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Estrogen receptor mediated regulation of brain-derived neurotrophic factor and its processing in CNS

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Title (English)

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Abstract

Brain-derived neurotrophic factor (BDNF) is an important protein in the nervous system that is synthesised as a proform and posttranslationally processed to its mature form. The mature BDNF is responsible for stimulation of neuronal growth, differentiation and cell survival. It has been found that the proform of BDNF can stimulate to the opposite biological effect from the mature form, by activating apoptotic pathways. The processing of the proform, which is thought to be mediated by estrogen, is therefore of high importance. In this study the BDNF protein pattern has been examined after stimulation with estrogen receptor agonists. A tendency to a higher expression of the mature BDNF can be seen in samples treated with an estrogen receptor β agonist. This upregulation could contribute to a higher level of cell survival, which could possibly result in an anti depressive like effect.

Keywords

Estrogen receptors, Brain-derived neurotrophic factor, depression, Western Blot, qPCR

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Sammanfattning

Depression är en sjukdom som har ökat kraftigt under de senare åren. Sjukdomen finns utbredd i alla folkgrupper och länder och drabbar människor av alla åldrar. Statistiken visar att kvinnor har dubbelt så hög risk som män att drabbas av sjukdomen. Tidpunkten för insjuknandet hos kvinnor är ofta sammankopplat med specifika händelser i livet, såsom efter en graviditet eller vid övergången till klimakteriet. Under dessa perioder utsätts kvinnor för stora variationer i hormonnivåerna, varför det kvinnliga könshormonet östrogen tros vara inblandad i uppkomsten av depressioner. Vid försök att tillföra kvinnor i menopausen extra hormon har en förhöjd risk att utveckla hjärtsjukdomar och bröstcancer visats. Östrogen verkar i kroppen på två olika typer av receptorer; östrogen receptorn α och östrogen receptorn β. Dessa två reglerar olika funktioner i kroppen och det är framförallt α-receptorn som tros ge de negativa effekterna vid behandlingen. Mer kunskap om den mekanism varpå östrogen utövar sin effekt på humöret skulle kunna användas för att modifiera östrogen så att bieffekterna vid behandlingen elimineras.

Brain-derived neurotrophic factor (BDNF) är ett protein i hjärnan som tros medverka i en kedja av reaktioner från östrogen till en anti-depressiv effekt. Proteinet stimulerar till bildning av nya celler och kopplingar mellan celler i nervsystemet. Det tillverkas som en proform och klyvs till en mogen aktiv form. Den prodel som klipps bort hjälper till vid transport och veckning av den mogna delen av proteinet. Studier har också visat att proformen kan stimulera till en motsatt biologisk effekt från vad den mogna delen gör. Då det mogna proteinet stimulerar till cellöverlevnad, kan proformen starta kedjor i cellen som leder till celldöd, vilket i sin tur kan ge upphov till en depression.

I detta arbete har nivåerna av proform och mogen form av BDNF studerats i hjärnan hos råttor som behandlats med olika typer av östrogener. Resultaten visade att östrogener som är modifierade att verka på β-receptorn ger en tendens till ökning av det mogna proteinet, vilket i så fall skulle stimulera till cellöverlevnad och en anti-depressiv effekt. De olika formerna av proteinet har även studerats i prover tagna från olika hjärnområden och från olika djur. Resultaten av dessa visade stor skillnad mellan hjärnområden och mellan könen.

Examensarbete 20 p Civilingenjörsprogrammet Molekylär bioteknik

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Abbreviations

AF	Activation function
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
DBN	DNA-binding domain
DPN	Diarylpropionitril
E2	17β-estradiol
ER	Estrogen receptor
HRE	Hormone response element
HRT	Hormone replacement therapy
LBD	Ligand binding domain
LTP	Long-term potentiation
MAOI	Monoamine oxidase inhibitor
NGF	Nerve growth factor
NT	Neurotrophin
PPT	Propyl pyrazole triol
SSRI	Selective-serotonin reuptake inhibitor
Trk	Tropomysin-related kinase

1 Introduction

Depression and other mood disorders have long been taken for being purely psychological in origin but are now known to be connected with molecular differences in certain brain regions. Statistics have shown that the illness affects twice as many women than men (Kesser *et al.* 1994). As the changes in mood often coincide with periods of life connected to fluctuations in the hormone level, like in the postpartum period and in the transition to menopause, the female sex hormone estrogen has been suggested to be correlated to the disease. This introduction intends to give the reader a theoretical background to the cause of mood disorders. It starts with a brief description of the brain function and anatomy, followed by an explanation of depressive disorders. Estrogens and their actions are thereafter described followed by a presentation of brain-derived neurotrophic factor.

1.1 The nervous system

The nervous system consists of billions of nerve cells linked together in a highly organized manner to form the rapid control system of the body. A nerve cell has a unique shape with long extensions that extend out from the cell body. These extensions, which can be longer than one meter, are either receiving incoming signals from connected cells (dendrites) or relaying outgoing information from the cell body (axons). The brain and the spinal cord make up what is called the central nervous system (CNS). It is responsible for integrating incoming information from the peripheral nervous system (PNS) and determines whether a response is needed. The information relayed in the nervous system is carried by neurons and consists of electrical impulses. An impulse is sent from the brain as an action potential that causes depolarisation along the axonal membrane. When the action potential reaches a synapse at the end of the neuron, calcium ion channels opens in respond to depolarisation of the membrane. The released calcium stimulates transport of vesicles with neurotransmitter to the synapse. Fusion of the vesicle membrane with the synapse membrane results in release of transmitter substances to the synaptic cleft between the nerve-end and the target cell. On the target cell there are receptors for neurotransmitters making it possible for the cell to receive the chemical signal and translate it into a desired response. By this mechanism an impulse is transferred from the brain by neurons to receiving target cells (figure 1)(Silverthorn 2004) .

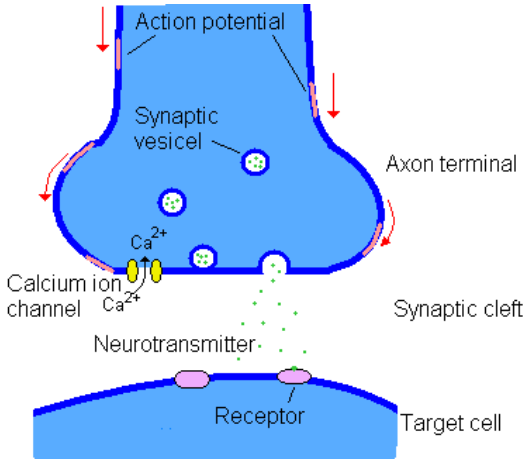
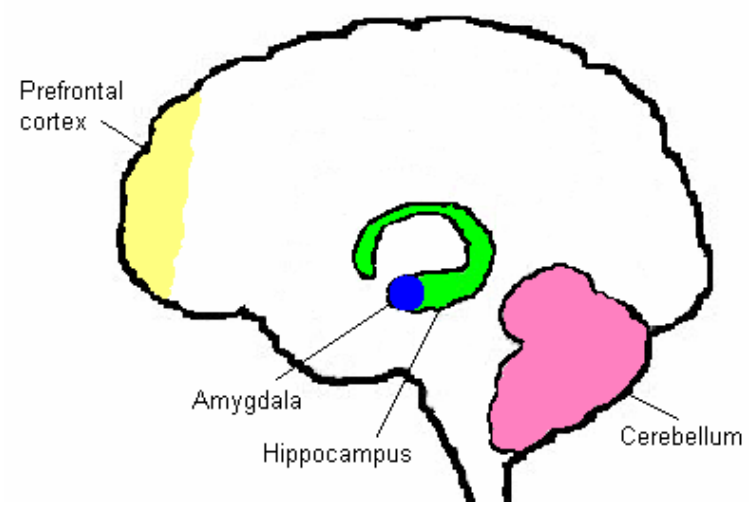


Figure 1 Communication in the nervous system. An action potential reaches the axon terminal of a nerve cell. The impulse causes depolarisation of the membrane and opening of calcium ion channels. Calcium is flowing into the cell where it stimulates synaptic vesicles to fuse with the membrane and release neurotransmitters into the synaptic cleft. This allowes the neurotransmitter to diffuse across the gap in the synaps and bind to receptors on the target cell. The binding of neurotransmitters to their receptors will in turn initiated a respons in the target cell.

The brain can be divided into different anatomical areas. Depressive states have been shown to be associated with increased activity in the limbic regions of the brain, such as the basal ganglia, amygdala and hippocampus (Haldane & Frangou 2006). The amygdala-hippocampal complex is of intense interest because of its role not only in the neuroanatomy of depression, but also for its involvement in memory, which is known to be impaired in depression (Doris *et al.* 1999). The limbic areas are tightly connected to the prefrontal cortex, making these areas of interest for studying of mood disorders (figure 2).



Figur 2 Brain areas connected to mood disorders. The brain can be divided into different areas. The Hippocampus and Amygdala is part of the limbic system that is associated with emotions, memory and learning. The limbic structure is in tight connections with the prefrontal cortex. The cerebellum is specialised in coordinating movements.

1.2 Depression

Depression is a common mental disorder that refers to a state of unhappiness or sadness accompanied with loss of interest, lack of energy, disturbed sleep, poor in concentration and feelings of guilt or low self-worth. It is distributed worldwide and occurs in persons of all genders, ages and backgrounds. A depression that lasts for more than two weeks and has advanced to be disruptive to the patients social functioning in daily living is defined as a clinical depression or a major depressive disorder (Schmidt 2005). A clinical depression is a long-term recurrent disease and can in serious cases lead to suicide. Major depression is a

leading source of disease-related disability in developing countries, and is predicted to be among the top three leading causes of disability worldwide by the year 2020 (Murray & Lopez 1997).

1.2.1 Anti-depressant drugs

The cause of depression is not known, but it is thought to be a combination of several factors such as biological effects, genetic background and social surrounding that can make rise to the illness. One theory of the molecular cause of depression is the monoamine deficiency hypothesis. It was formulated over 40 years ago and do still serve as the basis for the drug treatment today (Schildkraut 1965; Hirschfeld 2000). The theory proposes that depression is due to a deficiency in one or another of three signalling systems: serotonin, norepinephrine and/or dopamine. These are small molecules belonging to the group of neurotransmitters, which serves as chemical messengers in the nervous system (see brief description above). All the anti-depressants that are being used in the therapy today increase the amount of neurotransmitters in the synaptic cleft. The drugs perform their actions in two different ways; either by blocking the re-uptake of transmitter substance in the cleft or by inhibiting the enzyme that is responsible for degradation of the transmitters. The most widely used drugs are the selective-serotonin reuptake inhibitors (SSRIs), which use the first of the mechanism described. Monoamine oxidase inhibitors (MAOIs) use the other mechanism and were the first drug on the market (Stahl 1998). Even though the anti-depressants has been modulated to be safer and give less side-effects during the 40 years that has passed since the first anti-depressant medicament was invented, they still act through the same system by affecting the levels of neurotransmitters. As there are only ~50% of the individuals with depression that show full remission in response to treatment with the drugs presented today, there are a great need of anti-depressant drugs acting through different mechanisms (Berton & Nestler 2006).

1.3 Estrogens

Major depressive disorder affects nearly twice as many women than men, with a lifetime prevalence rate as high as 21 % in women compared to 13 % in men (Kesser *et al.* 1994). Women have a higher risk of developing the illness in certain periods of life. For example a few weeks after delivery when some women experience the postpartum syndrome or in the transition to menopause. As these periods of mental disturbances in women can be connected to fluctuations in the hormone levels, there is a large amount of evidence that the hormone estrogen, which is most abundant in women, is responsible for the mood disturbances.

1.3.1 Structure and functions of estrogens

Estrogens are a group of steroid hormones stimulating a wide range of functions throughout the body. They are referred to as female sex hormones because of their importance in female sexual maturation. They promote growth of the breast, uterus, vagina and ovaries and are also responsible for the estrous cycle in females of reproductive age. Although the levels of estrogens are higher in females, they are also present in males where they act on prostate and testis. In addition to the regulating functions in the reproductive system, estrogens are also present in the central nervous system (CNS) where they influence mood and behaviour (Wang *et al*. 2002). In the CNS they are also involved in the development, survival and ageing of neurons (Toran-Allerand 2005).

Estrogens are small lipophilic molecules that can freely diffuse through cell membranes and are able to cross the blood-brain barrier by lipid-mediated transport. In premenopausal women, estrogens are mainly produced in the ovaries from the precursor cholesterol. There are three naturally occurring estrogens: 17β-estradiol, estrone and estriol (figure 3). Of these, 17β-estradiol, commonly referred to as E2, is considered to be the most potent of them. Estrogens produced in the ovaries acts as circulating hormones that after synthesis are secreted and transported by plasma proteins to target tissues. In men and postmenopausal women the amount of circulating estrogens are very low. Instead the estrogens seem to be synthesised on a local level such as breast, brain and bone (Simpson *et al.* 2005).

Figure 3 Estrogens. Chemical structure of the three naturally occurring estrogens in the human body. Estradiol (E2), estrone and estriol.

1.3.2 Estrogens action

Estrogens perform its action by binding to estrogen receptors (ERs). The lipophilic estrogens permeate the membrane of the target cell and activate its receptor upon binding to the ligand-binding domain. Binding is causing a conformation change of the receptor, release of heat shock proteins and the subsequent translocation of the receptor into the nucleus. In the nucleus it binds as a dimer to specific hormone response elements (HREs) in the DNA that

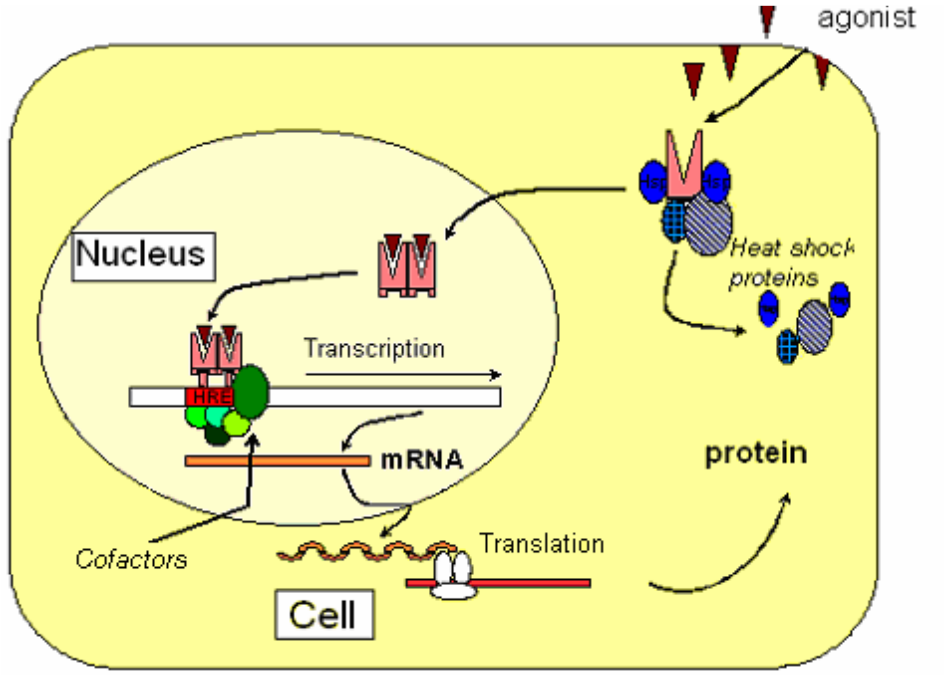


Figure 4 The mechanism of action for nuclear receptors. Estrogen agonists diffuse into the cell and binds to estrogen receptors. The receptors release heat shock proteins and translocate into the nucleus where they forms homodimers. A homodimer of estrogen receptors with its bound ligand recognises hormone response elements (HRE:s) on DNA and bind to these sequences. By recruiting cofactors it is able to initiate transcription of downstream genes. The transcripts are spliced to form mRNA, which are translated to proteins in the cytosol.

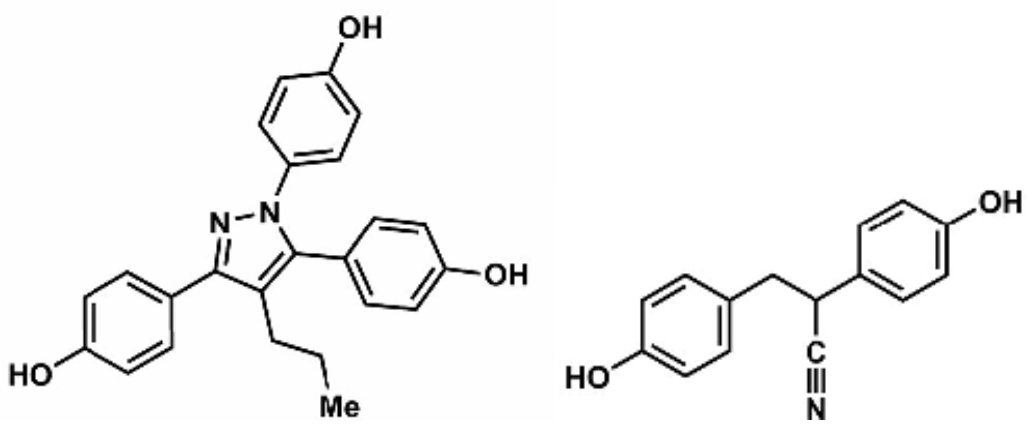
are usually palindromic repeats of the sequence 5'-AGGTCA-3' spaced by three nucleotides. The activated receptor attached to the specific site on DNA will recruit cofactors and initiate transcription of downstream genes (figure 4). By controlling the transcription rate of a wide range of genes, the estrogen receptors are able to affect a lot of functions in the cell (Mangelsdorf *et al.* 1995).

The ERs belongs to the family of nuclear receptors, also referred to as transcription factors. This superfamily of receptors consists of more than 150 known members with 48 of these present in humans (Cawla *et al.* 2001). These receptors all share a common structure; In the N-terminal there is a variable domain that has a transcriptional activation function termed AF1, which is followed by a DNA-binding domain (DBD) targeting the receptor to the HRE located upstream of a promoter sequence for a specific gene. A variable hinge region is separating the highly conserved DBD from the moderately conserved ligand-binding domain (LBD). The LBD ensures specificity and recognition of the receptor. At the C-terminal end of the protein there is an additional ligand-regulated transcriptional activation function domain (AF2), necessary for recruiting coactivating protein and responsible for dimerisation of the receptor (figure 5) (Bain *et al.* 2007).

Figure 5 The structure of a nuclear receptor. A nuclear receptor have two activation function domains (AF1 and AF2) located in both ends of the receptor. These domains are needed for the initiation of transcription and AF2 is also responsible for the dimerisation of two receptors. The DNA-binding domain (DBD) located after the AF1 unit recognises the specific sequence on DNA while the ligand-binding domain (LBD) contains the ligand binding pocket.

There are two major subtypes of ERs, ERα and ERβ. Although they are encoded by separated genes, located on different chromosomes, they share an important degree of homology due to the identity of their common endogenous ligand (Bodo & Rissman 2006). In the DBD, the α and β subtype share a 95% amino acid identity while the amino acid identity in the LBD is only 60% and in the N-terminal domain as low as 16% (Kuiper *et al.* 1997). Because of the similarity in the DBD, the two receptors recognise the same palindromic response element, which leads to up-regulation of the same genes and results in the same cellular response. Except from these similarities, a lot of factor differs between the receptors. An important difference is their tissue distribution. While ER-α is present in a higher rate in the uterus, ER-β seems to be wider distributed in the brain. The ER-α mRNA is expressed to a higher degree in the hypothalamus and the amygdala, areas involved in autonomic and reproductive neuroendocrine functions and the ER-β is dominating in the hippocampal formation, enthorinal cortex and thalamus, which is thought to be involved in cognition, non-emotional memory and motor functions (Österlund *et al.* 2000).

Although the endogenous estrogen 17β-estradiol (E2), has the same affinity for both the ER-α and ER-β, differences in the ligand-binding pocket of the receptors have made it possible to synthesise ligands with varying affinity for the two receptors. Propyl pyrazole triol (PPT) is a synthetic estrogen agonist that is highly selective for ER-α with an affinity for ER-α that is 410-fold higher than for ER-β (Stauffer *et al.* 2000). Diarylproponitrile (DPN) is a selective agonist for ER-β (figure 6) (Meyers *et al.* 2001). With the use of selective agonists for the different receptors, together with studies of knockout mice, it has been possible to study the different biological effects of the two receptors. ER-β in comparison to ER-α, seems to play a minor role in mediating estrogen action in the uterus and the skeleton, instead a dominant role for ER-β has been established in the ovary, cardiovascular system and brain (Harris 2007).



Propyl pyrazole triol (PPT) **Diarylproponitrile (DPN)**

Figure 6 Estrogen agonists. Chemical structure of two estrogen agonist with different affinity for the estrogen receptor (ER) α and β. Propyl pyrazole triol (PPT) has been reported to be 410-fold selective in binding ERα over ERβ while Diarylproponitrile (DPN) is an ERβ selective agonist.

1.4 Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) belongs to a family of neurotrophic factors. It is a group of four small basic secreted proteins: nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4) and BDNF. The first protein of these to be discovered was NGF, which was found in 1951 by scientists who studied the outgrowth of neurons in relation to the size of a target tissue. A larger target gave rise to a higher number of neurons presented, which indicated a metabolic exchange between the neurite and the substrate (Levi-Montalcini & Hamburger 1951). Fifteen years later BDNF was purified from pig brain as the second factor in the family (Barde *et al.* 1982). The early work led to the formation of the "Neurotrophic Factor Hypothesis". It proposes that a developing neuron, having extended processes into its target organ, would be in competition with other neurones for limited supplies of target-produced neurotrophic factors. Those that obtain the growth factor survive; those that do not die. The hypothesis has been of great importance for further studies of the neurotrophins but is today a simplistic way to describe their functions (Yuen *et al.* 1996).

The neurotrophins have a number of shared characteristics. They are expressed as preproprecursors with a signaling sequence and a prodomain that are processes to yield a mature protein of around 120 amino acids, 14 kDa. They share 50% identity in the primary structure and are all having six conserved cystein residues that form a distinct three-dimensional structure containing two pairs of antiparallel β-strands in a cystein motif (Lindsay 1996).

1.4.1 The function of BDNF

In addition to its action in neuronal survival and differentiation, BDNF has an important role in learning and memory (Thoenen 1995). The mechanism, in which the brain is able to change during life and form memory, is called synaptic plasticity. It involves formation of new connections between neurons (synapses) and changes in strength of existing synapses. The synaptic strength is increased or decreased by altering the level of post-synaptical depolarisation. This can be done in several ways; the receptors can be active a longer time and cause a higher depolarisation or the number of receptors can be increased. These processes occur in the whole brain but particularly in the hippocampus and associated limbic areas responsible for memory formation. Synaptic plasticity is characterised to be either short-term lasting between seconds and minutes or long-term, which may persist for several days. It is in the long-term potentiation (LTP) that BDNF is considered being most important (Figurov *et al.* 1996).

1.4.2 The BDNF genotype

The transcription apparatus of the BDNF gene is rather complex. With four different 5'-exons (exones I-IV) and one 3' exon (exon V) that contain two different polyadenylation sites there are eight different transcripts in human (Ohara *et al.* 1992). Because the 3' exon, which is present in all transcripts contains the only region coding for the mature protein, all of the transcripts give rise to the same peptide sequence (figure 7). The reason for use of different 5'-exons is the control of transcription by separate promoters. While three of the promoters are predominantly found in the brain, the fourth is found in lung and heart (Timmusk *et al.* 1993; Nakayama *et al.* 1994).

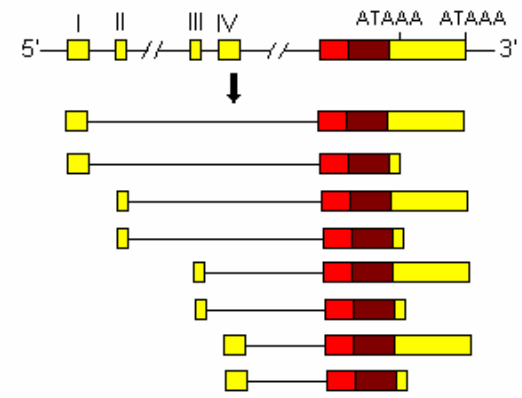


Figure 7 The gene coding for BDNF. A schematic presentation of the BDNF gene and its possible gene products. Boxes represent exons while lines are introns. Alternative transcripts can be formed by using different promoters transcribing four different 3' exons (I-IV) and by using two different polyadenylation sites (AATAAA). The yellow boxes stands for untranslated regions, the light red boxes are the proforms of the protein and the dark red are the sequence for the mature protein.

The BDNF gene contains a sequence with close homology to the hormone response element (HRE) recognised by the estrogen receptor-ligand complex (Sohrabji *et el.* 1995). It has been shown that the estrogen receptor with its bound ligand is able to recognise this sequence and promote the transcription of BDNF. The result has been discussed as the sequence differs from the typical HRE sequence. The two pentamers are in the BDNF gene separated by a spacer of nine nucleotides instead of three and one mismatch can also be seen with a guanine instead of a cytosine. Except from these differences the sequence is also containing the start codon for exon V (figure 8). One possible theory is that an HRE at this site is not a conventional transcriptional element but instead a site for estrogen receptor complexes to stabilize DNA during transcription, especially in genes with long intronic sequences, such as the one that codes for BDNF (Sohrabji & Lewis 2006).

Typical HRE sequence: **GGTCANNNTGACC**

Sequence found in BDNF gene: **GGTGAGAAGAGT*ATG*ACC**

Figure 8 The HRE found in BDNF. The typical hormone response element (HRE) for the estrogen receptor complex, compared to the sequence found in the BDNF gene. One mismatch can be seen in the first pentamer (C-G). The start-codon for exon five is present in the second pentamer (ATG).

1.4.3 Processing of the proBDNF to the mature BDNF

As described earlier, the BDNF gene gives rise to several transcripts that after splicing results in the same mRNA product. The first amino acid residues translated from the mRNA comtains a so-called signal peptide. It will guide the ribosome complex with the nascent protein to the endoplasmatic reticulum. The elongation of the peptide chain will automatically be transferred straight into the endoplasmatic reticulum while the synthesis occurs. The signal sequence is cleaved off when the whole chain is synthesised and the peptide is continuing to the *trans*-Golgi network. Most secretory proteins are posttranslationally modified and cleaved by furin-like enzymes in the *trans*-Golgi before they get packed into vesicles for constitutive secretion. This can be done without a need of a releasing signals. In neurons though, many proteins are secreted by the regulated pathway. In this pathway, the cleaving of the propeptide is done by prohormone convertases 1 and 2, and the processed protein is released in the extracellular space only in response to an appropriate signal (Lessman *et al.* 2003).

The translated BDNF contains 249 amino acid residues. The first 18 amino acids is the signal sequence that gets cleaved off already in the translational process. The following amino acids

from 19 to 136 are the pro-domain and residues 137-249 make up what is called the mature protein. At position 136 and 137 there is a multibasic site consisting of two arginine residues. This is a recognition site for cleaving by the protease furin in the *trans*-Golgi and indicates that BDNF is released by constitutive secretion. However, it has been shown that BDNF is using the regulated secreted pathway in some cells. This sorting is proposed to be depending on the efficiency of the furin cleavage (Mowla *et al.* 1999). As NGF and NT-3 are most often sorted to the constitutive pathway, BDNF appears to be sorted to the regulated pathway, indicating that BDNF is less efficiently cleaved by furin (Farhadi *et al.* 2000). Except from these two possible processing mechanisms, BDNF can also be secreted in its pro-form and get cleaved extracellularly by the protease plasmin (Lee *et al.* 2001). The proform of BDNF has been found in the extra cellular space in a higher level than the other neurotrophins.

Both the 28 kDa proBDNF and the 14 kDa mature BDNF has been found in the extracellular space in nervous tissues. Except from these two forms there has also been found a 32 kDa form of the protein that is indicating further processing of the chain. This processing is thought to be adding of a saccharide to the chain at an identified glycosylation site at amino acid 123. Glycosylation is an enzymatic process when sugar residues are added to the amine nitrogen of aspargine side chains in the purpose to stabilise the chain during transport and protect it from degradation (figure 9) (Mowla *et al.* 2001).

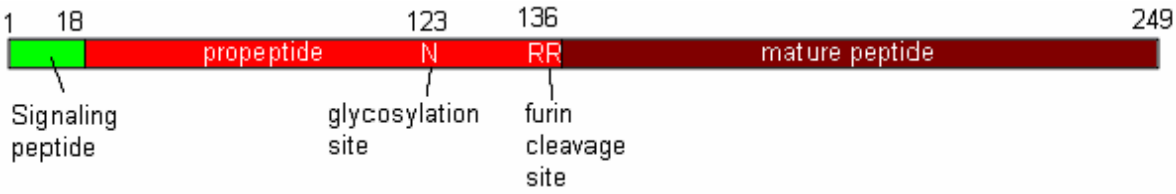


Figure 9 The BDNF protein. A schematic picture of the translated BDNF protein. Amino acid 1-18 is a signalling peptide that gets cleaved in the translational process. Amino acid 123 and 136 shows two sites for post-translational processing of the peptide, a glycosylation site and a cleavage site for the enzyme furin. The C-terminal end of the peptide is the mature protein.

1.4.4 Localisation of BDNF in the nervous system

Several studies have shown that BDNF is present in the entire nervous system but is most abundant in the CNS and particularly in the hippocampus (Ernfors *et al.* 1990; Michalski & Fahnestock 2003; Rosenfeld *et al.* 1995). According to the old "Neurotrophic Factor Hypothesis" BDNF can be released by target tissues and act on receptors on the axon of the postsynaptic neuron. BDNF with its bound receptor is thereafter becoming endocytosed and transported retrogradely in the axon to the cell soma where it activates cellular signalling

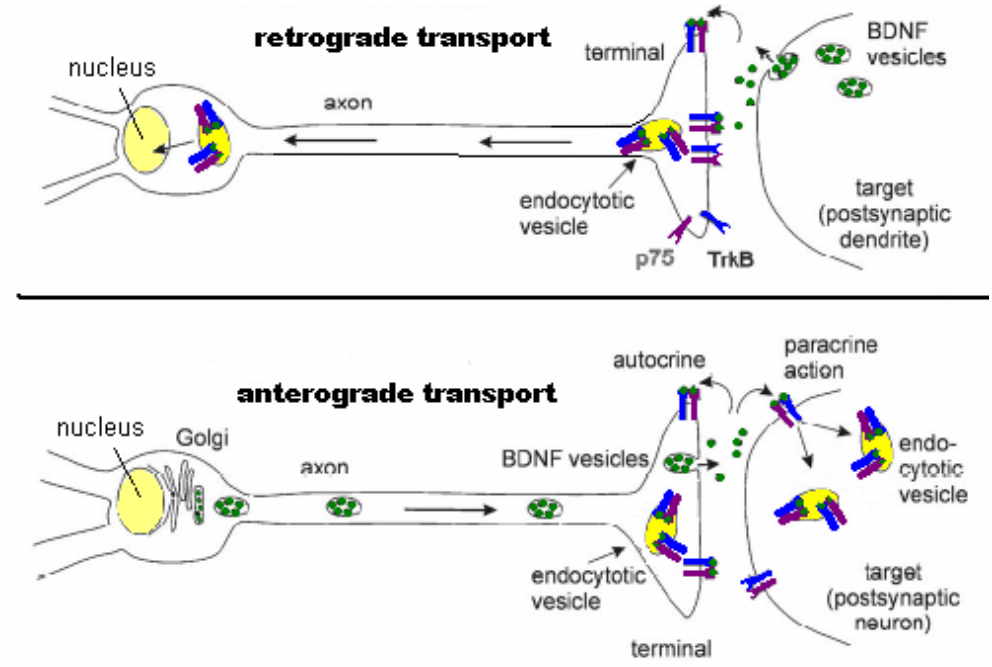


Figure 10 Transport of BDNF in the neuron. BDNF use both retrograde transport from the axon terminal to the nucleus and anterograde transport in the opposite direction from the nucleus to the axon terminal. The BDNF secreted by the neuron can act both paracrine and autocrine. There are two different receptors for BDNF, TrkB and $p75^{NTR}$.

cascades (Yuen *et al.* 1996). Except from this working mechanism, it has also been shown that BDNF can be synthesised by the neuron and anterogradely transported to the axon terminal. Following secretion into the synapse, BDNF can act either on receptors on the target cell (paracrine) or on receptors located on the cell from were it was released (autocrine) (figure 10) (Fawcett *et al.* 1998).

1.4.5 Receptors for BDNF

The neurotrophins perform their actions as homodimers on two different kinds of surface receptors. Their high affinity receptors belong to the family of tropomysin related kinase (Trk). NGF is activating the subtype of the receptor family called TrkA, BDNF and NT-4 activates TrkB and NT-3 activates TrkC. In addition, NT-3 can also activate TrkA and TrkB in certain cellular contexts (figure 11) (Patapoutian & Reichardt 2001). The Trk receptors share a conserved domain of 10 tyrosine residues. The neurotrophins act as homodimers and are able to pull together two receptors that dimerise. Dimerisation leads to activation and the tyrosine residues are phosphorylated. The phosphorylation act as docking sites for signalling molecules that initiate signalling pathways like PI-3 kinase-AKT and RAS-ERK, which in turn intercept nuclear and mitochondrial cell death programs (Ibáñez 2002). The result of Trk

receptor activation is a stimulation of neuronal differentiation, plasticity and survival (Frediman & Green 1999).

Except from the Trk receptors the neurotrophins aslo interacts with a low affinity receptor belonging to the tumour necrosis factor (TNF) receptor superfamily, the p75 neurotrophin receptor ($p75^{NTR}$). Like the Trk family receptors, this receptor is a transmembrane glycoprotein with an extracellular cysteine-rich domain. The function of this receptor is varying and it can either promote the effect of the Trk receptor, enhancing the cell survival properties, or guide the cell into the opposite pathway, apoptosis. The cell-survival promoting properties are achieved when the $p75^{NTR}$ is coexpressed with a Trk receptor. The coexpression of the two receptors increase the ligand binding affinity for the Trk and is also assisting the ligand in discriminating between the different Trk family members (Hempstead *et al.* 1991). In the absence of Trk receptors the neurotrophins can bind with a low affinity to only the $p75^{NTR}$. This binding is in contrast activating Jun-kinase pathways that in turn take the cell into cell death (Friedman 2000; Yoon *et al.,* 1998).

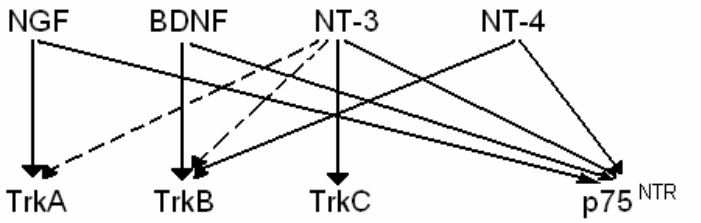


Figure 11 The neurotrophins and their receptors. All neurotrophins act on a receptor belonging to the tropomysin related kinase receptors (Trk) and the tumour necrosis factor receptor $p75^{NTR}$.

1.4.6 The opposite roles of proBDNF and mature BDNF

The neurotrophins are synthesised as proforms that are cleaved to their mature forms. The proforms have long been considered being inactive precursors and the role of the prosequence has been thought to be guiding of the protein into the right secretory pathway and contribution to the right folding of the mature protein. As the proforms of the neurotrophins were found extracellularly, an additional function for them was hypothesised. Studies showed that not only the mature form of the proteins activate receptors, but also the proform. In contrast to the mature protein, the proform is a high affinity ligand for the $p75^{NTR}$ receptor (Lee *et al.* 2001). As described above, activation of the $p75^{NTR}$ receptor is initiating apoptotic-signaling pathways in the cell, instead of cell survival pathways activated by the Trk receptor. The sum of these findings is that the mature forms is activating the Trk receptors, promoting cell survival, while the proforms are preferentially acting on $p75^{NTR}$, mediating apoptosis. The

processing of the neurotrophins is therefore essential for the regulation of their biological functions.

1.5 Estrogen, BDNF and depression

A connection between estrogen and depression has been suggested and hormone replacement therapy (HRT), in which women in the menopause receive estrogen and progestin as a complement for the drop in hormone levels, has been shown to give a positive anti-depressant effect. However, a big study done on HRT including 16 000 post-menopausal women was stopped in 2002 by the Women's Health Initiative (WHI) due to the found side effects. The study showed that long-term use (more than four years) of HRT increased the risk for suffering from heart disease and breast cancer (Russouw *et al.* 2002). The negative side effects of estrogen treatment are thought to be mediated by ER-α. A possible way to diminish the side effect would therefore be to create a selective estrogen agonist targeting the ER-β receptor (Österlund *et al.* 2005).

More research is needed to be able to understand the signalling pathway through which estrogen is exerting its anti-depressant effect. The involvement of BDNF in synaptic plasticity and memory formation makes it a putative member in the pathway. A hypothesis stated by Tsai (2007) proposes a mechanism of action of estrogen on BDNF. Estrogen, as well as anti-depressant treatment has been shown to up regulate the protein p11 (also called S100A10, Calpactin I light chain or annexin II light chain) (Svenningsson *et al.,* 2006) which is a small acidic protein interacting with a diverse set of target proteins in different cellular compartments. p11 in turn is found to act on tissue-type plaminogen activator (tPA) that converts plasminogen to its active form plasmin. As described above, plasmin is involved in the maturation of BDNF by cleaving of the pro-form to its mature form in the extracellular space. Through this complex signalling pathway, estrogen can stimulate an increased formation of the mature form of BDNF. As the proBDNF has been found to be a high affinity ligand for the $p75^{NTR}$ receptor, inducing apoptosis, and the mature BDNF stimulates cell-survival through the TrkB receptor; the pathway controlling the maturation of the protein is a powerful way to regulate the action of BDNF (figure 12).

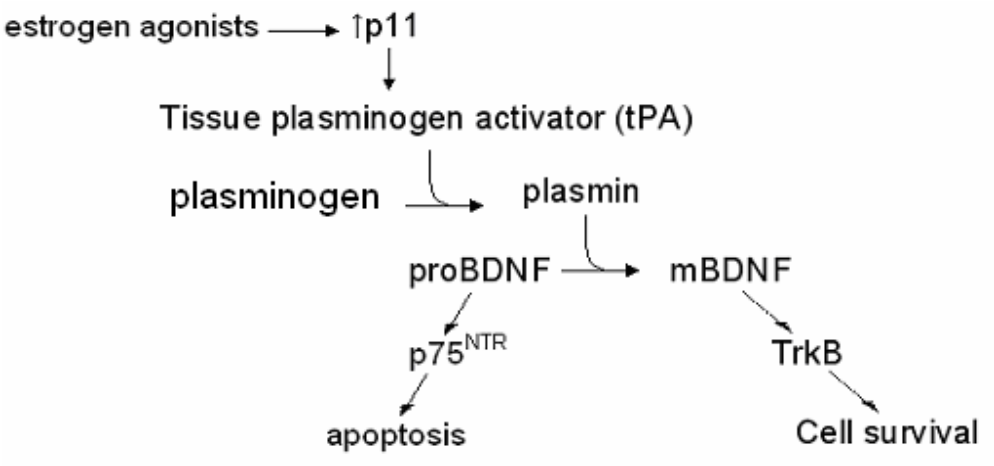


Figure 12 Signaling pathway for BDNF. A proposed signalling pathway in which estrogen agonists receive their anti-depressant activity. The processing of the proBDNF to its mature BDNF (mBDNF) is of crucial importance for its biological affect.

1.6 The aim of the thesis

The aim of the thesis was to study whether the processing of proBDNF to mature BDNF is upregulated in response to estrogen agonist stimulation, following the hypothesis stated by Tsai (2007) (figure 12). The processing of the protein has also been compared between different animals and brain areas.

2 Materials and Methods

2.1 Animals

In the animal experiments, all the rats and mice were housed under a 12 h light/dark cycle and had free access to food and water supply. All studies with animals were approved by the local Animal Experimental Ethics Committee.

2.1.1 Studies of BDNF in response to estrogen receptor agonists treatment

For the studies of the effects obtained on BDNF in the response to different estrogen receptor agonist stimulation, female SD rats weighting 133-146 g were used. The rats obtained subcutaneous doses during four days, with one dose of 200 µl per day, resulting in a total of four doses. 16 individuals were used divided into four groups. The first group received injections of only the vehicle (10% ethanol in sesame oil), the second group was treated with 17β-estradiol dissolved in the vehicle to a concentration of 0,14 mg/ml resulting in 0,2 mg/kg, group number three was treated with DPN of concentration 1,4 mg/ml dissolved in the vehicle resulting in doses of 2 mg/kg and the last group received PPT in the same concentrations as the DPN. Five hour after the last injection the animals were sacrificed by carbon dioxide inhalation. The brains were removed immediately and hippocampus, cerebellum and cortex were dissected and frozen in foil cooled on dry ice, and stored in -70^{o}C.

2.1.2 Studies of the processing of BDNF in different animals and brain areas

The animals used for the purpose of study differences in the processing of BDNF did not receive any injections. One male mouse of the strain OB/OB were used, one male SD rate and one female SD rat. The killing of the animals followed the same procedure as described above and the hippocampus, cerebellum and cortex were isolated, frozen and kept in -70^{o}C before homogenisation.

2.2 Western Blot

2.2.1 Tissue homogenisation

The brain tissues were taken from -70^{o}C and pieces between 50 mg and 90 mg were cut, weighted and put in centrifuge tubes of 2 ml. From cortex, the area taken for sample

preparation was located in the prefrontal cortex while from both hippocampus and cerebellum the pieces were taken randomly. The tissues were not allowed to thaw during the handling. Lysis buffer was added in the proportions 50 µl / 1 mg tissue. The lysis buffer contained 50mM Tris-HCl pH 7.5, 10 mM ethylendiamintetra acidic acid (EDTA), 0,5 % Tween-20, 100 µg/ml PMSF and one tablet of Complete^{TM} Protease Inhibitor Cocktail (Santa Cruz Biothecnology). It had been prepared in advance and kept in -20^{o}C until use when it was allowed to thaw on ice. The tissues were homogenized using a T8 Ultra-Turrax homogeniser (IKA) for approximately 15 seconds. The homogenates were incubated for 5 minutes and then centrifuged for 15 minutes at 14 000 rpm at 4^{o}C. The pellets were discarded while the supernatants were transferred to new tubes and frozen at -70^{o}C.

2.2.2 Recombinant protein

Recombinant expressed BDNF was used in the Western blot experiments as a positive control. It confirmed that the blotting of the proteins on the membrane and the antibody-detection had worked. The recombinant expressed BDNF was purchased from Sigma and diluted into 50 µl water to a concentration of 0,1 µg/ml. Before freezing in -20^{o}C it was aliquoted in volumes of 1 µl.

2.2.3 Sample preparation

The homogenates stored in -70^{o}C were let to thaw on ice and were vortexed and centrifuged on full speed one minute. To be able to load equal amount of protein in each well on the gel, the total protein concentration were measured on a ND-1000 Spectrophotometer (NanoDrop) that register the absorbance at 280 nm. After the measurements each homogenate was dissolved in 10% reducing agent, 25% sample buffer (both from Invitrogen) and an appropriate amount of water to receive a final protein concentration of 3,3 µg/µl. The recombinant expressed BDNF was diluted to a final concentration of 0,5 ng/µl (se table below). The reducing agent, dithiothreitol (DTT) breaks disulfide bridges and help to linearise the protein while the sample buffer contains sodium dodeocyl sulfate (SDS) that binds to the protein chain and donate a negative charge to it. The negative charge will cause the proteins to migrate towards the positive anode during electrophoresis. As smaller proteins travel faster through the gel matrix, a separation of the proteins depending on their size is achieved. The samples were mixed on a vortex and centrifuged to the bottom of the tube before heating in 70^{o}C during 10 minutes. Thereafter they were kept on the bench for cooling to room temperature and centrifuged shortly before loading onto the gel. All electrophoresis

equipment, reducing agent, sample buffer, running buffer and gels were purchased from Invitrogen.

	Sample (µl):	Water (µl)	Sample buffer (µl)	Reducing agent (µl)
Homogenate	**X**	**26-X**	**10**	**4**
Recombinant BDNF of concentration 1ng/ml	**2**	**24**	**10**	**4**

Table 1 Sample preparation for electrophoresis. The table shows the volume used for preparation of the samples for electrophoresis. X is the volume of the homogenized tissue needed to receive a final concentration in the well of 100 µg protein.

2.2.4 Gel electrophoresis and Western blotting

Polyacrylamide gradient gels with concentration 4-12% were used for separation of the proteins (NuPAGE® Bis-Tris, Invitrogen). Each gel had ten wells with a thickness of 1,5 mm. 5 µl of a ladder (SeeBlue® Plus Prestained standard, Invitrogen) was loaded in well number one followed by 15 µl of recombinant expressed BDNF of concentration 0,5 ng/µl in well number two. Well number 3-10 was loaded with 30 µl of the prepared samples with concentration of 3,3 µg/µl. The gel was run for 60 minutes at 200 V in a MOPS NuPAGE® running buffer (Invitrogen).

Two different transferring apparatuses were used. In both systems PVDF membranes with a pore size of 0,2 µm were used. The old traditional apparatus used a transfer buffer with 10% (v/v) methanol. The proteins were transferred from the gel to the membrane during 90 minutes at 30 V in room temperature. The new transfer apparatus used, Iblot^{TM} (Invitrogen) was run with 20V in 10 minutes and no transfer buffer was needed. After blotting the membrane was stained with ponceau solution (Pierce Biotechnology) to confirm a successful transferring of the proteins.

2.2.5 Antibody detection and development

The membranes were treated with 5% non fat milk in PBS-T in room temperature during one hour for blocking of unspecific binding sites. Thereafter they were treated with 10 ml primary antibody (N-20, Santa Cruz, CA, USA) diluted 1:200 in PBS-t with 5 % milk powder. The membranes were kept in the antibody-solution over night on a shaking table at 4^{o}C. The primary antibody was raised against an internal sequence of the mature protein, allowing for recognition of both the pro- and the mature-form of BDNF. Because of the high conservation of the protein between species, the antibody had 100% cross-reactivity between samples from human, mouse and rat. Two different batches from the manufacturer were used, batch A2207

and D1607. After washing with PBS-T three times quickly followed by 3 times five minutes, the membranes were probed with anti-rabbit horseradish peroxidase-conjugated antibody (Pierce Biotechnology). The conjugate enables for detection of the appropriate bands by using enhanced chemiluminiscence. The antibody was diluted 1:1000 in 5% non fat milk and was incubated on the membrane at a shaking table at room temperature during one hour. The membrane was washed in the same manner as after the primary antibody and incubated for five minutes in a developing solution (Pierce Biotechnology), dried and sealed in a plastic bag before development. The film was developed for 20 minutes with the old transferring apparatus, while for the Iblot-system only 30 seconds was needed.

To ensure equal loading of samples, the same membranes were also treated with an anti-actin antibody raised in mouse (Sigma). As the actin expression is often unchanged throughout different treatments, it is frequently used as a stable standard. The membranes were incubated with the actin antibody diluted 1:30 000 in PBS-T with 5% milk powder during 30 minutes. Washing of the membranes were done according to the instructions above and the membranes were probed with anti-mouse horseradish peroxidase conjugated antibody diluted 1:5000 during 30 minutes (Pierce Biotechnology).

2.3 Real time PCR

The activity of the gene for p11 and BDNF was studied with real time PCR. The standard curve method was used, which is a relative quantification method. Changes in gene expression of a treated sample can be detected in comparison to a reference.

2.3.1 Sample preparation

Approximately 30 mg brain tissue from each disserted hippocampus stored in -70^{o}C was homogenized with a T8 Ultra-Turrax homogeniser (IKA) in 1ml QIAzol Lysis Reagent. Chloroform was added and by centrifugation the homogenate was separated into an aqueous and an organic phase. The upper aqueous phase containing the RNA was extracted and mixed with ethanol to provide appropriate binding conditions before applying to an RNeasy^{TM} spin column. The total RNA was bound to the membrane in the column and contaminants were washed away. To ensure that no DNA was left in the samples, an RNase-Free DNase set was used for digestion of DNA during the purification step. All procedures were done according to the manual in the RNeasy^{TM} Lipid Tissue Mini Kit (Quiagen Inc., Valencia, CA, USA). Before cDNA synthesis, the quality and quantity of the RNA was measured with an agilent

(Agilent Technologies). 1 µg RNA of each sample was used in the reverse transcription to single stranded cDNA. An optimised mix of enzyme, nucleotides, primers and buffer was included in the High-Capacity cDNA Reverse Transcription Kit (Applied biosystems). A thermal cycle of 10 minutes in 25°C, 120 minutes in 37°C and 10 minutes in 70°C was used and the tube containing 20 µl of cDNA was stored in -20°C until use in the PCR reaction.

2.3.2 Determination of a housekeeping gene

In qPCR-assays the result is achieved by comparing data obtained for the gene of interest with data from a stably expressed gene (housekeeping gene). It is of outmost importance that the housekeeping gene is not affected by the different experimental conditions or treatment. Five different genes was studied in the finding of the best standard; Glucoronidase β, Hypoxanthine guanine phosphoribosyl transferase, Transferrin receptor, Actin- β and Albumin. The four samples in each test group (control, 17 β-estradiol, DPN and PPT) was pooled and run in a qPCR with the five genes. With a GeNorm-test (Vandesompele *et al*. 2002), the gene for Glucoronidase β was chosen for being least affected by the different treatments.

2.3.3 TaqMan® assays

The PCR reaction was done in the presence of a TaqMan® probe enabling to measure the amount of gene product after each thermal cycle. The TaqMan® probe contains a reporter dye in the 5' end and has a quencher dye in the 3' end. One PCR cycle starts with fusion of the specially designed probe and primers to the single stranded DNA of interest. It continues with elongation of the sequence from the primers. When the primers reach the sequence with a fused probe, an enzyme is able to cleave the probe, making further elongation possible. The cleaving of the probe will also separate the reporter dye and the quencher dye, which results in an increased fluorescence of the reporter. Accumulation of PCR product was detected directly by monitoring the increase in fluorescence of the reporter dye with a MX3000 qPCR instrument (Stratagene). TaqMan® probes, primers and optimised buffer was ordered from Applied Biosystems. The amount of thermal cycles needed to reach a certain threshold (Cτ) of fluorescence was determined and compared between the samples.

The PCR reactions were done in a 96 well format. On each plate a standard curve for the gene of interest (BDNF or p11) and for the housekeeping gene was done. The standard curves were done with a pool of all 16 samples. On the same plate, each of the 16 samples was also

studied with both the gene of interest and the housekeeping gene. All samples were done in duplicates. The relative quantification-standard curve method was used to analyse the results obtained from the PCR. A mean-value of the duplicates was used and in each sample the relation between the gene of interest and the housekeeping gene was calculated.

3 Results

3.1 Effect of estrogen receptor agonists treatment on BDNF

Four groups of female SD rats were treated with three different estrogen receptor agonists and one control sample consisting of the vehicle. The hippocampus was dissected from the removed brains and BDNF protein levels were studied together with the mRNA expression levels of both p11 and BDNF.

3.1.1 Changes in protein levels

Figure 13 shows the results from the Western Blot of the sixteen samples prepared from hippocampus in individual animals with the proteins BDNF and actin visible as dark bands. BDNF were detected by using of a polyclonal anti-BDNF IgG (Santa Cruz, batch number D1607) and actin was detected with the use of a monoclonal anti-actin antibody (Sigma). Actin corresponds to the band at the top around 42 kDa. It is a protein that is often unaffected through treatments and therefore used as an internal standard to verify that the same amount of proteins have been loaded in each well. The band at 28 kDa shows the pro-form of BDNF while the mature form corresponds to the band at the bottom, around 14 kDa. The double band shown around 19 kDa differs a lot between the samples and can possibly be a truncated form of the propeptide.

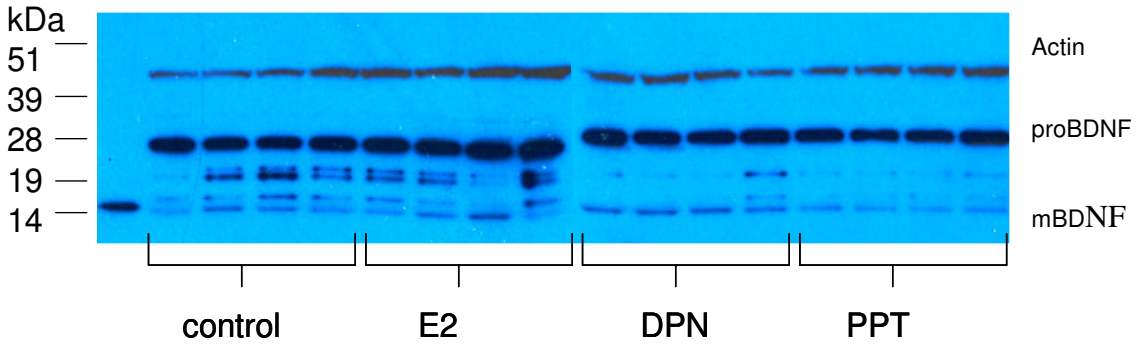


Figure 13 Western blot of BDNF. The different BDNF products have been separated on a polyacrylamide gel, transferred to a PVDF membrane with the IBlot system and detected with the use of an antibody raised against amino acid residues present in both the proBDNF and the mature form (batch number D1607). 100 ng total protein of each sample was loaded. The strong band at around 28 kDa correspond to the proform of the BDNF while the lowest band at 14 kDa is the mature form of BDNF. It is also a double band around 19 kDa present that might be a truncated form of the protein. The band around 42 kDa correspond to the protein actin, used as an intern standard, showing the amount of protein loaded in each well. Actin is detected with the use of an anti-actin antibody raised in mouse. The single band in the first well is a recombinant expressed BDNF serving as a positive control for the BDNF antibody. In the following 16 wells, the samples prepared from hippocampus in 16 animals treated with estrogen agonists, are loaded.

3.1.2 Expression of the genes for BDNF and p11

To determine the effects caused by the different estrogen receptor agonists on gene transcription, the mRNA levels of p11 and BDNF were studied using qPCR. The results are presented in diagrams with the mean value of the four samples in each group shown as bars (figure 14 and figure 15).

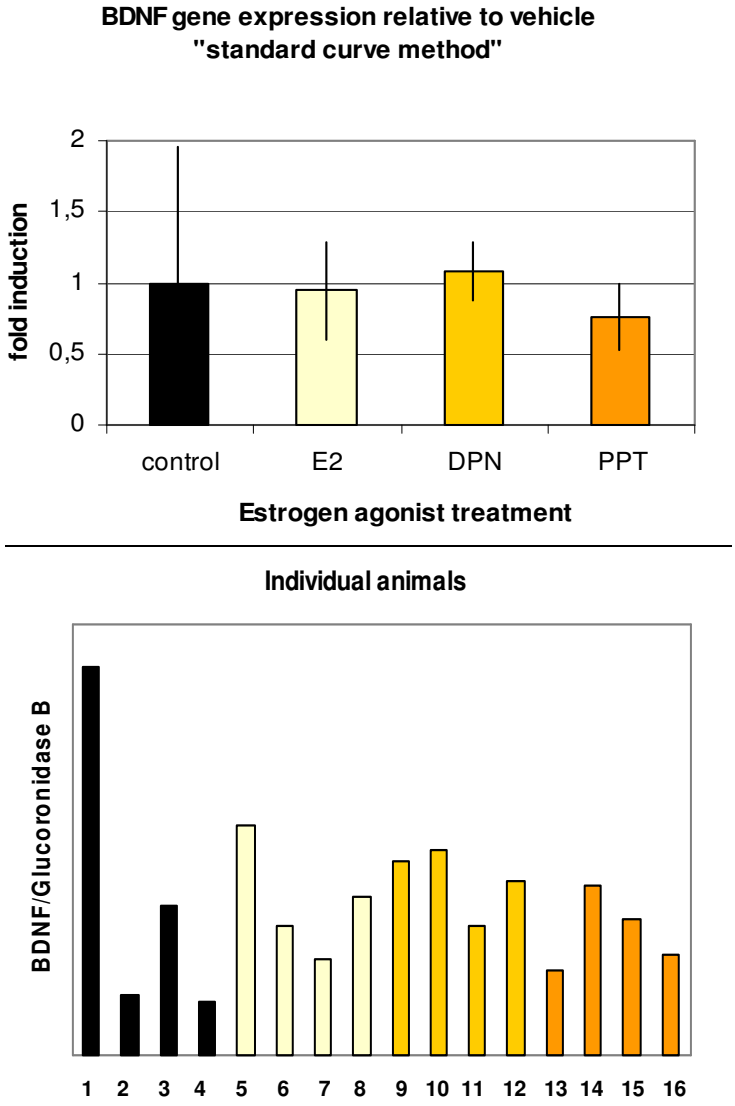


Figure 14 qPCR results. Samples prepared from hippocampal tissues of rats treated with different estrogen agonists were used to study the effects of estrogen on the transcription rate of BDNF. The mRNA levels were measured with qPCR. The diagram on top shows the mean values of the relation between the BDNF gene and the housekeeping gene (Glucoronidase β) of the four individuals in each group. The values are normalised against the control group to show the fold induction of the gene. Standard deviations between the individuals in the group are shown with bars. The diagram at the bottom shows the expression of the BDNF gene in relation to the housekeeping gene (Glucoronidase β) in each individual. Animal 1-4 are the controls, 5-8 have been treated with E2, 9-12 DPN and 13-16 PPT.

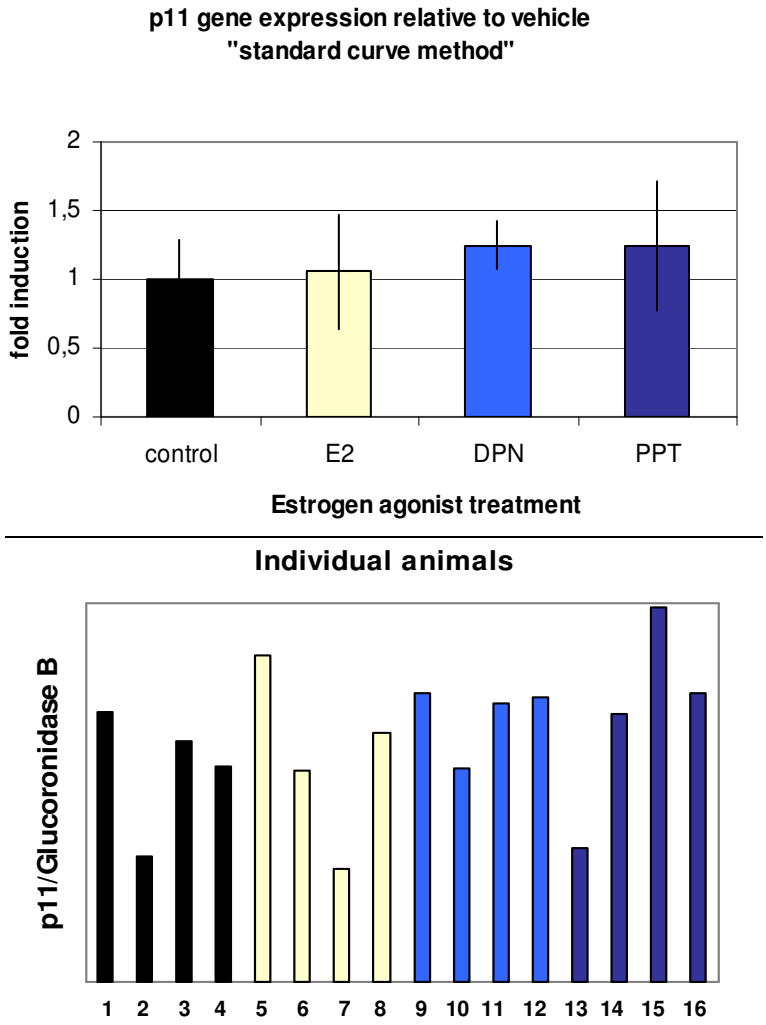


Figure 15 qPCR results Samples prepared from hippocampal tissues of rats treated with different estrogen agonists were used to study the effects of estrogen on the transcription rate of the gene for p11. The mRNA levels were measured with qPCR. The diagram on top shows the mean values of the relation between the p11and the housekeeping gene (Glucoronidase β) of the four individuals in each group. The values are normalised against the control group to show the fold induction of the gene. Standard deviations between the individuals in the group are shown with bars. The diagram at the bottom shows the expression of p11 in relation to the housekeeping gene (Glucoronidase β) in each individual. Animal 1-4 are the controls, 5-8 have been treated with E2, 9-12 DPN and 13-16 PPT.

The fold induction of the BDNF gene and the p11 gene is shown in the top diagram of figure 14 and figure 15 respectively. As Glucoronidase β was shown to be least affected by the different treatments, it was chosen as the housekeeping gene in the experiments. The gene expression of BDNF and p11 was measured in relation to the housekeeping gene and the fold induction was calculated by normalising to the control group treated with only the vehicle. The diagram at the bottom of the figures shows the variation between the individuals in each group. Numbers 1-4 are animals from the control group, number 5-8 has been treated with E2, 9-12 has received DPN and 13-16 were treated with PPT.

3.2 Processing of BDNF in different animals and brain regions

During studies of BDNF in estrogen agonist treated animals, the processing of BDNF in different species and different brain areas from untreated animals was observed. In figure 16 samples from a female SD rat is compared with a male SD rat and a male OB/OB mouse. The results shown are with the use of two different batches of the antibody; A2207 and D1607.

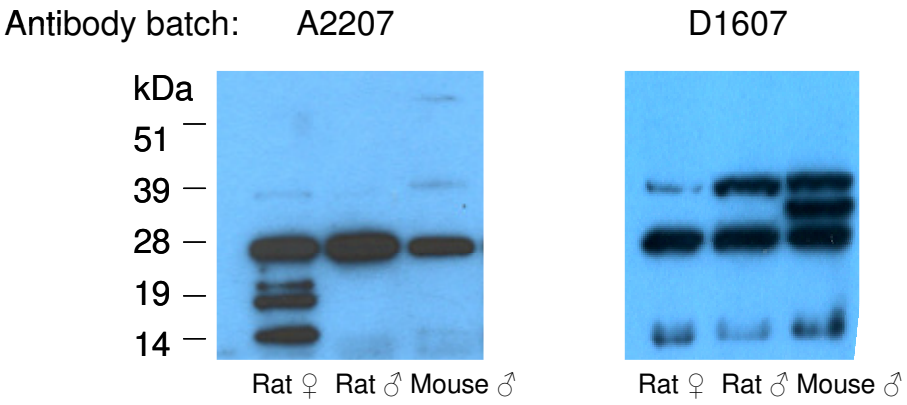


Figure 16 BDNF in different species. The figure shows the differences in processing of BDNF between species. It also shows the detection level of the two antibodies used, both obtained from Santa Cruz Biotechnology. The strong bands at 28 kDa correspond to the proBDNF while the band at 14 kDa is the cleaved mature form of BDNF. The other bands present might be the result of glycosylation, truncation or other post-translational processes on the protein chain. All samples were taken from cerebellum in untreated animals. The first well is sample from a female SD rat, the middle well is sample from a male rat, while the well to the right is sample taken from a male mouse.

Figure 17 shows samples prepared from one and the same female SD rat but taken from different brain areas. The sample taken from the prefrontal cortex (Cor) is showing extremely low levels of the mature protein at 14 kDa. Also the 19 kDa form of the protein, detected in the other brain areas of study, is hardly detected in the cortex.

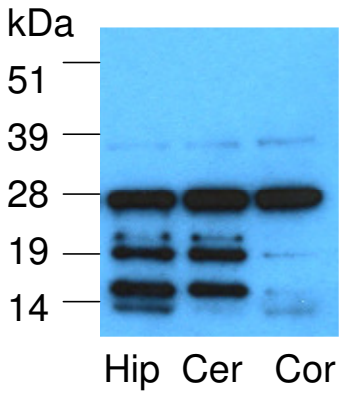


Figure 17 BDNF in different brain areas. Different brain areas show different processing of BDNF. The samples are taken from hippocampus (Hip), Cerebellum (Cer) and prefrontal cortex (Cor) from a female SD rat. BDNF is detected with the use of a polyclonal antibody (batch D1607 received from Santa Cruz). The dark band around 28 kDa shows de proform of BDNF, the band at 14 kDa shows the mature BDNF and the double band around 19 kDa shown in hippocampus and cerebellum might respond to a truncated form of the proBDNF.

4 Discussion

Difficulties in finding a good antidepressant drug are due to the poor insight into the molecular causes of the disorder. A change in the serotonergic levels of the brain has been confirmed in depressed patients and drugs that increase neurotransmitters in the synaptic clefts have been shown to give an anti depressive like effect. Although the most widely used antidepressants, the serotonin-selective reuptake inhibitors (SSRIs), increase the levels of serotonin as a direct response to medication, it is not giving a full therapeutic effect without chronic treatment in 3-4 weeks (Russo-Neustadt & Chen 2005). This long-time required medication indicates that the low levels of neurotransmitters are not the major cause behind the disease, but instead a consequence of something else, not yet known. In recent years several studies have implicated that the illness is caused by a decreased neurogenesis. BDNF is known to be involved in neuronal plasticity (Thoenen 1995) and may be the crucial factor for mediating the changes that are necessary for antidepressants to exert their therapeutic effect.

4.1 Studies of the differences in BDNF levels upon estrogen receptor agonist treatment

In the studies of the BDNF protein pattern after treatment with estrogen receptor agonists, a tendency to a higher expression of mature BDNF can be seen in the DPN treated animals compared to the animals treated with PPT (figure 13). As this change can not be seen in the studies of the amount of mRNA (figure 14), the increased level of mature BDNF is not likely to be caused by a change in the transcription activity, but instead an upregulation of the processing of the proform to the mature form of the protein. The difference between the estrogen receptor agonist named DPN and the agonist called PPT is the selectivity for two different receptor isoforms. DPN is an ERβ selective agonist (Meyers *et al.* 2001), while PPT is highly selective for ERα (Stauffer *et al.* 2000). This indicates that the hypothesis presented in this study, which suggest an upregulation of the processing of BDNF in respons to estrogen receptor agonist stimulation (Tsai 2007), is true for agonists acting on the β-isoform of the two receptors but not for the agonists on the α-isoform. Although the change in expression of the mature BDNF in ERβ agonist treated samples is not strong, it is important to understand that even a small shift in the equilibrium between the proform and the mature form can result in great biological changes as they act on different receptors and mediate opposite biological effects (Lee *et al.* 2001). If DPN but not PPT give an increased processing of BDNF to its

mature form, which is indicated in the results presented here, an activation of the estrogen receptor β, but not α would be able to increase the synaptic plasticity in the brain and possibly cause an anti-depressant like effect.

The results from the animals in the control group and the E2 treated group diverge, indicating a high variation between individuals. Differences among individuals in a test group are always a problem in doing experiments with animals and big groups are often needed to get significant results. The cyclic levels of endogenous estogen in females are a possible reason for the wide variations in this experiment. To create a more stable estrogen level between individuals in a group before a test is performed, the ovaries of the animals can be eliminated by a surgery. As the ovaries are the main producers of circulating estrogen, an elimination of them will create a more homogenised test group. The concentration of the substances given in the experiment, the amount of doses and the vehicle used are other factors that are also having strong influence on the results. Due to the time-limitation in this study, these parameters were not optimized in this study.

4.2 The importance of the processing of BDNF

The experimental work of this thesis has elaborated a method for studying the processing of BDNF. By the use of Western Blot with antibody detection of the different forms of the protein, the relation between the pro- and mature form can be studied together with differences in posttranslational modifications. As the unprocessed proform of BDNF and the cleaved mature form has been shown to act on different receptors that mediate opposite biological effect (Lee *et al.* 2001), the processing rate of the protein is of great importance to study. The results from these studies indicate great differences in the processing between genders, and also between different brain regions from one and the same animal (figure 16 and 17). This highlight the importance of choosing the right brain area in studies to be able to detect the possible differences achieved during treatments. The hippocampus is the area connected with memory formation and synaptic plasticity (Thoenen 1995), which makes it of interest for studying of BDNF. Hippocampus and the limbic system are found to be in tight connections with the prefrontal cortex. The results from this study show important differences between the processing of BDNF in these two brain areas (figure 17). While the pro- and the mature form of the protein are present in the same level in the hippocampus, the levels of the mature protein is almost undetectable in the prefrontal cortex. As the mature form of BDNF is responsible for the activities resulting in plasticity of neurons, and these changes are primary

done in hippocampal areas, a higher expression of the mature BDNF can be expected in these areas, which has also been shown (figure 17).

In the experimental work, two different batches of the same antibody delivered by Santa Cruz Biotechnology were used (figure 16). The differences in the results show the difficulties that can be achieved when working with antibodies. The 28 kDa form of the protein and the 14 kDa form, which are corresponding to the propeptide and the mature protein respectively, are detected by both of the batches used. However, the differences can be seen in the detection of band at 19 kDa for the antibody named D1607 and bands at 32 kDa and 37 kDa for the antibody called A2207 (figure 16). The propeptide contains a site for glycosylation that possibly could cause the 32 kDa form. A site for enzyme cleaving of the peptide has also been identified, enabling for a formation of the 19 kDa peptide (Mowla *et al.* 2001). Posttranslational processing mechanisms of BDNF might be an explanation for the detected BDNF forms of 32 and 19 kDa, even if they cannot be detected by any of the antibodies. As these modifications of the BDNF might have big influence on the final active protein, additional studies of the processing and the function of BDNF are of high relevance to understand the biological function of the protein.

4.3 Concluding remarks and future perspectives

The results presented in figure 13 indicate that an activation of the estrogen receptor β is increasing the amount of mature BDNF in hippocampal neurons. As the mature BDNF stimulates neuronal outgrowth and differentiation (Thoenen 1995), this could possibly give an antidepressant like effect according to the hypothesis by Tsai (2007). For further studies it would be of great interest to examine how the levels of BDNF vary in females with the estrous cycle and also look at gender difference of BDNF. The results shown in figure 16 indicate a higher expression of the mature protein in females that could further support the hypothesis of an estrogen regulated processing of BDNF. The receptor for the mature BDNF, TrkB is also of interest to study and the levels of phosphorylation of this receptor could give an additional view of the levels of activation of the pathway. If BDNF as proposed here, is the key molecule in the estrogen receptor mediated pathway to an anti depressive like effect, a lot of new drug targets can be identified that will contribute to the development of more safe and efficient antidepressant drugs.

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