

## CCL2 induces neural stem cell proliferation and neuronal differentiation in Niemann-Pick type C mice

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**ABSTRACT.** Niemann-Pick type C disease (NP-C) is a rare and ultimately fatal lysosomal storage disorder with variable neurologic symptoms. Loss of neuronal function and neuronal cell death occur in the NP-C brain, similar to the findings for other neurodegenerative diseases. Targeting of neuronal cells in the brain therefore represents a potential clinical intervention strategy to reduce the rate of disease progression and improve the quality of life. We previously reported that bone marrow stem cells show a neurogenic effect through CCL2 (also known as monocyte chemoattractant protein-1, MCP-1) secretion in the brains of NP-C mice. However, the direct effect of CCL2 on neurogenesis has not been ascertained. Here, to define neurogenic effects of CCL2 in NP-C, we applied human recombinant CCL2 to neural stem cells (NSCs) derived from NP-C mice. CCL2-treated NSCs showed significantly increased capacity for self-renewal, proliferation and neuronal differentiation. Similar results were observed in the subventricular zone of NP-C mice after CCL2 treatment. Furthermore, infusion of CCL2 into the NP-C mouse brain resulted in reduction of neuroinflammation. Taken together, our results demonstrate that CCL2 is a potential new therapeutic agent for NP-C.

**KEY WORDS:** CCL2, neural stem cell, neurogenesis, neuroinflammation, Niemann-Pick Type C

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Niemann-Pick type C disease (NP-C) is a neurovisceral autosomal recessive lysosomal storage disease characterized by cholesterol and glycolipid accumulation in the endosomal/lysosomal system that results in hepatosplenomegaly and progressive neurodegeneration [19]. NP-C is neuropathologically characterized by abnormal lysosomal storage in neurons and glia, which causes neuronal apoptosis and neuroinflammation [6]. Currently, no treatment is available to cure or prevent neuronal cell death, which results in inevitable decline in NP-C.

In the adult brain, neurogenesis from neural stem cells (NSCs) and progenitor cells occurs in two regions: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone of the hippocampus [12]. The discovery of neurogenesis in the adult brain has raised the possibility of potential therapeutic applications to overcome neurodegenerative diseases, such as Parkinson's disease, Huntington disease and Alzheimer's disease [12]. Recent studies have shown that the capacity of NSCs for self-renewal and differentiation significantly decreases in NP-C mice [8, 17]. These

findings indicate that neurodegenerative diseases, such as NP-C, are suitable targets for therapeutic stimulation and regulation of neurogenesis.

Our previous study showed that bone marrow mesenchymal stem cells (BM-MSCs) contribute to improving neurogenesis in the NP-C mice [8]. Furthermore, we have shown that the neurogenic effects of BM-MSCs on NP-C mice occur through paracrine activity of CCL2 (also known as monocyte chemoattractant protein-1, MCP-1), as evidenced by decreased neurogenic efficiency of BM-MSCs after CCL2 knockdown. CCL2 is best recognized for its chemotactic and activating actions on monocyte/macrophages, T lymphocytes and dendritic cells [13]. Both CCL2 and its receptor (CCR2) are widely expressed by astrocytes, microglia, neurons and NSCs [1, 3, 7, 15], which suggests that they may act as modulators of neuronal cell functions.

Based on these concepts and findings, we hypothesized that direct treatment with CCL2 could promote neurogenesis in the NP-C mouse brain. Here, we show for the first time that CCL2 increases neurogenesis and decreases neuroinflammation in the NP-C mouse brain.

### MATERIALS AND METHODS

**Animals:** A colony of BALB/c *Npc1*<sup>nh</sup> mice was maintained for this study by brother–sister mating of heterozygous animals. Polymerase chain reaction was performed to determine the genotype of each mouse [11]. Four-week-old mice were used for treatment of human recombinant CCL2

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(R&D Systems, Minneapolis, MN, U.S.A.). We choose the block randomization method to allocate the animals to experimental groups. 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 temperature-controlled room on a 12-hr-light/12-hr-dark circadian cycle.

**Isolation and culture of NSCs:** We used a previously described method with some minor modifications [5]. Briefly, dissociated SVZ neurosphere (NS) cultures were prepared using either homozygous NP-C mice or control littermates at 3 weeks of age. The SVZ was removed after mice were deeply anesthetized with Avertin (250 mg/kg ip) and kept in ice-cold HibernateA/2% B27/0.5 mM Glutamax medium (HABG) (all from Invitrogen, Carlsbad, CA, U.S.A.). Each brain was then mechanically dissociated in HABG. The single-cell suspensions were filtered through a 40- $\mu$ m cell strainer, centrifuged for 4 min at 200 g and resuspended with NSC culture medium. The recipe for NSC medium is detailed below: to Neurobasal-A add 2% B27 Supplement, 0.5 mM Glutamax, 10 mg/ml gentamycin, 5 ng/ml mouse FGF2 and 5 ng/ml mouse PDGFbb (all from Invitrogen Bioscience, Camarillo, CA, U.S.A.). These cells ( $2 \times 10^5$  cells/well) were seeded on six-well tissue culture plate (all from BD Biosciences, Bedford, MA, U.S.A.). NSCs proliferated in suspension and formed aggregates referred to as NSs. Every 2 days, half of the medium in each well was replaced with fresh culture medium. To determine the optimal concentration of CCL2, we added CCL2 to the NSC culture medium over a final concentration range of 1–100 ng/ml, and the cells were incubated for an additional 7 days (Fig. 1A).

**NS formation assay:** To examine the effect of CCL2 on the formation of NSs, NSs were mechanically dissociated, and the resulting viable individual cells were counted. These cells ( $1 \times 10^4$  cells/well in uncoated 24-well plates, BD Biosciences) were also used for assessment of self-renewal ability in NSC culture medium. After CCL2 treatment, the newly formed NSs were counted in each well using a microscope (IX71; Olympus Co., Tokyo, Japan). A minimum cutoff diameter of 50  $\mu$ m was used to define NSs.

**NSC proliferation assays:** The proliferative activity of NSCs was evaluated by 5-ethynyl-20-deoxyuridine (EdU, Invitrogen) immunocytochemistry. Single cell suspensions from NSs were seeded on glass coverslips (Fisher Brand, Fisher Scientific, Pittsburgh, PA, U.S.A.) coated with poly-L-ornithine (Sigma, St. Louis, MO, U.S.A.) /laminin (Invitrogen) at a density of  $1 \times 10^4$  cells/cm<sup>2</sup>. After CCL2 treatment, cells were labeled with a 10  $\mu$ M EdU and incubated for an additional 12 hr.

**NSC differentiation assays:** For NSC differentiation assays, single-cell suspensions cultured *in vitro* for 7 days were plated on glass coverslips coated with poly-L-ornithine/laminin at a density of  $1 \times 10^4$  cells/cm<sup>2</sup>, followed by incubation in Neurobasal-A medium supplemented with 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 10  $\mu$ g/ml of heparin, 2% B-27 supplement and 3% FBS (all from Invitrogen, Grand Island, NY, U.S.A.). At seven days after plating, the differentiated cultures were processed for

immunofluorescence staining.

**Immunocytochemistry:** The cells were fixed with PBS (0.1 M) containing 4% para-formaldehyde at room temperature for 15 min and permeabilized with 0.1% Triton X-100 (all from Sigma) in PBS for 5 min. The cells were preincubated for 10 min with 3% normal goat serum (Vector Laboratories, Burlingame, CA, U.S.A.) and 2% bovine serum albumin (BSA, Invitrogen) in PBS containing 0.4% Triton X-100 to block background immunostaining. For NSC differentiation assays, differentiated cultures were incubated overnight with  $\beta$ III-tubulin (monoclonal, mouse, diluted 1:400; Chemicon, Temecula, CA, U.S.A.) as a marker for neurons and GFAP (polyclonal, rabbit, diluted 1:1,000; Dako, Glostrup, Denmark) as a marker for astrocytes. For visualization, the primary antibody was developed by incubation with Alexa Fluor 488- or 594-conjugated secondary antibodies (diluted 1:1,000, Molecular Probes, Carlsbad, CA, U.S.A.) for 1 hr at room temperature against a corresponding species. The cells were analyzed using a laser scanning confocal microscope equipped with Fluoview SV1000 imaging software (Olympus FV1000, Olympus Co.) or with a microscope (BX51; Olympus Co.).

**Reverse-transcriptase PCR and quantitative real-time PCR:** The RNeasy Lipid Tissue Mini kit or RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) was used for extraction of RNA from brain homogenates and cell lysates. cDNA was synthesized from 5  $\mu$ g of total RNA using the cDNA Synthesis Kit (Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer's protocol. The reaction was incubated at 42°C for 1 hr followed by 70°C for 10 min to arrest cDNA synthesis. Quantitative real-time PCR was performed using a Corbett research RG-6000 real-time PCR instrument (Corbett Life Science, Sydney, Australia). The thermocycle programs had 40 cycles with the following steps: denaturation at 95°C for 10 sec, annealing at 58°C for 15 sec and elongation at 72°C for 20 sec. The primers used in this study were following; Tubb3 (sense: 5'-AAT GAC CTG GTG TCC GAG TA-3', antisense: 5'-GGA CAG ATG CTG CTT GTC TT-3'), GFAP (sense: 5'-GAG GTG GAG AGG GAC AAC TT-3', antisense: 5'-CTG TCT ATA CGC AGC CAG GT-3'), Interleukin (IL)-10 (sense: 5'- AAG GCC ATG AAT GAA TTT GA-3', antisense: 5'- TTC GGA GAG AGG TAC AAA CG-3'), IL-4 (sense: 5'-ATC CAT TTG CAT GAT GCT CT-3', antisense: 5'-GAG CTG CAG AGA CTC TTT CG-3'), IL-1 $\beta$  (sense: 5'-CCC AAG CAA TAC CCA AAG AA-3', antisense: 5'- CCC AAG CAA TAC CCA AAG AA-3'), IL-6 (sense: 5'- TTG CCA TTG CAC AAC TCT TT-3', antisense: 5'-CCG GAG AGG AGA CTT CAC AG-3'), tumor necrosis factor (TNF)- $\alpha$  (sense: 5'-GCT CCA GTG AAT TCG GAA AG-3', antisense: 5'- GAT TAT GGC TCA GGG TCC AA-3') and GAPDH (sense: 5'-AAC AGC CTC AAG ATC ATC AGC-3', antisense: 5'-TTG GCA GGT TTT TCT AGA CGG-3').

**Treatment of CCL2 into NP-C mouse brains:** NP-C mice at approximately 4 weeks of age were anesthetized with a combination of 100 mg/kg ketamine and 10 mg/kg xylazine, and an injection was administered using a stereotaxic injection apparatus (Stoelting, Wood Dale, IL, U.S.A.) as previously described [8]. A glass capillary (1.2 mm  $\times$  0.6 mm)

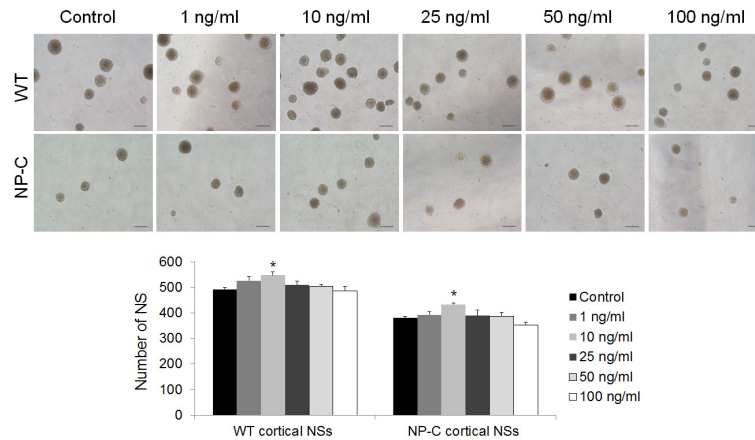


Fig. 1. Determination of the optimal CCL2 concentration for evaluation of neurogenic effects. To examine the neurogenic potential of CCL2, WT and NP-C NSCs were treated with different concentrations of human recombinant CCL2 (from 1 to 100 ng/ml), and self-renewal of NSCs was assessed. CCL2 increased the number of NSCs in WT and NP-C cultures at concentrations ranging from 1 to 25 ng/ml with maximum effect at 10 ng/ml (scale bar, 200 μm). All data are given as mean ± SEM. \**P*<0.05 compared with non-treated control.

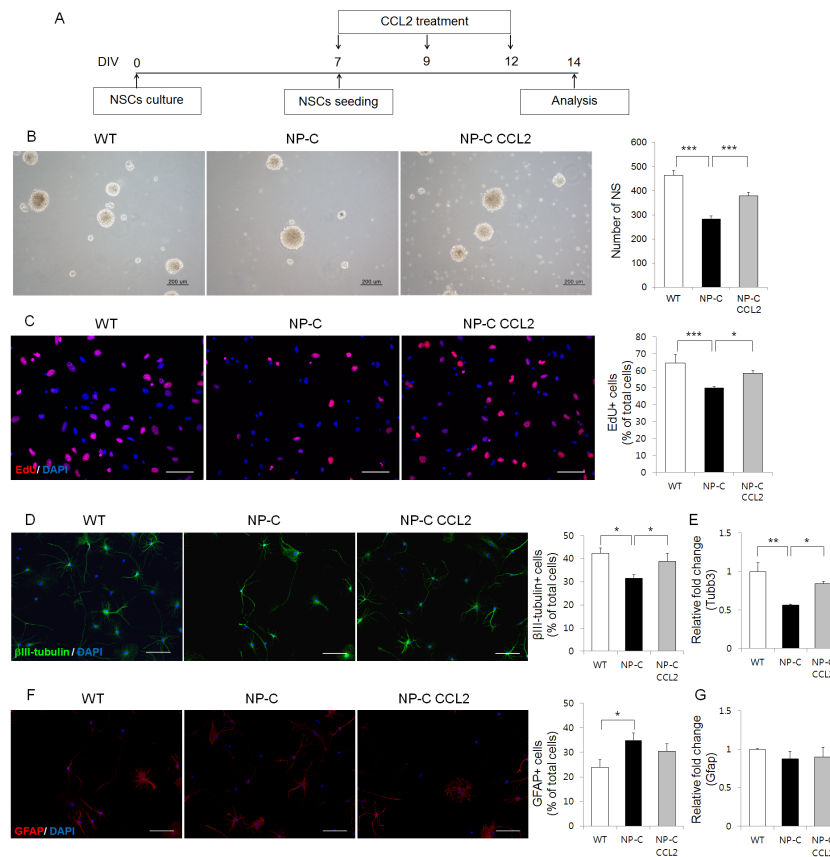


Fig. 2. CCL2 enhances the self-renewal, proliferation and neuronal differentiation of NP-C NSCs. (A) Experimental design to determine the effect of CCL2 on NP-C NSCs. (B) Light micrographs of NSs following CCL2 treatment in NP-C NSCs (scale bar, 200 μm). Self-renewal capacity was expressed as the number of NSs (n=3 per group). (C) Effect of CCL2 on the proliferation of NP-C NSCs (scale bar, 50 μm). Proliferation ability was assessed by the percentage of EdU-positive cells (n=3 per group). (D-G) CCL2 induced NP-C NSCs to undergo neuronal differentiation as measured by immunocytochemistry and quantitative real-time PCR. Representative fluorescence images and quantification of (D) βIII-tubulin (scale bar, 50 μm, n=3 per group) and (F) GFAP (scale bar, 50 μm, n=3 per group). Quantitative real-time PCR analysis of differentiation markers for (E) neurons (Tubb3) and (G) astrocytes (Gfap) (n=5 per group). All data are given as mean ± SEM. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.005.

was used for injection of CCL2 into the SVZ [8]. Approximately 3  $\mu$ l of CCL2 (10 ng/ml) was infused at a rate of 0.3  $\mu$ l/min at +0.74 anterior/posterior, -1.5 medial/lateral, -2.5 dorsal/ventral relative to the bregma in the SVZ. The same volume of PBS was injected at the same rate into the control mice. After treatment, the scalp was closed by sutures, and the animals were allowed to recover from the anesthesia before being returned to their cages. Age-matched normal littermates in the NP-C colony were used as controls.

**BrdU injection:** Mice received intraperitoneal injection of BrdU (Sigma) dissolved in 0.9% NaCl/0.007 M NaOH solution for 7 days (50 mg/kg).

**Tissue preparation:** Animals were deeply anesthetized with Avertin (250 mg/kg ip) and transcardially perfused with 4% paraformaldehyde in PBS at 7 and 30 days after CCL2 treatment. After perfusion, the brains were removed, postfixed overnight at 4°C and incubated in 30% sucrose at 4°C until equilibration. Sequential 30- $\mu$ m-thick transversal sections were obtained using a cryostat (CM3050S; Leica, NuBloch, Germany) and stored at -20°C.

**Immunohistochemistry:** Brain sections were treated with PBS containing 5% normal goat serum, 2% BSA and 0.4% Triton X-100 for 1 hr. In the same buffer solution, the sections were then incubated for 24 hr in anti-GFAP (polyclonal, rabbit, diluted 1:500; DAKO) antibodies at 4°C. Prior to BrdU histochemistry, cerebral sections were incubated in 2 N HCl for 1 hr at 37°C, followed by incubation in 0.1 M borate buffer for 10 min. The sections were then incubated overnight at 4°C in a mixture of anti-BrdU (monoclonal, mouse, diluted 1:50; BD Biosciences, San Jose, CA, U.S.A.) and anti-doublecortin (anti-DCX) (polyclonal, rabbit, diluted 1:1,000; Abcam, Cambridge, U.K.) antibodies. For visualization, sections were incubated in secondary antibodies for 1 hr at room temperature followed by washes. Alexa Fluor 488 and 594 were used as secondary antibodies.

**Stereology:** Cell proliferation and neuronal differentiation were quantified using stereologic counting of BrdU and DCX<sup>+</sup> cells in the SVZ. Systematic random sampling was obtained using every fifth section in a series of 30- $\mu$ m-thick coronal sections through the striatal SVZ. Equidistant sections (five per brain) were used for stereologic counting to assess cell proliferation and neuronal differentiation in the SVZ. All counting was performed through a 60 $\times$  objective lens on a microscope (BX51; Olympus Co.) equipped with a digital camera (DP70; Olympus Co.) and Visiomorph software (Visiopharm, Hoersholm, Denmark).

**Behavioral studies:** Motor coordination and balance were tested with the rotarod test. We performed the rotarod test using an accelerating rotarod (UGO Basile Accelerating Rotarod; Ugo Basile, Comerio VA, Italy) by placing a mouse on a rotating drum (3 cm diameter) and measuring the period that each animal was able to maintain its balance on the rod, as latency to fall (seconds). The machine was set to an initial speed of 4 rpm, and the acceleration was increased by 32 rpm every 25–30 sec. CCL2-treated NP-C mice were analyzed along with sham-transplanted NP-C and normal control mice. Scores were registered every 2 days beginning at 72 hr after treatment, and three independent tests were

performed at each measurement. Uniform conditions were carefully maintained for each test, and there was a rest time of 1 hr between trials. Each test was limited to 300 sec.

**Statistical analysis:** Comparisons between two groups were performed with Student's *t*-test. Tukey's HSD test and repeated measures analysis of variance test were used for multigroup comparisons using the SPSS statistical software. Differences were accepted to be statistically significant at  $P < 0.05$ .

## RESULTS

**CCL2 enhances the self-renewal, proliferation and neuronal differentiation of NP-C NSCs:** To examine the neurogenic potential of CCL2, we first evaluated the effect of human recombinant CCL2 on self-renewal of NSs at different concentrations. CCL2 increased the number of NSs in WT and NP-C cultures at concentrations ranging from 1 to 25 ng/ml with maximum effect at 10 ng/ml (Fig. 1). Therefore, we used a CCL2 concentration of 10 ng/ml for subsequent experiments (Fig. 2A). Compared with WT NSs, NP-C NSs showed significantly decreased self-renewal ability. However, CCL2-treated NP-C NSs showed increased formation of NSs (Fig. 2B). To assess proliferation, the percentage of EdU labeled cells was determined. CCL2 increased EdU incorporation in NP-C cultures relative to that in untreated NP-C cultures (Fig. 2C). In addition to self-renewal and proliferation, multilineage differentiation is a hallmark of stemness in NS cultures. To examine the effect of CCL2 on neuronal differentiation, NSs were dissociated and treated with CCL2 in differentiation media. After 7 days, expression of markers for neurons and astrocytes was evaluated in NSC-derived cells. Compared with WT cells, NP-C NSCs showed significantly decreased neuronal differentiation and increased astrocyte differentiation (Fig. 2D–2G). CCL2 treatment of NP-C NSCs resulted in increased efficiency of neuronal differentiation (Fig. 2D–2G). Taken together, our data suggest that CCL2 increases self-renewal, proliferation and neuronal differentiation of NP-C NSCs.

**CCL2 promotes SVZ neurogenesis in NP-C mice:** To examine the *in vivo* effect of CCL2 on neurogenesis, we injected human recombinant CCL2 into 4-week-old NP-C mouse brains. For assessment of the number of proliferating cells in the SVZ, BrdU was injected for the first 7 days (Fig. 3A). Similar to previous results [8], the total number of BrdU-positive cells significantly decreased in the brain of NP-C mice, and this effect was ameliorated in CCL2-treated NP-C mice (Fig. 3B). To confirm neuronal differentiation of BrdU-positive cells, we quantified new neurons by counting DCX and BrdU-double-positive cells at 1 week after CCL2 treatment (Fig. 3C). As expected, CCL2-treated NP-C mice showed a significantly increased number of DCX and BrdU-double-positive cells when compared with non-treated NP-C mice (Fig. 3C). These results demonstrate that CCL2 increases SVZ neurogenesis in NP-C mice.

**CCL2 ameliorates neuroinflammation in NP-C mice:** To investigate whether treatment of CCL2 affects glial cell activation, we examined astrocyte activation in NP-C mice

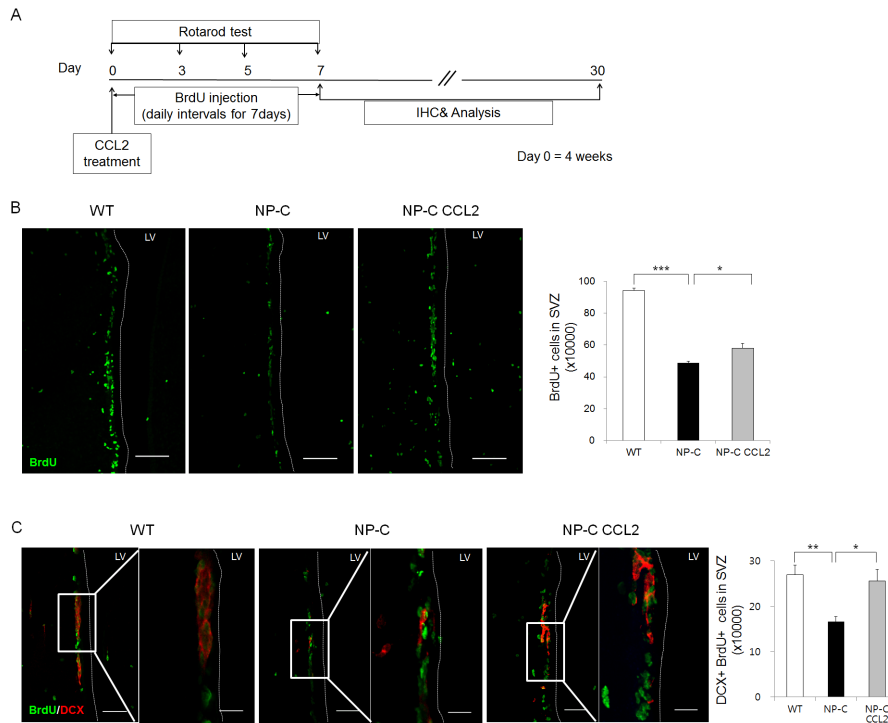


Fig. 3. CCL2 promotes SVZ neurogenesis in NP-C mice. (A) Experimental design to determine the neurogenic effect of CCL2 on the NP-C mouse brain. (B) Confocal images and quantification of BrdU-labeled cells in the SVZ after CCL2 treatment (scale bar, 100  $\mu$ m, n=3 per group). (C) Representative images and numerical density of BrdU/DCX-double-positive cells in CCL2-treated NP-C mice (scale bar, 100  $\mu$ m, n=3 per group). All data are given as mean  $\pm$  SEM. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.005.

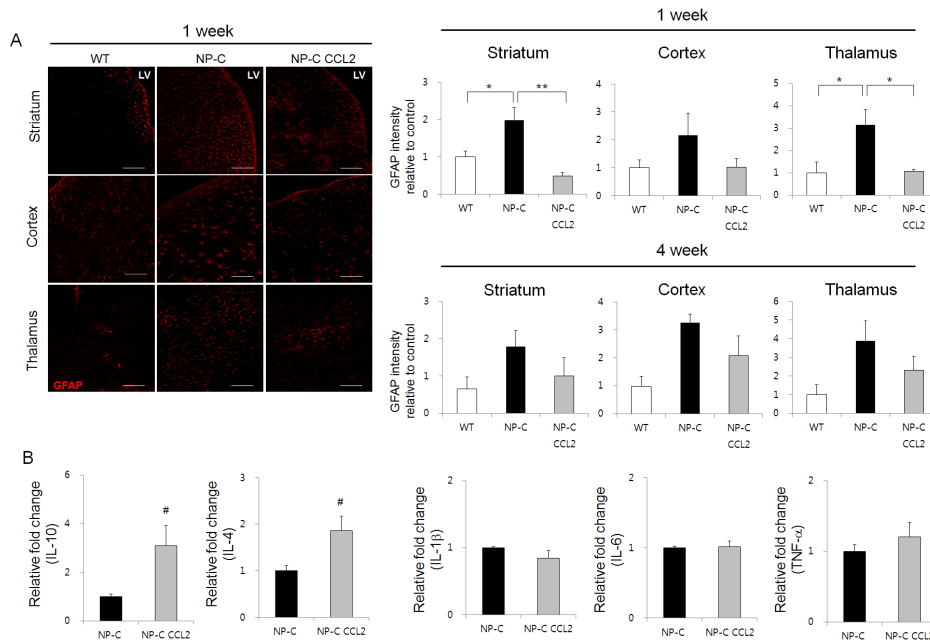


Fig. 4. CCL2 ameliorates neuroinflammation in NP-C mice. (A) Representative images and quantification of GFAP on days 7 and 28 after CCL2 treatment (scale bar, 100  $\mu$ m, n=3 per group). Quantification of GFAP immunostaining for activated astrocyte cells was performed in the striatum, cortex and thalamus. (B) Expression of anti-inflammatory cytokines (IL-10 and IL-4) and pro-inflammatory cytokines (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) was measured by quantitative real time PCR in the brain region of CCL2 injection (striatum close to the SVZ). The expression of the different genes was normalized by GAPDH (n=4 per group). All data are given as mean  $\pm$  SEM. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.005.

using GFAP staining. Astrocyte activation was significantly higher in the brains of NP-C mice than in those of WT mice, but decreased in the brains of CCL2-treated NP-C mice (Fig. 4A). Next, we examined whether CCL2 injection had a sustained effect on reduced astrocyte activation in NP-C mice. For this study, we assessed astrocyte activity at 4 weeks after CCL2 treatment and found that the activity of these cells was still decreased in CCL2-treated NP-C mice compared with non-treated NP-C mice, although this effect did not reach statistical significance (Fig. 4A). To further evaluate the influence of CCL2 on the inflammatory immune responses in NP-C mice, we examined the expression of the several anti-inflammatory cytokines (IL-10 and IL-4) and pro-inflammatory cytokines (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) mRNAs. The results revealed clear induction in the expression of IL-10 and IL-4 mRNA in the CCL2-treated NP-C mice compared with non-treated NP-C mice (Fig. 4B). The expressions of pro-inflammatory cytokines were not different between the two groups (Fig. 4B). These data indicate that the CCL2 treatment could modulate the inflammatory reactions by up-regulation of anti-inflammatory cytokines expression in NP-C mice.

There were also slight improvements in the rota-rod score of CCL2-treated NP-C mice compared to those of non-treated NP-C mice, although this difference did not reach statistical significance (Fig. 5).

## DISCUSSION

NP-C is a rare complex metabolic lysosomal storage disorder that leads to progressive deterioration of the nervous system and multiple organ systems in the body [18]. Neuropathologically, both human and murine NP-C is characterized by ballooned neurons distended with lipid storage, axonal spheroid formation and widespread neuronal loss [6]. Therefore, restoration of neuronal function and replenishment of injured neurons are therapeutic strategies for NP-C. We previously demonstrated that CCL2 was up-regulated in the brain of NP-C mice after BM-MSc treatment. We also found that CCL2 derived from BM-MSCs activated neurogenesis in NP-C mice [8]. In this study, we aimed to investigate the direct effects of CCL2 on neurogenesis in NP-C mice to confirm our previous findings.

CCL2 plays a role as an inflammatory mediator associated with the pathogenesis of inflammatory neurodegenerative and autoimmune diseases, including multiple sclerosis, Alzheimer's disease and stroke, in addition to viral and bacterial infections of the CNS [7]. Accumulating evidence has shown that independent of neuropathology, chemokines and their receptors are not only expressed in the developing brain but also play a role in orienting cell migration, trophic support, proliferation and differentiation [2, 10]. In a mouse model of stroke, CCL2 was shown to promote SVZ neuroblast migration toward the ischemic lesion [16]. In the present *in vitro* study, we observed a significant increase of self-renewal, proliferation and neuronal differentiation in CCL2-treated NP-C NSCs compared with non-treated cells (Fig. 2). Similarly, investigations of the neurogenic

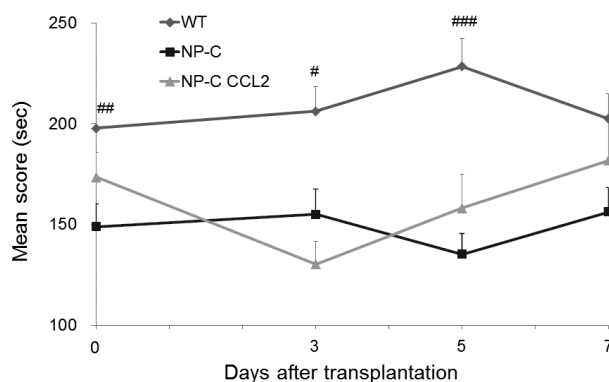


Fig. 5. CCL2 elicited functional improvement in NP-C mice. Rota-rod scores of mice were averaged and plotted beginning 3 days after CCL2 treatment (WT and NP-C,  $n=15$ ; CCL2-treated NP-C,  $n=9$ ). All data are given as mean  $\pm$  SEM. # $P<0.05$ , ## $P<0.01$ , ### $P<0.005$  compared to non-treated NP-C mice.

potential of CCL2 in NP-C mice revealed that treatment of CCL2 significantly increased the total number of new neurons in the SVZ compared with non-treatment (Fig. 3). Although the mechanisms of CCL2-induced neurogenesis in NP-C remain to be explored and whether CCL2-mediated newborn neurons are electrically active and functional, our data demonstrate that elevation of the CCL2 level improves proliferation and neuronal differentiation capacity of NSCs in a mouse model of NP-C, suggesting that CCL2 injection is a potential future therapy for NP-C patients.

Neuroinflammation is a common feature found in many disorders, particularly in those affecting the CNS. Pathological changes in glial cells have also been reported in mouse models of NP-C [14]. Glial cells have cytotoxic effects, and glial activation has been considered to be a key process leading to neuronal degeneration in lysosomal storage disorders, such as NP-C [4, 9]. In this study, we observed a significant decrease of neuroinflammation in the striatum and thalamus of NP-C mice after CCL2 treatment (Fig. 4A). CCL2-treated NP-C mice also showed slight decreased GFAP immunoreactivity in cortex compared with non-treated NP-C mice, but this did not reach statistical significance which might be related to the fact that the CCL2 was treated into the SVZ. In addition, we observed that the expression of IL-10 and IL-4 was increased in the SVZ of CCL2 treated NP-C mice compared with non-treated mice, although there were no differences in expression of pro-inflammatory cytokines. These results represent that reduced neuroinflammations after CCL2 treatment may have a relation, at least in part, with increased IL-10 and IL-4 expression by CCL2 treatment (Fig. 4B). As neurodegenerative diseases including NP-C are currently without treatment, targeting of secondary disease components, such as inflammation, is an attractive approach. Therefore, anti-inflammatory effects of CCL2 may be of potential benefit in NP-C, either alone or more likely in conjunction with other therapeutic agents.

Overall, the data presented here show that CCL2 can reduce NP-C pathology by increasing neurogenesis and

decreasing neuroinflammation in NP-C mice. Despite data supporting the neurogenic effects of CCL2 in NP-C, the clinical effectiveness of CCL2 treatment in the NP-C mouse model was modest (Fig. 5). There are several potential explanations for this finding. First, it must be recognized that in these animal model experiments, the primary lesion in the *Npc1* gene and protein remains in the treated mice, and thus, CCL2-induced neurogenesis may only slow progression and require additional, combinational therapies to achieve a more complete clinical effect. In addition, these positive but relatively moderate results in CCL2-treated NP-C mice may also have resulted from under-dosing of the animals. We speculate that these effects may be improved in the future by adjusting the dose or increasing the frequency of treatment. Lastly, the methods used to introduce CCL2 into the brain of the NP-C mice may need to be improved, and there are considerable research underway exploring different approaches to introducing proteins, such as CCL2, into the central nervous system. Despite the limitations of these animal model studies, however, the findings reported in the manuscript reveal a new potential approach for therapy via its neurogenesis-promoting properties.

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