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Transcriptional heterogeneity between primary adult grey and white matter astrocytes underlie differences in modulation of \textit{in vitro} myelination

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Abstract
Multiple sclerosis (MS) is an inflammation-mediated demyelinating disease of the central nervous system, which eventually results in axonal degeneration due to remyelination failure. Successful remyelination is, among others, orchestrated by astrocytes (ASTRs) and requires sequential activation, recruitment and maturation of oligodendrocyte progenitor cells (OPCs). In both MS and experimental models, remyelination is more robust in grey matter (GM) than in white matter (WM). This remarkable difference in remyelination efficiency is likely related to local differences between GM and WM lesions. Here, we investigated whether gmASTRs and wmASTRs differently modulate myelination. Our data revealed that adult wmASTRs were less supportive for in vitro myelination than gmASTRs, by secreting factors that reduced myelin membrane formation. Transcriptional profiling demonstrated that wmASTRs more abundantly express reactive ASTR genes and genes of a neurotoxic subtype of ASTRs, termed A1, while gmASTRs have more neuro-reparative A2-ASTR transcripts. A weighted gene network co-expression network analysis identified a gene network module containing cholesterol biosynthesis enzyme genes that positively correlated with gmASTRs; and a network module containing extracellular matrix (ECM)-related genes that positively correlated with wmASTRs. Adult wmASTRs and gmASTRs were heterogeneous at the transcriptional level, differed in their support of myelination and their pre-existing phenotype determined TLR3 agonist responses. Taken together, primary adult gmASTRs and wmASTRs were heterogeneous at the transcriptional level, differed in their support of myelination and their pre-existing phenotype determined TLR3 agonist responses. These findings point to a role of regional heterogeneity of ASTRs in remyelination differences between GM and WM lesions.

Keywords: astrocytes, multiple sclerosis, myelination, oligodendrocyte, region

Introduction
Remyelination is a natural regenerative process that follows upon demyelination by which new myelin membranes are formed around denuded axons. Remyelination in the central nervous system (CNS) is a multi-step process that involves activation, recruitment, and differentiation of oligodendrocyte progenitor cells (OPCs) into mature, remyelinating oligodendrocytes (OLGs). Remyelination is orchestrated by transient signaling of reactive astrocytes (ASTRs). Similar as during CNS development, ASTRs support oligodendroglial cell functioning and myelin membrane formation, among others, by extracellular matrix (ECM) remodelling and the supply of fatty acids and cholesterol. ASTRs become reactive upon demyelination, which is induced by a variety of inflammatory mediators as well as myelin debris and includes changes in ASTR morphology, gene expression, and function. ASTR responses towards injury vary and depend on the type of injury, and can be either beneficial or detrimental for remyelination. In this regard, two subtypes of reactive ASTRs have been described, A1- and A2-ASTRs. A1-ASTRs possess a neurotoxic phenotype and secrete factors that inhibit OPC proliferation, migration and differentiation, and are toxic to mature OLGs. By contrast, A2-ASTRs appear more neuroprotective and more supportive towards repair. At homeostatic conditions, ASTRs are not uniform, and multiple studies have described ASTRs diversity based on their morphology and function.

Historically, ASTRs are divided into two groups; fibrous ASTRs that reside in the white matter (WM) and the morphologically more complex protoplasmic ASTRs which are present in the grey matter (GM). Multiple single-nucleus RNA sequencing studies identify two to three groups of ASTRs dispersed throughout the adult GM and WM, while morphology and functional studies identify up to nine different groups of ASTRs in rodents. ASTR reactivity is also region dependent, i.e., upon cuprizone-induced demyelination ASTR reactivity is more apparent in ASTRs of the corpus callosum, a WM area, than in ASTRs in the cortex, a GM area. In addition, demyelination is delayed in the cortex compared to the corpus callosum, and remyelination occurs faster in GM than in WM areas. Also, in the chronic demyelinating disease multiple sclerosis (MS), remyelination is more efficient in GM than in WM, but ultimately fails in both areas, contributing to disease progression. As in toxin-induced demyelination rodent models, ASTR reactivity...
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...and around inflammatory WM lesions, but not around lesions in the GM. This difference in ASTR reactivity may contribute to the more efficient remyelination in GM MS lesions, while astrocytes in WM MS lesions do not. Whether intrinsic differences between gmASTR and wmASTR contribute to regional differences in ASTR reactivity and/or whether they differently respond to the same type of injury and inflammation, is currently not clear. By performing transcriptional profiling and the use of in vitro myelinating cultures that depend on ASTRs, we addressed here whether adult gmASTRs and wmASTRs are different cell populations that distinctly modulate myelination and display a differential response to inflammatory mediators.

## Results

### Cultured adult wmASTRs and gmASTRs are transcriptionally heterogeneous

Previous findings show that remyelination is more efficient in GM lesions than in WM lesions, OLGs depend on support from ASTRs to remyelinate denuded axons, and as gmASTRs and wmASTRs are morphologically and functionally different, we aimed to address whether gmASTRs and wmASTRs distinctly modulate myelination. As cultured neonatal gmASTRs are still considered as (reactive) ASTR progenitors, we used an in vitro culture model of ASTRs derived from young adult rat brains. These primary ASTRs exhibit properties like the expression of ASTR markers, glutamate uptake, and responses to injury, that are representative for their properties in the adult brain. To assess whether cultured gmASTRs and wmASTRs differ, their transcriptional profiles were compared (Fig. 1).

Analysis of cell type-specific genes revealed high abundance of transcripts of most ASTR-specific genes (e.g., Vim, Mfeg8, Gja1), low abundance of transcripts of OPC- (e.g., Cspg4, Pdgfra), newly-formed OLG- (e.g., Fyn), and mature OLG-specific (e.g., Mbp, Mog) genes. Transcript levels of genes typically expressed by microglia- (e.g., Irf8, Aif, Ncf1), endothelial cells (e.g., Cldn5, Egfl7, Vwa1) and neurons (e.g., Tubb3, Reln, Trp73) were low (Fig. 1a). These data indicate that both gmASTRs and wmASTRs cultures were highly pure. Principal component analysis (PCA) showed that 74% of the variance between the samples was explained by region of origin of the ASTRs (Fig. 1b), indicating that cultured adult gmASTR and wmASTRs were transcriptionally different. Between gmASTR and wmASTRs, 857 genes were differentially expressed (FDR <0.001), of which 183 genes met the threshold of an absolute logarithm of the fold change >2. Of these genes, 75 genes were more abundantly expressed in gmASTRs (Suppl. Table 1) and 108 genes in wmASTRs (Suppl. Table 2). Genes that were more abundantly expressed in wmASTRs include several ECM-related genes (Fbn1, Eln, Postn, Itga1), while in gmASTRs, genes involved in lipogenesis (Scd, Scd2) and Wnt signaling (Rspo2, Wnt6) were more abundantly expressed. Strikingly, two different members of the cadherin family are opposingly differentially expressed; Cdh2 encoding for N-cadherin was more abundantly expressed in wmASTRs, while transcripts for Cdh1 encoding for E-cadherin were more present in gmASTRs (Fig. 1c).

Most pan-reactive ASTR marker genes were more abundantly expressed in cultured adult wmASTRs compared to cultured adult gmASTRs (Fig. 1d, Lcn2; FDR=0.021;...
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Figure 1. Primary adult wmASTRs and gmASTRs are transcriptionally heterogeneous. RNA from 6 biological replicate cultures of adult grey matter astrocytes (gmASTRs) and adult white matter ASTRs (wmASTRs) was subjected to 3'RNA sequencing. (a) Heatmap of the logarithm of the gene million counts +1 (Log{CPM+1}) of genes specific for ASTRs, microglia (micro), endothelial cells (endo) neurons (neuron), oligodendrocyte progenitor cells (OPCs), newly-formed oligodendrocytes (nOLGs) and mature oligodendrocytes (mOLGs). Row Z-score represent relative expression of cell type-specific genes compared to each other within one biological sample, genes with a CPM>20 are depicted in bold. Note that most ASTR genes are highly expressed within the different batches compared other cell type specific genes. (b) Principal component analysis (PCA) show that 74% of variation between the samples is explained by their regional origin. (c) A volcano plot displaying all genes, where the dots for differentially expressed genes differ in color and size. The small black dots represent genes that were not considered to be differentially expressed. In white are genes more abundantly expressed in wmASTRs, in grey genes that are more abundantly expressed in gmASTRs (absolute logarithm of fold change (LogFC) >2, FDR<0.01). Large circles illustrate genes with a CPM>20 are depicted in bold. Notably, transcript levels for some A1-ASTR-associated genes as defined by Liddelow et al were more present in wmASTRs (Ggtai FDR=0.002; Fh153 FDR=0.005; Ugtta FDR=0.008), while transcripts of two A2-ASTR-associated genes (Sioota and Empi FDR<0.001) were more abundant in gmASTRs (Fig. 1d). Although Gap mRNA expression was low in both type of ASTRs, GFAP protein was expressed (Fig. 1e). Consistent with Gap mRNA levels (Fig. 1d), GFAP protein expression was significantly higher in wmASTRs compared to gmASTRs (Fig. 1f). This was caused by low expression of a GFAP splice variant by gmASTRs (Fig. 1e). Hence, cultured adult wmASTRs and gmASTRs were heterogeneous at the transcription level even upon prolonged time in culture. To assess whether gmASTRs and wmASTRs distinctly modulate myelination efficiency, their ability to regulate in vitro myelination was examined next.

Cultured adult wmASTRs are less supportive for myelination than cultured adult gmASTRs

To study a role of gmASTRs and wmASTR in myelination efficiency, we made use of an in vitro myelinating system of embryonic spinal cord cultures that relies on an ASTR feeding layer. Myelination efficiency was assessed by a double labeling of myelin and neurons, using myelin basic protein (MBP) as a marker for myelin, and neurofilament-H (NF-H) as a marker for neurons. The percentage of myelinated axons was higher on a feeding layer of gmASTRs than on a feeding layer of adult wmASTRs (Fig. 2a,b, p=0.016). This indicates that wmASTRs and gmASTRs differ in their ability to support myelination, which is either a result of wmASTR-derived inhibitory or gmASTR-derived stimulatory signals for myelination. ASTRs can signal to OPCs via secreted soluble factors, such as growth factors and cytokines, via insoluble factors, such as ECM proteins, and via adhesive interactions. The transcriptional profiles uncovered that wmASTRs expressed more transcripts of both positive and negative regulators of OPC proliferation (Pdgfra, Fgf251,420,421 and OPC maturation (Bmp4, Cntf, Timpi, Fn, Vcan, Tnc, Jag1, Igsf, Gja1, Tgm2235,295,315,412–415))
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Figure 2. wmASTRs are less supportive for in vitro myelination. (a,b) In vitro myelination cultures that depend on a feeding layer of astrocytes (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 (NF, red), an axonal marker. Representative images of myelinating spinal cord cultures on either adult white matter (wm) ASTRs or adult grey matter (gm) ASTRs are shown in (a) and quantification of the percentage of myelinated axons in (b) (n=3). (c) RNA from 6 biological replicate cultures of adult gmASTRs and wmASTRs is subjected to total 3’-RNA sequencing. Heatmap with literature-based genes of positive and negative regulators of OPC proliferation and differentiation by gmASTRs and wmASTRs. Column Z-score represent the relative expression of genes between different samples. Positive regulators of OPC differentiation are indicated in green; negative regulators in red. (*FDR<0.05, **FDR<0.01, ***FDR<0.001). (d-h) Assays performed to determine the effect of ASTR-conditioned medium (ACM) and ASTR-derived extracellular matrix (ECM) coatings on oligodendrocyte progenitor cells (OPCs) and oligodendrocytes (OLGs). A schematic representation is shown in (d). OPCs isolated from neonatal rat forebrain were cultured for 1 day in the presence of PDGF-AA and FGF-2 to assess proliferation (% KI67-positive cells of A2B5-positive cells), or differentiated for 6 days after growth factor withdrawal to assess metabolic activity (MTT), cytotoxicity (LDH), differentiation (% MBP-positive cells of total cells) and myelin membrane formation (% myelin membranes formed by MBP-positive cells). Representative images of MBP-positive OLGs (red) in the presence of NCM, wmACM or gmACM are shown in e; representative images of proliferation on PLL (no ECM), on wmECM coatings or gmECM coatings stained for KI67 (red) and with A2B5 (black) are shown in (g). Quantification of all assays in the presence of NCM, wmACM or gmACM are shown in (f) and when plated on PLL (no ECM), wmECM coatings and gmECM coatings in (h). Bars represent relative means to NCM (f) or no ECM (h, PLL), which were set to 1 in each independent experiment. Error bars 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 treatments and untreated control (NCM in f, no ECM in h), while a paired t-test (*p<0.05) was used to test for differences between effects of gmACM/gmECM coating versus wmACM/wmECM coatings. Absolute values of NCM are for proliferation 37.5% ± 22.4, cytotoxicity 25.9% ± 2.9, differentiation 48.8% ± 17.8 and myelin membrane formation 68.0% ± 14.4 and of no ECM (PLL) for proliferation 17.8% ± 4.8, differentiation 33.3% ± 4.7, and myelin membrane formation 65.3% ± 3.8. Note that upon exposure to wmACM, but not gmACM, myelin membrane formation is decreased compared to NCM (p=0.005), while metabolic activity is increased upon gmACM treatment compared to NCM (p=0.016) and wmACM treatment (p=0.033) (e,f). In addition, OPC proliferation is higher on gmECM coatings than on PLL (g,h, no ECM). Scale bars are 25 µm.than gmASTRs (Fig. 2c). To determine whether wmASTRs and gmASTR differentially support OPC behavior by a difference in secreted factors, the effect of ASTR-conditioned medium (ACM) of either ASTR on OPC proliferation, metabolic activity, and/or maturation was examined (Fig. 2d-f). OPC proliferation, as assessed by the number of K167-positive cells of A2B5-positive OPCs, was similar when OPCs were exposed to gmACM, wmACM and non-conditioned medium (NCM) (Fig. 2f). After 6 days of differentiation, the metabolic activity, as assessed with an MTT assay, was increased upon exposure to gmACM compared to both wmACM and NCM (Fig. 2e, gmACM versus NCM p=0.016; gmACM versus wmACM p=0.033). Remarkably, ACM from neither ASTR was toxic to the cells (Fig. 2e), despite wmASTRs more abundantly expressing specific genes for neurotoxic A1-ASTRs (Fig. 1d). In addition, while the percentage of MBP-expressing OLGs, reflecting their differentiation, remained similar, the percentage of OLGs that form myelin membranes was significantly decreased in the presence of wmACM compared to NCM (Fig. 2f p=0.005). Exposure to gmACM had no effect on OPC differentiation or myelin membrane formation (Fig. 2f). To examine the effect of ASTR-derived ECM on OPC behavior, OPCs were cultured on coatings of gmECM or wmECM. OPC proliferation was significantly increased when OPCs were plated on a gmECM coating compared to an inert PLL-coating (no ECM, Fig. 2g,h p=0.041). OPC proliferation was not affected by a wmECM coating. OPC differentiation and myelin membrane formation were not altered when plated on either gmECM or wmECM coatings compared to a PLL-coating (Fig. 2h).
Hence, these findings revealed that wmASTRs were less supportive for myelination than gmASTRs, which may be a net inhibitory effect of wmASTR-derived secreted factor(s) that preclude myelin membrane formation and/or a stimulatory effect of gmASTR-deposited ECM on OPC proliferation. To obtain more insight in the factors that drive the distinct regulation of myelination by gmASTRs and wmASTRs, a weighted gene co-expression network analysis (WGCNA)\(^47\) was performed on the RNA-seq data set.

**Gene co-expression networks differ between wmASTRs and gmASTRs**

WGCNA is a powerful method that determines in an unbiased manner networks based on correlation of changes in the expression of genes allowing for the identification of differentially expressed gene networks in modules that represent biological functions and regulatory mechanisms\(^48\). In this data set, 14 region-related gene modules were identified, of which two gene modules were significantly, and consistent among different biological samples, differentially correlated with gmASTRs and wmASTRs (Suppl. Fig. 1a-d). Cluster royalblue consisted of 844 genes and was positively correlated with gmASTRs (p<0.001) and negatively correlated with wmASTRs (Suppl. Data 1). Cluster darkgrey consisted of 110 genes and positively correlated with wmASTRs (p<0.001) (Suppl. Data 2).

Gene annotation revealed that many genes represented in the royalblue cluster were related to ‘cell division’ (Fig. 3a). As ASTR proliferation decreases with age\(^45\), this may indicate that gmASTRs were less mature than wmASTRs. In addition, genes that are present in the royalblue cluster were connected by gene ontology (GO) pathways for sterol, steroid, cholesterol and secondary alcohol biosynthesis (Fig. 3a,b). A heatmap of genes that are relevant for sterol, steroid and cholesterol biosynthesis (Suppl. Fig. 2) shows that most of these genes were more abundantly expressed in gmASTRs compared to wmASTRs, which predicts that there is more cholesterol biosynthesis in cultured adult gmASTRs. Notably, ASTRs supply cholesterol to OLGs, which supports myelination\(^47,48,372\).

Gene annotation of the darkgrey cluster revealed many genes that are relevant for ‘ECM formation and modification’, ‘atherosclerosis’, ‘hypoxia’ and ‘vascularization’ (Fig. 3c). Of interest, transient ECM remodeling plays an important role in successful remyelination\(^45,47,455,374\). A heatmap of genes with a CPM > 20 that differed significantly (FDR<0.05) and related to ECM formation and modification (Fig. 3d) shows that these genes are more abundantly expressed in wmASTRs compared to gmASTRs.

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*Figure 3. Gene co-expression networks differ between wmASTRs and gmASTRs. RNA from 6 biological replicate cultures of adult grey matter astrocytes (gmASTRs) and adult white matter ASTRs (wmASTRs) is subjected to total 3’-RNA sequencing. A weighted gene network co-expression analysis revealed that the royalblue module positively correlated with gmASTR and negatively with wmASTRs, and a darkgrey module that negatively correlated with gmASTRs and positively with wmASTR (Suppl. Fig. 1) (a) Gene annotation of the royalblue gene module revealed genes relevant to ‘cell division’ and cholesterol biosynthesis that are more abundantly expressed in gmASTRs. (b) Gene ontology (GO) visualization of sterol, steroid, secondary alcohol and cholesterol biosynthesis genes from the royalblue cluster which are more abundantly expressed in gmASTRs. (c) Gene annotation of the darkgrey gene module revealed genes relevant to ‘response to hypoxia’ and ‘extracellular matrix organisation’. (d) Heatmap of matrisome-core and -associated genes with a CPM > 20, an absolute LogFC > 2 and an FDR < 0.05 by gmASTRs and wmASTRs. Column Z-score represent the relative expression of genes between different samples. Note that more genes encoding for matrisome-core and associated genes are more abundantly expressed in wmASTRs. (*FDR<0.05, **FDR<0.01, ***FDR<0.001).*
Constituents are ECM-core proteins (e.g., Mfeg8, Ctgf, Spp1, Ltbpi) that are mainly matricellular proteins, and ECM-affiliated proteins (e.g., Bdnf, Hbegf, Mmp2, Ccl2, Ccl7), which are proteins that regulate ECM remodeling or secreted factors that associated with the ECM. Notably, transcripts of Sema3f, an OPC attractant, were more present in gmASTRs compared to wmASTRs (Fig. 3d). Several ECM genes that were more abundantly expressed in wmASTRs were associated with pathways previously implicated in OPC maturation and/or remyelination failure (Ctgf, Tnc, Spp1396,416,425,431,432) and/or expressed in reactive ASTRs in WM MS lesions (Mfeg8, Tnc, Spp171,397,431,433), and may relate to the more reactive ASTR profile of cultured adult wmASTRs (Fig. 1). Hence, the regulatory mechanisms that may account for the higher in vitro myelination potential of OLGs in the presence of gmASTRs, are more cholesterol biosynthesis in gmASTRs and increased deposition of constituents of the matrisome by wmASTRs. The different identities of gmASTRs and wmASTRs may result in distinct and specific responses towards inflammatory mediators that are increased upon injury. As demyelination involves innate immune activation, and as Toll-like receptor 3 (TLR3) and TLR4 are prominently present on reactive ASTRs in WM MS lesions29,35, we next examined whether gmASTRs and wmASTRs might respond differently to TLR3 and/or TLR4 agonists and as a consequence distinctly modulate OPC behavior. As TLR3 and TLR4 agonists interfered with the development of neurons, OPCs and OLGs in the in vitro myelinating cultures, we were not able to examine the role of ASTR reactivity on in vitro myelination.

**Poly(I:C) treatment of adult gmASTRs and wmASTRs result in different modulation of OPC proliferation and myelin membrane formation**

To assess whether gmASTRs and wmASTRs differently respond to TLR3 ligands, the effect of TLR3 agonist Poly(I:C) on ASTRs and their ability to modulate OPC behavior via secreted factors (ACM) or deposited ECM was determined. Secreted factors from Poly(I:C)-treated wmASTRs, but not from Poly(I:C)-treated gmASTRs decreased OPC proliferation compared to their respective untreated control ASTRs (Fig 4a, p=0.027). In contrast, OPC proliferation was enhanced when plated on ECM coatings of Poly(I:C)-treated wmASTRs, but not on ECM coatings of Poly(I:C)-treated gmASTRs (Fig. 4a, p=0.022). In addition, ACM of Poly(I:C)-treated gmASTRs consistently and significantly reduced myelin membrane formation by approx. 20% compared to untreated gm ACM (Fig. 4d p=0.016). By contrast, myelin membrane

![Figure 4. Poly(I:C) treated adult gmASTRs and wmASTRs differently modulate OPC proliferation and myelin membrane formation.](image-url)
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formation was unchanged when OPCs were exposed to ACM of Poly(I:C)-treated wmASTRs (Fig. 4d). Secreted factors from Poly(I:C)-treated gmASTRs or wmASTRs hardly affected metabolic activity of OLGs and OPC differentiation compared to secreted factors from their respective untreated control ASTRs (Fig. 4b,c). Hence, our findings demonstrate that Poly(I:C) treatment of adult gmASTR and wmASTRs results in different modulation of OPC proliferation and myelin membrane formation compared to untreated gmASTRs and wmASTRs, respectively. As secreted factors from Poly(I:C)-treated gmASTRs reduced myelin membrane formation, this may indicate that Poly(I:C)-treated gmASTRs are less supportive for myelination than untreated gmASTRs.

LPS treatment of adult gmASTRs and wmASTRs results in a decrease of myelin membrane formation

To assess whether gmASTRs and wmASTRs differently respond to TLR4 ligands, the effect of TLR4 agonist LPS on ASTRs and their ability to modulate OPC behavior was determined next. Secreted factors from LPS-treated wmASTRs and gmASTRs did not significantly change OPC proliferation (Fig. 5a) and differentiation (Fig. 5c). Secreted factors from both LPS-treated gmASTRs and wmASTRs inhibited myelin membrane formation by 40–50% compared to secreted factors from their respective untreated ASTRs (Fig. 5d, gmACM p=0.020, wmACM p=0.003), accompanied by a decrease in metabolic activity by 30–40% (Fig. 5b, wmACM p=0.039, gmACM p=ns). ECM coatings of LPS-treated gmASTRs or wmASTRs did not significantly alter OPC proliferation (Fig. 5a), differentiation (Fig. 5b) or OLG myelin membrane formation (Fig. 5c). Hence, our findings demonstrate that LPS treated wmASTRs and gmASTRs similarly modulate OPC behavior by secreting factors that reduced the percentage of OLGs that form myelin membranes.

Figure 5. LPS treatment of adult gmASTRs and wmASTRs results in a decrease of myelin membrane formation. (a-d) Primary grey matter astrocytes (gmASTRs) and white matter (wm) ASTRs were cultured for 24 hours in the presence or absence of TLR4 agonist LPS, after which conditioned medium (ACM) was collected in the next 24 hours. For ASTR extracellular matrix proteins (ECM), ASTRs were cultured for 48 hours in the presence or absence of LPS after which the cells were lysed, ECM was collected and used as coating. Oligodendrocyte progenitor cells (OPCs) isolated from neonatal rat forebrain were cultured in the presence of ACM or on ECM coatings, for 1 day in the presence of PDGF-AA and FGF-2 to assess proliferation (a, % KI67-positive cells of A2B5-positive cells), or differentiated for 6 days after growth factor withdrawal to assess metabolic activity (b, MTT), differentiation (c, % MBP-positive cells of total cells) and myelin membrane formation (d, % myelin membranes formed by MBP-positive cells). Bars represent relative means to their respective untreated control ACM or ECM coating, which were set to 1 in each independent experiment. Error bars 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 with untreated control ACM or ECM coating. Absolute values are 34.3 ± 17.4% proliferation, 55.1 ± 19.1% differentiation, and 56.8 ± 17.7% myelin membrane formation with control wmACM; 39.6 ± 13.5% proliferation, 61.6 ± 22.2% differentiation, and 67.4 ± 20.3% myelin membrane formation with control gmACM; 31.4 ± 12.5% proliferation, 51.9 ± 18.6% differentiation, and 56.7 ± 22.3% myelin membrane formation on control wmECM coatings and 41.0 ± 1.7% proliferation, 44.8 ± 15.1% differentiation, and 51.5 ± 12.5% myelin membrane formation on control gmECM coatings. Note that myelin membrane formation is decreased in the presence of ACM of both LPS-treated wmASTRs and gmASTRs (wmACM p=0.003, gmACM p=0.020), while metabolic activity was decreased in the presence of ACM of LPS-treated wmASTRs (wmACM p=0.039).
Discussion

ASTRs support myelination during development and are indispensable for successful remyelination upon demyelination\(^{13,14,15}\). As remyelination is more efficient in GM lesions than in WM (MS) lesions\(^{17,18,37,38,39}\), we aimed here to address whether gmASTRs and wmASTRs heterogeneity is reflected in distinct modulate of myelination. Our findings demonstrate that adult wmASTRs were less supportive towards in vitro myelination than adult gmASTRs. Transcriptional profiling demonstrated that even after prolonged time in culture, adult gmASTRs and adult wmASTRs have a distinct transcriptional profile, which may underlie their difference in modulating in vitro myelination. Moreover, when exposed to TLR4 agonist LPS, secreted factors of both gmASTRs and wmASTRs inhibited myelin membrane formation, while TLR3 agonist Poly(I:C) elicited distinct responses in gmASTRs and wmASTRs. Hence, our findings indicate that pre-existing regional heterogeneity in ASTRs may contribute to differences in remyelination efficiency in demyelinated GM and WM areas.

Our data revealed that adult rat ASTRs isolated from GM and WM were transcriptionally different. Previous profiling studies demonstrated that cultured neonatal gmASTRs isolated via a shake-off method, resemble reactive ASTRs (progenitors) at the proliferative and gene expression level, which is suggested to be induced by serum-derived components\(^{40-42}\). The adult gmASTRs and wmASTRs used in the present study were always generated at the same time from the same animal via a non-shake procedure, and cultured in serum, indicating that the observed differences in gmASTR and wmASTR reactivity were likely not acquired in vitro. Our data show that wmASTRs were more reactive than gmASTRs, which is consistent with a recent single nucleus RNA sequencing study of human control tissue that demonstrated heterogeneity between human gmASTRs and wmASTRs\(^6\), including a more reactive profile of wmASTRs compared to gmASTRs\(^9\). In addition, the cultured adult gmASTRs and wmASTRs differed in the subtype of ASTR reactivity. wmASTRs expressed more transcripts of several A1-ASTR-specific genes, while A2-ASTR-specific genes were more abundantly expressed in gmASTRs. Hence, our cultured adult gmASTRs and wmASTRs maintain some properties that are also observed in GM and WM areas.

Our findings further demonstrated that more axons were myelinated on a feeding layer of adult gmASTRs than on feeding layer of adult wmASTRs. As secreted factors from wmASTRs, but not gmASTRs, decreased myelin membrane formation, it is tempting to suggest that wmASTRs were less supportive to myelination. On the other hand, ECM components deposited by gmASTRs increased OPC proliferation, and as a higher density of OPCs augments myelination\(^{19}\), enhanced proliferation may contribute to the increased in vitro myelination on gmASTRs. The transcriptional data provided insight into differences in gene expression of signaling molecules in gmASTRs and wmASTRs that are relevant for (re)myelination. Analysis of literature-based modulators of OPC behavior, revealed that wmASTRs were equipped with higher transcript levels of genes of signaling molecules that either positively or negatively modulate OPC proliferation and differentiation\(^{20-22}\),\(^{43}\). These signaling molecules were soluble-derived (Bmp4, Fgf2, Cntf, Pdgfa)\(^{13,39,44-46}\) or adhesion-dependent (Jag1). Hence, it is likely a reflection of the net effect of these factors, that made wmASTRs less supportive for myelination than gmASTRs in our in vitro myelinating culture system.

An unbiased gene co-expression network analysis revealed that genes encoding for proteins that are relevant for ECM production and modification, i.e., the matrisome\(^{47,48}\) are more abundantly expressed in wmASTRs. ASTRs are known to play an essential role in transient ECM remodeling important for successful remyelination upon toxin-induced demyelination\(^{49,50,51}\). In addition, increased expression of ECM proteins usually marks ASTR reactivity\(^{11,49}\). Therefore, the enhanced expression of genes related to the matrisome are likely part of the more reactive phenotype of wmASTRs. Most of the matrisome-core enriched genes were matricellular proteins (Tbhs2, Postn, Ctgf, Fbln2, Spp1), which are non-structural ECM proteins that support matrix fibrillogenesis and/or that have important functions in tissue repair\(^{52}\). These proteins may both influence matrix formation and signal to OPCs by modulating cell functioning via interaction with cell-surface receptors as well as with the structural ECM proteins, such as fibronectin and proteoglycans that are present in demyelinated areas\(^{53,54}\). The effect of ECM coatings on OPC behavior presented in the present study may be underestimated, as ECM coatings do not reflect all properties of the ECM, such as the original topological ECM architecture and stiffness. Indeed, in addition to providing signals, the stiffness of the ECM influences OPC behavior; OPC proliferation and differentiation favor a stiff matrix, and myelination is supported...
White matter astrocytes are less supportive for myelination. Hence, a higher abundance of ECM-related genes in wmASTRs may contribute to less efficient remyelination in WM lesions compared to GM lesions. This is indeed observed in MS lesions, where ECM protein hyaluronan, a inhibitor of OPC differentiation\(^{298}\), and its receptor CD44, are significantly increased in the WM, but not in the GM areas of leukocortical lesions\(^{37}\). Also, wmASTRs form more remyelination-impairing fibronectin aggregates than gmASTRs\(^{302}\), and fibronectin expression is increased in marmoset EAE WM, but not GM lesions\(^{31}\). Thus, interference with the wmASTR-mediated role in ECM remodeling may prove a valuable target for the enhancement of remyelination in WM MS lesions.

Diversity between wmASTRs and gmASTRs was also observed in their modulating effect of OPC behavior in response to TLR3 agonist Poly(I:C). More specifically, secreted factors of Poly(I:C)-treated gmASTRs, but not of Poly(I:C)-treated wmASTRs, decreased myelin membrane formation compared to secreted factors from their respective untreated ASTRs. Previous studies revealed that in rodent ASTRs exposure to Poly(I:C) induced the expression of both pro-inflammatory mediators that are linked to A1-ASTRs, and inflammatory mediators that are secreted by A2-ASTRs\(^{21,239,240}\). In line with this reasoning, Poly(I:C) may distinctly interfere with gmASTR and wmASTR reactive subtypes. Thus, intrinsic differences between gmASTRs and wmASTRs may differently control remyelination by reacting in a region-specific manner to demyelinating injury. This is consistent with changes in gene expression between ASTRs from different regions in an animal model for MS\(^{286}\), as well as differential responses of ASTRs isolated from different regions in vitro\(^{193}\). On the other hand, secreted factors from both TLR4 agonist LPS-treated gmASTRs and wmASTRs reduced myelin membrane formation. Therefore, the ASTR responses in GM and WM lesions may not only depend on the pre-existing heterogeneity of gmASTRs and wmASTRs and their differential response to the same type of inflammatory mediator, but may also relate to local inflammatory the context, which is different in GM and WM lesions\(^{16,19,37,310,311}\).

Taken together, primary adult gmASTRs and wmASTRs are diverse cell types, are heterogeneous at the transcriptional level, and GFAP protein level, differ in their ability to modulate in vitro myelination and in their response to TLR3 agonists. As
Chapter 4

Methods

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

Adult ASTRs. Adult ASTRs were isolated from young adult female Wistar rats brains as described419, with minor modifications. To obtain gmASTRs, the cerebral cortices were dissected and meninges removed. Of the residual tissue, the amygdala was discarded and the remaining parts non-cortical parts (WM tracts including corpus callosum, mixed GM and WM tracts, including hippocampus and thalamus, and deep GM parts, including basal ganglia) were kept in HBSS and used to obtain non-cortical, mainly wmASTRs. All brain pieces were kept in Hank’s Balanced Salt Solution (HBSS, Life Technologies) and mechanically dissected using scissors. Then, 0.05% trypsin (Sigma) and 0.003% DNase (Roche) in 1 mL HBSS was added and incubated for 15 minutes at 37°C, followed by centrifugation for 7 minutes at 1500 rpm. The pellet was resuspended in papain digestion mix [24 μg/mL L-cysteine (Sigma), 40 μg/ml DNase I, 30 U/mL papain from papaya latex (Sigma) in MEM (Life Technologies)] incubated for 15 minutes at 37°C. The digestion was stopped using OVO [40 μg/mL DNase I, 1 mg/mL trypsin inhibitor (Sigma), 50 μg/ml bovine serum albumin (BSA, Sigma) in L15 medium (Sigma)] for 6 minutes at room temperature. The cell suspension was centrifuged for 7 minutes at 1500 rpm and resuspended in 15 mL HBSS. Hereafter, the cells were settled for 30 minutes before the upper layer was separated from the lower cell fraction. The cell fractions were diluted in 15 mL HBSS and centrifuged for 7 minutes at 1500 rpm. Cell pellets were resuspended in AA+ medium [10% fetal bovine serum (FBS), 15 mM Hepes (Gibco), 0.04‰ gentamicin (Life Technologies), 14.3 mM NaHCO₃ (Merck), 100 U/mL penicillin and streptomycin, 4 mM L-glutamine]. The cells were cultured for 18-20 days at 37°C in tissue flasks (Nunc T80; Thermo Fisher Scientific) that were coated with poly-L-lysine (PLL, 5 μg/mL, Sigma) Medium exchange occurred every 3-4 days. The cells were passaged at least once using trypsin and used for experiments after 2-3 weeks, or collected in RNA protect for 3’-RNA sequencing. To obtain ASTR conditioned medium (ACM), ASTRs were cultured for 24 hours either untreated or in the presence of 50 μg/mL TLR3 agonist Polynosine-polycytidylic acid (Poly(I:C), GE Healthcare) or 200 ng/mL TLR4 agonist lipopolysaccharide (LPS, Sigma), after which cells were washed and cultured for another 2 hour in defined SATO medium [5 μg/mL bovine insulin (Sigma), 50 μg/mL human holo-transferin (Sigma), 100 μg/mL BSA fraction V (Sigma), 62 ng/mL progesterone (Sigma), 16 μg/mL putrescine (Sigma), 5 ng/mL sodium selenite (Sigma), 400 ng/mL T3 (Sigma), 400 ng/mL T4 (Sigma), 4mM L-glutamine, 100 U/ml penicillin and streptomycin. Hereafter, ACM was collected and filtered using a 0.45 µm filter (GE Healthcare), to remove cell debris. To obtain ASTR extracellular matrix (ECM), ASTRs were cultured for 48 hours either untreated or in the presence of Poly(I:C) or LPS, after which cells were lysed by water (2 times 1 hour, cell lysis was checked by a microscope) and ECM was scraped in sterile PBS containing Complete protease inhibitors (Roche). After protein concentration determination using a BioRad DC-protein assay using BSA as standard, 8-well Permanox chamber slides (Nunc) were coated with 8 µg of ECM per chamber.

Oligodendrocyte progenitor cells (OPCs). OPCs were isolated from mixed glia cultures of the non-cortex of newborn rat forebrains using a shake-off procedure as described121,252,349. Briefly, contaminating microglia were removed from the flask by a pre-shake at 150 rpm for 1 hour at 37°C and OPCs were obtained after a 240 rpm overnight shake at 37°C. The detached OPCs were further purified by differential adhesion on non-tissue dishes121. The enriched OPCs were cultured on PLL-coated 13-mm coverslips (35,000 cells/coverlip) or on ECM-coated 8-well Permanox chamberslides (28,000 cells/chamber). For proliferation OPCs were cultured for 24 hours in defined SATO medium containing 10 ng/mL platelet-derived growth factor-AA (PDGF-AA, Peprotech) and 10 ng/mL fibroblast growth factor-2 (FGF2, Peprotech) in the presence or absence of ASTR conditioned medium (diluted 1:1) or on top of ECM coatings. For other assays, cells were cultured for 2 days in SATO medium supplemented with PDGF-AA and FGF2, followed by differentiation upon growth factors withdrawal were and culturing for 6 days in SATO supplemented with 0.5% FBS. Cells were cultured in the presence or absence of ASTR conditioned medium (diluted 1:3) or on top of ECM coatings, after which differentiation, myelin membrane formation and metabolic activity were determined.
Spinal cord cultures. Myelinating spinal cords were generated from 15-days old Wistar rat embryos (Harlan) as described with minor modifications\textsuperscript{50}. Meninges were removed from the dissected spinal cord, and mechanical dissociation of the tissue was performed in Leibowitz L-15 medium (Sigma). Hereafter, the tissue was enzymatically digested with a mixture of trypsin (2.5%, Sigma) and liberase DH(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), 40 μg/mL DNase (Roche) and 3 mg/mL BSA fraction V in Leibowitz's L15 medium). After centrifugation for 7 minutes at 1000 rpm, cells were resuspended in plating medium consisting of 50% DMEM (1500 mg/L glucose, Gibco), 25% horse serum (Invitrogen), 25% HBSS with calcium and magnesium (Gibco), and 2 mM L-glutamine (Invitrogen). Cells were plated at a density of 200,000 cells/well in a 24-wells containing a 2-day-old confluent feeding layer of adult gmASTRs or wmASTRs (120,000 cells/24 well) in 500 µl plating medium. After cells were attached, 500 µL growth medium [DMEM (4500 mg/L glucose, Gibco) supplemented with 5 mg/mL holotransferin (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)] was added. Every 2-3 days, half of the medium was replaced with new growth medium. Insulin was omitted from growth medium after 12 days in culture and cultures were analyzed at 26-30 days in culture.

Gene expression and WGCNA
Cells were scraped in RNA protect (Qiagen) and mRNA was isolated using the RNeasy Plus Micro Kit (Qiagen) according to manufacturer’s instructions, subsequently the quality of the mRNA was assessed using a BioRad Experion Highsense RNA kit. All RIN-values were higher than 7.9. Gene expression analysis was performed using Illumina Truseq reagents. Data was processed using MOLGENIS compute. Quality control of the data was performed on the raw fastq files with fastQC (0.11.3). Next, HiSat (0.1.5) was used for alignment of the sequenced reads against the Rattus norvegicus genome (38.82) allowing 2 mismatches and the aligned data was sorted with samtools (I.2). Finally, HTSeq (0.6.1p1) was used to quantify the data using the parameters: --stranded=no and --mode=union. High and low expressed genes were distinguished using Data-adaptive flag method for RNA-sequencing (DAFS). Normalization and processing of the raw reads were performed with EdgeR (3.20.9).

To determine differentially expressed genes, an absolute logarithm of the fold change (LogFC) of more than 2 and an FDR<0.01 were used. Vulcano plot was made using the ggplot2 package. The DAFS filtered genes were used as input to generate a signed network (with a soft power of 10) with the topological overlap matrix (TOM) function (TOMsimilarity). Cutting of the hierarchical clustered TOM with the function cutreeDynamic with a minimal cluster size of 100 and cutHeight 0.99 was used to determine the different modules. Afterwards, similar gene expression modules were merged together with mergeCloseModules (cutHeight 0.25). In the end, userListEnrichment was used to identify possible traits of the identified modules where the grey lists were omitted, with enabling of the parameters: useBrainLists, useBloodAtlases, useStemCellLists, useBrainRegionMarkers, usePalazzoloWang, useImmunePathwayLists. For Gene Ontology and Gene-Concept Network visualization, Clusterprofiler with the enrichplot package were used. All analyses were performed in R.

Western blotting
ASTRs were then scraped in 500 µL of lysis buffer (% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, and protease inhibitor cocktail (Roche), and the total protein concentration was determined using a BSA assay according to manufacturer’s instructions. For detection of GFAP 20 μg of protein a 7.5% or 15% SDS-polyacrylamide gel was used. Gel electrophoresis was performed and proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore,) by wet transfer. PVDF membranes were blocked for one hour with Odyssey blocking buffer (Li-Cor Biosciences), and incubated overnight with primary antibodies against GFAP (anti-GFAP (polyclonal, 1:5000, Dako, Z033430) at 4°C. After washing with PBS containing 1% Tween-20, membranes were incubated with IRDye-conjugated secondary antibodies (Li-Cor Biosciences, Lincoln; 1:3000) for one hour. As loading control β-actin (monoclonal mouse anti-β-actin; 1:2000, Sigma, A5441) was used. The membranes were scanned using the Odyssey Imaging System (Li-Cor), and GFAP was calculated relative to the amount of β-actin with densitometry using FIJI ImageJ (NIH).
Immunocytochemistry

Primary cells. Live and fixed immunostainings were performed as described\textsuperscript{65-67}. Live cell immunolabelling with A2B5, an antibody against c-series gangliosides (kind gift of Dr. Thijs Lopez-Cardozo, Utrecht, the Netherlands), which are enriched at the surface of OPCs\textsuperscript{65,66} was performed at 4°C. Non-specific antibody binding was blocked with 4% BSA for 10 minutes and cells were incubated with A2B5 antibody (1:5 in 4% BSA) for 30 minutes. Cells were rinsed twice with PBS and incubated with appropriate Alexa-conjugated antibody (1:500, Millipore). After another two washes with PBS, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 minutes at room temperature and incubated for 15 minutes with 1µg/ml DAPI (Sigma) to counterstain nuclei. For staining of internal components, PFA-fixed cells were 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 either anti-KI67 (1µg/ml; Abcam, cat. no. ab15580) or anti-myelin basic protein (MBP, 1:250 in BSA; Serotec, cat. no. MCA409S) antibodies at room temperature. Cells were washed three times with PBS before the appropriate Alexa-conjugated antibodies (1:500, Millipore) were added together with 1µg/ml DAPI for 30 minutes at room temperature. After washing with PBS, cells were mounted with mounting medium (Dako). Cells were analyzed using a conventional immunofluorescence microscope (Leica DMI 6000 B) equipped with Leica Application Suite Advanced Fluorescence software. In each independent experiment, approximately 150–250 Ki67-positive or A2B5-positive cells (proliferation), MBP-positive cells of DAPI-stained cells (differentiation), or the number of MBP-positive cells that form myelin membranes (‘myelination’) were scored per condition.

Spinal cord cultures. Spinal cord cultures were fixed with 4%PFA for 30 minutes, followed by blocking and permeabilization with 0.1% Triton X-100 in 4% BSA in PBS for 45 minutes. Cells were washed three times with PBS and incubated with anti-MBP antibody (1:250) and anti-neurofilament (NF, polyclonal chicken anti-neurofilament, 1:5000, EnCor Biotechnology Inc., 2796-7) for 90 minutes at room temperature. After washing twice with PBS, cultures were incubated with appropriate FITC- or TRITC-conjugated secondary antibodies (1:50, Jackson Immunolaboratories) combined with DAPI for 45 minutes at room temperature. Coverslips were mounted using mounting medium (Dako). Cultures were analyzed by confocal microscopy (SP8 AOBS Microscope, Leica Microsystems) using Leica Confocal 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\textsuperscript{328,412}. In each experiment, 5 images per coverslip and 2 coverslips per condition were analyzed.

Metabolic activity and cytotoxicity

Metabolic activity of OLGs in the presence of ACM was assessed by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma)-reduction, while cytotoxicity was analysed with a lactate dehydrogenase (LDH; Roche) assay. For the MTT-reduction assay, 500 µg/ml MTT was added to each well and left to incubate for 4 hours at 37°C. Cells were resuspended in dimethylsulfoxide and absorption was measured at 570nm. Data represent values relative to control. LDH assays were performed according to manufacturer’s instructions using the medium of the cells from the MTT-reduction assay and related to LDH in medium of lysed untreated cells.

Statistics

Data are expressed as mean ± standard error of the mean (SEM) for at least three independent experiments. When relative values of groups were compared to NCM (Fig. 2f), no ECM (Fig. 2f,h) control ACM (Figs. 4,5) or control ECM coatings (Figs. 4,5), statistical analysis was performed with a one-sample t-test by setting the untreated control values at 1 in each independent experiment. In all cases p-values of <0.05, <0.01, and <0.001 were considered significant and indicated with *, **, and ***, respectively. When values between two groups (Fig. 1b, Fig. 2f,h, wmACMs vs gmACM and wmECM vs gmECM) were compared, statistical significance was assessed using a paired two-sided t-test. Here, p-values of <0.05, <0.01, and <0.001 were considered significant and indicated with *, **, and ***, respectively. Statistics were performed using GraphPad Prism 6.0. In heatmaps of RNAseq data FDR-values of <0.05, <0.01, and <0.001 were considered significant and indicated with *, **, *** (Figs.1d, 2a, 3d, Suppl. Fig. 2).
Acknowledgments
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Supplementary information

Transcriptional heterogeneity between primary adult grey and white matter astrocytes underlie differences in modulation of *in vitro* myelination

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### Supplementary Table 1. Differentially expressed genes more abundantly expressed in wmASTRs

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**Chapter 4**

White matter astrocytes are less supportive for myelination...
### Supplementary Table 2. Differentially expressed genes more abundantly expressed in gmASTRs

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### Chapter 4

White matter astrocytes are less supportive for myelination Chapter 4
White matter astrocytes are less supportive for myelination

Supplementary Figure 1. Weighted gene co-expression network analysis of adult gmASTRs and wmASTRs. RNA from 6 biological replicate cultures of adult grey matter astrocytes (gmASTRs) and adult white matter astrocytes (wmASTRs) was subjected to total 3'-RNA sequencing and a weighted gene co-expression network analysis. (a) Cluster dendogram of genes modules containing genes that are co-expressed. Each color represents a different module and each module contains genes with similar expression patterns over all six sample sets. (b) Correlation of module eigengenes with experimental variables: region and sample. (c) The royalblue gene module is positively correlated with gmASTRs and negatively with wmASTRs. (d) The darkgrey gene module is positively correlated with wmASTRs and negatively with gmASTRs.
Supplementary Figure 2. Cholesterol biosynthesis genes are more abundantly expressed in grey matter astrocytes. RNA from 6 biological replicate cultures of adult grey matter astrocytes (gmASTRs) and adult white matter ASTRs (wmASTRs) is subjected to total 3'-RNA sequencing. Heatmap of identified sterol, steroid, secondary alcohol and cholesterol related gene ontology pathways derived from weighted gene co-expression network analysis (WGCNA). (Suppl. Fig. 1). Note that most cholesterol, steroid and sterol related genes are more abundantly expressed in gmASTRs. (*FDR<0.05, **FDR<0.01, ***FDR<0.001).