Gsta4 controls apoptosis of differentiating adult oligodendrocytes during homeostasis and remyelination via the mitochondria-associated Fas-Casp8-Bid-axis

Karl E. Carlström1✉, Keying Zhu1, Ewoud Ewing1, Inge E. Krabbendam1, Robert A. Harris1, Ana Mendanha Falcão2,3, Maja Jagodic1, Gonçalo Castelo-Branco2,4 & Fredrik Piehl1

Arrest of oligodendrocyte (OL) differentiation and remyelination following myelin damage in multiple sclerosis (MS) is associated with neurodegeneration and clinical worsening. We show that Glutathione S-transferase 4α (Gsta4) is highly expressed during adult OL differentiation and that Gsta4 loss impairs differentiation into myelinating OLS in vitro. In addition, we identify Gsta4 as a target of both dimethyl fumarate, an existing MS therapy, and clemastine fumarate, a candidate remyelinating agent in MS. Overexpression of Gsta4 reduces expression of Fas and activity of the mitochondria-associated Casp8-Bid-axis in adult oligodendrocyte precursor cells, leading to improved OL survival during differentiation. The Gsta4 effect on apoptosis during adult OL differentiation was corroborated in vivo in both lysolecithin-induced demyelination and experimental autoimmune encephalomyelitis models, where Casp8 activity was reduced in Gsta4-overexpressing OLS. Our results identify Gsta4 as an intrinsic regulator of OL differentiation, survival and remyelination, as well as a potential target for future reparative MS therapies.
Oligodendrocyte precursor cells (OPC) and oligodendrocytes (OL) are abundant throughout the central nervous system (CNS). They serve important roles in preserving the functionality and integrity of neuronal network connections, including providing metabolic support and enabling axonal signal transmission along myelinated fibers. OLs are affected directly or indirectly in many CNS diseases, of which Multiple Sclerosis (MS) is one of the most widely studied. Despite findings of proliferating OPCs around lesions, OLs largely fail to remyelinate axons leading to neurodegeneration.

Remyelination does occur in adulthood but the temporal interval within which the OLs receive crucial support in order to survive is restricted. As a consequence, as many as half of OLs undergo programmed cell death during pre-natal development and up to a third during the post-natal phase leading to neurodegeneration.

The transcription factor Nrf2 has been identified as the main transcription factor (bFGF) is essential in OPC in vitro expansion, as it promotes proliferation and blocks differentiation (Supplementary Fig. 1c). Gsta4 thus acts downstream of DMF and Clem-F to promote OL differentiation, suggesting an Nrf2-Gsta4-axis operating in the oligodendrocyte lineage.

Gsta4 leads to expression of genes involved in OL maturation. To study ex vivo effects of Gsta4 in the OL lineage we created a hemizygous rat (DA\textsuperscript{Gsta4}) over-expressing Gsta4 under a CAG promoter on a Dark Agouti (DA) background (Fig. 2a). This DA\textsuperscript{Gsta4} strain displayed approximately a two-fold higher levels of Gsta4 mRNA throughout the OL lineage compared to DA\textsuperscript{WT}, with the highest Gsta4 levels in both DA\textsuperscript{WT} and DA\textsuperscript{Gsta4} being observed in more mature cells (Fig. 2b). DA\textsuperscript{Gsta4} also displayed elevated Gsta4 mRNA levels in additional tissues, including brain, spinal cord and spleen (Supplementary Fig. 1g). From a physiological perspective, DA\textsuperscript{Gsta4} did not show obvious phenotypic characteristics in terms of behavior, general health, spontaneous tumors, weight, fertility or litter size as compared to wild type animals (DA\textsuperscript{WT}).

Initial characterization of DA\textsuperscript{Gsta4} was performed using mRNA microarray analysis of bulk tissue from adult (10–12 week old) brain cortex, corpus callosum and hippocampus. Consistent with our in vitro data (Fig. 1g) the transcripts involved in positive regulation inducing OL differentiation were significantly enriched in DA\textsuperscript{Gsta4} compared to DA\textsuperscript{WT}, as assessed by Gene Set Enrichment Analysis (GSEA) (Fig. 2c). To assess if this translated into differences in OL differentiation and maturation markers we determined the expression of Plp1 and myelin basic protein (Mbip) in primary oligodendrocyte cultures established from neonatal rats (Supplementary Fig. 1b). Basic fibroblast growth factor (bFGF) is essential in OPC in vitro expansion, as it promotes proliferation and blocks differentiation (Supplementary Fig. 1b). Upon withdrawal of bFGF, the DA\textsuperscript{WT} and DA\textsuperscript{Gsta4} OPC pools differentiated. However, DA\textsuperscript{Gsta4} expressed higher levels of Plp1 both in the presence of bFGF during extended in vitro expansion, and also in its absence upon differentiation (Fig. 2d).

Accordingly, there was a lower degree of proliferative (PDGFR\textsuperscript{α}/PDGFR\textsuperscript{α}+) DA\textsuperscript{Gsta4} OPCs compared to DA\textsuperscript{WT} OPCs in both presence or absence of bFGF (Fig. 2e). Nevertheless, while upon short in vitro expansion (Supplementary Fig. 1b) there was a similar decrease of proliferative DA\textsuperscript{Gsta4} OPCs upon differentiation (no bFGF) compared to DA\textsuperscript{WT}, there was slightly higher PDGFR\textsuperscript{α}/PDGFR\textsuperscript{α}+ in DA\textsuperscript{Gsta4} compared to DA\textsuperscript{WT} and decreased Plp1 when bFGF was present (Supplementary Fig. 1e, f). This suggests that long-term exposure of OPCs to bFGF might prime them to respond by triggering differentiation upon Gsta4 over-expression. To confirm that the effects on DA\textsuperscript{Gsta4} OPC differentiation upon extended in vitro expansion were dependent on Gsta4 we performed knock-down of Gsta4 using siRNAs. Indeed, Plp1 was dependent on Gsta4 (Fig. 2f). Moreover, the production of Plp protein during differentiation was decreased following addition of siRNA-Gsta4 compared to control siRNA.
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Fig. 1 DMF and Clem-F upregulate Gsta4 and promotes OL differentiation. a Transcription of Plp1 in primary OPC cultures following stimulation for 24 h with DMF in DMSO (10 μM) or DMSO control (n = 3). b Chemical structures of DMF, MMF, Clem-F and Clem-H. c OL stimulation with Clem-F and Clem-H (10 μg/mL) for 24 h (n = 4). d Nuclear activation of Nrf2 following stimulation for 24 h with DMSO Ctrl, DMF, Clem-H, and Clem-F dissolved in DMSO at 10 μM (scale bar 50 μm). Asterisk, comparisons to DMSO control. Number sign, comparison to Clem-H (n = 3). e Transcription of Gsta4 in primary OL cultures following stimulation for 48 h with DMF and Clem-F dissolved in DMSO (n = 3). f Transcription of Gsta4 in proliferative OPCs compared to differentiated OLs in vitro (n = 4). g Transcription of Plp1 in primary OL cultures following stimulation for 24 h with DMF and Clem-F dissolved in DMSO and lipofectamine-mediated knockdown of Gsta4 with siRNA-Gsta4 compared to scrambled sequence, siRNA-Neg (n = 4). Graphs show representative results from three (a, e) or two (d, g) independent experiments. All data are shown as mean and S.D. All statistical analyses were performed using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Fig. 2 OLs differentiate in a Gsta4-dependent manner. a Illustration of Dark Agouti (DA) rat over-expressing Gsta4 (DAGsta4). b In vivo transcription of Gsta4 across the OL lineage in adult DAGsta4 and DAWt (n = 4). c GSEA, analyzed using weighted enrichment statistics and ratio of classes for the metric as input parameters, for CNS bulk tissue (cortex, corpus callosum and hippocampus) from adult DAGsta4 and DAWt (n = 4). d Transcription of Plp1 following 48h differentiation of neonatal DAGsta4 or DAWt-derived OPCs in the presence or in absence of bFGF (n = 3). e In vitro proliferation of DAGsta4 and DAGsta4 OPCs for 72h in various concentration of Fgf (n = 4). f Transcription of Mbp and Plp1 following 48h differentiation of neonatal DAGsta4 or DAWt-derived OPCs in combination with Gsta4 knockdown with siRNA-Gsta4 or siRNA-Neg as a scrambled sequence (n = 4). g Immunocytochemistry of Plp protein expression following Gsta4 knockdown with siRNA-Gsta4 (n = 10). h Representative images at day 5, scale bar 50 μm. Graphs depict representative results from two (b, d-h) independent experiments. All data are presented as mean and S.D. All statistical analyses were performed using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
These observations strengthen the notion that Gsta4 regulates differentiation of rat OPCs while exerting context-dependent effects on their proliferation.

Gsta4 accelerates the passage of OPCs to OLs via Casp8-Bid. The effects of Gsta4 on the OL lineage were further evaluated in adult DA<sup>Wt</sup> and DA<sup>Gsta4</sup> rats as illustrated in Fig. 3a. In line with previous in vitro findings (Fig. 2) adult DA<sup>Gsta4</sup> had fewer mature OPCs, identified using IHC staining for PDGFR<sub>α</sub> (Fig. 3b).

Flow cytometric analysis of corpus callosum and grey matter regions (indicated in Fig. 3a, dotted line) corroborated these histological findings evident by decreased numbers of PDGFR<sub>α</sub> OPCs and pre-mature O4<sup>+</sup> OLs in DA<sup>Gsta4</sup> compared to DA<sup>Wt</sup>.
Fig. 3 Gsta4 promotes more efficient OL differentiation via Fas-Casp8-Bid. a Summary of experimental set-up. b Total number of PDGFRα+ OPCs per frame in corpus callosum of adult rats (n = 10–19). c Percentage of double-positive PDGFRα+Ki67+ OPCs of all PDGFRα+ OPCs in corpus callosum. d Representative images illustrating PDGFRα+ (empty arrows) and double-positive Ki67+ (filled arrows), scale-bar 10 μm. e Percentage of double-positive O4*EDU + of all O4 + and O1*EDU + of all O1 + after ten days of EdU administration (n = 5) analyzed using flow cytometry. f Percentage of double-positive O4*EDU + of all O4 + and O1*EDU + of all O1 + 6 weeks following EdU administration (n = 7 + 5). g Total number of CC1+ OLs per frame in corpus callosum (n = 5). h Top IPA Network from RNA-seq on O4* corpus callosum OLs (n = 5). i GSEA and heat map of transcripts promoting Fas/Casp8 signaling from RNA-seq on O4* corpus callosum OLs. j Heat-map based on transcript levels during OL maturation20. k Number of branched DA+ and DA+O4* OLs per image frame captured with live imaging during 36h of differentiation. The Fas inhibitor C75 was added after 18 h. l Representative images after 24 h. Overlay with contrast and CellROX staining (red), scale-bar 30 μm. m Transcription of Pipi at 36 h (n = 4). n Percentage of ex vivo Casp8 activity in O4+ and O1+ OLs from DA(Wt) (n = 10) and DA+Gsta4 (n = 7). o Quantification of active Casp8 derived from mitochondria of DA(Wt) and DA+Gsta4 OLs differentiated for 48h, without (n = 3) or with (n = 8) siRNA. p Quantification of Bid derived from mitochondria DA(Wt) and DA+Gsta4 OLs differentiated for 48h, without (n = 3) or with (n = 4) siRNA. Graphs show representative results from two (e, f, k-p) or one (b-d, g) independent experiment(s). All graphs depict mean and S.D., graph b-d shows box and whiskers indicating values outside 5–95 percentile. All statistical analyses were performed using one-way ANOVA apart from k, analyzed with two-way ANOVA. P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Gsta4 regulates mitochondrial 4-HNE load in OLs. 4-HNE is the only known substrate for Gsta4 enzymatic activity54. In addition, Gsta4 facilitates the extracellular transportation of 4-HNE via conjugation to glutathione (GSH), whereas additional scavenging enzymes show little overlap with Gsta4 function. Intracellular 4-HNE is thus a rate-limiting molecule for several cellular processes, interfering with normal function, protein aggregation and protein degradation55,56. We next investigated whether Gsta4 overexpression could work as a model for decrease in intracellular 4-HNE load, resulting in fewer biomolecules being covalently modified by 4-HNE. Indeed, staining for 4-HNE in the corpus callosum revealed the DA+Gsta4 strain to have a reduced load of 4-HNE compared to DA(Wt) (Fig. 4a, Supplementary Figure 2a). To identify mechanisms directly affected by Gsta4/4-HNE, we analyzed RNA-seq data of corpus callosum-derived O4+ OLs to identify crucial molecules involved in these processes, using the following criteria: (1) significant differential expression (P ≤ 0.001) between DA(Wt) and DA+Gsta4 (Supplementary Data 2); (2) the potential of the corresponding peptide to be modified by 4-HNE, as analyzed using high performance liquid chromatography screening57,58 (Supplementary Data 3). There were 1042 upregulated mRNAs in DA+Gsta4 OLs compared to in DA(Wt) in the naïve state, while only 105 mRNAs were downregulated (Fig. 4b left). Of these, 26 of these transcripts encoded proteins that are 4-HNE modified (Fig. 4b green). Transcripts elevated in DA+Gsta4 included Achoy and Mit2a, involved in processes of small carbon units during oxidative stress (Fig. 4b right). As previously observed, Fas and Bid involved in Casp8-induced apoptosis were downregulated in DA+Gsta4 in relation to DA(Wt).

We also assessed involvement of Gsta4/4-HNE in induced demyelination/remyelination, by using the lysoceilithin (LPC) model. Specifically, we applied stereotactic administrations of 0.1% LPC into the corpus callosum and sorted O4+ OLs during the remyelination phase. Bulk RNA-seq analysis indicated that transcripts such as Mecc2 and Vda3c, involved in mitochondrial gene expression, were differentially regulated between DA(Wt) and DA+Gsta4 (Fig. 4c down). Furthermore, the subcellular distributions of transcripts of potentially 4-HNE modified proteins (Fig. 4b, c green) suggested an induction of transcripts coding for proteins localized to the mitochondria during remyelination compared to during naïve conditions (Fig. 4d and Supplementary Figure 2b).

As mitochondrial stress and subsequent dysfunction is a typical outcome following high intracellular 4-HNE load30, we quantified (Supplementary Fig. 1h). Despite a smaller OPC pool in the DA+Gsta4, a similar number of double-positive PDGFRα+Ki67+ was observed in the corpus callosum (Fig. 3d and Supplementary Fig. 1i) suggesting a compensatory mechanism for OPC loss, possibly involving bFGF (Supplementary Fig. 1e). We also analyzed if Gsta4 regulated adult OPC differentiation by assessing EdU levels in O4+ and O1+ OLs following administration of EdU in drinking water for 10 days and 6 weeks after a single injection of EdU. The proportions of both double-positive O4+EdU+ and O1+EdU+ of all O4+ or O1+ were higher in DA+Gsta4 at both 10 days and 6 weeks (Fig. 3e, f), suggesting a faster differentiation rate in DA+Gsta4. However, the total number of CC1+ OLs in the adult corpus callosum was not different between DA+Gsta4 and DA(Wt) (Fig. 3g and Supplementary Fig. 1h).

While a compensation between proliferation and differentiation rates might contribute to the homeostatic levels of OLs in DA+Gsta4, this finding suggests that other cellular mechanisms might also be involved. To identify pathways contributing to the observed phenotype, O4+ OLs were sorted out from micro-dissected adult DA(Wt) (n = 5) and DA+Gsta4 (n = 5) corpus callosum followed by RNA-seq (Fig. 3a). Ingenuity Pathway Analysis (IPA) Network data suggested top networks to be implicated in cell survival and death (Myecn, Egfr, Fas) as well as carbohydrate and lipid metabolism (Bicic1, Pid, Slc25a14) (Fig. 3h, Supplementary Data 1). GSEA of the RNA from the sorted corpus callosum O4+ OLs also revealed lower expression of positive regulators of Fas and Caspase-8 (Casp8) signaling in DA+Gsta4 (Fig. 3i). A heat-map summarizing the levels of these regulators along the OL lineage also indicated that they were enriched in early myelin forming OLs (MPOL1) (Fig. 3i), indicating a role in the early stages of differentiation/myelination.

In order to address how Fas regulates OL differentiation the Fas antagonist C75 was added to DA(Wt) and DA+Gsta4 OPC cultures during differentiation. Under baseline conditions DA+Gsta4 OLs displayed more branches from the soma as compared to DA(Wt) OLs. DA+Gsta4 branching and Pipi1 expression were significantly increased upon addition of C75 (Fig. 3k-m). Fas signaling thus negatively regulates OL differentiation. When assessing intracellular mechanisms downstream of Fas by flow cytometry, Casp8 activity ex vivo was indeed lower in O1+ DA+Gsta4 as compared to in DA(Wt) O1+ OLs (Fig. 3n), while no differences were observed in O4+ OLs. Casp8 can either activate BH3 interacting-domain death agonist (Bid) or Caspase-3 (Casp3) via catalytic cleavage53. Upon cleavage of Bid, both Bid and active Casp8 are associated with the mitochondrial outer membrane53. We thus quantified the levels of active Casp8 and Bid in isolated mitochondria from differentiated OLs. We observed a decrease in active Casp8 and mitochondrial levels of Bid in DA+Gsta4 (Fig. 3o, p), in accordance with the ex vivo findings of reduced Casp8 activity in DA+Gsta4 OLs (Fig. 3n). In addition, knock-down of Gsta4 in differentiated OLs had the opposite effect, with an increase in mitochondrial levels of Casp8 and Bid (Fig. 3o, p). Taken together this indicates that Gsta4 reduces Fas-Casp8 signaling in differentiating OLs, which might regulate not only their survival but also their differentiation.
mitochondrial 4-HNE load by isolating mitochondria from OLs differentiated for 48h. DAGsta4 OLs had a reduced mitochondrial 4-HNE load compared to DA^Wt and the positive control BuOOH (Fig. 4e and Supplementary Fig. 2c, d), a potent inducer of lipid peroxidation (LPO) generating 4-HNE. Mitochondrial stress was measured using MitoSOX ex vivo in O4^+ and O1^+ OLs and in microglia/astrocytes. OLs had a higher MitoSOX signal compared to glial cells but DAGsta4 OLs had a reduced MitoSOX signal compared to DA^Wt (Fig. 4f). This was then increased upon addition of siRNA-Gsta4 in vitro (Fig. 4g). Furthermore, mitochondrial 4-HNE load showed a positive correlation with activated Casp8, suggesting that the beneficial effects of Gsta4 on OL apoptosis involves mitochondrial 4-HNE load (Fig. 4h). Taken together our data indicate that Gsta4 over-expression...
Fig. 4 Gsta4 regulates mitochondrial stress and intracellular 4-HNE load in OLs. a Staining for 4-HNE in corpus callosum of naïve adult DAWt and DAgsta4 (n = 3), scale-bar 10 μm. b Significant transcripts (gray) from RNA-seq of corpus callosum-derived O4+ OLs in naïve conditions. Transcripts corresponding to proteins potentially modified by 4-HNE (green)57,58. Heat-map indicates up (red) and down (blue) transcripts. c Significant transcripts (gray) from RNA-seq of corpus callosum-derived O4+ OLs during remyelination. d Subcellular distribution of difference in green versus gray transcripts in naïve conditions and during remyelination. e Mitochondrial 4-HNE load from differentiated DAWt and DAgsta4 OLs in culture and in vivo immunohistochemistry (IHC) with MitosOX in spinal cord and corpus callosum (Fig. 4i and Supplementary Fig. 3a). f Flow cytometric assessment of mitochondrial integrity with MitosOX in differentiated OLs following application of Gsta4 knockout with siRNA-Gsta4 (n = 8). g Flow cytometric assessment of mitochondrial integrity with MitoSOX in differentiated OLs following application of Gsta4 knockdown with siRNA-Gsta4 (n = 8). h Correlation between mitochondrial 4-HNE load and levels of active Casp8 associated to the mitochondria (n = 16). I Schematic illustration of Gsta4-mediated 4-HNE transport and possible binding sites for 4-HNE along the Fas-Casp8 pathway. Graphs depict representative results from two (f, h), three (e, g) independent experiments. Data in e–g are shown as mean and S.D. All statistical analyses were performed using two-tailed Student’s t test apart from h analyzed with two-tailed Pearson’s r test. *P < 0.05, **P < 0.01.

increased remyelination through enhanced OL differentiation. To address if Gsta4 contributes to repair and remyelination in the context of demyelination, we analyzed DAgsta4 rats upon induction of LPC-mediated demyelination/remyelination (Fig. 5a and Supplementary Fig. 3a). While Luxol fast blue (LFB) staining initially revealed a similar degree of demyelination between the two strains, examination at later time-points revealed a more efficient remyelination in DAgsta4 compared to in DAWt (Fig. 5b). The difference between strains was already evident at 72 h and persisted until 15 days post injection, a time point when DAgsta4 animals displayed minimal visible demyelinating damage, whereas such damage was still readily evident in the DAWt strain. No difference in corpus callosum size was evident between DAWt and DAgsta4 (Supplementary Fig. 3b). Since incomplete scavenging of myelin debris is known to limit OPC differentiation and remyelination, the possible contribution from Gsta4 in microglia during phagocytosis was assessed. pHrodo-labeled myelin was added to primary microglia cultures but no Gsta4 in microglia during phagocytosis was assessed. pHrodo-stained myelin was added to primary microglia cultures but no Gsta4 signal in microglia was observed (Supplementary Fig. 1c, 3c). Moreover, transcription of pro-inflammatory hallmark genes did not vary in bulk corpus callosum tissue at any time-point following LPC exposure (Supplementary Fig. 3d). Neither, was any strain difference in numbers of immune cells or astrocytes following LPC administration were observed (Supplementary Fig. 3e, h–j). Collectively, this indicates that Gsta4 over-expression had no or only a negligible effect on the inflammatory response in the LPC model. To evaluate remyelination of axons, corpus callosum in naïve state and 10 days following LPC injection were evaluated using transmission electron microscopy (TEM) (Fig. 5c and Supplementary Fig. 3f). A two-fold increase in the percentage of myelinated axons was recorded in DAgsta4 compared to DAWt during remyelination (Fig. 5c, d), where a difference in myelin thickness was also primarily evident in thinner axons (Supplementary Fig. 3f, g). The g-ratio describes the diameter of the transected axon in relation to the outer diameter of the surrounding myelin assessed using TEM. Using a software that randomly selects axons for analysis from every image frame29, DAWt axons were determined to be less myelinated after demyelination compared to DAgsta4 axons (Fig. 5c, e and Supplementary Fig. 3f). A small but significant difference was also evident between the strains during remyelization, as DAgsta4 axons had thinner myelin. We have previously reported that myelin sheath thickness correlates with axonal mitochondria size and that this is an indicator of advanced remyelination30, and we herein observed that at day 10 DAgsta4 animals re-established this correlation observed during homeostasis while DAWt did not (Fig. 5f). DAgsta4 also generated a larger number of O1+ and CC1+ cells compared to DAWt 10 days after LPC (Fig. 5g, h and Supplementary Fig. 3h, k). The total number of O4+ OLs and PDGFRA+ was decreased in DAgsta4 compared to DAWt following LPC, supporting the notion of a faster remyelinating process in DAgsta4. To validate this, animals were fed EdU in their drinking water for 10 days (Fig. 5j) showing a higher number of DAgsta4 OPCs differentiated into myelin producing O1+ OLs or CC1+ OLs compared to DAWt (Fig. 5j–l).

Gsta4 reduces demyelinated lesions and ameliorates EAE. To address the implications of Gsta4 over-expression in a model of autoimmune demyelination we induced experimental autoimmune encephalomyelitis (EAE) in DAWt and DAgsta4 rats by sub-cutaneous injection of recombinant myelin oligodendrocyte glycoprotein (MOG) in adjuvant. There were no differences in terms of disease incidence or day-of-onset between the groups (Supplementary Fig. 4a), suggesting that the triggering of an autoimmune response did not differ between the strains. However, after the onset of disease animals over-expressing Gsta4 displayed a milder disease course (Fig. 6a), with reduced disease duration and cumulative disability scores compared to DAWt (Fig. 6b). As observed in corpus callosum O4+ OLs during homeostasis (Fig. 3i), mRNAs of mediators partaking in the Fas-Casp8-Bid-axis were also differentially regulated in the spinal cord O4+ OLs comparing DAWt and DAgsta4 (Supplementary Fig. 4c). Since incomplete scavenging of myelin debris is known to limit OPC differentiation and remyelination, the possible contribution from Gsta4 in microglia during phagocytosis was assessed. pHrodo-labeled myelin was added to primary microglia cultures but no Gsta4 signal in microglia was observed (Supplementary Fig. 1c, 3c). Moreover, transcription of pro-inflammatory hallmark genes did not vary in bulk corpus callosum tissue at any time-point following LPC exposure (Supplementary Fig. 3d). Neither, was any strain difference in numbers of immune cells or astrocytes following LPC administration were observed (Supplementary Fig. 3e, h–j). Collectively, this indicates that Gsta4 over-expression had no or only a negligible effect on the inflammatory response in the LPC model. To evaluate remyelination of axons, corpus callosum in naïve state and 10 days following LPC injection were evaluated using transmission electron microscopy (TEM) (Fig. 5c and Supplementary Fig. 3f). A two-fold increase in the percentage of myelinated axons was recorded in DAgsta4 compared to DAWt during remyelination (Fig. 5c, d), where a difference in myelin thickness was also primarily evident in thinner axons (Supplementary Fig. 3f, g). The g-ratio describes the diameter of the transected axon in relation to the outer diameter of the surrounding myelin assessed using TEM. Using a software that randomly selects axons for analysis from every image frame29, DAWt axons were determined to be less myelinated after demyelination compared to DAgsta4 axons (Fig. 5c, e and Supplementary Fig. 3f). A small but significant difference was also evident between the strains during remyelization, as DAgsta4 axons had thinner myelin. We have previously reported that myelin sheath thickness correlates with axonal mitochondria size and that this is an indicator of advanced remyelination30, and we herein observed that at day 10 DAgsta4 animals re-established this correlation observed during homeostasis while DAWt did not (Fig. 5f). DAgsta4 also generated a larger number of O1+ and CC1+ cells compared to DAWt 10 days after LPC (Fig. 5g, h and Supplementary Fig. 3h, k). The total number of O4+ OLs and PDGFRA+ was decreased in DAgsta4 compared to DAWt following LPC, supporting the notion of a faster remyelinating process in DAgsta4. To validate this, animals were fed EdU in their drinking water for 10 days (Fig. 5j) showing a higher number of DAgsta4 OPCs differentiated into myelin producing O1+ OLs or CC1+ OLs compared to DAWt (Fig. 5j–l).
Fig. 5 Gsta4 stimulates axonal remyelination through more efficient OL differentiation. a Corpus callosum sections stained with LFB for evaluation of demyelination, scale-bar 1 mm. b LFB staining following administration of 0.1% LPC (naïve/24h/72h; n = 6, 15d; n = 5). c Representative TEM images of corpus callosum, myelinated axons pseudo-colored in blue, scale-bar 2 μm and 400 nm. d Percentage of myelinated axons in naïve DAWt and DAGsta4 and during remyelination (n = 3). e g-ratio in naïve DAWt and DAGsta4 and during remyelination (n = 3). f Correlation between g-ratio and axonal mitochondrial size during homeostasis (left) and during remyelination (right) (n = 30). g Illustration of experimental set-up. h Flow cytometric analysis of total number of PDGFRα+ OPCs (green) (n = 5) and O1+ and O1+ OLs (grey/blue) (n = 5 + 6). i Immunohistochemical validation of CC1+ OLs in corpus callosum (n = 5). j Representative corpus callosum images 10 days following LPC, scale-bar 10 μm. k Number of PDGFRα+, O4+, or O1+ double-positive for EdU+ 10 days after continuous EdU administration. l Number of CC1+ double-positive for EdU+ 10 days after EdU administration. All graphs show mean and S.D., apart from i, l showing box and whiskers, indicating values outside 5–95 percentile. All statistical analyses were performed with one-way ANOVA, apart from i, l performed with Students’ two-tailed unpaired t test and f analyzed with two-tailed Pearson’s r test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
over-expression contributes to a lowering of mitochondrial stress and activation of Casp8, which in turn results in less extensive demyelination and/or improved remyelination, as well as ameliorated clinical symptoms.

**Discussion**

There has been some progress in the identification of signaling pathways involved in OL maturation. These include the Wnt pathway, interfering with remyelination in the mammalian CNS\(^43\) and retinoid acid receptor RXR-\(\gamma\) signaling that enhances OPC differentiation and remyelination\(^62\). A large scale high-throughput screening of compounds with remyelinating effects identified a cluster of anti-muscarinic formulations, including clemastine, that enhance OPC differentiation and remyelination in vitro\(^43\). Clemastine is a first generation anti-muscarinic compound has been identified as a promising candidate for remyelinating therapy in clinical settings\(^30,43,44\). We herein demonstrate that both Clem-F and DMF promote OL differentiation in a Gsta4-dependent manner and that this involves Nrf2. The anti-muscarinic formulation contains fumarate, which represents the bioactive molecule following DMF metabolism. This was the basis for addressing the possible overlapping features between Clem-F and DMF to activate Nrf2 in OLs. We show that Clem-F activates Nrf2 and also Gsta4 to similar extent as does DMF, likely via the fumarate moiety. Importantly, we also observed a significant reduction in OL differentiation following DMF and Clem-F Application upon siRNA against Gsta4, which illustrates the importance of scavenging enzymes and cellular protection during differentiation. Collectively, our findings suggest that the
myelination-promoting effects of both DMF and Clem-F involve the Gsta4/4-HNE pathway and that Gsta4 is essential for mediating their beneficial effect on myelination. Further studies are needed to explore the role of Gsta4 in a clinical context and if manipulation of Gsta4 activity can be used to improve therapeutic outcomes.

Despite detailed knowledge of the molecular heterogeneity between juvenile and adult OL maturation, there are still large gaps in our understanding of what prevents OLs from remyelinating a damaged adult CNS. Our findings suggest that newly formed/myelinating-forming OLs represent a particularly sensitive state during adult OL differentiation. This may represent an important rate-limiting step during maturation that could benefit from enhanced Gsta4-activity.

Our approach to identify pathways directly affected by Gsta4 over-expression and sequent reduction of mitochondrial 4-HNE load consisted in overlapping two independent databases of 4-HNE modified proteins, together with assessing their transcription in the OL lineage. The transcripts for Fas and BID were the only significantly downregulated in DA^{Gsta4} OLs compared to DA^{WT}. Both Fas and Bid protein have been described to be 4-HNE-modified. Fas can be modified by 4-HNE at three different sites, including one modification within its active site. We confirmed that lowering of Fas (activity) was beneficial to OLs since addition of Fas inhibitor C75 led to an increased differentiation in DA^{WT} but not in DA^{Gsta4} OLs. The relevance of this pathway was further underlined by reduced activity of Casp8 in DA^{Gsta4} OLs ex vivo. Both mitochondrial Casp8 and Bid were negatively correlated with Gsta4 expression, further proving mitochondria as a relevant location for the intersection between 4-HNE and additional pathways, including the Fas-Casp8-Bid-axis. We thus speculate that the decline in 4-HNE load could be of importance for specific maturation checkpoints during OL differentiation and enables more efficient intracellular processes generally.

Interestingly, Gsta4 also affected OPC proliferation both in cultures derived from neonatal rats in vitro and adult animals in vivo. This effect appears to be context-dependent and may also involve compensatory mechanism to tackle the reduction of OPCs, possibly involving bFGF.

Collectively, our findings suggest that Gsta4 and physiological levels of 4-HNE regulate differentiation of OLs and that this has clinical relevance in in vivo models of inflammation- or toxin-induced demyelination. Tracking of maturing OLs by EdU incorporation after focal demyelination supports the notion that DA^{Gsta4} OLs more rapidly advance through intermediate states of myelination in order to enclose damaged corpus callosum axons. The corpus callosum of DA^{Gsta4} is nearly completely restored 15 days following focal demyelination whereas DA^{WT} still have tangible lesions. This was also supported by TEM analysis in which we observed that DA^{Gsta4} had double the number of myelinated axons 10 days following demyelination. This is despite a similar initial demyelination in both strains. Interestingly, DA^{Gsta4} axons in naïve adult animals appeared to have a slightly thinner myelin sheath. The OPC pool is also reduced in DA^{Gsta4}, an explanation for both of these observations possibly being that upon excess of extracellular 4-HNE, transportation cellular processes will operate more efficiently. Thus a smaller pool of precursor cells or thinner myelin is then sufficient to fulfill the function of metabolic support and axonal transmission.

Finally, we observed that Gsta4 over-expression leads to behavioral amelioration upon induction of MS-like disease (EAE). Several reports have suggested differences between species regarding adult remyelination or that precisely timed input is needed, however EAE is a widely accepted model for MS-like disease in rodents. Gsta4 did not affect time of onset or incidence but improved disease duration and maximum disability scores, also upon depletion of Gsta4 over-expression from the immune system. This could further be linked to mitochondrial dysfunction and Casp8, which were both reduced in DA^{Gsta4} OLs. EAE in non-chimeric animals displayed a difference in mortality, since this was not present in the chimeric EAE set-ups, so one cannot exclude Gsta4-dependent contributions from bone marrow-derived cells in non-chimeric EAE setting.

Facilitating and increasing remyelination has been a long-standing therapeutic goal in chronic demyelinating diseases such as MS. Until very recently successful examples, especially in clinical settings, have been lacking. Our study places Gsta4 as a key regulator of OL differentiation and remyelination in the context of cellular stress, ageing and demyelination in an experimental setting.

Methods

Study design. Our research object was to investigate the possible beneficial role of Gsta4, activated by DMF and Clem-F, in remyelination and in vivo OL differentiation in MS disease-like models. The DA^{Gsta4} rat was thus designed and used to over-express Gsta4 both in primary cultures and in vivo models in which siRNA towards Gsta4 was used as a knock-down tool. All primary OL cultures were established from neonatal rats (3–7 days) (Supplementary Fig. 1b) whereas adult animals at an age of 8–10 weeks were used for bone marrow transplantation and later EAE after an additional 8–10 weeks. For remaining experiments adult animals at an age of 10–12 weeks were used. Throughout this study littermate controls were used and all cages contained animals with different genotypes/treatment. OL differentiation was evaluated in the corpus callosum using PDGFRA+ to label OPC and CC1+ to label post-OPCs. Upon handling and evaluation with IHC and IFB the investigator was unaware of the genotype of the animals/sections.

Animals and genotyping. The rat DA^{Gsta4} strain over-expressing Gsta4 under a CAG-promoter was purchased from Taconic Bioscience. DA^{GFP} animals were kindly provided by Holger Reichardt’s laboratory. Animals were genotyped using PCR and vector-specific primers (F: ATCCACTTGTGCTTCGCCG, R: TTTCAAACATCGGAGTAACG), and primers toward G79bF (F: GACTCTGTGGCCTGCTCATCC, R: TTCAGCAAGACATGGGGAC) as wild-type allotype control. Animals were bred in the animal facility at Karolinska University Hospital (Stockholm, Sweden) in a pathogen free and climate-controlled environment with regulated 12 h light/dark cycles. All experiments were approved and performed in accordance with Swedish National Board of Laboratory Animals and the European Community Council Directive (86/609/EEC) under the permits N275-15 and N244-13.

Bone marrow transplantation and EAE. DA^{GFP} male animals were sacrificed with carbon dioxide and femurs was removed and rinsed with cold PBS in order to harvest hematopoietic stem cells (HSC). These were washed, passed through a 40μm strainer and erythrocytes were lysed using ACK buffer (Thermo Fisher Scientific, Waltham, MA) following the manufacturer’s protocol. At an age of 8–10 weeks, male BM recipients were subjected to lethal irradiation with 2×50 Gy and subsequently given 10×10^6 cells/in 300 μL PBS i.v. Successful transfer of the bone marrow graft was assessed by analyzing GFP+ cells in the peripheral blood 8 weeks after transplantation using flow cytometry. EAE in bone marrow transplanted male animals was induced in anesthetized 8–10-week-old animals after irradiation by subcutaneous injection at the tail base of 10 μg of recombinant MB CGM together with incomplete Freud's adjuvant 1:1 (Sigma, St Louis, MO). EAE was induced in 10–12-weeks-old female animals using the same protocol using 10 μg MOG. The animals were monitored daily from onset of disease and scored according to the following scheme: 0 = no clinical score, 1 = reduced tail tonus, 2 = hind leg paraparesis or hemiparesis, 3 = hind leg paralysis or hemiparesis, 4 = tetraplegia or moribund, 5 = death. Rats were sacrificed if they showed severe balance disturbance or abnormal behavior, >25% weight loss or prolonged tetraplegia. Removed animals were scored 4 throughout (Supplementary Fig. 4a). EAE symptoms were repeated twice (EAE1 = n = 17 + 26; EAE2 = n = 16 + 18) and twice in bone marrow transplanted animals (BM-EAE1 n = 12 + 16; BM-EAE2 n = 10 + 14) with comparable and significant results.

Lysoclethrin injections. Male rats animals 10–12 weeks were anaesthetized with isoflurane and subjected to a stereotactic injection into the corpus callosum (AP: 1.0 mm; L:1.2; DV: 2.2) (Supplementary Fig. 3a) with 2.5 μL 0.1% lysoclethrin (Sigma, St Louis, MO) in PBS over 5 min using a Hamilton syringe 17106RN (Scientific, St Louis, MO). Animals were kept for 24 h. Animals were kept for 24 h and subsequently killed; brains were harvested and evaluated with flow cytometry, qPCR, TEM or immunohistochemistry. For in vivo differentiation, 5-ethyl-2′-deoxyuridine (EdU; Thermo Fisher Scientific, Cambridge, MA) was injected intraperitoneally and the investigator was unaware of the genotype of the animals/sections.
Waltham, MA) was either injected i.p. 25 mg/kg, or added to the drinking water (0.2 mg/mL) for a period of 10 days following LPC injection or in naive animals for the same time period. Injected animals were kept for 6 weeks.

**Primary OPC cell cultures.** For rat primary OPC cell preparation brains were collected from neonatal rats (3–7 days). The tissue was incubated in 1 mL Accutase and 4 μL DNase/mL (Sigma, St Louis, MO) at +37 °C and passed through a fire-polished Pasteur pipette every 10 min, this being repeated three times with decreasing pipette gauge. The homogenate was directly labeled and isolated using magnetic anti-A2B5 beads (130-093-388) (Milteny Biotec, Bergisch Gladbach, Germany) instead of Percoll layering as for flow cytometry. The study was approved according to the institutional guidelines. Microglia were prepared by layering microglial cultures with 10 μM EdU for 72 h in media containing Neurobrew, N2 and PDGF-BB (Thermo Fisher Scientific, Waltham, MA) and analyzed using ImageJ default plugins for co-localized applications were acquired with a DMI6000 microscope (Leica Biosystems, Wetzlar, Germany) and analyzed using ImageJ default plugins for co-localized applications were acquired with a DMI6000 microscope (Leica Biosystems, Wetzlar, Germany).

Microglia were cultured from neonatal rats (3–7 days) in DMEM/F-12 media with 10% FCS (Sigma, St Louis, MO). Microglia were isolated using anti-A2B5 beads (130-093-388) (Milteny Biotec, Bergisch Gladbach, Germany) due to manufacturer’s exact instructions. The homogenate was filtered through a 70 μm strainer and myelin was removed by a 37% Percoll layer spun at 800 g for 10 min at +4 °C without any brake. The pellet was resuspended to a single cell suspension and stained for EdU Click-IT (Thermo Fisher Scientific, Waltham, MA) according to manufacturer’s instructions and then co-stained with anti-CD45 (103-095-825) (BD, Franklin Lakes, NJ). Dead cells were excluded using near IR Live/Dead (Thermo Fisher Scientific, Waltham, MA). Cell sorting was measured by ex vivo incubation of single cell suspension, prior to Ab-staining described above. The single cell suspension was incubated for 1 h at +37 °C in 300 μL DMEM/F12 media, supplemented with N2 and Neurobrew. FAM-LETD-FMK Caspase-8 reagent (Thermo Fisher Scientific, Waltham, MA) was added at the concentration described by the manufacturer and stained. Samples were analyzed using a 3-laser Beckman Coulter Gallios using Kaluza Software (Beckman Coulter, Brea, CA).

**Flow cytometry and Caspase-8 activity.** Animals were sacrificed with carbon dioxide and perfused with PBS followed by 4% formaldehyde and paraformaldehyde for 48 h followed by 3 days in 70% EtOH, snap frozen in isopentane, and kept at −80 °C. Sections were collected from neonatal rats (3–4) and DAWt (n = 4) animals, including cortex, hippocampus and corpus callosum was prepared for lyzing in Qiazol. Subsequent steps were performed similar to RNA extraction form sorted cells described above. Samples were analyzed using a RaGene-2_L1st array (Affymetrix, Santa Clara, CA) by the Array and Analysis facility at Uppsala University. GSEA was performed using http://www.genome-wide.com and gene set GO Process REGULATION and GO Process CYTE_DIFFERENTIATION and were calculated by GSEA with weighted enrichment statistics and ratio of classes for the metric as input parameters. Prediction of subcellular locations was generated based on Supplementary Data 2 and 3 using www.subcellbarcode.com.

**Histopathological analyses and immunohistochemistry.** Animals were sacrificed with carbon dioxide and perfused with PBS followed by 4% formaldehyde and paraformaldehyde for 48 h followed by 3 days in 70% EtOH, snap frozen in isopentane, and kept at −80 °C. Sections (12 μm) were prepared in a cryostat and kept at −20 °C. The EdU staining (Thermo Fisher Scientific, Waltham, MA) was done according to manufacturer’s instructions. For additional staining, sections were blocked in 3% normal goat serum (Sigma, St Louis, MO) for 1 h, repeatedly washed, fresh PBS and incubated with primary antibodies; anti-PDGFRα (ab85460) (1:100), anti-C3 (OP80) (1:100) (Millipore, Darmstadt, Germany), anti-Casp8 (1:200) (NB100-56116) (Novus Biologicals, Littleton, CO) or anti-HNE (1:50) (ab48506) (1:50) (Abcam, Cambridge, GB) overnight. All secondary antibodies were produced in goat and labeled with either Alexa Fluor 488, 594, Cy3 or Cy5 (Thermo Fisher Scientific, Waltham, MA).

**OPC proliferation.** Primary cultures of OPCs derived from neonatal rats were expanded and cultured at 0.000 cells/well in a 48-well plate. Cells were cultured with a 10 μM EdU for 72 h in media containing Neurobrew, N2 and PDGF-BB as described above. In addition, media was supplemented with full (1/1), half (1/2) of absent (0) of bFGF concentration referred to the concentration described above. Cells were harvested with Accutase, labeled with anti-PDGFRα (Milteny Biotec, Bergisch Gladbach, Germany). Dead cells were excluded using near IR Live/Dead (Thermo Fisher Scientific, Waltham, MA). Samples were analyzed with a 3-laser Beckman Coulter Gallios using Kaluza Software (Beckman Coulter, Brea, CA).

**Microglia phagocytosis.** For primary microglial cultures brains were collected from rats 10–12 weeks as described above. The cell pellet obtained after Percoll layering was plated in non-precoated culture wells for adherent cells and expanded for 4 days in DMEM/F-12 media with 10% FCS (Sigma, St Louis, MO). Microglia were isolated using anti-A2B5 beads (130-093-388) (Milteny Biotec, Bergisch Gladbach, Germany) due to manufacturer’s exact instructions. The homogenate was filtered through a 70 μm strainer and myelin was removed by a 37% Percoll layer spun at 800 g for 10 min at +4 °C without any brake. The pellet was resuspended to a single cell suspension and stained for EdU Click-IT (Thermo Fisher Scientific, Waltham, MA). Cell sorting was measured by ex vivo incubation of single cell suspension, prior to Ab-staining described above. The single cell suspension was incubated for 1 h at +37 °C in 300 μL DMEM/F12 media, supplemented with N2 and Neurobrew. FAM-LETD-FMK Caspase-8 reagent (Thermo Fisher Scientific, Waltham, MA) was added at the concentration described by the manufacturer and stained. Samples were analyzed using a 3-laser Beckman Coulter Gallios using Kaluza Software (Beckman Coulter, Brea, CA).

**Cell sorting, microarray, and RNA-seq.** Animals were sacrificed and perfused as described above, corpus callosum was dissected and prepared as samples for flow cytometry. The single cell suspension was labeled with magnetic anti-CD11b (130-049-601) and anti-Glial (130-095-825) beads at a dilution suggested by the manufacturer and incubated and separated using LS columns. The negative fraction was stained with anti-CD11b-04 beads (130-094-548) and isolated using LS columns. Reagents, and columns were provided by Milteny Biotec, Bergisch Gladbach, Germany. RNA was isolated using RNeasy Micro Kit (Qiagen, Venlo, Netherlands) according to the manufacturer’s instructions with minor modifications to the protocol. In brief, isolated cells were lysed in 500 μL Qiazol following sorting, 100 μL chloroform were added and samples spun at 2500 x g for 15 min at +4 °C in a Maxxtract column (Qiagen, Venlo, Netherlands). Mixed with 500 μL 70% EtOH and spun at 3000 x g for 30 s at +4 °C in a MiniElute column, washed with 350 μL RW1, on-column digestion for 15 min at room temperature, washed with 350 μL RW1, 500 μL RLT, followed by 500 μL 80% EtOH and spun for 2 min. RNA was dried in vacuum for 30 min and RNA was dissolved in 20 μL RNase-free water (Ambion, Life Technologies). RNA integrity was measured by ex vivo incubation of single cell suspension, prior to Ab-staining described above. The single cell suspension was incubated for 1 h at +37 °C in 300 μL DMEM/F12 media, supplemented with N2 and Neurobrew. FAM-LETD-FMK Caspase-8 reagent (Thermo Fisher Scientific, Waltham, MA) was added at the concentration described by the manufacturer and stained. Samples were analyzed using a 3-laser Beckman Coulter Gallios using Kaluza Software (Beckman Coulter, Brea, CA).

**Immunocytochemistry.** For immunocytochemistry cells were washed and fixed in 100 μL 4% PFA at +4 °C for 10 min, washed with PBS and incubated with 0.1% Triton X-100 for 5 min at +4 °C. For Pp-phenol staining was washed and stained with Cy3 (1:50) (Novus Biologicals, Littleton, CO) overnight, followed by anti-chicken-Cy3 (Thermo Fisher Scientific, Waltham, MA) and co-stained with CellMask and Hoechst (Thermo Fisher Scientific, Waltham, MA) for 5 min at +4 °C. Co-localization was also performed due to the manufacturer’s protocols using Puromycin (Sigma, St Louis, MO) as a negative control and anti-HNE (ab8506) (Abcam, Cambridge, GB) (1:50) and anti-Puff (PAS-54983) (Thermo Fisher Scientific, Waltham, MA) (1:200). Confocal images were for above described applications were acquired with a DMi6000 microscope (Leica Biosystems, Wetzlar, Germany) and analyzed using ImageJ default plugins for co-localization and staining intensity.

**OPC proliferation.** Primary cultures of OPCs derived from neonatal rats were expanded and cultured at 0.000 cells/well in a 48-well plate. Cells were cultured with 10 μM EdU for 72 h in media containing Neurobrew, N2 and PDGF-BB as described above. In addition, media was supplemented with full (1/1), half (1/2) of absent (0) of bFGF concentration referred to the concentration described above. Cells were harvested with Accutase, labeled with anti-PDGFRα (Milteny Biotec, Bergisch, Germany). Dead cells were excluded using near IR Live/Dead (Thermo Fisher Scientific, Waltham, MA). Samples were analyzed with a 3-laser Beckman Coulter Gallios using Kaluza Software (Beckman Coulter, Brea, CA).
Image overlay by measuring corpus callosum size and LFB intensity in black/white transposed images.

Transmission electron microscopy and analysis. For Transmission electron microscopy (TEM) evaluation of corpus callosum rats were perfused as described above using 2.5% glutaraldehyde, 1% paraformaldehyde in 0.1M PBS. Rinsed with 0.1PBS and post-fixed in 2% OsO₄ in 0.1M PBS at +4 °C for 2 h. Brains were dehydrated in 70%-OH for 30 min +4 °C, 95%-OH for 30 min +4 °C, 100%-OH 20 min, and critical point dried with liquid CO₂ at -70 °C. RT, LX-112/Acetone (1:2) 4h RT, LX-112/Acetone 1:1 overnight RT, LX-112/Acetone (2:1), overnight RT, LX-112 overnight RT. Embedding in LX-112 at +60 °C. Embedding and sectioning were performed and pictures taken by EMil, Clinical Research Center, Department of Laboratory Medicine, Karolinska Institutet, Huddinge, Sweden. Approximately 150 axons from three animals per condition were sub-sequentially analyzed randomly and blinded using ImageJ and G-ratio free plugin.

Mitochondria isolation and ELISA. Mitochondria was isolated from primary OL cultures using Mitochondria Isolation Kit for Cultured Cells (#89874) (Thermo Fisher Scientific, Waltham, MA) as per the manufacturer’s exact instructions. The lysed mitochondria were kept at −80 °C until analysis and diluted 1:1 in sample diluent. Proteins from whole primary OL cultures were obtained by lysing cells in RIPA buffer (Thermo Fisher Scientific, Waltham, MA) and sub sequential sonication after removal of media and repeated washes with PBS. The lysed cells were kept at −80 °C until analysis and diluted 1:1 in sample diluent. α-Tocopherol (50 µM) and tert-Butyl hydroperoxide (25 µM) (Sigma, St Louis, MO) were used as negative and positive controls and added for 2h to the cultures. The Casp8 ELISA (EKR1606) (Nordic Biosite, Sweden), Bid ELISA (NBP2-69968) (Novous Biologicals, Littleton, CO) and the competitive ELISA for detection of 4-HNE (CSB-EQ027232RA) (Cusabio Technology, Huston, TX) was performed according to the manufacturer’s exact instructions.

Quantitative real-time PCR. Total RNA was isolated from tissue or cells using QiAsol and RNeasy mini kit (Qiagen, Venlo, Netherlands) according to manufacturer’s instructions and with 15 min on-column DNase digestion (Qiagen, Venlo, Netherlands). cDNA was prepared with reverse transcriptase using iScript manufacturer’s instructions and with 15 min on-column DNase digestion (Qiagen, Venlo, Netherlands). 1:1 overnight RT, LX-112/Acetone (2:1), overnight RT, LX-112 overnight RT. Quantitative real-time PCR was performed due to the lack of commercially available primer sets. Primers for PCR were designed as follows:

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Author contributions

K.E.C. and F.P. designed experiments with input from A.M.F. and G.C.B. K.E.C. performed and analyzed all experiments, with input from A.M.F. and G.C.B. K.E.C., K.Z., and R.A.H. performed and analyzed qPCR experiments and I.H.C. K.E.C. and I.K. performed and analyzed EAE experiments. K.E.C., E.E., and M.J. analyzed RNA-seq. K.E.C. and F.P. wrote the manuscript with input from co-authors.

Competing interests

The authors declare no competing interests

Additional information

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Correspondence and requests for materials should be addressed to K.E.C.

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