ORIGINAL RESEARCH COMMUNICATION

Redox Indicator Mice Stably Expressing Genetically Encoded Neuronal roGFP: Versatile Tools to Decipher Subcellular Redox Dynamics in Neuropathophysiology

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Abstract

Aims: Reactive oxygen species (ROS) and downstream redox alterations not only mediate physiological signaling but also neuropathology. For long, ROS/redox imaging was hampered by a lack of reliable probes. Genetically encoded redox sensors overcame this gap and revolutionized (sub)cellular redox imaging. Yet, the successful delivery of sensor-coding DNA, which demands transfection/transduction of cultured preparations or stereotaxic microinjections of each subject, remains challenging. By generating transgenic mice, we aimed to overcome limiting cultured preparations, circumvent surgical interventions, and to extend effectively redox imaging to complex and adult preparations. **Results:** Our redox indicator mice widely express *Thy1*-driven roGFP1 (reduction–oxidation-sensitive green fluorescent protein 1) in neuronal cytosol or mitochondria. Negative phenotypic effects of roGFP1 were excluded and its proper targeting and functionality confirmed. Redox mapping by ratiometric wide-field imaging reveals most oxidizing conditions in CA3 neurons. Furthermore, mitochondria are more oxidized than cytosol. Cytosolic and mitochondrial roGFP1s reliably report cell endogenous redox dynamics upon metabolic challenge or stimulation. Fluorescence lifetime imaging yields stable, but marginal, response ranges. We therefore developed automated excitation ratiometric 2-photon imaging. It offers superior sensitivity, spatial resolution, and response dynamics. **Innovation and Conclusion:** Redox indicator mice enable quantitative analyses of subcellular redox dynamics in a multitude of preparations and at all postnatal stages. This will uncover cell- and compartment-specific cerebral redox signals and their defined alterations during development, maturation, and aging. Cross-breeding with other disease models will reveal molecular details on compartmental redox homeostasis in neuropathology. Combined with ratiometric 2-photon imaging, this will foster our mechanistic understanding of cellular redox signals in their full complexity. *Antioxid. Redox Signal.* 25, 41–58.

Introduction

SUBCELLULAR REDOX CONDITIONS modulate numerous target proteins, which not only contribute to the tight control of neural function but also to various neuropathologies (16, 22). This post-translational tuning involves especially cysteine thiol-based regulatory switches, which adjust to current redox conditions, for example, by reversibly forming intra-/intermolecular disulfide bonds, and thereby control protein conformation, interactions, and activity (38, 45). Hundreds of

proteins are assumed to respond to changes in their redox environment (54), suggesting highly complex signaling networks. Currently, we are just at the beginning of deciphering the full complexity of redox signaling. Pivotal tools in these efforts are reliable optical probes. Ideally, they should offer high spatial and temporal resolution, yield quantitative measures, and be applicable to various tissues, all maturational stages, and preparations of differing complexity.

Various genetically encoded redox sensors exist, which partly meet these criteria, and whose function is based on

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Innovation

Transgenic reduction–oxidation-sensitive green fluorescent protein 1 (roGFP1) mice circumvent transduction/transfection and efficiently extend quantitative redox imaging to adult and complex preparations. Being feasible for various imaging approaches at all postnatal stages, they are pivotal to decipher molecularly the redox signaling in central neurons during normal development and/or neuropathology. With widely expressed roGFP1, redox conditions can be mapped quantitatively throughout the brain, revealing regional characteristics and vulnerabilities. The introduced dual-laser excitation combines the advantages of 2-photon microscopy with ratiometric imaging and provides subcellular resolution to study the intercompartmental redox communication of mitochondria and cytosol. This will crucially foster our molecular understanding of physiological neural function and various neuropathologies.

engineered thiol switches (40, 48). They represent individual modified fluorescent proteins (29, 44), fluorescent proteins coupled to redox-sensing molecules (6), or fluorescence resonance energy transfer (FRET)-based constructs combining pairs of fluorophores *via* redox-sensitive linkers (28, 36, 60). We chose reduction–oxidation-sensitive green fluorescent protein 1 (roGFP1) (29), which is ratiometric by excitation (absorption maxima 400 nm and 490 nm) and enables true quantitative redox imaging (15, 29). Expressed in cells, native roGFP redox sensors almost exclusively report the ratio of reduced/oxidized glutathione (GSH/GSSG), which is mediated by cell endogenous glutaredoxins (39–40).

Previously, we verified in hippocampal cell and slice cultures that roGFP1 reliably reports cellular redox balance, is sufficiently sensitive to detect redox changes arising from altered cell endogenous reactive oxygen species (ROS) production, and is only negligibly affected by cellular pH and Cl^- changes (24, 26). In contrast, the only other ratiometric redox sensor, HyPer, senses H_2O_2 directly (6), but it markedly responds to pH changes (55).

Among the advantages of genetically encoded redox sensors is the specific targeting to any desired cellular location, organelle, or subcompartment. Yet, delivering the coding DNA to the cells of interest often is a major challenge. Furthermore, sufficient expression times of several days are mandatory before experiments can be run. This largely restricts an application of these sensors to cell and slice cultures, which can be obtained only from neonatal and immature brains. Expression in adult animals requires direct viral injections into the brain region of interest several days before experiments, and such surgical interventions may provoke additional complications.

To effectively extend redox imaging to complex preparations and more mature developmental stages, we generated transgenic mouse lines stably expressing roGFP1 in the cytosol or the mitochondrial matrix. Neuron-specific expression in large parts of the brain is controlled by the regulatory elements of the murine *Thy-1.2* gene (thymocyte differentiation antigen 1, CD90) (12, 46).

In this study, we present a first detailed characterization of these redox indicator mice. The expression patterns of roGFP1 were carefully mapped throughout the brain. Dy-

namic live cell imaging and response range calibrations verified that the expressed sensors are fully functional and sufficiently sensitive to detect redox changes arising from altered cell endogenous ROS production in mitochondria or cytosol. Neuron-specific expression and mitochondrial targeting were confirmed by counterlabeling. Detailed phenotyping of both mouse lines ruled out any adverse effects of roGFP1 expression on development, general appearance, blood parameters, life span, motor function, and exploratory behavior.

Our redox indicator mice are well suited for the entire range of preparations, spanning from cell cultures over acute tissue slices and the intact isolated hippocampal preparation to *in vivo* studies. Applicable quantitative redox imaging approaches include dynamic wide-field live cell imaging, the new developed excitation ratiometric 2-photon microscopy, and time-correlated single-photon counting fluorescence lifetime imaging microscopy (TCSPC FLIM). With this methodological diversity, our redox indicator mice strikingly extend the possibilities of optical redox analyses under physiological and pathophysiological conditions and will contribute markedly to the deciphering of subcellular redox signaling.

Results

By pronuclear DNA injections, we successfully generated transgenic mice stably expressing either cytosolic roGFP1 (roGFPc) or mitochondrial matrix-targeted roGFP1 (roGFPm) under the *Thy-1.2* promoter. Three stable founder lines for each model were established. For detailed analyses, we focused on that particular roGFPc and roGFPm line, which exhibited the preferred pronounced roGFP1 expression in the hippocampus as well as cortex, and which showed stable breeding by forwarding the transgene at the expected Mendelian probabilities and male-to-female ratios.

Brain expression pattern of roGFP

Detailed analysis of roGFP1 expression was performed on coronal and sagittal sections (30 μm thickness) of adult perfusion-fixed brains. In roGFPc mice, the redox sensor was detectable throughout the brain (Fig. 1A). Co-labeling of NeuN (neuronal nuclei antigen, Fox3) and 2-photon colocalization imaging confirmed a neuron-specific expression of cytosolic roGFP1 (Fig. 1B). Due to the *Thy-1.2* promoter, roGFP1 is not present in all neurons, but only in certain excitatory neurons. Very clear expression was found in the hippocampus (especially CA1 pyramidal neurons and weaker expression in CA3 pyramidal neurons and DG granule cells), cortex (especially layer V and layer II/III pyramidal neurons), thalamus, cerebellum (granule cells), inferior and superior colliculus, as well as some brainstem nuclei (trigeminal and facial nuclei, olivary complex).

A corresponding pattern was confirmed for roGFPm mice (Fig. 2A), in which dentate gyrus granule cells showed particularly intense roGFP1 expression. Based on the intracellular distribution of mitochondria and their small dimension, roGFP1 fluorescence in the sagittal slice overview appeared more diffuse. Neuron-specific expression of roGFPm was verified by NeuN counterlabeling (Fig. 2B), and in dissociated cell cultures from neonatal roGFPm hippocampus, we

FIG. 1. Brain expression pattern of roGFPc.

(A) The roGFPc indicator is almost ubiquitously expressed in the brain. In the hippocampus, particularly high levels are found in CA1. Images show an adult male mouse (P65); the sagittal view comprises six single CCD camera images taken with a 4×0.28 NA objective (XL Fluor; Olympus). (B) Counterlabeling with NeuN confirmed the neuron-specific targeting of roGFPc. Displayed 2-photon images were acquired using a $40 \times$ IR 0.8NA objective (Achromplan; Zeiss), scaling is identical for all images. BS, brainstem; CA1, *cornu ammonis* subfield 1; CA3, *cornu ammonis* subfield 3; CB, cerebellum; CCD, charge-coupled device; CTX, cortex; DG, dentate gyrus; HP, hippocampus; IC, inferior colliculus; roGFPc, cytosolically expressed roGFP; STR, striatum; S1, primary somatosensory cortex; TH, thalamus; VIS, visual cortex; Str. Or., *stratum oriens*; Str. Pyr., *stratum pyramidale*; Str. Rad., *stratum radiatum*; Lam. Mol., *lamina molecularis*; Lam. Gr., *lamina granularis*; Lam. Pl., *lamina plexiformis*. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

also confirmed by counterlabeling with MitoTracker Red its mitochondria-specific targeting (Fig. 3).

Both roGFP1 variants were also detected in the spinal cord (Fig. 4). At neonatal/juvenile and adult stages, neurons in the entire spinal gray matter, that is, motorneurons and sensory neurons, contain roGFPc and roGFPm. Yet, the density of roGFP1-expressing neurons is more sparse than in forebrain, generating a more inhomogeneous staining pattern especially in thin tissue sections.

Interference of cellular and tissue autofluorescence

Unspecific cellular/tissue autofluorescence with broad excitation/emission may arise from lipofuscin accumulation with aging, typically causing a speckled cytosolic staining (41, 49, 50). Other potential sources of autofluorescence, which even persist tissue fixation, are NADH and serotonin (50). As such background fluorescence may interfere with roGFP1 imaging, we compared nontransgenic wild-type

FIG. 2. Brain expression pattern of roGFPm. (A) Sagittal slice showing the expression in an adult female mouse (P55). The close-up view of the cortico-hippocampal areas is an overlay of roGFPm (green) and NeuN immunolabeling (red). (B) In addition, for other brain areas, NeuN counterlabeling confirmed neuron-specific expression of roGFPm. Displayed 2-photon images of an adult male mouse (P50) were taken with a 63×1.0 NA objective (Apochromat; Zeiss). OB, olfactory bulb; roGFPm, mitochondria-targeted roGFP. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(WT) with adult roGFPc and roGFPm tissue. Thin cortico-hippocampal sections ($30\ \mu\text{m}$) of perfusion-fixed brains were imaged successively with identical optical settings. At exposure times of 5 ms, roGFPc and roGFPm sections showed the expected structured expression, whereas noticeable fluorescence could hardly be detected in WT tissue (Fig. 5). Only at markedly prolonged exposure times of 35 ms, autofluorescence became detectable. It was distributed unspecifically rather than present in specific cells (Fig. 5). Hence, with settings applied for roGFP1 imaging, an interference of autofluorescence can be excluded.

Phenotypic characterization of mice

To screen for possible adverse effects of neuronal roGFPc and roGFPm on general phenotype and development, we monitored various systemic parameters, analyzed blood samples, and conducted behavioral tests. In general appearance, roGFPc and roGFPm mice were indistinguishable from WT siblings (Fig. 6A). Body weights were monitored until early adulthood (Fig. 6B), and minor growth and weight differences were evident only among males and females. Blood glucose level was slightly lower in most female mice

FIG. 3. Mitochondrial counterlabeling verifies proper subcellular roGFPm targeting. The 2-photon images were acquired with a 63× objective, and the overlay of roGFPm and MitoTracker fluorescence confirms a perfect mitochondrial colocalization. Images show hippocampal cell cultures prepared from a neonatal (P2) roGFPm mouse. This indicates that hippocampal neurons express roGFP already at the first postnatal days. MitoTracker was bulk loaded to the cell cultures ($1 \mu\text{M}$, 20 min). roGFPm, mitochondria-targeted roGFP. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(Table 1). Yet, compared with WT, neither roGFPc nor roGFPm had any obvious effects on growth, hematocrit, or blood glucose (Fig. 6B and Table 1).

Motor coordination and environmental exploration were rated by RotaRod and open field tests, respectively. RotaRod testing clearly excluded any negative effects of roGFPc and roGFPm on motor coordination; in fact, roGFPc females and, in part, also roGFPm females even did better than WT females (Fig. 6C). Neither did open field tests detect any marked genotypic differences in environmental exploration. Mean velocity, total distance traveled, and resting times were largely indistinguishable among WT and transgenic mice (Fig. 6D). Just some moderate gender-related differences were observed. In both mouse strains, WT females, in part, ran at slightly higher velocities and longer distances than WT males. This applied also to roGFPm females who, furthermore, rested less than roGFPm males (Fig. 6D).

Confirming roGFP functionality

Functional tests of the expressed roGFPc and roGFPm sensors were conducted in acute cortico-hippocampal tissue slices. First, response ranges were calibrated for various brain regions, using charge-coupled device (CCD) camera-based ratiometric wide-field imaging. Slices were exposed to saturating doses of H_2O_2 (5 mM) and DTT (10 mM, 7 min each) to determine in defined regions of interest (ROI) the fluorescence ratios corresponding to full oxidation (R_{ox}) and reduction (R_{red}) of roGFPc and roGFPm (Fig. 7A, B).

Once calibrated, the relative steady-state oxidation of roGFP can be calculated for different compartments and cell types (24, 29, 40), and redox maps of various brain regions may be defined. On average, in CA1 neurons, roGFPc was oxidized by $25.7\% \pm 3.8\%$ ($n=49$), and conditions were similar in dentate gyrus. Cortical neurons were only slightly

FIG. 4. Expression of roGFPc and roGFPm in the spinal cord. (A) CCD camera imaging of PFA-fixed spinal cord sections ($30 \mu\text{m}$ thickness) revealed that roGFPc is present in motor neurons and sensory neurons and that expression already starts at neonatal/ juvenile stages. Scaling of all images is identical. (B) The corresponding spinal expression was confirmed also for roGFPm mice. DH, dorsal horn; PFA, paraformaldehyde; VH, ventral horn. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

more oxidized ($28.4\% \pm 6.4\%$, $n=28$), whereas CA3 was most oxidized ($38.8\% \pm 7.5\%$, $n=31$; Fig. 7C). Based on these data, we calculated roGFP redox potentials (E_{roGFP1} , Fig. 7C). They show the corresponding conditions, but differ

less among brain regions than the relative oxidation levels. The roGFPc redox potential in CA1 neurons averaged -305.0 ± 2.6 mV ($n=49$) and it was most oxidized in CA3 neurons (-297.1 ± 4.2 mV, $n=31$, Fig. 7C). Comparing cytosolic and mitochondrial redox baselines revealed, for all brain regions, a markedly higher degree of roGFP oxidation and more oxidized (less negative) redox potentials in mitochondrial matrix. In accordance with what was seen with roGFPc, also roGFPm was more oxidized in CA3 ($82.7\% \pm 12.0\%$, $n=12$) than in CA1 neurons ($69.7\% \pm 12.0\%$, $n=13$) and the roGFPm redox potential was less negative (Fig. 7C). Mitochondrial redox conditions in cortex and dentate gyrus were comparable with CA1 (Fig. 7C).

Modifying endogenous ROS formation and redox regulation

Next, we determined in adult hippocampal slices ($>P50$) the responses of roGFPc and roGFPm to various stimuli modifying cellular ROS formation or scavenging to confirm that the expressed sensors are sufficiently sensitive to respond to cell endogenous changes in redox balance. As stimuli, we chose increased neuronal activity, blocking of superoxide dismutase, and mitochondrial challenge.

Neuronal activity was evoked by electrical stimulation of Schaffer collaterals, elevated extracellular K^+ levels, or neurotransmitters and the resulting redox responses were analyzed on the tissue level within rectangular ROIs ($\sim 40 \times 60 \mu\text{m}$) placed in CA1 *str. pyramidale*. Orthodromic stimulation of Schaffer collaterals by insulated steel microwire electrodes ($150 \mu\text{A}$, 50 Hz, 1 min) caused a tiny oxidizing shift of the roGFPc ratio, which averaged $0.8\% \pm 0.5\%$ ($n=9$; Fig. 8A, B). This response slightly intensified with 100 Hz stimulation for 1 min ($1.4\% \pm 1.1\%$, $n=8$) or 3 min ($2.9\% \pm 3.2\%$, $n=8$; Fig. 8A, B). High extracellular K^+ levels (50 mM, 5 min) increased the roGFP1 ratio by $4.4\% \pm 2.7\%$ ($n=10$), whereas norepinephrine ($500 \mu\text{M}$, 12 min) elicited a rise by $2.1\% \pm 1.5\%$ ($n=8$, Fig. 8B). Similar responses were observed in roGFPm mice. Electrical stimulation ($150 \mu\text{A}$, 50 Hz, 1 min) elicited an oxidizing shift in the roGFPm ratio, which averaged $0.4\% \pm 0.1\%$ ($n=9$) and was intensified by 100 Hz stimulation for 1 min ($0.6\% \pm 0.4\%$, $n=7$) and 3 min ($1.8\% \pm 1.0\%$, $n=7$; Fig. 8B). High extracellular K^+ levels (50 mM, 5 min) and norepinephrine ($500 \mu\text{M}$, 12 min) increased the roGFPm ratio by $2.2\% \pm 1.0\%$ ($n=7$) and $2.8\% \pm 2.0\%$ ($n=7$), respectively (Fig. 8B). The corresponding changes in roGFPc and roGFPm redox potentials are smaller than the normalized ratiometric changes, but still represent statistically significant changes (Fig. 8C).

As a crucial control, we asked whether NADH and FAD autofluorescence may interfere with roGFP1 imaging as

FIG. 5. Unspecific background fluorescence in non-transgenic slices. Comparison of adult cortico-hippocampal sections from roGFPc (P167), roGFPm (P139), and WT mice (P255). CCD camera images of all sections were taken successively with identical parameters. Note that in WT tissue, a background signal can hardly be detected. For orientation, some anatomical landmarks are indicated. Only with seven-fold prolonged exposure times, diffuse and nonspecific background fluorescence arises. LV, lateral ventricle; SC, superior colliculus; WT, wild-type. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 6. Redox indicator mice show normal phenotypic appearance. (A) WT and roGFPm siblings (P88) are virtually indistinguishable. (B) Monitoring body weights every 3–5 days confirms normal growth of transgenic mice compared with their WT siblings. Averages of 11–25 mice per group are plotted; for clarity, data labels and error bars were omitted. (C) RotaRod tests ruled out negative effects of roGFP expression on motor function. Female roGFPc mice did even slightly better than WT females; a similar trend was found for roGFPm females. Note that on the third day also male roGFPm mice did better than their WT siblings. Significant changes among genotypes are indicated by asterisks (* $p < 0.05$), and gender-based changes are indicated by crosshatches (# $p < 0.05$, ### $p < 0.001$). The number of mice tested is reported below the bars. (D) Open field testing did not detect any genotypic differences; only gender-based differences became obvious. Female WT and female roGFPm mice were more active than their male siblings. Crosshatches mark gender-based differences (## $p < 0.01$). Bar patterns are identical to (C).

neuronal activity and mitochondrial metabolism also modulate the levels of these electron carriers and since similar excitation wavelengths (NADH 360 nm, FAD 445 nm) are used to monitor their tissue autofluorescence (26, 32). Tests on WT slices with identical wavelengths and filter settings and similar ROIs used for roGFP imaging showed that electrical stimulation (100 Hz, 3 min) indeed increased NADH fluorescence (exc. 395 nm, 280 ms exposure) and decreased FAD fluorescence (exc. 470 nm, 160 ms exposure). As a result, the FAD/NADH fluorescence ratio increased by $3.9\% \pm 3.3\%$ in CA1 *str. pyramidale* ($n = 10$). This was, however, only detectable with 20-fold prolonged exposure times and full illumination intensity. The short exposure time and low intensity used for roGFP1 imaging were not sufficient to

detect tissue autofluorescence at all. Thus, interference of FAD/NADH autofluorescence with intense roGFP1 signals in roGFPc and roGFPm mice can be excluded.

Next, we recorded roGFP responses to severe hypoxia (95% N_2 , 5% CO_2 aerated artificial cerebrospinal fluid (ACSF) containing 1 mM of the O_2 scavenger Na_2SO_3). Ratiometric changes were quantified in different hippocampal subfields and cortex by placing ROIs in the soma of clearly identifiable neurons. In acute slices of adult roGFPc mice, 10 min of hypoxia increased the roGFPc fluorescence ratio of CA1 neurons by $6.2\% \pm 3.9\%$ ($n = 8$); the other brain regions responded similarly (Fig. 8D). During reoxygenation, the roGFPc ratio increased further in all regions, reaching after 10 min a total of $13.6\% \pm 6.6\%$ ($n = 8$) in CA1 neurons. Pretreating slices with

TABLE 1. STATISTICAL SUMMARY OF GROWTH AND BLOOD ANALYSES

Parameter	roGFPc				roGFPm			
	Male		Female		Male		Female	
	WT (n = 12)	roGFPc (n = 14)	WT (n = 12)	roGFPc (n = 14)	WT (n = 16)	roGFPm (n = 15)	WT (n = 12)	roGFPm (n = 14)
Body weight (g)	22.0 ± 1.3	23.3 ± 2.1 (n = 13)	17.2 ± 1.2 ^a	18.0 ± 1.5 ^a (n = 11)	22.7 ± 2.2	22.7 ± 1.7	18.7 ± 1.5 ^a	18.3 ± 1.2 ^a
Body length (cm)	8.3 ± 0.3	8.1 ± 0.3	7.8 ± 0.2 ^a	7.7 ± 0.4 ^b	8.0 ± 0.4	8.2 ± 0.4	7.7 ± 0.4	7.7 ± 0.3 ^b
Blood glucose (mg/dl)	238 ± 25	221 ± 41	198 ± 42 ^b	214 ± 22	208 ± 32	206 ± 22 (n = 12)	183 ± 22 ^c	189 ± 24 (n = 13)
Hematocrit (%)	44 ± 3	46 ± 3	45 ± 1	45 ± 2	45 ± 2	45 ± 2 (n = 12)	45 ± 2	45 ± 2 (n = 13)

Data were collected at ~P50, before the final experiments. In general, female mice had lower body weights than males, in part, were smaller, and showed lower blood glucose levels; this applied to WT and transgenic mice. Gender-matched phenotypic differences among WT and roGFP mice were not observed. Significant differences among male and females are indicated (^a $p < 0.001$, ^b $p < 0.01$, ^c $p < 0.05$). The number of observations slightly varies because not every parameter was recorded for each mouse.

WT, wild-type.

the antioxidant EUK-134 (50 μ M, 1 h), a synthetic superoxide dismutase/catalase mimetic (3), markedly dampened the hypoxia-induced redox changes. In the presence of EUK134, O₂ withdrawal increased the roGFPc ratio in CA1 neurons by only 1.9% ± 1.7%; further oxidation upon reoxygenation measured only 8.6% ± 3.8% ($n = 17$, Fig. 8D).

In contrast, in roGFPm mice, severe hypoxia caused in CA1 *str. pyramidale* a hardly detectable, but consistent, transient oxidizing shift (0.5% ± 0.4%, $n = 11$) that occurred right upon O₂ withdrawal and then turned into a more pronounced reducing shift in roGFPm ratio by 2.3% ± 1.5%, which slowly recovered upon reoxygenation (Fig. 8E).

Furthermore, the outcome of pharmacological mitochondrial inhibition was assessed. Blocking complex III by antimycin A (20 μ M, 10 min) elicited a marked increase in roGFP1 ratio, which was delayed by several minutes and then continued due to the irreversible effects of antimycin A. After 30 min of washout, the increase in roGFPc ratio measured 45.7% ± 13.6% ($n = 5$) and the increase in roGFPm ratio averaged 27.6% ± 6.5% ($n = 7$; Fig. 8F, G). This emphasizes that upon blocking of complex III, superoxide invades matrix space and cytosol, markedly affecting redox conditions in both compartments.

In addition, for mitochondrial matrix, the effect of radical scavengers was assessed. In slices pretreated with mito-Tempo (50 μ M, 1 h), a mitochondria-targeted superoxide dismutase mimetic (14), the effects of antimycin A were clearly dampened, and the roGFPm ratio increased by only 10.4% ± 3.1% ($n = 6$, Fig. 8G). In contrast, the mere pharmacological inhibition of superoxide dismutase by diethylthiocarbamic acid (DEDTC) (5 mM, 5 min) already elicited biphasic responses; an initial transient increase in roGFPm ratio (2.7% ± 1.3%, $n = 5$) and a secondary longer lasting increase by 4.6% ± 2.9%, ($n = 5$; Fig. 8H).

Time domain FLIM and excitation ratiometric 2-photon imaging

The roGFP1 mice extend redox imaging to more complex preparations. Accordingly, 2-photon imaging would be advantageous. Yet, quantitative analyses require ratiometric 2-

-photon excitation by repeatedly switching laser wavelengths. To circumvent this problem, we assessed the feasibility of roGFP1 for TCSPC-FLIM as this enables quantitative analyses independent of wavelength switching.

Proof-of-principle experiments were already performed in parallel to the initial breeding of the transgenic founder lines. In these first tests, we therefore used roGFP1-transfected hippocampal cell cultures to calibrate FLIM responses and to determine the instrument response function (IRF) from the background close to the recorded cells. Cytosolic roGFP1 was excited at 890 nm and line averaging (25–30 lines) was applied to ensure sufficient photon counts. Satisfactory exponential fits of the recorded fluorescence decays were obtained with a single exponential function (Fig. 9A), yielding an averaged roGFP1 lifetime of $\tau = 2.31 \pm 0.15$ ns ($n = 15$). Oxidation by H₂O₂ (5 mM, 5 min) only slightly decreased lifetime by 4.1% ± 2.1% ($n = 8$); reduction by DTT (10 mM, 5 min) slightly increased it by 4.0% ± 3.7% ($n = 6$, Fig. 9B).

As roGFP1 showed stable responses in cell cultures, we extended FLIM to adult roGFPc mice (Fig. 9C–E). In acute cortico-hippocampal slices, cortical neurons showed a lifetime of 2.45 ± 0.07 ns ($n = 14$) and CA1 pyramidal neurons exhibited a lifetime of 2.45 ± 0.10 ns ($n = 342$). The impact of redox challenge (5 mM H₂O₂, 10 mM DTT) was assessed in CA1. Unfortunately, in these treatments, the focal plane was lost regularly due to cell swelling. Therefore, roGFPc lifetimes under the various conditions were first analyzed from different cell populations. The corresponding histograms show a partial shift to shorter lifetimes upon roGFPc oxidation and a partial shift to longer lifetimes upon roGFPc reduction (Fig. 9C). The reason for this heterogeneous behavior within the analyzed populations is unclear. Most likely, the nonresponsive neurons represent those being damaged during brain slicing as they also exhibited very oxidized steady-state conditions already under control conditions.

We therefore readjusted the focal plane upon redox stimulation to assess its outcome in the identical cell population (Fig. 9D). Again, only very moderate changes in roGFPc lifetimes were obtained (Fig. 9E). Oxidation (5 mM H₂O₂, 5 min) decreased lifetime in CA1 pyramidal neurons by 2.5% ± 1.6% ($n = 19$) and reduction (10 mM DTT, 5 min)

increased it by $1.2\% \pm 1.1\%$ ($n = 20$); a repeated scan in ACSF with line averaging applied did not affect lifetimes ($n = 23$, Fig. 9E). Thus, in accordance to our findings in cultured cells, roGFP1 yields stable FLIM responses. The response range between full oxidation and full reduction is, however, only moderate and not sufficient to resolve reliably more subtle changes in cellular redox conditions.

Therefore, we next assessed the general feasibility of roGFP1 for ratiometric 2-photon imaging and recorded the 2-photon excitation spectrum in roGFP1-transfected hippocampal cell cultures. Facing the necessity of wavelength switching, we tuned excitation wavelengths manually from 700 to 1025 nm (12.5-nm increments) and confirmed a broad excitation of cytosolic roGFP1 between ~ 740 and 950 nm

(Fig. 10A); discrete absorption peaks were not observed. In this spectrum, we identified the wavelengths, 740 nm and 910 nm, for ratiometric excitation of roGFP1 and confirmed opposite roGFP1 responses to redox challenge. Oxidation by H_2O_2 ($500 \mu\text{M}$, 5 min) increased the roGFP1 ratio (F_{740}/F_{910}) by $152.6\% \pm 156.1\%$ ($n = 7$) and reduction by DTT (2 mM , 5 min) decreased it by $54.4\% \pm 25.3\%$ ($n = 13$; Fig. 10B, C). Transferring this approach to acute hippocampal slices of adult roGFPc mice (P50) yielded similar responses. In these tests, we increased drug concentrations and exposure times due to the diffusion barrier imposed by the tissue. Upon oxidation (5 mM H_2O_2 , 15 min), the roGFP1 ratio increased by $107.9\% \pm 38.9\%$ ($n = 28$) and, upon reduction (10 mM DTT, 15 min), it decreased by $31.9\% \pm 13.2\%$ ($n = 17$; Fig. 10C).

Based on these proof-of-principle experiments, we upgraded our 2-photon imaging system, adding a second optical port to the system's beam shaper unit and equipping both laser ports with fast mechanical shutters (Fig. 10D). This now enables the alternate use of two laser systems, each set to one of the desired excitation wavelengths, and thus fully automated, software-controlled, excitation ratiometric 2-photon imaging of roGFP1. Within the beam shaper unit, both lasers are aligned *via* a polarization beam combiner rather than a dichroic mirror. This guarantees full flexibility of each laser system, without restricting the available spectral range of both lasers. Verifying this novel approach in acute hippocampal slices from roGFPc mice showed very promising responses (Fig. 10E, F). In the CA3 subfield, pyramidal neurons exhibited an average steady-state 2-photon roGFP1 ratio of 3.9 ± 0.6 ($n = 15$) at resting baseline conditions. In slices exposed to H_2O_2 ($500 \mu\text{M}$, 8 min), it was increased by 66% (6.6 ± 1.1 , $n = 18$) and, in slices treated with DTT (2 mM , 8 min), it was decreased by 43% (2.3 ± 0.2 , $n = 22$; Fig. 10E). This confirms that compared with FLIM, the dynamic response range of ratiometric 2-photon excitation is much broader. Accordingly, ratiometric 2-photon excitation should

FIG. 7. Redox calibrations confirm full functionality of roGFPm and roGFPc. (A) Response range calibration of an acute hippocampal slice of a male roGFPm mouse (P52). Plotted is the roGFPm ratio as determined within an ROI in dentate gyrus. The displayed trace (and all others in the following figures) is a continuous recording. After calibration, the relative roGFP oxidation levels can be calculated. (B) The box plot summarizes the response ranges determined for the hippocampal subfields and cortex of roGFPc and roGFPm mice. The floor of each box represents full reduction (R_{red} , induced by 10 mM DTT), and the ceiling represents full oxidation (R_{ox} , induced by 5 mM H_2O_2). The corresponding standard deviations are indicated and the numbers report how many slices were calibrated. (C) Steady-state redox conditions of different brain regions. The upper diagram summarizes the relative levels of roGFPc and roGFPc oxidation ($\text{OxD}_{\text{roGFP1}}$, Equation 1) at rest. Redox balance is more oxidized in mitochondria than in cytosol, and highest oxidation was found in CA3. The lower diagram shows steady-state roGFP redox potentials calculated from the relative oxidation levels (Equation 2). Note that redox potentials differ less than the degrees of roGFP oxidation. Again, mitochondria are more oxidized (less negative redox potentials) than cytosol, and CA3 is most oxidized. In both diagrams, asterisks report significant differences compared with the CA1 subfield of each model. ROI, region of interest.

FIG. 8. Sensitivity of roGFPc and roGFPm to altered cell endogenous ROS formation. (A) Electrical stimulation of Schaffer collaterals (3 min) elicited a moderate oxidizing shift in roGFPc ratio in CA1 *str. pyramidale* of acute tissue slices. (B) Summary of activity-related roGFPc and roGFPm responses. Plotted are changes in the roGFP ratio (ΔR) normalized to pretreatment control conditions (R_o), that is, $\Delta R/R_o$ expressed as percentage changes. Ratiometric changes were quantified within rectangular ROIs ($\sim 40 \times 60 \mu\text{m}$) containing several neurons, and which were placed in CA1 *str. pyramidale*. Some effects (electrical stimulation) are quite small, nevertheless, all data represent statistically significant changes. (C) Neuronal activity evoked changes in roGFPc and roGFPm redox potentials (ΔE). (D) Severe hypoxia (N_2 , 1 mM Na_2SO_3) and the subsequent reoxygenation evoked an oxidation of roGFPc, whose magnitude was comparable in the hippocampal subfields and cortex. Due to marked cell swelling, the optical focus had to be readjusted repeatedly to monitor redox changes of selected neurons during hypoxia and reoxygenation (10 min each). Therefore, continuous recordings were not possible. In roGFPc slices pretreated with EUK-134 (1 h, 50 μM), the hypoxia/reoxygenation-induced oxidation was markedly dampened. Asterisks indicate statistically significant changes compared with CA1 neurons not treated with the antioxidant. (E) Effects of severe hypoxia on mitochondrial redox balance in the CA1 subfield. Note that roGFPm first responds with a hardly detectable oxidizing shift to O₂ withdrawal (arrow mark) and then shows a clear reduction. (F) Irreversible inhibition of mitochondrial respiration by antimycin A caused a marked oxidation of roGFPm, which continued upon washout. (G) Antimycin A-induced ROS formation affects mitochondrial and cytosolic redox balance. In roGFPm slices, pretreatment with mitoTEMPO (1 h, 50 μM) markedly decreased the antimycin A-mediated oxidation. Plotted changes were quantified in CA1 *str. pyramidale*. Asterisks indicate significant changes compared with roGFPm slices exposed to antimycin A only. (H) DEDTC, a blocker of superoxide dismutase, caused a biphasic increase in the roGFPm ratio. ROS, reactive oxygen species.

FIG. 9. FLIM and lifetime calibrations of roGFPc. (A) FLIM image of a cultured neuron transfected with roGFPc. The decay of roGFPc fluorescence (raw data) is corrected by the IRF and fitted exponentially (dashed line), yielding a lifetime of $\tau = 2.46$ ns. The low number of residual photons (<2) confirms the goodness of fit. (B) In dissociated hippocampal cultures, oxidation by H_2O_2 (5 mM) slightly decreased and reduction by DTT (10 mM; 5 min each) slightly increased lifetimes. Asterisks indicate statistically significant changes compared with pretreatment control conditions (ACSF). (C) Fluorescence lifetimes recorded in adult roGFPc hippocampal slices. Histograms show lifetime distributions of a population of CA1 neurons. Comparing redox conditions showed only moderate and also heterogeneous lifetime changes within the populations. H_2O_2 (5 mM, 5 min) induced a partial shift to shorter lifetimes, DTT (10 mM, 5 min) caused a partial shift to increased lifetimes. Averaged lifetimes of the three populations and the number of cells are indicated. (D) Intensity image of an acute roGFPc hippocampal slice (P74) and corresponding FLIM images at control conditions and upon oxidation (5 mM H_2O_2 , 5 min). The only moderate shortening of lifetimes (blue shift) in CA1 is hardly visible. (E) Normalized FLIM changes in adult hippocampal neurons ($\sim \text{P70}$). Defined CA1 neurons were imaged under control conditions and upon redox challenge (5 mM H_2O_2 , 10 mM DTT, 5 min each), and the resulting lifetime changes quantified. Displayed are normalized changes as referred and statistically compared with the lifetime of the respective cells in ACSF; significant differences are reported by asterisks. ACSF, artificial cerebrospinal fluid; FLIM, fluorescence lifetime imaging microscopy; IRF, instrument response function. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

be preferred to assess redox changes at the tissue level as it provides a sufficiently high level of sensitivity to detect also more subtle differences in (sub)cellular redox balance. Screening for subcellular differences in steady-state redox conditions, we found that in CA1 neurons, the roGFPc ratio in soma is $32.3\% \pm 18.1\%$ ($n=28$) higher, that is, more oxidized, than in dendrites (Fig. 10F).

Discussion

Due to their superior response properties, genetically encoded optical redox sensors revolutionized redox imaging in the past years. Among the various constructs, roGFP-based redox sensors (29) are considered especially reliable, which in particular is based on their excitation ratiometric properties.

FIG. 10. Excitation ratiometric 2-photon imaging of roGFPc. (A) Two-photon excitation spectrum of cultured roGFPc-transfected hippocampal neurons. The wavelengths chosen for ratiometric 2-photon excitation are identified. (B) Ratiometric image pair of a cultured hippocampal neuron. Laser excitation wavelengths were tuned manually to 740 and 910 nm and separate images were acquired. The overlay of these images confirms perfect colocalization of cellular structures, and the calculated ratiometric images indicate the redox status of roGFPc under control conditions and upon H₂O₂ treatment (500 μ M, 5 min). (C) Feasibility of roGFPc for excitation ratiometric 2-photon imaging. Comparable with ratiometric wide-field imaging, the 2-photon roGFPc ratio reliably increased upon oxidation (cell cultures 500 μ M H₂O₂; slices 5 mM H₂O₂) and decreased upon reduction (cell cultures 2 mM DTT; slices 10 mM DTT). Data were recorded from dissociated hippocampal cell cultures (roGFPc transfected) and adult hippocampal roGFPc slices. Plotted ratiometric changes are normalized and statistically compared with pretreatment control conditions (dashed line); asterisks indicate statistically significant changes. (D) By installing two separate laser systems, automated excitation ratiometric 2-photon imaging was established. Each laser is set to one of the desired excitation wavelengths and—aligned by a polarization beam combiner—coupled into the scanhead. With this development, true excitation ratiometric 2-photon imaging can be performed, fully software controlled and without further wavelength adjustments. (E) Steady-state redox balance of CA3 neurons under control conditions and H₂O₂ (500 μ M, 6–8 min) or DTT treatment (2 mM, 6–8 min). Dual-laser ratiometric 2-photon imaging was performed in acute hippocampal roGFPc slices (P30). Asterisks indicate statistically significant changes compared with control conditions (ACSF). (F) Ratiometric images of acute hippocampal tissue slices of an roGFPc mouse (CA1 str. pyramidale; P20). Note that the neuronal somata are consistently more oxidized than the dendrites. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Using these sensors demands, however, successful delivery of the coding DNA to the tissue of interest. Exactly this technical requirement often limits application of genetically encoded redox sensors to cultured preparations. The availability of transgenic redox indicator mice stably expressing roGFP1-based redox sensors effectively bridges these limitations. We demonstrate that redox balance can now be assessed at subcellular resolution in cytosol or mitochondria, in various brain regions, in multiple types of neurons, and at different maturational stages spanning from neonatal to aging mice. So far, only few redox sensor-expressing mouse models have been described [Thy1-mito-Grx1-roGFP2 (11), TH-mito-roGFP2 (27), EF-1 α -roGFP1 (58), β -globin-roGFP2 (59)]. Yet, they either carry different promoters and/or contain the more pH-sensitive roGFP2 variant, two of them express roGFP in mitochondria only, and none of them was used to study redox dynamics in the forebrain.

For our transgenic mice, we chose roGFP1 since it is less pH sensitive than roGFP2 (29) and only negligibly responds to changes in Cl⁻ concentration (24). Furthermore, based on its spectral properties, roGFP1 is well suited for 1- and 2-photon excitation. It shows a pronounced absorption at 395 nm (29), where polychromatic light sources become less intense and light transmission of the objectives is declining. The 470 nm absorption peak responds intensely to redox modulation, which ensures a wide dynamic response range also with single wavelength excitation, for example, non-ratiometric 2-photon excitation. In earlier studies on cell and slice cultures, we confirmed that roGFP1 responds reliably to subcellular redox changes arising from either external redox challenge or altered cell endogenous ROS formation and/or scavenging (24, 26). Being interested especially in redox homeostasis of hippocampal and cortical pyramidal neurons, we placed roGFP1 under the *Thy-1* promoter. It drives high expression levels of the inserted transgene predominantly in various excitatory neurons already toward the end of the first postnatal week (12, 25).

In terms of redox modulation of neuronal function and responsiveness, the cytosol is the central compartment to be analyzed. Yet, as mitochondria are major sources of cellular ROS formation (9, 10), only the deciphering of redox changes in both compartments and their mutual interactions will unveil detailed mechanistic insights into subcellular redox modulation of neuronal function. We therefore generated mice expressing roGFP1 in either cytosol or mitochondria.

In these transgenic mice, roGFPc and roGFPm are widely expressed especially in excitatory projection neurons throughout the forebrain and in spinal cord. This pattern matches other mice expressing *Thy-1*-driven reporter constructs (11–13, 21, 46). Response calibrations of roGFPc and roGFPm in various brain regions verified that both sensors are fully functional, responding reversibly to oxidation and reduction. Detailed phenotyping shows that despite brain-wide expression, roGFP sensors do not mediate any adverse effects on phenotypic appearance, growth and development, fertility, or general mouse behavior.

Another major advantage of the transgenic mice is that they provide the entire range of preparations. As roGFP1 expression starts in the first postnatal days, available *in vitro* preparations span from dissociated cell cultures, acute tissue slices of various developmental stages up to the more complex isolated intact hippocampal formation. Even *in vivo*

studies may be performed at different developmental stages. At the same time, we demonstrate that roGFP mice are feasible for an array of optical techniques, including live cell wide-field imaging at the cellular and tissue level, 2-photon imaging, and FLIM.

Ratiometric CCD camera imaging revealed clear cellular and subcellular differences in steady-state redox conditions. Cytosol and mitochondrial matrix were most oxidized in CA3 neurons, which may arise from their high metabolic demand. As indicated by neurometabolic coupling studies, the hippocampal CA3 subfield combines most pronounced γ -oscillations, highest O₂ consumption, almost maximal mitochondrial respiratory rates, and the highest expression of complex I subunits (33, 34).

In terms of (sub)compartmental differences, we found a more pronounced roGFPc oxidation in soma than in dendrites of CA1 neurons. In addition, mitochondrial matrix was more oxidized than cytosol in all regions analyzed, emphasizing the role of mitochondria as primary ROS sources (9, 10, 16). Similar compartmental differences were reported with roGFP2 in pulmonary and systemic smooth muscle (53), in which cytosolic roGFP2 was 19–22% oxidized, whereas mitochondrial roGFP2 was 49–63% oxidized. In contrast, in HeLa cells, matrix-targeted roGFP sensors were more reduced than in cytosol (15, 29). These differences are likely due to specifics of experimental conditions, culturing media, and especially O₂ levels, highlighting the importance of standardized experimental and analytic approaches in redox research.

Furthermore, our tests on roGFPc and roGFPm hippocampal slices confirm that the expressed sensors report cell endogenous redox changes arising from intensified neuronal activation or metabolic compromise. It is important to point out here that any interference of roGFP1 imaging with either cellular NADH and FAD autofluorescence in living slices or any unspecific autofluorescence still present in fixed tissue sections was clearly excluded. The oxidizing shifts observed in roGFPc and roGFPm hippocampus upon electrical stimulation and K⁺-mediated depolarization match the concept that strong neuronal activity drives mitochondrial superoxide production *via* a pronounced cytosolic Ca²⁺ load (8, 19, 30, 51). Adrenergic stimulation primarily activates hippocampal interneurons, and only prolonged application elicits a sustained increase in CA1 neuron excitability, which does, however, not involve massive Ca²⁺ influx (7, 18). Thus, further mechanisms may contribute to the norepinephrine-mediated oxidation of roGFPc and roGFPm.

Inhibiting mitochondrial respiration in acute slices oxidized roGFPm and roGFPc, corroborating our earlier observed oxidation of cytosolic roGFP1 in hippocampal cell and slice cultures by antimycin A (24, 26). As expected, the mitochondria-targeted antioxidant mitoTEMPO efficiently dampened the antimycin A-mediated oxidation of roGFPm. This follows the concept that antimycin A induces superoxide production at complex III, which then reaches both the matrix and cytosol (10, 37).

Hypoxia/reoxygenation is more complex as different phases can be distinguished (1). Mitochondria produce ROS only initially, until their membrane potentials collapse. Delayed activation of xanthine oxidase evokes a secondary ROS increase, and stimulation of NADPH oxidase upon reoxygenation dominates the postanoxic ROS burst, with only minor contribution of hyperoxidized mitochondria (1, 47).

Accordingly, in hippocampal roGFPm slices, a reducing shift occurred during hypoxia, which is in line with reports from smooth muscle (53). In contrast, in roGFPc hippocampus, oxidation markedly increased by hypoxia and increased further with reoxygenation. The dampening effect of the antioxidant EUK-134 on hypoxia/reoxygenation-induced oxidation of roGFPc in CA1 neurons emphasizes the role of the cytosolic compartment in hypoxia-related ROS formation. Differences in hypoxic roGFPc oxidation between the cortex and hippocampus were not found, corroborating earlier reports comparing ROS production in anoxic hippocampal and cortical neurons (1).

The fluorescence lifetimes of roGFPc determined in cell cultures and acute slices ranged between 2.31 and 2.45 ns, which matches the 2.63 ns lifetime of eGFP (20). FLIM responses were stable, but pronounced oxidation/reduction induced only a marginal 8% total lifetime change in transfected cell cultures and even less in roGFPc slices. Similar weak responses were reported for roGFP1 in HEK cells, whereas other roGFP variants offer larger dynamic FLIM response ranges (2, 56). Thus, the roGFP1 variant seems less suited for FLIM as its response range is not sufficient to detect moderate changes in redox balance. An identification of severely disabled and/or damaged cells may, however, be possible by roGFP1-based FLIM.

As confirmed by our proof-of-principle experiments, the method of choice to image roGFP in complex preparations is ratiometric 2-photon excitation. It may be achieved by manually tuning laser wavelengths and acquiring sequential image pairs, as we and others have shown earlier (11, 42). More advanced is the use of two separate lasers, which we have now introduced. It enables fully automated ratiometric imaging, as demonstrated in acute cortico-hippocampal slices. Not only redox changes but also baseline conditions can be quantified reliably, thereby yielding subcellular-resolution redox maps of selected brain regions or even entire brains. This will unveil subtle brain regional differences in redox homeostasis—both for steady-state conditions and evoked redox responses. Even different cell compartments can be compared and specifically analyzed, as indicated by the more oxidized conditions found in the soma region compared with dendrites (Fig. 10F).

Furthermore, the intense roGFP1 fluorescence allows addressing morphological alterations. As roGFPc homogeneously distributes in the cytosol, it also unveils changes in cell structure/shape, dimensions of cellular processes, and dendritic complexity in correlation with redox alterations. The same applies to roGFPm, which also reveals the shape of individual mitochondria, their motility, or fusion/fission events in close correlation with matrix redox conditions.

Materials and Methods

The generation of transgenic mice and all procedures involved have been approved by the Office of Animal Welfare of the University Medical Center Göttingen and the Lower Saxony State Office for Consumer Protection and Food Safety (file G12/0992).

Generation of transgenic mice

Mice stably expressing roGFP1 were generated by pronuclear DNA microinjections (31). The roGFP1 coding se-

quences were synthesized into the pTSC α 2A expression vector; mitochondrial roGFP1 expression is driven by the mitochondrial targeting sequence of subunit VIII of cytochrome C oxidase. The expression vector was amplified in competent DH5 α *Escherichia coli* cultures, and plasmid DNA was isolated using the PureLink HiPure Plasmid Maxiprep kit (Invitrogen), linearized by PvuI digestion, and separated on agarose gel. Isolated DNA was purified (Wizard SC Gel and PCR Clean-up System, Promega) and dissolved in TE (Tris EDTA) buffer (Invitrogen). Fertilized oocytes (zygotes) were harvested, DNA microinjected (~ 2.5 μ g DNA/ml, volume 1–2 pl), and transferred into the oviduct of pseudopregnant C57BL/6N recipient mice. The genotype of the founder mice was determined from tail biopsies to confirm successful gene transfer.

Behavioral testing

To rate motor performance and motor learning, RotaRod tests (17) were run on 3 consecutive days (P40–46). The treadmill (Ugo Basile) was used in accelerating mode, increasing rotation speed within 5 min from 5 to 50 rpm. The time was determined at which mice fell off or at which they clasped the rod for >5 full turns. Environmental exploration was assessed in an open field (52). Mice were placed in the center of the arena (45 $\times$ 45 cm), their motility tracked for 5 min by infrared beams, and analyzed with ActiTrack v2.7.13 software (Panlab, Harvard Apparatus).

Preparation

Acute tissue slices (400 μ m thickness) were prepared from ether-anesthetized male and female mice (23). Upon decapitation, the brain was rapidly isolated and cooled down in ice-cold ACSF. Blood was collected to measure hematocrit and blood glucose levels (Contour blood glucose meter; Bayer). Coronal cortico-hippocampal slices were cut using a vibroslicer (752M Vibroslice; Campden Instruments). The hemispheres of each slice were then separated and allowed to recover in oxygenated ACSF (22–24°C) for 1.5 h before experiments started.

Dissociated hippocampal cell cultures were prepared from 2- to 4-day-old mice of either sex (55). They were incubated at 37°C in a humidified 5% CO $_2$ -containing atmosphere, and the medium was refreshed every 2–3 days.

Transcardial perfusion and immunostaining

Expression of roGFP was mapped in perfusion-fixed brains. For transcardial perfusion, deep anesthesia was confirmed by the lack of foot pinch reflexes. The chest was quickly opened, the heart exposed, and an injection cannula inserted into the left ventricle. Remaining blood was removed from the vascular system by PBS perfusion, then perfusion fixation was performed with 4% paraformaldehyde (PFA) in PBS. Isolated brains were postfixed in 4% PFA for 3 days and then stored in PBS plus 0.02% Na $^+$ -azide.

For expression analyses and immunolabeling, thin coronal or sagittal sections (30 μ m thickness) were cut (VT1200S vibratome; Leica). Some were directly mounted (Dako Fluorescent Mounting Medium; Dako Cytomation) to analyze roGFP expression. Others were permeabilized (0.2% Triton X-100, 30 min), blocked with 20% donkey or 10%

goat serum (45 min), and incubated overnight at 4°C with primary anti-NeuN antibody (MAB377, Merck Millipore; 10 µg/ml in 2% BSA). Sections were then washed and incubated with secondary antibody [ab96880, Goat Anti-Mouse IgG H&L (Dylight550) preadsorbed; Abcam] for 2h (2 µg/ml, 2% BSA).

Solutions

Chemicals, unless specified differently, were obtained from Sigma-Aldrich. The ACSF contained (in mM) 130 NaCl, 3.5 KCl, 1.25 NaH₂PO₄, 24 NaHCO₃, 1.2 CaCl₂, 1.2 MgSO₄, and 10 dextrose; constant aeration with carbogen (95% O₂ - 5% CO₂) adjusted pH 7.4. In 50 mM K⁺-containing ACSF, NaCl was replaced stoichiometrically by KCl to maintain osmolarity. Antimycin A (AMC) was dissolved in absolute ethanol as 20 mM stock and stored at -20°C. Diethyldithiocarbamic acid (DEDTC), 1,4-dithio-DL-threitol (DTT; Fluka), H₂O₂ (30% aqueous stock solution), and the antioxidants, EUK-134 and mitoTEMPO, were directly added to ACSF at final concentration. MitoTracker Red (Invitrogen) was dissolved as 1 mM stock in DMSO.

Optical recordings

Ratiometric wide-field imaging was performed with computer-controlled fluorescence imaging systems. They comprised a monochromatic light source (Polychrome II or Polychrome V; Till Photonics) and a sensitive CCD camera (Imago QE, PCO Imaging, 62% quantum efficiency at 500 nm). Device synchronization, image acquisition, and online/offline analyses were performed with the imaging software (Till Vision 4.1 and 5.48; Till Photonics). Slices were placed in a submersion-style chamber at 33–35°C and, depending on the desired field of view, were imaged using a 10×0.3NA (Achromplan; Zeiss), 40×0.8NA (Achromplan, Zeiss), or 63×1.0NA water immersion objective (Apochromat; Zeiss).

As in our earlier studies, roGFP1 was excited ratiometrically at 395 nm and 470 nm (24, 26). The 395 nm excitation matches the short wavelength absorption peak, whereas the 470 nm excitation was slightly blue shifted from the 490 nm absorption peak, to use a broader emission bandpass and detect larger parts of the roGFP1 emission spectrum. Excitation and detection were optimized by a 492 nm excitation shortpass, a 495 nm dichroic, and a 525/50 nm emission bandpass. Exposure times of 15 ms, frame rates of 0.1 Hz, and 2×2 binning were used. For quantitation, the ratio F₃₉₅/F₄₇₀ was calculated from the averaged arbitrary fluorescence intensities in defined ROIs. Circular ROIs were placed manually on the soma of clearly identifiable roGFP-expressing neurons. For low-resolution imaging of tissue volumes, rectangular ROIs (~40×60 µm) were defined to obtain integrated responses of several roGFP-expressing neurons.

Ratios were calculated in real time during the recordings. As cellular NADH, FAD and lipofuscin autofluorescence were at least an order of magnitude less intense than roGFP1 emissions, and the majority of background fluorescence in fact represents out-of-focus roGFP1 fluorescence, background subtraction was not applied. The ratiometric responses of roGFPc and roGFPm were calibrated to full oxidation (R_{ox}, 5 mM H₂O₂) and reduction (R_{red}, 10 mM DTT) (24, 29) for each brain region. As confirmed in control

experiments, a 7-min-lasting treatment induced maximum responses of roGFPc and roGFPm. Both compounds were applied to a given slice to ensure reasonable readings of all calibration parameters (especially F470ox/F470red, see equation 1). Sufficiently long washout and recovery times were allowed to prevent direct interactions of H₂O₂ and DTT.

After calibration, the relative degrees of roGFP oxidation (OxD_{roGFP1}) were calculated (40), with adaptations to the roGFP1 sensor and our recording conditions:

$$\text{OxD}_{\text{roGFP1}} = \frac{R - R_{\text{red}}}{\frac{F_{470\text{ox}}}{F_{470\text{red}}} (R_{\text{ox}} - R) + (R - R_{\text{red}})} \quad (1)$$

Inserted into the Nernst equation, the relative roGFP1 oxidation levels together with the standard redox potential of roGFP1 (E⁰ = -291 mV) yield the corresponding roGFP1 redox potentials (29, 40):

$$E_{\text{roGFP1}} = E_{\text{roGFP1}}^0 - \frac{RT}{2F} \ln \left(\frac{1 - \text{OxD}_{\text{roGFP1}}}{\text{OxD}_{\text{roGFP1}}} \right) \quad (2)$$

For advanced redox imaging, 2-photon excitation microscopy was performed. Our 2-photon laser scanning microscope (TPLSM) is based on an upright microscope (BX51WI; Olympus) and two Ti:Sa laser systems (MaiTai eHP DS and Millennia Prime pumped Tsunami; Newport Spectra-Physics), which are coupled *via* separate, shutter-controlled optical ports into a TriMScope II scanhead with ImSpector control software (LaVision BioTec). Fluorescence was separated by a 670 nm beam splitter (670dcxxr) and an IR-block filter (HC680/SP) and detected in nondescanned mode. Green and red emissions were further split into two detection channels by a 570 nm dichroic mirror and 536/40 nm and 617/73 nm emission bandpass filters, respectively (35), and detected by highly sensitive photomultiplier tubes (PMTs, H7421/H7422; Hamamatsu). For 2-photon imaging of roGFPc, a 20X 0.95NA (XLUMPlanFI; Olympus) or a 40×0.8NA objective (IR Achromplan; Zeiss) was used; roGFPm was visualized using a 63×1.0NA objective (Apochromat; Zeiss). Overlay and ratio images were generated with Metamorph Offline 7.5 (Molecular Devices). Background subtraction was not required as with 2-photon excitation out-of-focus light is virtually nonexistent, but a scaling factor of 1000 ensured sufficient use of the ratiometric 12-bit pseudocolor palette. Quantitative analyses were performed within ROIs placed on the soma of clearly identifiable neurons.

During FLIM, fluorescence decay rates were monitored by a 76-MHz time-correlated single-photon counting detector (FLIM X16; LaVision BioTec) and underwent iterative reconvolution with the IRF, extracted from the background signal of cell cultures or the second harmonic generation emission of urea crystals (5, 55). Exponential fitting was performed using a nonlinear Levenberg–Marquardt least-square fitting algorithm (4, 43, 55, 57).

Statistics

To ensure independence of observations, each experimental paradigm was run on at least three different mice. All data are reported as mean ± standard deviation. The number

of experiments (n) refers to the quantity of specimen analyzed, that is, mice in phenotyping, slices in tissue-based assays, and cells in (sub)cellular imaging. Statistical significance of the changes observed was assessed by two-tailed, unpaired Student's *t*-tests. Normalized changes were rated by one-sample *t*-tests and compared with baseline or pretreatment conditions defined as unity.

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Author Disclosure Statement

No competing financial interests exist for any of the authors of this article.

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Abbreviations Used

ACSF = artificial cerebrospinal fluid
 AMC = antimycin A
 CA1 = *cornu ammonis* subfield 1
 CA3 = *cornu ammonis* subfield 3
 CCD = charge-coupled device
 DEDTC = diethyldithiocarbamic acid
 DH = dorsal horn
 DTT = 1,4-dithio-DL-threitol
 E_{roGFP1} = roGFP1 redox potential
 FLIM = fluorescence lifetime imaging
 microscopy
 FRET = fluorescence resonance energy transfer
 GSH = reduced glutathione

GSSG = oxidized glutathione (disulfide)
 HyPer = hydrogen peroxide-sensitive
 fluorescence sensor
 IRF = instrument response function
 LV = lateral ventricle
 NA = numerical aperture
 NeuN = neuronal nuclei antigen
 OB = olfactory bulb
 $\text{OxD}_{\text{roGFP1}}$ = relative degree of roGFP1 oxidation
 P = postnatal day
 PFA = paraformaldehyde
 PMT = photomultiplier tube
 R = fluorescence ratio
 roGFP1 = reduction–oxidation-sensitive
 green fluorescent protein 1
 roGFPc = cytosolically expressed roGFP
 roGFPm = mitochondria-targeted roGFP
 ROI = region of interest
 ROS = reactive oxygen species
 R_{ox} = fully oxidized fluorescence ratio
 R_{red} = fully reduced fluorescence ratio
 SC = superior colliculus
str. = *stratum*
 τ = fluorescence lifetime
 TCSPC = time-correlated single-photon counting
 Thy-1.2 = thymocyte differentiation antigen 1
 Ti:Sa = titanium–sapphire
 TPLSM = 2-photon laser scanning microscope
 VH = ventral horn
 WT = wild-type