Brain-derived neurotrophic factor:

Generation and characterization of adult mice lacking BDNF in the adult brain

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Summary

BDNF (brain-derived neurotrophic factor) plays an important role in neuronal survival, growth and maintenance of several neuronal systems, serves as neurotransmitter modulator and participates in plasticity important for learning and memory. BDNF is widely expressed in the CNS, beginning early in development and extending throughout life. In the mammalian brain, it is most abundant in the cerebral cortex and hippocampus. BDNF is of special interest because of the regulation of its expression and release as a function of the state of neuronal activity. In humans the most direct link between BDNF and pathological conditions is the recent discovery that the loss of one BDNF allele causes a strong obesity phenotype. In addition, there is accumulating evidence that in conditions such as Huntington (HD) decreased levels of BDNF may contribute to the disease. Finally, it is also associated with depression and anxiety. One key difficulty in understanding the role of BDNF in the adult brain has been the generation of suitable animal models as the germline deletion of BDNF leads to early postnatal death. To circumvent this difficulty, we generated conditional mutant mice in which BDNF is eliminated from the CNS through the use of the Cre-loxP recombination system. This was achieved by crossing 2 existing mouse lines, one carrying two floxed BDNF alleles and the other expressing Cre from the *tau* locus. Our results indicated that such conditional BDNF knockout mice (*cbdnf ko*) can survive for several months after birth with almost no detectable BDNF in their brain. This creates a novel opportunity to ask the general question of the role of BDNF in the adult CNS. Histological analysis indicated that the brain of these mutant mice is generally smaller, but in an unexpected region-specific manner affecting mostly the striatum and surprisingly not the hippocampus. The marked reduction in the volume of the striatum could not be explained by cell loss, defects of axonal diameter or hypomyelination, but most likely by a marked reduction in the volume of striatal neurons and their processes, which is currently quantified. The lack of hippocampal volume reduction could not be explained due to compensatory changes in the expression levels of NT4 and/or NT3. It thus seems that the postnatal increase of BDNF may be essential for the growth of striatal neurons, and perhaps other GABAergic neurons, but not for hippocampal pyramidal neurons. At the behavioural level *cbdnf ko* animals exhibit a variety of defects, including a clasping phenotype similar to that observed in mouse models of Huntington's disease.

The *cbdnf ko* mice also provided a key control for parallel studies examining the biosynthesis, processing and storage of endogenous BDNF in the CNS. We showed that in hippocampal neurons pro-BDNF is a transient intermediate that is converted intracellularly to the mature form of BDNF, which is stored and released by excitatory input (Matsumoto et al., 2008).

The new *bdnf* mutant animals generated during the course of this PhD thesis thus represent a new opportunity to study the role of BDNF in the adult mouse nervous system.

ABBREVIATIONS

Aβ	amyloid β
AChT	Acetylcholinetransferase
AKT/PKB	serine/threonine protein kinase (protein kinase B)
AMPA	α-amino-3-hydroxy-5-methyl4-isoxazole-propionic acid
APP	amyloid precursor protein
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
CA1/CA3	hippocampal subfields 1-3 of the ammons horn
CaMKII	Ca2+/calmodulin dependent kinase II
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
cbdnf ko	neuron specific conditional bdnf knockout mice
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanine monophosphate
CNS	central nervous system
CaRF	Ca^{2+} response factor
Cre	site-specific DNA recombinase
CREB	cAMP response element binding protein
DAG	diacylglycerol
DG	dentate gyrus
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
Erk	Ras/extracellular signal regulated kinase
ER	endoplasmic reticulum
EPSP	excitatory postsynaptic potential
Emx-BDNFKO	conditional BDNF knockout mice
FKHRL1	Forkhead 1
Frs-2	fibroblast receptor substrate-2
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
5-HT	5-hydroxytryptamine, serotonin
5-HIAA	5-hydroxyindoleacetic acid
IP3	inositol 1,4, 5 triphosphate
JNK	c-Jun-N-terminal kinase
K252	tyrosine kinase inhibitor
kb	kilobase
KCl	potassium chloride
KDa	kilodalton
LTD	long-term depression
LTP	long-term potentiation
L-LTP	late-LTP
MAP	microtubulus-associated protein
MAPK	mitogen-activated protein kinase
mAbs	monoclonal antibodies

MEK	MAPK/ERK kinase
mGluR	metabotropic glutamate recepor
mRNA	messenger ribonucleic acid
mIPSC	miniature inhibitory postsynaptic current
MMP	matrix metalloproteinase
NA	noradrenline
NFκB	nuclear factor κB
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NPG	nodose-petrosal ganglion complex
NRIF	neurotrophin receptor interacting protein
NO	nitric oxide
NT	neurotrophin
NT-3	neurotrophin-3
NT-4	neurotrophin-4
p75	low-affinity neurotrophin receptor
PCR	polymerase chain reaction
PI-3-K	phosphatidylinositol-3-kinase
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLCγ	phospholipase Cγ
PNS	peripheral nervous system
PSD	postsynaptic density
pY490	phosphotyrosine 490
Raf	MAP-kinase-kinase-kinase
Ras	GTP-binding protein
RGC	retinal ganglion cell
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of mean
Shc	adaptor protein with SH2 domain
T1	truncated trkB.T1
T2	truncated trkB.T2
Tau	microtubule associated protein
TBS	theta burst stimulation
Trk	tropomyosin-related kinase
TrkA	tyrosine kinase receptor for NGF
TrkB	tyrosine kinase receptor for BDNF and NT-4
TrkB-IgG	recombinant BDNF binding scavenger protein
TrkC	tyrosine kinase receptor for NT-3
VGCC	voltage-gated Ca2+ channel

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1. Introduction

1.1. Neurotrophin family

A hallmark of vertebrate evolution is the development of a complex nervous system. The enormous number of neurons makes coordination solely by cell intrinsic programs unfeasible. A common design principle in vertebrates is the sculpting of the nervous system during embryonic development from an initial excess of neurons with the elimination of the apparently redundant ones. Along with cell-intrinsic mechanisms, the final number is attained via degeneration of those neurons that fail to interact successfully with their intended targets. Specifically, they might fail in competition with their peers for survival signals in the form of secreted proteins that are provided in limited amounts by the target tissue. By this means, the number of innervating neurons is adjusted to the target tissue. Following the establishment of neuronal populations, neuronal processes are believed to grow or retract throughout ontogeny again as a function of target-derived support. This organizing principle, which became known as the “neurotrophic theory” (Purves, 1988), allows the nervous system to flexibly adjust to bodily changes in size and form via rather simple regulatory loops with a minimum of genetic instructions. This theory is generally recognized as one of the important principles explaining the development of the nervous system in vertebrates. Central to this concept was the identification and characterization of secreted proteins known as neurotrophic factors that act as intercellular survival signals between target tissue and neurons. Nerve growth factor (NGF) was not only the first neurotrophic factor but also the first polypeptide growth factor to be identified (Cohen and Montalcini, 1956) and has become a paradigm for trophic factor research. Brain-derived neurotrophic factor (BDNF) was purified as the second member of the neurotrophin family (Barde et al., 1982) and with the cloning of neurotrophin-3 (NT3) (Ernfors et al., 1990; Hohn et al., 1990; Jones et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990) and neurotrophin 4/5 (NT4/5) (Berkemeier et al., 1991; Ip et al., 1994) the small familiy of neurotrophins in higher vertebrates was complete.

Neurotrophins are initially synthesized as pre-pro-proteins by neuronal and non-neuronal cell types and the mature proteins, which are about 12-14 kDa in size, form stable, non-covalent dimers, and are expressed at very low levels. The pro-domain of the pro-neurotrophin is thought to be important for the proper folding and intracellular sorting of neurotrophins (also see below).

The members of the neurotrophin family show different binding specifity for two classes of receptor: the Trk (tropomyosin receptor kinase) family of RTKs (receptor tyrosine kinases) and the p75 neurotrophin receptor ($p75^{NTR}$). Upon ligand binding, Trk receptors dimerize and become catalytically active, resulting in receptor autophosphorylation and subsequent activation of a number of signaling cascades, including the Ras/Raf/MAPK (mitogen-activated protein kinase) (Thomas et al., 1992), PI3K (phosphoinositide 3-kinase) (Atwal et al., 2000), and phospholipase C-*γ* 1 pathways (Vetter et al., 1991). Trk receptor subtypes bind mature neurotrophins with different specificities: TrkA preferentially binds NGF, TrkB preferentially binds BDNF and to a lesser extent NT3 and NT4, while TrkC displays preference for NT3. $p75^{NTR}$ binds all mature neurotrophins with approximately equal affinity and has, in recent years, been demonstrated to bind the proneurotrophins with higher affinity (Lee et al., 2001). $p75^{NTR}$ can also interact with a number of receptors, including the Trks (Bibel et al., 1999), sortilin (Nykjaer et al., 2004), and NOGO (Bandtlow et al., 2004), and is capable of binding ligands other than the neurotrophins, e.g. the *β*-amyloid (Yaar et al., 1998) and prion peptides (Della-Bianca et al., 2001). Signaling mediators activated subsequent to $p75^{NTR}$ ligand binding include ceramide (Dobrowsky et al., 1994), NF-*κ*B (nuclear factor *κ*B) (Carter et al., 1996), Akt (also called protein kinase B) (Roux et al., 2001), JNK (c-Jun N-terminal kinase) (Friedman, 2000) and cysteine proteases termed caspases (Coulson et al., 2000). The complexity of this multiple ligand, multiple receptor signaling system is evidenced by the opposing actions of the neurotrophins. For example, Trk receptors are widely reported to promote cell survival, enhancement of process elongation and of the

efficacy of synaptic transmission, while strong evidence exists in support of a role for $p75^{NTR}$ in mediating inhibition of process elongation and cell death.

1.2 BDNF

1.2.1 BDNF gene, mRNA and protein

Brain-derived neurotrophic factor (BDNF) was identified as a second member of the neurotrophin growth factor family with close structural homology to NGF (Barde et al., 1982; Leibrock et al., 1989). The mouse BDNF gene (AY057907) has a complex structure and consists of 8 exons with only exon 8 being a protein coding exon. The other 5′ exons contain different promoters so that all splice variants mBDNF 1, 2, 3, 4, 5 and 6A were reported to result in the expression of the same protein encoded by exon 8. Exon 2 displays two intraexonic splice sites giving rise to three different mBDNF 2 subvariants called mBDNF 2A, 2B and 2C. In addition, a three-part transcript, mBDNF 6B is the result of splicing events incorporating exons 6, 7 and 8, so that altogether 9 different splicing variants could be detected (Liu et al., 2006). Most of these splice variants were found in different regions of the brain and additionally some of them in other peripheral organs including the heart, lung, muscle, kidney, liver, testis, and spleen. However, the functional relevance of these different splice variants remains unclear (Liu et al., 2006). It has been reported that the different promotors for the splice variants are localized in two clusters with one cluster encompassing exons I, II and III (covering splice variants 1, 2A, 2B, 2C and 3) whereas the second cluster encompasses exons IV, V and VI (covering splice variants 4, 5, 6A and 6B) (Liu et al., 2006). For cluster 2, some transcriptional regulatory elements that recognize calcium-responsive transcription factor (CaRF), cAMP/calcium-responsive element binding proteins (CREB) and methyl-CpG binding protein 2 (MeCP2) were described (Chen et al., 2003; Martinowich et al., 2003; Tao et al., 2002). Northern blot experiments revealed approximately equimolar amounts of two BDNF transcripts of about 1.6 kB and 4.2 kB in the brain. Hybridization with a probe from the 3`UTR of the coding exon showed that the main difference in the

length of the two transcripts is due to an alternative use of two polyadenylation sites in the 3`UTR of the coding exon (Hofer et al., 1990; Maissonpierre et al., 1990; Timmusk et al., 1993). In situ hybridization experiments have revealed that BDNF mRNA is strongly expressed in the brain. BDNF expression levels are low during fetal development, markedly increase after birth and then reduce to adult levels (Maisonpierre et al., 1990). In the adult animal, BDNF is expressed throughout the brain with the highest levels in the hippocampus, where BDNF mRNA was localized to pyramidal neurons of the CA1-CA3 regions and in granule cells of the dentate gyrus. Some CNS structures, such as the striatum and the spinal cord in particular express very low levels of BDNF mRNA (Hofer at al., 1990).

BDNF mRNA expression is regulated by neuronal activity. For example, epileptogenic activation of glutamatergic synapses increased the expression of BDNF mRNA in slices of rat hippocampus (Ernfors et al., 1991; Zafra et al., 1990), and increasing synaptic activity with AMPA receptor agonists induced a transient elevation in mRNA levels encoding BDNF and TrkB in the hippocampus and entorhinal cortex (Lauterborn et al., 2000). Conversely, reduction of electrical activity by blockade of glutamate receptors or stimulation of the GABAergic system reduced BDNF mRNA level in the hippocampus (Zafra et al., 1991). These studies demonstrate that an increase or a decrease of neuronal activity can enhance or reduce BDNF expression. Consistent with these observations light-induced physiological activity enhanced the expression of BDNF mRNA in the visual cortex and monocular deprivation elicits a striking decrease in BDNF mRNA in the visual cortex corresponding to the deprived eye (Bozzi et al. 1995; Rossi et al. 1999; Lein and Shatz 2000). Furthermore, sensory stimulation of whiskers enhances the expression of BDNF mRNA in the barrel cortex (Rocamora et al. 1996; Nanda and Mack, 2000).

There is some evidence that the nuclear transcript may be selectively transported to active dendrites and translated locally. Depolarization of hippocampal neurons

lead to increased levels of BDNF and TrkB transcripts in dendrites, which may allow for increased local protein synthesis (Righi et al., 2000; Tongiorgi et al., 1997).

Like all neurotrophins, BDNF exerts its biological effects as a homodimer (Jungbluth et al., 1994; Kolbeck et al., 1999) and is initially synthesized as pre-pro-protein both by neuronal and non-neuronal cell types, where the pre-pro-sequence is presumably cleaved in the endoplasmatic reticulum. Pro-BDNF then presumably transits through the Golgi apparatus where it becomes N-glycosylated. Because BDNF and neurotrophins in general are normally expressed at very low levels, little is known about their processing and secretion *in vivo*. However, recent studies have suggested that the precursor of NGF is released under pathological conditions (Harrington et al., 2004). proNGF may induce cell death through $p75^{NTR}$ in the presence of the type I transmembrane protein sortilin to mediate cell apoptosis (Lee et al., 2001; Nykjaer et al., 2001). Whether these findings reflect a unique property of proNGF or whether the proform of BDNF is similarly active in modulating $p75^{NTR}$ signaling is currently unclear. This question was addressed by overexpressing a cleavage-resistant mutant form of BDNF. These experiments revealed that proBDNF is an effective proapoptotic ligand for cultured sympathetic neurons, also requiring both $p75^{NTR}$ and sortilin to initiate cell death (Teng et al., 2005). However, no study has demonstrated so far that endogenous proBDNF is released under physiological or pathological conditions and that it acts through $p75^{NTR}$ and sortilin to mediate cell apoptosis. Very recent work from our laboratory (Matsumoto et al., 2008) indicates that proBDNF is rapidly processed intracellularly and that proBDNF is not released from neurons (see results 3.7).

Recently, a single nucleotide polymorphism in the *bdnf* gene leading to a valine (Val) to methionine (Met) substitution at codon 66 in the prodomain (BDNFMet) was found to be associated in humans heterozygous for the polymorphism with memory impairments (Egan et al., 2003). Further, the val66met polymorphism

leads to abnormal BDNF trafficking, distribution and activity-dependent release in neurons (Chen et al., 2004). A recent study suggests a mechanism for sorting BDNF to the regulated secretory pathway in neurons by the binding of its prodomain to sortilin, a membrane protein localized to the Golgi apparatus where sorting occurs. The study provides evidence that sortilin acts as a sorting receptor that preferentially binds proBDNF (val), but binds poorly to proBDNF(met) (Chen et al., 2004; Chen et al., 2006).

CNS neurons are thought to release BDNF by activity-regulated secretion pathways initiated via the excitatory neurotransmitters glutamate and acetylcholine. This process depends on calcium release from intracellular stores (Blöchl and Thoenen, 1995, 1996; Canossa et al., 1997; 2002; Griesbeck et al., 1999). More recently, it has been demonstrated that patterned electrical stimulation induces BDNF release in primary sensory neurons (Balkowiec and Katz, 2000). Analogous results have been obtained in the central neurons (Goodman et al., 1996; Gärtner and Staiger, 2002; Balkowiec and Katz, 2002). Additionally, these studies have suggested that BDNF can be released from both postsynaptic and presynaptic compartments (Haubensak et al., 1998; Balkowiec and Katz, 2002; Hartmann et al., 2001; Kohara et al., 2001). By comparison, little is known about potential pathways that downregulate BDNF secretion. So far only one study has described negative modulation of BDNF release. Canossa et al. (2002) have shown that endogenous production of nitric oxide (NO) downregulates BDNF secretion in hippocampal neurons by activating a cyclic guanosine monophosphate (cGMP)-dependent signal transduction pathway. Activation of this pathway leads to downregulation of Ca^{2+} release from IP3 sensitive intracellular stores by protein kinase G.

In general, the detection of BDNF protein matches the distribution of its mRNA (Nawa et al., 1995) and the levels of BDNF protein are known to increase postnatally (p1-p21) by about 10-fold in the cerebral cortex and in the hippocampus (Kolbeck et al., 1999). However, both in the striatum and the spinal

cord substantial levels of BDNF protein were found, while the mRNA studies revealed low levels of expression (Hofer et al., 1990; Schmidt-Kastner et al., 1996; Kolbeck et al., 1999).

The finding that BDNF is expressed in hippocampal neurons and that it is found in the cell bodies of cholinergic neurons projecting to the hippocampus that do not seem to express BDNF themselves suggest that BDNF is taken up by the axon terminals and retrogradely transported back to the cell bodies (Wetmore et al., 1991). This postulate was confirmed by DiStefano et al. (1992), who demonstrated that ^{125}I-labeled BDNF is retrogradely transported by distinct populations of neurons in the central nervous system. In addition, BDNF can undergo anterograde transport to presynaptic sites, thus explaining the presence of substantial amounts of BDNF in the striatum. In line with this, cortical neurons (layer III and V) projecting to the striatum contain high levels of BDNF mRNA (Altar et al., 1997). In addition, prevention of axonal transport in striatal afferents with colchicine results in elevation of BDNF in the cell bodies of cortical neurons and marked decrease in the striatal neuron, a result also observed after unilateral lesion of the cerebral cortex (Altar et al., 1997).

1.2.2 BDNF - dendritic growth

The first identified *in vivo* function of BDNF during normal development was its ability to promote survival of peripheral sensory neurons during programmed cell death (Hofer and Barde, 1988). BDNF also regulates dendritic growth of many different types of neurons in several different brain regions (Lom and Cohen-Cory, 1999; Segal et al., 1995; Shimada et al., 1998; McAllister et al., 1999). Exogenous BDNF application results in increased dendritic length and complexity of pyramidal neurons in the developing visual cortex in a layer specific manner (McAllister et al., 1995). The specificity of these effects suggests that BDNF does not act simply to enhance neuronal growth but rather acts to modulate particular patterns of dendritic arborization. Further, inhibition of spontaneous electrical activity, synaptic transmission, or L-type calcium channels, all prevent the large

increase in dendritic growth elicited by exogenous BDNF. These results indicate that neurons must be active in order to respond to the growth promoting effects of BDNF (McAllister et al., 1996). Accordingly, scavenging endogenous BDNF causes dendritic retraction, clearly demonstrating that endogenous BDNF influences the complexity of dendritic arbors of pyramidal neurons in the developing visual cortex (McAllister et al., 1997). Moreover, pyramidal neurons transfected to overexpress BDNF retract their existing dendritic spines and sprout more dendrites (Horch et al., 1999). Timelapse imaging shows that the dendrites of these cells are much more dynamic than non-transfected control dendrites, suggesting that BDNF induces structural instability and increases plasticity in both dendrites and spines (Horch et al., 1999). However, the mechanism by which BDNF exerts its stabilizing effect on dendrite morphology is uncertain, not least because some of these studies involved the use of BDNF overexpression (see below). BDNF-TrkB signaling might regulate the stability of dendrites directly. For example, TrkB signaling might regulate the activity of members of the Rho familiy of GTPases, known to modify dendrite number, form, and stability in a variety of neuronal types (Threadgill et al., 1997; Li et al., 2000; Tashiro et al., 2000; Hayashi et al., 2002; Luo et al., 2002). Alternatively, BDNF could influence dendritic structure indirectly through modulating synaptic activity (Cline, 2001; Kafitz et al., 1999; Poo, 2001). Regardless of whether BDNF directly or indirectly influences dendrite structure, conditional *bdnf* knockout mice (see also introduction 1.3.3) indicate that the essential function of BDNF is neither to support neuronal survival nor to regulate initial growth of dendritic trees, as suggested by *in vitro* studies (Gorski et al., 2003, Baquet et al., 2004). Instead BDNF appears to support the “survival” of dendritic structure generated through BDNF-independent mechanism. These results also suggest a possible role for BDNF in the etiology of neurodegenerative diseases.

1.3 Genetically modified mice

Transgenesis and ES cell technologies have opened a unique experimental access to the mouse genome and converted this rodent into the most advanced model organism for mammalian genetics. Indeed, gene-targeted knockout mice represent a powerful experimental system for studying development, behavior and physiology. They also may be useful to model certain human genetic diseases. The procedure for producing gene-targeted knockout mice involves several steps: Exogenous DNA containing a mutant allele of the gene being studied is introduced into embryonic stem (ES) cells by transfection. The targeting vector recombines with DNA sequences at homologous and in some cases at non-homologous sites. The small fraction of cells in which homologous recombination takes place can be identified by a combination of positive and negative selection: positive selection to identify cells in which any recombination occurs and negative selection to remove cells in which recombination takes place at non-homologous sites. For this selection scheme to work, the DNA constructs introduced into ES cells need to include, in addition to sequences used to selectively modifiy the gene of interest, two selectable marker genes. One of these additional genes (neo^r) confers neomycin resitance; it permits positive selection of cells in which either homologous (specific) or non homologous (random) recombination has occurred. The second selective gene, the thymidine kinase gene from herpes simplex virus (tk^{HSV}) confers sensitivity to ganciclovir, a cytotoxic nucleotide analog; this gene permits negative selection of ES cells in which non-homologous recombination has occurred. Only ES cells that undergo homologous recombination can survive this selection scheme. Once ES cells heterozygous for a knockout mutation are obtained, they are injected into a recipient mouse blastocyst, which subsequently is transferred into a surrogate pseudopregnant mouse. If the ES cells are homozygous for a visible marker trait (e.g. coat color) then chimeric progeny carrying the knockout mutation can be easily identified. These are then mated with mice homozygous for another allele of the marker trait to determine if the knockout mutation is incorporated into the germ line. Finally, mating mice, each heterozygous for the knockout allele, will

produce progeny homozygous for the knockout mutation. Although gene targeting by homologous recombination has revolutionized the way gene function is assessed *in vivo*, this strategy has certain limitations. For example, the early lethality of some gene disruptions prevents assessment of gene function in the adult and aging animal. Moreover, severe developmental defects can compromise other systems such as the primary effects of a null mutation are difficult to separate from secondary effects. Finally, the normal interactive relationships between different factors may be obscured due to the compensation of genes not principally affected by this mutation.

To circumvent these limitations conditional knockout strategies have been designed to inactivate genes in a spatiotemporally regulated manner. To obtain a tissue-specific gene knockout the Cre/loxP system is most widely utilized, for which two different mouse lines have to be separately generated (Sauer et al., 1998; Nagy, 2000). In one of these two lines a functionally relevant sequence of the gene of interest is flanked by two loxP sites using homologous recombination techniques in embryonic stem cells. The loxP sites are short sequence elements (34 bp) containing two 13-bp inverted repeats flanking an 8-bp asymmetric spacer. In the second mouse line, Cre recombinase expression is driven under control of defined tissue-specific promotors, which limits the recombination event to defined stages of the mouse development and to specific tissues, frequently preventing embryonic lethality or developmental adaption. Mating these two different mouse lines will yield offspring where Cre recombinase is solely expressed in the targeted tissue or cell type in which it will excise the floxed DNA fragment. The impact of this approach is reflected by the ever increasing number of mouse lines expressing the cre gene under the control of various tissue-specific promotors or contain a variety of genes equipped with Lox sites for gene inactivation or activation (http://www.mshri.on.ca/nagy). Obviously, Cre-induced recombination results in irreversible genetic alterations that reflect the activity “history” of the promoter driving the *cre* gene. Thus, this simple approach is limited not only by the irreversibility but also by the rigidity of the developmental and differentiation program of the organism, which, for example, prevents

recombination in a fully differentiated tissue at a later period in the mouse life. This difficulty is increasingly often circumvented by using forms of Cre that can be activated for example by tamoxifen that can also be administered in specific tissues. With regard to BDNF, several approaches with different Cre drivers have been used to address the role of BDNF in the mouse nervous system.

1.3.1 *Bdnf* knockout mice

Germ-line *Bdnf* knockout mice are born alive, exhibit reduced growth and die before reaching the second postnatal week. They show abnormal behavior including circling and spinning movements likely to be caused by defective innervation of the vestibular compartments of the inner ear (Jones et al., 1994). Ernfors et al. (1994) report neuron loss of more than 80% in the vestibular ganglia and that no vestibular axons innervate the sensory epithelium of the semicircular canals, saccule, or utricle in postnatal animals. These findings suggest that all vestibular ganglion cells innervating inner ear organs associated with sensing motion require BDNF for survival. In contrast, the gross appearance of structures related to hearing, the cochlea and spiral ganglion was normal. Interestingly, if the *bdnf* gene is replaced by *nt3*, the severe defects of the cochlea innervation and hearing observed in *bdnf* null mice are rescued, whereas the vestibular innervation is not (Agerman et al., 2003). Further analysis of mice lacking BDNF indicated a more than 50% loss of neurons in the nodose petrosal ganglion complex (NPG), implicating BDNF in the survival of NPGs. The NPG is comprised of neurons that relay sensory information critical for the regulation of respiration, heart rate and blood pressure. A sub-population of dopaminergic NPG neurons that innervate the carotid body are lost in *bdnf* knockout mice. The carotid body is a chemoreceptor organ that senses changes in arterial oxgen and carbon dioxide, provides tonic excitatory drive to ventilation, and is the primary site that initiates cardiorespiratory reflex responses to hypoxia. Further investigations showed that lack of BDNF compromises normal ventilatory responses to hypoxia, possibly contributing to premature death in *bdnf* knockout mice (Erickson et al., 1996). Additionally, Carroll et al. (1998) showed that slowly

adapting mechanoreceptors of *bdnf* knockout mice, which are required for tactile discrimination, show severely impaired mechanosensitivity and that this defect can be rescued by exogenous BDNF. Motor neurons, which were on the basis of pharmacological studies predicted to require BDNF, are surprisingly not affected in *bdnf* mutants (Ernfors et al., 1994; Jones et al., 1994), not even in the double *bdnf/nt4* null mice therefore suggesting that motorneuron survival is not controlled by this neurotrophins during normal development (Conover et al., 1995; Liu et al., 1995).

Neuronal populations in the CNS reported to be sensitive to BDNF deprivation include dopaminergic neurons in the substantia nigra, basal forebrain cholinergic neurons, hippocampal neurons, cortical neurons, cerebellar granule cells, and retinal ganglion cells (Huang and Reichardt, 2001). However, detailed analysis of *bdnf* knockout mice showed that BDNF does not affect neuronal survival in the postnatal brain, but reduces the expression of calbindin, parvalbumin, and neuropeptide Y in GABAergic interneurons, implying that BDNF is essential for normal differentiation, but not for the survival of most central nervous system neurons (Jones et al., 1994). Alternatively, central neurons may have acquired functionally compensating signaling pathways that protect them more efficiently than peripheral neurons from lack of BDNF (Alcántara et al., 1997; Minichiello et al., 1996). Additionally, Cellerino et al. (1997) demonstrated that BDNF plays a role in myelination of the CNS, as indicated by hypomyelinated retinal ganglion cell axons in mice lacking BDNF (but see below).

BDNF not only modulates neuronal differentiation in the central nervous system but also the number and formation of synapses and the efficacy of synaptic transmission. In mice overexpressing BDNF in sympathetic neurons increased synapse numbers were observed, whereas a decrease was seen in *bdnf* knockout animals (Causing et al., 1997). Other studies on *bdnf* knockout mice show presynaptic structural defects, including a decrease in the number of docked synaptic vesicles and reduced expression of the synaptic vesicle proteins synaptophysin and synaptobrevin, indicating impaired presynaptic transmitter

release in the absence of BDNF (Pozzo-Miller et al., 1999). Treatment of the mutant slices with BDNF reversed these deficits in the hippocampal synapses and BDNF appears to regulate high-frequency synaptic transmission by facilitating synaptic vesicle mobilization and docking into nerve terminals (Pozzo-Miller et al., 1999; Kafitz et al., 1999). One of the clearest indications that BDNF modulate synaptic transmission comes from the much studied paradigm of long-term potentiation (LTP). Hippocampal slice preparations from *bdnf* knockout mice exhibited impaired CA1 LTP (Korte et al., 1995). These deficits were reversible by either bath application of BDNF or adenovirus-mediated BDNF gene transfer (Korte et al., 1995; Patterson et al., 1996; Pozzo-Miller et al., 1999). Additional evidence to support the role of BDNF was provided by studies where LTP was attenuated if function-blocking BDNF antibodies or BDNF scavenging TrkB-IgG proteins are applied on *in vitro* slices (Chen et al., 1999; Figurov et al., 1996; Kang et al., 1997). Besides the immediate actions in the hippocampal potentiation, BDNF is essential during the late-phase LTP (L-LTP) that requires new protein synthesis (Bradshaw et al., 2003; Kang et al., 1997; Korte et al., 1998). Mutational analysis of phosphorylation sites of TrkB have demonstrated that inhibition of phosphorylation at the PLC-*γ*-binding site results in reduction in LTP in the hippocampus, whereas mutation of the Shc site, essential for activation of MAPK and PI3K, does not affect this (Minichello et al., 2002). These results were surprising since previous data connects Ras/MAPK pathway to synaptic potentiation (English and Sweatt, 1996; English and Sweatt, 1997; Patterson et al., 2001; Ying et al., 2002). Still, it is possible that cross-talk between TrkB downstream signaling pathways might rescue the Shc-deficit in terms of LTP induction.

1.3.2 *Bdnf* heterozygous mice

Studies on *bdnf* heterozygous mice indicated that they are viable and develop a phenotype characterized by increased anxiety, aggressiveness and hyperphagia accompanied by significant weight gain in early adulthood. Further, these mutants have deficits in spatial and contextual learning as well as impaired hippocampal LTP that is as affected as in homozygous mutants (Lyons et al., 1999; Linnarsson et al., 1997; but see Montkowski and Holsboer, 1997; Kernie et al., 2000; Liu et al., 2004; Korte et al., 1995; Patterson et al., 1996). *Bdnf* heterozygous mice show also different effects on two forms of cortical LTP: white matter-evoked, layer III LTP is normally inducible in *bdnf* heterozygous mice, whereas layer IV-evoked, layer III LTP is impaired. (Bartoletti et al.,2002).

One consequence of hyperphagia is that *bdnf* heterozygous mice display symptoms reminiscent of human obesity. Their adipocytes are enlarged and they have high plasma levels of leptin and insulin. Interestingly, Rios et al. (2001) showed that BDNF injection into the ventromedial hypothalamus reduce food intake in rats via activation of TrkB receptors and Xu et al. (2003) further demonstrated that BDNF is involved in the regulation of food intake as a downstream mediator of MC4R signaling. The role of BDNF in the regulation of food intake and its relation to MC4R signaling was further investigated by Nicholson et al. (2007), who demonstrated an acute hypothalamic release of BDNF after local MC4R stimulation with the selective MC4R agonist, MK1. Thus, BDNF appears to play an important role as a downstream mediator of the MC4R pathway.

Aggressiveness has been associated with dysfunction of the serotonin system (Lyons et al., 1999). At 18 months of age, *bdnf* heterozygous mice show decreased serotonin innervation, as well as decreased levels of 5-HT and 5-Hydroxyindol acetic acid (5-HIAA) in the hippocampus compared with age-matched wild-type mice. Further, significant decreases in 5-HT receptor (1A, 1B, 2A and 2C) mRNA levels in 6-9 months old heterozygous *bdnf* mice were found

in various brain regions (Lyons et al., 1999). To test whether age-accelerated loss of serotonergic innervation in *bdnf* heterozygous mice occurs in other brain regions, advances beyond 18 months or is associated with alterations in other neurotransmitter systems, Luellen et al., (2007) assessed serotonergic innervation in 26-month-old heterozygous *bdnf* mice. Age-related loss of serotonergic axons in the hippocampus was found in heterozygous *bdnf* mice compared with wild-type mice, particularly in CA1 subregion. By contrast, aging heterozygous *bdnf* mice showed increased serotonin innervation of the basomedial nucleus of the amygdala. *In vivo* zero microdialysis experiments in heterozygous *bdnf* mice showed a significant decrease in extracellular serotonin levels at 20 months of age. Thus, reduced BDNF seems to be associated with altered serotonergic and noradrenergic innervation in aging mice, and in particular, with accelerated loss of serotonergic innervation to the hippocampus that is manifest as a decrease in basal neurotransmission (Luellen et al., 2007). Interestingly, the heightened aggressiveness found in *bdnf* heterozygous mice can be ameliorated by the selective serotonin reuptake inhibitor fluoxetine indicating that endogenous BDNF is critical for the normal function of central 5-HT neurons and for the elaboration of behaviors that depend on these nerve cells (Lyons et al., 1999; Kernie et al., 2000).

1.3.3 Conditional *Bdnf* knockout mice

Obesity phenotypes have also been observed in CamKII-BDNF conditional knockout mice (CamKII-BDNF^{KO}) that have normal brain content of BDNF during perinatal development but a later depletion of BDNF in the postnatal period (Rios et al., 2001). These mutants also show hyperactivity and hyperaggression, suggesting a serotonin dysregulation. However, the presynaptic serotonin system in the adult CamKII-BDNF^{KO} mice appeared normal by histological, biochemical, and electrophysiological criteria (Rios et al., 2006). This was surprising as BDNF has been reported to support the differentiation of serotonin neurons (Mamounas et al., 2000). By contrast, a dramatic postsynaptic 5-HT$_{2A}$ deficit in CamKII-BDNF^{KO} mice was found. Electrophysiologically, serotonin neurons

appeared near normal, except for an almost complete absence of expected 5-HT_{2A}-mediated glutamate and GABA postsynaptic potentials normally displayed by neurons of the prefrontal cortex and dorsal raphe nucleus. Further analysis showed that BDNF mutants had much reduced 5-HT_{2A} receptor protein in dorsal raphe nucleus and a similar deficit in prefrontal cortex, a region that normally shows a high level of 5-HT_{2A} receptor expression. These findings suggest that postnatal levels of BDNF play a relatively limited role in maintaining presynaptic aspects of the serotonin system and a much greater role in maintaining postsynaptic 5-HT_{2A} receptors (Rios et al., 2006). A significant attenuation of 5-$HT1_A$ receptor function was also found in the dorsal hippocampus of NSE-tTA-TetOp-Cre-BDNF inducible knockout mice (Hensler et al., 2007;Monteggia et al., 2004), whereas no difference in 5-$HT1_A$ receptor function was found in the dorsal or median raphe nuclei or medial prefrontal cortex or anterior cingulate cortex, implying that deletion of BDNF expression in forebrain regions produces differential effects on distinct 5-$HT1_A$ receptor populations (Hensler et al., 2007).

Early-onset forebrain-restricted BDNF conditional knockout mice (*Emx-BDNF*^{KO}, Gorski et al., 2003) that develop in the absence of BDNF in the dorsal cortex, hippocampus, and parts of the ventral cortex and amygdala failed to learn the Morris Water Maze task, a hippocampal-dependent visuo-spatial learning task. Freezing during all phases of cued-contextual fear conditioning, a behavioral task designed to study hippocampal-dependent associative learning, was enhanced. These mice learned a brightness discrimination task well but were impaired in a more difficult pattern discrimination task. *Emx-BDNF*^{KO} mice did not exhibit altered sensory processing and gating, as measured by the acoustic startle response or prepulse inhibition of the startle response. Although they were less active in an open-field arena, they did not show alterations in anxiety, as measured in the elevated-plus maze, black-white chamber or mirrored chamber tasks. These data combined indicate that although an absence of forebrain BDNF does not disrupt acoustic sensory processing or alter baseline anxiety,

specific forms of learning are severely impaired. In addition, *Emx1-BDNF^{KO}* mice displayed a clasping phenotype, similar to that observed in mouse models of Huntington`s disease, and developed mild mature-onset obesity. Comparative volume measurements of major brain areas indicated that the striatum of postnatal Emx-BDNF^{KO} was reduced, explained by shrunken cell somas and thinner dendrites of striatal neurons, but not due striatal cell losses. However, although no significant striatal neuron losses were detected in young adult Emx-BDNF^{KO} mice, 35% of striatal neurons were lost in aged Emx-BDNF^{KO} mice. These findings indicated that BDNF has no significant survival role in the young adult brain, but that it is required for long term survival of neurons. Whether this loss of neurons is caused by a direct BDNF trophic survival function only apparent with aging, increased susceptibility to excitotoxic injury or some other type of insult, remains unclear (Gorski et al., 2003; Baquet et al., 2004).

Nestin-BDNF conditional knockout mice (Nestin-BDNF^{KO}) and CamKII-BDNF^{KO} mice with prenatal or postnatal depletion of central BDNF (Chan et al., 2006) were dramatically hyperactive during the light/dark cycles and hyperaggressive. They also exhibited a depression-like phenotype in the tail suspension test but not in the forced swim test. Interestingly, depletion of BDNF from the fetal brain had more pronounced effects on aggressive and depressive-like behaviors and led to deficits in 5-HT2$_A$ receptor content in the medial frontal cortex, highlighting the importance of this neurotrophin during development. It seems then that BDNF expression both pre- and postnatally is essential for normal modulation of behavior by neural circuits in the adult animal.

1.4 Aim of the thesis

To gain further insight into the function of BDNF in the adult brain and to complement previous studies by others using Cre lines causing area-specific excision of *bdnf,* we attempted a global BDNF deletion in the CNS by crossing floxed *bdnf* mice with mice expressing Cre from the *tau* locus. Our results show that mice survive several months after birth with nearly undetectable levels of BDNF protein in the CNS, which is analyzed in detail in the thesis. These mice also provided an essential tool in studies aiming at understanding the biosynthesis, storage and release of BDNF in the CNS.

2. Material and Methods

2.1 Generation of conditional *bdnf* knockout mice

The floxed *bdnf* (f*bdnf*) mouse line was a kind gift by Drs. Michael Sendtner and Stefan Wiese (University of Würzburg, Germany). Briefly, for the generation of mice with floxed *bdnf* alleles, loxP sites were inserted around exon 8, the single coding exon of *bdnf.* Thus, Cre-mediated recombination of floxed *bdnf* results in a null *bdnf* allele. Conditional *bdnf* knockout mice lacking BDNF in the entire mouse nervous system (*cbdnf ko*) were generated by breeding mice carrying floxed *bdnf* alleles with mice expressing Cre from the *tau* locus (Koreths-Smith et al., 2004). Genotypes of mice were determined by PCR (see 2.3) using a tail biopsie. Unless specifically mentioned, in all experiments 8 weeks old conditional mutants and wild-type littermate controls were used. The control mice for all experiments carried floxed *bdnf* alleles. The mice used in the present study were kept on a C57Bl6/Sv129 genetic background. The mice were maintained in the animal facility of the Biocenter, University of Basel, Switzerland. 3-4 mice were housed for 2 months before the start of experiments under conditions of controlled temperature (21–22 °C) and humidity (50%) under a 12:12 h light／dark cycle (lights on 06.00 h). The mice had free access to food and water. All experiments were performed in accordance with the Swiss regulations for animal experimentation.

2.2 Genotyping of *cbdnf* knockout mice

Mouse tails were incubated in 500 µl tail buffer (100 mM Trizma hydrochloride, pH 8.5, (Sigma, #T-3253); 5mM EDTA (Sigma, #E5134), 200mM NaCl (Fluka #71381), 0.2% SDS (Sigma, #L4390) containing 0.1µg/µl Proteinase K (Roche, 10mg/ml stock) on an Eppendorf shaker (400 rpm, 55°C over night). The solution was centrifuged (14000 rpm, 10 min) and the supernatant was transferred into 500 µl isopropanol (Fluka, #91237). The solution was centrifuged (14000 rpm, 10 min) and the supernatant was removed and precipitated DNA was subsequently dried and resuspended in 35 µl TE (10mM Tris-HCl, 1mM EDTA, pH 8). DNA was resuspended by agitation at 55°C over night before performing the PCR.

PCR Master Mix for 50 µl reaction volume:

	concentration
PCR buffer	**20 mM Tris-HCl; 50 mM KCl; 50 mM $MgCl_2$ (Sigma, #D1806-250UN)**
dNTPs	**200 nM (Sigma, #DNTP100-1KT)**
forward primer	**200 nM (Microsynth)**
reverse primer	**200 nM (Microsynth)**
Taq Polymerase	**2 units (Sigma, #D1806-250UN)**
DNA	**1 µl**

Primer pair *bdnf*13 and *bdnf*14 (for sequence and annealing temperature (AT) see Table below) detect the *bdnf* floxed (800 bp length) and *bdnf* wild-type allele (600 bp length); primer pair *bdnf*13 and *bdnf*19 detect the recombinant *bdnf* allele (1100 bp length). Primers *tko* forward and *tko* reverse detect the *tau* wild-type allele (200 bp length) and primer pair Cre forward and Cre reverse detect the Cre allele (400 bp length).

	Primer sequence 5` → 3`
***bdnf* 13**	**GTT GCG TAA GCT GTC TGT GCA CTG TGC**
***bdnf* 14**	**CAG ACT CAG AGG GCA CTT TGA TGG CTT G**
***bdnf* 19**	**CAT GGG CAG TGG AGT GTG AG**
***tko* forward**	**CTC AGC ATC CCA CCT GTA AC**
***tko* reverse**	**CCA GTT GTG TAT GTC CAC CC**
Cre forward	**GCC GAA ATT GCC AGG ATC AG**
Cre reverse	**AGC CAG CAG CTT GCA TGA TC**

PCR amplification protocol for genotyping:

step 1: 95°C 2 min
step 2: 95°C 30 sec
step 3: 61°C 30 sec Step 2-4 were repeated 34 times
step 4: 72°C 60 sec
step 5: 72°C 10 min

PCR products and 100 bp ladder (Roche, #11062590001)) were loaded on a 1.8 % agarose gel (Sigma #A5093) containing 0.5 µg/ml ethidiumbromide (Applichem, #A11520026). Gel detection was performed with Multi Image TM Light Cabinet detection system and print outs were done with a Mitsubishi video graphic printer CP770DW.

2.3 BDNF ImmunoAssay

Brains were freshly isolated and subdivided into sub-regions of the brain, weighed, collected on dry ice, and stored at -80°C. For the extraction of BDNF, 10 vol/wt of extraction buffer [0.05 *M* sodium acetate, pH 4.0, 1.0 *M* sodium chloride, 1% (vol/wt) BSA, 1% Triton X-100, 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.2 m*M* benzethonium chloride, 2 m*M* benzamidine hydrochloride was added and the tissues sonicated to homogeneity. The homogenates were kept on ice for 1 h and the sonication repeated three times. The homogenates were centrifuged (15 min, 25000 rpm), the supernatants collected and kept over-night at 4°C. A last centrifugation step was performed immediately preceding the use of the supernatants. For quantification of BDNF from tissue extracts, microtiter plates were coated with mAb#1 (0.4 µg/well) in 200 µl coating buffer (50 mM $NaHCO_3$, 50 mM Na_2CO_3, pH 9.7) over night at room temperature. Standard amounts of BDNF were diluted in extraction buffer (0.1–3.2 ng/ml), and 50 µl per well of each concentration was applied to 150 µl incubation buffer (0.1 *M* potassium/sodium phosphate, pH 8.0, 1% BSA, 0.2 m*M* phenylmethylsulfonyl-fluoride, 0.2 m*M* benzethonium chloride, 2 m*M* benzamidine hydrochloride). Fifty microliters per well of the extraction buffer alone was used as negative control. Similarly, 50 µl per well of the tissue extract supernatants was added to the wells. Subsequently, 200 µl per well of incubation buffer containing 100 mU/ml mAb#9 POD was applied. Plates were incubated for 3 h on a horizontal shaker at room temperature and washed with washing buffer (1x PBS, 0.1% Tween (Fluka, #P1379)). Two hundred microliters per well of the BM blue POD substrate (Roche, #114 84281001) was incubated for 12 min at room temperature on a horizontal shaker. The reaction was stopped with 50 µl of 1 *M* sulfonic acid (Fluka, #380075) per well, and the intensity of the reaction product was measured in a microplate reader (Biotek, PowerWave XS, KC4 software) at 450/650 nm. A standard curve was generated from the BDNF standard wells on each plate. BDNF levels in tissue extracts were determined by comparison with the standard curve using the GraphPad Prism software. All samples from an individual animal were run in triplicate, and the resulting quantities were averaged.

2.4 Tail suspension test

Wild-type and *cbdnf* knock-out mice at various ages were analyzed for limb clasping by suspending them from their tails at least 1 foot above a surface for 1 min. A clasping event was defined by the retraction of either or both hindlimbs into the body and toward the midline. Mice were scored on a simple “yes” or “no” basis.

2.5 Body Weight

Wild-type, heterozygous and mutant mice were maintained in a 12-h light/12-h dark cycle and fed a standard chow diet and water *ad libitum*. Growth curves for males and females were obtained by measuring body weight at 4, 6, 8, 10, 12 wk of age. Statistical significance was determined using a paired *t-test* and all values represent mean ± SEM.

2.6 Measurement of Locomotor Activity

Differences in locomotor activity were assessed during the light cycle by placing mutants 2 months of age and age-matched controls individually into cages and monitoring locomotor activity at baseline and after exposure to a novel chamber. Baseline activity was measured for 1 h subsequent to allowing animals to habituate to the activity monitor for 3 h. Activity was also monitored for 1 h (habituation period) immediately after placement into a fresh cage and for 1 h subsequent to habituation. Exposure to a novel cage has been used previously as a mild stressor (Baumgartner et al., 2001). Total activity was quantified using the Opto-Varimex-Mini infrared photocell activity monitor (Columbus Instruments, Columbus, OH). Statistical significance was determined using a unpaired *t*- test and values represent mean ± SEM.

2.7 Light/Dark Exploration Test

The light/dark exploration test is an accepted and frequently used anxiety test (van Gaalen et al., 2000). To test anxiety behavior, control and BDNF conditional mutant mice (n = 4), 4–8 wk of age, were placed in a box (20x20x45 cm) containing a light and dark chamber. The light chamber constructed of clear plastic material was two-thirds the size of the box and was brightly illuminated by a 150-W lamp. The dark compartment occupied the remaining third part of the box and was constructed of black plastic material that prevented the entrance of light. The two chambers were separated by a black plastic wall with a doorway (7x7 cm) to allow passage from one chamber to the other. Animals were placed in the dark compartment, and the latency for the first

transition to the light compartment, total time spent in the light compartment, and numbers of transitions from the dark compartment to the light compartment were monitored for a period of 5 min. The box was cleaned after testing each animal. Statistical significance was determined using an unpaired *t-test* and values represent mean ± SEM.

2.8 Volumetric analyses

Animals were heavily sedated by intraperitoneal injection of Ketalar (Parke Davies, 5mg/kg)/Rompun (Bayer Health Care, 100mg/kg) and perfused transcardially with 4% paraformaldehyde (Sigma, #P6148) in 1x PBS. The brains were removed and kept in fixative over night. Serial coronal 30-35 µm thick sections were obtained with a vibratome (Leica, VT 1000 S). Consecutive sections were submitted to cresyl-violet staining (Fluka, #255246). Stained sections were dehydrated in graded ethanol (Fluka, #459836) and xylene (Fluka, #95680), and coverslipped using Eukitt (Kindler, Freiburg, Germany). Sections were examined with a light microscope (Leica, 6x Ojective). Strict morphological criteria were used consistently in all mice to determine the boundaries of striatum, hippocampus and cortex (George Paxinos, The mouse bain). Briefly, the superior boundary of the striatum was defined by the corpus callosum, the lateral boundary by the external capsule, and the medial boundary by the lateral ventricle and the corpus callosum. The ventral boundary of the striatum was delineated by the anterior commissure, excluding the nucleus accumbens. For the cortex, the primary ventral boundary was the corpus callosum. A line connecting the rhinal fissure to the corpus callosum was used in more medial sections to define the anterior ventral portion of the neocortex. Entorhinal cortex was also included in cortical volume measurements. Hippocampal outlines encompassed the dentate gyrus, the CA1–3 fields of Ammon's horn, the subiculum, the presibiculum and the fimbria of the hippocampus**.** Starting with one of the four most anterior sections, selected on a random basis across brains, every fourth section through the anterior-posterior extent of both hemispheres of the brain was analyzed. Using this sampling strategy, 15 histological sections per brain were analyzed. All volumetric quantifications were performed with a Leica microscope (6x Objective) equipped with a camera. When calculating the volume of striatum, hippocampus, and cortex, the boundaries were defined and the volumes were determined with AnalysisD software according to the Cavalieri principle (Regeur and Pakkenberg, 1989).

2.9 Neuron/oligodendrocyte quantification

Mice were killed by cervical dislocation and the brains were extracted and embedded in O. C. T. medium (Electron Microscopy Sciences, #62550-01), and stored at -80°C. Serial coronal sections (10 µm) were obtained from *cbdnf* knockout and wild-type mice using a cryostat (Leica, CM 30505), and anti-NeuN/anti-Olig2 (Chemicon, MAB377, AB9600) immunhistochemistry was performed to identify neurons and oligodendrocytes. Briefly, after quenching of endogenous peroxidase activity with 0.3% H_2O_2/MeOH (Sigma, #32338-1) for 30 min, sections were blocked and permeabilized in 5% normal goat serum (Sigma, #G9023) and 0.5% NP-40 (Sigma, #74385) in 1x PBS for 1 hr. Sections were then washed and incubated overnight at 4°C with an antibody against NeuN (1:500)/Olig2 (1:500), followed by incubation with a secondary antibody, amplification using the Vectastain Elite ABC kit (Vector Laboratories, PK-6101, PK-6102), and incubation with AEC substrate (Vector Laboratories, SK-4200). To compare the number of neurons in the striatum of *cbdnf* mutants and wild-type mice, the number of NeuN-immunoreative cell bodies was counted in 8 (10 µm thick) sections containing the striatum and 120 µM apart using a 40x objective. NeuN positive cells were visualized using a Leica microscope equipped with a camera. Cell body counts in the acquired image were conducted using the measuring module of the AnalysisD software program. Statistical significance was determined using an unpaired *t*-test and all values represent mean ± S.E.M.

2.10 Electron microscopy

For electron microscopic analysis, mice were deeply anesthetized with Ketalar (Parke Davies, 5mg/kg) and Rompun (Bayer Health Care, 100mg/kg) and fixed by transcardiac perfusion with 3% glutaraldehyde/3% formaldehyde (Server, #23115; Sigma,#F-1635) in 0.1 M cacodylate buffer (0.2M Na(CH3)2 AsO2 • 3H2O, pH 7.4). Corpus callosum, spinal cord (L3-6) and optic nerve from 2 months old wild-type and *cbdnf ko* mice were dissected and kept over night for postfixation in 3% glutaraldehyde/3% formaldehyde in 0.1M cacodylate buffer at 4°C. Tissue sections were extensively washed with washing buffer (0.5% sodium chloride, 0.1M cacodylat buffer, pH 7.2) and then postfixed in 1% osmium tetroxide/1.5% potassium hexanoferrate (Sigma, #75632; Fluka, #480010) rinsed in 0.1 M cacodylate buffer for 3 h. Afterwards corpus callosum, spinal cord and optic nerve were dehydrated through graded ethanol solutions (70%, 80%, 96% and 100%), and soaked in propylene oxide (Fluka, #82320) for 1h. After infiltration for 2 h in

propylene oxide and Epon (Fluka, #45345) 1:1, samples were polymerized in propylene oxide and Epon 3:1 at room temperature overnight. Samples were then embedded with plastic capsules (Electron Microscopy Sciences, #70905-12) and polymerized at 37°C for 2h, followed by 45°C for 24h. Finally, the samples were polymerized at 65°C for 3 days. Semi-thin sections (1 µm) were cut in a plane orthogonal to the longitudinal axis of the optic nerve, spinal cord, corpus callosum with an ultramicrotome (Reichardt-Jung) and were stained with toluidine blue for light microscopy (DMRE Leica). Selected areas were further sectioned at 0.6 µm (ultrathin sections) for transmission electron microscopy. Ultrathin sections were collected on 200-mesh grids (Sigma, #G5901) and stained with lead citrate (Reynolds 1963). Immediately after staining, the grids were thoroughly washed with water. After removing the remaining water with filter paper (Schleicher & Schüll), the grids were air-dried before examination. Ultrathin sections were examined with an electron microscope (Philips EM400) at an accelerating voltage of 80 kV. Electron micrographs were randomly taken from the optic nerve, spinal cord and corpus callosum without knowing the genotype of the mice. The total area of the optic nerve and spinal cord was measured from semi-thin sections stained with toluidin blue using Analysis D software. Counts of axons were made at a final magnification of 8600x (optic nerve), 1600x (spinal cord) and 6500x (corpus callosum). For each probe, the sample consisted of 15 pictures corresponding to a total area of 18.33 μm^2 (optic nerve), 315 μm^2 (spinal cord), 18.35 μm^2 (corpus callosum). The axon size and thickness of myelin sheaths were measured by using Analysis D software at a final magnification of 8600x (optic nerve), 1600x (spinal cord) and 6500x (corpus callosum). Statistical analysis was performed with Microsoft Excel (*t-test*); all the graphs were generated in GraphPad Prism 4, with the exception of axonal size distribution, which was generated in Exel.

2.11 Quantitative RT-PCR

Total RNA was isolated by Trizol extraction (Invitrogen) from brains of 8 week old *cbdnf ko* and wild-type mice. 5 µg of RNA from each sample was used to generate cDNA, by using the SuperScriptTM First-Strand Synthesis System for RT-PCR kit (Invitrogen). Two microliter of the total RT product was used for each PCR reaction. Dye (Metabion) and HotStar Taq (Qiagen, #203203) were included in the PCR mix. Primers were as follows: *MAG*, sense 5`-CGCACGGTGGAGCTGAGT- 3`, antisense 5`-CCA CCA CCG TCC CAT TCA-3`, probe 5`-Fam-TCA TGT ATG CAC CTT GGA AGC CC-Tamra-3`;

MOG, sense 5`TGG CCC AGC TGC TTC CT- 3`, antisense 5`-GGA TGG GAT ACC CTG GTC CTA-3`, probe, 5`-Fam-TCA CTC TGA ATTGTC CTG CAT AGC-Tamra-3`, *PLP*, sense 5`-AGC GGG TGT GTC ATT GTT TG – 3`, antisense 5`-ACA ACA GTC AGG GCA TAG GTG AT – 3`, probe, 5`Fam –AAA CTT GTC GGG ATG TCC TAG CCA-Tamra-3`, *MBP*, sense 5`-GAC CCA AGA TGA AAA CCC AGT AGT-3`, antisense 5`-TTG GGA TGG AGG TGG TGT TC - 3, probe 5`-Fam-CAT TTC TTC AAG AAC ATT GTG ACA CCT – Tamra-3`, *CNPase*, sense 5`-TGT GCT GCA CTG TAC AAC CAA AT-3`, antisense 5`-AGG CCT TGC CAT ACG ATC TCT-3`, probe, 5`-CAC CAC CTC CTG CTG GGC GTA TTC T-Tamra-3`, *OSP*, sense 5`ACT GGT CTC TAC CAC TGC AAA CC-3`, antisense 5`-AGC AAT CAT GAG GGC TCT ACA AG-3`, probe 5`-Fam-CAT CCT CAT CCT TCC AGG CTA CGT-Tamra-3`, *MOBP*, sense 5`-CTG CTG CCC ACC CTT CAC-3`, antisense 5`-CGC TCT TGC AGA TGC TGT ACT T-3`, probe, 5`-Fam-CAA CTC CAA GCG TGA GAT CGT GGA CC-Tamra-3`, NGF, sense, 5`-GCC AAG GAC GCA GCT TTC TAT – 3`, antisense, 5`-AGT GAT CAG AGT GTA GAA CAA CAT GGA – 3`, probe, 5`-FAM-CTG GCC GCA GTG AGG TGC ATA GC – Tamra-3`; NT-3 (Mm_00435413_s1, Applied Biosystems); NT4/5 (Mm_01701592_g1; Applied Biosystems); GAPDH (Mm_99999915_g1; Applied Biosystems). Triplicate reactions were run for each sample for all myelin proteins, neurotrophins and GAPDH. Reactions were run on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Threshold cycle for each reaction was determined, using the ABI Prism 7000 SDS software (Applied Biosystems). The expression levels of myelin and neurotrophin genes were normalized against GAPDH.

2.12 Biosynthesis and processing of endogenous BDNF in hippocampal neurons that store and secrete BDNF, but not proBDNF

2.12.1 Generation of *bdnf*-myc knock-in mice

bdnf-myc knock-in (*bdnf-myc*) mice were generated by substituting exon V of the *bdnf* gene with a human *c-myc* tagged version of the gene. c-Myc was added at the C-terminus of wild-type BDNF following deletion of the last 3 amino acids. Recombinant BDNF-myc protein is indistinguishable from *wt* BDNF in survival assays using cultured chick nodose neurons and TrkB phosphorylation assay with mouse cortical neurons. Over several generations, the behavior and phenotype of *bdnf-myc* mice were

indistinguishable from those of wild-type animals and the levels of BDNF-myc protein were comparable with those found in wild-type animals in all regions tested.

2.12.2 Reagents

Recombinant cleavage-resistant pro-BDNF (3 arginine residues (R-4, R-3, and R-1 of the pro-domain), a site prohormone convertases recognize, were replaced with alanine) and BDNF-myc were produced using COS7 cells as in Fayard et al., 2005. Recombinant purified BDNF was a kind gift of Regeneron Pharmaceuticals (NY). Recombinant purified nonglycosylated pro-BDNF and polyclonal anti-pro-BDNF antibodies were a kind gift of Alomone Labs Ltd. (Israel). Monoclonal anti-BDNF antibodies (mAb#9 and mAb#1) were in Kolbeck et al., 1999. Polyclonal anti-BDNF (pAbN20) and anti-Myc antibodies (A14G) were purchased from Santa Cruz Biotechnology, Inc. (CA).

2.12.3 Extraction of BDNF from mouse hippocampus

BDNF extraction was performed as described in Kolbeck et al., 1999, with the following modifications. Hippocampi were dissected from 7-8-week old *wt*, *bdnf-myc*, and *cbdnf* KO mice, weighed, and then stored at –80°C. Five vol/wt of extraction buffer containing 0.05 M CH3COONa (pH4.0), 1 M NaCl, 0.1% Triton X-100 was added. For immunoprecipitation, twenty five vol/wt of RIPA buffer (50mM Tris-HCl (pH7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 1% Na deoxycholate, and 1% SDS) was added. To prevent protein degradation, especially pro-BDNF cleavage during extraction, freshly prepared protease inhibitors including protease inhibitor cocktail (Roche, Switzerland), 10 μM 1, 10-phenanthroline monohydrate, and 10 mM 6-aminohexanoic acid, 10 μg/ml aprotinin were added to the buffer. The tissues were sonicated, and the homogenates were centrifuged, followed by collection of the supernatants as in Kolbeck et al., 1999..

2.12.4 Cell culture

Cultured hippocampal neurons were prepared from embryonic day 16 mice (RCC, Switzerland). The dissected hippocampi were digested with trypsin and dissociated mechanically. Cells were plated in poly-D-lysine-coated dishes at $2x10^5$ cells/cm2 of cell density. One day after plating, serum (10% fetal calf serum)-containing medium was replaced with complete medium (Bibel et al., 2004). The cultures were then maintained until performing experiments.

2.12.5 Transfection

COS7 cells were transfected with constructs encoding rat *wt bdnf* or myctagged version of *bdnf* (*bdnf-myc*) as in Fayard et al., 2005. Constructs encoding *bdnf-myc* were a kind gift of Dr. Berengere Fayard (FMI, Basel, Switzerland), whereby human c-Myc was added at the end of C-terminus of wild-type BDNF, following deletion of the last 3 amino acids.

2.12.6 Pulse-chase experiments

COS7 cells or cultured hippocampal neurons were then preincubated with methionine/cysteine (Met/Cys)-free media for 2 hours, metabolically labeled for 2 hours with 200 mCi/ml of [35S]Met/Cys labeling mix (Amersham Pharmacia Biotech, IL), and rinsed with PBS. Cultures were incubated with fresh media and chased for indicated time. Cell lysates with RIPA buffer and conditioned media were then collected, followed by immunoprecipitation with indicated antibodies. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gels were fixed in 30% methanol and 10% acetic acid. Radioactive signals were detected with Photoimager (STORM 840; Amersham Pharmacia Biotech).

2.12.7 Western blotting

20 µg of total proteins in tissue lysates were separated on electrophoresis using 4-12% gradient SDS-polyacrylamide gels. After transfer of the proteins on the membrane, the membranes were blocked with 0.1% Tween 20/phosphate buffered saline (PBST) containing 5% skim milk, followed by incubating with polyclonal anti-BDNF, anti-pro-BDNF or anti-Myc antibody (1:500 with PBST containing 2% skim milk) at 4°C overnight. After 1-hour incubation with horseradish peroxidase-conjugated mouse anti-rabbit IgG antibody at room temperature, the signals were visualized using an ECL chemoluminescence system (Amersham Bioscience. NJ). Western blots following immunoprecipitation of tissue lysates (500 µg of total proteins) were performed using IP-Western Kit (GenScript) to eliminate non-specific signals from protein G (a protein with a molecular weight slightly higher than recombinant pro-BDNF) used for immunoprecipitation.

2.12.8 Electrophysiological experiments

Hippocampal transverse slices (400 µm thick) were prepared and maintained using standard procedure using a Vibratome (Leica VT 1000S, Wetzlar, Germany). Artificial cerebrospinal fluid (ACSF) at 4°C, containing (in mM) 124.05 NaCl, 2.95 KCl, 1.25 KH2PO4, 4.07 MgSO4, 1.87 MgSO4·7 H2O, 25.95 NaHCO3, 2.5 CaCl2·2 H2O and 9.98 glucose was used. All slices were prepared from 2-3-week old mice. For all experiments only littermates were used. All experiments were performed and analyzed without the experimenter knowing the genotype of the preparation. Monopolar tungsten electrodes were used for stimulation in the CA3 Schaffer-collateral region. Synaptic field potentials were elicited with frequency of 0.1 Hz. Responses were recorded with glass microelectrodes filled with 3 M NaCl (8-16 MOhm), and placed in the apical dendritic region (stratum radiatum) of the CA1 pyramidal neurons. LTD was induced with a low-frequency-stimulus (LFS) of 1Hz for 15 min (900 pulses). LTP was induced in the same slice that was used for the LTD experiments. For LTP induction three consecutive theta burst stimuli (TBS) were used. The TBS was delivered with 10 bursts of 4 pulses each (100 Hz, 200 µs duration, 200 ms inter-bust interval, 10 s inter-stimulus-interval [ISI]). As an indicator of synaptic strength the initial slope of the evoked fEPSPs was calculated, averaged across six consecutive measurements and expressed as percentages relative to the baseline mean. The normalized data of each experiment were then time-matched, averaged across experiments and expressed as means (±SEM). All statistical tests were unpaired *t-test*, two-tailed.

3.1 Generation of conditional *bdnf* (cbdnf) knockout mice

A floxed *bdnf* transgenic mouse line (a generous gift from Drs. Michael Sendtner and Stefan Wiese) was crossed with a mouse line expressing Cre from the *tau* locus (Korets-Smith et al., 2004; *fbdnfxtau*::Cre; Fig. 1a). Tau, a microtubule associated protein, is expressed in the vast majority, if not in all, post-mitotic neurons (E10.5) of the central and peripheral nervous system. Mice thus resulting from this breeding scheme (Fig. 1b; *bdnf* $^{\Delta/\Delta}$ *tau*$^{Cre/wt}$; *cbdnf ko*;) should develop in the absence of detectable BDNF in the central and peripheral nervous system assuming that Tau-expressing cells are the major sites of BDNF expression. A previous study revealed that, intercrosses between the *tau*::Cre mouse line and Cre-activatable reporter lines resulted in offsprings with recombination either restricted to the nervous system or throughout the entire embryo, indicating that Tau is expressed very early in development and that it is the context in which the loxP target alleles are expressed that contributes to differences in the pattern of excision (Korets-Smith et al., 2004).

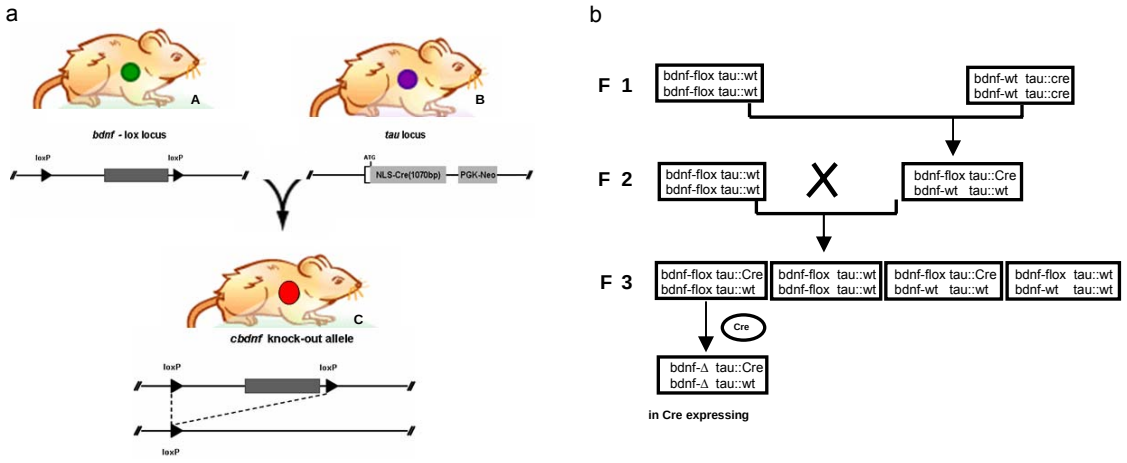

Fig. 1: Generating adult mice lacking BDNF in the CNS. **a:** *A,* Diagram of the *bdnf* lox allele. Exon VIII, the BDNF coding exon, is shown schematically flanked by lox sites (filled triangles).*B,* Targeted insertion of the Cre recombinase into the *tau* locus. Structure of the targeted allele; the Cre coding segment inserted into exon 1 provides the translation start site (ATG). *C,* Diagram of the *bdnf* lox allele after Cre-mediated recombination. BDNF-coding sequences in exon VIII are excised. **b:** Breeding scheme. Mice homozygous for the floxed *bdnf* allele are mated with mice homozygous for the *tau*::Cre allele to obtain mice heterozygous for both the floxed *bdnf* allele and *tau*::Cre allele (F2). The double heterozygous mice are mated with mice homozygous for the floxed *bdnf* allele to obtain *fbdnfxtau*::Cre mutants (F3). Δ: *bdnf* null allele

As determined by PCR using genomic DNA prepared from the progeny of *fbdnfx tau*::Cre (Fig. 2), the *fbdnfxtau*::Cre mutants fell into 3 categories: *bdnf* full knockout (19%), *bdnf* mosaic (5%) and tissue-specific *bdnf* knockout mice (1%) (Fig. 3A). *Bdnf* full knockout mice carried a *bdnf* null and no floxed *bdnf* allele, indicating that the levels of Cre present in the zygote is sufficient in many cases to catalyze excision of the floxed allele. *Bdnf* mosaic mice showed a floxed and a *bdnf* null allele, presumably as the result of a stochastic excision of the floxed *bdnf* allele in the preimplantation or early postimplantation embryo. Tissue-specific conditional *bdnf* knockout mice had a floxed *bdnf* allele and no *bdnf* null allele, indicating that Cre-mediated recombination did not take place in tissues contributing to the tail DNA (Fig. 2).

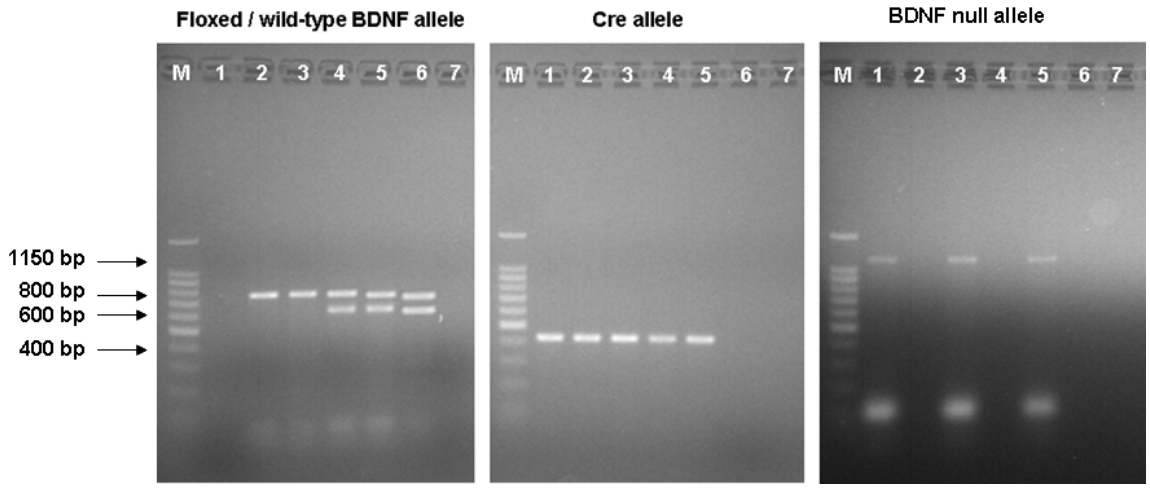

Fig. 2: Genotyping *fbdnfxtau*::Cre littermates. The genotype of each animal is assessed by PCR of genomic DNA isolated from tail (see Material and Methods for primer sequences and protocol). Primers for *bdnf* gene detection amplify a wild-type (*bdnf* +/+) fragment of 600 bp length and a floxed (*bdnf* flox/flox) fragment of 800 bp length. Primers for Cre gene amplify a fragment of 400 bp length. Primers for *bdnf* null gene detection amplify a fragment of 1150 bp length. Lane 1: *bdnf* full knockout mouse; Lane 2: tissue-specific *bdnf* knockout mouse; Lane 3: *bdnf* mosaic knockout mouse; Lane 4: heterozgous tissue-specific *bdnf* knockout mouse; Lane 5: heterozygous *bdnf* mosaic knockout mouse; Lane 6: *bdnf* wild-type mouse; Lane 7: negative control; M: 100bp DNA ladder

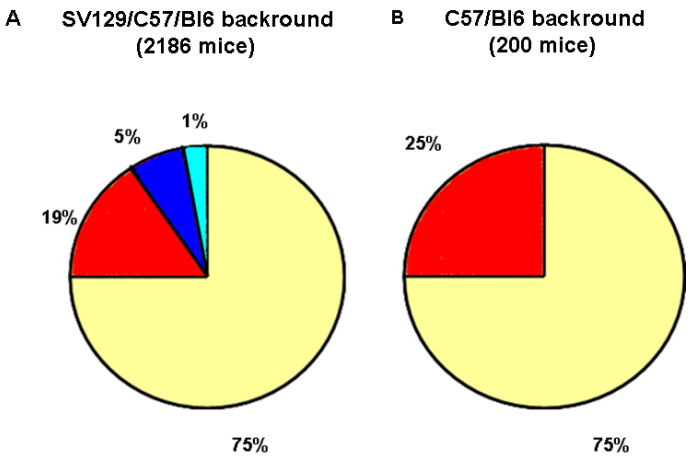

Fig. 3: Results of genotyping *fbdnf* x*tau*::Cre littermates. A) Total number of *fbdnf* x*tau*::Cre littermates (SV129/ C57/Bl6; 2186 mice) analyzed by PCR. B) Total number of analyzed *fbdnf* x*tau*::Cre littermates in C57/Bl6 backround (200 mice). As Korets-Smith et al. (2004) demonstrated that the percentage of tissue-specific deletion of the *tau*::Cre deleter was dependent on the Cre reporter used, we backcrossed the floxed *bdnf* as well as the *tau*::Cre mouse line, both Sv129/C57/Bl6, into a C57/Bl6 backround. To this end, the floxed *bdnf* mouse line was backcrossed 8 generations (~99% C57/Bl6), the *tau*::Cre mouse line 5 generations (94% C57/Bl6). As it turned out, all mutants generated in the C57/Bl6 backround were *bdnf* full knockout mice. Yellow: *bdnf* heterozygous and wild-type mice; red: *bdnf* full knockout mice; blue: mosaic *bdnf* knockout mice; turquoise: tissue-specific *bdnf* knockout mice.

We found the *bdnf* mosaics and tissue-specific *bdnf* knockout mice were able to survive up to eight months. To confirm that BDNF was depleted from their CNS, BDNF protein levels were measured by ELISA. A ≥ 95% reduction was found throughout the CNS, compared with wild-type mice (unpaired *t-test*, $p<0.001$, Fig. 4). Beside the CNS, BDNF is known to be also expressed in peripheral tissues such as heart, lung and muscle (Maissonpierre et al., 1990). Previous studies have also indicated that in the absence of BDNF, sensory axons fail to innervate the carotid body, contributing to the early death of *bdnf* full knockout mice (Hellard et al., 2004, Erickson et al., 1996). In our mosaic animals, BDNF protein levels were found to be unchanged in heart, lung and skeletal muscle (Fig. 5). Based on these findings, we assume that organs critical for postnatal survival such as the carotid body are likely to be innervated, such that vital functions, including breathing, develop adequately. It thus appears that *bdnf* mosaic and tissue-specific *bdnf* knockout mice (summarized as *cbdnf ko*) represent an interesting and novel model to study the role of BDNF in the adult mouse nervous system. As the postnatal increase in BDNF levels is completed by about 3 weeks after birth, we focused our analysis of *cbdnf ko* mutants at 8 weeks after birth when mice can be considered as sexually mature, young adult animals.

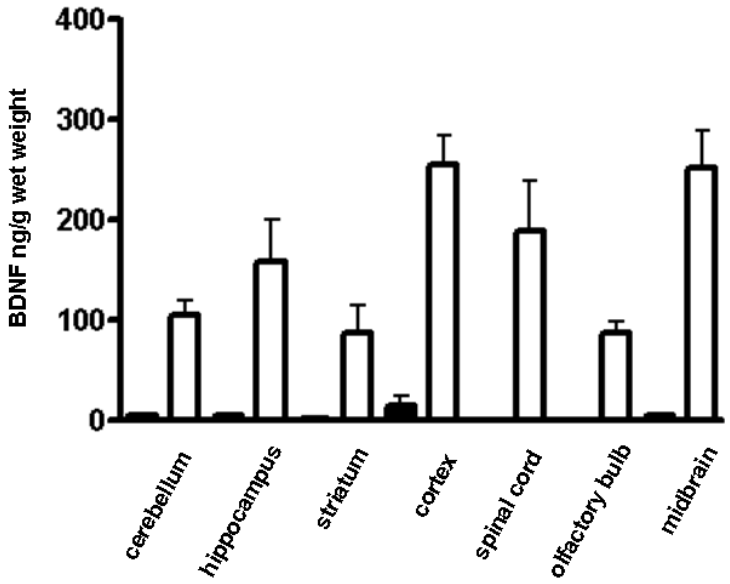

Fig. 4: Quantification of BDNF protein by ELISA at P56 in various CNS areas. All results are presented as a mean determined from the analysis of 4 mice per genotype ($p<0.001$, unpaired *t-test*). Black bars are *cbdnf ko*, white bars are wild-type.

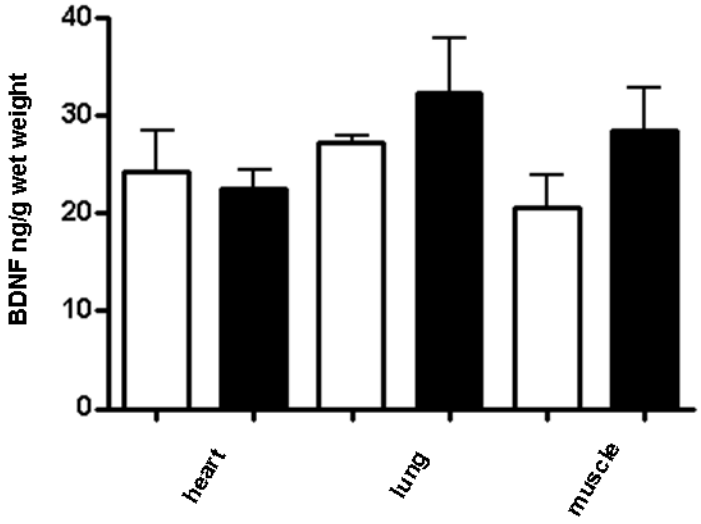

Fig. 5: Quantification of BDNF protein by ELISA at P56 in peripheral tissues. All results are presented as a mean determined from the analysis of 4 mice per genotype ($p>0.5$, unpaired *t-test*); Black bars are *cbdnf ko*, white bars are wild-type.

3.2 Phenotype of c*bdnf* knockout mice

3.2.1 General observations

At birth, *cbdnf ko* mice had the same size as their littermates, respond to a foot pinch, and display motor activity. After birth, conditional mutants lag behind littermates in growth, display periods of hyperactivity and of inactivity and can easily be recognized by their posture and movement. However, there was no significant difference in body length between 8 weeks old *cbdnf ko* and wild-type mice, indicating that skeletal growth was not affected (unpaired *t-test*, p=0.88, Fig. 6).

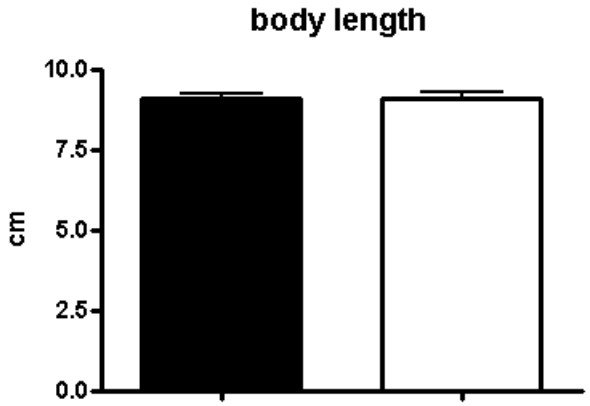

Fig. 6: Normal skeletal growth of 2 months old *cbdnf ko* mice. No significant differences in body length were detected between the genotypes. Results are presented as a mean ±SEM determined from the analysis of 6 mice per genotype (p>0.05; unpaired *t-test*). Black bars are *cbdnf ko* and white wild-type mice.

In their home cage environment, 8 weeks old *cbdnf ko* mice displayed no obvious movement abnormalities such as rotation, or alteration of stereotyped behaviours that would distinguish them from controls. However, typical movements associated with exploratory behaviours such as brief stopping, sniffing, rearing, or leaning against the wall were all reduced in *cbdnf ko* mice.

3.2.2 Body weight of *cbdnf ko* mice

Beyond 4 weeks of age, female conditional mutants had increased body weights compared with littermate controls, and this difference reached statistical significance at 6 weeks of age (43% increase in body mass compared with wild-type, (p<0.05, n=3; Fig. 7A). By 12 weeks of age, mutant females were 72% heavier than age-matched controls, respectively. The weight of mutant females at 12 weeks was 38.5g ±2.12 compared with 22.3g ±2.21 for the controls (unpaired *t-test*; P=0.003, n=3/genotype; Fig. 7A). Surprisingly, male *cbdnf ko* mice did not become obese. This is in contrast with previous reports on the phenotype of heterozygotes (Lyons et al., 1999; Kernie et al., 2000). Conditional mutant males had a mean weight value of 29.26g ±2.69 compared with 27.02g ±1.31 for the controls by 6 weeks of age (unpaired *t-test*; P>0.05, n=6 mice per genotype; Fig. 7B). We have no explanation for this surprising sex-specificity of the matured obesity phenotype (only observed in female animals), an observation that warrants further studies.

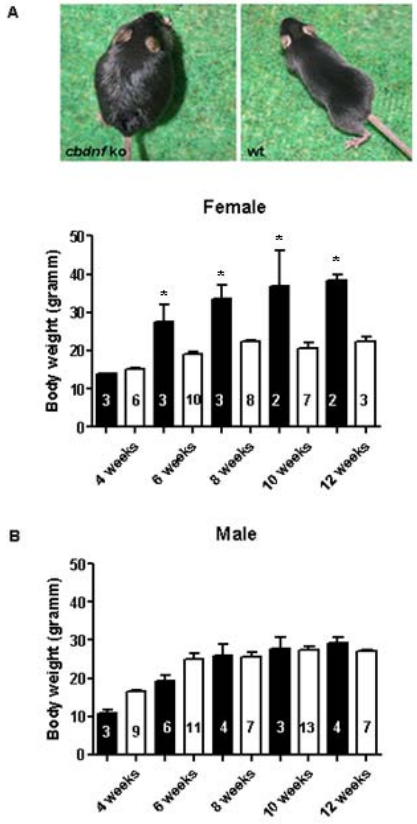

Fig. 7: Body weight and skeletal growth of *cbdnf ko* mice.
A) Growth of female *cbdnf ko* and control mice. Female *cbdnf ko* mice show maturity-onset obesity. B) Growth of male *cbdnf ko* and control mice. Male *cbdnf ko* mice show no maturity-onset obesity. All results are presented as a mean determined from the analysis of n mice/genotype (*, p<0.05, unpaired *t-test*). Black bars are *cbdnf ko* and white are wild-type.

3.2.3 Activity of *cbdnf ko* mice

cbdnf ko mice showed overall normal locomotor activity. However, they were hyperactive for brief periods of time when exposed to a novel environment.

3.2.4 Anxiety-like behavior of *cbdnf ko* mice

cbdnf ko mice showed an increase in anxiety-like behavior during the light/dark exploration test (Fig. 9). At all ages examined it took mutants longer to make the first transition from the dark to the light compartment, and they spent less time in the light zone compared with the controls, thus suggesting an anxiety-like behavior.

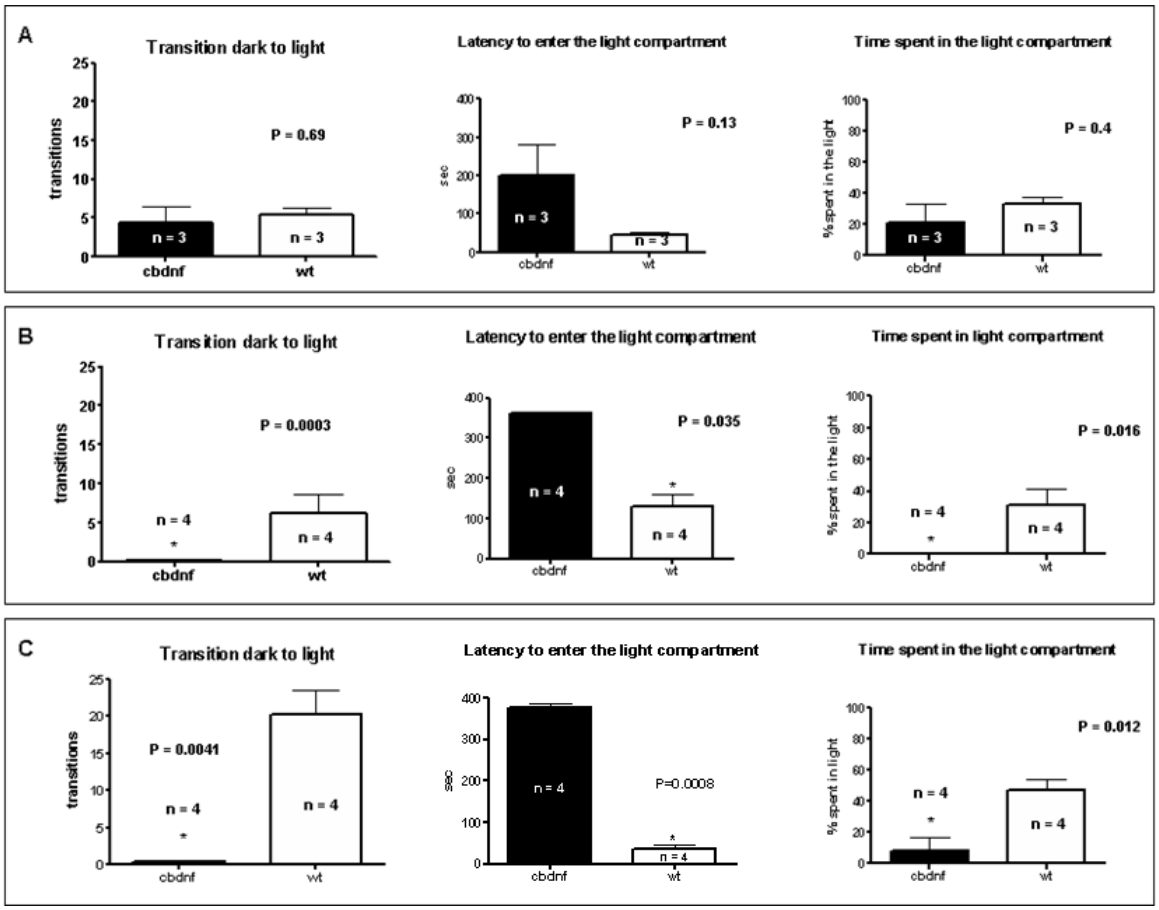

Fig. 9: Dark/light exploration test. Each *cbdnf ko* and control mouse was tested for a period of 5 minutes in a 2-chamber (dark v. light) test. A: 4 weeks old mice, B: 6 weeks old mice, C: 8 weeks old mice. All results are presented as a mean ±SEM determined from the analysis of n mice/genotype (*p<0.05; unpaired *t-test*). Black bars are *cbdnf ko* and white wild-type.

3.2.5 Brain weight of *cbdnf ko* mice

At 8 weeks, the brain weight of *cbdnf ko* mice was significantly reduced (0.37mg ±0.02) compared with that of wild-type mice (0.44mg ±0.01) (unpaired *t-test*, $p<0.05$; Fig. 10a) In addition, *cbdnf ko* mice exhibit an early onset clasping phenotype that is seen in several neurodegenerative mouse models including in particular Huntington`s disease (Lalonde, 1987; van den Akker et al., 1999; Auerbach et al., 2001; Guidetti et al., 2001; van Dellen et al., 2001). This phenotype was seen in 25% of *cbdnf ko* mice at 1 month and in all by 2 months (Fig. 10b).

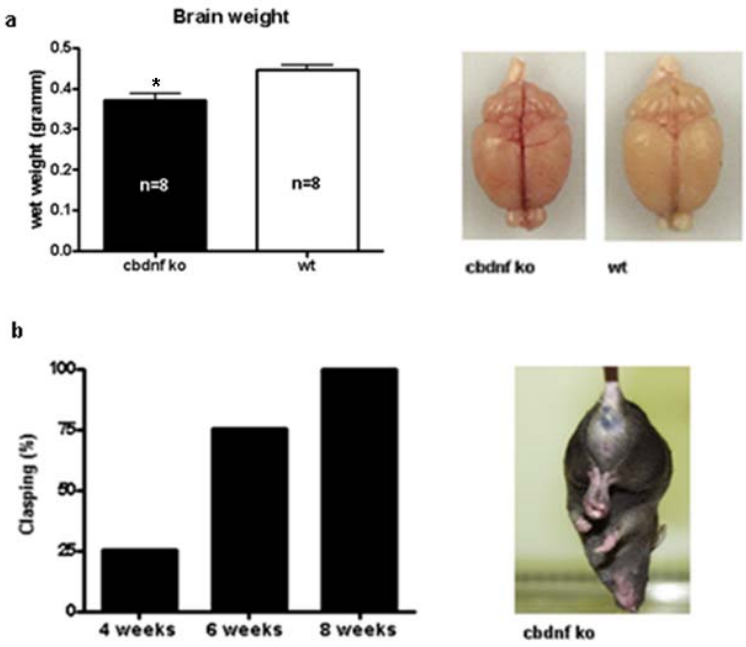

Fig. 10: *cbdnf ko* mice have reduced brain weight and show clasping phenotype. a: Brain wet weight of *cbdnf ko* mice was significantly different from control. Results are presented as a mean ±SEM determined from the analysis of n mice/genotype (*$p<0.05$; unpaired *t-test*). Black bars are *cbdnf ko* and white wild-type. b: Clasping phenotype. *cbdnf ko* and wild-type mice at 4, 6 and 8 weeks were suspended by their tails for 1 min. Clasping was defined as the balling up of both of the hindlimb paws.

3.3 Comparative CNS volume measurements of major brain areas in *cbdnf* knockout mice

To determine if there is a global or an area-specific reduction in brain size of *cbdnf ko* mice, comparative volume measurements were performed at P14, P35, and P56 on Nissl-stained sections of the hippocampus, cerebral cortex and striatum. The hippocampal volume of *cbdnf ko* mice was not significantly reduced compared with wild-type controls at all ages examined (Fig. 11), though a small (~10%) but not statistically significant reduction was observed at P14. At P35 and P56, average hippocampal volumes of *cbdnf ko* mice were comparable to wild-type values (unpaired *t-test*, p>0.05, Fig. 11). In contrast to the hippocampus, the volume of the cerebral cortex of *cbdnf ko* mice was smaller than controls at all ages examined (Fig. 11). At P14, a statistically significant 17% reduction was observed between *cbdnf ko* mice and wild-type controls (unpaired *t-test*, p<0.05, Fig. 11). At P35 and P56, the cortex of *cbdnf ko* mice was ~ 20% smaller than the cortical volumes of wild-types (unpaired *t-test*, p<0.05). Striatal volume was reduced in *cbdnf ko* mice compared with wild-type at all ages analyzed (Fig. 11). At P14 and P35, the striatal volume of *cbdnf ko* mice was ~16% less than wild-type littermates (unpaired *t-test*, p<0.05). At P56 the difference in striatal volumes between mutant and wild-type mice reached 36% and was significantly reduced in *cbdnf ko* mice compared with wild-type (unpaired *t-test*, p<0.05).

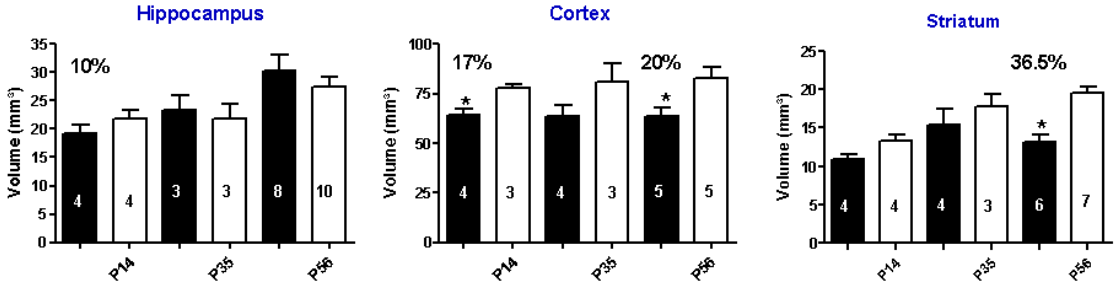

Fig. 11: The volume of both, the striatum and cortex, but not of the hippocampus fails to grow in the mutant between P14 and P56. Measurements were performed on Nissl stained sections from *cbdnf ko* and wild-type mice (P14; P35; P56) by Cavalieri analysis. All results are presented as a mean ±SEM determined from the analysis of n mice/genotype (*p< 0.05; unpaired *t-test*). Black bars are *cbdnf ko* and white wild-type.

Comparative volume measurements were also performed on Nissl-stained sections on the dentate gyrus and the olfactory bulb, as these brain areas are well-known sites involved in adult neurogenesis and BDNF has been repeatedly discussed in the context of adult neurogenesis (see discussion). However, neither the volume of the dentate gyrus nor that of the olfactory bulb of *cbdnf ko* mice were significantly reduced compared with wild-type controls at P56 (Fig. 12). While these results may suggest that endogenous BDNF does not play a significant role in adult neurogenesis, a recent report indicates that only a small portion of newly generated neurons stably integrate in the dentate gyrus (Ninkovic et al., 2007)

In sum then, comparative volume measurements of major brain areas in 8 weeks old *cbdnf ko* mice showed a region-specific volume reduction of the cortex (20%) and striatum (36%), but not in the hippocampus. Therefore, the loss of brain volume associated with an almost complete absence of BDNF in the CNS reveals a surprising regional specificity in the postnatal growth-promoting effects of BDNF when different brain areas are compared.

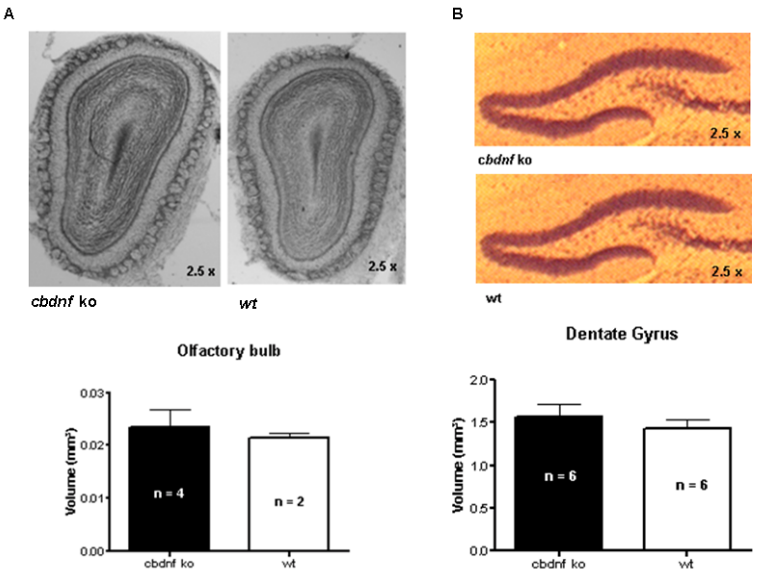

Fig. 12: Comparative volume measurements in the dentate gyrus and olfactory bulb at P56. Measurements were performed on Nissl stained sections of *cbdnf ko* and wild-type mice by Cavalieri analysis. All results are presented as a mean ±SEM determined from the analysis of n mice/genotype (*p<0.05; unpaired *t-test*). Black bars are *cbdnf ko* and white wild-type.

3.4 The striatal volume is reduced at 8 weeks, but cell losses are not apparent

To examine whether the decrease in striatal volume was caused by cell loss, neuronal numbers were determined using NeuN staining. No significant differences were found (unpaired *t-test*, p>0.05, Fig. 13 D), though neuronal density was increased (as expected) (p<0.05, Fig. 13 A, C). To determine whether the number of mature oligodendrocytes was affected, we also performed Olig-2 immunostainings on sections of 2-month old *cbdnf ko* mice. No significant decrease in the number of Olig-2 positive cells was found in the striatum of 2-month old *cbdnf ko* mice when compared to wild-type mice (unpaired *t-test*, p> 0.05, Fig. 13 F). Taken together, these data suggest that BDNF is not required for the survival of striatal neurons or of oligodendrocytes during the first 8 weeks after birth.

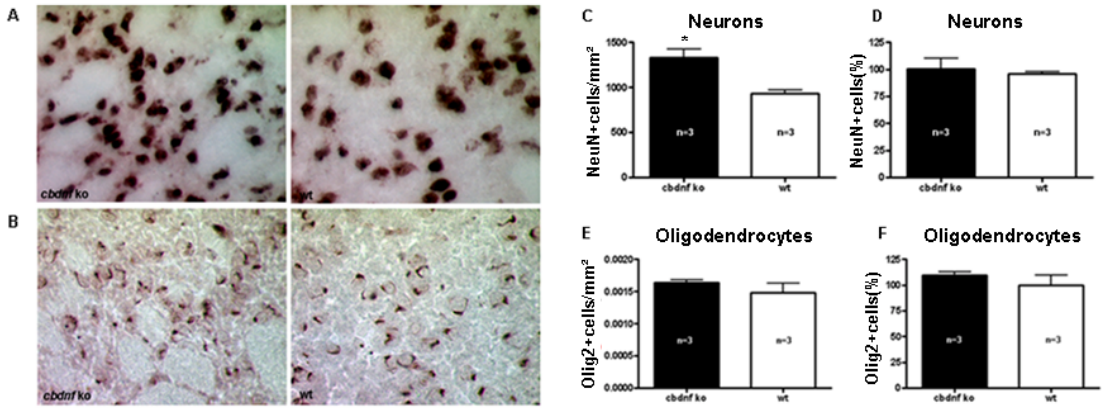

Fig. 13: The numbers of neurons and oligodendrocytes are analyzed in the striatum of 2-month old *cbdnf ko* mice. Representative images of the striatum of *cbdnf ko* and wild-type mice immunostained for NeuN (A) and for Olig-2 (B) (10 µm cryostat sections at 40x magnification). C) Neuronal density in the striatum of *cbdnf ko* and wild-type mice (n=3 per genotype). The neuronal density was higher in the striatum of *cbdnf ko* mice (p<0.05). D) Number of NeuN positive cells in the striatum of *cbdnf ko* and wild-type mice. No significant differences were detected between the genotypes (p>0.05; n=3 mice per genotype). E) Oligodendrocyte density in the striatum of *cbdnf ko* and wild-type mice (n=3 mice per genotype). Oligodendrocyte density in *cbdnf ko* mice appears indistinguishable from control (p>0.05). F) Number of Olig-2 positive cells in the striatum of *cbdnf ko* and wild-type mice. No differences between the genotypes were detected (p> 0.05; n=3 mice per genotype).

3.5 Axonal diameter and myelination in the optic nerve, corpus callosum and spinal cord of *cbdnf ko* mice

Based on the finding that by contrast with the striatum the volume of the hippocampus was unchanged, we then tested the possibility that the lack of BDNF may affect axonal diameter and myelination, as suggested by previous studies on BDNF and TrkB gene deletion (see Cellerino et al., 1997; Minichiello et al., 1999; Medina et al., 2004).

Fig. 14: Distribution of myelin in the adult brain. Parasagittal section (10µm) of wild-type brain at P56 were stained with an antibody to myelin oligodendrocyte glycoprotein (MOG). 1: Cortex, 2: Hippocampus, 3: Striatum, Arrow: cortico-striatal afferents

As the structure of the striatum does not lend itself to quantitative studies of axon diameter, axon numbers and the thickness of myelin sheaths, we turned to an electron microscopy (EM) study of the optic nerve, corpus callosum and spinal cord. These anatomical regions contain axons running in parallel, a feature that greatly facilitates the quantification of myelinated axons, axon density and diameter, as well as the thickness of the myelin sheath per axon. To ensure that

similar regions were analyzed in each animal, we generated 1 mm brain sections with a brain slicer, took always the very same brain region (“section 7/8”) and analyzed sagittal sections of the genu of the corpus callosum. In the spinal cord, cross sections were examined at the level of the cervical enlargement. The optic nerve is sufficiently small and homogenous to analyze entire cross sections of its intracranial segment.

Analysis of the optic nerve from *cbdnf ko* mice revealed no reduction in the cross –sectional area relative to wild-type mice (unpaired *t-test*, p=0.52; Fig. 15). Axons of the optic nerve were also counted and no significant differences in the number of axons were found in 2-month old *cbdnf ko* mice compared with wild-type mice (unpaired *t-test*, p=0.82, Fig. 15), indicating that RGC survival is not reduced in the absence of BDNF. Consequently, axon density of *cbdnf ko* mice appears indistinguishable from wild-type animals (unpaired *t-test*, p=0.54, Fig. 15). Morphometric analysis of retinal ganglion cell axons in the optic nerve of 2-month old *cbdnf ko* mice revealed no significant difference in the mean size of myelinated axons from wild-type mice (unpaired *t-test*, p>0.05 , Fig. 16). Further, the myelin sheaths of the optic nerve showed no signs of dysmyelination (Fig. 16) and the myelin sheath thickness of *cbdnf ko* animals appeared indistinguishable from wild-type (unpaired *t-test*, p>0.05, Fig. 16). The relationship between axonal diameter and myelin sheath thickness (g-ratio) revealed no significant differences in 2-month old *cbdnf ko* and wild-type optic nerves (unpaired *t-test*, p>0.05, Fig. 16). Moreover, the distribution of axonal diameter showed a unimodal pattern in both *cbdnf ko* and control animals (unpaired *t-test*, p>0.05, Fig. 16).

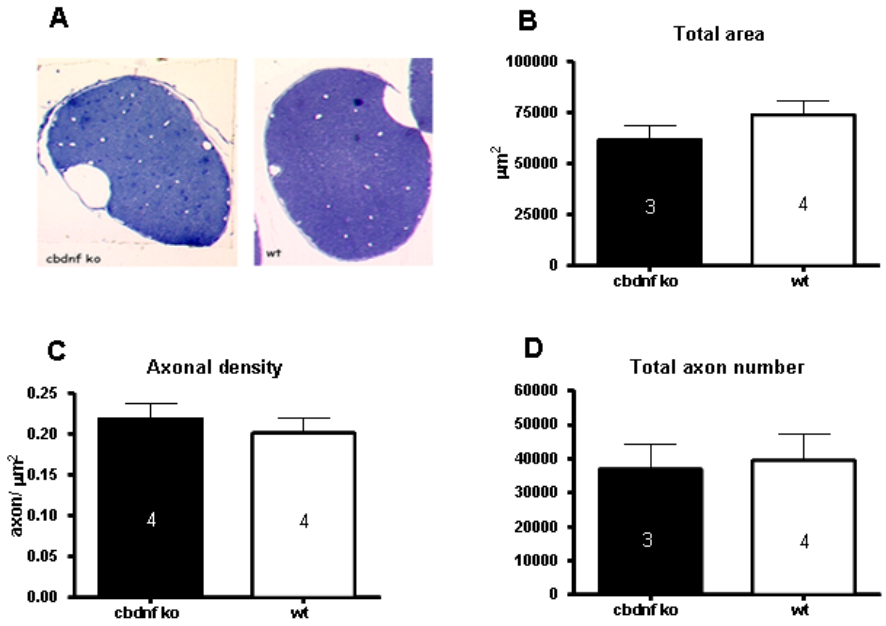

Fig. 15: Analysis of the optic nerve of *cbdnf ko* compared with wild-type animals. A) Light micrographs of semi-thin sections stained with toluidine blue of *cbdnf ko* and control mice. B) Total area of the optic nerve was not significantly different from controls (p=0.52). C) The axonal density in the optic nerve of *cbdnf ko* animals was not significantly different from control (p=0.54). D) The total number of retinal ganglion cell axons of *cbdnf ko* mice was not significantly different from controls (p=0.82). Note: Retinal Ganglion cell axons were counted on transverse ultrathin sections of the optic nerve. Random fields were photographed, axons were counted from prints, and the axonal density was multiplied by the total area of the nerve to determine the total number of axons. The data are presented as the mean ±95% interval confidence from the analysis of n mice/genotype.

Morphometric analyses of myelinated fibers in cross sections of the corpus callosum of 2-month old *cbdnf ko* mice also indicated that the size of axons and the axonal density was indistinguishable from control animals (unpaired *t-test*, p> 0.05, Fig. 17C, D). In addition, the myelin sheath thickness of *cbdnf ko* mice was not significantly different from control animals (unpaired *t-test*, p>0.05, Fig. 17E). Myelinated fibers from the corpus callosum of *cbdnf ko* and control animals revealed no significant increase in the ratio of axon diameter to fiber diameter (g-ratio) when compared to wild-type mice (unpaired *t-test*, p>0.05, Fig. 17F). Moreover, the frequency histogramm of *cbdnf ko* and control animals did not differ significantly (unpaired *t-test*, p>0.05, Fig. 17G). To determine whether there were structural abnormalities in myelin from the spinal cord of *cbdnf* ko mice, we analyzed white matter tracts at the level of the cervical enlargement. The size of axons, the axonal density as well as the myelin sheath of 2-month old *cbdnf ko*

mice was not significantly different from control animals (unpaired *t-test*; p>0.05; Fig. 18). These observations suggest that there are no clear cut differences in myelination of *cbdnf ko* mice and thus indicating, that BDNF does not contribute to the growth of axons and of their myelin sheath in the optic nerve, corpus callosum and spinal cord.

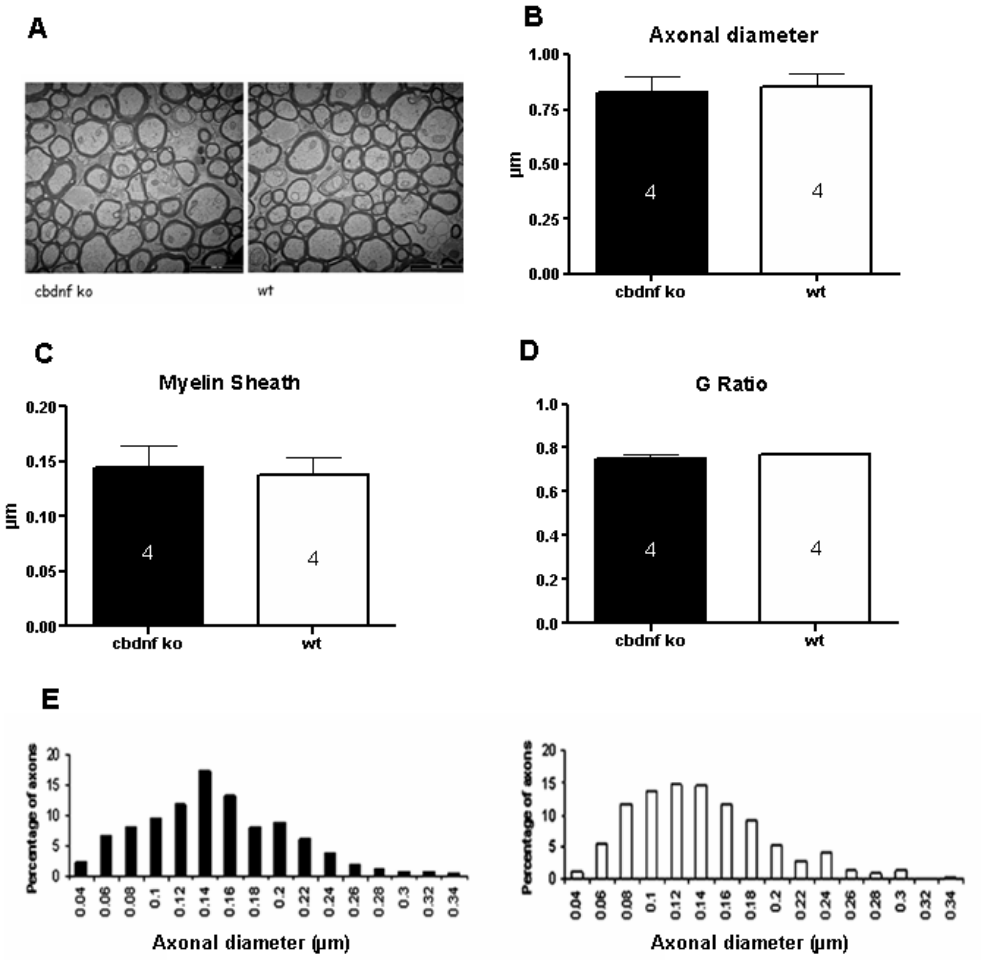

Fig. 16: Ultrastructure of the optic nerve in 2-month old *cbdnf ko* mice. A) Representative EM pictures of myelinated fibers in cross sections of the optic nerve of *cbdnf ko* and control animals. In the mutant the diameter of axons and their density appears indistinguishable from control animals. The myelin sheaths in the mutant show no signs of dysmyelination. Each EM picture is representative of sections from 4 different animals. B) Axonal diameters of *cbdnf ko* animals were not significantly different from control animals (0.83 ±0.16 vs. 0.85 ±0.12; p=0.78). C) The myelin sheath thickness of *cbdnf ko* animals was not significantly different from control animals (0.15 ±0.01 vs. 0.15 ±0.01; p=0.77). D) The myelinated axons of the optic nerve of *cbdnf ko* and control animals were not significantly different in G-ratio (0.75 ±0.04 vs. 0.77 ±0.02; p=0.33). E) Distribution of axonal diameter in the optic nerve. The percentage of axons within a particular size range is shown for a total of 455 axons. The frequency histogram showed a unimodal pattern in both *cbdnf ko* and control animals. Note: Axonal density was calculated by counting the total number of myelinated axons in 15 randomly selected non-overlapping fields of 18.33 µm² from the optic nerve and the axon density was multiplied by the nerve area to determine the total number of axons. Axonal diameters and myelin sheath thickness were measured from electronmicrographs using AnalysisD software and graphs were generated in GraphPad Prism. The G-ratio is the ratio between axonal diameter and total fiber diameter. Statistical analysis: the mean of each parameter for the single animals was calculated in Excel. Means obtained from *cbdnf ko* and control animals were compared using an unpaired *t-test*.

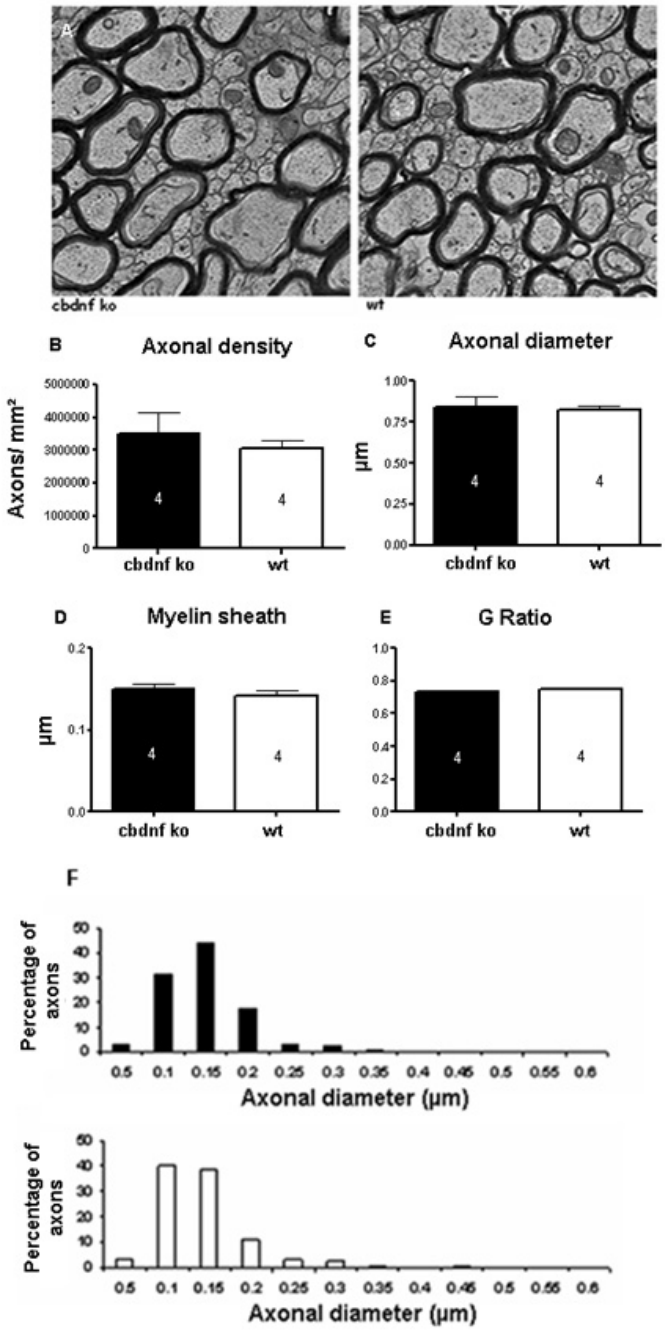

Fig. 17: Ultrastructure in the corpus callosum in 2-month old *cbdnf ko* mice. A) Representative EM pictures of myelinated fibers in cross sections of the corpus callosum. In the mutant the diameter of axons and their density appears indistinguishable from control animals. The myelin sheaths in the mutant show no signs of dysmyelination. B) The density of axons in *cbdnf ko* animals were not significantly different from control animals (p=0.57). The number of myelinated axons per mm² is presented. C) Axonal diameter of mutant mice was not significantly different from control animals (0.84 ± 0.16 vs. 0.85 ±0.12; p=0.88). D) Myelin sheath thickness of *cbdnf ko* mice was not significantly different from control animals (0.15 ±0.01 vs. 0.14 ±0.02; p=0.41). E) Myelinated fibers from the corpus callosum of *cbdnf ko* and control animals were not significantly different in G-Ratio (0.74 ±0.02 vs. 0.75 ±0.02; p=0.53). F) Distribution of axonal diameter in the corpus callosum. The percentage of axons within a particular size range is shown for a total of 463 axons. The frequency histogramm of *cbdnf ko* and control animals did not differ significantly. Note: Axonal density was calculated by counting the total number of myelinated axons in 15 randomly selected non overlapping fields of 18.35 µm² from coronal sections in the genu of corpus callosum. Axonal diameters and myelin sheath thickness were measured from electron-micrographs using AnalysisD software and graphs were generated in GraphPad Prism. The G-Ratio is the ratio between axonal diameter and total fiber diameter. Statistical analysis: the mean of each parameter for the single animals was calculated in Exel. Mean obtained from *cbdnf ko* and control animals were compared using an unpaired *t-tes*t. Black bars are *cbdnf ko* and white wild-type

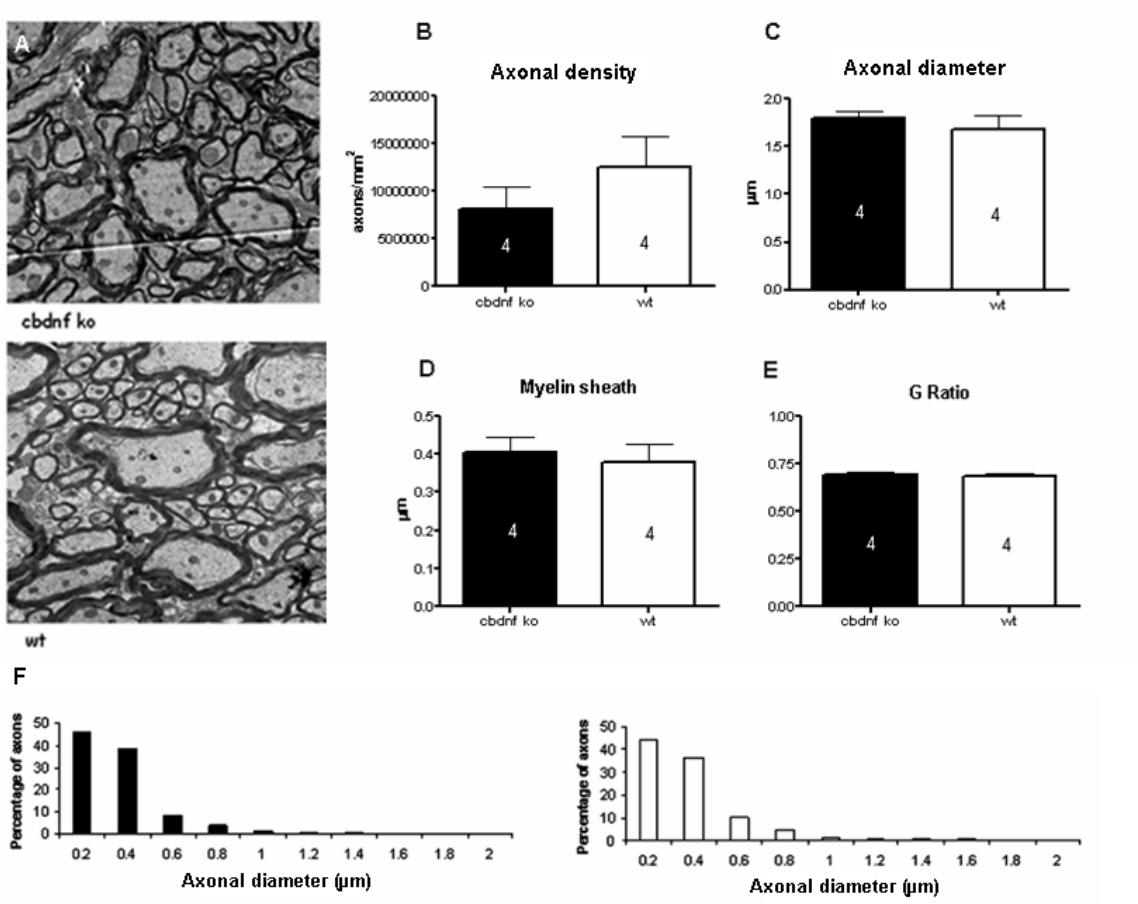

Fig. 18: Ultrastructure of the spinal cord in 2-month old *cbdnf ko* mice. A) Representative EM pictures of myelinated fibers in cross sections of the spinal cord. In the mutant the diameter of axons and the axonal density appears indistinguishable from control animals. The myelin sheaths in the mutant show no signs of dysmyelination. B) Axonal density of *cbdnf ko* animals was not significantly different from control animals (p=0.74). The number of myelinated axons per mm² is presented. C) Axonal diameter of mutant mice was not significantly different from control animals (p=0.5). D) Myelin sheath thickness of *cbdnf ko* mice was not significantly different from control animals (p=0.8). E) Myelinated fibers from the spinal cord of *cbdnf ko* and control animals were not significantly different in G-Ratio (p=0.74). F) Distribution of axonal diameter in the spinal cord. The percentage of axons within a particular size range is shown for a total of 1355 axons. The frequency histogram of *cbdnf ko* and control animals did not differ significantly. Note: Axonal density was calculated by counting the total number of myelinated axons in 15 randomly selected non overlapping fields of 315 µm² from the spinal cord. Axonal diameters and myelin sheath thickness were measured from electronmicrographs using AnalysisD software and graphs were generated in GraphPad Prism. The G-Ratio is the ratio between axonal diameter and total fiber diameter. Statistical analysis: the mean of each parameter for the single animals was calculated in Exel. Means obtained from *cbdnf ko* and control animals were compared using an unpaired *t-test*.

In addition to morphometric analyses, we also examined by qRT-PCR possible quantitative changes in mRNA expression of Myelin basic protein (MBP), Myelin-associated glycoprotein (MAG), Proteolipid protein (PLP), 3`cyclic-nucleotide-3`phosphodiesterase (CNPase), Myelin-associated oligodendrocyte basic protein (MOBP) and Myelin oligodendrocyte protein (MOG) in the CNS of 2-month old *cbdnf ko* mice compared with wild-type. In line with our morphometric analyses no significant differences were found in all tested structures with the exception of PLP and MAG in the striatum, and CNPase in the hippocampus (Fig. 19). The reasons for these expression profile changes in the mutant are unclear.

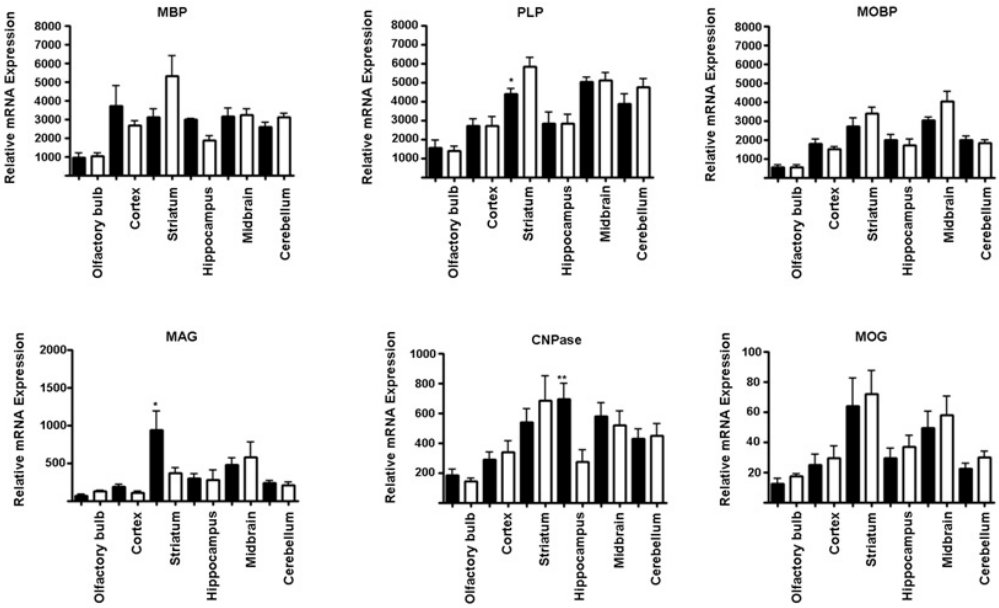

Fig. 19: mRNA expression levels of myelin genes in 2-month old *cbdnf ko* mice as determined by quantitative real-time PCR. The relative mRNA levels are normalized for GAPDH mRNA. No marked differences in the expression of myelin genes were detected in the CNS of 2-month old *cbdnf ko* mice compared with wild-type, except for PLP and MAG in the striatum, and CNPase in the hippocampus. All results are presented as a mean ±SEM derived from 4-7 individual *cbdnf ko* and control animals (*, $p<0.05$; **, $p<0.01$; unpaired *t-test*). Black bars are *cbdnf ko* and white wild-type. MBP: Myelin-basic protein; MAG: Myelin-associated glycoprotein; PLP: Proteolipid protein; CNPase: 3`cyclic-nucleotide-3`phosphodi-esterase; MOBP: Myelin-associated oligodendrocyte basic protein; MOG: Myelin oligodendrocyte protein.

NB: 1) No significant decrease in the expression of MBP was detected in the striatum of 2-month old *cbdnf ko* mice compared with wild-type, as the values obtained for different wild-type animals was unexplainably high. To test if the decrease in the expression of MBP mRNA in the striatum of 2-month old *cbdnf ko* animals is significant, the levels of MBP should be further investigated by increasing the number of animals. 2) mRNA expression of MBP, MAG, PLP, CNPase, MOBP, and MOG was also examined in the brainstem and spinal cord of 2-month old *cbdnf ko* and wild-type animals by qRT-PCR. As observed for MBP mRNA values in the wild-type animals, we also found a high variability both in wild-type and *cbdnf ko* mice in brainstem and spinal cord for all myelin marker. The reason for this high variability remains unclear. These data were not included into the graph. Clearly, additional measurements are needed.

3.6 mRNA levels of NGF, NT3 and NT4 in *cbdnf ko* mice

To test the possibility that the near complete absence of BDNF in the adult CNS may trigger compensatory changes in the levels of expression of the other neurotrophins, we determined mRNA levels of NGF, NT3 and NT4 by qRT-PCR in the brain of 2-month old *cbdnf ko* and wild-type animals. No significant differences in the expression of neurotrophin genes were detected in the CNS, with the exception of NGF in the olfactory bulb and cerebellum, and NT3 in the cortex (Fig. 20). The reasons for these expression profile changes in the mutant are unclear. The expression of NT3 and NT4 were not significantly changed in the hippocampus of 2-month old *cbdnf ko* mice compared with wild-type (Fig. 20). It is conceivable that NT4 and NT3 may not exert a compensatory role in the BDNF mutant and contribute to the lack of hippocampal volume reduction.

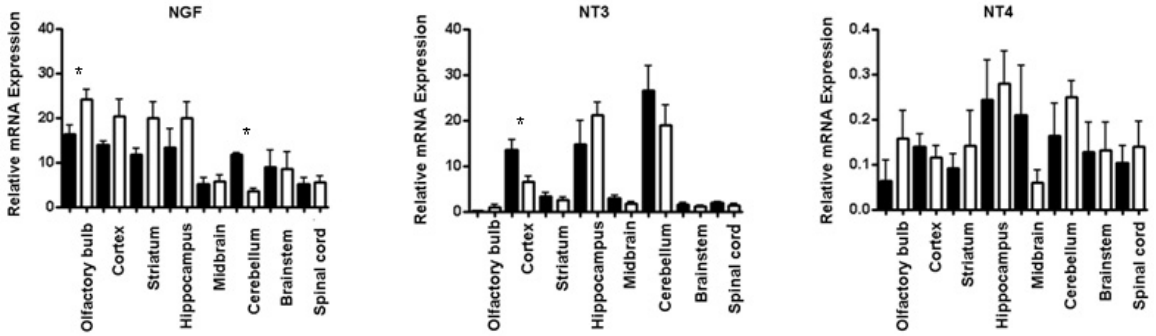

Fig. 20: mRNA expression levels of neurotrophin genes in 2-month old *cbdnf ko* mice as determined by quantitative real-time PCR. The relative mRNA levels are normalized for GAPDH mRNA. No marked differences in the expression of neurotrophin genes were detected in the CNS, with the exception of NGF in the olfactory bulb and cerebellum, and NT3 in the cortex. All results are presented as a mean ±SEM derived from 5-7 individual *cbdnf ko* and control animals (*, $p<0.05$; unpaired *t-test*). Black bars are *cbdnf ko* and white wild-type. NT3: neurotrophin-3; NT4: neurotrophin-4; NGF: nerve growth factor.

NB: No significant decrease in the expression of NGF was detected in cortex, striatum and hippocampus of 2-month old *cbdnf ko* mice compared with wild-type, as the values obtained for different control animals was unexplainably high. To test if the decrease in the expression of NGF mRNA in the cortex, striatum and hippocampus of 2-month old *cbdnf ko* animals is significant, the levels of NGF mRNA should be further investigated by increasing the number of animals. The same applies for NT4 in the olfactory bulb, striatum, midbrain and cerebellum, as the spread of values obtained for *cbdnf ko* and/or wild-type animals was unexplainably high.

3.7 Biosynthesis, storage and secretion of BDNF in the CNS

Over-expression studies suggested that pro-BDNF is released from central neurons in an activity dependent manner, that it is extracellularly cleaved via a TPA-dependent pathway and that it contributes to hippocampal LTD (Lee et al., 2001; Pang et al., 2004; Woo et al., 2005). In collaboration with my colleague Dr. Tomoya Matsumoto, we examined the question of endogenous pro-versus mature BDNF in the adult brain, as well as in cultured neurons (see also Matsumoto et al., 2008).

As BDNF is a protein of extremely low abundance, even in the adult brain, reliable detection of BDNF by Western blot techniques (WB) requires rigorous controls, including in particular the *cbdnf ko* mice generated in this study. In addition, due to the fact that BDNF induces numerous genes in the CNS the mere absence of BDNF may not be a sufficient specifity control. As an added control, we thus used a *bdnf*-myc mouse line previously generated in our laboratory by Dr. Johannes Klose (Fig. 22).

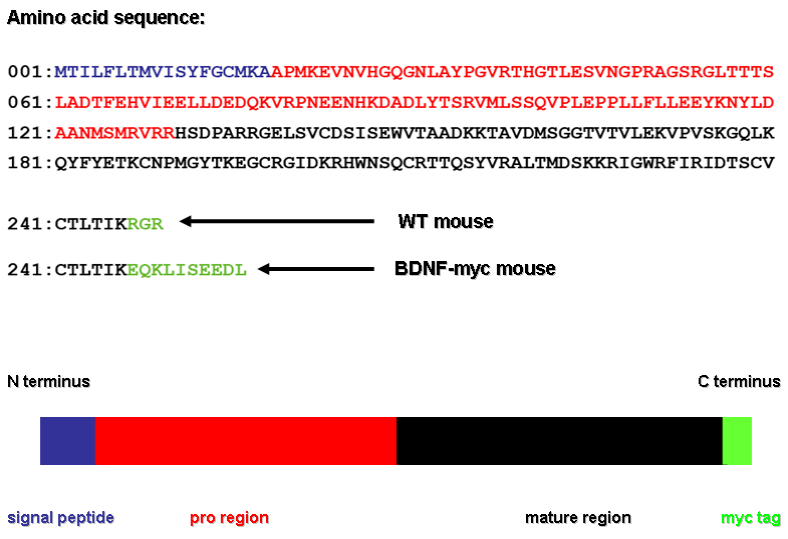

Fig. 22: Generating *bdnf*-myc knock-in mice: *bdnf*-myc knock-in (*bdnf*-myc) mice were generated by substituting exon VIII of the *bdnf* gene with a human c-myc tagged version of the gene. c-Myc was added at the C-terminus of wild-type BDNF following deletion of the last 3 amino acids.

Western blots performed with hippocampal extracts of 8-week old animals and BDNF antibodies showed a weak ~14 kDa signal that was absent in *cbdnf ko* or shifted in size in *bdnf-myc* extracts, while pro-BDNF was undetectable (Fig. 23A, left). We then compared the levels of BDNF determined by Western blot quantification with those obtained by a 2-site immunoassay (Kolbeck et al., 1999) and found the results to be in reasonable agreement (Fig. 23C). In an attempt to detect pro-BDNF, we first immunoprecipitated hippocampal lysates with a BDNF monoclonal antibody (mAb#9) that recognises both pro- and mature BDNF (Fig. 24A, B), followed by Western blot using BDNF polyclonal antibodies. This enrichment allowed the detection of a weak, but specific pro-BDNF signal of about 30-35 kDa, and quantification indicated that pro-BDNF levels are less than 10% of those of BDNF (Fig. 23B, D). These results were confirmed using either anti-pro-BDNF or anti-Myc antibodies (Fig. 23A, B). We next performed pulse-chase experiments using cultured hippocampal neurons and found that pro-BDNF is quantitatively converted to BDNF by the end of the chase period (Fig. 24A, right). Identical results were obtained when incubating the neurons with mAb#9 (Fig. 24B, left), indicating that pro-BDNF is a transient biosynthetic intermediate and undergoes intracellular processing. These results were confirmed using dissociated cultured neurons prepared from *bdnf-myc* animals (Fig. 24B, right). To examine the activity-dependent secretion of BDNF, we exposed the neurons to bicuculline, a GABA-A receptor inhibitor. This led to a marked increase of BDNF both in the medium and within the cells (Fig. 24C) in line with previous results indicating that the activation of synaptic NMDA receptors increases the transcription of *bdnf* (Hardingham et al., 2002). Under these conditions, barely detectable levels of pro-BDNF were observed in the cell lysates (Fig. 24C).

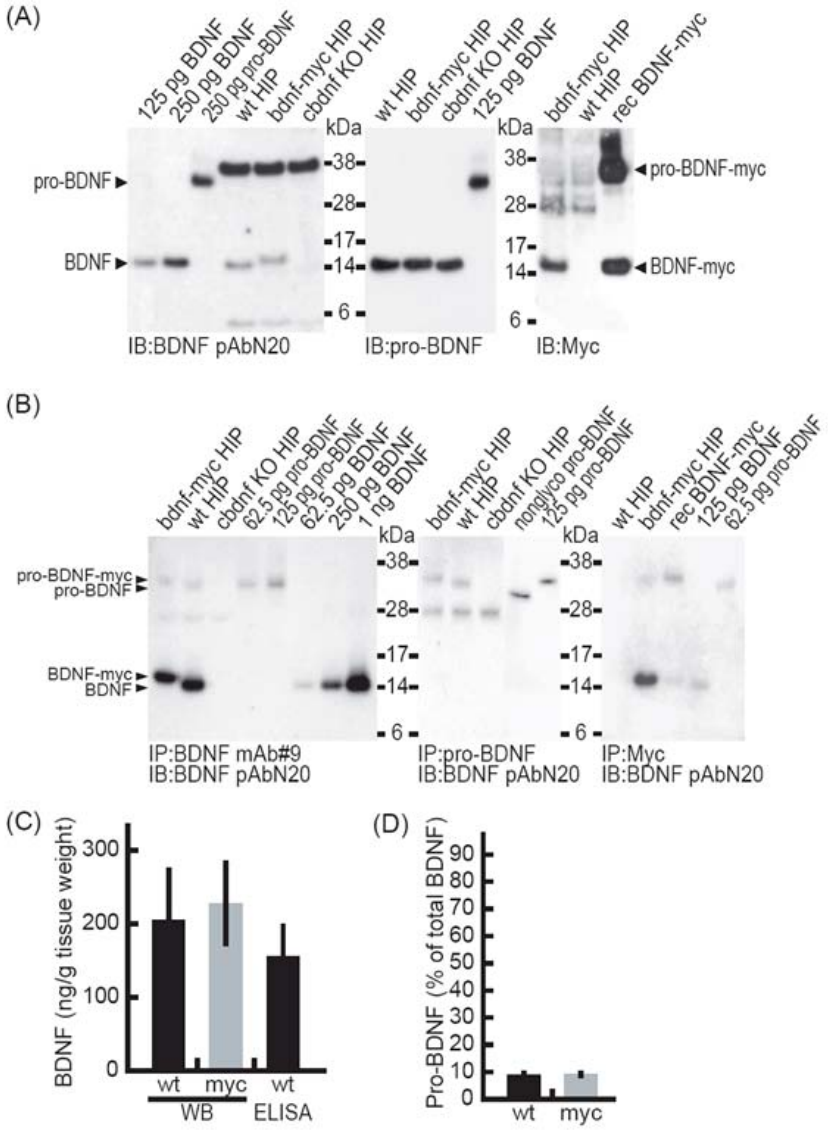

Fig. 23: Detection of BDNF in the mouse hippocampus. (A) Western blot with the indicated antibodies were performed with hippocampal extracts prepared from mice with the indicated genotypes. In the left or middle panel, note a signal of a size roughly corresponding to pro-BDNF or to mature BDNF, but also in present KO lysates. (B) Immunoprecipitation of hippocampal extracts from the indicated animals with the indicated antibodies followed by Western blot with the pAbN20 polyclonal antibody was performed. (C) BDNF concentrations were determined as in (A, left) and a 2-site immunoassay with 2 different monoclonal anti-BDNF antibodies (mAb#1 and mAb#9). (D) Ratio of pro-BDNF and BDNF. In (A, B), cleavage-resistant pro-BDNF and BDNF-myc (produced by COS7 cells), or recombinant purified BDNF and non-glycosylated pro-BDNF (produced in *E coli*) were used as a control. Error bars, SEM. IP: Immunprecipitation; IB: Immunoblot; WB: Western blot.

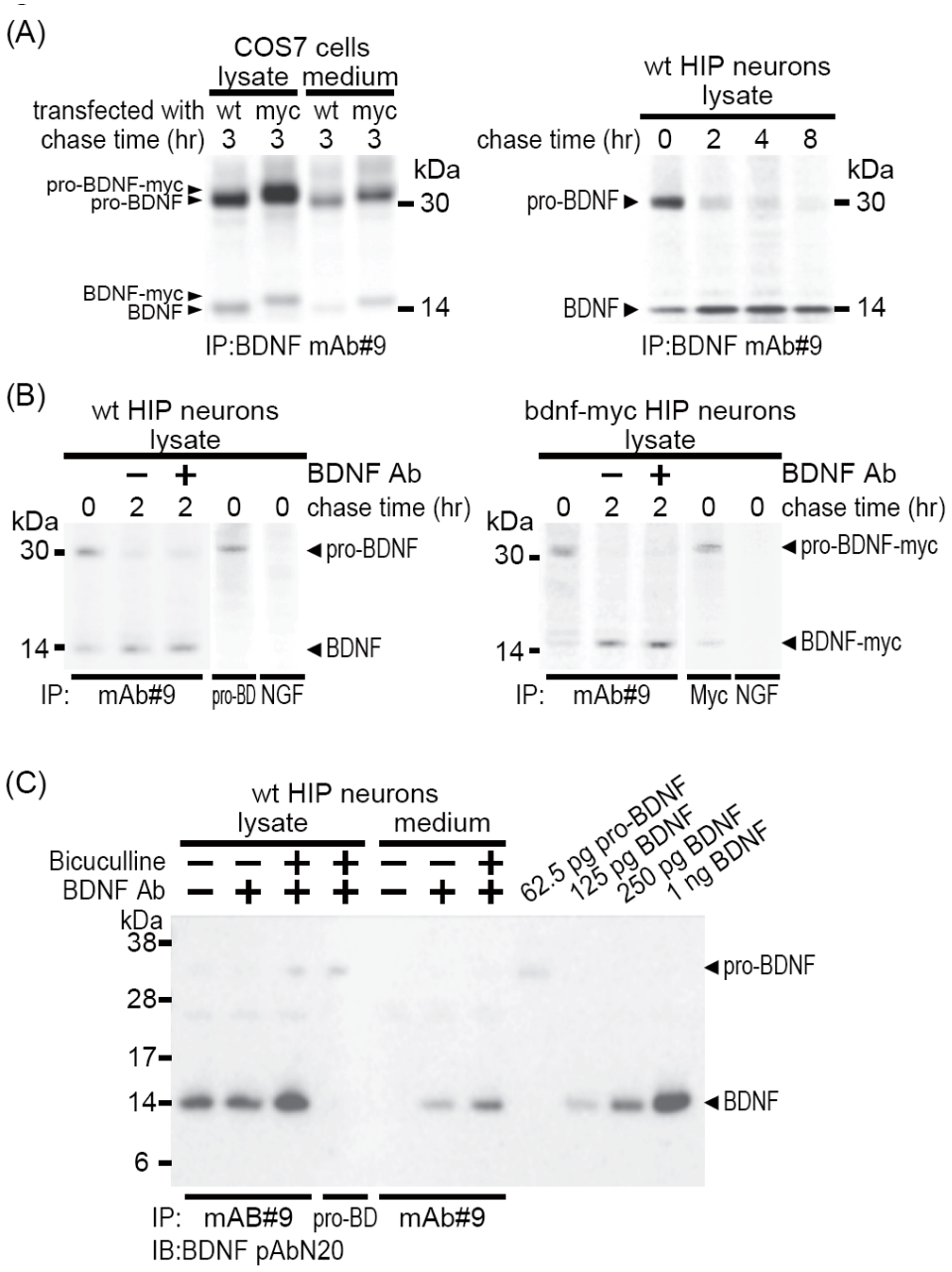

Fig. 24: Intracellular processing of pro-BDNF in neurons. A) COS7 cells transfected with the indicated constructs and cultured mouse hippocampal neurons (15 days *in vitro*, DIV) were radiolabeled with [^{35}S]methionine/cysteine for 2 hours and chased for the indicated times. Cell lysates and media were immunoprecipitated with mAb#9, followed by SDS-PAGE. (B) Using cultured *wt* and *bdnf-myc* hippocampal neurons (15DIV), pulse-chase experiments were performed as in (A), but in the presence of mAB#9. Cell lysates were immunoprecipitated with the indicated antibodies. (C) Cultured hippocampal neurons were treated with bicuculline (50 μM) in the presence or absence of mAb#9 (5 μg/ml) for 1 day (14-15 DIV). Cell lysates and media were immunoprecipitated with mAb#9, followed by WB with pAbN20. Recombinant purified BDNF and cleavage-resistant pro-BDNF were used as a control.

Finally, we tested a previous suggestion that pro-BDNF would play a crucial role in long-term depression (LTD) (Loo et al., 2005; Woo et al., 2005) through the neurotrophin receptor $p75^{NTR}$ (Woo et al., 2006; Rosch et al., 2006). If pro-BDNF were to be released from neurons, this would be an attractive possibility as recombinant pro-BDNF has been shown to bind to $p75^{NTR}$ with high affinity in the presence of sortilin (Teng et al., 2007). However, we found LTD to be unaffected in slices prepared from c*bdnf* KO animals (Fig. 25A). As expected (Poo et al., 2001), long-term potentiation (LTP) was compromised in c*bdnf* KO slices (*n*=3 slices from 2 mice) compared with *wt* samples (*n*=3 from 2) (Fig. 25B).

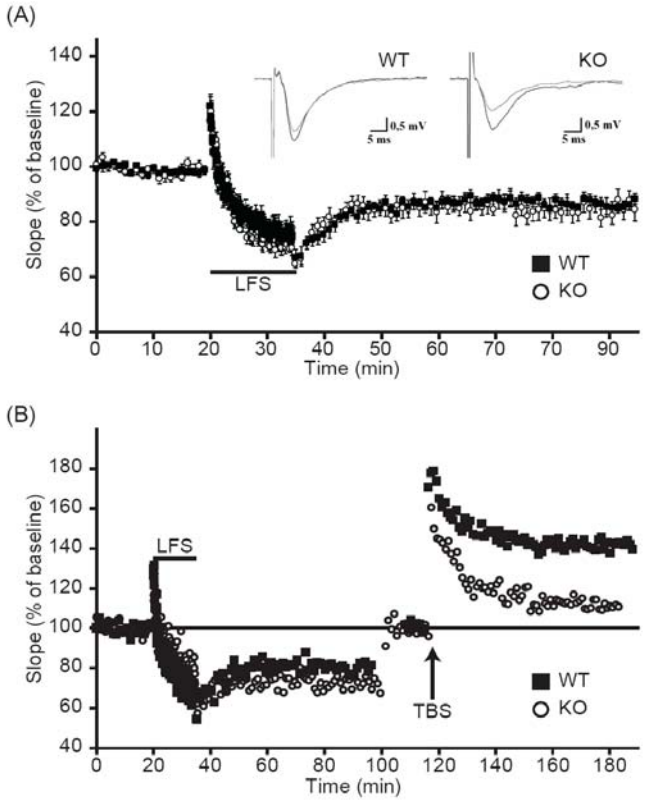

Fig. 25: BDNF is necessary for inducing hippocampal CA3→CA1 LTP, but not LTD. (A) Summary graph of LTD experiments with 2-3-week old *wt* (*n*=8 slices from 4 mice) and c*bdnf* KO mice (*n*=9; 4 mice). Symbols represent average responses plotted every minute. (*Inset*) Averaged single traces of an individual experiment just before and 55-60 minutes after low-frequency stimulation (LFS; 15 minutes, 1 Hz) of *wt* and c*bdnf* KO mice. (B) Single sample of an LTD/LTP experiment. Slope is plotted against time. After 20 minutes of baseline recording, the same LFS as in (A) was applied. After 60 minutes, the signal was adjusted back to the original fEPSP values (100% pre-LFS baseline). Three theta burst stimulation (TBS; 100 Hz, 200 µs duration, 200 ms inter-bust interval, 10 s inter-stimulus interval) were subsequently applied. fEPSP size and paired-pulse-facilitation was not altered in c*bdnf* KO mice (data not shown).

4. Discussion

In the present study we attempted a global BDNF deletion in the CNS by crossing floxed *bdnf* mice with mice expressing Cre from the *tau* locus. Our results show that mice survive several months after birth with nearly undetectable levels of BDNF protein in the CNS. As BDNF levels are very low even in the normal adult brain, lysate of *cbdnf ko* mice also served as a key control in biochemical experiments examining the molecular weight and quantity of BDNF in the brain (see below 4.4).

Two months old *cbdnf ko* mice were hyperactive when stressed, had higher levels of anxiety when evaluated in the light/dark exploration test and displayed a clasping phenotype similar to that observed in mouse models of Huntington`s disease. Additionally, female *cbdnf ko* mice developed mature onset obesity. Brain weight of two months old *cbdnf ko* mice was reduced by about 20% compared with wild-type. Comparative volume measurements of major brain areas indicated that the striatum (36.5%) and cortex (20%), but not the hippocampus of 2-month old *cbdnf ko* mice were significantly reduced in volume. To determine whether the decrease in striatal volume was caused by cell loss, neurons and oligodendrocytes were counted. No significant differences in their numbers were found, but the neuronal density was increased. Based on the finding that the volume of the hippocampus was unchanged, we initially hypothesized that the volume reduction of the striatum could be explained by a decrease in the size of axonal diameter of the cortico-striatal afferents, with a corresponding decrease in myelination. This possibility was suggested by previous findings, indicating a pro-myelinating role of BDNF through TrkB signalling (Cellerino et al., 1997; Medina et al., 2004). As the structure of the striatum does not lend itself to quantify axonal diameters, axonal numbers, and thickness of myelin sheaths of the cortico-striatal afferents, we turned to an electron microscopy study using the optic nerve, corpus callosum and spinal cord. However, no significant differences in axonal diameter and myelination were observed in these structures, suggesting that BDNF may not be required for the

growth and maintenance of axons and their myelin sheath. This result was further confirmed by a qRT-PCR analysis of myelin genes. In all tested structures, no consistent changes were observed, except for PLP and MAG in the striatum and CNPase in the hippocampus, for reasons that are unclear. mRNA levels of NGF, NT3 and NT4 were also determined by qRT-PCR and no significant increase of NT4 and/or NT3 mRNA expression was found in the hippocampus, indicating that NT4 (also known to activate TrkB) and NT3 may not exert a compensatory role and contribute to the lack of hippocampal volume reduction.

4.1 *cbdnf ko* mice

4.1.1 Tau

Tau is a low-molecular-weight microtubule associated protein that is abundant in the CNS, where it is expressed predominantly in axons of neurons (Cleveland et al., 1977; Binder et al., 1985) tho it is also found in oligodendrocytes (LoPresti et al., 1995). Tau regulates the assembly and stability of microtubules (Weingarten et al., 1975; Cleveland et al., 1977), and this microtubule-binding function of Tau is negatively regulated by phosphorylation (Drechsel et al., 1992; Biernat et al., 1993; Bramblett et al., 1993). However, a mouse mutant null for the *tau* gene did not reveal any defects in regard to axonal growth or maintenance (Harada *et al.*, 1994). Work from our laboratory has previously shown that the endogenous *tau* promoter drives expression of GFP soon, and long after neurons have become postmitotic (Tucker et al, 2001). Intercrosses between the *tau*::Cre and Cre-activatable reporter animals resulted in offsprings with recombination either restricted to the nervous system or throughout the entire embryo, indicating expression of Tau early in development. The percentage of neuron-specific excision was dependent on the Cre reporter system used. In spite of the observed variability, these data suggested that the *tau*::Cre mouse line can be used for pan-neuronal recombination of floxed alleles throughout the mouse nervous system (Korets-Smith et al., 2004). Based on these studies, we expected the elimination of BDNF specifically in most postmitotic neurons of the central and peripheral nervous system.

4.1.2 Generation of *cbdnf ko* mice

Mutants generated in the present study were in a mixed genetic backround (Sv129/C57/Bl6) and fell into 3 categories: *bdnf* full knockout (19%), *bdnf* mosaic (5%) and tissue - specific *bdnf* knockout mice (1%). This result indicated that in the majority of the progeny, the floxed *bdnf* allele was excised already at the zygote stage or in the early postimplantation embryo and only in a minority in a neuron-specific manner. However, mutants generated in a C57/Bl6 backround were exclusively *bdnf* full knockout mice. Recently, Korets-Smith et al. (2004) demonstrated that the genetic background of parental mice and the position in the genome of the *loxP* target allele can each contribute to differences in the exact pattern of recombination. In the present study, we confirmed that Cre-mediated recombination can be influenced by the strain background of the mice, raising the question of how strain background could exert this effect. One possibility is that modifier loci, present as different alleles in the different strains, alter a low basal level of Tau expression. Alternatively, these modifier loci might not influence expression of the wild-type *tau* locus, but instead alter that of the *tau*::Cre allele, which contains sequences not normally found at this locus. A final possibility is that the activity of the Cre recombinase itself can be modulated by factors present in some strains of mice but not in others. An alternative explanation is that the floxed *bdnf* gene in the C57/Bl6 backround itself is somehow more susceptible to recombination than the floxed *bdnf* gene in the Sv129/C57/Bl6 backround. Consequently, the levels of Cre known to be present early in development are sufficient to trigger recombination of the floxed *bdnf* allele in the C57/Bl6, but less efficient in the mixed backround. It is conceivable, although speculative, that local differences in chromatin structure can alter the efficiency of recombinase activity. If this were to be the case, great care should be exerted when extrapolating from results obtained with reporter lines when the reporter construct is not inserted in the gene of interest.

4.1.3 *cbdnf ko* mice compared with other conditional *bdnf* knockout mice

A fundamental difference between previously reported conditional *bdnf* knockout and the newly generated *cbdnf ko* mice is the extent of recombination of the floxed *bdnf* allele. In previous studies, the floxed *bdnf* allele was excised in an area- and cell type specific manner, like for example in the Emx-BDNF^{KO} mouse, in which BDNF was excised in excitatory neurons and glia cells of the cortex, hippocampus and amygdala (E10.5) (Gorski et al., 2002; Gorski et al., 2003; Baquet et al., 2004), or in the Wnt-BDNF^{KO} mouse, in which BDNF was excised in neurons of the midbrain-hindbrain (E8.5) (Baquet et al., 2005; Danielian et al., 1998), or in the CamKII-BDNF^{KO} mouse, in which BDNF was postnatally deleted in neurons of the hippocampus, cortex, hypothalamus and brainstem (P21) (Rios et al., 2001; Zakharenko et al., 2003; Monteggia et al., 2004; Monteggia et al., 2007). In the present study the floxed *bdnf* allele was globally deleted throughout the nervous system, already during development. Interestingly, Chan et al. (2006) used the Nestin-Cre deleter to generate mice with a virtual depletion of BDNF across the brain. However, although their approach is quite similar to ours, significant differences exist between these two genetic approaches. In particular the Cre construct used by Chan et al. (2006) directs the expression of Cre to the CNS, but not the PNS (Zimmerman et al., 1994). Therefore, BDNF expression is unaffected in neurons of the PNS. Tau, on the other hand is expressed in all postmitotic neurons of the CNS and PNS with the consequence that BDNF expression is absent from all CNS and PNS neurons of *cbdnf ko* mice. This is important, since BDNF can then not be imported by anterograde transport from the PNS to the CNS in *cbdnf ko* mice, while this is still the case in the Nestin-BDNF^{KO} mice.

4.1.4 BDNF protein measurement (ELISA)

A precise delineation of the numerous biological roles of BDNF necessitates reliable information on the localization and amount of BDNF protein in the nervous system. Measuring BDNF protein levels reliably is critically dependent on the availability of reagents such as monoclonal antibodies with established specificity. In addition to the problem of antibody specificity, it has been shown that the use of an acidic extraction buffer containing high salt and detergent increase the availability of BDNF in tissue extracts (Barde et al., 1982; Hofer and Barde, 1988; Nawa et al., 1995). It appears likely that upon basic or acidic pH treatment, this basic growth factor may dissociate from binding proteins present in the extracts, resulting in increased availability for antibody binding.

Most existing BDNF immunoassays are based on the use of polyclonal antisera to BDNF (Nawa et al., 1995; Zhou et al., 1996; Katoh-Semba et al., 1997), sometimes used in combination with a monoclonal antibody (mAb) (Radka et al., 1996; Inoue et al., 1997). Although BDNF is a homodimeric protein, it has not been possible to use the same mAb as a reagent in a sandwich immunoassay, as has been possible with NGF (Korsching and Thoenen, 1983) and NT3 (Shintani et al., 1993). Obtaining useful BDNF mAbs is a difficult task, in part due to the very high conservation of this protein between species: the sequence of processed, biologically active BDNF is identical in all mammals. However, Kolbeck et al. (1999) managed to produce two monoclonal antibodies recognizing two different epitopes of BDNF, which can be used in an immunoassay to measure BDNF protein levels with a satisfactory degree of sensitivity and specificity.

To measure the remaining levels of BDNF in the mutants of *fbdnfxtau*::Cre litters, we used in the present study the BDNF immunoassay system published by Kolbeck et al. (1999). We found a ≥ 95% reduction of BDNF protein levels throughout the brain of 2-month old mutants compared with wild-type littermates. The remaining levels of BDNF (≤ 5%) may be explained by the expression of

BDNF in endothelial cells, vasculature and astrocytes (Binder et al., 1985; LoPresti et al., 1995; Liem et al., 2001; Nakahashi et al., 2000; Donovan et al., 2000; Wang et al., 2006; Riley et al., 2004; Forman et al., 2005), since Tau is not expressed in those cell types. Conversely, due to the likely expression of *tau* in oligodendrocytes (Dai et al., 2003), we cannot exclude that BDNF is exclusively expressed in neurons though *in situ* hybridization experiments indicate that neurons are the primary sites of BDNF expression (Hofer et al., 1990). We note that the levels of BDNF measured in the CNS of 2-month old wild-type *fbdnfxtau*::Cre litters were similar to those published by Kolbeck et al. (1999), who measured BDNF protein levels in the CNS of 3 week old wild-type mice.

Further, our hippocampal values for BDNF (195 ng/g) are also similar to those previously determined in the adult hippocampus by Radka et al. (1996) (rat: 256 ng/g; mouse: 181 ng/g) and Narisawa-Saito and Nawa (1996) (rat: 270 ng/g) using BDNF immunoassays based on the combination of a mAb with a polyclonal antiserum or of two polyclonal antisera, respectively. However, our measured hippocampal BDNF protein levels are considerably higher than those reported by others in the adult rat hippocampus [14.5 ng/g (Zhou et al., 1996); 19.9 ng/g (Nawa et al., 1995); 5.4 ng/g (Katoh-Semba et al., 1997); 3 ng/g (Scaccianoce et al., 2003); 9 ng/g (Angelucci et al., 2000); 15 ng/g (Ickes et al., 2000); 60 ng/g (Rudge et al., 1998)]. Most likely, different types of BDNF extraction procedures and the correction of BDNF yields account for these discrepancies.

The relatively high levels of BDNF protein found in the striatum and the spinal cord, areas of the CNS known to express very low levels of BDNF mRNA, can be explained by the anterograde transport of BDNF (Altar et al., 1997; Zhou and Rush, 1996; Michael et al., 1997; Tonra et al., 1998).

4.1.5 Why do *cbdnf ko* mice survive and breath normally?

Erickson et al. (1996) demonstrated that BDNF is required for development of normal respiratory activity and that newborn *bdnf* knockout mice exhibit severely depressed and irregular breathing. This abnormal respiratory phenotype is related in part to the loss of BDNF-dependent chemoafferent neurons in the pedrosal ganglia that provide excitatory drive to the respiratory central pattern generator. In addition, BDNF is also known to modulate the responsiveness of second-order sensory neurons in the nucleus tractus solitarius (nTS) to glutamatergic stimulation, an effect of BDNF that can be mimicked by NT4, and which is blocked by the Trk tyrosine kinase inhibitor K252a, indicating the requirement for TrkB receptor activation. These findings could explain why the loss of BDNF may result in a depression of motor output from the brainstem respiratory rhythm generator and suggest that BDNF play an important role in regulating excitatory transmission at primary afferent synapses (Balkowiec and Katz, 1998; 2000; Thoby-Brisson et al., 2003; Baker-Herman et al., 2004). Therefore, the lethality associated with the *bdnf* knockout phenotype may result from deficits in central respiratory output (Balkowiec and Katz, 1998), as well as excitatory chemoafferent drive. Moreover, the absence of baroreceptor innervation in *bdnf* knockout mice suggests that cardiovascular homeostasis is likely to be disrupted in these animals as well (Brady et al., 1999).

A possible answer to the question why *cbdnf ko* mice survive in contrast to *bdnf* full knockout mice, is based on the results that the total amount of BDNF protein in the heart of *cbdnf ko* mice was not significantly altered compared with that of wild-type mice. This finding suggests that baroreceptor afferents from the nodose ganglia of *cbdnf ko* mice are likely to innervate the aortic arch as in wild-type animals and that cardiovascular homeostasis may be approximately normal in *cbdnf ko* mice (Erickson et al., 1996; Brady et al., 1999; Hellard et al., 2004; Tessarollo et al., 2004).

4.2 Histological analysis of 2 months old *cbdnf ko* mice

4.2.1 Volume reduction of the striatum

Our results indicate that striatal volume reduction of 2-month old *cbdnf ko* mice is not due a cell loss or a defect of axonal myelination. Similarly, Baquet et al. (2004) failed to detect neuronal losses in the striatum of 4 months old Emx-BDNF^{KO} mice. The lack of a consistent result clearly indicating that myelination is affected is unexpected in view of previous analysis of *bdnf* full knockout and TrkBNestinCre conditional knockout mice (Cellerino et al., 1997; Medina et al., 2004). In *bdnf* full knockout mice, a reduction in the size of retinal ganglion cell axons was shown to be accompanied by hypomyelination of the optic nerve. However, the interpretation of the phenotype of *bdnf* full knockout mice is complicated by their poor health, even if peripheral myelination was found to be normal (Cellerino et al., 1997). Our findings may be explained by a developmental delay of *bdnf* full knockout mice, leading to a retardation of CNS myelination, known to largely occur postnatally in rodents. TrkBNestinCre conditional knockout mice, in which TrkB is deleted during development from all neuronal and glial precursors of the CNS, were characterized by a significant reduction in the number of myelinated axons and myelin sheaths in the corpus callosum and CA1 region of the hippocampus (Medina et al., 2004). From this study it was concluded that TrkB signaling regulates myelination in the CNS and that this may occur, at least in part, through the regulation of oligodendrocyte differentiation. Interestingly, deletion of TrkB at P20 does not affect oligodendrocyte differentiation nor CNS myelination in the CA1 region of the hippocampus as observed in the TrkBCaMKIICre mutant mice (Minichiello et al, 1999), suggesting that TrkB signaling in axons regulates oligodendrocyte myelination before P20, and/or that TrkB signaling in oligodendrocytes is sufficient to mediate their proper differentiation and myelination function. A possible explanation for the observed difference between *cbdnf ko* and TrkBNestinCre mice may also be that alternate TrkB ligands compensate for the

loss of BDNF, and therefore supporting proper differentiation and myelination function of oligodendrocytes in *cbdnf ko* mice.

4.2.2 mRNA levels of NGF, NT3 and NT4 in *cbdnf ko* mice

In the present study, we tested the possibility that the virtual absence of BDNF in the adult CNS may trigger compensatory changes in the expression levels of other neurotrophins. We found that mRNA levels of NT3 and NT4 were not significantly changed in the hippocampus of 2-month old *cbdnf ko* mice compared with the wild-type. It is thus unlikely that NT4 (also known to activate TrkB) and NT3 may exert a compensatory role in the hippocampus of 2-month old *cbdnf ko* mice and contribute to the lack of hippocampal volume reduction. While Baquet et al. (2004) also found that the gross morphology of the hippocampus in 4 months old Emx-BDNF^{KO} mice was unchanged, they explained the lack of hippocampal volume reduction by 2-fold increase of NT3 expression as well as by the anterograde transport of BDNF from the amygdala, thalamus, hypothalamus and ventral tegmental area (Baquet et al., 2004).

Our findings on the lack of effects of BDNF deprivation on hippocampal volume are in accordance with that of Baquet et al. (2004), but contrast with that of Chen et al. (2006), who found a significant, but very small decrease of hippocampal volume in $BDNF_{Met/Met}$ knock-in mice. Importantly, $BDNF_{Met}$ protein levels were normal in the brain of 2-month old $BDNF_{Met/Met}$ mice compared with wild-type mice. However, the distribution of $BDNF_{Met}$ protein to neuronal dendrites and its activity-dependent secretion from neurons was reported to be decreased, though these difficult experiments would need to be independently confirmed. Clearly, more needs to be learned about the biosynthesis of wild-type BDNF versus $BDNF_{Met}$ protein (see also 4.4), while it has been suggested that $BDNF_{Met}$ may not interact as well as wild-type BDNF with sortilin, thought to be important for the intracellular trafficking of BDNF (Chen et al., 2005). It is surprising, that potentially small changes in the secretion of BDNF (Chen et al., 2006) lead to a

morphological hippocampal phenotype not seen in the almost complete absence of BDNF (this study).

4.2.3 Role of BDNF in adult neurogenesis

Because of the extensive literature indicating that granule cells of the hippocampus undergo neurogenesis throughout life, several studies have investigated whether BDNF might influence neurogenesis. For example, Benraiss et al. (2001) showed that increasing BDNF in the adult subventricular zone using an adenovirus approach increased the number of new neurons in several brain areas outside the hippocampus. Peneca et al. (2001) showed that i.c.v. BDNF led to increased numbers of new neurons in several areas adjacent to the ventricles, such as the striatum, septum, and the thalamus. In the dentate gyrus, Lee et al. (2000) showed that dietary restriction increased BDNF, and that there was an increase in the dentate gyrus neurogenesis as well. Katoh-Semba et al. (2002) reported that riluzole, a drug currently approved for amyotrophic lateral sclerosis, increased BDNF levels in the hippocampus, and increased proliferation of granule cells. Consistent with a role of BDNF, they further found that infusion of an antibody to BDNF into the ventricles blocked the increase in proliferation. In heterozygote *bdnf* knockout mice, Linnarsson et al. (2000) showed that the number of new cells in the dentate gyrus was decreased. Based on these studies it seems that BDNF may play an important role in adult neurogenesis. We found no significant volume reduction in the dentate gyrus of 2-month old *cbdnf ko* mice. Therefore it may appear unlikely that endogenous BDNF plays a major role in adult neurogenesis in the dentate gyrus. This conclusion relies on the assumption that newly born neurons in the dentate gyrus significantly contribute to maintaining the size of the adult dentate gyrus. New results suggest however that the contribution of newly generated neurons stably integrated in the dentate gyrus is quite small (Ninkovic et al., 2007).

4.3 Phenotype of *cbdnf ko* mice

4.3.1 Clasping behavior

cbdnf ko mice exhibit clasping of hindlimbs, a stereotyped behavioral phenotype indicative of striatal neuronal dysfunction that becomes increasingly severe as these mice age. A similar clasping phenotype has been seen in Huntington´s disease (HD) model mice (Carter et al., 1999; Auerbach et al., 2001; Guidetti et al., 2001), as well as in Emx-BDNF^{KO} mice (Baquet et al., 2004). Interestingly, Emx-BDNF^{KO} mice did not perform poorly on rotarod tests, whereas HD model mice exhibit both clasping phenotype and poor rotarod test performance. These similarities and subtle differences in behavioral phenotypes between HD model- and Emx-BDNF^{KO} mice opened the possibility to closely compare these two model systems to identify possible neurological foundations for BDNF dependent aspects of the HD phenotype. From microarray-based comparisons of striatal RNAs in early human Huntington´s disease with several rodent models of HD, Strand et al. (2007) reached the unexpected conclusion that depletion of BDNF provides the best murine mimic for the striatal changes observed in early HD, especially in mice, where the BDNF gene is specifically deleted in the cortex (Baquet et al., 2004; Gorski et al., 2003). Neither the R6/2 mouse, nor 3-NP-treated, MPTP-treated and PGC1-α knockout mice – all of which show HD-like pathology - do as well as Emx-BDNF^{KO} mice in mimicking HD-associated changes in gene expression. Strand et al. (2007) suggested that decreased synthesis and anterograde transport of BDNF may underlie the characteristic striatal pathology of HD. These data supported the conclusion that dysfunction and loss of striatal neurons does not result from the expression of mutant huntingtin (htt) in striatal neurons, but from the decreased trophic support from cortical cells that express mutant htt.

With regard to functional aspects, 2 ‘classic’ forms of long-term synaptic plasticity - LTD and LTP - have been described at corticostriatal synapses (Calabresi et al., 1992). Both seem to underlie motor skill learning, cognitive performances and reward mechanisms. Following LTP induction, it is possible to reverse the

previously potentiated synapse to pre-LTP levels. This last form of synaptic plasticity, termed 'depotentiation', appears to represent the neural correlate of 'forgetting' and and is thought to help improving circuit dynamics (Picconi et al., 2003). However, alterations in the induction and reversal of synaptic plasticity have been demonstrated in both 3-NP-treated rats and the genetic R6/2 mouse model of HD. Striatal medium-sized spiny neuron recordings from both of these models showed a normal LTP, but a failure to depotentiate their synapses after a low-frequency stimulation protocol (Picconi et al., 2006). This inability to reverse synaptic strength to pre-LTP levels leads to altered circuit efficiency during either information storage or physiological synaptic forgetting, and may account for the impaired behavioural flexibility described in HD patients at early clinical stages. Interestingly, although cholinergic interneuron recordings from control animals showed a robust LTP, they did not demonstrate LTP in either 3-NP animals or HD R6/2 mice (Picconi et al., 2006). As the synaptic depotentiation normally seen in medium-sized spiny neurons depends upon the activation of muscarinic receptors, it is conceivable that in HD models, the lack of potentiation of cholinergic interneurons accounts for the absence of depotentiation in spiny neurons. As Strand et al. (2007) described conditional *bdnf* knockout mice as the best murine mimic of striatal changes in early HD, *cbdnf ko* mice may offer an interesting additional opportunity to test this hypothesis.

4.3.2 Obesity

Obesity phenotypes have been observed in *bdnf* heterozygous mice and in mice in which the *bdnf* gene has been deleted in excitatory neurons in the adult brain (Lyons et al., 1999; Kernie et al., 2000; Rios et al., 2001). Even mouse mutants that express TrkB at a quarter of the normal amount showed hyperphagia and excessive weight gain (Xu et al., 2004). These studies indicated that the BDNF/TrkB signaling participates in the regulation of energy balance and feeding behavior. Surprisingly, in the present study, we found that only female *cbdnf ko* mice became obese over time, whereas males did not. These results were not in line with previous studies (Rios et al., 2001; Xu et al., 2004). However, most

mammals show gender dimorphisms in body mass and differently regulate energy homeostasis and substrate utilization, and our results suggest that BDNF might be involved in regulating these differences.

4.3.3 Activity

cbdnf ko mice had a substantial increase in locomotor activity when stressed but not at baseline, suggesting a role for BDNF in the regulation of anxiety-related behavior (4.3.4). Interestingly, increased spontaneous activity has been repeatedly observed in all BDNF mutants (Kernie et al., 2000; Lyons et al., 1999; Monteggia et al., 2004; Rios et al., 2001), except one (Gorski et al., 2003). Hyperlocomotion and impulsivity was also reported for the TrkB-CRE and TrkB-Shc mutants (Minichiello et al., 1998; Minichiello et al., 1999; Zorner et al., 2003). Although largely unknown, factors such as serotoninergic abnormalities, inner ear defect or aggravated stress response have all been suggested as possible origins explaining hyperactive behavior (Lyons et al., 1999; Minichiello et al., 1998; Zorner et al., 2003). Interestingly, hyperactivity is also a feature of mice lacking expression of 5-HT_{2C} (Nonogaki et al., 2003), a serotonin receptor subtype associated with depression (Gurevich et al., 2002; Lerer et al., 2001). As BDNF has been suggested to be important for proper serotonergic neurotransmission (Lyons et al., 1999; Mamounas et al., 1995; Siuciak et al., 1998), a careful examination of this system in the mutants is warranted.

4.3.4 Anxiety

Anxiety is a complex phenotype, mediated by multiple brain nuclei, including the amygdala, prefrontal and cingulate cortices, and the hippocampus (Gray, 1981; Sandford et al., 2000). Additionally, it is susceptible to alterations via numerous drugs that target multiple neurotransmitter receptor systems, including GABA and monoamines (Argyropoulos et al., 2000). BDNF is known to influence differentiation and function of GABAergic, serotonergic, dopaminergic and noradrenergic neurons suggesting that systems modulating anxiety could be affected by loss of BDNF (Altar et al., 1994; Sklair-Tavron and Nestler, 1995). In

our study, we used the light/dark exploration test to determine whether the lack of BDNF in *cbdnf ko* mice altered anxiety. Significant differences were found in the number of white box entries, and the time spent in the white chamber and we concluded that deleting BDNF early in development result in increased anxiety level. In line with this, postnatal, brain-restricted BDNF mutant mice (CamKII-$BDNF^{KO}$) exhibit higher levels of anxiety in the light/dark exploration test (Rios et al., 2001) and transgenic mice overexpressing TrkB exhibit reduced anxiety-like behavior in the light/dark exploration test (Koponen et al., 2004). As TrkB also serves as a receptor for NT4, which like BDNF augments monoaminergic systems (Altar et al., 1994), it is not clear whether those changes are entirely associated with BDNF signaling. A better understanding of the role of BDNF in anxiety related behavior could be achieved by testing the efficacy of anxiolytic agents in our *cbdnf ko* animals.

4.4 Biosynthesis, storage and secretion of BDNF

Recent studies have suggested that pro-BDNF is released from central neurons in an activity-dependent manner and that it is crucial in long-term depression (LTD) (Mowla et al., 1999; 2001; Chen et al., 2004; Woo et al., 2005). Pro-BDNF was also suggested to be cleaved extracellulary after release via a TPA-dependent pathway (Pang et al., 2004), with the suggestion that the secretion of TPA may also be activity dependent. However, our detailed studies revealed no evidence for pro-BDNF being released from cultured hippocampal neurons. In addition, *cbdnf ko* mice lacking all forms of BDNF in neurons exhibit normal LTD in acutely prepared hippocampal slices, while LTP induction was significantly affected. These results seem to exclude the model in which regulation of extracellular conversion of pro-BDNF to mature BDNF controls the extent of signaling via $p75_{NTR}$ or TrkB receptors, alternatively promoting LTD or LTP (Lu et al., 2005). While our studies focused on endogenous BDNF and intact neurons, it allows no prediction to be made as to the processing or the lack of pro-BDNF in injured neurons or in other cells. It is well possible that like it has been previously shown with NGF (Srinivasan et al., 2004; Harrington et al., 2004) cells such as

microglia may release substantial quantities of pro-BDNF after brain injury. However, our results also suggest that neurons may only have a limited capacity to process endogenous pro-BDNF, which may explain previous results indicating that neurons, transfected with BDNF cDNAs release substantial amounts of pro-BDNF.

4.5 Outlook

4.5.1 Reduction of striatal volume in *cbdnf ko* mice

In the present study, we examined the role of BDNF in the adult brain by using *cbdnf ko* mice lacking almost completely BDNF in the CNS. The striatum and cortex, but not the hippocampus of 2-month old *cbdnf ko* mice was reduced in volume compared with wild-type. We initially examined the possibility that the decrease in striatal volume could be explained by cell loss or by a decrease in the size of axonal diameter of the cortico-striatal afferents, with a corresponding decrease in myelination. However, the absence of BDNF neither caused the loss of neurons or of axons, nor were axonal growth and myelination measurably affected. Since within different brain regions the ratio of GABAergic and glutamatergic neurons varies tremendously, i.e. in the striatum GABAergic neurons compromise approximately 95%, in the cortex 20-30% and in the hippocampus 5-10%, another explanation for the disproportional reduction in the volume of the striatum of 2-month old *cbdnf ko* mice compared with the hippocampus could be that GABAergic neurons are more dependent on BDNF for their postnatal growth than is the case for glutamatergic neurons. To test this hypothesis, primary cultures of the striatum and the hippocampus (both wild-type) will be treated with BDNF, immunostained for GAD and MAP2 and subsequently the morphology of pyramidal and non-pyramidal neurons will be comparatively analyzed (in collaboration with Dr. Ruben Deogracias). In a parallel approach, brain slices of 8 weeks old *cbdnf ko* mice and wild-type mice will be labeled with DiO using a gene gun methodology and the morphology of

striatal medium spiny neurons and hippocampal pyramidal neurons will be comparatively analyzed (Martin Korte, TU, Braunschweig).

4.5.2 *cbdnf ko* mice - Model for Huntington´s disease and food intake regulation?

Despite methodological improvements with regard to the delivery of neurotrophins, many difficulties still remain, as these molecules have a number of suboptimal pharmacological properties, including rapid clearance with a serum half-life of minutes or less, poor oral bioavailability, and restricted CNS penetration (Poduslo and Curran, 1996; Pardridge, 2002). One potentially powerful approach overcoming these limitations is the development of neurotrophin mimetics with favourable stability and tissue penetration profiles that act via targeted neurotrophin receptors, such as TrkB. Researchers are therefore developing small-molecule BDNF analogues with improved pharmacokinetic properties and an ability to penetrate the blood brain barrier to an appreciable extent. One source of inspiration for these studies is the three-dimensional structure of BDNF, which has been used as a template to design cyclic peptides that mimic BDNF making use of loops 1, 2 and 4, which are required for binding to TrkB receptors. Preliminary studies have demonstrated that such BDNF mimetics act as BDNF agonists that promote the survival of cultured sensory neurons (O'Leary and Hughes, 2003; Fletcher and Hughes, 2006). As *cbdnf ko* mice show a shortend life-span and a massive striatal volume reduction at 2-month, it would be interesting to test in future studies if treatment of *cbdnf ko* mice with such mimetics extends their lifespan and rescues the morphology of the striatum. Likewise, it is conceivable, that antibodies activating TrkB may be developed and tested in our *cbdnf ko* animals, with regard to the clasping and obesity phenotypes in females.

4.5.3 *cbdnf ko* mice – Localization of pro- and mature BDNF

We showed that in hippocampal neurons pro-BDNF is a transient intermediate that is converted intracellularly to the mature form of BDNF, which is stored and released by excitatory input (Matsumoto et al., 2008). Recently, Egan et al. (2003) demonstrated that the Val66Met substitution in the pro-region of BDNF affects the sorting of BDNF into the nerve terminals, retards activity-dependent secretion and reduces learning functions of the hippocampus in humans. Therefore, detailed knowledge of the subcellular localization of endogenous pro- and mature BDNF is becoming increasingly important and would contribute to the understanding of the processing of BDNF in cell bodies and/or dendrites. In collaboration with my colleagues Drs. Sandra Dieni (Anatomy, Freiburg, Germany) and Tomoya Matsumoto, we plan to examine the endogenous localization of pro- and mature BDNF in the adult brain. Brain sections of 8 weeks old wild-type, *bdnf*-myc and *cbdnf ko* mice are immunostained for pro- and mature BDNF and the subcellular localization of pro- and mature BDNF is comparatively analyzed in pyramidal neurons of the hippocampus.

4.5.4 Generation of an inducible BDNFKO mouse by crossing the floxed *bdnf* mouse line with a *tau*::CreErt 2 mouse line

The generation of *cbdnf ko* mice circumvent the problem of postnatal lethality and allow a ≥ 95% reduction of BDNF protein levels throughout the brain compared with wild-type mice. However, this approach still suffers from the fact that *tau* is expressed very early in development with the consequence that the majority of animals generated results in the development of *bdnf* full knockout mice. To investigate the role of BDNF in the adult mouse brain more efficient, we are planning to generate a *tau*::CreERT2 mouse, which may allow us to delete BDNF after tamoxifen application. Further studies of these inducible BDNF KO mice will hopefully enable a more complete understanding of the role played by this neurotrophin in the adult and perhaps even aging brain.

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