

**PROTEIN LEVELS OF BRAIN-DERIVED NEUROTROPHIC
FACTOR IN THE HIPPOCAMPUS OF LOW/HIGH AERO-
BIC CAPACITY RATS**

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ABSTRACT

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Hippocrates and Plato have first documented the connection between a healthy mind and body, during a period which launched analytical thinking and philosophy in Ancient Greece. Modern research has also indicated the contribution of an active lifestyle to enhanced brain performance and decreased incidence of neurodegenerative diseases and mood disorders such as Alzheimer's disease and depression respectively. This has been hypothesized to emerge through mechanisms which are enhanced by exercise and contribute on brain plasticity and health.

The Neurotrophins hypothesis implicates several molecules in brain plasticity and healthy aging. Among them, Brain-derived Neurotrophic Factor (BDNF) has been shown to increase its expression levels in the most plastic regions of the brain in an activity-dependent manner both in development and adulthood, proposing its significance throughout lifespan.

We used Immunohistochemistry (IHC) to investigate the expression of BDNF protein levels in the hippocampi of Low/High aerobic capacity rats which have proposed a model of sedentary and active lifestyle. Rats were also subjected to Rotarod motor learning test where the high capacity group showed increased baseline performance and faster learning than the low capacity one. IHC in the hippocampi of the animals didn't detect any significant differences of BDNF protein levels in CA1 and CA3 areas. The failure to detect differences is believed to emerge due to the sedentary lifestyle in both groups, suggesting that genetic background may not determine the levels of the protein in sedentary conditions.

Keywords: BDNF, hippocampus, synaptic plasticity, muscle activation, aerobic capacity, metabolic syndrome, neurodegenerative disorders, depression

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LIST OF ABBREVIATIONS

ACC-beta	Acetyl coenzyme A carboxylase β
AD	Alzheimer's Disease
AMP	Adenosine Monophosphate
AMPK	AMP-activated Kinase
ARMS	Ankyrin-Rich Membrane Spanning domain
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
CAMKII	Calcium/Calmodulin protein Kinase II
CBF	Cerebral Blood Flow
CNS	Central Nervous System
CREB	cAMP-Response Element Binding
DG	Dentate Gyrus
DHA	Docosahexaenoic Acid
DMI	Dendritic Modification Index
EE	Environmental Enrichment
EPSPs	Excitatory Postsynaptic Potentials
ERK	Ras/Extracellular signal-regulated kinase
FAO	Fatty Acid Oxidation
FST	Forced Swim Test
GFP	Green Fluorescent Protein
HCR	High Capacity Runner
HFS	High Frequency Stimulation
IHC	Immunohistochemistry
L-LTP	Late-phase Long Term Potentiation
LCR	Low Capacity Runner
LFS	Low Frequency Stimulation
LTM	Long Term Memory

LTP	Long Term Potentiation
mBDNF	mature BDNF
MPFC	Medial Prefrontal Cortex
MRI	Magnetic Imaging Resonance
MWM	Morris Water Maze test
NGF	Nerve Growth Factor
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4
NTs	Neurotrophins
PA	Physical Activity
PBS	Phosphate-Buffered Saline
PC12 cells	cells derived from rat pheochromocytoma
PCr	Phosphocreatine
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein Kinase C
PLCγ	Phospholipase C γ
PNS	Peripheral Nervous System
SNP	Single Nucleotide Polymorphism
STZ	Streptozotocin
T2D	Type II Diabetes
TMS	Transcranial Magnetic Stimulation
TNF	Tumor Necrosis Factor
Trks	Tyrosine kinase receptors
TRPC3/6	Transient Receptor Potential Canonical subfamily 3/6
UCP-2	Uncoupling Protein 2
uMtCK	ubiquitous Mitochondrial Creatine Kinase
VE	Voluntary Exercise

1 INTRODUCTION

Documentation of the beneficial effects of exercise on brain health has been available since 5th Century BC, when Plato and Hippocrates stated the importance of a physically active lifestyle on “mind” performance and health.

In modern medicine the “mind” concept has been replaced from a set of processes carried out from the most complicated biological organ, the brain. Still far from revealing the exact mechanisms underlying brain functions, science has discovered and described numerous mechanisms related to them. The exponential advancing is characterized by the contribution of different sectors of science which develop methods enabling researchers to go deeper in the mysteries of life.

Methods in molecular Biology have rapidly increased the quantity and quality of the experimental setups available in Exercise Physiology and other health-related sectors of science. Investigation of the relationship between physical activity and brain health, which is the main purpose of the present work, can be more effective than any time before given the advances in Neuroscience and the already available literature which suggests a huge contribution of physical activity (PA) in brain plasticity and health.

From the exercise physiology point of view, acute and long term exercise evokes adaptations in several systems in both animals and human. The beneficial adaptations emerge through changes in mechanisms responsible for maintaining homeostasis and increase the possibilities of survival in a constantly changing environment. Exercise serves as a set of environmental stimuli which can potentially alter whole-body physiological properties.

Additionally, the hunting-based life of our ancestors has maybe contributed to mechanisms bridging physical activation and brain performance due to the involvement of both systems in acquiring and utilizing of food, a vital process to ensure survival. Natural selection and

evolution have the potential of imprinting favorable genetic changes originating from adaptations evoked by environmental factors.

Molecules and processes proposed to mediate the effects of physical activity on cognition and brain health will be discussed below with emphasis given on Brain-derived Neurotrophic Factor (BDNF), hippocampus functioning and skeletal muscle contraction.

A model of high/low aerobic capacity rats, which resulted from selective breeding (for this trait) of animals originating from the same strain, has been used in the present study to investigate the learning differences in relationship with aerobic capacity. Previous studies on this model have shown the contribution of the genetic component in aerobic capacity as a complex but inherited trait, so the present study aims in investigating molecules that have been implicated to learning and memory and seem to be up regulated by exercise.

2 NEUROTROPHINS

Neurotrophins (NTs) have emerged as candidate signaling molecules, mediating synaptic plasticity, supporting neuronal connectivity and protecting neurons from cell death (Lewin and Barde, 1996) . Their roles in Central Nervous System (CNS) and Peripheral Nervous System (PNS) have been well described compared to other trophic factors (Chao et al., 2006) . The family consists of 5 structurally related proteins: Nerve Growth Factor (NGF), Brain-derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3) and Neurotrophin-4/5 (NT-4/5), which share 50 % similar amino acid sequence. NTs exert their functions through 2 classes of receptors. All NTs bind with low affinity ($K_d \approx 10^{-9}$ M)¹⁰ to p75 receptor which belongs to the Tumor Necrosis Factor (TNF) super family of receptors and signals cell apoptosis (Lewin and Barde, 1996; McAllister et al., 1999). The beneficial effects of NTs binding occur due to their selective docking to the tyrosine kinase receptors (Trk) with high affinity ($K_d \approx 10^{-11}$ M)⁶ . Even though they are selective (NGF for TrkA, BDNF and NT-4/5 for TrkB and NT-3 for TrkC) they are not specific as for example NT-3 can bind to TrkA (but with lower affinity than to TrkC). (Pattarawarapan and Burgess, 2003) The phylogeny of NTs and Trk receptors suggest a parallel evolution of their sequence and is illustrated in figure 1.

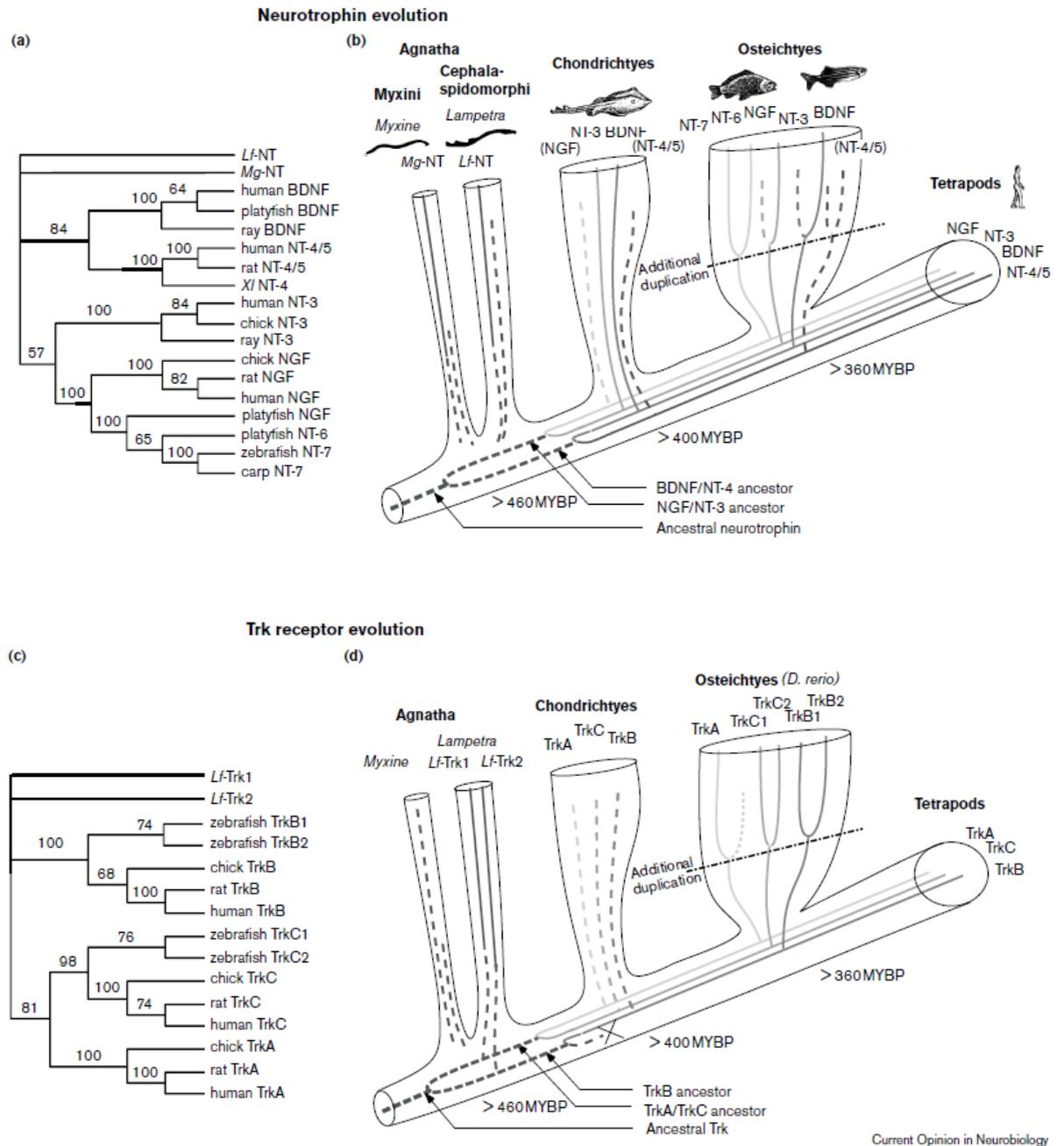


FIGURE 1. Phylogenetic relationship of NTs and their receptors from jawless fish to vertebrates, (adapted from Hallböök, 1999)

NTs are implicated to many biological functions in health and disease (Chao et al., 2006). Considering the 2 opposite pathways they can activate and the different tissues where they are expressed at, one can easily understand the complexity and possible combinations of functions that occur.

This dissertation focuses on the mRNA expression, protein level and localization of BDNF in the hippocampus and soleus muscle of rats. Therefore, other NTs will be briefly described. Contrarily, they will be further discussed when interactions and/or relationships with BDNF are cited.

2.1 Nerve Growth Factor

The neurotrophic properties of NGF were first described by Rita-Levi Montalcini (Levi-Montalcini and Hamburger, 1951) who was awarded with the Nobel Prize in Physiology or Medicine in 1986. Her group's experiments in chick embryos were crucial for the continuation and expanding of this sector of research, especially after the observations and further validation of the trophic effects of NGF in PNS. It was shown that NGF inhibits nerve terminals' death evoked pharmacologically in sympathetic ganglia of rodents treated with 6-hydroxydopamine, a substance known to destroy nerve terminals. Similarly, blocking of axonal transport, which leads to death the large majority of sympathetic nerve cells during the most active phase of differentiation and growth, by vinblastine had no effect in rodents which were treated with NGF. Further experiments supported the notion that NGF promotes the outgrowth and differentiation of sensory and sympathetic neurons leading to the expansion of the NT's field of research. (Levi-Montalcini, 1987).

After its discovery, NGF has been well studied for decades, and further evidence has occurred about its beneficial effects in PNS as well as in CNS. NGF and its receptors are expressed in different neural and non-neural tissues during all phases of life (development, adulthood, aging), striking the importance of the molecule on health and disease (Huang and Reichardt, 2001; Sofroniew et al., 2001; Salehi et al., 2004).

2.2 Brain-derived Neurotrophic Factor

BDNF followed the discovery of NGF and was purified from pig brain (Barde et al., 1982). The same group discovered that it promotes the survival of, and fibre outgrowth from, cultured embryonic chick sensory neurons. As mentioned above, BDNF shares ~50% homology with the other NTs. In this chapter, its structure will be further described as well as the molecular interactions with its high affinity receptor, TrkB.

2.2.1 Molecular Structure

As the rest of NTs, BDNF is first synthesized as precursor protein and then cleaved intracellularly to yield the mature, biologically active form of the protein which consists of 118-120 amino acids. The molecular weight of the mature form of BDNF is ~26 kDa, when the pre-protein's weight ~30-35 kDa (McDonald et al., 1991; Lewin and Barde, 1996; Chao et al., 2006). As a pro-protein BDNF contains a signal peptide, sites for glycosylation and pairs of basic amino acids which are recognized by processing enzymes (Seidah et al., 1996). Similarly to other NTs the pro region is implicated to folding, secreting and p75 receptor binding, therefore having significant biological value (Lee et al., 2001; Lu, 2003; Teng et al., 2005; Bergami et al., 2008; Silhol et al., 2008; Nagappan et al., 2009; Yang et al., 2009). BDNF structure is presented in figure 2.

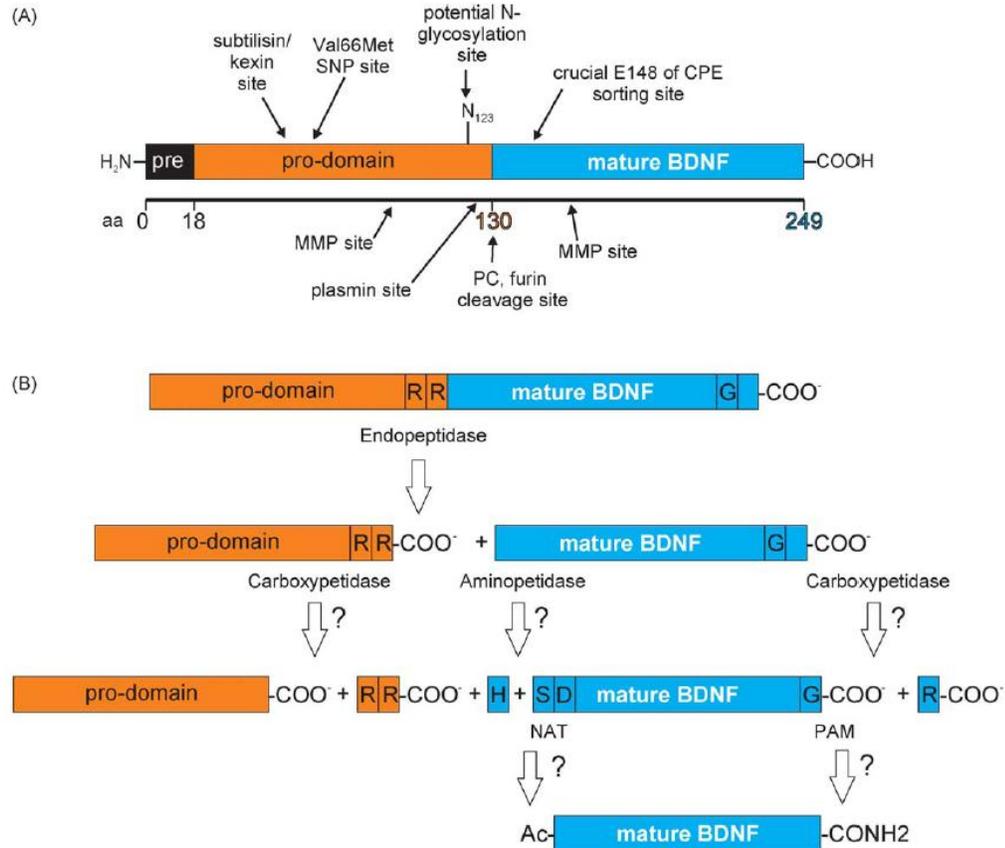


FIGURE 2. BDNF molecular structure. A. Protein domains and critical sites, B. Cleavage steps to mature protein (adapted from Leßmann and Brigadski, 2009)

It is noteworthy that the pro region of all NTs has been conserved through vertebrates' evolution, proposing important biological functions of that domain. The higher identity between the pro region in zebrafish and human BDNF compared to the mature protein provides evidence supporting that evolutionary conservation (Chao and Bothwell, 2002).

2.2.2 Pro region of BDNF

The pro region of NTs has been implicated in many functions, including non-specific binding to p75 receptor as well as folding and secretion of the mature form of biologically active BDNF (reviewed by Lu, 2003). Low frequency stimulation (LFS) has been shown to predominantly facilitate proBDNF secretion, while high frequency stimulation (HFS) facili-

tated extracellular secretion of mature BDNF (mBDNF) (Nagappan et al., 2009). Other studies have indicated activation of apoptosis through activation of p75 receptor after proBDNF low affinity binding, which evoked retraction of developing neuromuscular synapses (Yang et al., 2009) and activation of the same apoptotic pathway from mutant, cleavage-resistant proBDNF in cultured sympathetic neurons (Teng et al., 2005). The differential binding of BDNF and proBDNF is illustrated in figure 3.

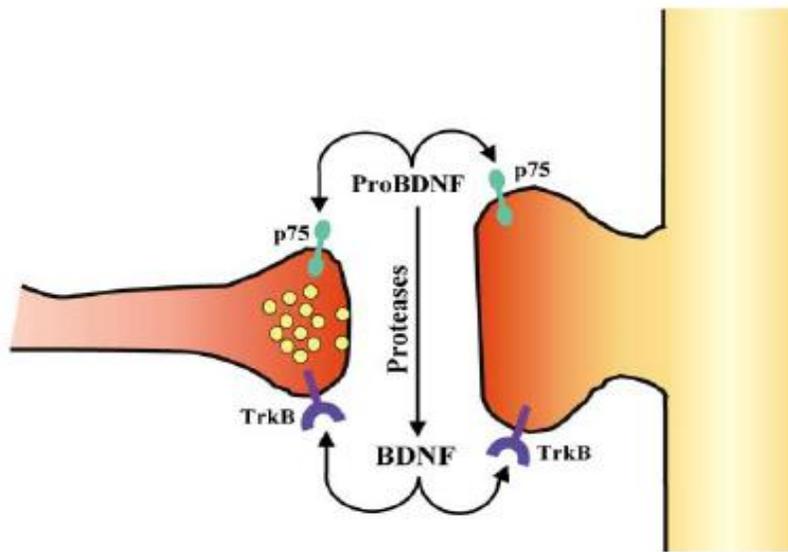


FIGURE 3. ProBDNF vs mBDNF binding (Lu, 2003).

Furthermore, there is evidence of variation in the expression of p75 receptor in different tissues, periods (development, adulthood and aging), and under pathological conditions (reviewed by Chen et al., 2009). Experimental designs are aiming in understanding the differential expression of the receptor in relation to NTs binding to it.

2.2.3 BDNF-trkB molecular interactions

NTs exert their beneficial for the cell cycle effects via activation of trK family receptors (McAllister et al., 1999; Huang and Reichardt, 2001; Hennigan et al., 2007; Jia et al., 2008; Leßmann and Brigadski, 2009) . Even though NTs' binding with high affinity is selective it is not specific (Pattarawarapan and Burgess, 2003) . A schematic representation is given below (figure 4).

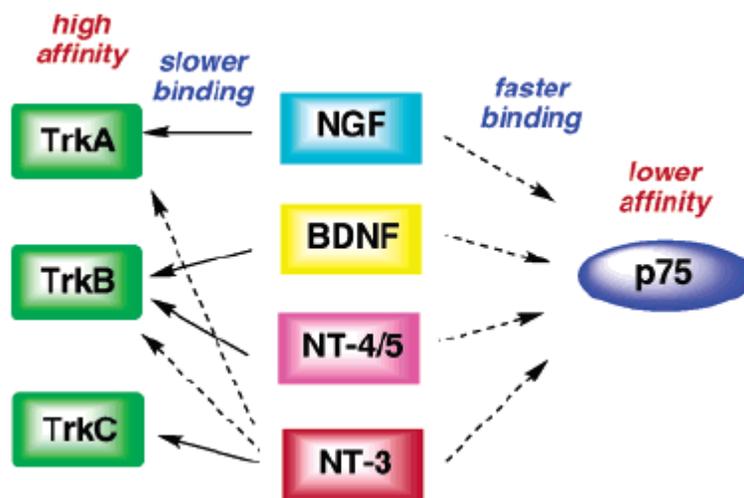


FIGURE 4. Selectivity but non-specificity of NTs (adapted from Pattarawarapan and Burgess, 2003)

The molecular pathway activated after BDNF-trkB binding has been well characterized from numerous studies. Below there is a description of the molecular events following trkB phosphorylation.

All Trk receptors are homologous in sequence in accordance with that NTs are structurally 50% similar as well. Their 3 dimensional structures are not exactly known but there are studies revealing the 5 sub domains which are localized outside the cell membrane. The intracellular domain encodes a tyrosine kinase active site. Activation of Trk results in the phosphorylation of the intracellular tyrosine residues that trigger downstream signaling

pathways that mediate neuron outgrowth, neuronal differentiation or survival. (Pattarawarapan and Burgess, 2003)

Factors that govern these specific cellular responses are not yet well understood. Nevertheless it has been shown that small-molecule ligands can selectively inhibit or promote one of these pathways (Pattarawarapan and Burgess, 2003). Binding to TrkB results in dimerization and autophosphorylation of the receptor and this leads to the activation of its tyrosine kinases. This creates docking sites for different adaptor proteins and signaling enzymes, setting in motion various parallel signal transduction cascades, with distinct functions. Phosphorylation of two tyrosine residues located outside the kinase activation domain of the Trk receptors mediates the interaction with Shc (Src homology 2-containing protein) and phospholipase C γ (PLC γ). Shc recruitment to the active Trk receptors is followed by phosphorylation of the adaptor protein, leading to the activation of the Ras/extracellular signal-regulated kinase (ERK) signaling pathway through recruitment of Grb2 and SOS. The Shc docking site on active Trk receptors may also allow binding of the adaptor protein fibroblast growth factor receptor substrate 2, which becomes phosphorylated on tyrosine residues, thus creating binding sites for the adaptor proteins Grb2 and Crk, the phosphatase SH-PTP2, the tyrosine kinase Src and the cyclin-dependent kinase substrate p13^{suc 1}. Crk binds and activates the exchange factor C3G, which in turn stimulates a small G protein, Rap-1, thereby activating the downstream kinase B-raf and the MEK/ERK signaling cascade. Alternatively, CrkL can be recruited to the activated Trk receptor through binding to the tyrosine-phosphorylated ankyrin-rich membrane spanning domain (ARMS)/Kidins220, resulting in the activation of Rap1 through C3G. Activation of ERK influences transcription events, such as the activation of cAMP-response element binding (CREB) transcription factor. Binding of Shc to the Trk receptor also activates the phosphatidylinositol 3-kinase (PI3K) pathway, either by direct interaction of Ras with PI3K or through recruitment of the adaptor protein Cab1. Activation of PI3K changes the composition of inositol phospholipids in the inner leaflet of the plasma membrane, resulting in the translocation of PKB (also known as Akt) to the plasma membrane, where it is activated through phosphorylation by upstream kinases, including phosphoinositide-dependent protein kinases 1 and 2 (possibly the mTOR complex). Akt may change transcription activity, but may also induce rap-

id and local changes in the proteome by regulating the translation machinery. This signaling pathway requires TrkB translocation to lipid rafts, possibly through a Fyn-dependent mechanism. PLC γ hydrolyses phosphatidylinositol 4,5-bisphosphate, giving rise to diacylglycerol, which activates protein kinase C (PKC), and inositol 1,4,5-trisphosphate (Ins(1,4,5)P $_3$), which releases Ca $^{2+}$ from intracellular stores. In cultures cerebellar granule cells, the BDNF-induced mobilization of intracellular Ca $^{2+}$ stores acts together with diacylglycerol generated by PLC γ in the activation of plasma membrane transient receptor potential canonical subfamily 3/6 (TRPC3/6) channels. The influx of Ca $^{+2}$ through these channels contributes to ERK and CREB activation, increasing cell survival. Activation of this pathway also plays a key role in synaptic plasticity. (Yamada and Nabeshima, 2003; Carvalho et al., 2008)

2.2.4 Distinct signal transduction and intracellular pathways

Following the identification of NTs and their high affinity receptors (trks), the intracellular pathways activated gained much experimental attention from several research groups. The use of PC12 cells (derived from a rat pheochromocytoma) as a model, boosted the identification of distinct intracellular pathways promoted from extracellular binding of NTs. Below is some evidence of the differential intracellular signaling followed by trk receptors in both PC12 and primary neurons. (Reviewed by Klesse and Parada, 1999)

While differentiation of PC12 cells occur as a result of NGF-trkA receptor binding and Ras/Raf cascade, constitutively expression of one of these has been shown to evoke differentiation in the absence of NGF. On the other hand, expression of dominant interfering forms of these blocks neurite outgrowth which is associated with NGF-induced differentiation. The above mentioned observations have pointed out the Ras/Raf mediated action of NGF. Different results were reported when the inhibition of Ras/Raf cascade using adenoviruses didn't inhibit the action of NGF in terms of survival, proposing a different and distinct intracellular mechanism promoting survival than differentiation (Klesse et al., 1999). A schematic summarizing the mechanism mentioned above is presents in figure 4.

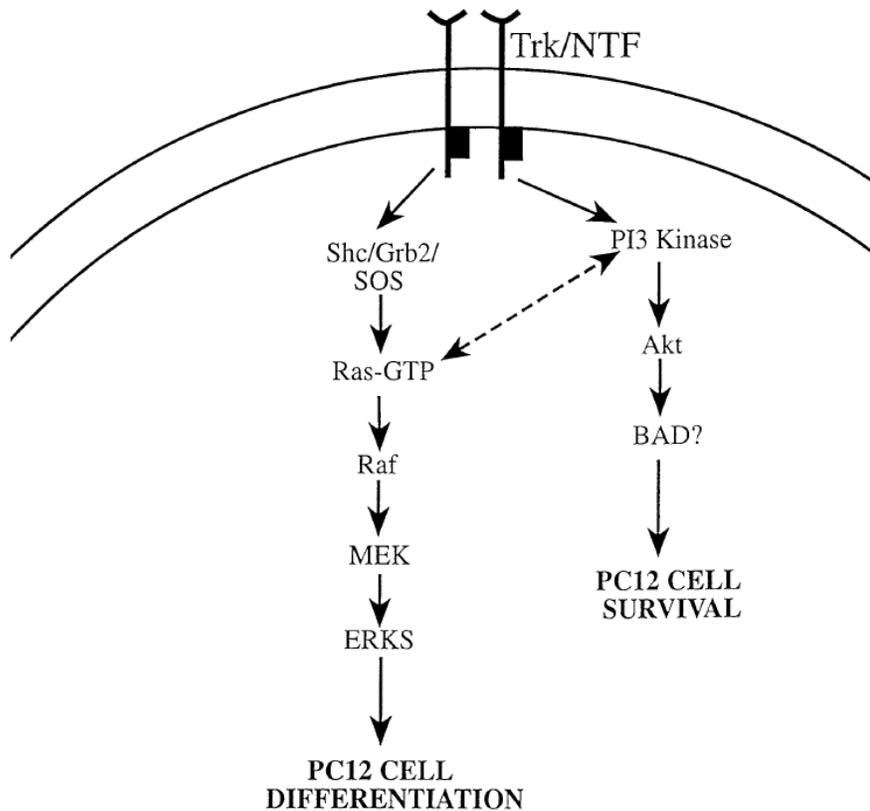


FIGURE 4. A distinct mechanism of differentiation and survival in PC12 cells (adapted from Klesse and Parada, 1999)

The same group reviews evidence of survival promotion by microinjection of activated PI 3 or its downstream effectors (Akt) in sympathetic neurons in the absence of NGF, while microinjections of dominant interfering forms of PI 3 and Akt inhibit survival in the presence of NGF. Taken together these data show the role of PI 3 activation in neuronal survival through activation of trk receptors for NTs. The main difference between PC12 and primary neurons seems to be that contrary to PC12 cells, neurons require the activation of both Ras/Raf and PI 3 cascade to survive. This notion is schematically represented in figure 5.

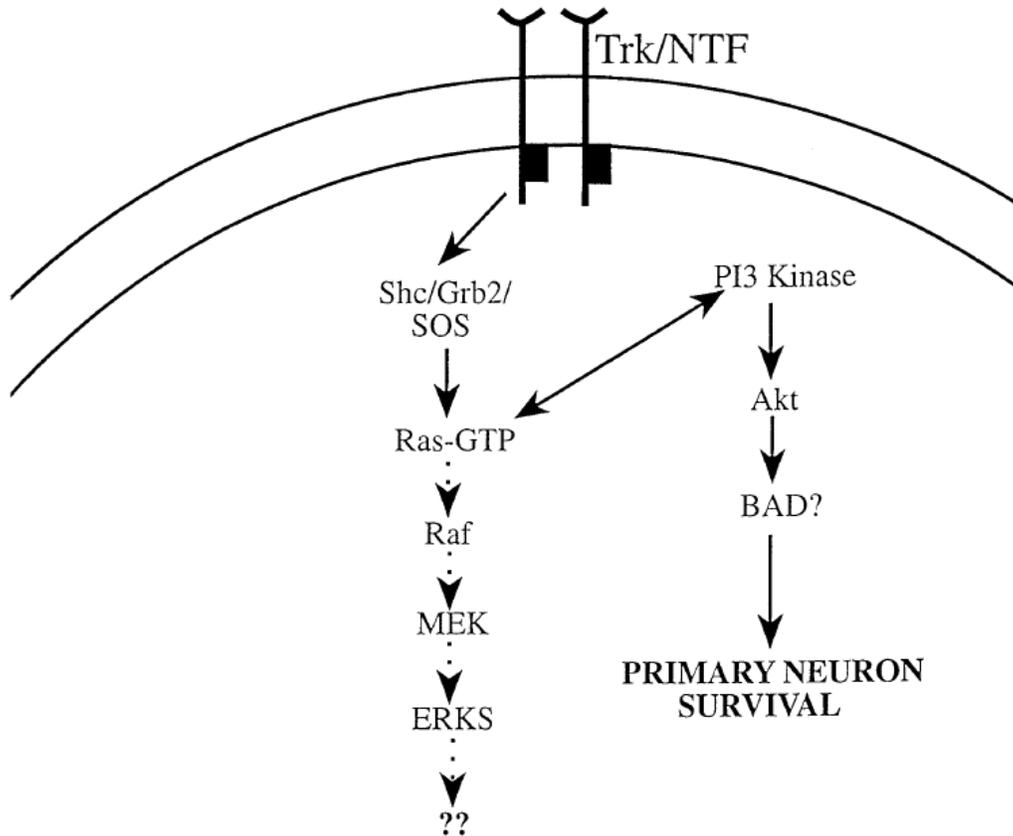


FIGURE 5. Schematic representation of the intracellular pathways activated after trk receptor's phosphorylation. (Adapted from Klesse and Parada, 1999)

2.3 Neurotrophin 3 (NT-3)

The sequence homology between the 2 first characterized NTs, NGF and BDNF, was used to identify the 3rd member of the family which was named NT-3. Its mRNA is present in different neuronal tissues and its biological functions distinguish it from the other NTs. (Maisonpierre et al., 1990). NT-3 activates TrkC receptor with high affinity but is also able to bind to TrkA, TrkB and p75 with lower affinity (Huang et al., 1999; McAllister et al., 1999; Pattarawarapan and Burgess, 2003). Early studies on NT-3 have proposed its implication in neuromuscular synapse formation and its acute application at the developing *Xenopus* neuromuscular synapses in culture has been shown to increase the frequency of excitatory postsynaptic potentials (EPSPs) (Lohof et al., 1993). Besides the structural similarity

with BDNF, observations have proposed similar expression patterns of NT-3 in respect to developmental periods of life with peak expression of the protein and its receptors in early postnatal age (reviewed by McAllister et al., 1999)).

2.4 Neurotrophin 4/5 (NT-4/5)

The last identified member of the family has been shown to be expressed in the brain and peripheral tissues with skeletal muscle included (Barde, 1989; McAllister et al., 1999). It binds with high affinity to TrkB receptor and with low affinity to p75 (Patapoutian and Reichardt, 2001; Pattarawarapan and Burgess, 2003) Its mRNA expression doesn't reach the levels of the other members of the family and is considered as the most divergent and least understood (Hallbook et al., 1991). Knock-out studies on NTs and their receptors have shown that in contrast with the rest members of the family, NT-4/5 is not crucial for the survival of the organism and mutations of its genes have resulted only in minor cellular deficits (Snider, 1994; Ibanez, 1996).

3 LEARNING AND MEMORY

BDNF has been characterized as a molecule implicated to learning and memory because of its expression in the adult brain (Bramham and Messaoudi, 2005; Chao et al., 2006; Bekinschtein et al., 2008; Gomez-Palacio-Schjetnan and Escobar, 2008; Kuipers and Bramham, 2006), and its relation with Long Term Potentiation (LTP) (Lessmann et al., 2003; Morris et al., 2003; Lu and Martinowich, 2008; Lu et al., 2008a; Hennigan et al., 2009). Additionally, BDNF has been shown to play critical roles in synaptic plasticity by regulating NMDA receptors and being expressed in glutamatergic neurons (Leßmann, 1998; Kerr et al., 1999; Hartmann et al., 2001; Yamada and Nabeshima, 2003; Lipsky and Marini, 2007; Carvalho et al., 2008; Crozier et al., 2008; Kuczewski et al., 2008; Madara and Levine, 2008; Mattson, 2008; Bustos et al., 2009).

3.1 BDNF role in Long Term Potentiation (LTP)

The activity dependent secretion of BDNF has led many research groups to hypothesize its relation with long term memory. Verification of the hypothesis would require the involvement of the BDNF in LTP, as a necessary process of long memory formation.

In vivo experiments have shown that local application of BDNF triggers LTP (BDNF-LTP) at medial perforant path-granule synapses, the induction of which requires MEK-ERK activation (Ying et al., 2002). Correlations of deficits in LTP with decreased BDNF expression and its receptor in the dentate gyrus suggest also the relation of the protein with LTP (Hennigan et al., 2009). Besides the induction of LTP BDNF has been described as a regulatory molecule for the late phase of LTP (L-LTP), which is an essential process for formation of long term memory (LTM) and requires local protein synthesis (reviewed by Pang and Lu, 2004). An illustration of BDNF activity in LTP is presented in figure 6.

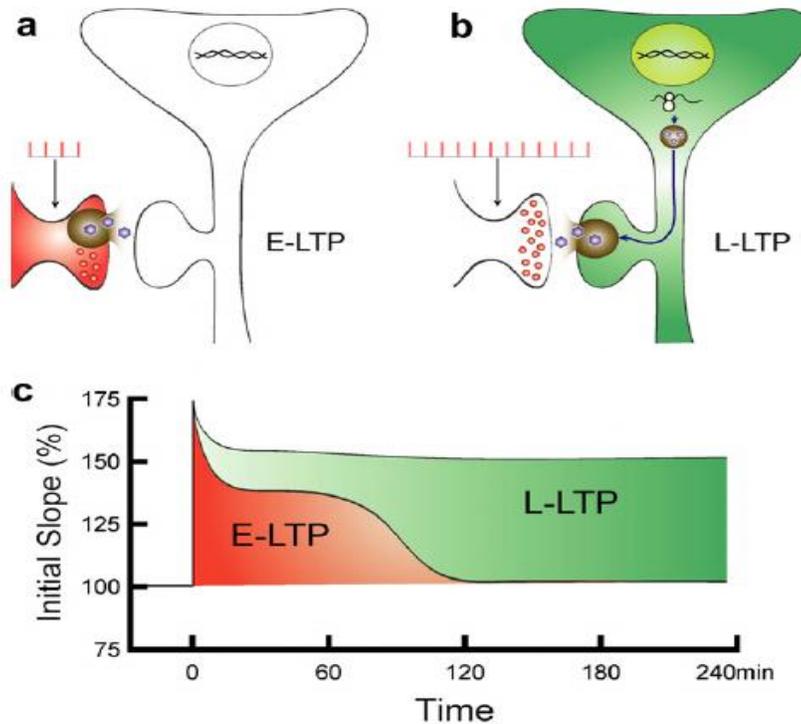


FIGURE 6. Activity dependent secretion of BDNF from presynaptic site (red in a and c) and in the postsynaptic site (green in b and c) is believed to be necessary for induction and maintenance of LTP (adapted from Lu et al., 2008b)

A recent review summarizes the most characteristic features of BDNF, which are relevant to memory processing and storage. Activity dependent secretion, the expression of the protein in the most plastic areas of the brain such as hippocampus, cerebellum and cerebral cortex, the beneficial (related to synaptic effects) molecular pathways activated by its binding to trkB receptor, the facilitation of glutamate release by BDNF administration, the increased phosphorylation of NR1 and NR2B subunits of the NMDA-receptor complex, the BDNF evoked up-regulation of GluR1 AMPA receptor and expression and phosphorylation, increased expression of its receptor in the plasma membrane and regulation of its insertion after exposure of hippocampal neurons to BDNF, and the BDNF related modulation of protein synthesis by transcriptional and translational mechanisms. (Bekinschtein et al., 2008) .

3.2 Synaptic transmission and plasticity

In line with the observations of the involvement of BDNF in late phase of LTP, independent groups have given evidence of enhancement of synaptic transmission and plasticity which results in the formation of neuronal circuits (reviewed by Lu and Chow, 1999). Treatment with BDNF in slices of the ferret developing visual cortex has shown to increase the number, length and branching of basal dendrites more than other NTs (McAllister et al., 1995). The same group has shown strongly enhanced dendritic growth of layer 4 pyramidal neurons of ferrets after application of BDNF in cortical slices. Furthermore, BDNF treatment increased the extent and complexity of both apical and basal dendrites, roughly doubling dendritic length and branching. BDNF also doubled the number of primary basal dendrites, resulting in a “halo” of short dendrites and protospines protruding from the cell body. The slices were also treated with activity inhibitors (APV) in order to assess the growth/branching effect of BDNF in relation to electric activity. (McAllister et al., 1996). A reconstruction of the images of the 4 groups (untreated, inhibited, BDNF-treated and BDNF + inhibited) are shown in figure 7. The results suggest the requirement of electrical/neuronal activity for BDNF-related neuronal growth.

In another set of experiment the same group showed opposed effects of BDNF and NT-3 in layers 4 and 6 of developing visual cortex of ferrets. The effects of BDNF were in line with their previous observations. The complexity of dendritic branching was assessed in terms of Dendritic Modification Index (DMI) and is presented in figure 8.

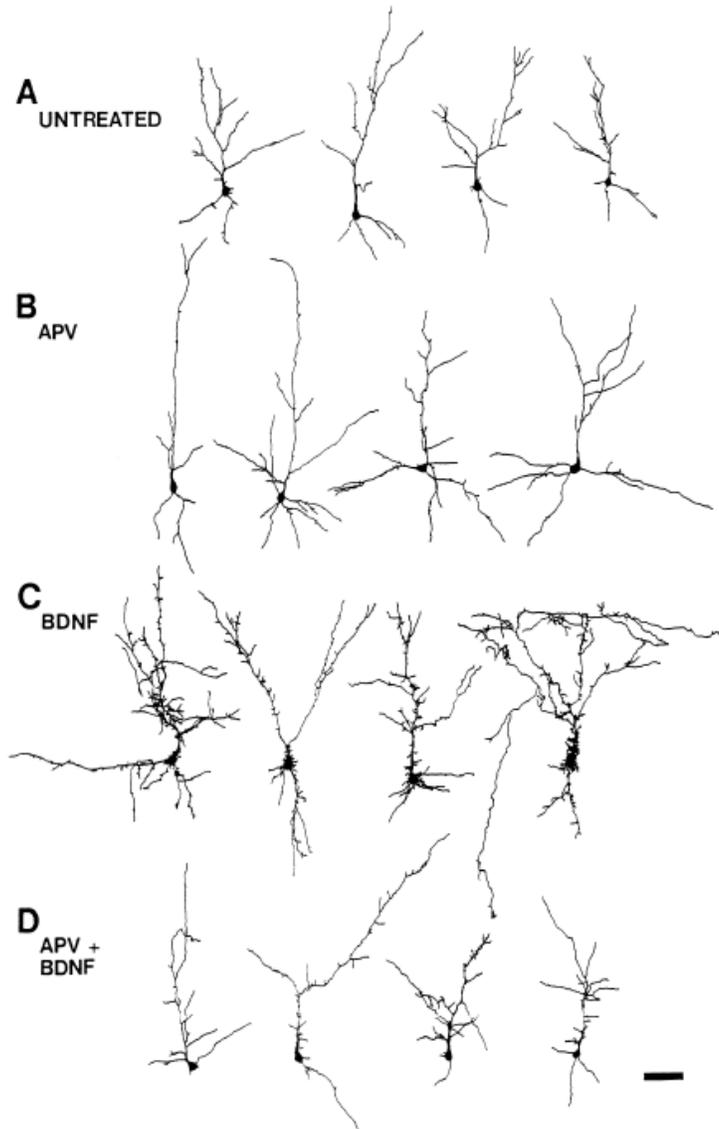


FIGURE 7. Reconstruction of images of layer 4 neurons from untreated (a), APV-treated (b), BDNF-treated (c), and BDNF+APV-treated cortical slices (adapted from McAllister et al., 1996)

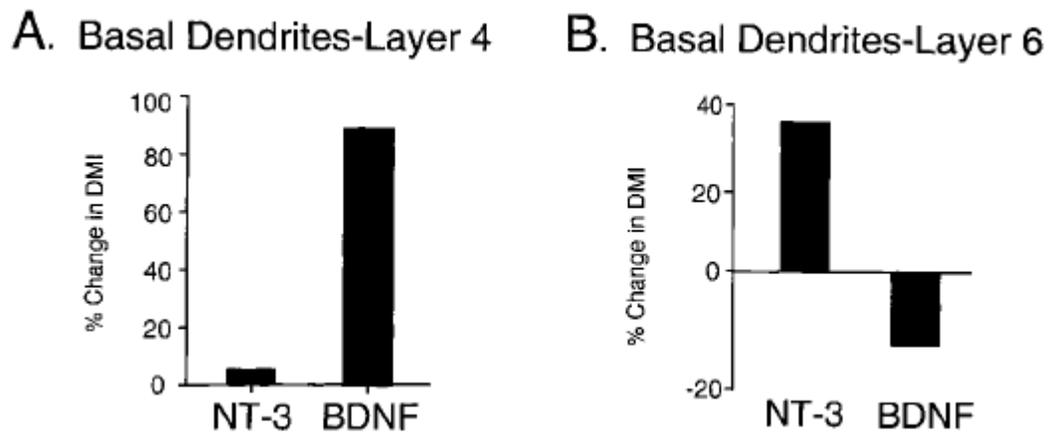


FIGURE 8. DMI after administration of exogenous of BDNF and NT-3. (Adapted from McAllister et al., 1997)

Exogenous administration of BDNF almost doubled DMI while NT-3 had almost no effect. Controversially, NT-3 enhanced branching and increased DMI in layer 6 Basal Dendrites while BDNF inhibited it.

The above mentioned observations suggest a differential role for neurotrophins in different cell population under different circumstances. BDNF and NT-3 evoked opposing results which were in layer 4 and 6. Possible explanation could be the competition of these 2 NTs for binding to TrkB receptor given in combination with the lower affinity binding of NT-3 to the receptor, which could lead to opposing effects on dendritic branching due to reduced promotion of the plasticity-related pathway which is activated after BDNF-TrkB binding (McAllister et al., 1997).

4 ENVIRONMENTAL FACTORS AND BDNF

The relationship between BDNF and environmental factors will be discussed in this chapter. Evidence on the up regulation of the protein due to Physical Activity (PA) and experiences in an Enriched Environment (EE) will be further discussed.

4.1 BDNF and Physical Activity (PA)

Many research groups have turned their research activities towards the investigation of the contribution of exercise and diet to brain health and plasticity. This “trend” seems reasonable if someone considers the importance of energy availability and optimization in every cell. Given the inability of the neurons to store glucose, their main energy source, the importance of its efficient delivery becomes evident for brain health and plasticity promotion. Literature on up regulation of plasticity-related molecules by exercise as well as energy metabolism promotion by an active lifestyle will be presented in this chapter.

PA has been shown to enhance learning in both animals and human studies (Fordyce and Wehner, 1993; Kramer et al., 1999; Mattson, 2000; Laurin et al., 2001; Mattson et al., 2002). Additionally regular PA prevents age related cognition impairment and dementias in humans (Larson et al., 2006; Komulainen et al., 2008; Richter and Ruderman, 2009).

Attention has been paid on the effects of exercise on molecular subsystems serving brain health and plasticity. Evidence of up regulation of specific neuroprotective and plasticity-related molecules is provided below.

4.1.1 Up regulation of BDNF through exercise

Classic studies on the effects of Voluntary Exercise (VE) on the expression of BDNF have shown up regulation of the molecule and other plasticity-related proteins in hippocampus (Farmer et al., 2004; Kim et al., 2005; Ding et al., 2006b; Radom-Aizik et al., 2007; Go-

mez-Pinilla et al., 2008) . VE has also been shown to enhance neurogenesis in the Dentate Gyrus (DG) of rats (Farmer et al., 2004; Pereira et al., 2007).

4.1.2 Exercise and metabolic proteins in the hippocampus

Energy management has a key role in the function of cell cycle as well as in the organism as a whole. Recent experiments have shown an up regulation of metabolic proteins in response to increased energy expenditure. The molecules under investigation are described briefly below.

1. AMP-activated kinase (AMPK) is activated in response to increased intracellular AMP/ATP ratio and therefore is used as an estimator of cellular energy availability (Fryer et al., 2002)
2. ubiquitous Mitochondrial Creatine Kinase (uMtCK) synthesizes Phosphocreatine (PCr) from ATP in the intermembranous space of mitochondria and plays a pivotal role in supplying energy to neurons during the highly demanding processes of higher brain functions (Boero et al., 2003)
3. Uncoupling Protein 2 (UCP-2) has been shown to modulate events related to energy supply and consumption which influence the substrates of neuroplasticity in the brain (Vaynman et al., 2006)
4. Ghrelin is a hormone secreted from the stomach as a result of energy depletion related to reduced food intake and has been shown to enhance memory retention in rats after local injections in the hippocampus (Carlini et al., 2002; Carlini et al., 2004)
5. Insulin-like Growth Factor has been associated with energy metabolism and cognitive function under homeostatic and challenging conditions. Additionally, insulin-like growth factor (IGF-I) shares downstream pathways with BDNF after binding to its receptor and its expression is abundant in the hippocampus (Yamada et al., 1997; Roudabush et al., 2000).

One week of VE, which elevated performance in learning and memory tests, also induced the mRNA up regulation of BDNF and all above described proteins in the hippocampus of rats suggesting the contribution of exercise through energy management to learning and memory. Following the up regulation of the mRNA expression, the group blocked the action of BDNF using a specific immunoadhesin chimera (TrkB-IgG) in order to determine how BDNF regulated these key factors during the exercise period. The up regulation of the molecules under investigation abolished after BDNF blockage, as well as the association between learning speed and the level of AMPK, uMtCK, IGF-I and ghrelin. (Gomez-Pinilla et al., 2008).

Other proteomic studies have evaluated the effect of acute exercise on the expression pattern and post-translational modification of multiple protein classes in the rat hippocampus. Increases of fructose-bisphosphate aldolase C, phosphoglycerate kinase 1, mitochondrial ATP synthase, uMtCK and glutamate dehydrogenase 1 was observed (Ding et al., 2006c) .

4.2 BDNF and nutrition

Nutrition has been implicated in proper neuronal excitability and therefore proper functionality. Evidence has occurred in the contribution of a balanced diet in terms of quality and quantity. Data on the relationship between food intake and synaptic efficacy have become available (Gomez-Pinilla, 2008). In the following lines, subsystems related to this theory will be presented. Additionally, a FIGURE illustrating the theory on the effects of diet and exercise on cognition through energy metabolism and is presented in figure 9.

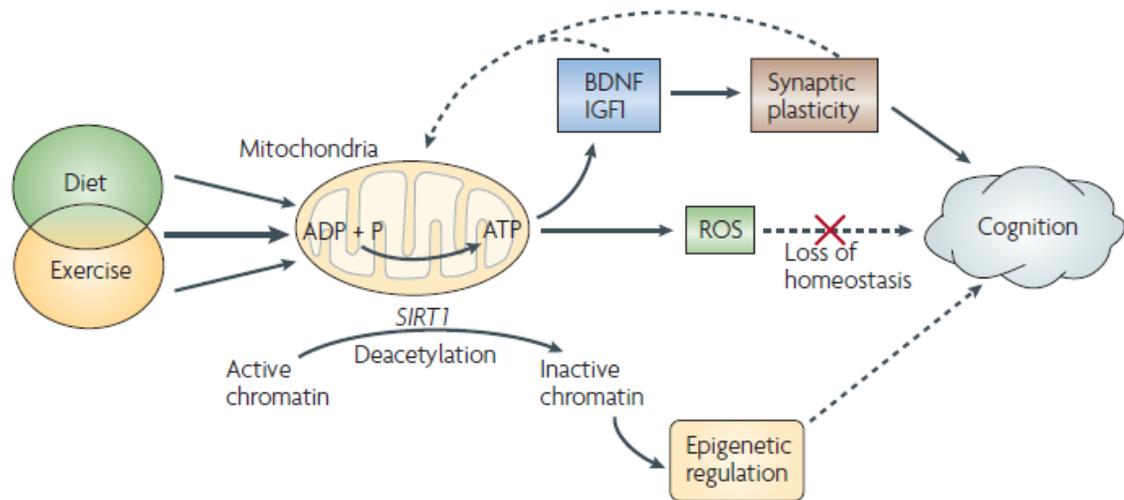


FIGURE 9. Schematic representation of the relationship between diet, exercise, and cognition through energy metabolism (adapted from Gomez-Pinilla, 2008)

4.2.1 Gut hormones associated with cognition

Several proteins have been associated with favorable effects in cognition. Leptin has been shown to convert Short-term potentiation to LTP through facilitation of NMDA receptor function because it rapidly enhances NMDA-induced increases in intracellular Ca^{+2} levels and facilitates NMDA receptor-mediated synaptic transmission. Impairment of this process may contribute to the cognitive deficits associated with diabetes mellitus. (Shanley et al., 2001). Orexin and leptin have also been shown to enhance LTP in the DG of rats in vivo (Wayner et al., 2004). In a study on leptin receptor-deficient mice, impaired LTP and spatial learning in Morris Water Maze test (MWM) (Li et al., 2002). Ghrelin is a peptide predominantly produced by stomach and stimulating appetite. Ghrelin has been implicated in many different functions which are summarized in figure 10. (Reviewed by van der Lely et al., 2004)

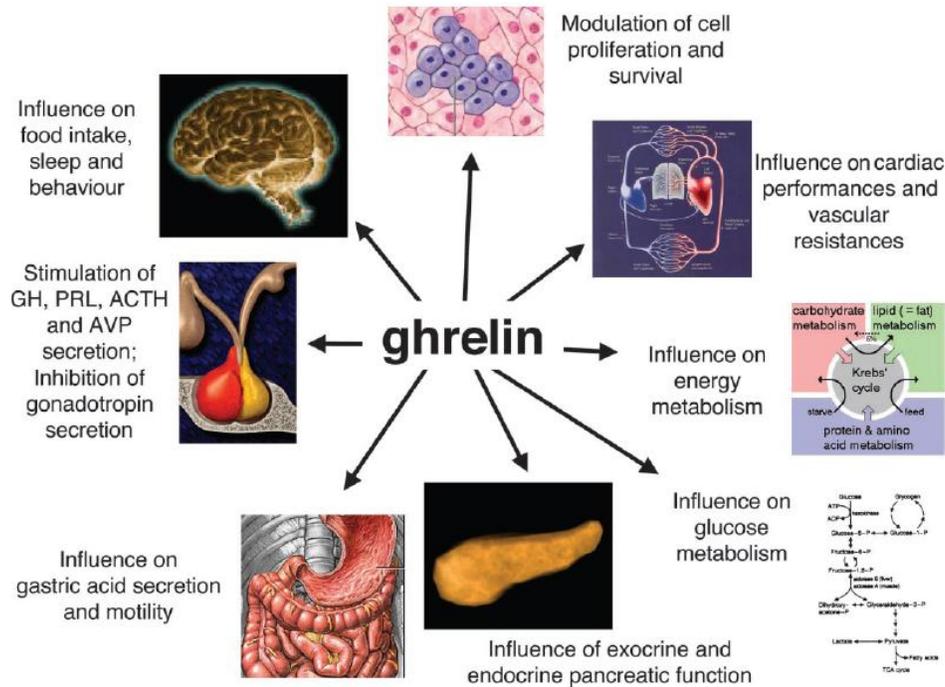


FIGURE 10. Direct and indirect actions of ghrelin (adapted from van der Lely et al., 2004).

4.2.2 Omega-3 fatty acids

Nervous system is the organ which comes second in fat concentration after adipose tissue (Bourre et al., 1989). In neural tissue, unlike adipose or muscle, fat has structural significance as it is the main constituent of the excitable membrane. A short description of the significance of docosahexaenoic acid (DHA), which humans obtain from dietary fish and consists more than 30 % of the phospholipid composition of the plasma membranes in the brain, is given below (Gomez-Pinilla, 2008).

As a structural element of the plasmatic membrane, DHA maintains its fluidity and integrity at synaptic regions of the neurons. These properties ensure the proper function of ion channels and membrane receptors, which in turn ensures survival and signaling of the cell (reviewed by Gomez-Pinilla, 2008). Figure 11 describes the functional relationship between DHA availability and cognition promotion by involvement of plasticity promoting molecules such as BDNF and IGF-I.

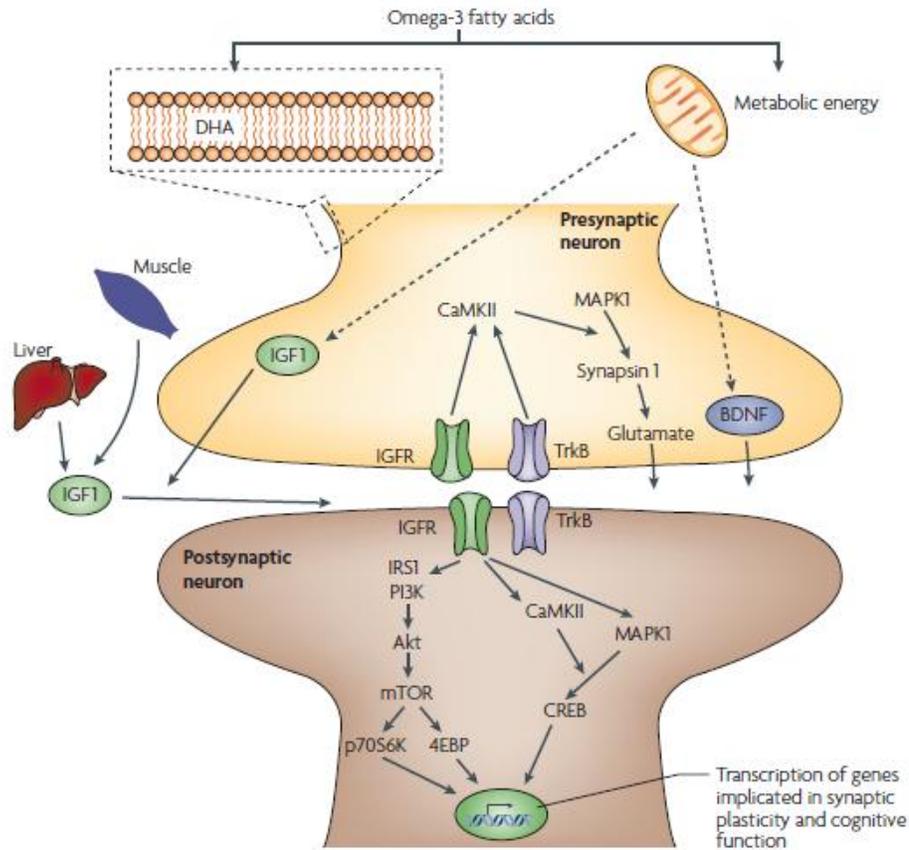


FIGURE 11. DHA can affect synaptic plasticity and cognition by supporting the structural integrity and functionality of the plasma membrane. (Adapted from Gomez-Pinilla, 2008)

5 BDNF AND THE “MIND-BODY CROSS TALK”

The concept “Healthy mind in a healthy body” (Hippocrates, 4th century BC) is of great significance nowadays, as the frequency of incidence of neurodegenerative and metabolic disorders increases. Mental exercise might not be enough to ensure brain health and plasticity, which is a hypothesis in line with the Hippocratic hypothesis. The evolved methods in molecular biology make possible the investigation of molecules which could be involved.

BDNF expression is widespread in mammals and abundantly expressed in the very plastic areas of the brain such as hippocampus, hypothalamus, cerebral cortex, and cerebellum (McAllister et al., 1999; Binder and Scharfman, 2004). In this chapter new evidence on BDNF expression in the skeletal muscle is being introduced. The relevance and believed mediation of BDNF effects on the brain through IGF-I are being discussed.

5.1 BDNF expression in skeletal muscle

BDNF has been described as a molecule that promotes survival of motor neurons throughout their lifespan and potentiation of neuromuscular transmission (Oppenheim et al., 1992; Sendtner et al., 1992; Lohof et al., 1993). Recent experiments have shown BDNF expression in muscle satellite cells, myoblasts and myotubes suggesting more functional roles (Mousavi and Jasmin, 2006). Another group has shown increased BDNF mRNA and protein expression in human skeletal muscle without release of protein into the circulation. Similar increment was observed after electrical stimulation of muscle cells. Additionally, BDNF increased phosphorylation of AMPK and Acetyl coenzyme A carboxylase β (ACC-beta) and enhanced Fat Acid Oxidation (FAO) both in vitro and ex vivo. When AMPK was infected using an AMPK dominant negative adenovirus or treated with compound C, an inhibitor of AMPK, the increase of FAO was abrogated, proposing an AMPK dependent role of BDNF in FAO. (Matthews et al., 2009).

The above mentioned observations propose a metabolic role of BDNF in the skeletal muscle connected with fat metabolism in an AMPK-dependent manner. A summary of the results is given in figure 12.

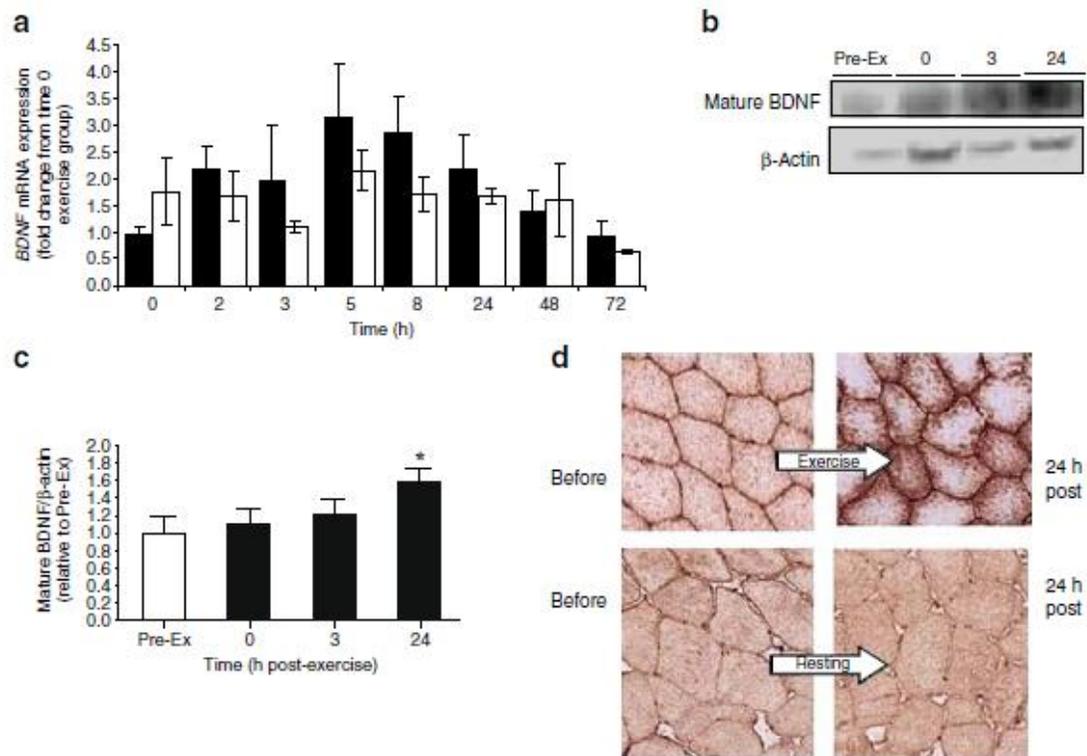


FIGURE 12. BDNF is increased in contracting skeletal muscle in vivo. BDNF mRNA levels (a) and protein production in muscle tissue measured by western blot (b, c) and Immunohistochemistry (d) at time points ranging from 0 to 72h after 2h of ergometer bicycle exercise of the volunteers at 60% of VO_{2max} . (Matthews et al., 2009)

5.2 Insulin-like Growth Factor I (IGF-I) and BDNF

Much research has been conducted on the relationship between molecules related to brain health and synaptic plasticity. IGF-I has been described as a molecule that is implicated in healthy ageing and longevity. Interestingly it has been described as a mediator of the beneficial effects of BDNF in the brain. Some evidence will be presented and discussed below.

5.2.1 IGF-I signaling and longevity

IGF-I has been correlated with extended lifespan in species ranging from yeasts to mammals. The mechanism by which this occurs is described as preserved through evolution. (Reviewed by Kenyon, 2001; Katic and Kahn, 2005)

A schematic representation of the common characteristics of IGF-I signaling pathway is presented in figure 13.

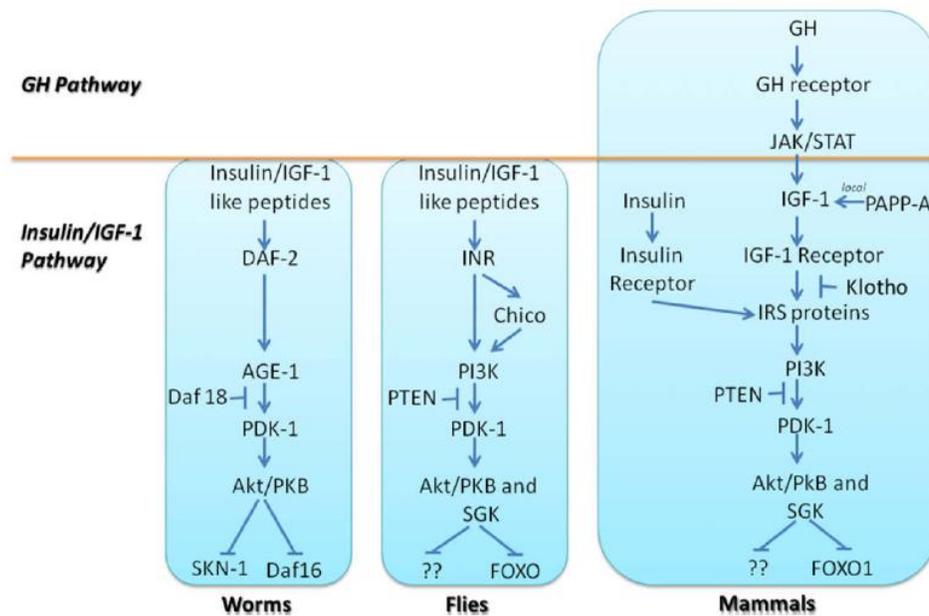


FIGURE 13. Comparison of IGF-I pathway in *C.Elegans*, *Drosophila Melanogaster* and mammals (adapted from Berryman et al., 2008).

5.2.2 IGF-I and the brain

Evidence of enhancement of neural survival and synaptic transmission by IGF-I has implicated this molecule as a regulator of synaptic plasticity and overall brain health throughout lifespan. Its uptake from neural cells and the abundant expression of its receptors in the brain has raised questions. Beside its trophic and growth-promoting effects in the periphery,

IGF-I is believed to exert favorable effects in cognitive processes such as neurogenesis in the brain (reviewed by Aleman and Torres-Alemán, 2009) . Figure 14 illustrates possible signaling mechanisms that regulate neurogenesis with IGF-I being among them.

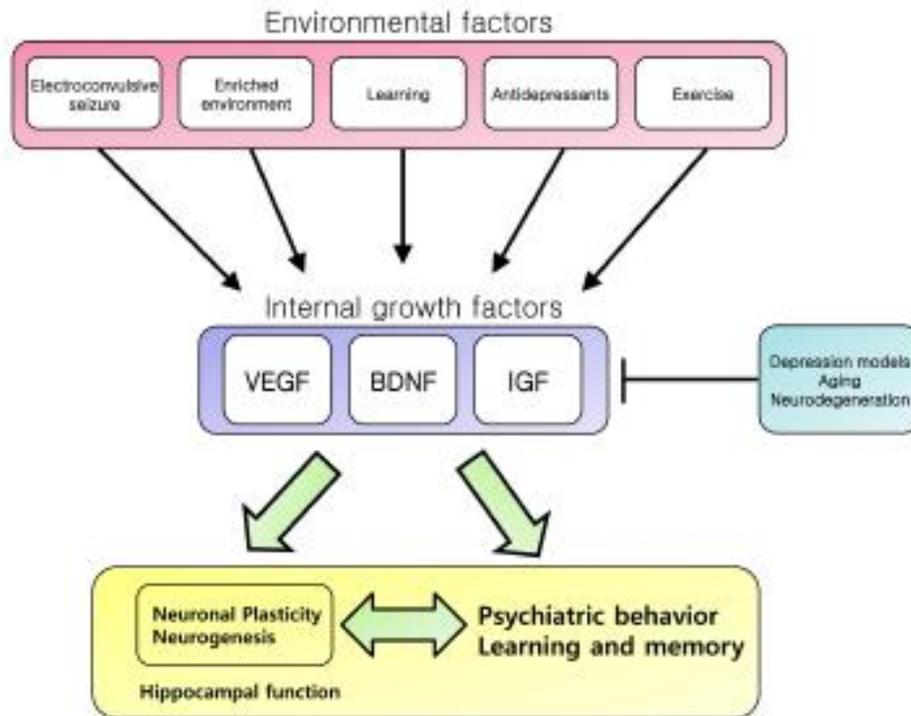


FIGURE 14. Signals regulating adult hippocampal neurogenesis (Lee and Son, 2009).

The importance of signaling of IGF-I in the brain is also underlined from observations of low plasma concentrations in humans suffering for dementias (Watanabe et al., 2005)

5.2.3 IGF-I-mediated BDNF effects on the brain

Experimental setups have tried to shed light on the functional relationship between IGF-I and BDNF. The permeability of IGF-I from circulation to the brain through Blood-Brain Barrier (BBB) (Reinhardt and Bondy, 1994) in combination with observations of activity dependent secretion of IGF-I have suggested a mediation mechanism of the protein on brain health and plasticity (Schwarz et al., 1996). BDNF has been related to this mechanism after observations of IGF-I mediated actions of BDNF. Evidence on this relationship is provided in this section.

Ding et al used learning tests in rats to assess the involvement of IGF-I in learning and memory retention before and after blocking its receptor activity. Additionally, they measured plasticity related molecules in the hippocampus of the subjects. They found out a significant decline in memory retention due to blockage of IGF-I action which was followed by down regulation of both mature BDNF and its precursor (pro-BDNF), suggesting a possible modification of the precursor to the mature protein in the presence of IGF-I. Molecular analysis showed a significant, exercise-induced elevation of proteins downstream to BDNF function as synapsin I, and signal transduction cascades associated with memory processing, i.e. phosphorylated calcium/calmodulin protein kinase II (CAMKII) and phosphorylated mitogen-activated protein kinase II. Importantly, blockage of IGF-I receptor abolished these exercise-induced increases illustrating a possible mechanism by which IGF-I interfaces with the BDNF system to mediate the effects of exercise on cognition and brain plasticity. (Ding et al., 2006a).

Another lab has shown antidepressant-like effects of BDNF and IGF-I in rats. The animals were exposed to a forced swim test (FST) which is widely used to assess antidepressant behavior (Petit-Demouliere et al., 2005). The tests were repeated 3, 6, and 12 hours after administration of exogenous BDNF and IGF-I. The results indicated a reduced immobility (antidepressant-like response) which lasted longer compared to the acute effects of known antidepressant (Hoshaw et al., 2005). More evidence is coming from studies on cellular and molecular mechanisms of long term action of antidepressants which show activation of pathways downstream to BDNF and IGF-I pathways (reviewed by D'Sa and Duman, 2002; Racagni and Popoli, 2008).

5.2.4 BDNF, IGF-I, and serotonin in the evolution context

Mattson et al have proposed a very attractive model of BDNF, IGF-I, and serotonin mediation of environmental inputs to organism adaptation and survival. Their review summarized here describes common molecular effects of the above mentioned molecules which regulate energy metabolism, stress responses and processes that are major determinants of ageing

and of the risk for development of age-related diseases. A diagram of their actions is presented in figure 15.

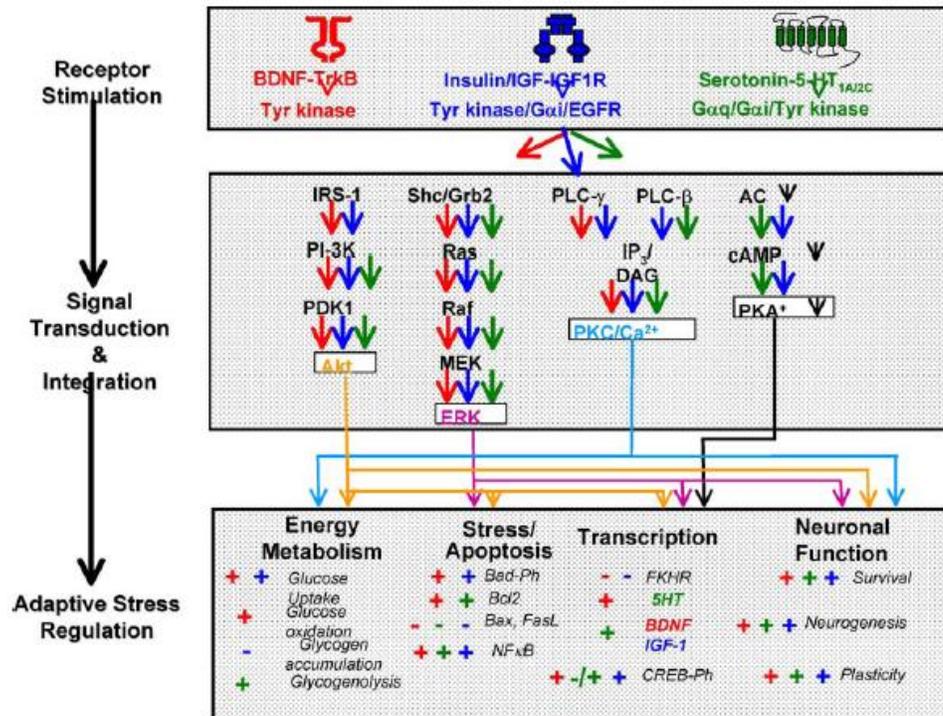


FIGURE 15. Overlap of actions of BDNF, IGF-I and serotonin after activation of their receptor (adapted from Mattson et al., 2004).

The model is based on the hypothesis of genetic adaptations that mammals went through evolution in order to avoid hazards and compete for limited food sources. In this context, the complex nervous system of mammals evolved to efficiently locate, capture and consume food in order to obtain the energy needed for the metabolic processes. Beside this, the energy had to be efficiently stored and dispensed in order to ensure survival during the periods of food unavailability (Mattson et al., 2004). Even complex cognitive function such as learning and memory, strategy formation and emotional states are derived from adaptations in energy metabolism adaptations. The importance of learning (e.g. hunting skills) and memory (e.g. places and periods of food availability/unavailability) in survival of multi cellular organisms supports the above described notion. A schematic representation of the model is presented in figure 16.

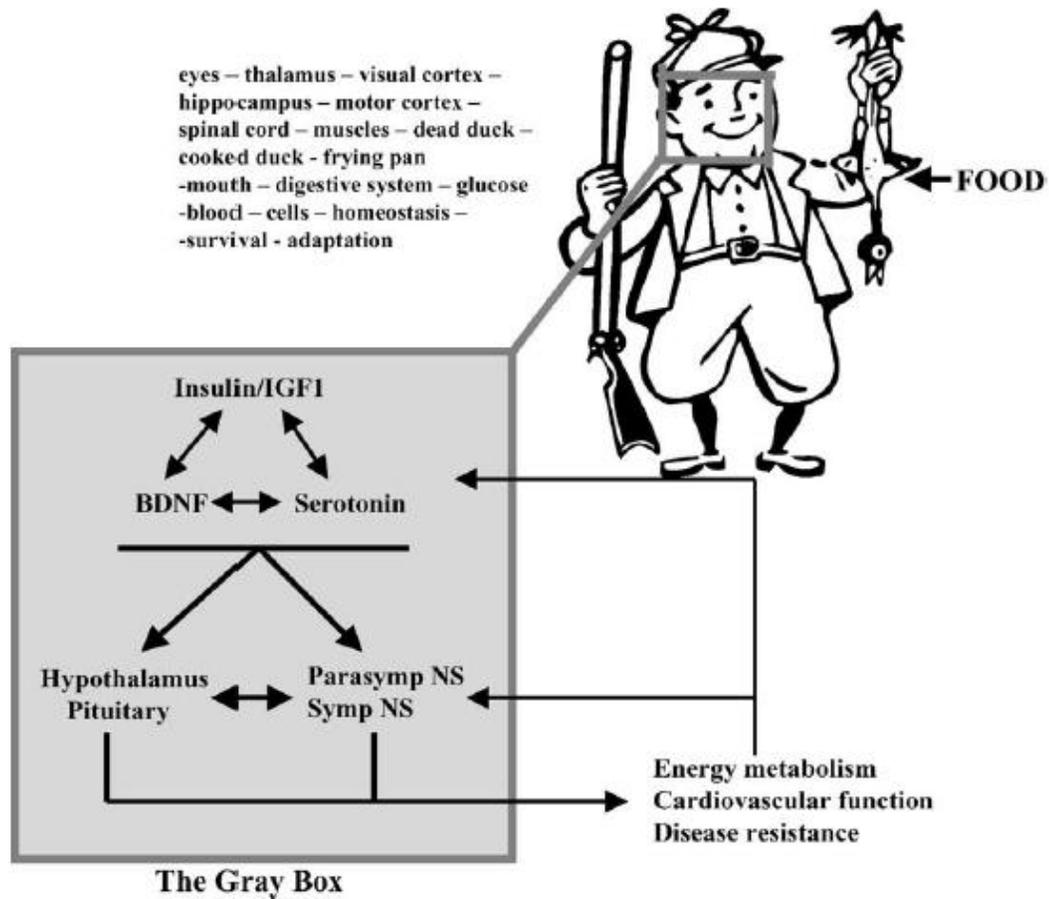


FIGURE 16. A model implicating BDNF, IGF-I and serotonin function in organism survival in the evolutionary context (Adapted from Mattson et al., 2004)

Consideration of the inverted modern lifestyle which is characterized by abundance of food and lack of physical activity has to be taken under consideration when wondering about the increased incidence of age and metabolism-related diseases which are discussed in the next chapter.

6 BDNF AND DISEASE

This chapter reviews literature on BDNF signaling in disease. Evidence is presented for the involvement of the molecule in neurological and metabolic disorders.

6.1 Neurodegenerative disorders

A longititude study on 1389 individuals has proposed BDNF as a biomarker for memory and general cognitive function in women (Komulainen et al., 2008). A similar 5-year follow-up study on 4615 65 years or older subjects concluded that physical activity could represent an important and potent protective factor for cognitive decline and dementia in elderly persons (Laurin et al., 2001). The above mentioned suggestions come in line with other studies on the relationship between BDNF, exercise and neuroprotection which are presented below.

6.1.1 BDNF and Alzheimer's disease (AD)

Postmortem studies on brains of patients with AD have shown decreased hippocampal BDNF mRNA and protein levels compared with control subjects of similar age (Hock et al., 2000; Lee et al., 2005; Phillips et al., 1991). The remarkable frequency of AD has led to epidemiological studies which focus on the identification of both genetic and environmental factors related to the pathophysiology of the disease. (Reviewed by Mayeux, 2003). Mattson et al have reviewed studies related to lifestyle habits such as exercise, restricted caloric intake and cognitive stimulation which up regulate BDNF signaling and correlate its expression with AD and other age-related neurodegenerative diseases (Mattson et al., 2004). Immunoreactivity measurements of the high affinity TrkB receptor in the temporal and frontal cortex have showed its decreased expression in patients with AD (Allen et al., 1999).

6.1.2 Parkinson's disease (PD)

PD is characterized from many pathophysiological changes in the brain characterizing the degeneration of neurons in substantia nigra (Gibb, 1989). Therefore many studies have tried to identify the role of different molecules in this process.

BDNF has been shown to be down regulated in postmortem samples of human brain tissue of patients suffering PD (Howells et al., 2000; Murer et al., 2001).

Evidence exists on the relationship between glutamate release, NMDA receptor activation and BDNF expression in the adult substantia nigra (Bustos et al., 2009). The abundant expression of the protein in the adult brain and its requirement for neuronal survival comes in line with the observations of its reduced expression in neurodegenerative disorders.

Possible cellular methods of coping with ageing-related insults of the brain have been described by Mattson et al and a representative diagram is presented in figure 17.

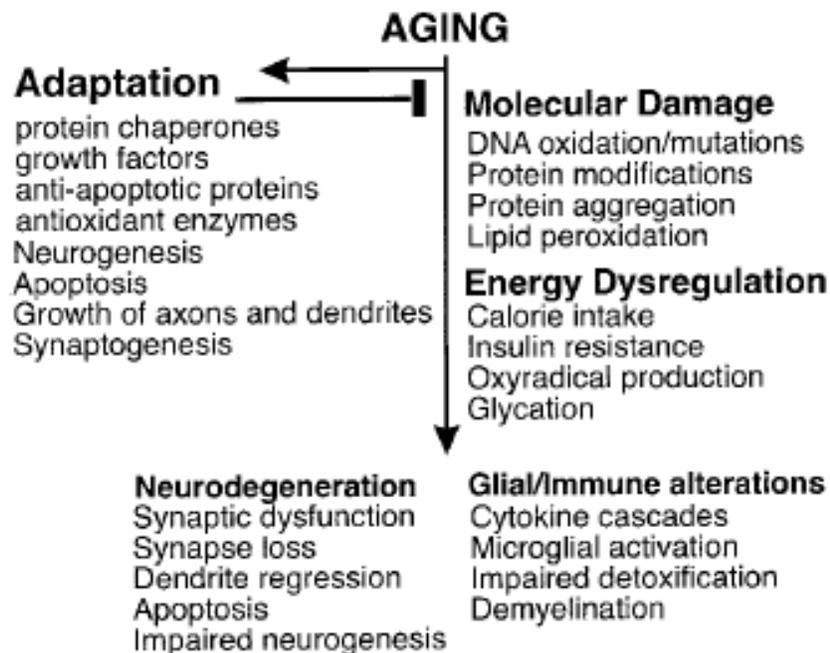


FIGURE 17. Coping methods to prevent ageing-related loss of function in the brain (adapted from Mattson et al., 2002)

6.2 BDNF and depression

As a disorder with complex pathophysiology, depression has been studied extensively. Many brain systems have been proven to be directly or indirectly affected in clinical occasions. Serotonergic, cholinergic, noradrenergic, GABAergic and dopaminergic systems show impaired function due to different biochemical changes which affect the properties of synaptic transmission and plasticity. (Reviewed by Manji et al., 2001)

Recent research on the actions of antidepressants in depression proposed the “Neuroplasticity hypothesis” which is based on the fact that the brain is able to rewire itself and change dramatically. The older “monoamine hypothesis” couldn’t explain the delay on the effects of antidepressants’ in mood increment as it was describing the symptoms of depression as the result of chemical imbalance. (Manji et al., 2001; Fuchs et al., 2004; Castren, 2005; Pitchot et al., 2008; Lanni et al., 2009).

Neuroimaging, neuropathological and lesion analysis studies have revealed a relationship between improper Cerebral Blood Flow (CBF) and glucose metabolism in brain areas related to emotional behavior as medial prefrontal cortex (MPFC) and closely related areas in the medial and caudolateral orbital cortex (medial prefrontal network), amygdala, hippocampus and ventromedial parts of the basal ganglia in animal models of depression and postmortem brains of depressive patients (Drevets, 2001; Drevets et al., 2008).

The “Neuroplasticity Hypothesis” strikes the importance of proper synaptic transmission and plasticity. As described in the current review, BDNF is believed to be crucial in both processes therefore its relationship with depression can be reasonably hypothesized.

Recent evidence show increased BDNF mRNA levels in rats’ brains after treatment with antidepressants (Musazzi et al., 2009) and antidepressants-like effects of increased BDNF signaling in mice over expressing the full length TrkB receptor (Koponen et al., 2005).

These data suggest a BDNF-mediated effect of antidepressants.

6.3 BDNF polymorphism

A single nucleotide polymorphism (SNP) has been identified in human BDNF gene. A methionine substitution for valine at codon 66 of the pro region of the protein impairs its functionality and is correlated with impairments in higher cognitive functions. It has been shown that carriers of the Met allele (Val/Met) have different hippocampal morphometry, revealed by using MRI scans. Val/Met individuals have smaller hippocampal volume in comparison with the homozygous for Val allele (Val/Val). This might be related to the important role of BDNF both during development and adulthood. (Reviewed by Chen et al., 2008).

Other experimental data have shown that val66met SNP affects the activity dependent secretion of BDNF and higher functions, as proposed from decreased hippocampal function in episodic memory tests by carriers of the Met allele. Observations on neurons transfected with Met-BDNF Green Fluorescence Protein (GFP) showed impaired intracellular trafficking and activity-dependent secretion. (Egan et al., 2003).

Impairment of cortical plasticity has been shown also using a Transcranial Magnetic Stimulation TMS protocol on humans and measuring the responses to it in humans heterozygous for the SNP (Cheeran et al., 2008).

The generation of mutant mice for the SNP showed normal expression of the protein in the neurons but impaired secretion. These data come in line with previous observations on secretion impairment due to the dysfunctional pro region of the protein. Additionally the mice carrying the mutation were showing increased anxiety behavior which was not inverted after administration of the antidepressant fluoxetine. (Chen et al., 2006).

Deficiency in NTs' and their impaired signaling has been proposed to be involved in the occurrence of psychosis through pathological alterations in embryonic neurogenesis which is considered as the etiology of the disease (reviewed by Thome et al., 1998) .

The maldevelopmental hypothesis for schizophrenia and psychoses has been described as inappropriate synaptic activity and cytoarchitecture which leads to the formation and maintenance of improper neuronal networks which are in charge for behavioral deficits as hallucinations, delusions and disorganized speech. Measurements on postmortem cortical and hippocampal samples of schizophrenic patients has shown altered expression of BDNF and NT-3 and strengthens the connection between NTs' and maldevelopmental hypothesis (reviewed by Durany et al., 2001).

Other studies have also shown correlation between the Val66Met SNP and psychiatric disorders and its age-related symptoms (Numata et al., 2006; Gratacos et al., 2007; Guillin et al., 2007; Lu and Martinowich, 2008).

6.4 Anti-diabetic properties of BDNF

Data from studies on models of diabetic animals have proposed a relationship between BDNF and glucose metabolism. In this section some evidence is presented on the effects peripheral administration of recombinant BDNF on the progression of type II diabetes (T2D).

The reversal of T2D symptoms by peripheral administration of recombinant BDNF was observed in diabetic (db/db) mice compared to non diabetic (db/m) ones (Yamanaka et al., 2008). The experimental setup aimed in determining the different effects on the progression of T2D in 4 weeks old db/db animals (prevention) and 8 weeks old db/db animals (early intervention). The pathophysiological markers under investigation were weight, blood glucose level, plasma insulin level, and pancreatic insulin level. The results on the pre diabetic group showed a protective effect of BDNF against the progression of the disease and delayed it on the early stage group by improving glucose metabolism and weight control. Additionally, reduced food intake was observed in the early stage diabetic BDNF-treated animals compared to vehicle-treated controls. Taken together the results propose favorable effects of peripheral administration of BDNF in glucose metabolism and body weight control. The mechanisms underlying these responses can be of great importance for prevention and treatment of the disease.

Previous studies have shown also regulatory effects of exogenously administrated BDNF on blood glucose control (Tonra et al., 1999). Of great importance is the observation that peripheral as well as intracerebroventricular administration of BDNF decreased food intake and lowered blood glucose by enhancing the action of peripherally administrated insulin in streptozotocin (STZ) – treated mice (Nakagawa et al., 2002).

Another group showed that peripheral administration of BDNF reduced blood glucose in obese and hyperinsulinemic mice (ob/ob) as well as in obese db/db mice during the period in which they showed hyperinsulinemia. Combined with the decrease of plasma insulin level without any change of blood glucose in normal db/m mice the results propose an enhancement and/or modulation of insulin actions in the periphery (Ono et al., 1997).

The above mentioned observations raise questions on the role of BDNF in the periphery and its possible bidirectional signaling with the brain which is discussed in the next section.

7 RESEARCH QUESTIONS

The broader question is how physical activity affects brain plasticity and health. Hippocampal plasticity which is related to learning and memory has been selected as the main brain area under investigation. The regulation and function of IGF-I which is proposed to mediate the effects of muscle activation on cognition through BDNF is also under investigation in the broader project on Exercise and NTs expression

The present thesis aims in identifying any correlations of the molecule under investigation and the aerobic capacity of the Low/High Capacity Rats (LCR/HCR), an animal model described below (Subjects section). A behavioral motor learning test is additionally performed to address the different motor learning capacity of the 2 groups. The literature review proposes an important role of BDNF in brain health as well as in learning and memory. The evidence on BDNF up regulation as a result of physical activity raises questions on a mechanism mediating the beneficial effects of exercise in the brain.

Given the increased oxygen and glucose demands of the brain, circulation properties are hypothesized to play critical role in the efficacy of its behavioral outputs. Additionally, neurons are relying in the blood for glucose since they can't store it. Taken together, the above mentioned facts propose that animals with higher aerobic capacity are more capable of delivering glucose through circulation.

Hypotheses

- a. Hippocampal BDNF protein levels are higher in HCR vs. LCR
- b. HCR show increased performance in motor learning compared to LCR

The measurements described in the thesis are part of a broader experimental setup which includes IHC for BDNF protein in skeletal muscle and in situ hybridization analysis for BDNF mRNA expression in the hippocampus and skeletal muscle of the same subjects.

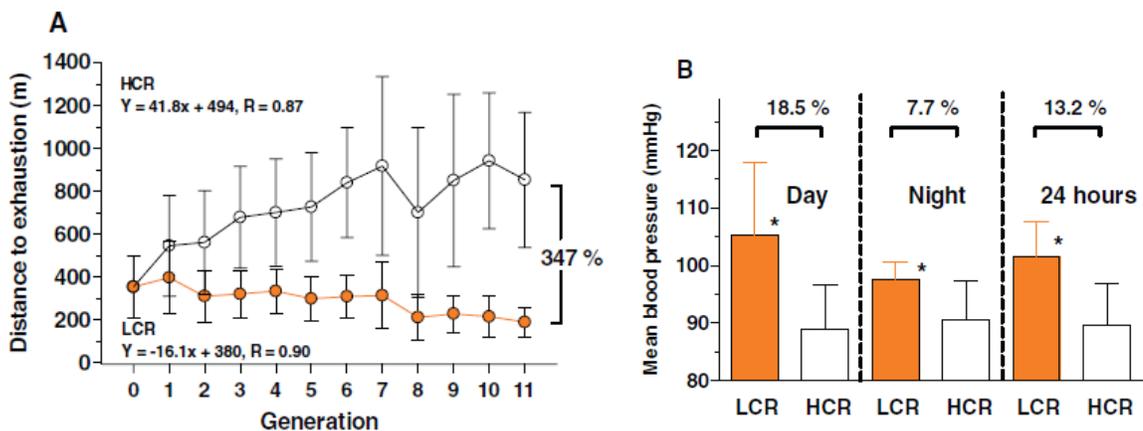
8 METHODS

8.1 Subjects

The 23rd generation of 13-months old N: NIH female rats (n=10) divided in LCR (n=5) and HCR (n=5) were used in this study. Rats were housed in pairs, in an environmentally controlled animal facility (12/12 h light-dark cycle, 22 °C, Animal house of University of Jyväskylä) and received water and standard food ad libitum. The study has been approved by the National Animal Experiment Board, Finland.

8.2 Animal model

The model of LCR/HCR has been developed after selectively breeding rats for their low/high capacity aerobic capacity. Significant differences in physiological values were evident for aerobic capacity (figure 18 A), blood pressure (figure 18 B) and artery response to exercise (figure 18 C) (Koch and Britton, 2001). The results of a series of studies proposed the emergence of 2 divergent strains after the 11th generation (Henderson et al., 2002; Gonzalez et al., 2006a; Gonzalez et al., 2006b;; Howlett et al., 2009; Palpant et al., 2009).



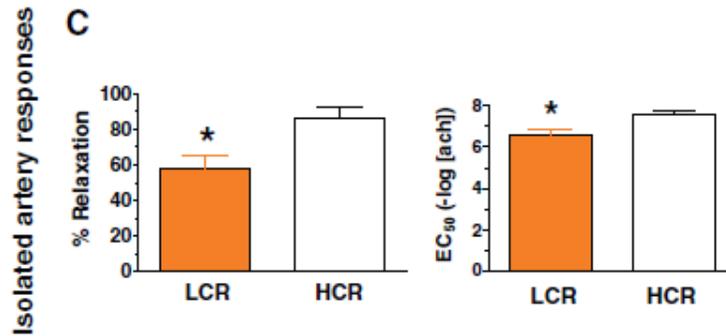


FIGURE 18. Physiological values after the 11th selectively bred generation of rats for A. aerobic capacity, B. blood pressure and C. artery response to exercise adapted from (Koch and Britton, 2001).

8.3 Immunohistochemistry (IHC)

Immunohistochemistry was performed using the N-Histofine[®] Simple Stain MAX PO staining method (Nichirei Biosciences Inc., Tokyo, Japan).

For IHC, the rats (5 animals from each group) were anesthetized with pentobarbital (30 mg/kg, i.p.) and perfused through the ascending aorta first with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.3) for 4-5 min. After perfusion the brains were excised and further fixed in the same solution for 60 min at RT. The tissues were cryoprotected with 20% sucrose in PBS. Frozen sections (6 μ m) were cut with Micron HM560 cryostat and thaw-mounted onto Polysine glass slides (Menzel, Braunschweig, Germany). The sections were incubated overnight with goat anti-BDNF antibody (dil. 1:200/400, Abcam, Cambridge, United Kingdom) followed with appropriate N-Histofine staining reagent for 30 min. ImmPACT[™] (Vector Laboratories, Burlingame, CA, USA) diaminobenzidine-solution was used as the chromogen. All antibodies were diluted in PBS containing 1% BSA and 0.3% of Triton X-100. Controls included omitting the primary antibody or replacing it with non-immune sera. No staining was seen in the controls.

8.3.1 Image analysis

The sections were assayed with image analysis system consisting of IBM PC, Nikon Microphot-FXA microscope, SensiCam digital camera (PCO Computer Optics GmbH, Germany) and Image-Pro Plus (Media Cybernetics, USA) program. BDNF expression was examined in hippocampal fields.

8.3.2 Analysis of cell expression

The BDNF-immunoreactive cells were quantified in the area of 500 μm in length in CA1, using Morphix program especially created by us for such analysis (Tugoy, Stroeve, 2006). The intensity of staining was expressed as conventional value of optical density scale from 0 (absolute white) to 100 (absolute black). We calculated all cells which maximal optical density was above the background. Then we express each individual value as a percentage to the average value of the first group.

8.3.3 Analysis of square expression

We compared the mean optical density value of the selected characteristic areas in CA1 and CA3 hippocampal fields in sections of both groups.

8.4 Motor learning measurements

Motor learning was assessed using Rotarod apparatus (Ugo Basile, Italy) consisting of a rotating drum with a grooved surface for gripping. The moving drum was set to accelerate from 4 to 40 rpm over 300 sec. The animals ($n=19$) had 2 familiarization trials on the rotating drum at 4 rpm for 1 min each with an interval of 10 min between them. The interval between the second familiarization and first test session was 30 min. Rats were subjected to

7 trials with 15 min interval between each trial. The time spend on Rotarod was assessed in each trial.

At the point of the measurements the animals were 11-months old and their mean mass was 312 ± 20.18 g (LCR) and $245.6 \pm$ (HCR).

8.5 Statistics

Statistical analysis for BDNF results was performed using the Wilcoxon 2 samples test. Analysis of variance (ANOVA) for repeated measures was used to examine the effects of training and group on dependent variable of motor learning. Mass and running distance to exhaustion (aerobic capacity) were expressed as mean \pm SD values of each group. Student T-tests were used to assess statistical differences between the 2 groups. The significance level was set to $p=0.05$ for all measurements.

9 RESULTS

9.1 Aerobic capacity

The aerobic capacity of the subjects was assessed using a treadmill running test until exhaustion. Mean distance run to exhaustion was $322,88 \pm 67,16$ for LCR and $1840,07 \pm 144,11$ for HCR ($p < 0,001$) (figure 19). Mean mass at the time of sacrificing was $312 \pm 20,18$ for LCR and $245,6 \pm 48,03$ for HCR ($p = 0,0013$) (figure 20).

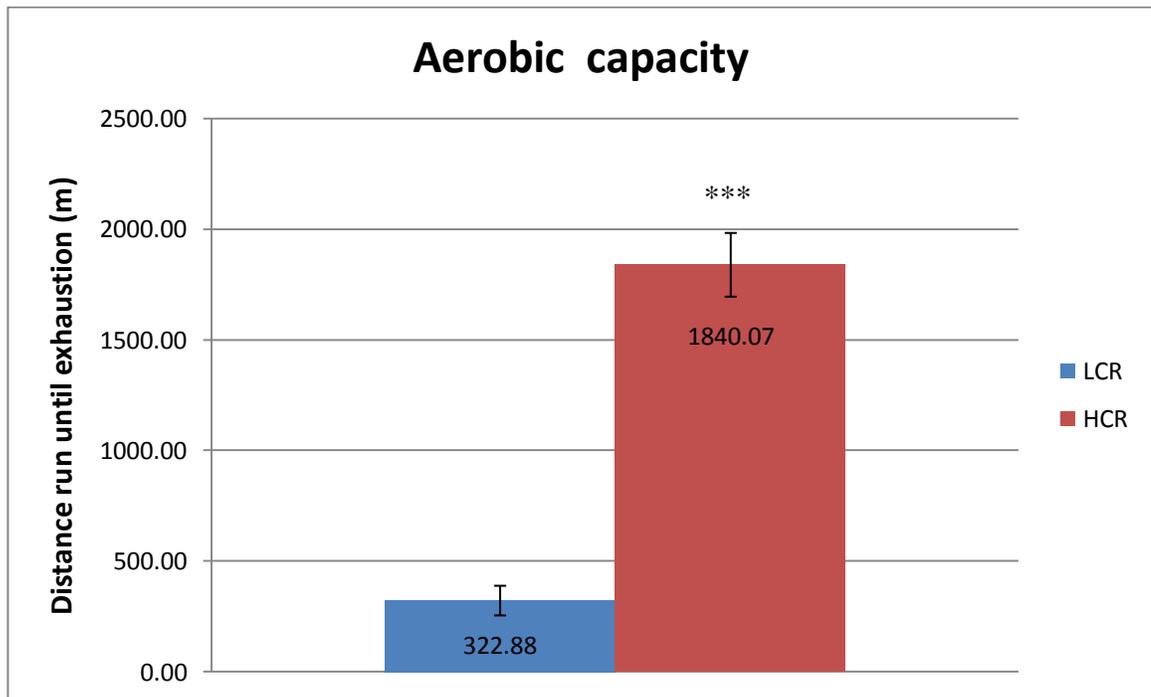


FIGURE 19. Aerobic capacity of LCR/HCR assessed by treadmill running test until exhaustion ($p < 0.001$).

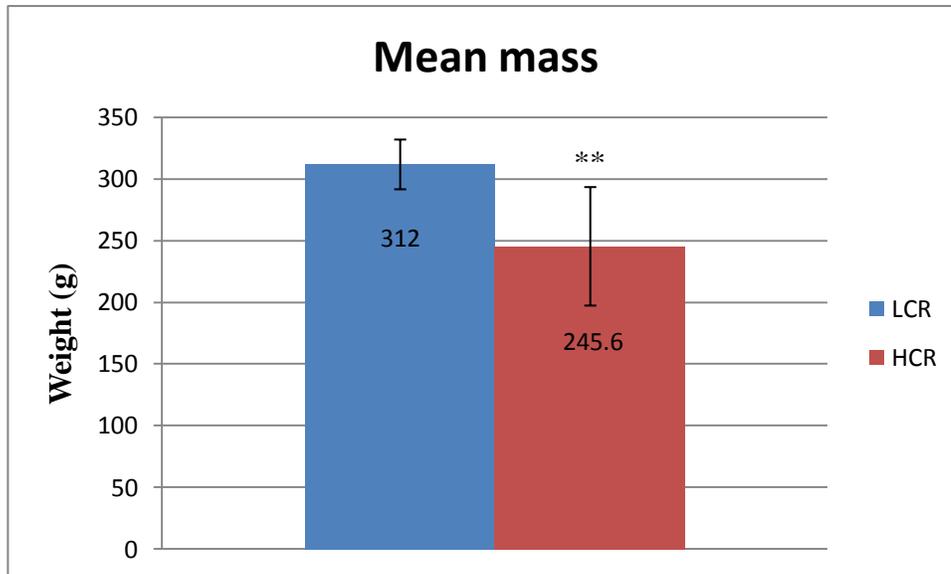


FIGURE 20. Mean mass of LCR/HCR ($p=0,0013$)

9.2 BDNF expression in CA1 and CA3 area of hippocampus

The percentage of mean cell expression in HCR ($n=5$) related to the mean of LCR ($n=5$) was 91.7 in CA1 sections (figure 21). The differences were not statistically significant ($p>0.05$). The percentage of mean square expression HCR ($n=5$) related to the mean of LCR ($n=5$) was 96% in CA1 sections (figure 22). The differences between the groups were not significant ($p>0.05$). The percentage of mean square expression in HCR ($n=5$) related to the mean of LCR ($n=5$) was 103 % in CA3 (figure 23) and is not considered statistically significant ($p>0.05$). Representative staining images from each area/group are presented in figure 24.

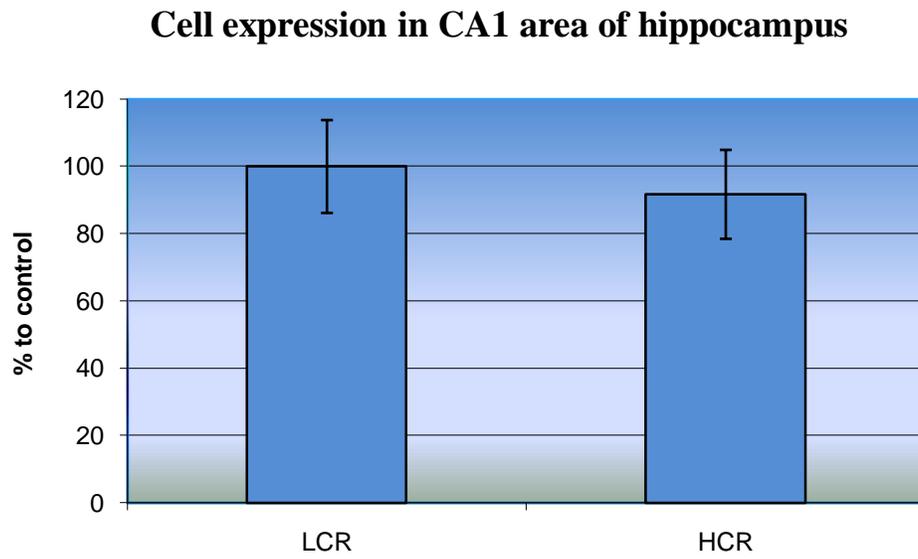


FIGURE 21. Mean cell expression in CA1 sections related to LCR mean.

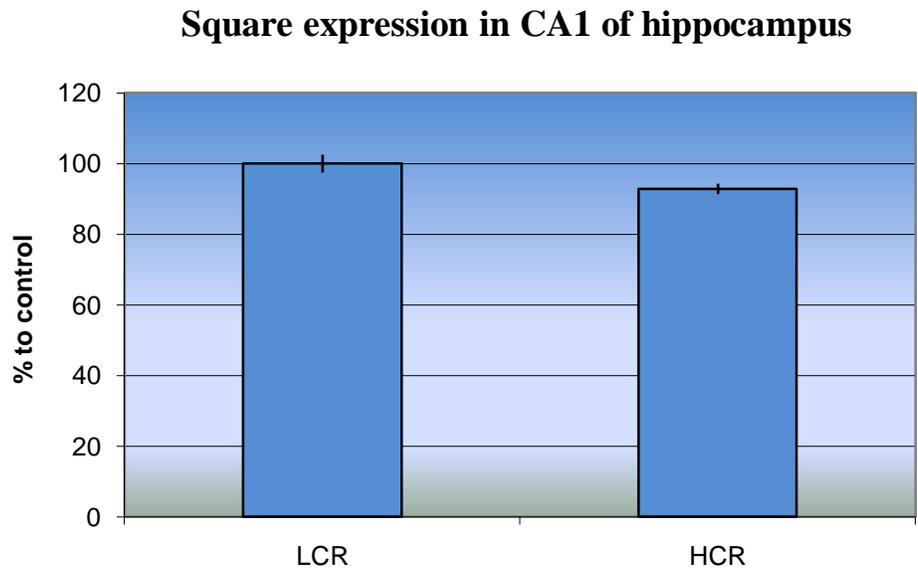


FIGURE 22. Mean square expression in CA1 sections related to LCR mean.

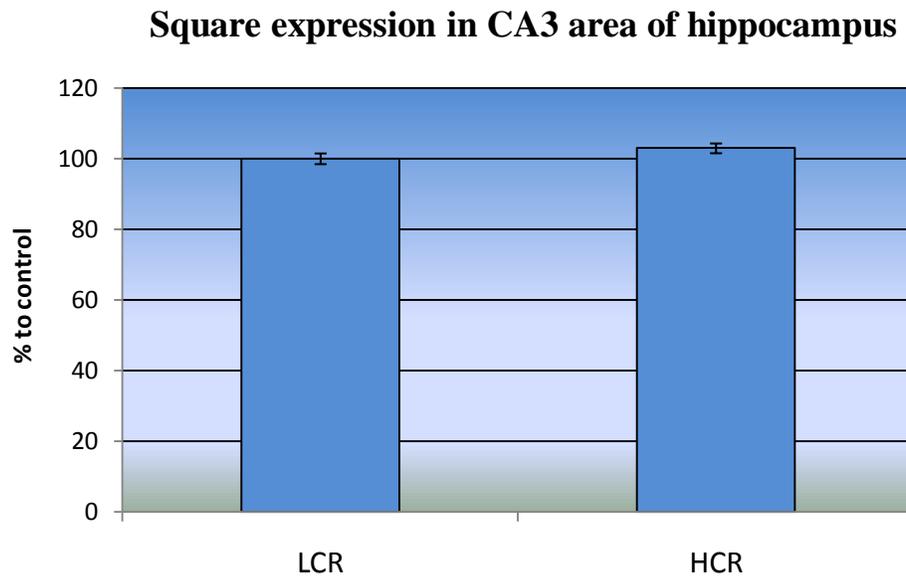


FIGURE 23. Mean square expression in CA3 sections related to LCR mean.

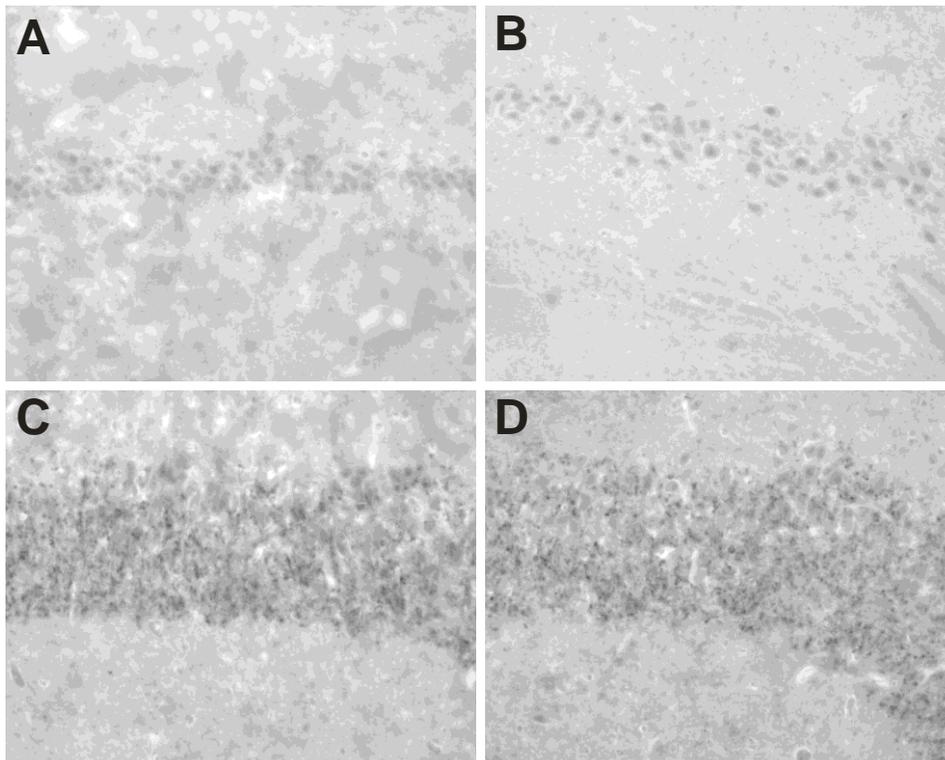


FIGURE 24. Immunohistochemistry staining for BDNF in CA1 (A,B) and CA3 (C,D). Images A,C correspond to LCR and B,D to HCR.

9.3 Balance test

The HCR animals were overall better at the Rotarod task as indicated by a significant main effect of group [$F(1,29) = 4.66$; $p < 0.05$; $\eta^2_{\text{partial}} = 0.138$]. Time stayed on the beam increased as a function of trial [$F(7,203) = 4.14$; $\eta^2_{\text{partial}} = 0.125$], but there was no significant interaction of trial and group. In separate analyses, trial had a significant effect on performance in HCR group [$F(7,91) = 2.37$; $p < 0.05$; $\eta^2_{\text{partial}} = 0.152$] and almost significant effect [$F(7,112) = 3.05$; $p = 0.06$], but significant linear contrast [$F(7,112) = 6.58$; $p < 0.05$; $\eta^2_{\text{partial}} = 0.291$] in the LCR group. Thus training had an equal effect on both groups but the HCR animals were overall better balanced.

Both LCR and HCR showed improvement between the trials and their performance was improving as a result of training. HCR showed an increased baseline performance and their learning curve was significantly higher compared to LCR. The results are summarized in figure 25.

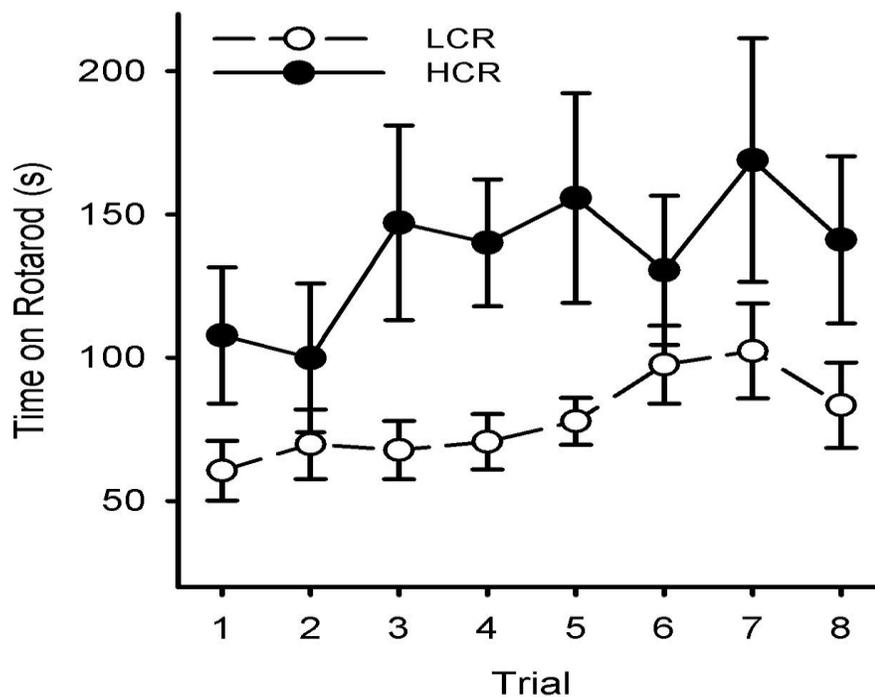


FIGURE 25. Learning curve for LCR and HCR on Rotarod balance test

10 DISCUSSION

HCR showed statistically significant higher aerobic capacity along with lower mean mass compared to LCR. These observations come in line with the phenotypic characteristics of the animal model of LCR/HCR. The IHC results verified the expression of BDNF in CA1 and CA3 areas of hippocampus which is crucial for learning and memory. Additionally, high capacity rats showed higher performance in the behavioral measurements which verifies the initial hypothesis. In qualitative terms, LCR tended to be less willing to participate in behavioral experiments compared to HCR. The hypothesis on elevated hippocampal BDNF levels in the high capacity groups was based on the neuroprotective properties of the protein. The non-significant differences between the 2 groups in combination with the literature-stated activity-dependent secretion of the protein and its acute elevation after a single bout of exercise propose an activation-related pattern of neuroprotective effects in neuronal networks. Our results propose that the different genetic background of the current rat model doesn't affect the protein levels of hippocampal BDNF. Possible reasons and suggestions for further research are discussed below.

10.1 Standard housing conditions

As described in the literature review of the present thesis, hippocampal expression of BDNF has been shown to be activity dependent. The LCR and HCR groups subjected to this study were caged in standard conditions without access to a running wheel or enriched environments. Quantification of BDNF protein levels failed to address significant differences between the two groups, a result expected if we consider the activity-dependent secretion of BDNF.

The hypothesis on long term effects on BDNF expression in hippocampus relied on the documented relationship of its effects with neuronal survival. In this context many molecules might serve as mediators of the acute BDNF up regulation to the survival of neuronal

structure and function. Binding to TrkB receptor may evoke local and acute effects related to survival promotion.

In behavioral terms, the animals subjected to the specific study showed different phenotypic characteristics and unpublished data from other behavioral experiments on learning and memory support the notion that LCR show lower performance which correlates with their low aerobic capacity and sedentary lifestyle. In this context, BDNF protein levels could be correlated with the amount of voluntary running and the behavioral scores of the animals of both groups.

10.2 Transcript variance and selectivity

The existence of multiple BDNF mRNA transcripts produced by alternative splicing of nine different 5'UTP exons (in the rat) which encode the protein have proposed an activity-dependent targeting to different cell compartments (Tongiorgi, 2008). This variance and selectivity is of great importance since protein targeting can fulfill the need of protein delivery to areas of the cell where it is required during synapse formation and/or synapse strengthening. Identifying the transcripts can increase our knowledge on BDNF selectivity during different processes and at different compartments of neurons. Additionally, it would be of great importance to correlate BDNF protein levels with acute neuronal events and/or single bouts of exercise. Processes known to require new dendritic synapses formation could be correlated with the presence of different mRNA transcripts. In respect to function, mRNA transcripts known to be trafficked to distal (compared to soma) dendrites could be correlated with new synapses formation in cell lines. The discovery of the presence of ribosomes in dendrites (Bodian, 1965) has suggested an important functional role of targeting different gene transcripts to dendrites which in turn participate in new synapses formation.

10.3 Behavioral measurements

Data on appetitive conditioning and T-maze tests (unpublished data) on the same subjects are in line with the results on the balance tests performed for this thesis. Animals from both groups learn and improve their scores as the trials number increases, but HCR started from a higher score and they exhibit faster learning than LCR. In qualitative terms, during the experimental setups, HCR were obviously more willing to participate in the measurements. That was clear since some LCR had to be excluded from the experiments due to their unwillingness to participate or their very poor overall performance in the tests.

10.4 Study limitations

Animals in both groups didn't have access to a running wheel which simulates a sedentary lifestyle. Thus, quantification of BDNF protein was only correlated with their initial aerobic capacity.

From a total number of 19 subjects only 10 were sampled for IHC due to different brain tissue preparation needed for the specific measurement (perfusion). Thus, 5 animals from each group were included in IHC measurements.

The proBDNF/mBDNF ratio could also have indicated a balance between apoptotic and survival promoting functions in hippocampal neurons. The cleavage of the pro-protein to its mature form could be correlated to behavioral data given their biologically opposite effects on the cell cycle. It should be taken under consideration that programmed cell death vs. survival/plasticity promotion depends on stimulation (activity dependent secretion of mBDNF).

10.4.1 TrkB binding

The biological significance of BDNF relies on its binding to TrkB receptor and the intracellular pathways it promotes. Therefore it would be of great importance to quantify the amount of bound BDNF on the cell membranes. Additionally, phosphorylation of the receptor can be quantified to assess the degree of functionality after protein-receptor binding. The amount of protein levels itself can't serve as an indicator of functionality since the above mentioned parameters contribute to the outcomes of BDNF binding to TrkB.

10.4.2 p75 binding

Low affinity binding of NTs to p75 receptors has been shown to promote programmed cell death (Chao and Hempstead, 1995; Roux and Barker, 2002; Teng et al., 2005). The pro-neurotrophins have been characterized as “the other half of Neurotrophins’ hypothesis” (Lee et al., 2001; Teng et al., 2005). The proteolytic cleavage of the pro-domain of proBDNF yields the mature form of the protein (mBDNF) which is considered as the biologically active form and important for plasticity-related processes as LTP (Pang et al., 2004). ProBDNF binding has also been shown to be essential for Long-term Depression (LTD) which is necessary for removal of unused synaptic connections (Lipsky and Marini, 2007; Yang et al., 2009). Thus, proBDNF IHC could be performed to assess the different localization pattern as well as protein levels in both groups.

10.5 Conclusions and further research

10.5.1 IGF-I and BDNF

The proposed mediation of BDNF action by IGF-I (Ding et al., 2006a) proposes the significance of its quantification in the areas of the brain where BDNF action is under investigation. Cell culture experiments investigating the synaptic plasticity and survival of neurons

under IGF-I action blockage could verify the proposed relationship between these molecules. Relatively recent research has shed light on Blood Brain Barrier (BBB) permeability by IGF-I and the enhanced binding of BDNF to TrkB while IGF-I is present (Reinhardt and Bondy, 1994; Carro et al., 2000; Niblock et al., 2000; Watanabe et al., 2005; Ding et al., 2006a). The above mentioned properties of IGF-I combined with its exercise-induced upregulation (Schwarz et al., 1996) propose the significance of further measurements on the model of HCR/LCR.

10.5.2 BDNF mRNA expression

It could be of functional importance to investigate BDNF mRNA expression in the areas under investigation. The quantity of mRNA present could be correlated with the amount of actual protein, potentially proposing differences in translation factors between the 2 groups. As literature on synaptic plasticity implicates BDNF, the mRNA/protein differences could argue different behavioral outcomes, in this case learning.

10.5.3 BDNF and skeletal muscle

The evidence on BDNF expression in skeletal muscle (Heinrich, 2003; Mousavi and Jasmin, 2006; Matthews et al., 2009; Clow and Jasmin, 2010) generates more questions on the molecular circumstances under which the protein is synthesized and secreted as well for its roles in the periphery. Gastrocnemius and soleus muscle samples from the same animals are currently under investigation as part of the wider project on differential expression of BDNF in this animal model.

10.5.4 Aerobic capacity and glucose delivery

An issue that has to be addressed is the differential ability of the animals in each group to deliver glucose in the brain, given its increased requirement in brain metabolism. The inability of neurons to store glucose emphasizes the importance of this process and long term

impaired glucose metabolism can be implicated in compromised brain performance and cognition.

10.5.5 BDNF and neuroendocrine system

The literature review proposes also a role of BDNF on the feeding behavior of mice and rats which can be connected with the regulation of leptin and ghrelin in the brain (Gomez-Pinilla, 2008; Saito et al., 2009). Correlations of feeding behavior and the expression of the above mentioned molecules might propose a relationship between physical activity and neuroendocrine system. Such correlations might shed light on the BDNF-mediated effects of physical activity on glucose metabolism and prevention of diabetes development in pre-diabetic mice (Ono et al., 1997; Tonra et al., 1999; Nakagawa et al., 2002; Yamanaka et al., 2008).

Since multiple biological systems have been proposed to affect and be affected by BDNF and its receptors, further research is required to expand our knowledge and reveal the molecular pathways which render exercise beneficial for brain health and plasticity.

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