred neurons per experimental group were analyzed. The total length of neurite outgrowth was measured using the perimeter/length function measured of the MCID system.

Statistical analysis

Two sample *t*-tests were performed to compare neurosphere formation, BrdU, telomerase activity, and TUJ1, GFAP, N-cadherin, β -catenin, Ngn1 mRNA expression between the young adult and retired breeder control SVZ cells. The N-cadherin, β -catenin and Ngn1, TUJ1 and GFAP mRNA expression, telomerase activity and BrdU incorporation were analyzed using two or three factor analysis of variance (ANOVA) models, with factors age (young and old) and treatment group (Control, 0.1 μ M NONOate and 0.4 μ M NONOate). The interaction between age and treatment group was tested. If there was significant interaction ($P<0.10$), all pair-wise comparisons were analyzed. If there was not a significant interaction, the main effect

for age and treatment groups was tested. Pair-wise comparisons for treatment group are present as mean±S.E. A value of $P<0.05$ was taken as significant.

RESULTS

SVZ neurosphere formation

Neural progenitor cells give rise to clonal aggregates of undifferentiated neural precursors, called neurospheres. These cells also reaggregate from dispersed cells in suspension culture and regenerate into histotypical three-dimensional spheres. SVZ cells are self-renewing and multi-potential progenitor cells (Reynolds and Weiss, 1996). In order to determine whether multi-potentiality and self-renewal of SVZ cells are altered with age, floating SVZ neurosphere formation was measured. Fig. 1 shows that SVZ cells from both young and retired breeder rats cultured in the growth medium (containing heparin, bFGF and EGF) form floating multicellular spheres (A and D). A significant decrease in neurosphere size and number was observed in SVZ neurosphere cultures from 14 month old retired breeder rats (D, G and H) compared with culture from 3 month young adult rats (A, G and H). DETA-NONOate treatment of neurospheres derived from both young (both doses, i.e. $0.1\ \mu\text{M}$ and $0.4\ \mu\text{M}$) and retired breeder (0.1 and $0.4\ \mu\text{M}$) rats significantly decreases floating neurosphere formation number and size, and induces neurosphere attachment to the flask. Both young (B and C) and retired breeder (E and F) rat SVZ neurospheres subsequently disperse and grow into sheets of largely flattened cells when cultured in growth medium.

Neurosphere cell viability

Decreased floating neurosphere formation number may be caused by an increase of cell adhesion, decrease of cell survival and cell proliferation (Chen et al., 2005). To measure whether DETA-NONOate treatment affects cell viability, total live and dead cells were measured. Neurosphere cells were mechanically dissociated to single-cells and were plated onto laminin-coated well. Neurosphere cells attached and dispersed. Cell viability was measured. Fig. 2 shows no significant difference between neurosphere cell viability in the DETA-NONOate-treated groups compared with the control group in both young adult (A–C) and retired breeder neurospheres (E–G). Fig. 2D and H shows the quantitative data of cell viability in young (D) and retired breeder (H) neurospheres. Fig. 2I shows that total live cell numbers were significantly increased in young adult neurosphere cells compared with retired breeder neurosphere cells (I, $P<0.05$).

SVZ neurosphere cell proliferation

To test SVZ neurosphere cell proliferation, BrdU ELISA assays were performed. There are no significant treatment group by age interactions ($P=0.414$) or treatment group main effects ($P=0.132$) observed in BrdU incorporation. Fig. 2J shows that retired breeder SVZ neurosphere cell proliferation significantly decreased compared with young adult rat SVZ neurospheres (J, $P<0.05$). DETA-NONOate added to young adult and retired breeder rat SVZ neurosphere culture did not alter cell proliferation compared with control non-treated SVZ neurospheres. This is consistent with BrdU expression measured by cell flow cytometry (data not shown).

SVZ neurosphere cell telomerase activity

Telomerase activity increases with cell proliferation, and rapidly decreases as cells differentiate (Cherif et al., 2003). To test whether the DETA-NONOate-induced decreases of young and aged SVZ neurosphere formation are related to the regulation of telomerase activity, telomerase activity was measured in both young adult and retired breeder rat SVZ neurospheres with or without DETA-NONOate. There is a significant age by treatment interaction ($P<0.05$) observed in the telomerase activity assay data. Fig. 2K shows that telomerase activity was

significantly decreased in retired breeder neurospheres compared with young adult neurospheres ($P<0.05$). In addition, DETA-NONOate treatment significantly decreases telomerase activity in both young and retired breeder neurospheres compared with non-treated neurospheres (Fig. 2K).

DETA-NONOate increases SVZ cell neuronal differentiation, but decreases glial differentiation

To test whether DETA-NONOate regulates SVZ neurosphere cell differentiation, RT-PCR was used to measure TUJ1 and GFAP mRNA expression in young adult and retired breeder rat SVZ neurospheres treated with or without DETA-NONOate (0.1 μ M and 0.4 μ M). A significant treatment by age interaction is found in the analysis of GFAP gene expression ($P=0.096$), but not in TUJ1 gene expression ($P=0.813$). Fig. 3A shows that there are no significant differences in TUJ1 mRNA expression in retired breeder neurospheres compared with young adult neurospheres. DETA-NONOate treatment significantly increases TUJ1 (A) mRNA expression in both young adult and retired breeder rats SVZ neurospheres. However, a significant increase of GFAP mRNA expression is found in retired breeder neurospheres compared with young adult neurospheres (B). DETA-NONOate significantly decreases GFAP mRNA expression in retired breeder neurospheres, but does not alter GFAP mRNA in young adult neurospheres compared with respective non-treatment group (B). These data suggest that DETA-NONOate promotes neuronal differentiation in both young and retired breeder neurospheres. However, DETA-NONOate selectively decreases the glial differentiation in retired breeder neurospheres.

To confirm that DETA-NONOate promotes neuronal differentiation in young and retired breeder rat SVZ neurospheres, neurospheres were passaged by mechanical dissociation and reseeded as single cells at a density of 2.0×10^4 cells/ml in laminin-coated glass coverslips and treated with or without DETA-NONOate (0, 0.1 and 0.4 μ M) for 7 days in differentiation medium. TUJ1 and GFAP protein immunostaining were performed. Fig. 3C–I shows that both doses of DETA-NONOate treatment (0.1 μ M and 0.4 μ M) significantly increase TUJ1 positive cell numbers compared with the respective non-treatment control group in both young adult and retired breeder neurospheres. In addition, GFAP expression significantly increases in retired breeder control neurospheres compared with young adult control neurospheres (J). Fig. 3J–M shows that DETA-NONOate decreases GFAP positive cells number in retired breeder neurospheres (K–M), but not in young adult neurospheres compared with respective non-treated controls (J). These data confirm that DETA-NONOate promotes neuronal differentiation in both young and retired breeder neurospheres, as well as decreases glial differentiation in retired breeder neurospheres.

DETA-NONOate promotes neurite outgrowth

To test whether DETA-NONOate regulates neurite outgrowth, total length of neurite outgrowth was measured from TUJ1 positive cells. Fig. 3N shows that neurite outgrowth significantly decreases in retired breeder control neurospheres compared with young adult control neurospheres. DETA-NONOate-treated groups show significantly increased neurite outgrowth compared with non-treatment controls in both young adult and retired breeder rat neurospheres, respectively.

N-cadherin mediates DETA-NONOate-induced cell adhesion, neuronal differentiation and neurite outgrowth

The above data show that DETA-NONOate decreases floating neurosphere formation and induces neurosphere adhesion to the flask and promotes neuronal differentiation. N-cadherin regulates cell adhesion and modulates the Wnt-induced nuclear activity of β -catenin (Modarresi et al., 2005), and β -catenin is required for neuronal differentiation (Otero et al., 2004).

Therefore, we first tested whether DETA-NONOate regulates N-cadherin mRNA expression. Fig. 4A shows that there are no significant differences in N-cadherin mRNA expression in retired breeder neurospheres compared with young adult neurospheres. However, DETA-NONOate treatment in both young adult and retired breeder rat neurospheres significantly increased N-cadherin mRNA expression (A) compared with the respective non-treatment controls.

To further investigate whether N-cadherin participates in DETA-NONOate-induced neuronal differentiation, N-cadherin ($5 \mu\text{g}/\text{cm}^2$) (recombinant human N-cadherin/Fc chimera, R&D System) and neutralized anti-N-cadherin antibody (monoclonal anti-N-cadherin/A-CAM; Sigma, St. Louis, MO, USA) treatment was employed. The young adult and retired breeder SVZ neurospheres were treated with or without DETA-NONOate, recombinant human N-cadherin/Fc chimera-coated ($5 \mu\text{g}/\text{cm}^2$) or an anti-N-cadherin antibody (80 ng/ml) in growth medium for 7 days. Neurosphere formation and cell differentiation were measured. Fig. 4B–G shows that N-cadherin significantly inhibits floating neurosphere formation (D), and significantly promotes neurosphere cell adhesion to the flask, dispersion and growth into sheets of largely flattened cells (D) compared with control young adult neurospheres (B). Anti-N-cadherin (F) reverses DETA-NONOate-induced neurosphere adhesion, and promotes neurosphere formation compared with DETA-NONOate ($0.4 \mu\text{M}$ NONOate) treatment alone (C). Fig. 4G shows the quantitative data of neurosphere formation from the different treatment groups. The retired breeder neurospheres have the same pattern of change as young adult neurospheres when treated with or without N-cadherin, or anti-N-cadherin (data not shown).

To test whether N-cadherin regulates β -catenin, and whether the N-cadherin/ β -catenin pathway plays a role in DETA-NONOate-induced neuronal differentiation, β -catenin and TUJ1 mRNA expression was measured using RT-PCR. Fig. 5A–D shows that N-cadherin promotes TUJ1 (A: retired breeder neurosphere C: young neurosphere) mRNA expression as well as upregulates β -catenin (B: retired breeder neurospheres; D: young neurospheres) mRNA expression in both young adult and retired breeder neurospheres. Anti-N-cadherin attenuates DETA-NONOate-induced TUJ1 (A and C) and β -catenin (B and D) mRNA expression. However, GFAP mRNA expression was not significantly different in N-cadherin- and anti-N-cadherin-treated groups (E).

DETA-NONOate has similar effects on regulating neuronal differentiation, and neurite outgrowth in both young and retired breeder neurospheres. Therefore, to test whether N-cadherin may also play a role in DETA-NONOate-induced neurite outgrowth, only young neurospheres were employed. Young adult neurospheres passaged by mechanical dissociation were reseeded as single cells at a density of 2.0×10^4 cells/ml in laminin-coated glass coverslips and treated with or without N-cadherin ($5 \mu\text{g}/\text{cm}^2$) and neutralizing anti-N-cadherin antibody for 7 days in differentiation medium. TUJ1 protein immunostaining was performed. Fig. 5F shows that N-cadherin treatment significantly increases TUJ1 positive cell neurite outgrowth compared with the control group. Anti-N-cadherin significantly decreases DETA-NONOate-induced neurite outgrowth (F) compared with DETA-NONOate treatment alone.

DETA-NONOate regulates Ngn1 gene expression (Fig. 6)

bHLH transcription factors such as Ngn1 play important roles in the regulation of neuronal differentiation and the inhibition glial differentiation (Sauvageot and Stiles, 2002). To evaluate the possible signal transduction pathways that may be involved in DETA-NONOate-induced neuronal differentiation and decreased glial differentiation, Ngn1 gene expression was measured in both young and retired breeder rat SVZ neurospheres. A significant two-way interaction between treatment groups ($P < 0.05$) was found. Fig. 6A shows that Ngn1 mRNA expression is significantly decreased in retired breeder neurospheres compared with young adult rat neurospheres. The decreased Ngn1 expression parallels the increase of GFAP mRNA

expression in retired breeder neurospheres (Fig. 3B). DETA-NONOate treatment significantly increases Ngn1 gene expression in both young adult (0.4 μ M NONOate treatment) and retired breeder rat SVZ neurospheres (0.1 μ M and 0.4 μ M NONOate treatment) compared with non-treated controls.

To test whether N-cadherin also regulates Ngn1 expression, SVZ neurospheres derived from both young adult and retired breeder rats were cultured in growth medium for 7 days and were treated with or without DETA-NONOate (0.4 μ M), N-cadherin (5 μ g/cm²) and anti-N-cadherin antibody (80 ng/ml) with DETA-NONOate (0.4 μ M). Ngn1 mRNA expression was measured. Fig. 6B–C shows that N-cadherin and DETA-NONOate significantly increase Ngn1 mRNA expression compared with control non-treatment neurospheres in both young adult and retired breeder rats. Anti-N-cadherin significantly inhibits DETA-NONOate-induced Ngn1 mRNA expression in both young adult and retired breeder neurospheres compared with DETA-NONOate (0.4 μ M) treatment alone.

DISCUSSION

Employing SVZ neurospheres derived from young adult and retired breeder rats, we demonstrate: 1) neurosphere formation, neural progenitor cell proliferation, and telomerase activity decrease, whereas, glial differentiation increases with age. 2) DETA-NONOate treatment decreases neurosphere formation and telomerase activity, but promotes neurosphere cell adhesion, neuronal differentiation and neurite outgrowth in both young adult and retired breeder SVZ neurospheres. 3) DETA-NONOate enhanced neuronal differentiation and neurite outgrowth parallels increased N-cadherin and β -catenin gene expression. Anti-N-cadherin reversed DETA-NONOate-induced neurosphere adhesion, neuronal differentiation, neurite outgrowth, and β -catenin mRNA expression. Thus, the N-cadherin/ β -catenin pathway may mediate DETA-NONOate induced neuronal differentiation and neurite outgrowth. 4) Ngn1 drives neuronal differentiation and inhibits glial differentiation. Decreased Ngn1 mRNA expression present in retired breeder neurospheres is consistent with increased gliogenesis with age. N-cadherin and DETA-NONOate treatment increased Ngn1 expression and neuronal differentiation. DETA-NONOate upregulation of Ngn1 gene expression may play an important role in promoting neuronal differentiation and decreasing glial differentiation in retired breeder neurospheres.

Neural progenitor cell self-renewal, proliferation and telomerase activity decrease with age

Endogenous neural progenitor cells may be important for the treatment of nervous system diseases (Wozniak, 1999). Neural progenitor cells provide a cellular reservoir for replacement of cells lost during normal cell turnover and after brain injury. However, neurogenesis does not compensate for neuronal loss in age-related neurodegenerative disorders, which may be caused by impaired neurogenesis. A reduction in basal levels of neurogenesis may contribute to the pathogenesis of such disorders (Darsalia et al., 2005; Haughey et al., 2002). Our data show that SVZ neural progenitor cells are capable of proliferation and self-renewal, but these functions are significantly decreased with age, and exhibit decreased neurosphere formation and BrdU incorporation. These data are consistent with the reported decrease of dentate neurogenesis by middle age (McDonald and Wojtowicz, 2005; Shetty et al., 2005). FGF-2, IGF-1, and VEGF decline in middle age and remain steady between middle age and old age (Shetty et al., 2005). These factors influence the proliferation of stem/progenitor cells in the dentate gyrus (Shetty et al., 2005) and possibly the progenitor and stem cells arising from the SVZ.

Our data also indicate that telomerase activity decreases in older SVZ neurospheres in parallel with reductions of SVZ neurosphere formation and cell proliferation in retired breeder rat SVZ cells compared with young adult rats. Cell growth and neuronal progenitor cell proliferation

decrease with age (Cameron and McKay, 1999; Darsalia et al., 2005; Kuhn et al., 1996; McDonald and Wojtowicz, 2005), which may be attributed to a decreased telomerase activity (Cherif et al., 2003). Neurogenesis declines dramatically with age, which in part is due to decreased levels of mitogens, and a decrease in the progenitor pool resulting from a reduction of factors which support brain plasticity (Shetty et al., 2005).

DETA-NONOate promotes neuronal differentiation

Increased neurogenesis may be caused by an increase in the neural progenitor cell pool or by an increase in neuronal differentiation. Neuronal differentiation requires factors that regulate the decision of dividing progenitors to leave the cell cycle and activate the neuronal differentiation program (Theriault et al., 2005). Decreased telomerase activity may be a prerequisite for neuronal precursors to differentiate (Kruk et al., 1996). Consistent with this idea, our data show that DETA-NONOate increases early neuronal marker TUJ1 mRNA and protein expression in both young adult and retired breeder SVZ neurospheres and decreases telomerase activity. However, DETA-NONOate decreases neurosphere formation number and size, but does not change cell survival and BrdU incorporation in both young and older neurospheres. Therefore, the DETA-NONOate-induced decrease of neurosphere formation may due to an increase in cell adhesion, and not an increase in cell death and/or a decrease of cell proliferation. Our data contrast with studies, that a decrease in NO production, using an NOS inhibitor, increases neural progenitor cell proliferation (Moreno-Lopez et al., 2004; Packer et al., 2003). It is however possible that this apparent inconsistency may be attributed to the fact that physiologically using a NOS inhibitor is not necessarily the converse of using an NO donor. Our data, are however consistent with observation that NO promotes neuronal differentiation (Cheng et al., 2003; Moreno-Lopez et al., 2004; Packer et al., 2003). In summary, our data suggest that DETA-NONOate-induced neurogenesis is likely mediated by an increase in neuronal differentiation, and not by an increase in the neural progenitor pool in both young adult and older neurospheres.

N-cadherin/ β -catenin pathway mediates DETA-NONOate-induced neuronal differentiation and neurite outgrowth

N-cadherin, a neural cell adhesion molecule, plays a role in neuronal differentiation of P19 cells, through the Wnt-1 signaling pathway (Gao et al., 2001). N-cadherin modulates the Wnt-induced nuclear activity of β -catenin (Modarresi et al., 2005). Increasing β -catenin signaling promotes neurogenesis (Otero et al., 2004). N-cadherin- β -catenin complex also regulates synaptogenesis and coordinates synaptic strength with presynaptic and postsynaptic organization, including the shape of dendritic spines (Benson and Tanaka, 1998; Goda, 2002). Overexpression of dominant-negative forms of N-cadherin induces abrogation of spine expansion (Suzuki et al., 2004). NO increases the cytosolic accumulation of β -catenin and nuclear formation of β -catenin/LEF-1 complex (Mei et al., 2002). Our data show that DETA-NONOate upregulates N-cadherin/ β -catenin expression and promotes neurosphere cell adhesion, neuronal differentiation and neurite outgrowth. Neutralized anti-N-cadherin antibody decreases DETA-NONOate-induced neurosphere cell adhesion, neuronal differentiation and neurite outgrowth, as well as decreases β -catenin expression. Therefore, DETA-NONOate-induced promotion of N-cadherin/ β -catenin expression may regulate the switch of neural progenitor cells from proliferation to neuronal differentiation and increase neurite outgrowth during neurogenesis. Although anti-N-cadherin decreases DETA-NONOate-induced neuronal differentiation, it does not influence glial differentiation. This suggests there may be a different signaling system responsible for the decrease observed in DETA-NONOate-regulated glial differentiation.

Glial activity increases with age; DETA-NONOate upregulates Ngn1 expression and decreases glial differentiation

Astrocytes maintain normal brain physiology during development and in adulthood. However, an age-related increase in glial reactivity causes reduced brain plasticity (Badan et al., 2003b). GFAP plays an important role in age-related deficits in learning and memory (Wu et al., 2005). Our data show that glial differentiation increased in retired breeder neurospheres compared with young adult neurospheres. The proneural bHLH factor, Ngn1, is involved in initiating neurogenesis and is expressed specifically at early stages of neurogenesis (Ma et al., 1997). Ngn1 participates in the specification of neural fate, promotes neurogenesis and suppresses glial differentiation (Sun et al., 2001). In addition, Ngn1 expression decreased in retired breeder neurospheres compared with young adult SVZ neurospheres, which parallels the decreased neuronal differentiation and increased glial differentiation. DETA-NONOate decreases SVZ neurosphere glial differentiation as well as increases Ngn1 expression in retired breeder neurospheres. In addition, N-cadherin also promotes Ngn1 mRNA expression, and inhibition of N-cadherin decreases DETA-NONOate-induced Ngn1 mRNA expression in young and old neurospheres. Therefore, the decreased Ngn1 expression in retired breeder SVZ neurospheres may cause the observed increase of glial differentiation in retired breeder neurospheres. DETA-NONOate decreases retired breeder rat SVZ neurosphere glial differentiation, but not young adult SVZ neurosphere glial differentiation. This disparity suggests that age-related increased gliogenesis is subject to modulation by Ngn1, whereas basal levels of gliogenesis in the young adult SVZ may be maintained by Ngn1 independent signaling.

CONCLUSION

In summary, neurogenesis decreases, whereas gliogenesis increases with age. DETA-NONOate decreases neurosphere formation, telomerase activity, promotes neuronal differentiation and neurite outgrowth, and upregulates N-cadherin/ β -catenin and Ngn1 mRNA expression. N-cadherin/ β -catenin may mediate DETA-NONOate-induced neuronal differentiation and neurite outgrowth. DETA-NONOate regulates Ngn1 expression, which may decrease glial differentiation in retired breeder SVZ neurospheres. DETA-NONOate, and other NO donors reduce neurological deficits after stroke and traumatic brain injury (Zhang et al., 2001), which may be attributed to its upregulation of neurogenesis and down-regulation of gliogenesis. However, the SVZ neurosphere cell culture and ex vivo data generated warrant confirmation *in vivo*.

Acknowledgments

This work was supported by NINDS grants PO1 NS23393 and RO1 NS047682.

Abbreviations

Abeta	beta-amyloid peptide
AM	acetoxymethyl ester
bFGF	basic fibroblast growth factor
bHLH	basic helix–loop–helix
BrdU	bromodeoxyuridine
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay

GFAP	glial fibrillary acidic protein
Ngn1	Neurogenin 1
NO	nitric oxide
SVZ	subventricular zone
TUJ1	β -tubulin III

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Fig. 1. SVZ neurosphere formation. Panels A–C show young rat SVZ neurosphere formation in non-treated control (A), treated with 0.1 μ M DETA-NONOate (B) and treated with 0.4 μ M DETA-NONOate (C). Panels D–F show retired breeder rat SVZ neurosphere formation in non-treated control (D), treated with 0.1 μ M DETA-NONOate (E) and treated with 0.4 μ M DETA-NONOate (F). Panels G and H show quantitative data of neurosphere formation number (G) and neurosphere size (H) in both young adult and retired breeder neurospheres treated with or without DETA-NONOate. Scale bar=200 μ m, F.

Fig. 2.

SVZ neurosphere cell viability, proliferation (BrdU ELISA) and telomerase activity measurements. Panels A–D show young adult SVZ neurosphere cell live–dead viability measurement (A for young adult neurosphere control; B for young neurosphere treated with 0.1 μ M DETA-NONOate; C for young neurosphere treated with 0.4 μ M DETA-NONOate; green color: live cell; red color: dead cell). Panel D shows quantitative data of cell viability for young adult neurosphere. Panels E–H show retired breeder rat SVZ neurosphere cell live–dead viability measurement (E for retired breeder neurosphere control; F for retired breeder neurosphere treated with 0.1 μ M DETA-NONOate; G for retired breeder neurosphere treated with 0.4 μ M DETA-NONOate; green color: live cell; red color: dead cell). Panel H shows quantitative data of cell viability for retired breeder neurosphere. Panel I shows results of total live cell numbers in both young and retired breeder neurospheres. Panel J shows quantitative data of a BrdU ELISA assay; panel K shows quantitative data of telomerase activity assay. Scale bar=100 μ m, G.

Fig. 3.

SVZ neurosphere cell differentiation and neurite outgrowth. Panels A and B show data from young and retired breeder neurospheres treated with or without DETA-NONOate regulate TUJ1 (A), and GFAP (B) mRNA expression. Panels C–H show TUJ1 (TUJ1 positive cells: red; DAPI: blue) immunostaining in young adult neurospheres (C for control; D for treated with 0.1 μ m DETA-NONOate; E for treated with 0.4 μ m DETA-NONOate) and retired breeder neurospheres (F for control; G for treated with 0.1 μ m DETA-NONOate; H for treated with 0.4 μ m DETA-NONOate). Panels I and J show quantitative data of TUJ1 (I) and GFAP (J) positive cells in young and retired breeder rat SVZ neurospheres treated with or without DETA-NONOate for 7 days. Panels K–M show GFAP (GFAP positive cells: green; DAPI: blue)

immunostaining in retired breeder rat SVZ neurospheres treated with or without DETA-NONOate for 7 days (K for control; L for treated with 0.1 μm DETA-NONOate; M for treated with 0.4 μm DETA-NONOate). Panel N shows quantitative data of TUJ1 positive cell neurite outgrowth. Scale bar=25 μm , H; M=100 μm .

Fig. 4.

N-cadherin regulates neurosphere formation and cell adhesion. Panel A shows quantitative data of N-cadherin mRNA expression in young and retired breeder rat SVZ neurospheres treated with or without DETA-NONOate for 7 days. Panels B–F show young adult neurosphere formation in control (B), treated with 0.4 μ M DETANONOate (C), treated with N-cadherin (5 μ g/cm²) alone (D), treated with anti-N-cadherin antibody (80 ng/ml) alone (E), treated with anti-N-cadherin antibody and 0.4 μ M DETANONOate (F). DETA-NONOate (C) and N-cadherin (D) increase cell adhesion and decrease neurosphere formation. Panel G shows quantitative data of neurosphere formation culture in the different treatment groups for 7 days in growth medium. Scale bar=200 μ m, F.

Fig. 5.

N-cadherin regulates SVZ neurosphere β -catenin mRNA expression, cell differentiation and neurite outgrowth. Panels A–D show quantitative data of TUJ1 (A for retired breeder neurosphere; C for young adult neurosphere), β -catenin (B for retired breeder neurosphere; D for young adult neurosphere) mRNA expression in young and retired breeder neurosphere treated with or without DETA-NONOate, N-cadherin, anti-N-cadherin or anti-N-cadherin with DETA-NONOate. Panel E shows quantitative data of GFAP mRNA expression in young neurospheres treated with or without DETA-NONOate and anti-N-cadherin. Panel F shows quantitative data of TUJ1 immunostaining positive cell neurite outgrowth in young adult neurosphere culture.

Fig. 6.

Ngn1 mRNA expression decreases with age; N-cadherin regulates Ngn1 mRNA expression. Panel A shows Ngn1 mRNA expression measured by RT-PCR in young adult and retired breeder neurospheres treated with or without DETA-NONOate for 7 days. Panels B and C show quantitative data of Ngn1 mRNA expression in young neurospheres (B) and retired breeder (C) neurosphere treated with or without DETA-NONOate, N-cadherin, anti-N-cadherin and DETA-NONOate combination with anti-N-cadherin.

Table 1

Experimental groups

Group	Young neurospheres	Group	Retired breeder neurospheres
1	Control	4	Control
2	Treatment with 0.1 μ M DETA-NONOate	5	Treatment with 0.1 μ M DETA-NONOate
3	Treatment with 0.4 μ M DETA-NONOate	6	Treatment with 0.4 μ M DETA-NONOate
7	Treatment with recombinant human N-cadherin/Fc chimera (5 μ g/cm ²)	10	Treatment with recombinant human N-cadherin/Fc chimera (5 μ g/cm ²)
8	Treatment with neutralized anti-N-cadherin antibody (80 ng/ml)	11	Treatment with neutralized anti-N-cadherin antibody (80 ng/ml)
9	Treatment with 0.4 μ M DETA-NONOate and anti-N-cadherin antibody (80 ng/ml)	12	Treatment with 0.4 μ M DETA-NONOate and anti-N-cadherin antibody (80 ng/ml)

Table 2

Primers for respective genes

Gene	Accession number and primers (5'–3')
B-tubulin III	FWD: CCC GAG GGC TCA AGA TGTC; REV: CGC TTG AAC AGC TCC TGGAT
GFAP	FWD: AAC TGA CAC GTT GTG TTC AAG CA; REV: AGC TTC CGA GAG GGT ACA CTAA
N-cadherin	FWD: GTG AGA AGC TTC CCT CTC TCT G; REV: CCA GGC TCA GGT TAA CAG CTA C
B-catenin	FWD: GTC AGC GAC TTG TTC AAA ACT G; REV: ACC CAG AAG CTG CAC TAG AGT C
Ngn1	FWD: CAG TAG TCC CTC GGC TTC AG; REV: AAG CAG GGT GTC GTA TGG AG
GAPDH	FWD: AGA GAG AGG CCC TCA GTT GCT; REV: TTG TGA GGG AGA TGC TCA GTG T