FACULTY OF HEALTH SCIENCES
UNIVERSITY OF COPENHAGEN

PhD thesis

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Brain-Derived Neurotrophic Factor (BDNF):

Interactions with the serotonergic system and its potential as a biomarker in neurological and neuropsychiatric diseases

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Submitted: 06/04/10

Title: Brain-Derived Neurotrophic Factor (BDNF): Interactions with the serotonergic system and its potential as a biomarker in neurological and neuropsychiatric diseases

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Date of submission: April 6, 2010

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Acknowledgements

The experimental work in this thesis was carried out at Neurobiology Research Unit, Copenhagen University Hospital, Rigshospitalet, and Department of Neuroscience, Tufts University, Boston, USA. Supervisors were Gitte Moos Knudsen and Susana Aznar.

The Neurocluster at University of Copenhagen and The Lundbeck Foundation Center for Integrated Molecular Brain Imaging (Cimbi) are sincerely acknowledged for financial support of this PhD project.

First, cordially thanks to my knowledgeable and inspiring supervisors, Gitte Moos Knudsen and Susana Aznar for endless answering of all my questions, and for scientific guidance and encouragement. I feel privileged to work in your lab. Jens D. Mikkelsen is acknowledged for great scientific advice and for involving me in the project on sleep disorders.

I would also like to thank Maribel Rios for letting me work in her lab at Dept. of Neuroscience, Tufts University, Boston, MA, and for thorough scientific discussions concerning BDNF knock-out mice and the serotonergic system. Similarly, thanks to German A. Calderon, Chris Parkin, Joshua Cordeira, Sarah Teillon, Jason Chan and TJ Unger from Rios lab for teaching me how to handle the crazy and unpredictable BDNF knock-out mice - and making my stay in Boston an enjoyable experience for life.

A special thanks to all my fellow PhD students, especially Anders Ettrup, Martin Santini and Christoffer Clemmensen for inspiring scientific and personal discussions, excellent team-work and ditto “happy hours”!

Hans-Jørgen Jensen, Christine Janssens and Astrid Beck are acknowledged for skilled technical assistance and for making Lab93 run smoothly, and thanks to all lab fellows for creating such a pleasant working environment. Also thanks to Dorthe Givard, Pia Farup and Dorte Frejwald for efficient administrative assistance.

Thanks to former PhD student Viktorija Trajkovska for teaching me the multifaceted methods for BDNF ELISA measurements. Also thanks to Klaus Holst, PhD student in biostatistics, for answering long, tiresome, and (for me) complex statistical questions.

Thanks to all co-authors for enthusiastic participation in the preparation of experiments and manuscripts.

And the biggest thanks of all to my wife and son, Ditte & Atle, for being the best family in the universe and for always being there and reminding me of life outside the BDNF galaxy.

Anders Bue Klein, Copenhagen, April 2010

Summary

Changes in brain-derived neurotrophic factor (BDNF) expression have been implicated in the pathophysiology of several brain disorders. This is based on the finding of decreased serum BDNF levels in patients with neurodegenerative disorders, such as Alzheimer's disease and Huntington's disease, and in neuropsychiatric disorders, e.g. major depression and anxiety. Likewise, the met-allele of a frequent polymorphism in the BDNF gene, *val66met*, has been associated with increased risk of developing neuropsychiatric disorders, such as schizophrenia, geriatric depression, and eating disorders. Moreover, lack of BDNF affects the development of serotonergic neurons and especially serotonin 2A (5-HT_{2A}) receptor expression and function. This is of much interest, as changes in 5-HT_{2A} receptor expression have been linked to neuropsychiatric disorders and anxiety-related behaviour.

The aims of this thesis were to further characterize 1) the interaction between BDNF and the serotonergic system 2) the validity of serum BDNF measurements as a useful biomarker for BDNF levels in brain and 3) the association between serum BDNF levels and brain disorders. To address these aims, we made use of human and animal studies, including a transgenic mouse model, where BDNF expression was manipulated.

We examined 1) whether central depletion of BDNF in a conditional BDNF knock-out mouse model results in changes in 5-HT_{2A} receptor binding and functionality 2) whether BDNF blood levels and val66met polymorphism status are associated with serotonin transporter (SERT) and 5-HT_{2A} receptor binding in healthy human subjects 3) whether blood BDNF levels can be used as a measure of brain BDNF levels validated across three different species (mice, rats and pigs) 4) whether hippocampal BDNF levels and serotonergic fibres are affected in an animal model of depression and finally, 5) whether blood BDNF levels are altered in patients with the sleep disorder narcolepsy.

The results revealed that in our conditional knock-out mice, removal of BDNF affects the expression and function of the 5-HT_{2A} receptor and expression of the 5-HT_{1A} receptor in hippocampus. In humans, however, we did not observe any effects of the BDNF val66met polymorphism on SERT and 5-HT_{2A} receptor binding, suggesting that the interaction between BDNF and the serotonergic system is more dependent on changes in total levels of BDNF. Blood BDNF levels correlate with BDNF levels in hippocampus across species,

which supports the potential of peripheral BDNF measurements as a biomarker for neurological and neuropsychiatric diseases associated with disturbances in neurotrophin regulation. For example, we showed that narcolepsy patients had a marked increase in serum BDNF levels compared to healthy controls suggesting a role for BDNF in sleep disorders.

In conclusion, the work comprised in this thesis further elucidates the close interaction between BDNF and the serotonergic system in general and the 5-HT_{2A} receptor in particular. Additionally, we found a correlation of BDNF in blood and brain suggesting that BDNF measurements in blood samples to a large degree reflect BDNF levels in brain.

Resumé in Danish

Ekspressionen af brain-derived neurotrophic factor (BDNF) menes at spille en rolle i patofysiologien bag flere hjernesygdomme. Man har hos patienter med neurodegenerative sygdomme, som f.eks. Alzheimers sygdom og Huntingtons sygdom, kunne påvise nedsat serum BDNF niveauer og dette gælder også for psykiatriske sygdomme, som f.eks. depression og angst. Endvidere er bærerstatus af met-allellen i en hyppigt optrædende BDNF polymorfi, *val66met*, forbundet med øget risiko for at udvikle en neuropsykiatrisk sygdom, såsom skizofreni, depression og spiseforstyrrelser. Endelig vides BDNF at være afgørende for udviklingen af det serotonerge system og særligt for ekspressionen og funktionen af hjernens serotonin 2A ($5\text{-}HT_{2A}$) receptor. Dette samspil er særligt interessant, da ændringer i ekspressionen af $5\text{-}HT_{2A}$ receptoren er observeret i visse hjernesygdomme.

Formålet med dette ph.d.-projekt var at afdække 1) BDNFs regulatoriske effekt på serotoninsystemet 2) validiteten af BDNF målinger i blodet som brugbar biomarkør for ændringer af BDNF niveauer i hjernen 3) associationen mellem BDNF niveauer i blodet og hjernesygdomme. Hertil indgik humane studier og dyreforsøg, herunder brug af en transgen musemodel, hvor BDNF-produktionen forsvinder efter fødslen.

Vi undersøgte 1) om en central depletering af BDNF påvirker $5\text{-}HT_{2A}$ receptor binding og funktionalitet i konditionelle BDNF knock-out mus 2) om der er en sammenhæng mellem BDNF niveauer og serotonin transporter (SERT) og $5\text{-}HT_{2A}$ binding i raske forsøgspersoner 3) om BDNF målinger i blodet kan bruges som et mål for BDNF niveauer i hjernen i forskellige arter (mus, rotter og grise) 4) om BDNF niveauer og serotonerge fibre i hippocampus i en dyremodel for depression er ændret, og endelig 5) hvorvidt serum BDNF koncentrationen hos patienter med narkolepsi er ændret.

Det påvistes, at der i de konditionelle BDNF knock-out mus var markante ændringer i $5\text{-}HT_{2A}$ receptorens funktions-, bindings- og ekspressionsniveau og ligeledes fandt vi ændringer i $5\text{-}HT_{1A}$ receptor binding i hippocampus. I forsøgspersonerne fandt vi ingen effekt af val66met polymorfien på SERT eller $5\text{-}HT_{2A}$ receptor bindingen, hvilket indikerer at serotoninsystemet primært er påvirket af ændringer i total BDNF. Endvidere fandt vi en signifikant sammenhæng mellem BDNF koncentrationen i blod og hjernevæv (hippocampus), hvilket støtter brugen af BDNF som biomarkør. Vi fandt en lavere

koncentration af BDNF i hippocampus i vores disponerede dyremodel for depression. Endeligt så vi, at narkolepsi patienter havde betydeligt højere serum BDNF niveauer sammenlignet med raske kontrolpersoner.

Resultaterne opnået i denne afhandling understreger det tætte samspil mellem BDNF og det serotonerge system og særligt mellem BDNF og 5-HT_{2A} receptoren. Metodemæssigt underbyggedes anvendelsen af blodkoncentrationen af BDNF som mål for koncentrationen i hjernen.

Table of Contents

Abbreviations

5-HT	5-hydroxytryptamine, serotonin
5-HT_{1A}	5-HT receptor 1A
5-HT_{2A}	5-HT receptor 2A
5-HT_{2C}	5-HT receptor 2C
A	adenine
AADC	amino acid decarboxylase
AC	adenylyl cyclase
AD	Alzheimer's Disease
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BDNF +/-	heterozygous BDNF knock-out mice
BDNF -/-	homozygous BDNF knock-out mice
cBDNF KO	conditional BDNF knock-out mice
C	cytosine
αCamKII	α-calcium/calmodulin-dependent protein kinase II
CSF	cerebrospinal fluid
CNS	central nervous system
DNA	deoxyribonucleic acid
DOI	2,5-dimethoxy-4-iodoamphetamine
DRN	dorsal raphe nucleus
ELISA	enzyme-linked immunosorbent assay
ESR	ear-scratch response
Fcx	frontal cortex
G	guanine
GPCR	G-protein coupled receptor
Hipp	hippocampus
HD	Huntington's Disease
HLA	human leucocyte antigen
HTR	head-twitch response
HPLC	high performance liquid chromatography
JNK	c-Jun N-terminal kinase
KO	knock-out
LH/cLH	learned helplessness/congenital LH
LSD	lysergic acid diethylamide
LTP	long-term potentiation
MAO	monoamine oxidase
MAP2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
Met	methionine
NAc	nucleus accumbens
NLH/cNLH	non-learned helplessness/congenital NLH
NGF	nerve growth factor
NT	neurotrophin
PD	Parkinson's Disease
PET	positron emission tomography

PI3K	phosphatidylinositol-3-kinase
PLA_2	phospholipase A2
PLCβ	phospholipase C beta
PLCγ1	phospholipase C gamma 1
PSD-95	postsynaptic density 95
RIA	radio-immuno assay
RNA	ribonucleic acid
RT	reverse transcription
RT-qPCR	RT-quantitative polymerase chain reaction
SERT	serotonin transporter
SNP	single nucleotide polymorphism
SPECT	single photon emission computed tomography
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant
TH	tryptophan hydroxylase
Trk	tropomyosin related kinase
Tryp	tryptophan
Val	valine
VTA	ventral tegmental area
WAGR	Wilms' tumor, aniridia, genitourinary anomalies and retardation

List of Manuscripts

This thesis is based on the following manuscripts and published papers, which in the text are referred to by their Roman numerals:

I. **Klein AB**, Santini M, Aznar S, Knudsen GM, Rios M (2010) Changes in 5-HT_{2A}-mediated behavior and serotonin receptor binding in conditional BDNF knock-out mice, *Neuroscience*, 169, 1007-1016

II. **Klein AB,** Trajkovska V, Erritzoe D, Haugbol S, Madsen J, Baaré W, Aznar S, Knudsen GM (2010) Cerebral 5-HT_{2A} receptor and serotonin transporter binding in humans are not affected by the val66met BDNF polymorphism status or blood BDNF levels, *Journal of Cerebral Blood Flow & Metabolism,* e1-e7

III. **Klein AB**, Williamson R, Santini M, Clemmensen C, Ettrup A, Rios M, Knudsen GM, Aznar S (2010) Blood BDNF concentrations reflect brain tissue BDNF levels across species, *International Journal of Neuropsychopharmacology*, p.1-7

IV. Aznar S, **Klein AB**, Santini MA, Knudsen GM, Henn F, Gass P and Vollmayr B (2010) Aging and depression vulnerability interaction results in decreased serotonin innervation associated with reduced BDNF levels in hippocampus of rats bred for learned helplessness, *Synapse* 64(7): 561-565

V. **Klein AB**, Jennum P, Knudsen S, Gammeltoft S, Mikkelsen JD (2010) Increased serum BDNF levels in hypocretin-deficient narcoleptic patients, *manuscript*

The following papers are related to the work described, but not included in the thesis:

1. Licht CL, **Marcussen AB**, Wegener G, Overstreet DH, Aznar S, Knudsen GM, 2009; The brain 5-HT4 receptor binding is down-regulated in the Flinders Sensitive Line depression model and in response to paroxetine administration. J. Neurochem., 109: 1363-74.

2. Trajkovska V, Santini MA, **Marcussen AB**, Thomsen MS, Hansen HH, Mikkelsen JD, Arneberg L, Kokaia M, Knudsen GM, Aznar S, 2009. BDNF downregulates 5-HT(2A) receptor protein levels in hippocampal cultures. Neurochem. Int. 55: 697-702.

3. Trajkovska V, **Marcussen AB**, Vinberg M, Hartvig P, Aznar S, Knudsen GM, 2007. Measurements of brain-derived neurotrophic factor: methodological aspects and demographical data. Brain Res. Bull. 73: 143-149.

Introduction

For more than a decade, the serotonin system has been the main research theme at the Neurobiology Research Unit. The last five years this has been supplemented with several studies on the neurotrophin BDNF (see e.g. Trajkovska et al., 2009; Trajkovska et al., 2007 - related papers #2 and #3). In one particular study, we looked at the interaction between BDNF and serotonin receptors using different *in vitro* systems (Trajkovska et al., 2009). To further elaborate on these findings, the main focus of this thesis was to investigate the interaction between BDNF and the serotonin system in brain using transgenic mice (Paper I) and human subjects (Paper II). Furthermore, we wanted to evaluate the potential of blood BDNF measurements to reflect BDNF expression in CNS (Paper III) and whether BDNF expression is changed in an animal model of depression vulnerability (Paper IV) as well as in the neurological sleep disorder narcolepsy (Paper V). To give the reader an opportunity to comprehend and interpret the latter results from these studies, the following sections contain a short theoretical introduction to this field of research. A description of BDNF and the serotonin system and their potential involvement in disease of the brain will be given, exemplified by depression and depression-like conditions and the sleep disorder narcolepsy, as these disorders in particular have been studied during the work presented in this thesis.

BDNF (brain-derived neurotrophic factor)

BDNF (brain-derived neurotrophic factor) is a 14 kDa protein belonging to the neurotrophin family, which also includes nerve growth factor (NGF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT4/5). The neurotrophins signal through their designated tropomyosin-related kinase (Trk) receptor (NGF via TrkA; NT-3 via TrkC; NT4/5 and BDNF via TrkB). Upon ligand binding, the matching Trk receptor dimerizes and undergoes autophosphorylation enabling signalling through the intracellular tyrosine kinase domain (Reichardt, 2006). Phosphorylation of specific tyrosine residues initiate at least three different pathways; the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol-3-kinase (PI3K) pathway, and the phospholipase C (PLCγ1) pathway (Chao, 2003)(Figure 1). Besides the full-length TrkB receptor, there is also two truncated isoforms of this receptor, TrkB.T1 and TrkB.T2, which lack the intracellular catalytic

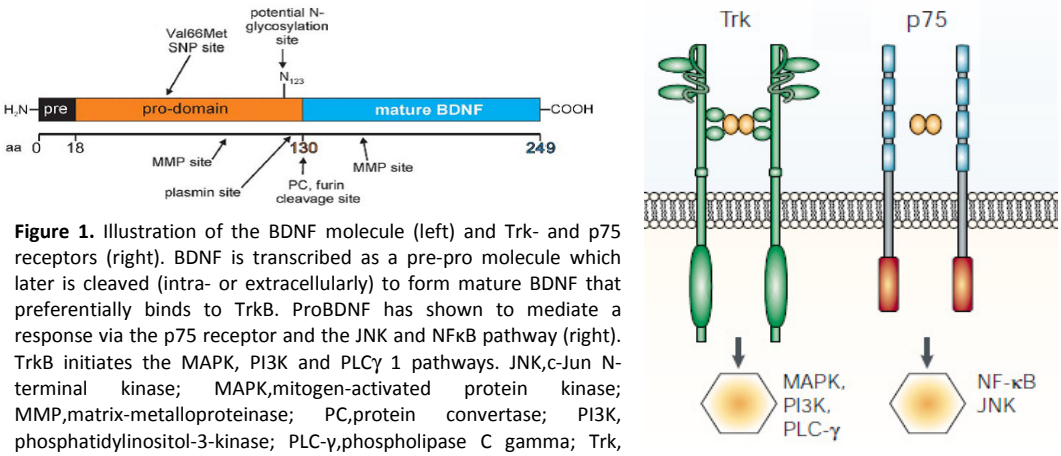

Figure 1. Illustration of the BDNF molecule (left) and Trk- and p75 receptors (right). BDNF is transcribed as a pre-pro molecule which later is cleaved (intra- or extracellularly) to form mature BDNF that preferentially binds to TrkB. ProBDNF has shown to mediate a response via the p75 receptor and the JNK and NFκB pathway (right). TrkB initiates the MAPK, PI3K and PLCγ 1 pathways. JNK,c-Jun N-terminal kinase; MAPK,mitogen-activated protein kinase; MMP,matrix-metalloproteinase; PC,protein convertase; PI3K, phosphatidylinositol-3-kinase; PLC-γ,phospholipase C gamma; Trk, tropomyosin-related kinase. Modified from Lessman and Brigadski, 2009 and Chao, 2003.

domain (Middlemas et al., 1991). The role of these truncated variants is not entirely clear, but an inhibiting and thereby regulatory effect of signalling through the full-length TrkB receptor has been described (Biffo et al., 1995). In cultured astrocytes, TrkB.T1 is known to execute important functions via Rho GDI1, which in turn regulates Rho GTPases controlling astrocyte morphology (Ohira et al., 2005). *In vivo*, TrkB.T1 knock-out mice do not display a noticeable phenotype, but have shown memory impairments, poorer regenerative response to ischemia-induced cell damage, increased anxiety in elevated plus maze and changes in dendritic length in hippocampus, all indicating antagonistic effects of TrkB.T1 on BDNF/TrkB signalling (Carim-Todd et al., 2009; Saarelainen et al., 2000). In addition to Trk receptor binding, all mature neurotrophins bind with low affinity to the p75 receptor (Chao, 2003). While Trk receptors all mediate pro-survival signals, the p75 receptor is coupled to the NFκB and c-JNK pathways which can mediate apoptosis (Reichardt, 2006)(Figure 1).

BDNF is translated as a pre-pro-molecule like the other neurotrophins. In the BDNF amino acid sequence, sorting signals are located in the pro part of the protein (Egan et al., 2003). This part is later cleaved off to form the mature neurotrophin (Figure 1)(Lessmann and Brigadski, 2009). Curiously, it was recently shown that proBDNF is secreted from neurons (Yang et al., 2009) and is able to mediate signalling via high-affinity binding and signalling through the p75 receptor. The antagonistic signalling via p75 is believed to be important for the maintenance of “synaptic homeostasis” (Woo et al., 2005). This duality in the BDNF signalling system, BDNF vs proBDNF and TrkB vs

TrkB.T1, adds significantly to the diversity in outcomes following transcriptional regulation of BDNF.

Evolutionarily, BDNF is a highly conserved protein across species (Tettamanti et al., 2010), and presumably, a prerequisite for more complex neuronal structures (Chao, 2000). In mammals, relatively high expression is seen in hippocampal neurons, but also in frontal cortex, amygdala and hypothalamus (Yan et al., 1997). In addition, BDNF is not solely expressed in the brain, but also produced in activated cells of the immune system (Kerschensteiner et al., 1999), endothelial cells (Donovan et al., 2000) and in liver and muscle tissue (Cassiman et al., 2001; Matthews et al., 2009). BDNF is transported in blood in high concentrations (ng/ml) in thrombocytes. The sources and significance of BDNF in the blood stream are heavily debated (Gass and Hellweg, 2010). Despite the fact that cells from many different tissues may add to these levels, it is believed that CNS is the main contributor to blood BDNF levels (Rasmussen et al., 2009). In CNS, BDNF is mainly released from neurons as a response to neuronal activity, but is also released via a constitutive secretory pathway (Figure 2)(Lu, 2003).

Several factors are known to stimulate BDNF expression including neuronal activation, enriched environment, antidepressant treatment, and exercise. More comprehensive studies of the overall effects of BDNF depletion and increased BDNF expression has been made possible after the creation of transgenic animals.

BDNF knock-out mice

To study the effects of perturbed BDNF signalling *in vivo*, BDNF knock-out mice have been created (Ernfors et al., 1994). However, since mice with global knock-out of BDNF (BDNF-/-) die within the first two postnatal weeks, BDNF heterozygous mice (BDNF+/-) were initially used. These animals are viable and express BDNF at levels corresponding to 50% of BDNF levels in wildtype mice (Ernfors et al., 1994; Lyons et al., 1999). These mice display significant changes in social behaviour (increased aggressiveness) and hyperphagia, which eventually results in obesity in adult animals (Lyons et al., 1999). Due to the limited survival of BDNF-/- animals and to more precisely address the significance of central BDNF depletion in adult animals, brain-specific BDNF knockout mice have been generated using the Cre-lox recombination system (Rios et al., 2001). This particular line of conditional knock-out animals was used in Paper I, hence a more detailed description

of the creation of these animals is provided in the Experimental Methods section. In brief, these mice are postnatally depleted of BDNF and have a distinctive phenotype: they are more prone to anxiety and easily stressed upon handling illustrated by a remarkable level of hyperactivity and aggressiveness (Chan et al., 2006). Occasionally, short periods of stereotypical behaviour can be observed (unpublished observations). Like BDNF+/- mice, these animals are hyperphagic and become obese in adulthood (Rios et al., 2001).

In parallel, mice overexpressing BDNF or full-length TrkB have been studied, but the data from these lines of mice are equivocal. In one study, TrkB full-length overexpressing mice showed improved learning and memory, but an impairment of long-term potentiation (LTP)(Koponen et al., 2004). In other studies, and somewhat surprising, BDNF overexpressing mice displayed hyperactivity, neuronal hyperexcitability and impairment of LTP (Croll et al., 1999). Moreover, mice overexpressing BDNF in the forebrain showed significant memory- and learning impairments (Cunha et al., 2009). Thus, BDNF may have beneficial effects on neurons, while too much (at least in adult animals) can be detrimental. Moreover, this is supposedly brain region specific, which further complicates potential therapeutic applications of systemic BDNF administration.

BDNF in humans

The level of BDNF expression in humans have been estimated using *in situ* hybridization, quantitative polymerase chain reaction (qPCR) for detection of mRNA levels, and measurements of BDNF protein levels in brain tissue, cerebrospinal fluid (CSF), serum and whole blood using immunohistochemistry, western blotting and ELISA (Chen et al., 2001; Laske et al., 2007; Trajkovska et al., 2007; Zuccato et al., 2008). The availability of commercially available BDNF ELISA kits for measurements of serum BDNF has resulted in a vast number of studies applying this as an indirect measurement of BDNF expression in brain. As an example, several studies have reported decreases in serum BDNF levels in patients with major depression, which was normalized following antidepressant treatment (Brunoni et al., 2008).

An alternative way to study effects of perturbed BDNF signalling is to study sporadic deletions or polymorphisms in the BDNF gene. The BDNF locus is located at chr11p14.1 and is often deleted in patients with the WAGR syndrome (Wilms' tumor, aniridia,

genitourinary anomalies and retardation). It is a rare genetic syndrome (~1:500.000), which is manifested in early childhood (Han et al., 2008). Remarkably, the phenotypic appearance of these patients shares some similarity with BDNF KO mice. WAGR patients are hyperphagic, obese, hyperactive and have impaired cognitive function (Gray et al., 2006; Han et al., 2008). The role of BDNF in obesity is particular evident, since all patients with a deletion in the BDNF locus were obese. Similarly, both BDNF+/-, conditional BDNF- and TrkB knock-out mice are obese (Lyons et al., 1999; Rios et al., 2001; Xu et al., 2003). Another way to study more subtle effects of perturbed BDNF function is to study individuals carrying polymorphisms in the BDNF gene, for example the single nucleotide polymorphism (SNP), val66met (Figure 2).

The BDNF val66met polymorphism

The extensively investigated polymorphism, val66met (rs6265/rs60760775) is relatively frequent in Caucasians (19-25%) and in Asians (45%)(Hwang et al., 2006; Shimizu et al., 2004). The val66met polymorphism is not present in animals, but its effects have been studied *in vitro* using transfection methods and *in vivo* using knock-in mice (Chen et al., 2004; Chen et al., 2006; Egan et al., 2003). The replacement of guanine (G) with adenine (A) causes an amino acid shift from a valine (val) to a methionine (met) in codon 66, which is localized in the pro-region of BDNF. Important sorting signals have shown to be positioned in this region, elegantly demonstrated by Egan and co-workers (2003)(Figure 1 and 2). They showed that introducing a methionine in codon 66 resulted in severe disturbance of BDNF vesicle sorting to the activity-regulated secretory pathway, while the constitutive secretory pathway was unaffected (Egan et al., 2003). These observations were later confirmed by Chen and colleagues, who replaced the val with met in either one or both alleles of the BDNF gene in a knock-in mouse strain (Chen et al., 2006). Primary cultures of neurons from these mice showed similar severe deficits in the activity-dependent secretion of BDNF, while total brain tissue BDNF levels were unaffected. Correspondingly, humans carrying this

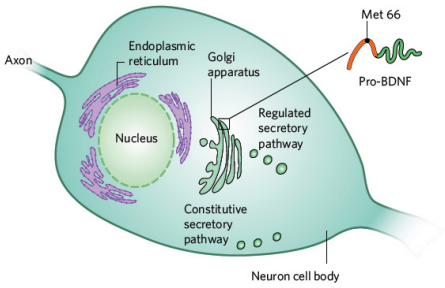

Figure 2. Illustration of the BDNF secretion pathways in the cell body of a neuron. ProBDNF carrying a methionine in codon 66 (Met66) is not appropriately sorted to the activity-dependent secretion pathway resulting in decreased amounts of released BDNF upon activation of the neuron. Modified from Krishnan & Nestler, 2008

substitution in either one or both alleles did not show any differences in total serum BDNF levels (Trajkovska et al., 2007; Tramontina et al., 2007). However, Lang et al. (2009) found an increase in serum BDNF in subjects with the val/met genotype compared to val/val. The authors of the latter study suggest that this is a compensatory mechanism in response to the decrease in activity-regulated secretion (Lang et al., 2009). Despite unchanged total BDNF levels (or a slight increase), effects of the altered BDNF secretion have been described. For example, *in vivo* neuroimaging studies have shown that in healthy subjects, the met-allele is associated with lower volumes of prefrontal cortex and hippocampus (Pezawas et al., 2004) and poorer performance in episodic memory tests (Egan et al., 2003; Hariri et al., 2003). In parallel, an altered susceptibility to several neuropsychiatric disorders have been associated with the met-allele (Gratacos et al., 2007). Finally, an interaction between val66met and the serotonergic system has been shown in healthy human subjects, where the val/val genotype resulted in significant higher SERT binding in men, while 5-HT_{1A} receptor binding showed no associations with val66met status (Henningsson et al., 2009).

Neuropsychiatric disorders and BDNF

In clinical studies, decreased levels of BDNF have been linked to various psychiatric disorders including schizophrenia, bipolar disorder and major depression (Kozisek et al., 2008). The exact role of BDNF in these diseases remains unknown, but the already proposed neurotrophic hypothesis of depression may account for most, if not all, psychiatric diseases (Figure 3)(Berton and Nestler, 2006; Castren, 2005; Duman, 2004).

The most studied neuropsychiatric disorder in relation to BDNF is depression. Compelling evidence has now established a role for BDNF in this disease (Brunoni et al., 2008; Sen et al., 2008). However, many of the same features are likely to be involved in other neuropsychiatric disorders (Castren and Rantamaki, 2010). One of the first comprehensive studies found that BDNF and TrkB mRNA was up-regulated following antidepressant treatment in rats and the treatment was moreover able to prevent the restraint stress-induced BDNF down-regulation seen in these animals (Nibuya et al., 1995). Later, several clinical studies have shown decreased serum BDNF levels in patients with major depression (Brunoni et al., 2008; Karege et al., 2005; Lee et al., 2007) and furthermore, most studies found that the majority of antidepressant treatment regimes

were capable of reversing the lowered serum BDNF levels (Meta analyses in Brunoni et al., 2008 and Sen et al., 2008).

The neurotrophic hypothesis of depression

Even though the exact mechanisms for regulation and function of BDNF in depression is not resolved, the proposed neurotrophic hypothesis of depression describes a possible pathophysiological mechanism (Figure 3)(Berton and Nestler, 2006; Duman, 2004). The hypothesis states that neurotrophic effects of antidepressant treatment can reverse or block the stress-induced atrophy of hippocampus, which is seen in many patients suffering from depression (Figure 3)(Castren, 2005; Duman, 2004; Neumeister et al., 2005). More specifically, the stress-induced deterioration of neural networks is re-established via newly formed synaptic connections mediated by an intervention-induced up-regulation of BDNF (Castren, 2005). Despite the existence of coherent findings supporting this theory, the hypothesis has also been met with critique. For example, BDNF knock-out mice do not show obvious signs of depressive-related behaviour and not all antidepressants up-regulate BDNF (Groves, 2007).

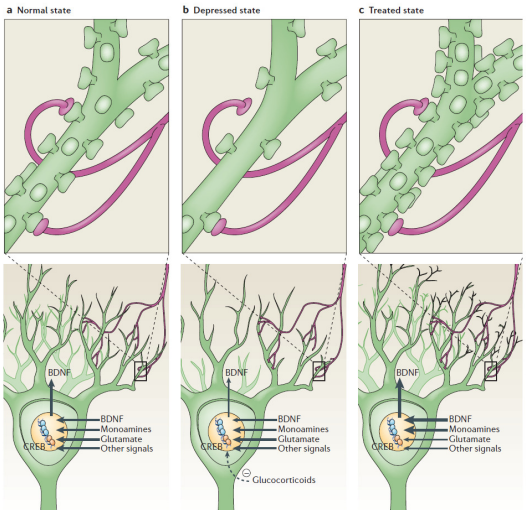

Figure 3. This cartoon and variants hereof is often used to describe the neurotrophic hypothesis of depression. During depression (and supposedly other disorders involving stress) synaptic connections are lost (b). Following antidepressant treatment, increased levels of BDNF will mediate the re-establishment of the synaptic contacts between neurons and thereby contributing to the observed clinical improvement (c). Modified from Berton & Nestler, 2006.

However, a study using site-directed knock-down of hippocampal BDNF in mice showed a depression-like phenotype, suggesting a specific role for hippocampal BDNF expression in relation to depression (Taliaz et al., 2010). Another study supporting this theory, found that knock-down of BDNF in dentate gyrus of hippocampus ameliorated antidepressants effects of desipramine (a TCA) and citalopram (a SSRI)(Adachi et al., 2008). Along this line, other studies have demonstrated different roles for BDNF depending on brain region (Berton et al., 2006; Eisch et al., 2003; Krishnan et al., 2007). For example, BDNF has shown to have a pro-depressant effect in the mesolimbic region, where stress increased BDNF expression in nucleus accumbens (NAc)(Berton et al., 2006). In addition, infusion of BDNF into the ventral tegmental area (VTA) resulted in increased depression-

related behaviour (Eisch et al., 2003). Thus, the up-regulation of BDNF in the mesolimbic region may have opposite effects compared to hippocampus, which could be important in future drug development. In addition, this may explain why a complete knock-out of BDNF does not provide a valid animal model of depression. Finally, depression or depression-like states in animals supposedly have to persist for a certain amount of time before the condition corresponds to what is seen in patients (Castren and Rantamaki, 2010; Gardier, 2009).

Neurological disorders and BDNF

Also in neurological disorders an important role for BDNF has been suggested. Especially, the neurodegenerative disorders have been in focus in BDNF research due to the neuro*re*generative potential of this molecule.

Neurodegeneration and BDNF

Phillips et al. (1991) showed a decrease in post-mortem BDNF mRNA levels in the hippocampus of Alzheimer patients and later, when antibodies against BDNF became available, this was also shown at protein level, and subsequently, several reports have observed lower BDNF levels in AD patients (reviewed in Zuccato and Cattaneo, 2009). The lowered BDNF levels could be a result of a significant loss of BDNF producing neurons. However, interestingly, a recent study showed that amyloid beta levels in transgenic mouse models of AD are negatively correlated with BDNF levels and depend on the size of the aggregated oligomers of amyloid beta (Peng et al., 2009). This suggests that BDNF is down-regulated as a result of the amyloid beta aggregation in AD patients, and that, the lower BDNF levels may be part of the etiology of AD. Also, BDNF is reported to be a fundamental regulator of cognition, memory formation and storage, which are functions that are severely affected in AD patients (Bekinschtein et al., 2007; Schindowski et al., 2008).

In another neurodegenerative disorder, Parkinson´s disease (PD), BDNF may play an important role as well. Most studies, but not all, have found lower BDNF immunoreactivity in substantia nigra of PD patients (Zuccato and Cattaneo, 2009). Additionally, experimental studies have shown that selective depletion of BDNF in the

midbrain results in a marked reduction in the number of dopaminergic neurons (Baquet et al., 2005). In another study, it was demonstrated that genetically modified fibroblasts, which produced BDNF, prevented cell loss caused by 6-hydroxydopamine (6-OHDA) in substantia nigra in rats (Levivier et al., 1995).

In another example of a neurodegenerative disorder, Huntington's disease (HD), a mechanistic role for BDNF is reported. *In vitro* experiments suggested that the mutations in the huntingtin gene (28-40 CAG (cytosine-adenine-guanine) repeats), which causes the disease, have a direct effect on BDNF regulation (Zuccato et al., 2001). Thus, BDNF is reported to be markedly decreased in a mouse model of HD and these results have also been confirmed in patients (Zuccato et al., 2008). An interesting mechanism causing this decrease has been described. Huntingtin is involved in BDNF vesicle transport along the microtubules, and mutations in huntingtin will impair the transport of BDNF significantly. This will result in lower release upon neuronal stimulation and maybe explain the lower levels of BDNF in HD patients (Gauthier et al., 2004).

In summary, BDNF is down-regulated in some of the common neurodegenerative disorders, even though diverse mechanisms are involved. A reduction of BDNF may further worsen the pathological conditions and a treatment potential of BDNF has been observed in animal models (Levivier et al., 1995). However, the constantly recurring problem of drug delivery (BDNF to CNS) may explain the limited success in clinical trials.

Narcolepsy and BDNF

Another neurological disorder, narcolepsy, is a chronic sleep disorder and by some people considered a "neurodegenerative disorder". Accepting this classification makes the disease the third most common of the neurodegenerative disorders, after AD and PD. In the current thesis, we investigated whether the disorder was associated with changes in neurotrophin levels in serum. To our knowledge, there are no reports in the literature on narcolepsy and neurotrophin response. A brief presentation of the known pathophysiological hallmarks in this disease is provided below.

Narcolepsy is a disabling neurological disorder with a prevalence of 1/2000 in Caucasians (Bourgin et al., 2008). The disease is often described clinically by the "narcoleptic tetrade" comprising excessive daytime sleepiness, cataplexy (sudden loss of muscle tonus triggered by strong emotions) and hypnagogic hallucinations (dream-like

hallucinations)(Longstreth Jr. et al., 2007). Narcolepsy has been ascribed to autoimmune mechanisms and virtually all narcolepsy patients are positive for the human leucocyte antigen (HLA) DQB1*0602-allele whereas only 25% in the general population are carriers (Bourgin et al., 2008).

In narcolepsy patients that present cataplexy, a very low CSF concentration of hypocretin-1 is found (Bourgin et al., 2008). Hypocretin-1 is a 33 amino acid neuropeptide with high homology across mammalian species. Hypocretin-producing neurons are mainly located in hypothalamus and have widespread projections to the forebrain and brain stem. From knock-out studies in mice and post-mortem analyses of humans, these neurons are known to control sleep/wake behaviour (Adamantidis and de Lecea, 2008; Kilduff and Peyron, 2000; Saper et al., 2005). Hypocretin-immunoreactivity is also dramatically reduced in lateral hypothalamus in patients with narcolepsy (Thannickal et al., 2009). However, it is unclear if this is a result of ceased hypocretin production or specific cell death of hypocretin neurons, even though a lack of signal from other markers for hypocretin neurons suggests a pronounced, but specific cell death of this subpopulation of neurons. Therefore, it has been speculated whether the disease should be categorized as a neurodegenerative disorder and in that case, whether changes in neurotrophin levels are present in patients with narcolepsy.

The potential role of BDNF as a biomarker

Several studies have in recent years investigated the potential of BDNF as a biomarker for disease, i.e. a substance used as an indicator for a biological state. Even though the independent diagnostic value of serum BDNF measurements needs to be established, peripheral BDNF levels can potentially provide us with information on the "BDNF regulatory potential" of new drugs. BDNF is also produced in the periphery and a major concern has been, whether BDNF levels measured in blood actually reflect BDNF levels in brain. There is some evidence suggesting that BDNF crosses the blood-brain barrier (BBB) in mice (Pan et al., 1998). On the other hand, Wu and colleagues did not observe any transport of BDNF across the BBB after an intravenous injection (Wu and Pardridge, 1999). However, when BDNF was pegylated and coupled to a monoclonal antibody directed against the transferrin receptor, transport across the BBB was made possible

and a marked neuroprotective effect was observed (Wu and Pardridge, 1999). Even so, these findings do not mutually exclude the possibility that BDNF levels in blood reflect BDNF levels in brain. There are three studies that have directly compared serum BDNF levels with brain tissue levels of BDNF in rat brain tissue. In the first study, the association was only evident in young rats (Karege et al., 2002) while in a later study, serum BDNF correlated with brain tissue BDNF also in adult animals (Sartorius et al., 2009). In contrast, Elfving and co-workers looked for an association in Flinders rats, but found a *negative* correlation between BDNF in blood and hippocampus (Elfving et al., 2009). To our knowledge, there are no studies available that have investigated this relation in other species than rats. Intriguingly, even though BDNF show a high-degree of homology between species, it was observed that BDNF was undetectable in mouse blood, despite comparable brain tissue levels of BDNF in rat and mouse (Paper III and Radka et al., 1996). This may explain the lack of correlation studies in mice. Thus, some species differences could exist regarding BDNF detection and/or transport between the different compartments.

Another field of research has contributed with interesting knowledge on BDNF in brain and blood. In an exercise study, where catheters were placed in the internal jugular vein and in the radial artery, BDNF concentrations were measured to determine the cerebral net flux (Rasmussen et al., 2009). They found a significant net BDNF efflux from the brain and this efflux increased during an exercise bout indicating BDNF flux from brain to blood. It remains, however, to be determined whether BDNF concentrations in human brain tissue are reflected in plasma and serum levels at all times. Further adding to the complexity, this association may be challenged during disease or changes in blood factors or changes in BBB properties.

The serotonin system

Serotonin (5-hydroxytryptamine, 5-HT) is mainly located in the intestines and blood. In the CNS, serotonin is produced in neurons located in the raphe nuclei in the brainstem, from where they project and innervate multiple brain regions (Figure 4)(Berger et al., 2009). The serotonin system is involved in the regulation of behaviour, cognition and mood, and plays an important role in brain development, regulating both neurite outgrowth, synaptogenesis and cell survival (Gaspar et al., 2003). Serotonin exerts its diverse effects through at least 15 different receptors, divided into 7 receptor subfamilies, 5-HT_{1-7} (Figure 5). They all belong to the G-protein-coupled receptor (GPCR) family, except for the 5-HT_3 receptor subfamily which are ligand-gated ion channels (Kroeze et al., 2002). In contrast to the diversity of the receptors, serotonin levels in the synaptic cleft are mainly controlled by the activity of a single protein, the serotonin transporter (SERT or 5-HTT). SERT mediates reuptake of serotonin from the synaptic cleft by transporting it back into the pre-synaptic neuron (Figure 5).

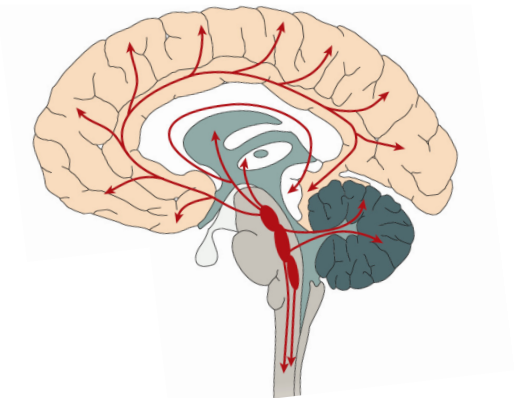

Figure 4. In CNS, serotonin is produced by neurons residing in the raphe nuclei which are located in the brain stem. These neurons have extensive projections to cerebrum and spinal cord. Modified from Berger et al., 2009.

In this thesis, we thoroughly investigated the association between BDNF levels and 5-HT_{2A} receptor expression and function, but we also looked at expression levels and/or protein binding of 5-HT_{2C}, 5-HT_{1A}, and SERT. Therefore, the following paragraphs contain descriptions of each of these members of the serotonin family including their reported interactions with BDNF.

The 5-HT$_{2A}$ receptor

The 5-HT$_{2A}$ receptor belongs to the GPCRs and is located in the plasma membrane, with a large fraction of receptors located as an intracellular pool (Gelber et al., 1999). The receptor is coupled to G_q/G_{11} proteins and signals through PLCβ and PLA_2, but also targets the ERK1/2 pathway via β-arrestin-dependent signalling (Schmid et al., 2008). The 5-HT$_{2A}$ receptor is mainly located on glutamatergic neurons and GABAergic interneurons (Willins et al., 1997) and is widely distributed in the CNS, with highest density in cerebral cortex, hippocampus, amygdala and hypothalamus (Leysen, 2004). The receptor is primarily located post-synaptically, but a pre-synaptic localization of the 5-HT$_{2A}$ receptor

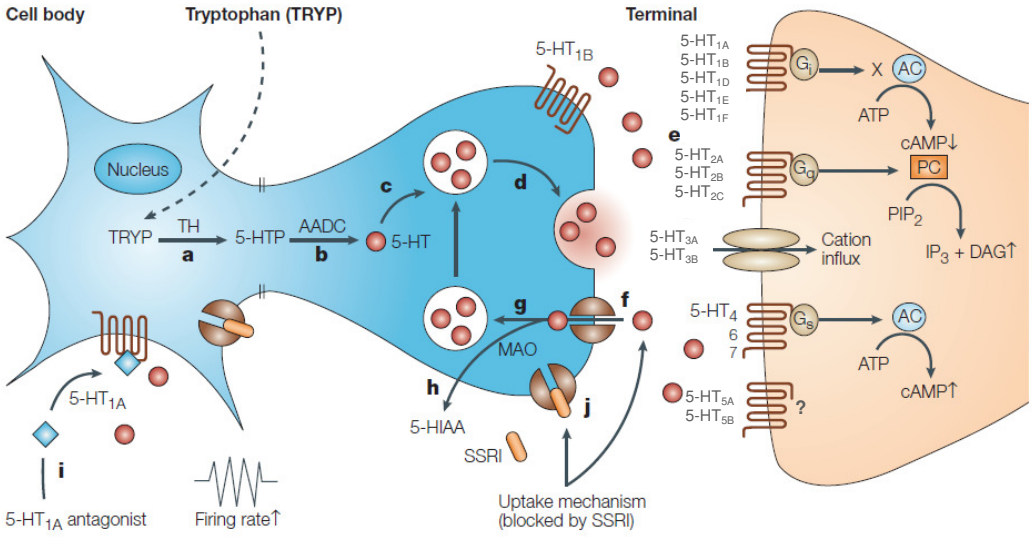

Figure 5. Schematic figure of a serotonergic synapse. **a.** Serotonin is produced from the amino acid tryptophan (TRYP) catalyzed by the enzymes tryptophan hydroxylase (TH) and aromatic amino acid decarboxylase (AADC), packed into vesicles (**c**) which fuses with the plasma membrane (**d**) and releases serotonin into the synaptic cleft (**e**). Here, serotonin can mediate its effect through 15 different receptor subtypes primarily located post-synaptically. Serotonin signaling is eliminated by reuptake of serotonin back into the pre-synaptic neuron by the serotonin transporter (SERT)(**f**) and by degradation via monoamine oxidase (MAO)(**g**). 5-HT$_{1A}$ receptors are also present as inhibitory pre-synaptic autoreceptors in dorsal raphe nuclei and 5-HT$_{1A}$ antagonists can block those thereby increasing serotonin neurotransmission (**i**). An increase in serotonergic neurotransmission can also be obtained by administration of SSRIs, blocking serotonin re-uptake and thereby increasing serotonin levels in the synaptic cleft (**j**). Modified from Wong et al., 2005.

has also been suggested (Jakab and Goldman-Rakic, 1998). The receptor has been implicated in a number of neuropsychiatric disorders, including major depression, schizophrenia, anxiety disorders, obsessive-compulsive disorder and eating disorders (reviewed in Allen et al., 2008) and several drugs mediate their actions through the 5-HT$_{2A}$ receptor, such as hallucinogenic drugs (e.g. LSD) and atypical antipsychotics (e.g. olanzapine)(Gonzalez-Maeso et al., 2007; Leysen, 2004).

The interest in 5-HT_{2A} receptor expression and function in major depression is illustrated by the vast number of studies in this research field (for review, see Stockmeier, 2003). *In vivo* imaging studies have shown a profound decrease in hippocampal 5-HT_{2A} binding in depressed subjects (Mintun et al., 2004), while other studies found an increase in depressive teenage suicide victims (Pandey et al., 2002). Our group has shown that frontolimbic 5-HT_{2A} receptor binding is positively correlated to neuroticism score, a risk factor for developing major depression (Frokjaer et al., 2008). Most, but not all, animal studies report a decrease in receptor binding following chronic antidepressant treatment (Licht et al., 2009 - related paper #1). *In vivo* PET studies in human subjects observed increased 5-HT_{2A} binding as a result of antidepressant treatment with SSRIs (Massou et al., 1997; Zanardi et al., 2001), while other found a decrease in receptor binding (Meyer et al., 2001; Yatham et al., 1999). In summary, the role for the 5-HT_{2A} receptor in depression and other neuropsychiatric disorders is not entirely clear. However, it is possible that this receptor mediates diverse responses depending on brain region.

The 5-HT_{2C} receptor

The 5-HT_{2C} receptor is also a GPCR, activating PLC via G_q/G_{11}, and located post-synaptically throughout the brain. The receptor is found in particular high concentrations on epithelial cells of the choroid plexus lining the ventricles and controlling production and secretion of CSF (Leysen, 2004). The 5-HT_{2C} receptor also plays an important role in the arcuate nucleus of hypothalamus, where the activation of proopiomelanocortin neurons inhibits appetite. Accordingly, 5-HT_{2C} knock-out mice are obese and 5-HT_{2C} agonists as appetite regulators are currently tested in clinical trials, e.g. Lorcaserin (APD356)(Smith et al., 2009). The 5-HT_{2C} receptor has been suggested as a pharmacological target in the development of antidepressant drugs. For example, pharmacological activation of 5-HT_{2C} receptors evoked feelings of anxiety and panic in humans and the anxiogenic effect of acute SSRI administration in rats can be blocked with selective 5-HT_{2C} receptor antagonists (Leysen, 2004). However, the lack of appropriate PET ligands for this receptor has complicated the *in vivo* evaluation of its role in neuropsychiatric disorders.

The 5-HT$_{1A}$ receptor

The 5-HT$_{1A}$ receptor also belongs to GPCR family, negatively coupled to adenylyl cyclase (AC)(Figure 5). Additionally, the receptor is able to activate K^+-channels and inhibiting Ca^{2+}-channels resulting in neuronal inhibition (Bockaert et al., 2006). The 5-HT$_{1A}$ receptor is unique in the sense of its dual function as both auto- and heteroreceptor. In the dorsal raphe nucleus (DRN) the receptor is pre-synaptically located on serotonergic neurons and functions as an autoreceptor, where it monitors serotonin levels and mediate autoinhibitory feedback to serotonergic neurons (Figure 5). The distribution of the post-synaptic 5-HT$_{1A}$ receptor is consistent with the role of this serotonin receptor subtype in cognitive functions and emotional states. The 5-HT$_{1A}$ receptor is present in high density in cortical and limbic areas (e.g. frontal cortex, entorhinal cortex, hippocampus), where the receptor inhibits firing of non-serotonergic neurons (Lanfumey and Hamon, 2004). The 5-HT$_{1A}$ receptor is involved in a large number of physiological and behavioural processes in CNS including control of body temperature, neuroendocrine responses and in regulation of sleep and mood (Lanfumey and Hamon, 2004). In parallel, 5-HT$_{1A}$ knock-out mice exhibit increased anxiety-like behaviour measured with open-field (Parks et al., 1998), while SSRI treatment (fluoxetine and paroxetine) failed to ameliorate immobility after stress exposure in these animals (Mayorga et al., 2001). Moreover, in depressive patients, 5-HT$_{1A}$ receptor binding has shown to be reduced in cortical and limbic areas (Drevets et al., 2000; Hirvonen et al., 2008). Selective 5-HT$_{1A}$ antagonists have been developed with the intention to block the autoinhibitory feedback occurring during antidepressant treatment with SSRIs (Figure 5). Newer compounds, e.g. WAY100635, are highly selective for the 5-HT$_{1A}$ receptor, but do not distinguish between the autoinhibitory component in DRN and the post-synaptic component in limbic areas leaving the mission regarding improved response time to SSRIs uncompleted.

SERT – the serotonin transporter

SERT is a highly conserved molecule belonging to the family of monoamine transporters also including the noradrenalin and dopamine transporters. SERT is located both in pre- and perisynaptic membranes and found in highest density in DRN and hippocampus (Murphy et al., 2004). The function of SERT is transport of serotonin back into the pre-

synaptic neuron after serotonin release into the synaptic cleft. The removal of serotonin terminates the synaptic action (Figure 5). SERT is a well-known protein due to its important role in depression and especially due to its position as target for well-known antidepressant drugs, such as Prozac™ (fluoxetine) and Cipralex/Lexapro™ (escitalopram). These antidepressants bind to and block SERT, while other antidepressants, e.g. venlafaxine, partly inhibit SERT and partly the noradrenaline transporter. The presence of the ss genotype (homozygosity for the short variant of the SERT promoter) in the 5-HTT-gene-linked polymorphic region (5-HTTLPR) results in decreased activity of the promoter causing lower expression efficiency, which has been associated with major depression in Caucasians (Caspi et al., 2003; Kiyohara and Yoshimasu, 2009). In addition, replicated studies have demonstrated that the ss genotype is associated with poorer therapeutic response following treatment with SSRIs (Yu et al., 2002).

BDNF and the serotonergic system

BDNF and serotonin has been presented as a "dynamic duo" (Mattson et al., 2004). This refers to studies showing that BDNF exerts trophic effects of BDNF on serotonergic neurons (Mamounas et al., 1995; Mamounas et al., 2000) and others describing a direct effect of BDNF on serotonergic neurotransmission (Siuciak et al., 1996; Siuciak et al., 1998). The term also implies the reciprocal effect, since several studies have shown that treatment with SSRI treatment increases BDNF levels (Sen et al., 2008). Furthermore, specific activation of 5-HT_{2A} receptors has shown to regulate BDNF expression in frontal cortex and hippocampus (Vaidya et al., 1997; Vaidya et al., 1999). We have previously shown that stimulation with BDNF selectively down-regulates 5-HT_{2A} receptor protein levels, without affecting 5-HT_{1A} receptor levels (Trajkovska et al., 2009). A pronounced impact of BDNF depletion on the serotonin system was illustrated by Lyons and co-workers (1999) describing serotonergic abnormalities in BDNF +/- mice. These mice showed decreased cortical levels of serotonin in aged animals and increased 5-HT_{2A} receptor mRNA levels in cortex, but a tendency towards the opposite in hippocampus. As mentioned previously, conditional depletion of BDNF resulted in a selective decrease in 5-HT_{2A} receptor expression and signalling without affecting 5-HT_{1A} expression levels (Rios

et al., 2006). Apparently, there are differences between these two models of BDNF depletion, despite the fact that both models show marked impact on serotonin receptor regulation. It should be kept in mind that changes in BDNF +/- mice is a combination of developmental effects and BDNF effects in adult animals since these animals have 50% BDNF from a very early embryonic stage. The mechanisms for this regulation in either model remains to be determined.

While Rios and co-workers primarily focused on 5-HT_{2A} receptor function and expression (Rios et al., 2006) others have investigated the effect of BDNF depletion on 5-HT_{1A} receptor function (Hensler et al., 2003; Hensler et al., 2007). In these studies it was shown that BDNF depletion through an inducible knock-down system (tetracycline-induced) does not affect 5-HT_{1A} receptor binding or function (Hensler et al., 2007), while a decreased function of the 5-HT_{1A} receptor without concomitant changes in binding, was reported in BDNF +/- mice (Hensler et al., 2003). *In vivo* imaging of human subjects did not reveal any interactions between the BDNF val66met polymorphism and 5-HT_{1A} binding in healthy subjects (Henningsson et al., 2009).

Only limited evidence describes an interaction between 5-HT_{2C} and BDNF, but a significant reduction in 5-HT_{2C} mRNA levels in hippocampus was observed in BDNF +/- mice (Lyons et al., 1999). On the other hand, the 5-HT_{2C} antagonist S32006 increased hippocampal BDNF expression (Dekeyne et al., 2008) and similarly another study showed increased BDNF expression in hippocampus in 5-HT_{2C} KO mice (Hill et al., 2010). Furthermore, agomelatine, a melatonin agonist and 5-HT_{2C} antagonist, has shown to have anxiolytic and antidepressant effects and recently it was shown that agomelatine acutely up-regulates BDNF expression in frontal cortex indicating a neurotrophic response of this compound (Molteni et al., 2010a). Collectively, also here a reciprocal function is reported for 5-HT_{2C} and BDNF.

Several reports have shown an interaction between SERT and BDNF. For example, Mössner et al (2000) demonstrated that BDNF administration to a lymphoblast cell line expressing SERT, decreased the activity of the transporter in a dose-dependent fashion (Mössner et al., 2000). Others have shown that BDNF tends to increase serotonin levels in raphe neurons probably by increasing expression of TH levels (Siuciak et al., 1998). This interrelation has also been investigated in BDNF +/- mice, demonstrating a lower activity

of SERT in these mice, without affecting the actual number of transporters (Daws et al., 2007). Similarly, Rios and co-workers found a small decrease in 5-HT levels in DRN of conditional BDNF KO mice, but no changes in hippocampus or frontal cortex (Rios et al., 2006). Reciprocally, a marked decrease in BDNF expression was observed in both hippocampus and prefrontal cortex in SERT knock-out rats (Molteni et al., 2010b)

In summary, a vast amount of evidence shows a close coalition of BDNF with the serotonergic system. Exactly how this interaction is mediated and whether it is involved in neuropsychiatric and/or neurological disorders is not known. Moreover, it is possible that the changes seen after perturbations of either BDNF- or serotonergic neurotransmission are compensatory mechanisms.

Aims of thesis

The aims of the current thesis were threefold:

- To investigate interactions between BDNF and the serotonin system and possible functional implications
- To evaluate the validity of serum BDNF measurements as a reflection of brain BDNF levels
- To investigate the association between serum BDNF levels and brain diseases

Experimental Methods

In this section, a short description of the methods used in this thesis is provided. More common techniques (e.g. immunohistochemistry and western blotting) are not described (see Materials and Methods in Paper I for a short outline).

BDNF knock-out mice

Traditional knock-out mice are created by introducing a deleting sequence into the genome of the blastocyst, thus completely removing the protein of interest. However, this is not an achievable model when studying BDNF. When BDNF knock-out mice (BDNF -/-) are engineered in this manner, they normally die during their second postnatal week making studies in adult mice unfeasible. Somewhat unexpectedly, the BDNF -/- animals die due to developmental abnormalities in the cardiovascular system (Donovan et al., 2000). To circumvent this problem, conditional BDNF knock-out mice have been generated using the Cre-lox recombination system (Rios et al., 2001). In one line of mice, the Cre-recombinase gene is driven by the neuron specific α-calcium/calmodulin-dependent protein kinase II (αCamKII) promoter. This line is then crossed with a second line of mice, where the coding exons of interest are flanked by two loxP sites, thereby enabling deletion in cells where cre-recombinase is expressed. Starting around P0, the αCamKII promoter is activated, driving Cre recombinase expression (Rios et al., 2001) and thereby progressively removing the single BDNF coding exon. The offspring shows extensive depletion of BDNF, and by P21, protein levels of BDNF are undetectable in the adult mouse brain (Figure 1 in Paper I and III). As a result of central BDNF depletion, mice display an anxiety-like phenotype and develop dramatic mature onset obesity (Rios et al., 2001).

A potential pitfall evaluating these mice in relation to disturbances in the serotonergic system may be their dramatic phenotype. Obviously, the phenotype is a result of the BDNF depletion, but many effects can be indirect, i.e. via changes in other neurotransmission systems. It complicates the work that these animals are so behaviourally challenged, making it hard to decipher which of the neurotransmission systems that are affected. Furthermore, a significant influence of compensatory

mechanisms is likely to occur. Despite these considerations, the conditional BDNF knock-out mice offer a unique opportunity to study the consequences of perturbed BDNF signalling on the serotonergic system *in vivo*.

cLH and cNLH rats

The creation of reasonably valid animal models of neurological and neuropsychiatric diseases has been a prerequisite to improve our understanding of the etiology behind these disorders. This is indeed not an easy task since diseases such as depression comprises a wide range of symptoms, which often are described verbally by the patients. However, it is known that stress and/or stressful life events often precede depression (Williamson et al., 1998). The animal model of learned helplessness (LH) is based on the fact that patients with depression often feel helpless or anxious in uncontrollable environments (Kendler et al., 2003). This scenario is possible to mimic in rodents when exposing them to unpredictable and inescapable stress (Henn and Vollmayr, 2005). Even though it is not possible to measure one of the central issues in depression, depressed mood, in these animals, there is an excellent concordance with other signs of depression, such as weight loss, agitation, sleep disturbances and associative-cognitive deficits (Henn and Vollmayr, 2005; Kendler et al., 2003).

To create a genetic animal model of depression, animals showing learned helplessness behaviour following inescapable stress can be selected and mated. After 20 generations the animals show signs of congenital learned helplessness (cLH) behaviour, i.e. appearing helpless without preceding uncontrollable shock. On the contrary, animals that are resistant to the effects of inescapable shock are termed congenital non-learned helpless (cNLH). In Paper IV, we made use of cLH and cNLH rats created from the following protocol: Sprague-Dawley rats were given a total of 20 min with uncontrollable and unpredictable 0.8 mA footshocks. Twenty-four hours later the rats were tested in an escape paradigm where the foot shock could be avoided with a single lever press: animals with more than 10 failures (of 15 trials) were considered helpless and animals with less than five failures were considered as nonhelpless. Helpless animals and nonhelpless animals, respectively, were mated for subsequent generations avoiding littermate crosses resulting in two selective strains: the cLH, demonstrating helpless

behaviour and cNLH which show no signs of this behaviour. Animals from the 49th generation were used in Paper IV.

ESR and HTR (ear-scratch response and head-twitch response)

Ear-scratch response (ESR) and head-twitch response (HTR) are characteristic movements in mice, originally discovered when hallucinogenic pharmacological compounds were analyzed (for review, see (Glennon et al., 1991). ESR is not seen in other species than mice, while rats demonstrate HTR and wet-dog shakes following administration of a 5-HT$_{2A}$ agonist. In rabbits, the reflex response is described as head-bobs (an up-down movement of the head) (Aloyo and Dave, 2007). ESR is defined as a scratching of the ear, head or neck with either hind limb. Each scratch response is defined as one single or several repetitive scratch movements. If these movements are interspersed by a period of more than 2 seconds they will be scored as individual responses (Darmani and Pandya, 2000). In addition, if the mouse alternates between right and left hind limb within one scratch response, they are scored as separate responses (Darmani and Pandya, 2000). HTR is a distinct, fast movement of the head, which can be distinguished from lateral head-jerks and head-bobs. In the experiments described in Paper I, ESR and HTR were evaluated as described above. In brief, mice were moved to a quiet room 1 hr before injection and either injected with 2,5-dimethoxy-4-iodoamphetamine (DOI)(2.5 mg/kg, i.p.) or saline (0.9% NaCl) and video taping was started immediately after. Scoring of HTR and ESR were done in 5 min bins for 20 min (5-25 min). The results are presented as ESR/20 min and HTR/20 min.

Autoradiography

In Paper I, we employed *in vitro* autoradiography as a method to quantify receptor binding for the 5-HT$_{2A}$ and 5-HT$_{1A}$ receptors and for SERT. In all cases we used ^{3}H-labelled ligands, which provide high spatial resolution due to their low-energy β-emission. However, traditionally this has been a very slow laboratory analysis method since the exposure to ^{3}H-sensitive films may be as long as 3-6 months. To circumvent this problem, we applied ^{3}H-sensitive phospho-image plates. In these, surface crystals store the energy from the β-emission and later release this as luminescence when scanned in a phosphoimager thereby providing a digital image of the radioactive decay (Kanekal et al.,

1995). This makes it possible to reduce the exposure time from several months to 7-14 days without compromising the resolution significantly.

The autoradiography applied in Paper I made use of ^{3}H-MDL100907 (5-HT_{2A} antagonist) ^{3}H-WAY100635 (5-HT_{1A} antagonist) and ^{3}H-escitalopram (SERT blocker), all highly specific ligands with low non-specific binding. Non-specific binding was analyzed after blocking 5-HT_{2A}, 5-HT_{1A} or SERT binding with high concentrations of ketanserin, serotonin and paroxetine, respectively. Pargyline (a MAO inhibitor) and ascorbic acid was added to the incubation buffer to minimize degradation of the ligand.

Traditionally, ligand concentrations in autoradiography studies are used in order to saturate all binding sites and thereby get a measurement of total binding (B_{max}). However, in certain conditions, for example using ^{3}H-MDL100907, a more robust quantification can be made using concentration of 0.4 nM, which supposedly correspond to 1-2 x K_d. This concentration was chosen due to optimal signal-to-noise ratio levels and has previously been used for similar experimental set-ups (Licht et al., 2009; Lopez-Gimenez et al., 1997). For SERT binding, we used concentrations of 2.0 nM ^{3}H-escitalopram (4-6 x K_d) and 1.5 nM ^{3}H-WAY100635 (4-6 x K_d). Comparisons of receptor binding were only performed within the same image plate, i.e. sections from all animals were located on the same image plate, to avoid inter-plate variation.

BDNF sandwich ELISA

The enzyme-linked immunosorbent assay (ELISA) has been one of the key methods in the work in this thesis (Paper I-V). This method can be used to detect very low levels of protein and the protein of interest can be detected in its native form, making it possible to analyze BDNF in both blood and tissue samples. We have previously validated this method for blood and serum samples (related paper #3). The ELISA method quantifies the concentration of the protein of interest via binding to specific antibodies in designated wells. Here, we made use of the sandwich ELISA method (also referred to as "two-

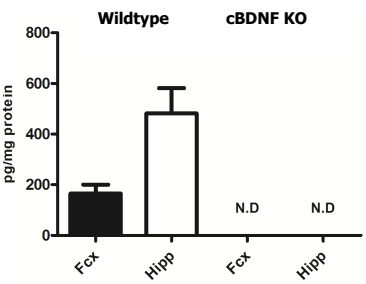

Figure 6. An estimation of cross-reactivity with other neurotrophins/related proteins in BDNF ELISA measurements of brain tissue levels. BDNF in brain tissue show the expected values for wildtype mice, while no signal was present in wells with tissue lysates from cBDNF KO mice. Modified from Figure 1 in paper III. N.D.;non-detectable

site ELISA"), which make use of a capture antibody (1st primary antibody) coated onto the bottom of the wells in the ELISA plate. After adding the samples containing BDNF, a second antibody is added (2nd primary), thereby forming a "sandwich" consisting of antibody-BDNF-antibody. Following this step, a secondary horse-radish peroxidase-coupled antibody directed against the second primary antibody is added to the complex, which can convert a substrate into a coloured substance, which can be read using an absorbance reader.

The great advantage using the sandwich method is the two primary antibodies, which increase the specificity considerably. The first capturing antibody will delimit non-specific binding to the ELISA plate. Since the antigen-antibody binding is much stronger than non-specific binding, a harsh washing protocol will eliminate most non-specific binding. The limitation of this method is the necessity of highly specific primary antibodies. However, in Paper III we present high specific binding of BDNF with negligible binding from other neurotrophins (Figure 6).

RT-qPCR

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a widely used method to quantitate mRNA levels in both tissue samples and cell culture systems (Nolan et al., 2006). RNA can be extracted from tissue using the TRIzol method (a phenol-chloroform extraction) or using column-based methods. Here, we used TRIzol to extract RNA from brain tissue. The RNA was reverse transcribed into cDNA using oligo(dT)15 primers. The reverse transcription reaction mixture contained 20% ImProm-II™ 5x reaction buffer, 6 mM $MgCl_2$, 0.5 mM dNTP mix and 20 units RNase inhibitor. The reverse transcription reaction was performed at 42 °C for 60 min, followed by heating at 70 °C for 15 min. The qPCR reactions were performed by adding the sample cDNA to a reaction mixture Brilliant II SYBR green 15 pmol of each primer. SYBR green preferentially binds to double stranded DNA (Zipper et al., 2004) providing a fluorescence signal corresponding to the amount of amplified cDNA. The qPCR was performed with a 10 minutes preincubation at 94°C followed by 40 cycles of 30 seconds at 94°C, 45 seconds at 60°C and 90 seconds at 72 °C. The real-time PCR method was validated using serially diluted cDNA to establish a standard curve. To quantify the gene expression profile of each sample, the efficiency of each standard curve was determined from its slope and

comparative threshold. For each sample, the amount of target mRNA was normalized to that of a reference gene, e.g. β-tubulin III.

A possible pitfall in RT-qPCR is the amplification of non-specific products. Since the amplification is exponential, a few "false" cDNA bits or DNA pieces (for example via genomic DNA contamination) will be amplified exponentially and may disturb the signal. However, before the RT reaction, samples are DNAse treated to prevent this. Moreover, if possible, we constructed our primers to span across a splice site. Doing this, an amplification of genomic DNA is unlikely because primers will only bind to sequences specific for the exons. Genomic DNA still have interspersed introns, while cDNA stems from coding sequences only. Moreover, we tested several sets of primers and made sure that we achieved similar results.

TaqMan genotyping

Some of the principles from the qPCR technique was applied to screen for the val66met polymorphism in Paper II using the TaqMan 5'-exonuclease principle (Livak et al., 1995). Here, we used fluorogenic probes comprising an oligonucleotide labeled with both a fluorescent reporter dye and a quencher dye (Livak, 2003). In the intact probe, proximity of the quencher eliminates the fluorescence signal observed from the reporter dye. Each probe is matched to either nucleotide (in this case) G or A. For the probes matching G, we used the reporter molecule VIC and for A we used the fluorescent molecule FAM. When running the PCR reaction, specific primers will bind to the site on the DNA strand close to the SNP of interest and the DNA polymerase will start to elongate the copy of the strand. When the DNA polymerase reaches the site of the probe, the 5´exonuclease activity of this enzyme will cleave the probe and thereby remove the quencher from the reporter dye. If only a VIC signal is detected, the person has the genotype G/G, which corresponds to the val/val genotype; if both VIC and FAM signals are detected, the genotype is val/met; and detecting only a FAM signal corresponds to the met/met genotype. There is no signal from the probes if not cleaved by the DNA polymerase during DNA amplification. The great advantage using this system is high degree of reliability with no false positives as well as the high-throughput – a single well in a PCR plate (96- or 384-wells) per subject.

Results & Discussion

Changes in the serotonergic system of cBDNF KO mice (Paper I)

In paper I, we investigated possible changes in 5-HT_{2A}, 5-HT_{1A}, 5-HT_{2C} and SERT in cBDNF KO mice. We mainly focused on frontal cortex and hippocampus, where relatively high expression levels of BDNF, 5-HT_{2A} and 5-HT_{1A} receptors are found. First, we evaluated whether BDNF was depleted to the same extent in these two regions. Our results showed very clearly that BDNF is completely removed from both regions in the cBDNF KO mice, illustrated by ELISA and western blotting (Figure 7). These findings correspond well to a previous study describing an almost complete ablation of the BDNF transcript in cortex (Chan et al., 2006). However, in another study, where they used the same promoter to drive CRE recombinase expression to remove TrkB, a depletion efficiency of 85% for hippocampus was observed (Minichiello et al., 1999). This minor discrepancy may be explained by shorter mRNA- or protein half-life for BDNF compared to TrkB, i.e. the residual brain TrkB transcripts will be present longer than BDNF, which results in a concentration detectable by western blotting and ELISA.

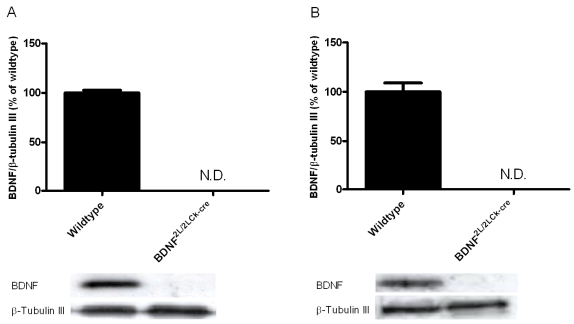

Figure 7. BDNF protein levels in frontal cortex (**A**) and hippocampus (**B**) in wildtype and $BDNF^{2L/2LCk\text{-}cre}$ mice. BDNF levels were not detectable in frontal cortex and hippocampus in the BDNF mutant mice using western blot, while wildtype levels corresponded to levels seen in other mouse strains. BDNF levels were related to β-tubulin III levels (n=7). Representative bands for BDNF (14 kDa) and β-tubulin III (55 kDa) are shown below the corresponding column. N.D.: not detectable. Error bars indicate SEM.

The serotonin receptor autoradiography studies revealed interesting findings. There was a marked decrease in 5-HT_{2A} binding in frontal cortex, but an increase in hippocampus (Figure 2 in Paper I). In contrast, and surprisingly, 5-HT_{1A} receptor binding was decreased in hippocampus and unchanged in frontal cortex (Figure 8). It was shown previously that 5-HT_{1A} receptor protein levels were unchanged in DRN in cBDNF KO mice (Rios et al., 2006). In DRN, the 5-HT_{1A} functions as autoreceptor, while in hippocampus, it mainly exists as post-synaptic heteroreceptor (Riad et al., 2001). Therefore, the data shown here suggests that BDNF affects 5-HT_{1A} receptors in a context-dependent manner. Hensler and colleagues (2007) found no change in hippocampal 5-HT_{1A} receptor binding using a

tetracycline-inducible model of BDNF depletion. However, the mice used in that study were depleted of BDNF in adulthood, while the mice used in the current work were gradually depleted during the first post-natal weeks starting at P0 (see Experimental Methods section). Therefore, the changes in 5-HT_{1A} binding seen here may reflect i) a long-term effect of BDNF depletion ii) effects of perturbed BDNF signalling during a critical developmental period or iii) a compensatory response to the changes in hippocampal 5-HT_{2A} receptor binding observed in these mice. A previous study elegantly showed counter-regulatory effects of 5-HT_{1A} and 5-HT_{2A} in the regulation of NMDA receptor function and trafficking (Yuen et al., 2008). In parallel, another study showed opposite responses on dendrite growth when stimulating with a 5-HT_{1A} or 5-HT_{2A} agonist, respectively (Kondoh et al., 2004). In summary, the observed receptor changes seen here is likely a collective compensatory response to overcome the challenge of BDNF depletion.

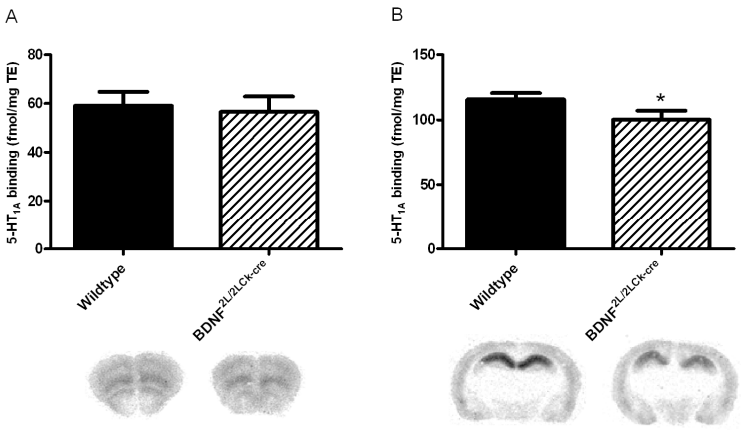

Figure 8. 5-HT_{1A} receptor binding (^{3}H-WAY100635) in $BDNF^{2L/2LCk\text{-}cre}$ mice (cBDNF KO mice) and wildtype littermates in frontal cortex (**A**) and dorsal hippocampus (**B**). There was a significant decrease in binding in dorsal hippocampus in $BDNF^{2L/2LCk\text{-}cre}$ mice vs wildtypes, while no change was observed in frontal cortex. (n=4, Student´s t-test, $*P < 0.05$). Representative autoradiograms are shown below the corresponding column. Error bars indicate SEM

The divergent results regarding the 5-HT_{2A} binding in frontal cortex and hippocampus may be a result of "cell type specificity" with respect to 5-HT_{2A} receptor localization. Vaidya et al (1997) observed opposite responses in BDNF regulation in these regions following administration of the 5-HT_{2A} agonist DOI and suggested that it might be due to the cell-type specific localization of the 5-HT_{2A} receptor. In cortex, the 5-HT_{2A} receptors are located in very high density in layer V on glutamatergic neurons, while in hippocampus the majority of receptors are located on GABAergic neurons (Vaidya et al., 1997). This suggests that an overall adjustment of 5-HT_{2A} signalling, e.g. following BDNF depletion, may be solved by a down-regulation of 5-HT_{2A} receptor expression in frontal cortex and an up-regulation in hippocampus. This is highly speculative, but the 5-HT_{2A} binding results in Paper I further illustrate the putative difference in function and

regulation of the 5-HT_{2A} receptor in frontal cortex vs hippocampus, which has been suggested previously (Dwivedi et al., 2005).

To assess the functionality of the observed decrease in 5-HT_{2A} receptor binding, we applied two specific tests for 5-HT_{2A}-agonist mediated behaviour, ear-scratch response (ESR) and head-twitch response (HTR). Following stimulation with a relatively high dose (2.5 mg/kg, i.p.) of the 5-HT_{2A} agonist, DOI, we observed a prominent HTR in both wildtype and cBDNF KO mice without any differences between genotypes. However, the ESR was markedly reduced in the cBDNF KOs indicating that this behaviour was influenced by the changes in 5-HT_{2A} receptor expression. This discrepancy between the two types of response may be explained by the complex "re-constituted receptor homeostasis" in the cBDNF KO animals. For example, the 5-HT_{2A} receptor binding appeared to be *increased* in hippocampus and it may be speculated that the two types of behaviour are differentially regulated by the two brain regions. Thorough studies have been performed in rats where 5-HT_{2A} agonists were injected in various brain regions. Specifically, the authors found medial prefrontal cortex to be the region responsible for HTR. But because rats do not show signs of ESR, a similar evaluation could not be made for this response (Willins and Meltzer, 1997). Future anatomical and functional studies may provide knowledge on the designated neuronal circuits responsible for ESR.

To verify whether the observed receptor changes were a mere result of general changes of the serotonin system, we investigated SERT binding and expression levels of synaptic and dendritic markers. We did not find any evidence indicating changes in SERT levels in either frontal cortex or hippocampus and previous data on serotonin levels provided no reason to believe that SERT is affected in these regions (Rios et al., 2006). However, it should be kept in mind that High Performance Liquid Chromatography (HPLC) analysis of serotonin levels does not provide any information on the dynamics of the system (secretion and re-uptake rates), but is an estimation of total levels. The activity of SERT, and thereby the clearance rate of serotonin form the synaptic cleft, was not analyzed in this study and we cannot exclude that serotonin transporter activity was changed. Previous studies found a decreased rate of serotonin transport in BDNF +/- mice, but no changes in SERT binding (Daws et al., 2007). In future studies this could be measured in cBDNF KO mice using microdialysis.

To ensure that the observed changes were not a result of dendrite or synapse loss, we verified that the decreased receptor densities were not accompanied by differences in dendritic and synaptic markers between BDNF knock-out animals and wildtype littermates. We investigated the protein levels of MAP2 (dendritic marker), synaptophysin (pre-synaptic marker) and PSD-95 (post-synaptic marker), but found no differences between wildtype and cBDNF KO animals, suggesting relative intact synaptic connections (see Figure 9 in Paper I).

A potential pitfall in Paper I is that we did not divide our animals according to gender. This may be a subject of criticism, but it is based on the following considerations: The breeding of cBDNF KO mice is not trivial and the yield of BDNF KO mice is low. Therefore, to compare knock-out animals with their wildtype littermates, it was necessary to collect both males and females. Alternatively, a very large mouse colony should be available or animals with different ages should be used. The latter bring in new problems regarding age-related changes in the serotonergic system (Paper IV and Lyons et al., 1999). However, we gender-matched the groups in all experiments, and when testing for gender-differences we did not find any evidence suggesting that gender should affect the results described in this study.

In conclusion, we demonstrated in Paper I that BDNF depletion results in context-dependent changes in 5-HT_{2A} and 5-HT_{1A} binding and expression. Furthermore, we showed that alterations in 5-HT_{2A} binding have functional consequences as illustrated by the diminished DOI-induced ESR in BDNF mutants. The diverse regulation of 5-HT_{2A} and 5-HT_{1A} receptors in these mice underlines the complex consequences of global BDNF depletion and illustrates a region-specific role for BDNF.

No effects of the BDNF val66met polymorphism on serotonergic markers (Paper II)

To further characterize the *in vivo* effects of perturbed BDNF signalling, we investigated whether BDNF levels in blood and the val66met BDNF polymorphism were associated with 5-HT_{2A} receptor- and SERT binding in healthy human subjects (Paper II). These studies involved PET scanning for 5-HT_{2A} receptor- and SERT binding, blood BDNF ELISA measurements, genotyping for val66met, and evaluation of personality scores using questionnaires.

We hypothesized that val66met would affect cortical 5-HT_{2A} receptor binding due to previous reports stating that BDNF depletion has a significant impact on 5-HT_{2A} receptor

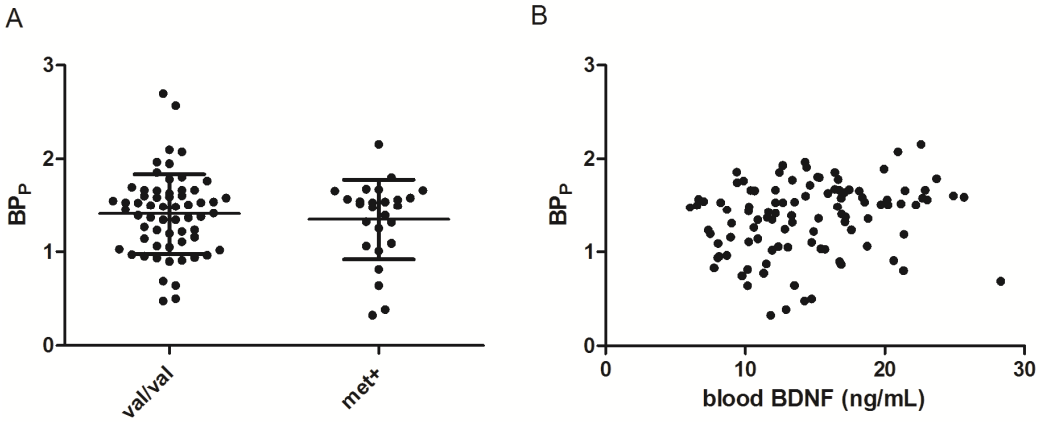

Figure 9. Neocortical 5-HT_{2A} binding in the val/val and met+ groups. There were no significant differences between the two groups (Mean: 1.41 ± 0.43 vs. 1.35 ± 0.43)(**A**). Correlation between BDNF levels in blood and neocortical 5-HT_{2A} binding (**B**). Even though there was a tendency towards a positive correlation, this was no longer present following adjustments for age, neuroticism and BMI. Error bars in **A** represents SD.

protein levels (Paper I and Rios et al., 2006). However, the 5-HT_{2A} receptor binding was not affected by val66met status (Figure 9A). In addition, we did not detect any association between BDNF levels in blood and 5-HT_{2A} receptor binding (Figure 9B). To further validate this, we made a small-scale, pre-liminary study in val66met knock-in mice (n=2-3), but did not see any tendencies towards changes in 5-HT_{2A} receptor binding (data not shown). The discrepancy between BDNF depletion and val66met-mediated effects may be because the val66met polymorphism does not lead to changes in total BDNF levels *per se* (Chen et al., 2006; Trajkovska et al., 2007), i.e. differences in the activity-dependent secretion of BDNF do not seem to have any effect on 5-HT_{2A} receptors, while changes in total BDNF levels alter 5-HT_{2A} receptor levels (Paper I & related paper #2). The val66met mice have in many respects normal behavioural

responses in baseline conditions, but respond differently to stressful situations than wildtype mice (Krishnan et al., 2007). Therefore, it is possible that the val66met-5-HT_{2A} receptor interaction only comes into play following stressful life events or under pathological conditions.

We did not find any association between the BDNF val66met polymorphism and SERT binding (Figure 10A). This is different from a recent study by Henningson and colleagues (2009), who found a significant difference in SERT binding between val/val and met-carriers in men using PET. They somewhat consolidate this using SPECT to determine

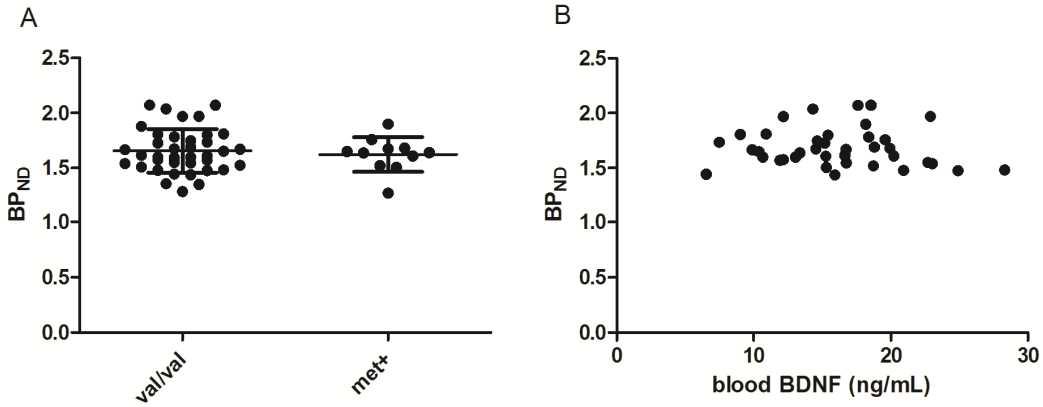

Figure 10. Mean SERT binding in a high-binding region in val/val and met+ carriers. There were no significant differences in SERT binding between val/val and met+ carriers (1.66 ± 0.20 vs 1.62 ± 0.16, respectively, *P*=0.61)(**A**). Correlations between blood BDNF levels and SERT binding (**B**). There were no significant associations between these parameters (r=-0.09, *P*=0.59).

SERT binding, where they see a tendency towards a lower SERT binding in male met carriers in a separate sample. However, the statistical power in the Henningson study is considered low to very low. For example, the female met-carrier group consisted of three subjects (Henningsson et al., 2009). Considering the biological variation in healthy humans as well as test-variability and possible confounders, such results should be interpreted with caution. Here, in a larger sample, we were not able to reproduce these findings. This illustrates a problem with imaging genetics – the risk for false positives and low reproducibility between studies. Larger sample sizes in future studies may improve these matters.

We also looked for a possible association between BDNF blood levels and SERT binding, since experimental studies have suggested that changes in SERT protein levels leads to changes in BDNF expression (Molteni et al., 2010b). On the other hand, we did not see any change in SERT binding in cBDNF KO mice (Paper I). Here, in healthy human subjects,

we did not detect any correlation between BDNF blood levels and SERT binding (Figure 10B).

In summary, the val66met polymorphism in the BDNF gene does not affect serotonergic markers in human subjects. Supposedly, the serotonergic system is only affected by more dramatic changes in blood BDNF levels, which may be seen in some patient groups, e.g. depressive patients. To answer whether such changes in serum and blood BDNF measurements reflects the actual brain BDNF status, we examined whether such a correlation exists between these two compartments.

Correlation of BDNF in blood and brain (Paper III)

To examine whether the serum and blood measurements used in paper II and V (and in numerous publications) reflect brain BDNF concentrations, we looked for a possible correlation between BDNF in blood and BDNF in brain in rats, mice and pigs. Unfortunately, a similar experiment in humans was not accessible. We obtained blood samples and dissected frontal cortex and hippocampus from rats, mice and pigs to investigate whether a possible correlation existed and to validate our BDNF ELISA kits for those species. Interestingly, we found that blood samples from mice do not contain detectable BDNF levels (Figure 11A & B). This is intriguing, since BDNF levels in brain are similar to the levels found in rats (Figure 11C). A similar observation has been presented before by Radka et al. (1996), where a home-made EIA assay was used to measure BDNF, while we used a well-validated commercially available ELISA kit.

We measured BDNF in lysed mouse blood samples as well as measurements on plasma

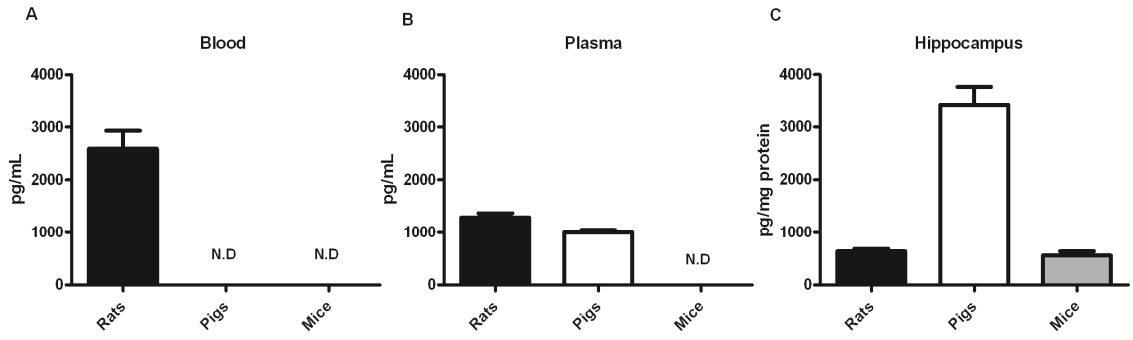

Figure 11. BDNF levels in blood (A), plasma (B) and hippocampus (C) in rats, pigs and mice. BDNF levels in whole blood could be measured in rats, but were undetectable in mice and pigs (a). In plasma, we were able to measure BDNF in rats and pigs, but not in mice (b). In hippocampus, pigs showed 4-5 fold higher BDNF concentrations than rats and mice (c). N.D: non-detectable. Error bars indicate SEM.

and undiluted serum. Nevertheless, we were not able to detect any signal above background measurements in plasma, serum or blood in several different mouse strains. Apparently, this is not due to lack of BDNF transport capacity, as it was shown that mice readily transport iodine-labelled BDNF and recombinant BDNF in the blood (Pan et al., 1998). It is possible that BDNF is rapidly cleared from mouse plasma, but this needs to be validated. The important message here is that blood BDNF in mice is below the detection limit in most commonly used ELISA kits.

In rats and pigs, we found a significant correlation between brain and blood BDNF (Figure 12), underlining the potential use of blood BDNF to monitor BDNF expression in brain. These results further support findings from previous studies. For example, Karege et al. (2002) reported a significant correlation between blood and brain BDNF in rats, but only in young animals. In a recent elaborate study, a significant correlation between serum BDNF and brain tissue in adult animals was presented (Sartorius et al., 2009). Curiously, the results from prefrontal cortex and hippocampus were not presented individually. In the current study, we could not detect a significant correlation between blood BDNF and BDNF in frontal cortex. A plausible explanation may be that BDNF is more difficult to extract from cortex and the total yield is therefore subject to higher variation. For example, Elfving et al. (2010) showed that the extraction efficiency of BDNF after spiking the homogenates was 30% for frontal cortex, but 70% for hippocampus. On the other hand, the data from the current study in mice tissue showed a highly significant correlation between BDNF levels in hippocampus and frontal cortex, suggesting a fairly low variation in extraction yield from these two compartments, at least in mice (Figure 1B in Paper III).

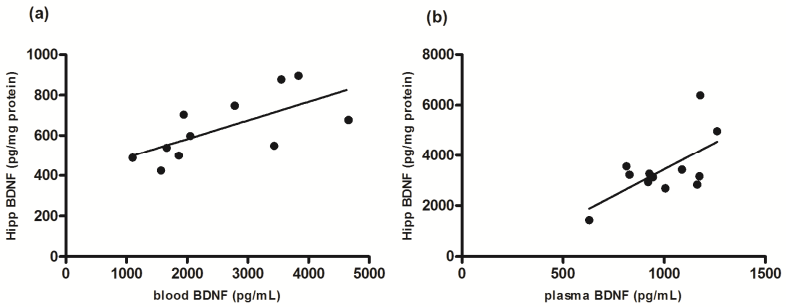

Figure 12. BDNF levels in hippocampus vs blood and plasma in rats (a) and pigs (b). There was a significant positive correlation between blood BDNF and BDNF in hippocampus in rats ($p=0.025$, $r^2=0.44$)(a) and a significant positive correlation between BDNF levels in plasma and hippocampus in pigs ($p=0.025$, $r^2=0.41$, $n=12$)(b), Pearson's correlation test. Hipp: Hippocampus.

BDNF and serotonin fibres in a congenital animal model of depression (Paper IV)

In this study, we looked for differences in the serotonergic system and association with BDNF and age in congenital learned helplessness (cLH) and congenital non-learned helplessness (cNLH) rats, respectively. Learned Helplessness has been described as the animal parallel to the composite condition often experienced by patients with major depression (Kendler et al., 2003). In the current study, Sprague-Dawley rats were exposed to inescapable foot shocks and animals, which showed behaviour of helplessness, were mated and similarly for animals that did not show the LH response. The animals used in this study were bred for 49 generations and then tested for helplessness again. Following 5 months (young) and 13 months (old), animals were analyzed for changes in serotonergic markers and BDNF levels.

We found decreased levels of BDNF in hippocampus in the cLH animals compared to cNLH animals, and this was unrelated to age (Figure 13). Previous studies have shown decreased BDNF levels in hippocampus in depression-like animal models. For example, in Flinders Rats (resistant and sensitive line) – another congenital animal model of depression – lower hippocampal BDNF levels were observed in the sensitive line (Elfving et al., 2009) which displays a depression-like phenotype (Overstreet et al., 1994). Furthermore, we found an age-dependent decrease in serotonergic fibre density in CA1 of the hippocampus of cLH animals compared with cNLH animals (Figure 1 in Paper IV). Interestingly, this finding corresponds well to previous studies in mice regarding serotonergic innervation in BDNF +/- animals. Here, an age-accelerated loss of serotonergic fibres was found, particular for the CA1 region (Luellen et al., 2007). Even though we did not find a significant age-related decrease in hippocampal BDNF levels in this study, it may be speculated that the same mechanisms are evident in the aged cLH rats. Unfortunately, the dissection method used in this study did not allow specific determination of BDNF protein levels in the CA1 region, while *in situ* hybridization and/or immunohistochemistry, detecting BDNF mRNA levels and protein levels, respectively,

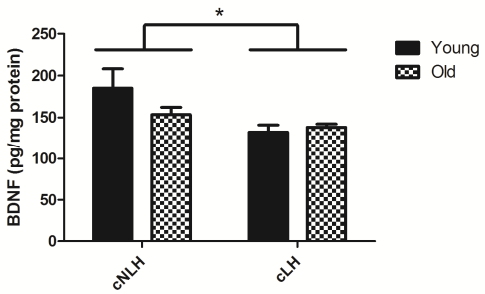

Figure 13. Hippocampal BDNF levels in young cNLH, old cNLH, young cLH and old cLH rats. Two-way ANOVA showed significant lower hippocampal BDNF levels in the cLH group ($P < 0.05$). There was no age effect.

could have provided the anatomical resolution. These analyses could have answered whether the observed BDNF loss is exclusively located in CA1. However, another study looking at BDNF mRNA levels in these animals were not able to detect differences between cLH and cNLH animals in baseline conditions, indicating that the observed differences in BDNF levels are a result of changed turnover or translational processing (Vollmayr et al., 2001).

In summary, the changes in hippocampal BDNF levels and serotonergic fibre density in a congenital animal model of depression underscores the importance and possible interrelation of these two proteins in depression and other stress-induced brain disorders.

Increased BDNF levels in narcolepsy: a biomarker for neuroregeneration? (Paper V)

In paper V, we investigated whether serum BDNF and NGF levels are altered in narcolepsy patients. Only patients with narcolepsy and cataplexy and with very low or undetectable levels of hypocretin were included (Figure 14A). We found a marked increase in serum BDNF levels in these patients (Figure 14B), which we suggest is the result of a neuronal regeneration response.

The importance of *in vivo* neuronal regeneration mediated by BDNF has previously been described (Sato et al., 2009; Song et al., 2008) and the potential of BDNF as a neuroprotective agent in neurodegenerative disorders has been intensively discussed (Zuccato and Cattaneo, 2009). However, the peripheral administration of BDNF has apparently some limitations. For example, in two different animal models of stroke,

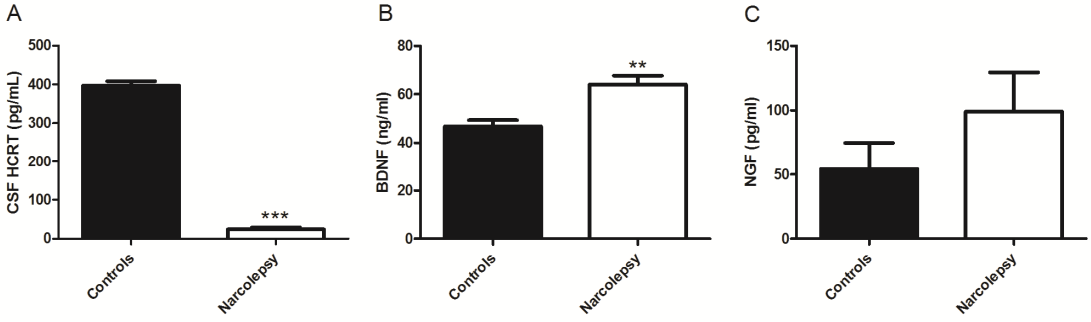

Figure 14. CSF levels of hypocretin (**A**) and serum levels of BDNF (brain-derived neurotrophic factor)(**B**) and NGF (nerve growth factor)(**C**) in healthy controls and patients with narcolepsy. Patients had significantly lower hypocretin-1 levels in CSF compared to healthy controls (24.44 ± 4.58 pg/ml, n=18, vs. 396.8 ± 11.31 pg/ml, n=12, t-test, ***P<0.0001)(**A**). Patients had significantly higher BDNF levels compared to controls (64.03 ± 3.69 ng/ml vs 46.79 ± 2.58 ng/ml, n=18 and n=15), respectively, t-test, **P<0.005)**(B)**. There was no significant difference between mean NGF levels in patients (99.35 ± 30.02 pg/ml, n=18) and healthy controls (54.15 ± 20.79 pg/ml, n=15)(non-parametric, Mann-Whitney test, p=0.34)**(C).** Error bars indicate SEM.

intravenous injections of BDNF did not have any effects on the integrity of hippocampal neurons or infarct size (Wu and Pardridge, 1999; Zhang and Pardridge, 2001). However, coupling of BDNF to an antibody against the transferrin receptor situated in the BBB resulted in a marked increase in transport and neuroregenerative response, illustrated by a reduction in infarct size (Wu and Pardridge, 1999; Zhang and Pardridge, 2001). In that regard, the initiatives to describe ways of boosting endogenous BDNF expression are highly relevant. Similarly, it is important to gain knowledge on a possible BDNF response in various disorders.

The increased BDNF levels indicate an important role for BDNF in narcolepsy and maybe also in sleep disorders in general. Several experimental studies have indirectly suggested a role for BDNF in the regulation of sleep. For example, studies have observed an up-regulation on both BDNF mRNA and protein levels following sleep deprivation in rats (Conti et al., 2007; Hairston et al., 2004). And in a recent clinical study, serum BDNF levels in sleep-deprived depressive patients were found to be increased (Gorgulu and Caliyurt, 2009). This implies a possible mechanism for the antidepressive effect of sleep deprivation and underscores the linkage between BDNF and sleep. Another possible explanation regarding the increased BDNF levels is the chronic fragmented sleep pattern in narcolepsy patients. A fragmented sleep pattern, illustrated by fast onset of rapid eye movement sleep phases, several nocturnal awakenings and long periods of intermittent wakefulness, has been reported in narcolepsy patients with cataplexy (Sturzenegger and Bassetti, 2004). It is possible that this fragmentation shares some common features with sleep deprivation paradigms and therefore contributes to the up-regulation of BDNF levels. Reciprocally, BDNF also regulates sleep. For example, cortical microinjections of BDNF have shown to increase slow-wave activity, which is a well-described marker for sleep need (Faraguna et al., 2008).

In summary, serum BDNF levels are markedly increased in narcolepsy patients, which could be a result of a neuroregenerative response, but the BDNF response could also be mediated by the sleep-fragmentation pattern seen in these patients. We are currently looking into the possible association between sleep behaviour and BDNF levels in humans. In addition, the findings in Paper V further add to the complexity and diversity in BDNF responses in neurological disorders.

Conclusions

Collectively, this thesis provides novel findings regarding the complex interactions between BDNF and the serotonergic system in both animal models and in human subjects. In Paper I, our results establish the significant interrelation between BDNF and the 5-HT_{2A} receptor and furthermore present results on hippocampal 5-HT_{1A} receptor expression not described before. In Paper II, we did not find any association between the val66met polymorphism and the serotonergic system, suggesting that in healthy subjects the subtle change in activity-dependent secretion is inadequate for mediating serotonergic changes comparable to those seen in Paper I. In Paper III, we suggest that total measurements of BDNF levels in blood can be used as a potential biomarker, i.e. a peripheral indicator of BDNF status in the brain. In paper IV, we show a possible interaction between BDNF and the serotonergic system in a congenital animal model of depression, which may be important for age-related risk for developing depression. In paper V, we describe a novel and still uncertain role for BDNF in the sleep disorder narcolepsy. The increase of BDNF in narcolepsy patients has not been reported previously and could represent a new BDNF research area.

Perspectives

The research perspectives in this line of research are many. First, a future interest is to determine whether the neurotrophic hypothesis of depression can be extended to include other neuropsychiatric and certain neurological disorders. The noticeable diverse functions of BDNF in different brain regions illustrate the difficulty in turning BDNF administration into a feasible pharmacological approach towards successful treatment of e.g. neurodegenerative disorders. For example, if BDNF is administered systemically, it will exert a global effect on the brain, which, supposedly, will create a complex and unpredictable outcome.

The val66met polymorphism in the BDNF gene does not seem to be associated with changes in serotonergic markers, but is speculated to play a role in combination with i) polymorphisms in the serotonin transporter and/or serotonin receptors or ii) following repeated stressful life events or in disease. Here, we only investigated the association in healthy subjects. The val66met polymorphism might affect the outcome in treatment regimes, where changes in the activity-dependent secretion of BDNF are involved in the mechanism of action. Collectively, this outlines an obvious design for future val66met experiments.

BDNF levels in blood can most likely be used as a reliable and representative marker of BDNF concentrations in CNS, even though this remains to be fully established in humans. However, it should be kept in mind that BDNF levels are subject to high interindividual variation in humans and correct use of sampling methods and timing are pivotal. Taking these factors into account, a human study is possible to undertake. However, important ethical concerns must be accounted for as well.

To establish the importance of BDNF in sleep regulation, further studies are needed. Through our collaboration with Danish Center for Sleep Medicine, we have initiated studies to elaborate on our initial findings. Future experiments will elucidate whether and how sleep regulates BDNF expression and vice versa. Novel findings in this area may contribute to a better understanding of the factors involved in narcolepsy and may provide new ways to develop pharmaceutical compounds that can either halt or limit the degeneration of hypothalamic neurons controlling this response.

It is established that BDNF is important for the mechanism of action in antidepressant treatment and we propose that the neurotrophic hypothesis of depression can be applied to a number of neurological and neuropsychiatric diseases. BDNF measurements may provide information on disease progression, mechanism of action of several drugs and may be used as a “screening tool” to assess new BDNF modulating treatment modalities.

References

1. Adachi M, Barrot M, Autry AE, Theobald D, and Monteggia LM (2008) Selective loss of brain-derived neurotrophic factor in the dentate gyrus attenuates antidepressant efficacy. *Biol Psychiatry* 63, 642-649

2. Adamantidis A and de Lecea L (2008) Sleep and metabolism: shared circuits, new connections. *Trends Endocrinol Metab* 19, 362-370

3. Allen JA, Yadav PN, and Roth BL (2008) Insights into the regulation of 5-HT2A serotonin receptors by scaffolding proteins and kinases. *Neuropharmacology* 55, 961-968

4. Aloyo VJ and Dave KD (2007) Behavioral response to emotional stress in rabbits: role of serotonin and serotonin2A receptors. *Behav Pharmacol* 18, 651-659

5. Baquet ZC, Bickford PC, and Jones KR (2005) Brain-derived neurotrophic factor is required for the establishment of the proper number of dopaminergic neurons in the substantia nigra pars compacta. *J Neurosci* 25, 6251-6259

6. Bekinschtein P, Cammarota M, Igaz LM, Bevilaqua LR, Izquierdo I, and Medina JH (2007) Persistence of long-term memory storage requires a late protein synthesis- and. *Neuron* 53, 261-277

7. Berger M, Gray JA, and Roth BL (2009) The expanded biology of serotonin. *Annu Rev Med* 60, 355-366

8. Berton O, McClung CA, Dileone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM, Bolanos CA, Rios M, Monteggia LM, Self DW, and Nestler EJ (2006) Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science* 311, 864-868

9. Berton O and Nestler EJ (2006) New approaches to antidepressant drug discovery: beyond monoamines. *Nat Rev Neurosci* 7, 137-151

10. Biffo S, Offenhauser N, Carter BD, and Barde YA (1995) Selective binding and internalisation by truncated receptors restrict the availability of BDNF during development. *Development* 121, 2461-2470

11. Bockaert J, Claeysen S, Becamel C, Dumuis A, and Marin P (2006) Neuronal 5-HT metabotropic receptors: fine-tuning of their structure, signaling, and roles in synaptic modulation. *Cell Tissue Res* 326, 553-572

12. Bourgin P, Zeitzer JM, and Mignot E (2008) CSF hypocretin-1 assessment in sleep and neurological disorders. *Lancet Neurol* 7, 649-662

13. Brunoni AR, Lopes M, and Fregni F (2008) A systematic review and meta-analysis of clinical studies on major depression and BDNF levels: implications for the role of neuroplasticity in depression. *Int J Neuropsychopharmacol* 11, 1169-1180

14. Carim-Todd L, Bath KG, Fulgenzi G, Yanpallewar S, Jing D, Barrick CA, Becker J, Buckley H, Dorsey SG, Lee FS, and Tessarollo L (2009) Endogenous truncated TrkB.T1 receptor regulates neuronal complexity and TrkB kinase receptor function in vivo. *J Neurosci* 29, 678-685

15. Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, 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, 386-389

16. Cassiman D, Denef C, Desmet VJ, and Roskams T (2001) Human and rat hepatic stellate cells express neurotrophins and neurotrophin receptors. *Hepatology* 33, 148-158

17. Castren E (2005) Is mood chemistry? *Nat Rev Neurosci* 6, 241-246

18. Castren E and Rantamaki T (2010) The role of BDNF and its receptors in depression and antidepressant drug action: Reactivation of developmental plasticity. *Dev Neurobiol*

19. Chan JP, Unger TJ, 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

20. Chao MV (2000) Trophic factors: An evolutionary cul-de-sac or door into higher neuronal function? *J Neurosci Res* 59, 353-355

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

22. Chen B, Dowlatshahi D, MacQueen GM, Wang JF, and Young LT (2001) Increased hippocampal BDNF immunoreactivity in subjects treated with antidepressant medication. *Biol Psychiatry* 50, 260-265

23. Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, Herrera DG, Toth M, Yang C, McEwen BS, Hempstead BL, and Lee FS (2006) Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science* 314, 140-143

24. Chen ZY, Patel PD, Sant G, Meng CX, Teng KK, Hempstead BL, and Lee FS (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, 4401-4411

25. Conti B, Maier R, Barr AM, Morale MC, Lu X, Sanna PP, Bilbe G, Hoyer D, and Bartfai T (2007) Region-specific transcriptional changes following the three antidepressant treatments electro convulsive therapy, sleep deprivation and fluoxetine. *Mol Psychiatry* 12, 167-189

26. Croll SD, Suri C, Compton DL, Simmons MV, Yancopoulos GD, Lindsay RM, Wiegand SJ, Rudge JS, and Scharfman HE (1999) Brain-derived neurotrophic factor transgenic mice exhibit passive avoidance deficits, increased seizure severity and in vitro hyperexcitability in the hippocampus and entorhinal cortex. *Neuroscience* 93, 1491-1506

27. Cunha C, Angelucci A, D'Antoni A, Dobrossy MD, Dunnett SB, Berardi N, and Brambilla R (2009) Brain-derived neurotrophic factor (BDNF) overexpression in the forebrain results in learning and memory impairments. *Neurobiol Dis* 33, 358-368

28. Darmani NA and Pandya DK (2000) Involvement of other neurotransmitters in behaviors induced by the cannabinoid CB1 receptor antagonist SR 141716A in naive mice. *J Neural Transm* 107, 931-945

29. Daws LC, Munn JL, Valdez MF, Frosto-Burke T, and Hensler JG (2007) Serotonin transporter function, but not expression, is dependent on brain-derived neurotrophic factor (BDNF): in vivo studies in BDNF-deficient mice. *J Neurochem* 101, 641-651

30. Dekeyne A, Mannoury la CC, Gobert A, Brocco M, Lejeune F, Serres F, Sharp T, Daszuta A, Soumier A, Papp M, Rivet JM, Flik G, Cremers TI, Muller O, Lavielle G, and Millan MJ (2008) S32006, a novel 5-HT2C receptor antagonist displaying broad-based antidepressant and anxiolytic properties in rodent models. *Psychopharmacology (Berl)* 199, 549-568

31. Donovan MJ, Lin MI, Wiegn P, Ringstedt T, Kraemer R, Hahn R, Wang S, Ibanez CF, Rafii S, and Hempstead BL (2000) Brain derived neurotrophic factor is an endothelial cell survival factor required for intramyocardial vessel stabilization. *Development* 127, 4531-4540

32. Drevets WC, Frank E, Price JC, Kupfer DJ, Greer PJ, and Mathis C (2000) Serotonin type-1A receptor imaging in depression. *Nucl Med Biol* 27, 499-507

33. Duman RS (2004) Role of neurotrophic factors in the etiology and treatment of mood disorders. *Neuromolecular Med* 5, 11-25

34. Dwivedi Y, Mondal AC, Payappagoudar GV, and Rizavi HS (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

35. Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Lu B, and Weinberger DR (2003) The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell* 112, 257-269

36. Eisch AJ, Bolanos CA, de WJ, Simonak RD, Pudiak CM, Barrot M, Verhaagen J, and Nestler EJ (2003) Brain-derived neurotrophic factor in the ventral midbrain-nucleus accumbens pathway: a role in depression. *Biol Psychiatry* 54, 994-1005

37. Elfving B, Plougmann PH, Muller HK, Mathe AA, Rosenberg R, and Wegener G (2009) Inverse correlation of brain and blood BDNF levels in a genetic rat model of depression. *Int J Neuropsychopharmacol*, 1-10

38. Ernfors P, Lee KF, and Jaenisch R (1994) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* 368, 147-150

39. Faraguna U, Vyazovskiy VV, Nelson AB, Tononi G, and Cirelli C (2008) A causal role for brain-derived neurotrophic factor in the homeostatic regulation of sleep. *J Neurosci* 28, 4088-4095

40. Frokjaer VG, Mortensen EL, Nielsen FA, Haugbol S, Pinborg LH, Adams KH, Svarer C, Hasselbalch SG, Holm S, Paulson OB, and Knudsen GM (2008) Frontolimbic serotonin 2A receptor binding in healthy subjects is associated with personality risk factors for affective disorder. *Biol Psychiatry* 63, 569-576

41. Gardier AM (2009) Mutant mouse models and antidepressant drug research: focus on serotonin and brain-derived neurotrophic factor. *Behav Pharmacol* 20, 18-32

42. Gaspar P, Cases O, and Maroteaux L (2003) The developmental role of serotonin: news from mouse molecular genetics. *Nat Rev Neurosci* 4, 1002-1012

43. Gass P and Hellweg R (2010) Peripheral brain-derived neurotrophic factor (BDNF) as a biomarker for affective disorders? *Int J Neuropsychopharmacol* 13, 1-4

44. Gauthier LR, Charrin BC, Borrell-Pages M, Dompierre JP, Rangone H, Cordelieres FP, De MJ, MacDonald ME, Lessmann V, Humbert S, and Saudou F (2004) Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. *Cell* 118, 127-138

45. Gelber EI, Kroeze WK, Willins DL, Gray JA, Sinar CA, Hyde EG, Gurevich V, Benovic J, and Roth BL (1999) Structure and function of the third intracellular loop of the 5-hydroxytryptamine2A receptor: the third intracellular loop is alpha-helical and binds purified arrestins. *J Neurochem* 72, 2206-2214

46. Glennon RA, Darmani NA, and Martin BR (1991) Multiple populations of serotonin receptors may modulate the behavioral effects of serotonergic agents. *Life Sci* 48, 2493-2498

47. Gonzalez-Maeso J, Weisstaub NV, Zhou M, Chan P, Ivic L, Ang R, Lira A, Bradley-Moore M, Ge Y, Zhou Q, Sealfon SC, and Gingrich JA (2007) Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling pathways to affect behavior. *Neuron* 53, 439-452

48. Gorgulu Y and Caliyurt O (2009) Rapid antidepressant effects of sleep deprivation therapy correlates with serum BDNF changes in major depression. *Brain Res Bull* 80, 158-162

49. Gratacos M, Gonzalez JR, Mercader JM, de CR, Urretavizcaya M, and Estivill X (2007) Brain-derived neurotrophic factor Val66Met and psychiatric disorders: meta-analysis of case-control studies confirm association to substance-related disorders, eating disorders, and schizophrenia. *Biol Psychiatry* 61, 911-922

50. Gray J, Yeo GS, Cox JJ, Morton J, Adlam AL, Keogh JM, Yanovski JA, El GA, Han JC, Tung YC, Hodges JR, Raymond FL, O'rahilly S, and Farooqi IS (2006) Hyperphagia, severe obesity, impaired cognitive function, and hyperactivity associated with functional loss of one copy of the brain-derived neurotrophic factor (BDNF) gene. *Diabetes* 55, 3366-3371

51. Groves JO (2007) Is it time to reassess the BDNF hypothesis of depression? *Mol Psychiatry* 12, 1079-1088

52. Hairston IS, Peyron C, Denning DP, Ruby NF, Flores J, Sapolsky RM, Heller HC, and O'Hara BF (2004) Sleep deprivation effects on growth factor expression in neonatal rats: a potential role for BDNF in the mediation of delta power. *J Neurophysiol* 91, 1586-1595

53. Han JC, Liu QR, Jones M, Levinn RL, Menzie CM, Jefferson-George KS, dler-Wailes DC, Sanford EL, Lacbawan FL, Uhl GR, Rennert OM, and Yanovski JA (2008) Brain-derived neurotrophic factor and obesity in the WAGR syndrome. *N Engl J Med* 359, 918-927

54. Hariri AR, Goldberg TE, Mattay VS, Kolachana BS, Callicott JH, Egan MF, and Weinberger DR (2003) Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance. *J Neurosci* 23, 6690-6694

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

56. Henningsson S, Borg J, Lundberg J, Bah J, Lindstrom M, Ryding E, Jovanovic H, Saijo T, Inoue M, Rosen I, Traskman-Bendz L, Farde L, and Eriksson E (2009) Genetic variation in brain-derived neurotrophic factor is associated with serotonin transporter but not serotonin-1A receptor availability in men. *Biol Psychiatry* 66, 477-485

57. Hensler JG, Advani T, and Monteggia LM (2007) Regulation of serotonin-1A receptor function in inducible brain-derived neurotrophic factor knockout mice after administration of corticosterone. *Biol Psychiatry* 62, 521-529

58. Hensler JG, Ladenheim EE, and Lyons WE (2003) Ethanol consumption and serotonin-1A (5-HT1A) receptor function in heterozygous BDNF (+/-) mice. *J Neurochem* 85, 1139-1147

59. Hill RA, Murray SS, Halley PG, Binder MD, Martin SJ, and van den BM (2010) Brain-derived neurotrophic factor expression is increased in the hippocampus of 5-HT(2C) receptor knockout mice. *Hippocampus*

60. Hirvonen J, Karlsson H, Kajander J, Lepola A, Markkula J, Rasi-Hakala H, Nagren K, Salminen JK, and Hietala J (2008) Decreased brain serotonin 5-HT1A receptor availability in medication-naive patients with major depressive disorder: an in-vivo imaging study using PET and [carbonyl-11C]WAY-100635. *Int J Neuropsychopharmacol* 11, 465-476

61. Hwang JP, Tsai SJ, Hong CJ, Yang CH, Lirng JF, and Yang YM (2006) The Val66Met polymorphism of the brain-derived neurotrophic-factor gene is associated with geriatric depression. *Neurobiol Aging* 27, 1834-1837

62. Jakab RL and Goldman-Rakic PS (1998) 5-Hydroxytryptamine2A serotonin receptors in the primate cerebral cortex: possible site of action of hallucinogenic and antipsychotic drugs in pyramidal cell apical dendrites. *Proc Natl Acad Sci U S A* 95, 735-740

63. Kanekal S, Sahai A, Jones RE, and Brown D (1995) Storage-phosphor autoradiography: a rapid and highly sensitive method for spatial imaging and quantitation of radioisotopes. *J Pharmacol Toxicol Methods* 33, 171-178

64. Karege F, Bondolfi G, Gervasoni N, Schwald M, Aubry JM, 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, 1068-1072

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

66. Kendler KS, Hettema JM, Butera F, Gardner CO, and Prescott CA (2003) Life event dimensions of loss, humiliation, entrapment, and danger in the prediction of onsets of major depression and generalized anxiety. *Arch Gen Psychiatry* 60, 789-796

67. Kerschensteiner M, Gallmeier E, Behrens L, Leal VV, Misgeld T, Klinkert WE, Kolbeck R, Hoppe E, Oropeza-Wekerle RL, Bartke I, Stadelmann C, Lassmann H, Wekerle H, and Hohlfeld R (1999) Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation? *J Exp Med* 189, 865-870

68. Kilduff TS and Peyron C (2000) The hypocretin/orexin ligand-receptor system: implications for sleep and sleep disorders. *Trends Neurosci* 23, 359-365

69. Kiyohara C and Yoshimasu K (2009) Association between major depressive disorder and a functional polymorphism of the 5-hydroxytryptamine (serotonin) transporter gene: a meta-analysis. *Psychiatr Genet*

70. Kondoh M, Shiga T, and Okado N (2004) Regulation of dendrite formation of Purkinje cells by serotonin through serotonin1A and serotonin2A receptors in culture. *Neurosci Res* 48, 101-109

71. Koponen E, Voikar V, Riekki R, Saarelainen T, Rauramaa T, Rauvala H, Taira T, and Castren E (2004) 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, 166-181

72. Kozisek ME, Middlemas D, and Bylund DB (2008) Brain-derived neurotrophic factor and its receptor tropomyosin-related kinase B in the mechanism of action of antidepressant therapies. *Pharmacol Ther* 117, 30-51

73. Krishnan V, Han MH, Graham DL, Berton O, Renthal W, Russo SJ, Laplant Q, Graham A, Lutter M, Lagace DC, Ghose S, Reister R, Tannous P, Green TA, Neve RL, Chakravarty S, Kumar A, Eisch AJ, Self DW, Lee FS, Tamminga CA, Cooper DC, Gershenfeld HK, and Nestler EJ (2007) Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* 131, 391-404

74. Kroeze WK, Kristiansen K, and Roth BL (2002) Molecular biology of serotonin receptors structure and function at the molecular level. *Curr Top Med Chem* 2, 507-528

75. Lanfumey L and Hamon M (2004) 5-HT1 receptors. *Curr Drug Targets CNS Neurol Disord* 3, 1-10

76. Lang UE, Hellweg R, Sander T, and Gallinat J (2009) The Met allele of the BDNF Val66Met polymorphism is associated with increased BDNF serum concentrations. *Mol Psychiatry* 14, 120-122

77. Laske C, Stransky E, Leyhe T, Eschweiler GW, 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, 387-394

78. Lee BH, Kim H, Park SH, and Kim YK (2007) Decreased plasma BDNF level in depressive patients. *J Affect Disord* 101, 239-244

79. Lessmann V and Brigadski T (2009) Mechanisms, locations, and kinetics of synaptic BDNF secretion: an update. *Neurosci Res* 65, 11-22

80. Levivier M, Przedborski S, Bencsics C, and Kang UJ (1995) Intrastriatal implantation of fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevents degeneration of dopaminergic neurons in a rat model of Parkinson's disease. *J Neurosci* 15, 7810-7820

81. Leysen JE (2004) 5-HT2 receptors. *Curr Drug Targets CNS Neurol Disord* 3, 11-26

82. Licht CL, Marcussen AB, Wegener G, Overstreet DH, Aznar S, and Knudsen GM (2009) The brain 5-HT4 receptor binding is down-regulated in the Flinders Sensitive Line depression model and in response to paroxetine administration. *J Neurochem* 109, 1363-1374

83. Livak KJ (2003) SNP genotyping by the 5'-nuclease reaction. *Methods Mol Biol* 212, 129-147

84. Livak KJ, Marmaro J, and Todd JA (1995) Towards fully automated genome-wide polymorphism screening. *Nat Genet* 9, 341-342

85. Longstreth WT, Jr., Koepsell TD, Ton TG, Hendrickson AF, and van BG (2007) The epidemiology of narcolepsy. *Sleep* 30, 13-26

86. Lopez-Gimenez JF, Mengod G, Palacios JM, and Vilaro MT (1997) Selective visualization of rat brain 5-HT2A receptors by autoradiography with [3H]MDL 100,907. *Naunyn Schmiedebergs Arch Pharmacol* 356, 446-454

87. Lu B (2003) BDNF and activity-dependent synaptic modulation. *Learn Mem* 10, 86-98

88. Luellen BA, Bianco LE, Schneider LM, and Andrews AM (2007) Reduced brain-derived neurotrophic factor is associated with a loss of serotonergic innervation in the hippocampus of aging mice. *Genes Brain Behav* 6, 482-490

89. Lyons WE, Mamounas LA, Ricaurte GA, Coppola V, Reid SW, Bora SH, Wihler C, Koliatsos VE, 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, 15239-15244

90. Mamounas LA, Altar CA, Blue ME, Kaplan DR, Tessarollo L, and Lyons WE (2000) BDNF promotes the regenerative sprouting, but not survival, of injured serotonergic axons in the adult rat brain. *J Neurosci* 20, 771-782

91. Mamounas LA, Blue ME, Siuciak JA, and Altar CA (1995) Brain-derived neurotrophic factor promotes the survival and sprouting of serotonergic axons in rat brain. *J Neurosci* 15, 7929-7939

92. Massou JM, Trichard C, ttar-Levy D, Feline A, Corruble E, Beaufils B, and Martinot JL (1997) Frontal 5-HT2A receptors studied in depressive patients during chronic treatment by selective serotonin reuptake inhibitors. *Psychopharmacology (Berl)* 133, 99-101

93. Matthews VB, Astrom MB, Chan MH, Bruce CR, Krabbe KS, Prelovsek O, Akerstrom T, Yfanti C, Broholm C, Mortensen OH, Penkowa M, Hojman P, Zankari A, Watt MJ, Bruunsgaard H, Pedersen BK, and Febbraio MA (2009) Brain-derived neurotrophic factor is produced by skeletal muscle cells

in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. *Diabetologia* 52, 1409-1418

94. Mayorga AJ, Dalvi A, Page ME, Zimov-Levinson S, Hen R, and Lucki I (2001) Antidepressant-like behavioral effects in 5-hydroxytryptamine(1A) and 5-hydroxytryptamine(1B) receptor mutant mice. *J Pharmacol Exp Ther* 298, 1101-1107

95. Meyer JH, Kapur S, Eisfeld B, Brown GM, Houle S, DaSilva J, Wilson AA, Rafi-Tari S, Mayberg HS, and Kennedy SH (2001) The effect of paroxetine on 5-HT(2A) receptors in depression: an [(18)F]setoperone PET imaging study. *Am J Psychiatry* 158, 78-85

96. Middlemas DS, Lindberg RA, and Hunter T (1991) trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. *Mol Cell Biol* 11, 143-153

97. Mintun MA, Sheline YI, Moerlein SM, Vlassenko AG, Huang Y, and Snyder AZ (2004) Decreased hippocampal 5-HT2A receptor binding in major depressive disorder: in vivo measurement with [18F]altanserin positron emission tomography. *Biol Psychiatry* 55, 217-224

98. Molteni R, Calabrese F, Pisoni S, Gabriel C, Mocaer E, Racagni G, and Riva MA (2010a) Synergistic mechanisms in the modulation of the neurotrophin BDNF in the rat prefrontal cortex following acute agomelatine administration. *World J Biol Psychiatry* 11, 148-153

99. Molteni R, Cattaneo A, Calabrese F, Macchi F, Olivier JD, Racagni G, Ellenbroek BA, Gennarelli M, and Riva MA (2010b) Reduced function of the serotonin transporter is associated with decreased expression of BDNF in rodents as well as in humans. *Neurobiol Dis* 37, 747-755

100. Mössner R, Daniel S, Albert D, Heils A, Okladnova O, Schmitt A, and Lesch KP (2000) Serotonin transporter function is modulated by brain-derived neurotrophic factor (BDNF) but not nerve growth factor (NGF). *Neurochem Int* 36, 197-202

101. Murphy DL, Lerner A, Rudnick G, and Lesch KP (2004) Serotonin transporter: gene, genetic disorders, and pharmacogenetics. *Mol Interv* 4, 109-123

102. Neumeister A, Wood S, Bonne O, Nugent AC, Luckenbaugh DA, Young T, Bain EE, Charney DS, and Drevets WC (2005) Reduced hippocampal volume in unmedicated, remitted patients with major depression versus control subjects. *Biol Psychiatry* 57, 935-937

103. Nibuya M, Morinobu S, and Duman RS (1995) Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *J Neurosci* 15, 7539-7547

104. Nolan T, Hands RE, and Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. *Nat Protoc* 1, 1559-1582

105. Ohira K, Kumanogoh H, Sahara Y, Homma KJ, Hirai H, Nakamura S, and Hayashi M (2005) A truncated tropomyosin-related kinase B receptor, T1, regulates glial cell morphology via Rho GDP dissociation inhibitor 1. *J Neurosci* 25, 1343-1353

106. Overstreet DH, Janowsky DS, Pucilowski O, and Rezvani AH (1994) Swim test immobility co-segregates with serotonergic but not cholinergic sensitivity in cross-breeds of Flinders Line rats. *Psychiatr Genet* 4, 101-107

107. Pan W, Banks WA, Fasold MB, Bluth J, and Kastin AJ (1998) Transport of brain-derived neurotrophic factor across the blood-brain barrier. *Neuropharmacology* 37, 1553-1561

108. Pandey GN, Dwivedi Y, Rizavi HS, Ren X, Pandey SC, Pesold C, Roberts RC, Conley RR, and Tamminga CA (2002) Higher expression of serotonin 5-HT(2A) receptors in the postmortem brains of teenage suicide victims. *Am J Psychiatry* 159, 419-429

109. Parks CL, Robinson PS, Sibille E, Shenk T, and Toth M (1998) Increased anxiety of mice lacking the serotonin1A receptor. *Proc Natl Acad Sci U S A* 95, 10734-10739

110. Peng S, Garzon DJ, Marchese M, Klein W, Ginsberg SD, Francis BM, Mount HT, Mufson EJ, Salehi A, and Fahnestock M (2009) Decreased brain-derived neurotrophic factor depends on amyloid aggregation state in transgenic mouse models of Alzheimer's disease. *J Neurosci* 29, 9321-9329

111. Pezawas L, Verchinski BA, Mattay VS, Callicott JH, Kolachana BS, Straub RE, Egan MF, Meyer-Lindenberg A, and Weinberger DR (2004) The brain-derived neurotrophic factor val66met polymorphism and variation in human cortical morphology. *J Neurosci* 24, 10099-10102

112. Phillips HS, Hains JM, Armanini M, Laramee GR, Johnson SA, and Winslow JW (1991) BDNF mRNA is decreased in the hippocampus of individuals with Alzheimer's disease. *Neuron* 7, 695-702

113. Radka SF, Holst PA, Fritsche M, and Altar CA (1996) 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, 122-301

114. Rasmussen P, Brassard P, Adser H, Pedersen MV, Leick L, Hart E, Secher NH, Pedersen BK, and Pilegaard H (2009) Evidence for a release of brain-derived neurotrophic factor from the brain during exercise. *Exp Physiol* 94, 1062-1069

115. Reichardt LF (2006) Neurotrophin-regulated signalling pathways. *Philos Trans R Soc Lond B Biol Sci* 361, 1545-1564

116. Riad M, Watkins KC, Doucet E, Hamon M, and Descarries L (2001) Agonist-induced internalization of serotonin-1a receptors in the dorsal raphe nucleus (autoreceptors) but not hippocampus (heteroreceptors). *J Neurosci* 21, 8378-8386

117. Rios M, Fan G, Fekete C, Kelly J, Bates B, Kuehn R, Lechan RM, and Jaenisch R (2001) Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. *Mol Endocrinol* 15, 1748-1757

118. Rios M, Lambe EK, Liu R, Teillon S, Liu J, Akbarian S, Roffler-Tarlov S, Jaenisch R, and Aghajanian GK (2006) Severe deficits in 5-HT2A -mediated neurotransmission in BDNF conditional mutant mice. *J Neurobiol* 66, 408-420

119. Saarelainen T, Lukkarinen JA, Koponen S, Grohn OH, Jolkkonen J, Koponen E, Haapasalo A, Alhonen L, Wong G, Koistinaho J, Kauppinen RA, and Castren E (2000) Transgenic mice overexpressing truncated trkB neurotrophin receptors in neurons show increased susceptibility to cortical injury after focal cerebral ischemia. *Mol Cell Neurosci* 16, 87-96

120. Saper CB, Scammell TE, and Lu J (2005) Hypothalamic regulation of sleep and circadian rhythms. *Nature* 437, 1257-1263

121. Sartorius A, Hellweg R, Litzke J, Vogt M, Dormann C, Vollmayr B, Danker-Hopfe H, and Gass P (2009) Correlations and discrepancies between serum and brain tissue levels of neurotrophins after electroconvulsive treatment in rats. *Pharmacopsychiatry* 42, 270-276

122. Sato Y, Chin Y, Kato T, Tanaka Y, Tozuka Y, Mase M, Ageyama N, Ono F, Terao K, Yoshikawa Y, and Hisatsune T (2009) White matter activated glial cells produce BDNF in a stroke model of monkeys. *Neurosci Res* 65, 71-78

123. Schindowski K, Belarbi K, and Buee L (2008) Neurotrophic factors in Alzheimer's disease: role of axonal transport. *Genes Brain Behav* 7 Suppl 1, 43-56

124. Schmid CL, Raehal KM, and Bohn LM (2008) Agonist-directed signaling of the serotonin 2A receptor depends on beta-arrestin-2 interactions in vivo. *Proc Natl Acad Sci U S A* 105, 1079-1084

125. Sen S, Duman R, and Sanacora G (2008) Serum brain-derived neurotrophic factor, depression, and antidepressant medications: meta-analyses and implications. *Biol Psychiatry* 64, 527-532

126. Shimizu E, Hashimoto K, and Iyo M (2004) Ethnic difference of the BDNF 196G/A (val66met) polymorphism frequencies: the possibility to explain ethnic mental traits. *Am J Med Genet B Neuropsychiatr Genet* 126B, 122-123

127. Siuciak JA, Boylan C, Fritsche M, Altar CA, and Lindsay RM (1996) BDNF increases monoaminergic activity in rat brain following intracerebroventricular or intraparenchymal administration. *Brain Res* 710, 11-20

128. Siuciak JA, Clark MS, Rind HB, Whittemore SR, and Russo AF (1998) BDNF induction of tryptophan hydroxylase mRNA levels in the rat brain. *J Neurosci Res* 52, 149-158

129. Smith SR, Prosser WA, Donahue DJ, Morgan ME, Anderson CM, and Shanahan WR (2009) Lorcaserin (APD356), a selective 5-HT(2C) agonist, reduces body weight in obese men and women. *Obesity (Silver Spring)* 17, 494-503

130. Song XY, Li F, Zhang FH, Zhong JH, and Zhou XF (2008) Peripherally-derived BDNF promotes regeneration of ascending sensory neurons after spinal cord injury. *PLoS One* 3, e1707

131. Stockmeier CA (2003) Involvement of serotonin in depression: evidence from postmortem and imaging studies of serotonin receptors and the serotonin transporter. *J Psychiatr Res* 37, 357-373

132. Sturzenegger C and Bassetti CL (2004) The clinical spectrum of narcolepsy with cataplexy: a reappraisal. *J Sleep Res* 13, 395-406

133. Taliaz D, Stall N, Dar DE, and Zangen A (2010) Knockdown of brain-derived neurotrophic factor in specific brain sites precipitates behaviors associated with depression and reduces neurogenesis. *Mol Psychiatry* 15, 80-92

134. Tettamanti G, Cattaneo AG, Gornati R, de EM, Bernardini G, and Binelli G (2010) Phylogenesis of brain-derived neurotrophic factor (BDNF) in vertebrates. *Gene* 450, 85-93

135. Thannickal TC, Nienhuis R, and Siegel JM (2009) Localized loss of hypocretin (orexin) cells in narcolepsy without cataplexy. *Sleep* 32, 993-998

136. Trajkovska V, Marcussen AB, Vinberg M, Hartvig P, Aznar S, and Knudsen GM (2007) Measurements of brain-derived neurotrophic factor: methodological aspects and demographical data. *Brain Res Bull* 73, 143-149

137. Trajkovska V, Santini MA, Marcussen AB, Thomsen MS, Hansen HH, Mikkelsen JD, Arneberg L, Kokaia M, Knudsen GM, and Aznar S (2009) BDNF downregulates 5-HT(2A) receptor protein levels in hippocampal cultures. *Neurochem Int* 55, 697-702

138. Tramontina J, Frey BN, Andreazza AC, Zandona M, Santin A, and Kapczinski F (2007) Val66met polymorphism and serum brain-derived neurotrophic factor levels in bipolar disorder. *Mol Psychiatry* 12, 230-231

139. Vaidya VA, Marek GJ, Aghajanian GK, and Duman RS (1997) 5-HT2A receptor-mediated regulation of brain-derived neurotrophic factor mRNA in the hippocampus and the neocortex. *J Neurosci* 17, 2785-2795

140. Vaidya VA, Terwilliger RM, and Duman RS (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

141. Vollmayr B, Faust H, Lewicka S, and Henn FA (2001) Brain-derived-neurotrophic-factor (BDNF) stress response in rats bred for learned helplessness. *Mol Psychiatry* 6, 471-4, 358

142. Williamson DE, Birmaher B, Frank E, Anderson BP, Matty MK, and Kupfer DJ (1998) Nature of life events and difficulties in depressed adolescents. *J Am Acad Child Adolesc Psychiatry* 37, 1049-1057

143. Willins DL, Deutch AY, and Roth BL (1997) Serotonin 5-HT2A receptors are expressed on pyramidal cells and interneurons in the rat cortex. *Synapse* 27, 79-82

144. Willins DL and Meltzer HY (1997) Direct injection of 5-HT2A receptor agonists into the medial prefrontal cortex produces a head-twitch response in rats. *J Pharmacol Exp Ther* 282, 699-706

145. Wong DT, Perry KW, and Bymaster FP (2005) Case history: the discovery of fluoxetine hydrochloride (Prozac). *Nat Rev Drug Discov* 4, 764-774

146. Woo NH, Teng HK, Siao CJ, Chiaruttini C, Pang PT, Milner TA, Hempstead BL, and Lu B (2005) Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. *Nat Neurosci* 8, 1069-1077

147. Wu D and Pardridge WM (1999) Neuroprotection with noninvasive neurotrophin delivery to the brain. *Proc Natl Acad Sci U S A* 96, 254-259

148. Xu B, Goulding EH, Zang K, Cepoi D, Cone RD, Jones KR, Tecott LH, and Reichardt LF (2003) Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. *Nat Neurosci* 6, 736-742

149. Yan Q, Rosenfeld RD, Matheson CR, Hawkins N, Lopez OT, Bennett L, and Welcher AA (1997) Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. *Neuroscience* 78, 431-448

150. Yang J, Siao CJ, Nagappan G, Marinic T, Jing D, McGrath K, Chen ZY, Mark W, Tessarollo L, Lee FS, Lu B, and Hempstead BL (2009) Neuronal release of proBDNF. *Nat Neurosci* 12, 113-115

151. Yatham LN, Liddle PF, Dennie J, Shiah IS, Adam MJ, Lane CJ, Lam RW, and Ruth TJ (1999) Decrease in brain serotonin 2 receptor binding in patients with major depression following desipramine treatment: a positron emission tomography study with fluorine-18-labeled setoperone. *Arch Gen Psychiatry* 56, 705-711

152. Yu YW, Tsai SJ, Chen TJ, Lin CH, and Hong CJ (2002) Association study of the serotonin transporter promoter polymorphism and symptomatology and antidepressant response in major depressive disorders. *Mol Psychiatry* 7, 1115-1119

153. Yuen EY, Jiang Q, Chen P, Feng J, and Yan Z (2008) Activation of 5-HT2A/C receptors counteracts 5-HT1A regulation of n-methyl-D-aspartate receptor channels in pyramidal neurons of prefrontal cortex. *J Biol Chem* 283, 17194-17204

154. Zanardi R, Artigas F, Moresco R, Colombo C, Messa C, Gobbo C, Smeraldi E, and Fazio F (2001) Increased 5-hydroxytryptamine-2 receptor binding in the frontal cortex of depressed patients responding to paroxetine treatment: a positron emission tomography scan study. *J Clin Psychopharmacol* 21, 53-58

155. Zhang Y and Pardridge WM (2001) Conjugation of brain-derived neurotrophic factor to a blood-brain barrier drug targeting system enables neuroprotection in regional brain ischemia following intravenous injection of the neurotrophin. *Brain Res* 889, 49-56

156. Zipper H, Brunner H, Bernhagen J, and Vitzthum F (2004) Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Res* 32, e103

157. Zuccato C and Cattaneo E (2009) Brain-derived neurotrophic factor in neurodegenerative diseases. *Nat Rev Neurol* 5, 311-322

158. Zuccato C, Ciammola A, Rigamonti D, Leavitt BR, Goffredo D, Conti L, MacDonald ME, Friedlander RM, Silani V, Hayden MR, Timmusk T, Sipione S, and Cattaneo E (2001) Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. *Science* 293, 493-498

159. Zuccato C, Marullo M, Conforti P, MacDonald ME, Tartari M, and Cattaneo E (2008) Systematic assessment of BDNF and its receptor levels in human cortices affected by Huntington's disease. *Brain Pathol* 18, 225-238

Appendices

Paper I-V

I. **Klein AB**, Santini M, Aznar S, Knudsen GM, Rios M (2010) Changes in 5-HT_{2A}-mediated behavior and serotonin receptor binding in conditional BDNF knock-out mice, *Neuroscience*, 169, 1007-1016

II. **Klein AB,** Trajkovska V, Erritzoe D, Haugbol S, Madsen J, Baaré W, Aznar S, Knudsen GM (2010) Cerebral 5-HT_{2A} receptor and serotonin transporter binding in humans are not affected by the val66met BDNF polymorphism status or blood BDNF levels, *Journal of Cerebral Blood Flow & Metabolism,* e1-e7

III. **Klein AB**, Williamson R, Santini M, Clemmensen C, Ettrup A, Rios M, Knudsen GM, Aznar S (2010) Blood BDNF concentrations reflect brain tissue BDNF levels across species, *International Journal of Neuropsychopharmacology*, p.1-7

IV. Aznar S, **Klein AB**, Santini MA, Knudsen GM, Henn F, Gass P and Vollmayr B (2010) Aging and depression vulnerability interaction results in decreased serotonin innervation associated with reduced BDNF levels in hippocampus of rats bred for learned helplessness, *Synapse* 64(7): 561-565

V. **Klein AB**, Jennum P, Knudsen S, Gammeltoft S, Mikkelsen JD (2010) Increased serum BDNF levels in hypocretin-deficient narcoleptic patients, *manuscript*

Paper I

Neuroscience 169 (2010) 1007–1016

CHANGES IN 5-HT$_{2A}$-MEDIATED BEHAVIOR AND 5-HT$_{2A}$- AND 5-HT$_{1A}$ RECEPTOR BINDING AND EXPRESSION IN CONDITIONAL BRAIN-DERIVED NEUROTROPHIC FACTOR KNOCK-OUT MICE

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Abstract—Changes in brain-derived neurotrophic factor (BDNF) expression have been implicated in the etiology of psychiatric disorders. To investigate pathological mechanisms elicited by perturbed BDNF signaling, we examined mutant mice with central depletion of BDNF (BDNF$^{2L/2LCk\text{-}cre}$). A severe impairment specific for the serotonin 2A receptor (5-HT$_{2A}$R) in prefrontal cortex was described previously in these mice. This is of much interest, as 5-HT$_{2A}$Rs have been linked to neuropsychiatric disorders and anxiety-related behavior. Here we further characterized the serotonin receptor alterations triggered by BDNF depletion. 5-HT$_{2A}$ ([^{3}H]-MDL100907) and 5-HT$_{1A}$ ([^{3}H]-WAY100635) receptor autoradiography revealed site-specific alterations in BDNF mutant mice. They exhibited lower 5-HT$_{2A}$ receptor binding in frontal cortex but increased binding in hippocampus. Additionally, 5-HT$_{1A}$ receptor binding was decreased in hippocampus of BDNF mutants, but unchanged in frontal cortex. Molecular analysis indicated corresponding changes in 5-HT$_{2A}$ and 5-HT$_{1A}$ mRNA expression but normal 5-HT$_{2C}$ content in these brain regions in BDNF$^{2L/2LCk\text{-}cre}$ mice. We investigated whether the reduction in frontal 5-HT$_{2A}$R binding was reflected in reduced functional output in two 5-HT$_{2A}$-receptor mediated behavioral tests, the head-twitch response (HTR) and the ear-scratch response (ESR). BDNF$^{2L/2LCk\text{-}cre}$ mutants treated with the 5-HT$_{2A}$ receptor agonist (±)-2,5-dimethoxy-4-iodoamphetamine (DOI) showed a clearly diminished ESR but no differences in HTR compared to wildtypes. These findings illustrate the context-dependent effects of deficient BDNF signaling on the 5-HT receptor system and 5-HT$_{2A}$-receptor functional output.

Key words: BDNF, serotonin, 5-HT$_{2A}$, 5-HT$_{1A}$, 5-HT$_{2C}$, behavior.

Brain-derived neurotrophic factor (BDNF) belongs to the family of neurotrophins and signals through the tropomyosoin-related kinase B (TrkB) receptor. It is important for neuronal survival and differentiation during development and synaptic plasticity in adulthood. In clinical studies, reductions in BDNF levels have been linked to various psychiatric disorders including schizophrenia, bipolar disorder and major depression (Kozisek et al., 2008). Studies involving rodent models of BDNF deficiency also lend support to a chief role of BDNF in the control of affective behavior. For example, BDNF$^{+/-}$ mice exhibited hyper aggressiveness, hyperphagia, excessive weight gain (Lyons et al., 1999; Kernie et al., 2000) and diminished responses to antidepressant treatment (Deltheil et al., 2008). In BDNF$^{2L/2LCk\text{-}cre}$ mice, BDNF is depleted in all brain regions, except cerebellum. The depletion begins during the first post-natal week and is completed around P21, when BDNF mRNA levels in hippocampus, cortex and hypothalamus range from very low to non-detectable (Chan et al., 2006; Rios et al., 2001). The adult conditional BDNF mutants have similar behavioral alterations and show increases in depressive and anxiety-like behavior (Rios et al., 2001; Chan et al., 2006).

Alterations in the serotonin receptor system might underlie some of the behavioral changes observed in BDNF$^{2L/2LCk\text{-}cre}$ mice. Deficient BDNF levels result in significant perturbations in serotonin receptor expression (Lyons et al., 1999) and in impairment of 5-HT$_{2A}$-mediated neurotransmission in the prefrontal cortex (Rios et al., 2006). The serotonin receptor 2A (5-HT$_{2A}$R) is a postsynaptic G protein coupled receptor widely expressed in the CNS. It is located on glutamatergic and GABAergic neurons, and expressed at high densities in pyramidal neurons in frontal cortex, amygdala and hippocampus (for review, see Leysen, 2004). Importantly, changes in 5-HT$_{2A}$R binding have been associated with schizophrenia, Alzheimer's disease and depression (Naughton et al., 2000). Moreover, a recent study showed that cortical depletion of 5-HT$_{2A}$Rs in mice resulted in decreased anxiety-like behavior (Weisstaub et al., 2006). Together, the collective data support an important role of the 5-HT$_{2A}$R in the regulation of affective behavior.

To further delve into the nature of the alterations in the serotonin receptor system in BDNF$^{2L/2LCk\text{-}cre}$ mutant mice and the impact on receptor functionality, we conducted an analysis of various 5-HT receptors in the hippocampus and frontal cortex of these mice. Autoradiography and RT-qPCR were used to quantify differences in receptor binding and expression, respectively, in these brain regions. Besides 5-HT$_{2A}$Rs, we also examined 5-HT$_{1A}$ and 5-HT$_{2C}$ receptors. 5-HT$_{1A}$Rs have been implicated in depression and anxiety phenotypes (for review, see Savitz et al., 2009

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Abbreviations: BDNF, brain-derived neurotrophic factor; CA1, cornu ammonis 1 of hippocampus; DOI, (±)-2,5-dimethoxy-4-iodoamphetamine; DRN, dorsal raphe nucleus; ESR, ear-scratch response; HTR, head-twitch response; MAP2, microtubule associated protein 2; RT, room temperature; SDS, sodium dodecyl sulfate; SERT, serotonin transporter; TE, tissue equivalents; TrkB, tropomysoin-related kinase B; 5-HT$_{1A}$R, 5-HT receptor 1A; 5-HT$_{2A}$R, 5-HT receptor 2A; 5-HT$_{2C}$R, 5-HT receptor 2C.

0306-4522/10 $ - see front matter
doi:10.1016/j.neuroscience.2010.05.054

and Akimova et al., 2009) and exert effects opposite to those of 5-HT_{2A}Rs (Yuen et al., 2008). 5-HT_{2C}Rs, for their part, resemble 5-HT_{2A} receptors in terms of functional activity and cellular distribution (Leysen, 2004). Levels of serotonin transporter (SERT) were also measured in BDNF mutant mice to ascertain whether the alterations in the postsynaptic 5-HT receptors could be due to primary changes in serotonergic projection. Finally, we assessed 5-HT_{2A}-functional output *in vivo* by measuring (±)-2,5-dimethoxy-4-iodoamphetamine (DOI) induced head-twitch and ear-scratch responses in $BDNF^{2L/2LCk\text{-}cre}$ mice. This was followed by an analysis of DOI-induced neuronal activation, measured by c-Fos protein induction, in frontal cortex and hippocampus. We found that central depletion of BDNF resulted in site-specific alterations in 5-HT receptor binding and expression. Furthermore, a deficit in 5-HT_{2A}-functional output was evident in BDNF mutants. The cumulative data indicate an important effect of BDNF depletion on serotonin receptor levels and 5-HT_{2A} functional output.

EXPERIMENTAL PROCEDURES

The following procedures were in accordance with the guidelines from the Animal Care and Use Committee at Tufts University and with the NIH Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals.

Animals

$BDNF^{2L/2LCk\text{-}Cre}$ mutants were generated as previously described (Rios et al., 2001). Briefly, mice containing floxed *Bdnf* alleles were crossed with transgenic mice in which expression of cre recombinase was driven by the CamKII (CamKII-cre93 mice). Animals used in the studies described here were in a mixed C57Bl6 and 129 background and wild type littermates used as controls to avoid problems interpreting the data due to differences in background. Animals were 10–14 weeks of age and the groups were age- and sex matched. All mice were individually housed in the Tufts University Behavioral Core Facility, habituated to the reverse 12-h light/dark cycle for a minimum of 1 week and had free access to water and standard chow. All of the procedures described here were approved by the Institutional Animal Care and Use Committee at Tufts University and were in compliance with the NIH guide for the care and use of laboratory animals.

Drugs and reagents

The 5-HT_{2A} agonist DOI hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was administered i.p. in a volume of 10 μL/g body weight. The dose used in the studies was 2.5 mg/kg. Saline was used as vehicle and injected in a corresponding volume in wild-types.

Western blotting

After cervical dislocation, the brain was quickly removed and hippocampus and frontal cortex dissected out (n=7–8 per group), homogenized and sonicated in ice-cold protein extraction buffer (Pierce, Rockford, IL, USA) and protein inhibitor cocktail (Pierce). The protein concentration was measured with modified Lowry method (DC Protein Assay, BioRad Laboratories, Herlev, Denmark). Samples were protein-matched and dissolved in 2xSDS sample buffer (125 mM Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol), heated for 5 min, 100 °C and run on 6–15% SDS-polyacrylamide gel (60 min, 150 V, RT). The proteins were transferred onto a PVDF membrane (300 mA, 50 min, RT, semi-dry transfer, BioRad Laboratories, Herlev, Denmark) and the membrane was blocked for unspecific binding with 5% non-fat dry milk (1 h, RT) and then incubated overnight at 4 °C with either primary antibody (β-actin, 1:10.000, Sigma; BDNF, 1:1000, SC-546, Santa Cruz, CA, USA; 5-HT_{2C}, 1:1000, Abcam, MAP2, 1:2000, M4403, Sigma; synaptophysin, 1:5000, MAB368, Millipore, MA, USA; PSD-95, 1:2000, Millipore; β-tubulin III, 1:10.000, Sigma). Washed 3×10 min in TBS, blocked with 5% BSA in TBS for 1 h at room temperature (RT), incubated with horseradish peroxidase-linked secondary antibody (GαM or GαR, DAKO Cytomation, Glostrup, Denmark) diluted at 1:5000 in blocking solution for 1 h at RT, and finally developed with ECL detection kit (RPN2106PL, GE Healthcare, Uppsala, Sweden). Band intensity was quantified using the NIH ImageJ software. For each sample, the optical density of each band was normalized by dividing with the optical density of the corresponding band of β-tubulin III or β-actin.

Autoradiography

To determine 5-HT_{2A} receptor binding in BDNF mutant mice and wild-types, animals (n=4 per group) were decapitated and brains were quickly dissected out and stored at −80 °C until sectioning. Brains were cut in 10 μm sections and mounted on gelatine-coated slides and stored at −80 °C until further processing. 5-HT_{2A} autoradiography was performed using [^{3}H]-MDL100907 [R(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorphenyl)-ethyl]-4-piperidin-methanol] (specific activity; 64 Ci/mmol, a gift from C. Halldin, Karolinska Institute, Stockholm) and non-specific binding was determined using 10 μM ketanserin tartrate ([3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]-ethyl]-2,4[1H,3H]quinazolinedione tartrate) (Tocris Cookson Ltd, Bristol, UK). For 5-HT_{1A} autoradiography we used [^{3}H]-WAY100635 (GE Healthcare, UK) and measured non-specific binding with 10 μM 5-HT (Sigma).

Briefly, sections were allowed to thaw for 1 h at RT and then pre-incubated with 50 mM Tris–HCl (Sigma), pH 7.4 containing 0.01% ascorbic acid (Sigma) and 10 μM pargyline hydrochloride (N-methyl-N-2-propynylbenzylamine hydrochloride) (Research Biochemicals International, MA, USA) for 30 min at RT under constant gentle shaking. Sections were then incubated for 60 min at RT using the same buffer containing 0.4 nM of [^{3}H]-MDL100907 (1.5 nM of [^{3}H]-WAY100635 for 5-HT_{1A} binding). Following incubation, slides were washed 2×20 s in ice-cold 50 mM Tris–HCl, pH 7.4, and 1×20 s in ice-cold dH_2O, dried for 1 h under a gentle stream of air.

The serotonin transporter (SERT) autoradiography protocol was modified from Thomsen and Helboe (2003). Briefly, sections were pre-incubated for 30 min in 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, pH 7.4 and subsequently incubated with 2.0 nM [[^{3}H]]-escitalopram (donated by H. Lundbeck A/S, Copenhagen, Denmark) for 60 min diluted in the same buffer. Nonspecific binding was determined in the presence of 10 μM paroxetine (GSK, Harlow, UK). After incubation, sections were washed for 3×2 min in 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, pH 7.4 (4 °C) and 1×20 s in ice-cold dH_2O.

All sections were placed at 4 °C overnight in a fixator containing paraformaldehyde vapour and then put in an excicator box for 3 h before slides were together with [^{3}H]-microscales (GE Healthcare, UK) exposed to a BAS-TR2040 Imaging Plate (Science Imaging Scandinavia AB, Nacka, Sweden) for 14 days at 4 °C. Finally, the imaging plate was scanned on a BAS-2500 scanner (Fujifilm Europe GmbH, Düsseldorf, Germany) and specific and non-specific binding was determined in frontal cortex and hippocampus using ImageJ and expressed as fmol/mg tissue equivalents (TE).

RT-qPCR

For total RNA extraction the mice were sacrificed by cervical dislocation and the brain quickly removed and frontal cortex and hippocampus were dissected on ice ($n=7$). Samples were frozen at −80 °C before homogenization. Total RNA was isolated with TRIzol Reagent (Sigma) according to the manufacturer's directions. The RNA samples were dissolved in RNase-free water and quantified with UV-spectrophotometry at 260 nm.

Extracted RNA was reverse transcribed into cDNA using the procedure of the supplier (ImProm-II™ reverse transcription system, Promega, Madison, WI, USA). The experimental RNA solution was combined with oligo(dT)15 primers and heated at 70 °C for 5 min. The reverse transcription reaction mixture contained of 20% ImProm-II™ 5× reaction buffer, 6 mM $MgCl_2$, 0.5 mM dNTP mix and 20 U RNase inhibitor. The reverse transcription reaction was performed at 42 °C for 60 min, followed by heating at 70 °C for 15 min.

The quantitative polymerase chain reaction (qPCR) was performed by adding the sample cDNA to a reaction mixture consisting of 1×Brilliant II SYBR green mastermix (Stratagene, La Jolla, CA, USA) and 15 pmol of each primer (DNA technology, Aarhus, Denmark) and adjusting the volume to 20 μl with DNase free water (Invitrogen, Carlsbad, CA, USA). PCR was performed with a 10 min preincubation at 94 °C followed by 40 cycles of 30 s at 94 °C, 45 s at 60 °C and 90 s at 72 °C (Roche Light Cycler 480, Roche, Indianapolis, IN, USA). The real-time PCR method was validated by using serially diluted cDNA to establish a standard curve. To quantify the gene expression profile of each sample, the efficiency of each standard curve was determined from its slope and comparative threshold according to the manufacturer's instructions. For each sample, the amount of targeted mRNA was normalized to that of the reference gene β-tubulin III.

Primers for mouse 5-HT_{1A}, 5-HT_{2A} receptors and β-tubulin III were designed for qPCR. The sequence of the specific primers designed based on gene bank data were as follows: 5-HT_{2A}: F: 5-ccgcttcaactccagaaccaaagc-3, R: 5-cttcgaatcatcctgtacccgaa-3; 5-HT_{2C}: F: 5-taatggtgaacctgggcactgcgg-3, R: 5-taaaagtgtcagttactatagctgc, 5-HT_{1A}, F: 5-ctgtttatcgccctggatg-3, R: 5-atgagccaagtgagcgagat-3; β-tubulin III, F: 5-ataggggccaagttctgggaggtc-3, R: 5-ctgggcacatacttgtgagaggag-3.

Behavioral studies

The head-twitch response (HTR) and ear-scratch response (ESR) scoring was performed as previously reported (Darmani, 2001) with minor modifications. In brief, animals ($n=7$ per group) were kept in their standard home cage (15×24 cm^2), but enrichment material was removed during the test period. Animals were injected with either DOI (2.5 mg/kg i.p.) or corresponding volume of saline 5 min prior to testing. The mice were video taped for 20 min and subsequently HTR and ESR were scored. One week later, the experiment was repeated so animals receiving DOI on the first test day, were given saline and vice versa.

Immunohistochemistry

Wild-type and cBDNF KOs mice were injected either saline or DOI (2.5 mg/kg) and after 1 h, transcardially perfused with 20 ml of cold saline followed by 50 ml of 4% paraformaldehyde (PFA). Brains were immediately removed, postfixed in 4% PFA overnight at 4 °C, cryoprotected in a 30% sucrose solution, and frozen in mounting media (Tissue-Tek, Torrance, CA, USA) until further use. 40 μm coronal sections representing frontal cortex and hippocampus were obtained and c-fos immunohistochemistry was performed as previously described (Unger et al., 2007). In brief, endogenous peroxidase activity was quenched in free-floating sections with 0.5% H_2O_2 in PBS for 15 min, permeabilized with 0.5% Triton X-100 in PBS for 20 min (Sigma), and blocked in 10% normal horse serum for 30 min at RT. Sections were then incubated for 48 h at 4 °C with a rabbit primary antibody against c-fos (1:50,000; Ab5; Oncogene Science, Cambridge, MA, USA), followed by washes and incubation with a biotinylated secondary antibody (1:200) and an avidin–biotin peroxidase complex as per the manufacturers instructions (ABC, Vector Elite Kit; Vector Laboratories, Burlingame, CA, USA). Sections were then incubated with substrate solution (0.025% diaminobenzidine/0.15% nickel ammonium sulfate/0.0036% H_2O_2 in 0.05 M Tris buffer) for 5 min, mounted on slides and left to dry for 2 h before placing cover slips. c-fos positive neurons were quantified in frontal cortex and hippocampus using the computer assisted stereological toolbox software (CAST) module (Visiopharm, Denmark).

Statistical analysis

All analyses were performed using GraphPad 5. Data are presented as mean±SEM if not otherwise stated. The data were analyzed using Student's *t*-test if comparing the two groups, and two-way ANOVA with Bonferoni as post hoc test, when comparing the groups with more than one variable. The level of statistical significance was set to $P<0.05$.

RESULTS

Total depletion of BDNF in frontal cortex and hippocampus of BDNF mutants

To confirm that adult $BDNF^{2L/2LCk\text{-}cre}$ mutant mice were depleted of BDNF protein in our brain regions of interest, we measured levels of BDNF in the mutant frontal cortex and hippocampus. Whereas BDNF protein was clearly detectable by Western blotting in wild-type mice, BDNF levels in BDNF mutants were below detectable levels (Fig. 1A, B). These results demonstrate that $BDNF^{2L/2LCk\text{-}cre}$ mice are depleted of BDNF protein in the frontal cortex and hippocampus.

5-HT_{2A} and 5-HT_{1A} receptor binding and expression is altered in BDNF mutant mice

The number of 5-HT_{2A} and 5-HT_{1A} receptors in frontal cortex and hippocampus was analyzed using autoradiography applying highly selective tritium-labeled ligands ([^{3}H]-MDL100907 and [^{3}H]-WAY100635, respectively). 5-HT_{2A} binding in frontal cortex of $BDNF^{2L/2LCk\text{-}cre}$ mice was lower when compared to wild-type littermates (45.6± 1.7 vs. 35.2±1.6 fmol/mg/TE, respectively) ($P=0.03$) (Fig. 2A). However, in hippocampus, 5-HT_{2A} receptor binding was increased by 31% in BDNF mutants (12.5±0.7 vs. 16.4±1.2 fmol/mg/TE, respectively) ($P=0.02$) (Fig. 2B). In frontal cortex 5-HT_{1A} receptor binding was similar in the two groups (59.15±2.9 (WT) vs. 56.55±3.2 (BDNF mutants) Fig. 3A), while in hippocampus there was a significant decrease in 5-HT_{1A} receptor binding in BDNF mutants compared to wild-types (115.7±2.5 vs. 100.2±3.5; Fig. 3B) ($P=0.01$).

The effect of perturbed BDNF signaling on 5-HT_{1A} and 5-HT_{2A} receptors was further evaluated by gene expression studies. Using RT-qPCR, we observed a decrease in 5-HT_{2A} mRNA levels in the frontal cortex of $BDNF^{2L/2LCk\text{-}cre}$ mutants that corresponded to the observed deficit in receptor binding (Fig. 4A). However, the increase in 5-HT_{2A} receptor binding in the BDNF mutant hippocampus was not associ-

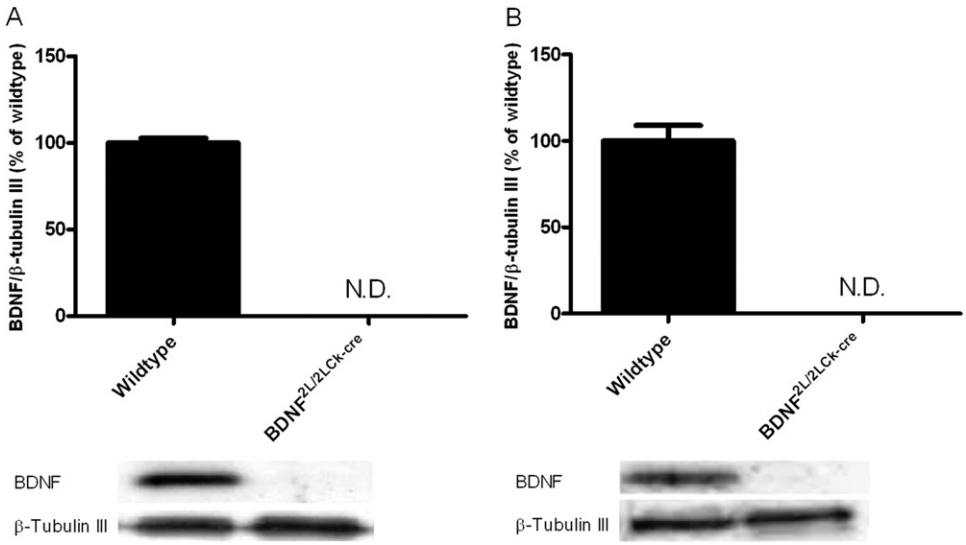

Fig. 1. BDNF protein levels in frontal cortex (A) and hippocampus (B) in wild-type and BDNF$^{2L/2LCk\text{-}cre}$ mice. BDNF levels were not detectable in frontal cortex and hippocampus in the BDNF mutant mice using western blotting, while wild-type levels corresponded to levels seen in other mouse strains. BDNF levels were related to β-tubulin III levels (n=7). Representative bands for BDNF (14 kDa) and β-tubulin III (55 kDa) are shown below the corresponding column. N.D., not detectable. Error bars indicate SEM.

ated with changes in 5-HT$_{2A}$ mRNA levels (Fig. 4D). Additionally, there was a trend towards a decrease in 5-HT$_{1A}$ mRNA expression in the BDNF mutant hippocampus but it did not reach statistical significance (P=0.08) (Fig. 4E). No differences in 5-HT$_{1A}$ mRNA levels were observed in the mutant frontal cortex (Fig. 4B). We also investigated whether transcript or protein levels of the 5-HT$_{2C}$ receptor were altered in BDNF mutant animals. No between-group differences were observed in frontal cortex or hippocampus (Figs. 4C, F, and 5). The cumulative data indicate that the changes in 5-HT$_{2A}$ and 5-HT$_{1A}$ receptor binding in BDNF mutants differ between brain regions and are apparently inversely regulated in hippocampus.

SERT binding is normal in BDNF$^{2L/2LCk\text{-}cre}$ mutant mice

To determine whether the observed alterations in 5-HT receptor binding and expression were related to upstream changes in the SERT, we used [^{3}H]-escitalopram autoradiography to quantify SERT binding sites. This analysis revealed that there were no differences in SERT binding between wild-types and BDNF mutants either in frontal cortex (Fig. 6A) or hippocampus (Fig. 6B).

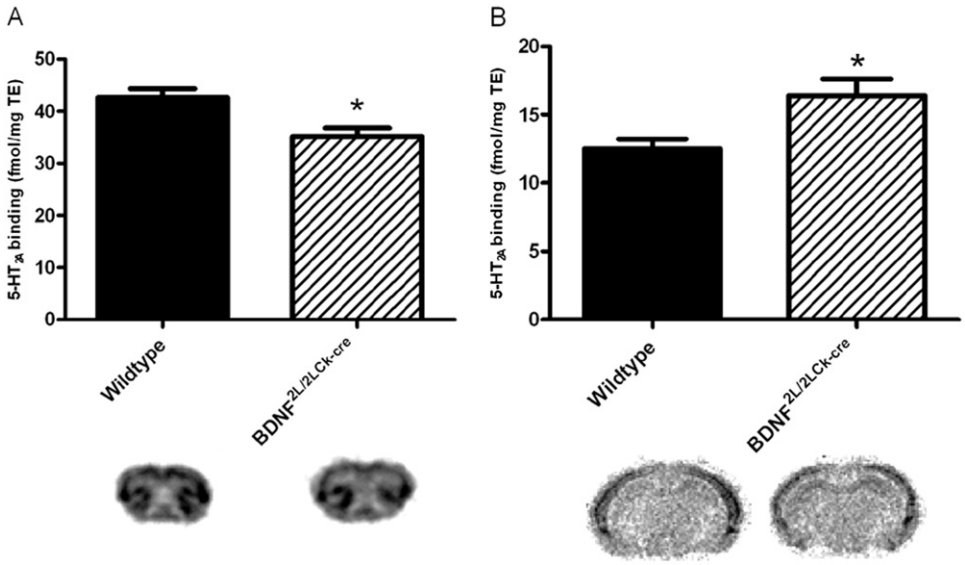

Fig. 2. 5-HT$_{2A}$ receptor binding ([^{3}H]-MDL-100907) in BDNF$^{2L/2LCk\text{-}cre}$ mice and wild-type littermates in frontal cortex (A) and dorsal hippocampus (B). There was a significant decrease in binding in frontal cortex in BDNF$^{2L/2LCk\text{-}cre}$ mice vs. wildtypes, while the opposite was observed for dorsal hippocampus (n=4, Student's t-test, * P<0.05). Representative autoradiograms are shown below the corresponding column. Error bars indicate SEM.

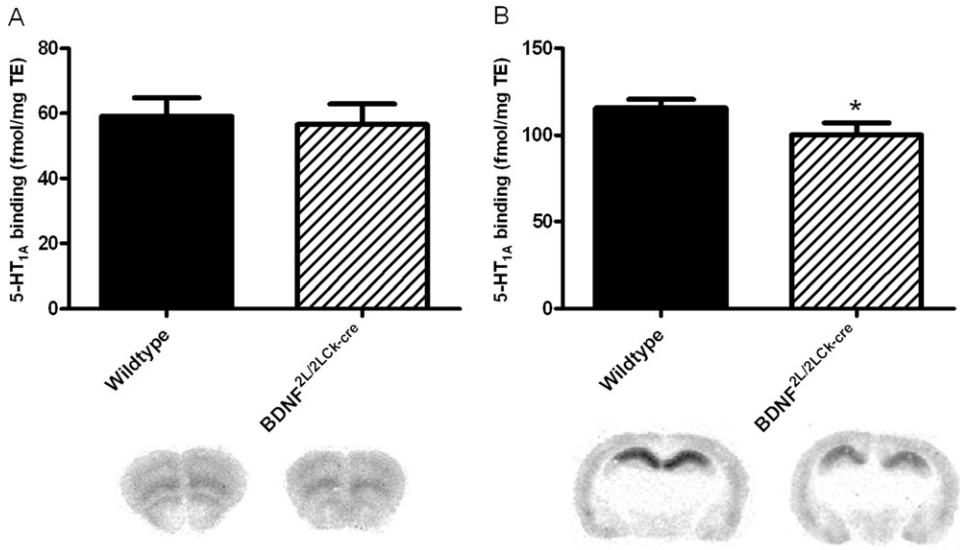

Fig. 3. 5-HT_{1A} receptor binding ([^{3}H]-WAY100635) in $BDNF^{2L/2LCk\text{-}cre}$ mice and wild-type littermates in frontal cortex (A) and dorsal hippocampus (B). There was a significant decrease in binding in dorsal hippocampus in $BDNF^{2L/2LCk\text{-}cre}$ mice vs. wildtypes, while no change was observed in frontal cortex ($n=4$, Student's t-test, * $P<0.05$). Representative autoradiograms are shown below the corresponding column. Error bars indicate SEM.

BDNF depletion leads to altered behavioral responses to 5-HT_{2A} receptor stimulation

Next, we tested the effect of the altered 5-HT_{2A} receptor expression in $BDNF^{2L/2LCk\text{-}cre}$ mutant mice on 5-HT_{2A}-mediated behaviors. For this, we measured the ESR and HTR following saline or DOI (2.5 mg/kg i.p.) administration. There was a significant interaction of genotype and drug treatment in the ESR (F, 12.39 and $P=0.002$, two-way ANOVA) (Fig. 7A). DOI induced a significant increase in ESR in wildtypes compared to their saline-treated counterparts (WT saline: 3±1.5; WT DOI: 18±5.5 head-twitches/20 min, Bonferroni post hoc test, $P<0.001$). In contrast, DOI did not induce an increase in ESR in $BDNF^{2L/2LCk\text{-}cre}$ mice compared to saline-treated mutants (Fig. 7A). However, DOI treatment induced similar levels of HTR in wild type and BDNF mutant mice (two-way ANOVA) (Fig. 7B). These results indicate that the 5-HT_{2A} mediated functional output measured by ESR is diminished in the BDNF mutant mice.

Neuronal activation following 5-HT_{2A} receptor stimulation in BDNF mutant mice

To further assess possible functional deficits in $BDNF^{2L/2LCk\text{-}cre}$ mutants related to 5-HT_{2A} receptor alterations, we measured neuronal activation by c-Fos immunoreactivity in frontal cortex and hippocampus 1 h after a single DOI or saline injection. DOI elicited similar increases in number of c-Fos positive cells in frontal cortex in both wild-types (saline: 1820; DOI: 4731 c-Fos+ cells; $P<0.05$) and BDNF mutants (saline: 1198; DOI: 4783 cells; $P<0.05$) (Fig. 8A) (two-way ANOVA, Bonferroni post hoc). In hippocampus, DOI did not induce a significant increase in c-Fos+ cells in either wild types (saline: 1930; DOI: 3257 cells), or BDNF mutants (saline: 4420; DOI: 4593 cells) (Fig. 8B). However, there was an effect of genotype indicating that the cell activation response following 5-HT_{2A}R-mediated cell activation is higher in hippocampus of the BDNF mutants compared to wild-types ($P=0.04$). This difference between genotypes was mainly driven by high baseline activation in the BDNF mutants. Collectively, these data indicate that the 5-HT_{2A} receptor changes in frontal cortex are not reflected in 5-HT_{2A}R-mediated cell activation, while the hippocampal cell activation is higher for the BDNF mutants.

Expression of synaptic markers in hippocampus and frontal cortex of $BDNF^{2L/2LCk\text{-}cre}$ mutant mice is normal

To examine the degree of synaptic integrity, we measured protein levels of synaptophysin (pre-synaptic), PSD-95 (post-synaptic) and MAP2 (dendritic) using western blotting. We found no change in protein levels between wild-types and BDNF mutants neither in frontal cortex nor in hippocampus (Fig. 9).

DISCUSSION

We hypothesized that alterations in the 5-HT system might underlie the behavioral alterations triggered by deficient BDNF signaling in the brain. One test of this hypothesis was to ascertain the viability of pivotal components of this neurotransmitter system in limbic regions of brain associated with anxiety and mood. We focused our investigations on 5-HT_{2A}, 5-HT_{2C} and 5-HT_{1A} receptors because human and animal model studies have linked them to behaviors known to be altered in BDNF mutant mice including anxiety-like behavior (Naughton et al., 2000; Heisler et al., 2007; Akimova et al., 2009). Our results confirm the previously described differences in cortical 5-HT_{2A} levels in BDNF mutant mice and reveal additional alterations in the 5-HT receptor system elicited by depleted BDNF stores. The decreased 5-HT_{2A} receptor content observed in frontal cortex of $BDNF^{2L/2LCk\text{-}cre}$ mice using western blotting was also reflected in lower receptor binding in this area.

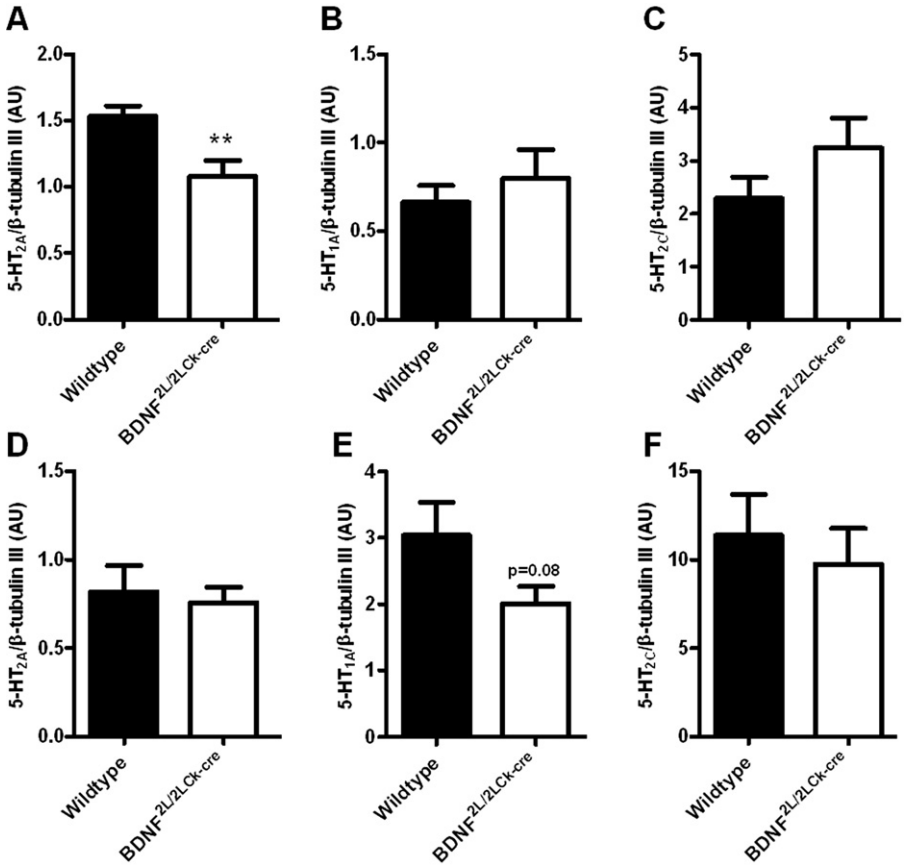

Fig. 4. RT-qPCR on 5-HT_{2A} (A, D), 5-HT_{1A} (B, E) and 5-HT_{2C} (C, F) mRNA in frontal cortex (upper panel) and hippocampus (lower panel) of wild-type and $BDNF^{2L/2LCk\text{-}cre}$ mice. There was a significant lower 5-HT_{2A} mRNA expression in $BDNF^{2L/2LCk\text{-}cre}$ mice in frontal cortex compared to wildtypes (A), while no differences could be observed in hippocampus (D) (n=7–8, Student's t-test, ** $P<0.01$). For the 5-HT_{1A} receptor, there was a tendency towards a decrease in expression in $BDNF^{2L/2LCk\text{-}cre}$ mice compared to wildtypes (E) (P=0.08) for hippocampus, while no change was observed in frontal cortex (B). For the 5-HT_{2C} receptor we did not find any differences in expression of mRNA between $BDNF^{2L/2LCk\text{-}cre}$ mice and wildtypes (AU, arbitrary units; Error bars indicate SEM).

Previously, we reported a 50–60% decrease in 5-HT_{2A} receptor protein levels in frontal cortex (Rios et al., 2006), and in the present paper we found a ~20% decrease in receptor binding in this region. Since the analyses were not

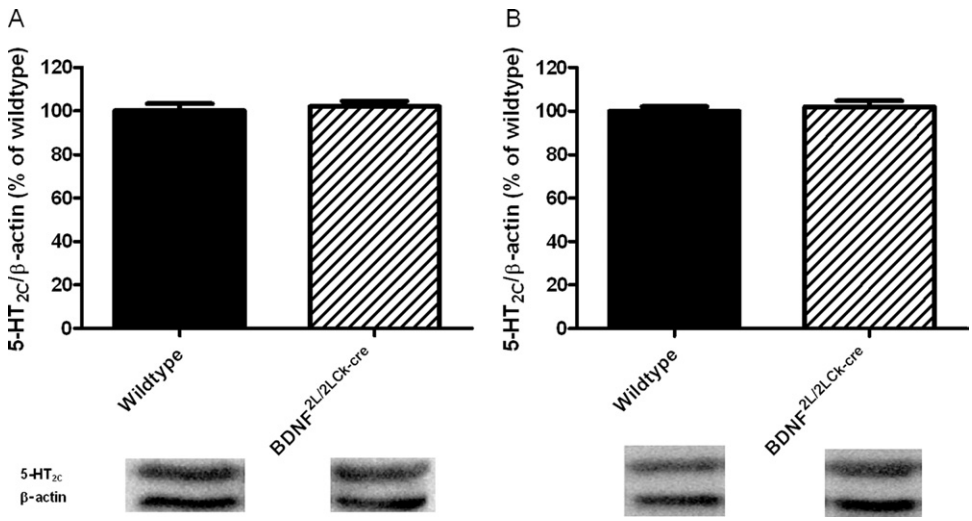

Fig. 5. 5-HT_{2C} receptor protein levels in wild-type and $BDNF^{2L/2LCk\text{-}cre}$ mice in frontal cortex (A) and hippocampus (B). No changes in protein levels of this serotonin receptor could be observed (n=7–8, Student's t-test). Representative bands for 5-HT_{2C} (55 kDa) and β-actin (40 kDa) are shown below the corresponding column. Error bars indicate SEM.

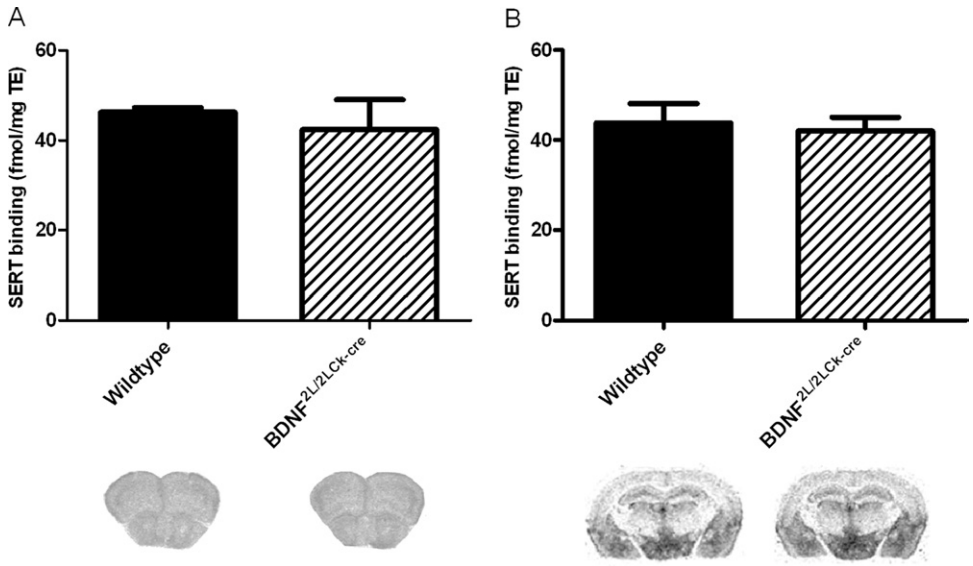

Fig. 6. SERT binding performed with [^{3}H]-escitalopram in frontal cortex (A) and hippocampus (B). We did not detect any differences in SERT binding between wildtypes and BDNF$^{2L/2LCk\text{-}cre}$ mice (n=4, Student's t-test). Representative autoradiograms are shown below the corresponding column. Error bars indicate SEM.

conducted on the same tissue, the quantitative outcomes cannot be directly compared. Differences in the receptor detection methods utilized could, however, explain eventual discrepancies. Total protein measurements reflect the entire pool of 5-HT$_{2A}$Rs, while receptor autoradiography reflects the binding of the antagonist radioligand to the intact receptor binding pocket. A special characteristic of the 5-HT$_{2A}$R is its high turnover rate, the largest receptor fraction being located intracellularly (Cornea-Hebert et al., 1999; Gray and Roth, 2001). Our results suggest that in BDNF mutant animals, it is primarily the intracellular fraction of receptors not available for ligand binding that is diminished. This is supported by the mRNA quantification results showing that indeed the rate of receptor synthesis is decreased in BDNF mutant mice.

Contrary to our observations in frontal cortex, we found that there was an increase in 5-HT$_{2A}$R binding in the BDNF mutant hippocampus. This is in accordance with our *in vivo* findings from BDNF+/− mice, where we also found increased hippocampal 5-HT$_{2A}$R protein levels (Trajkovska et al., 2009). The inverse observation, namely that exposure of BDNF to hippocampal cultures results in 5-HT$_{2A}$R downregulation is in also line with this (Trajkovska et al., 2009). The *in vivo* studies of BDNF$^{2L/2LCk\text{-}cre}$ mice described here indicate that the effects of BDNF on 5-HT$_{2A}$ receptor levels are context-dependent.

We extended our analysis to other 5-HT receptors to ascertain whether the effects of BDNF were restricted to the 5-HT$_{2A}$R. We found that 5-HT$_{2C}$R expression in frontal cortex and hippocampus of BDNF mutants was normal.

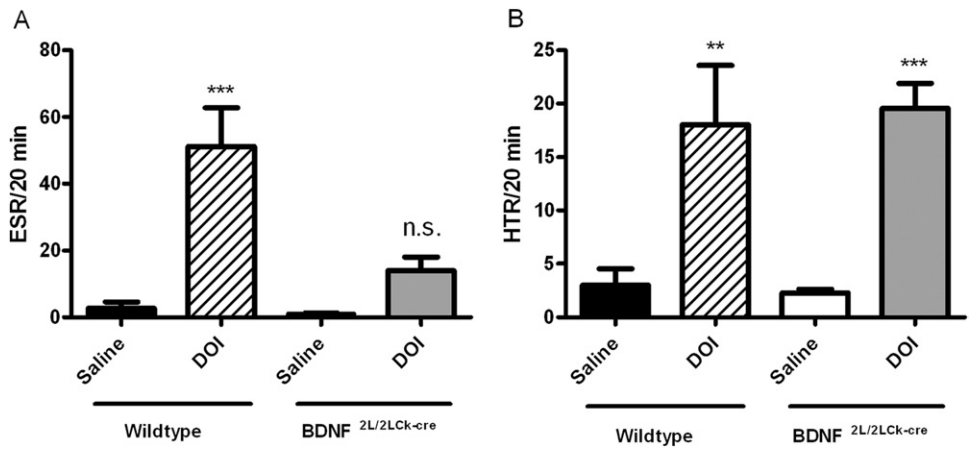

Fig. 7. Ear-Scratch Reponse (ESR) (A) and Head-Twitch Reponse (HTR) (B) in BDNF mutant mice and wild-type littermates after administration of DOI (2.5 mg/kg i.p.) or saline. The registration of ESR and HTR was started 5 min after administration and continued for 20 min. Bars: Black: wild-type, saline; Crossed: wild-type, DOI 2.5 mg/kg i.p.; White: BDNF$^{2L/2LCk\text{-}cre}$ mice, saline; Gray: BDNF$^{2L/2LCk\text{-}cre}$ mice (n=7, two-way ANOVA, Bonferroni post test, n.s., not significant; **P<0.01, ***P<0.001. Error bars indicate SEM.

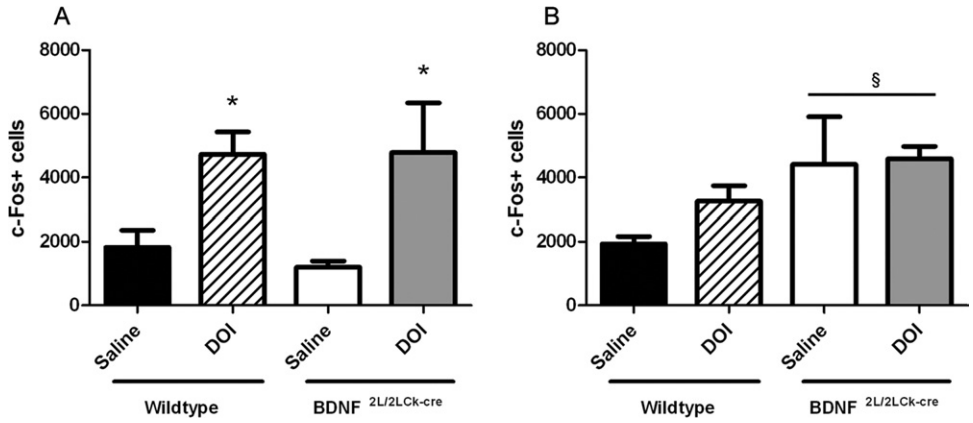

Fig. 8. c-Fos immunopositive cells in frontal cortex (A) and hippocampus (B). A single DOI (2.5 mg/kg) injection significantly induced c-Fos immunoreactive cells compared to a single saline injection in frontal cortex in both wildtypes and BDNF$^{2L/2LCk\text{-}cre}$ mice (A). There was a genotype effect in hippocampus (§) indicating higher cell activation response BDNF$^{2L/2LCk\text{-}cre}$ mice compared to wildtypes (B) (n=4, two-way ANOVA, Bonferroni post hoc test, *$P<0.05$. §$P<0.05$. Error bars indicate SEM.

We also measured expression and binding of the 5-HT$_{1A}$R in these brain regions. The 5-HT$_{1A}$R co-localizes with the 5-HT$_{2A}$R in dendrites in prefrontal cortex (Feng et al., 2001). Central BDNF depletion did not impact 5-HT$_{1A}$R binding in frontal cortex, suggesting that the lower 5-HT$_{2A}$R binding was a result from structural reorganization or retraction of dendrites. In support of this assertion, we found that expression of synaptophysin, a synaptic marker and of MAP2, a dendritic marker, were normal in BDNF mutants. In contrast to our findings in frontal cortex, 5-HT$_{1A}$R binding was decreased in the BDNF mutant hippocampus. Furthermore, we showed previously that 5-HT$_{1A}$R protein levels were unchanged in dorsal raphe nucleus (DRN) in BDNF$^{2L/2LCk\text{-}cre}$ mutants (Rios et al., 2006). The primary role of the 5-HT$_{1A}$Rs in DRN is as pre-synaptic autoreceptors, while in hippocampus they mainly exist as post-synaptic heteroreceptors (Riad et al., 2001). Therefore, the data shown here and previous findings provide additional examples of the context-dependent effects of BDNF. An intriguing study by Hensler et al. (2007) found no changes in hippocampal 5-HT$_{1A}$R binding using a tetracycline-inducible model of BDNF depletion (Hensler et al., 2007). It is important to note that whereas in those mutants BDNF was depleted in adulthood, in the mutant mice described here BDNF was gradually depleted during the first post-natal weeks (Rios et al., 2001). Therefore, the 5-HT$_{1A}$R alterations reported here might reflect the long-term effect of BDNF depletion, the effects of perturbing BDNF signaling during a critical developmental period or a compensatory response to the 5-HT$_{2A}$ receptor changes also observed in these mice. A "functional antagonism" between these two receptors was proposed previously as a mechanism to circumvent challenges on the serotonin system and restore proper neurotransmission (Yuen et al., 2008). Nonetheless, this alteration in 5-HT$_{1A}$Rs might contribute to the complex behavioral phenotype of these mice.

By examining 5-HT$_{2A}$-mediated behavioral output, we showed that the decrease in 5-HT$_{2A}$R levels was accompanied by a diminished functional response to pharmacological stimulation of this receptor *in vivo*. DOI-induced ESR was significantly attenuated in BDNF mutants, indicating a reduction in the pool of functional 5-HT$_{2A}$Rs that mediate this response. Interestingly, the DOI-induced HTR was not affected in the mutants. This was surprising as it is well established that both HTR and ESR are mediated by the 5-HT$_{2A}$R (Gonzalez-Maeso et al., 2007; Willins and Meltzer, 1997). Accordingly, both HTR and ESR were completely abrogated in 5-HT$_{2A}$ receptor knock-out mice (Gonzalez-Maeso et al., 2007, Suppl. mat). We suggest that the ESR is more sensitive to small changes in 5-HT$_{2A}$ receptor signaling, and that the 20% decrease in 5-HT$_{2A}$ receptor binding in frontal cortex is too small to be reflected in the HTR. Indeed, the ESR was demonstrated to be 30 times more sensitive than HTR (Darmani, 2001). Alternatively, the HTR and ESR might be mediated by different brain circuits that are differentially affected by perturbed BDNF signaling. Unfortunately, little is known regarding the exact networks underlying the HTR and ESR. In a thorough study, Willins and Meltzer (1997) delivered DOI to different regions of the rat brain and established that HTR could only be induced when the medial prefrontal cortex was activated. The ESR was not examined as this response had not been described in rats. If other brain areas besides the medial prefrontal cortex such as the hippocampus are involved in the ESR, the observed increase in hippocampal 5-HT$_{2A}$R binding in the BDNF mutants might exacerbate the diminished 5-HT$_{2A}$ frontal cortex output leading to impaired ESR. In support of this model, a previous study demonstrated a marked DOI-induced increase in motor-movements (head-bobs), which can be compared to HTR and ESR in rodents, in rabbits with increased 5-HT$_{2A}$ receptor binding in the CA1 region of the hippocampus (Dave et al., 2004). Future studies with intracerebral site-directed injections of 5-HT$_{2A}$ agonists in mice are needed to test this hypothesis.

Even though activation of the 5-HT$_{2A}$ receptor signaling pathway is necessary for both ESR and HTR, it has been suggested that other receptor systems can modulate the response. For example, Darmani (2001) showed that

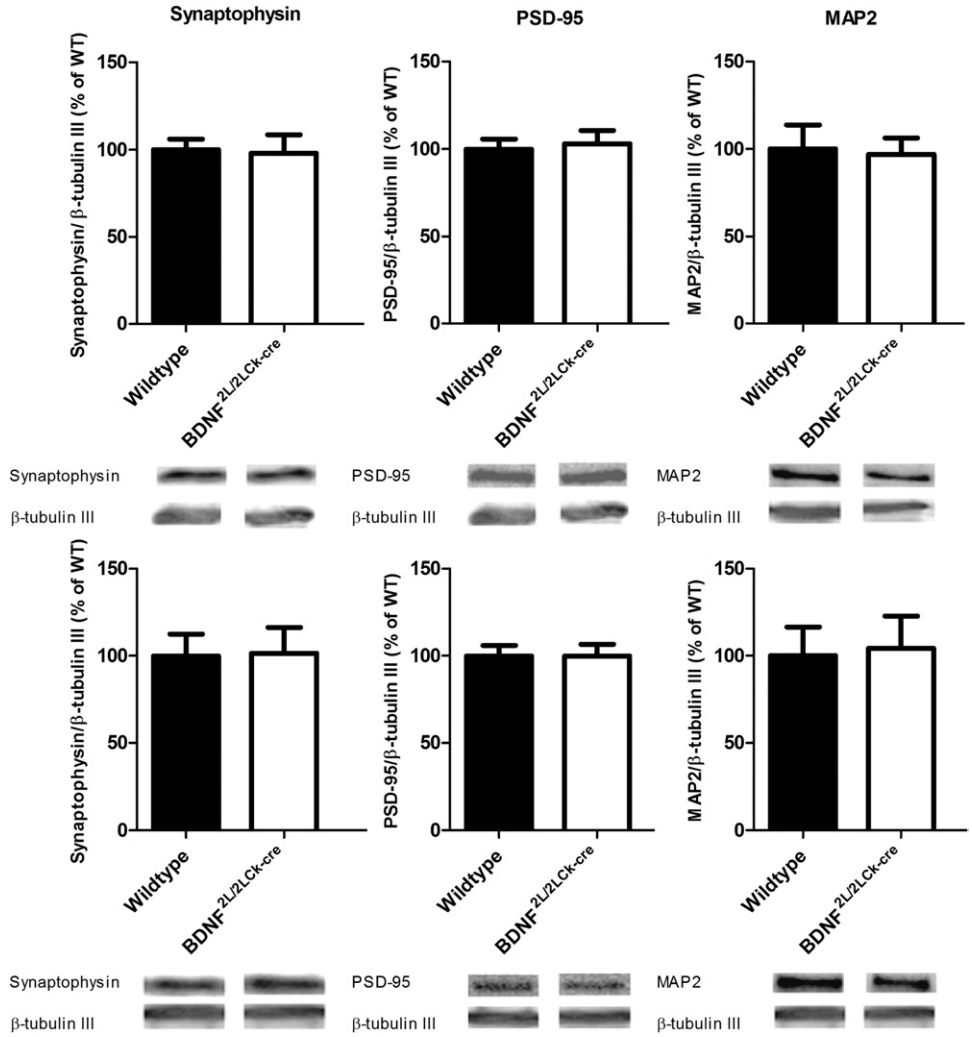

Fig. 9. Analysis of protein levels using western blotting for synaptophysin (38 kDa), PSD-95 (95 kDa) and MAP2 (250 kDa). Upper panel: frontal cortex, lower panel: hippocampus. BDNF mutants have normal contents of the synaptic markers synaptophysin and PSD-95 and the dendritic marker MAP2 when compared to wildtypes. β-tubulin III (55 kDa) was used as loading control (n=7–8, Student's t-test). Error bars indicate SEM.

HTR and ESR induced by DOI can both be inhibited by cannabinoids (e.g. CP55,940), but not to the same degree. This indicates that there are different thresholds for each response which can be modulated through other receptors. Other data suggest that 5-HT_{2A} mediated responses can be modulated by activation of the 5-HT_{1A} and 5-HT_{2C} receptors (Willins and Meltzer, 1997). Interestingly, we found that there was a significant decrease in hippocampal 5-HT_{1A} receptor binding in BDNF mutants. However, it remains to be elucidated whether this change is sufficient to impact the ESR and/or the HTR. Since BDNF depletion was not associated with changes in 5-HT_{2C} receptor expression, it is unlikely that deficits in this pathway contribute to the diminished ESR in BDNF mutants.

To further evaluate 5-HT_{2A} receptor functionality in the $BDNF^{2L/2LCk\text{-}cre}$ mutant mice, we quantified DOI-induced c-Fos expression as a measure of 5-HT_{2A}-mediated neuronal activation. In spite of decreased frontal 5-HT_{2A}-receptor binding, BDNF mutants had the same DOI-induced c-Fos expression in frontal cortex, as the wild-type mice. However, in hippocampus, BDNF mutants had a different cell activation response. This was partly driven by high levels of c-Fos+ cells in the hippocampus following the saline injection, suggesting that mutants might have elevated baseline levels of neuronal activity in this region at baseline. This may be mediated through the observed increase in 5-HT_{2A}Rs in hippocampus in these mice. Alternatively, BDNF mutants might have a higher basal level of arousal and are easily stressed when handled and accordingly, the saline injection *per se* may elicit a c-Fos response in this brain region.

CONCLUSION

In summary, we demonstrated that BDNF depletion results in context-dependent changes in 5-HT_{2A} and 5-HT_{1A} binding and expression. Furthermore, we showed that alterations in 5-HT_{2A} receptor levels have functional consequences as illustrated by the diminished DOI-induced ESR in BDNF mutants. The diverse regulation of these recep-

tors in $BDNF^{2L/2LCk\text{-}cre}$ mice underlines the complexity of neurotrophin and serotonin signaling interaction.

Acknowledgments—German A. Calderon, Hans-Jørgen Jensen and Christine B. Janssens are acknowledged for great technical assistance. This work was supported by the Lundbeck Foundation, the Augustinus Foundation, the EC-FP6-project DiMI, LSHB-CT-2005-512146 and the University of Copenhagen. MR was supported by NIH/NIDDK (DK073311) and NIH/NIMH (MH67817) research grants.

REFERENCES

Akimova E, Lanzenberger R, Kasper S (2009) The serotonin-1A receptor in anxiety disorders. Biol Psychiatry 66:627–635.

Chan JP, Unger TJ, Byrnes J, Rios M (2006) Examination of behavioral deficits triggered by targeting Bdnf in fetal or postnatal brains of mice. Neuroscience 142:49–58.

Cornea-Hebert V, Riad M, Wu C, Singh SK, 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.

Darmani NA (2001) Cannabinoids of diverse structure inhibit two DOI-induced 5-HT(2A) receptor-mediated behaviors in mice. Pharmacol Biochem Behav 68:311–317.

Dave KD, Fernando GS, Quinn JL, Harvey JA, Aloyo VJ (2004) Serotonin 5-HT2A receptors in the CA1 field of the hippocampus mediate head movements in the rabbit. Psychopharmacology (Berl) 176:287–295.

Deltheil T, Guiard BP, Cerdan J, David DJ, Tanaka KF, Reperant C, Guilloux JP, Coudore F, Hen R, Gardier AM (2008) Behavioral and serotonergic consequences of decreasing or increasing hippocampus brain-derived neurotrophic factor protein levels in mice. Neuropharmacology 55:1006–1014.

Feng J, Cai X, Zhao J, Yan Z (2001) Serotonin receptors modulate GABA(A) receptor channels through activation of anchored protein kinase C in prefrontal cortical neurons. J Neurosci 21:6502–6511.

Gonzalez-Maeso J, Weisstaub NV, Zhou M, Chan P, Ivic L, Ang R, Lira A, Bradley-Moore M, Ge Y, Zhou Q, Sealfon SC, Gingrich JA (2007) Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling pathways to affect behavior. Neuron 53: 439–452.

Gray JA, Roth BL (2001) Paradoxical trafficking and regulation of 5-HT(2A) receptors by agonists and antagonists. Brain Res Bull 56:441–451.

Heisler LK, Zhou L, Bajwa P, Hsu J, Tecott LH (2007) Serotonin 5-HT(2C) receptors regulate anxiety-like behavior. Genes Brain Behav 6:491–496.

Hensler JG, Advani T, Monteggia LM (2007) Regulation of serotonin-1A receptor function in inducible brain-derived neurotrophic factor knockout mice after administration of corticosterone. Biol Psychiatry 62:521–529.

Kernie SG, Liebl DJ, Parada LF (2000) BDNF regulates eating behavior and locomotor activity in mice. EMBO J 19:1290–1300.

Kozisek ME, Middlemas D, Bylund DB (2008) Brain-derived neurotrophic factor and its receptor tropomyosin-related kinase B in the mechanism of action of antidepressant therapies. Pharmacol Ther 117:30–51.

Leysen JE (2004) 5-HT2 receptors. Curr Drug Targets CNS Neurol Disord 3:11–26.

Lyons WE, Mamounas LA, Ricaurte GA, Coppola V, Reid SW, Bora SH, Wihler C, Koliatsos VE, 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 96:15239–15244.

Naughton M, Mulrooney JB, Leonard BE (2000) A review of the role of serotonin receptors in psychiatric disorders. Hum Psychopharmacol 15:397–415.

Riad M, Watkins KC, Doucet E, Hamon M, Descarries L (2001) Agonist-induced internalization of serotonin-1a receptors in the dorsal raphe nucleus (autoreceptors) but not hippocampus (heteroreceptors). J Neurosci 21:8378–8386.

Rios M, Fan G, Fekete C, Kelly J, Bates B, Kuehn R, Lechan RM, Jaenisch R (2001) Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. Mol Endocrinol 15:1748–1757.

Rios M, Lambe EK, Liu R, Teillon S, Liu J, Akbarian S, Roffler-Tarlov S, Jaenisch R, Aghajanian GK (2006) Severe deficits in 5-HT2A -mediated neurotransmission in BDNF conditional mutant mice. J Neurobiol 66:408–420.

Savitz J, Lucki I, Drevets WC (2009) 5-HT(1A) receptor function in major depressive disorder. Prog Neurobiol 88:17–31.

Thomsen C, Helboe L (2003) Regional pattern of binding and c-Fos induction by (R)-and (S)-citalopram in rat brain. Neuroreport 14:2411–2414.

Trajkovska V, Santini MA, Marcussen AB, Thomsen MS, Hansen HH, Mikkelsen JD, Arneberg L, Kokaia M, Knudsen GM, Aznar S (2009) BDNF downregulates 5-HT(2A) receptor protein levels in hippocampal cultures. Neurochem Int 55:697–702.

Unger TJ, Calderon GA, Bradley LC, Sena-Esteves M, Rios M (2007) Selective deletion of Bdnf in the ventromedial and dorsomedial hypothalamus of adult mice results in hyperphagic behavior and obesity. J Neurosci 27:14265–14274.

Weisstaub NV, Zhou M, Lira A, Lambe E, Gonzalez-Maeso J, Hornung JP, Sibille E, Underwood M, Itohara S, Dauer WT, Ansorge MS, Morelli E, Mann JJ, Toth M, Aghajanian G, Sealfon SC, Hen R, Gingrich JA (2006) Cortical 5-HT2A receptor signaling modulates anxiety-like behaviors in mice. Science 313:536–540.

Willins DL, Meltzer HY (1997) Direct injection of 5-HT2A receptor agonists into the medial prefrontal cortex produces a head-twitch response in rats. J Pharmacol Exp Ther 282:699–706.

Yuen EY, Jiang Q, Chen P, Feng J, Yan Z (2008) Activation of 5-HT2A/C receptors counteracts 5-HT1A regulation of N-methyl-D-aspartate receptor channels in pyramidal neurons of prefrontal cortex. J Biol Chem 283:17194–17204.

(Accepted 23 May 2010)
(Available online 1 June 2010)

Paper II

Journal of Cerebral Blood Flow & Metabolism (2010), e1–e7

www.jcbfm.com

Negative Result

Cerebral 5-HT$_{2A}$ receptor and serotonin transporter binding in humans are not affected by the val66met BDNF polymorphism status or blood BDNF levels

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Recent studies have proposed an interrelation between the brain-derived neurotrophic factor (BDNF) val66met polymorphism and the serotonin system. In this study, we investigated whether the *BDNF* val66met polymorphism or blood BDNF levels are associated with cerebral 5-hydroxytryptamine 2A (5-HT$_{2A}$) receptor or serotonin transporter (SERT) binding in healthy subjects. No statistically significant differences in 5-HT$_{2A}$ receptor or SERT binding were found between the val/val and met carriers, nor were blood BDNF values associated with SERT binding or 5-HT$_{2A}$ receptor binding. In conclusion, val66met *BDNF* polymorphism status is not associated with changes in the serotonergic system. Moreover, BDNF levels in blood do not correlate with either 5-HT$_{2A}$ or SERT binding.
Journal of Cerebral Blood Flow & Metabolism (2010) **e1–e7**; advance online publication, 25 August 2010; doi:10.1038/jcbfm.2010.114

Keywords: BDNF; 5-HT$_{2A}$; imaging; polymorphism; SERT; val66met

Introduction

Brain-derived neurotrophic factor (BDNF) belongs to the family of neurotrophins and is primarily produced in neurons and glia cells and to a lesser extent in endothelial cells (Leventhal *et al*, 1999), leucocytes (Kerschensteiner *et al*, 1999), and satellite cells in skeletal muscles (Mousavi and Jasmin, 2006). Brain-derived neurotrophic factor is important for growth of the central nervous system during fetal development, whereas in adults, BDNF is primarily involved in synaptic plasticity, neurogenesis, and neuronal survival (for review, see Waterhouse and Xu, 2009). Moreover, BDNF also affects synthesis and release of several neurotransmitters, including serotonin (Messaoudi *et al*, 1998).

Several studies point toward an involvement of BDNF in neuropsychiatric and neurologic disorders, such as depression and Alzheimer's disease (Connor *et al*, 1997; Karege *et al*, 2005). For example, serum BDNF levels are lower in patients with depression (Brunoni *et al*, 2008) and in women, genetically predisposed to depression and exposed to recent stressful life events (Trajkovska *et al*, 2008). In a recent meta-analysis study, it was shown that serum BDNF levels increased after antidepressant treatment (Brunoni *et al*, 2008).

Another method to investigate effects of perturbed BDNF signaling on the serotonin system is to study individuals carrying polymorphisms in the *BDNF* gene, e.g., the val66met polymorphism. This single-nucleotide polymorphism in the 5′-prodomain region of the *BDNF* gene leads to a nucleotide change in codon 66, where the amino acid valine (val) is substituted by methionine (met) in the propart of the BDNF protein (Egan *et al*, 2003). The propart is later cleaved off by proteases, such that the val66met substitution is not transferred to the mature form of BDNF. The presence of the met allele leads to a

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The study was sponsored by The Danish Medical Research Council; H:S (Copenhagen Hospital Cooperation) research council; Copenhagen University Hospital; The Faculty of Health Sciences, Neurocluster, Copenhagen University, and The Lundbeck Foundation.
Received 11 May 2010; revised 10 June 2010; accepted 19 June 2010

disturbance in the activity-dependent BDNF secretion (controlled by neuronal activity), while the constitutive secretion remains unaffected (Egan *et al*, 2003). The val66met polymorphism is relatively frequent in Caucasians (19–25%) and in Asians (45%) (Shimizu *et al*, 2004; Hwang *et al*, 2006). *In vivo* neuroimaging studies have shown that in healthy subjects, the met allele is associated with lower volumes of the prefrontal cortex and hippocampus and poorer performance in episodic memory tests (Hariri *et al*, 2003; Egan *et al*, 2003; Pezawas *et al*, 2004).

A close interaction between BDNF and the serotonergic system has previously been described (for review, see Mattson *et al*, 2004). Brain-derived neurotrophic factor provides neurotrophic support for serotonergic neurons and changes in BDNF levels affect the function of serotonin transporter (SERT) (Guiard *et al*, 2008), 5-hydroxytryptamine $(5\text{-HT})_{1A}$ (Hensler *et al*, 2007), and 5-HT_{2A} receptors (Rios *et al*, 2006). Functional studies of SERT function in heterozygous BDNF knockout mice showed a decreased rate of serotonin uptake in the ventral hippocampus (Daws *et al*, 2007; Guiard *et al*, 2008). In line with this, a positron emission tomography (PET) study of 25 healthy individuals showed that the val/val genotype was associated with increased SERT binding in men (Henningsson *et al*, 2009). In addition, a val66met interaction with the 5-HTTLPR (5-HT-transporter-linked promoter region) polymorphism in relation to neuroticism (a risk factor for developing major depression) has been reported (Terracciano *et al*, 2010).

Interactions with BDNF have also been reported for the 5-HT_{2A} receptor. For example, conditional BDNF knockout mice have severe impairment of cortical 5-HT_{2A} receptor function and decreased protein levels of the receptor in the frontal cortex (Rios *et al*, 2006). Furthermore, recent studies from our group showed that frontolimbic 5-HT_{2A} receptor binding was positively correlated with neuroticism score and that this association was particularly strong in individuals with high familial risk for developing depression (Frokjaer *et al*, 2008, 2010). These studies are suggestive of an interaction between BDNF and frontal cortex 5-HT_{2A} receptors, a claim which has been further substantiated by the finding of a direct regulatory effect of BDNF on the density of 5-HT_{2A} receptors *in vitro* (Trajkovska *et al*, 2009).

In this study, we investigated a large cohort of healthy individuals to determine whether the met allele of the *BDNF* val66met polymorphism or whole-blood levels of BDNF are associated with cerebral 5-HT_{2A} receptor binding measured by [^{18}F]altanserin PET and subcortical SERT binding measured by [^{11}C]DASB PET. On the basis of available experimental and clinical data, we hypothesized that the presence of the met allele would result in lower cortical 5-HT_{2A} binding and lower subcortical SERT binding. Finally, we expected a positive correlation between BDNF whole-blood levels and neocortical 5-HT_{2A} receptor and subcortical SERT binding.

Materials and methods

Subjects

A total of 133 individuals (88 men, median age 34 years, range, 18–81 years) were recruited from newspaper announcements and were included in the study after medical screening. The absence of psychiatric and neurologic disorders was ensured by obtaining a detailed interview, and by clinical evaluation. Subsets of the sample were included in previous publications (Frokjaer *et al*, 2008, 2009; Erritzoe *et al*, 2009).

Imaging

All subjects were PET scanned using an 18-ring GE-Advance scanner (General Electric, Milwaukee, WI, USA) in a three-dimensional acquisition mode, producing 35 image slices with an interslice distance of 4.25 mm. [^{18}F]altanserin was injected into the cubital vein as a combination of a bolus and a continuous infusion (ratio 1.75 hours) to obtain tracer steady state in blood and tissue according to Pinborg *et al* (2003). A maximal dose of [^{18}F]altanserin of 3.7 MBq/kg bodyweight, was administered with an average dose of 270 MBq. Dynamic three-dimensional emission scans (5 frames of 8 minutes each) started 120 minutes after administration of [^{18}F]altanserin. Blood samples were collected at mid-PET frame times and analyzed with high-performance liquid chromatography for determination of the activity of the nonmetabolized tracer in the plasma. Visual inspection of the time–activity curves was performed to ensure constant blood and tissue levels. The plasma metabolites of [^{18}F]altanserin were determined using a modification of previously published procedures (Pinborg *et al*, 2003).

For [^{11}C]DASB, a dynamic 90-minute-long emission recording was initiated during intravenous injection during 12 seconds of 485 ± 86 MBq (range, 279–601 MBq) [^{11}C]DASB, with specific activity of 29 ± 16 GBq/μmol (range, 9–82 GBq/μmol).

Magnetic resonance imaging (MRI) was performed on all subjects; magnetization prepared rapid gradient echo sequences were acquired on either a 1.5-T Vision scanner ($N = 69$) or a 3-T Trio scanner ($N = 64$) (both from Siemens, Erlangen, Germany).

Image Analysis

The MRI images were coregistered with the PET images using a Matlab-based (Mathworks, Natick, MA, USA) interactive program, which is based on visual identification of the transformation as described in the study by Adams *et al* (2004). The MRIs were segmented into gray matter, white matter, and cerebrospinal fluid tissue classes using SPM2 (Welcome Department of Cognitive Neurology, University College London, London, UK). A total of 35 regions of interest were automatically delineated on MRI

slices (Svarer *et al*, 2005) and transferred to PET images using the identified rigid body transformation. These PET images were partial volume corrected by means of the segmented MRI. A three-compartment model based on gray matter, white matter, and cerebrospinal fluid tissue was used (Quarantelli *et al*, 2004). The white matter value was extracted as the average voxel value from a white matter region of interest (midbrain) in the uncorrected PET image.

The volume of distribution (V_d) and binding potential (BP_p) for [^{18}F]altanserin and [^{11}C]DASB were calculated as described in the studies by Pinborg *et al* (2003) and Frokjaer *et al* (2009), respectively.

A volume-weighted mean of cortical 5-HT_{2A} binding was subsequently calculated and used as a measure of average brain cortical binding because we primarily tested the hypothesis that the association between the val66met polymorphism and *in vivo* 5-HT_{2A} would be reflected as a global effect on receptor density. The mean neocortical binding was calculated from the volume-corrected binding in the following areas: the orbitofrontal cortex, medial inferior frontal cortex, anterior cingulate, insular cortex, superior temporal cortex, parietal cortex, medial inferior temporal cortex, superior frontal cortex, occipital cortex, sensory and motor cortices, and posterior cingulate cortex. Values from left and right sides were averaged. A mean value for [^{11}C]DASB binding was calculated from a subcortical high-binding region consisting of a volume-weighted average of the caudate, putamen, and thalamus.

Assessment of Neuroticism Score

On the same day as the PET scanning, subjects completed the Danish version of the 240-item NEOPI-R (Neuroticism-Extraversion-Openness Personality Inventory Revised) self-report personality questionnaire as described previously (Costa and McCrae, 1992; Frokjaer *et al*, 2008). The neuroticism score is calculated as the sum of the score in each of the six subdimensions (facets) comprised in this personality trait.

Single-Nucleotide Polymorphism Genotyping

Blood samples for DNA analysis and BDNF measurements were collected at the day of the PET scanning and immediately frozen and stored at −20°C until further analysis. DNA was extracted from the blood with a Qiagen Mini kit using the guidelines included in the kit (Qiagen, Valencia, CA, USA).

Brain-derived neurotrophic factor val66met genotyping was performed using a TaqMan 5′-exonuclease allelic discrimination assay according to the instructions provided by the manufacturer (Applied Biosystems, Foster City, CA, USA, Assay-on-Demand single-nucleotide polymorphism product: C_11592758_10). The ABI 7500 multiplex PCR machine (Applied Biosystems) was used for this analysis.

Brain-Derived Neurotrophic Factor Measurements

To measure the blood BDNF concentration, blood samples were obtained at the day of the PET scanning session and 1.5 mL of whole blood was sampled in EDTA-containing tubes and immediately frozen at −20°C. On the day of analysis, blood samples were thawed on ice and 3% Triton-X 100 (Sigma-Aldrich, Copenhagen, Denmark) were added to the samples. Samples were then sonicated and centrifuged ($12{,}000 \times g$) for 10 minutes and 0.5 mL of the supernatant was aliquoted in new tubes. The following measurement of BDNF content in the lysated blood using enzyme-linked immunosorbent assay was conducted as described previously (Trajkovska *et al*, 2007).

Statistical Analysis

A genotype equilibrium test was performed using Pearson's χ^2 test. Correlation analyses between whole-blood BDNF and binding were performed using multiple regression analysis, including the covariates, age, neuroticism, body mass index, as well as free fraction of the radiotracer for [^{18}F]altanserin and gender, age, daylight minutes, and openness for [^{11}C]DASB data. Comparisons between val/val and met carriers were made using Student's *t*-test. Grubbs' outlier test was used for detecting outliers (an outlier was detected for BP_p in the met+ group, mean: 3.7; *Z*-value: 3.9, $P<0.05$, and therefore not included in the following BP_p-val66met analysis). Unless otherwise stated, the values are presented as mean ± s.d. The level of statistical significance was set at $P<0.05$. Graphs were created using GraphPad Prism 5 (La Jolla, CA, USA).

Results

The genotype distributions for subjects scanned with [^{18}F]altanserin and [^{11}C]DASB study are shown in Tables 1 and 2, respectively. The val/val and met frequencies were in Hardy–Weinberg equilibrium and correspond to those previously reported for Caucasians (Egan *et al*, 2003).

No differences were found in neocortical 5-HT_{2A} receptor binding between the val/val and met carrier groups (1.41 ± 0.43 versus 1.35 ± 0.43, respectively,

Table 1 Data on healthy subjects PET scanned with [^{18}F]altanserin

	PET-[^{18}F]altanserin (n = *133*)		
	val/val	*met+*	*Student's t-test*
n	90	43 (6)	
Age (years)	40 ± 19	41 ± 18	$P=0.86$
BMI (kg/m^2)	24.6 ± 3.3	24.7 ± 3.0	$P=0.95$
Neuroticism	72 ± 18	69 ± 19	$P=0.44$

BMI, body mass index; PET, positron emission tomography; 5-HT_{2A}, 5-hydroxytryptamine 2A.
val66met allele distribution in scanned healthy subjects. Allele distribution was in Hardy–Weinberg equilibrium (Pearson χ^2 test). Age, BMI, and neuroticism have been shown to affect cortical 5-HT_{2A} binding, but no differences were found between val/val and met carriers (Student's *t*-test). The number in brackets in met+ indicates the number of met/met subjects. Values represent mean ± s.d.

Table 2 Data on healthy subjects PET scanned with [^{11}C]DASB

	PET-[^{11}C]DASB					
(n = 49)	*Males*			*Females*		
	val/val	*met+*	*Student's t-test*	*val/val*	*met+*	*Student's t-test*
n	27	6 (1)		11	5 (2)	
Age (years)	34 ± 19	32 ± 18	$P = 0.78$	43 ± 21	40 ± 23	$P = 0.85$
BMI (kg/m^2)	23.9 ± 2.5	25.9 ± 3.2	$P = 0.09$	24.5 ± 3.4	27.3 ± 3.3	$P = 0.15$
Daylight minutes	746 ± 246	798 ± 281	$P = 0.65$	725 ± 200	635 ± 174	$P = 0.41$
Openness	117 ± 19	112 ± 12	$P = 0.53$	122 ± 18	125 ± 16	$P = 0.80$

BMI, body mass index; PET, positron emission tomography; 5-HT$_{2A}$, 5-hydroxytryptamine 2A.
A total of 49 subjects scanned with ^{18}F-altanserin were also scanned with ^{11}C-DASB. Here, possible confounders are age, BMI, daylight minutes, and openness, but no intergroup differences were found between val/val and met carriers (Student's *t*-test). The number in brackets in met+ indicates the number of met/met subjects. Values represent mean ± s.d.

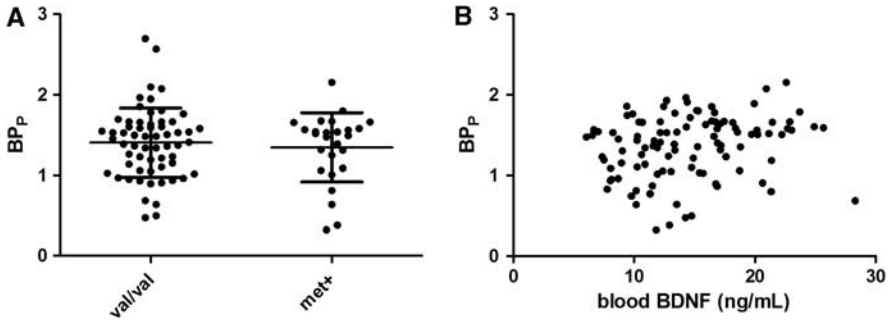

Figure 1 Neocortical 5-HT$_{2A}$ binding in the val/val and met groups. (**A**) There were no significant differences between the two groups (mean: 1.41 ± 0.43 versus 1.35 ± 0.43). (**B**) Correlation between BDNF levels in blood and neocortical 5-HT$_{2A}$ binding. Although there was a modest significant positive correlation ($P = 0.03$), this was no longer present after adjustments for age and BMI. BDNF, brain-derived neurotrophic factor; BMI, body mass index; 5-HT$_{2A}$, 5-hydroxytryptamine 2A.

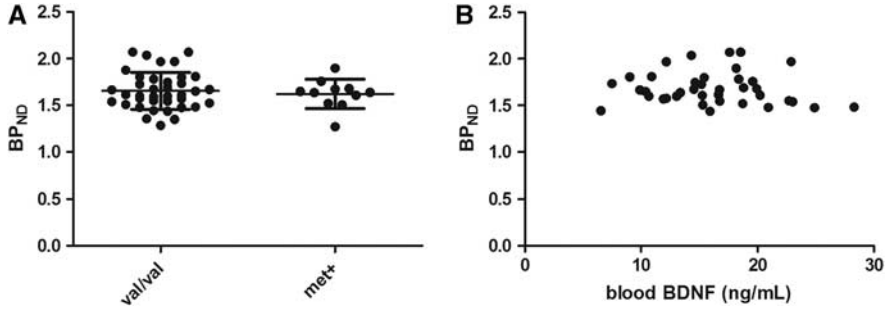

Figure 2 Mean SERT binding in the subcortical high-binding region in val/val and met carriers. (**A**) There were no significant differences in SERT binding between val/val and met carriers (1.66 ± 0.20 versus 1.62 ± 0.16, respectively, $P = 0.61$). (**B**) Correlations between blood BDNF levels and SERT binding. There were no significant associations between these parameters ($r = -0.09$, $P = 0.59$). BDNF, brain-derived neurotrophic factor; SERT, serotonin transporter.

$P = 0.56$) (Figure 1A). There was a tendency toward a positive correlation between BDNF blood levels and neocortical 5-HT$_{2A}$ receptor BP$_P$, but this was no longer evident after adjustment for age and body mass index in a multiple regression analysis (Figure 1B).

No differences in mean subcortical SERT binding were found between val/val and met carriers (1.66 ± 0.20 versus 1.62 ± 0.16, respectively, $P = 0.61$) (Figure 2A). For SERT binding, we further divided our val/val and met carriers groups in a gender-specific manner. This stratification did not reveal any effects of val66met on SERT binding in either men (1.67 ± 0.21 versus 1.65 ± 0.14, $P = 0.82$) or women (1.62 ± 0.17 versus 1.59 ± 0.19, $P = 0.74$). Moreover, we looked for an association between BDNF levels in blood and SERT binding in the

investigated subcortical high-binding region, but no significant correlations were observed ($r=-0.09$, $P=0.59$) (Figure 2B).

We controlled for differences between groups regarding age (Adams *et al*, 2004), body mass index (Erritzoe *et al*, 2009), and neuroticism (Frokjaer *et al*, 2008), both of which factors have earlier been shown to correlate with cerebral 5-HT_{2A} binding, but the val66met status was not associated with any of these (Table 1). In addition, there was no correlation between total BDNF levels in blood and the neuroticism score ($r=0.05$, $P=0.63$), nor was there any genotype-associated differences in the free fraction of the parent radiotracer, f_p (0.47 ± 0.42 versus 0.40 ± 0.17, respectively, $P=0.13$). The distribution of genotype groups scanned with either the 1.5 T or the 3 T was comparable (46 (1.5 T) versus 44 (3 T) for val/val and 23 (1.5 T) and 30 (3 T) for met carriers), excluding possible confounding effects related to differences in the MR scanner type.

To exclude possible confounders affecting SERT binding in the subcortical high-binding region (such as the caudate, putamen, and thalamus), we controlled for differences between groups regarding daylight minutes and openness, which was recently shown to be associated with SERT binding (Kalbitzer *et al*, 2009, 2010). No genotype-associated differences were found for these parameters (Table 2). All [^{11}C]DASB scanned subjects for SERT were MR scanned using the 3-T Trio MR scanner type.

Discussion

To the best of our knowledge, no studies have investigated the effect of val66met on 5-HT_{2A} binding. This is the first study examining the associations in healthy individuals between the val66met *BDNF* polymorphism carrier status, BDNF levels, and cerebral 5-HT_{2A} receptor and SERT binding. In contrast to our hypothesis, we could not observe any statistically significant association between any of these variables.

The close interaction between BDNF and the 5-HT_{2A} receptor is illustrated by a pronounced effect on the cortical 5-HT_{2A} receptor levels in conditional BDNF knockout mice during embryonic development and the first postnatal weeks (Chan *et al*, 2006; Rios *et al*, 2006). Furthermore, *in vitro* studies have shown a direct regulatory effect of BDNF on 5-HT_{2A} receptor levels (Trajkovska *et al*, 2009). In this study, we report that there is no interaction between val66met status and neocortical 5-HT_{2A} binding, indicating that the receptor regulations seen in animal studies are independent of activity-induced BDNF secretion, but may depend on total BDNF levels. To investigate this further, we examined the association between BDNF levels in blood and neocortical 5-HT_{2A} receptor binding. We found a tendency toward a positive correlation showing the association of low BDNF→low cortical 5-HT_{2A} as observed in animal studies (Rios *et al*, 2006). However, after adjusting the 5-HT_{2A} binding for age, neuroticism, and body mass index, this tendency was no longer evident, which illustrates the impact of covariates on measured parameters, in this case the neocortical 5-HT_{2A} binding.

One other study has investigated cerebral 5-HT_{1A} receptor and SERT binding and the BDNF val66met polymorphism in 53 and 25 healthy individuals, respectively (Henningsson *et al*, 2009). They found that whereas BDNF genotypes did not affect 5-HT_{1A} receptor binding in the entire population, male val/val carriers ($N=16$) had higher cerebral SERT binding than did met carriers. In our study of a two-fold larger group of males ($N=33$, ^{11}C-DASB-PET), we did not see the gender-dependent allelic differences. The reason for this discrepancy is not clear, which is why additional studies on the val66met–SERT association are required.

We did not see any association between serum BDNF levels and SERT binding, although experimental studies have shown an interaction between BDNF and SERT. However, a decrease in SERT efficiency in heterozygous BDNF knockout mice without concomitant changes in SERT binding has been reported (Daws *et al*, 2007). This may suggest a role of BDNF levels and val66met on SERT function rather than SERT expression. Alternatively, the BDNF–SERT interaction may be different if BDNF levels are decreased, e.g., during extended periods of major depression (Brunoni *et al*, 2008). Indeed, an investigation of SERT and val66met interaction in patients with major depression could be an interesting area of future research.

Decreased BDNF levels have also been associated with high neuroticism score in healthy subjects (Lang *et al*, 2004). We were unable to replicate this finding in our study. One explanation for this discrepancy could be that Lang *et al* (2004) measured BDNF in serum samples, whereas we measured BDNF in whole blood. However, we have previously shown that serum and whole-blood BDNF values correlate if storage time of the serum samples is <12 months, and that BDNF in whole blood is better preserved during storage (Trajkovska *et al*, 2007). Moreover, the carrier status of the BDNF val66met polymorphism in our cohort was not associated with the neuroticism score. This result is in accordance with results from a large meta-analysis (Terracciano *et al*, 2010) and suggests that changes in activity-dependent BDNF secretion in the brain is not linked to risk factors for developing depression *per se*, but rather to the ability of coping with external effectors, such as stressful life events. For example, in mice carrying the met allele of the val66met polymorphism, there were no changes in baseline stress levels, but the mice were severely affected when exposed to chronic social defeat (Krishnan *et al*, 2007). Similarly, in a study including human subjects, there was no detected interaction between val66met and early life stress predicted brain and arousal

pathways related to depression and anxiety response (Gatt *et al*, 2009). Unfortunately, in our cohort, we did not have access to this information, but in a previous study we showed a correlation between recent stressful life events and BDNF blood levels in individuals at high familial risk for depression (Trajkovska *et al*, 2008). Taken together, these studies suggest that the val66met polymorphism primarily gets manifested during processes that require an increase in BDNF secretion. Therefore, it could be speculated that other factors, such as stressful life events, should be taken into consideration when assessing effects of the val66met polymorphism.

In conclusion, we did not find evidence for an interaction between the val66met polymorphism in the BDNF gene, 5-HT_{2A} receptor binding, and SERT binding in healthy subjects or between total BDNF levels and 5-HT_{2A} receptor binding, even when gender-specific analyses were included. We suggest that the val66met polymorphism has a minor impact on the serotonin system in healthy subjects, but has a more significant role when the brain is exposed to stress or when val66met is present together with other polymorphisms in SERT or in serotonin receptors.

Disclosure/conflict of interest

The authors declare no conflict of interest.

References

Adams KH, Pinborg LH, Svarer C, Hasselbalch SG, Holm S, Haugbol S, Madsen K, Frokjaer V, Martiny L, Paulson OB, Knudsen GM (2004) A database of [(18)F]-altanserin binding to 5-HT(2A) receptors in normal volunteers: normative data and relationship to physiological and demographic variables. *Neuroimage* 21:1105–13

Brunoni AR, Lopes M, Fregni F (2008) A systematic review and meta-analysis of clinical studies on major depression and BDNF levels: implications for the role of neuroplasticity in depression. *Int J Neuropsychopharmacol* 11:1169–80

Chan JP, Unger TJ, Byrnes J, Rios M (2006) Examination of behavioral deficits triggered by targeting Bdnf in fetal or postnatal brains of mice. *Neuroscience* 142:49–58

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

Costa PT, McCrae RR (1992) Revised NEO Personality Inventory an NEO Five Factor Inventory, Professional Manual, Psychological Assessment Resources, Odessa, FL

Daws LC, Munn JL, Valdez MF, Frosto-Burke T, Hensler JG (2007) Serotonin transporter function, but not expression, is dependent on brain-derived neurotrophic factor (BDNF): *in vivo* studies in BDNF-deficient mice. *J Neurochem* 101:641–51

Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Lu B, Weinberger DR (2003) The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell* 112:257–69

Erritzoe D, Frokjaer VG, Haugbol S, Marner L, Svarer C, Holst K, Baare WF, Rasmussen PM, Madsen J, Paulson OB, Knudsen GM (2009) Brain serotonin 2A receptor binding: relations to body mass index, tobacco and alcohol use. *Neuroimage* 46:23–30

Frokjaer VG, Mortensen EL, Nielsen FA, Haugbol S, Pinborg LH, Adams KH, Svarer C, Hasselbalch SG, Holm S, Paulson OB, Knudsen GM (2008) Frontolimbic serotonin 2A receptor binding in healthy subjects is associated with personality risk factors for affective disorder. *Biol Psychiatry* 63:569–76

Frokjaer VG, Vinberg M, Erritzoe D, Baare W, Holst KK, Mortensen EL, Arfan H, Madsen J, Jernigan TL, Kessing LV, Knudsen GM (2010) Familial risk for mood disorder and the personality risk factor, neuroticism, interact in their association with frontolimbic serotonin 2A receptor binding. *Neuropsychopharmacology* 35:1129–37

Frokjaer VG, Vinberg M, Erritzoe D, Svarer C, Baare W, Budtz-Joergensen E, Madsen K, Madsen J, Kessing LV, Knudsen GM (2009) High familial risk for mood disorder is associated with low dorsolateral prefrontal cortex serotonin transporter binding. *Neuroimage* 46:360–6

Gatt JM, Nemeroff CB, Dobson-Stone C, Paul RH, Bryant RA, Schofield PR, Gordon E, Kemp AH, Williams LM (2009) Interactions between BDNF Val66Met polymorphism and early life stress predict brain and arousal pathways to syndromal depression and anxiety. *Mol Psychiatry* 14:681–95

Guiard BP, David DJ, Deltheil T, Chenu F, Le ME, Renoir T, Leroux-Nicollet I, Sokoloff P, Lanfumey L, Hamon M, Andrews AM, Hen R, Gardier AM (2008) Brain-derived neurotrophic factor-deficient mice exhibit a hippocampal hyperserotonergic phenotype. *Int J Neuropsychopharmacol* 11:79–92

Hariri AR, Goldberg TE, Mattay VS, Kolachana BS, Callicott JH, Egan MF, Weinberger DR (2003) Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance. *J Neurosci* 23: 6690–4

Henningsson S, Borg J, Lundberg J, Bah J, Lindstrom M, Ryding E, Jovanovic H, Saijo T, Inoue M, Rosen I, Traskman-Bendz L, Farde L, Eriksson E (2009) Genetic variation in brain-derived neurotrophic factor is associated with serotonin transporter but not serotonin-1A receptor availability in men. *Biol Psychiatry* 66:477–85

Hensler JG, Advani T, Monteggia LM (2007) Regulation of serotonin-1A receptor function in inducible brain-derived neurotrophic factor knockout mice after administration of corticosterone. *Biol Psychiatry* 62:521–9

Hwang JP, Tsai SJ, Hong CJ, Yang CH, Lirng JF, Yang YM (2006) The Val66Met polymorphism of the brain-derived neurotrophic-factor gene is associated with geriatric depression. *Neurobiol Aging* 27:1834–7

Kalbitzer J, Erritzoe D, Holst KK, Nielsen FA, Marner L, Lehel S, Arentzen T, Jernigan TL, Knudsen GM (2010) Seasonal changes in brain serotonin transporter binding in short serotonin transporter linked polymorphic region-allele carriers but not in long-allele homozygotes. *Biol Psychiatry* 67:1033–9

Kalbitzer J, Frokjaer VG, Erritzoe D, Svarer C, Cumming P, Nielsen FA, Hashemi SH, Baare WF, Madsen J, Hasselbalch SG, Kringelbach ML, Mortensen EL, Knudsen GM

(2009) The personality trait openness is related to cerebral 5-HTT levels. *Neuroimage* 45:280–5

Karege F, Bondolfi G, Gervasoni N, Schwald M, Aubry JM, 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

Kerschensteiner M, Gallmeier E, Behrens L, Leal VV, Misgeld T, Klinkert WE, Kolbeck R, Hoppe E, Oropeza-Wekerle RL, Bartke I, Stadelmann C, Lassmann H, Wekerle H, Hohlfeld R (1999) Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor *in vitro* and in inflammatory brain lesions: a neuroprotective role of inflammation? *J Exp Med* 189:865–70

Krishnan V, Han MH, Graham DL, Berton O, Renthal W, Russo SJ, Laplant Q, Graham A, Lutter M, Lagace DC, Ghose S, Reister R, Tannous P, Green TA, Neve RL, Chakravarty S, Kumar A, Eisch AJ, Self DW, Lee FS, Tamminga CA, Cooper DC, Gershenfeld HK, Nestler EJ (2007) Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* 131:391–404

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

Leventhal C, Rafii S, Rafii D, Shahar A, Goldman SA (1999) Endothelial trophic support of neuronal production and recruitment from the adult mammalian subependyma. *Mol Cell Neurosci* 13:450–64

Mattson MP, Maudsley S, Martin B (2004) BDNF and 5-HT: a dynamic duo in age-related neuronal plasticity and neurodegenerative disorders. *Trends Neurosci* 27:589–94

Messaoudi E, Bardsen K, Srebro B, Bramham CR (1998) Acute intrahippocampal infusion of BDNF induces lasting potentiation of synaptic transmission in the rat dentate gyrus. *J Neurophysiol* 79:496–9

Mousavi K, Jasmin BJ (2006) BDNF is expressed in skeletal muscle satellite cells and inhibits myogenic differentiation. *J Neurosci* 26:5739–49

Pezawas L, Verchinski BA, Mattay VS, Callicott JH, Kolachana BS, Straub RE, Egan MF, Meyer-Lindenberg A, Weinberger DR (2004) The brain-derived neurotrophic factor val66met polymorphism and variation in human cortical morphology. *J Neurosci* 24:10099–102

Pinborg LH, Adams KH, Svarer C, Holm S, Hasselbalch SG, Haugbol S, Madsen J, Knudsen GM (2003) Quantification of 5-HT2A receptors in the human brain using [18F]altanserin-PET and the bolus/infusion approach. *J Cereb Blood Flow Metab* 23:985–96

Quarantelli M, Berkouk K, Prinster A, Landeau B, Svarer C, Balkay L, Alfano B, Brunetti A, Baron JC, Salvatore M (2004) Integrated software for the analysis of brain PET/SPECT studies with partial-volume-effect correction. *J Nucl Med* 45:192–201

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

Shimizu E, Hashimoto K, Iyo M (2004) Ethnic difference of the BDNF 196G/A (val66met) polymorphism frequencies: the possibility to explain ethnic mental traits. *Am J Med Genet B Neuropsychiatr Genet* 126B:122–3

Svarer C, Madsen K, Hasselbalch SG, Pinborg LH, Haugbol S, Frokjaer VG, Holm S, Paulson OB, Knudsen GM (2005) MR-based automatic delineation of volumes of interest in human brain PET images using probability maps. *Neuroimage* 24:969–79

Terracciano A, Tanaka T, Sutin AR, Deiana B, Balaci L, Sanna S, Olla N, Maschio A, Uda M, Ferrucci L, Schlessinger D, Costa Jr PT (2010) BDNF Val66Met is associated with introversion and interacts with 5-HTTLPR to influence neuroticism. *Neuropsychopharmacology* 35:1083–9

Trajkovska V, Marcussen AB, Vinberg M, Hartvig P, Aznar S, Knudsen GM (2007) Measurements of brain-derived neurotrophic factor: methodological aspects and demographical data. *Brain Res Bull* 73:143–9

Trajkovska V, Santini MA, Marcussen AB, Thomsen MS, Hansen HH, Mikkelsen JD, Arneberg L, Kokaia M, Knudsen GM, Aznar S (2009) BDNF downregulates 5-HT(2A) receptor protein levels in hippocampal cultures. *Neurochem Int* 55:697–702

Trajkovska V, Vinberg M, Aznar S, Knudsen GM, Kessing LV (2008) Whole blood BDNF levels in healthy twins discordant for affective disorder: association to life events and neuroticism. *J Affect Disord* 108:165–9

Waterhouse EG, Xu B (2009) New insights into the role of brain-derived neurotrophic factor in synaptic plasticity. *Mol Cell Neurosci* 42:81–9

Paper III

International Journal of Neuropsychopharmacology, Page 1 of 7. Copyright © CINP 2010
doi:10.1017/S1461145710000738

ARTICLE

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Blood BDNF concentrations reflect brain-tissue BDNF levels across species

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Abstract

Brain-derived neurotrophic factor (BDNF) is involved in synaptic plasticity, neuronal differentiation and survival of neurons. Observations of decreased serum BDNF levels in patients with neuropsychiatric disorders have highlighted the potential of BDNF as a biomarker, but so far there have been no studies directly comparing blood BDNF levels to brain BDNF levels in different species. We examined blood, serum, plasma and brain-tissue BDNF levels in three different mammalian species: rat, pig, and mouse, using an ELISA method. As a control, we included an analysis of blood and brain tissue from conditional BDNF knockout mice and their wild-type littermates. Whereas BDNF could readily be measured in rat blood, plasma and brain tissue, it was undetectable in mouse blood. In pigs, whole-blood levels of BDNF could not be measured with a commercially available ELISA kit, but pig plasma BDNF levels (mean 994 ± 186 pg/ml) were comparable to previously reported values in humans. We demonstrated positive correlations between whole-blood BDNF levels and hippocampal BDNF levels in rats ($r^2 = 0.44$, $p = 0.025$) and between plasma BDNF and hippocampal BDNF in pigs ($r^2 = 0.41$, $p = 0.025$). Moreover, we found a significant positive correlation between frontal cortex and hippocampal BDNF levels in mice ($r^2 = 0.81$, $p = 0.0139$). Our data support the view that measures of blood and plasma BDNF levels reflect brain-tissue BDNF levels.

Received 18 February 2010; Reviewed 8 April 2010; Revised 12 April 2010; Accepted 28 May 2010

Key words: BDNF, blood, correlation, mouse, rat.

Introduction

Brain-derived neurotrophic factor (BDNF) is the most abundant neurotrophin in the brain and essential for neuronal survival during development and for integration of neurons in the adult brain (Waterhouse & Xu, 2009). BDNF is also expressed in liver (Cassiman *et al.* 2001), skeletal muscle (Matthews *et al.* 2009) and is pivotal for normal development of the cardiovascular system (Donovan *et al.* 2000). In blood, BDNF is mainly stored in thrombocytes, with only a minor free fraction in plasma (Fujimura *et al.* 2002). Whole-blood, serum and plasma BDNF protein levels can be determined with commercially available ELISA kits; these kits can also reliably be used to measure CSF and brain-tissue levels (Aznar *et al.* 2010; Laske *et al.* 2007; Trajkovska *et al.* 2007).

A large number of studies have examined and reported blood BDNF levels in patients with neurodegenerative and neuropsychiatric disorders, including major depression (reviewed in Alleva & Francia, 2009; Zuccato & Cattaneo, 2009; Martinowich *et al.* 2007, respectively). However, it is uncertain whether BDNF levels measured in blood reflect BDNF brain levels across species since BDNF is also produced in the periphery, although there is some evidence that BDNF crosses the blood–brain barrier in mice (Pan *et al.* 1998). Until now, only three studies have directly compared serum BDNF levels with brain-tissue levels of BDNF in rat brain tissue. In the first study, the association was only evident in young rats (Karege *et al.* 2002) while in a more recent study, it was reported that serum BDNF also correlated with brain-tissue

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BDNF in adult animals (Sartorius *et al.* 2009). In contrast, Elfving and co-workers looked for an association in Flinders rats, but found a negative correlation between BDNF in blood and hippocampus (Elfving *et al.* 2009). To our knowledge, there are no studies available that have investigated this relationship in species other than rats. Intriguingly, even though BDNF shows a high-degree of homology between species, it was observed that BDNF was undetectable in mouse blood, despite comparable brain-tissue levels of BDNF in rat and mouse (Radka *et al.* 1996). Thus, some discrepancies between species in BDNF detection and/or transport between the different compartments may exist.

There is a growing interest for incuding the pig (*Sus scrofa*) in neurobiological research due to the vast anatomical and genetic similarities between human and porcine neurobiology; furthermore, pigs are increasingly used to model human brain disorders (Lind *et al.* 2007). Contrary to the lisencephalic brain of rodents, the pig brain has sulci and gyri providing a structural organization which is more comparable to what is found in the primate brain. To our knowledge, no studies have measured BDNF in pig blood and only a few studies have investigated BDNF protein expression in pigs (e.g. Peiris *et al.* 2004). Moreover, in the present study, we included data from the pig to detect possible differences between rodents and other mammals.

Here, we investigated BDNF levels in the blood and brain in three different mammalian species to decide how accurately BDNF in the blood reflects BDNF expression in brain tissue.

Material and methods

Animals

Mice (NMRI, males, aged 8 wk) and rats (Sprague–Dawley, aged 8–12 wk) were housed 5–6 and 2 per cage, respectively, and maintained under a 12-h light/dark cycle (lights on 07:00 hours) with food and water available *ad libitum*. Following acclimatization for 1 wk before the start of the experiments, animals were euthanized by cervical dislocation (mice) or decapitation (rats), trunk blood was collected and the frontal cortex and hippocampus were dissected on a plate cooled with dry ice and immediately frozen at $-80\ ^{\circ}C$.

Pigs (Danish Landrace, females, aged 8–12 wk) were housed in pairs under standard conditions and were allowed to acclimatize for 1 wk before the start of experiments. Blood samples were taken after anaesthesia with 0.1 ml/kg i.m. injections of Zoletil veterinary mixture, and plasma was obtained and stored at $-20\ ^{\circ}C$. Following an injection of sodium pentobarbital (2000 mg), the animals were quickly decapitated, and the brain was removed for immediate dissection. Representative pieces from frontal cortex and hippocampus were obtained and immediately frozen at $-80\ ^{\circ}C$.

$BDNF^{2L/2LCk\text{-}Cre}$ mutants were generated as previously described (Rios *et al.* 2001). Animals were of a mixed C57Bl6 and 129 background and wild-type littermates were used as controls to avoid problems interpreting the data due to differences in background. Animals were aged 10–14 wk and individually housed in the Tufts University Behavioral Core Facility, habituated to a reversed 12-h light/dark cycle for a minimum of 1 wk with free access to water and standard chow.

All experimental procedures were approved by the Danish Animal Experiments Inspectorate and the experiments involving conditional BDNF knockout (cBDNF KO) mice were approved by the Institutional Animal Care and Use Committee at Tufts University and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Tissue and blood

Tissue extracts were homogenized in RIPA buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1% NP-40, 1% DOC, 0.1% SDS, protease inhibitor cocktail, 2 mm sodium orthovanadate, all reagents from Sigma, Denmark], sonicated for 5×6 s on ice followed by centrifugation in a cooled centrifuge (4 °C, 10000 *g* for 10 min). The supernatant was stored at $-80\ ^{\circ}C$ until further processing. The protein concentration was measured with the modified Lowry method (DC Protein Assay, Bio-Rad Laboratories, Denmark). Blood samples were collected in K_2EDTA-coated containers and either immediately frozen (whole blood) or centrifuged for 15 min at 3000 *g* to obtain plasma. For serum preparation, the blood was collected in additive-free containers and left to coagulate for 1 h. The lysation of whole-blood samples was performed as previously described (Trajkovska *et al.* 2007).

BDNF ELISA

Brain, blood and plasma samples were assayed for BDNF levels with two different immunoassay kits. For mouse samples we used a commercially available sandwich ELISA kit from Promega (Sweden) according to the manufacturer's instructions. In brief, 96-well Nunc-Immuno™ MaxiSorp™ plates (Denmark) were coated with anti-BDNF monoclonal antibody and

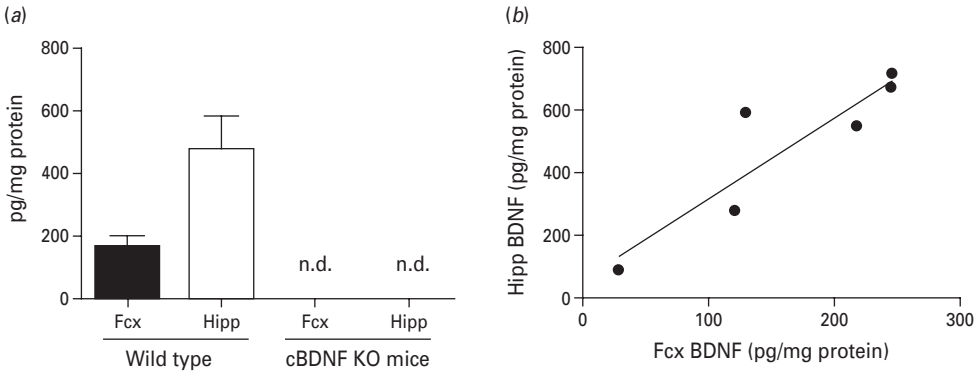

Fig. 1. (*a*) Brain-derived neurotrophic factor (BDNF) levels in the frontal cortex (Fcx) and hippocampus (Hipp) from wild-type and conditional BDNF knockout (cBDNF KO) mice, and (*b*) a correlation analysis of the levels in frontal cortex and hippocampus. BDNF levels were within the detection range in wild-type mice being ~3-fold higher in hippocampus than frontal cortex. In cBDNF KO mice, which are centrally depleted of BDNF, BDNF levels were not detectable in either the frontal cortex or hippocampus. There was a significant correlation between BDNF levels in the frontal cortex and hippocampus ($p = 0.0139$, $r^2 = 0.81$, Pearson's correlation test, $n = 6$). n.d., Non-detectable. Error bars indicate S.E.M.

incubated overnight at 4 °C. The plate was washed with TBS-T (Tris-buffered saline containing 0.1% Tween 20) (Sigma) and incubated with block and sample buffer at room temperature (RT) for 1 h before being washed again. All dilutions of samples and standard curve were made using block and sample buffer. Diluted samples and standards were added in duplicate. The plate was incubated with standards and samples for 2 h at RT, then washed and incubated with anti-human BDNF polyclonal antibody. After 2 h, the plate was washed and incubated with anti-IgY antibody conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. Finally, TMB (3,3′,5,5′-tetramethylebenzidine) and hydrogen peroxide solution was added to the plate and after 10 min, the reaction was stopped with 1 M HCl and the absorbance was immediately measured on an ELISA reader using a 450-nm filter (MicroPlate Reader 550, Bio-Rad Laboratories, Denmark).

For rat and pig samples we used a commercially available sandwich ELISA kit thoroughly described and validated in one of our previous studies (Trajkovska *et al.* 2007). Briefly, 100 µl of BDNF standards (7.82–500 pg/ml recombinant human BDNF) were applied in duplicate to the rabbit anti-human BDNF pre-coated 96-well plates. The samples were diluted (1:220) and 100 µl was added to the plate in duplicate. The plate was covered and incubated overnight at 4 °C. Subsequently, the plate was washed for 4 × 2 min in wash-buffer, incubated with another biotinylated primary antiserum for 3 h, washed, incubated with a streptavidin-HRP complex for 1 h and developed in a solution of TMB as described above. Samples and standards were run in duplicate and BDNF concentrations were calculated using the standard curve and the content was expressed as equivalent of human recombinant BDNF protein in the standards. The BDNF standards contain BDNF concentrations within the range 7.82–500 pg/ml. However, to obtain valid measurements all samples were diluted at least 1:2 in sample buffer resulting in a detection limit of ~16 pg/ml.

Statistical analyses

The correlation analyses were performed using Pearson's correlation. The level of statistical significance was set to $p < 0.05$. GraphPad Prism 5 (GraphPad software, USA) was used for statistical and graphical presentations.

Results

Validation of ELISA BDNF measurements

To ensure specificity of the ELISA method used in the present study, we obtained tissue samples from cBDNF KO mice to validate that the ELISA method can be used for detection of BDNF without cross-reactivity with related proteins. This is particularly important when measuring BDNF in brain tissue where the other neurotrophins are present as well. Unmistakably, we showed that while BDNF is detectable in the frontal cortex and hippocampus in wild-type mice, brain BDNF levels in KO mice were undetectable (Fig. 1*a*). Additionally, we found a significant correlation between BDNF levels in the frontal

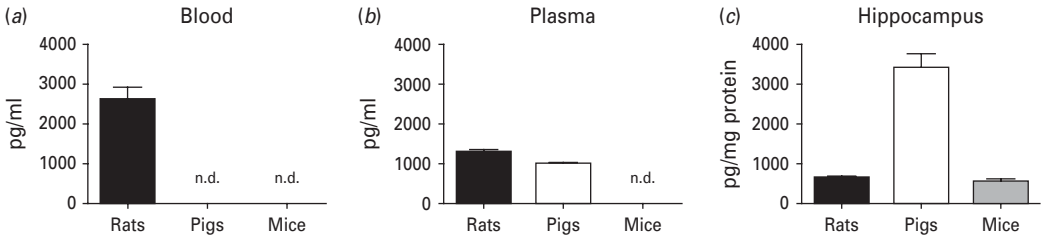

Fig. 2. Brain-derived neurotrophic factor (BDNF) levels in (*a*) blood, (*b*) plasma and (*c*) hippocampus in rats, pigs and mice. (*a*) BDNF levels in whole blood could be measured in rats, but were undetectable in mice and pigs. (*b*) In plasma, we were able to measure BDNF in rats and pigs, but not in mice. (*c*) In hippocampus, pigs showed 4- to 5-fold higher BDNF concentrations than rats and mice. n.d., Non-detectable. Error bars indicate S.E.M.

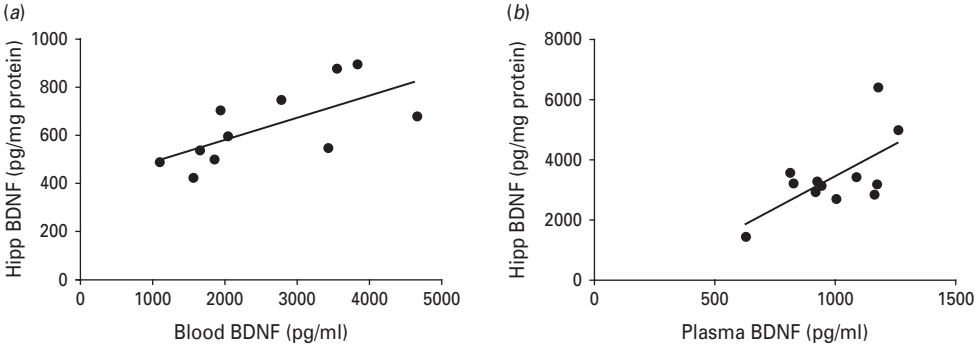

Fig. 3. Brain-derived neurotrophic factor (BDNF) levels in hippocampus (Hipp) *vs*. blood and plasma in rats and pigs. (*a*) There was a significant positive correlation between blood BDNF and BDNF in the hippocampus in rats ($p=0.025$, $r^2=0.44$), and (*b*) a significant positive correlation between BDNF levels in plasma and hippocampus in pigs ($p=0.025$, $r^2=0.41$, $n=12$); Pearson's correlation test.

cortex and hippocampus in wild-type mice ($p=0.0139$, $r^2=0.81$, $n=6$) (Fig. 1*b*). These findings support the use of sandwich ELISA to measure BDNF protein levels. Furthermore, it shows that cross-reactivity with other neurotrophins is negligible.

BDNF measurements in rats, pigs and mice

Using the ELISA kit from Millipore (USA) (rats and pigs) and Promega (rats, pigs, mice), BDNF levels could be measured in whole-blood samples from rats, but not in whole blood from mice and pigs (Fig. 2*a*). In plasma, we were able to measure BDNF in rats and pigs, but not in mice (Fig. 2*b*). In the hippocampus, a high-expression region for BDNF, BDNF was measurable in all three species when using the Millipore ELISA kit for rat and pig samples and the Promega ELISA kit for mouse samples. The hippocampal BDNF levels in rats and mice were comparable, while hippocampal BDNF levels in pigs were 5-fold higher (636.1 ± 158, 559.9 ± 171.2 and 3415 ± 1228 pg BDNF/mg total protein for rats, mice and pigs, respectively) (Fig. 2*c*).

Correlation of BDNF in blood and brain in rats and pigs

A correlation analysis of BDNF levels in blood and hippocampal tissue showed a significant positive correlation between BDNF in rat blood and BDNF in rat hippocampal brain tissue ($p=0.025$, $r^2=0.44$, $n=11$) (Fig. 3*a*). In pigs, we found a significant correlation between plasma BDNF levels and hippocampal BDNF levels ($p=0.025$, $r^2=0.41$, $n=12$) (Fig. 3*b*). Because BDNF is undetectable in mouse blood, plasma or serum, a correlation analysis was not obtainable.

Discussion

Our main findings in this study are (1) BDNF levels in blood and plasma correlate with BDNF in hippocampus, underlining the potential for peripheral measures of BDNF as a biomarker, (2) despite high homology in the protein sequence across species, the detection properties of blood BDNF in rats, mice and pigs are different.

The ELISA kits used in this study had previously been evaluated (Elfving *et al.* 2010; Trajkovska *et al.* 2007). In these studies, however, there was no evaluation of the specificity of the ELISA method for brain-tissue BDNF measurements. As stated in Gass & Hellweg (2010) it will be of great importance to obtain an estimation of the cross-reactivity with other neurotrophins, e.g. NGF, NT-3 and NT-4/NT-5. We found in our study that an analysis of tissue obtained from cBDNF KO mice and their wild-type littermates yielded the expected outcomes. In cBDNF KO mice, BDNF is gradually depleted post-natally and absent in adult mice (Rios *et al.* 2001). The signal we detected in tissue samples from the cBDNF KO mice was similar to background measurements, i.e. the absorbance without any added protein. This illustrates that the cross-reactivity with other members in the neurotrophin family in the applied ELISA kits is negligible.

We found a positive correlation between BDNF in hippocampus and BDNF in plasma and blood in pigs and rats, respectively. A correlation between BDNF in brain and BDNF in blood was first shown by Karege *et al.* (2002). In young rats, they found a correlation between BDNF in the cortex and serum, while serum BDNF and tissue levels in adults did not correlate. Recently, in a more elaborate study, a significant correlation between serum BDNF and brain tissue in adult animals was presented, but results from prefrontal cortex and hippocampus were not presented individually (Sartorius *et al.* 2009). The origin of BDNF in blood is not entirely clear, although supposedly the brain is the main contributor to blood BDNF. This was elegantly demonstrated in an exercise study, illustrated by a change in BDNF arterial-to-internal jugular venous difference after a single bout of exercise (Rasmussen *et al.* 2009).

We did not detect significant correlations in either rats or pigs when correlating BDNF in the frontal cortex with BDNF in blood and plasma, respectively. This could either be because the major BDNF output from the brain hypothetically originates from the hippocampus (Yan *et al.* 1997) and is thereby the key determinant for blood BDNF levels. However, it can also be due to intercortical differences in BDNF output leaving dissections from frontal cortex misleading for the whole cortex. Another more plausible explanation may be that BDNF is more difficult to extract from the frontal cortex and therefore the total yield is subject not only to a bias but also to higher variation. Elfving *et al.* (2010) showed that the extraction efficiency of BDNF after spiking the homogenates was $\sim$30% for frontal cortex, but $\sim$70% for hippocampus. On the other hand, the data from the present study in mice tissue showed a highly significant correlation between BDNF levels of hippocampus and frontal cortex, suggesting fairly low variation in extraction yield from these two compartments, at least in mice.

Intriguingly, we were unable to detect BDNF in blood from pigs albeit the method utilized has previously been validated for human samples (Trajkovska *et al.* 2007). Indeed, both ELISA kits used in this study were capable of detecting pig BDNF in plasma and brain tissue, with plasma values comparable to the values seen in most human studies (Brunoni *et al.* 2008). To our knowledge, no studies have quantified pig BDNF levels. Apparently, as exemplified in the present study, the transport of BDNF in blood is different across species. It may be speculated that BDNF binds strongly to a transporter protein in pig blood and this binding persists despite the relatively harsh lysis protocol applied to these samples. Another explanation could be non-specific binding of other proteins hindering binding of BDNF to the antibodies in the ELISA. Since this binding is suggested to be much weaker than specific binding of BDNF, it would be washed off during the subsequent comprehensive washing steps (see Methods section) leaving end-point measurements similar to background measurements (see above).

In mice, BDNF levels in blood, serum and plasma were undetectable using different commercially available BDNF ELISA kits. This is puzzling, however, since BDNF levels in brain tissue were easily detectable using the Promega ELISA kit. In a previous study, a similar observation was made using a home-made enzyme-linked immunoassay (EIA) for BDNF measurements (Radka *et al.* 1996). Correspondingly, these authors were unable to detect BDNF in either mouse serum or plasma, but did so readily in rats and humans and in mouse brain. Here, we elaborated on these findings using a well-validated ELISA kit. We analysed whole-blood lysates to get a complete release of BDNF from thrombocytes and we performed measurements on undiluted serum. Nevertheless, we were unable to detect any signal above background measurements in plasma, serum or blood. Apparently, this is not due to lack of BDNF transport capacity as it was shown that mice readily transport iodine-labelled BDNF and recombinant BDNF in blood (Pan *et al.* 1998). It is also possible that BDNF is rapidly cleared from plasma, but this needs to be validated. The important message here is that blood BDNF cannot be measured in mice with the most commonly used commercially available ELISA kit.

In conclusion, we show here that blood BDNF concentrations correlate positively with BDNF levels in

the hippocampus of rats and pigs. These findings further demonstrate the close linkage between blood and brain BDNF levels and heighten the potential of BDNF blood measurements as a predictor of BDNF levels in high-expression brain regions.

Acknowledgements

Astrid Beck, Hans-Jørgen Jensen, Christine B. Janssens and German A. Calderon are acknowledged for valuable technical assistance. This work was supported by the Lundbeck Foundation, the Augustinus Foundation, the EC-FP6-project DiMI, LSHB-CT-2005–512146 and the University of Copenhagen. M. R. was supported by NIH/NIDDK (DK073311) and NIH/NIMH (MH67817) research grants.

Statement of Interest

None.

References

Alleva E, Francia N (2009). Psychiatric vulnerability: suggestions from animal models and role of neurotrophins. *Neuroscience and Biobehavioral Reviews* **33**, 525–536.

Aznar S, Klein AB, Santini MA, Knudsen GM, *et al.* (2010). Aging and depression vulnerability interaction results in decreased serotonin innervation associated with reduced BDNF levels in hippocampus of rats bred for learned helplessness. *Synapse* **64**, 561–565.

Brunoni AR, Lopes M, Fregni F (2008). A systematic review and meta-analysis of clinical studies on major depression and BDNF levels: implications for the role of neuroplasticity in depression. *International Journal of Neuropsychopharmacology* **11**, 1169–1180.

Cassiman D, Denef C, Desmet VJ, Roskams T (2001). Human and rat hepatic stellate cells express neurotrophins and neurotrophin receptors. *Hepatology* **33**, 148–158.

Donovan MJ, Lin MI, Wiegn P, Ringstedt T, *et al.* (2000). Brain derived neurotrophic factor is an endothelial cell survival factor required for intramyocardial vessel stabilization. *Development* **127**, 4531–4540.

Elfving B, Plougmann PH, Muller HK, Mathe AA, *et al.* (2009). Inverse correlation of brain and blood BDNF levels in a genetic rat model of depression. *International Journal of Neuropsychopharmacology* **13**, 563–572.

Elfving B, Plougmann PH, Wegener G (2010). Detection of brain-derived neurotrophic factor (BDNF) in rat blood and brain preparations using ELISA: pitfalls and solutions. *Journal of Neuroscience Methods* **15**, 73–77.

Fujimura H, Altar CA, Chen R, Nakamura T, *et al.* (2002). Brain-derived neurotrophic factor is stored in human platelets and released by agonist stimulation. *Thrombosis and Haemostasis* **87**, 728–734.

Gass P, Hellweg R (2010). Peripheral brain-derived neurotrophic factor (BDNF) as a biomarker for affective disorders? *International Journal of Neuropsychopharmacology* **13**, 1–4.

Karege F, Schwald M, Cisse M (2002). Postnatal developmental profile of brain-derived neurotrophic factor in rat brain and platelets. *Neuroscience Letters* **328**, 261–264.

Laske C, Stransky E, Leyhe T, Eschweiler GW, *et al.* (2007). BDNF serum and CSF concentrations in alzheimer's disease, normal pressure hydrocephalus and healthy controls. *Journal of Psychiatric Research* **41**, 387–394.

Lind NM, Moustgaard A, Jelsing J, Vajta G, *et al.* (2007). The use of pigs in neuroscience: modeling brain disorders. *Neuroscience and Biobehavioral Reviews* **31**, 728–751.

Martinowich K, Manji H, Lu B (2007). New insights into BDNF function in depression and anxiety. *Nature Neuroscience* **10**, 1089–1093.

Matthews VB, Astrom MB, Chan MH, Bruce CR, *et al.* (2009). Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. *Diabetologia* **52**, 1409–1418.

Pan W, Banks WA, Fasold MB, Bluth J, *et al.* (1998). Transport of brain-derived neurotrophic factor across the blood-brain barrier. *Neuropharmacology* **37**, 1553–1561.

Peiris TS, Machaalani R, Waters KA (2004). Brain-derived neurotrophic factor mRNA and protein in the piglet brainstem and effects of intermittent hypercapnic hypoxia. *Brain Research* **1029**, 11–23.

Radka SF, Holst PA, Fritsche M, Altar CA (1996). Presence of brain-derived neurotrophic factor in brain and human and rat but not mouse serum detected by a sensitive and specific immunoassay. *Brain Research* **709**, 122–301.

Rasmussen P, Brassard P, Adser H, Pedersen MV, *et al.* (2009). Evidence for a release of brain-derived neurotrophic factor from the brain during exercise. *Experimental Physiology* **94**, 1062–1069.

Rios M, Fan G, Fekete C, Kelly J, *et al.* (2001). Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. *Molecular Endocrinology* **15**, 1748–1757.

Sartorius A, Hellweg R, Litzke J, Vogt M, *et al.* (2009). Correlations and discrepancies between serum and brain tissue levels of neurotrophins after electroconvulsive treatment in rats. *Pharmacopsychiatry* **42**, 270–276.

Trajkovska V, Marcussen AB, Vinberg M, Hartvig P, *et al.* (2007). Measurements of brain-derived neurotrophic factor: methodological aspects and demographical data. *Brain Research Bulletin* **73**, 143–149.

Waterhouse EG, Xu B (2009). New insights into the role of brain-derived neurotrophic factor in synaptic plasticity. *Molecular and Cellular Neurosciences* **42**, 81–89.

Yan Q, Rosenfeld RD, Matheson CR, Hawkins N, *et al.* (1997). Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. *Neuroscience* **78**, 431–448.

Zuccato C, Cattaneo E (2009). Brain-derived neurotrophic factor in neurodegenerative diseases. *Nature Reviews Neurology* **5**, 311–322.

Paper IV

SYNAPSE 64:561–565 (2010)

Short Communication

Aging and Depression Vulnerability Interaction Results in Decreased Serotonin Innervation Associated With Reduced BDNF Levels in Hippocampus of Rats Bred for Learned Helplessness

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KEY WORDS depression; serotonin; neurotrophins; aging; congenital; stereology

ABSTRACT Epidemiological studies have revealed a strong genetic contribution to the risk for depression. Both reduced hippocampal serotonin neurotransmission and brain-derived neurotrophic factor (BDNF) levels have been associated with increased depression vulnerability and are also regulated during aging. Brains from young (5 months old) and old (13 months old) congenital Learned Helplessness rats (cLH), and congenital Non Learned Helplessness rats (cNLH) were immunohistochemically stained for the serotonin transporter and subsequently stereologically quantified for estimating hippocampal serotonin fiber density. Hippocampal BDNF protein levels were measured by ELISA. An exacerbated age-related loss of serotonin fiber density specific for the CA1 area was observed in the cLH animals, whereas reduced hippocampal BDNF levels were seen in young and old cLH when compared with age-matched cNLH controls. These observations indicate that aging should be taken into account when studying the neurobiological factors behind the vulnerability for depression and that understanding the effect of aging on genetically predisposed individuals may contribute to a better understanding of the pathophysiology behind depression, particularly in the elderly. **Synapse 64:561–565, 2010.**

INTRODUCTION

The involvement of serotonin in depression has been established based on findings from both human (Butler and Meegan, 2008) and animal (Hellweg et al., 2007; Weisstaub et al., 2006) studies. Recent theories, however, have challenged the monoaminergic hypothesis of depression by claiming that diminished neuroplastic adaptation to stress stimuli and structural changes may be the primary triggers of depression (D'sa and Duman, 2002). Brain-derived neurotrophic factor (BDNF) is a key factor in neuroplasticity and has convincingly been demonstrated to be linked with major depression (Gonul et al., 2005; Karege et al., 2005; Pezawas et al., 2008).

Animal models with an inherent vulnerability for developing depression-like behavior are essential for investigating the neurobiology underlying the predisposition to depression. The learned helplessness paradigm initially described by Overmier and Seligman (Overmier and Seligman, 1967) is based on the fact that uncontrollable and unpredictable stress induces learned helplessness behavior in the rat. Some animals are more predisposed to developing learned helplessness than others, thus modeling the human

Contract grant sponsor: Lundbeck Foundation, the Danish Medical Research Council, sawmill owner Jeppe Juhl and Ovita Juhl Memorial Foundation, Augustinus Foundation, Simon Fougner Hartmanns Family Foundation, and the Foundation for Research in Neurology; Contract grant sponsor: Deutsche Forschungsgemeinschaft; Contract grant numbers: SFB636/B3, GA427/9-1.

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Received 20 August 2009; Accepted 8 December 2009

DOI 10.1002/syn.20773

Published online in Wiley InterScience (www.interscience.wiley.com).

susceptibility to depression. Two lines of learned helpless and not helpless rats have been created after selective breeding over >60 generations. Rats susceptible to the effects of uncontrollable stress show congenital learned helplessness (cLH) even without stress exposition, whereas control rats exhibit resistance to learned helplessness (cNLH). Several studies have shown that congenital learned helplessness animals show symptoms of depression-like anhedonia (Vollmayr et al., 2004), and a positive response to 5-HT_{2A} antagonists (Patel et al., 2004) as well as abnormalities in intracellular signaling (Kohen et al., 2003), and regulation of BDNF expression (Vollmayr et al., 2001), indicating that there are substantial congenital differences between the two strains that may account for their susceptibility for depression.

Both serotonin and BDNF levels also are affected by aging in normal individuals (Katoh-Semba et al., 2007; Yamamoto et al., 2002), and it has been known that aging poses a risk factor for depression (Paykel, 1994). Animal studies suggest that there are differences in the neurobiological substrates associated with stress induced depressive behavior in young versus old rats (Huston et al., 2009) involving the hypothalamic-pituitary-adrenal (HPA) axis (Topic et al., 2008b) and BDNF (Topic et al., 2008a). Furthermore, in old rats, the serotonergic system respond differently to experimentally induced stress compared to young rats (Slotkin et al., 1999, 2000, 2005). In a previous study, an age-related exacerbated decrease in serotonin immunopositive fibers was observed in the hippocampus of the Flinders Sensitive Line (Husum et al., 2006), suggesting an interaction between depression vulnerability and aging on the serotonergic system. In this study, we wanted to investigate whether the age-related decrease in hippocampal serotonin fibers also is present in the cLH rats and whether it may be associated with differences in BDNF levels. We made use of serotonin transporter immunodetection for measuring serotonergic innervation as this is a more reliable marker of serotonin fibers than immunodetection of serotonin itself (Nielsen et al., 2006).

LH and NLH strains

Sprague–Dawley rats were tested in the learned helplessness paradigm. Twenty-four hours after a total of 20-min uncontrollable and unpredictable 0.8 mA footshocks, the rats were tested in an escape paradigm where foot shock could be eliminated with a single lever press: animals with more than 10 failures (of 15 trials) were considered as helpless, animals with less than five failures were considered as nonhelpless. Helpless animals and nonhelpless animals, respectively, were mated for subsequent generations avoiding sib crosses and resulting in two selective strains: the cLH, demonstrating helpless behavior without prior inescapable shock, and the congenitally nonhelpless strain (cNLH), resistant to the development of learned helplessness. Rats from the 49th generation were used for the experiments and underwent learned helplessness testing at the age of 9 weeks. Animals were group housed in standard hanging rodent cages (12-h artificial light–dark cycle (lights on at 0600 h) 22°C ± 1°C, humidity: 55% ± 5%). Standard laboratory rat chow and water were provided ad libitum. The animals were treated in accordance with the European Communities Council Directive of November 24, 1986 and German Animal Welfare Act of May 25, 1998 experiments was approved by the Regierungspraesidium Karlsruhe.

Serotonin transporter immunodetection

Either 5 (young group) or 13 months (old group) after testing, animals were perfused intracardially with 150–200 ml of 4% cold paraformaldehyde. Forty-five-micrometer thick free-floating coronal slices were cut in a cryostat and slices immunostained for serotonin transporter (SERT). Briefly, free-floating sections were treated for 5 min with a solution of 10% methanol and 3% H_2O_2. They were subsequently blocked for 1 h at room temperature (RT) in a solution containing 2% goat serum, 2% bovine serum albumin, and 0.2% triton in TBS where after incubated overnight at 4°C with a monoclonal mouse antibody against serotonin transporter (Chemicon, 1:1500). The following day they were incubated for 1 h at RT with a biotin-coupled goat anti-mouse antibody (DAKO; diluted 1:600), washed and again incubated for 1 h at RT with streptavidin coupled to horseradish peroxidase (DAKO; diluted 1:600). Slices were developed with diaminobenzidine for 10 min, mounted on gelatin-coated slides, and covered with xylene-based mounting medium (Pertex©).

Stereological quantification

Every fourth section was systematically sampled from a defined anatomical start-point to a defined end point, corresponding to −1.80 mm and −4.52 mm from Bregma. The sections were analyzed using the Olympus DK CAST system. Counting was done blinded to the observer. The different hippocampal regions were outlined using a 1.5× objective. Counting was performed at a 100× magnification using a grid consisting of randomly angled parallel lines. The step length (x, y) of sampling was set at 250 μm. The counting frame consisted of the total area covered by the 100× oil immersion objective, and the total number of crossings between SERT positive fibers and the line grids were counted on a single plane 5 μm below the section's upper surface. The estimated total number of crossings for each animal was compared between groups.

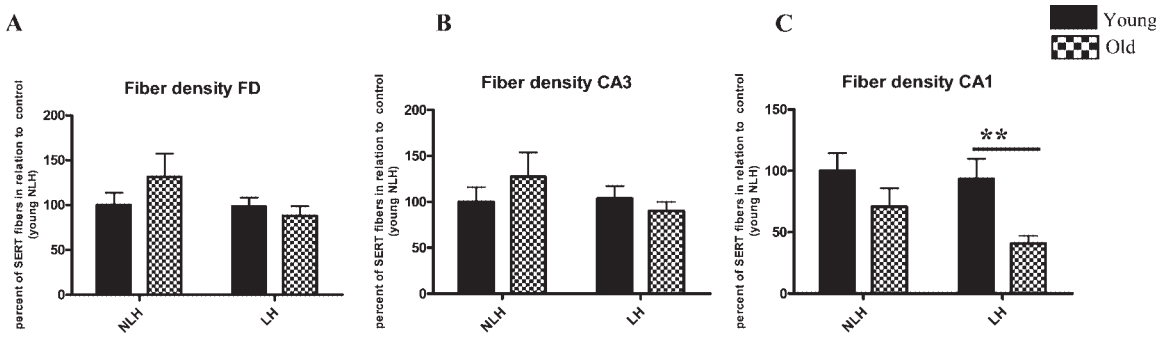

Fig. 1. Density (in percentage from control) of SERT positive fibers in FD (**A**), CA3 (**B**), and CA1 (**C**) hippocampal areas of young and old cLH and cNLH rats. $n = 3$ for adult rats in respectively cLH and cNLH and $n = 5$ for old rats in respectively cLH and cNLH. There was no strain or age effect of SERT fiber density in FD and CA3. In CA1, a Mann–Whitney test showed a significant reduction of SERT positive fibers in old cLH compared to adult cLH ($P < 0.01$).

Hippocampal BDNF ELISA measurement

Hippocampus from young and old cNLH ($n = 8$ and 5) and cLH ($n = 8$ and 6) rats were dissected and homogenized in buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 5 mM EDTA) containing a protease inhibitor cocktail (Sigma, P8340)). Samples were sonicated 6 × 5 s at low intensity, and protein concentration was measured with the modified Lowry method (Bio-Rad, DC Protein Assay). The BDNF content was measured by sandwich ELISA (Millipore, cat. no. CYT306) according to the manufacturer's instructions. Values are presented as pg BDNF per milligram total protein.

GraphPad was used for performing the statistical analysis. Nonparametric Mann–Whitney test for the fiber measurements and two-way ANOVA for the BDNF measurements were used. Differences were considered statistically significant at $P < 0.05$.

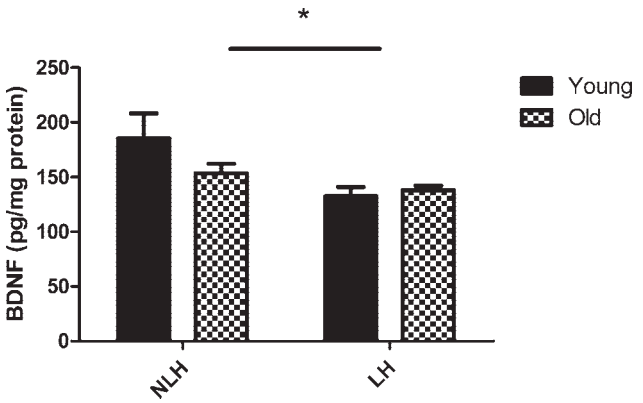

Fig. 2. Hippocampal BDNF levels in young cNLH ($n = 8$), old NLH ($n = 5$), young cLH ($n = 8$), and old cLH ($n = 6$). Two-way ANOVA showed significant lower hippocampal BDNF levels in the cLH group ($P < 0.05$). There was no age effect.

Age-dependent decrease in SERT positive fibers in cLH rats

Stereological estimation showed no significant age effect or differences in density of SERT positive fibers between cLH and cNLH rats in the Fascia Dentada region ($F = 1.111$; $df = 1$; $P = 0.31$) (Fig. 1A) and in the CA3 area ($F = 1.043$; $df = 1$; $P = 0.32$) (Fig. 1B). In the CA1 area, however, two-way ANOVA analysis showed a significant decrease of SERT fiber density in old cLH rats ($F = 0.76$; $df = 1$; $P < 0.01$) (Fig. 1C). The depression resistant cNLH rats, on the contrary, did not show a significant decrease in SERT fiber density in old animals when compared with the young cNLH group.

Lower hippocampal BDNF levels in cLH rats compared to cNLH

Two-way ANOVA analysis showed that cLH rats have significantly lower hippocampal levels of BDNF than the cNLH group ($P < 0.05$). This main effect, though, was independent of age; even though a tendency toward a decrease in BDNF levels was observed in the old group when compared with the young group in the cNLH group (Fig. 2).

Our results show an age-related loss of serotonergic innervation in CA1 hippocampal area in rats bred for learned helplessness. Furthermore, these animals show constitutive lower hippocampal BDNF levels than the depression resistant strain, independent of aging. Notably, these results corroborate previous findings in Flinders Sensitive Line, a different congenital animal model of depression vulnerability (Husum et al., 2006). In the first study, we found an exacerbated age-related loss in serotonin immunopositive fibers specific for the CA1 area in the Flinders Sensitive Line strain when compared with the depression resistant Flinders Resistant Line (Husum et al., 2006). We replicated this observation in this study but here using serotonin transporter immunodetection as a marker for serotonergic innervation, which

is shown to be a more reliable marker of serotonin fibers than immunodetection of serotonin itself (Nielsen et al., 2006). Even though not statistically significant, a tendency toward reduced serotonin positive fibers was observed in the cNLH rats posing the question whether the negative finding is just a matter of low-statistical power. However, other studies looking at age-related differences in hippocampal serotonergic innervation do not see decreases in normal animals up to 18-month of age (Lyons et al., 1999) supporting our observation of no decrease in serotonergic innervation in the 13-month-old cNLH rats.

These two congenital models of depression have been developed based on different grounds: the congenital Learned Helplessness by selective breeding for extreme stress responses (Henn and Vollmayr, 2005) and the Flinders Sensitive Line for increased pharmacologically induced cholinergic sensitivity (Overstreet et al., 2005). In both models, however, alterations in serotonin receptors (Neumaier et al., 2002, 2008; Nishi et al., 2009; Osterlund et al., 1999; Patel et al., 2004) have been described, suggesting that differences in the serotonergic system may mediate the increased depression vulnerability. The fact that we can replicate this initial observation in a different animal model strengthens the idea of an interaction between aging and genetic vulnerability. Contrary to the seronergic innervation, the difference in BDNF levels between the cNLH and cLH was not age-related but a constitutive feature of the vulnerable strain. Transgenic mice with constitutive reductions in BDNF expression show age-accelerated loss of serotonergic innervation particularly in the CA1 region (Luellen et al., 2006; Lyons et al., 1999), supporting that the reduction in BDNF levels in the cLH may be implicated in the age-related fiber loss seen in this strain. Alterations in BDNF expression have been reported previously in the congenital learned helplessness strain (Vollmayr et al., 2001). This alteration however was only manifested under stress conditions, with the cLH not showing the stress-induced reduction of BDNF expression in the hippocampus normally seen in control animals. Under basal conditions, no differences were seen in hippocampal BDNF mRNA levels (Vollmayr et al., 2001). In our study, we looked at protein levels, indicating that the lower BDNF levels observed here probably reflect differences in posttranslational processing or turnover rate in the cLH animals. Reduced levels of BDNF protein are also observed in several brain regions of Flinders Sensitive Line (Angelucci et al., 2000) and, similar to cLH rats, there is an absence of stress-induced regulation of BDNF expression in the hippocampus of Flinders Sensitive Line when compared with Sprague–Dawley and Flinders Resistant Line. We cannot exclude an exacerbated age-dependent decrease in BDNF levels specific for the CA1 area in the cLH rats, which may have been overseen as we only measured BDNF levels in total hippocampal lysates. Unfortunately, measuring BDNF levels in specific hippocampal subregions by ELISA was technically not possible and other detection techniques, like in situ hybridization for measuring mRNA levels, should be used in future studies to clarify this.

The age-related decrease in serotonin transporter immunoreactivity could also be related to impaired glucocorticoid feedback regulation in the old cLH rats. Aging attenuates glucocorticoid negative feedback in the rat brain (Mizoguchi et al., 2009) and interacts with high-glucocorticoid levels in affecting serotonin transporter expression (Fumagalli et al., 1996). Even though no difference in stress-induced glucocorticoid levels has been observed between young cLH and cLNH (Vollmayr et al., 2001), alterations in glucocorticoid-mediated gene expression have been associated with the learned helplessness behavior (Lachman et al., 1993). Thus, the cLH may have an inherent dysfunction of the HPA axis that may get exacerbated by aging resulting in a faster decrease in serotonin transporter expression. The lower hippocampal BDNF levels in the cLH further supports this idea as alterations in the HPA-axis affect BDNF levels (Molteni et al., 2009; Ridder et al., 2005). It would be interesting to see whether the decrease in serotonin fiber density is associated with age-related alterations in glucocorticoid receptor levels in hippocampus of cLH.

In conclusion, our results support the existence of an interaction between aging and genetic vulnerability, which is manifested as endophenotypic changes in hippocampal serotonergic innervation in the depression vulnerable individuals. Furthermore, these age-related changes may be associated with an impaired adaptive response of the vulnerable strain to stress factors like aging, presumably due to a more vulnerable HPA axis response, and constitutively lower levels of BDNF. Thus, aging and age-related changes are important factors to take into consideration when looking at the pathophysiology behind depression.

ACKNOWLEDGMENTS

Excellent technical assistance was provided by Anja Pedersen, Inge Møller, and Mette Søgård Hansen. We thank Arne Møller and Lars Christian Rønn for their scientific advice and valuable comments. There are no actual or potential conflicts of interest.

REFERENCES

Angelucci F, Aloe L, Vasquez PJ, Mathe AA. 2000. Mapping the differences in the brain concentration of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in an animal model of depression. Neuroreport 11:1369–1373.

Butler SG, Meegan MJ. 2008. Recent developments in the design of anti-depressive therapies: Targeting the serotonin transporter. Curr Med Chem 15:1737–1761.

D'sa C, Duman RS. 2002. Antidepressants and neuroplasticity. Bipolar Disord 4:183–194.
Fumagalli F, Jones SR, Caron MG, Seidler FJ, Slotkin TA. 1996. Expression of mRNA coding for the serotonin transporter in aged vs. young rat brain: Differential effects of glucocorticoids. Brain Res 719:225–228.
Gonul AS, 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.
Hellweg R, Zueger M, Fink K, Hortnagl H, Gass P. 2007. Olfactory bulbectomy in mice leads to increased BDNF levels and decreased serotonin turnover in depression-related brain areas. Neurobiol Dis 25:1–7.
Henn FA, Vollmayr B. 2005. Stress models of depression: Forming genetically vulnerable strains. Neurosci Biobehav Rev 29:799–804.
Huston JP, Schulz D, Topic B. 2009. Toward an animal model of extinction-induced despair: Focus on aging and physiological indices. J Neural Transm 116:1029–1036.
Husum H, Aznar S, Hoyer-Hansen S, Larsen MH, Mikkelsen JD, Moller A, Mathe AA, Wortwein G. 2006. Exacerbated loss of cell survival, neuropeptide Y-immunoreactive (IR) cells, and serotonin-IR fiber lengths in the dorsal hippocampus of the aged flinders sensitive line "depressed" rat: Implications for the pathophysiology of depression? J Neurosci Res 84:1292–1302.
Karege F, Bondolfi G, Gervasoni N, Schwald M, Aubry JM, 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.
Katoh-Semba R, Wakako R, Komori T, Shigemi H, Miyazaki N, Ito H, Kumagai T, Tsuzuki M, Shigemi K, Yoshida F, Nakayama A. 2007. Age-related changes in BDNF protein levels in human serum: Differences between autism cases and normal controls. Int J Dev Neurosci 25:367–372.
Kohen R, Neumaier JF, Hamblin MW, Edwards E. 2003. Congenitally learned helpless rats show abnormalities in intracellular signaling. Biol Psychiatry 53:520–529.
Lachman HM, Papolos DF, Boyle A, Sheftel G, Juthani M, Edwards E, Henn FA. 1993. Alterations in glucocorticoid inducible RNAs in the limbic system of learned helpless rats. Brain Res 609:110–116.
Luellen BA, Bianco LE, Schneider LM, Andrews AM. 2006. Reduced brain-derived neurotrophic factor is associated with a loss of serotonergic innervation in the hippocampus of aging mice. Genes Brain Behav 6:482–490.
Lyons WE, Mamounas LA, Ricaurte GA, Coppola V, Reid SW, Bora SH, Wihler C, Koliatsos VE, Tessarollo L. 1999. Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. Proc Natl Acad Sci USA 96:15239–15244.
Mizoguchi K, Ikeda R, Shoji H, Tanaka Y, Maruyama W, Tabira T. 2009. Aging attenuates glucocorticoid negative feedback in rat brain. Neuroscience 159:259–270.
Molteni R, Calabrese F, Chourbaji S, Brandwein C, Racagni G, Gass P, Riva M. 2009. Depression-prone mice with reduced glucocorticoid receptor expression display an altered stress-dependent regulation of brain-derived neurotrophic factor and activity-regulated cytoskeleton-associated protein. J Psychopharmacol (Epub ahead of print).
Neumaier JF, Edwards E, Plotsky PM. 2002. 5-HT_{1B} mRNA regulation in two animal models of altered stress reactivity. Biol Psychiatry 51:902–908.
Nielsen K, Brask D, Knudsen GM, Aznar S. 2006. Immunodetection of the serotonin transporter protein is a more valid marker for serotonergic fibers than serotonin. Synapse 59:270–276.
Nishi K, Kanemaru K, Diksic M. 2009. A genetic rat model of depression, Flinders sensitive line, has a lower density of 5-HT_{1A} receptors, but a higher density of 5-HT_{1B} receptors, compared to control rats. Neurochem Int 54:299–307.
Osterlund MK, Overstreet DH, Hurd YL. 1999. The flinders sensitive line rats, a genetic model of depression, show abnormal serotonin receptor mRNA expression in the brain that is reversed by 17β-estradiol. Brain Res Mol Brain Res 74:158–166.
Overmier JB, Seligman ME. 1967. Effects of inescapable shock upon subsequent escape and avoidance responding. J Comp Physiol Psychol 63:28–33.
Overstreet DH, Friedman E, Mathe AA, Yadid G. 2005. The Flinders Sensitive Line rat: A selectively bred putative animal model of depression. Neurosci Biobehav Rev 29:739–759.
Patel JG, Bartoszyk GD, Edwards E, Ashby CR. 2004. The highly selective 5-hydroxytryptamine (5-HT)2A receptor antagonist. EMD 281014, significantly increases swimming and decreases immobility in male congenital learned helpless rats in the forced swim test. Synapse 52:73–75.
Paykel ES. 1994. Life events, social support and depression. Acta Psychiatr Scand Suppl 377:50–58.
Pezawas L, Meyer-Lindenberg A, Goldman AL, Verchinski BA, Chen G, Kolachana BS, Egan MF, Mattay VS, Hariri AR, Weinberger DR. 2008. Evidence of biologic epistasis between BDNF and SLC6A4 and implications for depression. Mol Psychiatry 13:709–716.
Ridder S, Chourbaji S, Hellweg R, Urani A, Zacher C, Schmid W, Zink M, Hortnagl H, Flor H, Henn FA, Schutz G, Gass P. 2005. Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. J Neurosci 25:6243–6250.
Slotkin TA, Miller DB, Fumagalli F, McCook EC, Zhang J, Bissette G, Seidler FJ. 1999. Modeling geriatric depression in animals: Biochemical and behavioral effects of olfactory bulbectomy in young versus aged rats. J Pharmacol Exp Ther 289:334–345.
Slotkin TA, Seidler FJ, Ritchie JC. 2000. Regional differences in brain monoamine oxidase subtypes in an animal model of geriatric depression: Effects of olfactory bulbectomy in young versus aged rats. Brain Res 882:149–154.
Slotkin TA, Cousins MM, Tate CA, Seidler FJ. 2005. Serotonergic cell signaling in an animal model of aging and depression: Olfactory bulbectomy elicits different adaptations in brain regions of young adult vs aging rats. Neuropsychopharmacology 30:52–57.
Topic B, Huston JP, Namestkova K, Zhu SW, Mohammed AH, Schulz D. 2008a. Extinction-induced "despair" in aged and adult rats: Links to neurotrophins in frontal cortex and hippocampus. Neurobiol Learn Mem 90:519–526.
Topic B, Oitzl MS, Meijer OC, Huston JP, de Souza Silva MA. 2008b. Differential susceptibility to extinction-induced despair and age-dependent alterations in the hypothalamic-pituitary-adrenal axis and neurochemical parameters. Neuropsychobiology 58:138–153.
Vollmayr B, Faust H, Lewicka S, Henn FA. 2001. Brain-derived-neurotrophic-factor (BDNF) stress response in rats bred for learned helplessness. Mol Psychiatry 6:471–474.
Vollmayr B, Bachteler D, Vengeliene V, Gass P, Spanagel R, Henn F. 2004. Rats with congenital learned helplessness respond less to sucrose but show no deficits in activity or learning. Behav Brain Res 150:217–221.
Weisstaub NV, Zhou M, Lira A, Lambe E, Gonzalez-Maeso J, Hornung JP, Sibille E, Underwood M, Itohara S, Dauer WT, Ansorge MS, Morelli E, Mann JJ, Toth M, Aghajanian G, Sealfon SC, Hen R, Gingrich JA. 2006. Cortical 5-HT2A receptor signaling modulates anxiety-like behaviors in mice. Science 313:536–540.
Yamamoto M, Suhara T, Okubo Y, Ichimiya T, Sudo Y, Inoue M, Takano A, Yasuno F, Yoshikawa K, Tanada S. 2002. Age-related decline of serotonin transporters in living human brain of healthy males. Life Sci 71:751–757.

Paper V

Increased serum BDNF levels in hypocretin-deficient narcoleptic patients

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Title: 72 characters including spaces and punctuation

Abstract: 225 words

Introduction: 244 words

Main text: 1883

Number of references: 24

Figures: 3

Tables: 1

Search terms: [245] Narcolepsy, neurotrophins, neurodegeneration, neuroregenration

Study Funding: This study was supported by the NOVO Nordisk Foundation and the Lundbeck Foundation.

Statistical Analysis: The analyses were performed by ass.prof., MD DMsc Jens D Mikkelsen

The study was supported by the NOVO Nordisk Foundation and the Lundbeck Foundation.

Mr. Anders Bue Klein reports no disclosures.

Dr. Poul Jennum reports no disclosures.

Dr. Stine Knudsen reports no disclosures.

Dr. Steen Gammeltoft reports no disclosures.

Dr. Jens Damsgaard Mikkelsen reports no disclosures.

Abstract

Background: Several lines of evidence have shown that symptoms of narcolepsy are strongly correlated with a reduction in CSF hypocretin-1 levels and numbers of hypocretin neurons in the hypothalamus in post-mortem tissue. It has recently been proposed that the specific decline in hypocretin is caused by an autoinflammatory mechanism that may lead to a selective destruction of the hypocretin neurons. In case of neuronal death, we would assume that neuronal growth factors are released and may participate in neuronal plasticity processes.

This study was performed to measure serum levels of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in narcolepsy patients with cataplexy and low concentrations of CSF hypocretin-1. Healthy subjects without sleep disturbances were included as controls.

Methods: Blood and CSF samples were collected from patients with narcolepsy and healthy controls. BDNF and NGF levels were measured using ELISA. CSF hypocretin-1 was measured using RIA.

Results: Serum BDNF levels were found to be significant higher in narcolepsy patients than in healthy controls (64.0 ± 3.7 ng/ml in narcolepsy and 46.8 ± 2.6 ng/ml in controls, $P<0.005$). There were no significant changes in NGF levels and no correlation between serum BDNF and CSF hypocretin-1 was detected.

Conclusions: These results show that serum BDNF is increased in patients with narcolepsy supporting the hypothesis of an underlying neuroplastic and regenerative process taking place in affected individuals.

Introduction

Narcolepsy is a disabling neurological disorder which affects 1/2000 and characterized by excessive daytime sleepiness, cataplexy (sudden loss of muscle tonus triggered by strong emotions) and hypnagogic hallucinations (dream-like hallucinations).[1] Virtually all narcolepsy patients with cataplexy are positive for the HLA DQB1*0602-allele compared to 25% in the general population.[2] Narcolepsy has been ascribed to autoimmune mechanisms and a likely locus from where this arises has recently shown to be the T-cell receptor alpha locus.[1,3]

A low concentration of hypocretin-1 in the CSF is a reliable observation in narcolepsy with cataplexy[2] and a post-mortem analysis revealed a reduction in hypocretin-immunoreactive neurons in the lateral hypothalamus in patients with narcolepsy.[4] Supposedly, the neuronal loss may initiate an activation of neural plasticity processes. This hypothesis is supported by the demonstration of increased number of activated glial cells in the hypothalamus of narcolepsy patients.[4] However, any evidence of a subsequent neuronal re-establishment has not been demonstrated.

Brain-derived neurotrophic factor (BDNF) is involved in synaptic plasticity, neurogenesis and neuronal survival.[5] BDNF is primarily produced in neurons and glial cells, but also found in immune cells, e.g. leucocytes, macrophages and activated microglia.[6,7] Nerve growth factor (NGF), another member of the neurotrophin family, is produced in other neurons than BDNF and plays an important role in regeneration and protection of neurons as well.[8] To investigate the plasticity potential in the narcoleptic brain further, we investigated serum levels of BDNF and NGF with the overall aim to elucidate possible neuroplastic properties in these patients.

Methods

Participants

After approval (Jnr. number KA03119) from the Danish Ethical Committee and written informed consent from each patient, CSF and serum were collected from eighteen Danish Caucasian narcolepsy–cataplexy patients (6 males, 12 females) and fifteen Danish Caucasian healthy controls (7 males, 8 females). At the collection time, range of patient age was 15-75 years and range of disease duration was 1-59 years. The diagnosis of narcolepsy with cataplexy was defined according to the International Classification of Sleep Disorders, 2nd ed. 2005; the diagnosis was obtained from patient history of excessive daytime sleepiness and clear-cut cataplexy (triggered by laughter or jokes), polysomnography, and multiple sleep latency test criteria (mean sleep latency ≤8 min and ≥2 sleep onset rapid eye movement periods). All patients included in the study were HLA-DQB1*0602-positive and were hypocretin deficient. Healthy controls had no history of sleep disorders.

BDNF and NGF measurements

For serum samples, blood was collected in additive-free containers and left to coagulate for at least 1 hr. After centrifugation at 3000 rpm for 10 min, serum was collected and stored at −80°C until further analysis. For measurement of BDNF content in serum, a BDNF sandwich ELISA kit was applied according to instructions provided by the manufacturer (Millipore, Temecula, USA). Briefly, 100 µL of BDNF standards (7.82-500 pg/ml recombinant human BDNF) were applied in duplicates on the rabbit anti-human BDNF pre-coated 96-well plates. The samples were diluted (1:220) and 100 µL was added to the plate in duplicates. The plate was covered and incubated overnight at 4°C. Then, the plate was washed in wash-buffer (for 4 x 2 min), incubated with another

biotinylated primary antiserum for 3 hrs, washed for 4 x 2 min and then incubated with a streptavidin-HRP complex for 1 hr. Finally, the reaction was developed in a solution of TMB (3,3´,5,5´-tetramethylebenzidine) and hydrogenperoxide in the dark for 15 min. The reaction was stopped with 1 M HCl and the absorbance was immediately measured on an ELISA-reader using a 450 nm filter (MicroPlate Reader 550, Biorad Laboratories, Herlev, Denmark). Samples and standards were run in duplicates and BDNF concentrations were calculated using the standard curve and the content was expressed as equivalent of human recombinant BDNF protein in the standards. The determination of NGF was carried out as described above using a similar ELISA assay kit provided from the same source (Millipore, Temecula, USA).

Other biomarkers

Together with the blood sampling a lumbar puncture was performed and CSF was collected. CSF was kept at –80°C until CSF Hypocretin-1 was analyzed (duplicate measurements, mean result used) by a radio-immuno assay (RIA) as reported earlier. [9] Standard biochemical markers, weight and height were determined at the day of blood sampling.

Statistical Analysis

Mean values from patients with narcolepsy and healthy controls were compared using Student's t-test. In case of abnormally distributed data a Mann-Whitney test was used instead. Correlation analysis was performed using either Pearson's or Spearman's correlation analyses.

Results

Eighteen narcolepsy patients with cataplexy were included in the study together with 15 controls. As illustrated in Table 1, the narcoleptic patients were older, had higher BMI, and slightly higher leucocyte counts in blood. These confounding factors are important as other studies have suggested that BDNF levels may be affected by these (see below for discussion).

Interestingly, narcolepsy patients with cataplexy have significant higher serum BDNF levels compared with healthy controls (64.03 ± 3.69 ng/ml, n=18 vs 46.79 ± 2.58 ng/ml, n=15, $P<0.005$)(Fig. 1A). We found no sex differences regarding serum BDNF levels and the difference between narcolepsy with cataplexy and control was significant for both sexes (data not shown). NGF levels in serum were also found to be higher in the patient group, but were not found to be statistically different between narcolepsy patients and healthy controls (Fig. 1B). However, the biological variation in NGF levels is considerably larger than for BDNF and some subjects showed extraordinary high levels of NGF as previously reported[10].

The same group of patients have significantly lower CSF-hypocretin-1 levels compared to healthy controls (24.44 ± 4.58 pg/ml, n=18, vs. 396.8 ± 11.31 pg/ml, n=12, t-test, $P<0.0001$). Analysis of the interaction between CSF hypocretin-1 and serum BDNF revealed no direct correlation in either the control group (Fig.2A) or in narcolepsy patients (Fig. 2B). In another analysis, we correlated the serum BDNF levels with the number of years since diagnosis, but revealed no direct correlation between these two parameters (Fig. 3). However, in the group of relating old patients, a more stable level of BDNF was showed.

Discussion

The major novel finding of the present report is that the concentration of BDNF in serum, the most abundant neurotrophin in the brain, is markedly increased in patients with narcolepsy. No significant difference in NGF was detected. The most prominent pathological characteristics in the narcoleptic patients are reduced number of hypocretin neurons in the hypothalamus and this is also accompanied by infiltration of activated astroglial cells.[4] We assume that the higher level of serum BDNF is a consequence of a reorganisation of neuronal connections in the brain as a response to potential neuronal cells loss. Recent studies suggest that the brain is the primary contributor to blood BDNF levels illustrated by a change in BDNF arterial-to-internal jugular venous difference after a single bout of exercise.[11] Other studies have shown free passage of BDNF across the blood-brain-barrier in mice and a correlation between serum- and brain BDNF in rats.[12,13] Collectively, these studies support that changes in BDNF levels in blood correspond to changes in BDNF-dependent neurotransmission and release in the CNS.

We found no correlation between CSF hypocretin and serum BDNF levels eliminating the possibility that hypocretin during homeostasis inhibit BDNF expression in the human brain. If such a correlation existed, it may be speculated that narcolepsy patients will have high BDNF levels as a direct effect of low hypocretin levels. This was not evident in the current study and the BDNF regulating mechanism is likely to be found elsewhere.

Potential mechanisms that correlate increased BDNF with this disease are rather speculative, but several possibilities exist. However, previous studies have shown that BDNF is up-regulated in several conditions known to involve regeneration of brain tissue, such as stroke and damage to the spinal cord.[14,15] In contrast, BDNF is generally lower in chronic conditions such as depression, Huntington's, Alzheimer's and Parkinson's diseases.[16-18] The temporal correlation between the loss

of hypocretin-immunoreactive neurons and increased BDNF is uncertain, and as mentioned above, we did not find any correlation between CSF hypocretin and BDNF levels.

The exact cellular origin of BDNF is speculative, but inflammatory foci in multiple sclerosis have increased BDNF levels.[19] In the latter, it was shown that activated immune cells may be an important contributor to elevated BDNF levels. However, despite a small increase in blood leucocyte levels in narcolepsy patients, this cannot explain the fairly large change in BDNF levels. In a study by Grimsholm et al. (2008) they could not find any correlation between BDNF levels and leucocytes in the blood or any other inflammatory parameter indicating that these are not directly linked.[20] We therefore believe that the increased serum BDNF levels observed in the current study stems from neurons and glial cells in the CNS participating in the regenerative process surrounding damaged hypocretin-producing neurons and their projections.

Narcolepsy is connected with several confounding factors that might play a role when interpreting the current data. As illustrated in Table 1 the patients also display higher BMI and have a higher mean age. It has been reported previously that narcolepsy patients are less active and that their BMI is higher.[21,22] However, despite equivocal data on the relationship between BMI and BDNF, a recent large-scale study showed that BMI is not correlated to serum BDNF values.[23,24] In addition, less physical activity in the investigated group of narcolepsy patients compared to the controls will only underestimate the difference in BDNF levels observed in the present study. The same is evident for the age difference between the groups since BDNF levels are slightly negatively correlated with age.[24] In summary, it is unlikely that any of these possible confounders will affect the outcome of our results.

In conclusion; here, we report for the first time a marked increase in BDNF levels in hypocretin-deficient patients having narcolepsy with cataplexy. We excluded possible confounders via controlling for differences between the patient group and the control group. We believe that the increase in BDNF is a result of a regenerative process taking place in the microenvironment surrounding the damaged hypocretin neurons and their projections. This underlines the important role of BDNF as a regulator of regeneration in neurodegenerative disorders.

Table 1

Table 1. General characteristics of healthy controls and narcolepsy patients

	Controls	Narcolepsy
n	**15**	**18**
Gender		
Men	**8**	**6**
Women	**7**	**12**
Age	**27±4.7**	**44±18***
BMI	**22.4±2.4**	**28.2±4.8***
Blood leucocytes	**5.4±1.7**	**7.7±3.2***
CRP	**3.5±1.3**	**4.5±2.6**

*Statistical different from the controls. CRP, c-reactive protein, BMI, body mass index

Figure Legends

Figure 1

Serum levels of BDNF (brain-derived neurotrophic factor)(**A**) and NGF (nerve growth factor)(**B**) in healthy controls and patients with narcolepsy. Patients had significantly higher BDNF levels compared to controls (64.03 ± 3.69 ng/ml vs 46.79 ± 2.58 ng/ml, n=18 and n=15), respectively, t-test, **P<0.005)**(A)**. There was no statistical difference between mean NGF levels in patients (99.35 ± 30.02 pg/ml, n=18) and healthy controls (54.15 ± 20.79 pg/ml, n=15)(non-parametric, Mann-Whitney test, p=0.34)**(B).** Error bars indicate SEM.

Figure 2

Correlations of BDNF (brain-derived neurotrophic factor) serum levels and HCRT (hypocretin-1) CSF levels in healthy controls (**A**) and patients with narcolepsy (**B**). No correlations were found (p=0.93 and p=0.82, respectively). Spearman's correlation test.

Figure 3

Correlation of BDNF (brain-derived neurotrophic factor) serum levels and disease duration. No significant correlation was found (p=0.57). Spearman's correlation test.

References

1. Longstreth WT, Jr., Koepsell TD, Ton TG, Hendrickson AF, van BG. The epidemiology of narcolepsy. Sleep 2007;30:13-26.

2. Bourgin P, Zeitzer JM, Mignot E. CSF hypocretin-1 assessment in sleep and neurological disorders. Lancet Neurol 2008;7:649-62.

3. Hallmayer J, Faraco J, Lin L, et al. Narcolepsy is strongly associated with the T-cell receptor alpha locus. Nat Genet 2009;41:708-11.

4. Thannickal TC, Nienhuis R, Siegel JM. Localized loss of hypocretin (orexin) cells in narcolepsy without cataplexy. Sleep 2009;32:993-8.

5. Waterhouse EG, Xu B. New insights into the role of brain-derived neurotrophic factor in synaptic plasticity. Mol Cell Neurosci 2009;42:81-9.

6. Batchelor PE, Liberatore GT, Wong JY, et al. Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. J Neurosci 1999;19:1708-16.

7. Kerschensteiner M, Gallmeier E, Behrens L, et al. Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation? J Exp Med 1999;189:865-70.

8. Himeda T, Tounai H, Hayakawa N, Araki T. Postischemic alterations of BDNF, NGF, HSP 70 and ubiquitin immunoreactivity in the gerbil hippocampus: pharmacological approach. Cell Mol Neurobiol 2007;27:229-50.

9. Knudsen S, Jennum PJ, Alving J, Sheikh SP, Gammeltoft S. Validation of the ICSD-2 criteria for CSF hypocretin-1 measurements in the diagnosis of narcolepsy in the Danish population. Sleep 2010;33:169-76.

10. Lang UE, Gallinat J, Danker-Hopfe H, Bajbouj M, Hellweg R. Nerve growth factor serum concentrations in healthy human volunteers: physiological variance and stability. Neurosci Lett 2003;344:13-6.

11. Rasmussen P, Brassard P, Adser H, et al. Evidence for a release of brain-derived neurotrophic factor from the brain during exercise. Exp Physiol 2009;94:1062-9.

12. Pan W, Banks WA, Fasold MB, Bluth J, Kastin AJ. Transport of brain-derived neurotrophic factor across the blood-brain barrier. Neuropharmacology 1998;37:1553-61.

13. Sartorius A, Hellweg R, Litzke J, et al. Correlations and discrepancies between serum and brain tissue levels of neurotrophins after electroconvulsive treatment in rats. Pharmacopsychiatry 2009;42:270-6.

14. Sato Y, Chin Y, Kato T, et al. White matter activated glial cells produce BDNF in a stroke model of monkeys. Neurosci Res 2009;65:71-8.

15. Song XY, Li F, Zhang FH, Zhong JH, Zhou XF. Peripherally-derived BDNF promotes regeneration of ascending sensory neurons after spinal cord injury. PLoS One 2008;3:e1707.

16. Angelucci F, Spalletta G, di IF, et al. Alzheimer's Disease (AD) and Mild Cognitive Impairment (MCI) Patients are Characterized by Increased BDNF Serum Levels. Curr Alzheimer Res 2009.

17. Brunoni AR, Lopes M, Fregni F. A systematic review and meta-analysis of clinical studies on major depression and BDNF levels: implications for the role of neuroplasticity in depression. Int J Neuropsychopharmacol 2008;11:1169-80.

18. Zuccato C, Cattaneo E. Brain-derived neurotrophic factor in neurodegenerative diseases. Nat Rev Neurol 2009;5:311-22.

19. Stadelmann C, Kerschensteiner M, Misgeld T, Bruck W, Hohlfeld R, Lassmann H. BDNF and gp145trkB in multiple sclerosis brain lesions: neuroprotective interactions between immune and neuronal cells? Brain 2002;125:75-85.

20. Grimsholm O, Rantapaa-Dahlqvist S, Dalen T, Forsgren S. BDNF in RA: downregulated in plasma following anti-TNF treatment but no correlation with inflammatory parameters. Clin Rheumatol 2008;27:1289-97.

21. Kok SW, Overeem S, Visscher TL, et al. Hypocretin deficiency in narcoleptic humans is associated with abdominal obesity. Obes Res 2003;11:1147-54.

22. Nishino S, Ripley B, Overeem S, et al. Low cerebrospinal fluid hypocretin (Orexin) and altered energy homeostasis in human narcolepsy. Ann Neurol 2001;50:381-8.

23. Bullo M, Peeraully MR, Trayhurn P, Folch J, Salas-Salvado J. Circulating nerve growth factor levels in relation to obesity and the metabolic syndrome in women. Eur J Endocrinol 2007;157:303-10.

24. Ziegenhorn AA, Schulte-Herbruggen O, Danker-Hopfe H, et al. Serum neurotrophins--a study on the time course and influencing factors in a large old age sample. Neurobiol Aging 2007;28:1436-45.

Figure 1

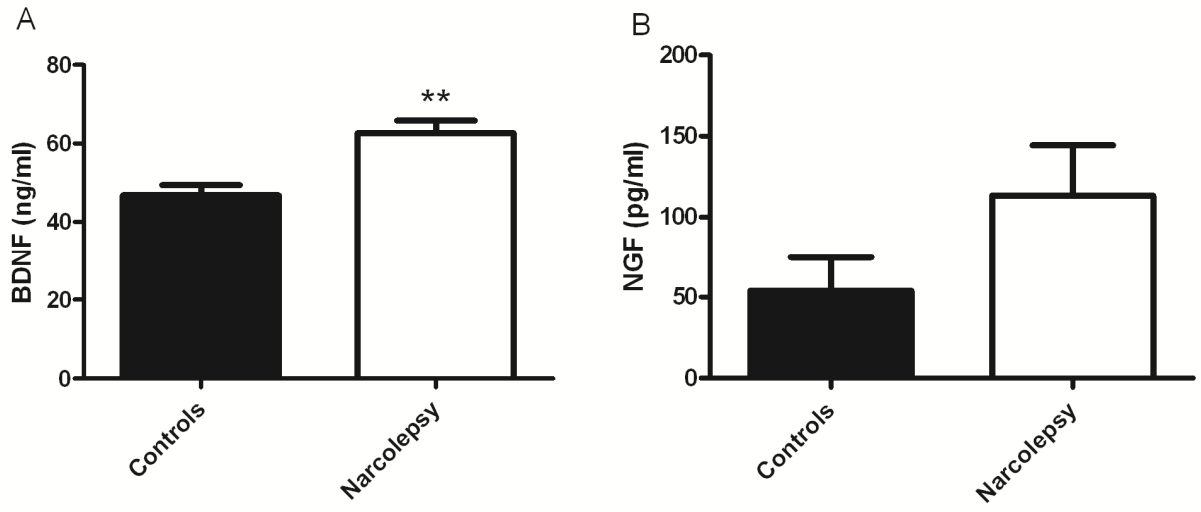

Figure 2

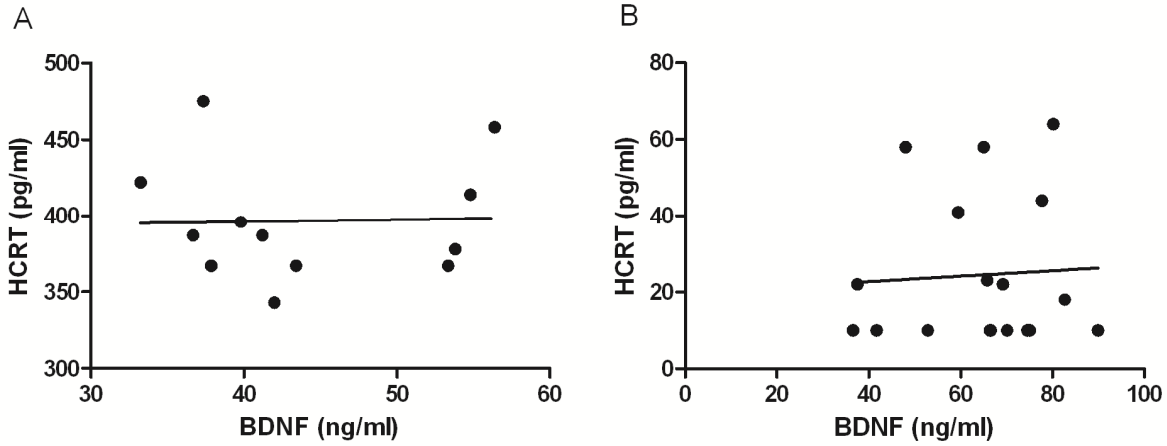

Figure 3