Donepezil Enhances Purkinje Cell Survival and Alleviates Motor Dysfunction by Inhibiting Cholesterol Synthesis in a Murine Model of Niemann Pick Disease Type C

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Abstract

Neurodegenerative processes are often accompanied by disruption of cholinergic systems; therefore, acetylcholinesterase (AChE) inhibitors (AChEIs) may have therapeutic potential in some neurological conditions. We evaluated the effects of administration of donepezil, a widely used AChEI, in the cerebellum in a murine model of Niemann-Pick disease type C (NPC). The NPC mice developed Purkinje cell loss at the age of 8 weeks; 4-week-old NPC mice given donepezil led to improvement of Purkinje cell survival that was associated with improvement of motor dysfunction in the mice. Because abnormal accumulation of cholesterol caused by impaired lipid homeostasis is the principal pathogenic mechanism underlying NPC, we investigated the effects of donepezil on cholesterol metabolism in the NPC mice. Donepezil treatment reduced cholesterol accumulation in adult neural stem cells in vitro, and it downregulated the expression of the cholesterol synthesis factors' sterol regulatory element-binding proteins and 3-hydroxy-3-methylglutaryl-CoA reductase in the cerebellum, implying that AChE activity might be associated with cholesterol homeostasis. Taken together, our findings suggest the role of a cholinergic pathway as a novel regulator of NPC progression and the potential application of AChEIs for the treatment of human NPC.

Key Words: Acetylcholinesterase inhibitor, Cholesterol, Cholinergic system, Donepezil, Neural stem cell Niemann Pick disease Type C, Purkinje neuron.

INTRODUCTION

Niemann-Pick disease type C (NPC) is a lysosomal lipid storage disorder that is caused by dysfunction of either NPC1 or NPC2 protein, each of which has important roles in sterol and lipid trafficking in mammalian cells (1). To date, NPC is incurable and typically leads to premature death by childhood. In most cases, neurological symptoms such as ataxia, tremor, and dementia develop in association with neurodegeneration (2). Because the involvement of the CNS is a decisive factor in determining the prognosis of the disease, many attempts have been made to elucidate the underlying mechanism of neurodegeneration (3). Given that maintaining the integrity of a neurotransmitter system is crucial for control of brain function (4), it would be worthwhile to investigate the relationships between neurotransmitter activity and NPC pathology. Although it is reported that the dopaminergic pathway is disrupted in the thalamus of NPC mice (5), other neurotransmitter systems including acetylcholine (ACh) have scarcely been investigated in NPC.

The “cholinergic hypothesis” is founded on the basis of several studies of neurodegenerative disease demonstrating that a decline in cholinergic activity is closely related to the severity of dementia and neurodegeneration (6). Indeed, the development of various acetylcholinesterase inhibitors (AChEIs), which prevent the breakdown of ACh, has provided a great advance in the therapeutic field of Alzheimer disease (7). Furthermore, positive outcomes have also been reported after the administration of AChEIs in other diseases, including myasthenia gravis (8), Parkinson disease (9), and Huntington disease (10). Therefore, consistent maintenance of the ACh concentration might be a principal strategy in the treatment of neurological disorders.

Here, we conducted a study of a 4-week-long administration of donepezil, one of AChEIs approved for the management of Alzheimer disease, into a murine model of NPC to evaluate the therapeutic potential of AChEIs in cerebellar degeneration. Importantly, donepezil delayed the loss of cerebellar Purkinje cells and improved motor function of NPC mice via regulating the cholesterol-related homeostasis. Thus, our data emphasize the importance of the cholinergic system in pathogenetic mechanisms of NPC and suggest a novel therapeutic approach to the disorder.

MATERIALS AND METHODS

Animal Model

Breeding pairs of BALB/c heterozygous (NPC1+/−) mice were purchased from Jackson Laboratories (Bar Harbor, ME), and genotyping was performed as previously described.

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Adult Neural Stem Cell Primary Culture

Adult neural stem cells (NSCs) isolated from the subventricular zone of 4-week-old mice were cultured in a Neurobasal-A medium supplemented with N2, B27, penicillin-streptomycin, epidermal growth factor, basic fibroblastic growth factor, and GlutaMax (all purchased from Gibco, Carlsbad, CA) for neurosphere formation. NSCs were then dissociated, and 5 × 10^3 cells were seeded on a poly-L-ornithine (Sigma-Aldrich, St. Louis, MO)/fibronectin (Becton Dickinson, Franklin Lakes, NJ)-coated coverslip for further experiments. To induce accumulation of cholesterol, NSCs were treated with an NPC-mimicking agent U18666A (1 μg/mL; Tocris Bioscience, Ellisville, MO) to impede intracellular cholesterol transport for 24 hours. To inhibit the cholinesterase activity, donepezil hydrochloride (Sigma-Aldrich) was added into the culture medium at the concentration of 10⁻⁵ mol/L for 24 hours. Inhibitory effect on NPC morphology was characterized using Image J software.

In Vivo Donepezil Administration

The effects of cholinesterase inhibition on NPC pathology were evaluated following a 4-week-long intraperitoneal administration of donepezil hydrochloride (0.33 mg/kg or 1.0 mg/kg, dissolved in normal saline) into 4-week-old NPC mice. Control groups received normal saline instead of donepezil.

Rota-Rod Test

Coordination ability of motor function was evaluated using a Rota-rod treadmill (7650 Accelerating model, Ugo Basile Biological Research Apparatus, Comerio, Italy). At 4 weeks of age, the mice were trained for 1 week, and their motor function was then tested once a week for 5 to 8 weeks of age at the speed of 10 rpm with the maximum duration of 180 seconds. The representative record of each subject was adopted as the mean performance time of 4 attempts.

Tissue Preparation

For histological analysis, mice were perfused with PBS and 4% paraformaldehyde at 8 weeks of age. Whole brain tissues were postfixed in 4% paraformaldehyde for 1 day, then in 30% sucrose until they set. The brains were then cryopreserved with an OCT compound (Sakura Finetek, Tokyo, Japan) and stored at −80°C. For RNA extraction, 8-week-old mice were perfused with PBS (pH 7.4) overnight with primary antibodies against calbindin (CBP; 1:500, Millipore, Billerica, MA), a reliable marker for Purkinje neurons [14]. Samples were then incubated for 2 hours with secondary antibody Alexa488 (1:1000, Invitrogen) at room temperature followed by nuclei staining for 2 hours with DAPI (1 μg/mL, Santa Cruz Biotechnology, Santa Cruz, CA). For Nissl staining, brain sections were mounted onto the gelatin-coated slides and dried overnight in the oven. Slides were soaked into a 1:1 alcohol/chloroform solution to reduce background and then rehydrated through 100% and 90% alcohol to distilled water. Nissl staining was performed by staining the samples in prewarmed 0.1% cresyl violet solution for 10 minutes. Slides were transferred into 95% alcohol for 20 minutes then dehydrated in 100% alcohol. Samples were mounted using Canada balsam after a xylene-clearing step. IHC- and Nissl-stained images were captured using a confocal microscope (Nikon, Eclipse TE2000, Tokyo, Japan). Only sections that contain all cerebellar layers (I to X; [Fig. 1]; 5 sections per animal, total of 4 mice) were used per group; they were sequentially selected (90-μm apart from each other), and whole cerebellum was classified into anterior (layer I–V) and posterior (layer VI–X) parts for Purkinje cell counting. Numbers of CBD-positive cells (IHC sample) or large amorphous cells with relatively pale soma (Nissl-stained samples) were normalized to the length of the Purkinje cell layer to calculate the mean density of Purkinje neurons, as previously described [15]. Total cell counting and measurement of the length of the Purkinje cell layer were conducted using Image J software by a researcher blinded to the experimental conditions.

Filipin Staining

Filipin staining was performed for the evaluation of intracellular cholesterol level. NSCs and sagittal brain sections (3 sections per animal, total of 3 mice per group) were stained with a filipin-working solution (50 μg/mL, Sigma-Aldrich) for 1.5 hours at room temperature; nuclei were then stained with propidium iodide (5 μM, Sigma-Aldrich) for 10 minutes. Images were captured immediately using a confocal microscope. All procedures were protected completely from light. The density of the filipin-positive area was measured using Image J software by a researcher blinded to the experimental conditions.

RNA Isolation and Quantitative Reverse Transcript-Polymerase Chain Reaction

Total RNA was isolated from whole cerebellum with a TRizol reagent (Invitrogen) according to the manufacturer’s protocol, and cDNA was synthesized by using a Maxime RT premix (Intron, Seongnam, Korea). Gene-specific primers were designed using Primer Express Software (PE-Applied Biosystems, Warrington, UK) as follows:

Liver X Receptor β (LXRB):
(F: 5’-TCCTGGGATGTCGACGGTAG-3’;
R: 5’-GACCTTG
TACCTCACACT-3’)
Sterol regulatory element-binding protein 1 (SREBP1):
(F: 5’-GTGACCTGGACCAAGCAATCA-3’;
R: 5’-GGTGCC
TAGAGCAAGAGG-3’),
Sterol regulatory element-binding protein 2 (SREBP2): (F: 5'-TGTGGAGCAGTCTCAACGTC-3'; R: 5'-TGGTAGGCTCTCACCCAGGAG-3'),
3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR): (F: 5'-TCTTTCCGTGCTGTGTTCTG-3'; R: 5'-TTTTAACCCACGGAGAGGTG-3'),
ATP-binding cassette transporter (ABC) A1: (F: 5'-CAACAGTTTGTGGCCCTTTT-3'; R: 5'-AGTTCCGGCTGGGGTACTT-3'),
ABCG1: (F: 5'-GAAGTGGCATCAGGGGAGTA-3'; R: 5'-AAAGAACGGGTTCACATCG-3'),
ABCG5: (F: 5'-TCACTTGCATTGCTTCTCTG-3'; R: 5'-TTGCTGAGGAGGTG-3'),
and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): (F: 5'-GGAAGGGCTCATGACCACAG-3'; R: 5'-GCAAGGGATGATGTTCTGGGC-3').

Polymerase chain reaction (PCR) electrophoresis was analyzed by GelDoc XR system (Bio-Rad, Hercules, CA), and quantitative RT-PCR was performed using SYBR Green (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. All amplifications were analyzed using Prism 7000 sequence detection system 2.1 software (Applied Biosystems).

Statistical Analysis
The results are shown as the mean ± SE of independent experiments. Except where noted, all statistical analyses were performed by one-way ANOVA followed by a Bonferroni posttest using GraphPad Prism (version 5.01, GraphPad Software, San Diego, CA).

RESULTS

Purkinje Cell Degeneration in NPC Mice
To confirm that specific loss occurs among the Purkinje cell population in the cerebellum of NPC mice (16), we assessed the cerebellar distribution pattern of Purkinje cells.
using immunohistochemistry in 8 week-old WT and NPC mice (Fig. 1B). As expected, considerable damage to the structural integrity of the Purkinje cell layer was found in the NPC mice. In addition, the loss of Purkinje cells was more severe in the anterior region (lobules I–V) than in the posterior region (lobules VI–X), as previously described (17). In WT mice, there were 26.22 and 26.37 CBD-positive cells per mm in the anterior and posterior Purkinje cell layer, respectively, whereas only 3.22 and 5.71 cells, respectively, were detected in the same regions in NPC mice (Fig. 1D). Similarly, evaluation with Nissl staining revealed the vulnerability of Purkinje cells in the NPC mice (anterior: 2.04 cells per mm; posterior: 4.10 cells per mm) versus WT mice (anterior: 23.51 cells per mm; posterior: 23.41 cells per mm) (Fig. 1C, E).

Inhibition of acetylcholinesterase improves the survival of Purkinje cells in the cerebellum and ameliorates motor dysfunction in NPC mice

Proper cholinergic system function is important for the maintenance of neurogenesis and neuronal survival (18, 19). It has also been reported that the number of Purkinje cells in the cerebellar cortex is regulated by ACh concentration (20). Therefore, we investigated whether enhanced ACh activity can promote the survival of Purkinje neuron in NPC mice. To prolong the presence of ACh in vivo, we administrated donepezil, which prevents the decomposition of ACh into acetate and choline, into 4-week-old NPC mice for 4 weeks. We then evaluated the protective effect of donepezil on the Purkinje neuronal degeneration using histological analysis. Importantly, CBD expression in the cerebellum was greater than in the control mice after chronic treatment with donepezil in a dose-dependent manner (Fig. 2A, B). In the posterior lobules, the total number of CBD-positive cells was increased approximately 2.0-fold and 2.3-fold after low dose (0.33 mg/kg; NPC-DL) and high dose (1 mg/kg; NPC-DH) donepezil treatment, respectively (Fig. 2C). Remarkably, donepezil provided an even greater protective effect on Purkinje cells in the anterior region of the cerebellum, where Purkinje cell loss occurs more severely compared with the posterior region (17). We detected a 4.4-fold (NPC-DL) and 6.6-fold (NPC-DH) higher number of Purkinje neurons in the anterior region versus saline-treated NPC mice (NPC-C) (Fig. 2C). Nissl staining also showed that the number of Purkinje neurons residing in the cerebellar cortex was increased in donepezil-treated NPC mice (Fig. 3A, B) (ratio to NPC-C anterior: 3.46 in NPC-DL and 7.01 in NPC-DH, posterior: 1.31 in NPC-DL and 1.74 in NPC-DH). Therefore, our results suggest that loss of Purkinje cells is partially rescued by donepezil treatment in the cerebellum in both the anterior and posterior regions.

Purkinje neurons conduct essential roles in motor coordination (4). Based on the protective effect of donepezil on Purkinje cell survival, we next conducted the Rota-rod test with control and donepezil-treated NPC mice to define the pharmacological effect of donepezil on motor function (Fig. 3C). All of NPC mice could endure the test for 180 seconds at 5 weeks of age, while the riding time of the control NPC group gradually decreased with the disease progression (127 seconds, 104 seconds, and 54 seconds at 6, 7, and 8 weeks of age, respectively). On the other hand, chronic treatment of donepezil delayed the loss of motor function in NPC mice. The average latencies of the NPC-DL and NPC-DH group increased almost 20% and 30% than the NPC-C group at 6 and 7 weeks of age, respectively. Moreover, the protective impact of high dose administration of donepezil on motor coordination ability lasted for 4 weeks; the performance time of the NPC-DH group was almost doubled at 8 weeks of age compared with the that of age-matched controls. Therefore, our results provide evidence that donepezil alleviates the motor dysfunction of NPC mice in a dose-dependent manner.

Donepezil Reduces Cholesterol Accumulation via Inhibition of Cholesterol Synthesis in NPC Mice

To explain how donepezil administration provides positive effects on the NPC pathology, we investigated whether donepezil can modify the disrupted cholesterol metabolism in an NPC state considering that abnormal accumulation of cholesterol is a key pathology of NPC. First, WT-derived adult NSCs were pretreated with U18666A for 24 hours to induce intracellular cholesterol accumulation as an NPC-like condition (21) and then further cultured with or without donepezil for 72 hours. Comparative measurement of cholesterol level was performed using filipin staining, a widely used method to detect cholesterol. U18666A-treated NSCs contained much more intracellular cholesterol compared with control NSCs (Fig. 4A). Thus, filipin staining revealed that donepezil significantly reduced the U18666A-induced cholesterol accumulation in vitro. The optical density of the filipin-positive area within the cytoplasm of control NSCs increased about 8.27-fold after U18666A treatment, whereas it is reduced by 40% after donepezil treatment (Fig. 4B).

We also evaluated in vivo the accumulation pattern of cholesterol in the cerebellum of the NPC-C and NPC-DH group using filipin staining (Fig. 4C). In line with in vitro experiments, chronic administration of donepezil led to a considerable decline in cholesterol level in the NPC cerebellum (Fig. 4D).

To clarify the mechanism of the beneficial action of donepezil on abnormal cholesterol increment, mRNA expression levels of cholesterol-metabolism-related genes in the cerebellum were screened using RT-PCR and quantitative RT-PCR. First, we examined the mRNA level of liver X receptors (LXRs), one of the principal intracellular cholesterol regulatory factors (22). We did not detect any effect of donepezil on the expression of LXRα (data not shown), whereas LXRβ was slightly upregulated in the high dose donepezil-treated group (Fig. 5A, B). Because LXRs can reduce cholesterol accumulation not only via stimulation of the cholesterol efflux system but also via inhibition of cholesterol synthesis through a negative feedback pathway, several downstream genes related to cholesterol transport or biosynthesis were also selected to examine their mRNA levels. We found that transcription of both sterol regulatory element-binding proteins (SREBPs), involved in cholesterol synthesis, and their downstream molecule 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) were downregulated in the cerebellum of the NPC-DH group (Fig. 5A, B), whereas no specific effect of donepezil was observed on mRNA level of cholesterol-trafficking-related genes such as ATP-binding cassette transporter (ABC) isoforms A1, G1, and G5 (Fig. 5A, C). These data suggest that donepezil reduced the cholesterol accumulation via inhibition.
DISCUSSION

In this study, we inhibited ACh breakdown via in vivo administration of donepezil, a widely used AChEI, and evaluated its effects on the neuropathology and motor dysfunction observed in NPC mice. Donepezil prevented Purkinje cell death and alleviated the impaired motor function of NPC mice. It readily decreased the level of U18666A- or NPC1-dysfunction-induced cholesterol accumulation both in vitro and in vivo. In addition, mRNA expression levels of several essential factors involved in cholesterol synthesis were downregulated in the cerebellum of NPC mice after 4 weeks of donepezil treatment. Therefore, we propose that maintenance of normal ACh activity might be important for management of NPC with respect to cholesterol homeostasis.

FIGURE 2. Donepezil contributes to maintaining calbindin (CBD)-expressing cells in the cerebellum. (A, B) Representative images showing distribution patterns of CBD-positive cells (Purkinje neurons) in donepezil-treated and untreated groups. Purkinje cell soma and neurites are more evenly distributed in the cerebellum of donepezil-treated mice compared with those of control mice. Magnifications: A, 200×; B, 40×; scale bars: 100 μm. (C) Counts of CBD-positive cells in anterior and posterior cerebellar regions demonstrating the protective effect of acetylcholinesterase activity on Purkinje cell survival. Results are shown as mean ± SE. **, p < 0.005; ***, p < 0.001. Abbreviations: NPC-C, normal saline-injected Niemann-Pick disease type C (NPC) group; NPC-DL, low dose (0.33 mg/kg) of donepezil-injected NPC group; NPC-DH, high dose (1 mg/kg) of donepezil-injected NPC group.
Progressive neurological disease is considered the most crucial factor that contributes to the severity of NPC (23). A recent study using a cell-type-specific NPC1 protein recovery technique has revealed that an NPC1 defect in neurons rather than in astrocytes or microglia is a prerequisite for the initiation of neurodegeneration in NPC (24, 25). In particular, the cerebellum is the most seriously affected brain region in NPC, and Purkinje cells lose from 2 to 3 weeks of age in a bait-free manner in mice (15), suggesting that Purkinje cells are a particularly susceptible population compared with other cerebellar neurons (26). Previous studies have reported that an overactivated autophagy process through Beclin 1 upregulation (27) and/or abundant apoptosis through the activation of proapoptotic transcription factor p73 (28) might be

**FIGURE 3.** Donepezil improves survival of Purkinje cells and motor performance in the Niemann-Pick disease type C (NPC) mice. (A) The beneficial impact of donepezil on Purkinje cell survival is confirmed using Nissl staining (100×). Purkinje cells were found more numerous in mice treated with Donepezil versus normal saline-injected mice (NPC-C). (B) Purkinje cell numbers in the anterior [A] and posterior [P] zones for each group. The loss of Purkinje cells was somewhat prevented both in the anterior and posterior lobules. (C) Locomotion ability was evaluated by Rota-rod test. The low dose (NPC-DL, n = 9) and high dose (NPC-DH, n = 12) donepezil-treated groups showed improved performance in a dose-dependent manner compared with control mice (n = 11). Scale bars: 100 μm. **, p < 0.01; ***, p < 0.001. Results are shown as mean ± SE.
responsible for this phenomenon, but the contribution of neuron-to-neuron signaling through various neurotransmitters to the cerebellar pathology of NPC has not been determined.

In this study, we demonstrate the role of ACh in the protection of Purkinje neuronal integrity in the cerebellum. Although cholinergic innervation is less distributed in the cerebellum compared with the cerebrum (29), ACh also plays pivotal roles in the cerebellum (30, 31). Indeed, it is the proportion of Purkinje cells expressing choline acetyl transferase, a marker of cholinergic neurons, that is gradually increased in

FIGURE 4. Administration of donepezil inhibited cholesterol accumulation in vitro and in vivo. (A) To determine the role of acetylcholinesterase activity in cholesterol metabolism, adult NSC were culture with the Niemann-Pick disease type C (NPC) mimicking agent U18666A for 24 hours followed by a 72-hour incubation with donepezil (10^{-5} mol/L) or dimethyl sulfoxide (DMSO) (control). The level of intracellular cholesterol in each group was analyzed using filipin staining. (B) Mean density of filipin-positive areas in the cytoplasm was quantified using densitometry; the value of DMSO-treated NSCs is set at 1. Donepezil treatment reduced U18666A-induced cholesterol accumulation. (C) Representative images of filipin staining (400×) showing in vivo cholesterol accumulation patterns after a 4-week administration of high dose donepezil (NPC-DH) or normal saline (NPC-C) in anterior [A] and posterior [P] cerebellar regions. Donepezil treatment decreased the level of cholesterol accumulation in vivo. (D) Mean density of filipin-positive areas in the cerebellum was quantified using densitometry; the value for control NPC mice was set at 1. Scale bars: A, 10 μm; C, 100 μm. ***, p < 0.001. Results are shown as mean ± SE. Unpaired t-test was used for statistical analysis in (D).
the developing rat brain, implying the contribution of ACh in Purkinje neuron maturation (20). Moreover, Takayasu et al suggested the role of ACh in Purkinje cell neurophysiology by reporting that ACh treatment stimulates cerebellar granule neurons, resulting in the upregulation of excitatory postsynaptic potentials in Purkinje cells (32). Our data support previous observations by demonstrating that enhanced ACh activity with donepezil treatment contributes to Purkinje cell survival of the NPC mice. Overall, these findings suggest a possible role of ACh in the maintenance of Purkinje neuronal integrity.

Abnormalities in cholesterol metabolism have been reported in many neurodegenerative diseases as well as in aging (33, 34). In NPC, dysfunction of NPC1 and/or NPC2 protein impairs proper cholesterol trafficking from the lysosome/late endosome to other organelles and leads to cholesterol accumulation in the brain (35). We found that treatment with donepezil readily decreased filipin-positive areas both in vitro and in vivo, implying that increased ACh activity could contribute to the recovery of cholesterol homeostasis in NPC mice. This is an important finding with respect to therapeutics for neurodegeneration because fine-tuned cholesterol concentration is critical for neuronal survival and function. In deed, U18666A-mediated blockage of cholesterol transport induces apoptosis and cholesterol accumulation of cultured neurons (36). On the other hand, inhibition of cholesterol biosynthesis using simvastatin (an HMGCR inhibitor) or squalestatin (an inhibitor of squalene, a precursor of cholesterol) provides protection against neurotoxic insults in vitro (37). Thus, we hypothesize that activation of ACh pathways protects Purkinje neurons by reducing intracellular cholesterol levels in the cerebellum in NPC. This is consistent with a previous study by Benny et al demonstrating that the removal of excessive cellular cholesterol using a single dose of 2-hydroxypropyl-β-cyclo-dextrin successfully alleviated cerebellar pathology, including Purkinje cell loss in NPC mice (38).

We have further revealed that ACh could regulate cholesterol level through activating the LXR pathway (Fig. 5). LXRs act as a dominant supervisor in cholesterol metabolism including cholesterol synthesis, uptake, and trafficking (22). It is reported that the gene expression level of LXR and its downstream molecules in human NPC fibroblast differ from

![Figure 5](image_url)

**FIGURE 5.** Donepezil inhibited cholesterol synthesis by modifying the liver X receptor β (LXRβ) pathway in vivo. (A–C) mRNA levels of LXRβ, sterol regulatory element-binding protein 1 and 2 (SREBP1 and SREBP2), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), and ATP-binding cassette transporters (ABCA1, ABCG1, and ABCG5) in the cerebellum of each group (n = 4) were screened using RT-PCR (A) and quantified using quantitative RT-PCR (B, C). The value of the NPC control group for each gene was standardized as 1. Donepezil reduced the mRNA expression of cholesterol synthesis factors by activating LXR-induced negative feedback of the SREBP-HMGCR axis. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Results are shown as mean ± SE.
those in WT cells (39). Moreover, NPC cells failed to increase transcription levels of cholesterol transporter ABCA1, one of the LXR target molecules, suggesting that a cholesterol-sensing system might be defective in NPC (40). Interestingly, it was found that artificial stimulation of the LXR pathway using LXR agonist T0901317 can reduce cholesterol accumulation and improve the survival of NPC mice (41). Therefore, enhanced activation of LXR pathway by ACh would likely play a primary role in the beneficial effects of donepezil in NPC mice.

In addition, ACh might perform neuroprotective roles via other mechanisms besides the cholesterol-regulating effect. Mount et al reported that treatment of an ACh agonist in combination with nerve growth factor directly increases the number of Purkinje cells in vitro (42). It is also interesting that nicotine-mediated stimulation of ACh release leads to cell survival in models of glutamate-induced excitotoxicity (43). We have suggested previously that glutamate-mediated cytotoxicity caused by the diminished activity of glutamate transporters EAAT2 and EAAT3 might be one of principal causes of Purkinje cell death in the murine NPC cerebellum (13). Therefore, further investigation is needed to elucidate the precise relationship between ACh activity and Purkinje cell protection in NPC.

Application of AChEIs has been widely tested to compensate the marked impairment of cholinergic projections in neurodegenerative processes (44, 45). Here, we evaluated the therapeutic impact of AChEIs on motor incoordination in NPC for the first time. As expected, increased survival of Purkinje neurons with donepezil treatment was associated with improved performance of NPC mice in the Rota-rod test, suggesting that AChEIs may be novel agents for NPC therapy. Considering that broad neuronal defect occurs as NPC progression, it would be also interesting to investigate the therapeutic effects of donepezil on other NPC-affected brain regions, including the hippocampus and the thalamus.

To our knowledge, this is the first report demonstrating the importance of the cholinergic pathway in NPC pathology. In the NPC brain, ACh preservation by donepezil prevented cholesterol accumulation and promoted Purkinje cell survival, leading to the alleviation of motor dysfunction in NPC mice. Therefore, our study not only provides a better understanding of NPC pathology with respect to this neurological disorder but also implies that various AChEIs, including donepezil, might be used as therapeutic agents for NPC.

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