THE EFFECTS OF β-ADRENORECEPTOR BLOCKADE DURING CHRONIC EXERCISE ON CONTEXTUAL FEAR CONDITIONING AND mRNA FOR BDNF

by

JACQUELINE DIERICKX VAN HOOMISSEN

(Under the direction of Rod K. Dishman)

ABSTRACT

Chronic exercise increases brain-derived neurotrophic factor (BDNF) messenger RNA (mRNA) in the brain and has been hypothesized as a biologically plausible explanation for the beneficial effects of exercise on brain function. The behavioral significance of the elevated levels of BDNF, however, is unknown. In addition, few studies have investigated the mechanism by which exercise leads to an increase in BDNF mRNA. Moreover, the regional specificity of the increase has not been examined. In the first experiment, we examined the regional specificity of the increase in BDNF mRNA after exercise and compared the effects of activity wheel running and antidepressant pharmacotherapy in the olfactory bulbectomy animal model of depression. Exercise or imipramine treatment alone increased BDNF mRNA in the hippocampal formation (HF) and ventral tegmental area/substania nigra regions of the brain. Exercise and imipramine combined did not potentiate BDNF mRNA expression above the levels observed with either treatment alone. These results indicate that the effect of exercise and antidepressant pharmacotherapy on BDNF mRNA is not limited to the HF and that the previously demonstrated potentiation of BDNF mRNA by exercise and pharmacotherapy may be time limited. In the second experiment, we explored the mechanism for, and the behavioral significance of, the increase in BDNF mRNA after exercise by examining the effect of activity wheel running and β-adrenoreceptor antagonism on contextual fear conditioning (CFC). Freezing behavior during the testing session of the CFC protocol was elevated above control levels in the activity wheel running animals that were treated with saline. This effect was attenuated by chronic treatment with propranolol. BDNF mRNA levels in the HF were elevated above home cage levels in all groups that underwent CFC, but were not different among the groups. These results suggest that aversively motivated learning is enhanced by chronic exercise and that CFC alters gene expression for BDNF in brain regions important in learning and memory.

INDEX WORDS: brain-derived neurotrophic factor, activity wheel running, norepinephrine, olfactory bulbectomy, contextual fear conditioning

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DEDICATION

This dissertation is dedicated to the best husband in the entire world, Mark Van Hoomissen. I would never have been able to accomplish as much as I have without his support over the past few years. I thank God that he chose to move to Georgia with me instead of staying in Oregon. He has and will always be my best friend, the love of my life, my sounding board, my counselor, my nighttime escort to the psychology building, my computer geek, my editor, my unskilled lab technician, and my handy man. May the next stage in our life together be as memorable and enjoyable as the "Athens, Georgia years."

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CHAPTER 1

INTRODUCTION

Acute and chronic physical activity influence brain function, resulting in alterations in neuronal parameters such as brain morphology (Black, Isaacs, Anderson, Alcantara, & Greenough, 1990), neuronal firing rates (Jacobs & Fornal, 1999; Rasmussen, Morilak, & Jacobs, 1986), neurotransmitter release (Soares et al., 1999), receptor level (Yoo, Tackett, Crabbe, Bunnell, & Dishman, 2000) and sensitivity (Chennoui et al., 2000), cellular metabolism (McCloskey, Adamo, & Anderson, 2001; Somani, 1996) and gene transcription (O'Neal, Van Hoomissen, Holmes, & Dishman, 2001). These changes may influence neural plasticity, which is a putative mechanism for the favorable effects of exercise on brain function and mental health (Dishman, 1997).

Maladaptations or deficits in neural plasticity have been hypothesized to be a possible cause of some neurodegenerative diseases, such as depression (Gage, 2000; Vaidya & Duman, 2001). Experimental manipulations to reverse or attenuate the changes in neural plasticity may represent potential clinical treatments for such diseases. Data from epidemiological and clinical studies have provided evidence that exercise can be effective in reducing symptoms of mild to moderate depression (Dunn, Trivedi, & O'Neal, 2001; O'Neal, Dunn, & Martinsen, 2000), but a biologically plausible mechanism by which this effect occurs has yet to be identified. Examination of the effects of exercise on neural plasticity might reveal such a mechanism.

The effects of acute and chronic physical activity on neural plasticity have not been fully examined. Some areas that have been investigated, however, include evaluation of the effects of exercise on neurogenesis (Van Praage, Christie, Sejnowski, & Gage, 1999; Van Praag, Kempermann, & Gage, 1999), neural protection (Carro, Trejo, Busiguina, & Torres-Aleman, 2001; Stummer, Weber, Tranmer, Baethmann, & Kempski, 1994), synaptic plasticity (Van Praag, Christie, et al., 1999), and structural changes within the brain (Anderson, Alcantara, & Greenough, 1996; Black et al., 1990; Kleim et al., 1998). Despite advances in the understanding of the influences of exercise on brain function, many questions remain to be addressed.

Neurotrophic factors represent a plausible mechanism by which exercise influences neural plasticity, given their role in cellular differentiation, development, and synaptic plasticity. Exercise has been shown to influence the level of gene expression and protein for neurotrophic factors in various brain regions including the hippocampal formation (HF), neocortex, and cerebellum (Carro, Nunez, Busiguina, & Torres-Aleman, 2000; Neeper, Gomez-Pinilla, Choi, & Cotman, 1995; Neeper, Gomez-Pinilla, Choi, & Cotman, 1996; Russo-Neustadt, Beard, Huang, & Cotman, 2000). The exercise-induced increase in neurotrophic factors in the brain has been hypothesized as a biological mechanism for the mental health benefits of physical activity (Cotman, & Engeser-Cesar, 2002), but no studies have examined the neurotrophic response to chronic exercise in an animal model of depression. The purpose of the first experiment, therefore, was to compare the effects of chronic activity wheel running and antidepressant pharmacotherapy on neurotrophic gene expression in multiple brain regions in an animal model of depression.

Mechanisms to explain how chronic exercise might increase expression of neurotophic factors have been understudied, but may involve the central noradrenergic neurotransmitter system. Evidence supporting a norepinephrine (NE)-mediated hypothesis include: (1) the anatomical relationship between the NE system and brain regions that express high levels of neurotrophic factors, such as the HF; (2) the reciprocal influential relationship between neurotrophic factors and the noradrenergic system; and (3) the direct evidence supporting the influence of acute and chronic physical activity on the NE system. The second study investigated whether the changes in neurotrophic factor mRNA in the HF could be attenuated by chronic antagonism of NE receptors.

The behavioral significance of the exercise-induced upregulation of BDNF mRNA in the HF is unknown. Of the studies conducted to date, few have concomitantly examined changes in brain function and behavior (Russo-Neustadt, Ha, Ramirez, & Kesslak, 2001). The second study examined whether the change in neurotrophic factor gene expression in the HF after chronic exercise was associated with changes in behavior. The HF is important in learning and memory (Maren, 2001) as well as the regulation of the hypothalamo-pituitary-adrenocortical (HPA)-axis response to stress (Herman, Prewitt, & Cullinan, 1996). Therefore, we examined the effect of chronic activity wheel running on these behaviors using contextual fear conditioning (CFC). CFC is a Pavlovian form of learning that has been hypothesized to involve the activity of the HF (Fanselow, 2000) and results in the activation of the HPA-axis (Cordero, Merino, & Sandi, 1998).

We hypothesized that chronic physical activity would enhance behavioral expression of CFC, and that chronic blockade of the NE system with a β-adrenoreceptor

antagonist (propranolol) during the exercise period would attenuate this behavioral effect. We also hypothesized that the increase in behavioral expression of CFC would be associated with an increased expression of BDNF mRNA.

CHAPTER 2

REVIEW OF LITERATURE

Exercise and Neural Plasticity

Introduction

Neural plasticity is defined as changes in neuronal properties and patterns of connections that lead to alterations in behavior (Levitan & Kaczmarek, 1997). The brain has the potential for structural plasticity as well, which is reflected in processes such as the creation of new neurons and the death of existing neurons (Gage, 2000). Although neural plasticity is generally defined as structural changes in the brain that occur in response to external stimuli (Kempermann, Van Praag, & Gage, 2000), it is understood that external stimuli influence neural plasticity through changes in internal neural activities, such as changes in afferent neural activity or changes in gene transcription. Because of the continuum between external and internal events, the effects of experimental manipulations on neural plasticity can be examined at various genetic, biochemical, or structural levels, with the ultimate goal of demonstrating a change in behavior that results from, or is associated with, alterations in neural plasticity.

Changes in neural or structural plasticity can be manifested at various levels of brain analyses, such as the morphological characteristics of a neuron (e.g. synapse number, dendritic length), the level of cellular division (neurogenesis) and differentiation, and the level of cellular communication. One form of neural plasticity is long-term changes in synaptic communication, or simply synaptic plasticity. Long-term potentiation (LTP) is a well-studied example of synaptic plasticity. In one common

experimental protocol, potentiated excitatory post-synaptic potentials in response to presynaptic stimulation can be induced by a train of high-frequency stimuli applied to presynaptic neurons. This phenomenon is most often studied in the hippocampal formation (HF), where LTP can be induced in the pyramidal neurons of Ammon's Horn area 3 (CA3) and Ammon's Horn area 1 (CA1) after stimulation of the mossy fiber pathway or the Shaffer collaterals, respectively.

Neurogenesis is a second form of neural plasticity that occurs in rodents (Altman & Das, 1965), non-human primates (Gould, Tanapat, McEwen, Flugge, & Fuchs, 1998), and humans (Eriksson et al., 1998) in specific brain regions that include the olfactory epithelium (Calof, Hagiwara, Holcomb, Mumm, & Shou, 1996), HF (Cameron, Woolley, McEwen, & Gould, 1993), and the subventricular zone of the lateral ventricles (Alvarez-Buylla, Herrera, & Wichterie, 2000; Privat & Leblond, 1972). Progenitor cells in these regions retain the capacity for mitotic cell division into adulthood, resulting in daughter cells that can differentiate into various cell types including neurons and glia. These cells are visualized by injecting animals, *in vivo,* with bromodeoxyuridine (BrdU), a thymidine analog that is incorporated into the nascent DNA strand during replication. Survival of the daughter cells is assessed by staining for BrdU after termination of injections. Analysis of the cell phenotypes is accomplished by co-staining for BrdU and other cell-type specific markers, such as NeuN, TuJ1, or MAP-2 to recognize neurons and S100b, GFAP, GalC, or PLP to recognize glial cells.

The structure of the brain is altered in response to experimental stimuli. Some methods used to investigate these changes include examination of the level of angiogenesis, the number of dendrites and axons per neuron, the level of axonal and

dendritic branching, the length of the dendrites and axons, and the number of synapses per neuron (synaptogenesis).

Neurogenesis

Experience and activity, such as the addition of an activity-wheel to an animal's cage or placement of an animal in an enriched environment, can induce changes in structural plasticity (Kempermann et al., 2000; Kolb & Whishaw, 1998). Although some cell generation occurs under baseline conditions in the central nervous system (Bayer, Yackel, & Puri, 1982), these experimental paradigms have been shown to alter neurogenesis and nascent cell survival (Gould, Beylin, Tanapat, Reeves, & Shors, 1999). Chronic activity wheel running and treadmill training are associated with an increase in cell proliferation in the HF (Trejo, Carro, & Torres-Aleman, 2001; Van Praag, Kempermann, & Gage, 1999). The level of neurogenesis observed after activity wheel running is greater than that observed after enriched environment living, swimming, learning, and control conditions. However, both wheel running and enriched living lead to similar levels of absolute cell survival, with approximately 88% of the new cells exhibiting neuronal phenotypes. The increase in neurogenesis that occurs with exercise may be beneficial in situations in which neural degeneration occurs, such as after chronic stress (Sapolsky, 2000).

Neural Protection

Any treatment that attenuates or blocks the negative impact of various forms of brain insult can be considered neuroprotective. Exercise is a beneficial prophylactic neural protectant for some forms of brain insult and damage in animal models. For example, access to running wheels for two weeks prior to 15 or 20 minutes of cerebral

ischemia decreases the mortality rate (Stummer, Weber, Tranmer, Baethmann, & Kempski, 1994). Whereas only 44% and 21% of the control animals in this experiment survived the 15 or 20 minute ischemia, respectively, 90% and 100% of the activity wheel running animals survived. Further, cerebral ischemia was associated with a decrease in neuronal density in the cortex, striatum, and HF, but prior exercise attenuated this effect. The most substantial effects occurred in CA3 region of the HF and in the dorsolateral striatum, where 50% of the neurons in the running group survived compared to only 10% survival in the non-runners. In the outermost layers of the parietal and frontoparietal cortex, neuronal density was not different between running animals and sham control animals.

The neural protective effect of chronic exercise is associated with an attenuation of neurotoxin-induced behavioral deficits. For example, Carro, Trejo, Busiguina, and Torres-Aleman (2001) reported improved motor coordination, spatial memory, and memory retention after neurotoxin injection in animals who received prior treadmill exercise training compared to sedentary control animals. However, treadmill training *after* neurotoxin injection resulted in a less substantial, gradual increase in motor coordination.

Structural Plasticity

In addition to neural protection, locomotory activity alters morphological characteristics of the brain, although relatively little data are available to address this topic. The changes observed after forced (treadmill walking) or voluntary (activity wheel running) exercise are distinct from those observed after skilled motor learning. For example, activity wheel running increases capillary density within the paramedian lobule

of the cerebellum, whereas skilled motor learning (e.g. balance beams, see-saws, rope bridges) increases the number of synapses per purkinjie cell (Anderson, Alcantara, & Greenough, 1996; Black, Isaacs, Anderson, Alcantara, & Greenough, 1990; Kleim et al.,1998). These data address the differential effects of motor behaviors on structural plasticity, with those skills that require a substantial learning component inducing different effects in the brain compared to those skills which require more repetitive movement.

Synaptic Plasticity

In addition to altering structural plasticity, chronic physical activity influences synaptic plasticity, defined as changes in communication between neurons at the level of the synapse. One well-studied example of synaptic plasticity is LTP, or the increased efficacy of synaptic communication after previous high-frequency stimulation of afferent fibers. Chronic wheel running alters the magnitude of LTP in the dentate gyrus of the HF, resulting in potentiated excitatory post-synaptic potentials in granule cell neurons after LTP induction compared to sedentary animals (Van Praag, Christie, Sejnowski, & Gage, 1999). These results have implications for the study of the effects of exercise on learning and memory, as LTP is a classic synaptic model of memory and studies show that chronic exercise improves performance in behavioral measures of learning and memory, such as the Morris Water Maze (Van Praage, Christie, et al., 1999) and radial arm maze (Anderson et al., 2000).

<u>Exercise and Neural Plasticity Mechanisms: Neurotrophic Factors</u>

The mechanisms to explain experience-induced changes in neural plasticity are unknown, but the actions of neurotrophic factors provide a plausible explanation.

Neurotrophic factors are families of proteins with a variety of functions throughout the nervous system during development and adulthood. The name "neurotrophic factor" describes a major function of these proteins, which is to nourish ("troph") nerves ("neuro"). One of the most widely recognized functions is facilitation of neuronal innervation of appropriate target cells during development. Neurotrophic factors are released from post-synaptic cells and diffuse across the synaptic cleft, where they bind to pre-synaptic membrane receptors. The binding and subsequent internalization of the neurotrophic factor and receptor activates a cascade of events that promotes neuronal survival and differentiation (Connor & Dragunow, 1998). The actions of neurotrophic factors are not confined to the developmental period, however, as these factors have additional functions in the adult nervous system, such as modulating synaptic efficacy and neurotransmission (Schuman, 1999).

Nerve growth factor (NGF) was the first neurotrophic factor isolated (Levi-Montalcini, 1987). This discovery led to subsequent discoveries of proteins with similar functions as NGF. These proteins share considerable homology with NGF, and collectively they have been named the NGF family of neurotrophic factors, which includes NGF, brain-derived neurotrophic factor (BDNF), neurotensin-3 (nt-3), and neurotensin-4/5 (nt-4/5).

Brain-Derived Neurotrophic Factor (BDNF)

BDNF was the second member of the NGF family to be purified and cloned (Barde, Edgar, & Thonen, 1982). The mature form of BDNF is translated from mRNA transcribed from the 40 kb gene. The BDNF gene contains 5 exons, regulated by 4 separate promoters and 2 different polyadenylation sites. This results in 8 different

BDNF mRNA transcripts, each containing exon V, the protein-coding region of the transcript (Timmusk et al., 1993). Like other neurotrophic factors, BDNF is transported through retrograde (Mufson, Kroin, Sendera, & Sobreviela, 1999) and anterograde mechanisms (Kohara, Kitamura, Morishima, & Tsumoto, 2001), although the anterograde mechanism has not been confirmed *in vivo*. The anterograde transport of these factors suggests that neurotrophic factors may be co-secreted with neurotransmitters in an activity-dependent fashion, thereby influencing neurotransmission.

Receptors for the NGF family include the high affinity tyrosine kinase receptor (trk A, B, and C receptors, pronounced "track" receptor) and the low affinity p75 receptor. The different neurotrophic factors bind with varying levels of affinity to the trk receptors; NGF binds primarily to trk A, BDNF to trk B, nt-3 to trk C, and nt-4/5 to trk B. Neurotrophic factor binding to the trk receptor induces dimerization of the receptor, resulting in activation of intracellular tyrosine kinase activity, leading to phosphorylation of tyrosine residues on cellular proteins (Barbacid, 1995; Bothwell, 1995; Chao & Hempstead, 1995).

BDNF influences numerous functions in the central nervous system, including cellular differentiation and survival (Ip, Yancopoulos, & Lindsay, 1993; Labelle & Leclerc, 2000), cellular communication (Kang & Schuman, 1995; Korte et al., 1995; Messaoudi et al., 1998; Schuman, 1999), monoaminergic activity (Altar et al., 1994; Martin-Iverson, Todd, & Altar, 1994; Siuciak, Boylan, Fritsche, Altar, & Lindsay, 1996) and neurogenesis (Pencea, Bingaman, Weigand, & Luskin, 2001). The importance of BDNF in normal organism growth, development, and survival is emphasized by the early

postnatal mortality of homozygote BDNF knock out mice, generally before postnatal day 2 (Jones, Farinas, Backus, & Reichardt, 1994).

Levels of BDNF vary throughout the neuroaxis, with high to moderate levels of BDNF mRNA present in the telencephalic regions (piriform cortex, neocortex, calustrum, HF, and amygdala) and lower levels found in the diencephalon, mesencephalon, metencephalon, mylencephalon, and spinal cord. Immunohistochemical studies using antibodies against BDNF show high levels of BNDF-like immunoreactivity in cortical regions (e.g. neocortex, piriform cortex, HF, basal forebrain, amygdala) and the inferior olive, and moderate levels of expression in other brain regions (cingulate cortex, striatum, septal area, thalamus, hypothalamus, midbrain, brainstem, cerebellum) (Friedman, Black, & Kaplan, 1998; Yan et al., 1997).

The level of BDNF expression is influenced by many different types of manipulations. BDNF gene expression in rats exhibits a diurnal regulation, with the highest levels detected during the dark-cycle, when rats are normally the most active. This suggests that BDNF gene expression is sensitive to changes in behavior and interactions with the environment (Berchtold, Oliff, Isackson, & Cotman, 1999; Liang, Walline, & Earnest, 1998). In addition, BDNF expression is regulated by various neurotransmitters (Gwag & Springer, 1993; Hutter, Johansson, Saria & Humpel, 1996; Zetterstrom et al., 1999), as well as pharmacological agents, such as antidepressant medications, which influence endogenous neurotransmitter system function (Nibuya, Morinobu, & Duman, 1995).

One of the transcription factors that regulate gene expression of BDNF is cyclic-AMP (cAMP) response element binding protein (CREB) (Shaywitz, & Greenberg, 1999;

Sheng, McFadden, & Greenberg, 1990). *In vitro* studies have identified a calcium/cAMP response element upstream of exon III in the BDNF gene that is recognized by CREB and plays an important role in the activity-dependent regulation of BDNF gene expression (Shaywitz, & Greenberg; 1998; Shieh, Hu, Bobb, Timmusk, & Gosh, 1998; Tao, Finkbeiner, Arnold, Shaywitz, & Greenburg, 1999). Therefore, CREB, is a potential downstream target of exogenous or endogenous stimuli, which influence cAMP activity and regulate BDNF gene expression.

The effects of BDNF at the cellular level may help explain the role of this neurotrophic factor in mediating aspects of behavior, including tests of learning and memory. Different types of behavioral learning paradigms, such as radial arm maze training, contextual learning, and avoidance learning, have been shown to increase BDNF mRNA and protein in the HF (Hall, Thomas, Everitt, 2000; Ma, Wang, Wu, Wei, & Lee, 1998; Mizuno, Yamada, Olariu, Nawa, & Nabeshima, 2000). In addition, performance in the radial arm maze, morris water maze, and inhibitory avoidance tests are impaired by either antisense oligoneucleotide injections or antibodies directed towards BDNF, suggesting a role for BDNF in learning and memory (Mizuno et al., 2000; Mu, Li, Yao, Zhou, 1999). The mechanism by which BDNF influences learning and memory may occur through its influence on LTP, as mice deficient in BDNF have impaired LTP, which is restored with viral-mediated transfer of the BDNF gene into the HF (Korte et al., 1996).

Exercise and Neurotrophic Factors

Physical activity influences BDNF gene expression. The first published study on the effects of exercise on neurotrophins within the central nervous system reported that

short-term (2 to 7 days) activity wheel running increased BDNF mRNA levels in the HF and caudal neocortex (Neeper, Gomez-Pinilla, Choi, & Cotman, 1995). This study led to additional investigations confirming the increase in BDNF mRNA after activity wheel running and treadmill training (Carro, Nunez, Busiguina, & Torres-Aleman, 2000).

The brain regions most often quantified, with regard to the effect of exercise on neurotrophins, are the cerebral cortex and the HF. Neeper, Gomez-Pinilla, Choi, & Cotman (1996) reported significant elevations (90% increase) of BDNF mRNA after activity wheel running in the caudal neocortex, the retrosplenial cortex, and CA1 region of the HF. Smaller changes (40% increase) were reported in CA4, the frontal cortex , the caudal neocortex (posterior to the thalamus) and the cerebellum.

The duration of physical activity affects BDNF mRNA expression, although the effects of an acute bout of exercise (hours) are mixed. Two days to six weeks of activity wheel running increases BDNF mRNA in the HF (Neeper et al., 1996; Widenfalk, Olson, & Thoren, 1999). However, when 3 days of wheel running are followed by a 10-day sedentary period and a subsequent reinstatement of wheel running, 6 hours, but not 12 hours of additional activity increases BDNF mRNA 30-56% in the HF compared to sedentary control animals (Oliff, Berchtold, Isackson, & Cotman, 1998). Interestingly, when activity wheels are taken away from running animals previously engaged in long term activity (6 weeks), BDNF mRNA in some regions of the HF decreases, suggesting that the locomotion may directly affect the increase in expression (Windenfalk et al., 1999).

The magnitude of the effect of physical activity on BDNF mRNA expression is transcript dependent. Oliff et al., (1998) examined specific exon-containing transcripts

after 6 hours of activity wheel running and reported significant elevations in exon I mRNA in the dentate gyrus (277%), hilus (133%), and CA3 (145%) regions of the HF, but exon IV mRNA was either slightly reduced or not affected in these areas. Longer exposure to the activity wheel (12 hours) significantly increased exon I mRNA in the dentate gyrus (680%), hilus (86%), and CA3 (135%) regions and exon II mRNA in CA1 (160%) region. The significance of the transcript-specific increase in BDNF after short-term exercise has not been determined, but the different transcripts may contain internal sequences that regulate the level of mRNA translation and/or degradation, thereby ultimately affecting the level of BDNF protein.

Although few studies have examined the influence of physical activity on gene expression, significant correlations between running distance and BDNF mRNA have been reported. For example, Neeper et al., (1995) reported a significant relationship between mean distance run per night and BDNF mRNA labeling in the HF (Pearson's r=0.967, 2 nights of activity wheel running; r=0.892 7 nights of activity wheel running). The small number of animals employed in this study (n=4/group) precludes a definite conclusion. Nonetheless, a subsequent study showed a significant correlation between the total running distance and BDNF mRNA expression in the HF using a larger sample size (n = 7-8/group; CA1, r = 0.894; CA3, r = 0.704; hillus, r = 0.866) (Oliff et al., 1998).

As with BDNF mRNA, chronic physical activity influences gene expression for other neurotrophins. NGF mRNA is increased significantly in the dentate gyrus and CA4 regions of the HF (approx. 20-30%), and in layers II and III of the caudal neocortex (approx. 60%), but not in the frontal or middle neocortex after 7-days of activity wheel

running (Neeper et al., 1995). In addition, fibroblast growth factor-2 mRNA and protein levels increase transiently in the HF, peaking after 4 days of running and subsequently returning to control levels with continued exercise (Gomez-Pinilla, Dao, & So, 1997).

Aside from examining the effects of exercise on neurotrophic gene expression, it is of interest to compare the combined effects of exercise and known antidepressant interventions. Both types of treatments decrease signs and symptoms of depression and may have a common or similar mechanism of action within the central nervous system. Pharmacotherapy and exercise have been shown to have similar effects on the monoamine neurotransmitter systems, such as the downregulation of β-receptor density (Yoo, Tackett, Crabbe, Bunnell, & Dishman, 2000), but few studies to date have examined the effects of exercise and drug treatment on neurotrophic factors. Chronic, but not acute, administration of antidepressant medications and other types of antidepressant treatment, such as ECT, increase BDNF mRNA levels in the HF (Nibuya et al., 1995). These changes may represent a common mechanism of action for various antidepressant medications that results in adaptive neuroprotective and neuroplasticity changes in the brain (Duman, 1998).

Studies that have examined the effect of exercise and drug treatment on neurotrophic factor gene expression report that exercise potentiates the effect of pharmacotherapy in a transcript-specific manner (Russo-Neustadt, Beard, Huang, & Cotman, 2000). For example, Russo-Neustadt, Beard, and Cotman (1999) reported that the administration of tranylcypromine or activity wheel running alone increased BDNF mRNA in the HF, but the combined treatment of medication and physical activity potentiated the effect. Imipramine administration, however, did not significantly elevate

BDNF mRNA expression, whereas the combination of imipramine and activity wheel running did. Because the effects of antidepressant treatment and exercise appear to be additive, the mechanism by which the potentiation occurs may involve either the maximization of a common pathway that is not fully altered by single treatment alone, or the combined effect of dissimilar pathways that converge at the level of BDNF gene transcription.

In addition to physical activity, neurotrophic factors are responsive to stressful situations. Studies have shown that laboratory stressors, such as immobilization and swim stress, downregulate the level of BNDF mRNA in the HF. This effect, however, is reversed by chronic antidepressant pharmacotherapy (Nibuya, et al., 1995; Russo-Neustadt, Ha, Ramirez, & Kesslak, 2001). Chronic exercise influences an animal's stress response during presentation of a heterotypic and homotypic stressor (Dishman et al., 1998; White-Welkely et al., 1996; White-Welkley, Bunnell, Mougey, Meyerhoff, & Dishman, 1995), but there are limited data available which have examined the interaction of exercise and antidepressant pharmacotherapy on the stress response in the brain (Dishman, Renner, White-Welkley, Burke, & Bunnell, 2000).

Only one study has examined the interaction of exercise and drug treatment on neurotrophic gene expression after acute stress (Russo-Neustadt et al., 2001). Exercise or antidepressant drug treatment alone was shown to protect against the stress-induced downregulation of BDNF mRNA in the HF observed after the Porsolt forced swim test. The combination of exercise and drug, however, elevated BDNF mRNA above control levels in the HF after stress. The relevance of these results for behavior is difficult to determine because the attenuation of BDNF in both the drug and exercise-alone

conditions was not associated with any change in swimming behavior during the test interval. The combination of exercise and drug, however, significantly elevated the time spent swimming. Although we can not conclude from this study that the increase in BDNF mRNA due to the combined treatments caused the change in behavior, it should be noted that midbrain infusions of human BDNF in the rat attenuates escape latency in the learned helplessness model of depression and reduces immobility time in the forced swim test (Siuciak, Lewis, Wiegand, & Lindsay, 1997).

The significance of the increase in neurotrophic gene expression after chronic exercise remains a primary topic of interest. Given the numerous functions of neurotrophins throughout the central nervous system, the exercise-induced upregluation of neurotrophic factors may impact a wide variety of functions including processes associated with neural and synaptic plasticity. Alterations in plasticity are hypothesized to be important factors in the etiology or therapeutic treatment for some neurological and neurodegenerative diseases, such as depression. Given the effects of exercise on neural plasticity, chronic exercise may be an effective method for treatment of these diseases.

Neural Plasticity and Depression

Alterations in aspects of structural plasticity have been associated with depression and are hypothesized to be a possible cause or correlate of this disease (Gage, 2000). Brain imaging studies in humans have reported structural changes in regions comprising the limbic-cortical-striatal-pallidal-thalamic tract, but volume loss in the HF remains the most commonly observed alteration (Sheline, 2000). For example, Sheline, Wang, Gado, Csernansky, and Vannier (1996), reported a decrease in hippocampal volume in depressed subjects, the magnitude of which was predicted by depression duration but not

age (Sheline, Shaghavi, Mintum, & Gado, 1999). In addition, studies have reported decreased cortical thickness, and neuronal size and density in the orbito-frontal region of depressed individuals (Rajkowska et al., 1999), suggesting that depression is associated with morphological changes in the brain

Although the mechanisms underlying these changes are unknown, possible causes include changes in neurodegeneration or neurogenesis, which may, in turn, be due to the stress-induced reduction in neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), or to repeated exposure to hypercortisolemia, which has been shown to result in the destruction of HF neurons (Sapolsky, 2000). Evidence from animal and clinical studies provides preliminary support for associations among neural plasticity, neurotrophic factors, stress, and depression.

Studies examining the relationship between neural or structural plasticity and depression have primarily utilized pharmacological models to examine the influence of antidepressant and non-antidepressant treatments on aspects of plasticity. Neurotrophic factors and intracellular signaling cascade proteins, such as CREB, may represent a potential common target for the therapeutic action of antidepressant medications (Duman, 1998; Altar, 1999). Chronic but not acute treatment with antidepressant medications, including tricyclics, SSRIs, and atypical medications, have been shown to increase gene expression for BDNF in the HF, whereas non-antidepressant medications such as morphine, cocaine and haloperidol seem to have no effect (Nibuya et al., 1995). Other types of antidepressant treatments, including repetitive transcranial magnetic stimulation (Muller, Toschi, Kresse, Post, & Keck, 2000) and electroconvulsive seizure (Nibuya et al., 1995), also increase BDNF mRNA in the HF. In addition to gene expression,

antidepressant medications have been reported to increase neurogenesis in the HF, an effect that occurs with chronic, but not acute, treatment (Malberg, Eisch, Nestler, & Duman, 2000)

Stress is associated with depression, and animal studies have provided evidence of the effects of a chronic elevation in gluccocorticoids on brain function, which may have deleterious consequences on mental health. Antidepressant treatments, however, prevent some of these changes. BDNF gene expression is stress-responsive, with acute and chronic stress resulting in differential effects on mRNA levels, depending upon the region of interest (Smith, Makino, Kim, & Kvetnansky, 1995). In the HF, stressors, such as immobilization, decrease BDNF mRNA (Ueyama, Kawai, Nemoto, Sekimoto, Tone, & Senba, 1997). This effect of stress, however, can be blocked by chronic pretreatment with antidepressant medication (Nibuya et al., 1995). These results may have implications for moderating some of the deleterious effects of stress on the HF. It is of interest, to determine if other effective treatments for depression, such as physical activity, may have similar effects.

Further investigation of the relationship between neurotrophic factors and depression has focused on the influence of BDNF on antidepressant-like behaviors in animals. Chronic administration of exogenous BDNF attenuates the escape latency in the learned helpless model of depression and decreases immobility in the forced swim test (Suiciak et al., 1997).

The role of neurotrophic factors in human depression is unknown, given that few studies have examined post-mortem brain tissue from depressed subjects and currently there are no available antidepressant medications that specifically target regulation of

BDNF in the central nervous system. However, the first clinical study to examine changes in post-mortem BDNF concentrations reported an increase in the level of BDNF in the brains of psychiatric subjects treated with antidepressant medications compared to other non-treated psychiatric patients. When the subjects were examined as separate groups (major depression, bipolar disorder, schizophrenia), antidepressant treatments appeared to increase BDNF levels only in the subjects with major depression, although the results were not statistically significant (Chen, Dowlatshahi, MacQueen, Wang, & Young, 2001).

Limitations of Current Research

Given the potential relationships among neural plasticity, depression, and the role of exercise in influencing neural plasticity and depression, comparison of the effects of known antidepressant medications and exercise on gene expression for neurotrophic factors and transcription factors that regulate neurotrophic factor expression, is an important research focus. There are many limitations to the present area of investigation that warrant additional experimental studies. The following paragraphs summarize these limitations.

Transcriptional Regulators of BDNF

The first limitation is the lack of data examining intracellular signaling cascades and transcriptional regulators of the BDNF gene, which may have therapeutic or regulatory effects. As previously mentioned, the transcription factor, CREB, binds to *cis*-elements upstream of the BDNF gene and acts as a potential regulator of BDNF gene transcription. The influence of CREB on gene transcription plays an important role in mediating changes in cellular function induced by extracellular signals. Intracellular

signaling cascades activate second messengers, such as cAMP or Ca^{+2}, and ultimately affect gene transcription through the interaction with protein kinases, which activate/inactivate transcription factors. The cAMP second-messenger cascade and its downstream target, CREB, are implicated as an intracellular mediator of the action and therapeutic effects of antidepressant medications (Duman, Heninger, & Nestler, 1997).

The role for intracellular signaling cascades and transcription factors (i.e. CREB) in mental health has been examined using animal models and human post-mortem tissue analysis. Preliminary evidence to support the role of intracellular signaling cascades in the etiology and treatment of depression has shown that chronic, but not acute, treatment with antidepressant medications alters the level or activity of components of the cAMP pathway. *In vitro* and *in vivo* studies have shown that antidepressant medications increase the availability of G proteins (Toki, Donati, & Rasenick, 1999), alter the level cAMP and metabolizing enzymes of cAMP (Shimizu, Nishida, Fukuda, Saito, & Yamawaki, 1995; Takahasi et al., 1999), increase binding of cAMP to protein kinase A (Mori et al., 1998), and increase the expression and phosphorylation of CREB (Nibuya, Nestler, & Duman, 1996; Thome et al., 2000). In addition, viral-mediated injections of CREB into the dentate gyrus improve performance in two animal models of depression (Chen, Shirayama, Shin, Neve, & Duman, 2001).

Post-mortem tissue analysis from the brains of depressed humans has provided additional information regarding the role of the cAMP pathway in the etiology and treatment of depression. These results indicate that adenylate cyclase activity and CREB levels are reduced in suicide victims (Cowburn, Marcusson, Eriksson, Weihager, & O'Neil, 1994; Dowlatshahi, MacQueen Wang, Reiach, & Young, 1999; Reiach, Li,

Warsh, Kish, & Young, 1999). Further, it was found that protein kinase A is influenced by antidepressant drugs (Lowther, Katona, Crompton, & Horton, 1997) and that CREB levels are reduced in unmedicated depressed subjects compared to control, but elevated in subjects treated with antidepressant medications (Dowlatshahi, MacQueen, Wang, & Young, 1998).

Lack of Use of Animal Models of Disease

A second limiting factor in the present area of research is the lack of use of animal models of depression. To study how treatments for depression, such as exercise, affect neurobiology and behavior an animal model of the disease is needed. It has previously been shown that changes in behavior in response to exercise may only occur in an animal model of depression, and not in control animals (Yoo et al., 2000; O'Neal, Van Hoomissen, Holmes, & Dishman, 2001). Therefore, data obtained may not extend to the animal model of depression used or have any practical implications in the clinical illness.

At the present time, the influence of exercise or antidepressant pharmacotherapy on BDNF levels in an animal model of depression is unknown. To our knowledge, only one study exists that has examined the effect of exercise on BDNF mRNA in an animal model of depression (Russo-Neustadt et al., 2001). The type of animal model used in this study, however (Porsolt swim test), is a behavioral animal model of depression that lacks construct validity and may be useful only in the prediction of effective antidepressant treatments, but not in the study of the neurobiological aspects of depression. This model may not provide an animal model of depression that mimics some of the endogenous neurobiological and behavioral aspects of depression. Therefore, without the use of an endogenous animal model of depression, it is not possible to determine the effects of

exercise on BDNF mRNA in an animal that has "depressive-like" behavioral and neurobiological features. Only one study has addressed the endogenous level of BDNF in an animal model of depression, Flinders Sensitive Line, and reported significant regional differences in the hypothalamus and occipital and frontal corticies (Angelucci, Aloe, Vasquez, & Mathe, 2000).

In the first study, we were interested in comparing the effects of exercise and antidepressant drug treatment on BDNF mRNA in the olfactory bulbectomy (OBX) animal model of depression. OBX leads to biochemical and behavioral effects that resemble depression and are reversed by chronic but not acute antidepressant treatment (Kelly, Wrynn, & Leonard, 1997). Some of the neurobiological alterations that occur after OBX that resemble observations in depressed humans include changes in brain structure (Wrynn et al., 2000), reduced levels of monoamines (Cairncross, Schofield, & Bassett, 1975; Redmond, Kelly, & Leonard, 1997), and elevated 5-HT2 (Early, Glenon, Lally, Leonard, & Junien, 1994), α- (Hong, Lee, & Rhim, 1987) and β-adrenergic receptor density (Richardson, & Tiong, 1999). In addition, OBX animals show behavioral and physiological similarities to depression, including reduced appetitively-motivated behavior (Lumia, Teicher, Salchi, Ayers, & Possidente, 1992), altered circadian rhythms (Lumia et al., 1992), dysregulated hypothalamic-pituitary-adrenocorticoid axis activity (Marcilhac, Anglad, Hery, & Siad, 1999; Marcilhac, Faudon, Genevieve, Hery, & Siaud, 1999), elevated locomotor activity (Redmond et al., 1997), and learning deficits (Hall, & Macrides, 1983; Van Rijzingen, Gispen, & Spruijt, 1995).

Lack of Examination of the Regional Specificity

A third limitation is the lack of investigation in additional brain regions that are involved in the regulation of emotion. In the first study we investigated gene expression in two main pathways; a cortical pathway (piriform cortex, HF) and the mesolimbic pathway (ventral tegmental area/substantia nigra, nucleus accumbens) in order to assess the effects of OBX and exercise. These pathways were chosen based on the idea that OBX could alter the level of BDNF mRNA in these areas as the result of (1) direct destruction of reciprocal connections between these areas and the olfactory bulbs, or (2) as a response to altered activity of neurotransmission from collateral axons of neurons projecting to these areas as well as to the olfactory bulbs. In addition, these regions were chosen because they have been shown to be responsive to either olfactory bulbectomy (Garris, Chamberin, & DaVanzo, 1984; Holmes, Davis, Masini, & Primeaux, 1998) and/or exercise (Emerson, Kappenmann, Ronan, Renner, & Summers, 2000; Tumer et al., 2001; Van Hoomissen, O'Neal, Holmes, & Dishman, 2001).

The piriform cortex and HF are interconnected with the olfactory bulbs (Scalia, & Winans, 1974; Van Groen & Wyss, 1990) and changes in gene expression within these regions have been observed after olfactory bulbectomy and/or exercise (Holmes et al., 1998; Van Hoomissen et al., 2001). The destruction of the olfactory bulbs leads to neurodegeneration of neurons connected with the olfactory bulbs (Nesterova, Gurevich, Nesterov, Otmakhova, & Bobkova, 1997). One of the primary efferent projections from the olfactory bulbs is the piriform cortex (Scalia & Winans, 1974). Previous studies have demonstrated an upregulation of neurotrophic factors after axotomy in the periphery, but these effects have not been fully investigated in the brain (Hughes et al., 1999). Therefore, the piriform cortex was chosen to investigate the influence of axotomy on

neurotrophic regulation in the brain, as well as the regional specificity of the effect of exercise on neurotrophic gene expression.

The olfactory bulbs are connected to the HF via inputs to the entorhinal cortex, which sends projections to the dentate gyrus, the primary input to the hippocampus. Granule cell neurons from the dentate gyrus synapse on pyramindal cells in the CA3 region, which send afferents to the CA1 pyramidal neurons. The CA1 neurons, the primary extrinsic projections of the hippocampus, send axons to various brain regions, including the olfactory bulbs (Van Groen, & Wyss, 1990). The destruction of the olfactory bulbs, therefore, might lead to changes within the HF through degeneration of these anatomical pathways.

The mesolimbic pathway (ventral tegmental area/substantia nigra, nucleus accumbens) was chosen because of the importance of these areas in mediating reward and motivated behavior and motor control. Projections from the ventral tegmental region terminate in the nucleus accumbens and this pathway is considered important in mediating aspects of appetitive behavior. Previous studies have shown decreased appetitively-motivated behaviors in the OBX animal (O'Neal et al., 2001), which may be due to alterations in the activity of the mesolimbic pathway. The major neurotransmitter in this system is dopamine, and dysregulation of this system may participate in these behaviors. Neurotrophins have been shown to influence the dopaminergic system, including elevating dopaminergic activity in vivo (Martin-Iverson et al., 1994; Siuciak et al., 1996) and controlling striatal dopamine receptor expression (Guillin et al., 2001). Olfactory bulbectomy, therefore, might lead to alterations in neurotrophin levels, which may influence dopaminergic neurotransmission and behavior.

Lack of a Proposed Mechanism: Norepinephrine

A fourth limitation at the present time is the lack of a proposed hypothesis to explain how physical activity might lead to an upregulation of the expression of BDNF. One hypothesis, however, has been proposed regarding the mechanism by which exercise influences neural plasticity, though it does not directly address the mechanism by which exercise influences neurotrophic gene expression. Carro et al., (2000) reported that insulin-like growth factor (IGF-1) mediates some of the effects of exercise on neural plasticity, as blockade of IGF-I uptake in the central nervous system during treadmill exercise training attenuated the exercise-induced increase in cFOS in the HF. Despite the fact that injections of IGF-I mimicked the effects of exercise on hippocampal BDNF mRNA, the authors failed to report the effects of IGF-I blockade during exercise on gene expression for BDNF. Consequently, the current question regarding the mechanism by which exercise influences neurotrophic gene expression has yet to be addressed.

Our hypothesis for the mechanism explaining an exercise-induced upregulation of BDNF mRNA, centers on the role of the central norepinephrine (NE) system in mediating the effect. NE is synthesized in noradrenergic neurons from the precursor amino acid, tyrosine, through three enzymatic steps. Initially, tyrosine is converted to 3,4-dihydroxyphenlyalanine (L-DOPA) by the rate-limiting enzyme, tyrosine hydroxylase. The enzyme, L-aromatic acid decarboxylase, then converts L-DOPA to dopamine. In noradrenergic neurons, a third enzyme, dopamine-β-hydroxylase, converts dopamine to NE (Flatmark, 2000). The NE system has been proposed to play a role in a variety of functions, including sensory modulation, stress, arousal, vigilance, attention, memory, and emotion (Valentino & Aston-Jones, 1995).

Several lines of evidence support the hypothesis for the role of NE; there is (1) a neuroanatomical relationship between the NE system and the HF, (2) a reciprocal influential relationship between BDNF and noradrenergic neuronal function (3) and direct evidence for the influence of physical activity on the NE system. A NE-mediated mechanism, therefore, represents a plausible hypothesis explaining the mechanism by which exercise could lead to changes in neurotrophic gene expression.

The noradrenergic containing neurons of the brain are bilaterally located along the medial portion of the brainstem and are divided into two populations: the locus coeruleus complex (nucleus A4 and A6) and the lateral tegmental system in the medulla (A1-3) and pons (A5, A7). The nucleus locus coeruleus (LC), located just ventral to the fourth ventricle, is about 1.2 mm long in the rat, contains between 1400-1800 neurons, and has a dorsal, more neuronally compact region, and a ventral, less compact region. The LC is an organized nucleus with morphologically distinct neurons (fusiform or multipolar neurons) that differ in the orientation of their dendrites and in the intrinsic organization, resulting in morphologically different subpopulations of neurons projecting to different efferent targets. This nucleus contains the greatest percentage of NE neurons in the brain and provides the majority of efferent NE pathways to the forebrain (Aston-Jones, Shipley, & Gruzanna, 1995).

The LC appears to be the sole noradrenergic input to the HF. Fibers from the LC neurons innervate the HF by way of the ansa peduncularis-ventral amygdaloid bundle system, the fornix, and the cingulum. Fibers synapse in all regions of the HF (dentate gyrus, CA1, CA2, CA3), but the most dense areas of innervation are in the dentate gyrus and CA3 region (Moore & Bloom, 1979; Foote, Bloom, & Aston-Jones, 1983). These

neuroanatomical studies highlight a neuroanatomical pathway by which the LC-NE system might influence the neurotrophic response to exercise.

The post-synaptic effects of NE depend upon the post-synaptic receptor and can be basic inhibitory, excitatory, or result in modulation of other neurochemical inputs. The two main types of NE receptors are the α- and β-adrenergic receptors. The receptors can be further subdivided into α_1, α_2, β_1, β_2, and β_3. NE receptors are G-protein coupled receptors that contain seven transmembrane domains (Flugge, 2000). Postsynaptic neurons in the HF express mRNA for both types of adrenergic receptors (α_1,α_2, β_1, β_2). Labeling for β_1 mRNA is strongest in the granule cell layer of the dentate gyrus, and in the molecular layer of regions CA1and CA2. Labeling is strongest for β_2 mRNA in the granule cell layer of the dentate gyrus and in the CA1, CA2, and CA3 (Nicholas, Pieribone, & Hokflet, 1993). In addition to the β-receptor mRNA labeling, the HF expresses mRNA for α-adrenergic subtype (Nicholas, Hokfelt, & Pieribone, 1996). Blockade of NE receptors with a receptor antagonist during chronic exercise would perturb normal noradrenergic neurotransmission, thereby influencing the adaptations that occur in this system and in NE target regions during and following chronic exercise training.

The NE system and neurotrophic factors have a reciprocal influential relationship. The influence of NE on HF expression of neurotrophins is demonstrated by the increase in BDNF mRNA levels in the HF after reductions in central NE levels by axonal lesion (DSP-4 or axotomy). In addition, treatment of HF slices with exogenous NE reduces BDNF mRNA expression (Hutter et al., 1996). BDNF influences NE neuron cell number and differentiation, as well as central NE concentration. Cultured embryonic dorsal pons

neurons (including the LC neurons) are responsive to exogenous BDNF, as revealed by the increase in the number of neurons that express tyrosine hydroxylase and the increase in extracellular NE uptake (Sklair-Tavron & Nestler, 1995). Injections of BDNF into the ventricular system results in a concomitant increase in NE concentrations in the cortex, nucleus accumbens, and locus coeruleus (Siuciak et al., 1996). These results suggest that NE activity *in vivo* could potentially influence BDNF gene expression.

The central NE system is altered by both acute and chronic exercise. An acute bout of exercise increases the firing rate of LC neurons (Rassmussen et al., 1986), increases extracellular concentrations of NE (Pagliari & Peyrin, 1995a, 1995b) and decreases post-exercise concentrations of NE (Barchas & Freedman, 1963). Chronic exercise, however, elevates baseline NE concentrations (Dunn, Reigle, Youngstedt, Armstrong, & Dishman, 1996), blunts the release of NE during novel stress (Soares et al., 1999), and downregulates β-adrenergic receptors in the frontal cortex (Yoo et al., 2000),

The changes in NE concentrations and NE-neuronal activity after exercise may result from alterations in the level or activity of synthetic enzymes for NE (e.g. tyrosine hydroxylase, L-aromatic acid decarboxylase, dopamine-β-hydroxylase) or other neuroactive substances that regulate NE-neuronal activity (e.g. galanin, neuropeptide Y, BDNF). The effects of acute exercise on enzymatic activity have not been fully investigated, but chronic activity wheel running and treadmill training do not appear to alter baseline levels of gene expression for tyrosine hydroxylase in the LC (O'Neal, Van Hoomissen, Holmes, & Dishman, 2000; Van Hoomissen, O'Neal, Holmes, & Dishman, 2001). Gene expression for galanin and neuropeptide Y, two neuropeptides that are co-localized in NE neurons and hyperpolarize LC neurons (Holmes & Crawley, 1995), is

responsive to exercise. For example, treadmill training, but not activity wheel running, increases baseline levels of galanin mRNA approximately 1 standard deviation (O'Neal et al., 2000; Van Hoomissen et al., 2001). Exercise alone does not alter baseline NPY mRNA levels in the LC, but levels are elevated above control when exercise is performed in an animal model of depression (O'Neal et al., 2001). These results may provide additional areas of investigation regarding adaptations of the NE-LC with exercise.

By integrating the data on the changes in neurotrophin gene expression after exercise with the changes already shown in other neurotransmitter systems (i.e. NE), a mechanism by which exercise could influence neurotrophin expression can be examined. Therefore, one of the purposes of the second study was to address the influence of the chronic adaptations in the NE system on stress-induced downregulation of BDNF gene expression during 3 weeks of activity wheel running using pharmacological blockade of β-adrenoreceptor receptors with propranolol. The influence of the NE system on baseline BDNF mRNA using pharmacological blockade will not be addressed in this study because recent data from other labs has already shown that daily injections of propranolol (i.p.) attenuates the exercise-induced increase in BDNF in the hippocampus (Ivy, Rodriguez, & Russo-Neustadt, 2001). Therefore, we are interested in testing the hypothesis that long-term changes in the NE system during chronic activity wheel running promotes the increase in BDNF mRNA, which subsequently mediates the protective effect of exercise on stress-induced downregulation of BDNF mRNA.

Lack of Use of Behavioral Assessment

A fifth limitation regarding the effect of exercise on neurotrophic gene expression involves the lack of use of behavioral testing or examination of additional physiological processes to assess the significance of the increased neurotrophic gene expression after chronic exercise or antidepressant treatment. Only one study to date has concomitantly examined behavior and BDNF mRNA, but did not find a significant effect of exercise alone on behavior, despite an increase in gene expression (Russo-Neustadt et al., 2001). Exercise consistently increases BDNF mRNA in the HF, and in order to determine if there is an association between neurotrophic gene expression and behavior, behavioral tasks that require the activity and involvement of the HF should be utilized.

Two important functions of the HF are to regulate the endocrine response to stress via its indirect influence on the hypothalamo-pituitary-adrenocortical-(HPA) and to influence learning and memory. The HF participates in a gluccocorticoid negative feedback loop, resulting in inhibition of the HPA axis through a multisynaptic connection between the HF and the paraventricular nuleus of the hypothalamus, which includes additional hypothalamic or extra-hypothalamic regions, such as the preoptic nucleus, the amygdala, or the bed nucleus of the stria terminalis (Sapolsky, Krey, & McEwen, 1984; Plotsky, 1991; Herman, Prewitt, & Cullinan, 1996; Chrousos, 1997). In addition to this function, the HF plays a prominent role in learning and memory (Maren, 2001).

Because exercise influences learning and memory, and the neuroendocrine responses to stress, a secondary purpose of the second study is to examine these phenomena after chronic physical activity. The effect of chronic physical activity on the hormonal stress response is dependent upon the type of physical activity utilized in the experiment (treadmill exercise training vs. activity wheel running), the nature of the

stressor (heterotypic vs. homotypic), and the specific stress hormone analyzed (adrenocorticotropic hormone [ACTH] vs. corticosterone [CORT]). For example, six weeks of treadmill exercise training potentiates the ACTH, but not CORT, response to heterotypic (novel) immobilization or foot shock stress, but attenuates the ACTH response to homotypic (familiar) treadmill running compared to sedentary animals (White-Welkley et al., 1995; White-Welkley et al., 1996). Chronic activity wheel running, however, does not affect ACTH or CORT responses to foot shock, but attenuates the ACTH response to foot shock if activity wheel running is combined with an additional stressor (e.g. cage switch) (Dishman et al., 1998).

The influence of physical activity on behavioral tests of learning and memory in animal models has received little attention, although studies exist that may suggest that exercise has a beneficial, although moderate, effect on some aspects of learning and memory, such as spatial memory. Spatial memory can be assessed using behavioral tasks, such as the Morris water maze test, the place learning set-task, and the radial arm maze test. Chronic physical activity has been shown to improve performance on all of these tasks (Anderson et al., 2000; Fordyce & Farrar, 1991a, 1991b; Fordyce & Wehner, 1993; Van Praag et al., 1999). Given these results, it is of interest to determine if physical activity differentially affects other forms of learning and memory, such as contextual memory, and more specifically contextual fear conditioning (CFC).

Context fear conditioning (CFC) is a Pavlovian form of learning, which can be used to examine the influence of a contextually conditioned aversive environment on subsequent physiological responses and aversively motivated behaviors (Fanselow, 2000). Animals that have been previously exposed to a specific environment (Skinner

box; conditioned stimulus) that is paired with an aversive stressor (foot shock; unconditioned stimulus) will display increased levels of freezing (somatomotor inhibition; conditioned response) and corticosterone, compared to control animals, when placed back into the environment without the shock (Sandi, Merino, Cordero, Touyarot, & Venero, 2001). This effect is maintained long-term, as it is observed immediately after the conditioning session and for several days after the initial training session. One of the brain regions hypothesized to mediate the behavioral expression of CFC is the HF, which, when lesioned, results in deficits in expression of stress-related behaviors (Kim, Rison, & Fanselow, 1993; Maren & Fanselow, 1997). One hypothesis suggests that the HF is important in forming a contextual fear memory. However, the HF is not hypothesized to be the site for long-term storage of the memory, which may occur in additional brain regions (Fanselow, 2000).

Consolidation and subsequent expression of CFC memory is influenced by circulating levels of corticosterone. Blockade of type II glucocorticoid receptors prior to or after conditioning attenuates the development of CFC (Pugh, Fleshner, Rudy, 1997; Cordero, & Sandi, 1998). In addition, CFC is impaired following the removal of circulating levels of adrenal hormones via adrenalectomy (Pugh, Tremblay, Fleshner, & Rudy, 1997). The level of plasma corticosterone during exposure to the chamber during the test session is positively correlated with freezing behavior ($r=0.51$) (Cordero, Merino, & Sandi, 1998). Therefore, experimental conditions, which influence levels of corticosterone in response to CFC training, might influence expression of CFC behavior.

Summary

The limitations presented above represent questions of interest for future research. Some of these questions were addressed by the two experiments presented in this dissertation. In the first study the main objective was to compare the effects of exercise and antidepressant medication on BDNF mRNA, in multiple brain regions, in an animal model of depression. The purpose of the second study was to address the effect of exercise on Pavlovian learning and the neuroendocrine stress response. A second purpose was to determine if the changes in behavior were associated with altered levels of BDNF in the HF.

CHAPTER 3

THE EFFECTS OF CHRONIC EXERCISE AND IMIPRAMINE ON mRNA FOR BDNF AFTER OLFACTORY BULBECTOMY IN RAT*

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Abstract

We examined the effects of chronic activity wheel running and antidepressant treatment on brain-derived neurotrophic factor (BDNF) messenger RNA (mRNA) in multiple brain regions (hippocampal formation [HF], ventral tegmental area/substantia nigra [VTA/SN], nucleus accumbens [NAc], and piriform cortex [PFx]) after bilateral olfactory bulbectomy (OBX). Male, Long-Evans rats (N=72) underwent either sham or OBX surgery and were randomly divided into 8 experimental groups in a 2 (sham vs. OBX) x 2 (sedentary vs. activity wheel) x 2 (saline vs. imipramine) factorial design. Animals were killed after 21 days of treatment. Drug x exercise interaction effects were observed for HF ($p=0.006$-0.023) and VTA/SN ($p=0.021$); exercise increased BDNF mRNA in the saline treated animals but not in the imipramine treated animals. OBX did not affect BDNF mRNA in the HF or VTA/SN ($p>0.05$). BDNF mRNA levels in the PFx were not altered by exercise, drug, or OBX ($p>0.05$). These results suggest that the effect of exercise on BDNF mRNA extends beyond the HF to specific mesolimbic regions and that the potentiation of BDNF mRNA by exercise and antidepressant pharmacotherapy reported by other investigators may be time limited.

Introduction

Physical activity has been inversely related to depression symptoms in prospective population-based cohort studies and randomized clinical trials (Dunn, Trivedi, & O'Neal, 2001). The biological mechanisms by which exercise exerts a positive effect on depression symptoms are unknown, but it is plausible that they involve alterations in the expression of neurotrophic factors (Duman, Heniger, & Nestler, 1997). This hypothesis stems from studies showing that brain-derived neurotrophic factor (BDNF) exerts antidepressant-like properties in animal models (Suiciak, Lewis, Wiegand, & Lindsay, 1997), is increased in the brain after treatment with antidepressant pharmacotherapy (Nibuya, Morinobu, & Duman, 1995), and is altered in post-mortem brain tissue after antidepressant pharmacotherapy (Chen, Dowlatshahi, MacQueen, Want, & Young, 2001).

Previous studies have indicated that chronic exercise has some similar central nervous system effects as observed after antidepressant drug treatment. These effects include elevation of monoamines (Dishman, Renner, White-Welkley, & Bunnell, 2000; Dunn, Reigle, Youngstedt, Armstrong, & Dishman, 1996), down-regulation of central β-adrenergic receptors (Yoo, Tackett, Crabbe, Bunnell & Dishman, 2000), and increased expression of BDNF messsenger RNA (mRNA) (Carro, Nunez, Busiguina, Torres-Alman, 2000; Neeper, Gomez-Pinilla, Choi, & Cotman, 1995, 1996; Oliff, Berchtold, Isackson, & Cotman, 1998; Windefalk, Olson, & Thoren, 1999). Exercise has also been shown to potentiate the effect of pharmacotherapy on BDNF mRNA (Russo-Neustadt, Beard, & Cotman, 1999; Russo-Neustadt, Beard, Huang, & Cotman, 2000). Therefore,

chronic exercise may exert antidepressant-like, or otherwise salutary effects on the brain through its influence on these systems.

The increase in BDNF mRNA observed after chronic exercise suggests a mechanism by which exercise might influence brain plasticity, given that BDNF (Jankowsky & Patterson, 1999; Korte, et al., 1995; Korte et al., 1996) and physical activity (Van Praag, Christie, Sejnowski, & Gage, 1999) both influence synaptic plasticity as well as morphological and structural properties of the central nervous system (Anderson, Alcantara, & Greenough, 1996; Black, Isaacs, Anderson, Alcantara, & Greenough, 1990; Kleim et al., 1998; Labelle & Leclerc, 2000). Studies have also reported a neurogenerative and neuroprotective influence of exercise on the brain by showing that exercise induces the growth and development of new cells (Van Praag, Kempermann, & Gage, 1999), and protects against neuronal damage in the HF (Stummer, Weber, Tranmer, Baethmann, & Kempski, 1994). These effects may have implications for the study of the health protective benefits of exercise for diseases such as depression, in which maladpatations in neural plasticity may be apparent (Vaidya & Duman, 2001; Gage, 2000).

Previous studies have speculated about the role of BDNF as a potential mediator of the antidepressant and cognitive benefits of exercise, yet have not used an animal model of the disease of interest, thereby limiting the generalizability of the results (Cotman & Engesser-Cesar, 2002). In the present study, we wanted to determine whether the level of BDNF gene expression would be altered at baseline after chronic exercise and antidepressant treatment in an animal model of depression. We examined these putative effects using the olfactory bulbectomy model (OBX) of depression, which

results in numerous biochemical and behavioral deficits that resemble several key features of depression and are reversed by chronic but not acute antidepressant pharmacotherapy (Kelly, 1996).

In addition to using an animal model of depression, we wanted to extend the current area of research by examining the regional specificity of the expected effects of exercise on mRNA for BDNF. The areas chosen included the ventral tegmental area/substantia nigra (VTA/SN), hippocampal formation (HF), piriform cortex (PFx), and nucleus accumbens (NAc). These regions were chosen because of their involvement in the regulation of emotional and motivated behavior, as well as motor control. Moreover, they have been shown to be responsive to either olfactory bulbectomy (Garris, Chamberin, & DaVanzo, 1984; Holmes, Davis, Masini, & Primeaux, 1998) and/or exercise (Emerson, Kappenmann, Ronan, Renner, & Summers, 2000; Tumer et al., 2001; Van Hoomissen, O'Neal, Holmes, & Dishman, 2001).

Materials and Methods

Animals and Experimental Design

Male, Long-Evans rats (N=72, 45 days old) were individually housed in polypropylene cages in a temperature (23±1°C) and humidity-controlled environment that was maintained on a 12 h light/dark schedule (lights on 6:00 a.m.-6:00 p.m.) with food and water provided *ad libitum*. All procedures were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and the U.S. National Institutes of Health Guide regarding the care and use of animals for experimental procedures and were approved by the University of Georgia Animal Care and Use Committee. Upon arrival, animals were

allowed to acclimatize to the vivarium for 2 weeks prior to the initiation of any experimental procedures. Animals were randomly divided into 8 groups in a 2 (sham vs. OBX) x 2 (sedentary vs. activity wheel) x 2 (saline vs. imipramine) factorial design.

Bilateral Olfactory Bulbectomy Surgery

Animals were anesthetized with Nembutal (25 mg/kg, i.p., Abbott Laboratories) and Ketamine Hydrochloride (40 mg/kg, i.p., Mallinckrodt) prior to the olfactory bulbectomy surgery. A 1 cm, rostral-caudal midline incision was made in the skin overlying the dorsal surface of the skull and two small burr holes (2 mm in diameter) were drilled in the skull 6 mm rostral of bregma and 1 mm lateral of the midline. Olfactory bulbs were removed using gentle aspiration and gel foam was inserted into the cavity to control bleeding. The wound was closed with vicryl sutures and animals were given 3 cc of saline to offset the loss of blood volume during surgery and Banamine for pain control. Animals were placed under a heat lamp until recovery and then returned to their home cage for the remainder of the study. Throughout the experiment all animals were weighed and handled daily. The success of the OBX surgeries was verified by dissection at the end of the study. Animals were excluded from the OBX condition if they contained olfactory bulb remnants that weighed more than 30% of the sham animal bulb tissue.

Exercise Protocol

Twenty-four hours after recovery from surgery, a stainless-steel activity wheel (circumference=105 cm) connected to an electromagnetic counter was placed in each animal's cage. Animals were given free access to the activity wheel 24 hours a day for 3

weeks, and the daily running distance was determined by multiplying the number of wheel revolutions by the circumference of the wheel.

Drugs

One-half of the animals received daily injections of the tricyclic antidepressant medication, imipramine hydrochloride (10 mg/kg, i.p., Sigma) beginning 24 hours after OBX. The remaining half of the animals received injections of sterile saline. The injections were continued for 21 days and were terminated at least 48 hours prior to death. Animals were killed by rapid decapitation a minimum of 48 hours after completion of the final running session and drug injection, between the hours of 8:00-10:00 a.m. Brains were quickly dissected, frozen in dry ice, and then stored at -80°C until sectioning.

In Situ Hybridization

A Microm Cryostat (Carl Zeiss, Waldorf, Germany) was used to slice the brains into 12 μm sections at the level of the VTA/SN, HF, PFx, , and NAc, in congruence with sections of the Rat Atlas (Paxinos & Watson, 1986). Sections were thaw-mounted onto gelatin coated glass microscope slides and approximately every tenth section was stained with 0.1% thionin to verify anatomical location. Four sections (2 slides) per animal were selected for hybridization. Sections were fixed in 4% formaldehyde in 0.12M sodium phosphate-buffered saline (PBS, pH 7.4) for 5 minutes, rinsed twice in PBS, and placed in 0.25% acetic anhydride in 0.1M triethanolamine HCl/0.9% NaCl (pH 8.0) for 10 minutes. Sections were dehydrated using a series of ethanol washes (70%, 80%, 95%, 100%), delipidated in chloroform for 5 minutes, rinsed in ethanol (100%, 95%), and allowed to dry.

Oligonucleotide probes were obtained from Oligos Etc. (Wilsonville, OR). The BDNF oligo probe was complementary to bases 650-699 of the mouse BDNF mRNA transcript, which is 98% homologous to rat (Hofer, Pagliusi, Hohn, Leibrock, & Barde, 1990; Maisonpierre et al., 1991). Probes were labeled at the 3'-end with [^{35}S]-dATP (1000 -1500μCi/mmol; New England Nuclear, Boston, MA), terminal deoxynucleotidyl transferase (TdT) (15 units/ml; Bethesda Research Lab, Gaithersburg, MD), and tailing buffer. Column separation was used to separate unincorporated nucleotides from the probes. Sections were hybridized with radiolabeled probes in solution containing 50% formamide, 600mM NaCl, 80 mM Tris-HCl, 4 mM EDTA, 0.1% sodium pyrophosphate, 0.2% SDS, 0.2 mg/ml heparin sulfate, and 10% dextran sulfate. Brain sections were incubated with the hybridization solution for 20 hours in a humid chamber at 37°C. Sections underwent a series of washes (3, 1X-SSC; 1, 2X-SSC/50% formamide; 3, 2X-SSC/50% formamide for 20 minutes at 40 °C; 2, 1X-SSC for 30 minutes at 22 °C) to reduce nonspecific binding. Slides were rinsed in deionized water and 70% ethanol and allowed to dry. Sections were exposed to autoradiograhpic film (BioMax MR, Eastman Kodak, Rochester, NY) and developed with Kodak GBX developer and fixer.

Autoradiographic films were analyzed using a computerized image analysis system (Image 1.38 software, Rasband, 1995, National Institute of Mental Health; Power Macintosh 8100 computer, Apple Computer, Cupertino, CA; light box and camera, Imaging Research, St. Catharines, Ontario; video interface, Data Translation, Marlboro, MA) to determine optical density (OD) within the VTA/SN, PFx, NAc, and the HF (granule cell layer of the dentate gyrus [DG], pyramidal cell layer of Ammon's Horn area 3 [CA3] and1 [CA1]). Cortical sections (HF and PFx) were analyzed by taking the

average OD of 10, 5 x 5 pixel circles, placed randomly throughout the brain region of interest. Subcortical sections (VTA/SN and NAc) were analyzed by measuring OD within a hand-drawn shape placed within each region. To verify accurate location in the dopaminergic cell region of the VTA/SN area, serial coronal sections containing the VTA/SN were hybridized with radiolabelled BDNF or tyrosine hydroxylase oligonucleotide probes. The computerized image analysis system was used to outline dopaminergic cells in the VTA/SN region that expressed mRNA for tyrosine hydroxylase. This area was then superimposed on the subsequent coronal section hybridized with the BDNF oligonucleotide probe. The investigator was blinded to subject condition at the time of quantification.

Data Analysis

Data from each brain region were analyzed separately using a 2 (sham vs. OBX) x 2 (sedentary vs. activity wheel) x 2 (saline vs. imipramine) ANOVA using SPSS Windows version 10.1 (SPSS, Inc., Chicago, IL). Significant interactions were decomposed using LSD post-hoc tests (2-tailed). The significance level was set at $p<0.05$. In addition, the effect size was estimated by Cohen's D (experimental mean–control mean/pooled standard deviation).

Results

BDNF mRNA

There was a significant main effect of exercise in the HF (DG, $F_{(1,64)}=19.530$, $p=0.001$, $d=1.0$; CA3, $F_{(1,64)}=14.358$, $p=0.001$, $d=0.89$; CA1, $F_{(1,64)}=11.998$, $p=0.001$, $d=0.77$). There was a main effect of drug in the DG ($F_{(1,64)}=4.610$, $p=0.036$, $d=0.42$) and CA3 region ($F_{(1,64)}=5.935$, $p=0.018$, $d=0.49$). There was significant drug x exercise

interaction in the HF (DG, $F_{(1, 64)}$=5.39, p=0.023; CA3, $F_{(1, 64)}$=6.26, p=0.015; CA1, $F_{(1,64)}$=7.970, p=0.006). Post-hoc analyses indicated that exercise increased BDNF mRNA in the HF of the saline treated rats (DG, $t_{(33)}$=-4.593, p=0.001, d=1.59; CA3, $t_{(33)}$=-4.565, p=0.001, d=1.59; CA1, $t_{(33)}$=-4.378, p=0.001, d=1.46), but not in the imipramine treated rats (DG, $t_{(35)}$=-1.570, p=0.125; CA3, $t_{(35)}$=-0.963, p=0.342; CA1, $t_{(35)}$=-0.509, p=0.614). There was a significant drug x exercise x surgery interaction in the CA1 region ($F_{(1,64)}$=8.265, p=0.005). Post-hoc analyses revealed a main effect of exercise in the saline treated animals ($F_{(1, 31)}$=18.584, p=0.001) and a exercise x surgery interaction in the imipramine treated animals ($F_{(1,33)}$=8.118, p=0.007), such that in the imipramine treated animals, exercise significantly increased BDNF mRNA in the CA1 region only in the OBX animals ($t_{(17)}$=2.628, p=0.018). Further post-hoc analyses verified the previously documented increase in BDNF mRNA in HF after chronic administration of imipramine (DG, $t_{(32)}$=-3.323, p=0.002, d=1.16; CA3, $t_{(32)}$=-3.889, p=0.001, d=1.42; CA1, $t_{(32)}$=-2.668, p=0.012, d=0.93)(Figures 3.1-3.3). OBX did not alter BDNF mRNA in the DG ($F_{(1,64)}$=1.322, p=0.255), CA3 ($F_{(1,64)}$=0.128, p=0.721), or CA1($F_{(1,64)}$=0.156, p=0.694) regions of the HF.

There was a significant main effect of exercise in the VTA/SN ($F_{(1,61)=}$6.977, p=0.010, d=0.67). There was a significant drug x exercise interaction in the VTA/SN ($F_{(1,61)}$=5.650, p=0.021). Post-hoc comparisons indicated that chronic activity wheel running elevated BDNF mRNA in the VTA/SN in the saline treated group ($t_{(33)}$=-3.681, p=0.001, d=1.29), but not in the imipramine treated group ($t_{(24.81)}$=0.071, p=0.944). Further post-hoc analyses indicated that imipramine treatment among the sedentary

animals increased BDNF mRNA in the VTA/SN ($t_{(33)}$=-2.187, p=0.036, d=0.77) (Figures 3.4-3.5). OBX did not alter BDNF mRNA in the VTA/SN ($F_{(1,61)}$=0.294, p=0.590).

BDNF mRNA in the PFX was not altered by activity wheel running ($F_{(1,61)}$=0.322, p=0.572), OBX ($F_{(1,61)}$=2.917, p=0.093) or imipramine treatment ($F_{(1,61)}$=0.187, p=0.667). The BDNF mRNA signal in the NAc was too low to be analyzed.

Discussion

The results of this experiment indicate that chronic activity wheel running increased BDNF mRNA levels in a regionally specific manner in the brain. BDNF mRNA was increased in the HF, which supports previous studies that have shown an increase in gene expression for BDNF in the brain after exercise (Neeper et al., 1995; Neeper et al., 1996; Oliff et al., 1998; Russo-Neustadt et al., 1999; Russo-Neustadt et al., 2000). However, the effect of exercise on BDNF gene expression was not specific to the HF. We also observed an upregulation of BDNF mRNA in the VTA/SN region. The magnitude of the increase in the VTA/SN was comparable to the effect of exercise in the HF (DG, d=1.0, CA3, d=0.89, CA1, d=0.77, VTA/SN, d=0.67).

The meaning of the increase in BDNF mRNA in the HF and VTA/SN regions after chronic exercise is currently unknown but might be related to observations that neuromodulatory peptides that co-exist with norepinephrine in the brain are activated by chronic exercise. For example, previous studies have shown that activity wheel running and treadmill exercise training alter gene expression for galanin, a neuromodulatory peptide co-localized with NE in the central noradrenergic neurotransmitter system (O'Neal, Van Hoomissen, Holmes, & Dishman, 2000; Soares et al., 1999).

The increase in BDNF mRNA in the VTA/SN region might result in the enhancement of mesolimbic dopamine function. Previous studies have indicated that BDNF mRNA is co-localized in dopaminergic neurons in the VTA/SN region (Seroogy et al., 1994). Some of the effects of BDNF on dopaminergic neurons include influencing dopamine release *in vitro* (Goggi, Pullar, Carney, & Bradford, 2002), altering dopaminergic cell electrophysiological activity (Shen, Altar, & Chiodo, 1994), and affecting the function of dopamine receptors in dopaminergic terminal fields (Guillin et al., 2001). The elevation of BDNF mRNA in the mesolimbic regions after chronic exercise might alter dopaminergic function, which may have relevance for the observed effects of exercise on appetitively-motivated behavior. For example, chronic activity wheel running has been shown to reverse deficits in masculine copulatory behavior in rats observed in two animal models of depression, the clomipramine (Yoo et al., 2000) and the olfactory bulbectomy models of depression (Van Hoomissen et al., 2001).

Chronic activity wheel running interacted with imipramine treatment to influence BDNF mRNA levels in the HF and VTA/SN; saline treated, but not imipramine treated, runners had increased BDNF mRNA. Those results do not support previous research indicating that exercise potentiates BDNF mRNA expression in animals treated with antidepressant pharmacotherapy. For example, Russo-Neustadt et al. (1999) reported that the combined treatment of activity wheel running and antidepressant pharmacotherapy (imipramine or tranylcypromine) potentiated the expression of BDNF mRNA levels in the HF, above the level observed with either exercise or drug treatment alone. Methodological differences between the current and previous studies, such as the latency between the last drug treatment or activity wheel running session and the isolation of the

brains, might explain the discrepancy. In the current study, we isolated brain tissue 48 hours after completion of the last nocturnal running session or drug injection. Previous studies reported isolation of brain tissue immediately after the last nocturnal running session and 24 hours after the last drug injection (Russo-Neustadt et al., 1999; Russo-Neustadt et al., 2000). The current results indicate that the previously observed potentiation of BDNF mRNA by exercise in antidepressant drug treated animals might be a short-term effect that dissipates 48 hours after completion of the last running session.

Our results confirm the previously documented increase in BDNF mRNA after chronic treatment with antidepressant pharmacotherapy. For example, Nibuya et al., (1995) reported that chronic treatment with imipramine elevated BDNF mRNA in the HF. In addition, we observed an increase in BDNF mRNA in the VTA/SN region, indicating that the antidpressant-induced upregulation was not specific to the HF but involved additional brain regions implicated in the motivation, emotion and motor control.

Because previous studies examining the effect of antidepressant pharmacotherapy on BDNF mRNA have not been conducted in animal models of depression, it is unknown whether the increase in BDNF mRNA in the HF after drug treatment extends to animal models. Post-hoc analyses of our data examining only the sedentary animals indicated that the imipramine increased BDNF mRNA in all regions of the HF in the sham animals ($p=0.001$-0.004), but did not increase BDNF mRNA in the HF of the OBX animals ($p=0.127$-0.625). This apparent attenuation of the imipramine-induced increase in mRNA in the OBX animals, however, was largely reversed by activity wheel running. Further investigations into the specificity of the increase in BDNF mRNA with

antidepressant pharmacotherapy are warranted, especially in studies using animal models of depression.

An additional finding of the present study was that the level of BDNF mRNA in the regions we examined was not altered by OBX alone. The OBX animal model of depression requires bilateral removal of the olfactory bulbs, the outcome of which is retrograde and anterograde neuronal degeneration of afferent and efferent neurons projecting to and from the olfactory bulb (Capurso et al., 1997; Nesterova, Gurevich, Nesterova, Otmakhora, & Bobkova, 1997). We hypothesized that OBX might alter neurotrophin expression in the brain because neuronal injury and axotomy are associated with an alteration in BDNF gene expression (Hughes et al., 1999), lesions of the olfactory bulb have been shown to alter BDNF expression in the olfactory bulb and other brain regions (Sohrabji, Feebles, & Marroquin, 2000), and previous studies have reported altered levels of BDNF in the brain of an animal model of depression, the Flinders Sensitive Line (Angelucci, Aloe, Vasquez, & Mathe, 2000).

These null findings of OBX alone on BDNF mRNA in the PFx, HF, and VTA/SN, might be explained in three ways. First, it is possible that the chosen time course of our present experiment, in which OBX animals were killed 3 weeks after surgery, prevented us from observing changes in BDNF gene expression that may have occurred immediately following removal of the olfactory bulbs. Second, it is possible that alterations in BDNF activity are not observable at the mRNA level, but only at the protein level. Third, it is possible that OBX does not alter levels of BDNF mRNA, rather the alteration in the levels of BDNF mRNA in the brain of animal models of depression is specific to the type of model examined.

One question that has not been addressed in the current literature is whether the increased hippocampal BDNF mRNA that accompanies chronic exercise is the result of the physical exertion of running, *per se*, or the consequence of previously sedentary animals learning a novel motor pattern (e.g., wheel running). At the present time, data are unavailable to support or refute this hypothesis, but it remains an important area for future research because other experimental behavioral learning protocols elevate BDNF levels (Hall, Thomas, & Everitt, 2000; Mizuno, Yamada, Olariu, Nawa, & Nabeshima, 2000). It should be noted, however, that long-term physical activity increases BDNF mRNA, and disengagement of this activity after this time period downregulates BDNF (Windenfalk et al., 1999). This occurs despite a presumed minimization of the learning component of physical activity after 6 weeks of practice.

In the present study, we isolated the brains at least 48 hours after completion of the last running session. This might have influenced the magnitude of the observed effect in the HF, as previous studies have reported >90% increase in BDNF mRNA after activity wheel running (Neeper et al., 1996). However, the smaller effect observed in the present study is a persistent, sustained effect of exercise that is still present more than 2 days after completion of the last running session, and represents a large effect by statistical standards (Cohen's d=0.77-1.0).

In conclusion, the present data confirm previous reports that chronic activity wheel running increases BDNF mRNA in the hippocampal formation, and they extend the evidence of a brain neurotrophinergic effect of chronic exercise to another brain region involved in motivation, emotion, and motor control; the VTA/SN. Because of the proposed role of the neurotrophins in depression, our results indicate a need for further

investigations of mesolimbic brain regions in the search for brain mechanisms that underly the antidepressant effects of exercise.

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Figure Captions

Figure 3.1. BDNF mRNA in the (A) DG, (B) CA3, and (C) CA1 regions of the HF. Post-hoc analyses (2-tailed independent samples t-tests) revealed a significant increase in BDNF mRNA expression in all regions of the HF after chronic exercise in the saline treated animals (p=0.001-0.05). In the imipramine treated animals, chronic exercise elevated BDNF mRNA in the dentate gyrus and CA1 in the OBX animals (p=0.07-0.018) but did not alter BDNF mRNA in the sham animals.

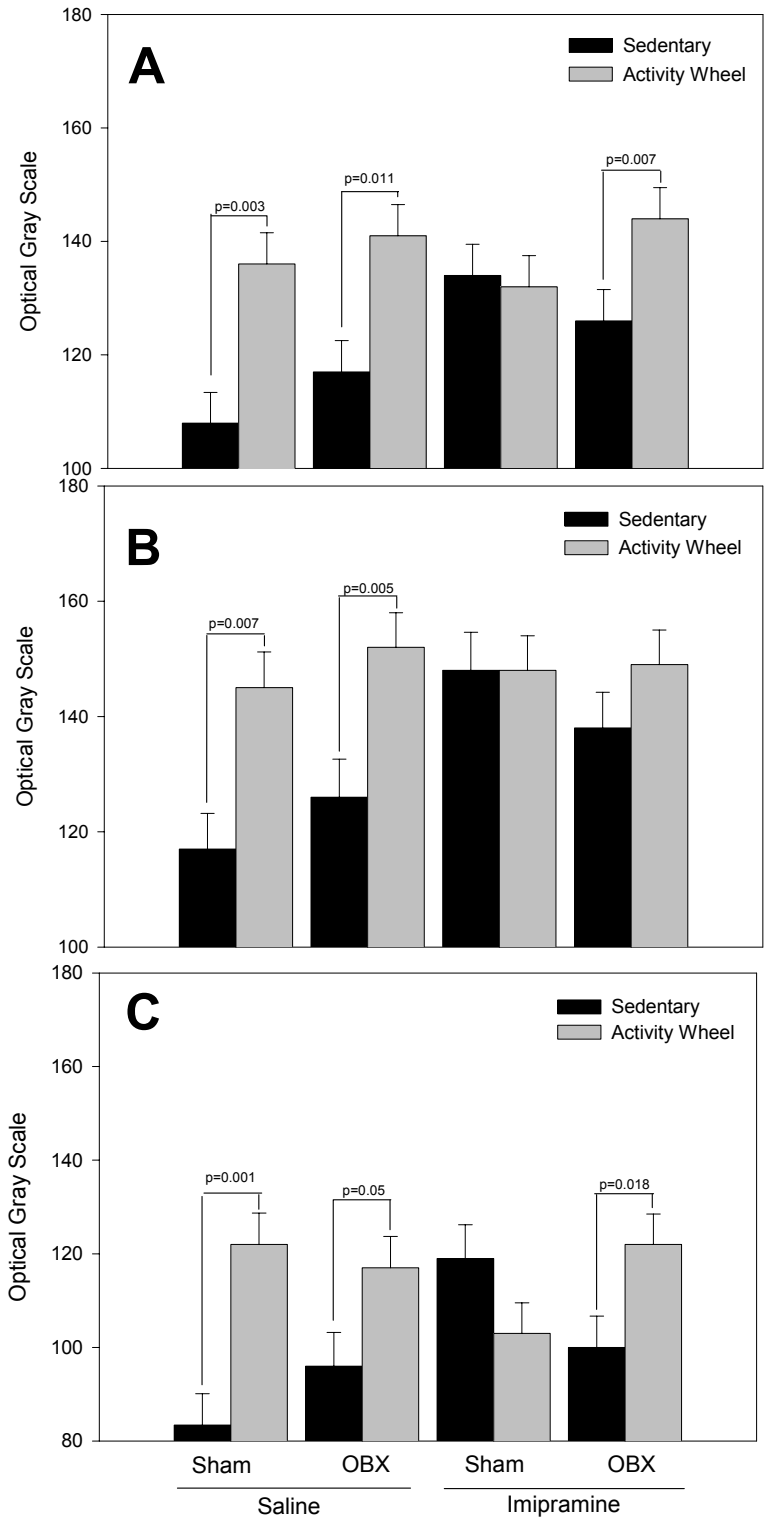
Figure 3.2. BDNF mRNA in the (A) DG, (B) CA3, and (C) CA1 regions of the HF. Chronic exercise elevated BDNF mRNA in the saline treated group (p=0.001-0.012), but not in the imipramine treated group.

Figure 3.3. Autoradiograms of BDNF mRNA in the HF depicting a drug x exercise interaction. (A) saline/sedentary, (B) saline/activity wheel, (C) imipramine/sedentary, (D) imipramine/activity wheel. Chronic activity wheel running elevated BDNF mRNA in the saline treated animals, but not in the imipramine treated animals.

Figure 3.4. BDNF mRNA in the (A) VTA/SN region. (B) Chronic exercise elevated BDNF mRNA in VTA/SN in the saline group, but not in the imipramine treated group. Imipramine treatment elevated BDNF mRNA in the VTA/SN in the sedentary animals.

Figure 3.5. Autoradiograms of BDNF mRNA in the VTA/SN of the rat brain. (A) Coronal section through the rat midbrain indicating (arrow) the area of enlargement in images B-G. (B) Enlarged view of BDNF mRNA in the VTA/SN region. (C) Enlarged view of tyrosine hydroxylase binding in the VTA/SN region (see methods for detailed description of BDNF mRNA analysis). BDNF mRNA in the (D) saline/sedentary, (E) saline/activity wheel, (F) imipramine/sedentary, and (G) imipramine/activity wheel

groups. Chronic exercise significantly elevated BDNF mRNA in the VTA/SN in the saline group, but not in the imipramine treated group.


A
Sedentary
Activity Wheel
Optical Gray Scale
180
160
140
120
100
p=0.003
p=0.011
p=0.007
B
Sedentary
Activity Wheel
Optical Gray Scale
180
160
140
120
100
p=0.007
p=0.005
C
Sedentary
Activity Wheel
Optical Gray Scale
180
160
140
120
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80
p=0.001
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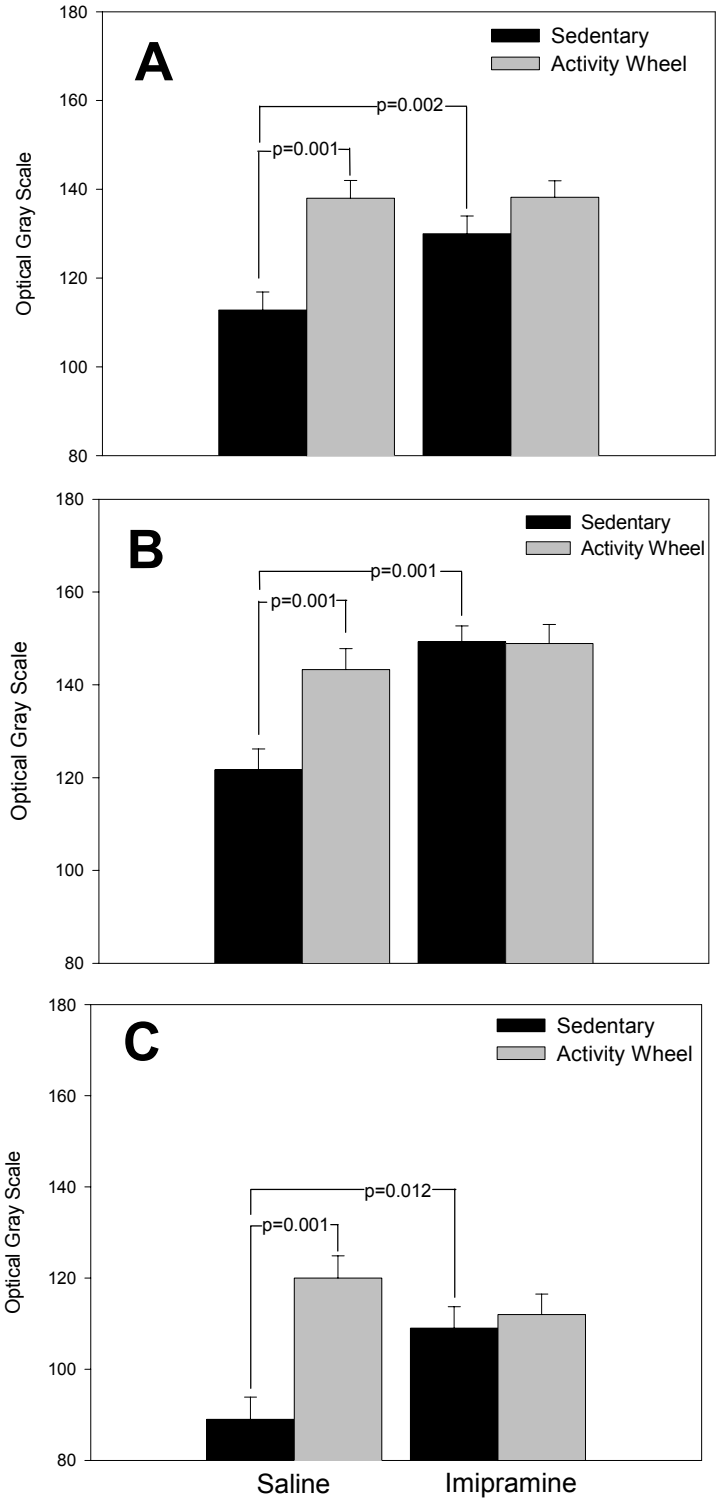
p=0.018
Sham
OBX
Sham
OBX
Saline
Imipramine

Figure 3.1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Sedentary
Activity Wheel
p=0.002
p=0.001
Optical Gray Scale
180
160
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100
80
B
Sedentary
Activity Wheel
p=0.001
p=0.001
Optical Gray Scale
180
160
140
120
100
80
C
Sedentary
Activity Wheel
p=0.012
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Optical Gray Scale
180
160
140
120
100
80
Saline
Imipramine

Figure 3.2

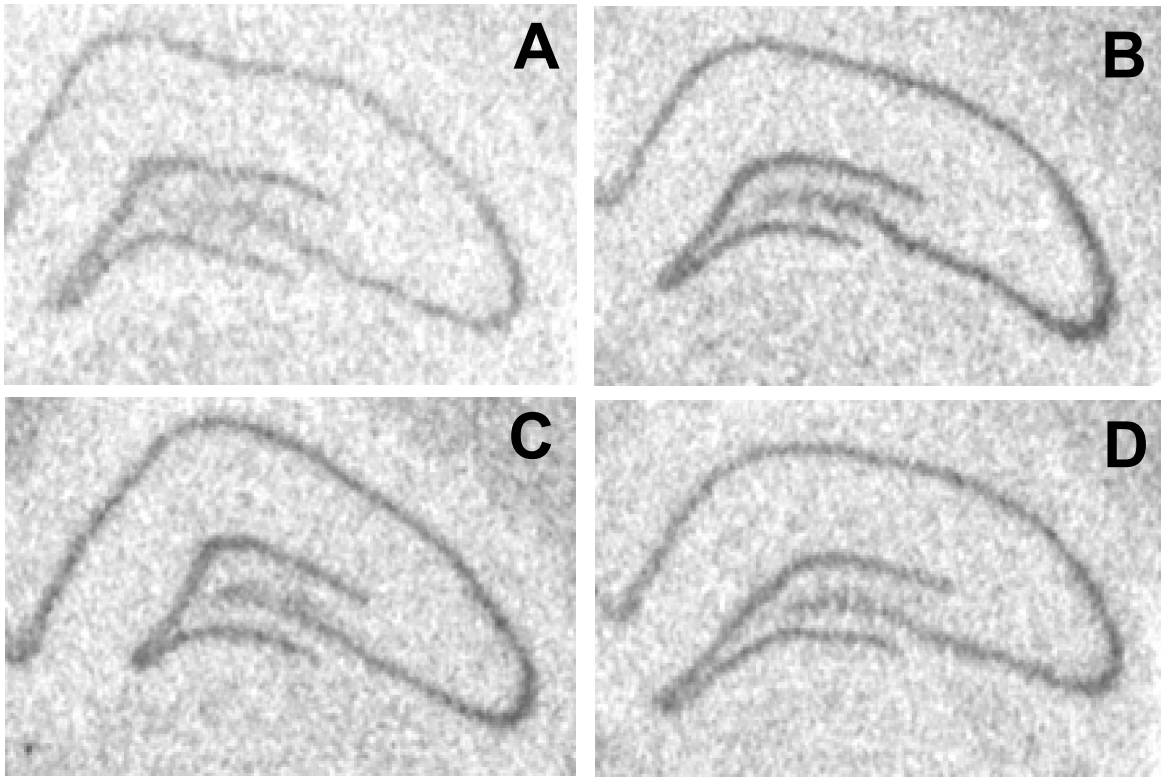
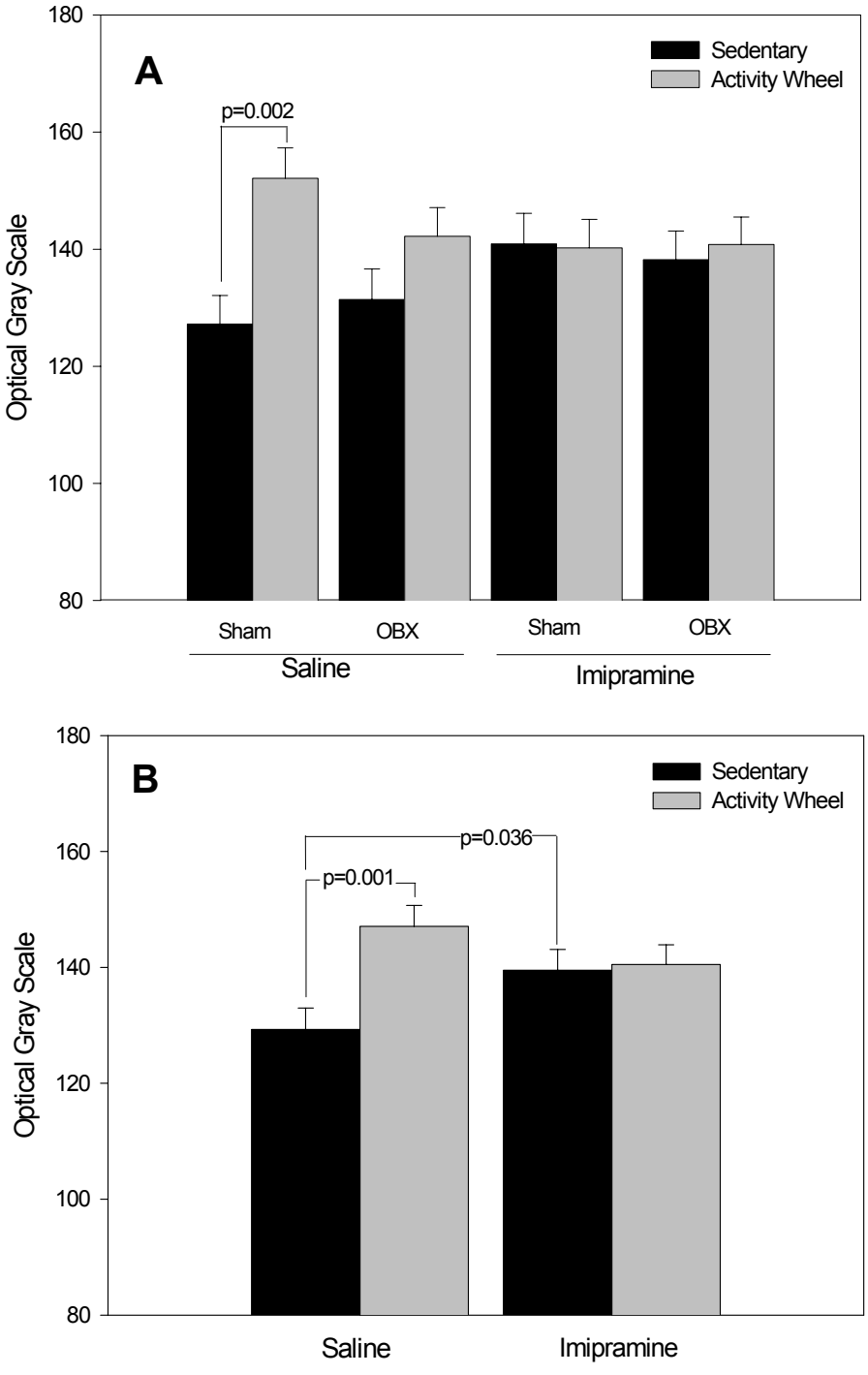


Figure 3.3


A
Sedentary
Activity Wheel
p=0.002
Optical Gray Scale
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Saline
Imipramine
B
Sedentary
Activity Wheel
p=0.036
p=0.001
Optical Gray Scale
180
160
140
120
100
80
Saline
Imipramine

Figure 3.4

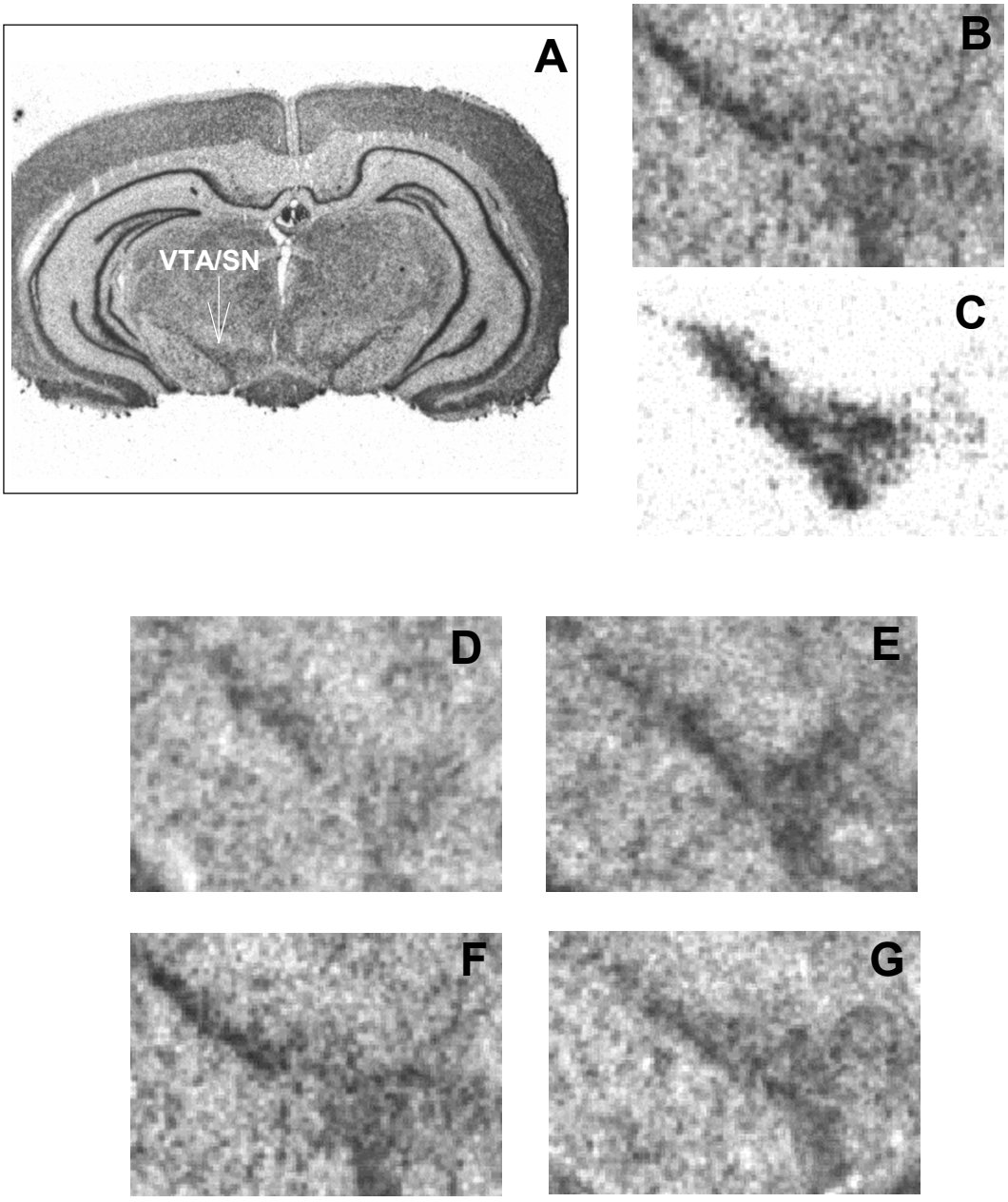

Figure 3.5

CHAPTER 4

THE EFFECTS OF β-ADRENORECEPTOR BLOCKADE DURING CHRONIC EXERCISE ON CONTEXTUAL FEAR CONDITIONING AND mRNA FOR BDNF *

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*Van Hoomissen, J.D., Holmes, P.V., Zellner, A.S., Poudevigne, A.M. & Dishman, R.K. Submitted to *Behavioral Neuroscience* June 25, 2002.

Abstract

The effects of β-adrenoreceptor blockade during chronic exercise on contextual fear conditioning (CFC), mRNA for brain-derived neurotrophic factor (BDNF) in the hippocampal formation (HF), and plasma corticosterone (CORT) and adrenocorticotropic hormone levels (ACTH) were examined. Rats were assigned to 5 groups: home cage (HC), sedentary/placebo (SED/PLBO), activity wheel/placebo (AW/PLBO), sedentary/propranolol (SED/PROP), activity wheel/propranolol (AW/PROP). Rats were implanted with subcutaneous 21-day time-release pellets of propranolol or placebo and remained sedentary or had access to an activity wheel in their cages for 21 days. All animals (except HC) underwent CFC and were killed immediately after the testing session. Freezing behavior during the testing session was elevated in the AW/PLBO compared to SED/PLBO ($p=0.025$) and AW/PROP ($p=0.05$) groups. CFC significantly elevated BDNF mRNA in the HF above HC levels in all of the experimental groups ($p<0.01$). CORT and ACTH levels were significantly elevated above the HC levels during the CFC testing session ($p<0.001$). These results suggest that exercise enhances aversively motivated behaviors and that antagonism of the β-adrenoreceptors attenuates this effect. In addition, neurotrophic factor mRNA is elevated by exposure to aversive conditioning.

Introduction

Previous studies have indicated that chronic activity wheel running or treadmill exercise training increase brain-derived neurotrophic factor (BDNF) messenger RNA (mRNA) in the hippocamapal formation (HF) (Barde, Edgar, & Thoenen, 1982; Carro, Nunez, Busiguina, & Torres-Aleman, 2000; Neeper, Gomez-Pinilla, Choi, & Cotman, 1995, 1996; Oliff, Berchtold, Isackson, & Cotman, 1998; Russo-Neustadt, Beard, & Cotman, 1999; Russo-Neustadt, Beard, Huang, & Cotman, 2000; Widenfalk, Olson, & Thoren, 1999). The mechanism by which chronic physical activity could lead to an upregulation of BDNF mRNA in the brain and the behavioral significance of the increase have not been fully examined (Russo-Neustadt, Ha, Ramirez, & Kesslak, 2001).

The exercise-induced increase in BDNF mRNA in the HF may be mediated by the adaptations in the central noradrenergic neurotransmitter system that occur after chronic exercise (Dishman, 1997). Acute exercise increases extracellular norepinepherine (NE) in brain cortex (Pagliari & Peyrin, 1995a, 1995b) but decreases post-mortem brain tissue concentrations of NE (Barchas & Freedman, 1963). Chronic exercise elevates baseline concentrations of NE in the brain (Dunn, Reigle, Youngstedt, Armstrong, & Dishman, 1996), protects against NE depletion during stress (Dishman et al., 1997; Soares et al., 1999), downregulates β-adrenoreceptors (Yoo, Tackett, Crabbe, Bunnell, & Dishman, 2000), and influences gene expression for neuromodulatory peptides of the NE-system (O'Neal, Van Hoomissen, Holmes, & Dishman, 2000).

There is a neuroanatomical pathway that links the NE system and the HF. Efferent fibers from the locus coeruleus, a brainstem nucleus containing the highest concentration of NE-containing neurons in the brain, provide the sole source of NE to the

HF (Moore & Bloom, 1979; Foote, Bloom, & Aston-Jones, 1983). Also, there is a reciprocal influential relationship between NE and BDNF. NE has an inhibitory effect on the level of BDNF in the HF, as evidenced by the increase in BDNF after axonal lesion of the NE system and the decrease in BDNF after treatment of HF slices with exogenous NE (Hutter, Johansson, Saria, & Humpel, 1996). In turn, BDNF influences noradrenergic cellular differentiation (Sklair-Tavron & Nestler, 1995) and NE concentrations (Siuciak, Boylan, Fritsche, Altar, & Lindsay, 1996). Because the available evidence is sufficient to hypothesize that NE mediates the increase in BDNF mRNA in the HF after exercise, we examined the effect of chronic antagonism of NE receptors on the exercise-induced upregulation of BDNF using the drug propranolol, a mixed β-adrenoreceptor antagonist.

The behavioral significance of the exercise-induced upregulation of BDNF mRNA in the HF is unknown. Previous studies have indicated that exercise alters behavioral measures of learning and memory (Fordyce & Farrar, 1991a, 1991b; Fordyce & Wehner, 1993; Van Praag, Christie, Sejnowski, & Gage, 1999; Anderson et al., 2000), but the mechanism behind the effect is unknown. It might involve elevations in BDNF in the HF, because studies have indicated that the HF is important in learning and memory (Maren, 2001). Moreover, various behavioral learning paradigms have been shown to influence BDNF mRNA and/or protein in the HF or are impaired by either antisense oligoneucleotide injections or antibodies directed towards BDNF (Hall, Thomas, & Everitt, 2000; Ma, Wang, Wu, Wei, & Lee, 1998; Mizuno, Yamada, Olariu, Nawa, & Nabeshima, 2000; Mu, Li, Yao, & Zhou, 1999). Therefore, in the present study, we examined the effect of chronic activity wheel running on contextual fear conditioning, a Pavlovian form of learning that involves the activity of the HF (Fanselow, 2000), and

determined whether changes in aversively motivated learning were associated with BDNF gene expression in the HF.

CFC is an aversive stressor that results in the activation of the hypothalamo-pituitary-adrenocortical (HPA)-axis (Cordero, Merino, & Sandi, 1998). Previous studies have indicated that chronic exercise alters neuroendocrine responses to acute stress (White-Welkley, Bunnell, Mougey, Myerhoff, & Dishman, 1995; White-Welkley et al., 1996; Dishman et al., 1998), but the meaning of those effects is unknown. One hypothesis suggests that some of the health benefits of chronic exercise result from a cross-stressor adaptation, in which chronic exercise alters the physiological response to a non-exercise stressor (Sothmann et al., 1996). In animal models, the types of non-exercise stressors examined have primarily been non-naturalistic, external stressors, such as immobilization and foot shock. The influence of exercise on the neuroendocrine hormone levels after exposure to an environment that produces "fear-like" behavior in the rat has not been examined. In addition, few studies have examined how chronic exercise influences the central nervous system response to stress (Dishman et al., 1997; Soares et al., 1999; Dishman, Renner, White-Welkley, Burke, & Bunnell, 2000). Therefore, we examined the corticosterone (CORT), adrenocorticotropic hormone (ACTH) and mRNA responses to a conditioned stressor.

We were interested in determining the effect of acute exposure to a contextually conditioned aversive environment that results in "fear-like" behavior on BDNF mRNA gene expression. The downregulation of neurotrophic factors after exposure to stress has been hypothesized to be a negative consequence of stress. This effect could lead to clinical pathological conditions, such as depression, through its influence on neural

plasticity (Gage, 2000; Nibuya, Morinobu, & Duman, 1995; Ueyama et al., 1997; Vaidya & Duman, 2001). The elevation of baseline BDNF mRNA in the brain after chronic exercise may represent a neuroprotective effect of exercise that protects against subsequent stress-induced downregulation of BDNF mRNA (Russo-Neustadt et al., 2001).

We hypothesized that chronic physical activity would enhance behavioral expression of CFC, and this result would be associated with increased levels of BDNF in the HF. In addition, we hypothesized that chronic blockade of β-adrenoreceptors would attenuate the increase in expression of CFC and BDNF mRNA in the HF observed after chronic exercise.

Materials and Methods

Animals

Male Long Evans (269±23 gm, 45 days of age) rats were purchased from Charles River (Raleigh, NC). Animals were given a 1-2 week accommodation period upon arrival to the vivarium, during which time the animals were individually housed in polypropylene cages, handled daily, and given free access to food and water. Ambient temperature was maintained at 23±1°C and a 12 hour light-dark cycle was initiated (lights on at 9:00 a.m.-9:00 p.m.). All procedures were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and the U.S. National Institutes of Health Guide regarding the care and use of animals for experimental procedures and were approved by the University of Georgia Animal Care and Use Committee.

Experimental Procedures

Animals were randomly divided into five groups (n=10-11/group): (1) home cage (HC), (2) sedentary/placebo (SED/PLBO), (3) activity wheel/placebo (AW/PLBO), (4) sedentary/propranolol (SED/PROP), (5) activity wheel/propranolol (AW/PROP). On day 0 of the experiment, animals were implanted with subcutaneous time-release pellets containing either 25 mg of propranolol, a lipophillic mixed β-adrenoreceptor antagonist, or placebo pellets, which contained no active drug (Innovative Research of America, Sarasota, FL). Pellets were designed for slow, continuous release of drug over a 21-day period. This method of drug delivery was chosen in order to maintain an elevated sustained concentration of propranolol throughout the light-dark cycle, and to avoid drug concentration peaks and valleys that occur after intraperitoneal injections. Pellets were inserted subcutaneously under Halothane anesthesia above the left scapula via a 1 cm rostral-caudal incision, which was 2 cm lateral of midline. The AW/PLBO and AW/PROP groups were allowed 24-hour free access to activity wheels (circumference=105 cm) placed within their cages for 21 days (day 0-day 21), beginning the day of the pellet implantation. Activity wheels were connected to an electromagnetic counter and daily running distance was determined by multiplying the number of revolutions by the circumference of the wheel. The SED/PLBO and SED/PROP groups remained sedentary throughout the entire experiment. The HC group served as a control group for comparisons of physiological responses to CFC and received placebo capsules, remained sedentary, and did not undergo CFC. Wheels were removed from the cages on day 21, 3 weeks after implantation of the pellets and the initiation of activity wheel running. Behavioral testing was conducted on day 22 and 23.

Contextual Fear Conditioning

The CFC protocol consisted of 2 days of behavioral assessment: a conditioning session on day 22, followed by a testing session 24 hours later on day 23. All behavioral testing was initiated 1 hour after lights on (10:00 a.m.) and was concluded within 2 hours (12:00 p.m.). The CFC sessions occurred within an enclosed chamber (43.3 x 43.3 x 30.5 cm) with clear plexiglass walls, which contained a grid floor (27 individual grids) that was electrified by an ENV-414S shock scrambler (Med Associates, Inc., St. Albans, VT). The chamber was illuminated by a 150-watt bulb located directly above the chamber and overhead room lights, resulting in 370 lux of illumination in the center of the chamber floor. A white noise generator (65 dB) was used during the conditioning and testing sessions to minimize the effects of extraneous room noise. A digital video camera (TRV-900, Sony Electronics Corp.) was placed next to the chamber in order to record behavior for later viewing.

Twenty-four hours after the discontinuation of running, all animals (except for the HC group) underwent the conditioning session of the CFC protocol, which lasted 6 minutes. Each animal was individually transported to the testing room and placed in one of the corners of the chamber, facing the chamber walls. During a 3-minute habituation period, the animals were allowed to freely explore the area. After the habituation period ended, animals received a 2 second, 0.8 mA scrambled foot shock that was repeated at minutes 4 and 5. The animal remained in the chamber for 1 minute after the final foot shock and was then returned to the vivarium. The chamber was cleaned with dilute Nolvasan prior to the introduction of the next animal to remove possible olfactory cues.

Twenty-four hours after the conditioning session, animals were individually placed within the chamber for an 8-minute testing session, during which time the animal was allowed to freely explore the chamber without foot shocks. Animals were killed by rapid decapitation immediately after the testing session. The HC group did not undergo contextual fear conditioning, but remained undisturbed in the vivarium on days 22 and 23, until they were killed by rapid decapitation on day 23.

Freezing behavior during the conditioning and testing sessions was monitored by two, blinded raters in order to calculate interrater reliability. One rater was present in the testing room during the behavioral testing, whereas the other rater assessed behavior from the videotapes. Freezing was defined as behavioral immobility except for the movement associated with respiration (Cordero, Merino, & Sandi, 1998) and was measured using a stop watch to determine the percent of each time interval (60 seconds) spent freezing during the conditioning and testing sessions.

Because freezing behavior changed over the 8-minute testing session, the percent of time spent freezing during each minute was examined to determine the number of stable, consecutive minutes of the testing session to use for hypothesis testing. A mixed model ANOVA decomposed by pairwise contrasts indicated a quadratic trend for time ($F_{(1,38)}=62.49$, $p<0.001$, $eta^2=0.62$), that was explained by increases in freezing behavior during the first 3 minutes of the testing session ($p<0.001$), with a plateau thereafter (Figure 4.1). Therefore, freezing behavior was determined during the first 3 minutes and the last 5 minutes of the testing session. This procedure also facilitated comparison of freezing behavior during the testing session to freezing behavior during the conditioning session, which consisted of an initial 3-minute pre-shock time interval**.** Interrater

agreement and intertrial reliabilities for freezing behavior during the CFC protocol were high (intraclass correlations ≥ 0.90).

Blood and Tissue Collection

Three-milliliters of trunk blood were collected immediately after decapitation in 2% EDTA in sterile saline. Samples were centrifuged at 8,000 x g and plasma was stored at -20°C. Brains were removed from the skull and immediately frozen using dry ice and stored at -80 °C.

In Situ Hybridization Histochemistry

Brains were sliced into 12 μm sections at the level of the dorsal HF using a Microm cryostat (Carl Zeiss, Waldorff, Germany). Sections were thaw-mounted onto gelatin coated glass microscope slides and approximately every tenth section was stained with 0.1% thionin to verify anatomical location. Four sections per animal were selected for hybridization based on anatomical congruence to sections from the Rat Brain Atlas (Paxinos & Watson, 1986). Sections were fixed in 4% formaldehyde in 0.12M sodium phosphate-buffered saline (PBS, pH 7.4) for 5 minutes, rinsed twice in PBS, and placed in 0.25% acetic anhydride in 0.1M triethanolamine HCl/0.9% NaCl (pH 8.0) for 10 minutes. Sections were dehydrated using a series of ethanol washes (70%, 80%, 95%, 100%), delipidated in chloroform for 5 minutes, rinsed in ethanol (100%, 95%), and allowed to dry.

Oligonucleotide probes were purchased from Oligos Etc. (Wilsonville, OR). The BDNF probe sequence was complementary to bases 650-699 of the mouse BDNF mRNA transcript, which is 98% homologous to rat (Hofer, Pagliusi, Hohn, Leibrock, & Barde, 1990; Maisonpierre et al., 1991). Probes were labeled at the 3'-end with [^{35}S]-dATP

(1000-1500mCi/mmol); New England Nuclear, Boston, MA), terminal deoxynucleotidyl transferase (TdT) (25 units/ml; Roche, Indianapolis, IN), and tailing buffer. Column separation was used to separate unincorporated nucleotides from the probes. Sections were hybridized with radiolabeled probes in solution containing 50% formamide, 600mM NaCl, 80 mM Tris-HCl, 4 mM EDTA, 0.1% sodium pyrophosphate, 0.2% SDS, 0.2 mg/ml heparin sulfate, and 10% dextran sulfate. Brain sections were incubated with the hybridization solution for 20 hours in a humid chamber at 37°C. Sections were then subjected to a series of washes to reduce nonspecific binding (3, 1xSSC; 1, 2xSSC/50% formamide; 3, 2xSSC/50% formamide for 20 minutes at 40°C; 2, 1xSSC for 30 minutes at 22°C). Slides were rinsed in deionized water and 70% ethanol and allowed to dry. Sections were exposed to autoradiograhpic film (BioMax MR, Eastman Kodak, Rochester, NY) and developed with Kodak GBX developer and fixer.

Autoradiographic films were analyzed using a computerized image analysis system to determine optical density (OD) within the HF (Image 1.38 software, Rasband, 1995, National Institute of Mental Health; Power Macintosh 8100 computer, Apple Computer, Cupertino, CA; light box and camera, Imaging Research, St. Catharines, Ontario; video interface, Data Translation, Marlboro, MA). The HF was analyzed by taking the average OD of 10, 8 x 8 pixel circles, placed randomly throughout the brain region of interest (dentate gyrus [DG], Ammon's horn area 1[CA1], Ammon's horn area 3 [CA3]), in each section, of each animal. The investigator was blinded to subject condition at the time of quantification

Radioimmunoassay

A commercially available radioimmunoassay kit was used to determine plasma concentrations of CORT and ACTH (Immunodiagnostic Systems Limited, Costa Mesa, CA). Samples of plasma CORT were run in triplicate (3 trials) on two separate days. Plasma levels of CORT for the HC group were virtually undetectable using the standard sample dilution (1:200) and were obtained from the third trial using a smaller sample dilution. Samples of plasma ACTH were run in duplicate. The intra- and inter-assay coefficients of variation were 3% and 11%, respectively. (1:100).

Data Analysis

Optical density values from the *in situ* hybridization experiments, freezing behavior, and CORT were analyzed by *a priori* planned multiple comparisons (1-tailed independent samples t-tests), with no adjustment made to control the familywise error rate (Keppel, 1991). The *a priori* planned comparisons tested included the following, listed in order of importance: (1) SED/PLBO vs. AW/PLBO to test the effect of exercise on freezing behavior, HPA-axis activation (CORT and ACTH), and gene expression (BDNF mRNA), (2) AW/PLBO vs. AW/PROP to test the hypothesis that adaptations in the norepinephrine system mediate the effects, (3) HC vs. SED/PLBO to test the effect of the CFC protocol on HPA-axis activation (CORT and ACTH) and gene expression (BDNF mRNA).

Effect sizes were estimated by Cohen's D (experimental mean-control mean/pooled standard deviation). Pilot data from our lab suggest that 3 weeks of AW results in a significant increase in BDNF mRNA expression in the HF (Van Hoomissen, O'Neal, Holmes, & Dishman, 2001). Based on the effect sizes obtained from these pilot

experiments (d=1.26–1.52) the sample size in the present experiment (n=10-11) was judged as adequate to provide a statistical power of .80 for detecting a large effect (d>0.8) of exercise on gene expression at an alpha level of $p<0.05$. The sample size needed to adequately power the hypothesis test for freezing behavior and CORT levels after chronic exercise was unknown because there were no available data from which to estimate the expected effect size.

A mixed model (groups x time) ANOVA with post-hoc comparisons (1-tailed independent samples t-tests) was used to analyze the differences in running distance between the two activity-wheel running groups. A one-way ANOVA, with post-hoc comparisons was used to analyze the effect of the experimental manipulations on overall weight gain. All statistical analyses were conducted using SPSS Windows version 10.1 (SPSS, Inc., Chicago, IL).

Results

Contextual Fear Conditioning

The difference in freezing behavior (mean±SD) between the AW/PLBO and SED/PLBO groups during the 3-minute pre-shock period of the conditioning session (3.1±2.6% vs. 5.9±5.1%, respectively) approached statistical significance ($t_{(19)}$=-1.601, p=0.06), but was not different during the post-shock period (AW/PLBO=69.8±12.9%, SED/PLBO=63.5±15.5%, $t_{(19)}$=1.009, p=0.16). The difference in freezing behavior between the AW/PLBO and AW/PROP was not significantly different between the groups during the 3-minute pre-shock period (5.9±5.1% vs. 9.4±7.7%, respectively, $t_{(18)}$=-1.204, p=0.122) or post-shock period (69.8±12.9% vs. 63.1±17.3%, respectively, $t_{(18)}$=0.986, p=0.17).

Freezing behavior was significantly elevated in the AW/PLBO group compared to the SED/PLBO group during the first 3 minutes of the testing session (63.6±17.5% vs. 43.6±26.4%, respectively, $t_{(20)}$=2.085, p=0.025, d=0.88) but not during the last 5 minutes of the testing session (70.2± 24.0% vs. 56.8±35.6%, respectively, $t_{(17.5)}$=1.031, p=0.158). Freezing behavior was significantly attenuated in the AW/PROP group compared to the AW/PLBO group during the first 3 minutes of the testing session (48.6±23.2% vs. 63.6±17.5%, respectively, $t_{(19)}$=-1.657, p=0.05, d=0.73), but not during the last 5 minutes of the testing session (63.4±24.4% vs. 70.2±24.0%, respectively, $t_{(19)}$=0.640, p=0.265) (Figure 4.2).

BDNF mRNA

BDNF mRNA levels in the HF were not different between the AW/PLBO and SED/PLBO groups (DG, $t_{(14.7)}$=0.464, p=0.325; CA3, $t_{(20)}$=0.034, p=0.487; CA1, $t_{(20)}$=0.195, p=0.424) or between the AW/PLBO and AW/PROP groups (DG, $t_{(19)}$=0.332, p=0.372; CA3, $t_{(19)}$=-0.212, p=0.418; CA1, $t_{(19)}$=-0.270, p=0.395). BDNF mRNA was significantly elevated above HC levels in the SED/PLBO group (DG, $t_{(12.4)}$=-2.707, p=0.009, d=1.15; CA3, $t_{(16.4)}$=-3.178, p=0.003, d=1.39; CA1, $t_{(13.2)}$=-3.637, p=0.001, d=1.56). Further post-hoc analyses indicated that BDNF mRNA levels were also elevated above HC levels in the AW/PLBO (DG, $t_{(20)}$=-5.551, p=0.001, d=2.36; CA3, $t_{(15.6)}$=-3.019, p=0.004, d=1.30; CA1, $t_{(16.6)}$=-4.003, p=0.001, d=1.70), SED/PROP (DG, $t_{(19)}$=-3.567, p=0.001, d=1.56; CA3, $t_{(19)}$=-2.890, p=0.005, d=1.28; CA1, $t_{(11.9)}$=-3.055, p=0.006, d=1.30), and AW/PROP (DG, $t_{(12.8)}$=-3.925, p=0.001, d=1.69; CA3, $t_{(19)}$=-3.707, p=0.001, d=1.60; CA1, $t_{(12.6)}$=-4.125, p=0.001, d=1.76) groups (Figures 4.3-4.4).

Corticosterone and ACTH

CORT and ACTH levels (mean±SD) were not different between the AW/PLBO and SED/PLBO groups (CORT, 368±70 ng/ml vs. 326±67 ng/ml, respectively, $t_{(20)}$=-1.422, p=0.08; ACTH, 212±63 pg/ml vs. 206±53 pg/ml, respectively, $t_{(18)}$=-0.247, p=0.404) or between the AW/PLBO and AW/PROP (CORT, 368±70 ng/ml vs. 345±65 ng/ml, respectively $t_{(19)}$=0.760, p=0.238; ACTH, 212±63 pg/ml vs. 251±92 pg/ml, respectively, $t_{(16)}$=-1.060, p=0.151) groups. CFC significantly elevated CORT and ACTH levels in the SED/PLBO group compared to the HC group (CORT, 326±67 ng/ml vs. 7 ng/ml, respectively, $t_{(10.091)}$=-15.716, p=0.001, d=6.72; ACTH 206±53 pg/ml vs. 54±10 pg/ml, $t_{(9.626)}$=-8.853, p=0.001, d=3.99). Further post-hoc analyses indicated that CORT and ACTH levels were also significantly elevated above HC levels (CORT, 7 ng/ml; ACTH, 54±10 pg/ml) in the AW/PLBO (CORT, 368±70 ng/ml, $t_{(10.082)}$= -16.836, p=0.001, d=7.19; ACTH, 212±63 pg/ml, $t_{(9.451)}$=-7.871, p=0.001, d=3.55), SED/PROP (CORT, 363±76 ng/ml, $t_{(9.058)}$=-14.714, p=0.001, d=6.61; ACTH, 201±71 pg/ml, $t_{(5.116)}$=-5.000, p=0.004, d=3.41) and AW/PROP (CORT, 345±65 ng/ml, $t_{(9.078)}$=-16.222, p=0.001, d=7.25; ACTH, 251±92 pg/ml, $t_{(7.130)}$=-6.004, p=0.001, d=3.21) groups. (Figure 4.5).

Running Distance and Weight Gain

There was a significant main effect of time on the running distance during the 3 weeks of the experiment ($F_{(2,36)}$=26.34, p=0.001). The difference in the overall average daily running distance between the AW/PLBO and AW/PROP approached statistical significance (6037±1233 vs. 4886±1847 meters/day, respectively, $F_{(1,18)}$=2.931, p=0.06) (Figure 4.6).

Weight gain over the course of the three-week experiment was significantly different among the groups ($F_{(4,47)}$=3.571, p=0.013). Post-hoc comparisons revealed a significantly larger average weight gain (mean ± SD) for the HC (95.8±28.7 gm) and SED/PLBO (103.1±24.9 gm) groups compared to AW/PLBO (73.0±25.8 gm), SED/PROP (75.8±12.4 gm) and AW/PROP (73.4±26.8 gm) groups (p=0.007-0.014, d=0.84-1.39).

Discussion

The results suggest that chronic activity wheel running enhances aversively motivated learning, as demonstrated by an increase in freezing behavior during the testing session of the CFC protocol, but the increased level of freezing in the activity wheel group is not associated with an increase in BDNF mRNA in the HF after the testing session of the CFC protocol. The effect of activity wheel running on freezing behavior may indicate that physical activity leads to enhanced conditioned fear memory formation, which supports other investigations which reported that physical activity influences behavioral tests of learning and memory in animal models. For example, chronic physical activity has been shown to improve performance in the Morris water maze, the place-learning set-task, and the radial arm maze test (Fordyce & Farrar, 1991a, 1991b; Fordyce & Wehner, 1993; Van Praag et al., 1999; Anderson, et al., 2000). The present experiment extends those findings to contextual fear conditioning.

Our results are in agreement with previous research in mice, reporting increased CFC after exposure to enriched environments, in which part of the environment contained an activity wheel (Tang, Wang, Feng, Kyin, & Tsien, 2001). We are reporting that environmental enrichment with an activity wheel alone is sufficient to enhance

contextually cued, aversively motivated behavior. The increase in "fear-like" behavior in the AW/PLBO group may represent a behaviorally appropriate and adaptive response that might serve to heighten an animal's awareness of the surrounding environment (Klemm, 2001). Instead of viewing the increase in freezing behavior in the AW/PLB O group as an effect of an "exercise condition" or "environmental enrichment" it may, instead, represent the behaviorally natural response to the environment. The behavior of the SED/PLBO group, however, may represent the effect of an unnatural "sedentary condition" devoid of any environmental enrichment.

The mechanism for the enhancement of aversively motivated behavior after chronic exercise was explored. We hypothesized that activity wheel running would increase freezing behavior during the testing session and that this effect would be associated with elevated levels of BDNF mRNA in the activity wheel running groups compared to the sedentary groups. Results from the *in situ* hybridization experiment did not support this hypothesis. BDNF mRNA levels in the HF were similar among the groups that underwent the CFC protocol, but were elevated compared to the HC group.

The meaning and significance of the increase in BDNF mRNA after exposure to an aversively conditioned environment is unknown. We know of only one study that has examined the effect of CFC on BDNF gene expression. Hall et al. (2000) reported an increase in the expression of BDNF mRNA in the CA1 region of the HF after the CFC conditioning session in rats, suggesting that BDNF is related to behavioral forms of hippocampal learning. In the present study, however, we are reporting an increase in BDNF mRNA in all regions of the HF after the *testing* session. The observed increase in BDNF mRNA immediately after the testing session (24 hours after the conditioning

session) suggests that the response is not specific to the acquisition or consolidation phase of the CFC protocol, but may be involved in fear-memory recall. Because we only examined mRNA levels after the testing session, we cannot conclude that the elevation in BDNF mRNA was a result of exposure to the testing session. There remains the possibility that the observed increase in mRNA in all of the groups was the result of a prolonged, sustained elevation of BDNF mRNA that occurred after the conditioning session. Future studies should address this hypothesis.

Because CFC confounds stress and learning, the increase in BDNF mRNA after the testing session may be the result of either aspect of the behavioral test. The increase in BDNF mRNA may represent a response to a specific type of active stressor. It is interesting to note, that immobilization has been shown to have the opposite effect, resulting in a downregulation in the levels of BDNF mRNA in the HF (Ueyama et al., 1997), but an upregulation of BDNF in other brain regions, such as the hypothalamus and pituitary (Smith, Makino, Kim, & Kvetnansky, 1995). In addition, BDNF mRNA levels are downregulated in the HF after forced swimming in the Porsolt swim test (Russo-Neustadt et al., 2001). It appears, then, that the BDNF mRNA response is stressor- and region-specific.

Chronic treatment with the β-adrenoreceptor antagonist, propranolol, resulted in the attenuation of the exercise-induced increase in freezing behavior during the testing session of the CFC protocol. These results may further emphasize the central role of the adaptations of the NE system after chronic exercise in influencing behaviors altered by exercise (Dishman, 1997). The attenuation of freezing behavior in the AW/PROP compared to the AW/PLBO most likely occurred, however, as a result of an overall

reduction in running behavior in the AW/PROP group. A post-hoc analysis of covariance, controlling for the overall average daily activity wheel running distance, supports this hypothesis. The difference in freezing behavior during the first 3 minutes of the testing session was similar between the two groups, when the overall average daily activity wheel running distance was included as a covariate in the statistical model (adjusted group means, AW/PLBO=61.0%, AW/PROP=51.5%, p=0.166). Therefore, it is possible that the observed reduction in freezing behavior in the AW/PROP was due to an overall reduction in running distance. This analysis, however, emphasizes the relationship between freezing behavior and activity wheel running, indicating that the distance an animal runs might determine the level of freezing behavior.

Our results showed an increase in the freezing behavior of the AW/PLBO group compared to the SED/PLBO group despite the unique characteristics of free-access activity wheel running as an exercise protocol. Because animals are allowed 24-hour free access to activity wheels, important aspects of physical activity, such as the exercise intensity, duration, and frequency are left uncontrolled by the experimenter. Nonetheless, 21 days of chronic activity wheel running significantly elevated freezing behavior during the testing session and was correlated with the average daily running distance overall (r=0.409, p=0.033) and during week 3 (r=0.528, p=0.008). It would be of interest to investigate other types of exercise training protocols, such as treadmill exercise training, which allows for greater control of the exercise stimulus.

Alternative explanations for the increase in freezing behavior, aside from an increase in contextually cued aversively motivated behavior in the AW/PLBO group compared to the SED/PLBO group, need to be addressed. One alternative explanation is

that activity wheel running may have altered the nociceptive response to the foot shock presented during the conditioning session, which may have consequently altered the behavioral response during the testing session. Previous studies have indicated that freezing behavior during the conditioning and testing sessions is positively related to the intensity of the foot shock (Cordero et al., 1998). If activity wheel running altered the nociceptive response to the foot shock during the conditioning session, altered post-shock freezing behavior would have been expected. In the present study, however, both of the activity wheel running groups displayed similar post-shock freezing behavior compared to the sedentary groups (SED/PLBO=63.5%, AW/PLBO=69.8%, SED/PROP=62.8%, AW/PROP=63.1%).

A second alternative hypothesis is that the increase in freezing behavior during the testing session was the result of an overall increase in freezing behavior in the activity wheel running group. In the present study, the difference in freezing behavior between SED/PLBO and AW/PLBO during the first 3 minutes of the conditioning session approached statistical significance (3.1% vs. 5.9%, $p=0.06$). The practical significance of this difference is limited because the results indicate a difference in freezing behavior of approximately 5 seconds out of 3 minutes. To further investigate this hypothesis we conducted a post-hoc analysis of covariance, including freezing behavior during the conditioning session as the covariate in the statistical model. Freezing behavior during the first 3 minutes of the testing session was still elevated in the AW/PLBO group compared to the SED/PLBO group when overall freezing during the conditioning session was statistically controlled (adjusted means, AW/PLBO=62.3% vs. SED/PLBO=45.4%, $p<0.05$).

In order to conclude that activity wheel running increased contextually cued, aversively motivated behavior it would be of interest to determine the context specificity of the behavioral response (freezing). Studies have indicated, that freezing behavior during the testing session of the contextual fear conditioning protocol is increased when animals are placed back into the environment in which they were conditioned, compared to an unconditioned environment (Fanselow, 1980; Fanselow, 2000). Further research is needed to determine whether the enhancement of aversively motivated behavior in the exercise group is specific to the conditioning chamber.

The increase in freezing behavior in the activity wheel running animals during the testing session might appear at odds with the literature showing that chronic exercise increases ambulatory activity in the open field behavioral test (Dishman et al., 1996; Tharp & Carson, 1975; Weber & Lee, 1968). Previous studies have shown that exercise increases ambulatory distance within this environment, which has been interpreted as an "anxiolytic-like" effect of exercise. In this experiment, the SED/PLBO and AW/PLBO groups displayed similar levels of overall ambulatory activity during the conditioning session (distance traveled, SED/PLBO=717±214 cm vs. 671±232 cm, $p=0.331$). In the testing session, the AW/PLBO group had significantly lower levels of overall ambulatory activity compared to the SED/PLBO group (134±167 cm vs. 370±397 cm, respectively, $p=0.045$), which was expected because of an increase in freezing behavior in the AW/PLBO group. We caution against comparing the present results to the open field behavioral data, as the testing environment in the CFC protocol represents an aversively conditioned environment to which the animal has been previously exposed, whereas the open field testing chamber is an unconditioned environment.

The reason for the lack of the hypothesized elevation in BDNF mRNA levels in the AW/PLBO group after CFC, compared to the other groups that underwent CFC, is unknown. At the current time, we are unaware of data that support the hypothesis that BDNF is directly involved in contextual fear conditioning (Hall et al., 2001). Studies that have explored the role of BDNF in learning and memory have focused on other types of behavioral tasks involving spatial memory, but have yet to explore its role in Pavlovian conditioning. Its probable that the exercise-induced elevation in freezing behavior occurred as the result of a BDNF-independent mechanism, involving other neurotransmitter systems, such as the glutamate system, that have been shown to be important in contextual fear conditioning (Maren, 2001). In addition, its also possible that exercise enhanced freezing behavior by elevating BDNF mRNA *prior to* or *immediately after* the conditioning session, which may have facilitated consolidation of the aversive memory and resulted in potentiated freezing behavior on the second day of testing. The use of antisense oligonucelotide technology to block BDNF protein expression or heterozygote mutant mice that contain depressed levels of BDNF are two possible methods for exploring the hypothesis of a BDNF-mediated mechanism for enhanced contextually cued aversively motivated behavior after chronic exercise.

Because we isolated brain tissue immediately after the testing session, and did not use a full factorial experimental design (i.e. including four additional groups that did not undergo CFC but received the same experimental conditions), we were unable to directly test the hypothesis for a NE-mediated mechanism responsible for the exercise-induced increase in BDNF mRNA in the HF. It is possible that chronic antagonism of the β-adrenoreceptors during the exercise period attenuated the exercise-induced increase in

BDNF mRNA in the HF, but this effect was not observable because of the increase in BDNF mRNA in the HF of all groups that underwent CFC.

Plasma CORT and ACTH levels were elevated above HC levels immediately following the testing session, but activity wheel running and chronic treatment with propranolol had no effect on these levels. We hypothesized that exercise would alter the level of CORT and ACTH after exposure to the aversively conditioned environment, thereby supporting the conclusion that physical activity influences the stress response to non-exercise stressors. Although the difference in CORT levels between the SED/PLBO and AW/PLBO groups did not reach statistical significance ($p=0.08$) the results represented a moderate effect ($d=0.61$). These results, along with previous research from our lab (Dishman et al., 1998; White-Welkley et al., 1995; White-Welkley et al., 1996), further support that exercise results in a stressor-specific cross-stressor adaptation.

In conclusion, we observed an increase in contextually cued aversively motivated behavior in rats after 3 weeks of chronic activity wheel running that was attenuated by blockade of β-adrenoreceptors. This effect was not associated with an increased expression of BDNF mRNA in the HF. Exposure to the contextual fear conditioning environment, however, increased levels of BDNF in the HF and elevated plasma levels of CORT. Future studies should further address mechanisms, in addition to a NE-mediated mechanism, responsible for the enhanced level of freezing behavior in the exercise group as well as the behavioral significance of the elevation in BDNF mRNA after contextual fear conditioning.

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Figure Captions

Figure 4.1. Freezing behavior as a percent of each one-minute time interval (100% maximum) during the (A) conditioning and (B) testing sessions of the CFC behavioral protocol.

Figure 4.2. Freezing behavior as a percent of each time interval (100% maximum) during the (A) conditioning and (B) testing sessions of the CFC behavioral protocol.

Figure 4.3. BDNF mRNA optical density in the (A) DG, (B) CA3, and (C) CA1 regions of the HF. * significantly different from HC (p=0.001-0.019)

Figure 4.4. Representative autoradiograms of BDNF mRNA binding in the HF of the (A) HC, (B) SED/PLBO, (C) AW/PLBO, (D) SED/PROP, (E) AW/PROP groups.

Figure 4.5. Corticosterone (A) and ACTH (B) levels after the testing session of the CFC protocol. *significantly different from HC (p=0.001)

Figure 4.6. Running distance. The difference in overall daily running distance between the AW/PLBO and AW/PROP groups approached statistical significance (p=0.06).

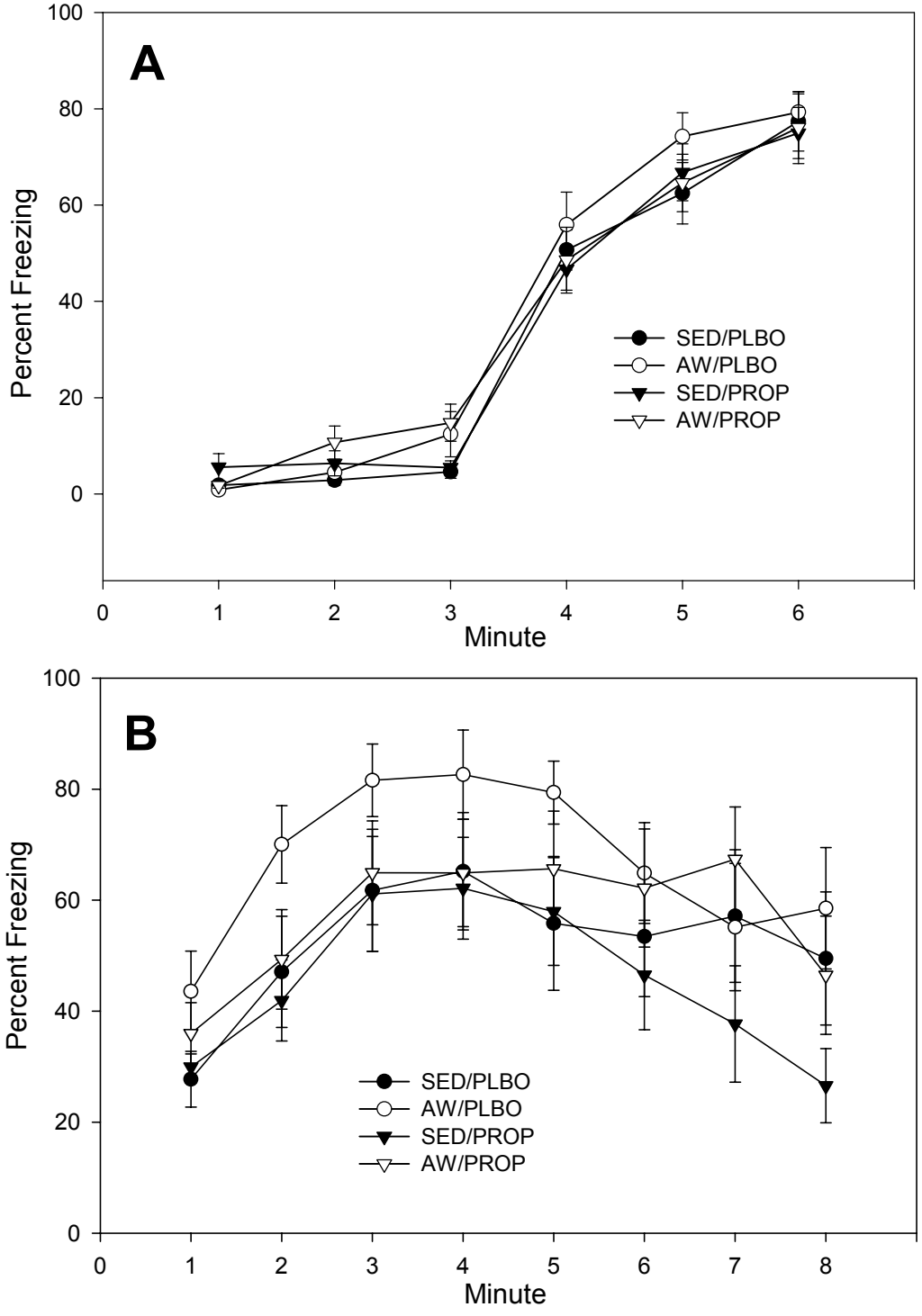
A
Percent Freezing
Minute
SED/PLBO
AW/PLBO
SED/PROP
AW/PROP
B
Percent Freezing
Minute
SED/PLBO
AW/PLBO
SED/PROP
AW/PROP

Figure 4.1

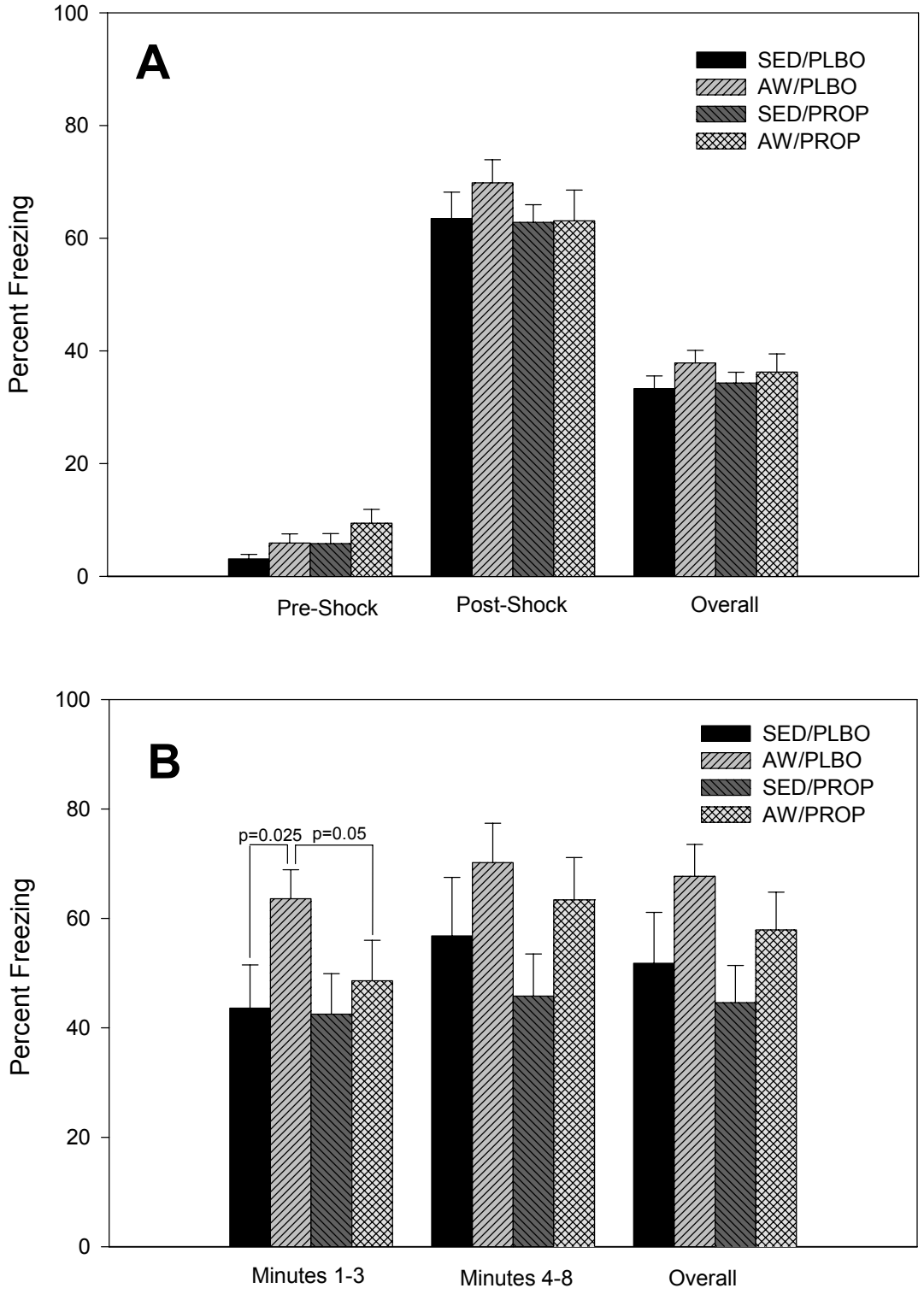
A
SED/PLBO
AW/PLBO
SED/PROP
AW/PROP
Percent Freezing
100
80
60
40
20
0
Pre-Shock
Post-Shock
Overall
B
SED/PLBO
AW/PLBO
SED/PROP
AW/PROP
p=0.025
p=0.05
Percent Freezing
100
80
60
40
20
0
Minutes 1-3
Minutes 4-8
Overall

Figure 4.2

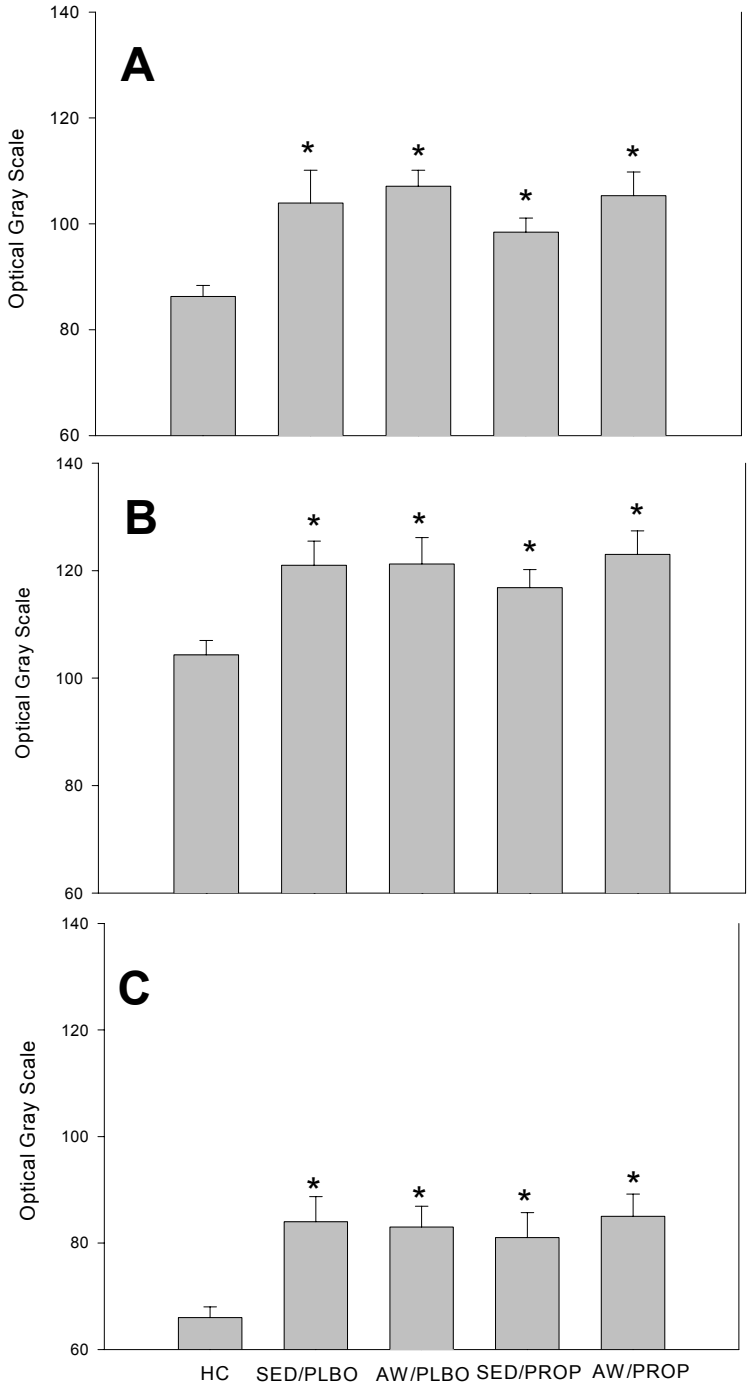
A
B
C
Optical Gray Scale
140
120
100
80
60
*
HC
SED/PLBO
AW/PLBO
SED/PROP
AW/PROP

Figure 4.3

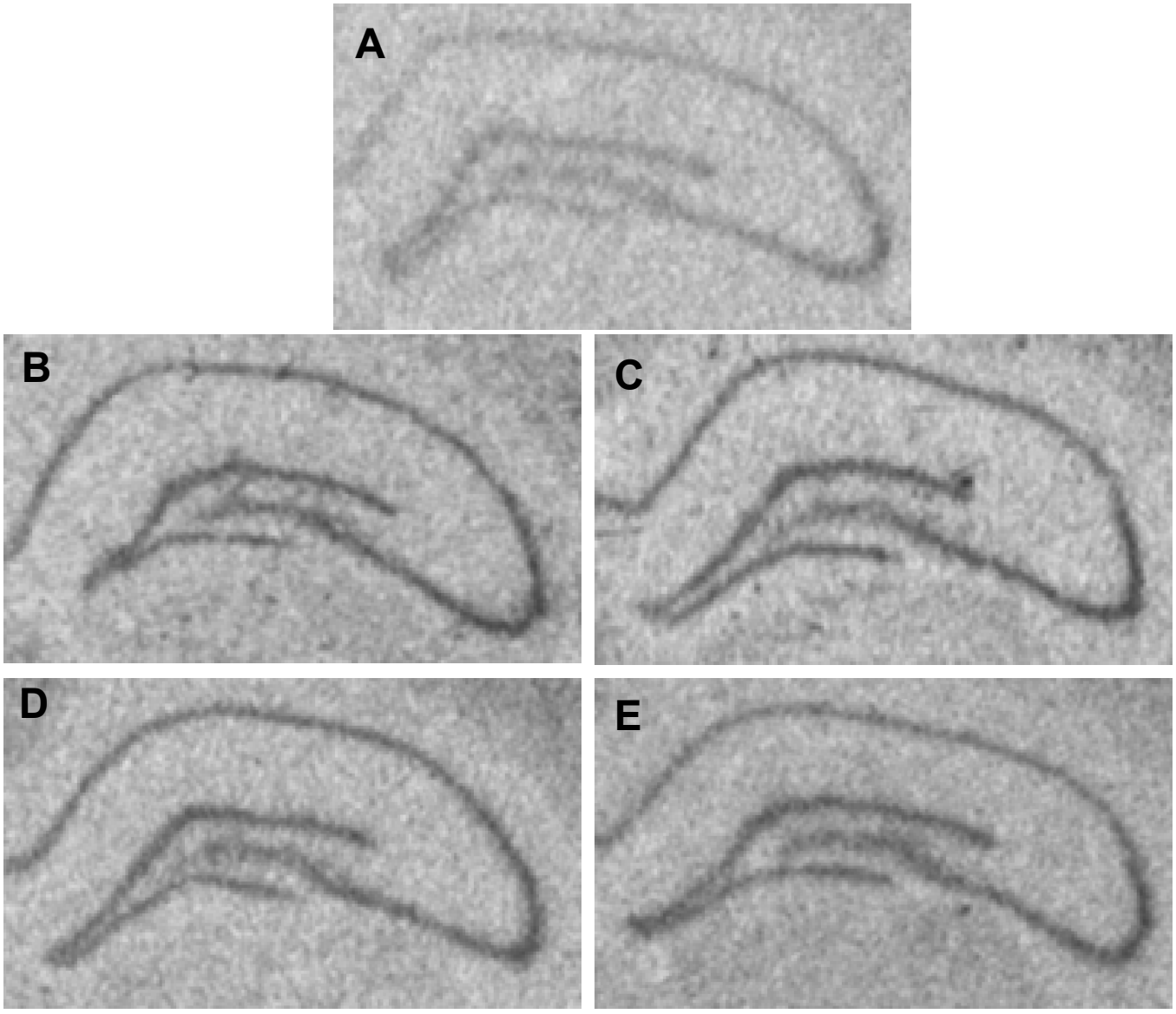

Figure 4.4

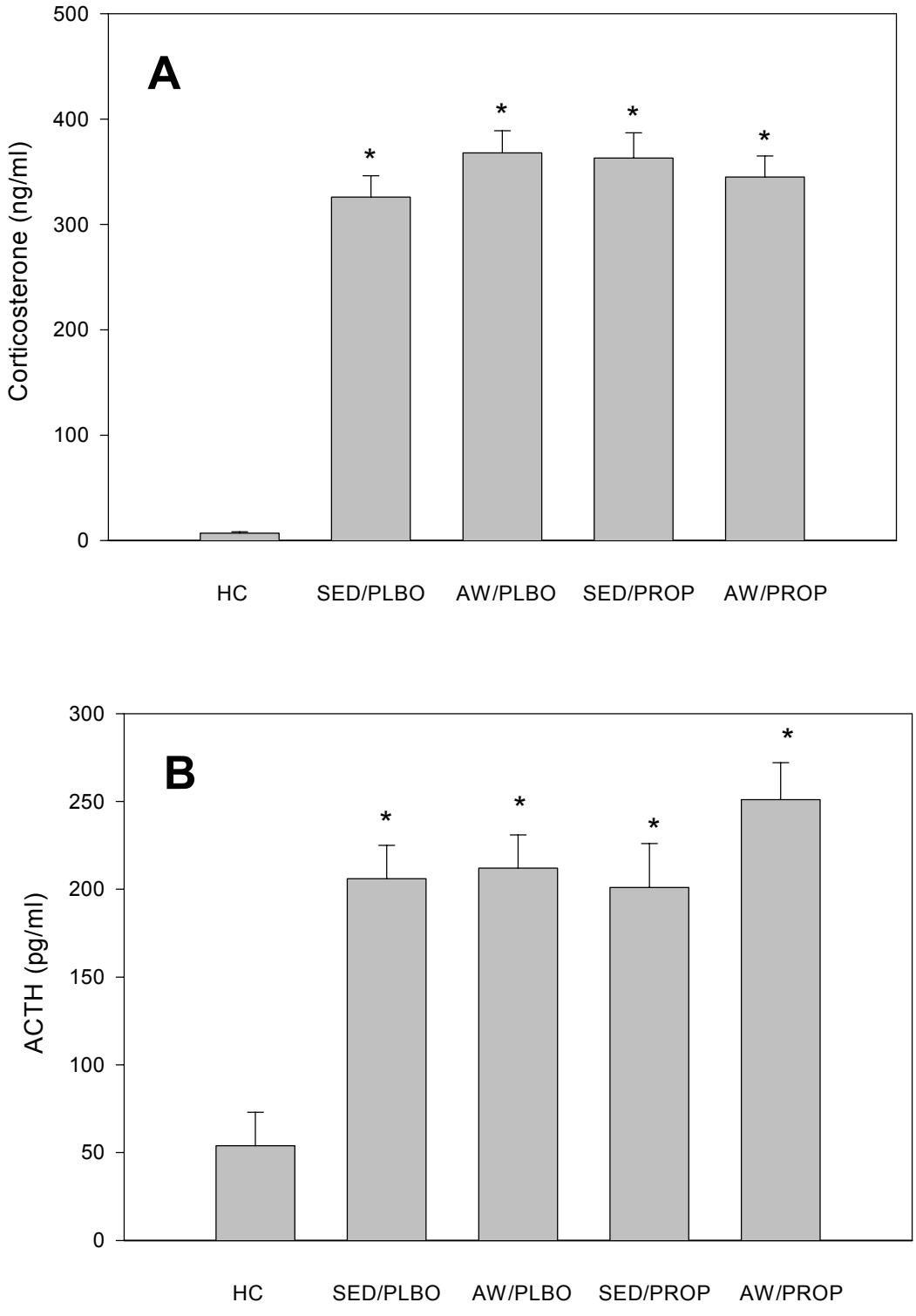
A
Corticosterone (ng/ml)
500
400
300
200
100
0
*
*
*
*
HC
SED/PLBO
AW/PLBO
SED/PROP
AW/PROP
B
ACTH (pg/ml)
300
250
200
150
100
50
0
*
*
*
*
HC
SED/PLBO
AW/PLBO
SED/PROP
AW/PROP

Figure 4.5

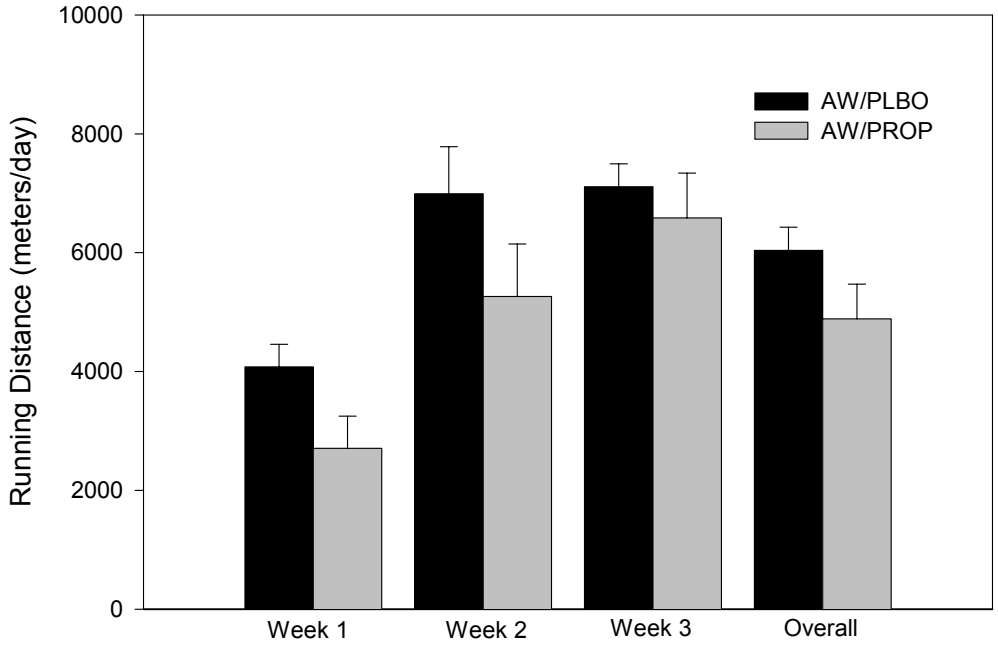
10000
8000
6000
4000
2000
0
Running Distance (meters/day)
AW/PLBO
AW/PROP
Week 1
Week 2
Week 3
Overall

Figure 4.6

CHAPTER 5

SUMMARY

The preceding two studies indicate that chronic physical activity and antidepressant pharmacotherapy increase gene expression for BDNF in the central nervous system. In the first study, we observed an increase in BDNF mRNA in the hippocampal formation (HF) and the ventral tegmental area/substantia nigra region of the rat brain after 3 weeks of activity wheel running and antidepressant pharmacotherapy. Contrary to previous findings, we did not find a potentiation of BDNF mRNA in the HF after combined experimental treatments of activity wheel running and antidepressant pharmacotherapy. Possible reasons for the discrepancy might include differences in experimental protocols, such as the latency between the experimental treatment and when brain tissue was isolated.

Previous studies examining the effect of antidepressant pharmacotherapy on BDNF mRNA have failed to use an animal model of depression that has neurobiological and behavioral features that resemble depression. In our first study, post-hoc analyses revealed an important finding emphasizing the need for the use of animal models. We found that the increase in BDNF mRNA in the HF after antidepressant pharmacotherapy was attenuated in the olfactory bulbectomy animal model of depression that has biochemical and neurobiological features that mimic depression. This finding suggests that the hypothesis that the increase in BDNF mRNA in the HF represents an intracellular

mediator of the therapeutic effects of antidepressant pharmcotherapy, cannot be extended to the animal model.

In our second study, we investigated the mechanism behind and the behavioral significance of the increase in BDNF mRNA in the HF. We examined the effects of activity wheel running and blockade of the central noradrenergic neurotransmitter system on contextual fear conditioning (CFC), a Pavlovian form of learning, that is hypothesized to involve the activity of the HF. We found that chronic activity wheel running increased the behavioral expression of CFC and that CFC increased BDNF mRNA levels in the HF. Chronic antagonism of β-adrenoreceptors, however, attenuated the increase in freezing behavior in the activity wheel running group.

The results suggest that environmental enrichment with an activity wheel enhances behavioral expression of an aversively conditioned response, possibly suggesting that exercise may facilitate some types contextual learning. Future studies should further address this hypothesis by examining the effects of different types of exercise (i.e. activity wheel running vs. treadmill training). In addition, other alternative hypotheses need to be addressed, such as the context specificity of the behavioral response, before a concrete conclusion can be drawn.

The mechanism for the increased freezing behavior that was demonstrated in the activity wheel running group was explored, although not directly tested in this study. Chronic antagonism of the β-adrenoreceptors with propranolol attenuated the increase in freezing behavior in the activity wheel running groups, possibly suggesting a norepinephrine mediated mechanism. Propranolol, however, reduced levels of overall activity wheel running, thereby limiting our conclusions about a norepinephrine mediated

mechanism. We also found that CFC elevated levels of BDNF mRNA in the HF but BDNF mRNA levels were not greater in the activity wheel groups and there was no relationship between the level of BDNF mRNA and freezing behavior. Future studies should further address the mechanism, other than a norepinephrine- or BDNF-mediated mechanism, by which exercise increases aversively conditioned behavioral responses.

Both of these studies emphasize the impact of chronic exercise on brain function *and* behavior. These results provide testable hypotheses for examining the mental health and cognitive benefits of exercise. Because physical activity and exercise have been shown to have beneficial effects for mental health, continued research is needed to further address the mechanisms by which these effects occur.

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