A dissertation for the Degree of Master

Donepezil enhances Purkinje cell survival and improves motor dysfunction by inhibiting cholesterol synthesis in a murine model of Niemann Pick type C disease

니이만 픽 타입 C 질병 마우스 모델에서 도네페질에 의한 콜레스테롤 합성 억제를 통한 퍼킨지 세포 생존 증가와 운동장애 개선효과

By

Yooyoung Shin

August 2013

Department of Zoonotic Animal Diseases

College of Veterinary Medicine,

Graduate School of Seoul National University

Donepezil enhances Purkinje cell survival and improves motor dysfunction by inhibiting cholesterol synthesis in a murine model of Niemann Pick type C disease

By

Yooyoung Shin

A dissertation submitted in partial fulfillment of

The requirement for the degree of

MASTER

Supervisor : Professor Kyung-Sun Kang, D.V.M., Ph.D.

August 2013

Approved by

Cho, Seong-Beom

Kang, Kyung-Sun **Yang, Se-Ran**

Department of Zoonotic Animal Diseases
College of Veterinary Medicine,
Graduate School of Seoul National University

ABSTRACT

Donepezil enhances Purkinje cell survival and improves motor dysfunction by inhibiting cholesterol synthesis in a murine model of Niemann Pick type C disease

Yooyoung Shin

Department of Zoonotic Animal Diseases

College of Veterinary Medicine

Graduate School of Seoul National University

Supervisor : Professor Kyung-Sun Kang, D.V.M., Ph.D.

Acetylcholine (ACh) plays various roles in the process of neural differentiation and maturation as well as in the maintenance of the cognitive function. The neurodegenerative process is often accompanied by the disruption of the cholinergic system. In this study, we aimed to elucidate the importance of cholinergic pathway to maintain cerebellar integrity using murine model of Niemann-Pick disease type C (NPC), one of the incurable lyosomal storage disorders accompanied with neurological symptoms. Choline acetyltransferase (ChAT) immunostaining has shown that ACh activity was significantly

reduced in the cerebellar cortex of NPC mice, where the pathognomonic Purkinje cell loss occurs. We then administrated acetylcholinesterase inhibitor (AChEI), Donepezil, for 4 weeks to compensate the attenuated cholinergic system. When NPC mice were treated with Donepezil, more Calbindin (CBD) positive Purkinje neurons survived during disease progress. In the rota-rod test, the performance of NPC mice was remarkably improved than that of control mice. To investigate the relationship between cholesterol accumulation and Purkinje neuronal survival, wild-type neural stem cells were treated with U18666A, a drug induces abnormal cholesterol accumulation similar to NPC state then were stained with filipin in vitro. It is noted that the number of filipin-positive cholesterol accumulated cells was reduced by Donepezil treatment. Improtantly, we found that Donepezil readily down-regulated mRNA levels of cholesterol synthesis molecules in vivo, implying that Ach might be related to the cholesterol homeostasis. Taken together, our findings suggest cholinergic pathway as another important regulator in NPC progress and propose the therapeutic application of AChEIs in NPC.

Keywords: donepezil, Niemann Pick disease Type C, acetyl cholinesterase inhibitor, Purkinje cell, cholinergic neuron, cholesterol

Student number : 2011-23723

LIST OF ABBREVIATION

NPC	Niemann-Pick type C disease
NSC	Neural stem cell
Ach	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
CBD	Calbindin
ChAT	Choline acetyltransferase
LXR	Liver X receptor
SREBP	Sterol Regulatory Element-Binding Proteins
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
ABC(A,G)	ATP-binding cassette transporter
WT	Wild type
AD	Alzheimer`s disease
PD	Parkinson`s disease
EAAT	Excitatory amino acid transporter

NO	Nitric Oxide
MAPK	Mitogen-activated protein kinases

TABLE OF CONTENTS

INTRODUCTION

Niemann-Pick disease type C (NPC) is one of a lysosomal lipid storage disorder and caused by dysfunction of either NPC1 or NPC2 protein, which has important roles in sterol and lipid trafficking in mammalian cells (1). To date, the disease is incurable, and usually leads to premature death by childhood. In most cases, the neurological symptoms such as ataxia, tremor and dementia develop followed by enlargement of liver or spleen, and remarkable neurodegenerative signs are also found in NPC affected brain (2). Since the involvement of neuropathy is suggested as a decisive factor to determine the prognosis of the disease (3), many attempts have been tried to reveal the underlying mechanism of the neurodegeneration. It has been reported that dopaminergic activity is disrupted in the thalamus of NPC mice (4), however, activity change of other neurotransmitters including acetylecholine (ACh) have scarcely been investigated.

The concept of ‘cholinergic hypothesis’ represents the strong correlation between cholinergic system and cognitive function. It is evolved on the basis of several findings with neurodegenerative disease showing that decline in cholinergic activity is closely related to the severity of dementia and other neurological symptoms (5). Therefore, consistent maintaining of the ACh concentration is the key strategy in the management of neurological disorder. The development of various acetylcholinesterase inhibitors (AChEIs) that prevent breaking down of ACh has provided a great advance in the field of Alzheimer’s disease (AD) therapeutics (6) and positive outcome of AChEIs application in

other diseases including myasthenia gravis (7), Parkinson`s disease (8) and Huntington`s disease (9) have also been reported. Considering that NPC shares some similar pathology with AD (10), we decided to verify the therapeutic potential of AChEIs in NPC.

In this study, we revealed decreased cholinergic activity in the cerebellum of the NPC mice by histochemical analysis. We then evaluated the effect of long-term administration of donepezil, one of the AChEIs approved for the management of AD, on the neuropathology and behavior dysfunction observed in NPC mice. Interestingly, donepezil treatment reduced Purkinje cell death and recovered the impaired motor function. We confirmed whether donepezil had effect on cholesterol accumulation which has been the main cause of NPC. We found that donepezil decreased the level of U18666A-induced cholesterol accumulation in neural stem cells. In addition, we found that mRNA expression levels of SREBP1, SREBP2, and HMGCR, key molecules for the cholesterol synthesis were also down-regulated by donepezil administration.

Our findings suggest the importance of cholinergic system for the establishment of new clinical approach to NPC as well as for the understanding of NPC pathology.

MATERIALS AND METHODS

Animal model

NPC mice were purchased from Jackson Laboratories (Bar Harbor, MA, USA) for breeding pairs of BALB/c heterozygous (NPC1+/-) mice. The genotyping was performed as described previously (11). The number of mice used in experiments is totally 12 of wild-type (WT) group, 18 of control NPC-/- (NPC) group, 18 of donepezil treated group 1 (0.33mg/kg), and 20 of donepezil treated group 2 (1mg/kg). All animal experiments were performed in accordance with the regulations of the Institute of Laboratory Animals Resources (SNU-110517-3, Seoul National University, Seoul, Korea).

Adult neural stem cell primary culture and experimental treatment

In 4-week-old mice, adult neural stem cells were isolated from the subventricular zone which was one of the neural stem cell origins. NSCs were cultured in Neurobasal-A medium (Gibco, CA, USA) supplemented with 1% N2 (Gibco), 2% B27 (Gibco), 1% penicillin-streptomycin (Gibco) and 1% GlutaMax (Gibco). The culture medium needed some growth factors such as 20ng/ml epidermal growth factor (Gibco) and 20n/ml basic fibroblastic growth factor (Gibco). After floating culture for neurosphere formation, cells were subcultured to expand the amount of NSCs. 5x103 cells were seeded on poly-L-ornithine (Sigma-Aldrich, MO, USA) and fibronectin (BD, CA, USA) coated coverslip for treatment. NSCs were treated with DMSO (Bioniche pharma, USA), U18666A (1 μg / ml ; Tocris Bioscience, MO, USA) and donepezil (10-5M ; Sigma-Aldrich) (12).

Donepezil administration in vivo

Two groups were adapted with different doses of donepezil hydrochloride (0.33 mg/kg, 1.0 mg/kg) (Sigma-Aldrich, MO, USA) dissolved in normal saline, which was used as the vehicle (13). Donepezil or a comparable volume of vehicle (10 mL/kg) was administered by intraperitoneal injection during weekdays at different doses for 4 weeks beginning at 4-week old.

Histological analysis

NPC mice were sacrificed at 8 weeks. Brain tissues were fixed in 4% paraformaldehyde for 1 day and sections of 30 μm thickness were prepared. Sections were blocked for 1 h with 5% normal goat serum (Invitrogen, CA, USA) and stained with antibodies against Calbindin(1:500, Millipore, MA, USA), Nestin(1:500, Millipore), TUJ1(1:500, abcam, Cambridge, UK), ChAT(1:500, Millipore), and MAP2(1:250, Abcam). Antigen retrieval was conducted with sodium citrate buffer at 85 C for 15 minutes before primary antibody incubation for ChAT. Overnight incubation with primary antibody was conducted at 4 C followed by 1.5 hr incubation with secondary antibody Alexa488 (1:1000, Invitrogen) or Alexa594 (1:1000, Invitrogen) at room temperature. Nuclei were stained with DAPI (1μg/mL, Santa Cruz Biotechnology, CA, USA) for 15minutes. Images were captured using a confocal microscope (Nikon, Eclipse TE200, Tokyo, Japan) (14).

Filipin staining

NSCs with experimental treatment, as described before, were performed filipin staining for detecting cholesterol level. NSCs were stained with filipin working solution (50μg/ml, Sigma-Aldrich, MO, USA) for 1.5 hours at room temperature. Nuclei were stained with propidium iodide (PI) (5uM, Sigma-Aldrich) for 10 minutes. Images were captured using a confocal microscope (Nikon, Eclipse TE200, Tokyo, Japan). All steps should be protected from light, and taken images immediately.

RNA isolation and qRT-PCR

Total RNA was isolated from whole cerebella with Trizol reagent(Invitrogen, CA, USA) according to the manufacturer's protocol. cDNA was synthesized by using Maxime RT premix(Intron, Kyung-ki do, Korea), and amplified with specific primers. Gene-specific primers were designed using Primer Express Software(PE-Applied Biosystems, Warrington, UK), and the primer sequences are provided as follows : Liver X receptor β (LXRβ) (F:5'-TCTGGGATGTGCACGAGTAG-3'; R:5'-GACCTGTGACCCTCACCACT-3'), Sterol regulatory element-binding protein 1 (SREBP1) (F:5'-GTGAGCCTGACAAGCAATCA-3'; R:5'-GGTGCCTACAGAGCAAGAGG-3'), Sterol regulatory element-binding protein 2 (SREBP2) (F:5'-TGTGGAGCAGTCTCAACGTC-3'; R:5'-TGGTAGGTCTCACCCAGGAG-3'), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (F:5'-TCTTTCCGTGCTGTG TTCTG-3'; R:5'-TTTTAACCCACGGAGAGGTG-3'), and GAPDH (F:5'-GGAAGGGCTCATGACCACAG-3'; R:5'-GCAGGGATGATGTTCT

GGGC-3'). PCR electrophoresis was analyzed by GelDoc XR system (Bio-Rad, USA). Real-time quantitative RT-PCR was performed using SYBRGreen (Applied Biosystems, CA, USA), according to the manufacturer's protocol. All amplications were analyzed using Prism 7000 sequence detection system 2.1 software (Applied Biosystems).

Rota-rod test

Motor function test was conducted using a Rota-rod treadmill (7650 Accelerating model, Ugo Basile Biological Research Apparatus, Comerio, Italy). 4 week old mice in each group were trained during the first week, and 5–8 week old mice were subjected to the Rota-rod test at 10 rpm. The test was performed once a week and the mean record was adopted as the performance time of 4 attempts (15).

Statistical analysis

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

RESULTS

The distribution pattern of Purkinje cells and cholinergic neurons in the cerebellum of WT and NPC mice

We investigated the pattern of cholinergic neuronal distribution in the cerebellum of NPC mice by staining for ChAT (Figure 1A). Only a few cells expressed ChAT along the cerebellar molecular layer in NPC mice, whereas a number of ChAT positive cells evenly distributed in the cerebellar molecular layer of WT mice. We next determined the CBD expression level by fluorescent microscopy to demonstrate the survival rate of Purkinje cells in the NPC cerebellum (Figure 1B). The number of Purkinje cells was remarkably decreased in the cerebellar sections of NPC mice compare to WT mice (WT=3, NPC=4). Interestingly, the pattern of cell distribution was different between the anterior and posterior cerebellar regions; the loss of Purkinje cells was gradually decreased from anterior to posterior region. Quantification of CBD-positive cells in anterior zone (lobule Ⅰ-Ⅴ) and posterior zone (lobuleⅥ-Ⅹ) of the cerebellum showed that 94.10 cells per mm squared were in the posterior zone of WT cerebellum while 5.23 cells per mm squared were found in the NPC cerebellum. Also, in the anterior zone, 92.56 cells per mm squared were in WT cerebellum while only 1.38 cells per mm squared were found in the NPC cerebellum (Figure 1C). In addition, the ratio of CBD+ cells in the anterior zone to total CBD+ cells in NPC mice cerebellum (20.9%) was lower than the ratio in WT mice cerebellum (49.5%). Taken together, our results indicate that the number of Purkinje cells and cholinergic neurons decreases in NPC mice, and they have different survival rate

considering their distribution in cerebellum of NPC mice.

Cholinergic neurons are activated by acetyl cholinesterase inhibition.

Several groups have reported that the number of Purkinje cells present in the cerebellar cortex is regulated by acetyl choline activity (16, 17). These findings indicate that Purkinje cells can be regulated by the expression of ChAT and AChE. To investigate whether the expression of ChAT is regulated by Acetyl cholinesterase activation, we compared the expression of ChAT in WT-, NPC control- and Donepezil treated mice by immunohistochemistry (Figure 2). Cholinergic neurons, which use acetyl choline for their neurotransmitter, were less activated in NPC mice than WT mice in accordance with their reduced ChAT expression level. However, the number and immunoreactivity of ChAT positive cells residing in the cerebellar cortex increased by 4-week treatment of donepezil in a dose-dependent manner. These results clarify that donepezil, which prevents the decomposition of acetyl choline into acetate and choline, improves impaired cholinergic system.

Acetyl cholinesterase inhibitor ameliorates motor function of adult NPC mice

Next, we conducted Rota-rod test with control- and donepezil treated NPC mice to define the pharmacological effect of donepezil on motor function (Figure 3). Both untreated and donepezil treated NPC mice completed the Rota-rod test for 180 seconds at 5weeks of age. However, follows the disease development, the riding time of control NPC mice group was progressively decreased. Importantly, high dose (1mg/kg) of -donepezil

treatment prevented the loss of motor function in NPC mice. Indeed, The difference of riding time between untreated and high dose donepezil treated NPC mice were 35.64s, 44.64s and 64.68s at 6,7 and 8 weeks of age, respectively. These findings suggest that donepezil at high dosage improves motor ability of NPC mice. Although low dose (0.33mg/kg) donepezil treated mice recorded less riding time at 8-weeks (75.39s) than high dose donepezil treated group, it also slightly improved motor function of NPC mice compared to untreated NPC mice. Therefore, our results support that donepezil contributes to ameliorate the motor function disorder in NPC disease in a dose dependent manner.

Inhibition of acetyl cholinesterase improves survival of Purkinje cells in NPC cerebellum.

In NPC mice, the patterns of Purkinje cell loss are diverse in each lobule (18) and the loss of Purkinje cells is more severe in the anterior region than the posterior region. To explain the protective effect of donepezil on motor deficit in NPC mice, we next investigated whether donepezil can regulate the survival of Purkinje cells. We sacrificed 4 mice from each group (donepezil treated and untreated), and determined CBD expression in cerebellum by immunohistochemistry on sagittal section. CBD staining revealed that the loss of Purkinje cells was reduced in donepezil treated group (Figure 4A) and CBD-positive cells were recovered starting from the posterior region to the anterior region (Figure 4B). We measured the number of CBD-positive cells by dividing the cerebellum into lobule Ⅰ-Ⅴ and lobuleⅥ-Ⅹ to compare the anterior region with the posterior

region. In the untreated NPC group, 6.31 CBD-positive cells per mm squared in the posterior zone and only 2.15 cells in the anterior zone were detected. Remarkably, treatment of donepezil increased the number of CBD+ cells in both regions (posterior: 25.13 CBD+ cells, anterior: 12.56 CBD+ cells in 0.33mg/kg-donepezil treated group; posterior: 31.54 CBD+ cells, anterior: 19.42 CBD+ cells in 1mg/kg-donepezil treated group) (Figure 4C). It is found that not only the total number of CBD+ cells was increased depending on the dosage, but also the ratio of Purkinje cells in the anterior zone to the whole cerebellar region was increased (22.8% in control, 33.3% in 0.33mg/kg-donepezil treated, and 38.1% in 1mg/kg-donepezil treated group). Therefore, our results suggest the loss of Purkinje cells is rescued by donepezil treatment in the cerebellum, especially in the anterior zone.

Donepezil reduces cholesterol accumulation by inhibition of cholesterol synthesis in NPC mice.

Since abnormal accumulation of cholesterol is a key pathology of NPC, we investigate whether donepezil delayed NPC-derived neural cell death by modifying cholesterol metabolism. We firstly treated U18666A on adult neural stem cells (NSCs) for mimicking the NPC condition by induction of cholesterol accumulation within the cells then donepezil was administrated to determine its cholesterol-regulating effect. Cholesterol specific filipin staining showed that U18666A-treated NSCs contained more filipin positive cells compared to control NSCs. As expected, donepezil significantly reduced the cholesterol level in NSCs (Figure 5A). In quantification analysis of filipin positive area in

each group of NSCs using optical intensity (Figure 5B), each mean positive area was 1±0.27%, 8.27±0.89% and 3.33±0.28% in control, U18666A-treated NSCs, and donepezil-treated NSCs, respectively. To determine whether donepezil-induced cholesterol reduction was caused by either prevention or recovery of cholesterol accumulation or both, cholesterol metabolism-related target genes were screened by PCR. Repa et al. have shown that Liver X Receptor (LXR) activation promotes cholesterol efflux to decrease in NPC1 mice (19). We found that donepezil had no effects on the expression of LXRα (Figure 5E, 5F), while LXRβ was slightly up-regulated in the high dose of donepezil treated group (Figure 5C, 5D). To confirm changed expression level in LXRβ pathway, target genes of LXR, such as Sterol Regulatory Element-Binding Proteins (SREBP) 1/2 and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), were detected. SREBP, which was involved in cholesterol synthesis, was down-regulated, and HMGCR activated by SREBP also decreased in the high dose of donepezil treated group. These target molecules were evaluated by quantitative RT-PCR only with the high dose of treated group except the low dose group which had no significant results (Figure 5D). The expression of LXRβ was up-regulated by 1.24 compared to NPC control, SREBP1 was slightly decreased by 0.9, SREBP2 also down-regulated by 0.48, and they led to the inhibition of HMGCR by 0.55. LXR mainly regulates cholesterol trafficking-related genes such as ATP-binding cassette transporter (ABC)A1, G1, and G5. Activation of these genes enhances intracellular cholesterol efflux; however, donepezil has no meaningful effects on these genes. These data suggest that donepezil activated LXRβ, and its target molecules were down-regulated through the LXRβ pathway. It was revealed that

donepezil decreased cholesterol accumulation by inhibiting cholesterol synthesis in NPC mice.

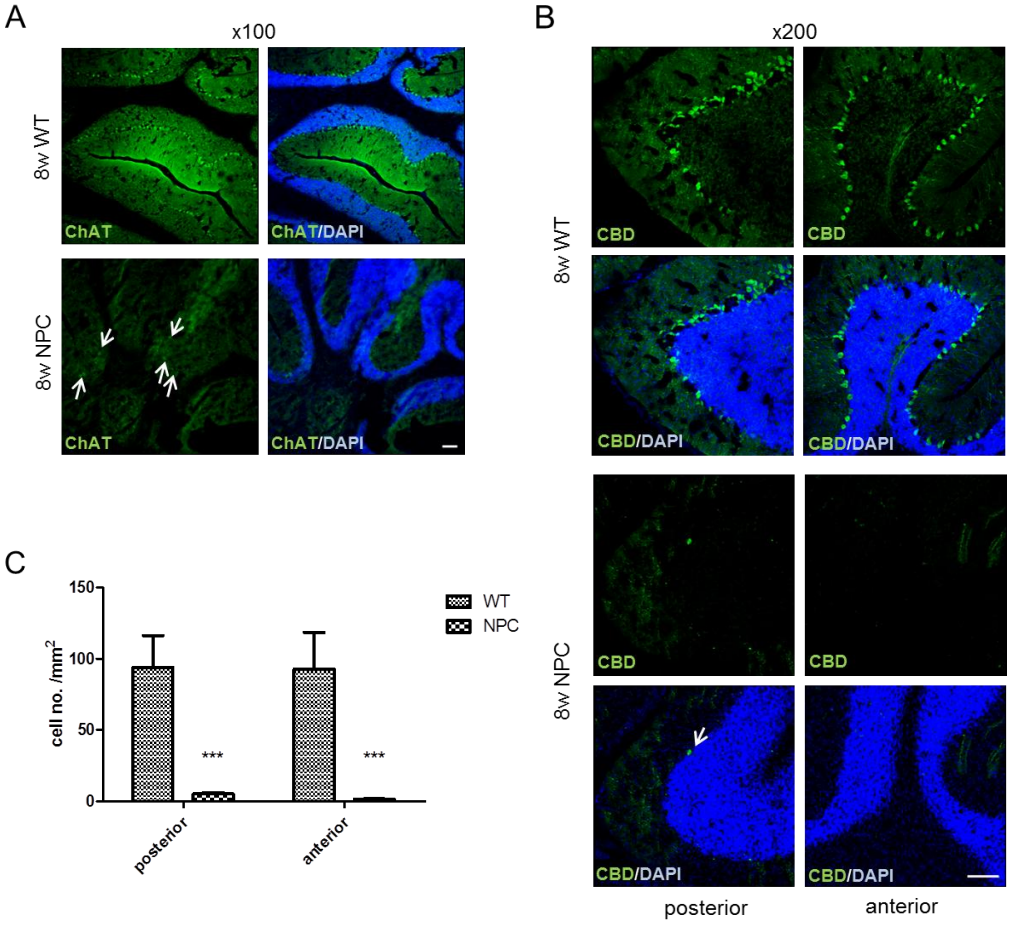

Figure 1. Pathological changes observed in 8-week old NPC cerebellum

(A) ChAT staining was performed to detect cholinergic neurons and ChAT+ cells are indicated by arrows. (B) Purkinje cells were stained for CBD. The number of Purkinje cells was gradually decreased from anterior to posterior region. (C) Quantitative analysis of CBD-positive cells between WT and NPC mice in the anterior and posterior zones of their cerebellum by blinded counting. Number of mice for each group; WT = 3, NPC = 4, *** $p<0.001$. Results are shown as mean ± SEM. Bar = 100 ㎛

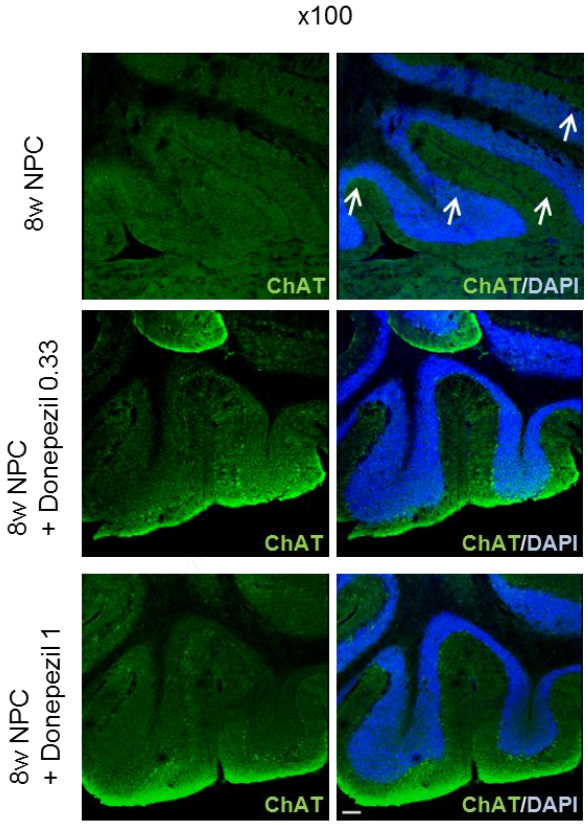

Figure 2. Donepezil stimulates activation of cholinergic neurons in the cerebellum of 8-week old NPC mice.

The expression of ChAT+ cells in donepezil-treated and untreated groups. Number of mice for each group; n=6 in NPC, n=6 in donepezil 0.33 mg/kg, n=4 in donepezil 1 mg/kg, *** $p<0.001$. Results are shown as mean ± SEM. Bar = 100㎛

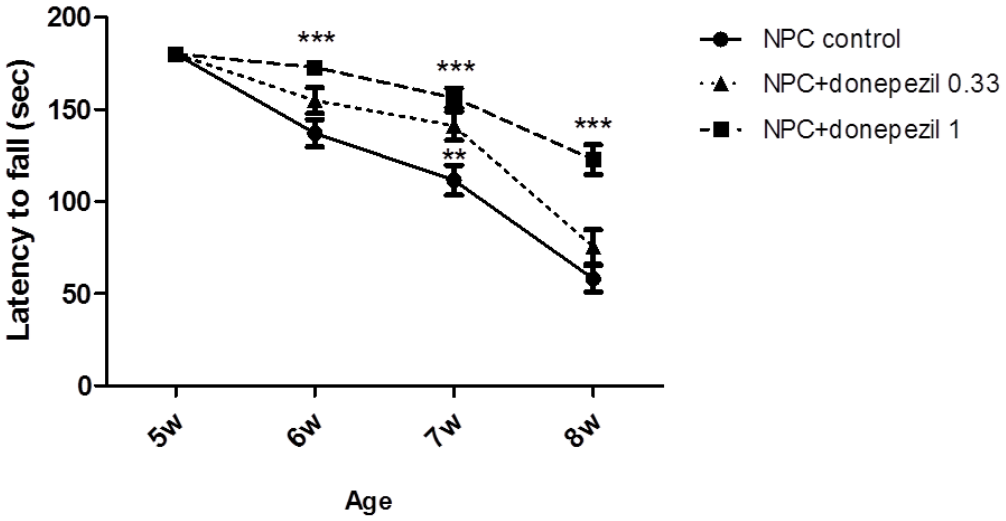

Figure 3. Donepezil recovers motor function deficits in NPC mice.

Locomotion ability was evaluated by rota-rod test. Donepezil treated group showed significant recovery. Number of mice for each group; NPC=11, donepezil 0.33 mg/kg=9, donepezil 1 mg/kg=12, ** $p<0.01$, *** $p<0.001$, (w; weeks, sec; seconds). Results are shown as mean ± SEM.

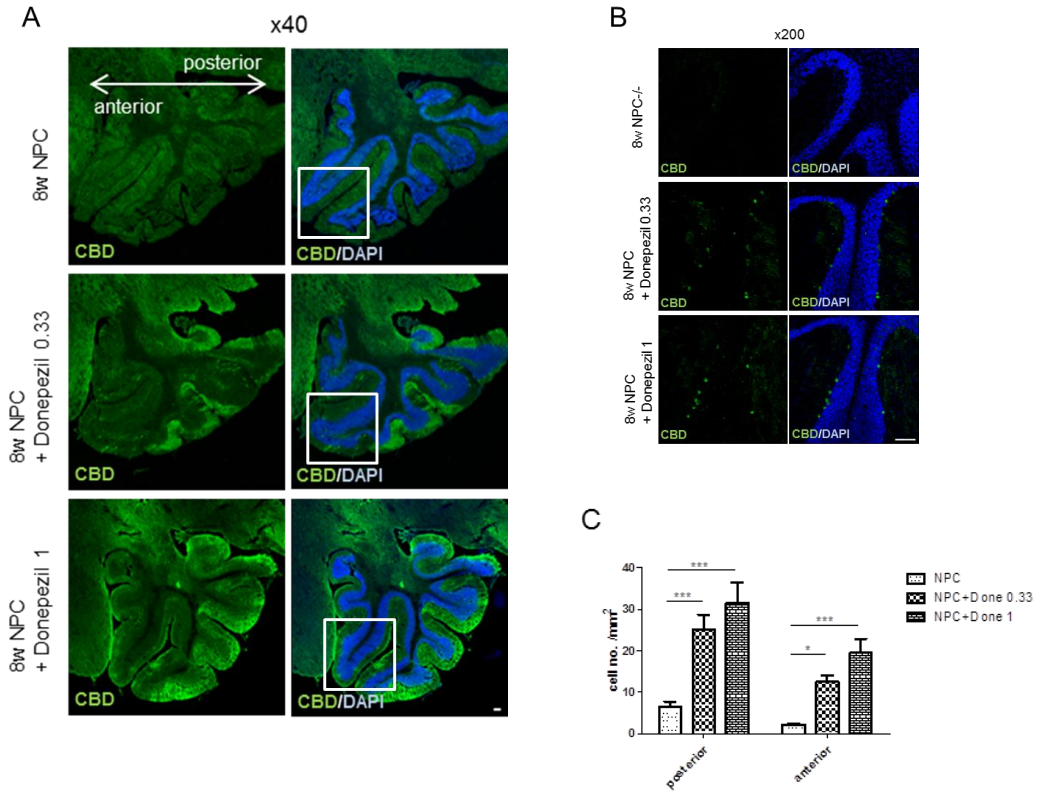

Figure 4. Donepezil improves survival of Purkinje cells in NPC cerebellum.

(A) Purkinje cell survival was improved gradually in the anterior region (left of the cerebellar area; square, under X 40). (B) The loss of Purkinje cells was repressed in donepezil treated groups, the images were observed under X 200. (C) Quantitative analysis of Purkinje cells in the anterior and posterior zones for each group by blinded counting. The loss of CBD-positive cells was recovered starting from the posterior region to the anterior region (n=6 in NPC, n=6 in donepezil 0.33 mg/kg, n=4 in donepezil 1 mg/kg), ***p <0.001, *p<0.05. Results are shown as mean ± SEM. Bar = 100㎛

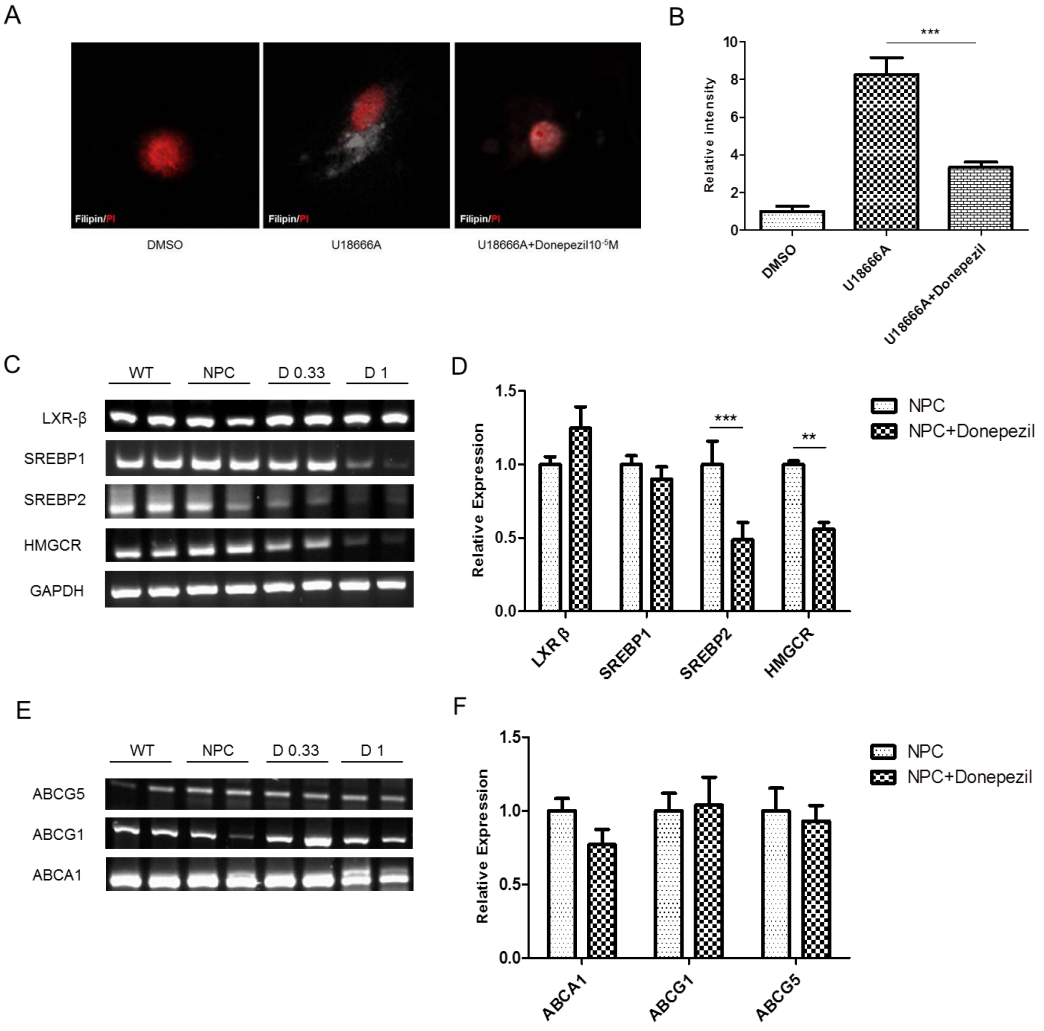

Figure 5. Donepezil inhibited cholesterol synthesis in NPC mice.

(A) U18666A-treated NSCs were remarkably filipin positive versus DMSO-control NSCs, and donepezil reduced accumulated cholesterol. The images were observed by zoom-in. (B) Quantitative analysis of the filipin positive areas in control and treated groups of NSCs (mean± SEM, ***p<0.0001) (C,E) mRNA level of LXRβ, SREBP1, SREBP2,

HMGCR, ABCA1, ABCG1 and ABCG5 using RT-PCR with NPC mice (D,F) Quantitative analysis of LXRβ, SREBP1, SREBP2, HMGCR, ABCA1, ABCG1 and ABCG5 by qRT-PCR with NPC mice (n=4 for each group, mean± SEM, Bar = 50㎛, **p<0.01, ***p<0.001)

DISCUSSION

Although the abnormal accumulation of unesterified cholesterol and sphingolipids in lysosome / late endosome is systemically observed, neurological pathology has been suggested as the most crucial factor contributed to the prognosis of NPC (2),(20). Although it is found that dopaminergic neuronal population residing in substantia nigra of mid brain is decreased as NPC progress (4), cerebellum is the most severely affected area in a murine model of NPC and Purkinje cells are exceedingly susceptible to the neurodegeneration compared to other neurons (21). Purkinje cell loss occurs from 2-3 weeks of age in an autonomous manner (22) and a recent study using cell type specific NPC1 rescue model mice (23) also suggested that defect in neuron rather than in astrocyte or microglia is a preceding condition in the neurodegenerative process in NPC. Previous works have revealed that over-activated autophagy process by beclin-1 up-regulation (22) and/or abundant apoptosis through the activation of proapoptotic p73 transcription factor (24) might be responsible for this phenomenon.

The cholinergic pathway is one of the fundamental modulatory systems in neuronal activity. Even though the cholinergic innervations are less distributed in the cerebellum compared to the cerebrum (25),(26), they also play various roles in neurophysiology and pathology (27),(28). Takayasu et al. has found that ACh treatment stimulates the granule neuron, resulting in up-regulated excitatory postsynaptic potential in Purkinje cells (29). In the developing rat brain, portion of Purkinje cell expressing ChAT, a marker for

cholinergic neuron, is gradually increased implying the contribution of ACh in Purkinje neuronal maturation (30). Here, we have compared the pattern of cholinergic neuron distribution between WT- and NPC model cerebellum for the first time. Interestingly, a massive number of cholinergic neurons were disappeared in the cerebellar cortex of the NPC mice (Figure 1A). Furthermore, similar to the pattern of Purkinje cell degeneration (31), this cell loss is more severely observed in the anterior cerebellar lobe than in the posterior. These findings suggest a possible role of the ACh in maintenance of Purkinje neuronal integrity.

Based on the fact that a marked impairment of cholinergic projections in the cerebral cortex is often observed in many neurodegenerative diseases such as Parkinson`s disease (PD) (32) and Alzheimer`s disease (AD) (33), administration of the acetylcholinesterase inhibitor(AChEI) to keep the Ach concentration has been widely tested in therapeutics (6), (34), (35). To our knowledge, our work is the first report demonstrating that inhibition of ACh degradation by long term treatment of donepezil, a widely used agent to AD patients, contributes to recover the impaired motor function and cerebellar pathology of the NPC mice model. Previous work has established that NPC mice perform poor motor behavior such as action tremor and unstable gait, when compared with age-matched wild type mice (36). Therefore, improved performance on the rota-rod test of the donepezil treated NPC mice (Figure 3.) is important evidence supporting the therapeutic potential of AChEI. Considering that survival rate of Purkinje neuron in the cerebellar anterior lobe is increased with donepezil administration, ACh might have protective effects on the neurodegeneration in NPC mice model. These findings are supported by previous works

demonstrating that activation of ACh release stimulated by nicotine leads to cell survival against glutamate- or amyloid β induced excitotoxicity (37),(38), because we have suggested glutamate-mediated cytotoxicity due to diminished glutamate transporters such as EAAT2 and EAAT3 as one of the main causes for Purkinje cell death in the NPC mice brain in our previous study (15). Additionally, Mount et al., reported that ACh agonist treatment with neuronal growth factor readily increases the number of Purkinje cells in vitro (39), implying the direct role of ACh on Purkinje neuron survival. Therefore, to further determine whether the effect of donepezil on NPC mice model works through direct or indirect signals, the exact underlying mechanism has to be elucidated. In this study, we have shown the importance of ACh circuit in NPC pathology for the first time. In NPC brain, cholinergic system is hypo-activated especially in the cerebellum in accordance to Purkinje cell loss. Interestingly, ACh preservation by donepezil promoted Purkinje cell survival, leading to improvement of motor function in NPC mice. Therefore, our study not only provides a better understanding of NPC pathology in aspect to neurological disorder, but also suggests various AChEIs including donepezil as novel therapeutic agents for NPC management.

REFERENCES

1. Subramanian K, Balch WE. NPC1/NPC2 function as a tag team duo to mobilize cholesterol. Proc Natl Acad Sci U S A 2008:105;15223-4.
2. Vanier MT, Millat G. Niemann-Pick disease type C. Clin Genet 2003:64;269-81.
3. Group N-CGW, Wraith JE, Baumgartner MR, et al. Recommendations on the diagnosis and management of Niemann-Pick disease type C. Mol Genet Metab 2009:98;152-65.
4. Luan Z, Saito Y, Miyata H, et al. Brainstem neuropathology in a mouse model of Niemann-Pick disease type C. J Neurol Sci 2008:268;108-16.
5. Perry EK, Tomlinson BE, Blessed G, et al. Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. Br Med J 1978:2;1457-9.
6. Terry AV, Jr., Buccafusco JJ. The cholinergic hypothesis of age and Alzheimer's disease-related cognitive deficits: recent challenges and their implications for novel drug development. J Pharmacol Exp Ther 2003:306;821-7.
7. Mehndiratta MM, Pandey S, Kuntzer T. Acetylcholinesterase inhibitor treatment for myasthenia gravis. Cochrane Database Syst Rev 2011;CD006986.
8. Aarsland D, Mosimann UP, McKeith IG. Role of cholinesterase inhibitors in Parkinson's disease and dementia with Lewy bodies. J Geriatr Psychiatry Neurol 2004:17;164-71.
9. Rot U, Kobal J, Sever A, et al. Rivastigmine in the treatment of Huntington's disease. Eur J Neurol 2002:9;689-90.
10. Nixon RA. Niemann-Pick Type C disease and Alzheimer's disease: the APP-endosome connection fattens up. Am J Pathol 2004:164;757-61.
11. Yang SR, Kim SJ, Byun KH, et al. NPC1 gene deficiency leads to lack of neural stem cell self-renewal and abnormal differentiation through activation of p38 mitogen-activated protein kinase signaling. Stem Cells 2006:24;292-8.

12. Miki A, Otori Y, Morimoto T, et al. Protective effect of donepezil on retinal ganglion cells in vitro and in vivo. Curr Eye Res 2006:31;69-77.
13. Alcantara-Gonzalez F, Mendoza-Perez CR, Zaragoza N, et al. Combined administration of cerebrolysin and donepezil induces plastic changes in prefrontal cortex in aged mice. Synapse 2012:66;938-49.
14. Kim SJ, Lim MS, Kang SK, et al. Impaired functions of neural stem cells by abnormal nitric oxide-mediated signaling in an in vitro model of Niemann-Pick type C disease. Cell Res 2008:18;686-94.
15. Seo Y, Yang SR, Jee MK, et al. Human umbilical cord blood-derived mesenchymal stem cells protect against neuronal cell death and ameliorate motor deficits in Niemann Pick type C1 mice. Cell Transplant 2011:20;1033-47.
16. De Bartolo P, Gelfo F, Mandolesi L, et al. Effects of chronic donepezil treatment and cholinergic deafferentation on parietal pyramidal neuron morphology. J Alzheimers Dis 2009:17;177-91.
17. Nagykery N, Terwilliger EF, Geula C. In vivo AAV-mediated expression of calbindin-D(2)(8)k in rat basal forebrain cholinergic neurons. J Neurosci Methods 2013:212;106-13.
18. Elrick MJ, Pacheco CD, Yu T, et al. Conditional Niemann-Pick C mice demonstrate cell autonomous Purkinje cell neurodegeneration. Hum Mol Genet 2010:19;837-47.
19. Repa JJ, Li H, Frank-Cannon TC, et al. Liver X receptor activation enhances cholesterol loss from the brain, decreases neuroinflammation, and increases survival of the NPC1 mouse. J Neurosci 2007:27;14470-80.
20. Vanier MT. Niemann-Pick disease type C. Orphanet J Rare Dis 2010:5;16.
21. Ong WY, Kumar U, Switzer RC, et al. Neurodegeneration in Niemann-Pick type C disease mice. Exp Brain Res 2001:141;218-31.
22. Ko DC, Milenkovic L, Beier SM, et al. Cell-autonomous death of cerebellar purkinje neurons with autophagy in Niemann-Pick type C disease. PLoS Genet 2005:1;81-95.

23. Lopez ME, Klein AD, Dimbil UJ, et al. Anatomically defined neuron-based rescue of neurodegenerative Niemann-Pick type C disorder. J Neurosci 2011:31;4367-78.

24. Alvarez AR, Klein A, Castro J, et al. Imatinib therapy blocks cerebellar apoptosis and improves neurological symptoms in a mouse model of Niemann-Pick type C disease. FASEB J 2008:22;3617-27.

25. Barmack NH, Baughman RW, Eckenstein FP, et al. Secondary vestibular cholinergic projection to the cerebellum of rabbit and rat as revealed by choline acetyltransferase immunohistochemistry, retrograde and orthograde tracers. J Comp Neurol 1992:317;250-70.

26. Turner JR, Kellar KJ. Nicotinic cholinergic receptors in the rat cerebellum: multiple heteromeric subtypes. J Neurosci 2005:25;9258-65.

27. De Filippi G, Baldwinson T, Sher E. Evidence for nicotinic acetylcholine receptor activation in rat cerebellar slices. Pharmacol Biochem Behav 2001:70;447-55.

28. Schmahmann JD, Caplan D. Cognition, emotion and the cerebellum. Brain 2006:129;290-2.

29. Takayasu Y, Iino M, Furuya N, et al. Muscarine-induced increase in frequency of spontaneous EPSCs in Purkinje cells in the vestibulo-cerebellum of the rat. J Neurosci 2003:23;6200-8.

30. Kwong WH, Chan WY, Lee KK, et al. Neurotransmitters, neuropeptides and calcium binding proteins in developing human cerebellum: a review. Histochem J 2000:32;521-34.

31. Sarna JR, Larouche M, Marzban H, et al. Patterned Purkinje cell degeneration in mouse models of Niemann-Pick type C disease. J Comp Neurol 2003:456;279-91.

32. Fujita M, Ichise M, Zoghbi SS, et al. Widespread decrease of nicotinic acetylcholine receptors in Parkinson's disease. Ann Neurol 2006:59;174-7.

33. Collerton D. Cholinergic function and intellectual decline in Alzheimer's disease. Neuroscience 1986:19;1-28.

34. Castro A, Martinez A. Targeting beta-amyloid pathogenesis through acetylcholinesterase inhibitors. Curr Pharm Des 2006:12;4377-87.

35. Krall WJ, Sramek JJ, Cutler NR. Cholinesterase inhibitors: a therapeutic strategy for Alzheimer disease. Ann Pharmacother 1999:33;441-50.

36. Voikar V, Rauvala H, Ikonen E. Cognitive deficit and development of motor impairment in a mouse model of Niemann-Pick type C disease. Behav Brain Res 2002:132;1-10.

37. Kaneko S, Maeda T, Kume T, et al. Nicotine protects cultured cortical neurons against glutamate-induced cytotoxicity via alpha7-neuronal receptors and neuronal CNS receptors. Brain Res 1997:765;135-40.

38. Kihara T, Shimohama S, Sawada H, et al. Nicotinic receptor stimulation protects neurons against beta-amyloid toxicity. Ann Neurol 1997:42;159-63.

39. Mount HT, Dreyfus CF, Black IB. Muscarinic stimulation promotes cultured Purkinje cell survival: a role for acetylcholine in cerebellar development? J Neurochem 1994:63;2065-73.

국 문 초 록

니이만 픽 타입 C 질병 마우스 모델에서 도네페질에 의한 콜레스테롤 합성 억제를 통한 퍼킨지 세포의 생존 증가와 운동장애 개선효과

서울대학교 수의대학원

협동과정 인수공통 동물질병학 전공

신 유 영

(지도교수 : 강 경 선)

아세틸콜린은 인지능력에 관여할 뿐만 아니라, 신경세포의 성숙과 분화 과정에 다양한 역할을 하는 것으로 알려져 있는데, 아세틸콜린이 작용하는 콜린성 시스템에 장애가 발생했을 때 신경계 퇴행이 수반되는 경우가 종종 있다. 본 연구에서는 불치성 신경계 질환 중 하나인 니이만 픽 타입C 질환 마우스 모델을 이용하여 콜린성 신경전달 경로의 중요성을 역설하고자 했다. NPC 질

환 특이적으로 퍼킨지 세포의 소실이 일어나는 소뇌 부위에서 콜린 아세틸트랜스퍼라아제 면역염색을 통해 아세틸콜린의 활성이 현저하게 감소되어 있는 것을 보였다. 그 다음으로는 아세틸 콜린에스테라제 저해제인 도네페질을 4주 동안 적용함으로써 약화되어 있는 콜린성 시스템을 회복시키고자 했다. NPC 질환 마우스에 도네페질을 적용했을 때, 질환이 진행되는 동안 칼빈딘 효소를 갖는 퍼킨지 뉴런의 생존율이 증가하고, 로타로드 검사를 통해서 운동 기능도 회복된 것을 확인할 수 있었다. 니이만 픽 타입 C 질병의 주요 원인인 콜레스테롤 침착과 퍼킨지 뉴런의 생존율과의 관계를 조사하기 위하여, 직접 정상 마우스에서 분리한 신경줄기세포에 임의로 콜레스테롤을 침착시켜서 필리핀 염색을 실시했다. 흥미롭게도, 콜레스테롤 침착된 세포에 도네페질을 처리한 결과 필리핀이 염색된 세포가 크게 감소된 것을 확인할 수 있었다. 또한 콜레스테롤 대사 관련 유전자들을 조사한 결과, 콜레스테롤 합성에 관여하는 유전자들의 발현이 도네페질 처리 후 감소된 것을 보여주었고, 이는 궁극적으로 도네페질에 의해 콜레스테롤 합성이 억제됨으로써 신경세포의 생존 기간을 연장 시켜줄 수 있다는 결과를 얻을 수 있었다. 위 결과를 종합하여 NPC 발병 과정에서 콜린성 경로가 중요한 조절체계임을 시사하고, 결과적으로 NPC 질환에서 아세틸 콜린에스테라제 저해제의 치료 효과를 처음으로 보이고자 한다.

주용어 : 도네페질, 니이만 픽 타입C 질환, 아세틸콜린에스테라제 저해제, 퍼킨지 세포, 콜린성 뉴런, 콜레스테롤

학번 : 2011-23723