Macroglial diversity and its effect on myelination
Werkman, Inge

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Impairing committed cholesterol biosynthesis in white matter astrocytes, but not grey matter astrocytes, enhances in vitro myelination.
Abstract

Remyelination is a regenerative process that is essential to recover saltatory conduction and to prevent neurodegeneration upon demyelination. Remarkably, remyelination is more efficient in grey matter (GM) than in white matter (WM) lesions. In the central nervous system, the formation of new myelin involves the differentiation of oligodendrocyte progenitor cells (OPCs) towards oligodendrocytes (OLGs), a process that, among others, is regulated by astrocytes (ASTRs). ASTRs supply lipids to myelin forming OLGs which requires high amounts of cholesterol. Here, we show that a feeding layer of gmASTRs was more supportive to in vitro myelination than a feeding layer of wmASTRs. Similar findings were obtained with gmASTR-conditioned medium on wmOPC, but not gmOPC, differentiation. In vitro analyses showed that gmASTRs secreted more cholesterol than wmASTRs. Cholesterol efflux from either ASTR was reduced upon exposure to pro-inflammatory cytokines, which was mediated via cholesterol transporter ABCA1, but not ABCG1, and correlated with a minor reduction of myelin membrane formation by OLGs. Surprisingly, ASTR knockdown of Fdft1 encoding for squalene synthase (SQS), an enzyme essential for the first committed step in cholesterol biosynthesis, enhanced myelination on a feeding layer of wmASTRs. Likely, reduced secretion of IL1β by enhanced isoprenylation and increased unsaturated fatty acid synthesis, both pathways upstream of SQS, masked the effect of reduced levels of ASTR-derived cholesterol. Hence, our findings indicate that gmASTRs export more cholesterol and are more supportive to myelination than wmASTRs, but that specific inhibition of cholesterol biosynthesis in wmASTRs is beneficial for wmASTR-mediated modulation of in vitro myelination.

Keywords: astrocytes, cholesterol, cytokines, myelin, oligodendrocyte

Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) characterized by recurring inflammation, loss of oligodendrocytes and myelin, astrogliosis, remyelination failure and neuronal loss. Most therapies for MS do not directly aim to promote remyelination, but are immunomodulatory and reduce the number and severity of attacks. Remyelination is a regenerative process in which new myelin membranes are formed and which is not only essential to recover saltatory conduction, but also to prevent neurodegeneration. MS lesions are present in both grey matter (GM) and white matter (WM), while GM remyelination is more efficient than WM remyelination. This is also observed in experimental toxin-induced demyelination models, where remyelination in the cortex is faster and more robust than remyelination in the corpus callosum. Revealing the underlying mechanism of this apparent discrepancy between GM and WM remyelination, may identify novel targets for therapy that promote remyelination.

Differences in regional remyelinating capacity may be due to intrinsic and/or extrinsic factors that differ between GM and WM areas. Recent findings, including our own, indicate that grey matter OPCs (gmOPCs) and white matter OPCs (wmOPCs) intrinsically differ. GMOPCs are less mature and more responsive to mitogens than WMOPCs, possibly rendering the gmOPCs better equipped for remyelination. In addition, WMOPCs are more susceptible to the detrimental effects of inflammatory mediators such as IFNγ. Also, the cellular composition of GM and WM differ in the type of astrocytes (ASTRs) that are present. More specifically, protoplasmic ASTRs are present in GM, and fibrous ASTRs reside in WM. These ASTR subtypes are morphologically and functionally distinct, i.e., protoplasmic gmASTRs are morphological more complex and ensheath synapses, whereas fibrous wmASTRs are specialized in providing structural support to myelinated axons and interact with the nodes of Ranvier. In addition, ASTRs play an important role in transient remodeling of the local signaling environment upon CNS injury. The role of ASTRs in remyelination in MS is still a matter of debate. A strong correlation exists between severe reactive glial scar formation and remyelination failure, while remyelination does not occur when ASTR numbers are low. In addition, ASTR malfunctioning can develop diseases that have a clear myelin pathology, including Alexander’s and Vanishing White Matter disease. In vitro studies revealed that
ASTRs derived from WM MS brain tissue form fibronectin aggregates that impair remyelination\(^46\), and reactive ASTRs form a dense scar in WM MS lesions that consists of persistent deposited extracellular matrix (ECM) molecules, including proteoglycans and fibronectin\(^46,293,351,352,357\). This deposition of ECM is not specific to MS, as also in experimental toxin-induced demyelination models ASTRs contribute to remyelination by transient deposition of these ECM molecules\(^46,293,351,352,357\). Although the presence of fibronectin aggregates has not been studied in the context of GM MS lesions, fibronectin expression is enhanced in WM, but not present in GM lesions of marmoset experimental autoimmune encephalitis (EAE), an immune-mediated experimental model of MS\(^248\). Hence, ASTRs from different brain regions may distinctly contribute to remyelination efficiency.

Differentiation of OPCs is enhanced by exogenously supplied cholesterol as well as by polyunsaturated fatty acids\(^371,372\). In the adult CNS, ASTRs are the main suppliers of these lipids\(^46,377–379\). Lipid biosynthesis, including cholesterol and unsaturated fatty acids, is regulated by the sterol regulatory element-binding protein (SREBP) transcription factor family\(^48,373\). Upon blocking lipid biosynthesis in ASTRs by inactivation of the SREBP-cleavage-activating protein (SCAP), an essential coactivator of SREBP, developmental myelination is severely retarded\(^48\). Moreover, when during developmental myelination OLGs are unable to produce cholesterol due to the absence of squalene synthase (SQS), the enzyme required for the first committed step in cholesterol biosynthesis\(^47\), myelin formation is reduced. However, cholesterol is still incorporated in myelin, which is likely supplied by ASTRs via lipoprotein particles\(^47\). Moreover, the ratio of cholesterol compared to other lipids in the myelin is comparable to control\(^47\). This indicates that cholesterol is an indispensable lipid component of myelin membranes. In addition, the availability of cholesterol accelerates OPC differentiation and is rate-limiting in oligodendrocyte myelin membrane growth\(^47–49\).

As the blood-brain barrier prevents the uptake of peripheral cholesterol, CNS cholesterol is primarily derived of de novo synthesis\(^377\). Thus, similar to developmental myelination, remyelination also requires an increase in cholesterol biosynthesis. Indeed, cholesterol synthesis is increased upon toxin-induced demyelination\(^371,375\).
Results

Cultured grey matter astrocytes are more supportive to myelination than cultured white matter astrocytes

To examine whether gmASTRs and wmASTRs distinctly modulate myelination efficiency, an in vitro myelinating system of embryonic spinal cord cultures that relies on an ASTR feeding layer was employed\(^{28}\). To this end, neonatal rat gmASTRs and wmASTRs were used. Neonatal gmASTRs and wmASTRs were positive for the ASTRs marker GFAP, and retained their difference in morphology (Fig. 1a)\(^{193}\). Thus, gmASTRs were more protoplasmic, while wmASTRs adopted a more fibrous stellate morphology. Assessment of the percentage of myelinated axons, as determined by a co-labeling of the myelin marker myelin basic protein (MBP) and the neuronal marker neurofilament-H (NF), revealed that myelination was higher on a feeding layer of gmASTRs than on a feeding layer of wmASTRs (Fig. 1d,c p=0.08). In monoculture, OPCs readily differentiate into MBP-positive OLGs that elaborate myelin-like membranes. To examine whether the distinct modulation of gmASTRs and wmASTRs on in vitro myelination relates to a difference in gmASTR- and wmASTR-derived secreted factors, the effect astrocyte conditioned medium (ACM) on differentiation and myelin membrane formation was examined in OPC monocultures. As gmOPCs and wmOPCs distinctly respond to injury signals\(^{26}\), and as both are present in our in vitro myelinating cultures, the effect non-conditioned medium (NCM), and ACM

Figure 1. Cultured grey matter astrocytes are more supportive for in vitro myelination than white matter astrocytes. (a) GFAP immunocytochemistry of primary neonatal grey matter (gm) and white matter (wm) astrocytes (ASTRs). Note the difference in morphology of wmASTRs and gmASTRs. (b,c) In vitro myelinating cultures that depend on a feeding layer of ASTRs are obtained from 15 days old rat embryo spinal cord cells and stained for myelin basic protein (MBP, green), a myelin marker, and neurofilament-H (NF, red), an axonal marker. Representative images of 4 independent myelinating spinal cord cultures on either neonatal wmASTRs or neonatal gmASTRs are shown in b and quantification of the percentage of myelinated axons in c. Note that a feeding layer of gmASTRs is more supportive for in vitro myelination that a feeding layer of wmASTRs (p=0.018). (d-h) Neonatal wmOPCs (d-f) and gmOPCs (g,h) were differentiated for 3 or 6 days in the presence of non-conditioned medium (NCM) or ASTR-conditioned medium (ACM) from gmASTRs or wmASTRs. MBP immunocytochemistry is performed to assess differentiation (% MBP-positive cells of DAPI-stained cells) and myelin membrane formation (% myelin membranes formed by MBP-positive cells). Representative images of MBP-positive wmOLGs (red) in the presence of NCM, wmACM or gmACM three days after initiating differentiation of 4-6 independent experiments are shown in d (arrow indicate MBP-positive cells; arrowheads point myelin membranes), quantification of OPC differentiation in the presence of NCM, wmACM or gmACM in e and of myelin membrane formation in f. Note that upon exposure to gmACM, but not wmACM, wmOPC differentiation is significantly increased three days after initiating differentiation (p=0.028) compared to exposure to NCM, while gmOPC differentiation is increased upon exposure to both wmACM and gmACM (wmACM p=0.002, gmACM p=0.037). Bars represent absolute values (c) or relative means compared to NCM (e-h), which is set to 1 in each independent experiment. Error bars represent standard error of the mean (SEM). Statistical analyses are performed using column statistics with a one-sample t-test (*p < 0.05) to test for differences between ACM treatments and NCM-treated control, while a paired t-test (*p < 0.05) was used to test for differences between effects of gmACM and wmACM (not significant). Absolute values of NCM are for wmOPC differentiation after 3 days 15.7 ± 3.5%, after 6 days 36.5 ± 9.1% and myelin membrane formation after 3 days 55.5 ± 5.6%; after 6 days 63.5 ± 16.9% and for gmOPC differentiation after 3 days 9.0 ± 2.7%, after 6 days 47.5 ± 6.1% and myelin membrane formation after 3 days 47.8 ± 8.9% and after 6 days 84.2 ± 2.1%. Scale bars are 25 µm (a,d) and 50 µm (b).
from gmASTR (gmACM) or wmASTRs (wmACM) on both gmOPC and wmOPC maturation was taken into account. Three days after initiating differentiation, exposure to gmACM, but not wmACM, increased the number of MBP-positive cells (Fig. 1e, \( p = 0.028 \)), a read-out for OPC differentiation, compared to NCM. Also, at six days after initiating wmOPC differentiation, the percentage of MBP-positive cells is reproducibly, but not significantly enhanced in the presence of gmACM (Fig. 1e), indicating that gmACM, but not wmACM, facilitated wmOPC differentiation. In contrast, three days after initiating differentiation the percentage of MBP-positive cells was significantly enhanced upon exposure to both gmACM and wmACM compared to NCM (Fig. 1e, \( p = 0.002 \); \( p = 0.037 \)). Remarkably, six days after initiating gmOPC differentiation, the percentage of MBP-positive cells was similar at all conditions, indicating that both gmACM and gmACM accelerated gmOPC differentiation. Both three and six days after initiating differentiation, no significant difference in myelin membrane formation was observed upon gmACM and wmACM treatment of either type of OPC (Fig. 1f). Hence, these findings demonstrated that gmASTRs were more supportive to myelination, likely by secreting (more) pro-OPC-differentiation factors that enhanced wmOPC differentiation. As ASTRs are the most important lipid suppliers in the adult for OLGs and exogenously supplied cholesterol accelerates OPC differentiation\(^{17}\), we next examine whether gmASTRs and wmASTRs differ in their capacity to supply cholesterol to differentiating OLGs.

**Grey matter astrocytes secrete more cholesterol than white matter astrocytes**

SREBPs are a family of membrane-bound transcription factors that modulate the transcription of genes of enzymes that are required for the synthesis of cholesterol and unsaturated fatty acids\(^ {40, 372} \). SREBP-2 drives the transcription of genes of enzymes involved in cholesterol biosynthesis, and SREBP-1c primarily drives unsaturated fatty acid synthesis pathway\(^ {45, 372} \) (Fig. 2a). qPCR analysis showed that mRNA of SREBP-2 (\( Srebf2 \)) levels were increased in cultured gmASTRs compared to cultured wmASTRs (Fig. 2b, \( p = 0.003 \)). In addition, cholesterol efflux from gmASTRs was higher than cholesterol efflux from wmASTRs (Fig. 2c \( p = 0.006 \)), while intracellular levels of gmASTRs and wmASTRs were comparable (Fig. 2d). Others have shown that exogenously supplied cholesterol facilitates gmOPC differentiation in vitro\(^ {17} \). To confirm this observation for rat OPCs, rat wmOPCs were differentiated in the presence of cholesterol. Three days after initiating differentiation, wmOPC differentiation (Fig. 2e, \( p = 0.008 \)) and myelin membrane formation (Fig. 2f, \( p = 0.048 \)) were enhanced upon cholesterol treatment. Labeling of ASTRs with the fluorescent cholesterol derivative, bodipy-cholesterol\(^ {38} \), followed by exposure of differentiating wmOPCs to ACM, showed that ASTR-derived bodipy-cholesterol was taken up by wmOLGs (Fig. 2f). Thus, gmASTRs effluxed more cholesterol, which strongly suggests

**Figure 2. Grey matter astrocytes secrete more cholesterol than white matter astrocytes.** (a) Schematic overview of several intermediates in the cholesterol and fatty acid synthesis pathway. SREBP-2 and SREBP-1c are transcription factors, and SQS and FASN are enzymes involved in respectively cholesterol and fatty acid synthesis. (b) qPCR analysis of \( Srebf2 \) in primary neonatal grey matter (gm) and white matter (wm)astrocytes (ASTRs) (\( e \)). (c,d) Cholesterol assays on gmASTRs and wmASTRs. Cholesterol efflux is shown in (c); intracellular cholesterol levels, shown as intracellular cholesterol/phosphate ratios, of gmASTRs and wmASTRs in (d). (e,f) Neonatal wmOPCs were differentiated for three days in the absence (ctrl) or presence of 10 µg/ml cholesterol (chol). MBP immunocytochemistry is performed to assess differentiation (\( \% \) MBP-positive cells of DAPI-stained cells) and myelin membrane formation (\( \% \) myelin membranes formed by MBP-positive cells). Note that exposure to cholesterol enhances OPC differentiation (\( p = 0.008 \)) and myelin membrane formation (\( p = 0.048 \)). (g) Oligodendrocytes (wmOLGs) three days after initiating differentiation that were exposed to ACM obtained from ASTRs that were pre-incubated with 10 µg/ml bodipy-cholesterol. Note that OLGs take up bodipy-cholesterol from ASTRs. Bars represent absolute values (\( c,d \)) or relative means compared to wmASTRs (\( b, w, m \)) or untreated wmOLGs (\( e,f, c, t \)), which are set to 1 in each independent experiment. Error bars represent standard error of the mean (SEM). Statistical analyses are performed using column statistics with a one-sample t-test (*\( p < 0.05 \), **\( p < 0.01 \)) to test for differences with wmASTRs (\( b, w, m \)) or ctrl wmOLGs (\( e,f,c \)), while a paired student t-test (**\( p < 0.01 \)) was used to test for differences between cholesterol efflux from wmASTRs and gmASTRs. Absolute values of ctrl OLGs are for OPC differentiation 20.5 ± 4.1% and for myelin membrane formation formation 46.7 ± 6.4%. Scale bar is 10 µm.
that the increased cholesterol secreted by gmASTRs may add to enhanced wmOPC differentiation in the presence of gmACM, but not wmACM. As inflammatory mediators also interfere with cholesterol efflux\(^2\), and to further explore the role of ASTR-derived cholesterol in myelination efficiency, the effect of MS-relevant inflammatory mediators on cholesterol efflux from ASTRs was examined next.

A mixture of pro-inflammatory cytokines reduces cholesterol efflux by grey matter and white matter astrocytes

MS is characterized by chronic inflammation\(^{3,4}\), and as ASTRs respond to inflammatory mediators, the effect of pro-inflammatory cytokines and Toll-like-receptor (TLR) agonists on cholesterol efflux was assessed. To this end, gmASTRs and wmASTRs were exposed for 24 hours to a mixture of pro-inflammatory cytokines

Figure 3. A mixture of pro-inflammatory cytokines reduces cholesterol efflux by grey and white matter astrocytes. Primary neonatal grey matter (gm) and white matter (wm) astrocytes (ASTRs) were either untreated (ctrl) or treated for 24 hours with TLR4 agonist LPS (200 ng/mL), TLR3 agonist Poly(I:C) (50 µg/mL), or a mixture of pro-cytokines IL1\(\beta\) (1 ng/ml), IFN\(\gamma\) (500 U/ml) and TNF\(\alpha\) (500 IU/ml). (a) Cholesterol assays on gmASTRs and wmASTRs. Relative cholesterol efflux is shown in a; intracellular cholesterol levels, shown as intracellular cholesterol/phosphate ratios of gmASTRs and wmASTRs in b. Note that treatment of gmASTRs with LPS (\(p=0.033\)) and either type of ASTRs with the cytokine mix (wmASTRs \(p=0.001\); gmASTRs \(p<0.001\)) results in a decrease of cholesterol efflux without changing intracellular cholesterol levels. (c,d) Neonatal wmOPCs were differentiated for 3 days in the presence of ASTR-conditioned medium (ACM) from untreated and cytokine (mixture)-treated gmASTRs or wmASTRs. MBP immunocytochemistry is performed to assess differentiation (c, % MBP-positive cells of DAPI-stained cells) and myelin membrane formation (d, % myelin membranes formed by MBP-positive cells). Note that membrane formation with gmACM, but not wmACM, is substantially and reproducibly reduced upon cytokine treatment of gmASTRs (\(p=0.048\), gmASTRs \(p=0.008\)) and for Fasn in wmASTRs (\(p=0.001\)). Bars represent relative means to exposure to control wmACM or gmACM (c,d) or untreated (ctrl) gmASTRs or wmASTRs (e-h), which are set to 1 in each independent experiment. Error bars represent standard error of the mean (SEM). Statistical analyses are performed using column statistics with a one-sample t-test (\(*p<0.05\), **\(p<0.01\), ***\(p<0.001\)) to test for differences with control wmACM or gmACM (c,d) or untreated (ctrl) gmASTRs or wmASTRs (e-h). Absolute values of OPC differentiation with control wmACM are 21.8 \pm 8.9% and with control gmACM 30.0 \pm 8.3% and or myelin membrane formation with control wmACM 59.7 \pm 8.3% and with control gmACM 70.5 \pm 12.9%.

Enhanced myelination upon impaired cholesterol biosynthesis

IFN\(\gamma\), IL1\(\beta\), TNF\(\alpha\) or Poly(I:C) or LPS, which are TLR3 or TLR4 agonists, respectively. Cholesterol efflux was significantly reduced from gmASTRs that were treated with LPS (Fig. 3a, \(p=0.033\)). In both gmASTRs and wmASTRs, the cholesterol efflux was reduced by 35-45% upon treatment with a mixture of the three pro-inflammatory cytokines, i.e., IFN\(\gamma\), IL1\(\beta\), and TNF\(\alpha\), compared to their respective untreated ASTRs (Fig. 3a, wm + cytokines \(p=0.001\); gm + cytokines \(p=0.001\)). For all ASTR treatments, intracellular levels of cholesterol were not significantly changed compared to the levels in untreated ASTRs (Fig. 3b). Exposure to ACM obtained from pro-inflammatory cytokine-treated gmASTRs reproducibly, but not significantly, reduced myelin membrane formation by wmOLGs, but not the number of MBP-positive cells compared to exposure to control gmACM (Fig. 3c,d). Exposure to ACM obtained from cytokine-treated wmASTRs did not alter wmOPC differentiation and myelin membrane formation (Fig. 3c,d). To determine whether the cytokines interfere with cholesterol biosynthesis, the mRNA levels of \(Fdf2\), a gene that encodes for squalene synthase (SQS), an enzyme in the cholesterol biosynthesis pathway (Fig. 2a), and

\(Fdf2\) mRNA levels in gmASTRs and wmASTRs that were either untreated (ctrl) or treated with a mixture of IL1\(\beta\), IFN\(\gamma\) and TNF\(\alpha\) for 24 hours. Note that exposure to cytokines decreases transcripts for Sreb12 in wmASTRs and gmASTRs (\(p=0.048\), gmASTRs \(p=0.008\)) and for Fasn in gmASTRs (\(p<0.001\)). Bars represent relative means to exposure to control wmACM or gmACM (c,d) or untreated (ctrl) gmASTRs or wmASTRs (e-h), which are set to 1 in each independent experiment. Error bars represent standard error of the mean (SEM). Statistical analyses are performed using column statistics with a one-sample t-test (\(*p<0.05\), **\(p<0.01\), ***\(p<0.001\)) to test for differences with control wmACM or gmACM (c,d) or untreated (ctrl) gmASTRs or wmASTRs (e-h). Absolute values of OPC differentiation with control wmACM are 21.8 \pm 8.9% and with control gmACM 30.0 \pm 8.3% and or myelin membrane formation with control wmACM 59.7 \pm 8.3% and with control gmACM 70.5 \pm 12.9%. 
Srebf2 were examined. In contrast to previous findings\(^{38,39}\), neither Srebf2 (Fig. 3e) nor Fdft1 (Fig. 3f) mRNA levels were decreased in cytokine-treated gmASTRs and wmASTRs. In fact, transcripts of both Fdft1 and Srebf2 tended to increase in cytokine-treated gmASTRs and wmASTRs, indicating a potential compensatory mechanism for the decreased cholesterol efflux by either ASTR. In line with such a negative feedback loop is that mRNA levels of Srebf2c were significantly decreased in cytokine-treated gmASTRs (Fig. 3g, \(p=0.048\)) and gmASTRs (Fig. 3g, \(p=0.008\)), whereas Fasn mRNA levels were significantly reduced in cytokine-treated gmASTRs, but not cytokine-treated wmASTRs (Fig. 3h, \(p=0.001\)). These findings indicate that also unsaturated fatty acid production and secretion may be enhanced in cytokine-treated ASTRs and may affect ASTR-mediated modulation of myelin membrane formation. Hence, the cytokine-induced decrease in cholesterol efflux from gmASTRs correlated with a decrease in ASTR-mediated modulation of myelin membrane formation, as well as a decrease in mRNA levels of genes encoding for a transcription factor and enzyme involved in unsaturated fatty acid synthesis. Also, as cytokine-treated wmASTRs effluxed less cholesterol and ACM from cytokine-treated wmASTRs did not reduce wmOPC differentiation, the remaining cholesterol levels were sufficient and not rate-limiting for OPC differentiation in vitro.

**Proinflammatory cytokines inhibit cholesterol efflux from ASTRs via an ABCA1-dependent pathway**

Cholesterol is effluxed by passive transfer over the plasma membrane as well as via facilitated transport\(^{34}\). The main transporters of cholesterol in ASTRs are ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1)\(^{35}\). To assess whether ABCA1 and/ or ABCG1 contributed to the cytokine-induced decrease in cholesterol efflux from ASTRs, glibenclamide, a specific inhibitor of ABCA1\(^{36}\), or thyroxine (T4), a specific inhibitor of ABCG1\(^{37}\), were added 1 hour prior to treatment with the mixture of cytokines. T4, but not glibenclamide, significantly inhibited cholesterol efflux from untreated gmASTRs and wmASTRs by 40-50\% (Fig. 4a, wm + ABCG1 inh. \(p=0.015\); gm + ABCG1 inh. \(p=0.029\)), which is in line with previous findings that ABCG1 is the main cholesterol transporter in ASTRs\(^{35}\). In contrast, glibenclamide, but not T4, prevented the cytokine-induced reduction in cholesterol efflux from gmASTRs (Fig. 4a: gm + cytokines versus gm + cytokines + ABCA1 inhibitor, \(p=0.027\)), and reproducibly, but not significantly from wmASTRs (Fig. 4a, \(p=0.074\), ns). Of note, treatment with both inhibitors simultaneously was toxic to ASTRs. Western blot analysis revealed that a mixture of pro-inflammatory cytokines significantly decreased ABCA1 expression in gmASTRs (Fig. 4c,d, gm + cytokines \(p<0.001\)). In contrast, ABCG1 expression was significantly higher in cytokine-treated gmASTRs than in untreated gmASTRs (Fig. 4c,e, gm + cytokines \(p=0.004\)). The effect of cytokines on ABCA1 and ABCG1 expression

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**Figure 4. Proinflammatory cytokines inhibit cholesterol efflux from ASTRs via an ABCA1-dependent pathway.** Primary neonatal grey matter (gm) and white matter (wm) astrocytes (ASTRs) were either untreated (ctrl) or treated for 24 hours with a mixture of pro-inflammatory cytokines IL1α (1 ng/ml), IFNγ (500 IU/ml) and TNFα (500 IU/ml) in the absence or presence of ABCA1 inhibitor glibenclamide (gli; 0.1 mM) or ABCG1 inhibitor T4 (50 µM), which were added to the cells 1 hour prior to addition of cytokines. (a,b) Cholesterol assays on gmASTRs and wmASTRs. Relative cholesterol efflux is shown in a and intracellular cholesterol levels, shown as intracellular cholesterol/phosphate ratios, of gmASTRs and wmASTRs in b. Note that the ABCA1 inhibitor glibenclamide counteracts the cytokine-induced reduction in cholesterol efflux from both wmASTRs and gmASTRs. (c-e) Western blot analyses of cholesterol transporters ABCA1 (c,d) and ABCG1 (c,e). Actin served as a loading control. Representative blots of 5-6 independent experiments are shown in c: quantification for ABCA1 in d and for ABCG1 in e. Note that exposure to cytokines decreases ABCA1 expression (\(p<0.001\)), and increases ABCG1 expression (\(p=0.004\)) in gmASTRs. Bars represent absolute values (b) or relative means (c,d,e) to control gmASTRs or wmASTRs, which are set to 1 in each independent experiment. Error bars represent standard error of the mean (SEM). Statistical analyses are performed using column statistics with a one-sample t-test (**\(p<0.01\), ***\(p<0.001\)) to test for differences between control gmASTRs or wmASTRs. A one-way ANOVA with a Šidák multiple comparisons post-test (\(9p<0.05\)) was used to test for differences between different treatments.
in wmASTRs was less pronounced (Fig. 4c-e). These findings demonstrate that while the cytokines decreased ABCA1 expression and increased ABCG1 expression, the pro-inflammatory cytokines-induced reduction in cholesterol efflux from gmASTRs, and to a lesser extent from wmASTRs, depended on ABCA1 activity.

**Inhibition of cholesterol biosynthesis by SQS knockdown in white matter astrocytes increases in vitro myelination**

To examine whether the increased secreted cholesterol levels by gmASTRs contribute to enhanced myelination, cholesterol was added to the in vitro myelinating system that depends on an ASTR feeding layer. Upon continuous cholesterol treatment, the percentage of myelinated axons did not increase on a feeding layer of wmASTRs and gmASTRs (Fig. 5a, b). As myelination on wmASTRs was still reduced compared to

*Figure 5. Inhibition of cholesterol biosynthesis by SQS knockdown in white matter astrocytes increases in vitro myelination. (a,b) In vitro myelinating cultures that depend on a feeding layer of ASTRs are obtained from 15 days old rat embryo spinal cord cells and either left untreated (ctrl) or treated with 10 µg/ml cholesterol for the duration of the experiment. Cultures were stained for myelin basic protein (MBP, green), a myelin marker, and neurofilament-H (NF, red), an axonal marker. Representative images of 4 independent control and cholesterol-treated myelinating spinal cord cultures on neonatal wmASTRs or neonatal gmASTRs are shown in a and quantification of the percentage of myelinated axons in b. Note that addition of cholesterol to myelinating spinal cord cultures does not affect myelination efficiency on either type of ASTR. (a–c,e) A lentiviral shRNA knockdown of Fdft1, encoding for squalene synthase (kdSQS), the first enzyme essential for dedicated production of cholesterol, was established in gmASTRs and in wmASTRs (kdSQS). A shRNA scrambled (scr) construct was used as negative control. A qPCR analysis of Fdft1 mRNA levels in control, scr and kdSQS gmASTRs and wmASTRs is shown in c, cholesterol efflux in d, and intracellular cholesterol levels, shown as intracellular cholesterol/phosphate ratios, in e. Note that Fdft1 mRNA levels (wmASTRs and gmASTRs p<0.001) and cholesterol efflux (wmASTRs p=0.041 and gmASTRs p=0.014) are decreased in kdSQS ASTRs. (a,f) In vitro myelinating cultures on a feeding layer of control (ctrl), scrambled (scr) and kdSQS ASTRs. Representative images are shown in a and quantification of the percentage of myelinated axons in f. (g) Neonatal wmOPCs were differentiated for 3 days in the presence of ASTR-conditioned medium (ACM) from control, scr and kdSQS gmASTRs or wmASTRs. MBP immunocytochemistry is performed to assess differentiation (% MBP-positive cells of DAPI-stained cells) and myelin membrane formation (% myelin membranes formed by MBP-positive cells). Bars represent relative means to control wmASTRs or gmASTRs (b), which are set to 1 in each independent experiment. Error bars represent standard error of the mean (SEM). Statistical analyses are performed using column statistics with a one-sample t-test (*p<0.05, **p<0.01, ***p<0.001) to test for differences between control wmASTRs or gmASTRs. Absolute values of the percentage of myelinated axons on a feeding layer of control ASTRs are for wmASTRs 3.2 ± 0.9% and for gmASTRs 6.7 ± 1.5%. Absolute values for control ACM of wmASTRs are for OPC differentiation 20.5 ± 5.4% and for myelin membrane formation 56.1 ± 7.9% and for control ACM of gmASTRs respectively 23.7 ± 2.4% and 49.9 ± 9.1%. Scale bar is 10 µm.
Enhanced myelination upon impaired cholesterol biosynthesis

Inhibition of cholesterol biosynthesis by SQS knockdown in white matter astrocytes increased Srebf1c mRNA levels and substantially decreases pro-IL1β secretion

Similar to cholesterol synthesis, unsaturated fatty acid synthesis is regulated by the SREBP transcription factor family (Fig. 2a). To examine whether an inhibition in cholesterol synthesis downstream of farnesyl-PP in white matter astrocytes (Fig. 2a) promoted, rather than inhibited, in vitro myelination. Upon kdSQS, pathways upstream of SQS-mediated conversion of squalene to cholesterol, such as isoprenylation and unsaturated fatty acid synthesis (Fig. 2a), may become more active in ASTRs and thereby compensate for the effect of reduced cholesterol levels, which was examined next.

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Similar to cholesterol synthesis, unsaturated fatty acid synthesis is regulated by the SREBP transcription factor family (Fig. 2a). To examine whether an inhibition in cholesterol synthesis downstream of farnesyl-PP in ASTRs results in an increase in fatty acid synthesis, the mRNA levels of Srebfic, encoding for SREBP-1c, the major transcription factor involved in unsaturated fatty acid synthesis, was examined by qPCR. An increase in Srebfic transcripts was observed in kdSQS ASTRs, but not in scrambled ASTRs, compared to control ASTRs (Fig. 6a, p=0.003), while mRNA levels of Srebfic were similar in control ASTRs and kdSQS ASTRs. Transcripts of Fasn, encoding for the enzyme fatty acid synthase (Fig. 2a), were not significantly changed in kdSQS ASTRs and kdSQS ASTRs (Fig. 6b). Hence, the increase in Srebfic mRNA levels suggest that unsaturated fatty acid production may be increased in kdSQS ASTRs. As polyunsaturated fatty acids are beneficial for OPC differentiation, this may contribute to increased myelination despite reduced cholesterol levels in the presence of kdSQS ASTRs. Like committed cholesterol biosynthesis, the generation of non-sterol isoprenoids is also downstream pathway of farnesyl-PP, the precursor of squalene, (Fig. 2a). Interestingly, when blocking upstream in the cholesterol biosynthesis pathway of farnesyl-PP with the HMG-CoA reductase inhibitor simvastatin (Fig. 2a), the secretion of several cytokines, including IL1β, is increased by inhibition of isoprenylation-dependent signaling pathways. In line with these observations, pro-IL1β levels in ASTR-conditioned medium (ACM) of 4 independent experiments in c, and quantification in d. Note that the kdSQS in ASTRs increases Srebfic mRNA levels in ASTRs (p=0.013), but not ASTRs, and that pro-IL1β secretion is substantially reduced in ACM of ASTRs (p=0.032). Bars represent relative means to control ASTRs or ASTRs, which are set to 1 in each independent experiment. Error bars represent standard error of the mean (SEM). Statistical analyses are performed using column statistics with a one-sample t-test (p<0.05) to test for differences between untreated control ASTRs or ASTRs.

Figure 6. (a-d) A lentiviral shRNA knockdown of Fdft1, encoding for squalene synthase (kdSQS), the first specific enzyme essential for the production of cholesterol, was established in ASTRs and in ASTRs (kdSQS). A shRNA scrambled (scr) construct was used as negative control. A qPCR analysis of Srebf1c and Fasn on control (ctrl), scrambled (scr) and kdSQS in ASTRs and ASTRs is shown in respectively a and b, representative Western blots of pro-IL1β levels in 80 µl ASTR-conditioned medium (ACM) of 4 independent experiments in c, and quantification in d. Note that the kdSQS in ASTRs increases Srebfic mRNA levels in ASTRs (p=0.013), but not ASTRs, and that pro-IL1β secretion is substantially reduced in ACM of ASTRs (p=0.032). Bars represent relative means to control ASTRs or ASTRs, which are set to 1 in each independent experiment. Error bars represent standard error of the mean (SEM). Statistical analyses are performed using column statistics with a one-sample t-test (p<0.05) to test for differences between untreated control ASTRs or ASTRs.

prominent in ASTRs than in ASTRs (Fig. 5d, p=0.044; ASTRs p=0.014). Intracellular cholesterol levels were unchanged (Fig. 5e). Against our expectations, the percentage of myelinated axons did not decrease on a feeding layer of either kdSQS ASTRs. In fact, an 2-fold increase in myelination was observed on a feeding layer of kdSQS ASTRs compared to control ASTRs (Fig. 5a, p=0.007). Also, OPC differentiation and myelin membrane formation remained similar upon exposure to ACM from both kdSQS ASTRs and kdSQS ASTRs (Fig. 5g), indicating that other factors may compensate for reduced cholesterol levels. Thus, rather unexpectedly, inhibition of cholesterol biosynthesis downstream of the SQS substrate farnesyl-PP in ASTRs (Fig. 2a) promoted, rather than inhibited, in vitro myelination.

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Chapter 3

Enhanced myelination upon impaired cholesterol biosynthesis

Discussion

ASTRs are recognized to be important players in the remyelination process. A potential malfunctioning of ASTR signaling in WM MS lesions, among others, via persistent deposition of remyelination-inhibitory ECM molecules has been described.46 As remyelination in toxin-induced demyelination models is faster in GM than in WM,17–19 and remyelination in GM MS lesions is more efficient than in WM lesions, we hypothesized that in addition to wmASTRs being more detrimental, gmASTRs may be more supportive for remyelination by supplying more cholesterol to developing OLGs. Our findings demonstrate that gmASTRs effluxed more cholesterol than wmASTRs and that this increase in cholesterol secretion correlated with enhanced gmOPC differentiation upon exposure to gmACM, but not wmACM, and a more supportive role of gmASTRs to in vitro myelination. Although a knockdown of SQS, the first enzyme to dedicated cholesterol biosynthesis, reduced cholesterol efflux from ASTRs, in vitro myelination remained similar on a feeding layer of kdSQS gmASTRs. Moreover, against our expectations, in vitro myelination enhanced rather than decreased on a feeding layer of kdSQS wmASTRs. Therefore, whether enhanced cholesterol supply by gmASTRs played a role in the more supportive role of gmASTRs on in vitro myelination cannot be confirmed using the kdSQS model in ASTRs. However, the kdSQS in wmASTRs did reveal that cholesterol supply by wmASTRs is not rate-limiting for in vitro myelination, and most importantly, accidentally uncovered that specific blocking of cholesterol biosynthesis in wmASTRs was in fact beneficial for in vitro myelination. This may open new ASTRs-based therapeutic strategies that aim to promote remyelination in WM MS lesions.

Our findings revealed that in vitro myelination was more efficient in the presence of neonatal gmASTRs than neonatal wmASTRs, indicating that gmASTRs and wmASTRs were also functionally diverse cells in vitro. Whether the more supportive role of gmASTRs contribute to faster remyelination in GM than WM remains to be determined in vivo. In our in vitro myelinating culture system both gmOPCs and wmOPCs were present. Importantly, wmACM, which thus contain less cholesterol than gmACM, did not accelerate wmOPC differentiation like gmACM. On the other hand, gmOPCs differentiation enhanced upon exposure to ACM from either type of ASTR. Given that gmOPCs are morphological more complex, differentiate faster115,121, and may produce more myelin membranes134, wmOPC differentiation may
depend more on secreted factors from ASTRs, including cholesterol, and/or are less susceptible for ASTRs-secreted factors, than gmOPC differentiation.

In addition, our findings showed that cholesterol efflux from gmASTRs and wmASTRs was differently regulated by inflammatory mediators. Thus, TLR4 agonist LPS more potently decreased cholesterol efflux from gmASTRs, than from wmASTRs. Also, while a mixture of pro-inflammatory cytokines decreased cholesterol efflux from both ASTRs to a similar extent, only ACM from cytokine-treated gmASTRs reproducibly decreased myelin membrane formation. Remarkably, Srebf2 and Fdft1 mRNA levels were not reduced by cytokine treatment of gmASTRs and wmASTRs, indicating that cytokines may interfere with the expression and/or function of cholesterol transporters. Indeed, cytokine treatment reduced cholesterol efflux via ABCA1. Moreover, ABCA1 expression was reduced in cytokine-treated gmASTRs. Also, the increased ABCG1 expression in cytokine-treated gmASTRs did not enhance cholesterol efflux, which is consistent with previous studies that demonstrate that an increased expression of ABCG1 does not enhance cholesterol efflux per se. Of note, treatment with an ABCA1 agonist increased cholesterol biosynthesis gene expression in ASTRs, and improved clinical outcome in EAE. A cytokine-induced reduction in expression of ABCA1 is in line with previous reports and shown to be mediated via a Liver-X-Receptor (LXR) related mechanism. LXRs belong to the nuclear receptor superfamily of ligand-activated transcription factors and are activated by endogenous oxysterols, i.e., oxidized derivatives of cholesterol. While SREBP2 increases cellular cholesterol, LXR family transcription factors have the opposite effect, i.e., LXR target genes include genes of cholesterol transporters Abca1 and Abcg1. Remarkably, LXRs also directly regulate the expression of genes encoding enzymes required for fatty acid synthesis, including Srebf1c. Our results revealed that exposure to a mixture of pro-inflammatory cytokines also reduced mRNA levels of Srebf1c in gmASTRs and wmASTRs. In addition, the mRNA expression of Fasn, encoding for the first enzyme required for unsaturated fatty acid synthesis, was reduced in cytokine-treated gmASTRs, but not cytokine-treated wmASTRs. Hence, exposure to pro-inflammatory cytokines likely interfered with cholesterol efflux and unsaturated fatty acid synthesis. Of relevance, inflammatory activity is lower in GM MS lesions than in WM MS lesions, and therefore, the cytokine-induced decrease in cholesterol secretion by ASTRs may of more relevance for the impaired remyelination capacity in WM MS lesions.

Cholesterol and unsaturated fatty acids are important components of cell membranes, including myelin membranes. Cholesterol and unsaturated fatty acids have acetyl-CoA as a common precursor and their synthesis is intertwined and tightly coordinated by the SREBP family of transcription factors. SREBPs are synthesized as inactive precursors and post-transcriptionally activated by SCAP, which activation is in turn regulated by sterols. A previous study showed that conditional deletion of SCAP in ASTRs resulted in hypomyelination, while developmental myelination is only delayed upon SCAP deletion in OLs. This indicates that myelination depends on ASTRs lipid metabolism. Of interest, SCAP deletion in ASTRs results in a decrease of WM volume of approx. 60%, while the reduction in the GM was only 10%. Whether this is due to the presence of less myelin in the GM, or that gmOPC differentiation is less dependent on ASTR-derived lipids is not studied. In favor of the latter, our observation that only gmACM enhanced wmOPC, while both gmACM and wmACM accelerated gmOPC differentiation. In addition, we show in the present study that by reducing cholesterol levels in ASTRs by knockdown of Fdft1, a gene that encodes for the first enzyme in the committed cholesterol biosynthesis pathway, in vitro myelination was enhanced on a feeding layer of kdSQS wmASTRs, despite the reduced levels of cholesterol. Concomitant with kdSQS, secreted pro-ILβ levels were severely decreased in ACM of kdSQS ASTRs. While findings on how IL1β affects OPC differentiation are conflicting, it has been shown that IL1β inhibits wmOPC differentiation in vivo. Of relevance, in macrophages, simvastatin, a HMG-CoA reductase inhibitor thus restricting synthesis of both cholesterol and non-sterol isoprenoids, i.e., hydrocarbon chains used to anchor several signaling proteins to cell membranes, increases the secretion of ILβ and IL-8 and inhibited the secretion of TNFα through an isoprenylation-dependent mechanism. In line with this reasoning, upon specifically blocking cholesterol biosynthesis in ASTRs, as established here with kdSQS, more substrate became available for non-sterol isoprenoid synthesis, which may explain the reduced ILβ secretion from ASTRs. In addition, treatment with simvastatin, and other statins, inhibits remyelination in the corpus callosum and induced an astroglia and microglia response, which hints to an altered inflammatory environment. Hence, it is tempting to suggest that statin-mediated inhibition of remyelination, may be in part unrelated to cholesterol.
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production, and be linked to altered ASTR reactivity and secretome as of reduced non-sterol isoprenoid synthesis. Similarly, hypomyelination as observed upon ASTR-specific deletion of SCAP, although not investigated yet, may, based on our findings and by others, partially relate to reduced biosynthesis of non-sterol isoprenoids and as a result increased secretion of several ASTR-derived cytokines that may delay and/or reduce OPC differentiation and myelination. Of relevance, upon SQS deletion in OLGs, i.e., making OLGs dependent on cholesterol supply by other cells, myelin appears of normal thickness in the GM, while being very thin in the WM. Although it cannot be excluded that this regional difference in the involvement of cholesterol in myelin membrane growth depends on an intrinsic difference in gmOPCs and wmOPCs, it is likely that a higher cholesterol efflux from gmASTRs, as is suggested and shown here, may also be involved. In addition, supplementation of cholesterol, or enhancing cholesterol efflux in toxin-induced demyelination models, accelerates wmOPC differentiation and WM remyelination, emphasizing the role of horizontal cholesterol transfer for remyelination in WM. The effect of cholesterol in GM remyelination has not been studied yet. Also, of relevance is our finding that in kdSQS wmASTRs, but not in kdSQS gmASTRs transcripts of Srebp1fc were increased. Exogenous addition of polyunsaturated fatty acids promotes OPC differentiation, implying that upon kdSQS in ASTRs, enhanced unsaturated fatty acid synthesis in ASTRs may also contribute to enhanced in vitro myelination on a feeding layer of kdSQS wmASTRs, but not kdSQS gmASTRs.

Taking together, here we show a correlation between cholesterol secretion and enhanced OPC differentiation and in vitro myelination. Nonetheless, whether cholesterol secretion by ASTRs plays a role in the increase in myelination efficiency by gmASTRs cannot be confirmed using the kdSQS model presented here. The addition of cholesterol to our in vitro myelinating cultures did not increase myelination efficiency at the end point of myelination. This indicates that the amount of supplied cholesterol may not be sufficient and be taken up by other cells like neurons, or that cholesterol is not the rate-limiting factor for in vitro myelination efficiency in these cultures. Intriguingly, this studied identified an under investigated role of non-sterol isoprenoids, which also share acetyl-coA as a common precursor with the cholesterol and fatty acid biosynthesis pathway, and regulate the secretion of cytokines and this way contribute to ASTR-mediated modulation of OPC differentiation and myelin membrane formation. Of interest, a lipid-enriched diet was sufficient to rescue hypomyelination and neurological deficits in the SCAP−/− ASTR experimental model, and dietary cholesterol accelerated WM remyelination in a toxin-induced demyelination model, indicating that an increased lipid supply is beneficial for, and may act on SREBPs, thus also involving isoprenoid synthesis. Hence, interference with non-sterol isoprenoid synthesis in ASTRs without interfering with cholesterol biosynthesis, may be a novel strategy to promote remyelination in WM MS lesions.
Materials and Methods

Primary cell cultures
Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Groningen (the Netherlands).

Oligodendrocytes. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Groningen (the Netherlands). All methods were carried out in accordance with national and local experimental animal guidelines and regulations. Oligodendrocyte progenitor cells (OPCs) were isolated from the neonatal cortex and neonatal non-cortical parts (WM tracts including corpus callosum, mixed GM and WM tracts, including hippocampus and thalamus, and deep GM parts, including basal ganglia, of rat forebrains using a shake-off procedure as described121-129. Briefly, contaminating microglia were removed by a pre-shake at 150 rpm for 1 hour at 37°C. To obtain the more firmly attached OPCs, flasks were shaken at 240 rpm overnight at 37°C. The detached OPCs were further purified by differential adhesion on non-tissue dishes for 15-20 minutes. The enriched OPC fraction contained 95-97% OPCs (Olig2-positive), less than 1% microglia (IB4-positive), 1-3% ASTRs (GFAP-positive) and less than 1% neurons (TuJ1-positive). OPCs were cultured on 13-mm poly-L-lysine (PLL; 5 µl/ml; Sigma) -coated glass slides in 24-well plates unless stated otherwise. GmOPCs were plated at a density of 35,000 cells per well and wmOPCs at a density of 40,000 cells per well, in defined Sato medium121. OPCs were synchronized to early OPCs by 10 ng/ml platelet-derived growth factor-AA (PDGF-AA; Peprotech) and 10 ng/ml human fibroblast growth factor-2 (FGF-2; Peprotech). After 2 days growth factors were removed and OPCs were allowed to differentiate in Sato medium supplemented with 0.5% fetal bovine serum (FBS, Capricorn) for 3 or 6 days in the absence or presence of ACM (1:1).

Astrocytes. The remaining ASTRs of the mixed glia cell culture flasks were shaken at 240 rpm overnight at 37°C, passaged once by trypsinization, transferred to 162 cm² flasks (Corning) and cultured in ASTR medium (100 U/ml penicillin and streptomycin, 4 mM L-glutamine, 10% heat-inactivated FBS (Bodinco) in DMEM). The enriched ASTR fraction yielded a highly pure >97% ASTR population (GFAP-positive) for both gmASTRs and wmASTRs. For cholesterol assays, ASTRs were cultured at a density of 1.0 x 10^6 cells/well in 6-wells-plate in ASTR medium. After 24 hours, cells were washed and serum-free ASTR medium was added. In case of kdSQS ASTRs, defined SATO medium was added. ASTRs were either left untreated or treated for 24 hours with Toll-like-receptor (TLR) 3 agonist polyinosinic-polycytidylic acid (Poly(I:C), 50 µg/ml, GE Healthcare), TLR4 agonist lipopolysaccharide (LPS, 200 ng/ml, Invivogen), or with a combination of pro-inflammatory cytokines interferon-γ (IFNγ, 500 IU/ml, Peprotech), interleukin-1β (IL1β, 1 ng/ml, Peprotech), tumor necrosis factor-α (TNFα, 10 ng/ml, Peprotech). To inhibit cholesterol transporters, 1 hour before addition of cytokines, ABCA1 inhibitor glibenclamide (0.1 mM, Sigma) or ABCG1 inhibitor thyroxine (T4, 50 µM, Sigma) were added. For immunoblot analysis, 1.0 x 10^6 ASTRs were plated on 10 cm dishes (Corning). Cells were left untreated or treated with a mixture of pro-inflammatory cytokines IFNγ, IL1β, and TNFα for 48 hours at 37°C. For collection of ASTR-conditioned medium (ACM), cells were plated in 6-well plates at 1x10^6 cells per well in ASTR medium. ASTRs were either left untreated or treated with a mixture of pro-inflammatory cytokines IFNγ, IL1β, and TNFα, or cultured in the presence of 10 µg/ml bodipy-cholesterol (Avanti). After 24 hours cells were washed with phosphate-buffered saline (PBS) and cultured for 24 hours in Sato medium. ACM was collected, filtered using a 0.45 µm filter (GE Healthcare) and stored at -20°C until further use.

Spinal cord cultures. Myelinating spinal cords were generated from 15 days old Wistar rat embryos (Harlan/Envigo) as described with minor modifications407. After removal of the meninges from the isolated spinal cords, the tissue was mechanically dissociated in Leibowitz L-15 medium (Sigma) followed by enzymatic digestion with a mixture of trypsin (2.5%, Sigma) and DH liberase (2.5 mg/ml, Roche) for 20 minutes at 37 °C. The enzymatic reaction was stopped by addition of Soybean trypsin inhibitor solution (0.52 mg/ml soybean trypsin inhibitor (Sigma), 0.04 mg/ml bovine pancreas DNase (Roche) and 3 mg/ml bovine serum albumin (BSA) fraction V made up in Leibovitz’s L-15 medium (Sigma)). Cells were centrifuged for 7 minutes at 1000 rpm, followed by resuspension in plating medium consisting of 50% DMEM (1500 mg/L glucose, Gibco), 25% horse serum (Invitrogen), 25% HBS with calcium and magnesium (Gibco), and 2 mM L-glutamine (Invitrogen). Cells were plated at a density of 200,000 cells/24 well or 160,000 cells/8 well permanox chamber
slides on a confluent feeding layer of gmASTRs or wmASTRs (80,000 cells/24 wells; 64,000 cells/8 well) in respectively 500 µl or 200 µl plating medium. After cell attachment, the volume of the medium was doubled with growth medium (DMEM (4500 mg/L glucose, Gibco) supplemented with 5 mg/ml holotransferrin (Sigma), 20 mM putrescine (Sigma), 4 µM progesterone (Sigma), 6 µM selenium (Sigma), 10 ng/ml biotin (Sigma), 50 nM hydrocortisone (Sigma) and 10 µg/ml insulin (Sigma)), and supplemented with 27.5 µM 2-mercaptoethanol (BME; Sigma) when cultured on permanox chamber slides. Every 2-3 days, half of the medium was replaced by new growth medium. Insulin was omitted from growth medium for 12 days in cultures onwards and cultures were analyzed at 28 days in culture.

**Lentivirus production and transduction**

For production of lentiviral particles, the constructs (pLKO.1-puro Sigma mission shRNA, construct SHCLNG-NM_010191, sequence TRCN0000099191, for rat Fdft1 shRNA, or SHC002 for control scrambled shRNA; 5 µg), packaging, and envelope plasmids (500 ng pMD2-VSVG; 5 µg pCMV-R8.91) were transfected into the HEK293T packaging cell line using Fugene (Promega). After 16 hours, medium was changed to 5 ml mixed glial culture medium (100 U/ml penicillin and streptomycin, 4 mM L-glutamine, 10% (v/v) FBS in DMEM). After 24 hours, viral particles were harvested and 5 ml new mixed glial culture medium was added to the HEK293T cells. After another 24 hours, viral particles were harvested, combined with the first batch and filtered through PVDF membrane-based 0.45-µm filter (Millipore) and either used immediately or stored frozen at −80°C.

**ASTRs were plated on a 6-well plate at a density of 0.8 × 10⁶ cells/well for production of ACM, cholesterol efflux measurements, or qPCR analysis, or at a density of 64,000 cells/well on a PLL coated 8-well chamber slide (Nunc) for spinal cord cultures. The following day, lentiviral particles were added to ASTRs (1:3 with ASTR medium) in the presence of hexadimethrine bromide (Polybrene, 8 µg/ml, Sigma). The next day, the cells were washed and used for spinal cord cultures or left for 5 days for qPCR analysis, cholesterol assays and production of ACM.**

Cholesterol assays

**Lipid extraction.** Lipids from cells and medium were extracted using the Blich and Dyer method⁴⁰⁸,⁴⁰⁹. Briefly, a methanol-chloroform-mixture (1:2) was added and the samples were vortexed and centrifuged at 2000 rpm for 5 minutes. The lower phase was collected and the upper phase was further processed by adding chloroform (1:1). After centrifugation at 2000 rpm for 5 minutes, the lower phase was added to previous collected lower phase and dried with a vacuum centrifuge and/or heating to 60 °C. After centrifugation, the dried lipid extracts of the cell samples were dissolved in chloroform mixture (1:1), of which half was used for phosphate concentration determination after which the samples were dried again.

**Cholesterol levels.** Cholesterol levels in cells (intracellular) and medium (extracellular) were quantified using a fluorescence-based method⁴¹⁰. The dried lipid extracts were dissolved in ethanol and a mixture of parahydroxy phenylacetic acid, phosphate buffer, sodium cholate, Triton-X100, water, cholesterol-oxidase (0.15 U/ml, Sigma), followed by peroxidase addition (0.95 U/ml, Sigma) and incubated for 20 minutes in the dark, including standard concentrations of 0-29 nmol cholesterol. Fluorescence was measured with an excitation of 325 nm and an emission of 415 nm (PerkinElmer instruments LS 55). The cholesterol efflux was calculated using the formula: efflux = extracellular cholesterol levels/ (intracellular + extracellular cholesterol levels).

**Phosphate determination.** As an internal control, the amount of phosphate in each sample was determined, which is an indication of the amount of protein in the sample⁴¹¹. To this end, a standard curve was prepared using 0-320 nmol phosphate concentrations. Then, 0.2 mL of 70% perchloric acid (Sigma) was added and the tubes were placed into a heating block at 180°C for 30 minutes. After cooling down, 2 mL of molybdate reagent (ammonium heptamolybdate tetrahydrate (Sigma), concentrated sulphuric acid (Sigma), water) and 0.25 mL of freshly made 10% ascorbic acid (Sigma) were added and the tubes were vortexed and placed in a heating block at 95°C for 10 minutes. To stop the reaction, the tubes were put in an ice-cold water bath and absorbance was measured at 812 nm. For intracellular cholesterol levels, the amount of cholesterol was normalized to phosphate, i.e., the ratio of cholesterol to phosphate was calculated (nmol/nmol).
Western blot analysis

For immunobLOTS of ABCA1 and ABCG1, ASTRs were washed with PBS and scraped in lysis buffer (6% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, and protease inhibitor cocktail (Roche). The total protein concentration was determined using a protein determination assay (Bio-Rad) according to manufacturer’s instructions and using BSA as a standard. Equal amounts of proteins (50 μg) were loaded onto 7.5% SDS-polyacrylamide gels. For detection of pro-Il1β in ACM, equal amounts of ACM (80 μl) were loaded onto 15% SDS-polyacrylamide gels. After gel electrophoresis, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore) via wet transfer. After blocking the membrane for one hour with Odyssey blocking buffer (1:1, Li-Cor Biosciences), the membranes were incubated with primary antibodies against ABCA1 (monoclonal mouse anti-ABCA1, 1:500, Novus Biologicals, NB100-2068), ABCG1 (polyclonal rabbit anti-ABCG1, 1 μg/ml, Novus Biologicals, NB400-132), or Il1β (monoclonal hamster anti-il1β, 1:200, Santa Cruz, sc-12742) overnight at 4°C. After washing with PBS containing 0.1% Tween-20, IRDye-conjugated secondary antibodies (Li-Cor Biosciences, Lincoln; 1:3000) were incubated for one hour. For pro-il1β, an additional incubation step with a rabbit-anti-hamster linker antibody was conducted, before secondary antibodies were applied. As loading control for ABCA1 and ABCG1 actin (monoclonal mouse anti-β-actin; 1:2000, Sigma, A5441) was used. The band were visualized with the Odyssey Imaging System (Li-Cor). The expression of each protein was calculated relative to the amount of β-actin with densitometry using FIJI (ImageJ).

Immunocytochemistry

Primary cell cultures. Cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature, and permeabilized with ice-cold methanol for 10 minutes. Non-specific antibody binding was blocked with 4% BSA for 30 minutes after which cells were incubated with primary antibodies against myelin basic protein antibody (MBP, 1:250 in BSA; Serotec, cat. no. MCA409S) or anti-GFAP (polyclonal rabbit anti-GFAP, 1:500, DAKO, Z033430) at room temperature. Cells were rinsed three times with PBS before the appropriate Alexa Fluor®-conjugated secondary antibodies (1:500) were added together with DAPI (Sigma, nuclear stain) for 30 minutes at room temperature. After three washes with PBS, coverslips were mounted using mounting medium (Dako). Samples were analyzed using a conventional immunofluorescence microscope (Leica DMI 6000 B) equipped with Leica Application Suite Advanced Fluorescence software. In each independent experiment, per condition approximately 150-250 cells per coverslip were scored for the number of MBP-positive cells of DAPI-stained cells (differentiation), or for the number of MBP-positive cells that form myelin membranes (myelin membrane formation).

Spinal cord cultures. Spinal cord cultures were fixed with 4% PFA for 30 minutes, and blocked and permeabilized with 0.1% Triton X-100 in 4% BSA in PBS for 45 minutes. After three washes with PBS, cells were incubated for 90 minutes with anti-MBP and anti-neurofilament-H (NF-H, polyclonal chicken anti-neurofilament, 1:5000, EnCor Biotechnology Inc., 2796-7) at room temperature. Cells were rinsed three times with PBS before the appropriate FITC- or TRITC-conjugated secondary antibodies (1:50, Jackson Immunolaboratories) or Alexa Fluor®-conjugated secondary antibodies (1:500) were added together with DAPI for 45 minutes at room temperature. Mounting of coverslips and slides was done with mounting medium (Dako). Samples were imaged using either a conventional immunofluorescence microscope (Leica DMI 6000 B) or a confocal microscope (TCS SP2 or SP8 AOBS Microscope, Leica Microsystems) using Leica Software. The percentage of myelinated axons was calculated in ImageJ as an area in pixels in each image occupied by both myelin and axons divided by the axonal density as described^32,41,2; or the percentage of myelinated axons was calculated using MATLAB software programmed to recognize only linear structures, thus including only myelin and axons and excluding OLG cell bodies^32. In each experiment, 5 images per coverslip and 2 coverslips per condition were analyzed.

qPCR analysis

Cells were scraped in RNA protect (Qiagen) and RNA was isolated using an RNA-isolation kit (Isolate II RNA Micro Kit; Bioline) according to manufacturer’s instructions. Of total RNA, 1 μg was reverse transcribed in the presence of oligo(dT)12–18 (Invitrogen) and dNTPs (Invitrogen) with M-MLV reverse transcriptase (Invitrogen) according to manufacturer’s instructions. mRNA levels of Fdft1, Fasn, Srebfc, and Srebfs2 were measured by real-time quantitative reverse transcriptase PCR (qPCR) using Absolute qPCR SYBR Green Master Mix (BioRad). Samples were analyzed using a conventional immunofluorescence microscope (Leica DMI 6000 B) equipped with Leica Application Suite Advanced Fluorescence software. In each independent experiment, per condition approximately 150-250 cells per coverslip were scored for the number of MBP-positive cells of DAPI-stained cells (differentiation), or for the number of MBP-positive cells that form myelin membranes (myelin membrane formation).
in a Step-One Plus Real-Time PCR machine. Each measurement was performed in triplicate and amplification data was processed using the LinRegPCR method\textsuperscript{413,414}. Primer sequences are shown in table 1. Expression was corrected for expression of housekeeping genes \textit{Eef1a1} or \textit{Hprt1}.

Table 1. Primer sequences used for qPCR.

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</table>

\textit{Statistical analysis}

Data are expressed as mean ± standard error of the mean (SEM) of at least 3 independent experiments. When comparing absolute values between groups (i.e., gmASTR versus wmASTR) statistical significance was assessed using a paired two-sided t-test. When relative groups were compared to control (i.e., NCM, control gmASTRs, control wmASTRs, control gmACM or control wmACM), statistical analysis was performed with a one-sample t-test, with indicated control set to 1 in each independent experiment. A paired t-test was used to test for differences between effects of wmACM and gmACM on OPC differentiation (#) with NCM was set to 1 in each independent experiment. A one-way ANOVA with a Šidák multiple comparisons post-test was performed to test for differences between different treatments. Statistics were performed using GraphPad Prism 6.0. In all cases, p-values of <0.05, <0.01, and <0.001 were considered significant and indicated with *, ** and *** or #, ##, and ###, respectively.

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