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Research Paper

Hippocampal neurons require a large pool of glutathione to sustain dendrite integrity and cognitive function

Seila Fernandez-Fernandez[a], Veronica Bobo-Jimenez[a,b], Raquel Requejo-Aguilar[a,c], Silvia Gonzalez-Fernandez[a], Monica Resch[a], Monica Carabias-Carrasco[a], Joaquim Ros[d], Angeles Almeida[a,b], Juan P. Bolaños[a,b,e,*]

[a] Institute of Functional Biology and Genomics (IBFG), Universidad de Salamanca, Spain
[b] Institute of Biomedical Research of Salamanca (IBSAL), Hospital Universitario de Salamanca, Spain
[c] Córdoba Maimónides Institute for Biomedical Research (IMIBIC), University of Cordoba, Spain
[d] Departamento de Ciències Mèdiques Bàsiques, IRBLleida, Universitat de Lleida, Spain
[e] CIBERFES, Instituto de Salud Carlos, III, Madrid, Spain

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ABSTRACT

Loss of brain glutathione has been associated with cognitive decline and neuronal death during aging and neurodegenerative diseases. However, whether decreased glutathione precedes or follows neuronal dysfunction has not been unambiguously elucidated. Previous attempts to address this issue were approached by fully eliminating glutathione, a strategy causing abrupt lethality or premature neuronal death that led to multiple interpretations. To overcome this drawback, here we aimed to moderately decrease glutathione content by genetically knocking down the rate-limiting enzyme of glutathione biosynthesis in mouse neurons *in vivo*. Biochemical and morphological analyses of the brain revealed a modest glutathione decrease and redox stress throughout the hippocampus, although neuronal dendrite disruption and glial activation was confined to the hippocampal CA1 layer. Furthermore, the behavioral characterization exhibited signs consistent with cognitive impairment. These results indicate that the hippocampal neurons require a large pool of glutathione to sustain dendrite integrity and cognitive function.

1. Introduction

When compared with astrocytes, neurons are highly dependent on oxidative phosphorylation for correct function [2,3], a situation linked with persistent production of reactive oxygen species (ROS) by the mitochondrial respiratory chain [4]. Being post-mitotic cells, neurons are exposed to ROS during longer time periods than dividing cells, which may contribute to aging and, if inappropriately controlled, to neurological diseases [5–9]. To modulate the possible deleterious actions of excess ROS, cells contain glutathione (γ-glutamyl-cysteinyl-glycine, GSH), an active and ubiquitous antioxidant cofactor responsible for ROS detoxification in neurons [10]. Accordingly, oxidative damage likely caused by progressive GSH deficiency is considered to be one of the earliest biochemical indicator of neuronal degeneration in aging, Parkinson's disease, and certain mental disorders [11–13]. In consistency with this notion, over-expression of glutamate-cysteine ligase (GCL), *i.e.* the first and rate-limiting step in GSH biosynthesis, in brain mitochondria protects neurons against neurological deficit *in vivo* [10].

Unfortunately, all clinical trials on antioxidants performed so far have not alleviated the neurological declines in a wide range of disorders [14], which might challenge the oxidative theory of aging and cognitive impairment [15–17]. Moreover, the lack of an experimental model that faithfully recreate, in neurons *in vivo*, the mild mode of oxidative damage occurring during neurological deterioration, has hampered understanding the actual role played by ROS in aging and

Abbreviations: CA1, *Cornu Ammonis* area 1; CA3, *Cornu Ammonis* area 3; CK2a, calmodulin kinase 2A; DG, dendate gyrus; DMEM, Dulbecco's modified Eagle's médium; DMSO, dimethylsulfoxide; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); FBS, fetal bovine serum; GCL, glutamate-cysteine ligase, catalytic subunit; GSH, glutathione, reduced form; GSSG, glutathione, oxidized form; MAP2, microtubule-associated protein-2; MEFs, mouse embryonic fibroblasts; shGCL, shRNA against GCL; $shGCL^{SFL}$, switched flox shGCL; $shGCL^{UFL}$, unswitched flox shGCL; shRNA, small hairpin RNA; SDS, sodium dodecyl sulfate; ROS, reactive oxygen species; PCR, polymerase chain reaction; TM, 4-hydroxy-tamoxifen; TUJ1, neuron-specific Class III β-tubulin

* Corresponding author at: Institute of Functional Biology and Genomics (IBFG), University of Salamanca-CSIC, C/Zacarías González, 2, 37007 Salamanca, Spain.
E-mail address: jbolanos@usal.es (J.P. Bolaños).

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neurological disorders. To address this issue, here we designed and generated a mouse model genetically engineered to knockdown GCL in neurons of the postnatal mouse brain *in vivo*. We found that GCL knockdown caused a moderate decrease in GSH in the neurons of the CA1 area of the hippocampus that led to dendrite disruption and cognitive impairment. These results reveal that mild oxidative damage in a discrete group of hippocampal neurons is sufficient to trigger the cellular and behavioral signs of neurological disorders.

2. Methods and materials

2.1. shGCL^{UFL} DNA construct generation

A DNA construct was designed to harbor a LoxP-flanked small hairpin RNA (shRNA) precursor against the catalytic subunit of glutamate-cysteine ligase (GCL) (hereafter shGCL). Before recombination (unswitched flox or *UFL*), the shGCL sequence (5′-GAAGGAGGCTACT TCTATA-3′) [18] was separated from the Pol-III promoter (H1) by the LoxP and Lox2272 sequences in the appropriate positions and orientations. Importantly, the shGCL has opposite strand orientation to the H1 promoter. To clone the DNA fragment elements, we used pIRES2-EGFP (Clontech), pSuper-neo/gfp (Oligoengine), pCDNA3.1(+) (Invitrogen) and pEGFP-C1 (Addgene). The small sequence adaptors were designed and chemically synthesized (Thermo) to introduce the LoxP and Lox2272 sites in the correct orientation and the appropriate restriction enzymes sites. To construct shGCL [18], the oligonucleotides were annealed and phosphorylated before ligation with the corresponding linearized and dephosphorylated plasmids. Elements from plasmids were excised by restriction enzyme digestion and directly cloned into the linearized plasmids. The correct sequence and orientations of the intermediary vectors were checked by restriction enzyme assay and, for the final clone containing shGCL (shGSL^{UFL}), by sequencing.

2.2. shGCL^{UFL}/+ and CK2a-Cre/shGCL^{SLF} mice generation

To generate mice harboring the shGCL^{UFL} construct, the linearized DNA was microinjected in the pre-fertilized pro-nucleus of a one-cell state oocyte from the hybrid C57Bl/6J-CBA lineage at the Animal Service of the University of Salamanca following standard protocols. A founder, identified to harbor a unique integration site for shGCL^{UFL} at the fourth (N4) generation, was successively backcrossed with C57Bl/6J until the congenic status (tenth generation) was achieved. Mice expressing Cre recombinase driven by the neuronal-specific promoter of mouse calcium-calmodulin kinase 2a (CK2a-Cre) were purchased from The Jackson Laboratories in a congenic C57BL/6 background. According to The Jackson Laboratories datasheet, Cre recombinase expression in this mouse is postnatal (from day 14–21 onwards) and confined mainly to the forebrain and hippocampus [19]. CK2a-Cre mice were backcrossed for two generations in a C57BL/6J background to obtain transgene homozygosis. To generate CK2a-Cre/shGCL^{SFL} mice, heterozygous shGCL^{UFL}/+ mice were bred with homozygous (CK2a-Cre/CK2a-Cre) mice. The efficacy of the Cre-mediated recombination in the CK2a-Cre/shGCL^{SFL} mice, but not in the CK2a-Cre/+, was tested in the hippocampus by PCR.

2.3. Tissue extraction

Male mice (9 months-old) were euthanized with CO_2 and tissues were extracted within the following 5 min. Mice were decapitated, and their brains were extracted and dissected. We collected the cerebellum, striatum, hippocampus and pre-frontal cortex. For DNA extraction, a small aliquot of tissue (tails or hippocampus) was placed in ice and frozen until further processing. DNA was extracted from tails, pelleted cells or tissue samples using the phenol-chloroform-isoamilic acid method followed by isopropanol precipitation. DNA concentrations were measured using NanoDrop 2000 (Thermo).

2.4. Genotyping

Genotyping was performed by PCR. To genotype the shGCL^{UFL} transgene, we used the following forward and reverse oligonucleotides 5′-AAGTCGTGCTGCTTCATGTG-3′ and 5′-ACGTAAACGGCCACAAG TTC-3′, which generated a 200 bp band from the fragment placed within the LoxP and Lox2272 sites of shGCL^{UFL}. An internal control was used to detect false negatives using the endogenous α-synuclein gene, the forward and reverse oligonucleotides of which were, respectively, 5′-ATCTGGTCCTTCTTGACAAAGC-3′ and 5′-AGAAGACCAAAGAGCAA GTGACA-3′, which generated a 150 bp band. To genotype the CK2a-Cre transgene, a 270 bp region of Cre recombinase was amplified by PCR. Forward and reverse oligonucleotides used were, respectively, 5′-GCA TTTCTGGGGATTGCTTA-3′ and 5′-CCCGGCAAAACAGGTAGTTA-3′, using the same α-synuclein oligonucleotides as above as the internal control. PCR conditions were: 3 min at 94 °C, 35 cycles of 1) 1 min at 94 °C, 2) 1 min at 58 °C, 3) 1 min at 72 °C and a final step 10 min at 72 °C. To detect the non-recombined state of the construct, we used the following forward and reverse oligonucleotides, respectively, 5′-CATC GAGCTGAAGGGCATC-3′ and 5′-CGGTGGGAGGTCTATATAAGCA-3′, which amplified a 900 bp band. The recombined state was detected using the forward and reverse oligonucleotides, respectively, 5′-GGTC AGGGTGGTCACGAG-3′ and 5′-CGGTGGGAGGTCTATATAAGCA-3′, and which amplified a 300 bp band. PCR conditions for both PCRs were: 3 min at 94 °C, 35 cycles of 1) 1 min at 94 °C, 2) 1 min at 55 °C, 3) 1 min at 72 °C and a final step 10 min at 72 °C.

2.5. Southern blotting

Genomic DNA from the tail (5–10 μg) was digested with *Hind*III, the fragments were separated by agarose gel electrophoresis and transferred to a nylon membrane (Immobilon™ NY+, Millipore). After UV crosslinking (Hoefer), the membrane was pre-hybridized with denatured ssDNA and hybridized with the radiolabelled oligonucleotide probe within the LoxP and Lox2272 sites of shGCL^{UFL}. The radioactive bands were visualized using a Phosphorimager screen (Fujifilm) and the images taken using a Bio-Rad Personal Molecular Imager.

2.6. Mouse embryonic fibroblasts (MEFs) immortalization

Fibroblasts were prepared from fetal shGCL^{UFL}/+ mice (E13.5). Embryos were processed individually, and the liver and brain tissue were disregarded. The carcass was mechanically disaggregated in 0.25 g/ml of trypsin/1 mM EDTA and seeded in a 60 cm2 cell culture dish containing DMEM (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Linus) and 25 mM D-glucose. Confluent cells were split 1:3 during 2 weeks, until the immortalization crisis occurred, which lasted for a further 2 weeks. During this period, fresh medium was replaced every 3 days. One week after the immortalization crisis, single cell colonies were spotted, picked and grew. MEFs were frozen in a mixture of FBS and DMSO (9:1). For experiments involving MEFs, dishes and plates were previously coated with poly-D-lysine (15 μg/ml). Cells were incubated at 37 °C in a humidified 5% CO2-containing atmosphere.

2.7. MEF transfections and treatments

Transfections of MEFs with plasmid vectors were performed using Lipofectamine2000™ (Invitrogen), following the manufacturer' instructions. After 6 h, fresh medium was added containing either 1 μM 4-OH-Tamoxifen (TM) (Sigma) or vehicle (ethanol).