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Bone Marrow-Derived Mesenchymal Stem Cells Prevent the Loss of Niemann-Pick Type C Mouse Purkinje Neurons by Correcting Sphingolipid Metabolism and Increasing Sphingosine-1-phosphate

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ABSTRACT

Niemann-Pick type C (NP-C) disease exhibits neuronal sphingolipid storage and cerebellar Purkinje neuron (PN) loss. Although it is clear that PNs are compromised in this disorder, it remains to be defined how neuronal lipid storage causes the PN loss. Our previous studies have shown that bone marrow-derived mesenchymal stem cells (BM-MSCs) transplantation prevent PN loss in NP-C mice. The aim of the present study was therefore to examine the neuroprotective mechanism of BM-MSCs on PNs. We found that NP-C PNs exhibit abnormal sphingolipid metabolism and defective lysosomal calcium store compared to wild-type mice PNs. BM-MSCs promote the survival of NP-C PNs by correction of the altered calcium homeostasis, restoration of the sphingolipid imbalance, as evidenced by increased sphingosine-1-phosphate levels and decreased sphingosine, and ultimately, inhibition of apoptosis pathways. These effects suggest that BM-MSCs modulate sphingolipid metabolism of endogenous NP-C PNs, resulting in their survival and improved clinical outcome in mice. STEM CELLS *2010;28:821–831*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Cerebellar Purkinje neuron (PN) loss begins early and progresses rapidly, correlating with gait ataxia, dysarthria, and dysphagia in Niemann-Pick type C (NP-C) disease [1–3]. A striking feature of NP-C disease is the broad range of lipids that accumulate in late endosomes and lysosomes, including sphingomyelin, cholesterol, glycosphingolipids, and usually, sphingosine [4, 5]. However, the actual mechanism(s) leading to this complex pattern of lipid storage remains unknown. It is also still a mystery as to why cerebellar neurons (CNs) degenerate in NP-C disease and how neuronal lipid storage is associated with neuronal loss.

In most cases (95%), NP-C disease is caused by mutations in the *NPC1* gene, which encodes a transmembrane protein of the late endosome and lysosome [6]. The NPC1 protein is involved in sphingosine efflux from lysosomes [7], and abnormal sphingolipid metabolism has been suggested as an initiating factor in NP-C disease pathology. However, the exact pattern of sphingolipid metabolism has not yet been investigated in NP-C mutant cerebellar PNs. Sphingolipid metabolites (including ceramide, sphingosine, and sphingosine-1-phosphate [S1P]) modulate apoptosis in response to stress. S1P is an anti-apoptotic molecule that mediates a host of cellular responses antagonistic to those of pro-apoptotic sphingolipids, such as ceramide and sphingosine [8]. Because NP-C neurodegeneration is known to involve apoptotic changes [9, 10], it stands to reason that sphingolipid signaling may play a role in the pathophysiology of NP-C disease.

Our previous studies have shown that bone marrow-derived mesenchymal stem cells (BM-MSCs) contribute to

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improved neurological function in the NP-C mouse brain following intracerebellar transplantation, correlated with increased numbers of PNs [11, 12]. However, the mechanism(s) by which the transplanted BM-MSCs elicit this effect, including preventing PNs loss, remains unknown.

On the basis of these considerations, we investigated the levels of sphingolipid metabolites in cerebellar PNs of NP-C and wild-type mice (WT), and changes in the levels of these metabolites following BM-MSC treatment in vitro and in vivo. We observed the elevation of sphingosine and ceramide, and reduction of S1P, in NP-C mutant PNs, and also defective lysosomal calcium compared to WT PNs. Interestingly, we found that BM-MSC treatment increased S1P levels and decreased sphingosine accumulation in NP-C mutant PNs. BM-MSCs also promoted survival of NP-C mutant cerebellar PNs and granule neurons (GNs) and reduced neuronal apoptosis of NP-C mutant CNs by correction of the altered calcium level. Notably, these effects did not require direct cell contact between cocultured PNs and BM-MSCs. We therefore postulate that NP-C mutant cerebellar PNs have deregulated sphingolipid metabolism and that replacement therapy by BM-MSCs ameliorates these abnormalities, leading to their increased survival through the release of tropic factors and alterations in the endogenous microenvironment.

Materials and Methods

Animals

A colony of BALB/c npc^{nih} mice has been maintained by brother-sister mating of heterozygous animals. The genotype of each mouse was determined by polymerase chain reaction (PCR) [13]. All procedures were performed in accordance with an animal protocol approved by the Kyungpook National University Institutional Animal Care and Use Committee. Animals were housed in a room maintained under controlled temperature and on a 12-hour light/12-hour dark cycle.

Isolation and Culture of BM-MSCs

Tibias and femurs were dissected from 4- to 6-week-old mice. Bone marrow was harvested, and single-cell suspensions were obtained using a 40-μm cell strainer (BD Biosciences, San Diego, http://www.bdbiosciences.com). Approximately 10^6 cells were plated in 25-cm^2 flasks containing MesenCult MSC Basal Medium and Mesenchymal Stem Cell Stimulatory Supplements (Stem Cell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) with antibiotics according to our previous report [12]. The cell cultures were grown for 1 week, and the plastic-adherent population (BM-MSC) was used for subsequent experiments.

Isolation and Culture of PNs

Dissociated cerebellar PNs cultures were prepared from individual embryonic (E) day 18 fetuses, either homozygous NP-C mice or control littermates (identified by PCR) as previously described [14–16] with some minor modifications. Briefly, the entire cerebellum was removed and kept in ice-cold Ca^{2+}/Mg^{2+}-free Hank's balance salt solution (HBSS) containing gentamicin (10 μg ml^{-1}; GIBCO, Carlsbad, CA, http://www.invitrogen.com). Each cerebellum was then dissociated into single cells using the SUMITOMO Nerve-Cell Culture System (SUMITOMO BAKELITE), and the cells were resuspended in seeding medium (Minimum Essential Medium; GIBCO) supplemented with L-glutamine (4 mM; Fluka, St. Louis, http://www.sigmaaldrich.com), D-glucose (11 mM; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), sodium pyruvate (500 μM; Sigma-Aldrich), gentamicin (10 μg ml^{-1}; GIBCO), and 10% heat-inactivated fetal bovine serum (FBS; GIBCO). The cell suspensions were seeded on poly-L-ornithine-coated (Sigma-Aldrich) glass coverslips (12 mm) at a density of 5×10^6 cells per milliliter, with each coverslip in an individual well of a 24-well tissue culture plate. After 3 hours of incubation in a CO_2 incubator (100% humidity, 37°C, 5% CO_2), 1 ml of culture medium further supplemented with B-27 supplement (GIBCO), N2 supplement (GIBCO), bovine serum albumin (100 μg ml^{-1}; Sigma-Aldrich), and tri-iodothyronine (0.5 nM; Sigma-Aldrich) was added to each culture well. After 7 days, half of the medium in each well was replaced with fresh culture medium additionally supplemented with cytosine arabinoside (4 μM; Sigma-Aldrich) to prevent proliferation of glia.

Methods for the isolation and purification of GNs were as previously described [16]. Briefly, GNs were obtained from cerebella of homozygous NP-C mice at postnatal (P) days 5-7. Cerebella were collected in a Ca^{2+}/Mg^{2+}-free HBSS supplemented with glucose (6 mg/ml; Sigma). The cerebella were then dissociated from the tissue into single cells using the SUMITOMO Nerve-Cell Culture System and resuspended in serum-free medium (SFM) containing 10% heat-inactivated FBS. SFM was composed of Neurobasal A-Medium (GIBCO) supplemented with GlutaMAX I (2 mM; GIBCO), penicillin-streptomycin (20 U ml^{-1}; GIBCO), and KCl (250 μM; Sigma-Aldrich). To generate monolayer cultures enriched in GNs, cells were preplated two times for 30 minutes on 35-mm dishes coated with poly-D-lysine (Sigma-Aldrich) and then strained through a 70-μm nylon cell strainer (BD Biosciences) before final suspension and counting. The cell preparations were resuspended at a density of 5×10^5 cells per milliliter in SFM containing the B-27 supplement, and 1 ml was plated in individual wells of a 24-well tissue culture plate that contained poly-D-lysine-coated glass coverslips (12 mm).

Coculture of BM-MSCs and CNs

In direct cell-mixing coculture experiments, BM-MSCs (3×10^4 cells per well), which were labeled with nanoparticles (0.1 mg ml^{-1}, NFP-STEM Silanol TMSR-RITC 50; BITERIALS, Seoul, Korea, http://www.biterials.com), were directly seeded on primary cultures of the PNs or GNs. For the indirect three-dimensional coculture experiments, 1.0-μm pore size Millicell Hanging Cell Culture Inserts (Millipore, Billerica, MA, http://www.millipore.com) were placed on top of PNs or GNs that had been previously plated. The BM-MSCs were seeded onto the insert at a density of 3×10^4 cells per insert. In this system there is no direct contact between the CNs and BM-MSCs.

Transplantation of BM-MSCs into NP-C Mouse Brains

NP-C mice (approximately 4 weeks of age, $n = 10$) were anesthetized with a combination of 100 mg/kg ketamine and 10 mg/kg xylazine and then injected using the styrofoam platform of a stereotaxic injection apparatus (Stoelting Company, Wood Dale, IL, http://www.stoeltingco.com) as described [12]. BM-MSCs were transplanted into the cerebellum by using a glass capillary (1.2 mm × 0.6 mm) [2]. The injection coordinates were 5.52-mm posterior to bregma and injection depth was 2.50 mm. Each recipient received approximately 1×10^5 cells in 3 μl of cell suspension at a rate of 0.15 μl/min. For sham transplantation, animals underwent the same transplantation procedure without cells. After transplantation, the scalp was closed by suture and the animals recovered from the anesthesia before they were returned to their cage. Age-matched normal littermates in the NP-C colony were used as controls (10 per group).

Lipid Extraction and Analysis

Cultured cerebellar PNs were washed with ice-cold phosphate buffered saline (PBS) and pelleted. For the brain samples, mice were sacrificed at 1 week after BM-MSC or sham transplantation. Brain samples were isolated from nearby the Purkinje cell layers of the cerebellar lobe IV/V and VI in BM-MSC or sham-transplanted NP-C and WT mice. Cell and tissue samples were

homogenized at 4°C in nine volumes of homogenization buffer containing 50 mM HEPES, 150 mM NaCl, 0.2% Igepal, and protease inhibitor. After brief centrifugation at 13,000 rpm for 10 minutes to remove large debris, the supernatant was collected and further centrifuged at 13,000 rpm at 4°C for 30 minutes. This second supernatant (from the 13,000 rpm spin) was collected. Quantification of acid sphingomyelinase (ASM), acid ceramidase (AC), ceramide, S1P, and sphingosine were performed as previously described [17].

S1P Treatment

S1P (Echelon Biosciences Inc., Salt Lake City, UT, http://www.echelon-inc.com) was initially prepared in methanol to a stock concentration of 2 mM and diluted in culture medium to a final concentration of 1 μM immediately before use. NP-C PNs were exposed to S1P for 3 days after 10 days in culture (i.e., days in vitro [DIV]). To ensure that the use of methanol did not mask S1P treatment results, a preliminary experiment was conducted using methanol alone. Cells treated with methanol did not show any differences as compared to control cells, indicating no solvent effect. For in vivo experiments, S1P was diluted in PBS to a final concentration of 10 μM immediately before use. Two microliters of the S1P solution was injected into the cerebullum of 4-week-old NP-C mice. For sham transplantation, animals underwent the same injection procedure without S1P.

Calcium Assay

We carried out lysosomal calcium measurements as previously described [18] with minor modification. Intracellular calcium was measured with the membrane-permeable calcium indicators Fura2-AM. Some of the procedures used for calcium imaging in this experiment have been described previously [19]. Fura2-AM (3 μmol L^{-1}) was added to the PNs in physiological saline solution (PSS) at room temperature (RT) for 30 minutes, and the cells were then washed in dye-free PSS for 30 minutes. Coverslips were placed on the stage of an inverted microscope (TM-100; Nikon, Tokyo, Japan, http://www.nikon.com) and imaging was performed using a dual-wavelength system (Intracellular Imaging Inc., Cincinnati, OH, http://www.intracellular.com). $[Ca^{2+}]_i$ was calculated as the relationship between the ratio of emissions at 510 nm from excitation at 340 and 380 nm. Ratio images were processed every 5 seconds, converted to $[Ca^{2+}]_I$, and compared with a calibrated range of such ratios versus known calcium concentrations (Calcium Calibration Buffer Kit; Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com). The experimental data represent the mean $[Ca^{2+}]_i$ calculated from at least 12 individually measured cells from three separate cultures. All imaging experiments were performed at RT.

Statistical Analysis

The Student's *t* test was used to compare the two groups, whereas the Tukey's HSD test and Repeated Measures Analysis of Variance test was used for multigroup comparisons according to the SAS statistical package (release 9.1; SAS Institute Inc., Cary, NC, http://www.sas.com). $p < .05$ was considered to be significant.

Other materials and technical procedure details are shown in the supporting information data.

RESULTS

BM-MSCs Enhance the Survival of NP-C CNs in Primary Culture

Our recent studies have shown that BM-MSCs contribute to improving neurological function following intracerebral transplantation into NP-C mice [11, 12, 20] (supporting information Fig. 1A). However, the mechanism(s) by which the transplanted BM-MSCs elicit this effect, including preventing PNs loss, remains unknown. To clarify this issue, we cocultured BM-MSCs with cerebellar PNs using two different methods, direct cell-mixing and an indirect, three-dimensional coculture system. The protocol is summarized in Figure 1A.

As compared with PNs from WT mice, mutant PNs from NP-C mice showed a marked decrease in survival ($p < .005$; Fig. 1C, 1E). Moreover, when the NP-C mutant PNs were directly cocultured with BM-MSCs, the survival of the PNs was increased (Fig. 1B, 1C) and reached the levels of WT PNs ($p < 0.05$). BM-MSCs did not affect the survival of WT PNs. To determine whether soluble bioactive factors secreted from BM-MSCs could promote the survival of NP-C mutant PNs, we also used an indirect three-dimensional coculture technique without cell-to-cell contacts. When the NP-C mutant PNs were cocultured with BM-MSCs in the transwells, the survival of the PNs also was increased ($p < .05$; Fig. 1D, 1E), similar to what had been observed from the cell-mixing experiments. BM-MSC coculture in this indirect system did not affect the survival of WT PNs, as was observed above (Fig. 1D, 1E).

In addition, we examined the effect of BM-MSCs on NP-C mutant cerebellar GNs. Seven DIV NP-C mutant GNs were cultured for 72 hours with BM-MSCs using the three-dimensional (indirect) coculture technique. GNs were stained with 4′,6-diamidino-2-phenylindole (blue) and TUNEL (green) to detect apoptosis (Fig. 1F, 1G) since the cerebellar neuronal cell death in the brain of NP-C disease has been shown to occur through apoptosis [9]. Confirming this observation, TUNEL staining demonstrated apoptotic change in 67.7% ± 15% of NP-C GNs. In contrast, 30.4% ± 14% of NP-C mutant GNs cocultured with BM-MSCs were apoptotic ($p < .05$; Fig. 1G).

Glial activation has been considered as a key process leading to neuronal degeneration in NP-C mice [21]. We reconfirmed that BM-MSC transplantation reduced the inflammatory response by suppressing astroglial activation in NP-C mice [20] (supporting information Fig. 1B). We also examined whether the astroglial activation was reduced by coculture with BM-MSCs using the three-dimensional system. In NP-C mutant cerebellar cell cultures, reactive astrocytes, as judged by increased glial fibrillary acidic protein immunoreactivity, were prominent compared to astrocytes of the WT cerebellar cultures. The increased astrogial activation in the NP-C mutant cerebellar cultures was slightly decreased after BM-MSC treatment (supporting information Fig. 1C). Overall, the results indicated that BM-MSCs could increase survival of NP-C mutant PNs and decrease the apoptosis of NP-C GNs, and also that these effects were not dependent on direct cell-cell contacts. To verify the indirect effects of BM-MSCs, the following experiments were performed using a three-dimensional coculture system.

BM-MSCs Upregulate S1P Levels of NP-C Mutant PNs in Primary Culture

In NP-C disease, abnormal sphingolipid metabolism is a primary defect that arises from mutations in the *NPC1* gene [7]. However, it remains to be determined whether these metabolites are directly relevant to the neuropathogenesis associated with NP-C disease. Herein, we investigated the levels of sphingolipid metabolites in WT and NP-C PNs, and also the levels and activities of several lipid-related enzymes. As shown in Figure 2A, 2C, no significant differences in ASM and AC activities were found between WT and NP-C PNs. However, both activities significantly decreased in the NP-C PNs (but not WT PNs) cocultured with BM-MSCs compared

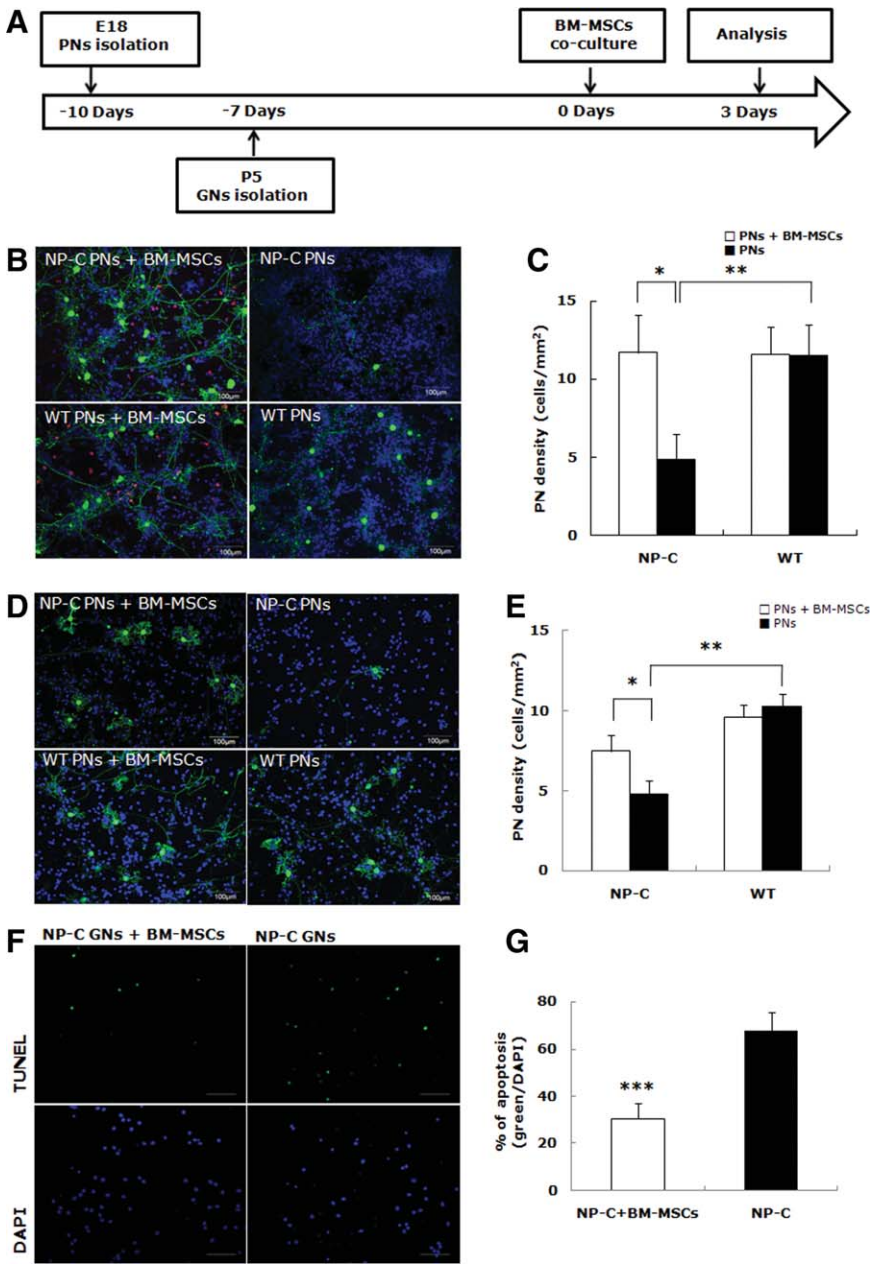

Figure 1. BM-MSCs promote the survival of NP-C mutant cerebellar neurons (CNs). **(A):** Experimental design for the coculture of BM-MSCs and CNs. **(B, D):** Immunofluorescence of PNs using a primary antibody (calbindin) in conjunction with an Alexa 488-conjugated secondary antibody. Nuclei were stained with DAPI. **(C, E)**: The mean number of calbindin-positive cells per squared millimeter was counted. Counting was done in square visual fields sequentially aligned on two lines crossing each other at the center of the cell layer. BM-MSCs, labeled with TMSR (red), were cultured with PNs either by direct cell-mixing **(B, C)** or by an indirect three-dimensional coculture in which there was no cell-cell contact **(D, E)**. Data in **(C)** and **(E)** are expressed as mean ± SEM ($n = 3$ or 9, respectively). The Anova, Tukey's HSD test was used for statistical analysis. *, $p < .05$; **, $p < .01$. Note that in both systems BM-MSCs coculture significantly increased the number of surviving NP-C PNs. **(F, G):** Apoptosis was determined by TUNEL assay in cultured NP-C mutant GNs. Green-stained cells were TUNEL-positive; all nuclei were stained with DAPI. The ratio of TUNEL-positive GNs among the total GNs was determined. Indirect coculture with BM-MSCs significantly reduced apoptosis of NP-C mutant GNs. Scale bar, 50 μm. Data are expressed as mean ± SEM; $n = 5$ for each group. Student's t test was used for statistical analysis. ***, $p < .005$. Abbreviations: BM-MSCs, bone marrow-derived mesenchymal stem cells; DAPI, 4′,6-diamidino-2-phenylindole; GNs, granule neurons; NP-C, Niemann-Pick type C; PNs, Purkinje neurons; WT, wild type.

to those of NP-C PNs without coculture ($p < .01$). We next examined the levels of ceramide, sphingosine, and S1P in NP-C mutant and WT PNs. No significant differences were found between the two groups (Fig. 2B, 2D, 2E). Consistent with the reduction of ASM activity (Fig. 2A), however, ceramide was modestly decreased in NP-C PNs cocultured with BM-MSCs as compared to that of NP-C mutant PNs without coculture (Fig. 2B). Sphingosine levels also were decreased in the cocultured NP-C PNs ($p < .05$; Fig. 2D), presumably because of the lower AC levels. Notably, and surprisingly, BM-MSC coculture significantly increased S1P levels in NP-C PNs ($p < .01$; Fig. 2E). In addition, S1P levels also were increased in WT PNs cocultured with BM-MSCs ($p < .01$). Overall, the sphingolipid changes observed in cocultured NP-C PNs (reduced ceramide and sphingosine; elevated S1P) are consistent with reduced apoptosis and increased survival.

To further investigate the role of S1P on the survival of PNs, we treated NP-C PNs with S1P and determined their survival (Fig. 2F, 2G). S1P treatment modestly increased survival of NP-C PNs, but the results did not reach statistical significance. Thus, the beneficial effects of BM-MSCs on the survival of NP-C PNs that we observed in vitro and in vivo seem to be mediated, at least in part, by enhancing S1P production within the PNs themselves, as opposed to simply an extrinsic effect of the S1P released by BM-MSC.

BM-MSC Transplantation Upregulates S1P Levels in the Cerebellum of NP-C Mice

To extend and confirm the above observations, we also investigated the levels of sphingolipid metabolites and activities of several lipid-related enzymes in the cerebellum of WT and NP-C mice before and after BM-MSC intracerebral transplantation. Animals were transplanted at 4 weeks of age as in our previous studies [11, 12], and at 1 week after transplantation they were sacrificed and evaluated. As shown in Figure 3A,

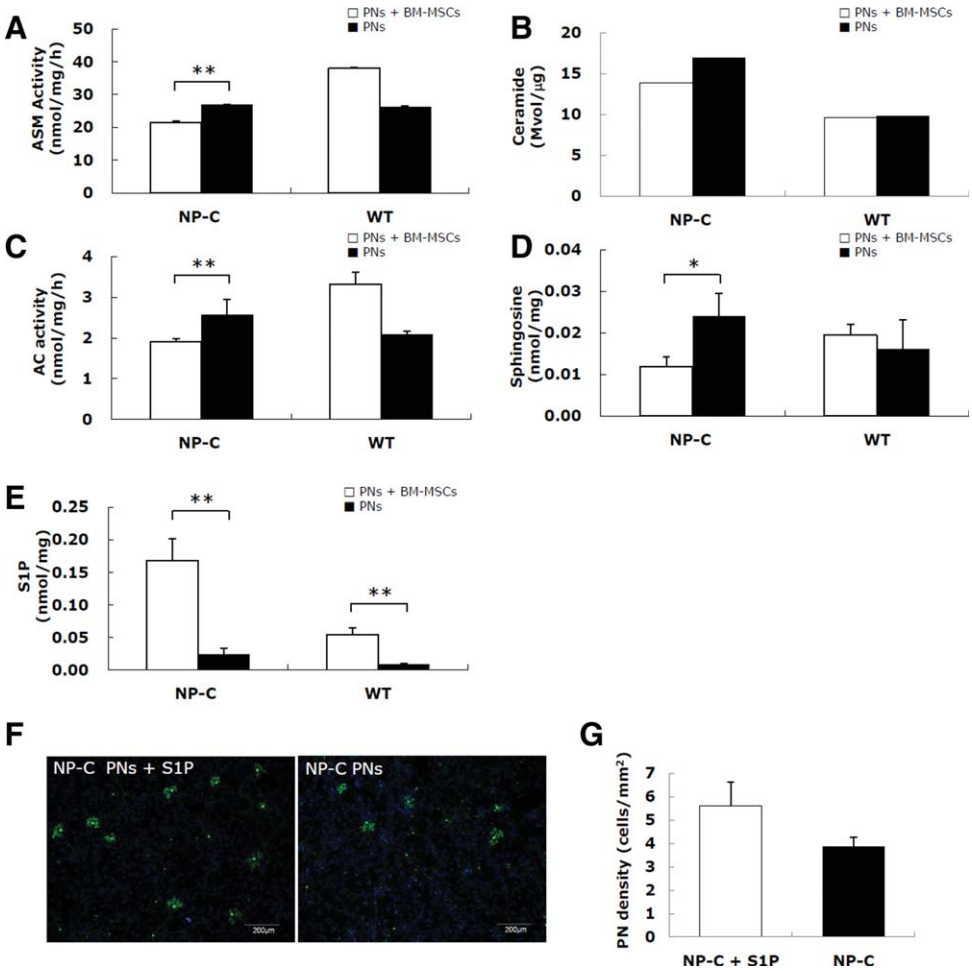

Figure 2. BM-MSCs indirect coculture upregulates S1P levels in NP-C PNs. At 10 days in vitro, NP-C PNs were indirect cocultured with or without BM-MSCs using the indirect three-dimensional system. Three days after coculture, the PNs were collected and prepared as described in the Materials and Methods. ASM **(A)** and AC **(C)** activities, ceramide **(B)**, sphingosine **(D)**, and S1P **(E)** were determined using high-performance liquid chromatography-based methods. **(A, C):** The activities of ASM and AC were decreased in NP-C mutant PNs cocultured with BM-MSCs. **(B, D):** The levels of ceramide and sphingosine were decreased in PNs cocultured with BM-MSCs. **(E):** The levels of S1P were significantly elevated in NP-C PNs cocultured with BM-MSCs as compared to controls. *, $p < .05$; **, $p < .01$. Values are expressed as the mean ± S.D. ($n = 3$). In WT, ASM, AC, ceramide and sphingosine go up after BM-MSCs coculture. **(F):** S1P treatment slightly increases the number of surviving NP-C mutant PNs. 10 DIV NP-C PNs were treated with S1P for 3 days and then stained with calbindin/4′,6-diamidino-2-phenylindole. Scale bar, 200 μm. **(G):** Mean number of calbindin-positive cells per squared millimeter were counted. Data are expressed as mean ± SEM. $n = 5$ for each group. Abbreviations: AC, acid ceramidase; ASM, acid sphingomyelinase; BM-MSCs, bone marrow-derived mesenchymal stem cells; GNs, granule neurons; NP-C, Niemann-Pick type C; PNs, Purkinje neurons; S1P, sphingosine-1-phosphate; WT, wild type.

3C, ASM and AC activities in and near the dissected Purkinje cell layers of NP-C and WT cerebella were similar in the absence of transplantation. However, these activities were significantly decreased in the treated NP-C group as compared to those of the untreated NP-C mice ($p < .01$). AC activity also was modestly decreased in BM-MSC-treated WT mice than in untreated WT mice, although ASM activity was not.

We next determined the levels of ceramide, sphingosine, and S1P. Elevated ceramide was detected in nontransplanted NP-C versus WT mice (Fig. 3B), as was sphingosine ($p < .01$; Fig. 3D). S1P, however, was markedly reduced in NP-C mice versus WT mice ($p < .05$; Fig. 3E). Notably, these changes were reversed in NP-C mice transplanted with BM-MSC (i.e., reduced ceramide and sphingosine [$p < .01$; Fig. 3B, 3D] and increased S1P [$p < 0.01$; Fig. 3E]). We also injected S1P directly into NP-C mouse cerebellum to verify whether S1P could increase the survival of PNs in a direct manner, and we found that it could not (Fig. 3A, 3B). Overall, these in vivo findings were consistent with the changes observed in vitro and support the hypothesis that NP-C neuropathology is caused, at least in part, by abnormal sphingolipid metabolism and that the neuroprotective mechanism of BM-MSC in the NP-C brain is mediated by alleviation of the sphingolipid imbalance.

BM-MSCs Restore Reduced Lysosomal Calcium of NP-C PNs

Mitogen-activated protein kinases (MAPKs), PI3K/Akt signaling pathways, and calcium signaling pathways are involved in a variety of cellular functions, including cell growth, differentiation, development, and apoptosis. To determine which signaling pathways are functionally involved in the increased survival rate of PNs by BM-MSCs, we treated NP-C mutant PNs with several inhibitors, including LY294002 (PI3K/Akt inhibitor), PD98059 (MEK/ERK inhibitor), SP600125 (JNK inhibitor), GF109203-X (PKC inhibitor), and BAPTA-AM (Ca^{2+} inhibitor). After pretreatment with the inhibitors for

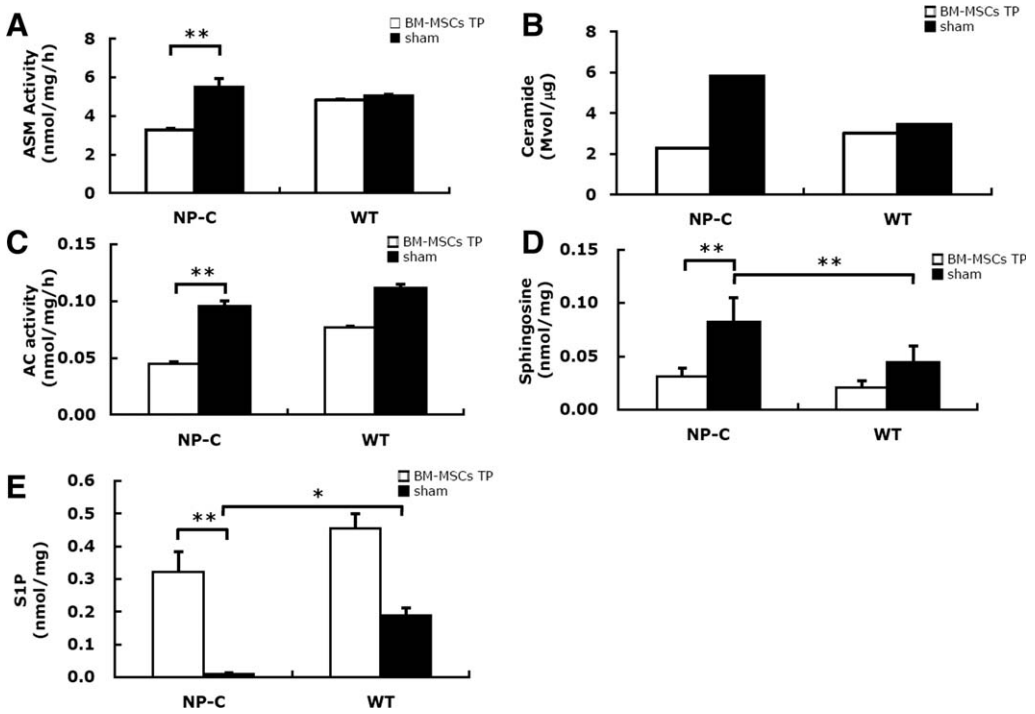

Figure 3. BM-MSCs intracerebellar transplantation decreases the levels of ceramide and sphingosine and increases the level of S1P in the cerebellum of NP-C mice. NP-C mice were transplanted with BM-MSCs at 4 weeks of age. One week after BM-MSCs transplantation, mice were sacrificed and analyzed. ASM **(A)** and AC **(C)** activities, ceramide **(B)**, sphingosine **(D)**, and S1P **(E)** contents were determined using HPLC. **(A, C):** The activities of ASM and AC were decreased in BM-MSCs transplanted NP-C mice. AC also was decreased in the BM-MSCs treated WT group compared to untreated WT mice. **(B, D):** The levels of ceramide and sphingosine were increased relative to WT mice, and these levels were significantly decreased following BM-MSCs transplantation in NP-C. **(E):** The level of S1P was markedly decreased in NP-C mice compared to WT mice, and was significantly elevated in BM-MSCs transplanted NP-C and WT mice as compared to nontransplanted controls. *, $p < .05$; **, $p < .01$. Values are expressed as the mean ± S.D. ($n = 3$). Significance not indicated for WT. Abbreviations: AC, acid ceramidase; ASM, acid sphingomyelinase; BM-MSCs, bone marrow-derived mesenchymal stem cells; NP-C, Niemann-Pick type C; S1P, sphingosine-1-phosphate; TP, transplantation; WT, wild type.

1 hour, mutant PNs were cocultured with BM-MSCs. Treatment with PI3K/Akt, MAPKs, JNK, and PKC signaling inhibitors did not affect the effect of BM-MSC on the survival of NP-C mutant PNs (supporting information Fig. 3), but treatment with the Ca^{2+} inhibitor (BAPTA-AM) did prevent the restorative effect of BM-MSC coculture (Fig. 4A). These results indicated that calcium signaling might be positively related to the therapeutic effect of BM-MSCs on NP-C mutant cerebellar PNs.

Consistent with this observation, alteration of calcium homeostasis in the endo-/lysosomal compartment has been predicted to result in defective endocytic transport, fusion, or both, potentially leading to some of the NP-C disease cellular phenotypes [7]. To determine whether calcium homeostasis is perturbed in NP-C PNs, we measured calcium release from the endo-/lysosomal compartment. PNs were loaded with the calcium probe, Fura2-AM, and treated with 200 mM Gly-Phe β-naphthylamide (GPN) to release endo-/lysosomal compartmentalized calcium. We observed a reduction in NP-C mutant PNs calcium release from the endo-/lysosomal compartment compared to WT PNs. Notably, this reduction was corrected in NP-C mutant cerebellar PNs cocultured with BM-MSCs, reaching WT levels (Fig. 4B). These results suggest that BM-MSCs can modulate defective endo-/lysosomal calcium homeostasis in NP-C mutant PNs.

BM-MSC Transplantation Inhibits Apoptosis of NP-C Mutant CNs

Ceramide and sphingosine, both of which are elevated in NP-C PNs, are associated with growth arrest and/or apoptosis. Many stress stimuli increase levels of ceramide and sphingosine, whereas suppression of apoptosis is associated with S1P through expression of the anti-apoptotic protein Bcl-2 [22–24]. We checked apoptotic cell death in NP-C CNs using TUNEL assays (Fig. 5A) and found that it was significantly increased as compared to WT ($p < .001$). However, the number of apoptotic cells was significantly reduced in the NP-C CNs cocultured with BM-MSCs compared with untreated NP-C CNs ($p < .001$). In addition to TUNEL assays, to evaluate the role of BM-MSC treatment on apoptosis, we performed immunoblotting. Western blotting with anti-phospho-p44/42 ERK, anti-phospho-p90RSK, and anti-Bcl-2, each of which is related to anti-apoptotic pathways closely associated with S1P, showed an increase in the BM-MSC-treated NP-C CNs (Fig. 5B).

To further confirm the above observations, BM-MSCs were transplanted into the cerebellum of 4-week-old WT and NP-C mice. One week after BM-MSC transplantation, mice were sacrificed and evaluated for apoptosis by TUNEL assay. Nontransplanted NP-C mice had higher levels of TUNEL-positive cells in both the Purkenje and granular layers of the cerebellum. However, BM-MSC-treated NP-C mice had reduced numbers of TUNEL-positive cells (Fig. 5C). Western blot analysis of cerebellar lysates indicated that antiapoptotic pathways were elevated in NP-C CNs by BM-MSC transplantation (Fig. 5D). Thus, overall, these results demonstrate that BM-MSCs inhibit apoptosis by activation of ERK/RSK and downstream anti-apoptotic signaling cascades, both in vitro and in vivo.

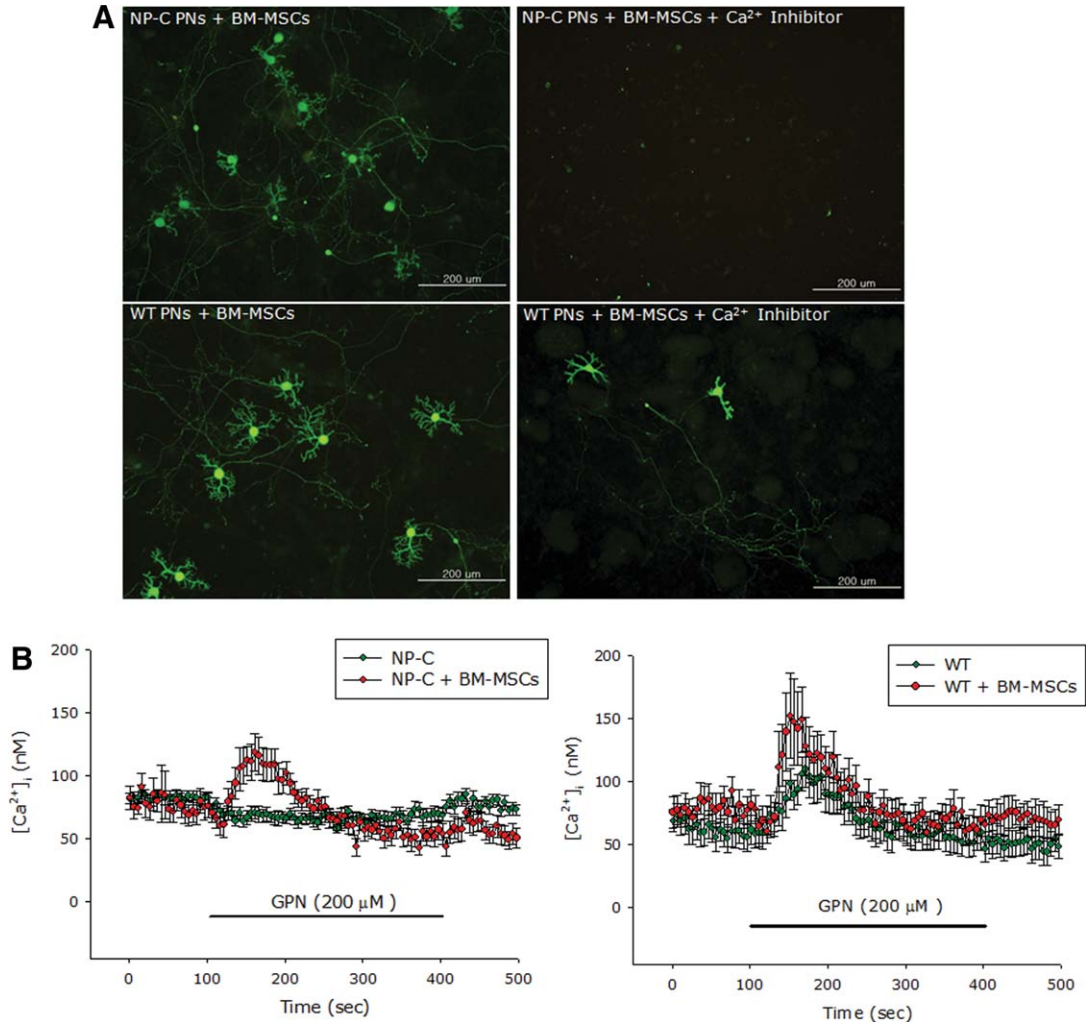

Figure 4. BM-MSCs overcome reduced lysosomal calcium defect in NP-C mutant PNs. **(A):** PNs were indirect cocultured with BM-MSCs ± pretreatment for 60 minutes with a Ca^{2+} inhibitor (50 μM). Note that the survival of NP-C and WT PNs was decreased by treatment with the Ca^{2+} inhibitor. **(B):** Representative time course of $[Ca^{2+}]_i$ changes in response to GPN. PNs, loaded with Fura2-AM, were treated with the GPN to release lysosomal calcium. We measured intracellular calcium ($[Ca^{2+}]_i$) with the membrane-permeable calcium indicators Fura2-AM. Calcium release from the endo-/lysosomal compartment of NP-C mutant PNs was reduced compared to that of WT PNs. This reduction was corrected in NP-C PNs. Data are the mean ± SEM of three independent experiments. n = 4-6 for each experiment. Abbreviations: BM-MSCs, bone marrow-derived mesenchymal stem cells; GPN, Gly-Phe β-naphthylamide; NP-C, Niemann-Pick type C; PNs, Purkinje neurons; WT, wild type.

Discussion

NP-C mice exhibit progressive neuronal loss, mainly of cerebellar PNs [4]. However, the mechanism(s) leading to this cell loss remain mostly unknown. Stem cell transplantation therapies of CNS pathologies are promising therapeutic strategies for neurodegenerative diseases, including NP-C disease [25–27], and our previous studies have shown that transplantation of BM-MSCs alleviates pathology in the murine NP-C cerebellum [11, 12, 20]. To date, the mechanism(s) underlying this observation has remained unknown. In the present manuscript we show for the first time that primary cultured mutant PNs isolated from fetal NP-C mice exhibited decreased survival compared with PNs from fetal WT mice (Fig. 1B-1E). These observations allowed us to examine the mechanism(s) underlying the therapeutic effects of BM-MSCs in NP-C disease, and also the causes of NP-C mutant PNs loss through a series of in vitro and in vivo experiments.

The potential of BM-MSCs to repair tissue through differentiation has been supported by experiments in which BM-MSCs were differentiated into cardiomyocytes, neurons, and other cell types with appropriate electrical activities [28–30]. Although the differentiation potential of BM-MSCs has been repeatedly demonstrated, one of the striking observations from several experiments was that these cells frequently produced functional improvements without much evidence of either engraftment or differentiation [31–33]. This finding suggests that BM-MSCs do not repair tissue solely by their stem cell-like ability to differentiate or fuse with existing cells, but only by releasing growth factors and other molecules that may elicit therapeutic effects [34, 35].

Supporting this concept, when BM-MSCs were labeled with nanoparticles and directly cocultured (by cell mixing) with the PNs, doubly positive PNs were hardly observed (supporting information Fig. 4). This suggested that little cell fusion of the PNs and BM-MSCs occurred in vitro. Recently, evidence has emerged that transplantation of BM-MSCs into a damaged brain promotes brain repair via trophic

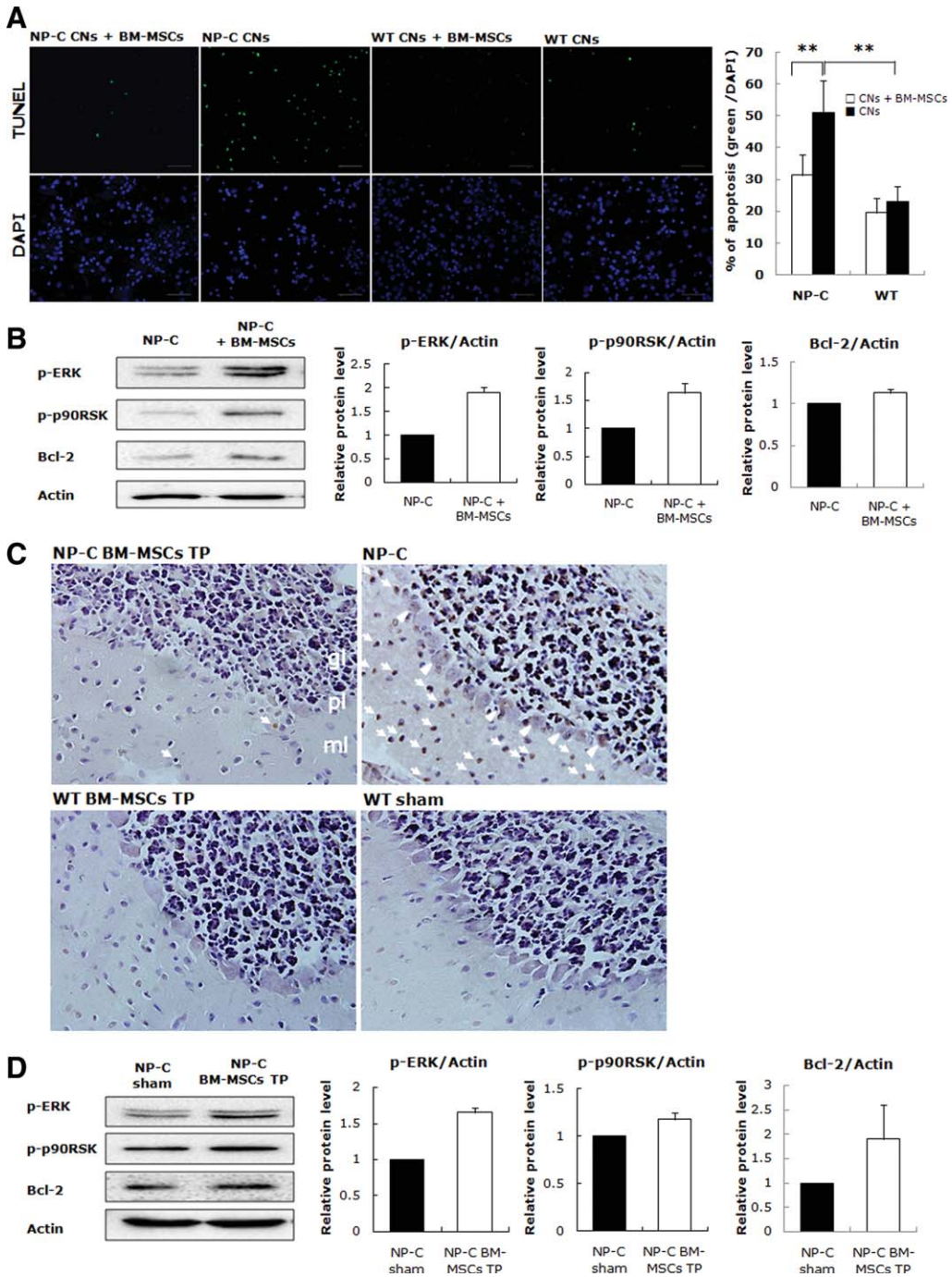

Figure 5. BM-MSCs inhibit apoptosis of NP-C mutant CNs. **(A):** Primary cultured CNs indirect cocultured with BM-MSCs or CNs alone were assessed using the TUNEL assay method. The percentage of apoptotic NP-C CNs were significantly increased compared to that of WT, and this was decreased when the NP-C CNs were cocultured with BM-MSCs. Data were expressed as mean ± SD; $n = 5$ for each group. Student's t test was used for statistical analysis. **, $p < .001$. **(B):** BM-MSCs treatment stimulates phosphorylation of extracellular signal-regulated kinase (ERK), ribosomal S6 kinase 1 (RSK-1), and expression of Bcl-2. Extracts from NP-C CNs cocultured with BM-MSCs or CNs alone were subjected to 12% SDS-PAGE and immunoblotted with antiphosphorylated ERK, antiphosphorylated RSK-1, anti-Bcl-2, and anti-β-actin. Similar results were obtained in three independent experiments. Values were normalized to β-actin levels. Values are mean ± SD. **(C):** BM-MSCs were transplanted into the cerebellum of 4-week-old NP-C or WT mice. The mice were sacrificed at 1 week after BM-MSC transplantation, and the brains were fixed and analyzed by TUNEL assay. Arrows indicate apoptotic cells in the molecular layer. Arrowheads show apoptotic cell death of PNs in Purkinje cell layer. BM-MSCs dramatically reduced the number of TUNEL-positive cells in the cerebellum of NP-C mice (×60) **(D):** BM-MSCs transplantation stimulates phosphorylation of ERK, RSK-1, and expression of Bcl-2. Extracts from the cerebellum of NP-C mice after BM-MSCs transplantation were analyzed by Western blot using antiphosphorylated ERK, antiphosphorylated RSK-1, anti-Bcl-2, and β-actin ($n = 3$ for each group). Similar results were obtained in three independent experiments. Values were normalized to β-actin levels. Values are mean ± SD. Abbreviations: BM-MSCs, bone marrow-derived mesenchymal stem cells; CNs, cerebellar neurons; DAPI, 4′,6-diamidino-2-phenylindole; gl, granular layer; GPN, Gly-Phe β-naphthylamide; ml, molecular layer; NP-C, Niemann-Pick type C; pl, Purkinje cell layer; PNs, Purkinje neurons; TP, transplantation; WT, wild type.

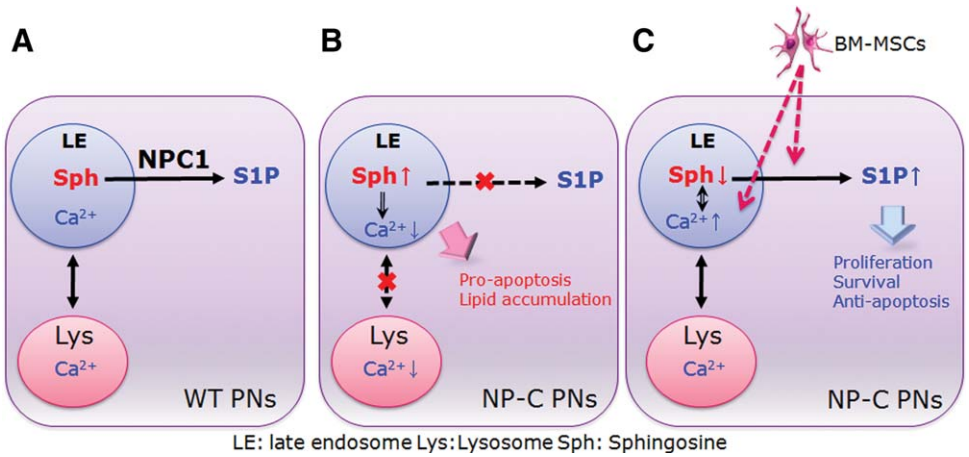

Figure 6. Schematic pathway proposed for the impact of BM-MSCs in the NP-C mutant cerebellar PNs. **(A):** Normal endocytic transport steps in WT PNs are a function of NPC1 and the transport of sphingosine. **(B):** Depletion of lysosomal calcium caused by sphingosine storage in NP-C mutant PNs leads to impaired endocytic function, apoptosis, and lipid storage. **(C):** BM-MSCs overcome defective lysosomal calcium release and correct sphingolipid metabolism, allowing endocytosis to continue in NP-C mutant PNs and promoting cell survival via the production of S1P. Abbreviations: BM-MSCs, bone marrow-derived mesenchymal stem cells; LE, late endosome; Lys, lysosome; NP-C, Niemann-Pick type C; PNs, Purkinje neurons; S1P, sphingosine-1-phosphate; Sph, sphingosine; TP, transplantation; WT, wild type.

mechanisms, resulting in the release of bioactive factors (e.g., cytokines, chemokines, and neurotrophins) [34]. In addition, the pattern of secreted cytokines and chemokines was dependent on the neurodegenerative microenvironment into which the BM-MSCs were transplanted. In accordance with previous reports, we wondered whether BM-MSCs could promote survival of NP-C mutant CNs, including PNs and GNs, without direct cell-to-cell contacts. We hypothesized that paracrine actions exerted by the BM-MSCs through the release of soluble factors might be an important mechanism of tissue repair and functional improvement in this disorder.

To examine this hypothesis, we cultured BM-MSCs with primary cultured CNs using a three-dimensional coculture system without direct contacts and confirmed the positive effect of BM-MSCs on the CNs. The number of surviving NP-C mutant PNs and GNs were significantly increased in the three-dimensional coculture system with BM-MSCs (Fig. 1D-1F), indicating that BM-MSCs may prevent loss of NP-C CNs by secreting bioactive trophic factors.

Understanding the exact mechanism(s) of neuropathology in NP-C should provide better insights for the development of treatment strategies for this disease. Biochemically, NP-C is characterized by extensive lysosomal accumulation of unesterified cholesterol in many tissues, and also lysosomal storage of sphingolipids in some tissues (e.g., liver and brain) [36]. Recently, several publications have raised the possibility that cholesterol may not be the primary toxic metabolite in NP-C disease [1, 6]. Supporting this concept, recent reports have shown that sphingosine storage is an initiating factor in NP-C disease pathogenesis [7]. The precise function of NPC1 is unknown, but when it is dysfunctional, sphingosine is rapidly accumulated in the lysosome due to reduced efflux. Normally, efflux from lysosomes is a key step in the salvage pathway leading to S1P generation [37] (Fig. 6A). Thus, cholesterol and other lipid abnormalities may be downstream events in NP-C disease pathogenesis caused by sphingosine storage [7].

Sphingolipids may affect neuronal differentiation, survival, and apoptosis [38], also suggesting that disturbed sphingolipid metabolism may contribute to the NP-C neurological phenotype (ataxia and tremor), and to the neuronal and glial loss observed in NP-C mice [39, 40]. So far, the relationship between NP-C pathophysiology and sphingolipid metabolism has been studied in NPC1 mutant fibroblasts and lymphocytes only [7], not in neuronal cells. Herein we investigated the levels of several sphingolipid metabolites in NP-C mouse mutant cerebellar PNs, and also the levels and activities of several lipid-related enzymes. For the first time we report the elevation of sphingosine and ceramide, and also reduction of S1P in dissected NP-C versus WT mouse PNs (Fig. 3). However, these patterns were not seen in primary cultured NP-C PNs (Fig. 2A-2E), likely because of differences in the degree of neuropathological progression (i.e., because the cultured NP-C cerebellar PNs were obtained from E18 fetuses, the lipid abnormalities had not yet progressed). Nonetheless, our findings clearly show the reduction of ceramide and sphingosine, and elevation of S1P in NP-C mice following BM-MSC treatment in vivo, correlating with increased survival of PNs.

S1P is an essential signaling lipid that regulates cell growth [41] and suppresses programmed cell death [42]. Therefore, our study suggested that the neuroprotective effect of BM-MSCs may be attributable to restoration of abnormal sphingolipid metabolism in NP-C mutant PNs, leading to increased S1P. In accordance with these results, we wondered whether direct S1P treatment promoted survival of PNs. To examine this hypothesis, we cultured primary NP-C mutant PNs with S1P and then injected S1P into the cerebellum of NP-C mice (Fig. 2F, 2G and supporting information Fig. 2). The survival rate of NP-C cerebellar PNs was increased slightly in the culture system, but the results did not reach statistical significance. There were also no significant differences in the survival rate of NP-C mutant PNs. These results suggested that the beneficial effects of BM-MSCs observed in vivo seem to be mediated by modulation of endogenous sphingolipid metabolism in NP-C CNs, not by a direct effect of secreted S1P.

Another emerging factor in the pathogenesis of several lysosomal sphingolipid storage disorders (Gauncher disease, Sandhoff disease, and GM1 gangliosidosis) is abnormal calcium homeostasis [43]. Sphingosine is a known inhibitor of protein kinase C, calcium/ATPases, and Na^+/Ca^{2+} exchangers [44], and sphingosine storage may inhibit calcium entry into the lysosome in NP-C PNs. As reported, sphingosine storage in the endo-/lysosomal compartment led to calcium depletion in these organelles, which then resulted in cholesterol, sphingomyelin, and glycosphingolipid storage in these compartments

(Fig. 6B). This calcium phenotype represents a new target for therapeutic intervention, as elevation of cytosolic calcium with curcumin normalized NP-C disease cellular phenotypes and prolonged survival of the NP-C mouse [7]. However, these previous studies did not investigate abnormal calcium homeostasis in NP-C cerebellar PNs. In this study we found that primary cultured NP-C mutant PNs have a large reduction in the endo-/lysosomal compartment calcium store compared to WT PNs, and that BM-MSCs correct the altered calcium levels in this compartment (Fig. 4B). The direct mechanism of BM-MSC-mediated correction of defective calcium and modulation of sphingolipid metabolism is not clear, although our experiments suggest that elevated calcium in the endo-/lysosomal compartment after BM-MSC treatment may be related to decreased sphingosine levels. Another potential explanation is an impact of upregulated S1P because this is also known to mobilize Ca^{2+} from internal stores primarily [45].

In NP-C mice, apoptotic cells could be detected in the cerebellum as early as 5 weeks of age, prior to the appearance of the neurological symptoms [11], and we have shown that the pro-apoptotic lipids, ceramide and sphingosine, also are elevated. It is also well known that stimulation by S1P can inhibit ceramide-induced apoptosis through the activation of ERK [42]. Thus, ceramide and S1P may exert opposing actions within cells via differential regulation of MAPK family members [46]. Our results demonstrate that BM-MSCs may inhibit apoptosis by activation of ERK/RSK and downstream antiapoptotic signaling cascades, in vitro and in vivo (Fig. 5). These results also suggested that increasing S1P following BM-MSC transplantation may exert its neuroprotective effect on NP-C mutant cerebellar PNs by regulating the expression of anti-apoptotic Bcl-2 protein in a MEK-dependent manner.

In summary, we have shown for the first time elevation of sphingosine and ceramide, and reduction of S1P, in NP-C mutant cerebellar PNs. Altered sphingolipid levels in NP-C mutant PNs were corrected after BM-MSC treatment, and in particular S1P levels were markedly elevated. These effects of BM-MSC on CNs could be observed without direct cell contacts in an in vitro culture system, suggesting that bioactive trophic factors may be released from BM-MSCs that modulate sphingolipid metabolism and calcium homeostasis in the brains of NP-C mice. These data therefore describe the mechanism underlying the therapeutic effect of BM-MSCs on the neuropathology of NP-C disease.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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