**Title**
Effects of psilocybin and related compounds on cerebroprotection during ischemic stroke (Stage 1 Registered Report)

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**Abstract**

Stroke remains a leading cause of death and long-term disability and stroke patients have only limited treatment options. Recent preclinical evidence suggests that psychedelics such as psilocybin may confer cerebroprotection by engaging serotonin (5-HT) receptor pathways, yet their cellular targets and translational potential remain poorly understood. Here, we propose to systematically evaluate psilocybin and related tryptamine analogs for cerebroprotective effects in ischemic mouse and human brain tissue. Using an ex vivo oxygen-glucose deprivation (OGD) model combined with automated imaging and single-nucleus RNA sequencing, we will quantify compound-specific protection, identify responsive neuronal and glial subtypes, and map conserved signaling networks in mice and human. This study will dissect the molecular and cellular pathways through which psilocybin and related tryptamines may promote cerebroprotection in stroke. Additionally, these findings may support the development of future psilocybin-related therapies.

**Lay summary:**

In this registered report we plan to test whether psilocybin-like compounds can protect brain cells after stroke. We use living brain slices from mice and from human surgical tissue, place them under low oxygen and glucose to mimic stroke, and then measure how many cells survive after treatment. We then use single nucleus RNA sequencing to determine which cell types respond and which biological pathways are involved.

# 1. Introduction

## 1.1 Research question, background, and relevance

Stroke is a leading cause of disability and death, affecting 1 in 4 people during their lifetime.[1] Although recanalization treatments exist for acute stroke,[2–4] many survivors are left with permanent motor, speech, and/or cognitive deficits that would benefit from new or adjunctive therapies.[5] Only a minority of patients even reach the hospital in time for recanalization. Psychedelics including psilocybin and related tryptamines have shown broad therapeutic potential for the treatment of various neuropsychiatric disorders in mice, rabbits, and humans.[6–8] More recently, animal studies now show significant cerebroprotection with psilocybin via activation of serotonin (5-HT) receptors. In rat models of stroke, a single post-stroke dose reduced infarct volume, lowered neuroinflammation, and restored locomotor function[9]. Yet, the cellular targets of this protection remain unknown, including which neuronal and glial subtypes respond most strongly. It is also unclear whether the same signaling pathways occur in the ischemic human brain or whether structurally related 5-HT-receptor ligands (e.g., natural compounds baeocystin, norbaeocystin, aeruginascin, and norpsilocin, or next-generation analogues like CYB003, PSIL 001, and PSIL 002), can provide similar or superior benefit.

Our groups have extensive preclinical and clinical stroke research experience that spans pharmacological, antibody and cell based therapeutics for stroke.[10–15] We recently built an oxygen glucose deprivation brain slice screening platform that maintains metabolic activity for high content screening, which we can combine with established single-cell RNA sequencing technology[14]. In collaboration with the neurosurgery department, we have access to cortical human tissue. In this proposed study, we will screen and evaluate how psilocybin and related compounds contribute to cerebroprotection in the ischemic mouse brain and translate the most promising candidates in a new ischemic ex vivo human brain slice model (**Fig. 1**).

## 1.2 Hypotheses

We hypothesize that psilocybin and related tryptamine analogs promote cerebroprotection in ischemic brain tissue by activating specific neuronal and glial signaling pathways that support cell survival and repair that will be mapped using agnostic screening. The **primary outcome** will be a Cerebroprotection Index derived from Propidium Iodide and/or Calcein-AM staining, combined with apoptotic/cell death markers such as cleaved-Caspase-3, TUNEL, and pMLKL. **Secondary outcomes** will include changes in neuronal, glial, and inflammatory markers (NeuN, GFAP, Iba1) and identification of activated cell populations using RNA sequencing.

We further hypothesize that some of the effective compounds identified in mouse stroke brain slices will show comparable neuroprotective effects in human brain slices exposed to ischemia. Conserved neuroprotective pathways and responsive cell types will be determined by ortholog mapping and ligand–receptor analysis. Quantitative outcomes will be defined per slice and aggregated by animal or donor using linear mixed-effects models, and molecular outcomes will be based on differentially expressed genes and enriched pathways between treatment and control groups across species.

## 1.3 Timeline

Experiments and data collection for Aim 1 is expected to be completed within the first 9 months, followed by validation in human tissue and transcriptomic analysis during months 10-18. If Stage 1 peer review is successful, we anticipate submitting the Stage 2 report with full results and analyses within 20-24 months of project initiation.

# 2. Methods

## 2.1 Experimental design

This study follows an experimental preclinical framework using ex vivo ischemic mouse and human brain slice models to test pharmacological interventions. It combines quantitative live/dead imaging, immunohistochemistry, and single-nucleus RNA sequencing to determine neuroprotective efficacy and underlying mechanisms. All procedures will be performed at the Keck School of Medicine of the University of Southern California (USC), within the Department of Physiology and Neuroscience, which provides access to state-of-the-art facilities for stroke modeling, brain slice preparation, high-resolution imaging, and transcriptomic profiling. All experimental protocols are well established in our laboratories and supported by institutional infrastructure and expertise. We highlight here core experimental procedures.

## 2.2 Interventions

**Stroke induction:** Transient focal ischemia will be induced using the intraluminal filament model of middle cerebral artery occlusion, which is routinely used in our lab. Male and female mice will be anesthetized with 1–2% isoflurane in oxygen and placed in a supine position with core body temperature maintained at 37°C via a servocontrolled heating pad. Following a ventral neck incision, the common, external, and internal carotid arteries will be exposed. A silicone-coated 6-0 monofilament (Doccol Corp.) will be inserted into the external carotid artery and advanced into the internal carotid to occlude the origin of the MCA. Laser Doppler flowmetry (Moor Instruments MOORLDI2-IR) will be used to confirm a ≥70% reduction in cerebral blood flow (CBF) and guide proper filament placement. MCA occlusion will be maintained for 60 minutes, after which the filament will be withdrawn to allow reperfusion. Mice will be kept on a heated pad during recovery, and receive postoperative analgesia. The procedures are established in our labs as shown previously.[10–15]

**Ex vivo mouse / human brain-slice screen:** Fresh 300 µm cortical slices are generated on a vibratome in chilled, carbogenated NMDG-aCSF following rapid perfusion (mouse stroke) or surgical resection (human). Slices recover 10 min in warmed NMDG-aCSF, then enter a controlled oxygen-glucose deprivation (OGD) chamber where perfusate and gas mixture are switched to OGD-aCSF (in 95 % $N_2$/5 % $CO_2$) to mimic ischemia. Lead psychedelic compounds, prepared at working dilutions immediately before use, are applied during the OGD or early reperfusion phase. After defined OGD (e.g., 60 min) slices are reperfused with normoxic aCSF under carbogen and incubated for 2 h. Viability is quantified by Propidium Iodide versus Calcein-AM (or LDH release). Surviving tissue is fixed (4% PFA), cryoprotected (30% sucrose), sectioned, and immunostained for neuron, astrocyte, microglia, and endothelial markers to map cell-type-specific protection and analysed with established automated pipelines. To identify and classify cell death, we will stain slices additionally with cleaved-Caspase-3 and TUNEL for apoptotic cells, and pMLKL or Fluoro-Jade C to detect necroptotic or degenerating neurons. All buffers are pH-titrated (7.30–7.40), gas flow and temperature are logged, and slice yield targets ≈ 20 high-quality sections per mouse brain, with identical workflows for human tissue.

**Single-nucleus RNA sequencing:** To study molecular changes, cell-cell interactions and identify receptor-ligand interactions, brain tissue will be snap-frozen, nuclei isolated, and processed for single-nucleus RNA-seq using 10x Genomics protocols. Cell hashing will preserve sample identity. Sequencing data will be processed with Cell Ranger v7, and downstream analysis including differential expression, pathway enrichment, and receptor-ligand

mapping will be performed in Seurat v5.0. Ligand-receptor interaction networks will be reconstructed using CellChat and other established pipelines to identify agnostically the key signaling pathways that drive cerebroprotection. The procedures and all downstream analysis are established in our labs as previously described.[13,14,16,17]

**Psilocybin and analogues**: Psilocybin and analogues will be purchased from licensed GMP vendors under USC controlled-substance approvals.

## 2.3 Sample size, randomization and blinding

For slice OGD, we will use adult (3–6-month-old) male and female mice. We will use in total 12 animals, yielding ~12-20 high-quality slices per brain, randomized to vehicle, psilocybin, and selected analogues. We will use intact mouse brain sections and stroke mouse brain sections under OGD. For human tissue, we aim to collect slices from ~3 neurosurgical donors, with 48 viable slices per donor. Sample size is based on prior studies using similar slice-based designs, allowing detection of moderate-high effects. Slice allocation will be randomized within each donor or animal and balanced across treatment conditions. The researchers performing the experiments and analysis will remain blinded to group assignment until quality control and analysis scripts are finalized.

## 2.4 Data collection, processing, and planned analysis

**Neuroprotective Screen of Psilocybin and related compounds in ischemic mouse brain slices (Aim 1)**

Cerebroprotection screen of psilocybin and related compounds in ex vivo mouse stroke

- We will calculate a composite Cerebroprotection Index that we infer from Propidium Iodide/Calcein-AM ratios and cleaved-Caspase-3, TUNEL, and pMLKL staining to rank the ability of each tested psychedelic compound to reduce ischemia-induced cell death. The Index is defined as the total # of PI positive cells, divided by the total # of DAPI positive cells seen in 5 randomly places 40x images from each section. A similar index will be estimated for each of the other cell injury markers. A hierarchy of Indices will be created based on the inverse of the overall variance seen for each marker.
- We will quantify microglial morphology using a ramification index and assess Iba1 signal intensity, measure GFAP signal intensity as a readout of glial scarring, and count NeuN positive nuclei to estimate neuronal survival.

Identify cell populations affected by psilocybin and related compounds and dissect underlying signaling networks using single nucleus RNAseq

- snRNA-seq data will be processed with Cell Ranger v7 and analyzed in Seurat for differential expression, pathway enrichment of individual brain cells
- CellChat ligand-receptor mapping to reveal cell-type-specific signaling cascades that correlate with protection.

**Validation of lead compounds in human ischemic brain slices (Aim 2)**

Validation of cerebroprotection of lead compounds from Aim 1 in human ischemic ex vivo brain tissue

- The same Cerebroprotection Index (see Aim 1) will confirm efficacy of top candidates in resected human cortical slices subjected to OGD.

Identify affected cell populations using single-nucleus RNAseq and cross species comparison

- Human snRNA-seq profiles will be integrated with the mouse dataset via ortholog mapping and label transfer to pinpoint conserved cell populations and pathways, highlighting the most translatable neuroprotective mechanisms.

## 2.5 Statistical methods

All data will be analyzed using linear mixed-effects models (with treatment as a fixed effect and animal or donor as a random intercept) to account for within-subject correlation across slices. Group differences in the Cerebroprotection Index and individual histology readouts will be assessed using two-sided tests, with Holm correction applied for multiple comparisons, where appropriate. Outliers will be defined as values exceeding 3 standard deviations from the group mean. All exploratory analyses, including pathway enrichment and ligand–receptor mapping from single-nucleus RNA-seq data, will be performed in R with validated specialized packages including Seurat v5 or CellChat v3.

## 2.6 Ethics and data management

Ethical approval for the use of human brain tissue and animal procedures has been obtained from the Institutional Review Board (IRB) and the Institutional Animal Care and Use Committee (IACUC) of the University of Southern California prior to the start of data collection. All experiments will be conducted in accordance with institutional and federal guidelines

All generated raw data will be deposited in permanent open-access repositories such as zenodo.org with assigned DOIs to ensure transparency and long-term availability. RNA sequencing data, including raw FASTQ files, count matrices, and metadata, will be deposited in specialized public repositories such as NCBI GEO, in compliance with NIH guidelines.

We commit to provide regular updates on ResearchHub and to uploading a preprint version of these findings to both bioRxiv and ResearchHub as soon as results are available. We will also report and share any negative results, which we believe are equally important for advancing science and improving reproducibility in the field.

## 3. Other required information

### 3.1 Acknowledgements

n/a

### 3.2 Funding

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### 3.3 Author contributions

R.R. conceived and designed the study. P.D.L. contributed to study design, clinical translation strategy, and manuscript preparation. R.R. and P.D.L. wrote the registered report. All authors reviewed and approved the final manuscript.

### 3.4 Ethical Approval

Ethical approval for the use of human brain tissue and animal procedures will be sought from the Institutional Review Board (IRB) and the Institutional Animal Care and Use Committee (IACUC) of the University of Southern California prior to the start of data collection. All experiments will be conducted in accordance with institutional and federal guidelines.

### 3.5 Conflicts of Interest

The authors declare that they have no conflicts of interest.

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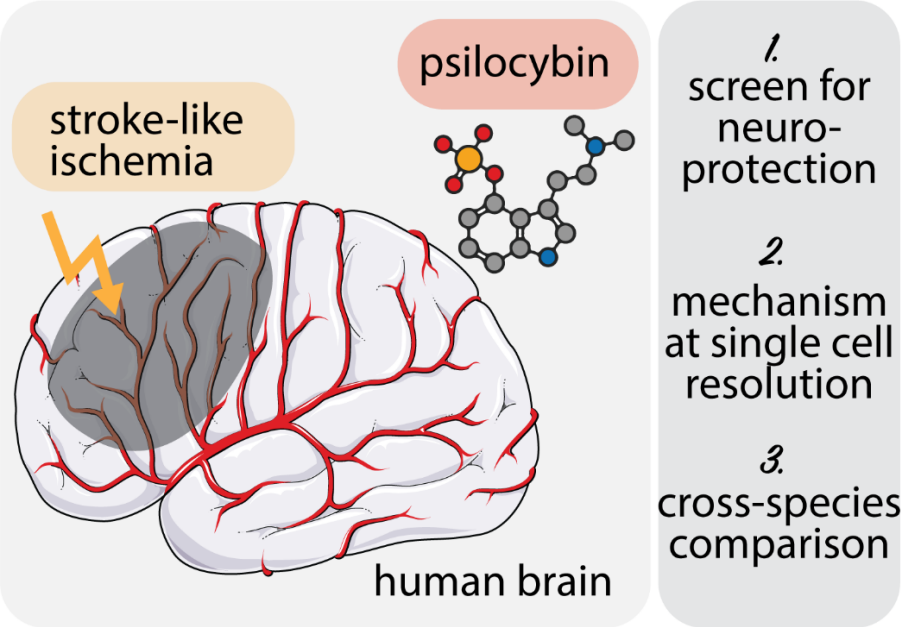


**Fig:1: Graphical abstract for proposed project:** 1) Screen psilocybin and related compounds for cerebroprotection in ischemic mouse brain slices, 2) Identify responsive cell types and signaling networks via single-nucleus RNA sequencing, 3) Validate lead compounds in ex vivo human brain slices and map conserved neuroprotective pathways.