Published in final edited form as:

Brain Behav Immun. 2017 March ; 61: 266–273. doi:10.1016/j.bbi.2016.09.001.

Myocardial Ischemia/Reperfusion impairs neurogenesis and hippocampal-dependent learning and memory

Kirsten S. Evonuk^{*,†}, Sumanth D. Prabhu[§], Martin E. Young[§], and Tara M. DeSilva^{*,†,||}

^{*}Center for Glial Biology in Medicine, University of Alabama at Birmingham, Birmingham, AL, 35294

[†]Department of Physical Medicine Rehabilitation, University of Alabama at Birmingham, Birmingham, AL, 35294

[§]Department of Medicine, Division of Cardiovascular Disease, University of Alabama at Birmingham, Birmingham, AL, 35294

^{||}Department of Neurobiology, University of Alabama at Birmingham, Birmingham, AL, 35294

Abstract

The incidence of cognitive impairment in cardiovascular disease (CVD) patients has increased, adversely impacting quality of life and imposing a significant economic burden. Brain imaging of CVD patients has detected changes in the hippocampus, a brain region critical for normal learning and memory. However, it is not clear whether adverse cardiac events or other associated co-morbidities impair cognition. Here, using a murine model of acute myocardial ischemia/reperfusion (I/R), where the coronary artery was occluded for 30 minutes followed by reperfusion, we tested the hypothesis that acute myocardial infarction triggers impairment in cognitive function. Two months following cardiac I/R, behavioral assessments specific for hippocampal cognitive function were performed. Mice subjected to cardiac I/R performed worse in the fear-conditioning paradigm as well as the object location memory behavioral test compared to sham-operated mice. Reactive gliosis was apparent in the hippocampal subregions CA1, CA3, and dentate gyrus 72 h post-cardiac I/R as compared with sham, which was sustained two months post-cardiac I/R. Consistent with the inflammatory response, the abundance of doublecortin positive newborn neurons was decreased in the dentate gyrus 72 h and 2 months post-cardiac I/R as compared with sham. Therefore, we conclude that following acute myocardial infarction, rapid inflammatory responses negatively affect neurogenesis, which may underlie long-term changes in learning and memory.

Keywords

myocardial infarction; neuroinflammation; cognition; reactive gliosis

Introduction

Cognitive deficits in patients with cardiovascular disease have become increasingly common in the Western population^{1–3}. Although technological advances have improved survival rates following adverse cardiac events, cognitive deficits remain and impose a significant, clinically measurable impact on memory. These outcomes interfere with quality of life and employment status, imposing a large financial burden on patients and their families. Ischemic heart disease is a primary cause of heart failure - a condition in which the heart is unable to efficiently supply blood to meet the needs of the body. In order to therapeutically address cognitive decline in this patient population, a basic understanding of the mechanisms that result in cognitive decline are required.

Recent studies suggest that patients with heart failure have damage to the hippocampus^{4–6}, a brain region critical for normal learning and memory, and reduced volume in the parahippocampal gyrus⁷ which projects to the hippocampus. The importance of these clinical assessments are supported by basic science research in rodents where selective lesioning of the hippocampus results in cognitive decline. Although experimental animal studies of cerebral vascular ischemia and stroke show pathological changes in the brain, the impact of myocardial ischemia-reperfusion (I/R), *i.e.*, reperused myocardial infarction, in the brain has not been explored.

Ischemic brain injury such as stroke results from cerebrovascular occlusion causing oxidative stress and inflammation to the surrounding brain parenchyma. Myocardial infarction, one of the most common forms of cardiovascular disease, results from an occlusion of the coronary arteries within the myocardium, causing oxidative stress and inflammation to the infarct and surrounding areas. Treatments with thrombolytic medications and/or angioplasty restore blood flow through the artery - a process termed reperfusion. Reperfusion of ischemic tissues is often associated with oxidative stress, microvascular injury, and inflammation, causing greater damage to the myocardium. However, whether myocardial I/R induces damage to extra-cardiac tissues is currently unknown.

The aim of the present study was to determine if acute cardiac I/R in the mouse (produced by occluding the coronary artery for 30 min followed by reperfusion) could recapitulate the cognitive deficits observed clinically. Our results demonstrate a specific impairment in the hippocampal-dependent object location memory task and contextual fear conditioning paradigm two months post-cardiac I/R. Recent studies have demonstrated that a decrease in neurogenesis can underlie spatial learning and memory deficits⁸. Therefore, we examined doublecortin positive neurons in the dentate gyrus and found a decrease at 72 h post-cardiac I/R that was sustained at two months. Consistent with other studies demonstrating that inflammation can impair neurogenesis, we also observed reactive microgliosis and astrogliosis in the dentate gyrus as well as the CA1 and CA3 sub-regions of the hippocampus. These findings suggest that reperused myocardial infarction can induce rapid and sustained hippocampal neuroinflammation and long-term impairments in cognition.

Methods

Animals

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23) and was approved by the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham. Male C57BL/6 mice (20 weeks old) were housed at the UAB Animal Research Program under controlled conditions (23 ± 1 °C; 12-h light/12-h dark cycle) and received standard laboratory chow and water ad libitum.

Animal Model and Surgery

Mice were anesthetized with 1.5–2.0% isoflurane, intubated, and ventilated on a Harvard ventilator. In the open-chest cardiac I/R group, a lateral thoracotomy was performed followed by immediate ligation of the left anterior descending artery (LAD), and the ligation was removed 30 minutes later as previously published by the authors ⁹. Studies for stereological assessment of hippocampal reactive gliosis also included closed-chest cardiac I/R mice. In this group, the occluding device was implanted subsequent to thoracotomy, followed by chest closure as previously described by the authors ⁹. One week later, the closed-chest I/R mice were subjected to a 30-minute occlusion followed by release. Sham mice for both groups were subjected to the same procedure respectively but without ligation. Open-chest and closed-chest cardiac I/R mice had similar inflammatory cell numbers in the CA1, CA3, and dentate gyrus subregions of the hippocampus at 72 h post-surgery, therefore we opted to use open-chest cardiac I/R mice and their corresponding sham controls for the remaining experiments.

Echocardiography

Mouse echocardiography was performed under anesthesia with tribromoethanol (0.25 mg/g IP), and isoflurane ($\approx 1\%$) as needed, using a VisualSonics Vevo 770 High-Resolution System with a RMV707B scan head as previously described ¹⁰. Mice were imaged on a heated, bench-mounted adjustable rail system (Vevo Imaging Station) that allowed steerable and hands-free manipulation of the ultrasound transducer.

Behavioral Procedures

Habituation—All animals were trained and tested during the same lighting and time of day conditions. Animals were given 30 minutes to habituate to the behavioral testing room before training or testing. All behavioral chambers were cleaned with 70% ethanol cleaning solution before and after mouse use.

Open field—Mice were placed in the center of an open-field chamber (42×42×30 cm). Movements were tracked with Ethovision (Noldus) for 5 min. Horizontal motor (distance traveled), movement speed, and central activity (distance traveled in central area/total distance traveled) were evaluated. Mean value and SEM were calculated in each group.

Zero maze—The zero maze consisted of a round track (65 cm diameter) divided into four zones of equal area by two sets of walls along the track, separated by 180 degrees around the

track. The animals were put in the center of the arena and observed for 4 min with a camera-driven tracker system, *i.e.*, Ethovision (Noldus, The Netherlands). The system recorded the position of the animal in the arena at 8 frames/second, and the data was analyzed for time spent in each open and closed zone, speed of locomotion, and frequency of entries into a zone.

Contextual Fear conditioning—Two months post-surgery animals were placed in a fear-conditioning chamber and given a 2 min interval of habituation followed by a 2 s, 0.6 mA mild electrical shock. This 2 min interval was then repeated followed by an additional 2 s, 0.6 mA shock. Animals were then returned to their home cage. Twenty-four hours after training, testing was performed by placing the animals back in the same conditioning chamber in which no foot shock was delivered. The freezing behavior was recorded for 5 minutes. FreezeFrame 4 (ActiMetrics) was used to analyze the freezing behavior in which the cutoff for freezing was while the animal was immobile excluding breathing.

Object Location Memory Task—During the acquisition phase, the animals were exposed to two identical Lego™ monkey toys in mirrored positions in the left and right corners of an arena (42×42×30 cm). The animals were allowed to explore both objects during a 10 min. training period, and interaction was measured by Ethovision (Noldus). After a delay of 24 h, one toy was moved to the opposite corner of the arena. The other toy was placed in its original location. The toy that was moved to the novel position was randomly switched in half of the test trials for each group to control for potential bias towards one object. Exploratory behavior was monitored for 5 min, and tracking was recorded with Ethovision (Noldus). The percentage of time the mouse spent with the object in the novel location as a percentage of total time spent with both the new and old object was reported.

Immunohistochemistry and stereology—Mice from each group (closed-chest sham, closed-chest I/R, open-chest sham, open-chest I/R) were anesthetized with 5% isoflurane before transcardiac perfusion with PBS (1.5 mM potassium dihydrophosphate, 2.7 mM sodium phosphate, and 150 mM sodium chloride, pH, 7.4) followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde for 24 h, cryoprotected in 30% sucrose, embedded in OCT, and cut (30 µm) coronally on a cryostat. For 3,3'-diaminobenzidine (DAB) immunohistochemistry, sections were washed in PBS, incubated in citrate buffer (pH 6.0) for antigen retrieval, and quenched in 3.0% H₂O₂ in PBS. Sections were blocked for 1 h at room temperature in 5% goat or horse serum (corresponding to the host of the secondary antibody) in PBS containing 0.3% Triton X-100. Primary antibodies for the following markers were used: glial fibrillary acidic protein (GFAP; 1:1000, BioLegend, SMI-22R), ionized calcium-binding adapter molecule 1 (Iba-1; 1:1000, Wako, 019-19741), Ki-67 (1:300, Millipore, AB9260), doublecortin (DCX; 1:200, Abcam, AB18723), and active caspase-3 (1:500, R&D Systems, AF835). Sections were incubated in 5% goat or horse serum in PBS containing 0.3% Triton X-100 and primary antibody at 4°C overnight. Sections were then washed and exposed to the respective biotinylated secondary antibodies diluted 1:200 in 5% goat or horse serum in 0.3% Triton X-100 in PBS for 1 h at room temperature. All stereological estimates were performed using an Olympus BX51 and

MicroFire A/R camera system with Stereo Investigator software (MicroBrightField, Williston, VT) using the optical fractionator method. The CA1, CA3, and dentate gyrus subfields of the hippocampus from bregma -1.06 mm to bregma -3.64 mm were defined using a commercially available atlas (Academic Press). Every 10th section for a total of 3–6 sections in the same region for each animal were processed for stereology. Animals were excluded if fewer than 3 sections were available for counting. Regions of interest (ROI) were traced using a 4X objective and cells were counted at high magnification (40X). A 45 μ m by 45 μ m counting frame was used in a 300 μ m by 100 μ m sampling grid. Cells were counted within a 20 μ m dissector height with a 5 μ m guard zone on both sides of the section's measured thickness. Only cells with a definable cell body were quantified to generate estimates. Gundersen $m = 0$ coefficient of error (CE) is reported for each hippocampal subregion, which has been shown to be more accurate for irregularly shaped tissue than Gundersen CE $m = 1$ ¹¹. For caspase-3-stained sections, a Leica DM5500 B upright microscope with 20X objective and Leica DFC425 C digital camera with Leica Application Suite (version 4.4.0) acquisition software were used.

Statistical analyses—Data are presented as mean $\pm$ SEM where n represents the number of mice. Statistical comparisons (significant for $p < 0.05$) were performed using two-tailed t -tests for all assessments.

Results

Myocardial ischemia-reperfusion decreased cardiac systolic function in mice

Cardiac function was assessed by echocardiography 72 h and 2 months following open-chest cardiac I/R or sham surgery. Left ventricular (LV) fractional shortening ($p = 0.091$, $t = 1.75$, $df = 29$) and ejection fraction ($p = 0.054$, $t = 2.01$, $df = 29$) at 72 h were reduced but not significantly decreased in myocardial I/R animals relative to sham controls (Fig. 1A and B). Cardiac I/R mice had a fractional shortening of $41.50 \pm 1.66\%$ (Fig. 1A) and an ejection fraction of $58.71 \pm 1.92\%$ (Fig. 1B) 72 h after cardiac I/R. Sham mice had a fractional shortening of $45.33 \pm 1.16\%$ (Fig. 1A) and an ejection fraction of $63.98 \pm 1.55\%$ (Fig. 1B). Two months after surgery, cardiac I/R mice exhibited statistically significant reductions in fractional shortening ($p = 0.018$, $t = 2.68$, $df = 14$) and ejection fraction ($p = 0.034$, $t = 2.35$, $df = 14$) relative to sham control with a fractional shortening of $33.12 \pm 2.74\%$ (Fig. 1C) and an ejection fraction of $50.57 \pm 3.33\%$ (Fig. 1D). Sham mice had a fractional shortening of $42.38 \pm 2.10\%$ (Fig. 1C) and an ejection fraction of $60.61 \pm 2.68\%$ (Fig. 1D). Hence, myocardial I/R was sufficient to produce sustained cardiac injury.

Myocardial ischemia-reperfusion impairs learning and memory

Two months following open-chest cardiac I/R, cognitive function was assessed relative to sham control mice. To rule out the potential contribution of differences in activity or anxiety-like behavior on cognitive assessments, open-field and zero maze assessments were performed. General activity was not different between sham and cardiac I/R groups as indicated by velocity ($p = 0.23$, $t = 1.24$, $df = 18$) or total distance ($p = 0.099$, $t = 1.33$, $df = 20$) in open field analysis (Fig. 2A and B), demonstrating no changes in exploratory behavior. Additionally, time in center ($p = 0.22$, $t = 1.26$, $df = 20$) and distance in center ($p =$

0.64, $t = 0.47$, $df = 21$), measurements of anxiety-like behavior, were not significantly different in cardiac I/R animals relative to sham in the open field assessments (Fig. 2C and D). This was further corroborated by no significant difference in number of entrances ($p = 0.52$, $t = 0.66$, $df = 21$) or time spent in the open area ($p = 0.22$, $t = 1.26$, $df = 21$) of the zero maze (Fig. 2E and F). With no changes in exploratory behavior or anxiety-like behavior evident in the cardiac I/R mice, cognitive performance could be appropriately ascertained.

Previous studies have shown lesions in the hippocampus of patients with heart failure in T2-weighted MRI scans⁴. We therefore utilized two hippocampal-dependent memory tasks to probe cognitive function: the object location memory task and contextual fear conditioning. The object location memory task revealed that sham animals spent significantly more time interacting with an object in a novel location relative to the cardiac I/R group (at 3 min., $p = 0.030$, $t = 2.42$, $df = 14$; at 4 min., $p = 0.013$, $t = 2.85$, $df = 14$; at 5 min., $p = 0.061$, $t = 2.04$, $df = 14$; Fig. 2G). Additionally, 24 h after training, the cardiac I/R animals spent significantly less time freezing in the contextual fear-conditioning assay ($p = 0.013$, $t = 2.71$, $df = 21$; Fig. 2H). Taken together these behavioral assessments suggest cardiac I/R animals have a hippocampal-dependent cognitive impairment relative to their sham counterparts.

Myocardial ischemia-reperfusion causes reactive gliosis in the hippocampus

We therefore decided to investigate if there was an inflammatory response within the hippocampus. To determine if neuroinflammation was present in the hippocampus, models of both open-chest and closed-chest cardiac I/R were used. The closed-chest model exhibits more circumscribed resolution of myocardial pro-inflammatory cytokine expression than the open-chest model, suggesting that this approach can more precisely define changes in inflammatory activation in comparison to sham¹². Coronal hippocampal sections from both the open- and closed-chest cardiac I/R models were DAB-stained for GFAP and Iba-1 72 h post-cardiac I/R. Using the optical fractionator method, stereological estimates were determined in the CA1, CA3, and dentate gyrus subfields throughout the entirety of the hippocampus. Both open-chest (Fig 3. A, B) and closed-chest (Fig 3. C, D) cardiac I/R models had significantly more GFAP-positive cells in all three subfields of the hippocampus when compared to sham controls (for open chest in CA1: $p = 0.0059$, $t = 4.17$, $df = 6$; in CA3: $p = 0.029$, $t = 2.74$, $df = 7$; in dentate gyrus: $p = 0.044$, $t = 2.45$, $df = 7$; for closed chest in CA1: $p = 0.011$, $t = 3.66$, $df = 6$; in CA3: $p = 0.0053$, $t = 4.26$, $df = 6$; in dentate gyrus: $p = 0.0042$, $t = 4.48$, $df = 6$). Similarly, the number of Iba-1 positive cells in all subfields of the open-chest (Fig. 3. E, F) and closed-chest (Fig. 3. G, H) animals was increased (for open chest in CA1: $p = 0.005$, $t = 4.41$, $df = 6$; in CA3: $p = 0.003$, $t = 4.67$, $df = 6$; in dentate gyrus: $p = 0.003$, $t = 4.94$, $df = 6$; for closed chest in CA1: $p = 0.002$, $t = 5.22$, $df = 6$; in CA3: $p = 0.06$, $t = 2.35$, $df = 6$; in dentate gyrus: $p = 0.0002$, $t = 8.27$, $df = 6$). Reactive astrocytes and microglia also appeared to have a more hypertrophic morphology in the hippocampi of cardiac I/R animals. Taken together these data indicate that neuroinflammation is present 72 h post-acute myocardial I/R.

Initially inflammation was probed at 72 h after surgery to investigate a time period in which acute inflammatory processes occur. We also determined if inflammation was sustained at 2 months post-cardiac I/R, a time in which changes in cognitive function were observed.

Using the optical fractionator method, stereological estimates were determined in the CA1, CA3, and dentate gyrus subfields throughout the entirety of the hippocampus in mice subjected to open-chest surgery. No statistically significant differences were found in the number of GFAP-positive cells in any subfield in cardiac I/R mice compared to sham (CA1: $p = 0.11$, $t = 1.87$, $df = 6$; in CA3: $p = 0.66$, $t = 0.46$, $df = 6$; in dentate gyrus: $p = 0.076$, $t = 2.14$, $df = 6$; Fig. 4A). A statistically significant increase in Iba-1 staining was observed in the dentate gyrus of cardiac I/R mice compared to sham (CA1: $p = 0.11$, $t = 1.91$, $df = 6$; in CA3: $p = 0.059$, $t = 2.33$, $df = 6$; in dentate gyrus: $p = 0.0024$, $t = 5.67$, $df = 5$; Fig. 4B). These data suggest that the reactive glial profile was reduced but partially maintained 2 months post-cardiac I/R when cognitive function was assessed.

Myocardial ischemia-reperfusion decreases neurogenesis

Our evidence of reactive gliosis demonstrates a significant and sustained inflammatory process in the CA3 and dentate gyrus subregions of the hippocampus. These data in conjunction with our behavioral data support a deficiency in spatial learning suggest impairment in the dentate gyrus. The dentate gyrus maintains proliferation of newborn neurons throughout life, which contribute to learning and memory. Previous literature has demonstrated that neuroinflammation inhibits neurogenesis in the dentate gyrus¹³. We therefore quantified the number of doublecortin-positive newborn neurons in the dentate gyrus in open-chest cardiac I/R and sham groups. At 72 h, the doublecortin immunopositive newborn neurons were significantly decreased in cardiac I/R animals relative to sham controls ($p = 0.043$, $t = 2.47$, $df = 7$; Fig. 5A). Additionally, the total number of doublecortin immunopositive cells was significantly reduced 2 months after cardiac I/R compared with sham ($p = 0.0027$, $t = 5.51$, $df = 5$; Fig. 5C).

To investigate if the decrease in newborn neurons was a result of apoptosis, caspase-3 immunoreactivity was assessed in the dentate gyrus and other regions of the hippocampus including CA1 and CA3 in sham and cardiac I/R mice (Fig. 6). Postnatal day 7 mouse hippocampus served as a positive control, since neuronal apoptosis is documented during development¹⁴. Caspase-3 immunoreactivity was observed in neuronal populations in the CA1, CA3, and dentate gyrus in the postnatal day 7 hippocampus (Fig. 6A). Caspase-3 expression decreases with aging, so postnatal day 30 mouse hippocampus was used as a negative control (Fig. 6A). In comparison, no caspase-3 immunoreactivity was observed in sham versus cardiac I/R mice from 72 hours post-surgery open and closed models (Fig. 6B) or the two months post-surgery open model (Fig. 6C). Since no staining was observed in postnatal day 30 hippocampus or in the sham or cardiac I/R hippocampus, contrast was enhanced to identify the architecture of hippocampal structures, thus resulting in increased background staining.

To further explore if the decrease in newborn neurons observed in cardiac I/R mice correlated with a change in proliferation, the number of Ki-67 positive cells (a marker of proliferation) was assessed in the subgranular zone of the dentate gyrus 72 h post-surgery ($p = 0.12$, $t = 1.82$, $df = 6$; Fig. 5B). Although the number of Ki-67 positive cells was decreased there was no statistically significant difference between sham and cardiac I/R groups. Similar results were observed at the 2 month time point ($p = 0.052$, $t = 2.42$, $df = 6$). These

data support a sustained reduction of neurogenesis induced by cardiac I/R in the presence of a neuroinflammatory state, and a measureable deficit in learning and memory.

Discussion

The key finding of our study is that a 30 min period of myocardial ischemia followed by reperfusion is sufficient to induce hippocampal-dependent cognitive decline in male C57BL/6 mice at 2 months post-cardiac injury. This conclusion is supported by changes in the hippocampal-dependent object location memory task and the contextual fear-conditioning paradigm. Additionally, neurogenesis in the dentate gyrus of cardiac I/R animals was significantly decreased both acutely and chronically after cardiac I/R, potentially contributing to the cognitive decline in our model. Mice subjected to myocardial I/R also display increased cellular localization within the CA1, CA3, and dentate gyrus subfields with a reactive inflammatory morphology that may contribute to the impaired proliferation of newborn neurons.

The cognitive decline and inflammation demonstrated in our model are consistent with clinical studies that observe worsening scores on mini-mental state exams in patients with myocardial infarction and heart failure^{1, 15–17} and T2-weighted lesions within the hippocampus⁴. We propose our model represents a continuum between acute adverse cardiac events in which reactive gliosis is observed within 72 h, and long-term cognitive decline in the context of progressive cardiac dysfunction and sustained cellular reactivity 2 months post-injury. While it is unclear how comorbidities may contribute to cognitive decline in patient populations, our data demonstrate that cardiac I/R alone is sufficient to induce cognitive impairment. Including reperfusion in this mouse model of acute myocardial infarction has particular clinical relevance, as the majority of acute infarctions in humans are reperfused with urgent coronary intervention. In a report using permanent ligation of the coronary artery without reperfusion, no changes in reactive microgliosis or astrogliosis were observed 3 months post-surgical event¹⁸. This suggests that the rapid and sustained reactive gliosis in our cardiac I/R model may result from reperfusion. Furthermore, the aforementioned study used the Morris water maze to assess cognition and observed a reduction in the time to find the platform during training. However, 24 hours after the acquisition phase when mice were subjected to the test phase, in which the platform was removed, no changes in platform crossings were observed¹⁸. These observations suggest either that permanent coronary ligation may not produce a robust change in learning and memory or that the Morris water maze is not a sensitive measure of cognitive outcomes in this model¹⁸. Another study using cryoinjury (nitrogen cooled probe) to the left ventricular free wall in rats reported microglial activation in the hippocampus measured by western immunoblotting of Iba-1; however, no cognitive assessments were performed¹⁹. Although reactive microgliosis was documented in that study, it is unclear if this resulted from vascular changes or freezing injury to the ventricle. While our model shows cognitive impairment in both the hippocampal-dependent object location memory and fear conditioning tasks after open-chest cardiac I/R, it is unclear if this impairment is due to failure of memory consolidation or failure to learn the tasks. Future studies can differentiate between these processes by including short-term memory tasks or non-hippocampal memory

tasks, wherein differences between experimental groups would indicate changes in ability to learn or retain short-term memory.

We are reporting for the first time, that ischemic myocardial injury inhibits neurogenesis. The dentate gyrus, a sub-section of the hippocampus, maintains proliferation of newborn neurons throughout life ²⁰. Neurogenesis in the dentate gyrus plays a critical role in spatial and object recognition memory in rodents ²¹. Furthermore, ablation of neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus ⁸. Importantly systemic injection (intraperitoneal) of lipopolysaccharide (a bacterial endotoxin released during inflammation) inhibits proliferation of neurogenesis without inducing cell death ²². We see similar results in our inflamed hippocampus in which no cell death was seen with caspase-3 staining at 72 h or 2 months post-surgery, but rather reductions in Ki-67 and DCX staining in the granular zone of the dentate gyrus suggesting impaired proliferation. However, further exploration on the mechanism of how cardiac I/R influences neurogenesis is necessary. It is possible that newborn neurons undergoing apoptosis are rapidly cleared by microglia via phagocytosis in the adult subgranular zone ²³. Furthermore, rats subjected to permanent ligation for 6 hours had reduced long-term potentiation of the perforant pathway leading to the dentate gyrus 6 hours post-surgery ²⁴. Although no cognitive measures were included in that study, the data demonstrate a decrease in synaptic activity in the dentate gyrus that is consistent with our observations of deficits in neurogenesis and cognition.

These observations warrant further study to determine if inhibition of inflammation ameliorates impaired neurogenesis after cardiac I/R and rescues subsequent cognitive decline. Our model recapitulates many of the observed phenotypes of patients with cardiovascular disease, and provides testable mechanisms for potential therapeutics in the clinical setting. Furthermore, our model provides evidence that reperfusion after the acute ischemic event may play an important role in hippocampal inflammation and cognitive decline that may not be present in permanent coronary ligation models. Taken together, these studies provide a strong rationale for clinical interventions at the time of cardiac reperfusion in the catheterization lab as a therapeutic window to prevent long-term cognitive impairment.

Cardiovascular disease affects approximately 70 million Americans. While survival rates have drastically improved over the past 2 decades, impaired cognitive function is becoming increasingly costly for healthcare systems and families. It is essential that more comprehensive therapies that target the central nervous system as well as the peripheral effects of cardiovascular disease be integrated into current treatment strategies. Our model provides a promising start to furthering our understanding of cognitive decline in CVD.

Acknowledgments

We would like to thank Terry Lewis, Ph.D. and the UAB Neuroscience Molecular Detection Core funded by NS047466 for their assistance in optimizing IHC protocols as well as the UAB Cellular and Molecular Neuropathology Core for assistance with StereoInvestigator software. We would also like to thank the UAB Behavioral Assessment Core (P30 NS47466) for use of behavioral testing facilities and advice on preliminary

behavioral assessments. This work was funded by NINDS P30-NS069324, The Civitan International Research Foundation, and The University of Alabama Health Services Foundation - General Endowment Fund.

References

1. Vogels RL, Scheltens P, Schroeder-Tanka JM, Weinstein HC. Cognitive impairment in heart failure: a systematic review of the literature. *European journal of heart failure*. 2007; 9:440–9. [PubMed: 17174152]
2. Nunes B, Pais J, Garcia R, Magalhaes Z, Granja C, Silva MC. Cardiac arrest: long-term cognitive and imaging analysis. *Resuscitation*. 2003; 57:287–97. [PubMed: 12804806]
3. Jefferson AL, Himali JJ, Au R, Seshadri S, Decarli C, O'Donnell CJ, Wolf PA, Manning WJ, Beiser AS, Benjamin EJ. Relation of left ventricular ejection fraction to cognitive aging (from the Framingham Heart Study). *The American journal of cardiology*. 2011; 108:1346–51. [PubMed: 21880293]
4. Woo MA, Kumar R, Macey PM, Fonarow GC, Harper RM. Brain injury in autonomic, emotional, and cognitive regulatory areas in patients with heart failure. *Journal of cardiac failure*. 2009; 15:214–23. [PubMed: 19327623]
5. Fujioka M, Nishio K, Miyamoto S, Hiramatsu KI, Sakaki T, Okuchi K, Taoka T, Fujioka S. Hippocampal damage in the human brain after cardiac arrest. *Cerebrovasc Dis*. 2000; 10:2–7.
6. Rauramaa T, Pikkarainen M, Englund E, Ince PG, Jellinger K, Paetau A, Parkkinen L, Alafuzoff I. Cardiovascular diseases and hippocampal infarcts. *Hippocampus*. 2011; 21:281–7. [PubMed: 20054813]
7. Woo MA, Macey PM, Fonarow GC, Hamilton MA, Harper RM. Regional brain gray matter loss in heart failure. *J Appl Physiol*. 2003; 95:677–84. [PubMed: 12716866]
8. Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia AD, Sofroniew MV, Kandel ER, Santarelli L, Hen R, Drew MR. Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc Natl Acad Sci U S A*. 2006; 103:17501–6. [PubMed: 17088541]
9. Durgan DJ, Pulinilkunnil T, Villegas-Montoya C, Garvey ME, Frangogiannis NG, Michael LH, Chow CW, Dyck JR, Young ME. Short communication: ischemia/reperfusion tolerance is time-of-day-dependent: mediation by the cardiomyocyte circadian clock. *Circulation research*. 2010; 106:546–50. [PubMed: 20007913]
10. Ismahil MA, Hamid T, Bansal SS, Patel B, Kingery JR, Prabhu SD. Remodeling of the mononuclear phagocyte network underlies chronic inflammation and disease progression in heart failure: critical importance of the cardiosplenic axis. *Circulation research*. 2014; 114:266–82. [PubMed: 24186967]
11. Slomianka L, West MJ. Estimators of the precision of stereological estimates: an example based on the CA1 pyramidal cell layer of rats. *Neuroscience*. 2005; 136:757–67. [PubMed: 16344149]
12. Nossuli TO, Lakshminarayanan V, Baumgarten G, Taffet GE, Ballantyne CM, Michael LH, Entman ML. A chronic mouse model of myocardial ischemia-reperfusion: essential in cytokine studies. *American journal of physiology Heart and circulatory physiology*. 2000; 278:H1049–55. [PubMed: 10749697]
13. Ekdahl CT, Claassen JH, Bonde S, Kokaia Z, Lindvall O. Inflammation is detrimental for neurogenesis in adult brain. *Proc Natl Acad Sci U S A*. 2003; 100:13632–7. [PubMed: 14581618]
14. Ahern TH, Krug S, Carr AV, Murray EK, Fitzpatrick E, Bengston L, McCutcheon J, De Vries GJ, Forger NG. Cell death atlas of the postnatal mouse ventral forebrain and hypothalamus: effects of age and sex. *J Comp Neurol*. 2013; 521:2551–69. [PubMed: 23296992]
15. Vogels RL, Oosterman JM, van Harten B, Scheltens P, van der Flier WM, Schroeder-Tanka JM, Weinstein HC. Profile of cognitive impairment in chronic heart failure. *Journal of the American Geriatrics Society*. 2007; 55:1764–70. [PubMed: 17727641]
16. Vogels RL, Oosterman JM, van Harten B, Gouw AA, Schroeder-Tanka JM, Scheltens P, van der Flier WM, Weinstein HC. Neuroimaging and correlates of cognitive function among patients with heart failure. *Dementia and geriatric cognitive disorders*. 2007; 24:418–23. [PubMed: 17938570]

17. Levine DA, Davydow DS, Hough CL, Langa KM, Rogers MA, Iwashyna TJ. Functional disability and cognitive impairment after hospitalization for myocardial infarction and stroke. *Circulation Cardiovascular quality and outcomes*. 2014; 7:863–71. [PubMed: 25387772]
18. Hong X, Bu L, Wang Y, Xu J, Wu J, Huang Y, Liu J, Suo H, Yang L, Shi Y, Lou Y, Sun Z, Zhu G, Behnisch T, Yu M, Jia J, Hai W, Meng H, Liang S, Huang F, Zou Y, Ge J. Increases in the risk of cognitive impairment and alterations of cerebral beta-amyloid metabolism in mouse model of heart failure. *PLoS One*. 2013; 8:e63829. [PubMed: 23737953]
19. Nural-Guvener HF, Mutlu N, Gaballa MA. BACE1 levels are elevated in congestive heart failure. *Neurosci Lett*. 2013; 532:7–11. [PubMed: 23127855]
20. Deng W, Aimone JB, Gage FH. New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? *Nat Rev Neurosci*. 2010; 11:339–50. [PubMed: 20354534]
21. Jessberger S, Clark RE, Broadbent NJ, Clemenson GD Jr, Consiglio A, Lie DC, Squire LR, Gage FH. Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. *Learning & memory*. 2009; 16:147–54. [PubMed: 19181621]
22. Fujioka H, Akema T. Lipopolysaccharide acutely inhibits proliferation of neural precursor cells in the dentate gyrus in adult rats. *Brain Res*. 2010; 1352:35–42. [PubMed: 20647006]
23. Sierra A, Encinas JM, Deudero JJ, Chancey JH, Enikolopov G, Overstreet-Wadiche LS, Tsirka SE, Maletic-Savatic M. Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell Stem Cell*. 2010; 7:483–95. [PubMed: 20887954]
24. Liu C, Liu Y, Yang Z. Myocardial infarction induces cognitive impairment by increasing the production of hydrogen peroxide in adult rat hippocampus. *Neurosci Lett*. 2014; 560:112–6. [PubMed: 24361546]

Figure 1. Acute myocardial infarction is sufficient to reduce left ventricular ejection fraction and fractional shortening 2 months post-surgery

Echocardiographic assessment of the left ventricle in sham and I/R groups for fractional shortening and ejection fraction 72 hours (A, B) and 2 months (C, D) post-surgery. At 72 hours, $n = 13$ sham and 18 I/R mice. At 2 months, $n = 8$ mice per group. Data are expressed as mean $\pm$ SEM. * $p < 0.05$, § = 0.054 using two-tailed t-test.

Figure 2. Acute myocardial infarction reduces cognitive function 2 months post-surgery
Using an open-field test, velocity (A), distance travelled (B), time in center (C), and distance in center (D) in sham (n=12) and cardiac I/R (n=11) groups were evaluated. A zero maze was utilized to assess anxiety-like behavior by measuring the number of entrances into open arms (E) and time in open arms (F), n = 11–12 mice per group. Cognitive function was evaluated by measuring time spent with an object in a novel location 24 hours after training in an object location memory task, n = 8 mice per group (G). Minutes on the x axis reflect total time from 0 to the number stated. Cognitive function was also assessed with contextual fear conditioning and freezing behavior was recorded, n = 11–12 mice per group (H). Data are expressed as mean ± SEM. * p < 0.05, § p = 0.061 using two-tailed t-test.

Figure 3. Acute myocardial infarction induces reactive astrocytosis and microgliosis in the hippocampus 72-hours post-surgery

DAB staining of GFAP for astrocytes (A, C) and Iba-1 for microglia (E, G) in the CA1, CA3, and dentate gyrus (DG) subfields in open- (A, E) and closed-chest (C, G) cardiac I/R animals compared to sham controls, $n = 4-5$ mice per group. Stereological estimates of the GFAP+ cells (B, D) and Iba-1+ cells (F, H) in the CA1, CA3, and dentate gyrus (DG) subfields of the hippocampus in open-chest and closed-chest groups. For GFAP staining in open-chest mice, the average coefficients of error (CEs) were 0.1 for CA1, 0.17 for CA3, and 0.13 for dentate gyrus. For GFAP staining in closed-chest mice, the average CEs were

0.11 for CA1, 0.15 for CA3, and 0.10 for dentate gyrus. For Iba-1 staining in open-chest mice, the average CEs were 0.10 for CA1, 0.15 for CA3, and 0.09 for dentate gyrus. For Iba-1 staining in closed-chest mice, the average CEs were 0.12 for CA1, 0.15 for CA3, and 0.10 for dentate gyrus. Data are expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $^{\text{£}}$ $p = 0.057$ using two-tailed t-test. Scale bar = 75 μm .

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. Reactive astrocytosis and microgliosis is sustained two months post- cardiac I/R
 Stereological estimates of GFAP+ (A) and Iba-1+ cells (B) in the CA1, CA3, and dentate gyrus (DG) subfields of the hippocampus in sham and cardiac I/R two months post-surgery, $n = 4$ mice per group. For GFAP staining, the average CEs were 0.14 for CA1, 0.14 for CA3, and 0.10 for dentate gyrus. For Iba1 staining, the average CEs were 0.12 for CA1, 0.15 for CA3, and 0.09 for dentate gyrus. DAB staining intensity of Iba-1 in the dentate gyrus subfield in sham and cardiac I/R (C). Data are expressed as means $\pm$ SEM. [§] $p = 0.059$, ^{**} $p < 0.01$ using two-tailed t-test with one outlier excluded using Grubbs' outlier test ($p < 0.05$). Scale bar = 50 μ m.

Figure 5. Acute myocardial infarction decreases the number of DCX-positive cells in the sub-granular zone of the dentate gyrus 72 hours and 2 months post-cardiac I/R

Representative images of DCX-positive cells in the dentate gyrus (DG) sub-granular zone of sham (left panel) and cardiac I/R (right panel) groups at 72 hours post-surgery (A).

Stereological estimates of DCX and Ki-67 cells in cardiac I/R mice compared to sham at 72 hours and 2 months post-surgery (B and C, respectively). For 72 hours, $n = 4-5$ mice per group. The average CEs for doublecortin and Ki-67 at 72 hours were 0.12. For 2 months, $n = 3-4$ mice per group. The average CEs for doublecortin and Ki-67 at 2 months were 0.10 and 0.11, respectively. Data are expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, § $p = 0.052$ using two-tailed t-test. Scale bar 100 μ m.

Figure 6. Acute myocardial infarction does not result in expression of caspase-3 in hippocampal neurons

Representative images of caspase-3-stained hippocampal regions of sham and cardiac I/R mice. The postnatal day P7 hippocampus serves as a positive staining control and postnatal day P30 hippocampus serves as a negative staining control (A). Representative images of caspase-3 staining in open- and closed-sham and cardiac I/R mice 72 h post-surgery (B). Representative images of open-cardiac I/R mice compared to sham 2 months post-surgery (C). Boxes indicated in the dentate gyrus (DG) are the magnified area represented in the inset. Scale bars 100 μm. Inset scale bars 25 μm. Of note, since no staining was observed in postnatal day 30 hippocampus or in sham or cardiac I/R hippocampus, contrast was enhanced to identify the architecture of hippocampal structures, thus resulting in increased background staining.