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Sialidase NEU4 Hydrolyzes Polysialic Acids of Neural Cell Adhesion Molecules and Negatively Regulates Neurite Formation by Hippocampal Neurons[S]

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Background: Despite crucial roles of polysialic acid (polySia) in neural functions, the enzyme involved in degradation of polysialic acid in its physiological turnover remains uncertain.
Results: Sialidase NEU4 catalytically degrades polySia and negatively regulates neurite outgrowth of hippocampal neurons.
Conclusion: Sialidase NEU4 is probably the major degradation enzyme for polySia in vertebrate.
Significance: The findings contribute to elucidation of the physiological turnover of polySia.

Modulation of levels of polysialic acid (polySia), a sialic acid polymer, predominantly associated with the neural cell adhesion molecule (NCAM), influences neural functions, including synaptic plasticity, neurite growth, and cell migration. Biosynthesis of polySia depends on two polysialyltransferases ST8SiaII and ST8SiaIV in vertebrate. However, the enzyme involved in degradation of polySia in its physiological turnover remains uncertain. In the present study, we identified and characterized a murine sialidase NEU4 that catalytically degrades polySia. Murine NEU4, dominantly expressed in the brain, was found to efficiently hydrolyze oligoSia and polySia chains as substrates in sialidase *in vitro* assays, and also NCAM-Fc chimera as well as endogenous NCAM in tissue homogenates of postnatal mouse brain as assessed by immunoblotting with anti-polySia antibodies. Degradation of polySia by NEU4 was also evident in neuroblastoma Neuro2a cells that were co-transfected with *Neu4* and *ST8SiaIV* genes. Furthermore, in mouse embryonic hippocampal primary neurons, the endogenously expressed NEU4 was found to decrease during the neuronal differentiation. Interestingly, GFP- or FLAG-tagged NEU4 was partially co-localized with polySia in neurites and significantly suppressed their outgrowth, whereas silencing of NEU4 showed the acceleration together with an increase in polySia expression. These results suggest that NEU4 is involved in regulation of neuronal function by polySia degradation in mammals.

Carbohydrate portions of glycoproteins and glycolipids are thought to play important roles in the development and maintenance of the nervous system (1). Polysialic acid (polySia)[2] is a homopolymer of α2,8-linked sialic acid, found almost exclusively associated with post-translational modification of the neural cell adhesion molecule (NCAM). Because of its polyanionic structure and large hydrated volume (2, 3), modulation of polySia levels influences a wide range of structural changes in cell position and shape in the nervous system. Numerous studies have provided evidence for crucial functions of polySia on NCAM (4, 5), especially with observation of NCAM and/or polysialyltransferase-deficient mice (6–10). In fact, polySia has been implicated in synaptic plasticity, neuronal differentiation, and cell migration (1, 11, 12). PolySia levels are high during embryonic development, whereas its expression in the adult is restricted to brain regions featuring persistent neural plasticity or neural generation, such as the hippocampus (13) and olfactory bulb neurons. For the biosynthesis of polySia in vertebrate, two polysialyltransferases, ST8SiaII (STX) and ST8SiaIV (PST), have been identified and well characterized (14–16). However, it is still unclear how polySia-NCAM is actually degraded physiologically and whether endogenous enzyme exists in vertebrate, although bacteriophage-derived endosialidase, specific for α2,8-linked sialylpolymer and essential for bacterial infection to penetrate the polySia capsule of the host, has been often used experimentally (17, 18).

Exo-sialidases catalyze the removal of sialic acid from nonreducing ends of glycoproteins and glycolipids. In mammals, four types have been identified (abbreviated to NEU1, NEU2, NEU3, and NEU4), differing in chromosomal location, subcellular localization and enzymatic properties (19, 20). Recent progress in sialidase research has provided compelling evidence that mammalian sialidases play crucial roles in various physiological functions, including cell differentiation, and growth, as

[S] This article contains supplemental Table S1.

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[2] The abbreviations used are: polySia, polysialic acid; NCAM, neural cell adhesion molecule; 4MU-NeuAc, 4-methylumbelliferyl neuraminic acid; TBA, thiobarbituric acid.

well as malignant transformation. Of the four types, NEU3 preferentially hydrolyzes gangliosides other than GM1 and GM2, and is up-regulated during neuronal differentiation with involvement in acceleration of neurite formation (21–24). In contrast, NEU4 possessing two isoforms, both catalyzing removal of sialic acid from oligosaccharides and glycoproteins as well as gangliosides, is likely to behave during neural differentiation in a way opposite to NEU3 (25). In the mouse, NEU4 is expressed predominantly in the brain (25, 26). We previously demonstrated that the levels are relatively low in the embryonic stage, rapidly increasing at 3–14 days after birth, while NEU3 shows high levels in embryonic stages and post-partum down-regulation. In the adult mouse brain, *in situ* hybridization exhibited NEU4 to be mainly present in the hippocampus, in which polySia-NCAM is rich and decreases after birth (25). In the present study, building on our previous findings, we investigated whether murine sialidases, especially NEU4, catalyze the degradation of polySia, and found that NEU4 efficiently hydrolyzes polySia-NCAM and negatively regulates neurite outgrowth of hippocampal neurons.

EXPERIMENTAL PROCEDURES

Cells and Materials—Mouse neuroblastoma Neuro2a cells (ATCC) and HEK293T cells (a gift from Dr. M. Sugai, Kyoto University School of Medicine) were maintained in Dulbecco's modified Eagle's medium with 10% heat-inactivated fatal bovine serum. Human neuroblastoma NB-1 cells (HSRRB, Osaka, Japan) were grown in 45% RPMI 1640 and 45% Eagle's medium with 10% heat-inactivated fatal bovine serum. Sialyloligomers with a degree of polymerization of 2 (DP2) and DP6 were obtained from Nacalai Tesque, (Kyoto, Japan), and DP18 oligomers were prepared as described previously (4). Antibodies were obtained from the following sources: anti-polySia (12F8) and anti-NCAM (N-CAM 13) and anti-calnexin (37/calnexin) from BD Bioscience (San Jose, CA), anti-polySia (2–2B) from Chemicon International (Hants, UK), anti-NCAM (H-300) and anti-Lamp-1 (C-20) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-FLAG (M2) and (FLA-1) from Sigma and MBL (Nagoya, Japan), respectively, anti-58K Golgi protein from Abcam (Cambridge, MA), and anti-TUJ1 (1–15-79) from Covance (Princeton, NJ). Anti-polySia (12E3) antibody was a gift from Dr. Tatsunori Seki (Tokyo Medical University). ER tracker (Invitrogen, Carlsbad, CA) was used as marker for endoplasmic reticulum.

Construction of a Soluble Chimeric PolySia- NCAM-Fc—To obtain a cDNA for a secreted form of NCAM, first strand cDNA was synthesized with oligo dT primers by reverse transcription from total RNA prepared from neuroblastoma NB-1 cells. The PCR fragment coding for the extracellular domain of NCAM was then amplified with a set of primers 5′-CCGATATCGCTGCAGGTGGATATTGTTCCCAGC-3′ and 5′-CCGATATCCCGCCTGAGGTGGGGCTGCCGTTG-3′ according to the available sequence information (NM_000615.5). PCR products were digested with EcoRV, subcloned into the EcoRV site of pBluescript (Stratagene) and the cloned cDNA sequences were confirmed by sequencing. pFUSE-NCAM-Fc encoding the extracellular domain of NCAM fused with the Fc portion of human IgG was constructed by inserting NCAM cDNA into the EcoRV site of the expression vector pFUSE-hIgG-Fc2 vector (InvivoGen). An expression plasmid for *ST8SiaIV,* α2,8-sialyltransferase, was constructed by inserting the entire open reading frame of the human gene into the pcDNA3.1 vector. For preparation of polySia-NCAM-Fc, these plasmids were co-transfected into HEK293T cells followed by culture in serum-free medium for 24 h. The medium was then collected and polySia-NCAM-Fc was purified with a protein A-Sepharose column.

Sialidase Activity—For sialidase enzymatic studies, HEK293T cells were transiently transfected with the respective expression plasmids for murine sialidase genes *Neu2* (21), *Neu3* (21), and two splicing isoforms *Neu4a* and *Neu4b* (25) and for rat *Neu1* gene (27), previously prepared. For NEU1 activity, the protective protein gene (*pp11*) was sometimes co-transfected (28). Two days after transfection, the cells were sonicated in a homogenizing buffer (phosphate buffered saline, pH 7.4, containing 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, and 0.5 μg/ml pepstatin), centrifuged at 1000 × *g* for 10 min, and the resulting supernatants (homogenates) were used for sialidase activity assays with 4MU-NeuAc (4-methylumbelliferyl *N*-acetylneuraminic acid) and ganglioside GM3 as substrates. Released 4-methylumbelliferone (4MU) and sialic acids were measured by fluorescence spectrophotometry and HPLC, respectively (29). Protein concentrations were estimated by dye-binding assay (Bio-Rad). According to the substrate preference, 4MU-NeuAc was used for NEU1, NEU2, and NEU4, and GM3 for NEU3 in sialidase activity assays. One unit of activity was defined as the amount of enzyme cleaving 1 nmol of sialic acid in 1 h. For assessing activity toward oligo- and polysialic acids, the reaction mixture contained 8 μg/ml substrate, 0.8 mg/ml BSA, 100 mM sodium acetate (pH 4.6, 5.5) or MES buffer (pH 6.5) and the same units of enzymes measured in advance toward 4MU-NeuAc or ganglioside GM3 in 50 μl. After incubation at 37 °C for 30 min, cleavage of α2,8-linkages was estimated by quantification of the reducing termini released using assays of amounted of thiobarbituric acid (TBA)-reactive products converted to nmol of free reducing ends. Values were corrected for the substrate-associated reducing ends determined without enzyme.

Fluorometric HPLC Analysis of DMB-derivatized PolySia—To analyze the chain length and the amount of polySia in enzyme reaction products, the DMB-HPLC method (30) was employed. Reaction mixture contained 100 μg/ml colominic acid (Nacalai Tesque), 100 mM sodium acetate (pH 4.6), 0.05% Triton X-100, and enzyme in 50 μl. After incubation at 37 °C, an equal volume of a solution of 40 mM trifluoroacetic acid containing 7 mM DMB, 18 mM $Na_2S_2O_4$ and 1.0 M 2-mercaptoethanol the solution was added, and then the mixture was kept at 4 °C for 24 h. After DMB derivatization, a one-quarter volume of 1.0 M NaOH was added to neutralize the solution, and insoluble material was removed by centrifugation. Released polySia chains were separated with a Shimazu HPLC system on a DNAPac PA-100 column (Dionex) with elution of a convex gradient of ammonium acetate (0–2 M; pH 8.0) at a flow rate of 1 ml/min. At 0, 5, 15, 20, 35, 55, 145, and 182.5 min, the concentrations of ammonium acetate were 0, 0, 20, 25, 32.5, 40, 62.5, and 100% (2.0 M), respectively. The elution profile

for DMB-derivatized colominic acid was monitored by fluorescence (excitation, 373 nm; emission, 448 nm).

Immunoblotting—Enzymatic degradation of polySia-NCAM was estimated by immunoblotting. Brains isolated from mice (C57BL/6J) on postnatal day 4 were homogenized in lysis buffer (the homogenizing buffer containing 1% Triton X-100), and after ultracentrifugation (100,000 × *g*, 60 min), the supernatants were used as substrate. Reaction mixtures contained polySia-NCAM-Fc or lysates of mouse brain (P4), 100 mM sodium acetate (pH 4.6, pH 5.5) or 100 mM MES (pH 6.5), 0.05% Triton X-100 and the same units of sialidases. Reaction products were dissolved in Laemmli buffer and placed at 65 °C for 15 min. Proteins separated by SDS-PAGE were then blotted, incubated with primary antibodies, and detected using an ECL detection kit (Amersham Biosciences) with the Versa Doc imaging system (Bio-Rad).

Immunofluorescence—Neuronal cells were fixed with 4% (w/v) paraformaldehyde for 15 min, permeabilized with 0.1% (w/v) Triton X-100 for 15 min, blocked with 1% (w/v) bovine serum albumin, and treated with primary antibodies followed by appropriate secondary conjugated antibodies. The cells were then washed and mounted under Fluoromount/Plus (Diagnostic BioSystems) as an anti-fading reagent. Fluorescent images were obtained using an optical microscope (Axioskop 2 plus, Carl Zeiss) or a confocal laser-scanning microscope (FluoView FV1000, Olympus). For cell surface staining, immunolabeling was performed on living cells. Briefly, after removal of medium, cultures were rinsed in PBS and incubated with primary antibodies for 1 h at 4 °C. Cells were then fixed with 4% paraformaldehyde and incubated with appropriate secondary antibodies.

Hippocampal Primary Culture—For primary cultures of neurons, hippocampi were dissected from fetal mice at 16 days gestation (embryonic day 16; E16) followed by treatment with 9 IU/ml of papain (31). Tissue fragments were resuspended and dissociated by pipetting in MEM supplemented with 10% FBS, 25 mM HEPES, 30 nM selenium, 100 μM L-serine, 3.9 mM glutamine, 500 μM sodium pyruvate, 100 μM putrescine, 11.1 mM glucose, 10 μg/ml gentamicin sulfate, and 0.1 mg/ml bovine serum albumin at final concentrations. Cells were plated on polyethylenimine-coated dishes or coverslips and after 1 h a serum-free medium was added. The serum-free medium was composed of Dulbecco's modified Eagle's medium supplemented with the reagents described above, and in addition, 10 μg/ml bovine insulin, 0.1 mg/ml human apo-transferrin, 20 nM progesterone, and B27 supplement instead of fetal bovine serum. After 24 h, the medium was replaced with fresh serum-free medium. Gene transfection was performed by nucleofection using a mouse neuron nucleofector kit (Amaxa) before the cells were plated.

Quantitative Reverse Transcription-PCR Analysis—The mRNA levels of the four endogenous mouse sialidases and two polysialyltransferases in hippocampal neuron were evaluated by quantitative RT-PCR (real-time PCR, Light Cycler, Roche Diagnostics) as described previously (25). Total RNA was isolated, and first-strand cDNA was synthesized by reverse transcription and used as a template. The primers for mouse four sialidases and two polysialyltransferases are listed in supplemental Table S1. To compare expression levels among sialidases, standard curves for respective sialidase cDNAs were generated by serial dilution of the pBluescript vector containing the gene encoding entire open reading frame as described earlier (32). For the polysialyltransferases, comparative evaluation was performed as described (33). To normalize for sample variation, expression of glyceraldehyde-3-phosphate dehydrogenase was determined as an internal control.

Transfection and RNA Interference—Neuro2a cells were transfected with expression plasmids for sialidases and ST8SiaIV at the ratio of 10:1 using Effectene (Qiagen). Hippocampal neurons were transfected with *Neu4* gene by a mouse neuron nucleofector kit (Amaxa Biosystems) before the cells were plated, according to manufacturer's instructions. For *Neu4* silencing, the neurons were co-transfected with 25 pmol of mouse NEU4 siRNA (L-055343-01, Darmacon, Lafayette) or control siRNA (siGENOME non-targeting siRNA #2, Dharmacon) in combination with 2 μg of GFP-plasmid (Amaxa Biosystems). siRNAs were assumed to be transfected with GFP-plasmid (34).

For quantification of polySia intensity, neurons were immunostained with polySia antibody (2–2B) at 2 days *in vitro* culture. The GFP-positive cells were randomly imaged by FV1000. Images of 50 cells were generated for each culture. All the parameters were kept constant for each session of measurements. The peak intensity measured over individual neurons was averaged for each culture.

RESULTS

To investigate how polySia-NCAM is degraded physiologically and whether endogenous enzyme exists in mammals, hydrolyzing activity toward oligo- and polysialic acid was determined using mouse four sialidases. We first performed sialidase activity assays with α2,8-linked sialic acid compounds with various of degrees of polymerization-2 (DP2), -6, and -18 as substrates using homogenates of sialidase gene-transfected cells, containing almost equal amounts of sialidase activity toward 4MU-NeuAc or GM3, as enzyme sources. After incubation, cleavage of α2,8-linkages was measured by quantification of released reducing termini using a TBA method (Fig. 1*A*). Among the four sialidases, NEU4, especially isoform NEU4b, gave the highest amount of TBA-reactive products with DP2, DP6, and DP18 substrates (7.35 ± 0.41, 5.44 ± 0.55, and 4.61 ± 0.14 nmol/h, respectively). Isoform NEU4a also yielded significant products (1.25 ± 0.10, 0.94 ± 0.05, and 1.46 ± 0.11 nmol/h, respectively). On the other hand, the other three sialidases generated only limited products even with high amounts of enzyme and in the presence of Triton X-100 (data not shown). NEU1 seemed to slightly increase the product up to 3–5% of that of NEU4b with DP2 and DP6, when the sialidase gene was co-transfected with the protective protein (carboxypeptidase A) *pp11* gene, known to be an essential factor for sialidase activity together with β-galactosidase in the case of the human NEU1 enzyme. It should be noted here that unlike the case with the human NEU1 enzyme, *pp11* co-transfection resulted in only a slight increase in sialidase activity even toward 4MU-NeuAc (120% of the activity without *pp11*), consistent with our previous report of less dependence of protective protein for the rat NEU1 enzyme (27). These results suggest

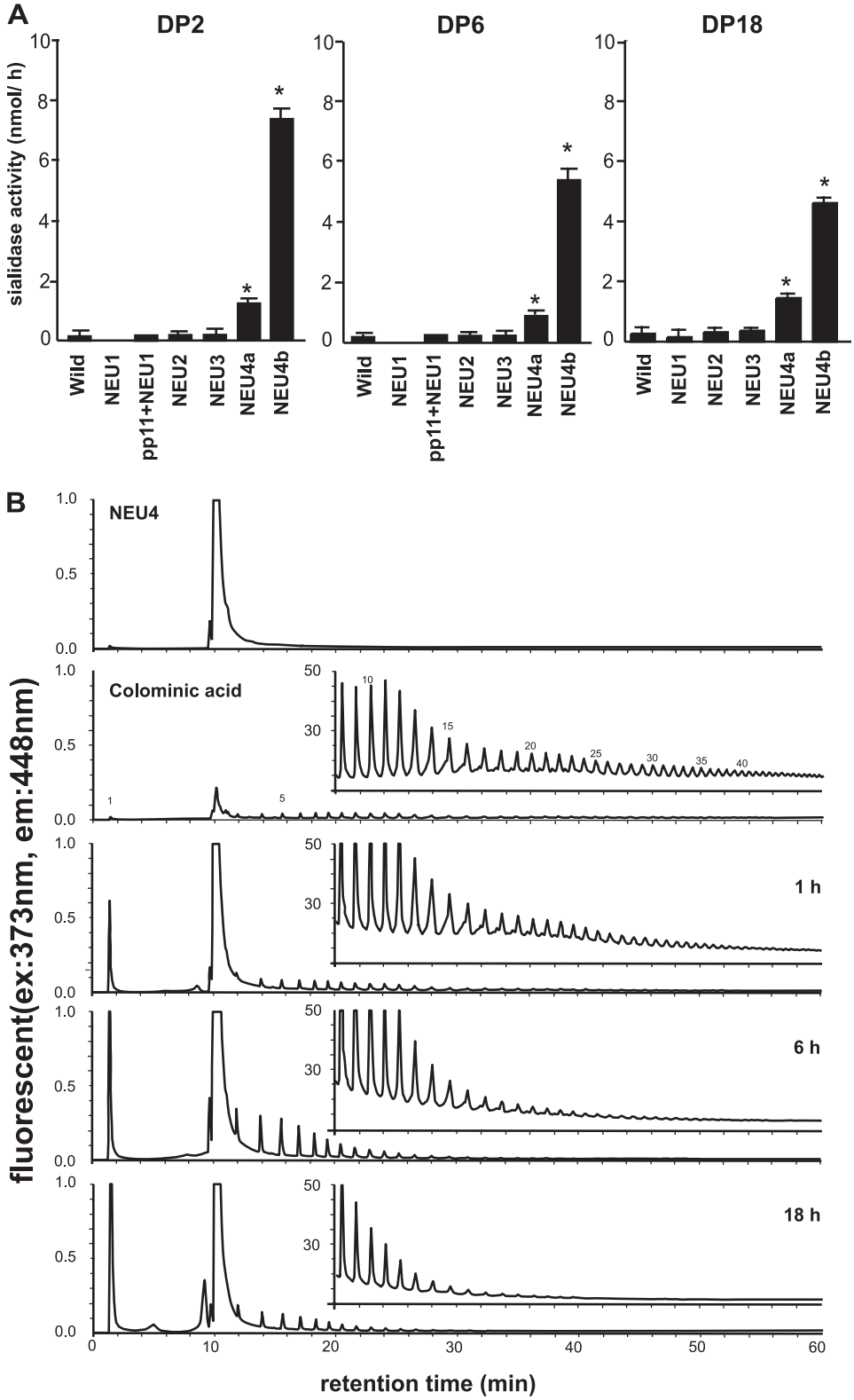

FIGURE 1. **Determination of hydrolyzing activity toward α2, 8-linked oligo- and polysialic acid using four mammalian sialidases.** *A*, activity was assayed with α2,8-linked sialic acid compounds with polymerization degrees of DP2, -6, and -18 as substrates using homogenates of sialidase gene-transfected cells containing almost equal amounts of sialidase activity toward 4MU-NeuAc or GM3 as the enzyme sources. After incubation, cleavage of α2,8-linkages was measured by a TBA method. Each value represents the mean $\pm$ S.D. of three independent experiments performed in triplicates. *, $p <$ 0.02. *B*, after incubation of colominic acid with Neu4b for the indicated time points, the products were analyzed by HPLC as described under "Experimental Procedures." *Numbers* above *peaks* indicate the DP of oligo- and polySia. The *insets* in each *panel* show a magnification of higher DP values, and the elution profiles are drawn at the same time scale. Products with DP (30 <) gradually decreased, concomitant with generation of DP1, but DP3 to DP6 products did not accumulate.

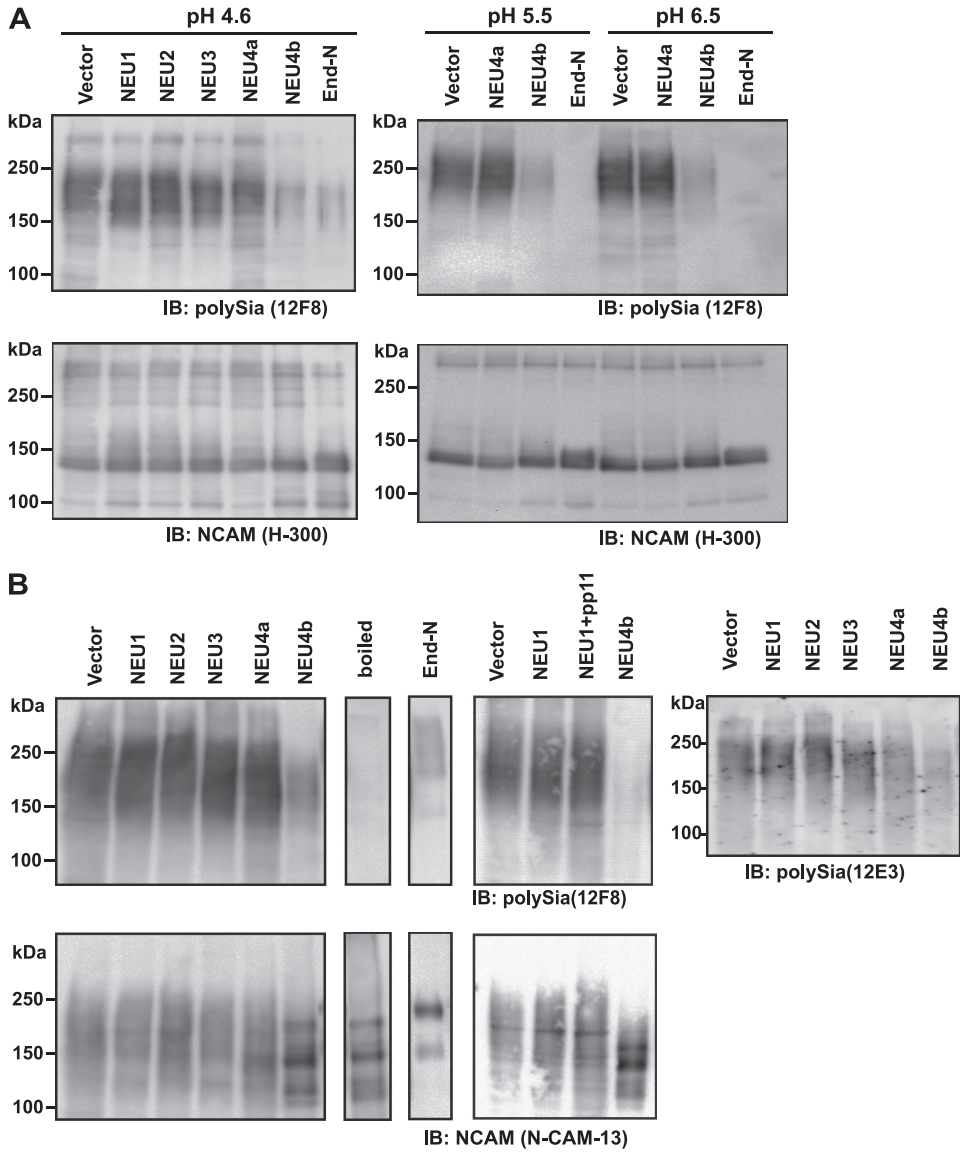

FIGURE 2. **Determination of hydrolyzing activity toward polySia-NCAM in an *in vitro* assay system.** *A*, hydrolyzing activity was assessed with polySia-NCAM-Fc as a substrate at acidic and nearly neutral pH, and after incubation, changes in polySia levels were estimated by immunoblotting with anti-polySia antibodies (12F8). NCAM was detected with anti-NCAM antibodies (H-300). *B*, hydrolyzing activity was estimated toward endogenous polySia-NCAM in lysate of postnatal mouse brain (P4) as substrate, assessed by immunoblotting with anti-polySia antibodies (12F8, 12E3) and anti-NCAM antibodies (N-CAM-13).

that NEU4 reacts on sialylpolymers and possesses exo-sialidase activity, although the possibility of endo-sialidase activity still remained. To characterize the manner of catalysis of NEU4b toward α2,8-sialylpolymers, the DP of the reaction products was analyzed by HPLC. Products having high DP (>30) gradually decreased, concomitant with increased DP1 (Fig. 1*B*), without sharp peaks not less than DP2 during incubation. These results indicate that NEU4 possesses exo-sialidase activity toward oligo- and polysialic acids *in vitro*, suggesting the possibility that the sialidase is an enzyme catalyzing cleavage of α2,8-linkage homopolymers in mammals.

The polySia moiety is predominantly associated with NCAM in vertebrates. To examine sialidase reactions on polySia-NCAM, we constructed an expression plasmid for NCAM-Fc chimera and transiently introduced the plasmid into HEK293T cells with co-transfection of the *ST8SiaIV* gene. The secreted polySia-NCAM-Fc was purified from the culture medium and was incubated with sialidases in the assay mixture. Degradation of the polySia-NCAM was assessed by immunoblotting with anti-polySia and anti-NCAM antibodies (Fig. 2*A*). Under the experimental conditions, NEU1, NEU2, and NEU3 hardly degraded the polySia-NCAM-Fc. As expected from the results for sialylpolymers, however, the NEU4b isoform clearly hydrolyzed polySia associated NCAM-Fc to a similar extent as endo-neuraminidase (endo-N) used as a positive control. Although the NEU4a isoform also significantly hydrolyzed sialylpolymers with the TBA method, only limited activity was observed with polySia-NCAM-Fc. Regarding effects of pH conditions on the hydrolyzing activity, NEU4b activity with polySia-NCAM-Fc was detected at acidic, and even at near neutral pH, similarly to the activity with 4MU-NeuAc at 4.4–4.6 and also significantly at pH 6.5. To further examine whether endogenous polySia-NCAM can be degraded by sialidases, homogenates of mouse postnatal brain tissues were used as substrates and changes in

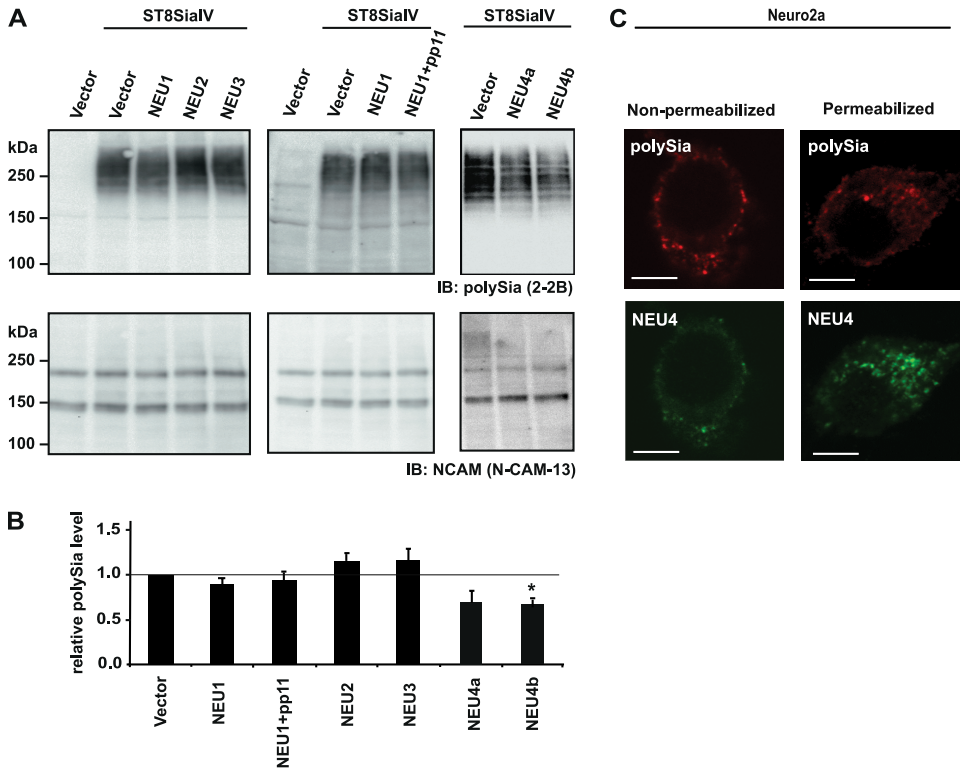

FIGURE 3. **Determination of hydrolyzing activity toward polySia-NCAM at the cellular level.** Neuro2a cells were co-transfected with *ST8SiaIV*- and sialidase-genes, and after 48 h, changes in cellular polySia levels and NCAM were determined by immunoblotting with anti-polySia (2–2B) and anti-NCAM (N-CAM-13) antibodies, respectively. Representative immunoblots are shown (*A*), and quantification of polySia levels relative to observed in the vector with ST8SiaIV transfectants was determined (*B*). Data present means ± S.E. $n = 3$. *, $p < 0.05$. After transfection of FLAG-*Neu4* and *ST8SiaIV* genes into Neuro2a cells, at 48 h of transfection, the cells were stained with anti-FLAG and anti-polySia antibodies under permeabilized or non-permeabilized condition (*C*) as described in the "Experimental Procedures." Scale bar, 50 μm.

polySia levels were assessed by immunoblotting with anti-polySia (12F8) and anti-NCAM (N-CAM-13) antibodies (Fig. 2*B*). Consistent with the data for polySia-NCAM-Fc chimera, NEU1, NEU2, NEU3, and the NEU4a isoform hardly degraded endogenous polySia on NCAM. Co-transfection of *Neu1* with *pp11* showed again no significant effect on polySia on NCAM from mouse postnatal brain tissues. On the other hand, polySia was considerably decreased by NEU4b as confirmed by immunoblotting with another anti-polySia antibody (12E3), which recognizes polySia with DP ≥5 (35, 36). Unlike the case with the anti-polySia (12F8) antibody, NEU4a showed a significant decrease with this antibody (12E3) (Fig. 2*B*, *right panel*), although we do not know the reason why the former antibody did not recognize the hydrolysis since there is no detailed epitope information available. Immunoblotting with anti-NCAM exhibited the protein bands of three isoforms (180, 140, and 120 kDa) after hydrolysis by NEU4b, similar to those with boiled polySia-NCAM that was almost completely non-polysialylated. These results indicate that NEU4 acts on endogenous polySia linked to three isoforms of NCAM in our *in vitro* assay system.

To obtain further evidence of *in vitro* hydrolysis, we examined whether sialidase hydrolyzes polySia-NCAM in cellular level on co-transfection of sialidase and *ST8SiaIV* genes into neuroblastoma Neuro2a cells expressing NCAM. After 48 h, polySia degradation was analyzed by immunoblotting with anti-polySia antibody (2–2B) recognizing polySia with DP ≥4 (36). Similar to the studies in sialidase assay system, NEU1, NEU2, and NEU3 gave no significant changes in polySia expression level, whereas NEU4b and even NEU4a caused considerable decrease (Fig. 3, *A* and *B*). These results together suggest that NEU4 possesses the highest activity toward polySia in the sialidase assay system applied as well as in cellular transfection studies.

We previously demonstrated that the human NEU4 short form, NEU4S, apparently present in almost all intracellular membranes including endoplasmic reticulum, but also partially in plasma membranes desialylating cell surface glycans such as sialyl-Le^a and sialyl-Le^x (37). To clarify in which cellular site murine NEU4b acts on the polySia, the subcellular localization studies were performed by immunofluorescence experiments of Neuro2a cells transfected with FLAG-NEU4b and ST8SiaIV. Co-localization studies with organelle markers including anti-LAMP-1 for lysosome, ER-Tracker, and calnexin for ER, and anti-58K Golgi protein for Golgi did not show clear results, and ~20–30% of NEU4b seemed to co-localize with calnexin and ER-Tracker (data not shown). In addition, interestingly, a part of NEU4b was found to be at the cell surface where polySia was detected under non-permeabilized condition (Fig. 3*C*). These results suggest that the NEU4b is likely to act on polySia at least partly at the cell surface.

To obtain insights into the functional significance of polySia hydrolysis by NEU4, we investigated involvement of the sialidase in development of mouse embryonic hippocampal neuron in cellular level, since our recent observations with *in situ* hybridization revealed NEU4 mRNA to be present mainly in

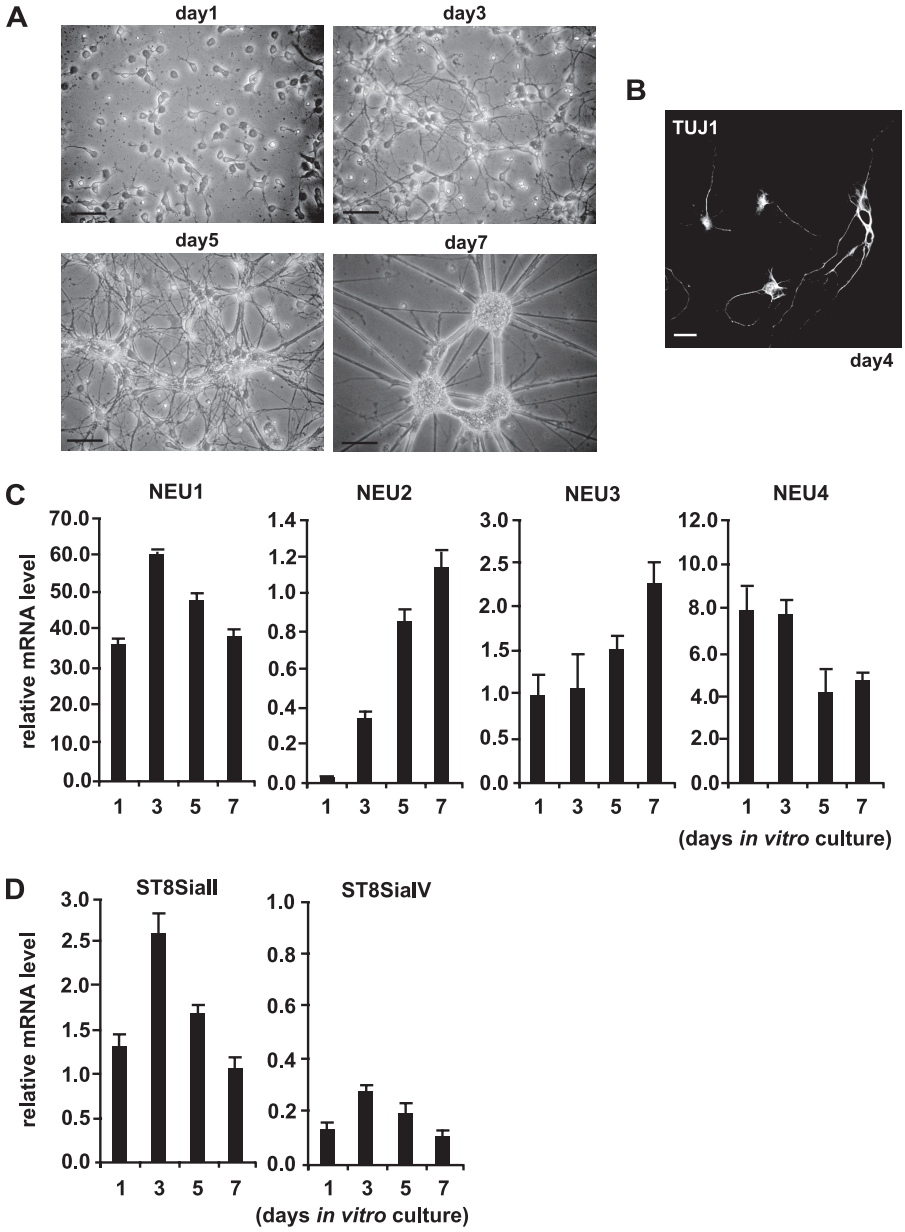

FIGURE 4. **Endogenous expression levels of four murine sialidases and two polysialyltransferases, ST8SiaII and ST8SiaIV, during differentiation of hippocampal culture neurons.** Primary neurons were isolated from fetal mice at 16 days gestation and cultured on polyethylenimine-coated dishes for 1–7 days (*A*) as described in the "Experimental Procedures." Hippocampal neurons were confirmed by immunostaining with neuronal marker, TUJ1 (*B*). Sialidase (*C*) and polysialyltransferase (*D*) mRNA levels were assessed by quantitative RT-PCR. The data given are mean values of two independent experiments performed in duplicates ± S.D. Scale bar, 50 μm (*A*) or 20 μm (*B*)

the hippocampus (25) where polySia-NCAM is expressed. When we prepared mouse embryonic hippocampal primary neurons (Fig. 4*A*), confirmed by immunostaining with neuronal marker, TUJ-1 (Fig. 4*B*), NEU4 showed a tendency of decreased expression during *in vitro* culture, and in contrast, NEU3 displayed increased expression (Fig. 4*C*), conforming our previous finding that NEU4 expression was down-regulated and NEU3 up-regulated during retinoic acid-induced neuronal differentiation of Neuro2a cells (25). The expression of NEU1 was about 7-fold that of NEU4 and increased after 3 days then declined, while NEU2 showed a remarkable increase during culture, even with a low level as compared with other sialidases. It is interesting that NEU2 may participate in neuronal differentiation, as previously reported (38), in the way of other than polySia degradation. With regard to the two polysialyltransferases, their expression was high after 3 days and then decreased, although ST8SiaIV expression was considerably lower than that of ST8SiaII. It has been shown that polysialyltransferase expression parallels polySia levels in many CNS regions (39), and both hippocampal tissue and cultured cells show a similar pattern, high at early postnatal stages or days *in vitro* and then down-regulated to minimal expression thereafter (40). In this context, although NEU4 expression did not show an inverse relation with physiological expression of polySia in this model during development, it is of interest that NEU4 still maintained in later days of culture nearly 60% of the level at early days, seemingly enough to degrade polySia.

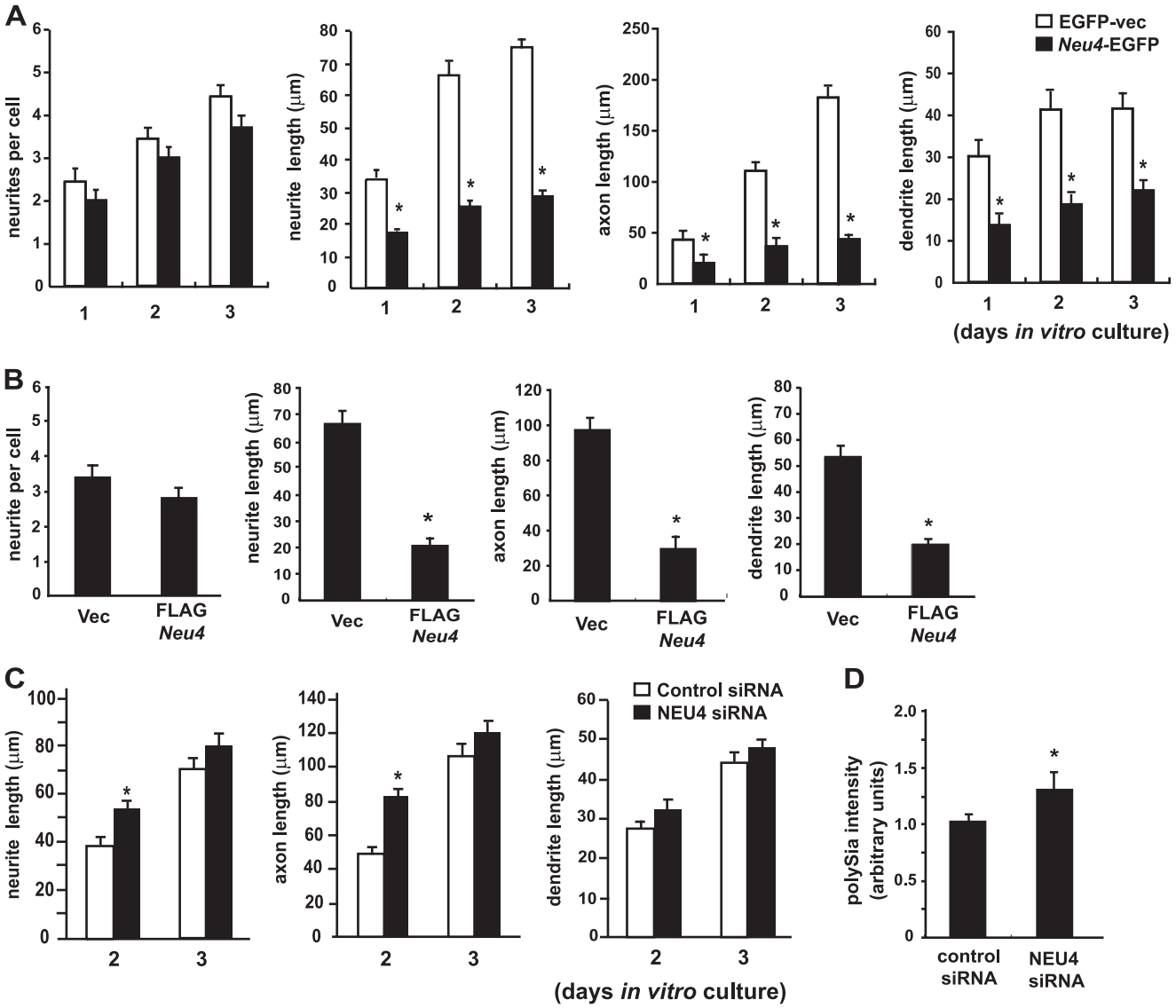

FIGURE 5. **Suppression of neurite outgrowth of hippocampal primary neuron by NEU4.** *Neu4*-EGFP (*A*), FLAG-*Neu4* (*B*) genes, and NEU4-siRNA together with GFP (*C*) were transfected into mouse hippocampal primary neurons by nucleofection using a mouse neuron nucleofector kit (Amaxa) before the cells were plated. At 1–3 days after gene transfection, cells bearing neurites were counted in culture (about 50 cells were evaluated per coverslips). Longest neurites were measured as axon, and other neurites as dendrite. *White bar*, EGFP-vector or control siRNA; *black bar*, *Neu4*-EGFP or NEU4-siRNA transfectants. The level of polySia expression was evaluated in hippocampal primary neuron transfected with NEU4-siRNA (*D*). Hippocampal neurons transfected with NEU4- or control siRNA together with GFP were fixed at 2 day *in vitro* culture, stained with polySia antibody (2–2B), and then measured the intensity by confocal microscopy (arbitrary units). All data represent mean ± S.E. *, $p < 0.05$. $n = 50$. Scale bar, 20 μm.

Next, we investigated the effect of NEU4 on neuronal function and the cellular site of action by introduction of EGFP- and FLAG-fused NEU4b. In *Neu4b*-transfected cells, significant suppression of neurite outgrowth occurred as compared with that in vector-transfected control cells, both in terms of the lengths of axons and dendrites, without affecting neurite number (Fig. 5, *A* and *B*). To verify these results, we also observed the effect of NEU4 on neurite outgrowth by its silencing. Neurons were co-transfected with GFP-plasmid and *Neu4*-targeting or control siRNAs. Two days after transfection, total NEU4 mRNA level was estimated by RT-PCR using β-actin as the internal control. The relative expression level with NEU4-siRNA was 74.1 ± 0.03% of that with controls, in line with transfection efficiency to be 27.5% for GFP-positive neurons. In contrast to *Neu4* overexpression, its silencing neurons showed enhancement of neurite outgrowth, especially in terms of axon length (Fig. 5*C*), and the quantitative analysis of polySia level demonstrated that NEU4-siRNA increases the polySia intensity of the neurons as compared with control siRNA (Fig. 5*D*). No significant difference on the intensity was observed between neurons transfected with control siRNA and non-transfected neurons (data not shown). These results together suggest that NEU4 regulates neurite outgrowth of hippocampal neuron, probably through degradation of polySia. We also examined the localization of NEU4b in the neurons. In the mouse hippocampal neuron, NEU4b-EGFP was found to be localized mainly in neuronal soma, co-localizing with ER-tracker as previously observed for Neuro2a (25), but interestingly, NEU4b was partially localized at the growth corns of neuronal cells in co-existence with polySia (Fig. 6*A*). Similar

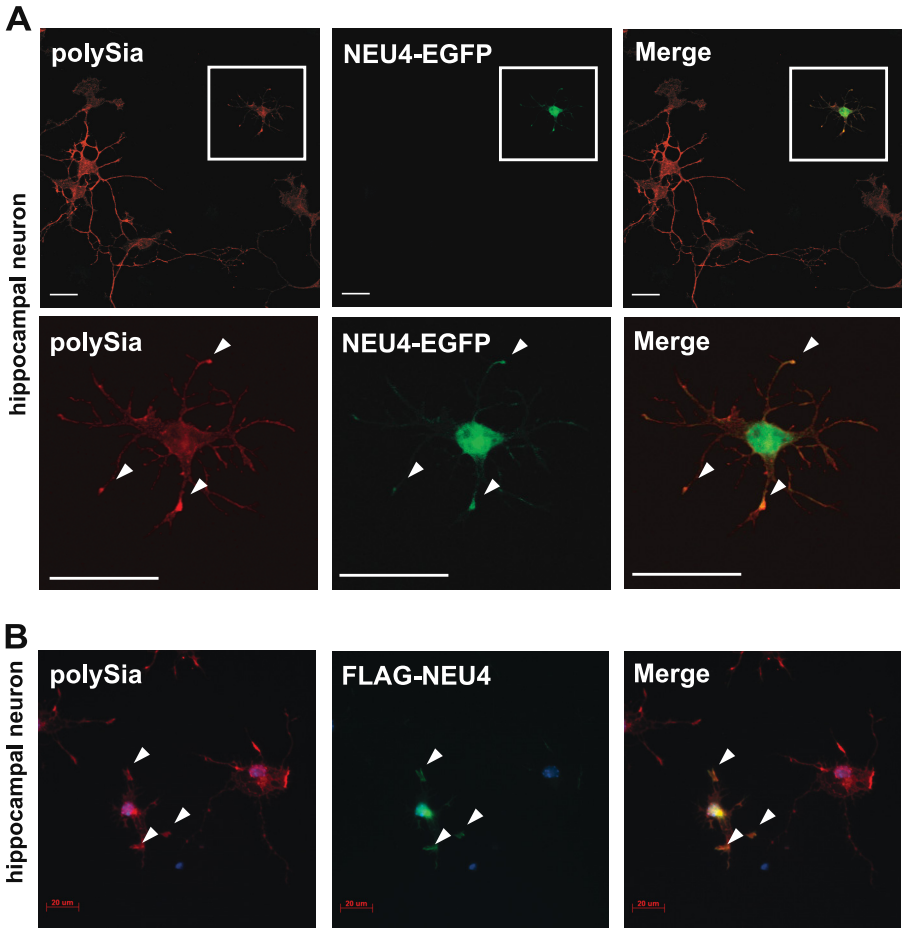

FIGURE 6. **Partial localization of NEU4 in the growth corns of neurons expressing polySia.** In mouse hippocampal neurons, at 3 days of transfection, NEU4-EGFP or FLAG-NEU4 and polySia were detected as fluorescent images as described in the "Experimental Procedures." Scale bar, 20 μm.

results were observed with FLAG-NEU4b by immunofluorescence staining with anti-FLAG antibodies (Fig. 6*B*). These results together suggest that NEU4b hydrolyzes polySia-NCAM at cell surface membranes and suppresses neurite outgrowth.

DISCUSSION

PolySia expression is known to be regulated by two polysialyltransferase, ST8SiaII and ST8SiaIV, responsible for the synthesis. The present study provided evidence that physiological degradation by NEU4 may also be important. Although numbers of studies on polySia functions using NCAM and/or polysialyltransferase-deficient mice and also bacteriophage-derived endosialidase have been reported, a physiological enzyme for polySia degradation has hitherto not been clearly identified. In mouse, sialidase NEU4 is dominantly expressed in the brain (25, 26), and whereas NEU4-deficient mice lack apparent pathological changes or behavioral deficits reflective of neuronal abnormalities, their ganglioside pattern in the brain features increased GD1a and decreased GM1 (41). In addition, mice with a double deficiency of NEU4 and β-hexosaminidase A exhibit epileptic seizures and rapid neuronal loss accompanied by GM2 accumulation (42). Although only a lack of NEU4 does not seem to apparently lead to abnormal expression of polySia-NCAM, the detailed observations on this point would be necessary in future. Our recent results indicated NEU4 expression to be relatively low in the embryonic stage, rapidly increasing at 3–14 days after birth with mRNA present mainly in the hippocampus (25), when polySia expression begins to decline (43). Based on these data, we hypothesized a functional role of NEU4 in brain development. Interestingly, we here could demonstrate that murine sialidase NEU4 efficiently catalyzed the hydrolysis of polysialic acid on NCAM *in vitro* while also negatively regulating outgrowth of hippocampal neurons. To our knowledge, this is the first clear evidence of polySia degradation by a sialidase in vertebrate.

Analysis of products of reactions, catalyzed by NEU4 with oligo- and polysialic acids as substrates, indicated probable action as an exo-enzyme (Fig. 1). As mammalian sialidases do not possess catalytic β-propeller domains, characteristic of endosialidases forming a homotrimeric molecule (44), it is feasible that NEU4 acts only as an exo-sialidase. NEU4 showed hydrolyzing activity toward polySia on NCAM *in vitro* under acidic conditions and also even near neutral pH. With regard to NEU4a, the isoform showed significant effects on polySia on NCAM from mouse postnatal brain tissues and also in cellular level in *ST8SiaIV*-transfected Neuro2a cells by immunoblotting with anti-polySia antibodies 12E3 and 2–2B, respectively (Figs. 2*B* and 3*A*). Furthermore, NEU4a exhibited low but sig-

nificant activity with α2,8-linked sialic acid compounds of DP2, DP6, and DP18, independent of the degree of polymerization, whereas NEU4b gave the much higher activity toward the substrates but the hydrolysis rate was decreased with higher degree of polymerization. As we previously reported (25), NEU4a hydrolyzes various substrates that NEU4b reacts on, although the hydrolytic rate was low. However, it is of interest that in comparison with NEU4b, the isoform shows more efficient hydrolysis of colominic acids, α2,8-linked sialic acid polymer, and that the expression level is higher in brain than in other tissues. These data together suggest that in addition to NEU4b, NEU4a may be a physiologically efficient enzyme for polySia degradation. NEU1 showed slight degradation of polySia in Neuro2a cells in one test (once in five separate experiments), independent of protective protein expression, although the results could not be confirmed in other *in vitro* analyses. However, it may not be excluded the possibility that NEU1 degrades polySia in cellular level together with some protein factors including β-galactosidase, as reported in facilitation of elastic fiber assembly (45). Compared with NEU4 levels, endogenous expression of NEU1 is very high in a number of tissues, including brain (25), where it was found to be ~7-fold higher (Fig. 4). Nevertheless, NEU4 hydrolyzed 3–4-fold more efficiently than NEU1 as assessed by TBA method (Fig. 1*A*). Thus, we conclude that NEU4 is probably the major degradation enzyme for polySia.

It has already been reported that polysialylation of NCAM plays a key role in neurite growth, cell migration and synaptic plasticity via enhancing flexibility of cell-cell and cell-substratum interactions (6, 7). Franceschini *et al.* (46) reported introduction of ST8SiaII to facilitate neurite growth, and Brocco *et al.* showed ST8SiaII silencing to cause opposite effect, probably due to premature NCAM interactions (47). Furthermore, recently, polySia-NCAM detected on microglia, the brain resident innate immune cells, has been suggested to participate in alleviation of neurotoxicity through function of Siglec 11 that binds to polySia on NCAM (48). From this point of view, it is feasible that NEU4 may play a role on immune function in microglia, in consistent with the observations by Seyrantepe *et al.* (41) that mouse NEU4 was expressed in scattered cells found in all regions of the brain with a distribution similar to that of microglia cells. Our results are generally in line with these findings. However, during neuronal development, endogenous NEU4 expression did not show an inverse correlation with expression of polysialyltransferases, but remained high when ST8SiaII was down-regulated in our cell model. Importantly, *Neu4* overexpression significantly shortened axonal and dendrite length in transfected hippocampal neurons, while being co-localized with polySia at the neurite, and *Neu4* knock down resulted in the opposite. Our results thus provide compelling evidence indicating that NEU4 may participate in regulation of expression of polySia in mammals.

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