Effect of voluntary exercise on \textit{BDNF/TrkB} gene expression and alcohol intake

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Title/Titel:
Effect of voluntary exercise on BDNF/TrkB gene expression and alcohol intake

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Abstract/Sammanfattning:
Voluntary wheel running is rewarding and believed to activate the same brain reward system as in alcohol and drug addiction. Brain-derived neurotrophic factor (BDNF), a well-known growth factor widely expressed in the brain, is modulated by both voluntary exercise and alcohol consumption. The aim of this study was to evaluate how voluntary exercise affects the expression levels of BDNF and its receptor TrkB in brain regions involved in positive and negative reinforcement. Additionally we wanted to evaluate the effect it may have on alcohol drinking behaviors in C57BL/6 mice, a mouse model which are naturally prone for engaging in voluntary exercise and voluntary alcohol consumption.

We found a small upregulation in DG and CA1 after three weeks of exercise, confirming findings by others, and a significant 3-fold downregulation of BDNF in NAc after both three weeks of exercise and exercise followed by a five week period of either ethanol intake or not. Interestingly, we here show a significant 100-fold increase in BDNF after exercise and a 120-fold increase after both exercise and alcohol consumption in amygdala, a region involved in regulation of anxiety-related behavior and negative reinforcement. Additionally a slightly lower 10-fold increase in BDNF was seen after exercise and a 15-fold increase after exercise followed by ethanol in prefrontal cortex, a structure contributing to reward-related behavior. Behaviorally, we could not either directly following exercise or at five weeks post-exercise detect any significant effect of wheel-running on depression-related behavior. However, we did find that exercise significantly increased the alcohol intake.

Keyword/Nyckelord:
Alcohol addiction, BDNF, C57BL/6 mice, Forced swim test, Gene expression, Neurotrophins, TrkB, Voluntary wheel exercise
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1 Abstract

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We found a small upregulation in DG and CA1 after three weeks of exercise, confirming findings by others, and a significant 3-fold downregulation of BDNF in NAc after both three weeks of exercise and exercise followed by a five week period of either ethanol intake or not. Interestingly, we here show a significant 100-fold increase in BDNF after exercise and a 120-fold increase after both exercise and alcohol consumption in amygdala, a region involved in regulation of anxiety-related behavior and negative reinforcement. Additionally a slightly lower 10-fold increase in BDNF was seen after exercise and a 15-fold increase after exercise followed by ethanol in prefrontal cortex, a structure contributing to reward-related behavior. Behaviorally, we could not either directly following exercise or at five weeks post-exercise detect any significant effect of wheel-running on depression-related behavior. However, we did find that exercise significantly increased the alcohol intake.

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2 List of abbreviations

AA- Amygdala
BDNF – Brain-derived neurotrophic factor
Ct – Cycle-threshold
EtOH – Alcohol / Ethanol
NAc – Nucleus Accumbens
PCR – Polymerase Chain Reaction
RT – Reverse Transcriptase
Trk – Tropomyosin-related kinase receptor
7,8-DHF – 7,8-dihydroxyflavone

AD – Alcohol dependence
CA1 – Cornu ammonis 1
DG – Dentate Gyrus
FST – Forced swim test
NTC – No template control
PFC – Prefrontal cortex
TrkB – Tyrosine kinase B receptor
qRT – Quantitative Real-Time
3 Introduction

Alcohol abuse and addiction
Alcohol dependence (AD) is a big health problem around the world, because of the difficulty to treat and the high rates of relapse (Davis 2008). According to the World Health Organization, alcohol is the second largest risk factor for disease in Europe, and results in 2.5 million deaths each year worldwide. Alcohol abuse is the cause of many social issues like violence, traffic accidents, child neglect and abuse (World Health Organization 2012). In addition, AD is often associated with underlying psychiatric disorders like anxiety, psychosis and mood disorders like depression (Bosse and Mathews 2011; Zanardini et al. 2011). The development of alcoholism depends on multiple factors, where the susceptibility to addiction depends on both genetic and environmental factors (Wong et al. 2011; Zanardini et al. 2011). Within the central nervous system, numerous neurotransmitters and other factors contribute to the addicted phenotype. Among those are the brain-derived neurotrophic factor (BDNF) whose expression can be modulated by alcohol and therefore appears to regulate reward as well as alcohol consumption behaviors (Bosse and Mathews 2011; Costa et al. 2011).

An addiction only occurs as a consequence of repeated exposure to an addictive substance, such as alcohol (Wong et al. 2011). Alcohol addiction is characterized by a three-step behavioral cycle that includes the anticipation of alcohol intake, intoxication or the drunken state and withdrawal with the negative effects of abstinence. In the beginning of an addiction, drug use is associated with positive reinforcement, where the pleasant effect of the drug leads to a drug-seeking behavior. After prolonged use, addiction becomes associated with negative reinforcement because the drug use becomes compulsive and its main effect is to suppress the emotional states caused by intoxication (Davis 2008). Chronic alcohol consumption causes neurobiological changes in neurotransmitter and neural signaling systems in the brain, including GABA, opiates, serotonin and dopamine (Davis 2008; Zanardini et al. 2011) and can also cause neurodegeneration (Davis 2008).

BDNF - background
Brain-derived neurotrophic factor (BDNF), is a secretory protein and a member of the neurotrophin growth factor family (Davis 2008; Zanardini et al. 2011). The neurotrophin family consists of structurally related growth factors and besides BDNF also include nerve growth factor (NGF), Neurotrophin (NT)-3 and NT-4/5. Neurotrophins are important to neuronal survival because they influence neuronal growth and differentiation during the development in both the central nervous system (CNS) and peripheral nervous system (PNS) (Davis 2008; Murray and Holmes 2011). BDNF and NT-4 are widely expressed throughout the brain, but most abundant in the hippocampus, cortex and cerebellum (Davis 2008; Bosse and Mathews 2011; Zanardini et al. 2011), whereas NGF is particularly important for the survival of sympathetic and cholinergic neurons in the CNS (Davis 2008). Production and secretion of BDNF are generally accomplished by neurons (astrocytes) and therefore regulated by neuronal activity (Bosse and Mathews 2011), but BDNF can also be produced by lymphocytes, platelets and vascular endothelium (Davis 2008).
Neurotrophins signal through and bind with high affinity to tropomyosin-related kinase (Trk) receptors. Although the Trks are not entirely selective the different neurotrophins prefer specific types of Trk receptors. NGF has a high affinity for TrkA, whereas NT-4 and BDNF bind TrkB, and NT-3 prefers TrkC. Neurotrophins can also bind with low affinity to the tumor necrosis factor receptor p75, inducing cell death and plasticity (Davis 2008; Murray and Holmes 2011). BDNF is produced as a pro-peptide that is cleaved by several proteolytic pathways into its mature form. It has been suggested that only the mature BDNF binds to the TrkB receptor, whereas the preferred receptor for pro-BDNF is p75 (Davis 2008).

BDNFs effects on neuronal growth, plasticity and survival are mediated through the activation of tyrosine kinase B (TrkB) receptors and its signaling pathways (Bosse and Mathews 2011). As for BDNF, the highest abundance of TrkB is found in the hippocampus, cortex and cerebellum (Davis 2008). Binding of BDNF to the TrkB receptor triggers dimerization and autophosphorylation of the receptor, that results in the activation of several signaling pathways involving mitogen-activated protein kinases (MAPK), phosphoinositide-3-kinase (PI3K) and phospholipase C (Figure 1) (Jang et al. 2010; Razzoli et al. 2011; Duman and Voleti 2012).

Figure 1. BDNF/TrkB signaling includes several signaling pathways. Binding of BDNF to TrkB induces dimerization and autophosphorylation of the receptor that leads to receptor activation. Several intracellular signaling pathways are activated including Ras/mitogen-activated protein kinases (MAPK), phosphatidylinositol-3 kinase (PI3K)/serine threonine kinase (Akt) and phospholipase C (PLC) pathways, which control different cellular functions, including neuroplasticity, survival and growth.

BDNF has been of therapeutic interest for a long time because of the many roles of the BDNF-TrkB system in memory, learning, brain plasticity and changes in this
BDNF system is associated with several disorders, like Alzheimer’s disease and Parkinson’s disease, and also in the development of AD (Jang et al. 2010; Andero et al. 2011). Since recombinant BDNF trials have not yielded any positive results because of some limitations like short half-life and poor delivery, there has been several attempts to find exogenous therapeutic agents that act as BDNF on the TrkB receptor. Recently a series of flavone compounds were discovered to activate the TrkB receptor, and the most potent compound being 7,8-dihydroxyflavone (7,8-DHF), an antioxidant found in fruits and vegetables (Jang et al. 2010; Andero et al. 2011). 7,8-DHF is a specific TrkB agonist that has the same function as BDNF to activate brain TrkB receptors. 7,8-DHF can activate the TrkB receptors and its signaling pathways in the absence of BDNF (Andero et al. 2011).

**BDNF in addiction**

BDNF has been shown to play a big role in the regulation of neurodevelopment, in homeostasis of the adult brain through neurogenesis and plasticity, and addiction (Davis 2008; Zanardini et al. 2011). There is also evidence to suggest that BDNF may play a protective role in the regulation of the reinforcing effects of alcohol, in both humans and animals (Bosse and Mathews 2011). Most studies show that the BDNF level in hippocampus is decreased with long-term exposure to alcohol. This suggests that the decrease of BDNF may be the consequence of neurodegeneration. Common biochemical substrates, like dopamine, that are used in memory-function are also associated with addiction. They cause long lasting alterations in the circuitry of the brain and these changes lead to compulsive drug seeking (Davis 2008). BDNF itself is important for long-term memory (Bekinschtein et al. 2008).

BDNF is an alcohol-responsive gene. In rodents both acute administration and voluntary consumption of alcohol activates the BDNF signaling pathway and increases BDNF mRNA levels. This shows that alcohol upregulates BDNF signaling that subsequently inhibits alcohol drinking. The positive effects of alcohol reinforcement are mediated mostly through the activation of dopaminergic pathways. BDNF has been shown to have a modulatory effect on dopamine transmission, and has the ability to alter the increased extracellular levels of dopamine that are induced by alcohol. This might be how BDNF can regulate alcohol drinking behavior (Ghitza et al. 2010; Bosse and Mathews 2011; Costa et al. 2011).

Susceptibility to develop alcohol dependence has been reported to be associated with polymorphisms of both the BDNF and TrkB gene (Bosse and Mathews 2011; Zanardini et al. 2011). Inhibition of TrkB receptors has been shown to increase the alcohol consumption in mice (Bosse and Mathews 2011). One well-known BDNF polymorphism Val66Met is clearly associated with alcohol use and also with a higher risk of relapse to drug-taking after treatment for alcoholism (Wojnar et al. 2009; Bosse and Mathews 2011; Costa et al. 2011; Zanardini et al. 2011). During withdrawal, BDNF has been suggested to have a role in neuroadaptation, because of the reports of increased BDNF levels after 24 hours of abstinence. Therefore it is possible that the levels of BDNF may be important for long-term alcohol abstinence (Costa et al. 2011).

Neurotrophins regulates the survival of the neurons in the brain, but if this regulated support does not function properly it may be involved in the susceptibility to alcohol dependence and in brain damage that often is the outcome from chronic alcohol intake.
Chronic alcohol exposure can impair the activation of transcription factors that regulate the expression of the BDNF gene. This indicates that the neuroprotective response to alcohol in the brain can be damaged by a lowering of BDNF levels due to chronic alcohol consumption. The reduced synthesis of BDNF therefore inhibits the protective mechanism and facilitates alcohol addiction (Costa et al. 2011; Zanardini et al. 2011). Regulatory impairment of the BDNF homeostasis may be an underlying factor of the progression and persistence of alcohol addiction (Crews and Nixon 2009; Costa et al. 2011).

Exercise and reward
Exercise has several health benefits, but also neural and cognitive effects in both humans and in laboratory animals. The brain reward system may play a role in wheel-running behavior, since voluntary exercise clearly seems to be rewarding. It is possible that the neural pathways that regulate the rewarding effects of food, sex and drugs of abuse also may be activated by voluntary exercise (Meeusen and De Meirleir 1995; Novak et al. 2012).

The reward system consists of several brain structures that are involved in mediating reinforcing and pleasurable behaviors. The most important pathway of the reward system, the mesocorticolimbic dopamine pathway, is formed in part by the ventral tegmental area (VTA) and NAc, but also includes prefrontal cortex and amygdala. Dopamine is released by dopamine-containing neurons in VTA onto neurons in the NAc and act as a signal for reinforcement. Consumption of alcohol or drugs of abuse leads to an increased release of dopamine in the VTA-NAc pathway, which leads to positive reinforcement of the rewarding behavior. A repeated exposure to drugs results in craving and addiction by producing plasticity and changing the level of neuronal excitation in this system. Reward and withdrawal affect the level of excitement by increasing the desire to consume more alcohol. The rewarding properties of drugs (morphine or cocaine) can be blocked by a direct infusion of BDNF into VTA, suggesting that BDNF may play a role in the VTA-NAc pathway in promoting addiction, craving and withdrawal (Davis 2008; Belujon and Grace 2011). It has been known for a long time that dopaminergic systems play a role in substance abuse, like alcohol addiction. The same systems are considered to be influenced by voluntary exercise, since an increase of the dopamine metabolism has been shown after just thirty minutes of wheel running (Meeusen and De Meirleir 1995; Novak et al. 2012).

Access to a running wheel also affects depression-like behaviors in rodents, by working as an antidepressant. This is most often assessed by the forced swim test (FST) where floating or immobility is seen as a measurement of depression (Cryan et al. 2005; Duman et al. 2008). It has been reported that voluntary exercise decreases the immobility, i.e. depression-like behavior, in rodents (Bjornebekk et al. 2005). An effect in BDNF is also seen after wheel running, where exercise increases the expression of BDNF mRNA and neurogenesis in hippocampus, the most studied brain area that are rich in growth factors (Neeper et al. 1996; Adlard et al. 2004; Novak et al. 2012). A less beneficial condition that also affects the levels of BDNF is stress. Most studies have demonstrated that stress and depression can lead to loss of neurons and cells in prefrontal cortex, amygdala and hippocampus, due to a decreased expression of BDNF (Duman and Monteggia 2006). However it has been shown that
chronic stress increases the levels of BDNF in the brain region NAc that are involved in the reward system (Krishnan et al. 2007).

**Experiments**
The first aim of this study was to look at the reward system after voluntary exercise and what kind of effects voluntary exercise (wheel running) has on both the BDNF and TrkB levels in different brain areas, i.e. Prefrontal cortex (PFC), Nucleus accumbens (NAc), Amygdala (AA), and two regions of the Hippocampus; dentate gyrus (DG) and cornu ammonis CA1. A second aim was to see if and how this would have an effect on depression-like behavior in mice. The third aim was to see if BDNF can be involved in the development of alcohol dependence in C57BL/6 mice, a model with the highest levels of voluntary ethanol drinking (Davis, 2008). Therefore, we investigated if the use of voluntary wheel exercise before exposure to alcohol (EtOH) in drinking water could have a protective effect against a development of alcohol dependency by an up regulation of BDNF. Really to see if natural reward (e.g. food, water and sex) from exercise, that induces neurogenesis, can protect the brain from neurodegeneration that occurs due to AD. The last and fourth aim was to observe if there were any changes in alcohol consumption after a period of alcohol deprivation (withdrawal).

**4 Material & methods**

**4.1 Animals**
Male C57BL/6 mice were obtained from Nova-SCB AB (Sollentuna, Sweden) and housed in a climate-controlled environment in the animal research facility at Linköping University. Mice were obtained at approximately 6-7 weeks of age and allowed a minimum of 1 week to acclimatize to the new environment. The mice were housed individually with free access to food and water, and maintained at standard conditions: 20 ± 2°C temperature, 33 ± 10% relative humidity and under a 12 h light/dark cycle (lights on at 7:00 am). Animal maintenance and all experimental procedures were approved by the Animal Care and Use Committee at Linköping University and performed in accordance to the Swedish regulations for animal experimentation.

**4.2 Experimental lay-out**
**Experiment 1: Voluntary exercise and brain BDNF/TrkB expression**
The experimental group (n = 10) were housed in standard rat cages with wire lid (Makrolon 365 x 205 x 145 mm, floor area 540 cm²) equipped with a running wheel (13 cm in diameter) and were given continual wheel access for three weeks, whereas the housing of the control group (n = 10) remained unchanged. The mice were checked and handled every other day to make sure that the wheels were working properly and to decrease stress. After three weeks of voluntary exercise, all mice were assessed for depression in a behavioural test, e.g. forced swim test, FST (see below). After the FST the mice were allowed back into their home cages for four days of voluntary exercise to lower their stress, before all mice, one by one, were euthanized by means of CO₂ and cervical dislocation (Figure 2).
Experiment 2: Voluntary exercise, alcohol intake and brain BDNF/TrkB expression
Two experimental groups were housed individually in rat home cages equipped with running wheels, and allowed to run for three weeks. After three weeks of voluntary exercise, one group (n = 12, Exercise + EtOH) were subjected to a two-bottle choice paradigm of increasing concentrations of EtOH solutions and water (H₂O) for five weeks. The other group (n = 12, Exercise) were not subjected to any EtOH, only two bottles with H₂O. Two additional groups were housed individually in standard mouse home cages with wire lid (Makrolon 265 x 205 x 140 mm, floor area 350 cm²) without a running wheel. After three weeks, one group (n = 12, EtOH) were subjected to a two-bottle choice paradigm of EtOH and H₂O, as previously described. The other group (n = 12, Control) acted as the control group, having no exposure to either a running wheel or EtOH. All mice were assessed for depression in a FST behavioural test, and allowed back into their home cage for a few days before being humanely euthanized by CO₂ followed by cervical dislocation (Figure 2). Each brain was dissected out and quickly frozen in isopentane on dry ice, and stored in a -80°C freezer.

<table>
<thead>
<tr>
<th>Exercise 3w</th>
<th>FST</th>
<th>end exp 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-bottle choice 5w</td>
<td>FST</td>
<td>end exp 2</td>
</tr>
</tbody>
</table>

Figure 2. The difference in the experimental lay-out for experiment 1 and experiment 2. In experiment 1, the exercising group had access to a running wheel for 3 weeks and then both groups were assessed in a FST. Animals were then euthanized directly following exercise. In experiment 2, the exercised groups had access to a running wheel for 3 weeks, then all wheels were removed and a five week long two-bottle choice paradigm was started. All groups were then assessed in a FST, before they were euthanized.

4.3 Behavioral testing
4.3.1 Voluntary exercise
Animals were allowed 24 hours free access to a running wheel in their home cage for three weeks. They were only deprived running for the short period of time it took to remove excessive sawdust under the wheel that stopped it from turning.

4.3.2 Alcohol intake
Two-bottle choice paradigm
Animals were subjected to a two-bottle choice paradigm, where two bottles were always present on each cage. The two groups with alcohol (EtOH) and H₂O, were subjected to different concentrations of EtOH, according to this scheme: Day 1-3 EtOH 3%, day 3-6 EtOH 6%, day 6-10 EtOH 9%, day 10-13 EtOH 12% and day 14-17 EtOH 15%. This was then followed by a period of absence/withdrawal, where the mice were denied any alcohol for four days before they were subjected again to EtOH 15% at day 22-38 (Figure 3). This was done to induce withdrawal, and to examine how this affected their drinking behaviour. Upon re-introduction of alcohol following a period of withdrawal, a deprivation induced increase in intake is generally observed. All the bottles were weighed every other day to record the alcohol drinking of the mice and the bottles position was altered to avoid place preference. For the two groups of mice that only had access to drinking water, the two bottles were weighed
once a week. One empty cage with two bottles of water was used as a control of spillage, since the bottles tended to drip when they were put back after weighing. These two bottles were weighed every day.

<table>
<thead>
<tr>
<th>3 weeks wheel or not</th>
<th>Days 1-3</th>
<th>Days 3-6</th>
<th>Days 6-10</th>
<th>Days 10-13</th>
<th>Days 14-17</th>
<th>Withdrawal</th>
<th>Days 22-28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3% EtOH</td>
<td>6% EtOH</td>
<td>9% EtOH</td>
<td>12% EtOH</td>
<td>15% EtOH</td>
<td>4 days</td>
<td>15% EtOH</td>
</tr>
</tbody>
</table>

*Figure 3. Schematic of two-bottle choice paradigm. Animals were allowed access to a running wheel or not for 3 weeks prior to continuous access to 15% alcohol.*

### 4.3.3 Forced swim test

Animals were acclimatized to the testing room for one hour before starting the tests. Four “drying-cages” were prepared with a couple of paper towels in the bottom. The cages were put half on and half off a heating pad (HabiStat Heat Mat, Euro Rep Ltd, UK). Glass beakers (low form, 5000mL) were filled with approximately 3500mL of 22-27°C water (on average 25°C). Mice was put gently in the water and assessed for six minutes. The behavior sampling was done by examining the mice every five seconds and categorizing it as swimming (I) or as immobile (-). A mouse was defined to be immobile when it ceased struggling and remained floating motionless in the water making only movements necessary to keep its head above the water, typically only small movements with one or two limbs (Eisch et al. 2003). After swimming the mouse was removed from the water, quickly dried using a towel and put into a “drying-cage”. Possible waste products were removed from the water and the next set of mice was assessed in the FST. Two mice, one subjected to voluntary exercise and one that was not, were allowed to swim in separate beakers, at a time. All swim tests were filmed using a digital video recorder (Canon LEGRIA FS406), and the recordings were later used to evaluate the scoring of the behaviors.

### 4.4 Brain tissue collection and dissection

The brain was dissected out and put in cold NaCl solution (Sodium chloride Braun 9mg/ml, B Braun, Germany) for a few seconds. In experiment 1, each brain was then placed in a mouse brain matrix (Stainless steel Mouse Brain Slicer Matrix, 1.0 mm coronal slice intervals, Zivic instruments, USA), and the cerebellum was removed using a razorblade. The rest of the brain was placed on and covered with dry ice for a quick freeze. The frozen brain were packed in parafilm (Pechiney Plastic Packaging Company, USA), with correct marking, and put in a Styrofoam box with dry ice. In experiment 2, the whole brain was dissected out, including the cerebellum. The brains were then stored in a -80°C freezer. In order to retrieve certain brain areas for further analysis, the brains were sliced in a cryostat (Leica 3050). Control slices were stained with cresyl violet, in order to better visualize the location of the different brain regions, according to this protocol; cresyl violet 1-2 min, rinsed with deionized water, dried first in 70% EtOH 1-2 min and then in 99,5% EtOH 1-2 min and then fixated in xylene 1-2 min. Prefrontal cortex (PFC) cingulate 1+2, nucleus accumbens (NAc) (Figure 4 A), amygdala (AA) (Figure 4 B) and two areas in the Hippocampus; dentate gyrus (DG) (Figure 4 B) and cornu ammonis (CA1) (Figure 4 C), were punched out, collected in Eppendorf tubes and stored in a -80°C freezer until they were used for
tissue homogenization and RNA isolation. RNA was then reversely transcribed into cDNA, and used for gene expression analysis by quantitative real-time PCR.

Figure 4. Visualization of the specific brain regions according to the Mouse brain atlas. PFC (A black circle), NAc (A red circles), AA (B red circles), DG (B black circle), and CA1 (C black rectangles) (Franklin and Paxinos, 2007).
4.5 RNA isolation from homogenized tissue

Micropunched tissues of left side brain regions of PFC, NAc, AA and DG (as dorsal sample) and CA1 (as ventral sample) were homogenized with a TissueLyser in order to isolate and purify RNA by using the RNAeasy® Micro Kit (cat. no. 74004, Qiagen®), following the manufacturers “Purification of total RNA from animal and human tissue” protocol. The RNA concentration and quality were determined using a Nanodrop ND-1000 Spectrophotometer, in order to calculate how much RNA to use for the transcription of cDNA. The RNA was stored in a -80 freezer.

4.6 Reverse Transcriptase PCR

RNA was reversely transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (200 reactions) from Applied Biosystems. Briefly, total RNA was mixed with RNase-free water, 10X RT Buffer, 10X RT Random Primers, 25X dNTP Mix (100 mM), and MultiScribe® Reverse Transcriptase. The samples were run, in the Veriti® 96-well Thermal Cycler, a 9700 GeneAmp® PCR instrument from Applied Biosystems, according to the BDNF setup; 25°C for 10 min, 37°C for 60 min, 85°C for 5 s. The cDNA was then diluted 1:10 by mixing 20 µL cDNA with 180 µL RNase free water, and stored in a -80 freezer.

4.7 Quantitative Real-Time PCR

Quantitative Real-time PCR was used to measure the levels of gene expression of BDNF, TrkB and the housekeeping gene β-actin in the different brain areas; PFC, NAc, AA, DG and CA1. All samples, including NTC as control, were run in duplicates for each gene on Fast optical 96-well reaction plates. Real-time PCR was performed on a 7900HT FAST Real-Time PCR system from Applied Biosystems using TaqMan® Fast universal PCR Master Mix (2X) No AmpErase® UNG according to the manufacturer’s instructions. The gene expression assays used were; BDNF: Mm 04230607_s1; TrkB (Ntrk2): Mm 00435422_m1 and β-actin: Mm 00607939_s1. Dilution curves for all probes used were performed to assure the quality and specificity of the assay. Gene expression was calculated using the $\Delta\Delta\text{Ct}$ method. The gene expression for each gene was normalized to the corresponding expression of β-actin, and the $2^{\Delta\Delta\text{Ct}}$ values for the experimental group(s) were compared to the values of the control group and demonstrated as fold difference.

4.8 Data analyses

Statistical analysis was performed for each brain area using STATISTICA 10 analytical software. Gene expression data was analyzed with Student’s t-test in Experiment 1 and one-way ANOVA in Experiment 2 (due to multiple groups). Drinking data was analyzed using a repeated-measures two-way ANOVA. The level of significance was set at p<0.05. All data are presented as mean ± SEM.
5 Results

5.1 Impact of voluntary exercise on BDNF and TrkB gene expression

We tested how voluntary wheel running activity would affect the mRNA levels of BDNF and its receptor TrkB following three weeks of exercise. Our hypothesis was that exercise, that most likely triggers the brain reward system and the dopaminergic pathways, would upregulate the expression of BDNF, at least in the two hippocampal areas. TrkB is most likely upregulated if BDNF is downregulated, in order for the receptor to be able to use the limited supply of BDNF. Some studies have however shown that TrkB also can be upregulated by exercise (Gomez-Pinilla et al. 2002; Klintsova et al. 2004).

Our expression study showed a significant 3-fold decrease of the BDNF expression in NAc compared to controls (Figure 5B). There were no significant difference in the expression of BDNF in the AA, PFC or DG (Figure 5A, C and D). For TrkB, no significant changes could be observed between running and controls in any examined brain-region (Figure 5A-E). A trend-level decrease could be seen in the DG, but this failed to reach statistical significance.

The only upregulation of BDNF expression directly following exercise-exposure could be seen in the CA1. Here, a 1.8-fold upregulation was demonstrated (Figure 5E). Hippocampus is rich in growth factors and is therefore the most studied brain region when it comes to BDNF. Many studies have shown that wheel running has an effect on BDNF expression, where exercise increases the expression of BDNF mRNA and neurogenesis in hippocampus, especially in DG, but also in CA1 (Neeper et al. 1996; Adlard et al. 2004; Novak et al. 2012).

These singled housed animals were euthanized directly after exposure to running wheels, so the results represent direct measurements after voluntary exercise.
Figure 5. Gene expression by quantitative real-time PCR (qRT PCR) of brain-derived neurotrophic factor (BDNF) and tyrosine kinase B receptor (TrkB) in PFC (A), NAc (B), AA (C), DG (D) and CA1 (E) following 3 weeks of voluntary exercise. qRT PCR of BDNF mRNA showed a significant 3-fold downregulation in NAc, and a trend level downregulation in AA compared to controls. However, a 1.8-fold upregulation of BDNF gene expression was demonstrated in CA1 after three weeks of exercise compared to the controls. No significant changes of BDNF gene expression in PFC, or of TrkB in either brain regions were observed. Each value represents the mean ± SEM. *p<0.05, vs. control.
5.2 Impact of voluntary exercise on depression-like behavior

To observe the impact of voluntary exercise and BDNF on depression-like behaviour, the mice were observed in a forced swim test (FST) following running wheel exposure (Figure 6). Our hypothesis was that the mice that were given access to continuous wheel running would have an increased swim time compared to the control mice.

However, our results from the FST showed no obvious difference in the total swimming time for the exercised mice and the non-exercised mice (Figure 6).

![Forced swim test](image)

*Figure 6. Total swimming time (sec.) in the forced swim test (FST). Testing was performed after 3 weeks of voluntary exercise (n=10) or no exercise/control (n=10). Mice were videotaped for the duration of the swim test (6 min) and scored by an observer. Exercised mice and control mice showed no significant difference in the latency of swimming. Each value represents the mean ± SEM.*
5.3 Impact of voluntary exercise and ethanol consumption on BDNF and TrkB gene expression

In addition to analysing how voluntary wheel exercise would influence the expression of BDNF and TrkB, we also tested what effect alcohol consumption with or without preceding running wheel exposure would have on BDNF and TrkB expression in PFC, NAc and AA. Our hypothesis was that alcohol alone may downregulate expression of BDNF, as a result of neurodegeneration. As for the mice subjected to exercise followed by alcohol, our hypothesis was that exercise either would have a protective effect against any influences by alcohol, or that voluntary exercise and subsequent alcohol intake would act together to further stimulate the reward system and therefore upregulate BDNF further.

Our results showed a 3-fold downregulation of BDNF in NAc after exercise (Figure 7 C), which is consistent with our previous findings (Figure 5 B). However in PFC, a 10-fold upregulation of BDNF was seen in mice subjected to exercise, and a 15-fold upregulation in the group with both exercise and alcohol consumption (Figure 7 A). For AA an even higher upregulation of BDNF expression after exercise as well as after combined exercise and alcohol consumption could be seen (Figure 7 E). Ethanol itself did not significantly affect BDNF expression. No significant changes in the expression levels of TrkB were observed for any of the three brain regions (Figure 7 B, D, and F).

These singled housed mice were euthanized after a five week period from the end of the voluntary exercise.
Figure 7. Gene expression by qRT PCR of BDNF and its receptor TrkB in PFC (A and B), NAc (C and D) and AA (E and F) following either 3 weeks of exercise, 5 weeks of continuous access to ethanol or 3 weeks of exercise followed by 5 weeks of continuous access to ethanol. qRT PCR of BDNF, showed a 10-fold upregulation after exercise and a 15-fold upregulation after exercise followed by ethanol consumption in PFC compared to controls. An even higher BDNF upregulation after exercise and exercise followed by ethanol consumption were demonstrated in AA. Additionally, similarly to the acute effect of exercise, gene expression of BDNF in NAc showed a 3-fold downregulation after exercise and, after exercise followed by ethanol consumption. No significant changes for BDNF in NAc after ethanol consumption, or for TrkB in either brain regions were observed. Each value represents the mean ± SEM. *p<0.05 vs. control, **p<0.01 vs. control, and ***p<0.001 vs. control.
5.4 Impact of voluntary exercise on ethanol consumption

After three weeks of wheel exercise C57BL/6 mice had continuous access to alcohol in a two bottle choice paradigm. During the first two weeks we introduced them to increasing concentrations of alcohol, followed by four days of withdrawal and then reintroduced them to 15% alcohol for a little more than two weeks. Our hypothesis was that exercise would have a protective effect against drinking so that the exercised mice would drink less alcohol than the non-exercised, or that exercise instead would upregulate the reward system so that they would drink more to be able to continue to stimulate the reward system. With the withdrawal period our hypothesis was that this would affect the alcohol drinking in both of those two groups, by increasing their intake as a result of abstinence and craving for ethanol.

Our results showed that C57BL/6 mice, on average had a higher intake of alcohol after exercise, and that they also had an increased intake after the withdrawal period (Figure 8 and Table 1). The increased ethanol intake of 9% EtOH is an indication that the animals have been introduced to drinking and started to build up a craving for ethanol. We can clearly see that the exercised mice had a higher ethanol intake. Since running clearly shares many of the reinforcing properties with addictive substances, it is not surprising that running appears to increase the ethanol consumption.

![Ethanol intake graph](image)

**Figure 8.** Alcohol intake in a two-bottle choice paradigm with continuous access after 3 weeks of voluntary exercise compared to non-exercised mice. Increasing concentrations of alcohol solutions were first administered (2-3 days/conc.) before a 4 day period of withdrawal, after which they were subjected to continuous access to a 15% alcohol solution (3-4 days/intake measurement). On average the exercised mice drank more ethanol than the non-exercised. After withdrawal the intake for both groups of mice were elevated, but the exercised mice drank on average more. Intake was measured over 24 h and calculated as an average value for each concentration period, and shown as g/kg (mean ± SEM.; n=12/group). *p<0.05 vs. control, and **p<0.01 vs. control.
Table 1. Average consumption of different concentrations of ethanol by C57BL/6 mice subjected to only ethanol or exercise followed by ethanol. On average the exercised mice consumed more ethanol than the non-exercised. A clear increase could be seen after withdrawal for both exercised and non-exercised mice. Each value represents the mean ± SEM.

<table>
<thead>
<tr>
<th>Ethanol intake for the different concentrations</th>
<th>EtOH (g/kg)</th>
<th>Exercise + EtOH (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3% EtOH</td>
<td>6.1 ± 0.6</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>6% EtOH</td>
<td>6.6 ± 0.4</td>
<td>6.3 ± 0.3</td>
</tr>
<tr>
<td>9% EtOH</td>
<td>16.7 ± 1.2</td>
<td>16.8 ± 1.7</td>
</tr>
<tr>
<td>12% EtOH</td>
<td>15.8 ± 0.7</td>
<td>17.8 ± 0.9</td>
</tr>
<tr>
<td>15% EtOH</td>
<td>11.4 ± 0.5</td>
<td>14.0 ± 0.7</td>
</tr>
<tr>
<td>15% EtOH after withdrawal</td>
<td>13.3 ± 0.4</td>
<td>17.2 ± 0.8</td>
</tr>
</tbody>
</table>
5.5 Impact of voluntary exercise and ethanol consumption on depression-like behavior

As for the acute wheel running experiment, we assessed depression-like behaviour using the FST in animals with or without exposure to running wheel combined with access to water or alcohol (Figure 9). Our hypothesis was, as previously, that exercised mice would have an increased swim time compared to control mice.

The results show a slight, non-significant increase in the swim time for the mice subjected to exercise or alcohol or both, compared to the controls.

![Forced swim test](image)

**Figure 9.** Latency of swimming (sec.) on one day of forced swim test (FST). Testing was performed after no exercise/water (n=12), voluntary exercise/water (n=12), no exercise/ethanol (n=12), or exercise/ethanol (n=12). Mice were videotaped for the duration of the swim test (6 min) and scored by an observer. Each value represents the mean ± SEM.
6 Discussion

Almost all studies of the involvement of voluntary wheel exercise on BDNF mRNA expression levels have been done in hippocampus, where the main focus has been on the ability of BDNF to affect neural plasticity, enhance learning and memory, and regulate stress and depression-like behaviors. There are in fact very limited studies on exercise-induced effects on BDNF in other brain areas than hippocampus (Davis 2008; Gustafsson et al. 2011). This is not very surprising since hippocampus is a brain area rich in growth factors, like BDNF, that are highly responsive to wheel running, thus increasing their expression and neurogenesis in this area (Novak et al. 2012). This is, to our knowledge, one of very few studies where exercise-induced effects on mRNA levels of both BDNF and its receptor TrkB has been investigated in different brain areas other than the hippocampus. Here we have looked at prefrontal cortex, nucleus accumbens and amygdala as well as two different areas of the hippocampus – dentate gyrus and CA1.

Our results show that voluntary exercise acutely upregulates BDNF mRNA expression in the hippocampus (CA1). The expression of TrkB however was not affected by exercise. BDNF protein can however come from the ventral tegmental area (VTA) since VTA-NAc forms a pathway that is involved in the reward system (Eisch, Bolanos et al. 2003).

Mice that are allowed to perform voluntary exercise show less depression-like behaviors and therefore tend to have a higher latency of swimming than non-exercised mice. This is due to the positive reinforcement they get from exercising and they are less likely to feel depressed and give up trying to escape from the water (Bjornebekk et al. 2005; Cryan et al. 2005; Novak et al. 2012). Maybe this upregulation of BDNF was too small to affect the depression-like behavior for these mice. FST is also somewhat difficult to assess and these mice may also have been more susceptible to stress, that would have influenced the outcome of this test.

BDNF has been reported to be an alcohol-responsive gene and most of the research on ethanol-induced effects on BDNF has also been done in hippocampus (Davis 2008). Short-term alcohol consumption has been shown to decrease BDNF mRNA levels, maybe as a result of the system trying to compensate with homeostasis or repair, whereas withdrawal may cause an upregulation, since presence of alcohol may inhibit the receptor of transducing its signals mediated by BDNF (Davis 2008). However, other studies have shown that both acute administration and voluntary consumption of alcohol activates the BDNF signaling pathway and increases BDNF mRNA levels (Ghitza et al. 2010; Bosse and Mathews 2011; Costa et al. 2011). The differential results obtained in these studies point to a complex relationship between BDNF and reward-related behaviors, with changes in expression being dependent on time-point as well as brain region examined. Here we directed our attention towards other brain areas – prefrontal cortex, nucleus accumbens and amygdala – and what effect voluntary exercise would have on alcohol drinking behaviors in C57BL/6 mice. Most studies on the effect of voluntary exercise on alcohol consumption, in mice and other rodents, have been done by introducing both wheel running and alcohol
simultaneously, in order to observe the relationship between natural reward and alcohol drinking. When these mice were denied any access to alcohol, their wheel running behavior increased. Interestingly, wheel running decreased upon the reintroduction of alcohol (Ozburn et al. 2008; Ehringer et al. 2009; Novak et al. 2012). We instead first introduced male C57BL/6 mice to three weeks of voluntary wheel exercise followed by five weeks of continuous access to alcohol, including a withdrawal period of four days. An interesting part was to see if exercise somehow would protect against the neurodegenerative effect of alcohol consumption, in terms of affecting the mRNA levels of BDNF and TrkB.

Our results showed that mice allowed utilizing of a running wheel for three weeks prior to continuous access to 15 % ethanol have a higher alcohol intake than control animals. Maybe this increased intake is a way of receiving the same level of reward after that running has triggered the natural release of dopamine/endorphins (natural reward). These enormously high expression levels of BDNF could be the result of an upregulation due to withdrawal. In fact BDNF has been suggested to have a role in neuroadaptation during withdrawal, because of the reports of increased BDNF levels after 24 hours of abstinence. It is possible that this may be important for long-term alcohol abstinence (Costa et al. 2011). Wheel running for rodents is clearly reinforcing and rewarding, but it may also be addictive as alcohol. Studies in rats have shown that wheel running can potentiate alcohol preference and increase their ethanol intake (Werme et al. 2002).

Some considerations must be made, on the difference between our first experiment, with only exercise, and our second experiment, with both exercise and alcohol consumption. During the first experiment, the mice were either subjected to wheels or not for three weeks before their behavior were tested in a FST and then animals were euthanized directly following access to running wheel. During the second experiment the mice were after three weeks of exercise, either subjected to a two-bottle choice paradigm with alcohol or only water for five weeks before being evaluated in a FST and euthanized (Figure 2). During these five weeks no animals were allowed any wheel exercise, and this could also be seen as a sort of withdrawal period from natural reward which could have influenced the high BDNF expression levels in PFC and AA after exercise in experiment 2.

In order to strengthen our trend results for both the running study and the drinking study, more samples must be run in qRT-PCR. It would also be of interest to look at the protein levels by using ELISA since mRNA expression changes may not necessarily result in changes in protein levels. However, mRNA levels may here be good indicator, since mRNA expression and protein levels for neuropeptides usually are comparable. Since we see a huge effect of exercise and exercise followed by ethanol consumption on BDNF expression in amygdala, it would be interesting to do an in situ hybridization to look at the neuronal activation and see if this could actually be the reason for our results. The 100-fold increase in BDNF gene expression is very high but plausible. It has been shown in other studies using real time PCR that this level of change can be detected (Dessie et al. 2007), however this has mainly been done in other kinds of tissues than brain. The results should therefore be interpreted with caution, although the results are supported by the maintained decrease detected in the NAc as well as the unchanged level of TrkB gene expression seen in the same material. This indicates that the very high fold-change is most likely not due to
methodological error. Still, the assay-results will need to be confirmed in a new set of animals.

Another interesting aspect would be to investigate the activity of the TrkB receptor, by using the agonist 7,8-DHF which activates the high affinity BDNF-receptor TrkB in the central nervous system (CNS). This would be done to see if the activation of TrkB receptors by 7,8-DHF would lead to an upregulation of BDNF and if 7,8-DHF could be a good therapeutic compound for depression, anxiety, or for example other brain disorders that break down neurons. It would also be interesting to see if voluntary exercise can increase the BDNF expression levels in heterozygote BDNF mice (BDNF<sup>+/−</sup>), that have a 50% reduction of BDNF expression. Homozygote BDNF<sup>−/−</sup> mice die after some weeks because of their inability to perform neurogenesis, whereas heterozygote BDNF<sup>+/−</sup> with a 50% reduction of BDNF levels can survive (Bosse and Mathews 2011).

### 6.1 Conclusions

Our results show a region as well as time-dependent plasticity for BDNF expression within the brain reward system following exercise as well as following exercise combined with alcohol intake. This possibility of the involvement of BDNF in regulation of alcohol reward is further strengthened by the recent finding of Koo et al. (2012) demonstrating that BDNF is a negative modulator of morphine action. The possibility of regulation of BDNF expression by exercise and that the changes in BDNF expression following exercise may contribute to subsequent effects of alcohol needs to be further established, however the results obtained here do implicate BDNF to be part of the mechanisms underlying alcohol dependence.

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8 References


