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By

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Abstract

Myocardial infarction (MI), often referred to as a heart attack, is a serious health issue in the United States. There is a well-documented link between MI and major depressive disorder (MDD), with a high incidence of MDD occurring after an MI. Overlapping pathologies have been observed within the hippocampus of the brain in animal models of MI and depression. These observations suggest that pathobiological cross-talk between the heart and brain could have a role in the etiology of MDD that occurs after an MI. Spinal cord stimulation (SCS) has previously been shown to have both cardioprotective and neuroprotective effects post-MI, and hence may protect individuals from developing depression post-MI. In this study, we examined the potential biochemical mechanisms that might underlie the neuroprotective actions of SCS following MI. Brain tissues were obtained from three groups of canines: sham-operated animals, animals subjected to experimental myocardial infarction/mitral regurgitation (MI/MR), and animals subjected to MI/MR that were simultaneously administered SCS. The whole hippocampus and hippocampal dentate gyrus were dissected from frozen brains. Quantitative endpoint-PCR and RT-qPCR techniques were employed to measure select biochemical mediators of neuroprotection, i.e. adenosine A_{2A} receptor, ghrelin, and ghrelin receptor expression in hippocampal samples. SCS induced a significant decrease in A_{2A} receptor expression and a dramatic increase in ghrelin expression in MI/MR canines as compared to the MI/MR group without SCS. These findings suggest that adenosine receptors and ghrelin may play a biochemical role in SCS-induced neuroprotection of the hippocampus. Understanding the neuroprotective actions of SCS has the potential to aid the development of new treatments or preventative measures for depression following a heart attack.

Background

Myocardial infarction (MI), commonly known as a heart attack, is a serious health issue in major industrialized countries with over two million people in the United States hospitalized with MIs between 2000 and 2008 (47). Of these patients, 65% are estimated to experience depressive symptoms and 20% will develop major depressive disorder (MDD) (49). Conversely, MDD has been linked with an increased risk of an MI, and patients with MDD have a fourfold increase in risk of death after an MI compared to those without depression (8). These clinical observations provide evidence for pathobiological cross-talk between the brain and the heart. Despite significant research in the field, the pathophysiological and biochemical link between MI and MDD remains poorly understood. However, several morphological and biochemical changes have been observed in experimental animal models following experimentally induced MI (17), including apoptosis in the amygdala and hippocampus (13, 48, 49). These two brain regions are part of the limbic system, a part of the brain that deals with emotion, and are known to play a distinct role in the pathobiology of depression (37). Hence, these brain regions are prime suspects for the study of pathobiological processes that may contribute to the linkage between MDD and MI.

During an MI, the damaged myocardium causes an increase in serum levels of purine nucleotides, including adenosine (1). Adenosine, a metabolite of adenosine triphosphate and a natural ligand of the adenosine receptor, is an endogenous cardioprotective agent that reduces the severity of ischemia and reperfusion injury (30). Previous research has linked adenosine imbalance to MDD, but studies have shown conflicting results (12). Adenosine binds to four different receptor subtypes, each with a different function and encoded by a separate gene. The subtype of interest in this thesis is the A_{2A} receptor, because A_{2A} receptors play a role in the heart by regulating blood flow and myocardial oxygen consumption (31) and have broader anti-inflammatory effects throughout the body (43). The A_{2A} receptor is extensively expressed within the hippocampus and in brain endothelial cells that comprise the blood brain barrier (BBB) (43, 38). Adenosine can increase the permeability of the BBB by binding to A_{2A} receptors on brain endothelial cells. Administration of an A_{2A} receptor agonist has been shown to increase BBB permeability enough to allow proteins up to 70 kDa in size to pass through the barrier (7). This increase in penetrability could allow pro-inflammatory cytokines released from the damaged area to enter the brain, as long as they are below 70 kDa in size.

One inflammatory cytokines that could potentially enter the brain following A_{2A} activation is tumor necrosis factor alpha (TNF-α), which has a size of 26 kDa (45). TNF-α is an inflammatory cytokine that plays a major role in activation of immune cells (35). Patients suffering from MDD have high serum levels of several pro-inflammatory cytokines, including TNF-α, pointing to the involvement of an inflammatory process in the pathology of MDD (41). Interestingly, TNF-α participates in apoptosis in the CA1, CA3, and dentate gyrus regions of the hippocampus via caspase-3 and caspase-8 activation following a MI (27, 13).

Adenosine and TNF-α have a complex signaling relationship. TNF-α increases the expression of adenosine A_{2A} receptors in human epithelial cells by acting as a transcription factor (40). However, administration of a potent A_{2A} receptor agonist reduces TNF-α levels and apoptosis in the hippocampus (5), suggesting the existence of a feedback inhibition mechanism whereby TNF-α increases A_{2A} receptors, which in turn reduce the amount of TNF-α.

Spinal cord stimulation (SCS) is a novel treatment for patients who have experienced an MI. SCS belongs to a new and growing field of therapeutics referred to as "electroceuticals". Electroceuticals are a class of medical devices that exert their effects via electrical modulation of the nervous system, or neuromodulation. In the case of SCS, a device is implanted in the patient that delivers electrical signals at a specified frequency to the spinal cord (46, 16). SCS works by modulating the existing neurochemical pathways within the autonomic nervous system (36). Sympatholytic effects, i.e. reducing peripheral sympathetic drive caused by MI and afferent sensory cardiac firing, are believed to be the primary mechanism of action of thoracic SCS (25). This has potential implications for depression research, as patients with MDD have shown an altered autonomic balance with sympathetic inputs predominating over parasympathetic inputs. It is believed that this is associated with both baroreflex sensitivity and heart rate variability (33). Despite this knowledge, the specific chemical mechanism that causes SCS's sympatholytic effects remains unknown.

Ghrelin may be involved in the biochemical mechanism of SCS-induced benefits post-MI. Ghrelin is a 28 amino-acid peptide that acts as an orexigenic hormone (24). Ghrelin is widely expressed throughout the human body (19), being primarily expressed in the gastrointestinal tract. Interestingly, ghrelin plays a distinct role within the central nervous system (CNS). Both ghrelin and the growth-hormone secretagogue receptor (GHSR), the only known ghrelin receptor, are extensively expressed throughout the CNS (20). Ghrelin has been shown to be involved in several areas of CNS physiology, including cognition (4), sleep (32), and mood (10). Abnormalities of ghrelin and GHSR expression have been previously suggested to play a role in several psychiatric disorders, (schizophrenia, anorexia nervosa, anxiety, and MDD) as well as being involved in stress regulation (50). Factors involved in the regulation of both ghrelin and GHSR remain unclear.

Intraperitoneal injection of ghrelin in rat models has been shown to improve cardiac function and remodeling (29) and to reduce TNF-α levels within the CNS (52). Furthermore, ghrelin has notable neuroprotective effects within the rat brain. Intraperitoneal ghrelin administration in rodent models caused upregulation of hippocampal neurogenesis, even independently of growth hormone and insulin-like growth factor (34). Rats injected with physiological doses of ghrelin have also shown significant improvement of performance on memory tests, which has been linked to a properly functioning hippocampus (28). Finally, serum ghrelin levels are increased by vagal nerve activity and reduced by sympathetic nervous system activity (22). Since SCS exerts sympatholytic effects by withdrawal of sympathetic tone or increase of vagal tone (25), it is anticipated that SCS may increase ghrelin levels which could mediate at least some of the beneficial effects of SCS after MI. Interestingly, adenosine has been shown to stimulate ghrelin release in the mouse stomach (51), as well as serve as a partial agonist to the GHSR receptor within the porcine hypothalamus (44), resulting in cross-talk between the adenosine and ghrelin signaling systems (9).

While previous research has shown intraperitoneal injections of ghrelin to have neuroprotective effects, the therapeutic potential for increasing local expression of ghrelin in the hippocampus has not been studied. SCS may cause an increase of local ghrelin release within the hippocampus, particularly since ghrelin and GHSR are widely expressed on the hippocampus and dentate gyrus. If ghrelin expression were upregulated during SCS, it could potentially reduce TNF-α within the hippocampus, reducing post-MI apoptosis.

Taken together, the previous findings implicate a possible relationship between ghrelin, A_{2A} receptors and TNF-α in MI and SCS. This thesis focuses on elucidating the potential therapeutic mechanism of SCS administered after MI by studying gene expression in the hippocampus from canines in a model of MI. Expression levels of adenosine A_{2A} receptors, GHSR receptors, and ghrelin were measured. My findings provide a possible explanation for the neuroprotective effects of SCS after MI, and have the potential to advance the discovery of new treatments or preventative measures for depression following a heart attack.

Methods

Approval of Animal Protocol

The use of animals in this study were approved and monitored by the Institutional Animal Care and Use Committee and the Division of Laboratory Animal Research.

Heart Failure and Spinal Cord Stimulation (SCS) Models

Three experimental groups were used in this study: 8 sham-operated control canines, 8 canines that had undergone a myocardial infarction and mitral regurgitation (MI/MR; a model of heart failure) and 6 canines that had undergone an MI/MR and SCS. For the MI/MR groups, myocardial infarction was created by performing 20-minute critical stenosis (~50%) on the left anterior descending coronary artery. After this, a 90-minute full occlusion and subsequent reperfusion of the left anterior coronary artery was performed. For the MI/MR + SCS group, a spinal cord stimulating device and pulse generator were implanted in the T1-T3 and T4-T5 levels of the spinal cord. The pulse generator was set on a schedule of 50 Hz, 200 μs. MR was produced two weeks following the MI by cutting roughly 20% of mitral valve chordae until an audible murmur was heard and appropriate left ventricular end diastolic pressure was met. SCS was activated in the SCS group one week following surgery. Canines were terminated four months following MI. Control canines were also maintained for four months and then terminated (13). Extensive measures of cardiac function were performed on all animals as a part of a separate study.

Tissue Acquisition

After animals were terminated in the course of the cardiac function study, we obtained brain tissue postmortem. Brain tissue was immediately removed to preserve RNA quality, and then dissected into smaller tissues in a cryostat at -20 °C. Finally, the tissues were stored at -80 °C.

Neuropathological Assessment

Frozen blocks of the left temporal and occipital cortex were sectioned into 50 μm sections using a Leica CM3050S cryostat microtome. The samples were then punched with a disposable 3.5 mm puncher. The whole hippocampus, amygdala, and occipital cortex gray matter was collected for gene expression analysis. Punched tissues from the ventral hippocampus were then collected at relatively the same place on every animal and stored in microcentrifuge tubes at -80 °C for the purpose of downstream assays

Total RNA Purification and Generation of cDNA

Total RNA was extracted from the dentate gyrus sample using the RNAqueous Micro Kit (includes DNase treatment; Ambion, USA). RNA samples were then stored at -90 °C according

to protocols previously published (42). SuperScript III cDNA was synthesized from RNA using the First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA) reverse transcriptase kit according to the manufacturer's recommendations.

Primers for RT-PCR and End-Point PCR Gene Expression Assays

Gene-specific primers were designed using the UNAFold Web Server software (53) (http://mfold.rna.albany.edu) for folding prediction and the Integrated DNA Technologies PrimerQuestSM Web Server software (http://www.idtdna.com/Scitools/Applications/Primerquest/).

Primers (Table 1) were designed to have a GC content of 45–55% and to span introns to prevent amplification of genomic DNA. The amplification conditions for each primer and gene set were standardized and optimized.

Quantitative Real-Time PCR (qPCR)

Each cDNA sample was checked against a sample of the RNA it was reverse transcribed from by running an identical mock RT reaction without the reverse transcriptase enzyme added. If no DNA was present at the end of this reaction (RT minus control), then it was concluded that genomic DNA contamination was not present in the sample. One µL of resulting cDNA was then used as a template for real-time PCR performed with a Stratagene MX3000P QPCR System (Agilent Technology, Inc., CA). Gene specific primers were used to carry out amplification at a final concentration of 400 nM for both reference and target genes. All quantitative PCR amplifications were performed, following the manufacturer's instructions, in triplicate wells using Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA). The quantity of the target gene was assessed using the cycle threshold (CT) value. The CT value was determined as the point at which the increased fluorescence exceeded background fluorescence, and the median CT value from each triplicate was used for expression analysis. For comparisons between sham and treated subjects, cDNAs from all canine samples were amplified simultaneously on the same plate.

End-Point PCR

End-point PCR was carried out in an Eppendorf Master Cycler ep (Eppendorf, Hamburg, Germany) using gene specific primers and Taq DNA Polymerase 100 U enzyme (5prime Technologies, Gaithersburg, MD). An Agilent Tape Station 2200 and Agilent Tape Station 2200 screen tapes (Agilent Technologies, Santa Clara, CA) were utilized using manufacturer's instructions for electrophoresis and analysis of gene amplification. The concentration [ng/µl] was used for gene expression analysis.

Data Analysis

Cycle threshold values from qPCR results were mathematically converted to ng/µl and the compared to expression of reference genes SuccDH and GADPH (included in supplemental materials). Endpoint PCR results were also compared to the same reference genes. Using these reference genes, relative expression values were calculated. Graphs were produced and significance was determined using a one-way analysis of variance (ANOVA) test with Tukey post-hoc analysis (PRISM GraphPad version 5), using a p-value <0.05 to determine statistical significance.

Results

A_{2A} Receptor

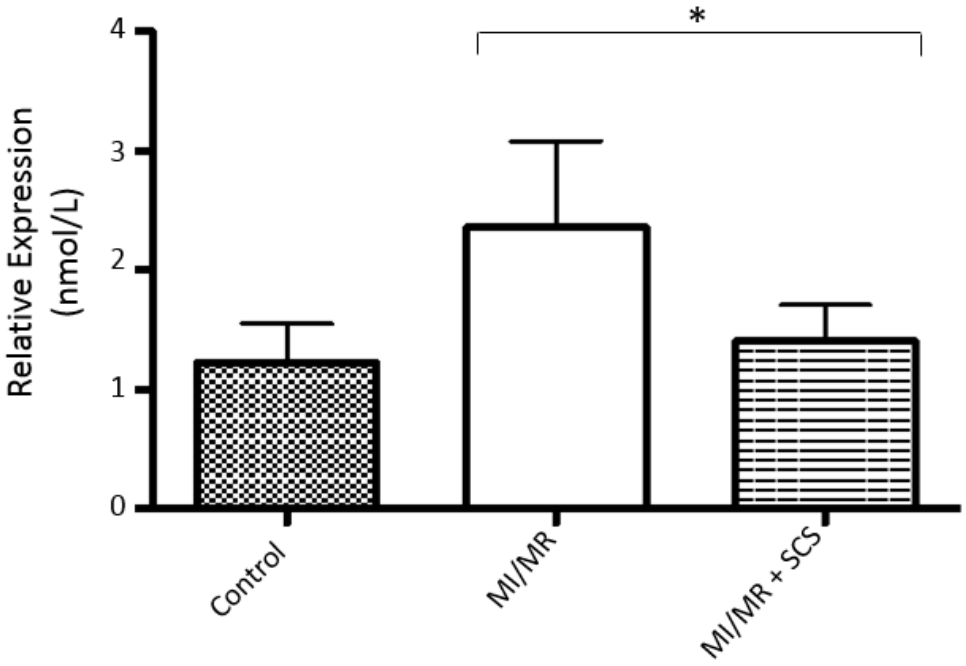

Figure 1: Relative Gene Expression of A_{2A} Receptor

A_{2A} receptor gene expression in the hippocampus is shown in Figure 1 for the three groups of canines. SCS therapy for MI/MR animals produced a significant decrease in A_{2A} receptor gene expression relative to the MI/MR group. MI/MR increased A_{2A} receptor gene expression relative to sham control animals, although the effect did not quite reach statistical significance ($p=0.053$). These data show that SCS has a negative effect on A_{2A} expression. Considering the previously discussed TNF-α-A_{2A} feedback inhibition loop, one might expect that SCS might also lower TNF-α expression. Future studies ought to consider directly quantifying the amount of TNF-α within the hippocampus.

Ghrelin

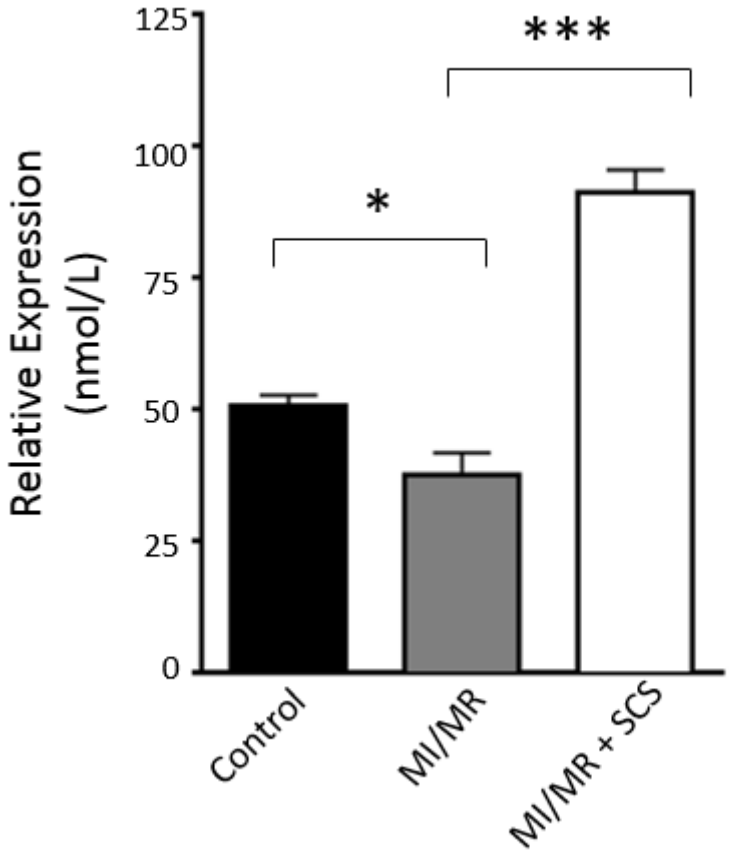

Figure 2: Relative Gene Expression of Ghrelin

Figure 2 shows the effects of MI/MR and SCS plus MI/MR on ghrelin gene expression. SCS significantly increased ghrelin gene expression levels in the hippocampus from MI/MR canines as compared to both control and untreated MI/MR canines. MI/MR alone induced a significant lowering of ghrelin gene expression. These data indicate that ghrelin plays a significant role in the actions of SCS. Additionally, expression levels of the GHSR receptor gene, the receptor which ghrelin binds to, where checked and no significant change was observed in any of the groups of canines (not shown).

GLUT-1 Expression

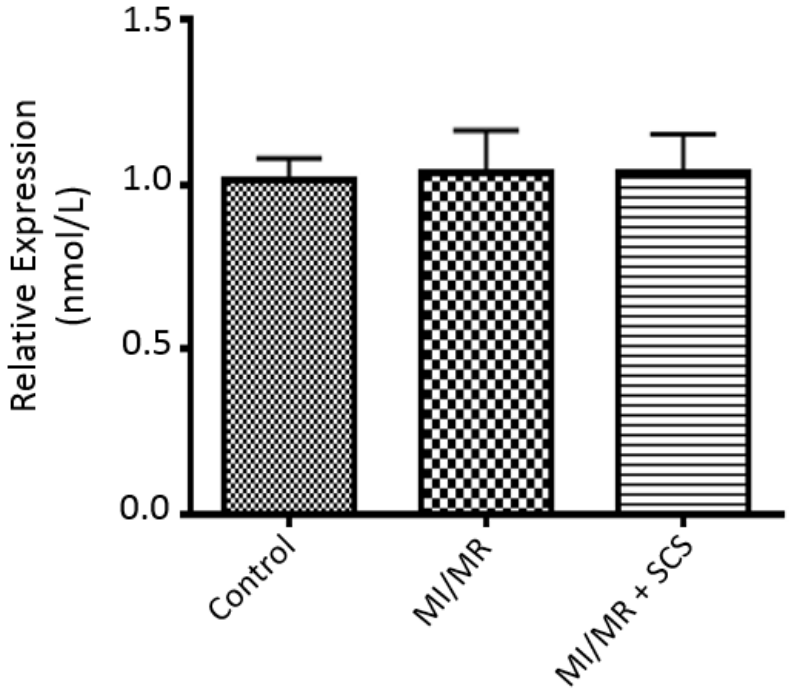

Figure 3: Relative Gene Expression of GLUT-1

To consider the possibility that SCS produces neuroprotection after MI by preventing hypoxia in MI/MR animals, a gene expression marker, GLUT-1, was measured in the three groups of animals (Figure 3). GLUT-1 gene expression levels suggest that canines in the MI/MR and MI/MR with SCS did not experience hypoxia.

Previous PCR analysis done on oxygen sensitive (6) hypoxia inducible factor 1-α (HIF-1α) revealed no significant difference between the three groups. However, HIF-1α is regulated at the protein level (18), so HIF-1α levels do not necessarily reflect a hypoxic response. To remedy this, expression of Glucose transporter protein type 1 (GLUT-1) was checked. GLUT-1 is a high-affinity glucose membrane transport protein regulated at the message level by the HIF-1α pathway (39, 15). Hypoxic conditions encourage greater expression of GLUT-16 and GLUT-1 has been shown to be a reliable endogenous marker of hypoxia (21, 11).

Results showed no significant difference in GLUT-1 expression in any of the experimental groups, indicating no difference in the hippocampal oxygen levels of the three models.

Discussion

It has been previously reported that SCS has cardioprotective effects. In animal models, SCS significantly reduced infarct size, reduced arrhythmias, and improved cardiac remodeling (46, 2, 3). Further research done by our lab has also given support to the hypothesis that SCS has

similar protective effects in the central nervous system (13). Results have indicated that MI/MR is associated with gross morphological deficits within the hippocampus. Our lab has also previously demonstrated that thoracic SCS is able to prevent the development of brain pathologies that could potentially contribute to the development of post-MI MDD (13).

This thesis attempted to determine at least part of underlying mechanism of SCS's neuroprotective effects. The region of interest within the brain was the hippocampus, due to its vital role in the limbic (emotional) system of the brain. The neuroanatomical networks between the hippocampus with other parts of the limbic system and also cardiovascular control centers have been implicated to hold a significant role in the pathology of MDD. In particular, the dentate gyrus of the hippocampus's involvement in both neurogenesis and the development of mood disorders following MI have made it a particular area of interest (14, 26, 23).

The relative sensitivity of A_{2A} receptors in the brain and the large increase in serum adenosine post-MI (1) made the A_{2A} receptor an ideal candidate for study in this thesis research. A_{2A} receptors are widely expressed in the hippocampus, and adenosine imbalances have previously been implicated in the development of MDD (12). This thesis research has shown that delivery of SCS to heart failure canines (modeled by MI/MR) significantly lowered A_{2A} receptor gene expression levels. This not only indicates that adenosine signaling may play a role in the mechanism of SCS, but, due to the proposed feedback inhibition relationship between TNF-α and A_{2A} receptors (5,40), may also indicate that TNF-α levels could be reduced within the hippocampus.

This thesis also explored the role that ghrelin, and its receptor GHSR, may play in the mechanism of SCS. Ghrelin imbalances have been linked to psychological disorders, and ghrelin has a poorly understood role in the functioning of the CNS (50). Ghrelin would be expected to be released when sympathetic neuronal activity is reduced, as has been shown to occur during SCS. These effects make ghrelin an ideal candidate for a role in SCS's effects (22). This thesis research showed ghrelin gene expression in the hippocampus was reduced by heart failure. In marked contrast, SCS treatment of heart failure animals robustly increased ghrelin gene expression in the hippocampus. These findings strongly indicate that ghrelin plays a major role in the neurochemical actions of SCS in the brain, and possibly contributes specifically to the neuroprotective actions of SCS in the treatment of heart failure in the canine model that this laboratory has previously observed. Because increasing sympathetic neuronal activity is known to reduce ghrelin levels, the ability of SCS to increase ghrelin gene expression lends further credence to the previously proposed sympatholytic mechanism of SCS.

Finally, the expression of GLUT-1 was checked because it is an endogenous biomarker of hypoxia. Hence, an increased gene expression of GLUT-1 could indicate that the positive effects of SCS are merely due to an improvement in circulatory health, rather than a distinct biochemical mechanism (21, 11). No noticeable change was detected in GLUT-1 expression in any experimental group, indicating that hypoxia was not a relevant factor in this study. This provides evidence that SCS's mechanism of action within the brain is due to more than improved cardiovascular circulation.

The results of this thesis research warrant further study of the mechanism of SCS. A direct measurement of TNF-α levels within the hippocampus is needed, as that would provide further support to the proposed role of A_{2A} receptors and ghrelin in the central actions of SCS. Additionally, serum levels of adenosine and TNF-α from the canines used in this study would

clarify the role of adenosine signaling in MI and SCS. Finally, investigation of other factors in the adenosine and ghrelin signaling systems, such as the A_1 receptor and leptin, would also contribute to a clearer understanding of SCS's mechanism of action.

The results of my thesis research have helped further elucidate the biochemical mechanisms underlying the neuroprotective effects of SCS. This thesis has shown that adenosine and ghrelin signaling likely play an important role in the mechanism of SCS, while effects on hypoxia do not. Knowledge of this mechanism could aid in the development of future treatments for MI and MDD, as well as provide new avenues for research into diseases that affect the hippocampus.

Supplemental Materials

Table 1: Primers Utilized in Study

GENE SYMBOL	ACCESSION #	SEQUENCE AMPLICON	LENGTH (BP)	TEMPERATURE (°C)
GAPDH	NC_006609.3	TGACACCCACTCTTCCACCTTCGAC CACCCGGTTGCTGTAGCCAAATTC	110	68
SDHA	NC_006616.3	AATCCGTGAAGGCAGAGGCTGTGG GCCGTCTCTGAAATGCCAGGCAGA	111	64
ACTB	NM_001195845	CACTATTGGCAACGAGCGGTTC GTAGTTTCATGGATGCCGCAGGA	90	68
18S rRNA	AY623831	TCGATGCTCTTAGCTGAGTGTCC GTAGTTTCATGGATGCCGCAGGA	125	58
A2AR	NM_001003278	GGTCCTCACTCAGAGCTCCATCTT GTGCCAGTCACCAAGCCATTGTA	102	66
GHSR	NM_001099945	GGCCTGCTCCGACCTGCTCATCTT GCAGCCCTCGCTCACGAACTGGAA	124	66
GHRL	NM_001003052	CTGCTGCTCTTCAGTGTGCTC GCGGCTTCTTGGACTCCTTTC	106	70
GLUT1	NM_001159326	CCATTGCTGTTGCTGGCTTCTC GAAGACGTACGGACCACACAGTT	95	62

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