8-2021

Micrornas As A Therapeutic Target To Regulate Microglial Activation In Post Stroke Isolation In Aged Male Mice

Anik Banerjee

MicroRNAs as a Therapeutic Target to Regulate Microglial Activation in Post-Stroke Isolation in Aged Male Mice

by

Anik Banerjee BS

APPROVED:

Louise McCullough, MD/Ph.D.
Advisory Professor

Sean Marrelli, Ph.D.

Andrey Tsvetkov, Ph.D.

Venugopal Venna, Ph.D.

Digitally signed by Arif Harmanci
DN: cn=Arif Harmanci, o=University of Texas Health Science Center, ou=School of Biomedical Informatics, email=arif.o.harmanci@uth.tmc.edu, c=US
Date: 2021.08.07 21:36:44 -05'00'

Arif Harmanci, Ph.D.

APPROVED:

Dean, The University of Texas
MD Anderson Cancer Center UTHealth Graduate School of Biomedical Science

MicroRNAs as a Therapeutic Target to Regulate Microglial Activation in Post-Stroke Isolation in Aged Male Mice

A

THESIS

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Anik Banerjee BS
Houston, TX

August 2021

MicroRNAs as a Therapeutic Target to Regulate Microglial Activation in Post-Stroke Isolation in Aged Male Mice

Anik Banerjee, BS

Advisory Professor: Louise McCullough, MD/PhD

Abstract

Introduction:

It is increasingly recognized that social Isolation (SI) leads to a wide array of behavioral and cognitive deficits. Isolation and loneliness are linked to all-cause mortality, as well as mortality from stroke and other vascular diseases. In addition, isolated or lonely individuals have significantly poorer cognitive and functional outcomes following stroke and have higher rates of stroke recurrence. These detrimental effects have also been recapitulated in animal models; animals isolated prior to, or at the time of stroke, have larger infarcts than pair-housed mice. However, the mechanisms mediating the effects of social factors on stroke recovery are unknown. An emerging subtype of microglia classified as “disease-associated microglia (DAM)” has been implicated in the progression of neurological diseases such as Alzheimer’s disease (AD) and amyotrophic lateral sclerosis (ALS). MicroRNAs (miRNAs), a class of 18-25 nucleotide non-coding RNAs, have been shown to regulate the activation of microglia from a resting state to “disease-associated microglia (DAM)”. Preliminary work in the laboratory has shown post-stroke SI significantly alters miRNA profiles in the brain. Targeted miRNA-based therapies reduced pro-inflammatory cytokines and restores functional recovery in aged male mice placed in isolation immediately after stroke.

Based on this preliminary data and previous reports, we hypothesize that differential expression of miRNAs may contribute to the deleterious effects of post-stroke SI through effects of accumulating presence of “disease-associated microglia”. The temporal profiles of miRNA changes were determined in mice placed in isolation three days after stroke.

Methods:

Aged (18-20 month) C57BL/6 male mice were used to examine the detrimental effects of post-stroke SI on miRNA profiles in the brain and to evaluate “disease-associated microglia (DAM)” phenotypes. Mice were randomly assigned to either continued pair housing (PH), or single housing (SI) three days after a 60-minute transient right middle cerebral artery occlusion (MCAO). At this time point (3d), the infarct is complete, and equivalent between groups, avoiding potential changes seen with differing infarct sizes. MiRNA profiling of the ipsilateral hemisphere was assessed at three time points (post-stroke SI D1, D4, and D27) using the QIAGEN NGS platform. Activation of “disease-associated microglia (DAM)” was determined by flow cytometry analysis at post-stroke SI D4.

Results:

Post-stroke SI results in a significant alteration of miRNA profiles within the brain across both acute and chronic time points. MiRNA-mRNA interactional analysis reveals miR-466i-3p, miR-10a-5p, and miR-10b-5p as pivotal nodes within the pool of miRNAs that interacted with the largest subset of miRNAs for post-stroke at SI D1, D4, and D27 respectively. Downstream pathway analysis utilizing two independent repositories showed four days of isolation results in microglial

activation and 27 days of isolation leads to long-term depressive phenotypes (FDR adjusted $p<0.05$). Independent validation cohorts demonstrated a significant activation of microglia at post-stroke SI D4 as measured by the expression of P2RY12 ($p<0.05$) on microglia. These results show that post-stroke SI results in microglial activation.

Summary and Conclusions:

In summary, these results support our hypothesis that post-stroke SI results in the activation of microglia that may be regulated by particular classes of miRNAs. Studies manipulating these discovered targets are needed to determine if the detrimental effects of SI can be reversed to enhance post-stroke recovery.

Table of Contents

I.	**Approval Page**	**i**
II.	**Title Page**	**ii**
III.	**Abstract**	**iii**
IV.	**List of Illustrations and Tables**	**vii**
V.	**Introduction**	**1**
VI.	**Methods and Materials**	**4**
	a. Animals	4
	b. Transient Stroke Model	5
	c. Euthanasia	6
	d. Sample Preparation for RNA Isolation and Real-Time Quantitative PCR of miRNA	6
	e. Quality Control, Small RNA library preparation, and Next generation sequencing	7
	f. Prediction and pathways analysis of target mRNAs for differentially expressed miRNAs	8
	g. Flow cytometry (Brain)	8
	h. Statistics	10
VII.	**Results**	**10**
VIII.	**Discussion**	**26**
IX.	**References**	**33**
X.	**Vita**	**41**

List of Illustrations

Figure #:

1. Schematic of experimental design for miRNA identification and analysis 11
2. Bioinformatics analysis of differential expressed miRNAs in post-stroke SI compared to PH conditions across three time points 12
3. miRNA–mRNA interaction networks 15
4. KEGG Pathway analysis of the predicted mRNA gene transcripts targeted by the differentially expressed miRNAs in the ipsilateral hemisphere of post-stroke SI 18
5. Reactome Pathway analysis of the predicted mRNA gene transcripts targeted by the differentially expressed miRNAs in the ipsilateral hemisphere of post-stroke SI 21
6. Validation of miRNA candidates within ipsilateral hemisphere of the brain in post-stroke SI D4 aged male mice 24
7. Post-stroke SI D4 induces microglial activation 25

Introduction

Ischemic stroke is initiated by the abrupt onset of energy depletion, cellular ionic imbalances, and oxidative stress primarily from arterial occlusions within the vasculature of the brain (Musuka et al., 2015). Phenotypic manifestations of cerebral ischemia are clinically observed as focal neurological deficits of variable severity of acute motor, sensory, and cognitive impairment (Samuelsson et al., 2021, Belova et al., 2021). Upon the onset of cerebral ischemia, differential blood supply within the cerebral environment results in an ischemic core and satellite penumbral regions (Yang and Liu, 2021).

The importance of microglia in maintaining homeostasis within the healthy brain and in various age-related neurodegenerative diseases are well documented (Streit et al., 2004, Butovsky and Weiner, 2018). However, studies show conflicting outcomes regarding the contribution of microglial activation in the onset of stroke and its disease progression (Santiago et al., 2017, Lin et al., 2021, Shi et al., 2021). Some reports show the transition of microglia from a resting state to an activated one within the acute phase of ischemic stroke promotes neuronal plasticity by mediating crosstalk and the clearance of damaged neurons (Zhang et al., 2021). Others show microglia activation results in the exacerbation of an inflammatory environment by transcriptional cascades leading to the acute production of detrimental cytokines resulting in behavioral deficits and delayed cognitive recovery in the chronic phase of ischemia (Frick et al., 2013). More recently, comprehensive single cell RNA analysis of central nervous system (CNS) immune cells have unraveled subtypes of microglia with distinct transcriptional and

functional signatures (Keren-Shaul et al., 2017). Disease-associated microglia are classified as immune cells with typical surface expression of ionized calcium binding adaptor molecule 1 (Iba1) and Cystatin C (cst3) (Deczkowska et al., 2018). One of these subtypes, “disease-associated microglia (DAM)” are predominantly present in Alzheimer’s disease (AD) and amyotrophic lateral sclerosis (ALS) may play a central role in the pathogenicity of neurodegenerative diseases (Keren-Shaul et al., 2017).

Disease-associated microglia were initially found in mouse models of AD which expressed five human familial AD mutations (5XFAD) (Ajami et al., 2018) and more recently in aging (Holtman et al., 2015). Accumulating evidence has shown that the presence of “disease-associated microglial (DAM)” in the brain may have mixed outcomes in disease progression and recovery thus the characterization and identification of these cell populations are essential. Many recent studies have suggested that there are robust markers that can identify “disease-associated microglial (DAM)” populations that can be used in parallel with a unique set of transcriptional signatures (Deczkowska et al., 2018). Two of these markers are trans-membrane protein 119 (Tmem119), of unknown function in the CNS, and the well-studied purinergic receptor, P2Y12 (P2RY12) (Bennett et al., 2016). Single cell sequencing studies have shown the downregulation of Tmem119 and P2RY12 expression in models of AD and ALS can identify “disease-associated microglia (DAM)” in the brain (Deczkowska et al., 2018). Investigating these regulators in relation to the modulation of these microglial phenotypes post-ischemic injury could emerge as a new avenue for novel therapeutics.

SI is a risk factor for a wide array of mental and psychological disorders which significantly enhances the chance of developing neurological diseases, especially within the aged population (House et al., 1988, Steptoe et al., 2013). Recent pre-clinical and clinical studies have found that SI not only increases the likelihood of having an ischemic stroke, but also exacerbates post-stroke inflammation and worsens functional recovery (Venna et al., 2012). Social interaction can overcome these detrimental effects of SI by promoting active coping behaviors and initiating anti-inflammatory mechanisms in the brain and systemically (Verma et al., 2014). Work from our laboratory has shown that mice placed into singly housed environments post-stroke have significantly poorer functional recovery, enhanced microglial activation, and significant astrogliosis (Verma et al., 2014). Although SI has been consistently found to influence post-stroke recovery, potential targeted interventions to mitigate these inflammatory events leading to impaired functional and cognitive recovery are extremely limited.

MiRNAs are classified as endogenous noncoding-RNAs around 18–25 nucleotides in length that mediate post-transcriptional gene modification by coordinately binding the 3′-untranslated regions (3′-UTR) (O'Brien et al., 2018). They have been increasingly recognized to be an effective diagnostic marker in stroke (Alhazzani et al., 2021). Multiple studies have shown that expression profiles of circulating miRNAs and miRNAs in the brain are altered through dynamic changes in the social environment that could potentially be harnessed to recapitulate the positive aspects of social interaction (Banerjee et al., 2021). Moreover, miRNAs have been shown to regulate microglial activation through

targeting genes involved in inflammatory signaling cascades, cytokine production, and transcriptional networks that determine microglia polarization (Verma et al., 2018). Our laboratory has identified classes of miRNAs that regulate key processes in the onset of stroke, which exacerbate inflammation induced through the production of deleterious pro-inflammatory cytokines (Verma et al., 2018). In addition, more recently, post-stroke mice housed in isolated environments have altered microglial activation states acutely, which may contribute to the production and the increased abundance of pro-inflammatory cytokines in the brain. This led us to hypothesize that the differential expression of miRNAs may play a deleterious role in post-stroke isolation via microglial activation.

Methods/ Materials

Animals

C57BL/6 aged male mice (18-20 months of age; National Institute on Aging, Bethesda, MD) were acclimated for 1 month in our animal care facility prior to use. Mice were housed in pairs for 2 weeks (2 mice/cage) with a daily compatibility monitoring (eg, weight gain and the absence of fight wounds) as previously shown (Verma et al., 2018). All mice were housed with 12-h light/dark schedule in a temperature- and humidity-controlled vivarium, with ad libitum access to food and water. Three days following middle cerebral artery occlusion (MCAO) surgery, mice were randomly assigned to either pair housing (PH; stroke mouse continued

to be paired with previous partner mouse); or singly housed (SI; stroke isolated). A previous study from our laboratory assigned housing conditions immediately following stroke (Verma et al., 2018), and data from an earlier report also showed that pre-stroke SI, or SI at the time of stroke resulted in a significant increase in infarction volume and in turn exacerbated stroke severity and behavioral deficits (Venna et al., 2012). Thus, to control for infarct size we waited for three days to place animals in their assigned housing conditions to ensure that the infarct was complete, and that infarct size was equivalent between the groups. This experimental paradigm ensures that differences seen are mediated by housing conditions, not stroke size. Based on a predefined criterion, if a pair-housed stroke mouse died, the other partner mouse was also excluded from the study. The Institutional Animal Care and Use Committee at The University of Texas McGovern Medical School approved all animal protocols. All studies were performed in accordance with the guidelines provided by the National Institute of Health (NIH) and followed RIGOR guidelines.

Transient Stroke Model

To induce middle cerebral artery (MCA) occlusion, mice were anesthetized with isoflurane, a midline neck incision was performed to expose external carotid artery, a 6.0 silicone rubber-coated monofilament (Doccol Corporation) was inserted into the external carotid artery stump and advanced to the root of MCA via the internal carotid artery and allowed to remain in place for 60-minute of occlusion. Following 60 minutes of occlusion, mice were re-anesthetized, and the monofilament suture was removed to restore the blood flow. Once mice were awakened from

anesthesia, the presence of an intra-ischemic deficit was evaluated. All mice were given wet mesh daily and 1.0 ml of subcutaneous saline once a day for 3 days following surgery. Each pair of animals were randomly assigned toward their housing condition three days following MCA occlusion into either isolation or continued pair housed groups (N=7-8/ group).

Euthanasia

Mice were euthanized at post-stroke SI Day 1, 4, and 27 respectively using an overdose of avertin (2,2,2- Tribromoethanol) injected intraperitoneally. Confirmation of anesthesia was done by paw pinch prior to tissue harvesting. Following tissue harvesting from the mice, the brain and blood were removed for analysis.

Sample Preparation for RNA Isolation and Real-Time Quantitative PCR of miRNA

RNA extraction for qPCR was performed using Qiagen miRNeasy Mini Kit from ipsilateral hemisphere samples from post-stroke SI day 1, 4, and 27. RNA was stored at −80°C until analysis by QIAGEN next generation sequencing (NGS) platform. 10 ng RNA was reverse transcribed in 10 µL reactions using the miRCURY LNA universal real-time miRNA complementary DNA system (QIAGEN, Germantown, MD). Resulting complementary DNA was diluted (1:50) and assayed in miRNA ready-to-use PCR mouse miRCURY LNA SYBR green master mix (QIAGEN) as per manufacturer’s protocol in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad).

Quality Control, Small RNA library preparation, and Next generation sequencing

NGS and bioinformatics analysis were conducted at QIAGEN Genomic Services. For sample preparation, RNA was isolated using miRNeasy kit from QIAGEN as per manufacturer's protocol. QIAseq miRNA Library kit was utilized for library preparation using 5 uL of RNA converted into miRNA NGS libraries. Once unique molecular identifiers (UMIs) were attached, the RNA was converted into complementary DNA (cDNA). Following the synthesis of cDNA, a total of 22 cycles in PCR was used to amplify the cDNA in which PCR indices were added at the time. Bio-Analyzer 2100 or Tape-Station 4200 from Agilent was used to perform quality control on the library preparation. All samples prepared for the library were pooled in an equimolar ratio based on the quality of the inserts and the concentration of RNA/ cDNA. The pooled samples were quantified using qualitative PCR (qPCR). Following quantification, the pooled samples were fed into a NextSeq 500 sequencing instrument as per manufacturer's instructions. FASTQ files were generated using bcl2fastq (bcl2fastq (RRID:SCR_015058)) software from Illumina Inc. Processing of FASTQ files were performed using various platforms; trimming to extract sequences from adapter and UMI raw reads were performed by Cutadapt (1.11) (DOI:10.14806/ej.17.1.200), non-coding mapping was performed using Bowtie2 (2.2.2) (Langmead et al., 2009) to align reads of abundant sequences toward miRbase (Kozomara et al., 2019) reference sequences. The criterion for mapping reads to spike-ins, abundant sequences, and the database from miRbase are essential to have perfect matches to reference

sequences. For mapping parameters to the genome, more than one mismatch within the first 32 bases of the read was not allowed. Further, no indels were allowed in the mapping. Unmapped reads were excluded from further differential expression analysis. Differential expression analysis was performed by EdgeR (Chen et al., 2016) statistical software package from Bioconductor for fold change analysis within CLC Workbench (QIAGEN).

Prediction and pathways analysis of target mRNAs for differentially expressed miRNAs

The mRNA targets of differentially expressed miRNAs were predicted using the publicly available miRNET 2.0 tool (Chang et al., 2020), which is a comprehensive platform with data integrated from 11 different programs (MicroT4, miRBridge, miRMap, miRNAMap, PICTAR2, RNA22, RNAhybrid, TargetScan, miRanda, miRDB and PITA). Target prediction tool within miRNET 2.0 (Chang et al., 2020) for mRNA targets and differentially expressed miRNAs were utilized for node and edge identification for miRNA-mRNA interactional network construction. KEGG and Reactome analyses were acquired using the functional feature in miRNET 2.0 utilizing all the identified genes and the hypergeometric test.

Flow cytometry (Brain)

Brain single-cell suspension was obtained using a previously published protocol (Honarpisheh et al., 2020, Lee et al., 2020). Briefly, mice were euthanized by i.p. avertin injection at post-stroke SI D4 (4 days post housing manipulation and 7 days post stroke). Following euthanasia, mice were trans-cardially perfused with 20-mL of ice-cold PBS prior to removal of brain tissues. A midline coronal section of the

ipsilateral hemisphere encompassing the perilesional infarct was flashed frozen in an Eppendorf tube and stored in -80C for following RNA isolation. The leftover brain tissue (from the ipsilateral hemisphere of both anterior and posterior sections) was placed in complete Roswell Park Memorial Institute (RPMI) 1640 (Lonza) medium prior to mechanically and enzymatically digesting the homogenate in Collagenase/Dispase (1 mg/mL) and DNase (10 mg/mL; Roche Diagnostics) for 45 min at 37 °C with gentle shaking (80-100 RPM). Following digestion, the cell suspension was filtered through a 70-μm filter. Leukocytes were harvested from the interphase of 70–30% Percoll gradients for the brain tissue to remove myelin. Cells were washed and blocked with mouse Fc Block (BioLegend, Lot: B298973) before staining with primary antibody-conjugated fluorophores: CD45-eF450 (eBioscience, Cat#: 48-0451-82, Lot: 2005853), CD11b-APC (BioLegend, Cat#: 101212, Lot: B279418), Ly6C-PerCP-Cy5.5 (BioLegend, Cat#: 128011, Lot: 292026), Tmem119-PE-Cy7 (eBioscience, Cat#: 25-6119-82, Lot: 2210260), P2RY12-PE (BioLegend, Cat#: 848003, B298459), and MHCII-APC-Fire750 (BioLegend, Cat#: 107652, Lot: B301025) pre-conjugated antibodies and Zombie Aqua (BioLegend, Cat#: 423102, Lot: B300004) to assess for cell viability. To minimize experimental variability, cell isolation and immunostaining steps were carried out all at once for both housing conditions (PH and SI). Data were acquired on Cytoflex-S (Beckman Coulter) and analyzed using FlowJo (Treestar Inc.). No less than 100,000 events were recorded for each sample. Unstained controls and tissue-matched fluorescence minus one (FMO) were used to aid in the gating strategy. Median fluorescence intensity (MFI) reported as arbitrary fluorescence

units [a.f.u] was used as measure of expression level on corresponding cell populations of interest.

Statistics

The evaluation of data and graphical representations was performed utilizing Graph Pad Prism 8.4.2 software, San Diego, California USA, www.graphpad.com. The data are shown as the means +/- standard error of the mean (SEM). Student t test was used for two-group comparisons set as non-parametric measures. A p-value of less than 0.05 was considered as a significant measure for two sample analyses. A false discovery rate (FDR) adjusted p-value of less than 0.05 was considered as a significant measure for multiple hypothesis testing.

Results

Identification of differentially expressed miRNAs in the ipsilateral hemisphere of Post-Stroke Isolated Mice

MiRNAs implicating the pathogenicity of stress-induced disorders have increasingly been recognized in the brain (Popa et al., 2020) and in the circulation (Banerjee et al., 2021). To investigate the role of miRNAs as a potential regulator of microglial activation resulting in the downstream detrimental effects of post-stroke SI; the miRNA abundance at three time points (Post-Stroke SI D1, D4, and D27) was examined. This stratification process led us to identify potential miRNA candidates that had differential expression over three different phases of SI which

potentially addressed the effect of isolation on miRNA profiles within the brain in a temporal manner. In the discovery phase, aged male mice were subjected to ischemic stroke and the ipsilateral hemisphere was collected following three time points of post-stroke SI D1, D4, and D27 in the corresponding housing conditions for QIAGEN-based NGS analysis as shown in **Figure 1**.

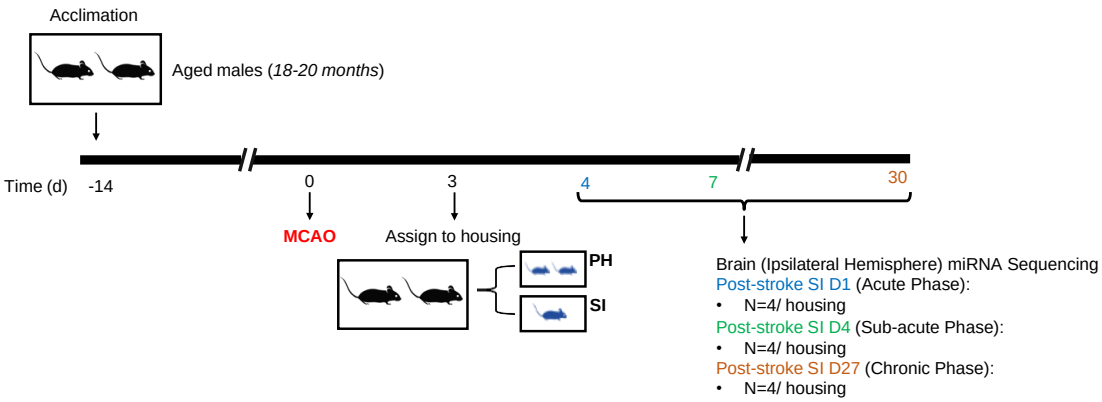


Figure 1. Schematic of experimental design for miRNA identification and analysis. Aged C57BL/6 male mice (18-20 months) were subjected to middle cerebral artery occlusion (MCAO) and were randomly assigned three days post-reperfusion to SI (individually housed) or continued pair housed conditions. At three distinct time points of post-stroke SI D1 (Acute), D4 (Sub-acute), and D27 (chronic); mice were euthanized, and the ipsilateral hemisphere were collected for further miRNA-based NGS sequencing from QIAGEN and downstream bioinformatics.

Differentially expressed miRNAs with a threshold criterion of (FDR adjusted $p < 0.05$) were identified through the QIAGEN CLC Workbench to filter 10 hits across all three points for appropriate fold change analysis as shown in **Figure 2 a, b and c**.

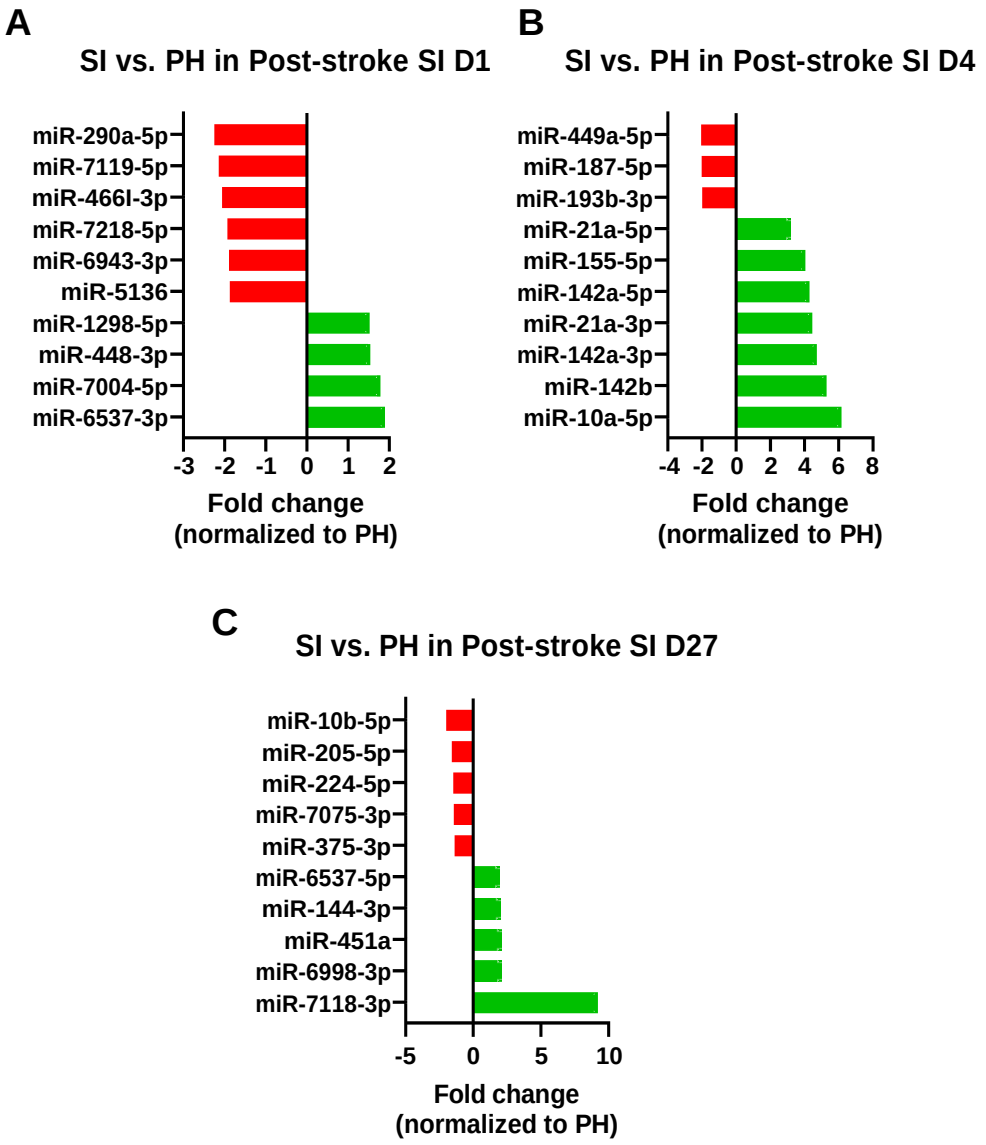


Figure 2. Bioinformatics analysis of differential expressed miRNAs in post-stroke SI compared to PH conditions across three time points.
(**A**, **B**, and **C**) highlight the significantly differential expressed miRNAs with a FC of 1 or higher for post-stroke socially isolated aged males at D1, D4, and D27, respectively. Positive fold change indicates an upregulation in SI, while negative fold change signifies a downregulation in SI.

Fold change was calculated based as a ratio from normalized transcripts per million (TPM) of PH mice to TPM values of SI mice. Of the 10 differentially expressed miRNAs identified within post-stroke SI D1 aged male mice, 4 (miR-6537-5p, miR-7004-5p, miR-448-3p, miR-1298-5p, and miR-1298-5p) were

upregulated and 6 (miR-5136, miR-6943-3p, miR-7128-3p, miR-466l-3p, miR-7119-5p, and miR-290a-5p) were downregulated relative to pair housing. Fold change analysis within the 10 miRNAs candidates identified from post-stroke SI D4 mice revealed 7 (miR-10a-5p, miR-142b, miR-142a-3p, miR-21a-3p, miR-142a-5p, miR-155-5p, and miR-21a-5p) were significantly upregulated in singly housed male mice while 3 (miR-193b-3p, miR-187-5p, and miR-449a-5p) were downregulated. Within the post-stroke SI D4 pool of candidate miRNAs, 3 of the 7 upregulated miRNAs belonged within the miR-142 family of miRNAs suggesting their potential synergistic biological function. At the chronic phase of isolation at post-stroke SI D27 mice, of the 10 differentially expressed miRNAs, 5 (miR-7118-3p, miR-6998-3p, miR-451a, miR-144-3p, and miR-6537-5p) were upregulated and 5 (miR-375-3p, miR-7075-3p, miR-244-5p, miR-205-5p, and miR-10b-5p) were downregulated based on our cutoff criteria of (FDR adjusted $p < 0.05$). Thus, these results demonstrate that SI has distinctive effects upon the brain in a temporal manner.

Construction of miRNA-mRNA interactional networks

In an effort to decipher the biological function of the identified miRNAs and to elucidate the downstream pathway involvement of these filtered candidates, mRNA genetic target analysis was performed incorporating an identification platform of differentially expressed targets through the miRNET 2.0 target feature. The differentially expressed miRNAs identified through the CLC Workbench feature from QIAGEN were fed into miRNET 2.0 and the pool of predictive mRNA targets were filtered utilizing a stringent criterion of 7 base points (bps) seed length

and a significance level of (FDR adjusted p < 0.05) (data not shown). Subsequently, 3 distinct miRNA-mRNA interactional networks were constructed integrating all miRNAs filtered from the given cutoff criteria (p < 0.05) or lower across the three time points of post-stroke SI as illustrated in **Figure 3 a, b, and c** using the miRNET 2.0 characteristic feature. Island networks encompassing one miRNA were not included in our analysis.

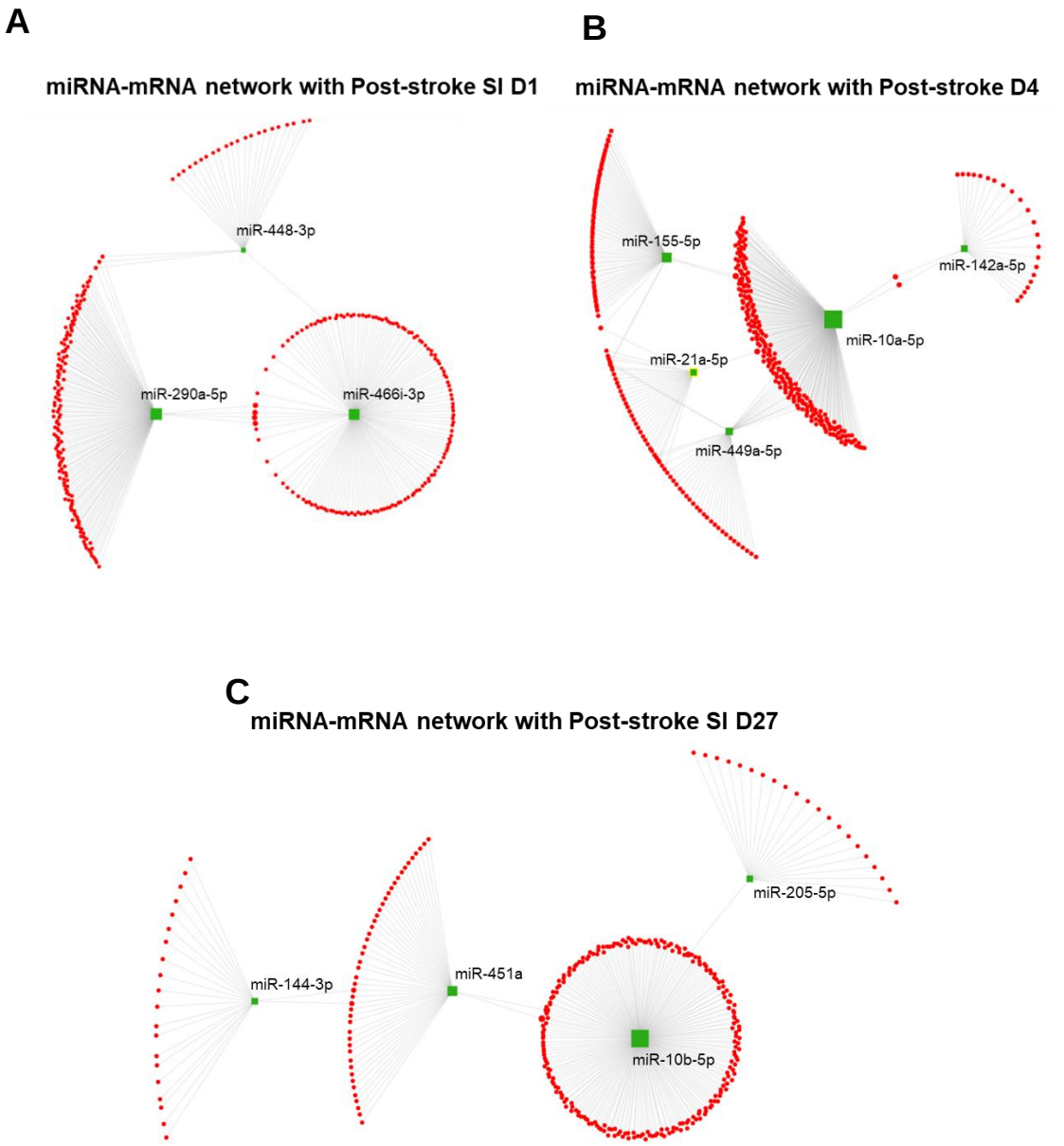


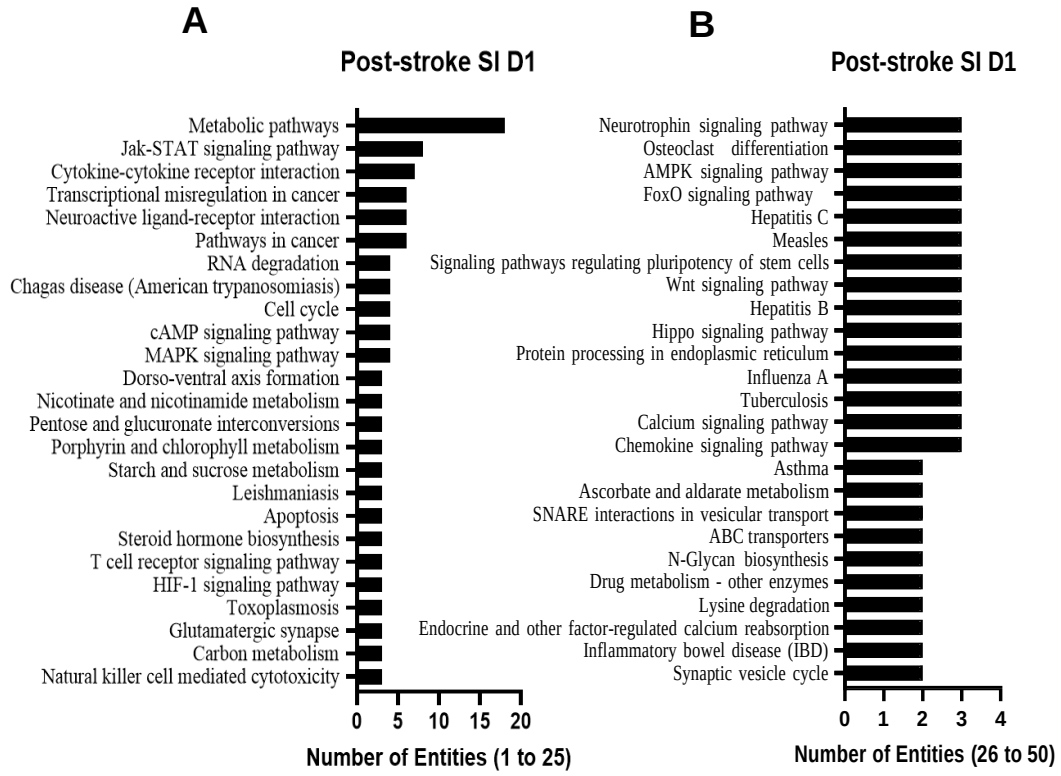
Figure 3. miRNA–mRNA interaction networks and miRNA gene targets.
(**A**, **B**, and **C**) The differentially expressed miRNAs and their predicted mRNA transcript targets are illustrated as networks across the three time points. Each individual node represents a miRNA or mRNA. Hubs of the 3, 5, and 4 differentially expressed miRNAs are shown in individual networks for Post-Stroke SI D1, D4, and D27 respectively. Nodes interacting with the miRNA are highlighted in green and individual target genes are highlighted in red.

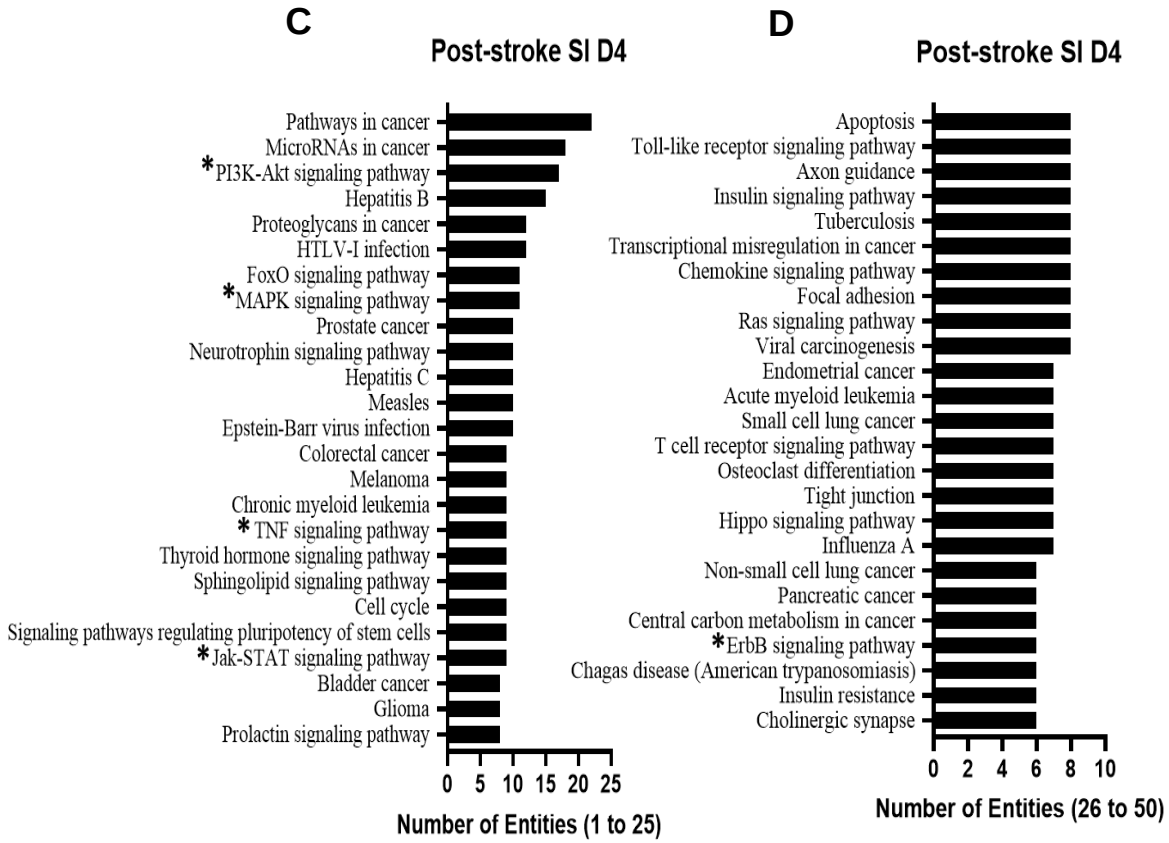
The miRNA-mRNA networks showed that several mRNA targets are shared by multiple differentially altered miRNAs at all three time points of post-stroke SI. Following interaction analysis, miR-466i-3p was identified as a pivotal node within the pool of miRNAs from post-stroke SI D1 mice indicating its role as a potential regulator of all other miRNAs from same time point. More notably, miR-10a-5p and miR-10b-5p were identified as a pivotal node across post-stroke SI D4 and D27, respectively, indicating a potential synergistic role and common pathways of involvement in the chronic stages of SI. In post-stroke SI D1 mice, miR-209a-5p had the highest predicted targets followed by miR-466i-3p, and miR-448-3p. Additionally, miR-10a-5p had the highest predicted targets followed by miR-155-5p, miR-449a-5p, miR-21a-5p, and miR-142a-5p within post-stroke SI D4 mice. In post-stroke SI D27 mice following interactional analysis, miR-10b-5p had the highest predicted targets followed by miR-451a, miR-205-5p, and miR-144-3p. The miR-10 family had the highest number of gene targets across the two time points of post-stroke SI D4 and D27, suggesting similar downstream pathway involvement and gene regulation. Among these pools of target mRNAs, a vast number of them were identified as pivotal regulators of depression, neuro-inflammatory processes, and microglial activation. Remaining miRNAs not found within the interactional network did not have overlapping gene targets across the

time points. As indicated, pivotal nodes among the networks were identified by centrality of degree over the peripheral regions of interaction. Interactional networks from post-stroke SI D1 and D27 had the relative same number of nodes and interactions. In contrast, differentially expressed miRNAs identified within the post-stroke SI D4 mice had the greatest number of nodes with higher gene interactions relative to the other time points suggesting a greater change in the overall pool of miRNAs within the same time point. Taken together following our network analysis, miR-446i-3p, miR-10a-5p, and miR-10b-5p were found to be crucial nodes within post-stroke SI D1, D4, and D27 groups respectively, that interacted with the largest subset of genes and miRNAs.

KEGG and Reactome analyses of mRNAs targeted by the differentially expressed miRNAs

Previous studies have shown that changes to environmental conditions and stressors can alter miRNA profiles within specific tissues and lead to downstream physiological changes (Miguel et al., 2020). Therefore, to examine the biological relevance of these miRNA alterations we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome analysis to further characterize their downstream functions and to investigate the underlying bio-molecular pathways (Lee et al., 2021). KEGG analysis revealed the top 50 signaling pathways within all three time points, all significantly enriched (FDR adjusted $p < 0.05$) (**Figure 4**).


A
Post-stroke SI D1
Metabolic pathways
Jak-STAT signaling pathway
Cytokine-cytokine receptor interaction
Transcriptional misregulation in cancer
Neuroactive ligand-receptor interaction
Pathways in cancer
RNA degradation
Chagas disease (American trypanosomiasis)
Cell cycle
cAMP signaling pathway
MAPK signaling pathway
Dorso-ventral axis formation
Nicotinate and nicotinamide metabolism
Pentose and glucuronate interconversions
Porphyrin and chlorophyll metabolism
Starch and sucrose metabolism
Leishmaniasis
Apoptosis
Steroid hormone biosynthesis
T cell receptor signaling pathway
HIF-1 signaling pathway
Toxoplasmosis
Glutamatergic synapse
Carbon metabolism
Natural killer cell mediated cytotoxicity
0 5 10 15 20
Number of Entities (1 to 25)
B
Post-stroke SI D1
Neurotrophin signaling pathway
Osteoclast differentiation
AMPK signaling pathway
FoxO signaling pathway
Hepatitis C
Measles
Signaling pathways regulating pluripotency of stem cells
Wnt signaling pathway
Hepatitis B
Hippo signaling pathway
Protein processing in endoplasmic reticulum
Influenza A
Tuberculosis
Calcium signaling pathway
Chemokine signaling pathway
Asthma
Ascorbate and aldarate metabolism
SNARE interactions in vesicular transport
ABC transporters
N-Glycan biosynthesis
Drug metabolism - other enzymes
Lysine degradation
Endocrine and other factor-regulated calcium reabsorption
Inflammatory bowel disease (IBD)
Synaptic vesicle cycle
0 1 2 3 4
Number of Entities (26 to 50)


C
Post-stroke SI D4
Pathways in cancer
MicroRNAs in cancer
*PI3K-Akt signaling pathway
Hepatitis B
Proteoglycans in cancer
HTLV-I infection
FoxO signaling pathway
*MAPK signaling pathway
Prostate cancer
Neurotrophin signaling pathway
Hepatitis C
Measles
Epstein-Barr virus infection
Colorectal cancer
Melanoma
Chronic myeloid leukemia
*TNF signaling pathway
Thyroid hormone signaling pathway
Sphingolipid signaling pathway
Cell cycle
Signaling pathways regulating pluripotency of stem cells
*Jak-STAT signaling pathway
Bladder cancer
Glioma
Prolactin signaling pathway
0 5 10 15 20 25
Number of Entities (1 to 25)
D
Post-stroke SI D4
Apoptosis
Toll-like receptor signaling pathway
Axon guidance
Insulin signaling pathway
Tuberculosis
Transcriptional misregulation in cancer
Chemokine signaling pathway
Focal adhesion
Ras signaling pathway
Viral carcinogenesis
Endometrial cancer
Acute myeloid leukemia
Small cell lung cancer
T cell receptor signaling pathway
Osteoclast differentiation
Tight junction
Hippo signaling pathway
Influenza A
Non-small cell lung cancer
Pancreatic cancer
Central carbon metabolism in cancer
*ErbB signaling pathway
Chagas disease (American trypanosomiasis)
Insulin resistance
Cholinergic synapse
0 2 4 6 8 10
Number of Entities (26 to 50)

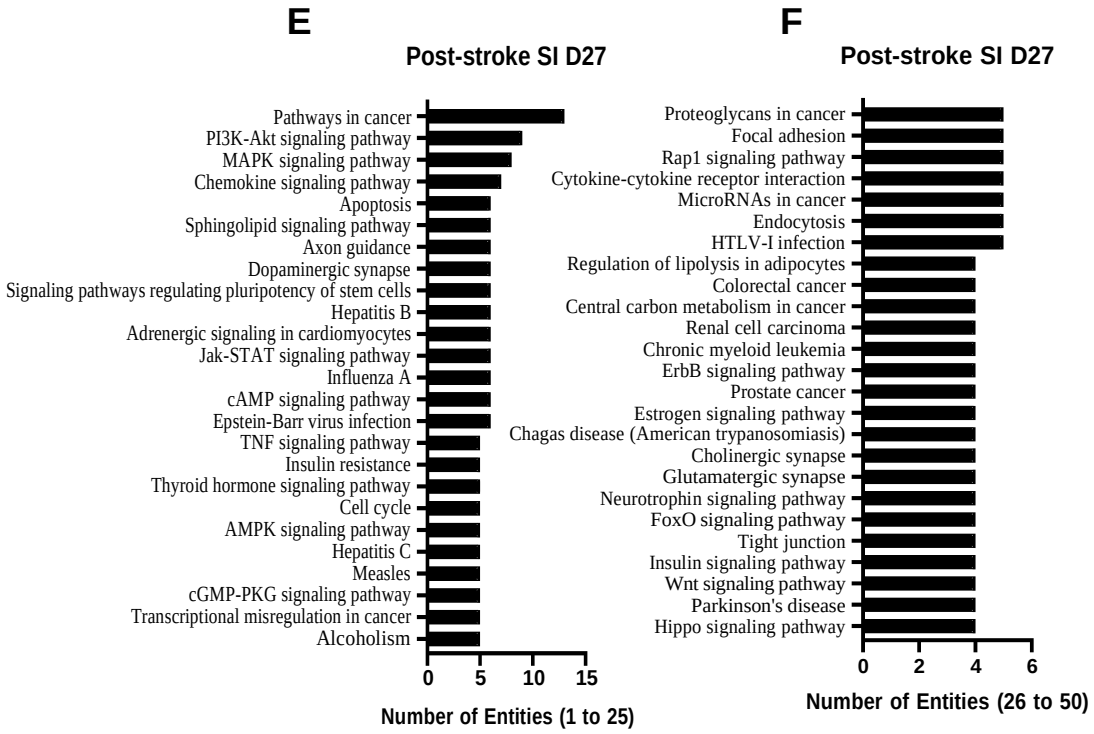
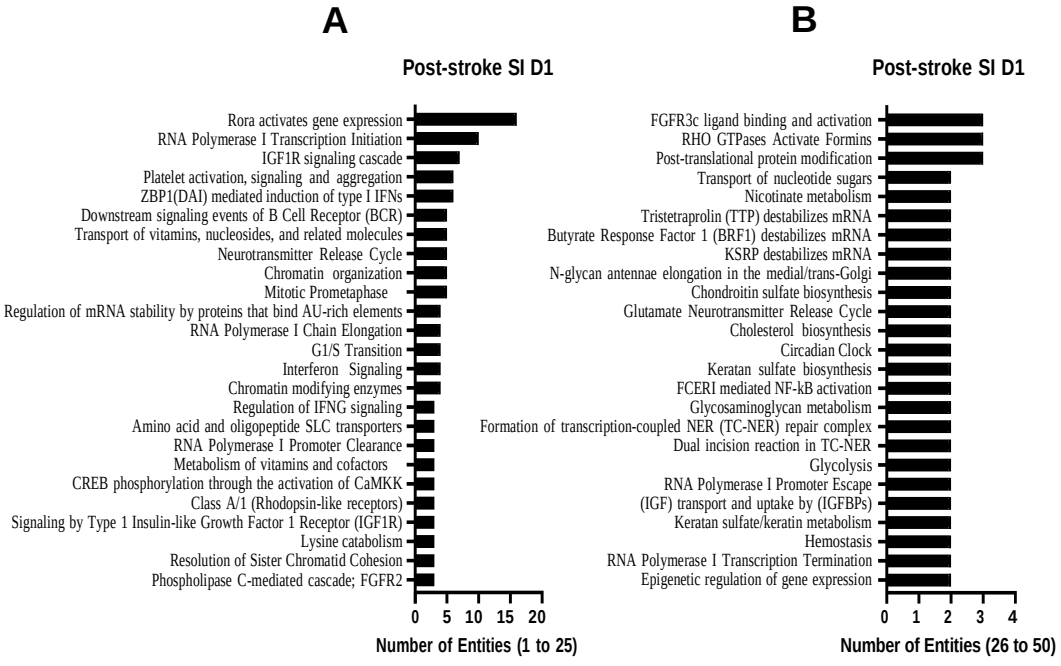


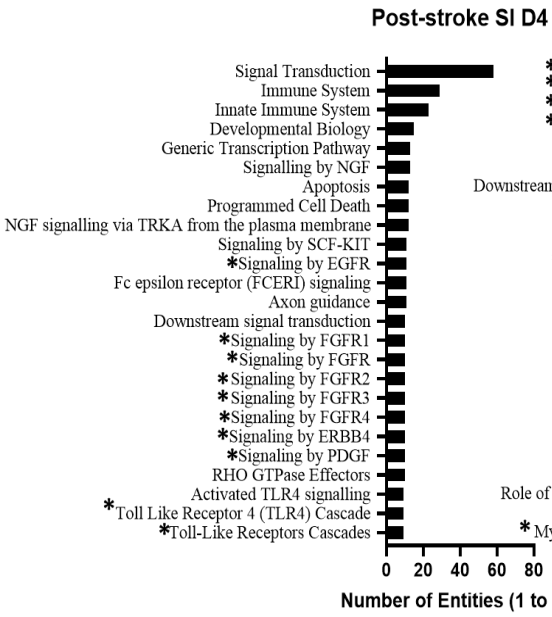
Figure 4. KEGG Pathway analysis of the predicted mRNA gene transcripts targeted by the differentially expressed miRNAs in the ipsilateral hemisphere of post-stroke SI.
D1 (**A** and **B**), D4 (**C** and **D**), and D27 (**E** and **F**). The top 50 identified pathways in post-stroke SI regulated the filtered miRNAs from interaction analysis were analyzed by KEGG and are shown based on the number of entities (i.e., the number of mRNA transcripts) involved. The * marks indicate pathways involved in microglial-related immune activation.

More notably, post-stroke SI D1 miRNAs were involved in pathways of cell cycle, RNA degradation, and calcium signaling pathways indicative of cell damage and ischemic injury at the acute phase of stroke which dominated the effects of SI on the miRNA profiles. The majority of the KEGG pathways identified within post-stroke SI D4 had many pathways involved with microglial-related immune activation that were not evident at the other time points. A few of the identified pathways related to microglial activation were the PI3K-Akt signaling pathway, the MAPK signaling pathway, the TNF signaling pathway, and the Jak-STAT signaling pathway. Furthermore, post-stroke SI D27 miRNAs were those involved in

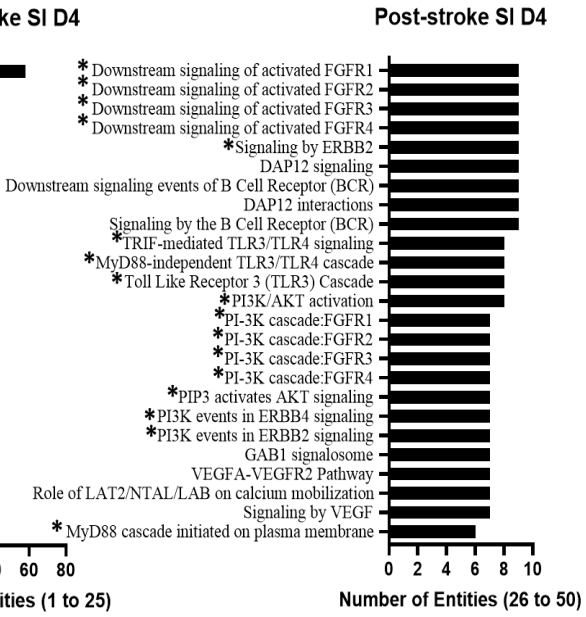
pathways related to neurobehavioral signaling and metabolic function suggesting the potential of the effect of SI on chronic behavioral impairment, cognitive recovery, and energy expenditure. Some of these pathways involved with neuro-behavioral and metabolic signaling included dopaminergic synapse, cholinergic synapse, glutamatergic synapse, and thyroid hormone signaling pathway. This significant difference between the time points suggests that the detrimental phenotypes induced by post-stroke SI manifest distinctly within the host over time. We further performed systemic enrichment analysis using the Reactome platform to pinpoint putative mechanisms on the effect of post-stroke SI toward the host through the miRNA changes observed. Reactome analysis revealed the top 50 signaling pathways within all three time points, all significantly enriched (FDR adjusted $p < 0.05$) (**Figure 5)**.



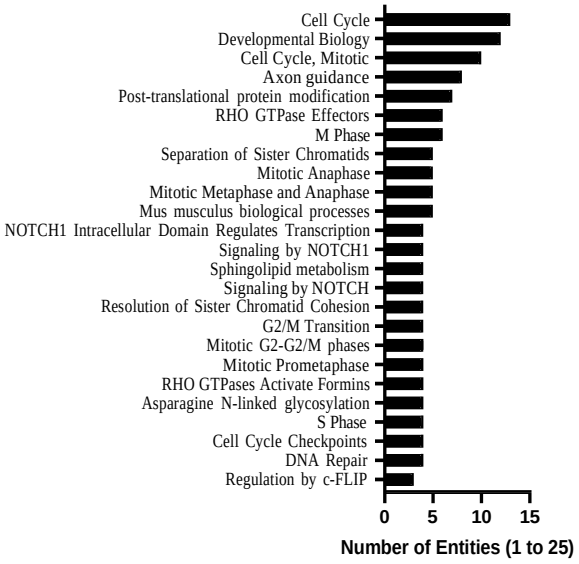
C


Post-stroke SI D4
Signal Transduction
Immune System
Innate Immune System
Developmental Biology
Generic Transcription Pathway
Signalling by NGF
Apoptosis
Programmed Cell Death
NGF signalling via TRKA from the plasma membrane
Signaling by SCF-KIT
*Signaling by EGFR
Fc epsilon receptor (FCERI) signaling
Axon guidance
Downstream signal transduction
*Signaling by FGFR1
*Signaling by FGFR
*Signaling by FGFR2
*Signaling by FGFR3
*Signaling by FGFR4
*Signaling by ERBB4
*Signaling by PDGF
RHO GTPase Effectors
Activated TLR4 signalling
*Toll Like Receptor 4 (TLR4) Cascade
*Toll-Like Receptors Cascades
0 20 40 60 80
Number of Entities (1 to 25)

D


Post-stroke SI D4
*Downstream signaling of activated FGFR1
*Downstream signaling of activated FGFR2
*Downstream signaling of activated FGFR3
*Downstream signaling of activated FGFR4
*Signaling by ERBB2
DAP12 signaling
Downstream signaling events of B Cell Receptor (BCR)
DAP12 interactions
Signaling by the B Cell Receptor (BCR)
*TRIF-mediated TLR3/TLR4 signaling
*MyD88-independent TLR3/TLR4 cascade
*Toll Like Receptor 3 (TLR3) Cascade
*PI3K/AKT activation
*PI-3K cascade:FGFR1
*PI-3K cascade:FGFR2
*PI-3K cascade:FGFR3
*PI-3K cascade:FGFR4
*PIP3 activates AKT signaling
*PI3K events in ERBB4 signaling
*PI3K events in ERBB2 signaling
GAB1 signalosome
VEGFA-VEGFR2 Pathway
Role of LAT2/NTAL/LAB on calcium mobilization
Signaling by VEGF
*MyD88 cascade initiated on plasma membrane
0 2 4 6 8 10
Number of Entities (26 to 50)

E


Post-stroke SI D27
Cell Cycle
Developmental Biology
Cell Cycle, Mitotic
Axon guidance
Post-translational protein modification
RHO GTPase Effectors
M Phase
Separation of Sister Chromatids
Mitotic Anaphase
Mitotic Metaphase and Anaphase
Mus musculus biological processes
NOTCH1 Intracellular Domain Regulates Transcription
Signaling by NOTCH1
Sphingolipid metabolism
Signaling by NOTCH
Resolution of Sister Chromatid Cohesion
G2/M Transition
Mitotic G2-G2/M phases
Mitotic Prometaphase
RHO GTPases Activate Formins
Asparagine N-linked glycosylation
S Phase
Cell Cycle Checkpoints
DNA Repair
Regulation by c-FLIP
0 5 10 15
Number of Entities (1 to 25)

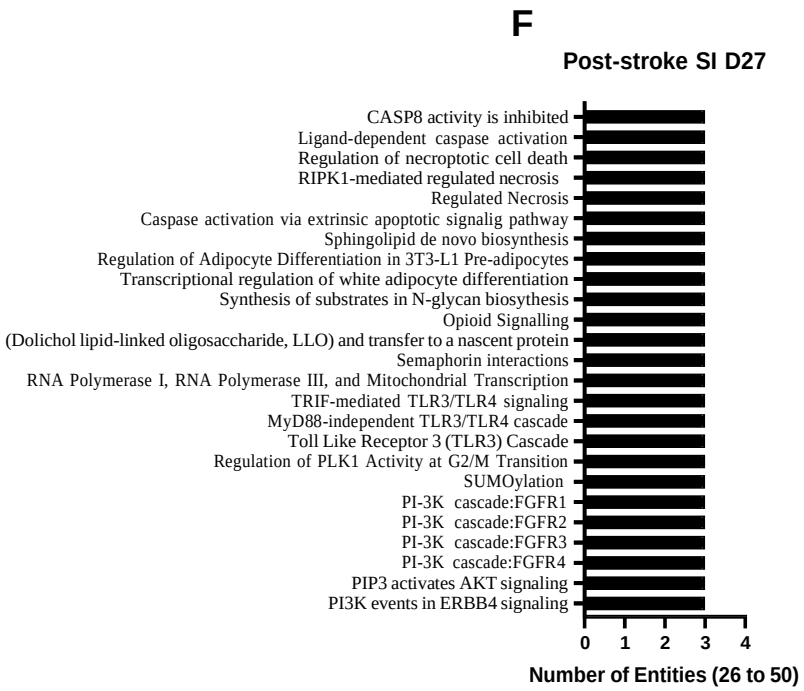


Figure 5. Reactome Pathway analysis of the predicted mRNA gene transcripts targeted by the differentially expressed miRNAs in the ipsilateral hemisphere of post-stroke SI.
D1 (**A** and **B**), D4 (**C** and **D**), and D27 (**E** and **F**) The top 50 identified pathways in post-stroke SI regulated the filtered miRNAs from interaction analysis were analyzed by Reactome and are shown based on the number of entities (i.e., the number of mRNA transcripts) involved. The * marks indicate pathways involved in microglial-related immune activation.

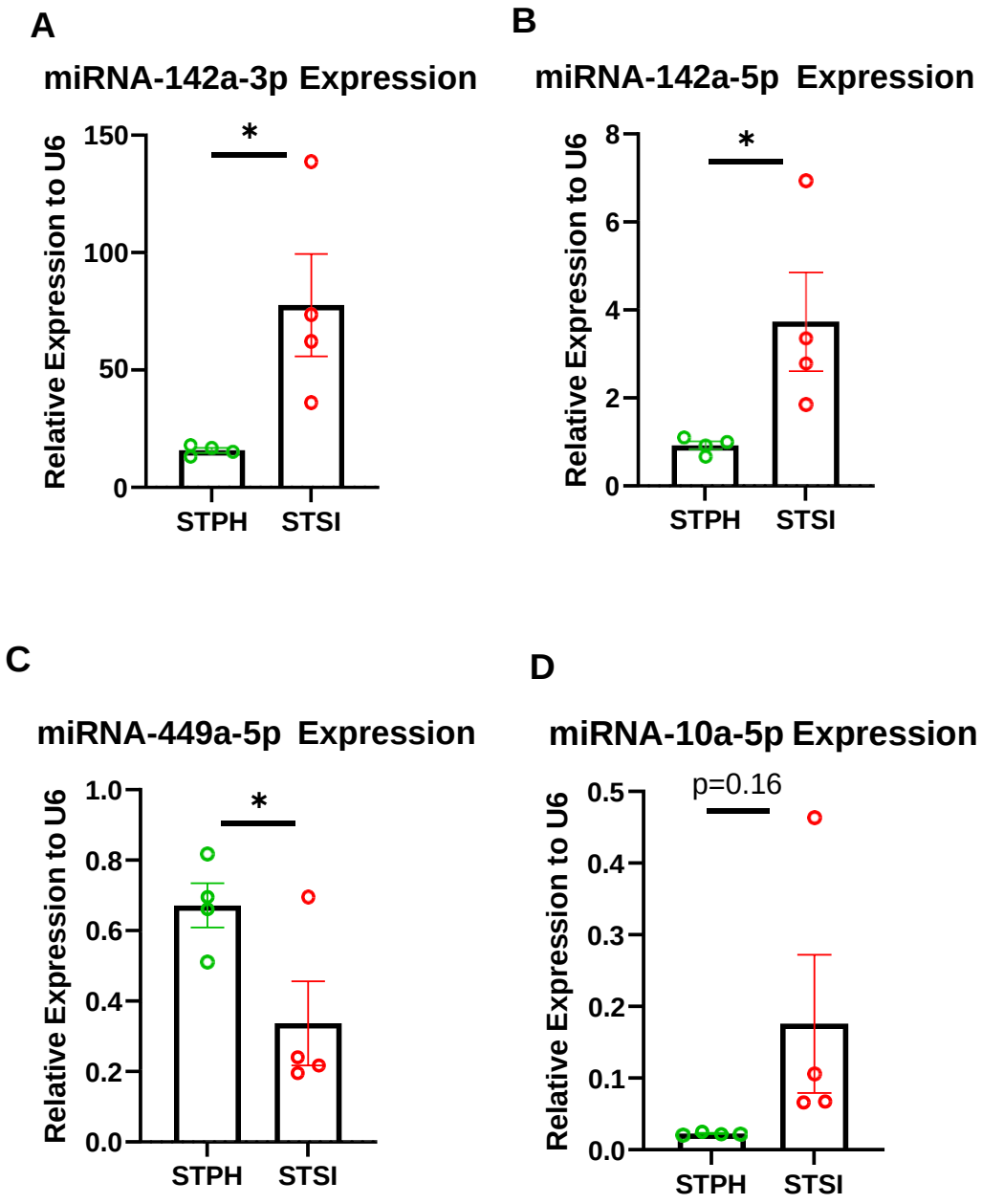
Reactome analysis included a majority of pathways involved in microglial-related immune activation within the altered miRNAs from post-stroke SI D4 mice. This suggested a putative connection among the effects of SI toward a response. Some of the identified putative pathways involved in microglial-related immune activation were the family of signaling by FGFR, activated TLR4 signaling, toll-like receptor cascade, and the family of PI3K cascades. In addition, Reactome analysis within the pool of filtered miRNAs from post-stroke SI D27 included many pathways involved in reparative mechanisms along with some pathways involved in

microglial activation indicative of potential crosstalk among neuronal cellular subtypes with other glial cells in the brain.

Post-Stroke SI Induces Microglial Activation

To validate our findings from our QIAGEN NGS and downstream pathway analysis we focused on post-stroke SI D4 as miRNA candidates and pathways involved in microglial activation were present across both enrichment analysis compared to the other time points. Furthermore, previous literature has shown the importance of the family of miR-10 miRNAs, a pivotal node identified across post-stroke SI D4 and D27, in regulating macrophage polarization within adipocyte tissue in models of glucose intolerance (Cho et al., 2019). Additionally, the miR-142-5p family of miRNAs, 3 of the 10 identified significantly differentially expressed targets within post-stroke SI D4 regulate macrophage polarization through targeting suppressor of cytokine signaling 1 (SOCS1) and prolonging signal transducer and activator of transcription 6 (STAT6) phosphorylation to induce IL-4 and IL-13 production as seen in a previous report (Su et al., 2015). In the validation phase, we examined the expression levels of the top 2 up-/ down-regulated miRNAs (miR-10a-5p, miR-142b, miR-187-5p, and miR-449a-5p) along with the candidate miRNAs that belonged to the miR-142 family (miR-142a-3p and miR-142a-5p) as a validation measure utilizing the QIAGEN NGS cohort of mice. As expected, miRNA-142a-3p and miR-142a-5p were significantly upregulated in post-stroke socially isolated mice as compared to paired house mice (**Figure 6a and b**). On the other hand, miR-449a-5p was significantly downregulated in post-stroke socially isolated mice

(**Figure 6c**). However, we didn't any changes in miR-10a-5p and miR-187-5p across the two housing conditions (**Figure 6d and e**).



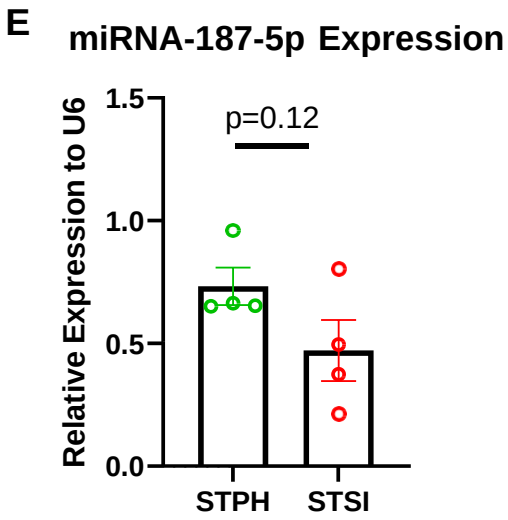


Figure 6. Validation of miRNA candidates within the ipsilateral hemisphere of the brain in post-stroke SI D4 aged male mice.
(**A, B, C, D, and E**) The expression levels of miRNA candidates were examined by real-time quantitative qPCR. miR-142a-3p and miRNA-142a-5p were significantly upregulated in a coronal section of ipsilateral hemisphere of the brain in post-stroke social isolation D4 aged male mice while miR-449a-5p was significantly downregulated. No change was observed for miRNA-10a-5p and miR-187-5p across the two housing conditions. Gene for U6 spliceosomal RNA was used as an endogenous control, in which the unpaired student's t-test was used. The data are presented as the means ± SEM. n = 4 per group. *p < 0.05.

Multitude of studies have shown the predominant surface markers for the identification of microglia are cluster of differentiation (CD) 45 (intermediate expression), CD11b, Tmem119, and P2RY12; the according markers were used to characterize microglial activation. **Figure 7a** shows the gating strategy used to identify microglial cells within the brain. To assess microglial activation and potentially identify "disease-associated microglia (DAM)" in our model of post-stroke SI at D4, independent validation cohorts were set up and flow cytometry was used to measure the expression level of Tmem119 and P2RY12 on microglial cells. We found that 4 days of isolation post-stroke led to a significant

downregulation of P2RY12 expression as a measure of median fluorescence intensity (MFI) compared to the paired housed controls (**Figure 7b**), however no change was observed in Tmem119 expression between the two groups (**Figure 7c**). Taken together, this suggests that SI may activate resting microglial cells to deviate from homeostasis and further may have a differential effect on their transcriptional signature due to their miRNA alterations.

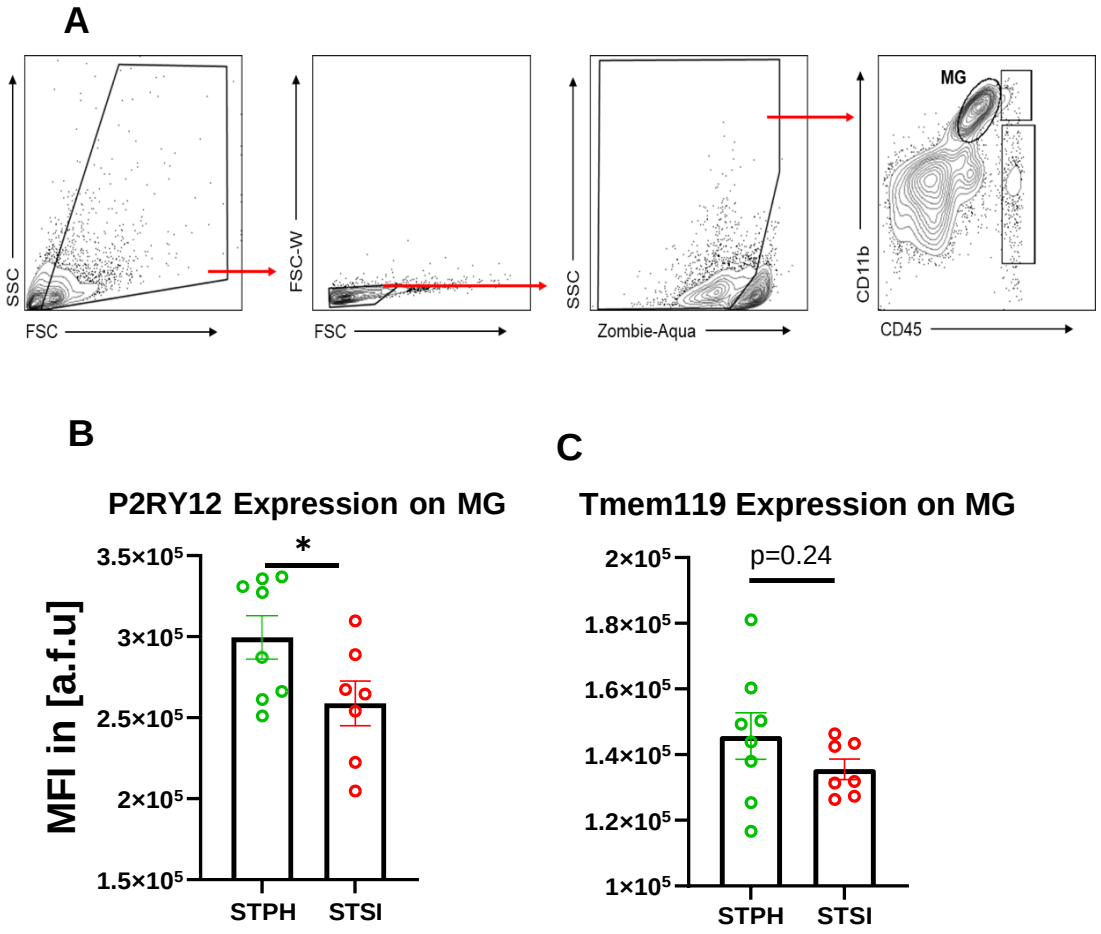


Figure 7. Post-stroke SI D4 induces microglial activation.
(**A**) Representative flow cytometry plots to identify microglia cell populations in the ipsilateral hemisphere of aged post-stroke SI D4 male mice. $CD45^{Int}CD11b^{+}$ cells in the ipsilateral hemisphere of post-stroke SI aged males were gated and analyzed as microglia cells. An amine-reactive Zombie-Aqua viability stain was used to identify live and dead cells. (**B** and **C**) Median fluorescence intensity (MFI)

of P2RY12, as a measure of expression, in $CD45^{Int}CD11b^{+}P2RY12^{+}$ cells showed significant decrease in post-stroke SI D4 aged mice. MFI of Tmem119 in $CD45^{Int}CD11b^{+}Tmem119^{+}$ cells showed no change among the two housing conditions post-stroke. MFI was reported as arbitrary fluorescence units [a.f.u]. (n = 7-8/gp, unpaired student t test. The data are presented as the means ± SEM. *p < 0.05).

Discussion

In this study, we have shown that post-stroke SI results in significant alterations of miRNAs profiles in the brain. Furthermore, we have shown that post-stroke SI can differentially effect miRNA profiles at differently among phases of SI suggesting distinct downstream molecular pathways at play. This was further corroborated by the putative interactional networks which showed different points of regulation and gene targets across the three time points. Many of the identified miRNA candidates in post-stroke SI D4 mice actively participated in the regulation of microglial activation shown through KEGG and Reactome analysis. This was further validated using qRT-PCR and flow cytometry as post-stroke SI D4 showed a significant alteration of miRNA candidates and a significant downregulation of P2RY12 expression, respectively. In this study, we determined that post-stroke SI in aged male mice at the acute, sub-acute, and chronic phases have a potential to influence the homeostatic miRNA profiles within the brain. These changes are reflective of potential mechanisms that facilitate the detrimental effects of post-stroke recovery and the exacerbate microglial activation, as SI is known to contribute to poorer outcomes following ischemic stroke (Steptoe et al., 2013). Moreover, several novel miRNAs candidates were identified in this study which may potentially regulate the expression of key genes involved in either promoting or worsening post-stroke recovery across housing conditions. This overview of

candidate miRNAs serves as an initial step in identifying targetable molecules, at the post-transcriptional level, to which give insight in potential downstream detrimental effects of post-stroke SI toward the host.

Previous reports have shown that SI immediately after stroke can result in the aberrant expression of miRNAs in the brain exacerbating cognitive impairment and promoting the expression of pro-inflammatory cytokines (Antony et al., 2020, Verma et al., 2018). However, the source of these pro-inflammatory cytokines as a contributor to the overall inflammatory landscape were not investigated in the study. This study was conducted to investigate the miRNAs that may potentially be associated with microglial activation resulting in the production of these pro-inflammatory cytokines investigated in previous studies. Following our initial exclusion criteria, 10 miRNAs were identified to be significantly differentially expressed in SI compared to PH mice across all time points of post-stroke SI D1, D4, and D27. Within post-stroke SI D1 mice, of the 10 identified differentially expressed miRNAs, 6 miRNAs (miR-290a-5p, miR-7119-5p, miR-466i-3p, miR-7218-5p, miR-6943-3p, and miR-5136) were downregulated while 4 miRNAs (miR-1298-5p, miR-448-3p, miR-7004-5p, and miR-6537-3p) were upregulated in SI compared to PH mice. In post-stroke SI D4 mice, 3 miRNAs (miR-449a-5p, miR-187-5p, and miR-193b-3p) were downregulated while 7 miRNAs (miR-21a-5p, miR-155-5p, miR-142a-5p, miR-21a-3p, miR-142a-3p, miR-142b, and miR-10a-5p) were upregulated in SI as compared to PH mice. In addition, 3 of the 10 identified miRNAs were a part of the miRNA-142 family indicative of a downstream synergic role toward the host. Within post-stroke SI D27 mice, 5 miRNAs (miR-

10b-5p, miR-205-5p, miR-224-5p, miR-7075-3p, and miR-375-3p) were observed to be significantly downregulated while 5 miRNAs (miR-6537-5p, miR-144-3p, miR-451a, miR-6998-3p, and miR-7118-3p) were upregulated within SI mice. The target finder feature within miRNET 2.0 was used to determine subsets of gene targets which were putatively regulated by the identified candidate miRNAs associated with post-stroke SI across the three time points (data not shown). Following target prediction, interactional analysis was performed to investigate how the identified pool of miRNAs in a given time point interacted among the pool of miRNAs and their gene targets. Interactional analysis showed miR-466i-3p, miR-10a-5p, and miR-10b-5p as central nodes for post-stroke SI D1, D4, and D27 mice, respectively. Central nodes were classified based on their degree of targeting the largest subset of genes and putatively regulating the largest number of miRNAs within the same network. More notably, across post-stroke SI D4 and D27, miR-10a-5p and miR-10b-5p, both belonging to the miR-10-5p family, were identified as central nodes suggesting a potential connective downstream gene network toward the host during the subacute and chronic phases of post-stroke SI. Sequencing studies from ischemic stroke models in mice have shown significant alterations of miR-466i-3p in the brain (Lusardi et al., 2014). More recently, miR-10-5p family of miRNAs has been shown to play an active role in regulating macrophage polarization within adipocytes in mice models of glucose intolerance (Cho et al., 2019) suggesting that differentially expressed miRNAs within the brain in the sub-acute and chronic phases of post-stroke SI may play a role in microglial activation. Further, the miRNA-10-5p family has also been shown to participate in

exacerbating depression within the dorsolateral prefrontal cortex by playing a role as an epigenetic modifier to genes that regulate the progression of major depressive disorders in patients (Dwivedi, 2018). MiRNAs regulating microglial activation following neuro-inflammation has been increasingly recognized (Guo et al., 2019). This modulation suggests its potential of therapeutic use as animal studies have shown the effective usage of agomiR/ antagomiR to target miRNA changes (Li et al., 2021).

In order to characterize the biological functionality of the filtered miRNAs from interactional analysis, two independent enrichment platforms were utilized, KEGG and Reactome. As expected, candidate miRNAs identified from post-stroke SI D1 mice were involved in pathways related to cell death and RNA degradation suggestive of a more profound physiological effect from the ischemic injury as compared to the alteration of the housing condition itself within the acute phase. Across both KEGG and Reactome analysis of miRNAs identified within post-stroke SI D4 mice, significant numbers of pathways were involved in microglial-related immune activation activation, such as PI3K-Akt signaling, TNF signaling, Jak-STAT signaling, and TLR signaling cascades. Numerous studies and through the efforts of single cell sequencing of microglia in mice models of Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) have shown that this microglial activation can be further characterized into distinct microglial subtypes having differential effects towards the overall neuro-immune landscape and the long term effects of cognition of the host (Deczkowska et al., 2018). Previous reports have shown that these microglial subtypes have unique signatures of transcriptional and

surface marker profiles (Bennett et al., 2018) which may be contributed by these miRNA alterations being observed. Common surface markers such as Tmem119 and P2RY12 have been used to characterize this differentiation of microglia into its subtypes (Bennett et al., 2016). Thus, future studies are warranted to investigate the role of miRNAs in inflammation and the differentiation of microglial subtypes. As characterizing microglial activation based on the alteration of functional and transcriptional markers have been well documented in various neuro-inflammation models (Honarpisheh et al., 2020, Bennett et al., 2018), independent validation cohorts were used to investigate the detrimental effects of post-stroke SI in the activation of microglia. We found that post-stroke SI D4 in aged male mice caused a significant activation of microglia, as a measure of the downregulation of P2RY12 expression (MFI) on ($CD45^{Int}CD11b^{+}$) microglia cells, through flow cytometry. However, no change was observed with Tmem119 expression (MFI) on the similar cell type ($CD45^{Int}CD11b^{+}$) suggesting a potential differential effect of SI toward the activation state of microglia. Further studies investigating the role of miRNAs in regulating the differentiation of microglial subtypes are warranted in the context of neuro-inflammation.

Previous reports have connoted the effects of post-stroke SI in cognitive impairment and facilitating depressive-like phenotypes (Venna et al., 2014). Similarly, KEGG and Reactome analysis from the miRNA candidates in post-stroke SI D27 mice revealed many pathways involved in reparative mechanisms such as cell cycle signaling along with pathways involved in learning, memory and cognition. A few pathways involved in microglial activation similar to those found

in post-stroke SI D4 were identified suggesting a prolonged presence of microglial activation in the chronic phases of SI. Future studies are essential to characterize the activation state of microglia at post-stroke SI D27. Recent reports have shown that long term social stress or social isolation can result in depressive-like phenotypes, anhedonia, and decreased urge of social interaction (Macdonald et al., 2021, Shirenova et al., 2021, Corbett et al., 2019). Taken together, our study shows that SI at the acute phase of D1 has no effect towards the host as ischemic damage is predominant, the sub-acute phase of SI has a significant effect toward the activation of microglia, and prolonged isolative conditions leads to the emergence of depressive-like phenotypes in post-stroke SI D27.

Despite our comprehensive approach in identifying potential miRNA targets and performing downstream target prediction/ pathway analysis a few limitations exist. One of which pertains to the concept of mRNA target validation through implementing luciferase assay since the target prediction in this study is putative. Further, Tmem119 expression showed no significant change as compared to P2RY12 expression within post-stroke SI D4 mice, further investigation examining the relation of SI toward the specificity of P2RY12 change is warranted. Further cytokine analysis is essential to characterize this activation state and its influence toward the host neuro-immune landscape. As miRNAs from post-stroke SI D27 mice showed pathways involved in cognitive impairment, memory, and depression; behavioral tests to capture potential cognitive deficits are essential at the chronic phase of SI. Further, as work from our laboratory have shown that miRNA profiles within the circulation of post-stroke SI mice are sexually dimorphic

(Banerjee et al., 2021) and recent reports highlight the role of sex differences in microglial activation (Liu et al., 2019), studies incorporating the biological variable of sex are essential.

In summary, the analysis from this study provides a wide overview of the miRNA alteration observed in the brain of post-stroke SI aged mice across three time points. The concept of social stress, social defeat, and SI have been shown to increase all-cause mortality, impair recovery, and exacerbate immune activation following ischemic injury (Verma et al., 2014, Verma et al., 2018). However standardized treatments options to alleviate the detrimental effects of SI are extremely limited. Our study provides a new avenue in investigating the effects of post-stroke SI toward the host at a post-transcriptional level. Our data supported our initial hypothesis that post-stroke SI leads to a significant alteration of miRNA profiles within the brain which may result in the activation of microglia that may be regulated by particular classes of miRNAs in SI mice. Future work will focus on modulating these miRNA changes through appropriate agomiR or antagomiR treatments to mediate the detrimental effects we see from post-stroke SI. Thus, the primary conclusion of this study is post-stroke SI significantly alters miRNA profiles within the brain to facilitate the activation of microglia.

References:

AJAMI, B., SAMUSIK, N., WIEGHOFER, P., HO, P. P., CROTTI, A., BJORNSON, Z., PRINZ, M., FANTL, W. J., NOLAN, G. P. & STEINMAN, L. 2018. Single-cell mass cytometry reveals distinct populations of brain myeloid cells in mouse neuroinflammation and neurodegeneration models. *Nat Neurosci,* 21**,** 541-551.

ALHAZZANI, A., VENKATACHALAPATHY, P., PADHILAHOUSE, S., SELLAPPAN, M., MUNISAMY, M., SEKARAN, M. & KUMAR, A. 2021. Biomarkers for Antiplatelet Therapies in Acute Ischemic Stroke: A Clinical Review. *Front Neurol,* 12**,** 667234.

ANTONY, M., SCRANTON, V., SRIVASTAVA, P. & VERMA, R. 2020. Micro RNA 181c-5p: A promising target for post-stroke recovery in socially isolated mice. *Neurosci Lett,* 715**,** 134610.

BANERJEE, A., CHOKKALLA, A. K., SHI, J. J., LEE, J., VENNA, V. R., VEMUGANTI, R. & MCCULLOUGH, L. D. 2021. Microarray Profiling Reveals Distinct Circulating miRNAs in Aged Male and Female Mice Subjected to Post-stroke Social Isolation. *Neuromolecular Med,* 23**,** 305-314.

BELOVA, A. N., BOGDANOV, E. I., VOZNYUK, I. A., ZHDANOV, V. A., KAMCHATNOV, P. R., KURUSHINA, O. V. & MASLOVA, N. N. 2021. [Therapy of moderate cognitive impairment in the early recovery period of ischemic stroke]. *Zh Nevrol Psikhiatr Im S S Korsakova,* 121**,** 33-39.

BENNETT, F. C., BENNETT, M. L., YAQOOB, F., MULINYAWE, S. B., GRANT, G. A., HAYDEN GEPHART, M., PLOWEY, E. D. & BARRES, B. A. 2018. A Combination of Ontogeny and CNS Environment Establishes Microglial Identity. *Neuron,* 98**,** 1170-1183.e8.

BENNETT, M. L., BENNETT, F. C., LIDDELOW, S. A., AJAMI, B., ZAMANIAN, J. L., FERNHOFF, N. B., MULINYAWE, S. B., BOHLEN, C. J., ADIL, A., TUCKER, A., WEISSMAN, I. L., CHANG, E. F., LI, G., GRANT, G. A., HAYDEN GEPHART, M. G. & BARRES, B. A. 2016. New tools for studying microglia in the mouse and human CNS. *Proc Natl Acad Sci U S A,* 113**,** E1738-46.

BUTOVSKY, O. & WEINER, H. L. 2018. Microglial signatures and their role in health and disease. *Nat Rev Neurosci,* 19**,** 622-635.

CHANG, L., ZHOU, G., SOUFAN, O. & XIA, J. 2020. miRNet 2.0: network-based visual analytics for miRNA functional analysis and systems biology. *Nucleic Acids Res,* 48**,** W244-w251.

CHEN, Y., LUN, A. T. & SMYTH, G. K. 2016. From reads to genes to pathways: differential expression analysis of RNA-Seq experiments using Rsubread and the edgeR quasi-likelihood pipeline. *F1000Res,* 5**,** 1438.

CHO, Y. K., SON, Y., KIM, S. N., SONG, H. D., KIM, M., PARK, J. H., JUNG, Y. S., AHN, S. Y., SAHA, A., GRANNEMAN, J. G. & LEE, Y. H. 2019. MicroRNA-10a-5p regulates macrophage polarization and promotes therapeutic adipose tissue remodeling. *Mol Metab,* 29**,** 86-98.

CORBETT, B. F., LUZ, S., ARNER, J., PEARSON-LEARY, J., SENGUPTA, A., TAYLOR, D., GEHRMAN, P., ROSS, R. & BHATNAGAR, S. 2019. Sphingosine-1-phosphate receptor 3 in the medial prefrontal cortex promotes stress resilience by reducing inflammatory processes. *Nat Commun,* 10**,** 3146.

DECZKOWSKA, A., KEREN-SHAUL, H., WEINER, A., COLONNA, M., SCHWARTZ, M. & AMIT, I. 2018. Disease-Associated Microglia: A Universal Immune Sensor of Neurodegeneration. *Cell,* 173**,** 1073-1081.

DWIVEDI, Y. 2018. MicroRNAs in depression and suicide: Recent insights and future perspectives. *J Affect Disord,* 240**,** 146-154.

FRICK, L. R., WILLIAMS, K. & PITTENGER, C. 2013. Microglial dysregulation in psychiatric disease. *Clin Dev Immunol,* 2013**,** 608654.

GUO, Y., HONG, W., WANG, X., ZHANG, P., KÖRNER, H., TU, J. & WEI, W. 2019. MicroRNAs in Microglia: How do MicroRNAs Affect Activation, Inflammation, Polarization of Microglia and Mediate the Interaction Between Microglia and Glioma? *Front Mol Neurosci,* 12**,** 125.

HOLTMAN, I. R., RAJ, D. D., MILLER, J. A., SCHAAFSMA, W., YIN, Z., BROUWER, N., WES, P. D., MÖLLER, T., ORRE, M., KAMPHUIS, W., HOL, E. M., BODDEKE, E. W. & EGGEN, B. J. 2015. Induction of a common microglia gene expression signature by aging and neurodegenerative conditions: a co-expression meta-analysis. *Acta Neuropathol Commun,* 3**,** 31.

HONARPISHEH, P., LEE, J., BANERJEE, A., BLASCO-CONESA, M. P., HONARPISHEH, P., D'AIGLE, J., MAMUN, A. A., RITZEL, R. M., CHAUHAN, A., GANESH, B. P. & MCCULLOUGH, L. D. 2020. Potential caveats of putative microglia-specific markers for assessment of age-related cerebrovascular neuroinflammation. *J Neuroinflammation,* 17**,** 366.

HOUSE, J. S., LANDIS, K. R. & UMBERSON, D. 1988. Social relationships and health. *Science,* 241**,** 540-5.

KEREN-SHAUL, H., SPINRAD, A., WEINER, A., MATCOVITCH-NATAN, O., DVIR-SZTERNFELD, R., ULLAND, T. K., DAVID, E., BARUCH, K., LARA-ASTAISO, D., TOTH, B., ITZKOVITZ, S., COLONNA, M., SCHWARTZ, M. & AMIT, I. 2017. A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell,* 169**,** 1276-1290.e17.

KOZOMARA, A., BIRGAOANU, M. & GRIFFITHS-JONES, S. 2019. miRBase: from microRNA sequences to function. *Nucleic Acids Res,* 47**,** D155-d162.

LANGMEAD, B., TRAPNELL, C., POP, M. & SALZBERG, S. L. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol,* 10**,** R25.

LEE, J., D'AIGLE, J., ATADJA, L., QUAICOE, V., HONARPISHEH, P., GANESH, B. P., HASSAN, A., GRAF, J., PETROSINO, J., PUTLURI, N., ZHU, L., DURGAN, D. J., BRYAN, R. M., JR., MCCULLOUGH, L. D. & VENNA, V. R. 2020. Gut Microbiota-Derived Short-Chain Fatty Acids Promote Poststroke Recovery in Aged Mice. *Circ Res,* 127**,** 453-465.

LEE, J., MOHSEN, A., BANERJEE, A., MCCULLOUGH, L. D., MIZUGUCHI, K., SHIMAOKA, M., KIYONO, H. & PARK, E. J. 2021. Distinct Age-Specific miRegulome Profiling of Isolated Small and Large Intestinal Epithelial Cells in Mice. *Int J Mol Sci,* 22.

LI, W., HOU, G., LV, J., LIN, F., SONG, G. & LI, R. 2021. MicroRNA-30d-5p ameliorates lipopolysaccharide-induced acute lung injury via activating AMPKα. *Immunopharmacol Immunotoxicol***,** 1-12.

LIN, X., ZHAN, J., JIANG, J. & REN, Y. 2021. Upregulation of Neuronal Cylindromatosis Expression is Essential for Electroacupuncture-Mediated Alleviation of Neuroinflammatory Injury by Regulating Microglial Polarization in Rats Subjected to Focal Cerebral Ischemia/Reperfusion. *J Inflamm Res,* 14**,** 2061-2078.

LIU, L. L., LI, J. M., SU, W. J., WANG, B. & JIANG, C. L. 2019. Sex differences in depressive-like behaviour may relate to imbalance of microglia activation in the hippocampus. *Brain Behav Immun,* 81**,** 188-197.

LUSARDI, T. A., MURPHY, S. J., PHILLIPS, J. I., CHEN, Y., DAVIS, C. M., YOUNG, J. M., THOMPSON, S. J. & SAUGSTAD, J. A. 2014. MicroRNA responses to focal cerebral ischemia in male and female mouse brain. *Front Mol Neurosci,* 7**,** 11.

MACDONALD, M., YU, Z., WEEKS, L. E., MOODY, E., WILSON, B., ALMUKHAINI, S., MARTIN-MISENER, R., SIM, M., JEFFERIES, K., IDUYE, D., NEEB, D. & MCKIBBON, S. 2021. Assistive technologies that

support social interaction in long-term care homes: a scoping review. *JBI Evid Synth*.

MIGUEL, V., LAMAS, S. & ESPINOSA-DIEZ, C. 2020. Role of non-coding-RNAs in response to environmental stressors and consequences on human health. *Redox Biol,* 37**,** 101580.

MUSUKA, T. D., WILTON, S. B., TRABOULSI, M. & HILL, M. D. 2015. Diagnosis and management of acute ischemic stroke: speed is critical. *Cmaj,* 187**,** 887-93.

O'BRIEN, J., HAYDER, H., ZAYED, Y. & PENG, C. 2018. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front Endocrinol (Lausanne),* 9**,** 402.

POPA, N., BOYER, F., JAOUEN, F., BELZEAUX, R. & GASCON, E. 2020. Social Isolation and Enrichment Induce Unique miRNA Signatures in the Prefrontal Cortex and Behavioral Changes in Mice. *iScience,* 23**,** 101790.

SAMUELSSON, H., VIKEN, J., REDFORS, P., HOLMEGAARD, L., BLOMSTRAND, C., JERN, C. & JOOD, K. 2021. Cognitive function is an important determinant of employment among young ischemic stroke survivors with good physical recovery. *Eur J Neurol*.

SANTIAGO, A. R., BERNARDINO, L., AGUDO-BARRIUSO, M. & GONÇALVES, J. 2017. Microglia in Health and Disease: A Double-Edged Sword. *Mediators Inflamm,* 2017**,** 7034143.

SHI, L., SUN, Z., SU, W., XU, F., XIE, D., ZHANG, Q., DAI, X., IYER, K., HITCHENS, T. K., FOLEY, L. M., LI, S., STOLZ, D. B., CHEN, K., DING,

Y., THOMSON, A. W., LEAK, R. K., CHEN, J. & HU, X. 2021. Treg cell-derived osteopontin promotes microglia-mediated white matter repair after ischemic stroke. *Immunity*.

SHIRENOVA, S. D., KHLEBNIKOVA, N. N. & KRUPINA, N. A. 2021. Long-Term Social Isolation Reduces Expression of the BDNF Precursor and Prolyl Endopeptidase in the Rat Brain. *Biochemistry (Mosc),* 86**,** 704-715.

STEPTOE, A., SHANKAR, A., DEMAKAKOS, P. & WARDLE, J. 2013. Social isolation, loneliness, and all-cause mortality in older men and women. *Proc Natl Acad Sci U S A,* 110**,** 5797-801.

STREIT, W. J., MRAK, R. E. & GRIFFIN, W. S. 2004. Microglia and neuroinflammation: a pathological perspective. *J Neuroinflammation,* 1**,** 14.

SU, S., ZHAO, Q., HE, C., HUANG, D., LIU, J., CHEN, F., CHEN, J., LIAO, J. Y., CUI, X., ZENG, Y., YAO, H., SU, F., LIU, Q., JIANG, S. & SONG, E. 2015. miR-142-5p and miR-130a-3p are regulated by IL-4 and IL-13 and control profibrogenic macrophage program. *Nat Commun,* 6**,** 8523.

VENNA, V. R., VERMA, R., O'KEEFE, L. M., XU, Y., CRAPSER, J., FRIEDLER, B. & MCCULLOUGH, L. D. 2014. Inhibition of mitochondrial p53 abolishes the detrimental effects of social isolation on ischemic brain injury. *Stroke,* 45**,** 3101-4.

VENNA, V. R., WESTON, G., BENASHSKI, S. E., TARABISHY, S., LIU, F., LI, J., CONTI, L. H. & MCCULLOUGH, L. D. 2012. NF-κB contributes to the detrimental effects of social isolation after experimental stroke. *Acta Neuropathol,* 124**,** 425-38.

VERMA, R., FRIEDLER, B. D., HARRIS, N. M. & MCCULLOUGH, L. D. 2014. Pair housing reverses post-stroke depressive behavior in mice. *Behav Brain Res,* 269**,** 155-63.

VERMA, R., RITZEL, R. M., HARRIS, N. M., LEE, J., KIM, T., PANDI, G., VEMUGANTI, R. & MCCULLOUGH, L. D. 2018. Inhibition of miR-141-3p Ameliorates the Negative Effects of Poststroke Social Isolation in Aged Mice. *Stroke,* 49**,** 1701-1707.

YANG, S. H. & LIU, R. 2021. Four Decades of Ischemic Penumbra and Its Implication for Ischemic Stroke. *Transl Stroke Res*.

ZHANG, Q., HE, L., CHEN, M., YANG, H., CAO, X., LIU, X., HAO, Q., CHEN, Z., LIU, T., WEI, X. E. & RONG, L. 2021. PSD-93 mediates the crosstalk between neuron and microglia and facilitates acute ischemic stroke injury by binding to CX3CL1. *J Neurochem,* 157**,** 2145-2157.

Vita:

Anik Banerjee completed high school at A&M Consolidated High School in College Station, TX within the class of 2015. Following high graduation, he entered Texas A&M University as an undergraduate in that same Fall. He received his Bachelor of Sciences in Chemistry from Texas A&M University in May of 2019. In August of 2019 he began working towards his Master's degree at The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences.

Permanent address:

1850 Old Main Street

Houston, Texas 77030

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

Jul 12, 2021

This Agreement between Anik Banerjee ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	5106811405638
License date	Jul 12, 2021
Licensed Content Publisher	Springer Nature
Licensed Content Publication	NeuroMolecular Medicine
Licensed Content Title	Microarray Profiling Reveals Distinct Circulating miRNAs in Aged Male and Female Mice Subjected to Post-stroke Social Isolation
Licensed Content Author	Anik Banerjee et al
Licensed Content Date	Oct 19, 2020
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute
Format	electronic
Portion	full article/chapter
Will you be	no

translating?

Circulation/distribution	1 - 29
Author of this Springer Nature content	yes
Title	MicroRNAs as a Therapeutic Target to Regulate Microglial Activation in Post-Stroke Isolation in Aged Male Mice
Institution name	MD Anderson UTHealth Graduate School of Biomedical Sciences
Expected presentation date	Aug 2021
Requestor Location	Anik Banerjee 6431 Fannin Street HOUSTON, TX 77030 United States Attn: Anik Banerjee
Total	0.00 USD

Terms and Conditions

Springer Nature Customer Service Centre GmbH
Terms and Conditions

This agreement sets out the terms and conditions of the licence (the Licence) between you and Springer Nature Customer Service Centre GmbH (the Licensor). By clicking 'accept' and completing the transaction for the material (Licensed Material), you also confirm your acceptance of these terms and conditions.

1. Grant of License

1. 1. The Licensor grants you a personal, non-exclusive, non-transferable, world-wide licence to reproduce the Licensed Material for the purpose specified in your order only. Licences are granted for the specific use requested in the order and for no other use, subject to the conditions below.

1. 2. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of the Licensed Material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of

another entity (as credited in the published version).

1. 3. If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. Scope of Licence

2. 1. You may only use the Licensed Content in the manner and to the extent permitted by these Ts&Cs and any applicable laws.

2. 2. A separate licence may be required for any additional use of the Licensed Material, e.g. where a licence has been purchased for print only use, separate permission must be obtained for electronic re-use. Similarly, a licence is only valid in the language selected and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence. Any content owned by third parties are expressly excluded from the licence.

2. 3. Similarly, rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee. Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

2. 4. Where permission has been granted free of charge for material in print, permission may also be granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

2. 5. An alternative scope of licence may apply to signatories of the STM Permissions Guidelines, as amended from time to time.

3. Duration of Licence

3. 1. A licence for is valid from the date of purchase ('Licence Date') at the end of the relevant period in the below table:

Scope of Licence	Duration of Licence
Post on a website	12 months
Presentations	12 months
Books and journals	Lifetime of the edition in the language purchased

4. Acknowledgement

4. 1. The Licensor's permission must be acknowledged next to the Licenced Material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book's homepage. Our required acknowledgement format is in the Appendix below.

5. Restrictions on use

5. 1. Use of the Licensed Material may be permitted for incidental promotional use and minor editing privileges e.g. minor adaptations of single figures, changes of format, colour and/or style where the adaptation is credited as set out in Appendix 1 below. Any other changes including but not limited to, cropping, adapting, omitting material that affect the meaning, intention or moral rights of the author are strictly prohibited.

5. 2. You must not use any Licensed Material as part of any design or trademark.

5. 3. Licensed Material may be used in Open Access Publications (OAP) before publication by Springer Nature, but any Licensed Material must be removed from OAP sites prior to final publication.

6. Ownership of Rights

6. 1. Licensed Material remains the property of either Licensor or the relevant third party and any rights not explicitly granted herein are expressly reserved.

7. Warranty

IN NO EVENT SHALL LICENSOR BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL OR INDIRECT DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND
WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

8. Limitations

8. 1. *BOOKS ONLY:*Where 'reuse in a dissertation/thesis' has been selected the following terms apply: Print rights of the final author's accepted manuscript (for clarity, NOT the published version) for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/).

8. 2. For content reuse requests that qualify for permission under the STM Permissions Guidelines, which may be updated from time to time, the STM Permissions Guidelines supersede the terms and conditions contained in this licence.

9. Termination and Cancellation

9. 1. Licences will expire after the period shown in Clause 3 (above).

9. 2. Licensee reserves the right to terminate the Licence in the event that payment is not received in full or if there has been a breach of this agreement by you.

Appendix 1 — Acknowledgements:

For Journal Content:
Reprinted by permission from [the Licensor]: [Journal Publisher (e.g. Nature/Springer/Palgrave)] [JOURNAL NAME] [REFERENCE CITATION (Article name, Author(s) Name), [COPYRIGHT] (year of publication)

For Advance Online Publication papers:
Reprinted by permission from [the Licensor]: [Journal Publisher (e.g. Nature/Springer/Palgrave)] [JOURNAL NAME] [REFERENCE CITATION (Article name, Author(s) Name), [COPYRIGHT] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].)

For Adaptations/Translations:
Adapted/Translated by permission from [the Licensor]: [Journal Publisher (e.g. Nature/Springer/Palgrave)] [JOURNAL NAME] [REFERENCE CITATION (Article name, Author(s) Name), [COPYRIGHT] (year of publication)

Note: For any republication from the British Journal of Cancer, the following credit line style applies:

Reprinted/adapted/translated by permission from [the Licensor]: on behalf of Cancer Research UK: : [Journal Publisher (e.g. Nature/Springer/Palgrave)] [JOURNAL NAME] [REFERENCE CITATION (Article name, Author(s) Name), [COPYRIGHT] (year of publication)

For Advance Online Publication papers:
Reprinted by permission from The [the Licensor]: on behalf of Cancer Research UK: [Journal Publisher (e.g. Nature/Springer/Palgrave)] [JOURNAL NAME] [REFERENCE CITATION (Article name, Author(s) Name), [COPYRIGHT] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM])

For Book content:
Reprinted/adapted by permission from [the Licensor]: [Book Publisher (e.g. Palgrave Macmillan, Springer etc) [Book Title] by [Book author(s)] [COPYRIGHT] (year of publication)

Other Conditions:

Version 1.3

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.