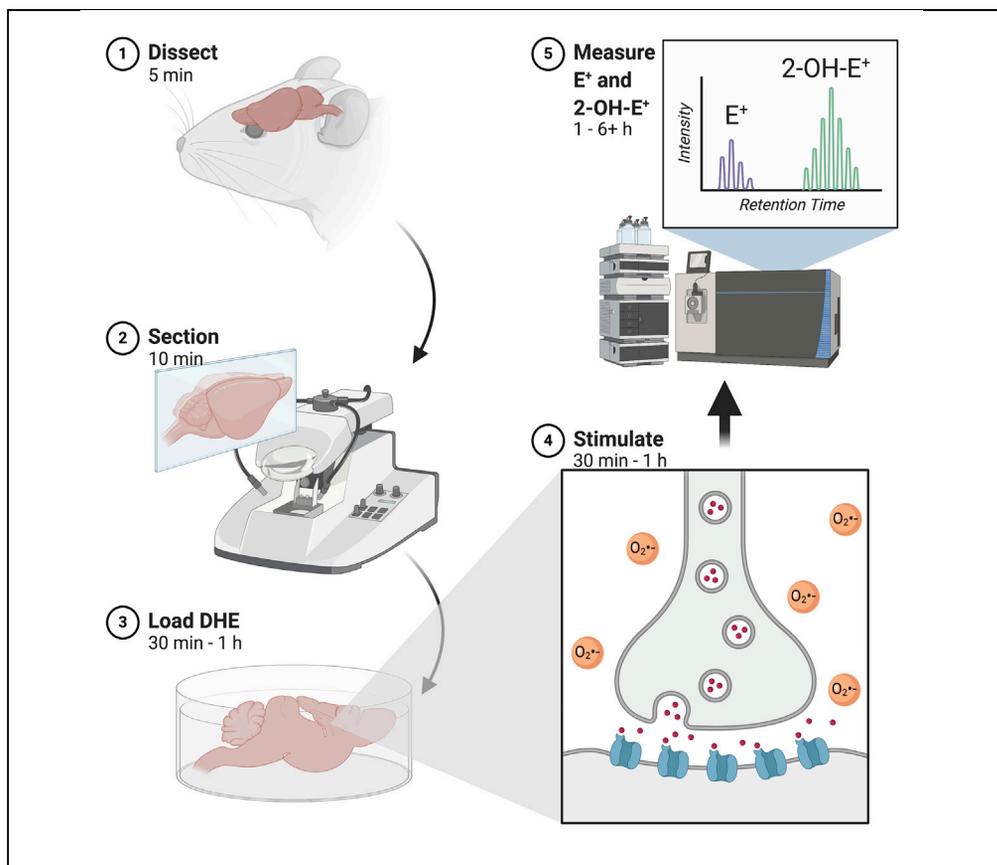


## Protocol

# Quantitative measurement of reactive oxygen species in *ex vivo* mouse brain slices



Evaluating redox homeostasis involves gauging the levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) directly in tissues and cells. The brain is especially metabolically active and is particularly vulnerable to excessive ROS and RNS. Here, we describe a methodology to quantitatively measure ROS in *ex vivo* mouse brain slices at baseline and after neural stimulation. Evaluating ROS in slices provides a more complete picture of neural redox signaling than when measured in isolated neurons or astrocytes.

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### HIGHLIGHTS

Quantitatively measure specific reactive oxygen and nitrogen species

Assess redox homeostasis in *ex vivo* mouse brain slices

Measure changes in reactive signaling in response to diverse neural stimuli

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## Protocol

Quantitative measurement of reactive oxygen species in *ex vivo* mouse brain slicesChirag Vasavda,<sup>1,4,\*</sup> Solomon H. Snyder,<sup>1,2,3</sup> and Bindu D. Paul<sup>1,5,\*</sup><sup>1</sup>The Solomon H. Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA<sup>2</sup>Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA<sup>3</sup>Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA<sup>4</sup>Technical contact<sup>5</sup>Lead contact\*Correspondence: [cvasavda@jhmi.edu](mailto:cvasavda@jhmi.edu) (C.V.), [bpaul8@jhmi.edu](mailto:bpaul8@jhmi.edu) (B.D.P.)<https://doi.org/10.1016/j.xpro.2021.100332>**SUMMARY**

Evaluating redox homeostasis involves gauging the levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) directly in tissues and cells. The brain is especially metabolically active and is particularly vulnerable to excessive ROS and RNS. Here, we describe a methodology to quantitatively measure ROS in *ex vivo* mouse brain slices at baseline and after neural stimulation. Evaluating ROS in slices provides a more complete picture of neural redox signaling than when measured in isolated neurons or astrocytes.

For complete details on the use and execution of this protocol, please refer to Vasavda et al. (2019).

**BEFORE YOU BEGIN**

Solutions are prepared following the recipes outlined in the [Materials and equipment](#) section. Solutions prepared in advance can be stored as indicated. A complete list of **Materials and Equipment** required are listed in the [Key resources table](#).

**Prepare the following solutions**

1. Sectioning artificial cerebrospinal fluid (S-aCSF).
2. Experimental artificial cerebrospinal fluid (E-aCSF).
3. Lysis Buffer.
4. Phosphate Buffer.
5. High-pressure Liquid Chromatography (HPLC) mobile phases.

**Prepare incubation chambers for brain slices**

6. Set up one apparatus ([Figure 1](#)) as a holding chamber for slices with ice-cold S-aCSF.
7. Set up another apparatus as an equilibration chamber for slices with E-aCSF at 20°C–25°C.

**Connect gas tanks to incubation chambers**

Connect both the holding and equilibration chambers to a gas tank with a supply of 95% O<sub>2</sub> and 5% CO<sub>2</sub>.



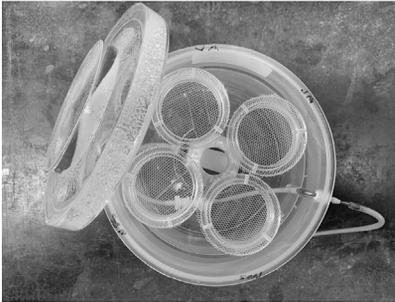


Figure 1. Incubation chambers for brain slices

### Equilibrate S-aCSF and E-aCSF with 95% O<sub>2</sub> and 5% CO<sub>2</sub>

Equilibrate aCSF buffers and holding/experimental chambers with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> for 30 min. Maintain S-aCSF on ice and E-aCSF at 20°C–25°C.

### Prepare vibratome

8. Position vibratome (Figure 2) near holding/experimental incubation chambers.
9. Fill the compartment immediately exterior to the partition in which the brains will be sectioned with ice in order to keep the brains cold while slicing.
10. Program the vibratome.
  - a. Section at 300 μm intervals at a controlled speed of 0.3 mm/s.
  - b. Set the vibratome frequency to its maximum at 100 Hz.
  - c. Position blade at a 15° to 20° angle from parallel.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
NaCl	Fisher Scientific	Cat #S271
NaH <sub>2</sub> PO <sub>4</sub>	Sigma	Cat# S0751
Na <sub>2</sub> HPO <sub>4</sub>	Sigma	Cat# 255793
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	Sigma	Cat# S9638
MgSO <sub>4</sub>	Sigma	Cat# 83266
CaCl <sub>2</sub>	Sigma	Cat# 21115
NaHCO <sub>3</sub>	Sigma	Cat# S6014
Glucose	Sigma	Cat# G8270
Trizma pre-set crystals, pH 7.4	Sigma	Cat# T7693
Sodium dodecyl sulfate (SDS)	Sigma	Cat# 436143
Sodium deoxycholate	Sigma	Cat# D6750
Triton X-100	Sigma	Cat# X100
Acetonitrile, HPLC grade	Sigma	Cat# 34851
Trifluoroacetic acid, HPLC grade	Sigma	Cat# 302031
Methanol, HPLC grade	Sigma	Cat# 34860
Water, HPLC grade	Sigma	Cat# 270733
Potassium nitrosodisulfonate (Fremy's salt)	Sigma	Cat# 220930
Diethylenetriamine pentaacetic acid (DTPA)	Sigma	Cat# D6518
5,5-Dimethyl-1-pyrroline N-oxide (DMPO)	Cayman Chemical	Cat# 10006436
(D-)-2-Amino-5-phosphonopentanoic acid) APV	Sigma	Cat# A8054
Dihydroethidium (DHE)	Thermo Fisher Scientific	Cat# D1168
Ethidium	Sigma	Cat# E1510
Glycine	Sigma	Cat# 410225

(Continued on next page)

<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
N-Methyl-D-aspartic acid or N-methyl-D-aspartate (NMDA)	Sigma	Cat# M3262
Dimethyl sulfoxide (DMSO)	Sigma	Cat# D5897
Potassium superoxide (KO <sub>2</sub> )	Sigma	Cat# 310492
1-Butanol	Sigma	Cat# 360465
Protease inhibitor cocktail	Sigma-Aldrich	Cat# P8340
Isoflurane	Covetrus	Cat# 11695-6777-2
N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)	Sigma	Cat# H3375
<b>Critical commercial assays</b>		
Pierce BCA Protein Assay Kit	Thermo Fisher	Cat# 23227
<b>Experimental models: organisms/strains</b>		
Mouse: WT: C57BL/6J; 8–12 week old males	Jackson Labs	Cat# 000664
Mouse: BVR <sup>-/-</sup> ; C57BL/6J-Blvra <sup>tm1Ozg</sup> ; 8–12 week old males	<a href="#">Vasavda et al. (2019)</a>	N/A
<b>Software and algorithms</b>		
GraphPad Prism	GraphPad Software, Inc	graphpad.com
<b>Other</b>		
Bottle top filters	Thermo Fisher Scientific	Cat#09-741-11
Superglue	Loctite	Cat# 1363589
HPLC scanning fluorescence detector	Waters	Cat# 474
HPLC instrument	Waters	Cat# 2690
Nitrogen gas	Airgas	Cat# NI NF200
Carbogen gas (95% O <sub>2</sub> and 5% CO <sub>2</sub> )	Airgas	Cat# X02OX95C2003102
Brain/tissue slice chamber	Scientific Systems Design Inc.	Cat# BSK 4
Vibratome	Leica Biosystems	Cat# VT1200S
LC-18 column, 150 × 3.9 mm	Waters	Cat# WAT046980
HPLC autosampler vials	Thermo Fisher Scientific	Cat# C4010-88AW
Benchtop pH meter	Thermo Fisher Scientific	Cat# STARA1117
Analytical balances	Thermo Fisher Scientific	Cat# 01-912-401

## MATERIALS AND EQUIPMENT

### Preparation of artificial cerebrospinal fluids: S-aCSF and E-aCSF

⌚ Timing: 30–45 min

Artificial cerebrospinal fluid (aCSF) is commonly used as an extracellular medium to bathe *ex vivo* or *in vitro* cells from the central nervous system. Sections are sliced in ice-cold sectioning-aCSF (S-aCSF) with high magnesium and low calcium to minimize excitotoxicity during sectioning. Slices are then transitioned to 20°C–25°C experimental-aCSF (E-aCSF) and allowed to equilibrate to experimental conditions.

- Preparation of sectioning artificial cerebrospinal fluid (S-aCSF) (1 L)

Reagent	Final concentration (mM)	Amount
NaCl	126 mM	7.36 g
KCl	3 mM	223.65 mg
NaH <sub>2</sub> PO <sub>4</sub>	1.4 mM	167.97 mg
MgSO <sub>4</sub>	10 mM	1.20 g
CaCl <sub>2</sub>	0.5 mM	55.5 mg
NaHCO <sub>3</sub>	26 mM	2.18 g
Glucose	10 mM	1.80 g

(Continued on next page)

**Continued**

Reagent	Final concentration (mM)	Amount
ddH <sub>2</sub> O	n/a	to 1,000 mL
<b>Total</b>	<b>n/a</b>	<b>1,000 mL</b>

- Prepare 500–600 mL of ultra-pure water in a beaker with a stir bar.
  - Add 7.36 g NaCl (final 126 mM NaCl).
  - Add 223.65 mg KCl (final 3 mM KCl).
  - Add 167.97 mg NaH<sub>2</sub>PO<sub>4</sub> (final 1.4 mM NaH<sub>2</sub>PO<sub>4</sub>).
  - Add 1.20 g MgSO<sub>4</sub> (final 10 mM MgSO<sub>4</sub>).
  - Add 55.5 mg CaCl<sub>2</sub> (final 0.5 mM CaCl<sub>2</sub>).
  - Add 2.18 g NaHCO<sub>3</sub> (final 26 mM NaHCO<sub>3</sub>).
  - Add 1.80 g glucose (final 10 mM glucose).
  - Adjust final pH to 7.4.
  - Bring solution up to a final volume of 1,000 mL with ultra-pure water.
  - Remove impurities and sterilize the buffer with a 0.2 μm bottle top vacuum filter.
  - Store buffer at 4°C until further use.
- Preparation of experimental artificial cerebrospinal fluid (E-aCSF) (1 L)

Reagent	Final concentration (mM)	Amount
NaCl	126 mM	7.36 g
KCl	3 mM	223.65 mg
NaH <sub>2</sub> PO <sub>4</sub>	1.4 mM	167.97 mg
MgSO <sub>4</sub>	0.8 mM	96.288 mg
CaCl <sub>2</sub>	1.4 mM	155.37 mg
NaHCO <sub>3</sub>	26 mM	2.18 g
Glucose	4 mM	720.60 mg
ddH <sub>2</sub> O	n/a	to 1,000 mL
<b>Total</b>	<b>n/a</b>	<b>1,000 mL</b>

- Prepare 500–600 mL of ultra-pure water in a beaker with a stir bar.
- Add 7.36 g NaCl (final 126 mM NaCl).
- Add 223.65 mg KCl (final 3 mM KCl).
- Add 167.97 mg NaH<sub>2</sub>PO<sub>4</sub> (final 1.4 mM NaH<sub>2</sub>PO<sub>4</sub>).
- Add 96.288 mg MgSO<sub>4</sub> (final 0.8 mM MgSO<sub>4</sub>).
- Add 155.37 mg CaCl<sub>2</sub> (final 1.4 mM CaCl<sub>2</sub>).
- Add 2.18 g NaHCO<sub>3</sub> (final 26 mM NaHCO<sub>3</sub>).
- Add 720.60 mg glucose (final 4 mM glucose).
- Adjust final pH to 7.4.
- Bring solution up to a final volume of 1,000 mL with ultra-pure water.
- Remove impurities and sterilize the buffer with a 0.2 μm bottle top vacuum filter.
- Store buffer at 4°C until further use.

△ **CRITICAL:** aCSF buffers may be reliably stored at 4°C for up to 2 weeks. If the solution becomes opaque or forms precipitates, discard the solution and prepare a fresh batch.

△ **CRITICAL:** It is important to ensure the osmolarity of the aCSF does not deviate significantly across experiments, since slices may respond differently in different osmotic environment. aCSF should be approximately 310 mOsm at a pH 7.4 when bubbled with O<sub>2</sub>.

Slices from older animals may be more sensitive to the osmolarity of the aCSF (Moyer and Brown, 1998).

### Preparation of lysis buffer

⌚ Timing: 15–20 min

- Preparation of lysis buffer (1 L)

Reagent	Final concentration	Amount
Tris-HCl pH 7.4	50 mM	7.58 g
NaCl	150 mM	8.77 g
SDS	0.1%	1 g
Sodium deoxycholate	0.5%	5 g
Triton X-100 (100%)	1%	10 mL
ddH <sub>2</sub> O	n/a	to 1,000 mL
<b>Total</b>	<b>n/a</b>	<b>1,000 mL</b>

- Prepare glass flask with 100 mL of ultra-pure water.
- Add 7.58 g Tris-HCl pH 7.4 pre-set crystals (final 50 mM Tris-HCl).
- Add 8.77 g NaCl (final 150 mM NaCl).
- Add 1 g SDS (final 0.1% SDS).
- Add 5 g sodium deoxycholate (final 0.5% sodium deoxycholate).
- Add 10 mL Triton X-100 (final 1% Triton X-100).
- Adjust final pH to 7.4.
- Bring solution up to a final volume of 1,000 mL with ultra-pure water.
- Remove impurities and sterilize the buffer with a 0.2 μm bottle top vacuum filter.
- Store buffer at 4°C until further use. Buffer can be stored for 2 months.
- Just prior to use, supplement with fresh protease inhibitors.

### Preparation of phosphate buffer

⌚ Timing: 15–20 min

- Preparation of phosphate buffer (1 L)

Reagent	Final concentration	Amount
Na <sub>2</sub> HPO <sub>4</sub>	84 mM	95.44 g
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	16 mM	17.64 g
ddH <sub>2</sub> O	n/a	to 1,000 mL
<b>Total</b>	<b>n/a</b>	<b>1,000 mL</b>

- Prepare glass flask with 100 mL of ultra-pure water.
- Add 95.44 g Na<sub>2</sub>HPO<sub>4</sub> (final 84 mM Na<sub>2</sub>HPO<sub>4</sub>).
- Add 17.64 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O (final 16 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O).
- Bring solution up to a final volume of 1,000 mL with ultra-pure water.
- Adjust final pH to approximately 7.4–7.6.
- Remove impurities and sterilize the buffer with a 0.2 μm bottle top vacuum filter.
- Store buffer at 4°C until further use. Buffer can be stored for 2 months.

### Preparation of high-pressure liquid chromatography (HPLC) mobile phases

⌚ Timing: 15–30 min

- Preparation of solvent A (1 L)

Reagent	Final concentration (%)	Amount
Acetonitrile	10%	100 mL
Trifluoroacetic acid	0.1%	1 mL
ddH <sub>2</sub> O	90%	899 mL
<b>Total</b>	<b>n/a</b>	<b>1,000 mL</b>

- Prepare glass flask with 100 mL of ultra-pure water.
- Add 100 mL of acetonitrile (final 10% acetonitrile).
- Add 1 mL trifluoroacetic acid (final 0.1% trifluoroacetic acid).
- Bring solution up to a final volume of 1,000 mL with ultra-pure water.
- Store solvent A at 20°C–25°C. Solvent can be stored for 2 months.

- Preparation of solvent B (1 L)

Reagent	Final concentration (%)	Amount
Acetonitrile	70%	700 mL
Trifluoroacetic acid	0.1%	1 mL
ddH <sub>2</sub> O	30%	299 mL
<b>Total</b>	<b>n/a</b>	<b>1,000 mL</b>

- Prepare glass flask with 100 mL of ultra-pure water.
- Add 700 mL of acetonitrile (final 70% acetonitrile).
- Add 1 mL trifluoroacetic acid (final 0.1% acetonitrile).
- Bring solution up to a final volume of 1,000 mL with ultra-pure water.
- Store solvent B at 20°C–25°C. Solvent can be stored for 2 months.

- Degas solvents A and B

- Transfer solvents A and B to vacuum flasks.
- Seal the top of the flask with rubber stopper .
- Attach one end of rubber tubing to the outlet of the vacuum flask and the other end to a vacuum source.
- Turn the vacuum on low to degas the solvents.



**Figure 2. Vibratome for sectioning brains**

- Intermittently stir and agitate the solvents gently to release the dissolved gases.
- After no further bubbling occurs, gently break the vacuum seal between atmosphere and flask by removing vacuum hose or rubber stopper.
- After the flask is no longer connected to the vacuum source, turn off the vacuum.

### Preparation of high-pressure liquid chromatography (HPLC) standards

⌚ Timing: 15–30 min

- Preparation of HPLC standards: dihydroethidium (DHE), 2-hydroxyethidium (2-OH-E<sup>+</sup>), and ethidium (E<sup>+</sup>)
  - Prepare standards the day of the experiment, preferably just before loading the experimental samples onto the HPLC columns.
  - Prepare a 10 mM DHE stock solution by dissolving 3.154 mg in 1 mL of 100% DMSO under anaerobic conditions.
  - Prepare a 1 mL standard solution of 25 μM DHE by diluting the DHE stock in phosphate buffer.
  - Prepare a 500 μL standard solution of 2-OH-E<sup>+</sup> by adding 50 μL of a superoxide (O<sub>2</sub><sup>•-</sup>) solution to 450 μL of the newly prepared 25 μM DHE standard.

**Note:** Prepare O<sub>2</sub><sup>•-</sup> solution by dissolving 1 mg of KO<sub>2</sub> in 1 mL of DMSO and vortex vigorously for 10 min.

- E<sup>+</sup> standards are readily obtained from commercial sources. Dilute the E<sup>+</sup> stock to 25 μM in phosphate buffer to use as a standard.

**Alternatives:** 2-OH-E<sup>+</sup> standards can also be prepared by reacting DHE with Fremy's salt (Zielonka et al., 2005). To use Fremy's salt, prepare a 100 mM stock solution by dissolving 26.833 mg Fremy's salt in 1 mL of phosphate buffer containing 0.2 mM diethylenetriamine pentaacetic acid (DTPA). Prepare a 500 μL standard solution of 2-OH-E<sup>+</sup> by adding 250 μL Fremy's salt solution to 250 μL of a 50 μM DHE standard. Fremy's salt is unstable in acidic solutions and should be prepared at a pH of 7.5 or greater.

## STEP-BY-STEP METHOD DETAILS

### Dissecting and sectioning mouse brain

⌚ Timing: 15 min to 1 h

The mouse brain is isolated and mounted for sectioning on a vibratome. Sections are then transferred to an incubation chamber in which the slices equilibrate before experimental manipulation.

1. Euthanize mice by cervical dislocation.
  - a. Anesthetize mouse with brief inhalation exposure to isoflurane.
  - b. Restrain the mouse on a firm, flat surface. While grasping the base of the tail with one hand, place a metal rod or the closed end of a pair of scissors against the back of the neck at the base of the skull.
  - c. To dislocate the spinal column from the skull or brain, quickly push the rod or scissors at the neck forward and downwards while pulling backward with the hand holding the tail.
  - d. To verify euthanasia, confirm both respiratory and cardiac arrest by observation and/or palpation.
2. Extract the brain from the skull.
  - a. Cut the head from the rest of the body.
  - b. Cut the skin off the skull with scissors in a posterior to anterior direction.
  - c. Using fine scissors, cut the skull first in a posterior to anterior direction on the superior surface and then on the lateral surfaces without damaging the underlying tissue.

- d. Using a scapula, extract the brain from the skull and into ice-cold S-aCSF.
3. Mount the brain onto the vibratome platform.
  - a. Trim the brain using a razor blade to generate a flat surface with which to mount the brain to the vibratome specimen disc.
    - i. For coronal sections, cut off the cerebellum and then mount the brain from the new flat, posterior surface.
    - ii. For sagittal sections, trim the lateral surface of one hemisphere just enough to generate a large enough flat surface to mount the brain.
  - b. Mount the brain onto the vibratome platform by applying superglue directly to the flat surface of the brain.

**Optional:** mount a block of supporting agar onto the vibratome specimen disc behind the brain to provide structural support while slicing.

4. Once the superglue has dried, place the specimen disk in the vibratome tissue partition.
5. Fill the vibratome tissue partition with ice-cold S-aCSF pre-equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The fluid level should be high enough to submerge the entire brain specimen.
6. Position the blade at a 15° to 20° angle from parallel.
  - a. This may be adjusted as needed to improve section quality.
7. Begin sectioning the brain at 300 μm intervals.
8. Using a transfer pipette or thin paint brush, transfer each brain slice from the vibratome tissue partition to the holding chamber with ice-cold S-aCSF maintained with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.
9. Continue sectioning as needed or until region of interest is fully sectioned.
10. Repeat steps 1–9 for each mouse as needed.
11. Using a transfer pipette or thin paint brush, transfer the brains slices from the holding chamber to the equilibration chamber for 1 h to allow the tissue to recover. The equilibration chamber should be filled with E-aCSF maintained with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 20°C–25°C.

**Note:** Cervical dislocation is a rapid means of euthanizing mice that does not require special equipment and minimizes potential neurochemical changes that result from injectable chemical euthanasia agents such as barbiturates. However, many institutional Animal Care and Use Committees require that cervical euthanasia only be performed on mice and small rats. Cervical dislocation should also be used secondarily after appropriate anesthesia such as with isoflurane. Cervical dislocation in unanesthetized animals may be permitted if there is an approved scientific justification.

**Note:** Mice are not typically perfused before sectioning in order to minimize the time between euthanasia and sectioning. However, perfusing the brain may be important depending on the experimental question and may outweigh the need for rapidly sectioning the brain. The effects of perfusing mice before sectioning should be evaluated empirically.

**Note:** As noted in step 3 above, this protocol be used to measure region-specific redox signaling by sectioning only the region of interest. We did not specifically evaluate differences in ROS between coronal and sagittal sections.

**Note:** Keep slices from the same mouse together as much as possible in order to keep track of biological replicates.

**△ CRITICAL:** It is important to keep the tissue cold while sectioning to minimize injury and/or degradation. Sections should ideally be cut and isolated within 5 min. Work as quickly as possible to prevent degradation. Please refer to [Troubleshooting 1](#) for solutions to maintain cellular integrity.

△ **CRITICAL:** Difficulties sectioning may indicate issues with the vibratome settings. If the vibratome is alternating between skipped sections and inappropriately thick sections, the blade angle may be too low. If the tissue is tearing while sectioning, please refer to [Troubleshooting 2](#) for potential solutions.

### Ex vivo stimulation

⌚ **Timing:** 30 min to 1 h

Slices are loaded with dihydroethidium (DHE) to capture ROS, which convert DHE to 2-hydroxyethidium (2-OH-E<sup>+</sup>) and ethidium (E<sup>+</sup>). Slices are then treated or stimulated depending on the experimental goals. Here, slices are treated with N-methyl-D-aspartic acid (NMDA) and glycine, which together activate the ionotropic glutamatergic NMDA receptor. When activated, NMDA receptors stimulate NADPH oxidase to generate superoxide (O<sub>2</sub><sup>•-</sup>) ([Brennan et al., 2009](#); [Lafon-Cazal et al., 1993](#)). O<sub>2</sub><sup>•-</sup> functions as a second messenger in long-term potentiation and other redox signaling axes ([Gao et al., 2007](#); [Heusler and Boehmer, 2004](#); [Klann, 1998](#); [Wang et al., 1996](#)).

12. Transfer slices with a pipette or brush to a chamber at 20°C–25°C with E-aCSF continuously bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> supplemented with 25 μM dihydroethidium (DHE).
13. Cover chambers with foil or maintain in dim light to minimize photooxidation of DHE.
14. Load slices with DHE for 30 min.
15. Briefly rinse slices by transferring to chamber at 20°C–25°C with fresh E-aCSF without DHE.
16. Rinse slices 1–2 more times as in step 15.
17. Transfer slices into separate experimental chambers. This will be unique to your experimental conditions. Chambers are continuously bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 20°C–25°C. Maintain chambers in dim light to minimize photooxidation of DHE.
  - a. A chamber with E-aCSF supplemented with vehicle.
  - b. A chamber with E-aCSF supplemented with 100 μM NMDA + 10 μM glycine.
18. Treat slices for 30 min.

**Optional:** As a negative control, treat slices with the NMDA receptor antagonist (2R)-amino-5-phosphonovaleric acid (APV) (100 μM) along with 100 μM NMDA + 10 μM glycine. Alternatively, treat slices with the spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO) (100 μM) along with 100 μM NMDA + 10 μM glycine. DMPO serves as a negative control by scavenging O<sub>2</sub><sup>•-</sup>, preventing it from reacting with DHE.

**Note:** The timing of when to load DHE can vary depending on the scientific question or experimental goals. DHE loaded before the experimental condition will result in E<sup>+</sup> or 2-OH-E<sup>+</sup> being generated during the treatment. For example, DHE loaded before NMDA treatment will react with ROS during stimulation to form E<sup>+</sup> or 2-OH-E<sup>+</sup>. If DHE is loaded before applying an oxidant rather than a stimulus, the levels of E<sup>+</sup> and 2-OH-E<sup>+</sup> are more likely to reflect the levels of the oxidant than the cellular response to it. In contrast, loading DHE after the experimental condition instead, the levels of E<sup>+</sup> and 2-OH-E<sup>+</sup> reflect ROS that remain after the treatment and may more accurately reflect the cellular response to the oxidant.

**Alternatives:** It may be valuable to monitor whether the levels of E<sup>+</sup> or 2-OH-E<sup>+</sup> decrease, rather than increase, with certain stimuli or treatments. For example, antioxidants may prevent or decrease the quantities of E<sup>+</sup> or 2-OH-E<sup>+</sup> measured.

△ **CRITICAL:** Treatments must be performed under dim light to minimize photooxidation of DHE ([Zielonka et al., 2006](#)).

△ **CRITICAL:** The chamber should not be so crowded with slices such that they begin to overlap one another. Slices should remain free-floating and non-overlapping in order to allow for adequate oxygenation of each slice.

### Extracting 2-OH-E<sup>+</sup> and E<sup>+</sup>

⌚ **Timing:** 1–2 h

DHE and its redox products 2-OH-E<sup>+</sup>, and E<sup>+</sup> are isolated from brain slices by liquid-liquid organic-aqueous extraction.

19. Transfer individual brain slices from experimental chambers into clean 1.6 mL microcentrifuge tubes with a brush or transfer pipette. Aspirate off any residual aCSF that follows the slices with a pipette. If evaluating multiple mice as biological replicates, pool slices from the same mouse to process them together.
20. Add 100  $\mu$ L of pre-chilled Lysis Buffer per pool of slices in the microcentrifuge tubes. Gently triturate the slices with a pipette in lysis buffer to facilitate lysis and extraction. Take care not to introduce bubbles or air.
21. Set aside 5  $\mu$ L of lysate from each sample in a clean 1.6 mL microcentrifuge tubes to quantify protein.
  - a. Measure protein by the bicinchoninic acid method (BCA) as per the manufacturer's instructions outlined here: <https://www.thermofisher.com/order/catalog/product/23225#/23225>.
22. Mix lysate with 1 volume equivalent of 1-butanol.
23. Gently invert the lysate/butanol mixture for 1 min to mix the solvents.
24. Centrifuge lysate/butanol mixture at 16,000  $\times$  g for 15 min at 4°C.
25. The aqueous and organic layers will separate after centrifugation, leaving the organic layer above the aqueous. Carefully collect the organic layer with a pipette and transfer to a clean 1.6 mL microcentrifuge tubes.
26. Concentrate the organic fraction by evaporation under nitrogen gas in a fume hood.
  - a. Connect a nitrogen tank to tubing with one end attached to the gas outlet and the other to a syringe.
  - b. Nitrogen will flow out of the syringe tip. Adjust the flow by testing the strength of the stream by inserting the syringe into a 1.6 mL microcentrifuge tube filled with 200  $\mu$ L of water. The gas should be flowing in the headspace above the solution but not bubbling through it. An excessively strong stream will push water out of the tube, whereas an inappropriately weak stream will not perturb the water surface tension.
  - c. After establishing an appropriate flow rate of nitrogen, flow nitrogen through the sample tubes. The flowing nitrogen will evaporate the excess solvent. Allow the nitrogen to flow until there is about 90% volume reduction.
27. Dilute the concentrated organic fraction with 10  $\mu$ L 100% DMSO and then bring up to 200  $\mu$ L in phosphate buffer.

△ **CRITICAL:** As with the treatments, extractions must be performed under dim light to minimize photooxidation of DHE (Zielonka et al., 2006). Do not lyse tissues with ultrasound or sonication, as both techniques induce *in situ* generation of O<sub>2</sub><sup>•-</sup> that can react with DHE to form 2-OH-E<sup>+</sup>.

### Detecting neural ROS by high-pressure liquid chromatography (HPLC)

⌚ **Timing:** 1–6+ h

DHE reacts with O<sub>2</sub><sup>•-</sup> to form the fluorescent product 2-OH-E<sup>+</sup>. As rigorously demonstrated through several independent studies, 2-OH-E<sup>+</sup> is the only product formed from the reaction between O<sub>2</sub><sup>•-</sup>

**Table 1. HPLC column equilibration method**

Time point (min)	Flow rate (mL/min)	Solvent A (%)	Solvent B (%)
0	1	100%	0%
10	1	100%	0%
15	1	80%	20%
20	1	100%	0%
30	1	100%	0%

and DHE. Other ROS such as  $\text{H}_2\text{O}_2$ , hydroxyl radical, peroxyxynitrite, and  $\text{HClO}$  do not oxidize DHE to 2-OH- $\text{E}^+$  (Fink et al., 2004; Zhao et al., 2005). Instead, these other oxidants react with DHE to form  $\text{E}^+$ . However, both 2-OH- $\text{E}^+$  and  $\text{E}^+$  exhibit similar fluorescence spectra and thus must be separated by HPLC in order to specifically quantify intracellular  $\text{O}_2^{\cdot-}$  (Zhao et al., 2003). Here, 2-OH- $\text{E}^+$  and  $\text{E}^+$  are resolved by HPLC. Extracted DHE, 2-OH- $\text{E}^+$ , and  $\text{E}^+$  are loaded onto analytical HPLC columns and measured by fluorescence.

28. Prepare HPLC mobile phases if not already done so as outlined in **Preparation of High-Pressure Liquid Chromatography (HPLC) Mobile Phases**.
29. Turn on the HPLC instrumentation and detectors at least 1 h prior to injecting the first samples to allow the instrument to equilibrate.
30. Verify that mobile phase supply lines are submerged in a flask containing 100% methanol.
31. Turn on degasser.
32. Prime the pumps according to the instrument manufacturer's instructions.
  - a. To confirm that no air is retained in the instrument, monitor the fluid entering the waste container. If bubbles persist in the waste line, re-prime the pumps.
33. Initiate slow flow with 100% methanol at 0.1 mL/min.
34. Replace storage column with reverse phase C18 150 × 3.9 mm column.
35. Pause flow and transfer HPLC mobile phase supply tubing into the flasks contained degassed solvents A and B.
36. Reinitiate flow and increase rate to 1 mL/min with 100% solvent A. Monitor for bubbles in the waste line. If bubbles return, re-prime the pumps.
37. Equilibrate the column as per the method parameters outlined in [Table 1](#).
38. Prepare DHE, 2-OH- $\text{E}^+$ , and  $\text{E}^+$  standards if not already done so as outlined in **Preparation of High-Pressure Liquid Chromatography (HPLC) Standards**.
39. Transfer the standards and dissolved sample pellets into HPLC autosampler vials.
40. Place autosampler vials in HPLC instrument.
41. To detect  $\text{E}^+$  and 2-OH- $\text{E}^+$ , set the fluorescence detector with excitation and emission at 510 and 595 nm, respectively.
42. Inject the standards and samples onto the column as per the method parameters outlined in [Table 2](#). In between each sample injection, re-equilibrate the column with the equilibration method outlined in [Table 1](#). It is critical to first evaluate whether the  $\text{E}^+$  and 2-OH- $\text{E}^+$  standards are clearly resolved before injecting the samples onto the column. If the two analytes are not distinguishable from one another, please refer to [Troubleshooting 3](#) for solutions.
43. After the runs have completed, power down the detector as per the manufacturer's recommendations.
44. Pause flow and return the mobile phase lines to flasks containing 100% methanol. Manually change the run parameters to the new mobile phase at 1 mL/min for 20 min to wash the column.
45. At the end of the column wash, slow flow to 0.1 mL/min with 100% solvent A.
46. Replace C18 column with storage column.
47. Return flow to 1 mL/min.
48. Wash storage column with 5 column volumes of 100% methanol.
49. Turn off the flow and degasser.

**Note:** The columns and buffers are all at 20°C–25°C while in use.

△ **CRITICAL:** Be sure to have enough of solvents A and B for all the runs needed. If the mobile phases run out, the instrument may inject air into the system, potentially damaging the column and the detector.

## EXPECTED OUTCOMES

When evaluating the analyte standards, DHE should remain undetected by HPLC whereas the  $E^+$  and 2-OH- $E^+$  standards should exhibit differential retention on a reverse phase column (Figures 3A–3D).  $E^+$  elutes off the column earlier than 2-OH- $E^+$ . 2-OH- $E^+$  in Figure 3B is the result of reacting DHE with  $KO_2$  directly as described in Preparation of High-Pressure Liquid Chromatography (HPLC) Standards. The absolute retention time will vary depending on the length and diameter of the analytical column, as well as the precise composition of the mobile phases.

Measuring DHE fluorescence alone is insufficient to evaluate NMDA redox signaling since raw fluorescence would not disambiguate between  $E^+$  and 2-OH- $E^+$ . The present methodology outlines an approach to quantitatively evaluate NMDA redox signaling by resolving and measuring  $E^+$  and 2-OH- $E^+$  by HPLC. Treating *ex vivo* brain slices from wild-type (WT) brain slices with NMDA should lead to significant 2-OH- $E^+$  formation from baseline since NMDA receptors signal in part through  $O_2^{\cdot-}$  (Figures 4A and 4B). If comparing slices from WT mice with mice lacking the enzyme biliverdin reductase ( $BVR^{-/-}$ ),  $BVR^{-/-}$  slices should accumulate more 2-OH- $E^+$  than WT slices (Figures 4A and 4B). Neural bilirubin scavenges NMDAR-associated  $O_2^{\cdot-}$  (Vasavda et al., 2019), and losing BVR disrupts NMDA receptor-dependent synaptic signaling (Vasavda et al., 2020).  $O_2^{\cdot-}$  can also react with other biomolecules or be converted to  $H_2O_2$  by superoxide dismutase, and these alternative ROS may react with DHE to produce  $E^+$ . As a result,  $E^+$  also increases in both WT and  $BVR^{-/-}$  slices after NMDA treatment, but to a greater extent in  $BVR^{-/-}$  slices. The greater increase in  $E^+$  in  $BVR^{-/-}$  slices could also be from reactions of intermediate DHE/ $O_2^{\cdot-}$  oxidation with other ROS that then also lead to  $E^+$ .

This protocol can be readily adapted to evaluate ROS signaling in neurodegenerative diseases or pathologies such as stroke. In several mouse models of Huntington's disease for example, loss of the enzyme cystathionine  $\gamma$ -lyase (CSE) mediates neurodegeneration (Paul et al., 2014). However, CSE synthesizes both cysteine and the gasotransmitter hydrogen sulfide ( $H_2S$ ), both of which exhibit antioxidant activity. The relative contributions of cysteine and  $H_2S$  to redox balance and neurodegeneration remains unclear, but could be further explored by monitoring how  $E^+$  and 2-OH- $E^+$  change in response to rescuing brains from CSE null mice with cysteine or  $H_2S$ .

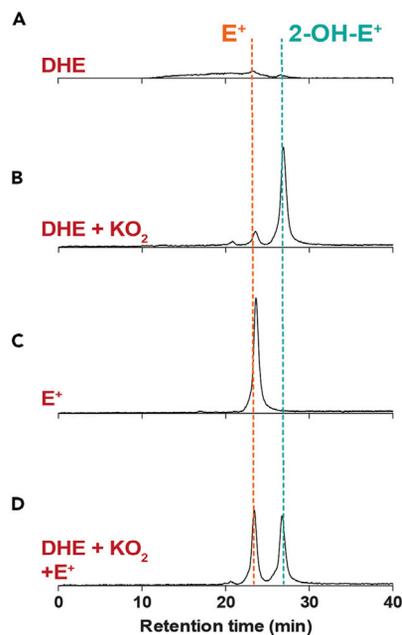
## QUANTIFICATION AND STATISTICAL ANALYSIS

To quantify relative levels of  $E^+$  and 2-OH- $E^+$  between samples, integrate the area under the curves for both  $E^+$  and 2-OH- $E^+$  using the software supplied with the HPLC instrument. If you prefer to quantify the absolute levels of  $E^+$  and 2-OH- $E^+$ , construct a standard curve by injecting a series of standards of each analyte at different concentrations, after which the concentrations in the samples can be interpolated. Normalize the quantities of  $E^+$  and 2-OH- $E^+$  per sample to the total protein of that sample.

Statistical analyses between group data should be performed using two-tailed unpaired Student's *t*-tests or ANOVA analyses, as appropriate. Differences can be considered significant at  $P < 0.05$ . Experiments should be performed concomitantly or in a blocked manner with consideration for both genotype and treatment.

## LIMITATIONS

Though the methodology outlined here may be applied to quantify ROS in response to diverse neural stimuli (Sbodio et al., 2018), it may be more amenable to certain experimental conditions than others. This workflow is best suited for acute paradigms over longer ones since *ex vivo* slices lose their reliability and viability after several hours. As a result, it may be difficult to evaluate the effects



**Figure 3. DHE,  $E^+$ , and 2-OH- $E^+$  standards by high-pressure liquid chromatography (HPLC)**

(A–D) Representative HPLC chromatograms of (A) DHE alone, (B) DHE +  $KO_2$ , (C) authentic ethidium ( $E^+$ ), and (D) DHE +  $KO_2$  spiked with authentic  $E^+$ . Figure reprinted with permission from Vasavda et al. (2019).

of chronic stimuli or treatments. Anecdotally, we have noticed that treatments that extend beyond 4 h are less consistent across technical replicates.

This methodology also outlines an approach to quantify two oxidation products of DHE,  $E^+$  and 2-OH- $E^+$ . 2-OH- $E^+$  is a unique DHE derivative that results from reacting with  $O_2^{\cdot-}$ . Unlike 2-OH- $E^+$  however, DHE reacts with several different physiologic oxidants to produce  $E^+$ , including hydrogen peroxide, hydroxyl radicals, and peroxynitrite. Thus, whereas 2-OH- $E^+$  can be monitored to quantify and track  $O_2^{\cdot-}$ , an increase in  $E^+$  may be the result of several different ROS and does not immediately point to the underlying molecule responsible. By extension, this protocol may fail to identify changes to redox signaling if the ROS does not react with DHE to form either  $E^+$  or 2-OH- $E^+$ . A comprehensive study of redox signaling in the brain should thus include additional diverse experimental approaches and could include methods involving cytochrome c electrodes (Fabian et al., 1994) or electron paramagnetic resonance spin trapping (Liu et al., 2004). Individual spin traps such as  $\alpha$ -phenyl-N-tert-butyl nitron, 5,5-dimethyl-1-pyrroline N-oxide, or 2-methyl-2-nitrosopropane exhibit selectivity toward particular ROS that DHE does not, permitting the measurement of more specific radicals.

## TROUBLESHOOTING

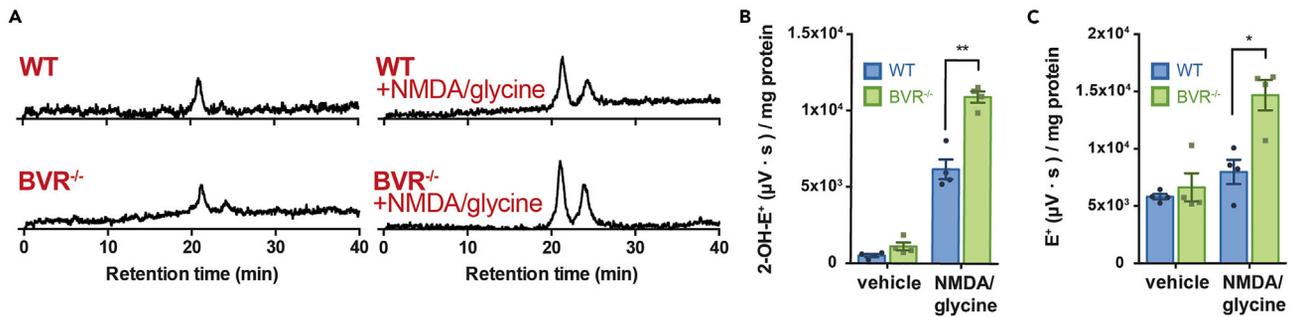
### Problem 1

No NMDA-dependent increase in  $E^+$  or 2-OH- $E^+$  (Quantification and statistical analysis).

### Potential solution

If stimulating the NMDA receptor does not result in an appreciable increase in  $E^+$  or 2-OH- $E^+$  despite loading adequate amounts of DHE, normal NMDA redox signaling may somehow be disrupted or blocked. This may occur due to several reasons, but may commonly be due to tissue degradation over the course of the experiment. Several steps can be taken to help maximize tissue integrity through the experiment:

While sectioning, continually top up the tissue partition with cold S-aCSF to keep the brain tissue cool.



**Figure 4. NMDA receptors signal via  $O_2^{\bullet-}$ , and NMDA receptor activity leads to more ROS in  $BVR^{-/-}$  mice**  
 (A) Representative normalized HPLC chromatograms of organic extracts from WT and  $BVR^{-/-}$  ex vivo brain slices loaded with DHE at baseline or after treatment with 100  $\mu$ M NMDA + 10  $\mu$ M glycine for 30 min.  
 (B and C) Relative quantification of  $E^+$  and 2-OH- $E^+$  extracted from WT and  $BVR^{-/-}$  brain slices by HPLC at baseline or after treatment with NMDA/ glycine. Points represent average of slices from individual mice. Mean  $\pm$  SEM depicted. Points represent normalized average of 2–3 slices from individual mice. \* $p < 0.05$  and \*\* $p < 0.01$  by two-way ANOVA followed by a post hoc Tukey test. Figure reprinted with permission from Vasavda et al. (2019).

Monitor the pH of the aCSF solutions to verify that bubbling with 95%  $O_2$  and 5%  $CO_2$  maintains the pH at around 7.4.

Work as quickly as possible to prevent degradation.

Consider adding N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) to osmotically balanced S-aCSF. HEPES has been reported to prevent edema while slicing (MacGregor et al., 2001). Ascorbate has similarly been reported to prevent edema in brain slices (Brahma et al., 2000), though ascorbate is an antioxidant and may confound ROS measurements.

Replacing NaCl in S-aCSF with equi-osmotic glycerol may prevent the possible neurotoxic effects of passive chloride entry during slicing, which can lead to vacuolization and lysis (Ye et al., 2006).

Alternatively, it may appear that there is no increase in either  $E^+$  or 2-OH- $E^+$  because the concentrations were below the limit of detection by the detector. To increase the quantity of  $E^+$  or 2-OH- $E^+$  injected over the column, dilute the products in less phosphate buffer after evaporating off the butanol. To increase the absolute quantity of  $E^+$  or 2-OH- $E^+$  in each sample, section more slices per mouse when evaluating biological replicates in order to pool together more tissue for analysis.

## Problem 2

Poor quality vibratome sections (Dissecting and Sectioning Mouse Brain).

### Potential solution

Fresh tissues are more difficult to section than when fixed. However, consistent difficulties with sectioning may indicate issues with the vibratome settings. If the vibratome is alternating between skipped sections and inappropriately thick sections, the blade angle may be too low. If the tissue is tearing while sectioning, the vibratome may be progressing too quickly or may not be vibrating at a high enough frequency. Alternatively, the blade angle may be too high. Loose knives and clamps or poorly attached tissue may also lead to poor quality sections. Adhering an agar tissue support block may help improve sections if adjusting any of these other parameters does not lead to more consistent sections.

## Problem 3

Poor separation of  $E^+$  from 2-OH- $E^+$  standards by HPLC (Detecting Neural ROS by High-Pressure Liquid Chromatography (HPLC)).

**Table 2. HPLC E<sup>+</sup> and 2-OH-E<sup>+</sup> separation method**

Time point (min)	Flow rate (mL/min)	Solvent A (%)	Solvent B (%)
0	1	100%	0%
40	1	80%	20%

### Potential solution

If the E<sup>+</sup> standard cannot be resolved from the 2-OH-E<sup>+</sup> standard with the method outlined in Table 2, the gradient may need to be altered empirically to suit the column and instrument being used. First evaluate whether isocratic elution at either 100% solvent A or 100% solvent B are able to separate the two analytes. If neither method is successful, evaluate whether widening the gradient between A and B from the original method improves or worsens the separation in a stepwise approach. If the separation improves, consider increasing the gradient further. If the resolution worsens, narrow the gradient.

### Problem 4

Chromatogram background fluorescence is too high relative to the signal from E<sup>+</sup> and 2-OH-E<sup>+</sup> (Detecting Neural ROS by High-Pressure Liquid Chromatography (HPLC)).

### Potential solution

If the baseline fluorescence is too high relative to the signal from E<sup>+</sup> and 2-OH-E<sup>+</sup>, it may be helpful to increase the absolute quantity of E<sup>+</sup> or 2-OH-E<sup>+</sup> in each sample. Section more slices per mouse and pool them together when extracting E<sup>+</sup> or 2-OH-E<sup>+</sup>. This will result in a greater concentration of E<sup>+</sup> or 2-OH-E<sup>+</sup> as it runs through the HPLC detector, improving the signal to noise ratio.

### Problem 5

High back pressure during HPLC (Detecting Neural ROS by High-Pressure Liquid Chromatography (HPLC)).

### Potential solution

Using an HPLC instrument under high pressure can deteriorate and overload the equipment. As a result, many instruments have automatic safeguards that stop runs when the pressure reaches above certain thresholds. High back pressure can cause retention times to fluctuate significantly between samples or can disrupt the flow of the sample through the column, potentially widening the analyte peak as it elutes less tightly off the column. This makes the inter-sample runs much more variable.

Back pressure can increase when debris clogs a column or from mobile phases that are incompatible with the stationary phase. Air bubbles can also result in back pressure.

To minimize back pressure, it is important to monitor column back pressure regularly while the samples are running through the instrument. Additionally, minimize the risk of introducing air by thoroughly degassing solvents A and B. Lastly, take care not to carry over the aqueous phase with the organic phase when isolating in E<sup>+</sup> or 2-OH-E<sup>+</sup>. Proteins or lipids from the aqueous phase will precipitate when injected over the C18 column, preventing the rest of the sample from flowing.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bindu Paul ([bpaul8@jhmi.edu](mailto:bpaul8@jhmi.edu)).

### Materials availability

All materials used in by this methodology are commercially and publicly available.

### Data and code availability

All data generated or analyzed during this study are included in this article. For complete details on the use and execution of this protocol, please refer to [Vasavda et al. \(2019\)](#).

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### AUTHOR CONTRIBUTIONS

C.V. and B.D.P. conceived the manuscript. C.V., B.D.P., and S.H.S. wrote the manuscript.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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