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# Linalool attenuates oxidative stress and mitochondrial dysfunction mediated by glutamate and NMDA toxicity

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## ARTICLE INFO

## ABSTRACT

Mitochondrial dysfunction and inflammation contribute to the initiation and development of several brain pathological conditions, including Alzheimer's disease and cerebral ischemia. Linalool is an aromatic plant-derived monoterpene alcohol with reported anti-inflammatory, and anti-oxidant properties. We investigated the role of linalool on glutamate-induced mitochondrial oxidative stress in immortalized neuronal HT-22 cells. Glutamate induced oxidative stress in neuronal cells, as detected by real-time cell impedance measurements, MTT assay, and analysis of Annexin V/PI. Administration of linalool 100 μM reduced cell death mediated by glutamate. Staining of glutamate-stimulated mitochondria by MitoTracker revealed improved morphology in the presence of linalool. Furthermore, we demonstrated a potential neuroprotective effect of linalool in conditions of oxidative stress by a reduction of mitochondrial ROS and mitochondrial calcium levels, and by preserving mitochondrial membrane potential. Experiments using both high-resolution respirometry and Seahorse Extracellular flux analyzer showed that linalool was able to promote an increase in uncoupled respiration that could contribute to its neuroprotective capacity. Linalool protection was validated using organotypic hippocampal slices as *ex vivo* model with NMDA as a stimulus to induce excitotoxity cell damage. These results demonstrate that linalool is protective in an *in vitro* model of glutamate-induced oxidative stress and in an *ex-vivo* model for excitotoxity, proposing linalool as a potential therapeutic agent against neurodegenerative brain diseases where oxidative stress contributes to the pathology of the disease.

## 1. Introduction

Oxytosis is a non-apoptotic form of cell death associated with increased oxidative stress and, according to recent findings, also with mitochondrial dysfunction [1,2]. The term oxytosis was first introduced on the basis of findings on oxidative cell death in neuronal cells [3]. Oxytosis is characterized by both cellular and mitochondrial ROS accumulation [4]. Emerging evidence links oxytosis cell death to the field of neuroscience with increasing indication for its contribution to brain injury and degeneration [5]. For example, oxytosis cell death mechanisms contribute to the pathology of many age-associated neurodegenerative diseases such as Alzheimer disease (AD) or acute events such as cerebral ischemia, where elevated production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), causes damage to all components of the cell, including proteins, lipids, and DNA [6–8].

Oxytosis-induced cell death can be initiated by compounds inhibiting the cysteine/glutamate antiporter system ($x_c-$), such as glutamate [3,9–11]. Blockage of the $x_c-$ system by excessive extracellular glutamate prevents the import of cystine, thereby reducing substrate availability for glutamate cysteine ligase (GCL), leading to depletion of intracellular glutathione (GSH) and decreased activity of glutathione peroxidase 4 (GPX4) [5]. GSH plays an essential role in several physiological processes acting directly as a co-factor in enzymatic reactions or indirectly as the major thiol-disulfide redox buffer in all mammalian cells. These properties allow GSH to provide a cellular critical defense

system against many forms of cellular stress, in particular, oxidative stress [12]. Reduced levels of cystine, in response to excessive glutamate application, leads to a depletion of GSH, GPX4 inhibition, and lipid peroxide formation. These events result in an increase of mitochondrial and cellular ROS levels, resulting in cell death.

Glutamate-mediated oxidative stress mechanisms have been extensively studied in HT-22 cells, a subclone of the hippocampal cell line HT4 [13]. This neuronal cell line has been specifically selected for its sensitivity to glutamate. Exposure of neuronal HT-22 cells to toxic concentrations of glutamate initiates a well-defined signaling cascade which mediates cell death through inhibition of $x_c-$ GSH depletion, excessive ROS production and mitochondrial damage [5]. Recent evidence supports that natural compounds (monoterpenes) might prevent oxidative stress, pathological features of neurodegenerative diseases and of acute events such as stroke [14,15]. Linalool, an enantiomer of the naturally occurring, aromatic plant-derived (e.g. *Lavandula angustifolia, Melissa officinalis, Rosmarinus officinalis, and Cymbopogon citratus*) monoterpene alcohol is a major component of essential oils [16]. Linalool exhibits several effects on the CNS, serving as antinociceptive, anticonvulsant, anxiolytic, and sedative agent [17–19]. Furthermore, various pharmacological properties such as antimicrobial, anti-inflammatory, antileishmanial, anti-atherogenic, and anti-depressant eff;ects have been reported [20–25]. Antioxidant properties of linalool were also demonstrated in a study, investigating oxidation reactions in unsaturated fatty acids extracted from guinea pig brains challenged with $H_2O_2$ to induce oxidative stress [26].

We have recently shown linalool being protective in neurodegenerative diseases, and being able to reduce the histopathological markers, including amyloid plaques and tau hyperphosphorylation in a triple transgenic mice model of AD (3xTg-AD) with improvements in behavioral assessments of learning and spatial memory [27]. Besides, in an *in vitro* model of glutamate excitotoxicity, we found that linalool protected neurons and astrocytes as indicated by reduced lactate dehydrogenase (LDH) release as well as recovered cellular ATP levels, a marker of improved mitochondrial functions. Moreover, neurological, motor, and cognitive impairments were ameliorated by oral linalool administration following global ischemia [28].

Linalool has been shown protective in several models of neurodegeneration, however, the mechanisms underlying the neuroprotective eff;ect of linalool in relation to mitochondrial functions still remain elusive and additional research is necessary in order to determine its precise eff;ects.

Our goal was to evaluate the effect of linalool on the physiological function of mitochondria and its potential neuroprotective properties in conditions of glutamate-induced oxytosis in HT-22 cells and in *ex vivo* organotypic hippocampal slices.

## 2. Materials and methods

### 2.1. Cell culture

HT-22 cells (kindly provided by Prof. Culmsee), which were used to model neurons, were cultured in Dulbecco's modified Eagle Medium (DMEM; Life Technologies, UK, #42340-025) supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Utah, USA, #SV30160.03), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco; Life Technologies, USA, #15070-063) and 1% sodium pyruvate (Gibco, Life Technologies Corporation, USA, #11360-070) at 37 °C and 5% $CO_2$. The passage number used were between 200-250 and the cells were mycoplasma negative. Additional substances used were linalool (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #L2602, Lot# STBG7505), and glutamate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #G1626, Lot#slbl6389 V).

### 2.2. Cell viability assay and xCELLigence measurement

Cell viability and metabolic activity of cells were investigated using the colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reduction assay. MTT solution (0.5 mg/ml) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany #M5655) was administered to cells in a 96-well plate format for maximum 1 h. Afterward, the MTT-containing medium was entirely removed and the plate was incubated for at least 2–4 h at − 80 °C. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #D8418, Lot# SHBH9942) was applied to the cells which allowed the formazan to uniformly disperse in the solution. DMSO was incubated for 1 h at 37 °C under continuous shaking conditions. The absorbance values were determined by Synergy™H1 (Bad Friedrichshall, Germany) Hybrid Multi-Mode Reader at 570 nm with a reference filter at 630 nm. Untreated healthy cells, defined as controls, were regarded as 100% cell viability. We dissolved the linalool in DMSO 0.1%, and this solvent had no effect on cellular activity or metabolic activity. HT-22 cells were co-treated with linalool or glutamate seeded onto 96 well plates in conditions as follows: control condition, linalool 100 μM, glutamate 6 mM and co-treatment with glutamate 6 mM plus linalool 100 μM.

Cell viability was measured in real-time using the xCELLigence ®RTCA MP system (ACEA BIO, The Netherlands) which is a label-free cell-based assay suitable for continuous monitoring of biological processes of living cells [29]. Continuous cell monitoring was performed using an E-plate 96 and measured by the xCELLigence ®RTCA MP Instrument. Cellular impedance was assessed every 30 min for 24 h and represented as Normalized Cell Index (NCI) which was defined before the application of the experimental conditions (control, linalool 100 μM, glutamate 6 mM and co-treatment of glutamate 6 mM with linalool 100 μM) as the starting point (t: 0 h) of the experiment.

### 2.3. Flow cytometry

Several intracellular parameters associated with oxidative stress, mitochondrial dysfunction, and cell death were determined by flow cytometry analysis using the CytoFLEXS benchtop flow cytometer (Beckman Coulter Life Sciences, Indianapolis, USA). HT-22 cells were seeded in 24-well plates with 40,000 cells/well. To allow the comparison between different treatment groups (control condition, linalool 100 μM, glutamate 6 mM, and co-treatment of glutamate 6 mM with linalool 100 μM) and the total amount of cells per condition. The supernatant of the medium was collected and washed with PBS, followed by trypsinization. For every condition, three wells were analyzed and the same number of cells (10,000)/well was counted by flow cytometry. Minimum of 3 independent experiments were performed, and the quantification of data was acquired using Kaluza Analysis 1.5 software.

#### 2.3.1. Annexin V/PI

In order to assess the amount of apoptotic as well as necrotic cells after stimulation, Annexin V FITC and propidium iodide (PI) (Invitrogen, Oregon, USA, #V13242, Lot#2008168) double staining kit was used. After treatment, HT-22 cells were trypsinized, and the cell suspension was incubated with Annexin V FITC and PI in binding buffer for 10 min at room temperature (RT). Detection of fluorescence was acquired at FITC: 494/518 and PI: 535/617.

#### 2.3.2. Mitochondrial ROS production

Mitochondrial ROS formation was evaluated by the MitoSOX dye (Invitrogen, Oregon, USA, #M36008, Lot#1924466). HT-22 cells were incubated with MitoSOX™ 1.25 μM dye for 30 min at 37 °C. Fluorescence was excited at 488 nm and detected at 690/50 nm.

#### 2.3.3. Mitochondrial membrane potential

Loss of mitochondrial membrane potential (ΔΨm) was determined by staining with TMRE™ dye (tetramethylrhodamine-ethyl ester;

Invitrogen, Oregon, USA, #T669). Cells were collected and incubated for 20 min with TMRE 0.2 µM at 37 °C. Fluorescence was excited at 488 nm and detected at 690/50 nm.

### 2.3.4. Measurement of mitochondrial calcium

For the measurement of mitochondrial calcium levels, HT-22 cells were incubated with of rhodamine-2-acetoxymethylester 2 µM dye (Rho2-AM) (Abcam, #ab142780, Lot# APN15176-1-1) in DMEM without serum for 30 min at RT, followed by incubation in DMEM for 30 min at RT in the dark and washed with PBS. Cells were excited at 552 nm wavelength and the emission intensities at 581 nm.

### 2.3.5. Lipid peroxidation

Lipid peroxidation was estimated with BODIPY 2 µM staining dye (Invitrogen, Karlsruhe, Germany, #D3861, Lot# 1890330) for 60 min at 37 °C. Cells were washed once with PBS followed by their analysis using the excitation channel at 488 nm and detection channel at 530 nm.

### 2.4. Mitochondrial morphology: Mito Tracker deep red

HT-22 cells ($4 \times 10^4$ cells) were treated with different conditions (control condition, linalool 100 µM, glutamate 6 mM and co-treatment of glutamate 6 mM with linalool 100 µM) for 18 h and incubated with 200 nM MitoTracker™Deep Red FM (Invitrogen, Oregon, USA, #M22426, Lot# 509441) for 30 min at 37 °C. After fixation with 4% Paraformaldehyde (PFA), the fluorescence was excited at 620 ± nm and detected at 700 ± 75 nm. 500 cells per condition were counted and classified into four different categories based on their mitochondrial morphology. This categorization was obtained by an investigator with no prior knowledge of the experimental conditions.

Category I represents healthy cells where mitochondria form an elongated tubular network distributed equally throughout the cytosol, Category II represents semi-viable cells comprising short yet tubular mitochondrial networks around the cytosol. Category II comprises mitochondria that appear short but are still forming long tubules. Cells which entail large, fragmented/ granulated mitochondria around the nucleus are referred as Category III. These cells do not show apoptotic features. On the contrary, damaged and dying cells were identified by small rounded mitochondria of diff ;erent sizes within proximity of the nucleus. The latter mitochondria are referred to as Category IV [30]. Three independent experiments were performed.

### 2.5. Mitochondrial respiration

HT-22 cells ($4–8 \times 10^7$) were harvested and mitochondria were isolated utilizing semi-automated pump-controlled cell rupture system (0.71 ml/min) (PCC) using syringes (SGE, Trajan© Scientific, Australia) attached to a cell homogenizer (Isobiotech, EMBL Heidelberg, Germany) superimposed on a pump (ProSense, Oosterhout, NL, #NE-1000) [31]. Mitochondria were suspended in isolation buff ;er (sucrose 250 mM, HEPES 20 mM, EDTA 3 mM adjusted to pH 7,5). The entire procedure was performed on ice.

### 2.5.1. High-resolution respirometry (Oroboros)

The amount of protein derived from the isolation procedure was 250 µg and it was determined using the Pierce™BCA Protein Assay Kit (Pierce™BCA Protein Assay Kit, Thermo Scientific, Rockford, USA, #23225). Mitochondrial respiration, of the organelles obtained from the HT-22, was analyzed via high-resolution respirometry oxygraph O2K (Oroboros Systems, Innsbruck, Austria). The isolated mitochondria prepared as previously described [31] were treated with linalool 100 µM or ethanol for 25 min, followed by Oroboros measurements. Mitochondrial state 1 was monitored under continuous stirring at 750 rpm in 1 ml MiR05 buff ;er (EGTA 0.5 mM, $MgCl_2$ 3 mM, lactobionic acid 60 mM, taurine 20 mM, $KH_2PO_4$ 10 mM, HEPES 20 mM, D-Sucrose

110 mM, BSA, essential fatty acid-free1g/L, at 7.4 pH). In order to perform the oxygen polarography, DatLab software (Oroboros Systems, Innsbruck, Austria) was implemented to record real-time oxygen flux per tissue mass (pmol $O2.s^{-1*}$ $mg^{-1}$) at 37 °C. Non-phosphorylating respiration (State 2) was assessed by addition of complex-I linked substrates (5 mM) pyruvate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany #P-2256), and (2 mM) malate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #374318). Thereafter, the oxidative-phosphorylation capacity (OXPHOS, State 3) of complex-I linked activity or basal respiration was initiated by means of adding saturating concentration (0.8 mM) of ADP (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #A5285). Subsequently, addition of oligomycin 0.1 µM/ml (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #O4876) was used to block *Fo* of the ATP-synthase, thereby inhibiting proton passage. Besides, maximal respiration (State 3 u) was assessed by incremental (steps 1 µM) addition of the protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #C2920). End of measurement by addition of Sodium dithionite (DTT, Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #15,795-3) to react with all the oxygen present in the chamberThe oxygen concentration as well as the initial derivate of the oxygen concentration which is a derivative of the oxygen consumption is provided as the $O_2$ slope (pmol $*$ (mL/s) of raw isolated mitochondria. The measurements were recorded in intervals (2 s) using instrumental background correction after calibration of the polarographic oxygen sensors.

### 2.5.2. Measurement of cellular oxygen consumption rate (OCR)

Oxygen consumption rate (OCR) was measured using XFe96 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA) with the mitochondrial stress test as previously described [1]. HT-22 cells were seeded in XFe96-well cell culture microplate at a density of 10,000 cell/well and incubated at 37 °C incubator with 5% $CO_2$. 24 h before the assay, the growth medium was replaced with a new medium containing linalool 100 µM or 200 µM. Cells were incubated for 1 h in 180 µL of assay medium (non-buffered DMEM) supplemented with 4.5 g/L glucose, 2 mM glutamine and 1 mM pyruvate, pH 7.35 in $CO_2$ free incubator at 37 °C. During the assay, basal respiration was measured before injecting the following inhibitors: oligomycin (4 µM; port A) as an ATP synthase inhibitor; 2,4-Dinitrophenol (DNP) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #34334) (50 µM; port B) as a mitochondrial oxidative phosphorylation uncoupler; and rotenone (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #R8875) /antimycin A (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #A8674) 0.1 µM and 1 µM; port C) as complex I and complex III inhibitors. The OCR measurements were normalized to the protein amount in each well using BCA assay. Three independent experiments with 6 wells/condition were performed, and One-way ANOVA test was used to determine statistical significance between different groups.

### 2.6. Animals

C57BL/6 J mice from the central animal laboratory at University of Groningen were housed and handled in accordance to Dutch standards and guidelines. All experiments were approved by the University of Groningen Committee for Animal Experimentation.

### 2.6.1. Organotypic hippocampal slice cultures and Maintenance

Organotypic hippocampal slice cultures (OHSC) were prepared from P0-3 mouse pups according to the mentioned protocol with slight modifications [32]. Briefly, after quick decapitation, brains were removed and the hippocampi were dissected out in ice cold serum-free Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #14170-088, Lot#1997485), supplemented with 0.5% glucose (Sigma) and 15 mM HEPES. Isolated hippocampi were transversally cut into 350–375 µm thick slices using a McIlwain tissue

chopper. OHSC were then transferred onto Millcell permeable membranes (0.4 μm pore size, Millipore, PICM03050) in six well plates. These culture plate inserts, containing 5 to 6 slices with culture medium: 0.5% Minimum essential medium- MEM (Gibco, #21430-020,), 25% Basal Medium Eagle (BME) (Gibco, 41010-026), 25% heat-inactivated horse serum (Gibco, 16050-122) supplemented with 0.65% glucose (Sigma, G8769) and 2 mM glutamax (Gibco, 35050-038), pH: 7.2. OHSC were maintained at 35 °C, 5% $CO_2$ and with culture medium being changed the first day after preparation and every second day. Experiments were performed after 7–8 days *in vitro*.

### 2.6.2. Drug treatment and cell damage in OHSCs

To induce excitotoxicity, OHSCs were exposed to 10 μM N-Methyl-D-aspartic acid (NMDA) ((Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #M3262) for 24 h (n:5). At the same time, the OHSCs were exposed to linalool 100 μM and a combination of NMDA with linalool 100 μM with its respective control. Following 8 days *in vitro* (DIV8), slice viability was determined using propidium iodide (PI) staining (5 μg/ml; Sigma- Aldrich, 81845). PI uptake was recorded using a Confocal Leica sp8 fluorescence microscope and analyzed by densitometry using Fiji-ImageJ software. The 3 main areas of the hippocampus where the analysis was performed included CA 1 (Cornu Ammonis 1), CA3 (Cornu Ammonis 3) and DG (Dentate gyrus).

### 2.6.3. RNA Extraction and quantitative real-time reverse- transcription polymerase chain reaction (RT-qPCR)

For RNA extraction, 6 OHSC per condition were pooled (one sample) and incubated in 500 μl using TriReagent (Invitrogen, Life Technologies, #AM9673) and trimethylene chlorobromide (BCP) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, #B9673) followed by wahing in isopropanol and elution in 30 μl RNAse-free $ddH_2O$ (n:4-6).The concentration and purity of the RNA was determined by measuring absorbance at 230, 160, and 280 nm with a Nanodrop ND-1000 spectrophotometer. Samples were considered to be sufficiently free of protein and phenol contamination and suitable for PCR analysis when the 260/280 and 260/230 ratios were between 1.8 and 2.2.

Total RNA was transcribed into cDNA using reverse transcription by AMV Reverse Transcriptase Kit (Promega, Madison, WI, #A3500) and was diluted with ddH2O proportionately according to concentration of input RNA. RT-qPCR was performed using an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories GmbH) in the presence of SYBR Green (Roche, #04913914001) using Illumina Eco Personal qPCR System (Westburg, Leusden, The Netherlands). PCR cycling was follows: 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, for 45 cycles. qRT-PCR data were analyzed with LinRegPCR analysis software [33]. Geometric mean of the reference genes ribosomal protein L13A (*Rpl3A*) and ribosomal RNA 18S was used to normalize qRT-PCR data. Mouse mRNA primers used in analysis were purchased from Biolegio and the sequences are listed in Table 1.

### 2.7. Statistical analysis

Statistical significance was evaluated using the unpaired Student´s t-test or ANOVA and Tukey`s test for post hoc multiple comparisons of the parametric data in between-group analyses. Non-parametric data were evaluated using Kruskal- Wallis test. Data were analyzed using GraphPad Prism software (version 7.0, GraphPad Software Inc., La Jolla, CA, USA), expressed as mean ± SD for xCELLigence measurements and SEM for the rest experiments. *P* values indicating statistical significance differences between mean values are defined as follows: $*p < .05$, $**p < .01$, $***p < .001$.

## 3. Results

### 3.1. Linalool ameliorated cytotoxicity and apoptosis in glutamate-stimulated HT-22 cells

Linalool had been reported protective in *in vitro* models of glutamate-induced excitotoxicity in primary cortical neurons [28]. Here, we evaluated the eff ;ect of linalool on glutamate-induced oxidative stress in neuronal HT-22 cells [2]. At first, the potential protection against glutamate-induced oxytosis was assessed using various linalool concentrations. Based on the previous experiments, we choose 100 μM concentration of linalool to investigate the underlying molecular neuroprotective mechanisms of linalool. Following the initiation of oxytosis, HT-22 cells change their morphological shape from the healthy spindle-shaped morphology to a more round-shaped morphology, culminating with the detachment of the cells from the bottom of the cell culture dish (Fig. 1a, upper panels). The protective effect of linalool was demonstrated by cell viability measurements using MTT assay following 18 h of glutamate challenge in the presence of linalool. We observed that glutamate stimulation leads to a significant cell death, whereas co-treatment with linalool in addition to glutamate significantly reduces cell death when compared to the glutamate condition. Application of linalool alone to cells did not significantly affect HT-22 cell viability at concentrations of 200 to 400 μM, however, 500 μM reduced cell viability (Fig. 1.b). Moreover, we observed that linalool at low nanomolar ranges did not mediate neuroprotection (Figure suppl 1.a). In addition, we performed pre-treatment experiments with 2 h or 8 h of linalool and detected that both pretreatment with linalool for 8 h and 2 h followed by glutamate challenge provided protection against glutamate toxicity. Interestingly, the presence of linalool during the glutamate challenge was a prerequisite for the observed protection, since the removal of linalool after the pretreatment was not able to mediate protection against glutamate (Figure suppl 1.b). To further confirm these neuroprotective effects of linalool, we quantified cell death by Annexin V/PI propidium iodide staining. Cells were treated with glutamate, with or without linalool for 18 h. As shown in Fig. 1.c and d, the number of double positive stained cells was increased after glutamate exposure, indicating cell death, and this effect was largely attenuated by linalool application.

The protective effect of linalool against glutamate toxicity in HT-22 cells was also confirmed by real-time cell impedance measurements using the xCELLigence system. Cell impedance measurements demonstrated that glutamate induces a strong decrease in cell index, an indication of cell detachment and cell death, approximately 10–14 h following the initiation of the glutamate treatment (Fig. 1.e). Co-treatment with linalool significantly delayed cell death, determined by

**Table 1**
List of primers used for qPCR experiments

| Gene | Forward | Reverse |
|------|---------|---------|
| *Bax* | AACTGGTGCTCAAGGCCC | TCCCGAAGTAGGAGAGGAG |
| *Cox-2* | TGAGTACCGCAAACGCTTC | CAGCCATTTCCTTCTCTCC |
| *Il-1β* | TGCCACCTTTTGACAGTGA | ATGTGCTGCTGCGAGATTT |
| *Nos2* | GGGACTGAGCTGTTAGAGACAC | TCTTGTATTGTTGGGCTGAGAACA |
| *Rpl3A* | AGAAGCAGATCTTGAGGTTACGG | GTTCACACCAGGAGTCCGTT |
| *18S* | AAACGGCTACCACATCCAAG | CCTCCAATGGATCCTCGTTA |
| *Tnf-α* | TACTGAACTTCGGGGTGATTGGTCC | CAGCCTTGTCCCTTGAAGAGAACC |
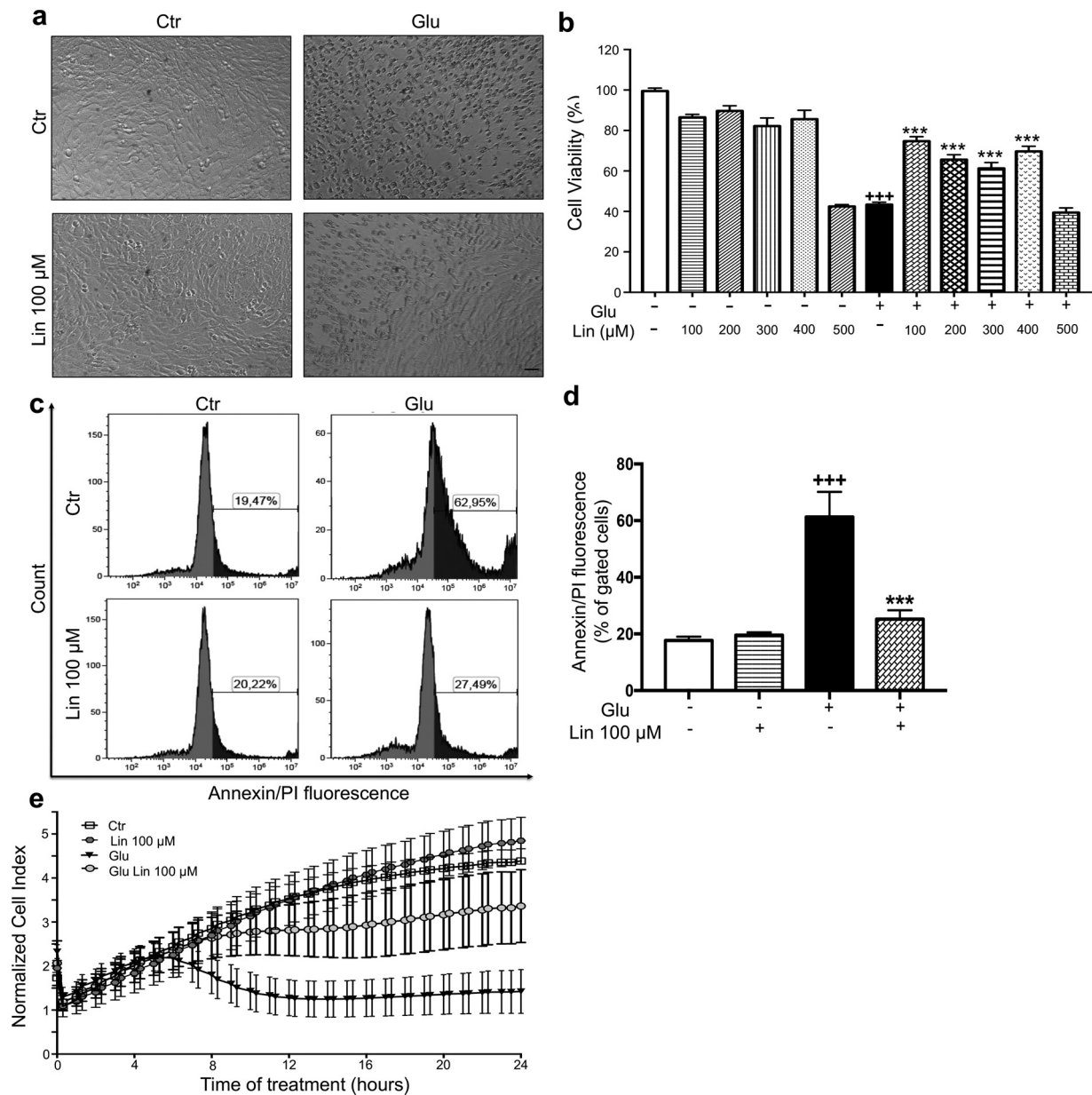
**Fig. 1.** Protective effects of linalool against glutamate in HT-22 cells. **(a).** Representative pictures of the cell morphology of HT-22 cells treated with glutamate (6 mM, 16 h) with and without linalool (100 µM) co-treatment. Magnification 10x (Scale bar: 100 µm) **(b).** Representative MTT assay of cells treated with glutamate and linalool in a range of 100 µM to 500 µM (n = 6 technical replicates; independent experiments repeated at least 3 times). **(c,d).** Measurement of the green Annexin V and the red PI fluorescence by FACS from HT-22 cells challenged with glutamate (6 mM) for 18 h in the absence and presence of linalool co-treatment (10,000 cells per condition; n = 3 technical replicates; $^{+++}p < 0.001$:untreated control versus glutamate, $^{***}p < 0.001$: glutamate versus linalool + glutamate-treated cells ANOVA, Tukey's test, independent experiments repeated at least 3 times). **(e).** Impedance-based real time detection of the morphological alterations of glutamate-treated HT-22 cells with and without linalool co-treatment (mean ± SD; n = 6 technical replicates). All experiments were repeated at least three times.

a right shift of the cell impedance curves. Treatment with linalool alone did not alter the cell index or morphological shape of the cells. Interestingly, linalool did not prevent cell death when cells were challenged with erastin instead of glutamate (Figure suppl 1.b). Erastin was shown to induce ferroptosis cell death and associated with mitochondrial dysfunction and lipid peroxide formation through iron-dependent oxidative enzymes [10]. Taken together, these results show that linalool is able to significantly reduce glutamate-induced cell death in neuronal HT-22 cells.

### 3.2. Linalool preserves mitochondrial integrity in glutamate-treated HT-22 cells

The cell death pathway of oxytosis involves mitochondrial fragmentation and dysfunction. Since mitochondrial fragmentation could be initiated by glutamate [30], we assessed whether linalool could preserve mitochondrial morphology. First, we identified changes in mitochondrial morphology after glutamate exposure compared to control cells (Fig. 2.a–b). In untreated control cells, mitochondria show an elongated and tubular-like shape distributed along the cytosol. This mitochondrial shape is considered as category I and is most frequently found in healthy cells; while category II mitochondrial networks are less extensive than category I mitochondria but are still forming long
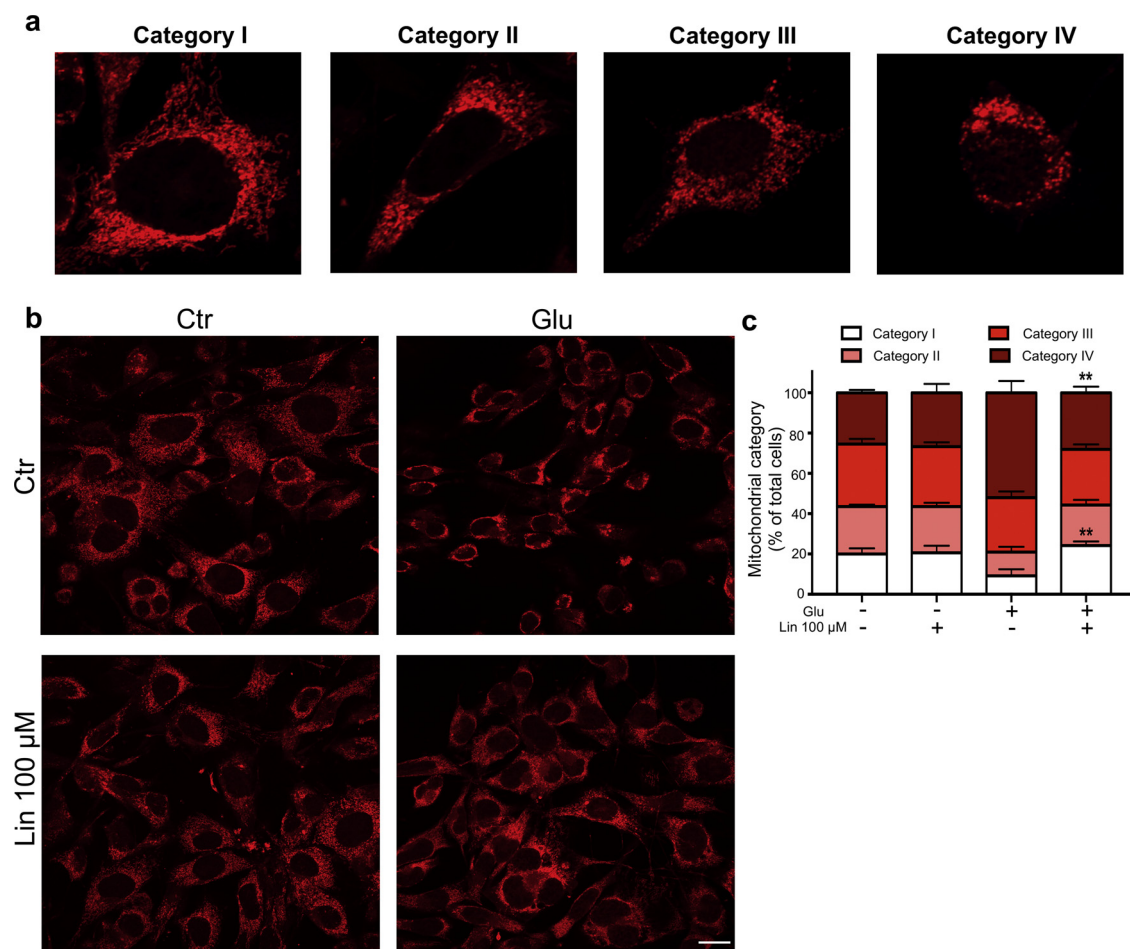
**Fig. 2.** Linalool attenuates mitochondrial fragmentation in glutamate-exposed HT-22 cells. **(a).** Mitochondrial morphology was classified into 4 categories of fragmentation (category I: mitochondria tubulin-like distributed equally throughout the cytosol; category II: short mitochondrial but still forming long tubules; category III: Large round mitochondria that begin to disrupt; category VI: Small rounded mitochondria of diff ;erent sizes within proximity of the nucleus. **(b).** Changes of mitochondrial morphology were visualized following glutamate exposition -challenge (18 h – 6 mM) using MitoTracker Red (scale bar: 20 μm). **(c).** Quantification of mitochondrial categories in all conditions. 500 cells per condition; n = 3 independent experiments; mean ± SEM, **$p <$ 0.01: glutamate *versus*linalool + glutamate-treated cells ANOVA, Tukey's test.

tubules. However, in cells treated with glutamate, mitochondrial fission and fragmentation was elevated and category III and IV are more abundant. Category III is characterized by round mitochondria spread throughout the cell and category IV exhibit round and disintegrated mitochondria surrounding the nucleus. Examination of the acquired confocal images and their categorization (Fig. 2.a–b) indicated a significant diff ;erence between glutamate-treated cells and the remaining conditions, explicitly pertaining to mitochondrial categorization. HT-22 cells exposed to toxic glutamate showed a significant reduction in mitochondria of categories I and II while mitochondria of category IV was significantly increased. In accordance with the cell viability data, co-treatment of glutamate with linalool showed similar mitochondrial categories as the control healthy cells, indicating that linalool is able to preserve mitochondrial shape and prevent its fragmentation.

Under physiological conditions, mitochondria represent the major endogenous source of ROS as a byproduct of the electron transfer system (ETS). ROS generation is enhanced upon mitochondrial dysfunction, subsequently leading to oxidative damage to lipids, proteins, and DNA [34]. We evaluated the mitochondrial ROS levels using flow cytometry of the fluorescent MitoSOX dye, following glutamate exposure in the presence or absence of linalool. Analysis of the FACS data indicated that HT-22 cells stimulated with glutamate yielded a significant increase in mitochondrial ROS levels when compared to vehicle-treated cells. HT-22 cells co-treated with glutamate and linalool showed

significantly decreased amounts of mitochondrial ROS levels when compared to the glutamate condition (Fig. 3.a–b).

Next, we evaluated the mitochondrial membrane potential ($\triangle\psi m$) by FACS analysis of the TMRE fluorescence, a dye specific for $\triangle\psi m$ measurements. Our results indicated that glutamate challenge provoked a significant decrease in $\triangle\psi m$. Linalool alone did not result in a noticeable diff ;erence compared to vehicle-treated cells (Fig. 3.c–d), while in the presence of glutamate, it significantly alleviated the loss of $\triangle\psi m$.

Mitochondria play an essential role in buffering the cytosolic calcium overload in stimulated neurons. Therefore, we evaluated Rhod2AM fluorescence, specific for assessing mitochondrial $Ca^{2+}$ levels by FACS measurements. Following glutamate treatment, we observed an increase in the Rhod2AM fluorescence intensities, while co-treatment with linalool reduced the increase in mitochondrial $Ca^{2+}$ levels (Fig. 3.e–f).

### 3.3. Linalool reduces lipid peroxidation in HT-22 cells

In HT-22 cells, glutamate exposure triggers an increase of detrimental ROS levels and also of lipid peroxides. To investigate the influence of linalool on lipid peroxide levels under oxidative stress, HT-22 cells were challenged with glutamate in the presence or absence of linalool for 18 h. Lipid peroxidation was examined by FACS analysis
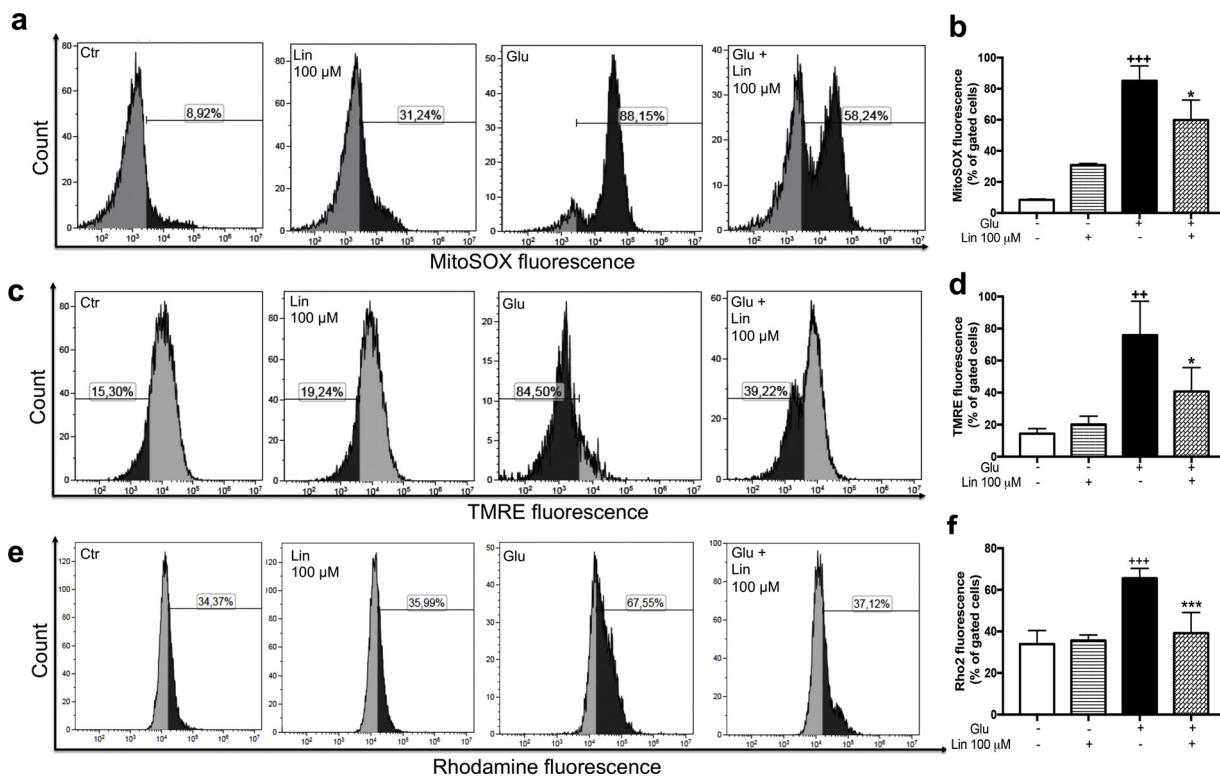
**Fig. 3.** Linalool preserved mitochondrial integrity. Mitochondrial integrity was analyzed by means of fluorescent-activated cell sorting using fluorescent probes for **(a–b).** mitochondrial ROS levels (MitoSOX), **(c–d)** mitochondrial membrane potential (TMRE) or **(e–f)** mitochondrial calcium (Rhod2 AM) following glutamate exposure (18 h) in the presence or absence of linalool 100 μM in HT-22 cells. Data are presented as mean ± SEM, n = 3–4 technical replicates, independent experiments repeated at least 3 times, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$: glutamate versus linalool + glutamate-treated cells ANOVA, Tukey's test.
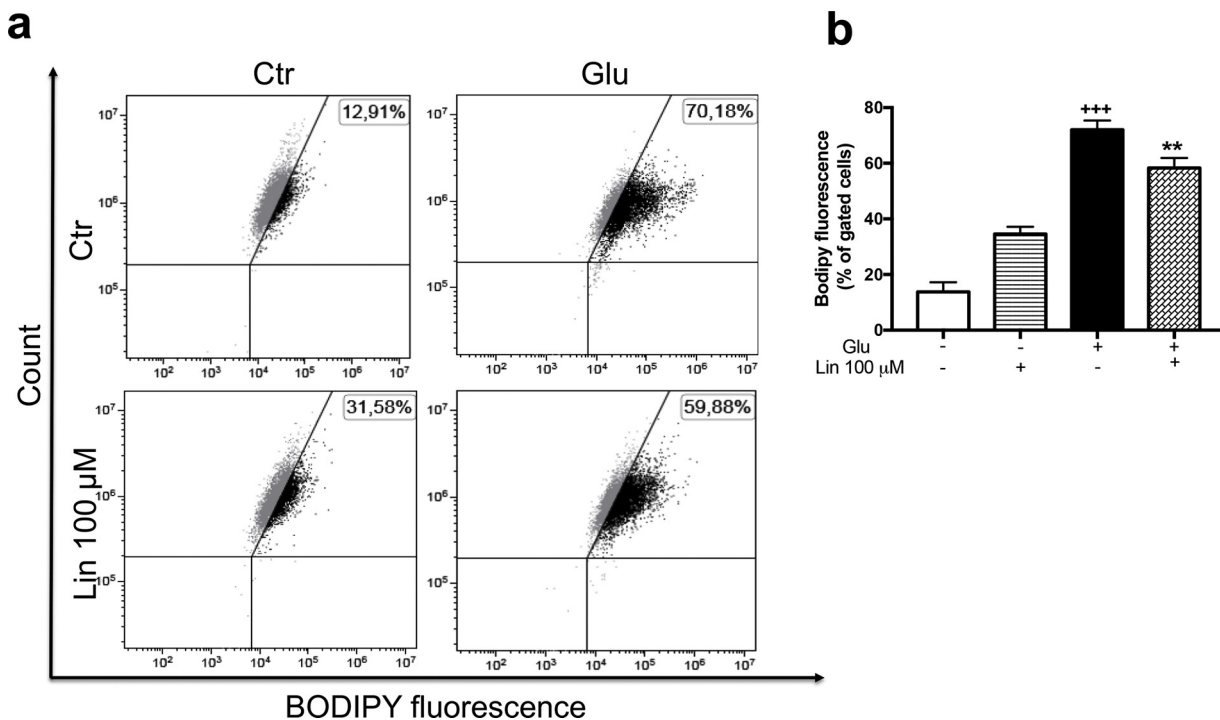


**Fig. 4.** Lipid peroxidation decreases with exposure of linalool after glutamate treatment. **(a).** Representative measurements of lipid peroxidation following glutamate exposure (18 h) in the presence or absence of linalool (Lin) in HT-22 cells. b. Bar graphs with statistics. Results are shown as mean ± SEM, n = 3 technical replicates. All experiments were repeated at least three times. **$p < 0.01$: glutamate versus linalool + glutamate-treated cells ANOVA, Tukey's test.
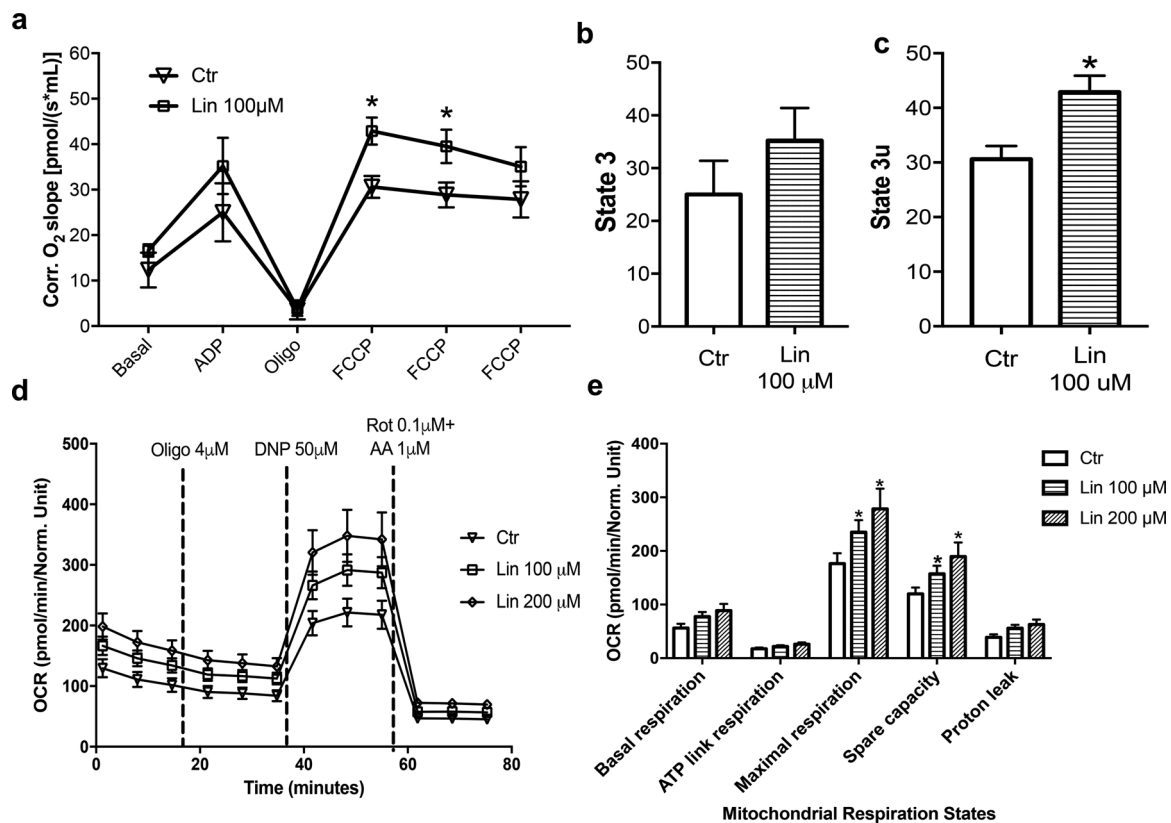
**Fig. 5.** Addition of linalool increases the uncoupled respiration in HT-22 cells. **(a).** Representative oxygen slope of isolated mitochondria from HT-22 cells. Corrected oxygen slope showing mitochondrial states after treatment with control (ethanol) or linalool 100 μM. **(b–c).** Quantification of state 3 and state 3 u of mitochondria pre-treated with control or linalool 100 μM in HT-22 cells (n = 3 technical replicates; independent experiments repeated at least 3 times). **(d).** Oxygen Consumption Rate (OCR) measurements in HT-22 cells treated with or without linalool 100 and 200 μM were obtained over time (min) using an extracellular flux analyzer (Seahorse Bioscience). The mitochondrial stress test was used to obtain bioenergetics parameters, by adding substrates **(e).** Maximal OCR was stimulated by DNP 50 u M addition and the spare respiratory capacity was calculated as the difference between maximal and basal OCR. Graphs show the OCR in pmoles per min. Data are shown as mean ± SEM, n = 3–6 technical replicates. All experiments were repeated at least three times. *$p < 0.05$ compared to indicated control.

using BODIPY fluorescence. Exposure of HT-22 cells to glutamate induced a significant increase in lipid peroxidation, while linalool co-treatment was able to reduce the lipid peroxidation. Additionally, treatment with linalool-only was unable to produce a significant increase of this marker (Fig. 4.a–b). Taken together, linalool application enhanced protection against glutamate-induced oxidative toxicity by preserving mitochondrial morphology and reducing the detrimental levels of mitochondrial ROS, lipid peroxides, and mitochondrial Ca$^{2+}$.

### 3.4. Linalool increases mitochondrial respiration in HT-22 cells

Oxidative phosphorylation is a central energy-conserving mechanism, coupling mitochondrial electron transfer to ATP synthesis and providing the energy required for the maintenance of cellular functions and cell survival [35]. Assessment of mitochondrial respiration represents a functional evaluation of mitochondrial homeostatic state [36,37]. In the next step, to study alterations of the main mitochondrial metabolic functions, we performed high-resolution respirometry experiments in purified mitochondria of HT-22 cells. Interestingly, we found that linalool application to isolated mitochondria for a period of 25 min showed a tendency towards improved complex I-linked respiration rate, induced by ADP (state 3) (Fig. 5.b). Analysis of OXPHOS in isolated mitochondria from HT-22 revealed that linalool also promotes an increase in maximum uncoupled respiration induced by FCCP (Fig. 5.a.b).

To complete the characterization of the respiratory capacity of the mitochondria (depicted as oxygen consumption rate; OCR), we used Seahorse Bioscience XF96 Extracellular Flux Analyzer. Fig. 5.d.e.

showed an increase in the mitochondrial respiration states, particularly, in the maximal respiration values as well as the spare respiratory capacity with the addition of linalool 100 μM to HT-22 cells, when compared with solvent-treated control cells. This increase in values is concentration dependent, as linalool 200 μM elicited a higher increase in respiration levels compared to linalool 100 μM. Considering these results, linalool improved mitochondrial respiration when compared to vehicle-treated HT-22 cells and also in isolated mitochondria.

### 3.5. Linalool significantly reduced NMDA-mediated cell death and cyclooxygenase-2 in organotypic brain slices

In order to corroborate the in vitro data (HT-22 cell line), potential neuroprotection effects of linalool were also evaluated ex vivo in OHSC. This ex vivo model is a good method for assessing neuronal death, microglial activation, neurogenesis, and drug screening. OHSC mainly preserves tissue structures, maintain neuronal activities and synapse circuitry, and mimic many aspects of the in vivo context [38]. After six days in culture, we exposed OHSC to 10 μM NMDA in the presence or absence of linalool for 24 h (Fig. 6.a). Cell death was measured by Propidium Iodide (PI) uptake using imaging quantification of the hippocampal slices. NMDA stimuli induced a strong increase in PI uptake in the CA1 and GD regions of the hippocampus, while the CA3 region of the hippocampus was less affected. Co-treatment with linalool significantly reduced the PI uptake in CA1 hippocampal region, although the NMDA-induced damage of GD and CA3 regions was less affected by linalool application (Fig. 6.b.c). Concerning apoptosis evaluation, hippocampal slices challenged with NMDA stimuli showed an increase,
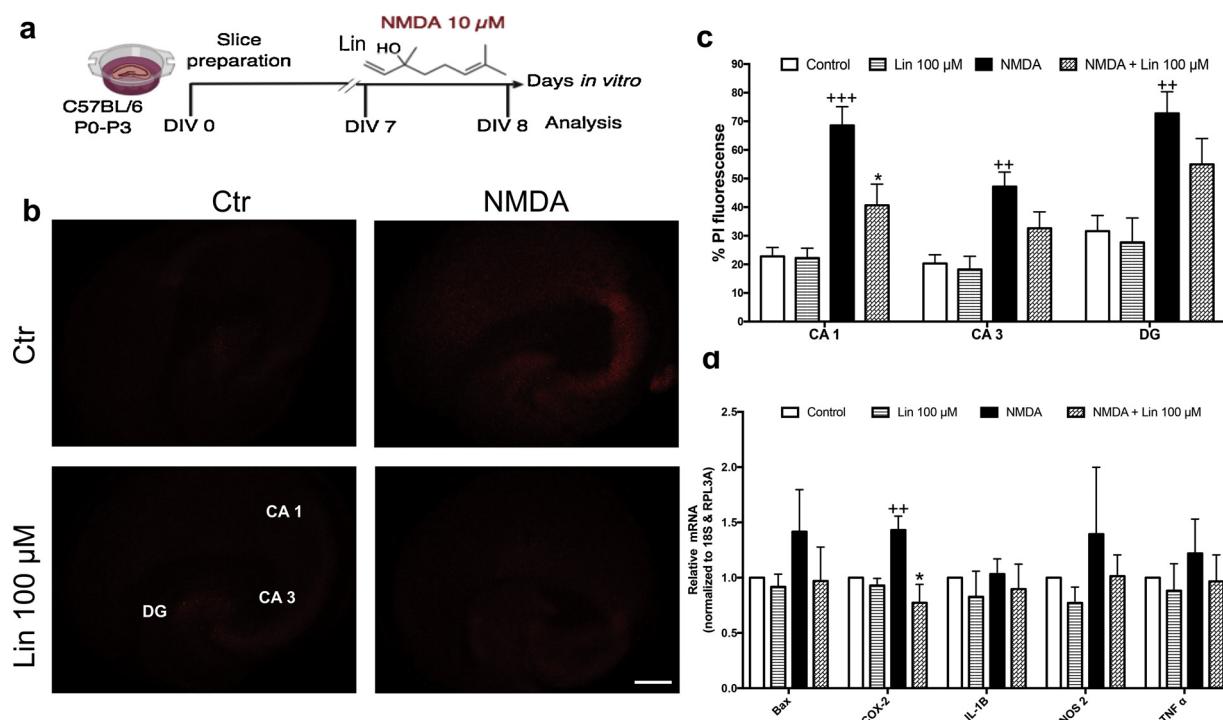
**Fig. 6.** The eff ;ect of linalool on NMDA excitotoxicity in mouse OHSCs. **(a).** Experimental time schedule for treatment with linalool after 10 μM NMDA challenge for 24 h. **(b).** PI staining images indicating the effects of vehicle, linalool and NMDA alone and NMDA + linalool on hippocampal cell damage. Magnification 5x (Scale bar: 50 μm) **(c).** PI fluorescence into CA1, CA3 and DG areas of each sample was expressed as % of total PI fluorescence. **(d).** qPCR analysis of different treatments in slice cultures revealed differences in the levels of *Cox-2* in the NMDA treatment compared with the control. No differences were observed in *Bax*, *Il-1β*, *Nos2* and *Tnf-α*. Data are shown as mean ± SEM n = 4–6 independent experiments). $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$: untreated control vs NMDA, $^*p < 0.05$: NMDA versus linalool + NMDA-treated OHSC ANOVA, Tukey's test.

although not significant to apoptotic Bax protein expression (Fig. 6.d).

To determine if any anti-/pro-inflammatory genes of the signaling systems were involved in the protective effect of linalool on NMDA-induced excitotoxicity in the hippocampus, we analyzed the expression of the following genes: Interleukin 1 beta (*Il-1β*), Nitric Oxide Synthase 2 (*Nos2*), Tumor necrosis factor alpha (*Tnfα*) and cyclooxygenase-2 (*Cox-2*) genes by RT-qPCR. *Cox-2* gene expression was increased after NMDA treatment and treatment with linalool reversed this proinflammatory marker to levels similar to the controls (Fig. 6.d). *Nos2* gene expression seems to increase, albeit no significant values were detected following NMDA treatment, while co-treatment of linalool showed a tendency to decrease the levels of *Nos2* genes. *Il-1β* and Tnfα did not show any differences between the treatments (Fig. 6.d). However, NMDA challenge of OHSC did significantly increase *Cox-2* levels when compared to OHSC-vehicle conditions (post hoc p < 0.01).

Data obtained in OHSC cultures validated the neuroprotective effects of linalool detected in neuronal HT-22 cell line. Furthermore, our results reinforce the hypothesis where the mitochondria are involved in the mechanism of protection when is activated by this monoterpene.

## 4. Discussion

Our findings demonstrate a neuroprotective effect of linalool in conditions of oxidative stress through mechanisms involving an increase in mitochondrial respiration, and preservation of several mitochondrial parameters, including ROS, calcium levels, and $\triangle\psi m$. Furthermore, linalool mediated protection against NMDA-induced excitotoxicity in hippocampal slices. Taken together, we showed that linalool induced neuroprotective effects in an *in vitro* and *ex vivo* models by preserving and restoring mitochondrial function.

In this study, we showed that linalool improves the viability of neuronal cells by 30% in conditions of oxidative stress, independent of excitotoxicity. The *in vitro* model of oxytosis was employed in neuronal

HT-22 cells, which lack the NMDAR, therefore, glutamate stimulation initiates a distinct pathway of cell death independent of NMDA-related increased neuronal excitability [5]. In this paper, we determined the potential timeframe of linalool's protection, which was detected following 10 h of glutamate challenge and was maintained longer than 24 h. Our results are in line with findings investigating the eff ;ects of linalool in glucose/serum deprivation (GSD)-induced cytotoxicity. Under GSD conditions, linalool was able to exert neuroprotective effects following 8 h of GSD in PC12 cells [16]. Similarly, in neuronal cultures has been shown that after oxygen-glucose deprivation/reoxygenation (OGD/R) or glutamate-mediated excitotoxity as *in vitro* model of ischemic stroke, linalool attenuated cell death. The neuroprotection was mediated by scavenging peroxyl-radicals, increasing SOD and catalase levels. We have previously demonstrated the role of linalool *in vitro* in a glutamate excitotoxicity model where linalool restored intracellular ATP levels in neurons and astrocytes [28]. Our results parallel another research using a different monoterpene, catalpol, that was shown to exert neuroprotection in the hippocampal CA1 area of a gerbil transient global cerebral ischemia model [39].

Mitochondrial fusion and fission processes are tightly coordinated by several dynamin protein family members (e.g. fission 1, dynamin related protein 1, endophilin B1), in response to environmental changes and cellular stress [30,40]. Based on previous research which showed that glutamate induces mitochondrial damage in HT-22 cells [2,41], we investigated whether linalool was capable of moderating this eff ;ect. Our results showed that linalool HT-22 cells prevent damaged or dying cells (mitochondrial category IV) stimulated with glutamate. Further, linalool prevented mitochondrial fragmentation and peri-nuclear accumulation of the organelles providing further evidence of its beneficial impact on mitochondria. Besides, we investigated the ability of linalool to modify the mitochondrial membrane potential ($\triangle\psi m$). The latter is a consequence of the proton gradient which exists across the inner mitochondrial membrane and is utilized for ADP phosphorylation. The

net negative charge across a healthy mitochondrion is essentially generated by cytochrome c, which shuttles electrons along the ETS. However, this function is impaired during oxytosis which not only leads to mitochondrial membrane permeabilization but also to release cytochrome c into the cytosol leading to immediate dissipation of $\triangle\psi$m [5]. Our findings showed that linalool enhanced mitochondrial respiration and preserved $\triangle\psi$m which might be a consequence of the conserved inner mitochondrial membrane (IMM).

The brain is particularly susceptible to oxidative stress due to its high content of unsaturated phospholipids [42]. Oxidative stress is highly associated with pathological conditions such as AD, in relation to Aβ plaque burden, and cerebral ischemia, initiated by excessive release of excitatory neurotransmitters and $Ca^{2+}$ leading to increased ROS formation [43,44]. Our results showed co-treatment of glutamate-stimulated HT-22 cells with linalool leads to a reduction of ROS levels. Previous studies showed that linalool reduced intracellular oxidative stress in the OGD/R condition [45]. Furthermore, linalool prevented glutamate-induced cell death alongside retraction of processes and actin cytoskeleton depolymerization in neurons [28]. However, glutamate-stimulation in HT-22 cells yields a well-defined program of cell death via oxytosis; therefore, our findings suggest that the reduction in ROS levels might be due to an increase of intracellular GSH availability. The latter is in line with previous research that showed linalool reduced oxidative stress and the levels of malondialdehyde in an animal model of neurotoxicity produced by acrylamide increasing the GSH levels [46].

We reported for the first time, the effect of linalool on mitochondrial respiration in a hippocampal cell line. Mitochondrial respiration is the power source of eukaryotic cells due to provision of ATP by means of OXPHOS [47]. We observed an increase in maximal oxygen consumption with linalool. Maximal oxygen consumption rate attained by adding the uncoupler FCCP. FCCP mimics a physiological "energy demand" by stimulating the respiratory chain to operate at maximum capacity, which causes rapid oxidation of substrates to meet this metabolic challenge [48]. This indicates that increased maximal respiration and spare capacity is associated with increased metabolic fitness as it makes the cell better able to cope with stress. There are few studies about monoterpenes involved in complex of mitochondrial respiration. It has been described by Usta et al., 2009 that linalool reduced cell viability of cancer cells (HepG2) by inhibition of complexes I and II and decreasing ATP [49]. Other monoterpenes as β-Pinene showed uncoupling effects at lower concentrations (100–200 μM) than the inhibition of respiration (400 μM) through an effect on the electron transport chain in the liver of Wistar rats [50]. In addition, a combination of monoterpenes camphene and geraniol (1:1) protected against nimesulide hepatotoxicity *in vivo*, reducing the mitochondrial swelling, inhibition in release of apoptotic proteins and prevented mitochondrial depolarization along with reduction in oxidized NAD(P)H and increased mitochondrial electron flow [51]. These findings demonstrated that monoterpenes have a potential to regulate mitochondrial respiration in different disease models.

To validate *in vitro* data, we evaluated the effects of linalool in OHSC. We observed an increase in cell death on response to NMDA. Linalool was found to protect areas in the hippocampus such as CA1, however, we did not detect significant differences between other regions of hippocampus due to the variability of cell damage. Additionally, we evaluated genes involved in inflammation and cell death which seemed to increase in response to NMDA. *Cox-2* is usually expressed at low levels under normal conditions, but is rapidly induced by an injury, triggers pro-inflammatory response with prostaglandins and can worsen neuronal degeneration in neurological diseases such as stroke [52,53]. We detected a significant increase in *Cox-2* gene expression after NMDA treatment while linalool reduced this increase. In previous research, we observed a reduction in the levels of inflammatory markers such as p38 MAPK and Cox-2 after linalool treatment for 3 months in aged 3xTg-AD mice [27]. Similarly, reduction of

Cox-2 levels was also observed post-linalool treatment for 1 month in a global ischemic model [28]. This result is further supported by other studies such as reduced levels of cyclooxygenases in other neurotoxicity models when stimulated with linalool and decrease in a dose-dependent manner in LPS-stimulated macrophages [54,55].

Recent studies have shown that cytokines such as TNF-α, IL-1β, IL-1α, IFN-γ are synthesized in the CNS and play an important role in inflammation. This release is promoted after glutamate excitotoxicity [17]. Linalool administered to mice inhibits behavioural nociceptive responses caused by proinflammatory cytokines Il-1β and TNF-α via inhibition of NMDA receptors [17,25]. However, we did not find any significant increase of these proinflammatory cytokines (NO2, IL-1β, TNF-α) due to variability in the cell damage in the hippocampus.

We have previously described the protective effects of linalool in a 3xTg-AD mice [27] and in cerebral ischemia [28]. Besides, linalool protects against acrylamide (ACR)-induced neurotoxicity in Wistar rats decreasing the ACR-induced lipid peroxidation in rat brain tissue [46]. Linalool protection can by mediated by the regulation of glutamatergic system [56,57], and axonal regeneration [58], among other properties described in mentioned literature.

## 5. Conclusion

Our results illustrate that linalool positively impacts the mitochondrial functioning of glutamate-stimulated HT-22 cells. We observed linalool reduced ROS, calcium production, and lipid peroxidation levels. Additionally, improved mitochondrial morphology, membrane potential, and respiration. This together results in an increase of cell viability. Importantly, this study is among the first to elucidate the particular underlying mechanism by which linalool exerts its eff ;ectiveness, precisely via a protective impact on the mitochondria. We validate the *in vitro* neuroprotective data with *ex vivo* OHSC slices where we showed a reduction of cell death in the hippocampus and modulation of various genes by linalool. Our data indicate that linalool may be therapeutically useful in treating neurodegenerative conditions marked by mitochondrial dysfunction and subsequent ROS production, due to its beneficial impact on mitochondrial integrity as well as ability to limit oxidative stress.

## Author contribution

A.M.S.G designed and realized the experiments, analyzed the data and wrote the paper; F.H help in high-resolution respirometry experiments; A.K performed the quantification of categories of mitochondria; A.O performed Seahorse experiments; E.B and A.K. interpreted data and review the manuscript; A.M.D. designed, analyzed and interpreted the data, prepared the manuscript and provided critical revision. All authors read and approved the final manuscript.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.109295.

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