Brain pericytes are highly vulnerable to oxidative stress

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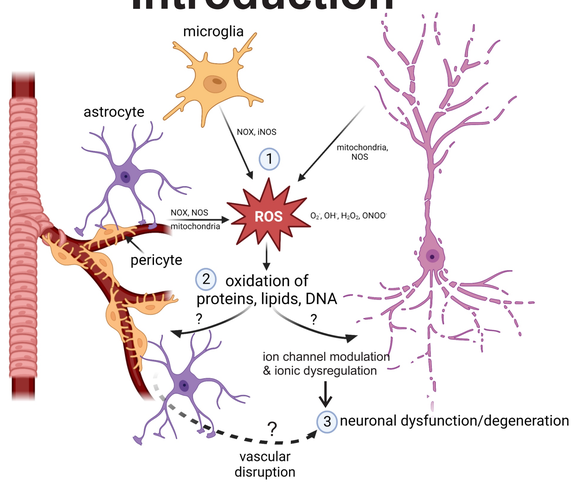
Introduction

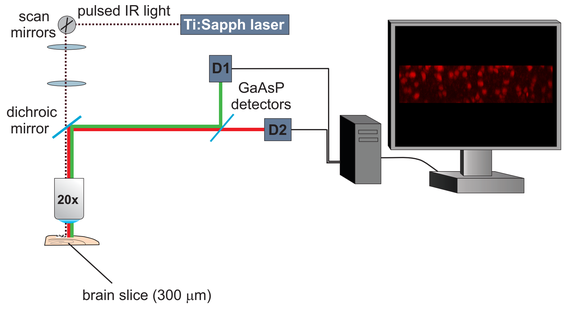


Background: Oxidative stress results from an excess of reactive oxygen or nitrogen species, which can disrupt neuronal function and lead to neurodegeneration. This process is implicated in a wide variety of neurological conditions including ischemic injury, inflammation, and neurodegenerative disorders, such as Parkinson's and Alzheimer's disease. Mechanistically, reactive oxygen species (ROS) can oxidize proteins, lipids, and DNA. As such, a **central problem is identify the key cell types and cell signalling pathways coupling oxidative stress to neuronal dysfunction.** This is a critical step necessary to disrupt the neurotoxic impacts of ROS in various brain disorders. The activity of many ion channel types is redox sensitive. As such, ion channel modulation and ionic dysregulation is likely to play a central role in coupling oxidative stress to neuronal dysfunction and death.

Hypothesis: Oxidative stress induces neuronal 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.

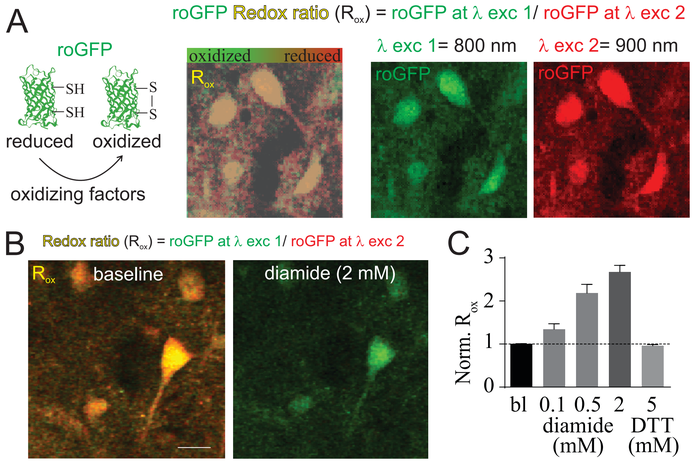
Imaging: Imaging of propidium iodide (PI) in live or fixed brain tissue was achieved using a two-photon laser scanning microscope (Zeiss LSM 7MP) coupled to a tunable Ti:Sapphire laser. For live imaging of pericytes, tissue was incubated in NeuroTrace or To-Pro3 (to label pericytes) and then placed in 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 either C57BL/6J mice or from *Hic1*^{CreERT2}; *Rosa26*$^{sLsl\text{-}tdTomato}$ mouse, which allows for tamoxifen-inducible expression of the Td-tomato reporter in mesenchymal cells (pericytes and perivascular fibroblasts).

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+}

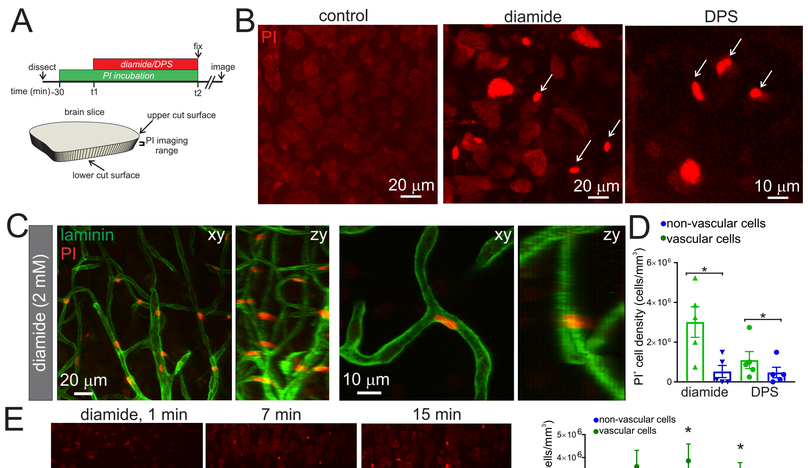
Results

Oxidation of thiols in cortical neurons with diamide.



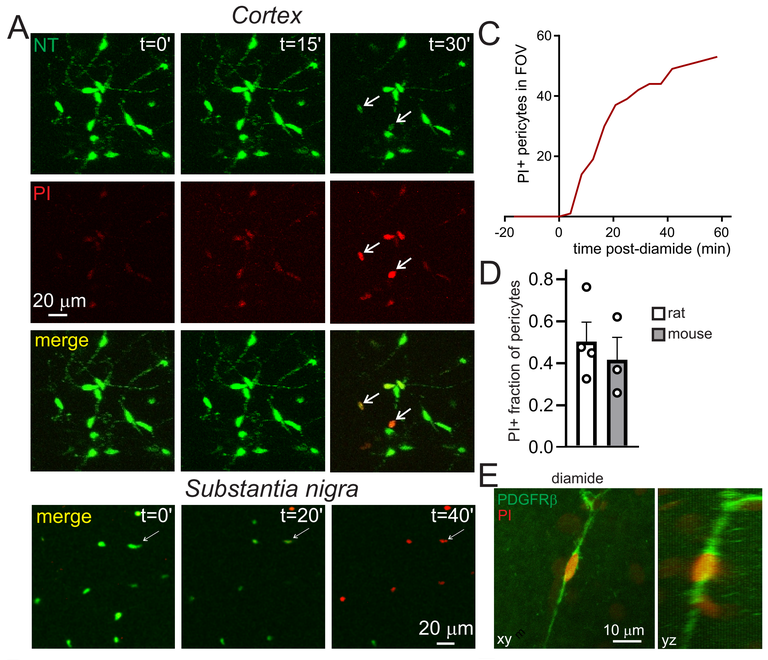
A) RoGFP is a genetically encoded redox sensitive fluorescent protein used to measure intracellular redox homeostasis. Oxidation of the RoGFP cysteine thiols shifts the excitation spectrum of the sensor- a property that can be measured as an increase in RoGFP fluorescence at excitation wavelength (λ) 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,C)** 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}). DTT, a reducing agent, reverses the oxidation of RoGFP.

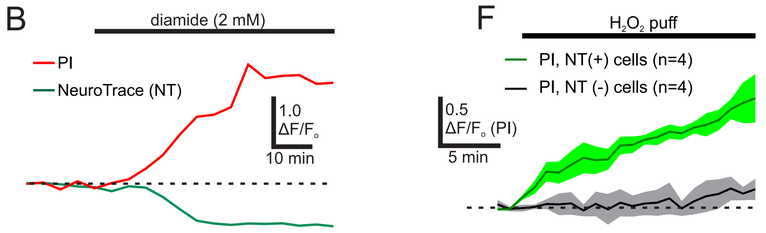
Thiol oxidation causes rapid death of blood vessel-associated cells



A) Acute brain slices were incubated in propidium iodide (PI) (20 μM), to measure cell membrane breakdown, and tissue oxidation was initiated with diamide or dipyridyl disulfide (DPS), which cause thiol oxidation. For analysis, PI labelled cells were only quantified in the middle 100 μm of the tissue slice. **B)** Diamide or DPS 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.

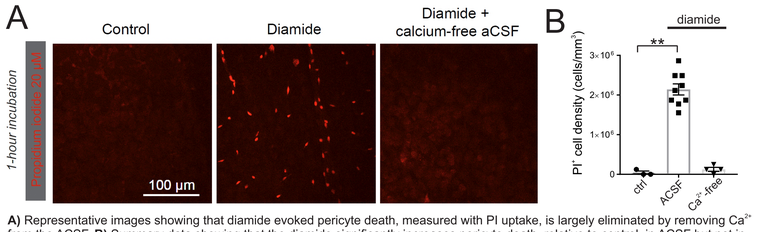
Rapid death of vascular pericytes following thiol oxidation





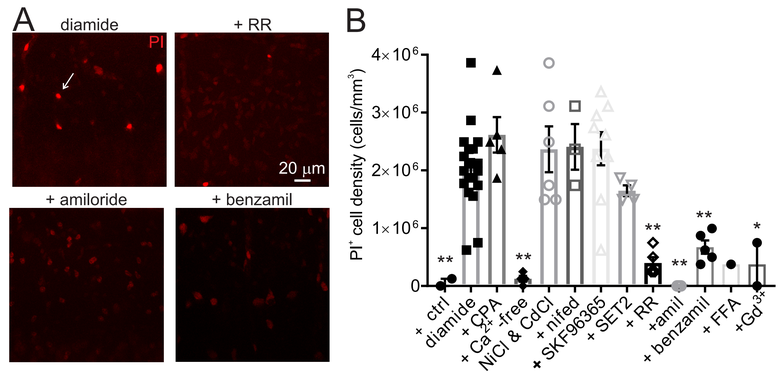
A) Live imaging of NeuroTrace (NT) labelled pericytes (Upper and lower) and propidium iodide (PI) (middle and lower) during thiol oxidation with diamide. PI loading occurs in a large fraction of NT positive cells. PI loading occurs primarily in NeuroTrace positive cells. After oxidation, pericytes rapidly 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)** Sample experiment showing the cumulative number 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)** PI loaded cells in diamide are positive for PDGFRβ. **F)** 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-).

Pericyte death induced by thiol oxidation requires extracellular Ca^{2+}



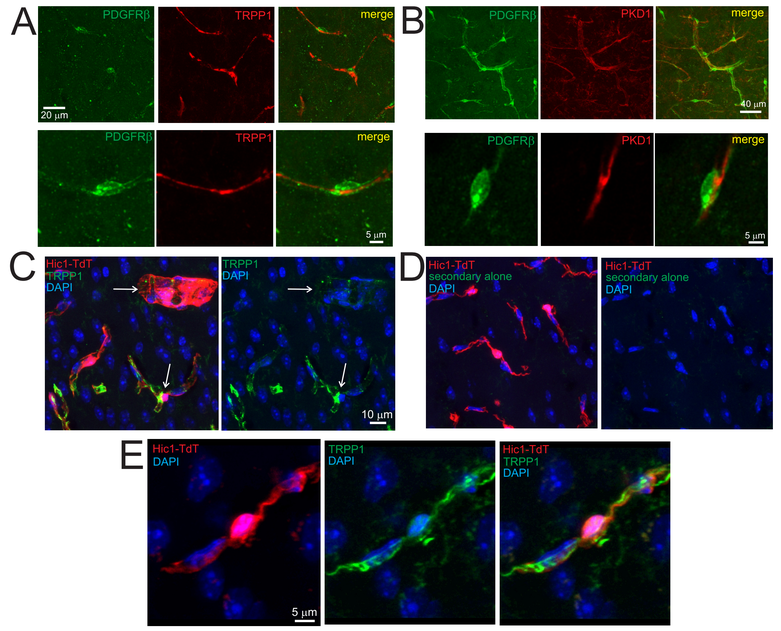
A) Representative images showing that diamide evoked pericyte death, measured with PI uptake, is largely eliminated by removing Ca^{2+} from the ACSF. **B)** Summary data showing that the diamide significantly increases pericyte death, relative to control, in ACSF but not in the absence of extracellular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

Pharmacological sensitivity of pericyte death evoked by thiol oxidation



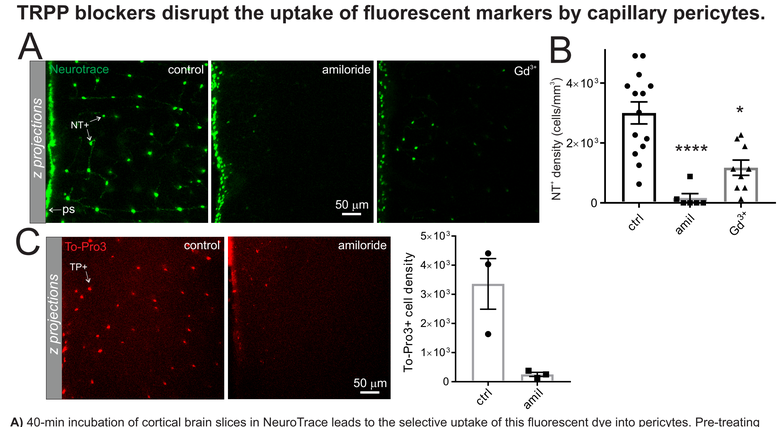
A) Images depict PI loading of pericytes in cortical tissue after oxidation with diamide (2 mM, 60 min) and block of diamide-evoked pericyte death in the presence of ruthenium red (20 μM), amiloride (2 mM), or benzamil (200 μM). **B)** Group data showing the impact of ion channel blockers on oxidation-induced 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).

TRPP1 and PKD1 are localized to capillary pericytes.



A) Brain tissue immunofluorescent labelling of pericytes, using a PDGFRβ antibody, and TRPP1. TRPP1 is strongly associated with PDGFRβ+ pericytes. **B)** Immunofluorescent imaging showing the distribution of PKD1, a subunit of the TRPP1 oligomeric channel complex. PKD1 distribution is similar to TRPP1 and is associated with pericytes. **C)** Localization of TRPP1 to TdT-positive cells in small, but not large, blood vessels. Brain tissue was taken from the *Hic1*^{CreERT2}; *Rosa26*$^{sLsl\text{-}tdTomato}$ mouse, which allows for tamoxifen-inducible expression of the Td-tomato reporter in mesenchymal cells (pericytes and perivascular fibroblasts). **D)** Control staining with secondary antibody alone (IgG-Alexa488) shows nominal staining in TdT-positive pericytes. **E)** Representative image of a TdT-positive pericyte showing the soma and thin-stranded processes along the length of a capillary. TRPP1 staining is strongly associated with pericyte processes.

TRPP blockers disrupt the uptake of fluorescent markers by capillary 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 vulnerable to oxidative stress induced by protein thiol oxidation.

2) Pericyte death occurs through a Ca^{2+} influx pathway with properties consistent with TRPP1.

3) TRPP1 is highly associated with capillary pericytes but not vascular cells on large blood vessels.

4) TRPP1 is a candidate pathway mediating Ca^{2+} entry and uptake of fluorescent markers in pericytes.

Our work suggests that pericyte dysfunction is an early response to brain oxidative stress and occurs through TRP channel activation. The over activation of TRPP1 in pericytes may contribute to vascular disruption in brain pathologies associated with oxidative stress.

Acknowledgments

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Parkinson Canada