THE ROLE OF THE DOPAMINERGIC SYSTEM IN THE REGULATION OF PHYSICAL ACTIVITY IN MICE

by

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ABSTRACT

AMY MARIE KNAB. The role of the dopaminergic system in the regulation of physical activity in mice. (Under the direction of DR. TIM LIGHTFOOT)

Physical activity (PA) is important to human health, and the genetic and biological regulating factors of physical activity are only beginning to be understood. The dopamine (DA) system has been shown to regulate motivation, and locomotor behavior in animals, and this research was designed to understand the dopaminergic factors important in regulating voluntary physical activity in mice. First, the repeatability of measuring exercise endurance vs. wheel running (WR) in different inbred strains of mice was investigated. It was found that WR behavior is a highly repeatable measurement, while exercise capacity measurements showed low repeatability in Balb/cJ mice. Next, expression levels of the five DA receptors, Tyrosine hydroxylase (TH), and the dopamine transporter (DAT) in the nucleus accumbens and striatum were studied in mice with or without wheel access in differentially active inbred strains of mice. No differences in expression levels of any DA receptors were found within strain between group, suggesting level of PA did not affect DA receptor expression. High active C57L/J mice had significantly decreased expression of *Drd1* and *TH* compared to low active C3H/HeJ mice indicating DA receptor, and enzyme expression/function may act independently to control level of PA. Pharmacological studies showed C57L/J mice significantly decrease WR in response to a D1 agonist, and C3H/HeJ mice significantly increase WR in response to a DAT inhibitor. These results suggest genetic differences in the DA system may mediate differences in PA behavior between inbred strains of mice.

DEDICATION

This dissertation is dedicated to my loving and supportive husband Brian.

Thank you for everything.

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INTRODUCTION

Physical inactivity has become more prevalent in today's society because technological advances have enabled people of Western cultures the freedom to do less work to accomplish the activities of daily living. Before industrialization, daily living still required a good degree of physical activity (22). Even though technological progress can be argued to have significantly advanced society, the burdens of physical inactivity can be experienced in other arenas, including human health (180). Lack of physical activity has been linked to the rising rate of obesity (297), and it is well known that regular physical exercise can improve the risk of heart disease (82), certain types of cancer (251), and depression (58, 134, 248). Thus, understanding the genetic and environmental factors regulating the amount of voluntary physical activity performed by a given individual is crucial to improving human health and standard of living, especially in Western cultures where physical activity is not necessarily required in daily living activities.

Environmental factors involved in physical fitness have been well studied (67, 281). However, genetic and non-genomic biological factors affecting voluntary physical activity have only recently begun to be studied, and are not well understood. Studies have shown that inheritance of physical activity traits in mice is anywhere from 20-80% (69, 119, 135, 149, 249, 262). The fact that there is a genetic component to physical activity behavior is no surprise; however, the actual genes regulating these behaviors have yet to be fully discovered and understood. Lightfoot and colleagues (2008) (153), investigated possible quantitative trait loci (QTL) [QTL are simply areas of the genome that are associated with a given trait] involved in physical activity

(distance, duration, and speed on a running wheel) in mice and found one significant QTL for distance, duration, and speed on chromosome 13, and one significant QTL for speed only, on chromosome 9 (138, 153). This work was expanded by Leamy and colleagues (138), in which it was found that single-locus QTL as well as epistatic interactions [epistatic interactions occur when one gene's action is affected by another gene] account for approximately 37-60% of the total variation between activity traits in mice. Thus, epistatic gene interactions may also play a major role in the genetic regulation of physical activity behavior in mice. Additionally, there have been early gene linkage studies in humans that have sought to find genes involved in fitness and performance phenotypes (193). Interestingly, several of the identified QTL in the animal models and at least one human study (235) have suggested the involvement of the dopamine system in the genetic/biological regulation of physical activity.

Recent evidence in animal studies has suggested a possible role of the dopamine system in regulating voluntary physical activity levels (29, 199-201). The dopamine system is an interconnected neuronal network located in the central nervous system that is primarily mediated by signaling from the neurotransmitter dopamine. Dopaminergic signaling in various areas of the brain is responsible for a wide array of functions including control of motor movement, motivation, reward, learning, and emotion (240). Malfunctions of the dopaminergic system are thought to be the cause of movement abnormalities manifested in Parkinson's disease patients, hyperactive behavior in Attention Deficit Hyperactivity Disorder, addictive behavior with drugs of abuse, and even behavioral abnormalities in eating disorders such as anorexia. It is therefore evident that the dopaminergic system has a clear independent relationship with

locomotor and motivational behavior; however the exact role of the dopamine system in regulation of voluntary physical activity is not known.

It is known that physical exercise causes changes in neurotransmitter systems such as the dopamine system. Specifically, depending on exercise intensity and duration, there is an acute rise in dopamine production (166), and theoretically dopamine signaling. In this case, dopamine signaling is a dependent variable, changing in response to exercise intensity and duration. However, recent evidence suggests that the dopamine system may also play an independent role in regulating physical activity levels in animals. The effects of dopaminergic acting drugs have been studied for their effects on locomotion in animals (70, 90, 92, 93, 121-123, 163, 191, 192, 211, 223, 226, 242, 260, 261, 275). Several studies have also shown locomotor response to dopaminergic drugs to be strain dependent (29, 56, 70, 191, 228, 238, 261) suggesting genetic differences in dopaminergic architecture and function between inbred strains of mice may mediate differences in locomotion response to dopaminergic drugs. This notion also suggests some of the genes involved in regulating physical activity may be located within the dopaminergic system. Interestingly, the suggestive QTL (post Haplotype analysis) on chromosome 13 found in Lightfoot's work contains *Drd1*, the gene which codes for the D1 receptor (153). Similarly, polymorphisms in the *Drd2* gene are associated with physical activity levels in white women (235).

The work done in locomotion experiments must be considered with care because there are many different methodologies and definitions of locomotor behavior in animal literature. Locomotion is generally defined as the act of movement, and animal locomotion is the study of how animals move. Thus, interpretation of general

locomotor behavior, although useful, is difficult in regard to understanding the role of the dopamine system in regulation of *voluntary physical activity*. Physical activity is generally defined as voluntary movement that significantly increases energy expenditure as well as increases fitness. Wheel running in animal models has been shown as a good measurement of voluntary physical activity, and is also suggested as a good correlate to human physical activity and/or exercise (61). Several experiments using mice selectively bred for high wheel running have sought to find the genetic differences causing the increased physical activity in selected animals compared to control line mice (29, 59, 84, 85, 199-201, 253, 255, 257). From these selective breeding experiments, though some peripheral differences do exist, a significant central component has been suggested as an important factor in mediating differences in wheel running (29, 200). Specifically, differences in the dopamine system have been identified in selectively bred mice for high wheel running, and these differences may act independently to regulate motivation for wheel running in the selected animals (198, 199).

Any trait is determined by the following set of variables:

Phenotype = genetic component + environmental component + interaction

In the case of physical activity, environmental factors are well known, but the possible genetic components have yet to be elucidated. Work done recently suggests that a significant central component, the dopamine system, may be an important genetic factor in the regulation of physical activity (29, 199-201). However, it is not known whether the dopamine system acts as an independent variable in the regulation of voluntary

physical activity, and if there is an independent mechanism, which dopaminergic genes may be involved in the regulation of physical activity.

In the following chapters, several studies will be addressed that attempted to determine the role of the dopaminergic system in regulation of physical activity. Chapter 1 contains an extensive literature review of the investigations that point toward a possible role of the dopamine system in regulation of physical activity (this chapter is currently in review for publication in *International Journal of Biological Sciences*). In Chapter 2, the repeatability of exercise behaviors was assessed using inbred mice to ensure measurement of wheel running was a repeatable phenotype and also examined the repeatability of treadmill exercise in mice. In this study it was found that in male and female Balb/cJ mice, wheel running behavior is a highly repeatable measure, while endurance treadmill testing is not repeatable (this chapter is currently in review for publication in *Physiology & Behavior*). Chapter 3 outlines a study that was designed to investigate whether the dopamine system acted in an independent fashion to regulate physical activity in inbred mice. Also, in this chapter, expression levels of seven vital dopaminergic genes in the nucleus accumbens/striatum area of the brain were analyzed to determine if expression of any dopaminergic receptors, transporter, or enzymes were different between differentially active inbred strains of mice. In this study we found that dopaminergic gene expression did not differ within strain, between mice with access to a wheel and mice without a wheel, suggesting there was no dependent mechanism through which wheel running affecting expression levels of the genes studied. However, significant differences were found between high active C57L/J mice and low active C3H/HeJ mice. High active mice expressed significantly lower amounts

of *Drd1* and tyrosine hydroxylase compared to low active mice, suggesting the dopamine system may be an independent variable in the regulation of physical activity (this chapter is currently in review for publication in *Behavioural Brain Research*). Finally, in Chapter 4, pharmacological studies were employed to confirm whether differences in expression of dopaminergic genes actually led to alterations in voluntary physical activity. This study sought to identify how genetic differences in the dopamine system between inbred strains of mice altered wheel running response to pharmacological agents. It was found that high active mice significantly reduced wheel running in response to a D1 agonist, while C3H/HeJ mice significantly increased wheel running in response to a dopamine re-uptake inhibitor, confirming that genetic differences in dopaminergic functioning may explain differences in physical activity levels in inbred strains of mice (this chapter is currently in review for publication in *Behavioural Brain Research*).

While these experiments were designed to determine whether dopaminergic functioning played a role in the regulation of physical activity, it is important to mention that dopaminergic signaling does not occur in isolation, and is affected biologically by other factors such as hormones, nutritional status, and exercise intensity. Thus, future studies will need to consider other factors to further investigate the genetic mechanisms of dopaminergic regulation of physical activity, and how this system can be altered biologically in order to improving motivation for physical activity and overall human health.

TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW
DOES THE DIFFERENCE BETWEEN PHYSICALLY ACTIVE AND COUCH POTATO LIE IN THE DOPAMINE SYSTEM?

Introduction

Voluntary physical activity is important to human health for many reasons, including the prevention of obesity (38, 224). The rate of obesity has steadily increased over the last 30 years (294), while at the same time the amount of voluntary physical activity has decreased (1). Increases in sedentary lifestyles in Western cultures has led to an increase in inactivity related diseases such as obesity, cardiovascular disease, Type II Diabetes, and certain types of cancer (189). Research has shown the benefits of physical activity to human health and its importance in increasing resting metabolic rate (241), prevention of certain types of cancer (18), prevention of age related muscle loss, or sarcopenia (54), and treatment of depression and anxiety (52). Although the physiology of exercise has been well studied, the factors controlling physical activity levels in humans are not fully understood. Thus, it is important to understand the regulating factors of voluntary physical activity in order to prevent inactivity related diseases and improve human health.

Biological Influence on Physical Activity

The manifestation of a particular phenotype (in this case voluntary physical activity level) is traditionally thought to be determined by the following equation:

Phenotype = environment + genetics/biological factor + environment/genetic interaction).

The relative contribution of each of these components differs depending on the phenotype in question. Several recent genetic studies have investigated the level of genetic association with physical activity in humans and in animal models. The estimated genetic component for physical activity from these studies ranges from 20-80% (69, 119, 135, 142, 149, 153, 184, 249, 262). Additional support for the genetic component of voluntary physical activity can be found in mice selectively bred for high wheel running activity (253). Even after just 10 generations of selective breeding for high wheel running, selected animals exhibited a 75% increase in wheel running activity (253). and after 35 generations selected animals ran 170% more than controls (197). Recently, Lightfoot et al. (2008) conducted single-gene quantitative trait loci (QTL) analysis to determine the genetic locations possibly involved in regulation of physical activity. QTL analysis allows for the investigation of specific areas of the genome that are associated with a given trait. Using three wheel running indices in mice as indicative of physical activity, one significant QTL for distance (Chr. 13), one significant QTL for duration (Chr. 13), and two significant QTL for speed (Chr. 13 and 9) were found, confirming a genetic component to the regulation of voluntary physical activity in mice (153). Further work from this group (138), in combination with the initial QTL analysis, showed that in the inbred F_2 model used, the single-gene and epistatic [gene-gene interactions] QTL together accounted for 84-100% of the genetically-related phenotypic variance.

Where does the genetic/biological regulation occur?

The site of action of possible genetic/biological components affecting physical activity may include either peripheral locations and mechanisms (e.g. fiber type,

number of mitochondria, cell metabolism components, oxygen consumption etc.), and/or central locations and mechanisms (e.g. brain signaling, neurotransmitters, motivational behaviors etc.). Interestingly, work done with animals selectively bred for high wheel running, has shown very few and/or minimal peripheral differences between mice selected for high wheel running, compared to control mice (59, 124, 196, 197, 255, 257, 264, 265). Peripheral differences alone cannot explain the huge differences in wheel running between selectively-bred high active mice and control mice suggesting that a significant portion of the genetic/biological component affecting physical activity likely comes from central factors. This hypothesis is supported by several studies. First, mice selectively bred for high activity had increased Brain Derived Neurotrophic Factor (BDNF) in the hippocampal area of the brain compared to control mice (118). Rhodes and colleagues also showed that mice selected for high wheel running had increased activity as measured by Fos immunoreactivity in specific areas of the brain including the mid-brain (200). Finally, Bronikowski et al. (2004) showed that mice selected for high wheel running had a 20% increase in dopamine 2 (D2) and dopamine 4 (D4) receptors in the hippocampus as compared to control line mice (29). The gene array used in this study did not contain the D1-like receptors, and the hippocampus is not known as a brain region mediating dopaminergic mediated motivation and reward, however the authors still suggested the data indicate a possible role of the dopamine system to an increased motivation to run in selected mice (29). Furthermore, given the fact that selected mice and control line mice respond similarly to D2-like antagonists (199), but respond differentially to D1-like antagonists suggests the D1-like receptors, and not the D2-like receptors, in certain areas of mid-brain are important in activity

regulation in selectively bred high active mice (199, 200). The results from studies on the central nervous system in the selectively bred mice are summarized in Table 1.

Supporting the hypothesis that the dopaminergic system is an appropriate genetic/biological candidate in the central control of voluntary physical activity are studies that have implicated dopamine functioning in the control of motor movement (213), reward (225), learning, motivation (181), and emotion (233). However, to this point, the majority of studies investigating physical activity in humans have treated changes in neurotransmitter systems, such as dopamine, as a dependent factor that responds to physical activity stimuli such as intensity or duration of exercise. Similarly, work done in animals has for the most part employed research designs focusing on neurotransmitter systems and "locomotion" in relation to diseases such as Parkinson's disease. However, extensive recent evidence presented by Garland and colleagues (29, 198-201) with mice selectively bred for high activity indicated a strong central component that may act in an independent fashion; i.e. the central component may control physical activity levels as part of a genetic/biological regulation scheme. This paper will review the literature implicating the dopaminergic system as an independent regulator of locomotion in animals, as well as the emerging effort to understand the role the dopamine system plays in the regulation of voluntary physical activity. A novel interpretation of the central biological regulation of voluntary physical activity with respect to the dopaminergic system will also be presented.

The Dopaminergic System

While an exhaustive review of the structure and function of the dopaminergic system is beyond the scope of this review, in order to place the potential function of the dopamine system within the context of the central regulation of physical activity, a short overview of the dopamine system is necessary.

The dopaminergic neurons in the brain originate from two distinct areas. The neurons originating from the substantia nigra pars compacta project into the dorsal striatum via the nigrostriatal tract (100), while those neurons originating from the ventral tegmental area project into the cortex and ventral striatum (nucleus accumbens) via the mesolimbic tract (60, 145). The dopaminergic neurons interconnect with many areas of the brain leading to the implication of the dopaminergic system in many central functions including reward, learning, motivation, response to stimuli, and movement (240). Figure 1 illustrates the important dopaminergic pathways in the brain. Potentially important for the regulation of physical activity is the striatum/nucleus accumbens area given this area is involved in motivation, reward, and motor movement.

There are two evolutionarily and genetically different subtypes of receptors for dopamine within the dopaminergic system, and a total of five known distinct receptors (34, 240). The dopamine D1-like receptor family includes the dopamine one (D1) and dopamine five (D5) receptors. These receptors contain no introns, act by way of Gs-proteins, and activate adenylyl cyclase, thus increasing cAMP production (139, 268). The D-2 like receptor family includes the dopamine two (D2), dopamine three (D3), and dopamine four (D4) receptors. These receptors contain introns, act via Gi-proteins, inhibit adenylyl cyclase activity, and thus decrease cAMP activity (139, 170). The two

dopamine receptor families do not appear to act in isolation however, because it has been shown that activation of D1 receptors in the rat striatum causes D2 receptors to shift to a “low binding state” for dopamine (229). Likewise, D1 and D2 receptors have been shown to physically interact in certain areas of the brain, possibly working synergistically to affect downstream signaling (60). Thus, the different dopamine receptors do not act independently; instead signaling from each of the dopamine receptors appears to affect the other dopamine receptors making the dopamine system a complicated signaling network.

Dopamine receptors differ in their anatomical locations on specific neurons, vary in density in specific regions of the brain, and can be found either presynaptically or postsynaptically depending on the type of tissue and/or neuron (170). The distribution of dopamine receptors in the brain is diverse; however, specific dopamine receptors are differentially expressed at higher or lower levels in particular areas of brain (60), exemplifying the complexity of the dopamine system. Dopamine receptor expression is found in nearly all areas of the brain, but receptors are most highly expressed in nigrostriatal and mesolimbic regions including the striatum, nucleus accumbens, and cortex (48, 114). The five known dopamine receptors differ in their affinity for dopamine, natural ligands, receptor activity, anatomical locations, genetic sequence, and thus, physiological activity (34); however, the dopamine receptors work in concert with each other to produce integrated responses and signals in the brain and body.

Expression levels of the dopamine receptors are important in mediating downstream behavioral responses including voluntary activity. Dopamine receptor

expression can be affected by the levels of dopamine in the system (90), level and length of treatment of pharmacological agents (31), as well as other external stimuli mediated through rewarding behavior such as sexual activity (167), or exercise (74). However, overall dopaminergic responses and signaling are also dependent on other factors such as the electrical response produced (dopamine signaling can act in both an excitatory manner, as well as an inhibitory manner depending on the circumstance) (36, 106, 145), as well as interactions with other neurotransmitters and signaling molecules. For example, the dopamine system has been shown to interact with glutamate (231), GABA (94), acetylcholine (221), and serotonin (64). Depending on the receptor involved and the anatomical location, dopamine receptors activate or repress a variety of signaling cascades including ERK/MAPK (156), CREB (204), and CAMKII (110), by affecting calcium and/or potassium channels in the nerve cell (170). A representative dopaminergic synapse is shown in Figure 2. Only possible signaling pathways for the D1-like receptors are illustrated. Possible signaling pathways in the dopaminergic neurons are extensively reviewed by Neve and colleagues (2004) (176).

Dopamine receptor signaling also affects downstream gene expression (170). Several immediate early genes that are activated in dopaminergic neurons following stimulation include those of the Fos family (107, 175, 200, 283). Fos is a transcription factor that is up-regulated in certain brain regions in response to stimulation from drugs, or other natural rewarding stimuli such as sexual behavior or exercise (200, 236). Fos is the product of the immediate early gene c-Fos, and Fos expression has been shown to be regulated by dopamine signaling (206). Pharmacological studies show that Fos immunoreactivity in the striatum and other key regions of the brain is increased

following administration of D1 and D2 agonists (97, 109, 115, 178, 205), suggesting Fos may be important as a downstream gene regulated by dopaminergic signaling. ΔFosB, a transcription factor and also a member of the Fos family of proteins, is likewise up-regulated in response to drugs of abuse and exercise. The expression of ΔFosB is usually longer lasting than Fos, and is thought to be involved in long term changes in behavior (175, 283). Brain Derived Neurotrophic Factor (BDNF) also appears to be regulated in part by dopamine signaling and has been shown to increase as a result of physical exercise (68). Additionally, it is thought that the antidepressant effect of exercise is mediated through the dopamine system, and increased expression of BDNF (63).

Thus, while Fos and BDNF are two examples of downstream transcription factors regulated by dopamine signaling, the dopamine system potentially affects a large number of downstream genes that may ultimately be important in the understanding of the genetic mechanisms involved in regulation of physical activity levels in animals and humans.

Fore example, dopamine signaling has also been shown to have direct affects on expression levels of certain neuropeptides including substance P (SP) (88), dynorphin (12, 76, 246), and enkephalin (136, 247). In addition to other functions, these neuropeptides can in-turn also modulate other gene expression and downstream signaling, highlighting the possible indirect effects of dopamine signaling on downstream gene expression changes. A detailed description of the interaction of neuropeptides and dopamine signaling is beyond the scope of this review; however, the point should be made that any regulation of voluntary physical activity by dopamine

signaling may be mediated through not only dopamine receptor expression levels, but also downstream signaling pathways including those that affect expression of transcription factors and other neuropeptides known to affect transcription and gene expression. Therefore, there are many aspects of the dopaminergic system, including expression of receptors, interaction with other neurotransmitters and signaling molecules, and downstream gene regulation that may be important in the genetic/biological regulation of voluntary physical activity.

Dopaminergic Regulation of Locomotion: Evidence from Human Disease States

Extensive studies have been conducted to assess the role of the dopamine receptors and the dopamine system in various behavioral functions (116, 290). Literature investigating disease states such as Parkinson's disease is available which emphasizes the role of the dopamine system in regulation of motor movement and/or "locomotion". It is important therefore, to make the distinction between "locomotion" and "physical activity". The term locomotion in scientific literature generally refers to any act of movement, which depending on methodology, can operationally differ significantly between studies. Conversely, physical activity is generally defined as purposeful exercise and/or movement that expends a significant amount of energy. While there are slight differences between operational definitions of locomotion and physical activity which are highlighted later in this review, it is still important to highlight the known dopaminergic involvement in locomotion to understand the possible role the dopamine system might play in regulating physical activity, especially since the preponderance of the available literature deals with 'locomotion' in disease states rather than physical activity.

Four major areas of disease research support a role of the dopamine system in the regulation of physical activity through control of motor movement and motivation including Parkinson's Disease, Attention Deficit Hyperactivity Disorder (ADHD), Anorexia, and Addiction. An overview of the role of the dopamine system in these four disease states is outlined in Table 2.

Parkinson's Disease

One area that has specifically highlighted the role of the dopamine system in the regulation of locomotion is Parkinson's Disease. Common characteristics of Parkinson's Disease include resting tremors, bradykinesia, rigidity, and overall difficulty in motor movement as a result of degradation and subsequent loss of dopaminergic neurons in the substantia nigra area of the brain (5, 301). Although the exact mechanisms that result in loss of dopaminergic neurons in Parkinson's disease are not well understood, it appears that misfolding and/or inherited mutations of the proteins alpha-synuclein and ubiquitin play an important role in the onset of the disease (125, 143, 160). Two types of animals models of Parkinson's symptoms give insight into the importance of the dopamine system in locomotor behavior. Toxin-induced models of Parkinson's commonly involve the use of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxin which when administered causes malfunction and loss of dopaminergic neurons in the brain. When MPTP is administered to mice, reduced locomotor function is evident through various tests including open field (228), and rotarod assessment (209). Interestingly, there appear to be strain differences in susceptibility to MPTP and this may be caused by genetic differences in the dopamine system between different strains of mice (103, 228). The second type of animal model

involves transgenic animals which either overexpress specific genes, or have genes "knocked out", and thus, do not express a particular gene involved in Parkinson's disease. Dopamine D2 receptor knock-out mice (11), as well as hybrid D1 receptor transgenic/D2 receptor deficient mice (55), display Parkinson's-like locomotor behavior; however, mice that have been genetically altered in some aspect of dopaminergic signaling usually display global behavioral changes, and thus are not ideal for studying specific aspects of locomotion in most cases. Regardless, it is clear from Parkinson's disease literature that the dopamine system plays a major role in motor deficiencies manifested in this disease.

Attention Deficit Hyperactivity Disorder

Another important line of evidence supporting the involvement of the dopaminergic system in regulation of voluntary physical activity is its well studied role in Attention Deficit Hyperactivity Disorder (ADHD) (8, 144). ADHD usually presents in childhood, but can also persist into adulthood (243), indicating that the central functioning mediating the symptoms may sometimes be irreversible. Genetic alterations of both the D4 and D5 receptors have been implicated as primary mechanisms in ADHD. *Drd4* polymorphisms have been found in both human and animal models of ADHD (169). Additionally, inheritance studies suggest an increased risk of ADHD associated with particular alleles (alternative forms of a gene) of *DRD4* and *DRD5* (8, 65, 147). Moreover, inheritance and allelic variant studies show an association between *DAT* , the dopamine transporter gene which is involved in transporting dopamine back into the neuron after it has been released into the synapse, and ADHD (65, 78). However, the most compelling evidence regarding dopaminergic

involvement with ADHD comes from pharmacological studies. Stimulants which block *DAT*, resulting in increased synaptic dopamine levels, have been shown to significantly reduce the hyperactive symptoms of ADHD (159, 286-288). The dopamine transporter (DAT) has been shown to be important in the control of many aspects of locomotion (81). The dopamine transporter is a key regulator of the dopamine system as it regulates the amount of dopamine signaling taking place with all the receptors. A complete review of the role of DAT in locomotion and parkinsonism can be found by GR Uhl, *Movement Disorders,* 2003 (263).

Mice exhibiting high amounts of wheel running after many generations of selective breeding have been suggested as a potential model of ADHD (269). Garland and colleagues have shown that these selectively-bred mice have altered dopamine profiles compared to control line mice, as well as responding more profoundly to dopaminergic acting drugs such as dopamine transporter inhibitors, suggesting similar mechanistic pathways as ADHD (200, 201). It has been suggested the selectively bred mice from Garland's group are a good model for ADHD (199), but they may also provide insight into the dopaminergic regulation of voluntary physical activity in mice.

Anorexia

Previous studies have suggested that the dopamine system is involved in regulation of feeding behavior in animals (182). In addition, recent studies have begun to investigate the increase in activity that results from the starvation characteristics of anorexia nervosa, which is sometimes labeled the "drive for activity" (35). Typically, reported symptoms of semi-starvation include slowing of motor movement and lethargy; however, in a significant percentage of anorexia nervosa patients quite the

opposite is observed with anorexic patients exhibiting increased physical activity levels (26, 117). In 2006, Davis and Kaptein suggested that anorexia nervosa patients who exhibit "excessive exercising" represent a subtype of the disorder closely linked to obsessive compulsive disorder (45). Whether the excessive exercising in a subgroup of anorexia patients represents co-manifestation of OCD is still controversial; however, the role of the dopamine system in mediating this behavior is relevant to this review. Several monoamine neurotransmitters including norepinephrine, serotonin (9), as well as dopamine have been suggested to play a role in this increased motivation for activity in anorexia nervosa (188). In animal models of "activity induced anorexia" the dopaminergic system is suggested as a mediator of the increased physical activity seen in this disorder (86, 188). Although the exact mechanism is still unclear, it has been shown that exercising intensely increases dopaminergic reward signaling (28), and subjects with anorexia may exercise excessively in order to relieve the "anhedonic state" created by insufficient nutrition (46, 75). Similarly, in a report by Frisch et al. (2001), it was reported that a polymorphism in the Catechol-O-methertransferase gene (COMT) was associated with risk of developing anorexia in humans (80). This gene confers an enzyme important for dopamine catabolism, and further suggests a role of the dopamine system in manifestation of anorexia. Additionally, Frank et al. (2005) studied dopamine D2/D3 receptor binding in the brain in women recovering from anorexia. Compared to controls, women with anorexia showed increased dopamine receptor binding in the striatum, and this suggested that decreased synaptic dopamine, or increased receptor expression may be associated with certain phenotypic characteristics of anorexia including increased physical activity (75).

Addiction

A complete review of the physiological underpinnings of addiction is beyond the scope of this review; however, it is well accepted that the dopamine system is a major mediator of addiction to drugs (reviewed extensively in Vetulani, 2001; Peirce and Kumaresan, 2006; and Di Chiara, 2007) (50, 187, 271). Specifically, the dopamine reward centers are known to involve the neurons in the ventral tegmental area which project into the nucleus accumbens and other forebrain regions. It has been hypothesized that people who are addicted to such things as risky behavior, drugs, and gambling may have genetic differences in their dopamine system that predispose them to such behavior (272). This hypothesis has been supported by results investigating the administration of methylphenidate (a psychoactive drug) to non-drug users whose D2 receptor expression was high in the brain. The administration of methylphenidate to these subjects produced a feeling of aversion, as opposed to what happened when methylphenidate was administered to people with low levels of D2 receptor expression; in these subjects the drug produced a pleasure feeling (273). Studies in animals also suggest a genetic component involving the dopamine system in the mechanism of addiction. For example, it has been found that C57BL/6J mice have increased expression of D1 and D2 receptors in the striatum compared to DBA/2J mice, and these differences are associated with ethanol preference in these mice and possible strain differences in tendency for alcohol addiction (177). Additional evidence in rodents has suggested both D1-like and D2-like dopamine receptors, and the dopamine transporter gene may be a mediator in addictive behavior (87, 101, 242). These results can be used to hypothesize that the dopaminergic system may play a role in the pleasurable feelings

associated with voluntary physical activity in humans and thus, might contribute to the observed variation in animals and humans in motivation for physical activity.

Evidence for dopaminergic involvement in locomotion alterations in diseases such as Parkinson's disease and ADHD, physical activity and the drive to exercise in Anorexia patients, as well as possible pleasure-fulfillment in addiction suggests that not only does the dopamine system regulate "motor movement" in the strict sense (see Table 2), but may also regulate motivational factors such as rewarding/pleasurable feelings involved in physical activity phenotypes.

Dopaminergic regulation of Physical Activity: Evidence from animal models in locomotion and wheel running studies

Locomotion Studies

The psychoactive drugs amphetamine and cocaine have been known to induce rewarding effects mediated through the dopamine system. Drug affects on locomotion through dopaminergic changes is relevant to this review because natural rewarding behaviors such as sexual behavior have also been shown to produce their effects through increased dopamine production in the midbrain (44). It can be argued that physical exercise is a naturally rewarding behavior as well, and the mechanism of this rewarding behavior may be important in the dopaminergic regulation of physical activity. Thus, to further illustrate the importance of the dopamine system in mediating locomotor behavior in animals it is necessary to briefly review both pharmacological studies, as well as studies using transgenic and/or knock-out mice investigating the effects of the dopamine receptors, as well as the dopamine transporter in mediating locomotion in animals.

A major pool of literature can be found linking the dopamine system to locomotor changes induced by psychoactive drugs (14, 16, 90-92, 97, 108, 123, 211, 223, 242, 296). For example, it has been shown that the dopamine system mediates differences in amphetamine induced locomotion between inbred strains of mice (270). The majority of studies involving amphetamine and locomotion implicate an increase in dopamine levels in the mid-brain as the main factor mediating the locomotor response to amphetamine (16, 51, 92), while studies also suggest this response is mediated downstream by BDNF (223). Similar studies using cocaine have implicated specifically the dopamine D1 receptors (299), as well as blockade of DAT (260), as being involved in mediating the cocaine induced changes in locomotion in animal models.

D1 and D2 receptors have been studied extensively in pharmacological studies investigating their role in locomotor behavior in animals with 3,4-Methylene-dioxymethamphetamine (MDMA) used as the primary stimulant increasing locomotion (91). When mice are pre-treated with a D2 receptor antagonist (eticlopride, 0.2mg/Kg), the locomotion response to MDMA was non-existent, while pre-treatment with a D1 antagonist (SCH-23390, 0.2mg/Kg) did not abolish the MDMA induced locomotion but did delay the onset of this effect. These results suggest that both D1 and D2 receptors are important in stimulant induced locomotion, yet serve different functions in this response (14). The suggestion of an important role for the dopaminergic receptors in the modulation of locomotion have been further confirmed by other pharmacological studies investigating the D1-like and D2-like receptors (171, 174, 244, 245).

Quantitative trait loci (QTL) analysis has also been used to provide initial genomic areas which may contain genes associated with baseline locomotor activity as

well as locomotor sensitivity to a D2-like agonist (quinpirole, 0.01-0.03mg/Kg) (30). In this study, a significant QTL was found on Chromosome 9, while suggestive QTL's on Chr 15, 13, and 5 (30). The authors suggested that the dopamine system was involved in the regulation of baseline locomotion because several dopamine related genes including *Drd2*, *Drd3*, and *DAT* fell within the QTL identified in this research (30).

Pharmacological studies also suggest the D3 receptors are important in the regulation of locomotor behavior in animals, specifically acting in an inhibitory manner in regard to locomotion in response to locomotor-stimulating amphetamine treatment (47, 222). McNamara and colleagues (164) investigated the role of the D3 receptor in locomotion in two distinct inbred strains of mice. They found that compared to DBA/2J mice, C57BL/6J mice had less inhibitory response to several locomotor-stimulating effects such as novelty, amphetamine treatments, and a D1 agonist (SKF38393, 5-20mg/Kg). In addition, C57BL/6J mice had less D3 receptor expression and/or binding density in several areas of the brain including the substantia nigra/ventral striatum, but greater expression in the hippocampus than the DBA/2J mice (164). These data suggest that another potential factor in the observed variation in locomotor response to pharmacological stimulants between strains of mice is the difference in locomotor inhibitory characteristics of dopamine receptor signaling.

It is apparent from studies involving D1-like and D2-like agonists and antagonists that the dopamine receptors play a role in locomotor behavior in mice or rats; however, there is not a consensus on the exact mechanism through which the dopamine system (including the dopamine receptors and transporter) is able to mediate the locomotor effects of these different dopaminergic acting drugs. This lack of

consensus is probably due to two reasons: First, it is hard to discern an exact definition of “locomotion” and methodologies for measuring such a complex behavior are hard to control and differ between studies; and second, the dopamine system is a complex system, and as noted earlier, receptor signaling may interact, as well as have different outcomes depending on the area of brain and type of neuron involved in the signaling. Thus, although it is clear that the dopamine system plays a key role in regulation of drug induced locomotion, the question still remains as to the mechanisms by which locomotion is altered and whether this system plays a role in regulating general physical activity patterns.

More recently, the development of knock-out and transgenic animals has enabled researchers to further study the role of specific dopaminergic genes (and thus, receptors) in regulating locomotion behaviors (300). Mice lacking the D1a receptor have been shown to have normal locomotion and coordination, but reduced exploratory activity (57). In another study, Xu et al. (295) showed D1a receptor knock-out mice actually had increased locomotor activity as measured by photo beam breaks and suggest D1 receptors are critical in the striatum for normal locomotor behavior. D3 receptor knock-out mice also show increased exploratory locomotor behavior (“hyperactivity”), suggesting this receptor has an inhibitory role in the regulation of exploratory locomotion (2). Antisense treatment targeting the D3 receptor (effectively turning this gene off temporarily) in rats induced an increase in spontaneous locomotion again suggesting an inhibitory role of this receptor in locomotor behavior (62). D1/D3 receptor knock-out mice also provide insight into the interactions the dopamine receptors may have in order to mediate locomotor behavior. D1/D3 receptor knock-out

mice have normal “baseline locomotion” but significantly reduced exploratory locomotion suggesting the D1 and D3 receptors work synergistically to manifest certain locomotor phenotypes (120).

D2 receptor knock-out mice typically exhibit reduced locomotor behaviors among other postural and growth abnormalities (11). Several studies have shown that mice lacking D2 or D4 receptors also show reduced spontaneous locomotor activity (11, 122, 211); however, these same mice showed variable responses to locomotor inducing drug stimulants such as the D1 agonist SKF38393,(122) the D2 agonist quinpirole, (122) ethanol, cocaine, and methamphetamine (211), making the exact mechanisms involved difficult to ascertain.

In addition to single receptor knock-out animals, Dracheva et al. (2001) studied locomotion in D1 receptor overexpressing animals, and found that mice that overexpressed D1 significantly reduced locomotion in response to a D1 agonist, but control mice increased locomotion in response to the same drug (56). The results of this study suggest that D1 receptor signaling may have inhibitory effects on certain types of locomotor activity, as do the D3 receptors as mentioned previously. A study of D1 overexpressing/D2 receptor deficient mice showed that decreased locomotion in hybrid D1 overexpressing/D2 receptor deficient mice appeared to be mediated by D1 receptors, and that reduced locomotion in the hybrid animals was not dependent on D1/D2 interactions (55). In this case, D1/D2 interaction was not necessary for dopaminergic regulation of locomotion.

DAT knock-out and knock-down mice have also been studied which show increased locomotor activity, and this hyperactivity can be reduced by psychostimulant

pharmacologic agents (83, 302). These data suggest DAT, and thus, overall presence of dopamine, and dopamine signaling are as important to locomotor behavior regulation as the dopamine receptors themselves.

From the transgenic and knock-out data available, it appears that D1 and D3 receptors may play an important inhibitory role in certain types of locomotor behavior, while D2-like receptors appear to facilitate certain aspects of locomotion. Because the dopaminergic system is complex and involved in many aspects of development, it is hard to discern if these conclusions are due to deletion of the targeted gene or whether the resultant effects on behavior are a result of other compensatory changes in the dopamine system. Thus, studies of dopamine receptor knock-out mice and locomotor behavior must be interpreted with care, and while knock-out models can be useful in studying gene function, this model may not be the best model for investigating dopaminergic regulation of physical activity. Temporary gene silencing methods such as RNAi technology could potentially be used in the future to study the effects of knock-down of dopamine genes on physical activity. Please refer to Appendix A for more information on gene silencing.

Wheel Running Studies

In addition to the general locomotor studies, evidence for involvement of the dopamine system with physical activity levels can also be found in wheel running studies conducted in animals. A strong case has been made that wheel running in animals is an appropriate model of voluntary physical activity in humans (61, 234). Thus, as opposed to the drug induced locomotion studies, wheel running studies may

give more accurate insights into the involvement of the dopamine system in general physical activity levels in humans.

Inbred mice strain differences in both dopaminergic anatomy and wheel running may prove useful in elucidating how genetic differences in dopaminergic signaling may differentially regulate physical activity in inbred mice. Lightfoot and colleagues screened 13 strains of mice for distance, duration, and speed on a running wheel, and found significant differences between strains in all running wheel indices, indicating a significant genetic component to regulation of physical activity behavior (149). Additionally, strain differences in dopamine anatomy and function have also been shown by various authors (13, 164, 168, 177, 227, 232, 252). For example, Fink and Reis, 1981, showed that BALB/cJ mice have more dopamine activity in both the nigrostriatal, and mesolimbic pathways in the brain compared to CBA/J mice (70). Combining the knowledge that CBA/J and Balb/cJ mice differ in dopaminergic anatomy in the mid-brain (70), as well as differ in wheel running indices (149), it is reasonable to suggest that genetic differences in the dopamine system between inbred strains of mice may translate into behavioral differences, including voluntary wheel running. Similarly, work done recently in our lab (126, 127) suggests expression differences of D1-like receptors as well and tyrosine hydroxylase between differentially active inbred strains may be important in mediating behavior differences in running wheel activity in differentially active inbred mice.

Supporting the hypothesis that genetic differences in the dopamine system may mediate behavioral differences in animal models is work done using selective breeding. Bronikowski and colleagues (2004) investigated gene expression changes in the

hippocampus region of the brain and found that mice selectively bred for high wheel running had a 20% increase in D2 and D4 receptor expression (D1-like receptors were not analyzed in this study) compared to control line mice (29). Also, Rhodes et al. (2003) investigated patterns of brain activity in mice selected for high wheel running, and found that certain areas of the brain exhibited increased activity (as measured by Fos expression) in selected animals compared to the control animals (200). Several of the regions identified in this research, including the nucleus accumbens, striatum, prefrontal cortex, and lateral hypothalamus are regions associated with high dopaminergic activity. Another study by Waters et al. (2008) in rats selectively bred for high aerobic capacity showed that the high capacity rats exhibited increased wheel running activity compared to controls while also exhibiting increased dopaminergic activity in the striatum area of the brain compared to low aerobic capacity rats (277). The authors suggested that artificial selection may have acted upon the dopamine system because the dopamine system is involved in motivation and that wheel running activity is a motivated behavior (277). Thus, combining the knowledge from genetic studies of dopamine and wheel running in both inbred and selectively bred mice it is warranted to investigate further the connection between the dopamine system and wheel running in animals.

Further elucidation of the role of the dopamine system in wheel running comes from investigations of the effects of pharmacological interventions (specifically psychoactive drugs) on wheel running in mice. The selectively bred mice mentioned above (see Garland et al. 2006 for a complete description of these selectively bred mice) (84) responded differently than controls to several dopaminergic acting drugs including

D1-like and D2-like agonists and antagonists, suggesting a dopaminergic involvement in regulation of wheel running in these selected animals (199, 201). Specifically, selected animals significantly reduced their wheel running by decreasing their speed as compared to control animals in response to cocaine and GBR 12909 (201). Both of these drugs act by inhibiting DAT which effectively increases the amount of dopamine in the synapse. In another study, Rhodes and colleagues (2003) showed that a DAT inhibitor (Ritalin, 15mg/Kg and 30mg/Kg) decreased wheel running in selected animals, but increased wheel running in control animals. A non-selective dopamine agonist (apomorphine, 0.25mg/Kg and 0.5mg/Kg) decreased wheel running more in control animals compared to selected animals at higher doses. Additionally, a selective D1-like antagonist (SCH-23390, 0.025-0.1mg/Kg) decreased wheel running in the control animals more than selected animals, while a selective D2-like antagonist (raclopride, 0.5-2.0mg/Kg) had similar effects on both selected and control animals (199). These results suggested that D1-like receptors and DAT were involved in mediating the differences seen in wheel running between the selected animals compared to controls, but not the D2-like receptors. Earlier studies by Schumacher and colleagues (1994) using mice classified as high active, or low active based on performance in a running wheel test, also showed differential locomotor responses to dopamine agonists such as apomorphine, bromocriptine, and amphetamine between the high active and low active mice. Specifically, bromocriptine and amphetamine stimulated physical activity more in the low active mice compared to the high active mice, suggesting a decreased functioning of the mesolimbic dopamine system in the high active mice (226). A study conducted in 2004 by Leng and colleagues showed that C57Bl/6 mice, after pre-

treatment with MPTP (a dopaminergic neurotoxin), exhibited significantly reduced wheel running after treatment with a tyrosine hydroxylase inhibitor which effectively reduced dopamine synthesis, highlighting the importance of dopamine itself, in addition to individual dopamine receptors, in the regulation of physical activity in the form of wheel running in mice (140). Additionally, it has been recently shown that C57L/J mice (high active) (149) significantly reduce wheel running in response to a D1-like agonist, but do not significantly change wheel running behavior in response to a D1-like antagonist, dopamine re-uptake inhibitor, or a tyrosine hydroxylase inhibitor (127). C3H/HeJ mice (low active) (149) did not respond to the D1-like agonist or antagonist, but did significantly increase wheel running in response to a dopamine re-uptake inhibitor (127). Genetic differences in the dopamine system between C57L/J mice and C3H/HeJ mice could explain the differential response to dopaminergic acting drugs. Specifically, it appears that signaling through D1-like receptors is important in mediating the high activity observed in C57L/J mice, while dopamine half-life and presence in the synapse is more important in mediating wheel running behavior in low active C3H/HeJ mice.

As is apparent from the above literature, a preponderance of evidence suggests that the dopamine system is involved in the regulation of wheel running behavior and general locomotion in mice. From a genetic aspect, studies suggest inbred strains of mice, as well as mice selectively bred for high amounts of wheel running differ not only in amount of physical activity performed, but also in dopaminergic anatomy, and thus function, in the mid-brain. Similarly, pharmacological studies provide insight into the possible role of the dopamine system in regulation of wheel running behavior.

However, it is still unclear whether the dopamine system is acting in an independent fashion to control physical activity or if there are possible dependent changes in the dopamine system due to physical activity which is in-turn mediating activity behavior.

Going Further: Linking the Dopamine System and Regulation of Physical Activity in Humans

It is known that exercise acts as an independent agent to cause changes in various neurotransmitter systems, specifically the dopamine system, noradrenergic systems, and the serotonergic system (165). For example, exercise increases the amount of dopamine released and metabolized in certain areas of the brain (276). In this respect, changes in the dopamine system act in a dependent fashion in response to exercise. However, this dependent change in the dopamine system is usually accompanied by a positive reinforcing response in which the dopamine system in-turn acts in an independent fashion causing changes in behavior to seek rewarding and/or pleasurable responses (290). Even though we can postulate that seeking rewarding and/or pleasurable responses in humans leads to increased physical activity, evidence is still lacking as to whether the dopamine system is actually working in an independent role in influencing voluntary physical activity. In other words, it is known that exercise causes changes in the dopaminergic system, but does the dopaminergic system itself also act as an independent variable to regulate overall physical activity levels? It has been shown that dopamine neurons in the striatum are primarily responsible for changes in motor activity (218), while dopaminergic function in the nucleus accumbens is involved in anticipatory behavior (anticipation of a reward or "motivation") (25, 186, 216). Dopamine depletion studies in the nucleus accumbens of rodents showed a decreased motor activity response to certain drugs (41), and dopamine depleted animals

showed lack of motivation for more effortful tasks (40, 215). Thus, there is overlap between the motivational aspects and motor control aspects of brain neurology (212), with the dopamine system mediating both portions. This multifaceted role of the dopamine system provides reason to investigate the relationship between dopaminergic activity in the brain and amount of *voluntary* physically activity that the organism undertakes.

The fact that exercise is often used as a treatment in depression also illustrates the dependent role of the dopamine system in response to physical activity. It has been shown that exercise alleviates symptoms of depression, most likely mediated through changes in the central nervous system in the brain (58). Along this same line of thought, the benefits of physical activity on the brain seem to be primarily mediated through catecholamine systems. Exercise and/or physical activity is known to increase neurotransmitter production and metabolism (52, 53, 157), which are thought to lead to changes at the molecular and cellular level that improve neuronal plasticity (73, 165), cognitive functioning (237), learning (289), and overall mood (53), all aspects that protect brain function. Mice that perform voluntary physical activity in the form of wheel running produce more brain-derived neurotrophic factor, causing an increase in synaptogenesis and neurogenesis, neuron survival, and increased learning capacity, all leading to possible protection from cognitive decline (39). Similarly, it has been shown that moderate physical activity decreases the risk of Parkinson's Disease (155, 258), as well as helps alleviate and slow the progression of symptoms of the disease (72, 133).

Training studies have also shed light on the dependent changes in the dopamine system in response to exercise in the form of training. Rats who underwent endurance

training showed increased D2 receptor binding over the lifespan compared to control animals, suggesting that endurance training provided some protection from age related loss of D2 receptor functioning (158). Likewise, rats exposed to treadmill running had increased Fos expression in the striatum area of the brain mediated through D1 receptors (154). Similarly human exercise training studies show dependent changes in neurotransmitter systems, including the dopamine system (19, 27, 37, 104, 128, 183), in response to exercise, and these cause and effect changes are likely due to dopamine's involvement in control of sympathetic nervous activity (161). In these particular studies dopamine was treated as the dependent variable in response to exercise, or training. However, some research suggests that not only is dopaminergic functioning altered in response to exercise, but perhaps the dopaminergic system also acts in an independent fashion on physical activity levels. For example, a study in humans using PET imaging showed no changes in dopamine D2 receptor availability in the caudate putamen after treadmill running (submax); however, the subjects used in this study were already persons with a history of regular exercise (274). It is plausible to assume that one reason no difference was seen from baseline, is that dopamine release in the striatum may not have been the true dependent variable in this methodology. It would be interesting to compare PET imaging of regular exercisers to non-exercisers in the case that dopamine signaling may work in an independent manner in relation to physical activity, and even training in some circumstances. Further support for an independent role of dopamine and physical activity comes from genetic studies linking single nucleotide polymorphisms in the *DRD4* (99), and *DRD2* genes (235), with physical activity levels in humans. Similarly, aging studies suggest an independent mechanism

of action for the dopamine system and regulation of physical activity levels. It is known that a decline in physical activity over the lifespan is most likely due in part to a decline in the functioning of the dopaminergic system (207). However, as mentioned, studies show that physical activity in the form of exercise can slow the rate of decline in functioning of the dopamine system, and increase quality of life. Thus, the benefits of physical activity on central nervous system functioning suggests that the dopamine system can have both a dependent and independent mechanism of action in regulation of physical activity levels.

It is clear that the dopaminergic system is affected by physical activity, and it is highly likely that the amount of *voluntary* physical activity is regulated at least in part by the dopamine system. The mechanisms behind this correlation are yet to be fully understood.

Dopamine, Reward, and possible implications for Physical Activity Regulation

A full neurobiological discussion of the role of the dopamine system in reinforcement and reward is outside the scope of this review; however, a brief discussion of the reward pathways is necessary to relate the proposed relationship of the dopamine system to regulation of physical activity. In the past several decades it has become increasingly clear from studies in drug addiction that dopaminergic signaling mediates behavioral responses to rewarding stimuli (225). Rewards, in and of themselves, provide three basic functions including eliciting a behavior, providing reinforcement (or positive feedback so as to increase the frequency or intensity of the behavior), and provision of some type of pleasurable feeling or response (225). With the context of these three basic functions, it is clear that drugs of abuse are “addictive”

because they provide all three functions of a "reward". It is generally accepted that the dopamine system is implicated in reward and reinforcing mechanisms as evidenced by the results of psychostimulant administration (49, 290). Specifically, the administration of psychostimulant drugs increases dopamine release and signaling in the mesolimbic areas of the brain, while withdrawal of these drugs causes a decrease in dopamine signaling in these areas and this response appears to be mediated by both D1 and D2 receptors (77, 131). Studies suggest that D2 receptors are responsible for mediating the self-reinforcing effect of drugs, while the D1 receptors act in a permissive fashion to facilitate the response. Both D1 and D2 agonists elicit a reinforcing response and have effects similar to cocaine administration; however, the D1-like receptors and D2-like receptors mediate different aspects of this self-reinforcing response (230). Cocaine self-administration studies suggest the D2 receptors are responsible for mediating further motivation to seek cocaine, while the D1 receptors may mediate a reduced drive to seek further cocaine reinforcement (230).

More recent evidence has led researchers to suggest that the dopamine system is specifically involved in the motivational aspect of reward for natural stimuli such as food. Dopamine depletion and dopamine antagonist studies in the nucleus accumbens of animals show that appetite for food is not reduced under these conditions; however, the motivation to engage in effortful tasks for food is significantly reduced (214). Thus, the dopamine system appears to regulate certain aspects of the "wanting" instead of the "liking" of natural rewards (23). Drugs of abuse are typically thought of as artificial rewards, while actions such as sexual behavior, food, and/or exercise can be termed "natural rewards." Traditionally, it has been assumed that drugs of abuse initiate the

natural reward system in the brain, mainly the dopamine system, and thus act in a similar fashion as natural rewards. This theory, which is based on the notion that the dopaminergic system mediates the reinforcing properties of natural rewarding stimuli, has been known as the "General Anhedonia Model" (217). As stated, this theory may not be the entire picture as it appears that the dopamine system may mediate the motivation for natural rewards, and not necessarily the reinforcement mechanism at least in the case of food rewards. Thus, the dopamine system and its role in mediating reward is complex, and the exact mechanisms through which the dopamine system mediates reward signaling to natural rewards such as physical activity is not known. However, it is increasingly clear from genetic studies involving locomotion and wheel running, as well as evidence from reward signaling in response to naturally rewarding behavior that the dopamine system plays a role in the regulation of physical activity in regard to mediating the natural rewarding properties of this behavior.

Proposed Model for Dopaminergic Regulation of Physical Activity

As already outlined in this review, it is well known that exercise induces changes in neurotransmitter systems as well as endorphin release and signaling. These changes typically depend on intensity and duration of exercise. To date, most studies investigating changes in neurotransmitters due to exercise treat the neurotransmitter changes as the dependent variable. Studies involving motor movement and/or locomotion, wheel running, and addiction however, provide evidence for a regulatory role of the dopaminergic system on voluntary physical activity. Furthermore, it is warranted to propose a dual role for the dopamine system in the genetic and biological regulation of physical activity. First, it appears that physical activity in the form of

exercise itself and/or training produces beneficial changes in the dopamine system including increased dopamine signaling as well as increased BDNF levels in the brain. In this role, dopamine signaling is acting in a dependent fashion to mediate central changes in response to physical activity. Second, it is also apparent from the growing amount of literature on the role of the dopamine system in motivation for natural rewards, that the dopamine system creates a positively reinforcing condition in which the dopamine system acts in an independent fashion controlling the "wanting" and/or motivation for natural rewarding stimuli such as physical activity. Thus, it is proposed that dopaminergic signaling acts in both a dependent and independent fashion in the regulation of physical activity (proposed schematic outlined in Figure 3).

Going back to the equation mentioned in the first part of this review, any phenotype is affected by both genetic and environmental components, as well as biological interactions:

Phenotype = environment + genetics/biological factor + environment/genetic interaction).

Genetic studies involving dopamine and locomotion outlined in this review provide a solid basis for genetic differences in the dopamine system mediating behavioral differences in regard to physical activity in animals. Not covered in this review, but still very important, are the biological interactions that may also be playing a role in dopaminergic regulation of physical activity. The dopamine system does not act in isolation, and is affected by interaction with other neurotransmitter systems such as serotonin. Other biological and/or environmental factors such as hormonal influences may also play an important role in this regulation. A proposed model for this regulation is outlined in Figure 3. The dopamine system appears to be a central component determining the phenotype of physical activity in that dopaminergic signaling is

determined in part by genetics, is also influenced by the environment, and can interact with the environment and other biological components. Thus, the dopamine system appears to act in a dual role – both dependently and independently to regulate levels of physical activity performed by a given animal. As a result, it is important to take a multifaceted approach for future research to seek out the underlying mechanisms of this genetic/biological regulation of physical activity in order to improve human health and prevent disease.

Figure 1: Model of brain dopaminergic tracts.

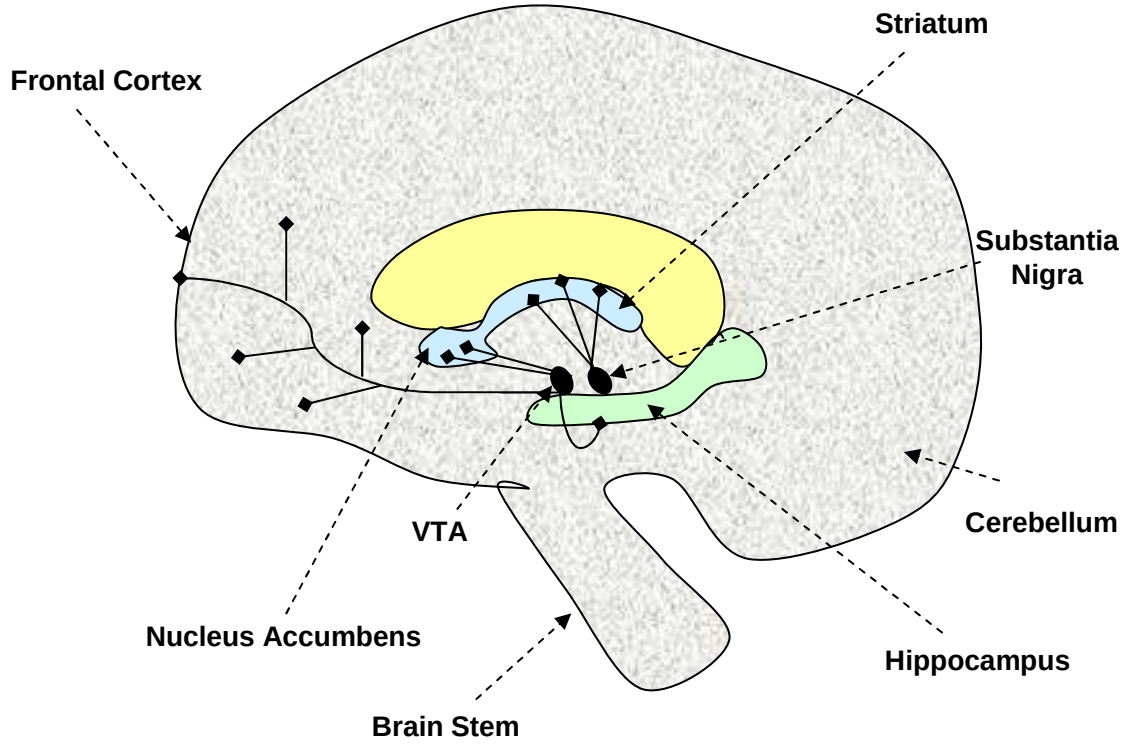

Figure 1: This figure illustrates the known dopaminergic neuronal tracts. The nigro-striatal tract consists of dopaminergic neurons originating from the substantia nigra, and projecting into the striatum. This tract is thought to be involved in control of motor movement. The mesolimbic tract is made of dopaminergic neurons projecting from the ventral tegmental area (VTA) into the nucleus accumbens, frontal cortex, and hippocampus. This area is thought to be involved in motivation, reward, and learning. Thus, the striatum and nucleus accumbens may play an important role in regulating the motivation for physical activity. Dashed arrows indicate specific brain regions, while blunt ended solid line arrows indicate dopaminergic neuronal tracts.

Figure 2: Representative dopaminergic synapse

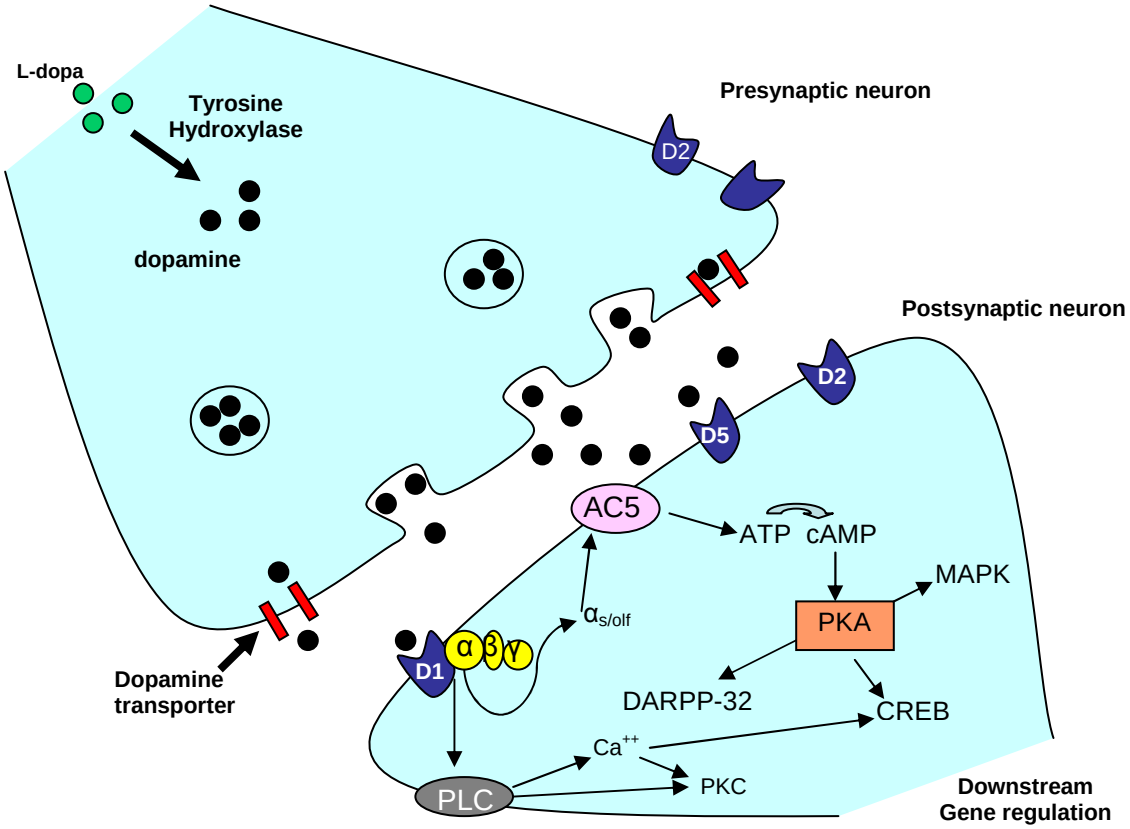

Figure 2: The above illustration is a representative dopaminergic synapse. The signaling pathways in the postsynaptic neuron are only representative of D1-like receptor signaling (which increases cAMP). D2-like receptors are known to have opposite affects on cAMP activity, and thus slightly different downstream signaling cascades. Dopaminergic signaling effects on ion channels and membrane permeability are not shown however, may be important in the regulation of behavior such as physical activity. For a full review of the signaling cascades proposed to be involved in D1-like and D2-like receptor signaling please refer to Neve et al. 2004 (176). Abbreviations: AC5 – adenylate cyclase 5; ATP – adenylyl tri-phosphate; CREB – cyclic AMP response element binding protein; DARPP-32 – dopamine and cyclic AMP-regulated phosphoprotein (thought to be important in positive feedback signaling); D1 – dopamine receptor 1; MAPK – mitogen-activated protein kinase; PKA – protein kinase A; PKC – protein kinase C; PLC – phospholipase C.

Figure 3: Proposed Schematic of the role of dopamine system in the central regulation of physical activity

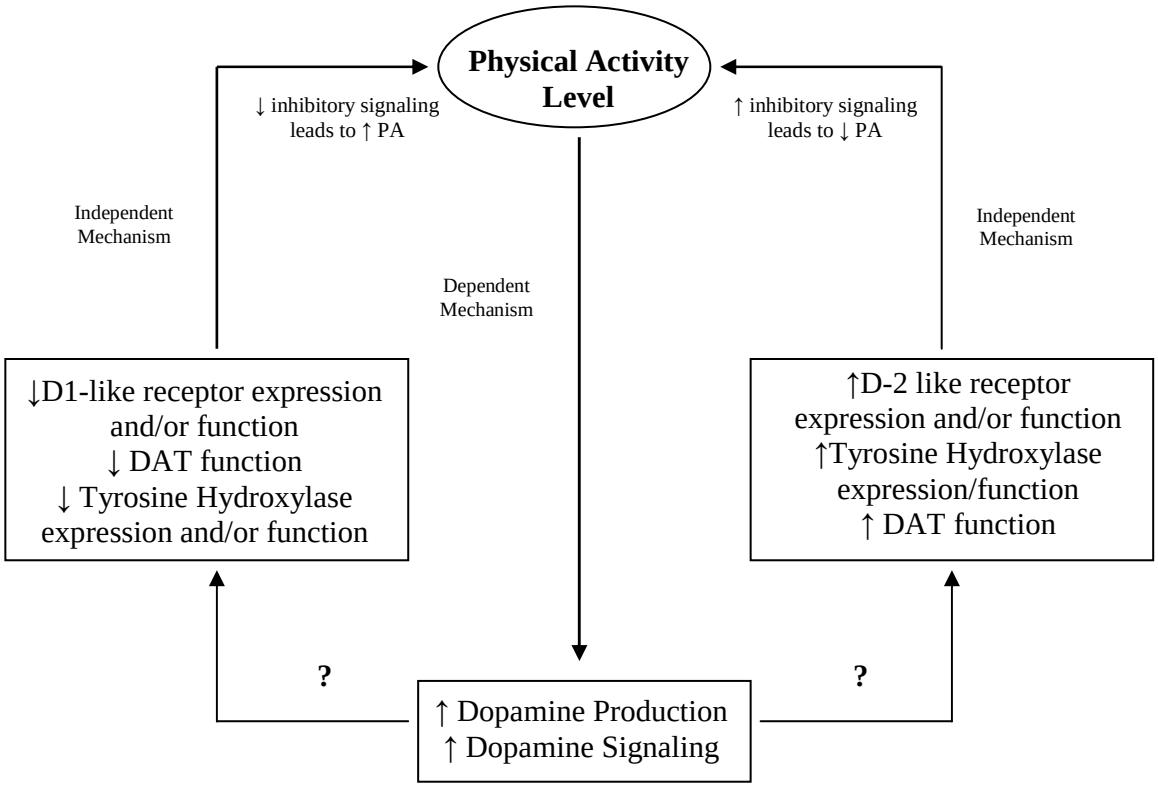

Figure 3: It is proposed that the dopamine system can act in both an independent and dependent manner in regard to regulation of physical activity. Both genetic factors, and biological factors that interact with the genetic machinery, are important in second messenger signaling, and downstream gene expression changes to dopaminergic neuronal signaling. Likewise, it is also possible that physical activity (i.e. intensity and duration of exercise) can cause changes in neuronal signaling as well, possibly mediating a reinforcing behavioral mechanism. Proposed differential effects on physical activity of D1-like vs. D2-like receptor expression, DAT function, and Tyrosine Hydroxylase function are included. "?" indicates unknown signaling pathways.

Table 1: Summary of dopaminergic findings in selectively bred mice for high WR

Area of Brain	Methods	Finding	Conclusions	Reference
Hipocampus	Gene Array	24% ↑ D4 receptors 19% ↑ D2 receptors	small changes in gene expression in the brain can cause large phenotypic changes. D1 receptors were not analyzed.	Bronikowski et al., 2004
Lateral Hypothalamus, Medial Frontal Cortex, Striatum	Fos expression in selected mice blocked from wheel	↑ Fos expression	Different brain regions in control of intensity of running vs. motivation for running	Rhodes et al., 2003
N/A	Agonists, Antagonists, re-uptake inhibitor	Differential responses in WR in selected mice vs. controls	D1-like receptors likely involved in mediating high WR in selected mice	Rhodes and Garland, 2003

Table 1: Evidence from studies in selectively bred mice for high wheel running suggest the central regulation of physical activity likely involves the dopamine system.

Table 2: The dopamine system and locomotion in disease states

Disease	Parkinson's Disease	ADHD	Anorexia	Addiction
Possible Mechanism	loss of DA neurons	DRD4/DRD5 and DAT	D2/D3? Altered signaling	D1/D2, DAT, altered signaling
locomotor outcome	lack of motor control	Hyperactive Phenotype	↑ drive for activity (other OCD tendencies)	mediates motivation for pleasure/reward seeking

Table 2: Dopamine signaling plays a prominent role in locomotor dysfunction in several disease states. Possible mechanisms are listed based on the described literature

CHAPTER 2:
REPEATABILITY OF EXERCISE BEHAVIORS IN MICE

Abstract:

Purpose: Measurements of exercise behaviors in rodents such as maximal treadmill endurance and physical activity are often used in the literature; however, minimal data are available regarding the repeatability of measurements used these exercise behaviors. This study assessed the repeatability of a commonly used maximal exercise endurance treadmill test as well as voluntary physical activity measured by wheel running in mice. **Methods**: Repeatability of treadmill tests were analyzed for both inbred and outbred mice in addition to a 10 week repeatability analysis using Balb/cJ mice (n=20). Voluntary daily physical activity was assessed by; distance, duration, and speed of wheel running (47). Physical activity measurements on days 5 and 6 of WR in a large cohort (n=739) of both inbred and outbred mice were compared. **Results**: No significant differences ($p>0.05$) in exercise endurance were found between different cohorts of Balb/cJ and DBA/2J mice; however, significant differences were seen within $BaD2F_2$ animals ($p<0.001$). Weekly endurance testing over 10 weeks in Balb/cJ mice showed significant differences among weeks for female mice ($p = 0.04$), no significant differences among weeks in male mice ($p = 0.33$), and no significant correlations between paired endurance measures within each mouse. Within mouse comparisons of exercise endurance tests showed large average percentage differences between tests in all mice (404±463%, mean±SD). No significant differences were found

for WR measurements within mouse between days (p=0.99). High correlations between days within mouse for WR was found (r=0.74-0.85). **Conclusions**: High intra-mouse variability between repeated endurance tests suggests that treadmill testing in an enclosed chamber with shock grid for motivation to run in mice is not repeatable. Conversely, high correlations and low percent differences between consecutive measurements of WR suggest that measurements of voluntary activity are repeatable and stable within individual mice.

Key Words: running wheel, endurance, treadmill, physical activity

Introduction:

Most measurements of exercise behavior in humans (e.g. exercise endurance, $VO_{2max,}$ activity level) have been shown to be repeatable within subject (24, 162, 282). With this precedence, measurements of exercise endurance and daily physical activity in rodents are often used to investigate regulating mechanisms associated with exercise that are difficult to measure in humans (142, 149, 151). Given the high test-retest repeatability for human exercise behavior measurement, it is natural to assume that endurance tests in rodents would also be repeatable and stable. However, repeatability of exercise measurements in rodents must be established to ensure valid physiological conclusions from such studies.

Exercise behavior testing in rodents usually consists of either the determination of exercise endurance/capacity and/or voluntary daily activity. Forced exercise capacity tests in rodents generally use small treadmills encapsulated by a chamber to assess maximal exercise endurance and/or VO_{2max} (129, 141, 150, 255, 279). These treadmill protocols typically use a variety of stimuli (e.g. shock grid, tail tapping, or high pressure

bursts of air) to motivate the animal to run. Treadmill testing for assessment of endurance/aerobic capacity in rodents has been generally preferred to swimming tests since rodents do not display consistent swimming behaviors (e.g. animals will bob, float, and/or dive) and these behaviors skew any data investigating aerobic capacity (132). Several variations of exercise treadmill protocols have been used with rodents (15, 129, 142, 150, 151, 195, 255, 279); however, in the current literature, limited studies report a measure of repeatability of forced treadmill testing within animal (20, 79, 195). These studies report within animal repeatability of VO_{2max} measurements, using enclosed treadmill protocols ranging from r=0.42 to 0.97 (20, 79, 195). In spite of the wide use of exercise endurance treadmill testing in rodents, no repeatability measures of maximal running time using enclosed chambers without VO_{2max} measurement have been reported. Koch and colleagues used a protocol consisting of five consecutive endurance tests on consecutive days (129) and have reported that "120 runs in 24 female rats were found not to be different from a normal distribution as assessed by the Kolmogorov-Smirnov test". Unfortunately, it was not noted whether the five tests differed significantly from each other, and it is not clear whether this is a good indicator of repeatability. Thus, although some papers present some form of repeatability of VO_{2max} measurements in rodents, no studies have systematically analyzed the within subject repeatability of forced exercise treadmill tests in rodents.

The other most common measurement of exercise behavior in rodents involves the determination of daily voluntary activity levels using wheel running (69, 149, 152, 253, 257, 262, 298). Much like exercise endurance, day-to-day wheel running within strains of rodents has been assumed to be repeatable; however, little data is published

regarding this assumption. Friedman and colleagues (79) evaluated several locomotor behaviors including wheel running in 35 random bred male ICR mice and reported a r-value=0.852 (with deletion of one outlier) between days 6 and 7 of wheel running. Additionally, Swallow et al. (255) tested 577 male and female mice selectively bred for high-wheel running activity and reported a r-value=0.787 for females, and a r-value=0.868 for males for repeatability of wheel running between days 5 and 6 of data collection.

Given the relative paucity of the data regarding the repeatability of rodent exercise behaviors in the literature, the goal of this study was to examine the repeatability of commonly used forced exercise treadmill tests and daily voluntary physical activity measurements in several cohorts of inbred and outbred mice.

Methods:

Overview

A variety of different mouse cohorts were used in the completion of this study. Archived, unpublished data from several previous studies (149-152) as well as data collected specifically for this project are reported in this paper. All procedures were reviewed and approved by the University of North Carolina Charlotte Institutional Animal Care and Use Committee, conformed to the animal care policies of the U.S. Department of Agriculture (USDA), and conformed to the *Resource Book for the Design of Animal Exercise Protocols* (132). All animals were housed in the University Vivarium with 12 hour light/dark cycles, were provided standard rodent chow (Harlan Teklad) and water *ad libitum*, and were weighed weekly. Mice used in maximal exercise treadmill tests were group housed with 4 mice per cage and identified using ear

punches. Mice used during wheel running experiments were single housed in rat size cages and identified using a unique mouse number as well all other identifying information on cage cards.

Animals Used

Exercise Endurance repeatability: The first question we sought to answer was whether exercise endurance was similar within inbred strain between different mouse cohorts separated in time. This question directly addressed whether exercise endurance within a particular strain of mouse was stable over time and was determined by comparing exercise endurance from two cohorts of Balb/cJ and DBA/2J inbred mice tested in the same manner in 1999 (150) and in 2005 (unpublished data). With both cohorts, we used an open treadmill, which allowed manual stimulation of the animal (tapping the tail) in conjunction with a shock grid to encourage running. Otherwise, the procedures used were the same as that addressed below. The strains tested in 1999 consisted of eight female Balb/cJ (weight = 19.0±1.2g) and seven female DBA/2J mice (weight = 16.9±1.4g), while the 2005 cohort consisted of 10 female Balb/cJ (weight = 20.6±0.8g) and 10 female DBA/2J mice (weight = 20.4±1.6g).

To determine repeatability of exercise endurance in outbred mice at two distinct time points, we compared exercise endurance from 80 BaD2F_2 outbred mice that were tested using a sealed metabolic chamber that used a shock grid as the sole means to motivate exercise. These 80 mice were chosen from a cohort of 300 F_2 mice because they exhibited either high (n=40) or low (n=40) endurance during a maximal endurance test conducted using methods outlined below and previously published (150). These mice were developed by reciprocally crossing high endurance Balb/cJ and low

endurance DBA/2J inbred strains (150), and exercise endurance of the $BaD2F_2$ mice was measured at 86.3±7.2 days (weight = 23.1±3.1g) and 140.1±5.3 days of age (weight = 24.9±2.7g).

Finally, to investigate the actual within mouse repeatability of exercise endurance across shorter time spans, but without intervening exercise training, 20 Balb/cJ mice (10 female, 10 male), were exercise endurance tested using the sealed metabolic treadmill approximately every seven days after two orientations to the treadmill (see below). Balb/cJ mice were chosen for this protocol because previous studies have shown this strain to perform well on forced treadmill tests (150). The males were tested every seven days starting at age 41.5±0.5 days. To eliminate possible sex hormone effects on exercise endurance, the female mice were tested during the diestrous phase of the estrous cycle which was determined by the presence of cornified epithelial cells in a vaginal smear (6). This testing began when the females were 44.6±0.5 days of age and given the normal length of the estrous cycle (≈4-5 days, with diestrous lasting 2-2.5 days), endurance treadmill testing was accomplished approximately once every seven days.

Physical Activity repeatability: We also determined if measurement of voluntary physical activity using a running wheel were repeatable. The data used to determine the repeatability of physical activity were taken from a large dataset using a base cohort of 739 mice from 22 inbred strains (n= 367; 129s1/SvImJ, A/J, AKR/J, Balb/cJ, C3H/HeJ, C3Heb/FeJ, C57BL/10J, C57BL/6J, C57BLKS/J, C57L/J, CAST/Ei, CBA/J, CE/J, DBA/2J, LP/J, MRL/MpJ, NZB/BinJ, PL/J, SM/J, SPRET/Ei, SWR/J, WSB/Ei) and from 2 outbred strains developed in our laboratory (n=372, $C3C5F_1$,

$C3C5F_2$). Within this large cohort, there were 324 females and 415 males. Given that the highest activity levels for mice generally occur between 9 and 12 weeks of age (256), we attempted, where possible, to draw data for the day 5/day 6 repeatability comparison when the mice were 68-69 days of age (i.e. 9 weeks + 5 days). Thus, the average age of the mice for the day 5-6 comparison was 69.7±7.4 days. In 34 cases, data for the repeatability comparison was shifted from day 5-6 to day 4-5 or to day 6-7 because of equipment sensor failure on either day 5 or 6 of wheel running exposure. It is common in wheel running literature to report repeatability based on day 5 and 6 of wheel running exposure (254).

Forced Maximal Endurance Testing

Similar methods were used to determine exercise endurance for all mice (150, 151) with the exception of the use of an open treadmill or a sealed, metabolic treadmill (5.08 cm x 38 cm; Columbus Instruments, Columbus, OH). All mice, regardless of the treadmill used, had one or two orientation exposures to the treadmill, each separated by at least 48 hours from the other orientation exposure or an exercise endurance test. In all cases, the front eight cm of the treadmill chamber was covered to provide a dark area for the mice to run toward. The first orientation exposure consisted of placing the mouse on the treadmill and letting the mouse walk on the treadmill at 16 m/min for 15 minutes. A shock grid mounted at the back of the treadmill delivered a 3.0 mA current (142, 255) to provide motivation for exercise. The treadmill endurance protocol consisted of a series of stages and has been described previously (150, 151). Briefly, each stage was three minutes long with the initial stage being a period of rest. At the end of the first three minutes, the speed was increased to 16 m/min and then increased

by four m/min every three minutes until a maximum speed of 40 m/min. If the mouse was still running at this stage the grade was increased every three minutes by five percent. The test was ended when the mouse sat on the shock grid at the back of the treadmill for five seconds, or if the protocol was maxed out at 36 minutes, 40 m/min, and 15% grade.

To determine if exercise endurance measurement was repeatable over a longer period when tested weekly, each Balb/cJ mouse was endurance tested once a week for a period of ten weeks. As noted earlier, female mice were only tested during the diestrous phase of the estrous cycle when estrogen levels are lowest. To eliminate technician bias, five male and five female mice were randomly assigned to one of two technicians and these technicians conducted the endurance tests on the same ten mice each week throughout the study.

Voluntary Physical Activity Measurement

Daily running on the wheel was measured using methods described previously (137, 149, 152). Briefly, mice were housed individually, with a running wheel (circumference 450mm; Ware Manufacturing, Phoenix, AZ) mounted in each cage. The wheels were equipped with a magnet mounted on the outside surface and the top of the cage was equipped with a magnetic sensor (BC500; Sigma Sport, Olney, IL). Each cage computer was calibrated for the wheel circumference allowing for accurate measurement of distance (km) and time the animals ran on the wheel (duration = mins). Speed of activity (m/min) on both days was calculated by dividing daily distance by daily duration of exercise. The data were collected every 24 hours for 7-21 days and data collected on days 5 and 6 were used for repeatability testing. The wheels were

checked manually each day to assure sensor alignment and free-turning of the wheel. "Coasting" by the mice, where the mice stopped running while the wheel continued to turn with the mouse still on the wheel, was not a concern due to three factors: 1) The running wheels used had a metal solid-surface and thus, they could not grip the wheel to coast unlike if the treadmill surface were mesh; 2) the wheels had a diameter that was too small for the mouse to run up one side and then coast as the wheel re-centered from the unequal weight on one side of the wheel; and 3) two cross axis bars attaching the wheel to the axle prevented the mice from jumping off the wheel while it was still turning, thus requiring that the mouse stop the wheel before getting off and removing any excess wheel spinning. In addition, anecdotally our research team has not ever observed the mice coasting the running wheels we use to measure daily activity.

Statistical Analysis

All analyses were conducted using JMP software (ver. 7.0, SAS Institute, Cary, NC) and the alpha value was set *a priori* at 0.05. Several analyses were used depending upon the questions being examined. A two way ANOVA (factors = strain and year tested) was used to determine the overall stability of exercise endurance between different mouse cohorts separated by time. A two way ANOVA (factors = endurance classification and time of measure) with a repeated measure on one factor (time of measure) was used to determine the repeatability of exercise endurance within a cohort of F_2 mice that were classified on the basis of one exercise endurance test. Determination of the repeatability of exercise endurance every week for 10 weeks within the same cohort of animals was accomplished using a two way ANOVA (time of measurement and sex) with time of measurement being a repeated factor. Additionally,

due to previous concerns, we conducted pairwise correlations between all 10 weeks of endurance testing to determine the association of endurance test results across the 10 repeated endurance tests. In all analyses, Tukey's post-hoc analysis was used where significant main effects were observed.

A two-way ANOVA (day of measurement and sex) was used to initially determine if sex played a role in the repeatability of any of the physical activity measurements. If sex exerted a non-significant main effect, the analysis was repeated using paired t-tests with each running wheel index (i.e. distance run, duration of exercise, and speed of exercise) to determine if activity level measurement was repeatable between days 5 and 6 of exposure to a running wheel.

Results:

Different groups of Balb/cJ and DBA/2J mice were endurance tested in 1999 and 2005. Results in Figure 1 show that endurance test performance was not different between these measurements, within strains of mice (Balb/cJ mice, $p=0.55$; DBA/2J mice, $p=0.51$) despite being separated by approximately six years. A large cohort of F_2 outbred mice ($n=300$) were exercise endurance tested at 12 weeks of age and the top 40 performing animals were classified as "high endurance" and the lowest 40 performing animals were classified as "low endurance". A second endurance test was conducted on these 80 mice within seven weeks of the original test. Figure 2 shows a comparison of the average endurance of the high and low endurance mice between the first and second exercise test. In the second test, the high endurance mice exhibited significantly less endurance ($p<0.001$) than on their first test. Conversely, the low endurance mice exhibited significantly higher endurance ($p<0.001$) than on their first test.

In comparing the 10 weeks of endurance testing among male and female mice, no difference in association between max endurance tests were attributed to sex. Thus, all animals were combined, and pairwise correlations were completed for all 20 Balb/cJ mice for each week of endurance testing (Table 1). When compared using repeated measures analysis, starting at week four, significant differences were found between males and females in overall average run time with males running a significantly longer duration than females ($p=0.035$; data not shown). Repeated measures also showed significant differences between exercise endurance tests across weeks in the female mice ($p=0.041$). The coefficient of variation within each mouse between exercise endurance tests over the 10 weeks was very high for both males and females (average CV= 37.0, CV= 51.0 respectively). Further, there were large average percent differences within mice between endurance tests for both males (287±316%, mean±SD), females (521±568%), and the total group (404±463%) (Fig. 3). No technician bias was found to have been associated with the variation in endurance scores ($p>0.05$, $t=1.97$) and body weight was not correlated with endurance performance (males, $r=0.26$; females, $r= -0.15$).

In regard to wheel running repeatability, female and male mice exhibited similar repeatability measures in distance, duration, and speed (data not shown). Thus, when all mice were pooled, there were no significant differences found between days 5 and 6 in distance, duration, or speed (Fig. 4). Additionally, high correlations between days 5 and 6 (distance, $r=0.74$; duration, $r=0.74$; speed, $r=0.85$) indicate repeatability within mouse for physical activity measurements.

Discussion

Over the past several years, studies examining both maximal endurance phenotypes and physical activity phenotypes in rodents have been reported in an effort to assess the genetic/biological factors involved in the regulation of these exercise behaviors (59, 129, 142, 149, 151, 153, 195, 200, 253, 279). Given the relative consistency of these measures of exercise behaviors in humans (e.g. VO_{2max} tests) and in smaller reported cohorts of mice, all of which assessed repeatability of VO_{2max} measurements (20, 79, 195), it has been natural to assume that these measures were repeatable in mice. In addition, given the fact that VO_{2max} is a good predictor of exercise endurance in humans (17, 42), and has been shown repeatable, the assumption could be made that maximal endurance tests used to assess endurance in rodents (15, 129, 150, 280) would also be repeatable. Our finding of within strain stability of overall endurance in different cohorts of mice over a six year period (Fig. 1) and the repeatability of voluntary physical activity measurements (Fig. 4) support this assumption. However, over the course of several years, and a number of studies, a lack of consistency in repeat testing of mouse maximal endurance became apparent in our lab (Fig. 2). This evidence, led us to conduct the 10 week repeatability of max endurance outlined in Table 1, and combined, these data raise questions regarding the repeatability of this method of maximal endurance measurement in mice.

Forced Maximal Endurance Tests

Conducting endurance treadmill tests in rodents can be difficult. It has been noted (20, 132) that anywhere from 10-25% of rodents will refuse to run on a treadmill, even with orientation exposures. Given the difficulty of having rodents perform forced

endurance tests, it is surprising that relatively few studies have reported repeatability results of maximal exercise endurance or VO_{2max} using a graded treadmill protocol in mice. Rezende and colleagues (195) measured VO_{2max} during endurance treadmill tests in mice (n=48) selectively bred for high wheel running and reported repeatability of VO_{2max} during treadmill tests as r=0.42. Uniquely, Rezende and colleagues also reported using a subjective scale to assess the quality of the treadmill tests. Any "poor trials" were not included in the analysis (195) suggesting that there was some acknowledgment that animals may not repeatedly run to exhaustion.

Other interpretations of rodent exercise capacity repeatability may be hampered by methodological limitations. Bedford and colleagues (20) tested the repeatability of a ten-stage graded treadmill test in rats (n=18) and reported a reliability coefficient of 0.97. However, Bedford and colleagues operationally defined VO_{2max} as "one in which there is less than a 5% increase in VO_2 with increase in work intensity." This operational definition was different than what is normally used in literature - allowing the rodent to run to exhaustion - and this operational definition difference may contribute to their observation of higher repeatability values compared to other studies. We have noted that even in using four different rodent metabolic carts and three different forced exercise modalities, that oxygen consumption values in rodents often peak very early in a forced endurance test and then decline in spite of continued increases in workload. Speculatively, this type of response is most likely due to the common set-up of most commercially available rodent exercise metabolic chambers which allows the animal to remove their ventilatory stream from the gas sampling airstream when the mouse runs farther back on the treadmill. Support for this

suggestion comes from earlier work by Friedman et al. (79) that tested the repeatability of several locomotor behaviors in random bred ICR mice (n=38) and reported a repeatability for VO_{2max} of r=0.809. In this study, the authors used the peak VO_2 measurement during a test as the VO_{2max} regardless of whether this peak measurement occurred at the end of the test when mice were exhausted and unwilling to run farther or if the peak was reached earlier in the test but the animal continued to run beyond this point. Thus, our observations, combined with both Friedman and colleagues' (79) and Bedford et al.'s (20) studies suggest that repeatability of a forced exercise test in a rodent may depend upon the operational definition of the primary measure (e.g. VO_{2max}) used as well as the testing equipment used.

Since measurement of maximal aerobic capacity in rodents can be challenging, graded treadmill protocols have also been used to measure maximal endurance without measurement of VO_{2max} (15, 129, 150, 280). To date, repeatability of exercise endurance measures using this type of protocol has not been reported. Koch et al. (129) initially implemented an endurance testing protocol which consisted of a week of increasing orientation bouts on a treadmill, followed by endurance max testing in the second week for five consecutive days to assess heritability of exercise endurance in rats. These authors reported that within sex, variation in the five consecutive max endurance tests "was found not to be different from a normal distribution". However, in none of the publications where this endurance testing model has been used, has it been noted whether the five tests differed significantly from each other, nor whether possible physiological training effects of the five consecutive max tests occurred. Regardless of whether these items were considered, the exhibition of a normal distribution across

repeated testing does not indicate repeatability. For example, in the current study, the repeated testing we did over a ten-week time period (Table 1, and Fig. 3), was still normally distributed (Shapiro-Wilk W test, p=0.12) in spite of exhibiting an approximately 400% difference in day to day results and virtually no test-test significant association. Therefore, a set of repeated measures can have a normal distribution, yet be significantly different within-subject and thus, not be a repeatable test-test. While it appears that measurement of VO_{2max} in rodents may be repeatable under specific conditions, the data found in our study (Table 1, Fig. 2, and Fig. 3) indicate that measurement of endurance in mice (as assessed by time to exhaustion in a sealed treadmill chamber using shock grid for motivation) may not be repeatable.

Indeed, one possibility for the lack of repeatability within our studies was the use of a sealed metabolic chamber with shock grid. During our early use of an open treadmill which allowed manual encouragement of running (using tail tapping – see Fig. 1) we observed significant repeatability within strain, even across several years. The use of an enclosed treadmill, while necessary for metabolic measures, eliminates the possibility of using manual encouragement, as a supplement for electric shock, for the mice to continue running. While we do not have repeated measures of exercise endurance within mice using an open treadmill, this is an observation that bears further explanation.

Another possibility to explain the lack of repeatability we observed using the sealed treadmill with electric shock is that this type of testing may be more of a psychological stressor to the animal than other exercise measurements such as voluntary wheel running. This hypothesis is supported indirectly by several studies. First, the use

of various means of motivation for running during forced treadmill tests (e.g. electric shock, puffs of air, tapping of the tail) may induce a negative response in the animal similar to that of chronic psychological stress, and this could mask true exercise behaviors (172). One such negative response is the observation that brain derived neurotrophic factor (BDNF) decreases after forced exercise in animal models similar to the effects seen during immobilization stress (3, 4). However, in humans, treadmill exercise has been shown to have beneficial effects on the brain including increased BDNF levels (68) contributing to an antidepressant effect (63). Thus, treadmill exercise in rodents may not be an appropriate model for the comparison of the response to treadmill exercise in humans due to the psychological stress to the rodent, which may in turn contribute to this measurement being non-repeatable in mice.

The difference observed between the repeatability of exercise endurance in male and female mice during the repeated 10 week endurance study was unexpected, but may be related to the time of measurement within the estrous cycle. Female mice were only endurance tested during the diestrous phase of their cycles which corresponded to periods of low estrogen. There have been no studies investigating the effects of the estrous cycle on exercise endurance in rodents; however, numerous other studies have suggested that estrogen may play a role in the regulation of overall physical activity patterns (202). Thus, while it cannot be definitively concluded that the low estrogen levels are responsible for the sex difference seen in average exercise endurance in this study, the wide test-to-test variation seen in both females and males across time (averaged 404±463%, Fig. 3) and the lack of significant test-test association (Table 1) lends support to the finding that exercise endurance measured in a sealed treadmill is

not repeatable. Furthermore, the observation of no significant differences in exercise endurance between the 10 repeated tests in the male mice may have occurred because the variation between tests were so large that statistical significance may have been undetectable. This hypothesis is supported by the large test-to-test variation in both the male and female mice and was further mirrored in the large average percent differences between endurance tests for both males and females (Fig. 3). Additionally, it is worth noting that none of the animals in this maximal treadmill protocol actually reached the end of the protocol before stopping; thus, variation in the endpoints of the protocol did not contribute to the overall variation observed. Therefore, although males and females were significantly different in average run time on the endurance tests, both sexes were similar in their lack of repeatability in this measure. The overall average percent difference of 404±463% in maximal exercise endurance we observed with repeated testing is relevant given the repeatable nature of maximal endurance testing in humans (8-10%) (162) and the growing number of studies that are using maximal endurance testing without repeatability monitoring to distinguish between treatments in animals (173, 291).

Voluntary Physical Activity (Running Wheel)

Our large cohort data in addition to the available literature suggest that physical activity as measured by running wheel activity in rodents is a repeatable phenotype (Fig. 4). Swallow and colleagues (253) reported high repeatability of running wheel activity on days 5 and 6 of measurement in selectively bred mice (n=287 females r=0.787; n=273 males r=0.868). In addition, Rezende and colleagues measured VO_{2max} during wheel running in selectively bred female mice (n=48) and reported repeatability

of VO_{2max} measurements during running wheel activity as r=0.844 (195), indicating both running wheel activity, and VO_{2max} achieved during wheel activity are repeatable. Similar to humans, levels of BDNF increase in the brain following voluntary physical exercise in mice (21), possibly helping to explain the repeatability of this phenotype in rodent models.

It is also warranted to speculate that the repeatability of wheel running in rodents is due to the voluntary and perhaps innate nature of this activity. Rowland (208) described the idea of an intrinsic biological control of energy expenditure in animals. From an evolutionary standpoint, it would be beneficial for organisms to maintain energy balance, and he proposed this was done by an "activity-stat" mechanism. Rowland proposed several lines of evidence, including genetics, for this "activity-stat" mechanism which would theoretically work centrally to control amount of intrinsic physical activity, and thus, energy expenditure (208). Supporting the hypothesis of an "activity-stat" is the observation that genetically different strains of mice differ in the level of voluntary wheel running (149). Because this "activity-stat" would be regulated centrally and would be intrinsic to individual animals, this could explain why the measurement of voluntary physical activity has been shown to be repeatable in the rodent literature.

Conclusions

In conclusion, while average exercise endurance within strain measured with an open treadmill across time appears to be stable, exercise endurance measurements using sealed treadmills repeated on the same mouse are not repeatable. Crabbe and colleagues (43) employed a well designed study to show that inbred strains of mice

differ in behavioral phenotypes depending on the laboratory setting. Even though different technicians and slightly different laboratory settings were employed for the different cohorts of mice outline in Figure 1, these two strains, as groups, tested the same over time. The different reported values for repeatability of VO_{2max} testing in rodents in the literature could be partially explained by the evidence presented by Crabbe and colleagues; however, in the current study, even when repeated maximal endurance testing in the same lab, under the same conditions, with the same technicians was employed (Table 1) the results indicate high variability in this behavioral test. It may be possible to reliably endurance test rodents using other methods; however, the results in this study indicate using an enclosed treadmill with a shock grid for aversive stimuli that produces a negative stimulus to encourage mice to run to "exhaustion" is not a repeatable measure for assessing exercise endurance in mice. In contrast, daily physical activity as assessed by distance, duration, and speed on a running wheel appears highly repeatable in both inbred and outbred mice. The level of voluntary physical activity an animal performs appears to be both genetically and biologically regulated possibly influencing the high repeatability of this phenotype. The observations in this study are critical in considering results from current and future exercise behavior literature that investigates the role of various biological factors involved in the regulation of exercise behaviors in rodents.

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Footnotes:

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Figure 1: Average time run of two different cohorts of Balb/cJ and DBA/2J mice

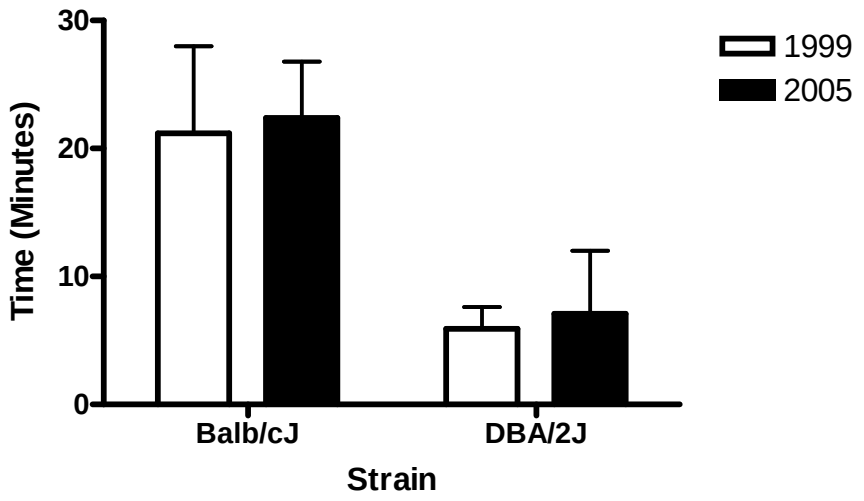

Figure 1: Average Time (and standard deviations) in minutes of two different cohorts of Balb/cJ mice and DBA/2J mice. No significant differences were found between years within either strain (Balb/cJ mice, p=0.55; DBA/2J mice, p=0.51).

Figure 2: Comparison of endurance Test 1 vs. Test 2 in 80 F_2 mice

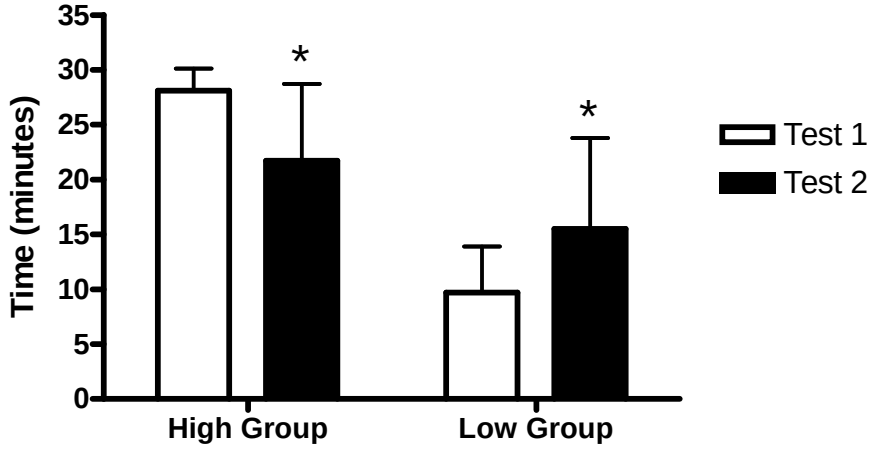

Figure 2: F_2 animals classified as high runners were significantly higher than those classified as low runners in each test ($p<0.001$). Test 2 endurance results were significantly different than test 1 endurance results within each group ($p<0.001$). * indicates a significant difference between test 1 and test 2, within group.

Table 1: Correlation Values of Endurance for Each Week of Testing in 20 Balb/cJ mice

Week	1	2	3	4	5	6	7	8	9	10
1	---	.27	.34	-.15	.23	.02	-.08	-.08	-.04	.25
2		---	.35*	.35	.21	.16	.47	.03	.05	.41
3			---	.08	.44	.12	.30	.10	-.08	.34
4				---	.16	.75	.76	.66	.41	.38
5					---	.33	.30	.10	.22	.44
6						---	.51	.52	.62	.33
7							---	.55	.24	.51
8								---	.42	.47
9									---	.18
10										---

* = significance at $p<0.05$

Table 1: Ten Balb/cJ male and ten Balb/cJ female mice were endurance tested once a week for ten weeks. No differences in association between max endurance tests were attributed to sex, thus matched pairs correlation values are shown in the table for all mice for all ten weeks. In addition, average coefficient of variation: males CV=63.5, females CV=118.5. Repeated measures ANOVA showed no significant differences in male mice across weeks ($p=0.33$), but there were significant differences in female mice across weeks ($p=0.04$) (data not shown).

Figure 3: Average percent differences between 10 consecutive endurance tests in male and female Balb/cJ mice.

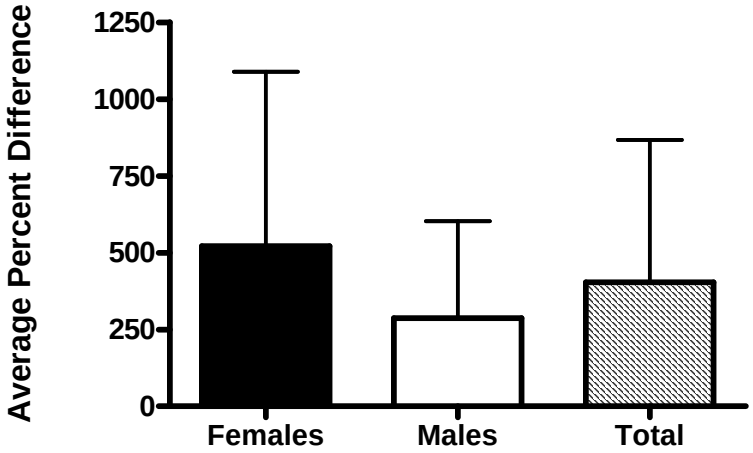

Figure 3: Average percent differences (± standard deviations) between endurance tests in male Balb/cJ mice (n=10), and female Balb/cJ mice (n=10).

Figure 4: Comparison of wheel running indices between day 5 and 6 of wheel running exposure in inbred and outbred mice.

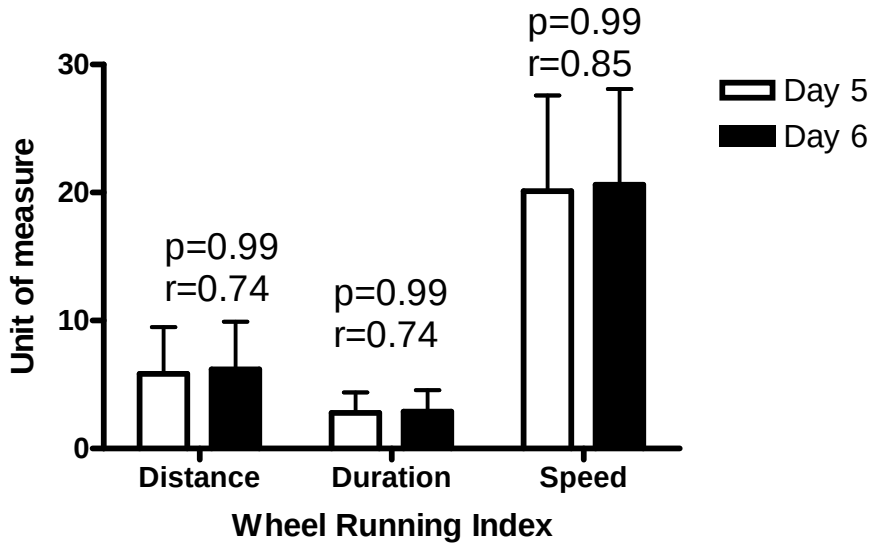

Figure 4: Comparison of wheel running indices between day 5 and 6 of wheel running exposure in inbred and outbred mice (n=739). No significant differences were found between the two days of measurement for any index (Distance, Km/day; duration, min/day*100; and speed, m/min). Correlation values are also reported for each index.

CHAPTER 3:
ALTERED DOPAMINERGIC PROFILES: IMPLICATIONS FOR THE REGULATION OF VOLUNTARY PHYSICAL ACTIVITY

Abstract:

The biological regulating factors of physical activity in animals are not well understood. This study investigated differences in central mRNA expression of seven dopamine genes (*Drd1*, *Drd2*, *Drd3*, *Drd4*, *Drd5*, *TH*, and *DAT*) between high active C57/LJ (n=17) male mice and low active C3H/HeJ (n=20) male mice, and between mice with access to a running wheel and without running wheel access within strain. Mice were housed with running wheels interfaced with a computer for 21 days with distance and duration recorded every 24 hours. On day 21, the striatum and nucleus accumbens were removed during the active period (~9pm) for dopaminergic analysis. On average, the C57L/J mice with wheels ran 99% farther, 98% longer, and 65% faster than the C3H/HeJ mice with wheels over the 21 day period. No differences in gene expression were found between mice in either strain with wheels and those without wheels suggesting that access to running wheels did not alter dopaminergic expression. In contrast, relative expression for two dopamine genes was significantly lower in the C57L/J mice compared to the C3H/HeJ mice. These results indicate that decreased dopaminergic functioning is correlated with increased activity levels in mice and suggests that D1-like receptors as well as Tyrosine Hydroxylase (an indicator of

dopamine production), but not D2-like receptors are associated with the regulation of physical activity in inbred mice.

Key Words: dopamine, locomotion, running wheel, mice, dopamine receptor, striatum, nucleus accumbens

Introduction:

It is axiomatic that physical activity is important to human health. Given the known benefits of physical activity, it is imperative to understand the mechanisms that regulate this behavior. It has been well established in both human and animal models that genetic factors significantly influence physical activity levels (69, 119, 135, 138, 142, 149, 153, 249, 262). However, the identity of which systems or genes are involved in the regulation of activity level is currently unclear.

The central function of the dopaminergic system is to control motivation for natural rewards and motor movement (285), and several studies in rodents suggest certain aspects of dopaminergic functioning may contribute to the genetic/biological regulation of physical activity (29, 198, 200, 235). The dopamine system has also been implicated in movement disorders such as Attention Deficit Hyperactivity Disorder (190), and Parkinson's Disease (130), making it a likely candidate to be involved in regulating voluntary activity.

Several studies have linked D1-like and D2-like dopamine receptors to various aspects of locomotion in animals (10, 11, 29, 55, 99, 122, 164, 235). However, the term "locomotion" in animal literature simply refers to the act of movement, which can encompass a wide variety of specific definitions depending on the methodology used. Voluntary physical activity, which is commonly defined as purposeful exercise or

movement that expends a significant amount of energy, appears to be a separate phenotype from locomotion, and no studies have been conducted to investigate the role of the dopaminergic system in the regulation of *voluntary* physical activity in inbred strains of mice. Artificial selection studies in mice have shown that mice bred for high wheel running activity not only have high motivation for natural rewards such as exercise, food, and sex, they also respond differently than controls to drugs such as Cocaine or Ritalin which act by blocking the dopamine transporter (200). In addition, Rhodes and colleagues found that D1-like antagonists reduced wheel running more in control line mice compared to selected animals, while D2-like antagonists had similar effects on both selected and control mice, suggesting D1-like receptors may be important in mediating the increased wheel running in the selected animals (199). Fink and Reis, 1981, showed that Balb/cJ mice have more dopamine activity in both the nigrostriatal, and mesolimbic pathways in the brain compared to CBA/J mice (70) suggesting that differences in the dopamine system are genetically determined in mice, and that these differences may translate into behavioral differences in motivation for physical activity.

Whilst dopaminergic functioning may act as an independent variable to regulate physical activity, it has also been shown that changes in the dopamine system such as increased dopamine activity and/or neural synthesis can be dependent upon physical activity (212, 259, 276, 290). From the current studies available (29, 70, 199-201, 235) it is unclear if dopamine functioning is acting independently on physical activity levels or if physical activity is affecting dopaminergic functioning. Therefore, this study investigated whether the dopamine system acts as the dependent or independent

variable in the regulation of physical activity by assessing expression differences in seven dopamine related genes in the striatum/nucleus accumbens area of the brain.

Methods:

Animals:

C57L/J mice, previously shown to be high active animals and C3H/HeJ mice, previously shown to be low active animals were used in this study (149). Both strains have been inbred past 130 generations, were purchased from The Jackson Laboratory (Bar Harbor, ME), and have no phenotypic abnormalities that would confound this study. Only male mice were used in this study to avoid possible confounding effects of the menstrual cycle on daily physical activity in female mice (7). All mice were housed in the University Vivarium with 12 hour light/dark cycles and were provided food (Harlan Teklad 8604 Rodent Diet, Madison, WI) and water *ad libitum*. All procedures were approved by the University of North Carolina Charlotte Institutional Animal Care and Use Committee.

In order to investigate whether the dopamine system is acting in a dependent or independent fashion in the regulation of physical activity, mice from each strain were randomly assigned to experimental groups housed with running wheels (C57L/J, n=10; C3H/HeJ, n=10), or control groups housed with no running wheels (C57L/J, n=7; C3H/HeJ, n=10). Each group was housed and treated the same other than the presence of a wheel in the experimental group. All mice were approximately 9 weeks of age at the beginning of the study. Only 7 control C57L/J mice were used because of difficulty in supply availability from The Jackson Laboratory.

Measurement of Voluntary Daily Activity Level:

Daily wheel running in mice was chosen as the model of human voluntary physical activity level (61) and was measured using methods described previously (149, 153). Briefly, mice were housed individually with a running wheel (450mm circumference; Ware Manufacturing, Phoenix, AZ) mounted in each cage. The wheels were equipped with a magnet mounted on the outside surface and the top of the cage was equipped with a magnetic sensor (BC500; Sigma Sport, Olney, IL). Each cage computer was calibrated for the circumference of the cage wheel allowing for accurate measurement of distance (km) and time the animals ran on the wheel (duration in min). The data were collected every 24 hours for 21 days and the wheels were checked manually each day to assure sensor alignment and free-turning. Speed of activity (m/min) was calculated by dividing daily distance by daily duration of exercise. Additionally, weight of all animals was recorded weekly.

Molecular Analysis:

Brains were harvested whole as described previously (29) and the striatum and nucleus accumbens area was dissected over ice and immediately flash frozen in liquid nitrogen and stored at -80°C. Tissues were harvested between 9 pm and 12 am, corresponding to hours 4 through 6 of the active cycle (12 hour light/dark cycle with the dark cycle between 6pm and 6am) in order to capture dopaminergic activity during the active period.

Quantitative real time RT-PCR was conducted using standard protocols to analyze mRNA expression of the following dopaminergic genes: dopamine receptor 1 (*Drd1),* dopamine 2 receptor *(Drd2),* dopamine 3 receptor *(Drd3),* dopamine 4 receptor

(Drd4), dopamine 5 receptor *(Drd5)*, tyrosine hydroxylase (*TH*), and the dopamine transporter (*Slc6a3* also known as *DAT*). Primers were designed using Primer 3 (Steve Rozen and Helen J. Skaletsky) (210) and ordered from Integrated DNA Technologies Inc (San Diego, CA). Total mRNA from the striatum and nucleus accumbens samples were isolated using trizol reagent (Sigma-Aldrich, Saint Louis, MO), and cDNA was prepared using QuantiTect Rev. Transcription Kit (QIAGEN, Valencia, CA). Real time analysis was conducted using QuantiTect SYBR Green PCR Kit (QIAGEN, Valencia, CA) and the LightCycler®1.5 Carousel-Based System (Roche Applied Science, Indianapolis, IN). All dopamine receptor mRNA expressions were normalized to an endogenous positive control (beta-actin) using methods as described previously (185).

Statistics:

Two-way ANOVA (JMP 7.0, SAS Institute, Cary, NC) was used to compare expression of all seven genes for the main effects of strain (C57L/J high active or C3H/HeJ low active) and group (wheel-running or non-wheel running). The alpha value was set at 0.05 and Tukey's HSD *post-hoc* tests were used when significant main effects were present to evaluate strain by group interactions.

Results

Voluntary Physical Activity

As expected from past research, the C57L/J mice were significantly more active than the C3H/HeJ mice (Figure 1). C57L/J mice with wheel access ran significantly farther (10.25±1.37 km/day vs. 0.01±0.09 km/day, $p<0.001$), longer (329.73±30.52 mins/day vs. 7.81±6.32 mins/day, $p<0.001$), and faster (31.27±3.13 m/min vs. 11.81±1.08 m/min, $p<0.001$) than C3H/HeJ mice with wheel access during 21 days of

wheel running data collection. There was no difference ($p=0.67$) in starting weights between C57L/J mice (25.6±1.0g) and C3H/HeJ mice (25.6±1.1g). There were also no significant differences ($p>0.05$) in weight within C3H/HeJ mice between group or over time [Control group: beginning weight=25.8±0.9g, end weight=26.1±1.5g; Running wheel group: beginning weight=25.8±1.2g, end weight=27.0±1.4g]. Additionally, within the C57L/J mice, no significant changes in weight were seen over time within group ($p>0.05$); however, C57L/J control mice weighed significantly more ($p<0.05$) at the end of the study than C57L/J running wheel mice at the beginning of the study [Control group: beginning weight=26.1±1.5g, end weight=26.8±1.3g; Running group: beginning weight=25.3±0.7g, end weight=26.5±0.9g].

mRNA Expression

No significant differences in expression of any of the genes were found between wheel-running and non-wheel-running groups within each strain (Figure 2 and Figure 3). However, significant differences were found between strains in the expression of the dopamine genes. The expression of *Drd1* ($p<0.0001$, power=0.90), and *TH* ($p=0.0008$, power=0.90) (Figure 4) were markedly different between the high active and low active mice. C57L/J mice (high active) expressed significantly lower amounts of mRNA of each of these genes in the striatum/nucleus accumbens than did the C3H/HeJ mice (Figure 4). Expression of *Drd5* ($p=0.05$; power = .44) bordered on significance between strains; however, this marginal difference in *Drd5* is not surprising considering that *Drd1* and *Drd5* are in the same sub-family of dopamine receptors. No differences in gene expression between strains were found for *Drd2* ($p=0.01$; power =0.4), *Drd3* ($p=0.21$; power =0.2), *Drd4* ($p=0.27$; power =0.2), and *DAT* ($p=0.83$; power =0.05).

Discussion

The genetic and biological regulating factors of physical activity are only beginning to be understood. This study showed that genetically different strains of mice not only differ in their physical activity levels, but that these differences are perhaps mediated at least in part by the dopamine system. Specifically, it was shown that C57L/J male mice run significantly farther, longer, and faster than C3H/HeJ male mice (Figure 1). No differences in expression of any of the dopamine receptors, as well as *TH*, and *DAT* genes were found as a result of access to a running wheel thus suggesting that activity was not altering dopaminergic expression levels. Finally, significant differences were found between the high and low active animals for both *Drd1* and *TH* dopaminergic genes. Both *Drd1* and *TH* were expressed at significantly lower levels in C57L/J (high active) mice compared to the C3H/HeJ (low active) mice. In conjunction with past literature relating dopaminergic functioning with activity, our results further support the hypothesis that the dopaminergic system independently regulates physical activity possibly through the *Drd1* receptors and tyrosine hydroxylase.

The results of this study highlight an important first step in the understanding of the genetic/biological regulation of physical activity. Voluntary physical activity has been shown to have a significant genetic component underlying the manifestation of this trait. Heritability studies estimate the genetic contribution to physical activity ranges from 20-80% (69, 135, 142, 149, 184, 262). Recent studies by Lightfoot and colleagues have also begun to elucidate possible quantitative trait loci (QTL) associated with regulation of physical activity in mice including a QTL that contains the *Drd1* gene (153). With this being said, biological (non-genomic) factors have also been

proposed as possible regulators of physical activity. For example, the sex hormones have been shown to significantly affect physical activity in rodents (96, 148, 179). It is warranted to speculate that the dopaminergic system may act in both a genetic and biological (non-genomic) manner in the regulation of physical activity. From a genetic standpoint, the current study highlights the possible importance of differences of overall expression of various dopaminergic genes (in particular *Drd1* and *TH*) in the mid-brain in mediating differences in physical activity levels between genetically different inbred strains of mice. However, it has also been proposed that the dopamine system may also be influenced by biological factors such as the sex hormones and this interaction may also be important to the regulation of physical activity (148).

Within the genetic component, it is unclear as to whether the genes regulate differences in physical activity levels between individuals through peripheral or central mechanisms. Several studies conducted using mice bred for high wheel running activity indicate a possible "central" regulation of physical activity as opposed to peripheral factors such as mitochondrial number, and/or muscle fiber type differences (59, 85, 89, 111, 112). Specifically, mice bred for high wheel running have altered regional brain activation profiles compared to control mice (200), as well as respond differently to dopaminergic acting drugs (199, 201). Thus, in this paper we hypothesized the dopamine system is an important central genetic factor involved in the regulation of physical activity behavior in inbred strains of mice. Similarly, the nucleus accumbens/striatum were investigated in this study because this area of the midbrain has been implicated in motor movement as well as motivation and reward behaviors (213).

It is well known that the dopamine system is important in mediating certain aspects of locomotion in animals. In addition to the dopamine system's known role in the motor movement disabilities manifested in Parkinson's disease (114), dopaminergic functioning has also been implicated in the hyperactive phenotype typical of Attention Deficit Hyperactivity Disorder (ADHD). The results of the current study suggest that high active mice have lower overall dopamine production and decreased dopamine signaling through D1-like pathways compared to low active mice. This result corresponds to research showing that the hyperactive phenotype appears to be a result of lower dopamine presence in the synapse and thus altered overall dopamine signaling in ADHD (144, 159). Ritalin improves symptoms of ADHD by blocking the dopamine transporter (DAT) and effectively increasing the amount of dopamine in the synapse. Similarly, when given cocaine or GBR 12909, both DAT inhibitors, mice selectively bred for high amounts of wheel running decreased their wheel running more than controls (201). We did not find a difference in the expression of DAT in the current study; however, this differential finding may be due to differences between the mechanistic underpinnings of high activity versus ADHD. Nevertheless, it is intriguing that the high active mice in this study had significantly lower amounts of *TH* in the striatum and nucleus accumbens area compared to low active mice indicating the amount of dopamine production and turnover is lower in high active inbred mice, and may be important for overall physical activity levels.

In addition to the dopaminergic role in general motor movement and locomotion aspects such as Parkinson's disease and ADHD, studies have begun to suggest the dopamine system may play a key role in motivation for movement as well. Rhodes and

Garland investigated the effects of several dopaminergic acting drugs on wheel running in mice bred for high amounts of wheel running and compared their responses to control line mice. They found that apomorphine (a non-selective dopamine agonist) and SCH 23390 (a selective D1-like antagonist) decreased wheel running more in the control lines compared to the selected lines, while treatment with raclopride (a selective D2-like antagonist) had similar effects on wheel running in both the selected and control lines (199). The authors suggested these results indicated the selected animals had a decreased function of the D1-like receptors, but not the D2-like receptors, and these differences may mediate motivational differences for high voluntary amounts of running in the selected animals. Our results correspond with the results from Rhodes and Garland, in that a decreased function of the D1-like receptors in a high active inbred strain compared to a low active inbred strain of mice was apparent, and suggests these receptors are important in the regulation of physical activity behavior, possibly in the form of motivation for this voluntary behavior.

It has been unclear whether the dopamine system acts as a dependent or independent factor in the regulation of physical activity. It has been shown that exercise causes changes in neurotransmitter systems, including an increase in dopamine production, and these responses lead to beneficial changes at the molecular and cellular levels including increased neuronal plasticity, cognitive functioning, learning, and overall mood (53). The neurotransmitter alterations is a primary reason that exercise is often used in the treatment of depressive disorders (28). While activity may influence dopaminergic functioning independently, the previous work, especially that from Garland's group as well as the known role of the dopamine system in regulation of

motivation and reward (50, 214), led us to hypothesize an independent role of the dopamine system in the genetic/biological regulation of physical activity. Our findings of similarities in brain dopaminergic gene expression within strain, regardless of whether the mice were exposed to a running wheel, suggests that expression levels of these genes are not necessarily subject to fluctuation based on activity levels and thus do not act in a dependent fashion in this case. The differences we observed between strains in *Drd1* and *TH* suggest that these particular genes may be acting in an independent fashion in mediating the large differences seen in activity levels between these two strains of mice.

The evidence presented in this study is an important first step to understanding the multifaceted genetic and biological regulation of voluntary physical activity levels. As mentioned previously, the genetic contribution to regulation of physical activity ranges from 20-80%; however, we are only beginning to understand the genetic regulating factors of this behavioral trait. The present study suggests the dopamine system may be an important central genetic factor involved in regulation of physical activity. In addition, this study is the first to highlight the fact that the dopamine system appears to act as an independent variable in the regulation of physical activity in mice, and specifically lower expression of the D1 receptor, as well as tyrosine hydroxylase in the mid-brain, may possibly mediate the high activity seen in the C57L/J strain. Given that the dopamine system itself is influenced by factors such as nutritional status, and hormones and that the dopamine system also regulates several downstream signaling pathways leading to differential gene expression, the central regulation of voluntary

physical activity is an intriguing avenue of study and certainly bears significance in the prevention of inactivity related diseases.

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Footnotes:

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Figure 1: Average distance, duration, and speed for C57L/J and C3H/HeJ mice over 21 days.

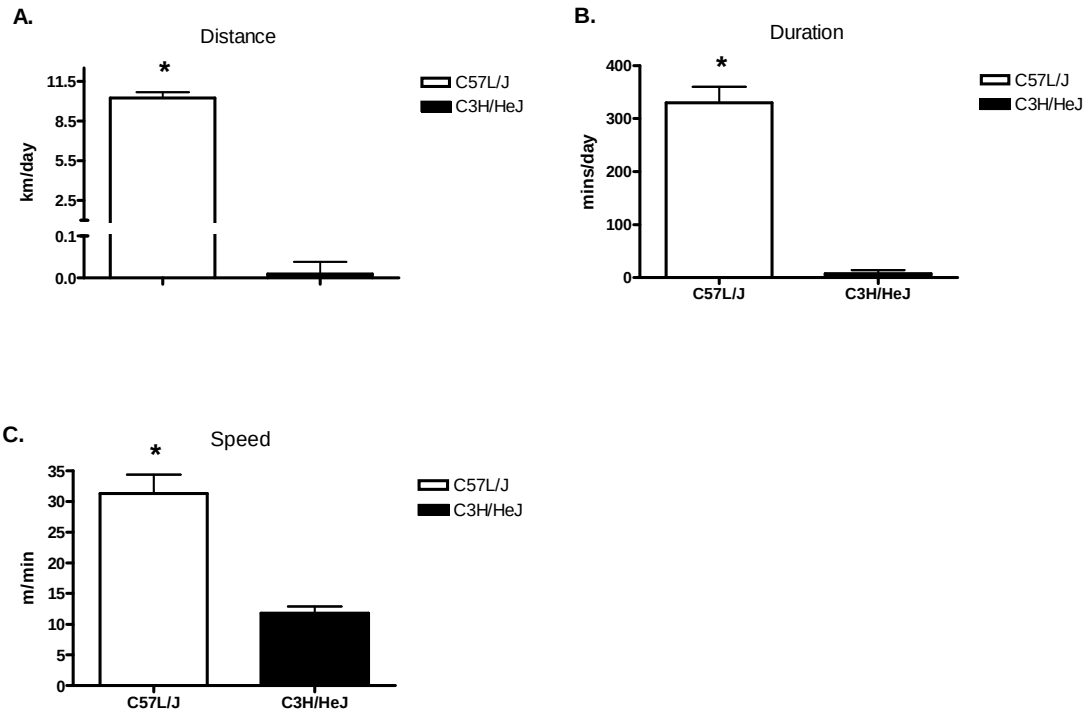

Figure 1: Average distance, duration, and speed for C57L/J (n=10) and C3H/HeJ (n=10) mice over 21 days. **A.** Average distance (Km/day) for C57L/J (10.25±1.37) and C3H/HeJ (0.01±0.09). **B.** Average distance (mins/day) for C57L/J (329.73±30.52) and C3H/HeJ (7.81±6.32) mice**. C.** Average speed (m/min) for C57L/J (31.27±3.13) and C3H/HeJ (11.81±1.08) mice. * designates significantly higher than C3H/He mice at $p<0.001$.

Figure 2: Gene expression in the striatum/nucleus accumbens tissue of C3H/HeJ mice housed with or without a running wheel.

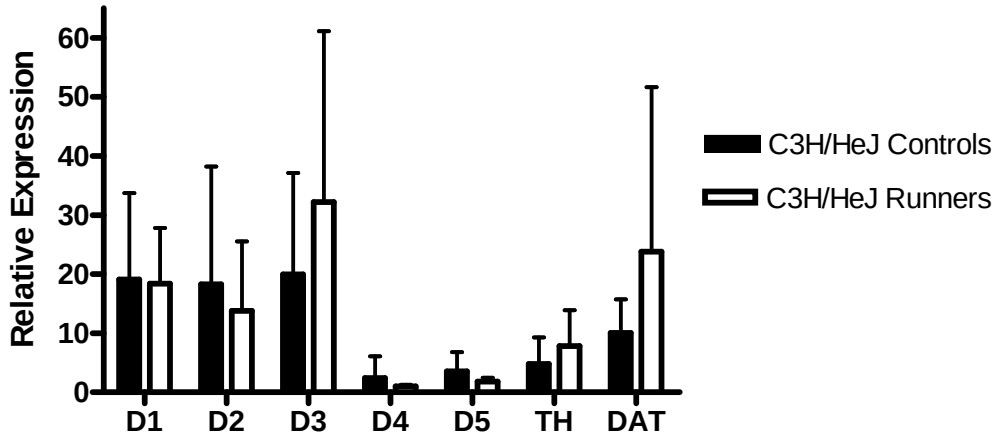

Figure 2: Gene expression in the striatum/nucleus accumbens tissue of C3H/HeJ mice housed with a running wheel and C3H/HeJ mice housed without a running wheel. No expression differences ($p>0.05$) were found between control ($n=10$) and running ($n=10$) C3H/HeJ mice for any of the seven dopaminergic genes.

Figure 3: Gene expression in the striatum/nucleus accumbens tissue of C57L/J mice housed with or without a running wheel.

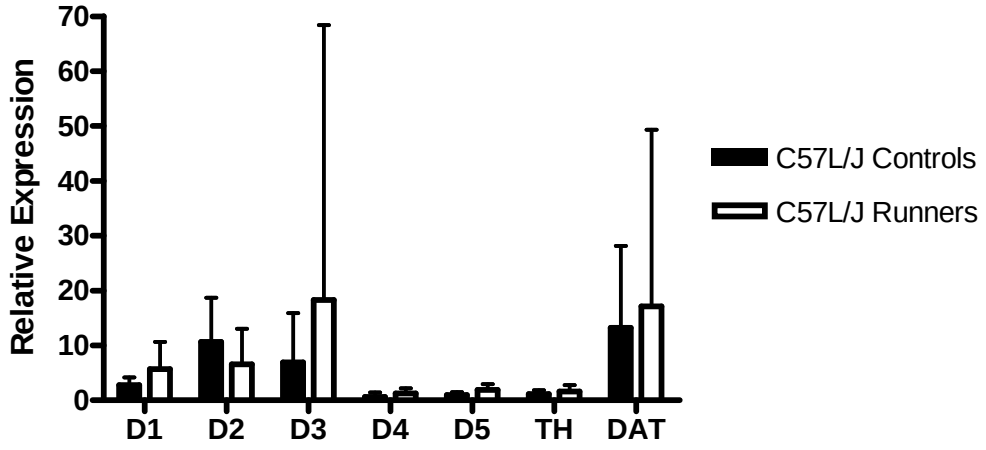

Figure 3: Gene expression in the striatum/nucleus accumbens tissue of C57L/J mice housed with a running wheel and C57L/J mice housed without a running wheel. No expression differences ($p>0.05$) were found between control ($n=7$) and running ($n=10$) C57L/J mice for any of the seven dopaminergic genes.

Figure 4: Comparison of dopaminergic gene expression between low active C3H/HeJ mice and high active C57L/J mice.

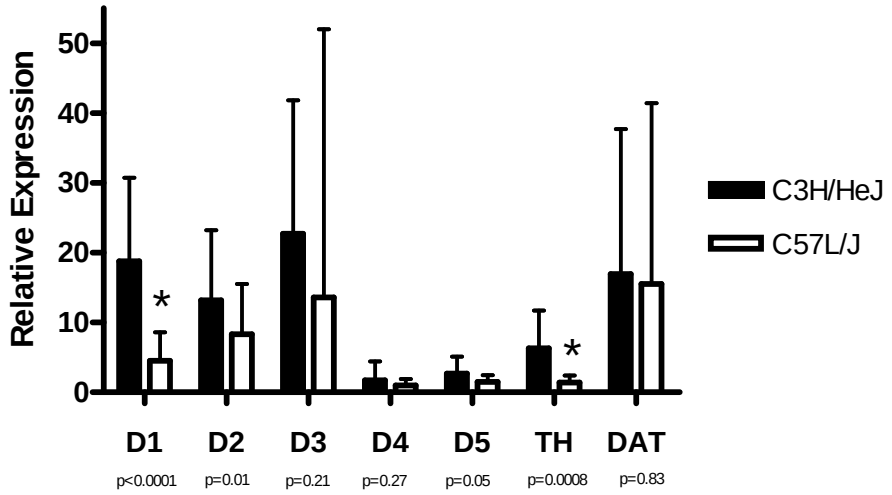

Figure 4: A significant main effect of strain was found for two dopaminergic genes (*Drd1, and Tyrosine Hydroxylase*). The data in this figure represent all mice from each strain (control and experimental). For each of these genes, C57L/J mice had significantly lower expression than C3H/HeJ mice. p values are reported for each gene in the figure.

CHAPTER 4:
PHARMACOLOGICAL MANIPULATION OF THE DOPAMINERGIC SYSTEM AFFECTS WHEEL RUNNING ACTIVITY IN DIFFERENTIALLY ACTIVE MICE.

Abstract

The genetic factors involved in the regulation of physical activity are not well understood. The dopamine system has been implicated in the control of voluntary locomotion and wheel running (WR) in mice and is thus a likely candidate as a genetic/biological system important to the regulation of physical activity. **Purpose**: This study evaluated the effects of four different dopaminergic acting drugs on WR in differentially active inbred strains of mice. **Methods:** High active C57L/J (n=7, 3-controls, 5-experimental) and low active C3H/HeJ (n=8, 3-controls, 5-experimental) were analyzed for baseline wheel-running indices of distance (km/day), duration (mins/day), and speed (m/min) for 21 days. Experimental mice received increasing doses over four days of each of the following drugs: SKF 81297 (D1 agonist), SCH 23390 (D1 antagonist), GBR 12783 (DAT inhibitor), and AMPT (tyrosine hydroxylase inhibitor). Each drug dose response treatment was separated by three days of recovery (no drug injections). WR indices were monitored during drug treatments and during drug wash-out phases. **Results**: SKF 81297 significantly reduced (p=0.0004) WR in the C57L/J mice, but did not affect WR in the C3H/HeJ mice. GBR 12783 significantly increased (p=0.0005) WR in C3H/HeJ mice, but did not affect WR in C57L/J mice. Only duration (not overall WR) was significantly reduced in C57L/J mice in response to

SCH 23390 (p=0.003) and AMPT (p=0.043). SCH 23390 (p=0.44) and AMPT (p=0.98) did not significantly affect WR in C3H/HeJ mice. **Conclusions**: These results suggest that genetic differences in dopamine signaling are important in the WR response to dopaminergic acting drugs in inbred strains of mice. The high activity in the C57L/J strain is primarily mediated by D1-like receptors, while in the C3H/HeJ strain, activity is mediated through overall dopamine signaling determined by dopamine re-uptake.

Key Words: Dopamine, dopamine signaling, physical activity, inbred mice, genetics, regulation

Introduction

It is well known that physical activity improves human health by decreasing risk of obesity (82, 146, 297), cardiovascular diseases (66), Type II Diabetes (250), depression (248), certain types of cancer (102, 251, 293), and overall mortality (105). Although the physiology of exercise has been well studied over the past 40 years, the genetic and biological regulating factors of physical activity have yet to be fully investigated and understood. It has been estimated that physical inactivity is a leading cause of mortality, and contributes to increasingly higher health care costs in developed countries (180). Therefore, in order to prevent disease and improve human health it is vital to understand the regulating factors of physical activity.

It has been shown that physical activity patterns are significantly regulated by genetic factors, with the estimated genetic component ranging from 20-80% (69, 119, 135, 142, 149, 153, 184, 249, 262). At least two studies have identified both single-gene and epistatic quantitative trait loci (QTL) involved in the regulation of physical

activity in mice; in particular, significant single-gene QTL have been found on chromosomes 9 and 13 (138, 153). However, the exact genes involved in regulation of physical activity are yet to be discovered. Selective breeding studies conducted by Garland and colleagues also illustrate a significant genetic component involved in the regulation of physical activity. After 35 generations of selective breeding for running wheel activity, selected animals ran over 170% farther than control mice (197). Selection acting on genetic variation in the original outbred population of mice highlights a definite genetic component to the regulation of voluntary physical activity in mice.

Furthermore, it appears from several studies that factors in the central nervous system may play a key role in the genetic/biological regulation of physical activity in rodents (29, 199-201). The dopamine system, part of the central nervous system, located in the mid-brain, plays a role in mediating locomotion (213) and motivation (214). For example, it is known that depletion of dopamine neurons in the mid-brain are a major cause of the motor deficits seen in Parkinson's disease (301). Also, the hyperactive phenotype common in Attention Deficit Hyperactivity Disorder (ADHD) is also mediated through dysfunctions in dopamine signaling in the brain (8). Pharmacological studies in rodents confirm dopaminergic involvement in locomotor behavioral responses to stimuli such as psychostimulant drugs (14, 16, 91, 92, 108, 211, 223, 242); however, compelling evidence from wheel running studies in mice also implicates the dopamine system in mediating general voluntary physical activity levels. Specifically, Rhodes and Garland (2003) investigated the effects of Ritalin (a DAT inhibitor), apomorphine (a non-selective dopamine agonist), SCH 23390 (a selective

D1-like antagonist), and raclopride (a selective D2-like antagonist) on wheel running in both selected and control animals (199). A differential response to Ritalin was seen where the selected animals decreased wheel running in response to Ritalin, while the control animals increased wheel running. At high doses of apomorphine, and all doses of raclopride, both control and selected animals markedly decreased their wheel running by the same proportion. However, in response to SCH 23390 control line mice decrease wheel running more than selected animals (199). Additionally, recent results from our lab exhibiting an independent relationship of dopamine D1 receptors and tyrosine hydroxylase genes with differentially active inbred mice in the nucleus accumbens and striatum area of the brain (126) indicate that D1-like receptors as well as the amount of dopamine present in the mid-brain are involved in regulating wheel running in mice.

Wheel running in animals has been suggested as a good model for daily physical activity in humans (61, 234). Thus, studying wheel running responses to dopaminergic drugs may prove useful in elucidating the proposed independent mechanism by which the dopamine system mediates physical activity behavior. Therefore, the purpose of this study was to investigate the wheel running responses to several dopaminergic acting drugs in differentially active inbred mice. This study is another step in the understanding of the central genetic and biological regulation of physical activity, and will be important for future studies investigating the mechanisms of this regulation and importance to human health and performance.

Methods

Animals

Differentially active strains of inbred mice were used in this study: C3H/HeJ mice (n=8 males) previously identified as low active (30), and C57L/J mice (n=6 females, n=1 male) previously identified as high active (30). The use of primarily female C57L/J mice, while not optimal, was unavoidable due to the extremely limited supply of these highly active mice (see below). However, whereas comparisons are made primarily within mouse and versus control mice of the same sex, appropriate conclusions can be drawn from the use of both male and female mice in this study. The C3H/HeJ mice were purchased from Jackson Laboratories; however, given that C57L/J mice are no longer available from Jackson Laboratories (nor from other suppliers), the C57L/J mice used in this study were taken from a small breeding colony our lab maintains. These mice were the first generation inbred offspring from C57L/J breeder pairs purchased from Jackson Laboratories in Spring 2008.

Running wheel data were collected from the mice beginning at 63 days (9 weeks) of age which corresponds to the most active period in the lifespan for mice (255). All mice were housed in the University Vivarium with 12 hour light/dark cycles (light 6am-6pm, dark 6pm-6am) and were provided food (Harlan Teklad 8604 Rodent Diet, Madison, WI) and water ad libitum. All procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee. Additionally, all animals were weighed twice weekly.

Measurement of Voluntary Activity (Wheel Running)

Daily wheel running was measured using methods described previously (149, 153). Briefly, mice were housed individually in standard rat sized cages, each equipped with a solid surface running wheel (450mm circumference; Ware Manufacturing,

Phoenix, AZ) mounted on the cage top. A magnet was mounted on the outside surface of each wheel and the cage top was equipped with a magnetic sensor (BC500; Sigma Sport, Olney, IL). Each computer was calibrated with wheel dimensions to allow for accurate measurement of distance (km/day) and time (duration-mins/day) each mouse ran on the wheel. Speed of running (m/min) was then calculated from the distance and duration data. Mice were monitored and data was collected every 24 hours at approximately 9am during baseline and drug wash-out phases of the protocol. During drug treatments, data was collected immediately before drug treatment at 6pm (the beginning of the dark/active phase for mice), at 12am (6hrs post drug treatment), and again at 6am (12hrs post drug treatment).

Drug Treatment

Evidence from our lab (126) and others (199, 201) suggest physical activity in the form of wheel running in mice is at least partially regulated by the D1-like receptors, the dopamine transporter (DAT), as well as possibly the expression and/or function of the tyrosine hydroxylase enzyme. Thus, in this study, we used four different drug treatments: SKF 81297 (D1-like agonist; Tocris Bioscience, Ellisville, MO), SCH 23390 (D1-like antagonist; Tocris Bioscience, Ellisville, MO), GBR 12783 (DAT inhibitor; Tocris Bioscience, Ellisville, MO), and DL-2-Methyl-3-(4-hydroxyphenyl) alanine (AMPT) (Tyrosine Hydroxylase inhibitor; Sigma Aldrich, St. Louis, MO). All drugs have been shown to be centrally active after intraperitoneal (IP) injection and were administered IP in a volume of 0.3mL per mouse. Dose responses were investigated using the following consecutive drug doses (mg/kg): SKF 81297 (0.5, 0.75, 1.0, 1.25), SCH 23390 (0.5, 0.75, 1.0, 1.25), GBR 12783 (15, 20, 25, 30), and

AMPT (85, 90, 95, 100). All doses were based on previous literature investigating locomotion responses in mice to these particular drugs.

Treatment procedures: At nine weeks of age, mice were housed with a wheel, and baseline activity patterns was assessed for 21 consecutive days in all mice. Five mice from the C3H/HeJ strain and 4 mice (3 females, and 1 male) from the C57L/J strain were randomly chosen for the experimental drug treatment group, leaving three mice in each strain serving as controls. Control mice received saline injections only. The experimental animals received one injection (according to the dose schedule described above) at 6pm, at increasing doses for 4 consecutive days, followed by three full days of drug wash-out (i.e. no injections). Wheel running was monitored at 12am and 6am during drug treatment, and every 24 hours during drug wash-out. This pattern was repeated for all four drugs in succession.

Injection methods: Each drug injection solution was made fresh each day immediately prior to injections and all drugs were dissolved in 0.9% sterile saline. Once the appropriate dose was dissolved, the solution was placed in a sterile syringe and filtered through a 0.2 micron filter during injection. The C57L/J mice received the drugs in the following order: SKF 81297 (83-87 days old), SCH 23390 (90-94 days old), GBR 12783 (97-101 days old), and finally AMPT (103-106 days old). Due to age differences upon arrival and the need to keep drug injections sterile the C3H/HeJ mice received the drug treatments in the following order: GBR 12783 (83-87 days old), AMPT (90-94 days old), SKF 81297 (97-101 days old), and SCH 23390 (103-106 days old).

Statistics

Given the differential drug injections at differing ages, (e.g. the C57L/J mice received SKF 81297 at 83-87 days old, but the C3H/HeJ mice received this drug at 97-101 days old), each strain was analyzed in a separate ANOVA for the effects of the four drugs on wheel running indices. The alpha value was set *a priori* at 0.05. Within strain, each drug was analyzed separately with a two-way ANOVA with group (control vs. experimental) and dose (repeated measure) as main effects. Three dependent variables were analyzed including distance (km/day), duration (mins/day), and speed (m/min). Tukey's HSD *post-hoc* tests were used to evaluate main effects and group by dose interactions within the ANOVA model. There were no statistical differences between wheel running indices taken at 6 hours post-injection or 12 hours post-injection (data not shown) and thus, only wheel-running data from 12 hour post-injection will be presented. Differences in weight at baseline measurements between strains, as well as differences in weights between group within strains, were analyzed using independent t-tests, and correlation analysis was used to investigate relationships between weight and distance run.

Results

Weights

Mice were weighed twice weekly during this study to encompass one weight measurement during each drug treatment, as well as one weight measurement during drug wash-out. C3H/HeJ (n=8 males) mice as a whole group were significantly heavier than C57L/J (n=6 females, n=1 male) mice at baseline, and at all time points throughout the study ($p<0.001$). Weight of the control versus the experimental animals did not

differ across the treatments (C3H/HeJ, p=0.20; C57L/J, p=0.66). As has been shown in previous studies (149, 153) during baseline activity measurements, weight was not correlated with distance run in either strain (C3H/HeJ: p=0.11, r^2=0.43; C57L/J: p=0.12, r^2=0.36). Speed was also not correlated with weight in either strain (C3H/HeJ: p=0.66, r^2=0.03; C57L/J: p=0.93, r^2=0.002). Duration was significantly correlated with weight in both strains (C3H/HeJ: p=0.04, r^2=0.54; C57L/J: p=0.02, r^2=0.69). Weight did not significantly increase over the course of the study in C3H/HeJ mice (p=0.69; beginning: 28.0±1.6g; end: 29.9±2.2g), while weight did significantly increase in C57L/J mice over the course of the study (p=0.02; beginning: 23.6±1.1; end: 25.1±1.0).

Baseline Physical Activity Results

Baseline wheel running indices for both strains of mice are illustrated in Figure 1. As was expected from previous literature, the C57L/J mice ran 191% farther, 177% longer, and 84% faster than C3H/HeJ mice (p<0.0001). There was no difference between control and experimental mice at baseline in distance (p=0.52), duration (p=0.52), or speed (p=0.74) in the C57L/J mice. Likewise, there was no difference between groups of C3H/HeJ mice at baseline in distance (p=0.22), duration (p=0.33), or speed (p=0.16).

Drug Effects on WR in C57L/J Mice

Wheel-running distance, the product of duration of activity and speed of activity, responses in C57L/J mice to all four drugs are shown in Figure 2. No significant dose response was seen in distance run after treatment with the D1 agonist SKF 81297 (p=0.72); however, SKF 81297 significantly reduced wheel running distance regardless of dose (Fig. 2; p=0.0004). No significant differences in distance

were observed between group or by dose for the D1-antagonist SCH 23390 (p=0.12), the DAT inhibitor GBR 12783 (p=0.89), or the TH inhibitor AMPT (p=0.37). Similar responses for duration and speed for all four drugs were observed and are reported in Table 1.

Drug Effects on WR in C3H/HeJ Mice

Wheel-running distance responses in C3H/HeJ mice (low active) to all four drugs are shown in Figure 3. No significant dose response was seen in distance run after treatment with the DAT inhibitor GBR 12783 (p=0.73); however, injection of GBR 12783 did significantly increase wheel running independent of dose (Fig. 3; p=0.0005). No other drugs used in this study significantly affected wheel running the C3H/HeJ mice: the D1-agonist SKF 81297 (p=0.91), the D1-antagonist SCH 23390 (p=0.44), and the TH-inhibitor AMPT (p=0.98). Data for duration and speed for all four drugs for C3H/HeJ mice showed similar responses as distance and are reported in Table 2.

Discussion

The purpose of this study was to investigate four different dopaminergic acting drugs on a high active strain of mice and a low active strain of mice to determine the role of D1-like receptors, DAT, and tyrosine hydroxylase in regulating physical activity level. As designed, we observed a significant difference in all baseline wheel running indices between C57L/J mice and C3H/HeJ mice but no differences within strain between control and experimental groups. Interestingly, we observed strain dependent effects of the D1-like receptor agonist (SKF 81297) and the DAT inhibitor (GBR 12783). The D1-like agonist significantly reduced overall distance, duration, and speed

in C57L/J mice (high active), while the DAT inhibitor significantly increased overall distance, duration, and speed in the C3H/HeJ (low active). Surprisingly, none of the drugs increased activity in the high active mice (C57L/J) or decreased activity in the low active mice (C3H/HeJ).

It is becoming well accepted that a significant genetic component exists in the regulation of physical activity in both rodents (69, 142, 149, 153, 262) and humans (193). Since wheel running in mice has been proposed as a good model for physical activity in humans (61, 234), it is warranted to study genetic components of physical activity in mice with the probable translation to a human health benefit. Using wheel running as a model of physical activity in mice, both single-gene and epistatic QTL associated with physical activity have been found (138, 153). However, the genes and gene interactions involved in the regulation physical activity behavior are still unclear. Interestingly, haplotype analysis conducted in the study by Lightfoot and colleagues identified a suggestive QTL on chromosome 13 that contains the *Drd1* gene which codes for the D1 receptor (153). In humans, at least one study has suggested that *DRD2*, which codes for the D2 receptor, is associated with physical activity patterns in white women (235). Limited studies are beginning to link genes to physical activity phenotypes; however the mechanistic pathways by which these genes may function to regulate physical activity behavior are not understood.

Research by Garland and colleagues presented evidence for a substantial genetic/biological influence on physical activity levels in mice (85). Their results suggest central factors such as neurotransmitter systems may be primary in mediating the phenotypic differences seen in the selectively bred animals. For example, Rhodes et

al. (200) investigated differences in patterns of brain activity between mice selectively bred for high wheel running compared to control mice. Selectively bred mice, when blocked from the wheels, showed increased Fos expression in several areas of the brain including the striatum compared to control mice, indicating these areas of the brain may be important in motivation for running (200). Bronikowski et al (29). found that mice selectively bred for high wheel running have approximately 20% increased expression of D2 and D4 receptors (D1-like receptors were not analyzed in this study) in the hippocampus compared to control line mice. Finally, pharmacological studies with mice selectively bred for high wheel running indicate a strong influence of dopamine signaling in mediating the difference in running wheel activity between selected animals and control line animals (199, 201). In addition, the dopaminergic influence on physical activity appears to be strain dependent. Several studies in inbred mice have shown differential motor responses to dopaminergic acting drugs in different inbred strains of mice (32, 33, 194, 228, 238, 239, 261), suggesting genetic differences in the dopamine system may mediate behavioral differences in motor response and/or physical activity.

Similarly, research conducted in our lab (126) indicates C57L/J inbred mice (high active) were found have significantly lower expression of *Drd1* mRNA as well as tyrosine hydroxylase compared to low active C3H/HeJ inbred mice. Differences in expression of key dopamine genes in the striatum and nucleus accumbens between high active C57L/J mice and low active C3H/HeJ mice, combined with the data from Rhodes and Garland (199), provides evidence for the involvement of the D1-like receptors, as well as overall dopamine signaling in the mid-brain in the regulation of physical activity

in mice. Thus, the current study sought to elucidate this possible mechanism further by studying the effects of both D1-like agonists and antagonists (both of which affect dopamine signaling by manipulation of the receptor), as well as a DAT inhibitor and a tyrosine hydroxylase inhibitor (which alter dopamine signaling by manipulating presence of dopamine in the synapse) on wheel running distance, duration, and speed in differentially active inbred mice.

Wheel running in response to DAT and Tyrosine Hydroxylase Inhibitors

GBR 12783 and AMPT were used in this study to investigate wheel running responses to drugs affecting either dopamine re-uptake or dopamine production respectively. The dopamine re-uptake inhibitor increases the length of time dopamine molecules are present in the synapse, while the tyrosine hydroxylase inhibitor would theoretically inhibit dopamine production via this enzymatic pathway and essentially decrease overall dopamine in the brain.

Strain dependent responses to GBR 12783 (a dopamine re-uptake inhibitor): Strain dependent responses were observed in the current study in response to GBR 12783. Specifically, low active C3H/HeJ mice significantly increased wheel running distance, duration, and speed independent of dose compared to control mice (Figure 3, Table 2). However, no significant changes in wheel running indices were observed in high active C57L/J mice (Figure 2, Table 1). The fact that C3H/HeJ mice did increase wheel running in response to a dopamine re-uptake inhibitor corresponds to previous research with animal models of ADHD and treatment with Ritalin, also a dopamine re-uptake inhibitor. Differential responses to Ritalin have been shown in both animals (201) and humans (98, 203, 219). Specifically, it has been proposed, that the response

to drugs such as Ritalin depends largely on baseline values of the response in question (219). Rhodes and colleagues (2001 and 2003) demonstrated this differential response in mice selectively bred for high wheel running (199, 201). The mice selectively bred for high wheel running ("hyperactive mice") reduced wheel running, while control line mice increased wheel running in response to dopamine re-uptake inhibitors. This same differential response is seen in "normal" humans, where re-uptake inhibitors appear to increase activity (98), while a decrease in activity in response to re-uptake inhibitors is seen in humans diagnosed with ADHD (278). In the current study, the dopamine re-uptake inhibitor significantly increased wheel running in C3H/HeJ mice suggesting these mice respond similarly as "normal" subjects, and that this particular pathway is important in the regulation of physical activity in this strain of mice.

Genetic differences in the dopamine system, and thus dopamine signaling in response to pharmacological agents, could explain the lack of response to the DAT inhibitor in the high active C57L/J mice compared to the increased wheel running observed in the low active C3H/HeJ mice. It has been suggested that synergistic activity between D1 and D2 receptors is necessary for normal behavior such as locomotion (113, 245, 284) and overall receptor balance may be important to locomotor responses (56). Because C57L/J mice have been shown to have decreased expression of both D1 receptors and tyrosine hydroxylase in the mid-brain (126), it is possible that the overall balance of D1/D2 in these animals would compensate for an increase in dopamine, and thus, override any affects on locomotion by a dopamine re-uptake inhibitor. Thus, even though dopamine signaling should increase due to treatment with a DAT inhibitor, the decreased expression of D1 receptors, the decreased dopamine

production due to decreased expression of tyrosine hydroxylase, and/or possible synergistic compensation from D2 receptors in the C57L/J mice may override any affect of a re-uptake inhibitor on wheel running in this strain.

Wheel Running responses to AMPT (a tyrosine hydroxylase inhibitor): We hypothesized that treatment with AMPT, which would have decreased overall dopamine levels would have resulted in an increased activity level. However, the administration of a tyrosine hydroxylase inhibitor did not affect wheel running indices in the low active C3H/HeJ mice (Figure 3, Table 2). However, the only effect of the TH inhibitor was a slight, but significant decrease in duration in the high active C57L/J mice (Table 1). We observed no significant group by dose interactions for this drug in C57L/J mice, with no difference reflected in distance or speed (Figure 2, Table 1). In our previous study, we observed decreased expression of tyrosine hydroxylase mRNA in the mid-brain of C57L/J mice compared to C3H/HeJ mice (126). If decreased expression of tyrosine hydroxylase, and subsequent decreased dopamine production and downstream dopamine signaling mediate the high active phenotype, inhibiting this enzyme further would theoretically lead to further increased physical activity. However, one explanation to why this result was not seen could be that this high active inbred strain is already running at a physiological maximum, and any further increase in activity would be impossible. This limitation by a physiological maximum is supported by Rhodes and Garland (199) who suggested a possible “ceiling effect” in response to high doses of apomorphine in mice selectively bred for high wheel running. Thus, there is a possible “ceiling effect” with the C57L/J mice, in that antagonist treatment may not increase

wheel running because these mice cannot physiologically increase distance, duration, or speed significantly higher than baseline.

Strain differences in tyrosine hydroxylase activity have been shown previously (266, 267), suggesting genetic background may be important in baseline tyrosine hydroxylase activity and subsequent behavioral effects. Genetic differences in baseline tyrosine hydroxylase activity could explain why C3H/HeJ mice did not respond to AMPT treatment. In the current study, C3H/HeJ mice did increase wheel running in response to a dopamine re-uptake inhibitor, which increased the available pool of dopamine for signaling in the mid-brain. However, inhibiting tyrosine hydroxylase in these mice would essentially decrease the pool of available dopamine for signaling, which would theoretically induce an opposite response (e.g. decreased wheel running). However, it is also possible there is a "floor effect" in the low active C3H/HeJ mice. The concept of a floor effect is supported by other studies that show that even when all factors are controlled and the effects of sex hormones are removed, in spite of a marked decrease in daily activity, there is a baseline minimum below which the mice will not become 'less active' (i.e. activity levels can not be reduced to zero, RS Bowen, personal correspondence). Given that the activity levels of the C3H/HeJ mice we observed are similar to the minimum baseline levels observed by controlled, gonadectomized mice, we suggest that the C3H/HeJ may naturally be running at a physiological floor, and any drug induced reductions in wheel running may not be possible. Thus, genetic differences in dopaminergic signaling between high active C57L/J mice and low active C3H/HeJ mice – strains of mice that were intentionally chosen for this investigation due to their marked differences in activity levels as compared to the standard reference

strain C57Bl/6J (149) - may be mediating differential responses to both dopamine increases and decreases resulting in undetectable responses due to physiological "ceiling/floor" effects.

Wheel running in response to D1-like agonist and antagonist

In contrast to determining the response to generalized alteration in dopamine levels through the use of reuptake inhibitors or dopamine synthesis inhibitors, we used SKF 81297 (D1-like agonist) and SCH 23390 (D1-like antagonist) to investigate the affects of manipulation of dopamine signaling specifically through the D1-like receptors. Previous research from our lab suggested that expression of D1 receptors were significantly decreased in the striatum and nucleus accumbens in C57L/J mice compared to C3H/HeJ mice independent of wheel running exposure (126).

Strain dependent responses to SKF 81297 (a selective D1-like agonist): With the application of a D1-like receptor agonist, the dopamine signaling should increase, thus, hypothetically decreasing activity levels if the D1 receptors are involved in regulation of activity. We confirmed this hypothesis in only the C57L/J mice with the significant reduction in distance, duration, and speed. However this D1-like receptor agonist had no effect on wheel running indices in C3H/HeJ mice (Figures 2 and 3, Tables 1 and 2). Evidence from our lab indicates that reduced function of the D1-like receptors in high active inbred C57L/J mice is at least partly explained by reduced expression of these receptors in the mid-brain compared to other low active inbred strains (126). It has also been suggested that mice selectively bred for high wheel running have reduced function of D1-like receptors, but not the D2-like receptors (199). Thus, our observation that high active C57L/J mice in the current study reduced wheel

running in response to a D1 agonist supports the hypothesis that decreased function of D1-like receptors may mediate running wheel activity in high active inbred strains.

In contrast to the high active strains, the low active C3H/HeJ mice did not decrease wheel running in response to the D1 agonist used in this study. However, two plausible explanations could explain this differential strain response to the D1 agonist. First, as mentioned previously, it is possible that the low active C3H/HeJ strain may already run at a physiological "floor". If this were the case, any drug treatment hypothesized to decrease activity levels (e.g. D1-agonist, TH inhibition) would not decrease wheel running any lower than baseline. Secondly, the D1-receptor pathway is likely not the only pathway regulating low activity in this strain. Others have shown strain dependent responses in locomotion to different dopamine acting drugs (32, 33, 228, 238, 239, 261, 266) and differences in dopaminergic anatomy between strains has also been demonstrated (70). Further, other investigators have suggested that low activity may be a different phenotype than high activity and thus, probably has differing regulating mechanistic pathways (JT Lightfoot, personal correspondence). Thus, due to differences in genetic make-up of C3H/HeJ mice, it is possible that regulation of physical activity in this low active inbred strain is still mediated in part by the dopamine system, but regulated through different pathways compared the clear D1-like receptor regulation of physical activity in C57L/J mice.

Wheel running responses to SCH23390 (a selective D1-like antagonist): Given the evidence of the importance of D1-like receptors in regulating the high active phenotype in C57L/J mice, we hypothesized that a D1-like antagonist would further increase wheel running in this strain by blocking the inhibitory D1-like receptors in the

mid-brain. However, SCH 23390 (a selective D1-like antagonist) had no significant effect on wheel running in C3H/HeJ mice, and only a slight effect on duration in C57L/J mice in the current study (Figure 2 and 3, Tables 1 and 2). However, we do not believe that this slight change in duration in the C57L/J mice is physiologically significant because *post-hoc* analysis did not reveal any significant group by dose interactions suggesting that the overall main effect significant difference was seen only when data from all four doses were combined. Additionally, no differences in distance or speed were found after administration of the D1 antagonist in C57L/J mice indicating that the difference in duration did not significantly affect total distance. Thus, in spite of the effect of the D1-agonist in reducing activity, the earlier proposed "ceiling effect" in the C57L/J mice when given a dopamine reuptake inhibitor may also be active with the administration of the D1-like antagonist.

The low active C3H/HeJ mice did not increase wheel running in response to the D1 antagonist which was similar to the response seen when these mice were given the D1-like agonist. As we suggested earlier, it is possible that signaling through other pathways in the dopamine system is able to "compensate", and thus the D1 receptors may not be the primary signaling pathway through which physical activity responses of low active C3H/HeJ mice are regulated.

Summary

Strain differences in the response to a D1 receptor agonist demonstrate that D1-like receptors may play a role in mediating the high active phenotype in C57L/J mice. Likewise, differential strain responses to a dopamine re-uptake inhibitor suggest that the amount of dopamine present in the synapse may be important in mediating the low

active phenotype in C3H/HeJ mice. However, full elucidation of the role of dopaminergic functioning in these strains purposely selected for their divergent activity responses is difficult because of the possibility of physiological ceiling and floor effects in physical activity levels. Thus, genetic differences in dopamine signaling between inbred strains are a potential explanation for the differences in wheel running responses to dopaminergic drugs.

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Footnotes:

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Figure 1: Baseline values of distance, duration, and speed in control and experimental mice

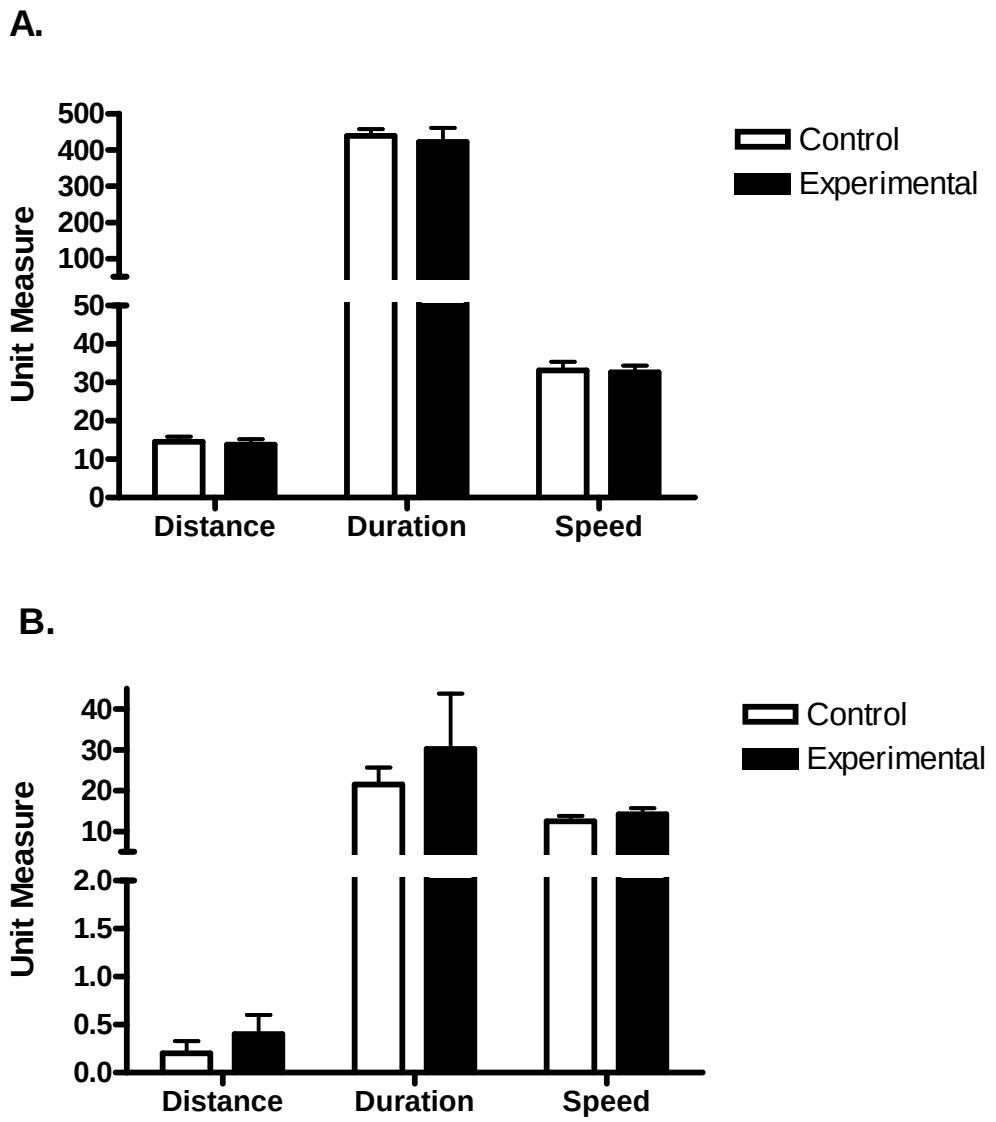

Figure 1: **A.** Running wheel data at baseline for C57L/J mice (n=7) is shown. No difference in distance (km) ($p=0.52$), duration (mins) ($p=0.52$), or speed (m/min) ($p=0.74$) were found between control and experimental groups; however, C57L/J mice ran significantly farther, longer, and faster than C3H/HeJ mice at baseline ($p<0.0001$). **B.** Running wheel data at baseline for C3H/HeJ mice (n=8). No differences in distance ($p=0.22$), duration ($p=0.23$), or speed ($p=0.44$) were found between control and experimental groups.

Figure 2: Distance responses to all four dopaminergic drugs in C57L/J mice.

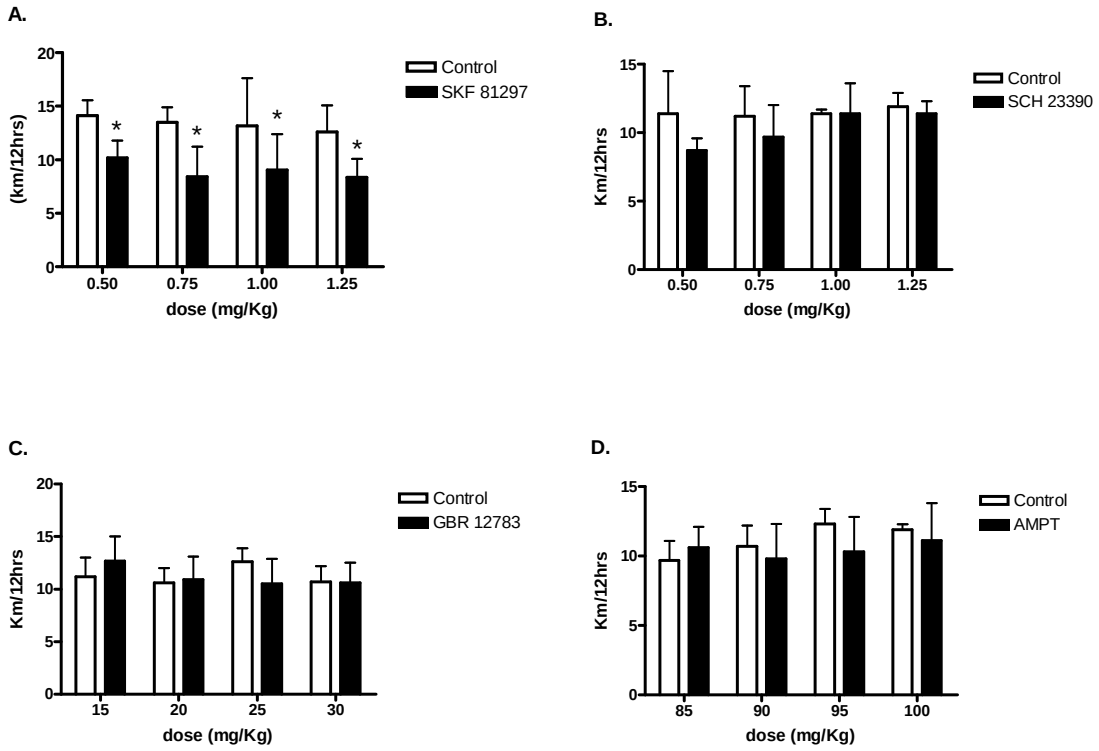

Figure 2: Distance responses to all four drugs in the C57L/J mice. **A**. Dose response after administration of SKF 81297 is shown. No significant dose response was seen; however, all four doses significantly reduced wheel running distance in experimental mice compared to controls (p=0.0004). **B**. Dose response to SCH 23390 is shown. No significant changes in distance run between groups were seen for any dose (p=0.12). **C**. Dose response to GBR 12783 is shown. No significant differences in distance run were seen between groups for any dose (p=0.89). **D**. Dose response to AMPT is shown. No significant differences in distance run between group was seen for any of the doses (p=0.37).

Figure 3: Distance responses to all four dopaminergic drugs in C3H/HeJ mice.

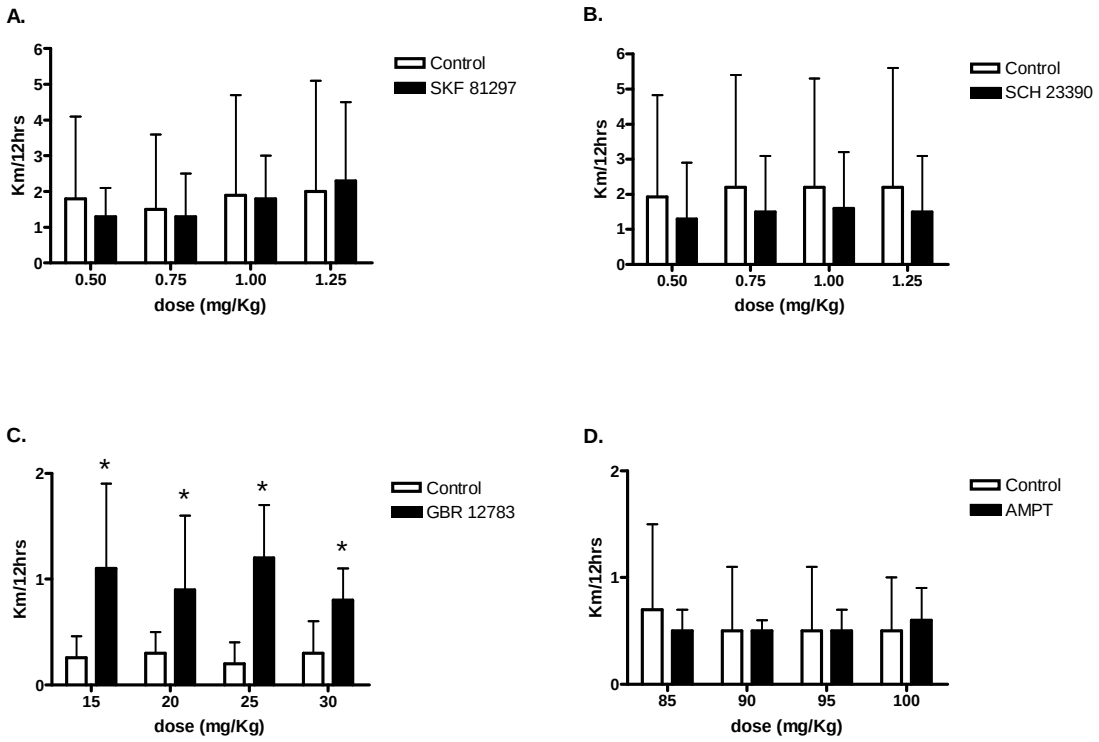

Figure 3: Distance responses to all four drugs in the C3H/HeJ mice. **A**. Dose response after administration of SKF 81297 is shown. No significant differences in distance between groups were seen for any dose (p=0.91). **B**. Dose response to SCH 23390 is shown. No significant changes in distance run between groups were seen for any dose (p=0.44). **C**. Dose response to GBR 12783 is shown. No significant dose response was observed, however, distance was significantly increased in the experimental group compared to control following treatment with all four doses (p=0.0005). **D**. Dose response to AMPT is shown. No significant differences in distance run between group was seen for any of the doses (p=0.98).

Table 1: Duration and speed responses to dopaminergic drugs in C57L/J mice

Drug	Dose (mg/Kg)	Duration (mins/12hrs)		Speed (m/min)	
		Control	Experimental	Control	Experimental
SKF 81297	0.5	425±13	358±82	33.3±4.4	29.1±5.7
	0.75	416±17	327±65	32.5±4.5	25.3±4.6
	1	389±50	346±69	33.3±7.7	25.7±5.8
	1.25	401±13	294±45	31.4±5.4	28.3±3.6
			p=0.002*		p=0.015*
SCH 23390	0.5	396±63	312±47	28.5±4.1	28.2±5.4
	0.75	389±45	324±42	28.7±3.7	30.0±5.7
	1	395±29	364±15	29.0±2.8	31.1±5.3
	1.25	402±41	371±24	29.7±3.0	30.7±3.1
			p=0.003*		p=0.54
GBR 12783	15	392±12	382±35	28.4±4.0	33.1±3.5
	20	375±27	339±39	28.4±4.5	31.9±4.2
	25	431±24	369±70	29.3±2.4	28.4±1.3
	30	378±10	373±60	28.5±3.4	28.5±2.0
			p=0.091		p=0.18
AMPT	85	341±28	343±36	28.6±4.6	30.9±4.0
	90	362±27	320±82	29.6±3.4	30.7±2.8
	95	396±25	323±67	31.0±2.4	32.0±3.1
	100	392±37	342±40	30.6±2.5	32.2±4.2
			p=0.043*		p=0.29

Table 1: Duration and speed data for C57L/J mice. p values reported indicate significant differences between group within strain.

Table 2: Duration and speed responses to dopaminergic drugs in C3H/HeJ mice.

Drug	Dose (mg/Kg)	Duration (mins/12hrs)		Speed (m/min)	
		Control	Experimental	Control	Experimental
SKF 81297	0.5	97±106	73±33	15.2±4.8	17.2±2.6
	0.75	83±111	75±56	14.4±3.6	16.4±2.6
	1	101±145	103±56	14.5±4.2	16.5±2.8
	1.25	104±150	124±99	15.0±4.4	17.1±2.9
		p=0.95		p=0.11	
SCH 23390	0.5	95±133	74±79	15.7±5.0	16.0±2.8
	0.75	110±141	84±78	15.3±5.5	16.3±2.5
	1	111±142	91±80	15.6±4.6	16.3±2.9
	1.25	110±147	89±80	15.5±5.6	16.1±2.6
		p=0.56		p=0.63	
GBR 12783	15	20±16	66±41	12.5±1.4	16.3±1.7
	20	21±14	53±36	12.5±1.0	15.4±2.0
	25	19±12	69±25	12.1±1.2	16.4±1.8
	30	23±19	53±15	13.1±1.0	15.7±1.1
		p=0.0005*		p<0.0001*	
AMPT	85	39±43	35±12	14.3±3.4	15.3±1.6
	90	34±32	36±6	14.1±2.9	15.0±1.3
	95	33±30	35±10	14.2±2.7	15.3±1.4
	100	29±26	37±15	15.3±3.0	15.5±1.6
		p=0.82		p=0.31	

Table 2: Duration and speed data for C3H/HeJ mice. p values reported indicate significant differences between group within strain.

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APPENDIX A
siRNA TECHNOLOGY TO KNOCK-DOWN GENES INVOLVED IN PHYSICAL ACTIVITY

As mentioned throughout this dissertation, the use of knock-out animals is not always ideal in that deleterious side effects of completely knocking out a gene would likely obscure the true functions of the gene of interest with relation to the phenotype in question. This is particularly apparent with the genes coding for the dopamine receptors. Knock-out of the D1 and D2 receptors causes severe developmental problems in mice. Recently, the development of technology that allows for transient knock-down of a gene holds promise in the study of gene function in all areas of science. The process of RNAi (RNA interference) in animals was originally reported in 1998 by Fire and Mello (71). Since then, several methods have been developed to try and use this process to selectively knock-down expression of a gene of interest. This appendix will briefly summarize pilot studies from our lab using siRNA (short interfering RNA) techniques to knock-down genes of interest in mice in vivo. Although early studies using siRNA in our lab were not successful, this technology (and methods of administration) are constantly evolving, and will hopefully be useful in future studies of genes involved in the regulation of physical activity in mice.

Hydrodynamic Tail Vein Procedure

In-vitro work with siRNA has been widely successful, but in-vivo silencing using this technology is typically hindered by unsuccessful delivery methods. Several methods using siRNA in combination with a vector have been developed. These techniques include but are not limited to lipid-siRNA complexes (220), cationic

polymer complexes with siRNA (95), plasmids, and viral vectors. Although these techniques have been used with limited success, access to these types of vectors was a limiting factor in our lab. Delivery of "naked siRNA" (just the siRNA nucleotide sequence without a vector) has also been studied, and several techniques including local delivery, intranasal/inhalation, and hydrodynamic tail vein procedure have been used with some success in rodents. We decided to attempt the hydrodynamic tail vein procedure (292) in a small number of mice to target knockdown of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, a typical housekeeping gene), and lipoprotein lipase (LPL).

Method A: The hydrodynamic tail vein procedure is described in detail in the protocol for TransIT Delivery Solution (Mirus Bio, Madison, WI). Briefly, siRNA (Dharmacon Inc., Chicago, IL) targeting GAPDH, and LPL were mixed with TransIT Delivery Solution (1ug/mL). This solution was administered via the tail vein in a volume of 3mL in less than 10 seconds. Two mice received experimental injections (siRNA targeting LPL), while one mouse received a control injection (siRNA targeting GAPDH). 2 days after injections, mice were sacrificed and tissues were harvested. Liver mRNA expression was analyzed using semi-quantitative PCR. The liver is the most likely tissue to take up siRNA after systemic injection, thus we investigated liver mRNA levels first. Ultimately we were interested in knocking-down the gene in muscle tissue.

Results Method A: No differences were seen in mRNA expression in the liver for either gene between mice receiving siRNA and mice with no injections, suggesting this

method was unsuccessful in silencing the GAPDH gene, or the LPL gene (data not shown).

Comments Method A: It was extremely difficult to inject that much volume into the tail vein in less than 10 seconds. In fact in some mice the injection needed to be split into 3 separate injections using a smaller gauge needle.

Method B: In our second attempt to use siRNA to knock-down genes in vivo in mice, we used two different strategies in combination with the methods described above. First, we increased the dose of siRNA to 40ug/mL. Second, we also exercised a group of the mice on the treadmill immediately following injection in hopes that more blood circulation would stimulate more uptake of the siRNA in the tissues. Because of technical difficulties using the large volume for the hydrodynamic tail vein procedure we also tried simple intraperitoneal (IP) injections using 40ug/mL siRNA in a volume of 0.5mL TransIT solution injection in a separate group of mice. Thus, in these procedures 2 mice received the standard hydrodynamic tail vein procedure (siRNA targeting GAPDH, 40ug/mL) [one of these mice was run on the treadmill immediately following recovery from injection (approx. 15 min)]. Additionally, 2 mice received 0.5mL IP injection of siRNA targeting GAPDH (40ug/mL) [one of these mice was run on the treadmill immediately following injection (approx. 15 min)]. Tissues were harvested 2 days post injections and liver and muscle mRNA levels were evaluated using semi-quantitative PCR.

Results Method B: No differences in mRNA expression of GAPDH in liver or muscle tissue was found between siRNA injected animals vs. controls, or between animals who

ran on the treadmill following injection vs. animals who did not run, in either the hydrodynamic tail vein method, or the IP injection method (Data not shown).

Comments Method B: At this point we were very disappointed; however research in the literature does show that knock-down methods in vivo are quite difficult using standard methods. The main hurdle remains getting the tissues to take up the siRNA. Thus we decided to try a different method of delivery.

Method C: We attempted a transient isolation hind limb procedure in which we attempted to systemically deliver the siRNA via the femoral vein, however we were unable to successfully complete this procedure in mice. Thus, we ended up trying direct intra-muscular injections of siRNA targeting GAPDH in TransIT Solution. Two mice were injected with siRNA (10ug/mL) directly into the hind limb muscle in the right leg (left leg muscle was used as control). Additionally, 3 mice were injected with siRNA (40ug/mL) directly into the hind limb muscle of the right leg (left leg control). Tissue was harvested two days post injection and muscle GAPDH mRNA levels were assessed using semi-quantitative PCR.

Results Method C: Results of direct intra-muscular injection of siRNA targeting GAPDH at a low concentration, and a high concentration are illustrated in Figure 1 below.

Figure 1: Knock-down of GAPDH in hind limb muscle using direct intra-muscular injection of two different doses.

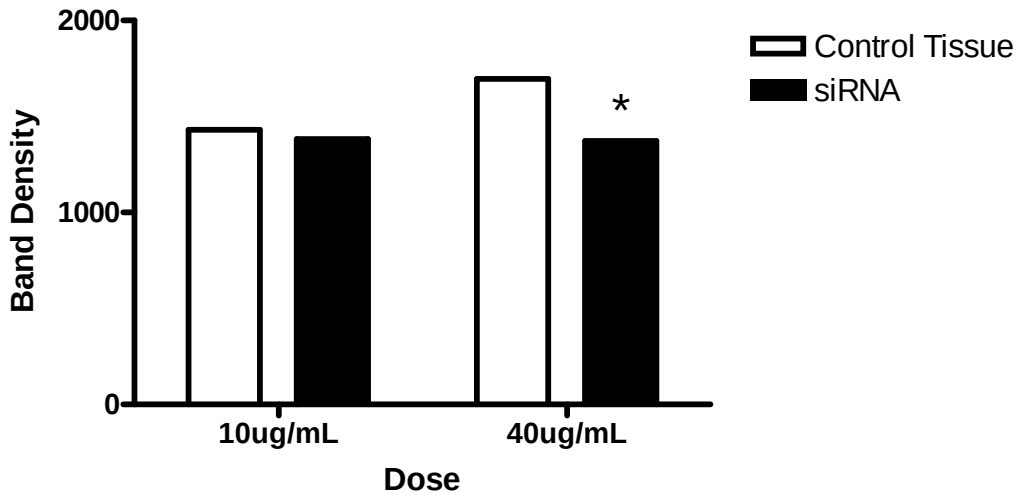

Figure 1: No differences were found between the injected leg muscle, and control leg muscle using 10ug/mL injection of siRNA (p=0.81). However, 40ug/mL siRNA injected intra-muscularly significantly reduced GAPDH expression (p=0.03) in muscle tissue.

Conclusions Method C: Knocking down GAPDH using direct intra-muscular injection of high doses of siRNA in TransIT Solution appears to be possible. However, at lower doses no differences were observed. Thus, intra-muscular injection of high doses of siRNA may be useful in knocking down genes of interest in muscle tissue only. As apparent in the figure however, the knock-down, although statistically significant, may not have been biologically significant. Only a 19% reduction in expression was achieved.

Overall Conclusions

These studies demonstrate the already known fact that siRNA knock-down of genes in vivo is a difficult and delicate process. Limited resources in our lab prevented us from studying further more complicated methods of gene knock-down using siRNA

technology. It is certainly possible that in the future siRNA may be used to study the role of the dopamine genes in regulation of physical activity, however direct injection into the brain is not a current option for these genes in mice. Thus, siRNA targeting methods for gene knock-down in brain tissue need to be developed to further study the role of genes located in brain tissue.