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Metabolic gene expression changes in astrocytes in Multiple Sclerosis cerebral cortex are indicative of immune-mediated signaling

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ABSTRACT

Emerging as an important correlate of neurological dysfunction in Multiple Sclerosis (MS), extended focal and diffuse gray matter abnormalities have been found and linked to clinical manifestations such as seizures, fatigue and cognitive dysfunction. To investigate possible underlying mechanisms we analyzed the molecular alterations in histopathological normal appearing cortical gray matter (NAGM) in MS. By performing a differential gene expression analysis of NAGM of control and MS cases we identified reduced transcription of astrocyte specific genes involved in the astrocyte–neuron lactate shuttle (ANLS) and the glutamate–glutamine cycle (GGC). Additional quantitative immunohistochemical analysis demonstrating a CX43 loss in MS NAGM confirmed a crucial involvement of astrocytes and emphasizes their importance in MS pathogenesis. Concurrently, a Toll-like/IL-1β signaling expression signature was detected in MS NAGM, indicating that immune-related signaling might be responsible for the downregulation of ANLS and GGC gene expression in MS NAGM. Indeed, challenging astrocytes with immune stimuli such as IL-1β and LPS reduced their ANLS and GGC gene expression *in vitro*. The detected upregulation of *IL1B* in MS NAGM suggests inflammasome priming. For this reason, astrocyte cultures were treated with ATP and ATP/LPS as for inflammasome activation. This treatment led to a reduction of ANLS and GGC gene expression in a comparable manner. To investigate potential sources for ANLS and GGC downregulation in MS NAGM, we first performed an adjuvant-driven stimulation of the peripheral immune system in C57Bl/6 mice *in vivo*. This led to similar gene expression changes in spinal cord demonstrating that peripheral immune signals might be one source for astrocytic gene expression changes in the brain. *IL1B* upregulation in MS NAGM itself points to a possible endogenous signaling process leading to ANLS and GGC downregulation. This is supported by our findings that, among others, MS NAGM astrocytes express inflammasome components and that astrocytes are capable to release Il-1β *in-vitro*. Altogether, our data suggests that immune signaling of immune- and/or central nervous system origin drives alterations in astrocytic ANLS and GGC gene regulation in the MS NAGM. Such a mechanism might underlie cortical brain dysfunctions frequently encountered in MS patients.

1. Introduction

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). MS predominantly affects young adults and leads to substantial disability in a high proportion of patients (Compston and Coles, 2002). Pathologically, MS is characterized by multiple demyelinated plaques in the white and gray matter. Besides that, diffuse white and gray matter

Abbreviations: ANLS, astrocyte–neuron lactate shuttle; GGC, glutamate–glutamine cycle; NAGM, normal appearing cortical gray matter.

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abnormalities in non-lesional normally myelinated areas have been gaining increasing attention (Graumann et al., 2003; Stadelmann et al., 2008; Zeis et al., 2008, 2009). In the case of the non-lesional normal appearing gray matter (NAGM) of MS patients little is known about the molecular changes which possibly precede gray matter lesion formation (Dutta et al., 2006, 2007) and potentially contribute to clinical features encountered in MS. Of those, gray matter pathology has been linked with physical disability and cognitive impairment (Pirko et al., 2007) as well as fatigue (Roelcke et al., 1997; Filippi et al., 2002; Niepel et al., 2006), which are common manifestations of MS (Wishart and Sharpe, 1997; Blinkenberg et al., 2000; Lazeron et al., 2000; Amato et al., 2004; Benedict et al., 2004; Morgen et al., 2006; Sanfilipo et al., 2006; Houtchens et al., 2007).

One of the most abundant cell types of the gray matter is the astrocyte, which fulfills many important tasks ensuring brain function (Sofroniew and Vinters, 2010). A key assignment of astrocytes is the removal of neurotransmitters released by neurons via the glutamate–glutamine cycle (GGC; Schousboe et al., 2014). The disturbance thereof is strongly suggested to be involved in the pathogenesis of neurological disorders (Seifert et al., 2006). A reduction of the astroglial excitatory amino acid transporter 2 (EAAT2) was reported in neuromyelitis optica (NMO; Hinson et al., 2008), an inflammatory demyelinating disease earlier thought to be a variant of MS (Brosnan and Raine, 2013). Further, in amyotrophic lateral sclerosis (ALS), a decreased glutamate transport (Rothstein et al., 1992) as well as a selective loss of EAAT2 was shown (Rothstein et al., 1995). This suggests that a failure of the GGC by astrocytes might be crucial for excitotoxic damage and the subsequent pathogenic process in ALS (Seifert et al., 2006).

Increasing evidence indicate that astrocytes are also important contributors to CNS metabolism (Sofroniew and Vinters, 2010). In 1994, Pellerin and Magistretti proposed an astrocyte–neuron lactate shuttle (ANLS) based on existing experimental data (Pellerin and Magistretti, 1994). This model includes the following sequence of molecular events: Following increased synaptic activity glutamatergic neurons release the neurotransmitter glutamate into the synaptic cleft. Glutamate is avidly taken up by the astrocytes surrounding the synaptic cleft, via specific glial glutamate transporters (EAAT1 and EAAT2). EAATs co-transport glutamate with sodium ions increasing intracellular sodium concentration in the astrocyte and activating the energy dependent Na+/K+ ATPase pump (specifically the recruitment of the Na+/K+ ATPase alpha 2 subunit, ATP1A2). The corresponding hydrolysis of ATP leads to activation of astrocytic glycolysis, i.e. the degradation of glucose to pyruvate, which is then converted to lactate via lactate dehydrogenase (LDH). Lactate is then released via astrocytic monocarboxylate transporters (MCT1 and 4) into the extracellular space and from there taken up by the neurons (via MCT2 transporter), where it serves as an energy substrate following its intracellular conversion to pyruvate (Fig. 1B; Sofroniew and Vinters, 2010; Pellerin and Magistretti, 2012). This activity dependent supply of energetic metabolites from astrocytes to neurons is further enabled by a gap-junction mediated astrocyte network allowing intercellular trafficking of metabolites throughout the astrocyte syncytium (Rouach et al., 2008). Recently, it was shown that the provision of lactate to neurons is important for long- as well as short-term memory (Newman et al., 2011; Suzuki et al., 2011). This suggests that astrocyte dysfunction might play an important role in the reported cognitive impairments of MS patients (for review see Ferreira, 2010).

Inflammasomes are signaling platforms activated by signs of tissue damage and metabolic dysfunction (e.g. extracellular ATP), which lead to the maturation of cytokines such as IL-1β and thereby drive inflammation (Schroder and Tschopp, 2010). They are composed of an inflammasome sensor molecule (usually a Nod-like Receptor, NLR) and a caspase effector, often coupled via a signaling adaptor (apoptosis-associated speck-like protein containing CARD, ASC). Inflammasomes can be activated by pathogen associated molecular patterns (PAMPs) and/or danger-associated molecular patterns (DAMPs; Schroder and Tschopp, 2010). Activation of inflammasomes and secretion of active IL-1β requires two sequential steps; "signal 1" induces the expression of the inactive cytokine precursor (pro-IL-1β), while "signal 2" triggers its caspase-1-dependent processing via inflammasomes (Schroder and Tschopp, 2010). In innate immune cells, "signal 1" is usually provided by pro-inflammatory signaling by TLRs, or by IL-1β itself, whereas "signal 2" might among others be provided by host-derived molecules indicative of injury e.g. extracellular ATP (Schroder and Tschopp, 2010). A recent publication by Kawana et al., 2013 showed that in MS active demyelinating lesions astrocytes express all necessary components of the NLRP3 inflammasome (Kawana et al., 2013). Further, animals lacking Casp1, Nlrp3 or Asc develop a mild clinical course of in experimental autoimmune encephalomyelitis (EAE; Furlan et al., 1999; Gris et al., 2010; Inoue et al., 2012), suggesting an involvement of inflammasomes in MS pathogenesis.

In our study, we discovered a downregulation of astrocyte specific genes involved in the ANLS and the GGC in the MS NAGM. Additionally, a gene expression signature indicative of activated Toll-like/Il-1β signaling as well as an upregulation of *IL1B* was detected. To investigate a potential interconnection between Toll-like/Il-1β signaling and the downregulation of astrocyte GGC and ANLS genes we treated pure astrocyte cultures with IL-1β, LPS (a TLR4 agonist), ATP (as a potential endogenous danger/degeneration signal) and with ATP/LPS (inflammasome activation protocol for Il-1β release). These treatments led to a similar downregulation of genes as found *in vivo* in the MS NAGM, demonstrating an interconnection between the Toll-like/IL-1β signaling and the astrocytic ANLS as well as GGC genes. We further demonstrated *in vivo* in C57Bl/6 mice that such an astrocyte reaction in the MS NAGM can be a consequence of peripheral immune-system signaling. On the other hand, a downregulation of GGC and ANLS genes might also be a consequence of an endogenous brain reaction. Supporting this hypothesis, we demonstrated that *IL1B* is upregulated in the MS NAGM. Further, astrocytes were shown to express the necessary inflammasome proteins in the MS NAGM and to be capable of releasing Il-1β *in-vitro*. Altogether, this indicates that in MS, immune-or danger related signals induce ANLS and GGC changes in astrocytes of the NAGM. As astrocyte dysfunction was shown to play an important role in the reported cognitive impairments of MS patients (for review see Ferreira, 2010), ANLS or GGC changes might contribute to the encountered cognitive impairments in MS.

2. Material and methods

2.1. Tissue collection

MS and control brain tissue samples were provided by the UK Multiple Sclerosis Tissue Bank (UK Multicentre Research Ethics Committee, MREC/02/2/39), funded by the Multiple Sclerosis Society of Great Britain and Northern Ireland (registered charity 207495). All brain tissues were routinely screened by a neuropathologist to confirm diagnosis of MS and to exclude other confounding pathologies (Reynolds et al., 2011). Tissues used for this study were immunohistochemically analyzed for MOG (myelin marker), NeuN (neuronal marker) and CD68 (microglial marker). Tissues showing demyelination (Supplementary Fig. 1), neuronal degeneration or strong microglia activation were not included in the study. In total 31 tissue samples from 17 control as well as 31 tissue samples from 18 MS cases were used for the study (Table 1).

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Table 1
Patient data and tissue used. Clinical and pathological information about the 17 control and 18 MS cases investigated in this study. The number of different tissue blocks used per case is indicated in parenthesis. (PPMS= primary progressive MS, SPMS= seconday progressive MS, RPMS= relapsing progressive MS)

Patient	Gender	Age at death	p.m. time	Disease duration	MS type	Cause of death	IHC	qRT-PCR	Array	In-situ
Control patients										
C1	m	69	10			Acute cardiac death			X (1)	
C2	f	59	22			Acute pancreatitis			X (1)	
C3	m	89	9			Cancer of the rectum	X (1)	X (1)		
C4	f	95	10			Bronchopneumonia		X (1)		
C5	f	85	9			Cancer of the esophagus	X (1)	X (1)		X (1)
C6	f	93	9			Unknown		X (1)		
C7	m	73	21			Cardiogenix shock		X (1)		
C8	m	77	26			Carcinoma of the lung	X (1)	X (1)		
C9	m	64	18			Cardiac failure	X (1)	X (4)	X (1)	
C10	f	84	24			Congestive cardiac failure, ischemic heart disease	X (1)	X (1)	X (2)	
C11	m	35	22			Carcinoma of the tongue	X (4)	X (2)	X (2)	X (1)
C12	f	78	33			Myeloid leukemia	X (1)			
C13	f	60	13			Ovarian cancer	X (1)	X (1)	X (2)	
C14	m	75	17			Aspiration pneumonia	X (4)	X (1)	X (1)	X (1)
C15	m	88	22			Prostate cancer, bone metastases	X (1)	X (1)		
C16	f	88	20			Bronchopneumonia	X (1)	X (1)		
C17	m	84	5			Bladder cancer, pneumonia	X (2)	X (3)	X (1)	X (1)
Multiple Sclerosis patients										
MS1	f	56	8	31	SPMS	Breast carcinoma, pneumothorax	X (1)	X (1)	X (1)	
MS2	f	58	16	22	PPMS	Peritonitis, Multiple Sclerosis	X (1)	X (1)		
MS3	f	78	18	33	SPMS	Myocardial infarction, acute abdomen			X (1)	
MS4	f	58	6	21	PPMS	Bronchopneumonia	X (3)	X (1)	X (1)	X (1)
MS5	f	20	17	17	PPMS	Pulmonary embolus, pneumonia	X (2)	X (1)		
MS6	f	69	11	31	SPMS	Acute pyelonephritis, Multiple Sclerosis	X (1)	X (1)		
MS7	f	78	9	47	PPMS	Lung infection	X (2)	X (1)	X (1)	
MS8	f	78	5	42	SPMS	Metastatic carcinoma of bronchus	X (4)	X (3)	X (3)	X (1)
MS9	f	54	22	20	SPMS	Bronchopneumonia	X (2)	X (2)	X (2)	
MS10	m	53	12	11	SPMS	Urinary tract infection, Multiple Sclerosis	X (1)	X (1)	X (1)	
MS11	m	38	19	17	RPMS	Aspiration pneumonia, pulmonary edema	X (1)	X (1)	X (1)	
MS12	f	50	12		SPMS	Multiple Sclerosis	X (1)	X (1)	X (1)	
MS13	m	37	12	27	PPMS	Intestinal obstruction, Multiple Sclerosis	X (1)	X (2)	X (1)	
MS14	f	78	24	47	SPMS	Aspiration pneumonia, Multiple Sclerosis	X (2)	X (1)	X (1)	X (1)
MS15	f	49	12	27	PPMS	Bronchopneumonia, Multiple Sclerosis		X (1)	X (2)	
MS16	m	45	24	25	SPMS	Bronchopneumonia, Multiple Sclerosis	X (1)	X (1)		
MS17	f	47	16	17	SPMS	Septicaemia, bronchopneumonia, Multiple Sclerosis	X (1)	X (2)	X (1)	X (1)
MS18	m	45	9	17	SPMS	Multiple sclerosis		X (1)		

2.2. RNA profiling and statistical analysis

Total RNA was isolated using the RNeasy Kit (Qiagen, Hilden, Germany) together with Qiazol as lysis reagent. RNA quality control was done by using the Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, USA) and by RNA gel electrophoresis or by the RNA 6000 Nano total RNA Kit on an Agilent 2100 Bioanalyzer. Degraded (RIN < 6) and/or contaminated (260/280 nm ratio < 1.5; 230/280 nm ratio < 1.5) samples were excluded from the study. Gene expression profiling using our custom microarrays with 1176 representative cDNA sequences was performed as described before (Graumann et al., 2003; Zeis et al., 2008). Quantification of differential hybridization signal intensities was done with the AtlasImage™ 2.0 software program. Normalization of the gene expression array was performed by using the 10% trimmed mean as suggested for Human 1.2 Arrays from Clontech (Kroll and Wolfl, 2002). Microarray data analysis was performed with Partek Genomics Suite software. Differentially expressed genes were identified by performing a three-way ANOVA (disease, patients, tissue type). For this study, genes with a p-value of <0.05 (t-test on disease) and a mean fold difference of >1.2 were considered to be significantly differentially expressed between MS and controls. Significantly differentially expressed genes were further analyzed with the use of IPA (Ingenuity Systems, www.ingenuity.com). IPA upstream regulator analysis was performed to detect possible transcriptional regulators (TR) leading to the detected differential gene expression in MS NAGM. An activation z-score was calculated by IPA showing whether a potential TR was in an activated (z-score > 2) or inhibited (z-score < −2) or uncertain state.

2.3. Quantitative RT-PCR and statistical analysis

Real-time RT-PCR was performed in 384-well plates (VWR, Radnor, USA, Cat.No. 82006-678) using the ABI ViiA Fast Real-Time PCR system (Applied Biosystems, Life technologies Ltd., Paisley, UK). Primer sequences (Supplementary Table 1) were designed from unique sites over exon–intron junctions to prevent amplification of genomic DNA. Real-time RT-PCR was performed according to the manufacturer's protocol using the KAPA SYBR Fast Universal 2x qPCR Master mix (KAPA Biosystems, Woburn, USA). RNA amounts were calculated with relative standard curves for all mRNAs of interest. Optimal normalization factor for each experiment was identified among a set of detectable housekeeping genes by the NormFinder Software (Andersen et al., 2004). Data of the qRT-PCR expression analysis are shown as the mean of relative mRNA levels with error bars representing the standard deviation (SD). For all experiments, statistical significance was determined using the student's t-test, $^{*}p < 0.05$, $^{**}p < 0.0001$. Unless otherwise stated, all values were adjusted to the corresponding control group set to 1.

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2.4. Immunohistochemistry

Cryostat sections (10 μm) from fresh frozen tissue blocks or cell culture dishes were fixed for 10 min in 10% formalin. For myelin staining, sections were delipidated after fixation with methanol at −20 °C for 8 min. Tissue sections and dishes were blocked for 2 h at room temperature (RT) in blocking buffer (PBS pH 7.4, 0.2% Triton X-100, 0.1% Tween-20, 5% normal donkey serum) and incubated with primary antibodies in blocking buffer overnight at 4 °C. Tissue sections and dishes were then treated with 0.3% hydrogen peroxide in PBS for 20 min. Sections subjected to immunofluorescence were instead incubated for 1 h in 10 mM $CuSO_4$, 50 mM CH_3COONH_4, pH 5.0. Next, sections and dishes were incubated with secondary antibodies in blocking buffer for 1 h at RT. Sections for peroxidase stainings were processed with the Vectastain ABC Kit (Vector Laboratories, Burlingame, USA) as described by the manufacturer. Finally, immunofluorescence sections and dishes were counterstained with DAPI and mounted using Fluorosave (Calbiochem, Merck Millipore, Merck KGaA, Darmstadt, Germany). Antibodies used for immunohistochemical and immunofluorescence stainings are listed in Table 2.

2.5. Astrocyte cultures

Primary cultures of cerebral cortical astrocytes are prepared from newborn (1- to 2-days-old) OF1 mice (Charles River Laboratories, L'Arbresle, France) as previously described (Allaman et al., 2004; Gavillet et al., 2008). Culture experiments were approved by the Veterinary Office of the State of Vaud. For IL-1β treatments, culture medium was renewed 24 h before cytokine treatment induced by addition of IL-1β at 0.25 ng/ml (R&D systems, Abington, UK) in the culture medium (Gavillet et al., 2008). Treatment with IL-1β was performed on confluent 21-day-old cultures for 24 h. For ATP/LPS induced inflammasome activation, astrocytes were incubated in serum-free DMEM (D5030) supplemented with 5 mM glucose, 44 mM $NaHCO_3$ and 10 mL/L of an 100× antibiotic–antimycotic solution (Invitrogen, Life technologies Ltd., Paisley, UK) (DMEM) 24 h before treatments. Cells were then prestimulated with LPS (10 ng/ml) and 4 h later 5 mM ATP was added for an additional 1 h or 24 h. At the end of the treatment cells were harvested and total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). RNA quality control was done by using the Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, USA) and by the RNA 6000 Nano total RNA Kit on an Agilent 2100 Bioanalyzer. Degraded (RIN < 6) and/or contaminated (260/280 nm ratio < 1.5; 230/280 nm ratio < 1.5) samples were excluded from the study. For protein analysis cells were lysed in 62.5 mM Tris–HCl pH 6.8, 50 mM DTT and 0.3% SDS, sonicated for 10 s, boiled for 5 min at 100 °C and stored at −80 °C.

Using this culture procedure, >99% of the cells were immunoreactive for the astrocytic marker glial fibrillary acidic protein (GFAP), and microglia (CD68) were almost complete absent (<0.01%). No significant cell death upon any treatment was detected by the calcein survival test (data not shown).

2.6. Western blot analysis

Protein samples from the astrocyte cultures were subjected to gel electrophoresis using a 4–12% NUPAGE gradient gel (Invitrogen, Life technologies Ltd., Paisley, UK). After blotting on 0.2 μm Immun-Blot PVDF membranes (BIO-RAD, Hercules, USA), blots were incubated with blocking buffer (3% TOP-BLOCK in TBS) for 1 h at RT. Membranes were incubated with primary antibodies in blocking buffer overnight at 4 °C. After several washing steps membranes were incubated with secondary fluorescence-labeled antibody for 1 h at RT, washed four times with TBST and scanned using the Odyssey Western System (LICOR Systems, Lincoln, USA). Antibodies used for Western blot analysis are listed in Table 2.

2.7. CX43 analysis

CX43 analysis was performed on cerebral cortex using 19 gray matter tissue blocks from 12 control cases and 24 NAGM tissue blocks from 15 MS cases (Table 1). Average staining intensity was measured for 20 segments throughout the cortical layers (an example is given in Fig. 3A and C). For every tissue block all possible gyri were measured and an average staining intensity for all 20 segments for every tissue block was calculated. Intensity values of each block were normalized to the staining intensity of the non-lesioned adjacent normal appearing white matter (NAWM; showing no significant differences between control and MS patients) to correct for possible minor staining artefacts. Values from different blocks from the same patients were then combined

Table 2
Antibodies used in this study. Table shows all antibodies, their origin and use in the study. (MOG = myelin oligodendrocyte protein, NeuN = RNA binding protein, fox-1 homolog (*C. elegans*) 3 (RBFOX3), GFAP = glial fibrillary acidic protein, CD68 = CD68 molecule, OLIG2 = oligodendrocyte transcription factor 2, dk = donkey, gt = goat, rb = rabbit, m = mouse, a = anti).

Antibody	Company	Cat.Nr.	Used for IHC	Dilution	Used for WB	Dilution
Primary antibodies						
anti-MOG (Clone Z12)	Kindly provided by Prof. R. Reynolds	–	X	1:200		
anti-NEUN	Millipore	MAB377	X	1:500		
anti-GFAP	Sigma–Aldrich	G-3893	X	1:2000		
anti-CD68	Abcam	ab845	X	1:500		
anti-OLIG2	Millipore	ab9610	X	1:500		
anti-MCT1	Kindly provided by Dr. Merenzhinskaya, Washington DC, USA	–	X	1:500	X	1:1000
anti-GJA1 (Cx43)	Abcam	ab11370	X	1:500	X	1:10000
anti-EAAT2	Abcam	ab49645	X	1:500		
anti b-Actin (AC-74)	Sigma–Aldrich	A2228			X	1:5000
Secondary antibodies						
dk-a-m-Biotin	Jackson ImmunoResearch	715-065-150	X	1:500		
gt-a-rb-Biotin	Jackson ImmunoResearch	111-065-144	X	1:500		
dk-a-rb-Cy2	Jackson ImmunoResearch	711-225-152	X	1:500		
dk-a-rb-Cy3	Jackson ImmunoResearch	711-165-152	X	1:500		
dk-a-m-Cy2	Jackson ImmunoResearch	715-225-150	X	1:500		
dk-a-m-Cy3	Jackson ImmunoResearch	715-165-150	X	1:500		
gt-a-m-Alexa680	Molecular Probes	A21048			X	1:10000
gt-a-rb-IRDye 800	Rockland Immunochemicals, Inc.	611-132-002			X	1:10000

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and an average value was calculated. Statistical analysis over the 20 segments was then calculated by performing a two-way ANOVA (Disease, Segments). To further assess a possible reduction of CX43 in control gray matter tissue, a hypothetic "evenly stained" group was created by calculating the average intensity for all 20 segments by using data from all tissue blocks without a reduction in CX43 immunoreactivity. Differences between this virtual group and control and MS cases were then evaluated by using the Holm-Sidak method, with alpha = 5.000%.

2.8. In situ hybridization

Synthetic digoxigenin-labeled riboprobes (cRNA) were generated from recombinant pCRTMII-Topo® plasmid containing a 723 bp cDNA insert of human *MCT1* sequence (5′-11-733-3′) and a 836 bp cDNA insert of human *IPAF* sequence (5′-50-865-3′). Transcription was done from both sides with either SP6 or T7 RNA polymerase, generating antisense or sense (control) cRNA probes. Further, synthetic digoxigenin-labeled riboprobes (cRNA) were generated from recombinant pBSKSII plasmid containing a 823 bp cDNA insert of human *CASP4* sequence (5′-56-878-3′), a 130 bp cDNA insert of human *ASC* sequence (5′-600-729-3′), a 539 bp cDNA insert of human *NALP1* sequence (5′-2541-3079-3′), and a 713 bp cDNA insert of human *NALP3* sequence (5′-2693-3405-3′). Transcription was done from both sides with either T3 or T7 RNA polymerase, generating antisense or sense (control) cRNA probes. In situ hybridization was performed on 14 µm cryosections of freshly frozen tissues as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993; Graumann et al., 2003). In situ hybridization signal was revealed by alkaline phosphatase with BCIP and NBP as substrate. Immunohistochemistry was performed as described above. Antibodies used for immunohistochemical stainings are listed in Table 2.

2.9. ELISA

For determination of Il-1β secretion, astrocytes were stimulated with LPS, ATP or ATP/LPS for either 1 or 24 h (see above for treatments conditions). Il-1β concentration was measured by ELISA using the ELISA MAX Deluxe Set Mouse Il-1β Kit (Biolegend, San Diego, USA) according to the manufacturer's instructions.

2.10. CFA-induced peripheral immune stimulation in C57/Bl6 mice

8 weeks old C57Bl/6 mice were bred in-house and kept together in groups of five. Four independent experiments were made with 5 (pilot study) or 10 mice per treatment group. Mice were anaesthetized with 3% isoflurane and immunized subcutaneously in the flanks with either 0.1 ml PBS with 0.1 ml complete Freund's adjuvant (CFA) containing 0.4 mg Mycobacterium tuberculosis (CFA/PT) or with 0.2 ml PBS (Control). 200 ng pertussis toxin was injected intraperitonally on the day of immunization and two days later. Injections were made in the SPF animal facility. Animals were euthanatized using carbon dioxide and subsequent decapitation. Spinal cord tissue was dissected at day 14 after immunization. All experiments were approved by the Veterinary Office of the State of Basel. Sample size was estimated by using the following assumptions derived from earlier experiments (Difference of means = 20%; common standard deviation = 30%; alpha = 0.05; power = 80%). Both, male and female animals were randomly assigned to the treatment groups, and the analysis was performed by a nonblinded investigator. In total, 35 control and 34 CFA/PT injected mice were analyzed. One animal of the CFA/PT group was excluded due to bad RNA quality. No adverse events were noted during all experiments.

3. Results

3.1. Metabolic genes are downregulated in chronic MS NAGM

To identify molecular alterations in cortical MS NAGM, a differential gene expression analysis was performed. 8 control and 13 MS cases (Table 1) were investigated using a custom microarray containing 1176 representative cDNA sequences. In MS NAGM, we found a significantly decreased expression of genes involved in the ANLS and in the GGC; for example the monocarboxylate transporter 1 (*MCT1*; Solute carrier family 16, member 1, SLC16A1) and the excitatory amino acid transporter 2 (*EAAT2*; GLT-1; solute carrier family 1, member 2, SLC1A2; Fig. 1A and B). Additionally, a downregulation of connexin 43 (*CX43*, gap junction protein, alpha 1, GJA1) was detected (Fig. 1A and B).

In order to validate and substantiate our observations we analyzed additional NAGM tissues of up to 14 control and 17 MS cases by qRT-PCR. We further enlarged our analysis by additional genes functionally connected to the ones identified by the microarray, e.g. genes from the ANLS to which MCT1 belongs to, as well as additional genes of the GGC (Fig. 1A and B). Results from the qRT-PCR confirmed the transcriptional downregulation of *MCT1*, *CX43*, *EAAT2*, glutamine synthetase (*GLUL*) and glucose transporter type 1 (*GLUT1*; solute carrier family 2 (facilitated glucose transporter, member 1, SLC2A1). Additionally, we detected a significant downregulation of N-system amino acid transporter 1 (*NAT1*; solute carrier family 38, member 3, SLC38A3) and ATPase, Na+/K+ transporting, alpha 2 polypeptide (*ATP1A2*). In contrast, an upregulation of lactate dehydrogenase A (*LDHA*) was found. All genes of interest (Fig. 1A) were further evaluated whether their expression pattern is influenced by confounding factors such as post-mortem delay time, age at death, disease duration, clinical course or gender. Neither significant correlation nor differential expression due to any of these confounding factors could be identified.

3.2. MCT1, CX43 and EAAT2 are expressed in astrocytes in MS NAGM

MCT1, CX43 as well as EAAT2 are expressed by astrocytes in human brain (Milton et al., 1997; Rouach et al., 2002; Pellerin et al., 2005; Pierre and Pellerin, 2005; Chiry et al., 2006). To confirm their expression pattern in MS NAGM we performed immunohistochemistry and immunofluorescence co-localization studies. MCT1 staining was strongest on blood vessels and, additionally, a diffuse staining of the neuropil was evident (Fig. 2A, inset shows a blood vessel and the surrounding neuropil at higher magnification). Immunofluorescence co-localization microscopy of MCT1 and GFAP demonstrates MCT1 expression in human cortical astrocytes (Fig. 2B, arrows). The expression pattern of CX43 in MS NAGM was variable within the cortical layers (Fig. 2C, inset shows a single CX43-positive astrocyte at higher magnification). Immunofluorescence co-localization microscopy of CX43 and GFAP confirmed the exclusive cellular expression of CX43 in human astrocytes (Fig. 2D, arrow). Finally, the glutamate transporter EAAT2 was also detected to be expressed exclusively in astrocytes of the MS NAGM (Fig. 2E and F).

Currently, a controversial discussion is taking place in the scientific community concerning the localization of MCT1 expression in brain tissue. As IHC stainings of MCT1 showed an overall diffuse staining of the neuropil, we have performed an additional in situ hybridization analysis to further validate the cellular expression pattern of MCT1 within the human cerebral cortex. We found a widespread MCT1 expression in many different cell types such as in endothelial cells of blood vessels (Fig. 2G and H, arrowhead), in astrocytes identified by immunohistochemistry for GFAP

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A.

Symbol	Gene	Microarray Fold Change	Microarray p-value	qRT-PCR Fold Change	qRT-PCR p-value
	ANLS and GGC genes				
MCT1	Monocarboxylate transporter 1	-2.04	0.001	-2.01	<0.001
MCT4	Monocarboxylate transporter 4			-1.06	0.793
CX43	Gap junction protein, alpha 1, 43 kDA	-1.34	0.042	-1.56	<0.001
EAAT1	Excitatory amino acid transporter 1 (GLAST)			-1.33	0.161
EAAT2	Excitatory amino acid transporter 2 (GLT-1)	-1.34	0.024	-1.60	<0.001
GLUL	Glutamine synthetase	-1.28	0.112	-1.52	<0.001
NAT1	N-system amino acid transporter 1			-1.25	<0.001
LDHA	Lactate dehydrogenase A			1.31	0.011
LDHB	Lactate dehydrogenase B			1.10	0.265
ATP1A2	ATPase, Na+/K+ transporting, alpha 2 polypeptide			-1.77	<0.001
GLUT1	Glucose transporter type 1	-1.29	0.207	-1.15	0.001

B.

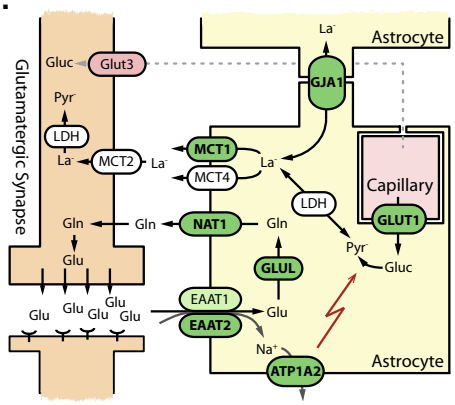

Fig. 1. Differential gene expression in normal appearing cortical gray matter of chronic MS. (A) Table shows microarray and qRT-PCR expression data of selected genes, which are differentially expressed in MS NAGM. Genes belonging to the ANLS and the GGC were in general downregulated in MS NAGM. Data are presented as mean fold change and unadjusted *p*-value. *P*-values were calculated by performing a three-way ANOVA (Microarray: $n = 11$ control, $n = 16$ MS cases; qRT-PCR: $n = 20$ control, $n = 22$ MS cases). (B) Schematic drawing of genes belonging to the ANLS and GGC. This model includes the following sequence of molecular events: Following increased synaptic activity glutamatergic neurons release the neurotransmitter glutamate into the synaptic cleft. Glutamate is avidly taken up by the astrocytes surrounding the synaptic cleft, via specific glial glutamate transporters (EAAT1 and EAAT2). EAATs co-transport glutamate with sodium ions increasing intracellular sodium concentration in the astrocyte and activating the energy dependent Na+/K+ ATPase pump (through the recruitment of the alpha 2 subunit). The corresponding hydrolysis of ATP leads to activation of astrocytic glycolysis, i.e. the degradation of glucose to pyruvate, which is then converted to lactate via lactate dehydrogenase (LDH). Lactate is then released via astrocytic monocarboxylate transporters (MCT1 and 4) into the extracellular space and from there taken up by the neurons (via MCT2). In neurons it serves as an energy substrate following its intracellular conversion to pyruvate by LDH. Genes which were found by qRT-PCR to be highly significantly ($p \leqslant 0.001$) downregulated in MS NAGM are shown in dark green (in bold), genes with a higher *p*-value showing a tendency to be downregulated are shown in light green. Genes showing a tendency to be upregulated are shown in light red. Red bolt indicates coupling of glutamate transport with glucose utilization. *Abbreviations:* Gluc = glucose, Pyr^- = pyruvate, La^- = lactate, Glu = glutamate, Gln = glutamine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2H, arrows), in a subpopulation of cortical neurons identified by NeuN (Fig. 2I, arrow) and in Olig2-positive cells (Fig. 2J, arrows). Altogether, our data confirm the expression of MCT1, CX43 and EAAT2 in astrocytes in the MS NAGM.

3.3. CX43 protein reduction in MS NAGM is characterized by its loss in individual astrocytes

Reduced expression of *CX43* as well as the inhomogeneous CX43 protein expression pattern in MS NAGM (Fig. 2C) prompted us to investigate the expression pattern of CX43 in control and MS cortex in more detail. Whereas the NAGM of most of the MS cases was characterized by a selective absence of CX43 in individual astrocytes (Fig. 3A and B), such a CX43 loss was found to a much lesser extent in the gray matter of control cases (Fig. 3C and D).

The reduction of CX43 was not homogenous throughout the cortical layers. Instead the reduction was observed in individual astrocytes within layers III–V with single astrocytes still expressing CX43 (e.g. Figs. 3A, B and 2C inset). In some cases the reduction of CX43 was so profound that only few Cx43 positive astrocytes remained as a thin rim in layer I and layer VI (Fig. 3B as an example). To quantify the CX43 reduction in MS NAGM, we measured CX43 staining intensity over all layers of the gray matter from 12 control and 14 MS cases (Fig. 3E and F). For each case, we measured the average CX43 staining intensity of 20 segments reaching from layer I to layer VI (Fig. 3A and C) and normalized it to the adjacent white matter staining intensity to account for minor staining artifacts (no statistical significant difference was detected between control and MS white matter CX43 staining). Statistical analysis over the segments from MS NAGM versus controls revealed that the CX43 expression was significantly more reduced in MS NAGM than in control gray matter ($p < 0.0001$, Two-way ANOVA) with an average reduction of about 32% throughout all layers (Fig. 3E). If compared to a virtual "evenly stained" group, generated from tissues with an even CX43 staining throughout all layers (e.g. Fig. 3C), MS NAGM but also control gray matter were statistically significantly different ($p < 0.0001$ for both, Holm-Sidak's multiple comparisons test; Fig. 3F). As in a study of human autopsy tissue additional factors may affect CX43 immunoreactivity, we investigated whether the degree of CX43 reduction correlates with post-mortem delay time, age at death, disease duration or gender. No significant correlation or difference was found due to these possible confounding factors (data not shown). A comparable immunohistochemical staining pattern as for CX43 was also detected for EAAT2 (Fig. 2E). This suggests that EAAT2 expression might be reduced in individual astrocyte as observed for CX43. Taken together, our analysis demonstrates that CX43, the major gap junction protein expressed by astrocytes and involved in the formation of the astrocyte syncytium, is downregulated in MS NAGM most prominently in layers III–V. A recent study identified a higher CX43 expression in MS cortical NAGM in comparison to control samples (Markoullis et al., 2014). Whether this discrepancy is due to the smaller sample number investigated or to the heterogeneous expression of CX43, which we also observed in control cases, cannot be verified.

3.4. Differential gene expression in MS NAGM is indicative of immune-related signaling

Simultaneously to the downregulation of metabolic genes in MS NAGM, the inflammasome associated cytokine, interleukin-1beta (IL1β), was significantly upregulated in MS (Fig. 4A). In keeping with this, Ingenuity pathway upstream analysis (Ingenuity Systems, www.ingenuity.com) of the microarray data identified a Toll-like/IL-1β signaling expression signature to be present in the differential gene expression pattern of MS NAGM (IL1 group activation *z*-score 4.748, IL1B activation *z*-score 3.538; LPS/TLR4 activation *z*-score 4.441; Fig. 4A). Further, additional immune-related signaling signatures were found in the differential gene expression pattern of MS NAGM (e.g. TNF, IL2, IFNG; Fig. 4A). Together, this strongly suggests that gene expression in MS NAGM is influenced by immune-related signaling.

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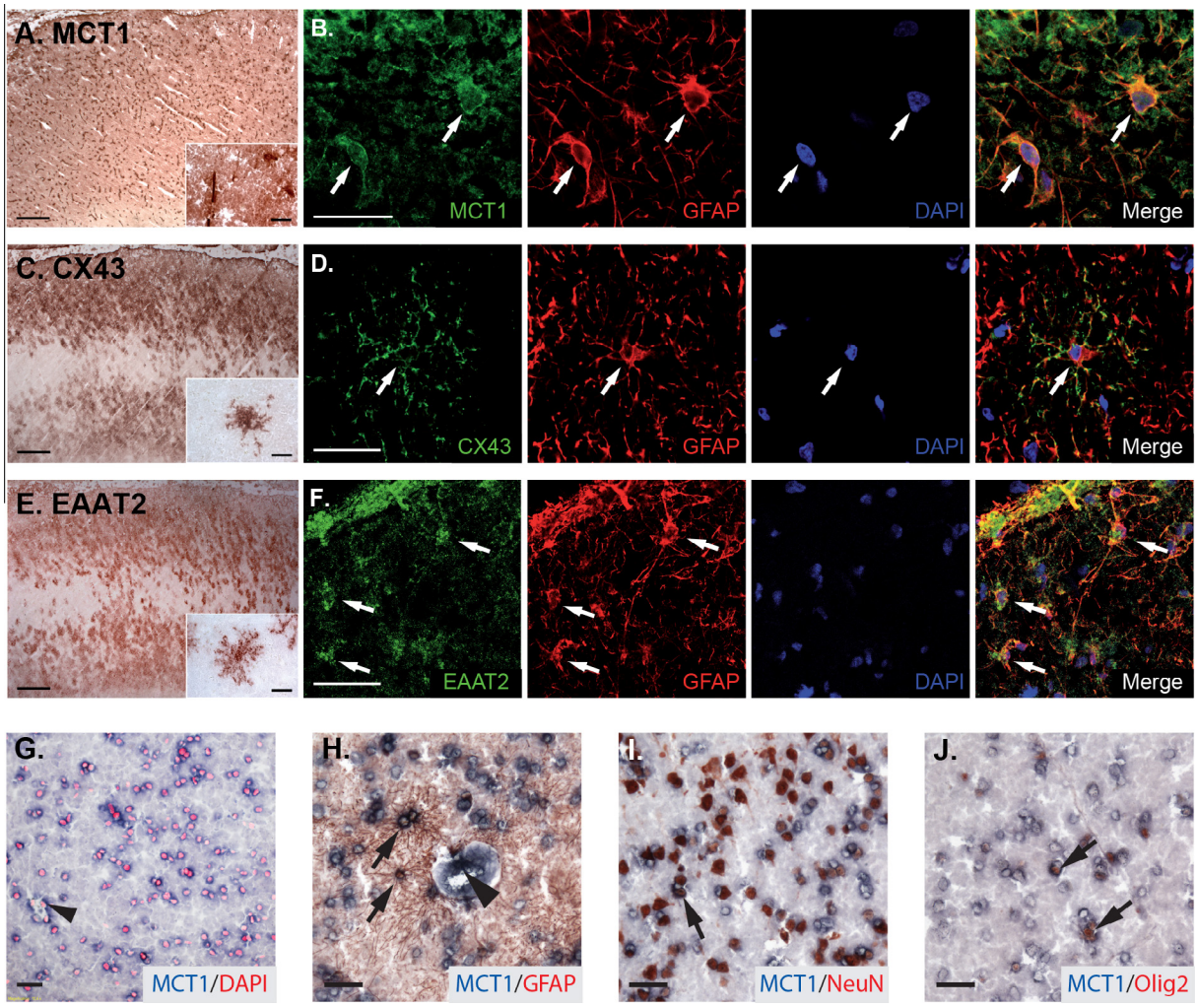

Fig. 2. Expression pattern of MCT1, CX43 and EAAT2 in NAGM of MS cerebral cortex. (A and B) MS NAGM showing immunoreactivity for MCT1. (A) Immunoreactivity to MCT1 was strongest on blood vessels and a diffuse staining of the neuropil was evident. (B) Immunofluorescence colocalization shows astrocytes (arrows) co-expressing MCT1 (green) and GFAP (red). (C and D) Immunoreactivity for CX43 in the MS NAGM. (C) Single astrocytes positive for CX43 are visible in the MS NAGM with reduced CX43. (D) Astrocyte (arrow) expressing CX43 (green) and GFAP (red). (E and F) Immunoreactivity to EAAT2 in MS NAGM. (E) Single astrocytes are expressing EAAT2 in the MS NAGM. (F) Double-labeling of EAAT2 (green) and GFAP (red) showing astrocytes (arrows) expressing EAAT2 in the MS NAGM. (G–J) In situ hybridization for MCT1 in control cortex counterstained with DAPI (G), and in MS NAGM counterstained with GFAP (H), with NeuN (I) and with Olig2 (J). Scale bars: A, C, E = 500 μm, insets = 20 μm, B, D, F = 20 μm, G–J = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. ANLS and GGC genes in astrocytes are downregulated upon immune-related signaling

The upregulation of *IL1B* as well as the immune-related signaling signatures and the downregulation of ANLS and GGC genes in MS NAGM lead to the question whether these phenomenons are interconnected. Therefore, we investigated whether immune-related signaling directly affect astrocyte ANLS and GGC gene expression. For that, we chose IL-1β, which was upregulated in MS NAGM and whose signaling signature was detected in the MS NAGM, and the mediator of the identified second most significant signaling signature, the TLR4 agonist LPS (Fig. 4A). We treated mouse primary cortical astrocyte cultures with either 0.25 ng/ml IL-1β or 10 ng/ml LPS for 24 h. qRT-PCR analysis of treated astrocytes revealed significant transcriptional downregulation of *Cx43*, *Glul*, *Ldhb*, *Glut1*, *Nat1* (IL-1β and LPS treatment), and *Eaat1* and *Atp1a2* (LPS treatment only), whereas the expression of *Eaat2* and the astrocyte specific monocarboxylate transporter *Mct4* (Solute carrier family 16, member 3, Slc16a3) (both upon LPS treatment) were significantly upregulated (Fig. 4B). Western blot analysis as well as immunofluorescence stainings confirmed strong downregulation of Cx43 on the protein level (Fig. 4C and F). In the case of Mct1, a significant upregulation could be detected (Fig. 4B) whereas its protein expression was rather the opposite (Fig. 4C). The unchanged or slightly upregulated expression of EAAT2 and the induction of MCT1 mRNA expression in pure astrocyte cultures might be due to the lack of their normal cellular counterparts such as neurons and oligodendrocytes in the cerebral cortex. Our findings show that cultured astrocytes exposed to immune-related stimuli such as IL-1β and LPS alter the expression of their ANLS and GGC genes substantially.

3.6. ANLS and GGC genes are differentially expressed upon inflammasome activation

The upregulation of *IL1B* in MS NAGM suggests priming or even activation of inflammasomes in the tissue. To investigate whether inflammasome activation can also take place in astrocytes and whether this influences gene expression of the ANLS and GGC components, we treated cortical astrocyte cultures with ATP alone, mimicking possible tissue damage in MS, as well as with ATP plus LPS in a similar manner as described for inflammasome activation (Schroder and Tschopp, 2010; Zhou et al., 2011). In general,

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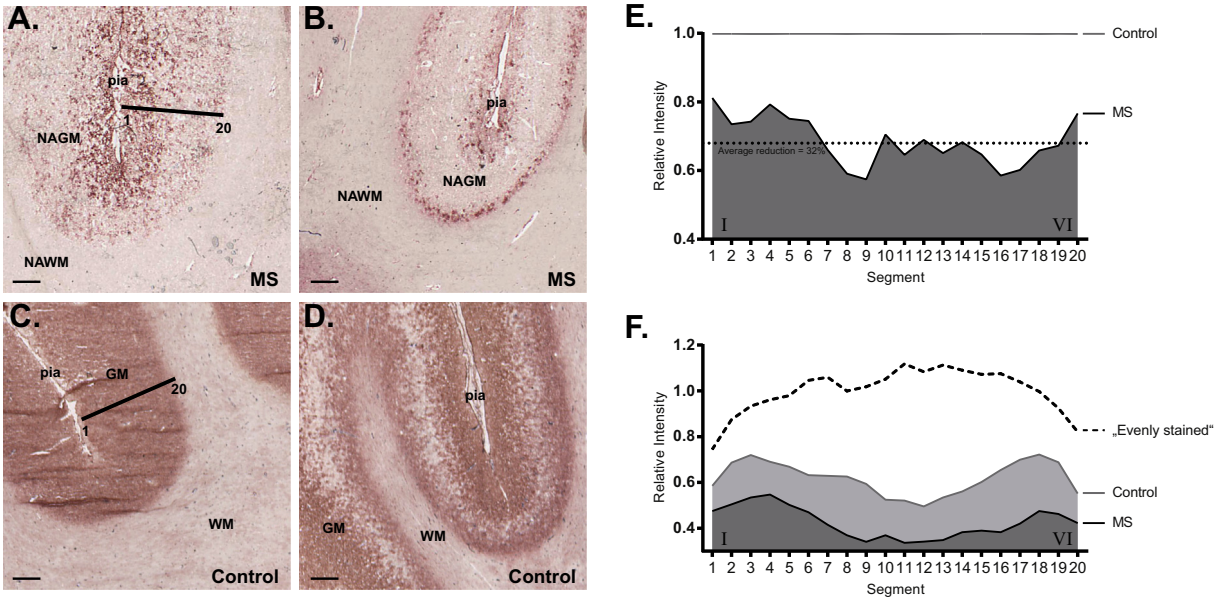

Fig. 3. CX43 protein loss in NAGM of MS cerebral cortex. (A–D) Representative images of CX43 stained cortical brain areas from MS (A and B) and control (C and D) cases showing different CX43 staining patterns. (E) Quantification of the average CX43 intensity in MS relative to control cases (set to 1) over all cortical segments (1–20) is shown. MS tissues show a reduction in CX43 staining intensity over all segments ranging from 19% in segment 1 to 43% in segment 9 with an average overall reduction of 32%. (F) Average relative CX43 intensity in MS and control tissue samples in comparison to intensity values from evenly stained cortices over all cortical segments is shown (all segments normalized to the overall average intensity of evenly stained cortices set as 1). MS but also control patients show a reduction in CX43 staining intensity over all segments. Scale bar$_{(A–D)}$ = 1 mm.

treatment with ATP or ATP/LPS led to significant downregulation of *Cx43*, *Ldhb*, *Glut1*, *Nat1*, *Eaat1* and *Mct4* as well as *Atp1a2* if treated by ATP/LPS (Fig. 4D). In contrast, *Glul* expression was significantly upregulated by both treatments (Fig. 4D) as well as *Atp1a2* if treated with ATP alone. Whereas *Eaat2* and *Mct1* where downregulated by ATP treatment, ATP/LPS treatment led to an upregulation of these genes. For *Mct1*, this is in line with the data obtained from the LPS and IL-1β treatment (Fig. 4B). Western blot analysis as well as immunofluorescence stainings confirmed the strong downregulation of Cx43 on the protein level (Fig. 4E and F), and also Mct1 protein expression was slightly but significantly downregulated (Fig. 4E). Taken together, our data demonstrate that astrocytes challenged with inflammasomal triggers respond by changing the expression of genes involved in the ANLS and GGC.

3.7. Peripheral inflammation signals reduce Mct1 and Cx43 expression in the spinal cord of C57/Bl6 mice

In MS, immune-related signaling might origin from immunological processes (e.g. lesion formation) in regions far away of the investigated MS NAGM tissue. Therefore, we asked the question whether signaling from the activated immune system can lead to a response in the gray matter tissue similar to that observed in MS NAGM brain tissue and in astrocyte cultures. To test this hypothesis we treated C57Bl/6 mice with a subcutaneous injection of 100 µl CFA with 100 µl PBS in the base of the tail, and twice pertussis toxin i.p. according to the standard EAE protocol. Injection of myelin auto-antigen was omitted to exclude possible T cell infiltration and to limit the immunological reaction to the outside of the CNS. In order to detect CNS gene expression changes also due to possible signaling by primed T cell whose accumulation in the meninges peaks around d11-12 after EAE induction (Kivisakk et al., 2009), spinal cord tissues were dissected at day 14 after CFA injection. Gene expression in cervical spinal cord was then analyzed by qRT-PCR. This protocol, which does not lead to the development of demyelinating lesions in the CNS, caused a small but persistent significant downregulation of *Mct1* and *Cx43* (Fig. 5A). No changes in gene expression were seen at that stage for *Eaat1* and *2*, *Glul*, *Ldha*, *Ldhb*, *Atp1a2*, *Nat1* as well as *Glut1* (data not shown). Whether the expression of these genes has already normalized after the CFA injection or whether they will be changed later or not at all has to be further elucidated. However, our data show that a CNS-unspecific inflammation in the periphery can lead to alterations of CNS metabolic gene expression.

3.8. Inflammasome components are expressed in human cerebral cortex

The upregulated *IL1B* expression, together with the found IL-1β signaling signature in the MS NAGM raised the question whether inflammasome activation might participate in MS NAGM alterations. Therefore, we first investigated if specific NLR inflammasome sensor molecules (NLRC4, NLRP1, NLRP3), the inflammasome adaptor ASC, and caspase effectors (CASP1, CASP4) are expressed in control and MS NAGM. In situ hybridization analysis demonstrated that these inflammasome components are expressed throughout the human cortex (Fig. 5B). We could demonstrate that CASP4, ASC, NLRC4, NLRP1, and NLRP3 were expressed in astrocytes (Fig. 5B, arrows). This indicates that astrocytes *in vivo* are capable to elicit inflammasome activation.

3.9. Cortical astrocytes are capable to release IL-1β upon inflammasome activation

The upregulation of *IL1B* in MS NAGM further provides evidence that immune-related signaling leading to a disturbance in astrocyte metabolic processes may take place in the tissue itself. As *IL1B* was upregulated and astrocytes expressed the necessary components for inflammasome activation we investigated whether these cells could be induced to trigger inflammasome-dependent IL-1β release. The expression profiling of NAGM indicated that *IL1B* gene expression was induced in MS patients as compared healthy controls (Fig. 4A), suggesting that "signal 1"of the activation cascade has already occurred in MS NAGM. We thus

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A.

Symbol	Gene	Microarray Fold Change	Microarray p-value	qRT-PCR Fold Change	qRT-PCR p-value
IL1B	Interleukin 1, beta	1.87	0.009	2.09	0.016

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
IL1	group	Activated	4.748	1.65E-15
Lipopolysaccharide	chemical drug	Activated	4.441	1.55E-29
TNF	cytokine	Activated	4.003	1.84E-26
IL1B	cytokine	Activated	3.538	4.43E-22
IL2	cytokine	Activated	3.526	2.47E-14
IFNG	cytokine	Activated	3.493	3.03E-30
TLR9	transmembrane receptor	Activated	3.384	1.32E-07
TLR3	transmembrane receptor	Activated	3.382	1.62E-07
NR3C1	ligand-dependent nuclear receptor	Inhibited	-3.349	2.19E-12

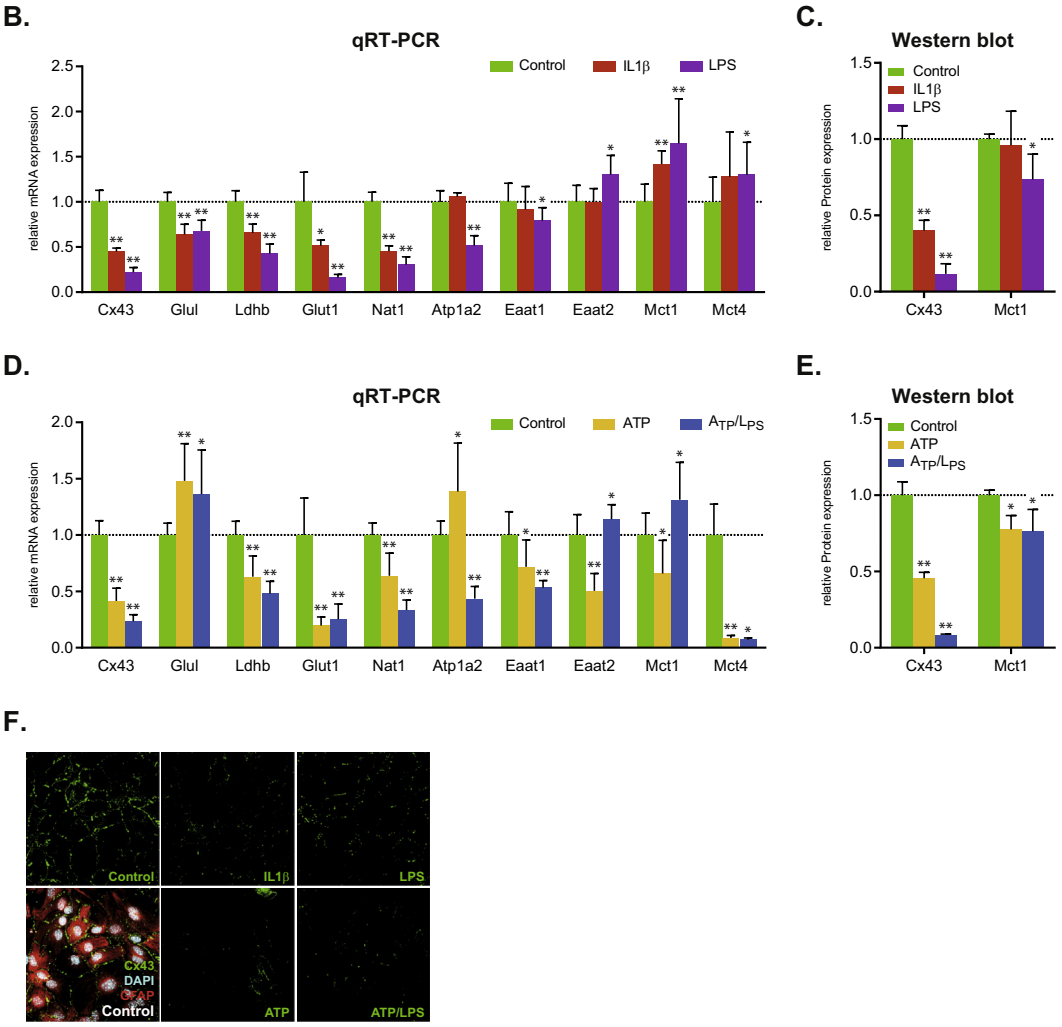

Fig. 4. Differential expression pattern of ANLS and GGC genes of cultured mouse astrocytes treated with IL-1β, LPS, ATP and ATP/LPS. (A) *IL1B* is upregulated in cortical MS NAGM tissue in comparison to control gray matter. Further, IPA upstream regulator analysis suggested possible upstream signaling by immune related regulators such as e.g. IL1B, LPS, TNF, IL2 and IFNG. (B) qRT-PCR analysis of mouse primary astrocyte cultures treated with IL-1β or LPS for 24 h. Mean values ± SD of relative mRNA levels (normalized to control cultures set to 1) for differentially expressed ANLS and GGC genes are shown ($n = 18$ for control, $n = 6$ for IL-1β and $n = 8$ for LPS, 3 independent experiments). (B) Quantitative Western blot analysis showing mean values ± SD of relative protein levels (normalized to control cultures set to 1) are shown for Mct1 and Cx43 ($n = 12$ for each, 3 independent experiment). (D) qRT-PCR analysis of mouse primary astrocyte cultures treated with ATP or ATP and LPS (ATP/LPS) for 24 h. Mean values ± SD of relative mRNA levels (normalized to control cultures set to 1) for selected ANLS genes are shown ($n = 18$ for controls, $n = 8$ for ATP and $n = 11$ for ATP/LPS treated cultures, 3 independent experiments). (E) Quantitative Western blot analysis of mouse primary astrocyte cultures treated with ATP and LPS (ATP/LPS) for 24 h. Mean values ± SD of relative protein levels (normalized to control cultures set to 1) are shown for Mct1 and Cx43 ($n = 12$ for both conditions, 3 independent experiments). (F) Immunofluorescence staining for Cx43 (green) of mouse control astrocyte cultures and astrocyte cultures treated with IL-1β, LPS, ATP and ATP/LPS shows the reduction of Cx43 in the treated cultures. All cultures looked as shown in the bottom left control picture stained for Cx43, GFAP (as a marker for astrocytes) and DAPI. For all experiments, statistical significance was determined using the student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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hypothesized that LPS would also trigger expression of the *Il1b* gene in primary astrocytes, as is already established for immune cells. Indeed, LPS strongly upregulated *Il1b* gene expression, compared to control astrocyte cultures where *Il1b* gene expression was below the detection limit (data not shown).

We then treated cortical astrocyte cultures with LPS alone (signal 1 alone), ATP alone (signal 2 alone), or LPS plus ATP (both signals 1 and 2) as described for Nlrp3 inflammasome activation (Schroder and Tschopp, 2010; Zhou et al., 2011) and measured IL-1β release. While LPS strongly induced the expression of the *Il1b* gene in primary astrocytes (data not shown), IL-1β release was weak and present only after 24 h treatment (Fig. 5C). Treatment with ATP alone did neither induce *Il1b* expression nor IL-1β release. However, strong IL-1β release, although only minor *Il1b* induction, required cell stimulation with both LPS and ATP (Fig. 5C), which is in line with studies in immune cells.

4. Discussion

Beside lesions, which are considered to be the pathological hallmark of MS, numerous molecular alterations have been identified in the so-called normal-appearing tissue (Graumann et al., 2003; Lindberg et al., 2004; Dutta et al., 2006, 2007; Zeis et al., 2008, 2009). Among these processes, alterations of the gray matter are of particular interest since they appear to be associated with clinical deficits and disease course of MS. Magnetic resonance imaging (MRI) studies for example revealed a strong correlation between gray matter atrophy and future disability progression (Amato et al., 2007; Fisher et al., 2008). Gray matter pathology has further been linked with physical disability and cognitive impairment (Pirko et al., 2007) as well as fatigue (Roelcke et al., 1997; Filippi et al., 2002; Niepel et al., 2006), which are common manifestations of MS (Wishart and Sharpe, 1997; Blinkenberg et al., 2000; Lazeron et al., 2000; Amato et al., 2004; Benedict et al., 2004; Morgen et al., 2006; Sanfilipo et al., 2006; Houtchens et al., 2007). However, to date the precise pathological alterations underlying these manifestations is not known. To investigate molecular mechanisms possibly underlying these impairments in MS we analyzed the molecular alterations in histopathological normal appearing gray matter in MS.

Transcriptional profiling revealed that MS NAGM is associated with the downregulation of gene transcripts involved in the GGC and in the ANLS (Fig. 1B), an experimentally-based framework proposed by Magistretti and coworkers (Pellerin and Magistretti, 1994; Bittar et al., 1996; Pellerin et al., 1998; Magistretti et al., 1999). In the ANLS concept, neuronal activity is coupled to astrocytic glucose utilization involving an activation of aerobic glycolysis in astrocytes and lactate consumption by neurons (Pellerin et al., 2007). Reduction of the ANLS might lead to a decrease in activity-dependent lactate delivery to neurons, and thus to a decrease in neuronal activity as suggested in the study of Dutta and coworkers (Dutta et al., 2006). Such a mechanism was hypothesized as a possible cause for central or mental fatigue (Ronnback and Hansson, 2004), which is observed in the majority of MS cases. Astrocyte–neuron lactate transport is also required for memory formation (Newman et al., 2011; Suzuki et al., 2011). Hence, chronic reduction of this particular energy supply, as suggested by our findings, might lead to some of the observed cognitive impairments in MS (for review see Ferreira, 2010). Further, they might also have an impact on synaptic activity in the cerebral cortex especially in layer III–V, the major in- and output of the cerebral cortex. Exactly in these regions we detected the strongest reduction of CX43 and potentially of the glutamate transporter EAAT2. A similar reduction of the astroglial EAAT2 was also found in neuromyelitis optica (NMO; Hinson et al., 2008), an inflammatory demyelinating disease earlier thought to be a variant of MS (Brosnan and Raine, 2013). Changed expression of CX43 as well as EAAT2 was suggested to be crucially involved in memory processes (Frisch et al., 2003; Stehberg et al., 2012; Poletti et al., 2014). In summary, a reduced ANLS and GGC gene expression in the MS NAGM might lead to many of the encountered clinical features of MS such as fatigue, cognitive impairment or physical disability as discussed before.

The biological activity of IL-1β is controlled by signaling complexes called inflammasomes (Schroder and Tschopp, 2010), which are emerging as central drivers of innate immune function (Martinon et al., 2009). Concomitant to ANLS and GGC gene downregulation, the expression of the gene encoding the pro-inflammatory cytokine IL-1β was upregulated. Further, and in keeping with *IL1B* gene induction, *in silico* upstream regulator analysis revealed gene signatures indicative of Il-1β signaling, suggesting innate immune activation in MS NAGM. This is supported by reports showing elevated expression of the purinergic receptor P2X ATP-gated ion channel 7 (P2X7R), mediating ATP-dependent NLRP3 activation, in the spinal cord of MS patients (Yiangou et al., 2006). Also, elevated levels of the NLRP3 agonist uric acid were found in the CSF of MS patients (Amorini et al., 2009). As the receptor for IL-1β is ubiquitously expressed among all brain cell types (Allan et al., 2005), a chronic exposure of gray matter tissue to IL-1β in MS patients as suggested by our differential gene expression analysis may be a crucial factor driving MS pathogenesis. In rats, intracerebral administration of IL-1β led to oligodendrocytes apoptosis (Fan et al., 2009), and subarachnoid injection of proinflammatory cytokines such as TNF and IFNγ lead to subpial demyelination (Gardner et al., 2013). Chronic IL-1β release, as suggested by the global upregulation of *IL1B* in MS NAGM, may be further pathologically relevant as it was reported to lead to neuronal death mediated by astrocytes (Thornton et al., 2006).

The concomitant presence of immune-related signaling signatures, potential inflammasomal activation as well as downregulation of ANLS and GGC genes in MS NAGM suggests that these phenomenons are interconnected. Treatment experiments of cultured cortical astrocytes demonstrated that this is indeed the case *in-vitro*. We found that in all treatments made (IL-1β, ATP, LPS and ATP/LPS), genes from the ANLS as well as the GGC have been downregulated *in vitro*. This demonstrates a direct link between immune-related signaling and the expression of ANLS and GGC genes in astrocytes, resembling our observations for MS NAGM *in vivo*. These findings are in line with reports showing that pro-inflammatory cytokines can modulate the astrocytic metabolic phenotype (Gavillet et al., 2008). This suggests that in MS, immune-related signaling, either from brain exogenous or endogenous sources lead to a reduction in ANLS and GGC genes. Finally, this might result in cognitive impairments as discussed before. A possible impact of immune-related signals on cognition is further supported by the findings of long-term cognitive impairment among survivors of severe sepsis (Iwashyna et al., 2010). Additionally, peripheral inflammation was reported to acutely impair human spatial memory (Harrison et al., 2014).

Although gene expression regulation of treated astrocytes resembles gene regulation in MS NAGM, some genes showed a different expression behavior. This is mostly true for MCT1 and EAAT2. The use of pure astrocyte cultures to investigate immune mediated changes in their gene expression offers methodological advances. However, they are likely to exhibit different properties than those *in vivo* (Halim et al., 2010). We suspect that in a controlled culture environment with constant glutamate supply, downregulation of EAAT2 might be overruled. In the case of MCT1, Nijland et al., 2014 showed that MCT1 is strongly upregulated in MS white matter lesions whereas its expression is reduced in perilesional areas (Nijland et al., 2014). We therefore speculate that MCT1 expression in astrocytes is dependent on their reactivity

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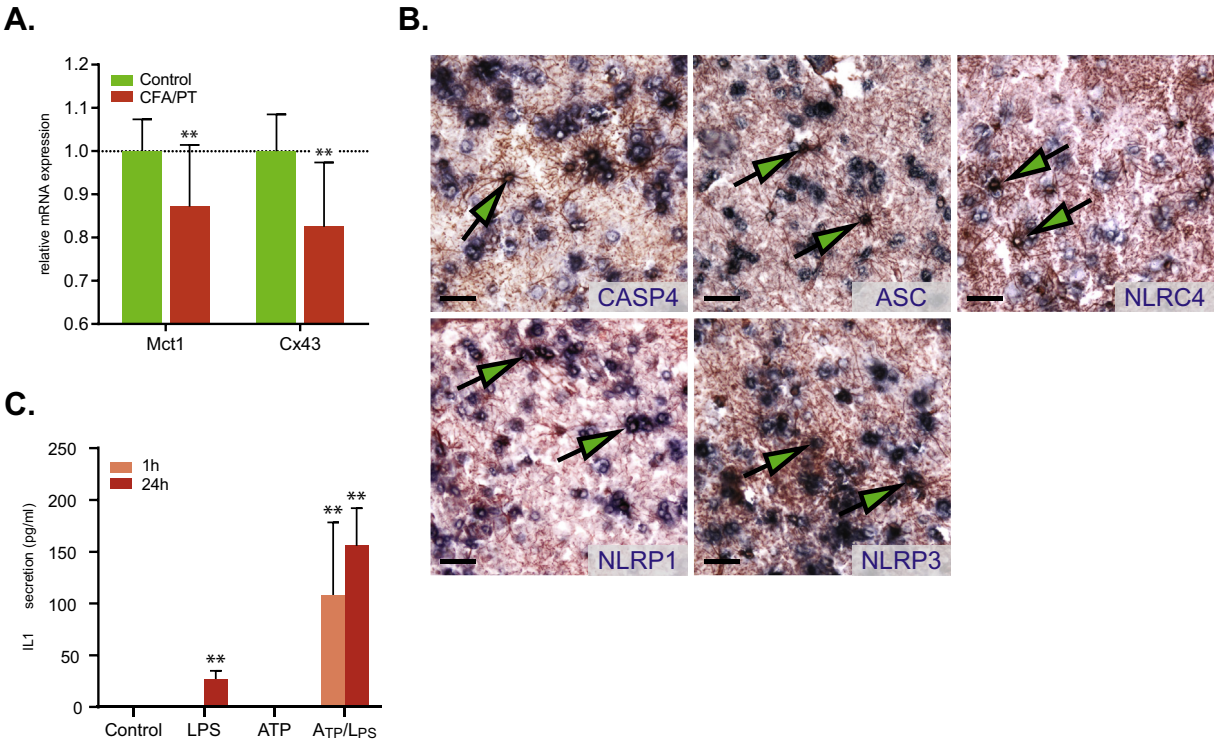

Fig. 5. Exogenous and endogenous signaling as a source for ANLS and GGC downregulation. (A) qRT-PCR analysis of spinal cord tissues of C57/Bl6 mice immunized with CFA and pertussis toxin showing the mean value ± SD of relative mRNA levels (normalized to control mice set to 1) for Mct1 and Cx43 ($n = 35$ for controls, $n = 34$ for CFA/PT, 4 independent experiments). (B) In situ hybridization of cortical gray matter tissue sections was performed for CASP4, ASC, NLRC4 (IPAF), NLRP1 (NALP1) and NLRP3 (NALP3). The hybridization signal was revealed by alkaline phosphatase with NBT/BCIP leading to a blue precipitate and an immunohistochemical staining for GFAP was made for collocation with astrocytes. In situ hybridization signals revealed that components of the inflammasome complex are expressed, among others, in astrocytes of the human cortex. Bars = 50 μm. (C) ELISA of primary astrocyte cultures showing the mean values ± SD for IL-1β secreted into the medium of cultures treated for 1 h or 24 h without (control) or with LPS, ATP or ATP/LPS ($n = 9$ at 1 h, $n = 18$ at 24 h, 3 independent experiments). For all experiments, statistical significance was determined using the student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

state. Our treatment might, in the case of MCT1, activate astrocytes to a higher degree so that MCT1 gets upregulated as e.g. in MS white matter lesions. In contrast, astrocytes of the NAGM might be activated to a lesser extent, downregulating MCT1 as shown in MS perilesional areas (Nijland et al., 2014).

Immune-related signaling in MS NAGM can be of brain exogenous but also endogenous origin. Downregulation of Cx43 and Mct1 in the *in vivo* adjuvant driven peripheral immunization experiment provides evidence that ANLS gene expression alterations in the MS NAGM might result from peripheral events and subsequent immune-related signaling to the brain tissue. Recent investigations detected TNF and IFNγ in inflamed meninges and in cerebrospinal fluid of MS cases (Gardner et al., 2013), which could act as such proinflammatory stimuli. Further, Dujmovic et al., 2009 demonstrated elevated IL-1β levels in the CSF and sera of MS patients (Dujmovic et al., 2009). As it was shown that cytokines can be transported through the blood brain barrier (for review see Banks et al., 1995), continuous and/or repetitive inflammatory events, such as lesion formation, might lead to the observed downregulation of ANLS and GGC genes in MS NAGM. However, immune-related signaling in MS NAGM might also be of endogenous origin. This notion is supported by the finding of a CX43 loss in the middle of the NAGM in layer III-V. Immune-related signaling from exogenous sources as e.g. from the meninges as recently suggested (Magliozzi et al., 2010; Gardner et al., 2013) would most probably lead to a gradient of CX43 loss from layer I towards layer VI as e.g. shown for neuronal loss (Magliozzi et al., 2010). Loss of CX43 due to immune-related signaling from blood vessels would further be expected to strike the whole NAGM rather than a demarcated zone in layer III–V. An endogenous signaling is also supported by the upregulated expression of *IL1B* in the MS NAGM itself, lacking immune cell infiltration as a source for this gene expression. Finally, besides microglia known to express inflammasomes and release cytokines and chemokines (Benveniste, 1997), an intrinsic signaling by activated astrocytes might be the source of MS NAGM ANLS and GGC downregulation as we could show that astrocytes themselves express the necessary inflammasome components and can be stimulated to release IL-1β.

5. Conclusion

Although recent progress has been made in MS treatment to date (Kappos et al., 2010), clinical disability continues to rise in MS patients over time. Our study demonstrates that in MS patients, astrocytes display a differential ANLS and GGC gene expression phenotype that is a likely cause for encountered clinical features in MS such as fatigue, cognitive impairment or physical disability. Further, we posit that the observed astrocyte differential gene expression in MS can be caused by immune-related signaling (e.g. inflammasomes) of exogenous but also endogenous origin. Our findings highlight the importance of subtle gray matter changes in MS pathogenesis, and the need to develop therapies to interfere with the responsible molecular pathways (e.g. IL-1β) to ameliorate clinical symptoms of MS.

Conflict of interest

The authors declare no competing financial interests.

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Author contributions

Study concept and design: NSW, qRT-PCR, microarray analysis, immunofluorescence, immunohistochemistry and analysis, Western blot analysis: TZ, immunohistochemistry, in situ hybridization: MG, primary astrocyte culture experiments: IA and PM, inflammasome related experiments: KS, JT; drafting of the manuscript: TZ, IA, KS, PM, and NSW.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2015.04.013.

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