Biosynthesis and release of Brain-derived neurotrophic factor: a study using neurons derived from embryonic stem cells.

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Stefano Olivieri

aus Mailand, Italien

Basel, 2014

Genehmigt von der Philosophisch Naturwissenschaftlichen Fakultät

<u>Auf Antrag von:</u>

Prof. Markus Rüegg

Prof. Yves-Alain Barde

Basel den 18 Juni, 2013

Prof. Dr. Jörg Schibler

Dekan

Namensnennung-Keine kommerzielle Nutzung-Keine Bearbeitung 3.0 Schweiz
(CC BY-NC-ND 3.0 CH)

Sie dürfen: **Teilen** — den Inhalt kopieren, verbreiten und zugänglich machen

Unter den folgenden Bedingungen:

Namensnennung — Sie müssen den Namen des Autors/Rechteinhabers in der von ihm festgelegten Weise nennen.

Keine kommerzielle Nutzung — Sie dürfen diesen Inhalt nicht für kommerzielle Zwecke nutzen.

Keine Bearbeitung erlaubt — Sie dürfen diesen Inhalt nicht bearbeiten, abwandeln oder in anderer Weise verändern.

Wobei gilt:

- **Verzichtserklärung —** Jede der vorgenannten Bedingungen kann **aufgehoben** werden, sofern Sie die ausdrückliche Einwilligung des Rechteinhabers dazu erhalten.
- **Public Domain (gemeinfreie oder nicht-schützbare Inhalte) —** Soweit das Werk, der Inhalt oder irgendein Teil davon zur Public Domain der jeweiligen Rechtsordnung gehört, wird dieser Status von der Lizenz in keiner Weise berührt.
- **Sonstige Rechte —** Die Lizenz hat keinerlei Einfluss auf die folgenden Rechte:
  - Die Rechte, die jedermann wegen der Schranken des Urheberrechts oder aufgrund gesetzlicher Erlaubnisse zustehen (in einigen Ländern als grundsätzliche Doktrin des **fair use** bekannt);
  - Die **Persönlichkeitsrechte** des Urhebers;
  - Rechte anderer Personen, entweder am Lizenzgegenstand selber oder bezüglich seiner Verwendung, zum Beispiel für **Werbung** oder Privatsphärenschutz.
- **Hinweis —** Bei jeder Nutzung oder Verbreitung müssen Sie anderen alle Lizenzbedingungen mitteilen, die für diesen Inhalt gelten. Am einfachsten ist es, an entsprechender Stelle einen Link auf diese Seite einzubinden.

Quelle: http://creativecommons.org/licenses/by-nc-nd/3.0/ch/ Datum: 12.11.2013

In memory of my mother Emanuela

Summary

Brain-derived neurotrophic factor (BDNF) is a secreted growth factor widely expressed in all major areas of the CNS where it regulates a number of different functions. In animal models of diseases, reduced levels of BDNF have been associated with several conditions, like Rett syndrome, Huntington's disease and depression. Moreover, reduced BDNF expression in humans has been recently linked with metabolic and neurocognitive impairments, including obesity, episodic memory loss and depression. Like other members of the neurotrophin family, BDNF is synthetized as a N-glycosylated precursor (pro-BDNF) that is post-translationally converted to mature protein. In CNS neurons, mature BDNF is subsequently sorted in large dense core vesicles, transported anterogradely to the synapses and released upon stimulation. As endogenous BDNF is expressed at extremely low levels, most previous studies on BDNF processing and release were performed using acute overexpression, an approach that can lead to the intracellular accumulation and secretion of unprocessed pro-BDNF. To test this possibility, an engineered ES cell line was generated in our laboratory by targeting *Bdnf* cDNA on *Mapt* locus. Since the expression of *Mapt* gene starts when progenitors exit from cell cycle, neurons derived from *Mapt::Bdnf* ES cells overexpress *Bdnf* in a controlled fashion throughout their maturation in culture.

My results indicate that BDNF overexpression is incompatible with complete processing and leads to a progressive accumulation and constitutive-secretion of pro-BDNF. By contrast, in wild-type neuronal cultures pro-BDNF is fully converted to mature BDNF that is released in an activity dependent fashion.

Regarding the important question of the release of endogenous BDNF from neurons, I observed that basal BDNF release is fully dependent on extracellular calcium influx through specific voltage gate calcium channels. By contrast, calcium efflux from sarcoplasmic reticulum, which triggers BDNF release during specific stimulation patterns, does not affect the basal BDNF secretion.

In order to identify the mechanisms underlying BDNF release during elevated neuronal activity, I derived a new ES cell line from *Bassoon* mutant mice ($Bsn^{m/m}$), which develop epileptic seizures and exhibit higher BDNF protein levels in various brain areas. Neurons derived from the $Bsn^{m/m}$ ES cell lines show

significantly higher levels of BDNF secretion. In addition, the release of BDNF observed in *Bassoon* mutant ES cell-derived neurons activates TrKB in these cells and down-regulates the expression of *KCC2*, a gene encoding for the major neuronal Cl^-/K^+ co-transporter.

My results thus shed new light on physiological mechanisms of endogenous BDNF biosynthesis and release–and invite a critical re-consideration of data obtained using overexpression paradigms.

Acknowledgements

Foremost, I would like to thank Prof. Barde for his continuous support during my PhD, for our discussions and for the example he has provided as a successful scientist and professor.

My sincere thank goes to Ruben Deogracias and Martijn Dekkers, for their helps and advices, and for all the stimulating conversations we had. A special thanks to Daniela Klewe-Nebenius for teaching me how to isolate ES cells from blastocyst, and to Kaspar Vogt for our important discussions about calcium channels.

I gratefully thank Mihai Constantin Ionescu for his incredible help to set and perform the BDNF ELISA assay, and for his precious suggestions.

I would like to acknowledge Chantal Rencurel and Valerie Crotet for their precious technical support and for our coffee breaks, during them I had the opportunities to improve my poor French skills.

Finally, I would like to say *grazie* to Anna, the most important woman in my life.

Abbreviations

AMPA	α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid
APV	(2*R*)-amino-5-phosphonovaleric acid
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid tetrakis(acetoxymethyl ester)
BDNF	Brain-derived neurotrophic factor
BSN	Bassoon
CaMKIV	Calcium/calmodulin-dependent protein kinase IV
CIRC	Calcium-induced calcium release
CNS	Central nervous system
CREB	cAMP responsive element-binding protein
EB	Embryoid body
ERK	Extracellular signal-regulated kinase
ES cells	Embryonic Stem cells
HVA	High voltage activated channel
KCC2	K+/Cl- co-transporter
LIF	Leukaemia inhibitory factor
LTD	Long-term depression
LTP	Long-term potentiation
LVA	Low voltage activated channel
mAb	Monoclonal Antibody
Mapt	Microtubule-associated protein tau
MeCP2	Methyl-CpG binding protein 2
MRI	Magnetic resonance imaging
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NGF	Nerve growth factor
NMDA	N-methyl D-aspartate

NT3	Neurotrophin-3
NT4	Neurotrophin-4
$p75^{NTR}$	Neurotrophin receptor p75NTR
pAb	Polyclonal antibody
PDGF	Platelet-derived growth factor
PNS	Peripheral nervous system
PKA	Protein kinase A
RA	Retinoic acid
RTT	Rett syndrome
SM proteins	Sec1 and Munc18 proteins
SNARE	SNAP (Soluble NSF Attachment Protein) Receptor
TGFβ	Transforming growth factor β
TLE	Temporal lobe epilepsy
TrK	Tropomyosin receptor kinase
TTX	Tetrodotoxin
VGCC	Voltage-gated calcium channels
WAGR	Wilms tumor, Aniridia, Genitourinary anomalies, mental retardation

Table of Contents

1. Introduction

The discovery of nerve growth factor (NGF), the first growth factor ever to have been discovered, introduced the concept that secretory proteins play a critical role during the development of multicellular organisms (Cohen et al., 1954; Levi-Montalcini, 1966; Levi-Montalcini and Hamburger, 1951). The extraordinary NGF antibody experiment performed by Levi-Montalcini and Cohen allowed the demonstration that such factors are essential for specific parts of the nervous system, the sympathetic ganglia in the original experiments, which are absent in animals deprived of NGF (Cohen, 1960; Cohen and Levi-Montalcini, 1957). By now, a few dozens of different growth factors have been shown to act on the developing nervous system and they are subdivided into distinct families based on structure similarities. One of these families comprises the neurotrophins and includes NGF, Brain-derived neurotrophic factor (BDNF), Neurotrophin-3 (NT3) and Neurotrophin-4 (NT4). The neurotrophins are not only structurally related, they also bind to similar receptors. All neurotrophins bind to related tyrosine kinase receptors of the Trk family with similar affinity and selectivity. NGF binds exclusively to TrkA (Kaplan et al., 1991), while both BDNF and NT4 bind to TrkB and NT3 to TrkC (Barbacid, 1994). Neurotrophins are homodimers (not linked by disulphide bridges, but by hydrophobic interactions) and these dimers activate Trk receptors by causing trans-phosphorylation following ligand-induced dimerization (Cunningham and Greene, 1998; Jing et al., 1992). Most, if not all trophic actions of neurotrophins can be accounted for by binding to Trk receptors. All neurotrophins also bind with similar nanomolar affinity to one other receptor designated the neurotrophin receptor p75 ($p75^{NTR}$), a member of the tumour necrosis factor family (Rodriguez-Tebar et al., 1990). Activation of the $p75^{NTR}$ typically antagonizes the “positive” effects elicited by Trk receptor activation and, in extreme cases, even causes cell death (Frade et al., 1999; Roux et al., 1999). Importantly, pro-neurotrophins bind to $p75^{NTR}$ with even higher affinity than processed (or mature) neurotrophins (Lee et al., 2001).

My Thesis work focuses on the expression and release of BDNF, in particular mature versus pro-BDNF. BDNF is a protein of extremely low abundance first characterized in brain extracts in 1982 (Barde et al., 1982) with its cDNA sequence reported in 1989 (Leibrock et al., 1989). For a number of reasons, including in particular results from human genetics and association with common conditions such as depression (see below), BDNF is receiving increasing attention, with on average in 2013, more than 5 publications a day dealing with BDNF according to PubMed. The total number of publications referring to BDNF exceeded those dealing with NGF a few months ago, and some of the reasons for this recent surge in interest for BDNF are summarized in the following paragraphs.

1.1 BDNF and human genetics

The first direct link in humans between BDNF and brain function was in the context of memory formation and retention following the discovery of the association between a nucleotide polymorphism leading to a valine (val) to methionine (met) substitution in pro-BDNF (Fig. 1) (Egan et al., 2003).

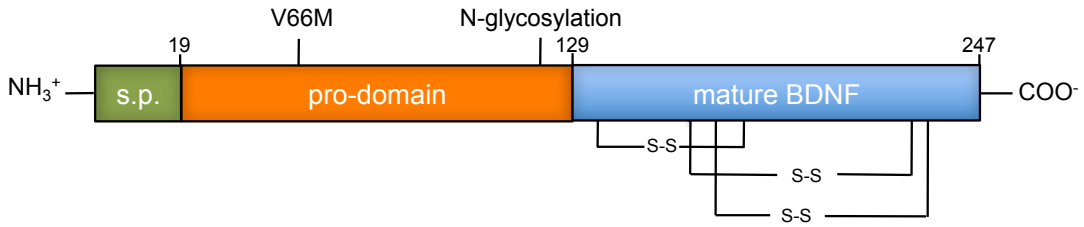


Figure 1. Domain organization of BDNF protein based on the cDNA sequence.

BDNF is composed of signal peptide (*green*), pro-domain (*red*) and mature protein (*blue*). The pro-domain undergoes N-glycosylation at the consensus sequence located six residues upstream to the cleavage site. The pro-domain is thought to exert a chaperon-like function allowing the formation of disulphide bridges of the mature protein. The polymorphism at amino acid 66 leads to a valine substitution with a methionine on pro-domain sequence (V66M) (see text). All neurotrophins known so far

have a similar domain organisation, including a recently reported neurotrophin identified in Aplysia (Kassabov et al., 2013)

The analysis of Met-carriers revealed that this polymorphism is associated with poorer episodic memory performance and abnormal hippocampal activation, as measured by functional magnetic resonance imaging (MRI). Given the very low abundance of BDNF and the resulting difficulties with the understanding of its biosynthesis and processing (see below), it is still very unclear how this amino acid substitution in pro-BDNF causes an impairment of memory performance. While the literature on the topic is quite controversial, what appears to be the most reliable study on quantification of BDNF in blood indicates that the levels of BDNF in heterozygous carriers are unchanged (Vinberg et al., 2013)(see below) with no published studies yet on homozygote carriers. A very interesting mouse model, aimed at mimicking the Val/Met substitution, has been published (Chen et al., 2006), but a detailed examination of the methodology indicates that the animal used in that study may differ by more than the Val/Met amino acid exchange in pro-BDNF. Thus, the causality between the anxiety phenotype observed in these animals and the Val/Met substitution cannot be firmly established. In general, a large number of publications attempt to correlate the BDNF polymorphism with a various conditions including depression, obesity and diseases of the nervous system including autism, Parkinson's and Alzheimer's diseases (Karamohamed et al., 2005; Ventriglia et al., 2002), but so far it is only the link with memory that could be independently confirmed in Basel by the group of Andreas Papassotiropoulos (Cathomas et al., 2010). The link between BDNF and memory is also interesting in the context of a large body of previous works in rodents linking BDNF with long-term potentiation (see below), thus creating a need for a better understanding of the cell biology of this protein, including the mechanisms involved in the release from neurons.

The loss of one *BDNF* allele in humans leads to a strong and early phenotype in children characterized by severe obesity and mental retardation, indicating that BDNF is involved in the regulation of food intake, in line with previous observations in rodents (Rios et al., 2001). Patients with a *de novo* inversion involving the *Bdnf* locus exhibit severe hyperphagia and obesity, associated with

impaired cognitive functions and hyperactive behaviours (Gray et al., 2006). Interestingly, very similar symptoms were observed in patients with a *de novo* missense mutation in TrkB gene, the tyrosine kinase receptor that mediates the trophic effects of both BDNF and NT4 (Yeo et al., 2004). In the mouse, both BDNF and TrkB are prominently expressed in the ventro-medial nucleus of the hypothalamus known to regulate food intake (Xu et al., 2003), and various *Bdnf* mouse mutants have been consistently associated with obesity, especially in females (Chang et al., 2006; Rauskolb et al., 2010).

The BDNF haploinsufficiency was recently associated with more severe neurocognitive impairments in subjects with WAGR (Wilms tumor, Aniridia, Genitourinary anomalies, mental retardation) syndrome, which is a rare genetic disorder caused by heterozygous chromosome 11p deletions of variable size (Han et al., 2008; Xu et al., 2008). Given that the loss of single BDNF allele frequently occurs in subjects with WAGR syndrome, these patients were recently used as model for studying human BDNF deficiency. Interestingly, subjects with BDNF haploinsufficiency suffer from severe impairments of adaptive behaviour and cognitive functions, supporting the hypothesis that BDNF plays an important role in human neurocognitive development (Han et al., 2013).

1.2 BDNF and depression

In addition to numerous results obtained in the mouse correlating BDNF levels and signalling with models of depression, there is also a growing literature in humans (Dwivedi et al., 2003; Lee and Kim, 2010). As it turns out in humans (but not in mice), significant levels of BDNF can be measured in the blood, mostly in serum, following the release of BDNF from platelets (Aydemir et al., 2006). While the function of BDNF in platelets remains enigmatic at this point, BDNF levels have been consistently reported to negatively correlate with depression (Brunoni et al., 2008; Karege et al., 2002). Moreover, treatment with various antidepressants has also been consistently reported to restore close to normal serum levels of BDNF (Chen et al., 2001). At present these findings remain very difficult to understand, as it appears implausible that BDNF levels measured in

blood samples may directly reflect the levels of the protein in the brain. Not only are the levels exceedingly low anywhere in the brain, but also the biochemical characteristics of BDNF, a very basic protein, makes it *a priori* unlikely that it could diffuse from its sites of secretion and through the blood-brain barrier.

1.3 Rett syndrome and role of BDNF in animal models of the disease

Rett syndrome (RTT) is a neurological disorder caused in most cases by a mutation on the X-linked methyl-CpG binding protein 2 gene (*MeCP2*) (Amir et al., 1999). In human, the predominant symptoms are deceleration of head growth, social withdrawal, loss of previously acquired skills and gait ataxia (Chahrour and Zoghbi, 2007). Male mice lacking *MeCP2* exhibit RTT-like symptoms starting about a month after birth including reduced brain weight, decreased neuronal size, hind limb clasping and impaired locomotor functions (Chang et al., 2006). Not only some of these symptoms are reminiscent of those observed in mice lacking BDNF in the CNS (see below), but also, in mice lacking *MeCP2*, the levels of BDNF fail to increase normally during the first weeks after birth. In addition, a genetic link has been established between BDNF and MeCP2 whereby decreasing BDNF levels aggravates, and increasing BDNF levels ameliorates the symptoms observed in males lacking *MeCP2* (Chang et al., 2006). In a similar vein, our laboratory recently showed that the administration of fingolimod, a sphingosine-1 phosphate receptor modulator, to male mice lacking *MeCP2* increased BDNF levels in brain areas affected by the lack of *MeCP2* and significantly improve RTT-related symptoms, in particular loco-motor symptoms (Deogracias et al., 2012). The same study also noted that the size of the striatum, a brain structure consisting largely of GABAergic neurons, is markedly reduced in mice lacking *MeCP2* mutant mice, and could be restored following fingolimod treatment. As the *Bdnf null* mutation is early lethal (Ernfors et al., 1994), meaningful comparisons with other mouse mutants mimicking neurological diseases such as Rett syndrome, could only be made following the conditional excision of *Bdnf*. Our laboratory found that using the *Mapt* (or *Tau*) to drive the expression of the Cre recombinase, mice essentially devoid of BDNF in the CNS

can be generated (Rauskolb et al., 2010). No evidence for neuronal death could be observed in these animals, but BDNF depletion caused severe symptoms broadly resembling what are observed in mice lacking *MeCP2* (see above), including obesity in females. Also, the number of dendrites, their length and spine density of GABAergic neurons was markedly decreased, thus explaining the reduction of the total striatal volume.

1.4 BDNF and long-term potentiation, a cellular model of memory

Long before a link between BDNF and memory performance could be established in humans (see above), the role of BDNF in the induction of long-term potentiation (LTP) was examined in the mouse hippocampus as this area was early identified as a major site of BDNF expression (Hofer et al., 1990). Learning and memory have long been thought to result from long-term changes in the strength of synaptic connections in specific brain areas such as the hippocampus. LTP is defined as an enduring change of synaptic strength resulting from specific stimulation patterns of presynaptic neurons and represents the most commonly used cellular model of memory (Bliss and Collingridge, 1993). Importantly, LTP can last not only for a few hours but also for days or even months. Short-lasting modifications are often referred to as early-phase LTP (E-LTP) and are typically caused by a single, high frequency tetanic stimulation. Long-lasting changes are referred to as long-phase LTP (L-LTP) or simply LTP (Kandel, 2004; Sweatt, 1999). They are triggered by repeated high frequency stimulations and typically involve mRNA and protein synthesis. During L-LTP, the protein kinase A (PKA), calcium/calmodulin-dependent protein kinase IV (CaMKIV) and extracellular signal-regulated kinase (ERK) signalling activate key transcription factors, including cAMP responsive element-binding protein (CREB), which triggers the synthesis of proteins that underlie L-LTP maintenance (Kandel, 2012; Minichiello, 2009). One of these genes is *Bdnf*, which is one of the genes typically regulated by activity and CREB activation in excitatory neurons. In addition, BDNF, now known to be stored in pre-synaptic terminals (see below) has long been known to be required for LTP induction (Korte et al., 1995): the loss of one

allele in the mouse was shown to severely compromise the induction of LTP in CA1 following high frequency of the Schaffer collaterals.

1.5 BDNF biosynthesis, processing and trafficking

Like all neurotrophins, BDNF is synthesized as a precursor protein composed of 3 distinct domains: a signal sequence, a pro-domain and the mature protein carrying the best-known biological properties of neurotrophins. The first 18 amino acids are thought to be cleaved during the transfer of the nascent protein into the lumen of the rough endoplasmic reticulum and the resulting pro-BDNF transferred to the Golgi apparatus, where it undergoes N-glycosylation at the single consensus sequence located six residues upstream to the cleavage site used by yet uncharacterized proteases to generate mature BDNF (Fig. 1).

Like a large number of secretory proteins and peptide hormones, all neurotrophins are initially synthesized as precursors, like, for example, the members of the transforming growth factor β family, insulin or enkephalins (Harrison et al., 2011; Peinado et al., 2003). The pro-domain is translated ahead of the mature domain and exerts an essential chaperone-like function allowing the formation of complex disulphide bridges in the mature protein (Fig. 1) (Eder and Fersht, 1995). In the case of the neurotrophins, PDGF and TGFβ, the arrangement of the bonds involving the cysteine residues is designated the *cysteine knot motif* (Swindells, 1992; Sun PD, 1995, review). With regard to neurotrophins, it is well established that mature, biologically active neurotrophins cannot be generated in physiologically relevant quantities in the absence of the pro-domain. However, this domain does not seem to be neurotrophin-specific, i.e. it can be swopped between for example BDNF and NT3 (Jungbluth et al., 1994). In CNS neurons, a work from our laboratory has directly demonstrated the presence of BDNF and pro-BDNF in the Golgi apparatus of hippocampal principle neurons, as well as in their axons and in pre-synaptic large dense core vesicles (Dieni et al., 2012). However, where exactly the cleavage of pro-BDNF takes place and by which protease cleaved at the consensus sequence RVRR preceding mature BDNF, is still unclear. This would

be important to know in order to better understand the role of BDNF, especially in overexpression paradigms (see below).

1.6 BDNF localization and release

Unlike with hormones such as insulin, that are stored in large quantities in secretory vesicles in specialized cells and organs, growth factors are stored in and released from neurons in only very limited amounts. Beyond very special neurons dedicated to secretion as the so-called magno-cellular cells of the hypothalamus, storing and secreting hormones such as vasopressin and oxytocin (Luckman et al., 1994), the vast majority of neurons does not store secretory protein in large amounts, as they only seem to act on post-synaptic structures closely apposed to BDNF-containing terminals. This makes a reliable detection of BDNF anywhere in the brain exceedingly difficult. The neurotrophin field has been very slow at recognizing that ultimately, the discovery of NGF and the key results about its physiological role were all made possible by the providential discovery of extremely large amounts of NGF in secretory cells of the adult male mouse submandibular gland. Up to this day, it is still entirely unclear why this should be so and restricted to the mouse. By contrast, but not surprisingly from a physiological standpoint, BDNF in the adult brain of all mammals tested is presented in very limited quantities, in the order of one part in a million based on tissue wet weight (Barde et al., 1982), so that its detection by straightforward techniques such as Western blotting is difficult and necessitates adequate controls. A large number of published results can be explained by insufficiently controlled experiments and/or by the use of overexpression strategies. The latter in particular are based on the underlying assumption that the enzyme(s) cleaving pro-BDNF are present in transfected cells at levels such that overexpressed pro-BDNF can be accommodated in neurons, i.e. properly cleaved and routed to physiological compartments. A major goal of my Thesis work was to test this hypothesis in neurons under defined conditions, with one of very few possible alternatives being the use of genetically engineered neurons (see below). Briefly, my results provide evidence that the hypothesis of extra processing capacity of pro-BDNF is not founded and that overexpression

paradigms cannot be used to study the processing and release of BDNF. These new results challenge the conclusions of a number of previous studies with regard to over-expression of BDNF not only *in vitro*, but also *in vivo*.

Utilizing *in vitro* over-expression paradigm typically using BDNF delivered by viruses and/or neurons transfected with BDNF cDNAs and tagged with (for example) GFP, numerous published results indicate that pro-BDNF is found in the conditioned medium of these cells to then get processed by extracellular tissue plasminogen activator. Indeed, much has been made of the observation that as the secretion of plasminogen activator is activity dependent, the role of BDNF in LTP may be explained by extracellular cleavage of pro-BDNF following its secretion during the course of stimulation leading to LTP at hippocampal synapses (Pang et al., 2004). This notion was later challenged by observations from our laboratory using pulse-chase analysis and immunoprecipitation of neuronal lysates, (endogenous) pro-BDNF is rapidly and fully processed intracellularly (Matsumoto et al., 2008). Similar results were obtained upon incubation with BDNF antibodies during the pulse-chase experiment, confirming that the conversion of pro-BDNF is an intracellular process. Accordingly, no evidence for the release of pro-BDNF could be obtained in the same experiments by analyzing the culture medium (Matsumoto et al., 2008).

For the same reason of very low abundance, the distribution and release mechanisms of BDNF from neurons, the main objective of my work, have been very difficult to study. The question of the sub-cellular localization of BDNF, i.e. axon terminal and/or dendrites is of special importance in the context of BDNF, especially in view of the attractive model propagated for years on the basis of convincing result obtained with NGF and the peripheral nervous system. In this regard, the structural relatedness of BDNF with NGF has been misleading since NGF has been traditionally considered in the context of the "neurotrophic theory" (Purves et al., 1988), according to which neurons compete for a limiting amount of NGF secreted from targeted tissues, with the prediction that only the successful competitors survive to establish new functional connections. This notion was so appealing that it was then applied to essentially all growth factors subsequently discovered, including BDNF. But as it turned out, in what appears to be the first series of rigorously controlled immunohistochemical experiments,

including localization by electron microscopy and gold-labeled BDNF antibodies, the localization of BDNF turned to be entirely presynaptic, with no specific staining in dendrites, not even under conditions of marked increase activity and BDNF levels (Dieni et al., 2012). Fully consistent with the view that BDNF accumulates in pre-synaptic terminals, BDNF has been proposed to be anterogradely transported by cortical afferents projecting to the striatum, a demonstration based on the selective excision of *Bdnf* in the developing cortex. As the *Bdnf* gene is not expressed in the striatum, the logical explanation for the presence of the protein in this structure is anterograde transport. Similar results following unilateral ablation of the cerebral cortex in the adult rat led to a marked decrease of BDNF levels in the ipsilateral striatum (Altar et al., 1997).
With regard to release mechanisms and for the reasons discussed in the above, still very little is known about the conditions necessary for the release of endogenous BDNF. The release mechanism is also unknown: it is not clear if there is a "constitutive" i.e. activity-independent release, if it is calcium-dependent and, in this case, if extracellular calcium is involved. So far, there is only one published study (Balkowiec and Katz, 2000) dealing with the release of endogenous BDNF using new-born, but immature hippocampal neurons. As these results are directly relevant to my work, they are mentioned and compared with my own data in the Discussion.

1.7 Embryonic stem cells and neuronal differentiation

As the major objective of my Thesis work was to perform biochemical and cell biological studies with neurons, both wild-type and engineered, there was little choice but to use neurons derived from mouse embryonic stem (ES) cells. These cells can be expanded indefinitely, solving the quantity problem neurobiologists have been facing for decades and ES cells can of course be engineered to express cDNAs such as BDNF introduced into specific loci, thus allowing for controlled and reproducible levels of BDNF. What greatly facilitated my work is that our laboratory developed a few years ago a method based on the use of ES cells allowing the generation of essentially homogenous populations of neurons (Bibel

et al., 2007) . After about 10 days following plating of neural progenitors, the neurons are electrically active and form functional synapses using glutamate as neurotransmitter (Bibel et al., 2004). These characteristics make these neurons ideally suited to examine basic aspects of BDNF biochemistry and release both under basal conditions and after controlled overexpression. Cultured ES cells multiply indefinitely in culture while retaining their ability to differentiate into all cell types including germ cells. The addition of leukaemia inhibitory factor (LIF), an interleukin class 6 cytokine expressed and secreted by embryonic fibroblasts, has long been known to allow the pluripotency of ES cells to be maintained possibly mimicking an arrested developmental state found in rodents designated diapause (Smith et al., 1992; Williams et al., 1988). More recently, work by the laboratory of Austin Smith demonstrated that pluripotency may be a "ground state", meaning that self-renewal and pluripotency represent a default state that can also be achieved when the action of external differentiating signals such as fibroblast growth factors is prevented (Ying et al., 2008). This worked greatly facilitated the isolation of ES cells from different mouse strains, making researchers independent from the Sv129 strain used in all ES cell-related experiments for about 20 years. My work (see Results) also benefited from these improvements and made it possible to isolated neurons from the *Bassoon* mutant in a marked increased of BDNF levels is observed, presumably as a result of increased activity.

A main advantage of our differentiation method is that it is robust and simple, for example unlike most other related differentiation protocols it does include fluorescent cell sorting. Briefly, our protocol starts with the culture of mouse ES cells on a feeder layer of embryonic fibroblasts in presence of LIF and serum to maintain their pluripotency. ES cells are then deprived of feeders and the key step of our procedure is to ensure homogeneity of the ES cells by splitting them frequently. This procedure progressively dilutes cells dividing more slowly, which is one of the characteristics of ES cells that begin to differentiate. In addition, the procedure also helps diluting the levels of differentiating secretory factors such as the FGF4 generated by ES cells as this gene is under the control of SOX2 and Oct4 (Yuan et al., 1995), two of the characteristic transcription factors

expressed by pluripotent ES cells. The subsequent ES cells aggregates, initially cultured in the absence of retinoic acid, are treated with retinoic acid for 4 days to induce neural commitment as monitored by the expression of Pax6, a useful marker of radial glial cells (Gotz et al., 1998). As shown with the developing mouse cortex about 10 years ago (Bibel et al., 2004), Pax6 progenitors essentially all (>90%) differentiate into glutamatergic neurons, the other cells representing a mixture of GABAergic neurons, oligodendrocytes and astrocytes.

Objectives of the Thesis

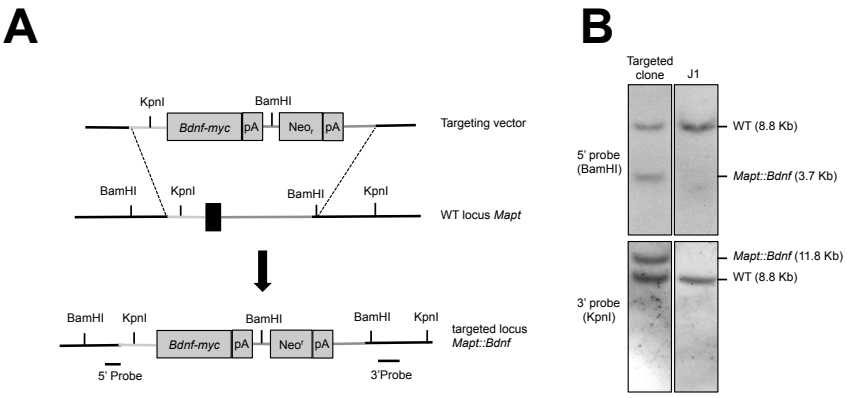
Using neurons generated from ES cells expressing either wild-type or increased levels of BDNF, the focus of my work is to determine the proportion of processed versus unprocessed pro-BDNF at different time points, as well as the mechanisms involved in the release of BDNF, including the role of activity and calcium.

2. Results

2.1 Generation of engineered ES cells

As the endogenous levels of BDNF are extremely low, most studies dealing with processing, release and even with the physiological functions of BDNF were performed using acute over-expression. Nevertheless, BDNF over-expression in neurons can lead to an excess of unprocessed and secreted pro-BDNF, a possibility that seems not to be sufficiently considered in the field. To directly test it, our laboratory generated a new ES cell line targeting the neuron-specific *Mapt* locus of J1 ES cell with cDNA expressing mouse *Bdnf* (Fig.2A). The *Mapt* locus is particularly suitable for gene targeting and its expression starts when neuronal progenitors exit the cell cycle. As observed in mutant mice, the deletion of *Mapt* gene does not interfere with the axonal growth and maintenance (Harada et al., 1994). In addition, *Mapt* expression levels rise during neuronal maturation and, more importantly, are independent of neuronal activity.

From the targeted *Bdnf* cDNA the last 9 base pairs were removed at the 3' end of the protein coding sequence to eliminate a putative cleavage site. In addition, a Myc tag sequence was added on the new 3' end to allow discrimination of endogenous versus exogenous BDNF. After electroporation and antibiotic selection, 100 ES clones were picked and their genomic DNA analysed by non-radioactive Southern blot to identify the recombination. Using both 5' and 3' probes, 5 clones were identified as positive for a correct recombination in the *Mapt* locus (Fig.2B). These clones were subsequently differentiated in a pure population of glutamatergic neurons using a protocol established in our laboratory (Fig. 2C).



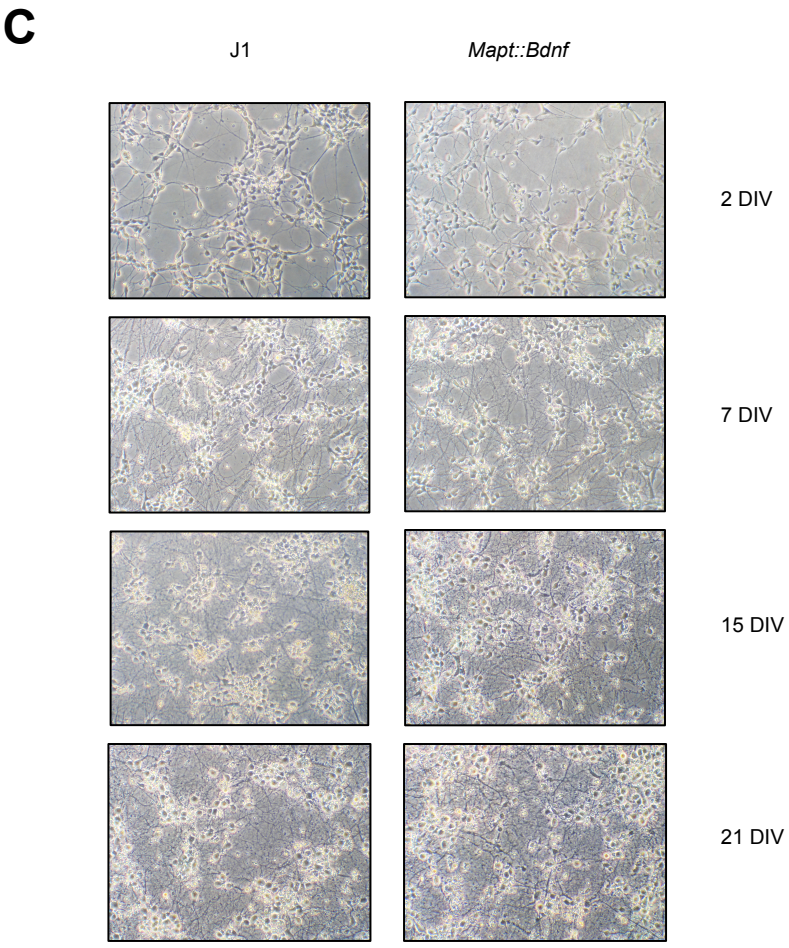


Figure 2. Generation and neuronal differentiation of *Mapt::Bdnf* ES cell line.

A) Scheme of targeting strategy to insert BDNF cDNA in the exon 1 of *Mapt* gene B) Southern blot analysis on genomic DNA extracted from picked ES clones confirms the homologous recombination. C) Neurons obtained after differentiation of J1 and *Mapt::Bdnf* ES cells do not show any morphological difference by phase contrast microscopy analysis.

In order to determine BDNF expression levels, cell extracts were collected at 6, 9 and 21 DIV from J1 and *Mapt::Bdnf* ES cell-derived neurons and analysed by real-time PCR and Western blot (Fig. 3). The analyses revealed that in both J1 and *Mapt::Bdnf* ES cell derived neurons, there is a progressive increase of BDNF protein levels. In addition, as the *Mapt* locus begins to be expressed early during the process of neuronal differentiation, an increase of BDNF over wild-type cells is already evident in engineered cells a few days after progenitors plating (Fig. 3A). The mRNA quantification results by real time PCR are in line with the protein data and a progressive increase was observed in both neuronal cultures (Fig. 3B). In *Mapt::Bdnf* ES cell-derived neurons, the increase was between 2.3 and 2.6-fold compared with wild-type neurons at day 6 and day 21 after progenitor plating, respectively.

Note that the values obtained for *Mapt::Bdnf* ES cell-derived neurons correspond to the mRNA transcribed from both the endogenous and exogenous *Bdnf* templates.

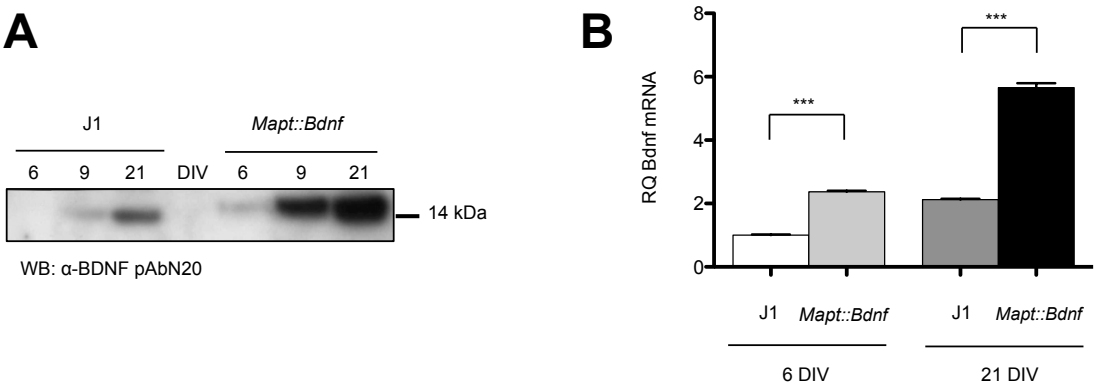


Figure 3. BDNF protein and mRNA levels in J1 and *Mapt::Bdnf* ES cell-derived neurons

A) Western blot analysis of cell extracts (20 µg) obtained from ES cell-derived neurons confirms overexpression in *Mapt::Bdnf* cells and shows a progressively increase of BDNF protein levels in both wild type and engineered neurons. B) Relative mRNA quantification shows a progressive increase of *Bdnf* transcription during neuronal maturation in both J1 and *Mapt::Bdnf* ES cell-derived neurons. (Student t-test analysis: mean ± SEM; n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

2.2 BDNF processing increases during neuronal maturation

To investigate whether BDNF processing changes during the course of neuronal maturation and to explore how this may be altered by gene overexpression, we analysed cell extracts of neurons at 6, 9 and 21 DIV by Western blot (Fig. 4). After 6 DIV, the ratio between endogenous pro-BDNF versus the total BDNF revealed an absence of detectable processing (J1 ESCDN 6DIV: ratio pro-BDNF/total-BDNF = 1). However, in the *Mapt::Bdnf* ES cell-derived neurons, a weak signal corresponding to the mature BDNF is detectable at 6 DIV, indicating a modest processing capacity (*Mapt::Bdnf* ESCDN 6DIV : ratio pro-BDNF/total-BDNF = 0.86).

The analysis of the cell extracts obtained from 9 and 21 DIV neuronal cultures revealed that BDNF processing changes dramatically within few days during neuronal maturation. In J1 ES cell-derived neurons, the ratios pro-BDNF/total BDNF observed after 9 and 21 days were respectively 0.34 and 0.02, indicating a progressive increase in the capacity of developing neurons to process pro-BDNF. A similar increase was observed in *Mapt::Bdnf* ES derived neurons, with the ratio pro-BDNF/total BDNF corresponding to 0.57 and 0.24 after 9 and 21 DIV respectively.

These results indicate that both J1 and *Mapt::Bdnf* ES cell-derived neurons develop a detectable capacity to process pro-BDNF during neuronal differentiation but that even a relatively moderate degree of overexpression is sufficient to exhaust the processing capacity in differentiating neurons.

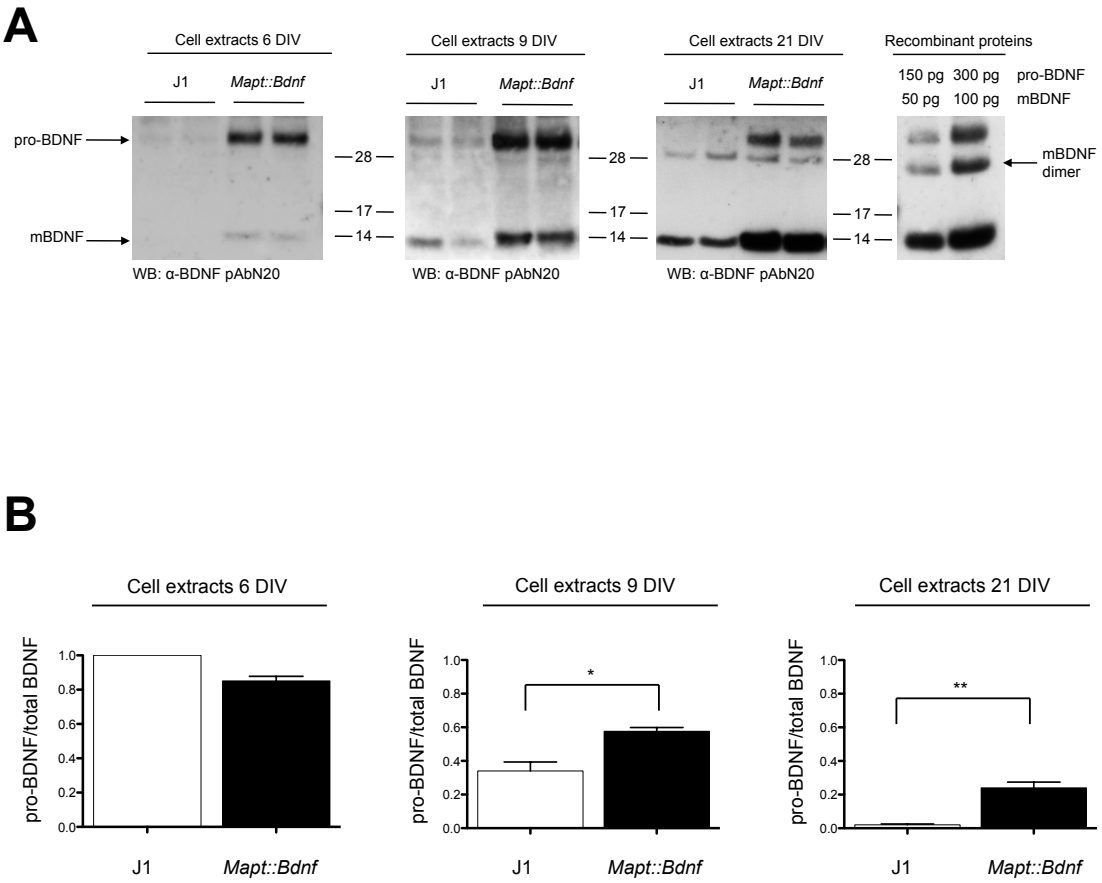


Figure 4. Analysis of pro-BDNF/total BDNF ratio during ES cell-derived neuronal maturation

A) Western Blot analysis of cell extracts (20 µg) from J1 and *Mapt::Bdnf* ES cell-derived neurons collected at 6, 9 and 21 DIV shows a progressive increase of pro-BDNF cleavage capacity during neuronal maturation. B) Optical density analysis of Western blot bands and quantification of pro-BDNF/total BDNF ratio. (Student t-test analysis: mean ± SEM; n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

2.3 Evaluation of BDNF overexpressing levels in Mapt::Bdnf ES-cell derived neurons

To further explore the impact of BDNF over-expression on processing, I then quantified the levels of pro- and mature BDNF stored in both wild-type and engineered ES cell-derived neurons using recombinant proteins as standard. Western blot analyses were performed with cell extracts obtained from J1 and *Mapt::Bdnf* ES cell-derived neurons cultured at 21 DIV (Fig. 5A). A standard curve was generated with various concentrations of recombinant pro- and mature BDNF and its linearity allowed a quantitative analysis of the results (mature BDNF standard curve: R^2=0.9941; pro-BDNF standard curve: R^2=0.9870) (Fig. 5B,D).

After 21 DIV, in 20 µg of cell extract obtained from J1 and *Mapt::Bdnf* ES cell-derived neurons I determined 7.83 pg (n=3, SEM ±1.980) and 95.8 pg (n=3, SEM ±6.853) of BDNF respectively (Fig. 5C). This analysis also revealed a larger difference in pro-BDNF levels between the 2 lines: 5.409 pg (n=3, SEM ±1.401) versus 583 pg (n=3, SEM ±7.702) in 20 µg of cell extracts of wild-type versus engineered neurons (Fig. 5E).

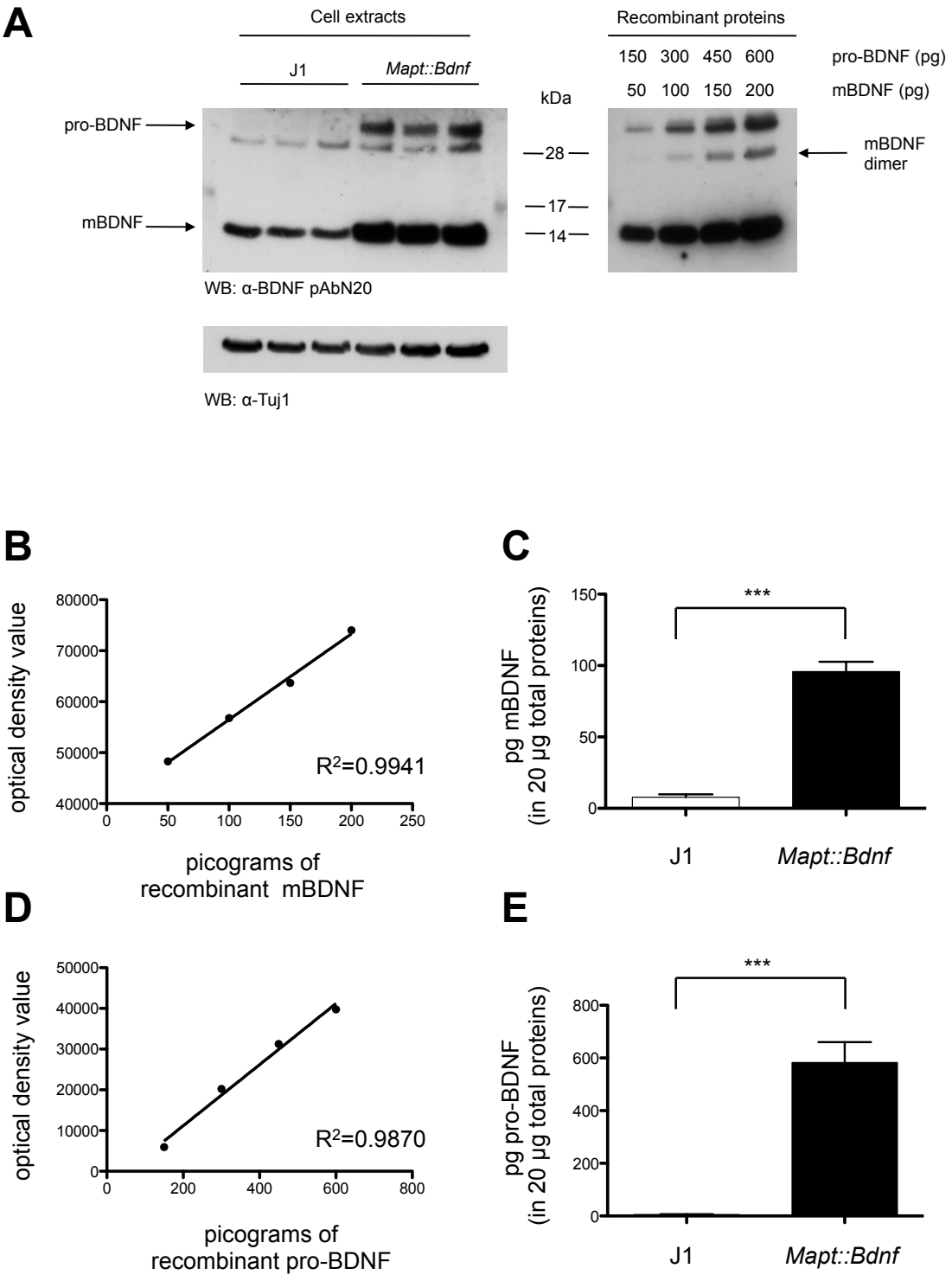


Figure 5. Quantification of pro- and mature BDNF in J1 and *Mapt::Bdnf* ES cell-derived neurons (21 DIV)

A) Western blot analysis of cell extracts (20 µg) of J1 and *Mapt::Bdnf* ES cell-derived neurons. B and D) Standard curves based on known amounts of recombinant pro- and mature BDNF pro- and mature BDNF. C and E) Quantification of pro- and mature BDNF in J1 and *Mapt::Bdnf* ES cell-derived neurons. (Student t-test analysis: mean ± SEM; n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

2.4 BDNF overexpression causes pro-BDNF release

The results of my experiments indicate that BDNF overexpression causes an accumulation of pro-BDNF, presumably saturating the regulated secretory pathway leading to an activity-independent release of pro-BDNF. To directly test whether pro-BDNF is released and detected in the conditioned medium of neurons over-expressing BDNF, an immunoprecipitation (IP) was performed allowing the detection of released mature and pro-BDNF from J1 and *Mapt::Bdnf* ES cell-derived neurons at 21 DIV (Fig. 6A). In these experiments, neurons were incubated in the presence of a monoclonal antibody (Mab#9) capturing secreted BDNF and preventing its re-uptake of BDNF by neurons. The conditioned medium was subsequently examined by Western blot, an experiment including the use of different concentrations of recombinant pro- and mature BDNF to allow the generation of a standard curve for protein quantification (Fig. 6B,D). In all conditioned media collected from J1 ES cell-derived neurons, these analyses failed to reveal any detectable signal corresponding to pro-BDNF. By contrast, in the conditioned media from *Mapt::Bdnf* ES cell-derived neurons, I could detect a clear signal corresponding to pro-BDNF (n=3, 486.2 ± 48.50 pg) (Fig. 6E). This analysis also revealed that the amount of mature BDNF released from *Mapt::Bdnf* ES cell-derived neurons (21 DIV) is 3.14-fold higher compared with the endogenous mature BDNF released from J1 cell line (n=3; J1 ESCDN: 18.66 ± 3.3312 pg; *Mapt::Bdnf* ESCDN: 58.75 ± 6.004 pg; unpaired t-test analysis: p= 0.0043) (Fig. 6C). These results further support the notion that the endogenous pro-BDNF is fully processed intracellularly with no detectable pro-BDNF released in the medium. They also show that a relatively moderate increased of pro-BDNF levels in the same neurons at identical stage of differentiation do release of pro-BDNF in the incubation medium.

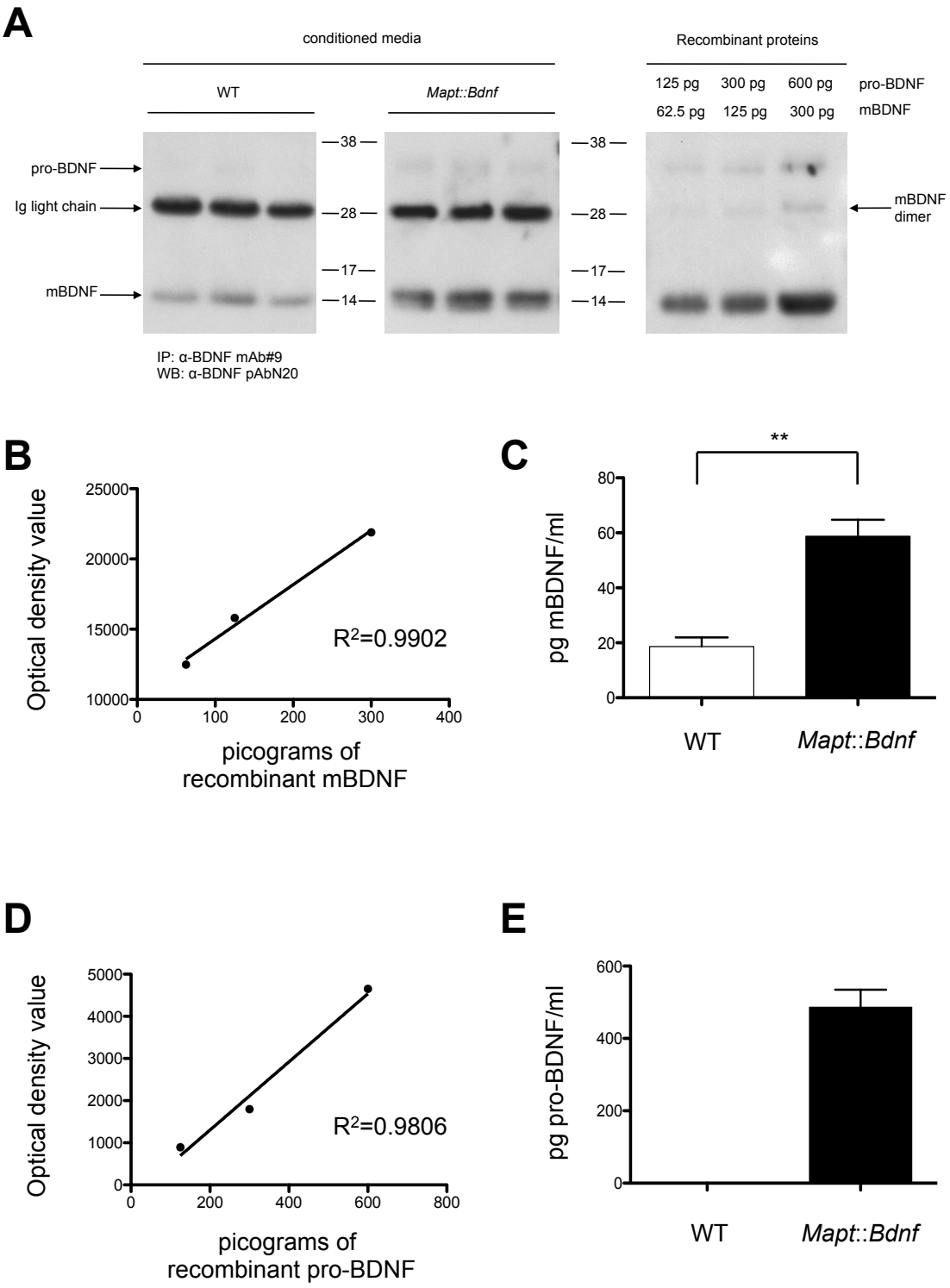


Figure 6. Quantification of BDNF and pro-BDNF in conditioned media of J1 and *Mapt::Bdnf* ES cell-derived neurons at 21 DIV.

A) Western blot analysis of BDNF released overnight by J1 and *Mapt::Bdnf* ES cell-derived neurons. Conditioned media (1 ml) were immunoprecipitated with α-BDNF mAB#9 and resolved by SDS-PAGE. B and D) Standard curves were established with recombinant pro- and mature BDNF. C and D) Quantification of mature and pro-BDNF released overnight from J1 and *Mapt::Bdnf* ES cell derived neurons (Student t-test analysis: mean ± SEM; n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

2.5 Constitutive BDNF release from Mapt::Bdnf ES cell-derived neurons

To determine if BDNF is released from ES cell derived neurons via a regulated pathway and to test whether the release is affected by BDNF overexpression, I treated J1 and *Mapt::Bdnf* ES cell-derived neurons overnight with 100 μM bicuculline, GABAa receptor antagonist, 50 mM potassium chloride (KCl) and with 1 μM tetrodotoxin (TTX), a specific sodium channel blocker (Fig. 7). Since previous results revealed that spontaneous electrical activity begins to emerge in J1 ES cell-derived neurons between 8 to 10 DIV (data not shown), I performed experiments with "inactive" neurons at 6 DIV and compared the results with 15 DIV neurons. BDNF was immunoprecipitated and subsequently analysed by Western blot. While no signal could be detected in J1 (wild-type) neuronal cultures, I observed a faint, but detectable BDNF band already in the medium conditioned by *Mapt::Bdnf* ES cell derived neurons at 6 DIV. Interestingly, neither bicuculline, KCl or TTX treatment affected the release from the *Mapt::Bdnf* cells, suggesting that BDNF secretion from these young, engineered neurons is independent of their activity status. By contrast, at 15 DIV, TTX treatment decreased BDNF release in both J1 and *Mapt::Bdnf* ES cell-derived neurons. In addition, increased BDNF release was observed with both types of neurons following bicuculline treatment. Strikingly, KCl addition led to a massive BDNF release, both from wild-type and engineered neurons.

Together, these results reveal that the secretion of both native and exogenous BDNF from mature ES cell-derived neurons is dependent on neuronal activity. However, young *Mapt::Bdnf* ES cell-derived neurons also release BDNF in activity-independent fashion, further suggesting that BDNF overexpression affects BDNF trafficking leading to its unregulated secretion.

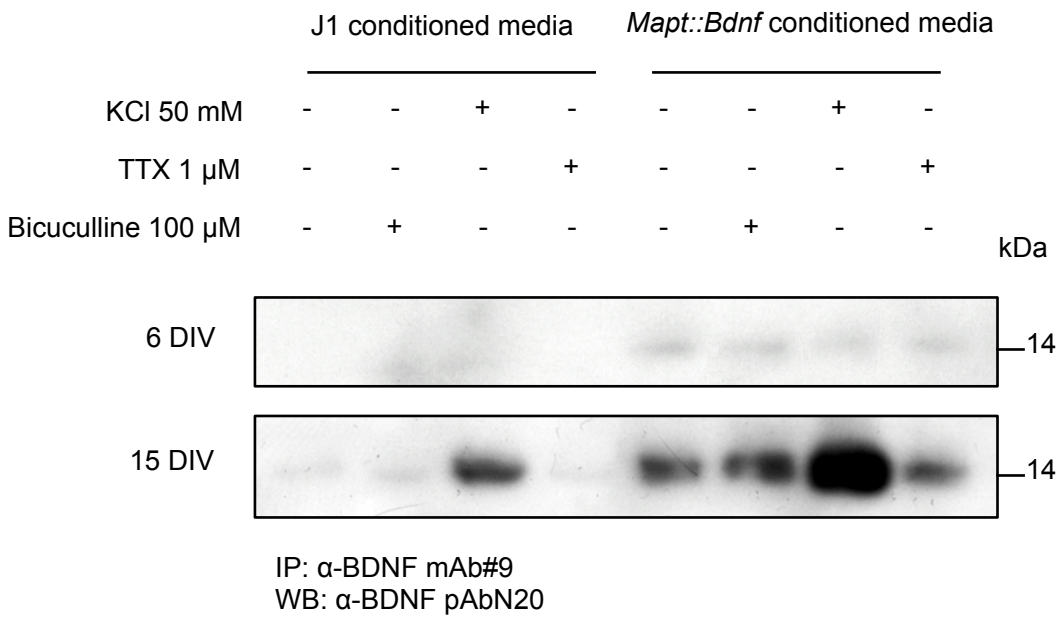


Figure 7. Analysis of activity-dependent release of BDNF from young (6 DIV) and adult (15 DIV) neurons derived from J1 and *Mapt::Bdnf* ES cells.

Western blot analysis of BDNF released after o/n treatment of J1 and *Mapt::Bdnf* ES cell-derived neurons (6 and 15 DIV) with 50 mM KCl, 1 µM TTX and 100 µM Bicuculline. Conditioned media (1 ml) were immunoprecipitated with α-BDNF mAB#9 and analysed by SDS-PAGE. After 6 DIV, only *Mapt::Bdnf* neurons release BDNF in an activity-independent manner while activity-dependent BDNF release is observed with 15 DIV neurons and is regulated by drug addition.

2.6 Expression levels and release of endogenous BDNF during chronic depolarization

So far, essentially all studies dealing with the mechanisms of release of BDNF from neurons used primary cultures of neurons transfected with BDNF cDNA constructs. However, it is very unclear whether the results obtained analysing the release and the processing of exogenous BDNF can be extrapolated to the endogenous neurotrophin. As depolarizing stimuli such as increased KCl concentrations typically also increase BDNF transcription (Zafra et al., 1990), I first examined the impact of overnight depolarization with 20 mM KCl on BDNF

levels in J1 and *Mapt::Bdnf* ES cell-derived neurons at 21 DIV (Fig. 8). As expected, the Real time PCR assay revealed a strong *Bdnf* up-regulation (unpaired t-test analysis J1 ESCDN: w/o treatment vs KCl p=0.0009, N=3; unpaired t-test analysis *Mapt::Bdnf* ESDN: w/o treatment vs KCl p=0.0010, N=3). Note that the relative increase was more marked in WT neurons compared with engineered neurons.

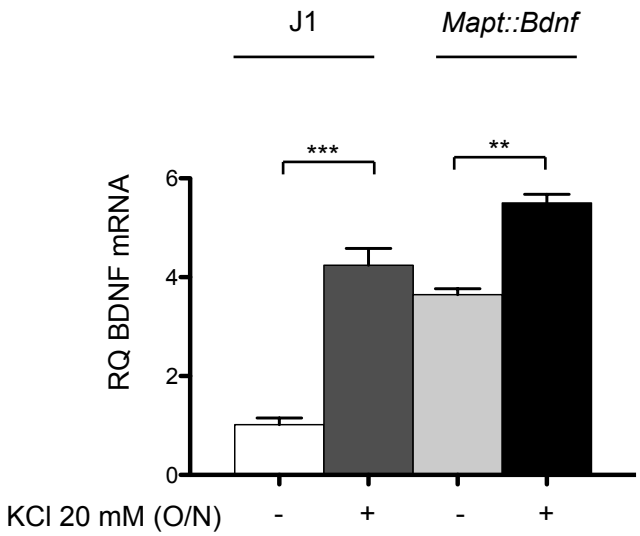


Figure 8. BDNF mRNA levels after overnight KCl exposure of J1 and *Mapt::Bdnf ES* cell-derived neurons at 21 DIV.

Student t-test analysis: mean ± SEM; n = 3. *P < 0.05; **P < 0.01; ***P < 0.001.

As the addition of 50 mM KCl to the medium significantly increases its osmotic pressure (measured by cryoscopy) to 83 osm/L, I tested the effect of 50 mM NaCl on BDNF release (Fig. 9A). In these experiments, the conditioned medium was first supplemented with anti-BDNF #9 antibody to collect and remove BDNF released by cultured neurons up until then. The medium was then removed either after 3 hours or overnight incubation in the presence of fresh biotinylated antibody. BDNF levels were then assessed with a modified ELISA (Kolbeck et al., 1999). While NaCl addition did not trigger BDNF release [21 DIV; w/o treatment: 9.443 ± 0.5354 pg (3h) and 83.272 ± 1.9031 pg (o/n); 50 mM NaCl 10.048 ± 0.5557 pg (3 h) and 63.639 ± 0.70 pg (o/n)], KCl addition significantly BDNF

released both at short (3 h) and long (18 h) time intervals: 50 mM KCl: 41.871 ± 0.8942 pg (3 h) and 180.144 ± 3.6492 pg (o/n). The mechanisms underlying BDNF release trigged by KCl treatment were further investigated by removing extracellular calcium during KCl depolarization. At 21 DIV, J1 ES cell-derived neurons were treated for 2 hours in presence of the biotinylated anti-BDNF #9 antibody with KCl added to either standard or calcium-free medium (Fig. 9B). The results of the BDNF quantification by ELISA revealed that in the absence of extracellular calcium, BDNF release is abolished: control: 6.455 ± 0.0913 pg; 50 mM KCl 22.729 ± 0.0050 pg; 50 mM KCl and without extracellular calcium: 2.014 ± 0.0435 pg.

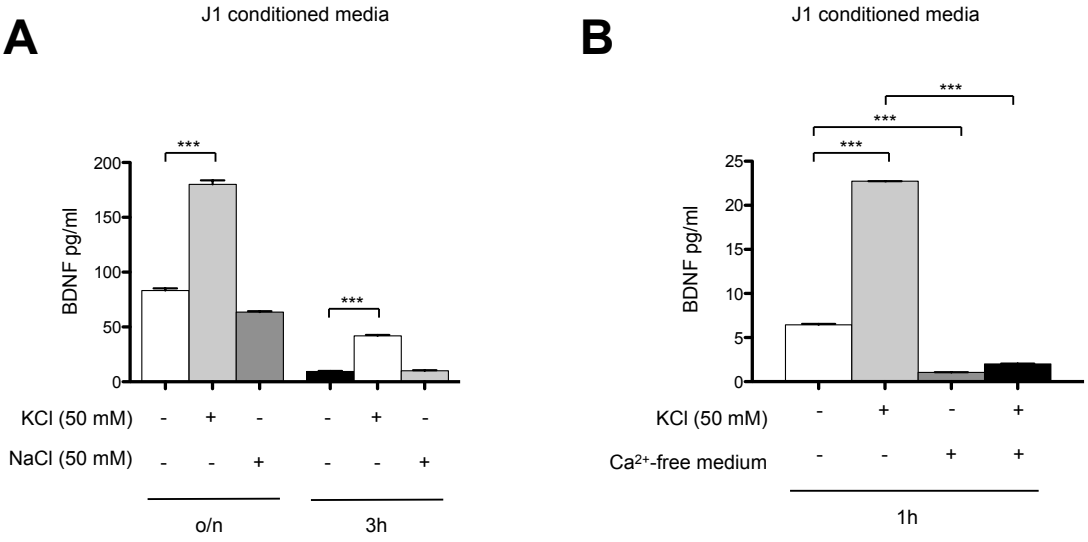


Figure 9. ELISA quantification of BDNF released from J1 ES cell-derived neurons (21 DIV) in the presence of KCl, NaCl and with or without extracellular calcium.

A) The conditioned media were collected after overnight exposure to KCl (50 mM) and NaCl (50 mM) treatment of J1 ES cell-derived neurons (21 DIV). B) BDNF basal secretion is decreased and KCl-induced release suppressed in the absence of extracellular calcium (Student t-test analysis: mean ± SEM; n = 3;). *P < 0.05; **P < 0.01; ***P < 0.001.

2.7 Opposite effects of neuronal activity on pro- and mature BDNF secretion in engineered ES cells

I then tested if the release not only of mature, but also of pro-BDNF from *Mapt::Bdnf* ES cell-derived neurons (21 DIV) would be modulated either by 20

mM KCl or 1 μM TTX using J1 neurons as controls (Fig. 10 and 11). As described, BDNF release from J1 ES cell-derived neurons increases in presence of KCl ($p=0.0015$, n=3) and decreases during the TTX treatment ($p=0.0463$, n=3; w/o treatment: 38.88 ± 3.897 pg; KCl: 107.5 ± 8.034 pg; TTX: 24.01 ± 3.464 pg) (Fig. 10C). Upon simultaneous treatment with both TTX and KCl, BDNF release was moderately decreased (KCl-TTX: 85.83 ± 7.124 pg; n=3). Pro-BDNF realised from J1 neurons remained undetectable during chronic depolarization with 20 mM KCl. The corresponding Western blots on cell lysates reveal that mature BDNF levels is less abundant in neurons (21 DIV) treated with KCl (w/o treatment: 109.4 ± 2.811 pg; KCl: mean 59.09 ± 1.734 pg; unpaired t-test analysis: $p<0.001$; n=3) (Fig. 10B), indicating that BDNF release impacts the levels remaining in the neurons. TTX treatment did not change the levels of BDNF stored intracellularly (TTX: 103.1 ± 5.542 pg, n=3, see Discussion for interpretation of these results). In addition, I found that TTX treatment during chronic depolarization caused the levels of mature BDNF to return to those observed in neurons without treatment (KCl-TTX: mean 105.9 ± 2.461 pg, n=3; unpaired t-test analysis: $p<0.001$). These results suggest that the changes caused by KCl treatment are primarily mediated by increased neuronal activity.

The analysis of *Mapt::Bdnf* ES cell-derived neurons (21 DIV) revealed increased release of mature BDNF compared with wild-type neurons (Fig. 11A) that was further increased by KCl treatment ($p=0.0002$), but remained unaffected by TTX treatment (w/o treatment: 71.63 ± 2.025 pg; KCl: 104.5 ± 1.650 pg; KCl-TTX: mean 110.6 ± 7.748 pg; n=3) (Fig. 11C). TTX treatment of the *Mapt::Bdnf* ES cell-derived neurons partially reduced the amount of released mature BDNF ($p=0.0016$ TTX: 47.01 ± 2.256 pg, n=3). With regard to the secretion of pro-BDNF from these engineered neurons, neuronal activity turned out to have the opposite effects compared with mature BDNF (Fig. 11D). During chronic depolarization, a slight decrease ($p=0.341$) of pro-BDNF release was observed in the *Mapt::Bdnf* ES cell derived neurons (21DIV; w/o treatment: 478.3 ± 26.38 pg; KCl: 387.8 ± 11.10 pg, n=3) that remained unaffected by pairing chronic depolarization with TTX treatment (TTX-KCl: 371.2 ± 16.30 pg, n=3). Surprisingly, TTX treatment alone increased the release of pro-BDNF ($p=0.010$)

in the conditioned medium (TTX: mean 642.1 ± 24.08 pg). The analysis of cellular extracts revealed a significant decrease of mature BDNF after KCl (p=0.0012) and TTX (p=0.0406) treatment on *Mapt::Bdnf* ES cell-derived neurons (21 DIV, w/o treatment: 147.9 ± 3.740 pg; KCl: 98.63 ± 4.641 pg; TTX: mean 128.6 ± 5.806 pg; n=3). As observed with J1 neurons, TTX treatment of *Mapt::Bdnf* ES cell-derived neuron did not affect the amount of mature BDNF stored intracellularly. The same result were observed treating the culture with TTX and KCl simultaneously (KCl-TTX: mean 129.1 ± 5.419 pg; n=3).

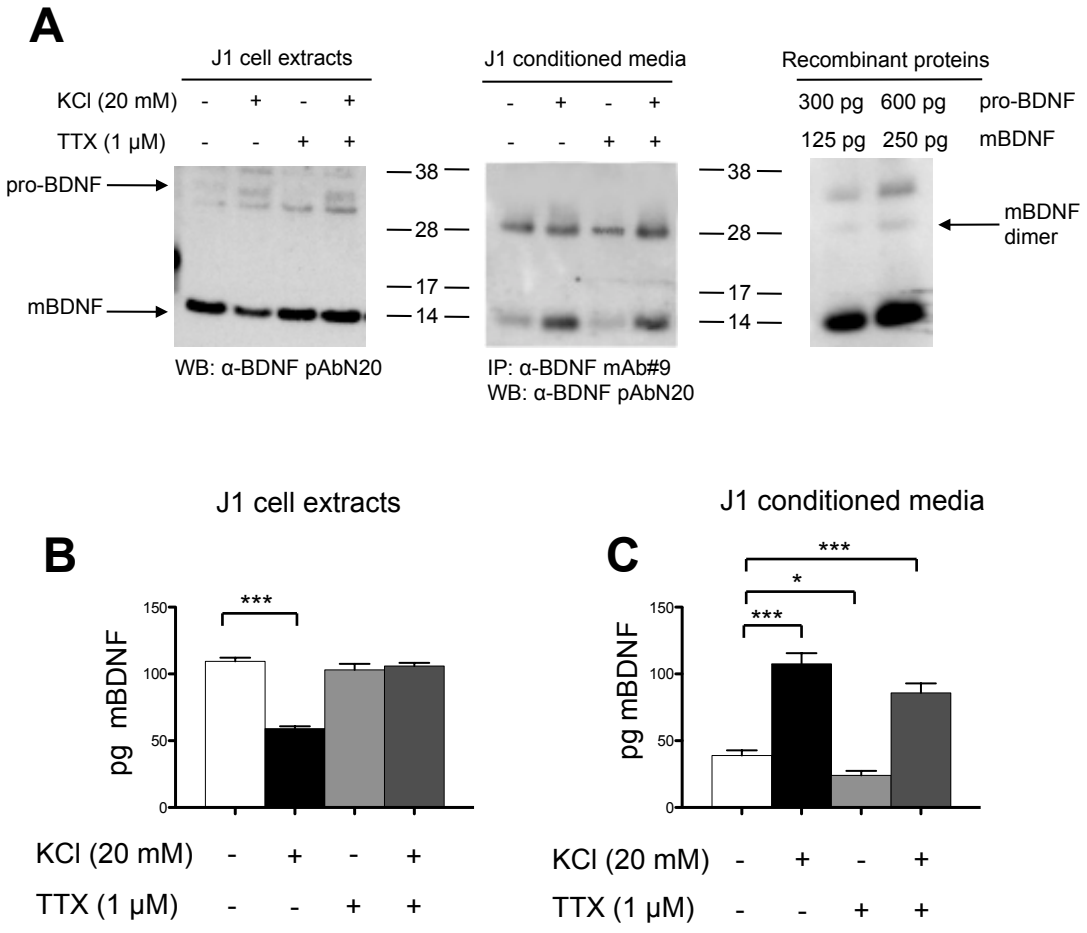


Figure 10. Activity-dependent release of BDNF from J1 ES cell-derived neurons (21 DIV)

A) Western blot analysis of BDNF stored (left panel) and released (middle panel) from J1 ES cell-derived neurons treated overnight with KCl (20 mM) or TTX (1 µM). Conditioned media (1 ml) were immunoprecipitated with α-BDNF mAB#9 and resolved by SDS-PAGE. B, C) Densitometric analysis of wild-type cell extracts (B) or conditioned media (C) after addition of KCl or TTX or both. Chronic depolarization increases BDNF release, which is not prevented by TTX treatment. (Student t-test analysis: mean ± SEM; n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

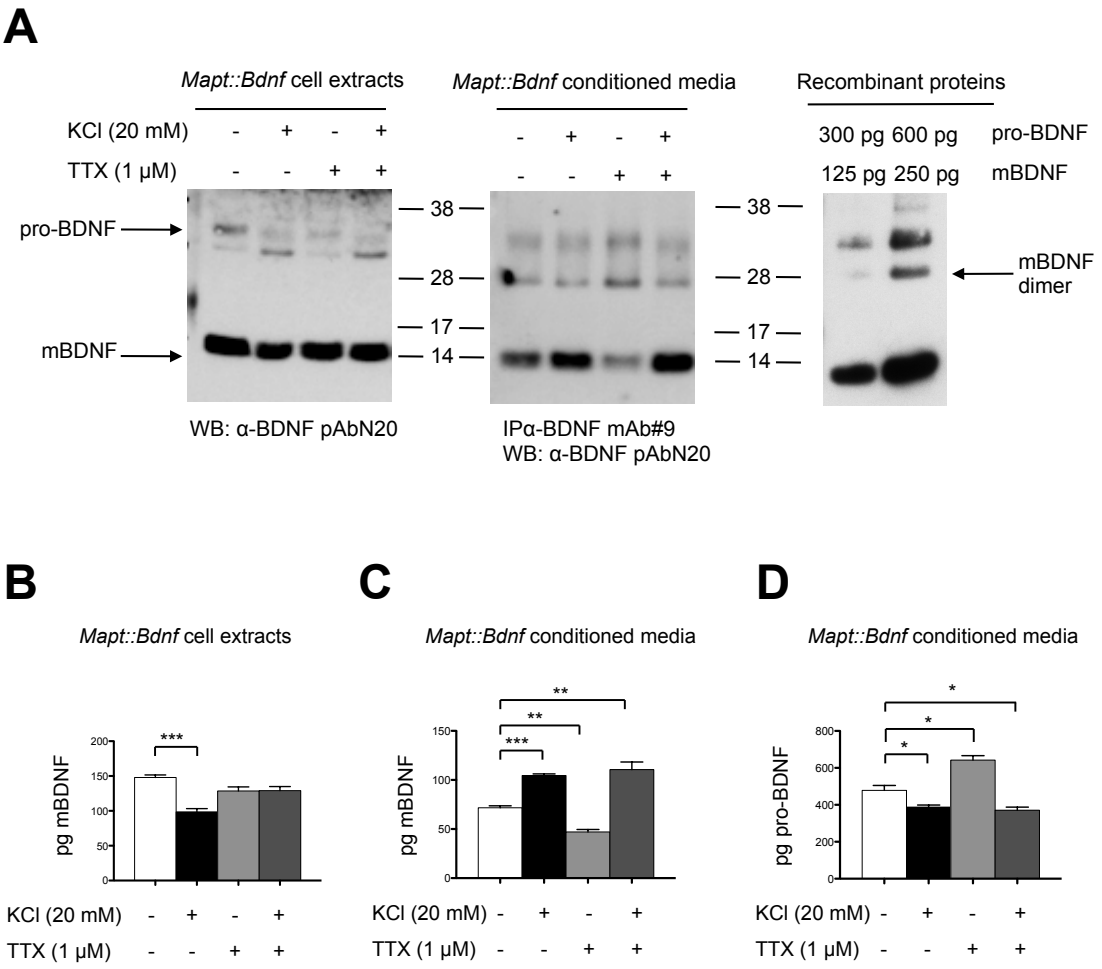


Figure 11. Characterization of activity dependent release of BDNF from *Mapt::Bdnf* (21 DIV)

A) Western blot analysis of BDNF stored (left panel) and released (middle panel) following overnight treatment of *Mapt::Bdnf* ES cell-derived neurons with KCl (50 mM) and TTX (1 µM). Secreted BDNF was immunoprecipitated from conditioned media (1 ml) and resolved by SDS-PAGE B, C, D) Densitometric analysis of mBDNF and pro-BDNF stored in and released from neurons treated with KCl. (Student t-test analysis: mean ± SEM; n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

2.8 The basal BDNF release is activity dependent

To begin to identify mechanisms underlying the basal release of BDNF from wild-type and engineered neurons overexpressing BDNF, J1 and *Mapt::Bdnf* ES cell-derived neurons (29 DIV) were treated with 1 µM TTX for 1 hour (Fig. 12).

The conditioned medium, supplemented with biotinylated anti BDNF #9 antibody, was subsequently collected and an ELISA assay performed as above. I observed that TTX treatment is sufficient to abolish the BDNF release from wild-type neurons, indicating that its secretion is exclusively activity-dependent (w/o treatment: 1.377 ± 0.1128 pg; TTX: 0 pg; n=4) (Fig. 12A). This result was further confirmed treating the cells for 1 hour with 10 µM APV and 50 µM NBQX, which inhibit neuronal activity in this culture system (Bibel, 2004) (w/o treatment: 1.203 ± 0.0083 pg; APV-NBQX: 0.0396 ± 0.032; unpaired t-test analysis: p=0.0028, N=4) (Fig. 13). By contrast, BDNF release was decreased but not abolished by TTX treatment of BDNF overexpressing neurons, indicating that a significant fraction of overexpressed BDNF is secreted independently from neuronal activity (w/o treatment: 3.652 ± 0.2167 pg ; TTX: 1.920 ± 0.4686 pg; N=4, unpaired t-test analysis: p=0.015) (Fig. 12B).

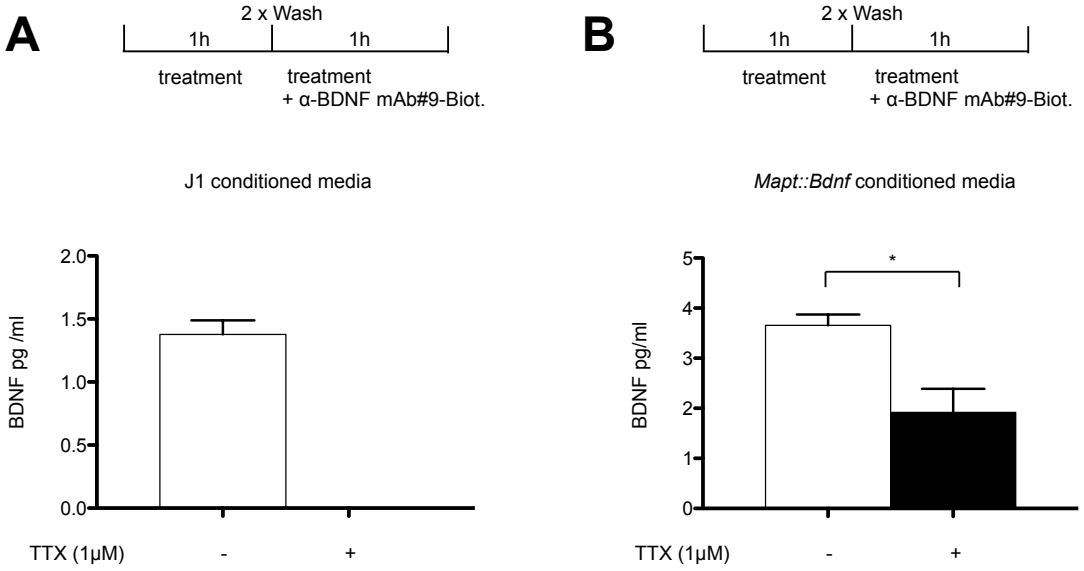


Figure 12. Basal BDNF release from J1 and *Mapt::Bdnf* ES cell-derived neurons (29 DIV) is suppressed by TTX

ELISA assay on BDNF released from J1 (A) and *Mapt::Bdnf* (B) ES cell-derived neurons (21 DIV) after treatment with or without 1 µM TTX. TTX completely abolishes the basal BDNF release from wild type neurons but not from BDNF-overexpressing cells. ELISA assays were performed on 1 ml conditioned medium collected after 1 hour of treatment. Cells were pre-treated with the drugs for 1 hour to avoid possible interference due to different binding kinetics. (Student t-test analysis: mean ± SEM; n = 4). *P < 0.05; **P < 0.01; ***P < 0.001.

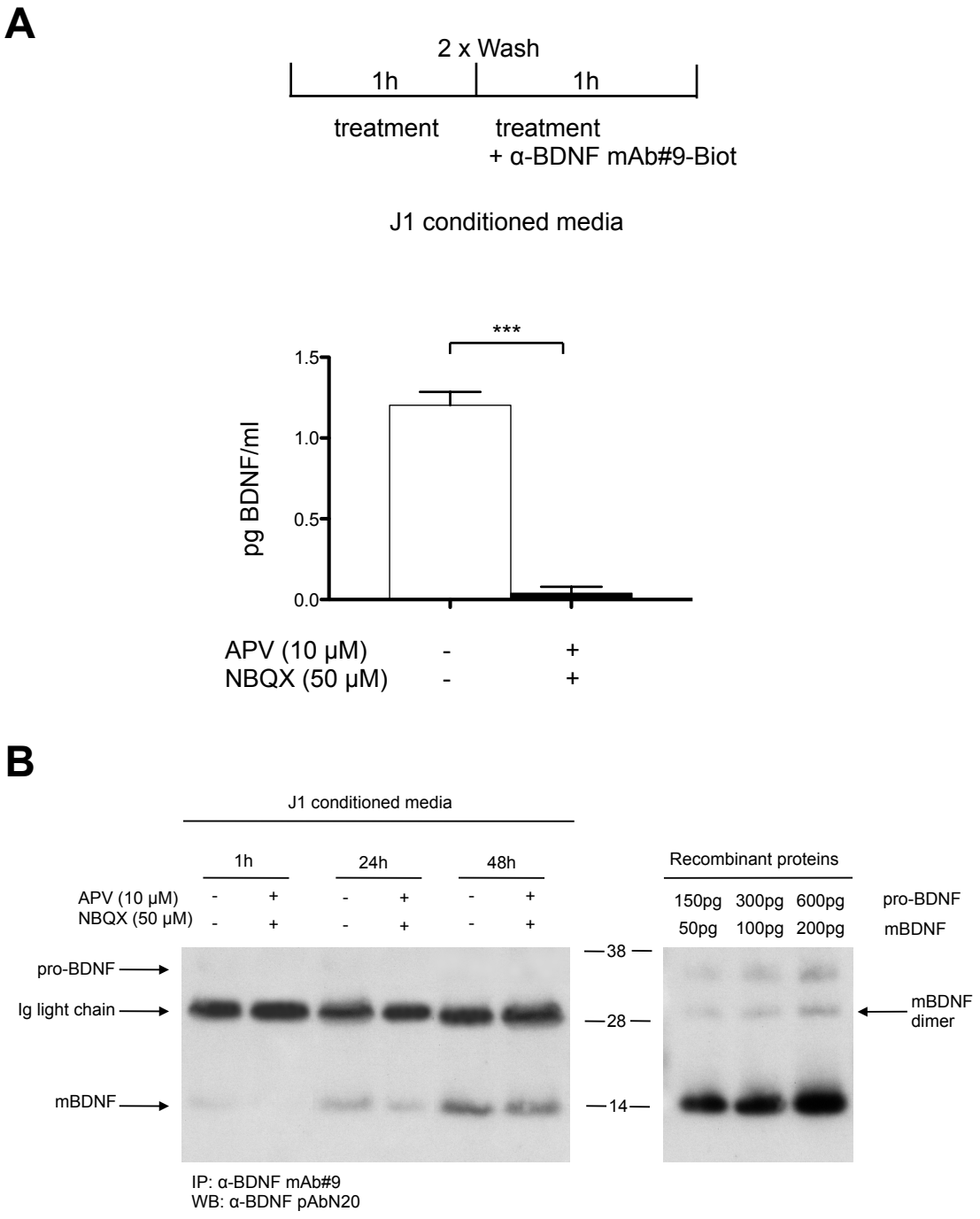


Figure 13. Basal BDNF release is suppressed in J1 ES cell-derived neurons (21 DIV) by APV and NBQX

A) ELISA assay on conditioned media after treatment (1 h) of J1 ES cell-derived neurons (21 DIV) with 10 μM APV and 50 μM NBQX shows that BDNF release is abolished in absence of neuronal activity. Cells were pre-treated with the drugs for 1 hour to avoid possible interference due to different binding kinetics. B) Western blot analysis of BDNF released from J1 ES cell-derived neurons after treating (1, 24 and 48 h) with 10 μM APV and 50 μM NBQX. Conditioned media (1 ml) were immunoprecipitated with α-BDNF mAB#9 and resolved by SDS-PAGE (Student t-test analysis: mean ± SEM; n = 4). *P < 0.05; **P < 0.01; ***P < 0.001.

2.9 BDNF release depends on extracellular calcium

In addition to the role of calcium in KCl-induced release (see Fig. 9), I also tested the role of extracellular calcium in basal BDNF release. To this end, I incubated wild-type ES cell-derived neurons in a calcium-free medium. ELISA quantification indicated that BDNF release was completely abolished in absence of extracellular calcium: J1 ESCDN 29 DIV: 2.989 ± 0.235 pg; w/o calcium: 0.1140 ± 0.1146 pg; unpaired t-test analysis: p=0.0028; n=4 (Fig. 14A). By contrast, in *Mapt::Bdnf* ES cell-derived neurons, BDNF secretion was reduced but not abolished in absence of extracellular calcium (*Mapt::Bdnf* ESCDN 29 DIV : 7.914 ± 0.2566 pg; w/o calcium: 3.192 ± 0.619 pg; unpaired t-test analysis: p=0.0021; n=4) (Fig. 14B).

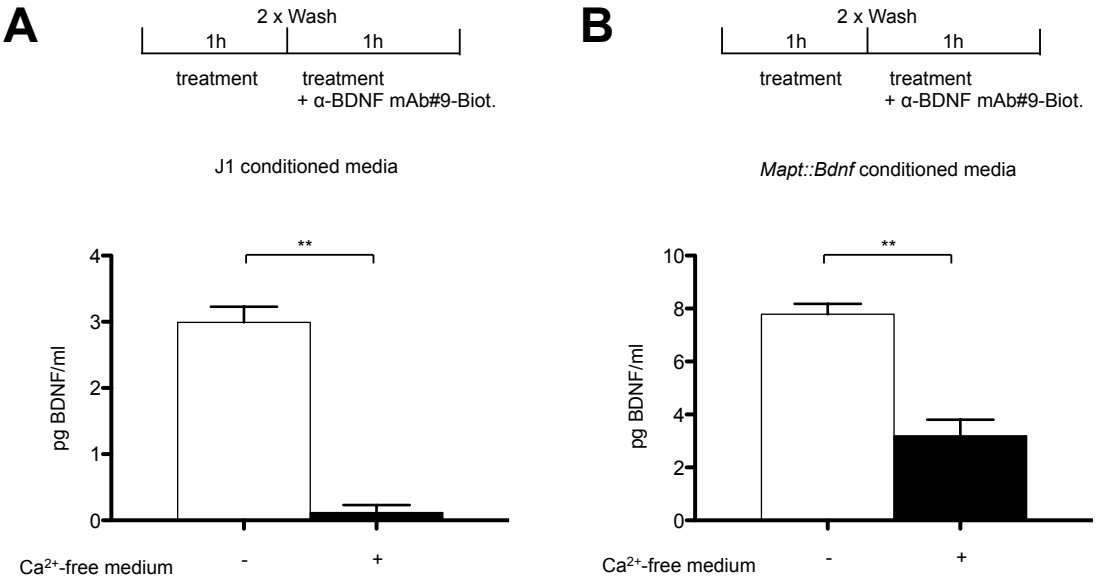


Figure 14. Role of extracellular calcium on BDNF release from J1 and *Mapt::Bdnf* ES cell-derived neurons (29 DIV)

A) ELISA assay reveals that basal BDNF secretion from J1 ES cell-derived neurons after 1 hour is completely abolished in the absence of extracellular calcium. B) The absence of extracellular calcium partially reduces the basal BDNF release from *Mapt::Bdnf* ES cell-derived neurons. ELISA assays were performed on 1 ml conditioned media collected after 1 hour of treatment (Student t-test analysis: mean ± SEM; n = 4). *P < 0.05; **P < 0.01; ***P < 0.001.

2.10 Endogenous BDNF release requires extracellular calcium influx through specific voltage-activated calcium channels

To begin to explore which calcium channel subtype is involved in BDNF release, I analysed the expression levels of all types of VGCCs in hippocampal and cortical primary cultures and compared those with ES cell-derived neurons. Quantitative PCR experiments revealed that the N- and P/Q- and R-types are the most abundant channels in all examined cultures (Fig. 15). The levels of the N-type channels (Cav2.2) and the two classes (Cav3.1 and Cav3.2) of T-type channels turned out to be much higher in ES cell-derived neurons compared to the levels determined in hippocampal and cortical neurons (one-way ANOVA: Cav2.2, Cav3.1 and Cav3.2 $p<0.0001$; n=3).

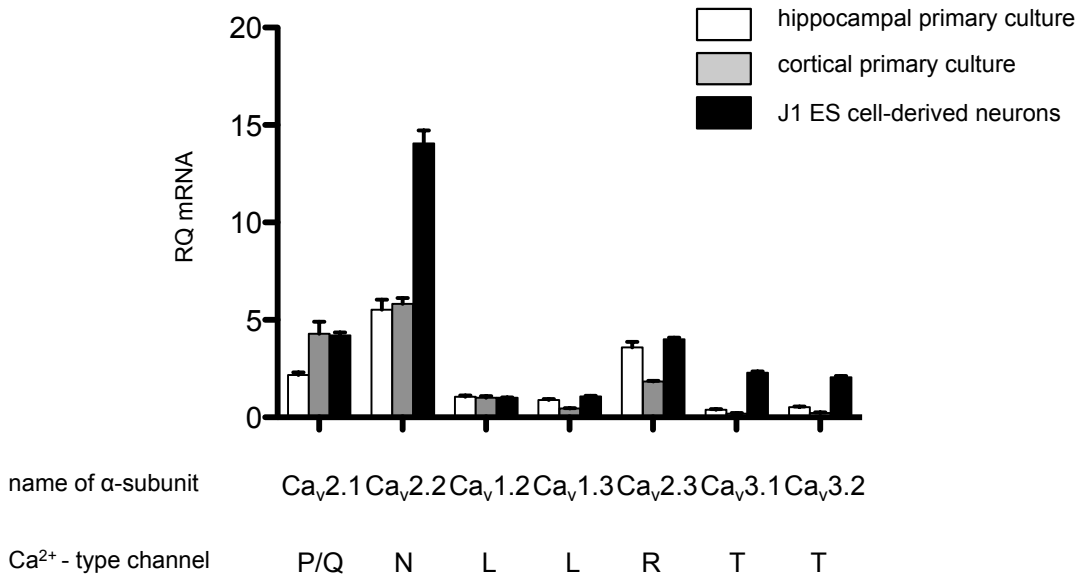


Figure 15. Comparison of VGCCs expression levels in primary cell cultures and in ES cell-derived neurons

The mRNA levels of all VGCCs were analysed in J1 ES cell-derived neurons (21 DIV), hippocampal (23 DIV) and cortical (23 DIV) primary cell cultures by real-time PCR and normalized by *Gadph* expression. N-, P/Q- and R-type channels are most abundantly expressed in all the tested cultures.

The functional implication of the various VGCCs in the release of BDNF was then examined using various blockers. While treatment of J1 ES cell-derived neurons with ω-conotoxin GVIA, a specific blocker of N-type channel, partially reduced the BDNF release (32 DIV, w/o treatment: 1.463±0.286 pg; conotoxin: 0.482 ± 0.3688 pg, n=4) (Fig. 16A), ω-agatoxin, a specific blocker for P/Q-type channels, was even more effective at blocking the basal release of BDNF (31 DIV, w/o treatment: 1.463±0.286 pg; agatoxin: 0.1494 ± 0.1232 pg; unpaired t-test analysis: p=0.0149, n=4) (Fig. 16B). Neither nifedipine, a blocker for L-type channels nor Nickel, an unspecific blocker for R-type channels, had any significant effect on BDNF release (29 DIV, w/o treatment: 2.242±0.3756 pg; nifedipine: 2.004 ± 0.4114 pg; nickel: 2.591 ± 0.4774; n=4) (Fig.16 C,D).

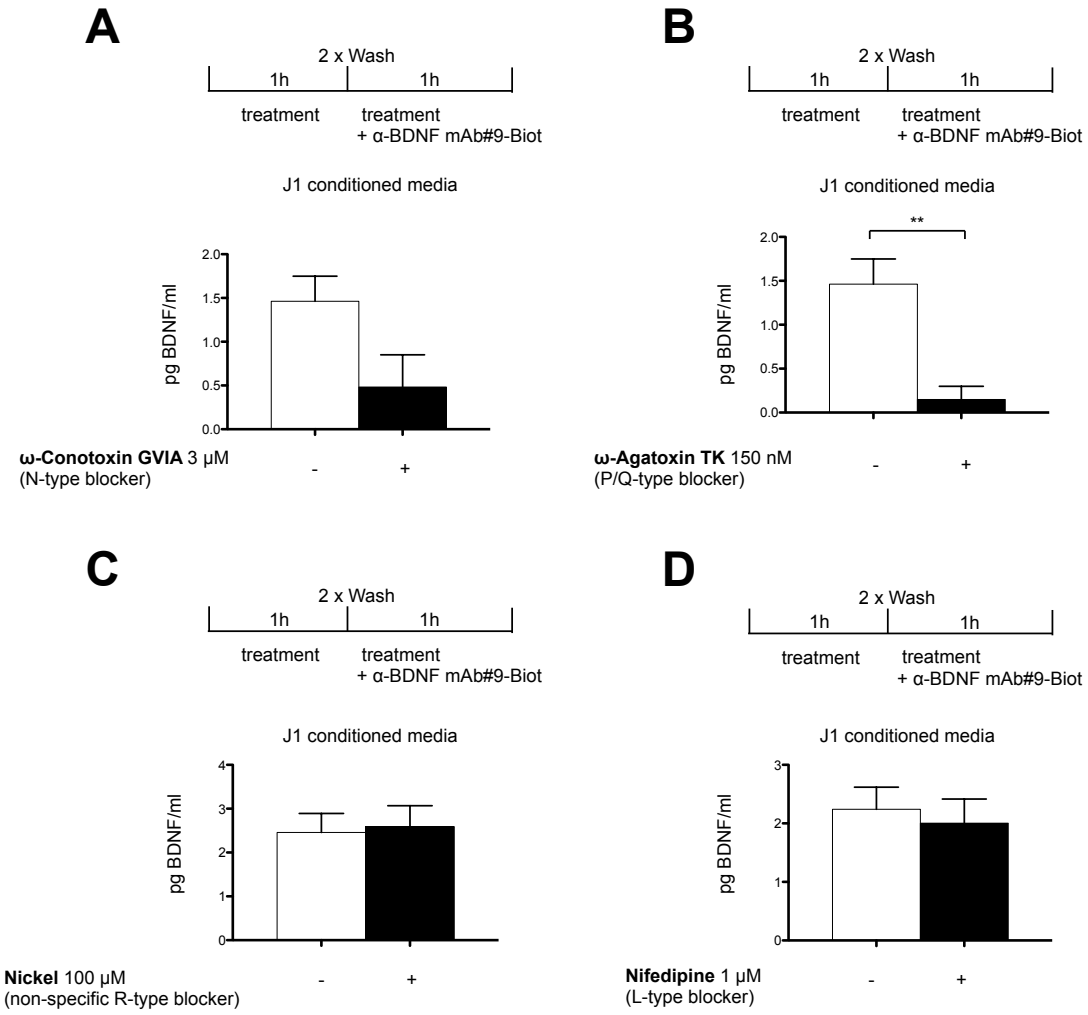


Figure 16. Effects of different calcium channel blockers on BDNF released from J1 ES cell-derived neurons (29 DIV)

J1 ES cell-derived neurons were treated for 1 hour with 3 μM Conotoxin (A), 150 nM ω-Agatoxin-TK (B), 100 μM Nickel (C) and 1 μM Nifedipine (D) to block N-, P/Q, R and L type channels respectively. ELISA assay was performed on 1 ml of conditioned medium to detect released BDNF. Cells were pre-treated with the drugs for 1 hour to avoid possible interference due to different binding kinetics. (Student t-test analysis: mean ± SEM; n = 4). *P < 0.05; **P < 0.01; ***P < 0.001.

In addition, I observed that cells treated with mibefradil, a T-type channel blocker, fully abolished BDNF release (Fig.17A). As previous reports indicate that mibefradil may lack specificity blocking other calcium channels, NNC-550 was also tested. This molecule also completely blocked BDNF release from J1 ES cell-derived neurons (Fig.17B).

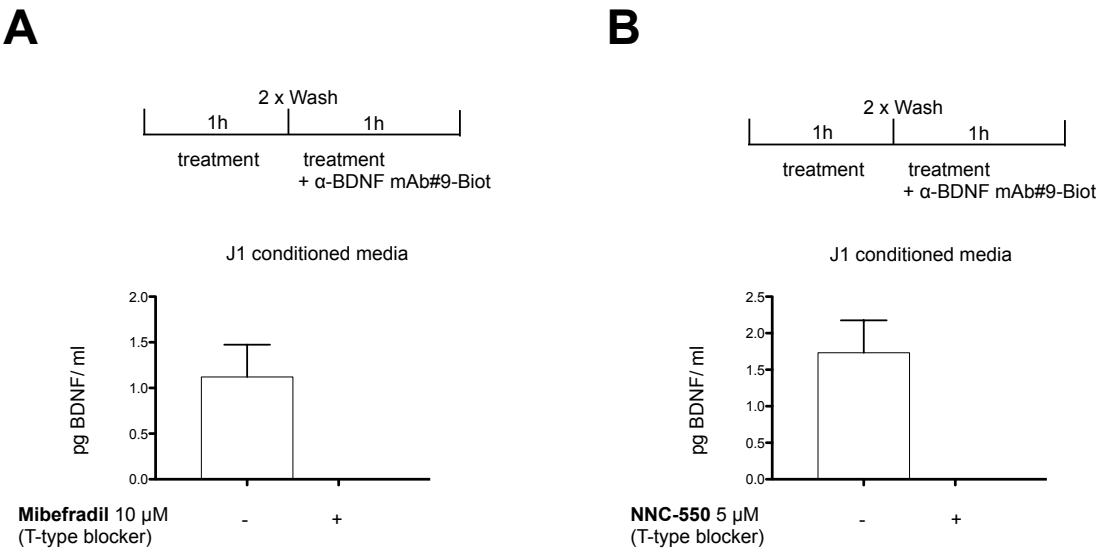


Figure 17. Effects of two LVA calcium channel blockers on BDNF released from J1 ES cell-derived neurons (29 DIV)

J1 ES cell-derived neurons were treated with Mibefradil (A) and NNC-550 (B) to block the T-type channels. ELISA assay on treated media reveals that both tested drugs fully abolish BDNF secretion. Cells were pre-treated with the drugs for 1 hour to avoid possible interference due to different binding kinetics (mean ± SEM; n = 4).

2.11 Basal BDNF release is independent of intracellular calcium

Ryanodine receptor mediates the release of calcium from endoplasmic reticulum (ER) through a mechanism called calcium-induced calcium release (CIRC). To examine if calcium mobilization from the ER contributes to BDNF release, I treated neurons with 90 µM caffeine, an agonist of the ryanodine receptor (Fig. 18). I first established that caffeine does increase BDNF release (29 DIV, w/o treatment: 1.257 ± 0.047 pg; caffeine: 4.972 pg ± 0.964 pg; unpaired t-test analysis: p= 0.0184; n=4), and that the co-addition of 90 µM BAPTA-AM, a

chelator of intracellular calcium, prevented the effect of caffeine on BDNF release (caffeine-BAPTA-AM: 1.898 ± 0.369 pg; unpaired t-test analysis: p= 0.0409; n=4). These results showed that the effect on caffeine is dependent on the increase of calcium release from the endoplasmic reticulum into the cytoplasm. To explore if the basal BDNF release requires the mobilisation of intracellular calcium, I treated the neurons with BAPTA-AM only and found that the basal release of BDNF was not affected. Therefore, mobilization of calcium from the endoplasmic reticulum does not appear to be involved in the basal release of BDNF (BAPTA-AM: 1.261 ± 0.0339 pg, n=4). Summarizing, the basal release of BDNF is entirely calcium-dependent, but the calcium is provided from extracellular sources through voltage-activated calcium channels.

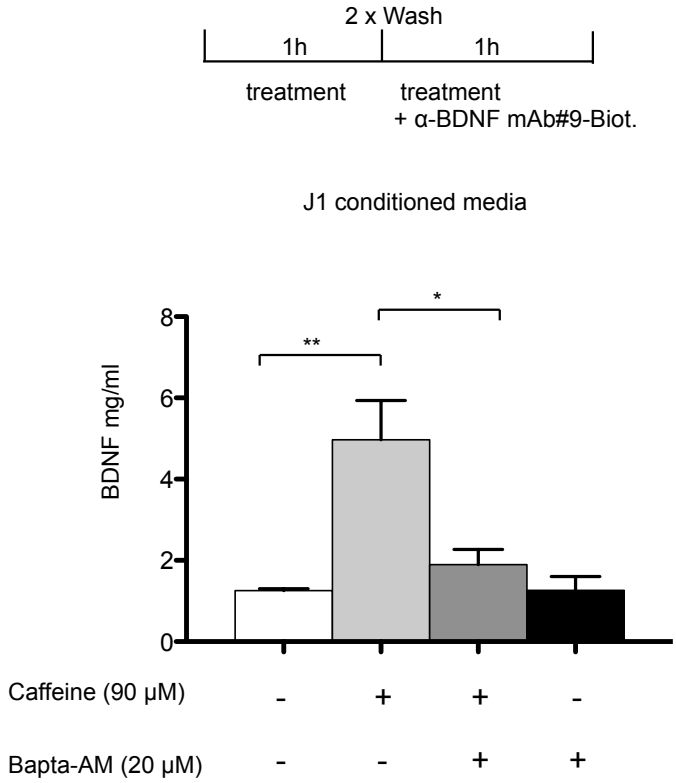


Figure 18. Role of intracellular calcium mobilization on BDNF release

The ELISA assay was performed on medium collected from J1 ES cell-derived neurons treated for 1 hour with 90 µM caffeine and/or 20 µM BAPTA-AM. Cells were pretreated with the substances for 1 hour to avoid possible interference due to different binding kinetics. BDNF release is affected by caffeine, which triggers the ER calcium release into the cytoplasm. However, basal BDNF is not affected when the intracellular calcium is buffered by BAPTA-AM (Student t-test analysis: mean ± SEM; n = 4). *P < 0.05; **P < 0.01; ***P < 0.001.

2.12 Isolation of Bassoon mutant ES cell lines

To explore BDNF release in an additional model, I isolated ES cells lines from mutant mice obtained from the Gundelfinger's laboratory (Altrock et al., 2003) expressing a truncated version of the pre-synaptic protein Bassoon. These mutants are known to develop seizure episodes and markedly increased BDNF levels in various brain areas including the hippocampus, which increases in size as much as 40% in 3 months. To explore whether or not increased BDNF levels could be observed in *Bassoon* mutant neurons, I proceeded to isolate ES cells from *Bassoon* blastocysts. After crossing two mice heterozygote for the *Bsn* mutation (*Bsn* $^{m/+}$), several blastocysts were isolated and cultured on a feeder layer of mouse embryonic fibroblasts (Fig. 19B). Two lines turned out to be homozygote for the *Bassoon* mutation (designated 7.1 and 28.1: *Bsn* $^{m/m}$). A line wild type for *Bassoon* gene was also isolated (A4: *Bsn* $^{+/+}$) from the same animals for control purposes. All ES lines were then analysed for the expression of pluripotency markers (Fig.19B, D, F).

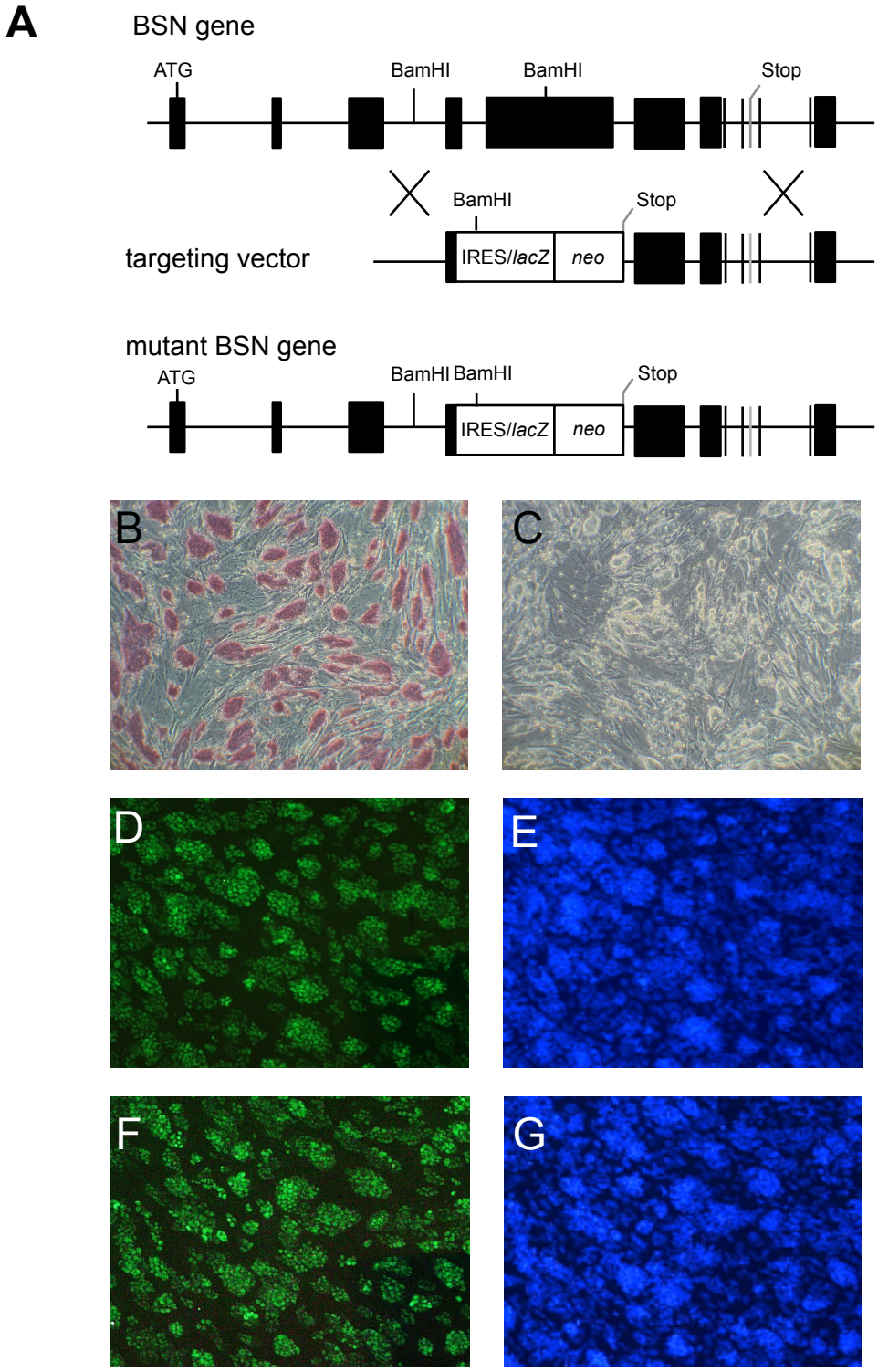


Figure 19. Isolation of *Bsn*$^{m/m}$ ES cell line

A) Scheme of *Bsn* gene, targeting vector and resulting mutant gene, in which part of exon 4 and the entire exon 5 were replaced by an internal ribosomal entry site (IRES) containing lacZ/neo cassette. Heterozygous mice for the mutant gene were used for mating and sacrificed for blastocyst collection. The pluripotency of new *Bsn* $^{m/m}$ stem cells was analysed by the alkaline phosphatase histochemical reaction (B), Nanog (D) and Oct-4 (F) staining. Corresponding phase contrast images are illustrated (C) and DAPI staining (E and G).

These ES cell lines were subsequently differentiated in neurons using our standard protocol (Bibel et al., 2007) and the morphology subsequently analysed at different time-points after plating the progenitors (Fig. 20). While the *Bsn* $^{m/m}$ ES cell-derived neurons did not show any striking phenotype after 7 DIV, a less dense network was observed after 15 DIV. In addition, morphological changes begun to appear in some mutant cells (Fig 20D).

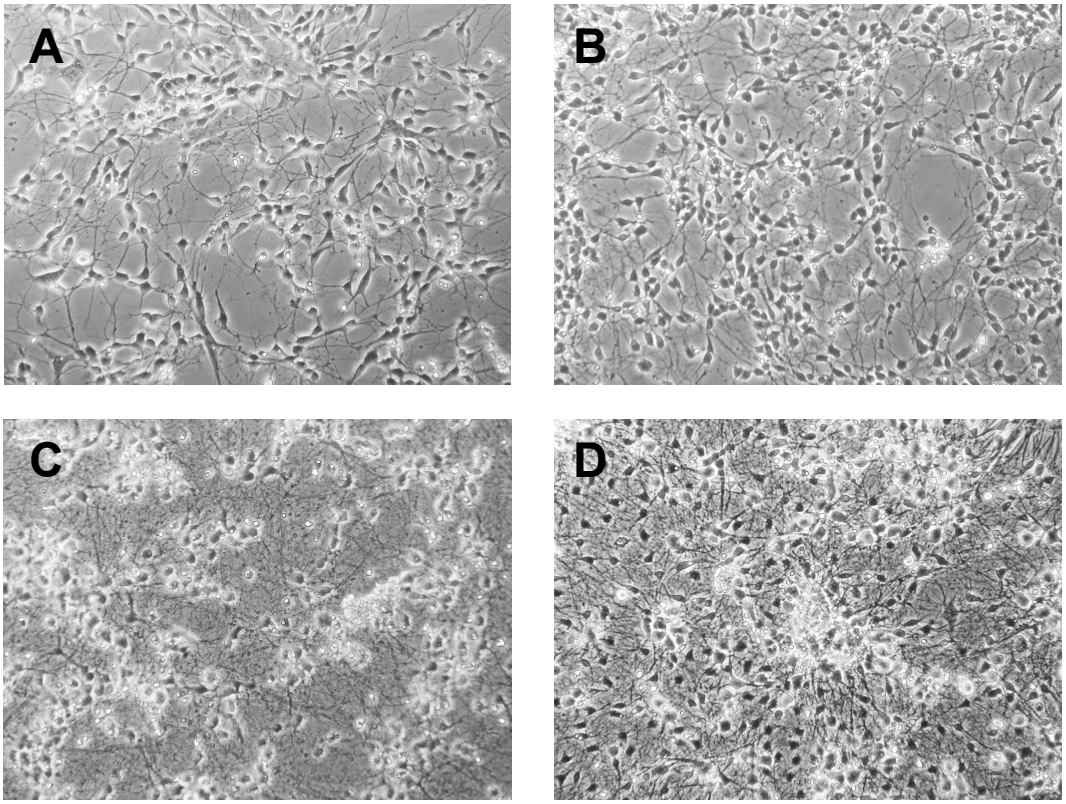


Figure 20. Neuronal differentiation of *Bsn*$^{+/+}$ (clone A4) and *Bsn*$^{m/m}$ (clone 7.1) ES cell lines.

A) *Bsn*$^{+/+}$ ES cell-derived neurons after 2 days *in vitro*. B) *Bsn*$^{m/m}$ ES cell-derived neurons after 2 days in vitro. C) *Bsn*$^{+/+}$ ES cell-derived neurons after 15 days in vitro D) *Bsn*$^{m/m}$ ES cell-derived neurons after 15 days in vitro.

2.13 Analysis of BDNF mRNA and protein levels in Bsn $^{m/m}$ mutant neurons

As our (still unpublished) electrophysiological experiments indicate that neurons derived from *Bassoon* mutant ES cells displayed increased activity compared with wild-type neurons (data not shown), mRNA levels were determined by Real-time PCR in these cells at 15 DIV (Fig. 21C). Unexpectedly, Western blot analyses of the same extracts revealed that the amount of BDNF in *Bsn* $^{m/m}$ (mean 55.28 ± 7.284 pg, n=3) were actually lower than those found in *Bsn* $^{+/+}$ control neurons (mean 141 ± 9.790 pg, n=3; unpaired t-test analysis: p=0.0022) (Fig. 21A,B). These results are also surprising in view of *in vivo* results indicating considerably higher levels of BDNF in the presynaptic terminals of hippocampal neurons in the same mutant. They suggest that, like observed in the KCl experiments, *Bsn* $^{m/m}$ ES cell-derived neurons can only store a very limited amount of BDNF *in vitro* and that increased activity causes higher release.

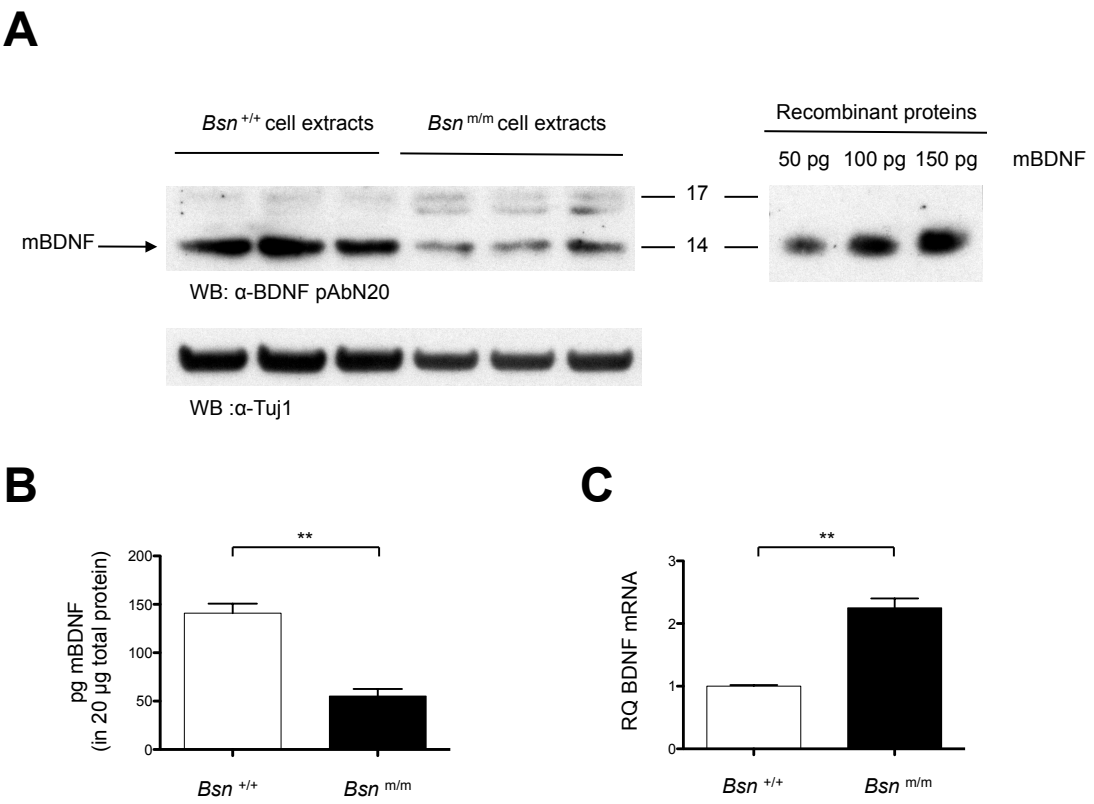


Figure 21. mRNA and protein levels of BDNF in *Bsn*$^{m/m}$ ES cell-derived neurons

A) Western blot analysis of cell extracts (20 µM) of WT and *Bsn*$^{m/m}$ ES cell-derived neurons (15 DIV) shows a decrease of BDNF levels in mutant Bassoon cells. B) Optical density analysis and quantification of BDNF after Western blot analysis. Protein levels were normalized using Tuj1 protein. C) Analysis of mRNA levels of BDNF in WT and *Bsn*$^{m/m}$ ES cell-derived neurons using real-time PCR. The obtained results were normalized using *Gapdh* expression levels (Student t-test analysis: mean ± SEM; n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

2.14 Increased BDNF secretion in Bassoon mutant neurons

To quantify BDNF secretion in *Bassoon* mutant neurons, ELISA measurements were performed on conditioned medium supplemented with biotinylated #9 anti-BDNF antibody and the values normalized with Tuj1 levels determined by Western blot analysis. After 1 hour, BDNF secretion from *Bsn* $^{m/m}$ neurons (15 DIV) was found to be strongly elevated: 7.248 ± 0.004 pg (n=4) compared with

wild-type neurons: 0.95 ± 0.007 pg (n=4, unpaired t-test analysis: p<0.0001) (Fig. 22A). After 24 hours, the corresponding values were 31.56 ± 1.430 pg BDNF (n=3) for mutant neurons compared with 12.41 ± 0.707 pg BDNF (n= 3, unpaired t-test analysis: p= 0.0003) for wild-type neurons (Fig. 22B).

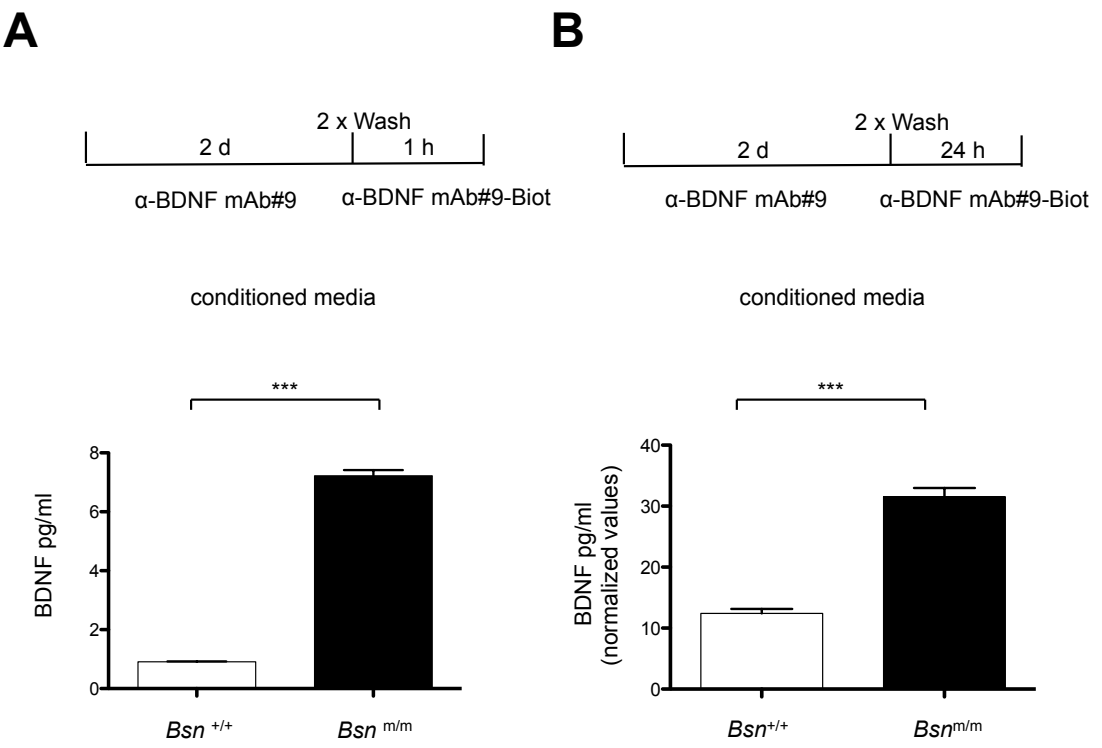


Figure 22. Increase of BDNF release in *Bsn$^{m/m}$* neurons

BDNF release was determined by ELISA after 1 hour (A) or 24 hours (B) in medium conditioned by WT and *Bsn$^{m/m}$* neurons. The protein levels were normalized using Tuj1 protein detected by Western blot analysis (Student t-test analysis: mean ± SEM; n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

2.15 The expression of the potassium-chloride co-transporter KCC2 is decreased in Bsn $^{m/m}$ and Mapt::Bdnf ES cell-derived neurons

The impact of GABA-A receptor activation on the neuronal membrane potential is modulated by several factors, including the electrochemical gradient of chloride (Ben-Ari, 2002). In particular, the low concentration of intracellular Cl^- ions, largely determined by the chloride-potassium co-transporter KCC2, is

critical for GABA-A receptor that increases Cl^- influx leading to intracellular hyperpolarization. Previous reports indicate that BDNF can induce hyper-excitability of cortical neurons, thus interfering with the inhibitory signals (Rivera, 2002) and that exogenous BDNF down-regulates the expression of KCC2 gene (Rivera, 2004). As Bassoon ES cell-derived neurons express and release increased levels of BDNF, I examined the levels of KCC2 mRNA in wild-type and Bsn $^{m/m}$ and ES cell derived neurons by Real time PCR (Fig. 23A). In these experiments, both ES cells were derived from littermates. Consistent with increased BDNF release from Bsn $^{m/m}$ cell ES cell-derived neurons, I also observed increased levels of TrkB phosphorylation (data not shown). To confirm that BDNF causes the down regulation of KCC2 gene in ES cell-derived neurons, I repeated the analysis using the mRNA obtained from J1 and Mapt::Bdnf ES-derived neurons. The real time PCR showed a decrease of KCC2 gene expression in Mapt::Bdnf ES cell-derived neurons, confirming the results obtained with the Bassoon mutant cell line (unpaired t-test analysis: $p=0.0103$). It appears then that the release of BDNF, presumably through TrkB activation, reduces KCC2 expression leading to increased intracellular chloride concentrations. This mechanism may be responsible for an impairment of inhibitory signals and for increased neuronal activity of Bassoon mutant neurons observed in our study (Fig. 23B,C).

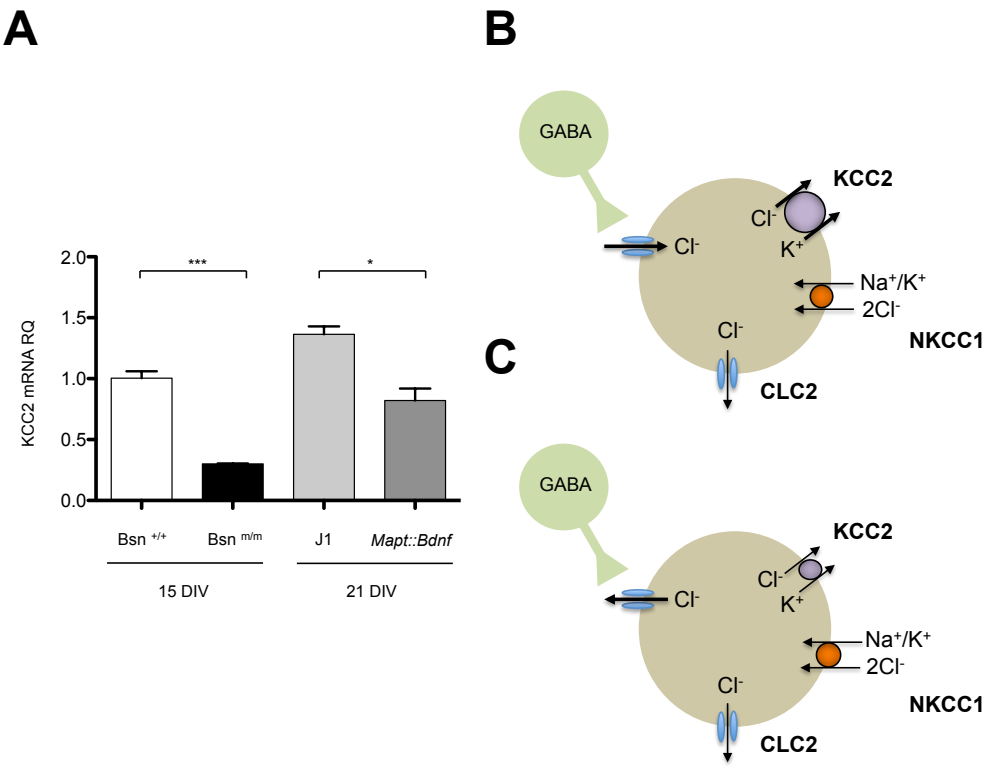


Figure 23. Decrease of KCC2 mRNA levels in *Bsn*$^{m/m}$ ES cell-derived neurons

A) Analysis of KCC2 mRNA levels in *Bsn*$^{+/+}$ (15 DIV), Bsn$^{m/m}$ (15 DIV), J1 (21 DIV) and *Mapt::Bdnf* (15 DIV) ES cell-derived neurons by real-time PCR. B) In mature neurons, *KCC2* is the main pump that exports Cl^-, reducing its intracellular concentration. C) *KCC2* expression is downregulated by BDNF-induced TrkB signalling (Rivera et al., 2002). For this reason, a chronic increase of BDNF release can affect the Cl^- extrusion from GABA receptor impairing the amplitude and the polarity of GABAergic response (Student t-test analysis: mean ± SEM; n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

3. Discussion

In this study, I could show that a controlled, relatively mild overexpression of BDNF in neurons is incompatible with complete processing and leads to a progressive accumulation of pro-BDNF and to its secretion. My results help to understand why the overexpression of BDNF cDNAs *in vivo* failed to indicate any role of BDNF on overgrowth of the post-natal brain in overexpression paradigms in transgenic animals. It also suggests that viral delivery of BDNF, currently the most often discussed way of using BDNF in a therapeutic setting, is unlikely to be effective. In addition, I found that the basal release of BDNF from wild-type neurons is fully dependent on neuronal activity and of external calcium. By contrast, I could confirm that in the absence of overexpression, endogenous pro-BDNF is not released from neurons. These results are significant as the release of pro-BDNF activates the $p75^{NTR}$-dependent pathway, leading to effects that typically counteract those resulting from the activation of the TrkB, the BDNF tyrosine kinase receptor that cannot be activated by pro-BDNF (Teng et al., 2005). While mature BDNF also binds to $p75^{NTR}$, pro-BDNF binds to $p75^{NTR}$ with significantly higher affinity (about 10^{-11} M) but is unable to bind to TrkB, suggestion that co-secretion of both pro-BDNF and mature BDNF from neurons would significantly tilt the balance towards "non-neurotrophic" effects of BDNF release from overloaded neurons.

With regard to mechanisms of release of (mature) BDNF, I could show by treating neurons with specific VGCC blockers that the extracellular calcium influx through P/Q- and T-type channels is necessary for basal BDNF secretion. In addition, calcium release from sarcoplasmic reticulum through ryanodine receptors increases BDNF exocytosis. This mechanism has been previously described after theta-burst stimulation of hippocampal neurons (Balkowiec and Katz, 2002; Blochl and Thoenen, 1995; Canossa et al., 2001; Goodman et al., 1996), but it is not involved in basal BDNF release as my results indicate.

To analyse BDNF release under conditions of increased neuronal activity, I isolated a new ES cell line from *Bassoon* mutant mice known to develop epileptic seizures and increased BDNF levels in various brain areas such as the

hippocampus and the cerebral cortex (Altrock et al., 2003; Heyden et al., 2011). I found that neurons generated from these ES cells show increased expression levels of BDNF expression and secretion. Interestingly, the *KCC2* gene encoding a Cl^-/K^+ co-transporter expressed at lower levels in *Bassoon* mutant neurons. Given that TrkB activation decreases *KCC2* expression (Rivera et al., 2002), increased BDNF secretion may impair the response to the neurotransmitter GABA that can only act as inhibitory neurotransmitter when chloride extrusion is operated by the Cl^-/K^+ exchanger (Rivera et al., 2004). These results are likely to explain why the *Bassoon* neurons show increased activity, as the GABAergic neurons, also present in our culture, are known to decrease spontaneous activity shown by the addition of bicuculline (Bibel et al., 2004).

3.1 Processing and release of pro-BDNF

In ES cell-derived neurons, as previously described in pulse-chase experiments with hippocampal neurons (Matsumoto et al., 2008), endogenous pro-BDNF is a transient biosynthetic intermediate that is fully processed intracellularly to generate the mature form of BDNF. Efficient processing seems to need neuronal maturation as my results indicate that the ratio pro-BDNF/mature BDNF changes significantly during maturation (see Fig. 4), presumably ensuring that when neurons become excitable by about 10 DIV in our cultures, low-levels of intracellular pro-BDNF are reached and the secretion of pro-BDNF prevented. My results challenge a number of previous reports in the field indicating that pro-BDNF is secreted and cleaved extracellularly by metalloproteinase or tPA, through plasminogen activation (Pang et al., 2004). Released pro-BDNF was even described as being required for LTD induction (Lu et al., 2005; Woo et al., 2005), but our laboratory showed that LTD indication is unaffected in the absence of the *Bdnf* gene (Matsumoto et al., 2008). It seems that the simple explanation accounting for these previous results is the use of overexpression paradigms leading to incomplete pro-BDNF processing as my results indicate (see above). Interestingly, the release of pro- and mature BDNF from overexpressing BDNF neurons is differently modulated by the neuronal activity. While the increase and

decrease of neuronal activity result in the augmentation and reduction of the amount of mature BDNF release, the opposite effects were observed with pro-BDNF release (see Fig. 11). These observations suggest that the secretion of mature-BDNF is activity-dependent, while pro-BDNF is constitutively secreted.

3.2 Constitutive and regulated BDNF secretion

An important, unresolved question in the field relates to what has been designated the "constitutive" release of BDNF. However, I found that there is indeed a basal BDNF secretion, but that this can be accounted for by the on-going activity. The release of mature and/or pro-BDNF reported in previous studies in the absence of activity is likely to be accounted for by overexpression and subsequent leakage from compartments that are unable to store BDNF (Mowla et al., 2001). To facilitate a precise quantification of secreted BDNF, we modified an existing ELISA assay to increase its sensitivity (Kolbeck et al., 1999). This allowed to measure BDNF release at short time intervals. This was particularly critical since depolarising stimuli typically lead to increase BDNF transcription and intracellular levels (Tao et al., 2002; Zafra et al., 1990). The only hope then to determine if BDNF release is truly dependent on activity is to be able to capture freshly released BDNF. This was made possible by first incubating the culture with an anti-BDNF antibody and than, at time zero, changing the medium with a fresh biotinylated antibodies to bind BDNF released for 1 hour. In the subsequent 2-phases ELISA, the antibody used to coat the plate specifically retains the BDNF already bound to biotinylated antibody. I also found that BDNF release is massively increased during chronic depolarization following KCl addition. However, I found that KCl-induced release cannot be blocked by TTX (see Fig. 10 and 11). As suggested by previous studies, it likely appears that a chronic depolarization induced by KCl eventually prevents the generation of action potentials generated by voltage-dependent sodium channels (Grubb and Burrone, 2010). Supporting this view was the observation that at short time periods (1 hour), BDNF release was completely blocked by preventing activity

with TTX or with APV and NBQX, two selective antagonists for NMDA and AMPA receptors, respectively (see Fig. 12 and 13).

3.3 Role of calcium in BDNF release

The activity-dependent release of secretory vesicles is a highly dynamic process spatially and temporally regulated by protein-protein interactions. The Sec1/Munc18 protein (SM) and SNAP receptors (SNARE) emerge as central components of the exocytosis apparatus (Rizo and Sudhof, 2002; Sudhof, 2012). SNAREs proteins directly mediate membrane fusion, forming a tight complex bringing secretory vesicles and plasma membrane together (Gundelfinger and Fejtova, 2012). The SM proteins were suggested to assist the formation of this complex and modulate the function of different proteins involved in the exocytosis process. The interaction between SNARE proteins located on target plasma membrane (t-SNAREs, like Synataxin and SNAP25) and those in vesicle membranes (v-SNARE, like Synaptobrevin/VAMP) generate the so-called core complex, which is required for the membranes fusion (Ziv and Garner, 2004). The local calcium entry supports the formation of the core complex and modulates the exocytosis binding to Synaptotagmin, a vesicle protein that penetrates in the plasma membrane to induce the fusion (Chapman, 2008). However, while these mechanisms are well established for vesicles containing small molecular weight neurotransmitters, those allowing the release of proteins from large dense core vesicles, in which BDNF has been shown to be localized, are essentially unknown (Voets et al., 2001). In particular, which of the many synaptotagmin proteins are associated with such vesicles is unclear. In fact, even if the role of calcium in activity-dependent release of BDNF is controversial, it appears plausible *a priori*. Nevertheless several previous reports using chronic depolarization to induce BDNF release suggested a mechanism independent of calcium entry, but rather dependent on intracellular calcium mobilisation (Griesbeck et al., 1999). By contrast, my results indicate that BDNF secretion is entirely dependent on extracellular calcium as calcium-free media completely abolished the basal BDNF release from wild type, but not from BDNF

overexpressing neurons (see Fig. 14). Most previous results concluding the opposite can be explained by the use of overexpression paradigms, with one based on investigations relating to endogenous BDNF performed with very young, immature neurons presumably using different secretion mechanism dependent on intracellular calcium (Griesbeck et al., 1999; Mowla et al., 1999).

3.4 Role of intracellular calcium on BDNF release

In the past decade, confocal and two-photon microscopy revealed that stimulation of Shaffer collateral in hippocampal slices induces a release of calcium from intracellular store and a secretion of glutamate in the synaptic clefts (Sandler and Barbara, 1999). In CA3, spontaneous vesicle exocytosis can be triggered by pharmacological treatment inducing calcium release from ER stores and treatment with specific blocker revealed that a significant fraction of post-synaptic EPSC involves ryanodine receptors controlling calcium stores in the ER (Emptage et al., 2001). Calcium release from the ER is critical for LTP induction and thapsigargin, which depletes intracellular calcium stores by inhibiting ATP-dependent Ca2+ uptake, blocks LTP induction (Harvey and Collingridge, 1992). Interestingly, calcium release from the ER is dependent on the increase of free cytoplasmic calcium activating the ryanodine receptor through a mechanism called calcium-induced calcium release (CIRC) (Emptage et al., 2001). The increase in intracellular free calcium is triggered by repetitive activation of VGCCs allowing influx from extracellular calcium. Single or paired action potentials are not sufficient to trigger calcium release from the ER, while tetanic or theta burst stimulation paradigms do (Balkowiec and Katz, 2002). In view of the previous literature on a possible role of intracellular calcium in the release of BDNF, I used BABTA-AM to complex intracellular calcium and failed to observe any change of basal BDNF secretion.

Taken together, my results indicate that BDNF release does not depend on intracellular calcium mobilisation (see Fig. 18). However, when intracellular calcium is mobilised by caffeine and released from the ER through ryanodine receptor, BDNF secretion was induced and prevented by the concomitant

addition of BAPTA-AM. It is therefore plausible that two different calcium-dependent mechanisms involved in BDNF secretion exist. On one hand, in the absence of frequent stimulations, basal BDNF release is exclusively dependent on extracellular calcium influx through VGCCs; those generate a rapid increase of calcium concentration localised in the area where exocytosis of large dense core vesicles occurs. On the other hand, repeated stimuli increase the influx of extracellular calcium through VGCCs to a level sufficient to raise the concentration of free cytoplasmic calcium, eventually triggering calcium mobilisation from the ER through ryanodine receptors.

3.5 Role of different Voltage gate calcium channels on BDNF secretion

During the course of action potentials, the presynaptic calcium influx is triggered by the activation of the Voltage Gated Calcium Channels (VGCCs) (Hofmann et al., 1999; Lacinova, 2005). The VGCCs constitute a class of trans-membrane ion channels expressed widely in all brain areas and in several non-neuronal cells. The classification of the calcium channels is based on electrophysiological and pharmacological properties (Lacinova, 2005). The low voltage channels LVA correspond to the calcium channels that are activated at the membrane voltage of -70mV. Because of the small amplitude of conductance and fast decay, these channels were also called T-type calcium channel (T for transient). Conversely, the high-voltage calcium channels HVA have an activation threshold of -20mV and for their large amplitude of conductance and slow decay, they are called L-type channel (L for long-lasting). Further experiments with neuronal cells revealed novel calcium channels with a conductance characteristics between those of L and T-type channels: The N-type (N for neuronal), P/Q -type (P for Purkinje cells, where they were characterized) and R-type calcium channels (R for resistant, since a specific blocker could not be identified). Individual neurons often express all types of VGCCs, which are differently distributed among cell compartments (Kamp et al., 2012). The extracellular calcium influx is mainly associated with P/Q-, N- and R- type channels, which are predominantly located on the pre-synaptic membranes (Li et al., 2007; Trimmer and Rhodes, 2004). My

results show that these 3 types are the most highly expressed calcium channels in hippocampal, cortical primary culture as well as in neurons derived from ES cells (see Fig. 15). In spite of several previous studies on the topic, the role of different types of VGCCs on activity-dependent BDNF release is not clear yet. A previous study with 3-day old hippocampal neurons showed that BDNF secretion during theta bust stimulation requires calcium influx and in particular N-type channels (Balkowiec and Katz, 2002). Given the fact that these young neurons lack excitatory glutamatergic synapses, this *in vitro* model is not ideal to investigate the role of VGCCs on BDNF release. In my work I used mature ES cell-derived neurons and selective calcium channel antagonists to characterize the role of different VGCCs. In these neurons, the P/Q-type channels seem to be primarily involved in basal BDNF secretion, in absence of external stimuli. Only a small reduction of basal BDNF release could be observed by treating neurons with Conotoxin, a potent N-type channels blocker. By contrast, the L- and R- type channels blockers did not have any effect on BDNF secretion, indicating that these calcium channels are not involved in the process (see Fig. 16). Whereas previous electrophysiological studies indicate action potentials of short duration activate high affinity P/Q- channels, action potentials generated by theta burst stimulation lead to selective recruitment of N- and R-type channels (Li et al., 2007). Therefore, it appears plausible that P/Q channels mainly modulate activity dependent BDNF release and that N-type channels are only activated upon repetitive stimulation. In addition, my results also show that blocking T-type channels with both mibefradil and NNC-550 completely abolished the BDNF secretion, indicating that sub-threshold changes of resting potential critically affect basal BDNF release (see Fig. 17). A role for T-type channels in vesicle exocytosis was previously suggested. As observed for N-type channels, T-type channels interact with neuronal SNARE proteins syntaxin-1A and SNAP25, which modulate the channel activity and support low-threshold exocytosis (Weiss et al., 2012a; Weiss et al., 2012b). Recent studies revealed that T-type channels are important in controlling basal vesicular release in different synapses of nociceptive dorsal horns neurons, entorhinal cortex pyramidal neurons and hippocampal CA1 (Huang et al., 2011; Jacus et al., 2012). In addition, the functional role of T-type channels in generating low-threshold spikes and

rebound burst firing was demonstrated in neurons from thalamus, hippocampus and neocortex (Huguenard, 1996).

Summarising, my results on calcium channels support the view that there is a differential regulation of spontaneous and evoked release of BDNF from neurons. Basal BDNF release is the result of the single action potential and of sub-threshold activation. Single action potentials result in the extracellular calcium influx through P/Q-type channels, whereas the sub-threshold resting potential changes open transiently the T-type channels. Future experiments with ES cell-derived neurons will provide more data on calcium influx during repetitive stimulation.

3.6 The Bassoon mutant ES cell-derived neurons

At chemical synapses, vesicle exocytosis is restricted in a specific region of the presynaptic plasma membrane, called active zone (Sudhof, 2012). This is characterized by an electron dense structure composed by specific proteins that define the site of neurotransmitter release. Among them, Bassoon is a 420 KDa presynaptic cytomatrix protein potentially involved in the assembly of functional active zones (Fenster et al., 2000; Richter et al., 1999; Wang et al., 1999). Mice lacking Bassoon protein develop epileptic seizures starting at about 3 weeks post-natally and altered pattern of neuronal activity for reasons that are still unknown. In addition, the size of the brain of these mutant mice is enlarged (the hippocampus is about 40% bigger at 3 months). Similarly to the *Bsn* $^{m/m}$, the megencephaly mice (mceph/mceph) that lack the Kv1.1 channel have altered neuronal activity and abnormal brain growth (Almgren et al., 2008; Diez et al., 2003). This spontaneous germ line mutation makes potassium channel non-functional causing temporal lobe epilepsy (TLE) (Petersson et al., 2003). A few weeks after birth, these mice begin to develop epileptic seizures of increasing severity over time, in parallel with brain overgrowth just like with the *Bassoon* mutant. This excessive brain enlargement is restricted to the hippocampus and ventral cortical structures, including the piriform/entorhinal cortex and amygdala (Petersson et al., 2003) (Diez et al., 2003). Increased cell proliferation and reduced apoptosis has been noted within the dentate gyrus and CA3 region,

presumably accounting in part for the megencephaly phenotype (Almgren et al., 2007; Almgren et al., 2008). However, there is still not direct evidence indicating that hypertrophy observed in both *Bsn* $^{m/m}$ and mceph/mceph brains is caused by increased BDNF levels, as increased activity, most likely the explanation for the BDNF increased levels, may obviously change the expression pattern of many genes including growth factors others than BDNF. Our laboratory found that in 3 week-old *Bassoon* mutants BDNF levels were markedly increased in the hippocampus, cortex and striatum (Heyden et al., 2011). However, the distribution of the protein was found to be identical compared with control animals, without any evidence for dendritic localization, but with a very marked pre-synaptic increase of BDNF levels (Dieni et al., 2012). Also, the *in vivo* work failed to reveal any evidence for increased BDNF release in these animals. I noticed that during their maturation, the *Bsn*$^{m/m}$ ES cell-derived progenitors showed a higher degree of cell proliferation compared to wild type (see Fig. 20), with apparently newly generate neurons appearing as late as 15 DIV (data not shown). Both the nature and the origin of these newly born neurons need to be further investigated the *Bsn* $^{m/m}$ ES cell-derived neurons also show increased neuronal activity developing in parallel with increased BDNF release (see Fig. 22). Whether BDNF is involved in supporting extended progenitor proliferation of these cells will be investigated in future experiments.

3.7 Role of BDNF in regulation of inhibitory signals

During neuronal development, GABA initially acts an excitatory neurotransmitter as result of elevated intracellular chloride concentrations (Ben-Ari, 2002). The developmental expression of the K^+-Cl^- co-transporter KCC2 seems to be the key factor allowing GABA to develop its well-known inhibitory activity and in mutant mice lacking the *KCC2* gene, GABA does not become an inhibitory neurotransmitter (Hubner et al., 2001). A few years ago, the surprising observation that BDNF is a major regulator of *KCC2* expression through TrkB activation was reported (Rivera et al., 2002). Thus, hippocampal slices exposed for 2 hours to BDNF showed a massive decrease of *KCC2* and a

progressive increase of intracellular Cl^-. In addition, a complete block of activity-induced *KCC2* down-regulation can be observed in hippocampal neurons treated with the antibody TrKB-Fc. The decrease of KCC2 expression I observed in both *Mapt::Bdnf* and *Bsn* $^{m/m}$ ES cell-derived neurons suggests that elevated BDNF release reduces the expression of this ion exchanger in these cells (see Fig. 23). However, it might be that the low expression levels of *KCC2* in *Bassoon* mutant neurons are due to the presence of an increased proportion of immature neurons, known to express low levels of *KCC2* as mentioned.

4. Conclusion

My results indicate that overexpression paradigms are not suitable to study key aspects of the biology of BDNF. In particular, over-expression prevents full pro-BDNF processing and suggests mechanisms of release that do not faithfully mimic the release of the endogenous protein. Perhaps the most important consequence of these findings is that the role of BDNF, especially in the adult will need to be re-considered. In particular, the role of BDNF in overall brain growth needs to be investigated, as it may be a particularly interesting aspect of its biology, as suggested by observations with the *Bassoon* and the *Kv1.1* mutants (Heyden et al., 2011; Lavebratt et al., 2006). In both cases, very significant brain overgrowth develops most probably as a result of increasing episodes of activity bursts, eventually leading to seizures. This pattern of increased activity has long been known to be particularly efficient at increasing transcription of the *Bdnf* gene (Cho et al., 2007; Pencea et al., 2001; Scharfman et al., 2005). While causality still needs to be established, my results suggest a plausible explanation for the lack of brain overgrowth in apparently related paradigm of BDNF overexpression in transgenic mice. For example, even if *Bdnf* expression under the *CaMKII* promoter in adult neocortex and hippocampus increases *Bdnf* mRNA levels 2 to 3-fold (Huang et al., 1999), these brain areas were not reported to be increased in size. A possible explanation for these findings, as shown in my controlled overexpression paradigm, is that the processing capacity of neurons overexpressing BDNF is not sufficient to ensure a complete processing of the

precursor protein. This presumably leads to the release of pro-BDNF *in vivo*, and to an activation of $p75^{NTR}$, thus opposing any growth effect mediated by mature BDNF. By contrast, in the *Bassoon* mutant, our laboratory showed that the processing capacity is increased, presumably as a result of higher neuronal activity, maintaining therefore the pro-BDNF/mature BDNF ratio comparable with control animals (Dieni et al., 2012). The growth effects of BDNF observed in these mutants could be plausibly explained by the ability of BDNF to prevent the death of large populations of newly generated neurons in the adult brain originating from the sub-ventricular zone and the dentate gyrus (Li et al., 2012). A possible role of BDNF in regulating the growth of the adult brain is of special interest given recent results indicating that fingolimod, the first oral treatment of multiple sclerosis, increases BDNF levels in some brain areas in the mouse (Deogracias et al., 2012). Importantly, fingolimod prevents the reduction of brain rodent areas, like striatum, lacking the gene *MeCP2.* Recent human studies indicate that in chronic forms of multiple sclerosis, fingolimod prevents the reduction of brain volume (visualized by MRI) more efficiently than the standard of care drug beta-interferon (Cohen et al., 2013).

In the future, it will be of special interest to explore the possibility to use BDNF either directly or else with the help of drugs such as fingolimod to prevent brain shrinkage as observed in several neurodegenerative diseases. My work contributes to understand why this could not possibly be identified earlier.

5. Materials and Methods

5.1 Animals

The mutant mice, lacking the central part of the Bassoon protein (*Bsn*$^{\Delta Ex4/5}$), were generated as previously described (Altrock et al., 2003), backcrossed with C57/B16 mice, and then crossed with SV129 strain obtaining a mixed C57/B16/SV129 genetic background for mutants and wild types. The mutant mice were identified by Polymerase Chain Reaction (PCR) using the following primers:

Mutant allele primer pair:
KOS2: GGT ATC CTG TTC TGA AAG ACT TTC
pWHAS2: AAG CTT GAT ATC GAA TTT GGC CTG
mutant band: ~ 400bp

Wild-type allele primer pair:
V2-Maus: AGT TGT CAA GCC TGT TCC AGA AGC
V3-Maus: ACA CCG TCG GAG GAG TAG CCT GT
wild-type band: ~ 600bp

5.2 Isolation of blastocyst-derived Stem Cells

Mutant *Bsn*$^{m/+}$ female mice were intraperitoneally injected with 4U of Pregnant mare's serum gonadotropin (PMSG, Sigma #4527) and, after 48 hours, with 3U Human Chorionic Gonadotropin (hCG, Sigma #C8554) to induce superovulation. After administration of hCG, females were crossed with *Bsn*$^{m/+}$ male and sacrificed after 3.5 days. The abdominal cavity was opened and uterus was isolated keeping the junction with the oviduct intact. The uterus was placed in a small volume of KSR medium [Knockout DMEM (Invitrogen # 10829-018) with 20% Knockout Serum Replacement (Invitrogen # 10828-028), 0.1 mM non-essential amino acids (Sigma #), 2 mM L-Glutamine (Sigma #M7145), 0.001% β-mercaptoethanol (Sigma#M6250) and 4 ng/ml bFGF (Sigma #F0291)] the utero-

tubal junction was cut lengthwise, and each horn was flushed from the cervix. The blastocyst were collected, counted and plated on immortalized mouse feeder with ES medium supplemented with 2 μM glycogen synthase kinase 3β (GSK-3β) and mitogen-activated protein kinase (MAPK/ERK kinase or MEK) inhibitors (CHIR99021 and PD0325901, Stemgent, # 04-0004 and 04-0006). After 5 days, the inner cell masses were picked using a mouth pipette and disaggregated in a drop of Trypsin (0.025% in 0.04% EDTA/PBS). ES cells were cultured on immortalized mouse feeder and different homozygous and wild-type cell lines were identified by PCR using the primer indicated above.

5.3 Cell culture

Undifferentiated ES cells (J1, *Mapt::Bdnf* clone G3, $Bsn^{+/+}$ clone A4 and $Bsn^{m/m}$ clone 7.1) were grown on immortalized mouse fibroblasts in ES medium [DMEM medium (Sigma, # D-5546) supplemented with 15% Fetal Bovine Serum (Biological Industries, # 04-002-1A), 0.1 mM Non-Essential Amino Acids (Sigma, # 7145), 2 mM Glutamine (Sigma, # G-7513), 10^5 U/ml Leukemia Inhibitor Factor (LIF) and 0.001% β-mercaptoethanol (Sigma, # M-7522)]. Cells were split 1:10 or 1:20 every two days and plated on new 60 mm cell culture plate with immortalized fibroblasts at lest for 1 week. Then, ES cells were plated on 100 mm gelatin-coated plates and split 1:3 or 1:6 to obtain a homogeneous monolayer of stem cells. For aggregates formation, 4 x 10^6 ES cells were plated for 8 days on 100 mm non-adherent bacteria dishes in EB medium (D-MEM medium supplemented with 10% FCS, 0.1 mM Non-Essential Amino Acids, 2 mM Glutamine and 0.001% β-mercaptoethanol). Medium was changed every two days and 5 μM retinoic acid (Sigma, # R2625) was added after 4 days. Aggregates were dissociated after 2 washes with PBS (Sigma, # D-8537) using fresh prepared 0.05% trypsin solution (Sigma, # T-8802) in 0.04% EDTA (Sigma, # E-6758) / PBS and gently resuspended in EB medium. After centrifugation (5 minutes at 1000 rpm), cells were resuspended in N2 medium [1:1 DMEM / F-12 (Sigma# D-6421), 25 mg/ml insulin (Sigma, # I-6634), 50 mg/ml human transferrin (Sigma, # T-8158), 20 nM progesterone (Sigma, # P-8783) 100 nM putrescine (Sigma, # P-5780), 30 nM sodium selenite (Sigma, # S-5261) and BSA

50 mg/ml and filtered through 40 μm nylon cell strainer (BD, # 352340). Cells were plated in 12 wells-plates ($1x10^6$ cells per well), which were previously treated over night with poly-D-ornitine (Sigma, # P-8638) and for 2 hours with laminin (Roche, # 11234217001). After 2 days the N2 medium was replaced by complete medium [D-MEM medium supplemented with 2 μg/ml L-alanine (Sigma, # A-7627), 0.1 μg/ml biotin (Sigma, # B-4501), 2.5 mg/ml BSA, 5 μg/ml human transferrin, 4 μg/ml insulin, 2 μg glutamine, 2 μg/ml L-carnitine (Sigma, # C-0283), 1 μg/ml ethanolamine (Sigma, # E-9508), D-galactose (Sigma, # G-0625) 15 μg/ml, 7.76 μg/ml L-proline (Sigma, # P-0380), 16.1 μg/ml putrescine (Sigma, # P-7505), 25 μg/ml Na-pyruvate (Sigma, # P-5280), 0.016 μg/ml Na-selenite (Sigma, # S-1382), 0.34 μg/ml vitamin B12 (Sigma, # V-2876), 0.194 μg/ml zinc sulfate (Sigma, # Z-4750), 2.56 μg/ml catalase (Sigma, # C-40), 1 μg/ml glutathione (Sigma, # G-6013), 2.5 μg/ml superoxide dismutase (Sigma, # S-2515), 1 μg/ml linoleic acid (Sigma, # L-1376), 1 μg/ml linolenic acid (Sigma, # L-2376), 6.3 ng/ml progesterone (Sigma, # P-8783), all-trans retinol (Sigma, # R-7632) 100 ng/ml, 100 ng/ml retinylacetate (Sigma, # R-7882), 1 μg/ml tocopherol (Sigma, # T-3251), 1 μg/ml tocopherolacetate (Sigma, # T-3001).

5.4 Immunofluorescence analysis

ES cells were grown on glasses coverslip with immortalized mouse feeder and fixed for 30 minutes with 4% paraformaldehyde solution. Unspecific signals were prevented incubating the fixed cells with blocking solution (10% horse serum, 0.1% Tween 20 in PBS) for 1 hour at room temperature. Cells were subsequently incubated overnight at 4°C in a humid chamber with the primary antibody diluted in the blocking solution. Antibodies used are as follows: mouse monoclonal anti-OCT-4 (Abcam, # ab19857), rabbit polyclonal anti-Nonog (Abcam, # ab21603). All secondary antibodies, conjugated with a fluorophore, were used at 1:10000 dilution followed by nuclear staining with 10 mg/ml Hoechst 33342 (Sigma, # B-2261).

5.5 Immunoprecipitation (IP)

Rabbit polyclonal anti-BDNF #9 antibody was added (12 µg/ml) overnight to the culture media of ES cell-derived neurons to bind released BDNF and to avoid its internalization. Culture medium was subsequently incubated for 2 days at 4°C with 12.5 µg Protein A-Agarose (Roche, # 05015979001). After short centrifugation, the proteins G were washed two times with two different buffers (Buffer 1: 50 mM Tris-HCl pH 7.5, 500 mM sodium chloride; 0.1% Nonidet 40, 0.05% sodium deoxycholate; Buffer 2: 50 mM Tris-HCl, pH 7.5, 0.1% Nonidet 40, 0.05% sodium deoxycholate) and boiled at 95°C for 10 minutes in sample buffer. Proteins obtained after immunoprecipitation were analyzed by Western blot using the One-HOUR IP-Western kit (GeneScript, # L00231) to reduce the background originated from light and heavy chains of the antibody.

5.6 Western Blot analysis

ES cell-derived neurons were washed twice with cold PBS and lysate on ice with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche, # 11697498001) and phosphatase inhibitor (Roche, # 04 906 845 001). After centrifugation at 13.000 g for 20' at 4° C supernatants were collected and proteins were quantified using BCA assay (Pierce # 23225). Samples were boiled in 1X NuPAGE sample buffer (Invitrogen, # NP0007) and 20 µg of proteins were loaded in NOVEX 4-12% gradient SDS-polyacrylamide gels (Invitrogen # EC60352BOX). Proteins were subsequently transferred on 0.2 µm nitrocellulose (GE Healthcare, # RPN3032D) using the NOVEX semi-dry blotter (Invitrogen, # SD1000). Membranes were blocked with 5% milk (or 3% BSA) to prevent non-specific binding of the antibody and then incubated overnight with the primary antibody and 1 hour with the secondary antibody. Signal detection was performed using ECL Advance (Invitrogen, # RPN2135) and optical density analysis was realized using Image J software. The antibodies used for our analysis were: rabbit polyclonal anti-BDNF N-20 (Santa Cruz, # SC-546) 1:500, mouse monoclonal anti-phospho-Trk (Cell Signalling, # 9141L) rabbit polyclonal anti-TrkB (Cell

Signalling, # 4606) 1:500, mouse monoclonal anti-phospho-S6 ribosomal protein (Cell Signalling, #2211) rabbit polyclonal anti-SP1 (Cell Signalling, # 2212), mouse monoclonal antibody anti-β-III-Tubulin (Covance, # MMS-435P) 1:10000. Secondary antibodies coupled with horseradish peroxidase were used at a final concentration of 1:2000.

5.7 BDNF ELISA

The monoclonal anti-BDNF #9 antibody was biotinylated using the EZ-Link Sulfo-NHS-LC Biotin reagent (Thermo Scientific, # 21435) according to the protocol of the manufacturer. ES cell-derived neurons were incubated for 2 days with anti-BDNF #9 antibody, washed twice with PBS and then a new medium containing the biotinylated antibody #9 was added to the cells. To analyze the short and long-time BDNF release, medium was collected after either 1 hour or overnight incubations. Micro 96 Well Plates (Nunc) were coated overnight at room temperature with 2 µg/ml of anti-BDNF #1 antibody diluted in coating buffer (0.05M Na_2CO_3, 0.05M Na_2HCO_3, pH 9.7). After blocking for 2 hours with 4% BSA in PBS, 200 µl of cell medium was added and plates were incubated for 3 hours on rotating platform (300 rpm) at room temperature. A standard curve was also generated for each plate adding complete medium containing recombinant BDNF with a concentration ranging from 0.05 pg/ml to 25 ng/ml, pre-incubated with biotinylated anti-BDNF #9 antibody. After removing the samples and standards, the plate was washed 5 times with PBS containing 0.1% Tween20, and then incubated for 3 hours at room temperature, on the same rotating platform, with Horseradish Peroxidase conjugated Neutravidin (Invitrogen) diluted 1:25000 (40ng/ml final concentration) in incubation buffer (100mM KH_2PO_4, 100mM Na_2HPO_4, pH 7.6) containing 4% BSA. After 5 washes, 100 µl of BM chemioluminescence ELISA substrate POD (Roche) was added into each well and luminescence was measured using an Infinite M1000 PRO microplate reader (Tecan).

5.8 Mapt locus targeting

One day before electroporation, J1 ES cells were split from 60 mm to 100 mm dish on immortalized feeder. Cells were subsequently collected, resuspended in 550 µl of PBS and transferred in a 0.2 cm gap MicroPulser Cuvette (Bio-Rad, # 165-2086). Cells were electroporated at 0.4 kV and 25 µF in a MicroPulser Electroporator (Bio-Rad, # 165-2100) with 30 µg of linearized pTIgBFmyc plasmid contained the Bdnf cDNA flanked by two sequences of 7 and 2.2 kb homologous to the exon 1 of *Map*t locus. The electroporated cells were plated on Neomycin resistant feeder and grown in medium supplemented with 50 µg/ml G-418 (Sigma, # A1720***)***. Antibiotic resistant ES cell colonies were picked and plated in 96 well plates with immortalized mouse feeder.

5.9 Southern Blot analysis

Picked ES clones were dissociated using a 0.05 % trypsin solution and plated on gelatin coated 24-well plates in ES medium. After 2 days, cells were lysate using the tail buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2 % SDS, 200 mM NaCl, 100 ⌠g/ml Proteinase K) and the genomic DNA was precipitated adding 1 volume of 2-Propanol. After 2 washes with 70% Ethanol, DNA was digested using BamHI and KpnI restriction enzymes for 7 hours at 37°C. The DNA fragments were resolved in 0.7 % agarose gel, subsequently denatured (0.5 M NaOH, 1.5 M NaCl) and neutralized (0.5 M Tris-HCl pH 7.5, 1.5 M NaCl). The genomic DNA was transferred overnight on Hybond-XL membrane (Amersham, # RPN2020S), which was subsequently cross-linked and pre-hybridized with DIG Easy Hyb buffer (Roche, # 11603558001). Then, the membrane was incubated overnight in DIG Easy Hyb buffer with the DIG-labeled DNA probes for the *Mapt* locus. After 1 hour of incubation with anti-Digoxigenin antibody conjugated with alkaline phosphatase (Roche, # 11093274910) the signal was developed using CDP-star (Roche, # 12041677001) and visualized using X-ray films.

5.10 Quantitative PCR

Total RNA was extracted from neuronal cultures using RNeasy Plus Mini Kit (Qiagen, # 74134), and converted in cDNA using SuperScript III (Invitrogen, # 1182467) with random primers (Promega, # C-1181). The cDNA solution was subsequently diluted 20 times in water and 5 µl were used in qRT-PCR reaction containing TaqMan Universal Master Mix (Roche # 4304437), 0.9 µM *Bdnf* (exon VIII) or *Gapdh* primers and 0.25 µM TaqMan probes. The quantification of BDNF mRNA was performed in MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems, # P/N N801-0560) using the ABI Prism 7'000 Sequence Detection System (Applied Biosystems), and normalized to the amount of *Gapdh* mRNA. The primers and probes were as follows: *Bdnf*, forward: 5'-GGG AGC TGA GCG TGT GTG A-3´, reverse: 5'-CGT CCC GCC AGA CAT GTC-3', TaqMan probe: 5'-CGA GTG GGT CAC AGC GGC AGA-3'; *Gapdh,* forward: 5'-TGT GTC CGT CGT GGA TCT GA-3', reverse: 5'-CCT GCT TCA CCA CCT TCT TGA-3', TaqMan probe: 5'- CCG CCT GGA GAA ACC TGC CAA GTA TG-3'.

5.11 Statistical analysis

After checking the Gaussian distribution using the Kolmogorov-Smirnov test, data obtained from optical density analysis were evaluated by Student's t-test. Parametric one-way ANOVA was used to analyse the statistical difference between data obtained from three or more independent groups. In all analysis, $p<0.05$ was considered statistically significant. The statistical analyses were performed using Prism 5.0b (GraphPad).

6. References

Almgren, M., A.S. Persson, C. Fenghua, B.M. Witgen, M. Schalling, J.R. Nyengaard, and C. Lavebratt. 2007. Lack of potassium channel induces proliferation and survival causing increased neurogenesis and two-fold hippocampus enlargement. *Hippocampus*. 17:292-304.

Almgren, M., M. Schalling, and C. Lavebratt. 2008. Idiopathic megalencephaly-possible cause and treatment opportunities: from patient to lab. *European journal of paediatric neurology : EJPN : official journal of the European Paediatric Neurology Society*. 12:438-445.

Altar, C.A., N. Cai, T. Bliven, M. Juhasz, J.M. Conner, A.L. Acheson, R.M. Lindsay, and S.J. Wiegand. 1997. Anterograde transport of brain-derived neurotrophic factor and its role in the brain. *Nature*. 389:856-860.

Altrock, W.D., S. tom Dieck, M. Sokolov, A.C. Meyer, A. Sigler, C. Brakebusch, R. Fassler, K. Richter, T.M. Boeckers, H. Potschka, C. Brandt, W. Loscher, D. Grimberg, T. Dresbach, A. Hempelmann, H. Hassan, D. Balschun, J.U. Frey, J.H. Brandstatter, C.C. Garner, C. Rosenmund, and E.D. Gundelfinger. 2003. Functional inactivation of a fraction of excitatory synapses in mice deficient for the active zone protein bassoon. *Neuron*. 37:787-800.

Amir, R.E., I.B. Van den Veyver, M. Wan, C.Q. Tran, U. Francke, and H.Y. Zoghbi. 1999. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nature genetics*. 23:185-188.

Aydemir, C., E.S. Yalcin, S. Aksaray, C. Kisa, S.G. Yildirim, T. Uzbay, and E. Goka. 2006. Brain-derived neurotrophic factor (BDNF) changes in the serum of depressed women. *Progress in neuro-psychopharmacology & biological psychiatry*. 30:1256-1260.

Balkowiec, A., and D.M. Katz. 2000. Activity-dependent release of endogenous brain-derived neurotrophic factor from primary sensory neurons detected by ELISA in situ. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 20:7417-7423.

Balkowiec, A., and D.M. Katz. 2002. Cellular mechanisms regulating activity-dependent release of native brain-derived neurotrophic factor from hippocampal neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 22:10399-10407.

Barbacid, M. 1994. The Trk family of neurotrophin receptors. *Journal of neurobiology*. 25:1386-1403.

Barde, Y.A., D. Edgar, and H. Thoenen. 1982. Purification of a new neurotrophic factor from mammalian brain. *The EMBO journal*. 1:549-553.

Ben-Ari, Y. 2002. Excitatory actions of gaba during development: the nature of the nurture. *Nature reviews. Neuroscience*. 3:728-739.

Bibel, M., J. Richter, E. Lacroix, and Y.A. Barde. 2007. Generation of a defined and uniform population of CNS progenitors and neurons from mouse embryonic stem cells. *Nature protocols*. 2:1034-1043.

Bibel, M., J. Richter, K. Schrenk, K.L. Tucker, V. Staiger, M. Korte, M. Goetz, and Y.A. Barde. 2004. Differentiation of mouse embryonic stem cells into a defined neuronal lineage. *Nature neuroscience*. 7:1003-1009.

Bliss, T.V., and G.L. Collingridge. 1993. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*. 361:31-39.

Blochl, A., and H. Thoenen. 1995. Characterization of nerve growth factor (NGF) release from hippocampal neurons: evidence for a constitutive and an unconventional sodium-dependent regulated pathway. *The European journal of neuroscience*. 7:1220-1228.

Brunoni, A.R., M. Lopes, and F. Fregni. 2008. A systematic review and meta-analysis of clinical studies on major depression and BDNF levels: implications for the role of neuroplasticity in depression. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum*. 11:1169-1180.

Canossa, M., A. Gartner, G. Campana, N. Inagaki, and H. Thoenen. 2001. Regulated secretion of neurotrophins by metabotropic glutamate group I (mGluRI) and Trk receptor activation is mediated via phospholipase C signalling pathways. *The EMBO journal*. 20:1640-1650.

Cathomas, F., C. Vogler, J.C. Euler-Sigmund, D.J. de Quervain, and A. Papassotiropoulos. 2010. Fine-mapping of the brain-derived neurotrophic factor (BDNF) gene supports an association of the Val66Met polymorphism with episodic memory. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum*. 13:975-980.

Chahrour, M., and H.Y. Zoghbi. 2007. The story of Rett syndrome: from clinic to neurobiology. *Neuron*. 56:422-437.

Chang, Q., G. Khare, V. Dani, S. Nelson, and R. Jaenisch. 2006. The disease progression of Mecp2 mutant mice is affected by the level of BDNF expression. *Neuron*. 49:341-348.

Chapman, E.R. 2008. How does synaptotagmin trigger neurotransmitter release? *Annual review of biochemistry*. 77:615-641.

Chen, B., D. Dowlatshahi, G.M. MacQueen, J.F. Wang, and L.T. Young. 2001. Increased hippocampal BDNF immunoreactivity in subjects treated with antidepressant medication. *Biological psychiatry*. 50:260-265.

Chen, Z.Y., D. Jing, K.G. Bath, A. Ieraci, T. Khan, C.J. Siao, D.G. Herrera, M. Toth, C. Yang, B.S. McEwen, B.L. Hempstead, and F.S. Lee. 2006. Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science*. 314:140-143.

Cho, S.R., A. Benraiss, E. Chmielnicki, A. Samdani, A. Economides, and S.A. Goldman. 2007. Induction of neostriatal neurogenesis slows disease progression in a transgenic murine model of Huntington disease. *The Journal of clinical investigation*. 117:2889-2902.

Cohen, J.A., F. Barkhof, G. Comi, G. Izquierdo, B. Khatri, X. Montalban, J. Pelletier, B. Eckert, D.A. Haring, and G. Francis. 2013. Fingolimod versus intramuscular interferon in patient subgroups from TRANSFORMS. *Journal of neurology*.

Cohen, S. 1960. Purification of a Nerve-Growth Promoting Protein from the Mouse Salivary Gland and Its Neuro-Cytotoxic Antiserum. *Proceedings of the National Academy of Sciences of the United States of America*. 46:302-311.

Cohen, S., and R. Levi-Montalcini. 1957. Purification and properties of a nerve growth-promoting factor isolated from mouse sarcoma 180. *Cancer research*. 17:15-20.

Cohen, S., R. Levi-Montalcini, and V. Hamburger. 1954. A Nerve Growth-Stimulating Factor Isolated from Sarcom as 37 and 180. *Proceedings of the National Academy of Sciences of the United States of America*. 40:1014-1018.

Cunningham, M.E., and L.A. Greene. 1998. A function-structure model for NGF-activated TRK. *The EMBO journal*. 17:7282-7293.

Deogracias, R., M. Yazdani, M.P. Dekkers, J. Guy, M.C. Ionescu, K.E. Vogt, and Y.A. Barde. 2012. Fingolimod, a sphingosine-1 phosphate receptor modulator, increases BDNF levels and improves symptoms of a mouse model of Rett syndrome. *Proceedings of the National Academy of Sciences of the United States of America*. 109:14230-14235.

Dieni, S., T. Matsumoto, M. Dekkers, S. Rauskolb, M.S. Ionescu, R. Deogracias, E.D. Gundelfinger, M. Kojima, S. Nestel, M. Frotscher, and Y.A. Barde. 2012. BDNF and its pro-peptide are stored in presynaptic dense core vesicles in brain neurons. *The Journal of cell biology*. 196:775-788.

Diez, M., P. Schweinhardt, S. Petersson, F.H. Wang, C. Lavebratt, M. Schalling, T. Hokfelt, and C. Spenger. 2003. MRI and in situ hybridization reveal early disturbances in brain size and gene expression in the megencephalic (mceph/mceph) mouse. *The European journal of neuroscience*. 18:3218-3230.

Dwivedi, Y., H.S. Rizavi, R.R. Conley, R.C. Roberts, C.A. Tamminga, and G.N. Pandey. 2003. Altered gene expression of brain-derived neurotrophic factor and receptor tyrosine kinase B in postmortem brain of suicide subjects. *Archives of general psychiatry*. 60:804-815.

Eder, J., and A.R. Fersht. 1995. Pro-sequence-assisted protein folding. *Molecular microbiology*. 16:609-614.

Egan, M.F., M. Kojima, J.H. Callicott, T.E. Goldberg, B.S. Kolachana, A. Bertolino, E. Zaitsev, B. Gold, D. Goldman, M. Dean, B. Lu, and D.R. Weinberger. 2003. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell*. 112:257-269.

Emptage, N.J., C.A. Reid, and A. Fine. 2001. Calcium stores in hippocampal synaptic boutons mediate short-term plasticity, store-operated Ca2+ entry, and spontaneous transmitter release. *Neuron*. 29:197-208.

Ernfors, P., K.F. Lee, and R. Jaenisch. 1994. Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature*. 368:147-150.

Fenster, S.D., W.J. Chung, R. Zhai, C. Cases-Langhoff, B. Voss, A.M. Garner, U. Kaempf, S. Kindler, E.D. Gundelfinger, and C.C. Garner. 2000. Piccolo, a presynaptic zinc finger protein structurally related to bassoon. *Neuron*. 25:203-214.

Frade, J.M., P. Bovolenta, and A. Rodriguez-Tebar. 1999. Neurotrophins and other growth factors in the generation of retinal neurons. *Microsc Res Tech*. 45:243-251.

Goodman, L.J., J. Valverde, F. Lim, M.D. Geschwind, H.J. Federoff, A.I. Geller, and F. Hefti. 1996. Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. *Molecular and cellular neurosciences*. 7:222-238.

Gotz, M., A. Stoykova, and P. Gruss. 1998. Pax6 controls radial glia differentiation in the cerebral cortex. *Neuron*. 21:1031-1044.

Gray, J., G.S. Yeo, J.J. Cox, J. Morton, A.L. Adlam, J.M. Keogh, J.A. Yanovski, A. El Gharbawy, J.C. Han, Y.C. Tung, J.R. Hodges, F.L. Raymond, S. O'Rahilly, and I.S. Farooqi. 2006. Hyperphagia, severe obesity, impaired cognitive function, and hyperactivity associated with functional loss of one copy of the brain-derived neurotrophic factor (BDNF) gene. *Diabetes*. 55:3366-3371.

Griesbeck, O., M. Canossa, G. Campana, A. Gartner, M.C. Hoener, H. Nawa, R. Kolbeck, and H. Thoenen. 1999. Are there differences between the secretion characteristics of NGF and BDNF? Implications for the modulatory role of neurotrophins in activity-dependent neuronal plasticity. *Microsc Res Tech*. 45:262-275.

Grubb, M.S., and J. Burrone. 2010. Activity-dependent relocation of the axon initial segment fine-tunes neuronal excitability. *Nature*. 465:1070-1074.

Gundelfinger, E.D., and A. Fejtova. 2012. Molecular organization and plasticity of the cytomatrix at the active zone. *Current opinion in neurobiology*. 22:423-430.

Han, J.C., Q.R. Liu, M. Jones, R.L. Levinn, C.M. Menzie, K.S. Jefferson-George, D.C. Adler-Wailes, E.L. Sanford, F.L. Lacbawan, G.R. Uhl, O.M. Rennert, and J.A. Yanovski. 2008. Brain-derived neurotrophic factor and obesity in the WAGR syndrome. *The New England journal of medicine*. 359:918-927.

Han, J.C., A. Thurm, C. Golden Williams, L.A. Joseph, W.M. Zein, B.P. Brooks, J.A. Butman, S.M. Brady, S.R. Fuhr, M.D. Hicks, A.E. Huey, A.E. Hanish, K.M. Danley, M.J. Raygada, O.M. Rennert, K. Martinowich, S.J. Sharp, J.W. Tsao, and S.E. Swedo. 2013. Association of brain-derived neurotrophic factor (BDNF) haploinsufficiency with lower adaptive behaviour and reduced cognitive functioning in WAGR/11p13 deletion syndrome. *Cortex; a journal devoted to the study of the nervous system and behavior*.

Harada, A., K. Oguchi, S. Okabe, J. Kuno, S. Terada, T. Ohshima, R. Sato-Yoshitake, Y. Takei, T. Noda, and N. Hirokawa. 1994. Altered microtubule organization in small-calibre axons of mice lacking tau protein. *Nature*. 369:488-491.

Harrison, C.A., S.L. Al-Musawi, and K.L. Walton. 2011. Prodomains regulate the synthesis, extracellular localisation and activity of TGF-beta superfamily ligands. *Growth factors*. 29:174-186.

Harvey, J., and G.L. Collingridge. 1992. Thapsigargin blocks the induction of long-term potentiation in rat hippocampal slices. *Neuroscience letters*. 139:197-200.

Heyden, A., M.C. Ionescu, S. Romorini, B. Kracht, V. Ghiglieri, P. Calabresi, C. Seidenbecher, F. Angenstein, and E.D. Gundelfinger. 2011. Hippocampal enlargement in Bassoon-mutant mice is associated with enhanced neurogenesis, reduced apoptosis, and abnormal BDNF levels. *Cell and tissue research*. 346:11-26.

Hofer, M., S.R. Pagliusi, A. Hohn, J. Leibrock, and Y.A. Barde. 1990. Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *The EMBO journal*. 9:2459-2464.

Hofmann, F., L. Lacinova, and N. Klugbauer. 1999. Voltage-dependent calcium channels: from structure to function. *Reviews of physiology, biochemistry and pharmacology*. 139:33-87.

Huang, Z., R. Lujan, I. Kadurin, V.N. Uebele, J.J. Renger, A.C. Dolphin, and M.M. Shah. 2011. Presynaptic HCN1 channels regulate Cav3.2 activity and neurotransmission at select cortical synapses. *Nature neuroscience*. 14:478-486.

Huang, Z.J., A. Kirkwood, T. Pizzorusso, V. Porciatti, B. Morales, M.F. Bear, L. Maffei, and S. Tonegawa. 1999. BDNF regulates the maturation of inhibition and the critical period of plasticity in mouse visual cortex. *Cell*. 98:739-755.

Hubner, C.A., V. Stein, I. Hermans-Borgmeyer, T. Meyer, K. Ballanyi, and T.J. Jentsch. 2001. Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. *Neuron*. 30:515-524.

Huguenard, J.R. 1996. Low-threshold calcium currents in central nervous system neurons. *Annual review of physiology*. 58:329-348.

Jacus, M.O., V.N. Uebele, J.J. Renger, and S.M. Todorovic. 2012. Presynaptic Cav3.2 channels regulate excitatory neurotransmission in nociceptive dorsal horn neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 32:9374-9382.

Jing, S., P. Tapley, and M. Barbacid. 1992. Nerve growth factor mediates signal transduction through trk homodimer receptors. *Neuron*. 9:1067-1079.

Jungbluth, S., K. Bailey, and Y.A. Barde. 1994. Purification and characterisation of a brain-derived neurotrophic factor/neurotrophin-3 (BDNF/NT-3) heterodimer. *European journal of biochemistry / FEBS*. 221:677-685.

Kamp, M.A., D. Hanggi, H.J. Steiger, and T. Schneider. 2012. Diversity of presynaptic calcium channels displaying different synaptic properties. *Reviews in the neurosciences*. 23:179-190.

Kandel, E.R. 2004. The molecular biology of memory storage: a dialog between genes and synapses. *Bioscience reports*. 24:475-522.

Kandel, E.R. 2012. The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. *Molecular brain*. 5:14.

Kaplan, D.R., B.L. Hempstead, D. Martin-Zanca, M.V. Chao, and L.F. Parada. 1991. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science*. 252:554-558.

Karamohamed, S., J.C. Latourelle, B.A. Racette, J.S. Perlmutter, G.F. Wooten, M. Lew, C. Klein, H. Shill, L.I. Golbe, M.H. Mark, M. Guttman, G. Nicholson, J.B. Wilk, M. Saint-Hilaire, A.L. DeStefano, R. Prakash, S. Tobin, J. Williamson, O. Suchowersky, N. Labell, B.N. Growdon, C. Singer, R. Watts, S. Goldwurm, G. Pezzoli, K.B. Baker, M.L. Giroux, P.P. Pramstaller, D.J. Burn, P. Chinnery, S. Sherman, P. Vieregge, I. Litvan, J.F. Gusella, R.H. Myers, and A. Parsian. 2005. BDNF genetic variants are associated with onset age of familial Parkinson disease: GenePD Study. *Neurology*. 65:1823-1825.

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

Kassabov, S.R., Y.B. Choi, K.A. Karl, H.D. Vishwasrao, C.H. Bailey, and E.R. Kandel. 2013. A Single Aplysia Neurotrophin Mediates Synaptic Facilitation via Differentially Processed Isoforms. *Cell reports*. 3:1213-1227.

Kolbeck, R., I. Bartke, W. Eberle, and Y.A. Barde. 1999. Brain-derived neurotrophic factor levels in the nervous system of wild-type and

neurotrophin gene mutant mice. *Journal of neurochemistry*. 72:1930-1938.

Korte, M., P. Carroll, E. Wolf, G. Brem, H. Thoenen, and T. Bonhoeffer. 1995. Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proceedings of the National Academy of Sciences of the United States of America*. 92:8856-8860.

Lacinova, L. 2005. Voltage-dependent calcium channels. *General physiology and biophysics*. 24 Suppl 1:1-78.

Lavebratt, C., A. Trifunovski, A.S. Persson, F.H. Wang, T. Klason, I. Ohman, A. Josephsson, L. Olson, C. Spenger, and M. Schalling. 2006. Carbamazepine protects against megencephaly and abnormal expression of BDNF and Nogo signaling components in the mceph/mceph mouse. *Neurobiology of disease*. 24:374-383.

Lee, B.H., and Y.K. Kim. 2010. The roles of BDNF in the pathophysiology of major depression and in antidepressant treatment. *Psychiatry investigation*. 7:231-235.

Lee, R., P. Kermani, K.K. Teng, and B.L. Hempstead. 2001. Regulation of cell survival by secreted proneurotrophins. *Science*. 294:1945-1948.

Leibrock, J., F. Lottspeich, A. Hohn, M. Hofer, B. Hengerer, P. Masiakowski, H. Thoenen, and Y.A. Barde. 1989. Molecular cloning and expression of brain-derived neurotrophic factor. *Nature*. 341:149-152.

Levi-Montalcini, R. 1966. The nerve growth factor: its mode of action on sensory and sympathetic nerve cells. *Harvey lectures*. 60:217-259.

Levi-Montalcini, R., and V. Hamburger. 1951. Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *The Journal of experimental zoology*. 116:321-361.

Li, L., J. Bischofberger, and P. Jonas. 2007. Differential gating and recruitment of P/Q-, N-, and R-type Ca2+ channels in hippocampal mossy fiber boutons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 27:13420-13429.

Li, W., G. Calfa, J. Larimore, and L. Pozzo-Miller. 2012. Activity-dependent BDNF release and TRPC signaling is impaired in hippocampal neurons of Mecp2 mutant mice. *Proceedings of the National Academy of Sciences of the United States of America*. 109:17087-17092.

Lu, B., P.T. Pang, and N.H. Woo. 2005. The yin and yang of neurotrophin action. *Nature reviews. Neuroscience*. 6:603-614.

Luckman, S.M., R.E. Dyball, and G. Leng. 1994. Induction of c-fos expression in hypothalamic magnocellular neurons requires synaptic activation and not simply increased spike activity. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 14:4825-4830.

Matsumoto, T., S. Rauskolb, M. Polack, J. Klose, R. Kolbeck, M. Korte, and Y.A. Barde. 2008. Biosynthesis and processing of endogenous BDNF: CNS neurons store and secrete BDNF, not pro-BDNF. *Nature neuroscience*. 11:131-133.

Minichiello, L. 2009. TrkB signalling pathways in LTP and learning. *Nature reviews. Neuroscience*. 10:850-860.

Mowla, S.J., H.F. Farhadi, S. Pareek, J.K. Atwal, S.J. Morris, N.G. Seidah, and R.A. Murphy. 2001. Biosynthesis and post-translational processing of the

precursor to brain-derived neurotrophic factor. *The Journal of biological chemistry*. 276:12660-12666.

Mowla, S.J., S. Pareek, H.F. Farhadi, K. Petrecca, J.P. Fawcett, N.G. Seidah, S.J. Morris, W.S. Sossin, and R.A. Murphy. 1999. Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 19:2069-2080.

Pang, P.T., H.K. Teng, E. Zaitsev, N.T. Woo, K. Sakata, S. Zhen, K.K. Teng, W.-H. Yung, B.L. Hempstead, and B. Lu. 2004. Cleavage of proBDNF by tPA/Plasmin Is Essential for Long-Term Hippocampal Plasticity. *Science*. 306:487-491.

Peinado, J.R., H. Li, K. Johanning, and I. Lindberg. 2003. Cleavage of recombinant proenkephalin and blockade mutants by prohormone convertases 1 and 2: an in vitro specificity study. *Journal of neurochemistry*. 87:868-878.

Pencea, V., K.D. Bingaman, S.J. Wiegand, and M.B. Luskin. 2001. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 21:6706-6717.

Petersson, S., A.S. Persson, J.E. Johansen, M. Ingvar, J. Nilsson, G. Klement, P. Arhem, M. Schalling, and C. Lavebratt. 2003. Truncation of the Shaker-like voltage-gated potassium channel, Kv1.1, causes megencephaly. *The European journal of neuroscience*. 18:3231-3240.

Purves, D., W.D. Snider, and J.T. Voyvodic. 1988. Trophic regulation of nerve cell morphology and innervation in the autonomic nervous system. *Nature*. 336:123-128.

Rauskolb, S., M. Zagrebelsky, A. Dreznjak, R. Deogracias, T. Matsumoto, S. Wiese, B. Erne, M. Sendtner, N. Schaeren-Wiemers, M. Korte, and Y.A. Barde. 2010. Global deprivation of brain-derived neurotrophic factor in the CNS reveals an area-specific requirement for dendritic growth. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 30:1739-1749.

Richter, K., K. Langnaese, M.R. Kreutz, G. Olias, R. Zhai, H. Scheich, C.C. Garner, and E.D. Gundelfinger. 1999. Presynaptic cytomatrix protein bassoon is localized at both excitatory and inhibitory synapses of rat brain. *The Journal of comparative neurology*. 408:437-448.

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

Rivera, C., H. Li, J. Thomas-Crusells, H. Lahtinen, T. Viitanen, A. Nanobashvili, Z. Kokaia, M.S. Airaksinen, J. Voipio, K. Kaila, and M. Saarma. 2002. BDNF-induced TrkB activation down-regulates the K+-Cl- cotransporter KCC2 and impairs neuronal Cl- extrusion. *The Journal of cell biology*. 159:747-752.

Rivera, C., J. Voipio, J. Thomas-Crusells, H. Li, Z. Emri, S. Sipila, J.A. Payne, L. Minichiello, M. Saarma, and K. Kaila. 2004. Mechanism of activity-dependent downregulation of the neuron-specific K-Cl cotransporter

KCC2. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 24:4683-4691.

Rizo, J., and T.C. Sudhof. 2002. Snares and Munc18 in synaptic vesicle fusion. *Nature reviews. Neuroscience*. 3:641-653.

Rodriguez-Tebar, A., G. Dechant, and Y.A. Barde. 1990. Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron*. 4:487-492.

Roux, P.P., M.A. Colicos, P.A. Barker, and T.E. Kennedy. 1999. p75 neurotrophin receptor expression is induced in apoptotic neurons after seizure. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 19:6887-6896.

Sandler, V.M., and J.G. Barbara. 1999. Calcium-induced calcium release contributes to action potential-evoked calcium transients in hippocampal CA1 pyramidal neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 19:4325-4336.

Scharfman, H., J. Goodman, A. Macleod, S. Phani, C. Antonelli, and S. Croll. 2005. Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats. *Experimental neurology*. 192:348-356.

Smith, A.G., J. Nichols, M. Robertson, and P.D. Rathjen. 1992. Differentiation inhibiting activity (DIA/LIF) and mouse development. *Developmental biology*. 151:339-351.

Sudhof, T.C. 2012. The presynaptic active zone. *Neuron*. 75:11-25.

Sweatt, J.D. 1999. Toward a molecular explanation for long-term potentiation. *Learning & memory*. 6:399-416.

Tao, X., A.E. West, W.G. Chen, G. Corfas, and M.E. Greenberg. 2002. A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. *Neuron*. 33:383-395.

Teng, H.K., K.K. Teng, R. Lee, S. Wright, S. Tevar, R.D. Almeida, P. Kermani, R. Torkin, Z.-Y. Chen, F.S. Lee, R.T. Kraemer, A. Nykjaer, and B.L. Hempstead. 2005. ProBDNF Induces Neuronal Apoptosis via Activation of a Receptor Complex of p75NTR and Sortilin. *The Journal of Neuroscience*. 25:5455-5463.

Trimmer, J.S., and K.J. Rhodes. 2004. Localization of voltage-gated ion channels in mammalian brain. *Annual review of physiology*. 66:477-519.

Ventriglia, M., L. Bocchio Chiavetto, L. Benussi, G. Binetti, O. Zanetti, M.A. Riva, and M. Gennarelli. 2002. Association between the BDNF 196 A/G polymorphism and sporadic Alzheimer's disease. *Molecular psychiatry*. 7:136-137.

Vinberg, M., J.D. Bukh, B. Bennike, and L.V. Kessing. 2013. Are variations in whole blood BDNF level associated with the BDNF Val66Met polymorphism in patients with first episode depression? *Psychiatry research*.

Voets, T., R.F. Toonen, E.C. Brian, H. de Wit, T. Moser, J. Rettig, T.C. Sudhof, E. Neher, and M. Verhage. 2001. Munc18-1 promotes large dense-core vesicle docking. *Neuron*. 31:581-591.

Wang, X., M. Kibschull, M.M. Laue, B. Lichte, E. Petrasch-Parwez, and M.W. Kilimann. 1999. Aczonin, a 550-kD putative scaffolding protein of presynaptic active zones, shares homology regions with Rim and Bassoon and binds profilin. *The Journal of cell biology*. 147:151-162.

Weiss, N., S. Hameed, J.M. Fernandez-Fernandez, K. Fablet, M. Karmazinova, C. Poillot, J. Proft, L. Chen, I. Bidaud, A. Monteil, S. Huc-Brandt, L. Lacinova, P. Lory, G.W. Zamponi, and M. De Waard. 2012a. A Ca(v)3.2/syntaxin-1A signaling complex controls T-type channel activity and low-threshold exocytosis. *The Journal of biological chemistry*. 287:2810-2818.

Weiss, N., G.W. Zamponi, and M. De Waard. 2012b. How do T-type calcium channels control low-threshold exocytosis? *Communicative & integrative biology*. 5:377-380.

Williams, R.L., D.J. Hilton, S. Pease, T.A. Willson, C.L. Stewart, D.P. Gearing, E.F. Wagner, D. Metcalf, N.A. Nicola, and N.M. Gough. 1988. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature*. 336:684-687.

Woo, N.H., H.K. Teng, C.J. Siao, C. Chiaruttini, P.T. Pang, T.A. Milner, B.L. Hempstead, and B. Lu. 2005. Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. *Nature neuroscience*. 8:1069-1077.

Xu, B., E.H. Goulding, K. Zang, D. Cepoi, R.D. Cone, K.R. Jones, L.H. Tecott, and L.F. Reichardt. 2003. Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. *Nature neuroscience*. 6:736-742.

Xu, S., J.C. Han, A. Morales, C.M. Menzie, K. Williams, and Y.S. Fan. 2008. Characterization of 11p14-p12 deletion in WAGR syndrome by array CGH for identifying genes contributing to mental retardation and autism. *Cytogenetic and genome research*. 122:181-187.

Yeo, G.S., C.C. Connie Hung, J. Rochford, J. Keogh, J. Gray, S. Sivaramakrishnan, S. O'Rahilly, and I.S. Farooqi. 2004. A de novo mutation affecting human TrkB associated with severe obesity and developmental delay. *Nature neuroscience*. 7:1187-1189.

Ying, Q.L., J. Wray, J. Nichols, L. Batlle-Morera, B. Doble, J. Woodgett, P. Cohen, and A. Smith. 2008. The ground state of embryonic stem cell self-renewal. *Nature*. 453:519-523.

Yuan, H., N. Corbi, C. Basilico, and L. Dailey. 1995. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. *Genes & development*. 9:2635-2645.

Zafra, F., B. Hengerer, J. Leibrock, H. Thoenen, and D. Lindholm. 1990. Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. *The EMBO journal*. 9:3545-3550.

Ziv, N.E., and C.C. Garner. 2004. Cellular and molecular mechanisms of presynaptic assembly. *Nature reviews. Neuroscience*. 5:385-399.