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NB-3/Notch1 Pathway via Deltex1 Promotes Neural Progenitor Cell Differentiation into Oligodendrocytes*

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Neurons and glia in the vertebrate central nervous system arise in temporally distinct, albeit overlapping, phases. Neurons are generated first followed by astrocytes and oligodendrocytes from common progenitor cells. Increasing evidence indicates that axon-derived signals spatiotemporally modulate oligodendrocyte maturation and myelin formation. Our previous observations demonstrate that F3/contactin is a functional ligand of Notch during oligodendrocyte maturation, revealing the existence of another group of Notch ligands. Here, we establish that NB-3, a member of the F3/contactin family, acts as a novel Notch ligand to participate in oligodendrocyte generation. NB-3 triggers nuclear translocation of the Notch intracellular domain and promotes oligodendrogliogenesis from progenitor cells and differentiation of oligodendrocyte precursor cells via Deltex1. In primary oligodendrocytes, NB-3 increases myelin-associated glycoprotein transcripts. Thus, the NB-3/Notch signaling pathway may prove to be a molecular handle to treat demyelinating diseases.

Neural progenitor cells (NPCs)[1] are self-renewing multipotent cells that can give rise to all types of neural cells, namely neurons, oligodendrocytes (OLs), and astrocytes. Increasing evidence suggests that this fate commitment of NPCs requires molecular cues provided by extracellular molecules and intrinsic signaling involving various transcription factors (1, 2). Our recent study (3) has demonstrated that the F3/Notch signaling pathway via Deltex1 (DTX1) promotes oligodendrocyte precursor cell (OPC) differentiation into oligodendrocytes (OLs) and up-regulates myelin-associated glycoprotein (MAG) expression in both primary OLs and OLN-93 cells, an OL cell line. F3/contactin is a glycosylphosphatidylinositol-anchored neural cell adhesion molecule of the immunoglobulin superfamily (4, 5). However, the extracellular molecules involved in the progression from NPCs to OLs have been defined poorly.

The Notch signaling pathway mediates gliogenesis from NPCs from embryonic day (E) 17 to 18 (6). Notch1 is a transmembrane molecule characterized by a novel signaling paradigm, regulated intramembrane proteolysis (RIP) (7). Upon activation by the typical ligands, Delta, Serrate/Jagged, and Lag2 (DSL), Notch undergoes two programmed proteolytic cleavages and releases the intracellular domain (NICD) into the nucleus where together with transcription factors CSL/RBP-J and DTX1 NICD modulates the expression of different target genes, such as *Hes* (8). Notably, NB-3, a close homologue of F3/contactin (9, 10), is expressed from E17 onwards with an abrupt increase in the postnatal period and reaches the peak between postnatal day 7 (P7) and P21, which corresponds to the time frame for oligodendrogliogenesis from progenitor cells and OL maturation. In the present study, we demonstrate that NB-3 is a neuronal molecule and activates the Notch signaling pathway to direct OL generation from NPCs and OPCs via DTX1.

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal GFAP (DAKO), Notch1 (kind gifts from Dr. U. Lendahl), NICD (20), F3 (11), and MAG (3) antibodies, monoclonal Notch1 epidermal growth factor (EGF) repeats (Neomarker), c-*myc* (9E10), HA probe (Santa Cruz Biotechnology), FLAG, β-tubulin, neurofilament 200, CNPase, MAP2 (2a+2b)(Sigma), V5 epitope (Invitrogen), galactocerebroside, glial fibrillary acidic protein (GFAP), and CNPase (Chemicon) antibodies were used. NB-3 antibodies were generated by immunizing rabbits for antiserum and immunizing BALB/c mice for the monoclonal antibody (12) with recombinant protein encoding rat NB-3 Ig domains I-II (amino acids 30–227) expressed in *Escherichia coli*.

Western Blot Analysis of Developmental Expression Patterns—The striatum, hippocampus, cerebellum, spinal cord, and brain stem were dissected from embryos at E17 or neonates between P0 and P21 of

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[1] The abbreviations used are: NPCs, neural progenitor cells; OL, oligodendrocytes; DTX1, Deltex1; OPC, oligodendrocyte precursor cell; MAG, myelin-associated glycoprotein; NICD, Notch intracellular domain; E, embryonic; P, postnatal; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; BSA, bovine serum albumin; GST, glutathione *S*-transferase; DAPI, 4,6-diamidino-2-phenylindole; dn, dominant-negative; RIP, regulated intramembrane proteolysis; HA, hemagglutinin; CNpase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase.

Wistar rats. The specimens were homogenized in 9 volumes of reducing sample buffer and boiled for 5 min. Each 10-μl aliquot of the homogenates was subjected to a Western blot. Detection was carried out with ECL Western blotting System (Amersham Biosciences).

Cell Culture—Neurons, OLs, and astrocytes of E17 Wistar rats were isolated and cultured according to Itoh's methods (13). OLs of P5–7 Wistar rat cerebella were obtained by Percoll gradient centrifugation (14), and OPCs were purified from P5–7 Wistar rat optic nerves (15).

Culture of Neural Progenitor Cells—Murine neural progenitor cells were isolated from striatum, hippocampus, cerebellum, and the spinal cord of 14-day-old BALB/c mouse embryos (IFFA Credo, L'Arbresle, France) and cultured in Dulbecco's modified Eagle's medium/F12 (1:1) with N2 supplement and EGF (20 ng/ml) (Invitrogen) (16). Cells from passages 6–15 were used to induce differentiation. The spheres were dissociated mechanically into single cells and treated with NB-3 (12.5 nM) or Jagged1 (50 nM) in culture medium without growth factor for 24 h. The cells were then plated onto poly-L-lysine-coated coverslips (Nunc) in 4-well dishes at a density of 3×10^4/well in growth factor-free culture medium with 1% fetal calf serum.

Immunocytochemistry—This was performed mainly as described (3). For detection of primary neurofilament 200, GFAP, and galactocerebroside antibodies, Alexa Fluor 488-conjugated anti-mouse IgG or Alexa Fluor 546-conjugated anti-rabbit IgG (1:1500; Molecular Probes) were used. NPCs, OPCs, and OLN were first transfected with plasmid caN1, DTX1-D1, DTX1-D2, dominant-negative (dn)-N1 and pcDNA4/LacZ-V5 (Invitrogen) using LipofectAMINE 2000 (Invitrogen). The NPCs were treated with or without NB-3 in serum-free culture medium without growth factor for 24 h, and then cells were allowed to differentiate on 13-mm coverslips in 1% fetal calf serum medium for 7 days. The OPC and OLN were treated with NB-3 for 48 h. Cells were fixed with 4% paraformaldehyde and blocked with 1% bovine serum albumin (BSA). Cells were then incubated with primary antibodies in 0.2% BSA for 1 h followed by Cy3- or Cy2-labeled secondary antibody (Amersham Biosciences). After mounting in fluorescent mounting medium (DAKO), cells were visualized with a Leica DM RXA2 fluorescent microscope. The photos were taken using the same optical parameters to ensure the comparable luminosity. At least 10 different viewing fields from three independent experiments were used to calculate the percentage of cells showing NICD translocation or differentiation. In the case of γ-secretase inhibitor (Sigma) treatment, OLN cells were incubated with a 200 μM concentration of this inhibitor for 2 h prior to NB-3 treatment. Two-hundred cells from at least three independent experiments were quantified for fluorescence intensities by Adobe Photoshop™ (17). The raw data were analyzed by Student's t test with $p < 0.05$ and $p < 0.01$ being considered a significant or highly significant difference, respectively.

Cell Adhesion Assay—The cell adhesion assay was performed as described (4). Briefly, 35-mm tissue culture Petri dishes (Becton Dickinson) were coated with methanol-solubilized nitrocellulose and then proteins (12 μM) for 2 h at 37 °C in a humidified atmosphere. Subsequently, the dishes were washed and blocked overnight with 2% heat-inactivated fatty acid-free BSA (Sigma). After rinsing the dishes, the cells were plated in 2 ml of chemically defined medium at a density of 1.5×10^6cells/ml. At 0.5 h, the cells were gently washed and fixed with 2.5% glutaldehyde. Blockage of adhesion was carried out using anti-F3 (1:100), anti-NB-3 (1:100), and anti-Notch1 (1:200) antibodies. Cells adhering to the various spots were photographed and counted. The results were analyzed with a Newman-Keuls test with $p < 0.05$ and $p < 0.01$ being considered a significant or highly significant difference, respectively.

Production of Recombinant NB-3-Fc Proteins—The signal sequence of the glycosylphosphatidylinositol anchor was substituted with human IgG Fc followed by a termination codon. The cDNA thus manipulated was inserted into pREP4 between the Hind III and BamHI sites and transfected into HEK293 cells. NB-3-Fc was purified from the conditioned medium using protein A-agarose (Roche Applied Science).

The production of Notch1 GST fusion proteins has been described previously (3). Four sequential equal-sized fragments (EGF repeats 1–13), N1.2 (EGF repeats 11–24), N1.3 (EGF repeats 22–34), and N1.4 (EGF repeats 32–36, LNR repeats), were produced in *E. coli*.

Co-immunoprecipitation and GST Pull-down Assay—These assays were carried out as described (3). In brief, rat brain membrane samples were incubated overnight at 4 °C with protein A-agarose beads (coupled with NB-3-Fc or indicated antibodies, or glutathione-agarose beads) bound to GST-N1.1, N1.2, N1.3, or N1.4. Captured proteins were separated and detected in SDS-PAGE and Western blot.

Flow Cytometric Analysis—After 7 days *in vitro* culture, the differentiated NPCs were trypsinized, washed with phosphate-buffered saline, and treated with FACSPerm (Becton Dickinson). Cells were stained with antibody MAP2 (2a+2b), GFAP, CNPase, and fluorescein isothiocyanate-conjugated anti-mouse IgG1 and anti-rabbit IgG, then analyzed by flow cytometer (FACScalibur, BD Biosciences) with Cell Quest software (BD Biosciences). Raw data were analyzed and compared with Student's t test.

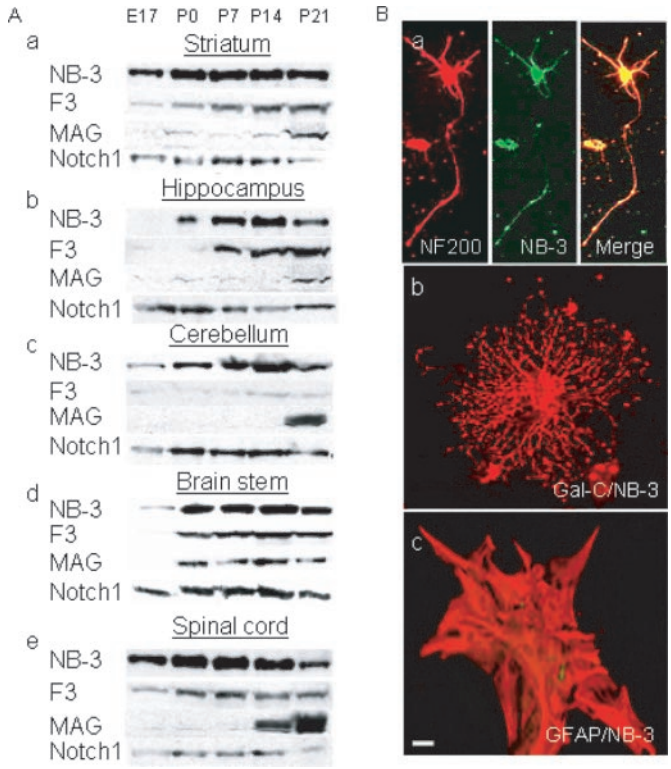

FIG. 1. **Characterization of NB-3 expression.** *A*, NB-3 is expressed from E17. Striatum (*a*), hippocampus (*b*), cerebellum (*c*), brain stem (*d*), and spinal cord (*e*) from rats with indicated ages were homogenized and subjected to immunoblot for NB-3, F3, MAG and Notch1. *B*, NB-3 is expressed by neurons. Purified neurons (*a*), OLs (*b*), and astrocytes (*c*) from E17 cerebella were cultured and double-stained for NB-3 and respective markers: neurofilament 200 (*NF200*) for neuron, galactocerebroside (*GAL-C*) for oligodendrocyte, and *GFAP* for astrocyte. *Scale bar* in c = 30 μm.

Transient and Stable Transfection—Cells were transfected with various plasmids using LipofectAMINE 2000. Stable transfectants were selected with 400 μg/ml G418 (Sigma) and identified by Western blot and immunocytochemistry.

Luciferase Reporter Assay—The luciferase reporter assay was performed as described (3). NPCs (1.5×10^5/well) in 12-well dishes were used for Hes1 luciferase reporter assays. Cells were transiently transfected using LipofectAMINE and LipofectAMINE Plus reagents. Each well received 0.2 μg of pGVB/Hes1 luciferase reporter plasmid together with various expression plasmids (0.2 μg of caN1, 0.6 μg of dnN1, DTX1-D1-HA, and DTX1-D2-FLAG). For dnN1, cells were then treated with 50 nM Jagged1. The β-galactosidase expression plasmid pCMV/β-Gal was included to monitor the transfection efficiency. Cells were lysed at 24-h post-transfection and assayed using the Steady-Glo luciferase assay kit (Promega). The raw data from at least four independent experiments were used to determine the relative reporter activity.

Real Time PCR Analysis—Real time PCR analysis was performed as described (3). Total RNA from primary OLs was extracted using the Qiagen RNeasy kit and treated with RNase-free DNaseI (Invitrogen). Samples were used for reverse transcription with random hexamer primers using *Taq*Man RT kit (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Real time PCR was performed using the *Taq*Man system on an ABI PRISM 7700 sequence detection system. The primers and *Taq*Man probes were designed using Primer Express Software (ABI), and sequences are available upon request. The raw data from at least four independent experiments were used to determine the relative expression levels of each transcript by employing the comparative C_T method (34).

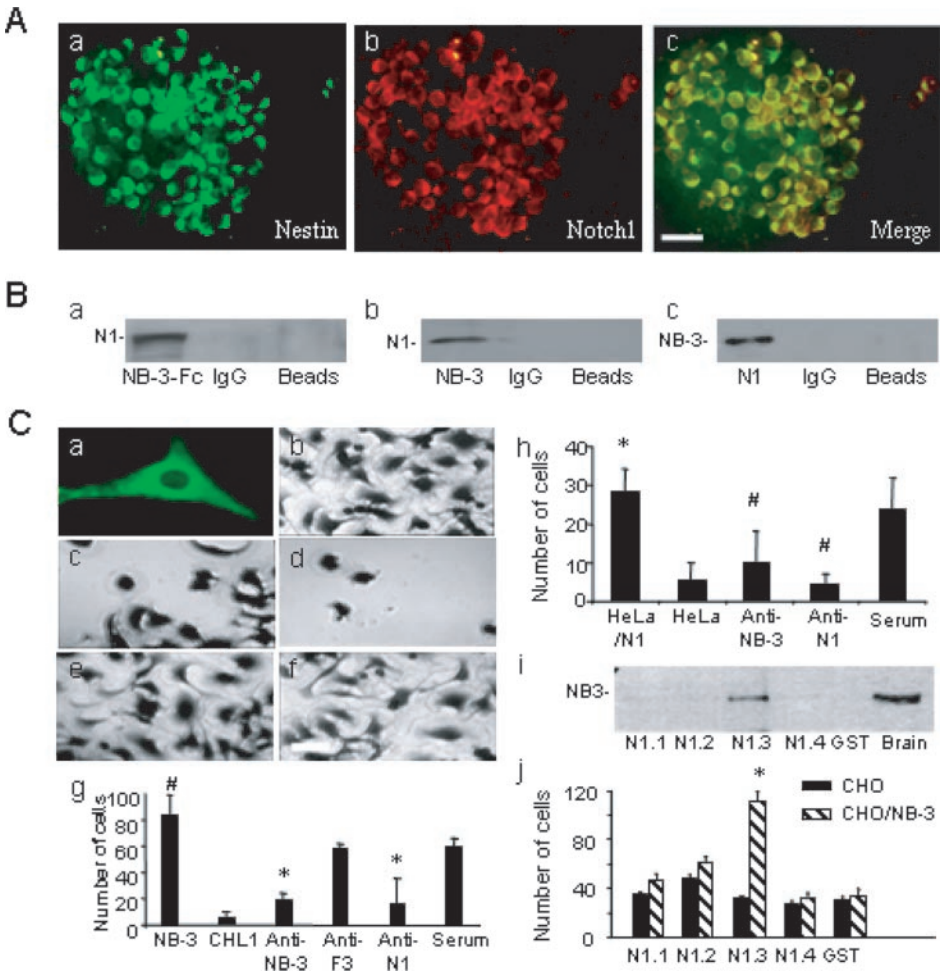

FIG. 2. **NB-3 is a binding partner of Notch1.** *A*, NPCs express Notch1. NPCs were double-stained for precursor marker nestin (*a*) and Notch1 (*b*); *c* is the merged picture. *Scale bar* in $c = 60$ μm for *a–c*. *B*, NB-3 binds to Notch1. NPCs samples were precipitated with protein A beads coupled with NB-3-Fc (*a*), P0 rat brain samples were immunoprecipitated using α-NB-3 or α-Notch1, and the precipitates were blotted as indicated (*b* and *c*).*C*, cell adhesion assay. OLN cells were labeled with α-Notch1 (*a*). OLN cells were seeded on NB-3 substrate (*b*) or in the presence of α-Notch1 (*c*), α-NB-3 (*d*), α-F3 (*e*), or pre-immune serum (*f*). *g*, quantification of OLN cell adhesion on the NB-3 substrate and the effects of blocking antibodies. #, $p < 0.05$ compared with CHL1; *, $p < 0.05$ compared with preimmune serum. *Scale bar* in $f = 8$ μm for *a–f*. *h*, quantification of mouse N1-transfected HeLa cell adhesion on NB-3 substrate and the effects of antibody blockade. Mouse N1-transfected HeLa cells or mock-transfected HeLa cells were plated on NB-3 or in the presence of α-NB-3, α-Notch1, or pre-immune serum. *, $p < 0.05$ compared with mock-transfected cells; #, $p < 0.05$ compared with preimmune serum. *i*, NB-3 binds to a specific region on Notch1. The GST-Notch1 extracellular fragments (N1.1, N1.2, N1.3, and N1.4) or GST alone were used in a GST pull-down assay with rat brain lysate. The precipitates and brain lysates were probed for NB-3. *j*, quantification of adherent NB-3- and mock-transfected Chinese hamster ovary (*CHO*) cells to the four Notch1 GST fusion fragments. *, $p < 0.05$ compared with GST.

RESULTS

NB-3 Expression Parallels OL Development—To better understand the potential role of both NB-3 and Notch in the context of oligodendrogliogenesis from progenitor cells, we investigated the developmental expression patterns of NB-3 and Notch1. A Western blot of different rat brain parts, striatum, hippocampus, cerebellum, the brain stem, and spinal cord, showed that both NB-3 and Notch1 were present at E17, and in hippocampus, cerebellum, and the brain stem, NB-3 showed an abrupt increase after birth and reached a maximum level between P0 and P21, which corresponds to the time frame of oligodendrogliogenesis (Fig. 1*A*). On the other hand, the expression of F3 is generally later than NB-3, which was most obviously seen in the cerebellum and hippocampus. In the striatum, cerebellum, and spinal cord, the F3 expression level was apparently lower than NB-3 at comparable developmental stages. As expected, MAG showed observable expression rather late (from P14) except in the brain stem.

NB-3 Is a Neuronal Molecule—To determine the cell type(s) that express NB-3, purified neurons, OLs, and astrocytes from E17 rat cerebella were separately cultured and double-stained for NB-3 and specific markers, neurofilament 200 for neurons (Fig. 1*B*, *a*), galactocerebroside for OLs (Fig. 1*B*, *b*), and GFAP for astrocytes (Fig. 1*B*, *c*). Only the neurons expressed NB-3.

NB-3 and Notch Are Binding Partners—F3 has been shown to interact with Notch (3). We were interested in determining whether NB-3 also bound to Notch. First, we studied the expression of Notch1 on NPCs by immunofluorescence. The progenitor cells were isolated from embryonic day 14 BALB/c mouse embryo striatum (16). These cells expressed the intermediate filament protein nestin (Fig. 2*A*, *a*), a progenitor marker, and Notch1 (Fig. 2*A*, *b* and *c*). Then NB-3-Fc fusion protein coupled to protein A beads was used to precipitate potential binding partners of NB-3 from progenitor cell membrane extracts. The precipitate was positively blotted with Notch1 antibody (Fig. 2*B*, *a*). Further, we immunoprecipitated P0 and adult (not shown) rat brain membrane extracts using NB-3 antibody and blotted the precipitates with Notch1 antibody and vice versa. Western blot showed reciprocal co-immunoprecipitation of NB-3 and Notch1 (Fig. 2*B*, *b* and *c*) suggesting a potential interaction between NB-3 and Notch1.

To confirm the NB-3/Notch interaction, OLN-93 (OLN) cells, a permanent cell line resembling maturing OLs (18) were used in cell adhesion assays as described (3, 4). Immunocytochemistry showed that OLN cells expressed Notch1 (Fig. 2*C*, *a*). Cells adhered to the coated NB-3-Fc (NB-3) substrate (Fig. 2*C*, *b*) but not to CHL1-Fc, another neural cell adhesion molecule (19) (Fig. 2*C*, *g*). The adhesion was blocked by preincubation with NB-3 or Notch1 antibodies, but not with F3 antibody or preimmune serum (Fig. 2*C*, *e* and *f*). Interestingly, upon contact with NB-3, cells enlarged rapidly to form an expansive cytoplasmic sheet (Fig. 2*C*, *b*) suggesting that NB-3 might signal to OLN cells to induce a morphological change. In addition, murine Notch1-transfected HeLa cells also adhered to NB-3, but mock-transfected HeLa cells did not. Adhesion was reversed by the addition of NB-3 or Notch1 antibodies but not by preimmune serum (Fig. 2*C*, *h*).

To map the binding site(s) on Notch1, four subcloned sequential equal-sized portions of the mouse Notch1 extracellular domain, labeled as N1.1, N1.2, N1.3, and N1.4 (3), were used in a GST pull-down assay from rat brain lysates. Immunoblotting showed that NB-3 associated only with N1.3, a region contain-

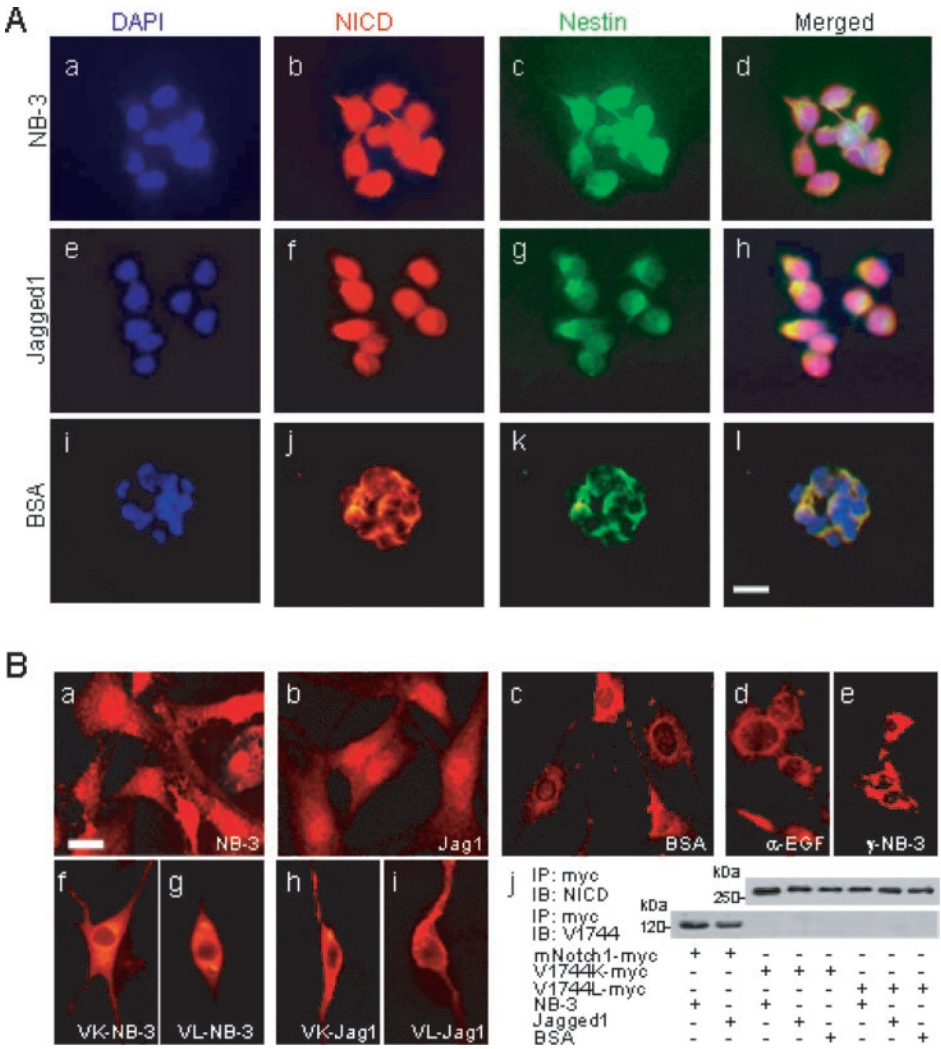

FIG. 3. **NB-3 is a functional ligand of Notch1.** *A*, NB-3 induces NICD nuclear translocation. NPCs were individually treated with NB-3 (12.5 nM) (*a–d*), Jagged1 (50 nM) (*e–h*), or BSA (*i–l*) for 24 h then fixed and triple-stained for nestin (*green*), NICD (*red*), and DAPI (*blue*) to locate NICD. *Scale bar* in $l = 25$ μm for *a–l*. *B*, NB-3/Notch interaction induces NICD nuclear translocation in OLN cells. Mouse Notch1-transfected OLN cells treated with NB-3 (*a*), Jagged1 (*b*), and BSA (*c*) were immunostained for NICD. Some cells were treated with EGF antibody (*d*) or γ-secretase inhibitor (*e*) before NB-3 stimulation. OLN cells were also transfected with V1744K-myc (*f* and *h*) or V1744L-myc (*g* and *i*), treated with NB-3 (*f* and *g*) or Jagged1 (*h* and *i*), and immunostained with c-*myc* antibody to locate NICD. *Scale bar* in $a = 20$ μm for *a–i*. *j*, after NB-3 or Jagged1 treatment, α-c-*myc* precipitates from mouse Notch1-myc-, V1744K-myc-, or V1744L-myc-transfected OLN cells were immunoblotted by α-NICD (*top panel*) or α-V1744 (*bottom panel*).

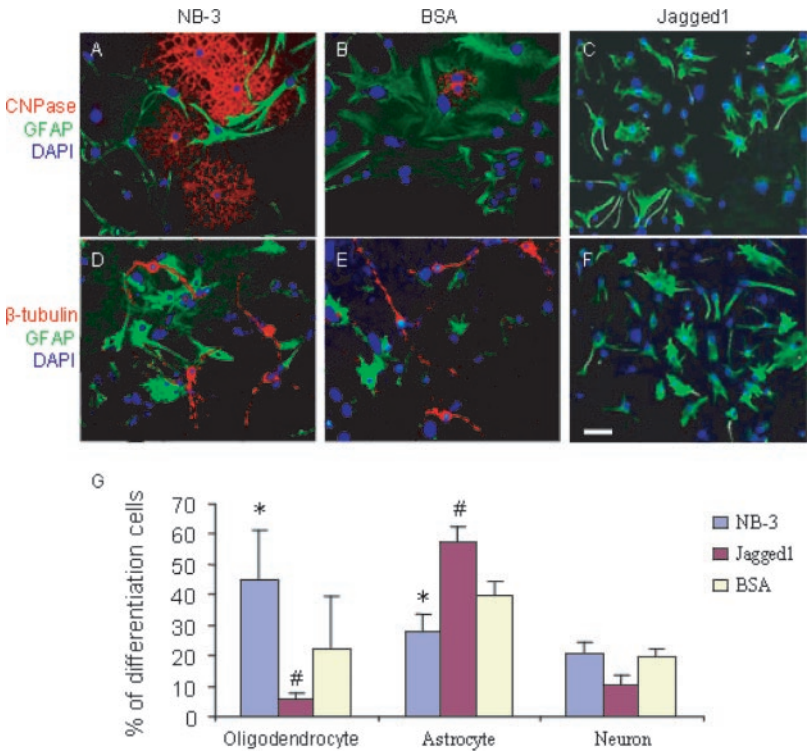

FIG. 4. **NB-3 promotes OL generation.** NPCs were passaged into mitogen-withdrawn culture medium that was supplemented with NB-3 (*A* and *D*), BSA (*B* and *E*), or Jagged1 (*C* and *F*). After 7 days *in vitro* culture differentiation, the cells were triple-stained for CNPase (*A–C*, *red*), β-tubulin (*D* and *F*, *red*), or GFAP (*A* and *F*, *green*) and DAPI (*A–F*, *blue*). Other NPCs were individually immunolabeled with marker antibody and subjected to flow cytometry. The percent of each type of cell, OLs, neurons, and astrocytes, were counted (*G*). Data are mean ± S.D. * and #, $p < 0.01$ in Student's *t* test, compared with BSA. *Scale bar* in $F = 40$ μm for *A–F*.

ing EGF repeats 22–34 (Fig. 2*C*, *i*). The specificity was confirmed by the observations that NB-3-transfected Chinese hamster ovary cells plated upon the individual recombinant fragments bound predominantly to N1.3 (Fig. 2*C*, *j*), whereas mock-transfected Chinese hamster ovary cells did not bind. Together, these results support the concept that NB-3 is a binding partner of Notch1.

NB-3 Triggers Notch RIP at the S3 Site and Transports NICD to the Nucleus—Notch activation by ligand binding is characterized by NICD nuclear translocation (20). We studied whether NICD nuclear translocation in progenitor cells could occur in response to NB-3 stimulation. Merged triple staining showed that NB-3 treatment of nestin-positive (*green*) progenitor cells resulted in NICD clustering (*red*) in the nucleus visualized by 4,6-diamidino-2-phenylindole (DAPI) (*blue*) (Fig. 3*A*, *a–d*), which was similar to Jagged1 stimulation (Fig. 3*A*, *e–h*). However, BSA failed to trigger this event (Fig. 3*A*, *i–l*). To confirm this, we investigated whether NB-3 could activate Notch in OLN cells. Cells were transfected with mouse Notch1-myc encoding myc-tagged full-length mouse Notch1 and treated with NB-3 (Fig. 3*B*, *a*) or Jagged1 (Fig. 3*B*, *b*). In both cases NICD concentrated in the nucleus. BSA failed to affect this (Fig. 3*B*, *c*). An antibody against EGF repeats on Notch1 prevented NB-3-induced NICD nuclear clustering (Fig. 3*B*, *d*). These observations indicated that NB-3 interacts with Notch1 to effect typical NICD nuclear translocation.

To study the properties of NB-3-induced NICD release, OLN cells were preincubated with γ-secretase inhibitor before exposure to NB-3 (Fig. 3*B*, *e*) or Jagged1 (not shown). This inhibitor specifically targets aspartyl protease. In both cases, nuclear NICD clustering was abolished, indicating that NB-3-induced NICD release involves γ-secretase. Moreover, cells transfected with two S3 mutants, myc-tagged V1744K and V1744L, which abolished S3 cleavage (20), successfully prevented NB-3-induced (Fig. 3*B*, *f* and *g*) as well as Jagged1-induced (Fig. 3*B*, *h* and *i*) NICD nuclear translocation. In the Western blot (Fig. 3*B*, *j*), c-*myc* antibody precipitates from either NB-3 or Jagged1-treated V1744K-myc or V1744L-myc-transfected OLN cells could only be blotted by the NICD antibody, which also recognizes intact Notch1 (~250 kDa) (*upper panel*) but not by V1744 antibody, which only recognizes released NICD from the S3 site. In contrast, mouse Notch-myc transfected OLN cells generated NICD (~120 kDa) that was blotted by V1744 antibody after NB-3 or Jagged1 treatment (Fig. 3*B*, *j*, *bottom panel*). Together, these results demonstrated that NB-3 induced γ-secretase-dependent Notch1 RIP at the S3 site.

NB-3 Promotes Oligodendrogliogenesis—Given the parallel expression of both NB-3 and Notch1 during development, we explored whether the NB-3/Notch interaction was involved in oligodendrogliogenesis from progenitor cells. To do this, embryonic neural progenitors were allowed to differentiate for 7 days in the absence of mitogen and in the presence of serum and NB-3 (Fig. 4, *A* and *D*), BSA (Fig. 4, *B* and *E*), or Jagged1 (Fig. 4, *C* and *F*), respectively. The cells, identified by DAPI (Fig. 4, *A–F*, *blue*), were immunostained for OL marker CNPase (Fig. 4, *A–C*, *red*), neuronal marker β-tubulin (Fig. 4, *D–F*, *red*), and astrocyte marker GFAP (Fig. 4, *A–F*, *green*). The results

TABLE I

NB-3 induces oligodendrogliogenesis from neural progenitor cells in different parts of the central nervous system

For every cell line treated with individual proteins, CNPase-positive oligodendrocytes in each of the 50 fields taken from 10 coverslips (5 fields/coverslip) were counted and represented as the mean percentage ± S.D. of the total cells visualized by DAPI labeling in each field. At least 40–60 cells were counted in each field.

	NB-3	BSA	Jagged1
	%	%	%
Fetal striatum	45.1 ± 16.3[a]	22.2 ± 17.5	5.6 ± 1.8[a]
Fetal hippocampus	37.5 ± 12.3[a]	21.9 ± 12.7	8.7 ± 6.3[a]
Fetal cerebellum	36.5 ± 8.6[a]	17.3 ± 8.7	6.9 ± 5.5[a]
Fetal spinal cord	39.1 ± 11.3[a]	19.3 ± 13.5	8.6 ± 7.6[a]

[a] $p < 0.01$ in Student's t test, compared with BSA treatment.

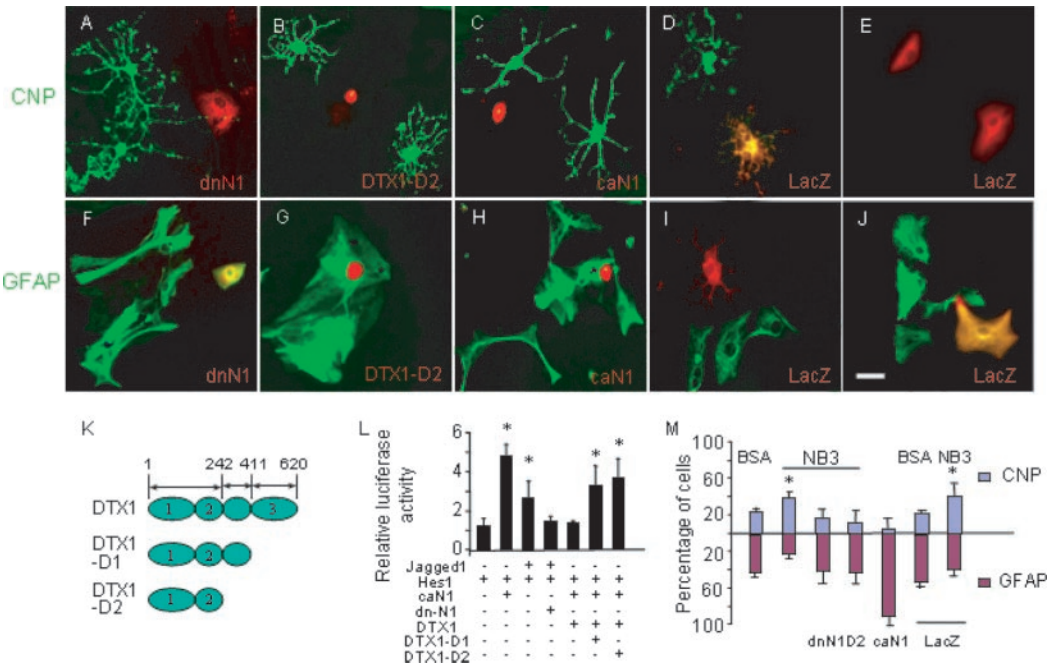

FIG. 5. **NB-3/Notch interaction promotes oligodendrogliogenesis from NPCs via DTX1.** NPCs were transfected with dn-N1 (*A* and *F*), DTX1-D2 (*B* and *G*) followed by NB-3 (12.5 nM) treatment, or caN1 transfection (*C* and *H*). As a control vector, pcDNA4/LacZ-V5 was introduced into NPCs, which were then treated with NB-3 (*D* and *I*) or BSA (*E* and *J*). Cells were double-stained afterward for appropriate tags and CNPase (*A–E*) or GFAP (*F–J*). *K*, schematic structure of DTX1 and its two deletion mutants. Numbers *1*, *2*, and *3* correspond to N-terminal, praline-rich region, and Ring-H2 finger motif, respectively. The validity of the constructs utilized here was confirmed in Hes1 luciferase reporter assays (*L*). *Bars* are mean ± S.D. *, $p < 0.01$ compared with Hes1 single transfection in Student's t test. The percents of transfected cells that were positive for CNPase or GFAP were counted (*M*). Data are mean ± S.D. *, $p < 0.01$ compared with BSA in Student's t test. *Scale bar* in *J* = 40 μm.

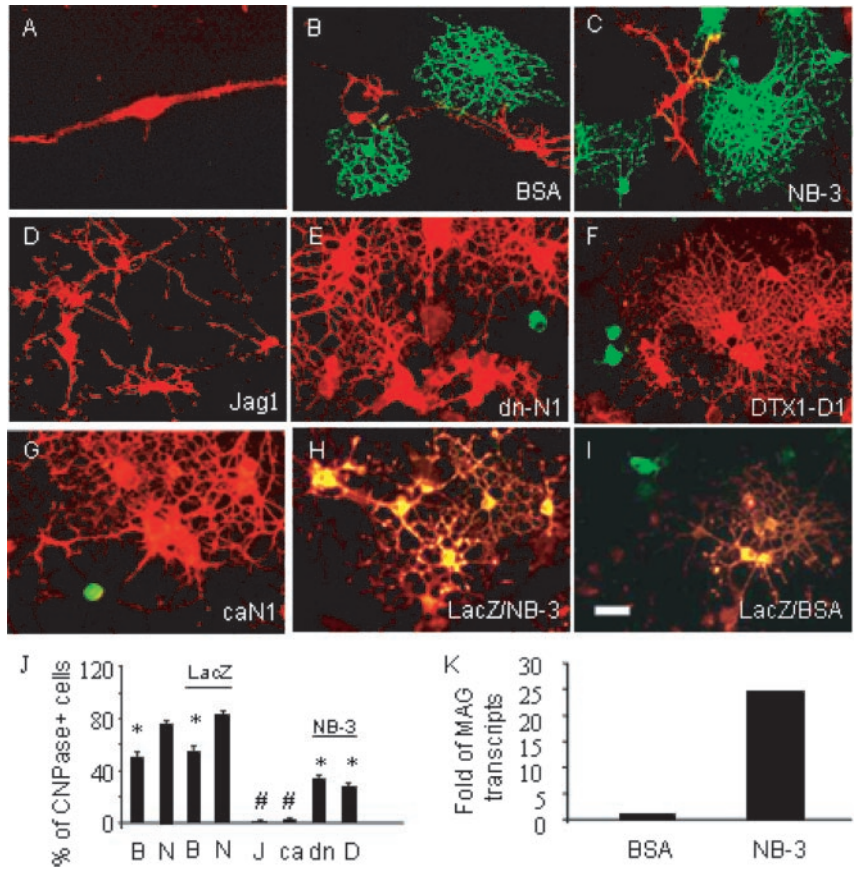

FIG. 6. **NB-3/Notch signaling promotes OL maturation from OPCs.** Purified Ng2-positive OPCs from P7 rat optic nerve (*A*) were treated with BSA (*B*), NB-3 (*C*), or Jagged1 (*D*) for 2 days and double-labeled for Ng2 (*red*) and CNPase (*green*). Other cells were transfected with dn-N1 (*E*), DTX1-D1 followed by NB-3 stimulation (*F*), or caN1 alone (*G*). As a vector control, OPCs were transfected with pcDNA4/LacZ-V5 and treated with NB-3 (*H*) or BSA (*I*). Cells were then immunostained for tags (*green*) and CNPase (*red*). The percentage of CNPase-positive cells was counted (*J*). Data are mean ± S.E. *, $p < 0.01$, compared with BSA; #, $p < 0.001$, compared with BSA in Student's *t* test. *B*:,BSA; *N*, NB-3; *J*, Jagged1; *ca*, caN1; *dn*, dnN1; *D*, DTX1-D1. Isolated OLs from P5–P7 rat cerebella were treated with BSA or NB-3 and subjected to real time PCR analysis (*K*). The *scale bar* in *I* = 40 μm.

showed that, distinct from BSA and Jagged1, NB-3 promoted OL generation from progenitor cells. On the other hand, compared with BSA, NB-3 had little impact on neurogenesis, whereas Jagged1 inhibited neurogenesis, which is consistent with the function of the typical ligands of Notch (21). Flow cytometry confirmed these observations in that NB-3 induced a 2-fold increase in OL generated compared with BSA treatment, whereas Jagged1 inhibited OL development (Fig. 4*G*). Furthermore, by using other NPCs isolated from different parts of the brain, we obtained similar results in immunofluorescence assays (Table I). For example, in NPCs from the fetal striatum, NB-3 treatment results in 45.1 ± 16.3% CNPase-positive OLs, which is significantly higher than BSA (22.2 ± 17.5%) or Jagged1 (5.6 ± 1.8%) treatment. For the other three cell lines from the fetal hippocampus (37.5 ± 12.3%), cerebellum (36.5 ± 8.6%), and spinal cord (39.1 ± 11.3), the results are quite consistent with the notion that NB-3 treatment specifically favors OL generation from NPCs.

NB-3/Notch Signaling via DTX1 Is Involved in Oligodendrogliogenesis—To confirm the involvement of Notch1 in this event, the embryonic progenitor cells from the striatum were transfected with a V5-tagged dominant-negative Notch1 (dn-N1) (22) that lacks the intracellular domain but can still bind to extracellular ligand. Hes1 luciferase reporter assay showed that dn-N1 failed to respond to Jagged1 treatment to activate Hes1, compared with Jagged1 treatment of normal NPCs (Fig. 5*L*). Double labeling for V5 and CNPase or GFAP showed that the dysfunction of Notch1 by dn-N1 abolished NB-3-promoted oligodendrogenesis, while favoring astrocyte formation (Fig. 5, *A*, *F*, and *M*). To further investigate whether DTX1 participates in NB-3-induced OL formation, cells were transfected with HA-tagged DTX1-D1 (23) and FLAG-tagged DTX1-D2 (24) (Fig. 5, *B* and *G*). The Hes1 luciferase reporter assay confirmed that DTX1 inhibited Hes1 activation by caN1, whereas DTX1-D1 and DTX1-D2 restored Hes1 elevation by caN1 (Fig. 5*L*). After differentiation in the presence of NB-3, cells were immunostained for FLAG and CNPase or GFAP. The results showed that DTX1-D1- (not shown) and DTX1-D2-transfected cells failed to differentiate into OL after NB-3 stimulation but were directed to astrocytes (Fig. 5, *B*, *G*, and *M*). And consistent with a previous study (25) the introduction of caN1 into progenitor cells resulted in astrogliogenesis while inhibiting OL generation (Fig. 5, *C*, *H*, and *M*). pcDNA4/LacZ-V5 was used as a control vector, and the introduction of this vector into the progenitor cells did not affect the effects of NB-3 (Fig. 5, *D* and *I*) and BSA (Fig. 5, *E* and *J*) on oligodendrocyte generation and astrogliogenesis. These observations indicate that NB-3 promoted oligodendrogliogenesis via the Notch/DTX1 signaling pathway.

NB-3 Promotes OPC Differentiation into OLs via Notch/DTX1—To investigate whether the NB-3/Notch interaction is involved in OL maturation, purified OPCs from P7 Wistar rat optic nerve (Fig. 6*A*) were treated with BSA, NB-3, or Jagged1 for 2 days and then immunostained for Ng2 (*red*), a progenitor marker, and CNPase (*green*), a young OL marker (Fig. 6, *B–D*). Statistical counting showed that NB-3 promoted OPC differentiation into CNPase-positive OLs (~75%), compared with BSA (~50%) or Jagged1 (~0%) treatment (Fig. 6*J*). To confirm the involvement of Notch1/DTX1 signaling in NB-3-promoted OPC differentiation, OPCs were transfected with dn-N1 (Fig. 6*E*) or DTX1-D1 (Fig. 6*F*) and treated simultaneously with NB-3. Double labeling for tags (*green*) and CNPase (*red*) showed that introduction of either construct significantly blocked NB-3-promoted OPC differentiation into OLs (Fig. 6*J*). And consistent with a previous study (26), caN1-transfected OPCs remained undifferentiated (Fig. 6, *G* and *J*). As a vector control, pcDNA4/LacZ-V5 was introduced into OPCs but did not affect either NB-3- or BSA-induced OPC differentiation progress (Fig.

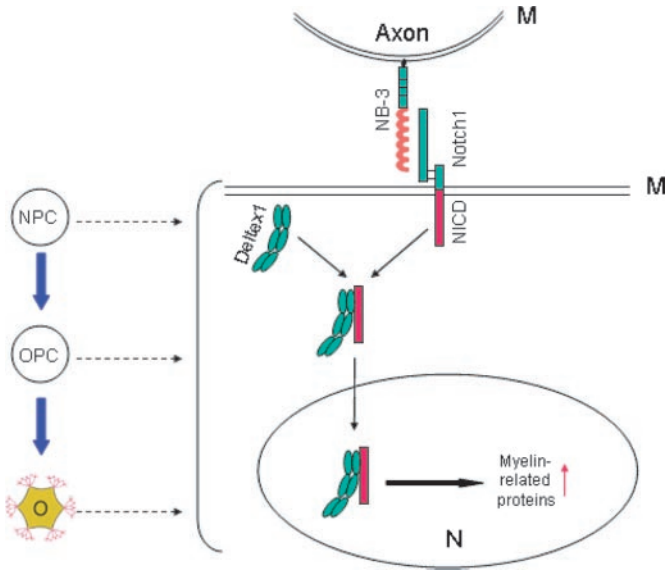

FIG. 7. **The putative model.** The extracellular NB-3/Notch interaction releases NICD from the membrane, which recruits DTX1 and translocates into the nucleus where the complex mediates the expression of myelin-related proteins directly or indirectly, such as CNPase and MAG, thus promoting oligodendrogliogenesis and oligodendrocyte maturation. *O*, oligodendrocyte; *M*, membrane; *N*, nucleus.

6, *H*, *I*, and *J*). In primary OLs, we analyzed the mRNA levels of MAG, a hallmark of OL maturation, by real time PCR. The analysis showed that NB-3 treatment increased MAG mRNA ~24-fold compared with BSA treatment (Fig. 6*K*). These results indicated that NB-3/Notch is also involved in OPC maturation.

DISCUSSION

We have shown that NB-3, a neuronal molecule, is a functional ligand of Notch1. NB-3 activation of Notch releases NICD at the S3 site via RIP and triggers the Notch/DTX1 signaling pathway, thus promoting oligodendrogliogenesis from NPCs and OPCs and OL maturation by up-regulating myelin-related proteins in young OLs (Fig. 7).

A recent study (27) shows that conditional ablation of Notch1 in OPCs results in the appearance of ectopic premature OLs and subsequent apoptosis. Moreover, in multiple sclerosis, the effort of remyelination is partly thwarted by the inflammation-induced Jagged1 expression by astrocytes that surround OPCs (28). These observations indicate that the spontaneous development of OPCs could be disordered when Notch1 is absent or inadequately activated by Jagged1, and other instructive pathways via Notch, besides the inhibitory Jagged1/Notch signaling pathway, may contribute to correct OPC differentiation into OLs. This notion is supported by our recent findings that the F3/Notch signaling pathway promotes OL maturation (3) and by the present study involving NB-3.

In the NB-3 null mice, the cerebellum-related motor coordination is impaired (29), and in F3/contactin mutants, severe ataxic phenotype consistencies with defects in the cerebellum (30) are present, indicating that both NB-3 and F3/contactin contribute to cerebellar development. Notably, in the striatum, hippocampus, cerebellum and spinal cord, the NB-3 protein level was higher than F3 from embryonic day 17 to postnatal day 14. And observable F3 expression generally started from around birth or even later (Fig. 1*A*). Thus NB-3 appears to function under physiological conditions from an earlier stage than F3 does, especially in embryonic days and may play a predominant specific role in some parts of the brain. Thus NB-3/Notch signaling may function coordinately with F3/Notch either spatially (different regions of the brain) or temporally (embryonic or postnatal stage). Of course, future work is needed to justify the differential usage of NB-3/Notch and F3/Notch *in vivo*.

Increasing evidence indicates that OL differentiation is mediated by neuron derived signals, such as DSL and F3/contactin (3, 4, 31). In a similar fashion to F3/contactin (3), NB-3 binds to N1.3, a region containing EGF repeats 22–34, whereas DSL binds to EGF repeats 11–12. As a result of the binding, NICD is released and translocated into the nucleus, a well established feature of Notch activation. The blockage of this phenomenon by the EGF antibody confirms that Notch is cleaved by NB-3 binding. This event can also be prevented by γ-secretase inhibitor, implying the involvement of presenilin-dependent γ-secretase in NB-3/Notch interaction. Furthermore, NB-3-induced NICD production is abolished in two S3 mutants, V1744K and V1744L, indicating that NB-3 binding triggers RIP at the typical S3 site. In summary, these observations have demonstrated that NB-3, a F3/contactin homologue, and Notch are functional binding partners.

NPCs possess the capability to generate all types of neural cells when given the proper molecular cues (2). One mechanism that is involved in fate selection is the lateral inhibition, exemplified by the typical Notch signaling (32). Cells expressing classical ligands DSL signal to apposing Notch-expressing cells to suppress the differentiation of these cells. Our recent finding that F3/contactin acts as a functional Notch ligand to promote OL generation from OPCs and OL maturation suggests the multiple roles that Notch may play during OL development (3). However, the exact scenario from NPCs to OLs remains obscure. Here, we have demonstrated that NB-3, as a neuronal cue, promotes OLs differentiation from both NPCs and OPC. Blockage of the NB-3-induced oligodendrogenesis by DTX1-D1 and DTX1-D2 indicates that one Notch signaling component, DTX1, participates in NB-3/Notch signaling pathway. Thus, NB-3 may activate the Notch receptor and release NICD, which then interacts with and recruits transcription factors, such as DTX1, other than the typical factor RBP-J. This process may start even earlier than the OPC stage; in other words, it may start from progenitor cell stage. To support this notion, we have demonstrated that NB-3 and Notch1 are distributed in the striatum, hippocampus, cerebellum, brain stem, and spinal cord from E17 suggesting that NB-3/Notch interaction is expected to occur widely in the central nervous system spatiotemporally.

Multiple sclerosis is a chronic demyelinating disease of the central nervous system. Transplantation of NPCs is a promising treatment for such diseases (28, 33). It will be of significance to establish a reliable method for inducing the selective differentiation of appropriate donor progenitor cells to OPCs or OLs. Thus, this study involving NB-3/Notch interaction is of clinical significance, because it is desirable that one be able to preferentially activate NB-3/Notch signaling in endogenous or exogenous NPCs to treat multiple sclerosis.

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NB-3/Notch1 Pathway via Deltex1 Promotes Neural Progenitor Cell Differentiation into Oligodendrocytes

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