Aus dem Institut für integrative Neuroanatomie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Zerebelläre Schnittkulturen zur Darstellung und Beeinflussung des Morbus Niemann-Pick Typ C in-vitro

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Doctor medicinae (Dr. med.)

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von

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Inhaltsverzeichnis

1. Abstrakt

Zerebelläre Schnittkulturen zur Darstellung und Beeinflussung des Morbus Niemann-Pick Typ C in-vitro

Niemann-Pick Typ C (NPC) ist eine letal verlaufende, hereditäre, lysosomale Speichererkrankung, welche durch Mutationen des NPC1- oder NPC2-Gens verursacht wird. Typischerweise entwickeln Patienten bereits in einem jungen Alter eine schwere Ataxie aufgrund eines massiven Zelltods von Purkinje-Zellen im Kleinhirn. Bislang ist es nicht möglich diesen Zellverlust in-vitro darzustellen. Ziel dieser Studie war es zu untersuchen, ob organotypische zerebelläre Schnittkulturen geeignet sind, den natürlichen Verlauf der Erkrankung darzustellen und ggf. den Einfluss von verschiedenen pharmakologischen Wirkstoffen zu zeigen.

Dazu wurden ebensolche Zellkulturen eines bekannten NPC-Maus-Modells über einen Zeitraum von 6 Wochen kultiviert und verschiedene Wirkstoffe getestet.

Es konnte gezeigt werden, dass organotypische zerebelläre Schnittkulturen den Verlauf der Purkinje-Zell-Degeneration in NPC zuverlässig darstellen und dass 2-hydroxypropyl-ß-cyclodextrin, wie bereits in anderen Tierversuchen gezeigt, das Absterben von Purkinje-Zellen vermindert. Zudem konnte nachgewiesen werden, dass 3-Methyladenin den Purkinje-Zelltod durch Korrektur des autophagischen Flusses vermindert.

Als weiterer potenzieller Wirkstoff wurde Geranylgeranyl-Pyrophosphat (GGPP) untersucht. Verschiedene Studien haben eine mögliche Hypersensitivität von NPC1-Zellen gegenüber zytotoxischen Effekten von Statinen (HMG-CoA Reduktase Inhibitoren) gezeigt und suggerieren einen möglichen Mangel von GGPP. GGPP ist ein nicht-steroidales Isoprenoid, welches essenziell für Zellüberleben und –differenzierung ist. In einem Partnerlabor wurden GGPP-Konzentrationen in Zerebella von NPC1-Mäusen und ihren Wildtyp-Wurfgeschwistern gemessen. Hier konnte ein physiologischer Anstieg zwischen dem postnatalen Tag 21 und Tag 49 in Wildtyp-Mäusen festgestellt werden, welcher in NPC1-Mäusen nicht zu entdecken war. Dieses Ergebnis unterstützt die Theorie, wonach die Purkinje-Zelldegeneration zwischen Tag 21 und Tag 49 durch einen niedrigen GGPP-Spiegel verursacht werden könnte. Um dieser Hypothese weiter nachzugehen, wurde in unserer Arbeitsgruppe der Einfluss von GGPP auf Langzeit-Schnittkulturen von Kleinhirnen von NPC1-Mäusen untersucht. Es konnte jedoch kein positiver Effekt nachgewiesen werden.

Diese Studie zeigt, dass organotypische zerebelläre Schnittkulturen von NPC-Tieren ein wirkungsvolles Werkzeug sind, um den Purkinje-Zellverlust zu untersuchen.

Organotypic cerebellar slice cultures to monitor Niemann-Pick type C disease in-vitro

Niemann-Pick type C (NPC) disease is a fatal hereditary lysosomal lipid storage disease caused by mutations in NPC1 or NPC2. Typically, patients develop severe cerebellar ataxia due to progressive Purkinje cell loss. Hitherto, in-vitro studies did not allow monitoring this cell loss. Aim of this study was to evaluate whether organotypic slice cultures are usable to monitor the natural process of NPC-associated Purkinje-cell degeneration and if so to show the influence of different pharmacological agents.

Therefore organotypic cerebellar slice cultures of a well-established NPC mouse model were cultivated for a time period of 6 weeks. Moreover several therapeutic candidates were evaluated due to their effect on Purkinje-cell survival.

In our study it is shown that it is possible to monitor and to prevent NPC-related Purkinje cell death reliably in-vitro. As to be expected from the results of previous animal experiments, 2-hydroxypropyl-β-cyclodextrin rescued Purkinje cells. It was discovered that 3-methyladenine preserved Purkinje cell numbers by adjusting the autophagic flux in NPC slices.

Moreover geranylgeranyl pyrophosphate (GGPP) was tested because different laboratories pointed to hypersensitivity to cytotoxic effects of statins (HMG-CoA reductase inhibitors) in NPC1 and suggested an underlying lack of GGPP. GGPP is a non-sterol isoprenoid essential for cell survival and differentiation. GGPP levels in cerebella of a NPC1 mouse model and of wild-type littermates were measured and a physiological increase of GGPP levels between post-natal days 21 and 49 in wild-type mice but not in NPC mice was found. This supports the hypothesis that Purkinje cell loss may be due to an extremely low level of GGPP. The progressive Purkinje cell loss in NPC starts between p21 and p49. To prove the hypothesis, organotypic slice cultures of NPC1 mice were cultivated and it was tested if chronic administration of GGPP might prevent Purkinje cell loss. However we did not see a beneficial effect.

This study shows that cerebellar slice cultures are a powerful in-vitro tool to study NPC-associated Purkinje cell death in an organotypic setting.

2. Eidesstattliche Versicherung

„Ich, Nils Marschalek, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema:

"Zerebelläre Schnittkulturen zur Darstellung und Beeinflussung des Morbus Niemann-Pick Typ C in-vitro"

selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Meine Anteile an den ausgewählten Publikationen entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst."

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Datum Unterschrift

Anteilserklärung an den erfolgten Publikationen

Nils Marschalek hatte folgenden Anteil an den folgenden Publikationen:

1. Marschalek N., Albert F., Meske V. and Ohm T.G.: **The natural history of cerebellar degeneration of Niemann-Pick C mice monitored in vitro**; *Neuropathology and applied Neurobiology* (2014), 40: 933-945

2. Marschalek N., Albert F., Afshordel S., Meske V., Eckert G.P., Ohm T.G.: **Geranylgeranyl pyrophosphate is crucial for neuronal survival but has no specific role in Purkinje cell degeneration in Niemann Pick type C1 disease;** *Journal of neurochemistry* (2015), 133: 153-161

Beitrag im Einzelnen:

- Erlernen, Mithilfe bei Mauszucht und Verpaarung
- Markierung und Genotypisierung (PCR) von Neugeborenen Mäusen
- Präparation der Mäuse und Anlegen der Slice-Kulturen
- Regelmäßiger Mediumwechsel der Kulturen
- Behandlung der Kulturen mit unterschiedlichen Wirkstoffen
- Färbung und Eindeckelung der Schnittkulturen
- Mikroskopieren und Aufnahme/Bearbeitung von Fotographien (Photoshop)
- Zählung der Purkinje-Zellen (Image J)
- Statistische Auswertung und Erstellung von Graphen und Tabellen (Excel)
- Verfassen des Artikels

Unterschrift des Doktoranden/der Doktorandin

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4. „Geranylgeranyl pyrophosphate is crucial for neuronal survival but has no special role in Purkinje cell degeneration in Niemann Pick type C1 disease"

Journal of Neurochemistry

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ORIGINAL ARTICLE

Geranylgeranyl pyrophosphate is crucial for neuronal survival but has no special role in Purkinje cell degeneration in Niemann Pick type C1 disease

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Abstract

Niemann Pick type C (NPC1) is a rare fatal hereditary cholesterol storage disease associated with a massive Purkinje cells loss. The mechanisms leading to neurodegeneration are still poorly understood. Different laboratories pointed to hypersensitivity to cytotoxic effects of statins (HMG-CoA reductase inhibitors) in NPC1 and suggested an underlying lack of geranylgeranyl pyrophosphate (GGPP). GGPP is a non-sterol isoprenoid essential for cell survival and differentiation. We measured GGPP levels in cerebella of a NPC1 mouse model and of wild-type littermates and found a physiological increase of GGPP levels between post-natal days 21 and 49 in wild-type mice but not in NPC mice. This further supports the hypothesis that Purkinje cell loss may be due to an extremely low level of GGPP. The progressive Purkinje cell loss in NPC starts between p21 and p49. To test the hypothesis, we used long-term organotypic slice cultures of NPC1 mice that display the natural history of NPC1 disease *in vitro* and tested if chronic administration of GGPP might prevent Purkinje cell loss. We did not see a beneficial effect. This suggests, in contrast to the expectations, that the relative lack of GGPP may not significantly contribute to mechanisms of Purkinje cell loss in NPC1.

Keywords: cerebellum, GGPP, mevalonate pathway, Niemann Pick type C1, organotypic slice culture, Purkinje cell.
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Niemann Pick type C (NPC) is a rare disease caused by mutations in NPC1 or NPC2 (Carstea *et al.* 1997; Naureckiene *et al.* 2000). These two genes encode proteins acting sequentially in the transfer of free cholesterol out of the lysosomes (Infante *et al.* 2008; Kwon *et al.* 2009; Goldman and Krise 2010; Vance 2010; Wang *et al.* 2010). This explains why cholesterol accumulates in the endosomal/lysosomal compartment of NPC cells and why both disease subtypes develop the same cellular phenotype (Treiber-Held *et al.* 2003; Sleat *et al.* 2004; Dixit *et al.* 2011). Patients suffering from the disease usually die within the first three decades of their life. There is no established cure yet. Currently, the most promising therapeutic tool is 2-hydroxypropyl-β-cyclodextrin which was shown to partially correct the NPC1 phenotype *in vitro* and *in vivo* (Davidson *et al.* 2009; Ramirez *et al.* 2010; Aqul *et al.* 2011; Peake and Vance 2012; Marschalek *et al.* 2014). However, it was also found that cyclodextrins promote aggregation of proteins involved in Alzheimer's disease and may increase their neurotoxic effects (Wang *et al.* 2009). This is relevant because at least some Alzheimer's disease-like neuropathology related to protein tau is an early and common feature in NPC patients (Zhang *et al.* 2010). Up until now, there are no available data regarding the long-term safety of cyclodextrins, and the information regarding other side effects like hearing loss caused by hair cell death (Crumling *et al.* 2012) is limited. Another intriguing finding is that cyclodextrins may have organ-specific effects, e.g., cyclodextrins do not reduce cholesterol storage in the lung (Liu *et al.* 2010). Considering the potential problems of cyclodextrin treatment,

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Abbreviations used: GGPP, geranylgeranyl pyrophosphate; NPC1, Niemann Pick type C1.

it is still necessary to improve our knowledge about the pathogenesis of NPC and develop new therapeutic approaches.

Several studies with animal models of the disease indicate that the metabolic consequences of this gene defect are crucial especially in neurons (Ko *et al.* 2005; Elrick *et al.* 2010; Yu *et al.* 2011). Redistribution of free cholesterol and demyelination eventually lead to neurodegeneration, causing clinical signs like motor disturbances and a progressive cognitive decline (Võikar *et al.* 2002; Treiber-Held *et al.* 2003). Understanding the underlying pathogenetic mechanisms would be valuable in finding therapeutic means which might also be useful in human patients of NPC. Studies carried out with non-neuronal and neuronal cells point to hypersensitivity of NPC1-deficient cells, the cytotoxic effects of statins and some other inhibitors of the HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase (Ohno *et al.* 1993; Corey and Kelley 2007). This is the rate-limiting enzyme of endogenous cholesterol biosynthesis and it controls the formation of mevalonate, which is the precursor of sterol and non-sterol isoprenoids. We found that NPC1-deficient primary neuronal cultures died preferentially when treated with Lovastatin (Ohm and Meske 2006). This suggests that they are more vulnerable to the toxic effects of the statin. An *in situ* microfluorointensitometrical analysis of brain sections of NPC1 mice showed an inverse relationship between accumulated free cholesterol and HMG-CoA reductase mRNA (Ohm *et al.* 2003). Notably, whereas most organs show an increase in cholesterol biosynthesis, the brain exhibits a decrease in cholesterol biosynthesis (Xie *et al.* 1999; Quan *et al.* 2003). By analyzing cerebella from NPC1 mice at different ages (i.e., 3 weeks (before onset of Purkinje cell loss) and after 7 weeks (after severe Purkinje cell loss)) we found decreases in genes of the mevalonate pathway (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE5944). Others found statistically significant decreases in other genes that encode enzymes of the mevalonate pathway in 3-week-old NPC1 mice cerebellum using two different microarray platforms (Liao *et al.* 2010). This indicates that the mevalonate pathway of cholesterol-loaded cells may be down-regulated. Moreover, this does not seem to reflect secondary signs of neurodegeneration because some of these genes are also down-regulated in NPC1 fibroblasts (De Windt *et al.* 2007). However, in the liver, several genes of the mevalonate pathway showed a biphasic behavior over time. An early increase (already at 1 week) was followed by a late decrease (at 11 weeks) (Cluzeau *et al.* 2012). This indicates the existence of some tissue-specific effects.

Inhibition of HMG-CoA reductase does not only block the formation of cholesterol but also reduces the synthesis of geranylgeranyl pyrophosphate (GGPP), because both molecules derive from mevalonate. Interestingly, GGPP completely prevented the toxic effects of Mevastatin on NPC1-deficient fibroblasts (Corey and Kelley 2007) and that of Lovastatin on cultured primary NPC1-neurones (own unpublished data). GGPP serves as a lipid donor for a post-translational process called prenylation. Many intracellular proteins, especially members of the small GTPase superfamily or heterotrimeric G proteins, undergo prenylation. These prenylated proteins are involved in almost all fundamental cellular processes. Among these prenylated proteins is RhoA, which is only prenylated by GGPP (Hooff *et al.* 2008). After inhibition of RhoA with the specific inhibitor C3, survival in wild-type fibroblasts was significantly higher than in NPC1 fibroblasts (Corey and Kelley 2007). Together, the studies suggest a relative lack of GGPP in NPC1 either because of down-regulated genes or because of the change in the flux of the mevalonate pathway, eventually resulting in a deficiency of GGPP. GGPP deficiency or reduced geranylgeranylation was shown to result in reduced synaptic function, formation, and lifetime. Moreover, it leads to reduced cell division (Terano *et al.* 1997; Fuse *et al.* 2004), decreased secretion of the cholesterol transporter apoE, and increased levels of interleukins, nitric oxide, and microglial activation (Koudinov and Koudinova 2001; Cordle and Landreth 2005), changes of the cytoskeleton (Meske *et al.* 2003) and amyloid precursor protein processing and Aß1-42 formation and secretion (Zhou *et al.* 2003, 2008; Cordle and Landreth 2005). Thus, it is not surprising that GGPP is considered as a tool with which to treat or prevent neurodegeneration (Li *et al.* 2012). Evidence from yeast and mammalian cells also suggest a geranylgeranylated protein as a potent feedback regulator of endoplasmic reticulum degradation of HMG-CoA reductase, which may have an impact on the flux in the mevalonate pathway (Garza *et al.* 2009; Ling *et al.* 2009; Leichner *et al.* 2011). However, up until now, there are no data pertaining to the average level of GGPP in NPC1 disease. We reasoned that if there was indeed a lack of GGPP in NPC1 neurons, addition of GGPP might prevent neuronal death not only in statin-challenged neurones but also during the natural history of NPC1. To address these questions, we measured GGPP levels in cerebella of wild-type and NPC1 mice before and after onset of Purkinje cell loss, and used long-term organotypic cerebellar slices to test the effects of GGPP on the natural history of Purkinje cell loss *in vitro*.

Material and methods

Animals

BALB/cNctr-Npc1^{m1N}/J (The Jackson Laboratory, Bar Harbor, Maine 04609 USA) spontaneous mutant mice were fed and bred according to the German Animal Care Committee guidelines. At post-natal day 2–4 tail tips were removed for DNA preparation. PCR as described elsewhere was used to determine genotypes (Treiber-Held *et al.* 2003). All animal experiments were approved by the Charité University medicine Berlin and Goethe University Frankfurt.

Determination of GGPP and cholesterol

We used cerebella of post-natal day 21 (p21) (before onset of Purkinje cell loss) and post-natal day 49 (p49) (after onset of

Purkinje cell loss) of NPC1-deficient animals and age- and gender-matched wild-type littermates. Number of each group was between 5 and 7. Quantification of GGPP was performed as described previously (Hooff *et al.* 2008). Because of the limit of quantification of the analytical method, a minimum quantity of brain tissue that refers to the size of the cerebellum had to be used for analysis. Briefly, GGPP brain tissue samples were homogenized in 100 mM Tris buffer (pH 8.5) containing 5 μL Halt® (Thermo scientific, Pittsburgh, Pennsylvania, USA) and 10 μL Phosstop® (Indianapolis, IN, USA) phosphatase inhibitors. The homogenate was vigorously mixed with 1 mL 100 mM Tris buffer (pH 8.5) and then spiked with 15 μL 2.8 μM solution of 5-(dimethylamino)naphthalene-1-(4-nonylphenol)-sulfonic acid ester used as the internal standard (IS). The mixture was loaded onto Merck Extrelut® NT1-columns (Darmstadt, Germany) and eluted with 1-butanol–ammonium hydroxide–water mixture. The filtrate was centrifuged and the supernatant was evaporated under reduced pressure. After sonication, the solution was applied to Oasis® (Waters, Milford, MA, USA) HLB solid-phase extraction cartridges. The extract was washed with methanol and finally eluted with an ammonium hydroxide–propanol–n-hexane mixture. The filtrate was vacuum-dried and re-dissolved in an assay buffer for the enzymatic reaction. For pre-column dansyl-labeling, the dried residue was dissolved in Tris-HCl assay buffer and spiked with D*-GCVLS and D*-GCVLL (dansyl-labeled peptides) as well as with the coupling enzyme GGTase, respectively. The chromatographic separation was carried out on a Jasco HPLC-system (LG-980-02, PU-980, AS-950; Gross-Umstadt, Germany) with a gradient elution on an Ascentis® Express C-18 reversed-phase analytical column from Supelco (150 × 2.1 mm, 2.7 μm; Munich, Germany) protected by a Phenomenex Security guard column (C-18, 4 × 2.0 mm; Aschaffenburg, Germany). Total cholesterol levels were determined enzymatically, using the CHOD-PAP method (Cholesterol + oxygen –(enzyme cholesterol oxidase)–> cholestenone + hydrogen peroxide Hydrogen peroxide + 4-aminophenazone + phenol –(enzyme peroxidase)–> colored complex + water) (Kirsch *et al.* 2003). Protein concentrations were measured using the bicinchoninic acid Protein Assay Kit from Thermo-Fisher/Pierce (Bonn, Germany). Samples were measured in triplicates.

Organotypic slice cultures

Cultures were prepared from BALB/cNctr-Npc1^{m1N}/J mice following previous protocols (Stoppini *et al.* 1991; Falsig *et al.* 2008). Briefly, mice were decapitated at post-natal days 9–11, their brains were aseptically removed and the cerebellum was dissected in ice-cold Krebs-medium. The cerebellum was included in 4% low melting agarose (Peqlab, Erlangen, Germany) and cut into 350-μm-thick sagittal sections with a vibratome (Integraslice 7550 MM, Campden (Instruments, Lafayette, IN 47903 USA)) under aseptic conditions. Four to six sagittal slices of the cerebellar vermis were separated from the low melting agarose and transferred onto transparent Biopore CM membranes with 0.4 μm pore size (Millicell, Millipore, Billerica, MA, USA). The slices were incubated on 1.1 mL of incubation medium in a humidified atmosphere with 5% CO_2 at 37°C. The complete incubation medium was changed every 2–3 days. The pH value was adjusted between 7.2 and 7.4 properly. Slices were kept in culture for a maximum of 42 days.

Pharmacological compounds

Treatment generally started after 10 days *in vitro*. 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, Steinheim, Germany) was added to the medium weekly for 18 h at a concentration of 0.25% (w/v). GGPP (Sigma-Aldrich) was solved in methanol. In the first approach, it was added to the medium weekly from Friday to Monday at a concentration of 10 μM. This concentration was previously shown to rescue from cell death after a statin-induced fatal decrease of GGPP in both wild-type and NPC1 neurones (Meske *et al.* 2003). In the second regime, 10 μM GGPP was added to the medium with every medium change, i.e., every 2–3 days. From previous studies, we experienced that neuronal death after a statin-induced complete block of GGPP formation can be prevented even when adding GGPP within 2–3 days. Lovastatin (Sigma-Aldrich) was solved in dimethylsulfoxide and added to the medium from 12th to 16th day *in vitro* at different concentrations (10, 5, 1 μM). These concentrations cover the previously determined range from no effect on neuronal survival in murine primary neuronal cultures to 100% loss. At 16th day *in vitro* slice were fixed with 4% paraformaldehyde (w/v).

Immunohistochemistry

For immunohistochemical analysis, slices attached at their membranes were fixed with 4% paraformaldehyde (w/v) in phosphate-buffered saline (PBS) for 30 min at 22°C. They were stored for one night at 4°C in PBS. On the next day, slices were incubated in blocking buffer (8.5 mL phosphate buffer, 1 mL normal goat serum, 0,5 mL 10% Triton X) for a minimum of 1 h to permeabilize the tissue and block unspecific antigen binding. Anti-Calbindin D-28k antibody (Swant, Marly, Switzerland, anti-rabbit) was added to the slice in fresh incubation solution (1 : 2500) and incubated for a minimum of 48 h at 4°C. After washing in PBS secondary antibody Alexa 488 (Invitrogen, Darmstadt, Germany) was added in fresh incubation solution (1 : 500) for 3 h. After washing in PBS, the membranes were cut out of their framework and mounted on coverslips with Immu-Mount (Thermo Scientific, Pittsburgh, Pennsylvania, USA). Cultures were viewed and recorded on a confocal microscope (Leica SL, Wetzlar, Germany). For excitation, we used the argon laser (480 nm) with identical intensity.

Quantitative analysis of Purkinje cell survival

Purkinje cells were counted with Image J (National Institute of Health, Bethesda, Maryland, USA) in the following manner: pictures were recorded with a resolution of 2048 × 2048 pixels, 2048 pixel correlate with 3000 μm, threshold of the recorded images was adjusted so that the immunostained and thus highlighted Purkinje cells were detected and could be counted with the 'count particles' button. An object surpassing the detection threshold and with a circularity of 0.5–1.0 and larger than 100 μm² in the picture were defined as 'Purkinje cell' and counted. Compared to manual count it revealed similar results. Almost all Purkinje cells were detected.

Statistical analysis

For statistical analysis, a *t*-test was performed to compare the Purkinje cell numbers. The number of animals (n) for NPC1 wild type is 11 (53 slices); for NPC1$^{-/-}$ it is 9 (42 slices); for CD-treated slices it is 7 (31 slices); for GGPP-treated slices it is 7 (33 slices) for the first attempt and 6 (29 slices) for the second attempt.

For Lovastatin treatment, we used five animals for each concentration.

Results

To get the total levels of GGPP, we made measurements of GGPP in cerebella of wild-type mice and determined a statistically significant ($p < 0.05$) increase between p21 and p49, i.e., from a mean of 444 pg/μg protein at p21 (SD = 48) to 562 pg/μg protein at p49 (SD = 101) (Fig. 1). At p21 the GGPP concentration in NPC1 cerebella (433 pg/μg protein; SD = 93) is almost identical to the wild-type values (Fig. 1). At this timepoint no neuronal loss can be detected in cerebella of NPC1. Purkinje cell loss in NPC1 mice starts between 40 and 60 days after birth (Higashi *et al.* 1993). At p49 the GGPP concentration in NPC1 cerebella was 410 pg/μg protein (SD = 83). This is significantly lower than in wild-type cerebella of this age ($p < 0.05$) and is almost equivalent to the level of p21 animals. This implies that the physiological increase of GGPP levels seen in wild-type mice has not taken place (Fig. 1).

Cholesterol determinations resulted in a different pattern. At p21 the NPC1 cerebella had statistically significant lower cholesterol levels (124 μg/mg protein; SD = 25) than the wild-type controls (163 μg/mg protein; SD = 24) (Fig. 2). At p49 the cholesterol values were similar between NPC1 (164 μg/mg protein; SD = 30) and wild-type cerebella (183 μg/mg protein; SD = 44) (Fig. 2).

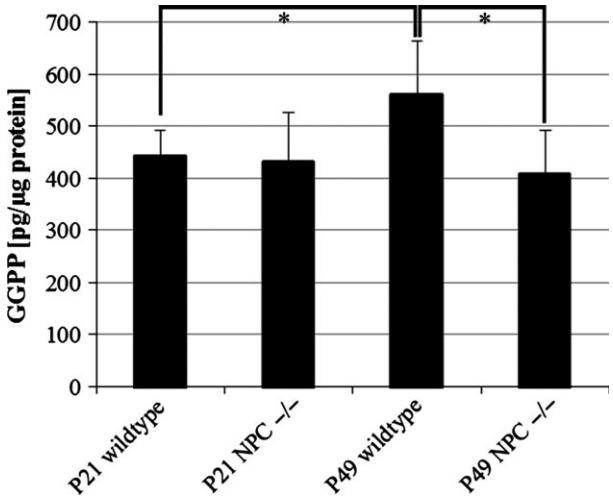

Fig. 1 Shows geranylgeranyl pyrophosphate (GGPP) concentration in cerebella of wild-type and Niemann Pick type C (NPC) mice at p21 and p49. In wild-type mice, there is a significant increase in GGPP levels between p21 and p49. At p21 the GGPP levels in NPC cerebella are almost identical to the wild-type values. At p49 the GGPP concentration in NPC cerebella was about the level of p21 animals. This implies that the physiological increase of GGPP levels seen in wild-type mice has not taken place. Asterisk indicates significant difference ($p < 0.05$), i.e., the increase in wild-type animals between p21 and p49 and the difference between wild-type and $NPC^{-/-}$ at p49.

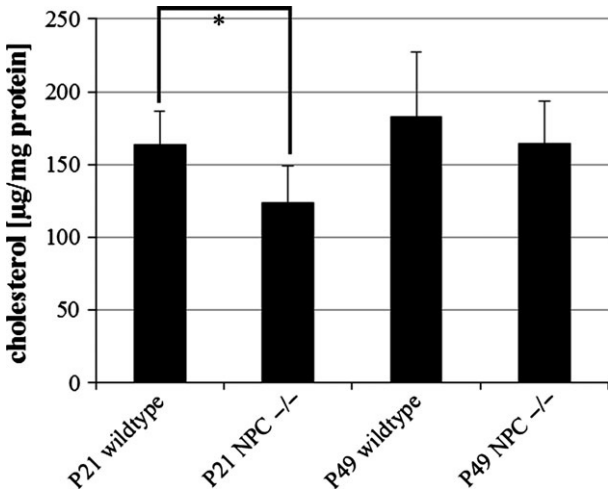

Fig. 2 Shows total cholesterol levels of wild-type and Niemann Pick type C (NPC) mice cerebella at p21 and p49. At p21 the NPC cerebella had statistically significant lower cholesterol levels than the wild-type controls. At p49 the cholesterol values were similar between NPC and wild-type cerebella. Asterisk indicates significant difference compared to wild-type control ($p < 0.05$).

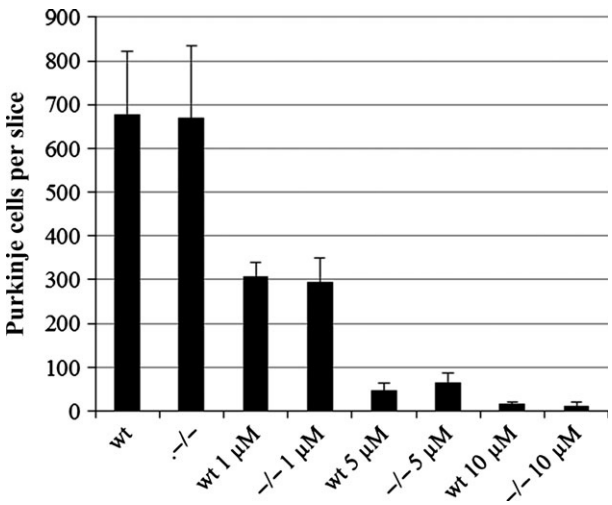

Fig. 3 Shows surviving Purkinje cells after treatment with different Lovastatin concentrations from 12th to 16th day *in vitro*, which refers to post-natal days 22–26 *in vivo*. At 10 μM [17 (wt) versus 12 (Niemann Pick type C, NPC) Purkinje cells per slice] and 5 μM [46 (wt) vs. 65 (NPC) Purkinje cells per slice] Lovastatin killed almost all Purkinje cells. 1 μM Lovastatin resulted in nearly 50% survival [308 (wt) vs. 295 (NPC) Purkinje cells per slice]. Mean values of untreated slices are 678 (wt) and 670 (NPC) Purkinje cells per slice.

Previous studies pointed to hypersensitivity of NPC1-deficient non-neuronal and neuronal cells toward the cytotoxic effects of statins (Ohno *et al.* 1993; Corey and Kelley

2007). We thus reasoned that Lovastatin might also evoke a different degree of neurodegeneration in cerebellar slice cultures of wild-type and NPC mice. To test this, we applied Lovastatin at different concentrations to 12-day-old organotypic slice cultures. Staining with Anti-Calbindin D-28k antibody, however, revealed no differences between the Purkinje cell loss of wild-type and NPC1 mice (for details see Fig. 3, Figure S1). This is in line with our above outlined results when, at post-natal day 21 *in vivo*, which refers to 11 days *in vitro*, no difference in GGPP levels of cerebella of wild-type and NPC1 mice was found.

Recently, we found that organotypic slice cultures live up to 6 weeks *in vitro* and thereby displaying the natural history of Purkinje cell death in NPC1 disease (Marschalek *et al.* 2014). If there was a relative lack of GGPP during the progress of the disease that leads to neuronal loss, it might be prevented by adding GGPP to cerebellar slice cultures of NPC1 mice. However, we did not find a beneficial effect on Purkinje cell shape and survival in slice cultures of NPC1 mice, irrespective of the application mode of GGPP (Fig. 4). Untreated NPC1 mouse cerebellar slice cultures displayed a mean number of 472 Purkinje cells per slice (SD = 104), whereas GGPP given by regime 1 showed 477 (SD = 74) and only 178 (SD = 22) under regime 2. In contrast to that treatment with 2-hydroxypropyl-β-Cyclodextrin, an agent which was able to correct the NPC1 phenotype *in vitro* and *in*

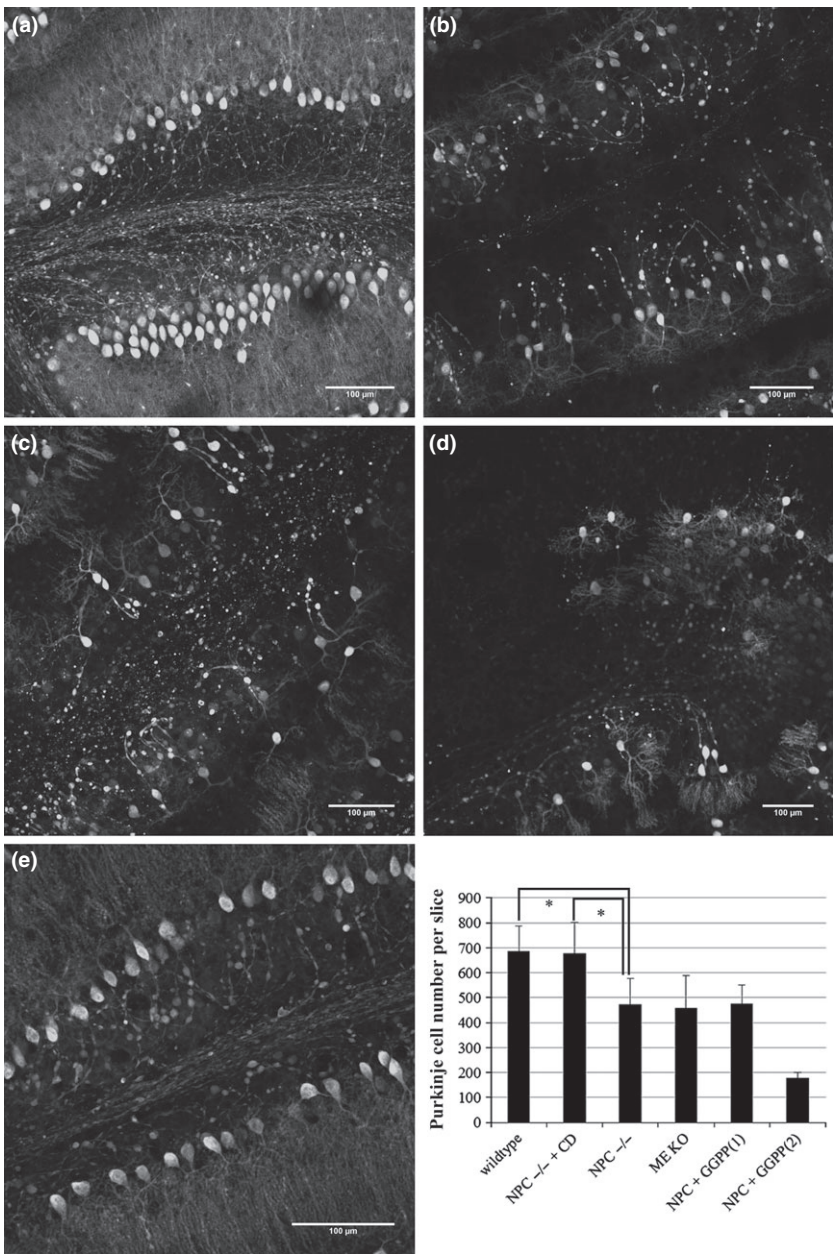

Fig. 4 Shows detailed images of cerebellar slice cultures stained with Anti-Calbindin D28-k antibody after 42 days *in vitro*. Wild type (a) and CD-treated Niemann Pick type C (NPC) slices (e) show almost identical Purkinje cell numbers after 42 days *in vitro*. The typical architecture of the cerebellum is preserved. Untreated NPC slices (b) revealed reduced Purkinje cell numbers. Geranylgeranyl pyrophosphate (GGPP) failed to prevent Purkinje cell death in both approaches. In the first attempt (c) GGPP was administered from Friday to Monday with a concentration of 10 μM. In the second attempt (d), it was added with every medium change (Monday, Wednesday, Friday). * $p<0.05$

vivo (Ramirez *et al.* 2010; Aqul *et al.* 2011; Peake and Vance 2012; Marschalek *et al.* 2014), rescued the Purkinje cells from death in organotypic slice cultures. Mean Purkinje cell numbers of NPC1 untreated versus NPC1 Cyclodextrin-treated slice cultures were 472 (SD = 104) versus 679 (SD = 123), respectively ($p < 0{,}05$). The Cyclodextrin-treated NPC1 slices showed highly similar mean Purkinje cell numbers than the wild-type control slices [679 (SD = 123) versus 687 (SD = 100)] (Fig. 4).

To rule out that GGPP failed to reach its intracellular target, we explored whether GGPP can prevent the toxic effects of Lovastatin on cerebellar slice cultures. Therefore, we added GGPP to Lovastatin-treated cerebellar slice cultures and found a statistically significant increase of Purkinje cell survival. NPC1 slices treated with 1 μM Lovastatin had a surviving mean Purkinje cell number per slice of 295 (SD = 55) which was much more when GGPP was added: 577 (SD = 52) Purkinje cells per slice ($p < 0.05$), but did not reach the level of untreated NPC slices [670 (SD = 164)] (Fig. 5).

Discussion

Despite our substantially increased knowledge as to how NPC1 and NPC2 act in concert to shuttle free cholesterol within lysosomes (Infante *et al.* 2008; Kwon *et al.* 2009; Goldman and Krise 2010; Vance 2010; Wang *et al.* 2010), we are still far from knowing how an impairment of this process translates into the cellular phenotype, eventually resulting in neuronal loss. Previous findings of different laboratories demonstrated an increased sensitivity of NPC1-deficient neuronal and non-neuronal cells toward the toxic effects of statins (Ohno *et al.* 1993; Corey and Kelley 2007) (own unpublished data). A lack of GGPP in NPC1 is suggested because adding GGPP prevented the statin-induced cell loss in NPC fibroblasts (Corey and Kelley 2007) and in primary neuron cultures (own unpublished data). This view is also supported by gene expression data (Ohm *et al.* 2003; De Windt *et al.* 2007; Liao *et al.* 2010; Cluzeau *et al.* 2012). The observed decrease in gene expression of mevalonate pathway-associated enzymes in 21-day-old NPC1 mice cerebellum suggested a decline in cerebellar cholesterol. In agreement with this, we can see a significantly reduced cholesterol level at this age. Thereafter, at post-natal day 49, the cholesterol took the level of the wild-type mice, a result which is in line with a previous report (Xie *et al.* 2000). In sum, previous studies indicate the possibility that neuronal death in NPC1 occurs when the GGPP level drops below the level necessary for survival. This raises the question whether adding GGPP may prevent cell death, serving as the basis for a new therapeutic strategy.

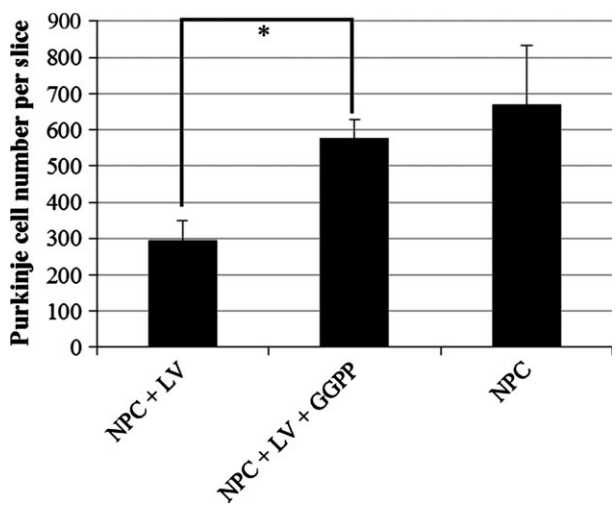

Fig. 5 Shows impact of geranylgeranyl pyrophosphate (GGPP) on Lovastatin-challenged slice cultures. GGPP treatment significantly improved Purkinje cell survival which implies that GGPP reached its intracellular target. Asterisk indicates significant difference compared to control ($p < 0.05$).

For the first time, we determined brain levels of GGPP in NPC1 mice. Under physiological conditions we detected an increase in GGPP levels by ~ 25% between p21 and p49. This is in line with previous data reporting down-regulated GGPP during differentiation and maturation. Under normal conditions, the cerebellum develops its structure and connectivity until p21 (Foran and Peterson 1992). It is tempting to speculate that the observed lack of increase in GGPP levels in NPC1 cerebellum is due to remodeling of neuronal connectivity. Whereas Purkinje cell loss was reported to start not before p 40–60, first signs of neurodegeneration of their dendritic tree can be seen in the cerebellum earlier (Ong *et al.* 2001). The associated process of remodeling might have induced a down-regulation similar to that seen during differentiation and modeling of the mature structure of the cerebellum. Further studies are required to test this hypothesis in detail.

However, our present findings suggest that GGPP is not the crucial factor in NPC1-related neurodegeneration. Although we detected a relatively lower level of GGPP in cerebella of NPC mice at p49, we failed to prevent Purkinje cell death by adding GGPP to organotypic cerebellar slice cultures. This could be due to different reasons: Firstly, Purkinje cells may already have entered a one-way path to death at the stage of investigation. But since CD was able to rescue Purkinje cells, we can be sure that this has not happened. Secondly, because of technical limitations of the GGPP determination assay, we have not been able to determine GGPP levels on a cellular level. Therefore, we cannot rule out the possibility that Purkinje cells did not participate in the GGPP decrease which we observed in homogenates of the cerebellum of NPC1 mice. In this context, it is important to consider that NPC1-specific cell loss seems to be a cell autonomous process. Ko *et al.* (2005)

found that it cannot be prevented by neighboring wild-type cells in a chimeric mouse model that have functional npc1 in only some cells. Thus, it is most likely that Purkinje cells also show a relative lack of GGPP and should have responded to the GGPP treatment. Thirdly, adding GGPP might fail to prevent Purkinje cell loss in our slice cultures because GGPP has not been available for the intracellular geranylgeranyl transferases. This is highly unlikely because we have been able to rescue Lovastatin-challenged Purkinje cells in organotypic slice cultures by adding GGPP. This proves that GGPP has the capacity to rescue neurons from a provoked lack of GGPP and suggests that GGPP can enter neurons also in organotypic slice culture conditions.

The results of GGPP treatment are surprising and contradict the hypothesis derived from the previous findings. We can give two possible explanations as to why GGPP does not prevent neuronal death. Firstly, although the NPC brain shows a reduced cholesterol biosynthesis (Xie *et al.* 1999; Quan *et al.* 2003), this is not necessarily indicative of a reduced non-sterol isoprenoid synthesis. The simplest explanation is that the level of GGPP, independent of its decrease in NPC1 mice cerebellum as measured by us for the first time, is still high enough to maintain cellular needs, and additional GGPP does not affect survival. The second explanation is related to the possibility that cellular metabolism depends not only on cell type but also on the age of a given cell population. The latter is reported for neurons: Myelination of Purkinje cell axons is a cholesterol-dependent process which is largely completed within the first 3 weeks *in vivo* and in rodent cerebellar slices *in vitro* (Jaeger *et al.* 1988; Foran and Peterson 1992; Notterpek *et al.* 1993; Schnädelbach *et al.* 2001; Bouslama-Oueghlani *et al.* 2003; Birgbauer *et al.* 2004). The stage of myelination may modulate the sensitivity of Purkinje cells against statins. A study using organotypic cerebellar slices showed that younger slices with lower myelination are significantly more vulnerable to statins than those at an age equivalent to 20 days after birth. Interestingly, the Purkinje cell loss could be prevented completely by mevalonate, partially (about 50%) by farnesyl pyrophosphate (FPP), but not by GGPP or Low density lipoprotein (LDL)-cholesterol at 10 days *in vitro* (Xiang and Reeves 2009). Although we have not examined the effects of FPP (our own unpublished data did not show a statistically significant reduction of FPP in NPC1 cerebella), the finding that GGPP treatment at 12 days *in vitro* rescued Lovastatin-challenged Purkinje cells reveals a discrepancy to these findings. However, with regard to NPC1, our *in vitro* data imply that the Purkinje cell loss is not initiated by a relative lack of GGPP.

Surprisingly, however, there was not only the lack of a beneficial effect of GGPP on Purkinje cell survival, but there was also a negative effect under regime 2, i.e., with the addition of GGPP when medium change took place every third day. This regime had been chosen because we wanted to be sure that there would be enough GGPP at any time point, and from the lovastatin experiments we had calculated that 3-day intervals would be appropriate. For the time being, we do not have a sound explanation for the observed negative effect, but it seems that it is likely due to a toxic effect of either accumulating GGPP itself or that of a metabolite. In sum, our data show GGPP as an essential molecule for neuronal survival but possibly with no special role in the process of NPC1-related neuronal loss.

Acknowledgments and conflict of interest disclosure

This study was part of the doctoral thesis of Nils Marschalek. We thank Nina Schreyer for secretarial help. Nils Marschalek, Frank Albert, Volker Meske, Sarah Afshordel, Gunter Eckert and Thomas Ohm declare that they have no conflict of interest. Nils Marschalek, Frank Albert carried out all experiments except GGPP measurements. GGPP was measured by Sarah Afshordel and Gunter Eckert. Nils Marschalek, Volker Meske, and Thomas Georg Ohm conceived the experiments. All authors were involved in writing the manuscript and had final approval of the submitted and published version.

All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Shows exemplary pictures of wild-type and NPC slice cultures treated with Lovastatin in different concentrations (a–f).

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5. Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

6. Publikationsliste

2015 Marschalek N., Albert F., Afshordel S., Meske V., Eckert G.P. and Ohm T.G.: **Geranylgeranyl pyrophosphate is crucial for neuronal survival but has no special role in Purkinje cell degeneration in Niemann Pick type C1 disease** *Journal of neurochemistry, 133: 153-161*

2014 Marschalek N., Albert F., Meske V. and Ohm T.G.: **The natural history of cerebellar degeneration in Niemann Pick C mice monitored in vitro**. *Neuropathology and applied Neurobiology*, 40: 933-945

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