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Simvastatin induces cell death in a mouse cerebellar slice culture (CSC) model of developmental myelination

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Abstract

Statins (inhibitors of HMG-CoA reductase) have shown promise in treating multiple sclerosis (MS). However, their effect on oligodendrocyte remyelination of demyelinated axons has not been clarified. Since developmental myelination shares many features with the remyelination process, we investigated the effect of lipophilic simvastatin on developmental myelination in organotypic cerebellar slice cultures (CSC). In this study, we first characterized developmental myelination in CSC from postnatal day (P)5 and P10 mice that express enhanced green fluorescence protein (eGFP) in oligodendrocyte-lineage cells. We then examined the effect of simvastatin on three developmental myelination stages: early myelination (P5 CSC, 2DIV), late myelination (P10 CSC, 2DIV) and full myelination (P10 CSC, 10DIV). We found that treatment with simvastatin (0.1 μ M) for 6 days decreased the survival of Purkinje cells and oligodendrocytes drastically during the early myelination stage, while moderately during the late and full myelination stages. Oligodendrocytes are more resistant than Purkinje cells. The toxic effect of simvastatin could be rescued by the product of HMG-CoA reductase mevalonate but not low-density lipoprotein (LDL). Additionally, this toxic effect is independent of isoprenylation since farnesyl pyrophosphate (Fpp) but not geranylgeranyl pyrophosphate (GGpp) provided partial rescue. Our findings therefore suggest that inhibition of cholesterol synthesis is detrimental to neuronal tissue.

Keywords

Statin; toxicity; cerebellar slice culture; oligodendrocytes; Purkinje cells; myelination; eGFP; mevalonate; LDL; isoprenylation

Introduction

In multiple sclerosis (MS), autoimmune reactions in the central nervous system (CNS) lead to the loss of myelin as well as myelinating oligodendrocytes (Steinman, et al., 2002, Zamvil and Steinman, 2003). As a natural repair mechanism, oligodendrocyte precursor (OP) cells proliferate and differentiate within the demyelination sites to replenish the lost myelinating oligodendrocytes (Levine, et al., 2001, Ruffini, et al., 2004). Current therapeutic strategies include suppression of immunoreactions and inflammation, which are designed to halt the destruction myelin.

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Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting enzyme for cholesterol synthesis, and have shown promise in treating neurological diseases (Menge, et al., 2005, Weber, et al., 2005). Statins protect against glutamate excitotoxicity (Zacco, et al., 2003) or A β toxicity (Cordle and Landreth, 2005), and have been shown to be beneficial in the experimental autoimmune encephalomyelitis (EAE) model for MS (Paintlia, et al., 2005, Youssef, et al., 2002) and in a limited MS clinical trial (Vollmer, et al., 2004). As recently reviewed, the beneficial effect of the statins appears to be immunomodulatory and anti-inflammatory, and not due to their cholesterol lipid-lowering properties (Menge, et al., 2005, Weber, et al., 2005). Despite the reported beneficial effect, statins have been associated with arrest of DNA synthesis and proliferation (Langan and Volpe, 1986), cell death in primary neuronal culture (Marz, et al., 2007, Michikawa and Yanagisawa, 1999), neuroblasts (Garcia-Roman, et al., 2001), PC12 cells (Kumano, et al., 2000), and cardiac myocytes (Demyanets, et al., 2006). Synaptic loss (Mauch, et al., 2001) and cognitive deterioration in clinical studies (Padala, et al., 2006) are also reported.

Pertinent to remyelination in MS, it is thus important to evaluate the effect of the statins more thoroughly concerning their overall actions in the CNS tissue, especially during critical cellular development as occurs in the remyelination process. In the absence of a suitable remyelination model *in vitro*, we investigated the effect of simvastatin on developmental myelination using organotypic cerebellar slice culture (CSC) as a model system. Indeed, much of our understandings of the remyelination process have been gained from developmental myelination studies (Dubois-Dalcq, et al., 2005). Myelination in the cerebellum occurs postnatally and CSC from early postnatal animals replicates this *in vivo* myelination process (Birgbauer, et al., 2004, Bouslama-Oueghlani, et al., 2003, Jaeger, et al., 1988, Notterpek, et al., 1993, Schnadelbach, et al., 2001). CSC thus provides an *in vitro* model system for neuronal tissue in a semi-*in vivo* setting.

In this study, we first characterized Purkinje cell myelination in CSC from P5 and P10 mice, using transgenic mice expressing enhanced green fluorescence protein (eGFP) under the control of the phospholipid protein (PLP) promoter (Mallon, et al., 2002). We then tested the effect of the lipophilic simvastatin on CSC. We found that treatment with simvastatin at clinically relevant concentrations is detrimental to the survival of Purkinje cells and oligodendrocytes during myelination.

Results

Appearance and distribution of oligodendrocytes in the mouse cerebellum

In the mouse cerebellum neurogenesis is complete at birth (Thompson and Potter, 2000). Progressive myelination of Purkinje cell axons follows and is largely complete by P21 (Bouslama-Oueghlani, et al., 2003, Foran and Peterson, 1992). We used transgenic mice that express eGFP under the control of the oligodendrocyte-specific PLP promoter (Mallon, et al., 2002) to facilitate identifying oligodendrocyte-lineage cell types.

First brain sections of the cerebellum at different postnatal days were compared. At postnatal day 5 (P5) eGFP⁺ oligodendrocyte-lineage cells were faint in fluorescence intensity and appeared only in the white matter (cerebellar peduncle) close to the brain stem (Figure 1A). At P10, eGFP⁺ cells extended into each folium and increased in fluorescence intensity (Figure 1B). At P25, bright eGFP⁺ cells were distributed throughout the white matter except for within the deep nuclei of the white matter where only scattered eGFP⁺ cells were observed (Figure 1C). A small number of eGFP⁺ cells were observed in the granule cell layer at this developmental time point. The pattern of eGFP fluorescence observed at P25 was grossly comparable to that of a 4month adult mouse (not shown).

Appearance and distribution of oligodendrocytes in CSC

We then selected P5 and P10 mouse pups for generation of CSCs and examined *in vitro* development of oligodendrocyte-lineage cells by their eGFP fluorescence. In P5-derived CSC that had been cultured for 10DIV, most of the eGFP⁺ cells were limited to the root of the white matter and sparsely distributed along the axis of each folium (Figure 1D). Comparing with P5 brain (Figure 1A), eGFP⁺ cells migrated into each folium. It should be noted that in CSC not all Purkinje cell axons are distributed along the axis of each folium. Some axons appear to have reverted back to the Purkinje cell layer.

In P10-derived CSC at 12DIV there was a significant increase in eGFP⁺ cells in the whiter matter and axis of each folium (Figure 1E). eGFP⁺ cells within each folium extended processes from the axis. These processes can only be seen in the much thicker CSC (~100 μ m) but not in a thin brain section (10 μ m, Figure 1C). Some eGFP⁺ cells were also distributed in the granular cell layer and molecular layer of the CSC. In summary, P5 and P10 CSC both preserved the general organotypic structure of cerebellum (Figure 1G); however, oligodendrocyte-lineage cells develop much better in P10 CSC. The pattern of eGFP fluorescence (Figure 1E) is comparable to the pattern of myelin basic protein (MBP) immunostaining (green) in a P10 CSC derived from a wild-type mouse (Figure 1F), suggesting that eGFP fluorescence may be used to monitor myelination.

Characterization of myelination of Purkinje cell axons in CSC

Further confirmation of myelination of Purkinje cell axons in CSC was obtained by examining the co-localization of immunostainings for β III-tubulin (Figure 2A, 2E, Red), and MBP (Figure 2C, 2G, Blue), and observing eGFP fluorescence (Figure 2B, 2F, Green) in a P10 CSC cultured for 14DIV. The cytoarchitecture within a folium is maintained in these CSCs because the molecular layer, Purkinje cell layer, granule layer and white matter are clearly identifiable. eGFP fluorescence and MBP immunostaining co-localized well in the processes of the same cells, although within the soma / nucleus eGFP fluorescence was strong while MBP immunostaining was weak. This result suggests that eGFP fluorescence in the processes can be used as an indication for MBP presence. Purkinje cell bodies and axons were visualized by β III-tubulin immunostaining. It is of note that instead of a single Purkinje cell layer as observed in cerebellar tissue sections, Purkinje cells in CSCs are in multiple layers due to realignment of these cells after collapse of the original cerebellar slice from a thickness of 350 μ m to about 100 μ m after 14DIV. β III-tubulin immunostaining in the processes co-localized with eGFP fluorescence and MBP immunostaining demonstrating that eGFP⁺ oligodendrocytes myelinate Purkinje cell axons (Figure 2D, 2H). These results confirm that in CSC eGFP fluorescence identifies both oligodendrocyte processes and myelinated Purkinje cell axons.

Monitoring eGFP⁺ cells and myelination in CSC

CSC from PLP-eGFP mice allows continuous monitoring of oligodendrocyte-lineage cells during the course of culture. As shown in Figure 3, eGFP fluorescence from the same folium of a P10 CSC was examined every two days up to 18DIV (only selective days are shown). At 2DIV eGFP fluorescence was detectable halfway along the axis of the folium. With continued culture of the CSC, eGFP⁺ cells expanded toward the distal end of the folium and by 10DIV emanated thin processes from the axis, indicating myelination of Purkinje cell axons. From 10DIV on up to 18DIV, there was a small increase in eGFP fluorescence intensity but the pattern remained the same suggesting that myelination is complete by 10DIV.

Simvastatin toxicity at early myelination stage (P5 CSC, 2DIV)

We next examined the effect of simvastatin on developmental myelination in CSC. We chose three progressive stages of myelin development arbitrarily defined as early myelination (P5,

2DIV), late myelination (P10, 2DIV) and full myelination (P10, 10DIV). CSCs were treated with simvastatin (0.1 or 1 μ M) for 6 days, and the status of Purkinje cells and oligodendrocytes (eGFP⁺) was assessed by axonal immunostaining (neurons) and observing eGFP fluorescence (oligodendrocytes). The viability of these cells was scored on a scale of 0 to 3 (worst to best) (see Methods).

At early myelination stage, simvastatin treatment induced nearly complete death of Purkinje cells and oligodendrocytes compared to untreated CSC (Figure 6A-C). For Purkinje cells, immunostaining was largely absent or was detectable only in short fragmented pieces. For oligodendrocyte-lineage cells, eGFP⁺ processes were fragmented into beads. Shown in Figure 4, similar damage was observed at both concentrations (0.1 or 1 μ M) indicating that simvastatin at 0.1 μ M induces maximal damage of both Purkinje cells and oligodendrocytes. A few dying eGFP⁺ oligodendrocytes were observed mostly limited to the root of the white matter and displayed no processes.

Simvastatin toxicity at late myelination stage (P10 CSC, 2DIV)

At late myelination stage, simvastatin treatment at 0.1 μ M induced moderate damage of Purkinje cells and oligodendrocytes, while at 1 μ M induced significantly more Purkinje cell death (Figure 4 and 6D-F). However, even at 1 μ M some eGFP⁺ oligodendrocytes, but not neurons, survived suggesting that Purkinje cells are more vulnerable to simvastatin than oligodendrocytes at this myelination stage. In addition, a comparison with simvastatin-treated P5 CSC (see Figures 4 and 6A-C) suggests that neurons and oligodendrocytes in P10 CSC are more resistant to simvastatin treatment.

Simvastatin toxicity at full myelination stage (P10 CSC, 10DIV)

At full myelination stage, simvastatin treatment at 0.1 μ M caused moderate damage to neurons and eGFP⁺ oligodendrocytes. At 1 μ M there was death of virtually all neurons and most eGFP⁺ oligodendrocytes (Figures 4 and 6G-H).

Mevalonate, but not LDL rescues CSC from simvastatin toxicity

Statins inhibit the activity of HMG-CoA reductase, so we tested whether supplementation with mevalonate, the enzymatic product of HMG-CoA reductase, rescues the detrimental effect of simvastatin. CSCs at full myelination stage were supplemented in the medium with mevalonate (100 μ M) during a 6-day treatment with simvastatin. CSCs supplemented with mevalonate alone were comparable to controls. Mevalonate at 100 μ M largely rescued the neuronal and oligodendrocyte death induced by 1 μ M simvastatin treatment (Figure 5 and 6I). These results suggest that the detrimental effect of simvastatin is due to inhibition of cholesterol synthesis.

Next, we examined whether extracellular uptake of cholesterol-rich low-density lipoprotein (LDL) particles via the LDL receptor (LDLR) rescues simvastatin-treated CSCs. CSCs were supplemented in the medium with LDL (10 ng/ml) during a 6-day treatment with simvastatin. CSCs supplemented with LDL alone were comparable to controls. There was no improvement in neuronal and oligodendrocyte survival in simvastatin-treated and LDL supplemented CSCs (Figures 5-6D). These results suggest that LDL/LDLR-mediated uptake of cholesterol during developmental myelination does not compensate for the detrimental effect of simvastatin.

Simvastatin toxicity is not due to blockade of isoprenylation in CSC

Simvastatin inhibits cholesterol synthesis and results in a shortage of the intermediate product farnesylpyrophosphate (Fpp). Since Fpp is the precursor for geranylgeranylpyrophosphate (GGpp), which is involved in protein isoprenylation in signal transduction, we tested whether blockade of isoprenylation contributed to simvastatin toxicity in CSC.

CSCs were supplemented in the medium with Fpp or GGpp (all 10 μ M) during a 6-day treatment with simvastatin (1 μ M). CSCs supplemented with Fpp or GGpp alone were comparable to controls. While Fpp supplement partially rescued neuronal survival and fully rescued oligodendrocyte survival in simvastatin-treated CSCs, GGpp provided no statistically significant improvement (Figure 5 and 6K-L). These results suggest that simvastatin toxicity is not due to blockade of isoprenylation in CSC.

Discussions

CSC as a model

Myelination of Purkinje cell axons in the cerebellum occurs postnatally and is largely completed by P21 (Bousslama-Oueghlani, et al., 2003, Foran and Peterson, 1992). In CSC-derived from early postnatal rat or mouse this myelination is replicated *in vitro* (Birgbauer, et al., 2004, Bousslama-Oueghlani, et al., 2003, Jaeger, et al., 1988, Notterpek, et al., 1993, Schnadelbach, et al., 2001). CSC thus provides a suitable model for studying myelination in a semi-*in vivo* fashion. The majority of CSCs used in previous studies were from rat rather than mouse. CSC from a P0 mouse has been reported (Bousslama-Oueghlani, et al., 2003) and CSC from older mice (P6 to P12) have been used in short-term culture (< 3DIV) for virus infections (Inamura, et al., 2000, Sato, et al., 2004). Our studies shown here characterizing long-term mouse CSC facilitates the usage of the vast resource of genetically manipulated mice.

Taking advantage of PLP-eGFP mouse which expresses eGFP in oligodendrocyte lineage cells (Mallon, et al., 2002), we characterized CSC from P5 and P10 mouse. P5 CSC undergoes clear development of oligodendrocytes, which extend from white matter toward Purkinje cell bodies along Purkinje cell axons, consistent with *in vivo* myelination, which starts from the distal end of axons. P5 CSC is prone to extensive cell proliferation and sprouting, and indeed we observed that in P5 CSC Purkinje cell axons are spreading out and some axons appear to have reverted back to the Purkinje cell layer. P10 CSC, however, has better organotypic features, excellent Purkinje cell survival, and myelination as demonstrated by co-localization of eGFP fluorescence with neurofilament and MBP immunostaining. Full myelination in P10 CSC is mostly achieved by 10DIV, similar to that reported in rat CSC (Birgbauer, et al., 2004, Dusart, et al., 1997). With older mice, Purkinje cell survival in CSC decreases drastically, as the more mature neurons are vulnerable to the traumatic damage sustained during preparation (data not shown).

CSC allows experimental manipulation of the environment (culture medium) or the cells by genetic manipulation (i.e. RNAi; viral infection) or pharmacological means. Mouse CSC therefore can be used for studying myelination and inflammation as well as the effect of various differentiation factors and tropic factors on these processes. In addition, slice culture (not restricted to cerebellum) can be used for tissue-based drug screening, which would facilitate pre-clinical screening of therapeutic drug candidates. Lastly, although CSC is an immune-independent setting, immune cells or factors can be introduced to adapt to specific research goals.

Statin effect on CSC

Cholesterol is synthesized locally in the CNS and not obtained from the peripheral system (Bjorkhem and Meaney, 2004, Dietschy and Turley, 2004). In the CNS there is an exceptionally high amount of cholesterol enriched in myelin. In addition to being a critical structural component of all plasma membranes cholesterol has an integral role in membrane trafficking and signal transduction and disruption of cholesterol metabolism is associated with diseases such as Niemann-Pick disease (Maxfield and Tabas, 2005). In mice with deficient squalene synthase (a key enzyme in cholesterol synthesis) in oligodendrocytes, CNS myelination is

retarded (Saher, et al., 2005) and constitutional knockout of squalene synthase is lethal at an early embryonic stage (Tozawa, et al., 1999). Brain cholesterol accumulation is most rapid in the first few postnatal weeks (Dietschy and Turley, 2004) when myelination (Bousslama-Oueghlani, et al., 2003, Foran and Peterson, 1992) and synaptogenesis (Thompson and Potter, 2000) are at their peak. Interruption in the supply of cholesterol would disturb these processes.

Using our CSC model, we investigated the effect of simvastatin in isolated CNS tissue. We found that simvastatin induced neuronal and oligodendrocyte death in CSC at concentrations as low as 0.1 μ M, which is in the similar range for cholesterol inhibition by simvastatin in hepatic tissue (IC₅₀ around 20 nM) (Bergstrom, et al., 1998, Mosley, et al., 1989). This concentration (0.1 μ M) is lower or comparable to the concentrations used in numerous *in vitro* studies (Demyanets, et al., 2006, Garcia-Roman, et al., 2001, Kumano, et al., 2000, Marz, et al., 2007, Michikawa and Yanagisawa, 1999, Miron, et al., 2007) examining various biological functions of statins including protective effects against toxicity of glutamate (Zacco, et al., 2003) or A β (Cordle and Landreth, 2005). In mice chronically treated with simvastatin (50mg/Kg) the level of simvastatin in the brain reaches comparable levels (50-500pmol/g or approximately 50-500 nM) (Johnson-Anuna, et al., 2005). In clinical studies using a much smaller dose of these HMG CoA reductase inhibitors (<80mg / daily), similar plasma concentrations occur in patients (Corsini, et al., 1999, Desager and Horsmans, 1996). While a direct comparison between *in vitro* and *in vivo* studies is indeed a difficult comparison because statins *in vivo* have different pharmacokinetics, we feel our studies do suggest that the effect observed *in vitro* may have clinical relevance and warrant further investigation.

In a previous report using primary cultures of rat and human oligodendrocyte precursor cells (OPC), while short-term treatment with simvastatin induced OPC process outgrowth and differentiation, prolonged treatment induced OPC death after 4 days and differentiated OPC death after 8 days (Miron, et al., 2007). Our results using CSC are consistent with these findings in that oligodendrocytes at early developmental stages are more sensitive to cholesterol inhibition. In addition, we found that neurons in CSC are more sensitive to statin treatment than oligodendrocytes. It remains to be determined if specific cerebellar neuronal cell types (i.e. Purkinje cells, granule neurons) are more sensitive to statin treatment. However, it has been shown previously that primary cultures of cerebellar granule neurons die when treated with 0.1 μ M simvastatin (Marz, et al., 2007).

In our study, supplement with LDL did not provide rescue. In mice with genetically blocked cholesterol synthesis in oligodendrocytes, myelination, although at a slower pace, can still be completed suggesting cholesterol uptake is important at later stages of development (Saher, et al., 2005). Although uptake through LDLR is implied as one of the major means to acquire cholesterol besides synthesis in oligodendrocytes, the exact mechanism and physiological relevance of cholesterol uptake in the CNS are not clear. In our experimental setting, all cells in CSC are affected by simvastatin treatment. The lack of rescue by LDL probably implies that uptake through LDLR has much slower kinetics than cholesterol synthesis and cannot keep up with the demand. Alternatively, cholesterol synthesis in astrocytes may be indispensable for their survival and support of neurons and oligodendrocytes (Pfrieger, 2003).

In conclusion, our results show that simvastatin-mediated cholesterol synthesis inhibition is detrimental to neurons and oligodendrocytes during developmental myelination. It should be noted that our developmental myelination model might not completely reflect the remyelination process that can occur in MS, where the latter occurs in an inflammatory environment. However, our results are still relevant since both these processes involve proliferation of oligodendrocyte precursor cells and differentiation into myelinating oligodendrocytes. Our results thus warrant more investigation in considering statins as a therapeutic for treatment of neurodegenerative disease such as MS.

Materials and Methods

Materials

Antibodies for MBP were from Santa Cruz Biotechnology (Santa Cruz, CA), β III tubulin from R&D (Minneapolis, MN), and pan-axonal neurofilament marker (SM312) from Dr. Bradley Hyman (Massachusetts General Hospital, Alzheimer's unit). Tissue culture reagents were from Invitrogen (Carlsbad, CA). Membrane inserts (30 mm diameter, 0.4 μ m pore size) from Millipore (Billerica, MA). Simvastatin was from Calbiochem (San Diego, CA) and activated according to the manufacturer's instructions. Mevalonate, human LDL, farnesylpyrophosphate (Fpp) and geranylgeranylpyrophosphate (GGpp) were from Sigma (St. Louis, MO).

Animals

Transgenic mice expressing enhanced green fluorescence protein (eGFP) under the control of the phospholipid protein (PLP) promoter (Mallon, et al., 2002) were kindly provided by Dr. Wendy Macklin (Cleveland Clinic Foundation, Ohio). Wild type (C57BL/6J) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animal use was in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and was approved by the subcommittee on Research Animal Care at Massachusetts General Hospital.

Cerebellar slice culture

CSC were established as previously described for hippocampal slice culture (Xiang, et al., 2000). In brief, PLP-eGFP transgenic or wild type mouse pups at the age of P5 or P10 were decapitated and the cerebella were separated and placed in modified Gey's balanced salt solution. Cerebella were then cut into 350 μ m parasagittal slices using a McIlwain tissue chopper. Two to three slices were plated onto each membrane insert, and the inserts were placed in a 6-well plate containing 1 ml serum-free slice culture medium and cultured at 37°C in 5% CO₂. Medium was changed every 3 days. Serum-free slice culture medium consists of Neurobasal A medium, B27 supplement, 2.5 mM L-glutamine and 5mM glucose. All media contained 100 U/ml penicillin and 100 μ g/ml streptomycin.

Immunostaining

CSC grown on membrane inserts were fixed in 4% paraformaldehyde for 30 min and then rinsed in phosphate buffered saline (PBS). After permeabilization for 1 h at RT with 0.2% Triton X-100 in PBS and 10% normal donkey serum to block non-specific binding, CSC inserts were incubated for 4 h with primary antibodies diluted in PBS with 1% normal donkey serum. The antibodies used and dilutions were goat anti-MBP (1: 100), mouse anti- β III tubulin (1:1000) and rabbit anti-neurofilament (SMI312, 1:1000). After three 30 min washes with PBS, CSC inserts were incubated with the appropriate Cy2-, Cy3- or Cy5-conjugated secondary antibodies at RT for 1 h in the dark. CSC inserts were mounted on slides with fluorescence mounting medium containing the nuclear dye DAPI and examined using an Olympus BX60 microscope equipped with epifluorescence optics.

Quantification

Damage to neurons and oligodendrocytes in each CSC were assessed based on immunostaining of pan-axonal neurofilament (Purkinje cells) and eGFP fluorescence (oligodendrocytes), and scored on a scale of 0-3 (worst to best). For each slice, the folium with the best quality was scored as follows: Neuron scoring; 3, all fibers are intact that run from the Purkinje cell layer to the axis of the folium with no fragments; 2, most of the fibers are long intact fibers mixed with some short fragments; 1, most of the fibers are short fragments mixed with very little long intact fibers; 0, fragments and debris only. Oligodendrocyte scoring; 3, All eGFP⁺ processes are intact and long with no fragments; 2, most of processes are intact and long mixed with some

eGFP⁺ fragments; 1, most of processes are in fragments mixed with very little long intact processes; 0, eGFP⁺ fragments only.

Statistics

Statistical analysis was performed using Prism software (GraphPad Software, San Diego, CA). One-way ANOVA (for statin effect) or two-way ANOVA (for statin and rescue agent or Age) and Bonferroni posttest were used to assess the difference among groups. Significance levels were set at $p < 0.05$ (*); 0.01 (**); 0.001 (***).

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Abbreviations

CSC	cerebellar slice culture
DIV	days <i>in vitro</i>
MS	multiple sclerosis
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
eGFP	enhanced green fluorescence protein
PLP	phospholipid protein
MBP	myelin basic protein
LDL	low-density lipoprotein
Fpp	farnesyl pyrophosphate
GGpp	

geranylgeranyl pyrophosphate

Figure 1.

Myelination in cerebellar slice culture (CSC) mimics developmental myelination *in vivo*. Mouse cerebella from P5 (**A**), P10 (**B**) and P25 (**C**) PLP-eGFP mice were sectioned and nuclei were then stained with DAPI to illuminate the general cytoarchitecture. At P5, weak eGFP fluorescence (green) first appears in the root of the cerebellum. At P10, eGFP⁺ cells spread along the axis of each folium, and increase in number and fluorescence intensity. By P25, eGFP⁺ oligodendrocytes reach the tip of each folium and spread into the gray matter. CSC from a P5 PLP-eGFP mouse cultured for 10DIV retains the general folia structure (**D**). Purkinje cell fibers are immunostained with β III tubulin (red). Sparse eGFP⁺ oligodendrocytes can be observed and are limited to white matter regions (**D**). A P10 PLP-eGFP mouse CSC cultured for 12DIV still retains a conserved folia structure, with eGFP⁺ oligodendrocytes distributed throughout the white matter generating a brush-like appearance in the folia (**E**). The pattern of eGFP fluorescence is very similar to the immunofluorescence observed in CSC from a P10 WT mouse at 12DIV immunostained with MBP (green) and β III tubulin (red) (**F**). Scale bar, 250 μ m. The diagram in (**G**) shows the general structure of a folium in the cerebellum. M, molecular layer; G, granular cell layer, P, Purkinje cell layer; WM, white matter.

Figure 2.

Myelination of Purkinje cell axons in CSC. A P10 CSC from PLP-eGFP mouse was cultured for 14DIV, and subjected to immunostaining for β III tubulin (**A**, **E**, Cy3 in Red) and MBP (**C**, **G**, Cy5 but shown in Blue), Co-localization with eGFP (**B**, **F**, Green) is displayed in the merged pictures (**D**, **H**). A folium is shown in the top row (**A-D**). Bottom row (**E-G**) are higher magnification images intended to show more detail. Scale bar, 50 μ m.

Figure 3.

Myelination is progressive in CSC. A P10 CSC from a PLP-eGFP mouse was imaged during the course of culture. The left most panel is a phase contrast image showing a folium. The remaining panels are live eGFP fluorescent images of the same folium at different DIVs. Note the extension of eGFP fluorescence along the axis of the folium. After 10DIV, eGFP⁺ cells appear in the distal end of the folium. Some scattered eGFP⁺ cells appear within the gray matter. This pattern of eGFP fluorescence was sustained up to 18DIV. Scale bar, 200 μ m.

Figure 4.

Simvastatin is detrimental to Purkinje cells and oligodendrocytes in all three stages of developmental myelination in CSC. CSC at early myelination (P5 CSC, 2DIV, empty bars), late myelination (P10 CSC, 2DIV, bars with horizontal lines) and full myelination (P10 CSC, 10DIV, bars with diagonal lines) stages were treated with simvastatin (0.1 or 1 μM) for 6 days. The status of Purkinje cells (NF immunostaining, **A**) and oligodendrocytes (eGFP fluorescence, **B**) after the treatment period were scored on a scale of 0 (worst) to 3 (best) (see Methods for details). Data were shown as Mean $\pm$ SEM, the number within each bar indicates the total number of slices from three separate experiments. One-way ANOVA (for statin effect) or two-way ANOVA (for statin and age) and Bonferroni posttest were used. Unless indicated

otherwise, asterisks indicate significance compared to untreated group at specific stage with * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 5.

Simvastatin toxicity is due to cholesterol inhibition and is independent of isoprenylation. CSCs at full myelination stage (P10 CSC, 10DIV) were treated simvastatin (1 μ M) for 6 days in the absence or presence of mevalonate (100 μ M), LDL (10 ng/ml), Fpp (10 μ M) or GGpp (10 μ M). Following treatment, CSCs were immunostained with the axonal marker NF (neurofilament) and the status of neurons (**A**) and eGFP⁺ oligodendrocytes (**B**) were scored on a scale of 0 (worst) to 3 (best) (see Methods). Data are shown as Mean $\pm$ SEM, the number within each bar indicates the total number of slices from three separate experiments. Two-way ANOVA (for statin and age) and Bonferroni posttest were used. Simvastatin group is

significantly different from control group, all other asterisks indicate significance levels compared to simvastatin group, *** $p < 0.001$.

Figure 6.

Typical images showing simvastatin toxicity and rescue in CSC. CSC at early myelination (A-C), late myelination (D-F) and full myelination (G-L) stages were treated with 0.1 μ M (B, E) or 1 μ M (C, F, H) simvastatin for 6 days, in the presence of mevalonate (100 μ M) (I), LDL (10 ng/ml)(J), Fpp (10 μ M) (K) or GGpp (10 μ M) (L). Shown are merged pictures of NF immunostaining (red) and eGFP fluorescence (green). Scale bar, 50 μ m. Slices treated alone with mevalonate, LDL, Fpp or GGpp were not different from non-treated (Con) and the images were not shown.