PRECLINICAL ASSESSMENT OF MICRORNA THERAPY FOR RECOVERY OF LONG TERM STROKE OUTCOME

A Dissertation

by

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ABSTRACT

Menopause not only signifies the end of the reproductive age for women, but also marks the time period of higher risk for cardiovascular disease such as stroke. In older females, stroke leads to greater disabilities in physical, communicative, emotional and cognitive functions. About a third of stroke survivors develop depression or cognitive impairment over time and there is no specific drug approved for these behavior impairments. This dissertation focused on testing therapeutic effects of small non-coding (micro)RNA for post stroke depression and cognitive impairment. Middle-aged female rats were used to best resemble the post-menopausal female population. In the experiments included in this dissertation we tested the therapeutic effects of a microRNA, mir363-3p in long-term stroke outcome such as depression and cognitive decline. In experiment 1, we found that a depressive-like phenotype develops in middle-aged female rats 1-3 months after stroke and is accompanied by transient increases in pro-inflammatory cytokines and decrease in the circulating levels of Brain Derived Neurotrophic Factor (BDNF). The mesostriatal dopaminergic pathway, a part of the reward pathway, showed retrograde degeneration in the ischemic hemisphere. Mir363-3p treatment improved the depressive-like phenotype as well as normalized the cytokines and growth factors in the blood. In addition to this, the mesostriatal pathway was preserved in the mir363-3p treated group. Cognitive impairment did not occur up to 3 months in experiment 1. Experiment 3 found that cognitive impairment develops by 6 months after stroke, and the impairment was in the spatial as well as non-spatial memory. Mir363-3p improved both aspects of memory. In experiment 2, we tested if the depressive phenotype displayed by rats from experiment 1 was associated with changes in the gut microbiome. We found that stroke animals had lower circulating level of tryptophan, a

metabolite from the gut. Furthermore, gut dysbiosis was also evident in this group in the form of an increased ratio of Firmicutes: Bacteroidetes (F:B) ratio and reduced expression of specific bacterial families associated with the depressive phenotype. Collectively, these data show that mir363-3p has a therapeutic role for not only acute sensorimotor deficit after stroke, but also long-term depression and delayed cognitive impairment.

DEDICATION

I would like to dedicate this work to my family and all the mentors in my life who have inspired me to always pursue my dreams.

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Contributors

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CHAPTER I

INTRODUCTION

Stroke

Stroke is the fifth leading cause of death in the US and a major cause of disability for adults [1, 2]. It is a neurological disorder, which is vascular in origin and leads to death of brain tissue. There are two different ways the tissue dies: from lack of oxygen and glucose supply to the brain tissue (ischemic stroke) when a blood vessel is blocked, or from mechanical pressure of the pooled blood on the brain parenchyma (hemorrhagic stroke) when a blood vessel ruptures. Either way, a stroke leads to long-term disabilities or death. The most common clinical symptom of a stroke is sensory loss, neglect, muscle weakness and loss of control, dysarthria, dysphagia or death [3]. A diagnosis is made by focusing on the symptoms, performing physical exams for sensorimotor skills and imaging by CT scans or MRI.

About 87% of strokes occurring in the US are ischemic in nature[4]. A thrombus (blood clot that forms locally over time) or embolus (a blood clot, gas or fat that is formed elsewhere in the body) gets dislodged from the original vessel, enters circulation and blocks another blood vessel interrupting nutrient supplies to the tissue. When the oxygen and glucose supply to tissue is compromised (a condition known as ischemia), the neurons undergo stress, which initiates a cascade of cellular responses such as free-radical formation or rapid release of toxic levels of excitatory neurotransmitters [3]. If the ischemia is prolonged, the stressed neurons undergo cell death in the form of apoptosis or necrosis. Stress signals and cell death activate local microglia and astrocytes, which then triggers immune infiltration into the brain and contributes to further injury to the brain [5]. About 75% of the ischemic stroke occurs due to a blockage of Middle

Cerebral Artery (MCA) which is the largest branch of common carotid artery and supplies motor cortex, sensory cortex, Wernicke area and broca area [6]. An occlusion of this artery leads to hemiparesis or hemiparalysis of the contralateral lower half of the face and extremities (upper and lower), sensory loss of the contralateral face and extremities, ataxia and speech impairment.

Treatment

There are currently two treatment options for ischemic stroke: Tissue Plasminogen Activator (tPA) and Thrombectomy. tPA is delivered iv (or intra-arterially)and acts to dissolve the clot and reestablishing the perfusion in the blood vessel [7]. However, it is also capable to activating matrix metalloproteinases (MMPs) [8] that degrade the extracellular matrix of the blood vessel, possibly leading to a hemorrhage. As a result, an optimal therapeutic window for administration (1-4.5h after onset of symptoms) is established for tPA [9]. If administered within the window, the recovery outweighs the adverse outcomes and tPA reduces mortality and morbidity[9]. Currently about 6-8% of stroke patients qualify for tPA, however studies has shown that half of these patients do not recover or even die after tPA treatment [10, 11]. Thrombectomy is an endovascular procedure that removes the clot directly from the blood vessel. However, this procedure being an invasive one has more strict criteria. Mechanical thrombectomy is indicated for stroke due to a large artery occlusion in the anterior circulation and has a window of 24h. However only about 10 percent of the patients have occlusion in the large artery. Since majority of stroke patients do not qualify for this treatment [12], there is a need for an effective therapy that could go beyond the therapeutic window and get accessible to a larger population.

Differences in stroke outcome: Age and Sex

Stroke is a common disease among the elderly population [13]. Young adults generally have a better outcome after stroke especially young women[14]. This is likely due to the neuroprotective role of estrogen in women. Even though men are more likely to get stroke as compared to women, the lifetime risk for stroke, however, is greater for women due to their longer lifespan [15-18]. Also, middle-aged women (around the age for menopause) have a higher risk for stroke [19] . This led to an idea that estrogen treatment would protect older women from stroke. However, a clinical trial called the Women's Health Initiative (WHI) which was designed to compare women ages 50-89 who were administered either conjugated equine estrogen (CEE) or placebo had to be stopped prematurely since CEEs were found to increase the risk for myocardial infarction and stroke instead of improving them [20]. This unexpected effect was successfully replicated in animal studies as well. Estrogen administration to ovariectomized young females was neuro-protective, but in middle-afed female rats, it led to worse stroke outcome [21]. This dissertation work focuses on novel therapeutic for stroke outcomes in adult middle-aged female rats.

MicroRNA therapy for stroke

MicroRNA are a class of small non-coding RNAs that regulate the expression of multiple target genes through sequence-specific hybridization to the 3' Un-Translated Region (UTR) of messenger RNAs [22], thus playing an integral role in several biological processes such as immune modulations, cell cycle, metabolic control, stem cell differentiation and neuronal survival [23] . MiRNA expressions are altered in several diseases, and manipulation of these molecules has emerged as novel therapeutics for various pathological conditions such as cancer,

metabolic disorders, cardiovascular diseases and neurological disorders/neurodegenerative diseases including stroke [24-26]. Many preclinical studies are now focused on using microRNA as a therapeutic for stroke [27]. Our lab was among the first group to show that miRNA profiles in circulation and the brain are altered in an age- and sex-specific manner and that manipulating miRNA using either mimic sequence or inhibitory sequence can improve stroke outcomes [28-30]. In the case of mir363-3p, the neuroprotective effect appears to involve the caspase-3 mediated cell death pathway that is activated after stroke.

A number of promising results with microRNA as neuroprotectants (reviewed in Sohrabji and Selvamani, 2019), along with the ease of administration and sufficient uptake in the tissue without any off-target effects make microRNA therapy very attractive. This dissertation consists of a series of studies which show that mir363-3p injection delivered once 4h after stroke, improves not only early sensorimotor deficits after stroke, but also long term development of depression and cognitive impairment.

Long term consequences of stroke: Depression and Cognition

Stroke leads to disabilities in not only physical and communication functions but also emotional and cognitive functions in the long term. Over one third of the survivors eventually develop depression and cognitive impairment that do not respond well to therapeutics. A meta-analysis of 43 studies [31] reported a cumulative incidence of depression of 39-52% five years within stroke and prevalence of 29% within 10 years. Another meta-analysis of 61 studies [32] reported that the incidence of depression was 31% within 5 years of stroke. Post stroke depression severely affects physical and cognitive recovery after stroke. It presents with anhedonia, social

dysfunctioning, feeling of despair, greater dependency on others for day-to-day activities, psychomotor retardation, weight gain or weight loss and inability to focus. Compliance to medication as well as rehabilitation therapy is also affected by the state of depression [33]. This severely affects the quality of life of the survivor. Studies have shown that caregivers of the depressed survivors also tend to develop depression over time [34].

There are multiple theories indicated for pathogenesis of depression. The 'Biogenic monoamine' theory suggests low monoamine neurotransmitters like serotonin and catecholamine are responsible for development of depression [35]. Most prescribed anti-depressants work by increasing serotonin or catecholamine in the synaptic cleft by either inhibiting their re-uptake or inhibiting Monoamine oxidase (MAO) [36]. The 'Neurotropic' theory suggests that shrinkage of the frontal- cortex and hippocampus, resulting in low expression of the neurotrophin BDNF in these regions may be an underlying cause of depression [37]. This is supported by evidence that electroconvulsive therapy as well as anti-depressants such as fluoxetine, sertraline and desipramine increase BDNF expression in frontal-cortex and hippocampus [38, 39]. The 'Inflammation' theory defines depression as an inflammatory disorder in which pro-inflammatory cytokines such as CRP, IL-6, IL-1, IFN and TNF are elevated [40] [41]. TNF and IFN activate Indole-amine 2,3 dioxygenase (IDO) that metabolizes tryptophan, a precursor for serotonin, leading to a decrease in serotonin production [42]. Serotonin signaling across the synapse is also affected by IL-1 and TNF which stimulate serotonin uptake and IFN-mediated decreases of serotonin receptor 1A [43]. The 'Neural circuitry disruption' theory suggests a disruption in the reward pathways, featuring midbrain-striatum-cortex circuitry, leads to development of depression [44]. Natural rewards like food, liquid, sex and many drugs of abuse

increase extracellular dopamine in ventral striatum (Nucleus Accumbens, NAc) [45] [46] by stimulating VTA Dopaminergic (DA) neurons, which is then relayed to the dorsal striatum, via a synapse on Substantia Nigra (SN) neurons [47]. Disruption of the VTA-Striatum circuitry as well as the striato-nigro-striatal connection between ventral and dorsal striatum is reported to disrupt the reward system (Fig 1) and addiction [48].

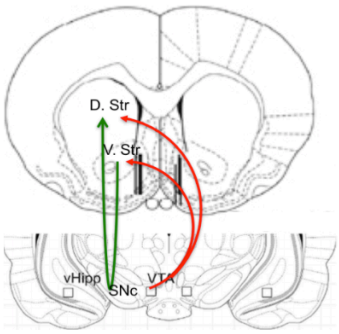

Figure 1: Schematic representation of meso-striatal reward pathway. D. Str: Dorsal Striatum; V. Str: Ventral Striatum; SNc: Substantia Nigra compacta; VTA: Ventral Tegmental Area

Like post stroke depression, cognitive impairment is also a common consequence after stroke that significantly reduces quality of life. In fact cognitive impairment usually co-occurs with depression [49]. More than half of stroke patients suffer from some levels of cognitive impairment by 6 months after stroke [50, 51]. Patients who do not develop cognitive impairment directly after stroke have a 9-fold increased risk of developing delayed cognitive impairment [52]. Cognitive impairment could present as deficit in any of these given aspects: complex attention, executive functioning, memory, language, perceptual-motor/visuospatial function and social cognition. A diagnosis is usually made when there is a significant impairment in at least one of these domains. The exact mechanisms for the post stroke cognitive dysfunction are still

not well known. Studies have suggested it could be due to the ischemic insult to various region of the brain. The prefrontal cortex is an important region for cognitive functioning. It provides an executive, 'top-down' control on cognition by suppressing old distracting memories from interfering with new information that is being learned [53-55]. Accordingly, ischemic injury to the pre-frontal cortex induces cognitive dysfunction [56]. Similarly, entorhinal cortex connects the hippocampus to other associated cortices to form spatial as well as non-spatial memory [57, 58]. These regions are easily vulnerable to global as well as focal ischemia in the brain. In addition to the direct injury from ischemia, increased inflammation after stroke also leads to cognitive deficit. Increased inflammatory response in the form of activated microglia has been observed in hippocampus of the rats that have cognitive deficit after stroke [59].

Despite the occurrence of depression and cognitive impairment after stroke, we still do not have effective therapeutics for these disorders. SSRI fluoxetine is not efficacious for PSD until at least a year of administration and the TCA family of drugs such as imipramine, amitriptyline and amoxapine are not as effective for PSD in older (65+ years) women compared to age-matched men or young females [60, 61]. For cognitive impairment after stroke, we still do not have a specific drug approved. Clinicians sometimes prescribe drugs approved for Alzheimer's disease such as donepezil and mementine for cognitive deficits. These drugs have shown some improvement for cognition but not in the global functioning of the patients[62-64]. These results suggest a unique pathogenesis for depression and cognitive impairment after stroke. This dissertation is focused on modeling depression and cognitive impairment in middle-aged rats, and finding an effective therapeutics for these long-term disorders that occur after stroke.

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CHAPTER II

MIR363-3P ATTENUATES POST-STROKE DEPRESSIVE-LIKE BEHAVIORS IN MIDDLE-AGED FEMALE RATS[1]

Introduction

In addition to physical and cognitive impairments, post-stroke depression (PSD) is a critical, long-term consequence of stroke. PSD is characterized by anxiety, anhedonia, social dysfunction and feelings of despair[1-3]. About one-third of stroke survivors develop PSD[4], which not only affects their quality of life but also adversely influences their recovery[5, 6]. Women are more likely to develop PSD than men[7]. Although the mechanistic explanation for this sex difference is poorly understood, it may be related to greater disability after stroke in women, which makes them less independent in their daily activities and consequently more likely to be in assisted-living facilities[8, 9]. Social isolation and lower quality of life resulting from institutional living also contributes to the development of PSD.

Depression is associated with several central and peripheral biochemical markers, including changes in monoamine neurotransmitter levels [10, 11], inflammatory cytokines [12-14], and neurotrophic factors [15, 16]. Anti-depressant therapies therefore include tricyclic drugs (TCA) that increase levels of norepinephrine and serotonin, or newer drugs such as the serotonin reuptake inhibitors (SSRI). However, the SSRI fluoxetine is not efficacious for PSD until at

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least a year of administration[17] and the TCA family of drugs such as imipramine, amitriptyline and amoxapine are not as effective for PSD in older (65+ years) women compared to age-matched men or young females[18]. This resistance to conventional anti-depressants suggests the likelihood of a unique pathophysiology of PSD.

In animal models, occlusion of the middle cerebral artery (MCAo), which is the most commonly affected artery in ischemic stroke, can result in a depressive phenotype[19-21]. MCAo damages the striatum and overlying cortex and loss of striatal growth factors such as BDNF may contribute to the depressive phenotype[20, 22]. Accordingly, BDNF treatment after stroke has been shown to reduce post stroke depression [23]. The striatum also receives projections from midbrain dopaminergic neurons, and loss of these 'reward' pathways may constitute another substrate for post stroke depression.

We hypothesize that if ischemic cell loss contributes to the pathology of PSD, then drugs that reduce infarct volume in the acute stage of stroke may also reduce PSD. Our recent studies show that a small non-coding RNA mir363-3p is inversely correlated with infarct size and stroke outcome [24], and a single IV injection of mir363-3p, delivered 4h after MCAo, reduces infarct volume and improves sensory-motor performance in middle-aged female rats [25]. Remarkably, mir363-3p treatment to age-matched males had no effect on infarct volume or sensory motor impairment, adding the growing literature on stroke neuroprotectants that display sex specific effects. Hence this study focused on the long-term effects of mir363-3p treatment in females only. Middle-aged female rats were subject to MCAo and treated 4h later with mir363-3p and assessed for motor impairment and depressive behaviors for a period of 3 months. We report that

MCAo leads to sensory motor impairment in the acute stage of ischemia, which resolves over time, and depressive-like behaviors, which are observed at 100+ days post stroke. Treatment with mir363-3p attenuates both acute motor impairment and long term depressive behaviors in middle-aged females. These data are consistent with the hypothesis that ischemic pathology contributes to PSD and that neuroprotectants, that reduce infarct volume, may act as anti-depressant therapies for PSD.

Methods and Materials

Animals and estrous cycle determination: Middle-aged female rats (10-12 months, 230-340g) were purchased from Envigo (IN). All animals were housed in a temperature (22°C) and humidity (45-55%) controlled AAALAC-accredited vivarium facilities on a 12/12 h light and dark cycle. Water and food were available ad libitum. Three weeks after arrival, the rats underwent daily vaginal smearing for up to 21 days to determine estrous status as described previously [26-29]. Cotton swabs were used to obtain vaginal smears and cells were deposited on glass slides. When the animals displayed cell cytology indicating constant diestrus for at least 7 days, they were included in the study. Our previous studies have shown that females in constant diestrus have low estradiol levels and elevated FSH levels, which resembles salient hormonal aspects of the post-menopausal stage in women ([26, 30]). A total of 40 animals were used in these studies. A timeline of the procedures and behavioral assays is shown in Fig 2. All procedures were performed under approved protocols in accordance with institutional guidelines for humane treatment of animals. Animals were randomly assigned to scrambled control or mir363-3p treatment groups after middle cerebral artery occlusion. All behavioral tests were

performed between 8:00h and 12:00h, and experimenters were blind to the treatment conditions while performing and analyzing tests.

Middle Cerebral Artery Occlusion (MCAo): Animals were subjected to MCAo by stereotaxic injection of a vasoconstrictor, Endothelin-1 (ET-1), using our previously established procedures[24, 25, 31, 32]. Animals were anesthetized (20mg/ml/kg xylazine and 100 mg/kg ketamine) and placed in a stereotaxic equipment. Endothelin-1 (ET-1) (American Peptide Company INC; 0.5ug/ul, 600 pmol; 3 uls) was microinjected to occlude the left middle cerebral artery at the following coordinates relative to bregma: AP: +0.9, ML: -3.4, DV: -8.5. Body temperature was maintained at 37 °C throughout the surgery and in the home cage until recovery from anesthesia. For Sham surgeries, animals were subject to all other surgical procedures (such as anesthetic, scalp incision, drilling of the skull), but did not receive ET-1.
Four hours after ET-1 or Sham surgery, animals received either control oligos (scrambled) or miR-363-3p delivered via tail vein injection. Scrambled and Mir363-3p mimic (AAUUGCACGGUAUCCAUCUGU) oligonucleotide sequences were purchased from Thermo Fisher, Grand Island, NY and packaged in In-vivo RNA-LANCEr II (Bio-Scientific, Austin, TX).

Behavioral Assays: All the behavior tests were performed between 8:00 am-12:00 pm in the light cycle. A timeline of these tests is shown in Fig 2 and described here. For assessing sensorimotor function, adhesive-tape removal test was done before (-2d) and after (2d, 5d, 60d and 100d) stroke. Similarly, assays for depressive-like phenotype were performed before and after stroke. Forced Swim Test and Social Interaction: -9d and 100d. T Maze cost/benefit task: -10d, 30d, 60d

and 98d. Grip strength, Locomotion and Novel object recognition was performed at 100d after stroke. All the tests were performed and scored blinded.

Adhesive-tape removal test: This test was performed prior to and after MCAo using our previously published methods[24, 25, 31, 33]. Adhesive-backed foam tape (12.7 X 12.7 mm) was attached to the palmar surface of the paw of each forelimb. For each limb, the time taken to remove the tape was recorded for three trials. Animals were allowed to rest for 5 min between trials and each trial had a maximum time limit of 120s.

Forced Swim Test: This test was used to determine helplessness[20]. Rats were placed in an open cylinder (diameter 30 cm, height 60cm) filled with water (23-25^{o}C) to a height of 40 cm for 5 min. Sessions were video-recorded with an overhead camera and the duration of immobility was scored by a blind observer. A rat was judged to be immobile if it was passively floating in water with only movements necessary to keep its head above water.

Social Interaction Test: A three-chambered Plexiglass box was used for the social interaction test. Each chamber had a dimension of 40”x13”and all chambers had open access to each other. For habituation, the three chambers were closed off from each other, and the test rat was placed in each chamber for 2 min. Thereafter, the rat was allowed to freely explore the 3 chambers for additional 10 min and then returned to the home cage. For the testing session, a conspecific (stranger) rat was placed within a plastic mesh cylinder in one of the end chambers, while the test rat was placed back in the middle chamber and allowed to explore for 10 min. The time spent in

each chamber was recorded and sociability was scored as the total time (in seconds) spent by the test rat in the chamber with the stranger rat.

T-maze cost/benefit task: This test was used to determine anhedonia. The physical apparatus consisted of a T-maze, where the 2 end arms were 20" long and 5" wide, and the central limb was 6" long and 6" wide. Each arm was baited with sucrose pellets during the test session. For habituation, the rats were allowed to freely explore the maze for 2 consecutive days for 30 min each day and sucrose pellets placed on the floor of the home-cage for 2 days. For training (daily for 6 weeks), rats were deprived of food for 12 hours before the training on each alternate day for the first 2 weeks and were given 10 trials to choose an arm. After each trial, the rat was placed in its home cage, for one minute, and then placed back to the start arm of the maze. For the first week, a small barrier (6 cm rectangular box) was placed midway on one arm of the T-maze and a sucrose pellet placed in the far corner of the arm. The rats were trained for to go over the small barrier to retrieve the sucrose pellet. From the second week, a large barrier (12 cm rectangular box) was also added midway down the other arm of the T-maze and 4 sucrose pellets placed in the far corner of this arm. Arm choice was recorded for each trial up to 10 trials. After 6 weeks of training, the test was repeated 10 days before the stroke as a pre-stroke baseline measure. Following stroke, the test was repeated at 30d, 60d and 98d after MCAo.

Open Field Test: Locomotor activity was assessed using the Open Field Arena (Kinder Scientific, CA). Rats were placed in the middle of a clear acrylic box (16" by 16") and allowed to freely explore for 30 min. General locomotor activity was quantified by the total number of

beam breaks over 30 min using MotorMonitor Software, as well as the percent of beam breaks in the center of the field.

Novel Object Recognition Test: This test was used to assess cognition. This test consisted of three phases: habituation, familiarization, and test phase. In the habituation phase, the rat was placed in 16"x16" open field and allowed to freely explore the arena for 10 min each day for 2 days. On the third day (familiarization phase), the animal was again placed in the open-field apparatus, which now contained two identical objects (*A* + *A*) placed diagonally from each other. The rat was allowed to explore the arena and the objects for 10 minutes. The rat was then returned to its home cage for 1h (retention interval) and then placed again in the open-field arena for the test phase. For the test phase, the arena contained two objects in the same location, one that was previously available (*A*) and the other that was novel (*B*). The rats' behavior was recorded for 5 mins and the amount of time spent exploring the novel object was determined from these recordings by an investigator blind to the experimental condition. Exploration of an object was defined as the animal's snout directed to the object, sniffing or touching the object with its snout at a distance <2 cm to the object and/or touching it with the nose. Running around the object, sitting or climbing on it was not recorded as exploration. The discrimination index (%) was calculated as: (Time spent exploring Novel object)/(Time spent exploring Novel + Familiar object) *100.

Grip Strength Test: Grip strength was measured to ensure that MCAo-induced physical disability did not contaminate performance on the depressive behaviors. Rats were held by the tail and lowered towards the rod attached to the grip strength meter. Once the animal grabbed

the bar, it was pulled backwards in a horizontal plane. The force applied to the bar just before it loses grip was recorded as the peak tension. Three such trials were performed, and the mean peak tension was normalized to body weight in grams.

Cytokine Assay: Rat cytokine/chemokine assay kit (MAP kit, Millipore, CA) was used to quantify a panel of inflammatory cytokines/chemokines in serum, using manufacturer's instructions and our previously established procedures [29]. Plates were read on a Bio-Plex suspension array system (Bio-Rad Laboratory, CA).

BDNF expression: BDNF levels were quantified in serum samples using a Rat BDNF ELISA Kit (ThermoScientific, MA) and manufacturer's instructions. BDNF was detected by a sandwich ELISA and a colorimetric readout. Absorbance was measured on ELISA microplate reader set to 450nm. Sample unknowns were interpolated from a standard curve.

Assessment of the meso-striatal pathway: Retrograde labeling by fluorogold was used to assess meso-striatal pathway 100d+ after stroke. Animals were anesthetized and placed in a stereotaxic frame. Fluorogold (0.2ul of 2% (dissolved in de-ionized water), Santa Cruz Biotechnology, TX) was injected into both the left and right striatum at 2 depths (0.1ul in each depth) using 1-ul Hamilton microsyringe. The coordinates for the injection from bregma were as follows: 1mm anterior, 3mm lateral, 4.5mm and 5.5mm ventral from dura. The needle was slowly retracted 5 minutes after injection to prevent backflow. Five days later, rats were deeply anesthetized and perfused transcardially with saline followed by 4% formaldehyde. The brain was removed and submerged in 4% paraformaldehyde overnight. Thereafter brains were prepared for block

embedding and sectioning (30um) (NeuroScience Asssociates, TN). Sections through the striatum were inspected for Flg label and the rostro caudal extent of the Flg injection was calculated for each hemisphere for each animal. Animals where the injections did not cover 75% of the rostrocaudal extent of the striatum would be excluded from further analysis. No animal met this criterion and thus all injected animals were analyzed. Subsequently, sections through the SNc and VTA were imaged under fluorescent illumination (4X magnification) for Flg and photographed using Q-capture (QImaging, BC, Canada). Three sections per animal, 240 micrometers apart, were selected and brightly fluorescent neurons in the SNc and VTA region was counted in both hemispheres using ImageJ. The total number of cells in each region was added together for each hemisphere.

Statistical Analyses: For all assays, group mean +SEM are reported. Group differences were determined by a two-way ANOVA performed for surgery (Sham/MCAo) and treatment (Scrambled/Mir363-3p), with planned comparisons. For analyses where only 2 groups were compared (mesostriatal projections), a student t-test was used. All group differences were considered significant at $p<0.05$. Statistics were analyzed using Prism GraphPad (GraphPad, San Diego, CA) and SPSS (IBM Corporation).

Results

Mir363-3p improves sensory-motor deficit after stroke: Sensory motor performance was evaluated by the Adhesive-tape removal test, which was performed at 5 different time points: prior to MCAo (baseline/pre), 2d, 5d, 60d and 100d after MCAo. As shown in Fig 3, animals removed the tape rapidly at the pre-stroke time point but were significantly delayed in the early

acute stroke phase (2-5d) in both the MCAo+scrambled and MCAo+mir363-3p group as compared to pre-stroke performance ($F_{(4,68)}$: 5.189, p=0.0010). At 5d post stroke, planned comparison showed that the MCAo+scrambled group was significantly delayed compared to its pre-stroke timings (p=0.0101), while the mir363-3p treated group was not different from baseline. Both groups recovered substantially in the chronic phase where their performance was not different from baseline at 60d (p=0.7518), or 100d (p>0.9999) after stroke. Thus, mir363-3p treatment accelerates the rate of sensory motor recovery as compared to controls. This is consistent with previous work that showed that mir363 treatment reduced sensorimotor deficit and reduced infarct volume in the acute phase following MCAo[25].

Mir363-3p treatment abrogates the development of depressive-like behaviors after stroke: Depressive behaviors were assessed by a battery of tests including T-maze cost/benefit task, social interaction and forced swim test (Fig 2) to capture the following domains of depressive-like behaviors: anhedonia, reduced sociability and vulnerability to helplessness in stressful events, respectively.

T-maze cost/benefit task: Anhedonia was assessed using a T-maze cost/benefit task, which gives rats two options: Climb a high barrier for high reward (4 sucrose pellets) or climb a low barrier for low reward (1 sucrose pellet). This test was performed at 4 different time points: Before MCAo (baseline/pre), 30d, 60d and 98d after MCAo. At baseline, animals displayed a preference for the high barrier-high reward over 80% of the time (Fig 4). After MCAo, both groups (MCAO+scrambled and MCAo+mir363-3p) showed a gradual decrease in their choice of high barrier-high reward (main effect of time, $F_{(1,27)}$: 77.82, p<0.0001), compared to sham controls.

Moreover there was a significant effect of treatment ($F_{(1,27)}$: 4.35, p=0.047). In the MCAo+scrambled group, this preference dropped from 87% at baseline to 22% at 30d, 14% at 60d and 5.5% at 98d. In the MCAO+mir363-3p group, high barrier preference decreased from 90% at baseline to 44% at 30d, 40% at 60d and 30% at 98d. At each time point after stroke, the MCAo+mir363-3p group showed a greater preference for the high barrier/high reward than MCAo+scrambled (30d: p=0.08, 60d: p=0.024 and 98d: p=0.034). Since the behavioral assay spanned 3 months, sham surgery groups, treated with scrambled oligos or mir363-3p were also compared to assess if time/age would affect performance. As shown in Fig 4, sham groups treated with either mir363 or scrambled oligos did not show any decrease in high barrier/high reward choice up to 98 days.

Social Interaction test: The total amount of time spent in the chamber containing the conspecific stranger rat was recorded as a measure of sociability. This test was performed at 2 time points: Before MCAo (Pre) and 100d after MCAo and at equivalent time points for the Sham (no stroke) group. There was an overall effect of stroke and drug treatment ($F_{(3,28)}$: 7.46, p=0.001) Before MCAo, the test rats interacted with the stranger rat for over 400 seconds out of 600 seconds. At 100d after stroke, sociability decreased by 67% in the MCAo+scrambled group as compared to baseline, while the MCAo+mir363 group was not different from baseline (Fig 5). Social interaction was therefore significantly higher in the MCAo+Mir363 group than the MCAo+scrambled group at 100d (p<0.0001). Sham groups treated with either mir363-3p or scrambled oligos had similar baseline values and did not show any reduction in sociability at 100d.

Forced Swim Test (FST): Vulnerability to helplessness was assessed using the Forced Swim Test (Fig 6), defined as the total time a rat spends passively floating in the water. Greater immobility time indicates that the rat has 'given up' in the stressful environment. This test was performed before MCAo (Pre) and 100d after MCAo and at equivalent time points for the Sham (no stroke) group. There was an overall effect of MCAo and treatment ($F_{(3,28)}$: 3.48, p=0.0290). Sham groups treated with either scrambled oligos or mir363-3p had similar baseline values and showed no significant changes in immobility at 100d (p=0.6891). In groups subject to MCAo, the duration of immobility in the scrambled group was 185.5 secs and was elevated to 246.33 secs after MCAo, an increase in 36% over baseline (p=0.0026). In contrast, the mir363-3p treated group had a higher immobility at baseline (224.27 secs) which rose after stroke to 238.8 secs, which was not significantly different from baseline (p=0.385).

Locomotion, motor strength and cognition 90d after stroke: Animals were tested for locomotion, motor strength and cognition at 3+ months (99d) after stroke to ensure that performance on the tests of depression were not confounded by motor or memory impediments. Locomotion was assessed by total X/Y beam breaks in the open field chamber during a 30 min test period. There were no differences in the number of beam breaks in the Sham+scrambled, Sham+mir363-3p, MCAo+scrambled and MCAo+mir363-3p groups over the 30 min testing period (Fig 7A; $F_{(3,25)}$: 1.616, p=0.211). There was also no difference in the proportion of time spent in the center between the groups at 3+ months ($F_{(3,25)}$: 0.2579, p=0.8550). Similarly, the Grip Strength Test showed that there was no difference in motor strength in the Sham or MCAo groups treated with either scrambled or mir363-3p (Fig 7B; $F_{(3,26)}$: 0.295, p=0.82). The force of the grip normalized to the body weight ranged from 2.47 - 2.65 g/g for all the groups, indicating that, similar to

sensory motor function (Fig 3), motor strength is also completely recovered by 3 months after stroke. The Novel Object Recognition Test revealed that Sham+scrambled (80.04%), Sham+mir363-3p (70.63%), MCAo+scrambled (69.24%) and MCAo+mir363-3p (78.3%) groups all showed a greater preference for the novel object compared to the familiar object, and there were no group differences in novel object preference (Fig 7C; $F_{(3,26)}$: 0.513, p=0.67). Collectively, these data suggests that impaired performance on the depressive-behavior tests was not contaminated by impaired locomotion, motor deficits or cognitive decline at the time of the test.

Growth factor and inflammatory changes after stroke: Low levels of BDNF and elevated levels of inflammatory cytokines IL-6 and TNF-a have been implicated in depression in both preclinical and clinical studies[20, 34, 35].

Cytokines: As shown in Fig 8A, there was a time effect ($F_{(3,27)}$: 7.805, p=0.0007) and treatment effect ($F_{(1,27)}$: 6.273, p=0.0186). IL-6 levels were no different in each group at baseline but were significantly elevated above baseline at 30d after stroke (p=0.0018). This increase was mainly due to elevated levels of IL-6 in the MCAo+scrambled group (planned comparisons, p=0.0023). At 60d and 100+d there were no differences between the groups. A similar pattern was seen in TNF-alpha (Fig 8B, time effect $F_{(3,65)}$: 6.915, p=0.0004), with a significant elevation over baseline at 30d in the MCAo+scrambled group (p=0.0160). At 60d and 100d there were no differences over baseline or between the groups. BDNF: Due to limited volume of serum samples, pre-stroke levels of BDNF are not available. BDNF levels were measured at 30, 60 and 100d after stroke. As shown in Fig 8C, BDNF expression decreased over this time course in the

MCAo+scrambled group, such that neurotrophin expression at 100+d after stroke was one third of the level seen at 30d levels (p=0.0009). There was a time X treatment interaction effect $F_{(2,34)}$: 5.4, p=0.0092. By contrast, BDNF expression in the MCAo+mir363-3p group showed a scalloped pattern; BDNF levels decreased at 60d compared to 30d after stroke, and then recovered to 30d levels when measured at 100d post stroke. At 100d post stroke, BDNF levels were significantly higher in the MCAo+mir363-3p group as compared to the MCAo+scrambled group (p=0.0170).

Mesostriatal projections after stroke: Since BDNF levels and inflammatory cytokines are both known to affect neuroplasticity, we next investigated whether striatal infarction would, in the long term, affect reward circuitry. FluoroGold (Flg) was injected bilaterally into the striatum at 102 days after stroke to detect changes in the mesostriatal pathway. Shown in Fig 9A is a representative striatal injection site from the MCAO+scrambled and MCAo+mir363-3p groups. Visual inspection indicated that Flg label was detected throughout the striatum, spanning a rostrocaudal extent 1.7-1.9 mm (Paxinos Bregma interaural 10.70 mm to 8.70 mm). There were no differences in the mean rostrocaudal extent of tracer distribution between the ischemic and non-ischemic hemispheres, or between the MCAO+scrambled and MCAo+mir363-3p groups (Fig 9B). Fig 9C shows retrogradely-labeled neurons in the VTA and SNc in the ischemic and non-ischemic hemisphere of MCAo+scrambled and MCAo+mir363-3p animals. The proportion of retrogradely-labeled cells in these regions was significantly lower in the ischemic hemisphere ($F_{(1,15)}$: 5.068; p=0.04), compared to the non-ischemic hemisphere. Furthermore, there was a significant hemisphere X treatment interaction ($F_{(1,15)}$: 9.042, p=0.009), indicating lower numbers of retrogradely-labeled neurons in the ischemic hemisphere of the MCAo +scrambled group

compared to the MCAo+mir363-3p group. These data indicate that the mesolimbic reward pathway undergoes stroke induced plasticity, in association with depressive behaviors.

Discussion

The present studies employed middle-aged female rats to assess the development of post stroke depression. Post stroke depression occurs in a subset of stroke patients, and disproportionately occurs in older female stroke patients. Preclinical studies thus far have not focused on this demographic. Our studies have three major findings: first, MCAo causes an early loss of sensory motor function that resolves in the chronic phase. Second, stroke results in depressive-like behavioral changes as measured by a cluster of tests, that are observed between 30d and 3+ months after MCAo. Finally, treatment with mir363-3p, administered i.v. 4h after MCAo, reduces sensory motor impairment in the acute phase, and attenuates depressive-like behaviors in the chronic phase. Moreover, mir363-3p treatment attenuates the loss of BDNF in the chronic phase. MCAo also resulted in decreased numbers of retrogradely-labeled cells in the mesostriatal reward pathway, which was attenuated in the mir363-3p tested group. Overall, these results indicate that a microRNA that is neuroprotective for middle-aged females in the acute phase of stroke [25] also improves PSD among the same population. In contrast, early sensory motor deficits and post-stroke depressive-like symptoms are worse in scrambled controls, suggesting that early neuroprotection (by mir363-3p) improves later consequences of stroke.

MiRNAs are important regulators of mRNA transcript stability[36] and gene translation[37] and play a significant role in disease processes. MiRNA profiles are altered with stroke in both human[38, 39] and experimental[40-43] models, and specific miRNA are associated with pathogenic processes that contribute to or exacerbate stroke, such as hyperlipidemia (mir33),

hypertension (mir155), atherosclerosis (mir21, mir126) plaque rupture (mir222) (reviewed in[44]). We were among the first to show that miRNA can serve as stroke neuroprotectant. ICV injections of antagomirs to Let7f and mir1, which are predicted/validated targets for IGF-1, a neuroprotective factor, reduced infarct volume in adult female rats[32]. Mir181 and mir29 have been shown to reduce ischemia-induced cell death[45, 46], by targeting members of the Bcl-2 family of pro- and anti-apoptotic proteins. Subsequent studies have shown that exosomes from mesenchymal stromal cells [47] enriched with mir133b[48], mir124[49], mir17-92 [50]or their upregulation in neural progenitor cells are neuroprotective for stroke and leads to functional recovery in rodents[42, 47-49, 51]. Expression of mir363-3p, identified through miRNA profiling analysis[25], was negatively correlated with infarct volume. Subsequently, we reported that iv injections of mir363-3p mimics effectively reduced infarct volume and behavioral impairment in middle-aged females, but not males. Only a handful of validated targets are known for mir363-3p, which includes caspase-3, a cell death effector, [52], and genes associated with tumor suppression such as proliferating cell nuclear antigen (PCNA) [53], high mobility group AT-Hook 2 (HMGA2) [54], and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) [55]. Our studies showed that mir363-3p treatment after stroke reduces caspase-3 expression and activation in middle-aged females but not males. This is consistent with previous studies that have defined caspase-3 pathway as a sex-specific stroke pathway [56, 57], and underscores the importance of sex-specific therapies for stroke. Few studies have examined the effect of miRNA treatment on long-term consequences of stroke. An exception to this is a recent study which showed that mir137 decreased depressive behaviors caused by stroke combined with chronic mild stress at 3 weeks, although it is not clear if mir137 is also

neuroprotective[58]. The current studies show that mir363-3p, which exerts a neuroprotective effect in the early phase of stroke, also attenuates long-term consequences of stroke.

The present studies add to a growing literature showing that depressive behaviors develop after stroke and social isolation in a rodent model[19-21]**.** While it is a challenge to study depression in a rodent model, some inferences are possible using a cluster of tests that assess cardinal signs of the disease. A clinical (DSM-V) diagnosis of depression requires at least 5 symptoms over 2 weeks that show impaired social or occupational functioning. Anhedonia, reduced interest in social interactions and propensity for negative mood are some of the major hallmarks of depression and these attributes were assessed in our behavioral tests. Reduced social interaction and despair behavior were measured by the social interaction test[19] and by immobility in the FST[59, 60] respectively. Anhedonia is usually assessed by the sucrose preference test, although recent work suggests that this test *per se*, is not as sensitive a measure of depressive behavior as "effort based" behaviors for palatable rewards[61]. As a refinement to the anhedonia test[62], we adapted the T-maze cost/benefit task where the test subject has to expend effort (climbing over a barrier) to obtain a food-reward, a measure of diminished interest and low energy. On these tests, performance after stroke (in the non-drug treated group) was worse when compared to baseline. The mir363-3p treated group either showed no change from baseline (as in the social interaction test and the FST) or a blunted response (as in the T-maze cost/benefit task). We further ensured that impairment on these depressive-behavior tests were not due to loss of motor strength or dexterity or sensory-motor loss**.** Moreover, it is possible that performance on the T-maze cost/benefit task may be impaired at 3+ months due to cognitive decline rather than affective decline. However, in the novel object recognition test, both the scrambled and the

mir363-3p treated stroke groups displayed a greater interest in the novel object to a similar extent, indicating that group differences in the T-maze cost/benefit task is likely indicative of anhedonia and not loss of memory. Thus, by modeling three different components of depression, our data shows that depressive-like behavior develops in the long term in middle-aged female rats, which can be improved by mir363-3p treatment.

Multiple mechanisms have been proposed to explain the pathogenesis of depression. These include biochemical alterations, such as low levels of the monoamine neurotransmitters serotonin and catecholamine[10, 11], low expression of the neurotrophin BDNF in the frontal cortex and hippocampus[15, 16], or elevated expression of pro-inflammatory cytokines[14, 63]. Depression has also been linked to disruption of neural circuits that mediate forebrain reward pathways, including midbrain-striatum-cortex circuits[64, 65]. The present study shows partial support for several of these theories. Middle-aged female rats who received scrambled oligo treatment after stroke had increased levels of pro-depressant[66-69] cytokines (IL-6, TNF-alpha) at 30d post stroke, lower levels of circulating BDNF and greater disruption of meso-striatal projection in the ischemic hemisphere by 100d. Loss of trophic support may lead to aberrant plasticity in neural circuitry[70] and the gradual development of depressive-like behavior. Most of these measures were alleviated in animals that received mir363-3p injections, including higher levels of circulating BDNF, less IL-6 at 30d and virtually no disruption of meso-striatal projection in the ischemic hemisphere. Our present study design does not allow us to define which events are primary and which events are secondary. It is possible that ongoing degeneration of the neural circuitry occurs first, which primes inflammation. Alternately, loss of trophic support from the ischemic striatum may be the primary event that leads to retrograde degeneration in mesostriatal

circuits. Glial-cell derived neurotrophic factor (GDNF), for example, is retrogradely transported from the striatum to the VTA and SNc and protects dopaminergic neurons against injury[71, 72]. Similarly, BDNF is also retrogradely transported by dopaminergic projections from the midbrain to the striatum, and loss of BDNF-trkB signaling leads to progressive degeneration of the nigrostriatal pathway[73]. This loss of meso-striatal projection develops gradually after stroke, such that it is not observed in the acute phase of stroke (5d after stroke) but is evident as early as 45 days after stroke (unpublished observations). Our previous work showed that administration of mir363-3p mimics localizes to neurons and reduces the expression and activation of Caspase-3 in middle-aged female brain, suggesting an anti-apoptotic role for this miRNA [25]. We hypothesize that the changes in the level of the trophic factors and inflammatory cytokines over time may be a direct effect of infarction, and that mir363-3p stabilizes these changes by reducing the initial infarct volume. Future imaging studies would be important in evaluating initial infarction and consequent changes in meso-striatal projections at 3+ months.

In conclusion, these studies and others [74-76] indicate that post stroke depression may be more tractable to neuroprotectant therapy as compared to conventional anti-depressants. Our previous work showed that a single dose of mir363-3p injected intravenously after stroke reduced infarct volume in the cortex and striatum and attenuated the early loss of sensory motor behavior in middle-aged females[25]. The present study shows that this early treatment may be sufficient to alleviate post stroke depression. While the precise mechanism underlying this outcome needs further study, we propose that neuroprotectants may be a better option for older women after stroke instead of agonist/transmitter-based therapies, possibly due to their pleitrophic actions on other stroke-induced changes in the body.

Figures

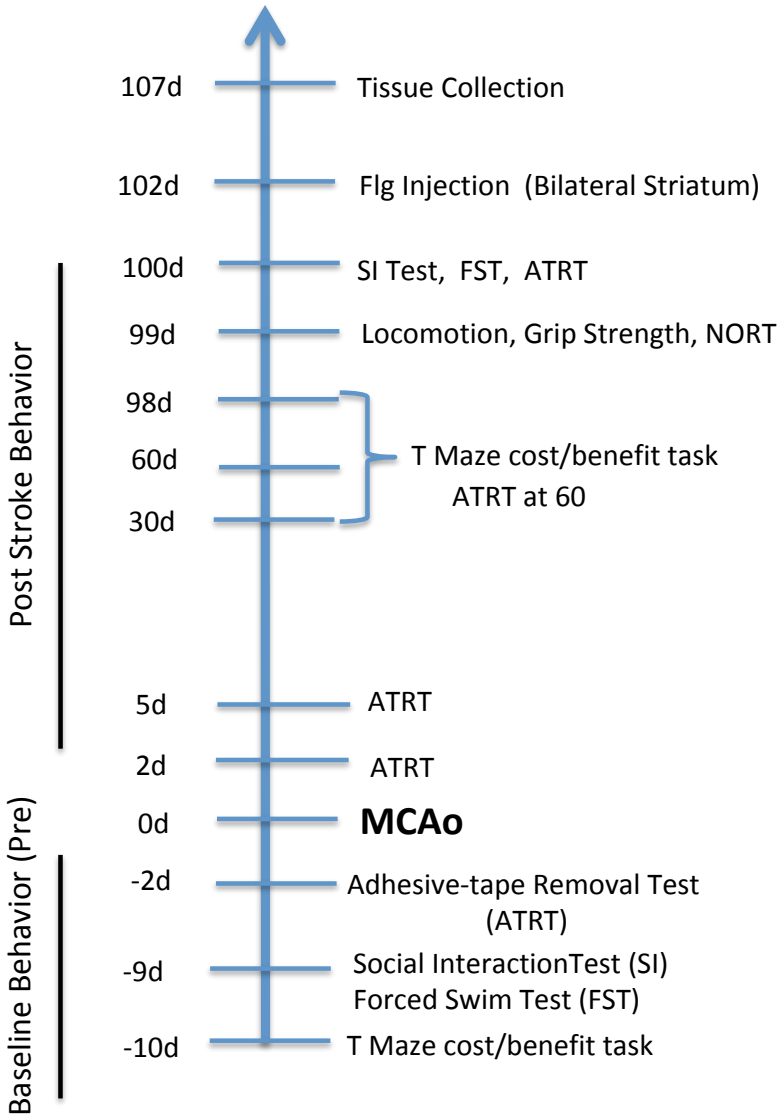

Figure 2: Experimental timeline of behavioral tests in relation to stroke (MCAo). MCAo is demarcated as 0d (day 0). All animals were injected with Fluorogold (Flg) after the last test and terminated 5 days later. ATRT: Adhesive Tape Removal Test; FST: Forced Swim Test; SI: MCAo: Middle Cerebral Artery occlusion; NORT: Novel Object Recognition Test; Social Interaction test.

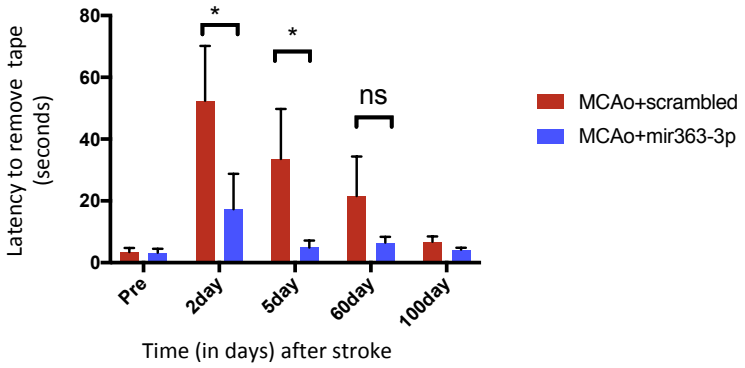

Figure 3: Assessment of stroke-associated sensorimotor impairment. Histogram depicts mean+SEM of the latency (in seconds) to remove an adhesive tape from the forepaw of the limb contralateral to the ischemic hemisphere. This test was performed before (Pre) and at 2, 5, 60 and 100d after stroke. Key: #: $p<0.05$, comparison of the group with its baseline levels; *: $p<0.05$, comparison of treated vs control groups at the same time point. ns: not significant.

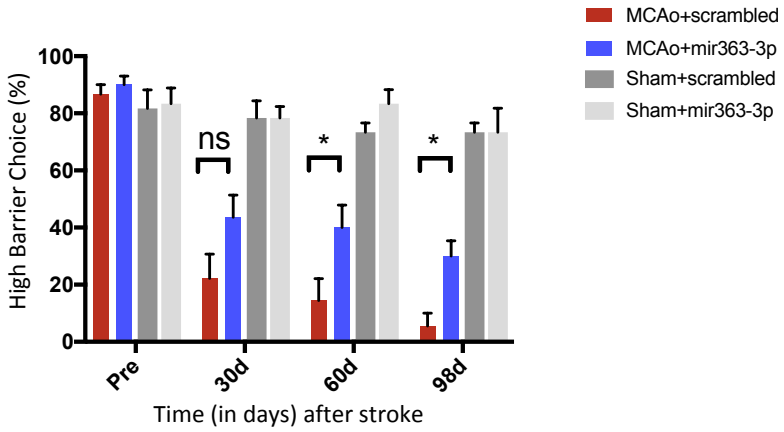

Figure 4: T-maze cost/benefit task test. Stroke and sham animals, treated with either scrambled oligos or mir363-3p, were assessed on this test prior (Pre) to stroke (or sham) surgery and 30d, 60d, 98d after stroke. Histogram depicts mean±SEM % preference for the high barrier/high reward high. Key: #: $p<0.05$, comparison of the group with its baseline levels. *: $p<0.05$, comparison of treated vs control groups at the same timepoint. ns: not significant.

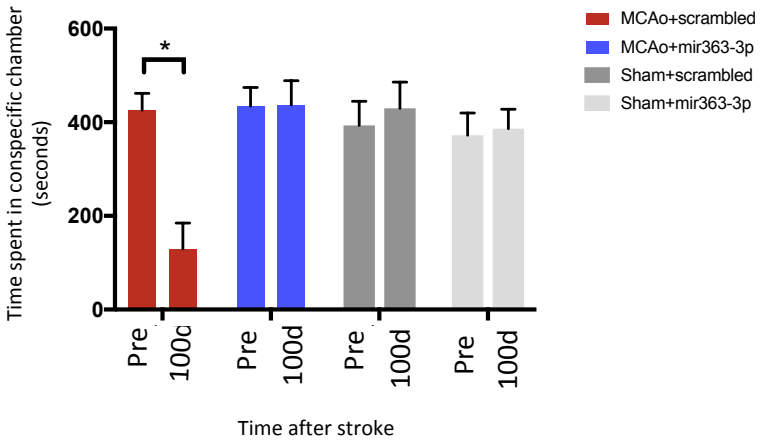

Figure 5: Social Interaction test. Stroke and sham animals, treated with either scrambled oligos or mir363-3p, were tested for social interaction with a conspecific stranger prior (pre) and 100d after stroke. Histogram depicts mean±SEM time spent with the conspecific. Key: *: $p<0.05$, comparison of the group with its baseline levels.

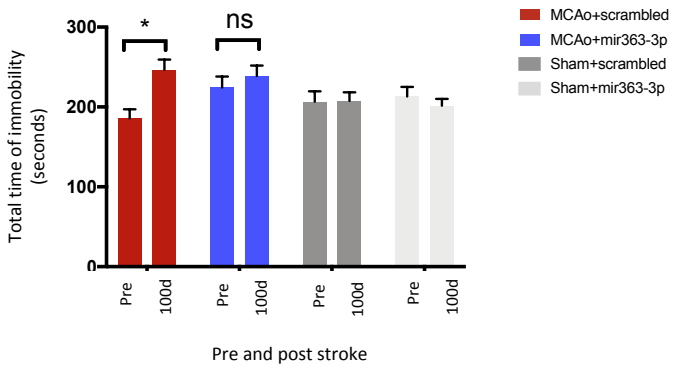

Figure 6: Forced Swim Test. Stroke and sham animals, treated with either scrambled oligo or mir363-3p, were tested for immobility in the Forced Swim Test. Histogram depicts the mean±SEM amount of time spent immobile. *:$p<0.05$, comparison of the group with its baseline levels; ns: not significant.

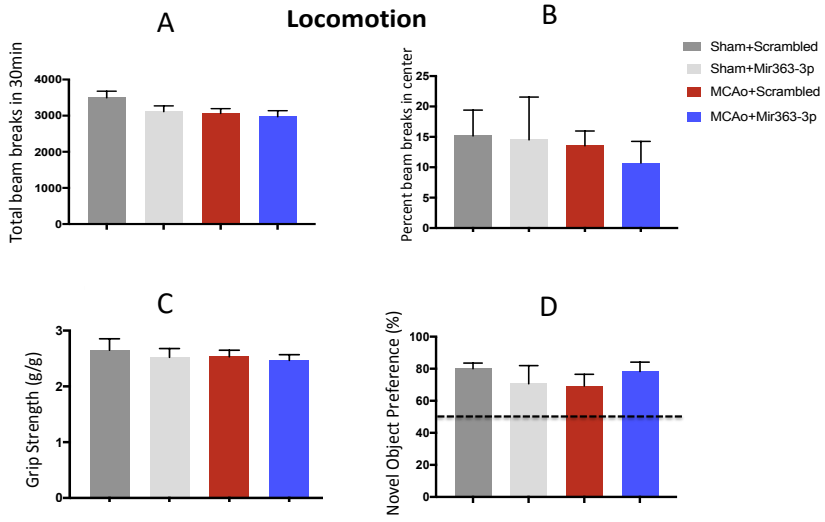

Figure 7: Locomotion, motor strength and cognitive changes 90d after stroke. (A) Locomotor impairment was assessed by the total number of beam breaks and beam breaks in the center in an open field apparatus. The total number of beam breaks was no different between sham or MCAo groups treated with either scrambled oligos or MCAo+mir363-3p at 99d after stroke. (B) The percent time spent in the center of the open field was not different in any of the groups. (C) Motor impairment was tested using the grip strength meter. The peak tension force for forelimb grip was no different between the sham or MCAo groups treated with either scrambled oligos or MCAo+mir363-3p at 99d after stroke. (D) Novel Object Recognition: Novel object recognition test was used to assess loss of cognitive capacity. All groups showed a greater preference (>50%; dotted line) for the novel object than the familiar object and were statistically no different from each other.

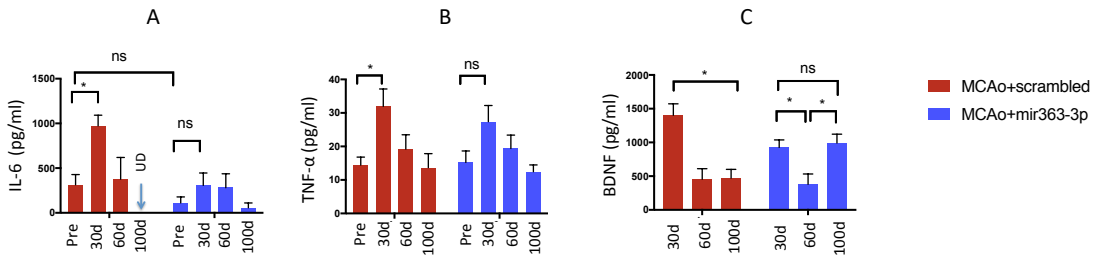

Figure 8: Time course of cytokine and neurotropin expression after stroke. Circulating levels of IL-6 (A) and TNF-alpha (B) were significantly elevated at 30d after MCAo as compared to baseline in the MCAo+scrambled group, but not the MCAo+mir363-3p treated animals. By 100d after stroke, both cytokines returned to baseline levels. C) Circulating BDNF levels decreased at 60 and 100d after stroke compared to 30d after stroke in the MCAo+scrambled group. In the MCAo+mir363-3p treated group, BDNF levels decreased at 60d compared to 30 days after stroke but returned to 30d levels by 100d after stroke. *: $p<0.05$; ns: not significant.

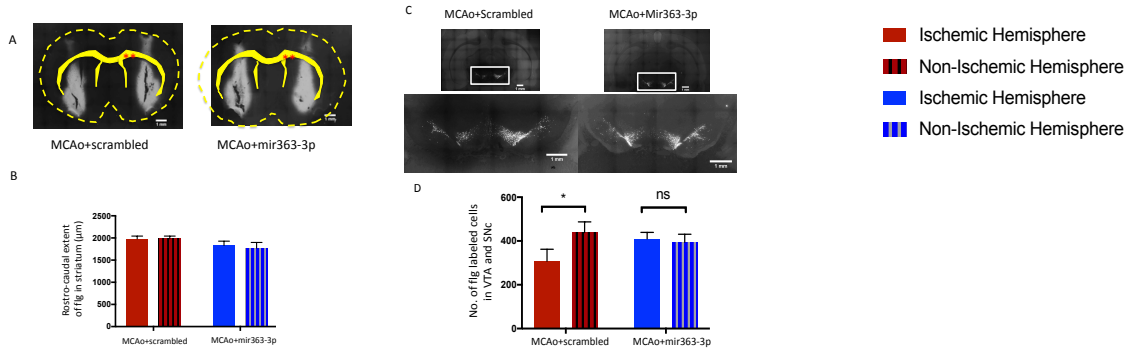

Figure 9: Assessment of the mesostriatal pathway at 3+ months (107d) after stroke. A) Photomicrograph depicting bilateral injections of Fluorogold into the striatum. The outline of the brain and key landmarks are shown in stippled yellow. B) The rostrocaudal extent of Fluorogold dye was similar in both hemispheres and in both groups, indicating similar amount of dye at the target site. C) Fluorogold labeling of SNc and VTA neurons: Photomicrograph of a coronal section depicting Flg-labeled neurons in the VTA and SNC from the MCAo+scrambled group (Ci) and MCAo+mir363-3p treated group (Cii). Boxed area in each image is shown in higher magnification below. D) Histogram depicts mean (+SEM) number of Flg-labeled cells in SNc and VTA in the ischemic and non-ischemic hemisphere. *: $p<0.05$, ns: not significant. Single red asterisk: Lateral ventricle, double red asterisk: Corpus callosum

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CHAPTER III

MIDDLE CEREBRAL ARTERY OCCLUSION REDUCES LEVELS OF THE GUT METABOLITE TRYPTOPHAN AND RESULTS IN PERSISTENT GUT DYSBIOSIS IN MIDDLE-AGED FEMALE RATS

Introduction

Post Stroke Depression (PSD) is one of the long-term outcomes of stroke that affects about one-third of stroke survivors. Depression is characterized by anhedonia, social dysfunction and feeling of despair [1-3]. PSD affects the quality of life of the patient and hampers recovery after stroke. Several factors contribute to the pathogenesis of depression, including the loss of neurotrophic factors, neurotransmitters as well as elevated inflammatory cytokines. Low expression of BDNF in the hippocampus and the frontal cortex has been indicated as an underlying cause for depression which is further supported by the evidence that BDNF expression is up-regulated in these regions after treatment with anti-depressants and electroconvulsive therapy [4-6]. Similarly, low levels of monoamine neurotransmitters, including serotonin, norepinephrine and dopamine are included in the pathophysiology of depression [7]. Most prescribed anti-depressants target the levels of catecholamine and serotonin in synaptic clefts which improves the depression [8]. Inflammation also plays a critical role in the development of depression. IL-6, IFN-γ, IL-1 and TNF-α are some of the pro-inflammatory cytokines that are elevated during depression [9]. TNF-α and IFN-γ activates Indole-amine 2,3 dioxygenase (IDO), which metabolizes tryptophan, a precursor for serotonin [10]. Accumulating data indicates that the availability of tryptophan as well as inflammatory mediators is significantly influenced by the gut microbiota.

Commensal bacteria in the gut and their metabolites critically shape the gut-brain axis and contribute to neurological as well neuropsychiatric disorders. Gut microbiome communicates with the brain via nervous, endocrine and immunological signals, which are mediated by microbial derived metabolites such as short chain fatty acids and tryptophan [11-13]. Beside increasing the risk for Parkinson's disease [14] and regulating symptoms in ASD [15] [16], gut microbiota have also been shown to induce depression and anxiety [17, 18]. Altered microbiota composition has been found in depressed patients [19]. A study showed that E coli outbreak in Canada and Germany led to increased depression and anxiety in the affected population [20]. Similarly, fecal microbial transplant from depressed human to rodents have induced depressive-like behavior in the rodents [21]. Gut dysbiosis may affect the development of depression via changes in the tryptophan metabolism [22, 23] or by inducing inflammatory responses in the blood [24, 25]. The "leaky gut" due to aging or stress may translocate bacterial products such as endotoxin (lipopolysaccharide (LPS)) into the blood, which are known to elevate the pro-inflammatory cytokines in the blood[26].

In this study, we show that middle-aged females rats who were subject to stroke and exhibit a depressive-like behavioral phenotype [27] display decreased levels of the gut metabolite tryptophan as compared to sham treated animals. This is also accompanied by microbiota dysbiosis as measured by an elevated ratio of Firmicutes to Bacteroidetes, and decreased levels of SCFAs. While recent studies have shown that gut dysbiosis occurs in the acute phase of stroke [28, 29], to our knowledge this is the first evidence of persistent gut dysbiosis in a chronic phase of stroke. Our data also suggest that gut metabolites may be mechanistically linked to post stroke depression.

Methods

Animals: Fecal samples for this study were obtained from a subset of rats from a previously published data set demonstrating that middle cerebral artery occlusion (MCAo) increases depressive like symptoms as compared sham (non-stroke) animals [27]. Briefly, middle-aged female rats (10-12 months age; Envigo) were characterized as reproductive senescent by cytology of vaginal lavage [27]. Ischemic stroke was induced by stereotaxic delivery of endothelin-1 to the MCA [27, 30-32]. Depressive-like behavior was assessed by the Effort-based sucrose consumption test, Social Interaction (SI) and Forced Swim Test (FST) [27]. Blood samples and fecal samples were collected at termination.

Tryptophan assay: Tryptophan levels in the blood were quantified using a rat Tryptophan ELISA Kit (Abnova, CA), using manufacturer's instructions. Absorbance was measured on ELISA microplate reader set to 450nm.

Fecal Samples: Fecal samples collected 100 days after stroke and from age matched sham animals were stored frozen. DNA was extracted using MoBIO Power soil DNA isolation kit (MoBio Laboratories, USA) followed by illumine sequencing of the bacterial 16S rRNA genes using primers 515F (50-GTGCCAGCMGCCGCGGTAA-30) to 806R (50-GGACTACVSGGGTATCTAAT-300) at MR DNA lab (Shallowater, TX). Quantitative Insights Into Microbial Ecology (QIIME) v 1.8 was used to analyze the sequence. The raw sequences were uploaded to NCBI Sequence Read Archive and then demultiplexed and quality filtered using QIIME. Using USEARCH, chimeras were detected and filtered against 97% clustered representative sequences from the Greengenes v 13.8 database.

SCFA assay: SCFAs including acetic acid, proprionic acid and butyric acid were analyzed by Gas Chromatography-Mass Spec (Creative Proteomics, NY)

Statistics: For all assays, group differences were analyzed by Student t-test (SPSS, IBM). Group differences were considered significant at $p<0.05$.

Results

Tryptophan: Tryptophan is the precursor molecule for serotonin. Serum levels of tryptophan were detected by ELISA in both groups (Fig 10A). Tryptophan levels were reduced by 35% in animals that were subject to MCAo 3+ months prior to sampling as compared to the sham surgical group ($p=0.0002$), indicating a persistent decrease in this amino acid.

Bacterial composition: Abundance: Since tryptophan is a gut metabolite, we next examined gut dysbiosis in these groups. Gut dysbiosis was determined by the ratio of Firmicutes to Bacteroidetes (F:B ratio), two major bacterial phyla, and elevated F:B ratio is indicative of gut dysbiosis [28, 33, 34]. The F:B ratio in sham animals was 3.79 while this ratio was 5.54 in the MCAo group, representing a 46% increase in the F:B ratio in the MCAo group ($p=0.0087$; Fig 10B). Alpha diversity, assessed by Shannon and observed OTUs, indicate no differences between groups (Fig 10C and 10D). Beta diversity (UniFrac) indicated no significant difference in unweighted branches (Fig 10F), but significant difference in weighted branches between the 2 groups ($p<0.04$; Fig 10E). N=8-10

We next determined the expression of specific bacterial families that are implicated in clinical Major Depressive Disorder (MDD) or preclinical depressive-like behaviors. As shown in Fig 11A, at least three bacterial families, including Proteobacteria, Actinobacteria and Prevotella have been implicated in MDD [19, 21]. Abundance of Proteobacteria ($p=0.4280$) and Actinobacteria ($p=0.5156$) were similar in both groups. However, Prevotella was significantly less abundant in the MCAo group as compared to sham animals ($p<0.0267$). In preclinical studies, low abundance of Bacteroidetes and Lactobacillus are associated with depressive like symptoms [35, 36]. MCAo treated animals had significantly lower abundance of Bacteroidetes as compared to sham animals ($p=0.0042$). There was a trend toward decreased abundance of Lactobacillus in the MCAo treated group ($p=0.0728$).

Short chain fatty acids (SCFAs): Acetic acid, proprionic acid and butyric acid are the most abundant SCFAs. Levels of acetic acid ($p=0.047$) and proprionic acid ($p=0.020$), synthesized by Bacteroidetes, were significantly decreased in MCAo treated animals as compared to age matched sham controls. Butyric acid, synthesized by Firmicutes, was not significantly different between the two groups ($p=0.080$).

Discussion

Several recent studies show that the gut microbiome is altered after ischemic stroke, and that these changes are associated with stroke recovery as well [28] . To the best of our knowledge, this study provides the first evidence of gut dysbiosis in the chronic phase of stroke, persisting several months after the initial ischemic event. Gut dysbiosis was observed in the form of an elevated F:B ratio, as well as changes in specific bacterial families, and gut metabolites. In view

of the effect of the gut microbiome on inflammatory cells and cytokines, this persistence in gut dysbiosis suggests a mechanism whereby stroke may lead to chronic inflammation, and other neuropsychiatric conditions that are affected by inflammation.

Gut microbiota synthesize neurotransmitters such as serotonin [37] that modulates behavior and short chain fatty acids (SCFA) that regulates the blood-brain barrier and brain maturation [38, 39], and profoundly regulate central and peripheral inflammation [40]. Via disruption of the hypothalamo-pituitary-adrenal gland (HPA) axis and increased norepinephrine secretion, stroke affects motility, mucus regulation, acid/bicarbonate secretion, gut permeability and eventually the composition of the gut by disrupting its autonomic regulation [41, 42] thus exacerbating the inflammatory response. Gut microbe regulates both γδ T cells, which produce neurotoxic cytokines, as well as T-reg cells which suppress γδ T cells [29]. Gut leakiness is a principal contributor to post stroke bacterial infection [43]. The importance of gut metabolites is underscored by the observation that antimicrobial therapy to treat a post-stroke infection is not beneficial [44, 45]. In fact, the anti-microbial therapy made stroke recovery even worse by disrupting the gut microbiome. Thus, novel therapeutics have been focusing on improving stroke outcomes by targeting the overall gut microbiome health [28, 46] [47].

Ischemic stroke can result in several long-term consequences, including but not limited to depression, epilepsy, addiction and cognitive dysfunction. In a recent study, we showed that middle-aged female rats subjected to MCAo display depressive-like behaviors such as anhedonia, social isolation and helplessness as compared to non-stroke sham controls [27]. The animals used in this study are a subset of the group in Panta et al 2019. Thus, the changes observed in the microbiome may also provide clues to bacterial populations that are implicated

for depression. Several piece of evidence shown here suggest the link between PSD and gut health and add to the growing literature on alterations in the gut microbiome and the development of depression [18, 19]. Peripheral tryptophan was significantly decreased 3 months after stroke as compared to the rats subjected to sham surgery. Tryptophan, a precursor to serotonin (5-HT), is principally extracted from diet by gut microbiota. Reduced levels of this amino acid suggests that MCAo may cause persistent gut dysbiosis, which is supported by the elevated F:B ratio seen in this group. Firmicutes and Bacteroidetes are the two most prominent phyla, and an increased F:B ratio is an indicator of dysbiosis of gut microbiome in both rodent and human studies [28, 48, 49]. Gut dysbiosis is also seen in depressive disorders [19, 21, 35, 36]. Fecal microbiome transfer from depressed patients has been shown to induce depressive like behaviors in germ free mice as compared to mice that receive fecal transfer from healthy controls [21, 36].

In addition to global gut dysbiosis, alterations in specific bacterial families have also been implicated in clinical or experimental depression. In patients with MDD, Proteobacteria, and Actinobacteria were more abundant as compared to non-depressed controls [19], while levels of Prevotella were decreased [19, 21]. In the case of Bacteroidetes, one study reported elevated levels [19] while another reported decreased levels of this family [36]. Declining levels of Lactobacillus were noted in depressed patients as well as in a stress-induced model of despair in mice [35]. Reciprocally, Lactobacillus-containing probiotic causes improvement in patients with MDD [50] . Our data show some commonalities with these clinical and preclinical populations, such as a decrease in Prevotella, Bacteroidetes and a declining trend of Lactobacillus. There is also a decline in Bacteroidetes synthesized SCFAs, proprionic acid and acetic acid.

This work adds to the growing evidence of stroke-induced changes in the gut microbiome and extends the field by reporting that gut dysbiosis persists for months after stroke. These data also underscore the importance of gut health after stroke, especially in the context of post-stroke depression. Through its regulation of tryptophan availability and the inflammatory responses [24, 25], the gut microbiome represents a unique therapeutic target that can be modified by diet, especially in populations such as older female that do not respond well to anti-depressants.

Figures

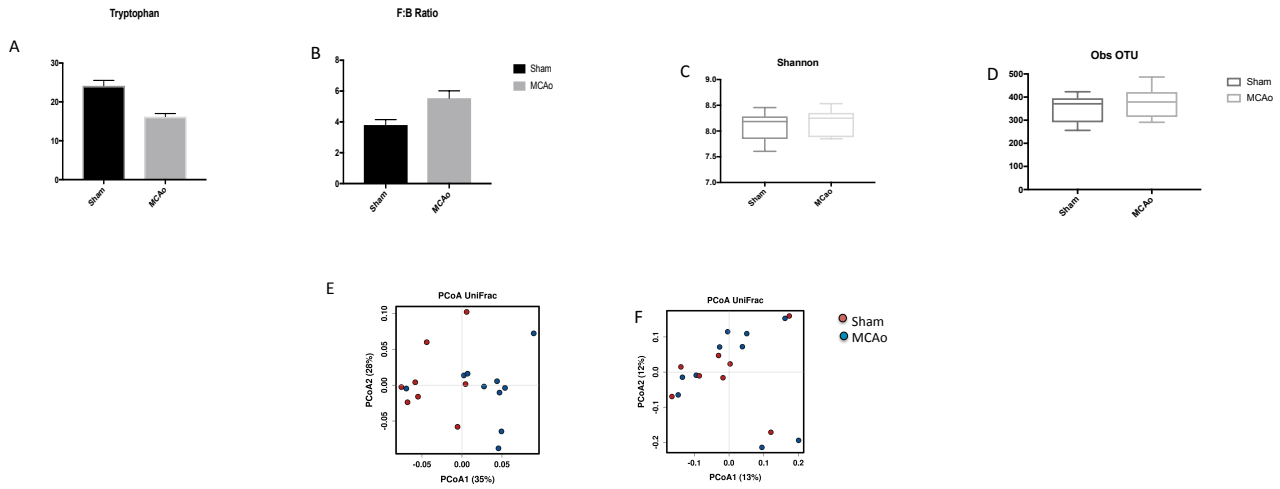

Figure 10: Gut dysbiosis persists in the chronic phase after stroke. Blood and fecal samples were obtained from middle aged females that received an ischemic stroke or sham surgery. (A) Three months after stroke, animals displayed lower serum levels of the amino acid tryptophan compared to age matched sham animals ($p<0.0002$). (B) Fecal samples from animals with MCAo displayed an elevated ratio of Firmicutes to Bacteroidetes (F:B) as compared to age-matched sham controls ($p<0.0087$). Bacterial diversity as calculated by Shannon (C) and Observed OTUs (D) was similar in both groups ($p>0.05$). Weighted (E), but not unweighted (F) principal coordinate analysis (PCoA) show middle-aged and adult female rats grouped into two significantly distinct clusters ($p=0.009$). N=8-10.

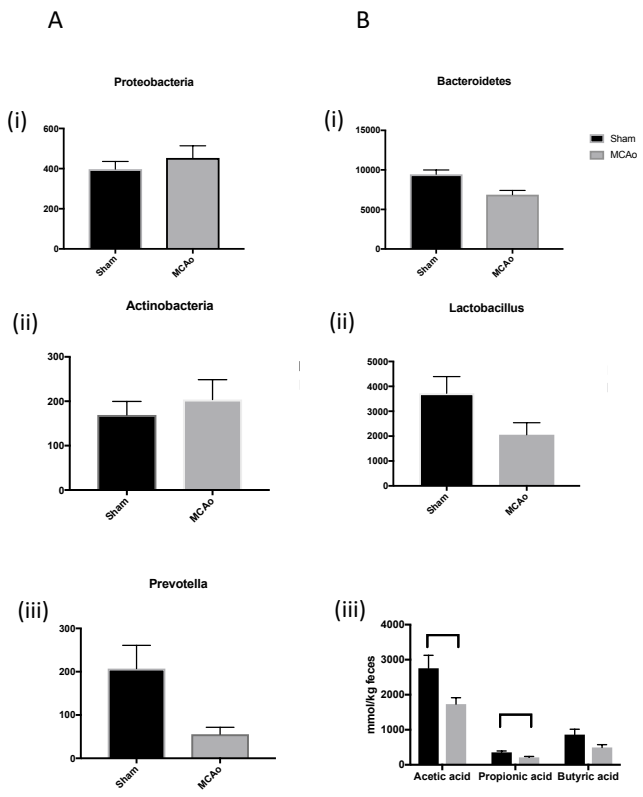

Figure 11: Bacterial abundance is altered in the chronic phase after stroke. (A) Bacterial species that are altered in patients with Major Depressive Disorder: Fecal samples from stroke and sham animals were no different in the abundance of (Ai) Proteobacteria (p=0. 4280) or (Aii) actinobacteria (p=0.5156), while (Aiii) Prevocetella levels were significantly decreased in animals with MCAo (p=0.0267). (B) Bacterial species that are altered in preclinical studies of depression: Fecal samples from animals with MCAo had reduced abundance of (Bi) Bacteroidetes (p=0.0042) and (Bii) Lactobacillus (p=0.0728) as compared to age-matched sham animals. N=8-10, Students t-test. (Biii) SCFAs are selectively altered by MCAo: Acetic acid (p=0.047) and proprionic acid (p=0.020) levels were significantly decreased in animals that received MCAo as compared to sham controls. Butyric acid was not altered by MCAo (p=0.080). N=7-10.

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CHAPTER IV

COGNITIVE DEFICITS OCCUR LONG TERM AFTER STROKE IN MIDDLE-AGED FEMALE RATS AND ARE ATTENUATED BY MIR363-3P TREATMENT[2]

Introduction

Cognitive impairment is a common consequence after stroke that significantly impairs the patient's functional recovery as well as quality of life. More than half of stroke patients suffer from some level of cognitive impairment by 6 months after stroke [1, 2]. Patients who do not develop cognitive impairment directly after stroke have a 9-fold increased risk of developing delayed cognitive impairment [3]. Age is an important risk factor for both stroke as well as cognitive decline and studies have shown that prevalence of cognitive decline after stroke increases exponentially with age [4]. In addition to age, previous or recurrent stroke, volume of infarction, location of the stroke and female sex are all strongly associated with post stroke cognitive impairment [5] . In fact, among these survivors, women are more likely to require assisted living facilities because of deteriorating cognitive function [6-8].

The precise mechanisms underlying post stroke cognitive dysfunction are still not well known. Studies have suggested it could be due to an ischemic insult to brain parenchyma, vasculature as well as the white matter. The prefrontal cortex is an important region for cognitive functioning. It provides an executive, 'top-down' control on cognition by suppressing old distracting memories

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[2] Part of this chapter is reprinted with permission from an open access publication Panta A, Montgomery K, Nicolas M, Mani K, Sampath D, Sohrabji F. Mir363-3p Treatment Attenuates Long-Term Cognitive Deficits Precipitated by an Ischemic Stroke in Middle-Aged Female Rats. Frontiers in Aging Neuroscience. September 2020. Volume 12.

from interfering with new information that is being learned [9-11]. Accordingly, ischemic injury to the pre-frontal cortex induces cognitive dysfunction [12]. Similarly, the entorhinal cortex connects the hippocampus to other associated cortices. Medial entorhinal cortex (MEC) provides input to hippocampus for spatial information, whereas lateral entorhinal cortex (LEC) provides input for nonspatial (contextual) information [13, 14]. However, these remote regions are vulnerable not only to focal ischemia, but also to distal ischemia. Middle cerebral artery occlusion (MCAo), which comprises 75% of all strokes, primarily affects sensorimotor cortex and striatum but can also affect remote brain regions in a delayed fashion. Delayed neuronal death (DND) in the CA1 region of hippocampus, reduced activity in the prefrontal cortex and thinning of the entorhinal cortex have been reported after middle cerebral artery ischemia [15-19]. Li and colleagues have shown that MCAO leads to increased GABAergic neurotransmission and reduced activity of extracellular regulated protein kinase (ERK) in both hippocampi resulting in cognitive deficit. Similarly, an increased inflammatory response, in the form of activated microglia, is observed in hippocampus of the rats that have cognitive deficit after MCAO [20]. These results were supported by the findings of Gemmell et. al where hippocampal atrophy was observed in the post mortem brains of patients with delayed post stroke dementia [21]. Collectively, delayed neuronal death in remote brain regions after MCAO ischemia may explain delayed onset of cognitive impairment in the stroke patients.

Our previous studies showed that in the chronic phase (1-3 months later) after MCAo, middle-aged female rats exhibited depressive-like symptoms in the form of increased anhedonia, increased feeling of despair and decreased social interaction [22]. This was accompanied by transient elevated levels of inflammatory cytokines and lower circulating levels of BDNF, and attrition of the projection pathway between midbrain neurons of the ventral tegmental

area/substantia nigra to the striatum [23]. In addition, intravenous injection of mir363-3p administration 4 hour after stroke significantly reduced infarct volume measured at 5d post stroke and improved sensory motor function at 2d and 5d (acute phase) after stroke [24], and also reduced the depressive phenotype that develops in the chronic phase after stroke [22]. However, non-spatial memory as assessed by the Novel Object Recognition Task (NORT) was not impaired after MCAo at 3 months. In view of the delayed onset of cognitive impairment reported for stroke patients, the present study tested the hypothesis that MCAo would affect cognitive function much later in the chronic phase of stroke and that early treatment with mir363-3p would attenuate these cognitive losses as it did for the depressive phenotype. We report that cognitive function assessed using Novel Object Recognition Test (NORT) (non-spatial memory) and Barnes Maze (spatial memory) declined six months after stroke, but were attenuated in the group that received mir363-3p treatment. Social interaction, which is already impaired at 3 months after stroke [22] remained impaired at 6 months after stroke. These data support the hypothesis that cognitive impairment after MCAo occurs in a delayed manner in middle-aged female rats and can be attenuated by a neuroprotectant treatment delivered in the early hours after stroke.

Methods and Materials

Animals: Middle-aged female rats (12 months old, 260-320 gm) were purchased from Envigo (IN) and were housed in an AAALAC-accredited vivarium on a 12/12 light/dark cycle with a controlled temperature (22C) and humidity (45-55%). Food and water were available ad libitum. The rats were allowed to acclimatize to the vivarium for three weeks after arrival and daily vaginal swabs were performed up to 21 days to determine the estrus cycle as described in our

previous work [25, 26]. When the animals displayed cell cytology consistent with diestrus phase for at least 7 consecutive days, they were included in the study. Our previous works have shown that hormonal profile of female rats in constant diestrus phase resembles the hormonal profile of post-menopausal stage in women (low estradiol and elevated FSH levels) [25, 26]. A total of 30 animals were used. Animals were randomly assigned to two treatment groups (Scrambled or mir363-3p) after middle cerebral artery occlusion and sham surgeries. The behavioral tests performed on the animals are included in the timeline as shown in Fig 12. All the behavioral tests were performed between 8:00am and 12:00pm and experimenters were blinded to the treatment condition while performing and analyzing the tests.

Middle Cerebral Artery Occlusion (MCAo): Animals were subjected to MCAo by stereotaxic injection of a vasoconstrictor (Endothelin-1), using our established protocol [22, 27-30]. Animals were anesthetized with a mixture of ketamine (100mg/kg) and xylazine 20mg/ml/kg) and placed in a stereotaxic equipment. Endothelin-1 (American Peptide Company; 0.5 ug/ul, 600 pmol; 3uls) was microinjected at the coordinates relative to bregma: AP: +0.9, ML: -3.4, DV: -8.5 to occlude left middle cerebral artery. Throughout the anesthesia duration, body temperature was maintained at 37C. For sham surgeries, animals were subject to all the surgical procedures (anesthetic, scalp incision, drilling of the skull), but did not receive Endothelin-1.

MiRNA treatment: Four hours of MCAo, animals received either scrambled oligos (control) or mir363-3p (7mg/kg, 300ul) administered via tail vein injection. Scrambled and mir363-3p mimic (AAUUGCACGGUAUCCAUCUGU) oligonucleotide was purchased from Thermo Fisher, Grand Island, NY and packaged in In-vivo RNA-LANCEr II kit (Bio-Scientific, Austin, TX).

Behavioral assays: All behavior tests were performed between 8:00am and 12:00 pm in the light cycle. For assessing sensorimotor function, adhesive-tape removal tape was performed before (Pre, -2d) and after (2d and 5d) stroke. Assay for non-spatial memory and social interaction were performed before (-10d) and after (6 months) stroke. Spatial memory was tested only after (6 months) stroke. Gait was assessed at the end of the study using DigiGait equipment. All tests were performed and scored blinded.

Adhesive-tape removal test: Adhesive-tape removal test was performed as described in our previous works [22, 27-29]. A square adhesive tape (12.7 x 12.7 mm) was attached on the palmar side of the paw of each forelimb. The time taken to remove the tape was recorded for three trials. Each trial had a maximum time limit of 120 seconds and the animals were allowed to rest for at least 5 min between the trials.

Novel Object Recognition Test (NORT): This test consisted of three phases: habituation, familiarization and test phase. In the habituation phase, rat was placed in 16" x 16" open field and allowed to freely explore the arena for 10 min each day for 2 days as done in our earlier work [22]. On the third day (familiarization phase), the animal was again placed in the open-field apparatus, which now contained two identical objects (A+A) placed diagonally from each other. The rat was allowed to explore the arena and the objects for 10 min. The rat was then returned to its home cage for 1 hour (retention interval) and then placed again in the open-field arena for the test phase. For the test phase, the arena contained two objects in the same location, one that was previously available (A) and the other that was novel (B). The rat's behavior was recorded for 5 mins and the amount of time spent exploring the novel object was determined from these

recordings by an investigator blind to the experimental condition. Exploration of an object was defined as the rat sniffing or touching the object with its snout at a distance of <2cm from the object. Climbing or sitting on the object was not defined as exploration. The preference for novel object was calculated as: (Time spent exploring novel object) / (Time spent exploring novel object + familiar object) * 100.

Barnes Maze: Barnes Maze was performed on the rats to assess spatial memory [31]. A circular maze (diameter of 48") consisting of 20 holes was used. Each hole consisted of either a small square box (4" x 4" x 2") or a bigger escape box (8" x 4" x 4"). The test was divided into 2 phases spanning 4 days: Habituation (1 day) and learning (3 days). For habituation, rats were first placed in an escape box for 2 minutes covered with a lid. This allowed habituation to the escape box. After 2 minutes in the escape box, rats were placed inside a dark tube at the center of the Barnes Maze. Bright lights (for aversion) were turned on and the center tube gently lifted off allowing the rats to freely explore the maze for a maximum of 5 min to find the escape box. If the rat did not find or enter the escape box in 5 min during the day of habituation, the experimenter manually guided the rat to the escape box. Once the rat entered the escape box, lights were turned off. For the learning phase, the escape box was placed at a certain location, and the rats were allowed maximum of 2 min to find the escape. Each rat underwent 3 trials at the interval of 15 min. Ethovision software was used to analyze the latency to find the escape box and velocity during each trial.

Digigait: Gait dynamics were recorded using ventral plane videography, as described in other works [32, 33] [34]. Briefly, rats were exposed to the treadmill compartment (7cm X 30cm, DigiGait Imaging System, Mouse Specifics, Inc., Boston, MA) to acclimate. The following day,

animals were allowed to explore the compartment for 1 min with the motor speed set at 20cm/s . Video images of the rat were collected at ~125 frames per second by a high-speed digital video camera mounted below the transparent treadmill belt. Most animals walked comfortably at this speed and it was sufficiently fast to prevent the rats from rearing or turning around during videography. Digigait indices such as brake and propel were analyzed as done earlier by Piesla, M.J., et al [34] using the proprietary software.

Statistics: For all assays, group mean + SEM are reported. Group differences were determined by a two-way ANOVA performed for surgery (Sham/ MCAo) and treatment (Scrambled/Mir363-3p), with planned comparisons. For analyses where only 2 time points were compared (Social interaction) or each time compared to chance (Novel object recognition test) , a student t-test was used. All group differences were considered significant at $p < 0.05$. Statistics were analyzed using Prism GraphPad (GraphPad, San Diego, CA) and SPSS (IBM Corporation).

Results

In previous studies [24] [22] we found no differences in sham animals that received either the scrambled control or mir363-3p, on either sensory motor tests, tests of depression or NORT. Thus, for this study, the sham-scrambled and sham-mir groups were combined and reported as the 'Sham' group.

Sensorimotor deficit after stroke and improvement by Mir363-3p: Adhesive tape removal test was performed before and after MCAo to assess sensorimotor deficits acutely after stroke (Fig 12). Animals with a stroke typically take a longer time to remove the tape from the paw

contralateral to the MCAo. As shown in the Fig 12, animals removed the tape rapidly before stroke but were significantly delayed in the early acute phase, i.e. 2 day and 5 day ($F(2,30)=47.13$, $p<0.0001$) with a treatment effect ($F(1,15)=4.95$, $p=0.0419$). Post hoc analysis shows significant recovery in MCAo+Mir363-3p group as compared to MCAo+Scrambled at day 5 ($p=0.0411$). This data was consistent to our two previous studies that showed Mir363-3p acutely improves sensorimotor deficits [23] [24].

Social Interaction: Interaction with a conspecific was obtained for all three groups before and at 6 months after stroke (Fig 13). Reduced interaction with a conspecific is indicative of social disinterest, and may also indicate loss of social cognition. Prior to stroke (or sham surgery), Sham (496 secs), MCAo+Mir363-3p (387 sec) and MCAo+Scrambled (463 sec) groups had similar amounts of baseline interaction with the conspecific. Six months later, social interaction for the Sham group decreased slightly (13%), which was not statistically different from prior interaction levels (paired ttest, $p=0.543$). Six months after stroke, the MCAo+Scrambled showed a 39% decline in interaction, which was significantly lower than pre-stroke levels (paired ttest, $p=0.035$). In contrast, the MCAo+Mir363 showed a statistically insignificant decline (32%, $p=0.106$) from baseline levels at 6 months after stroke and This is consistent with our earlier work showing social interaction declines after stroke (3 months) and is attenuated in the group that received mir363-3p [23].

Novel Object Recognition Test: Preference for a novel object over a familiar object, indicated by the amount of time spent exploring the object, was assessed before and 6 month after MCAo/sham surgery (Fig 14). A 50% preference indicates that the animals do not discriminate

between novel and familiar object. Increased time spent exploring the novel object indicates retention of the memory of the familiar object and thus ability to discriminate between the two objects. All three groups showed higher preference (higher than 50%) for novel object before sham or MCAo surgery : Sham 69.2% ($p=0.0531$), MCAo+Scrambled 69.4% ($p=0.042$) and MCAo+Mir363-3p 66.6% ($p=0.034$). Six months later, the Sham group spent more time with the novel object (67.2%, $p=0.049$ as compared to chance), while the MCAo+Scrambled group spent virtually similar amount of time with the novel and familiar object (50.7%, $p=0.907$ as compared to chance). However, animals treated with mir363-3p 4h after stroke (MCAo+Mir363-3p) were able to discriminate the novel object above chance (60.7%, $p=0.014$). This data shows that mir363-3p preserves delayed non-spatial cognitive loss in animals after stroke.

Barnes Maze: Spatial learning in the animals was assessed by latency to find an escape hole in a circular maze over 3 days (Fig. 15a). Decreased latency over the testing days is indicative of learning, while longer latencies indicate impaired ability to locate the escape hole. All three groups showed gradual learning over time ($F(2, 54) = 13.37$, $p<0.001$), as well as a main effect of treatment on latency ($F(2, 54) = 7.64$, $p=0.0014$) (Fig 15b). Planned comparisons showed that both MCAo groups had a higher latency over the learning period, as compared to Shams. At day 3, MCAo+Scrambled group took significantly longer than sham animals to find the escape hole ($p=0.0141$), while latency for the MCAo+Mir363-3p was not different from Sham ($p=0.7894$). In order to ensure that differences in latency was not due to impaired physical ability, velocity measurements were also analyzed for each trial day. As shown in Fig 15b, velocity was not different for 3 groups ($F(2, 14) = 0.02341$, $p=0.9769$).

Gait dynamics: Long term motor coordination after stroke was assessed by ventral plane videography (Digigait, Quincy, MA) at the end of the study (Fig 16). Gait dynamics are crucial for regular behavior and are impaired in neurological diseases.

Brake time: Defined as the duration between the initial paw contact and maximum paw contact while walking on the belt, longer brake times indicate precise control and distribution of the body load while walking. Brake time was no different for forelimbs ($p=0.6618$) among the groups and across the limbs (Fig 16a).

Propel: Defined as the duration of the maximum paw contact on the belt before swinging the limb again to take the next stride, shorter propel times indicate better strength and control over the body in motion. Propel duration was not different in the forelimbs ($p= 0.5716$) or hindlimbs among the groups (Fig 16b).

Paw angle: Defined as the angle that the long axis of a paw makes with the direction of motion of the animal during peak stance, a wider paw angle is associated with ataxia, spinal cord injury and demyelinating diseases [35]. Planned comparison showed significantly smaller forelimb paw angle in MCAo+Mir363 animals as compared to MCAo+Scrambled ($p=0.0458$) and Sham ($p=0.0007$) (Fig 16c). A similar improvement was found in the right forelimb paw angle of MCAo+Mir363 as compared to Sham ($p=0.0018$). This indicates that mir363-3p administration improves paw angle after stroke.

Discussion

This study focused on long term recovery after stroke, specifically, cognitive impairment in middle-aged female rats. After menopause, women are at a higher risk for stroke as well as worse stroke outcomes as compared to young females and age-matched males [36]. Over one-third of these stroke survivors develop depression and some form of cognitive deficit over time, which significantly lowers their quality of life. Our previous work showed that depressive-like behaviors develop 1-3 months after stroke, and this outcome can be improved by mir363-3p treatment [23]. However, at this time point, cognitive decline as assessed by the novel object recognition task was not observed. Thus, in this study, we chose to investigate delayed cognitive impairment along with social interaction at an extended time point after stroke (6 months). Our main findings: 1) confirm that MCAo results in an early deficit in sensorimotor skills and long term (6 months) impairment in cognition, including spatial and non-spatial memory, 2) show that social interaction which is impaired at 3 months remains impaired at 6 months and 3) that Mir363-3p treatment improved the early deficits in sensorimotor skills and abrogated cognitive deficits and social interaction impairment.

MicroRNAs have emerged as an attractive therapy for a wide range of diseases because of their role in regulating gene transcription and translation. These 18-25 nucleotide noncoding RNAs function by regulating the methylation of target genes [37], stability of mRNA transcript [38]or their translation to proteins[39]. MicroRNA profiles alter with both stroke as well as risk factors for stroke as shown in clinical and preclinical studies [40-43]. Many preclinical studies have focused on microRNAs as a treatment for stroke, however, a vast majority of these studies used male animals exclusively [44]. We were among the first to use microRNA in both males and

females, and have shown that neuroprotection from microRNAs have sex-specific effects. ICV administration of antagomirs to mir1 and let7f reduced infarct volume in only female rats only, presumably by targeting the IGF-1 pathway [29]. Similarly, IV administration of mir363-3p reduced infarct volume in middle-aged female rats but not males. Mir363-3p caused a sex-specific regulation of caspase-3 [28], which has been shown previously as an effective target in for stroke recovery in females but not males [45].

Furthermore, very few studies have focused on the role of microRNA in improving stroke outcomes beyond the acute phase. A clinical study in Chinese cohort has shown that miR-132 expression in the blood is up-regulated in the post stroke cognitively impaired patients both males and females [46]. However, microRNA-based treatments for post stroke cognition have mostly focused on males. Liu et al showed that miR134 improved cognition after stroke in males (age not specified) [47]. Similarly, inhibition of miR27b was shown to improve post stroke cognition in 10wk old male mice [48]. To the best of our knowledge, this present study is the first to address delayed cognitive deficits in a middle-aged female animal model, and to show that post-stroke microRNA treatment improves cognitive function long term after stroke.

Six domains of cognition are included in DSM V to make a positive diagnosis for a Neurocognitive disorder [49]. They are: complex attention, executive functioning, memory, language, perceptual-motor/visuospatial function and social cognition. A diagnosis is usually made when there is a significant impairment in at least one of these domains. Changes in personality and social behavior can also occur with cognitive impairment [50-52]. In the present study, memory and visuospatial functions were modeled by the NORT and Barnes Maze test

respectively. The NORT task showed that animals were able to discriminate between novel and familiar objects before stroke, but did not retain this ability 6 months after stroke, in the scrambled treated group. Remarkably, recognition of the novel object was retained in the mir363-3p treated groups. In a previous study, we reported that this task was not affected at 3 months after stroke [23], indicating that this non-spatial memory function deteriorates between 3 to 6 months. This pattern was consistent with the results in a test of spatial memory. Performance on the Barnes Maze at 6 months indicated a decline in a spatial learning task (shown by longer latency to reach the escape hole) for animals with a stroke as compared to sham group over the 3 test days. Interestingly, performance on the test plateaued in the stroke group that received scrambled oligos, learning in the mir363-3p treated stroke group continued to improve and was no different from sham at the end of the learning trials.

Recognizing that motor impairment may confound some of the results on tests of neurocognitive assessment, our tests show that velocity in the Barnes maze was similar for all groups. Impairment on the adhesive removal test does not persist after 30 days post stroke [23], indicating that general sensory motor function returns to normal. Furthermore, the gait analysis, using the Digigait apparatus, showed that all groups had similar gait and did not have any ambulatory difficulties pre and post stroke.

This study also underscores the importance of aging as a factor in post stroke neuropsychiatric deficits. As indicated above, no impairment was seen in the NORT task at 3 months post stroke when the rats were 15 months old chronologically [23], however, at 6 months after stroke (chronological age 18 months) impairment was evident in both the NORT and Barnes maze task.

Even in the mir363-3p treated groups, preference for the novel object declined at 18 months of age, as compared to their performance at 15 months of age, although in both cases the novel object was more preferred than the familiar object. Social behavior as assessed by social interaction was significantly impaired after stroke in the scrambled treated animals at 3 months after stroke and remained reduced 6 months after stroke, indicating that the depressive phenotype in the scrambled treated animals persisted even months after stroke.

Despite the absence of any major motor deficits at 18 months of age (6 months post stroke), gait analysis yielded an unexpected difference in paw angle, which was larger in both sham and scrambled-treated stroke animals as compared to the mir363-3p-treated stroke group. Greater paw angle is related to ataxia and demyelinating diseases [35] and the increased paw angle in Sham and MCAo+Scrambled group may indicate white matter injury that occurs with age. White matter pathways are critical for cognitive functions [53-55]. Studies have suggested myelin breakdown as a consequence of stroke and a contributing factor for aging [56-58]. Oligodendrocytes, in particular, are highly vulnerable to ischemia, undergoing demyelination after stroke [59, 60]. The observation that Mir363-3p-treat stroke animals have a narrow angle suggest a possible neuroprotective effects on myelin health due to aging. Future studies would focus more on the effects of mir363-3p on myelination.

The tasks included in this study assess hippocampal function directly or indirectly. Barnes Maze assesses spatial memory, which is heavily dependent on the hippocampus, while hippocampal involvement in the Novel object recognition test is still debatable [61-66]. Social interaction, which is a measure of social interest and of social cognition is also hippocampus-dependent [67, 68] and can occur with cognitive impairment [50-52].

The stroke model in this study, MCAo, is the most common stroke type in human patients and focal ischemia due to MCAo does not directly affect hippocampus. However, studies have shown that MCAo triggers apoptosis in hippocampus in as early as 3 days via activation of caspase-3 [15]. This distant injury could be a result of the mechanical compression of hippocampus from edema in the brain or spread of excitotoxicity from the cortical region to hippocampus. CA1 and CA3 regions of the hippocampus have high expression of NMDA receptors that make them highly vulnerable to the glutamate toxicity [69] [70]. In our earlier studies, we show that exogenous mir363-3p is internalized by neurons in the ischemic cortex and striatum, and downregulated the expression and activation of caspase-3 in the ischemic hemisphere of middle-aged female rats [24]. It is possible that this microRNA is also internalized by hippocampal neurons, likely suppressing caspase-3 activation and restricting delayed injury to the hippocampus. Future studies should focus on possible localization of mir363-3p in hippocampus and local caspase-3 activation acutely after stroke.

Effective therapy for cognitive decline after stroke still remains an unmet medical need. No drugs are approved specifically for cognitive impairment. Clinicians sometimes prescribe drugs approved for Alzheimer's disease such as donepezil and mementine for cognitive deficits. These drugs have shown some improvement for cognition but not in the global functioning of the patients [71-73]. Social interaction as well as depression are critical aspects of global functioning in the patient population [74]. This underscores the possibility that cognitive impairment after stroke may be different from cognitive impairment from AD, and that it is important to focus on the pathophysiology of stroke to target therapies for the cognitive deficit that follows. We show that microRNA 363-3p which has neuroprotective effects against stroke in middle-aged female

rats is capable of improving long-term post-stroke cognitive impairment. Mir363-3p not only improved spatial and non-spatial memory, but also improved social interaction in the rats. Since there is no specific therapy approved for cognitive impairment after stroke, and the dementia medications do not address global functioning, our data showing improved social interaction and cognitive function with a single dose of microRNA treatment after stroke suggest that neuroprotectant molecules could be better for cognitive treatment purpose.

Figures

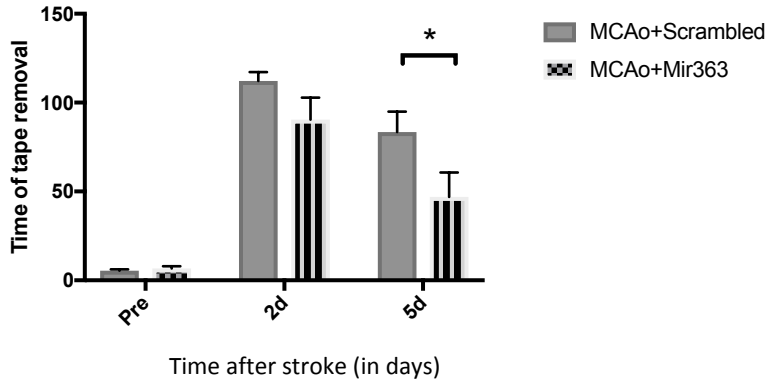

Figure 12: Adhesive-tape removal test. Histogram depicts mean + SEM of the latency (measured in seconds) to remove a tape attached to palmar side of the forepaw contralateral to the ischemic hemisphere. Animals were at 3 time points: Before MCAo (Pre), 2 and 5 day after MCAo. Key: *: $p<0.05$, comparison of treated vs control group at the same time.

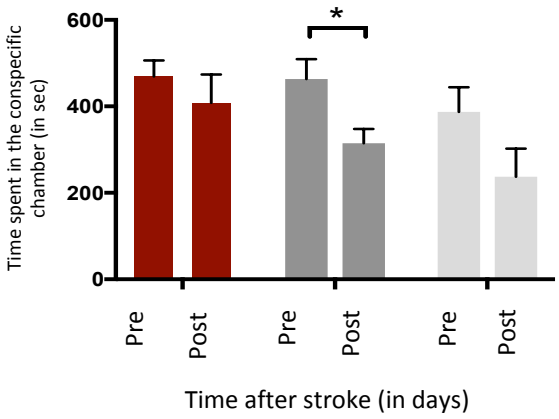

Figure 13: Sociability Test. Sham animals and stroke animals treated with either scrambled oligos (MCAo+Scrambled) or mir363-3p (MCAo+Mir363-3p) were tested for social interaction with a stranger rat in a 3-chamber apparatus before (Pre) and 6 months after (Post) MCAo. Histogram depicts mean + SEM of interaction time (in seconds). Key: *: $p<0.05$, comparison of the group with its baseline.

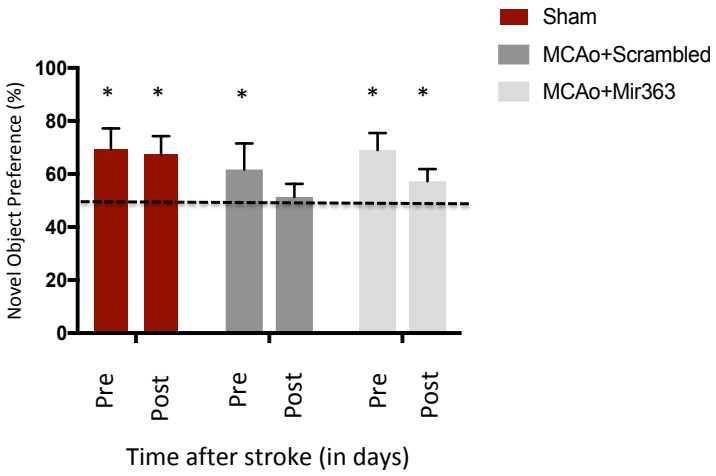

Figure 14: Novel object Recognition Test. Sham animals and stroke animals treated with either scrambled oligos (MCAo+Scrambled) or mir363-3p (MCAo+Mir363-3p) were tested on their preference for novel objects before (Pre) and 6 month after (Post) MCAo. Histogram depicts mean + SEM preference (in %) for the novel object. Key: *: $p<0.05$, comparison of actual preferences to chance (50%) (as shown by the dotted line).

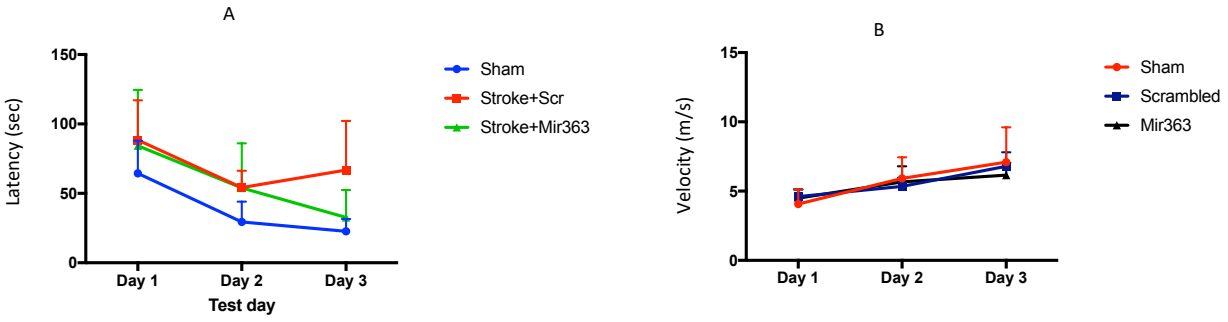

Figure 15: Barnes Maze Test. Sham animals and stroke animals treated with either scrambled oligos (MCAo+Scrambled) or mir363-3p (MCAo+Mir363-3p) were tested for (A) spatial memory and (B) velocity in the Barnes maze 6 months after stroke. Data are presented as the mean + SEM latency (in seconds) and velocity as m/sec. Key: a: main effect of time; b: main effect of treatment; *: $p<0.05$, comparison of MCAo+scr and sham at day 3.

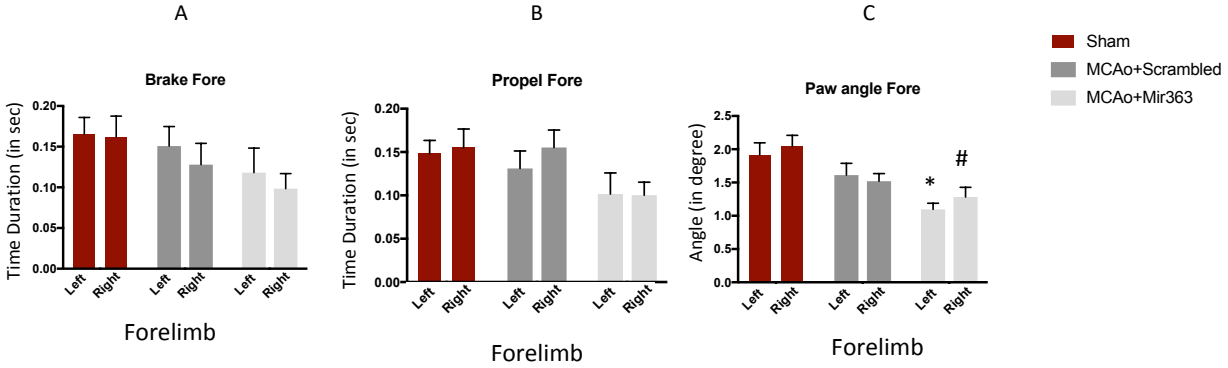

Figure 16: Gait Dynamics. Sham animals and stroke animals treated with either scrambled oligos (MCAo+Scrambled) or mir363-3p (MCAo+Mir363-3p) were tested for brake time (in sec), propel time (in sec) and paw angle (in degrees) for left and right forelimbs 6 month after stroke. Histogram depicts mean + SEM. Key: *:$p<0.05$, comparison of treated (MCAo+Mir363-3p) and control (MCAo+Scrambled) group for left forelimb. #: $p<0.05$, comparison of treated (MCAo+Mir363-3p) and Sham for right forelimb.

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CHAPTER V

CONCLUSION

Mir363-3p improves acute sensory motor function and long-term stroke outcomes

MicroRNA are important regulators of mRNA transcript stability and gene translation [1, 2] and their profile has been found to be altered in stroke in clinical as well as preclinical studies [3-5]. We found in our earlier studies that expression of mir363-3p was reduced with age in female rats and was negatively correlated with infarct volume [6]. When exogenous mir363-3p was administered in middle-aged female rats 4 hour after stroke, it significantly improved acute sensory motor functions and reduced the infarct volume [7]. These results encouraged us to investigate if the therapeutic effects of this microRNA also extended to long-term stroke outcomes which is presented in this dissertation.

Since depression and cognitive impairment are common long-term consequences of stroke, we were interested in testing whether mir363-3p treatment improved these disorders. In the first study, post-stroke depression (PSD) was seen to develop in middle-aged female rats by 1-3 months after stroke, and mir363-3p improved it. To model depressive-like phenotype in rats, we tested anhedonia, social interaction and despair/learned helplessness. In the rats that developed depressive-like phenotype, peripheral inflammatory cytokines were transiently up-regulated, and peripheral BDNF was down-regulated. Moreover the meso-striatal component of the reward pathway underwent retrograde degeneration in the ischemic hemisphere of the brain. In the groups that were treated with mir363-3p, peripheral inflammatory cytokines and BDNF were found to be normalized and meso-striatal projections were rescued. Cognitive function, however,

was intact at 3 months after stroke. In the second study, we investigated changes in the microbiome of stroke rats that would shed light in the depressive phenotype. Tryptophan, a gut metabolite and also a precursor to serotonin, was decreased in the blood of depressed rats 3 months after stroke as compared to the sham animals. Since tryptophan is cleaved from nutrients by gut bacteria, this observation suggested that gut dysbiosis may be present in stroke rats that exhibit depression. A global indicator of dysbiosis, i.e., the ratio of Firmicutes:Bacteroidetes was elevated in stroke rats, and short chain fatty acids, another gut metabolite was also decreased. This data suggested that stroke results in chronic changes in the gut.

In the final study, we examined the effect of stroke on cognitive impairment and the effect of mir363-3p on post stroke cognitive impairment. While we did not observe cognitive impairment in middle-aged female rats at 3 months after stroke, we tested the impairment at 6 months. Deficit in spatial as well as non-spatial memory was detected at 6 months time, and mir363-3p improved performance in both domains.

All these results support the hypothesis that mir363-3p improves not only acute sensory motor functions, but also long-term consequences such as post stroke depression and cognitive impairment. Currently, treatment for stroke is limited to only tPA and thrombectomy, which is focused on dissolving/removing the clot and reperfusing the brain. For stroke survivors that develop depression and cognitive impairment, there is no specific drug approved. The anti-depressants and AD drugs that are generally prescribed are not too effective and sometimes even complicate the quality of life with the side effects that follow. We do not yet know from our animal study whether mir363-3p has its side effects. However, since all these improvements

were brought by just a single dose 4 hour after stroke, it is possible that this dose may not have continuing side effects.

Localization in the brain

Mir363-3p is packaged in a lipid-based bilayer vehicle and administered IV vial tail vein. Our previous work shows that it is seen in the blood up to 48 hours and is taken up by specific brain cells [7]. In the brain, it is preferentially located in the ischemic hemisphere and is found localized in the neurons as well as the endothelial cells of middle cerebral artery by 5 days. We have not followed the localization beyond 5 days in our work. It is also possible that mir363-3p may be improving long term cognitive function as shown in chapter 3 by being up taken hippocampus acutely after administration. Future work would focus on the localization in other forebrain areas. Since mir363-3p also improves the inflammatory response as shown in chapter 1, we can not ignore the possibility that it might be modulating inflammatory responses via the spleen or liver.

Limitations: Male vs Female

Mir363-3p reduces infarct volume and improves sensorimotor functions in middle-aged female rats, but not male rats. Death pathway after stroke shows dramatic sexual dimorphism[8]. One of the death pathway is mediated by neuronal Nitric Oxide (nNOs) which leads to DNA damage and activated DNA repair enzyme poly ADP ribose polymerase-1 (PARP-1)[9]. When the nNOS pathway was inhibited in male rats, it led to better stroke outcome. However, the same inhibition in female rats led to worsening of the ischemic injury. Similarly, the other death pathway involves Caspase-3. Post stroke, Caspase-3 cleavage occurs in both males and females, but

predominantly in females. However, inhibitor to Caspase has shown neuroprotection for stroke in females, but not males [8]. This suggests that males primarily undergo nNOS/PARP-1 induced neuronal death, whereas females primarily undergo Caspase-3 induced neuronal death. This could be the reason that mir363-3p is not effective for males. Hence, we did not include male studies in any of our long-term studies. We believe that mir363-3p may not be effective in improving depression and memory in male rats after stroke, because it does not improve short-term deficits.

Therapeutic window for Mir363-3p

The main goal of this study is to find an alternate therapeutics for stroke that can be more accessible to stroke patients. Currently only about 6-8% of stroke patients qualify for the tPA treatment because of its therapeutic window of 4.5h after the onset of symptoms. Majority of people do not get to the medical facility in time as they may misinterpret the symptoms. We have therapeutic effects from mir363-3p when administered at 4h. Future work would focus on a longer time point of administration.

Mir363-3p for depression and cognitive decline only in the context of stroke

Finally, we would like to emphasis that mir363-3p improves long term depression and cognitive impairment only in the context of stroke. This study focused only on post-stroke depression (PSD) and cognitive impairment (PSCI). There are different types of depression and a wide range of cognitive impairment. Our study does not model other forms of depression or cognitive impairment.

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