PhD thesis

Brain-Derived Neurotrophic Factor (BDNF) and glucocorticoids: Influence on serotonin 2A receptors and relation to major depression

Viktorija Trajkovska

Neurobiology Research Unit
The Neuroscience Centre
Department of Neurology
Rigshospitalet
Copenhagen University Hospital
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Supervisors

- Professor Gitte Moos Knudsen, MD, Dr. Med., Department of Neurology, Neurobiology Research Unit, Rigshospitalet, Copenhagen University Hospital, Denmark
- Senior researcher Susana Aznar Kleijn, M. Sci., PhD, Department of Neurology, Neurobiology Research Unit, Rigshospitalet, Copenhagen University Hospital, Denmark

Review committee

- Associate professor Gitta Wortwein, PhD (Chairman), Department of Health Psychology, Laboratory of Neuropsychiatry, Rigshospitalet, Copenhagen University Hospital, Denmark
- Associate professor Maribel Rios, PhD, Tufts University School of Medicine, Department of Neuroscience, Boston, MA, USA
- Head of department Torsten Meldgaard Madsen, MD, PhD, International Clinical Research - Mood and Anxiety Disorders, H. Lundbeck A/S, Denmark

Summary

Major depression is associated with both dysregulation of the hypothalamic pituitary adrenal (HPA) axis and serotonergic deficiency, not the least of the 5-HT_{2A} serotonin receptor. There is also accumulative evidence that brain-derived neurotrophic factor (BDNF) is involved in the pathophysiology of major depression. However, whether the changes in 5-HT_{2A} receptor levels are a direct effect of the hyperactive HPA axis and the reduced levels of BDNF seen in depression is unknown. Furthermore, if the altered BDNF level is a state or a trait marker of depression remains unclear.

The aims of this thesis were to study the net effects of a dysregulatory HPA axis, namely high levels of corticosterone and altered glucocorticoid receptor (GR) signalling, and the effects of BDNF and TrkB on 5-HT_{2A} receptor protein levels. For this we used organotypic hippocampal cultures and mice models under- and over-expressing GR (GR+/-, YGR), BDNF (BDNF+/-) and TrkB (TrkB TK). We also investigated whole blood BDNF levels as trait or state marker of depression in unaffected twins discordant for affective disorder.

The results show that increased GR signalling, by high levels of corticosterone or high GR expression, lead to increased hippocampal 5-HT_{2A} receptor levels. The increase in 5-HT_{2A} receptor levels was counteracted by specific blockers for GR and mineralocorticoid receptor (MR). Decreased GR signalling, by contrast, decreased 5-HT_{2A} receptor levels, indicating that a dysfunction in HPA axis directly regulates 5-HT_{2A} receptor levels. BDNF also had an effect on the 5-HT_{2A} receptor; high levels of BDNF decreased hippocampal 5-HT_{2A} receptor levels and BDNF+/- mice displayed increased hippocampal 5-HT_{2A} receptor levels. That the BDNF-induced alterations in 5-HT_{2A} receptor levels might be dependent on TrkB levels was shown by TrkB TK mice having increased frontal cortex and hippocampal 5-HT_{2A} receptor levels. Furthermore, adult organotypic cultures exposed to BDNF for seven days also had decreased TrkB levels.

The methodological investigations established that whole blood BDNF levels are measured with accuracy and high reproducibility and that female gender is associated with higher whole blood BDNF levels, adding important information on what considerations should be done when measuring blood BDNF. Furthermore, the combination of genetic risk, female gender and number of recent stressful life events

decreased whole blood BDNF levels, indicating that low blood BDNF levels are a trait marker of depression.

In conclusion, this thesis sheds light on how BDNF and glucocorticoids regulate 5-HT_{2A} receptor protein levels, and it associates whole blood BDNF levels to *in vivo* brain 5-HT_{2A} receptor binding levels and to risk factors for development of depression.

Resumé

Flere studier har påvist, at brain-derived neurotrophic factor (BDNF) er nedsat ved affektive sygdomme, ligesom der også ses dysregulering af hypothalamus hypofyse-binyreaksen (HPA) samt serotonerge deficits, herunder ændringer i serotonin 2A (5-HT_{2A}) receptorbindingen. Det er imidlertid uvist, om ændringerne i 5-HT_{2A} receptorbindingen forårsages direkte af enten den hyperaktive HPA eller nedsat BDNF-niveau, og om det nedsatte niveau af BDNF udgør en state eller trait markør for depression.

I denne PhD-afhandling undersøges effekten af en dysreguleret HPA, BDNF og TrkB på 5-HT_{2A} receptorproteinniveauerne i hjernen. Til det formål anvendtes organotypiske hippokampale kulturer og genetisk modificerede mus, der henholdsvis under- og overudtrykker glukokortikoidreceptoren (GR) (GR+/-, YGR), BDNF (BDNF+/-) og TrkB (TrkB TK). Fuldblods-BDNF-niveauer blev endvidere undersøgt i raske tvillinger diskordante for affektiv sygdom.

Resultaterne viser, at øget GR signalering medfører øgede hippokampale niveauer af 5-HT_{2A} receptoren; en effekt, der modvirkes af specifikke antagonister for GR og mineralokortikoid-receptoren. Omvendt medfører tilstande med reduceret GR signalering en reduktion i 5-HT_{2A} receptorniveauerne. Højt BDNF niveau nedsatte endvidere det hippokampale 5-HT_{2A} receptorniveau, og i BDNF+/- mus fandtes forøget hippokampalt 5-HT_{2A} receptorniveau. Det er sandsynligt, at disse BDNF associerede ændringer i 5-HT_{2A} receptorniveauet afhænger af TrkB niveauet, idet TrkB TK mus udviste forøget 5-HT_{2A} niveau i frontale cortex og hippocampus.

I et metodemæssigt valideringsstudie påvises det, at fuldblods-BDNF-niveauer kan måles med høj nøjagtighed og høj reproducerbarhed. Det påvises, at der findes højere fuldblods-BDNF-koncentration hos kvinder end hos mænd, og at kombinationen af genetiske risikofaktorer for depression, kvindeligt køn og antallet af nylige stressfulde oplevelser er associeret med nedsat fuldblods-BDNF-niveau, hvilket tyder på, at lavt fuldblods-BDNF er en trait markør ved depression.

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List of abbreviations

ACTH	Adenocorticotrophin
AVP	Arganine vasopressin
BDNF	Brain-derived neurotrophic factor
BDNF+/-	Mice heterozygous for BDNF
CRF/CRH	Corticotrophin-releasing factor/hormone
DOI	2,5-dimethoxy-4-iodoamphetamine
DZ	Dizygotic
ELISA	Enzyme-linked immunoabsorbent assay
GABA	Gamma-aminobutyric acid
GR	Glucocorticoid receptor
GR+/-	Mice heterozygous for GR
HPA	Hypothalamic-pituitary-adrenal
HRP	Horseradish peroxidase
LSD	Lysergic acid diethylamine
MAPK	Mitogen-activated protein kinase
MR	Mineralocorticoid receptor
MZ	Monozygotic
NE	Norepinephrine
PCR	Polymerase chain reaction
PET	Positron emission tomography
PLC	Phospholipase C
PVN	Paraventricular nucleus
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
SSRI	Selective serotonin reuptake inhibitors
TrkB	Tropomyosin-related kinase B
TrkB TK	Mice overexpressing TrkB
WT	Wild-type
YGR	Mice overexpressing GR
5-HT	Serotonin
5-HT_{2A}	Serotonin receptor 2A

List of original papers

The thesis is based on the following published papers or manuscripts:

I. Trajkovska V, Krey G, Thomsen MS, Chourbaji S, Brandwein C, Ridder S, Gass P, Knudsen GM, Aznar S. Activation of glucocorticoid receptors increases hippocampal 5-HT_{2A} receptor levels. *Submitted (J Neurochem, resubmission encouraged).*

II. Trajkovska V, Thomsen MS, Marcussen MS, Hansen HH, Mikkelsen JD, Arneberg L, Kokaia M, Castren E, Knudsen GM, Aznar S. 5-HT_{2A} serotonin receptor levels are associated with brain-derived neurotrophic factor (BDNF) and TrkB levels. *Manuscript.*

III. Trajkovska V, Marcussen AB, Vinberg M, Hartvig P, Aznar S, Knudsen GM. Measurements of Brain-derived neurotrophic factor: Methodological aspects and demographical data. *Brain Research Bulletin 73(2007);143-9* .

IV. Trajkovska V, Vinberg M, Aznar S, Knudsen GM, Kessing LV. Whole blood BDNF levels in healthy twins discordant for affective disorder: Association to life events and neuroticism. *Accepted (J Affect Dis).*

Background

Major depression

Major depression is a serious neuropsychiatric illness, probably the western world's largest public health burdens. It is predicted to be the second leading cause of premature death or disability by 2020 (World Health Organisation 2001; Murray & Lopez 1997). Major depression is a largely heterogenous disease and is characterized by depressed mood, irritability, low self esteem, feelings of hopelessness and worthlessness, altered appetite and weight, decreased interest in pleasurable stimuli, and recurrent thoughts of death and suicide.

Risk factors

Major depression is a highly familial disease; heritability for depression is approximately 40% (Sullivan et al. 2000; Levinson 2006). Heritability seems to be more pronounced in women than in men (Marcus et al. 2005), but genes alone are not predictive for development of affective disorders. It is well acknowledged today that affective disorders result from an interaction between genetic liability and environmental risk factors (Kendler et al. 1995; Caspi et al. 2003; Farmer et al. 2005). An important environmental risk factor is stress and stressful life events often precipitate the onset of major depression (Caspi et al. 2003; Williamson et al. 1998).

Brain areas involved in depression

Given the diversity of the symptoms in major depression it is likely that multiple brain regions are involved in this disease. This is supported by brain imaging studies that have reported changes in blood flow (Drevets 2001; Liotti & Mayberg 2001) and anatomical abnormalities (Miguel-Hidalgo & Rajkowska 2002; Rajkowska 2000; Rajkowska et al. 1999) in prefrontal and cingulated cortex, hippocampus, striatum, amygdala, and thalamus. Of course, these various brain regions would operate in a complex neural circuitry, together yielding the symptomatic picture seen in major

depression. Even so, the large majority of studies on major depression point out frontal cortex and hippocampus as key structures involved in affective disorders. The hippocampus is of particular interest in depression as it is a key member of the limbic system controlling emotional behavior and in regulating the hypothalamic-pituitary-adrenal (HPA) axis (De Kloet et al. 1998).

The HPA axis

Regulation of the HPA axis

All disturbances in the body evoke a stress response which serves to restore homeostasis and to facilitate adaptation to this disturbance. Essential to the stress response is the HPA axis and the neurons in the paraventricular nucleus (PVN) that regulate the activity of this axis. Besides PVN the HPA axis is also controlled by several brain pathways including excitatory afferents from the amygdala and inhibitory afferents from the hippocampus and prefrontal cortex (Herman et al. 2005).

The main driving force of the HPA axis is corticotrophin-releasing factor/hormone (CRF/CRH) which acts with in synergy with arganine vasopressin (AVP), and leads to the release of glucocorticoids (cortisol in man and corticosterone in rodents) (Figure 1). All vertebrates have a circadian rhythm of glucocorticoid secretion, with the highest peak during the active period of the diurnal cycle.

Corticosterone maintains the basal activity of the HPA axis and controls sensitivity or threshold of the system's response to stress. By this, corticosterone coordinates other circadian events like the sleep/awake cycle, food intake, etc. (De Kloet et al. 1998). Corticosterone also exerts an inhibitory feedback primarily on the pituitary and PVN, terminating the stress-induced HPA axis activation.

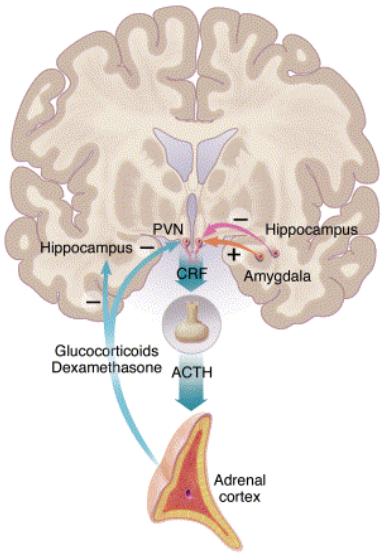

Figure 1. The HPA axis. Activated neurons in the PVN of the hypothalamus secrete corticotrophin-releasing factor (CRF) which stimulates the synthesis and release of adenocorticotrophin (ACTH) from the anterior pituitary. Through the blood stream ACTH reaches the adrenal glands and leads to the release of glucocorticoids. Glucocorticoids then exert a potent feedback on the HPA axis by affecting CRF and ACTH synthesis and release, thereby inhibiting their own synthesis. (Adapted from Nestler et al. 2002).

Corticosteroid receptors

The actions of corticosterone are mediated by the glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) (McEwen et al. 1986). GR is expressed throughout the whole brain, while MR is predominately expressed in limbic areas (De Kloet et al. 1998). Both receptors highly colocolize in the hippocampus and hypothalamus (Han et al. 2005). The high affinity receptor MR is occupied during basal corticosterone levels, and is believed to be responsible for maintaining the basal tone of the HPA axis (Ratka et al. 1989; Barden et al. 1995). The low affinity receptor GR on the other hand is activated when corticosterone levels are high, i.e, at circadian peak or during stress, and mediates the negative feedback effects of corticosterone on the HPA axis (Barden et al. 1995; Moldow et al. 2005).

Stress and depression

The HPA axis and depression

A large proportion of patients with severe depressive episodes display hyperactivity of the HPA axis (Weber et al. 2000; Holsboer-Trachsler et al. 1991; Lesch et al. 1988). Furthermore, there is an association between HPA axis hyperactivity and depressive symptoms (Holsboer-Trachsler et al. 1991). The hyperactivity of the HPA axis may result from an impaired feedback regulation due to altered GR (reviewed in Pariante &

Miller 2001) expression or function, or by hyper drive of CRH (reviewed in Claes 2004).

Stress and sustained high levels of glucocorticoids decrease brain-derived neurotrophic factor (BDNF) (Jacobsen & Mork 2006; Nair et al. 2007), induce cell damage and reduce neurogenesis (Cameron & Gould 1994) and dendritic aborization (Alfonso et al. 2004), yielding structural changes and damage to the hippocampus. The stress-related changes may be responsible for the reductions in hippocampal volume reported in depression (Colla et al. 2007; Saylam et al. 2006). Elevated glucocorticoid levels secondary to hippocampal damage could produce further injury to the hippocampus, and consequently, even greater increase in glucocorticoid levels, all due to loss of normal hippocampal feedback inhibition. There are indications that a persistent HPA axis dysregulation may increase the risk of depression relapse (O'Toole et al. 1997; Zobel et al. 2001), but whether hyperactivity of the HPA axis precedes a depressive episode or is a consequence of it remains unsolved (Steimer et al. 2007; Holsboer et al. 1995; Mannie et al. 2007).

GR and depression

Because patients with major depression exhibit impaired HPA axis negative feedback in the context of elevated circulating cortisol levels (Bhagwagar et al. 2003; Weber et al. 2000), a number of studies have considered the possibility that the expression or function of GR is reduced in depressed patients. This motion has been supported through post mortem studies of major depressed patients where reduced GR levels have been found in several brain areas, including the hippocampus (Lopez et al. 1998; Webster et al. 2002a).

In the brain, GR has been proposed to modulate emotional behaviour, cognitive functions and addictive states (Tronche et al. 1999). Successful antidepressant treatment normalizes HPA axis hyperactivity (Heuser et al. 1996), possibly through an increase in GR expression and function (Pariante et al. 2001; Lai et al. 2003; Seckl & Fink 1992) leading to a restoration of the impaired negative feedback inhibition (Pariante et al. 2004).

There are also indications that GR may also be involved in depression liability. A recent clinical study has provided evidence that two specific polymorphisms of the GR gene are associated with an increased risk of developing depression (van Rossum et al.

2006). Furthermore, mice with genetically decreased GR function display depressive-like behaviour (Ridder et al. 2005; Boyle et al. 2005), suggesting that impaired GR signalling may lead to increased vulnerability to stress-induced depression.

The neurotrophin BDNF

BDNF in brain

BDNF is a 14 kDa protein and belongs to the neurotrophin family that also includes nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). BDNF is widely distributed in the central nervous system with the highest levels in the hippocampus (Yan et al. 1997). It is predominantly produced by neurons, but is also synthesized and released by astrocytes under the control of neuronal activity (Juric et al. 2006; Balkowiec & Katz 2000). Early on it was believed that BDNF, like other neurotrophins, only was secreted from post synaptic neurons, acting retrogradely on presynaptic terminals (Wetmore et al. 1991). However, recent studies indicate that the most important way of BDNF secretion is through anterograde transport to the axons with subsequent release to postsynaptic neurons (Kohara et al. 2001; Adachi et al. 2005).

The expression of BDNF in the brain is regulated by a variety of factors, such as stress (Jacobsen & Mork 2006; Nair et al. 2007), exercise (Berchtold et al. 2005), enriched environment (Rossi et al. 2006) and ischemia (Lee et al. 2004).

BDNF in blood

Through a saturable transport system, BDNF passes the blood-brain barrier (Pan et al. 1998). In the blood BDNF is stored in platelets (Yamamoto & Gurney 1990) and released upon platelet agonist stimulation by thrombin, Ca^{2+}, collagen or shear stress (Fujimura et al. 2002). But blood BDNF is not only brain derived; other peripheral sources of BDNF include the endothelial cells (Nakahashi et al. 2000), smooth muscle cells (Donovan et al. 1995), and eosinophils (Noga et al. 2003).

Animal studies indicate that there is a strong correlation between blood and brain BDNF during maturation and aging (Karege et al. 2002b). Indeed, blood and

brain BDNF levels are similarly altered by antidepressant treatment (Shimizu et al. 2003; Chen et al. 2001), in schizophrenia (Toyooka et al. 2002; Durany et al. 2001), Alzheimer's disease (Laske et al. 2007; Connor et al. 1997), etc.

BDNF receptors

BDNF mediates its neurotrophic effect through binding to a specific high affinity receptor, the tropomyosin-related kinase B (TrkB), and to the low affinity neurotrophin receptor p75NTR. The TrkB receptor is expressed in a similar pattern as BDNF in the brain (Fryer et al. 1996; Kokaia et al. 1993; Yan et al. 1997) and is present in three isoforms; the full length TrkB receptor, and two truncated forms lacking the intracellular cytoplasmic tyrosine kinase domain; TrkB T1 and TrkB T2. The TrkB T1 receptor has been implicated as a dominant negative receptor (Haapasalo et al. 2001) or as a protein that traps BDNF, limiting the available BDNF concentration for the TrkB full length receptor. However, there are also indications that TrkB T1 might have some functional properties (Rose et al. 2003).

When BDNF binds to the TrkB full length receptors they dimerize and the intracellular tyrosine residues become auto-phosphorylated. In addition, three specific signaling pathways that mediate the effects of BDNF are activated: the mitogen-activated protein kinase (MAPK) pathway, the phosphotidylinositol-3 kinase/ Akt pathway and the phospolipase C (PLC) gamma pathway (Chao 2003).

Effects of BDNF

By promoting neurogenesis, synaptic plasticity and cell survival (Schinder & Poo 2000; McAllister et al. 1999; Pencea et al. 2001), BDNF plays an important role in the development and plasticity of the brain. Furthermore, with BDNF deficient mice the involvement of BDNF in learning (Linnarsson et al. 1997), obesity (Lyons et al. 1999b), anxiety (Rios et al. 2001) and depression (Chan et al. 2006) has been shown. The peripheral effects of BDNF are only sparsely investigated but indicate that BDNF is involved in neuronal regeneration after nerve injury (Novikova et al. 1997) and in regulating the immune system (Schuhmann et al. 2005; Schenone et al. 1996).

BDNF and depression

Depression-related changes in BDNF

There is compelling evidence of BDNF being an important player in depression. Clinical studies have shown that patients with major depression have decreased serum and plasma BDNF concentrations (Karege et al. 2002a; Gonul et al. 2005; Karege et al. 2005; Lee et al. 2007). Blood BDNF levels are negatively associated with the clinical severity of depression (Shimizu et al. 2003). In addition, depressed patients on antidepressant medication have increased brain BDNF levels at time of death (Chen et al. 2001). Animal studies also indicate that BDNF and increased BDNF signalling mediates antidepressant-like effects (Shirayama et al. 2002; Siuciak et al. 1997; Koponen et al. 2005; Saarelainen et al. 2003). However, the exact involvement of BDNF in the pathophysiology of depression remains unresolved.

The neurotrophin hypothesis of depression

One hypothesis for the involvement of BDNF in depression is that a stress-induced deficiency in BDNF contributes to hippocampal pathology and to the development of depression in vulnerable individuals (Duman et al. 1997). The hippocampus appears to be particularly vulnerable, as chronic stress results in CA3 pyramidal atrophy and debranching, while neurons in the amygdala exhibit enhanced dendritic aborization in response to the same stressor (Vyas et al. 2002). According to the neurotrophin hypothesis the increase in BDNF after antidepressant treatment (Nibuya et al. 1995; Altar et al. 2003) helps to repair and protect the hippocampus from further damage (Figure 2). Thus the inability of neuronal systems to exhibit appropriate, adaptive plasticity would contribute to the pathogenesis of depression. This hypothesis is supported by the role of BDNF in attenuating corticosterone-induced cell death (Nitta et al. 1999), inducing neurogenesis (Pencea et al. 2001) and increasing dendritic aborization. A recent study indicates that BDNF has a role in regulating hippocampal volume (Pezawas et al. 2004). In addition, antidepressant treatment increase brain BDNF levels only after chronic treatment (De Foubert et al. 2004), corresponding to the time course for the therapeutic action of the medication. Further, the most clinically effective antidepressants also have the largest inducing effect of BDNF expression (Nibuya et al. 1995).

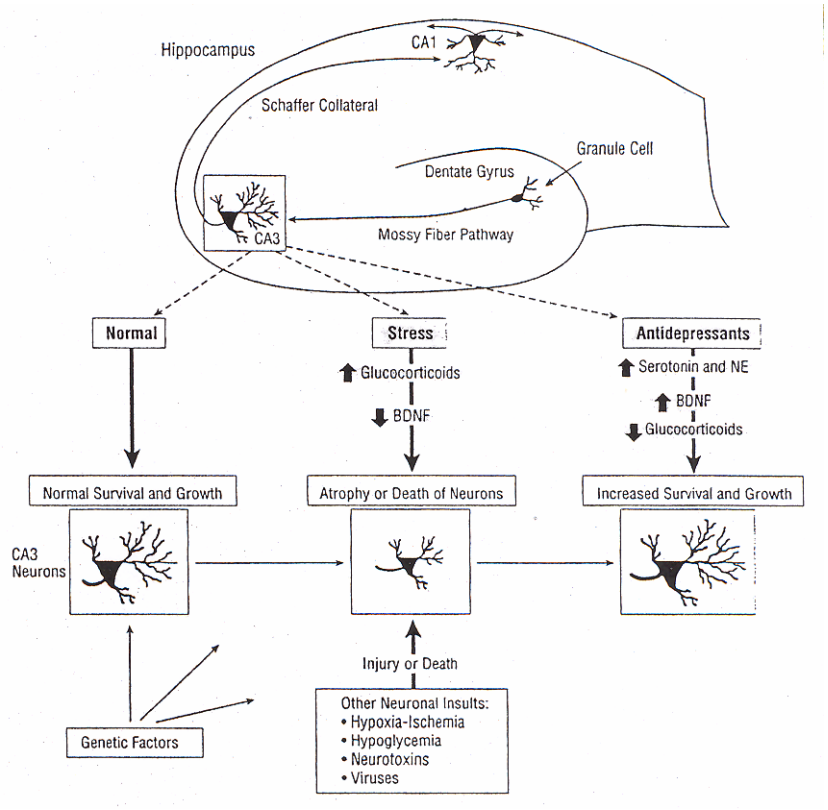

Figure 2. The neurotrophin hypothesis of depression. Chronic stress and high levels of glucocorticoids decrease BDNF levels and lead to atrophy and damage of hippocampal CA3 neurons, thereby increasing the vulnerability of these neurons to a variety of potential insults (hypoxia, hypoglycaemia, neurotoxins, and viral infections). High levels of glucocorticoids also decrease neurogenesis in the hippocampus. Antidepressant treatment increases BDNF and results in increased growth and survival of neurons. The neurons may also be repaired and protected against further damage. By these actions, antidepressants may reverse and prevent the stress-induced changes in the hippocampus, relieving certain symptoms of depression. (Adapted from Duman et al. 1997).

There are, however, some limitations for this hypothesis. There is some inconsistency in the antidepressant-induced increase in BDNF (Vinet et al. 2004; De Foubert et al. 2004; Dias et al. 2003; Conti et al. 2002), and not all studies using mutant mice display a role for BDNF in depression (Lyons et al. 1999; MacQueen et al. 2001). Furthermore, infusion of BDNF into the ventral-tegmental area-nucleus accumbens (VTA-NaC) pathway yields depressive-like behaviours (Eisch et al. 2003). The study

of Eisch and co-workers (2003) could indicate that BDNF may produce different effects on depression-like behaviour depending on the brain region, accounting for different aspects of the disease phenotype.

It is possible that a deficiency in BDNF *per se* is not enough to yield a depressive-like phenotype, and that a combination of other factors, such as changes in the serotonin system, would lead to the expression of depressive-like behaviour (Ren-Patterson et al. 2005).

The serotonin system

The serotonin (5-hydrotryptamine; 5-HT) system is involved in multiple physiological processes including sleep (Sharpley et al. 1994), appetite (McCann et al. 2007), memory (Liy-Salmeron & Meneses 2007), learning (Hirst et al. 2006), temperature regulation (Schwartz et al. 1995), mood (Staley et al. 1998) and sexual behaviour (Hull et al. 2004). Furthermore, disturbances in this system have been implicated in several disorders, such as depression (Bhagwagar et al. 2006), anxiety (Serretti et al. 2007) and dementia (Lai et al. 2005).

Within the raphe nuclei and the brainstem are clusters of 5-HT neurons that extend their fibers through the central nervous system and are responsible for releasing 5-HT. The effects of 5-HT are mediated through multiple pre- and postsynaptic receptors divided into seven distinct receptor families. To date there are 15 known 5-HT receptor subtypes.

The 5-HT_{2A} receptor

The 5-HT_{2A} receptor is a G-protein coupled postsynaptic receptor. In the rat brain 5-HT_{2A} receptor is expressed in the cortex, olfactory system, septum, hippocampus, basal ganglia, amygdala, diencephalon and brain stem (Cornea-Hebert et al. 1999). In the hippocampus and cortex 5-HT_{2A} receptor is predominantly expressed on pyramidal neurons, on granule cells and GABAergic interneurons (Luttgen et al. 2004; Willins et al. 1997). Activation of 5-HT_{2A} receptors evokes both neuronal excitation and inhibition (Zhou & Hablitz 1999) as it leads to the release of both glutamate

(Aghajanian & Marek 1999) and GABA (Zhou & Hablitz 1999). In addition, 5-HT_{2A} receptor agonists like lysergic acid diethylamine (LSD) and 2,5-dimethoxy-4-iodoamphetamine (DOI) have a hallucinogenic effect, while 5-HT_{2A} receptor antagonists are atypical antipsychotic drugs (Celada et al. 2004).

The 5-HT_{2A} receptor and its association to depression

The monoamine hypothesis of depression is based on the observation that antidepressant treatment increase brain norepinephrine (NE) and 5-HT levels. Although selective serotonin reuptake inhibitors (SSRI) increase 5-HT levels within days, the clinical effects appear only after weeks of treatment. Furthermore, monoamine depletion does not produce depression in healthy subjects (Heninger et al. 1996), indicating that a serotonin deficiency is not sufficient to elicit a depressive state.

Both from human (Sheline et al. 2004; Mintun et al. 2004) and animal studies (Weisstaub et al. 2006; Dwivedi et al. 2005) there is compelling evidence that the 5-HT_{2A} receptor is involved in depression and anxiety modulation. Disruption of cortical 5-HT_{2A} receptor signaling in mice is associated with reduced conflict behavior, and restoration of 5-HT_{2A} receptor signaling leads to normalized behavior (Weisstaub et al. 2006). Some studies have indicated that patients with major depression have lower hippocampal 5-HT_{2A} receptor binding, as shown either by *in vivo* imaging (Mintun et al. 2004) or post-mortem (Rosel et al. 2004; Rosel et al. 2000). Meyer et al. (2003) reported that patients with severe depression and high levels of dysfunctional (more pessimistic) attitudes have increased prefrontal cortex 5-HT_{2A} receptor binding and the level of dysfunctional attitudes correlates with receptor binding. This conclusion was corroborated by Bhagwagar et al. (2006) that, in addition, showed that recovered, non-medicated patients with a history of recurrent unipolar depression have increased cortical 5-HT_{2A} receptor binding in extensive parts of the cortex.

Blockade of 5-HT_{2A} receptors inhibits the effects of stress on BDNF expression (Vaidya et al. 1999) and confers antidepressant-like improvement in treatment-resistant depression (Shelton et al. 2001). Therefore, the study of the interaction between the 5-HT_{2A} receptor and BDNF in relation to depression is highly relevant.

Glucocorticoids, BDNF and 5-HT_{2A} receptors in depression?

BDNF has been reported to be a potent neurotrophic factor for the NE and 5-HT system (Altar 1999; Mamounas et al. 2000), and thus, BDNF may also affect the disordered monoamine systems in depression. Unpublished data suggest a strong co-localization of 5-HT_{2A} and TrkB receptors in the brain (Figure 3). Therefore, elucidating whether BDNF may affect 5-HT_{2A} receptor levels is highly interesting. It is known that stress and high glucocorticoid levels are associated with changes in 5-HT_{2A} receptor levels (Dwivedi et al. 2005; Schiller et al. 2003), but the mechanism of this regulation is still unresolved. The scope of this thesis is to study the effects of BDNF and glucocorticoids on 5-HT_{2A} receptor levels and their relationship on depression vulnerability.

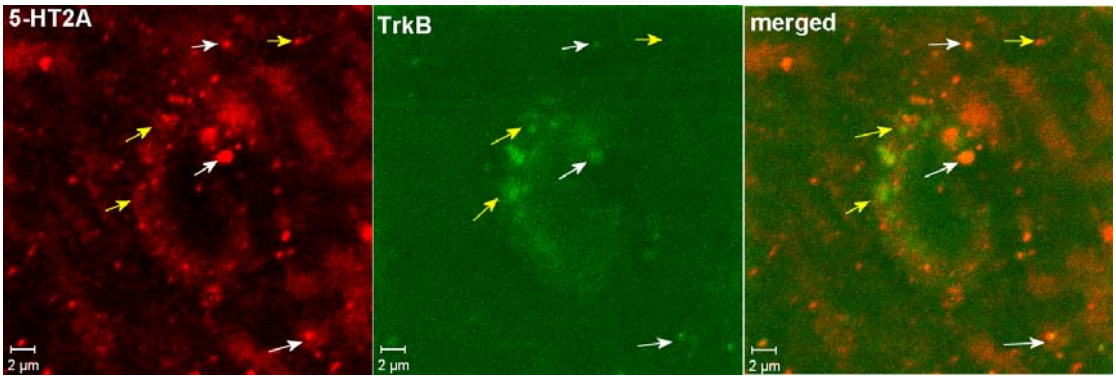

Figure 3. Co-localization of 5-HT_{2A} and TrkB receptors. Immunohistochemistry and co-localization of 5-HT_{2A} receptors and TrkB in the hippocampus. The white arrows indicate co-localization and the yellow arrows indicate no co-localization.

Aims of the thesis

The general aims of this thesis were to study the effect of glucocorticoids and BDNF on 5-HT_{2A} receptor levels and to elucidate whether low blood BDNF levels is a state or a trait marker of depression.

The specific aims were:

I. To investigate the effects of a dysregulatory HPA axis, namely high levels of corticosterone and altered glucocorticoid receptor (GR) signalling, on hippocampal 5-HT_{2A} receptor levels.

II. To elucidate the effects of BDNF and TrkB on frontal cortex and hippocampal 5-HT_{2A} receptor levels.

III. To study whole blood BDNF levels in healthy subjects and in people predisposed to depression through the combination of various risk factors: personality profile, stressful life events, and gender.

Materials and Methods

For detailed description of the methodological experiments, we refer to the corresponding manuscript (appendices).

Experimental analyses

Western blot analysis

Western blot is a technique that is used to detect the expression of a specific protein in tissue homogenates or cell extracts after size separation by using gel electrophoresis. The protein homogenate is dissolved in a buffer containing sodium dodecyl sulphate (SDS). This is an anionic detergent that binds quantitatively to proteins, giving them linearity and uniform charge, so that they can be separated solely on the basis of their size (Burnette 1981). Mercaptorethanol is also added in the buffer to reduce any disulphide bonds within the protein. Detection of the protein of interest is conducted after electroblotting the separated proteins onto a nitrocellulose membrane and by using high-quality primary antibodies directed against the desired protein (Burnette 1981). The detection reaction is accomplished with horseradish peroxidase-conjugated (HRP) secondary antibodies and chemiluminescence, yielding a highly sensitive analytical system (Kricka 1991).

Western blot is a fast and high throughput technique for investigating protein expression in any given tissue. The disadvantage of using western blot analysis to other protein analyses is that information regarding the cellular and anatomical localization within the region of interest is not obtained. Furthermore, the quantification is only semiquantitative.

Immunoprecipitation

In order to enrich a given protein to some degree of purity from tissue or cell extracts, a process called immunoprecipitation is conducted. Immunoprecipitation is a technique where any antigen is precipitated by using specific antibodies directed against the antigen of interest. As the densities of TrkB in the organotypic

hippocampal cultures are below the detection limit of the analysis, immunoprecipitation was performed previous to western blot analysis. The immunoprecipitation was carried out by incubating tissue homogenate together with a pan-Trk antibody (sc-11, Santa Cruz Biotechnology). The pan-Trk antibody recognizes a C-terminal portion that is highly conserved in all Trk receptor isoforms (Ip et al. 1993), thereby immunoprecipitating all Trk receptors. The TrkB receptor is then detected by using a specific TrkB antibody.

Whole blood sample preparation and BDNF ELISA

During coagulation BDNF is released from the thrombocytes (Fujimura et al. 2002). In whole blood samples coagulation is inhibited by the addition of EDTA. In order to empty the thrombocytes of BDNF, whole blood was lysed with 3 % triton X-100 and sonication (Toyooka et al. 2002). Disrupted cell membranes were discarded by centrifugation.

Lysed blood samples were assessed for BDNF concentrations using a commonly used commercially available two-site sandwich enzyme-linked immunoabsorbent assay (ELISA) kit (Chemicon, Temecula, USA) (Yang et al. 2006; Palomino et al. 2006; Altar et al. 2003).

The ELISA analysis quantifies the concentration of the protein by specifically immobilizing it to a surface by using specific primary antibodies (Engvall et al. 1971), in this case antibodies directed towards BDNF. In sandwich ELISA primary antibody is applied a second time creating an antibody-protein-antibody complex (Maiolini et al. 1978). The major advantage of a sandwich ELISA is the ability to use crude or impure samples and still selectively bind any antigen that may be present. Without the first layer of "capture" antibody, any proteins in the sample may competitively adsorb to the plate surface, lowering the quantity of antigen immobilized. In the detection step, an enzymatic substrate is added that reacts with the HRP-conjugated antibody, creating a colorimetric reaction. The limitation of ELISA lies in the specificity of the primary antibody.

DNA genotyping of BDNF

Real-time polymerase chain reaction (PCR) is a technique that is used to amplify and quantify a specific part of a given DNA molecule by using specific oligonucleotide probes (Fregeau & Fourney 1993). With this technique it is possible to determine whether or not a specific sequence or polymorphism is present in the DNA. The BDNF genotyping was performed using a TaqMan 5'-exonuclease allelic discrimination assay (Applied Biosystems, Foster City, California) and a multiplex real-time PCR machine. The assay allows genotyping of the two possible variants at the single-nucleotide polymorphism (SNP) site in a target template sequence, and recognises the Val/Val, Val/Met and Met/Met phenotypes by using a unique pair of fluorescent dye detectors. The actual quantity of target sequence is not determined.

In vitro and in vivo models

Adult organotypic cultures

Adult organotypic hippocampal cultures were prepared from 20-30 day-old Sprague Dawley rat pups (Xiang et al. 2000). The pups were decapitated and the brains quickly taken out under sterile conditions. Neuronal damage is minimized by rapid isolation and prolonged chilling of the brain. The hippocampi were dissected and cut transversely into 400 μm-thick slices. Intact and undamaged slices were cultured on membrane inserts at 5% CO_2 atmosphere at 32°C. The first week of culturing is critical, and to inhibit slice degeneration the slices are cultured in a high potassium medium during the two first days. Within the first seven days the cell death and changes in protein levels that occurs initially in the cultures stabilize (Xiang et al. 2000; Finley et al. 2004) and they can be used experimentally.

The advantages of using adult organotypic cultures are that the slice cultures retain the connective organization found *in situ* that is reached after adult developmental maturation and they can be subjected to prolonged manipulations. Further, since the cultures are isolated from the serotonergic projections from raphe nuclei, 5-HT_{2A} receptor changes unrelated to alterations in 5-HT levels can be

assessed. The disadvantage is that this is an *in vitro* system where the hippocampus is isolated from the normally present synaptic connections with other brain areas.

The use of adult organotypic hippocampal cultures is relatively new (Xiang et al. 2000; Wilhelmi et al. 2002), but already several studies have made use of this strong and valuable *in vitro* model e.g. (Finley et al. 2004; Leutgeb et al. 2005; Leutgeb et al. 2003).

Transgenic mice models

Mutant mice with altered GR expression

In accordance with the recommendations of the Banbury conference (1997) all mice used were F1 hybrids from two commonly used inbred strains, C57BL/6N and FVB/N (Ridder et al. 2005). GR +/- mice were generated by homologous recombination in embryonic stem cells (Tronche et al. 1999), and YGR mice that carry two additional copies of the GR were generated by a transgenic approach using a yeast artificial chromosome (Reichardt et al. 2000).

Several other mice strains with altered GR expression of function have been generated; mice with point mutations of GR (Reichardt et al. 1998), brain-specific GR knock-outs (Tronche et al. 1999) and a GR antisense models (Pepin et al. 1992). The advantage of using the GR+/- mice to the other mice models is that in these mice, the decreased GR expression may mimic the situation of patients with affective disorder more accurately than complete GR knock-out. Furthermore, in the GR+/- and YGR mice the regulatory principles governing the normal gene are maintained (Tronche et al. 1999; Reichardt et al. 2000).

BDNF +/- mice

BDNF heterozygote and wild-type mice were from a breeding colony originating from heterozygote mice from Jax strain B6.129S4-*Bdnf*^{tm1Jae}/J, as described in (Nygren et al. 2006). The mice are generated by deleting a part of exon 5 of the gene replaced by a neomycin cassette (Ernfors et al. 1994), leading to a 50 percent reduction of BDNF expression. Homozygote knock-out mice generated with this procedure do not survive

until adulthood. Mice with total BDNF depletion that do survive can be generated by a conditional knock-out approach (Chan et al. 2006) and give the advantage of studying the effects BDNF depletion separately from the developmental effects of BDNF. In the BDNF +/- mice we have used, developmental compensatory mechanisms to the reduced BDNF levels can not be discriminated to those that arise in maturation and aging.

TrkB full length (TrkB TK) overexpressive mice

Mice overexpressing the full-length form of rat TrkB receptor under the Thy1.2 promoter have been previously described (Koponen et al. 2004b). Briefly, transgenic mice generated by pronucleus injection of purified TrkB.TK+-Flag cDNA Thy1.2 construct into hybrid embryos from CD2F1 females mated with CD2F1 males. Transgenic male founders were mated with CD2F1 females to create a stable heterozygous mice line. Transgenic male mice were mated with CD2F1 female mice to produce both transgenic and wild-type control mice from the same litters.
To our knowledge this is the only mouse model with an overexpression of TrkB.

Clinical studies

Registers and cohort

By cross comparison between the Danish civil registration system, the Danish Psychiatric Central Research Register and the Danish Twin Registry, 408 high risk and low risk twins were identified. Of the 408 invited, 271 wished to participate. Subsequently, 37 twins were excluded according to the exclusion criteria, resulting in 234 participants. Whole blood was collected from 206 subjects. The final cohort of 206 participants, 122 women and 84 men, were divided into four groups according to risk of affective disorder. Additionally, a fifth group was defined as participants with another first generation family history of affective disorder or schizophrenia (n= 14).

In- and exclusion criteria

The inclusion criterium for high risk twins was having a monozygotic (MZ) or a dizygotic (DZ) co-twin with a diagnosis of affective disorder (co-twin history of affective disorder). The inclusion criterium for low risk twins was no family history of affective disorder among the MZ or DZ co-twin or other severe psychiatric illness among other first- degree relatives.

Exclusion criteria included a personal history of severe to moderate depression, earlier medical treatment for an affective episode, severe organic brain disease or schizophrenia.

Participants and non-participants

	Participants (n= 206)	**Non-participants (n= 131)**
Women (%)	59.2	66.7
Age	44.3 ± 13.3	47.2 ± 13.0
Zygosity mono (%)	31.0	33.3
Education years	12.8 ± 3.2	12.4 ± 3.2

Table 1. Characteristics of participants and non-participants (mean ± SD).

Table 1 reflects the characteristics of participants and non-participants. These two groups are very similar in sex and zygosity distribution, and education years. The sex distribution reflects the known prevalence of affective disorder for each sex (Kendler et al. 2006). Also the distribution of zygosity for monozygotic twins lays around one third, which is the normal distribution for twins. The only variable that discriminated the two groups is that the non-participants are slightly older ($p < 0.05$) than the participants.

Assessment of life events and neuroticism

Participants were asked about life events in the year prior to the interview (recent life events) and life events in their lifetime before that, using a Danish version of the questionnaire used in the studies of Kendler and colleagues (Kendler et al. 1995). Nine personal events (events that happened to the participants) were assessed. I addition, 22

network events were assessed i.e. events that occurred primarily to, or in interaction with, an individual in the participant's social network.

Personality dimensions were assessed using the EPQ, Danish version. The EPQ comprises 101 items intended to measure a broad dimension of neuroticism, extroversion and psychotism (Eysenck 1975).

Results and Discussion

Increased GR signaling increases 5-HT_{2A} receptor levels (Paper I)

To study the effects of altered glucocorticoid signalling on 5-HT_{2A} receptor levels in the hippocampus, mice models over- and under-expressing GR were used. Mice under-expressing GR (GR+/-) display a depression-like phenotype upon stress, while GR over-expressing mice (YGR) are stress resistant and do not reveal depression-like features (Ridder et al. 2005). Protein analysis on hippocampal tissue homogenate revealed that GR+/- mice had significantly decreased 5-HT_{2A} receptor levels (Figure 4a). YGR mice on the other hand showed increased 5-HT_{2A} receptor levels (Figure 4b). Our results indicate that an increased vulnerability to depressive-like behaviour is associated with reduced 5-HT_{2A} receptor levels. This corroborates earlier findings where depressed patients display reduced hippocampal 5-HT_{2A} receptor binding (Sheline et al. 2004; Mintun et al. 2004; Rosel et al. 2004; Rosel et al. 2000). Our results on altered hippocampal 5-HT_{2A} receptor levels in mice with reduced GR expression contrast, however, an earlier animal study where hippocampal 5-HT_{2A} receptor binding was increased (Farisse et al. 2000), but are in line with a another showing decreased hippocampal 5-HT_{2A} receptor mRNA levels (Cyr et al. 2001). Differences between animal studies may be due to strain differences or differences in the generation of the animal models used.

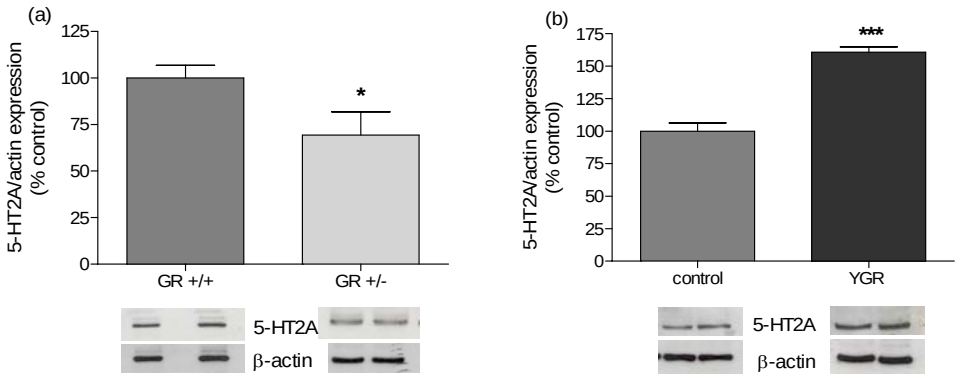

Figure 4. Hippocampal 5-HT_{2A} receptor expression in mice under- and overexpressing GR. (a) Mice underexpressing GR (GR +/-) show significantly reduced hippocampal 5-HT_{2A} receptor protein levels. (b) In contrast, mice over expressing GR (YGR) show significantly increased 5-HT_{2A} receptor levels. Scale bars represent mean ± SEM, * $p< 0.05$, *** $p< 0.001$.

To verify that a long-term activation of GR by corticosterone directly relates to 5-HT_{2A} receptor changes, adult organotypic hippocampal cultures were subjected to different concentrations of corticosterone, with or without specific blockers of GR and MR. Hippocampal slice cultures exposed to corticosterone for one day did not display any changes in 5-HT_{2A} receptor levels. After seven days exposure on the other hand, application of corticosterone lead to a significant increase in 5-HT_{2A} receptor levels. The increase was blocked by the addition of the specific GR and MR blockers, mifepristone and spironolactone, respectively (Figure 5). Both GR+/- and YGR mice have no changes in basal corticosterone levels (Ridder et al. 2005). We therefore show both in cultures and in transgenic mice with an incorporated additional copy of the GR gene that increased GR signalling up-regulates hippocampal 5-HT_{2A} receptor levels. Conversely, in GR+/- mice with decreased GR levels the opposite effect is seen on hippocampal 5-HT_{2A} receptor levels. Furthermore, increased GR agonism, as a regulatory mechanism for 5-HT_{2A} receptor levels, is in line with what has been suggested before (Garlow & Ciaranello 1995).

The alterations in 5-HT_{2A} receptor levels seen in the different slice culture experimental set ups were not due to changes in cell death. Further, since hippocampal slice cultures are entirely denervated from serotonergic projections we can conclude that the corticosterone-induced changes in 5-HT_{2A} receptor levels occur independently of changes in 5-HT levels (Gartside et al. 2003). In addition, it has been consistently shown that mice genetically modified to express GR differently have normal baseline extracellular and total 5-HT and 5-HIAA levels (Farisse et al. 1999; Linthorst et al. 2000; Schulte-Herbruggen et al. 2006).

In conclusion, increased GR expression is associated with increased hippocampal 5-HT_{2A} receptor levels, and decreased GR expression yields decreased hippocampal 5-HT_{2A} receptor levels. Furthermore, the corticosterone-induced increase

in 5-HT_{2A} receptor levels appears after chronic exposure, is independent of 5-HT levels and is blocked by specific GR and MR antagonists.

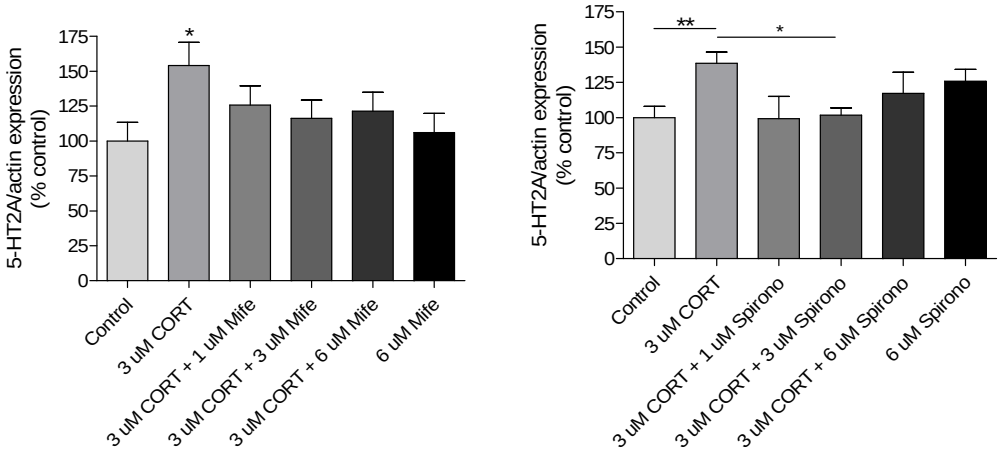

Figure 5. 5-HT_{2A} receptor protein expression in adult organotypic cultures exposed to corticosterone (CORT) with and without the addition of mifepristone (Mife) or spironolactone (Spirono). Scale bars represent mean ± SEM.

5-HT_{2A} receptor levels are associated with BDNF and TrkB levels (Paper II)

To study whether BDNF and TrkB has a regulatory effect on 5-HT_{2A} receptor levels, adult hippocampal organotypic cultures exposed to different concentrations of BDNF, BDNF heterozygous mice (BDNF +/-) and mice over-expressing the TrkB full length (TrkB TK) receptor form were used.

When hippocampal slice cultures were exposed to BDNF in a chronic paradigm (5-7 days), 5-HT_{2A} receptor protein levels were decreased (Figure 6). No changes in 5-HT_{2A} receptor levels were seen after a 1-3-day paradigm. In addition, the alterations in 5-HT_{2A} receptor levels in the cultures were not accompanied by increased cell death or synaptic degeneration.

The role of BDNF in inducing 5-HT_{2A} receptor changes was corroborated in BDNF +/- mice, which showed increased levels of this receptor in the hippocampus.

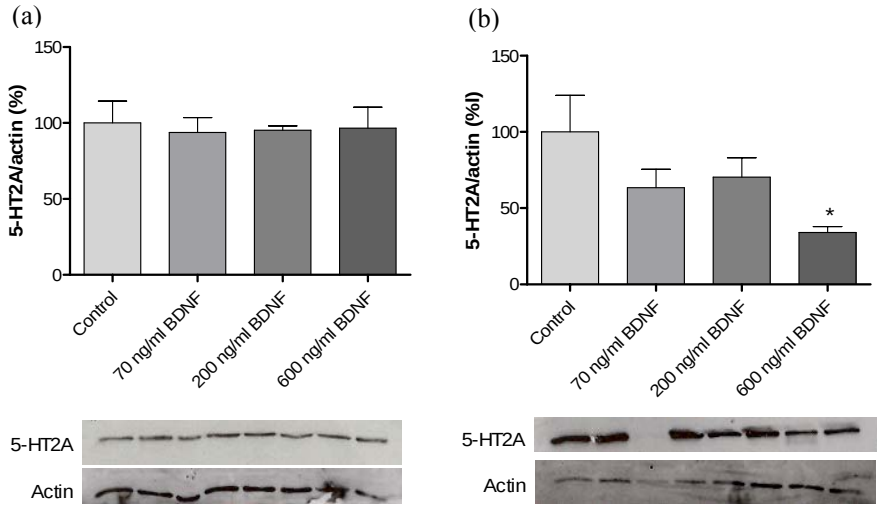

Figure 6. A seven-day (b), and not one-day (a), exposure to BDNF (600 ng/ml) in the hippocampal slice cultures lead to a decrease in 5-HT_{2A} receptor levels (* $p < 0.05$). Scale bars represent mean ± SEM.

We suggest that the changes in 5-HT_{2A} receptor levels may be related to TrkB levels since TrkB TK mice displayed increased 5-HT_{2A} receptor levels in the hippocampus and frontal cortex (Figure 7). This is supported by the finding that the increase in 5-HT_{2A} receptor levels in BDNF +/- mice was accompanied by a tendency to increased TrkB levels. In addition, a decrease in TrkB levels was observed in organotypic cultures exposed to the same concentration of BDNF that induces reduced 5-HT_{2A} receptor levels. Chronic, but not acute, BDNF exposure decreases TrkB levels (Frank et al. 1997), as well as 5-HT_{2A} receptor levels. Our results thus suggest that the level of TrkB and not BDNF may regulate 5-HT_{2A} receptor levels (Table 2). Further, TrkB TK mice show no changes in BDNF mRNA or protein levels (Koponen et al. 2004a; Koponen et al. 2004b). However, whether or not TrkB directly regulates 5-HT_{2A} receptor levels needs to be investigated in further studies.

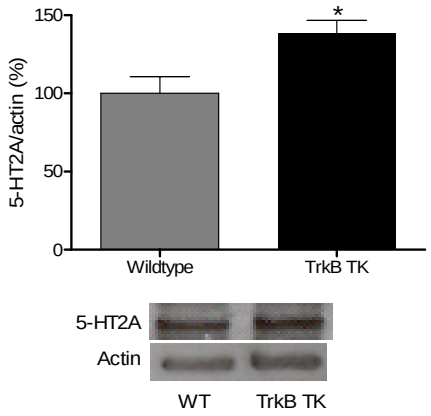

Figure 7. Mice overexpressing the full length form of TrkB (TrkB TK) display increased hippocampal (graph) and frontal cortex 5-HT2A receptor levels (* $p < 0.05$) compared to wild-type littermates (WT). Scale bars represent mean ± SEM

Our result on the increased hippocampal 5-HT_{2A} receptor levels in BDNF +/- mice are in apparent contrast to earlier studies (Rios et al. 2006; Chan et al. 2006), where BDNF conditional knock-out mice displayed decreased 5-HT_{2A} receptor levels in dorsal raphe nucleus and frontal cortex. In those studies, however, TrkB levels were not measured and it is likely that TrkB levels are differently altered after total BDNF depletion. Also, region specific differences in 5-HT_{2A} receptor regulation by BDNF may also be present. No information about hippocampal 5-HT_{2A} or TrkB receptor levels was presented in the papers of Rios et al. (2006) and Chan et al. (2006).

	BDNF	**TrkB**	**5-HT_{2A} receptor**
Organotypic cultures + BDNF	High	Low	Low
BDNF +/- mice	Low	High	High
TrkB TK mice	Unchanged	High	High

Table 2. Hippocampal BDNF, TrkB and 5-HT_{2A} receptor levels in the organotypic slice cultures and in two mouse models.

BDNF increases 5-HT synthesis and sprouting (Siuciak et al. 1998; Celada et al. 1996), and increased 5-HT levels lead to 5-HT_{2A} receptor desensitization (Hanley & Hensler 2002) and down-regulation (Gray & Roth 2001). However, no changes in 5-HT levels are known to occur in the TrkB TK mice (Koponen et al. 2005; Saarelainen et al. 2003), and three months old BDNF +/- mice (Lyons et al. 1999). This suggests that the BDNF-induced changes in 5-HT_{2A} receptor levels are independent of 5-HT levels. Furthermore, in the adult organotypic hippocampal cultures the 5-HT

innervation is removed upon dissection, excluding the possibility that presynaptic serotonin mechanism is involved in the BDNF induced changes.

In conclusion, chronic BDNF exposure decreases hippocampal 5-HT_{2A} receptor levels. Conversely, a decreased BDNF expression is associated with increased hippocampal 5-HT_{2A} receptor levels. Furthermore, the BDNF-induced changes in 5-HT_{2A} receptor protein levels are independent of 5-HT levels and might be mediated through alterations in TrkB levels.

Validation of BDNF measurements in blood and the effect of demographical data (Paper III)

Although numerous clinical studies have identified changes in serum or blood BDNF concentrations in patients with neuropsychiatric disorders (Toyooka et al. 2002; Nakazato et al. 2003; Shimizu et al. 2003; Laske et al. 2007), methodological issues regarding measurements of BDNF concentration have only been superficially encountered. Therefore, we felt that a thorough evaluation of storage conditions and of a commonly used ELISA based assay regarding the reproducibility and accuracy of blood BDNF measurement was warranted. The relation between serum and whole blood BDNF levels, inter-subject variability and the effect of demographical data on whole blood BDNF was also investigated.

We found that the BDNF ELISA assay measures BDNF levels accurately (91.6 ± 3.0 %) and with high reproducibility (Ngeh et al. 2004; Blacksell et al. 2006). The inter-assay variability was below 10 %, which is considered low in comparison to other assays (Ngeh et al. 2004; Upreti et al. 2003). Furthermore, intra-subject variability was modest and did not vary much with the time between blood sampling.
BDNF levels are better assessed in whole blood than in serum, particularly when the BDNF measurements are not carried out immediately. Storage of whole blood samples for up to five years at –20°C was not associated with any significant decrease in whole blood BDNF levels. Also, repeated freezing and thawing did not affect whole blood BDNF levels significantly. By contrast, long-term storage of serum samples was associated with significantly lower serum BDNF levels (Figure 8). Already within

eighth months some serum samples had BDNF levels below the detection limit of the BDNF ELISA kit. This indicates that when stored in platelets BDNF is protected against degradation (Pardridge et al. 1994). The weakness of this study on the effect of storage time on BDNF levels was that is was cross-sectional. A more direct way of assessing the effect of storage and degradation would be by using a longitudinal study design.

Storage of whole blood samples in 4°C is not recommended as it is associated with a decrease in BDNF levels.

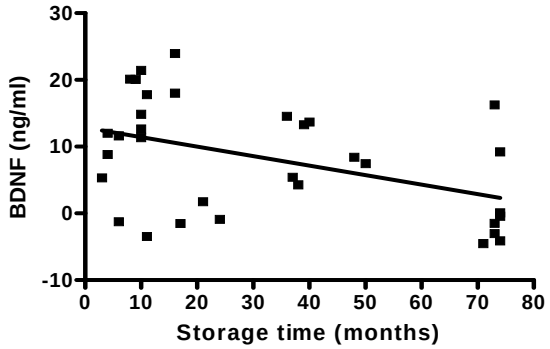

Figure 8. Storage of serum samples at -20°C is associated with a significant reduction in BDNF levels.

The effect of demographical data on whole blood BDNF levels was also assessed. In a sample of 206 healthy subjects the effect of age, gender, thrombocyte count and the BDNF Val66Met functional polymorphism on whole blood BDNF levels was investigated. We found that female gender (Figure 9) is associated with higher whole blood BDNF levels than male. This is in line with another study showing increased plasma BDNF levels in women (Lommatzsch et al. 2005), but contrast two earlier studies that found no differences in serum BDNF levels between men and women (Lang et al. 2004; Ziegenhorn et al. 2007). Further, women and not men displayed a right-shrewd BDNF distribution possibly explained by variations in BDNF levels during the different stages in the menstrual cycle (Lommatzsch et al. 2005; Scharfman et al. 2003).

We found no influence of age, thrombocyte count or the BDNF Val66Met polymorphism on whole blood BDNF levels, which is supported by other studies on the effect of age (Lommatzsch et al. 2005; Webster et al. 2002b) and genotype (Lee et al. 2005; Chen et al. 2004) on BDNF levels.

In conclusion, this study shows that the BDNF ELISA assay measures BDNF accurately and with high reproducibility. Serum samples should be analysed immediately, whereas whole blood can safely be stored at –20 °C for at least five years. Whole blood samples can be thawed up to three times without any impact on the whole blood BDNF determinations. The intra-individual variation over time in whole blood BDNF levels is modest. Women display significantly higher whole blood BDNF levels than men, and display a right-shrewd BDNF distribution. We failed to identify any association between whole blood BDNF and thrombocyte count, age, or BDNF Val66Met genotype.

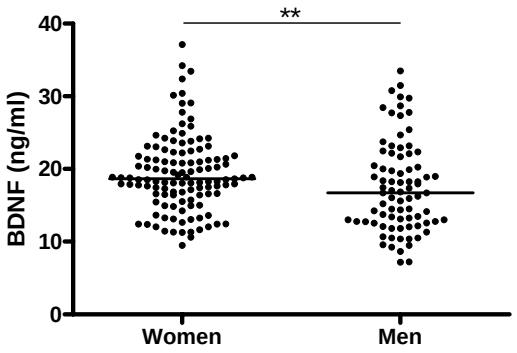

Figure 9. Healthy women display higher whole blood BDNF levels than healthy men. A normal distribution of whole blood BDNF levels was found in men only. ** $p < 0.01$

Genetic predisposition, female gender and stress decreases whole blood BDNF levels (Paper IV)

To assess whether whole blood BDNF levels are decreased in subjects at genetic high risk of developing depression, whole blood BDNF concentrations in a twin population including both subjects highly predisposed and protected against affective disorder was studied. Four different twin risk groups (206 subjects) were included: high risk monozygotic predisposed twins (co-twin depressed), moderately predisposed dizygotic twins, moderately protected dizygotic twins (co-twin unaffected), and highly protected monozygotic twins. Whole blood samples were collected and assessed for BDNF concentrations, and correlated to risk status, gender, neuroticism, and number of stressful life events.

We found that whole blood BDNF levels were similar in healthy twins with or without a genetic predisposition to depression, indicating that the genetic risk of developing depression is insufficient to induce changes in whole blood BDNF levels. Also the number of stressful life events or the degree of neuroticism themselves did not affect whole blood BDNF levels.

It is well acknowledged today that affective disorders are a result of an interaction between genetic liability and environmental risk factors (Kendler et al. 1995; Caspi et al. 2003; Farmer et al. 2005). Experiencing three or more events is associated with a great increase in depression liability, as to experiencing two or less (Caspi et al. 2003). We found that the combination of female gender, high genetic risk and high number of recent stressful life events is associated with lower whole blood BDNF levels. Women highly genetically predisposed to depression that had experienced three or more recent stressful events had decreased whole blood BDNF levels compared to high risk women with two or less recent events (Figure 10). This association was not found in low risk women or in high and low risk men, and supports the notion that females with genetic predisposition to affective disorders are particularly vulnerable to depression (Kendler et al. 2007).

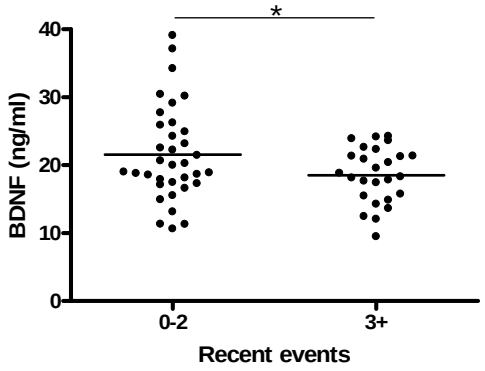

Figure 10. The combination of high genetic risk, female gender and three or more recent stressful life events leads to decreased whole blood BDNF levels. High risk women with two or less events (0-2) vs. high risk women with three or more events (3+), 21.6 ± 7.0 vs. 18.5 ± 4.1 ng/ml, * $p < 0.05$.

Not only the genetic risk but also a combination of several environmental risk factors increases depression liability. Subjects with high neuroticism and high level of stress are at higher risk of developing depression (Kendler et al. 2004). In accordance with this we found a tendency ($p = 0.07$) for women with high neuroticism score (score over five) and three or more recent events displaying decreased whole blood BDNF levels. By contrast, men with high neuroticism score and more than two recent life stressful events displayed increased whole blood BDNF levels as compared to low neuroticism

men with less than three recent stressful life events (Figure 11). This association was irrespective of their genetic risk. It seems that in men BDNF levels are appropriately counteracting two risk factors for depression, thereby rendering these at-risk men less likely to develop depression.

In conclusion, the genetic risk of developing depression itself does not induce changes in whole blood BDNF levels. In females that are genetically disposed for depression and subjected to high number of recent stressful life events whole blood BDNF levels are lower, indicating that BDNF dysregulation may be more important in women's vulnerability to depression. Thus low whole blood BDNF levels are a trait factor for female depression. By contrast, in men BDNF levels seem to appropriately counteract the risk factors high neuroticism and high number of recent stressful life events.

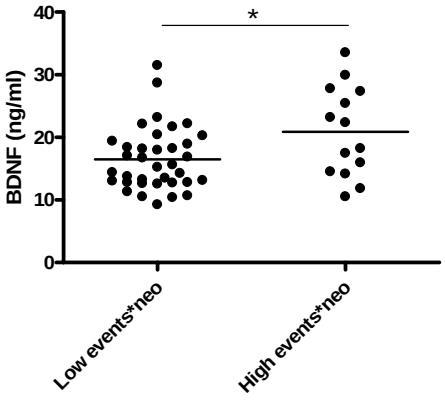

Figure 11. Men with high number of stressful life events (three or more) and high neuroticism display increased whole blood BDNF levels compared to men with low number of events (two or less) and low neuroticism. * $p < 0.05$.

General discussion

This thesis seeks to identify key changes involved in depression vulnerability. The focus for the investigation was to study the effects of BDNF and glucocorticoids on the 5-HT_{2A} receptor, and further to evaluate the role of BDNF as a state or a trait marker of depression.

5-HT_{2A} receptor levels and depression vulnerability

In mouse models with altered GR, TrkB and BDNF expression we found differences in 5-HT_{2A} receptor levels that seem to be associated with a higher or lower vulnerability to depression. We found that the YGR and TrkB overexpressing mice that display reduced depression-like behaviour (Ridder et al. 2005; Koponen et al. 2005; Koponen et al. 2004b), both had increased hippocampal 5-HT_{2A} receptor levels. Similarly, GR+/- mice that have a predisposition to depression-like behaviour (Ridder et al. 2005) display lower hippocampal 5-HT_{2A} receptor levels, suggesting that low hippocampal 5-HT_{2A} receptor levels is a trait marker of depression vulnerability. The BDNF+/- mice used in our study also had increased hippocampal 5-HT_{2A} receptor levels, but data on whether BDNF+/- mice display alterations in anxiety and depression-like behaviour have been inconsistent, showing both behavioural changes (Chan et al. 2006) and no changes (Lyons et al. 1999). Our results from the clinical study (paper IV) point towards low BDNF levels being a trait factor for female depression, and when taking gender into account female BDNF+/- mice display depression-like behaviour while male do not (Monteggia et al. 2007). Furthermore, supportive of low 5-HT_{2A} receptor levels being an expression of increased depression vulnerability, another study showed that fetal BDNF depletion is associated with a pronounced increase in depressive-like behaviour and decreased frontal cortex 5-HT_{2A} receptor levels (Chan et al. 2006).

In a recent positron emission tomography (PET) study of 83 healthy subjects a significant correlation between frontolimbic 5-HT_{2A} receptor binding and the personality trait neuroticism (known to infer a higher risk of developing major depression) was found (Frokjaer et al. 2007). In that study, a trend for positive association was also seen for hippocampus ($p= 0.07$). In a subset of the subjects

predisposed to affective disorder enrolled in paper IV preliminary data however show no differences in cortical 5-HT_{2A} receptor binding, as measured with PET (Frokjaer et al. *In preparation*.). Our results indicate that a genetic predisposition to depression is not firmly associated with a decrease in whole blood BDNF levels, and it is suggested that the various risk factors for depression (high heritability, gender, stressful life events), need to be taken into account when evaluating alterations in 5-HT_{2A} receptor binding. It is currently unknown if a decrease in whole blood BDNF induce changes in 5-HT_{2A} receptor binding in humans. In fact, a recent study from our laboratory indicates that in healthy humans cortical 5-HT_{2A} receptor binding does not correlate with whole blood BDNF levels, whereas the BDNF Val66Met functional polymorphism is associated with lower cortical 5-HT_{2A} receptor binding in healthy subjects (Marcussen et al. *Submitted*). This observation suggests that 5-HT_{2A} receptor binding in humans may be determined during development. The BDNF Val66Met polymorphism is associated with decreased hippocampal volume (Pezawas et al. 2004; Chen et al. 2006), however, in genetic association studies for depression, there is little consensus as to whether this allele confers increased susceptibility (Strauss et al. 2005; Strauss et al. 2004; Hong et al. 2003; Schumacher et al. 2005).

Our observations support the idea that when looking at trait markers for depression vulnerability one has to look not only on a single factor (e.g. genetic) but on the interaction between several factors (environmental as well as endophenotypes). That is probably why many studies until now have failed in showing a direct correlation between specific endophenotypes, genetic or environmental factors and depression. Furthermore, whether differences in 5-HT_{2A} receptor levels is a trait marker *per se* or the result of an interaction between several factors and one of the main triggering factors for depression has to be further investigated.

Atypical antipsychotic drugs and antidepressant drugs that act through blocking 5-HT_{2A} receptor responses are used to increase the clinical effect of SSRI in treatment-resistant depressed patients (Shelton et al. 2001; Marek et al. 2003). Furthermore, chronic antidepressant treatment of rodents leads to a down-regulation of 5-HT_{2A} receptors although not with the more selective SSRI's (Gray & Roth 2001). TrkB TK mice, that display increased 5-HT_{2A} receptor levels, however, also display antidepressant-like behaviour in the forced swim test (Koponen et al. 2005), and further, an intact TrkB signalling is required for mediating an antidepressant response

(Saarelainen et al. 2003). Recently, polymorphisms in the 5-HT_{2A} receptor gene have been associated with the outcome of antidepressant treatment (McMahon et al. 2006; Choi et al. 2006). One of these polymorphisms yields intronic variants and the functional relevance of it is unknown. The other, however, lies close to the promoter region of the gene (Spurlock et al. 1998) and would therefore affect 5-HT_{2A} receptor gene transcription (Du et al. 1995), indicating that 5-HT_{2A} receptor expression levels are directly related to depression.

A polymorphism in the GR gene, the Bcl 1 SNP, that is associated with increased HPA axis activity (van Rossum et al. 2002; Brouwer et al. 2006) due to a decrease in GR receptor functionality and thereby impaired negative feedback inhibition (van Rossum et al. 2002), has also been coupled to the outcome of antidepressant treatment. Carriers of the Bcl1 SNP GR polymorphism respond poorly to antidepressants (Brouwer et al. 2006). Increase in GR expression and function is believed to be one of the effects of antidepressant treatment (Pariante et al. 2001; Lai et al. 2003; Seckl and Fink 1992), and according to our results increased GR signaling up-regulates 5-HT_{2A} receptor levels. Also lower levels of the GR receptor was associated with lower 5-HT_{2A} receptor levels, therefore based on this an explanation to the lower antidepressant response in the carriers of the the Bcl 1 SNP might be low 5-HT_{2A} receptor levels in this group. It would certainly be interesting to investigate whether carrying the Bcl 1 SNP is associated with lower 5-HT_{2A} receptor binding.

It seems from the above observations that the 5-HT_{2A} receptor is involved in the antidepressant effect of SSRIs and makes it very plausible that differences in this receptor is of importance when considering the main factors involved in depression vulnerability.

Corticosterone and BDNF as regulatory factors for 5-HT_{2A} receptor levels

In our adult organotypic hippocampal cultures, both corticosterone and BDNF induce opposing changes in hippocampal 5-HT_{2A} receptor levels. It has been consistently found that corticosterone alters BDNF expression (Jacobsen & Mork 2006; Nair et al. 2007), indicating that the effect of corticosterone on 5-HT_{2A} receptors might be mediated through these changes. The corticosterone-induced increase in 5-HT_{2A} receptor levels was blocked by both GR and MR antagonist, but acute GR blockade also inhibits the corticosterone-induced changes in BDNF levels (Yang et al. 2004;

Hansson et al. 2000). The corticosterone-induced changes in 5-HT_{2A} receptor levels appear only after a seven-day exposure, and even though there are no studies on the effects of chronic GR blockade, we can not exclude the possible involvement of BDNF in the observed changes in 5-HT_{2A} receptor levels in the organotypic hippocampal slice cultures.

Recent studies on the GR+/- and YGR mice show that both of these mice models have diurnal changes in BDNF levels (Schulte-Herbruggen et al. 2006; Schulte-Herbruggen et al. 2007). In the slice culture studies we showed that BDNF induces 5-HT_{2A} receptor changes only after a seven day exposure, indicating that the diurnal fluctuations of BDNF in these mice are not sufficient in inducing the observed changes in hippocampal 5-HT_{2A} receptor levels. Therefore, it is highly probable that the observed effects on 5-HT_{2A} receptor levels in these two mice models are through altered GR expression and are not secondary to changes in BDNF levels. Furthermore, other studies have earlier shown that GR signaling might be involved in 5-HT_{2A} receptor regulation (Garlow & Ciaranello 1995; Cyr et al. 2001; Farisse et al. 2000). Whether GR or BDNF signaling is the most important mechanisms for determining 5-HT_{2A} receptor protein expression in the hippocampus needs to be further investigated though.

Whole blood BDNF as trait marker of depression

As there is no method available of measuring *in vivo* brain BDNF levels in humans, studies on BDNF have been done by assessing blood BDNF levels. In addition, numerous studies have identified changes in blood BDNF levels in different neuropsychiatric disorders (Toyooka et al. 2002; Shimizu et al. 2003; Azoulay et al. 2005). We assessed whole blood BDNF levels in a cohort of healthy subjects and found a gender effect. Men display lower levels and seem to be better at regulating their whole blood BDNF level than women, as show by the large variability of whole blood BDNF levels in women (paper III) and that only high risk women show decreased BDNF levels (paper IV). The gender-specific differences in BDNF levels may be due to differences in HPA axis regulation (Young 1998; Handa et al. 1994), as estrogen enhances HPA axis function (Handa et al. 1994) while testosterone seems to protect against HPA axis hyperactivity (Handa et al. 1994; Rubinow et al. 2005). In

addition, estrogen seems to be involved in mood regulation and major depression (Young et al. 2007; Geng et al. 2007; Douma et al. 2005).

Our gender-specific observation is well in line with depressed women showing a greater reduction in serum BDNF levels than depressed men (Karege et al. 2002a). In addition, recent genetic studies confirm our finding by linking BDNF to increased vulnerability to depression (Kaufman et al. 2006; Gatt et al. 2007). Early life stress renders BDNF levels in rodents when subjected to subsequent late life stress (Faure et al. 2007; Nair et al. 2007). It may be that the vulnerability to stress, and therefore also to depression, is displayed in the individual's ability of regulating their BDNF levels (Shelton 2007). As BDNF levels are affected throughout the different stages of the menstrual cycle (Lommatzsch et al. 2005), it is possible that women would be more sensitive to disturbances in the regulation of BDNF levels and therefore more vulnerable to developing depression.

One concern when measuring blood BDNF levels is whether blood levels actually reflect the levels in the brain. Event though brain and blood BDNF levels are similarly changed by antidepressant treatment (Shimizu et al. 2003; Chen et al. 2001) and in different disease states (Toyooka et al. 2002; Durany et al. 2001; Laske et al. 2007; Connor et al. 1997), to our knowledge there is only one animal study directly assessing the association between blood and brain BDNF levels (Karege et al. 2002b). In order to understand the significance of the altered blood BDNF levels, the association between blood and brain BDNF levels in humans needs to be further investigated.

Conclusions

This thesis was undertaken to investigate the effects of BDNF and glucocorticoids on 5-HT_{2A} receptor levels and their relationship on depression vulnerability. Taken together this thesis showed that:

- Increased GR agonism increases hippocampal 5-HT_{2A} receptor levels. Conversely, a decreased GR expression leads to a down-regulation of hippocampal 5-HT_{2A} receptor levels. The impact of corticosterone on 5-HT_{2A} receptor levels takes place independently of 5-HT levels and can be reverted by both GR and MR blockade.

- Chronic BDNF exposure independently of 5-HT levels decreases hippocampal 5-HT_{2A} receptor levels without affecting cell death. Reduced BDNF expression on the other hand increases hippocampal 5-HT_{2A} receptor levels. The BDNF-induced changes in 5-HT_{2A} receptor levels might be mediated through changes in TrkB levels.

- The BDNF ELISA assay determines BDNF levels accurately and with high reproducibility. Long-term storage of serum, and not whole blood, samples in -20◦C is associated with a decrease in BDNF levels. Intra-subject variability in whole blood BDNF is modest and does not vary with time between blood sampling. Women display higher whole blood BDNF levels than men, while whole blood BDNF levels and age, thrombocyte count or BDNF Val66Met polymorphism are un-associated.

- Whole blood BDNF levels were similar in healthy twins with or without a genetic predisposition to depression. Also the number of stressful life events and degree of neuroticism themselves and whole blood BDNF levels are un-

associated. The combination of female gender, high genetic risk and high number of stressful life events lowered whole blood BDNF levels. In men, high neuroticism (score over four) and high number of stressful life events lead to higher whole blood BDNF levels, irrespective of the genetic risk status.

Mice models with altered glucocorticoid or BDNF signalling and of increased and decreased depression vulnerability showed changed 5-HT_{2A} receptor levels. From this we can conclude that low hippocampal 5-HT_{2A} receptor levels may be a trait factor of depression. There are, however, to date limited number of human studies supportive of this. The precise etiology and pathophysiology of depression is unknown, and the diagnosis of depression is purely based on the presence of some core symptoms which makes is difficult, if not impossible, to replicate in animal models. Currently there is no available animal model that includes all aspects of depression. Therefore, even though conducting translational research may be a strength, drawing any firm conclusions from the results is difficult.

The results from the clinical study show that when taking genes, stress and gender into account, low BDNF levels were found to be a trait marker of depression. We therefore conclude that when looking at trait markers for depression vulnerability one has to look not only on a single factor but on the interaction between several factors; genetic, as well as environmental.

Research perspectives

The findings presented in this thesis add to the current literature documenting on different factors that increase depression vulnerability. The focus of this thesis was on BDNF, glucocorticoids and 5-HT_{2A} receptor expression. To further study the effect of BDNF, TrkB and altered GR expression on 5-HT_{2A} receptor levels it would be interesting to investigate the anatomical and cellular localization of the 5-HT_{2A} receptor changes with autoradiography, and to expand the study by looking at other depression-related brain areas. Furthermore, it is highly relevant to elucidate whether there are any effects on 5-HT_{2A} receptor mRNA levels and on the functional properties of 5-HT_{2A} receptors.

Further studies are needed to elucidate the mechanism of the BDNF-induced changes in 5-HT_{2A} receptor levels. It needs to be confirmed that the changes are mediated through TrkB and furthermore the intracellular pathways that are activated could be studied by using specific blockers.

To confirm that the 5-HT_{2A} receptor protein levels are mediated through altered GR expression, independent of corticosterone levels, 5-HT_{2A} receptor levels should be measured in adrenalectomised GR +/- and YGR mice. It is also important to expand the results to human studies and to elucidate whether cortisol levels and different GR polymorphisms may alter 5-HT_{2A} receptor binding as measured with PET.

The involvement of 5-HT_{2A} receptor levels in anxiety and depression modulation should be further studied in animal experiments using specific 5-HT_{2A} receptor antagonist and behavioral assessment models, and in human studies where 5-HT_{2A} receptor binding is measured in individuals protected and vulnerable to affective disorder.

In our cohort of individuals genetically vulnerable to depression it would be interesting to confirm the involvement of GR by investigating the allelic distribution of different GR polymorphisms. The same could also be done for TrkB polymorphisms and future potential genes and polymorphisms that may uncover the genetic mechanism underlying the susceptibility to major depression.

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References

(1997) Mutant mice and neuroscience: recommendations concerning genetic background. Banbury Conference on genetic background in mice. *Neuron* **19,** 755-9.

Adachi N., Kohara K., and Tsumoto T. (2005) Difference in trafficking of brain-derived neurotrophic factor between axons and dendrites of cortical neurons, revealed by live-cell imaging. *BMC Neurosci* **6,** 42.

Aghajanian G. K. and Marek G. J. (1999) Serotonin, via 5-HT2A receptors, increases EPSCs in layer V pyramidal cells of prefrontal cortex by an asynchronous mode of glutamate release. *Brain Res* **825,** 161-71.

Alfonso J., Pollevick G. D., Van Der Hart M. G., Flugge G., Fuchs E., and Frasch A. C. (2004) Identification of genes regulated by chronic psychosocial stress and antidepressant treatment in the hippocampus. *Eur J Neurosci* **19,** 659-66.

Altar C. A. (1999) Neurotrophins and depression. *Trends Pharmacol Sci* **20,** 59-61.

Altar C. A., Whitehead R. E., Chen R., Wortwein G., and Madsen T. M. (2003) Effects of electroconvulsive seizures and antidepressant drugs on brain-derived neurotrophic factor protein in rat brain. *Biol Psychiatry* **54,** 703-9.

Azoulay D., Vachapova V., Shihman B., Miler A., and Karni A. (2005) Lower brain-derived neurotrophic factor in serum of relapsing remitting MS: reversal by glatiramer acetate. *J Neuroimmunol* **167,** 215-8.

Balkowiec A. and Katz D. M. (2000) Activity-dependent release of endogenous brain-derived neurotrophic factor from primary sensory neurons detected by ELISA in situ. *J Neurosci* **20,** 7417-23.

Barden N., Reul J. M., and Holsboer F. (1995) Do antidepressants stabilize mood through actions on the hypothalamic-pituitary-adrenocortical system? *Trends Neurosci* **18,** 6-11.

Berchtold N. C., Chinn G., Chou M., Kesslak J. P., and Cotman C. W. (2005) Exercise primes a molecular memory for brain-derived neurotrophic factor protein induction in the rat hippocampus. *Neuroscience* **133,** 853-61.

Bhagwagar Z., Hafizi S., and Cowen P. J. (2003) Increase in concentration of waking salivary cortisol in recovered patients with depression. *Am J Psychiatry* **160,** 1890-1.

Bhagwagar Z., Hinz R., Taylor M., Fancy S., Cowen P., and Grasby P. (2006) Increased 5-HT(2A) receptor binding in euthymic, medication-free patients recovered from depression: a positron emission study with. *Am J Psychiatry* **163,** 1580-7.

Blacksell S. D., Newton P. N., Bell D., Kelley J., Mammen M. P. Jr, Vaughn D. W., Wuthiekanun V., Sungkakum A., Nisalak A., and Day N. P. (2006) The comparative accuracy of 8 commercial rapid immunochromatographic assays for the diagnosis of acute dengue virus infection. *Clin Infect Dis* **42,** 1127-34.

Boyle M. P., Brewer J. A., Funatsu M., Wozniak D. F., Tsien J. Z., Izumi Y., and Muglia L. J. (2005) Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes

in adrenal axis regulation and behavior. *Proc Natl Acad Sci U S A* **102,** 473-8.

Brouwer J. P., Appelhof B. C., van Rossum E. F., Koper J. W., Fliers E., Huyser J., Schene A. H., Tijssen J. G., Van Dyck R., Lamberts S. W., Wiersinga W. M., and Hoogendijk W. J. (2006) Prediction of treatment response by HPA-axis and glucocorticoid receptor polymorphisms in major depression. *Psychoneuroendocrinology* **31,** 1154-63.

Burnette W. N. (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* **112,** 195-203.

Cameron H. A. and Gould E. (1994) Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. *Neuroscience* **61,** 203-9.

Caspi A., Sugden K., Moffitt T. E., Taylor A., Craig I. W., Harrington H., McClay J., Mill J., Martin J., Braithwaite A., and Poulton R. (2003) Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* **301,** 386-9.

Celada P., Puig M., Amargos-Bosch M., Adell A., and Artigas F. (2004) The therapeutic role of 5-HT1A and 5-HT2A receptors in depression. *J Psychiatry Neurosci* **29,** 252-65.

Celada P., Siuciak J. A., Tran T. M., Altar C. A., and Tepper J. M. (1996) Local infusion of brain-derived neurotrophic factor modifies the firing pattern of dorsal raphe serotonergic neurons. *Brain Res* **712,** 293-8.

Chan J. P., Unger T. J., Byrnes J., and Rios M. (2006) Examination of behavioral deficits triggered by targeting Bdnf in fetal or postnatal brains of mice. *Neuroscience* **142,** 49-58.

Chao M. V. (2003) Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat Rev Neurosci* **4,** 299-309.

Chen B., Dowlatshahi D., MacQueen G. M., Wang J. F., and Young L. T. (2001) Increased hippocampal BDNF immunoreactivity in subjects treated with antidepressant medication. *Biol Psychiatry* **50,** 260-5.

Chen Z. Y., Jing D., Bath K. G., Ieraci A., Khan T., Siao C. J., Herrera D. G., Toth M., Yang C., McEwen B. S., Hempstead B. L., and Lee F. S. (2006) Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science* **314,** 140-3.

Chen Z. Y., Patel P. D., Sant G., Meng C. X., Teng K. K., Hempstead B. L., and Lee F. S. (2004) Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. *J Neurosci* **24,** 4401-11.

Choi M. J., Kang R. H., Lim S. W., Oh K. S., and Lee M. S. (2006) Brain-derived neurotrophic factor gene polymorphism (Val66Met) and citalopram response in major depressive disorder. *Brain Res* **1118,** 176-82.

Claes S. J. (2004) CRH, stress, and major depression: a psychobiological interplay. *Vitam Horm* **69,** 117-50.

Colla M., Kronenberg G., Deuschle M., Meichel K., Hagen T., Bohrer M., and Heuser I. (2007) Hippocampal volume reduction and HPA-system activity in major depression. *J Psychiatr Res* **41,** 553-60.

Connor B., Young D., Yan Q., Faull R. L., Synek B., and Dragunow M. (1997) Brain-derived neurotrophic factor is reduced in Alzheimer's disease. *Brain Res Mol Brain Res* **49,** 71-81.

Conti A. C., Cryan J. F., Dalvi A., Lucki I., and Blendy J. A. (2002) cAMP response element-binding protein is essential for the upregulation of brain-derived neurotrophic factor transcription, but not the behavioral or endocrine responses to antidepressant drugs. *J Neurosci* **22,** 3262-8.

Cornea-Hebert V., Riad M., Wu C., Singh S. K., and Descarries L. (1999) Cellular and subcellular distribution of the serotonin 5-HT2A receptor in the central nervous system of adult rat. *J Comp Neurol* **409,** 187-209.

Cyr M., Charbonneau C., Morissette M., Rochford J., Barden N., and Di Paolo T. (2001) Central 5-hydroxytryptamine-2A receptor expression in transgenic mice bearing a glucocorticoid receptor antisense. *Neuroendocrinology* **73,** 37-45.

De Foubert G., Carney S. L., Robinson C. S., Destexhe E. J., Tomlinson R., Hicks C. A., Murray T. K., Gaillard J. P., Deville C., Xhenseval V., Thomas C. E., O'Neill M. J., and Zetterstrom T. S. (2004) Fluoxetine-induced change in rat brain expression of brain-derived neurotrophic factor varies depending on length of treatment. *Neuroscience* **128,** 597-604.

De Kloet E. R., Vreugdenhil E., Oitzl M. S., and Joels M. (1998) Brain corticosteroid receptor balance in health and disease. *Endocr Rev* **19,** 269-301.

Dias B. G., Banerjee S. B., Duman R. S., and Vaidya V. A. (2003) Differential regulation of brain derived neurotrophic factor transcripts by antidepressant treatments in the adult rat brain. *Neuropharmacology* **45,** 553-63.

Donovan M. J., Miranda R. C., Kraemer R., McCaffrey T. A., Tessarollo L., Mahadeo D., Sharif S., Kaplan D. R., Tsoulfas P., Parada L., and et a. l. (1995) Neurotrophin and neurotrophin receptors in vascular smooth muscle cells. Regulation of expression in response to injury. *Am J Pathol* **147,** 309-24.

Douma S. L., Husband C., O'Donnell M. E., Barwin B. N., and Woodend A. K. (2005) Estrogen-related mood disorders: reproductive life cycle factors. *ANS Adv Nurs Sci* **28,** 364-75.

Drevets W. C. (2001) Neuroimaging and neuropathological studies of depression: implications for the cognitive-emotional features of mood disorders. *Curr Opin Neurobiol* **11,** 240-9.

Du, Y-L., Wilcox, E. D., and Jeffrey, J. J. (1995) Regulation of rat 5-hydrotryptamine type 2A receptor gene activity: identification of cis elements that medicate basal and 5-hydrotryptamine-dependent gene activation. *Mol Pharmacol* **47**, 915-22.

Duman R. S., Heninger G. R., and Nestler E. J. (1997) A molecular and cellular theory of depression. *Arch Gen Psychiatry* **54,** 597-606.

Durany N., Michel T., Zochling R., Boissl K. W., Cruz-Sanchez F. F., Riederer P., and Thome J. (2001) Brain-derived neurotrophic factor and neurotrophin 3 in schizophrenic psychoses. *Schizophr Res* **52,** 79-86.

Dwivedi Y., Mondal A. C., Payappagoudar G. V., and Rizavi H. S. (2005) Differential regulation of serotonin (5HT)2A receptor mRNA and protein levels after single and repeated stress in rat brain: role in learned helplessness behavior. *Neuropharmacology* **48,** 204-14.

Eisch A. J., Bolanos C. A., de Wit J., Simonak R. D., Pudiak C. M., Barrot M., Verhaagen J., and Nestler E. J. (2003) Brain-derived neurotrophic factor in the ventral midbrain-nucleus accumbens pathway: a role in depression. *Biol Psychiatry* **54,** 994-1005.

Engvall E., Jonsson K., and Perlmann P. (1971) Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. *Biochim Biophys Acta* **251,** 427-34.

Ernfors P., Lee K. F., and Jaenisch R. (1994) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* **368,** 147-50.

Eysenck H. J. (1975) Manual of the Eysenck Personality Questionnaire. London: Hodder and Staugton.

Farisse J., Boulenguez P., Semont A., Hery F., Barden N., Faudon M., and Hery M. (1999) Regional serotonin metabolism under basal and restraint stress conditions in the brain of transgenic mice with impaired glucocorticoid receptor function. *Neuroendocrinology* **70,** 413-21.

Farisse J., Hery F., Barden N., Hery M., and Boulenguez P. (2000) Central 5-HT(1) and 5-HT(2) binding sites in transgenic mice with reduced glucocorticoid receptor number. *Brain Res* **862,** 145-53.

Farmer A., Eley T. C., and McGuffin P. (2005) Current strategies for investigating the genetic and environmental risk factors for affective disorders. *Br J Psychiatry* **186,** 179-81.

Faure J., Uys J. D., Marais L., Stein D. J., and Daniels W. M. (2007) Early maternal separation alters the response to traumatization: resulting in increased levels of hippocampal neurotrophic factors. *Metab Brain Dis* **22,** 183-95.

Finley M., Fairman D., Liu D., Li P., Wood A., and Cho S. (2004) Functional validation of adult hippocampal organotypic cultures as an in vitro model of brain injury. *Brain Res* **1001,** 125-32.

Frank L., Wiegand S. J., Siuciak J. A., Lindsay R. M., and Rudge J. S. (1997) Effects of BDNF infusion on the regulation of TrkB protein and message in adult rat brain. *Exp Neurol* **145,** 62-70.

Fregeau C. J. and Fourney R. M. (1993) DNA typing with fluorescently tagged short tandem repeats: a sensitive and accurate approach to human identification. *Biotechniques* **15,** 100-19.

Frokjaer V. G., Mortensen E. L., Nielsen F. L., Haugbol S., Pinborg L. H., Adams K. H., Svarer C., Hasselbalch S. G., Holm S., Paulson O. B., and Knudsen G. M. (2007) Frontolimbic serotonin 2A receptor binding in healthy subjects is associated with personality risk factors for affective disorder. *Biol Psychiatry, Accepted.*

Frokjaer V. G., Vinberg M., Baare W., Madsen J., Kessing L. V., and Knudsen G. M. Serotonin 2A receptor binding as measured with [18F]Altanserin PET in healthy twins at high risk of developing mood disorders in comparison with twins at low risk . *In prep.*

Fryer R. H., Kaplan D. R., Feinstein S. C., Radeke M. J., Grayson D. R., and Kromer L. F. (1996) Developmental and mature expression of full-length and truncated TrkB receptors in the rat forebrain. *J Comp Neurol* **374,** 21-40.

Fujimura H., Altar C. A., Chen R., Nakamura T., Nakahashi T., Kambayashi J., Sun B., and

Tandon N. N. (2002) Brain-derived neurotrophic factor is stored in human platelets and released by agonist stimulation. *Thromb Haemost* **87,** 728-34.

Garlow S. J. and Ciaranello R. D. (1995) Transcriptional control of the rat serotonin-2 receptor gene. *Brain Res Mol Brain Res* **31,** 201-9.

Gartside S. E., Leitch M. M., and Young A. H. (2003) Altered glucocorticoid rhythm attenuates the ability of a chronic SSRI to elevate forebrain 5-HT: implications for the treatment of depression. *Neuropsychopharmacology* **28,** 1572-8.

Gatt J. M., Clark C. R., Kemp A. H., Liddell B. J., Dobson-Stone C., Kuan S. A., Schofield P. R., and Williams L. M. (2007) A genotype-endophenotype-phenotype path model of depressed mood: integrating cognitive and emotional markers. *J Integr Neurosci* **6,** 75-104.

Geng Y. G., Su Q. R., Su L. Y., Chen Q., Ren G. Y., Shen S. Q., Yu A. Y., and Xia G. Y. (2007) Comparison of the polymorphisms of androgen receptor gene and estrogen alpha and beta gene between adolescent females with first-onset major depressive disorder and controls. *Int J Neurosci* **117,** 539-47.

Gonul A. S., Akdeniz F., Taneli F., Donat O., Eker C., and Vahip S. (2005) Effect of treatment on serum brain-derived neurotrophic factor levels in depressed patients. *Eur Arch Psychiatry Clin Neurosci* **255,** 381-6.

Gray J. A. and Roth B. L. (2001) Paradoxical trafficking and regulation of 5-HT(2A) receptors by agonists and antagonists. *Brain Res Bull* **56,** 441-51.

Haapasalo A., Koponen E., Hoppe E., Wong G., and Castren E. (2001) Truncated trkB.T1 is dominant negative inhibitor of trkB.TK+-mediated cell survival. *Biochem Biophys Res Commun* **280,** 1352-8.

Han F., Ozawa H., Matsuda K., Nishi M., and Kawata M. (2005) Colocalization of mineralocorticoid receptor and glucocorticoid receptor in the hippocampus and hypothalamus. *Neurosci Res* **51,** 371-81.

Handa R. J., Burgess L. H., Kerr J. E., and O'Keefe J. A. (1994) Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. *Horm Behav* **28,** 464-76.

Hanley N. R. and Hensler J. G. (2002) Mechanisms of ligand-induced desensitization of the 5-hydroxytryptamine(2A) receptor. *J Pharmacol Exp Ther* **300,** 468-77.

Hansson A. C., Cintra A., Belluardo N., Sommer W., Bhatnagar M., Bader M., Ganten D., and Fuxe K. (2000) Gluco- and mineralocorticoid receptor-mediated regulation of neurotrophic factor gene expression in the dorsal hippocampus and the neocortex of the rat. *Eur J Neurosci* **12,** 2918-34.

Heninger G. R., Delgado P. L., and Charney D. S. (1996) The revised monoamine theory of depression: a modulatory role for monoamines, based on new findings from monoamine depletion experiments in humans. *Pharmacopsychiatry* **29,** 2-11.

Herman J. P., Ostrander M. M., Mueller N. K., and Figueiredo H. (2005) Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. *Prog Neuropsychopharmacol Biol Psychiatry* **29,** 1201-13.

Heuser I. J., Schweiger U., Gotthardt U., Schmider J., Lammers C. H., Dettling M.,

Yassouridis A., and Holsboer F. (1996) Pituitary-adrenal-system regulation and psychopathology during amitriptyline treatment in elderly depressed patients and normal comparison subjects. *Am J Psychiatry* **153,** 93-9.

Hirst W. D., Stean T. O., Rogers D. C., Sunter D., Pugh P., Moss S. F., Bromidge S. M., Riley G., Smith D. R., Bartlett S., Heidbreder C. A., Atkins A. R., Lacroix L. P., Dawson L. A., Foley A. G., Regan C. M., and Upton N. (2006) SB-399885 is a potent, selective 5-HT6 receptor antagonist with cognitive enhancing properties in aged rat water maze and novel object recognition models. *Eur J Pharmacol* **553,** 109-19.

Holsboer F., Lauer C. J., Schreiber W., and Krieg J. C. (1995) Altered hypothalamic-pituitary-adrenocortical regulation in healthy subjects at high familial risk for affective disorders. *Neuroendocrinology* **62,** 340-7.

Holsboer-Trachsler E., Stohler R., and Hatzinger M. (1991) Repeated administration of the combined dexamethasone-human corticotropin releasing hormone stimulation test during treatment of depression. *Psychiatry Res* **38,** 163-71.

Hong C. J., Huo S. J., Yen F. C., Tung C. L., Pan G. M., and Tsai S. J. (2003) Association study of a brain-derived neurotrophic-factor genetic polymorphism and mood disorders, age of onset and suicidal behavior. *Neuropsychobiology* **48,** 186-9.

Hull E. M., Muschamp J. W., and Sato S. (2004) Dopamine and serotonin: influences on male sexual behavior. *Physiol Behav* **83,** 291-307.

Ip N. Y., Li Y., Yancopoulos G. D., and Lindsay R. M. (1993) Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. *J Neurosci* **13,** 3394-405.

Jacobsen J. P. and Mork A. (2006) Chronic corticosterone decreases brain-derived neurotrophic factor (BDNF) mRNA and protein in the hippocampus, but not in the frontal cortex, of the rat. *Brain Res* **1110,** 221-5.

Juric D. M., Miklic S., and Carman-Krzan M. (2006) Monoaminergic neuronal activity up-regulates BDNF synthesis in cultured neonatal rat astrocytes. *Brain Res* **1108,** 54-62.

Karege F., Bondolfi G., Gervasoni N., Schwald M., Aubry J. M., and Bertschy G. (2005) Low brain-derived neurotrophic factor (BDNF) levels in serum of depressed patients probably results from lowered platelet BDNF release unrelated to platelet reactivity. *Biol Psychiatry* **57,** 1068-72.

Karege F., Perret G., Bondolfi G., Schwald M., Bertschy G., and Aubry J. M. (2002a) Decreased serum brain-derived neurotrophic factor levels in major depressed patients. *Psychiatry Res* **109,** 143-8.

Karege F., Schwald M., and Cisse M. (2002b) Postnatal developmental profile of brain-derived neurotrophic factor in rat brain and platelets. *Neurosci Lett* **328,** 261-4.

Kaufman J., Yang B. Z., Douglas-Palumberi H., Grasso D., Lipschitz D., Houshyar S., Krystal J. H., and Gelernter J. (2006) Brain-derived neurotrophic factor-5-HTTLPR gene interactions and environmental modifiers of depression in children. *Biol Psychiatry* **59,** 673-80.

Kendler K. S., Gardner C. O., Gatz M., and Pedersen N. L. (2007) The sources of co-morbidity between major depression and generalized anxiety disorder in a Swedish national twin sample. *Psychol Med* **37,** 453-62.

Kendler K. S., Gatz M., Gardner C. O., and Pedersen N. L. (2006) A Swedish national twin study of lifetime major depression. *Am J Psychiatry* **163,** 109-14.

Kendler K. S., Kessler R. C., Walters E. E., MacLean C., Neale M. C., Heath A. C., and Eaves L. J. (1995) Stressful life events, genetic liability, and onset of an episode of major depression in women. *Am J Psychiatry* **152,** 833-42.

Kendler K. S., Kuhn J., and Prescott C. A. (2004) The interrelationship of neuroticism, sex, and stressful life events in the prediction of episodes of major depression. *Am J Psychiatry* **161,** 631-6.

Kohara K., Kitamura A., Morishima M., and Tsumoto T. (2001) Activity-dependent transfer of brain-derived neurotrophic factor to postsynaptic neurons. *Science* **291,** 2419-23.

Kokaia Z., Bengzon J., Metsis M., Kokaia M., Persson H., and Lindvall O. (1993) Coexpression of neurotrophins and their receptors in neurons of the central nervous system. *Proc Natl Acad Sci U S A* **90,** 6711-5.

Koponen E., Lakso M., and Castren E. (2004a) Overexpression of the full-length neurotrophin receptor trkB regulates the expression of plasticity-related genes in mouse brain. *Brain Res Mol Brain Res* **130,** 81-94.

Koponen E., Rantamaki T., Voikar V., Saarelainen T., MacDonald E., and Castren E. (2005) Enhanced BDNF signaling is associated with an antidepressant-like behavioral response and changes in brain monoamines. *Cell Mol Neurobiol* **25,** 973-80.

Koponen E., Voikar V., Riekki R., Saarelainen T., Rauramaa T., Rauvala H., Taira T., and Castren E. (2004b) Transgenic mice overexpressing the full-length neurotrophin receptor trkB exhibit increased activation of the trkB-PLCgamma pathway, reduced anxiety, and facilitated learning. *Mol Cell Neurosci* **26,** 166-81.

Kricka L. J. (1991) Chemiluminescent and bioluminescent techniques. *Clin Chem* **37,** 1472-81.

Lai M., McCormick J. A., Chapman K. E., Kelly P. A., Seckl J. R., and Yau J. L. (2003) Differential regulation of corticosteroid receptors by monoamine neurotransmitters and antidepressant drugs in primary hippocampal culture. *Neuroscience* **118,** 975-84.

Lai M. K., Tsang S. W., Alder J. T., Keene J., Hope T., Esiri M. M., Francis P. T., and Chen C. P. (2005) Loss of serotonin 5-HT2A receptors in the postmortem temporal cortex correlates with rate of cognitive decline in Alzheimer's disease. *Psychopharmacology (Berl)* **179,** 673-7.

Lang U. E., Hellweg R., and Gallinat J. (2004) BDNF serum concentrations in healthy volunteers are associated with depression-related personality traits. *Neuropsychopharmacology* **29,** 795-8.

Laske C., Stransky E., Leyhe T., Eschweiler G. W., Maetzler W., Wittorf A., Soekadar S., Richartz E., Koehler N., Bartels M., Buchkremer G., and Schott K. (2007) BDNF serum and CSF concentrations in Alzheimer's disease, normal pressure hydrocephalus and healthy controls. *J Psychiatr Res* **41,** 387-94.

Lee B. H., Kim H., Park S. H., and Kim Y. K. (2007) Decreased plasma BDNF level in depressive patients. *J Affect Disord* **101,** 239-44.

Lee J., Fukumoto H., Orne J., Klucken J., Raju S., Vanderburg C. R., Irizarry M. C., Hyman B. T., and Ingelsson M. (2005) Decreased levels of BDNF protein in Alzheimer temporal cortex are independent of BDNF polymorphisms. *Exp Neurol* **194,** 91-6.

Lee T. H., Yang J. T., Kato H., Wu J. H., and Chen S. T. (2004) Expression of brain-derived neurotrophic factor immunoreactivity and mRNA in the hippocampal CA1 and cortical areas after chronic ischemia in rats. *J Neurosci Res* **76,** 705-12.

Lesch K. P., Laux G., Schulte H. M., Pfuller H., and Beckmann H. (1988) Corticotropin and cortisol response to human CRH as a probe for HPA system integrity in major depressive disorder. *Psychiatry Res* **24,** 25-34.

Leutgeb J. K., Frey J. U., and Behnisch T. (2003) LTP in cultured hippocampal-entorhinal cortex slices from young adult (P25-30) rats. *J Neurosci Methods* **130,** 19-32.

Leutgeb J. K., Frey J. U., and Behnisch T. (2005) Single cell analysis of activity-dependent cyclic AMP-responsive element-binding protein phosphorylation during long-lasting long-term potentiation in area CA1 of mature rat hippocampal-organotypic cultures. *Neuroscience* **131,** 601-10.

Levinson D. F. (2006) The genetics of depression: a review. *Biol Psychiatry* **60,** 84-92.

Linnarsson S., Bjorklund A., and Ernfors P. (1997) Learning deficit in BDNF mutant mice. *Eur J Neurosci* **9,** 2581-7.

Linthorst A. C., Flachskamm C., Barden N., Holsboer F., and Reul J. M. (2000) Glucocorticoid receptor impairment alters CNS responses to a psychological stressor: an in vivo microdialysis study in transgenic mice. *Eur J Neurosci* **12,** 283-91.

Liotti M. and Mayberg H. S. (2001) The role of functional neuroimaging in the neuropsychology of depression. *J Clin Exp Neuropsychol* **23,** 121-36.

Liy-Salmeron G. and Meneses A. (2007) Role of 5-HT(1-7) receptors in short- and long-term memory for an autoshaping task: Intrahippocampal manipulations. *Brain Res* **1147,** 140-7.

Lommatzsch M., Zingler D., Schuhbaeck K., Schloetcke K., Zingler C., Schuff-Werner P., and Virchow J. C. (2005) The impact of age, weight and gender on BDNF levels in human platelets and plasma. *Neurobiol Aging* **26,** 115-23.

Lopez J. F., Chalmers D. T., Little K. Y., and Watson S. J. (1998) A.E. Bennett Research Award. Regulation of serotonin1A, glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: implications for the neurobiology of depression. *Biol Psychiatry* **43,** 547-73.

Luttgen M., Ove Ogren S., and Meister B. (2004) Chemical identity of 5-HT2A receptor immunoreactive neurons of the rat septal complex and dorsal hippocampus. *Brain Res* **1010,** 156-65.

Lyons W. E., Mamounas L. A., Ricaurte G. A., Coppola V., Reid S. W., Bora S. H., Wihler C., Koliatsos V. E., and Tessarollo L. (1999) Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. *Proc Natl Acad Sci U S A* **96,** 15239-44.

MacQueen G. M., Ramakrishnan K., Croll S. D., Siuciak J. A., Yu G., Young L. T., and Fahnestock M. (2001) Performance of heterozygous brain-derived neurotrophic factor

knockout mice on behavioral analogues of anxiety, nociception, and depression. *Behav Neurosci* **115,** 1145-53.

Maiolini R., Ferrua B., Quaranta J. F., Pinoteau A., Euller L., Ziegler G., and Massaeyeff R. (1978) A sandwich method of enzyme-immunoassay. II. Quantification of rheumatoid factor. *J Immunol Methods* **20,** 25-34.

Mamounas L. A., Altar C. A., Blue M. E., Kaplan D. R., Tessarollo L., and Lyons W. E. (2000) BDNF promotes the regenerative sprouting, but not survival, of injured serotonergic axons in the adult rat brain. *J Neurosci* **20,** 771-82.

Mannie Z. N., Harmer C. J., and Cowen P. J. (2007) Increased waking salivary cortisol levels in young people at familial risk of depression. *Am J Psychiatry* **164,** 617-21.

Marcus S. M., Young E. A., Kerber K. B., Kornstein S., Farabaugh A. H., Mitchell J., Wisniewski S. R., Balasubramani G. K., Trivedi M. H., and Rush A. J. (2005) Gender differences in depression: findings from the STAR*D study. *J Affect Disord* **87,** 141-50.

Marcussen, A. B., Trajkovska, V., Erritzoe, D., Haugbol, S., Madsen, J., Baaré, W., Aznar, S., and Knudsen, G. M. The val66met BDNF polymorphism is associated with decreased cortical 5-HT2A receptor binding. *Submitted.*

Marek G. J., Carpenter L. L., McDougle C. J., and Price L. H. (2003) Synergistic action of 5-HT2A antagonists and selective serotonin reuptake inhibitors in neuropsychiatric disorders. *Neuropsychopharmacology* **28,** 402-12.

McAllister A. K., Katz L. C., and Lo D. C. (1999) Neurotrophins and synaptic plasticity. *Annu Rev Neurosci* **22,** 295-318.

McCann U. D., Szabo Z., Vranesic M., Seckin E., Wand G., Duval A., Dannals R. F., and Ricaurte G. A. (2007) Quantitative positron emission tomography studies of the serotonin transporter in humans previously treated with the appetite suppressants fenfluramine or dexfenfluramine. *Mol Imaging Biol* **9,** 151-7.

McEwen B. S., De Kloet E. R., and Rostene W. (1986) Adrenal steroid receptors and actions in the nervous system. *Physiol Rev* **66,** 1121-88.

McMahon F. J., Buervenich S., Charney D., Lipsky R., Rush A. J., Wilson A. F., Sorant A. J., Papanicolaou G. J., Laje G., Fava M., Trivedi M. H., Wisniewski S. R., and Manji H. (2006) Variation in the gene encoding the serotonin 2A receptor is associated with outcome of antidepressant treatment. *Am J Hum Genet* **78,** 804-14.

Meyer J. H., McMain S., Kennedy S. H., Korman L., Brown G. M., DaSilva J. N., Wilson A. A., Blak T., Eynan-Harvey R., Goulding V. S., Houle S., and Links P. (2003) Dysfunctional attitudes and 5-HT2 receptors during depression and self-harm. *Am J Psychiatry* **160,** 90-9.

Miguel-Hidalgo J. J. and Rajkowska G. (2002) Morphological brain changes in depression: can antidepressants reverse them? *CNS Drugs* **16,** 361-72.

Mintun M. A., Sheline Y. I., Moerlein S. M., Vlassenko A. G., Huang Y., and Snyder A. Z. (2004) Decreased hippocampal 5-HT2A receptor binding in major depressive disorder: in vivo measurement with. *Biol Psychiatry* **55,** 217-24.

Moldow R. L., Beck K. D., Weaver S., and Servatius R. J. (2005) Blockage of glucocorticoid, but not mineralocorticoid receptors prevents the persistent increase in circulating basal

corticosterone concentrations following stress in the rat. *Neurosci Lett* **374,** 25-8.

Monteggia L. M., Luikart B., Barrot M., Theobold D., Malkovska I., Nef S., Parada L. F., and Nestler E. J. (2007) Brain-derived neurotrophic factor conditional knockouts show gender differences in depression-related behaviors. *Biol Psychiatry* **61,** 187-97.

Murray C. G. L. and Lopez A. D. (1997) Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet* **349,** 1498-504.

Nair A., Vadodaria K. C., Banerjee S. B., Benekareddy M., Dias B. G., Duman R. S., and Vaidya V. A. (2007) Stressor-specific regulation of distinct brain-derived neurotrophic factor transcripts and cyclic AMP response element-binding protein expression in the postnatal and adult rat hippocampus. *Neuropsychopharmacology* **32,** 1504-19.

Nakahashi T., Fujimura H., Altar C. A., Li J., Kambayashi J., Tandon N. N., and Sun B. (2000) Vascular endothelial cells synthesize and secrete brain-derived neurotrophic factor. *FEBS Lett* **470,** 113-7.

Nakazato M., Hashimoto K., Shimizu E., Kumakiri C., Koizumi H., Okamura N., Mitsumori M., Komatsu N., and Iyo M. (2003) Decreased levels of serum brain-derived neurotrophic factor in female patients with eating disorders. *Biol Psychiatry* **54,** 485-90.

Nestler E. J., Barrot M., DiLeone R. J., Eisch A. J., Gold S. J., and Monteggia L. M. (2002) Neurobiology of depression. *Neuron* **34**, 13-25.

Ngeh J., Gupta S., and Goodbourn C. (2004) The reproducibility of an enzyme-linked immunosorbent assay for detection of Chlamydia pneumoniae-specific antibodies. *Clin Microbiol Infect* **10,** 171-4.

Nibuya M., Morinobu S., and Duman R. S. (1995) Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *J Neurosci* **15,** 7539-47.

Nitta A., Ohmiya M., Sometani A., Itoh M., Nomoto H., Furukawa Y., and Furukawa S. (1999) Brain-derived neurotrophic factor prevents neuronal cell death induced by corticosterone. *J Neurosci Res* **57,** 227-35.

Noga O., Englmann C., Hanf G., Grutzkau A., Seybold J., and Kunkel G. (2003) The production, storage and release of the neurotrophins nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 by human peripheral eosinophils in allergics and non-allergics. *Clin Exp Allergy* **33,** 649-54.

Novikova L., Novikov L., and Kellerth J. O. (1997) Effects of neurotransplants and BDNF on the survival and regeneration of injured adult spinal motoneurons. *Eur J Neurosci* **9,** 2774-7.

Nygren J., Kokaia M., and Wieloch T. (2006) Decreased expression of brain-derived neurotrophic factor in BDNF(+/-) mice is associated with enhanced recovery of motor performance and increased neuroblast number following experimental stroke. *J Neurosci Res* **84,** 626-31.

O'Toole S. M., Sekula L. K., and Rubin R. T. (1997) Pituitary-adrenal cortical axis measures as predictors of sustained remission in major depression. *Biol Psychiatry* **42,** 85-9.

Palomino A., Vallejo-Illarramendi A., Gonzalez-Pinto A., Aldama A., Gonzalez-Gomez C., Mosquera F., Gonzalez-Garcia G., and Matute C. (2006) Decreased levels of plasma BDNF in

first-episode schizophrenia and bipolar disorder patients. *Schizophr Res* **86,** 321-2.

Pan W., Banks W. A., Fasold M. B., Bluth J., and Kastin A. J. (1998) Transport of brain-derived neurotrophic factor across the blood-brain barrier. *Neuropharmacology* **37,** 1553-61.

Pardridge W. M., Kang Y. S., and Buciak J. L. (1994) Transport of human recombinant brain-derived neurotrophic factor (BDNF) through the rat blood-brain barrier in vivo using vector-mediated peptide drug delivery. *Pharm Res* **11,** 738-46.

Pariante C. M., Makoff A., Lovestone S., Feroli S., Heyden A., Miller A. H., and Kerwin R. W. (2001) Antidepressants enhance glucocorticoid receptor function in vitro by modulating the membrane steroid transporters. *Br J Pharmacol* **134,** 1335-43.

Pariante C. M. and Miller A. H. (2001) Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment. *Biol Psychiatry* **49,** 391-404.

Pariante C. M., Papadopoulos A. S., Poon L., Cleare A. J., Checkley S. A., English J., Kerwin R. W., and Lightman S. (2004) Four days of citalopram increase suppression of cortisol secretion by prednisolone in healthy volunteers. *Psychopharmacology (Berl)* **177,** 200-6.

Pencea V., Bingaman K. D., Wiegand S. J., and Luskin M. B. (2001) Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. *J Neurosci* **21,** 6706-17.

Pepin M. C., Pothier F., and Barden N. (1992) Impaired type II glucocorticoid-receptor function in mice bearing antisense RNA transgene. *Nature* **355,** 725-8.

Pezawas L., Verchinski B. A., Mattay V. S., Callicott J. H., Kolachana B. S., Straub R. E., Egan M. F., Meyer-Lindenberg A., and Weinberger D. R. (2004) The brain-derived neurotrophic factor val66met polymorphism and variation in human cortical morphology. *J Neurosci* **24,** 10099-102.

Rajkowska G. (2000) Postmortem studies in mood disorders indicate altered numbers of neurons and glial cells. *Biol Psychiatry* **48,** 766-77.

Rajkowska G., Miguel-Hidalgo J. J., Wei J., Dilley G., Pittman S. D., Meltzer H. Y., Overholser J. C., Roth B. L., and Stockmeier C. A. (1999) Morphometric evidence for neuronal and glial prefrontal cell pathology in major depression. *Biol Psychiatry* **45,** 1085-98.

Ratka A., Sutanto W., Bloemers M., and de Kloet E. R. (1989) On the role of brain mineralocorticoid (type I) and glucocorticoid (type II) receptors in neuroendocrine regulation. *Neuroendocrinology* **50,** 117-23.

Reichardt H. M., Kaestner K. H., Tuckermann J., Kretz O., Wessely O., Bock R., Gass P., Schmid W., Herrlich P., Angel P., and Schutz G. (1998) DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* **93,** 531-41.

Reichardt H. M., Umland T., Bauer A., Kretz O., and Schutz G. (2000) Mice with an increased glucocorticoid receptor gene dosage show enhanced resistance to stress and endotoxic shock. *Mol Cell Biol* **20,** 9009-17.

Ren-Patterson R. F., Cochran L. W., Holmes A., Sherrill S., Huang S. J., Tolliver T., Lesch K. P., Lu B., and Murphy D. L. (2005) Loss of brain-derived neurotrophic factor gene allele exacerbates brain monoamine deficiencies and increases stress abnormalities of serotonin

transporter knockout mice. *J Neurosci Res* **79,** 756-71.

Ridder S., Chourbaji S., Hellweg R., Urani A., Zacher C., Schmid W., Zink M., Hortnagl H., Flor H., Henn F. A., Schutz G., and Gass P. (2005) Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. *J Neurosci* **25,** 6243-50.

Rios M., Fan G., Fekete C., Kelly J., Bates B., Kuehn R., Lechan R. M., and Jaenisch R. (2001) Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. *Mol Endocrinol* **15,** 1748-57.

Rios M., Lambe E. K., Liu R., Teillon S., Liu J., Akbarian S., Roffler-Tarlov S., Jaenisch R., and Aghajanian G. K. (2006) Severe deficits in 5-HT2A -mediated neurotransmission in BDNF conditional mutant mice. *J Neurobiol* **66,** 408-20.

Rose C. R., Blum R., Pichler B., Lepier A., Kafitz K. W., and Konnerth A. (2003) Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. *Nature* **426,** 74-8.

Rosel P., Arranz B., San L., Vallejo J., Crespo J. M., Urretavizcaya M., and Navarro M. A. (2000) Altered 5-HT(2A) binding sites and second messenger inositol trisphosphate (IP(3)) levels in hippocampus but not in frontal cortex from depressed suicide victims. *Psychiatry Res* **99,** 173-81.

Rosel P., Arranz B., Urretavizcaya M., Oros M., San L., and Navarro M. A. (2004) Altered 5-HT2A and 5-HT4 postsynaptic receptors and their intracellular signalling systems IP3 and cAMP in brains from depressed violent suicide victims. *Neuropsychobiology* **49,** 189-95.

Rossi C., Angelucci A., Costantin L., Braschi C., Mazzantini M., Babbini F., Fabbri M. E., Tessarollo L., Maffei L., Berardi N., and Caleo M. (2006) Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. *Eur J Neurosci* **24,** 1850-6.

Rubinow D. R., Roca C. A., Schmidt P. J., Danaceau M. A., Putnam K., Cizza G., Chrousos G., and Nieman L. (2005) Testosterone suppression of CRH-stimulated cortisol in men. *Neuropsychopharmacology* **30,** 1906-12.

Saarelainen T., Hendolin P., Lucas G., Koponen E., Sairanen M., MacDonald E., Agerman K., Haapasalo A., Nawa H., Aloyz R., Ernfors P., and Castren E. (2003) Activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects. *J Neurosci* **23,** 349-57.

Saylam C., Ucerler H., Kitis O., Ozand E., and Gonul A. S. (2006) Reduced hippocampal volume in drug-free depressed patients. *Surg Radiol Anat* **28,** 82-7.

Scharfman H. E., Mercurio T. C., Goodman J. H., Wilson M. A., and MacLusky N. J. (2003) Hippocampal excitability increases during the estrous cycle in the rat: a potential role for brain-derived neurotrophic factor. *J Neurosci* **23,** 11641-52.

Schenone A., Gill J. S., Zacharias D. A., and Windebank A. J. (1996) Expression of high- and low-affinity neurotrophin receptors on human transformed B lymphocytes. *J Neuroimmunol* **64,** 141-9.

Schiller L., Jahkel M., Kretzschmar M., Brust P., and Oehler J. (2003) Autoradiographic analyses of 5-HT1A and 5-HT2A receptors after social isolation in mice. *Brain Res* **980,** 169-78.

Schinder A. F. and Poo M. (2000) The neurotrophin hypothesis for synaptic plasticity. *Trends Neurosci* **23,** 639-45.

Schuhmann B., Dietrich A., Sel S., Hahn C., Klingenspor M., Lommatzsch M., Gudermann T., Braun A., Renz H., and Nockher W. A. (2005) A role for brain-derived neurotrophic factor in B cell development. *J Neuroimmunol* **163,** 15-23.

Schulte-Herbruggen O., Chourbaji S., Ridder S., Brandwein C., Gass P., Hortnagl H., and Hellweg R. (2006) Stress-resistant mice overexpressing glucocorticoid receptors display enhanced BDNF in the amygdala and hippocampus with unchanged NGF and serotonergic function. *Psychoneuroendocrinology* **31,** 1266-77.

Schulte-Herbruggen O., Hellweg R., Chourbaji S., Ridder S., Brandwein C., Gass P., and Hortnagl H. (2007) Differential regulation of neurotrophins and serotonergic function in mice with genetically reduced glucocorticoid receptor expression. *Exp Neurol* **204,** 307-16.

Schumacher J., Jamra R. A., Becker T., Ohlraun S., Klopp N., Binder E. B., Schulze T. G., Deschner M., Schmal C., Hofels S., Zobel A., Illig T., Propping P., Holsboer F., Rietschel M., Nothen M. M., and Cichon S. (2005) Evidence for a relationship between genetic variants at the brain-derived neurotrophic factor (BDNF) locus and major depression. *Biol Psychiatry* **58,** 307-14.

Schwartz P. J., Wehr T. A., Rosenthal N. E., Bartko J. J., Oren D. A., Luetke C., and Murphy D. L. (1995) Serotonin and thermoregulation. Physiologic and pharmacologic aspects of control revealed by intravenous m-CPP in normal human subjects. *Neuropsychopharmacology* **13,** 105-15.

Seckl J. R. and Fink G. (1992) Antidepressants increase glucocorticoid and mineralocorticoid receptor mRNA expression in rat hippocampus in vivo. *Neuroendocrinology* **55,** 621-6.

Serretti A., Mandelli L., Lorenzi C., Pirovano A., Olgiati P., Colombo C., and Smeraldi E. (2007) Serotonin transporter gene influences the time course of improvement of "core" depressive and somatic anxiety symptoms during treatment with SSRIs for recurrent mood disorders. *Psychiatry Res* **149,** 185-93.

Sharpley A. L., Elliott J. M., Attenburrow M. J., and Cowen P. J. (1994) Slow wave sleep in humans: role of 5-HT2A and 5-HT2C receptors. *Neuropharmacology* **33,** 467-71.

Sheline Y. I., Mintun M. A., Barch D. M., Wilkins C., Snyder A. Z., and Moerlein S. M. (2004) Decreased hippocampal 5-HT(2A) receptor binding in older depressed patients using. *Neuropsychopharmacology* **29,** 2235-41.

Shelton R. C. (2007) The molecular neurobiology of depression. *Psychiatr Clin North Am* **30,** 1-11.

Shelton R. C., Tollefson G. D., Tohen M., Stahl S., Gannon K. S., Jacobs T. G., Buras W. R., Bymaster F. P., Zhang W., Spencer K. A., Feldman P. D., and Meltzer H. Y. (2001) A novel augmentation strategy for treating resistant major depression. *Am J Psychiatry* **158,** 131-4.

Shimizu E., Hashimoto K., Okamura N., Koike K., Komatsu N., Kumakiri C., Nakazato M., Watanabe H., Shinoda N., Okada S., and Iyo M. (2003) Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. *Biol Psychiatry* **54,** 70-5.

Shirayama Y., Chen A. C., Nakagawa S., Russell D. S., and Duman R. S. (2002) Brain-

derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *J Neurosci* **22,** 3251-61.

Siuciak J. A., Clark M. S., Rind H. B., Whittemore S. R., and Russo A. F. (1998) BDNF induction of tryptophan hydroxylase mRNA levels in the rat brain. *J Neurosci Res* **52,** 149-58.

Siuciak J. A., Lewis D. R., Wiegand S. J., and Lindsay R. M. (1997) Antidepressant-like effect of brain-derived neurotrophic factor (BDNF). *Pharmacol Biochem Behav* **56,** 131-7.

Spurlock G., Heils A., Holmans P., Williams J., D'Souza U. M., Cardno A., Murphy K. C., Jones L., Buckland P. R., McGuffin P., Lesch K. P., and Owen M. J. (1998) A family based association study of T102C polymorphism in 5HT2A and schizophrenia plus identification of new polymorphisms in the promoter. *Mol Psychiatry* **3,** 42-9.

Staley J. K., Malison R. T., and Innis R. B. (1998) Imaging of the serotonergic system: interactions of neuroanatomical and functional abnormalities of depression. *Biol Psychiatry* **44,** 534-49.

Steimer T., Python A., Schulz P. E., and Aubry J. M. (2007) Plasma corticosterone, dexamethasone (DEX) suppression and DEX/CRH tests in a rat model of genetic vulnerability to depression. *Psychoneuroendocrinology* **32,** 575-9.

Strauss J., Barr C. L., George C. J., Devlin B., Vetro A., Kiss E., Baji I., King N., Shaikh S., Lanktree M., Kovacs M., and Kennedy J. L. (2005) Brain-derived neurotrophic factor variants are associated with childhood-onset mood disorder: confirmation in a Hungarian sample. *Mol Psychiatry* **10,** 861-7.

Strauss J., Barr C. L., George C. J., King N., Shaikh S., Devlin B., Kovacs M., and Kennedy J. L. (2004) Association study of brain-derived neurotrophic factor in adults with a history of childhood onset mood disorder. *Am J Med Genet B Neuropsychiatr Genet* **131,** 16-9.

Sullivan P. F., Neale M. C., and Kendler K. S. (2000) Genetic epidemiology of major depression: review and meta-analysis. *Am J Psychiatry* **157,** 1552-62.

Toyooka K., Asama K., Watanabe Y., Muratake T., Takahashi M., Someya T., and Nawa H. (2002) Decreased levels of brain-derived neurotrophic factor in serum of chronic schizophrenic patients. *Psychiatry Res* **110,** 249-57.

Tronche F., Kellendonk C., Kretz O., Gass P., Anlag K., Orban P. C., Bock R., Klein R., and Schutz G. (1999) Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet* **23,** 99-103.

Upreti V. V., Mamidi R. N., Katneni K., and Srinivas N. R. (2003) Quantitative determination of DRF-1042 in human plasma by HPLC: validation and application in clinical pharmacokinetics. *Biomed Chromatogr* **17,** 385-90.

Vaidya V. A., Terwilliger R. M., and Duman R. S. (1999) Role of 5-HT2A receptors in the stress-induced down-regulation of brain-derived neurotrophic factor expression in rat hippocampus. *Neurosci Lett* **262,** 1-4.

van Rossum E. F., Binder E. B., Majer M., Koper J. W., Ising M., Modell S., Salyakina D., Lamberts S. W., and Holsboer F. (2006) Polymorphisms of the glucocorticoid receptor gene and major depression. *Biol Psychiatry* **59,** 681-8.

van Rossum E. F., Koper J. W., Huizenga N. A., Uitterlinden A. G., Janssen J. A., Brinkmann

A. O., Grobbee D. E., de Jong F. H., van Duyn C. M., Pols H. A., and Lamberts S. W. (2002) A polymorphism in the glucocorticoid receptor gene, which decreases sensitivity to glucocorticoids in vivo, is associated with low insulin and cholesterol levels. *Diabetes* **51,** 3128-34.

Vinet J., Carra S., Blom J. M., Brunello N., Barden N., and Tascedda F. (2004) Chronic treatment with desipramine and fluoxetine modulate BDNF, CaMKKalpha and CaMKKbeta mRNA levels in the hippocampus of transgenic mice expressing antisense RNA against the glucocorticoid receptor. *Neuropharmacology* **47,** 1062-9.

Vyas A., Mitra R., Shankaranarayana Rao B. S., and Chattarji S. (2002) Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J Neurosci* **22,** 6810-8.

Weber B., Lewicka S., Deuschle M., Colla M., Vecsei P., and Heuser I. (2000) Increased diurnal plasma concentrations of cortisone in depressed patients. *J Clin Endocrinol Metab* **85,** 1133-6.

Webster M. J., Knable M. B., O'Grady J., Orthmann J., and Weickert C. S. (2002a) Regional specificity of brain glucocorticoid receptor mRNA alterations in subjects with schizophrenia and mood disorders. *Mol Psychiatry* **7,** 985-94, 924.

Webster M. J., Weickert C. S., Herman M. M., and Kleinman J. E. (2002b) BDNF mRNA expression during postnatal development, maturation and aging of the human prefrontal cortex. *Brain Res Dev Brain Res* **139,** 139-50.

Weisstaub N. V., Zhou M., Lira A., Lambe E., Gonzalez-Maeso J., Hornung J. P., Sibille E., Underwood M., Itohara S., Dauer W. T., Ansorge M. S., Morelli E., Mann J. J., Toth M., Aghajanian G., Sealfon S. C., Hen R., and Gingrich J. A. (2006) Cortical 5-HT2A receptor signaling modulates anxiety-like behaviors in mice. *Science* **313,** 536-40.

Wetmore C., Cao Y. H., Pettersson R. F., and Olson L. (1991) Brain-derived neurotrophic factor: subcellular compartmentalization and interneuronal transfer as visualized with anti-peptide antibodies. *Proc Natl Acad Sci U S A* **88,** 9843-7.

Wilhelmi E., Schoder U. H., Benabdallah A., Sieg F., Breder J., and Reymann K. G. (2002) Organotypic brain-slice cultures from adult rats: approaches for a prolonged culture time. *Altern Lab Anim* **30,** 275-83.

Williamson D. E., Birmaher B., Frank E., Anderson B. P., Matty M. K., and Kupfer D. J. (1998) Nature of life events and difficulties in depressed adolescents. *J Am Acad Child Adolesc Psychiatry* **37,** 1049-57.

Willins D. L., Deutch A. Y., and Roth B. L. (1997) Serotonin 5-HT2A receptors are expressed on pyramidal cells and interneurons in the rat cortex. *Synapse* **27,** 79-82.

World Health Organisation (2001) The World Health Report 2001. Mental Health: new understanding, new hope.

Xiang Z., Hrabetova S., Moskowitz S. I., Casaccia-Bonnefil P., Young S. R., Nimmrich V. C., Tiedge H., Einheber S., Karnup S., Bianchi R., and Bergold P. J. (2000) Long-term maintenance of mature hippocampal slices in vitro. *J Neurosci Methods* **98,** 145-54.

Yamamoto H. and Gurney M. E. (1990) Human platelets contain brain-derived neurotrophic factor. *J Neurosci* **10,** 3469-78.

Yan Q., Rosenfeld R. D., Matheson C. R., Hawkins N., Lopez O. T., Bennett L., and Welcher A. A. (1997) Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. *Neuroscience* **78,** 431-48.

Yang C. H., Huang C. C., and Hsu K. S. (2004) Behavioral stress modifies hippocampal synaptic plasticity through corticosterone-induced sustained extracellular signal-regulated kinase/mitogen-activated protein kinase activation. *J Neurosci* **24,** 11029-34.

Yang Z. F., Ho D. W., Lau C. K., Tam K. H., Lam C. T., Poon R. T., and Fan S. T. (2006) Platelet activation during tumor development, the potential role of BDNF-TrkB autocrine loop. *Biochem Biophys Res Commun* **346,** 981-5.

Young E. A. (1998) Sex differences and the HPA axis: implications for psychiatric disease. *J Gend Specif Med* **1,** 21-7.

Young E. A., Kornstein S. G., Harvey A. T., Wisniewski S. R., Barkin J., Fava M., Trivedi M. H., and Rush A. J. (2007) Influences of hormone-based contraception on depressive symptoms in premenopausal women with major depression. *Psychoneuroendocrinology*.

Zhou F. M. and Hablitz J. J. (1999) Activation of serotonin receptors modulates synaptic transmission in rat cerebral cortex. *J Neurophysiol* **82,** 2989-99.

Ziegenhorn A. A., Schulte-Herbruggen O., Danker-Hopfe H., Malbranc M., Hartung H. D., Anders D., Lang U. E., Steinhagen-Thiessen E., Schaub R. T., and Hellweg R. (2007) Serum neurotrophins-A study on the time course and influencing factors in a large old age sample. *Neurobiol Aging* **28,** 1436-45.

Zobel A. W., Nickel T., Sonntag A., Uhr M., Holsboer F., and Ising M. (2001) Cortisol response in the combined dexamethasone/CRH test as predictor of relapse in patients with remitted depression. a prospective study. *J Psychiatr Res* **35,** 83-94.

Appendices

I Activation of glucocorticoid receptors increases hippocampal 5-HT_{2A} receptor levels.

II 5-HT_{2A} serotonin receptor levels are associated with brain-derived neurotrophic factor (BDNF) and TrkB.

III Measurements of Brain-derived neurotrophic factor: Methodological aspects and demographical data.

IV Whole blood BDNF levels in healthy twins discordant for affective disorder: Association to life events and neuroticism.

V Declarations of co-authorship

Paper I

Activation of glucocorticoid receptors increases hippocampal 5-HT2A receptor levels

Viktorija Trajkovska[*], Gesa Krey[*], Morten S. Thomsen[*], Sabine Chourbaji†, Christiane Brandwein†, Stephanie Ridder†, Peter Gass†, Gitte M. Knudsen[*], Susana Aznar[*]

[*]*Neurobiology Research Unit and Centre for Integrated Molecular Brain Imaging (CIMBI), Copenhagen University Hospital, Unit 9201, Blegdamsvej 9, 2100 Copenhagen, Denmark.*

†*Central Institute of Mental Health Mannheim, University of Heidelberg, Mannheim, Germany.*

Correspondence:

Viktorija Trajkovska
Neurobiology Research Unit 9201
Rigshospitalet
9 Blegdamsvej
DK- 2100 Copenhagen
Denmark
Phone: +45 3545 6704
Fax: +45 3545 6713
E-mail: viktorija.trajkovska@nru.dk

Abbreviations: 5-HT, 5-hydroxytryptamine, serotonin; 5-HT2A receptor, serotonin receptor 2A; 5-HIAA, 5-hydroxyindoleacetic acid; GR, glucocorticoid receptor; $GR^{+/-}$, heterozygote for GR; HPA, hypothalamic-pituitary adrenal; MR, mineralocorticoid receptor; YGR, overexpressing GR.

Abstract

Major depression is associated with both dysregulation of the hypothalamic pituitary adrenal axis and serotonergic deficiency, not the least of the 5-HT2A serotonin receptor. However, how these phenomena are linked to each other, and whether a low 5-HT2A receptor level is a state or a trait marker of depression is unknown. In adult organotypic hippocampal cultures and mice with altered glucocorticoid receptor (GR) expression we investigated 5-HT2A receptor protein expression. Adult organotypic hippocampal cultures were exposed to corticosterone with or without GR antagonist mifepristone and mineralocorticoid receptor (MR) antagonist spironolactone, and 5-HT2A receptor levels were quantified by Western blot.

In GR underexpressing mice, hippocampal 5-HT2A receptor levels were decreased by 26.3 ± 1.6 % ($p< 0.05$) as compared to wild-type mice. Conversely, in overexpressing GR mice hippocampal 5-HT2A receptor levels were increased by 60.8 ± 4.0 % ($p= 0.0001$) as compared to wild-type mice. Long-term exposure of 3 μM corticosterone to organotypic hippocampal cultures increased 5-HT2A receptor levels by 91.2 ± 28.7 % ($p< 0.05$). The corticosterone-induced 5-HT2A receptor up-regulation was blocked by addition of either spironolactone or mifepristone.

In conclusion, corticosterone-stimulated activation of GR increases hippocampal 5-HT2A receptor levels directly, and conversely, a decreased GR stimulation is associated with a decrease in 5-HT2A receptor levels.

Key words: corticosterone, spironolactone, mifepristone, adult organotypic culture, glucocorticoid receptor transgenic mice.

Introduction

Clinical studies have suggested that depressive episodes are triggered by the combined effects of a genetic predisposition and exposure to stress (Kendler *et al*. 1995; Jacobs *et al*. 2006). A large proportion of patients with severe depressive episodes display hyperactivity of the hypothalamic pituitary adrenal (HPA) axis (Weber *et al*. 2000; Holsboer-Trachsler *et al*. 1991; Lesch *et al*. 1988) which may result from an impaired feedback regulation due to altered glucocorticoid receptor (GR) (Barden 2004) or mineralocorticoid receptor (MR) (Heuser *et al*. 2000; Young *et al*. 2003) expression or function. Some studies have also suggested that an imbalance between GR and MR (Lopez *et al*. 1998; Young *et al*. 2003; Holsboer-Trachsler *et al*. 1991) leads to an overactive HPA axis. A recent clinical study has provided evidence that two specific polymorphisms of the GR gene are associated with an increased risk of developing depression (van Rossum *et al*. 2006). Also, when exposed to stress, mice with pre-existing decreased GR function display depressive-like behaviour (Ridder *et al*. 2005; Boyle *et al.* 2005), suggesting that impaired GR signalling may lead to increased vulnerability to stress-induced depression.

Repeated stress leads to decreased hippocampal serotonin receptor 2A (5-HT2A) levels (Schiller *et al*. 2003; Dwivedi *et al*. 2005). It is currently unclear how this regulation takes place, but the 5-HT2A receptor is clearly of importance since it is specifically implicated in anxiety modulation (Weisstaub *et al*. 2006), in the treatment response to antidepressants (McMahon *et al*. 2006), and depressed patient show lower hippocampal 5-HT2A receptor binding (Sheline *et al*. 2004; Rosel *et al*. 2004; Mintun *et al*. 2004; Rosel *et al*. 2000).

In this study we investigated how changes in glucocorticoid signalling evoke alterations in 5-HT2A receptor levels in the hippocampus, a key structure for both emotional behaviour as well as control area of the HPA axis. Mice under-expressing GR display a depression-like phenotype upon stress, while GR over-expressing mice are stress resistant and do not reveal depression-like features (Ridder *et al*. 2005). In these models we investigated how a genetic predisposition to depression is accompanied with changes in hippocampal 5-HT2A receptor levels. To verify that a long-term activation of GR by corticosterone directly relates to 5-HT2A receptor levels, adult organotypic hippocampal cultures were subjected to different concentrations of corticosterone, with or without specific blockers of GR and MR. Adult organotypic hippocampal cultures are isolated from the serotonergic projections from raphe nuclei and thus allows for studies of assessment of 5-HT2A receptor changes unrelated to corticosterone-induced alterations in 5-HT levels. Furthermore, by using adult organotypic cultures as opposed to using cultures from premature animals, it is possible to directly correlate the results from the cultures studies with those from the GR under- and over-expressing mice.

Materials and methods

Mutant mice with altered GR expression

In accordance with the recommendations of the Banbury conference (1997) all mice used in our experiments were F1 hybrids from two commonly used inbred strains, C57BL/6N and FVB/N, as described previously (Ridder *et al*. 2005). This results in a so-called hybrid vigour that can reduce the impact of genetic alterations compared to a mixed or inbred genetic background (Wolfer *et al*. 2002). On the other hand, the detection of phenotypic alterations or pathologies in mice on a hybrid background

implies that the consequences of the underlying mutations are robust and reliable. $GR^{+/-}$ mice were generated by crossing heterozygous C57BL/6N males (backcrossed with the mutation for >10 generations) with wild-type FVB/N females. For all experiments, male $GR^{+/-}$ mice and their male wild-type littermates were used. YGR mice that carry two additional copies of the GR were generated by a transgenic approach using a yeast artificial chromosome (Reichardt *et al*. 2000). YGR mice were also bred as F1 hybrids from C57BL/6N and FVB/N (Ridder *et al*. 2005). Animals were supplied with food and water *ad libitum* and were kept single caged on a 12h:12h dark-light cycle (lights on at 6:00 in the morning), similarly as for the behavioural and biochemical studies described elsewhere (Ridder *et al*. 2005; Schulte-Herbruggen *et al*. 2006a). For the experiments described here, five-months-old GR and seven-months-old YGR male mice were decapitated and the hippocampi dissected out, frozen on dry ice and stored in –80 °C until further processing. German animal welfare authorities (Regierungspräsidium Karlsruhe) had approved all animal experiments.

Animals for culture

Sprague Dawley rat pups were purchased from Charles Rives, Germany and housed under standard conditions. After arrival they were allowed to acclimatise for at least 5 days. The rats were treated in compliance with the European Communities Council Resolves of 24th November 1986 (86/609/ECC) and the study was approved by Danish State Research Inspectorate (J No 2002/561-527).

Adult organotypic hippocampal culture

Adult organotypic hippocampal cultures were prepared from 20-30 day-old Sprague Dawley rat pups (Xiang *et al*. 2000), as adult organotypic hippocampal cultures from

mouse pups are not established in our laboratory. The pups were decapitated and the brains quickly taken out under sterile conditions. The hippocampi were dissected in ice-cold, pre-aerated (95% O_2, 5% CO_2) Gey´s Dissection Buffer (1.5 mM $CaCl_2$, 5.0 mM KCl, 0.2 mM KH_2PO_4, 10 mM $MgCl_2$, 0.12 mM $MgSO_4$, 0.12 M NaCl, 27 mM $NaHCO_3$, 0.7 mM Na_2HPO_4, 32 mM D-glucose, 24 mM HEPES), and cut transversely into 400 μm-thick slices. Intact and undamaged slices were selected under microscope and cultured on membrane inserts (Millipore, Bredford, USA) at 5% CO_2 atmosphere at 32°C, and in culture medium containing a high concentration of potassium (50% basal medium Eagle, 25% inactivated horse serum, 25% Earle´s basal salt solution, 25mM HEPES (pH 7.2), 27 mM D-glucose, 1 mM L-glutamine, all from Invitrogen, Denmark). After two days they were cultured in a medium with physiological concentrations of potassium (50% basal medium Eagle, 25% inactivated horse serum, 25% home made solution (2.2 mM $MgSO_4$, 0.96 mM Na_2HPO_4, 34 mM $NaHCO_3$, 57 mM NaCl, 1.8 mM $CaCl_2$), 25mM HEPES (pH 7.2), 27 mM D-glucose, 1 mM L-glutamine).

Exposure of organotypic hippocampal slice cultures

After one week in culture the hippocampal brain slices were exposed to three different concentrations of corticosterone (0.1, 0.9, 3μM, Sigma Aldrich, St Louise, USA) for one or seven days. Corticosterone was dissolved in 70% ethanol and diluted in culture media (50% basal medium Eagle, 10% inactivated horse serum, 40% Homemade Solution, 25mM HEPES (pH 7.2), 27 mM D-glucose, 1 mM L-glutamine). Control cultures and cultures stimulated with corticosterone were subjected to ethanol concentration below 0.0175%. Addition of corticosterone to the medium every second day was needed in order to maintain a constant concentration. The corticosterone

concentration in the medium was monitored and measured with ELISA (data not shown here).

In another experimental set up, cultures were exposed to 3 μM corticosterone alone or in combination with GR antagonist mifepristone (RU-38486, 1, 3, 6 μM Sigma Aldrich) or MR antagonist spironolactone (1, 3, 6 μM, Sigma Aldrich). Mifepristone (6 μM) and spironolactone (6 μM) were also added to cultures alone. Control cultures and cultures stimulated with spironolactone or mifepristone were at no time subjected to ethanol concentrations over 0.0175%. At the end of each experimental set up the hippocampal slice cultures were frozen in liquid nitrogen and kept in -80°C until further processing.

Western blot analysis

Adult organotypic hippocampal slices cultures and hippocampus from mice with altered GR expression mice were homogenized in ice-cold homogenate buffer containing a cocktail of protease inhibitors (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 70 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 0.05 mM aprotinin, 1 mM leupeptin, 2 mM bestatin, 1 mM pepestatin A, 1 mM E-64 (Sigma, Saint Louis, Missouri, USA)), and centrifuged at 13,000 g, 20 minutes, 4 °C. The pellet was dissolved in 2x SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulphate (SDS), 20% glycerol, 12% 2-mercaptoethanol) and the protein concentration measured with modified Lowry method (Hartree 1972)(DC Protein Assay, BioRad Laboratories, Herlev, Denmark). Protein-matched samples were heated for 5 min, 100 °C and run on 6 % SDS-polyacrylamide gel (60 minutes, 150 V, 4 °C). The proteins were electro blotted (350 mA, 1 h, and 4 °C) on to a nitro-cellulose membrane, and the membrane was blocked for unspecific binding with 5% bovine serum albumin or 5%

non-fat dry milk (1 h, RT). Incubation with anti-5-HT2A receptor (1:1000, BD Bioscience, Brøndby, Denmark), anti-stress-activated protein kinase/Jun-N-terminal kinase (SAPK/JNK; 1:2000, Cell Signaling, Danvers, MA, USA), anti phospho-SAPK/JNK (1:1000) or cleaved caspase-3 (1:1000, Cell Signaling) was conducted over night at 4 °C. Protein band detection was done by 1 h incubation with horseradish peroxidase-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark) and chemiluminescence detection reagent (Amersham Bioscience, Buckinghamshire, UK) on hyperfilm (Amersham Bioscience, Buckinghamshire, UK). The membrane was stripped to remove the antibodies (62.5 mM Tris-HCl (pH 6.7), 2% sodium dodecyl sulphate, 0.7% 2-mercaptoethanol, 30 min, 55 °C) and re-incubated with rabbit anti-β-actin (1:5000, Sigma). The optical density of the protein band was quantified with Scion Image (Scion Corporation, www.scioncorp.com). The 5-HT2A receptor optical density was expressed as percentage of the corresponding β-actin protein band.

Statistics

For group comparison, two-tailed unpaired t-test and one-way ANOVA was used. Kolmogorov-Smirnov´s normality test was used to investigate normal distribution. In case of the absence of a normal distribution, the data was ln-transformed to accommodate to a normal distribution or a non-parametric test was used. All data are presented as means ± SEM. Significance level was set at $p < 0.05$ (two-sided).

Results

5-HT2A receptor receptor densities in GR transgenic mice

Western blot analysis on hippocampal protein extracts showed a 26.3 ± 1.6 % reduction of 5-HT2A receptor protein content in $GR^{+/-}$ mice (Fig. 1a, n= 4-5, t= 2.527,

p=0.039) compared to wild-type littermates. The YGR mice, on the other hand, demonstrated a 60.8 ± 4.0 % increase (Fig. 1b, n=5, t= 7.610, p=0.0001) in 5-HT2A receptor levels in comparison to their wild-type controls.

5-HT2A receptor densities in organotypic hippocampal cultures exposed to corticosterone

Organotypic hippocampal cultures exposed to corticosterone (0.1, 0.9, or 3 μM) for one day did not show any changes in 5-HT2A receptor protein content (n= 7-8). However, after seven days exposure to 3 μM corticosterone, 5-HT2A receptor receptor levels were significantly increased by 91.2 ± 28.7 % as compared to the control condition (Fig. 2, n= 8, t= 2.67, p= 0.019).

Blockage of GR with mifepristone

In a new series of experiments corticosterone was added to the organotypic cultures for a period of seven days with or without mifepristone, a commonly used blocker of GR. Addition of 3 μM corticosterone again led to a significant increase in 5-HT2A receptor levels (Fig. 3, n= 7-8, t= 0.027, p= 0.027); the increase was blocked by addition of mifepristone (at all doses, 1, 3 and 6 μM). One-way ANOVA showed a tendency (p= 0.078) towards 6 μM mifepristone significantly blocking the increase in 5-HT2A receptor levels compared to cultures treated with 3 μM corticosterone. This tendency is significant when applying a t-test (n= 8, t= 2.473, p= 0.04). Mifepristone (6 μM) alone did not influence 5-HT2A receptor levels (n=8).

There was no apoptosis in organotypic cultures treated with corticosterone or mifepristone, as shown by analysis of pSAPK/JNK and cleaved caspase -3 (Fig. 4).

Blockage of MR with spironolactone

In a third series of culture experiments we investigated the role of the MR in the 5-HT2A receptor corticosterone response. As before, addition of 3 μM corticosterone to the organotypic cultures resulted in the expected increase of 5-HT2A receptor levels (Fig. 5, n= 7, sum of ranks 32, 73, p=0.007). One-way ANOVA showed that addition of 3 μM spironolactone in combination with 3 μM corticosterone significantly decreased 5-HT2A receptor levels when compared with the cultures treated with corticosterone only (Fig. 5, n= 7-8, F= 3.721, p= 0.043). Spironolactone (6 μM) alone had no effect on 5-HT2A receptor levels (n= 8).

There was no apoptosis in organotypic cultures treated with corticosterone or spironolactone, as shown by analysis of pSAPK/JNK and cleaved caspase -3.

Discussion

Here we demonstrate that congenital under- or over-expression of GR causes characteristic alterations of 5-HT2A receptor protein levels in the adult mouse hippocampus. Specifically, we are the first to show that GR over-expressing YGR mice display a significant increase in hippocampal 5-HT2A receptor levels, while GR+/- mice display significantly lower levels. The GR+/- mice display increased helplessness after a stress exposure (Ridder *et al*. 2005) and they also have the lowest hippocampal 5-HT2A receptor levels. Induced helplessness is considered a manifestation of depressive behaviour in rodents (Henn and Vollmayr 2005). By contrast, mice over-expressing GR are less helpless in the same stress paradigm (Ridder *et al*. 2005) and they also had the highest 5-HT2A receptor levels. Interestingly, low frontal cortex 5-HT2A receptor binding has in humans been

associated to the personality trait hopelessness, which is associated with a higher vulnerability to depression (van Heeringen *et al.* 2003). Our observations suggest that low hippocampal 5-HT2A receptor levels potentially constitute an endogenous manifestation of a vulnerability to depression rather than being a consequence of it. Future studies must, however, reveal whether decreased hippocampal 5-HT2A receptor levels are associated with susceptibility to depressive-like behaviour.

Currently, there is some evidence for a genetic component in the involvement of the HPA-axis in mood disorders. Impaired feedback regulation and mild hypercortisolism pre-exist in healthy subjects at genetic risk for depression (Holsboer *et al*. 1995; Zobel *et al*. 2004) and polymorphisms of the GR gene (van Rossum *et al*. 2006; van West *et al*. 2006) are associated with a vulnerability to depression. This suggests that an imbalance in the HPA axis may precede the manifestation of clinical symptoms. In this study we show that altered corticosterone levels, the net outcome of imbalances in the HPA axis, have a direct regulatory role on hippocampal 5-HT2A receptor levels. Also, a positive association between GR expression and 5-HT2A receptor levels support that it is the total GR signaling that regulates 5-HT2A receptor levels. Interestingly, carriers of the Blc1 GR polymorphism respond poorly to antidepressants (Brouwer *et al*. 2006), and we now suggest that this may be due to lower hippocampal 5-HT2A receptor levels in this group of non-responders. This notion is supported by the identification of a connection between antidepressant efficacy and 5-HT2A receptor functionality (Choi *et al*. 2005; McMahon *et al*. 2006). In *in vivo* imaging studies, cortical 5-HT2A receptor binding has both been reported to be increased (Bhagwagar *et al*. 2006) or decreased (Messa *et al.* 2003) in depression. For hippocampus, however, there is converging evidence that 5-HT2A receptor binding is

decreased in depressed subjects (Sheline *et al*. 2004; Rosel *et al*. 2004; Mintun *et al*. 2004; Rosel *et al*. 2000).

Repeated stress in rats is associated with a decrease in hippocampal 5-HT2A receptor levels (Dwivedi *et al.* 2005; Schiller *et al*. 2003)**,** but it is not known to what degree such a stress-associated down-regulation of 5-HT2A receptor is mediated through the HPA axis. We here show both in cultures and in transgenic mice with an incorporated additional copy of the GR gene, that increased GR and MR signaling up-regulates hippocampal 5-HT2A receptor levels. Conversely, in $GR^{+/-}$ mice with decreased GR levels the opposite effect is seen on hippocampal 5-HT2A receptor levels. The GR transgenic mice in our study had normal basal corticosterone concentrations (Ridder *et al*. 2005) supporting the notion that the observed effects on 5-HT2A receptor levels are related to altered GR levels and not to changes in corticosterone levels. Repeated stress decreases hippocampal GR mRNA and protein levels (Kim *et al*. 1999; Dronjak *et al*. 2004), and GR levels remain decreased also after normalisation of corticosterone levels (Sebaai *et al*. 2002; Paskitti *et al*. 2000). Based on our results the decreases in hippocampal 5-HT2A receptor levels after long-term stress in the study of Dwivedi *et al*. 2005 might be due to a net decrease in GR transmission. In our hippocampal culture the increase of 5-HT2A receptor by addition of corticosterone is the result of long-term occupancy of hippocampal GR. Even though long-term exposure to high corticosterone levels may have an effect on hippocampal GR levels in our cultures, the net result of sustained high corticosterone concentrations is an increased GR signalling. Furthermore, an increased GR and/or MR agonism, as a regulatory mechanism for 5-HT2A receptor levels, is in line with what has been suggested before (Garlow and Ciaranello 1995).

In the mice genetically modified in terms of GR expression, some effect on the MR density is also likely to occur, at least according to Farisse and co-workers that observed a 20-30 % decrease in MR density in GR deficient mice (Farisse *et al*. 2000). It is known that a balanced activation of GR and MR is important to maintain homeostasis and to protect against stress-related brain disorders (De Kloet *et al*. 1998). Accordingly, the ratio between GR and MR activation may be important for determining 5-HT2A receptor levels. When activated, GR and MR are translocated from the cytoplasm to the nucleus where they influence gene transcription (De Kloet *et al*. 1998). Heterodimers of GR/MR are suggested to regulate the transcription of the 5-HT1A gene (Ou *et al*. 2001). Only after seven days exposure to corticosterone an increase in hippocampal culture 5-HT2A receptor levels was seen. This supports that the GR and MR mediated increase in 5-HT2A receptor density takes place at the transcriptional level. Reduced hippocampal 5-HT2A receptor mRNA expression in the CA3 and hilus of mice with reduced GR expression has been earlier found (Cyr *et al*. 2001).

The change in 5-HT2A receptor receptor density in the GR transgenic mice might also be mediated through changes in serotonin levels. It has been reported that corticosterone increases extracellular 5-HT (Gartside *et al*. 2003). It has, however, been consistently shown that mice genetically modified to express GR differently have normal baseline extracellular and total 5-HT and 5-HIAA levels (Farisse *et al*. 1999; Linthorst *et al*. 2000; Schulte-Herbruggen *et al.* 2006a; Schulte-Herbruggen *et al.* 2006b). Changes in 5-HT and 5-HIAA only seem to occur in mice with altered GR expression after exposure to stress (Farisse *et al.* 1999; Linthorst *et al*. 2000) and accordingly, corticosterone levels increase above basal levels only when GR modified

mice are subjected to stress (Ridder *et al.* 2005). The mice included in our study had not previously been exposed to any stress paradigm, and when housed under standard conditions they do not display any changes in corticosterone levels (Ridder *et al*. 2005). Again, the advantage of the organotypic hippocampal cultures is that it is entirely denervated from serotonergic projections. Our results point thus to a direct effect of GR and MR activation in regulating 5-HT2A receptor levels.

In conclusion, an increased GR expression increases hippocampal 5-HT2A receptor protein levels directly. Conversely, a decreased GR expression leads to a down-regulation of hippocampal 5-HT2A receptor protein levels. The impact of corticosterone on 5-HT2A receptor receptor levels takes place independently of 5-HT levels and can be reverted by both GR and MR blockade.

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References

Banbury Conference (1997) Mutant mice and neuroscience: recommendations concerning genetic background. Banbury Conference on genetic background in mice. *Neuron* **19,** 755-759.

Barden N. (2004) Implication of the hypothalamic-pituitary-adrenal axis in the physiopathology of depression. *J. Psychiatry Neurosci.* **29,** 185-193.

Bhagwagar Z., Hinz R., Taylor M., Fancy S., Cowen P., and Grasby P. (2006) Increased 5-HT(2A) receptor binding in euthymic, medication-free patients recovered from depression: a positron emission study with. *Am. J. Psychiatry* **163,** 1580-1587.

Boyle M. P., Brewer J. A., Funatsu M., Wozniak D. F., Tsien J. Z., Izumi Y., and Muglia L. J. (2005) Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. *Proc. Natl. Acad. Sci. U S A* **102,** 473-478.

Brouwer J. P., Appelhof B. C., van Rossum E. F., Koper J. W., Fliers E., Huyser J., Schene A. H., Tijssen J. G., Van Dyck R., Lamberts S. W., Wiersinga W. M., and Hoogendijk W. J. (2006) Prediction of treatment response by HPA-axis and glucocorticoid receptor polymorphisms in major depression. *Psychoneuroendocrinology* **31,** 1154-1163.

Choi M. J., Kang R. H., Ham B. J., Jeong H. Y., and Lee M. S. (2005) Serotonin receptor 2A gene polymorphism (-1438A/G) and short-term treatment response to citalopram. *Neuropsychobiology* **52,** 155-162.

Cyr M., Charbonneau C., Morissette M., Rochford J., Barden N., and Di Paolo T.

(2001) Central 5-hydroxytryptamine-2A receptor expression in transgenic mice bearing a glucocorticoid receptor antisense. *Neuroendocrinology* **73,** 37-45.

De Kloet E. R., Vreugdenhil E., Oitzl M. S., and Joels M. (1998) Brain corticosteroid receptor balance in health and disease. *Endocr. Rev.* **19,** 269-301.

Dronjak S., Gavrilovic L., Filipovic D., and Radojcic M. B. (2004) Immobilization and cold stress affect sympatho-adrenomedullary system and pituitary-adrenocortical axis of rats exposed to long-term isolation and crowding. *Physiol. Behav.* **81,** 409-415.

Dwivedi Y., Mondal A. C., Payappagoudar G. V., and Rizavi H. S. (2005) Differential regulation of serotonin (5HT)2A receptor mRNA and protein levels after single and repeated stress in rat brain: role in learned helplessness behavior. *Neuropharmacology* **48,** 204-214.

Farisse J., Boulenguez P., Semont A., Hery F., Barden N., Faudon M., and Hery M. (1999) Regional serotonin metabolism under basal and restraint stress conditions in the brain of transgenic mice with impaired glucocorticoid receptor function. *Neuroendocrinology* **70,** 413-421.

Farisse J., Hery F., Barden N., Hery M., and Boulenguez P. (2000) Central 5-HT(1) and 5-HT(2) binding sites in transgenic mice with reduced glucocorticoid receptor number. *Brain Res.* **862,** 145-153.

Garlow S. J. and Ciaranello R. D. (1995) Transcriptional control of the rat serotonin-2 receptor gene. *Brain Res. Mol. Brain Res.* **31,** 201-209.

Gartside S. E., Leitch M. M., and Young A. H. (2003) Altered glucocorticoid rhythm attenuates the ability of a chronic SSRI to elevate forebrain 5-HT: implications for the

treatment of depression. *Neuropsychopharmacology* **28,** 1572-1578.

Hartree E. F. (1972) Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* **48,** 422-427.

Henn F. A. and Vollmayr B. (2005) Stress models of depression: forming genetically vulnerable strains. *Neurosci. Biobehav. Rev.* **29,** 799-804.

Heuser I., Deuschle M., Weber B., Stalla G. K., and Holsboer F. (2000) Increased activity of the hypothalamus-pituitary-adrenal system after treatment with the mineralocorticoid receptor antagonist spironolactone. *Psychoneuroendocrinology* **25,** 513-518.

Holsboer F., Lauer C. J., Schreiber W., and Krieg J. C. (1995) Altered hypothalamic-pituitary-adrenocortical regulation in healthy subjects at high familial risk for affective disorders. *Neuroendocrinology* **62,** 340-347.

Holsboer-Trachsler E., Stohler R., and Hatzinger M. (1991) Repeated administration of the combined dexamethasone-human corticotropin releasing hormone stimulation test during treatment of depression. *Psychiatry Res.* **38,** 163-171.

Jacobs N., Kenis G., Peeters F., Derom C., Vlietinck R., and van Os J. (2006) Stress-related negative affectivity and genetically altered serotonin transporter function: evidence of synergism in shaping risk of depression. *Arch. Gen. Psychiatry* **63,** 989-996.

Kendler K. S., Kessler R. C., Walters E. E., MacLean C., Neale M. C., Heath A. C., and Eaves L. J. (1995) Stressful life events, genetic liability, and onset of an episode of major depression in women. *Am. J. Psychiatry* **152,** 833-842.

Kim C. K., Yu W., Edin G., Ellis L., Osborn J. A., and Weinberg J. (1999) Chronic intermittent stress does not differentially alter brain corticosteroid receptor densities in rats prenatally exposed to ethanol. *Psychoneuroendocrinology* **24,** 585-611.

Lesch K. P., Laux G., Schulte H. M., Pfuller H., and Beckmann H. (1988) Corticotropin and cortisol response to human CRH as a probe for HPA system integrity in major depressive disorder. *Psychiatry Res.* **24,** 25-34.

Linthorst A. C., Flachskamm C., Barden N., Holsboer F., and Reul J. M. (2000) Glucocorticoid receptor impairment alters CNS responses to a psychological stressor: an in vivo microdialysis study in transgenic mice. *Eur. J. Neurosci.* **12,** 283-291.

Lopez J. F., Chalmers D. T., Little K. Y., and Watson S. J. (1998) A.E. Bennett Research Award. Regulation of serotonin1A, glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: implications for the neurobiology of depression. *Biol. Psychiatry* **43,** 547-573.

McMahon F. J., Buervenich S., Charney D., Lipsky R., Rush A. J., Wilson A. F., Sorant A. J., Papanicolaou G. J., Laje G., Fava M., Trivedi M. H., Wisniewski S. R., and Manji H. (2006) Variation in the gene encoding the serotonin 2A receptor is associated with outcome of antidepressant treatment. *Am. J. Hum. Genet.* **78,** 804-814.

Messa C., Colombo C., Moresco R. M., Gobbo C., Galli L., Lucignani G., Gilardi M. C., Rizzo G., Smeraldi E., Zanardi R., Artigas F., and Fazio F. (2003) 5-HT(2A) receptor binding is reduced in drug-naive and unchanged in SSRI-responder depressed patients compared to healthy controls: a PET study. *Psychopharmacology (Berl)* **167,** 72-78.

Mintun M. A., Sheline Y. I., Moerlein S. M., Vlassenko A. G., Huang Y., and Snyder

A. Z. (2004) Decreased hippocampal 5-HT2A receptor receptor binding in major depressive disorder: in vivo measurement with. *Biol. Psychiatry* **55,** 217-224.

Ou X. M., Storring J. M., Kushwaha N., and Albert P. R. (2001) Heterodimerization of mineralocorticoid and glucocorticoid receptors at a novel negative response element of the 5-HT1A receptor gene. *J. Biol. Chem.* **276,** 14299-14307.

Paskitti M. E., McCreary B. J., and Herman J. P. (2000) Stress regulation of adrenocorticosteroid receptor gene transcription and mRNA expression in rat hippocampus: time-course analysis. *Brain Res. Mol. Brain Res.* **80,** 142-152.

Reichardt H. M., Umland T., Bauer A., Kretz O., and Schutz G. (2000) Mice with an increased glucocorticoid receptor gene dosage show enhanced resistance to stress and endotoxic shock. *Mol. Cell Biol.* **20,** 9009-9017.

Ridder S., Chourbaji S., Hellweg R., Urani A., Zacher C., Schmid W., Zink M., Hortnagl H., Flor H., Henn F. A., Schutz G., and Gass P. (2005) Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. *J. Neurosci.* **25,** 6243-6250.

Rosel P., Arranz B., San L., Vallejo J., Crespo J. M., Urretavizcaya M., and Navarro M. A. (2000) Altered 5-HT(2A) binding sites and second messenger inositol trisphosphate (IP(3)) levels in hippocampus but not in frontal cortex from depressed suicide victims. *Psychiatry Res.* **99,** 173-181.

Rosel P., Arranz B., Urretavizcaya M., Oros M., San L., and Navarro M. A. (2004) Altered 5-HT2A receptor and 5-HT4 postsynaptic receptors and their intracellular signalling systems IP3 and cAMP in brains from depressed violent suicide victims. *Neuropsychobiology* **49,** 189-195.

Schiller L., Jahkel M., Kretzschmar M., Brust P., and Oehler J. (2003) Autoradiographic analyses of 5-HT1A and 5-HT2A receptor receptors after social isolation in mice. *Brain Res.* **980,** 169-178.

Schulte-Herbruggen O., Chourbaji S., Ridder S., Brandwein C., Gass P., Hortnagl H., and Hellweg R. (2006a) Stress-resistant mice overexpressing glucocorticoid receptors display enhanced BDNF in the amygdala and hippocampus with unchanged NGF and serotonergic function. *Psychoneuroendocrinology* **31,** 1266-1277.

Schulte-Herbruggen, O., Hellweg, R., Chourbaji, S., Ridder, S., Brandwein, C., Gass, P. & Hortnagl, H. (2006b) Differential regulation of neurotrophins and serotonergic function in mice with genetically reduced glucocorticoid receptor expression. *Exp. Neurol., in press.*

Sebaai N., Lesage J., Vieau D., Alaoui A., Dupouy J. P., and Deloof S. (2002) Altered control of the hypothalamo-pituitary-adrenal axis in adult male rats exposed perinatally to food deprivation and/or dehydration. *Neuroendocrinology* **76,** 243-253.

Sheline Y. I., Mintun M. A., Barch D. M., Wilkins C., Snyder A. Z., and Moerlein S. M. (2004) Decreased hippocampal 5-HT(2A) receptor binding in older depressed patients using. *Neuropsychopharmacology* **29,** 2235-2241.

van Heeringen C., Audenaert K., Van Laere K., Dumont F., Slegers G., Mertens J., and Dierckx R. A. (2003) Prefrontal 5-HT2A receptor receptor binding index, hopelessness and personality characteristics in attempted suicide. *J. Affect. Disord.* **74,** 149-158.

van Rossum E. F., Binder E. B., Majer M., Koper J. W., Ising M., Modell S.,

Salyakina D., Lamberts S. W., and Holsboer F. (2006) Polymorphisms of the glucocorticoid receptor gene and major depression. *Biol. Psychiatry* **59,** 681-688.

van West D., Van Den Eede F., Del-Favero J., Souery D., Norrback K. F., Van Duijn C., Sluijs S., Adolfsson R., Mendlewicz J., Deboutte D., Van Broeckhoven C., and Claes S. (2006) Glucocorticoid receptor gene-based SNP analysis in patients with recurrent major depression. *Neuropsychopharmacology* **31,** 620-627.

Weber B., Lewicka S., Deuschle M., Colla M., Vecsei P., and Heuser I. (2000) Increased diurnal plasma concentrations of cortisone in depressed patients. *J. Clin. Endocrinol. Metab.* **85,** 1133-1136.

Weisstaub N. V., Zhou M., Lira A., Lambe E., Gonzalez-Maeso J., Hornung J. P., Sibille E., Underwood M., Itohara S., Dauer W. T., Ansorge M. S., Morelli E., Mann J. J., Toth M., Aghajanian G., Sealfon S. C., Hen R., and Gingrich J. A. (2006) Cortical 5-HT2A receptor receptor signaling modulates anxiety-like behaviors in mice. *Science* **313,** 536-540.

Wolfer D. P., Crusio W. E., and Lipp H. P. (2002) Knockout mice: simple solutions to the problems of genetic background and flanking genes. *Trends Neurosci.* **25,** 336-340.

Xiang Z., Hrabetova S., Moskowitz S. I., Casaccia-Bonnefil P., Young S. R., Nimmrich V. C., Tiedge H., Einheber S., Karnup S., Bianchi R., and Bergold P. J. (2000) Long-term maintenance of mature hippocampal slices in vitro. *J. Neurosci. Methods* **98,** 145-154.

Young E. A., Lopez J. F., Murphy-Weinberg V., Watson S. J., and Akil H. (2003) Mineralocorticoid receptor function in major depression. *Arch. Gen. Psychiatry* **60,**

24-28.

Zobel A., Barkow K., Schulze-Rauschenbach S., Von Widdern O., Metten M., Pfeiffer U., Schnell S., Wagner M., and Maier W. (2004) High neuroticism and depressive temperament are associated with dysfunctional regulation of the hypothalamic-pituitary-adrenocortical system in healthy volunteers. *Acta. Psychiatr. Scand.* **109,** 392-399.

Figure legends

Fig. 1 Hippocampal 5-HT2A receptor protein expression in mice under- and overexpressing GR. (a) Mice underexpressing GR (GR +/-) show significantly reduced (26.3 ± 1.6 %, * $p < 0.05$) hippocampal 5-HT2A receptor protein levels. (b) In contrast, mice over expressing GR (YGR) show significantly increased (60.8 ± 4.0 %, *** $p < 0.001$) 5-HT2A receptor levels. Scale bars represent mean ± SEM.

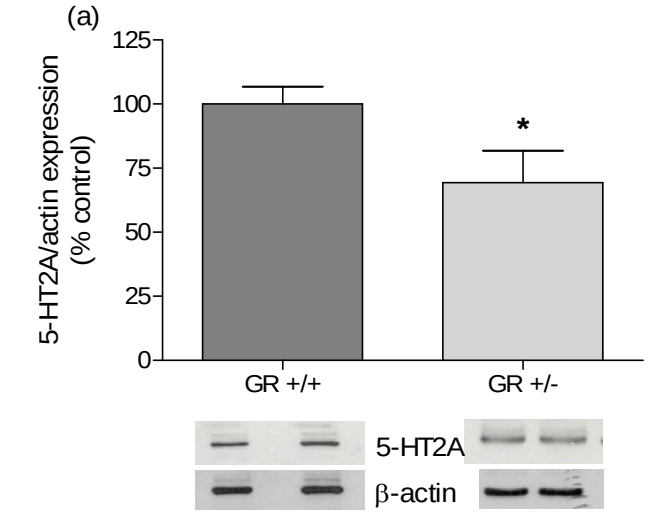
Fig. 2 5-HT2A receptor protein expression in adult organotypic hippocampal cultures exposed to corticosterone. Long-term, and not acute, exposure of high corticosterone concentrations (3 μM) increase hippocampal 5-HT2A receptor protein levels (* $p < 0.05$). Grey bars represent one-day exposure to corticosterone, and black seven-day exposure. Scale bars represent mean ± SEM.

Fig. 3 5-HT2A receptor protein expression in adult organotypic cultures exposed to corticosterone with and without the addition of mifepristone. Blockade of GR by mifepristone inhibits the corticosterone-induced increase in hippocampal 5-HT2A receptor levels. (CORT; corticosterone, Mife; mifepristerone). Scale bars represent mean ± SEM.

Fig. 4 Analysis of tissue mortality in adult organotypic cultures. (a) Adult hippocampal slices exposed to corticosterone (3 μM) and mifepristone for seven days do not express pJNK nor cleaved caspase 3 (b). Neither is there any apoptosis in cultures exposed to corticosterone and spironolactone (blot not shown here). The blot for JNK displays the two characteristic protein bands at 54 and 46 kDa. The samples were blottet together with a positive and negative control for pJNK (c) and cleaved caspase 3 (d), respectively, consisting of commercially bought cell lysates treated with UV light or staurosporine (Cell Signaling).

Fig. 5 5-HT2A receptor protein expression in adult organotypic cultures exposed to corticosterone with and without the addition of spironolactone. Blockade of MR by spironolactone normalizes hippocampal 5-HT2A receptor levels, and inhibits the corticoterone-induced increase. (CORT; corticosterone, Spirono; spironolactone). Scale bars represent mean ± SEM.

Figure 1


(a)
5-HT2A/actin expression
(% control)
125
100
75
50
25
0
*
GR +/+
GR +/-
5-HT2A
β-actin

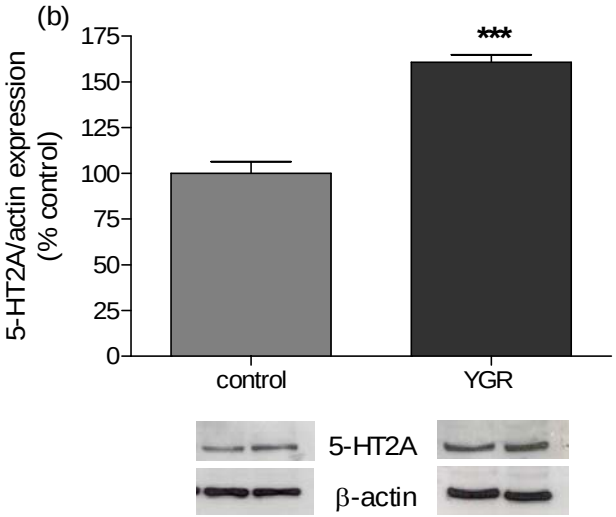

(b)
5-HT2A/actin expression
(% control)
175
150
125
100
75
50
25
0
***
control
YGR
5-HT2A
β-actin

Figure 2

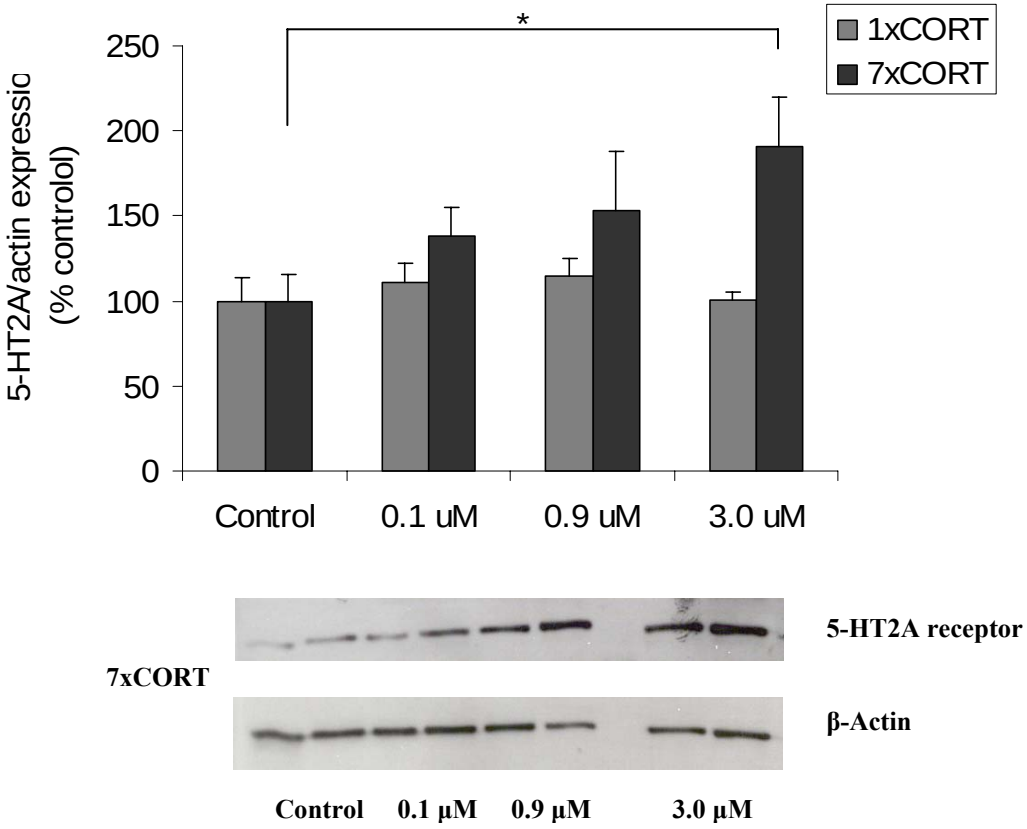

*
1xCORT
7xCORT
5-HT2A/actin expressio
(% controlol)
250
200
150
100
50
0
Control
0.1 uM
0.9 uM
3.0 uM
5-HT2A receptor
7xCORT
β-Actin
Control
0.1 µM
0.9 µM
3.0 µM

Figure 3

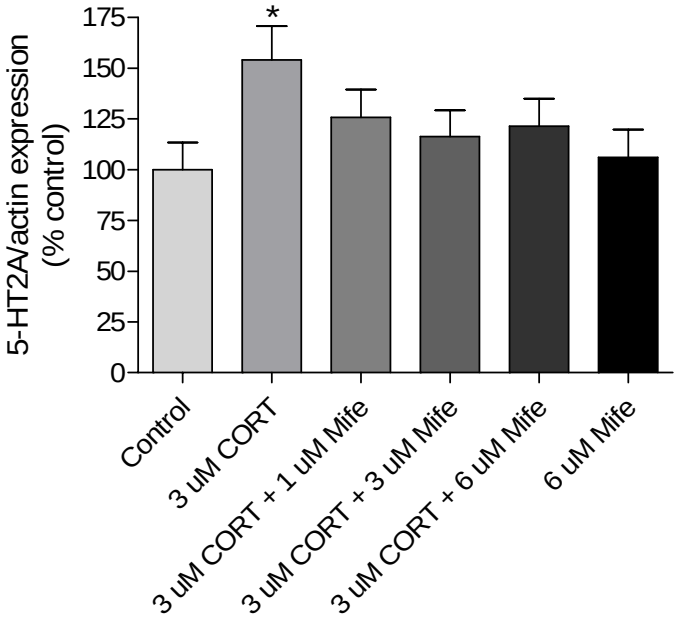
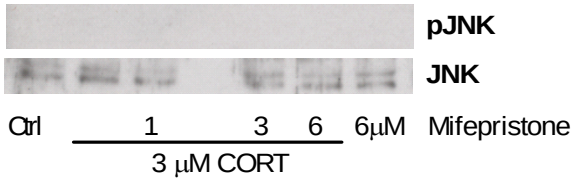
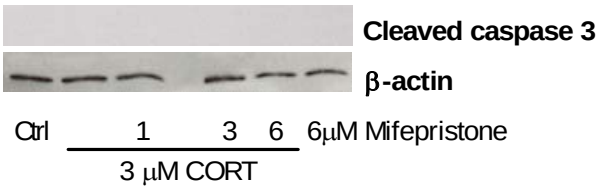

5-HT2A/actin expression
(% control)
175
150
125
100
75
50
25
0
*
Control
3 uM CORT
3 uM CORT + 1 uM Mife
3 uM CORT + 3 uM Mife
3 uM CORT + 6 uM Mife
6 uM Mife

Figure 4


pJNK
JNK
Ctrl
1
3
6
6μM
Mifepristone
3 μM CORT

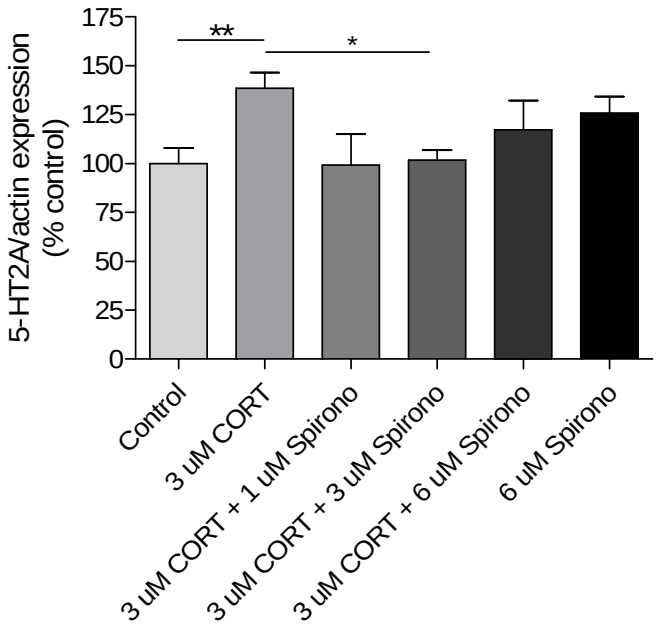
(b)


Cleaved caspase 3
β-actin
Ctrl
1
3
6
6μM Mifepristone
3 μM CORT

(c)

(d)

Figure 5


5-HT2A/actin expression
(% control)
175
150
125
100
75
50
25
0
**
*
Control
3 uM CORT
3 uM CORT + 1 uM Spirono
3 uM CORT + 3 uM Spirono
3 uM CORT + 6 uM Spirono
6 uM Spirono

Paper II

5-HT2A serotonin receptor levels are associated with brain-derived neurotrophic factor (BDNF) and TrkB levels

V Trajkovska[1,*], MS Thomsen[1], AB Marcussen[1], HH Hansen[2], JD Mikkelsen[2], L Arneberg[1], M Kokaia[3], E Castren[4], GM Knudsen[1], S Aznar[1]

1. *Neurobiology Research Unit and Centre for Integrated Molecular Brain Imaging (CIMBI), Copenhagen University Hospital, Unit 9201, Blegdamsvej 9, 2100 Copenhagen, Denmark.*
2. *Neurosearch A/S, Ballerup, Denmark.*
3. *Section of Restorative Neurology, Wallenberg Neuroscience Center, BMC, University Hospital, Lund, Sweden.*
4. *Neuroscience Center, University of Helsinki, Helsinki, Finland.*

* Correspondence:

Viktorija Trajkovska

Neurobiology Research Unit 9201

Rigshospitalet

9 Blegdamsvej

DK- 2100 Copenhagen

Denmark

Phone: +45 3545 6704

Fax: +45 3545 6713

E-mail: viktorija.trajkovska@nru.dk

Abstract

Both brain-derived neurotrophic factor (BDNF) and serotonin receptor 2A (5-HT2A) have been associated with depression. The nature of the interaction between BDNF and its receptor tropomyosin-related kinase B (TrkB) and 5-HT2A receptor levels is, however, unknown.

5-HT2A receptor levels were analysed by Western blot in mature hippocampal organotypic cultures exposed to BDNF (1, 3, 5 and 7 days) and BDNF deficient mice (BDNF +/-). To elucidate whether 5-HT2A receptor levels depend on TrkB levels, hippocampal and frontal cortex 5-HT2A receptor levels were measured in mice overexpressing TrkB full length receptor (TrkB TK), and TrkB levels were assessed in BDNF +/- mice and slice cultures exposed to BDNF.

Seven days exposure to BDNF (600ng/ml) decreased hippocampal 5-HT2A receptor levels (66 ± 3.8%, $p < 0.05$) without affecting synaptic protein expression or inducing cell death. Less than seven days exposure did not have any significant effect on 5-HT2A receptor levels. BDNF+/- mice showed increased 5-HT2A receptor levels in hippocampus (206 ± 43%, $p < 0.05$). TrkB TK mice had increased hippocampal and frontal cortex 5-HT2A receptor levels compared to wild-type, 139 ± 8.2% and 121 ± 7.3% ($p < 0.05$), respectively. Further, cultures exposed to BDNF (600 ng/ml) for seven days had decreased TrkB levels ($p < 0.05$) and BDNF +/- mice showed a tendency towards increased TrkB levels.

In conclusion, BDNF induces changes in 5-HT2A receptor levels and we suggest that that these changes are mediated through alterations in TrkB levels. The specificity of this regulation needs to be further studied.

Keywords: brain-derived neurotrophic factor (BDNF), 5-HT2A, TrkB, mature organotypic cultures, hippocampus, frontal cortex

Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family. It is widely distributed in the brain with the highest levels in the hippocampus (Yan et al. 1997) and it mediates its neurotrophic effect through binding to a high affinity receptor, the tyrosine receptor kinase B (TrkB). Through its promotion of neurogenesis, synaptic plasticity and cell survival (Schinder and Poo 2000; McAllister et al. 1999; Pencea et al. 2001), BDNF plays an important role in the development and plasticity of the brain.

Numerous studies implicate BDNF in major depression. Depressed patients display decreased serum and plasma BDNF levels (Karege et al. 2002; Gonul et al. 2005; Karege et al. 2005; Lee et al. 2006) and BDNF levels are negatively correlated with disease severity (Shimizu et al. 2003). Furthermore, brain BDNF levels are increased in post-mortem patients treated with antidepressant medication (Chen et al. 2001) and in rats an infusion of BDNF has antidepressant effects (Shirayama et al. 2002). Exactly how BDNF is involved in the pathophysiology of depression remains unresolved.

The serotonin receptor 2A (5-HT2A) is involved in both depression and anxiety modulation as has been well established in human (Sheline et al. 2004; Mintun et al. 2004) and animal studies (Weisstaub et al. 2006; Dwivedi et al. 2005; Trajkovska et al. 2007). Depressed patient show lower hippocampal 5-HT2A receptor binding (Sheline et al. 2004; Mintun et al. 2004; Rosel et al. 2004; Rosel et al. 2000) and animals with disruption of the 5-HT2A receptor display reduced conflict behaviour (Weisstaub et al. 2006). Similarly, animals genetically engineered to have a hyper- or hypofunctional hypothalamic-adrenal-pituitary axis with resulting higher or lower predisposition to depression-like behavior also show differences in hippocampal 5-

HT2A receptor levels (Trajkovska et al. 2007). Identifying the factors behind 5-HT2A receptor regulation is therefore of importance for understanding the neurobiological mechanisms preceding depression.

In this study we investigated whether BDNF has a regulatory effect on 5-HT2A receptor levels. We exposed mature hippocampal organotypic cultures to different concentrations of BDNF for respectively between one day and one week. Mature organotypic hippocampal cultures offer the advantages of retaining the connective organization found *in situ* that is reached after adult developmental maturation. Further, since they are isolated from the serotonergic projections from raphe nuclei, one can assess 5-HT2A receptor changes unrelated to BDNF-induced alterations in 5-HT levels. To assess whether the BDNF induced changes in 5-HT2A receptor levels also applied *in vivo* we determined 5-HT2A receptor levels in BDNF heterozygous mice (BDNF+/-). Further, since increased BDNF-TrkB signalling is associated with antidepressant-like effects (Koponen et al. 2005), we also investigated whether changes in TrkB receptor levels could be involved in the 5-HT2A receptor regulation by BDNF. To determine this we made use of mice overexpressing the TrkB full length (TrkB TK) receptor form. Changes in TrkB levels in BDNF +/- mice and BDNF treated organotypic cultures were also analyzed.

Materials and Methods

Animals for culture

Sprague Dawley rat pups were purchased from Charles Rives, Germany and housed under standard conditions. After arrival they were allowed to acclimatise for at least 5 days. The rats were treated in compliance with the European Communities Council

Resolves of 24th November 1986 (86/609/ECC) and the study was approved by Danish State Research Inspectorate (J No 2002/561-527).

Mature organotypic hippocampal culture

Mature organotypic hippocampal cultures were prepared from 20-30 day-old Sprague Dawley rat pups (Xiang et al. 2000). The pups were decapitated and the brains quickly taken out under sterile conditions. The hippocampi were dissected in ice-cold, pre-aerated (95% O_2, 5% CO_2) Gey´s Dissection Buffer (1.5 mM $CaCl_2$, 5.0 mM KCl, 0.2 mM KH_2PO_4, 10 mM $MgCl_2$, 0.12 mM $MgSO_4$, 0.12 M NaCl, 27 mM $NaHCO_3$, 0.7 mM Na_2HPO_4, 32 mM D-glucose, 24 mM HEPES), and cut transversely into 400 μm-thick slices. Intact and undamaged slices were selected under microscope and cultured on membrane inserts (Millipore, Bredford, USA) at 5% CO_2 atmosphere at 32°C, and in culture medium containing a high concentration of potassium (50% basal medium Eagle, 25% inactivated horse serum, 25% Earle´s basal salt solution, 25mM HEPES (pH 7.2), 27 mM D-glucose, 1 mM L-glutamine, all from Invitrogen, Denmark). After two days they were cultured in a medium with physiological concentrations of potassium (50% basal medium Eagle, 25% inactivated horse serum, 25% home made solution (2.2 mM $MgSO_4$, 0.96 mM Na_2HPO_4, 34 mM $NaHCO_3$, 57 mM NaCl, 1.8 mM $CaCl_2$), 25mM HEPES (pH 7.2), 27 mM D-glucose, 1 mM L-glutamine).

Exposure of organotypic hippocampal slice cultures

After one week in culture the hippocampal brain slices were exposed to three different concentrations of BDNF (70, 200, 600 ng/ml, PeproTech, USA) for one or seven days. In a subsequent hippocampal brain slices were exposed to 600 ng/ml BDNF for 3, 5 or

7 days. To maintain a constant BDNF concentration in the medium, a fresh BDNF and medium was added to the cultures every day. Stability of BDNF concentration in the culture media was monitored in a pilot experiment by ELISA (data not shown here) and these results confirmed that the media had to be changed on a daily basis.
At the end of each experimental set up the hippocampal slice cultures were frozen in liquid nitrogen and kept at -80°C until further processing. For analysis of TrkB levels four wells containing 12 hippocampal slices exposed for 7 days to the highest BDNF concentration were pooled, and kept at -80°C until further processing.

BDNF +/- mice

BDNF heterozygote and wild-type mice were bred from heterozygote mice from Jax strain B6.129S4-*Bdnf*^{tm1Jae}/J, as described in (Nygren et al. 2006). Homozygote knock out mice do not survive until adulthood. The pups were genotyped as described previously (Ernfors et al. 1994). The mice were housed with a 12 hr light-dark cycle and given food and water ad libitum. Animals included in this study were male and female BDNF+/- and wild-type mice, all at the age of 10-11 weeks. Hippocampus and frontal cortex was dissected, immediately frozen and stored at -80°C until further processing.

TrkB overexpressive mice

Mice overexpressing the full-length form of rat trkB receptor under the Thy1.2 promoter have been previously described (Koponen et al. 2004b). Hippocampus and prefrontal cortical tissues were dissected from 4-5 week-old transgenic mice and wild type littermates and immediately frozen and stored at -80°C until further processing.

Protein extract preparation

Mature organotypic hippocampal slices cultures and hippocampus or frontal cortex from mice with altered TrkB and BDNF expression were sonicated in ice-cold homogenate buffer containing a cocktail of protease inhibitors (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), 5 mM EDTA, 70 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 0.05 mM aprotinin, 1 mM leupeptin, 2 mM bestatin, 1 mM pepestatin A, 1 mM E-64 (Sigma, Saint Louis, Missouri, USA) and the protein concentration measured with modified Lowry method (Hartree 1972) (DC Protein Assay, BioRad Laboratories, Herlev, Denmark).

Immunopercipitation of Trk

For analysis of TrkB levels in the organotypic cultures Trk immunoprecipitation was performed previous to the western blot analysis to increase the amount of detectable TrkB levels. Protein (180 μg) was incubated on a rotator (15 rpm) overnight at 4°C with immunopercipitation buffer (67% Triton X-100, 7mM Tris, pH 8.0, 46 mM NaCl, 16 mM NaF, 0.3% NP-40, 3.3 % glycerol, 2mM Na_3VO_4) and pan-Trk antibody (sc-11, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein G-linked Sepharose beads (Zymed, San Fransisco, CA, USA) were added and the samples re-incubated on the rotator, followed by centrifugation at 16,100 x g, 3 min, 4°C. The pellet was re-suspended, washed several times and analyzed by western blot.

Western blot analysis

Protein-matched samples were dissolved in 2x SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethano), heated for 5 minutes, 100 °C

and run on 6-8 % SDS-polyacrylamide gel (60 minutes, 150 V, 4 °C). The proteins were electro blotted (350 mA, 1 h, and 4 °C) on to a nitro-cellulose membrane, and the membrane was blocked for unspecific binding with 5% bovine serum albumin or 5% non-fat dry milk (1 h, RT). Incubation with anti-5-HT2A receptor (1:1000, BD Bioscience, Brøndby, Denmark), anti-TrkB (1:2000, BD Bioscience), anti-Synaptophysine (1:2000, Chemicon, Temecula, LA, USA), anti-Synaptotagmin (1:1000, BD Bioscience), anti-Syntaxin (1:10 000, Chemicon), anti-stress-activated protein kinase/Jun-N-terminal kinase (SAPK/JNK; 1:2000, Cell Signalling, Danvers, MA, USA), anti-cleaved caspease 3 (1:1000, Cell Signalling) or anti-phospho-SAPK/JNK (1:2000, Cell Signalling) was conducted over night at 4 °C. Protein band detection was done by 1 hour incubation with horseradish peroxidase-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark) and chemiluminescence detection reagent (Amersham Bioscience, Buckinghamshire, UK) on hyperfilm (Amersham Bioscience, Buckinghamshire, UK). After detection with the primary antibody, the membrane was stripped (Restore Western blot Stripping Buffer, Pierce Biotechnology, Rockford, IL, USA) and re-incubated with rabbit anti-β-actin (1:5000, Sigma). The optical density of the protein bands was quantified with Scion Image (Scion Corporation, www.scioncorp.com). The 5-HT2A optical density was expressed as percentage of the corresponding β-actin protein band.

Statistics

For group comparison, two-tailed unpaired t-test and one-way ANOVA was used. Kolmogorov-Smirnov´s normality test was used to investigate normal distribution. In case of the absence of a normal distribution, the data was logarithmically transformed

to accommodate to a normal distribution or a non-parametric test was used. All data are presented as means ± SEM. Significance level was set at $p < 0.05$ (two-sided).

Results

Decreased 5-HT2A receptor levels in slice cultures exposed to BDNF

Organotypic hippocampal cultures were exposed to BDNF (70, 200 or 600 ng/ml) for one and seven days. One day of BDNF exposure did not alter 5-HT2A receptor levels (Fig. 1A, n= 7-8). However, after seven days exposure of BDNF (600ng/ml) 5-HT2A receptor protein expression was decreased by 65.9 ± 3.8 % as compared to the control condition (Fig. 1D, n= 6-7, F= 3.556, p= 0.033). To establish when 5-HT2A receptor changes were detectable, a new set up was established were slice cultures were subjected to BDNF (600 ng/ml) for three and five days. Three days of BDNF exposure did not alter 5-HT2A receptor protein content (Fig 1B, n= 7-8), but a five-day of exposure to 600 mg/ml BDNF, yielded a tendency towards a decrease in 5-HT2A receptor levels (Fig. 1C, n= 8, p= 0.06).

BDNF does not induce apoptosis nor change synaptic protein levels

To exclude that the observed 5-HT2A receptor changes were the result of BDNF-mediated changes in synaptic density or to an apoptotic effect of the high BDNF concentrations, cultures were processed for synaptic protein levels and apoptotic markers. Seven days of exposure of BDNF (600 ng/ml) did not alter hippocampal slice culture densities of the synaptic proteins synatophysin (Fig 2A, n= 7-8), syntaxin (Fig. 2B, n= 6-8), or synaptotagmin (Fig. 2C, n= 6). Furthermore, BDNF (70, 200, 600 ng/ml) exposure was not associated with formation of the apoptotic proteins pJNK and cleaved caspase -3 (Fig. 3, n=8).

Increased 5-HT2A receptor levels in BDNF +/- mice

Western blot analysis on hippocampal protein extracts showed a 206 ± 43 % increase of 5-HT2A protein content in BDNF +/- mice (Fig. 4A, n= 8-9, t= 2.233, p=0.04) compared to wild-type littermates. In frontal cortex protein extracts from BDNF +/- mice there was a tendency to increased 5-HT2A levels (157 ± 48 %, Fig. 5B, n= 8-9).

5-HT2A receptor levels in TrkB TK overexpressive mice

In TrkB full length receptor (TrkB TK) overexpressive mice 5-HT2A receptor levels were significantly increased in the hippocampus (139 ± 8.2 %, Fig 5A, n=8-9, t= 2.898, p= 0.011) and frontal cortex (121 ± 7.3 %, Fig 5B, n=9, t= 2.362, p= 0.031) compared to wild-type littermates.

TrkB levels in BDNF treated organotypic cultures and BDNF (+/-) heterozygous mice

To determine TrkB levels in cultures treated with BDNF a new set up was established. Pooled slice cultures were immunopercipitated prior to TrkB analysis; this was not needed for TrkB analysis in total brain tissue from the BDNF +/- mice.

Seven days exposure of BDNF (600ng/ml) resulted in a small decrease (14 ± 5.1 %) in total TrkB levels (Fig 6, n= 5, t= 2.737, p= 0.05). In hippocampus and frontal cortex of BDNF +/- mice there was a tendency towards increased TrkB levels, 125.5 ± 13.2 % and 115.4 ± 8.2 %, respectively, compared to their wild-type littermates.

Discussion

We found that one week of exposure to high concentrations of BDNF induces a decrease in 5-HT2A receptor protein levels in mature organotypic hippocampal

cultures. This effect of BDNF corroborated in BDNF +/- mice, where increased hippocampal 5-HT2A receptor levels were found. Further, we show for the first time that 5-HT2A receptor levels are associated with TrkB levels since TrkB TK mice display increased 5-HT2A receptor levels in the hippocampus and frontal cortex. In addition, a decrease in TrkB levels was observed in organotypic cultures exposed to concentrations of BDNF sufficient to induce a 5-HT2A receptor down-regulation.

Chronic, but not short-term, BDNF (600 ng/ml) exposure decreased 5-HT2A receptor levels in the mature organotypic cultures. BDNF has acute and long-term effects on synaptic transmission (Tyler and Pozzo-Miller 2001), synapse number (Pozzo-Miller et al. 1999) and on the levels of hippocampal synapse proteins (Tartaglia et al. 2001). Syntaxin, synatophysin and synaptotagmin are markers of total number of synapses (Tartaglia et al. 2001), and of synaptic activity (Chavis and Westbrook 2001). As the densities of these proteins do not change after chronic BDNF (600 ng/ml) exposure, this indicates that the decrease in 5-HT2A receptor levels is not due to synaptic degeneration. We also excluded that the decrease in 5-HT2A receptor levels was due to cell death as the highest concentration of BDNF did not induce the formation of pJNK and cleaved caspase-3.

Previous studies have shown that BDNF increases 5-HT synthesis and sprouting (Siuciak et al. 1998; Celada et al. 1996), and increased 5-HT levels lead to a desensitisation and down-regulation of 5-HT2A receptor levels (Gray and Roth 2001). Serotonin levels are however unchanged in the TrkB TK mice (Koponen et al. 2005; Saarelainen et al. 2003). In BDNF +/- mice show forebrain 5-HT levels are reduced at the age of 12-18 months (Lyons et al. 1999), however, the BDNF +/- mice used in our study were three months old and at this age BDNF +/- mice have normal 5-HT levels

(Lyons et al. 1999). Furthermore, in the mature organotypic hippocampal cultures where the 5-HT innervation is absent, a presynaptic serotonergic mechanism cannot mediate the BDNF-induced changes. This supports the notion that the changes in 5-HT2A receptor levels we identified in the two different mice models and in the slice cultures are not caused by changes in 5-HT levels.

For the first time we show that alterations in TrkB levels are associated with similar changes in 5-HT2A receptor levels. The TrkB TK mice display higher hippocampal and frontal cortex 5-HT2A receptor levels than wild-type littermates, which suggests that increased TrkB signalling might increase 5-HT2A receptor levels. This is further supported by the observation that the increase in TrkB levels in BDNF +/- mice was accompanied with increased 5-HT2A receptor levels and that chronic BDNF exposure in the organotypic cultures simultaneously decreases TrkB and 5-HT2A receptor levels. In the cultures, a seven-day BDNF exposure decreased 5-HT2A receptor levels, in accordance with an earlier study where BDNF after chronic exposure decreases TrkB levels (Frank et al. 1997). These results suggest that the level of TrkB, not BDNF, regulate 5-HT2A receptor levels. Also, the TrkB TK mice have normal BDNF mRNA and protein levels (Koponen et al. 2004a; Koponen et al. 2004b).
Our data do, however, contrast to earlier studies (Rios et al. 2006; Chan et al. 2006), where BDNF conditional knock-out mice displayed decreased 5-HT2A receptor levels in dorsal raphe nucleus and frontal cortex. But in those studies TrkB levels were not measured and it is not unlikely that TrkB levels are altered differently after total BDNF depletion. The BDNF knock down model we used has a 50 percent reduction in BDNF levels during development, while Rios and co-workers used a mice model completely depleted of BDNF in development and adulthood (Rios et al. 2006; Chan

et al. 2006). Another explanation to the discrepancies found might be region specific differences in 5-HT2A receptor regulation by BDNF.

The TrkB TK mice display reduced anxious behaviour and better stress tolerance (Koponen et al. 2004b) a behavioural effect potentially attributed to increased 5-HT2A receptor levels (Weisstaub et al. 2006). Low hippocampal 5-HT2A receptor levels have been found in major depressed patients (Sheline et al. 2004; Mintun et al. 2004; Rosel et al. 2004; Rosel et al. 2000), while the 5-HT2A receptor alterations in the cortex have been found to be both increased (Bhagwagar et al. 2006) and decreased (Messa et al. 2003). Again, differences in 5-HT2A receptor levels seem to be associated with the susceptibility to develop depression. Mice with high predisposition to depression-like behavior also have reduced 5-HT2A receptor levels; whereas mice with low genetic vulnerability to depression have increased hippocampal 5-HT2A levels (Trajkovska et al. 2007).

Increased BDNF-TrkB signalling is associated with antidepressant-like effects (Koponen et al. 2005), and normal TrkB signalling is required for mediating an antidepressant response (Saarelainen et al. 2003). Here we show that increased TrkB signalling is accompanied with increased 5-HT2A receptor levels. Recently, a polymorphism in the 5-HT2A receptor gene has been associated with the outcome of antidepressant treatment (McMahon et al. 2006), indicating that the increase in 5-HT2A receptor levels in the TrkB TK mice might be involved in mediating the antidepressant response.

In conclusion, chronic BDNF exposure decreases hippocampal 5-HT2A receptor levels. Low BDNF expression on the other hand increases 5-HT2A receptor levels.

We suggest that the BDNF-induced 5-HT2A receptor changes are mediated through changes in TrkB levels, although this hypothesis needs to be examined in future studies.

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References

Bhagwagar Z., Hinz R., Taylor M., Fancy S., Cowen P., and Grasby P. (2006) Increased 5-HT(2A) receptor binding in euthymic, medication-free patients recovered from depression: a positron emission study with. *Am J Psychiatry* **163,** 1580-7.

Celada P., Siuciak J. A., Tran T. M., Altar C. A., and Tepper J. M. (1996) Local infusion of brain-derived neurotrophic factor modifies the firing pattern of dorsal raphe serotonergic neurons. *Brain Res* **712,** 293-8.

Chan J. P., Unger T. J., Byrnes J., and Rios M. (2006) Examination of behavioral deficits triggered by targeting Bdnf in fetal or postnatal brains of mice. *Neuroscience* **142,** 49-58.

Chavis P. and Westbrook G. (2001) Integrins mediate functional pre- and postsynaptic maturation at a hippocampal synapse. *Nature* **411,** 317-21.

Chen B., Dowlatshahi D., MacQueen G. M., Wang J. F., and Young L. T. (2001) Increased hippocampal BDNF immunoreactivity in subjects treated with antidepressant medication. *Biol Psychiatry* **50,** 260-5.

Dwivedi Y., Mondal A. C., Payappagoudar G. V., and Rizavi H. S. (2005) Differential regulation of serotonin (5HT)2A receptor mRNA and protein levels after single and repeated stress in rat brain: role in learned helplessness behavior. *Neuropharmacology* **48,** 204-14.

Ernfors P., Lee K. F., and Jaenisch R. (1994) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* **368,** 147-50.

Frank L., Wiegand S. J., Siuciak J. A., Lindsay R. M., and Rudge J. S. (1997) Effects

of BDNF infusion on the regulation of TrkB protein and message in adult rat brain. *Exp Neurol* **145,** 62-70.

Gonul A. S., Akdeniz F., Taneli F., Donat O., Eker C., and Vahip S. (2005) Effect of treatment on serum brain-derived neurotrophic factor levels in depressed patients. *Eur Arch Psychiatry Clin Neurosci* **255,** 381-6.

Gray J. A. and Roth B. L. (2001) Paradoxical trafficking and regulation of 5-HT(2A) receptors by agonists and antagonists. *Brain Res Bull* **56,** 441-51.

Hartree E. F. (1972) Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal Biochem* **48,** 422-7.

Karege F., Bondolfi G., Gervasoni N., Schwald M., Aubry J. M., and Bertschy G. (2005) Low brain-derived neurotrophic factor (BDNF) levels in serum of depressed patients probably results from lowered platelet BDNF release unrelated to platelet reactivity. *Biol Psychiatry* **57,** 1068-72.

Karege F., Perret G., Bondolfi G., Schwald M., Bertschy G., and Aubry J. M. (2002) Decreased serum brain-derived neurotrophic factor levels in major depressed patients. *Psychiatry Res* **109,** 143-8.

Koponen E., Lakso M., and Castren E. (2004a) Overexpression of the full-length neurotrophin receptor trkB regulates the expression of plasticity-related genes in mouse brain. *Brain Res Mol Brain Res* **130,** 81-94.

Koponen E., Rantamaki T., Voikar V., Saarelainen T., MacDonald E., and Castren E. (2005) Enhanced BDNF signalling is associated with an antidepressant-like behavioral response and changes in brain monoamines. *Cell Mol Neurobiol* **25,** 973-80.

Koponen E., Voikar V., Riekki R., Saarelainen T., Rauramaa T., Rauvala H., Taira T., and Castren E. (2004b) Transgenic mice overexpressing the full-length neurotrophin receptor trkB exhibit increased activation of the trkB-PLCgamma pathway, reduced anxiety, and facilitated learning. *Mol Cell Neurosci* **26,** 166-81.

Lee B. H., Kim H., Park S. H., and Kim Y. K. (2006) Decreased plasma BDNF level in depressive patients. *J Affect Disord*.

Lyons W. E., Mamounas L. A., Ricaurte G. A., Coppola V., Reid S. W., Bora S. H., Wihler C., Koliatsos V. E., and Tessarollo L. (1999) Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. *Proc Natl Acad Sci U S A* **96,** 15239-44.

McAllister A. K., Katz L. C., and Lo D. C. (1999) Neurotrophins and synaptic plasticity. *Annu Rev Neurosci* **22,** 295-318.

McMahon F. J., Buervenich S., Charney D., Lipsky R., Rush A. J., Wilson A. F., Sorant A. J., Papanicolaou G. J., Laje G., Fava M., Trivedi M. H., Wisniewski S. R., and Manji H. (2006) Variation in the gene encoding the serotonin 2A receptor is associated with outcome of antidepressant treatment. *Am J Hum Genet* **78,** 804-14.

Messa C., Colombo C., Moresco R. M., Gobbo C., Galli L., Lucignani G., Gilardi M. C., Rizzo G., Smeraldi E., Zanardi R., Artigas F., and Fazio F. (2003) 5-HT(2A) receptor binding is reduced in drug-naive and unchanged in SSRI-responder depressed patients compared to healthy controls: a PET study. *Psychopharmacology (Berl)* **167,** 72-8.

Mintun M. A., Sheline Y. I., Moerlein S. M., Vlassenko A. G., Huang Y., and Snyder A. Z. (2004) Decreased hippocampal 5-HT2A receptor binding in major depressive

disorder: in vivo measurement with. *Biol Psychiatry* **55,** 217-24.

Nygren J., Kokaia M., and Wieloch T. (2006) Decreased expression of brain-derived neurotrophic factor in BDNF(+/-) mice is associated with enhanced recovery of motor performance and increased neuroblast number following experimental stroke. *J Neurosci Res* **84,** 626-31.

Pencea V., Bingaman K. D., Wiegand S. J., and Luskin M. B. (2001) Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. *J Neurosci* **21,** 6706-17.

Pozzo-Miller L. D., Gottschalk W., Zhang L., McDermott K., Du J., Gopalakrishnan R., Oho C., Sheng Z. H., and Lu B. (1999) Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. *J Neurosci* **19,** 4972-83.

Rios M., Lambe E. K., Liu R., Teillon S., Liu J., Akbarian S., Roffler-Tarlov S., Jaenisch R., and Aghajanian G. K. (2006) Severe deficits in 5-HT2A -mediated neurotransmission in BDNF conditional mutant mice. *J Neurobiol* **66,** 408-20.

Rosel P., Arranz B., San L., Vallejo J., Crespo J. M., Urretavizcaya M., and Navarro M. A. (2000) Altered 5-HT(2A) binding sites and second messenger inositol trisphosphate (IP(3)) levels in hippocampus but not in frontal cortex from depressed suicide victims. *Psychiatry Res* **99,** 173-81.

Rosel P., Arranz B., Urretavizcaya M., Oros M., San L., and Navarro M. A. (2004) Altered 5-HT2A and 5-HT4 postsynaptic receptors and their intracellular signalling systems IP3 and cAMP in brains from depressed violent suicide victims.

Neuropsychobiology **49,** 189-95.

Saarelainen T., Hendolin P., Lucas G., Koponen E., Sairanen M., MacDonald E., Agerman K., Haapasalo A., Nawa H., Aloyz R., Ernfors P., and Castren E. (2003) Activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects. *J Neurosci* **23,** 349-57.

Schinder A. F. and Poo M. (2000) The neurotrophin hypothesis for synaptic plasticity. *Trends Neurosci* **23,** 639-45.

Sheline Y. I., Mintun M. A., Barch D. M., Wilkins C., Snyder A. Z., and Moerlein S. M. (2004) Decreased hippocampal 5-HT(2A) receptor binding in older depressed patients using. *Neuropsychopharmacology* **29,** 2235-41.

Shimizu E., Hashimoto K., Okamura N., Koike K., Komatsu N., Kumakiri C., Nakazato M., Watanabe H., Shinoda N., Okada S., and Iyo M. (2003) Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. *Biol Psychiatry* **54,** 70-5.

Shirayama Y., Chen A. C., Nakagawa S., Russell D. S., and Duman R. S. (2002) Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *J Neurosci* **22,** 3251-61.

Siuciak J. A., Clark M. S., Rind H. B., Whittemore S. R., and Russo A. F. (1998) BDNF induction of tryptophan hydroxylase mRNA levels in the rat brain. *J Neurosci Res* **52,** 149-58.

Tartaglia N., Du J., Tyler W. J., Neale E., Pozzo-Miller L., and Lu B. (2001) Protein synthesis-dependent and -independent regulation of hippocampal synapses by brain-

derived neurotrophic factor. *J Biol Chem* **276,** 37585-93.

Trajkovska V., Krey G., Thomsen M.S., Chourbaji S., Brandwein C., Ridder S., Gass P., Knudsen G.M., Aznar S. (2007) Activation of glucocorticoid receptors increases hippocampal 5-HT2A receptor levels. *Submitted.*

Tyler W. J. and Pozzo-Miller L. D. (2001) BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses. *J Neurosci* **21,** 4249-58.

Weisstaub N. V., Zhou M., Lira A., Lambe E., Gonzalez-Maeso J., Hornung J. P., Sibille E., Underwood M., Itohara S., Dauer W. T., Ansorge M. S., Morelli E., Mann J. J., Toth M., Aghajanian G., Sealfon S. C., Hen R., and Gingrich J. A. (2006) Cortical 5-HT2A receptor signalling modulates anxiety-like behaviors in mice. *Science* **313,** 536-40.

Xiang Z., Hrabetova S., Moskowitz S. I., Casaccia-Bonnefil P., Young S. R., Nimmrich V. C., Tiedge H., Einheber S., Karnup S., Bianchi R., and Bergold P. J. (2000) Long-term maintenance of mature hippocampal slices in vitro. *J Neurosci Methods* **98,** 145-54.

Yan Q., Rosenfeld R. D., Matheson C. R., Hawkins N., Lopez O. T., Bennett L., and Welcher A. A. (1997) Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. *Neuroscience* **78,** 431-48.

Figure legends

Fig. 1. A seven-day (D), and not one- (A), three- (B) and five-day (C) BDNF (600 ng/ml) exposure decreases 5-HT2A receptor levels (65.9 ± 3.8 %, $p < 0.05$) compared to the control condition.

Fig. 2. Chronic BDNF (600 ng/ml) does not alter protein density of synaptophysine (A), syntaxin (B) or synaptotagmine (n= 6-8). Scale bars represent mean ± SEM.

Fig 3. Mature hippocampal slices exposed to BDNF (70, 200, 600 ng/ml) for seven days do not express elevated pJNK (A) or cleaved caspase 3 (B). The blot for JNK displays the two characteristic protein bands at 54 and 46 kDa (A). A control blot with a positive and negative signal for pJNK (C) and cleaved caspase 3 (D), respectively, from commercially bought cell lysates treated with UV light or staurosporine (Cell Signalling).

Fig. 4. The BDNF +/- mice have significantly increased (206 ± 44 %, $p < 0.05$) hippocampal 5-HT2A receptor protein levels, as measured with Western blot (A). In protein extracts from frontal cortex there was a tendency towards increased 5-HT2A receptor levels (157 ± 48 %, Fig. 4B, n= 8-9) in BDNF +/- mice. Scale bars represent mean ± SEM.

Fig. 5. Mice overexpressing the TrkB full length (TrkB TK) receptor display increased 5-HT2A receptor levels in the hippocampus ($p < 0.05$, n= 8-9) compared to wild-type littermates (WT). Also in frontal cortex TrkB TK mice showed a 121 ± 7.3 % increase in 5-HT2A receptor protein levels (Fig. 5B, n=9, $t = 2.362$, $p = 0.031$). Scale bars represent mean ± SEM.

Fig. 6. Seven day exposure to BDNF (600ng/ml) decreased TrkB levels ($p = 0.05$). Scale bars represent mean ± SEM.

Figure 1

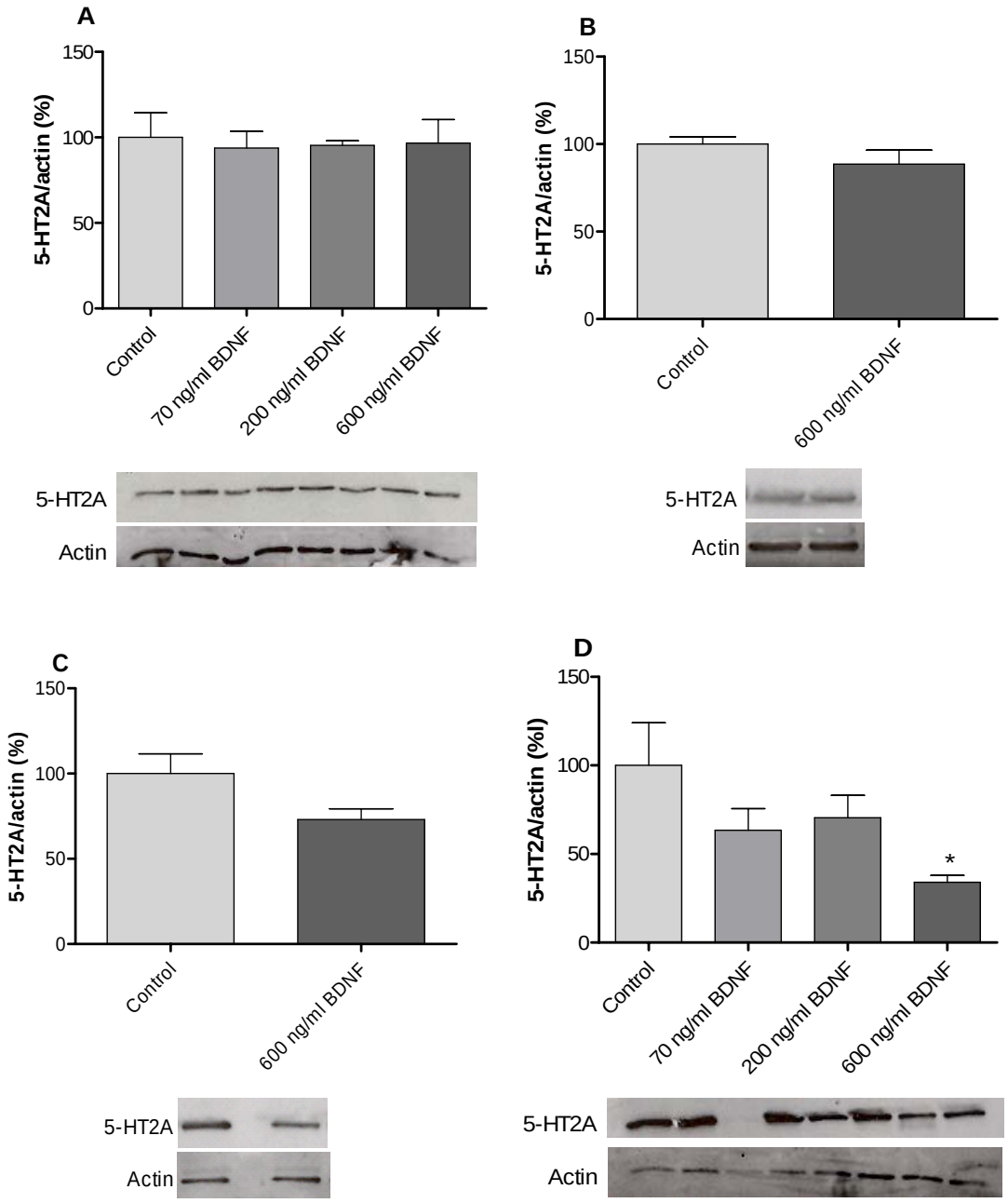

A
150
100
50
0
5-HT2A/actin (%)
Control
70 ng/ml BDNF
200 ng/ml BDNF
600 ng/ml BDNF
5-HT2A
Actin
B
150
100
50
0
5-HT2A/actin (%)
Control
600 ng/ml BDNF
5-HT2A
Actin
C
150
100
50
0
5-HT2A/actin (%)
Control
600 ng/ml BDNF
5-HT2A
Actin
D
150
100
50
0
5-HT2A/actin (%I)
*
Control
70 ng/ml BDNF
200 ng/ml BDNF
600 ng/ml BDNF
5-HT2A
Actin

Figure 2

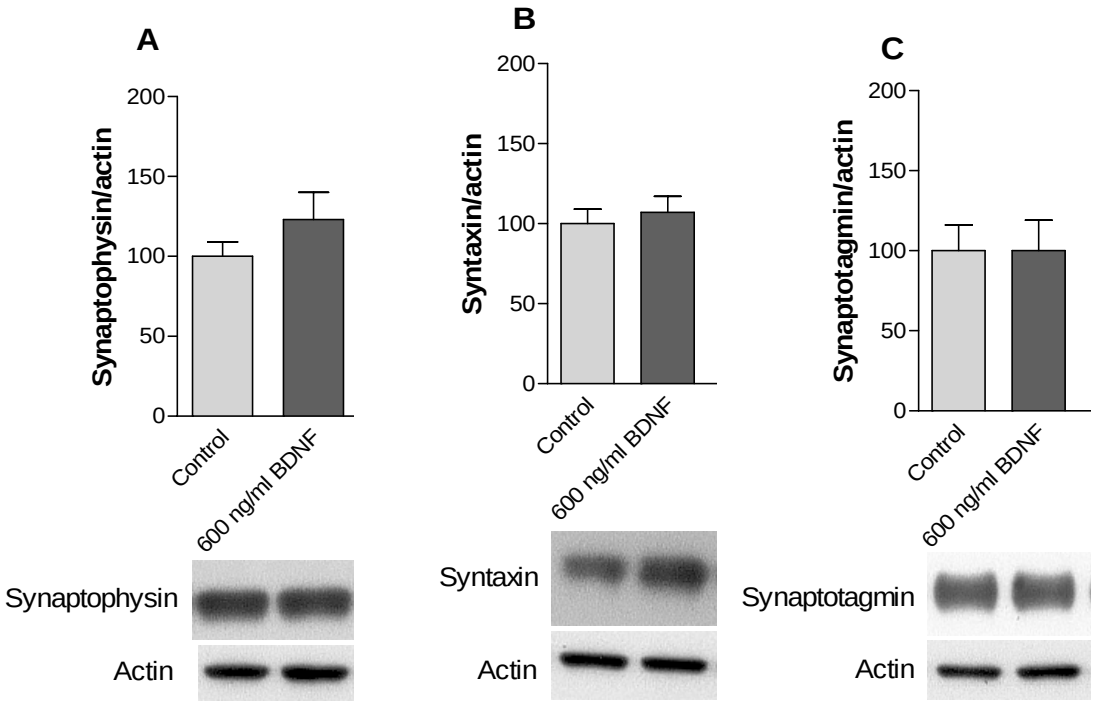

A
Synaptophysin/actin
200
150
100
50
0
Control
600 ng/ml BDNF
Synaptophysin
Actin
B
Syntaxin/actin
200
150
100
50
0
Control
600 ng/ml BDNF
Syntaxin
Actin
C
Synaptotagmin/actin
200
150
100
50
0
Control
600 ng/ml BDNF
Synaptotagmin
Actin

Figure 3

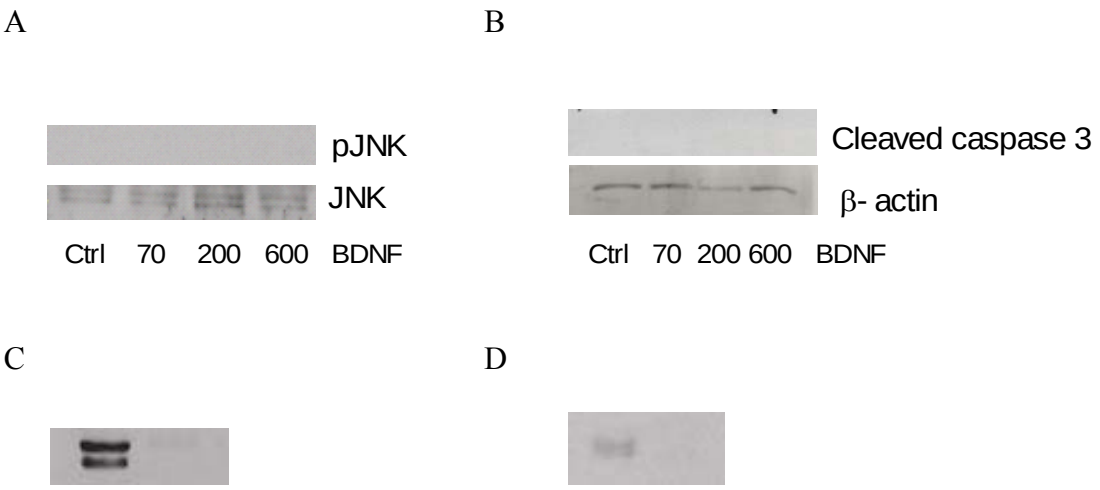

A
B
pJNK
JNK
Ctrl 70 200 600 BDNF
Cleaved caspase 3
β- actin
Ctrl 70 200 600 BDNF
C
D

Figure 4

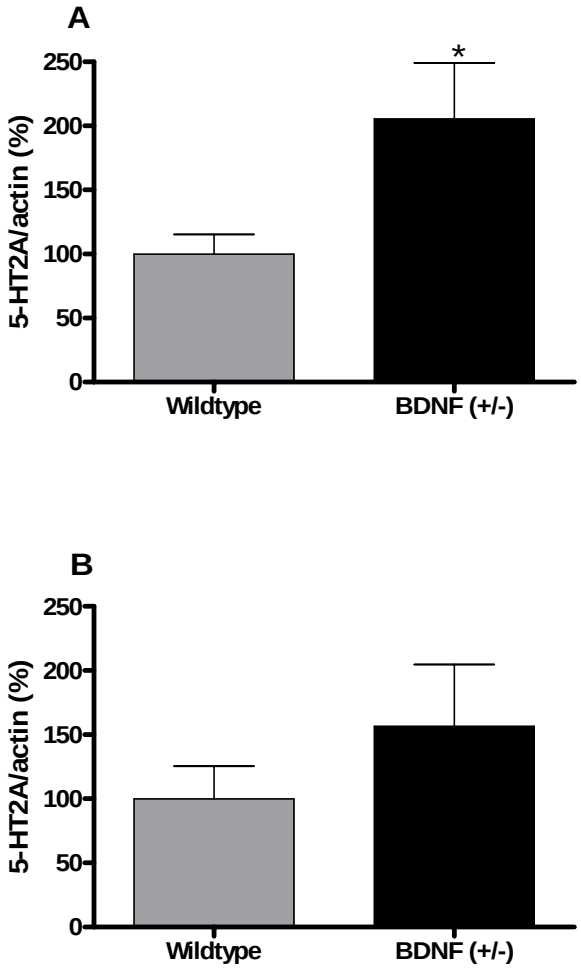
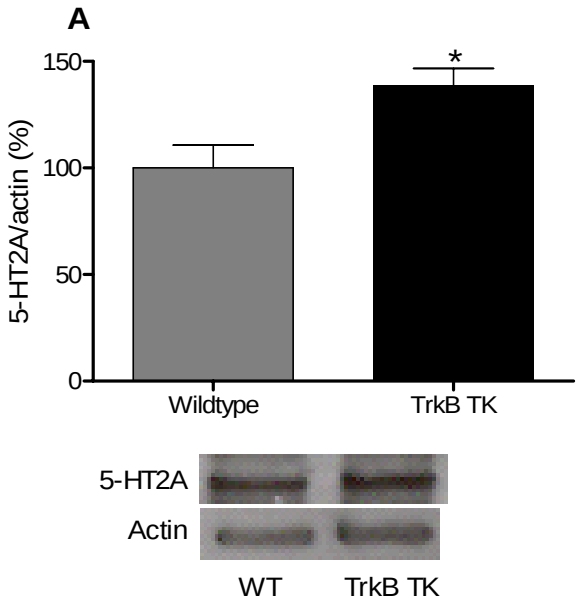

A
5-HT2A/actin (%)
250
200
150
100
50
0
*
Wildtype
BDNF (+/-)
B
5-HT2A/actin (%)
250
200
150
100
50
0
Wildtype
BDNF (+/-)

Figure 5


A
150
100
50
0
5-HT2A/actin (%)
*
Wildtype
TrkB TK
5-HT2A
Actin
WT
TrkB TK

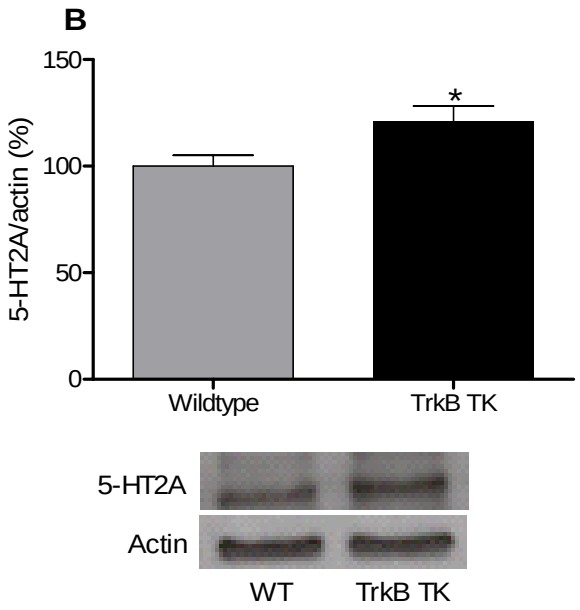
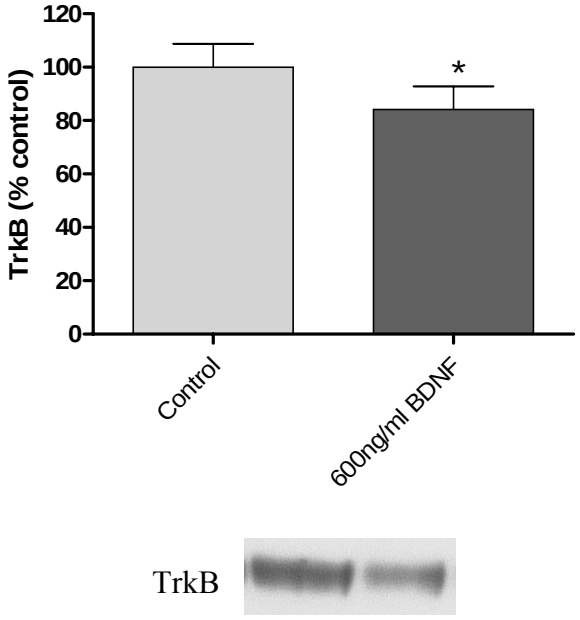

B
150
100
50
0
5-HT2A/actin (%)
*
Wildtype
TrkB TK
5-HT2A
Actin
WT
TrkB TK

Figure 6



Paper III

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Technical comment

Measurements of brain-derived neurotrophic factor: Methodological aspects and demographical data

Viktorija Trajkovska [a,*], Anders B. Marcussen [a], Maj Vinberg [b], Per Hartvig [a], Susana Aznar [a], Gitte M. Knudsen [a]

[a] *Neurobiology Research Unit and Center for Integrated Molecular Brain Imaging, Rigshospitalet, Copenhagen University Hospital, Section 9201, Blegdamsvej 9, DK-2100 Copenhagen, Denmark*

[b] *Department of Psychiatry, Rigshospitalet, Copenhagen University Hospital, Section 6233, Blegdamsvej 9, DK-2100 Copenhagen, Denmark*

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Abstract

Although numerous studies have dealt with changes in blood brain-derived neurotrophic factor (BDNF), methodological issues about BDNF measurements have only been incompletely resolved. We validated BDNF ELISA with respect to accuracy, reproducibility and the effect of storage and repeated freezing cycles on BDNF concentrations. Additionally, the effect of demographic characteristics in healthy subjects on BDNF was verified. Whole blood and serum was collected from 206 healthy subjects and a subgroup was genotyped for BDNF Val66Met polymorphism. The effect of age, gender, BDNF genotype and thrombocyte count on whole blood BDNF was assessed. The BDNF ELISA measurement was accurate, 91.6 ± 3.0%, and showed high reproducibility, whereas inter-assay and intra-subject variations were modest, 8.4 ± 5.2% and 17.5 ± 14.1%, respectively. Storage of whole blood samples at 4 °C significantly decreased BDNF concentration, while repeated freezing cycles and storage at −20 °C was without any effect. Storage at −20 °C of serum, but not whole blood, was associated with a significant decrease in BDNF concentration. Women had significantly higher whole blood BDNF concentrations than men (18.6 ± 1.3 ng/ml versus 16.5 ± 1.4 ng/ml), and showed a right-skewed BDNF concentration distribution. No association between whole blood BDNF concentrations and thrombocyte count, age, or BDNF genotype was found. In conclusion, the BDNF ELISA assay determines whole blood BDNF accurately and with high reproducibility. Female gender is associated with higher whole blood BDNF concentrations whereas age, thrombocyte count and BDNF Val66Met polymorphism were un-associated.

Keywords: Brain-derived neurotrophic factor (BDNF); Age; Gender; BDNF Val66Met polymorphism; Thrombocyte count; Storage conditions

1. Introduction

Brain-derived neurotrophic factor (BDNF) was characterised for the first time more than a decade ago [48] and it belongs to the neurotrophin family that also includes nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). The neurotrophin BDNF is crucially involved in brain development, neurogenesis, neural circuit formation, and plasticity [40]. It has an activity dependent release and is transported across the blood–brain barrier [33]. In the blood, BDNF is stored in platelets and released upon agonist stimulation by thrombin, Ca^{2+}, collagen or shear stress [11] and the BDNF concentration can be measured with a commercially available assay [37]. Other peripheral sources of BDNF include the endothelial cells, smooth muscle cells, and eosinophils [8,30,38].

The peripheral effects of BDNF are only sparsely known; but it is believed to be involved in regeneration of neurons during nerve injury [31] and affect the immune system [13,39,41]. Animal studies indicate that brain and blood BDNF concentrations undergo similar changes during maturation and aging, suggesting that blood BDNF levels may reflect the BDNF levels in brain [18]. Indeed, rodent studies show a tight correlation between brain and blood BDNF values [18].

Numerous clinical studies have identified changes in serum or blood BDNF concentrations in patients with neuropsychiatric disorders such as depression [43], schizophrenia [44], Alzheimer's disease [21], multiple sclerosis [2], and anorexia [27]. Furthermore, in Alzheimer's disease protein and mRNA

* Corresponding author. Tel.: +45 3545 6704; fax: +45 3545 6713.
E-mail address: viktorija.trajkovska@nru.dk (V. Trajkovska).

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levels of BDNF are changed in the same direction, [12,21,26], and the same applies to exposure to stress [15,26] and antidepressants [7,26]. Methodological issues regarding measurements of BDNF concentration have, however, only been superficially encountered. Measurements of BDNF is highly valuable since it may be a marker of disease development in Alzheimer's disease [35] and it correlates with disease severity in major depression [43]. The aim of this study is, firstly, to validate a commonly used ELISA based assay. The reproducibility and accuracy of blood BDNF measurement as well as the influence of various storage conditions was assessed. Secondly, the relation between serum and whole blood BDNF concentrations was investigated to assess if studies on serum or whole blood are directly comparable. Thirdly, whole blood BDNF concentrations were investigated in healthy controls and related to gender, age, and the BDNF Val66Met functional polymorphism.

2. Materials and methods

2.1. Subjects

Two-hundred and six healthy subjects, 122 women and 84 men, with a mean age of 44.3 ± 13.3 (range 20–70) years were recruited, mainly from newspaper advertisements. None of the subjects had a history of neuropsychiatric disorders, current alcohol abuse, and they were all drug free. Written informed consent was obtained according to the declaration of Helsinki II, and the study was approved by the Ethics Committee of Copenhagen and Frederiksberg [(KF)02-058/99, (KF)12-091/00, (KF)12-113/00, (KF)12-152/01, (KF)12-029/02, (KF)01-001/02, (KF)01-156/04, (KF)11-061/03].

2.2. Blood samples from subjects

A total of 206 whole blood and 37 serum samples were collected from the subjects. For serum samples, blood was collected in additive-free containers and was left to coagulate for at least 1 h. After centrifugation (Eppendorf, Denmark) at 3300 rpm for 10 min, serum was collected and stored at −20 °C until further analysis. Whole blood was collected in EDTA containing tubes and immediately frozen. At the time of examination whole blood was lysed with 3% triton X-100 (Merck, Germany) and sonication [44]. Disrupted cell membranes were discarded by centrifuging the samples at 12,000 rpm for 10 min at 4 °C and the supernatant was aliquoted and kept at −20 °C until further processing.

2.3. Determination of BDNF concentration with ELISA

Lysed blood samples were assessed for BDNF concentrations using a commonly used [1,32,49] commercially available two-site sandwich enzyme-linked immunoabsorbent assay (ELISA) kit (CYT 306, batch number 0511016132, Chemicon, Temecula, USA). The samples were processed according to the manufacturer's specifications: the samples and standards were added in duplicate when serially diluted, and in triplicate when diluted in one single concentration and added to rabbit anti-human BDNF antibody pre-coated 96-well plates, and incubated at 4 °C over night. The following day the wells were washed with buffer and incubated with biotinylated mouse anti-BDNF antibody for 3 h at room temperature. In order to amplify the signal, streptavidin-horseradish peroxidase conjugate was added to each well and allowed to incubate for 1 h. The addition of 3,3′,5,5′-tetramethylebenzidine started the colour reaction. The colour reaction was stopped 15 min later with HCl solution, and the absorbency was immediately measured at 450 nm (BioRad Laboratories, Herlev, Denmark). The concentration of the samples was either calculated from the standard curve that came with each plate or by averaging six standard curves. According to the manufacturer, the standard curve is linear in the concentrations range of 7.8–500 pg/ml BDNF and the BDNF ELISA kit has no significant cross-reactivity to the neurotrophins NGF, NT4/5 or NT3.

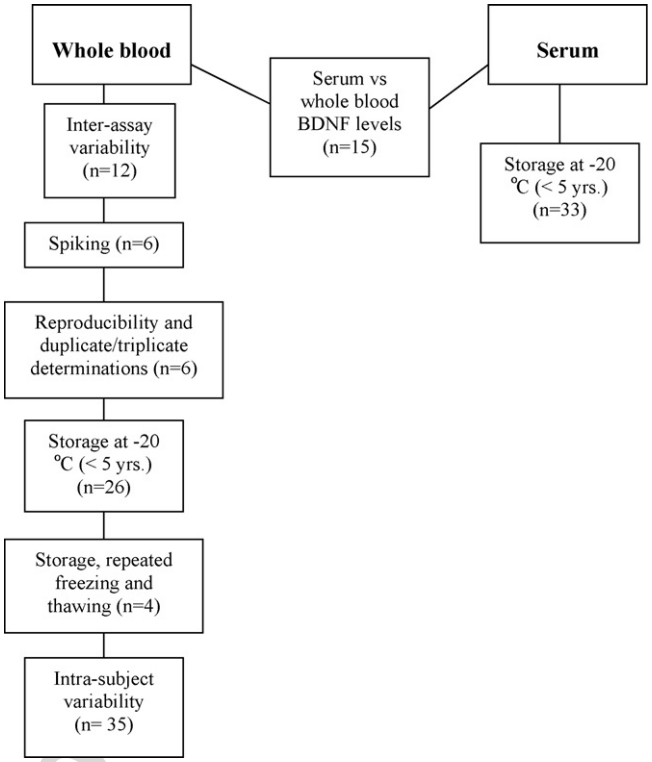

Fig. 1. Chart over the various validations with a commercial ELISA BDNF assay.

2.4. Validation of the BDNF ELISA assay

For a schematic overview of all methodological experiments see Fig. 1.

2.4.1. Accuracy of assay determined in spiked samples

To assess the accuracy of the BDNF measurement at a known concentration, BDNF was added to the samples, a procedure commonly referred to as spiking. Sonicated whole blood samples were spiked by adding 100, 200 or 400 pg/ml human BDNF (PeproTech, London, UK) and run together with an un-spiked portion of the same sample. The resulting BDNF concentration (%) was compared in the non-added sample with the spiked sample ($n = 6$).

2.4.2. Internal standards for the BDNF ELISA kit

As an inter-plate/assay control in addition to the standard curves calculated for each ELISA kit, two standards were every time included among the other samples. The internal standards consisted of blood samples from two different subjects with BDNF concentrations in the lower and upper range of the standard curve. The values of the internal standards were extracted from six BDNF standard curves obtained from their corresponding plate. Additionally, a common single BDNF standard curve was determined by averaging the six BDNF standard curves. The analysis yielding the smallest variation was collected for further analysis.

Whole blood samples were also run in duplicates and triplicates without serial dilutions ($n = 6$).

Duplicate determinations of absorbency with a coefficient of variance above 10% were excluded from the comparison studies.

2.4.3. Test–retest variation

To test the reproducibility of the BDNF measurements of the same sample, samples were measured twice with BDNF ELISA kits from two different batches ($n = 6$). The measurements were done at two different time points. Furthermore, to assess intra-subject variability over time whole blood BDNF concentrations were measured in two samples taken from the same individuals, at a time intervals of 0–8 months (mean 1.9 ± 1.7, $n = 35$).

2.4.4. Stability of BDNF on storage, freezing and thawing

Following blood collection ($n = 4$) and lysis, the blood taken from the same individual was divided into four test tubes that were treated differently. Three of the tubes were placed at −20 °C, one tube was kept stored at −20 °C, a second was thawed and refrozen twice and the third sample was exposed to three freeze-thaw cycles. Between the thawings, the samples were entirely frozen. The fourth tube was stored at 4 °C for 48 h before being analyzed.

Additionally, in a cross-sectional design whole blood ($n = 26$) and serum ($n = 33$) samples stored for up to 5 years in −20 °C were analyzed for BDNF concentrations and compared to sample storage time in order to assess possible degradation.

2.5. Whole blood BDNF ELISA on 206 healthy subjects

In order to make sure that the measurements of BDNF were done under optimal conditions several precautions were undertaken. Care was taken not to freeze and thaw the aliquot of the samples more than one time after lysis and before BDNF analysis, and the samples were all stored at −20 °C. All samples were processed within 1 week after lysis.

When using the kit-specific standard curve for calculation of BDNF concentrations (as recommended by the manufacturer) some variation was found in BDNF concentrations of the internal standards when different kits were used. In order to minimize inter-sample variance resulting from experimental variation, all 206 samples were measured simultaneously and with BDNF ELISA kits from the same batch. The determinations of the BDNF concentrations in the samples were done in duplicate. Duplicate determinations of absorbency with a co-efficient of variance above 10% were excluded from the comparison studies. Three kits (six plates) were used in this set-up. Each plate had its corresponding BDNF standard and contained our two internal control standards. A single average BDNF standard curve was calculated on the basis of all the BDNF standard curves included in the set-up. Since this approach yielded the smallest inter-assay variation (see internal standards and repeated measurement in the result section), the sample concentration was determined from this averaged standard curve.

2.6. DNA genotyping of BDNF

The DNA was extracted from blood samples using the QIAamp DNA Blood Mini Kit (Qiagen, Inc.). The BDNF genotyping was performed using a TaqMan 5′-exonuclease allelic discrimination assay according to the instructions provided by the manufacturer (Applied Biosystems, Foster City, California). The multiplex real-time PCR machine used for this analysis was an ABI 7500 (Applied Biosystems, Foster City, California). This analysis recognises the Val/Val, Val/Met and Met/Met phenotypes. Because of the limited number of the Met/Met phenotype ($n = 3$), data from these subjects were not included in the analysis.

2.7. Statistics

For comparison between groups, two-tailed paired and unpaired *t*-test were used. D'Agostino and Pearson omnibus normality test was used to test for the presence of a normal distribution. In the absence of a normal distribution, data were either logarithmically transformed to accommodate to a normal distribution or a non-parametric Mann–Whitney test was applied. Linear regression was applied on log-transformed data to investigate the effect of age on BDNF concentration. Spearman's correlation analysis was applied for whole blood BDNF concentration and time between blood sampling, and storage time of serum. All data are presented as means ± S.D. Significance level was set at $p < 0.05$.

3. Results

3.1. Accuracy and yield of the BDNF assay

The average yield for samples spiked with three different concentrations of BDNF (100, 200, or 400 pg/ml), was high: 91.6 ± 3.0% ($n = 6$).

3.2. Internal standards and repeated measurements

When the individual standard curves from three kits were applied, the two internal standard samples BDNF concentrations varied by 34.0 ± 20.9%. This was due to a large variation in the absorbance values of the standard that was applied in each kit and used to yield the standard curve for each plate. For comparison, the manufacturer claims an inter-assay variation of 8.5% (at 125 pg/ml). However, when averaged standard curves were applied, the variability was reduced to 8.4 ± 5.2% ($n = 12$, $t = 3.766$, $p = 0.003$). In addition, when internal standards were used in the repeated measurement the test–retest variability was reduced from 22.2 ± 15.5% to 18.6 ± 13.3% ($n = 33$, $t = 3.375$, $p = 0.002$).

Triplicate determinations of whole blood BDNF concentration did not reduce the standard deviation of the measurements significantly as compared to duplicate measurements (10.9 ± 2.4 ng/ml versus 11.2 ± 2.5 ng/ml, $n = 6$, $r^2 = 0.99$).

3.3. Inter-assay and intra-subject variation

Inter-assay variation between repeated assays was determined in six samples with two different ELISA kits, measured on two different days. A paired *t*-test showed no significant difference between the two measurements (11.7 ± 4.6 ng/ml versus 10.9 ± 2.4 ng/ml, $n = 6$).

Intra-subject variation was 12.0 ± 2.6% ($n = 35$), and the variability did not differ between women and men, 10.9 ± 2.9% and 13.9 ± 2.3%, respectively. There was no significant correlation between intra-subject variation and time between blood sampling (Fig. 2, $n = 35$).

3.4. Stability on storage, freezing and thawing

Aliquots stored at 4 °C for 48 h showed a significant decline in whole blood BDNF concentration of 15.8 ± 9.3% (Fig. 3,

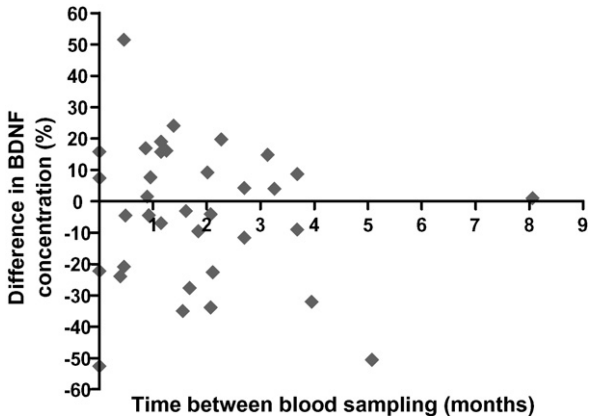

Fig. 2. Whole blood BDNF concentrations were measured twice in the same subject. The figure shows the percentual difference between first and second measurement (in relation to the first whole blood BDNF value) plotted vs. the time interval between the two blood samplings. Each point thus represents one individual. Intra-subject variability in terms of percentual difference in whole blood BDNF concentrations was modest, 12.0 ± 2.6%, and did not vary with the time between blood sampling ($n = 35$).

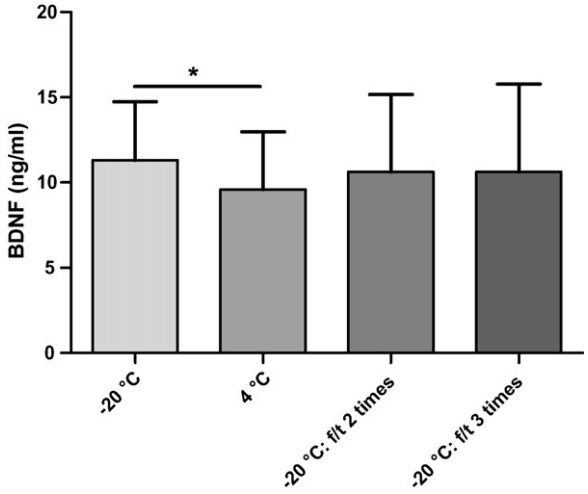

Fig. 3. Storage at 4 °C for 48 h was associated with a decrease in whole blood BDNF concentrations. By contrast, up to three freezing and thawing cycles did not change whole blood BDNF concentrations. f/t: freezing-thawing. Scale bars represent mean ± S.D.

$n = 4$, $t = 3.441$, $p = 0.04$), compared to aliquots stored at −20 °C. Two or three freezing and thawing cycles did not alter the measured BDNF concentration in whole blood in a small sample of four.

In a cross-sectional design, samples stored for up to 5 years were analyzed for BDNF and the outcome compared to the sample storage time. There was no sign of decreased BDNF concentration in whole blood samples stored at −20 °C for up to 5 years ($n = 26$). For serum samples, however, BDNF concentrations were significantly and negatively associated with storage duration at −20 °C (Fig. 4, $n = 33$, Spearman's $r = -0.3883$, $p = 0.0256$).

3.5. Serum and whole blood BDNF concentrations

When serum samples stored for less than 12 months were compared to their corresponding whole blood sample, there was no significant difference in BDNF concentrations ($n = 15$), 12.9 ± 8.0 ng/ml versus 12.1 ± 4.4 ng/ml, serum and whole blood, respectively. The variance in BDNF concentration, however, was significantly higher for serum than for whole blood samples ($n = 15$, $F = 0.28$, $p = 0.02$).

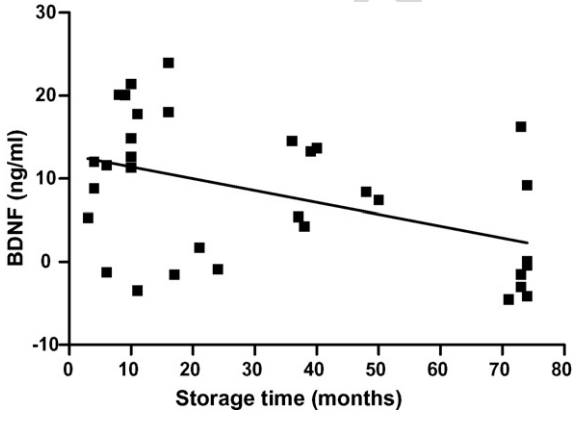

Fig. 4. Storage of serum samples at −20 °C is associated with a significant reduction in serum BDNF concentration. Already after 6–10 months some serum samples have BDNF concentration below the detection limit of the BDNF ELISA assay.

3.6. Thrombocyte count and BDNF concentration in whole blood

Within the reference interval of normal thrombocyte count (150–400 × 10^9/L) there was no significant correlation between thrombocyte number and whole blood BDNF concentration ($n = 19$).

3.7. Whole blood BDNF concentrations related to gender

In the 200 healthy volunteers, mean whole blood BDNF concentration was 17.7 ± 1.4 ng/ml. The distribution of whole blood BDNF values did not conform to a normal distribution but was right-skewed ($n = 200$, K2 = 12.4, $p = 0.002$). However, when separating the sample into women and men, only the female distribution was right-skewed ($n = 119$, K2 = 14.9, $p = 0.0006$). Furthermore, women had higher average whole blood BDNF concentrations than males, 18.6 ± 1.3 ng/ml versus 16.5 ± 1.4 ng/ml (Fig. 5, $n = 200$, $t = 2.794$, $p = 0.0057$).

3.8. Whole blood BDNF concentrations related to age

Within the age range studied, no age-related changes in blood BDNF concentrations was found (Fig. 6, $n = 200$). Neither when taking gender into account, no age-related associations were present. The gender effect on BDNF concentrations remained, however, significant ($n = 200$, $t = -2{,}656$, $p = 0.009$). When dividing into men and women, a weak association with age ($r = 0.08$, $t = 2.052$, $p = 0.04$) was found in women only. Further, the linear model was confirmed when testing for an association in the quadratic term ($p = 0.84$).

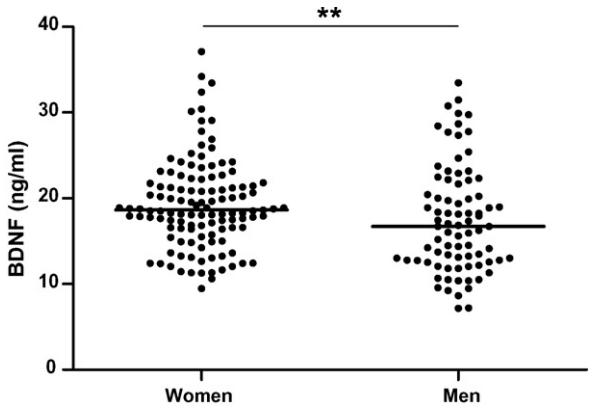

Fig. 5. Women have higher whole blood BDNF concentrations than men (18.6 ± 1.3 ng/ml vs. 16.5 ± 1.4 ng/ml, $n = 200$, $p = 0.0057$). A normal distribution of whole blood BDNF concentrations is found in men only.

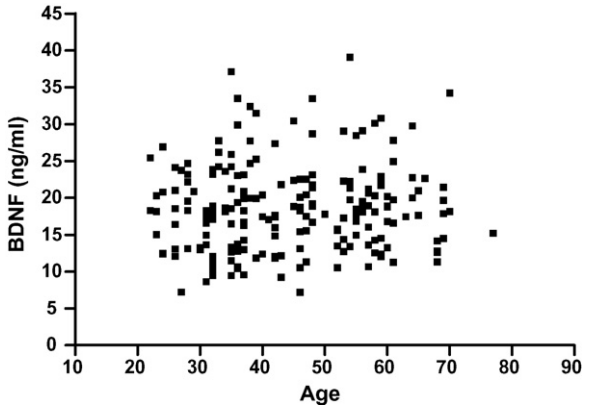

Fig. 6. No significant age effect on whole blood BDNF concentration was found in healthy subjects (age 44.5 ± 13.1 years, $n = 200$).

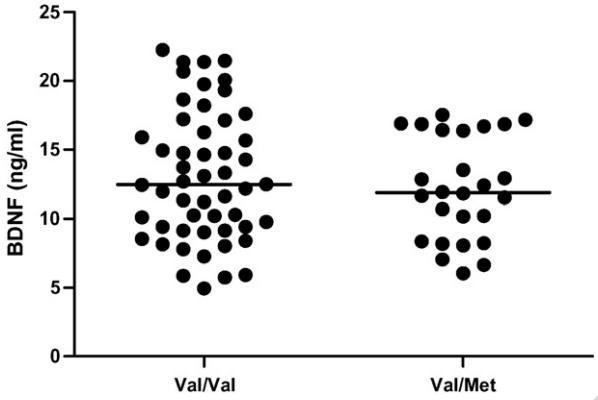

Fig. 7. In a sample of 78 healthy subjects we did not identify any influence of the Val66Met polymorphism on whole blood BDNF concentrations. The two groups are gender matched.

3.9. Whole blood BDNF concentration and BDNF Val66Met polymorphism

Seventy-eight of the individuals were genotyped for the BDNF Val66Met functional polymorphism. Men and women had similar allele frequencies (1:3). Whole blood BDNF concentrations were similar in Val/Val as compared to Val/Met carriers, 12.2 ± 1.5 ng/ml versus 11.6 ± 1.4 ng/ml (Fig. 7, $n = 78$).

4. Discussion

This is the first study to systematically validate the BDNF ELISA assay with respect to accuracy, yield, inter-assay, and intra-subject variability. In addition, the stability of BDNF in samples under various storage conditions and the relation between serum and whole blood BDNF was assessed. We found that the BDNF ELISA assay is accurate and highly reproducible [3,29]. Based on our data we recommend that duplicate determinations are carried out. Further, to ensure stability of the assay for e.g., comparisons over time we recommend including at least two internal blood standards. The inter-assay variability was below 10%, which is considered low in comparison to other assays [29,45]. The longitudinal BDNF measurements in the same subject also showed that on an individual level, whole blood BDNF concentrations were relatively stable, at least within a time period of up to 8 months.

Storage of whole blood samples for up to 5 years at −20 °C was not associated with any significant decrease in whole blood BDNF concentration. By contrast, long-term storage of serum samples was associated with significantly lower serum BDNF concentrations. In fact, already after 6–10 months storage at −20 °C, 15% of the measured serum BDNF concentrations were below zero (Fig. 4). This finding suggests that BDNF, when stored in platelets, is protected from degradation. After intravenous BDNF injection plasma BDNF is cleared with a half-life of less than 60 min [19,34]. A small fraction of BDNF binds to alpha-2-macroglobulin [47] and brain capillaries, and some is stored in platelets, but the most important cause of the rapid plasma clearance is degradation [34]. Accordingly, BDNF is better assessed in whole blood than in serum, particularly when the BDNF measurements are not carried out immediately. Most studies [16,23,28] do not, however, contain information about time lapsed between sampling and analysis and storage conditions. Based on our data, this may be a critical issue.

For serum samples stored for less than 12 months we found no significant difference in whole blood versus serum BDNF, but serum BDNF values had a higher variance. Karege and coworkers have earlier found a serum: blood BDNF ratio of 74% [28], but it is not stated if this difference is statistically significant, neither is there any information on the possible confounding effect of the time elapsed between serum sampling and analysis.

A right-skewed whole blood BDNF concentration distribution was observed in our sample of 206 healthy subjects; this was due to a few subjects having very high BDNF concentrations. This finding corroborates the outcome from another large-scale study [50] and suggests that transformation of BDNF data may be needed prior to statistical analysis. The non-normality distribution may eventually remain uncovered in small sample size studies.

In accordance with a previous study where no correlation between age and serum or platelet BDNF [24] was found, we did not identify any age effect on whole blood BDNF concentrations. By contrast, one other study identified a weak age associated decline in serum BDNF in a group of elderly subjects, aged between 70 and 103 years ($r = -0.15$) [50]. Animal studies point at serum BDNF concentrations as reflecting brain BDNF levels well [18] and there is no evidence that brain BDNF concentrations change with aging [46].

In our study of 200 healthy subjects, whole blood BDNF was higher in women than men, similar to what has been reported for plasma BDNF in a previous study of 140 subjects [24]. In contrast, two earlier studies did not reveal any gender effects on serum BDNF [20,50]. We also found that whole blood BNDF displayed a right-skewed distribution in women only. This distribution may be explained by variation in BDNF concentration during the different stages in the menstrual cycle, as suggested from rodent [38] and clinical studies [24]. Some might argue that gender differences in blood BDNF concentrations are due to differences in BMI since some studies are suggestive of a positive correlation between body mass index (BMI) and BDNF [6,10,27]. But this association is most strongly found in studies

including extreme overweight and underweight subjects. In our sample, where most subjects were normal-weighted we did not, however, detect any significant correlation between BMI and whole blood BDNF (data not shown here).

The BDNF Val66Met polymorphism affects activity-dependent secretion of BDNF in neuronal cultures [5,9] and is associated with poorer performance in episodic memory tests in humans [9,14]. This polymorphism is frequent (19–25%) in Caucasians and has been reported to be associated with a decreased hippocampal volume [36]. Here we show that the BDNF Val66Met genotype is not associated with differences in whole blood BDNF concentrations. One can hypothesize that the antibodies in the BDNF ELISA may have a different affinity for Val/Val and Val/Met, but generally epitopes encompass several amino acids so small differences in the BDNF sequence would be left undetected by the antibody. Furthermore, there is no clinical evidence that BDNF Val66Met polymorphism alter BDNF concentrations. In a post-mortem study of patients with Alzheimer's disease brain BDNF protein levels did not differ between various BDNF genotypes and alleles [22]. The previously reported effect of the BDNF Val66Met polymorphism on cognitive performance or hippocampal volume may thus be explained by the polymorphism having a high impact on brain development. Alternatively, local activity-dependent secretion from neurons may be influenced by the BDNF polymorphism whereas the constitutive secretion and average BDNF concentrations in brain and blood are unaffected by the genotype [4]. Interestingly, both major depression [17,42] and Alzheimer's disease [21,25] have been associated with both the functional Val66Met polymorphism and lower BDNF concentrations in blood, but according to our data there is not any clear association between the two measures.

In conclusion, this study shows that the BDNF ELISA assay yields accurate and highly reproducible BDNF measurements. It is recommended to conduct the analysis in duplicate and to include at least two internal blood standards. Serum samples should be analysed immediately, whereas whole blood can safely be stored at −20 °C for at least 5 years. Whole blood samples can be thawed up to three times without any impact on the whole blood BDNF determinations. The intra-individual variation over time in whole blood BDNF levels is modest. We failed to identify any association between whole blood BDNF and thrombocyte count, age, or BDNF Val66Met genotype. Women had significantly higher whole blood BDNF concentrations than men and in addition, showed a right-skewed BDNF concentration distribution.

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References

[1] C.A. Altar, R.E. Whitehead, R. Chen, G. Wortwein, T.M. Madsen, Effects of electroconvulsive seizures and antidepressant drugs on brain-derived neurotrophic factor protein in rat brain, Biol. Psychiatry 54 (2003) 703–709.
[2] D. Azoulay, V. Vachapova, B. Shihman, A. Miler, A. Karni, Lower brain-derived neurotrophic factor in serum of relapsing remitting MS: reversal by glatiramer acetate, J. Neuroimmunol. 167 (2005) 215–218.
[3] S.D. Blacksell, P.N. Newton, D. Bell, J. Kelley, M.P. Mammen Jr., D.W. Vaughn, V. Wuthiekanun, A. Sungkakum, A. Nisalak, N.P. Day, The comparative accuracy of 8 commercial rapid immunochromatographic assays for the diagnosis of acute dengue virus infection, Clin. Infect. Dis. 42 (2006) 1127–1134.
[4] Z.Y. Chen, D. Jing, K.G. Bath, A. Ieraci, T. Khan, C.J. Siao, D.G. Herrera, M. Toth, C. Yang, B.S. McEwen, B.L. Hempstead, F.S. Lee, Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior, Science 314 (2006) 140–143.
[5] Z.Y. Chen, P.D. Patel, G. Sant, C.X. Meng, K.K. Teng, B.L. Hempstead, F.S. Lee, Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons, J. Neurosci. 24 (2004) 4401–4411.
[6] A. Chiaretti, M. Piastra, G. Polidori, C. Di Rocco, E. Caresta, A. Antonelli, T. Amendola, L. Aloe, Correlation between neurotrophic factor expression and outcome of children with severe traumatic brain injury, Intens. Care Med. 29 (2003) 1329–1338.
[7] G. De Foubert, S.L. Carney, C.S. Robinson, E.J. Destexhe, R. Tomlinson, C.A. Hicks, T.K. Murray, J.P. Gaillard, C. Deville, V. Xhenseval, C.E. Thomas, M.J. O'Neill, T.S. Zetterstrom, Fluoxetine-induced change in rat brain expression of brain-derived neurotrophic factor varies depending on length of treatment, Neuroscience 128 (2004) 597–604.
[8] M.J. Donovan, R.C. Miranda, R. Kraemer, T.A. McCaffrey, L. Tessarollo, D. Mahadeo, S. Sharif, D.R. Kaplan, P. Tsoulfas, L. Parada, et al., Neurotrophin and neurotrophin receptors in vascular smooth muscle cells. Regulation of expression in response to injury, Am. J. Pathol. 147 (1995) 309–324.
[9] M.F. Egan, M. Kojima, J.H. Callicott, T.E. Goldberg, B.S. Kolachana, A. Bertolino, E. Zaitsev, B. Gold, D. Goldman, M. Dean, B. Lu, D.R. Weinberger, The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function, Cell 112 (2003) 257–269.
[10] A.H. El-Gharbawy, D.C. Adler-Wailes, M.C. Mirch, K.R. Theim, L. Ranzenhofer, M. Tanofsky-Kraff, J.A. Yanovski, Serum brain derived neurotrophic factor concentrations in lean and overweight children and adolescents, J. Clin. Endocrinol. Metab. 91 (2006) 3548–3552.
[11] H. Fujimura, C.A. Altar, R. Chen, T. Nakamura, T. Nakahashi, J. Kambayashi, B. Sun, N.N. Tandon, Brain-derived neurotrophic factor is stored in human platelets and released by agonist stimulation, Thromb. Haemost. 87 (2002) 728–734.
[12] D. Garzon, G. Yu, M. Fahnestock, A new brain-derived neurotrophic factor transcript and decrease in brain-derived neurotrophic factor transcripts 1, 2 and 3 in Alzheimer's disease parietal cortex, J. Neurochem. 82 (2002) 1058–1064.
[13] A. Gielen, M. Khademi, S. Muhallab, T. Olsson, F. Piehl, Increased brain-derived neurotrophic factor expression in white blood cells of relapsing-remitting multiple sclerosis patients, Scand. J. Immunol. 57 (2003) 493–497.
[14] A.R. Hariri, T.E. Goldberg, V.S. Mattay, B.S. Kolachana, J.H. Callicott, M.F. Egan, D.R. Weinberger, Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance, J. Neurosci. 23 (2003) 6690–6694.

[15] J.P. Jacobsen, A. Mork, Chronic corticosterone decreases brain-derived neurotrophic factor (BDNF) mRNA and protein in the hippocampus, but not in the frontal cortex, of the rat, Brain. Res. 1110 (2006) 221–225.
[16] F. Karege, G. Bondolfi, N. Gervasoni, M. Schwald, J.M. Aubry, G. Bertschy, Low brain-derived neurotrophic factor (BDNF) levels in serum of depressed patients probably results from lowered platelet BDNF release unrelated to platelet reactivity, Biol. Psychiatry 57 (2005) 1068–1072.
[17] F. Karege, G. Perret, G. Bondolfi, M. Schwald, G. Bertschy, J.M. Aubry, Decreased serum brain-derived neurotrophic factor levels in major depressed patients, Psychiatry Res. 109 (2002) 143–148.
[18] F. Karege, M. Schwald, M. Cisse, Postnatal developmental profile of brain-derived neurotrophic factor in rat brain and platelets, Neurosci. Lett. 328 (2002) 261–264.
[19] A. Kishino, N. Katayama, Y. Ishige, Y. Yamamoto, H. Ogo, T. Tatsuno, T. Mine, H. Noguchi, C. Nakayama, Analysis of effects and pharmacokinetics of subcutaneously administered BDNF, Neuroreport 12 (2001) 1067–1072.
[20] U.E. Lang, R. Hellweg, J. Gallinat, BDNF serum concentrations in healthy volunteers are associated with depression-related personality traits, Neuropsychopharmacology 29 (2004) 795–798.
[21] C. Laske, E. Stransky, T. Leyhe, G.W. Eschweiler, W. Maetzler, A. Wittorf, S. Soekadar, E. Richartz, N. Koehler, M. Bartels, G. Buchkremer, K. Schott, BDNF serum and CSF concentrations in Alzheimer's disease, normal pressure hydrocephalus and healthy controls, J. Psychiatr. Res. 41 (2007) 387–394.
[22] J. Lee, H. Fukumoto, J. Orne, J. Klucken, S. Raju, C.R. Vanderburg, M.C. Irizarry, B.T. Hyman, M. Ingelsson, Decreased levels of BDNF protein in Alzheimer temporal cortex are independent of BDNF polymorphisms, Exp. Neurol. 194 (2005) 91–96.
[23] M. Lommatzsch, K. Schloetcke, J. Klotz, K. Schuhbaeck, D. Zingler, C. Zingler, O. Schulte-Herbruggen, H. Gill, P. Schuff-Werner, J.C. Virchow, Brain-derived neurotrophic factor in platelets and airflow limitation in asthma, Am. J. Respir. Crit. Care Med. 171 (2005) 115–120.
[24] M. Lommatzsch, D. Zingler, K. Schuhbaeck, K. Schloetcke, C. Zingler, P. Schuff-Werner, J.C. Virchow, The impact of age, weight and gender on BDNF levels in human platelets and plasma, Neurobiol. Aging 26 (2005) 115–123.
[25] S. Matsushita, H. Arai, T. Matsui, T. Yuzuriha, K. Urakami, T. Masaki, S. Higuchi, Brain-derived neurotrophic factor gene polymorphisms and Alzheimer's disease, J. Neural. Transm. 112 (2005) 703–711.
[26] A. Nair, K.C. Vadodaria, S.B. Banerjee, M. Benekareddy, B.G. Dias, R.S. Duman, V.A. Vaidya, Stressor-specific regulation of distinct brain-derived neurotrophic factor transcripts and cyclic AMP response element-binding protein expression in the postnatal and adult rat hippocampus, Neuropsychopharmacology, doi:10.1038/sj.npp.1301276.
[27] M. Nakazato, K. Hashimoto, E. Shimizu, C. Kumakiri, H. Koizumi, N. Okamura, M. Mitsumori, N. Komatsu, M. Iyo, Decreased levels of serum brain-derived neurotrophic factor in female patients with eating disorders, Biol. Psychiatry 54 (2003) 485–490.
[28] A. Neumeister, P. Yuan, T.A. Young, O. Bonne, D.A. Luckenbaugh, D.S. Charney, H. Manji, Effects of tryptophan depletion on serum levels of brain-derived neurotrophic factor in unmedicated patients with remitted depression and healthy subjects, Am. J. Psychiatry 162 (2005) 805–807.
[29] J. Ngeh, S. Gupta, C. Goodbourn, The reproducibility of an enzyme-linked immunosorbent assay for detection of Chlamydia pneumoniae-specific antibodies, Clin. Microbiol. Infect. 10 (2004) 171–174.
[30] O. Noga, C. Englmann, G. Hanf, A. Grutzkau, J. Seybold, G. Kunkel, The production, storage and release of the neurotrophins nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 by human peripheral eosinophils in allergics and non-allergics, Clin. Exp. Allergy 33 (2003) 649–654.
[31] L. Novikova, L. Novikov, J.O. Kellerth, Effects of neurotransplants and BDNF on the survival and regeneration of injured adult spinal motoneurons, Eur. J. Neurosci. 9 (1997) 2774–2777.
[32] A. Palomino, A. Vallejo-Illarramendi, A. Gonzalez-Pinto, A. Aldama, C. Gonzalez-Gomez, F. Mosquera, G. Gonzalez-Garcia, C. Matute, Decreased levels of plasma BDNF in first-episode schizophrenia and bipolar disorder patients, Schizophr. Res. 86 (2006) 321–322.
[33] W. Pan, W.A. Banks, M.B. Fasold, J. Bluth, A.J. Kastin, Transport of brain-derived neurotrophic factor across the blood–brain barrier, Neuropharmacology 37 (1998) 1553–1561.
[34] W.M. Pardridge, Y.S. Kang, J.L. Buciak, Transport of human recombinant brain-derived neurotrophic factor (BDNF) through the rat blood–brain barrier in vivo using vector-mediated peptide drug delivery, Pharm. Res. 11 (1994) 738–746.
[35] S. Peng, J. Wuu, E.J. Mufson, M. Fahnestock, Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the pre-clinical stages of Alzheimer's disease, J. Neurochem. 93 (2005) 1412–1421.
[36] L. Pezawas, B.A. Verchinski, V.S. Mattay, J.H. Callicott, B.S. Kolachana, R.E. Straub, M.F. Egan, A. Meyer-Lindenberg, D.R. Weinberger, The brain-derived neurotrophic factor val66met polymorphism and variation in human cortical morphology, J. Neurosci. 24 (2004) 10099–10102.
[37] S.F. Radka, P.A. Holst, M. Fritsche, C.A. Altar, Presence of brain-derived neurotrophic factor in brain and human and rat but not mouse serum detected by a sensitive and specific immunoassay, Brain Res. 709 (1996) 122–301.
[38] H.E. Scharfman, T.C. Mercurio, J.H. Goodman, M.A. Wilson, N.J. MacLusky, Hippocampal excitability increases during the estrous cycle in the rat: a potential role for brain-derived neurotrophic factor, J. Neurosci. 23 (2003) 11641–11652.
[39] A. Schenone, J.S. Gill, D.A. Zacharias, A.J. Windebank, Expression of high- and low-affinity neurotrophin receptors on human transformed B lymphocytes, J. Neuroimmunol. 64 (1996) 141–149.
[40] A.F. Schinder, M. Poo, The neurotrophin hypothesis for synaptic plasticity, Trends Neurosci. 23 (2000) 639–645.
[41] B. Schuhmann, A. Dietrich, S. Sel, C. Hahn, M. Klingenspor, M. Lommatzsch, T. Gudermann, A. Braun, H. Renz, W.A. Nockher, A role for brain-derived neurotrophic factor in B cell development, J. Neuroimmunol. 163 (2005) 15–23.
[42] C. Schule, P. Zill, T.C. Baghai, D. Eser, P. Zwanzger, N. Wenig, R. Rupprecht, B. Bondy, Brain-derived neurotrophic factor Val66Met polymorphism and dexamethasone/CRH test results in depressed patients, Psychoneuroendocrinology 31 (2006) 1019–1025.
[43] E. Shimizu, K. Hashimoto, N. Okamura, K. Koike, N. Komatsu, C. Kumakiri, M. Nakazato, H. Watanabe, N. Shinoda, S. Okada, M. Iyo, Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants, Biol. Psychiatry 54 (2003) 70–75.
[44] K. Toyooka, K. Asama, Y. Watanabe, T. Muratake, M. Takahashi, T. Someya, H. Nawa, Decreased levels of brain-derived neurotrophic factor in serum of chronic schizophrenic patients, Psychiatry Res. 110 (2002) 249–257.
[45] V.V. Upreti, R.N. Mamidi, K. Katneni, N.R. Srinivas, Quantitative determination of DRF-1042 in human plasma by HPLC: validation and application in clinical pharmacokinetics, Biomed. Chromatogr. 17 (2003) 385–390.
[46] M.J. Webster, C.S. Weickert, M.M. Herman, J.E. Kleinman, BDNF mRNA expression during postnatal development, maturation and aging of the human prefrontal cortex, Brain Res. Dev. Brain Res. 139 (2002) 139–150.
[47] B.B. Wolf, S.L. Gonias, Neurotrophin binding to human alpha 2-macroglobulin under apparent equilibrium conditions, Biochemistry 33 (1994) 11270–11277.
[48] H. Yamamoto, M.E. Gurney, Human platelets contain brain-derived neurotrophic factor, J. Neurosci. 10 (1990) 3469–3478.
[49] Z.F. Yang, D.W. Ho, C.K. Lau, K.H. Tam, C.T. Lam, R.T. Poon, S.T. Fan, Platelet activation during tumor development, the potential role of BDNF-TrkB autocrine loop, Biochem. Biophys. Res. Commun. 346 (2006) 981–985.
[50] A.A. Ziegenhorn, O. Schulte-Herbruggen, H. Danker-Hopfe, M. Malbranc, H.D. Hartung, D. Anders, U.E. Lang, E. Steinhagen-Thiessen, R.T. Schaub, R. Hellweg, Serum neurotrophins-A study on the time course and influencing factors in a large old age sample, Neurobiol. Aging, doi:10.1016/j.neurobiolaging.2006.06.011.

Paper IV

Whole blood BDNF levels in healthy twins discordant for affective disorder: Association to life events and neuroticism

Viktorija Trajkovska[a,*], Maj Vinberg[b], Susana Aznar[a], Gitte M. Knudsen[a], Lars V. Kessing[b]

[a] *Neurobiology Research Unit and Center for Integrated Molecular Brain Imaging, Copenhagen University Hospital, Section 9201, Blegdamsvej 9, 2100 Copenhagen, Denmark.*

[b] *Department of Psychiatry, Copenhagen University Hospital, Section 6233, Blegdamsvej 9, DK-2100 Copenhagen, Denmark.*

* Correspondence:

Viktorija Trajkovska, M. Med, PhD student

Neurobiology Research Unit,

Rigshospitalet 9201

Blegdamsvej 9

DK- 2100 Copenhagen

Denmark

Phone: +45 3545 6736

Fax: +45 3545 6713

e-mail: viktorija.trajkovska@nru.dk

Abstract

Background: Depression has been associated with decreased blood BDNF concentrations; but it is unclear if low blood BDNF levels are a state or a trait marker of depression.

Methods: We investigated blood BDNF concentrations in a twin population including both subjects highly predisposed and protected against affective disorder. Four different twin risk groups were included: high-risk monozygotic predisposed twins (co-twin depressed), moderately predisposed dizygotic twins, moderately protected dizygotic twins (co-twin unaffected), and highly protected monozygotic twins. Whole blood was collected and assessed for BDNF concentrations, and correlated to risk status, neuroticism, and number of stressful life events.

Results: Between the groups, we found no significant difference in whole blood BDNF levels. Women at high risk for depression who had experienced three or more recent stressful events (n= 26) had decreased whole blood BDNF levels compared to high-risk women with less than three recent stressful events (n= 35), 21.6 ± 7.0 vs. 18.5 ± 4.1 ng/ml, respectively, ($p < 0.05$). No such association was found in low-risk women or in men. In men, however, low neuroticism scores and two or less recent stressful events were associated with decreased whole blood BDNF levels (n= 50, $p < 0.05$).

Limitations: The cross-sectional design limits the possibility of drawing firm conclusions on causatility of the findings.

Conclusion: This is the first study to show that blood BDNF may be a trait marker for depression as BDNF is decreased in females with many recent stressful events who are genetically predisposed for affective disorder.

Keywords: genetic predisposition; major depression; brain-derived neurotrophic factor (BDNF); neuroticism; stressful life events; vulnerability.

1. Introduction

Major depression is a serious neuropsychiatric illness that is one of the world's greatest public health burdens (World Health Organisation 2001). There is accumulative evidence that brain-derived neurotrophic factor (BDNF) may be involved in the pathophysiology of major depression. BDNF belongs to the neurotrophin family that also includes nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), and is crucially involved in brain development, neurogenesis, neural circuit formation and plasticity (Schinder et al., 2000; McAllister et al., 1999; Pencea et al., 2001).

Experimentally, it has been established that acute and repeated stress reduces brain BDNF levels in rodents (Gronli et al., 2007; Murakami et al., 2005) and that intracerebral infusion of BDNF elicits antidepressant-like effects in animal model of depression (Shirayama et al., 2002). Brain and blood BDNF concentrations undergo parallel changes during maturation and aging (Karege et al., 2002, b) and blood and brain BDNF levels at a given time strongly correlate (Karege et al., 2002, b), which indicates that measurements of blood BDNF levels reflects brain BDNF levels well. Furthermore, the Val66Met BDNF polymorphism is associated with increased anxiety-related behaviour in mice (Chen et al., 2006), human liability to mood disorders (Strauss et al., 2005; Neves-Pereira et al., 2002) and may affect the response to antidepressant treatment (Choi et al., 2006).

Patients with major depression have decreased serum and plasma BDNF concentrations (Karege et al., 2002, a; Gonul et al., 2005; Karege et al., 2005; Lee et al., 2006) that both correlate negatively with the clinical severity of their depression (Karege et al., 2002, a; Shimizu et al., 2003), and with the depression-related personality trait neuroticism (Lang et al., 2004). It is yet unclear, however, if low

blood BDNF level is a state or a trait of human depression. Since a high score on neuroticism is associated with an increased susceptibility to mood disorders (Kendler et al., 2004; Vinberg et al., 2006), we hypothesized that an increased genetic and personality profile risk of developing mood disorders would also be associated with low blood BDNF concentration.

In this study we examined whole blood BDNF levels in twins genetically predisposed to mood disorder. The primary aim of this study was to assess whether whole blood BDNF levels are decreased in subjects predisposed to affective disorder, and secondly, whether whole blood BDNF levels are associated with neuroticism score or the number of adverse stressful life events in men and women.

2. Method

2.1 The registers

2.1.1 The Danish Civil Registration System

The Danish Civil Registration System assigns a unique personal identification number for all Danish residents. This number is linked to information about name, address, date of birth, death, emigration and immigration that all are recorded in the system. All other Danish registers use the same unique identifier and thus Danish residents can be tracked in all the public registers through record linkage.

2.1.2 The Danish Psychiatric Central Research Register

The Danish Psychiatric Central Research Register is nationwide, with registration of all psychiatric admissions and outpatients' hospital contacts in Denmark for the country's 5.3 million inhabitants. From April 1969 to December 1993, diseases were classified according to the International Classification of Diseases,"8th"(ICD-8) (World

Health Organisation 1967) and from January 1994, International Classification of Diseases, "10th" (ICD-10) (World Health Organisation 2005).

2.1.3 The Danish Twin Registry

The Danish Twin Registry was initiated in 1953 and contains information on 75,000 twin pairs born from 1870 to 2003. The completeness varies with the birth cohort and is approximately 70 % for the period before and close to 100 % for the period after the Civil Registration System was established in 1968 (Kyvik et al., 1996; Harvald et al., 2004).

2.2 The linkage

Through record linkage between the Danish Twin Registry, The Danish Psychiatric Central Research Register and the Danish Civil registration system, a cohort of "high risk" twins was identified. This linkage identified 204 twin pairs (same sex, age 22 to 70 years) in which one twin had been treated in a psychiatric hospital setting for an affective episode (the proband) and one had not been treated for affective disorder, the High-Risk healthy twin. Further four control-twins (low risk) were identified, without known personal or co-twin history of hospital contact with affective disorder, and matched on age, sex and zygosity for each high risk twin.

Probands were identified as twins who on their first admission, in the period between 1968 and 2005, were discharged from a psychiatric hospital with a diagnosis of depression or recurrent depression (ICD-8-codes 296.09, 296.29, 296.89, 296.99; ICD-10-codes: F32-33.9) or a first diagnosis of manic/mixed episode or bipolar affective disorder (ICD-8-codes 296.19, 296.39, ICD-10-codes: F30-31.6, F34.0, F38.00). Control-twins (low risk) were identified as twins without known personal or co-twin

history of hospital contact with affective disorder, and matched on age, sex and zygosity for each high-risk twin.

2.3 Inclusion and exclusion criteria for High-Risk twins

Inclusion criteria: having a monozygotic (MZ) or a dizygotic (DZ) co-twin with a diagnosis of affective disorder (co-twin history of affective disorder) according to information from the Danish Psychiatric Central Research Register. Exclusion criteria: a personal history of severe to moderate depression, earlier medical treatment for an affective episode, severe organic brain disease or schizophrenia.

2.4 Inclusion and exclusion criteria for Low-Risk Twins

Inclusion criteria: no family history of affective disorder among the MZ or DZ co-twin or other severe psychiatric illness among other first- degree relatives. Exclusion criteria: same as for high risk persons. The control twins and their co-twins were not recorded in the Danish Psychiatric Central Research Register with a diagnosis of mania or depression. However, it is possible that they or their co-twin had received another diagnosis. If a low risk twin had been admitted to a psychiatric hospital they were excluded. If they at the end of the interview answered that a first-degree relative had a history of severe psychiatric illness they were reclassified to the group of twins with another family history.

2.5 Ethics

The Danish Ministry of Health, The Danish Ethical Committee ((KF)-12-122/99 and (KF)-01-001/02) and the Data Inspection approved the study. The study was conducted in accordance with the latest version of the Declaration of Helsinki. All procedures were carried out with the adequate understanding and written informed

consent of the participants.

2.6 Assessment

During the recruitment period (May 2003 to September 2005) 204 high risk and 204 low risk twins were invited to participate in the study. A total of 408 letters containing an invitation, informed consent and a return envelope were posted to the twins. A total of 271 twins agreed to participate; subsequently 37 persons were excluded according to the mentioned exclusion criteria and blood samples were not collected in another 28 subjects. The 206 participants, 122 women and 84 men, were divided into four groups according to risk of affective disorder. Additionally, a fifth group was defined as participants with another first generation family history of affective disorder or schizophrenia (n= 14). For detailed presentation of the study design, inclusion and exclusion of subjects see (Christensen et al., 2006).

Participants were rated in a face-to-face interview using semi-structured interviews: diagnoses were made using Schedules for Clinical Assessment in Neuropsychiatry (SCAN) version 2.1 (Wing et al., 1990). All persons with a lifetime (current or past) diagnosis of affective disorder, schizoaffective disorder or schizophrenia according to SCAN interview were excluded from the study. Self rating of psychopathology was assessed using Symptom Scale for Depression and Anxiety including assessment of depressive symptoms using the 21-item Beck Depression Inventory, BDI 21 (Beck et al., 1961).

2.7 Life events

Participants were asked about life events in the year prior to the interview (recent life events) and life events in their lifetime before that, using a Danish version of the

questionnaire used in the studies of Kendler and colleagues (Kendler et al., 1995). Participants were asked about personal and network events. Nine personal events (events that happened to the participants) were assessed including assault, serious marital problems, divorce/break-up, job loss, and loss of a confidant, serious illness, major financial problem, being robbed, and serious legal problems. I addition, 22 network events were assessed i.e. events that occurred primarily to, or in interaction with, and individual in the participant's social network. Included were death or severe illness of the participant's spouse, child, parent, co-twin, other sibling, other relative or other individuals close to the participant and serious trouble getting along with the participant's parent, child, co-twin, sibling, in-laws, other relatives, neighbour, or close friend. Recent life events were categorized in five groups (no events, one event, two events, three events, four or more events).

2.8 Personality measure

Personality dimensions were assessed using the EPQ, Danish version. The EPQ comprises 101 items intended to measure a broad dimension of neuroticism, extroversion and psychotism (Eysenck 1975). The Danish version of the EPQ has shown coefficient alpha values of 0.87 for neuroticism and 0.84 for extraversion (Mortensen et al., 1996).

2.9 Whole blood samples

A total of 206 whole blood samples were collected from the participants. Whole blood was collected in EDTA containing tubes and immediately frozen. At the time of examination whole blood was lysed with 3 % triton X-100 (Merck, Germany) and sonication (Toyooka et al., 2002). Disrupted cell membranes were discarded by

centrifuging the samples at 12000 rpm for 10 minutes at 4 °C and the supernatant was aliquoted and kept at -20 °C until further processing.

2.10 Determination of BDNF concentration with ELISA

Lysed blood samples were assessed for BDNF concentrations using a commercially available two-site sandwich enzyme-linked immunoabsorbent assay (ELISA) kit (Chemicon, Temecula, USA). The samples were processed as earlier described (Trajkovska et al., 2007). According to the manufacturer, the BDNF ELISA kit has no significant cross-reactivity to the neurotrophins NGF, NT4/5 or NT3.

2.11 Whole blood BDNF ELISA

In order to make sure that the measurements of BDNF were done under optimal conditions several precautions were undertaken (Trajkovska et al., 2007): Care was taken not to freeze and thaw the aliquot of the samples more than one time after lysis and before BDNF analysis, and the samples were all stored at -20 ˚C. All samples were processed within one week after lysis.

In order to minimize inter-sample variance resulting from experimental variation, all 206 samples were measured simultaneously and with BDNF ELISA kits from the same batch. The determinations of the BDNF concentrations in the samples were done in duplicate. Duplicate determinations of absorbancy with a coefficient of variance above 10 % were excluded from the study resulting in a total of 200 samples.

2.12 Statistical analysis

D´Agostino and Pearson omnibus normality test was used to test for the presence of a normal distribution. Since whole blood BDNF concentrations did not comply with a

normal distribution they were either logarithmically transformed to accommodate to a normal distribution or the Spearman´s correlation test was used. For comparison between groups, two-tailed unpaired t-test or analysis of variance (ANOVA) was used. Linear regression was applied on log-transformed data to investigate the effect of neuroticism, recent events and life events on BDNF concentration. Univariate analysis of variance was applied with sex as a covariate when studying the effect of risk, neuroticism and number of events on BDNF levels. Multivariate analysis was applied to investigate the influence of sex, genetic risk, age, and 21-item Beck Depression Inventory (BDI 21) on whole blood BDNF levels. Data was analysed using the Statistical Package for the Social Sciences (SPSS) and GraphPad. Significance level was set at $p< 0.05$ (two-sided). All data are presented as means ± SD.

3. Results

3.1 Blood BDNF levels in high-risk and low-risk twins

No difference in whole blood BDNF levels was found between high risk (n= 106) and low risk (n= 80) twins, 19.5 ± 6.4 vs. 18.5 ± 5.0 ng/ml. Similarly, when high risk and low risk twins were separated into monozygotic and dizygotic groups, no differences in whole blood BDNF levels were identified (Figure 1). As earlier reported (Trajkovska et al., 2007), female gender is associated with higher whole blood BDNF levels (n=200, F= 7.854, p= 0.006). Gender also interacted with the association between group risk and whole blood BDNF (n=200, F= 4.554, p= 0.001), while no association with age and BDI 21 was found. When high risk twins predisposed to bipolar disorder were excluded (n= 6 monozygotic, n=13 dizygotic) from the data analysis, similar results were obtained.

3.2 Whole blood BDNF and life events

Recent events were defined as stressful life events in the year prior to the interview, while lifetime life events were defined as stressful life events in their lifetime before that. Twenty nine percent of the subjects had experienced no recent events; 22% experienced one event; 17% two events; and 32% three or more events. For the lifetime life events the corresponding numbers were; 18% no life events; 27% one event; 15% two events; 40% three or more events.

No significant association between whole blood BDNF and recent events (n= 196) or lifetime life events (n= 192) was identified in the total sample. High risk twins reported a higher number of recent events than low risk twins, 2.8 ± 2.8 vs. 1.4 ± 1.6 (n= 196, Mann Whitney U= 2788, p= 0.0003), but no association between recent events and whole blood BDNF was found neither in the high nor in low risk ($p > 0.05$).

Experiencing three or more events is associated with a great increase in depression liability, as to experiencing two or less (Caspi et al., 2003), therefore the effect of life events on whole blood BDNF was studied in two groups of two or less and three or more events. High risk women with three or more recent events (n=26), however, had lower whole blood BDNF levels than women experiencing 2 or less recent events (n=35) (Figure 2, t= 2.126, p= 0.038) whereas such an association was not present in low risk women (n= 48) nor in high risk (n= 43) and low risk men (n= 29).

3.3 Whole BDNF levels and neuroticism

No association between whole blood BDNF levels and neuroticism in the total sample (n= 195, p= 0.57) was found, nor was there any significant association between whole blood BDNF and neuroticism in high risk and low risk twins or in men and women,

respectively. As expected (Vinberg et al., 2006), high risk twins displayed higher neuroticism scores (n= 181, F= 3.275, p= 0.023) compared to low risk twins. Further, a positive association between neuroticism and number of recent events (n= 181, F= 2.317, p= 0.01) was found. As high risk twins displayed a mean neuroticism score around five, the effects of neuroticism and life events on whole blood BDNF levels were studied in two subgroups of subjects with neuroticism score over and under five. Men with high scores on neuroticism (scores over five) that have experienced three or more recent events (n= 14) had significantly higher BDNF levels than men with low neuroticism (scores under 5) *and* less than three recent events (n= 36), 20.9 ± 5.0 versus 16.5 ± 5.0 ng/ml (t= 2.442, p= 0.02). By contrast, women with high scores on neuroticism *and* with three or more recent events (n= 23) showed a tendency towards decreased whole blood BDNF levels compared to low neuroticism scoring women with two or less recent events (n= 49, p= 0.07).

4. Discussion

This is the first study to investigate BDNF level in healthy first-degree relatives of patients with affective disorder. Apart from the genetic predisposition we also took into account two additional factors known to be predictive for development of affective disorder, namely more than two recent stressful life events (Caspi et al., 2003) and a high score on the personality trait neuroticism (Kendler et al., 2006; Kendler et al., 2004, b).

We found that whole blood BDNF levels were similar in healthy twins with or without a genetic predisposition to depression. When recent stressful life events, however, were taken into account it was found that women highly genetically predisposed to depression that had experienced three or more recent stressful events had decreased

whole blood BDNF levels compared to high risk women with two or less recent events. The absence of such an association in men and in women genetically protected against depression supports the notion that females with genetic predisposition to affective disorders are particularly vulnerable to depression (Kendler et al., 2006, a). Interestingly, men with high neuroticism (score over four) that had experienced three or more recent events had higher whole blood BDNF levels than men with low neuroticism and less than three recent events, irrespective of their genetic makeup. We interpret this finding such that in men BDNF levels are appropriately counteracting two risk factors for depression, thereby rendering these at-risk men less likely to develop depression.

Mood disorder is a highly familial disease; heritability for depression is approximated to 40% (Sullivan et al., 2000; Levinson 2006). Kendler and colleagues were the first to describe a study design that identified twins in four categories of risk by crossing zygosity with family history of affective disorder (Kendler et al., 1995) similar to our sample. The predisposed twins included in this study show increased self-reported scores on neuroticism (Vinberg et al., 2006), higher rates of subclinical affective symptoms (Christensen et al., 2007) and signs of discrete cognitive dysfunction (Christensen et al., 2006) in comparison to twins at low risk, but no changes in whole blood BDNF levels. The lack of associations between genetic risk and whole blood BDNF is likely to be due to a large heterogeneity in the total sample, and that genetic risk of developing depression is insufficient to induce changes in whole blood BDNF. It is well acknowledged today that affective disorders are a result of an interaction between genetic liability and environmental risk factors (Kendler et al., 1995; Caspi et al., 2003; Farmer et al., 2005), and stressful life events often precipitates the onset of major depression (Caspi et al., 2003; Williamson et al., 1998). Animal studies have

shown that stress leads to decreased BDNF levels (Xu et al., 2006; Christensen et al., 2007), but until now it has been unknown whether stress is associated with decreased whole blood BDNF in humans. We find that the number of recent events or the number of life events by themselves do not influence whole blood BDNF levels. The combination, however of female gender, high genetic risk and high number of recent stressful life events is associated with lower whole blood BDNF levels. This gender-specific difference in regulation of whole blood BDNF levels might be caused by gender differences in hypothalamic-pituitary adrenal (HPA) axis regulation (Young 1998; Handa et al., 1994). Estrogen enhances HPA axis function (Handa et al., 1994), thereby lowering BDNF levels, while testosterone seems to have a protective effect against HPA axis hyperactivity (Handa et al., 1994; Rubinow et al., 2005). Gender differences also exist in major depression. There is evidence that heritability is more modest in men than in women (Marcus et al., 2005; Bierut et al., 1999). Our gender-specific observation is well in line with the notion that in major depression women show a greater reduction in serum BDNF levels than men (Karege et al., 2002, a). Depletion of forebrain BDNF leads to depression-like behaviour in female mice only (Monteggia et al., 2007). Furthermore, in an animal model of depression vulnerability, female rats show increased vulnerability during estrus when estrogen levels are highest (Sun et al., 2006), and BDNF levels the lowest (Cavus et al., 2003). This together with our results indicates that BDNF dysregulation may be more important in women's vulnerability to depression, thus lower whole blood BDNF levels being a trait factor. Since we report data from a cross-sectional study we cannot determine whether low BDNF levels are a predictor of a later affective episode.

Kendler and co-workers have shown that subjects with high neuroticism and high stress are at higher risk of developing depression (Kendler et al., 2004). In accordance with this observation we found a tendency ($p= 0.07$) for women with high neuroticism score and three or more recent events displaying decreased whole blood BDNF levels. By contrast, men with high neuroticism score and more than two recent life stressful events have increased whole blood BDNF levels as compared to low neuroticism men with less than three recent stressful life events. Again, men seem to be better at regulating their whole blood BDNF levels than women.

In contrast to earlier studies where a negative association between serum BDNF and neuroticism was identified (Lang et al., 2004; Sen et al., 2003) we were unable to replicate this finding. Even though our high risk twins have increased neuroticism as compared to the low risk twins (Vinberg et al., 2006), they still had lower neuroticism scores than subjects included in other studies (Lang et al., 2004; Sen et al., 2003), possibly because inclusion of subjects with previous depressive episodes favours the inclusion of subjects with high neuroticism. A negative association between BDNF and neuroticism is more likely to be revealed in a cohort with a large variance in neuroticism scores (Maier et al., 1995).

In conclusion, the genetic risk of developing depression does not translate directly into whole blood BDNF levels. In females that are genetically disposed for depression and subjected to recent stressful life events whole blood BDNF levels are lower. By contrast, in men BDNF levels seem to appropriately counteract the risk factors high neuroticism and high number of recent stressful life events.

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References

Beck, A.T., Ward, C.H., Mendelson, M., Mock, J., Erbaugh, J., 1961. An inventory for measuring depression. Arch. Gen. Psychiatry 4, 561-571.

Bierut, L.J., Heath, A.C., Bucholz, K.K., Dinwiddie, S.H., Madden, P.A., Statham, D.J. et al.., 1999. Major depressive disorder in a community-based twin sample: are there different genetic and environmental contributions for men and women? Arch. Gen. Psychiatry 56, 557-563.

Caspi, A., Sugden, K., Moffitt, T.E., Taylor, A., Craig, I.W., Harrington, H. et al., 2003. Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science 301, 386-389.

Cavus, I. and Duman, R.S., 2003. Influence of estradiol, stress, and 5-HT2A agonist treatment on brain-derived neurotrophic factor expression in female rats. Biol. Psychiatry 54, 59-69.

Chen, Z.Y., Jing, D., Bath, K.G., Ieraci, A., Khan, T., Siao, C.J. et al., 2006. Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. Science 314, 140-143.

Choi, M.J., Kang, R.H., Lim, S.W., Oh, K.S., Lee, M.S., 2006. Brain-derived neurotrophic factor gene polymorphism (Val66Met) and citalopram response in major depressive disorder. Brain Res. 1118, 176-182.

Christensen, M.V., Kyvik, K.O., Kessing, L.V., 2006. Cognitive function in unaffected twins discordant for affective disorder. Psychol. Med. 36, 1119-1129.

Christensen, M.V., Kyvik, K.O., Kessing, L.V., 2007. Subclinical psychopathology

and socio-economic status in unaffected twins discordant for affective disorder. J. Psychiatr. Res. 41, 229-238.

Eysenck, H.J., 1975. Manual of the Eysenck Personality Questionnaire. London: Hodder and Staugton.

Farmer, A., Eley, T.C., McGuffin, P., 2005. Current strategies for investigating the genetic and environmental risk factors for affective disorders. Br. J. Psychiatry 186, 179-181.

Gonul, A.S., Akdeniz, F., Taneli, F., Donat, O., Eker, C., Vahip, S., 2005. Effect of treatment on serum brain-derived neurotrophic factor levels in depressed patients. Eur. Arch. Psychiatry Clin. Neurosci. 255, 381-386.

Gronli, J., Bramham, C., Murison, R., Kanhema, T., Fiske, E., Bjorvatn, B. et al.., 2007. Chronic mild stress inhibits BDNF protein expression and CREB activation in the dentate gyrus but not in the hippocampus proper. Pharmacol. Biochem. Behav. 85, 842-849.

Handa, R.J., Burgess, L.H., Kerr, J.E., O'Keefe, J.A., 1994. Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. Horm. Behav. 28, 464-476.

Harvald, B., Hauge, G., Kyvik, K.O., Christensen, K., Skytthe, A., Holm, N.V., 2004. The danish twin registry: past and present. Twin Res. 7, 318-335.

Karege, F., Bondolfi, G., Gervasoni, N., Schwald, M., Aubry, J.M., Bertschy, G., 2005. Low brain-derived neurotrophic factor (BDNF) levels in serum of depressed patients probably results from lowered platelet BDNF release unrelated to platelet

reactivity. Biol. Psychiatry 57, 1068-1072.

Karege, F., Perret, G., Bondolfi, G., Schwald, M., Bertschy, G., Aubry, J.M., 2002, a. Decreased serum brain-derived neurotrophic factor levels in major depressed patients. Psychiatry Res. 109, 143-148.

Karege, F., Schwald, M., Cisse, M., 2002, b. Postnatal developmental profile of brain-derived neurotrophic factor in rat brain and platelets. Neurosci. Lett. 328, 261-264.

Kendler, K.S., Gatz, M., Gardner, C.O., Pedersen, N.L., 2006, a. A Swedish national twin study of lifetime major depression. Am. J. Psychiatry 163, 109-114.

Kendler, K.S., Gatz, M., Gardner, C.O., Pedersen, N.L., 2006, b. Personality and major depression: a Swedish longitudinal, population-based twin study. Arch. Gen. Psychiatry 63, 1113-1120.

Kendler, K.S., Kessler, R.C., Walters, E.E., MacLean, C., Neale, M.C., Heath, A.C. et al.., 1995. Stressful life events, genetic liability, and onset of an episode of major depression in women. Am. J. Psychiatry 152, 833-842.

Kendler, K.S., Kuhn, J., Prescott, C.A., 2004. The interrelationship of neuroticism, sex, and stressful life events in the prediction of episodes of major depression. Am. J. Psychiatry 161, 631-636.

Kyvik, K.O., Christensen, K., Skytthe, A., Harvald, B., Holm, N.V., 1996. The Danish Twin Register. Dan. Med. Bull. 43, 467-470.

Lang, U.E., Hellweg, R., Gallinat, J., 2004. BDNF serum concentrations in healthy volunteers are associated with depression-related personality traits. Neuropsychopharmacology 29, 795-798.

Lee, B.H., Kim, H., Park, S.H., Kim, Y.K., 2006. Decreased plasma BDNF level in depressive patients. J. Affect. Disord. DOI:10.1016/j.jad.2006.11.005.

Levinson, D.F., 2006. The genetics of depression: a review. Biol. Psychiatry 60, 84-92.

Maier, W., Minges, J., Lichtermann, D., Franke, P., Gansicke, M., 1995. Personality patterns in subjects at risk for affective disorders. Psychopathology 28 Suppl. 1, 59-72.

Marcus, S.M., Young, E.A., Kerber, K.B., Kornstein, S., Farabaugh, A.H., Mitchell, J. et al.., 2005. Gender differences in depression: findings from the STAR*D study. J. Affect. Disord. 87, 141-150.

McAllister, A.K., Katz, L.C., Lo, D.C., 1999. Neurotrophins and synaptic plasticity. Annu. Rev. Neurosci. 22, 295-318.

Monteggia, L.M., Luikart, B., Barrot, M., Theobold, D., Malkovska, I., Nef, S. et al., 2007. Brain-derived neurotrophic factor conditional knockouts show gender differences in depression-related behaviors. Biol. Psychiatry 61, 187-197.

Mortensen, E.L., Reinisch, J.M., Sanders, S.A., 1996. Psychometric properties of the Danish 16PF and EPQ. Scand. J. Psychol. 37, 221-225.

Murakami, S., Imbe, H., Morikawa, Y., Kubo, C., Senba, E., 2005. Chronic stress, as well as acute stress, reduces BDNF mRNA expression in the rat hippocampus but less robustly. Neurosci. Res. 53, 129-139.

Neves-Pereira, M., Mundo, E., Muglia, P., King, N., Macciardi, F., Kennedy, J.L., 2002. The brain-derived neurotrophic factor gene confers susceptibility to bipolar disorder: evidence from a family-based association study. Am. J. Hum. Genet. 71,

651-655.

Pencea, V., Bingaman, K.D., Wiegand, S.J., Luskin, M.B., 2001. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. J. Neurosci. 21, 6706-6717.

Rubinow, D.R., Roca, C.A., Schmidt, P.J., Danaceau, M.A., Putnam, K., Cizza, G. et al., 2005. Testosterone suppression of CRH-stimulated cortisol in men. Neuropsychopharmacology 30, 1906-1912.

Schinder, A.F. and Poo, M., 2000. The neurotrophin hypothesis for synaptic plasticity. Trends Neurosci. 23, 639-645.

Sen, S., Nesse, R.M., Stoltenberg, S.F., Li, S., Gleiberman, L., Chakravarti, A. et al., 2003. A BDNF coding variant is associated with the NEO personality inventory domain neuroticism, a risk factor for depression. Neuropsychopharmacology 28, 397-401.

Shimizu, E., Hashimoto, K., Okamura, N., Koike, K., Komatsu, N., Kumakiri, C. et al., 2003. Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. Biol. Psychiatry 54, 70-75.

Shirayama, Y., Chen, A.C., Nakagawa, S., Russell, D.S., Duman, R.S., 2002. Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. J. Neurosci. 22, 3251-3261.

Strauss, J., Barr, C.L., George, C.J., Devlin, B., Vetro, A., Kiss, E. et al., 2005. Brain-derived neurotrophic factor variants are associated with childhood-onset mood

disorder: confirmation in a Hungarian sample. Mol. Psychiatry 10, 861-867.

Sullivan, P.F., Neale, M.C., Kendler, K.S., 2000. Genetic epidemiology of major depression: review and meta-analysis. Am. J. Psychiatry 157, 1552-1562.

Sun, M.K. and Alkon, D.L., 2006. Differential gender-related vulnerability to depression induction and converging antidepressant responses in rats. J. Pharmacol. Exp. Ther. 316, 926-932.

Toyooka, K., Asama, K., Watanabe, Y., Muratake, T., Takahashi, M., Someya, T. et al., 2002. Decreased levels of brain-derived neurotrophic factor in serum of chronic schizophrenic patients. Psychiatry Res. 110, 249-257.

Trajkovska, V., Marcussen, A.B., Vinberg, M., Hartvig, P., Aznar, S., Knudsen, G.M., 2007. Measurements of brain-derived neurotrophic factor: Methodological aspects and demographical data. Brain Res. Bull. 73, 143-149.

Vinberg, V., Mortensen, E.L., Kyvik, K.O., Kessing, L.V., 2006. Personality traits in unaffected twins discordant for affective disorder. Acta. Psychiatry Scand. DOI:10.1111/j.1600-0447.2006.00909.x

Williamson, D.E., Birmaher, B., Frank, E., Anderson, B.P., Matty, M.K., Kupfer, D.J., 1998. Nature of life events and difficulties in depressed adolescents. J. Am. Acad. Child Adolesc. Psychiatry 37, 1049-1057.

Wing, J.K., Babor, T., Brugha, T., Burke, J., Cooper, J.E., Giel, R. et al.., 1990. SCAN. Schedules for Clinical Assessment in Neuropsychiatry. Arch. Gen. Psychiatry 47, 589-593.

World Health Organisation., 1967. World Health Organisation: Manual of the

International Classification of Diseases, Eight Revision (ICD-8). Genova, Switzerland.

World Health Organisation., 2001. The World Health Report 2001. Mental Health: new understanding, new hope.

World Health Organisation., 2005. World Health Organisation: International Classification of Diseases, 10th Revision (ICD-10). Genova, Switzerland.

Xu, Y., Ku, B., Tie, L., Yao, H., Jiang, W., Ma, X. et al.., 2006. Curcumin reverses the effects of chronic stress on behavior, the HPA axis, BDNF expression and phosphorylation of CREB. Brain Res. 1122, 56-64.

Young, E.A., 1998. Sex differences and the HPA axis: implications for psychiatric disease. J. Gend. Specif. Med. 1, 21-27.

Figure Legends

Fig. 1. Whole blood BDNF levels in monozygotic (MZ) and dizygotic (DZ) twins; MZ low risk (n= 33), DZ low risk (n= 47), DZ high risk (n= 77), MZ high risk (n= 29).

Fig. 2. High risk women with three or more recent events (n= 26) have decreased whole blood BDNF levels as compared with high risk women with two or less recent events (n= 35), 21.6 ± 7.0 vs. 18.5 ± 4.1 ng/ml, * $p < 0.05$.

Figure 1

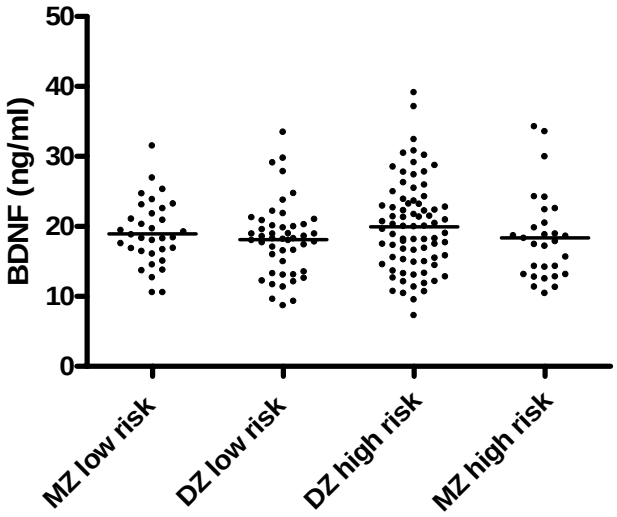
BDNF (ng/ml)
50
40
30
20
10
0
MZ low risk
DZ low risk
DZ high risk
MZ high risk

Figure 2