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Neuromodulation Therapy Mitigates Heart Failure Induced Hippocampal Damage

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Cardiovascular disease (CVD) is the leading cause of death in the United States. Nearly half of the people diagnosed with heart failure (HF) die within 5 years of diagnosis. Brain abnormalities secondary to CVD have been observed in many discrete regions, including the hippocampus. Nearly 25% of patients with CVD also have major depressive disorder (MDD), and hippocampal dysfunction is a characteristic of both diseases. In this study, the hippocampus and an area of the hippocampal formation, the dentate gyrus (DG), were studied in a canine model of HF. Using this canine HF model previously, we have determined that myocardial infarction with mitral valve regurgitation (MI/MR) + spinal cord stimulation (SCS) can preserve cardiac function. The goal of this study was to determine if the SCS can also protect the brain in a similar fashion. Both the entire hippocampus and the DG tissues were dissected from canine brains and analyzed. These findings provide strong evidence that, in addition to the cardioprotective effects observed previously, SCS following MI/MR induces neuroprotective effects in the brain.
1. INTRODUCTION

In the United States, cardiovascular disease (CVD) is the most prominent disease and the leading cause of death (21). Within this population, there are a greater percentage of people with psychiatric diseases as compared to the population as a whole, and major depressive disorder (MDD) is one of the most prominent (12). Between 15-30% of patients who have had a myocardial infarction (MI) are diagnosed with MDD, and a greater percentage experiences depressive symptom at some point during their lifetime (1). In the event of an MI, the body experiences oxidative stress, which can lead to MDD and a variety of other diseases (17). The development of MDD post-MI increases mortality in CVD patients (12). Imaging studies in depressed patients have demonstrated a reduction in limbic system volume, which is hypothesized to be related to apoptosis and reduction in neurogenesis (4). In studies evaluating brain pathology in CVD, the limbic system is a major point of interest and in particular, the hippocampus (1). Inflammatory diseases are associated with higher incidences of MDD, and the administration of cytokines is an independent risk factor for depressive pathology (3). After an MI, pro-inflammatory cytokines are released from the myocardium and levels of tumor necrosis factor-α, interleukin-1β, and interleukin-6 (IL6) are elevated in blood (12). Cardiac disease progression is hypothesized to have a role in depressive symptomatology present in CVD patients, and proinflammatory cytokines have been demonstrated to induce atrophy in the hippocampus in an MI animal model (1). Evidence suggesting a pathological link between depression and CVD has been shown extensively in animal models for CVD (1, 2). In an MI rat model, increased stress on the forced swim test and alteration of sucrose preference, which is a behavioral test
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of anhedonia, have been observed are similar to those observed in models for depression, and the degree of these deficits were significantly correlated to the degree of hippocampal apoptosis (2). Interestingly, the administration of the selective serotonin reuptake inhibitor (SSRI) antidepressant sertraline prevented apoptosis in the limbic system following MI (2). In addition to hippocampal damage, an increase in caspase-3 activity (which plays a central role in the execution phase of the cell apoptosis cascade) has been observed in other limbic brain regions such as the temporal cortex, amygdala, and dentate gyrus (DG), a subregion of the hippocampus (2, 4).

The subgranular zone (SGZ) of the DG within the hippocampus is one of the two regions in the adult mammalian brain where neurogenesis can occur (9). In MDD, there is significant evidence indicating a reduction in neurogenesis, and the increase in neurogenesis after the administration antidepressant SSRIs is thought to be a mediating factor in disease treatment (4). Brain derived neurotropic factor (BDNF) is a neurotrophic factor involved in cell survival and is reduced in patients MDD, and is elevated by antidepressant treatments in the brain (4). An important modulator in neurogenesis is the excitatory amino acid glutamate and the NMDA receptor (10). Both the NR1 and NR2B subunits of the NMDA receptor are expressed in the dentate granule cell layer from birth to early postnatal life, underlining the role of excitatory amino acids and NMDA receptors in the differentiation of granule neurons (Monyer 1994). While the role of the NMDA receptor in neurogenesis is not fully understood, the administration of multiple NMDA antagonists has been shown to increase levels of neurogenesis in the DG (10). Suppressing the expression of NR1, a necessary subunit
for the NMDA receptor, has been shown to increase cell proliferation in transgenic mice (11).

Endothelial dysfunction resulting from peripheral inflammation is hypothesized to be a mediator in the development of post-MI depression (12). Blood brain barrier (BBB) breakdown has also been observed independently of CVD in patients with MDD and has been linked to inflammation (20). Damage to the BBB can cause leakage and lasting cellular damage (14). In CVD, inflammatory factors that are released have been implicated in BBB breakdown (19).

A characteristic of CVD and the progression of HF is the abnormal activation of the sympathetic nervous system (SNS), which is often a result of pro-inflammatory cytokine signaling (5). In the periphery, an increase in SNS activity is associated with a variety of pathological changes, included increased heart rate, sodium retention, and vasoconstriction (5). Another component to HF is the activation of the renin-angiotensin-aldosterone system (RAAS), which contributes to cytokine release in the myocardium and endothelial dysfunction (5). Traditional HF drug therapies aim to control the autonomic nervous system (ANS) and particularly sympathetic outflow through beta-receptor blockade (15). Autonomic regulation therapies currently being studied for heart failure, such as spinal cord stimulation (SCS), attempt to control the ANS by modulating the existing neurochemical system through neural stimulation (16). High-frequency dorsal SCS has been shown to reduce infarct size in myocardial ischemia and protect against arrhythmias in a heart failure animal model (7). Thoracic SCS has been shown to reduce peripheral sympathetic drive and reduce afferent sensory cardiac neuron firing, and the sympatholytic effects of thoracic SCS are
hypothesized to be the reason for its efficacy (8). Interestingly, patients with MDD show an alteration in autonomic balance where sympathetic inputs predominate parasympathetic inputs, and this is associated with heart-rate variability and baroreflex sensitivity (13).

The aim of this study was to evaluate brain pathologies in areas implicated in MDD pathophysiology in an animal model for HF. Additionally, a goal of this study was to determine if high thoracic SCS could protect the central nervous system (CNS) similarly to the manner in which it protects the peripheral cardiac nervous system and myocardium in HF. In the brain, gene expression analysis was used to investigate pathological differences in regions associated with MDD (hippocampus, DG). Pathways were investigated with significant pathology in both MDD and HF, including apoptosis, neurogenesis, endothelial dysfunction, and glutamate toxicity. Histology was completed to qualitatively view differences in the DG and tissue surrounding the BBB between treatment groups. Additionally, area fraction analysis was performed to quantitatively determine difference in density of the granule cell layer of the DG in each group of canines. Investigating the complex interactions between the heart, peripheral cardiac nervous system, and higher control centers as well as the manner in which modulating the autonomic nervous system affects the CNS will allow researchers and clinicians to better design therapies and treat diseases of the brain and the heart simultaneously.

2. METHODS

2.1 Heart failure model and spinal cord stimulation

A total of 25 canines were utilized for this study. Canines were randomly assigned to the groups sham control (CO), myocardial infarction with mitral regurgitation
(MI/MR), and myocardial infarction with mitral regurgitation and spinal cord stimulation (MI/MR + SCS). For the heart failure groups, myocardial infarction was produced by a 20 minute critical stenosis (~50%) of the left anterior descending (LAD) coronary artery followed by a 90 minute full occlusion and subsequent reperfusion. For the MI/MR + SCS group, a spinal cord stimulation device was implanted during the MI surgery. Two leads were implanted in the T1-T3 and T4-T5 level of the spinal cord and the pulse generator was implanted and set to a pulse schedule (50Hz, 200µs). Two weeks following the MI, MR was produced by cutting select chordae on the mitral valve (~20%) until appropriate left ventricular end-diastolic pressure was met and an audible murmur was induced. One week following the MR surgery, SCS was activated in the MI/MR + SCS group. Approximately four months post-MI, the canines were terminated. Control canines were maintained for the same period of time. Canine brain tissues were surgically removed, dissected in a cryostat at -20C, and stored at -80C.

2.2 Brain tissue dissection

Canine brain tissue blocks from the left temporal cortex were sectioned (50µm) using a cryostat microtome. The ventral hippocampus was collected by punch dissection (3.5 mm) for gene expression analysis. For laser capture microdissection (LCM), tissue from the adjacent hippocampal layer was sectioned (10µm) and placed on Arcturus PEN Membrane Glass Slides. After the slides were made, they were desiccated for 5 minutes and stored at -80°C for further use. For histochemical processing, the tissue was sectioned coronally (10µm), desiccated overnight, and stored at -80°C for further use.
2.3 Laser capture microdissection

The dentate gyrus (DG) of the hippocampus was dissected by laser capture microdissection (LCM) using the Veritas Microdissection Instrument Model 704 with CapSure Macro Caps. Settings optimized for the dissection were 50mW pulse power, 1,700 μsec pulse duration, 11-12 laser power, and 2 μm spot diameter. Relative humidity was maintained at <25%. The DG was located on the tissue slides by comparison to rapid (10 min) cresyl violet stained slides at 10x magnification. The staining to capturing timeframe was limited to 2 hours to ensure high quality RNA. After sample collection, the LCM DG samples were suspended in lysis buffer.

2.4 Area fraction analysis

Tissue samples (20 μm) from the hippocampus were stained with Cresyl Violet. Two-dimensional areal fraction (AF) was carried out using Image J Software to calculate cell density in the DG. Three nonadjacent slides were selected from equal anatomical levels. Frame-by-frame images of the entire DG were taken with a light microscope at 40X magnification. Sizes of the images were set to 342 x 257 μm and arranged to the middle of the microscope field and 12-14 images were taken. The photographs of the DG contained portions of the molecular layer and the polymorphic cell layer. Using Image J Software, the DG images were cropped to the same size to only contain the granule cell layer. Prior to analysis, the subjects were arranged into groups with the same standard threshold. The AF was calculated by dividing the Nissel-positive area (μm²) from the binary image by the total area of the image frame.
2.5 Total RNA purification and cDNA synthesis

Total RNA was purified from the hippocampus punch tissue and laser captured DG followed manufacture protocol. The RNAques Microkit (Ambion) was used to purify total RNA, which included a DNase treatment to remove contaminated genomic DNA. Following RNA purification, samples were stored at -80°C. To synthesize cDNA, the Superscript III First-Strand Synthesis Supermix reverse transcriptase kit was used (Invitrogen). Following cDNA synthesis, an RNase treatment was performed to remove any contamination of RNA.

2.6 Primer design and optimization for gene expression assays

Primers were designed for genes of interest using Integrated DNA Technologies PrimerQuest SM Web Server Software (Zuker). The UNAFold Web Server software was also used to predict nucleic acid folding. Primers were designed to contain a GC content of 45-55% and span introns, limiting the amplification of genomic DNA. RT-PCR was utilized to optimize the conditions for each primer set using 5-Prime MasterMIX (Eppendorf).

2.7 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was used to measure the gene expression of target genes. cDNA samples were screened for genomic DNA contamination by using RT minus controls. A Stratagene MX3000P QPCR System or Biorad MiQ qPCR was used for running qPCR reactions. One microliter of cNDA from each subject was used as a template for the reaction, and each reaction was carried out in triplicate. Gene specific primers used for amplification were at a final concentration of 400 nM. Platinum Quantitative PCR SuperMix-UDG (Invitrogen) was used, and the SYBR signal was
normalized to the ROX signal. The cycle threshold (Ct) value was used for quantification of gene product, and is determined by the point which fluorescence exceeds background fluorescence. cDNAs from all subjects (CO, MI/MR, MI/MR + SCS) were ran on the same plate for each gene of interest. Each target gene was normalized to the geometric mean of two or three control genes.

2.8 Statistical Analysis

To analyze gene expression and area fraction results, a one-way analysis of variance (ANOVA) followed by a Tukey’s Multiple Comparison Test was conducted.

3. RESULTS

3.1 Apoptosis (CASP3)

To determine the degree of apoptosis in the hippocampus in a heart failure animal model, caspase-3 (CASP3) gene expression was measured. Caspase-3 is a protease involved in apoptosis and serves as the final mediator in the apoptotic pathway, making it a reasonable biomarker for this pathway. Through mRNA expression analysis, it was determined that there was a significant increase in hippocampal CASP3 expression in the heart failure group as compared to sham operated controls (+97%; p<0.0001). Additionally, in the heart failure group that received spinal cord stimulation (SCS) therapy, CASP3 mRNA levels in the hippocampus were maintained at sham control levels (-98%; p<0.0001). CASP3 mRNA upregulation indicates an increase in apoptosis in the MI/MR group that is maintained at control levels in the MI/MR + SCS group. Figure 1.
3.2 Blood brain barrier (eNOS, GFAP)

Blood brain barrier disruption has been observed in cardiac disease, and this disruption is observed on the level of endothelial cells. To investigate endothelial dysfunction, endothelial nitric oxide synthase (eNOS) gene expression was measured. eNOS dysfunction is characteristic of cardiac disease and can result in deleterious vascular and neuronal defects. Through mRNA expression analysis, it was determined that there was a significant increase in hippocampal eNOS expression in the heart failure group as compared to sham operated controls (+98%; p=0.0185). Additionally, in the heart failure group that received spinal cord stimulation (SCS) therapy, eNOS mRNA levels in were not elevated to the same degree (+61%; ns). Figure 2.
In addition to endothelial cells, astrocytes, a type of glial cell, are essential to the maintenance and structure of the blood brain barrier. Glial dysfunction is observed in a variety of psychiatric diseases. Glial fibrillary acidic protein (GFAP) is a protein expressed predominantly in astrocytes, and GFAP expression is an accepted biomarker for reactive astrocytes. Through mRNA expression analysis, the data suggested that there was a decrease in hippocampal GFAP expression in the heart failure group as compared to sham operated controls (-22%; ns). This effect did not appear to be mitigated by SCS therapy. These results indicate that the trend in heart failure induced GFAP reduction within the hippocampus is not mitigated by SCS. Figure 3.
3.3 Neurogenesis (NMDA, BDNF)

The dentate gyrus (DG) of the hippocampus is one of the regions where neurogenesis can occur in the adult mammalian brain. A number of biochemical pathways influence the dynamic process of neurogenesis, including neurotrophic factors, glial cells, and glutamatergic neurons. Brain derived neutrophic factor (BDNF) is a neurotrophic factor related to hippocampal atrophy, stress, and neurogenesis regulation. Through mRNA expression analysis, the data suggested that there was a decrease in hippocampal BDNF expression in the heart failure group as compared to sham operated controls (DATA). This effect did not appear to be mitigated by SCS therapy. Figure 4.
N-methyl-D-aspartate receptor (NMDAR) is a glutamate receptor subtype and is in high concentrations in the hippocampus and in particular, the DG. A necessary subunit of the NMDAR is NR1, which binds the co-agonist glycine. To investigate the NMDAR function in a heart failure model, NR1 expression was measured from the DG. Through mRNA expression analysis, it was determined that there was a disruption in DG NR1 expression in the heart failure group as compared to sham operated controls (+106%; ns). Additionally, in the heart failure group that received spinal cord stimulation (SCS) therapy, NR1 mRNA expression levels in the hippocampus were maintained at sham control levels. Figure 5.
3.4 Area fraction of the dentate gyrus

Area fractal analysis was used to determine the cell density in the granule cell layer of the DG. It was determined that there was a significant decrease in granule cell layer density in the heart failure group as compared to sham operated controls (-35%; $p=0.0054$). The granule layer cell density was maintained at control levels post-SCS. Figure 6.
3.5 Hippocampus morphology

Figure 7.

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3.6 Blood brain barrier morphology

Figure 8

Control

MI/MR

MI/MR + SCS
4. DISCUSSION

This study shows that brain pathologies observed in HF in brain regions associated with MDD are mitigated by high thoracic SCS. This is the first study to evaluate brain pathologies after SCS therapy in a heart failure model. These results indicate that SCS has both cardio protective effects and neuro protective effects. Volume reduction in limbic regions has been observed in patients with MDD and apoptosis observed in the hippocampus in this study provides evidence for overlapping pathology in HF (4). A possible explanation for this observation could that the hippocampus is susceptible to oxidative stress damage and has a high concentration of glutamate receptors, which can cause neural damage in the event of ischemic induced glutamate release (23). Qualitative histological data on the hippocampus supports the observed gene expression changes. The maintenance of caspase-3 mRNA expression levels indicates that SCS prevents HF induced hippocampal apoptosis. In the MI/MR group, there was a significant increase in endothelial nitric oxide synthase (eNOS) mRNA expression level in the hippocampus as compared to controls, indicating blood brain barrier disruption. eNOS-derived NO is an antihyperintensive, and functions to relax smooth muscle and inhibit platelet aggregation (14). Whereas the upregulation of eNOS mRNA may appear to be positive due to the anti-atherosclerotic properties of NO, downstream of mRNA expression, there is an inhibition of eNOS activity (14). SCS did not fully reverse the eNOS dysfunction observed in the heart failure animals, but a trend of reduction was observed across the samples. Hippocampus histology indicates that there is damage to the tissue around the blood brain barrier (BBB), and we hypothesize that this is due to endothelial dysfunction. GFAP is expressed in reactive astrocytes,
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making it an accepted marker for reactive astrocytes. While there were no significant changes in GFAP mRNA expression across the groups that were studied, a negative trend was observed between control and MI/MR groups, and this trend was not altered by SCS. It is likely that by increasing the number of subjects in this study that the observed trend would reach statistical significance. Similarly to GFAP, there were no significant changes in BDNF mRNA expression between groups, though there was a negative trend between control and MI/MR groups that was not mitigated by SCS. In the DG laser captured sample, NR1 gene expression was measured. NR1 is a subunit of NMDAR, a subtype of glutamate receptor. A number of studies imply that NR1 expression is a vital component of the complex process of neurogenesis. The expression levels of NR1 were altered drastically in the MI/MR group as compared to controls, and this disruption was prevented by SCS. Results from AF analysis support the hypothesis that the DG and the process of neurogenesis are disrupted in HF and mitigated by SCS. These data suggest that in addition to cardioprotective effects, there are neuroprotective effects of SCS in brain regions associated with MDD. Considering that many patients develop MDD that have CVD and mortality is increased as a result of it, therapies that benefit both the brain and the heart will reduce overall mortality and benefit the population.
5. REFERENCES


14. Li, Huige, and Ulrich FÃ¶rstermann. "Uncoupling of endothelial NO synthase in


### 6. SUPPLEMENTAL MATERIALS

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