

Pro-homeostatic Astrocyte and Microglial Genes in Ipsilesional Ischemic Core and Penumbra in Rats after Ischemic Stroke: Neuroprotection by NPD1 plus RvD1.

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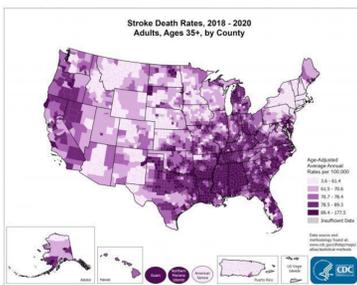
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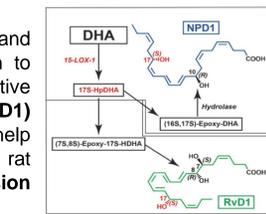
Introduction

Background:

- Stroke is the second leading cause of death worldwide, causing **1 in 4** people over the age of 25 to have a stroke
- Every year, more than **795,000** people in the United States, suffer a stroke
- About **610,000** of these cases are new strokes
- About **185,000** strokes – nearly **1 in 4** – are in people who have had a previous stroke
- In 2021, **1 in 6** deaths from cardiovascular diseases was due to stroke
- Every 40 seconds, someone in the United States has a stroke
- About **87%** of all strokes are ischemic strokes, in which blood flow to the brain is blocked
- Stroke-related costs in the United States came to nearly **\$56.5 billion** between 2018 and 2019
- Stroke is a leading cause of serious long-term disability and reduces mobility in more than **50%** of stroke survivors in **age 65 and older**
- Currently, there is no effective neuroprotective therapy available to mitigate ischemic stroke
- Tissue plasminogen activator (tPA)** is the only FDA approved treatment for stroke
- It has been previously shown that **Omega-3 polyunsaturated fatty acids (PUFAs)** have positively impacted cerebrovascular diseases, including carotid stenosis, vertebral and intracranial stenosis, aneurysms, and vascular malformations

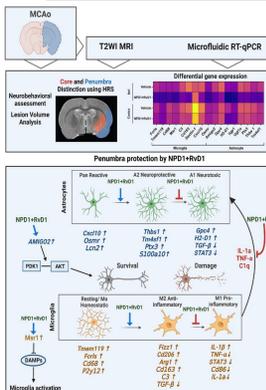


- By limiting inflammatory responses and promoting specific mechanisms to return to homeostasis, certain endogenous protective docosanoids, **Neuroprotection D1 (NPD1)** and **Resolvin D1 (RvD1)**, have proven to help protect against cerebral ischemia in a rat model of middle cerebral occlusion (MCAo) (Reid *et al*, PMID: 37270727)



Objective and Hypothesis:

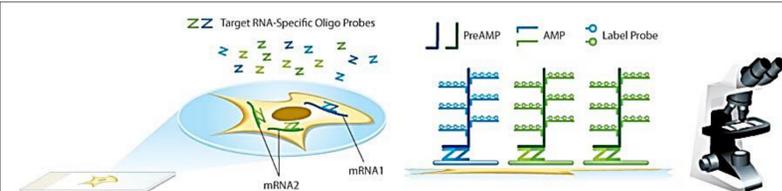
- Recent results from the lab using bulk RNA extraction and high throughput qPCR using Fluidigm Biomark HD system, showed NPD1+RvD1 mediated ischemic stroke penumbra protection increases expression of pro-homeostatic microglia and astrocyte genes
- We hypothesized that we would see similar transcriptomic profiling in ischemic core and penumbra brain regions *in situ* by analyzing through RNA fluorescence *in situ* hybridization (FISH)



Methods – MCAo & Brain Slices

- Ischemic stroke was induced in rats using the transient middle cerebral artery occlusion (MCAo) for 2h, 1h after, rats were re-anesthetized and treated with either vehicle (0.9% saline) or **NPD1 + RvD1 (Dose – 222 µg/kg body weight)**
- 24h later, rat was euthanized, and brain slices were flash-frozen in methyl butane pre-chilled with dry ice
- Brain sections were embedded with an Optimal Cutting Temperature (OCT) medium in a cryomold, and cryo-sectioned into 10-micron slices and placed onto positively charged Superfrostplus slides
- Slides were then fixed with neutral buffered formalin, followed by alcohol dehydration, and subjected to multiple RNAscope probes (for multiplexing), counterstained with DAPI, and coverslip mounted with Prolong Gold antifade mountant
- Slides were imaged and analyzed the data in Olympus Fluoview and CellSens software followed by unbiased image analysis using FIJI ImageJ

Methods – RNA FISH (RNAScope)



1: Tissue section Start with properly prepared tissue sections and pretreat to allow access to target RNA.

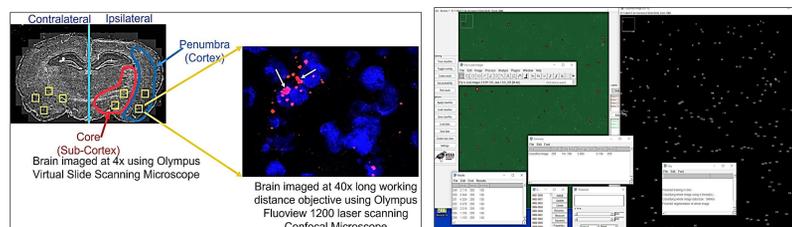
2: Hybridize to target RNA Hybridize multiple sets of gene-specific probe pairs to target mRNAs.

3: Amplify signal Use up to four signal amplification systems to detect multiple target RNAs. Hybridize probes to a cascade of signal amplification molecules, culminating in binding of dye-labeled probes visible in different fluorescent channels.

4: Image Visualize target RNA using a standard fluorescent microscope.

- RNAscope is a multiplex fluorescent *in situ* hybridization (FISH) that allows simultaneous signal amplification and background suppression to achieve exceptional sensitivity allowing for single-molecule visualization of RNA targets while preserving tissue morphology and spatial context
- Target RNAs are hybridized to single stranded DNA “z-probes” composed of a complementary ~20 nucleotide sequence to the RNA of interest, a spacer sequence, and a **14-nucleotide tail region**. **28-nucleotide re-amplifier oligos** bind to the tail region of the z-probe pairs bound to adjacent sequences in the RNA, which are then bound to amplifiers that are labeled with horseradish peroxidase (HRP) enzyme molecules
- Tyramide-conjugated fluorophores** are added, leading to HRP enzymatic conversion of tyramide into a highly oxidized intermediate which covalently binds to proteins near the HRP label, thus depositing a large number of detectable fluorophores – **20 z pairs x 20 Amplifiers x 20 labels = 8000 labelled molecules per 1 kb region of the gene**

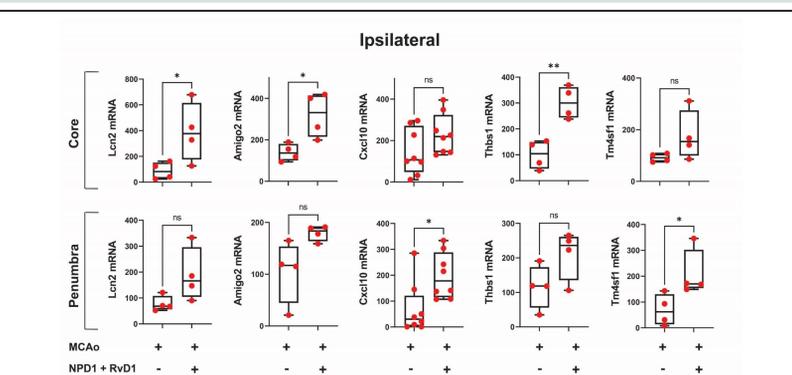
- Multiple RNA targets can be labeled with the use of detection probes in different channels with distinct tail sequences which allow for the generation of unique amplification trees for each target. Each target/probe is then sequentially developed using **tyramide signal amplification (TSA)** by using channel specific HRP labels and conjugated dyes **Vivid 520, 570, and 650 for channels C1, C2, and C3 respectively**. At the end of signal development, sections are counterstained with **DAPI**



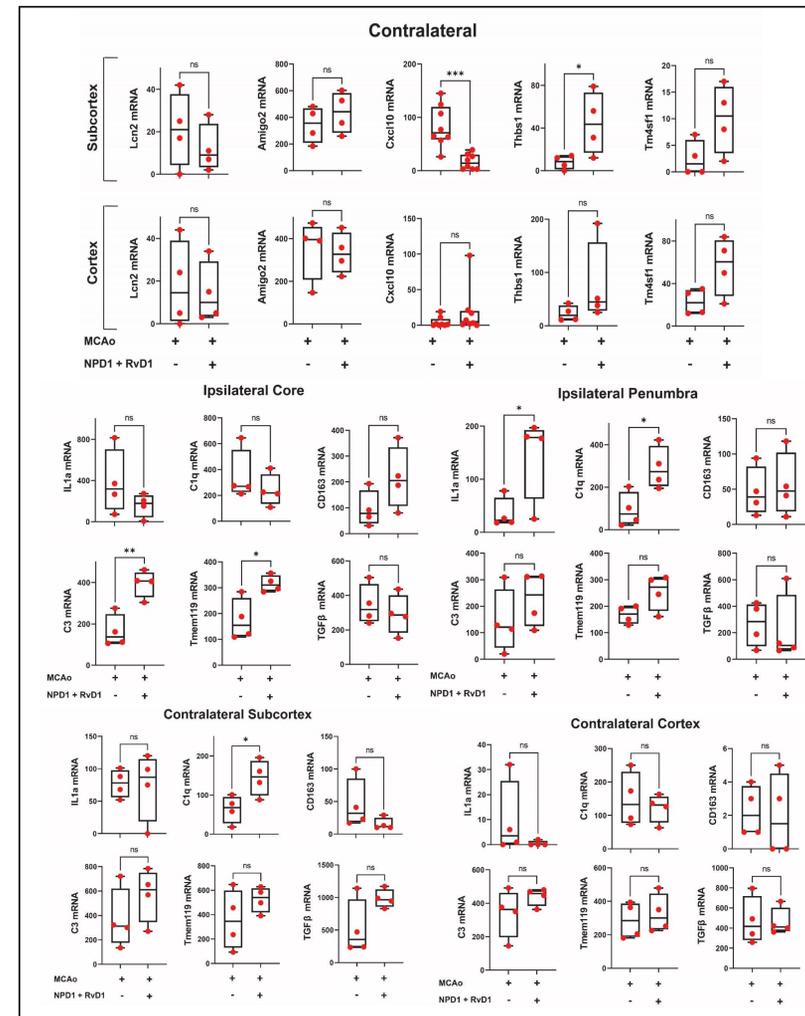
DNA probes used for RNA-FISH experiments

- Lcn2** – lipocalin2 (pan reactive astrocyte gene)
- Amigo2** – adhesion molecule with Ig like domain 2 (A1 neurotoxic gene)
- Cxcl10** – C-X-C motif chemokine ligand 10 (pan reactive astrocyte gene)
- Thbs1** – thrombospondin 1 (A2 neuroprotective astrocyte gene)
- Tm4sf1** – transmembrane 4 L six family member 1 (A2 neuroprotective astrocyte gene)
- IL1a** – interleukin 1 alpha (M1 pro-inflammatory gene)
- C1q** – complement (C1q) (central complement pathway gene)
- C3** – complement (C3) (central complement pathway gene)
- CD163** – CD163 molecule (M2 anti-inflammatory microglia gene)
- Tmem119** – transmembrane protein 119 (resting/ Mo homeostatic microglia gene)
- TGFβ** – transforming growth factor beta (M2 anti-inflammatory microglia gene)

Results



Results



Conclusion

- Lcn2** – lipocalin2 upregulated in ipsilateral core and penumbra
- Amigo2** – upregulated in ipsilateral core and penumbra
- Cxcl10** – upregulated in ipsilateral core and penumbra
- Thbs1** – thrombospondin 1 upregulated in ipsilateral core
- Tm4sf1** – upregulated in ipsilateral core
- IL1a** – interleukin 1 alpha upregulated in ipsilateral penumbra
- C1q** – complement (C1q) upregulated in ipsilateral penumbra
- C3** – complement (C3) upregulated in ipsilateral core
- Most genes are *not significant* in either contralateral subcortex or cortex except **Thbs1** and **C1q**
- Our *in-situ* single molecular RNA FISH results confirm the previously reported results from the lab using bulk RNA extraction followed by high density quantitative PCR after subjecting rats to transient MCAo, followed by treatment with Docosanoids – NPD1 + RvD1
- This is the first time that DNA probes were demonstrated to work for the groups of selected genes using RNA *in situ* hybridization, aiming to understand further the mediative and protective affects NPD1 and RvD1 have on the brain through analysis of neuroprotective and anti-inflammatory gene expressions in the ischemic core and penumbra
- These observations uncover spatially distributed specific genes being expressed during neuroprotection elicited by the lipid mediators and will be important to guide potential therapeutics.

References

- Reid *et al*, PMID: 37270727
- Bhattacharjee *et al*, PMID: 28959727
- Bazan, PMID: 30244005
- Stroke Facts, National Center of Statistics, CDC. <https://www.cdc.gov/stroke/facts.htm>