AN ATTEMPT TO ENHANCE NEUROGENESIS OF MDX MICE VIA AEROBIC EXERCISE AND MYOSTATIN INHIBITION

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ABSTRACT

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Duchenne`s muscular dystrophy (DMD) is an inherited disorder that occurs with 1 out of every 3600 male infants. It involves muscular weakness, dystrophy and cognitive dysfunction. The disorder is caused by a defective gene for dystrophin protein. It has been hypothesized that cognitive deficit occurs because DMD downregulates patients´ neurogenesis. Neurogenesis is a process where new brain cells are continuously generated at subventicular zone and dentate gyrus of hippocampus. Cures for DMD are mainly researched with mdx mice. Etiology of the mdx is close enough to that of patients with DMD. This research focuses on an attempt to enhance the neurogenesis of mdx mice via aerobic exercise and myostatin inhibition. Myostatin inhibition enhances mammals' muscle growth. Research has three main focus: 1. Does aerobic exercise enhance neurogenesis in mdx mice? 2. Do bigger muscles enhance neurogenesis in mdx mice? 3. Does aerobic exercise with bigger muscles enhance neurogenesis in mdx mice? Also the effect of myostatin blocking on running readiness was examined. Myostatin inhibition affected running by decreasing running distances compared to pbs treated runners at the beginning of the experience. Against expectations there where no significant differences in proliferating new born cell count between any of the groups. It seems to be that neither exercise nor bigger muscles or their combination are factors that are sufficient or efficient enough to affect the neurogenesis of mdx mice. The other reasons for not finding any differences between cell counts could be that the neurogenesis of the mdx mice was not affected by the disease.

Keywords: Neurogenesis, Duchenne muscular dystrophy, mdx mice, aerobic exercise, myostatin blocker, nitric oxide

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Duchennen lihasdystrofia (DMD) on perinnöllinen sairaus, jonka esiintyvyys on noin 1/3600 poikavauvasta. Siihen liittyy lihasten heikkoutta, rappeutumista ja kognitiivista vajavaisuutta. Taudin aiheuttaa mutatoitunut geeni dystrophiini proteiinille. On esitetty, että kognitiivinen vajavaisuus johtuu taudin vaikutuksesta ehkäistä neurogeneesiä. Neurogeneesi on prosessi, joka jatkuvasti synnyttää uusia hermosoluja pääasiallisesti subventikulaari alueella ja hippokampuksen dentate gyruksella. Hoitoa ja parannusta tautiin tutkitaan yleensä mdx-hiirillä, joiden taudin etiologia on riittävän lähellä ihmisten tautia. Tämä tutkimus keskittyy pyrkimykseen vaikuttaa mdx-hiirten neurogeneesiin aerobisen liikunnan ja myostatiinin estäjän välityksellä. Myostatiinin estäjä lisää nisäkkäiden lihaskasvua. Tutkimuksella on kolme pääfokusta: 1. Vaikuttaako aerobinen harjoittelu mdx-hiirten neurogeneesiin? 2. Vaikuttavatko kasvaneet lihakset mdx-hiirten neurogeneesiin? 3. Vaikuttavatko aerobinen liikunta ja kasvaneiden lihasten yhteisvaikutus mdx-hiirten neurogeneesiin? Lisäksi myostatiinin estäjän vaikutusta juoksemisvalmiuteen arvioitiin. Myostatiinin ehkäisy vaikutti juoksemiseen lyhentämällä juoksumatkoja kokeen alkupuolella. Vastoin odotuksia lasketuista jakautuvista uusien hermosolujen lukumääristä ei löytynyt tilastollisesti merkitseviä eroja ryhmien välillä. Liikunta, suuret lihakset tai niiden yhdistelmä eivät tämän tutkimuksen mukaan ole riittäviä tai tarpeeksi vaikuttavia tekijöitä muuttamaan mdx-hiirten neurogeneesiä. Toinen mahdollinen syy erojen löytymättömyyteen solujen lukumääristä voi olla, että tauti ei ollut vaikuttanut mdx-hiirten neurogeneesiin.

Avainsanat: Neurogeneesi, Duchennen lihasdystrofia, mdx hiiret, aerobinen liikunta, myostatiinin estäjä, typpioksidi

CONTENTS

1. INTRODUCTION

Duchenne muscular dystrophy (DMD) is a disease that slowly destroys human muscle tissue. The absence of a protein called dystrophin causes the life-threatening and progressive degeneration and death of human skeletal dystrophin deficit muscle fibers (De Luca et al., 2003). DMD gene's short arm mutations cause the disease (Blake & Kröger, 2000). As one of the most common genetic diseases DMD affects approximately 1/3600 boys. DMD gene is a recessive hereditary (Nardes, Araújo & Ribeiro, 2012) gene which means that it very rarely affects female since there would have to be two mutation chromosomes in the same gene to get a female foetus infected and male DMD patients do not usually become fathers. DMD is characterized by lacking all, or almost all protein dystrophin which causes degeneration of muscle fibers leading to loss of strength and functional abilities. As a subsarcolemmal protein dystrophin links the intracellular cytoskeleton of the skeletal fiber to the extracellular matrix via the interaction of glycoproteins, in the so called dystrophin-glycoprotein complex (DGC) that spans the plasma membrane (cell membrane) (e.g. Blake & Kröger, 2000; Nardes et al., 2012). Dystrophin deficiency destroys DGC which leads to crucial mechanical and signal processing problems in muscles (De Luca, 2012). Dystrophin stabilizes muscular contraction (Nardes et al., 2012) and protects them from all mechanical powers. Finally, DMD leads to a state where myofibres are replaced by fat and fibrosis (De Luca, 2012). Almost all patients suffer from scoliosis that worsens after the individual loses the ability to walk, which occurs usually around 11-12 years from birth (Nardes et al., 2012). Scoliosis significantly reduces the respiratory systems' vital capacity (Nardes et al. 2012). There is no cure and the mean life expectancy is around 25 years after which death is caused by respiratory or cardiac-muscle failure (e.g. Blake & Kröger, 2000; Nardes et al., 2012).

Although muscle weakness is the main symptom of the DMD, other organs are affected by the disease too. The idea that DMD causes also abnormality in brain function was first introduced by the French Physician Duchenne de Boulogne in the latter half of the 19th century (Blake & Kröger, 2000). Mdx mice are used in DMD research because they are the most suitable animal models for preclinical therapy testing (e.g. Willmann, Possekel, Dubach-Powell, Meier, & Ruegg, 2008). Cxmd dog´s muscular disease resembles DMD more than that of mice but because of the low cost of mdx mice and short gestation time, it remains the most widely used animal model for DMD (Wang, Chamberlain, Tapscott, & Storb, 2009).

1.1. Building muscle mass

When muscles lack dystrophin their dystrophin-glycoprotein complex gets destroyed which causes deficits in the muscles´ mechanical stability and in the mechano-transduction signaling (De Luca, 2012). These muscles are more susceptible to contraction injuries which will lead to myofibre necrosis and replacement of the muscle fibres by fibrosis and fat tissue (De Luca, 2012).

The most common treatment for DMD is to increase the patient's muscle mass (De Luca, 2012). To gain more muscle mass there has been trials which use different mechanisms for building muscle strength such as anabolic steroids, β2 –agonists (enhanced protein synthesis) and myostatin blockers.

β2 –agonist that is known as an asthma drug has an anabolic effect in high doses. Radley et al. (2007) point out that classical β2 –agonists have many undesirable side-effects e.g. tremors and higher heart rate which naturally limits their therapeutic potential.

Before there is a cure for DMD, anabolic steroids such as corticosteroids will be used as alleviators for cardiac and breathing problems and also for reducing inflammations that are believed to cause myofibril necrosis typical to DMD (Radley et al., 2007). Anabolic steroids (testosterone and nandrolone) have given controversial results about their therapeutic potential when treating DMD in both dystrophic patients and mdx mice (De Luca, 2012). They also affect the tissues that are not wanted to be affected. Yet they are the only medication shown to be effective in delaying the progression of the disease (Beytía, Vry, & Kirschner 2012). Anabolic steroids that affect only the target tissue are under development (De Luca, 2012).

There have been good results with IGF-1 (insulin-like growth factor) which stimulates muscle regeneration, a process that fails in DMD patients (De Luca et al., 2003), and is also involved in anabolic action caused by growth hormone in muscles (De Luca 2012). In chronic use IGF-1 causes significant metabolic actions and pro-tumours that are a major concern (De Luca, 2012).

In this research yet another solution is used in building more muscle mass for disease weakened muscles, without devastating side-effects. Myostatin is an endogenous inhibitor on muscle growth (McPherron, Lawler, & Lee, 1997). It inhibits muscle stem cell proliferation (Taylor et al., 2001; Thomas et al., 2000) and differentiation (Langley et al., 2002) and attenuates adult muscle fiber protein accretion (Trendelenburg et al., 2009) resulting in limited muscle size and mass. Genetic loss or pharmacological inhibition of myostatin leads to increased muscle growth (Thomas et al., 2000).

1.2. DMD and brain functioning

It has been shown in many experiments that there is a connection between DMD and cognitive capacity. However, the nature of the connection remains unresolved (Blake & Kröger, 2000). Dystrophin deficiency causes cognitive defects that are difficult to relate to the loss of dystrophin (Deng, Glanzman & Tidball, 2009).

1.2.1. Human brain

It is not well known why DMD also affects cognitive deficits. Does loss of dystrophin also affect brain functioning or do weak muscles also cause changes in brain function or via reduced ability and readiness to exercise?About 20-30% of DMD boys fall in to the group of mild-retardation in IQ tests (e.g. Blake & Kröger, 2000; Nardes et al. 2012). DMD patients' average IQ is 85 when in normal population it varies between 90-120 (Nardes et al., 2012).

Anatomically, the gross morphology of DMD patients' brains appears normal (Blake & Kröger, 2000). It might be that specific brain areas are selectively affected by loss of dystrophin (Deng et al., 2009). Some isoforms of dystrophin are found in the soma and dendrites of cortical and hippocampal neurons, and in cerebellar Purkinje cells (Blake & Kröger, 2000). Blake and Kröger (2000) hypothesize that dystrophin may have a role in synapse structure or functioning since it is found at high levels in postsynaptic densities that have different functions in synaptic events. Abnormalities in dendritic development and arborization of cortical pyramidal neurons have also been found in DMD patients (Jagadha & Becker, 1988). Jagadha and Becker (1988) also found neuronal loss and gliosis. Gliosis is the proliferation or hypertrophy of glial cells which leads to glial scarring. Dystrophin-deficient neurons are also more susceptible to hypoxia-induced loss of synaptic transmission (Blake & Kröger, 2000). This research focuses on adult neurogenesis which abnormalities could be another major contributor to the deficits in cognition caused by DMD.

1.2.2. Mouse brain

Hippocampus-dependent learning and memory processes with dystrophin deficit mice is largely intact (Blake & Kröger, 2000). Some behavioral abnormalities such as retention impairment has been reported in mdx mice (Vaillend, Rendon, Misslin, & Ungerer, 1995), but they have normal spatial learning and hippocampal long term potentiation (LTP) (Blake & Kröger, 2000). Some alterations in their metabolism have also been reported which may affect the cognitive capacity of the mdx mice. (Tracey, Dunn, & Radda, 1996). Even though cognitive deficits seem to be milder in mdx mice they are still valuable in studying the role of dystrophin in the central nervous system.

1.3. Neurogenesis and learning

Many genetic and environmental factors that contribute to neurogenesis cause corresponding changes in cognitive performance (Zhao et al., 2008). Although memory cannot be localized to any certain part of the brain, it is widely approved that there is one specific part that certainly has its contribution on learning, memory and cognition, the hippocampus. Hippocampal formation participates in the acquisition and retention of memories but the specific mechanism it uses remains unknown (Leuner, Gould, & Shors, 2002). It could be that neurogenesis provides a mechanism through which synaptic plasticity and associated memory may be promoted in the hippocampus (e.g. van Praag et al., 1999; Shors et al., 2001).

New neurons are produced on a continuous basis in healthy adult brain, with neural stem/progenitor cells residing in two major brain regions: the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus. Eriksson et al. (1998) were first to demonstrate that neuroblasts are produced throughout adult life in human dentate gyrus. These neuroblasts proliferate locally and become incorporated into dentate gyrus circuitry, phenomenon that is common to most, if not all, mammal species (Gould et al. 1999). Presumably, these neurons are involved in cognitive functions such as learning, attention and processing speed (Déry et al., 2013; Nokia, Anderson, & Shors, 2012; Shors et al., 2002). DG produces 250 000 new neurons a month in rat, which is a very significant amount, given that the structure possesses only 1-2 million granule cells(Shors, Anderson, Curlik, & Nokia, 2012; Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002). Human brain may produce even more new cells. These new neurons have a threshold for the

induction of long-term potentiation (LPT) that is lower than that of mature cells (Schmidt-Hieber & Bischofberger, 2004) which possibly makes them more adaptive for acquiring new information and forming new dendrites and neural connections. One of the most effective ways to keep these new neurons alive is learning new things (e.g. Shors et al., 2012).

Cognitive deficits in DMD patients may occur because dystrophin mutation disrupts adult neurogenesis by promoting cell proliferation in dentate gyrus and suppressing neuronal differentiation (Deng et al., 2009). It is likely that neurogenesis and newborn cells affect memory. Elevated levels of proliferating cells are useless without differentiation since undifferentiated newborn cells die soon. Furthermore, changes in neurogenesis have been proposed to be the underlying cause of mental disorders like depression and schizophrenia (Yau, Lau, & So, 2011).

Elevation in adult neurogenesis has been connected especially to enriched environment and voluntary exercise. Enriched environment probably enhances neurogenesis because it offers a lot to learn and to memorize. It is assumed that abnormally low or high levels of neurogenesis result to abnormal synaptic connectivity or neural activity that contributes to impairments in cognition in DMD patients (e.g. Deng et al., 2009). Still we do not fully understand the mechanisms between learning and enhanced neurogenesis and even negative regulators of hippocampal neurogenesis sometimes result in enhanced learning (Lee, & Son, 2009).

1.4. The contribution of aerobic exercise to neurogenesis

It is well known that exercise enhances learning and memory processes (e.g. van Praag, Christie, Sejnowski, & Gage, 1999) and counteracts age-related mental decline but mechanisms underlying these benefits are not well understood. Aerobic exercise (AE) possibly increases hippocampal perfusion and also contributes to greater connectivity in the hippocampus which means better information transfer throughout the brain. (Burdette et al., 2011). Researchers have found a positive correlation between AE and hippocampus size, especially anterior hippocampus and dentate gyrus, which is linked to better memory capacity (Erickson et al., 2011). Also, it has been shown that exercise selectively increases dentate gyrus cerebral blood flow which is probably due to exercise-induced angiogenesis in the dentate gyrus (Pereira et al. 2007). Increased angiogenesis correlates with increased neurogenesis in rodents. Aerobic exercise enhances not only neurogenesis but also LTP in the dentate gyrus (Farmer et al., 2004; van Praag et al., 1999).

It has been hypothesized that the effects of exercise on mood and cognition would be partly mediated by the fact that voluntary exercise strongly induces hippocampal neurogenesis (Yau et al, 2011). First of all, exercise has to be voluntary because forced exercise causes stress and chronic stress creates oxidative stress which creates a neurotoxic environment (Gerecke, Kolobova, Allen, & Fawer, 2013). There is substantial evidence that the stress hormone glucocorticoids suppress hippocampal neurogenesis, perhaps because stress hormones change the electrophysiological properties of the hippocampus (de Kloet, 1992) and influence negatively on plasticity of hippocampus (Reagan & McEwen, 1997; Snyder, Soumier, Brewer, Pickel, & Cameron, 2011). Glucocorticoids inhibit cell proliferation and decrease cell survival and differentiation but there is also evidence that in stressful events glucocorticoids actually promote neurogenesis if a rewarding experience is included in the event (Schoenfeld, & Gould, 2013). Chronic stress also significantly decreases the expression of vascular endothelial growth factor (VEGF) which is a neurogenesis supporting factor (Lee, & Son, 2009). The best results of the effects of exercise are found from subjects with voluntary moderate exercise (Yau et al., 2011). This might be because too moderate exercise is not sufficient enough to cause the wanted effect and too hard exercise causes stress which suppresses neurogenesis.

There is also evidence that the brain derived neurotrophic factor (BDNF) along with trkB receptor have an important role in modulating hippocampal plasticity that contributes to learning and memory (Erickson et al., 2011; Vaynman, Ying, & Gomez-Pinilla, 2004; Lee, & Son, 2009). trkB receptor´s role is to regulate synaptic strength and plasticity in the mammalian nervous system. It has been shown that exercise can raise BDNF levels (Cotman & Berchfold, 2002; Marlatt, Potter, Lucassen, & van Praag, 2012; Vaynman et al., 2004). Higher BDNF serum levels are connected to better memory and larger anterior hippocampal volume (Erickson et al., 2010) which includes dentate gyrus. In addition to earlier mentioned effects of stress it has been shown that acute or chronic stress decreases the expression of BDNF in hippocampus (Duman, & Monteggia, 2006; Lee, & Son, 2009).

1.5. Research questions and hypotheses

This research focuses on the possible treatment of cognitive deficits associated with DMD via the enhancement of neurogenesis. We have read from above that neurogenesis may have a major role in cognitive capacity of mammals and muscle degenerative diseases downregulate adult neurogenesis.

DMD patients or mdx mice lack muscle strength and stamina to exercise, so different methods have been introduced to increase their muscle size and power. The main focus of the present study is attempting to enhance the neurogenesis of mdx mice. The effect of myostatin inhibition on running readiness of mdx mice is also examined. The research questions are: Do myostatin blocking affect running readiness of mdx mice? Does aerobic exercise alone affect neurogenesis of mdx mice? Does muscle size have any effect on neurogenesis in mdx mice, and if it does, do bigger muscles increase neurogenesis of sedentary animals or do they also need aerobic exercise to be effective?

It could be expected that readiness to voluntary exercise will deteriorate because myostatin inhibition may decrease muscle oxidative capacity and thus may decrease voluntary physical activity (Hulmi et al, 2013). Based on this assumption it is hypothesized that the running readiness of mdx + act + rw may deteriorate. Based on previous research it is hypothesized that pbs treated animals have abnormalities in neurogenesis and their cell proliferation rate is expected to be the highest. It is also hypothesized that running alone is not a factor that is sufficient to enhance mdx mice neurogenesis. The disease causes abnormalities in the neurogenesis of mdx mice but it is assumed that weakened muscles do not cause it directly. Based on this assumption, it is hypothesized that mdx + act treatment is not going to enhance the neurogenesis of mdx mice. Because it has been proven in many previous research that aerobic exercise has an neurogenerative function, it is hypothesized that mdx mice with chemically enhanced muscle capacity along with running treatment would enhance their neurogenesis if their running readiness is not significantly reduced. Also the use of ImageJ as a cell counting program is evaluated.

2. MATERIALS AND METHODS

2.1. Subjects

Mice with X-chromosome linked muscular dystrophy (mdx) were used as subjects. Mdx mutant mice and Duchenne muscular dystrophy (DMD) patients share several histopathological features (Bulfield, Siller, Wight, & Moore, 1984). Both diseases are X chromosome-linked and they have a similar gene order on X chromosome (Bulfield et al., 1984).

In this experiment, 6- to 7 week-old male mice from the Jackson Laboratory were used (Bar Harbor, Maine, USA). The modestly dystrophic mdx mice were from a C57Bl/10ScSnJ background

and healthy C57Bl/10ScSnJ served as controls. The mice were housed in standard conditions (temperature 22°C, light from 8:00 AM to 8:00 PM) and had free access to tap water and food pellets (R36, 4% fat, 55.7% carbohydrate, 18.5% protein, 3 kcal/g, Labfor, Stockholm Sweden). The treatment of the animals was in strict accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The protocol was approved by the National Animal Experiment Board (Permit Number: ESLH-2009-08528/Ym-23). A total of 34 mice were used as subjects.

2.2. Experimental design

The mdx mice were randomly divided into 4 groups: 1) mdx/pbs (n=8), 2) mdx/act (n=6), 3) mdx/pbs + running (n=8), and 4) mdx/act + runnings (n=7). Wild-type mice served as healthy controls (n=5). All animals were housed in their individual cages. sActRIIB-Fc or PBS was injected intraperitoneally once a week with a 5-mg/kg dose of sActRIIB-Fc. During the experiment all the conditions were standardized.

2.2.1. Voluntary wheel running

The chosen exercise modality was wheel running for seven weeks (mice age from 7 weeks to 14 weeks). To allow the treatment take effect, the running wheels were locked during the first injection day and the following day preventing mice from exercising. On the last two days, the mice did not have access to running wheels, so that the outcome effects would not reflect the acute effects of exercise. The mice in running treatment were housed individually in cages where they had free access to custom-made running wheels (diameter 24 cm, width 8 cm) 24 h/day. Monitored running enabled to determine whether the myostatin blocking affects running activity during the treatment period. Total running distance was recorded 24 hours daily. Sedentary animals were housed individually in similar cages without the running wheel.

2.2.2. Immunohistochemistry

The mice were sacrificed after seven weeks by cervical dislocation and brains were collected. The brains were kept in 4% paraformaldehyde for 48 hours. Then the brains were switched to 0.1 M phosphate buffered saline before cutting them. The brains were then cut with a vibratome to 35 micrometer slices. The left or the right hippocampus was randomly selected. Every tenth slice was harvested, which formed a representative sample through the hippocampus. Five to seven slices from each mouse were used in final analysis. Slices were stored in -20 °C and cryoprotectant was used to prevent them from freezing. Doublecortin (DCX) was used as a biological marker for new neurons**.** DCX is a protein expressed by neuronal precursor cells and immature neurons. Neuronal precursor cells begin to express DCX while actively dividing. Neuronal daughter cells continue to express DCX for 2-3 weeks as the cells mature into neurons. The brain samples were kept 30 minutes in a heated citrate liquid (pH 6). Then they were washed with tris-buffered saline with Triton X-100 (TBS-T (pH 6)) three times for five minutes. After the wash the samples were soaked in doublecortin antibody in room temperature. After this they were soaked in bionytilated rabbit anti-goat IgG antibody for two hours in room temperature. Then they were kept two hours in streptavidin-horseradish peroxidase conjugate (HRP). After all these antibody treatments the samples were TBS-T washed again. Finally, they were soaked for three minutes in diaminobenzidine in room temperature. The samples were then attached to a regular microscope slide using gelatin and a coverslip was glued on top of it.

2.2.3. Data analysis

Primo Star microscope with 10x zoom was used to detect new cells. Powershot G10 digital camera was attached to a microscope to take pictures of hippocampi. Camera settings were: 5x zoom (maximum zoom), ISO 400 M and macro. ImageJ graphics program was used to study the newborn cells. Pictures were cropped so that only subgranular zone (SGZ), granular cell layer (GCL) and molecular layer (ML) were left in the picture. Hilus was removed from the pictures because healthy newborn neurons do not usually migrate there. The distance from which the cells were counted was measured with segmented line option of ImageJ.

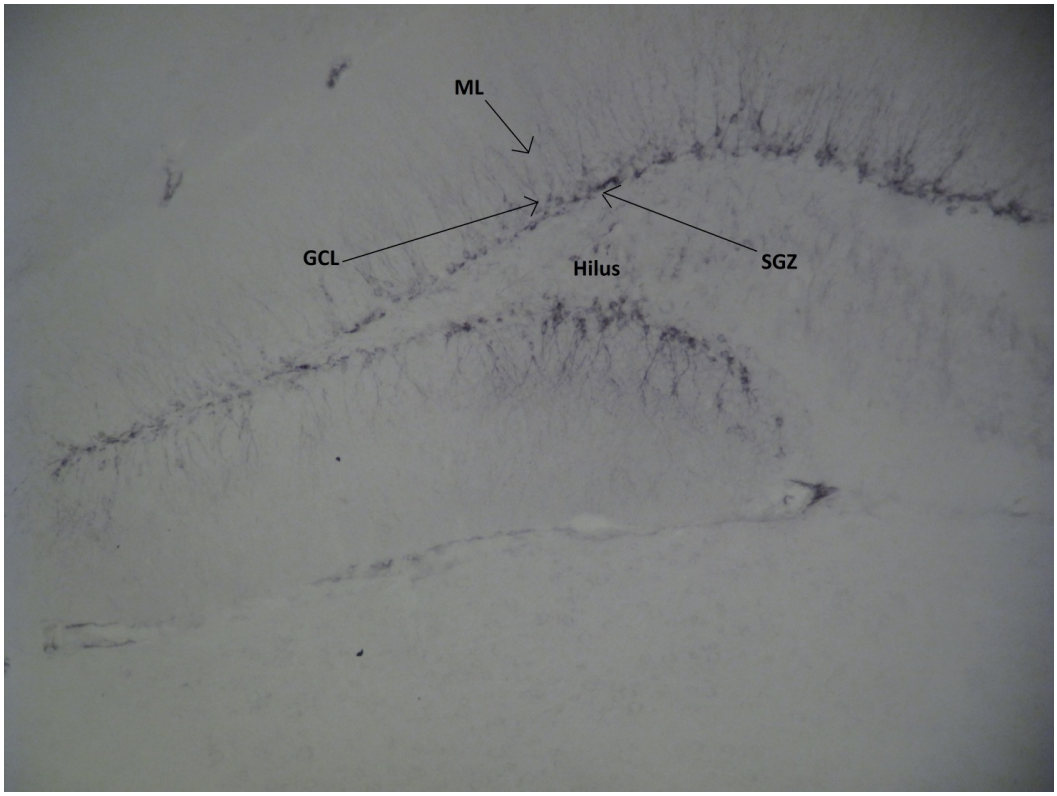

Figure 1. *An example of the original picture of mouse hippocampus. Dark-coloured parts at subgranular zone (SGZ), granule cell layer (GCL) and molecular layer (ML) are DCX+ cells (newborn neurons).*

Figure 2. *Cropped photo. Only SGZ, GCL and ML are left from the original picture.*

The RGB image was split into three 8 bit grayscale images containing the red, green and blue components of the original. Red pictures were used in final analyze. Smooth continuous background was removed from the images, rolling ball radius was set to 30 with light back ground and dark objects. A reversed image was created (invert), similar to a photographic negative. To discriminate unwanted particles from wanted particles (subtract background) the threshold level was set from 0 to12. Outliers were removed (remove outliers) and the picture was reversed againg. Particles that touched each other were separated (watershed) to be counted as individual particles. A macro for ImageJ to do all this can be seen in appendix 1. With a multitresholder the image can be segmented based on 15 different features. Changing the threshold and other values, different objects will be counted as qualified particles. The number of DCX+ cells in SGZ, GCL and ML, which are dividing neuronal precursor cells, and their neuronal daughter cells in hippocampus, were counted (analyze particles)**.**

2.3. Statistical Analysis

Statistical analysis was performed by SPSS v.20 for Windows program. Repeated measures analysis was used to ascertain if there was a difference in the running distances through time between pbs and act groups. Independent sample t-test was used to analyse if the running distances between the pbs treated mice and the sActRIIB-Fc mice differed from each other in any given week. In the analysis of the cell counts a one way analysis of variance (ANOVA) was used. A new variable was created by dividing the sum of new cells in SGZ, GCL and ML by the length of the area where they were counted from as it was assumed that new born cells are divided evenly through the neurogenerative zone. Differences were considered statistically significant if the *P*-value was less than 0.05.

3. RESULTS

Repeated measures analysis showed that the running distances did not differ from each other through time F(1, 13) = 3.812, p = 0.073. If sample sizes had been a little larger the p-value would have gone to a significant level, so an independent sample t-test was used to test if running distances differed in any given week from each other. Independent sample t-test showed that pbs treated mice ran significantly more than sActRIIB-Fc treated mice at week 2 t(13) = 2.540, p = .031, and week 3 t(13) = 2.301, p = .44. During weeks 1 and 4-7 there was no significant difference between running distances t(13) = .46 – 1.972, p = .07 - .653.

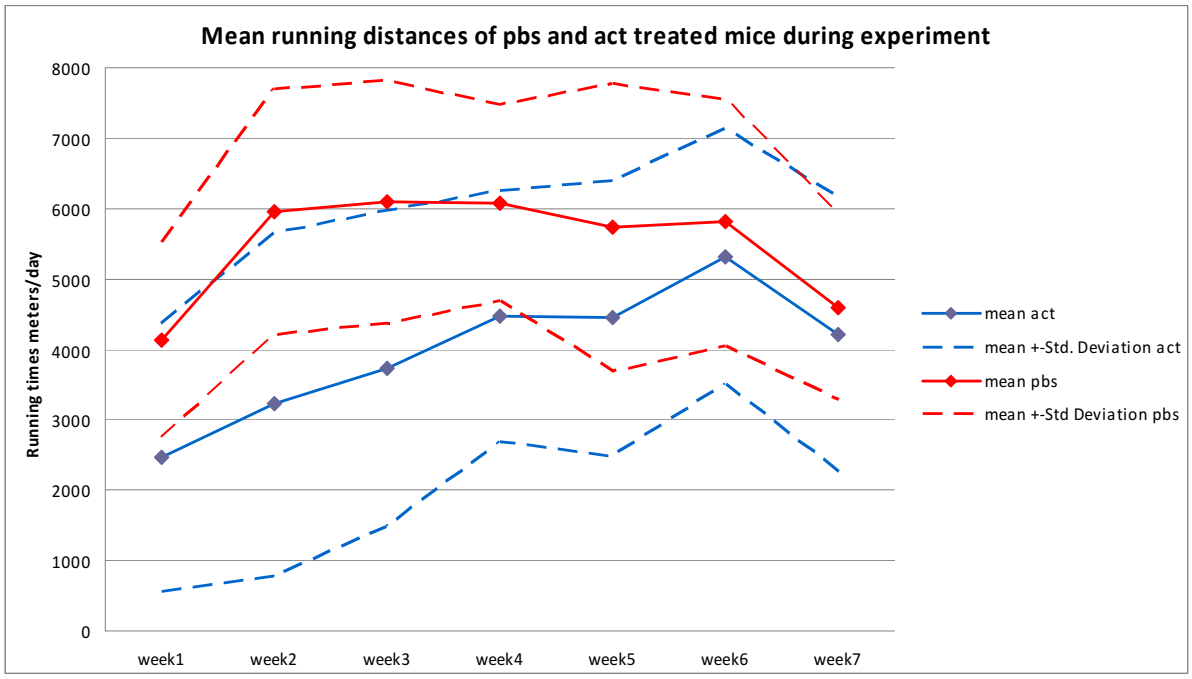

Figure 3. *Running distances of pbs and sActRIIB-Fc treated mice and their standard deviations.*

A one-way ANOVA was used to test cell count mean differences between mice. There was no significant difference between groups counted by ImageJ program [F(4,29) = 0,928, p = 0.461]. Tukey post-hoc comparisons of the groups indicate that the mdx + pbs group (M = 16.11, SD = 3.4218) and the wild type control (M = 9.62, SD = 3.6121) had the highest mean difference.

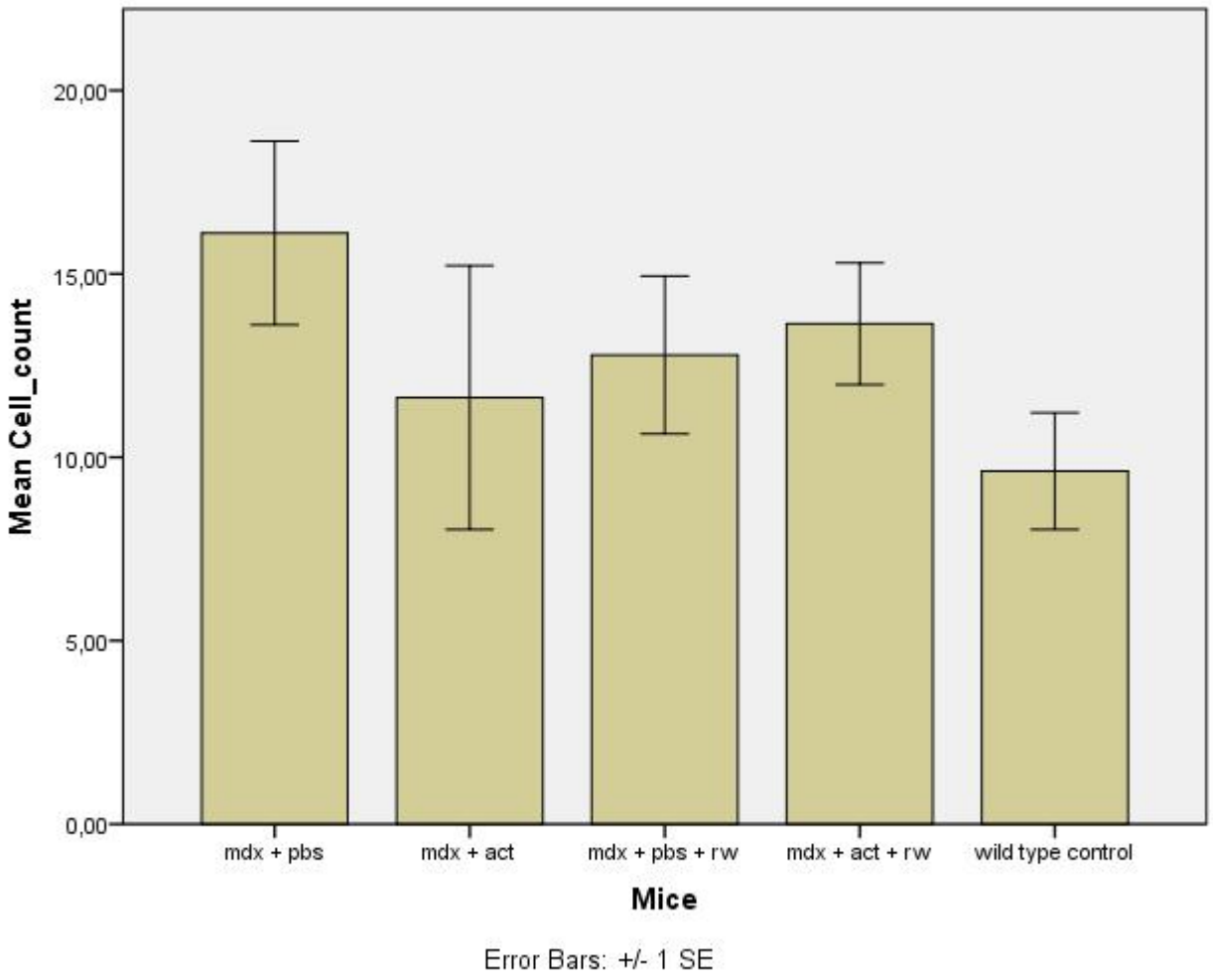

Figure 4. *Mice dentage gyrus´s newborn cells means in different groups counted with ImageJ with 1 standard error of mean (SEM) error bar.*

4. DISCUSSION

The present study had 4 different groups where aerobic exercise and myostatin blocking treatment were the changing factors when trying to affect the neurogenesis of mdx mice. First it was analysed if myostatin inhibition affected running readiness of the mdx mice. It was expected that readiness to voluntary exercise will deteriorate because of myostatin inhibition. Contrary to expectations the running distances of the whole treatment period showed no differences between act and pbs groups. Newborn cell counts were analysed and contrary to expectations pbs treated mdx mice did not differ from the control group or any other group. Running treatment alone was not a factor that would

enhance the neurogenesis of the mdx mice as was hypothesized. Since it is expected that abnormalities in the neurogenesis of mdx mice are not directly caused by degenerated muscles, it was hypothesized that bigger muscles alone are not a factor that would enhance the neurogenesis of mdx mice. Mdx + act treatment did not enhance the neurogenesis of mdx mice as was hypothesized. It was also hypothesized that mdx + act + rw mice would show enhanced neurogenesis if their running readiness would not be significantly reduced. Contrary to expectations they did not show enhanced neurogenesis and there were no significant differences between any of the groups when the cell counts were analysed.

Neurogenesis is a fairly new area in the field of neuroscience. Much is known but many aspects of neurogenesis still remain unresolved. The present study arises from a hypothesis that an enhanced neurogenesis could possibly improve cognitive capacity of Duchenne muscular dystrophy patients, which is usually suppressed by the disease. Mdx mice were used in the research, since they are found to be the best laboratory animals to conduct DMD research, even though their etiology of the disease is not exactly the same as that of patients with DMD.

It is commonly known that aerobic exercise is a factor that promotes neurogenesis so that was the method used in an attempt to enhance mdx mice´s neurogenesis. Also, myostatin inhibition was used to strengthen the muscles of mdx mice. At first it was analyzed if myostatin inhibition had any effect on running behavior of the mdx mice. It was hypothesized that myostatin blocked mdx mice would show limited rates of running readiness. When the distances the mice ran were analysed for the whole period of the experiment, the groups did not differ from each other. Blocking of myostatin seemed to diminish running readiness of mdx mice but only at the beginning of the experience. They ran significantly less than pbs treated mice at weeks 2-3, but their running readiness came back to normal levels as the experiment proceeded. It seems to be that it takes some time from mdx mice to adjust to the myostatin inhibition before their normal running behaviour returns.

Pbs treated mdx mice had the highest rate of cell proliferation, as it was hypothesized but this difference was not significant. Mdx is supposed to enhance cell proliferation and reduce cell differentiation. The reasons for this may be due to raised nitric oxide levels of mdx mice which will be discussed later. Mdx + rw treated mice did not show enhanced neurogenesis as was expected based on previous research. In their research Deng et al. (2009) did not found any improvements in neurogenesis of the mdx mice when they were treated with running only. There were also expectations that bigger muscles alone are not able to enhance the neurogenesis of mdx mice, since it is assumed that muscle atrophy is not directly causing the abnormalities in the neurogenesis. It is expected that when exercising, muscles produce chemicals that contribute to neurogenesis. So

bigger muscles in the sedentary condition have no or only little effect on the neurogenesis of mdx mice. It was also hypothesized that running treated mdx mice with bigger muscles (mdx + act + rw) would show enhancement in their process of neurogenesis if their running readiness would not be significantly reduced. Mdx + act + rw treated mice did not differ from the other groups when cell counts where analyzed. From figure 1 we can see that sActRIIb-Fc treated running mice showed lower activity for the whole 7-week period than pbs treated mice, especially at the beginning of the experiment. During two week rapid muscle growth period sActRIIb-Fc treated exercising mdx mice showed decreased running activity, but after seven weeks their exercise activity restored to the level of PBS-treated mice. It could be argued that their amount of exercise was not sufficient enough to affect positively on neurogenesis. In future, treatment time should be extended or the running treatment should start later until the mice have got used to myostatin blocking and their running readiness is normalized. It can also be that aerobic exercise with stronger muscles is not a factor that would alter the damage that mdx does to neurogenesis.

The research question was if AVCR2B blocker injectection or the exercising (or combination of these two) would enhance the neurogenesis of mdx mice. There was no significant difference in newborn cell count between any of the groups. Neurogenesis was not enhanced, at least not significantly. Possible reasons for this and the major expected contributors on neurogenesis, will be discussed next. Also, the limitations of the study will be considered.

There are a many things that contribute to neurogenesis so there are plenty of possible reasons why differences in the cell counts between the groups were not found. We believe that enriched environment and aerobic exercise promote neurogenesis and survival of newborn neurons but we don't know all the intrinsic and extrinsic variables that regulate the process of neurogenesis. In some research even the effect of exercise is questioned. In Curlik et al's. (2013) experiment aerobic exercise did not enhance neurogenesis but learning a tricky physical new skill did. Notable is also that learning a new physical skill was not a hippocampal-dependent task but it still helped newborn hippocampal neurons to survive and mature.

There has been found some contributors to neurogenesis that affect in cell and molecular level but information of them is rather limited. Also, the mechanisms how mdx (or DMD) affects neurogenesis are still unknown even though there are speculations of them.

One hypothesis is that mdx mice and DMD patients have lower serum nitric oxide (NO) levels than wild-type mice. NO is a membrane-permeant gas which in animals originates after activation of nitric oxide synthase (NOS), and plays many important roles in human body as a signal molecule or intercellular messenger (Garthwaite, & Bulton, 1995; Weitzdoerfer et al., 2004). nNOS is expressed by specific neurons widely distributed in the mammalian nervous system

(Estrada & Murillo-Carratero, 2005) and it is also localized in the sarcolemma of muscles, especially in myotendinous junctions, the same areas where dystrophin is distributed (Chang et al., 1996). It has been shown that serum (Gücüyener, Ergenekon, Erbas, Pinarli, & Serdaroğlu, 2000) and expired gases (Straub, Ratjen, Amthor, Voit, & Grasemann, 2002) NO levels in DMD patients are significantly lower than in healthy people. Dystrophin deficiency in DMD patients leads to secondary loss of nNOS (80% less NOS activity) (Chang et al., 1996; Deng et al., 2009) and it correlates with low nNOS levels although the connection between them remains unknown (Chang et al., 1996). Recent studies have shown that muscle-derived NO may have an important role in regulation of adult neurogenesis (Deng et al. 2009).

It has been reported that NO inhibits the proliferation of various cell types and facilitates cell differentiation (Ciani, Severi, Bartesaghi, & Contestabile, 2004). This is why it was hypothesized that pbs treated mdx mice would show the highest level of cell proliferation. Nitric oxide promotes cell differentiation by downregulating oncogenic N-Myc (Ciani et al., 2004), which has a promoting role in cell proliferation, and it may also have a role in newborn cell survival, although there is no evidence of that in vivo yet (Deng et al., 2009. It could also be that NO has no direct effect on neurogenesis but it causes significant changes in blood pressure and in cerebral blood flow that may indirectly affect neurogenesis (Estrada & Murillo-Carretero, 2005). Nitric oxide's contribution to learning and memory is one of the main neurobiological nNOS-related questions still open (Weitzdoerfer et al. 2004) but it is expected that an animals neurogenesis is atypical if it shows abnormal NO levels.

It may be that serum nitric oxide levels of mdx mice cannot be changed via aerobic exercise or the effects of exercise are very temporary. Deng et al. (2009) managed to raise acute and cronic levels of serum NO levels of mdx mice, but only when they were treated with the muscle-specific nNOS transgene. Chang et al. (1996) reported that there is no substantial recovery of nNOS in regenerated mdx muscles. That is why the present study hypothesized that bigger muscles alone are not able to affect neurogenesis of mdx mice and can also explain why there were no enchanged neurogenesis in mdx + act + rw group. If aerobic exercise is not a factor that could alter the NO levels damaged by mdx then it does not matter how long they run or how big their muscles are. Even if serum NO levels would have elevated via aerobic exercise or bigger muscles it could be that dentate gyrus's progenitors are not sensitive to NO action (Moreno-López et al., 2004) meaning less significant effects on neurogenesis of the dentate gyrus. That would indicate that changes in NO serum levels are not causing increased cell proliferation or decreased cell differentiation with mdx mice at all. In future research serum NO levels should be measured so that these questions could be answered. Deng et al. (2009) found elevated serum NO levels with wild-type mice only when

measured immediately after treadmill running. In the present study mice were kept two days without exercise before collecting the data, so it could be that the effects of the exercise on NO level had already vanished if there was any. In future research part of the data should be collected immediately after the exercise period. We are not sure if neurogenesis of DMD patients or mdx mice is dysfunctional because of low NO levels. The question remains, if lower levels of NO have no significant effect on neurogenesis of mdx mice, then what would it be?

It is not well known what kind of signals control cell profileration and survival (Estrada & Murillo-Carretero, 2005). Furthermore, there are also other messenger molecules and transcription factors than NO that are present in niches where new cells are born, so there are other factors that have been suggested to be important contributors to adult neurogenesis. In SVZ neural stem cells divide in response to fibroblast growth factor 2 (FGF-2) (Gritti et al. 1999) and transforming growth factor α (TGF-α) knockout mice present reduced cell profileration (Tropepe, Craig, Morshead, & van der Kooy, 1997). Probably there are multiple morphogens that are concurrently influencing on adult neural precursor cells and the amount of new neurons in the adult brain. Vascular endothelial growth factor (VEGF) (Schänger et al., 2004), brain-derived neurotrophic factor (BDNF) (Pencea, Bingaman, Wiegand, & Luskin, 2001; Waterhouse et al., 2012) and erythropoiesis (EPO) (Lee, & Son, 2009) enhance neurogenesis. Continuous infusion of epidermal growth factor (EGF) and FGF-2 also increased the number of new neurons in the hippocampus after induction of ischemia, resulting in a recovery of approximately 40% of the lost neurons four weeks later (Nakatomi et al. 2002). Both factors promote cell proliferation in subgranular zone in vivo. Infusion of vascular endothelial growth factor (VEGF) promotes cell proliferation (Cao et al., 2004). VEGF is required so that increased neurogenesis occurs via exercise and enriched environment (Lee, & Son, 2009; Zhao, Deng, & Gage, 2008). So if VEGF levels of the mdx mice of the present study were significantly lower than normal, then aerobic exercise had no promoting effect on neurogenesis. Aerobic exercise can raise BDNF levels which has been shown to enhance memory, promote proliferation and growth of the cells in the hippocampus (Erickson et al., 2010) and is also connected to larger hippocampal volume, including dentage gyrus. BDNF may also be the reason for enhanced LTP of newborn neurons (Farmer et al., 2004). Also, there might be other growth factors and neurotransmitters that contribute to neurogenesis.

There are many extrinsic factors and also intracellular pathways that may contribute to the regulation of adult neuron stem cells and progenitors. It is also possible to chemically enhance neuronal differentiation. In Tozuka, Fukuda, Namba, Seki, & Hisatsune´s (2005) experiment they found that systemic injection of GABAergic agonists caused 50% increase in the number of new neurons. They concluded that GABAergic inputs to hippocampal progenitor cells promote activity-

dependent neuronal differentation. Our understanding is rather limited in terms of molecular mechanisms underlying cell proliferation, differentiation, survival, maturation and integration during the process of turning from progenitor cell to integrated mature cell. In future, these factors should be investicated more closely, so that we would have an accurate description of molecular mechanisms of adult neural progenitor regulation and cell maturing.

The mechanisms governing the enhanced neurogenesis via exercise and enriched environment still remains elusive. Hippocampal circuits seem to have a pivotal role in adult neurogenesis. With pharmaceutical treatments we can affect the hippocampus. Theta-rhythm is typical network activity in hippocampus. Hisatsune et al. (2011) propose that neurogenesis could be mediated by the dynamics of theta-rhythm which could be tuned by environmental stimuli and pharmaceutical treatment.

It is possible that mdx mice used in this study had not yet any brain effects caused by the disease since they were only 7-14 weeks old. For example in Deng et al.´s (2009) research where they found differences in neurogenesis between mdx, wild-type and treatment groups they used mice aged between 4-6 months. So it could be that the mdx mice used in the present research had already normal levels of neurogenesis and that is why sedentary pbs treated mdx mice did not differ from the healthy control group, which were also in sedentary condition. In case that the control group would have conducted aerobic exercise there should have been differences based on theory about aerobic exercise´s effects on neurogenesis. Also in Sesay, Errington, Levita, & Bliss´s (1996) experiment they found no differences in hippocampal LTP or spatial learning between mdx mice (aged 10-12 weeks) and healthy control group mice which supports the hypothesis that mdx mice of this research were not significantly affected by the disease. DMD patients and mdx mice demonstrate the absence of full-length 427 kDa dystrophin in brain but mdx mice have a normal expression of dystrophin referred to as DP71 and DP140 (Sesay et al., 1996). It could be that mdx does not cause abnormalities in behavior and neurogenesis unlike DMD, at least not in its early stage. More severely impaired mdx^{3cv} mice lack all production of dystrophin and they could be a more suitable mouse model in DMD research.

Genetic backround has an effect on hippocampal neurogenesis (Hisatsune et al., 2011; Kempermann et al., 1997). For example Kempermann et al. (1997) found differences between all four different mouse strains used in their experiment in profileration, differentiation and survival of newborn cells that manifested in total cell counts and volymetric parameters of the dentate gyrus. Mdx mice in the present study were all from the same strain. If the mice from this strain were very productive when it comes to neurogenesis, maybe their neurogenesis would seem to be normal even if the disease would downregulate it.

4.1. Limitations of the research

This research could only detect that there were no differences in cell counts between the five different groups. Reasons for this are considered but nothing reliable can be said because there are many unmeasured variables that may contribute to neurogenesis. As no blood samples were collected, we cannot conclude whether the levels of nitric oxide, BDNF, the other growth factors or neurotransmitters changed during the experiment or if they were deteriorated by the disease. In future, blood samples should be collected for the better understading of the mechanisms covering the neurogenesis and possible effect of mdx on them.

It was hypothesized that there would be differences between different treatment groups, so there was only a sedentary control group. By adding an exercising control group there should have been at least this one group that would have shown a significant difference in newborn cell count. Otherwise, the method of this research would be false, assuming that the theory of the effect of aerobic exercise on neurogenesis is valid. If this kind of research is committed in the future an exercising control group should be added to the setting.

Mice from different strains should be used. Mice used in the present study were all from the same strain so it is possible that these mice have a particularly high level of neurogenesis. Mice used in the experiment should also be older than in this study, so that we could ensure the disease has already affected their neurogenesis. Also, there were only 5 mice in control group which lowers statistical validity of the experiment. By increasing sample sizes, a significant difference, at least between mdx + pbs and the control group, would have possible been seen.

We have to remember that mdx is a much milder form of muscle degenerative disease than DMD. Instead of mdx mice, mdx^{3cv} mice or Cxmd, dogs could be better research animals when conducting DMD research since their pathology resembles DMD more than that of mdx mice. In their experiment Blake and Kröger (2000) found only a small effect upon the hippocampal-dependent learning and memory processes with mdx mice. With DMD patients the cognitive impairment is usually definite.

It seems that mdx mice used in this research could have had already normal levels of proliferating cells, since mdx + pbs groups did not differ significantly from the control group, even though they did have the highest level of proliferating cells. Mice used in this research might be too young to show significant negative effects in neurogenesis caused by the disease. This kind of research would need some more adjustment to produce more precise information.

4.2. Conclusions

Contrary to expectation no differences between cell count were found between any of these five groups. The only statistically significant difference in this reasearch was running distances of pbs and act treated mdx mice in week 2 and 3. These findings suggest that at least mdx mice require extra time to adjust to the effects of the myostatin blocking in order to tolerate a large amount of voluntary running.

When looking at brain slices of mice hippocampi through microscope you could clearly see differences in newborn cell count between mice. In some samples there were many more doublecortin labelled cells than the others. It raised a question if there was something wrong with the cell counting procedure with the ImageJ program. Newborn cells were counted once more but this time manually. Altough ImageJ and manually counted cell counts differed from each other there were still no significant differences between any of the groups. According to this research ImageJ seems to be reliable tool when counting newborn cells if the parameters are carefully adjusted.

It is possible that mdx had not significantly downregulated the neurogenesis of mice used in this research but it still might have had an effect which prevents the enhancement of neurogenesis via aerobic exercise. In future it would be valuable to define the onset when the disease starts to affect neurogenesis of mdx mice, not forgetting the differences of the different strains. It is also possible that mdx had downregulated neurogenesis and there were actually some enhancement of the neurogenesis via treatments but they did not made statistically significant difference compared to pbs group. All of these groups had statistically normal level of proliferating cells of healthy sedentary mice. There is also a chance that treatment periods were too narrow. Treatments started to have an affect but not long enough to make a statistical difference. Also, we have to remember that all the proliferating cell data was collected two days after the running had stopped, which could have reduced the effects of the treatment on cell proliferation.

It must be noted that mdx + pbs treated mice did have the biggest newborn cell count, as could have been expected, if mdx had downregulated their neurogenesis, although this difference was not statistically signicant. If the treatments actually had an effect by lowering the progenitor cell´s proliferation rate, it could also have an enhancing effect on the survival rate of newborn cell and differentiation and promote the final outcome of neurogenesis. In future, it would be valuable to determine all the standard values concerning neurogenesis. Also all standard values of intrinsic and

extrinsic factors in healthy mice or men that affect neurogenesis should be determined so that we could compare these values for animals or individuals with abnormalities in neurogenesis.

Finally, As this is a fairly new area of research and the information of neurogenesis and all the intrinsic and extrinsic factors contributing to it there were many variables that this research setting did not take into account. This was a pioneering experimentation of this kind of research setting and therefore other settings, with more sophisticated methods and measurements should be concluded before we can make direct conclusions that aerobic exercise or bigger muscles or their combination are not factors that possibly enhance the neurogenesis of the mdx mice.

Not finding enhancement on neurogenesis in this study does not mean that aerobic exercise or bigger muscles would not effect on neurogenesis, cognition and mental health of DMD patients. The data cannot be transferred directly from mice to men.

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APPENDIX

A macro used for cell counting with ImageJ

```
open("c:\\h\\M1\\m1_cropped\\m1_1_c.jpg");        (directory where the picture file is)
run("Split Channels");
close();
close();
run("Subtract Background...", "rolling=30 light");
run("Invert");
setThreshold(0, 12);
run("MultiThresholder", "Triangle apply");
run("Remove Outliers...", "radius=13 threshold=1 which=Bright");
run("Invert");
run("Watershed");
run("Analyze Particles...", "size=100-Infinity circularity=0.15-1.00 show=[Overlay Outlines]
display exclude clear summarize");
close();
```