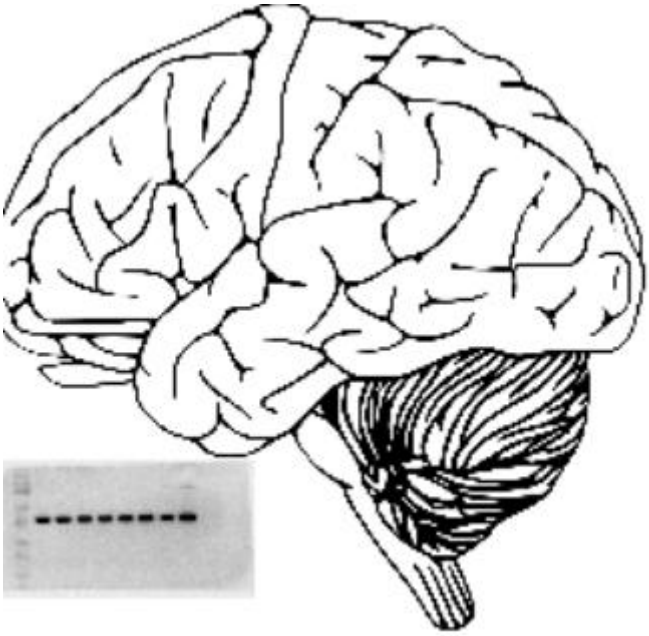
RAM MANOHAR BASNET



Biochemical correlates of synaptic plasticity in exercise

Master's thesis in Neuroscience

August 2012

Preface

This Master's thesis was carried out at laboratory of synaptic plasticity, Centre for Molecular Biology and neuroscience, Department of Anatomy, IMB, University of Oslo.

I am extremely grateful to Norwegian University of Science and Technology (NTNU) for providing me with the opportunity to study in Norway under quota scheme.

Thanks to my principal supervisor Professor Ursula Sonnewald for the collaboration and connection with NTNU.

This thesis is done under the guidance of my subject supervisor Professor Svend Davanger of University of Oslo. I am very indebted for his excellent guidance throughout the year.

Thanks to all the members of my research group for the help and encouragement you have provided while carrying out this thesis. Also, I would take this opportunity to thank senior lab engineers Karen Marie Gujord, Bjørg Riber and Jorunn Kuntsen for the sincere help they have provided.

Thanks to Professor Ivar Walaas, Marit Nielsen, and Bjørg Riber at biochemical department for good guidance and teaching.

Last, but not the least, thank you to all my dear friends and family. Everything I do is incomplete without you all.

Oslo, August 17th 2012

Ram Manohar Basnet

Abstract

Current views of the brain are shifting towards a more changeable, plastic brain than previously envisaged. Physical training has been shown to be one of few strategies that seems able to increase the plasticity of the brain. Little is known, however, about the molecular mechanisms that regulate synaptic plasticity during training. The aim of the project is to explore training based synaptic plasticity at a molecular level, focusing primarily on the excitatory glutamatergic synapses. Most of the research done on exercise has been focused on a specific brain region. I have tried to investigate global effects of exercise on the synapse.

Crude synaptosomes were prepared from trained and sedentary groups of mice, and semi-quantitative western blotting was done to determine possible changes in synaptic expression of functionally crucial proteins, i.a. glutamate receptors.

I found increased level of syntaxin, GluR1 AMPA receptor subunits, and the 2A/2B subunits of the NMDA receptor. There was also a decreased level of Arc in trained compared to sedentary mice. There was no significant change in the expression of the neuronal marker, beta tubulin, of the synaptic markers synaptophysin and PSD-95, or in the AMPA receptor subunit GluR2. Taken together, my results may indicate that physical exercise may lead to an increased level of ongoing hebbian plasticity, a reduction in homeostatic plasticity, an increased presynaptic release capacity, with no apparent change in the density of neurons or synapses.

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1) INTRODUCTION

1.1) Exercise

"Exercise is just as important to provide against disease in the healthy man as to cure him who was already attacked." (Herodicus, Greek physician, 500BC;(Schaub & Marian, 2011)

"If we could give every individual the right amount of nourishment and exercise, not too little and not too much, we would have found the safest way to health" (Hippocrates,400BC; (Voss, Nagamatsu, Liu-Ambrose, & Kramer, 2011)

"On order for man to succeed in life, God provided him with two means, education and physical activity. Not separately, one for the soul and the other for the body but for the two together. With these two means, men can attain perfection" (Plato,4th century BC; (Ströhle, 2008)

These quotes carry the importance of exercise from early period of time immemorial.

Physical activity has been the key to human survival since evolution. Ancient man travelled long distances searching for food and shelter. Neuroplasticity due to physical activity might be the reason behind hominid evolution during which brain size of primates increased rapidly before reaching the stable weight in homo sapiens (Knöchel et al., 2012). The therapeutic role of exercise in maintaining good health and treating diseases started long ago. Susruta, a 600 BC physician in India prescribed exercise as a therapeutic purpose to his patients. Hippocrates (460–377 BC) wrote "in order to remain healthy, the entire day should be devoted exclusively to ways and means of increasing one's strength and staying healthy, and the best way to do so is through physical exercise." Plato (427–347 BC) referred to medicine as a sister art to physical exercise and the famous ancient Greek physician Galen (129–217 AD) wrote several essays on aerobic fitness and strengthening muscles. Hugh Blair, a 18th century Scottish theologian said that "Strong body makes the mind strong" ((Voss, et al., 2011)The Royal College of Physicians have recommended doctors to ask about exercise when they see patients, particularly when they come for routine health checks, and should be made aware of and advise on suitable exercise programmes (Fentem, 1994).

All these evidences and quotes underline the importance of exercise and physical activity.

“Physical activity is broadly defined as any bodily movement generated by skeletal muscles resulting in energy expenditure” (Knöchel, et al., 2012; Sullivan, Scheman, Venesy, & Davin, 2012). Physical activity and exercise are similar term that can be interchanged with each other and includes activities varying in type, frequency, intensity, and mode (Sullivan, et al., 2012). However, according to Fentem, exercise is a biochemical, physical, psychological and social phenomenon. “It is a planned, structured, and repetitive movement to improve or maintain one or more components of physical fitness” (Knöchel, et al., 2012). Exercise can be broadly subdivided into 3 types:1)aerobic exercise; 2)anaerobic exercise and 3)flexibility, coordination and relaxation exercise (Sullivan, et al., 2012). In aerobic exercise, there is physical activity for long time increasing the capacity of oxygen transport system of body, e.g., walking, marathon running, playing football. Anaerobic exercise consists of short duration with high intensity of physical activity like sprinting and weight lifting. Flexibility, coordination and relaxation exercise consists of stretching, ballet and yoga (Glenister, 1996). Based on the heart rate and age, exercise can be classified into various zones as shown below by Fox and Haskell formula. I have taken into account every types of physical activity and exercises while writing this thesis.

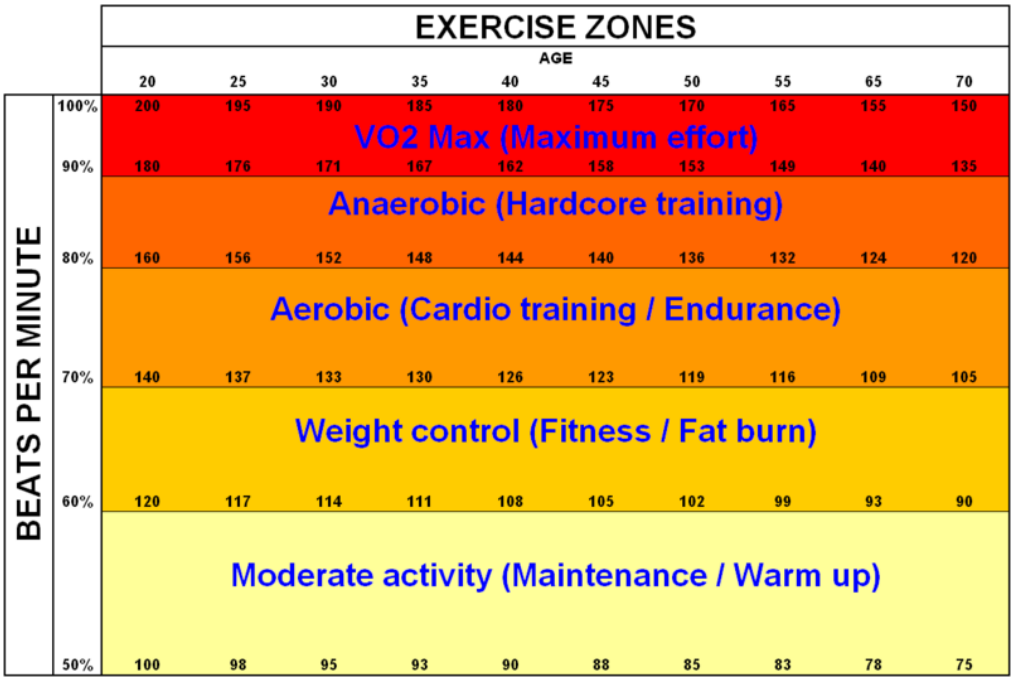


Figure 1: Fox and Haskell formula showing the split between aerobic (Light orange) and anaerobic (dark orange) exercises and the heart rate (Source: Wikipedia)

1.2) The general health benefits of exercise

Discoveries and emergence of newer technologies since last century have made human no longer dependent on physical activity for survival (Knöchel, et al., 2012). As a result, majority of the world population have increasingly adopting sedentary lifestyles that are closely related to various risk factors for negative health outcomes (Penedo FJ., 2005). Physical inactivity causes a wide range of diseases affecting almost every systems of our body. Several studies have led to the conclusion that effect of physical activity in society is almost at the level of smoking, obesity and hypertension. Abstinence from exercise in middle age might lead to shortening of life (Penedo FJ., 2005). Regular physical activity is a preventive measure for many chronic diseases (Fentem, 1994; Kruk, 2007). Doing physical training regularly reduces the risk of cardiovascular diseases like coronary artery diseases, hypertension and stroke. It also helps in reducing several cardiovascular risk factors including obesity, dyslipidemia, hypertension, metabolic syndrome and diabetes mellitus (Agarwal, 2012). Physical activity might help the patients with non-insulin dependent diabetes mellitus (T2DM) by increasing insulin sensitivity and improving glucose tolerance (Fentem, 1994**)**. Exercise also reduces the risk of several tumors such as breast, colorectal and prostate (Penedo FJ., 2005). It helps in enhancing the skeletal muscle function, tendon and connective tissue functions and joint functions (Fentem, 1994). Bone pathologies like osteoporosis(Fentem, 1994) , osteoarthritis and arthritis (Penedo FJ., 2005) can be prevented by exercise.

Moderate physical exercise increases immunity of our body (Knöchel, et al., 2012). However, intense exercise is shown to have negative effect for the immune system (Knöchel, et al., 2012; Stephen A. Martin, 2009). The effect of physical exercise in different systems of the human is shown in figure below.

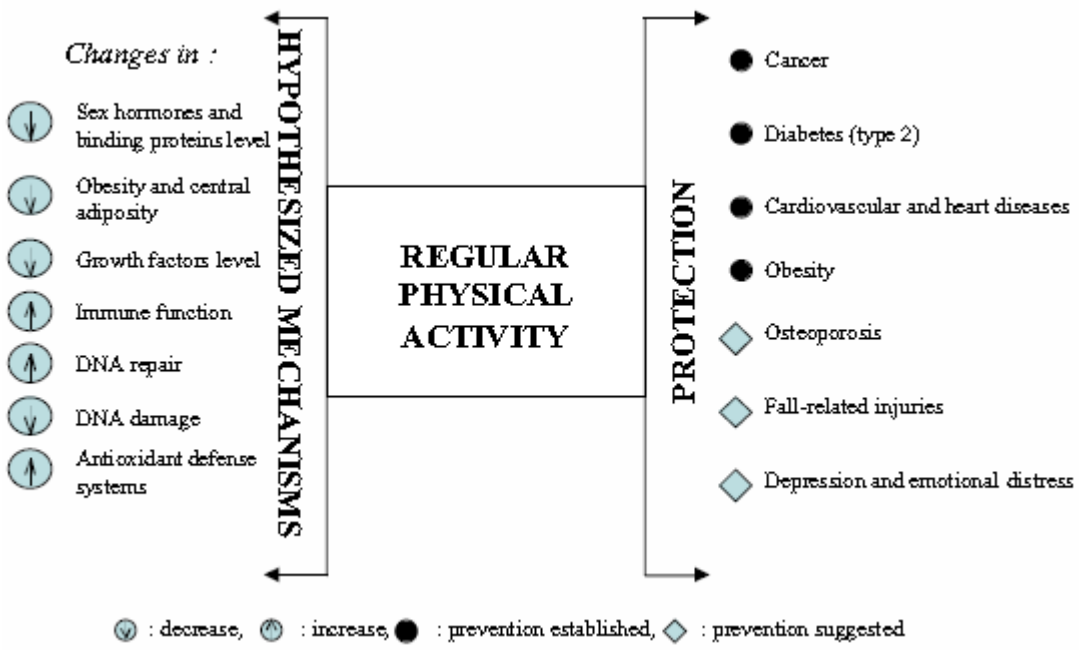


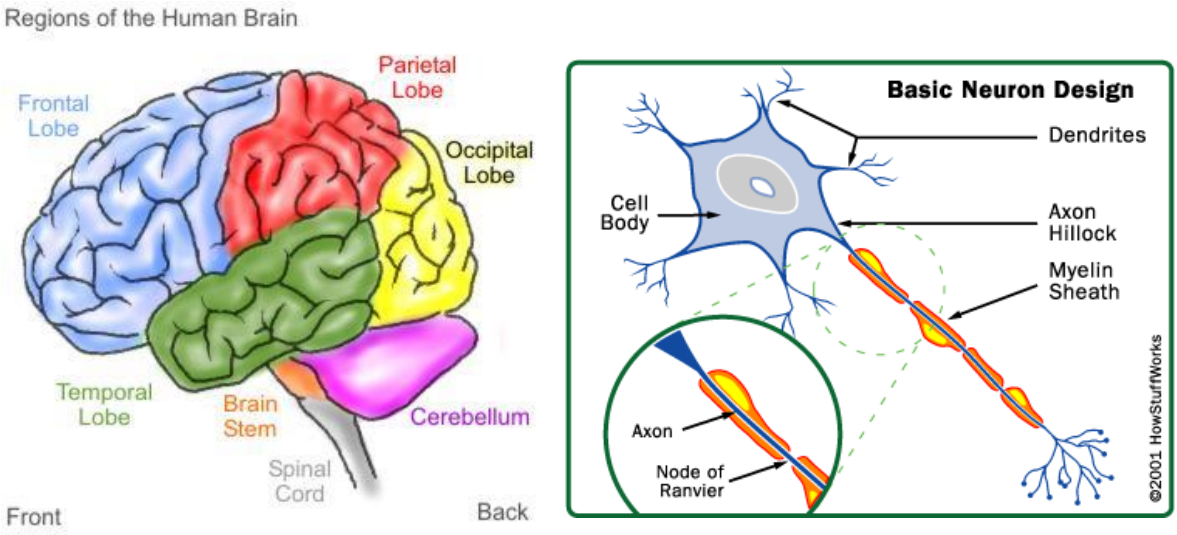
Figure 2: Protective Effects of Physical Activity on Chronic Diseases and Hypothesized Biological Mechanisms for its health benefits (Kruk, 2007).

Besides physical wellbeing, exercise contributes to mental health across all ages, sex and population both healthy and with disease. In normal people, it helps in maintaining positive mood besides reducing anger and tension (Fengju & Witzmann, 2007; Penedo FJ., 2005). By decreasing stress perception and negative effect, exercise helps a person to become psychologically fit (West, Otte, Geher, Johnson, & Mohr, 2004). In patients with psychiatric illnesses, regular physical exercise could stop the onset of depression (Paffenbarger R, 1994). It also decreases the symptoms of depression and anxiety (Penedo FJ., 2005) and is helpful in patients with major depression (Babyak et al., 2000). During old age, it prevents the cognitive decline acting as a buffer that try to decrease the age related cognitive decline (Penedo FJ., 2005)**.**

1.3) Human Nervous System

The nervous system is divided into central nervous (CNS) and peripheral nervous system. Central nervous system consists of the brain and spinal cord and peripheral nervous system consists of sensory neurons and motor neurons that act as a bridge between CNS and muscles and glands. The brain can be further subdivided into: cerebral hemispheres,

diencephalon, midbrain, cerebellum , pons and medulla as shown in the figure below (Purves et al., 2008).



and

Figure 3: Regions of Human Brain (Left) [Source:http://www.healthilluminationproducts.com/page/neurotransmitters] and Basic Neuron Design (Right)[Source: http://mail.colonial.net/~hkaiter/brainlogics.html]

The basic fundamental units of nervous system are the nerve cells or neurons. Human brain has about 100 billion neurons. Neurons are involved in transmitting information by generating action potential. This function is dependent on the unique structure of neurons. Neurons are anatomically divided into: dendrites, cell body, axon and axon terminals (see figure 3). Dendrites receive information from neighboring neurons; the information travels through cell body and may elicit a new action potential in the axon and axon terminal from where it is again transmitted to other neuron(Purves, et al., 2008).

Synapses are electrical or chemical communicative contacts between neurons. They are of two types; electrical and chemical. Electrical synapses are formed by neuronal gap junctions and communication occurs between neurons through propagation of electrical impulses by direct contact. With its relatively simple organization, the function and molecular structure of these synapses are less likely to change thus showing little plasticity (Fengju & Witzmann, 2007).

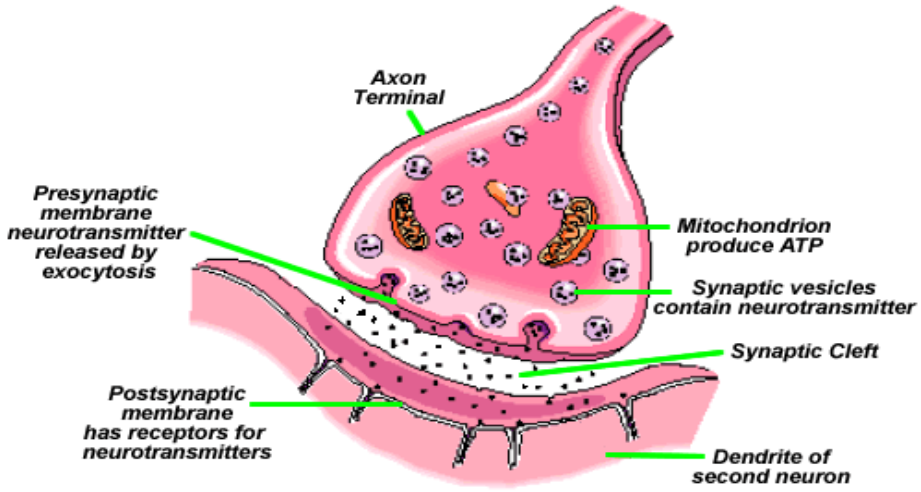


Figure 4: Synapse [Source: http://cognitivephilosophy.net/brain-research/neuroplasticity-in-brief/]

Chemical synapses consists of a wide range of range of chemical neurotransmitters and neuropeptides for interneuronal communication. These synapses also contain localized translational machinery which is coupled directly to different signaling molecules . They are composed of three main constituents: a presynaptic component (presynaptic ending, axon terminal), a synaptic cleft, and a postsynaptic component (dendritic spine). The pre- and the postsynaptic membranes are distinguished by visible densities along their corresponding plasma membranes. The space between pre and post synaptic membranes is called synaptic cleft. Pre and post synaptic membranes together with synaptic cleft form the synapse (Figure 4). The subcellular fraction containing the synapse is called synaptosome. These are the artificial, membranous sacs containing synaptic components that are obtained after subcellular fractionation (Fengju & Witzmann, 2007). Details about synaptosome are discussed in the method section.

1.4) Exercise and Brain

Brain regions that are affected by exercise include, inter alia, the frontal cortex, parietal cortex and temporal cortex. The cellular density in the prefrontal cortex and temporal cortex increases after aerobic exercise. Increase in neuronal density is even more prominent in the hippocampus. Most of the research on exercise and brain is focused on hippocampus (Hooghiemstra, Eggermont, Scheltens, van der Flier, & Scherder, 2012).

The number of scientific articles showing positive effects of exercise on brain is ever growing. Research done in both animals and humans has shown that physical activity improves learning and memory (van Praag, 2009). By increasing physical fitness it also helps people remain psychologically fit. Similarly, it helps in the improvement of mood and decreases anxiety level (Ströhle, 2008). Also, an active lifestyle might prevent or delay loss of cognitive function with aging or neurodegenerative disease (Ahlskog, Geda, Graff-Radford, & Petersen, 2011; van Praag, 2009). Exercise training also helps in improving hind limb movements and helps in bringing the withdrawal reflexes back to normal following spinal cord injury (Ilha et al., 2011). Physical activity and exercise training also changes the plasticity of brain by altering neurogenerative, neuroadaptive, and neuroprotective processes (Dishman, 2006). It encourages vascularization and neurogenesis in brain as well as alters neuronal structure and increases neuronal resistance to injury (Dishman, 2006).

Exercise has a beneficial action on brain function affecting fundamental and broad aspect of brain plasticity (Gomez-Pinilla, 2011). It causes increased synthesis of neurotrophins and growth factors, enhancing neuroplasticity. Its effect on hippocampal function is such that it causes significant improvement in hippocampal function even with advancing age and disease (Intlekofer & Cotman, 2012). The effect of exercise on brain can be broadly classified into peripheral and central effect (Figure 5). While peripheral effect affects the brain indirectly, central effect acts directly on brain.

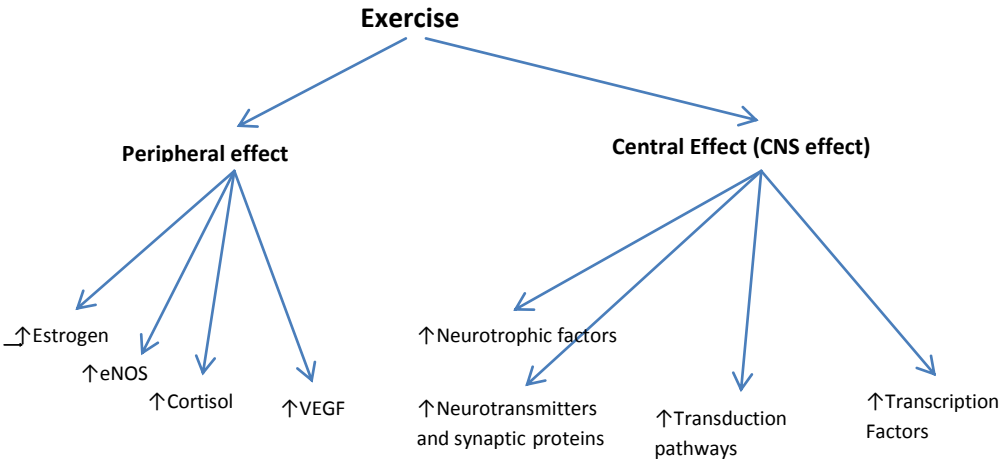


Figure 5: Overview of effect of exercise

Below, I will discuss the effect of exercise under different headings and try to elaborate the mechanisms behind exercise induced brain changes.

1.5) Exercise and Cognition

Cognition is defined as the ability to attend, identify, and plan meaningful responses to external stimuli or internal motivation. It's the collective function of association cortices of parietal, temporal and frontal lobes constituting almost 25% of total human brain tissue. Parietal areas are important for attention and awareness to a stimuli, temporal area helps in the recognition of highly processed sensory information, and frontal regions are involved in guiding complex behavior by planning responses to ongoing stimulation(Purves, et al., 2008).

Different subjective studies have shown that exercise benefits brain. Aerobic exercise, more than high intense exercise is likely to help in the cognitive process (Dik, Deeg, Visser, & Jonker, 2003). A study conducted by Kramer et al. showed that older people doing aerobic training exercise have significant increase in gray matter volume in frontal and superior temporal lobes compared to those without exercise (A. F. Kramer, K. I Erickson, & S. J Colcombe, 2006). Colcombe and colleagues also found out that older people participating in a specific exercise protocol showed increased activity in the frontal and parietal regions of the brain that are important in attention control and performance task (Colcombe et al., 2004). An inverse relationship was found between physical activity and cognitive decline by

Yaffe et al. (Yaffe K, 2001). These subjective studies are supported by objective studies, which measure the peak oxygen consumption. Objective measures of physical fitness study showed the existence of significant inverse relationship between physical activity and cognitive decline (Barnes, Yaffe, Satariano, & Tager, 2003). However, some other studies have not found any relationship between exercise and cognition (Wison et al., 2002; Yamada et al., 2003).

Aerobic training during old age has the dvantge that it improves executive functions such as multi-tasking, planning, and inhibition in which prefrontal cortex is involved (Voss, et al., 2011). Colcombe et al. (2006) noted that exercise modulates cortical capillary blood supply, the number of synaptic connections, and the development of new neurons. They suggested that in comparison to sedentary situation, regular exercise results in greater brain plasticity, efficiency and adaptability. In this way physical activity could play an important role in influencing cognitive brain functions
including learning and memory (A. F. Kramer, K. I. Erickson, & S. J. Colcombe, 2006). This is supported by the metanalysis conducted by Eggermont et al. (2006) which showed that visual memory, working memory, executive control processes, multitasking, cognitive flexibility and information processing were all found to be influenced by exercise (Eggermont, Swaab, Luiten, & Scherder, 2006). Exercise also increases the cognitive performance in neurological diseases like Alzheimer's disease, schizophrenia, cerebral ischemia and metabolic diseases (A. G. K. Ferreira et al., 2011). Following specific diet and exercise routines makes the brain more resistant to damage. It also increases synaptic transmission, cognitive abilities (Gomez-Pinilla, 2011).

Besides human studies, animal studies also support the role of exercise in maintaining cognitive function. Studies done in animals indicate that aerobic training stimulates neurogenesis in the hippocampus (Van Praag, Christie, Sejnowski, & Gage, 1999; Van Praag, Kempermann, & Gage, 1999). It also prevents the decline of hippocampal neurogenesis with normal aging (Kim et al., 2004; van Praag, 2005). Trained rats doing aerobic exercise reached higher levels of acquisition, consolidation, and maintenance of spatial, motor, and procedural memory than controls (Pietrelli, Lopez-Costa, Goñi, Brusco, & Basso, 2012). Doing

exercise on a regular basis helps elderly rats in counteracting detrimental effects of inactivity and helps prevent cognitive decline (Pietrelli, et al., 2012).

Overall, exercise training show very strong effects in improving cognitive performance although much investigation is needed regarding its effect on patients with schizophrenia (SZ) and major depressive disorder (MDD).

1.6) Exercise and Neuropsychiatric disorders

Exercise is beneficial in persons with neuropsychiatric disorders (Tanaka et al., 2009). Inadequate exercise is taken as a risk factor for various neurodegenerative disorders like Alzheimer's disease (Chytrova, Ying, & Gomez-Pinilla, 2010) and psychiatric disorders like depression (Gomez-Pinilla, 2011).

Brain volume measurements by MRI in Alzheimer's disease patients have shown a progressive loss of neurons affecting the temporal lobe in particular. This loss of tissue in Alzheimer's disease can be decreased by regular fitness exercise with high peak VO_2 during exercise (Ahlskog, 2011). Exercise is coming out as a treatment strategy in in Alzheimer's disease as it decreases the neurodegeneration associated with Alzheimer's disease and advancing age and it also improves the function of mitochondria and the immune system (Intlekofer & Cotman, 2012). Most of the research in this field has shown that exercise increases spatial memory and hippocampal function in animal models of Alzheimer's disease (Intlekofer & Cotman, 2012). It also decreases the formation of amyloid plaque in the animal models (Ahlskog, 2011; Intlekofer & Cotman, 2012).

Including exercise as a part of treatment regimen in Parkinson's disease can improve prognosis (Ahlskog, 2011). It improves executive functions i.e. volition, planning, purposive action and effective performance (Tanaka, et al., 2009). It also decreases the anxiety and depression that commonly occurs in Parkinson's disease (Ahlskog, 2011). Exercise also counteracts the cognitive decline in patients with Parkinson's disease by increasing hippocampal volume (Weintraub & Morgan, 2011). Various studies in animal models have shown that exercise reduces the toxic effect of 6-hydroxydopamine and 1-methyl,4-phenyl,1,2,3,6-tetrahydropyridine (MPTP), providing neuroprotection (Ahlskog, 2011).

Exercise may be helpful in major depressive disorder. Morris et al. (1992) and Russkanen and Ruoppila (1995) have shown low incidence of depression in adolescent people and elderly people, respectively, with habit of doing regular physical exercise (Ströhle, 2008). In patients with major depression that are refractory to standard treatment procedures, walking in the treadmill 30 minutes per day for 10 days regularly have shown to reduce the depression level prominently (Dimeo, Bauer, Vahram, Proest, & U., 2001). Exercise training for sixteen weeks was as effective as treatment with antidepressant sertraline in older patients with major depression (Blumenthal et al., 1999). The ability of exercise in reducing depression is probably equal to that of antidepressant drugs (Blumenthal et al., 2007).

Exercise as an acute bout has been shown to reduce panic in patients with panic disorder (Ströhle et al., 2005) and in healthy subjects (Ströhle, 2008). Exercise can also be helpful in persons with Posttraumatic stress disorder (PTSD) (Ströhle, 2008). A 10 to 12weeks program of exercise training in schizophrenic patients is shown to reduce both positive and negative symptoms (Acil, Dogan, & Dogan, 2008; Pajonk et al., 2010) Recently, aerobic exercise is shown to improve short-term memory in schizophrenia by increasing hippocampal volume, and increasing the level of N-acetyl-aspartate in the hippocampus (Wobrock, Hasan, & Falkai, 2010). However, the total evidence supporting the importance of exercise in the treatment of these disorders is still weak (Wolff et al., 2011).

1.7) Mechanisms by which exercise changes the brain

Exercise influences the ability of the neurons to communicate with each other. It is involved in modification of synaptic plasticity. A study of granule cells in the dentate gyrus of the hippocampus has shown that exercised animals have more complex dendritic architecture with longer dendrites and dendritic spines than the control animals. This shows that the exercised animals have a tendency to increased levels of synaptic plasticity (Christie et al., 2008).

Voluntary physical exercise has been shown to change the expression of 94 genes that are involved in hippocampal neuronal plasticity. These genes include ones coding for neurotrophic factors, synapse and signal transduction pathways, neurotransmitter systems and transcription factors. (Molteni, Ying, & Gomez-Pinilla, 2002).

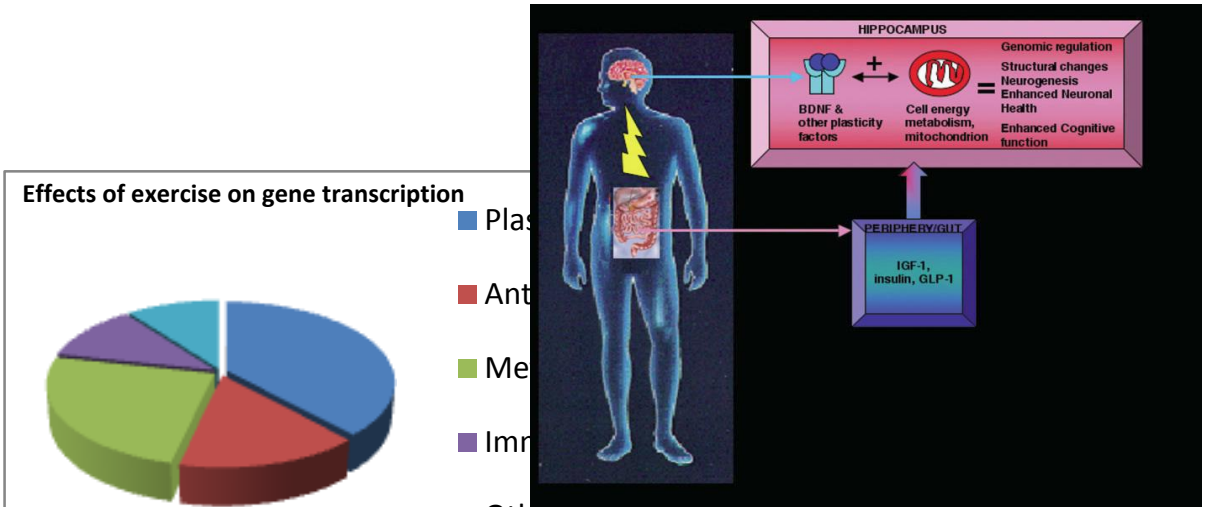


Figure 6: Left side: Effects of exercise in gene transcription (Cotman & Berchtold, 2002). Right side: Body and brain work together to influence neuronal and cognitive health (Shoshanna Vaynman & Gomez-Pinilla, 2006)

Exercise exerts its effect on brain through various changes. These include changes in:

1. Blood vessels
2. Neurotrophins
3. Synapses and signal transduction pathways

4. Neurotransmitters
5. Transcription factors.

1. Changes in Blood vessels:

Cerebral vasodilation and up regulation of endothelial nitric oxide synthase (eNOS) following exercise might play a role in synaptic plasticity. Endothelial nitric oxide synthase (eNOS) is involved in increased expression of brain derived neurotrophic factor (BDNF) in the brain. BDNF plays a key role as a controller of exercise induced synaptic plasticity by modulating the pre-synaptic release of neurotransmitters, postsynaptic phosphorylation of AMPA receptors and controlling the polymerization of actin in the dendritic spines (Christie, et al., 2008).

2. Changes in Neurotrophic Factors:

Different animal experiments have shown a continuous up regulation of BDNF mRNA both in acute (3 days) and chronic exercise (28 days) (Cotman & Berchtold, 2002; Molteni, et al., 2002). The changes in BDNF mRNA level were seen in dentate gyrus, hilus and CA3 regions and the elevation was persistent even after the cessation of exercise (Cotman & Berchtold, 2002). Other areas of brain, like the lumbar spinal cord, cerebellum and cortex also showed increase in BDNF levels (Cotman & Berchtold, 2002). There is also an increase in genes encoding neurotrophic factors nerve growth factor (NGF) and fibroblast like growth factor-2 (FGF-2), although these were just transient and not strong (Molteni, et al., 2002).

BDNF is the one of the most widely studied neurotrophins implicated to physical activity. It is regarded as a crucial factor in exercise induced benefits on learning and memory (Hillman, Erickson, & Kramer, 2008) inducing long term potentiation (LTP) and facilitating synaptic plasticity **(Farmer et** al).Exercise increases BDNF mRNA level in hippocampus thus increasing BDNF level. BDNF induces cellular genesis in dentate gyrus (DG) (van Praag, 2009)

Exercise also up-regulates the expression of different molecular systems involved in cellular energy metabolism that ultimately alters the capacity of synapses. A week of exercise causes an up-regulation of hippocampal mRNA levels of metabolic regulators (AMPK, uMtCK and

UCP-2). UCP-2, located inside the presynaptic and postsynaptic mitochondria is involved in calcium homeostasis, generation of ATP and prevention of oxidative stress. UCP-2 is the point where cellular metabolism and signal transduction cascades interact with each other controlling the capacity of BDNF to regulate CREB and synapsin I after exercise.(Shoshanna Vaynman & Gomez-Pinilla, 2006)

Gomez-Pinilla et al. formulated a hypothesis suggesting that BDNF acts as a metabotrophin in hippocampus connecting neuronal energy metabolism with synaptic plasticity. BDNF activates these metabolic regulators, thus regulating the signal processing capacity of the synapse. How BDNF activates the metabolic regulators is not clear. BDNF receptors (TrkB) present in hippocampal neurons and mitochondria could be responsible for the BDNF dependent activation of metabolic regulators . BDNF activates AMPK by increased phosphrylation of AMPK and AMPK activates BDNF through NF-kappa B dependent mechanism suggesting the occurrence of mutual activation between AMPK and BDNF (Gomez-Pinilla, Vaynman, & Ying, 2008).

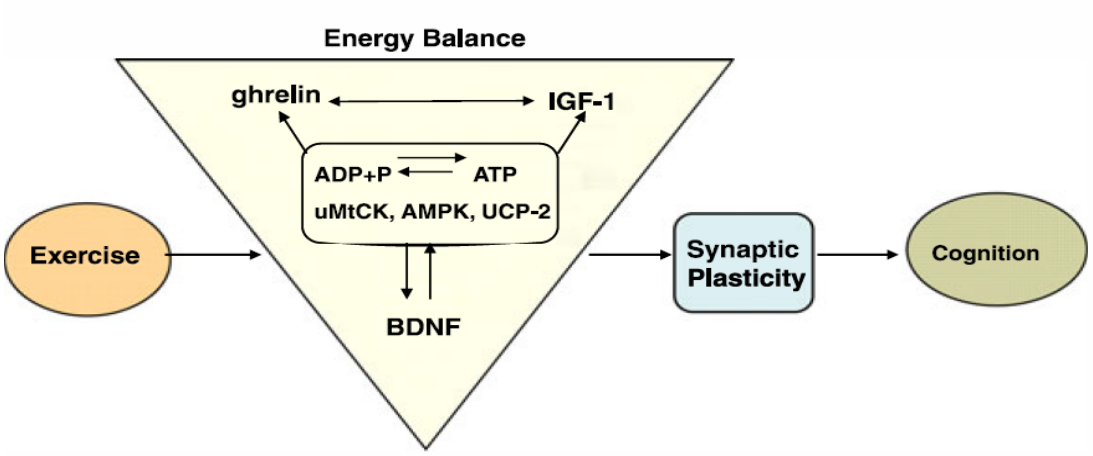


Figure 7: Proposed mechanism by which exercise enhances cognitive function by engaging aspects of cellular energy metabolism (Gomez-Pinilla, et al., 2008)

Lack of BDNF causes synaptic fatigue (Christie, et al., 2008; S. Vaynman, 2005). Synaptic fatigue occurs due to the reduction of synaptic vesicle proteins, namely Synapsin I and Synaptophysin. Synapsin I is a synaptic protein regulated by exercise under the effect of

BDNF (S. Vaynman, 2005). BDNF phosphorylates synapsin I, thus activating the protein (Jovanovic, Czernik, Fienberg, Greengard, & Sihra, 2000). Phosphorylated synapsin I fastens synaptic vesicles to the actin cytoskeleton, thereby forming a reserve pool of synaptic vesicles in synapses. Lack of BDNF reduces vesicular reserve pools of synapsin I, causing impairment in neurotransmitter release (S. Vaynman, 2005). Exercise also activates transcription factor cyclic response element binding protein (CREB). CREB is involved in the regulation of BDNF transcription which itself is regulated by BDNF (Christie, et al., 2008; S. Vaynman, 2005).

Besides BDNF, another neurotrophic factor, Insulin like growth factor-1 (IGF-1) also plays a role in exercise induced brain changes. IGF-1 production increases in both the central and peripheral nervous system following aerobic exercise. IGF-1 is important for proper BDNF function . Without IGF-1, BDNF will not be able to increase the hippocampal BDNF mRNA production and thus BDNF protein.(EECB-12,30) Similarly, synaptic fatigue occurs as the exercise induced increases in Synapsin-I is also blocked (Hillman, et al., 2008).

3.Changes in synapse and signal transduction pathways:

Experiments conducted by Molteni, et al. showed an increase in synapsin I, synaptotagmin and syntaxin after exercise. Exercise upregulated genes coding proteins involved in synaptic function in the hippocampus (Molteni, et al., 2002).

BDNF activates signal transduction cascades (MAP kinase and CAMKII) which in turn activate CREB and synapsin I mediated plasticity. BDNF activates the nerve terminal TrkB receptors causing downstream activation of MAP-K present in synaptosomes (Jovanovic et al1996). Activation of MAP-K in synaptosomes causes phosphorylation of synapsin I at MAP-K dependent phosphorylation sites which subsequently results in the release of neurotransmitters from synapses (Jovanovic, et al., 2000). TrkB receptors are also activated by MAP-K pathways and CaM-K pathways (Segal & Greenberg, 1996). These genes coding the enzymes of intracellular signal pathways which includes MAP-K pathways and CaM-K pathways are up-regulated following exercise. Thus exercise increases genes encoding the

signal transduction system increasing the TrkB receptors making it favorable for BDNF to phosphorylate synapsin via the increased TrkB receptors.

4.Changes in Neurotransmitter systems:

Exercise has shown to up-regulate mRNA level of NR-2A and NR-2B even after 3 days of exercise although the level of NR-2B returns to normal within 7 days (Molteni, et al., 2002). NR-2A receptor plays a crucial role in maintaining synaptic plasticity modulating long term potentiation and depression(Sprengel et al., 1998).BDNF phosphorylates NR-2B subunits thus increasing the chance of opening up of NMDAR ion channels upon glutamate binding (Molteni, et al., 2002).

Exercise also up-regulates the glutamate receptors in brain (Real, Ferreira, Hernandes, Britto, & Pires, 2010) and glutamate transporter EAAC1 in the hippocampus (Molteni, et al., 2002). Increased EAAC1 might act as protective mechanism for the brain against excitotoxicity by removing glutamate from synaptic cleft. Besides the excitatory neurotransmitters, exercise reduces the subunits of inhibitory neurotransmitter GABA receptors in the hippocampus. Also, it reduces the level of the enzyme GAD65 which is required for GABA synthesis. Furthermore, GABA suppresses BDNF expression in the hippocampus. This might lead to an increase of BDNF post exercise (Molteni, et al., 2002). All put together, the upregulation of EAAC1 along with the downregulation of GABA receptor and GAD65 sheds some light on how exercise provides a protective shield to our brain although the detailed mechanism behind the process are yet to be clarified.

5.Changes in Transcription factors:

Exercise induces the expression of the transcription factor CREB (Molteni, et al., 2002). CREB and BDNF are involved in mutual interactions, activating each other. CREB controls the transcription of the BDNF gene, whereas by activating the MAP-K (signal transduction) pathway, BDNF phosphorylates CREB causing its activation and thus CREB mediated transcription of the gene (Molteni, et al., 2002; Shoshanna Vaynman & Gomez-Pinilla, 2006).

To sum up the mechanism, the following picture gives an overview of the effect of exercise at molecular level although it does not cover the detail described above.

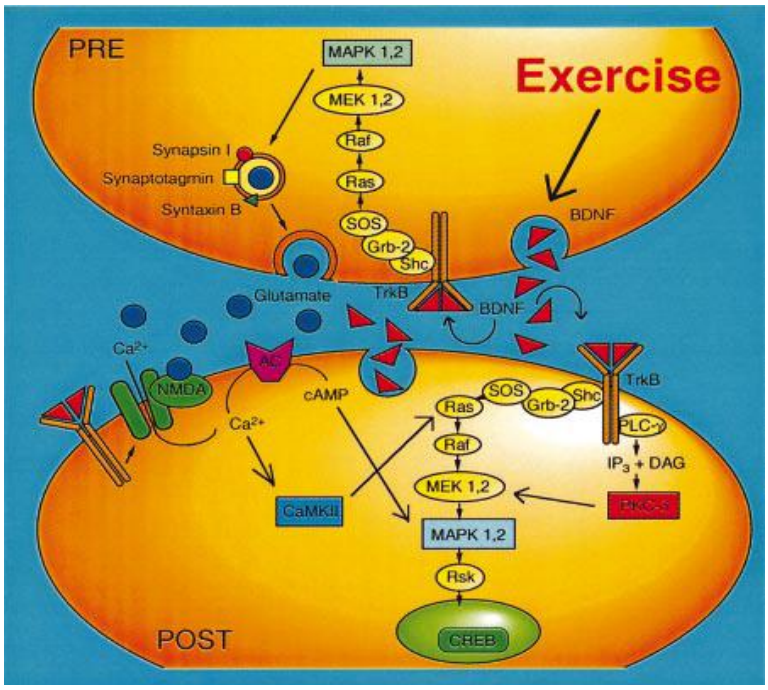


Figure 8: Potential mechanisms by which exercise can modulate neuronal plasticity in the hippocampus (R. Molteni et al.)

BDNF acts through TrkB receptors, which are present at both pre and post synaptic membranes. Exercise increases the level of BDNF along with its receptor TrkB. BDNF binding to TrkB results in the activation of intracellular cascades in both pre and post synaptic membranes (Molteni, et al., 2002) .

At presynaptic membrane, it causes upregulation of several downstream genes, MAP-KI and MAP-KII, PKC-d and CaM-KII. Also it acts on synaptic proteins to modulate the release of neurotransmitter. At the post synaptic membrane, it causes upregulation of several downstream genes MAP-KI and MAP-KII, PKC-d and CaM-KII. It also causes an increase in NMDA receptors in the postsynaptic membrane, causing an influx of calcium ions, activating the MAP-K cascade which activates the transcription factor CREB which itself is upregulatted during exercise (Molteni, et al., 2002).

1.8) Exercise and Synaptic proteins

Exercise increases the synaptic efficiency by the increased expression of molecules involved in learning and memory (Farmer et al., 2004). Synapsin I (SYN) and synaptophysin (SYP) are shown to increase following exercise (S. S. Vaynman, Ying, Yin, & Gomez-Pinilla, 2006). Short term, moderate intensity treadmill exercise causes an alteration of synaptophysin and GluR1 in rat hippocampus indicating its role in hippocampal synaptic plasticity. Moderate exercise can also bring changes in plasticity mediated by different subunits of glutamate receptors like GluR1 and GluR2+3 (Real, et al., 2010)**.** Moreover, exercise also increases the proteins that are specifically related to synaptic plasticity and these includes cytoskeletal proteins alpha internexin and molecular chaperones such as Neuronal protein 22,heat shock protein (HSP)1 and HSP 8 (Ding, Vaynman, Souda, Whitelegge, & Gomez-Pinilla, 2006).

As mentioned above, physical activity has been shown to be one of the few strategies available that may increase growth of neurons and synapses. Physical training may have an impact on cognitive function, and may counteract CNS decline seen in different neuropsychiatric diseases. Little is known, however, of the molecular mechanisms actually taking place to regulate synaptic plasticity during training. Such information would be valuable in order to know what training actually does and what it cannot do, under different conditions. In order to explore training based on synaptic plasticity at molecular level, we have focussed on excitatory synapses. Glutamate is the neurotransmitter in the majority of excitatory synapses. Regulation of these synapses underlies the importance of brain functions such as learning and memory. Thus, we will focus on the change in concentration of glutamate receptor subunits and synaptic proteins in the brains of exercising versus sedentary mice.

The excitatory neurotransmitter glutamate acts through glutamate receptors (GluRs). GluRs act in the postsynaptic membrane through two types of receptors; ionotropic and metabotropic. While metabotropic recptors are involved in the transduction pathways, it is through ion gated channels that the signal transmission occurs (Chua, Kindler, Boyken, & Jahn, 2010). GluRs are very dynamic, as these receptors are continuously inserted and removed from the postsynaptic plasma membranes as part of the processes which are collectively called synaptic plasticity. Ionotropic glutamate channels are further subdivided

into: N-methyl D-aspartic acid(NMDAR), 2-amino3-(5-methyl-3-oxo-1,2-oxzol-4-yl) propanoic acid (AMPAR)s and kainate receptors (KAR)s (Chua, et al., 2010). In my project, priority is given to ionotropic glutamate receptors NMDARs and AMPARs. Furthermore, 2A /2B subunits of NMDAR are studied, and GluR1 and GluR2 subunits of the AMPA receptors are studied. NR2A and NR2B are selected in our study because they are broadly present in the brain, predominate in the postnatal cortex, and are believed to play important role in synaptic plasticity (Yashiro & Philpot, 2008). Also, these subunits are responsible for biophysical and pharmacological properties of NMDARs (Lau & Zukin, 2007). GluR1 and GluR2 are selected as they are directly involved in synaptic transmission. An increase in the synaptic concentration of these proteins would be believed to underlie an increase in synaptic strength (Real, et al., 2010).

Insertion of GluR1 subunits in postsynaptic membrane is associated with long term potentiation (LTP) and it is also needed for the conversion of silent synapses into active synapses. GluR1 homomers are permeable to calcium and are responsible for LTP in a similar manner to NMDAR (Lee & Kirkwood, 2011). GluR2 subunits, present in the majority of AMPARs, is responsible for the calcium impermeability of AMPARs (Lee & Kirkwood, 2011).

I used beta tubulin as a neuronal marker, and synaptophysin and PSD-95 as synaptic marker proteins, being present in the pre and post synaptic terminals, respectively. The concentrations of thee proteins in brain tissue would be assumed to correspond to the density of neurons (beta-tubulin) and synapses (synaptophysin and PSD-95). I have used syntaxin as a core representative of the pre-synaptic vesicle fusion machinery. It is inserted in the active zone and functions as a target SNARE molecule, attaching synaptic vesicles to the presynaptic plasma membrane and facilitating fusion and exocytosis. A change in the synaptic expression of this protein would be assumed to underlie a change in the transmitter release capacity at the synapse.

Synaptophysin is the most abundant synaptic vesicle (SV) protein by mass, accounting for ~10% of total vesicle protein. Each SV harbors ~32 copies of synaptophysin, which is second only to VAMP. As synaptophsin is exclusively localized to SVs, it is widely used as a marker for presynaptic terminals. The role of synaptophysin is closely linked to the finction of the

synapse in general, including exocytosis, synapse formation, biogenesis, and endocytosis of SVs. Rrecent research has shown that synaptophysin is required for the efficient endocytosis of synaptic vesicles in neurons and it is also needed for VAMP recycling during endocytosis(Chua, et al., 2010).

Synaptophysin and syntaxin are seen to explore if there are any changes in these presynaptic proteins after exercise causing the change in plasticity of brain.

Beta tubulin is a microtubule protein and is part of the cytoskeleton. Six of the seven beta tubulin isotypes are expressed in the brain. Class III beta tubulin is largely expressed in testis and brain. In the adult brain, it is expressed exclusively by the neuronal cells (Katsetos, Legido, Perentes, & Mork, 2003). Besides a marker of neuronal protein, class III beta-tubulin is also used as a loading control in our experiment.

Postsynaptic terminal contains a specialized area called the postsynaptic density (PSD). PSD functions, inter alia, in bringing together the glutamate receptors needed for the propagation of signal. Crucual to this function is the protein PSD-95, which is a scaffolding protein present inside the postsynaptic density. PSD-95 through PSD-95 associated proteins interacts with proline rich synapse associated protein (ProSAP). Together they bind to and cluster NMDARs and Kainate-type glutamate receptors (KARs) and potassium channels. PSD-95 also interacts with AMPARS through AMPA receptor regulatory proteins (also called stargazin)(Chua, et al., 2010). So PSD-95 is selected to see if there is any change in the post synaptic density zone which is important for the postsynaptic transfer of signal.

Besides the presynaptic and postsynaptic proteins and glutamate receptors, I have looked upon the changes in Arc (activity regulated cytoskeletal-associated protein). Arc is an immediate early gene (IEG) that moves rapidly to dendrites and accumulates at sites of synaptic activity along with the Arc proteins. Arc protein alters the expression of AMPA type glutamate receptors. Neuronal plasticity in hippocampus crucially depends on Arc-mediated endocytosis of AMPA receptors. Arc is also involved in homeostatic plasticity (Bramham, Worley, Moore, & Guzowski, 2008). The main objective for selecting Arc in our study is to see if there are any changes in synaptic plasticity following training.

1.9)Aims and Hypothesis

The overall objective of this project is to investigate some aspects of physical exercise based synaptic plasticity in the brain. Specifically, my aim has been to document the change in expression of functionally important synaptic proteins in the whole brain as a result of physical exercise. I have used a mouse exercise model that has been used and well characterized for cardiovascular research at NTNU. As mice share almost 90 % of their genes with human beings (University of Oxford), this might shed some light on synaptic plasticity in humans as well**.** I have investigated changes in the synaptic concentration of neuronal and pre- and post-synaptic marker proteins, glutamate receptor subunits, and a homeostatic synaptic plasticity related protein. My method of choice has been quantitative Western blotting.

Overall hypotheses:

1. Synaptic plasticity is induced in the brain upon exercise training.
2. The density of synapses in the brain changes as a result of exercise, as shown by altered concentrations of common synaptic proteins.

Specified hypotheses:

1) Mice undergoing physical training show increased density of synapses in brain tissue, thus showing increased concentrations of the pre-synaptic protein synaptophysin, and the post-synaptic protein PSD-95.
2) Mice undergoing physical training up-regulate post-synaptic glutamate receptors of both the AMPA type and NMDA type. Up-regulation of AMPA receptors underlies increase in synaptic strength, and is involved in both homeostatic and hebbian synaptic plasticity. Up-regulation of NMDA receptors may increase the potential for synaptic plasticity, e.g. long-term potentiation, and is crucial in hebbian forms of synaptic plasticity.

3) Mice undergoing physical training up-regulate the pre-synaptic vesicle fusion protein syntaxin.

4) Mice undergoing physical training up-regulate synaptic plasticity related immediate early gene Arc.

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2) MATERIALS AND METHODS

2.1) Animals

Total 14 mice were used. They were of Cg-m+/+ Leprdb/ BomTac type. The mice came to the lab on 05.01.2011.On arrival, they were 8 weeks old. Among the 14 mice, seven were trained for 8 weeks. The exercise protocol of trained mice is described in "Theory of Material and Method" section.

Table 1 and 2 below gives the information about the mice used in my experiment.

T: Trained group	Fix 4%FA+0,1% GA	Frosset i lqN2	Mus ID	Vekt g	Kjønn
Nr 1	V hemisfære	1 hemisfære	T1-0	24,90	M
Nr 2	V hemisfære	1 hemisfære	T1-1	23,93	M
Nr 3	H hemisfære	1 hemisfære	T1-2		M
Nr 4	H hemisfære	1 hemisfære	T2-0	25,47	M
Nr 5	V hemisfære	1 hemisfære	T2-1	21,96	M
Nr 6	H hemisfære	1 hemisfære	T2-2	25,04	M
Nr 7	V hemisfære	1 hemisfære	T2-3	22,88	M

Table 1: Trained mice

S: Untrained mice	Fix 4%FA+0,1%GA	Frosset	Mus ID	Vekt	M
Nr 1	H hemisfære	1 hemisfære	SED1-1	25,30	M
Nr 2	V hemisfære	1 hemisfære	SED1-2	27,25	M
Nr 3	H hemisfære	1 hemisfære	S1-3	25,49	M
Nr 4	H hemisfære	1 hemisfære	S2-0	25,38	M
Nr 5	V hemisfære	1 hemisfære	S2-1	25,58	M
Nr 6	V hemisfære	1 hemisfære	S2-2	24,18	M
Nr 7	V hemisfære	1 hemisfære	S2-3	24,60	M
Nr 1	H hemisfære	1 hemisfære	SED1-1	25,30	M

Table 2: Untrained mice

2.1.1) Exercise training and testing

Maximal oxygen uptake was measured every second week in the exercise group until the exercise training period is finished in order to assure the relative intensity throughout the exercise period. In the sedentary groups maximal oxygen uptake was only measured prior and after the intervention period. In brief, the mice warmed up for at least 10 minutes at 50-60% of VO2max, whereupon treadmill velocity increased by 0.03 m · s-1 every 1-2 minutes until VO2 plateaued despite of increased workload or the mice is exhausted. The test was performed as uphill (25º) running on a treadmill in a metabolic chamber enabling us to control the air volume and the fraction of O2 and CO2 in and out of the chamber.

High intensity aerobic interval training was performed as uphill running (25º), alternating between 4 min at 85%-90% of VO2max and 2 min at 50% of VO2max for 60 min/day, 5 days/week, for 8 weeks.

The efficacy and relevance of this exercise regime have been demonstrated by both clinical trials and experimental studies in cardiovascular research, e.g.(Tjonna et al., 2008). Interval training normalizes cardiomyocyte function, diastolic Ca2+ control, and SR Ca2+ release synchronicity in a mouse model of diabetic cardiomyopathy (Stolen et al., 2009). However, the relevance of this training regime in neurological research have not been explored.

2.1.2) Dissection

14 mice brains, 7 each from trained and control mice were dissected rapidly (within 3-5 minutes) after decapitation and the brains were immersed immediately in liquid nitrogen. A hemisphere of each mice brain was obtained for our project. Priority was not given to the side of hemisphere and they were taken out randomly. The mice brains were stored at -80^{o}C till the homogenization.

2.2) CRUDE SYNAPTOSOME PREPARATION

Crude synaptosomes were prepared from each mice brain with protocol modified from the procedure described in Gylys et el. (Gylys, Fein, & Cole, 2000)**.** Differential centrifugation was used to obtain crude synaptosomes. Beckmann centrifuge was used and fixed angle rotor was used.

2.2.1) Preparation of 500mL solution of 0,32M sucrose in 4mM HEPES

Pour approx. 250mL of Hepes buffer in a beaker with magnet in it for stirring. Put 54.77grams of sucrose in the beaker under stirring. When sucrose is completelty dissolved, fill up till approx. 400mL with Hepes buffer and check that the pH is correct. i.e. 7.4.

Transfer the buffer to 500mL measuring flask and fill with Hepes buffer til the level reaches the 500mL line. The buffer can be stored for months at -20 ^{o}C.

Add Protease inhibitor to the Homogenization buffer before homogenizing the tissues. Per 10mL of homogenizing buffer , 400µl 25X stock solution is added .(This process can be done just before the start of tissue homogenization)

2.2.2) Homogenization and Fractionation

I. Prepare a 10% homogenisate in 0.32M sucrose in 4mM HEPES ,pH 7.4. Add proteaseinhibitor. For 1g of brain add 10mL of homogenizing buffer and for 10mL of buffer ,add 400 µl of pretease inhibitor.

II. Homogenize the brain tissue in a motor driven glass-teflon homogenizer(overhead stirrer) at ~465rpm(ideally 900rpm): Use special homogenizing tube with pestel made of Teflon and Shaft made of steel. The pestle(piston) should not be loose while its in the tube.

III. Homogenize the tissues with 8 strokes up and down until the tissues are evenly dissolved. Work in cold and use ice box. Be aware that water in the base of the tubes might fall into the buffer causing it to be hypotonic.

IV. Take out ~ 50 µl. Measure the protein concentration from each brain homogenisate.

V. Transfer the homogenisate into the Centrifuge tubes. Wipe out the ice and water present at the bottom of the tube before putting them in the fixed angle rotor (JA-20) of Beckmann centrifuger. Centrifuge at 1000Xg (2900rpm) for 10 minute at 4degrees to remove the pelleted nuclear fraction (P1). The centrifuge tubes and the rotor should all be maintained at 4 degrees. Adjust temperature to 4^{o}C and close the door of centrifuge. The centrifuge stops itself once the temperature reaches 4^{o}C. Then put the centrifuge tubes inside the rotor. Adjust speed, time and temperature (4^{o}C) of rotor and press start. In case of only one tube, put the equal weight of water or buffer in the other tube so as to balance the rotor while spinning.

VI. Take out the Supernatant (S1) with the help of pipette .Try to take as much volume of supernatant as possible but never take out the pellet. Handle gently so that the pellet won't mix with the supernatant.

VII. Spin the Supernatant (S1) at 10,000Xg (9200 rpm) for 15 minutes to yield the crude synaptosomal pellet (P2).

VIII. Re-suspend P2 into 10 volumes of homogenisation buffer and re-spin at 10,000Xg for next 15 minutes to yield what washed crude synaptosomal fraction(P2').

IX. Suspend pellet from step 3 in Homogenization buffer 10 times the volume. Measure the volume of the final solution .Put the solution in the ependorf tube and add SDS immediately(1-2%). Heat the solution at 37 ^{o}C for 30 minutes. Store at -20 ^{o}C.

The crude synaptosomes were stored at -80^{o}C until the western blotting. Some of the samples were used for the measurement of protein concentration.

2.3) PROTEIN CONCENTRATION MEASUREMENT

Protein concentration of crude synaptosomes was measured using pierce BCA protein assay kit. Bicinchoninic acid (BCA) is used for calorimetric detection of total protein in a sample.

Seven dilutions of standard protein samples were prepared from BSA (Bovine serum albumin) as shown in the table below. Note that number 1 dilution contains only distilled water.

	Standard BSA(mg/ml)	µL BSA(2mg/mL)	µLmQH_2O
1	0,000	0	500
2	0,125	32	468
3	0,250	62	438
4	0,500	125	375
5	0,750	188	312
6	1,000	250	250
7	1,500	375	125

Table 3: Standard protein dilutions *(Note that 1 is not a protein standard as it contains only water)*

For protein samples(Crude synptosomes), two dilutions were prepared. The samples were diluted in 0,1M NaOH. Each dilution was replicated 3 times.

10µL of the standards and the test samples were pipetted into the wells of 96 well tissue culture plate by reverse pipetting. BCA reagent A and reagent B were mixed in a ratio of 50:1 and 200µL of the mixture was added into each well. The tissue culture plate was incubated at 37^{o}C for half an hour. After half an hour, the plate was cooled at room temperature for five minutes. Then the plate is exposed to 562nm of light in spectrophotometer. The reading is entered into the excel sheet and the protein concentration calculated. From each sample we have two different readings. The standard error of the mean was less than 10% for each sample.

The total protein concentration of each mice is shown in table in Appendix B.

2.4) WESTERN BLOTTING

2.4.1) Casting Gels

Assemble gel sandwich according to the manufacturer's instructions. Lay the longer spacer-plates down first, then place the shorter glass-plate on top of the spacers. For Mini-gel, be sure that the bottom of both gel plates and spacers are perfectly flush against a flat surface before tightening clamp assembly. A slight misalignment will result in leak.

Align the glass plates and spacers of the gel sandwich in the alignment slot of the casting stand and then snapp the glass plate assembly into one of the casting slots. The rubber gasket provides a leak-proof seal. To be even safer, one can add small amounts of butan-2-ol between the two plates and observe if any leakage occur. If not, then the gel sandwiches are now ready for casting.

Prepare the resolving/separating gel solution in a falcon tube by combining all reagents listed on the table except ammonium per sulfate (APS) and TEMED. The composition of the various reagents depends on the gel-type one is making, i.e. acrylamide percentage. Use low acrylamide composition if the protein of interest has a high molecular weight.

Place a comb of appropriate thickness into the assembled gel sandwich. With a marker pen, place a mark on the glass plate 5 mm below the teeth of the comb. This will be the level to which the separating gel is poured. Remove the comb.

Add APS and TEMED to the solution and carefully pour the solution to the mark, using a glass pipette. Pipette solution so that it descends along a spacer. This minimizes the possibility of air bubbles becoming trapped within the gel.

When appropriate amount of separating gel solution has been added (i.e. to the mark), gently layer about 1-5 mm of butan-2-ol on the top of the separating solution. This keeps the gel surface flat.

Allow the gel to polymerize for 45 minutes to 1 hour. It is a good idea to keep some of the unused/remaining separating gel solution in the falcon tube as it serves as a check for polymerization.

Once polymerized, a distinct interface will appear between the separating gel and the alcohol. Pour off the butanol covering the separating gel. Do not allow alcohols to remain on the gels for more than 1 hour, or the top of the gel will dehydrate.

Prepare the *stacking gel solution*. Combine all reagents, saving APS and TEMED until the end. Once these two are added and the tube mixed, (quickly) pippette the stacking solution into separating gel until solution reachs top of the front plate.

Now carefully insert a comb of appropriate thickness into gel sandwich until bottom of teeth reaches top of the front plate. Be sure no bubbles are trapped on ends of teeth. Tilting the comb at a slight angle is helpful for insertion without trapping air bubbles. Allow the gel to polymerize (about 30 minutes).

After stacking gel has polymerized, remove comb carefully, making sure not to tear the well ears, and place gel into electrophoresis chamber. If the gel is to be used later, comb need not be removed. It can be wrapped in plastic (with the comb intact) for few days at 4^0 C.

2.4.2) Gel electrophoresis

After measuring the protein concentration, the samples of western blotting were prepared.

Prepare the samples by mixing the sample with *loading buffer* and ddH_2O in correct amounts. Vortex regularly. Heat the samples to 37^0 C for 20-30 minutes (for resolubilization of the precipitated SDS).

Assemble the casted gel into the eletrophoresis chamber and fill the chamber with 1X *Electrophoresis buffer* (also called *running buffer*). If the buffer is stored in a stock solution (say 10X), it should be diluted 10 times in ddH_2O. A buffer dam has to be made independent of the nr of gels that are to be run.

The heated sample solutions are now ready for loading. Before loading can begin, the comb has to be removed. Now, introduce samples into well using a disposable gel loading tip. Layer protein solution on bottom of well and raise syringe tip as dye level rises. The first well is usually preserved for a molecular weight standard. Use 5 µl of the standard. For rest of the wells, the volume that is to be loaded depends on the concentration of samples.

Minimum protein loading per well: 0.1 µg

Maximum protein loading per well: 20-40 µg

To avoid edge effects, add 1X loading buffer to unused wells.

Once loaded, the gel is ready to run. Attach electrode plugs to proper electrodes and place the lid on top of the chamber to fully enclose the cell. Current should flow towards the anode.

Run the gels at constant 150 V for 75 min if they are homemade.

For precast gels, use 200V for 50 minutes.

Never disconnect electrodes before first turning off the power source. After electrophoresis, gels may stand for a few hours before staining without harm except for gels with low percentage acrylamide in which protein will start to diffuse. Thus, once the electrophoresis is finished, first turn off the power supply. Remove the electrode plugs from electrodes. Pour off the buffer. Remove gel plates from electrode assembly. The gels are now ready for blotting.

After use, rinse the electrophoresis chamber and the clamps with distilled water. For glass plates, spacers and combs was with a laboratory detergent, then rinse thoroughly with distilled water.

2.4.3) Blotting

Prepare the Blotting/transfer buffer: 350 mL ddH_2O, 50 mL 10X *Blotting buffer*, and 100mL methanol. Assemble the gel and membrane sandwich in the transfer apparatus in correct orientation to ensure the transfer of proteins to the membrane. From bottom to top, the sandwich should consist of: filter paper, PDVF membrane, gel, filter paper:

- Soak a thick filter paper in blotting buffer (since it comes into direct contact with the anode). Activate the PVDF membrane by submerging it in 100% methanol (10 seconds) and then in the blotting buffer (30 seconds). Lay the soaked-filter paper with the membrane (anode stack) in

the centre of the cassette base. Ensure that the stack is not overlapping the green rubber molding in the base.

- Carefully align the gel on the membrane. [When taking out the gel do the following: use a gel releaser to carefully remove a spacer from the gel sandwich; insert the releaser in one corner between the plates, and gently pry apart the gel plates. The gel will stick to one of the plates.] If necessary, gently use the blot roller to remove air bubbles between the gel and membrane. If transferring two mini gels, place them on the membrane so that the feet of the gels are facitng toward each other.

- Soak the other filter paper in the buffer and gently place in on the gel. Use the blot roller to remove any air bubbles in the assembled transfer pack and provide consistent contact between the layers.

- Once the pack is assembled, place the cassette lid (cathode). Press the lid down firmly and turn the dial clockwise to engage the lid pins into the locking slots.

Slide the cassette (with the dialing facing up) into one of the Trans-Blot Turbo instrument bays until it makes contact with the magnetic interlock in the back of the instrument tub and you hear a click. The cassette can be inserted into the bays with or without power to the system. One or both cassettes can be used for a blotting run. If both cassettes are run, they must use the same protocol and have the appropriate combination of gels. The cassettes can be run individually or simultaneously with independent start times as long as the same protocol is being used for both.

Once the cassette(s) is/are mounted, turn on the Trans-blot apparatus. If two mini format gels are being run per cassette, use the protocol that is preprogrammed in the machine - i.e., 25 V for 30 minutes. This protocol can be accessed from the home screen: LIST > BIORAD > 2-MINI GELS > STANDARD SD. Press the Navigation button that corresponds to A: Run for the cassette in upper bay or B:Run for the cassette in the lower bay. The protocol will run automatically.

2.4.4) Blocking, washing and antibody incubation

While the transfer protocol is running, prepare 5% blocking solution made of skim milk powder in TBS-T buffer. [Unoccupied binding sites on the membranes must be blocked to prevent nonspecific binding of probes; failure to completely block these sites can lead to high background]. Depending on the number of membranes one is using, 200 mL solution (with 10 g milk) should be sufficient.

Once the transfer protocol is complete, remove the cassette from the transblot by pulling it straight out of the instrument. Unlock the cassette by turning the dial counterclockwise to the Unlock position. Disassemble the blotting sandwich and place the membrane in a suitable container with the blocking solution. [If a PVDF membrane is being used, place it immediately into a storage solution (either ddH_2O, blocking solution, or staining solution) as the membrane will quickly dry out. If a PVDF memrbane requires rewetting, dip it in methanol or ethanol until uniformly opaque, then wash with deionized water.

The container(s) containing the membranes are shaken for ½-1 hour in a shaker.

Discard the filter paper and the gel after one use. Empty residual liquid from the blotting cassette. If no additional transfer will be performed immediately, rinse the base and lid of the cassette with deionized water and dry them with a paper towel. Turn off the Trans-Blot Turbo system with the power switch if it is no longer required.

Prepare primary antibody solution (2.5 % skim milk in TBS-T) in a falcon tube (~5 mL). Once blocking is finished, carry out three quick washes with TBS-T buffer. Cut the membrane if necessary, and transfer them into antibody-containing tubes. Incubate the primary anitbody overnight on a rotator (room temperature).

Next day, transfer the membranes to small containers/boxes and perform three quick washes (just to get rid of any remaining blocking solution). Then wash 4 X 15 minutes with TBS-T buffer on a horizontal shaker (120 rpm). Change the buffer in between the washes. [Washing the blots prior to detection removes excess antibody and prevents nonspecific binding.]

Prepare secondary antibody solution in falcon tubes (1.25% skim milk in TBS-T). Once the washing is finished, incubate the membranes with secondary antibody for 1 hour on a rotator.

Wash the membranes 4 X 15 minutes again with TBS-T buffer.

Incubate the washed membranes with ECF substrate in dark for about 3 minutes. Use about 1000 µl per membrane. Put the membranes in between a plastic sheet and into a hypercassette. Take for scanning.

2.4.5) Scanning

Scanning was done in typhoon scanner in microbiology department of Rikshospitalet. The table below shows the settings of the scanner during the scanning of western blots

f-stop	2,75
Focal plane	Platen or zero
Field of vision (fov)	103,75
Exposure time	Different antibodies were exposed for differer
Number of exposures	1
Excitation wavelength	460nm
Emission wavelength	530nm
Illumination source	Multi-wavelength
Pixels	X-binning:4pixels Y-binning:4pixels
Image settings	Image maximum: 99,5%-99,9% Image minimum: zero Gamma: 1

Table 4: Settings of typhoon scanner

2.5) QUANTITATION AND STATISTICAL ANALYSIS

Quantitation of the western blots was done using Photoshop. The scanned membranes were opened in adobe Photoshop CS 5.1. The blots were inverted and zoomed in to enlarge. With a rectangular box, the mean value of each band were measured. Then the mean value along with the pixel were entered in excel sheet The intensity of each band were calculated by dividing the product of its mean and pixel by 1000. The background of the scanned blots was also calculated in similar way. Then the intensities of bands were corrected by subtracting their intensities from the respective background.

The corrected band intensites from sedentary and trained mice were entered in excel sheet and the data were analysed and tested for the significance.

2.6) LOADING CONTROLS IN WESTERN BLOTS

Three different methods were employed as loading control in western blotting. These are:

1) Loading of each sample in three different lanes. The minimum criteria was the intensity of any two of the values obtained should be less than 10% of standard error of mean.

2) Two to three control curves/lines were obtained for each antibody . Loading control curve is made by plotting band intensity in y-axis against protein concentration in y-axis. Different amount of proteins were loaded in 2-3 wells of randomly selected 2-3 gels in an experiment. The bands obtained from these wells are quantitated and a curve is made. Any experiments with downward going loading control curve were excluded and the experiments were repeated again.

3) The bands of each of the antibodies were correlated with that of B-tubulin, a neuronal marker protein.

2.7)ANTIBODIES

Antibodies used are:

- **GluR1**: Anti-glutamate receptor 1(AMPA subtype) antibody, Abcam(ab31232) , Lot :GR56603-1
- **GluR2**: Anti-glutamate receptor 2 antibody(extracellular), Alomone labs(AGC-005), Lot:AN-03
- **$NMDAR_{2A/2B}$**: Rbbit anti-NMDA receptor 2A/B affinity purified polyclonal antibody, Millipore(AB1548), Lot: LV179226
- **Synaptophysin**: Mouse monoclonal to Synaptophysin ,Abcam(ab18008), Lot:GR49452
- **Syntaxin**: Anti-Syntaxin 1, Alomone labs (ANR-002), Lot: AN-04, Polyclonal
- **PSD-95**: Mouse monoclonal to PSD95, Abcam(ab13552), Lot: GR42550-2
- **Beta-tubulin**: Class III β-Tubulin(TUJ1) monoclonal antibody, Purified, Covance(MMS-435P),Lot: E10082CF
- **Arc**: Arc(H-300): sc-15325, Polyclonal, Santa Cruz Biotechnology , Inc., Lot :G2110

The table below shows the antibody concentration, amount of protein and concentration of gels used in western blotting**:**

Antibody	Dilution of antibody	Molecular weight(KDa)	%gel used	Proteins loaded in lane(µg)
GluR1	1:1200	100	12	20
GluR2	1:3000	99	12	10
$NMDAR_{2A/2B}$	1:1350	180	12	10
Synaptophysin	1:20000	38	7,5	5
Syntaxin	1:30000	35	7,5	5
PSD-95	1:6000	95	12	10
Beta-tubulin	1:8000	50	7,5	5
Arc	1:1000	55	12	10

Table 5

2.8) OVERVIEW OF EXPERIMENT

I prepared crude synaptosomes from7 trained mice and 7 sedentery mice by using the crude synaptosome preparation protocol described above. Protein concentration of each of the crude synaptosomes were measured by using BCA assay kit. Then the crude synaptosomes were stored at -75^{0}C till the experiment day.

For quantitative western blotting, I used 3 wells of the gel for each brain per antibody. The loaded proteins were separated by gel electrophoresis and the proteins from the gels were transferred into PVC membrane by semi-dry blotting. The blots were incubated with primary and secondary antibody and the bands were visualized by fluorescent detection method.

Intensities of the bands were calculated using photoshop and were tested for significance of results.

Three different methods of loading controls were used in the experiment as described above.

3) BACKGROUND OF MATERIALS AND METHODS

3.1) Exercise training

To determine maximal oxygen uptake the mice will run on a customized treadmill in a metabolic chamber where the volume and percentage of O2 and CO2 is continuously measured. (This is performed at the start and at the end of the intervention period for the sedentary groups and every week in the exercise groups.) On the back of the treadmill there is an electrical grid, giving electrical pulses of 0,2mA. This leads to distaste but not much pain. The electrical grid is used during testing, during the first exercise session and just occasionally during the rest of the exercise period. Normally they learn quickly not to touch the electrical grid and leads to controlled exercise intensity which is crucial to our experiments. No mice will stand on the grid longer than about 3 seconds before the electricity is turn off. Usually, during exercise the power on the electrical grid is turned off and a brush is used to tickle the feet on the animals if they stand on the grid. If the mouse touches the electrical grid 3 times for more than 3 seconds within 30 seconds, the mice will be taken out of the study. This method have been used several times for last 9 years in cardiovascular research and all have been approved by FDU.

3.2) BCA Protein Assay

The BCA Protein Assay combines reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. The first step is the chelation of copper with protein in an alkaline environment to form a light blue complex. In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate.

In the second step of the color development reaction, bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-colored reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm

with increasing protein concentrations. The BCA reagent is approximately 100 times more sensitive (lower limit of detection) than the pale blue color of the first reaction.

The reaction that leads to BCA color formation is strongly influenced by four amino acid residues (cysteine or cystine, tyrosine, and tryptophan) in the amino acid sequence of the protein. However, unlike the Coomassie dye-binding methods, the universal peptide backbone also contributes to color formation, helping to minimize variability caused by protein compositional differences. (Source:Pierce protocol)

3.3) Crude Synaptosomes

Synaptosomes are membranous sacs containing presynaptic membrane, synaptic cleft and postsynaptic membranes. They are artificial sacs and are generated by subcellular fractionation of homegenized brain tissue. Once the synaptosome is teared off from the axon terminal, the lipid bilayers naturally seal off creating the synaptosomal sac. Crude synaptosomes contains myelin and mitochondria as well whereas pure synaptosomes contains only synaptosomes. As all the molecules that is involved in the release, uptake and storage of neurotransmitter are intact in synaptosome, they are used for the study of synapse and synaptic transmission.(Fengju & Witzmann, 2007)

The purity of synaptosomes can be assessed by using electron microscopy where a typical morphology ocan be visualized or it can also be assessed biochemically using enzymatic markers.(Fengju & Witzmann, 2007)

3.4) Centrifugation

Centrifugation is one of the widely applied research techniques in biochemistry, cellular and molecular biology, and in medicine.

Centrifuge machine uses centrifugal force(also called g-force) to separate the particles from their surrounding medium .Centrifugal force causes the particles in a suspension to move away from its axis of rotation. The force on the individual particle is called relative centrigugal force. RCF of 200g means that the centrifugal force applied is 200times greater than the gravitational force.It is this RCF that causes the isolation of different particles from the suspension. Thus centrifuge uses centrifugal force that magnifies the gravitational power in the particles causing them to separate.

There are two types ofCentrifugation:- Differential: separation based on the size of the particles and density gradient: separation based on the density gradients creatd through various density gradient medium like sugars, polysaccharides.*(http://www.coleparmer.com/techlibraryarticle/30)*

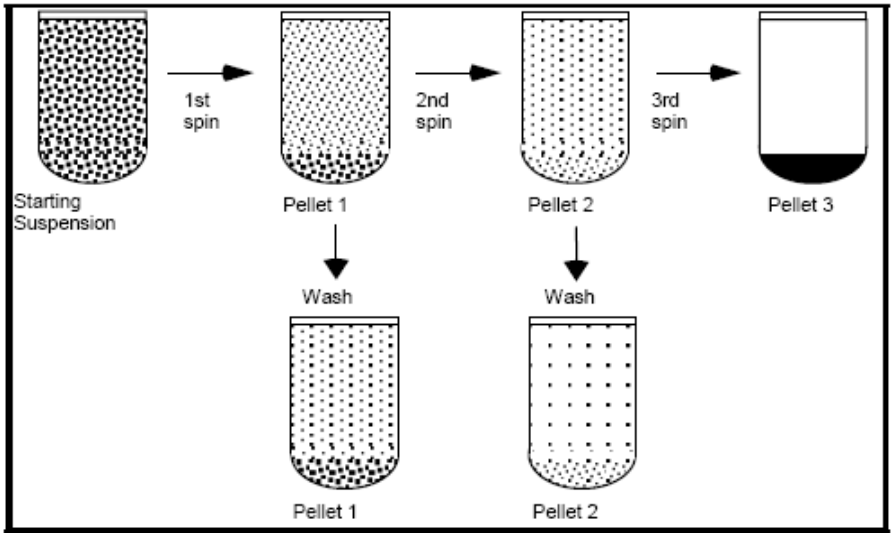


Figure 9:Differential Centrifugation (source*:* *http://www.coleparmer.com/techlibraryarticle/30*)

To process the sample in centrifuge,rotors are used.There are three different types of rotor. Among them, fixed angle rotor is the most widely used rotor for pelleting of samples. The

tubes containing the samples are placed in the chambers and rotated at a constant angle giving a very effective pelleting. *(http://www.coleparmer.com/techlibraryarticle/30)*

4) RESULTS

Western blots were performed with antibodies against GluR1, GluR2, Synaptophsyin, Syntaxin 1A, class III beta-tubulin, PSD-95, $NMDAR_{2A/2B}$ and Arc. The band intensities were quantitated using the software Adobe Photoshop CS 5.1. Mean intensities of trained mice and untrained mice were calculated along with standard deviation and standard error of the mean. I have graphically presented my data with histograms. For each antibody, I made one histogram comparing trained and sedentary mice. Mean intensities of trained and sedentary mice were used for making histogram. Standard error of mean was used for making the error bar in the histogram.

I used two-tailed t-test to see the statistical significance of my results with alpha 0, 05. The calculated p-values are highlighted in red color in the tables below.

The results showed significant difference in GluR1, $NMDAR_{2A/2B}$, syntaxin, and Arc between trained and sedentary mice. Significant differences were not seen in GluR2, PSD-95, synaptophysin and beta-tubulin.

For the results section, for each antibody, I have included a loading control curve, histogram, one scanned blot, and t-test table.
I prepared loading control curve by plotting intensities of loading control bands along the y-axis and the amount of protein loaded along the x-axis. For each experiment, 2-3 gels were selected randomly for loading controls. This curve should be moving upward with increase in protein concentration. Any experiment with downward curve was repeated again. Although, I have mostly loaded three different amounts of proteins in three different wells as loading control, in some cases I have only used two different masses of proteins in two different lanes. For gels with two controls, I have made a loading control line and compared it with the expected loading control line.

For the convenience, p-values of each antibody are shown below in table:

Antibody	p-value
GluR1	0,049
GluR2	0,81
$NMDAR_{2A/2B}$	0,041
Synaptophysin	0,33
Syntaxin	0,01
PSD-95	0,96
Class III beta-tubulin	0,34
Arc	0,02

Table 6

The results for each antibody are shown below.

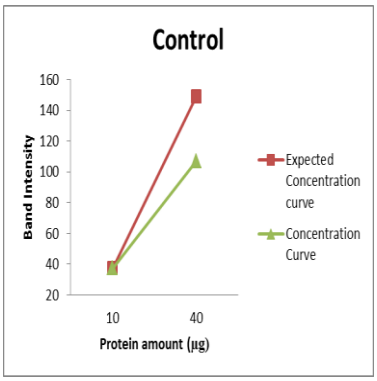
4.1 AMPA Receptors

I found an increase in the GluR1 AMPA receptor (p-value=0,049) while the GluR2 level was not changed significantly (p-value=0,81). The results for GluR1 and GluR2 are shown in Figure 1 and 2 respectively. The loading control curves, bar diagram, and statistical analysis are shown in figures 10 and 11 respectively.

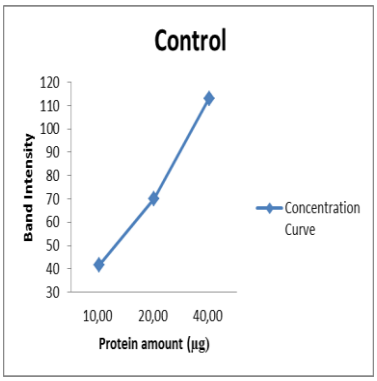
The GluR2 blots additionally shows two different bands at around 75 KDa and 50 KDa. This might be either due to cross reactivity or posttranslational modification or proteolysis of the receptors inside the samples. However, the intensities of both extra bands are proportional to their corresponding intensities of GluR 2 bands, indicating that they are the results of proteolysis of the real receptor protein.

4.1.1) GluR1:

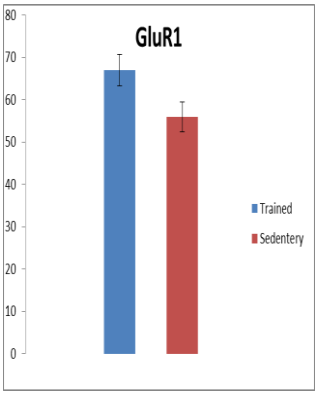
A



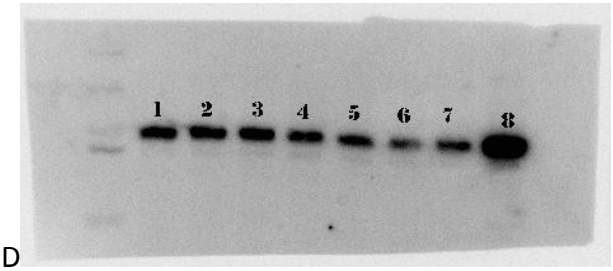
B



C



D



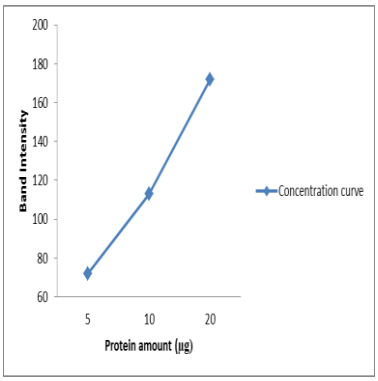
t-Test: : Two-Sample Assuming Unequal Variances		
	Variable 1	*Variable 2*
Mean	66,94714286	55,897
Variance	84,97459048	94,99877
Observations	7	7
Hypothesized Mean Difference	0	
df	12	
t Stat	2,17927881	
P(T<=t) one-tail	0,024979149	
t Critical one-tail	1,782287556	
P(T<=t two-tail	**0,049958299**	
t Critical two-tail	2,17881283	

E

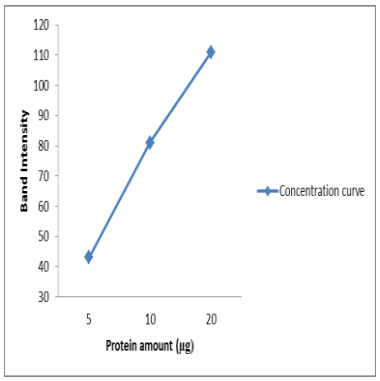
Figure 10: A, B) Loading control curve , concentration of loaded protein is shown in x-axis and band intensity on y-axis C) Histogram D) Western Blot : 1, 2, 3 are bands from trained mice ; 4, 5, 6 are bands from sedentary mice and 7, 8 are control bands E) t-test

4.1.2) GluR2:

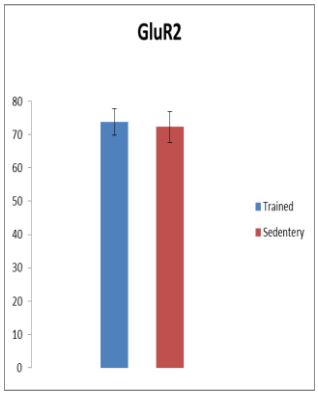
A



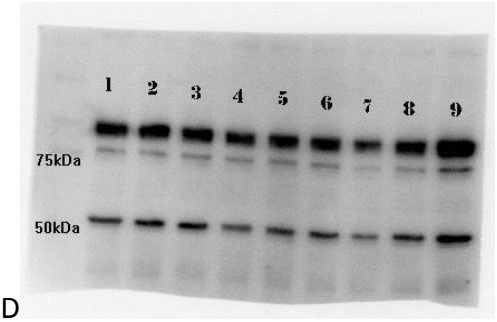
B



C



D



t-Test: Two-Sample Assuming Unequal Variances		
	Variable 1	*Variable 2*
Mean	73,75785714	72,32428571
Variance	109,0159668	153,4250952
Observations	7	7
Hypothesized Mean Difference	0	
df	12	
t Stat	0,234127519	
P(T<=t) one-tail	0,4094164	
t Critical one-tail	1,782287556	
P(T<=t) two-tail	**0,8188328**	
t Critical two-tail	2,17881283	

E

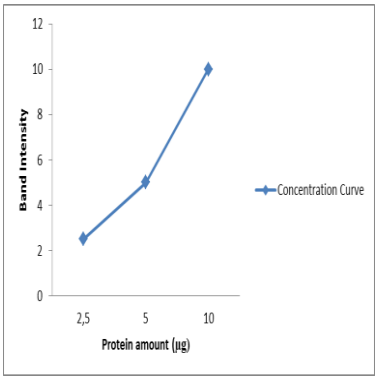
Figure 11: A, B) Loading control curve C) Histogram D) Western Blot 1, 2, 3 are bands from trained mice ; 4, 5, 6 are bands from sedentary mice and 7, 8, 9 are control bands E) t-test

4.2) NMDAR$_{2A/2B}$

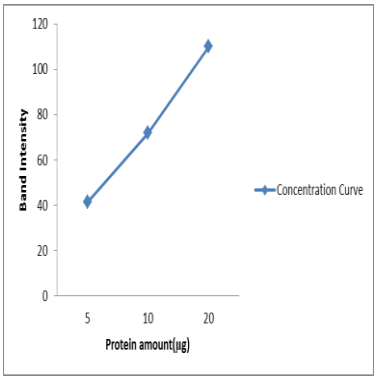
I performed western blotting against 2A/2B subunits of the NMDA receptor. As mentioned earlier, the bands I got, did not correspond to the expected molecular weight of 165 KDa. Other colleagues in my lab have also got similar results with different brain samples. The low molecular weight in my experiment might be due to proteolysis of samples. It might possibly be due to incomplete inhibition of metalloproteinase, which are involved in the proteolysis of NR1 and NR2 subunits (Szklarczyk et al., 2008). However, following proteolysis, we should at least be able to see two to three different bands at different molecular weights, which is not the case in my experiment. I am including this finding in my report on the basis of use of specific NMDAR$_{2A/2B}$ antibody.

My result showed a significant rise in 2A/2B subunits of NMDA receptors (p-value=0,04). The results with NMDAR is shown in figure 12.

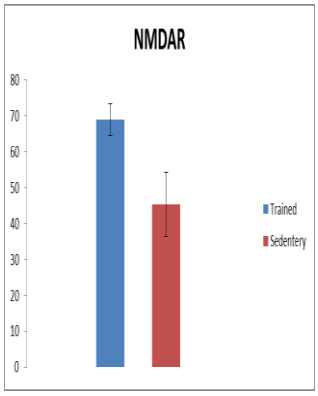
A



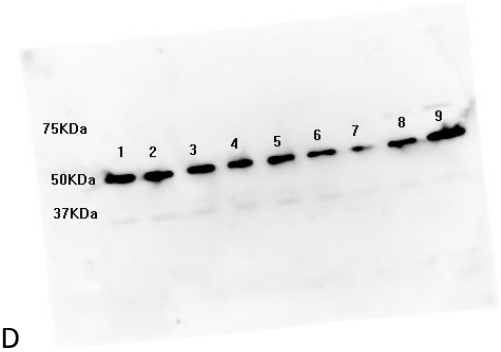
B



C



D



t-Test: Two-Sample Assuming Unequal Variances		
	Variable 1	*Variable 2*
Mean	68,89745714	45,37891429
Variance	134,2448341	552,6445315
Observations	7	7
Hypothesized Mean Difference	0	
df	9	
t Stat	2,374193077	
P(T<=t) one-tail	0,020810977	
t Critical one-tail	1,833112933	
P(T<=t) two-tail	**0,041621954**	
t Critical two-tail	2,262157163	

E

Figure 12: A, B) Loading control curve C) Histogram D) Western Blot: 1, 2, 3 are bands from trained mice ; 4, 5, 6 are bands from sedentary mice and 7, 8, 9 are control bands E) t-test

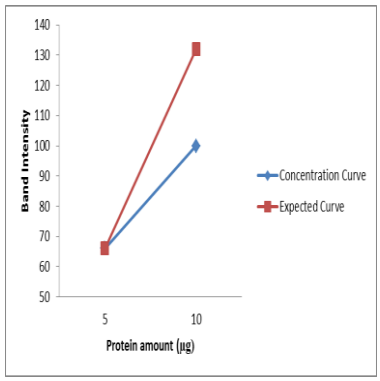
4.3) Neuronal and synaptic marker proteins

Neuronal and synaptic marker proteins:

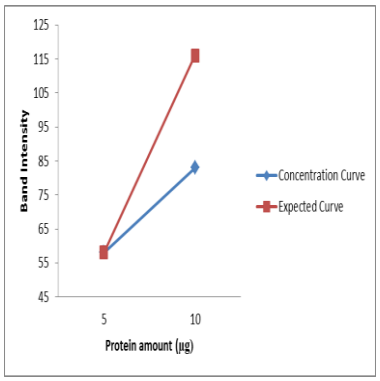
I found a significant rise in syntaxin after exercise (p-value=0,015). But there was no significant change in other synaptic proteins synaptophysin (p-value=0,33) and PSD-95 (p-value=0,96). Significant change was not found either with beta-tubulin (p-value=0,34), which I have used here as a loading control and a neuronal marker protein.

The blots of syntaxin showed two bands. (figure 14 D; lanes 4,5,6).It might be due to more rapid stacking of some proteins. I have included both bands for quantitation as there is no distinct separation of bands in some lanes (Lanes 1,2,3 of figure 14 D). PSD-95 blots showed streaking in the blots (figure 15 D). It might be due to increased protein loading. So, I decreased the protein concentration. But there was still streaking in the blots. It might also be due to antibody, as my colleagues using different PSD-95 antibody did not get streaking. I quantitated the blots as the streaking was uniform across all lanes. The most prominent band present at the end of streak correspond to the weight of PSD-95.

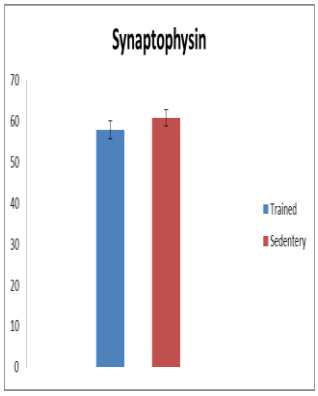
4.3.1) Synaptophysin



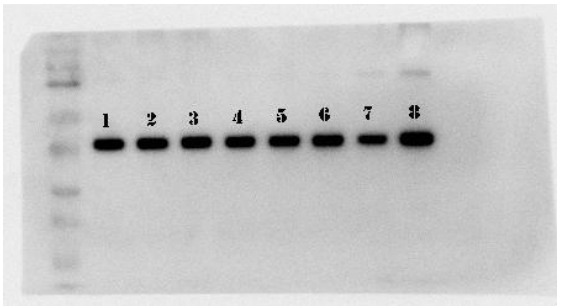
A



B



C



D

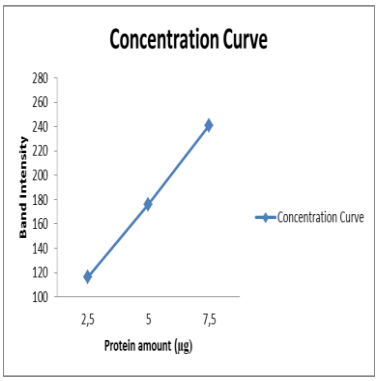
t-Test: Two-Sample Assuming Unequal Variances		
	Variable 1	*Variable 2*
Mean	57,83142857	60,73
Variance	31,18998095	27,41023333
Observations	7	7
Hypothesized Mean Difference	0	
df	12	
t Stat	-1,001805531	
P(T<=t) one-tail	0,168105608	
t Critical one-tail	1,782287556	
P(T<=t) two-tail	**0,336211217**	
t Critical two-tail	2,17881283	

E

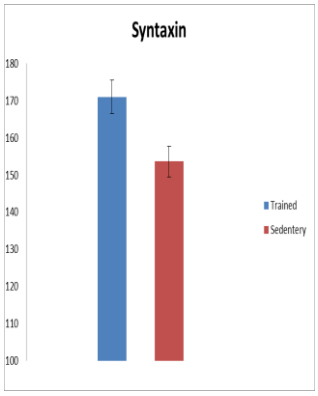
Figure 13: A, B) Loading control curve C) Histogram D) Western Blot: 1, 2, 3 are bands from trained mice ; 4, 5, 6 are bands from sedentary mice and 7, 8 are control bands E) t-test

4.3.2) Syntaxin

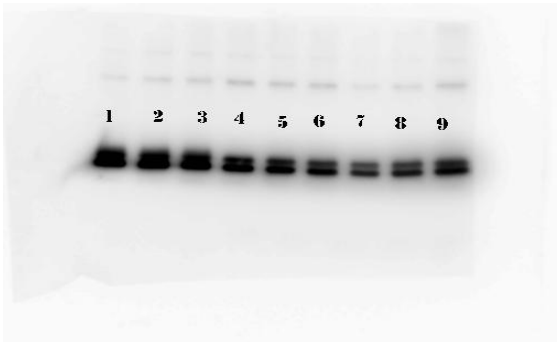
A



B



C



t-Test: Two-Sample Assuming Unequal Variances		
	Variable 1	*Variable 2*
Mean	175,515	157,7985714
Variance	121,54363	123,8430476
Observations	6	7
Hypothesized Mean Difference	0	
df	11	
t Stat	2,87590962	
P(T<=t) one-tail	0,007540619	
t Critical one-tail	1,795884819	
P(T<=t) two-tail	**0,015081238**	
t Critical two-tail	2,20098516	

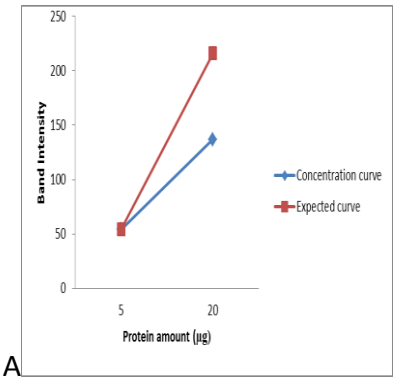
D

D

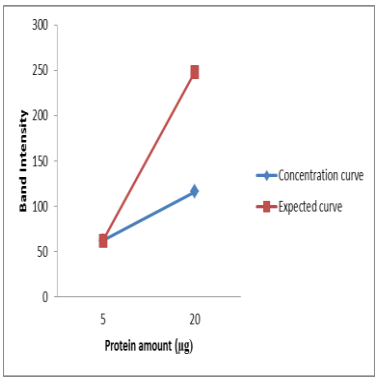
Figure 14: A) Loading control curve B) Histogram C) Western Blot: 1, 2, 3 are bands from trained mice ; 4, 5, 6 are bands from sedentary mice and 7, 8, 9 are control bands D) t-test

4.3.3) PSD-95

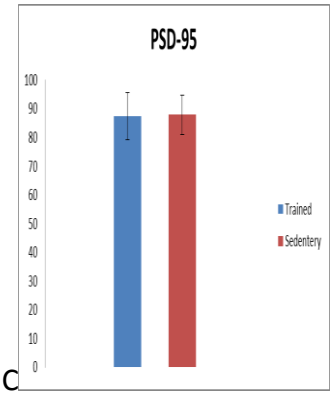
A



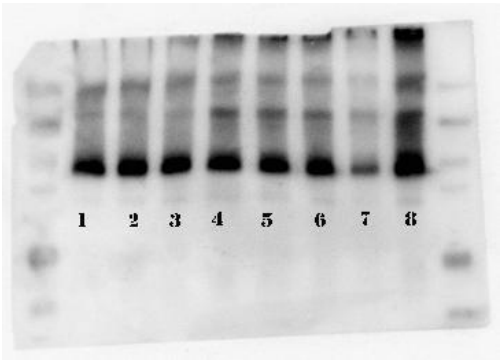
B



C



D

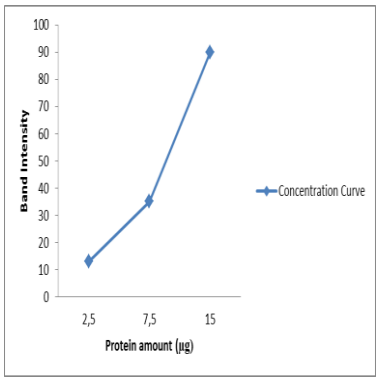


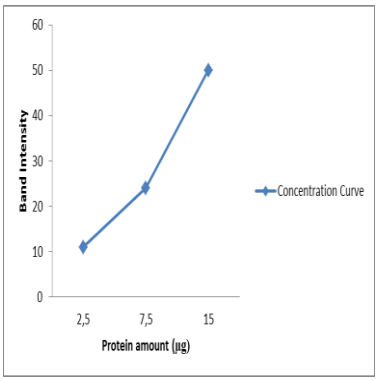
t-Test: Two-Sample Assuming Unequal Variances		
	Variable 1	*Variable 2*
Mean	87,36774286	87,83265464
Variance	457,1763877	331,9516312
Observations	7	7
Hypothesized Mean Difference	0	
df	12	
t Stat	-0,043787066	
P(T<=t) one-tail	0,482897162	
t Critical one-tail	1,782287556	
P(T<=t) two-tail	**0,965794324**	
t Critical two-tail	2,17881283	

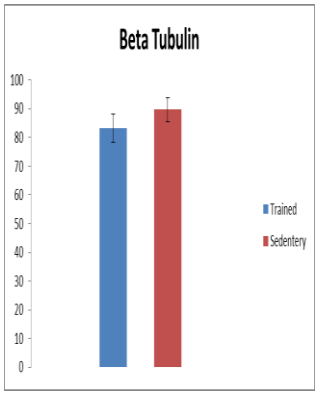
E

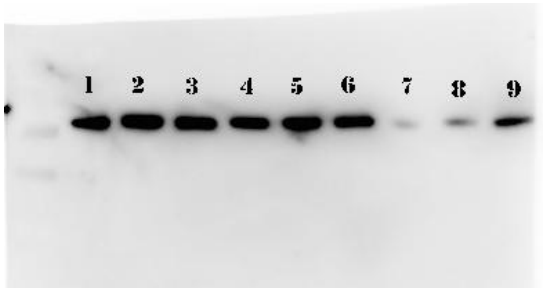
Figure 15: A, B) Loading control curve C) Histogram D) Western Blot: 1, 2, 3 are bands from trained mice ; 4, 5, 6 are bands from sedentary mice and 7, 8 are control bands E) t-test

4.3.4) Beta- tubulin

A 

B 

C 

D 

t-Test: Two-Sample Assuming Unequal Variances		
	Variable 1	*Variable 2*
Mean	83,16	89,65428571
Variance	182,0650667	116,7697619
Observations	7	7
Hypothesized Mean Difference	0	
df	11	
t Stat	-0,993950612	
P(T<=t) one-tail	0,170804278	
t Critical one-tail	1,795884819	
P(T<=t) two-tail	**0,341608556**	
t Critical two-tail	2,20098516	

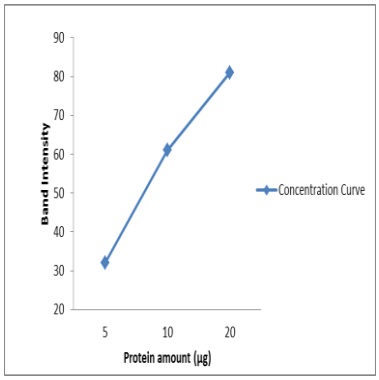
E

Figure 16: A, B) Loading control curve C) Histogram D) Western Blot: 1, 2, 3 are bands from trained mice ; 4, 5, 6 are bands from sedentary mice and 7, 8 are control bands E) t-test of class III beta-tubulin

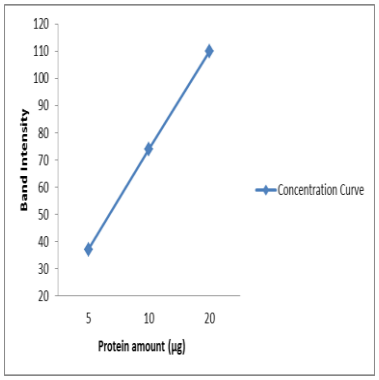
4.8) Arc

Arc was significantly lowered in trained mice (p-value=0,018). It was a difficult antibody to work with. The blots had lots of background and there were presence of overlapping bands in some lanes. The results are shown in figure 17.

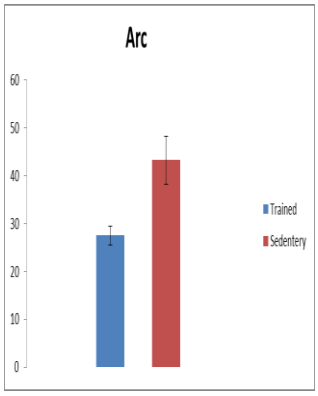
A



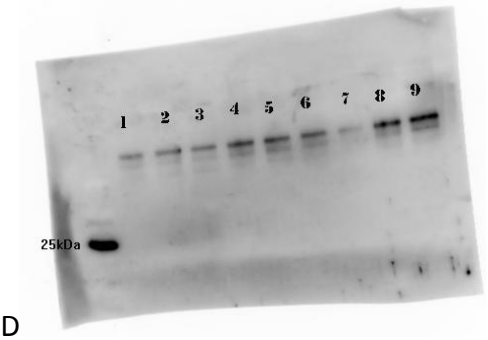
B



C



D



E

t-Test: Two-Sample Assuming Unequal Variances		
	Variable 1	*Variable 2*
Mean	27,5482	43,27714286
Variance	26,37179921	174,3078571
Observations	7	7
Hypothesized Mean Difference	0	
df	8	
t Stat	-2,93762855	
P(T<=t) one-tail	0,009388823	
t Critical one-tail	1,859548038	
P(T<=t) two-tail	0,018777647	
t Critical two-tail	2,306004135	

Figure 17: A, B) Loading control curve C) Histogram D) Western Blot: 1, 2, 3 are bands from trained mice ; 4, 5, 6 are bands from sedentary mice and 7, 8, 9 are control bands E) t-test

5) DISCUSSION

5.1) Discussion of materials and methods

Before discussing the results, several things regarding my materials and methods below need to be considered.

Different exercise protocols have been used recently to study the effect of exercise on brain. Exercise can be voluntary or forced. It can be treadmill running, swimming or acrobatic exercise. The duration may vary from minutes to months. The importance of all these voluntary versus forced, type, and duration of exercise, is important as it has been seen recently that difference in exercise protocol can cause difference in changes it brings about synaptic and structural proteins (Garcia et al., 2012). In this project, high intensity aerobic interval training was performed as uphill treadmill running with maximum VO2 reaching 90% for four minutes alternating between 4 min at 85%-90% of VO2max and 2 min at 50% of VO2max for 60 min/day, 5 days/week. This training is not voluntary and the training period is of 8 weeks duration. This protocol has been a success in human cardiovascular research, where it has been shown to improve cardiovascular health in both clinical and experimental trials. With this protocol that brings about the changes in cardiovascular health, we wanted to see if this type of high intensity interval aerobic exercise could bring the changes in brain plasticity as well.

Research dealing with exercise and synapses are mostly focused on specific regions of brain, often in the hippocampus. Given the function of the hippocampus and its role in learning and memory/long term potentiation, its natural that hippocampus is often the target. However, research has been done in other specific brain regions like cerebral cortex, spinal cord, cerebellum etc. In my own study, I have focused on global changes, i.e. in whole brain, not in any single isolated region. I have included the entire brain which consists of the cerebral hemisphere, midbrain, cerebellum and brain stem while preparing samples. As we are looking for synaptic proteins, the samples we have prepared is crude synaptosomes. Due to the fear of insufficient sample mass, we could not prepare pure synaptosome. Many other studies have also used crude synaptosomes for the study of synaptic proteins. To study synaptic proteins and synapses, several methods can be used. We have used quantitative

western blotting to study the synaptic proteins as it's a widely used and proven method. However, we could have used other immunostaining techniques like enzyme linked immunosorbent assay (ELISA), flow cytometry, mass spectrometry, immunohistochemistry and immunogold electron microscopy to characterize synaptic proteins present in synaptosomes. Similarly, we could have done immunocytochemistry to visualize synapses and count them in vivo.

The purity of crude synaptosomes was not specifically controlled. It can be controlled by electron microscopy, or by checking the presence of enzymatic markers such as: LDH, MAO, AChE and cytochrome-c oxidase (Lachowicz, Janiszewska, Wojtkowiak, & Wojtkowiak, 1983). However, there are studies in which synaptophysin and PSD-95 have been used as presynaptic and postsynaptic markers , respectively, as a control of purity the of crude synaptosomes. Having said all these, Pellet-2(P2) obtained from differential centrifugation also contains mitochondria and myelin fragments besides synaptosomes (Whittaker, Michaelson, & Kirkland, 1964). Moreover, the presence of glial cells should also be considered, as it is involved in the metabolism of glutamate receptor in neurons (Henn, Anderson, & Rustad, 1976) and a part of tripartite synapse (Santello, C., & P., 2012). Although the effect of glial cells should be taken into account, we are not going to discuss it while interpreting the results of the present study.

The exercise protocol in our research has made it difficult to compare the results directly with other studies. Research studies on whole mice brain with similar exercise protocol could not be found. However efforts have been made to compare results with different studies with different exercise protocols and different samples.

5.2) Discussion of results

Overall, the results indicate that treadmill running for 8 weeks has a modulatory effect on synapses through various mechanisms. It increases significantly the synaptic concentration of syntaxin, which is involved in the release of neurotransmitters. In the postsynaptic membrane, there is a similar increase in AMPA receptors, suggesting an increase in synaptic transmission following exercise.

The results can be discussed into three parts based on the proteins that have been studied.

1) Effect on AMPA receptor subunits.
2) Effect on NMDA receptor subunits.
3) Effect on neuronal marker proteins.
4) Effect on synaptic marker proteins.
5) Effect on synaptic plasticity related protein Arc.

5.2.1) Effect on AMPA receptor subunits.

Synaptic GluR1 was significantly raised in the trained mice, whereas GluR2 was unchanged. AMPA receptors are involved in fast synaptic transmission at glutamatergic synapses with consequent depolarization of cellular membrane, and promoting the opening of NMDA receptors by removing the magnesium block, a mechanism that has been related to long term potentiation (Purves, et al., 2008). The results show that forced treadmill running for 8 weeks increases the expression of GluR1 receptors in synapses compared to the sedentary mice. However, there was no increase in the level of GluR2. The increase in GluR1 receptor following exercise has been seen in various studies. Dietrich et al showed an increase in GluR1 and GluR2/3(to lesser extent) in post-synaptic densities of cortical mice brain following a month of voluntary treadmill running (Dietrich et al., 2005). Experiment conducted by Kamakura et al 2005 showed an upregulation of gene expression of GluR1 AMPA receptor subunit following a forced swim test (Isaac, Ashby, & McBain, 2007; Kamakura, Tamaki, Sakaki, & Yoneda, 2005). Increase in GluR1 with unchanged GluR2 in trained suggests that there is either an increase in the total concentration of AMPA receptors or an increase in GluR1 containing AMPA receptors only. Most of the AMPARs present in the brain have GluR2 subunits (Isaac, et al., 2007). GluR2 is responsible for the

biophysical properties of AMPA receptor and its presence makes AMPAR impermeable to calcium (Isaac, et al., 2007) . The unaltered level of GluR2 with an increase in GluR1 following training in my results shows that exercise induced synaptic plasticity may be accompanied by a higher AMPA receptor calcium conductance.

5.2.2) Effect on NMDA receptors

$NMDAR_{2A/B}$ was also increased in trained mice. But the molecular weight of $NMDAR_{2A/B}$ did not match with its expected molecular weight which is about 165 KDa. The bands in my experiment correspond to around 50-75 KDa. This was replicated by other members of the lab with other brain samples. It might be due to the proteolysis of NMDA receptor by proteases in samples in our lab or cross reactivity of antibodies or posttranslational modification of proteins. The company profile for anti-$NMDAR_{2A/B}$ states that no cross-reaction is seen with NMDAR1 or other glutamate receptor subunits.

NMDA receptor subunits 2A/2B are significantly increased in trained mice compared to sedentary mice in my experiment. This might be important for synaptic plasticity. Our findings correlate with several other studies which have also shown an increased expression of different subunits of NMDA receptor in different areas of rodent brain. Dietrich et al showed an increase in NMDAR in the post synaptic density in cortices of mice brain after voluntary exercise (Dietrich, et al., 2005). Similarly, voluntary exercise has been shown to increase the mRNA level of NR_{2B} subunits in dentate gyrus (Farmer, et al., 2004) and $NMDAR_{2A/2B}$ in hippocampus in rats (Molteni, et al., 2002). My results indicate that this increase may be a global brain response.

5.2.3) Effect on Neuronal marker proteins

Class III β-tubulin was not significantly altered in trained compared to sedentary mice after exercise. Tubulins, which are the major component of neuronal cytoskeleton, make 15-20% of total cell protein in brain (Laferrière, MacRae, & Brown, 1997) . They are needed for the growth and maintenance of neuronal structure, and are essential for the transport of intracellular cargoes containing cellular organelles and proteins (Laferrière, et al., 1997).They are concentrated in axons and dendrites of developing neurons. Among the tubulins, class III isotype of β-tubulin are present in very high amount in nervous system making them a marker of developing neurons (Richard P tucker) My results with class III β-tubulin shows that the concentration of neurons does not change after exercise. Unchanged β- tubulin after exercise also rules out the possibility of exercise being crucial in the growth and development of neurons and transfer of intracellular cargoes. Although, studies done in hippocampus have shown neurogenesis in the dentate gyrus of rats (Farmer et al), my results show that such exercise induced neurogenesis is a local change rather than the global effect.

5.2.4) Effect on synaptic marker proteins

Syntaxin was significantly increased following exercise, compared to sedentary mice. Other synaptic proteins, synaptophysin and PSD-95, were not increased. Increase in syntaxin mRNA has been shown in hippocampus of rodents (Molteni, et al., 2002). However, these results were quite opposite compared to the study done by Vayman et al which has shown that exercise increases synaptophysin, but not syntaxin in rodents (S. S. Vaynman, et al., 2006). The differences might be due to use of different exercise protocol (voluntary exercise for 3 days), different tissue (hippocampus) and different sample (Whole hippocampal homogenate). Syntaxin is a t-SNARE protein that is involved in the synaptic release of neurotransmitters. It functions with other SNARE proteins like VAMP and SNAP-25 in the release of neurotrasmitter by vesicle docking and fusion (S. S. Vaynman, et al., 2006). I would expect that an increase in syntaxin causes an increase in release of neurotransmitter from the presynaptic terminal.

Synaptophysin acts as a key protein in the biogenesis of synaptic vesicles from cholesterol and may possibly facilitate membrane retrieval during vesicle recycling. Increase in synaptophysin points toward either an increase in synapse formation or increase in number of vesicles in existing synapses(S. S. Vaynman, et al., 2006). As synaptophysin is not increased in exercising mice, it indicates that the number of synaptic vesicles and the number of synapses remains constant irrespective of exercise. This result contrast with other previous studies. Forced treadmill running in rats causes an increase in synaptophysin in striatum (A. F. Ferreira, Real, Rodrigues, Alves, & Britto, 2010), and motor cortex (Garcia, et al., 2012).

So in exercising mice, higher synaptic concentrations of syntaxin might be one of the mechanisms through which exercise increases the release of neurotransmitters, and thus also increases synaptic strength.

Post synaptic density protein of 95KDa (PSD-95) is unchanged in trained compared to sedentary mice. As PSD-95 is an important scaffolding protein in the PSD protein complex, the result might suggest that the proteins for holding of AMPA and NMDAR receptors in the postsynaptic density zone is not affected by exercise. Although Dietrich et al have shown increased PSD proteins including PSD-95 in the post synaptic density fractions prepared from cortices after voluntary exercise. I have not investigated other PSD proteins like SAP-97 and GRIP-1, so my findings need further investigation. On the other hand, the absence of change in PSD-95 concentrations fits well with the results of synaptophysin. They support each other: the overall concentration of synapses is not significantly changed.

5.2.5 Effect on Arc

Arc is a growth factor and an activity regulated gene that encodes cytoskeletal-associated protein found in neuronal dendrites(Lyford et al., 1995). It's a marker for neuronal activation (Clark, Bhattacharya, Miller, & Rhodes, 2011). It is accumulated in synapses in an activity dependent manner (Tzingounis & Nicoll, 2006). Arc is involved in maintaining homeostatic synaptic plasticity by endocytosis of AMPARs (Rial Verde, Lee-Osbourne, Worley, Malinow, & Cline, 2006). Increase in Arc means an increase in homeostatic synaptic plasticity, i.e. neurons have more capacity of maintaining homeostasis inside the neurons following induction of LTP or LTD.

Somewhat surprisingly, I found that Arc in exercising mice was significantly lower compared to sedentary mice. Arc is a marker of neuronal activation, and study conducted by clark et al reported that Arc increases immediately following exercise in adult mouse hippocampal granule neurons (Clark, et al., 2011). The down-regulation of Arc in trained as compared to sedentary mice in my result suggests that there is a decrease in homeostatic synaptic plasticity following exercise. However, trained mice in my experiments have undergone a full eight weeks of training. So we need to look after the long term effects of exercise rather than short term. The transcription of Arc is affected by NMDA and AMPA receptors with increase in AMPA receptors decreasing the level of Arc by inhibiting its gene transcription (Tzingounis & Nicoll, 2006). So, as the trained mice have undergone training regularly for eight weeks, the decrease in Arc protein in trained mice could be due to an increase in GluR1 AMPA receptors providing a negative feedback to maintain the homeostasis inside synapses (homeostatic synaptic plasticity). However, NMDA receptor activation causes an increase in Arc.

Overtraining might also be the reason behind decrease in Arc in trained mice, as Kelly and deadwyler (2002) showed that expression of Arc is decreased in overtrained rats compared to newly trained rats in an operant task (Kelly & Deadwyler, 2002). So forceful high intense exercise training might have caused overtraining in trained mice leading to decrease in Arc.

5.2.6 Exercise and Synaptic Plasticity

The overall effect of exercise on synapses can be viewed on two perspectives: Hebbian synaptic plasticity and Homeostatic synaptic plasticity. Hebbian synaptic plasticity consists of long term depression(LTD) and long term potentiation(LTP).

My research shows increase of both AMPAR(GluR1) and NMDAR(NMDAR $_{2A/2B}$) after 8 weeks of treadmill running. The increased level of GluR1 causes strengthening of synapses as it causes more magnesium release from NMDAR thus activating more NMDAR. Increased amount of calcium ions enter through NMDAR. The fate of plasticity is controlled mostly by the kinetics and amount of calcium influx through NMDARs. And the level of calcium influx through NMDARs is partly controlled by the level of postsynaptic membrane depolarization as this is responsible for the release of magnesium block from NMDARs (Yashiro & Philpot, 2008). Postsynaptic depolarization is caused by the opening of AMPA receptors. Thus AMPA and NMDA receptor both act together causing an increase in hebbian plasticity in trained mice and taking the direction of plasticity towards LTP.

There are two main school of thoughts regarding the maintenance of long term potentiation (LTP). One school of thought proposes that LTP is maintained by sustained increase in transmitter release by presynaptic terminals, most probably by the modification of proteins involved in exocytosis. The other school of thought proposes that LTP is maintained by addition of new AMPA receptors in the postsynaptic membrane by an NMDA receptor dependent mechanism or by increased current flow through the already present channels (Purves, et al., 2008). My results are compatible with both schools, which are mutually exclusive. Presynaptically, my results find increase in syntaxin, a t-SNARE protein which is involved in vesicular fusion and exocytosis of synaptic vesicles. And postsynaptically, exercise causes an increase GluR1 and NMDAR$_{2A/2B}$ receptors which are critical for the establishment of LTP and the subsequent increase in AMPA receptors (GluR1). NMDARs are highly permeable to calcium ions and influx of calcium ions triggers 2nd messenger system which results in synaptogenesis, experience-dependent synaptic remodeling and long lasting changes in synaptic efficacy such as LTP and LTD. Thus, according to my results, hebbian

plasticity increases after exercise. However, the decrease in Arc following exercise suggests that homeostatic plasticity is reduced after exercise.

The increase in GluR1 receptors in trained mice can also be related to the decrease of Arc protein which is involved in the endocytosis of GluR1 receptors from postsynaptic membrane. Arc downregulates AMPARs by increasing the rate of endocytosis and the long term effect of Arc appears to be preferential for endocytosis of GluR1, although GluR2 endocytosis also occur in the short term (Chowdhury et al., 2006). This might also explain the increased GluR1 receptors in trained as compared to sedentary mice. Sedentary mice having more Arc have lower GluR1 receptors because of the action of Arc. GluR2 level in both groups are not changed significantly, supporting the idea of preferential endocytosis of GluR1 by Arc.

On the other hand, Arc itself is induced following LTP form of hebbian synaptic plasticity and is involved in maintaining balance inside the neurons by endocytosis of AMPA receptors . However, it is not known if increase in AMPAR after exercise decreases Arc or it's the decrease in Arc following exercise that increases the AMPAR in synapses. Although a study by Shepherd et al. (2006) showed that GluR1 surface expression is significantly increased in Arc knockout neurons with GluR2 showing no change (Shepherd et al., 2006).

5.2.7 Putting the synaptic changes together

The absence of significant difference in β-tubulin in trained compared to sedentary mice shows that there is no change in the number of neurons after exercise. Likewise, the absence of significant difference between PSD-95 and synaptophysin shows that there is no alteration in the synaptic structure and number of synapses after exercise. However, exercise brings the changes in synapse by altering different proteins and receptors present inside them. It increases the presynaptic protein syntaxin and the GluR1 AMPA receptors and $NMDAR_{2A/2B}$ receptors. Exercise also brings about the decrease in plasticity protein Arc, which in my case might be to control the increased synaptic plasticity after exercise.

6) CONCLUSION

My study demonstrates a differences between studies done in whole brain and on specific brain tissue like the hippocampus. It also supports the previous studies that differences in exercise protocol might have important effects on the way the brain changes.

Trained mice have upregulation of GluR1 type AMPA receptors and NMDAR in the postsynaptic terminal. This correlates with my hypothesis that trained mice have increased hebbian synaptic plasticity. The number of neurons and synapses are not altered significantly after exercise. So my hypothesis of increase in number of neurons and synapses after exercise is rejected. The increase in syntaxin after exercise supports my hypothesis that exercise causes an upregulation of synaptic proteins involved in the release of neurotransmitters. The hypothesis that exercise increases the homeostatic synaptic plasticity by the upregulation of Arc is rejected, as it has been decreased in my experiment.

7) FUTURE CHALLENGES

The effect of Arc in synaptic plasticity needs to be explored further. I would also want to determine if the increase in GluR1 receptor is either due to an increase of total AMPA receptors or whether it represents an increase of GluR1 homomeric AMPA receptors only. Other synaptic proteins, like synapsin-1, GRIP-1, PICK, can also be studied to get further understanding of molecular changes in synapse after exercise. In addition to my western blot findings, postembedding immunogold electron microscopy would be a good idea to further confirm my findings.

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9) APPENDIX

9.1 Appendix A

Chemicals

Chemical	*Supplier*	*Catalogue number*
30% Acrylamide/Bis solution, 37.5:1 (2,6%C)	Bio-Rad	161-0158
Trizma base	Sigma	T1503-1KG
Sodium dodecyl sulphate	Sigma	L4390-500G
HCl	AnalaR NORMAPUR	20252.290
Ammonium persulphate	Sigma	A3678-25G
Tris-HCl	Sigma	T3253-1KG
Glycerol	AnalaR NORMAPUR	24388.295
2-mercaptoethanol	Sigma	M7154-25ML
ECF	GE Healthcare	1067873
NaCl	AnalaR NORMAPUR	27810.295
Tween 20	Sigma	P5927-500ML
Methanol	AnalaR NORMAPUR	20847.307
Glycine	Sigma	G7126-1KG
Skim milk powder	Fluka	70166-500G
Sodium azide	Sigma	S8032-100G
NaOH	AnalaR NORMAPUR	28244.295
Hepes	Sigma	H3375-500G

Protein inhibitor cocktail	Complete	11 697 498 001
D(+)-Saccahrose	AnalaR NORMAPUR	27480.260
Bromphenol blue – xylene cyanole dye solution	Sigma	B3269-5ML
Monoclonal anti-rabbit IgG (γ-chain specific) – Alkaline phosphatase, antibody produced in mouse	Sigma	A2556-5ML
Anti-mouse IgG (whole molecule) F(ab)$_2$ fragment – Alkaline phosphatase	Sigma	A3563-5ML

9.2 APPENDIX B

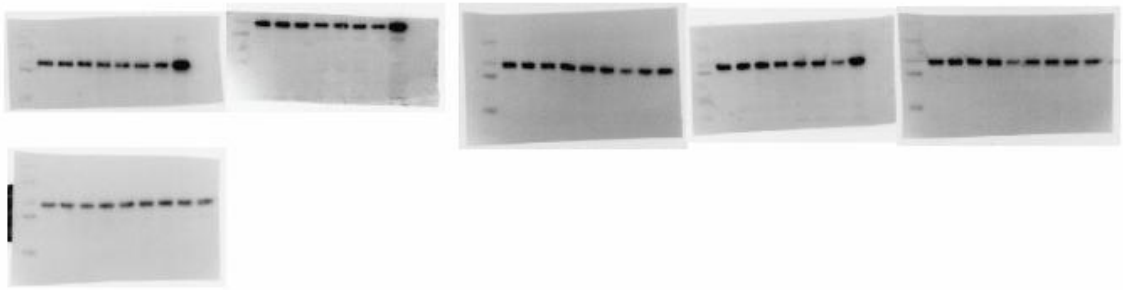
Weight of mice brain and the total protein concentration of the crude synaptosomes obtained from each mice brain.

Serial No	Mouse Identification	Weight of Mouse Brain	Protein Concentration(µg/µL)
1	Mouse Trained Nr 1	0,143	0,98
2	Mouse Untrained Nr 1	0,15	1,21
3	Mouse Trained Nr 2	0,207	2,44
4	Mouse Untrained Nr 2	0,198	2,92
5	Mouse Trained Nr 3	0,1858	2,67
6	Mouse Untrained Nr 3	0,2062	1,87
7	Mouse Trained Nr 4	0,2164	2,492=2,5
8	Mouse Untrained Nr 4	0,2	2,196=2,2
9	Mouse Trained Nr 5	0,1756	1,91
10	Mouse Untrained Nr 5	0,1972	2,379=2,38
11	Mouse Trained Nr 6	0,1948	2,628=2,63
12	Mouse Untrained Nr 6	0,1478	2,2
13	Mouse Trained Nr 7	0,1852	2,37
14	Mouse Untrained Nr 7	0,1769	2,239=2,24

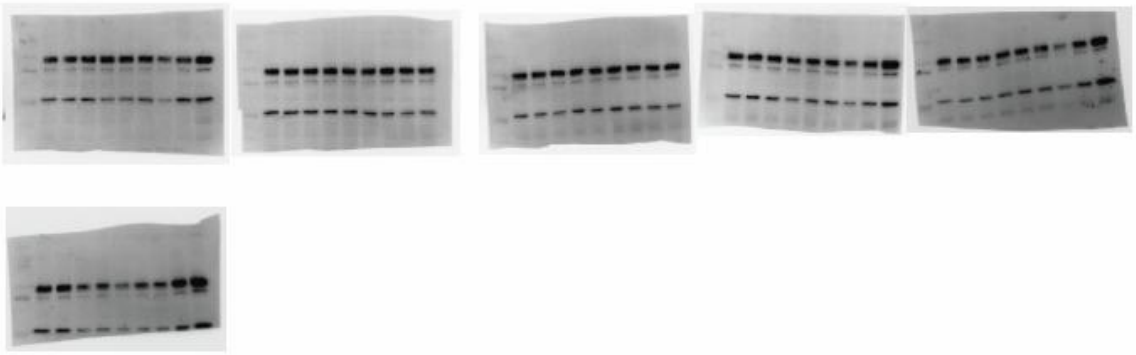
9.3 APPENDIX C

Western Blots

GluR1

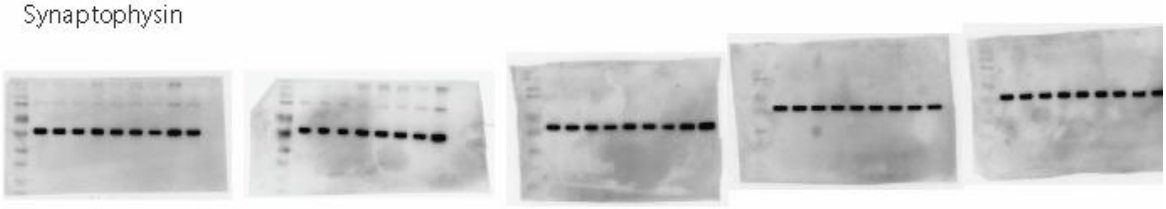


GluR2



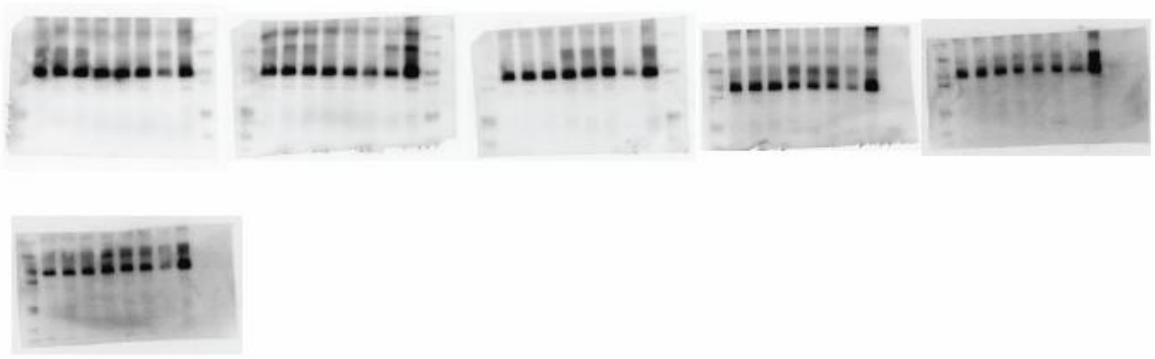
NMDAR

Synaptophysin

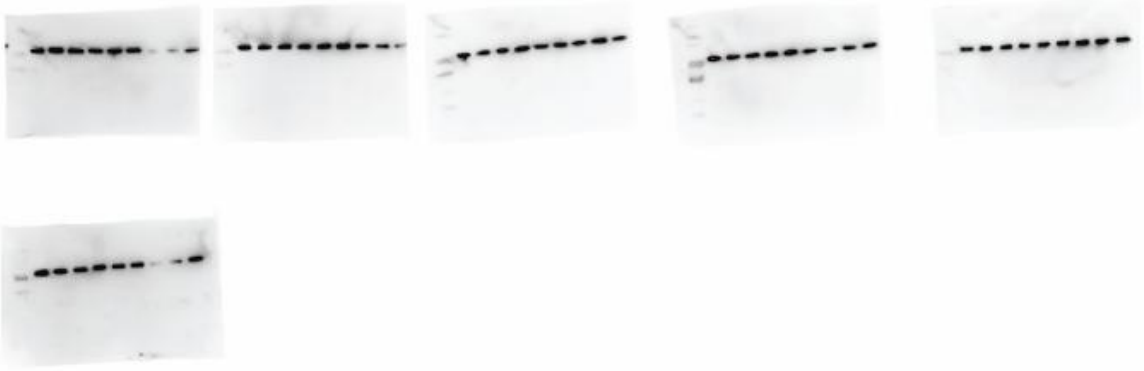


Syntaxin

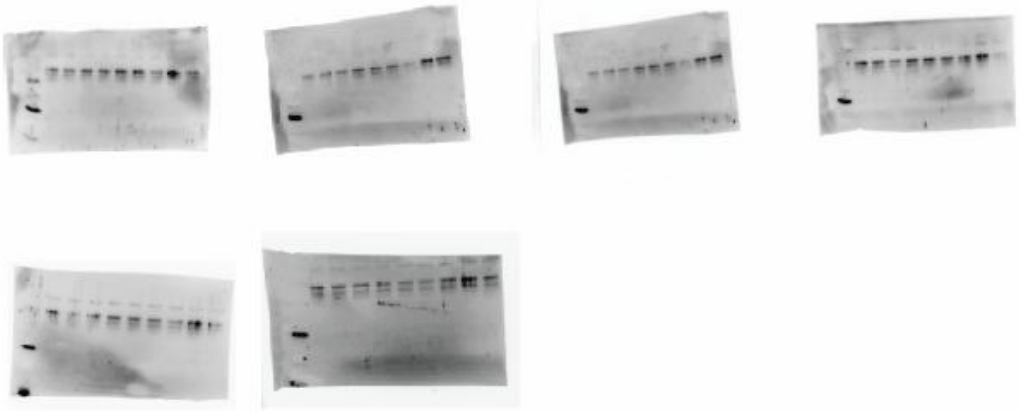
PSD-95



Beta-tubulin



Arc



9.4 Appendix D

Calculation of protein concentrations.

Feb 14, 2012

MULTISKAN PICHROMATIC VERSION 1.03

ABSORBANCE MODE
CONTINUOUS MOVEMENT
FILTER 562

ABSORBANCES
3X. XXX 19XX XX:XX:XX

$y = 0,4992x + 0,106$

$R^2 = 0,9943$

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.081	0.506	0.319	0.325	0.331	0.089	0.179	0.152	0.146	0.167	0.165	0.158
B	0.080	0.488	0.262	0.249	0.248	0.169	0.201	0.163	0.173	0.160	0.176	0.179
C	0.162	0.613	0.305	0.296	0.304	0.184	0.193	0.181	0.163	0.160	0.183	0.182
D	0.159	0.602	0.216	0.219	0.217	0.184	0.179	0.175	0.168	0.169	0.172	0.170
E	0.227	0.836	0.315	0.306	0.309	0.153	0.115	0.190	0.179	0.186	0.192	0.174
F	0.240	0.632	0.231	0.224	0.226	0.115	0.058	0.153	0.179	0.178	0.193	0.191
G	0.400	0.172	0.188	0.182	0.190	0.174	0.211	0.189	0.171	0.185	0.155	0.186
H	0.375	0.176	0.158	0.210	0.149	0.193	0.189	0.169	0.158	0.177	0.179	0.160

A
B
C
D

C => Mouse 7 Trained :-

1:5 -> 2,044
1:10 -> 2,438
} Mean = 2.241

% S.E. of mean = 8.8 %

A => Mouse 3 Trained = 1:5 ->, 1:10 ->
B => Mouse 6 Untrained = 1:5 ->, 1:10 ->
} => Already calculated.

Jan 10, 2011, 4th exp.

MULTISKAN BICHROMATIC VERSION 1.03

ABSORBANCE MODE
CONTINUOUS MOVEMENT
FILTER 562

$y = 0,48772x + 0,1193$
$R^2 = 0,9895$

ABSORBANCES
3X. XXX 19XX XX:XX:XX

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.130	0.620	1.149	0.539	0.528	0.521	0.085	0.531	0.220	0.218	0.225	0.039
B	0.123	0.574	1.111	0.409	0.393	0.455	0.100	0.488	0.161	0.167	0.163	0.040
C	0.250	0.700	0.042	0.667	0.672	0.643	0.178	0.602	0.334	0.348	0.347	0.040
D	0.256	0.754	0.038	0.456	0.458	0.475	0.160	0.577	0.243	0.251	0.243	0.039
E	0.400	1.084	0.043	0.065	0.075	0.068	0.232	0.819	0.358	0.390	0.374	0.041
F	0.392	0.412	0.043	0.044	0.041	0.043	0.247	0.852	0.325	0.296	0.270	0.040
G	0.390	1.050	0.048	0.045	0.046	0.046	0.413	0.045	0.043	0.045	0.042	0.042
H	0.139	0.132	0.045	0.045	0.037	0.048	0.410	0.041	0.043	0.042	0.041	0.043

CS→
Mouse 1 Trained:-

1% 5 → 1,043
1% 10 → 0,910
Mean → 0,9765.
S.D. → 0.09045.
S.E.of mean $= \frac{SD}{\sqrt{n}} = \frac{0.09045}{\sqrt{2}} = 0.06325.$
∴ % S.E.of mean $= \frac{0.06325}{0,9765} = 0.064 = 6.4\%$.

CS→
Mouse 2 Trained:-

1% 5 → 2,293.
1% 10 → 2,591.
Mean → 2,442.
S.D. = 0,210718.
S.E.of mean $= \frac{0,21}{\sqrt{2}} = \frac{0.14}{}$.
∴ % SE.of mean $= \frac{0.14}{2,442} = 0.057 = 5.7\%$

Mouse 2 Untrained:-
1% 5 → 2,611
1% 10 → 3,664
% S.E.of mean = 16%

Jan 11, 2nd exp.

$y = 0,5014 + 0,1225$

$R^2 = 0,9877$

MULTISKAN BICHROMATIC VERSION 1.03

ABSORBANCE MODE
CONTINUOUS MOVEMENT
FILTER 562

ABSORBANCES
3X. XXX 19XX XX:XX:XX

	1	2	3	4	5	6	7	8	9	10	11	
A	0.115	0.551	0.638	0.647	0.661	0.052	0.357	0.366	0.369	0.037	0.036	0.
B	0.084	0.508	0.407	0.393	0.421	0.104	0.241	0.236	0.278	0.045	0.039	0.
C	0.159	0.633	0.670	0.675	0.686	0.041	0.329	0.352	0.329	0.040	0.038	0.
D	0.154	0.611	0.446	0.460	0.442	0.142	0.230	0.245	0.232	0.040	0.039	0.
E	0.239	0.841	0.713	0.697	0.703	0.040	0.322	0.307	0.327	0.041	0.094	0.
F	0.264	0.852	0.456	0.449	0.458	0.122	0.215	0.216	0.217	0.039	0.142	0.
G	0.432	0.041	0.630	0.622	0.616	0.038	0.343	0.342	0.031	0.349	0.055	0.
H	0.409	0.038	0.435	0.433	0.433	0.040	0.259	0.247	0.244	0.037	0.040	0.

(5) ~~Mouse~~ - Crude Synaptosome (CS), Mouse - 4, Trained.

1:5 -> 2.408
1:10 -> 2.576
} 2.492 ~ 2.5 or 2.49 -> %age standard error of mean = ~~3.37~~ 3.37 %

(6) CS, ~~Mouse~~ = 4, ~~Untrained~~ Untrained.

1:5 -> 2.136
1:10 -> 2.257
} 2.1965 ~ 2.2 -> %age S.E. of mean = ~~2.4~~ 2.73 %.

(7) CS, Mouse - 5 Trained.

1:5 -> 1.956
1:10 -> 1.865
} 1.9105 -> %age S.E. of mean = 2.37 %.

(8) CS, ~~Mouse~~ 5, Untrained.

1:5 -> 2.215
1:10 -> 2.543
} 2.379 = 2.38 -> %age S.E. of mean = 6.9 %.

[Jan 11, 2012] 1st exp.

MULTISKAN BICHROMATIC VERSION 1.03

ABSORBANCE MODE
CONTINUOUS MOVEMENT
FILTER 562

ABSORBANCES
SN. XXX 19XX XX:XX:XX

$y = 0{,}5126x + 0{,}107$
$R^2 = 0{,}9913$

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.061	0.533	0.721	0.697	0.700	0.097	0.404	0.395	0.398	0.096	0.035	0.042
B	0.087	[illegible]	0.407	0.404	0.413	[illegible]	[illegible]	0.262	0.262	0.120	[illegible]	0.049
C	0.156	0.615	0.640	0.637	0.648	0.126	0.040	0.043	0.067	0.125	0.105	0.079
D	0.152	0.644	0.440	[illegible]	0.419	0.039	[illegible]	0.352	0.363	[illegible]	0.142	0.042
E	0.243	0.657	0.040	0.063	0.039	0.116	0.246	0.249	[illegible]	0.161	[illegible]	0.123
F	0.224	0.836	0.039	0.038	0.029	0.120	0.145	0.135	0.152	0.179	0.173	0.116
G	0.356	0.115	0.119	0.041	0.039	0.137	0.298	[illegible]	[illegible]	[illegible]	0.133	0.135
H	0.392	0.039	0.042	0.029	0.042	0.115	0.207	0.201	0.195	0.153	0.040	0.043

(3) -> Crude Synaptosome, Mouse-2, Untrained ~~Trained~~.
1:5 -> 2,848 } 2.918 ~ 2.92 -> % SE of mean = 2.40 %.
1:10 -> 2,988 }

(4) -> Crude Synaptosome, Mouse-3, Trained:
1:5 -> 2,432 } -> 2.6725 ~ 2.67 -> % standard error of mean = 9.02 %
1:10 -> 2,913 }

(5) -> Crude Synaptosome, Mouse-3, Untrained
1:5 -> 1,912 ~~[illegible]~~ } -> 1.873 ~ 1.87 -> % standard error of mean = 2.08 %.
1:10 -> 1,834

Trained [Jan 11, 2012] 1st exp.

MULTISKAN BICHROMATIC VERSION 1.03

ABSORBANCE MODE
CONTINUOUS MOVEMENT
FILTER 562

ABSORBANCES
SN. XXX 19XX XX:XX:XX

$y = 0{,}5126x + 0{,}107$
$R^2 = 0{,}9913$

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.061	0.533	0.721	0.697	0.700	0.097	0.404	0.395	0.398	0.096	0.035	0.042
B	0.087	[illegible]	0.407	0.404	0.413	[illegible]	[illegible]	0.262	0.262	0.120	[illegible]	0.049
C	0.156	0.615	0.640	0.637	0.648	0.126	0.040	0.043	0.067	0.125	0.105	0.079
D	0.152	0.644	0.440	[illegible]	0.419	0.039	[illegible]	0.352	0.363	[illegible]	0.142	0.042
E	0.243	0.657	0.040	0.063	0.039	0.116	0.246	0.249	[illegible]	0.161	[illegible]	0.123
F	0.224	0.836	0.039	0.038	0.029	0.120	0.145	0.135	0.152	0.179	0.173	0.116
G	0.356	0.115	0.119	0.041	0.039	0.137	0.298	[illegible]	[illegible]	[illegible]	0.133	0.135
H	0.392	0.039	0.042	0.029	0.042	0.115	0.207	0.201	0.195	0.153	0.040	0.043

(3) -> Crude Synaptosome, Mouse-2, Untrained ~~Trained~~.
1:5 -> 2,848 } 2.918 ~ 2.92 -> % SE of mean = 2.40 %.
1:10 -> 2,988 }

(4) -> Crude Synaptosome, Mouse-3, Trained:
1:5 -> 2,432 } -> 2.6725 ~ 2.67 -> % standard error of mean = 9.02 %
1:10 -> 2,913 }

(5) -> Crude Synaptosome, Mouse-3, Untrained
1:5 -> 1,912 ~~[illegible]~~ } -> 1.873 ~ 1.87 -> % standard error of mean = 2.08 %.
1:10 -> 1,834

9.5) Appendix E

Calculation of the intensities of bands.

Quantitation

Sample	Crude synaptosomes
Antibody	GluR2

Background calculation

Spor nr.	Mean	Pixels	MeanxPixels/1000	Average
Trained1	79,75	1026	81,8235	84,41928
	81,24	1026	83,35224	
	85,85	1026	88,0821	
Untrained1	89,77	1026	92,10402	91,40634
	89,54	1026	91,86804	
	87,96	1026	90,24696	
Trained2	63,76	962	61,33712	63,86397
	64,63	962	62,17406	
	70,77	962	68,08074	
Untrained2	78,03	962	75,06486	74,93018
	78,29	962	75,31498	
	77,35	962	74,4107	
Trained3	75,35	962	72,4867	70,29334
	74,72	962	71,88064	
	69,14	962	66,51268	
Untrained3	72,31	1116	80,69796	86,7504
	76,65	1116	85,5414	
	84,24	1116	94,01184	
Trained4	87,62	1116	97,78392	99,4728
	90,55	1116	101,0538	
	89,23	1116	99,58068	
Untrained4	88,19	1116	98,42004	96,62328
	87,72	1116	97,89552	
	83,83	1116	93,55428	
Trained5	55,15	1102	60,7753	66,41139
	59,9	1102	66,0098	
	67,32	1102	74,18664	
Untrained5	74,47	1102	82,06594	85,22501
	79,3	1102	87,3886	
	78,24	1102	86,22048	
Trained6	104,87	1292	135,492	137,3051
	107,14	1292	138,4249	
	106,81	1292	137,9985	
Untrained6	109,94	1292	142,0425	143,7436
	112,42	1292	145,2466	
	111,41	1292	143,9417	
Trained7	91,17	792	72,20664	75,96072
	95,19	792	75,39048	
	101,37	792	80,28504	
Untrained7	105,9	792	83,8728	83,8464
	106,57	792	84,40344	

Band Intensity calculation

Spor nr.	Applisert p	Mean	Pixels	Mean x Pixels/1000	Snitt av 3	Bakgrunn	Korrigert for bakgrunn
	Trained1	136,19	1026	139,7309	149,6831	84,41	65,27314
		141,06	1026	144,7276		0	0
		160,42	1026	164,5909		0	0
	Untrained1	166,14	1026	170,4596	169,2763	91,4	77,87632
		164,79	1026	169,0745		0	0
		164,03	1026	168,2948		0	0
	Trained 2	142,2	962	136,7964	142,8538	63,86	78,99379
		147,68	962	142,0682		0	0
		155,61	962	149,6968		0	0
	Untrained 2	165,08	962	158,807	156,1422	74,93	81,21222
		161,89	962	155,7382		0	0
		159,96	962	153,8815		0	0
	Trained3	170,23	962	163,7613	156,0621	70,29	85,77205
		160,96	962	154,8435			
		155,49	962	149,5814			
	Untrained3	147,45	1116	164,5542	166,9015	85,75	81,15152
		147,93	1116	165,0899		0	0
		153,28	1116	171,0605		0	0
	Trained4	158,42	1116	176,7967	179,0176	99,47	79,54756
		161,03	1116	179,7095		0	0
		161,78	1116	180,5465		0	0
	Untrained4	151,66	1116	169,2526	168,2891	96,62	71,66908
		148,53	1116	165,7595			
		152,2	1116	169,8552			
	Trained 5	138,16	1102	152,2523	156,2085	73,004	83,2045
		141,38	1102	155,8008		0	0
		145,71	1102	160,5724		0	0
	Untrained5	140,84	1102	155,2057	160,6128	85,225	75,38783
		148,28	1102	163,4046		0	0
		148,12	1102	163,2282		0	0
	Trained6	153,21	1292	197,9473	199,2436	137,3	61,94363
		152,5	1292	197,03		0	0
		156,93	1292	202,7536		0	0
	Untrained6	169,25	1292	218,671	217,2843	143,74	73,54425
		170,07	1292	219,7304		0	0
		165,21	1292	213,4513		0	0
	Trained7	181,96	792	144,1123	137,5519	75,96	61,59192
		183,46	792	145,3003		0	0
		155,61	792	123,2431		0	0
	Untrained7	168,19	792	133,2065	129,3072	83,84	45,4672
		150,72	792	119,3702		0	0
		170,89	792	135,3449		0	0

Quantitation

Sample	Crude synaptosomes
Antibody:	PSD 95

Intensity calculation of four trained mice and four untrained mice for PSD antibody

	Trained 1	Untrained 1	Trained 2	Trained 3	Trained 4	Untraind 2	Untrained 3	Untrained 4
	122,811	126,23	100,78	74,21	81,6	102,59	83,05	86,64
	129,56	127,34	102,82	79,28	84,86	94,01	82,98	83,84
	130,065	120,82	104,22	81,79	83,35	89,65	81,49	83,18
Mean	127,4787	124,7966667	102,6067	78,42667	83,27	95,4166667	82,50666667	84,55333333
Background	11,2	11,2	15,09	9,34	19,79	15,09	9,34	19,79
Corrected Intensity	116,2787	113,5966667	87,51667	69,08667	63,48	80,3266667	73,16666667	64,76333333

Intensities of 6 mice were calculated using software

Sample	Crude synaptosomes
Antibody:	PSD 95

Bakgrunnsavlesninger:

Spor nr.	Mean	Pixels	MeanxPixels/1000	Average
Trained 5	52,74	986	52,00164	54,86761333
	58,36	986	57,54296	
	55,84	986	55,05824	
Untrained 5	53,11	986	52,36646	53,4412
	53,18	986	52,43548	
Trained 6	56,31	986	55,52166	
	90,33	1020	92,1366	
	93,61	1020	95,4822	
Untrained 6	91,65	1020	93,483	95,7525
	96,1	1020	98,022	
Trained 7	67,68	992	67,13856	81,57216
	85,96	992	85,27232	
	93,05	992	92,3056	
Untrained 7	92,46	992	91,72032	90,53653333
	89,57	992	88,85344	
	91,77	992	91,03584	

Band Intensity Quantitation

Spor nr.	Applisert prøve	Mean	Pixels	Mean x Pixels/1000	Snitt av 3	Bakgrunn	Korrigert for bakgrunn
2	Trained 5	168,19	986	165,8353	171,071	54,8676	116,2034
3		176,37	986	173,9008		0	0
4		175,94	986	173,4768		0	0
5	Untrained 5	163,98	986	161,6843	163,8436	53,44	110,4036
6		167,41	986	165,0663		0	0
7		167,12	986	164,7803		0	0
2	Trained 6	157,67	1020	160,8234	168,6672	88,1858	80,4814
3		166,78	1020	170,1156		0	0
4		171,63	1020	175,0626		0	0
5	Untrained 6	163,73	1020	167,0046	166,8346	96,0364	70,7982
6		160,8	1020	164,016			0
7		166,16	1020	169,4832		0	0
2	Trained 7	138,66	992	137,5507	156,3822	81,57216	74,81003
3		156,34	992	155,0893		0	0
4		177,93	992	176,5066		0	0
5	Untrained 7	191,43	992	189,8986	180,4944	90,5365	89,9579
6		182,87	992	181,407		0	0
7		171,55	992	170,1776		0	0

Quantitation:

Sample	Crude synaptosomes
Antibody	GluR1

ajoner:	**Bakgrunnsavlesninger:**			
Spor nr.	Mean	Pixels	MeanxPixels/1000	
Trained 1	64,48	936	60,35328	59,47032
	62,85	936	58,8276	
	63,28	936	59,23008	
Untrained 1	62,64	936	58,63104	55,37064
	58,49	936	54,74664	
	56,34	936	52,73424	
Trained 2	73,25	1014	74,2755	77,70958
	76,78	1014	77,85492	
	79,88	1014	80,99832	
Untrained 2	84,33	1014	85,51062	83,03308
	83,3	1014	84,4662	
	78,03	1014	79,12242	
Trained3	60,17	1014	61,01238	66,34602
	66,52	1014	67,45128	
	69,6	1014	70,5744	
Untrained3	68,78	1014	69,74292	68,80666
	67,18	1014	68,12052	
	67,61	1014	68,55654	
Trained4	88,73	858	76,13034	71,79172
	83,96	858	72,03768	
	78,33	858	67,20714	
Untrained 4	94,77	858	81,31266	80,46038
	93,96	858	80,61768	
	92,6	858	79,4508	
Trained5	88,8	1408	125,0304	117,0846
	83,93	1408	118,17344	
	76,74	1408	108,04992	
Untrained 5	44,28	1394	61,72632	61,86572
	44,27	1394	61,71238	
	44,59	1394	62,15846	
Trained6	78,39	900	70,551	73,392
	81,33	900	73,197	
	84,92	900	76,428	
Untrained6	88,15	900	79,335	77,442
	85,93	900	77,337	
	84,06	900	75,654	
Trained7	44,78	1394	62,42332	62,48373
	45,03	1394	62,77182	
	44,66	1394	62,25604	
Untrained7	45,2	1394	63,0088	59,48663
	43,12	1394	60,10928	
	39,7	1394	55,3418	

Band quantitation:

Applisert p	Mean	Pixels	Mean x Pixels/1000	Snitt av 3	Bakgrunn	Korrigert for bakgrunn
Trained 1	115,56	912	105,3907	110,0541	55,37064	54,68344
	119,68	912	109,1482		0	0
	126,78	912	115,6234		0	0
Untrained	125,81	912	114,7387	115,3558	59,4702	55,88564
	123,28	912	112,4314		0	0
	130,37	912	118,8974		0	0
Trained 2	141,8	1014	143,7852	146,7089	77,70958	68,99932
	145,59	1014	147,6283		0	0
	146,66	1014	148,7132		0	0
Untrained 2	141,44	1014	143,4202	135,9673	83,03308	52,93418
	138,19	1014	140,1247		0	0
	122,64	1014	124,357		0	0
Trained 3	141,02	1014	142,9943	141,4091	66,34602	75,06304
	142,95	1014	144,9513		0	0
	134,4	1014	136,2816		0	0
Untrained 3	119,01	1014	120,6761	122,5892	68,80666	53,78256
	122,77	1014	124,4888		0	0
	120,91	1014	122,6027		0	0
Trained 4	153,18	858	131,4284	134,1369	71,79172	62,34514
	155,41	858	133,3418		0	0
	160,42	858	137,6404		0	0
Untrained 4	174,21	858	149,4722	148,9145	80,46308	68,4514
	176,99	858	151,8574		0	0
	169,48	858	145,4138		0	0
Trained 5	135,45	1408	190,7136	185,5697	117,0846	68,48511
	135,17	1408	190,3194		0	0
	124,77	1408	175,6762		0	0
Untrained 5	69,48	1394	96,85512	102,1151	61,86572	40,24943
	74,87	1394	104,3688		0	0
	75,41	1394	105,1215		0	0
Trained 6	168,62	900	151,758	154,257	73,392	80,865
	171,3	900	154,17		0	0
	174,27	900	156,843		0	0
Untrained 6	160,37	900	144,333	143,739	77,442	66,297
	160,9	900	144,81		0	0
	157,86	900	142,074		0	0
Trained 7	85,28	1394	118,8803	120,0188	62,48373	57,53502
	84,94	1394	118,4064		0	0
	88,07	1394	122,7696		0	0
Untrained 7	86,87	1394	121,0968	109,6056	59,48663	50,11894
	79,26	1394	110,4884		0	0
	69,75	1394	97,2315		0	0

Quantitation

Sample	Crude synaptosomes
Antibody	Arc

	Backgrou	**calculation**		
	Mean	Pixels	MeanxPixel	Avg
Gel 1	52,08	1073	55,88184	68,15428
	84,73	1073	90,91529	
	56,77	1073	60,91421	
	60,49	1073	64,90577	
Gel 2	49,33	1225	60,42925	68,17431
	73,96	1225	90,601	
	52,26	1225	64,0185	
	47,06	1225	57,6485	
Gel 3	62,59	840	52,5756	62,4435
	70,87	840	59,5308	
	76,14	840	63,9576	
Gel 4	87,75	840	73,71	
	75,62	770	58,2274	47,86705
	58,72	770	45,2144	
	57,99	770	44,6523	
	56,33	770	43,3741	
Gel 5	39,02	992	38,70784	43,49589
	52	992	51,584	
	40,52	992	40,19584	
	63,96	992	63,44832	61,14688
	64,02	992	63,50784	
	56,94	992	56,48448	
	50,28	992	49,87776	48,15499
	50,55	992	50,1456	
	44,8	992	44,4416	
Gel 6	39,02	992	38,70784	43,49589
	52	992	51,584	
	40,52	992	40,19584	
	38,65	899	34,74635	35,06699
	39,62	899	35,61838	
	38,75	899	34,83625	
	50,28	992	49,87776	48,15499
	50,55	992	50,1456	
	44,8	992	44,4416	

Band Quantitation

Spor nr.	Mean	Pixels	Mean x Pixels/1000	Average	Backgroun	Correction of Background
Trained 1	91,71	962	88,22502	96,47577333	68,154	28,3217733
	96,11	962	92,45782		0	0
	113,04	962	108,7445		0	0
Untrained	118,79	962	114,276	120,3205467	68,154	52,1665467
	118,94	962	114,4203		0	0
	137,49	962	132,2654		0	0
Trained 2	72,44	1225	88,739	95,92975	68,2	27,72975
	74,66	1225	91,4585		0	0
	87,83	1225	107,5918		0	0
Untrained 2	91,39	1225	111,9528	119,2700833	68,2	51,0700833
	103,43	1225	126,7018		0	0
	97,27	1225	119,1558		0	0
Trained 3	88,44	840	72,6096	79,3128	62,44	26,7428
	96,41	840	80,9844		0	0
	100,41	840	84,3444		0	0
Untrained 3	124,69	840	104,7396	105,6356	62,44	53,0656
	128,27	840	107,7468		0	0
	124,31	840	104,4204		0	0
Trained 4	114,56	770	88,2112	79,12263333	47,86	31,2626333
	105,46	770	81,2042		0	0
	88,25	770	67,9525		0	0
Untrained 4	125,37	770	96,5349	93,12636667	47,86	45,2663667
	118,45	770	91,2065		0	0
	119,01	770	91,6377		0	0
Trained 5	112,14	1428	160,1359	158,746	132,21	26,536
	116,29	1428	166,0621		0	0
	105,07	1428	150,04		0	0
Trained 6	113,68	1428	162,335	161,9114	132,21	29,7014
	113,27	1428	161,7496		0	0
	113,2	1428	161,6496		0	0
Untrained 5	130,65	1428	186,5682	188,12948	132,21	55,91948
	145,33	1428	207,5312			
	119,25	1428	170,289			
Untrained 6	69,34	992	68,78528	67,44277333	43,49	23,9527733
	77,32	992	76,70144		0	0
	57,3	992	56,8416		0	0
Trained 7	62,5	899	56,1875	55,03977667	35,06	19,9797767
	65,46	899	58,84854		0	0
Untrained	55,71	899	50,08329		0	0
	55,46	992	55,01632	54,98656	48,154	27,4
	58,88	992	58,40896			
	51,95	992	51,5344			

Quantitation

Sample	Crude synaptosomes
Antibody	class III beta-tubulin

Bakgrunnsavlesninger:

Spor nr.	Mean	Pixels	MeanxPixels/1000	
Trained 1	31,37	1140	35,7618	35,606
	31,4	1140	35,796	
	30,93	1140	35,2602	
Untrained	31,13	1140	35,4882	33,0562
	29,09	1140	33,1626	
	26,77	1140	30,5178	
Trained 2	17,98	780	14,0244	16,6738
	21,94	780	17,1132	
	24,21	780	18,8838	
Untrained	25,1	780	19,578	19,5702
	25,15	780	19,617	
	25,02	780	19,5156	
Trained 3	12,96	1080	13,9968	14,6304
	13,29	1080	14,3532	
	14,39	1080	15,5412	
Untrained	14,15	1080	15,282	14,3028
	12,78	1080	13,8024	
	12,8	1080	13,824	
Trained 4	9,62	1080	10,3896	8,9856
	7,44	1080	8,0352	
	7,9	1080	8,532	
Untrained	19,54	700	13,678	13,16233
	18,1	700	12,67	
	18,77	700	13,139	
Untrained	18,29	700	12,803	11,235
	16,21	700	11,347	
	13,65	700	9,555	
Trained 6	10,67	700	7,469	7,497
	8,93	700	6,251	
	12,53	700	8,771	
Untrained	37,13	888	32,97144	32,97144
	34,11	888	30,28968	
	38,79	888	34,44552	
Trained 5	11,89	888	10,55832	10,77144
	11,46	888	10,17648	
	13,04	888	11,57952	
Trained 7	30,65	1254	38,4351	40,6087
	32,48	1254	40,72992	
	34,02	1254	42,66108	
Untrained	40,62	1254	50,93748	41,55756
	31,37	1254	39,33798	
	27,43	1254	34,39722	

Band Quantiation

Spor nr.	Applisert p	Mean	Pixels	Mean x Pixels/1000	Snitt av 3	Bakgrunn	Korrigert for bakgrunn
	Trained1	103,98	1190	123,7362	137,3657	35,606	101,7597
		121,08	1190	144,0852		0	0
		121,24	1190	144,2756		0	0
	Untrained1	109,9	1190	130,781	134,3827	33,056	101,3267
		122,78	1190	146,1082		0	0
		106,1	1190	126,259		0	0
	Trained2	105,32	891	93,84012	102,2036	16,67	85,53364
		114,7	891	102,1977		0	0
		124,1	891	110,5731		0	0
	Untrained2	123,84	891	110,3414	116,3676	19,97	96,39757
		130,62	891	116,3824		0	0
		137,35	891	122,3789		0	0
	Trained3	88,95	1080	96,066	89,6976	14,63	75,0676
		76,83	1080	82,9764		0	0
		83,38	1080	90,0504		0	0
	Untrained3	100,82	1080	108,8856	102,0852	14,3	87,7852
		87,37	1080	94,3596		0	0
		95,38	1080	103,0104		0	0
	Trained4	84,33	1080	91,0764	92,0304	8,98	83,0504
		103,08	1080	111,3264			
		68,23	1080	73,6884			
	Untrained4	127,69	700	89,383	86,44067	13,16	73,28067
		119,36	700	83,552		0	0
		123,41	700	86,387		0	0
	Untrained5	133,05	700	93,135	90,64767	11,235	79,41267
		141,21	700	98,847		0	0
		114,23	700	79,961		0	0
	Trained6	90,15	700	63,105	69,15767	7,49	61,66767
		99,28	700	69,496			
		106,96	700	74,872			
	Untrained6	151,22	888	134,2834	134,4432	32,97	101,4732
		163,78	888	145,4366		0	0
		139,2	888	123,6096		0	0
	Trained5	95,33	888	84,65304	88,95096	10,77	f
		105,52	888	93,70176		0	0
		99,66	888	88,49808		0	0
	Trained7	109,53	1254	137,3506	137,5011	40,6087	96,8924
		109,01	1254	136,6985		0	0
		110,41	1254	138,4541		0	0
	Untrained7	115,9	1254	145,3386	129,4922	41,5575	87,93472
		100,82	1254	126,4283		0	0
		93,07	1254	116,7098		0	0

Quantitation

Sample	Crude synaptosome
Antibody	NMDAR

Bakgrunnsavlesninger:

Spor nr.	Mean	Pixels	MeanxPixels/1000	Average
Trained 1	49,72	703	34,95316	36,86532
	51,67	703	36,32401	
	55,93	703	39,31879	
Untrained 1	51,08	703	35,90924	31,92089
	43,54	703	30,60862	
	41,6	703	29,2448	
Trained 2	12,83	756	9,69948	10,32192
	13,85	756	10,4706	
	14,28	756	10,79568	
Trained 3	15,43	756	11,66508	10,28664
	13,91	756	10,51596	
	11,48	756	8,67888	
Untrained 2	8,68	756	6,56208	4,88124
	4,18	756	3,16008	
	6,51	756	4,92156	
Trained 4	14,81	722	10,69282	10,69282
	19,64	722	14,18008	
	25,12	722	18,13664	
Untrained 4	29,75	722	21,4795	21,4795
	29,09	722	21,00298	
	28,36	722	20,47592	
Trained 5	16,43	798	13,11114	13,11114
	20,87	798	16,65426	
	24,98	798	19,93404	
Untrained 5	26,67	798	21,28266	21,28266
	27,95	798	22,3041	
	26,01	798	20,75598	
Untrained 3	53,44	828	44,24832	44,24832
	54,54	828	45,15912	
	50,53	828	41,83884	
Trained 6	61,78	720	44,4816	44,4816
	55,53	720	39,9816	
Untrained 6	39,02	720	28,0944	28,0944
	29,85	720	21,492	
Trained 7	60,23	1008	60,71184	60,71184
	51,97	1008	52,38576	
Untrained 7	63,37	1008	63,87696	63,87696
	64,14	1008	64,65312	

Band Quantitation

Spor nr.	Applisert prøve	Mean	Pixels	Mean x Pixels/1000	Snitt av 3	Bakgrunn	Korrigert for bakgrunn
2	Trained 1	153,01	703	107,56603	115,0881	36,86532	78,22281
3		160,76	703	113,01428		0	0
4		177,36	703	124,68408		0	0
5	Untrained 1	117,98	703	82,93994	69,6673	31,92	37,7473
6		91,58	703	64,38074		0	0
7		87,74	703	61,68122		0	0
2	Trained 2	100,51	756	75,98556	86,51412	10,32192	76,1922
3		128,03	756	96,79068		0	0
4		114,77	756	86,76612		0	0
5	Trained 3	94,41	756	71,37396	75,3858	10,28664	65,09916
6		101,72	756	76,90032			0
7		103,02	756	77,88312		0	0
	Untrained 2	18,05	756	13,6458	15,12	4,88124	10,23876
		20,61	756	15,58116			
		21,34	756	16,13304			
2	Trained 4	105,82	722	76,40204	85,49202	10,69282	74,7992
3		121,41	722	87,65802		0	0
4		128	722	92,416		0	0
5	Untrained 4	137,27	722	99,10894	96,80576	21,4795	75,32626
6		134,2	722	96,8924		0	0
7		130,77	722	94,41594		0	0
2	Trained 5	109,2	798	87,1416	83,4974	13,11114	70,38626
3		106,83	798	85,25034		0	0
4		97,87	798	78,10026		0	0
5	Untrained 5	91,92	798	73,35216	71,98226	21,28266	50,6996
6		97,14	798	77,51772		0	0
7		81,55	798	65,0769		0	0
2	Untrained 3	137,92	828	114,19776	117,7057	44,24832	73,4574
3		149,95	828	124,1586		0	0
4		138,6	828	114,7608		0	0
2	Trained 6	115,61	720	83,2392	81,864	44,48	37,384
3		111,79	720	80,4888		0	0
4				0		0	0
5	Untrained 6	72,81	720	52,4232	47,6712	28,0944	19,5768
6		59,61	720	42,9192		0	0
7				0		0	0
2	Trained 7	103,83	1008	104,66064	133,817	60,71184	73,1052
3		130,47	1008	131,51376		0	0
4		135,04	1008	136,12032		0	0
5	Untrained 7	108,91	1008	109,78128	105,9811	63,87696	42,10416
6		101,37	1008	102,18096		0	0
7		86,53	1008	87,22224		0	0

Quantitation

Sample	Crude synaptosomes
Antibody	Synaptophysin

Band intensity quantitation

	Background calculation				Spor nr.	Applisert prøve	Mean	Pixels	Mean x Pixels/1000	Snitt av 3 avlesn	Bakgrunn	Korrigert for bakgrunn
Spor nr.	Mean	Pixels	MeanxPixels/1000			Trained 1	131,95	736	97,1152	100,8369067	35,42	65,416907
Trained1	47,69	736	35,09984				137,78	736	101,40608		0	0
	48,79	736	35,90944				141,29	736	103,98944		0	0
	47,9	736	35,2544			Untrained 1	139,08	736	102,36288	106,4942933	36,39	70,104293
Untrained1	46,07	736	33,90752				143,42	736	105,55712		0	0
	48,35	736	35,5856				151,58	736	111,56288		0	0
	53,92	736	39,68512			Trained2	132,14	744	98,31216	97,11432	40,282	56,83232
Trained2	44,63	744	33,20472				130,63	744	97,18872		0	0
	55,97	744	41,64168				128,82	744	95,84208		0	0
	61,83	744	46,00152			Untrained2	147,2	744	109,5168	114,92568	52,784	62,14168
Untrained2	67,37	744	50,12328				146,39	744	108,91416		0	0
	69,82	744	51,94608				169,82	744	126,34608		0	0
	75,65	744	56,2836			Trained3	159,25	504	80,262	82,81728	34,43	48,38728
Trained3	47	504	23,688	34,4316			169,02	504	85,18608		0	0
	83,78	504	42,22512				164,69	504	83,00376		0	0
	74,17	504	37,38168			Untrained3	188,22	504	94,86288	86,06808	25,65	60,41808
Untrained 3	70,65	504	35,6076	25,6536			163,19	504	82,24776		0	0
	42,76	504	21,55104				160,9	504	81,0936		0	0
	39,29	504	19,80216			Trained4	154,63	570	88,1391	90,1588	27,68	62,4788
Trained4	46,21	570	26,3397	27,6868			155,1	570	88,407		0	0
	49,63	570	28,2891				164,79	570	93,9303		0	0
	49,88	570	28,4316			Untrained4	157,54	570	89,7978	93,6548	32,74	60,9148
Untrained4	50,64	570	28,8648	32,7446			171,01	570	97,4757		0	0
	62,09	570	35,3913				164,37	570	93,6909		0	0
	59,61	570	33,9777			Trained5	170,46	540	92,0484	94,0662	35,51	58,5562
Trained5	59,31	540	32,0274	35,5158			174,21	540	94,0734		0	0
	64,6	540	34,884				177,92	540	96,0768		0	0
	73,4	540	39,636			Untrained5	180,32	540	97,3728	97,8246	45,59	52,2346
Untrained5	90,41	540	48,8214	45,5922			181,89	540	98,2206		0	0
	86,73	540	46,8342				181,26	540	97,8804		0	0
	76,15	540	41,121			Trained6	173,2	540	93,528	91,3338	37,41	53,9238
Trained6	75,16	540	40,5864	37,4166			163,78	540	88,4412			
	68,28	540	36,8712				170,43	540	92,0322			
	64,43	540	34,7922			Untrained6	146,66	640	93,8624	92,6336	32,94	59,6936
Untrained 6	49,13	640	31,4432	32,94933			144,56	640	92,5184		0	0
	54,12	640	34,6368				143	640	91,52		0	0
	51,2	640	32,768			Trained7	152,62	640	97,6768	99,30026667	40,04	59,260267
Trained7	55,6	640	35,584	40,04693			155,09	640	99,2576		0	0
	62,94	640	40,2816				157,76	640	100,9664		0	0
	69,18	640	44,2752			Untrained 7	156,42	640	100,1088	101,8837333	42,25	59,633733
Untrained7	61,19	640	39,1616	42,2592			147,81	640	94,5984			
	57,12	640	36,5568				173,35	640	110,944			.
	79,78	640	51,0592									

Quantitation

Sample	Crude synaptosomes
Antibody	Syntaxin

Band Intensity Quantitation

	Background Calculation					Spor nr.	Applisert p	Mean	Pixels	Mean x Pixels/1000	Snitt av 3 a	Bakgrunn	Korrigert for bakgrunn
Spor nr.	Mean	Pixels	MeanxPixels/1000	Average			Trained 1	144,52	1404	202,9061	211,2224	31,59	179,63244
Trained 1	22,5	1404	31,59	31,59				155,11	1404	217,7744		0	0
	23,78	1404	33,38712					151,7	1404	212,9868		0	0
	21,67	1404	30,42468										
Untrained	21	1404	29,484	29,484			Untrained	147,13	1404	206,5705	196,56	29,484	167,076
	18,87	1404	26,49348					139,64	1404	196,0546		0	0
	16,42	1404	23,05368					133,23	1404	187,0549		0	0
Trained2	25,35	864	21,9024	21,9024			Trained 2	206,16	864	178,1222	175,6253	21,9	153,72528
	26,59	864	22,97376					202,96	864	175,3574		0	0
	23,45	864	20,2608					200,69	864	173,3962		0	0
Untrained2	22,92	864	19,80288				Untrained 2	201,93	864	174,4675	165,4877	21,9	143,58768
	22,75	864	19,656					192,51	864	166,3286		0	0
	23,78	864	20,54592					180,17	864	155,6669		0	0
Trained3	14,95	1190	17,7905	19,8254			Trained3	127,8	1190	152,082	163,026	19,8254	143,2006333
	17,1	1190	20,349					142,1	1190	169,099		0	0
	17,93	1190	21,3367					141,09	1190	167,8971		0	0
Untrained3	19,1	1190	22,729	21,7294			Untrained3	143,3	1190	170,527	165,6361	21,7294	143,9067
	18,13	1190	21,5747					136,65	1190	162,6135		0	0
	17,55	1190	20,8845					137,62	1190	163,7678		0	0
Trained 4	25,26	1656	41,83056	41,83056			Trained 4	136,86	1656	226,6402	223,0466	41,83	181,21664
	24,64	1656	40,80384					136,17	1656	225,4975		0	0
	24,61	1656	40,75416					131,04	1656	217,0022		0	0
Untrained4	25,09	1656	41,54904	41,54904			Untrained	138,27	1656	228,9751	213,8558	41,54	172,31584
	23,74	1656	39,31344					127,65	1656	211,3884		0	0
	23,34	1656	38,65104					121,5	1656	201,204		0	0
Trained5	19,78	1120	22,1536	20,94027			Trained5	175,29	1120	196,3248	196,0075	20,94	175,0674667
	18,16	1120	20,3392					174,65	1120	195,608		0	0
Untrained5	18,15	1120	20,328					175,08	1120	196,0896		0	0
	19,13	1120	21,4256	19,09227			Untrained5	164,01	1120	183,6912	175,0149	19,09	155,9249333
	16,6	1120	18,592					158,48	1120	177,4976		0	0
	15,41	1120	17,2592					146,3	1120	163,856		0	0
Trained 6	19,93	1365	27,20445	27,20445			Trained6	149,67	1365	204,2996	207,248	27,2	180,04795
	21,14	1365	28,8561					156,76	1365	213,9774		0	0
	22,54	1365	30,7671					149,06	1365	203,4669		0	0
Untrained	19,86	1365	27,1089	27,1089			Untrained	142,66	1365	194,7309	191,7734	27,1	164,6734
	19,99	1365	27,28635					138,31	1365	188,7932		0	0
	20,79	1365	28,37835					140,51	1365	191,7962		0	0
Trained7	17,12	1369	23,43728	24,31344									
	17,76	1369	24,31344				Trained7	152,09	1369	208,2112	207,7412	24,31	183,4311867
	18,4	1369	25,1896					153,04	1369	209,5118		0	0
Untrained7	18,93	1369	25,91517	26,72288				150,11	1369	205,5006		0	0
	19,74	1369	27,02406				Untrained7	138,71	1369	189,894	183,8658	26,72	157,1458267
	19,89	1369	27,22941					134,85	1369	184,6097		0	0
								129,36	1369	177,0938		0	0