# The Neuroprotective Effects of Exercise in an Immobilization Chronic Stress Model in the Mouse Hippocampus

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## ABSTRACT

A number of environmental factors have been indicated to increase vulnerability to neurodegenerative disorders, and one possible environmental factor is chronic stress, which triggers the release of glucocorticoids in the brain. Glucocorticoids are known toxic factors that may cause cell death (apoptosis) in the hippocampus. Exercise has been shown to increase the secretion of factors that may help to protect the brain against the toxic effects of glucocorticoids. Therefore, we hypothesize that chronic restraint stress will increase neurodegeneration in the brain as measured by apoptosis and astrogliosis. In addition, exercise will decrease the expression of these markers and will protect against neurodegeneration. To test this, female mice were randomly assigned to standard housing (SH) or placed in cages equipped with running wheels (Exercise). Half of the mice from each group were exposed to chronic restraint stress for two hours each day for two weeks prior to sacrifice. To analyze the expression of toxic effects, sections of the hippocampus will be labeled for markers of apoptosis (Bax) and glial activation (GFAP) using immunohistochemistry techniques. We predict that chronic restraint stress will induce toxic effects in the hippocampus and that exercise will protect against these effects. However, our hypotheses were not supported and the Bax staining was inconsistent, so no definite conclusions can be drawn from this data. Our only significant finding was that in the no stress conditions exercise increased astrocyte expression as compared to standard housing.

## INTRODUCTION

Because American society revolves around fast food, sweetened sodas, large portion sizes, and sedentary activities, obesity has become an epidemic (Brannon & Feist, 2007). Since the 1950's what constitutes "normal" body weight consistently increased, and since the early 1980's adult obesity increased by 50% with currently 35% of all American adults being obese and another 29.8% being overweight (Brannon & Feist, 2007). One cultural trend that

contributes heavily to America's increasing waistlines is the overall decrease in physical activity. Instead of blue-collar jobs involving manual labor, more Americans work white-collar jobs behind computers or in cubicles; budget cuts cause physical education programs to disappear from schools; personal automobiles replace walking or riding a bike as a mode of transportation; and parents who fear for their children's safety discourage them from playing outside, so instead children remain inside in front of a television or computer screen. As Americans remove exercise from their daily lives they endanger not only their bodily health by increasing their risk for a variety of diseases and pathological conditions, such as cardiovascular diseases, Type II diabetes, muscular atrophy, and obesity, they also endanger the health of their brains by increasing their risk for Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases (Booth & Lees, 2007). For centuries human survival was dependent on mobility (see Radak *et al*., 2008b for review), so the human body evolved to expect, even need, a certain level of physical activity. Hence, physical inactivity leads to impairment of physiological processes and reduces the whole body's resistance to the damaging effects of oxidative stress (see Radak *et al*., 2008a for review).

Oxygen is metabolized in all parts of the body, and although this process is necessary for life, it also comes at a cost. Oxygen consumption involves oxidative phosphorylation of different materials; this process produces superoxide and electronegative, unstable free radicals that tend to damage and inactivate other molecules by striping them of electrons (see Kiraly & Kiraly, 2005 for review). Normally, superoxide and free radicals are neutralized by the body's natural stores of antioxidants; however, when oxidative agents reach such an overwhelming concentration that they cannot be neutralized by antioxidants present in the tissue, a condition known as oxidative stress occurs (see Madrigal *et al*., 2006 for review). The brain is particularly vulnerable to oxidative stress because: 1) the brain utilizes about one-fifth of all the oxygen

consumed by the body, and greater oxygen consumption means greater production of free radicals and oxidants; 2) neuronal membranes are made of lipids enriched with easily oxidizable polyunsaturated fatty acids, 3) the brain has naturally low levels of antioxidant enzymes, and 4) the brain contains high concentrations of iron, which is a potent catalyst for oxidant formation (see Liu & Mori, 1999 for review). Oxidative stress is a natural, inescapable process, but unfortunately the brain is especially vulnerable to the potentially damaging effects of this natural process.

Glucocorticoids are hormones secreted by the body that are responsible for many of the detrimental effects of oxidative stress. Glucocorticoids damage neurons by causing the accumulation of glutamate (Stein-Behrens *et al.*, 1992; Moghaddam *et al*., 1994; Stein-Behrens *et al*., 1994), which binds to an N-methyl-D-aspartate neuronal receptor. Glutamate binding increases the number of open calcium ion channels, which greatly increases calcium influx, and thus, the calcium concentration inside the neuron (Choi, 1992; Hertz *et al*., 1999; Kandel & O'Dell, 1992; Mawatari *et al*., 1996), and high internal calcium concentration initiates free radical production by activating the xanthine dehydrogenase/oxidase system (Mawatari *et al*., 1996). Not only do glucocorticoids contribute to the production of free radicals, they also decrease already low neuronal antioxidant capacity (see Kiraly & Kiraly, 2005 for review). Glucocorticoids can cause potent long-term damage because after they bind to their receptors, chaperone proteins transport them into the nucleus where they influence gene transcription. If gene transcription is altered over a period of time, the protein content of a neuron will change, affecting its function (see Joëls, 2008 for review).

Human bodies evolved the oxidative stress response as a way to deal with acute stressors, or any immediate threat, whether real or perceived, that disrupts homeostasis and requires a

compensatory response (see Radley & Morrison, 2005 for review). For example, if a person suddenly meets a bear in the woods, their heart rate and breathing rate immediately increase, pupils dilate, and overall metabolism speeds up in order to provide the body with the energy needed to flee. The acute stress response is adaptive but when multiple, often uncontrollable and unpredictable, stressors are presented consecutively with little time for recovery in between, the chronic stress response becomes maladaptive (see Joëls, 2008 for review). Modern society has achieved living conditions in which the acute stress response is not necessarily important for survival. Instead, people experience chronic stress, such as in response to daily problems experienced at work or with friends or family (see Sapolsky, 1998 for review).

Chronic stress experienced by most people today has been linked to maladaptive changes in the brain. For example, studies have shown that chronic stress leads to dendritic retraction, loss of synapses, and less postsynaptic density in the hippocampus; it also reduces neuronal proliferation, survival, migration, and differentiation (see Joëls, 2008 for review). Chronic stress also decreases levels of brain-derived neurotrophic factor (BDNF), a substance necessary for neuronal growth and survival (see Kiraly & Kiraly, 2005 for review). Moreover, chronic stress increases brain metabolism and oxygen consumption (Landfield & Eldridge, 1994), which increases the brain's oxidative burden and the potential for oxidative damage (see Liu & Mori, 1999 for review). Compounding this effect is the fact that chronic stress also decreases neurons' natural defense capacity (see Kiraly & Kiraly, 2005 for review). Finally, chronic stress increases the amount of damaging glucocorticoids in the brain (see Joëls, 2008 for review), and there is also evidence of these hormones causing cytoskeleton destabilization, which effectively kills neurons by destroying their internal structure (see Radley & Morrison, 2005 for review). Overall, chronic stress exacerbates the damaging effects of oxidative stress, making the flow of

information within the brain less efficient, making it harder to induce the basic mechanisms of memory, and preventing the normalization of activity after a stressful event, all of which can lead to cognitive impairment (see Joëls, 2008 for review).

Since recognizing the damaging effects of chronic stress and oxidative stress, researchers have begun looking for therapies to protect the brain against these threats, and one promising possibility is exercise. Studies suggest that exercise protects the brain from many different insults, facilitates learning and memory, dampens the effects of brain injury, and delays the onset of neurodegenerative disorders and slows symptom progression. Exercise has these benefits because it increases and makes more efficient the mechanisms that support neuronal plasticity, such as synaptic stabilization, neurogenesis, and vascular development and function (see Cotman *et al*., 2007 for review). Studies have found that mice allowed to run on an exercise wheel showed increased neurogenesis in the hippocampus and the survival of these newly formed neurons increased (see Kiraly & Kiraly, 2005 for review; van Pragg *et al*., 2005). In human studies, cardiovascular training before and after stroke reduced neuronal damage and improved recovery time (see Cotman *et al*., 2007 for review). Exercise can even slow generalized neuronal death due to aging and help protect areas of the brain associated with cognitive function from age-related atrophy (see Cotman *et al*., 2007 for review); for example, aged rats allowed to exercise for four weeks expressed new neurons with the same dendritic length, dendritic branching, and spine density as those in young rats (van Pragg *et al*., 2005). Even in human studies involving Alzheimer's disease, exercise reduced the number of cytotoxic amaloid-beta plaques in both the cortex and the hippocampus (see Kiraly & Kiraly for review, 2005; see Cotman *et al*., 2007 for review).

Since exercise is neuroprotective in such debilitating conditions as stroke and Alzheimer's disease, perhaps it would also protect neurons against the damaging effects of oxidative stress induced by chronic stress. Since examination of this research question would be unethical in humans, we chose to instead use mice immobilization as a model of human emotional stress, and previous studies have established that limb immobilization and tube immobilization stress in mice produces oxidative agents which result in oxidative damage (Radak *et al*., 2001b; Fontella *et al*., 2005). We chose to focus on the hippocampus specifically because of its high vulnerability to oxidative damage due to its low antioxidant stores (see Kiraly & Kiraly, 2005 for review) and high number of glucocorticoid receptors (see Radley & Morrison, 2005 for review). Moreover, the hippocampus is essential to the integration of information from all areas of the cortex and to the storage and retrieval of long-term memories. Because of this, atrophy, degeneration, or malfunction of the hippocampus is often implicated in disorders of memory, such as dementias and Alzheimer's disease (see Miller & O'Callaghan, 2005 for review).

In this experiment, damage to the hippocampus will be measured by the presence of astrocytes, glial cells that normally provide neurotrophic support to neurons and aid in the maintenance of neuronal integrity and function by connecting neurons to blood vessels (see Miller & O'Callaghan, 2005 for review). Because of their role in removing waste and damaged tissue, astrocytes usually increase in number in response to neuronal damage or injury (Anderson, 2004; see Miller & O'Callaghan, 2005 for review). However, an over abundance of astrocytes, as occurs in oxidative stress, can actually contribute to neuronal damage because the astrocytes begin to release neurotoxic factors, such as pro-inflammatory cytokines, reactive nitrogen species, proteases, reactive oxygen species, eicosanoids, and excitatory amino acids (see

Gao *et al*., 2003 for review). Another measure used to assess hippocampal damage will be the presence of apoptotic neurons. It has been observed that characteristics of neuronal death due to oxidative stress are similar to characteristics of neuronal death caused by apoptosis (see Satoh *et al*., 1998 for review), suggesting that oxidative stress causes tissue damage by triggering neurons to begin apoptosis. Indeed, in a PC12 cell *in vitro* neuronal model, cells exposed to conditions conducive to oxidative stress showed marked degeneration and any observed cell death was triggered by apoptosis (Kubo *et al*., 1995; see Satoh *et al*., 1998 for review).

Therefore, we hypothesize that subjecting mice to immobilization stress will increase oxidative stress in the hippocampus as measured by increased astrocyte expression and increased apoptotic neuron number. Secondly, we expect exercise to provide protection against the harmful effects of oxidative stress as measured by a decrease both in the number of astrocytes and in the number of apoptotic neurons in animals allowed to exercise as compared to those living in standard housing conditions. If our hypotheses are supported by the data then this will add to the growing body of research suggesting the importance of physical activity. Americans are bombarded with information about how exercise is beneficial for the heart, weight maintenance, etc. but it must also be stressed that exercise is crucial for optimum brain function.

## MATERIALS AND METHODS

### Animals

Thirty-two, eight-week-old, female mice (C57/B16J) were obtained from Jackson Laboratories (Bar Harbor, ME) and kept in a room at constant temperature (68 ± 2°C) and humidity with a light set to a 12-hour light/12-hour dark cycle (6AM-6PM). Two mice were placed in each cage and given standard mouse chow and water *ad libitum* while in home cages. Mice were monitored daily to ensure maintenance of proper living conditions. Six mice were randomly assigned to each experimental condition: standard housing (SH), standard housing plus stress (SH + S), exercise (Ex), and exercise plus stress (Ex + S). Mice in the SH and SH + S conditions were housed in a standard colony cage and mice in the Ex and Ex + S conditions were housed in standard breeding cages equipped with two running wheels for voluntary exercise. At all times the animals were maintained and handled according to guidelines approved by the Rhodes Institutional Animal Care and Use Committee and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

## Chronic Restraint Stress Paradigm

The mice were habituated into their new living conditions for one week. Stress was administered by placing the mice in Broome Restraint tubes (Harvard Apparatus; Holliston, MA) for two hours a day for fourteen consecutive days. Each day, during the time the other mice were being stressed, the mice not receiving stress were picked up in order to avoid confounding effects of handling stress.

## Immunocytochemistry Techniques

### *Tissue Preparation*

After the two weeks of experimental manipulation, mice were euthanized using tribromoethanol (250 mg/kg (i.p.)). After euthanization the brains were transcardially perfused

with 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS; pH 7.4). Whole brains were extracted and postfixed overnight at 4°C in the same fixative for preservation purposes. The next day the brain was processed for embedding in paraffin for sectioning. A microtome was used to make eight-micron-thick sections in the coronal plain which were mounted into SuperFrost Plus Slides (Fisher Scientific; Atlanta, GA).

*GFAP Staining Technique*

Stains that mark the presence of glial fibrillary acidic protein (GFAP) were used to visualize astrocytes. Once the hippocampus was clearly visible, every third slide was selected for staining. The slides were then deparaffinized in xylenes and rehydrated in graded alcohols before being rinsed and rehydrated in PBS-Tween (PBS-T; PBS containing 0.05% Tween 20). After removing the excess PBS-T around the tissue, the tissue was encircled using a PAP pen (Research Products International; Mt. Prospect, IL) and blocked in of PBS-T/Blocking Buffer (PBS-T/BB; 0.5% Bovine Serum Albumin, 0.3% Triton, in PBS) for 30 minutes at room temperature. After blocking, slides were incubated in the primary antibody (anti-GFAP; clone G-A-5; Sigma Chemical Company; St. Louis, MO) at a 1:1000 dilution in PBS-T/BB overnight at 4°C.

The next day the slides were washed in three changes of PBS-T for five minutes each. The secondary donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories; West Grove, PA) was added to the slides in a 1:250 dilution in PBS-T/BB and incubated in a humid chamber for two hours at room temperature. The slides were washed in PBS-T as before and visualized by peroxidase-mediated deposition of diaminobenzidine (DAB; DAB Immunoperoxidase Substrate kit; Vector Laboratories; Burlingame, CA) per manufacturer's

instructions. The slides were washed in running tap water for 10 minutes and dehydrated though a series of graded alcohols and xylenes before being coverslipped using Permount.

*Bax Staining Technique*

Bax was used to detect apoptosis. The slides were deparaffinized and rehydrated though xylenes and graded alcohols and placed in tap water for 5 minutes. Antigen retrieval was performed by gently boiling the slides in a 10mM citrate buffer (0.1M citric acid, 0.1M sodium citrate, distilled water) for 15 minutes. The slides were allowed to cool to room temperature and were then rehydrated in PBS-T for 30 minutes. The tissue was encircled using a PAP pen as described above. Nonspecific binding was blocked by preincubating sections for 30 minutes at room temperature in blocking buffer [TNB (0.1M TrisHCL, 0.15M NaCl, 5% blocking buffer); pH 7.5; Renaissance TSA-Indirect kit, NEN Life Science Products; Boston, MA]. The primary anti-Bax antibody (clone 6A7; BD Biosciences; San Jose, CA) was diluted 1:250 in TNB and placed on the slides which were incubated overnight in a humid chamber at 4°C.

The slides were washed in PBS-T as before. The same secondary donkey anti-mouse antibody was diluted 1:250 in TNB and added to the slides before incubating for 2 hours in a humid chamber at room temperature. The slides were washed in PBS-T as previously described. In order to amplify the stain, the slides were incubated in biotinyl tyramide (BT; 1:50 dilution in Amplification Diluent from the Renaissance TSA-Indirect kit) for 10 minutes at room temperature. The slides were washed in PBS-T as before and incubated in a 1:100 dilution of streptavidin conjugated to HRP in TNB for 1 hour at room temperature. Again the slides were washed in PBS-T and visualized with DAB per instructions on the kit. The slides were next counterstained by placing the slides in hematoxylin for 15 seconds followed by 10 minutes in

running tap water and 30 seconds in acid alcohol (3% acetic acid in 70% EtOH). The slides were then dehydrated through graded alcohols and xylenes and coverslipped using Permount.

**Data Analysis**

To quantitatively analyze the amount of astrocytes detected with GFAP staining, images of the hippocampus on the prepared slides were taken with Leica microscope equipped with a digital camera. Five images of tissue sections containing CA 1-3 fields were randomly chosen from each animal in each condition. The images were coded so as to make the quantification process blind to the raters. Astrocytes in each image were counted by three independent reporters and their data compiled to achieve an average amount of astrocytes for each image. These averages were used to perform statistical analysis to determine if there were significant differences among the variables. Statistical Program for Social Sciences (SPSS) was used to perform a 2 x 2 factorial Analysis of Variance (ANOVA) and a one-way ANOVA with Tukey and LSD post-hoc analyses.

Images of the CA fields of the hippocampus were also taken of the prepared tissue stained with Bax. The slides photographed were chosen in the same way as GFAP imaging. These images were qualitatively analyzed for high, medium, or low levels of apoptotic nuclei marked by the expression of the Bax protein.

## RESULTS

*Astrogliosis in the Hippocampus*

One marker of degeneration in the hippocampus is enhanced glial expression. To asses glial recruitment, photomicrographs were taken of the CA fields of animals from each condition, and the number of GFAP labeled astrocytes was assessed (Figure 1). The numbers observed from three independent reporters were averaged for each condition and the marginal means (±SEM) were: SH = 36.80 (±5.02); SH + S = 46.40 (±3.55); Ex = 51.30 (±4.46); Ex + S = 39.80 (±4.24) (Figure 2). There were no significant main effects in either the housing or the stress conditions, [$F(1, 85) = 0.820$, $p = 0.368$; $F(1,85) = 0.045$, $p = 0.832$]. A factorial ANOVA revealed a significant housing by stress interaction [$F(1,85) = 5.87$, $p<.05$]. In order to further examine the interaction, the factorial ANOVA was followed up with a one-way ANOVA and post hoc analyses using Fisher LSD and Tukey HSD for pair-wise comparisons. The second series of tests revealed that the only significant simple effect was between the SH condition and the Ex condition ($ps<0.05$). Mice in the Ex condition expressed significantly more astrocytes ($M = 51.26$) than mice in the SH condition ($M = 36.80$).

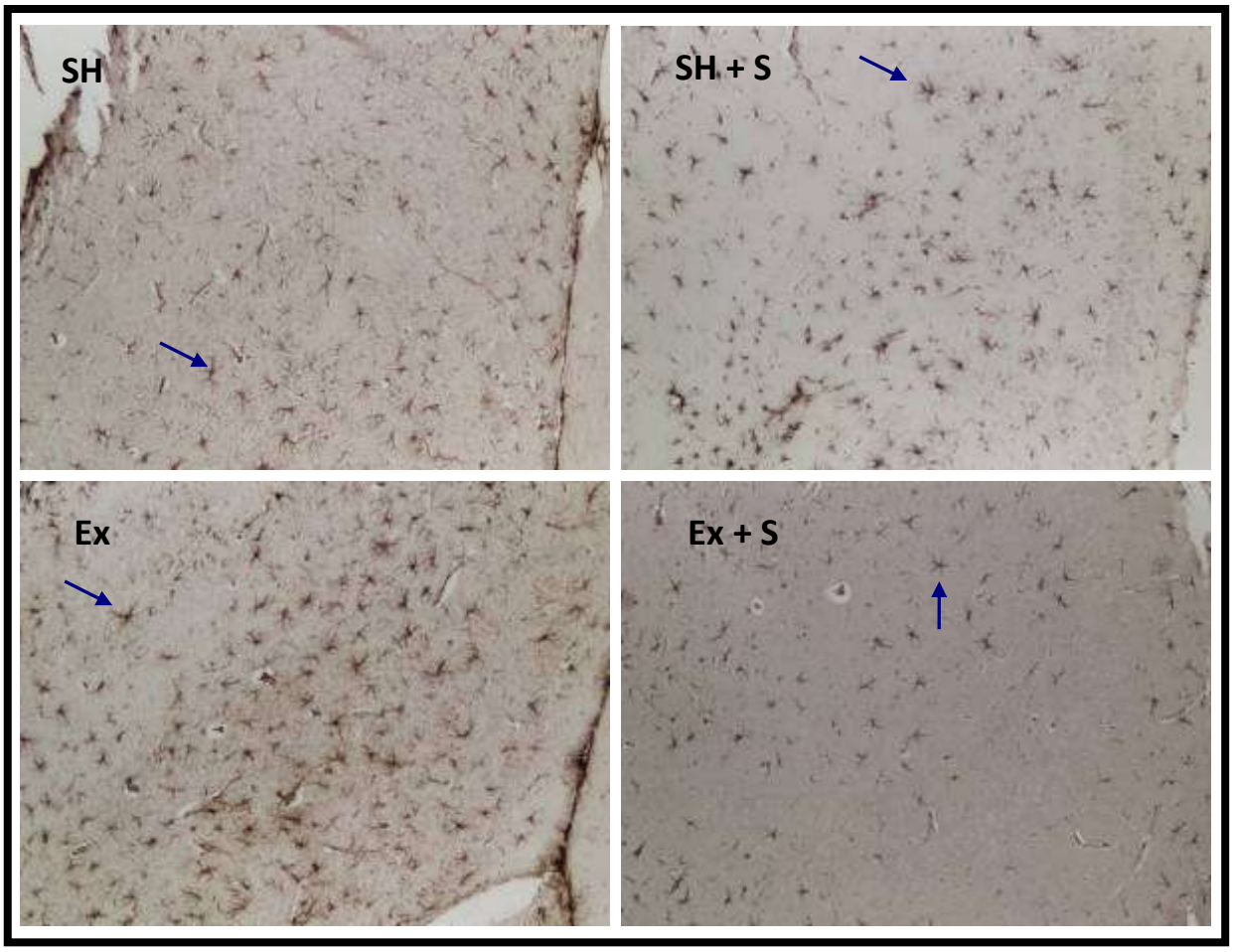


**Figure 1**: Low power photomicrographs (10X) of GFAP labeled astrocytes in the CA fields of the hippocampus. The number of astrocytes in each image is representative of the average for each group and arrows point to an astrocyte with well defined cell soma and processes.

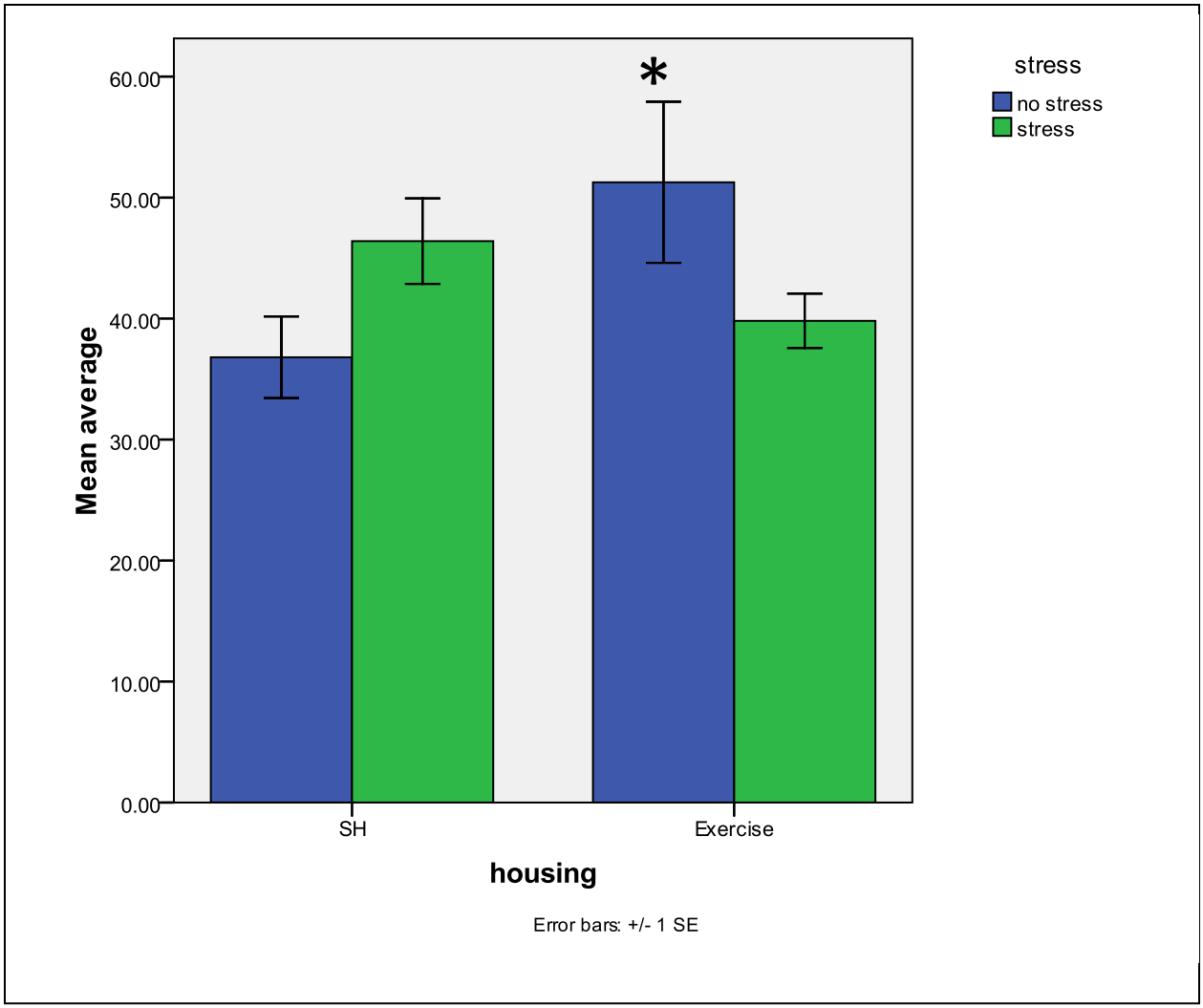


**Figure 2**: A bar graph showing the average number of astrocytes expressed in the CA region of the hippocampus of mice in each experiemental condition. A factorial ANOVA revealed a significant housing by stress interaction. Bars represent means ± SEM; * represents $p<0.05$ as compared to standard housing.

*Expression of the Apoptotic Marker Bax in the Hippocampus*

Initial observation seemed to indicate that the Bax staining was optimized and appropriate; however, upon further analysis the staining proved to be inconsistent and therefore unquantifiable. Qualitative analysis was attempted but since the quality of the staining varied between slides from the same animal, the results were inconclusive. Further optimization is

needed to obtain quantifiable results. Selected images are representative of slides with appropriate staining for Bax labeled neurons (Figures 3 and 4).

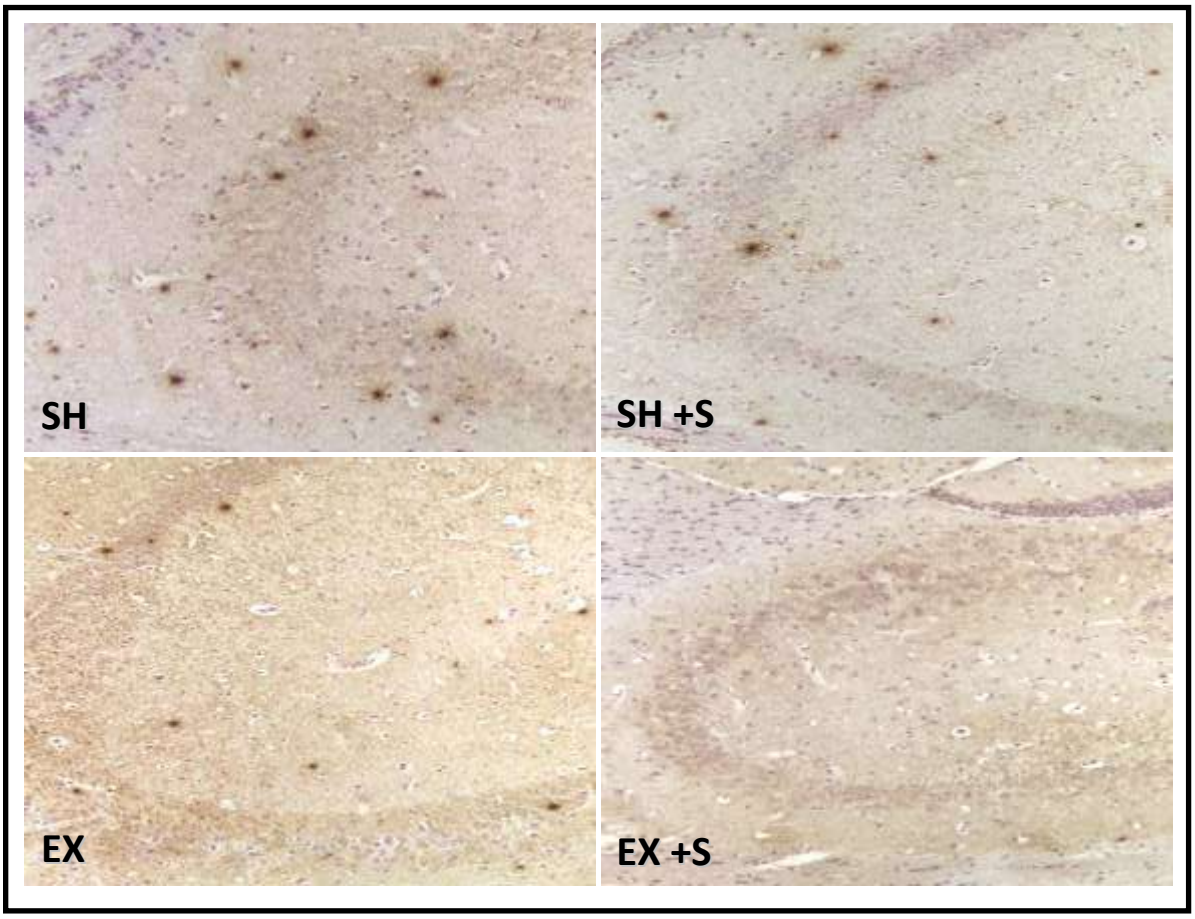


**Figure 3**: Low power photomicrograph (10X) of Bax labeled apoptotic nuclei in the CA fields of the hippocampus.

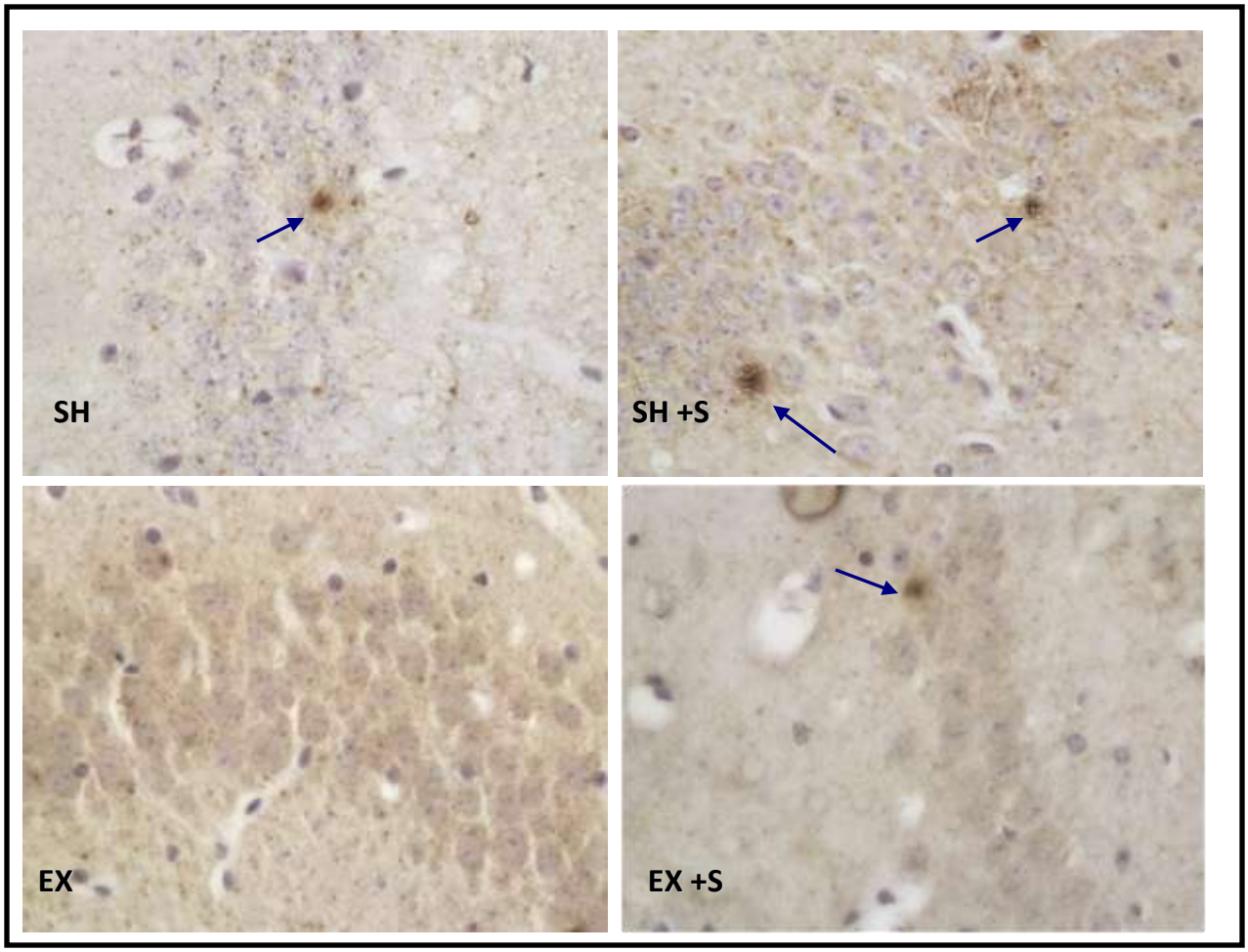


**Figure 4**: High power photomicrograph (40X) of Bax labeled apoptotic neurons in the CA fields of the hippocampus. Arrows point to cells that phenotypically appear to be prominent apoptotic neurons.

## DISCUSSION

The data from this experiment did not support our original hypothesis that immobilization stress would increase oxidative stress, in that there was not a significant increase in astrocyte expression in either of the stress conditions as compared to the conditions that did not experience stress. Nor did the data support the hypothesis that exercise would protect against oxidative stress because there was no significant difference between the number of astrocytes in the exercise conditions as compared to the standard housing conditions.

Our only significant finding was that exercise increased astrocyte expression in animals that did not experience stress, which contradicted our hypothesis. One possible explanation for this finding is that exercise causes oxidative stress and the increase in astrocyte number is a result of astrogliosis in response to exercise-induced neuronal injury. Some previous research does suggests exercise is a stressor and causes the production of reactive oxygen species (Powers *et al*., 1999; Ji *et al*., 2006). Reactive oxygen species are involved in oxidative stress and can be harmful to tissue, so it is possible that the mice engaged in enough exercise to increase the levels of reactive oxygen species in their hippocampi. Since activated astrocytes help rescue injured neurons by eliminating toxic substances, releasing neuotrophins, and promoting tissue repair (see Gao *et al*., 2003 for review), more astrocytes where recruited to alleviate the damage caused by the oxidative challenge of exercise.

However, this explanation is probably not accurate because the reactive oxygen species created during exercise tend to only be harmful to tissue unaccustomed to this type of stressor (Powers *et al*., 1999; Ji *et al*., 2006; see Radak *et al*., 2008b for review). Mice in the exercise conditions of our experiment were placed in cages with exercise wheels and allowed to run for two weeks prior to the two-week experimental stress manipulation as well as run during the time of the stress. Thus, the mice had been exercising for almost a month, so the tissue can hardly be considered unaccustomed to the stress of exercise. Instead, these mice were regular exercisers and regular exercise has been shown to upregulate the body's defense mechanisms against oxidative stress (see Radak *et al*., 2008b for review). Indeed, the reactive oxygen species generated during regular exercise can actually serve as messengers that help change gene expression so as to increase the activity of antioxidant enzymes and promote more effective

housekeeping by DNA repair enzymes and proteasome complexes (see Radak *et al*., 2008a, b for review).

A more likely explanation of the increase in astrocyte expression in the exercised animals is that additional astrocytes were recruited as increased metabolic support. Muscular activity during exercise increases heart rate, which increases blood flow to all areas of the body including the brain. As more blood is pumped though the brain, metabolism increases and more oxygen is taken up by the neurons (see Cotman & Berchtold, 2002 for review; Fabel *et al*., 2003; Radak *et al*., 2001a, 2006). In order to maintain the high level of metabolism, as well as the resulting growth of new neurons, expression of enzymes involved in glucose metabolism and growth of new blood vessels must be increased (see Cotman *et al*., 2007 for review). Trophic factors, such as BDNF, insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor, are increased and all work in concert to promote both neurogenesis and angiogenesis (see Cotman *et al*., 2007 for review).

Therefore, the observed increase in astrocyte number in the exercised animals must have been due to increased astrocyte recruitment to help maintain the exercise-induced metabolic changes. Astrocytes are a primary source of neurotrophic factors (see Gao *et al*., 2003 for review), without which the growth of new neurons would be severely hampered. Secondly, astrocytes are involved in the transportation of materials between blood vessels and neurons (Rosenzweig *et al*., 1972); thus, more astrocytes would be needed to meet the metabolic demands of not only new neurons but also established ones. Moreover, a study examining mice in an enriched environment, a part of which was exercise, observed more glial cells, one type of which is astrocytes, in the enriched environment animals as compared to mice in an impoverished environment (Rosenzweig *et al*., 1972). This suggests that astrocyte upregulation

can happen in response to neuronal growth and angiogenesis. We suggest this is what happened in our animals and the increase in astrocyte number in the exercise group was due to an increased metabolic need, not due to oxidative stress.

Astrogliosis is only part of the complicated oxidative stress process. In order to get a better idea of the overall process, we chose to combine our astrocyte investigation with an examination of apoptotic neurons. The Bax protein staining used to mark apoptotic neurons would have shown us whether the increase in astrogliosis was associated with an increase in apoptosis, both of which are indicative of oxidative stress. Had expression of both astrocytes and the Bax protein increased in the hippocampus, then we would have been confident in concluding that astrogliosis was indicative of a response to oxidative stress.

The Bax staining, however, proved inconclusive and one possible reason is that we waited to harvest the tissue until twenty-four hours after the last stress, in hopes of observing long-term changes caused by chronic stress. This time period might have been too long, allowing Bax expression to normalize. A study performed by Krajewski *et al*. (1995) looked at Bax expression from thirty minutes to three hours after the last stress and found that Bax expression was markedly increased in the CA1 region of the hippocampus. However, this study not only used adult rats instead of mice but used a completely different model: generalized ischemia following induced transient cardiac arrest as opposed to chronic immobilization. In future studies investigating exercise and the immobilization stress model, researchers might look into harvesting the tissue at intervals beginning at thirty minutes after the last stress. At these intervals increased Bax expression might be at its height and consistent staining could be obtained before protein expression normalizes, as it seems to do within twenty-four hours.

Oxidative stress is a complex, global phenomena in the brain that involves a lot more than just astrogliosis and apoptosis, so in the future researchers should look for different markers of oxidative stress. One possible marker is nitric oxide (NO) because stress has been shown to generate high levels of NO in the brain (Olivenza *et al*., 2000; see Madrigal *et al*., 2006 for review). In rodent immobilization stress models it was observed that enzymes that degrade NO were upregulated following the stress (Madrigal *et al*., 2002) and the longer an animal was stressed, the higher the expression of NO-degrading enzymes (Olivenza *et al*., 2000). NO is a gas produced in the brain that normally crosses cell membranes and, at moderate concentrations, has a similar function to neurotransmitters and can even serve as an antioxidant. However, at high concentrations, NO interacts with superoxide, another chemical increased during oxidative stress, to form peroxynitrite ($ONOO^-$), a very reactive, highly cytotoxic substance that can damage neuronal lipids, proteins and DNA (Beckman *et al*., 1990; Pacher *et al*., 2007). In future research, perhaps during Western blotting for which we harvested and prepared tissue this summer, one element to be looked for could be the concentrations of NO-degrading enzymes because increased NO-degrading enzyme concentrations would indicate oxidative stress.

Another indicator of oxidative stress that could be analyzed is the presence of glucocorticoids in the brain. Glucocorticoids are normally involved in the metabolism of sugars, proteins, and fats and are released after stressors because they play a role in the stress-induced fight-or-flight response (see Liu & Mori, 1999 for review). In response to stress, the hypothalamic-pituitary-axis in the brain signals the adrenal glands, which sit just above the kidneys, to secrete glucocorticoids. These hormones provide the body with a readily available energy supply by stimulating the release of energy substrates into the blood stream and inhibiting glucose uptake and fatty acid storage while also suppressing unessential anabolic processes, such

as protein synthesis (Cannon, 1915; Baxter, 1976; see Munck *et al*., 1984 for review; see Joëls, 2008 for review).

Sustained high concentrations of glucocorticoids, such as is the case during chronic stress, can damage neurons, and hippocampal neurons are especially vulnerable because they contain a high number of glucocorticoid receptors (see Radley & Morrison, 2005 for review). When glucocorticoids bind to their receptors, chaperone proteins take them into the cell and into the cell's nucleus where they influence gene transcription. Overtime, chronic exposure to elevated levels of glucocorticoids will change gene expression and thus change the protein content of the cell which will eventually change the cell's function (see Joëls, 2008 for review). Furthermore, glucocorticoids increase the accumulation of glutamate (Stein-Behrens *et al*., 1992; Moghaddam *et al*., 1994; Stein-Behrens *et al*., 1994), which binds to its receptor, opening more calcium ion channels and letting massive amounts of calcium into the cell (Choi, 1992; Hertz *et al*., 1999; see Kandel & O'Dell, 1992 for review; Mawatari *et al*., 1996). Increased internal calcium concentration can initiate free radical and NO generation, mitochondria dysfunction, protein misfolding and cytoskeletal damage (Mawatari *et al*., 1996). Finally, increased glucocorticoids can decrease a neuron's antioxidant capacity (see Kiraly & Kiraly, 2005 for review), make neurons more vulnerable to damage from which they would normally be able to recover (Muhlen & Ockenfels, 1969; Sapolsky, 1985a; Sapolsky *et al*., 1986; Tombaugh *et al*., 1992; see Lupien & Meaney, 1998 for review), and cause the loss of synapses, dendritic atrophy, and a decrease in neurogenesis (Sapolsky *et al*., 1985a; Magariños & McEwen, 1995; Eriksson *et al*., 1998; Gould *et al*., 1998; see Miller & O'Callaghan, 2005 for review). Thus, it would be interesting to examine the levels of glucocorticoids in the brain following chronic stress to get a better understanding of how chronic stress relates to oxidative stress.

Another marker that would be worth examining is the amount of growth factors present in brain tissue following chronic stress and exercise. Previous studies have established that growth factors, especially BDNF, are downregulated in response to stress (see Kiraly & Kiraly, 2005 for review). However, exercise has been shown to increase growth factor levels or at least maintain them (see Cotman *et al*., 2007 for review). If our research continued to look specifically at the hippocampus, it would be beneficial to examine the levels of BDNF and IGF-1 because BDNF is important for hippocampal function, especially in maintaining synaptic plasticity that is essential for learning, and IGF-1 is important for neurogenesis, an essential part of memory formation (see Cotman *et al*., 2007 for review).

Overall, our hypothesis was not supported and the inconclusive Bax staining prevents us from drawing definite conclusions about the meaning of the observed increase in astrocyte expression in exercised animals. However, future studies in this lab could look for other markers of oxidative stress that would give a more complete picture of the consequences of immobilization stress. It is important to note that restraint stress is used as a model for human emotional stress (Fontella *et al*., 2005), so conclusions drawn from this research can be somewhat translated to the impact of emotional stress on humans. Indeed, studies that have looked at repeated stress in animals were able to recreate in the animals the same types of behavioral abnormalities characteristic of human mental illnesses, making stress experiments with animals a reliable model for stress-related mental illness in humans (Ottenweller *et al*., 1989; Willner, 1997). Further supporting the link between our research and human experience is that our research concentrated on the detrimental effects of stress in the hippocampus, a brain structure highly conserved throughout all levels of animal brains, and results from research on

the hippocampi of both animals and humans tends to be consistent (see Kiraly & Kiraly, 2005 for review).

Stress is an unavoidable part of the human experience and unfortunately it is also associated with neurodegenerative diseases such as Huntington's disease, Parkinson's disease, as well as Alzheimer's disease and other forms of dementia (see Madrigal *et al*., 2006 for review). These diseases are especially terrifying because they involve the loss of one's body, one's memories, and one's personality, all of which are characteristics that make us undeniably "human." Currently, there is no cure for neurodegenerative diseases, only treatments that slow symptoms or alleviate suffering. Thus, it is worthy to look for something that might offer protection or maybe even prevent the development of such catastrophic diseases. Because of exercise's success with delaying the onset and slowing symptom progression of neurodegerative diseases, it is scary to think of the implications of eliminating exercise as part of one's daily routine. More sedentary lifestyles lead to impairment of physiological functioning and reduce the entire body's ability to combat oxidative stress (see Radak *et al*., 2008a for review), making a person more vulnerable to stress-related, neurodegenerative diseases. As Americans eliminate exercise, they are cutting out one of the most reliable forms of neuronal protection and as a result the incidence of stress related neurodegenerative diseases is increasing. If this trend continues, we may see younger and younger people develop these diseases and deteriorate more rapidly.

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