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Nrf2 Signaling in Sodium Azide-Treated Oligodendrocytes Restores Mitochondrial Functions

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Abstract

Mitochondrial dysfunctions mark a critical step in many central nervous system (CNS) pathologies, including multiple sclerosis (MS). Such dysfunctions lead to depolarization of mitochondrial membranes and imbalanced redox homeostasis. In this context, reactive oxygen species (ROS) are potentially deleterious but can also act as an important signaling step for cellular maintenance. The transcription factor nuclear erythroid2 like 2 (Nrf2), the key regulator in the cellular oxidative stress-response, induces a battery of genes involved in repair and regeneration. Here, we investigated the relevance of Nrf2-signaling for the prevention of cellular damage caused by dysfunctional mitochondria. We employed sodium azide (SA) as mitochondrial inhibitor on oligodendroglial OliNeu cells *in vitro*, and the cuprizone model with wild type and GFAP-Cre+:Keap1$^{loxP/loxP}$ mice to induce mitochondrial defects. The importance of Nrf2 for cellular functions and survival after SA-treatment was elucidated by *in vitro* knockdown experiments with shRNA directed against Nrf2 and its inhibitor Keap1 as well as by methysticin treatment. Metabolic activity, cytotoxicity, and depolarization of the mitochondrial membrane were analyzed after SA-treatment. The expression of Nrf2 target genes as well as endoplasmic reticulum stress response genes was additionally measured by real-time PCR (*in vitro*) and PCR gene arrays (*in vivo*). Treatment of OliNeu cells with SA resulted in significant depolarization of the mitochondrial membrane, decreased metabolic activity and increased cytotoxicity. This was partly counteracted in Nrf2-hyperactivated cells and intensified in Nrf2-knockdown cells. Our studies demonstrate a key of Nrf2 in maintaining cellular functions and survival in the context of mitochondrial dysfunction.

Keywords: Nrf2, oligodendrocytes, mitochondrial dysfunction, complex IV, sodium azide, depolarization

Introduction

Although the pathomechanisms of MS are not fully understood there is ample evidence that mitochondrial dysfunctions play a crucial role in the neurodegenerative and neuroinflammatory aspects of this disease (Campbell et al. 2014; Witte et al. 2014). Underlying processes that initially cause mitochondrial dysfunctions have not been completely investigated. However, distinct mutations in mitochondrial (mt) DNA are known to increase the risk of MS development, and the majority of these mutations concern subunits of the mitochondrial electron transport chain (Patergnani et al. 2017). Also of note, mtDNA is prone to mutations with a mutation rate that is about 10-fold higher than in chromosomal DNA (Linnane et al. 1989). This finding might be explained by the close spatial relation of mtDNA with the inner mitochondrial membrane, where endogenous ROS are generated. In addition, neuroinflammatory processes observed in MS lesions are increasingly recognized to be

associated with mitochondrial dysfunctions, thereby contributing to neuronal injury and degeneration (Carvalho 2013).

Mitochondria are primarily involved in adenosine triphosphate (ATP) synthesis, Ca^{2+} regulation, and the production of endogenous ROS. Consequently, mitochondrial damage in the course of MS may lead to both inadequate energy production as well as oxidative stress. Elevated ROS-levels are harmful to both neurons and glia, however, oligodendrocytes, the myelin producing cells of the CNS, are especially vulnerable to both oxidative stress and mitochondrial dysfunctions (Juurlink et al. 1998; Lan et al. 2018). This vulnerability can be explained by the unique oligodendrocyte physiology. A high lipid synthesis rate of oligodendrocytes leads to elevated auto-oxidation of cholesterol to 7-keto- (7KC), 7a-hydroxy- and 7b-hydroxy-cholesterol. This lipid molecule is involved in demyelination by inducing oligodendrocyte cell death via a disruption of mitochondrial membrane structures, the activation of caspase-3-dependent apoptosis and the induction of oxidative stress (Leoni et al. 2016). Furthermore, oligodendrocytes display a very high metabolic rate, store large amounts of intracellular iron and possess only low concentrations of the anti-oxidative enzyme glutathione (Bradl and Lassmann 2010; Lan et al. 2018), all of them characteristically contributing to oligodendroglial vulnerability to oxidative damage. Thus, mitochondria-related mechanisms such as decreased activity of the mitochondrial electron transport chain by damage or mutation, concomitant impairment of mitochondrial energy metabolism and inefficient removal of ROS might contribute to oligodendrocyte loss during MS lesion formation and disease progression (Licht-Mayer et al. 2015).

In general, all eukaryotic cells possess highly effective mechanisms to counteract and prevent oxidative challenges. The main regulation of these responses is orchestrated by the nuclear factor (erythroid-derived 2)-like 2/antioxidant response element system (Nrf2/ARE system) that recently has come into the focus of MS research (Lu et al. 2016). Upon induction, Nrf2 is stabilized in its “closed” state bound to two molecules of its inhibitor Kelch ECH associating protein 1 (Keap1). As a consequence, newly synthesized Nrf2 is not further scavenged by Keap1 and can thereby bind to the ARE sequence within the promoter region of its target genes (Baird et al. 2013). With respect to MS, the targeting of Nrf2/ARE signaling by dimethyl fumarate promotes mitochondrial biogenesis, restores gene expression and function (Hayashi et al. 2017), and patients treated with dimethyl fumarate benefit from a significant reduction in relapse rate, disability progression and brain lesions (Al-Sawaf et al. 2015; Bomprezzi 2015). In oligodendrocytes, the activation of the Nrf2/ARE system triggers the expression of endoplasmic reticulum-stress related genes such as Ddit3 and ATF3 (Teske et al. 2018).

In this study, we investigated a particular aspect of oligodendrocyte physiology related to MS pathophysiology, i.e. the potential of this glial cell type to counteract mitochondrial challenges by activating the Nrf2/ARE pathway.

Materials and Methods

Cell culture

The murine oligodendroglial cell line OliNeu was cultured in SATO medium containing 2% fetal bovine serum (FBS). SATO is composed of DMEM (Gibco Life Technologies, #41966-029) with 1% bovine serum albumin (BSA, Carl ROTH, #CP84.2), 1% N2 Supplement (Gibco Life Technologies, #17502-048), 1% Penicillin/Streptomycin 100x10.000 U/ml (Gibco Life Technologies, #15140-122), 0.1% N-Acetylcystein (Sigma-Aldrich, #9165), 0.002% Biotin (Sigma-Aldrich, #B4639).

Cells were treated with concentrations in a range from 1 mM to 100 mM of sodium azide (SA) for up to 24 h. Methysticin pre-treatments were done with 50 µM for 6 h prior to SA stimulation. For qRT-PCR experiments, OliNeu cells were seeded on poly-D-lysine (PDL, Sigma #P6407) coated 6-well plates at a density of 3 x 10^5 cells/well and exposed to starvation conditions (SATO containing 0.5% FBS) for 48 h. After the treatment regimen, the cells were washed once with 1x PBS and lysed in PeqGold (peqlab, #30-1010) for RNA isolation and subsequent qRT-PCR (see below). Methysticin treatment (LKT Laboratories, #M1679) served as a positive control for Nrf2-Induction.

Lactate dehydrogenase release (LDH) measurement was performed using CytoTox 96 Non-Radioactive Cytotoxicity Assay (LDH, Promega, #G1780). Metabolic activity was determined via CellTiter-Blue Cell Viability Assay (CTB, Promega, #G8080). Both assays were used according to the manufacturer´s instruction. For experiments, cells were plated in 96-well plates at a density of 1.5 x 10^4 cells/well and grown in phenol red-free SATO. Methysticin pre-treatments were done with 50 µM for 6 h prior to SA treatments. After this, the absorbance (LDH) and the fluorescence (CTB) of the supernatant were measured using the Infinite M200 microplate reader (Tecan, Switzerland). CytoTox 96 results were normalized to lysed cells (100% dead cells) and in case of shRNA experiments additionally related to shNT group. CTB data was normalized to untreated control cells (=100% viability).

Animal experiments

The relevance of Nrf2 activity during mitochondrial inhibition *in vivo* was investigated by applying the cuprizone model to wild type (WT) as well as astrocyte-specific Keap1-KO mice (GFAP-Cre+::Keap1$^{loxP/loxP}$). Therefore, mice were either fed with normal chow (n = 4) or with chow containing cuprizone for 1 week (n = 3). The mice were euthanised by cervical dislocation, and brain tissue was isolated for gene array analysis (see below). The experiments were conducted in accordance with the Directive 2010/63/EU of the European Parliament and were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen.

Gene silencing

Nrf2 and Keap1 gene expression was silenced in OliNeu cells by lentiviral shRNA delivery (see Fig. S1 in the supplemental material). For that purpose, we used commercially available pLKO.1 vectors encoding shRNA sequences for either Nrf2 (TRC clone ID: TRCN0000054659) or Keap1 (TRC clone ID: TRCN0000099447). These vectors are part of the MISSION® shRNA contingent distributed by Sigma Aldrich (Munich, Germany). For virus production HEK293T cells (ATCC®CRL-11268™) were co-transfected with the shRNA expression vector pLKO.1, the VSV-G envelope expressing pMD2.G construct (addgene #12259) and the 2nd generation lentiviral packaging plasmid psPAX2 (addgene #12260). Transfection was conducted by using jetPEI® (Polypus Transfection™, #101) transfection reagent according to the manufacturer´s instruction (Teske et al. 2018). For transduction, cells were re-plated on poly-D-lysin coated culture dishes in SATO supplemented with 2% FBS. The cells were exposed to the virus containing supernatant for 16 h, afterwards they were washed with PBS and cell culture medium was replaced. After 72 h, puromycin (Carl ROTH, #0240.1) was constantly supplemented to the medium at a concentration of 2 µg/mL to assure sufficient selection of transduced cells. After positive selection, the gene expression levels of Nrf2 and Keap1 were measured by qRT-PCR to determine knockdown efficiency. Control cells were transduced with a pLKO.1 construct expressing a shRNA without any target in mammals (pLKO.1-shNonTarget; #SHC216) to avoid data misinterpretation based on transduction side effects (Teske et al. 2018).

Measurement of mitochondrial membrane potential levels

Mitochondrial membrane potential was quantified using the Muse™ MitoPotential Kit (Merck, Germany, #MCH100110) together with the benchtop flow cytometry device *Muse Cell Analyzer* (Merck, Germany). To this end, cells were seeded onto 9.6 cm^2 (~$5x10^5$ cells) plastic culture dishes pre-coated with 10 µg/mL poly-D-lysine in modified SATO-medium. Modified SATO is composed of DMEM with 1% N2 supplement (Gibco Life Technologies, #17502-048), 0.1% Tri-Iodo-thyronine (Sigma-Aldrich, #T6397), 0.016% L-thyroxin (Sigma-Aldrich, #T1775) and 0.05 % Gentamicin (Gibco Life Technologies, #15710049). After 47 h, cells were exposed to 100 mM SA for another 60 min and then harvested using TrypLE™ Express enzyme solution (Gibco Life Technologies, #12604039). Thereafter, cells were stained and flow cytometry analyses were performed according to the manufacturer´s instruction. To avoid any bias, gating of the different cell populations was performed in a blinded manner (NT). Experiments were performed with six biological and one technical replicate.

Gene expression

Gene expression levels were measured by real-time reverse transcription-PCR (qRT-PCR). For this purpose, cells were homogenized in PeqGold RNA Pure (PeqLab, #30-1010) and RNA

isolated according to the manufacturer's protocol. The measurement of the RNA concentration und purity was performed by the NanoDrop 1000 (Thermo Fisher Scientific, USA). For reverse transcription the MMLV reverse transcriptase kit (Invitrogen, #28025013) was used. The qRT-PCR was performed by SYBR Green SensiMixTM (Bioline, #QT615-05) and carried out on the MyIQ RT-PCR detection system (Biorad, Germany) applying a standardized protocol as published previously (Clarner et al. 2011). Relative quantification was performed using the ΔCq method with hypoxanthine guanine phoshoribosyl transferase (Hprt) as a reference gene. Primer sequences were as follows:

Hprt1 (F: TCAGTCAACGGGGGACATAAA, R: GGGGCTGTACTGCTTAACCAG),
Ddit3 (F: GCATGAAGGAGAAGGAGCAG, R: CTTCCGGAGAGACAGACAGG),
Atf3 (F: TTTGCTAACCTGACACCCTTTG, R: ATGGCGAATCTCAGCTCTTCC),
Nrf2 (F: CCCAGCAGGACATGGATTTGA, R: AGCTCATAGTCCTTCTGTCGC),
Keap1 (F: GGCAGGACCAGTTGAACAGT, R: CATAGCCTCCGAGGACGTAG),
Nqo1 (F: AGAGAGTGCTCGTAGCAGGAT, R: CTACCCCCAGTGGTGATAGAAA).

***In vivo* gene array**

RNA from the *corpus callosum* of cuprizone- and normal chow-fed WT as well as GFAP-Cre+::Keap1$^{loxP/loxP}$ mice were isolated using the RNeasy Lipid Tissue Mini Kit (Cat.#: 74804, Qiagen) following the manufacturer's instructions. RNA was quantified as above and subsequently reverse-transcribed using the RT^2 First Strand Kit (Cat.#: 330404, Qiagen) as recommended. Obtained cDNA samples were used for mouse Unfolded Protein Response RT^2 Profiler PCR arrays (Cat.#: PAMM-089, Qiagen) comprising 84 key genes involved in ER stress response.

Statistics

Statistical analyses were performed using Prism 5 (GraphPad Software Inc., San Diego, CA, USA). All data are given as arithmetic means + SEM. A p value of < 0.05 was considered to be statistically significant. Applied statistical tests including the number of individual independent experiments (n) are given in the respective figure legends. No outliers were excluded from the analyses.

Results

Inhibition of complex IV by SA induces mitochondrial dysfunction *in vitro*

Wild type OliNeu cells were stimulated with different concentrations of SA for 24 h to specifically inhibit the mitochondrial complex IV and to investigate the impact on mitochondrial membrane polarization. Methysticin pre-treatment was conducted to investigate whether pharmacological Nrf2 induction prevents SA-mediated cytotoxicity and induces the expression

of the stress-related genes Ddit3 and Atf3. SA treatment with 1 mM of SA neither affected metabolic activity nor cell viability (Fig. 1A and B, 2nd column) in OliNeu cells. Stimulation with 10 mM of SA decreased metabolic activity by 18 % in comparison to untreated cells despite any signs of cytotoxicity (Fig. 1A and B, 3rd column). Increasing the SA concentration to 100 mM resulted in significant reduction of metabolic activity by 82 % and increased cytotoxicity respectively (Fig. 1A and B, 4th column). Moreover, this treatment regimen significantly increased the percentage of cells displaying mitochondrial membrane depolarization (Fig. 1C). Methysticin pre-treatment did not counteract the early cytotoxic effects of SA as shown in Fig. 1D (6h time point). However, it significantly reduced cytotoxicity when OliNeu cells were stimulated for 24 h (Fig. 1D, 24h time point). The qRT-PCR experiments revealed that Ddit3 gene expression was significantly increased after methysticin and methysticin + SA but not after SA single treatment (Fig. 1E). Both methysticin as well as SA treatment induced the gene expression of Atf3 significantly. Notably, Atf3 expression was even more increased after co-treatment with both substances (Fig. 1F).

ER stress responses in mice with Nrf2-hyperactivation

The induction of ER-stress related factors such as Ddit3 was further investigated *in vivo* using mice with an GFAP-specific Keap1-deletion (see Materials and Methods) applying Mouse Unfolded Protein Response RT^2 Profiler PCR arrays. Results of this study are summarized in supplementary data 1. Note that Ddit3 was induced in both WT and Keap1-deficient animals in response to cuprizone. In total, 13 ER-stress related genes were altered (7 up- and 6 down-regulated) in response to cuprizone in WT mice. In Keap1-deficient animals 21 genes were regulated (19 up- and 2 down-regulated). However, none of these cuprizone-regulated genes showed a significant difference in between WT and Keap1-KO, indicating comparable response to cuprizone for both genotypes.

Confirmation of shRNA–mediated knockdown of *Keap1* and *Nrf2* in OliNeu cells

After transduction of OliNeu cells with lentiviral particles containing shRNA constructs against either Keap1 (shKeap1) or Nrf2 (shNrf2), knockdown effects and efficiency were analyzed. Control cells were transduced with a construct encoding for a non-target shRNA sequence (shNT). Selected cultures were viable and displayed comparable densities under normal growth conditions. Representative images of each are depicted in Fig. 2A. To test for shRNA-mediated knockdown efficiency, we conducted qRT-PCR experiments. For this purpose, the gene expression of *Keap1, Nrf2* as well as the Nrf2 target gene NAD(P)H quinone dehydrogenase 1 (*Nqo1)* was analyzed. Nqo1 has been chosen as readout because to our knowledge, Nrf2 is the only described regulator of its gene expression. These experiments revealed knockdown efficiencies of 72 % for *Keap1* (Fig. 2B) and 67 % for *Nrf2* (Fig. 2C). This was further confirmed by the analysis of *Nqo1* expression as seen in Fig. 2D. Methysticin treatment, a potent Nrf2 inducing substance (Fragoulis et al. 2017; Wruck et al. 2008), was

used as positive control. Besides from significant differences of *Nqo1* expression in between unstimulated cultures, methysticin effectively induced the expression of *Nqo1* in both shNT and shKeap1 cells. In contrast, Nrf2-deficient cells did not display a significant induction of *Nqo1*.

Nrf2 prevents SA-mediated cell death and restores mitochondrial functions

To analyze the effects of Nrf2-expression on SA-induced mitochondrial dysfunctions, cells were treated with 100 mM SA for 2, 6 and 24 h and the metabolic activity, LDH-release and mitochondrial depolarization were measured. Results are summarized in Fig. 3. As shown in A, the metabolic activity decreased in a time-dependent manner in all cell lines. Differences between the shNrf2 and the other cell lines were only found after 2 h and 6 h SA-treatment. SA-treatment for 2 h already decreased the metabolic activity of shNT cells to 71 % of untreated control cells. The Keap1 knockdown tended to be beneficial in this context (79 %, without statistical significance). In contrast, Nrf2 knockdown clearly decreased metabolic activity after 2 h SA-treatment (44 %) compared to shNT as well as shKeap1 cells (Fig. 3A, 2 h panel). The decrease of metabolic activity was further intensified after 6 h SA-treatment. Although the Keap1 knockdown did not display any benefit, Nrf2 silencing resulted in worsening of SA-toxicity (Fig. 3A, 6 h panel; 39 % vs. 43 % vs. 13 %). Cell viability assays (Fig. 3B) highlighted the necessity of Nrf2-signalling for cell survival after SA-treatment. Similar to the observed SA effects on metabolic activity, LDH-release showed a significant genotype effect (*** $p < 0.001$) which was highest in the shNrf2-group at all investigated time points. Of note, Nrf2 hyper-activation due to Keap1 knockdown only slightly improved cell viability after SA-treatment, since LDH-release was almost comparable to treated shNT cells independent from the stimulation time point. Since the observed protective effects of Nrf2-signalling might be due to restored mitochondrial functions, amount of depolarized cells after SA stimulation was investigated in the next step. As shown in Fig. 3C, Keap1 knockdown significantly reduced the amount of depolarized cells after 1 h SA-stimulation compared to shNT cells. Additionally, the total amount of living cells was increased (Fig. 3D). The opposite effect was observed in SA-stimulated Nrf2 knockdown cells (Fig. 3E and F).

Discussion

Mitochondria are regulators of the intracellular redox as well as Ca^{2+} homeostasis. Furthermore, they are a relevant source of ROS which are crucial for the control of a variety of intracellular signaling pathways (Guzman-Villanueva and Weissig 2017) such as Nrf2/ARE. Imbalances of mitochondrial homeostasis have been described to be critically involved in pathophysiological different diseases such as Morbus Alzheimer (Fragoulis et al. 2017), Morbus Huntington (Hroudova et al. 2014), Morbus Parkinson (Das and Sharma 2016),

Amyotrophic lateral sclerosis (ALS) (Faes and Callewaert 2011) and MS (Campbell and Mahad 2018; Campbell et al. 2014).

Mitochondrial dysfunctions are characterized by decreased respiratory enzyme activity and ATP production and contribute to the age-related risk to develop neurodegenerative diseases (Lin and Beal 2006). Lower respiration rates lead to lower intracellular ATP concentrations which directly affect and decrease the mitochondrial potential. This can initiate mitochondrial swelling and ultimately trigger apoptotic cascades (Irvin et al. 2015; Safiulina et al. 2006). The impact of lower ATP concentrations on oxidative imbalances is bi-directional and further impairs mitochondrial function (Cassina et al. 2008; Cozzolino and Carri 2012). l. The mitochondrial respiratory chain consists of five complexes (I–V). Most of the oxygen (90 %) is consumed by complex IV, the rate limiting step of electron transport chain. Complex IV defects in acute MS lesions impair cellular functions of oligodendrocytes, astrocytes, and axons (Ziabreva et al. 2010). Oligodendrocytes are known to be particularly susceptible to pathologic events such as hypoxia, excitotoxicity, reactive oxygen, and nitrogen species, and the subsequent loss of oligodendrocytes has been functionally linked to mitochondrial dysfunctions (Ziabreva et al. 2010). The use of SA as an inhibitor of mitochondrial complex IV (Bennett et al. 1996) is a well-characterized model to induce “chemical hypoxia” (Ziabreva et al. 2010) and membrane depolarization in oligodendrocytes (Teske et al. 2018). Since SA-treatment leads to both a decrease in cellular ATP levels (Harvey et al. 1999) and an increase in net ROS production (Gao et al. 2018), it can be considered as suitable model to investigate mitochondrial dysfunctions *in vitro*. Furthermore, SA has been described to induce mitochondrial swelling in neurons, thereby inhibiting axonal transport (Kaasik et al. 2007).

Other processes by which mitochondrial dysfunctions are thought to contribute to the development of neurodegenerative diseases are the disturbance of the production and removal of endogenous ROS leading to a net ROS over-production and thereby to oxidative stress. This promotes then a vicious cycle in which ROS can further damage mitochondria, causing more free-radical generation and a loss of the antioxidant capacity (Lin and Beal 2006). With respect to multiple sclerosis, mitochondrial dysfunctions and oxidative stress are likely to critically contribute to lesion development and progression, and oxidative stress markers can be found in the blood plasma, saliva, liquor and brain tissue of MS patients (De Riccardis et al. 2018; Karlik et al. 2015; Mao and Reddy 2010; Morel et al. 2017; Shu et al. 2017). Oxidized lipids (phospholipids), proteins and DNA have been demonstrated in astrocytes, macrophages, myelin proteins, damaged axons and in particular in oligodendrocytes (Cross et al. 1998; Haider et al. 2011; Liu et al. 2001; Qin et al. 2007; van Horssen et al. 2008).

Nrf2 is the main regulator of cellular defense-mechanisms preventing oxidative damage (Bellezza et al. 2018). A recent study by Licht-Mayer and colleagues demonstrated the

presence of high levels of nuclear Nrf2 within damaged oligodendrocytes in MS lesions, indicating the relevance of the oligodendroglial Nrf2/ARE system during MS lesion formation (Licht-Mayer et al. 2015). In their study, the authors speculated that the pharmacological induction of Nrf2 by fumarate might cause additional cell stress in oligodendrocytes or neurons already exposed to severe oxidative injury (and thereby already displaying high endogenous Nrf2-activity).To further investigate this assumption *in vitro*, the cell lines described by Teske and colleagues (Teske et al. 2018) were utilized. These cells exhibit a stable and functional expression of shRNAs directed against both Nrf2 and its endogenous inhibitor Keap1. These novel cell lines are therefore useful tools to study the role of Nrf2 in the context of demyelinating diseases or oligodendrogliopathy (Draheim et al. 2016).

We recently demonstrated that oxidative stress in oligodendrocytes activates an ER stress response in a Nrf2-dependent manner (Teske et al. 2018). In the current study, we show that the pharmacological activation of Nrf2 by methysticin leads to a similar inductive effect *in vitro*. Furthermore, we used the cuprizone animal model to induce oligodendrocyte specific stress and death by inhibiting mitochondria (Acs et al. 2013). As expected, such treatment induced ER stress related genes in wild type animals. In animals displaying astrocyte-specific hyperactivation of Nrf2, a higher induction of ER stress related genes was expected. However, the induction of these genes was comparable in between both genotypes. This might be explained by the fact that due to Nrf2-hyperactivation the cuprizone-induced damage and neuroinflammation is reduced (Draheim et al. 2016) and the activation of a potentially protective ER stress response is moderated or not required.

Additionally, our results demonstrate the relevance of Nrf2-signaling for the maintenance of mitochondrial function in oligodendrocytes. While Nrf2-deficiency intensifies both cell death as well as membrane potential breakdown in an SA-mediated manner, both genetic, as well as the pharmacological hyper-activation of Nrf2 lead to a (partial) restoration of mitochondrial functions, improving cell survival. Therefore, Nrf2-activation in oligodendrocytes might represent a possible approach to counteract mitochondrial dysfunctions even under circumstances of prevalent oxidative damage. Further studies using oligodendrocyte-specific hyperactivation of Nrf2 may elucidate its potential to counteract mitochondrial dysfunctions in brain diseases that involve oligodendrocyte damage or loss.

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Compliance with Ethical Standards

Conflict of interest

All authors declare that there are no financial or other relationships that might lead to a conflict of interest.

Figures

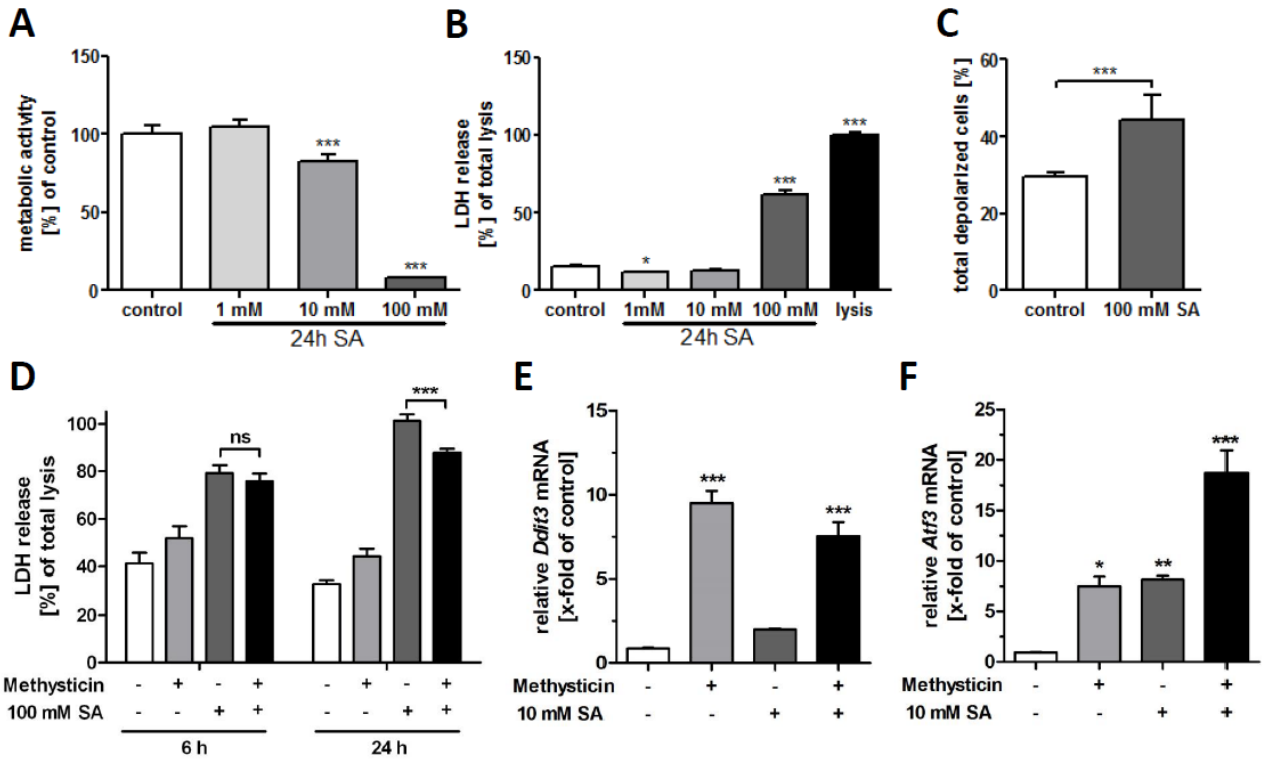
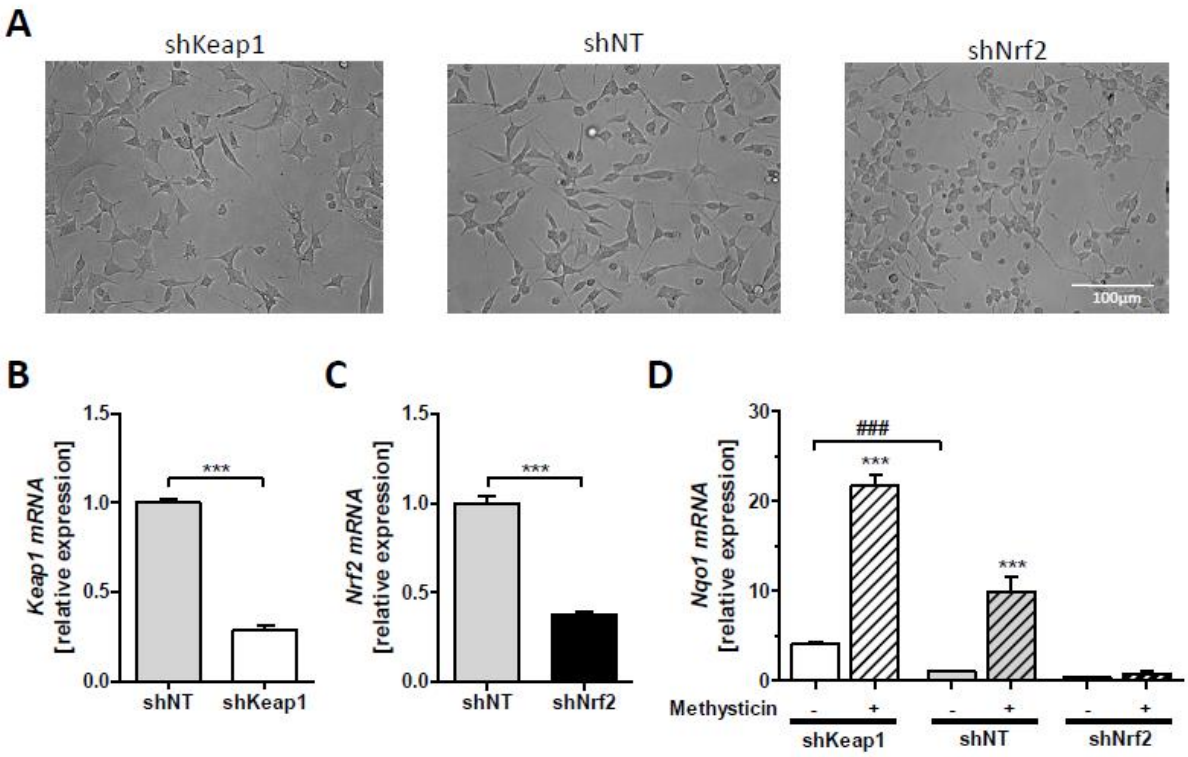


Fig. 1: Inhibition of complex IV by SA induces cellular stress and mitochondrial dysfunction which are partly prevented by methysticin pre-treatment

In (A) results from cell viability measurements of OliNeu cells treated with SA for 24 h are shown. SA decreases metabolic activity (A) and increases cytotoxicity (B) in a dose-dependent manner. (C) Mitochondrial membrane potential in OliNeu cells after 1 h SA (100 mM) exposure is shown. (D) LDH release after SA treatment for 6 and 24 h with or without methysticin pre-treatment. Gene expression of (E) Ddit3 and (F) Atf3 after methysticin and SA treatment was analyzed by qRT-PCR. Comparison of control vs. SA-treated cultures was performed by students t-test (C, n=6) or 1way ANOVA followed by Bonferroni post hoc test (A, B, D & E n=4). Comparisons of multi-parametric data were achieved by 2way ANOVA followed by Bonferroni *post-hoc* test (D, n=6). Significant differences with respect to controls or as indicated by brackets are depicted as * $p < 0.05$ & *** $p < 0.001$.



368 **Fig. 2: shRNA–mediated knockdown of *Keap1* and *Nrf2* in OliNeu cells**

369 (A) Representative pictures of shRNA-transduced cells are shown. In (B & C) the relative
370 *Keap1* or *Nrf2* mRNA expression of the respective shRNA clones is shown in comparison to
371 non-target shRNA cells (shNT); n=9. Students t-test was performed to test for statistical
372 significance. In (D), the transcription levels of the Nrf2-target gene *Nqo1* is shown in the distinct
373 cell lines. Methysticin treatment (50 µM) was used to induce Nrf2-dependent induction of Nqo1
374 mRNA expression; n=4. Note that basal Nqo1 expression was elevated in shKeap1 cells in
375 comparison to shNT cells. Methysticin failed to induce Nqo1 expression in shNrf2 cells.
376 Significant differences were calculated by 1way ANOVA followed by Bonferroni *post-hoc* test
377 and are indicated as * $p < 0.05$, & *** $p < 0.001$.

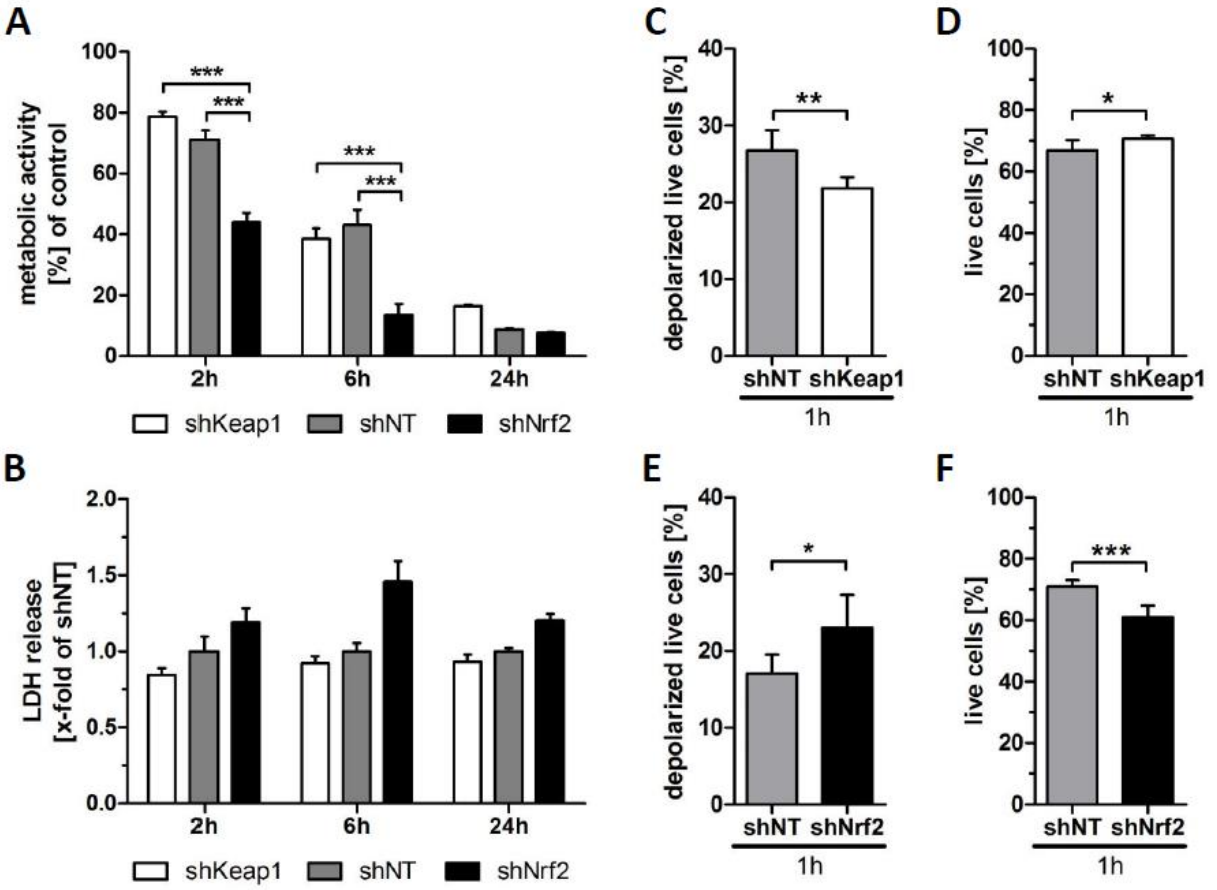


Fig. 3: Nrf2 prevents SA-mediated cell death and restores mitochondrial functions

Metabolic activity (A) and cytotoxicity (B) of shKeap1, shNT and shNrf2 OliNeu cells after 2 h, 6 h and 24 h SA treatment (n≥6) are shown. Note that Nrf2-deficient cells display reduced metabolic activity after 2 h and 6 h treatment (A). Furthermore, Nrf2-deficiency led to higher cell death (LDH-release) after 6 h SA exposure. Statistical significance was evaluated using 2way ANOVA with subsequent Bonferroni *post-hoc* test in case of significant interaction (significant differences are indicated). Mitochondrial membrane potential measures in shNT, shNrf2 and shKeap1 OliNeu cells after 1 h SA (100 mM) exposure are shown in C-F. Comparison of differences between shNT and shKeap1 as well as shNT and shNrf2 cells (n=6) was done applying Students t-test. Significant differences with respect to control cultures are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Viable cells with a breakdown of the mitochondrial membrane potential (C, E) and the sum of viable cells with or without a breakdown of the mitochondrial membrane potential (D, F) were separately compared. Note that the hyper-activation of Nrf2 by Keap1-knockdown protects cells from mitochondrial depolarization and death, whereas Nrf2-deficiency led to increased depolarization and death.

Supplementary Figure

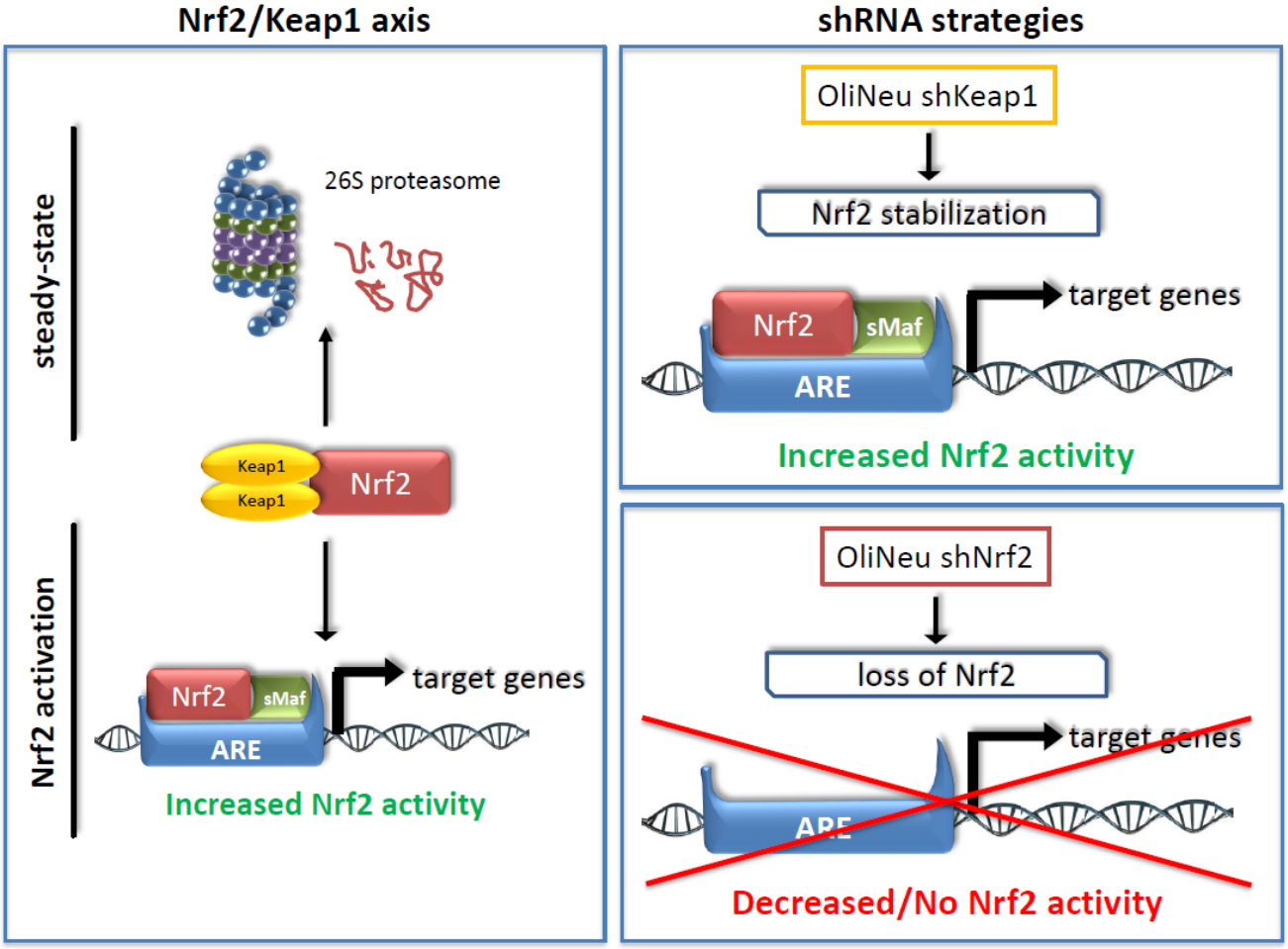


Fig. S1: The Nrf2/Keap1 axis and applied shRNA strategies for KD experiments (left side) Under steady-state conditions, Nrf2 is scavenged by its intracellular inhibitor Keap1. This interaction leads directly to 26S proteasome-mediated degradation of Nrf2. In the presence of Nrf2 activating stimuli, Keap1 dissociates from Nrf2 and enables its nuclear translocation. There, Nrf2 binds to the anti-oxidant response elements (ARE) within the promoter region of its target genes and thereby induces or enhances their gene expression. (right side) In our experiments, we applied shRNA against Keap1 to decrease Keap1 protein content and thereby boost the Nrf2 activation in OliNeu cells even under steady-state conditions. The use of shRNA directed against Nrf2 was chosen to decrease Nrf2 activity in OliNeu cells.

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