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Restoration of FcRγ/Fyn Signaling Repairs Central Nervous System Demyelination

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Disruption of myelin causes severe neurological diseases. An understanding of the mechanisms that control myelination and remyelination is needed to develop therapeutic strategies for demyelinating diseases such as multiple sclerosis (MS). Our previous finding indicating the critical involvement of the γ chain of immunogloblin Fc receptors (FcRγ) and Fyn signaling in oligodendrocyte differentiaion and myelination demands a fundamental revision of the strategies used for MS therapy, because antigen–antibody complexes in MS patients may induce the direct dysregulation of myelination process as well as the inflammatory destruction of myelin sheath. Here we show that the FcRγ/Fyn signaling cascade is critically involved in cuprizone-induced demyelination/remyelination, with no lymphocytic response. The levels of phosphorylated myelin basic proteins (p-MBPs), especially the 21.5-kDa isoform, but not the levels of total MBPs, decreased markedly during demyelination induced by aging, cuprizone treatment, and double knockout of FcRγ/Fyn genes. We also showed that the recovery from demyelination in cuprizone-treated and aged mice is achieved after administration of the herbal medicine Ninjin'yoeito, an effective therapy targeting the FcRγ/Fyn—Rho (Rac1)—MAPK (P38 MAPK)—p-MBPs signaling cascade. These results suggest that the restoration of FcRγ/Fyn signaling represents a new approach for the treatment of demyelinating diseases.

Key words: myelin basic protein; cuprizone; aging; Rho protein; herbal medicine

Mutiple sclerosis (MS) is a demyelinating autoimmune disease in which cell-mediated and antibody-mediated immune responses are critically involved (Sospedra and Martin, 2005). Various immunomodulating therapies have been developed, but they lack sufficient efficacy. We have reported that cross-linking of the common γ-chain of immunoglobulin Fc receptors (FcRγ) triggers Fyn tyrosine kinase signaling, which plays a critical role in the differentiation of oligodendrocyte progenitor cells (OPCs) and the production of myelin basic proteins (MBPs; Nakahara et al., 2003). This finding may demand a fundamental reevaluation of the strategies used for MS therapy, insofar as antigen–antibody complexes produced during the immune response lead not only to the inflammatory destruction of myelin sheath but also, inevitably, to the direct dysregulation of the signaling pathways for oligodendrocyte differentiation and myelination. Although the reasons for the eventual failure of remyelination in MS are unknown, the presence of OPCs and immature oligodendrocytes in some nonrepairing lesions suggests that these cells fail to drive relevant differentiation signal machinery (Wolswijk, 1998; Chang et al., 2002; Back et al., 2005). Restoration of the signaling pathways for oligodendrocyte differentiation and/or myelination is thus emerging as a novel and potentially important therapeutic strategy for demyelinating diseases.

To elucidate the intracellular signaling cascade underlying the myelination process in CNS, we used, in addition to rodents with demyelination resulting from aging, a well known neurotoxin, cuprizone, which induces extensive corpus callosum demyelination without a lymphocytic cell response (Matsushima and Morell, 2001). C57BL/6 mice fed a diet containing 0.2% cuprizone show decreased expression of myelin-related genes, apoptosis of mature oligodendrocytes, accumulation of

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OPCs, and activation of microglia/macrophages, successively, and massive and synchronous demyelination occurs within 5 weeks (Mason et al., 2001). If the cuprizone challenge is terminated, an almost complete remyelination takes place within an additional 2 weeks. In this model, various endogenous factors, including tumor necrosis factor-α (Arnett et al., 2001), insulin-like growth factor-1 (Mason et al., 2000), major histocompatibility complex class II (Arnett et al., 2003), and basic helix-loop-helix transcription factor Olig1 (Arnett et al., 2004), have been shown to be required in the remyelination process. However, no remedial agent has been reported.

Ninjin'yoeito (NYT), a "Kampo" (Japanese traditional) medicine, which is the hot-water extract of a combination of 12 medicinal plants, has long been used for the treatment of various diseases, including dementia and MS in east Asia (Supplementary Table I and Supplementary Fig. 1). We have previously demonstrated that oral administration of NYT potently increases oligodendrocytes and OPCs in aged rat brain (Kobayashi et al., 2003). In the present study, we provide evidence that NYT significantly ameliorates aging- and cuprizone-induced demyelination via promoting remyelination. Furthermore, the combined use of NYT and FcRγ/Fyn doubly deficient (dKO) mice in the cuprizone model has enabled us to provide a detailed analysis of the signaling pathway triggered by FcRγ-Fyn in isolated myelin sheath. The present study addresses the possibility that restoration of the FcRγ-Fyn signaling pathway involved in myelination is a promising therapeutic strategy for the treatment of MS and other demyelinating diseases.

MATERIALS AND METHODS

Mice

Three- to thirty-one-month-old C57BL/6 mice were supplied by the Department of Animal Science of Tokyo Metropolitan Institute of Gerontology. The dKO mice were obtained by crossing FcRγ-deficient (Takai et al., 1994) and Fyn-deficient (Yagi et al., 1993) mice as described previously (Nakahara et al., 2003). All mice, including the mutants, were of the C57BL/6 background strain. All procedures used in this study were in accordance with our institutional animal care and use committee.

Induction of Demyelination by Cuprizone

Demyelination was induced in 8-week-old male C57BL/6J mice by 5 weeks of feeding with a diet containing 0.2% cuprizone (Sigma, St. Louis, MO). For remyelination, cuprizone-fed animals were switched to a normal diet for up to 2 additional weeks.

NYT Treatment

NYT, manufactured by Tsumura & Co. (Tokyo, Japan) as "TJ-108" under strict scientific and quality control and with approval for ethical use by the Ministry of Health, Labor, and Welfare of Japan, is a spray-dried extract of a mixture of 12 raw medicinal plants. The composition of plants and three-dimensional high-performance liquid chromatography profile of NYT are presented in Supplementary Table I and Supplementary Figure 1. In the cuprizone-induced demyelination model, NYT was mixed with the CE-2 diet (CLEA Japan, Tokyo, Japan) at a concentration of 1% and was fed throughout the experiments. In the aging-induced demyelination model, 1% NYT was administered in the drinking water for 2 months before sacrifice.

Immunohistochemistry

Brains were fixed overnight in an acid-alcohol solution (95% ethanol/5% acetic acid, v/v). Paraffin-embedded brains were sectioned into 10-μm-thick slices. The sections were mounted and stained with anti-MAG pAb (from Dr. Y. Matsuda, National Center for Neurology and Psychiatry, Japan) as primary antibody and subsequently with horseradish pereoxidase (HRP)-conjugated anti-rabbit IgG Ab (MBL, Nagoya, Japan). Diaminobenzidine (DAB) solution (Wako, Osaka, Japan) was used for visualization.

Electron Microscopy

The cerebrum was fixed with 2.5% glutaraldehyde and then postfixed with OsO_4. After dehydration in ethanol, specimens were embedded in Quetol 812 (Nisshin EM, Tokyo, Japan). Ultrathin sections stained with 2% uranyl acetate and leaded solution were observed with a JEOL 100C electron microscope (JEOL, Tokyo, Japan) as described elsewhere (Seiwa et al., 2000). For the G-ratio measurement, three to five mice per group were used. Microphotographs of the corpus callosum (coronal) at midline at high magnification (×5,000, ×10,000) were taken from each mouse, and the G-ratio (a ratio of the diameter of the axon to the diameter of the axon and surrounding myelin, Fig. 1C) was measured until the number of counted axons reached at least 50. Axons with aberrant morphology such as myelin reduplication, abnormal splitting of myelin sheath, vacuolization of myelin lamellae, and myelin balloon formation were excluded from G-ratio measurement, because the aberrant morphogenesis of myelin makes it impossible to evaluate the degree of myelination accurately. For example, the extraordinarily large myelin sheath shown in axons with abnormal morphology resulting from repetition of unaccomplished remyelination should not be estimated in the same manner as the sheath of normally developed myelinated axons. Another quantitative study was undertaken to assess the number of myelinated fibers per 400 μm^2 with three animals per group.

Preparation of Myelin

Myelin was prepared as described previously (Seiwa et al., 2000). Briefly, cerebrum (0.2 g) was homogenized with a Teflon homogenizer in 20 volumes (w/v) of 0.32 M sucrose, and the homogenate was layered over 0.85 M sucrose. After centrifugation at 25,000 rpm for 30 min, the layer of crude myelin formed at the interface of the two sucrose solutions was collected. The crude myelin layers were resuspended in water by homogenization and washed by repeated centrifu-

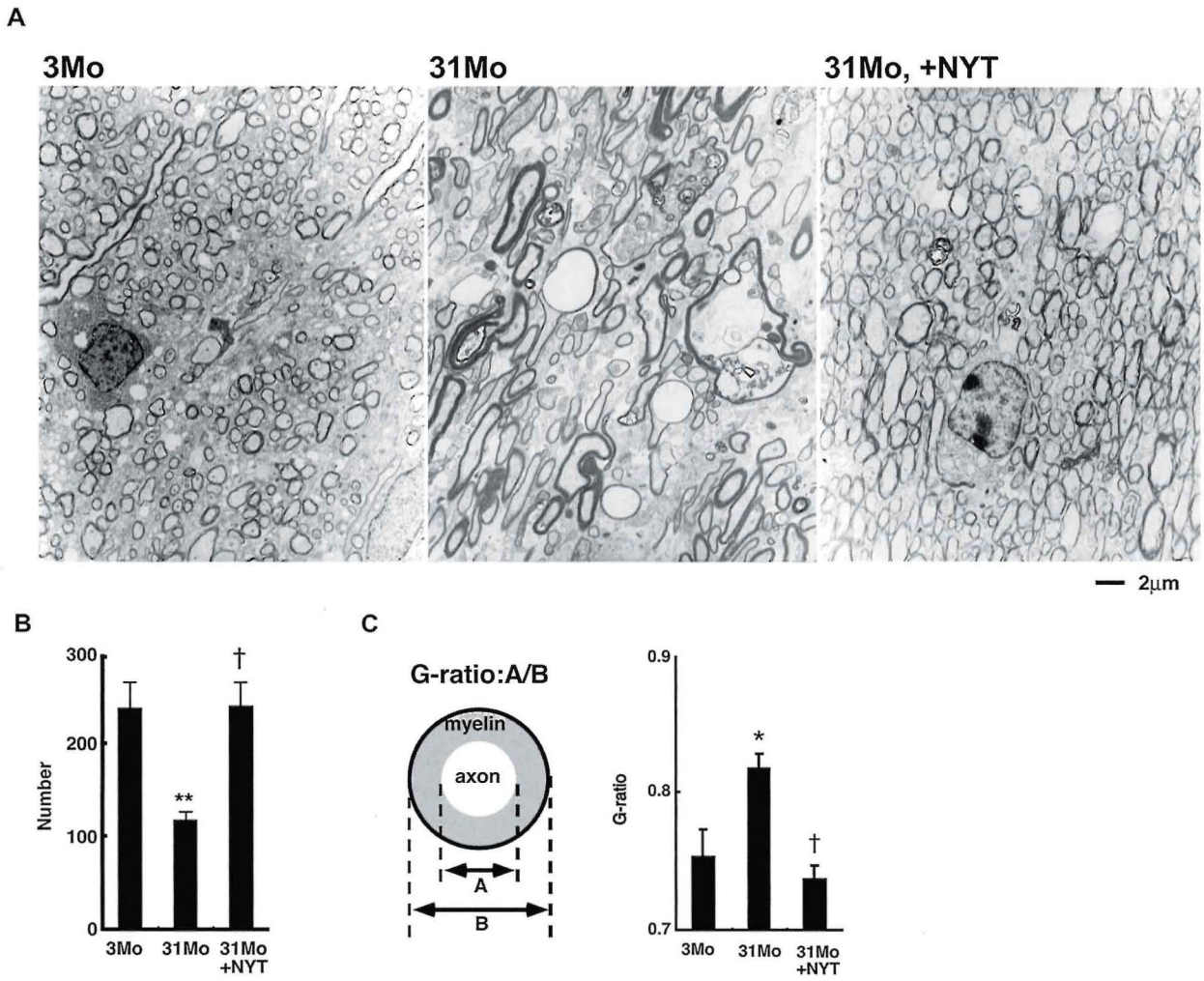

Fig. 1. Suppression of aging-induced demyelination by NYT. **A:** Electron micrographs of cross sections of the corpus callosum showed aging-induced demyelination in 31-month-old (designated as 31 Mo) mice compared with 3-month-old (3 Mo) mice. Myelination status was greatly improved after NYT treatment for 2 months (+NYT). **B:** The number of myelinated fibers per 400 μm^2 was lower in 31-month-old mice than in 3-month-old mice. NYT led to significant recovery of myelinated fiber density. Data represent mean ± SEM (n = 3). $^{**}P < 0.01$ vs. 3 Mo, $\dagger P < 0.01$, vs. 31 Mo. **C:** The G-ratio, an indicator of demyelination defined as the ratio of the diameter of axon to the diameter of the axon and the surrounding myelin (the formula is shown in the **inset**), shows that elevation of G-ratio in aged mice was abrogated by NYT treatment. Data represent mean ± SEM. $^{*}P < 0.05$ vs. 3 Mo, $\dagger P < 0.01$, vs. 31 Mo.

gation. The homogenate was again centrifuged on a 0.85 M sucrose bed, and the purified myelin was removed.

GeneChip Analysis

White matter was homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) with a Physcotron (NITI-ON, Chiba, Japan), and total RNA was isolated, labeled, and prepared for hybridization with Mouse Genome 430A 2.0 Array (Affymetrix, Santa Clara, CA) according to the recommended protocol. Details of data processing and statistical analysis are described in the footnote to Supplementary Table II.

Western Blot

Western blot analysis was performed as described previously (Seiwa et al., 2000). Membranes were probed with antibodies to FcRγ (Takai et al., 1994), Fyn (Dr. K. Senzaki National Institute for Physiological Sciences, Japan), v-Src (Calbiochem, San Diego, CA), p190 RhoGAP (Upstate Biotechnology, Charlottesville, VA; UB), p120 RasGAP (Santa Cruz Biotechnology, Santa Cruz, CA; SC), RhoGDI (SC), p115 RhoGEF (SC), RhoA (SC), RhoG (SC), Rac1 (Sigma), Cdc42 (BD Transduction Laboratories, Lexington, KY; BD), total and phosphorylated MAPKs (p38 MAPK, ERK1/2, JMK/SAPK; Cell Signaling Technology, Danvers, MA), PY20 (Calbiochem), MBP (Nichirei, Tokyo, Japan), phosphorylated MBP (UB), and actin (Sigma). Quantification of bands was performed by densitometry in ImageJ. The difference in the amount of loaded proteins among the lanes in a single experiment was normalized to the amount of actin loaded.

TABLE I. Top 20 Probe Sets Decreased by 3-Week Cuprizone Treatment*

Probe set ID	Definition	Gene ID	Fold change water/cup
1450483_at	**Gap junction membrane channel protein alpha 12**	118454	28.1
1418086_at	Protein phosphatase 1, regulatory (inhibitor) subunit 14A	68458	9.9
1417275_at	**Myelin and lymphocyte protein, T-cell differentiation protein**	17153	7.8
1448982_at	Protease, serine, 18	19144	7.2
1448768_at	**Myelin oligodendrocyte glycoprotein**	17441	5.0
1426960_a_at	Fatty acid 2-hydroxylase	338521	4.9
1460219_at	**Myelin-associated glycoprotein**	17136	4.8
1451961_a_at	**Myelin basic protein**	17196	4.8
1425467_a_at	**Proteolipid protein (myelin) 1**	18823	4.6
1432558_a_at	**Myelin and lymphocyte protein, T-cell differentiation protein**	17153	4.5
1433543_at	Anillin, actin binding protein (scraps homolog, *Drosophila*)	68743	4.5
1420968_at	Hyaluronan and proteoglycan link protein 2	73940	4.3
1418472_at	Aspartoacylase (aminoacylase) 2	11484	4.1
1419063_at	**UDP-glucuronosyltransferase 8**	22239	4.0
1416003_at	**Claudin 11**	18417	3.8
1451718_at	**Proteolipid protein (myelin) 1**	18823	3.7
1418406_at	Phosphodiesterase 8A	18584	3.6
1423946_at	PDZ and LIM domain 2	213019	3.6
1454651_x_at	**Myelin basic protein**	17196	3.5
1450088_a_at	**Myelin-associated oligodendrocytic basic protein**	17433	3.3

*Significant analysis of microarray (SAM; Tusher et al., 2001) detected 138 genes whose expression associated negatively with cuprizone treatment (median number of false significant = 10.09, delta = 1.95939, median of false discovery rate = 4.73). The genes were sorted by the order of -fold-change, and the top 20 genes are listed. Genes listed in boldface are known myelin sheath components. A more detailed analysis of genes that showed a decrease or increase in expression after 3-week cuprizone treatment and after 7 weeks (5-week cuprizone treatment and an additional 2 weeks for recovery) is available online as Supplementary Table II.

Rho Family GTPase Activities

Pull-down assay for Rho-GTPase were performed according to the affinity precipitation protocol (UB). In brief, myelin fractions were resuspended in ice-cold lysis buffer (UB) supplemented with complete protease inhibitor (Roche, Basel, Switzerland). Lysates were incubated with PAK-1 PBD agarose or GST-tagged Rhotekin Rho-binding domain bound to glutathione agarose. The beads were washed with lysis buffer and heated for 5 min at 100°C in reducing SDS-PAGE sample buffer, and the released proteins were electrophoresed and probed with anti-RhoA, anti-Rac1, and anti-Cdc42 antibodies.

Statistical Analysis

Morphometric data are shown as mean ± SEM. Differences among groups were analyzed by Student's *t*-test corrected using Bonferroni's method for multiple comparisons. $P < 0.05$ was considered significant. Microarray data were analyzed by SAM (Tusher et al., 2001) and Welch's *t*-test (see Tables I, II, Supplementary Table II). The statistical analysis of immunoblot data was performed by the closed testing procedure (Marcus et al., 1976), with Dunnett's multiple comparison test and Welch's *t*-test with Bonferroni's correction.

TABLE II. Top 10 Probe Sets Increased by NYT Treatment in the Cuprizone-Induced Demyelination Model (Week 3)*

Probeset ID	Definition	Gene ID	-Fold change NYT/control
1438403_s_at	Receptor (calcitonin) activity-modifying protein 2	54409	2.2
1452751_at	Early B-cell factor 3	73115	2.2
1460219_at	**Myelin-associated glycoprotein**	17136	2.2
1418517_at	Iroquois-related homeobox 3 (*Drosophila*)	16373	2.0
1417153_at	BTB (POZ) domain containing 14A	67991	2.0
1454651_x_at	**Myelin basic protein**	17196	2.0
1436201_x_at	**Myelin basic protein**	17196	1.9
1424567_at	Tetraspan 2	70747	1.8
1437341_x_at	**Cyclic nucleotide phosphodiesterase 1**	12799	1.8
1451718_at	**Proteolipid protein (myelin) 1**	18823	1.8

*Significant changes in gene expression were determined by Welch's *t*-test ($P < 0.05$).
The genes were sorted by the order of -fold change, and the top 10 genes are listed. Genes listed in boldface are known myelin sheath components and were decreased by 3-week cuprizone treatment. A more detailed analysis of the genes that showed a decrease or increase in expression resulting from NYT treatment with or without cuprizone feeding is available online as Supplementary Table II.

RESULTS

Recovery of Demyelination in NYT-Treated Mice and Lack of Cuprizone-Induced Demyelination/Remyelination in FcRγ-Fyn Doubly Deficient (dKO) Mice

The myelination status assessed from electron micrographs of young (3-month-old) and aged (31-month-old) mice are demonstrated in Figure 1A and Supplementary Figure 2. As previously reported (Knox et al., 1989), the brains of aged mice showed many abnormal findings in the myelin sheath, such as myelin balloon formation, splitting of myelin sheath, vacuolization of myelin lamellae, paranodal retraction, and abnormal tight junctions, which are rarely found in younger mice. Large, electron-dense inclusion bodies characteristic of dying axons, active phagocytic cells (macrophages), and axons with thinly myelinated fibers and active remyelination have also been noted. In the brains of mice treated with NYT (1% in drinking water) for 2 months, marked improvement of these abnormal histological findings was observed. The density of myelinated fibers per 400 μm^2 significantly decreased in 31-month-old mice and recovered as a result of NYT treatment (Fig. 1B). The G-ratio, an indicator of demyelination, defined as the ratio of the diameter of axon to the diameter of the axon and the surrounding myelin, was increased with age, but NYT treatment caused the ratio to recover to the same level as that of young animals (Fig. 1C).

We next examined demyelination in the cuprizone model by using methylene blue staining (data not shown), immunohistochemical staining by antimyelin associated glycoprotein (MAG) antibody (Fig. 2A), and electron microscopy (Fig. 2B) of the corpus callosum sections. Consistent with previous reports (Mason et al., 2001; Matsushima and Morell, 2001), myelinated axons decreased dramatically, and demyelination reached a maximal level after 5 weeks of cuprizone treatment. In FcRγ/Fyn double-knockout (dKO) mice, large areas of hypomyelination were observed even before cuprizone treatment (Supplementary Fig. 3); however a certain degree of myelination was still observed. As shown in Figure 2C, the number of myelinated axons in dKO mice is larger than that of 5-week-cuprizone-treated mice. Therefore, it is possible to evaluate whether cuprizone induces demyelination or not in dKO mice, both by quantitative and by histological means. As a result, no indication of further demyelination after cuprizone feeding (Fig. 2A,B), or remyelination after cuprizone removal (data not shown), was noted. Feeding of 1% NYT in the diet markedly ameliorated the demyelination in WT mice, but no change was observed in cuprizone-treated dKO mice (Fig. 2A,B). The density of myelinated fibers and G-ratio of NYT-treated mice recovered to near-normal levels, whereas those of dKO mice remained aberrant throughout the experimental period, with or without NYT administration (Fig. 2C,D). High-magnification electron micrographs from NYT-treated mice showed recruitment and accumulation of microglia/macrophages and phagocytosis of myelin debris, which have been postulated as prerequisites for the initiation of remyelination (Morell et al., 1998; Arnett et al., 2003; Arancibia-Carcamo et al., 2004), and the presence of many small myelinated axons, which are presumed to be newly remyelinated (Supplementary Fig. 4a–g). The amelioration of myelination status has been observed by NYT posttreatment for 3 days in which administration of NYT began after the establishment of demyelination by 5-week cuprizone treatment (unpublished observations). These results suggest that NYT promotes remyelination rather than preventing demyelination. In NYT-treated or untreated dKO mice, no histopathological change was shown.

To characterize further the effect of cuprizone and NYT, we analyzed gene expression with an oligonucleotide microarray, the Affymetrix GeneChip system (Tables I, II, Supplementary Table II-1–8). Consistent with previous reports (Morell et al., 1998; Jurevics et al., 2002; Arnett et al., 2003), the expression of various oligodendrocyte- and myelin-related genes markedly decreased at week 3 of cuprizone treatment, when very minimal demyelination was histologically observed. Among the top 20 probe sets whose expression was most decreased by cuprizone, 12 sets were for eight genes whose products constitute myelin sheath, including myelin and lymphocyte protein (MAL), myelin-oligodendrocyte-glycoprotein (MOG), MAG, MBP, proteolipid protein 1, and claudin 11 (Table I, Supplementary

Fig. 2. Suppression of cuprizone-induced demyelination by NYT. **A:** Immunohistochemistry of coronal sections of the cerebrum with anti-myelin-associated glycoprotein antibody (anti-MAG). White matter from the cingulate cortex of cuprizone-treated (+Cup), wild-type (WT) or FcRγ/Fyn doubly deficient (dKO) mice with NYT treatment (+NYT) at 0 and 5 weeks (0W, 5W) and controls are shown. Myelin stained with anti-MAG antibody forms the vertically oriented bundles of myelinated nerve fibers and was extensively decreased by 5-week cuprizone treatment. NYT recovered myelin. Myelin is present also in dKO mice but in lower amounts than in WT mice. Cuprizone treatment had no effect. cg, Cingulum; cc, corpus callosum. **B:** Electron microscopy performed on cross-sections of the corpus callosum. The results of cuprizone-treated (+Cup), wild-type (WT) or FcRγ/Fyn doubly deficient (dKO) mice at 0, 3, and 5 weeks (0W, 3W, 5W) were shown. Extensive demyelination was observed by week 5 of cuprizone treatment, and coadministration of NYT (+NYT) suppressed the demyelination in WT mice. Cuprizone and/or NYT treatment had no effect in dKO mice. **C:** The number of myelinated fibers per 400 μm^2 was lower in dKO mice and cuprizone-treated WT mice. NYT treatment led to significant recovery of myelinated fiber density in cuprizone-treated WT mice. Data represent the mean ± SEM (n = 3). $^{**}P < 0.01$ vs. 0W WT, $^{\dagger}P < 0.01$, vs. 5W WT. **D:** The G-ratio increased to nearly 1.0 (the value signifying complete demyelination) by week 5 of cuprizone treatment. The ratio in NYT-treated WT mice recovered to near-normal levels, whereas in dKO mice the levels remain high throughout the experimental period whether or not NYT was administered. Data represent mean ± SEM. $^{**}P < 0.01$ vs. 0W WT, $^{\dagger}P < 0.01$ vs. 5W WT. Scale bars = 112 μm.

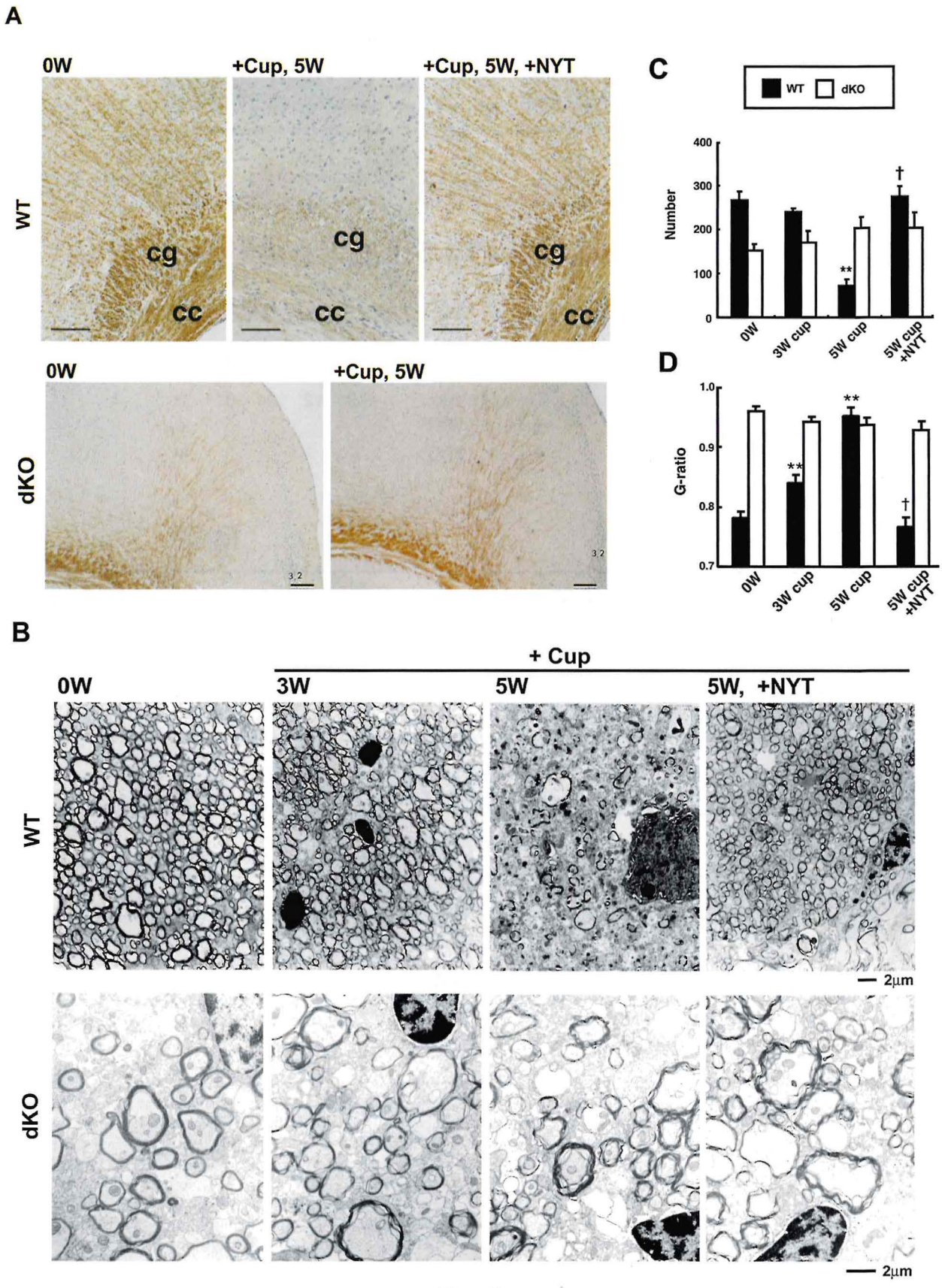
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Figure 2.

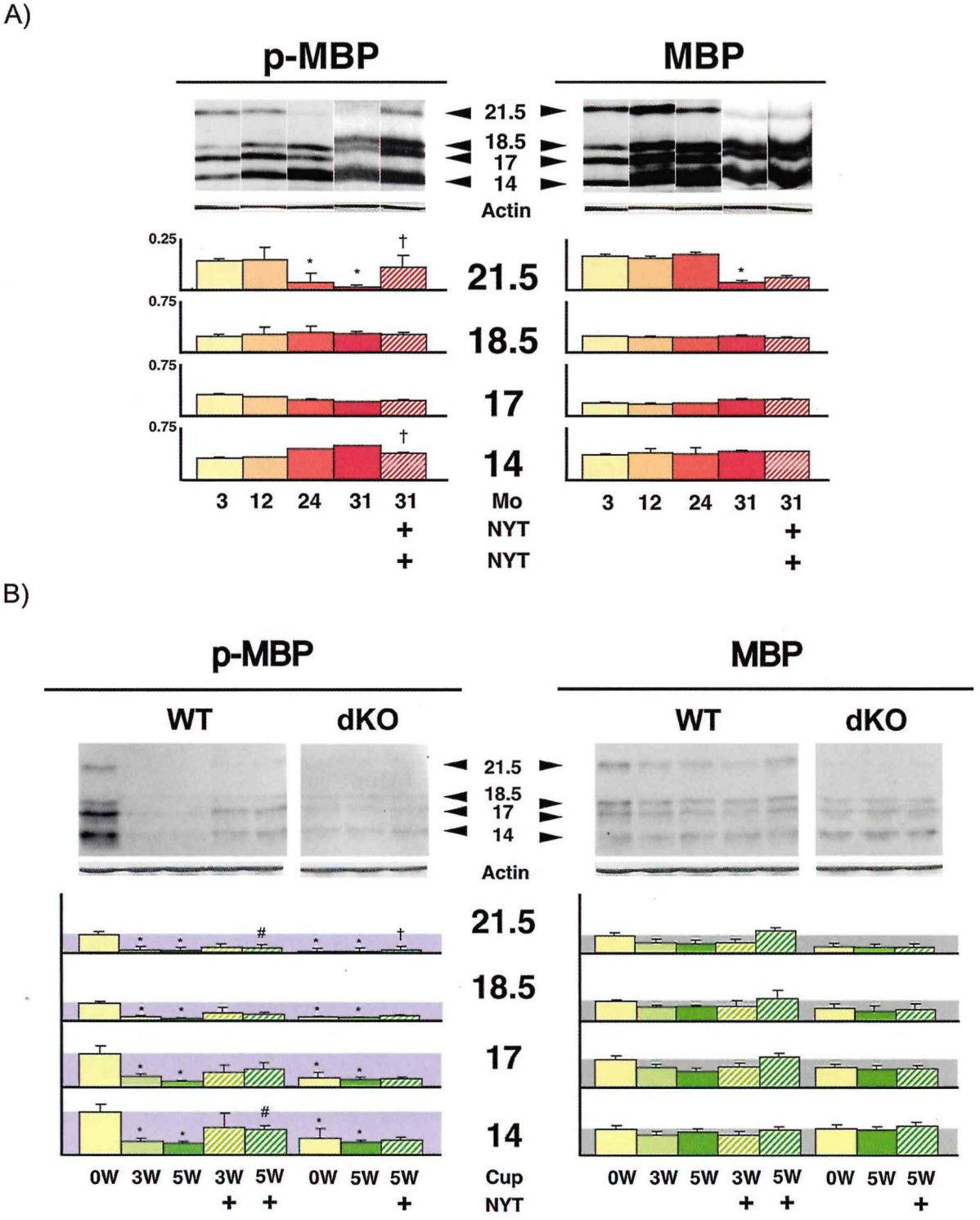


Fig. 3. 21.5-kDa phosphorylated MBP ("p-MBP") levels were closely related to myelination status. **A:** Aging-induced demyelination and its improvement by NYT were accompanied, respectively, by the specific disappearance and reappearance of 21.5-kDa p-MBP protein. The levels of the 21.5-, 18.5-, 17-, and 14-kDa isoforms (21.5, 18.5, 17, and 14) of total MBPs (MBP) and phosphorylated MBP (p-MBP) were quantitated by immunoblot analyses of isolated myelin sheaths. Because the basal levels of p-MBPs and MBPs fluctuated during aging, the quantitative ratios among four isoforms at each age are calculated. The results from 3-, 12-, 24-, and 31-month-old mice (3 Mo, 12 Mo, 24 Mo, 31 Mo) with NYT treatment (+NYT) and controls are shown. Quantitation and statistical analysis were performed as described in Materials and Methods. Data represent mean ± SEM (n = 3). $\star P < 0.05$ vs. 3 Mo, $^{\#}P < 0.05$ vs. 12 Mo, $\dagger P < 0.05$ vs. 31 Mo. **B:** Cuprizone-induced demyelination and recovery following NYT treatment were accompanied by a decrease and recovery of p-MBP isoforms. The immunoblot analyses were performed as described above. Thirty micrograms of protein was analyzed, and the MBP and p-MBP levels in wild-type (WT) and FcRγ/Fyn doubly deficient (dKO) mice after various treatment (cuprizone, +Cup; NYT+NYT) and time intervals (0, 3, 5 weeks, 0W, 3W, 5W, respectively) were quantitated as described in Materials and Methods. The levels at 0W of WT mice are indicated by the heights of purple-gray (for p-MBP) and gray (for MBP) zones in the graphs, respectively. Although cuprizone treatment and dKO did not induce significant change in the amount of total MBPs, the bands of p-MBPs, especially those of the 21.5-kDa isoform, markedly decreased in dKO mice and cuprizone-treated WT mice. NYT led to partial restoration of the amount of 21.5-kDa p-MBP. Data represent mean ± SEM (n = 3). $\star P < 0.05$ vs. 0W WT, $^{\#}P < 0.05$ vs. 5W WT, $\dagger P < 0.05$ vs. 5W dKO.

Table II-1). Among the top 10 probe sets whose expression was most increased by NYT at 3 weeks, five sets corresponded to myelin-related genes whose expression was substantially down-regulated by cuprizone (Table II, Supplementary Table II-5). These gene expression data were consistent with the ameliorative effect of NYT on demyelination.

Phosphorylated MBPs Are Closely Related to Myelination Status

A substantial number of MBPs (four isoforms translated from alternatively spliced transcripts) in CNS are phosphorylated under physiological conditions, and the phosphorylation state of MBPs has been assumed to regulate myelin sheath integrity (Boggs et al., 1997; Ridsdale et al., 1997; Kim et al., 2003), although the precise mechanism has not been determined. To investigate this area further, we analyzed phosphorylated and total MBPs by immunoblot analysis of myelin sheath isolated from the corpus callosum of young (3 months old), adult (12 monthsd old), and aged (24–31 months old) rats (data not shown) and mice (Fig. 3A). Although the brains of aged mice showed increased demyelination (Fig. 1A), significant decreases in total MBPs were not noted. In contrast, 21.5-kDa phosphorylated MBP (p-MBP) dramatically decreased in aged mice. In 31-month-old mice treated with NYT for 2 months, in which aging-induced demyelination was substantially improved, the level of 21.5-kDa p-MBP increased to the same level as that of 12-month-old mice.

Cuprizone treatment induced only a marginal decrease in the total amount of MBP (Fig. 3B) when normalized to the amount of actin protein, whereas the levels of p-MBP isoforms significantly decreased during the pre- and middemyelination period (3 and 5 weeks, respectively). In particular, the 21.5-kDa p-MBP band had disappeared almost completely at 5 weeks. Partial but significant recovery in the levels of the 21.5- and 14-kDa p-MBP isoforms was observed following NYT treatment at week 5 (Fig. 3B).

As demonstrated in Figure 2, naïve dKO mice exhibited severe hypomyelination, and myelination status did not change with cuprizone feeding or withdrawal. The amounts of total MBPs showed no significant decrease relative to untreated wild-type (WT) mice, although the levels of p-MBPs, especially those of the 21.5-kDa isoform, were markedly lower. Treatment with cuprizone and/or NYT had little effect on the levels of p-MBPs except for the 21.5-kDa isoform, which was slightly increased by NYT treatment at week 5. These data suggest that the amount of p-MBPs, especially of the 21.5-kDa isoform, essentially correlate with whether myelination/remyelination will proceed.

Investigation of the Signal Transduction Pathway Downstream of the FcRγ-Fyn Complex in the Myelin Sheath

The evident hypomyelination and unresponsiveness to cuprizone treatment in dKO mice strongly indicated that the FcRγ-Fyn signaling cascade is crucially involved in cuprizone-induced demyelination. To obtain the detailed understanding of the cascade, we investigated the molecular candidates involved in the cascade by using a combination of immunoblot, immunoprecipitation, and pull-down assays. Previous reports using in vitro cultures of OPCs and oligodendrocytes have suggested that Fyn regulates Rho GTPases, members of the Ras superfamily proteins that cycle between an active, GTP-bound state and an inactive, GDP-bound state. Three types of regulatory proteins that influence Rho activity by altering the balance between the amounts of GTP-bound vs. GDP-bound Rho proteins have been described. GTPase-activating proteins (GAPs), which promote GTP hydrolysis, and guanine nucleotide dissociation inhibitors (GDIs), which bind to GTPases, inactivate Rho GTPases. Guanine nucleotide exchange factors (GEFs) act as positive regulators by stimulating the exchange of GDP for GTP (Hakoshima et al., 2003). Rho proteins, such as RhoA, Cdc42, and Rac1, have been reported to play different roles in proliferation and several stages of differentiation of oligodendrocyte-lineage cells (Ishikawa et al., 2002; Niederost et al., 2002; Liang et al., 2004; Mi et al., 2005). It has been suggested that MBPs are phosphorylated by specific MAPKs (Erickson et al., 1990), and several MAPKs, including ERK1/2, JNK/stress-activated protein kinase (JNK/SAPK), and p38 MAPK, play important roles in various cellular processes of OPCs and oligodendrocytes (Fragoso et al., 2004).

Immunoblot analyses of FcRγ, Src family proteins (Fyn, v-Src), Rho regulators (p190 RhoGAP, p120 RasGAP, RhoGEF, RhoGDI), Rho GTPases (RhoA, RhoB, RhoE, RhoG, Cdc42, Rac1), and MAPK family proteins (ERK1/2, JNK/SAPK, p38 MAPK) revealed that all of these proteins tested might be present in the myelin sheath (data not shown). As expected, the decrease (or absence due to gene knockout) in the amount of Fyn seemed to occur in parallel with demyelination (Fig. 4A), whereas another Src family protein, v-Src, showed no change. FcRγ levels were not affected by cuprizone treatment but were significantly increased by NYT treatment (Fig. 4A). Among the tested proteins, the active forms of Rac1 and p38MAPK showed quantitative relationships with the degree of myelination status, i.e., the decrease in dKO and cuprizone-treated WT mice and recovery by NYT administration (Fig. 4C,D).

DISCUSSION

Cuprizone has been thought to inhibit mitochondrial function and to interfere with energy-generating mechanisms (Matsushima and Morell, 2001); however, the exact molecular mechanisms involved in demyelination and remyelination in the cuprizone model remained undetermined. The present study demonstrated that the Fyn cascade is severely defective in cuprizone-treated mice (Fig. 4A). It seems reasonable that v-Src

was not related to myelination status, insofar as knockout mice that lack the Src family of genes other than Fyn (v-Src, Lyn, and Yes) did not show detectable aberrations in CNS myelination (Sperber et al., 2001). Furthermore, in dKO mice, cuprizone treatment did not induce demyelination or subsequent remyelination. As we have previously reported, dKO mice have a certain amount of myelinated fibers (Nakahara et al., 2003), which is quite a contrast to the almost complete absence of myelin sheath in genetically MBP-deficient shiverer mice, and OPC isolated from dKO mice retain the ability to differentiate and produce MBPs in response to triiodothyronine or dibutyryl cyclic AMP (Seiwa et al., 2004), suggesting that dKO mice do not lack the basic machinery necessary for myelination. Therefore, the present data indicate that a deficit in the FcRγ-Fyn cascade is critically involved in cuprizone-induced demyelination. Most of the remaining myelinated axons had morphological abnormalities such as loosening, splitting, duplication, and infoldings of myelin and destroyed major dense lines (Nakahara et al., 2003), suggesting that the FcRγ-Fyn cascade plays a crucial and presumably indispensable role in the compaction of myelin sheath.

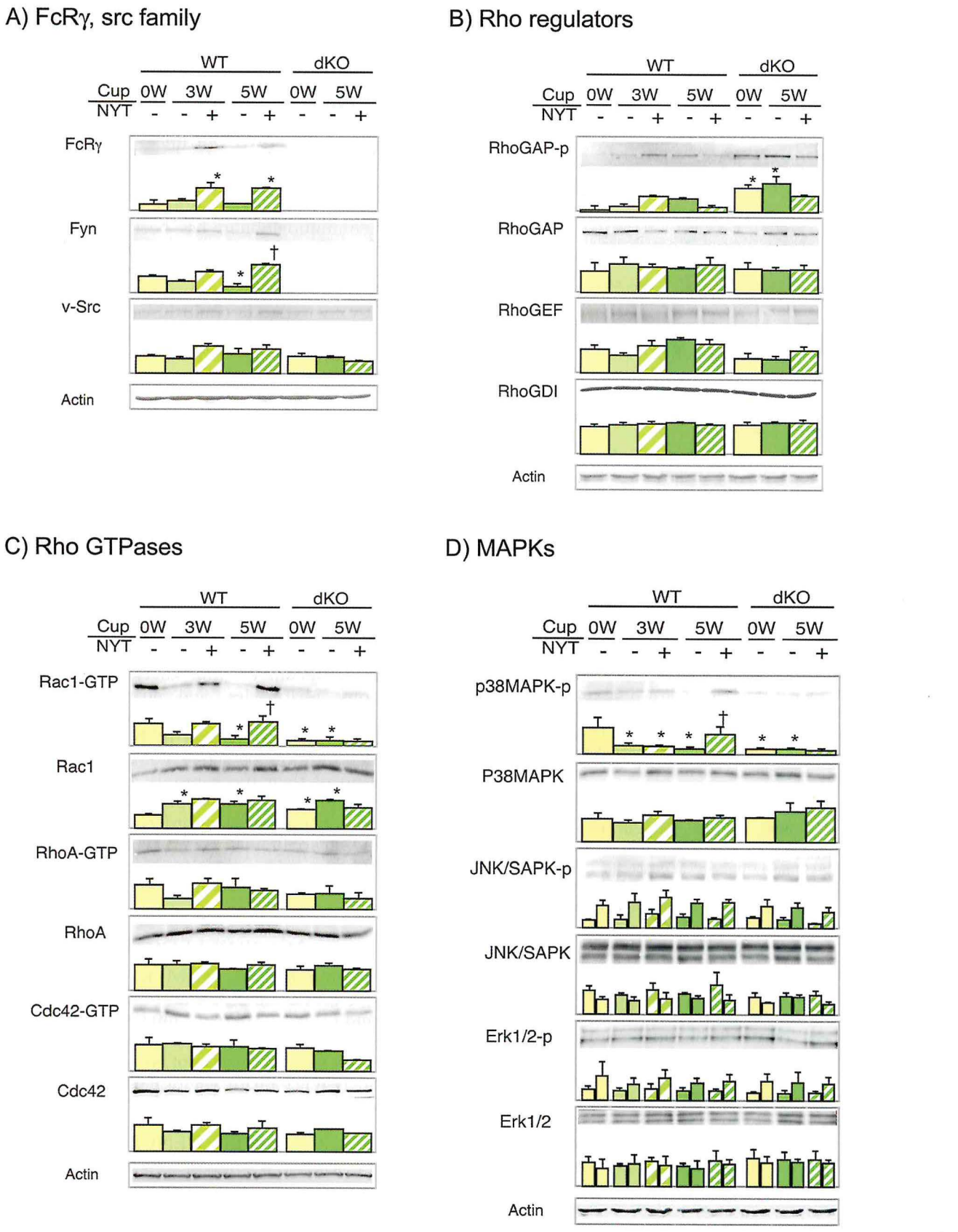
The second important contribution of this study is the elucidation of the signal transduction pathway downstream from the FcRγ/Fyn signaling complex in the myelin sheath. In the present study, we analyzed Rho family proteins (Rac1, Cdc42, RhoA) Rho regulators (RhoGAP, GDI, GEFs), and MAPKs (p38MAPK, ERK1/2, JNK/SAPK) and found that many of these molecules are present in myelin sheath and are associated with FcRγ and Fyn (unpublished data of immunoprecipitation analysis). Among them, a quantitative relationship between myelination status and the amount of activated forms of Rac1 and p38 MAPK was clearly demonstrated. These findings suggest that, among various signaling molecules associated with Fyn in oligodendroglial cells, a restricted subset might be involved in myelin compaction (Fig. 5). However, because of the distinct and complex spatiotemporal pattern of activation of various Rho regulators and Rho GTPases during the execution of cellular processes (Rogers et al., 2003; Aoki et al., 2004; Hoppe and Swanson, 2004), other molecules that have not yet shown a clear-cut relationship with myelination status may still play an important role.

MBP binds to the cytoplasmic surfaces of the membrane and brings them close together so that myelin can form a tight spiral around the axon. MBP is, therefore, an indispensable component for myelination. However, the mere presence of MBP protein does not appear to be sufficient to complete the process of myelin compaction, since substantial amounts of total MBPs were found in demyelinated brain tissue from aged rodents, cuprizone-treated mice, and dKO mice. In contrast, the level of p-MBPs in demyelinated brains was clearly lower than that in normal brains (Fig. 2). In these, the decrease in 21.5-kDa p-MBP was extensive and consistent, wherease the levels of the other three isoforms varied depending on the means of demyelination induction. These data suggest that 21.5-kDa p-MBP is necessary for proper compaction of myelin sheath. NYT-mediated amelioration of aging-induced and cuprizone-induced demyelination was accompanied by a recovery in the levels of 21.5-kDa p-MBP. The recovery resulting from NYT treatment in the cuprizone model was rather modest; however, the specific and extensive decrease of 21.5-kDa p-MBP during demyelination suggests that even low amounts of this isoform may potently drive the myelination process.

It has been suggested that phosphorylation can decrease the positive charge of MBPs, which alters their affinity for the cytoplasmic membrane (Eichberg and Iyer, 1996; Ridsdale et al., 1997; Takai, 2005). Others have assumed that the phosphorylation status of MBPs can affect compaction by triggering the opening and closing of tight junction pores (Dyer, 2002), which is consistent with our finding that p-MBP can bind to claudin 11, a known myelin tight junction protein (unpublished observation). Dephosphorylation of MBPs might be involved in keeping cytoplasmic incisures open in the compact myelin structure prior to complete demyelination. Furthermore, the exon II-containing 21.5- and 17-kDa MBP isoforms have been reported to be translocated into the nucleus in a phosphorylation-dependent manner, whereas the two other MBP isoforms are not transported to the nucleus (Staugaitis et al., 1990). These reports suggest that exon II-containing MBPs have phosphorylation-dependent regulatory effects on the transcription of certain genes. The disappearance of 21.5-kDa p-MBP from demyelinated brain tissue may indicate a loss of the regulatory action of MBPs on myelin compaction.

Our findings have important implications for the treatment of demyelinating diseases. Recent studies

Fig. 4. Analysis of the signaling pathways of the FcRγ-Fyn cascade. Immunoblots of FcRγ and Src family proteins (**A**), Rho regulators (**B**), RhoGTPases (**C**), and MAPKs (**D**) are presented. The amounts of FcRγ, Fyn, v-Src, RhoGEF, RhoGDI, Rac1, RhoA, Cdc42, ERK1/2, phosphorylated ERK1/2 (ERK1/2-p), JNK/SAPK, phosphorylated JNK/SAPK (JNK/SAPK-p), p38 MAPK, and phosphporylated p38 MAPK (p38 MAPK-p) were detected on immunoblots of electrophoresed myelin sheath. RhoGAP and phosphorylated RhoGAP (RhoGAP-p) were immunoprecipitated, and the precipitates were electrophoresed, blotted, and analyzed with anti-RhoGAP antibody for detection of total RhoGAP and with anti-PY22 antibody for RhoGAP-p. Activated (GTP-bound) forms of RhoA (RhoA-GTP), Cdc42 (Cdc42-GTP), and Rac1 (Rac1-GTP) were affinity-precipitated using Rho activation assay kits and Rac/cdc42 assay reagent kits (Upstate), respectively. The precipitates were analyzed by immunoblotting with antibodies raised against the proteins described above. The levels of each protein in WT and dKO mice after various treatments and time intervals were normalized to those of actin, and quantitation and statistical analysis were performed as described in Materials and Methods. Data represent mean ± SEM (n = 3). $*P < 0.05$ vs. 0W WT, $\dagger P < 0.01$ vs. 5W WT.


A) FcRγ, src family
WT
dKO
Cup 0W 3W 5W 0W 5W
NYT - - + - + - - +
FcRγ
Fyn
v-Src
Actin
B) Rho regulators
WT
dKO
Cup 0W 3W 5W 0W 5W
NYT - - + - + - - +
RhoGAP-p
RhoGAP
RhoGEF
RhoGDI
Actin
C) Rho GTPases
WT
dKO
Cup 0W 3W 5W 0W 5W
NYT - - + - + - - +
Rac1-GTP
Rac1
RhoA-GTP
RhoA
Cdc42-GTP
Cdc42
Actin
D) MAPKs
WT
dKO
Cup 0W 3W 5W 0W 5W
NYT - - + - + - - +
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P38MAPK
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JNK/SAPK
Erk1/2-p
Erk1/2
Actin

Figure 4.

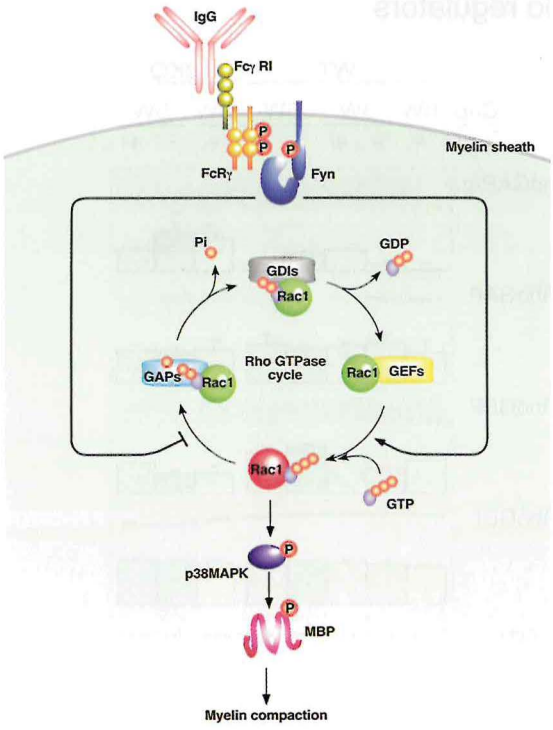

Fig. 5. Diagram of the proposed FcRγ-Fyn signaling cascade involved in myelin compaction. Cross-linking of FcRγ recruits and activates Fyn kinase. Fyn impairment in dKO mice showed increased amounts/activity of p190 RhoGAP in the myelin sheath. RhoGAP might play a suppressive role on Rac1 activity in the stage of myelin compaction, as in the case of morphological differentiation of oligodendrocytes (Liang et al., 2004). Reciprocal regulation of RhoA and Cdc42-Rac1 has been noted in epithelial cells (Rogers et al., 2003) and OPCs (Liang et al., 2004), although it is unknown whether Fyn modulates the balance directly or indirectly. Distinct functions and spatiotemporal activation patterns between Cdc42 and Rac1 have been noted in macrophages (Hoppe and Swanson, 2004), neuroblastomas (Aoki et al., 2004), and epithelial cells (Rogers et al., 2003). Activation of Rac1 resulted in the activation of p38 MAPK, which phosphorylates, presumably directly (Yon et al., 1996), MBP. Phosphorylation of MBP may play a definitive role in the compaction of myelin via the interaction with a tight junction protein, claudin 11 (unpublished observation).

have suggested that, at least in some MS patients, dysfunction of oligodendrocyte and/or microglial function, rather than immune responses, are critically involved in the pathogenesis of demyelinating lesions (Barnett and Prineas, 2004; Matute and Perez-Cerda, 2005; Zhao et al., 2005). Furthermore, the direct involvement of FcRγ in oligodendrocyte differentiation and myelination suggests that, even for MS accompanied with inflammation and immune responses, the efficacy of conventional therapeutic strategies designed to inhibit inflammatory tissue destruction may be limited. Consistent with findings from the cuprizone model, studies in MS patients have demonstrated the presence of MBP proteins, a decrease in phosphorylated MBPs (mainly at Thr98), and the accumulation of OPCs in demyelination lesions (Yon et al., 1996). FcRγ is expressed in OPCs from MS patients (Nakahara and Aiso, 2006), and gene microarray analysis of chronic MS plaques has revealed elevated FcRγ expression (Lock et al., 2002). It should be noted that, in experimental autoimmune encephalomyelitis (EAE) in mice, which is a frequently used "autoimmune" MS model, knocking out of FcRγ has been reported to influence the disease recovery stage (Lock et al., 2002). Efficacy of intravenous injection of immunoglobulin has been demonstrated in an EAE model (Achiron et al., 1994) and in MS patients (Achiron et al., 2004). These reports, along with the findings of the present study, suggest that restoration of the FcRγ-Fyn cascade could be an effective therapeutic approach in some MS patients. This possibility was supported in the present study by the beneficial effect of NYT. NYT is a Kampo medicine (Japanese traditional medicine developed from traditional herbal medicines originating in ancient China) and is now manufactured in Japan on a modern industrial scale in which the quality and quantity of ingredients are standardized under strict, scientific quality controls (Egashira et al., 2003; Kobayashi et al., 2003). NYT has been approved ethically as a drug and is used for the treatment of various diseases in Japan by physicians who have been educated in Western medicine. Although appropriate toxicological and pharmacological studies have not been carried out, empirical knowledge about NYT's efficacy and safety has been accumulated over the centuries. Furthermore, NYT is now in use under the extensive surveillance system of drug safety covering all medical institutions, pharmacies, and pharmaceutical manufacturers in Japan. This should encourage future research to establish the effectiveness of NYT in MS. In addition, identification of active compounds in NYT and elucidation of their mechanism(s) of action may lead to the development of new pharmacotherapeutic agents for various neurological disorders, such as MS, dementia, and schizophrenia, these being diseases in which demyelination may play an important role (Hakak et al., 2001; Marner et al., 2003; Stewart and Davis, 2004).

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