The Effect of Exercise and Stress on Neurodegeneration in the Mouse Hippocampus

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Abstract

A number of environmental factors have been indicated to increase the brain's vulnerability to neurodegenerative disorders, such as Parkinson's disease. Chronic stress triggers the release of glucocorticoids in the brain, which are known toxic factors that may cause apoptosis (cell death) in the hippocampus. Exercise has been shown to increase the secretion of protective factors that may also 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 reactive gliosis. 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 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. To analyze the expression of toxic effects, sections of the hippocampus were labeled for a marker 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.

Introduction

There are an extremely large number of advertisements in the media that promote ways to improve people's overall health; from eating Cheerios™ to help protect the heart by lowering cholesterol to eating yogurt with probiotics to regulate the digestive system. However, there are hardly any advertisements telling us how to protect the most important organ in the body—the brain. Without the brain the rest of the body would not be able to function; the medulla oblongata in the brain stem regulates heart rate and breathing; the cerebellum controls complex movement and refines signals from the motor cortex; other parts of the brain are crucial for cognition, vision, and speech production as well as speech comprehension. Since the brain is an extremely crucial organ for survival, it would seem that there would be highly publicized ways to protect the brain from degeneration and disease. Although there has been an increase in the rate of incidence and number of fatalities from neurodegenerative

diseases, such as Parkinson's disease and Alzheimer's disease (Centers for Disease Control), there has not been a substantial increase in the amount of advertisements that advise people on how to prevent these diseases.

Since the rate of people diagnosed with neurodegenerative diseases is rising every year, it is important to understand more about these diseases in order to be able to prevent them. Two of the main neurodegenerative diseases are Alzheimer's disease and Parkinson's disease. Alzheimer's disease affects a specific part of the brain called the *hippocampus,* which is responsible for learning and memory, and causes symptoms including memory loss. Unfortunately, there are only symptomatic treatments for this devastating disease (Kiraly et al., 2005). Parkinson's disease causes the degeneration of dopamine-producing neurons in a part of the brain stem called the *substantia nigra,* which controls movement; symptoms of this disease include tremors, balance impairment, and difficulty coordinating movement (NIH Senior Health). The Centers for Disease Control (CDC), using data from 2006, reported that there were 72,432 deaths from Alzheimer's disease in the United States. This is a large increase from what the CDC reported in 2004 when the number of fatalities from Alzheimer's disease was 65,965 and 49,558 in 2000. The CDC also reported that in 2005 the number of fatalities from Parkinson's disease was 19, 544 compared to 17,997 in 2003 (Centers for Disease Control). This increase in the rates of neurodegenerative diseases has momentous implications for the elderly population because, at this time, there is no cure for either of these diseases.

As the average lifespan of humans continues to increase due to significant advances in medicine and technology, the rates of neurodegenerative diseases could also continue to increase because people would be exposed to environmental toxins that affect brain health for a longer period of time. In 1996, there was a prediction that brain disease would be the leading cause of death in future decades (Lopez et al., 1998). Thus, it is important to find ways to protect the brain to help prevent or postpone the onset of these debilitating diseases and the resulting neurodegeneration. The hippocampus, which is affected during Alzheimer's disease, is also particularly vulnerable to other causes and forms of neurodegeneration and is one of only two areas in the brain where the majority of neurogenesis occurs (Miller et al., 2005).

The hippocampus has the greatest amount of receptors for steroid hormones called glucocorticoids of any region in the brain. Neurodegeneration can be caused by the chronic release of glucocorticoids, which are adrenal steroids that are secreted during stress from the hypothalamus-pituitary-adrenal (HPA) axis. This system regulates the physiological and behavioral responses to stress and also tells the adrenal cortex, from where glucocorticoids are secreted, to stop producing the steroid

(Swaab et al., 2005). Physiological stress and the release of these steroids causes the body to redirect oxygen and nutrients to the brain and detoxify toxic products (Liu et al., 1999). The parts of the hippocampus that are particularly vulnerable to the effects of glucocorticoids are the dentate gyrus and the CA1 regions because they have a large amount of receptors for the steroid (Joels, 2008). The CA1 region of the hippocampus is one of the first areas of the brain that is affected in Alzheimer's disease (West et al., 2004). Thus, this region is particularly interesting for studying if glucocorticoids are one possible cause of its degeneration.

The presence of glucocorticoids can be beneficial in small amounts because they mobilize energy to tissues during an emergency and stop unnecessary anabolic functions (Fontella et al., 2005). Interestingly, in the absence of corticosterone, granule neurons in the dentate gyrus lose distal dendrites (Wossink et al., 2001). These cells alter their gene expression profiles to protect themselves against the apoptosis that occurs in the absence of stress hormones. The vulnerable neurons in the dentate gyrus become more sensitive to the influx of calcium in the absence of stress hormones, such as corticosteroine, and therefore are more vulnerable to apoptosis (Nair et al., 2004; Joels, 2008). Another benefit from the acute release of glucocorticoids includes the increased activity of CA1 neurons that are thought to be involved in the process of encoding information. Pyramidal neurons also strengthen synaptic connections during this time and may help preserve previously encoded information (Joels, 2008).

During acute stress the effects of the hormones that are released subside after a short period of time and do not seem to have devastating effects. The release of glucocorticoids causes an increase in certain toxins in the brain such as calcium and free radicals, which are substances that can cause damage to the brain by contributing to oxidative stress (Joels, 2008). The effects of calcium and free radical accumulation are not damaging in acute stress because there are systems in place to clear these toxins, such as the antioxidant system. However, prolonged secretion of glucocorticoids and the resulting accumulation of toxins leave no time for the body to clear these substances resulting in cellular disintegration (Fontella et al., 2005). As mentioned previously, the release of glucocorticoids causes the accumulation of calcium. In the CA1 region of the hippocampus, prolonged calcium accumulation increases the neuron's vulnerability to other assaults (Joels, 2008). The acute release of glucocortioids and calcium influx may temporarily impair neuronal function but the chronic accumulation of calcium causes structural decline in the brain (Liu et al., 1999). When stress is chronic and glucocorticoids are constantly released, the effects can be devastating since glucocorticoids starve cells by limiting the amount of glucose that enters and disturbs the ionic balance in the cell due to the increase of calcium

(Gao et al., 2003). Glucocorticoids also starve the neurons by disrupting energy metabolism by reducing glucose uptake (Liu et al., 1999). With a lack of glucose for energy, the neurons do not have the necessary materials to function. Thus, the acute release of glucocorticoids has beneficial effects for the brain, but the problem arises when the threshold of glucocorticoid concentration is passed and toxins begin to accumulate faster than the body can clear them.

Research has also shown that neurons undergo many morphological changes in response to chronic stress including dendritic atrophy and spine reduction (Cotman et al. 2002). Chronic stress causes the neurons in the CA3 region to retract their dendrites and curbs neurogenesis in the dentate gyrus as well as reduces neuron number in this region (Joels, 2008; Sapolsky et al., 1985). This level of stress also causes a decrease in synaptic connections in the CA3 region and lowers the cellular complexity of this area (Joels, 2008). The loss of dendrites in the CA3 region leaves the neurons vulnerable to toxins and may represent compromised neurons (Conrad et al., 2007). Granule cells in the dentate gyrus become more sensitive to the effects of subsequent episodes of stress after exposure to chronic stress (Joels, 2008). Taken together, these studies indicate that the release of glucocorticoids alters neurons and makes the cells vulnerable to the effects of other assaults on the brain by lowering a neuron's ability to overcome oxidative stress (Kiraly et al., 2005). It is believed that there are three main causes of neuron death following damage to the brain: accumulation of glutamate, chronic influx of calcium, and oxidative stress (Tyurin et al., 2000).

The brain is particularly vulnerable to oxidative stress because it uses a large amount of oxygen from the blood and this high concentration has been shown to damage neurons in the central nervous system (Satoh et al., 1998). The brain also contains a low level of antioxidants, which help ameliorate oxidative damage, and has a large concentration of iron, which is thought to be a catalyst for oxidative damage (Lui et al., 1999). The accumulation of calcium from chronic glucocorticoid secretion has been shown to produce free radicals (Dykens, 1994) and the increased levels of glutamate have been known to increase oxidative damage (Stein-Behrens et al., 1994; Radak et al., 2001). Oxidative damage occurs with the accumulation free radicals and generation of reactive oxygen species (ROS) which are products of aerobic metabolism (Nino-Cabrera et al., 2002; Pani et al., 2000). These substances are necessary for many important processes in the body such as initiating apoptosis, metabolizing xenobiotics, and starting the cellular repair process; however, these substances are also involved in many pathological processes, including neurodegenerative diseases (Pani et al., 2000). The production of free radicals has been described as a factor that causes many diseases, especially age-related degenerative ailments (Liu et al., 1999).

Reactive oxygen species, such as free radicals disrupt the homeostasis that is crucial for body systems to survive (Tyurin et al., 2000). These substances, which are naturally found in metabolism and inflammation as well as through environmental toxins (Nino-Cabrera et al., 2002; Lovell et al.,2007), can damage the DNA as well as important proteins in the body (Lovell et al., 2007). Free radicals and ROS can also disturb the functional balance of CA3 pyramidal neurons leaving them vulnerable to damage. Chronic restraint stress, which is the model in this study, also produces free radicals and, as such, is a major factor in oxidative damage (Ejchel-Cohen et al., 2006). With the cells vulnerable from the production of free radicals and the toxins in the cells from the chronic release of glucocorticoids, oxidative stress can effectively destroy neurons. Oxidative stress is marked by reactive astrogliosis, reactive microgliosis, inflammatory cytokines, and apoptosis (Gao et al., 2003). As previous research has shown, chronic stress, through the production of toxic substances, can have devastating effects on the hippocampus. However, there have been promising findings that show that there is a way to protect the brain from the degeneration due to toxic insults, including stress.

Exercise has been shown to help the body ameliorate the effects of ROS and alleviate oxidative stress (Radak et al., 2001). This alleviation of oxidative stress may explain why studies have shown that regular exercise decreases the rates of neuronal apoptosis in the dentate gyrus of the hippocampus (Lee et al., 2003). This may also be due to the fact that, during exercise, the brain secretes neurotrophic factors which help protect the brain from damage and promote neurogenesis in the hippocampus (Cotman et al., 2002). A recent study indicated that mice that were allowed to run had a substantially greater number of newly formed neurons in the hippocampus than a non-running control. This trend of neuron growth, although attenuated, was still much higher in the running group after four weeks of training than in the control group (van Praag et al., 2005). Other studies have shown that the number of mitotic cells, which indicate neurogenesis, was much higher in running mice compared to sedentary ones (Fabel et al., 2003). Not only does exercise increase the number of newly formed neurons, it also has been shown to increase the survival of newly formed neurons as well as increase synaptic plasticity, and neurotransmission (Spires et al., 2005; van Praag et al., 2005). Research by Rosenzweig indicates that rats in an environment that includes exercise had more dendritic spines than those in an impoverished environment, suggesting an increase in synaptic capabilities (1972). Many of the synaptic contacts between neurons are made on dendrites or dendritic spines, which are small projections from the dendrite (Rosenzweig et al., 1972). Previous studies have shown that exercise, although beneficial for the structure and morphology of neurons, is not limited to neurogenesis and neuron protection.

Exercise has been shown to protect the brain from neurodegeneration and prevent the onset of neurodegenerative diseases and other cognitive impairments (Miller et al., 2005). Exercise can improve cognitive function and learning as well as help lower the risk of developing Alzheimer's disease and dementia in general (Cotman et al., 2002). Studies have shown that exercise can help patients suffering from stroke recover more brain function compared to sedentary stroke patients (Kiraly et al., 2005). Through the production of neurotrophic factors, exercise strengthens neural structure and facilitates transmission in the synapses. Exercise also acts to prime the neurons for encoding and preserve information that has already been encoded (Cotman et al., 2002). Thus, since exercise has been shown to protect the brain, it may be able to protect the brain from the deleterious effects of stress because exercise has been shown to help the body handle a variety of stressors (Radak et al., 2008a). However, it is important to have exercise in moderation since there is some amount of ROS and toxin production during exercise (Radak et al., 2008a).

At extreme levels exercise can be detrimental to brain health. Studies have shown that long periods of heavy exercise in rodents show more damaged mitochondria and shrunken neurons than in rodents in a mild exercise condition (Sumitani et al., 2002; Kim et al., 2003). On the other hand, a single bout of exercise has been shown to increase concentrations of substances that increase oxidative damage. Regular training, however, has been shown to decrease the accumulation of oxidative proteins and amount of DNA damage (Radak et al., 2001). Exercise also increases the uptake of glutamate that accumulates in response to glucocorticoid production (Radak et al., 2001). This uptake would help ameliorate the deleterious effects of glucocorticoid influx from chronic stress. The majority of current research supports the idea that exercise is highly beneficial for brain health.

The noted studies have shown that the chronic release of glucocorticoids damages the brain and increases its vulnerability to various toxins and exercise has been shown to protect the brain from degeneration. To further examine this question we hypothesize that chronic restraint stress will cause neurodegeneration in the hippocampus of mice and exercise will help protect against the degeneration that is caused by chronic stress. To determine if stress and the release of glucocorticoids causes apoptosis in neurons of the hippocampus of mice we will look at markers of apoptosis, specifically reactive astrogliosis and the expression of the Bax protein. If these markers are upregulated in response to chronic stress, it would suggest that chronic stress does cause apoptosis. We also will examine the effects of exercise on cell degeneration. If these markers are lower in the exercising group, we may be able to conclude that exercise protects against the deleterious effects of chronic restraint stress.

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 two weeks. Stress was administered by placing the mice in Bromme 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 profused 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 plane 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 a 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).

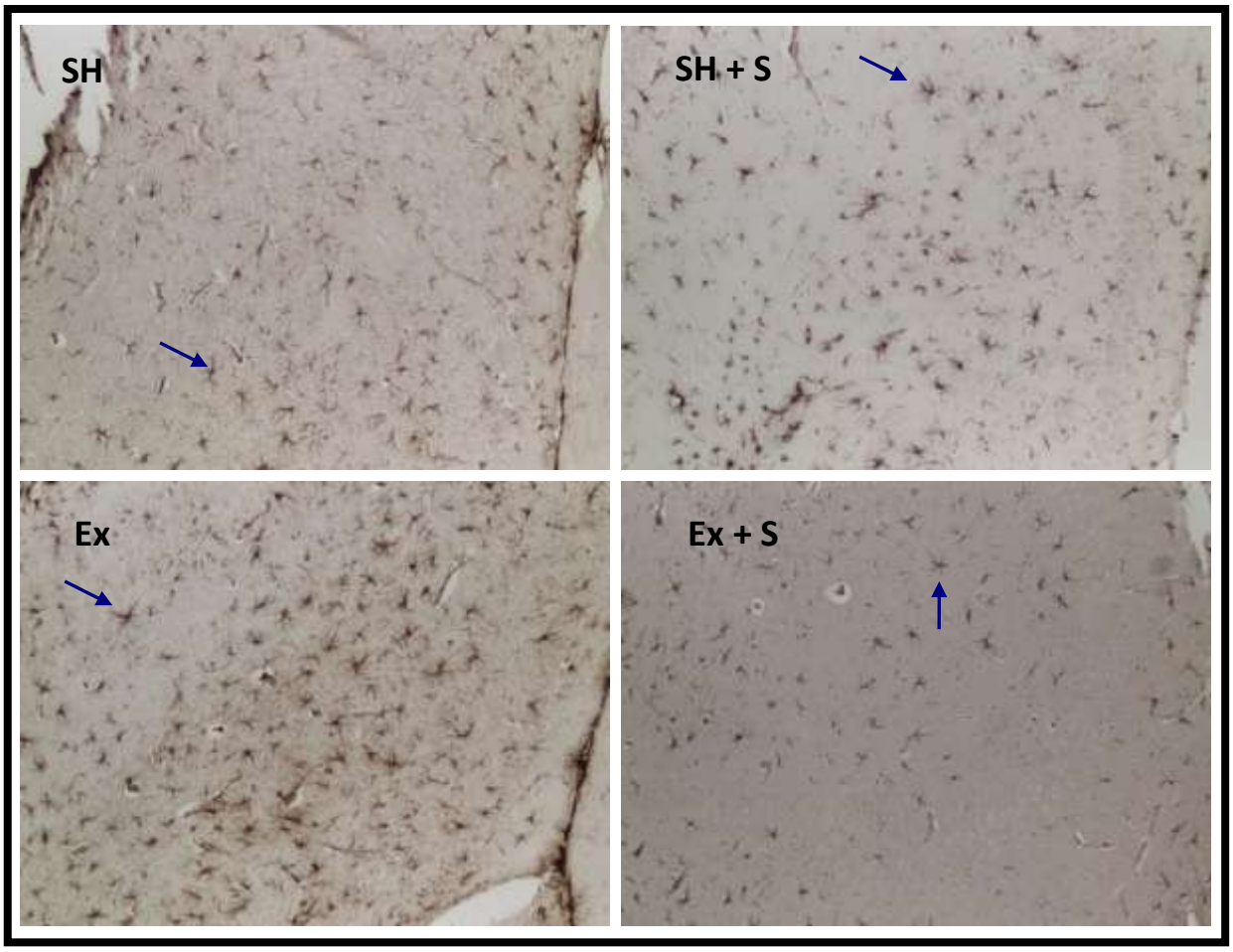

Figure 1: Low power photomicrographs (10X) of GFAP labeled astrocytes in the CA fields of the hippocampus. The number of astrocytes in each image is representative of the average for each group and arrows point to an astrocyte with well defined cell soma and processes.

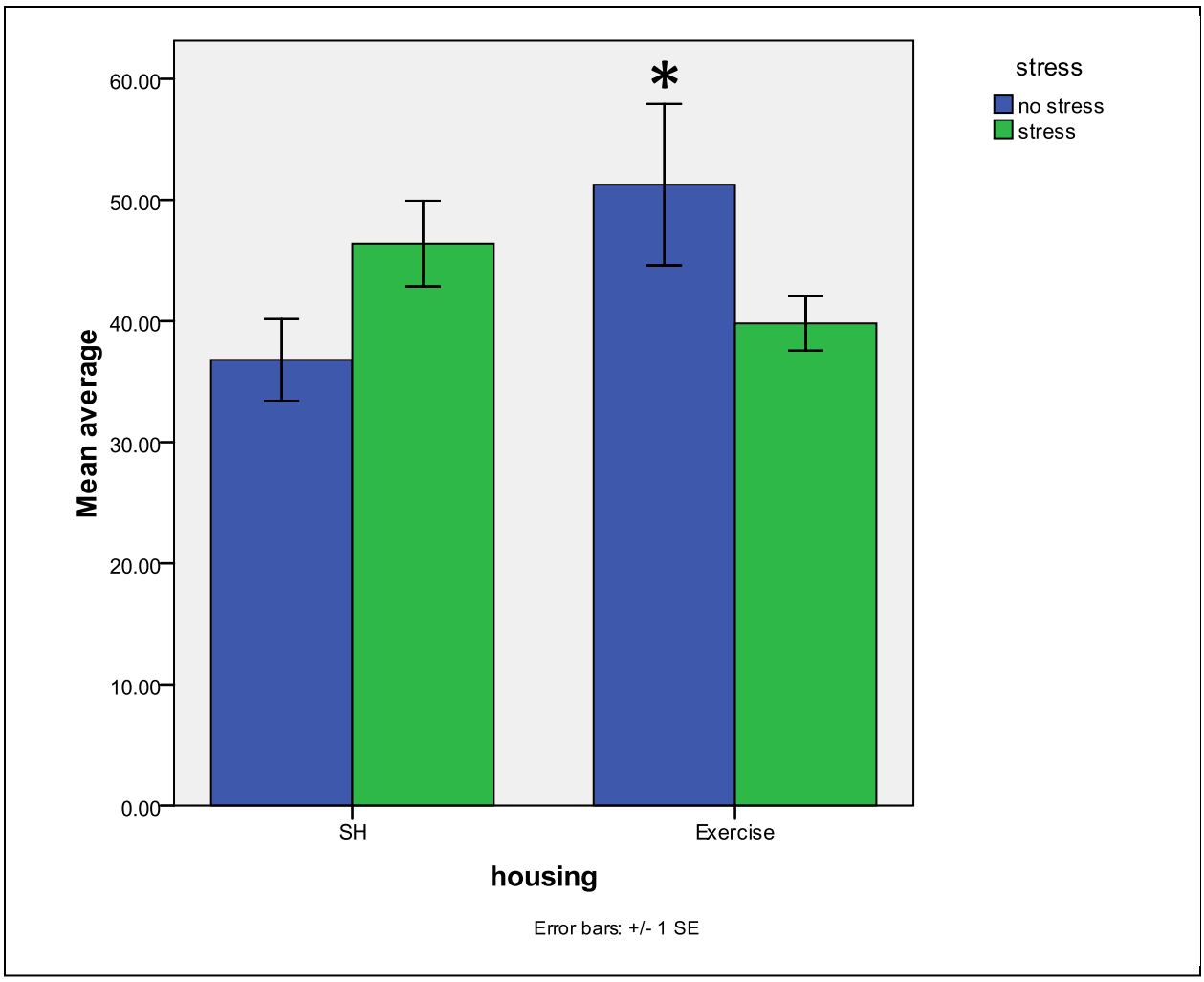

Figure 2: A bar graph showing the average number of astrocytes expressed in the CA region of the hippocampus of mice in each experiemental condition. A factorial ANOVA revealed a significant housing by stress interaction. Bars represent means ± SEM; * represents $p<0.05$ as compared to standard housing.

Expression of the Apoptotic Marker Bax in the Hippocampus

Initial observations 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).

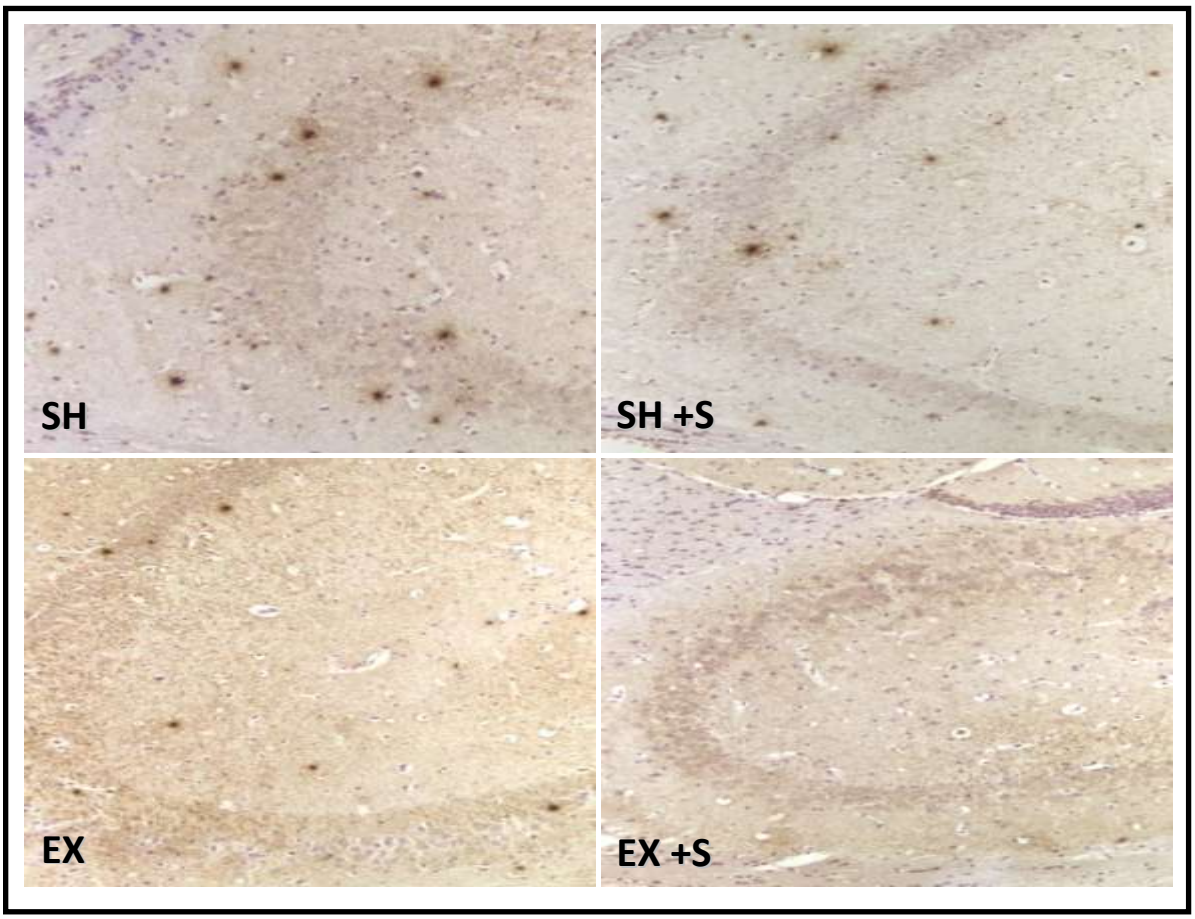

Figure 3: Low power photomicrograph (10X) of Bax labeled apoptotic nuclei in the CA fields of the hippocampus.

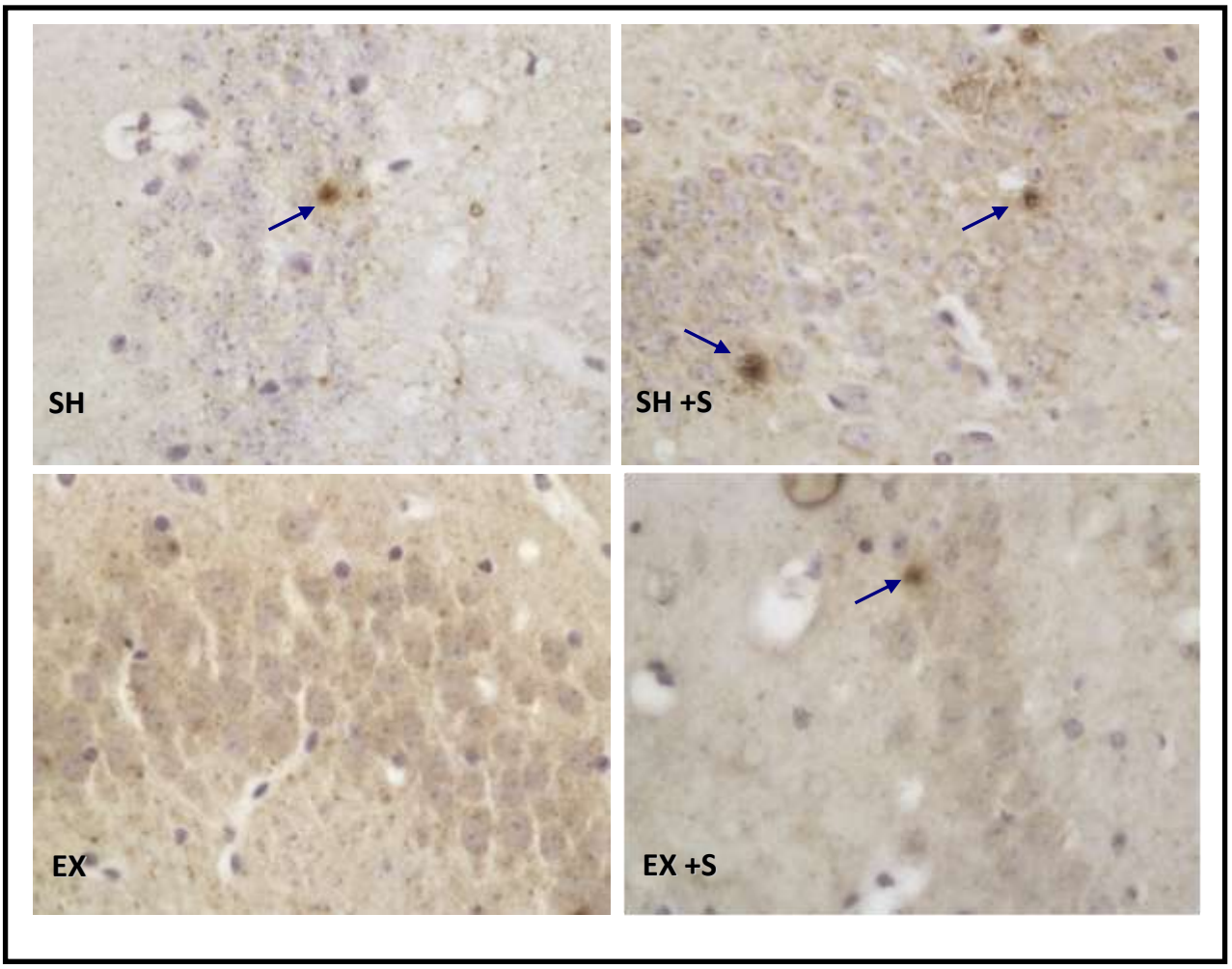

Figure 4: High power photomicrograph (40X) of Bax labeled apoptotic neurons in the CA fields of the hippocampus. Arrows point to cells that are phenotypically prominent apoptotic neurons.

Discussion

GFAP

Although previous studies indicate that stress should cause significant damage in the brain such as dendritic atrophy and spine reduction (Cotman et al., 2002), neuronal cell death (Marini et al., 2008), and morphological changes in the microtubule network (Bianchi et al., 2003), the examination of markers for cell death and damage in this experiment did not yield results that were consistent with the literature. Statistical analyses revealed that the SH condition did not significantly differ from the SH+S

condition in the number of astrocytes. We expected to find that there would be more astrocyte expression in the SH+S condition than the SH condition because astrocytes have been implicated in oxidative stress and neuronal death; their function is to release neurotrophic factors to protect the brain from assault, eliminate toxic factors, and promote tissue repair. However, they are not the only indicator of neuronal death and damage (Gao et al., 2003). There are several other markers that are crucial to fully understand the complex process of neuronal death that are necessary to further examine this hypothesis of stress causing neuronal damage.

One key factor that marks neurodegeneration that would be beneficial to examine is microglial activation. Microglia are the immune cells in the central nervous system. During cell death the microglia are transformed into phagocytic cells that remove debris from the cells (Koutsilieri et al., 2002). Activated microglia also produce factors that are crucial in the inflammatory process, such as inflammatory cytokines, that are thought to contribute to neurodegeneration (Gao et al., 2003). Activated microglia and astroglia are markers for neurodegeneration but are in themselves beneficial for brain function. They clean and protect the neurons from toxins and hazardous debris as well as destroy pathogens (Kreutzbery et al., 1996). Thus, although reactive astrogliosis, or activated astroglia, are a marker for neuron death and degeneration, it is necessary and beneficial to also examine the expression of microglia and inflammatory cytokines in order to obtain a complete representation of what is happening during chronic stress to determine if stress causes apoptosis.

The results of the GFAP staining of astrocytes indicate that there were more astrocytes in the EX condition than any other condition. This was the only finding that was statistically significant but it did not coincide with the original hypothesis. The hypothesis predicted that there would be more astrocytes in the SH+S condition than the EX+S condition. Although this finding did not coincide with the hypothesis, it can still be explained by the literature. Glial cells help transport materials from the capillaries to the nerves, and remove damaged neural tissue (Rosenzweig et al., 1972). During exercise the body is in need of increased levels of oxygen and glucose to compensate for the high demands of the body during stress. The activation of astrocytes helps transport these much needed nutrients from the blood to the brain. Since exercise requires greater metabolic support and activates glial transport for this support (Radak et al., 2001), it is not surprising that there are more astrocytes in the EX condition. Exercise also helps make the cellular repair process more efficient and helps protect against oxidative stress (Radak et al., 2000). As mentioned previously, astrocytes aid in cellular repair and rescue injured neurons (Gao et al., 2003). This function also aids in explaining the increased number of astrocytes in the EX condition because there is more need for cellular repair and protection during the

demanding process of exercise. The role of astrocytes in cellular repair can help explain the finding that exercise has been shown to help patients suffering from brain injury recover (van Pragg et al., 1999). Since the results of this study show that astrocytes are increased during exercise, they could help repair the damaged tissue in brain injury. A mentioned previously, however, astrocytes are a small portion of what happens during neuronal degradation. To fully understand the effects of exercise on stress, many other markers need to be examined.

Bax

The results for Bax were inconclusive due to the fact that the stain has not yet been optimized. This stain would have been very helpful in determining the effects of stress and exercise because it is a marker for apoptosis. Knowing which group had the most apoptotic neurons would help the overall results be more conclusive because there would be another piece to represent neuronal death instead of relying solely on reactive astrogliosis. If this stain cannot be optimized to produce consistent staining, another marker for apoptosis can be used to look for apoptotic neurons. The next step for looking at the Bax protein is to use Western Blotting where fresh tissue is used as opposed to tissue embedded in paraformaldehyde. The tissue for Western Blotting has already been gathered and this study will be ongoing. A problem with using tissue that is not fresh is that staining tissue for Bax that is embedded with paraformaldehyde is highly problematic and requires many extra procedures such as antigen retrieval and amplification. With Western Blotting there is no interference from the paraformaldehyde and thus is far less problematic. The tissue will be analyzed for Bax protein concentrations in various brain regions including the hippocampus. The Western Blotting procedure will not yield as specific results because it will look at the entire hippocampus rather than the specific regions, such as the dentate gyrus and the CA fields, during immunocytochemistry. Once the Western Blotting for Bax is complete, more data will be available to analyze the effects of exercise and stress on neurodegeneration and the implications of this data could be momentous.

Further directions

In addition to looking for other markers of apoptosis, such as reactive microgliosis, inflammatory cytokines, and Western Blotting for the Bax protein, it would be interesting to examine Bax at an earlier time after the last episode of chronic stress. Although Bax has not been studied in the chronic stress model, it has been studied in ischemia models, where there is a deficiency of oxygen and blood flow to the vital organs. An ischemia study has revealed that the Bax protein is upregulated 0.5 to 3 hours after an ischemic episode (Krajewski et al., 1995). In the current study, the brains were extracted for immunocytochemistry 24 hours after the final episode of stress. By this time, the Bax protein may have

already been downregulated and may not have been concentrated enough for detection. The next step for this study is to repeat the stress and gather the tissue at an earlier time after the final stress. As in the current study, the tissue will be stained using immunocytochemistry techniques and Western blotting.

Implications

As further evidence emerges describing how exercise helps protect the brain from various toxins and factors that increase neurodegeneration, the benefits may become known to the general public and influence people to live healthier lives. This has particular relevance for our society due to our dependence on automobiles. Automobiles have virtually replaced walking as a daily routine, except in metropolitan areas. People must specifically plan to exercise and since the benefits of exercise are not highly publicized, people may not have the motivation to force themselves to exercise. This may be fixed by increased advertisements on television and in the media that explore the benefits of exercise for the brain and not just the benefits for cardiovascular health. Our increased dependence on automobiles has other implications besides a decrease in exercise. The exhaust fumes from automobiles release toxins in the air that can cause oxidative stress. As mentioned previously, oxidative stress can cause neurons to die. This could help explain a growing number of statistics indicating that neurodegenerative disorders are on the rise.

A major implication of this study and its results showing the effects of exercise could be to influence people to raise their children to appreciate exercise. With the increase in computers, televisions, and video games, children are spending more time inside on the couch rather than playing outside and learning to build healthy lifestyle habits. Perhaps if the benefits of exercise for the brain were better known people will encourage their children to be more active and learn habits that will enable them to life a long and healthy life. Another trend that is increasing the sedentary lifestyle of children is that during times of economic struggle, when a school's budget is lowered, physical education is one of the first things to be eliminated. This required exercise may be the only time that some children are physically active. With these programs cut children have a smaller opportunity to learn that exercise is extremely important for health. If education administrators were more aware of the benefits of exercise they may encourage schools to keep required physical education in elementary and middle schools as well as encourage children to participate in physically demanding activities outside of school.

The deleterious effects of stress should also not be overlooked because as mentioned previously, stress can be very damaging to brain health. Although some stress cannot be avoided,

excessive stress is also unnecessary. The emphasis that our culture places on success adds superfluous stress that, if continued, may add to the number of incidences of neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Although it is nearly impossible to change the trends and beliefs of an entire culture, the findings that have emerged from studying the effects of exercise on neurodegeneration and neuroprotection may help protect people from the damage that is caused from constantly being under stress to succeed.

Knowledge acquired from studies such as this one may be a vital piece to aid the discovery of a cure or another way to alleviate the symptoms for many neurodegenerative diseases. It has been shown that exercise has helped reduced the risk of developing Alzheimer's Disease (Friedland et al., 2001; Laurin et al., 2001) and helped the brain recover from brain injury as well as help the prognosis of people who have already developed Parkinson's Disease (Cadet et al., 2003; Garza et al., 2004). Since exercise has been shown to help patients with these neurodegenerative diseases, it would be beneficial for physicians and the general public to know about these effects to help their patients and loved ones. With the increasing rates of these diseases it is vital to find another way to alleviate the devastating symptoms that accompany them and search for a cure to prevent the death of thousands of people a year.

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