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Maastricht University

# Faculty of Medicine and Life Sciences
## *School for Life Sciences*
## Master of Biomedical Sciences

***Master's thesis***

***Modulation of EAAT3 in oligodendrocyte precursor cells: inhibitor hampers differentiation into mature oligodendrocytes***

**Evelien Nouboers**

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

**SUPERVISOR :**

Prof. dr. Tim VANMIERLO

**MENTOR :**

Mevrouw Lieve VAN VEGGEL

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# Inhibition of excitatory amino acid transporter 3 hampers differentiation of oligodendrocyte precursor cells

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*Running title: *EAAT3 modulation in oligodendrocyte precursor cells*

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## ABSTRACT

**In multiple sclerosis, the immune system produces reactive oxygen species (ROS) that attack and damage oligodendrocytes and their produced myelin. Differentiation of oligodendrocyte precursor cells (OPCs) is crucial to replenish the oligodendrocyte pool and improve remyelination. However, OPCs are vulnerable to ROS due to their low basal level of the antioxidant glutathione. Glutathione production is limited by the amount of cysteine available in the cell. We aimed to modulate cysteine transport in OPCs by regulating the excitatory amino acid transporter 3 (EAAT3). In this study, the effect of modulating EAAT3 on OPC viability and differentiation was investigated. Two viability assays were used to demonstrate the effect of a cysteine free environment and an EAAT3 modulation on the viability of OPCs and an OPC-like cell line, Oli-neu; both stimulated with ROS. Two differentiation markers, O4 and MBP were investigated to illustrate the effects on OPC differentiation. To quantify the amount of cysteine, a cysteine probe was optimised. We demonstrated that a cysteine free environment decreased cell viability in Oli-neu cells and OPCs. This indicates a vital role of cysteine in the cells. An EAAT3 inhibition decreased cell viability and hampered differentiation in OPCs, suggesting a crucial role of EAAT3 in OPCs. The EAAT2 agonist derivative D8 did not affect the function and viability of OPCs. In conclusion, EAAT3 modulation could be an interesting target for stimulating differentiation, which could enhance remyelination.**

## INTRODUCTION

Multiple sclerosis (MS) is a chronic neuroinflammatory disease of the central nervous system (CNS) (1,2). With a median prevalence of 33 per 100 000 people, MS is one of the most common causes of neurological disability in young adults worldwide (2,3). It is characterised by demyelination, axonal loss and neurodegeneration in the CNS (1,4-6). In MS, neuroinflammation causes damage to both oligodendrocytes and their produced myelin, leading to decreased functional, mechanical, and metabolic support of neurons. This pathology is called demyelination of the CNS. Consequently, demyelinated axons are prone to injury and degeneration. This process manifests itself clinically in neurological deficits and disability (6-9). The majority of patients start with a relapsing-remitting multiple sclerosis (RRMS) disease course. RRMS is characterised by periods of neurological dysfunction followed by periods of remission (1,5,10). 80% of RRMS patients experience disease progression, called secondary progressive multiple sclerosis (SPMS) within 20 years. 10-20% of the patients show progression from the onset of the disease, skipping the RRMS stage. This is called primary progressive multiple sclerosis (PPMS) (1,5,11).

While immunomodulatory and anti-inflammatory treatments can suppress neuroinflammation and reduce the severity and frequency of relapsing episodes, they are less effective in the progressive stage of MS. A reason for this is that neurodegeneration, as a consequence of demyelination, is the biggest factor of disease progression in progressive MS. Furthermore,

neuroinflammation is often decreased in the progressive stage (1,3). Therefore, new treatments focusing neurodegeneration by modulating remyelination in the progressive stage of MS are needed.

Current research focusses on the role of oligodendrocyte precursor cells (OPCs) in remyelination. Differentiation of resident OPCs into mature oligodendrocytes is crucial to replenish the oligodendrocyte pool in the CNS. Differentiation is triggered by various stimuli, including damage to, or the loss of mature oligodendrocytes (6,9,12). Furthermore, the differentiation of OPCs into new oligodendrocytes promotes remyelination of demyelinated axons. However, oligodendrocyte differentiation diminishes when OPC function is impaired (6,8,12).

Previous studies have reported that while the total OPC pool is not affected in MS patients, OPC function is impaired in the chronic phase of MS (7,13,14). It has been hypothesised that elevated pro-oxidant levels, present in MS, suppress the capacity of OPCs to differentiate. An imbalance between the production of pro-oxidants, such as reactive oxygen species (ROS), and endogenous antioxidant defences leads to oxidative stress. This can eventually lead to cell cycle arrest, proliferation inhibition, and cell death (15,16). ROS may induce damage to OPCs by epigenetic and metabolic changes that could lead to a stress-induced differentiation block (8). In contrast, ROS can alter the expression of differentiation regulators and reduce mature oligodendrocyte and myelin markers (8). OPCs are vulnerable to oxidative stress due to their low antioxidant defence and low basal levels of the antioxidant glutathione (GSH) (6,17). GSH is a metabolite synthesised from glutamic acid, cysteine, and glycine in two steps. First, cysteine and glutamic acid are combined by glutamate-cysteine ligase. This dipeptide is then combined with glycine by glutathione synthase. GSH has two main roles. First, it can directly react with free radicals such as superoxide (O2-) non-enzymatically. Secondly, it acts as a reducing agent in the presence of glutathione peroxidase (GPx) (15,17-20). GSH reduces hydrogen peroxide (H2O2) by donating an electron leading to the formation of H2O and glutathione disulphide (GSSG) (18,19). GSSG can then be reduced back into GSH by glutathione reductase. In the CNS, GSH is crucial for the prevention of oxidative stress-induced damage to cells. However, GSH in the brain declines with ageing, which increases ROS levels in the cell. This can contribute to the pathogenesis of neurodegenerative disorders, such as MS (20,21).

While GSH levels are low in OPCs, it can potentially protect the cells from oxidative stress (17). However, disrupting the ROS/GSH balance can lead to damage to biomacromolecules in the cell (15,20). Interestingly, H2O2 is known to deplete GSH in OPCs, further lowering its antioxidative defence (8). Currently, there is increasing interest in regulating OPC homeostasis in an environment of oxidative stress in the field of MS. However, it is still unknown whether stimulating endogenous defences against oxidative stress, such as GSH, will be sufficient to protect OPCs and restore differentiation into oligodendrocytes.

This study will focus on stimulating the production of GSH, limited by the availability of intracellular cysteine. By increasing intracellular cysteine, the production of GSH will also be enhanced (17). Previous research suggests that the excitatory amino acid transporter 3 (EAAT3), a transporter of glutamate and cysteine, is the major transporter of cysteine for the synthesis of GSH in neurons (22,23). EAAT3, encoded by the SLC1A1 gene, belongs to the EAAT family, which are high-affinity Na+-dependent carriers important in maintaining glutamine homeostasis (23-25). The function of EAAT3 is to regulate glutamatergic transmission. Unlike other transporters of this family, EAAT3 also transports cysteine (23-26). Furthermore, cysteine uptake mediated by EAAT3 is considered to be essential for GSH synthesis. A knockdown of EAAT3 reduced both cysteine uptake and intracellular levels of GSH in neurons, which increased $H_2O_2$ vulnerability in neurons (26,27). Furthermore, oxidative stress-induced brain damage was greater and brain GSH level was lower in EAAT3 deficient mice (26).

While EAAT3 is also present in the oligodendrocyte lineage, it is unknown whether the transporter is also the primary provider of cysteine for GSH synthesis in OPCs. This transporter may provide a cysteine pool for GSH synthesis in OPCs, potentially increasing the survival rate and antioxidant defences of OPCs (24).

The goal of this study is to determine whether modulation of EAAT3 can protect OPCs under oxidative stress. Because there is no EAAT3 agonist yet, a derivative of an EAAT2 activator will be tested to assess its protective effect. The viability and differentiation potential of OPCs with an EAAT3 modulation are examined *in vitro* after stimulation with glucose oxidase (an $H_2O_2$ producing enzyme). **We hypothesise that modulation of EAAT3 in OPCs will protect the cells from oxidative stress and improve differentiation *in vitro*.**
This research will elaborate on the role of EAAT3 in OPC viability and differentiation in an environment with oxidative stress. Our study will provide important insights into the endogenous oxidative defences in OPCs, especially in diseases of the CNS involving oxidative stress.

## EXPERIMENTAL PROCEDURES

*Primary mouse OPCs* – Primary OPCs were harvested from mouse cortices, using the shake-off method (28). OPCs were isolated from postnatal day 0 or 1 mice. All cell cultures had a purity of 95%. The OPCs were seeded onto 24- or 96-well plates and maintained in differentiation medium previously described, called SATO medium (28). Depending on the experiment, cysteine free medium (DMEM, high glucose, no glutamine, no methionine, no cystine (Gibco); 4 mM glutamine and 201.34 µM methionine was added) was used instead of normal DMEM (Sigma-Aldrich). Thereafter, this was supplemented with 300 µM L-cysteine (Sigma-Aldrich) to obtain a culture medium with cysteine. For viability assays, cells were seeded with a density of 15 000, 30 000 or 50 000 cells per well in a 96 well plate. For a 24 well plate, cells were seeded with a density of 150 000 cells per well for immunocytochemistry and 250 000 cells per well for qPCR.

*Treatments* – 2-(Furan-2-Yl)-8-Methyl-N-(2-Methylphenyl)Imidazo[1,2-A]Pyridin-3-Amine is an inhibitor for EAAT3 (29). The cells were treated with 30 µM of the inhibitor (MolPort) dissolved in DMSO (Merck) at day 0 or day 1 after seeding for the viability assays. For immunocytochemistry and qPCR, cells were treated with inhibitor at day 0, 2 and 4.

A derivative of the EAAT2 agonist GT949 was produced by Dr. Hanne Diliën from Maastricht University. This derivative, called D8, binds near the active site of the EAAT3 transporter. After testing the toxicity of D8 with an MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) assay, cells were treated with a concentration of 100 nM or 10 µM at day 2 and 4 after seeding for immunocytochemistry.

$H_2O_2$ (Supleco) was dissolved in phosphate buffered saline (PBS) to obtain a concentration of 30 and 100 µM. At day 1 after seeding, Oli-neu cells were treated with both concentrations of $H_2O_2$.

Glucose oxidase (GO, G7141-10KU, Sigma) was used to provide a continuous production of $H_2O_2$. 1 mg GO was dissolved in 1 mL 50 mM sodium acetate. After testing the toxicity of GO in Oli-neu cells, the cells were treated with 0.3 µM and 1 µM. For viability assays, the cells were treated at day 1 after seeding. For immunocytochemistry and qPCR, cells were treated with inhibitor at day 0, 2 and 4.

*Oli-neu cells* – The Oli-neu cells (neu-immortalised OPC cells) were maintained in DMEM supplemented with 10% FCS, Gibco, and 50U/ml penicillin and 50 mg/ml streptomycin (P/S, Invitrogen), further called 'culture medium', at 37°C and 5% $CO_2$. Oli-neu cells were seeded in a 96- or 24- well plate in cysteine free medium with addition of 10% FCS and 1% P/S (adding 300µM cysteine if needed), or culture medium. For viability assays, cells were seeded with a density of 15 000 cells per well in a 96 well plate. For a 24 well plate, cells were seeded with a density of 150 000 cells per well for immunocytochemistry and 250 000 cells per well for qPCR.

*MTT assay* – At day 1 after seeding, the treatment of interest was added to the medium of the cells and at day 2 after seeding, the colorimetric MTT assay was performed. Cells seeded on a 96 well plate were incubated with 100 µL culture medium and 12.5µL MTT solution (5mg/ml in PBS, Sigma) per well, for 4 hr at 37°C and 5 or 8.5% $CO_2$, for Oli-neu and OPCs respectively. The supernatant was discarded and 175 DMSO/glycine (1:7 solution) was added to each well to dissolve the produced formazan crystals. The absorbance of each well was measured at 540 nm using a microplate reader, CLARIOstar$^{PLUS}$, BMG LABTECH.

*PI assay*– At day 1 after seeding, the treatment of interest was added to the medium of the cells, and at day 2 after seeding, PI (propidium iodide) assay was performed. The medium of the cells in a

96 well plate was discarded. 75 µL Lysis buffer reagent A100 (chemometec) was added to the cells. After a short incubation of 5 minutes at room temperature, 75 µL PI in stabilisation buffer reagent B (1:50, chemometec) was added. The cells were incubated for 15 minutes. The suspension was then pipetted in the wells of a black 96 well plate. The well plate was analysed with a microplate reader (FLUOstar OPTIMA, BMG Labtech).

*Immunocytochemistry of OPCs* – At day 2 and 4 post seeding, treatment of interest was added to the medium of the cells. At day six post seeding, OPCs were fixed with 4% paraformaldehyde (PFA, Merck) for 30 minutes at room temperature. Immunocytochemistry was done following the protocol of Assia T. *et al. (28).* Primary and secondary antibodies are provided in the supplementary (**Table S1**). Nuclei were stained with 4'6-diamidino-2-phenylindole (DAPI, Thermofisher**)**. Cells were detected using a fluorescence microscope (Leica DM2000). Images were analysed using Fiji, ImageJ (5 images per coverslip). The area percentage of MBP and O4 was quantified and divided by the count of nuclei in the image.

*Cysteine probe* – The cysteine probe was made by Dr. Hanne Diliën from Maastricht University. The production is described in the study of Luping Hu, *et al*. (30). Oli-neu cells were incubated with 1, 1.25, 2.5, 5, 10 and 20 µM cysteine probe (dissolved in DMSO (Merck)) in cysteine free medium after 3 washing steps with PBS. Incubation was at 37°C and 5% CO2, for 15 or 30 minutes. The medium was discarded, and cells were washed 3 times with PBS. For an MTT, the cells were incubated with an MTT solution as described previously. To detect the probe under a microscope, the cells were first fixated with 4% PFA (Merck) for 30 minutes at room temperature. Depending on the experiment, cells were incubated for 10 minutes with 5 µM SYTO™ 61 Red Fluorescent Nucleic Acid Stain (Invitrogen) diluted in PBS. Cells were detected using a fluorescence microscope (Nikon ECLIPSE 80i).

*qPCR* – Total RNA was isolated from Oli-neu cells. Lysis was performed with 350 µL Qiazol Lysis reagent (Qiagen). Cell suspension was separated by adding 70 µL chloroform and incubation for 5 minutes at room temperature. After 15 minutes of centrifugation at 13 200 rpm, the upper phase (±180 µL) was pipetted on 2 µL glycogen. 180 µL 2-isopropanol was added to the samples, following incubation for 30 minutes at room temperature. After 10 minutes of centrifugation, supernatant was discarded. The RNA was washed with 75% ethanol two times. The RNA pellet was dried and dissolved in RNAse free water. RNA concentration and quality were analysed with a Nanodrop spectrophotometer (Isogen Life Science). RNA was converted to cDNA using qScript cDNA SuperMix (Quanta Biosciences). Quantitative PCR was conducted on a StepOnePlus detection system (Applied Biosystems). The PCR reaction mix contained SYBR green master mix (thermo fisher biosystems), cDNA template (12.5 ng), 10 µM forward and reverse primers and MiliQ. Primer pairs are listed in Supplementary (**table S2**). The qPCR experiment failed, due to a high amount of RNA present in the cDNA samples. Therefore, results were not analysed.

*Mice* – C57/BL6 mice were maintained on a 12 h light/dark cycle with free access to water and a standard chow diet (Ssniff). All animal procedures were conducted in accordance with the institutional guidelines and approved by the Ethical Committee for Animal Experiments of Hasselt University.

*Statistical analysis* – Statistical analysis was performed using GraphPad Prism 9.3.1 software (GraphPad software Inc., CA, USA). Differences between group means were determined using an ordinary one-way ANOVA test for normally distributed data and a Kruskal Wallis test for not normally distributed data. Sample size is provided in each figure as "n"; amount of repeated experiments is indicated as "N". All data are depicted as mean ± SEM, $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$; $\#p \leq 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ compared to control.

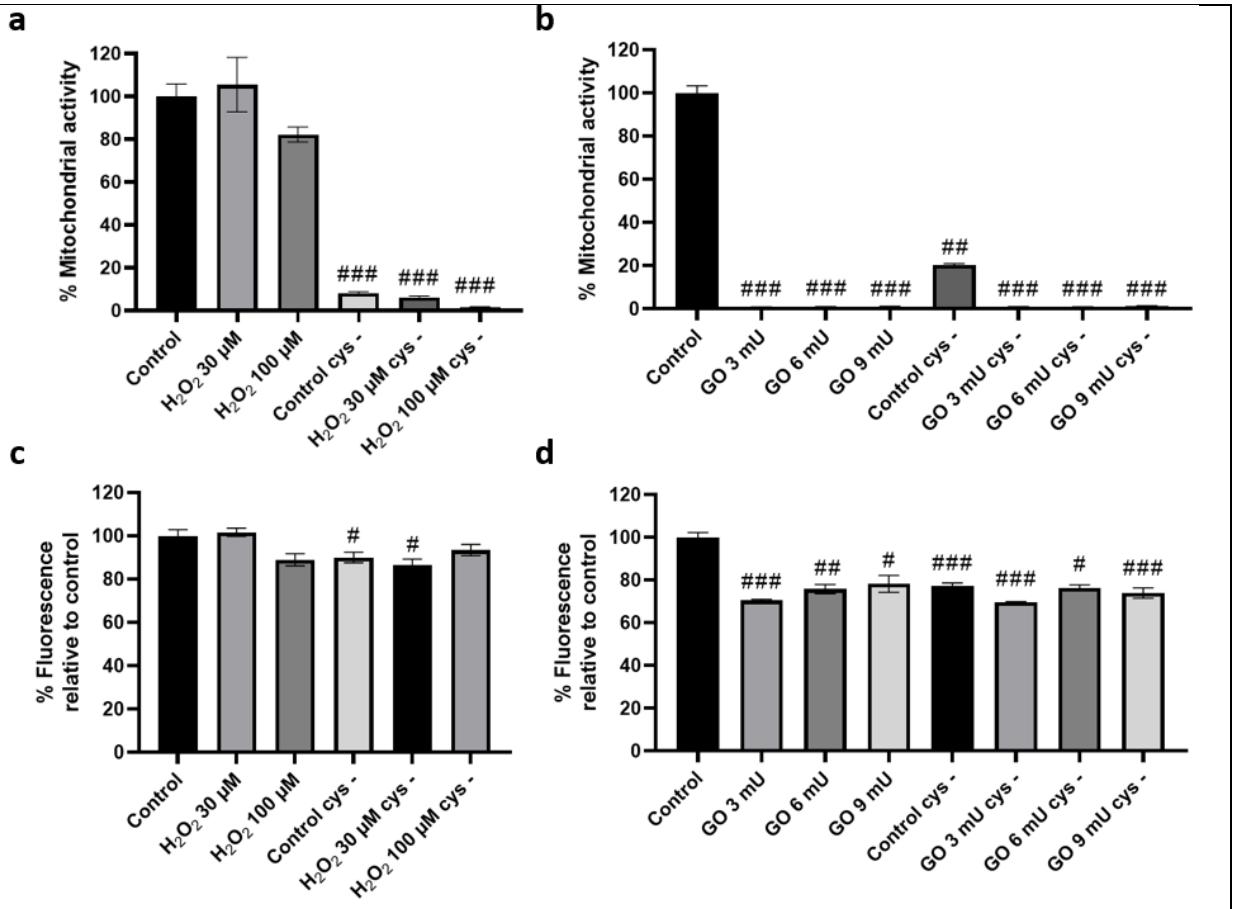


**Figure 1** – **cell survival and mitochondrial activity after stimulation with different concentrations of GO and H2O2 in Oli-neu cells.** (a) Percentage mitochondrial activity relative to control determined by an MTT assay in Oli-neu cells after exposure to 30 µM and 100 µM $H_2O_2$. (b) Percentage mitochondrial activity relative to control determined by an MTT assay in Oli-neu $H_2O_2$ after stimulation with 3, 6, and 9 mU GO. (c) Percentage fluorescence relative to control determined by a PI assay after exposure to 30 µM and 100 µM $H_2O_2$ analysis in Oli-neu cells. (d) Percentage fluorescence relative to control determined by a PI assay in Oli-neu cells after stimulation with 3, 6, and 9 mU GO. (a-d) Oli-neu cells were treated with cysteine supplemented (300 µM) medium and cysteine free medium. Data are represented as mean ±SEM; n=6, N=1,. # $p<0.05$ compared to control, ## $p<0.01$ compared to control, ### $p<0.001$, compared to control. Cys -, cysteine depleted medium; PI, propidium iodide; $H_2O_2$, hydrogen peroxide, GO, glucose oxidase.

## RESULTS

*Glucose oxidase as a stressor for Oli-neu cells* – First, we determined which stressor could provide oxidative stress to the Oli-neu cell line. The pro-oxidant $H_2O_2$ was tested, a well-known reactive oxygen species that is reduced by GSH. The cells were cultured in cysteine depleted medium to evaluate the vulnerability to $H_2O_2$ when GSH production is hampered. Incubation with 30 µM and 100 µM $H_2O_2$ in a cysteine supplemented medium did not significantly decrease cell survival and mitochondrial activity (**fig. 1 a, c**). However, the cysteine depleted medium did significantly decrease the mitochondrial activity and cell survival in Oli-neu cells (**fig. 1 a, b**). Glucose oxidase is an enzyme that reacts with β-D-glucose and oxygen to produce $H_2O_2$ continuously (31). Following the research of FitzGerald UF, *et al.*, three concentrations of GO (3 mU, 6 mU, and 9 mU) were tested for the effect on the viability of Oli-neu cells (32). The mitochondrial activity and survival of the cells significantly decreased after incubation with all concentrations of GO, with or

without the presence of cysteine in the medium. The absorbance measured for all concentrations of GO was close to 0% (**fig. 1 b, d**). A lower concentration of GO was tested to not completely impair cell viability. The dose-response curve of GO in Oli-neu demonstrates that a concentration of 0.3 mU is enough to lower viability significantly in Oli-neu with cysteine free and supplemented medium (**fig. S1 a-b**). A repeated experiment supports the data of the dose-response curve. The cell viability decreased almost completely with 1 mU GO, with and without the presence of cysteine. 0.3 mU GO only decreased the mitochondrial activity in the absence of cysteine (**fig. S1 c-d**).

*Glucose oxidase as a stressor for primary OPCs* – The GO concentration for OPCs was chosen following the experiments of Oli-neu cells. This cell line was chosen due to the similarity to primary OPCs. 3 mU of GO decreased the mitochondrial activity significantly. However, the optical density (OD) Values were very low and thus unreliable (**fig. 2 a**). 1 mU and 0.3 mU GO did not decrease the mitochondrial activity and cell survival of OPCs in the presence of cysteine (300 µM). Cysteine depletion decreased mitochondrial activity and cell

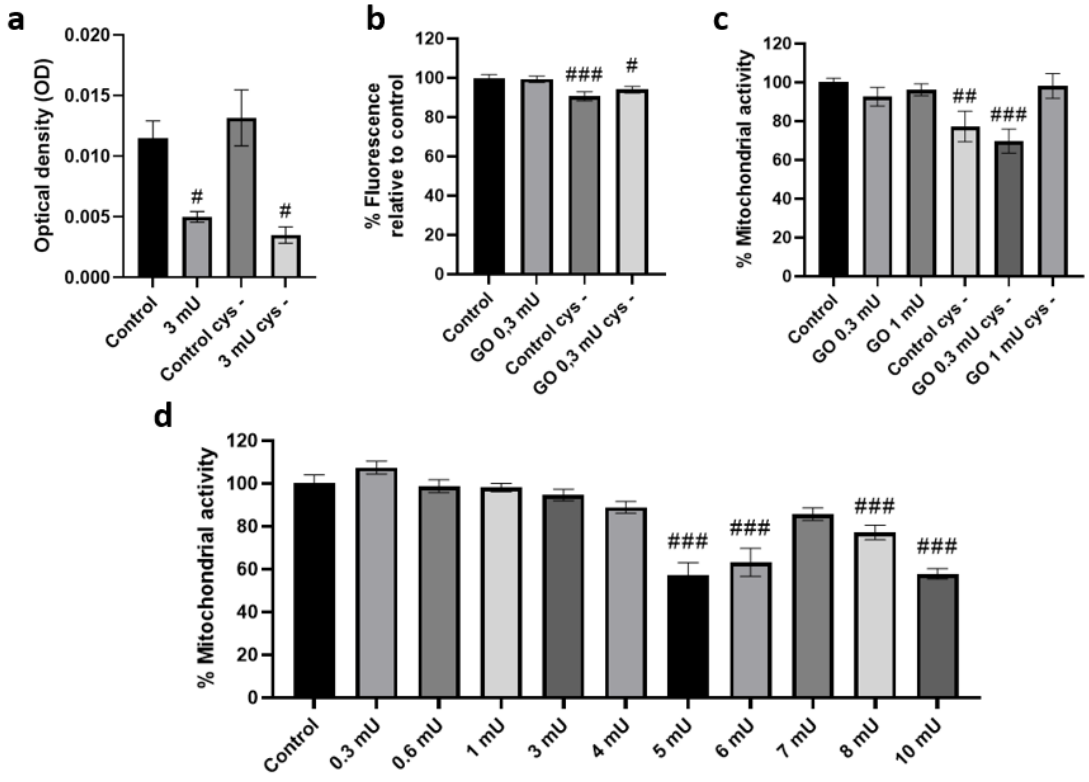


**Figure 2 – cell survival and mitochondrial activity after stimulation with different concentrations of GO in OPCs.** (a) mitochondrial activity determined by an MTT assay after stimulation with 3 mU GO. OPCs were seeded for 15 000 cells per well. (b) Percentage fluorescence relative to control determined by a PI assay on OPCs stimulated with 0.3 mU GO. OPCs were seeded for 50 000 cells per well. (c) Percentage mitochondrial activity relative to control determined by an MTT assay after stimulation with 0.3 mU and 1 mU GO. OPCs were seeded for 50 000 cells per well. (d) Percentage mitochondrial activity relative to control determined by an MTT assay. Cells were seeded for 50 000 cells per well and stimulated with 0.3, 0.6, 1, 3, 4, 5, 6, 7, 8, and 10 mU GO. (a-d) OPCs were treated with cysteine supplemented (300 µM) medium and cysteine free medium. Data are represented as mean ±SEM; n=6, N=1 for a and d, N=2 for b and c. # $p<0.05$ compared to control, ## $p<0.01$ compared to control, ### $p<0.001$ compared to control. Cys -, cysteine depleted medium; PI, propidium iodide; GO, glucose oxidase.

survival in OPCs, regardless of the presence of GO (0.3 mU). However, 1 mU GO did not decrease the mitochondrial activity in the absence of cysteine (**fig. 2 b, c**). An MTT assay demonstrates that the viability of OPCs reduced in the presence of 5, 6, 8 and 10 mU GO (**fig. 2 d**).

*EAAT3 inhibition reduces cell viability of primary OPCs, but not Oli-neu cells* – viability assays were performed to investigate whether an EAAT3 inhibitor hinders antioxidant defences,. Cell survival and mitochondrial metabolism of Oli-neu cells did not significantly change after stimulation with the EAAT3 inhibitor added on day one after seeding. (**fig. S2 a, b**).

Exposure to 30 µM inhibitor at the same time of seeding did not decrease cell survival and mitochondrial activity either (**fig. 3 a, b**). A combination of 0.3 mU or 1 mU GO and 30 µM EAAT3 inhibitor decreased the cell viability significantly. Cysteine depletion decreased the mitochondrial activity in all treated cells except the EAAT3 inhibitor (**fig. 3 b**). The viability of OPCs

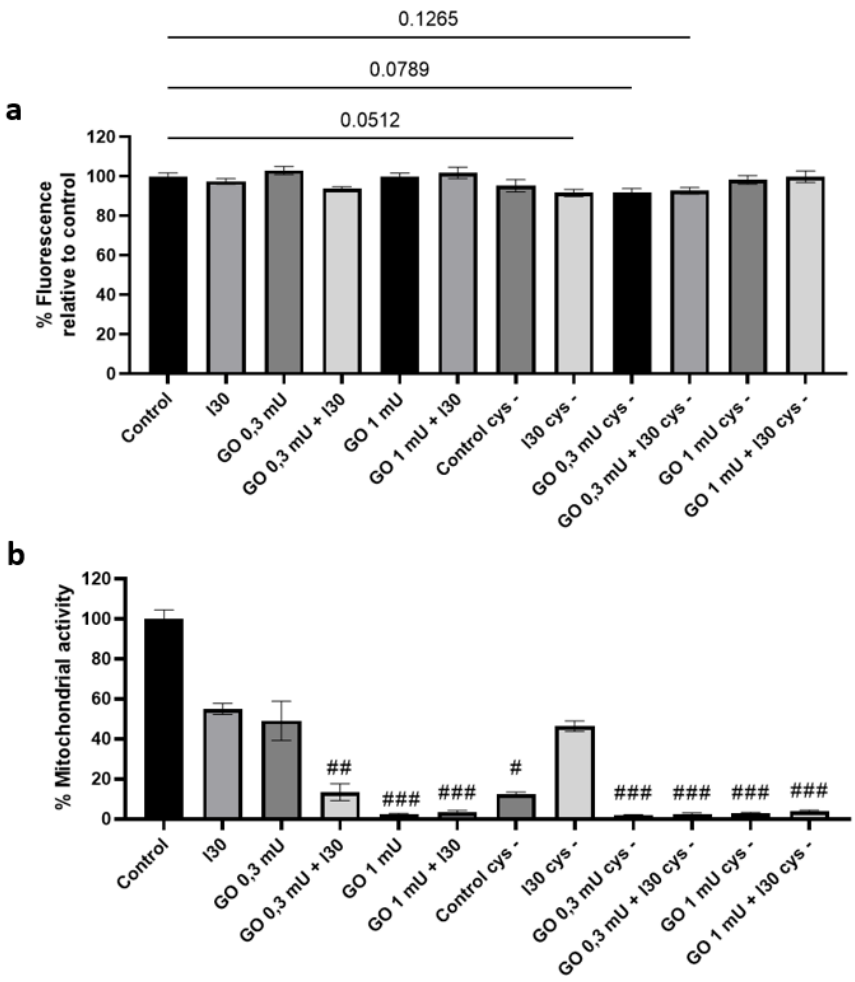


**Figure 3** – **cell survival and mitochondrial activity after stimulation with an EAAT3 inhibitor and different concentrations of GO in Oli-neu cells.** (a) Percentage fluorescence relative to control determined by a PI assay of Oli-neu cells stimulated with 0.3 mU, 1 mU GO, and 30 µM EAAT3 inhibitor. (b) mitochondrial activity determined by an MTT assay after stimulation with 0.3 and 1 mU GO, and 30 µM EAAT3 inhibitor. (a-b) Oli-neu cells were treated with cysteine supplemented (300 µM) medium and cysteine free medium. Data are represented as mean ±SEM; n=6, N=2. # $p<0.05$ compared to control, ## $p<0.01$ compared to control, ### $p<0.001$ compared to control. Cys -, cysteine depleted medium; PI, propidium iodide; GO, glucose oxidase; I30, 30 µM EAAT3 inhibitor.

did not change after exposure to 30 µM of the EAAT3 inhibitor one day after seeding (**fig. 4 a**). However, adding the inhibitor at the time of seeding did significantly decrease the cell survival and mitochondrial activity of the cells. A cysteine depleted medium decreased mitochondrial activity and cell survival.

*EAAT3 inhibition hampers differentiation of OPCs* – To determine whether the inhibitor and GO affect OPC differentiation, an immunocytochemistry with antibodies for O4 (a marker for early-phase differentiation) and MBP (a marker for late-phase differentiation) was performed. O4 did not significantly change after treatment with GO (0.3 mU) (**fig. 5 a, b**). MBP significantly decreased after treatment with 30 µM EAAT3 inhibitor, but not with 0.3 mU GO. A combination of inhibitor and 0.3 mU GO decreased MBP (**fig. 5 a, c**). MBP and O4 per cell did not decrease significantly with GO treated cells with or

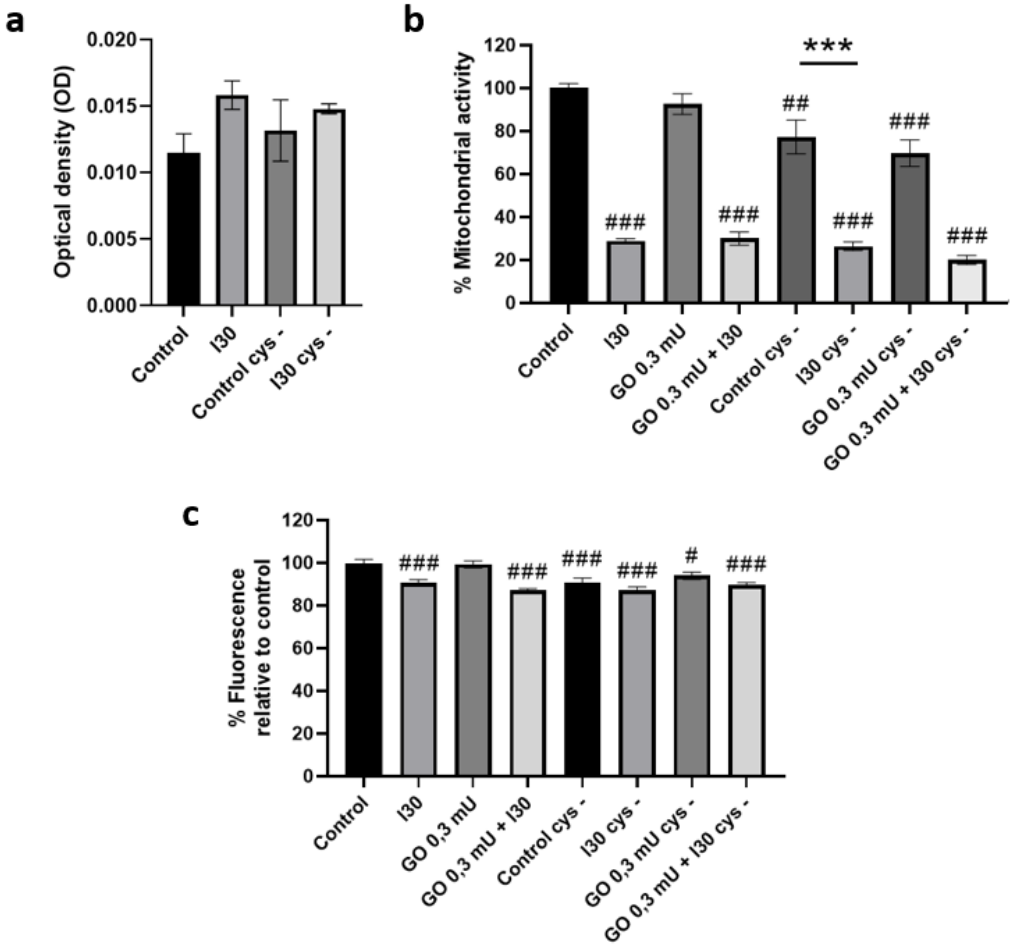


**Figure 4** – **cell survival and mitochondrial activity after stimulation with an EAAT3 inhibitor and different concentrations of GO in primary OPCs.** (a) mitochondrial activity determined by an MTT assay after exposure to 30 µM EAAT3 inhibitor. OPCs were seeded for 15 000 cells per well. (b) Percentage mitochondrial activity relative to control determined by an MTT assay after exposure to 30 µM EAAT3 inhibitor and 0.3 mU and 1 mU GO. OPCs were seeded for 50 000 cells per well. (c) Percentage fluorescence relative to control determined by a PI assay of OPCs stimulated with 0.3 mU GO. OPCs were seeded for 50 000 cells per well. (a-c) OPCs were treated with cysteine supplemented (300 µM) medium and cysteine free medium. Data are represented as mean ±SEM, n=6. # $p<0.05$ compared to control, ## $p<0.01$ compared to control, ### $p<0.001$ compared to control;*** $p>0.001$. Cys -, cysteine depleted medium; PI, propidium iodide; GO, glucose oxidase; I30, 30 µM EAAT3 inhibitor.

without the presence of cysteine. A combination of 0.3 mU GO, and EAAT3 inhibitor decreased the ratio of MBP/O4. The MBP and O4 did not change significantly in a cysteine depleted medium, but it increased the MBP/O4 (**fig. 5 a, d**).

*A derivative of EAAT2 does not affect OPC differentiation* – A derivative of the EAAT2 allosteric agonist GT949, D8, was tested to establish whether it can activate EAAT3 and enhance differentiation. Different concentrations of the activator were tested for its toxicity to Oli-neu cells and primary OPCs. The viability of the cells was not affected by D8 (**fig. S3 a-b**). To determine whether D8 could provide OPCs with enhanced antioxidant capacity, an MBP and O4 staining was performed. 10 µM and 100 nM D8 did not change

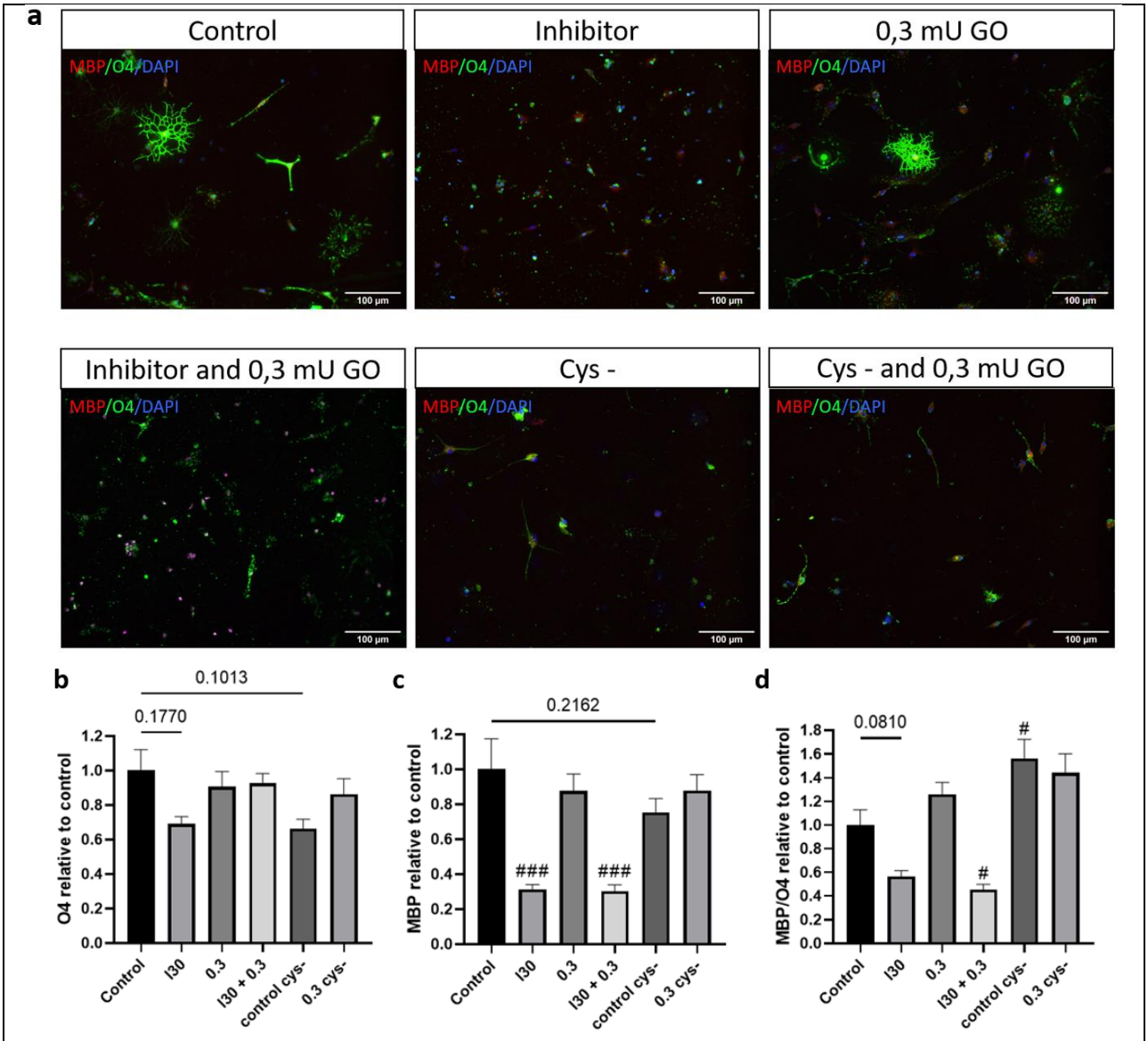


**Figure 5** – **MBP expression in OPCs and MBP/O4 ratio decreased after treatment with EAAT3 inhibitor.** (a) Representative images of primary OPCs treated with 0.3 GO and 30 µM EAAT3 inhibitor. (b-c) Quantification of O4 and MBP respectively of fluorescence area per nucleus count relative to control. (d) MBP/O4 ratio of fluorescence area quantified. OPCs were treated with cysteine supplemented (300 µM) medium and cysteine free medium. Data are represented as mean ±SEM. N=4; 5 pictures per coverslip. # $p<0.05$ compared to control, ### $p<0.001$ compared to control. Cys -, cysteine depleted medium, MBP, myelin basic protein; GO, glucose oxidase; I30, 30 µM EAAT3 inhibitor.

MBP and O4 expression in OPCs independently. However, 10 µM D8 increased the MBP in the presence of 0.3 mU GO (**fig 6 a-c**). The ratio MBP/O4 remained unchanged after treatment with D8 and GO (**fig 6 a, d**).

*Optimisation of a cysteine probe* – A cysteine probe was tested in Oli-neu cells to quantify the amount of cysteine in the cells. Following the protocol of Luping Hu, *et al*., Oli-neu cells were incubated with 20 µM of the cysteine probe (30). The morphology of the cells with cysteine depletion in the presence of 20 µM probe resembled stressed or dying cells (round shaped cells indicated with red arrows, **fig. 7 a**). The cells incubated with the probe in the presence of cysteine resulted in less round shaped cells and more spindle forming cells (green arrows, **fig. 7 b).** An MTT was performed to test the toxicity of the cysteine probe. 10 and 20 µM of the cysteine probe, incubated for 15 minutes, reduced the mitochondrial activity significantly in both cysteine supplemented and depleted medium changes. A medium change with a cysteine depleted medium reduced the mitochondrial activity of the control and 5 µM probe (**fig. 7 c**). A 30-minute incubation with 10 µM and 20 µM of the probe lowered the mitochondrial activity in cells with a cysteine supplemented medium change. A cysteine depleted medium change reduced the activity with 1.25 µM and 20 µM of the probe (**fig. 7 d**). Immunocytochemistry shows that the probe detection overlapped with the red nuclear staining, SYTO™ 61 red, in the presence of 1 µM and 2.5 µM cysteine probe (**fig. S4 a, b**).

## DISCUSSION

*Establishing a stressor to induce oxidative stress in Oli-neu cells* – An MTT assay was performed to measure mitochondrial activity, which is an indication of cell viability. Mitochondrial reductases reduce MTT to purple formazan (33). Cells in later stages of apoptosis will have less metabolic and enzymatic activity than viable cells, and thus less MTT is reduced (34,35). A PI assay was used to determine the amount of cell survival and, therefore, cell viability.

To identify the importance of cysteine transport for OPCs and Oli-neu cells, the cells were cultured in a cysteine depleted medium. The cell viability of Oli-neu cells decreased significantly (an 80 to 90 % reduction in mitochondrial activity, **fig. 1 a-b**) in the absence of cysteine. This is most likely related to the crucial roles of cysteine. Cysteine has the ability to form disulphide bonds, which is important in protein structure and folding (36,37). It is also the main component of the antioxidant GSH. A reduction in GSH production limits the protection against pro-oxidants. As a result, pro-oxidants accumulate in the cell. This induces oxidative stress (17,36,37). While cysteine depletion reduced the cell viability of OPCs, the mitochondrial activity did not decrease as much in comparison with the Oli-neu cells (20 to 30% reduction, **fig. 2 c**). We suggest that primary OPCs can still obtain cysteine despite the cysteine depleted DMEM. The SATO medium used in primary OPC culture contains horse serum that could still contain free cysteine.

Our results demonstrate that $H_2O_2$ did not reduce mitochondrial activity and cell survival of Oli-neu cells. This is possibly due to several factors. First, cellular catalases and glutathione peroxidases can reduce $H_2O_2$ rapidly. This indicates that the exposure to $H_2O_2$ is less intense and probably shorter than 24 hours (8,38). Secondly, $H_2O_2$ could produce damage-inducing oxygen radicals by Fenton's reaction, but the damage is already repaired after 24 hours. Lastly, 100 µM could

damage the cells irreparablyare in the first stages of apoptosis (38). However, it is still not detectable by the MTT assay after 24 hours. The early stages of apoptosis do not affect the mitochondria's enzymatic activity much (34).

The enzyme glucose oxidase (GO) was utilized as a treatment to provide continuous production of $H_2O_2$ to the cells. The concentration of $H_2O_2$ remains relatively constant as long as beta-D-glucose and oxygen are present (31,39). Our data show that GO (3, 6 and 9 mU) drastically reduced the viability of Oli-neu cells. The mitochondrial activity was close to 0% relative to control (**fig. 1 b**), suggesting that almost all cells lost their mitochondrial function. The detected fluorescence in the PI assays decreased by 20 to 30%. However, the background detection could not be reduced for PI assays. Therefore, a 30% reduction could already be a complete impairment of cell survival. Since future experiments do not need a full impairment of the viability, a lower concentration of GO was needed. A dose-response curve of GO shows that above 0.3 mU GO the mitochondrial activity is impaired in Oli-neu cells. However, other experiments showed that 0.3 mU did not decrease the mitochondrial activity significantly. Therefore,

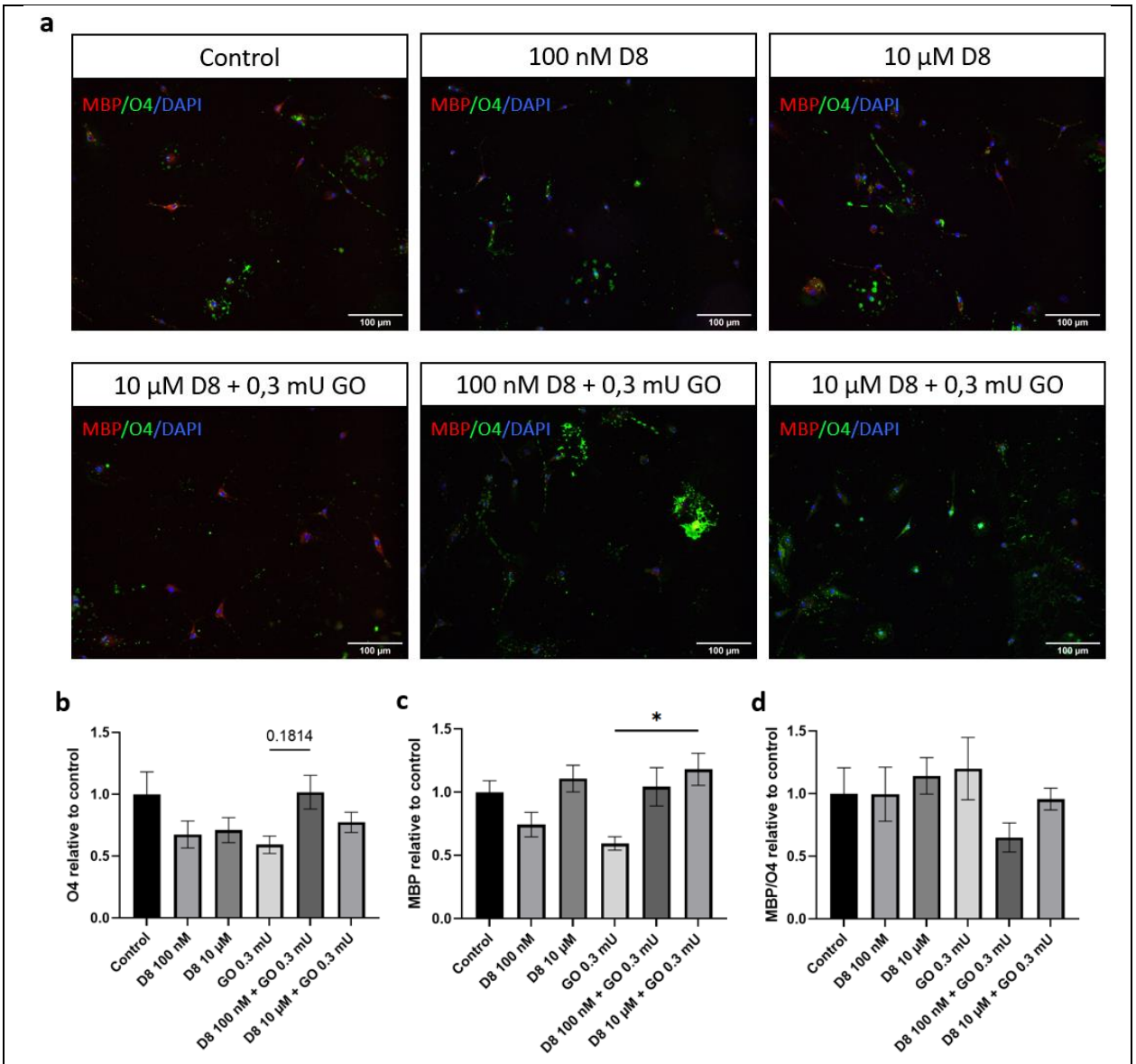


**Figure 6** – **O4 and MBP expression in OPCs did not change after treatment with D8 and GO.** (a) Representative images of primary OPCs treated with 0.3 GO, 10 µM and 100 nM D8. (b-c) quantification of O4 and MBP respectively of fluorescence area per nucleus count relative to control. (d) MBP/O4 ratio of fluorescence area quantified. Data are represented as mean ±SEM. N=3, except for 10 µM D8: n=6; 5 pictures per coverslip. * $p<0.05$. MBP, myelin basic protein; GO, glucose oxidase; D8, derivative of GT949.

the effect of 0.3 mU GO on mitochondrial activity remains inconclusive. It is important to note that the data are more variable from the mean for 0.3 mU GO (SEM: 9.78, **fig S1**). The mitochondrial activity was close to 0% after exposure to 1 mU GO, indicating a full impairment of cellular viability. Therefore, the optimal concentration for reducing mitochondrial activity is between 0.3 and 1 mU GO.

*Establishing a stressor to induce oxidative stress in primary OPCs* – 3 mU of GO decreased the mitochondrial activity significantly. However, the cells were not densely plated, resulting in extremely low OD values for all conditions (**fig. 2 a**). Plating cells at low density can affect OPC survival greatly (40). The results were, therefore, unreliable. Hereafter, OPCs were plated with a high density (**fig. 2 b-d**). The cell survival of these OPCs

in the presence of 0.3 mU GO did not decrease. The mitochondrial activity of the cells did not decrease after exposure to GO (0.3 and 1 mU) either. In the absence of cysteine, cell viability does decrease with or without the presence of GO (0.3 mU). However, GO (0.3 mU) did not significantly decrease compared to the control condition without cysteine. This indicates that 0.3 and 1 mU GO was insufficient to affect the cell viability in OPCs compared to Oli-neu cells. Primary OPCs are cultured in SATO medium, containing a high concentration of growth factors and components important for survival and differentiation (28). One of the reasons why primary OPCs are less vulnerable to ROS could be due to the protective properties of the medium. To evoke a drop in cell viability, an increased concentration of GO was needed. The mitochondrial activity of OPCs decreased significantly after exposure with a concentration of 5 mU GO and higher. Due to lack of time, this was not pursued in further experiments. Future research will explore this further.

Studies show that OPC death is not a significant factor in neurodegeneration. Sublethal oxidative stress in OPCs does not affect cell viability, but can still impair the differentiation pathways of OPCs (8,41,42). Therefore, while 0.3 mU GO did not affect the mitochondrial activity or cell survival, it could affect the differentiation of OPCs in mature oligodendrocytes. OPC differentiation was examined in the presence of 0.3 mU GO with immunocytochemistry. O4 and MBP expression and the ratio MBP/O4 remained unchanged after exposure to GO (0.3 mU). This suggests that 0.3 mU GO did not affect the differentiation of OPCs. Posssibily, 0.3 mU GO was insufficient to provide oxidative stress. A future study should investigate whether a higher concentration of GO can affect the capacity to differentiate OPCs.

*Inhibition of EAAT3 did not reduce cell viability of Oli-neu cells* – A selective inhibitor (2-(Furan-2-Yl)-8-Methyl-N-(2-Methylphenyl) Imidazo[1,2-A]Pyridin-3-Amine) was tested in Oli-neu cells to block EAAT3 (29). Cell survival of the cells exposed to the inhibitor did not change Significantly in the presence of cysteine (**fig. S2 a-b**). The mitochondrial activity did not significantly reduce after exposure to the inhibitor either. Thus, the inhibitor was not sufficient to reduce cell viability. It is possible that exposure the EAAT3 inhibitor one day after seeding did not have a significant effect on the cells. The cells were already provided with cysteine for 24 hours, which could diminish the effect of the inhibitor on the cells. Therefore, in subsequent experiments, the inhibitor was added immediately at seeding time (**fig. 3 a-b**). This, however, did not decrease the cell viabilityin both viability assays either. Interestingly, a combination of inhibitor and 0.3 mU GO did decrease the enzymatic activity significantly. This suggests that the combination has an additive effect on cell viability. The fact that cysteine depletion had a more significant impact on the cell viability than exposure to inhibitor suggests that EAAT3 might not be the most important cysteine transporter in Oli-neu cells. Oli-neu cells can possibly obtain cysteine through other pathways. One possible transporter is the cystine/glutamate antiporter system $x_c^-$. Cystine, the oxidized form of cysteine, is transported in the cell via the $x_c^-$ antiporter system and reduced by thioredoxin reductase 1 or GSH (43-45). This could then provide the cells with enough cysteine to survive. Interestingly, a cysteine depletion in the presence of the EAAT3 inhibitor did not decrease mitochondrial activity significantly. It is possible that there are off target effects due to the EAAT3 inhibitor.

*Inhibition of EAAT3 reduced cell viability and hampers OPC differentiation* – Similar to Oli-neu cells, the viability of OPCs did not change after exposure to the EAAT3 inhibitor one day after seeding (**fig. 4 a**). However, exposing cells to the inhibitor at the same time of seeding did decrease the mitochondrial activity and cell survival significantly (**fig. 4 b-c**). A cysteine depletion in the presence of the EAAT3 inhibitor decreased the mitochondrial activity of OPCs more than a cysteine depletion alone. This suggests that OPCs might still be provided cysteine despite a cysteine depletion in DMEM. The large decrease in cell viability in OPCs shows that cysteine uptake is essential for cell functions. Because an EAAT3 inhibition will prevent the uptake of cysteine, GSH production will diminish. This results in less protection against ROS, which increases the risk of oxidative stress. Moreover, cysteine is an important amino acid for protein structure and folding (36,37). Therefore, the production of essential proteins containing cysteine will also decrease when cysteine uptake is prevented. The substantial

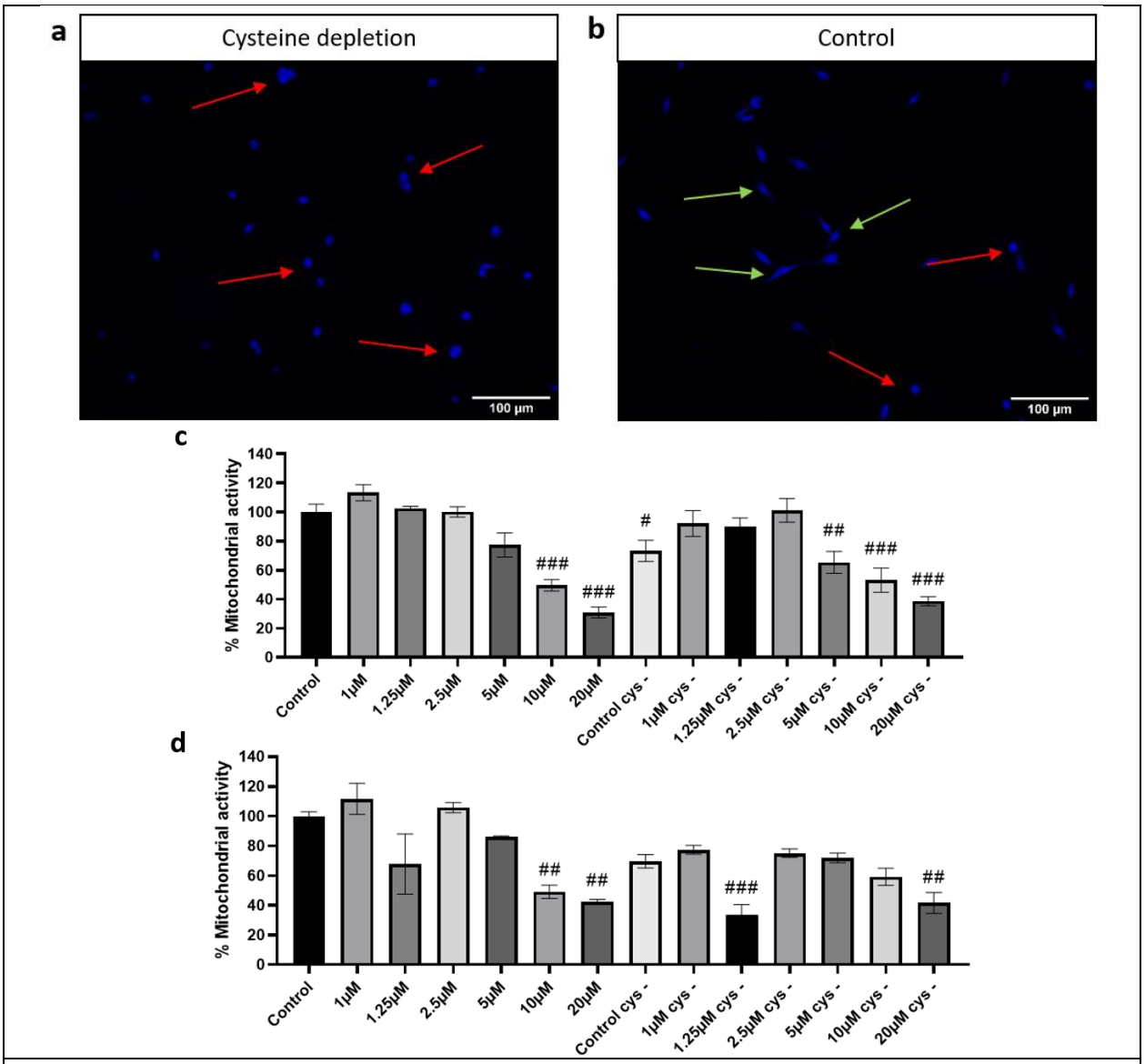


**Figure 7** – **The detection of the cysteine probe and its toxicity in Oli-neu cells.** (a) Fluorescence detection of the cysteine probe in Oli-neu cells, incubated with a cysteine depletion. Red arrows point to a circle-like morphology. (b) Fluorescence detection of the cysteine probe in Oli-neu cells. Red arrows point to a round shaped morphology, green arrows point to a spindle-like morphology. (c) Percentage mitochondrial activity relative to control determined by an MTT assay after stimulation with 1, 1.25, 2.5, 5, 10 and 20 µM of the cysteine probe. Oli-neu cells were incubated for 15 minutes with culture medium or cysteine free medium. (d) Percentage mitochondrial activity relative to control determined by an MTT assay after stimulation with 1, 1.25, 2.5, 5, 10 and 20 µM of the cysteine probe. Oli-neu cells were incubated for 30 minutes with culture medium or cysteine free medium. Data are represented as mean ±SEM. # $p<0.05$ compared to control, ## $p<0.01$ compared to control, ### $p<0.001$ compared to control. Cys -, cysteine depleted medium.

decrease in mitochondrial activity (70% reduction, **fig. 4 b**) indicates that cysteine uptake is largely diminished and that EAAT3 is one of the most crucial cysteine transporters in OPC survival. Therefore, it plays an important role in maintaining the function of OPCs in neurodegenerative and neuroinflammatory diseases, such as MS.

Interestingly, the OPC-like cell line Oli-neu did not show the same results. It is possible that the Oli-neu cells, while similar to OPCs do not have the same cell membrane properties as OPCs. Cell lines can sometimes express gene patterns not found in any cell type *in vivo.* Moreover, cell characteristics can change after periods of continuous proliferation. This could contribute to a decrease in EAAT3 expression in Oli-neu cells (46).

Immunocytochemistry of OPCs demonstrated that the protein expression of MBP decreased significantly with exposure to the EAAT3 inhibitor (**fig. 5 a-d**). The expression of O4 remained unchanged, while MBP/O4 ratio had an insignificant trend to decrease after EAAT3 inhibition, and a combination of inhibitor and GO (0.3 mU) decreased the ratio significantly. This indicates that EAAT3 inhibition decreases the expression of differentiation markers and suggests that inhibition of EAAT3 can reduce the capacity to differentiate in OPCs. The decrease in MBP expression can be explained by two reasons. First, our data shows that the EAAT3 inhibitor decreases viability. The apoptotic cells have a different morphology than viable cells. This morphology includes shrinking and blebbing of the cell. Because cell debris is washed away, the number of proteins per cell will decrease due to this morphology. A second factor could be an increase of intracellular pro-oxidants . Cysteine uptake is prevented, hence GSH production is reduced. ROS accumulate resulting in oxidative stress, which reduces mature oligodendrocyte and myelin markers, including MBP. Oxidative stress can also alter gene expression of cell differentiation regulators, such as a reduction in Sox10 expression (8,42). Studies have shown that Sox10 directly regulates myelin gene expression, including MBP (47,48). Therefore, oxidative stress could reduce MPB expression. In this way, OPCs could lose their ability to differentiate. The capacity to differentiate into oligodendrocytes is crucial to maintain remyelination, especially in neurodegenerative and neuroinflammatory diseases. Therefore, it is meaningful to stimulate cysteine EAAT3 uptake in OPCs.

*Stimulation of OPCs with D8 did not change differentiation capacity of OPCs* – Next, we investigated whether increased activity of EAAT3 can protect OPCs. There are no known compounds that positively modulate cysteine transport by EAAT3. Therefore, a derivative of an EAAT2 activator, GT949, was tested. GT949 increases the activity of EAAT2 by allosteric modulation (49). In silico, molecular docking demonstrated that D8 binds to an allosteric site of EAAT3. We established that D8 did not induce toxicity to the cells. In this study, we tested whether the derivative of EAAT2, D8, could enhance differentiation by activating EAAT3. We expected that D8 can protect OPCs in the presence of GO by increasing production of GSH. Immunocytochemistry of OPCs established that O4 and MBP did not change after exposure to D8 (100 nM and 10 µM) (**fig. 6 a-d**). However, 10 µM D8 increased the expression of MBP in the presence of GO (0.3 mU). MBP/O4 ratio did not change. This suggests that while MBP increased, the amount of differentiation did not change. Despite in silico docking, it is possible that D8 does not bind to an allosteric site of EAAT3 resulting in no activation of the transporter. To test whether D8 truly activates EAAT3, the amount of cysteine in the cells should be measured. If D8 activates EAAT3, an increase of cysteine uptake would be observed.

*A cysteine probe for quantification of cysteine in cells* – Until now, only indirect effects of EAAT3 modulation were investigated, such as cell viability and differentiation markers. A direct effect of EAAT3 modulation is an increase or decrease in cysteine uptake. To investigate this direct effect, the amount of intracellular free cysteine can be measured. In this study, we tested a cysteine probe on Oli-neu cells (**fig. 7 a-d**). Following the protocol of Luping Hu *et al*., the probe (20 µM) was incubated for 30 minutes in culture medium (30). Here, the probe could bind to free cysteine in the medium, enabling fluorescence emission. The cysteine-probe molecule is structurally larger than free cysteine. This could hamper the uptake of cysteine through EAAT3. Therefore, Oli-neu cells were also incubated in the absence of cysteine. Cysteine depletion resulted in a round and shrunken cell morphology, resembling dying cells. The cells cultured in the presence of cysteine only had a round morphology. Other cells were spindle-like, indicating viable cells. This suggests that a cysteine incubation of 30 minutes can already affect the viability of the cells. Because the probe itself could also have a toxic effect on the cells, mitochondrial activity was assessed. The incubation time was reduced because the probe can bind to cysteine and

emit fluorescence after 5 minutes and might be toxic. Our data establishes there was no difference in mitochondrial activity between an incubation of 15 or 30 minutes. However, the suggested 20 µM of the cysteine probe did decrease the mitochondrial activity significantly by ±70%. Furthermore, incubation with the probe in the absence of cysteine, decreased mitochondrial activity significantly after 15 minutes of incubation. To be safe the probe did not provide stress to the cells, 1 µM and 2.5 µM seemed the best fit. To determine differences in detected fluorescence between conditions, a nuclear staining was performed. This is used to determine the amount of fluorescent area per cell nucleus counted. A red nucleus staining, SYTO™ 61 red, was tested on Oli-neu cells (**fig. S4 a-b**). Here, it is demonstrated that the nucleus staining also binds to other proteins in the cells. Therefore, it could not be used to count nuclei. However, the detected probe overlapped with the red staining, suggesting that the detected probe was located in the cells. To quantify the detection of cysteine probe, another nucleus staining should be tested in the future.

*Future directions* – While this study elucidated interesting properties of EAAT3, future experiments should be performed to validate our findings. First, A new potential allosteric agonist should be acquired through in silico docking. Moreover, the cysteine probe should be optimised to quantify cysteine uptake in OPCs. Next, expression of *SLCa1*, the gene encoding for EAAT3, should be quantified. Although it has been demonstrated that oxidative stress promotes transcriptional upregulation of neuronal EAAT3 (23,50), it is unknown whether the same applies to OPCs. Therefore, it is interesting to investigate whether expression *SLC1a1* changes after oxidative stress induction with GO. In addition, the expression of differentiation-associated genes, such as MBP and myelin-associated glycoprotein (MAG), could be examined. While there was an attempt to investigate gene expression with qPCR, the experiments were unsuccessful thus far. EAAT3 protein expression and membrane trafficking could also be examined after exposure to oxidative stress. The complexity of the cell branching could also be considered. While the MBP/O4 ratio is a measure for differentiation, it does not represent the complex branching of differentiated oligodendrocytes. Therefore, a sholl analysis could be performed to compare the morphology of the OPCs in oxidative stress and in the presence of an EAAT3 modulator (51). An EAAT3 overexpression could be established *in vivo* with an MS mouse model. Here, remyelination of the corpus callosum could be assessed. Furthermore, visual evoked potentials could be examined. This measures the latency and amplitude of the recording, corresponding to myelination and axonal damage respectively (52).

## CONCLUSION

In conclusion, we showed that GO decreases the cell viability of Oli-neu cells and OPCs *in vitro*. A specific inhibitor of EAAT3 reduces cell viability in both *in vitro* models. However, primary OPCs are more vulnerable against the effect of the inhibitor, suggesting an important role of EAAT3 in OPC survival. The difference between primary OPCs and the cell line Oli-neu indicates that Oli-neu cells do not represent primary OPCs fully. This should be considered in future studies using Oli-neu cells. Inhibiting EAAT3 hampered differentiation, further proving the importance of EAAT3. Therefore, EAAT3 can play a protective role in OPCs in neurodegenerative and neuroinflammatory diseases, such as MS. Stimulating cysteine uptake by EAAT3, could preserve the differentiation capacity of OPCs in the brain. An activation of EAAT3 could not be confirmed in this study. While the derivative of GT949 increased MBP expression in the presence of GO, the effect of D8 needs to be validated with a higher concentration of GO. While our data points to the importance of EAAT3 in OPCs, underlying effects of modulation of EAAT3 on cysteine uptake remain to be elucidated.

**REFERENCES**
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1. Lassmann, H., van Horssen, J., and Mahad, D. (2012) Progressive multiple sclerosis: pathology and pathogenesis. *Nature Reviews Neurology* **8**, 647-656
2. Howard, J., Trevick, S., and Younger, D. S. (2016) Epidemiology of Multiple Sclerosis. *Neurol Clin* **34**, 919-939
3. Oh, J., Vidal-Jordana, A., and Montalban, X. (2018) Multiple sclerosis: clinical aspects. *Curr Opin Neurol* **31**, 752-759
4. Correale, J., Gaitán, M. I., Ysrraelit, M. C., and Fiol, M. P. (2017) Progressive multiple sclerosis: from pathogenic mechanisms to treatment. *Brain* **140**, 527-546
5. Lassmann, H. (2018) Pathogenic Mechanisms Associated With Different Clinical Courses of Multiple Sclerosis. *Front Immunol* **9**, 3116
6. Cunniffe, N., and Coles, A. (2021) Promoting remyelination in multiple sclerosis. *J Neurol* **268**, 30-44
7. Gruchot, J., Weyers, V., Gottle, P., Forster, M., Hartung, H. P., Kury, P., and Kremer, D. (2019) The Molecular Basis for Remyelination Failure in Multiple Sclerosis. *Cells* **8**
8. Spaas, J., van Veggel, L., Schepers, M., Tiane, A., van Horssen, J., Wilson, D. M., 3rd, Moya, P. R., Piccart, E., Hellings, N., Eijnde, B. O., Derave, W., Schreiber, R., and Vanmierlo, T. (2021) Oxidative stress and impaired oligodendrocyte precursor cell differentiation in neurological disorders. *Cell Mol Life Sci* **78**, 4615-4637
9. Tiane, A., Schepers, M., Rombaut, B., Hupperts, R., Prickaerts, J., Hellings, N., van den Hove, D., and Vanmierlo, T. (2019) From OPC to Oligodendrocyte: An Epigenetic Journey. *Cells* **8**
10. Baecher-Allan, C., Kaskow, B. J., and Weiner, H. L. (2018) Multiple Sclerosis: Mechanisms and Immunotherapy. *Neuron* **97**, 742-768
11. Ruiz, F., Vigne, S., and Pot, C. (2019) Resolution of inflammation during multiple sclerosis. *Semin Immunopathol* **41**, 711-726
12. Skaper, S. D. (2019) Oligodendrocyte precursor cells as a therapeutic target for demyelinating diseases. *Prog Brain Res* **245**, 119-144
13. Kuhlmann, T., Miron, V., Cui, Q., Wegner, C., Antel, J., and Bruck, W. (2008) Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis. *Brain* **131**, 1749-1758
14. Kotter, M. R., Li, W. W., Zhao, C., and Franklin, R. J. (2006) Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. *J Neurosci* **26**, 328-332
15. Liu, T., Sun, L., Zhang, Y., Wang, Y., and Zheng, J. (2022) Imbalanced GSH/ROS and sequential cell death. *J Biochem Mol Toxicol* **36**, e22942
16. Chen, X., Guo, C., and Kong, J. (2012) Oxidative stress in neurodegenerative diseases. *Neural Regen Res* **7**, 376-385
17. Forman, H. J., Zhang, H., and Rinna, A. (2009) Glutathione: overview of its protective roles, measurement, and biosynthesis. *Mol Aspects Med* **30**, 1-12
18. Flohé, L. (1978) Glutathione peroxidase: fact and fiction. *Ciba Found Symp*, 95-122
19. Brigelius-Flohé, R., and Maiorino, M. (2013) Glutathione peroxidases. *Biochim Biophys Acta* **1830**, 3289-3303

20. Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R., and Turner, N. D. (2004) Glutathione metabolism and its implications for health. *J Nutr* **134**, 489-492
21. Ni, G., Hu, Z., Wang, Z., Zhang, M., Liu, X., Yang, G., Yan, Z., and Zhang, Y. (2022) Cysteine Donor-Based Brain-Targeting Prodrug: Opportunities and Challenges. *Oxid Med Cell Longev* **2022**, 4834117
22. Shanker, G., Allen, J. W., Mutkus, L. A., and Aschner, M. (2001) The uptake of cysteine in cultured primary astrocytes and neurons. *Brain Res* **902**, 156-163
23. Bianchi, M. G., Bardelli, D., Chiu, M., and Bussolati, O. (2014) Changes in the expression of the glutamate transporter EAAT3/EAAC1 in health and disease. *Cell Mol Life Sci* **71**, 2001-2015
24. Bjørn-Yoshimoto, W. E., and Underhill, S. M. (2016) The importance of the excitatory amino acid transporter 3 (EAAT3). *Neurochem Int* **98**, 4-18
25. Escobar, A. P., Wendland, J. R., Chávez, A. E., and Moya, P. R. (2019) The Neuronal Glutamate Transporter EAAT3 in Obsessive-Compulsive Disorder. *Front Pharmacol* **10**, 1362
26. Aoyama, K., and Nakaki, T. (2013) Neuroprotective properties of the excitatory amino acid carrier 1 (EAAC1). *Amino Acids* **45**, 133-142
27. Himi, T., Ikeda, M., Yasuhara, T., Nishida, M., and Morita, I. (2003) Role of neuronal glutamate transporter in the cysteine uptake and intracellular glutathione levels in cultured cortical neurons. *J Neural Transm (Vienna)* **110**, 1337-1348
28. Tiane, A., Schepers, M., Riemens, R., Rombaut, B., Vandormael, P., Somers, V., Prickaerts, J., Hellings, N., van den Hove, D., and Vanmierlo, T. (2021) DNA methylation regulates the expression of the negative transcriptional regulators ID2 and ID4 during OPC differentiation. *Cell Mol Life Sci* **78**, 6631-6644
29. Wu, P., Bjørn-Yoshimoto, W. E., Staudt, M., Jensen, A. A., and Bunch, L. (2019) Identification and Structure-Activity Relationship Study of Imidazo[1,2-a]pyridine-3-amines as First Selective Inhibitors of Excitatory Amino Acid Transporter Subtype 3 (EAAT3). *ACS Chem Neurosci* **10**, 4414-4429
30. Hu, L., Zheng, T., Song, Y., Fan, J., Li, H., Zhang, R., and Sun, Y. (2021) Ultrasensitive and selective fluorescent sensor for cysteine and application to drug analysis and bioimaging. *Analytical Biochemistry* **620**, 114138
31. Bankar, S. B., Bule, M. V., Singhal, R. S., and Ananthanarayan, L. (2009) Glucose oxidase--an overview. *Biotechnol Adv* **27**, 489-501
32. FitzGerald, U. F., Gilbey, T., Brodie, S., and Barnett, S. C. (2003) Transcription factor expression and cellular redox in immature oligodendrocyte cell death: effect of Bcl-2. *Mol Cell Neurosci* **22**, 516-529
33. Kumar, P., Nagarajan, A., and Uchil, P. D. (2018) Analysis of Cell Viability by the MTT Assay. *Cold Spring Harb Protoc* **2018**
34. Sumantran, V. N. (2011) Cellular chemosensitivity assays: an overview. *Methods Mol Biol* **731**, 219-236
35. Ghasemi, M., Turnbull, T., Sebastian, S., and Kempson, I. (2021) The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis. *Int J Mol Sci* **22**
36. Netto, L. E. S., de Oliveira, M. A., Monteiro, G., Demasi, A. P. D., Cussiol, J. R. R., Discola, K. F., Demasi, M., Silva, G. M., Alves, S. V., Faria, V. G., and Horta, B. B. (2007) Reactive cysteine in proteins: protein folding, antioxidant defense, redox signaling and more. *Comp Biochem Physiol C Toxicol Pharmacol* **146**, 180-193

37. Brosnan, J. T., and Brosnan, M. E. (2006) The sulfur-containing amino acids: an overview. *J Nutr* **136**, 1636s-1640s
38. Ransy, C., Vaz, C., Lombès, A., and Bouillaud, F. (2020) Use of H(2)O(2) to Cause Oxidative Stress, the Catalase Issue. *Int J Mol Sci* **21**
39. Khatami, S. H., Vakili, O., Ahmadi, N., Soltani Fard, E., Mousavi, P., Khalvati, B., Maleksabet, A., Savardashtaki, A., Taheri-Anganeh, M., and Movahedpour, A. (2021) Glucose oxidase: Applications, sources, and recombinant production. *Biotechnol Appl Biochem*
40. Yang, Z., Watanabe, M., and Nishiyama, A. (2005) Optimization of oligodendrocyte progenitor cell culture method for enhanced survival. *J Neurosci Methods* **149**, 50-56
41. Lim, J. L., van der Pol, S. M., Baron, W., McCord, J. M., de Vries, H. E., and van Horssen, J. (2016) Protandim Protects Oligodendrocytes against an Oxidative Insult. *Antioxidants (Basel)* **5**
42. French, H. M., Reid, M., Mamontov, P., Simmons, R. A., and Grinspan, J. B. (2009) Oxidative stress disrupts oligodendrocyte maturation. *J Neurosci Res* **87**, 3076-3087
43. Soria, F. N., Zabala, A., Pampliega, O., Palomino, A., Miguelez, C., Ugedo, L., Sato, H., Matute, C., and Domercq, M. (2016) Cystine/glutamate antiporter blockage induces myelin degeneration. *Glia* **64**, 1381-1395
44. Lewerenz, J., Hewett, S. J., Huang, Y., Lambros, M., Gout, P. W., Kalivas, P. W., Massie, A., Smolders, I., Methner, A., Pergande, M., Smith, S. B., Ganapathy, V., and Maher, P. (2013) The cystine/glutamate antiporter system x(c)(-) in health and disease: from molecular mechanisms to novel therapeutic opportunities. *Antioxid Redox Signal* **18**, 522-555
45. Zhang, Y., Chen, K., Sloan, S. A., Bennett, M. L., Scholze, A. R., O'Keeffe, S., Phatnani, H. P., Guarnieri, P., Caneda, C., Ruderisch, N., Deng, S., Liddelow, S. A., Zhang, C., Daneman, R., Maniatis, T., Barres, B. A., and Wu, J. Q. (2014) An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci* **34**, 11929-11947
46. Carter, M., and Shieh, J. C. (2010) Chapter 13 - Cell Culture Techniques. in *Guide to Research Techniques in Neuroscience* (Carter, M., and Shieh, J. C. eds.), Academic Press, New York. pp 281-296
47. Wei, Q., Miskimins, W. K., and Miskimins, R. (2004) Sox10 acts as a tissue-specific transcription factor enhancing activation of the myelin basic protein gene promoter by p27Kip1 and Sp1. *J Neurosci Res* **78**, 796-802
48. Stolt, C. C., Rehberg, S., Ader, M., Lommes, P., Riethmacher, D., Schachner, M., Bartsch, U., and Wegner, M. (2002) Terminal differentiation of myelin-forming oligodendrocytes depends on the transcription factor Sox10. *Genes Dev* **16**, 165-170
49. Falcucci, R. M., Wertz, R., Green, J. L., Meucci, O., Salvino, J., and Fontana, A. C. K. (2019) Novel Positive Allosteric Modulators of Glutamate Transport Have Neuroprotective Properties in an in Vitro Excitotoxic Model. *ACS Chem Neurosci* **10**, 3437-3453
50. Escartin, C., Won, S. J., Malgorn, C., Auregan, G., Berman, A. E., Chen, P. C., Déglon, N., Johnson, J. A., Suh, S. W., and Swanson, R. A. (2011) Nuclear factor erythroid 2-related factor 2 facilitates neuronal glutathione synthesis by upregulating neuronal excitatory amino acid transporter 3 expression. *J Neurosci* **31**, 7392-7401

51. Bird, A. D., and Cuntz, H. (2019) Dissecting Sholl Analysis into Its Functional Components. *Cell Rep* **27**, 3081-3096.e3085
52. Ridder, W. H., 3rd, and Nusinowitz, S. (2006) The visual evoked potential in the mouse--origins and response characteristics. *Vision Res* **46**, 902-913

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*Author contributions* – **Who did what**? JH and JdR conceived and designed the research. JH and BvH performed experiments and data analysis. EvS and DD provided assistance with FLIM-FRET. JH and JdR wrote the paper. All authors carefully edited the manuscript.

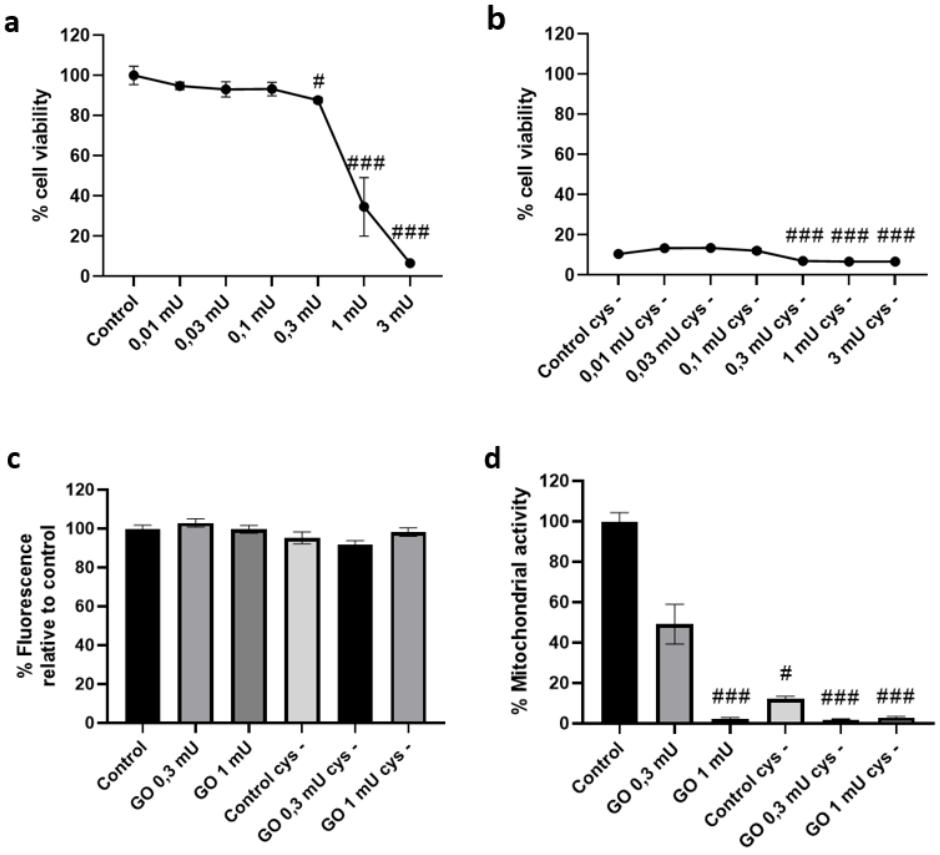
## SUPPLEMENTARY

**Table S1: Antibody list**

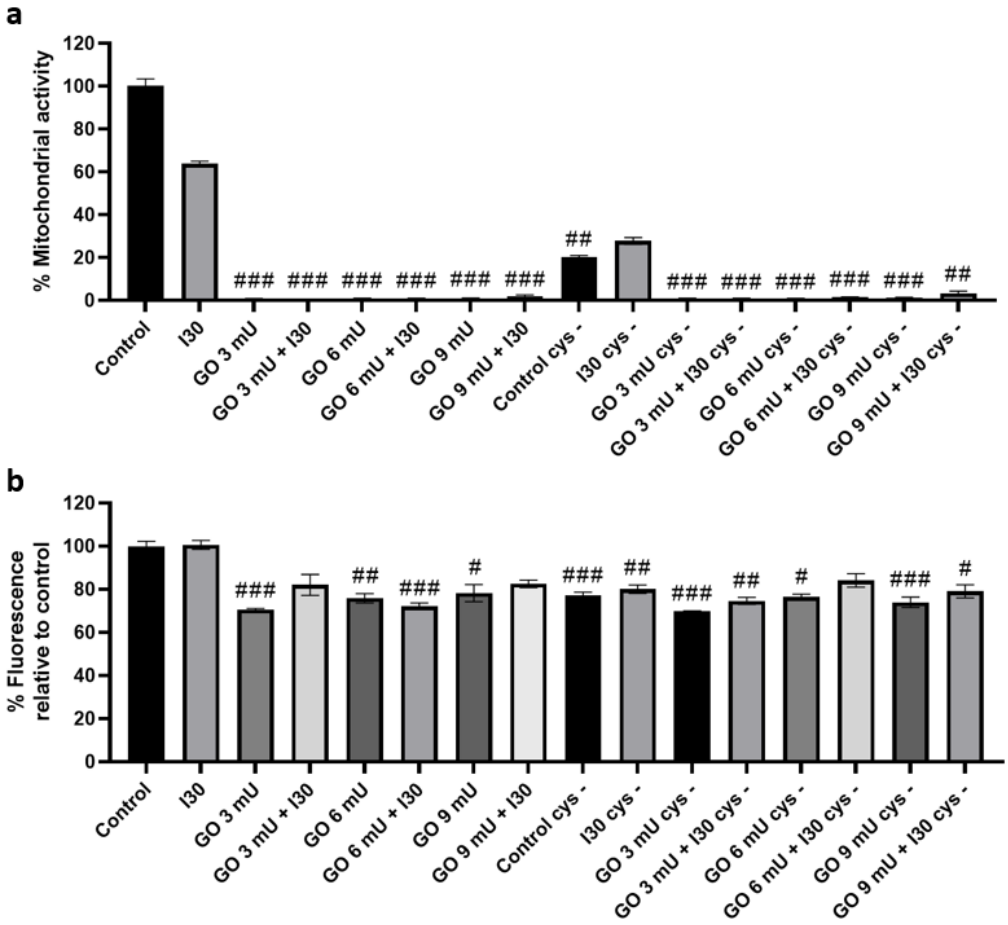
| Antigen | Company | Dilution |
|---|---|---|
| O4 | R&D Systems (MAB1326) | 1:1000 |
| MBP | Merck (MAB386) | 1:500 |
| Goat anti-mouse IgM | Invitrogen (A21042) | 1:600 |
| Goat anti-rat IgG | Invitrogen (A21434) | 1:600 |

**Table S2: qPCR primer list**

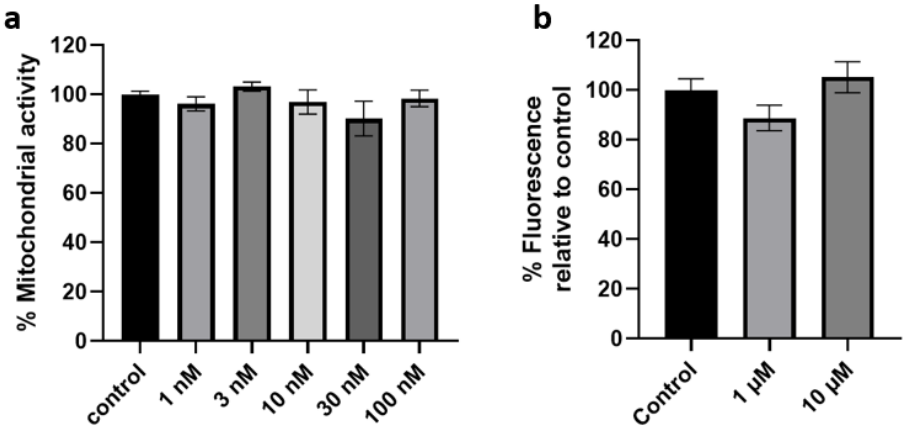
| Antigen | Forward primer (5'-3') | Reverse primer (3'-5') |
|---|---|---|
| Ltris3 (mouse) | ATT GCT GGA ACC CAC TAT GC | GCC CCG AGT AGC TGA AG |
| Cypa (mouse) | GCG TCT CCT TCG AGC TGT T | AAG TCA CCA CCC TGG CA |
| Iap1 (mouse) | TGG GAG GTA TGT CTG ATT GCA | TGA TCC CCA GTG TGC AGA AA |
| Rltr4 (mouse) | GTA ACG CCA TTT TGC AAG GC | CCA TCT GTT CTT TGG CCC TG |
| Gpx1 (mouse) | CTC ACC CGC TCT TTA CCT TCC TT | ACAA CCG GAG ACC AAA TGA TGT ACT |
| RPL13a (mouse) | GGA TCC CTC CAC CCT ATG ACA | CTG GTA CTT CCA CCC GAC CTC |
| MOG (mouse) | TAC AAC TGG CTG CAC CGA AG | GCT CCA GGA AGA CAC AAC CA |
| SLC1A1 (mouse) | GCT ACA TGC CGA TTG TTG GCA TT | TAC CCA AGG CAA AGC GGA AA |




**Supplementary figure S1: cell survival and mitochondrial activity after stimulation with different concentrations of GO.** (a-b) Dose-response curve of the percentage of cell viability relative to control with cysteine supplemented (300 µM) medium (a), and cysteine depleted medium (b). (c-d) Analysis of cell survival and mitochondrial activity after stimulation with 0.3 mU and 1 mU GO in cysteine supplemented (300 µM cysteine) medium and cysteine free medium. (c) PI assay analysis in Oli-neu cells. (d) Percentage cell viability relative to control determined by an MTT assay in Oli-neu cells. Dotted line indicate negative control (d; 25.40%). Data are represented as mean ±SEM, n=6, N=1 for a and b, N=2 for c and d . # $p<0.05$ compared to control, ### $p<0.001$ compared to control. Cys -, cysteine depleted medium; PI, propidium iodide; GO, glucose oxidase.




**Supplementary figure S2: cell survival and mitochondrial activity after stimulation with an EAAT3 inhibitor and different concentrations of GO in Oli-neu cells.** (a) Percentage mitochondrial activity relative to control determined by an MTT assay in Oli-neu cells after exposure to 3, 6, and 9 mU GO and 30 µM EAAT3 inhibitor. (b) Percentage fluorescence relative to control determined by a PI assay in Oli-neu cells after stimulation with 3, 6, and 9 mU GO and 30 µM EAAT3 inhibitor. (a-b) Oli-neu cells were treated with cysteine supplemented (300 µM) medium and cysteine free medium. Data are represented as mean ±SEM, n=6.# $p<0.05$ compared to control, ## $p<0.01$ compared to control, ### $p<0.001$, compared to control. Cys -, cysteine depleted medium; PI, propidium iodide; GO, glucose oxidase; I30, 30 µM EAAT3 inhibitor.




**Supplementary figure S3: Mitochondrial activity after stimulation with D8, a derivative of EAAT2, in Oli-neu cells and OPCs.** (a) Percentage mitochondrial activity relative to control determined by an MTT assay in Oli-neu cells after exposure to 1, 3, 10, 30, and 100 nM D8. (b) Percentage mitochondrial activity relative to control determined by an MTT assay in Oli-neu cells after exposure to 1 and 10 µM D8. Data are represented as mean ±SEM, n=6.# $p<0.05$ compared to control, ## $p<0.01$ compared to control, ### $p<0.001$, compared to control. Cys -, cysteine depleted medium; PI, propidium iodide; GO, glucose oxidase; I30, 30 µM EAAT3 inhibitor.

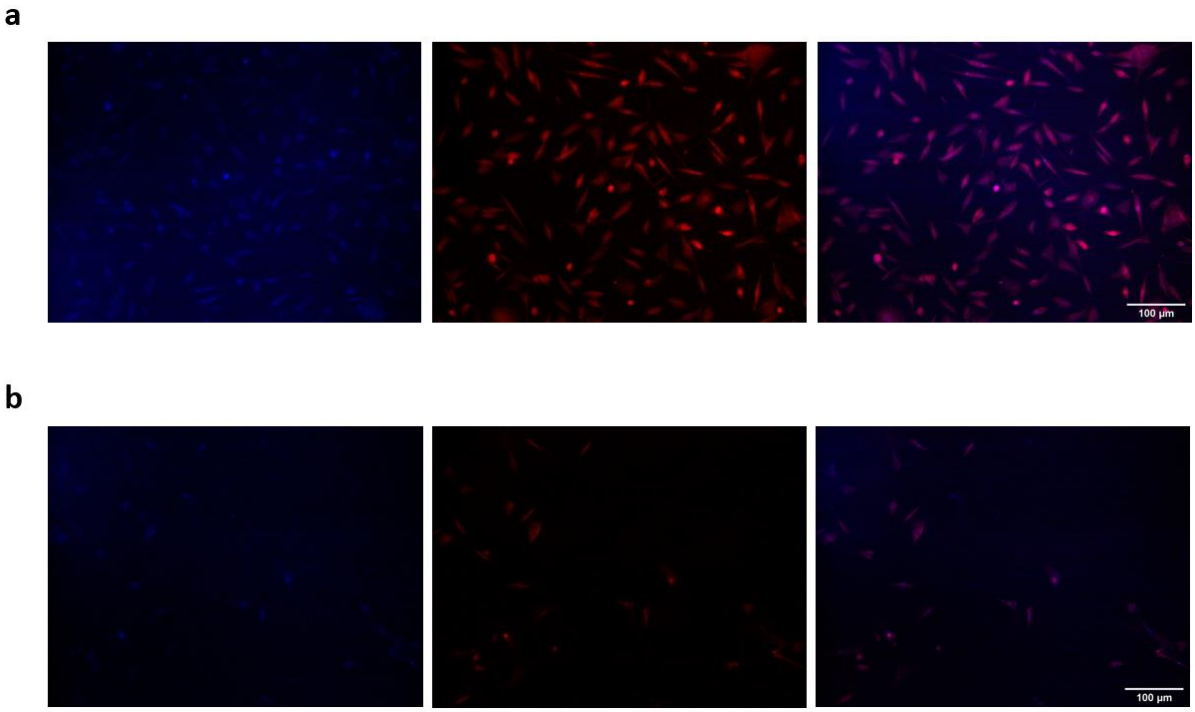



**Figure S4: The detection of the cysteine probe and nucleus staining SYTO™ 61 red.** (a) Representative images of fluorescence detection of the cysteine probe (1µM, blue) and SYTO™ 61 red in Oli-neu cells. Right picture is an overlap. (b) Representative images of fluorescence detection of cysteine probe (2.5µM, blue) and SYTO™ 61 red in Oli-neu cells. Right picture is an overlap.