Oxidative stress sensitivity in pericytes: exploring underlying mechanisms

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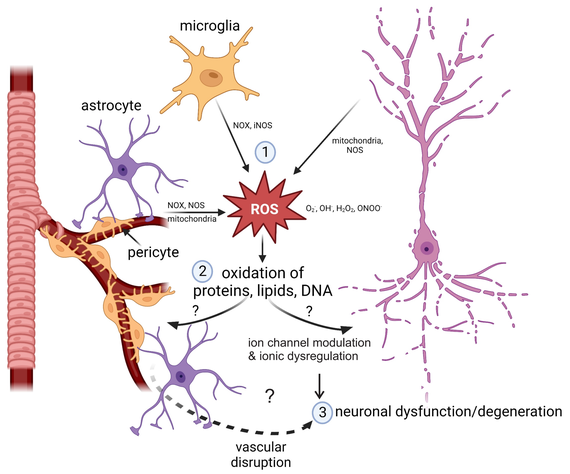
Introduction

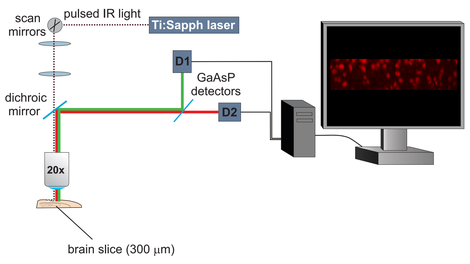


Background: Oxidative stress results from an excess of reactive oxygen or nitrogen species, which can disrupt neuronal function and lead to neurodegeneration. This process is implicated in a wide variety of neurological conditions including ischemic injury, inflammation, and neurodegenerative disorders, such as Parkinson's and Alzheimer's disease. Mechanistically, reactive oxygen species (ROS) can disrupt cellular function by oxidizing proteins, lipids, and DNA. **However, it remains unclear which cell types and signalling pathways are central to brain tissue dysfunction and degeneration in response to oxidative stress.** Addressing this problem is a critical step towards developing strategies to prevent the neurotoxic impacts of oxidative stress during brain disorders. Recent work from our lab demonstrated that neuronal death during excitotoxicity is driven by the activation of ROS-dependent ion channels. This suggests that redox sensitive ion channel modulation and downstream ionic dysregulation are likely to play a central role in coupling oxidative stress to cellular dysfunction and death in a variety of pathologies.

Hypothesis: Oxidative stress induces necrosis through oxidative modulation of ion channel function and subsequent ionic dysregulation.

Objective: We will test the impact of oxidative stress on cell death in brain tissue slices and test for the involvement of key ion channels classes in this process.

Methods



Adenoassociated virus (AAV): The rAAV gene construct encoding for RoGFP, expressed under the control of the neuronal synapsin promoter, was delivered to the cortex of adult mice via intracranial AAV delivery.

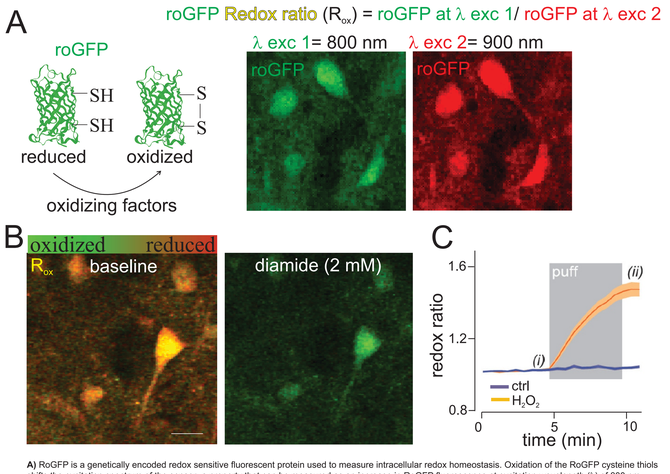
Imaging: Imaging of propidium iodide (PI) in live or fixed brain tissue was achieved using a two-photon laser scanning microscope (Zeiss LSM 7MP) coupled to a tunable Ti:Sapphire laser. For live imaging of pericytes, tissue was incubated in NeuroTrace or To-Pro3 (to label pericytes) and then placed in the imaging chamber and perfused with carbogen (95% O_2 / 5% CO_2) bubbled artificial cerebrospinal fluid (ACSF). For imaging of immunostained fixed brain tissue, sections were imaged using a Zeiss confocal microscope.

Animals: SD rats (p17-30) and mice (p30-300) were used for all experiments. Mouse brain tissue was taken from C57BL/6J mice.

Drugs and Solutions: ACSF composition in mM: 126 NaCl, 2.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 1.5 $MgCl_2$, 1.25 NaH_2PO_4, 10 glucose, pH=7.3, 310 mOsm. For ion replacement experiments Ca^{2+} and NaCl were removed and solution supplemented with EGTA and sodium-isothionate respectively. The following drugs were used, concentration in mM: 0.02 propidium iodide (PI), 2 diamide, 0.5 dipyridal disulphide (DPS), 5 H_2O_2, 2 amiloride, 0.4 benzamil, 0.03 CPA, 0.1 NiCl, 0.05 CdCl, 0.04 nifedipine, 0.1 SKF96365, 0.02 SET2, 0.02 ruthenium red, 0.2 flufenamic acid, 0.2 Gd^{3+}

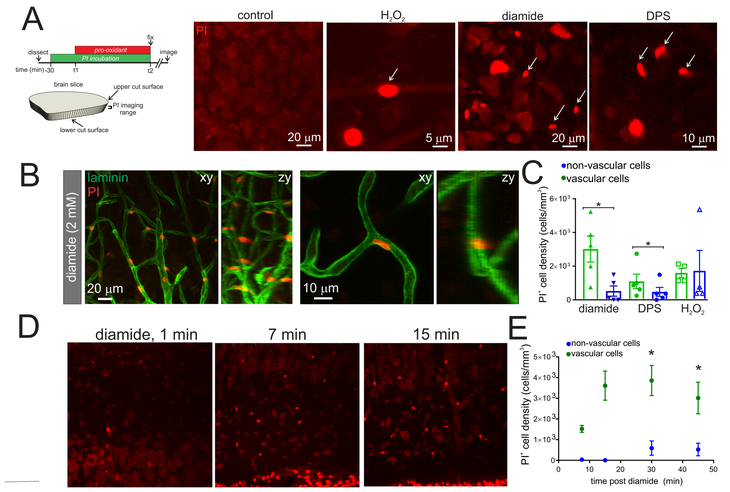
Results

Measuring the impact of pro-oxidants on redox state in brain tissue



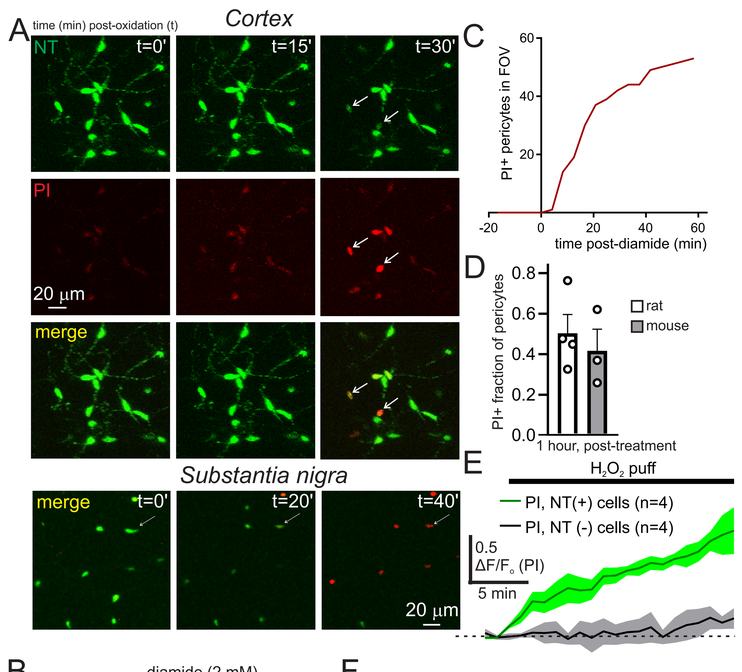
A) RoGFP is a genetically encoded redox sensitive fluorescent protein used to measure intracellular redox homeostasis. Oxidation of the RoGFP cysteine thiols shifts the excitation spectrum of the sensor- a property that can be measured as an increase in RoGFP fluorescence at excitation wavelength (λ) of 800 nm relative to excitation at 900 nm. The ratio of RoGFP fluorescence at 800 nm and 900 nm excitation (R_{ox}) is used to quantify intracellular redox state. **B)** Diamide, which oxidizes thiol groups, potently oxidizes cytosolic RoGFP in neurons of an acute brain slice, as measured by the oxidative shift in RoGFP redox ratio (R_{ox}). **C)** Puff application of hydrogen peroxide (H_2O_2) causes acute oxidation of neurons in a brain slice.

Pro-oxidants cause rapid death of blood vessel-associated cells



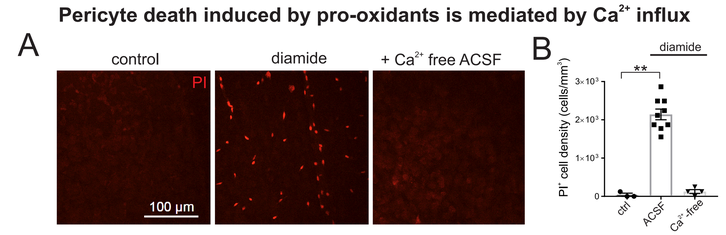
A) Acute brain slices were incubated in propidium iodide (PI) (20 μM), to measure cell membrane breakdown, and tissue oxidation was initiated with diamide, dipyridyl disulfide (DPS), which cause thiol oxidation, and H_2O_2. For analysis, PI labelled cells were only quantified in the middle 100 μm of the tissue slice. **B)** Diamide (2 mM), DPS, or H_2O_2 (1 mM) application results in a large number of small PI loaded cells, and less frequently, larger cells, most likely neurons. **C)** PI positive cells primarily associate with blood vessels, as shown with laminin immunostaining. **D)** Summary data showing that either diamide or DPS cause significantly greater death of vessel associated cells relative to non-vascular cells (two-tailed paired Student's t tests, *p<0.05). **E)** Following diamide application, vascular cell death begins within 7 min, with minimal non-vascular death. (two-tailed paired Student's t tests, *p<0.05). All presented data represent the mean ± SEM. **F) Following incubation with proxidants, treating with dithiothreitol (DTT)**

Rapid death of vascular pericytes during oxidative stress



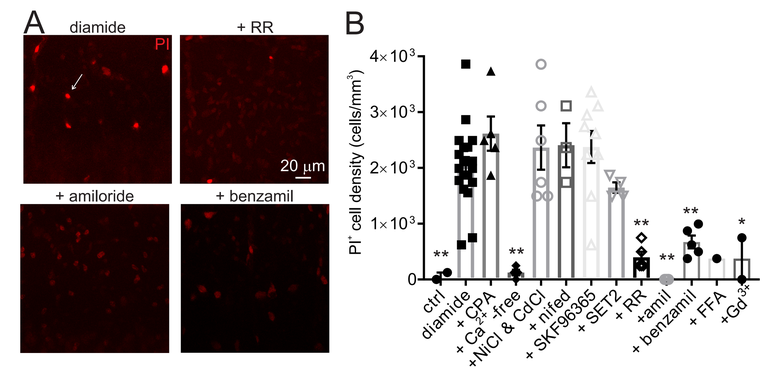
A) Live imaging of NeuroTrace (NT) labelled pericytes (Upper and lower) and propidium iodide (PI) (middle and lower) during thiol oxidation with diamide. PI loading occurs in a large fraction of NT positive cells. After oxidation, pericytes rapidly lose NT label, simultaneous with PI uptake in both the cortex and substantia nigra. **B)** Representative experiment showing simultaneous uptake of PI and loss of NeuroTrace from a pericyte following diamide application. **C)** Representative experiment showing the cumulative number of pericytes in the imaging FOV which load with PI following diamide application. **D)** Summary data showing the high fraction of NT labelled pericytes which load with PI following diamide application. **E)** Puff applying hydrogen peroxide (H_2O_2) to brain tissue causes a greater increase in PI uptake in NeuroTrace labelled pericytes (NT+) relative to neighboring NeuroTrace negative cells (NT-). **F)** Example image of a PI loaded cell in diamide that is positive for PDGFRβ and displays a dystrophic, blebbing morphology.

Pericyte death induced by pro-oxidants is mediated by Ca^{2+} influx



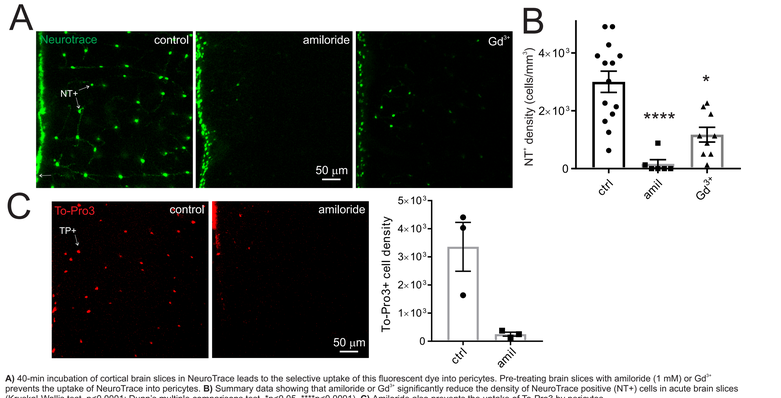
A) Representative images showing that diamide evoked pericyte death, measured with PI uptake, is largely eliminated by removing Ca^{2+} from the ACSF. **B)** Summary data showing that the diamide significantly increases pericyte death, relative to control, in ACSF but not in the absence of extracellular Ca^{2+} (Ca^{2+}-free) (Kruskal-Wallis test, p<0.0001; Dunn's multiple comparisons test, **p<0.001).

Pharmacological disruption of pericyte death evoked by oxidative stress



A) Images depict PI loading of pericytes in cortical tissue after oxidation with diamide (2 mM, 60 min) and block of diamide-evoked pericyte death in the presence of ruthenium red (20 μM), amiloride (2 mM), or benzamil (200 μM). **B)** Group data showing the impact of ion channel blockers on oxidation-induced pericyte death. Depleting the endoplasmic reticulum Ca^{2+} store with CPA had no impact on diamide-evoked death. Blocking voltage-gated Ca^{2+} channels with nifedipine (nifed) or NiCl and CdCl had no significant impact on pericyte death. The TRPV2 blocker, SET2, and the TRPC/TRPV2 channel blocker SKF96365 had no impact on pericyte death. Conversely, pericyte death was prevented by ruthenium red, amiloride, benzamil, Gd^{3+}, or flufenamic acid (FFA).(One-way ANOVA, p=<0.0001; Dunnett's post-test, *p<0.05, **p<0.01).

A putative TRP channel mediates selective uptake of small molecules by pericytes.



A) 40-min incubation of cortical brain slices in NeuroTrace leads to the selective uptake of this fluorescent dye into pericytes. Pre-treating brain slices with amiloride (1 mM) or Gd^{3+} prevents the uptake of NeuroTrace into pericytes. **B)** Summary data showing that amiloride or Gd^{3+} significantly reduce the density of NeuroTrace positive (NT+) cells in acute brain slices (Kruskal-Wallis test, p<0.0001; Dunn's multiple comparisons test, *p<0.05, ***p<0.0001). **C)** Amiloride also prevents the uptake of To-Pro3 by pericytes.

Conclusion

1) Brain vascular pericytes are highly sensitive to pro-oxidants.

2) Pericyte death occurs through a Ca^{2+} influx pathway involving a putative TRP channel.

3) The putative TRP channel regulates the selective uptake of small molecule markers by brain pericytes.

4) The over activation of this pathway may contribute to the pericyte dysfunction and downstream vascular disruption seen in the many brain pathologies associated with oxidative stress.

Acknowledgments

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