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The epileptogenic trinity

Oxidative stress, brain inflammation and iron in epilepsy

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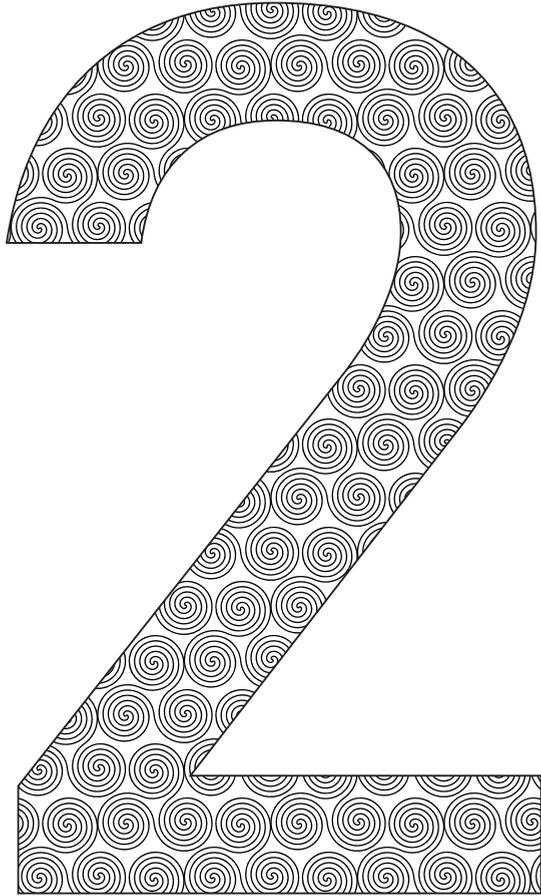
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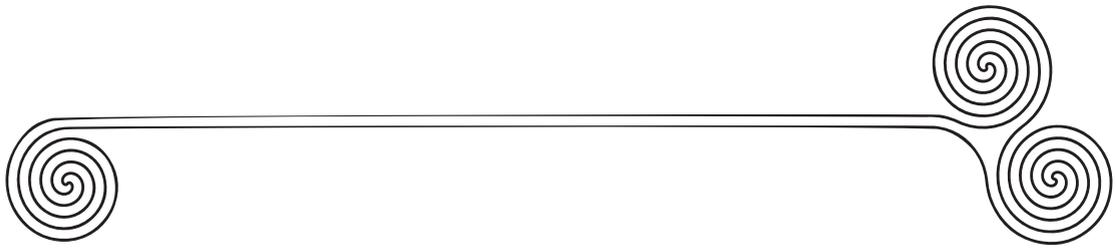
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OXIDATIVE STRESS AND INFLAMMATION IN A SPECTRUM OF EPILEPTOGENIC CORTICAL MALFORMATIONS: MOLECULAR INSIGHTS INTO THEIR INTERDEPENDENCE

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Abstract

Oxidative stress (OS) occurs in brains of patients with epilepsy and coincides with brain inflammation, and both phenomena contribute to seizure generation in animal models. We investigated whether expression of OS and brain inflammation markers co-occurred also in resected brain tissue of patients with epileptogenic cortical malformations: hemimegalencephaly (HME), focal cortical dysplasia (FCD) and cortical tubers in tuberous sclerosis complex (TSC). Moreover, we studied molecular mechanisms linking OS and inflammation in an *in vitro* model of neuronal function. Untangling interdependency and underlying molecular mechanisms might pose new therapeutic strategies for treating patients with drug-resistant epilepsy of differing etiologies. Immunohistochemistry was performed for specific OS markers xCT and iNOS and brain inflammation markers TLR4, COX-2 and NF- κ B in cortical tissue derived from patients with HME, FCD IIa, IIb and TSC. Additionally, we studied gene expression of these markers using the human neuronal cell line SH-SY5Y in which OS was induced using H₂O₂. OS markers were increased in dysmorphic neurons, and balloon/giant cells in cortex of patients with FCD IIb or TSC. Expression of OS markers was positively correlated to expression of brain inflammation markers. *In vitro*, 100 μ M, but not 50 μ M, of H₂O₂ increased expression of TLR4, IL-1 β and COX-2. We found that NF- κ B signaling was activated only upon stimulation with 100 μ M H₂O₂ leading to upregulation of TLR4 signaling and IL-1 β . The NF- κ B inhibitor TPCA-1 completely reversed this effect. Our results show that OS positively correlates with neuroinflammation and is particularly evident in brain tissue of patients with FCD IIb and TSC. *In vitro*, NF- κ B is involved in the switch to an inflammatory state after OS. We propose that the extent of OS can predict the neuroinflammatory state of the brain. Additionally, anti-oxidant treatments may prevent the switch to inflammation in neurons thus targeting multiple epileptogenic processes at once.

Keywords: Epilepsy, inflammation, oxidative stress, focal cortical dysplasia, tuberous sclerosis complex, hemimegalencephaly

Background

Oxidative stress (OS) and brain inflammation are closely related phenomena and they are often found concomitantly in various neurological disorders including epilepsy (2, 12, 68). Various studies support the existence of a self-sustaining cycle between OS generation and brain inflammation (9, 12, 67). Neurons are specifically vulnerable to oxidative damage due to their post-mitotic state and high metabolic demand (66). Recently, the presence of OS was shown in resected brain tissue of patients with temporal lobe epilepsy and hippocampal sclerosis (TLE-HS). Furthermore, antioxidant treatment in models of acquired epilepsy significantly improved disease outcomes (44, 49, 50, 52). This suggests that patients with epileptic syndromes might benefit from antioxidant therapies. To test if OS is also a common mechanism induced in other epileptogenic pathologies, we studied the presence of OS markers in resected cortical tissue from patients suffering from hemimegalencephaly (HME), focal cortical dysplasia (FCD) type IIa, type IIb or tuberous sclerosis complex (TSC). Moreover, we investigated the molecular mechanisms potentially linking OS and brain inflammation.

OS is defined as an imbalance in the cellular redox state due to impairment of antioxidant scavenging mechanisms and an increase in reactive oxygen (ROS) and nitrogen species formation. In physiological conditions ROS can serve different roles depending on the cellular context as they are important sensors in many cellular processes (61). High concentrations of ROS act as cytotoxic agents causing irreversible damage via oxidation of lipids, protein and DNA (60), and they may contribute to drug-resistance in epilepsy (22). Additionally, nitric oxide (NO) derived from the inducible NOS (iNOS) can further exacerbate OS by reacting with superoxide anions O_2^- to form highly reactive oxidant peroxynitrite ($ONOO^-$) that damages cell macromolecules similar to ROS (47). The cell possesses a multitude of antioxidant mechanisms that can buffer ROS, in order to protect the cell from oxidative damage. These include the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). SOD catalyzes the dismutation of superoxide anions (O_2^-) originating from cellular respiration to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), which can be converted by CAT and GPx to H_2O (70). In addition, other endogenous antioxidant signaling pathways exist like the redox sensitive nuclear factor erythroid 2 like 2 (Nrf-2) (46). Nrf-2 is a transcription factor that under increased OS conditions dissociates from its cytoplasmic repressor protein Kelch like ECH associated protein 1 (KEAP1) thereby moving to the nucleus where it binds to antioxidant response elements (ARE) in the promoter region of antioxidant genes such as heme oxygenase 1 (HO-1) and solute carrier family 7 member 11 (xCT) (23, 39, 46). Importantly, activation of the aforementioned antioxidant defense system is closely associated with inflammatory signaling in the cell.

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) transcription factor family enables cells to respond to a wide variety of stimuli with an inflammatory response. The NF- κ B pathway is facilitated by stimulation of Toll like receptor 4 (TLR4) via a well characterized pathway. TLR4 plays an important role in initiating inflammatory responses and has been shown to critically contribute to seizure development (42). Importantly, disulfide high mobility group box-1 (HMGB-1), a protein associated with iktogenesis, is generated under OS and it was shown to bind TLR4 to promote seizures and excitotoxicity (42). Upon HMGB-1 stimulation, TLR4 associates with the adapter myeloid differentiation primary response 88 (MyD88) via the Toll/interleukin-1 receptor (TIR) domain followed by the recruitment and activation of signaling molecules like interleukin 1 receptor associated kinase 1 (IRAK1), IRAK2, TNF receptor associated factor 6 (TRAF6) and TGF- β activated kinase 1/ MAP3K7 binding protein 2 (TAB2). These signaling molecules promote the activation and translocation of the transcription factor NF- κ B and consequently expression of pro-inflammatory mediators such as interleukin-1 β (IL-1 β) and cyclooxygenase-2 (COX-2) (13, 26, 31). Although it is known for a long time that OS modulates NF- κ B activation, the underlying mechanisms, especially concerning at what check point ROS can switch a cell from a homeostatic to a detrimental inflammatory state have not yet been clearly elucidated (6, 29, 65). More importantly, activation of NF- κ B driven genes seems to be highly cell type dependent and its relationship with OS in neurons is poorly understood.

The aim of the current study was twofold. First, to investigate the correlation between OS and brain inflammation in brain tissue of patients with HME, FCD II or TSC and intractable epilepsy. The FCD subtypes investigated were focal cortical dysplasia type IIa (FCD IIa) and type IIb (FCD IIb). Second, we investigated the magnitude of the oxidative insult required to lead to a transition from homeostatic cellular ROS signaling to initiation of pathologic inflammatory responses *in vitro*, and the mechanisms implicated. We evaluated therefore the acute effect of sub-toxic concentrations of the oxidizing agent H₂O₂ on TLR4 gene expression, inflammatory mediators, and TLR4 signaling components and regulators in human neuronal SH-SY5Y cells. In addition, to clarify the potential link between OS and NF- κ B activation, the aforementioned targets were also investigated in SH-SY5Y cells in the presence of the NF- κ B inhibitor TPCA-1.

Methods

Subjects

The cases included in this study were obtained from the archives of the Departments of Neuropathology of the Amsterdam UMC, (Amsterdam, The Netherlands). Cortical brain samples were obtained from patients undergoing surgery for intractable epilepsy and who were diagnosed with HME (n=5; aged 6-23 months; with features

of FCD type IIa), FCD IIa (n=5; aged 18-45 years), FCD IIb (n=5; aged 21-45 years) or TSC (n=4; aged 2-16 years) (Table 1; Suppl. Fig. 1). Surgical material was processed according to the consensus standard operational procedure for tissue inspection, distribution, and processing, using specific markers as recommended by the international consensus FCD classification system (7). All cases were reviewed independently by two neuropathologists, and the diagnosis of FCD was confirmed according to the international consensus classification system proposed for grading FCD (7). FCD Type II is characterized by disrupted cortical lamination and cytological abnormalities and includes two subtypes, FCD Type IIa (with dysmorphic neurons, but without balloon cells) and FCD Type IIb (with dysmorphic neurons and balloon cells), (7). The HME cases included are characterized by the enlargement of one cerebral hemisphere and the resected cortical specimens display features of FCD type IIa. All patients with cortical tubers fulfilled the diagnostic criteria for TSC (59). Cortical tubers consist of areas of cortical dyslamination that contain different cell types, including dysmorphic neurons and giant cells (3, 15). None of the FCD patients fulfilled the diagnostic criteria for TSC. Control material was obtained at autopsy from cortex of 12 controls (aged 2-62 years), without a history of seizures or other neurological diseases (Suppl. Table 2). All autopsies were performed within 24h after death. Tissue was obtained and used in accordance with the Declaration of Helsinki and the AMC Research Code provided by the Medical Ethics Committee. The clinical information of each patient is reported in Table 1.

Immunohistochemistry of epilepsy associated pathologies

Human brain tissue was fixed in 10 % buffered formalin and embedded in paraffin. Paraffin embedded tissue was sectioned at 5 μ m, mounted on pre-coated glass slides (Star Frost, Waldemar Knittel, Braunschweig, Germany) and processed for immunohistochemical staining. Sections were deparaffinated in xylene, rinsed in ethanol (100 %, 95 %, 70 %) and incubated for 20 min in 0.3 % hydrogen peroxide diluted in methanol to block endogenous peroxidase activity. Antigen retrieval was performed using a pressure cooker in 0.01 M sodium citrate buffer (pH 6.0) at 120°C for 10 min. Slides were washed with phosphate buffered saline (PBS, pH 7.4) and incubated overnight with primary antibodies against xCT (polyclonal rabbit, gift from Prof. Vezzani, Milan, Italy, 1:250), iNOS (polyclonal rabbit, Abcam, Cambridge, UK; 1:250), COX-2 (clone D5H5, monoclonal rabbit, Cell signaling Technology, Beverly, MA, USA; 1:600), TLR4 (polyclonal rabbit, Santa Cruz Biotechnology, Dallas, TX, USA; 1:20) and NF- κ B p65 (rabbit polyclonal, Abcam, Cambridge, UK; 1:100) in PBS at 4°C. Sections were washed in PBS and then stained with a polymer based peroxidase immunohistochemistry detection kit (Brightvision plus kit, ImmunoLogic, Duiven, The Netherlands) according to the manufacturer's instructions. Staining was performed using Bright 3,3'-diaminobenzidine (DAB) substrate solution (ImmunoLogic, Duiven, The Netherlands). Reaction was stopped by washing in distilled water. Sections were counterstained with Haematoxylin-Mayer solution (Klinipath, Breda, The Netherlands), dehydrated in alcohol and xylene and coverslipped.

Table 1: Clinical information of patients with epilepsy.

Pathology	Gender	Age	Duration epilepsy	Seizure types	Brain area	Seizures/month
HME/FCDIIa	f	8mo	8mo	FIA, FB	I	>200
HME/FCDIIa	f	13mo	12mo	FIA, FB	T	>100
HME/FCDIIa	m	23mo	22mo	FB/TC	T	>200
HME/FCDIIa	m	16mo	16mo	FIA, FB	T	>200
HME/FCDIIa	f	6mo	6mo	FB/TC	F	>100
FCD IIa	m	44y	26y	FIA	T	6
FCD IIa	f	45y	19y	FIA	F	375
FCD IIa	m	32y	29y	FIA, FB/TC	F	90
FCD IIa	f	32y	24y	FIA, FB/TC	T	9
FCD IIa	m	18y	14y	FA, FIA, FB (absence)	F	20
FCD IIb	F	23y	22y	FB (absence), FB/TC	F	40
FCD IIb	f	41y	40y	FIA (hypermotor)	F	105
FCD IIb	f	37y	30y	FB (tonic)	O	20
FCD IIb	m	21y	15y	FIA (hypermotor)	F	450
FCD IIb	f	45y	19y	FIA (hypermotor)	F	375
TSC	f	4y	10mo	FIA	F	600
TSC	m	2y	2y	FIA	F	600
TSC	f	13y	13y	FIA	F	84
TSC	m	3y	3y	FIA	T	128

FCD= focal cortical dysplasia, HME=hemimegalencephaly, TSC=tuberous sclerosis complex, FIA = focal impaired awareness, FB = focal to bilateral, TC = tonic-clonic, FA = focal aware, F=frontal, T=temporal, O=occipital, I=insular, y = years, mo = months, m = male, f = female

Evaluation of immunohistochemistry

All tissue sections selected for investigation of OS and inflammatory markers were evaluated by two independent observers, blind to clinical data, for the presence or absence of various histopathological parameters and specific immunoreactivity (IR) for the different markers used for the diagnosis of HME, FCD IIa, FCD IIb, and TSC tubers. In the case of discordant evaluations, independent re-evaluation was performed by both observers to define the final score. The intensity of immunoreactive staining (IR) for iNOS, xCT, TLR4 and COX-2 was evaluated using a 0-3 scale (0=absent, 1=weak, 2=moderate, 3=strong staining). All areas of the lesion were examined and the score represents the predominant staining found in each case. In addition, the number of cells showing immunoreactivity for the different markers (0=absent, 1=rare, 2=sparse, 3=high) was also scored. As described previously (72), the immunoreactivity score (IRS) was calculated for each case by multiplying the intensity score by the number score and compared between groups.

Cell culture and treatment

SH-SY5Y neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/HAM F12 (1:1) (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 50 units/ml penicillin, 50 µg/ml streptomycin, 1 % L-Glutamine and 10 % fetal calf serum (FCS; Gibco, Life Technologies, Grand Island, NY, USA). The cultures were maintained in a 5 % CO₂ incubator at 37°C. For experiments cells were seeded into 96 and 12-well cell culture plates (Greiner Bio-One, Kremsmünster, Austria) and allowed to adhere for 24h. The culture medium was then replaced with fresh medium containing 1 % FCS, treated with different amount of hydrogen peroxide (H₂O₂, 50 µM or 100 µM, Sigma-Aldrich, St Louis, MO, USA, #16911) and incubated for 3 h. For TPCA-1 treatment culture medium was replaced with fresh medium containing either 0.05 % dimethyl sulfoxide (DMSO) or 1 µM TPCA-1 (Selleck Chemicals, Munich, Germany) in DMSO (0.05 % final DMSO concentration) one hour prior to H₂O₂ stimulation.

MTT and DCF assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, St Louis, MO, USA) cell viability assay. Subsequent to H₂O₂ treatment, 15 µl of 5 mg/ml MTT reagent was added to each well and the plate was incubated for 2 hours at 37 °C in a 5 % CO₂ incubator. The reaction mixture was discarded and 100 µl acid isopropanol (4mM HCl, 0.1 % Nonidet P-40 in isopropanol) was added to each well to stop color development. Plates were kept in agitation at room temperature for about 15–20 min in the dark. Cell density was determined by measuring optical density at 570 nm wavelength using a microplate reader (BMG Labtech, Ortenberg, Germany). Absorbance of treated cells was plotted relative to control cells.

Intracellular ROS levels were measured by the 2',7'-dichlorofluorescein (DCF, Sigma-Aldrich, St Louis, MO, USA) method. The cells were incubated with 10 µM 2',7'-dichlorofluorescein diacetate (H₂DCF-DA) in DMEM without phenol red for 30 min at 37 °C in the dark. After H₂DCF-DA staining, cells were washed twice with pre-warmed PBS and then suspended in DMEM without phenol red. The formation of DCF was measured with a microplate reader with excitation and emission wavelength at 485 nm (bandwidth 5 nm) and 535 nm (bandwidth 5 nm), respectively. Levels of DCF fluorescence were directly proportional to intracellular ROS levels and the increase of signal due to H₂O₂ treatment was reported as a fold-change compared to untreated samples. All assays were performed in triplicate for each condition.

RNA isolation

For RNA isolation, after H₂O₂ stimulation and/or TPCA-1 treatment the cells were washed twice with PBS and homogenized in 700 µl Qiazol Lysis Reagent (Qiagen Benelux, Venlo, the Netherlands). Total RNA fraction was isolated using the miRNeasy

Mini kit (Qiagen Benelux, Venlo, the Netherlands) according to manufacturer's instructions. The concentration and purity of RNA was determined at 260/280 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Quantitative real-time PCR analysis

Quantitative real-time PCR was used to evaluate gene expression of antioxidant enzymes, inflammatory mediators, and TLR4 signaling components (primer sequences in Suppl. Table 1). Briefly, 250 ng of total RNA was reverse-transcribed into cDNA using oligo dT primers. Quantitative real-time PCR was performed in 384-well optical plates and conducted on a Roche Lightcycler 480 thermocycler (Roche Applied Science, Basel, Switzerland). Quantification of data was performed using the computer program LinRegPCR in which linear regression on the Log(fluorescence) per cycle number data is applied to determine the amplification efficiency per sample (54). The starting concentration of each specific product was divided by the geometric mean of the starting concentration of the reference genes (elongation factor 1 α (EF1 α) and chromosome 1 open reading frame 43 (C1ORF43)) and this ratio was compared between groups.

Statistical Analysis

Statistical analyses were performed with Graphpad Prism[®] software (Graphpad software Inc., La Jolla, CA, USA). For H₂O₂ regulation non-parametric Kruskal–Wallis test followed by the Dunn's *post hoc* test was used to assess differences between groups. Non-parametric Mann-Whitney U test was used for TPCA-1 data. Correlations between OS and inflammation markers in human tissue were assessed using the Spearman's rank correlation test. Comparison of staining intensity of immunohistochemistry was done using non-parametric Kruskal–Wallis test followed by the Dunn's *post hoc* test. A p-value < 0.05 was considered to indicate a significant difference.

Results

Expression of oxidative stress and inflammatory markers in malformations of cortical development

The expression of iNOS, xCT, TLR4 and COX-2 was investigated in surgically resected cortical tissue of patients with HME, FCD IIa, IIb or TSC (see methods for diagnostic criteria) using immunohistochemistry (see summary of quantification in Table 2). Overall, expression of all investigated markers was mainly restricted to the cytoplasm in all cell types. In control tissue, weak staining was observed for the OS markers iNOS and xCT in neurons and cells with glial morphology (glia) (Fig. 1 A, B). For the inflammatory marker TLR4, weak staining could be detected in neurons and glia (Fig.

1 C). COX-2 expression could not be detected in neurons or glia of control tissue (Fig. 1 D). In normally appearing neurons in HME and FCD IIa, the expression of iNOS, xCT, TLR4 and COX-2 was not different from control tissue displaying weak staining (Fig. 1 E-L). However, dysmorphic neurons in HME and FCD IIa displayed increased expression of xCT, TLR4 and COX-2, but not iNOS compared to normally appearing neurons (Fig. 1 E-H and I-L; arrows). Expression of COX-2 in HME was specifically increased in a subset of dysmorphic neurons scattered throughout grey and white matter (Fig. 1, H; $p=0.0002$). In comparison, iNOS staining was enhanced in FCD IIb in both dysmorphic neurons ($p=0.0002$) and glia ($p=0.001$) and in dysmorphic neurons in TSC as compared to HME, FCD IIa and controls (Fig. 1 M, Q versus A, E, I). xCT staining was increased in dysmorphic neurons ($p<0.0001$) in both FCD IIb and TSC

Table 2: Immunoreactivity scores of iNOS, xCT, COX-2 and TLR4 in control and surgical tissue of HME, FCD IIa, FCD IIb and TSC.

		(Dysmorphic) neurons	Glia	Giant/Balloon cells
iNOS	Control	2 (0-4)	2 (0-2)	-
	HME	2 (2-3)	1 (0-2)	-
	FCD IIa	2 (2-4)	2 (2-4)	-
	FCD IIb	9 (6-9)**	9 (4-9)**	7.5 (6-9)**
	TSC	7.5 (6-9)*	6 (4-9)*	7.5 (6-9)**
xCT	Control	3 (3-6)	3 (3)	-
	HME	3 (2-3)	3 (2-3)	-
	FCD IIa	3 (3-6)	3 (3-6)	-
	FCD IIb	9 (9)***	6 (3-9)	9 (9)**
	TSC	9 (9)**	6 (3-6)	9 (9)**
TLR4	Control	3 (0-3)	3 (3)	-
	HME	3 (2-6)	3 (2-3)	-
	FCD IIa	3 (2-6)	3 (2-3)	-
	FCD IIb	9 (3-9)	6 (3-9)	9 (6-9)**
	TSC	9 (9)**	6 (3-9)	6 (6-9)**
COX-2	Control	0 (0-1)	0 (0)	-
	HME	3 (3-6) **	0 (0-3)	-
	FCD IIa	1 (0-4)	0 (0-1)	-
	FCD IIb	3 (1-6)*	2 (1-4)*	4 (3-6)**
	TSC	3.5 (3-6)**	2 (1-4)*	2.5 (2-6)*

The immunoreactivity score (IRS) is given as median with the range in brackets. Immunoreactivity was evaluated using a 0-3 scale (0=absent, 1=weak, 2=moderate, 3=strong staining). In addition, the number of positive cells was evaluated (0=absent, 1=rare, 2=sparse, 3=high). The product of these two scores (the immunoreactivity score, IRS) was calculated for each case and compared between groups. Kruskal–Wallis test followed by the Dunn’s *post hoc* test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$; HME n=5, FCD IIa n=5, FCD IIb n=5, TSC n=4

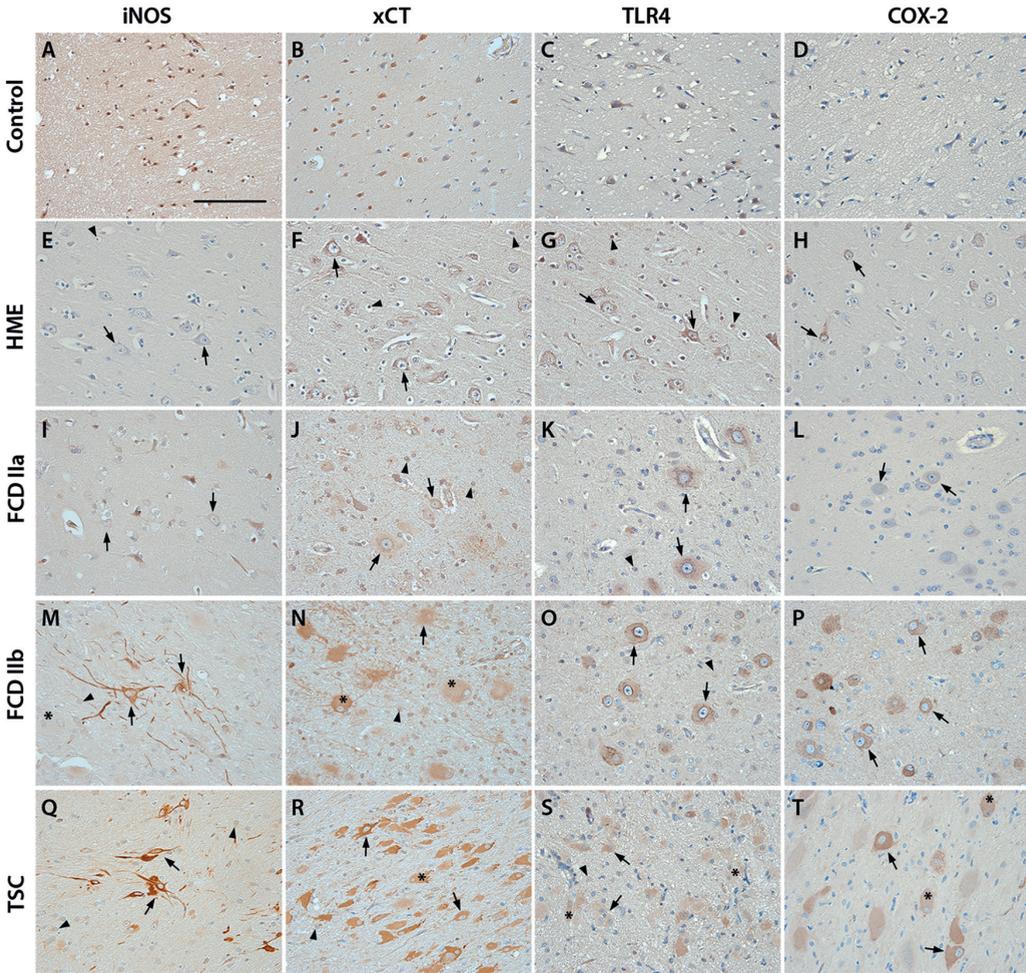


Figure 1: Representative images of oxidative stress markers (iNOS, xCT) and inflammatory markers (TLR4, COX-2) in control and surgical tissue of HME, FCD IIa, FCD IIb and TSC. Panels A-D show expression of all markers in cortical tissue of autopsy controls. TLR4, iNOS and xCT display (very) weak expression in cortical neurons (A-C), COX-2 staining is absent (D). HME and FCD IIa tissue show very weak staining for iNOS in dysmorphic neurons (E, I). TLR4 and xCT expression in HME and FCD IIa dysmorphic neurons and glia is increased but not different to control tissue (F, G, J, K). COX-2 in dysmorphic neurons in HME is weak with sparsely distributed cells displaying strong reactivity (H, arrows). Very weak staining for COX-2 in FCD IIa could only be detected in dysmorphic neurons (L). In FCD IIb iNOS, xCT, TLR4 and COX-2 were strongly increased compared to control in dysmorphic neurons and glia (M-P). Balloon cells had a very strong expression of all markers. In TSC, a similar pattern was observed: iNOS, xCT, TLR4 and COX-2 being expressed in dysmorphic neurons and glia (Q-T). Giant cell expression of all markers was strong as for balloon cells in FCD IIb (M-T, asterisks). Sections were counterstained with hematoxylin. Scale bar: 100 μ m in A; arrows=dysmorphic neurons, arrowheads=glia, asterisk*=balloon/giant cells

(Fig. 1 N, R). Increased expression of iNOS ($p=0.0005$) and xCT ($p<0.0001$) was also detected in balloon cells in FCD IIb (Fig. 1 M, N) and giant cells in TSC (Fig. 1 Q, R). In accordance with OS markers, TLR4 (dysmorphic neurons $p=0.0025$, glia $p=0.014$) and COX-2 (dysmorphic neurons $p=0.0002$, glia $p=0.005$) were both upregulated in dysmorphic neurons and glia of FCD IIb and TSC (Fig. 1 O, P, S, T). Upregulation of inflammatory markers TLR4 ($p=0.0003$) and COX-2 ($p=0.0005$) was also seen in giant cells (Fig. 1 S, T; asterisk) and balloon cells.

Expression of NF- κ B p65 in malformations of cortical development

NF- κ B expression was detected in cortical neurons and cells with glial morphology in control tissue (Fig. 2 A). Pathological tissue displayed the same staining intensity in dysmorphic neurons and glia, showing no difference to control specimens (Fig. 2 B-E). Expression in neurons was restricted to the cytoplasm and did not show any overlap with nuclear hematoxylin staining in control cortex and normally appearing neurons. As for most normally appearing neurons, the majority of dysmorphic neurons displayed cytoplasmic staining (Fig. 2 B, C, D₁, E₁, arrow), however nuclear NF- κ B p65 staining could be detected for a small subpopulation of dysmorphic neurons in all pathologies (Fig. 2 B for HME, arrowhead). Moreover, some balloon and giant cells also displayed NF- κ B p65 accumulation in the nucleus (Fig. 2 D₂, E₂; arrowheads) suggesting translocation of NF- κ B p65 from the cytoplasm to the nucleus.

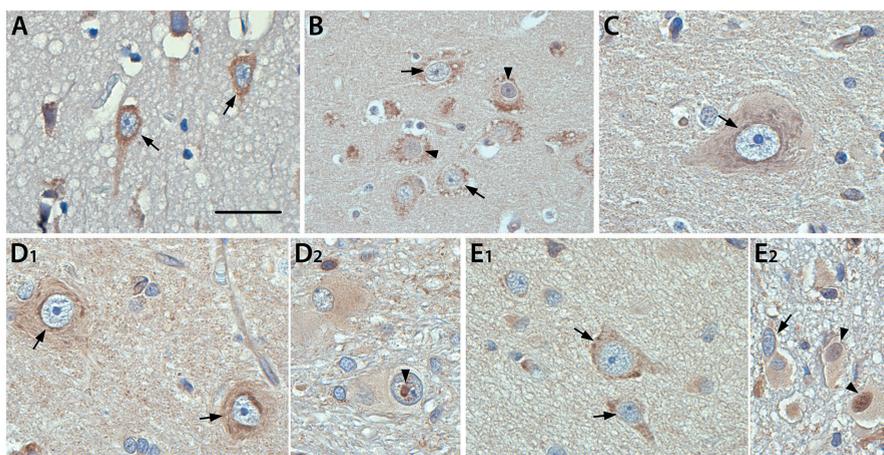


Figure 2: Representative images of cytoplasmic and nuclear expression of NF- κ B p65 in control and surgical tissue of HME, FCD IIa, FCD IIb and TSC. The expression of NF- κ B p65 in cortical neurons of control tissue (A) and dysmorphic neurons in HME and FCD IIa was mainly restricted to the cytoplasm (B, C). Similarly, dysmorphic neurons in FCD IIb and TSC expressed NF- κ B p65 in the cytoplasm (D₁, E₁). Occasional nuclear staining in dysmorphic neurons could be detected (representative for HME in B, arrowheads). In comparison some balloon cells (D₂) and giant cells (E₂) displayed nuclear staining, suggesting translocation of NF- κ B p65 from the cytoplasm to the nucleus. Arrows= cytoplasmic staining, arrowheads= nuclear staining. Sections were counterstained with hematoxylin. Scale bar A, C, D₁, D₂, E₁ = 25 μ m; Scale bar B, E₂ = 50 μ m.

Effects of H₂O₂ treatment on OS and viability in SH-SY5Y cells

We evaluated the intracellular ROS levels of SH-SY5Y cells treated with H₂O₂ (50 μM or 100 μM) for 3 hours. As expected, stimulation with both H₂O₂ concentrations promoted a significant elevation of DCF formation (Fig. 3 A); no differences in cell viability were evident between the treated and the control groups (Fig. 3 B). In order to evaluate whether H₂O₂ stimulation was associated with the dysregulation of antioxidant enzymes, we measured the mRNA expression levels of SOD1, CAT and GPx. Interestingly, we observed a consistent decrease ($p=0.0004$) of mRNA levels for CAT at both 50 μM and 100 μM of H₂O₂ (Fig. 3 C) while we did not detect differences in SOD and GPx expression between treated/control groups (data not shown). As additional proof that the cells were stimulated with subtoxic doses of H₂O₂, we analyzed the expression of Nrf-2 and its target HO-1 (Fig. 3 D, E). Both genes were upregulated (Nrf-2 $p=0.0028$; HO-1 $p=0.0002$) after 50 μM and 100 μM H₂O₂ treatment (Fig. 3 D, E).

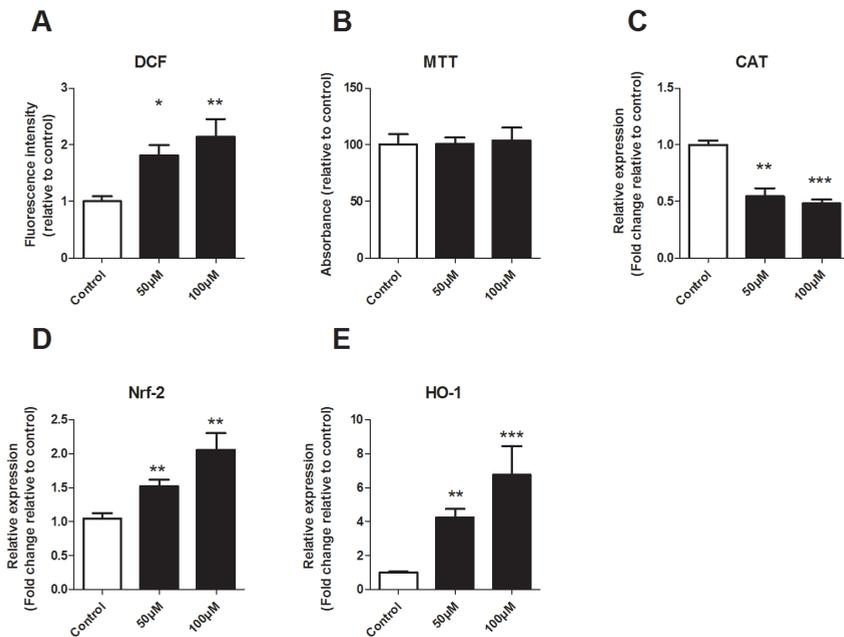


Figure 3: The effect of H₂O₂ on intracellular ROS accumulation, cell viability and gene expression of oxidative stress markers. SH-SY5Y cells were treated with different amounts of hydrogen peroxide (H₂O₂, 50 μM or 100 μM) for 3h. (A) The intracellular accumulation of ROS was assessed by DCF fluorescence intensity (A). Cell viability was determined using MTT assay (B). The mRNA level of catalase was reduced at both concentration whereas the expression of Nrf-2 and HO-1 was increased (C-E). mRNA expression was normalized to the geometric mean of reference genes C1ORF43 and EIF1α. Kruskal–Wallis test followed by the Dunn's *post hoc* test. Error bars represent standard error of mean (SEM); * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Data are representative of three independent experiments with 3 replicates for each group.

Effects of H₂O₂ treatment on pro-inflammatory mediators

To assess the potential contribution of OS to the development of an inflammatory response, we measured the mRNA levels of the inflammatory mediators IL-1 β , COX-2 and TLR4 after 50 μ M or 100 μ M H₂O₂ incubation for 3 hours in SH-SY5Y cells (Fig. 4 A, B, C). We observed an increased COX-2 mRNA expression ($p=0.0004$) at both H₂O₂ concentrations (Fig. 4 B) whereas the mRNA expression of IL-1 β ($p=0.001$) and TLR4 ($p=0.0055$) was increased only at 100 μ M H₂O₂ compared to respective control group (Fig. 4 A, C). Next, we focused on TLR4 signaling components in cells exposed to 100 μ M H₂O₂ and we found that the mRNA expression of both TAB2 ($p=0.0059$) and IRAK2 ($p=0.0003$) were increased compared to control (Fig. 4 D, E).

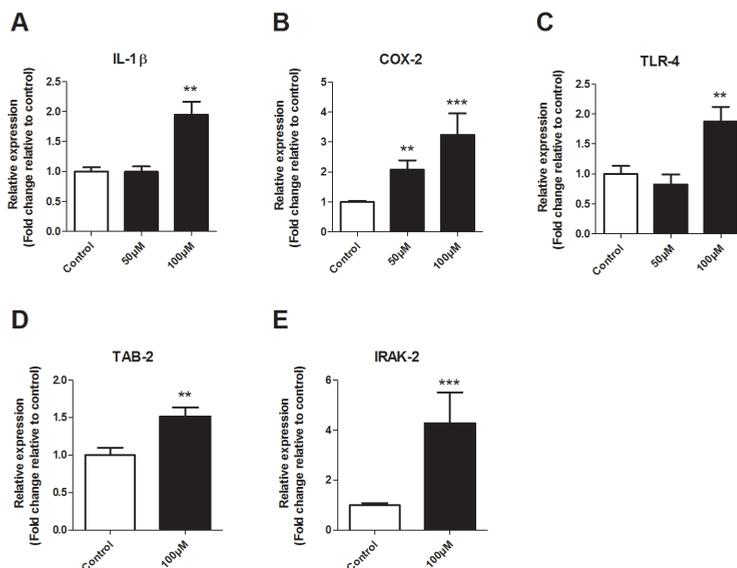


Figure 4: Expression of IL-1 β , COX-2 and TLR4 (50 μ M or 100 μ M) and TAB2 and IRAK2 (100 μ M) in response to H₂O₂ treatment for 3h. Quantitative real-time PCR of IL-1 β (A), COX-2 (B) and TLR4 (C) mRNA after 50 μ M and 100 μ M H₂O₂ as well as TAB2 (D) and IRAK2 (E) after 100 μ M H₂O₂. Data are expressed relative to levels observed in control groups; mRNA expression was normalized to the geometric mean of reference genes C10RF43 and EIF1 α . Kruskal–Wallis test followed by the Dunn’s *post hoc* test. Error bars represent standard error of mean (SEM); ** $P<0.01$, *** $P<0.001$. Data are representative of three independent experiments with 3 replicates for each group.

The role of NF- κ B signaling in the development of an inflammatory response after H₂O₂ exposure

In order to investigate whether NF- κ B is involved in the molecular mechanisms by which H₂O₂ upregulates IL-1 β , COX-2 and TLR4, we evaluated the effect of the NF- κ B inhibitor TPCA-1 on gene expression of these inflammatory mediators in SH-SY5Y cells treated with 100 μ M H₂O₂ for 3 hours. The H₂O₂-mediated upregulation of COX-2 ($p<0.0001$) and TLR4 ($p=0.0206$) (Fig. 5 B, C) was reversed by TPCA-1 pretreatment (IL-1 β $p=0.014$, TLR4 $p=0.0016$, COX-2 $p=0.004$ vs H₂O₂ alone; Fig. 5 A-C).

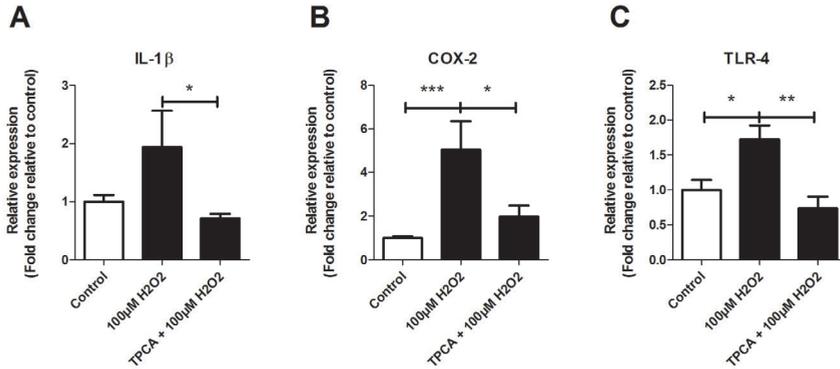


Figure 5: Expression of IL-1 β , COX-2 and TLR4 after stimulation with 100 μ M H₂O₂ with or without TPCA-1- pretreatment. Quantitative real-time PCR of IL-1 β (A), COX-2 (B) and TLR4 (C) in SH-SY5Y cells TPCA-1- pretreated with or without 100 μ M H₂O₂. Data are expressed relative to expression observed in control groups; mRNA expression was normalized to the geometric mean of reference genes C1ORF43 and EF1 α . Non-parametric Mann-Whitney U test. Error bars represent standard error of mean (SEM); * P<0.05, ** P<0.01, *** P<0.001. Data are representative of three independent experiments with 3 replicates for each group.

Effects of H₂O₂ treatment on NF- κ B regulators SHIP-1 and SOCS-1

In order to further clarify the mechanisms by which OS modulate TLR4/NF- κ B signaling, we measured the mRNA expression of SOCS-1 and SHIP-1 in SH-SY5Y cells after H₂O₂ pretreated with or without TPCA-1 (Fig. 6). The mRNA expression of SOCS-1 was increased after 50 μ M ($p=0.0002$) and 100 μ M ($p<0.0001$) H₂O₂ compared to the control group (Fig. 6 A, B). In addition, we found an increase in mRNA expression for SHIP-1 ($p<0.0001$ in both conditions) in all experimental conditions compared to the control group (Fig. 6 C, D). TPCA-1 pre-treatment inhibited the H₂O₂-induced increase of SOCS-1 (50 μ M $p=0.0421$, 100 μ M $p=0.0011$; Fig. 6 A, B) but not SHIP-1 (Fig. 6 C, D) expression.

Discussion

This study shows an increased expression of OS and inflammatory markers in resected brain tissue of patients with HME, FCD II or TSC, as compared to autaptic control specimens. In addition, we show that the expression of OS markers positively correlates with the expression of inflammatory markers in FCD IIb and TSC. To explain the relationship between OS and inflammation we provide evidence in SH-SY5Y neuronal cells that NF- κ B mediates OS induction of inflammatory genes with icogenic and excitotoxic properties.

Expression of OS markers, inflammatory markers and NF- κ B p65 in HME, FCD II and TSC

We report a consistent upregulation of OS markers, primarily in dysmorphic neurons and giant/balloon cells, but also glia, in FCD IIb and TSC. The presence of OS was

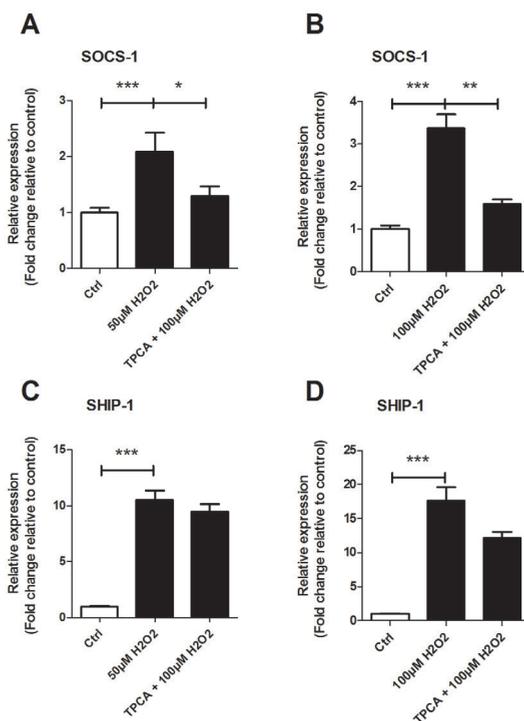


Figure 6: Expression of SOCS-1 and SHIP-1 after stimulation with or without TPCA-1- pretreatment and treated with H₂O₂. Quantitative real-time PCR of SOCS-1 (A, B) and SHIP-1 (C, D) in SH-SY5Y cells TPCA-1- pretreated and/or H₂O₂-treated. Data are expressed relative to expression observed in control groups; mRNA expression was normalized to the geometric mean of reference genes C1ORF43 and EF1α. Non-parametric Mann-Whitney U test. Error bars represent standard error of mean (SEM* P<0.05, ** P<0.01, *** P<0.001. Data are representative of three independent experiments with 3 replicates for each group.

previously reported in patients with TLE (36, 40, 49, 57), progressive myoclonic epilepsy (5), acute encephalopathy with refractory epilepsy (62) and genetic generalized epilepsy (35). FCD and TSC represent the most frequent developmental disorders leading to refractory epilepsy in the pediatric population. The FCDs comprise brain lesions displaying cortical dyslamination, cytoarchitectural lesions and abnormalities in white matter in any combination (8). The presence of OS has not been characterized yet except for one study showing that peroxiredoxin 6, an endogenous antioxidant molecule, was decreased in FCD patients (53). TSC is a genetic disorder due to a loss of function mutation in the TSC1 or TSC2 gene that together act as inhibitor of the mTOR pathway (55). A link with OS has been suggested by examining resected angiomyolipomas of TSC patients and in models of TSC1/2 deficient cells and mice (17, 24, 25). However, OS presence and extent has not been addressed in detail yet. HME is a rare malformation of cortical development (associated with developmental delay and severe epilepsy), characterized by the enlargement of one cerebral hemisphere displaying different

features of FCD type II and TSC cortical lesions, such as cortical dyslamination and the presence of hypertrophic and dysmorphic neurons (for review see (3, 15)). All three pathologies (HME, FCD II and TSC) present with aberrant mTOR signaling, thus share similar pathomechanisms and accumulating evidence suggests that they represent a spectrum of disorders involving hyperactivation of the mTOR pathway (1, 4, 14, 16, 38). However, the role and interplay of OS and inflammation in HME has not been investigated in detail. We show increased iNOS and xCT expression in brain tissue of FCD IIb and TSC indicating the presence of OS. We also identified an increased expression of TLR4 and COX-2 in the same brain specimens, in accordance with previous evidence of altered, ictogenic TLR4 signaling and increased brain inflammation (72). Notably, upregulation of TLR4 signaling was positively correlated with the expression of OS markers in neurons and glia.

The analyzed markers of OS and inflammation were increased in dysmorphic neurons, glia, giant cells and balloon cells, and iNOS expression was especially upregulated in balloon/giant cells. Although neuronal NO production has essential physiological functions in neurons excessive levels by reacting with ROS can potentially lead to the production of peroxynitrite which is a highly reactive radical species (63). The increased production of xCT in the same cells is an index of ROS generation as xCT is Nrf-2 dependent and can be induced by ROS (23). Interestingly, glutamate-cysteine ligase catalytic subunit (GCLC), an enzyme crucial in the synthesis of antioxidant glutathione, is considered to be pivotal for the redox adaptation leading to positive selection of mutant cells in TSC (41). Combined with the specific increase in iNOS expression, this set of data suggest that mutant cells in TSC drive OS but are also more resistant to it via glutathione dependent ROS reduction. Thus, in TSC, OS could be a key element in the progression of the disease by damaging healthy neurons via excessive ROS production and by selecting resistant cells carrying the pathologic mutation. Concerning neuroinflammation, the accumulation of NF- κ B p65 in the nuclei of selected subpopulations of dysmorphic neurons in all pathologies and balloon/giant cells in FCD IIb and TSC suggests that these cells may produce excessive inflammatory mediators via the canonical NF- κ B pathway (32). Indeed, previous studies reported upregulation of TLR4 in giant cells of fetal TSC brains as early as 23 gestational weeks indicating a prolonged exposure of the developing brain to a pro-inflammatory environment already before birth (51). Moreover, this study concluded that giant cells appeared prior to the development of dysmorphic neurons. Combining inflammation and OS would impose damaging molecules to the surrounding neurons.

Inflammation and OS can be cause and consequence of each other and both processes were shown to be involved in epileptogenesis (43, 69). Although they are concomitantly expressed in resected FCD type II and TSC epileptogenic tissues, it is noteworthy that dysmorphic neurons in HME and FCD IIa did not show increased

expression of iNOS and COX-2 compared to control. The expression of xCT and TLR4 in these cells was increased to some extent but much less than in FCD IIb and TSC. Since balloon cells are lacking in FCD IIa and in most HME cases, the overall expression of OS and inflammatory markers was consequently less than in FCD IIb and TSC. It is noteworthy however, that although dysmorphic neurons in HME displayed similar staining to that seen in FCD IIa for iNOS, TLR4 and xCT, a few sparsely distributed neurons in the grey and white matter exhibited very strong COX-2 expression compared to FCD IIa. Thus, the staining pattern of HME for the investigated markers lies between FCD IIa and FCD IIb, possibly explained by the occasional presence of balloon cells, which are lacking in FCD IIa, acting as drivers of inflammation and/or OS in HME. To elucidate the mechanisms that could underlie the differential expression pattern in dysmorphic neurons we investigated the neuronal inflammatory response to OS *in vitro*.

SH-SY5Y cells possess a dose dependent intracellular switch to an inflammatory state after OS

To understand the contribution of OS to the inflammatory state of dysmorphic neurons in the epileptogenic lesions, we used the human neuronal cell line SH-SY5Y and induced OS using H_2O_2 . Since SH-SY5Y cells express human-specific proteins we used this cell line to study the neuronal response to OS *in vitro*. SH-SY5Y is a neuroblastoma-derived cell line that resembles neurons in an undifferentiated state, mostly catecholaminergic, which is easily maintained and produces reliable, reproducible results (34). It is noteworthy though that these cells are derived from a tumor, hence they may not express neuronal markers to the same extent as neurons in the human brain. However, for the study of basic mechanisms we assume that general responses to OS are comparable between SH-SY5Y cells *in vitro* and neurons *in vivo*. Moreover, we decided not to differentiate SH-SY5Y cells into specific neuronal lineages since the investigated malformations of cortical development are characterized by neuronal cells that display aberrant differentiation states (45). We observed that H_2O_2 induced a significant ROS overproduction, while not being cytotoxic. The increase in ROS was associated with activation of the antioxidant response. To investigate if a sustained elevation of OS could promote the development of an inflammatory state, we analyzed changes in expression levels of TLR4, TLR4 signaling components and its inflammatory mediators IL-1 β and COX-2 in SH-SY5Y cells treated with H_2O_2 . Interestingly, we observed an increase in expression of COX-2 at both H_2O_2 concentrations used while mRNA expression of TLR4 and IL-1 β were only increased significantly at the highest H_2O_2 concentration together with increased signaling proteins such as TAB2 and IRAK2. Thus, sub-lethal OS mediates increased transcription of pro-inflammatory genes and downstream signaling molecules in neurons. TLR4 signaling activation in neurons was previously reported after oxidative insults like ischemia and reperfusion (20, 28, 64). Since TLR4 deficiency in mice can reduce oxidative damage to the brain (11, 33) and IL-1 β

activates NF- κ B (37), OS and neuroinflammation could induce a self-perpetuating cycle of inflammatory signaling via autocrine/paracrine signaling (Fig. 7) (27). This would be in line with the positive correlation of OS and inflammation in FCD IIb and TSC. Since we could not observe this correlation in HME and FCD IIa one might expect different mechanisms, secondary to mTOR activity, driving the pathology between HME/FCD IIa and FCD IIb/TSC. Interestingly, mouse models of inflammation via intrauterine lipopolysaccharide infusion (10) and peripheral influenza infection (30) were shown to have altered neuronal morphology, proving that a pro-inflammatory environment could have a direct effect on neuronal morphology. Moreover, longer durations of epilepsy in FCD IIb was shown to affect cell morphology in dysplastic areas directly (19). These findings support the idea that additional factors other than the genetic drivers in malformations of cortical development, like OS and the resulting inflammation, could contribute to the histopathological features in FCD IIb and cortical tubers in TSC. In line with this, a recent study addressing the ontogenetic processes at the origin of the complex cytological alteration in FCD type II suggests the concomitant occurrence of two different events: the first very early during cortical development related to the somatic gene mutation acting on mTOR pathway and the second one, promoted by the presence of inflammatory processes that could be responsible for a second wave of cytoarchitectural abnormalities (56). It is noteworthy that FCD IIa and IIb are only discriminated on the histopathological level whereas in patients both FCD subtypes display a similarly high degree of intrinsic epileptogenicity (48). Thus, seizures alone may not be the only determinants of the observed prominent increased expression of OS and inflammatory markers in FCD type IIb and cortical tubers. Therefore, the lesion *per se* with its specific cytoarchitectural and functional abnormalities, or the concomitant presence of the lesion and the epileptic activity, is likely to play a role in modulating OS and pro-inflammatory molecules in these malformations of cortical development.

SHIP-1 might be a mediator of the switch to an inflammatory state in SH-SY5Y cells. To better understand the potential role of OS in modulating TLR4/NF- κ B pathway and understand the concentration dependent switch to an inflammatory state, we evaluated the changes in the expression of two regulatory proteins, namely SOCS-1 and SHIP-1. While SHIP-1 positively regulates NF- κ B through the inactivation of protein kinase B (AKT), which suppresses mitogen activated protein kinase (MAPK) activation (18), SOCS-1 has been proposed as negative regulator by binding to the p65 subunit of NF- κ B. Binding facilitates ubiquitination and degradation of p65 (58, 71). We observed that both SOCS-1 and SHIP-1 were increased upon stimulation with H₂O₂. SHIP-1 expression and regulation of NF- κ B seems to be counterbalanced at low H₂O₂ concentrations via negative regulation of e.g. SOCS-1, which prevents transcription of NF- κ B targets including IL-1 β (Fig. 7 A). The dose-dependent increase of SHIP-1 promotes a disequilibrium between SOCS-1 and SHIP-1 in favor of SHIP-1, thus initiating self-perpetuating NF- κ B dependent transcription of pro-inflammatory

genes (Fig. 7 B). Moreover, TPCA-1 can prevent SOCS-1 upregulation which does not occur for SHIP-1. Interestingly, this effect was also shown in other cell lines (21) making SHIP-1 a key player in the translation of OS to inflammation in neurons as well.

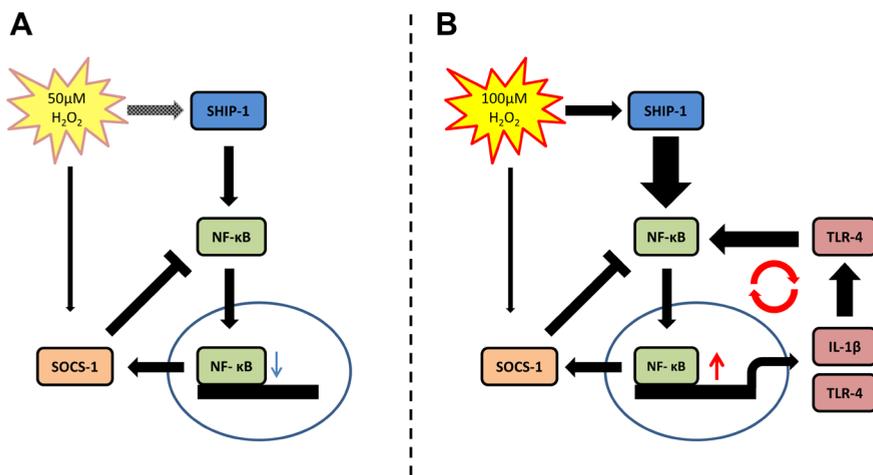


Figure 7: Proposed mechanism of OS-mediated switch to an inflammatory state. Low OS at 50 μM H_2O_2 stimulates SHIP-1 and, mediated via NF- κB , SOCS-1 expression alike creating an equilibrium that does not induce expression of inflammatory genes. High OS at 100 μM H_2O_2 leads to increased SHIP-1 expression and NF- κB activation and subsequently increased IL-1 β and TLR4 expression which reinforces NF- κB signalling further. In this condition SOCS-1 cannot equilibrate the NF- κB signal leading to a self-perpetuating inflammatory signal.

Conclusion

Our study shows that OS is common to FCD type IIb and TSC and is positively correlated to neuroinflammation in epileptogenic tissue. We also showed that the neuronal cell line SH-SY5Y responds to OS with dose dependent induction of inflammatory mediators. We propose that the extent of OS is pivotal for inducing or perpetuating an inflammatory process. Based on our hypothesis, anti-oxidant therapy could potentially prevent the switch to inflammation in neurons, and possibly other cell types, thereby preventing the consequent cascade of events contributing to the epileptogenic process.

List of abbreviations

ARE, antioxidant response element; AKT, protein kinase B; C1ORF43, chromosome 1 open reading frame 43; CAT, catalase; COX-2, cyclooxygenase 2; DAB, 3,3'-diaminobenzidine; DCF, 2',7'-dichlorofluorescein; DMEM, Dulbecco's modified Eagle's medium; EF1 α , elongation factor 1 α ; F, frontal; FA, focal aware; FB, focal to bilateral; FCD, focal cortical dysplasia; FCS, fetal calf serum; FIA, focal impaired awareness; GPx, glutathione peroxidase; HO-1, heme oxygenase; H2DCFH-DA, 2',7'-dichlorofluorescein diacetate; HME, hemimegalencephaly; HMGB-1, high mobility group box 1; I, insular; iNOS, inducible nitric oxide synthase; IL-1 β , interleukin 1 β ; IRAK1/2, interleukin 1 receptor associated kinase 1/2; IRS, immuno reactivity score; KEAP-1, Kelch like ECH associated protein; MAPK, mitogen activated protein kinase; MCD, malformations of cortical development; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MYD88, adapter myeloid differentiation primary response 88; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; Nrf-2, nuclear factor erythroid 2 like 2; O, occipital; OS, oxidative stress; PBS, phosphate buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase; T, temporal; TAB2, TGF- β activated kinase 1/MAP3K7 binding protein 2; TC, tonic-clonic; TIR, Toll/interleukin-1 receptor; TLE-HS, temporal lobe epilepsy with hippocampal sclerosis; TLR4, Toll like receptor 4; TRAF6, TNF receptor associated factor 6; TSC, tuberous sclerosis complex; xCT, solute carrier family 7 member 11

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Authors' contributions

AA and TZ performed quantitative real-time PCR analysis. TZ and JA performed immunohistochemical analyses. Data analysis was performed by AA and TZ. EV, JM and AI contributed to the data analysis and interpretation. Cell culture experiments were performed by AA and TZ. JS and AK contributed to cell culture experiments. AV provided the antibodies for oxidative stress markers and MP the SH-SY5Y cell line. WS, PR, FJ, WH, JB, SI and EA helped with the selection and collection of the brain tissues. EA conceived the study and participated in its design and coordination together with EV. TZ, AA, JM, AM, EV, EA drafted and finalized the manuscript. All authors read, revised, and approved the final manuscript.

Disclosures

The authors declare that they have no competing interests.

Ethics approval and consent to participate

The tissue was obtained and used in accordance with the Declaration of Helsinki and the Amsterdam UMC Research Code provided by the Medical Ethics Committee.

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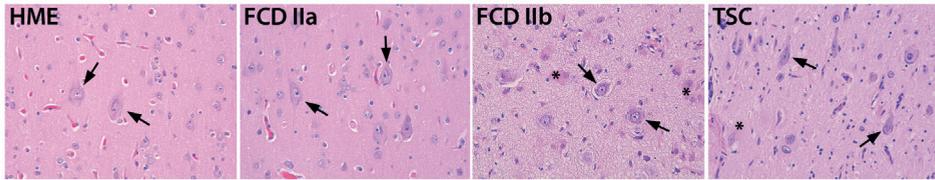
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Supplementary material



Suppl. Figure 1: Representative haematoxylin and eosin staining for the classification of HME, FCD IIa, FCD IIb and TSC. HME and FCD IIa were classified based on cortical dyslamination with cytoarchitectural abnormal dysmorphic neurons (arrows). In FCD IIb and TSC the same features apply, however with additional presence of balloon and giant cells, respectively (asterisks) (see also “Subjects” section in Methods).

Suppl. Table 1: Primer sequences used for quantitative real-time PCR.

Gene	Forward primer	Reverse primer	Amplicon size (nt)
SOD	tcatcaatttcgagcagaagg	gcaggccttcagtcagtc	78
CAT	tcatcagggatccatattgtt	ccttcagatgtgtctgaggattt	72
GPx	ttccctgcaaccagttt	ggacgtacttgagggaattctg	73
Nrf-2	acacgggtccacagctcatc	tgctcctcaaagtatgtcaatca	96
HO-1	ggccagcaacaaagtgaag	agttaaggaccatcggaga	105
IL-1 β	gcatccagctacgaatctcc	gaaccagcatcttctcagc	99
COX-2	gaatggggtgatgagcagtt	gccactcaagtgttcacat	99
TLR4	aatcccctgaggcatttagg	aaactctggatggggttcc	161
IRAK2	cctcctctgaggcctgtgt	tgatctcaatttgccacgaa	72
TAB2	gacagcaccagctcaagttc	tgctccttgctgcctaagt	112
SHIP-1	gtccaaggatggttctgagg	gtgactaatgagctgcaggatt	65
SOCS-1	gcccctctgtaggatggta	ctgctgtggagactgcattg	63
C1ORF43	gatttcctgggttccagt	attcgactcctcagggttca	94
EF1 α	atccaccttgggtcgcttt	ccgcaactgtctctcatatcac	51

Suppl. Table 2: Clinical information on autopsy control specimens.

Gender	Age (years)	Brain area	Cause of death
m	48	F	Myocardial infarction
m	18	F	Myocardial infarction
m	62	F	Pneumonia
f	35	F	Cardiac arrhythmia
m	47	F	Aortic dissection
f	45	F	Myocardial infarction
f	27	F	Cardiac arrhythmia
m	15	T	Respiratory failure
f	2	F	Respiratory failure
m	2	F	Pneumonia
f	17	F	Congenital heart defects
m	10	T	Congenital heart defects

F=frontal, T=temporal, m = male, f = female