Università degli Studi di Ferrara

LOCAL SUPPLEMENTATION OF BRAIN-DERIVED NEUROTROPHIC FACTOR FOR THE TREATMENT OF NEURONAL DAMAGE

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Local supplementation of brain-derived neurotrophic factor for the treatment of neuronal damage

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Abstract

The importance of nerve growth factors, especially brain-derived neurotrophic factor (BDNF) in the regulation of neuronal survival and plastic changes in morphology and function has been increasingly studied during the recent years. It has been proposed that the pathogenesis of some neurological diseases may be due to an alteration in neurotrophic factor and/or Trk receptor levels. The use of neurotrophic factors as therapeutic agents is a promising approach aimed at restoring and maintaining neuronal function in the central nervous system (CNS). This study is undertaken to develop a novel stem cell-based gene therapy to deliver neurotrophic factors to vulnerable regions of the CNS. Stem cell-based gene therapy is a potential delivery option by which cells are engineered to produce neurotrophic factors in vitro and then transplanted to the target area where neurotrophic factors are secreted to exert protective and/or restorative effects on the host tissue. A recently isolated mesodermal stem cell, mesoangioblast (MAB), has a high adhesin-dependent migratory capacity and may selectively cross the blood-brain barrier and home in the lesioned areas. Therefore, MABs provide an ideal cellular source for BDNF delivery. In this study, we generated a genetically modified mesoangioblast producing BDNF (MABs-BDNF). These engineered MABs maintained transgene expression and secretion of bioactive BDNF in time. We investigated the protective effects of MABs-BDNF in vitro using primary cultures and organotypic cultures of hippocampal slices. The viability of the cultured slices was assessed in several ways: fluorescein diacetate (FDA) hydrolysis assay, lactate dehydrogenase (LDH) release assay, immunohistochemistry for MAP2, immunoblot for neurofilament 68, and field potential recordings. Direct exposure of recombinant BDNF to primary cultured neurons and adult slices resulted in a concentration-dependant protective effect. The conditioned medium from MABs-BDNF highly promoted cell survival, while the conditioned medium from control cells (MABs) or an equivalent amount of rBDNF showed beneficial effects on cell survival to a lesser extent. The protective effects of MABs-BDNF were attenuated by adding either with the TrkB receptor blocker K252a or the BDNF scavenger TrkB-IgG.. This indicates that the conditioned medium from MABs-BDNF can foster the adult slice culture through secreting the engineered BDNF and unknown pro-survival factors produced intrinsically by MABs. The MABs-BDNF conditioned medium was optimal for retention of morphologic characteristics and viability in organotypic cultures from adult hippocampal slices. Moreover, MABs-BDNF were found to promote

neurogenesis and glia proliferation. Treatment with the MABs-BDNF conditioned medium was found to increase the number of BrdU-labeled and BrdU/NeuN double labeled cells in the dentate gyrus of cultured slices. These in vitro findings demonstrate the beneficial effects of MABs-BDNF on neurons and provide a rationale for transplanting MABs-BDNF in the damaged brain as a therapeutic approach. Thus, we tested the transplantation of MABs-BDNF in an animal model of neuronal loss, the hippocampal sclerosis induced by status epilepticus. So far, we have not detected a deposition of MABs-BDNF in the epileptic brain after their systemic administration. Future experiments will aim at optimizing the transplanting conditions, changing the delivering routes, and assessing their therapeutic value in other neurological diseases associated with cell death. In terms of their prominent beneficial by-stander effects on neurons, MABs-BDNF hold substantial promise as therapeutic agents in the treatment of neurological diseases.

Keywords: BDNF, mesoangioblasts, gene therapy, delivery, brain damage

Rilascio localizzato di *brain-derived neurotrophic factor* per il trattamento del danno neuronale

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Abstract

L'importanza dei fattori neurotrofici, in particolare del *brain-derived neurotrophic factor* (BDNF), nel controllo della sopravvivenza neuronale e nelle modificazioni plastiche morfo-funzionali del sistema nervoso è diventata sempre più rilevante negli ultimi anni. Si è infatti ipotizzato che un'alterazione di questo fattore neurotrofico e/o dei livelli del suo recettore Trk possano rappresentare tappe patogenetiche importanti nello sviluppo di alcune patologie neurologiche. Da ciò deriva che l'utilizzo dei fattori neurotrofici come agenti terapeutici sia un approccio farmacologico promettente per il recupero ed il mantenimento delle funzioni neurali del sistema nervoso centrale (SNC). Scopo dello studio intrapreso è stato quello di cercare di sviluppare una nuova terapia genica basata sulle cellule staminali per ottenere il rilascio di fattori neurotrofici in aree vulnerabili del SNC. La terapia genica basata sull'impiego di cellule staminali è una delle possibili tecniche di *bio-delivery* per mezzo della quale le cellule, ingegnerizzate per produrre fattori neurotrofici in vitro, vengono trapiantate nell'area bersaglio dove rilasciano i fattori neurotrofici con effetti protettivi e/o rigenerativi sul tessuto ospite. Il mesoangioblasto (MAB) è una cellula staminale, recentemente isolata, che possiede un'alta capacità migratoria adesina-dipendente, può attraversare selettivamente la barriera emato-encefalica e raggiungere l'area neurale lesionata. I MAB, perciò, offrono una sorgente cellulare ideale per il rilascio di BDNF. Nella nostra ricerca sono stati generati mesoangioblasti geneticamente modificati per la produzione di BDNF (MABs-BDNF). I mesoangioblasti in questione mantengono nel tempo l'espressione del transgene e le proprietà secretorie di BDNF biologicamente attivo. Successivamente abbiamo studiato i loro effetti neuroprotettivi in vitro, su colture primarie e su colture organotipiche ippocampali. Si è valutata la vitalità delle fettine tissutali ippocampali mantenute in coltura mediante diverse metodiche: test di idrolisi della Fluoresceina DiAcetato (FDA), saggio di rilascio della LattatoDeidrogenasi (LDH), immunoistochimica per MAP2 (*Microtubule Associated Protein 2*, proteina associata ai microtubuli di tipo 2), immunoblot per il neurofilamento 68, registrazione dei potenziali di campo. Si è visto che l'esposizione diretta delle colture primarie neuronali e delle colture d'ippocampo adulto al BDNF ricombinante (rBDNF) produce effetti protettivi dose-dipendente. Il medium condizionato dai MABs-BDNF promuove in maniera rilevante la sopravvivenza cellulare, mentre sono assai minori gli effetti benefici sulla sopravvivenza cellulare indotti dal medium condizionato ottenuto dalle cellule di controllo (MABs) o da un equivalente quantità di rBDNF. Gli effetti protettivi

dei MABs-BDNF vengono attenuati dall'aggiunta sia di K252a, bloccante recettoriale di TrkB, sia da TrkB-IgG, sottrattore anticorpale di BDNF. Questo sta ad indicare che il medium condizionato dai MABs-BDNF può proteggere le colture organotipiche di adulto non solo mediante la liberazione di BDNF ma anche tramite il rilascio di non ancora noti fattori neuroprotettivi prodotti intrinsecamente dai MABs. Il medium condizionato MABs-BDNF è risultato ottimale per il mantenimento delle caratteristiche morfologiche e di vitalità delle colture organotipiche ippocampali di adulto. I MABs-BDNF, inoltre, sono risultati promuovere la neuronogenesi e la proliferazione gliale. Il medium condizionato dai MABs-BDNF ha incrementato, infatti, il numero di cellule marcate con la BromoDeossiUridina (BrdU) e delle cellule doppiamente marcate, BrdU/NeuN (BromoDeossiUridina/*Neuronal Nuclear protein*, proteina nucleare neuronale) nel giro dentato delle fettine in coltura. Queste scoperte ottenute in vitro dimostrano gli effetti positivi dei MABs-BDNF sui neuroni e forniscono un razionale per l'utilizzo terapeutico del trapianto di MABs-BDNF nelle aree cerebrali danneggiate. In modo preliminare abbiamo testato il trapianto di MABs-BDNF in un modello animale di perdita neuronale, la sclerosi ippocampale indotta dallo stato epilettico, non avendo purtroppo riscontrato una localizzazione di MABs-BDNF nel cervello epilettico, in seguito alla loro somministrazione sistemica. Successivi esperimenti saranno necessari per ottimizzare le condizioni del trapianto, modificando le vie di *delivery* e valutando il loro valore terapeutico in altre patologie neurologiche associate alla morte cellulare. I MABs-BDNF, per il loro preminente effetto benefico, seppur indiretto, a tipo "spettatore innocente" sui neuroni, risultano promettenti agenti terapeutici per il trattamento delle malattie neurologiche.

Parole chiave: BDNF, mesoangioblasti, terapia genica, *delivery*, danno cerebrale

Chapter I

BACKGROUND

Preface

Back in the early 1950s, a group of embryologists discovered that a series of signalling molecules secreted by the target tissues in the developing nervous system, which were called neurotrophic factors (NTFs). NTFs were defined as target-derived, antiapoptotic molecules that maintain embryonic or adult neuronal cells. The word 'trophic' is derived from Greek 'trophé' meaning nourishment or taking up of nutrients. Various families of neurotrophic factors exist, e.g., the neurotrophin (NT) family, the fibroblast growth factor (FGF) family, the epidermal growth factor (EGF) family, the insulin-like growth factor (IGF) family, the vascular-endothilial growth factor (VEGF) faimily, the glial cell-derived neurotrophic factor (GDNF) family, and the ciliary neurotrophic factor (CNTF). Currently, six neurotrophins have been isolated: NGF, BDNF, NT-3, NT-4 (also known as NT-5), NT-6, and NT-7. The NT-6 and NT-7 genes have been identified only in fish and probably do not have mammalian or avian orthologues (Gotz et al., 1994; Nilsson et al., 1998). The FGF family includes 22 known members in human, which initially were found to influence the growth of fibroblasts and later their ability in many other cell types were demonstrated (Burgess and Maciag, 1989). For example, basic FGF (bFGF, also called FGF-2, EDGF, or HBGF-2) can influence the differentiation and migration of neurons and glia (Walicke et al., 1986). All these NTFs signal via factor specific multicomponent receptor complexes. The NGF-superfamily of receptors includes p75 and the receptor protein tyrosine kinases (Trk): TrkA, TrkB and TrkC. The mammalian FGF receptor family has 4 members, FGFR1, FGFR2, FGFR3, and FGFR4. The GDNF family of receptors includes a receptor complex of Ret and growth factor receptor α1-4. The neurokine superfamily ligands act via the receptors gp130 and leukemia inhibitory factor receptor-β (LIFR-β).

Since their discovery, research in the field of NTFs has provided a vast amount of data not only on their biochemical properties but also on how they function during development and how they maintain neuronal activity in the adult nervous system. It has been evidenced that NTFs regulate almost all aspects of neuronal development and function, including precursor proliferation and commitment, cell survival, axon and dendrite growth, membrane trafficking, synapse formation and function, neurotransmitter release, synaptic plasticity, as well as glia differentiation and interaction with neurons. It is well accepted that NTFs are critical to the development and maintenance of the mammalian CNS. Alterations in NTF levels have profound effects on a wide variety of phenomena, including myelination, regeneration, pain, aggression, depression and substance abuse. The influence of neurotrophic factors spans from developmental neurobiology to neurodegenerative and psychiatric disorders. Recently, NTFs have received considerable interest as possible means for the treatment of neurodegenerative diseases, psychiatric disorders and CNS trauma. There are many successful paradigms with respect to pre-clinical and clinical applications of NTFs, especially neurotrophin family member (e.g. NGF, BDNF) to prevent, slow the progression of, or even reverse the effects of a number of neurodegenerative diseases and other types of insults in both the central and peripheral nervous system, since the idea that degenerative diseases of the nervous system may result from insufficient supply of these neurotrophins has generated great interest in these NTFs as potential therapeutic agents.

Besides the neurotrophin family, many other NTFs show significant therapeutic potential in a variety of diseases states, from neurological diseases, Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), stroke, schizophrenia, and depression to diabetic neuropathies, neuropathic pain, macular degeneration, and obesity. These factors include members of the fibroblast growth factor (FGF) family, GDNF family, CNTF family, and insulin-like growth factors I and II (IGF-I and -II). Here, we will focus primarily on the neurotrophin family and their cognate receptors.

In this chapter, we will summarize the knowledge acquired on the gene and protein structure of neurotrophins and their molecular and cellular biology during the last decade. Because of the importance of BDNF on neuronal survival and regeneration, we will review particularly the distribution and alteration of BDNF and its therapeutic potential in the neurological diseases. In addition, the various therapeutic strategies against neurodegenerative diseases are introduced. Among these strategies, a novel cell source (mesoangioblast) for cell based therapy attracted our interest.

1 Neurotrophins

The neurotrophins are a family of structurally related secretory proteins that are widely expressed in neurons and their target cells (Alderson et al., 1990). These secreted proteins play a crucial role in the control of neuronal numbers and of dendritic growth. To date five members have been identified. They are called nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin (NT) 3, 4/5, and 6. The first neurotrophin identified was the NGF (Levi-Montalcini, 1966). It was found, however, that only a few CNS neurons were responsive to NGF. The second neurotrophin, BDNF, initially purified from the pig brain as a trophic factor for dorsal root ganglion cells, helped to establish the concept that the diffusible growth factors could regulate the fate and the shape of most vertebrate neurons (Hofer and Barde, 1988). Molecular cloning of BDNF revealed that its amino acid sequence had a strong homology to that of NGF. By using the contiguous regions between NGF and BDNF to design oligonucleotides for polymerase chain reaction cloning, a third related protein called neurotrophin 3 (NT-3) was cloned (Maisonpierre et al., 1991; Hallbook et al., 1993; Dawson et al., 1995). This is located on human chromosome 12 (12p13) (Maisonpierre et al., 1991). Finally, the last member of the neurotrophin family (NT-4) was identified and cloned in Xenopus (Hallbook et al., 1991). The equivalent human cDNA is different from Xenopus NT-4 and is thought to be a separate gene and called NT-5(Berkemeier et al., 1991; Berkemeier et al., 1992). Subsequently it was realized that they are homologueous genes and this neurotrophin is often termed NT-4/5. NT-4/5 is present on human chromosome 19 (19q13.3) (Berkemeier et al., 1992; Ip et al., 1992). NT-6 has only been found in fish (Gotz et al., 1994).

To better exploit the NTFs therapeutic potential, we need to understand the details of the molecular biology of the neurotrophin system and integrate them with the physiology of neurotrophins. In the following sections, we review their gene and protein structure, biosynthesis and secretion, receptors, signaling pathways, and regulation of neuronal activity. The data on the molecular mechanisms of gene regulation from other fields of biomedical science, together with the physiological data on neurotrophic factor action and known intracellular signal transduction pathways help us to get insight into the function and modulation of these NTFs, thereby contributing to the exploitation of potential therapies for neurological disorders.

1.1 Gene and protein structure

The recent explosion in genomics research has given us dozens of amino acid sequences of NTFs from dozens of species. In addition, the crystal structures of many NTFs are now available after a decade of intense research. Comparing the amino acid sequences and structural features of the NTFs can give insight into the evolutionary history of these families and can help reveal functionally important regions of the molecules.

Sequence alignments

All genes encoding neurotrophins have a basically similar structure. A single discontinuous exon contains all the information for encoding the prepropeptide. The coding exon is preceded by several noncoding exons that are subject to alternative splicing and give rise to alternative 5'- untranslated regions (UTRs) of mRNA. Multiple promoters have been demonstrated for all neurotrophins.

The BDNF gene (*bdnf*), mapped to chromosome 11p in humans, has four upstream exons (exons I-IV) that are associated with distinct promoters, and one 3' exon (exon V) that encodes the mature BDNF protein (Metsis et al., 1993; Timmusk et al., 1993). Recently, some novel exons had been identified in the upstream noncoding exons (Aid et al., 2007). According to the new nomenclature, BDNF transcripts contain 9 exons (I-IXA) in the upstream unstranslated region, while the common coding region previously called exon V is now named as exon IX. The usage of different promoters gives rise to mRNAs that contain only one of the upstream noncoding exons spliced to the coding exon. Upstream exons of the BDNF gene do not contain a splicing acceptor site thus preventing the splicing of the more upstream exons to those located proximally to the coding exon. As many as 11 different BDNF transcripts can be generated in both humans and rodents by alternative splicing. They differ only in their upstreanm untranslated region but share a common exon IX (Aid et al., 2007). All these BDNF mRNAs are expressed in the brain at very different levels and generating a spatial diversity of expression. Moreover, different promoters in the BDNF gene have specific developmental patterns of expression and differential activation capacities. For instance, a 5-kb region of promoter Ⅲ is involved in BDNF gene induction after sciatic nerve transaction (Timmusk et al., 1995).

Following the detailed description of BDNF gene structure, many attempts have been made to localize structural elements of the gene governing mRNA regulation in vivo or in vitro. For instance, the first intron of the BDNF gene contains a neuron-restrictive silencer element (NRSE)-type regulatory sequence (Timmusk et al., 1999). Alterations in the NRSE affected expression levels of promoters II and I that are located nearby. In the brain, mutation of the NRSE modulated the responsiveness of BDNF promoters I and II to activation of the glutamatergic system (Timmusk et al., 1999). Short regions flanking promoters I, II, and III in the BDNF gene have been localized as putative calcium responsive regions (Bishop et al., 1997). Two different regulatory sequences involved in the activation of transcription were localized in the proximal promoter region. One is a novel calcium response element required for calcium-dependent BDNF expression in both embryonic and postnatal cortical neurons. The second element matches the consensus sequence of a cAMP response element (CRE) and is required for transactivation of the promoter in postnatal but not in embryonic neurons (Tabuchi et al., 2002). Differential cell-specific activation of different BDNF promoters via activation of the glutamatergic system most likely reflects involvement of specific kinase pathways in the cells (Marmigere et al., 2001).

The human NGF gene is located on the short arm of chromosome 1 (1p22) (Francke et al., 1983) and codes for a polypeptide of 307 amino acids. It contains three upstream exons and the promoter activity has been linked to the first and the third exon. The NGF gene has the most complex splicing pattern. The majorities of transcripts are transcribed from the distal promoter and contain exon I, while the

other noncoding exons are combined by alternative splicing and give rise to a variety of mRNAs with different 5'-UTRs. In addition to *ngf* and *bdnf*, two other neurotrophin genes, neurotrophin-3 (*nt3*) and neurotrophin-4/5 (*nt4/5*), have been identified in mammals (Sekimoto et al., 1998). The NT-3 gene contains two upstream exons flanked by promoters.

Molecular evolution

The evolution of the NTFs has been studied using phylogenetic trees that organize the relationships between their amino acid sequences (Kullander et al., 1997). The works have divided neurotrophin residues into two categories: conserved and variable, based on early sequence alignments (Thoenen, 1991). With the exception of NT4/5, neurotrophin sequences are highly conserved. For instance, BDNF shares about 50% amino acid identity with NGF, NT-3. Based on sequence comparisons and on the isolation of neurotrophin genes in various vertebrates, it is thought that *ngf* / *nt3* and *bdnf* / *nt4/5* evolved from separate duplication events (Hallbook, 1999). The most primitive neurotrophin genes have been isolated from jawless fishes, a river lamprey and the Atlantic hagfish. In bony fishes, more neurotrophin and receptor genes have been isolated than in mammals (Hallbook, 1999).The neurotrophin receptors of the trk family seem to have coevolved with the neurotrophin genes (Hallbook, 1999). So far, no neurotrophin-like sequences have been detected in invertebrates (Bargmann, 1998). No ortholog of the NT-4 gene has been found in teleost fishes, and no ortholog of the NT-6 or NT-7 genes has been found in tetrapods (although the names are similar, the human NT-6 genes are closer to NGF).

Proteins structure

All the neurotrophins are synthesized as precursor proteins a little over 200 amino acids long (approximately 30 kDa in size), containing a signal sequence for secretion. Following proteolytic cleavage, a mature C-terminal active peptide slightly shorter than 120 amino acids long (approximately 13 kDa) is released containing six cysteine residues at identically spaced positions in all mammalian neurotrophins (Chao and Bothwell, 2002). The mature part is very well conserved and approximately 50% of the amino acids are common to all neurotrophins. The neurotrophins are secreted as noncovalent-linked dimers, containing a signal peptide following the initiation codon and a pro-region containing an N-linked glycosylation site. They are all basic proteins with isoelectric points above 9.0, a somewhat unusual property for secreted proteins, which may serve the purpose of limiting their range of action (Chao and Bothwell, 2002).

Three-dimensional structures of most of the human neurotrophins have been determined. These structures can be aligned to reveal that the regions of similarity are much larger than was suggested from sequence alignments. Most of the residues that participate in secondary structure are strongly conserved both within and between sub-families, but there are numerous exceptions.

The core structure consists of two pairs of intertwined two-strand beta sheets, joined by three disulfide bonds. There are also three shorter beta strands leading to beta turns and loops (**Fig. 1**). The four core beta strands are virtually

superimposable across all the structures. The characteristic formation of a double loop formed by two disulphide bonds, penetrated by a third disulphide bond, is named cysteine 'knot' (McDonald and Hendrickson, 1993). The neurotrophins and GDNF family members are members of a large superfamily of growth factors that contain a cysteine knot motif (Holland et al., 1994; Saarma and Sariola, 1999). This reveals strong conservation, especially within secondary-structure elements. This family includes transforming growth factor (TGF)- β , human chorionic gonadotropin, platelet-derived growth factor, vascular endothelial growth factor, and many others (McDonald and Hendrickson, 1993). The cysteine knot consists of three cysteine disulphide bonds. The crystal structures of the neurotrophins show the classic cysteine knot growth factor structure with head-to-head sub-units forming a noncovalently linked dimer. These residues implicated in binding that are conserved across families are thought to represent a common interface to the Trk receptors, while the unique ones may represent elements of specificity (Robinson et al., 1995).

The only significant differences between neurotrophins are found in the loops and turns between beta strands (McDonald and Hendrickson, 1993), especially loop 3. Loop 3 is the most different among the four neurotrophin structures. NT-3 has a single loose helical turn in this region. NT-4 and NT-6 sub-families have a large insertion in this portion, including a small eight-residue beta turn. Interestingly, two cysteines that make up the knot are missing from the human NT-6 molecules (Lai et al., 1998). The N- and C-termini are highly variable in both sequence and structure among the neurotrophins, and the temperature factors of these loosely structured residues are comparatively high. The N-terminus assumes a helical structure upon binding the Trk receptor. The role of the C-terminal residues is not known, although they may participate in p75 binding in NT-3 (Carraway and Mitra, 1987) and NGF (Bax et al., 1997).

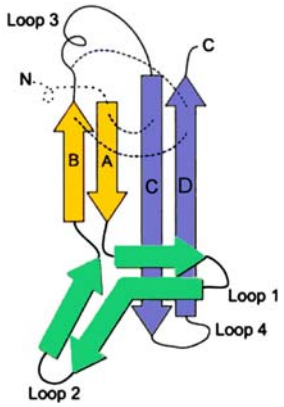

Fig. 1. Schematic of the neurotrophin molecule. Dashed blue lines represent the three disulfide bonds of the cysteine knot. The N terminus is disordered in the unbound structures and is shown by a dashed line. *(Butte et al., Cell. Mol. Life Sci. 58 (2001) 1003–1013)*

By comparing the residues involved in the interface between TrkA-d5 and NGF with the corresponding sequence alignments for the neurotrophins and the Trk receptors, they can be seen to form two groups of conserved and non-conserved residues (Wiesmann et al., 1999). One set of residues making up a large patch and centered on Arg 103 form a common set across all the neurotrophins and receptors. As these residues are highly homologous and consist mainly of hydrophobic and aromatic side chains, they probably mediate the bulk of common binding affinity between neurotrophin and receptor. The other portion of the TrkA interface centers

on the N-terminal residues of the neurotrophin. These residues show little conservation, either on the neurotrophin or the receptor side (Hallbook et al., 1991). The N-terminus, which was disordered in all the unbound neurotrophin structures, forms a one-and-a-half-turn helical arrangement in the complex between TrkA and NGF (Wiesmann et al., 1999). NGF binding buries two helical hydrophobic residues and creates a salt bridge across the interface. BNDF, NT-3, and NT-4 do not share the same pattern of residue types at the N-terminus, and TrkB and TrkC also differ in their corresponding interacting residues (Robinson et al., 1995). Together, these results suggest that the N-terminal residues help determine receptor binding specificity and that each neurotrophin probably uses a different specific interface with its cognate receptor in this region (Kullander et al., 1997). Other specificity-determining residues appear to lie scattered within the common binding site.

The neurotrophins are initially synthesized as precursors or pro-neurotrophins, which are cleaved to produce the mature proteins. Pro-neurotrophins are cleaved intracellularly by FURIN or pro-convertases at a highly conserved dibasic amino-acid cleavage site to release carboxy-terminal mature proteins (Mouri et al., 2007). The mature proteins exist exclusively as dimers, though a variety of arrangements are seen, including heterodimers and homodimers, covalent and non-covalent association of the promoters, and different spatial configurations (head-to-toe, head-to-head, and skew). They are normally expressed at very low levels during development. The amino-terminal half of the pro-neurotrophin is believed to be important for the proper folding and intracellular sorting of neurotrophins (Mouri et al., 2007; Nomoto et al., 2007). It is worthy to note that the discovery that pro-neurotrophins are biologically active has revolutionized the field of neurotrophin research, necessitating the re-evaluation of much published data (Nomoto et al., 2007). Biological activity has also been attributed to two other peptides produced as a result of proNGF cleavage, LIP1 and LIP2 (Dicou et al., 1997), which have been shown to protect against excitotoxin-induced cell death (Dicou, 2006).

1.2 Neurotrophin receptors: identification and structure

Neurotrophins bind to two different classes of transmembrane receptor proteins: the Trk (tropomyosin receptor kinase) family of RTKs (receptor tyrosine kinases) and $p75^{NTR}$ (p75 neurotrophin receptor). This dual system allows the transduction of very different signals following ligand binding, which can be as contrasting as signaling cell death through $p75^{NTR}$ or cell survival through the Trk receptors. These two classes of receptors also directly interact, allowing fine tuning and cross talk (Chao and Hempstead, 1995). Different neurotrophins show binding specificity for particular receptors: TrkA preferentially binds NGF; TrkB preferentially binds BDNF and NT4, while TrkC displays preference for NT3. These interactions have generally been considered to be of high affinity. However, in reality, the binding of NGF to TrkA, and of BDNF to TrkB is of low affinity (Kaplan and Miller, 1997), but it can be regulated by receptor dimerization, structural modifications or association with the p75 receptor (Heumann, 1994). The $p75^{NTR}$ binds all mature neurotrophins with approximately equal low affinity and

has, in recent years, been demonstrated to bind the proneurotrophins with high affinity (Massa et al., 2006). The ability of Trk and p75 receptors to present different binding sites and affinities to particular neurotrophins determines both their responsiveness and specificity. The ratio of receptors is important in dictating the number of surviving cells, and interactions between p75 and Trk receptors provide greater discrimination between different neurotrophins (Chao and Hempstead, 1995). Upon ligand binding, Trk receptors dimerize and become catalytically active, resulting in receptor autophosphorylation (Jing et al., 1992) and subsequent activation of a number of signalling cascades, including the Ras/ Raf/MAPK (mitogen-activated protein kinase) (Thomas et al., 1992), PI_3K (phosphoinositide 3-kinase) (Soltoff et al., 1992), and phospholipase C-γ 1 pathways (Widmer et al., 1993). Independent of Trk, the binding of neurotrophins to the $p75^{NTR}$ receptor results in activation of NF-κb, a transcription factor (Carter et al., 1996), and c-Jun N-terminal kinase (Harrington et al., 2002).

P75 ^{NTR} receptor

The receptors for NGF were first identified on chick sensory ganglia and dorsal root ganglia (DRG) using receptor binding techniques (Sutter et al., 1979). For many years this was believed to be a low-affinity receptor specific for NGF. It was eventually established that $P75^{NTR}$ binds to all of the neurotrophins with a very similar affinity (Rodriguez-Tebar et al., 1991). The gene is present on the human chromosome 17 (17q21－22) (Huebner et al., 1986). $P75^{NTR}$ is a transmembrane glycoprotein receptor of ~75 kD. There are four cysteine repeats (CR1-CR4) in the extracellular domain. It contains a 28 amino acid signal peptide, a single transmembrane domain and a 155 amino acid cytoplasmic domain. The cytoplasmic domain of this receptor contains a "death" domain, known to be involved in apoptosis (Liepinsh et al., 1997). Signaling of P75 occurs through cytoplasmic interactors. $P75^{NTR}$ is a distant member of the tumor necrosis factor receptor family (Chao, 1994; von Bartheld et al., 1995). The defining motifs of this receptor family are cysteine repeats in the extracellular domain, which form the ligand-binding domain. With the exception of the neurotrophins, all other known ligands of this receptor family are trimeric proteins that lead to the trimerization of the receptor following ligand binding. Neurotrophin binding to $p75^{NTR}$ has been shown to affect cell survival (Barrett and Bartlett, 1994) and axonal outgrowth (Walsh et al., 1999; Bentley and Lee, 2000). Signaling by this receptor is discussed at length below.

Trk receptors

In 1986 a human oncogene was isolated from colon carcinoma and was called trk (tyrosine receptor kinase) (Martin-Zanca et al., 1986). The protooncogene TrkA was first identified as an NGF receptor (Kaplan et al., 1991; Klein et al., 1991a), followed by TrkB and TrkC through screening of cDNA libraries (Barbacid, 1994). TrkB protein was shown to be a receptor for BDNF (Klein et al., 1991b) and NT-4/5 (Klein et al., 1992) and TrkC was found to be a receptor for NT-3 (Lamballe et al., 1991). These specificities are not absolute, and NT3 is also a ligand for TrkA and TrkB. Members of other neurotrophic factor families have also been shown to

activate Trk. These include GDNF, CNTF and other neuropoietic cytokines (Neet and Campenot, 2001). These Trk activate many of the same intracellular signaling pathways regulated by the receptors for mitogens. The protein Trk domains are highly homologueous (~80% amino acid identity), whereas the extracellular domains are more divergent (~30%). The TrkA gene is located on chromosome 1 (1q21 – 22) near *ngf*; TrkB is on chromsome 9 (9p22.1) and TrkC is on chromosome 15 (15q25). The Trk receptors are transmembrane glycoproteins of ~ 140 kD, comprising about 800 amino acids with half of the residues at the amino terminus forming the extracellular portion of the receptor. Examination of sequence motifs in the extracellular region of the Trk receptors showed that there are five distinct domains. They are tyrosine kinases with an extracellular ligand-binding domain containing multiple repeats of leucine-rich motifs (LRR1-3), two cysteine clusters (C1, C2), two immunoglobulin-like domains (Ig1, Ig2), and a single transmembrane domain (Schneider and Schweiger, 1991). The most important site at which Trk receptors interact with neurotrophins has been localized to the most proximal immunoglobulin (Ig) domain of each receptor (Urfer et al., 1995; Urfer et al., 1998). The three-dimensional structures of each of these Ig domains has been solved (Ultsch et al., 1999), and the structure of NGF bound to the TrkA membrane proximal Ig domain has also been determined (Wiesmann and de Vos, 1999). This exciting structural information has provided detailed information about interactions that regulate the strength and specificity of binding between neurotrophins and Trk receptors.

1.3 Signaling pathway

Binding of neurotrophins to the Trk receptors causes signaling events that promote neuron survival, whereas activation of the $p75^{NTR}$ pathway triggers apoptosis and cell death (Kaplan and Miller, 2000). Through Trk receptors, neurotrophins activate Ras, phosphatidyl inositol-3 (PI3)-kinase, phospholipase C-g1 and signalling pathways controlled through these proteins, such as the MAP kinases. Activation of $p75^{NTR}$ results in activation of the nuclear factor-κB (NF-κB) and Jun kinase as well as other signalling pathways. Neurotrophins can bind both Trk and $p75^{NTR}$ and activate the different singalling pathways simultaneously, and the signaling pathways through Trk receptors and $p75^{NTR}$ may interact with each other (Gargano et al., 1997).

Signaling through the $P75^{NTR}$ receptor

The mechanisms of transduction mediating the biological effects of $p75^{NTR}$ in neurons are poorly understood. On the one hand, $p75^{NTR}$ can modulate cellular responses to neurotrophins, by interacting with Trk. Modulation of Trk interaction with neurotrophins has been considered as the main $p75^{NTR}$ mechanisms of action since the discovery of Trk receptors (Barbacid, 1994; Chao, 1994; Chao and Hempstead, 1995). On the other hand, ligand engagement of $p75^{NTR}$ has been shown to promote survival of some cells and apoptosis of others (Barrett and Bartlett, 1994) and affects axonal outgrowth (Yamashita et al., 1999; Bentley and

Lee, 2000) through interacting with the intracellular binding proteins. To date, several proteins had been identified that directly interact with $p75^{NTR}$, such as RhoA, a member of the Ras superfamily of GTP-binding proteins (Yamashita et al., 1999), tumor necrosis factor receptor-associated factor-6 (TRAF6) (Khursigara et al., 1999), and a zinc finger protein named NT receptor interacting factor (NRIF) (Casademunt et al., 1999). RhoA has been shown to control the organisation of the actin cytoskeleton in many cell types (Ridley, 2006). Like other members of the Ras superfamily, RhoA cycles between active, GTP-bound and inactive, GDP-bound states. These proteins are presumptively the mediators of $p75^{NTR}$ -induced changes in NF-κB and c-Jun kinase activities, and activation of the sphingomyelin cycle, which result in the biological effects of $p75^{NTR}$ activation (Chao, 1994). An important pathway promoting cell survival of many cell populations involves activation of NF-κB (Barrett and Bartlett, 1994). As is the case with TNF receptor 1 signaling, it appears that activation of NF-κB prevents cell death (Liu et al., 1996; Hamanoue et al., 1999). Beyond the activation of NF-κB, there are also clear indications that $p75^{NTR}$ activation by neurotrophins causes programmed cell death. Cell death signaling involves caspase activation, as well as Bax/Bad, Bcl-2, and Bcl-xL (Coulson et al., 1999; Soilu-Hanninen et al., 1999), but the details of the pathway linking $p75^{NTR}$ with cell death execution are far from clear. Inhibition of Jun kinase (JNK) activity blocks apoptosis through $p75^{NTR}$, suggesting that JNK plays a significant role in p75-mediated apoptosis (Yoon et al., 1998; Bhakar et al., 2003). It is assumed that the different use of cytoplasmic interactors for signal transduction causes the considerably various consequences of $p75^{NTR}$ activation associated with the cellular context (Barker, 1998).

Signaling through the Trk receptors

Binding of the neurotrophins activates the Trk receptors by a two-step process: ligand-induced receptor dimerization, and autophosphorylation of tyrosine residues in its autoregulatory loop (Schlessinger and Ullrich, 1992). The activated receptors become able to interact and phosphorilate several intracellular targets (Segal and Greenberg, 1996). Among the proteins that can be activated by autophosphorilated Trk receptors are phospholipase C-1g (Obermeier et al., 1994; Stephens et al., 1994); the adapter proteins Shc, rAPS and SH2-B (Obermeier et al., 1994; Stephens et al., 1994; Qian et al., 1998); phosphatidylinositol-3' kinase (PI_3K) (Stephens et al., 1994); Fyn, a protein tyrosine kinase involved in regulation of cell adhesion and synaptic plasticity (Iwasaki et al., 1998); the brain immunoglobulin-like molecule with tyrosine-based activation motifs (BIT), also known as SHPS-1 and SIRPa (Ohnishi et al., 1999); and fibroblast growth factor receptor substrate 2 (FRS2) (Easton et al., 1999). In turn, these activated proteins lead to activation of the Ras/mitogen-activated protein kinase (MAPK) signaling pathway and phosphorylation of extracellular signal-regulated kinases (ERKs) (Kaplan and Stephens, 1994), to an increase in intracellular calcium concentration and subsequent activation of calcium/ calmodulin-dependent kinases and casein kinase 2 (Finkbeiner et al., 1997; Blanquet, 1998), CREB phosphorylation (Finkbeiner et al., 1997), and further activation of phosphatidylinositol-3 ' kinase (Baxter et al., 1995). Three main signaling cascades are activated by the Trk receptors and their substrates. First, the activation of the Ras/Raf/MEK/MAPK pathway results from the formation of a variety of complexes of adapter molecules. Phosphorylated Shc

leads to the activation of the Ras/Raf/MEK/MAPK pathway (Kaplan and Miller, 2000). Phospho-Shc binds to the Grb2 – SOS complex, which activates Ras, and MAP kinase is activated through Raf and MEK. Similarly, phosphorylated FRS2 recruits a complex of the tyrosine phosphatase SHP-2/Grb2/SOS and activates Ras/Raf/MEK/MAPK (Kouhara et al., 1997; Hadari et al., 1998). Also, SNT seems to activate this pathway, which mediates neuritogenesis (Stephens et al., 1994). Other docking molecules seem to form complexes with Grb2/SOS, such as rAPS and SH2-B (Qian et al., 1998). They were identified in developing neurons and may be involved in neuronal differentiation. This complexity of activation allows a sustained activation of the MAPK pathway in response to neurotrophins, ultimately leading to activation of gene expression, neuronal survival and neurite outgrowth (Kaplan and Stephens, 1994).

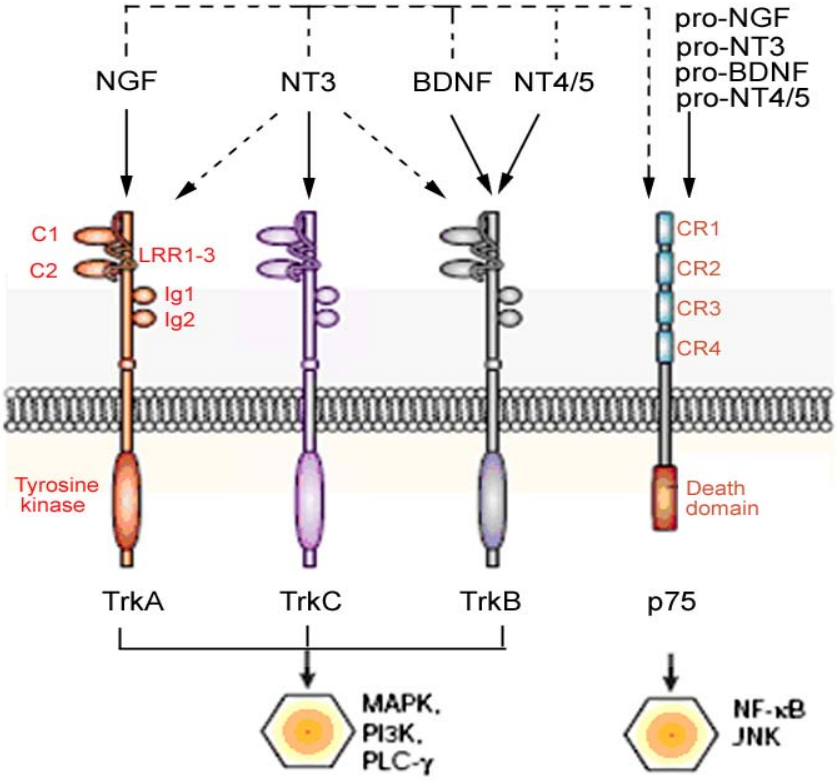

Fig. 2. Multicomponent receptor systems of neurotrophic factors. The neurotrophins family of NGF, BDNF, NT-3 and NT-4 bind to specific Trk receptors. Cross-talk is also apparent in these interactions as more than one neurotrophin may bind to the same Trk receptor, and more than one Trk may bind to the same neurotrophin with less efficiency (e.g. NT3 activate TrkA and TrkB) in some cellular contexts. All members of the neurotrophin family interact with $p75^{NTR}$. Each proneurotrophin also binds $p75^{NTR}$, but not the Trk receptors. Each Trk receptor activates several signal transduction pathways. Neurotrophin binding to the Trk receptors mediates survival, cell migration and myelination through several signalling pathways. The Trk receptor extracellular domain consists of two cysteine rich domains (C1,C2), three leucine-rich motifs (LRR 1-3), and two IgG-like (Ig 1-2) domains; the intracellular domain is a protein tyrosine kinase. The extracellular portion of $p75^{NTR}$ contains four cysteine-rich repeats, and the intracellular part contains one or two death domains. Additional details are provided in the text. *(Modified from Chao, Nature, 2003)*

2 BDNF

As reviewed above, neurotrophins are critical molecules that support the development, differentiation, maintenance and plasticity of brain function throughout life. Among these molecules, BDNF is the most abundantly expressed and widely distributed in the central nervous system (CNS), and has survival promoting actions on a variety of CNS neurons including hippocampal and cortical neurons (Ghosh et al., 1994; Lindholm et al., 1996; Lowenstein and Arsenault, 1996), cholinergic neurons (Alderson et al., 1990; Nonomura and Hatanaka, 1992), and nigral dopaminergic neurons (Hyman et al., 1991; Ostergaard et al., 1996). Moreover, BDNF regulates almost all aspects of neuronal development and function including precursor proliferation and commitment (Davies, 1994), axon and dendrite growth (Davies, 2000), membrane trafficking, synapse formation and function (Li et al., 1998), as well as glial differentiation and interactions with neurons (Lykissas et al., 2007). Recent research suggests that alterations in the levels of BDNF or its main receptor TrkB can lead to neuronal death and contribute to the pathogenesis of many CNS disorders such as Parkinson disease, Alzheimer disease, Huntington disease, amyotrophic lateral sclerosis, and also aging (Dawbarn and Allen, 2003). There is widespread interest in the use of BDNF to treat these CNS disorders. To better exploit its potential application in the therapy of CNS disorders, here we describe in detail BDNF distribution, regulation, secretion and function.

2.1 Distribution and cellular localization

BDNF mRNA and protein levels have been detected in the hippocampus, amygdala, thalamus, projection areas of the olfactory system, inner and outer pyramidal layers of the neocortex, claustrum, septum, cerebellum and the superior colliculus (Murer et al., 2001), indicating that BDNF has a more widespread distribution than NGF. In the rat (Nishio et al., 1994; Yan et al., 1997) and human (Quartu et al., 1999) hippocampus, BDNF mRNA and protein levels have been visualized in both the pyramidal and granule cell layers. BDNF-immunoreactivity has also been observed in the hilar region of the dentate gyrus as well as the pyramidal and apical dendritic processes of the CA3, CA2, CA1 and subiculum regions (Goutan et al., 1998). There are only few brain areas like the striatum that completely lack BDNF mRNA (Yan et al., 1997). BDNF expression is low in developing regions of the CNS and increases as these regions mature (Maisonpierre et al., 1990). TrkB, receptor for BDNF, is also found in neuronal perikarya in neocortex and striatum and in reactive astrocytes (Soontornniyomkij et al., 1998). The presence of both receptor and factor in the neocortical perikarya suggests an autocrine function in those neurons while the presence of factor in neurites of striatum suggests that the factor is not synthesized locally in striatum but is in the process of being taken up by retrograde transport (Canals et al., 2001). It is important to keep in mind that BDNF-IR can result either from targeting of the newly synthesized NT in BDNF producing cells or from BDNF uptake and

redistribution of endocytosed BDNF, which was originally released from neighboring cells.

In the cortex, BDNF-immunostained cell bodies with a pyramidal morphology were present in all regions, including the primary visual cortex and other occipital areas, the motor and somatosensory cortex, the insular cortex, and cortex of the temporal pole (Murer et al., 1999). Non-pyramidal neurons were rarely stained. Pyramidal BDNF-immunoreactive neurons were preferentially located in layers V and III. The distribution is more abundant in the insular and temporal cortices than primary motor and sensory cortices. Scattered pyramidal neurons show intense labeling in the cells of deeper pyramidal layers. BDNF-immunoreactive neurons were also noticeable in the white matter immediately adjacent to the cortex (see (Conner et al., 1997) and(Yan et al., 1997), for a detailed description in the rat brain).

In the hippocampal formation of the adult human brain, a minority of neurons displayed a strong cell body BDNF-immunoreactivity (Goutan et al., 1998). The density of perikaryal staining seemed higher in the granule cell layer of the dentate gyrus than in the Ammon's horn (Nishio et al., 1994). Within the latter, the density of immunoreactive pyramidal cell bodies was lowest in CA1. Intensely labeled axon-like fibers were present in the polymorph layer and hilar region of the dentate gyrus, and in the pyramidal layer of fields CA3 and CA4. In fields CA3 and CA4, the intensity of neuropile staining revealed the location of non-labeled pyramidal neuronal cell bodies. The disposition of BDNF-immunoreactive axon-like fibers in the CA3 and CA4 fields strongly suggested that they were in close contact with the proximal dendrites of pyramidal neurons (Dawson et al., 2001). In conjunction, the anatomical distribution of BDNF in the hippocampal formation supports that a large part of hippocampal BDNF is contained in the mossy fiber system, that is, in the axons of dentate gyrus granule cells which make synaptic contacts on the proximal dendrites of pyramidal neurons in the Ammon's horn after traversing the polymorph layer and hilar region of the dentate gyrus (Wetmore et al., 1994). Moderately intense neuropile staining was also found in the inner molecular layer of the dentate gyrus. The two outer thirds of the molecular layer were almost devoid of labeling, however, suggesting scarce or no BDNF in afferent fibers arriving from the entorhinal cortex (the perforant path). In the molecular layer, labeled neuronal profiles were rarely found (Conner et al., 1997).

With respect to the subcellular localization of endogenous BDNF, (Fawcett et al., 1997) showed BDNF-IR in the vesicular fraction of isolated synaptosomes from rat cortex homogenates, and Western blot analysis revealed colocalization with the synaptic marker synaptotagmin, which is associated with small synaptic vesicles as well as with dense core vesicles (DCV) (Berg et al., 2000). A postsynaptic localization of BDNF at glutamatergic synapses is directly evident in the postsynaptic density of rat cortical synapses (Aoki et al., 2000). Interestingly, Berg et al. (2000) reported about the ultrastructural localization of BDNF in DCV, which had been biochemically isolated by density gradient centrifugation from the axons of the optic nerve, from tissue of the superior colliculus and from the lateral geniculate nuclei of the thalamus, respectively. Taken together, the ultrastructural localization of endogenous BDNF in central neurons is not yet completely resolved, which might in part be due to the low abundance of BDNF protein in central neurons under basal conditions. The available data are consistent with a vesicular

storage in both axons and presynaptic structures as well as in dendrites and postsynaptic structures (Altar and DiStefano, 1998).

Earlier efforts showed that BDNF is retrogradely transported by peripheral and CNS neurons (Altar and DiStefano, 1998; Mufson et al., 1999). Locally applied BDNF can be taken up by axons and retrogradely transported to neuronal cell bodies in the adult mammalian CNS (Mufson et al., 1999). Both $p75^{NTR}$ and TrkB seem to be able to mediate retrograde transport of BDNF (Curtis et al., 1995; Pease et al., 2000; Hibbert et al., 2006; Iwabe et al., 2007). The distribution of exogenous BDNF protein following localized brain injections, is compatible with BDNF anterograde transport from cell bodies to nerve endings. There are many studies that demonstrated the existence of anterograde axonal transport of BDNF (Spalding et al., 2002; Butowt and von Bartheld, 2005). Evidence regarding a functional role of anterogradely transported BDNF in the CNS, however, is scarce. There are studies showing a functional role of BDNF in the adult mossy fiber and CA3 pyramidal neuron synapse, suggesting that anterogradely transported BDNF can be involved in normal synaptic function (Scharfman, 1997) and epileptogenesis (Binder et al., 1999b; Scharfman et al., 1999). A report by Fawcett et al. (1998) provided evidence supporting a trophic action of anterogradely transported BDNF in the developing rodent CNS (Fawcett et al., 1998). Another means of delivering BDNF would be via dendritic targeting of BDNF protein or mRNA. Such locally synthesized BDNF could regulate synaptic strength in a site-specific manner. Indeed, dendritic accumulation of BDNF mRNA and protein was found selectively in discrete dendritic lamnias during epileptogenesis (Tongiorgi et al., 2004).

2.2 Expression regulation

The expression of the neurotrophins in the mammalian brain is regulated during development and by neuronal electrical activity. BDNF expression levels are low at birth but increase dramatically during the first weeks of postnatal development (Dieni and Rees, 2002). BDNF expression shows distinct modifications during fetal and early postnatal development. Expression of the BDNF gene in the adult CNS can be regulated by a series of events, either physiological signals or insults, including an increased expression following hypoxia-ischemia and hypoglycemic coma (Lindvall et al., 1992; Merlio et al., 1993; Korhonen et al., 1998; Hughes et al., 1999), an increased expression in interneurons located close to axotomized cortical projection neurons (Wang et al., 1998), and a reduced expression after stress (Smith et al., 1995; Altar, 1999). Some spontaneous changes in bioelectrical activity, or changes in activity induced by pharmacological manipulations can modify BDNF mRNA expression in vitro (Rutherford et al., 1997; Rutherford et al., 1998; Gorba et al., 1999). The regulation of BDNF expression occurs in an activity dependent manner. Activity-induced changes in BDNF expression have a very short latency, suggesting that BDNF can function as an immediate-early gene. BDNF expression can also be regulated by neurotransmitters and hormones (Cosi et al., 1993; Vaidya et al., 2001). Other synaptic mediators and hormones involved in the regulation of BDNF expression in the CNS are acetylcholine (Lapchak et al., 1993; French et al., 1999), serotonin (Vaidya et al., 1999; Zetterstrom et al., 1999), nitric oxide (Xiong et al., 1999),

thyroxine (Luesse et al., 1998), glucocorticoids and mineralocorticoids (Schaaf et al., 1998), and sexual steroids (Gibbs, 1999).

Zafra et al. showed that a balance between the glutamatergic and GABAergic systems controls the physiological levels of BDNF and NGF mRNA within hippocampal neurons both in vitro and in vivo (Zafra et al., 1990; Zafra et al., 1991). Blockage of glutamatergic receptors and/or stimulation of the GABAergic system results in a reduction of BDNF and NGF mRNA levels in the hippocampus and NGF protein in the septum and hippocampus. The refined and rapid regulation of both BDNF and NGF mRNA by glutamate and GABA transmitter systems suggests that BDNF and NGF may be involved in activity-dependent synaptic plasticity. Hippocampal neurons receive glutamatergic afferents from the entorhinal cortex via the angular bundle perforant pathway. The maintenance of basal NGF and BDNF mRNA levels appears to be mediated by excitatory neurotransmitters acting via NMDA/nonNMDA receptors (Rocamora et al., 1994; Brandoli et al., 1998). Supporting these findings, transynaptic stimulation of hippocampal afferents was observed to increase the level of BDNF mRNA within the hippocampus (Falkenberg et al., 1992).

Recent molecular cloning of the BDNF gene also revealed how the BDNF promoters I, II and III regulate BDNF expression (Hayes et al., 1997; Dennis and Levitt, 2005). These promoters are predominantly detected in the brain. The alternative usage of these promoters and different splicing can result in four forms of BDNF mRNA with different 5' untranslated exons and a common exon encoding the mature part of BDNF. The BDNF exons have been reported to be regionally and specifically induced by different forms of brain insult (Kokaia et al., 1994b). Falkenberg et al. reported that activation of entorhinal cortical afferents resulted in the regulation of different BDNF promoters within the hippocampus in a differential and dose-dependent manner (Falkenberg et al., 1993). Functionally, it is possible that the multipromoter system of the BDNF gene regulates BDNF synthesis in different cell types at both the transcriptional and translational levels.

2.3 BDNF secretion

The first two studies that addressed qualitatively the release of endogenous BDNF from central neurons were by Wetmore et al. (1994) and Ghosh et al. (1994)(Ghosh et al., 1994). The study by Wetmore et al. showed a shift of BDNF-IR from intracellular compartments to the extracellular neuropil of CA2 and CA3 pyramidal neurons, following kainate-induced depolarization of the hippocampus in vivo (Wetmore et al., 1994). Direct detection of released BDNF protein was accomplished by Goodman et al. (1996) who showed a depolarization-induced accumulation of BDNF in the extracellular medium using Western blot analysis of cultured hippocampal neurons overexpressing BDNF (i.e. bulk secretion) (Goodman et al., 1996). BDNF has been shown to be released both constitutively and in a regulated manner by different cells. Subsequently, BDNF was demonstrated to be present in a synaptosomal vesicular fraction obtained from rat cortical extracts (Fawcett et al., 1997). Localization of BDNF to vesicles was found in primary cultured rat cortical neurons (Haubensak et al., 1998) and further studies in hippocampal neurons addressed the mechanism of neuronal BDNF

secretion (Lessmann et al., 2003).

Release of endogenous BDNF could be elicited by electrical field stimulation, 50 mM K^+ or glutamate-induced depolarization in a calcium-dependent manner (Canossa et al., 1997; Griesbeck et al., 1999). Extracellular BDNF protein generally peaked within 50 – 250 s after the start of the stimulation (Canossa et al., 1997; Griesbeck et al., 1999; Hartmann et al., 2001). Glutamate-induced secretion of BDNF from hippocampal neurons has been suggested to be mediated by AMPA receptors and/or metabotropic glutamate receptors (Canossa et al., 2001). Application of AMPA can trigger depolarization-induced Ca^{2+} influx via voltage-gated calcium channels (VGCCs). It is of note that electrically evoked (as high K^+ induced) bulk secretion of BDNF is also dependent on intact intracellular Ca^{2+} stores (Balkowiec and Katz, 2002). Ca^{2+} release from internal stores could be an important amplification mechanism to reach an amplitude and a time course of intracellular Ca^{2+} at the plasma membrane that can trigger efficient peptide secretion (Gartner and Staiger, 2002).

Interestingly, neurotrophin secretion can also be triggered by neurotrophins themselves: using NT-4/5- or NT-3-induced stimulation of neuronal TrkB and TrkC receptors, Canossa et al. (1997) showed secretion of endogenous and of overexpressed BDNF from acute hippocampal slices. This NT-induced secretion of neurotrophins is mediated by mobilization of Ca^{2+} from internal stores. This is a common signal cascade downstream of Trk receptor activation (Berninger et al., 1993; Li et al., 1998; Patapoutian and Reichardt, 2001), and it is mediated by activation of PLC via a specific intracellular tyrosine in Trk receptors (Canossa et al., 2001). In CNS neurons, which are known to coexpress BDNF and TrkB receptors, the BDNF-induced release of BDNF could be important for prolonged BDNF release by this autocrine loop and could account for sustained NT secretion even when the triggering stimulus has ceased.

The axonal localization and dendritic targeting of BDNF (Kohara et al., 2001) had been clearly observed. In line with the axonal and dendritic localization of endogenous BDNF in different brain regions, pre- and postsynaptic secretion of BDNF had both been proven. The mechanism of secretion, however, was not addressed. Hartmann et al. (2001) showed that synaptic secretion of BDNF is dependent on the presence of extracellular Ca^{2+} and can be either mediated via activation of VGCC upon high K^+-induced depolarization or via activation of synaptic glutamate receptors following electrical stimulation (Hartmann et al., 2001). Interestingly, axonal as well as dendritic release of BDNF has been shown to depend on action potential generation, whereas steady depolarization alone was inefficient (Kohara et al., 2001; Gartner et al., 2006; Taniguchi et al., 2006). The relief of Ca^{2+} channel inactivation by intermittent post-spike hyperpolarizations is an important prerequisite for BDNF secretion (Kolarow et al., 2007).

3 The role of BDNF in the pathogenesis of some neurological diseases

As mentioned earlier, BDNF is vital for nervous system development, as indicated by the lethality of homozygous knockout mice for BDNF or severe developmental abnormalities in the nervous system and other organs of homozygous and heterozygous knockout animals (Pozzo-Miller et al., 1999; Hall et al., 2003; Zhu et al., 2009). Abnormal expression or signaling of BDNF can also have consequences on synaptic function, neuronal gene expression, and behavior, leading to neuronal dysfunction and neurological disorders such as neurodegenerative diseases, epilepsy, pain, hypoxia-ischemia, and hypoglycemia, depression etc (See review (Kuipers and Bramham, 2006). Recent studies suggest that changes in retrograde transport, decreased synthesis, altered processing of pro-neurotrophins or signaling through $p75^{NTR}$ may contribute as a secondary event to neuronal degeneration in some CNS disorders (Zhou et al., 2004; Iwabe et al., 2007; Wang et al., 2008). Although loss of BDNF signaling during development and adulthood has clearly been shown to result in developmental deficits and neuronal degeneration, no direct evidence has been provided to link BDNF to the etiology of any specific neurological disease. The possible involvement of BDNF in neurodegenerative diseases, epilepsy, pain, depression will be reviewed blow.

3.1 BDNF in neurodegenerative diseases

There is growing evidence that reduced neurotrophic support is a significant factor in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD), Huntington disease (HD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS).

Alzheimer's disease

Growing evidence suggests that a decrease in BDNF levels could be associated with AD pathogenesis. AD is characterized by a progressive decline in cognitive function that correlates with a number of pathological changes in several different brain regions. Selective reduction of BDNF mRNA in the hippocampus has been reported in Alzheimer's disease specimens (Phillips et al., 1991; Ferrer et al., 1999). The BDNF transcript in AD patients was reduced to 50% relative to control individuals. Decreased BDNF protein has been demonstrated in individuals with Alzheimer's disease by cell counting, optical density measurements (Connor et al., 1997) and western blot assay (Ferrer et al., 1999). Murer et al. (1999) demonstrated in AD brains that neurons showing neurofibrillary tangles are usually devoid of BDNF-immunoreactive material, whereas most intensely BDNF labeled neurons were devoid of tangles (Murer et al., 1999). Besides, in the hippocampus of AD patients, high amounts of truncated (95 kDa) TrkB receptors have been found in amyloid plaques (Connor et al., 1996). These data suggest a progression of

changes with loss of BDNF and conversion of full length to truncated TrkB during the amyloid plaque formation. It was also suggested that BDNF signaling might be impaired early in the course of AD (Murer et al., 1999). Connor et al. (1997) reported that immunoreactive TrkA and TrkB receptors were increased in astrocytes in the CA1 region in AD brains and to some degree were associated with amyloid plaques. In the basal forebrain, immunostaining showed a large reduction in TrkA, TrkB and TrkC expression in AD (Salehi et al., 1996). Allen et al. (1999) reported a profound decrease in catalytic TrkB protein expression in the frontal and temporal cortices of individuals with Alzheimer's disease (Allen et al., 1999). CREB, a crucial BDNF signaling molecule, has been reported to be impaired in AD patients (Yamamoto-Sasaki et al., 1999). The precursor form of BDNF and mature BDNF (Michalski and Fahnestock, 2003; Peng et al., 2005) or its mRNA (Holsinger et al., 2000) were also decreased in the parietal cortex and hippocampus, even in pre-clinical stages of AD. In summary, the available evidence suggests that AD patients have a reduced mRNA and protein content of BDNF, pro-BDNF or BDNF-containing molecules in the hippocampal formation, temporal cortex, and probably other cortical areas. These alterations vary in the course of the disease and are correlated with the severity of dementia (Tapia-Arancibia et al., 2008).

Huntington's disease

Interestingly, recent work has implicated BDNF in Huntington's disease as well. In Huntington's disease the mutant protein huntingtin leads to a down-regulation of BDNF in the basal ganglia, and loss of huntingtin-mediated BDNF transcription leads to loss of trophic support to striatal neurons which subsequently degenerate in the hallmark pathology of the disorder (Zuccato et al., 2001). These data demonstrated a role of huntingtin in influencing BDNF transport and BDNF expression (Wu et al., 2009b). A recent study has demonstrated that huntingtin normally inhibits the neuron restrictive silencer element (NRSE) involved in tonic repression of transcription from BDNF promoter II (Zuccato et al., 2003; Rigamonti et al., 2007). BDNF protects the majority of striatal projection neurones from excitotoxin lesions. Reduced BDNF levels or TrkB receptors have been found in brain regions most affected in HD patients (caudate and putamen) (Gines et al., 2006; Ciammola et al., 2007) and in transgenic mice that express human mutant huntingtin (Zuccato et al., 2005). In animals treated with the excitotoxin quinolinic acid, cells genetically engineered to release BDNF, NT-3 or NT-4/5 were implanted into the striatum. All these neurotrophins were shown to promote the survival of striatal projection neurones, with BDNF being most effective as a survival agent (Perez-Navarro et al., 2000). BDNF has been proposed for the clinical treatment of HD patient (Roze et al., 2008).

Parkinson's disease

Parkinson's disease is characterized by the selective degeneration of nigral dopaminergic neurons and subsequent depletion of striatal dopamine which leads to motor dysfunction. One hypothesis of the cause of degeneration of the nigrostriatal dopaminergic neurons is that PD is caused by apoptosis due to decreased neurotrophins. Decreased BDNF protein has been demonstrated in the substantia nigra (SN) in Parkinson's disease (Parain et al., 1999; Howells et al., 2000). In

substantia nigra pars compacta (SNc) of PD brains compared to age-matched controls, the number of pigmented neurons containing BDNF was reduced to 9.6% of the control value while the number of pigmented neurons non-immunoreactive for BDNF was reduced only to 23.9% of the control value, thus suggesting that SNc pigmented neurons not expressing BDNF have a greater probability of surviving than BDNF-positive pigmented neurons. All the dopaminergic neurons observed to have Lewy bodies were immunoreactive for BDNF (Parain et al., 1999). These data suggest that BDNF actually confers a greater sensitivity to degeneration in PD. In a quantitative assessment of NTFs content within individual neurons based on density of staining with either immunoperoxidase or immunofluorescent labeling (Nagatsu et al., 2000), small decreases in BDNF and CNTF (5 to 10%) were observed, but little or no significant changes in NGF, NT-3 or NT-4/5 in surviving neurons of SNc in PD. To date, supplementation of some specific NTFs (e.g. BDNF) has been used as potential therapy against PD in experimental animal and in vitro models (Jiang et al., 2006; Singh et al., 2006; Sadan et al., 2009). Further elucidation on the relationship between BDNF and PD will be need to reach a clinical application.

Amyotrophic lateral sclerosis

ALS is a neurodegenerative disease that usually attacks both upper and lower motor neurons and causes degeneration throughout the brain and spinal cord. A majority of spinal cord motor neurons in humans exhibit immunoreactive BDNF and their respective receptors, TrkB and TrkC, thus suggesting autocrine regulation by BDNF and NT-3 (Kawamoto et al., 1998). In the spinal cords of patients died of ALS, three-quarters of the motor neurons were lost and the remaining motor neurons demonstrated decreased BDNF/TrkB and increased NGF and TrkA (Koliatsos et al., 1993; Mutoh et al., 2000). Other studies showed that a number of NTF promote survival of spinal motor neurons, including BDNF (Kishino et al., 1997), CNTF, IGF and glial-derived neurotrophic factor. However, only IGF-I slowed the efficiency in the progression of functional impairment in patients with ALS when administered with these NTFs (Turner et al., 2001). BDNF therapy produced no significant overall survival effect or benefit for the primary end points but did show statistically significant benefits in subgroups of patients with early respiratory impairment in a phase III trial (Kalra et al., 2003). A recent report showed that BDNF enhances branching and arborization but not axon outgrowth in corticospinal motor neurons (Ozdinler and Macklis, 2006).

3.2 BDNF and epilepsy

The discovery that limbic seizures increase NGF mRNA levels (Gall and Isackson, 1989) led to the idea that seizure-induced expression of NTFs may contribute to the lasting structural and functional changes underlying epileptogenesis (Gall et al., 1991; Simonato et al., 2006). It is well known that epileptogenic insults increase the synthesis of BDNF and the activation of trkB receptors (Binder et al., 1999b; Binder et al., 2001). Increases in BDNF mRNA expression were found not only after long-lasting or recurrent seizures, but also

after brief episodes of hippocampal epileptiform activity, and were not restricted to the hippocampal formation, but involved widespread regions of the cerebral cortex and the amygdaloid complex (See review Simonato et al. 2006). Remarkably, increased BDNF expression in the hippocampus is found in specimens from patients with temporal lobe epilepsy (Mathern et al., 1997; Takahashi et al., 1999). In temporal lobes resected for treatment of refractory epilepsy and showing hippocampal sclerosis, compared to autopsy material or resections for mass lesions, granule cells contain increased levels of mRNAs for BDNF, NGF and NT-3. Interestingly, BDNF is down-regulated under chronic epileptic conditions (Shetty et al., 2003). Nevertheless, it was suggested that there were functional associations among granule cell neurotrophins, mossy fiber synaptic plasticity and hippocampal neuron damage in hippocampal sclerosis and epilepsy. Many studies convincingly support the notion that BDNF have a proepileptogenic role. Infusion of anti-BDNF agents (Binder et al., 1999b) or use of BDNF knockout (Kokaia et al., 1995) or truncated trkB-overexpressing (Lahteinen et al., 2002) mice inhibits epileptogenesis in animal models. Direct application of BDNF induces hyperexcitability in vitro (Scharfman et al., 1999), overexpression of BDNF in transgenic mice leads to spontaneous seizures (Croll et al., 1999), and intrahippocampal infusion of BDNF into intact adult rats initiates mossy fiber sprouting and seizure activity (Scharfman et al., 2002a). However, BDNF also seems to have some beneficial effects during the course of TLE. For instance, it has been shown that the BDNF amplifies GABA currents in oocytes expressing $GABA_A$ receptors transplanted from surgically removed specimens of human epileptic brains (Palma et al., 2005). Furthermore, our previous studies shows that the BDNF favors survival and regeneration of hippocampal neurons damaged by the SE (Simonato et al., 2006; Paradiso et al., 2009). Thus, the role of BDNF in epilepsy needs to be elucidated much more profoundly.

3.3 BDNF and other neurological disorders

Neurotrophins control survival, differentiation and synaptogenesis, and play important roles in activity-dependent forms of synaptic plasticity in the nervous system. It is well accepted that alterations in the levels of NTFs or their receptors contribute to the pathogenesis of neuropathy, pain and psychiatric disorders. Studies performed from human nerve biopsies in cases of neuropathy showed that the neurotrophin receptors were upregulated in $p75^{NGR}$ while downregulated in TrkB and TrkC in nerves with axonal pathology (Goettl et al., 2004). The mRNA levels for NGF, BDNF and NT-3 are increased in diseased nerves (Rodriguez-Pena et al., 1995; Anand, 2004).

BDNF signaling may also be involved in affective disorders (Altar, 1999). A recent series of studies has linked a polymorphism in the pro-domain of BDNF with depression, bipolar disorders and schizophrenia (Skibinska et al., 2004; Tsai, 2004; Kolarow et al., 2007). This polymorphism, which was identified from a single nucleotide polymorphism screen, is caused by a single amino-acid change, in the pro-domain of the BDNF protein (Lu and Martinowich, 2008). However, a recent meta-analysis did not support BDNF genotype as a major influence on the development of depression (Chen et al., 2008). Several lines of evidence have

implicated neurotrophins in depression (Castren and Rantamaki, 2008). Environmental stresses such as immobilization induce depression and also decrease BDNF mRNA (Smith et al., 1995; Murakami et al., 2005). In animal models, restraint stress leads to decreased expression of BDNF in the hippocampus (Rasmusson et al., 2002; Chourbaji et al., 2004). The administration of BDNF to the midbrain or hippocampus results in antidepressant effects in animal models of depression (Shirayama et al., 2002). Existing pharmacological antidepressants are thought to act primarily by increasing endogenous monoaminergic (i.e. serotonergic and nor-adrenergic) synaptic transmission, and recent studies have shown that effective antidepressants increase BDNF mRNA (Dias et al., 2003) and protein (Chen et al., 2001a; Altar et al., 2003). BDNF has been shown to have trophic effects on 5-HT and noradrenergic neurons. Mutant mice with decreased levels of BDNF show a selective decrement in the function of 5-HT neurons and behavioural dysfunctions that are consistent with serotonergic abnormalities (Lyons et al., 1999). Interestingly, lithium, a major drug for the treatment of bipolar disorder, increases BDNF and trkB activation in cerebral cortical neurons (Hashimoto et al., 2002).

BDNF also may play an important neuromodulatory role in pain transduction (Malcangio and Lessmann, 2003). BDNF is synthesized by dorsal horn neurons and markedly upregulated in inflammatory injury to peripheral nerves (along with NGF) (Fukuoka et al., 2001). BDNF acutely sensitizes nociceptive afferents and elicits hyperalgesia which is abrogated by BDNF inhibitors (Kerr et al., 1999; Pezet et al., 2002). Spinal neurons show increased excitability to nociceptive inputs after treatment with exogenous BDNF (Thompson et al., 1999). Electrophysiological and behavioural data demonstrate that inhibition of BDNF signal transduction inhibits central pain sensitization (Kerr et al., 1999; Pezet et al., 2002).

4 BDNF as a potential therapeutic agent for neurological diseases

Substantial evidence indicates that NTFs can protect and even restore impaired functions resulting from trauma, aging, toxic agents, and genetically linked neurodegenerative disorders. The use of NTFs as therapeutic agents is becoming a promising approach in the treatment of CNS disorders. Clinical trials are now in progress to assess the therapeutic efficacy of NTFs in a variety of neurodegenerative diseases such as AD, PD, HD or ALS. Many studies were undertaken to determine whether members of the neurotrophic factor family have potential therapeutic roles in preventing and/or reducing the neuronal cell loss and atrophy. For example, NGF administration prevents cholinergic neuron atrophy caused by lesions of the septohippocampal and basal forebrain or cortical systems (Tuszynski et al., 2005).. The compiled evidence led to the suggestion that NGF administration might produce some benefit to individuals with Alzheimer's disease (Tuszynski et al., 2005). Clinical trials with GDNF in PD patient showed a marked reduction of symptoms when GDNF was administered directly into the parenchyma of the dorsal putamen via a pump and a catheter (Salvatore et al., 2006). On the contrary, clinical trials with CNTF in amyotrophic lateral sclerosis have showed either no improvement in neurological outcome (Penn et al., 1997) or a worsening (Miller et al., 1996b), with deleterious side effects (Miller et al., 1996a). Systemically administered bFGF decreased brain injury resulting from experimental focal ischemia (Bethel et al., 1997). In peripheral neuropathies caused by chemotherapeutic agents, systemically given neurotrophins have been demonstrated to be of benefit in some instances (Apfel and Kessler, 1996).

The important role of BDNF in the CNS, mentioned above, provides possibilities for predicting therapeutic approaches in the treatment of psychiatric and neurological disorders. BDNF have the potential to protect diseased and injured neurons from dying, to induce neuronal sprouting and to increase neuronal metabolism and function. The neurotrophic actions of BDNF can be used to reduce neuronal loss and mobilize the internal resources of an organism in the rearrangement of neural circuits to cure the diseases. BDNF has survival promoting actions on a variety of CNS neurons including hippocampal and cortical neurons, cholinergic neurons, and nigral dopaminergic neurons. These facts raised keen interest in BDNF as a potential therapeutic agent for many injury-related neurological diseases. This interest has been intensified by in vitro and in vivo studies demonstrating the essential role of BDNF and Trk receptors in the pathogenesis of neurological disorders. The idea that neuronal death in degenerative disorders can result from the lack of an endogenous trophic molecule highlights the therapeutic use of BDNF to prevent and/or reduce selective neuronal loss in the diseased human brain. Indeed, BDNF has been observed to increase the survival of ventral dopaminergic neurons and prevent the rotational asymmetry in an animal model of PD (Singh et al., 2006; Sadan et al., 2009). BDNF has also been shown to promote the survival of motorneurons both in culture and in neonatal rats as well as to rescue motorneurons from axotomy-induced degeneration (Canals et al., 2004). The effects of BDNF in treating motorneuron disorders has been investigated in the progressive wobbler mouse, a natural murine

model of motorneuron disease (Tsuzaka et al., 2001). The administration of exogenous BDNF can retard motor dysfunction and diminish denervation muscle atrophy and motor axon loss (Ikeda et al., 1995). It has also been proposed a potential benefit of BDNF in HD. The promising suggestion came from in vitro studies by Saudou et al. (1998), who reported that cultured mammalian neurons bearing the mutant huntingtin were prevented from dying when BDNF was administered (Saudou et al., 1998). Intrathecal administration of BDNF increased the survival of enkephalin-expressing striatal cells, the most affected cells in HD (Canals et al., 2004).Following the discovery that AD pathology may primarily be due to a deficit in neurotrophic factor protein and/or trk receptor expression, the protective effect of BDNF was demonstrated against toxicity induced by Aβ peptides on neuronal survival of primary cultures of cortical neurons (Yao et al., 2005) and applied in an animal model of AD (Singh et al., 2006). In the clinical trials in treatment of ALS patients, BDNF appeared to increase survival rate and retard loss of pulmonary function (BDNF-study-group-Phase-III, 1999).

Interestingly, BDNF has received considerable attention for being modulated in the brain by antidepressant treatments, such as drugs, electroconvulsive shocks, physical exercise, although there is still no direct evidence that endogenous BDNF plays a role in the mechanism of antidepressant drug action. The potential BDNF application in the treatment of neuropsychiatric disorders has been initiated.

To date, however, clinical trials of BDNF have not been particularly successful. For better results, it will elementary to improve the delivery strategies.

5 Strategies of NTFs therapy

As stated above, evidence from preclinical and clinical studies indicates that it may be possible to use NTFs to prevent, slow the progression of, or even reverse the effects of a number of neurodegenerative diseases and other types of insults in both the central and peripheral nervous system. Despite the encouraging results from these studies, progress in adaptation of this treatment strategy to human disease has been slow and sometimes disappointing. The clinical failure of many candidate trophic factors is often ascribable to poor delivery methods (Thorne and Frey, 2001). NTFs need to be specifically targeted and regionally restricted to the area of interest to achieve significant results without widespread, unwanted adverse effects. The direct injection of NTFs such as BDNF to the brain is, however, problematic due to: (i) their inability to cross the blood－brain barrier; (ii) brain diffusion and pharmacodynamic issues; and (iii) side-effects associated with binding to extra-target receptors.

In an attempt to overcome such problems, other therapeutic approaches have been developed to aim at searching drugs capable of increasing the synthesis of endogenous BDNF or simulating BDNF action to activate its signaling pathway. These options include: (i) activation of receptors by small, nonpeptidyl molecules amenable to chemical synthesis; (ii) rigid peptides interacting with the ligand binding domain (LeSauteur et al., 1995); (iii) Trk receptor antibodies to engage and dimerize Trk, thereby mimicking NTFs-like trophic effects (Lucidi-Phillipi et al., 1996); (iv) neurotrophic factor－transferrin receptor antibody fusion proteins, to facilitate transferrin receptor-mediated cellular entry (Friden and Walus, 1993). Moreover, the improved methods in molecular biology allow a local and regulated supply of BDNF by either *in vivo* or *ex vivo* gene transfer. In this section, we will review the delivery strategies of NTFs.

5.1 Direct infusion

Since the discovery of the effects of BDNF on the survival of peripheral and CNS neurons, and the demonstration of its deficit in diseased brains, BDNF received considerable interest as possible means for the treatment of neurodegenerative diseases and CNS trauma. For example, infusion of the neurotrophin BDNF (10±30 μg/day) for 14 days proximal to the substantia nigra in PD patients is effective in partially rescuing axotomized dopaminergic neurons previously retrogradely labeled with dye (Hagg, 1998). Supranigral BDNF (7.2 mg/day) for 21 days also rescues cresyl violet identified nigral neurons that have undergone transneuronal degeneration due to removal of their striatal target neurons (Volpe et al., 1998). BDNF (168 mg total beginning 6 days prior to lesion) was effective in attenuating turning behavior but not in restoring dopamine levels (Altar et al., 1994).

Despite the promising results, BDNF supplementation raises a number of problems. The major problem is how to assess the amount of BDNF that reaches the affected neurons, and this is compounded by the fact that BDNF is relatively

unstable and only a small amount can cross the blood-brain barrier (Betz et al., 1989; Knusel et al., 1992; Pardridge, 2002). If the supplyed amount of BDNF is too small, it may not be sufficient to produce the required effects; if it is too large, it may be dangerous because, besides regulating survival, differentiation and maintenance of neuron-specific characteristics, BDNF is also important in modulating activity-dependent neuronal plasticity, which is essential for the functional and structural refinement of neuronal circuits, as well as in learning and memory (Binder and Scharfman, 2004). Uncontrolled BDNF administration may interfere with these mechanisms and even give rise to serious side effects such as epileptic activity (Binder et al., 2001). It has also been reported that high BDNF levels may downregulate the expression of TrkB receptors, thus reducing the signalling activated by BDNF and therefore blocking any possible beneficial effect (Frank et al., 1996; Knusel et al., 1997; Sommerfeld et al., 2000). It is probably for these reasons that subcutaneous and intrathecal BDNF administrations had little clinical success (BDNF-study-group-Phase-III, 1999; Ochs et al., 2000).

Intracerebroventricular (ICV) administration of BDNF has been explored as a possibility to bypass the blood – brain barrier. These studies suggest that infusion with i.c.v administration of neurotrophins (NGF/BDNF) will probably result in hyperplasia in man that, although probably reversible and nonmalignant, it may be partly responsible for pain (Cirulli et al., 2000). Intracranial infusion of NGF to a single Alzheimer's disease patient had positive clinical effects (Seiger et al., 1993), but a subsequent study with three patients proved negative (Eriksdotter Jonhagen et al., 1998). There are the other routes of administration reports in which I^{125}-NGF was injected into the olfactory bulb of rats, the radio labeled NGF was found to be retrogradely transported specifically to basal forebrain cholinergic nuclei (Altar and Bakhit, 1991). Similarly, it has been shown that radio labeled NGF can be transported into the brain following administration as nose drops (Chen et al., 1998). This may be a alternative mode of administration for BDNF delivery.

Although the exogenous addition of BDNF can rescue neurons from death *in vitro* and *in vivo*, neurotrophin administration in the human brain induces side effects such as pain and weight loss (Schulte-Herbruggen et al., 2007), limiting its possible therapeutic utilization. The situation underscores the need for the development of BDNF mimetics.

5.2 BDNF mimetics

Neurotrophins have a number of suboptimal pharmacological properties, including poor stability with a serum half-life of minutes or less, poor oral bioavailability, and restricted CNS penetration (Poduslo and Curran, 1996; Pardridge, 2002). The pharmacokinetic properties of the neurotrophins are probably the key factor in the therapeutic success or failure of these proteins. The production of small molecule agonists acting via targeted neurotrophic receptors would help to circumvent this problem. The small-molecule neurotrophin mimetics possess favorable stability and tissue penetration profiles. With the mass screening of large libraries of synthetic chemicals, researchers have developed small-molecule BDNF analogues with improved pharmacokinetic properties and an ability to penetrate the

blood brain barrier to an appreciable extent. The discovery of the small-molecule BDNF analogues has been achieved largely through a detailed knowledge of the three-dimensional structures of BDNF and its receptors. Cyclic peptides were designed to mimic BDNF specific domains, which are required for binding to TrkB receptors (Fletcher et al., 2008). In combination with the X-ray crystal structure of the Trk-binding domains, it is possible to develop useful therapeutic compounds by screening the BDNF mimetics from very large virtual libraries of molecules with the aid of high performance computers. Preliminary studies have identified the peptides capable of interacting with erythropoietin receptors and mimicking the actions of the protein granulocyte colony stimulating factor (G-CSF). In the case of G-CSF, Tian et al. (1998) have now demonstrated that a small, nonpeptidyl molecule (SB 247464) is capable of triggering the activation of a large receptor protein that requires dimerization for activation, through a domain not involved in binding the natural ligand (Tian et al., 1998). Application of this strategy to Trk receptors should be particularly interesting.

Also, there is also the possibility of developing small molecules that activate Trk receptors in the absence of neurotrophins, acting through G protein-coupled receptors (GPCRs) to activate neuroprotective pathways (Jeanneteau and Chao, 2006; Skaper, 2008). For example, two GPCR ligands, adenosine and pituitary adenylate cyclase ‑ activating polypeptide, activate Trk receptor activity and increase cell survival through stimulation of the neuroprotective Akt pathway. These alternative approaches to enhance neuronal survival may lead to new treatment for those neurological diseases that are associated with neuronal death.

It was demonstrated that such BDNF mimetics act as BDNF agonists that promote the survival of cultured sensory neurons, although their drug-like properties, particularly their proteoliyic stability, their ability to function via TrkB receptors, and their capacity of crossing the cell membrane, still require analysis (O'Leary and Hughes, 2003; Fletcher and Hughes, 2006). A considerable amount of work remains to be done, but this approach may represent a valuable step towards the development of BDNF mimetics for clinical use. The compounds that have been found to be able to increase BDNF levels in the brain raise another possibility for the BDNF mimetics strategies. For instance, the antidepressants known to affect BDNF levels are selective serotonin reuptake inhibitors (SSRIs) (Tardito et al., 2006) and lithium (Chuang, 2004). The latter induces the expression of BDNF and the subsequent activation of TrkB in cortical neurons (Fukumoto et al., 2001). In addition, memantine and riluzole (a non-competitive antagonist of ionotropic glutamate NMDA receptor) have been shown to upregulate BDNF and TrkB mRNA (Marvanova et al., 2001; Katoh-Semba et al., 2002), and also GDNF (Mizuta et al., 2001; Katoh-Semba et al., 2002). They are clinically used as neuroprotective agents to treat AD, PD and ALS. Similarly, cystamine and cysteamine have recently been shown to increase BDNF levels in the CNS and their potential therapeutic effects on HD have been analyzed (Borrell-Pages et al., 2006).

5.3 Local delivery

Despite the improvements that have been made to strategies for neurotrophin production and delivery, many difficulties still remain. Perturbations in BDNF – TrkB signaling in the brain, such as decreased signaling in the hippocampus but increased signaling in the mesolimbic circuit, may baffle the systematic application of BDNF or BDNF mimetics. Therefore, it has become increasingly clear that the successful implementation of NTF therapy requires a targeted, localized, sustained, and safe delivery of NTFs to avoid unwanted adverse effects resulting from widespread receptor activation. Several promising strategies of local administration of NTFs have been described in preclinical studies. These include:

1) Local delivery of the polypeptide interstitially within the brain parenchyma;
2) Surgical implantation of cannulae into the cerebroventricular space (Williams et al., 1997);
3) Implantation of encapsulated NTFs in microspheres (Maysinger et al., 1994);
4) Stereotaxic injection of virus mediating NTF gene transfer (Paradiso et al., 2009);
5) Local implantation of transfected cells secreting NTFs (Frim et al., 1994);
6) Local implantation of encapsulated cells secreting NTFs (**Fig. 3**)(Lindner et al., 1995).

These methods of local delivery of NTFs offer the advantages of bypassing the BBB and achieving high levels of drug at the therapeutic site, while reducing systemic exposure and avoiding unwanted side effects.

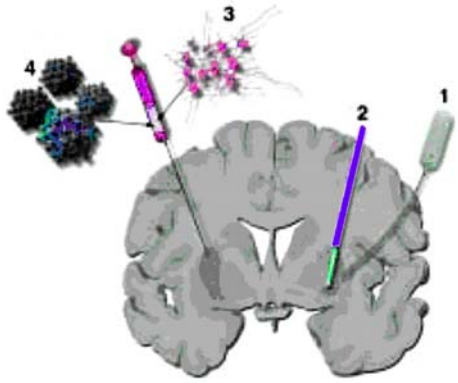

Fig. 3. The strategy of local NTF delivery with the encapsulated cell secreting NTFs into the brain: (1) mechanical pump; (3) encapsulated cells; (2) cell transplantation; and (4) direct injection of viral vectors. *(Patrick Aebischer. TRENDS in Neurosciences. 2001)*

5.4 Gene therapy

The dramatic advances made in the application of developments in molecular biology have offered new approaches to BDNF delivery. These involve gene therapy techniques. These techniques may be used to replace a faulty gene, or to introduce a new gene whose function is to cure or to favorably modify the clinical course of a disease. Researchers are testing several approaches to gene therapy, including: (i) replacing a mutated gene that causes disease with a healthy copy of the gene; (ii) inactivating, or "knocking out" a mutated gene that is functioning improperly; (iii) introducing a gene into the body to help fight a disease.

To deliver the working copy of the gene to the target cells, a selection of *in vivo* and *ex vivo* approaches is available. The method for *in vivo* gene delivery into the brain is that a vector with targeting genes is administered directly to the host while, in the process of *ex vivo* technique, DNA is delivered to cells in vitro for subsequent transplantation into a target tissue.

In vivo gene therapy

One of the most promising methods currently being developed is the use of harmless viruses that can be used to carry genes into cells. The therapeutic DNA is injected directly into the body cells, usually via one of two types of viruses (Rex, 2008). The most frequently used type is the very simple retrovirus. Using retroviruses is very safe and provides long-lasting effects. The second type of virus used for the in vivo technique is the adenovirus, the common cold virus. Nonviral vehicles for directly delivering genes into cells are also being explored, including the use of plain DNA and DNA wrapped in a coat of fatty molecules known as liposomes.

Research is currently being undertaken to determine potential therapeutic effects with delivering NTFs to vulnerable regions of the CNS in neurodegenerative diseases. Preclinical studies using adeno-associated virus vectors for delivery of neurotrophic proteins in models of Parkinson's disease (Bilang-Bleuel et al., 1997) and motor neuron disease (Haase et al., 1997) have been described, but do not appear to be viable strategies at present. Later studies confirmed that the viral delivery of BDNF can be protective in animal models of HD. In an elegant study, an adenovirus encoding BDNF was intrastriatally injected into the rat brains lesioned by a toxin quinolinic acid. One month after the lesion, histological analyses revealed that striatal neurons were protected only in the rats treated with the BDNF adenovirus, indicating that transferring the BDNF gene is of therapeutic value (Bemelmans et al., 1999). In a subsequent study, a delivery of the BDNF gene, by means of an adeno-associated viral (AAV) vector, to the striatum in the QUIN rodent model of HD also showed a significant increase in striatal neuron survival (Kells et al., 2004). Similarly, our previous BDNF gene therapy using lentivirus as a vector inhibited epileptogenesis after inoculating the recombinant virus into an epileptic rat brain (Paradiso et al., 2009).

Ex vivo gene therapy

In the *ex vivo* approach cells are cultured and used for gene transfer, by injecting or splicing the DNA that will correct the disease into the cells and letting them divide in cultures. These transfected cells are then introduced in a targeted

tissue. As compared with *in vivo* gene therapy, there are some advantages of an *ex vivo* approach: (i) gene transfer efficiency is generally high and retroviral vectors are particularly effective; (ii) the transduced cells can be enriched if the vector has a selectable marker gene and (iii) transduction efficiency can be assessed before re-implantation.

Ex vivo gene therapy has been applied in the treatment of neurological diseases by introducing genes coding for members of the neurotrophin family. For instance, studies have shown that retroviral transfected fibroblasts, expressing NGF, rescue cholinergic neurons after a fimbria fornix lesion in the rat (Stromberg et al., 1990). In a phase I trial of *ex vivo* NGF gene delivery, eight individuals with mild Alzheimer disease were implanted autologous fibroblasts genetically modified to express human NGF into the forebrain (Tuszynski et al., 2005).

In summary, gene therapy offers an alternative to administration of exogenous NTFs. We can use *in vivo* or *ex vivo* gene therapy to transfer and express NTFs in neurons. The expression of the NTFs genes is essentially an intervention that alters the instruction set of a cell, consequently correcting the gene defect for some disorders or influencing the cell fate. It might compensate for an ineffective NTF gene, and provide an alternative approach for NTFs protein delivery as well. The *ex vivo* gene therapy allow introduction of cells that naturally, or through genetic modification, produce NTFs. After the transplantation, the NTFs secreted by the graft could produce favorable effects for the regeneration or neuroprotection and promote brain repair.

5.5 Stem cell-based gene therapy

To date, about 40 percent of the more than 450 gene therapy clinical trials are via *ex vivo* method, i.e. cell-based gene therapy. Of these, approximately 30 percent have used human stem cells as the means for delivering transgenes into patients. Stem cells are distinguished from other cell types by two important characteristics. First, they are unspecialized cells capable of renewing themselves through cell division, sometimes after long periods of inactivity. Second, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions. The reasons for the extensive application of stem cells in cell-based gene therapy include: (i) the ability to self-renew, which means that the need to provide repeated administrations of gene therapy can be reduced or even eliminated; (ii) the natural ability to produce a rich variety of growth factors, cytokines, and other bioactive molecules that have the potential to facilitate the survival of the graft, and foster regeneration, neuroprotection, and other compensatory and repair mechanisms in the host tissue; (iii) stem cells can ‘home’ or migrate, to a number of different spots in the body, which suggests the possibility of a local, targeted gene therapy.

Given these unique abilities, stem cells offer new potentials for gene therapy. Moreover, stem cells have a great therapeutic potential in repopulating damaged tissues besides being genetically manipulated and used in cell-based gene therapy. To date, the transplantation of some types of stem cells has emerged as a restorative therapy for CNS disorders such as ischemic stroke (Kameda et al., 2007) and spinal

cord injury (Lu et al., 2003). In the CNS, stem cells could exert not only long-term functional integration (by giving rise to large numbers of properly functioning cells integrating into the brain circuitries) but also repair capabilities. Stem and progenitor cell-mediated gene delivery is emerging as a strategy to improve the efficacy and minimize the toxicity of current gene therapy approaches.

There are two broad types of mammalian stem cells: embryonic stem cells that are isolated from the inner cell mass of blastocysts, and adult stem cells that are found in adult tissues. Embryonic stem cells could provide better maintenance of therapeutic effects in comparison with adult stem cells, because a greater potential for self renewal in embryonic stem cells make the repeated administrations of gene therapy less necessary. However, embryonic cell sources are confronted with ethical and legal complications (Joannides and Chandran, 2008).

There are diverse types of stem cells that are studied as gene-delivery-vehicle candidates, such as myoblasts (Mohajeri et al., 1999), osteoblasts (Yudoh and Nishioka, 2004), fibroblasts (Stromberg et al., 1990), mesenchymal stem cells (Harper et al., 2009), hematopoietic stem cell (Rizvanov et al., 2008) and neural stem cells (Lu et al., 2003). For instance, the transplantation of the myoblasts containing the transgene for a human NGF into the muscles of the ALS mice successfully delayed the onset of disease symptoms, slowed muscle atrophy, and delayed the deterioration of motor skills (Mohajeri et al., 1999). Neural stem cells genetically modified to create a protein that activates a precursor drug appear to be effective in treating gliomas, which is a difficult type of brain tumor to treat (Li et al., 2005). Embryonic stem cells, neural stem cells, or neural progenitors constitute the most promising source for neural transplantation because they have a potential ability to differentiate into neurons (Kim and de Vellis, 2009). Embryonic stem cells isolated from developing blastocysts theoretically possess the greatest potential for uses in neural transplantation, as long as their differentiation fate can be controlled. Neuronal progenitors in human adults have been proposed as a source of transplantable neural cells. The adult derived transplantation approach is, however, limited by low in vitro proliferation potential of these cells (Kim and de Vellis, 2009). Human embryo-derived neuronal progenitors do not show the same limitations, with the exception of the ethical issue related to the use of human fetal tissue. So far, it had been revealed that most stem cells can be genetically engineered without modification of their fate after transplantation into the rodent brain (Sabate et al., 1995), opening the possibility of *ex vivo* gene therapy approaches. The promising results obtained from *ex vivo* gene therapy in CNS diseases have increased the efforts to find an ideal stem cell and generate modified stem cells producing NTFs or other substances (Martinez-Serrano and Bjorklund, 1997; Boucherie and Hermans, 2009). These cells should readily survive transplantation and allow introduction of recombinant NTFs genes so that they can produce and deliver adequate amounts of growth factors in the brain.

5.6 Mesoangioblast: an alternative cell source for NTF delivery

To date, multiple types of stem cells and progenitor cells have been identified as potential sources of NTF delivery, including autologous and allogeneic embryonic cells (Li et al., 2008; Rizvanov et al., 2008; Makar et al., 2009), fetal and adult somatic cells from neural (Martinez-Serrano and Bjorklund, 1996; Lu et al., 2003; Kameda et al., 2007) and mesenchymal tissues (Harper et al., 2009). Here we describe a novel type of vessel associated stem cell that may be developed as a vehicle for NTF delivery. These stem cells can differentiate into different mesoderm cell types and derive from the vasculature, probably a primitive luminal angioblast, therefore named "mesoangioblast" (MAB). MABs are firstly isolated from explants of dorsal aorta or other embryonic or juvenile postnatal vessels (De Angelis et al., 1999; Cossu and Bianco, 2003). The MABs clonal isolates can be grown and proliferated to at least 50 passages in vitro under certain conditions without obvious alterations of morphology and gene expression profile. MABs express two well-known stem cell markers: CD34, a cell surface sialomucin-like adhesion molecule expressed in hematopoietic stem cells, and Thy-1, a 25 kDa glycosylphosphatidylinositol-linked membrane glycoprotein, which is expressed in hematopoietic stem cells, T lymphocytes, neural cells and, during angiogenesis, in endothelial cells, pericytes and smooth muscle cells (Minasi et al., 2002). Moreover, MABs exhibit stem cell features, such as pluripotency and self-renewal ability, and can differentiate in vivo and in vitro into different mesoderm cell types, such as muscle, bone, and adipocytes, in response to specific extracellular cues (De Angelis et al., 1999; Cossu and Bianco, 2003). They are supposed to be involved in the development of the vasculature, and probably in the development of the nervous system since many of the genes expressed in MABs are neural genes, for example GPRC5B, which is expressed in brain and spinal cord; Tm4sf2, which has been implicated in activity-dependent brain plasticity and others (Tagliafico et al., 2004). Among genes selectively expressed by mesoangioblasts are many cytokines, chemokines and their receptors. This indicates a role of these cells in tissue regeneration and first inflammatory response to damage (Tagliafico et al., 2004).

A significant improvement in understanding MAB biology in recent years has paved the way to their potential clinical use. There are several reasons for MABs being valuable candidates for cell-based gene therapy: (i) MABs are non-tumorigenic and stable cell lines capable of unlimited clonal expansion in vitro, and with the capacity of longterm survival, which allows the gene manipulation to the cells; (ii) they can be isolated from humans, cultured in vitro, and autologously transplanted into patients, thus overcoming the difficulties related to immune rejection of transplanted cells; (iii) the ability of migrating and homing to damage sites because of the expression of different cytokines and chemokines; (iiii) the multipotent differentiation.

Owing to their general availability, higher abundance and ease of isolation, MABs may be convenient delivery vehicles for localized NTF delivery.

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Chapter II

THE FINDINGS

1. Overview of the research program

1.1 Rationale

As described above, neurotrophic factors are critical to survival, development and function of neurons in the mammalian central nervous system. Alterations in neurotrophic factors or their receptors can lead to neuronal death and contribute to the pathogenesis of neurodegenerative diseases such as Alzheimer disease (Connor et al., 1997), Huntington disease (Zuccato and Cattaneo, 2007), Parkinson disease (Bradford et al., 1999), and multiple sclerosis (De Santi et al., 2009). Many NTFs have been also implicated in diseases associated with damage, like stroke and some forms of epilepsy (Simonato et al., 2006). Based on these studies, the neurotrophic factors (NTFs) emerge as very strong candidates for a therapeutic role: not only they have trophic properties that suggest an involvement in cell death, neurogenesis and axonal sprouting but, also, they exert functional effects at synaptic level (Thoenen and Sendtner, 2002; Simonato et al., 2006). The use of neurotrophic factors as therapeutic agents is a novel approach aimed at restoring and maintaining neuronal function in the CNS.

Among these NTFs, particular attention has been devoted to brain derived neurotrophic factor (BDNF). Compelling preclinical data show that BDNF exerts trophic and neuroprotective effects on neurons. However, its clinical use is hindered by a short biological half-life and a poor blood-brain barrier permeability (Thoenen and Sendtner, 2002; Boado, 2008). Given these difficulties, the attention turned into the development of techniques that allow localized and regulatable BDNF delivery. These may be based on gene therapy (Paradiso et al., 2009) or on cell-mediated approaches, in which engineered cells deliver the therapeutic biomolecules to the brain.

1.2 Research Basis

We have already evidenced that viral vector-mediated supplementation of FGF-2 and BDNF favors proliferation of neural stem cells and/or progenitors and their differentiation into neurons in vitro (Paradiso et al., 2009). In this previous experiment, the neural precursors isolated by buoyant density gradients were infected with the FGF-2 and/or BDNF vectors and maintained for one month in the same plate, in a medium deprived of any other supplement. The data show that FGF-2 and BDNF together potently favor proliferation, survival and neuronal differentiation of neural progenitors. The vector expressing FGF-2 and BDNF was also tested in an animal model in which an epileptogenic insult (an episode of SE induced by the peripheral administration of the muscarinic agonist pilocarpine) causes damage in the hippocampus and, in time, the occurrence of spontaneously recurrent seizures (SRSs), i.e. epilepsy. We injected the FGF-2 and BDNF-expressing vector in the hippocampus after the establishment of damage and

before the occurrence of SRSs. In keeping with our in vitro findings, we found that this approach prompts neuronogenesis, allowing a significant recovery from damage. A behavioral observation and the video EEG analysis conducted in a time span of several weeks during the chronic period following pilocarpine-induced SE also suggest a reduction in SRSs (Paradiso et al., 2009). These previous results from our group indicate that supplementation of FGF-2 and BDNF in the hippocampus lesioned by prolonged seizures leads to repair of existing damage and to highly significant improvement of the "clinical" outcome (prevention of epilepsy). The local NTF delivery based on in vivo gene therapy is an effective treatment strategy to prevent brain damage, and mobilize self-recovery, consequently slow the progression of epileptogenesis. The strategy of local NTF delivery to exert trophic effects in the lesion area has also been applied in a number of neurodegenerative diseases and other types of insults in both the central and peripheral nervous system (Schulte-Herbruggen et al., 2007).

The use of viral vectors in the local NTF delivery strategy of our previous studies has several advantages over any pharmacological approach: (i) it circumvents the blood – brain barrier to reach the targeted area; (ii) the high-level NTFs gene expression obtained with viral vectors provides a robust and long-term supplementation of the NTFs of interest in the lesion area without implant of permanent devices; (iii) it avoids pharmacodynamic issues; (iiii) the small administration volume of vectors makes it possible to limit brain diffusion, reducing side-effects associated with binding to extra-target receptors.

1.3 Unsolved issues

These studies demonstrated the efficiency of local NTF delivery in an acquired epilepsy model using an *in vivo* gene therapy strategy. However, the local NTF delivery using viral vectors are not totally satisfactory because of: (i) the potential risk of viral infection, even if it is demonstrated that gene therapy mediated by most of viral vectors are not toxic; (ii) the immunogenic properties of the virus (in our previous work, we found that the injected viral vectors produce a small, but clearly detectable, infiltrate of lymphocytes at the very site of inoculation); furthermore, (iii) the vector inoculation can be performed only in highly specialized centers, equipped for stereotaxical surgery, and it is, therefore, a very costly and time-consuming approach; (iiii) it is an invasive treatment which causes new damage to the brain.

Based on these considerations, it is necessary to explore alternative approaches. Cell-based gene delivery, namely *ex vivo* gene therapy, is emerging as a strategy to treat chronic neurological disorders and injury related neural degeneration. In this strategy, cells are genetically modified with NTFs and then transplanted into the brain, where engineer cells continuously express and release the desired NTFs at safe doses. Cell-based delivery strategies may offer some degree of CNS targeting with minimal invasiveness. Stem and progenitor cell-mediated gene delivery is potentially interesting for drug manipulation, with improvement of the efficacy and minimization of the toxicity of current gene therapy approaches. The strategy has already been used and validated in several

lines of stem cells, including neural stem cells (Martinez-Serrano and Bjorklund, 1996; Lu et al., 2003; Kameda et al., 2007), embryonic stem cells (Li et al., 2008; Rizvanov et al., 2008; Makar et al., 2009), and mesenchymal stem cells (Harper et al., 2009), which were engineered to produce neurotrophic factors and capable of ameliorating neurodegenerative disease pathology. Using genetically modified stem cells as grafts could exert not only long-term functional integration but also repair capabilities. As opposed to systemic delivery of therapeutic agents, stem cell-based therapy can as well offer a continuous and concentrated local delivery of secretable therapeutic molecules like BDNF, thus reducing the non-selective targeting, and allowing higher treatment efficiency and potency for a longer time period.

These findings prompt the search for alternative cell sources that, unlike neural or embryonic stem cells, may be readily used in the clinics. Here, we explored the feasibility of a new approach for the local supplementation of BDNF in lesion brain areas. This approach is the use of multipotent, mesodermal stem cells (mesoangioblasts, MABs) that constitutively produce a subset of NTFs (e.g. bFGF, VEGFB, HGF, IGF) and can be engineered to produce others. MABs are an affordable cellular source. They can be isolated from the perivascular human adult tissue, and are already utilized for regenerative purposes in complex animal models, other than rodents. Their principal feature is the ability to differentiate, under appropriate conditions, in the different mesodermic tissues. The MAB-based therapy is now approaching the clinics (Sampaolesi et al., 2006). MABs have a high adhesin-dependent migratory capacity and, therefore, can reach perivascular targets. Their chemotactic ability is increased under inflammatory conditions, when the adhesin-integrin system and diapedesis moving are activated (Galvez et al., 2006). Therefore, when peripherally administered, MABs may selectively cross the blood-brain barrier and home in the lesioned areas. These cells have many neuro-ectodermal genes, but they do not differentiate into neurons (Tagliafico et al., 2004). Thus, we do not plan to use them for a restorative engraftment, but to exploit their migratory ability for targeting the lesion area: after infusion in the peripheral blood, MABs are expected to act as a reservoir and delivery system for NTFs.

1.4 Aim

In this context, we aim exploiting MABs as a novel delivery system by genetically engineering BDNF into MABs. The specific aims of this study are: (i) to examine the by-stander effects of MABs engineered to produce BDNF; (ii) and to perform a pilot transplantation into the lesion brain induced by status epilepticus.

1.5 Scheme

We began examining the by-stander effects of MABs in an *in vitro* culture systems, because the culture system allows maintenance of a constant level and a defined concentration of exogenously supplied BDNF during the entire treatment period. Considering the facts that BDNF can not only promote cell survival but also

regulate precursor proliferation and commitment, we studied the by-stander effects of MABs in two respects: neuroprotection and neurogenesis. Finally, we performed an attempt to systemically transplant MABs engineered with BDNF into the lesion brain induced by status epilepticus. Therefore, the program is divided into three parts:

1) Part I: Assess the neuroprotection effects of MABs-BDNF in primary hippocampal neuron culture and organotypic hippocampal slice culture.

2) Part II: Assess the neurogenesis effects of MABs-BDNF in organotypic hippocampal slice culture.

3) Part III: MABs transplantation in seizure-induced damage brain.

2. PART I - The neuroprotective effects of MABs-delivered BDNF

2.1 Introduction

Recent evidence suggests that neurotrophic factors that promote the survival or differentiation of developing neurons may also protect mature neurons from neuronal atrophy in the degenerating human brain (Schulte-Herbruggen et al., 2007). Furthermore, it has been proposed that the pathogenesis of human neurodegenerative disorders may be due to an alteration in neurotrophic factors (NTFs) and/or Trk receptor levels (Dawbarn and Allen, 2003). The use of neurotrophic factors as therapeutic agents is a novel approach aimed at restoring and maintaining neuronal function in the central nervous system. Research is currently being undertaken to determine potential mechanisms to deliver neurotrophic factors to selectively vulnerable regions of the CNS.

The importance of NTFs, especially brain-derived neurotrophic factor (BDNF) in the regulation of neuronal survival has been increasingly studied since their discovery. For example, shortly after BDNF discovery, Alderson et al. (1990) showed that BDNF promotes survival of rat embryonic septal cholinergic neurons in culture (Alderson et al., 1990). Other studies indicate that exogenous BDNF protects hippocampal and cortical neurons from injury (Kume et al., 1997; Nakagami et al., 1997; Wu et al., 2009a). Administration of BDNF protects hippocampal/cortical neurons from ischemic (Galvin and Oorschot, 2003) and excitotoxic injury (Lindholm, 1994). Howerver, a significant challenge to its clinical use is the difficulty associated with delivering these proteins to the CNS. Here we constructed modified MABs genetically engineered with BDNF in order to develop a cell-based BDNF delivery strategy.

To assess the suitability of MABs for cell trophic factors delivery strategies, in vitro test is a necessary prerequisite to manifesting their efficiency on neurons and optimizing the intending cell-based therapeutic strategies as well. These results form the basis for developing potential MABs-based gene delivery of BDNF to treat neurological diseases associated with cell death. Therefore, the initial part of the study is to examine the by-stander effects of MABs engineered to produce and secrete BDNF. In this part, the media collected from MAB cultures (containing BDNF and other constitutively produced NTFs) was applied to two in vitro systems, primary hippocampal neuronal cultures and organotypic hippocampal slice culture (OHSC).

We focused on the effects of MABs conditioned media in the OHSC because it preserves the morphological and physiological features of the hippocampal neuronal network and allows easy access and precise control of extracellular environment for a short or long period (Stoppini et al., 1991; Kristensen et al., 2001). Organotypic cultures made from slices of explanted tissue provide an ideal in vitro model system to assess toxic or trophic effects of the test agents. In general, the OHSC is prepared from early postnatal animals not older than postnatal day 10

(P10) (Gahwiler et al., 1997), whereas the organotypic cultures from adult hippocampal slices lead to spontaneous and continuous neuronal degeneration (Wilhelmi et al., 2002). A slice culture from adult animals would however be desirable to serve as a model system to assess neuroprotective or neurodegenerative influence, considering its better simulation of brain pathophysiology of adult patients. We elected to employ hippocampal cultures prepared from adult animals because they do not survive well in standard culture media (Xiang et al., 2000) and, thus, may provide a very convincing evidence of the favorable effects of the supplementation of NTFs if they survive when cultured in the MAB media.

2.2 Methods

Animals

Swiss mice (Morini Co., Italy) were used in this study. Animals were housed in a 12-h light/dark cycle with food pellets and water available ad libitum. All procedures for animal care and treatment were in accordance with European Community and national laws and policies. All efforts were made to minimize animal suffering.

MABs-BDNF and cell culture

In order to achieve ex vivo cell-based gene delivery, MABs was transfected with a reporter green fluorescent protein (GFP) and with the human BDNF gene, obtaining a stable cell line (MABs-BDNF) that can constantly express BDNF. The control MABs were only engineered with GFP. These experiments were performed by Raffaella Scardigli and Antonino Cattaneo at the EBRI institute in Rome.

Pure cultures of MABs were grown using a culture media (Medium-1) consisting of: 88% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 0.5% GlutaMaxII, 22 mM mg/ml glucose, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin (all from Gibco, Invitrogen). After 2 days in culture, the medium was collected and centrifuged for 5 min at 1000 rpm, and then the supernatant was collected. Thus, the supernatant consist of the media containing the soluble substances secreted by MABs, including BDNF in the case of MABs-BDNF.

Enzyme-linked immunosorbent assay (ELISA)

The soluble BDNF levels for either MABs-BDNF, MABs, and control media were quantified by ELISA at different culture days. An ELISA Kit (Chemicon) was used to measure the amount of BDNF in the supernatant according to the manufacturer's instructions. Briefly, culture supernatants were divided equally into two Eppendorf tubes and stored at -80℃ till further processing. In order to standardize the total amount of protein from each experimental group used for ELISA, the content of one Eppendorf tube from each sample was used for measurements of protein content using Bio-Rad protein assay with BSA as standard.

ELISA microtiter plates were coated with anti-BDNF antibody (dilution 1:2000) in carbonated coating buffer ($NaHCO_3$ 0.025 M, Na_2CO_3 0.025 M) 14 – 18 h at 4℃. After washing in Tris – HCl-buffered saline with Tween-20 (TBST), the plates were incubated with blocking buffer (supplied by the manufacturer) for 1 h at RT and then washed in TBST, before the samples and standards (standards ranging from 40 to 4000 pg/ml) were added in duplicates. The samples and standards were incubated for 2 hrs, the plates washed in TBST, and anti-human BDNF antibody (diluted 1:2000 in blocking buffer) applied for 2 h at room temperature. After gentle, but thorough washing in TBST, anti-Ig coupled to horseradish peroxidase (diluted 1:2000 in blocking buffer) was added for 1 h at room temperature, and washed again in TBST before the color developer (supplied by the manufacturer) was added. The color reaction was stopped after 10 min by 1 M phosphoric acid, and the intensity of individual wells measured in an ELISA reader (wavelength 450 nm). BDNF levels in the media are reported in pg/ml ± standard error of the mean (SEM).

Primary hippocampal neuron cultures

Primary hippocampal neuron cultures were derived from P0 newborn Swiss mice. Hippocampi were dissected and minced with forceps, and then completely dissociated into a single-cell suspension using trypsin digestion. Isolated hippocampal cells were plated at a low density of approximately $5 \times 10^4 \cdot cm^{-2}$ viable cells in 24-well plates, which have been coated with poly-L-lysine (Sigma). Cells were grown in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin 50 U/ml and streptomycin 50 mg/ml. The culture media was replaced with the different conditioned media (shown below) supplemented with 5μM Ara-C at the day after plating. The cells were maintained at 37 ℃ under a humidified atmosphere of 95% air and 5% CO_2.

Organotypic cultures from adult hippocampal slice

Hippocampal organotypic slice cultures were prepared as described by Stoppini et al. (1991) with slight modifications. Young adult male Swiss mice (30 – 50 g, 4 weeks old, Morini, Italy) were briefly anesthetized by diethyl ether and decapitated. The hippocampi rapidly dissected out in ice-cold artificial cerebrospinal fluid (aCSF) consisting of (in mM): NaCl 118, KCl 2.5, $MgSO_4$ 3, NaH_2PO_4 1.1, $NaHCO_3$ 26, $CaCl_2$ 1 and glucose 11 (all reagents from Sigma) bubbled with 95% O_2/5% CO_2. The corpus callosum was bisected above the thalamus and the posterior margin of each cortical hemisphere rolled back. Subsequently, 300 μm thick coronal slices were cut with a vibrotome (MA752, Campden Instruments). The hippocampi were carefully dissected in cold, oxygenated Hank's balanced salt solution (Gibco, Invitrogen), transferred onto sterile porous membrane confetti (Millicell, Millipore), and cultured with their standard media (Medium-2) or with the MAB-conditioned media (see below). The incubation conditions were maintained in a humidified 5% CO_2 atmosphere at 37℃. The culture media was changed the day after preparation and then every 2 – 3 days for the course of the experiment.

Preparation of the conditioned media

The effects of the MABs delivering BDNF on cell survival were evaluated in several experimental groups cultured with a special conditioned media. In the groups "MABs" and "MABs-BDNF", the condition media was composed by the mixture of equal volumes of fresh Medium-1 and of the 2-day-culture supernatant from MABs and MABs-BDNF, respectively. The group called Medium-1 served as a control, being challenged with the fresh Medium-1. Medium-2 is a standard culture media for OHSC from postnatal animals, consisting of 50% MEM, 25% horse serum, 18% HBSS, 4 mM L-glutamine, 12 mM glucose, 4.5 mM NaHCO3, 20 mM sucrose, 100 U/ml penicillin and 100 mg/ml streptomycin (from Gibco or Sigma). The other conditioned media were based on the above media with different supplementations of reagents, like the recombinant human BDNF (ranged from 0.03 ng/ml to 300 ng/ml, Immunological Sciences), the BDNF antagonist K252a (50 nM, Sigma) or TrkB-IgG (2 μg/ml, R&D Systems), a recombinant tyrosine kinase receptor B (TrkB) engineered as an immunoadhesin to sequester BDNF. Sister slices were randomly assigned to the different groups.

FDA hydrolysis assay of cell survival

The viability of the neurons in primary culture and the slices in organotypic culture was assessed in two ways. First, the fluorescein diacetate (FDA) hydrolysis assay was used to measure enzyme activity in cells. Living cells actively convert the non-fluorescent FDA into the green fluorescent compound "fluorescin", an indication of cell viability. Slices were incubated with 10 μg/ml of FDA (Sigma) for 30 min, then images were taken using a optical microscope (Leica, Germany) and the fluorescence intensity was quantified using the software Image-Pro Plus 6.0 (Media Cybernetics, USA).

Lactate dehydrogenase (LDH) assessment of cell death

The lactate dehydrogenase (LDH) release assay was used to measure cell death in vitro. LDH is a stable cytoplasmic enzyme present in all cells that is rapidly released into the culture media upon damage of the plasma membrane; thus, LDH leakage to the media is a marker for cell damage. We measured LDH activity to quantify cell injury, according to the method outlined in (Noraberg et al., 1999). The culture media during the OHSC was all collected and then quantified the LDH leakage by using a LDH Cytotoxic Test kit (Clontech) according to the manufacturer's instructions. In brief, samples culture media was collected from each well, whilst maintaining the total volume of media. Samples were stored at -20 ℃ until analysis. Samples of media were added to pyruvate and nicotinamide adenine dinucleotide (all provided from the kits) in TBS, and the absorbence of the mixture was measured, using a spectrophotometer (DU 530; Beckman, High Wycombe, UK), at 30 ℃.

Tissue processing

Cultured slices were fixed with 4% paraformaldehyde (PFA) in phosphate

buffer solution (PBS) for 1 h at room temperature. Cultures were transferred to a 30% sucrose solution for 24 h. Single cultures were then placed on the flat base of a dish, and the excess liquid removed using filter paper. The membrane on the bottom of slices was detached carefully under optical microscope. The slices were then placed onto the stage made of OCT embedding matrix (Kaltek, Italy). A drop of OCT embedding matrix was applied to cover the slice until frozen. Slices were sectioned to a thickness of 30 μm in a cryostat (Leica, Germany), where the chamber temperature was maintained below -20 ℃. The embedded frozen culture was then mounted on an orientating chuck and careful approach sections were taken, correcting the orientation of the culture until cutting blade and culture lay in parallel planes. The initial two sections were discarded as they generally were incomplete and the topmost part of OHSC has a glial scar. The next sections were collected on separate gelatinised slides, this process was repeated collecting another section on each slide, and the sections stored at -20 ℃ for further processing.

Immunohistochemistry

Adjacent sections from each culture were taken from -20 ℃ and put in 4 ℃ for 30 min, and then incubated in PBS (pH 7.4) for 30 min. Sections were incubated with 0.3% triton in PBS for 1 h. PBS with 5% BSA and 5% normal goat serum was used to block the slices for 30 min, and after that they were incubated overnight with the primary antibodies anti mouse microtubule-associated protein (MAP2abc; 1:50; Immunological Sciences) Finally, they were rinsed with PBS and incubated with the secondary antibody Alexa 488 goat anti mouse IgG (1:100). The slices were mounted onto slides after staining with DAPI (1:1000).

Western blot

To identify the density of the survival neurons within a slice, we performed immunoblot analysis of the neuron-specific marker neurofilament 68. After 14 days in culture, slices were rinsed with ice-cold PBS and then lysed in sample buffer (ECL western blotting kit, Amersham). Aliquots from each sample (15 μg protein/lane) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidine fluoride membranes (Millipore). The blots were blocked in blocking buffer (20 mM Tris-HCl, 137 mM NaCl, and 5% skim milk) for 1 h at room temperature and then treated with anti-neurofilament 68 antibody (diluted 1:500; Sigma) overnight at 4 ℃. Membranes were washed repeatedly in Tris-buffered saline containing 0.05% Tween 20, and then the HRP-conjugated secondary antibody (diluted 1:20000) was added for 1 h. Immunoreactive bands were detected using enhanced luminol-based chemiluminescence (ECL). The membranes were stripped and then immunoblotting for beta-actin (1:1000, Sigma) performed, as a loading control. Bands were scanned into digital images and analyzed with the software of Image-Pro Plus (Media Cybernetics, USA).

Field potential recording

After 7 days in culture, slices were transferred to a holding chamber for 1 hour at room temperature in aCSF, while continuously aerated with 95% O_2 and 5% CO_2.

Slices were then placed in a submerged in vitro recording chamber and perfused with oxygenated aCSF. The temperature in the recording chamber was kept at 36±1 ℃. Bipolar wire electrodes (tungsten with a tip diameter of 90 μm; WPI Inc.) were used for stimulation of the Schaffer collateral pathway in the CA_3 region (70 μs duration rectangular pulses at 0.05 Hz for 120 s). Glass microelectrodes filled with 0.9% NaCl were placed on the CA1 stratum radiatum to record field excitatory postsynaptic potential (fEPSP). A conventional electrophysiological technique of extracellular recordings was employed to identify the maximal response and to adjust the stimulus strength. The stimulus intensity that could repeatedly evoke the maximal synaptically evoked response/excitatory postsynaptic current (up to 900 μA) was used in each slice. Signals were acquired under constant conditions, and off-line processed using the Patchmaster software (HEKA Instruments Inc., Germany).

Statistical analysis

All values are expressed as mean±S.E.M. Analysis of variance (ANOVA) and post hoc Bonferroni test or Newman-Keuls test were employed.

2.3 Results

2.3.1 Recombinant BDNF exhibits concentration-dependent protective effects on cell survival

First, we tested if continuous application of recombinant BDNF (rBDNF) in the media exerted trophic effects on cell survival in low-density primary cultures of hippocampal neurons (PCs) and in organotypic cultures of adult hippocampus slices (OCs). Thus, culture media were supplemented with increasing concentrations of rBDNF, ranging from 0.03 ng/ml to 300 ng/ml.

In PCs, neurons seeded at low density can hardly survive without the support of rBDNF. Quantification of survival, based on cell counting and on LDH release, showed that BDNF produces a significant beneficial effect in a concentration-dependent manner **(Fig. 1A and 1B)**. The maximal effect was reached at a concentration of approximately 2×10^{-5} M (slightly more than 300 ng/ml) and the EC50 was approximately 5×10^{-6} M (slightly less than 100 ng/ml). These data are in keeping with previous findings (Cheng and Mattson, 1994; Kokaia et al., 1994a).

We also examined the neurotrophic activities of rBDNF in OCs. In the absence of rBDNF supplementation, OCs displayed degeneration aspects (like white spotted cell debris and uneven surface) after 6-8 DIV, that were clearly visible under phase contrast microscopy. Similar to PCs, rBDNF concentration-dependently reduced these signs of degeneration. Viability was quantified using two assays: FDA fluorescence intensity, to identify viable cells; LDH release, to identify the degree

of cell death. As shown in **Fig. 1C** and **1D**, BDNF concentration-dependently increased FDA fluorescence, in line with previous observations reporting that BDNF (50-100 ng/ml) enhances the cell tolerance to serum-deprivation of organotypic culture from postnatal slices (Pringle et al., 1996; Nakagami et al., 1997), and reduced LDH release. These effects were obtained in the same range of concentrations that proved effective for PCs.

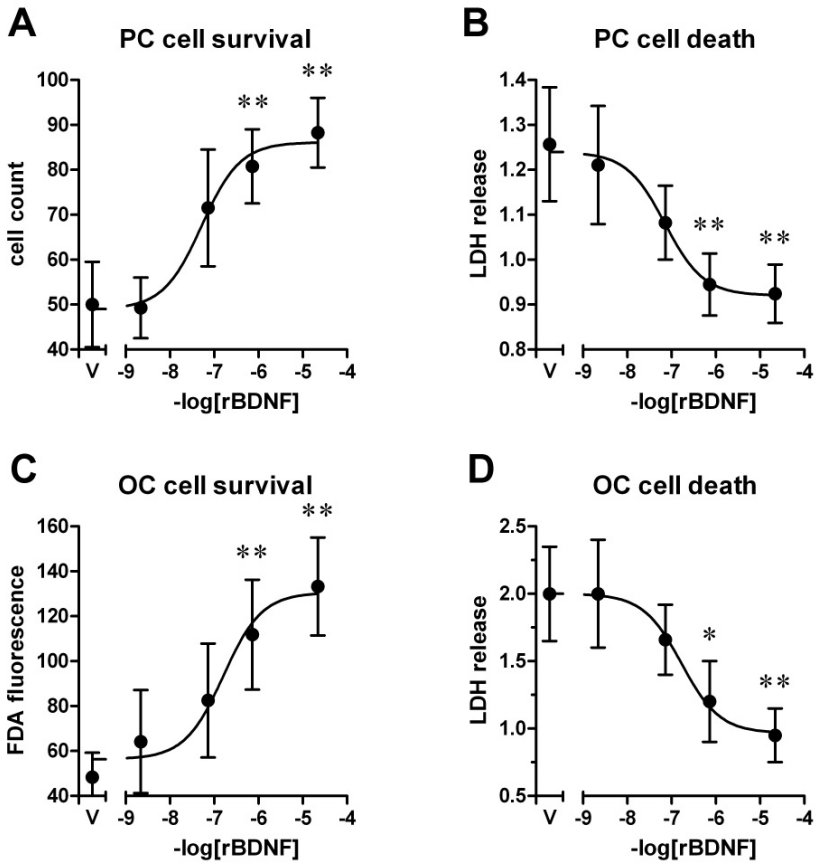

Fig. 1. Recombinant BDNF enhances cell survival in primary hippocampal neuronal cultures (PC, panels **A** and **B**) and in organotypic adult hippocampus slice cultures (OC, panels **C** and **D**) in a concentration-dependant manner. Neurons were cultured with the indicated concentration of BDNF for 7 days, slices for 14 days. Panels **A** and **C** indicate viable cells, as quantified using cell counting for PCs and fluorescence intensity of the marker FDA for OCs. Panels **B** and **D** represent cell death, as estimated measuring the levels of released LDH. Data are expressed as the mean±SEM (n=6). * P<0.05, ** P<0.01 vs. control (0 ng/ml); ANOVA and *post-hoc* Newman-Keuls test.

2.3.2 Soluble mediators from MABs engineered to produce BDNF highly improve cell survival

We engineered a line of MABs (MABs-BDNF) to stably produce and secrete human BDNF and the green florescence protein (GFP) as a tracer. These cells can be cultured long-term in vitro and constantly express BDNF and GFP (**Fig. 2A and 2B**). The amount of BDNF secreted into the media (Medium-1) from MABs-BDNF after 2 days in culture was approximately 2 ng/ml, as measured using an ELISA assay (**Fig. 2C**). If MABs-BDNF were cultured with the media usually employed to culture OCs (Medium-2), the amount of secreted BDNF was approximately halved (**Fig. 2C**). Therefore, although controls were also performed using Medium-2, the conditioned media that we used in all experiments was based on Medium-1: 50% culture supernatant from 2-day-cultured MABs-BDNF and 50% fresh Medium-1. Thus, according to the ELISA measures, this conditioned media contained approximately 1 ng/ml BDNF, whereas no detectable of BDNF was present in the media conditioned with control, conventional MABs (**Fig. 2C**).

We first screened the trophic effects of the MABs-BDNF conditioned media in the PC system. The MABs conditioned media increased the number of surviving neurons by about 80%, a level similar to the effect of 300 ng/ml rBDNF. The effect of the MABs-BDNF conditioned media was much greater (it enhanced neuronal survival by approximately 170%). We used the LDH assay to measure these effects: as shown in **Fig. 2D**, both 300 ng/ml rBDNF and the MABs media significantly (and to a similar extent) reduced cell death, but the MABs-BDNF media had a significantly greater effect. Interestingly, 1 ng/ml rBDNF (i.e. the concentration of BDNF present in the media) alone did not produce a significant effect on neuronal survival. Together with the observation of a significant effect of the MABs media, this indicates that other trophic factors are secreted by MABs that can synergize with BDNF to produce neuroprotection.

We then extended and deepened this analysis in OCs. Adult OCs were cultured with different media and, after 14 DIV, cell death was measure using the LDH release assay. As shown in **Fig. 2E**, cell death was remarkable in control media (Medium-1 and Medium-2, see also **Fig. 3A**). As with PCs, the MABs-conditioned media significantly decreased cell death, to nearly the level of rBDNF 300 ng/ml, and the MABs-BDNF-conditioned media produced a significantly more pronounced effect. This extra-effect is mediated by the low concentrations (1 ng/ml) of BDNF produced by the MABs-BDNF, because (i) adding 1 ng/ml rBDNF to the MABs media produced an effect similar to the one produced by the MABs-BDNF media; (ii) the TrkB inhibitor K252a and the BDNF scavenger TrkB-IgG to the media conditioned by MABs-BDNF abolished the extra effect observed in the MABs-BDNF group, while these two BDNF blockers had no significant influence on the media conditioned by MABs (**Fig. 2E**). These data support the notion that the pro-survival effect of the MABs-BDNF media is mediated by a synergy between BDNF and other, yet unidentified, soluble substances constitutively produced by MABs. Accordingly, 1 ng/ml rBDNF alone did not reduce cell death at all, and much higher concentrations (300 ng/ml) were needed to almost reach the effect of the MABs-BDNF conditioned media.

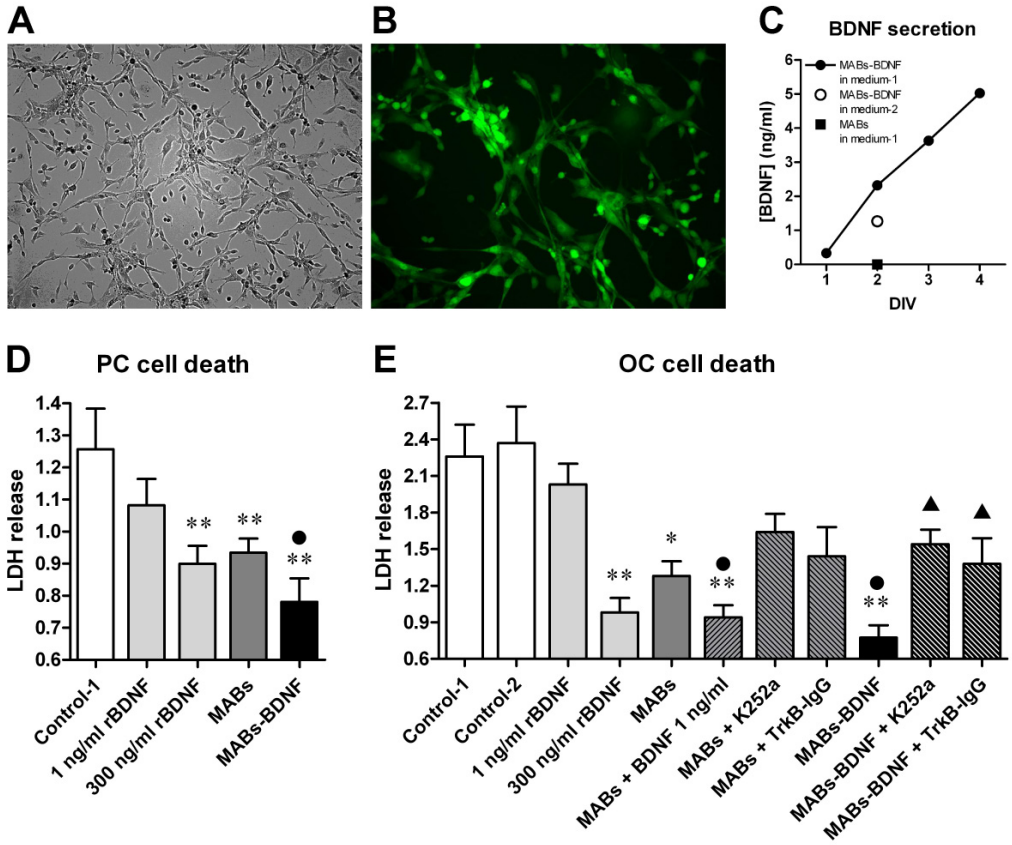

Fig. 2. MABs-BDNF deliver BDNF and enhance cell survival in primary hippocampal neuronal cultures (PC) and in organotypic adult hippocampus slice cultures (OC). (**A**) and (**B**): genetically modified MABs-BDNF proliferate and constantly express GFP. (**C**) MABs-BDNF secrete BDNF. Concentration of BDNF in the medium of MABs and MABs-BDNF at various days in vitro (DIV), as measured using ELISA. MABs-BDNF were cultured in 2 different types of medium (Medium-1 and Medium-2, see text for details) as indicated. (**D**) Effect of different treatment procedures on the viability of hippocampal PCs, as estimated using the LDH release assay. Data are the mean±SEM of 6 replicates in separate experiments. ** P<0.01 vs. Control-1; ● P<0.05 vs. MABs; ANOVA and *post-hoc* LSD test. (**E**) Effect of different treatment procedures on the viability of adult hippocampus OCs, as estimated using the LDH release assay. Data are the mean±SEM of 6 replicates in separate experiments. * P<0.05 and ** P<0.01 vs. Control-1; ● P<0.05 vs. MABs; ▲ P<0.05 vs. MABs-BDNF; ANOVA and *post-hoc* Newman-Keuls test. Control-1 are slices treated with the typical medium for MABs culture (Medium-1). Control-2 are slices treated with the typical medium for postnatal slice culture (Medium-2).

2.3.3 The MABs-BDNF-produced mediators sustain neuronal structural integrity in adult organotypic slices

The above findings have been confirmed at the light microscope and using FDA fluorescence, an indicator of viable cells. At the bright field observation, as compared with slice cultures in the control media (which, as described, display obvious signs of degeneration, **Fig. 3A**), the slices cultured in the media conditioned on MABs-BDNF remained thicker and preserved their structural organization for a long period, up to 25 days (**Fig. 3C**). Slices cultured in the MABs media appeared healthier that those cultured in control media but less healthy that those cultured in the MAB-BDNF media (**Fig. 3B**). In line with these findings and paralleling to the high levels of LDH release described above, FDA fluorescence was weak and uneven in OCs cultured with control media (**Fig. 3D**), indicating that most of the cells are indeed degenerating. OCs cultured with the MABs media displayed a better labeling quality **(Fig. 3E**), which was further improved in OCs cultured with the MABs-BDNF media (**Fig. 3F**).

Cell counting, performed by using the DAPI nuclear stain, confirmed that, even if some cell loss was present in slices cultured with MABs-BDNF conditioned media (**Fig. 3I**), the number of surviving cells was much higher than in the control group (**Fig. 3G**). Slices treated with the media conditioned in MABs performed a little better that controls, but much worse than those treated with the media conditioned in MABs-BDNF (**Fig. 3H**).

We also performed other morphological examinations and electrophysiological recordings. For morphological analysis, slices were fixed and immunostained with microtubule-associated protein 2 (MAP2). MAP2 is a cytoskeletal protein primarily found in neuronal dendrites. As shown in Fig. 3J, adult slices cultured in the control media for 14 days exhibit not only extensive cell loss, but also a grossly altered structure, with many lacunae in the pyramidal cell layer and just a few remaining, exclusively pyknotic neurons. MAP2 staining was essentially absent in control slices. While the MABs media only slightly improved this situation (**Fig. 3K**), the one conditioned in MABs-BDNF clearly attenuated the neuronal loss (**Fig. 3L**). The dendritic network and the cytoarchitectonic characteristics were essentially maintained in adult OCs treated with the MABs-BDNF conditioned media, although signs of neuronal degeneration could still be detected, with loss of neurons, decreased dendrites, and many condensed or swelling pyramidal cells. These signs of degeneration progressively increased in time. Nonetheless, this morphological analysis further confirms that the media conditioned in MABs-BDNF enhances neuronal survival and preserves structural organization.

The density of neurons in the slices cultured with the different media was also assessed using Western blot analysis for the neuron-specific marker neurofilament 68. We found that OCs cultured on MABs-BDNF conditioned media contained more neurofilament 68, thus more neurons, as compared with the slices cultured in the control media or in media from conventional MABs (**Fig. 4**). A statistically significant difference was also found between the MABs and the control group, indicating that supplementation of the soluble substances produced by MABs can slightly (but significantly less efficiently than with BDNF) attenuate neuronal loss.

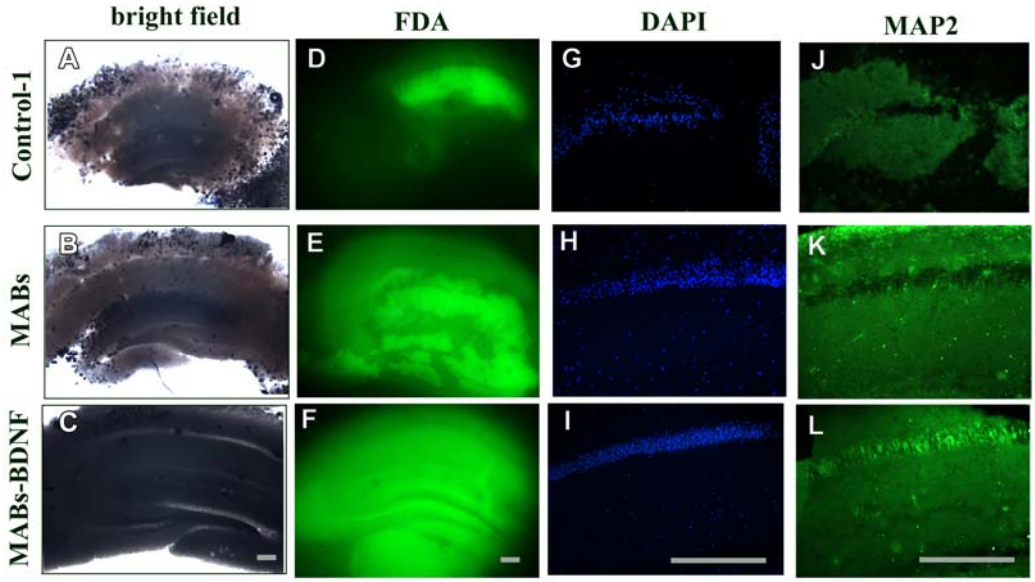

Fig. 3. Morphological evidence of the beneficial effects of the medium conditioned in MABs-BDNF on adult organotypic cultures. Adult slices were cultured for 14 days with the control Medium-1, with medium conditioned in MABs, or with medium conditioned in MABs-BDNF. Note that slices cultured in the MABs-BDNF conditioned medium, compared with the other two groups, better maintain their integrity based on bright field observation (**A-C**), remain more viable based on FDA (**D-E**), DAPI staining (**G-I**) and MAP2 immunohistochemistry (**J-L**). Nuclei in blue in **G-I**, neurons in green in **J-L**. Scale bar: 200 μm.

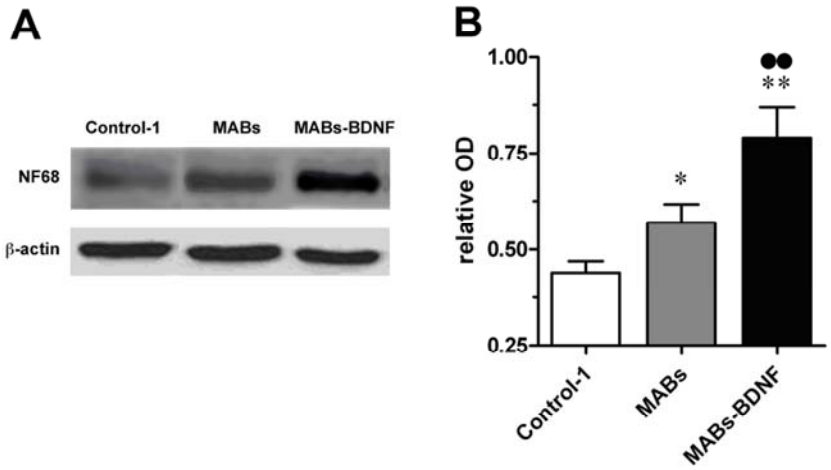

Fig. 4. Density of neurons surviving in adult slices after 14 days in vitro, under the different experimental conditions. Neuronal density was quantified performing Western blot analysis for a neuron-specific marker, neurofilament 68. (**A**) Representative blot. (**B**) Data quantification. The data are mean±SEM of 4 separate experiments. * P<0.05 and ** P<0.01 vs. Control-1; ●● P<0.01 vs. MABs; ANOVA and *post-hoc* LSD test.

2.3.4 Functional benefits afforded by the MABs-BDNF conditioned media in the cultured slices

We performed electrophysiological recordings to demonstrate the viability of the surviving neurons and the persistence of synaptic connections at a functional level. We measured fEPSPs in CA1 pyramidal neurons after stimulation of the Schaffer collaterals. We chose 7 DIV as a checking time point, because adult slices in culture, even those cultured with the MABs-BDNF conditioned media, display a progressive decrease in the amplitude and stability of the extracellular field potentials in time (data not shown), such that currents could not be reliably measured at 14 DIV, when neurons are still visible using immunofluorescence: this is an indication that, as expected, the functional damage precedes the morphological one. As shown in **Fig. 5A**, adult slices cultured with the typical media exhibited very small (if any) synaptic response at 7 DIV, while relatively stable fEPSPs could be recorded in those cultured with the media conditioned in MABs or MABs-BDNF. However, the mean amplitude of the evoked fEPSPs in the MABs-BDNF group was much higher than in the MABs group (**Fig. 5B**). The presence of evoked synaptic currents, albeit lower and less stable than in acute slices or in cultured postnatal slices, in adult OCs cultured with the MABs-BDNF-conditioned media demonstrates that the neuronal activity and the hippocampal circuitry remain functional by virtue of the beneficial effects of the mediators secreted by MABs-BDNF.

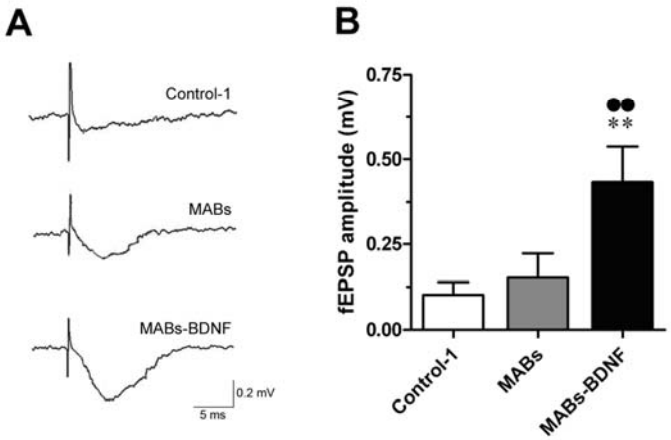

Fig. 5. Maintenance of synaptic connections in adult slices after 7 days in culture with the MABs-BDNF conditioned medium. Synaptic connections were identified by recording evoked fEPSP in CA1 after stimulation of the Schaffer collaterals. (A) Representative recordings of the evoked fEPSP in the different groups. (B) Peak amplitude of the fEPSPs, measured and statistically analyzed as described in the Materials and Methods. The data are the means SEM of 5 separate experiments. ** P<0.01 vs. Control-1; ●● P<0.01 vs. MABs; ANOVA and post-hoc LSD test.

2.4 Discussion

2.4.1 Protective Effects of rBDNF on Neurons

We first showed that continuous application of recombinant BDNF promoted the survival of the cultured neurons seeded in low-density. These results are consistent with those of Lindholm et al. (1996), who showed that cultured hippocampal neurons die spontaneously when are plated at low (but not at high) density (Lindholm et al., 1996). Spontaneous death of hippocampal neurons plated at low density can be prevented by administration of BDNF. It was reported that addition of anti-BDNF antibodies (but not of antibodies to other NTs) to the culture media reduced survival of cultured cortical neurons (Matsumoto et al., 2003), but could not prevent the survival-promoting action of high potassium-induced depolarization (Armanino et al., 2005). The results highlight the pronounced promotion of neuronal survival by BDNF. However, several reports hold different opinions about the effect of BDNF on cultured hippocampal neurons. For instance, Ip et al. did not find any survival promoting effect of BDNF on embryonic hippocampal neurons, even though cultured hippocampal neurons express TrkB and show phenotypic changes after exposure to BDNF, such as an increased expression of c-Fos, calbindin, and AChE (Ip et al., 1993). Similar findings, in which BDNF-induced intracellular events lack survival-promoting effect, have been reported by Marsh and Palfrey (1996) in highly enriched cultures of rat hippocampal pyramidal neurons (Marsh and Palfrey, 1996). Lowenstein and Arsenault (1996) found evidence of increased survival of hippocampal dentate granule neurons in cultures treated with exogenous BDNF when dentate granule cells were plated at low density (Lowenstein and Arsenault, 1996). Furthermore, the authors found increased death rates for cultured hippocampal neurons from BDNF knockout mice relative to wild-type mice, and a survival promoting action of exogenous BDNF on cultured pyramidal neurons lacking the BDNF gene, suggesting an autocrine/paracrine effect of endogenous BDNF on survival of hippocampal pyramidal neurons (Lindholm et al., 1996). It seems that hippocampal neurons can survive and differentiate when plated at high density, thank to the existence of functional autocrine/paracrine trophic loops in the culture. Exogenous BDNF displays a noticeable effect only when hippocampal neurons are plated at low density, when autocrine/paracrine loops are presumptively not functional (Lindholm et al., 1996). All in all, the presence of either exogenous or endogenous BDNF is indispensable for neuronal survival.

Moreover, we showed that continuous application of recombinant BDNF provided protective effects on the adult organotypic hippocampal slice culture in a concentration-dependent manner. The effective dose of the rBDNF is similar to the one in primary neuron culture (Lowenstein and Arsenault, 1996) and organotypic hippocampal slice culture from postnatal slices (Pringle et al., 1996; Nakagami et al., 1997). We here used the organotypic hippocampal slice culture from adult mice as a model of neurodegeneration. As we known, the age of the animal for OHSC is crucial for the maintenance of the cultured slices. Adult slices cultured in a typical media always result in rapid and continuous cell death within a slice. The spontaneous cell degeneration in cultured adult slices may be used as a model to

screen trophic or neuroprotective substances. There are also other models based on organotypic hippocampal slice culture from postnatal animals, in which cell death is induced by exogenous insults such as neurotoxins (Noraberg et al., 1998), oxygen-glucose deprivation (Wise-Faberowski et al., 2009), and trauma (Morrison et al., 2006). As opposed to these models based on postnatal slices, the cultured slices from adults display good regional morphology with less cell reorganization, and resemble the adult brain in gene expression profile, despite the unclear mechanisms of the spontaneous cell death. The consistent results in two independent assays, FDA hydrolysis and LDH release, suggest the feasibility of adult organotypic hippocampal slice culture as a model of degeneration. The mechanism underlying the pro-survival effects of BDNF in adult hippocampal slice cultures remains unclear. Nevertheless, these results suggest that hippocampal neurons may depend on BDNF support derived from their targets or exogenous supplementation for continued survival, not only during development but also in the adult nervous system. The pro-survival effects of BDNF found in adult neurons may be of great significance particularly for age-related neurodegeneration.

2.4.2 Protective effects of MABs and MABs-BDNF produced factors

The protective effect of rBDNF is of great interest for therapeutic purpose. To overcome the difficulties of rBDNF delivery in the brain, we pursue cell-base gene delivery strategy and generated the MABs-BDNF. We found that MABs-BDNF can be grown extensively in culture and stably secrete BDNF into the culture media. Although the secreting BDNF is at relative low concentrations, the conditioned media from the culture supernatant of MABs-BDNF still exerted extensive protective effects on the adult OHSC. According to our concentration-response assay with rBDNF, the concentration of rBDNF (1 ng/ml) equivalent to that of MABs-BDNF secreted BDNF, and even the highest dose of rBDNF (300 ng/ml) could not extend cell survival as much as MABs-BDNF, indicating the MABs-BDNF produced not only BDNF but also some other unknown survival factors. Indeed, the conditioned media from control MABs did enhance cell survival of the cultured adult slices when compared with the control media. The result that the MABs-BDNF beneficial effects could be simulated by the combination of MABs and 1 ng/ml of BDNF reinforced the assumption that MABs-BDNF can produce two pro-survival components: the secreted BDNF, and the unknown soluble substances inherently secreted by MABs. It also seems that there is a synergistic effect between BDNF and the MABs produced unknown factors. As seen in **Fig. 2**, MABs-BDNF increased cell survival to a much higher extent, while the equivalent dose of BDNF alone or MABs just show a minor increase. The synergic effect of NTFs is a general phenomenon in the nervous system. A proper combination of different NTFs could enhance the trophic abilities and reduce possible side effects. MABs seem to be ideal for delivering BDNF since the delivery was effective at a low dose, which would reduce the side effects in future clinical application. So far, the effective components in the culture supernatant of MABs have not been identified. A micro-array analysis of gene

expression in MABs revealed that several growth factors are expressed at high level in these cells, including vascular endothelial growth factor B (VEGFB), basic fibroblast growth factor (bFGF), FGF7, platelet-derived growth factor AA (PDGF AA), hepatomaderived growth factor, and stromal derived factor 1 (Galli et al., 2005). It remains unknown which which of these factors may exert neuroprotective effects. It has been reported that various NTFs, growth factors, and cytokines have protective effects against neuronal degeneration (Lindsay, 1994). The benefit of MABs on adult slice survival is probably multifactorial, relating with various growth factors and chemokines, many of which may not even be known to us. It is worthy to note that MABs preferentially expressed many neural genes including receptors and transcription factors in spite of their mesodermal origin (Tagliafico et al., 2004). Therefore, it is reasonable that MABs could express some specific survival factors for neurons.

Together with the morphological analysis and the immunoblot assay for neuron-specific markers, the beneficial effects of the conditioned media from MABs-BDNF on the adult slice culture are solid. Moreover, the cultured adult slices in the conditioned media of MABs-BDNF showed a better synaptic response with higher peak amplitudes of fEPSP compared to the groups of MABs and the control media. The facilitated synaptic responses may ascribe to the better preservation of synaptic connection or the potentiated synaptic transmission. It is reported that application of BDNF could modulate the strength of existing synaptic connections (Huber et al., 1998; Jiang et al., 2001) and acts in the formation of new synaptic contacts within the hippocampal circuit (Lauterborn et al., 2007). In the morphological examination using immunohistochemistry for MAP2, only cultured adult slices in the group of MABs-BDNF retained low density of dendrites in the stratum radium after 14 days in vitro culture. It's necessary to know that the conditioned media from MABs-BDNF can reduce cell degeneration but completely prevent it. The cultured adult slices, even those in the MABs-BDNF conditioned media, exhibited continuous signs of degeneration during culture, such as PI-uptake, pyknotic cells, decreasing MAP2 signals and so on. The electrophysiological recordings also indicate that the cultured adult slices were not as healthy as the typically cultured postnatal slices, since the fEPSPs in adult slices were difficult to evoke relative to postnatal slices and almost disappeared after 10 days in vitro culture (data not shown). In the future, an improved culture method together with supplementation of the MABs-BDNF conditioned media might make it possible to achieve a successful organotypic culture from adult hippocampus slices, which is a technical bottleneck for many studies.

2.4.3 The possible mechanism of BDNF protective effects

Several inestigations indicate a pivotal role of BDNF in neuroprotection in different models of neurotoxicity, such as in vivo and in vitro ischaemic neuronal damage (Larsson et al., 1999; Kano et al., 2002), glucose deprivation (Tong and Perez-Polo, 1998) or glutamate-induced neuronal damage in neuronal cultures (Cheng and Mattson, 1994), kainate acid-induced excitotoxicity in the striatum (Gratacos et al., 2001) and hippocampal kindling (Larmet et al., 1995). In this study, we demonstrated the beneficial effects of rBDNF or MABs delivered BDNF on cell

survival in adult slice cultures. In fact, neuronal cell death as a result of neurotrophic factor deprivation is a physiological cell death mechanism which is commonly observed during neuronal development (Rich, 1992; Suzuki and Koike, 1997; Vizuete et al., 2001; Roceri et al., 2004). Many neurons within the vertebrate nervous system require the presence of NTFs such as NGF or BDNF for survival (Koike and Tanaka, 1991; Roceri et al., 2004). Neurons which fail to obtain a sufficient quantity of the necessary neurotrophic factor die by a process of programmed cell death (Wozniak, 1993). This process is thought to regulate the number of neurons and neuronal connections within the developing CNS. While the mechanisms involved in the neuronal loss that occurs following a reduction in neurotrophic support have been determined in the developing nervous system, researchers are still uncertain as to what implications deficits in trophic support may have in the adult CNS. It has been observed that several neuronal populations within the adult CNS require the presence of neurotrophic factors to maintain neuronal function and phenotype (Binder, 2004). If mature neurons are still dependent upon the presence of neurotrophic factors for survival and function then the loss of transcriptional suppression of the 'suicide programme' due to a reduction in neurotrophic factor expression may result in the neuronal atrophy seen in normal aging or the neuronal loss observed in neurodegenerative disorders such as Alzheimer's disease (Johnson et al., 1989). Indeed, it has been proposed that AD pathology may primarily be due to a deficit in neurotrophic factor protein and/or Trk expression (Hefti, 1983; Soontornniyomkij et al., 1999; Savaskan et al., 2000). Although it remains unclear if a similar reduction of BDNF and/or Trk expression occurs in the adult slice culture, the benefit of supplementation of exogenous BDNF might be a hint to reveal the mechanism underlying the cell death of adult neuron cultures.

It is presumed that BDNF is an essential constituent of the microenvironment for neuronal survival in the adult hippocampus. Actually, BDNF administration to cultured slices potentiates presynaptic release (Li et al., 1998) and promotes dendritic and axonal growth (Labelle and Leclerc, 2000). Independently, other studies have provided insight into the effects of BDNF on potentiating excitatory transmission (Huber et al., 1998; Jiang et al., 2001). Moreover, BDNF have the potential to protect diseased and injured neurons from dying, induce neuronal sprouting and to increase neuronal metabolism and function (Labelle and Leclerc, 2000; Chapleau et al., 2008). The signaling mechanisms underlying the pro-survival effects of BDNF appear to involve TrkB activation. A large part of hippocampal pyramidal and dentate granule cells co-express both BDNF and TrkB (Kokaia et al., 1993). In our results, the TrkB blocker K252a attenuated the protective effects of MABs-BDNF, indicating that the protection conferred by MABs-BDNF is mediated in part by the TrkB pathway. TrkB receptors have long been known to mediate trophic support of adult neurons (Ferrer et al., 1999), and a number of studies show that decreased expression of the receptor is associated with cell loss (Mesquita et al., 2002). TrkB regulates neuronal proliferation, survival, axonal and dendritic growth, assembly of the cytoskeleton, synapse formation and function (Barbacid, 1994). BDNF-TrkB signals have actions as anti-excitotoxins, antioxidants and can improve mitochondrial function. They upregulate calcium buffering proteins, antioxidant enzymes, and anti-apoptotic signals (Cui, 2006). High levels of intracellular Ca^{2+} which occuring during neurotoxic insults can cause mitochondrial dysfunction, free radical production, apoptotic signals, and activation

of many destructive proteases and lipases. By acting on intracellular calcium buffering proteins, BDNF can provide cells with a superior capacity to withstand dangerous cytosolic calcium effluxes and potential cell death (Cheng and Mattson, 1994; Nonner et al., 2000). However, there is still considerable controversy regarding action of BDNF. Many potential effects of BDNF are still unresolved. Our data showing that the continuous presence of BDNF is required in the adult slice culture to sustain neuronal survival will at least contribute to elucidating its roles in neuronal activity.

2.4.4 Neuroprotective strategies with NTFs

In this study, we demonstrate that soluble mediators secreted by the MABs-BDNF have profound survival effects on neurons. We found a potential merit of using MABs to deliver BDNF, which were the survival factors inherently secreted by MABs. The coaction of BDNF and MABs-produced survival factors led to a higher resistance to cell death and a better maintenance of neuronal network, and allowed the delivered BDNF exert a profound protective effect at a low dose. Besides, MABs possess the other natural advantages for cell-based therapy: they can be expanded to virtually unlimited numbers in vitro; they are amenable to genetic modification; they have self-renewal and multipotent capacities; they may specifically home in the damage area. These advantages raise the possibility that the MABs-BDNF might be developed into a new cell-based strategy for BDNF delivery into CNS, thereby promoting neuronal survival and restoring network function in those neurological diseases associated with brain damages. Transplantation of MABs-BDNF might become an alternative and possibly more efficacious therapy for brain diseases caused by neurodegeneration, including multiple sclerosis, stroke, epilepsy and trauma. The transplanted MABs-BDNF may protect neurons from damage through a “bystander ” mechanism, by which MABs-BDNF chronically secrete low doses of BDNF and other survival factors to provide an optimal environment for neuronal survival and tissue repair in the damaged brain.

There are a number of neuroprotective, as well as neurorestorative, strategies which are becoming available. Neuroprotection refers to the potential for reinforced defence against cell death, whereas neurorestoration refers to the recovery of functional variables, or symptoms, to levels comparable with pre-insult or "steady-state" levels. The objective of our study is to create a neuroprotective agent (MABs-BDNF) and meet the clinical necessities. Although the study just provides groundwork for future clinical application, it is conceivable that the MABs-BDNF will become a promising agent for neuroprotective strategies.

3. PART II - Effects of MABs-delivered BDNF on neurogenesis

3.1 Introduction

In the adult mammalian brain, new neurons are continuously generated from a proliferating population of neural progenitor/stem cells and become incorporated into the existing neuronal circuitry via a process termed adult neurogenesis (Lee and Son, 2009). The existence of active functional adult neurogenesis raises the exciting possibility that manipulating endogenous neural progenitors, or transplanting the progeny of exogenously expanded neural progenitors, may lead to successful cell replacement therapies for various degenerative neurological diseases. Significant effort is being made to decipher the mechanisms regulating adult neurogenesis, which may allow us to translate this endogenous neuronal replacement system into therapeutic interventions for many neurological diseases (Kuhn et al., 2001; Ormerod et al., 2008).

In rodents, there are two known neurogenic regions in the adult: the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Eriksson et al., 1998; Palmer et al., 2001; Alvarez-Buylla and Garcia-Verdugo, 2002). Growth factors are important components of stem cell niches (Horner and Palmer, 2003). Cells in the SVZ and DG respond to multiple extracellular factors, including EGF, FGF2, PDGF, BMPs, noggin, prolactin and erythropoietin, which influence SVZ proliferation and neurogenesis (Kuhn et al., 1997; Lim et al., 2000; Shingo et al., 2001; Zheng et al., 2004; Jackson et al., 2006). Recent findings indicate that neurotrophic factors play a fundamental role in adult neurogenesis (Lee and Son, 2009).

Particularly, BDNF has **b**een hypothesized to play an important role in hippocampal neurogenesis. Evidentiary are reports indicating that BDNF enhances neurogenesis in the different regions of the brain (Benraiss et al., 2001; Chmielnicki et al., 2004; Scharfman et al., 2005). A tremendous amount of correlative data suggests that BDNF is critical for regulation of mammalian adult neurogenesis in the SGZ of the hippocampus (Xu et al., 2004b). Intraventricular (Yan et al., 1994; Anderson et al., 1995) and intra-hippocampal infusions of BDNF resulted in enhancement of neurogenesis (Scharfman et al., 2005). Altered cell proliferation and diminished enhancement of neurogenesis by antidepressant treatment or dietary restriction were found in the hippocampi of BDNF+/- mice (Lee et al., 2002a; Sairanen et al., 2005). In vitro, BDNF promotes proliferation of NPCs (Barnabe-Heider and Miller, 2003) and increases neuronal production in neurosphere cultures (Young et al., 2007). Our previous data also showed that FGF-2 and BDNF, when supplied together but not when supplied individually, potently favor proliferation, survival and neuronal differentiation of neural progenitors in vitro in neurosphere cultures, and in vivo, in an epilepsy model (Paradiso et al., 2009). Nonetheless, controversy still exists about how BDNF influences proliferation and differentiation in neural precursor cells (Lee et al.,

2002b; Sairanen et al., 2005). More research is clearly needed to understand the role of this neurotrophin and its interplay with other growth factors in endogenous neurogenesis before it can be considered promising for therapeutic purposes.

In addition to the neuroprotection effects described in Part I, the present study was also undertaken to investigate the neurogenesis effects of the MABs-BDNF conditioned media in the organotypic hippocampal slice cultures (OHSC). OHSC offers an attractive model for studying neurogenesis both in the early postnatal and in the adult hippocampus, as they retain much of their anatomical circuitry and intrinsic functional activity for a number of weeks (Gahwiler et al., 1997; De Simoni et al., 2003). A slice culture containing various neural and nonneural elements is a more suitable *ex vivo* model for postnatal neurogenesis than a neurosphere culture. The easy access to in vitro environment allows precise experimental manipulation that is not possible in vivo and the visualization of cell morphology or function using fluorescent markers/probes within the same cultures over prolonged periods (Kamada et al., 2004). Hippocampal slice cultures can generate new neurons in vitro, as suggested by the observation that neural progenitor-like cells are found in the slice culture (Miyaguchi, 1997). Recent studies have shown that OHSC from postnatal day 5 – 10 pups preserved endogenous neuronal progenitor cells in the dentate gyrus, in which new neurons are spontaneously generated, and therefore are particularly suitable for studying neurogenesis (Kamada et al., 2004; Raineteau et al., 2004).

The neurogenic abilities in hippocampal slice cultures were identified using the 5-bromodeoxyuridine (BrdU) labelling method (Raineteau et al., 2004). Here we also assessed cell division by measurement of the uptake of BrdU, which is incorporated into the nuclear DNA of dividing cells. In these cultures, BrdU uptake will, of course, reflect gliogenesis as well as neurogenesis (Dehghani et al., 2004; Kamada et al., 2004). To distinguish between the two, the neuronal maturation of the BrdU labeled cells was assessed using a neuronal marker, the neuron-specific DNA-binding protein neuronal nuclear (NeuN). The brain slice culture system was here applied to study the effects on neurogenesis of BDNF and FGF2, and more importantly the by-stander effect of MABs-BDNF. As it turns out that the neural progenitor cells in hippocampus may repair a damaged brain (Paradiso et al., 2009), the possible neurogenesis effects of the transplanted MABs-BDNF may serve as "regenerative medicine" in which hippocampal stem cells are coaxed into forming neurons and migrating to regions of injury or degeneration to treat disease.

3.2 Methods

Animal

Adult pregnant females or male Swiss mice (Morini, Italy) were housed under constant temperature (21±1 ℃) and lighting (light on from 07:00 am to 07:00 pm) regimens. Food pellets and water were freely available throughout the experiment. Procedures involving animals and their care were conducted in conformity with European Community and national laws and policies. All efforts were made to minimize animal suffering.

Bromodeoxyuridine administration.

To label the immediately post-mitotic cells, the DNA synthesis marker 5-bromo-2-deoxyuridine (BrdU) was administered twice as previously described (Namba et al., 2007) with small modifications. Firstly, BrdU (Sigma) dissolved in 0.9% NaCl was injected intraperitoneally at a dose of 50 mg/kg into postnatal (P6) mice 30 min before slice preparation; the second administration was at 3 days before slice fixation at a concentration of 0.5 μM BrdU in the culture media, that was maintained for 3 days.

Organotypic hippocampal slice culture from postnatal mice

Hippocampal organotypic slice cultures were prepared similar to the adult slice culture in the PART I except the animals are postnatal (P6) swiss mice. The pups were decapitated without anesthesia and the hippocampi were rapidly dissected out in ice-cold artificial cerebrospinal fluid (aCSF) as described in PART I. Subsequently, 400 μm thick coronal slices were cut with a vibrotome (MA752, Campden Instruments). 1-2 slices with dorsal hippocampal in each pup were collected for culture. The number of granule cells and proliferating cells in the dentate gyrus of slice cultures taken from the full septo-temporal extent of the hippocampus, with granule cell layer size increasing from temporal to septal ends. To correct for possible confounding effects of the site of origin of the culture on the number of proliferating cells, position-matched cultures were taken from the full extent of each hippocampus from each animal. Position-matched slices from each hippocampus were then grown in either Medium-2 or the condition media so that each animal contributed a slice from one position (coronal section with dorsal hippocampus) matched to the culture condition groups. The allocation of matched slices to each group was also randomized with respect to hippocampal side.

The hippocampal slices were softer and more transparent in paleness. And they have to be carefully dissected in cold, oxygenated Hank's balanced salt solution (Gibco, Invitrogen) under stereo microscope. Those slices without visible blemish were transferred onto sterile porous membrane confetti (Millicell, Millipore), and cultured with their standard media (Medium-2) or with the MAB-conditioned media (see below). The incubation conditions were maintained in a humidified 5% CO_2 atmosphere at 37 ℃. The culture media was changed the day after preparation and then every 2-3 days for the course of the experiment.

Preparation of the conditioned media

As desribed in the PART I, the condition media in the groups "MABs" and "MABs-BDNF" was composed by the mixture of equal volumes of fresh Medium-2 and of the 2-day-culture supernatant from MABs and MABs-BDNF, respectively. The MABs and MABs-BDNF were cultured according to the methods described in PART I but replaced the Medium-1 with Medium-2, because we found Medium-1 is not as good as Medium-2 for the postnatal slice culture. Medium-2 is a standard culture media for OHSC from postnatal animals, consisting of 50% MEM, 25% horse serum, 18% HBSS, 4 mM L-glutamine, 12 mM glucose, 4.5 mM

NaHCO3, 20 mM sucrose, 100 U/ml penicillin and 100 mg/ml streptomycin (from Gibco or Sigma). The other conditioned media were based on the above media with different supplementations of reagents, like the recombinant human BDNF (Immunological Sciences), human FGF2 (Immunological Sciences) or their combination. Sister slices were randomly assigned to the different groups.

Culture sectioning

Following 1 hour in 4% PFA at room temperature, cultures were transferred to a 30% sucrose solution for 24 hours. The section protocol is described in PART I/Methods except the thickness was 10 μm.

BrdU/NeuN immunostaining.

Sections of control cultures and cultures exposed to different conditioned media were thawed and washed in PBS for 15 min. After washing in PBS, slices were exposed to 2 M HCl at 37 ℃ for 30 minutes and were subsequently washed in boric acid (0.1 M, pH 8.5,) for 10 minutes. Slices were then washed thoroughly in PBS, and blocked with 5% Goat serum and 5% BSA for 30 min. Subsequently, slices were incubated with rat anti-BrdU (1:50, Sigma) and mouse anti-NeuN (1:50, Chemicon) in 0.1% Triton PBS overnight. After thoroughly wash in PBS 3 times for 10 min, second antibodies goat anti-rat Tritc (1:50, Chemicon) and goat anti-mouse Alexa 488 (1:50, Chemicon) in PBS. Appropriate negative controls showed no nonspecific secondary antibody staining. Following multiple washes in PBS, the slices were cover-slipping with DPX (Sigma).

Cell counting

Unbiased stereological cell counting was carried out using a software (Leica) connected to a DMRA2 microscope (Leica) with a DFC300FX video camera (Leica) to obtain images at 10×, 20×, and 40× magnification. The number of proliferating of cells or newly born neurons was determined by counting the number of BrdU-positive cells and their colabeled with NeuN, respectively. The counting areas included the whole section, CA1, CA3, but mainly in the dentate gyrus. The border of interested area was first drawn with the mouse cursor. Within the counting frames, either BrdU single labeled cells or NeuN/BrdU double staining cells were counted. Cells in the granule cell layer that were obviously pyknotic were not counted when generating estimates of normal cell number. For all quantification, slides were coded and counts carried out with the examiner blind to the treatment of each section.

Statistics

Statistical analysis and graph composition was performed using the SPSS software (USA). All values are presented as means±SEM. A One-way ANOVA and post hoc Bonferroni test were used for multiple group comparisons. Statistical significance was established at $P < 0.05$.

3.3 Results

3.3.1 Neurogenesis in hippocampal slice cultures

Organotypic cultures from the postnatal mouse hippocampal slices were maintained in vitro for 14 days, when they are believed to have matured to a near adult state. The cultured slices displayed well defined neuronal cell layers resembling sections of the hippocampus in vivo, except that the neuronal cell layers were more spread out. The structural changes in OHSC took place largely within the first week and were stable in the following 2 weeks. No discernable differences were observed among the groups cultured with different media in any of the hippocampal subfields under bright field microscopy. Similarly, no overall structural differences were observed between control cultures and the cultures treated with 300 ng/ml BDNF and FGF2, or the MABs-BDNF conditioned media by using anti-MAP2 immunohistochemistry (data not shown).

To investigate in more detail neurogenesis and maturation of newly born neurons, BrdU was administered twice according to the Namba's protocol (Namba et al., 2007): BrdU was first injected at a dose of 50 mg/kg 30 min before sacrifice; the second administration was at 3 days before slice fixation with a concentration of 0.5 μM, and maintained for 3 days. Cultured in vitro for two weeks, the slices were processed for BrdU and NeuN immunoreactivity. Numerous BrdU-positive cells were observed at the surface of all regions of the slices (**Fig. 6**). An average of 213±29 BrdU-positive cells per 2500 μm^2 were detected in the DG region, 178±25 cells over the CA1 pyramidal cell layer and 189±24 over the stratum moleculare of the same region (n = 5). As already described by others (del Rio et al., 1991), a large proportion of the BrdU positive cells (>80%) was immunoreactive for the astroglial marker GFAP. These proliferating astrocytes were present as flat type-1 astrocytes at the border of the culture facing the glass coverslip, but also as reactive astrocytes (type-2 astrocytes) at the tissue/media interface, forming a protective shield around the slice. Proliferation of astrocytes was less pronounced but also presented within the slices in the areas CA1 and CA3 as well as in the DG. The proliferation of astrocytes is, started very early and persisted during the entire culturing period, leading to the formation of a thin cell layer covering the slice, analogous to a glia limitans (del Rio et al., 1991; Gahwiler et al., 1997).

Double immunostaining of the mitotic marker BrdU with the neuronal marker NeuN showed that BrdU/NeuN-positive cells were consistently observed in slices. On average, there were 9.2±1.8 BrdU/NeuN-positive cells in 10 μm thickness sections of the cultured slice. The cell bodies of these BrdU/NeuN-positive cells looked round and healthy, and the BrdU-immunoreactive signals were relatively light in contrast to other BrdU-positive cells (**Fig. 6**). These BrdU/NeuN-positive cells, representing the newly born neurons, were found throughout the entire depth of the hippocampal slices. The vast majority (over 90%) were distributed in the DG, especially in the inner layer, while a small proportion scattered in the other regions of the hippocampus such as hilus, CA1 and CA3. The distribution of these newly born neurons closely reflects the in vivo regional distribution in the adult hippocampus. On the basis of their morphological appearance and characteristic

location within the slice, these newly born neurons were mostly identified as granule cells.

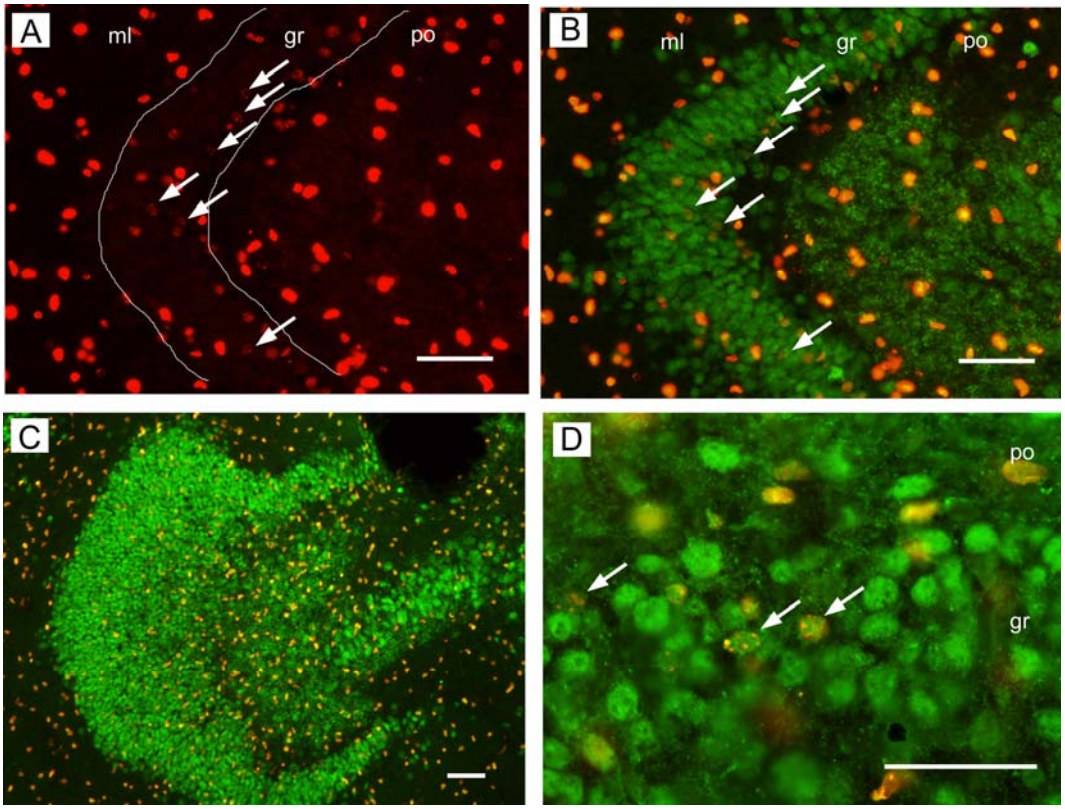

Fig. 6. Fluorescent immunohistochemistry for BrdU/NeuN in organotypic culture of postnatal mouse hippocampal slices, indication of spontaneous neurogenesis and proliferation of cells in the dentate gyrus at DIV 14. (A) shows the BrdU labeled cells. Note that only the cells with punctuate signals in nuclei are newly born neurons, indicated by arrows. (B) BrdU/NeuN double labeled cells (new born neurons), indicated by arrows. (C) Low magnification showing the numerous BrdU labeled cells, most of which are glia cells in proliferation. (D) New born neurons with punctuate BrdU signals in NeuN-positive nuclei. Abbreviation: po, polymorphic cell layer; gr, granule cell layer; ml, molecular layer. Scale bar: 150 μm.

3.3.2 Modulation of neurogenesis by NTFs and MABs-BDNF

We previously demonstrated BDNF and FGF2 gene delivery mediated by viral vectors could promote the survival of neural progenitors and induce more progenitors to differentiate into neurons in neurosphere culture. The pro-neurogenesis effects of the combined BDNF and FGF2 gene delivery were also shown in the hippocampus in an animal model of epilepsy. Here we confirmed the actions of BDNF and FGF2 on neurogenesis in the OHSC system by administration

of a defined dose of exogenous recombinant BDNF and FGF2. Moreover, we validated the hypothesis that the BDNF delivered by engineered MABs contribute to the proliferation and differentiation of endogenous neural stem cells, which suggests that MABs-BNDF might play a regenerative role in brain repair in future MABs-based therapy. To pursue the aims, different conditioned media (see the methods) were used in the OHSC, and maintained for 14 days.

First, the overall proliferation rates of glial cells were determined by counting the number of BrdU positive cells (as stated above, over 80% of BrdU positive cells are glial cells)(**See Fig. 7**). BrdU positive cells were counted in the dentate gyrus from non-overlapping pictures. In control cultures, the mean number of BrdU-positive cell was 72 ± 4.0 (n=5). Chronic combined supplementation of rBDNF and rFGF2 (300 ng/ml each) for 14 days upregulated the number of BrdU-positive cells to 102 ± 6.6 cells. Similarly, significantly more BrdU-positive cells were observed in the slices cultured with the MABs-BDNF conditioned media ($P<0.01$), whereas slices cultured with the conditioned media from MABs without genetically expressed BDNF displayed a small increase without statistical significance ($P>0.05$). There was significant difference between the group of MABs-BDNF and the group of MABs ($P<0.05$), even though the concentration of MABs delivered BDNF was as low as 1 ng/ml. Interestingly, the single supplementation of rBDNF at low concentration (1 ng/ml) failed to significantly increase the number. While a high concentration (300 ng/ml) slightly increase the BrdU+ cells ($P<0.05$) indicating that BDNF alone at a high dose could promote proliferation of glia cells to a minor extent. If 300 ng/ml rBDNF was administered with FGF2 (300 ng/ml), the proliferation rate was highly increased ($P<0.01$). Thus, BDNF exerted a stronger mitogenic effect if combined with other factors like FGF2 or MABs inherently produced factors.

Next, more importantly, BrdU/NeuN double labeled cells were also counted. The number of BrdU/NeuN positive cells in dentate gyrus in control media were 9.2 ± 1.8 (n = 5). Supplementation of 1 ng/ml rBDNF did not induce a significant change in the number of BrdU/NeuN positive cells. The higher concentration (300 ng/ml) induced a significant increase in the number of newly born neurons ($P<0.01$). The additional supplementation of 300 ng/ml rFGF2, did not further increase the effect of rBDNF. However, MABs delivered BDNF in the group of MABs-BDNF doubled the number of newly born neurons in control media, and also showed a significant increase compared to the MABs group ($P<0.05$). The conditioned media from MABs showed a moderate pro-neurogenesis effect compared to control. When comparing the MABs-BDNF group (consisting of 1 ng/ml BDNF and other inherently produced factors) and the 1 ng/ml rBDNF group, a significant increase was found ($P<0.05$), confirming the pro-neurogenesis effect of soluble mediators inherently produced by MABs. There was no difference among MABs-BDNF, MABs, 300 ng/ml rBDNF, and 300 ng/ml rBDNF/rFGF2.

These results demonstrate that both rBDNF and MABs-delivered BDNF can increase proliferation of glia cells and the number of newly born neurons differentiated from endogenous neural stem cells in the OHSC system. The additional supplementation of FGF2 to 300 ng/ml BDNF appears to be effective in proliferation of glia cells but not in the number of newly born neurons. The soluble mediators secreted from MABs contribute to proliferation of glia cells and neurogenesis to a small extent, but the effects can be augmented by the transgene

expression of BDNF. The conditioned media from the MABs-BDNF culture showed effects of gliosis and neurogenesis in the OHSC system, which may be due to the transgene expression of BDNF and inherently produced factors.

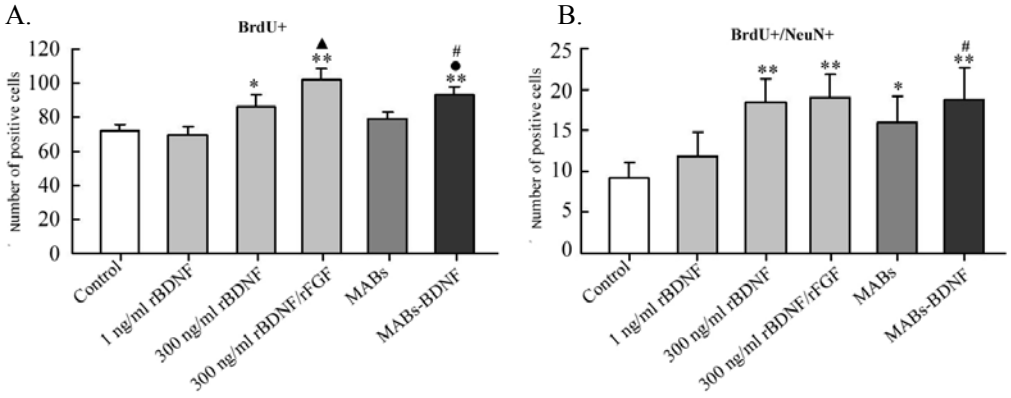

Fig. 7. Cell counting of BrdU+ and BrdU+/NeuN+ cells in the dentate gyrus of slices cultured with different conditioned media. (A) Number of cells incorporating BrdU per 10 µm section in the dentate gyrus of the slices cultured with the indicated medium. (B) Number of new born neurons labeled with BrdU and NeuN in the dentate gyrus. Data are the mean±SEM of 5 replicates in separate experiments. * $P<0.05$ and ** $P<0.01$ vs. Control; ● $P<0.05$ vs. MABs; ▲ $P<0.05$ vs. 300 ng/ml rBDNF; # $P<0.05$ vs. 1 ng/ml rBDNF; ANOVA and post-hoc Student-Newman-Keuls test.

3.4 Discussion

3.4.1 Neurogenesis in OHSC

In the present study, we used long-term hippocampal organotypic cultures as an ex vivo system for the study of neurogenesis, in which neurogenesis occurs spontaneously and maturation and regional distribution closely resemble those observed in living animals. Application of hippocampal organotypic slice cultures for the story of neurogenesis is relatively rare (Kamada et al., 2004; Raineteau et al., 2004; Chechneva et al., 2005; Laskowski et al., 2005; Poulsen et al., 2005; Hoareau et al., 2006; Sadgrove et al., 2006; Lossi et al., 2009). These studies examined neurogenesis through BrdU incorporating method in the GCL/SGZ of OHSC in different species using different culture methods, demonstrating the presence of neurogenesis in the OHSC. Several lines of evidence indicate that the incorporation of BrdU in NeuN-positive cells reflects the birth of new neurons (Palmer et al., 2000). Here we used BrdU/NeuN double positive cells to identify the newly born neurons.

Consistent with the previous observations, we found that the vast majority of the newly born neurons were in the innermost layer of the DG, less in the outermost region. The distribution of newly born neurons closely matches that reported in adult rodents in vivo (Kempermann et al., 2003). In this study, we observed a few

newly born neurons (1~3) in regions other than the dentate gyrus (i.e., areas CA3 and CA1), while the previous reports showed that neurogenesis was absent in these regions (Kamada et al., 2004; Raineteau et al., 2004). The discrepancy might be due to the reorganization of the cells during slice culture or the BrdU administration method. It was indicated that the three-dimensional structure of the neurogenic niche in the slice culture may be somewhat reorganized (Kamada et al., 2004). The rearrangements are probably a result of afferent deprivation (Robain et al., 1994; Gutierrez and Heinemann, 1999). It is worthy to note that some variables such as culture media composition, culture age, structural changes within cultures, and dorsoventral differences in the number of neuronal precursor cells, may affect neurogenesis in this in vitro model system. The highly pronounced proliferation of cells indicated by BrdU incorporation was different from the situation in vivo, in which proliferation of glia cells was much lower. Therefore, BrdU positive cells are mostly glia cells, rather than neural stem cells. Another variable is the BrdU administration regimen. To determine whether it could influence the identification of newly born neurons, we repeated this experiment following a different BrdU administration protocol. We found that the dose or timing of BrdU delivery must be controlled strictly.

Generally speaking, hippocampal slice cultures retain endogenous neural progenitors in the dentate gyrus throughout the cultivation period that maintain the potential to differentiate spontaneously into neurons. The time course and pattern of maturation and incorporation into the dentate gyrus of these newly born neurons is highly comparable to the one described in vivo (Kuhn et al., 1996; Eriksson et al., 1998). In vivo, the hippocampus is a region where neurons are generated intrinsically and spontaneously throughout postnatal life. Neurogenesis in the hippocampus is largely confined to the dentate gyrus, where it appears to play an important role in certain types of memory formation (Gould et al., 1999; Shors et al., 2001; Garthe et al., 2009), the behavioral effects of antidepressant drugs (Santarelli et al., 2003), and may serve as potential regenerative source for hippocampal damage (Ge et al., 2008).

These results establish organotypic hippocampal slice as a intesting model to study neurogenesis. All types of neurons and glia are preserved with their specific morphologies and localizations, and the main network organization is very similar to the one of living animals. The culture permits direct chronic treatment with drugs and various factors to modulate neurogenesis and facilitates the use of electrophysiological recordings from newly born neurons, together with their target cells. Therefore, hippocampal slice cultures offer the attractive possibility of studying the mechanisms of modulation of neurogenesis.

3.4.2 Neurogenesis modulated by NTFs

For neurogenesis to occur, it has been hypothesized that an appropriate set of signals need to be present within the neurogenic regions (Gage, 2000). To date, however, the microenvironment present in neurogenic regions of the adult brain still remains largely unknown. Several growth factors are known to affect proliferation of endogenous neural stem cells and their differentiation into neurons

and glia. A decade of work with such neural stem cells both *in vivo* and *ex vivo* has revealed that exposure to various individual (or combinations of) growth factors [e.g., FGFs, epidermal growth factor (EGF), transforming growth factors (TGFs), IGF-1] and/or neurotrophic factors (e.g., BDNF and CNTF) influences cell proliferation and differentiation along either neuronal or astrocytic lineages. For example, FGF, EGF, and IGF all promote neurogenesis when added to cultures of precursor cells from hippocampus, forebrain, cerebellum, and spinal cord (Gensburger et al., 1987; Ray et al., 1993; Vescovi et al., 1993; Pixley et al., 1998; Jin et al., 2003b). Our previous study also revealed that overexpression of FGF2 and BDNF mediated by herpes vectors promotes the survival, proliferation of neural stem cells, and induced more of them differentiate into neurons (Paradiso et al., 2009).

Several lines of evidence show that BDNF promotes the survival, differentiation and/or maturation of neurons derived from adult neural progenitor cells both in vitro and in vivo (Kirschenbaum and Goldman, 1995; Goldman et al., 1997; Pincus et al., 1998; Whittemore et al., 1999; Pencea et al., 2001; Scharfman et al., 2005; Chan et al., 2008) and can direct the neuronal differentiation of endogenous adult neural progenitor cells into region-specific neuronal phenotypes in the normal adult rat (Benraiss et al., 2001).

Consistent with these findings, we observed that exogonenous administration of BDNF in the OHSC system induced the number of newly born neurons in the dentate gyrus as indicated by the presence of BrdU and NeuN double labeling. We also observed an increase in glial proliferation in BDNF-treated slices, indicated by the presence of BrdU labeled cells when compared with the control group. We propose that this reflects the ability of BDNF and/or other NTFs (e.g. FGF2, MABs-produced growth factors) to create a mitogenic and neurogenic environment. However, there is some inconsistency among previous studies about BDNF effects on neurogenesis. For example, intra-ventricular infusion of BDNF yielded different results depending on the animal species, having no effect on neuron production from mouse SVZ, while decreasing it in rats (Galvao et al., 2008). BDNF germline heterozygous mice have been reported to have decreased (Lee et al., 2002b) or increased (Sairanen et al., 2005) proliferation of neural progenitor cells, as well as decreased (Sairanen et al., 2005) or unaltered (Rossi et al., 2006) long-term neurogenesis. Intra-cerebroventricular (i.c.v.) infusions of BDNF stimulated progenitor proliferation only on the side of the infusion (Givalois et al., 2006). Exposing the SVZ to BDNF increases production of olfactory bulb interneurons (Zigova et al., 1998; Berghuis et al., 2006) and induce new neurons from resident progenitor cells (Pencea et al., 2001; Chmielnicki et al., 2004). From these facts, it appears that the effects of BDNF on neurogenesis or cell proliferation are complicated.

BDNF has high levels of expression in the dentate gyrus and has an established role in the regulation of proliferation in the hippocampus (Nibuya et al., 1995). Using the OHSC, we demonstrated that chronic supplementation of BDNF can enhance proliferation of glia cells and neurogenesis in the dentate gyrus. The low concentration of BDNF (1 ng/ml) is not as efficient as 300 ng/ml BDNF in terms of mitogenic and neurogenic effects. However, 1 ng/ml BDNF produced by the MABs seemed to be much more efficient in increasing proliferation of glia cells but not the neurogenesis, indicative of a synergistic promitogenic effect between BDNF and MABs inherently produced factors. The combined administration of BDNF and FGF2 also highly elevated proliferation of glia cells. FGF2 is a potent

mitogen for adult neural progenitor cells in vitro (Gensburger et al., 1987; Vescovi et al., 1993; Gritti et al., 1999). In vivo, administration of FGF2 into both neurogenic or non-neurogenic regions increases cell proliferation (Kuhn et al., 1997; Kojima and Tator, 2000; Martens et al., 2002; Hagood et al., 2006). FGF2 has also been shown to alter the default glial state of adult neural progenitor cells from nonneurogenic regions by activating the neurogenic potential of these progenitor cells (Palmer et al., 1995; Shihabuddin et al., 1997; Palmer et al., 1999). In vivo, chronic infusion of FGF2 into the adult rat lateral ventricle results in an increase in proliferating cells in the SVZ and striatum and a subsequent increase in the number of neurons migrating from the SVZ to the olfactory bulb (Kuhn et al., 1997). Consistent with these results, we found that BDNF increased the number of BrdU/NeuN double positive cells much more when in the presence of FGF2, indicative of the neurogenic effects of FGF2 on the endogenous progenitor cells. Indeed, FGF2 was found to not only increase the population of progenitor cells but also induce an increased number of neurons in the olfactory bulb, which is the normal destination of neuronal progenitors (Kintner, 2002). When comparing the number of newly born neurons between the group of 300 ng/ml BDNF and 300 ng/ml BDNF/FGF2, we did not find any significant difference, indicating that FGF2 did not contribute to the neurogenic effect. This suggests that FGF2 may not have the same potential as BDNF to induce neuronal differentiation of neural progenitor cells in the OHSC system. In vivo, newly generated cells resulting from FGF2 administration have been shown to differentiate into neurons in neurogenic regions of the adult brain including the SVZ, SGZ and olfactory bulb (Kuhn et al., 1997; Wagner et al., 1999; Jin et al., 2003a) but differentiate predominantly into astrocytes in non-neurogenic regions such as the spinal cord and striatum (Kojima and Tator, 2000; Martens et al., 2002; Hagood et al., 2006). It was proposed that BDNF creates a neurogenic environment allowing for neuronal differentiation of adult progenitor cells, while FGF2 appears to support the proliferation and multipotentiality of progenitor cells in vivo. The interplay between BDNF and FGF2 is worthy to be further elucidated.

Besides the results of BDNF and FGF2, we found that the MABs-BDNF conditioned media (which consisted of 1 ng/ml BDNF and MABs inherently produced factors) induces a shift of neurogenesis, whereas the MABs conditioned media leaved the hippocampal neurogenesis unaffected. However, individual supplementation of 1 ng/ml rBDNF was insufficient to induce an increase of neurogenesis in the dentate gyrus. A possible hypothesis to explain these results is the interplay between BDNF and MABs inherently produced factors. A micro-array analysis of gene expression in MABs revealed that several growth factors are expressed at high level in these cells, including vascular endothelial growth factor B (VEGFB), basic fibroblast growth factor (FGF-2), FGF7, platelet-derived growth factor AA (PDGF AA), hepatomaderived growth factor, and stromal derived factor 1 (Galli et al., 2005). It had been shown that many growth factors like EGF, FGF-2 and IGF-1 increase neuro- and gliogenesis, just as BDNF can modulate dentate neural precursor cell proliferation, as illustrated by increased incorporation of BrdU into glia cells and NeuN positive cells in the dentate gyrus. These growth factors include FGFs, IGF, and EGF also promote the proliferation of stem cells isolated from the adult brain and may direct them toward specific fates (Craig et al., 1996; Kuhn et al., 1997), which is worthy of more extensive study.

We observed that supplementation of either rBDNF or MABs delivered BDNF

in the media increased the population of BrdU positive cells (representing glia cell proliferation) and BrdU/NeuN positive cells (representing newly born neurons) in the OHSC system. At this stage we are unable to conclude that these factors can direct the neural progenitors to neurons. The increased number of BrdU/NeuN positive cells may be either due to more neural progenitors proliferating and/or differentiating along neuron lineage, or due to more progenitors surviving from the culture procedure. Most of the growth factors, including BDNF and FGF2, are implicated in regulation of survival, proliferation, differentiation of progenitor cells. The regulation of BDNF on hippocampal neurogenesis is presumably via its primary receptor, TrkB. There are studies showing that BDNF-TrkB signaling impacts survival rather than proliferation (Lowenstein and Arsenault, 1996; Sairanen et al., 2005), but controversy exists about how BDNF affects neurogenesis (e.g. proliferation vs. survival/differentiation). From out data, it just appears that both BDNF and FGF2 induce dentate granule cell neurogenesis in cultured slices. It is known that BDNF and FGF2 by activation of their respective receptors can activate both common and separate intracellular pathways. Neural progenitor cell-derived neurospheres express genes for many growth factors and their receptors (Einstein et al., 2006), indicating that many growth factors and signaling pathways were involved in neural progenitor proliferation and survival.

In conclusion, these results suggest that regional environmental cues present in the OHSC play a strong role in the proliferation of cells and neurogenesis. Growth factors including BDNF and FGF2 have been implicated in the control of adult neurogenesis based on effects on proliferation and fate choice of adult neural progenitor cells both in vitro and in vivo. Here we demonstrated that BDNF delivered by MABs was effective on neurogenesis. FGF2 is an essential mitogen for multipotent neural progenitor maintenance and proliferation in vitro. Here we also demonstrated that supplementation of FGF2 augmented the mitogenic effect of BDNF. These observations suggest that BDNF and FGF2 play a prominent role in the generation and maintenance of a mitogenic and neurogenic environment in the OHSC system.

3.4.2 Potential application

The data presented here suggest that (i) the controlled and bioactive delivery of BDNF by engineered MABs is technically feasible; (ii) MABs might secrete factors promoting neurogenesis and cell proliferation; and (iii) the BDNF secreted by MABs contribute to promoting neurogenesis from endogenous progenitor cells. Together with the intriguing properties of MABs (e.g. general availability, specific homing etc., detailed in Research Background), transplantation of MABs-BDNF may be attractive to enhance endogenous regeneration as a strategy for the treatment of injure-related neurological disorders, like neurodegenerative disorders, epilepsy, stroke etc.

Cell therapeutic interventions might involve both transplantation of stem cells and stimulation of the endogenous neurogenic response. The persistent neurogenesis and the presence of quiescent neuronal progenitors in human adults have been proposed as a source of transplantable neural cells. The neuronal

progenitors derived transplantation approach is, however, limited by the invasiveness of the procedure and low in vitro proliferation potential of these cells (Marshall et al., 2006). Another strategy is to stimulate endogenous neurogenesis by manipulating the environmental cues around neural progenitors. For instance, administration of neurotrophic factors, which differs from cell-replacement therapies based on delivery of ex vivo-derived neural cells to areas of injury or degeneration, could promote proliferation, survival of endogenous neural stem cells, or direct them to differentiate into neurons or glia. Neurons derived from such neural stem cells are capable of migrating to various regions of the CNS, receiving afferent innervation, forming axonal projections, and expressing neurotransmitters (Goh et al., 2003; Garzon-Muvdi and Quinones-Hinojosa, 2009). Thus, these neural stem cells may serve as a self-repair mechanism by enabling the restoration of damaged CNS circuitry. Stimulation of endogenous neurogenesis by neurotrophic factors (e.g. BDNF) delivered through MABs-BDNF transplantation might be an intriguing therapeutic option to be explored.

4. PART III - Transplantation of MABs-BDNF

4.1 Introduction

Stem cells are emerging as an encouraging strategy for repopulating damaged tissues, and can also be genetically manipulated and used in cell-based gene therapy. Cell-based therapy may offer some degree of organ targeting with minimal invasiveness. The vessel associated stem cell, the mesoangioblasts described here, hold a great therapeutic promise because of their special properties.

For instance, transplantation of MABs, delivered intra-arterially, has been shown to restore to a significant extent muscle structure and function in animal models of muscular dystrophy (Sampaolesi et al., 2003; Sampaolesi et al., 2006). Muscle fibers that have incorporated mesoangioblasts, as well as surrounding fibers, are protected from damage (Berry et al., 2007). MABs are known to functionally express various chemokine receptors, which might be responsible for the homing in different organs following tissue damage (Cossu and Bianco, 2003). The specific homing to tissue damage of transplanted MABs was not only demonstrated in dystrophic mice and dogs but also in heart failure, in which MABs homed in the damage heart and protected the cardiac muscle by inherently secreting NTFs after myocardial infarction (Galli et al., 2005).

These special properties of MABs received great interest as a cell-based NTF delivery strategy. To exploit the therapeutic potential of MABs in treating CNS disorders, we engineered MABs to continuously secrete BDNF (MABs-BDNF). MABs-BDNF are non-tumorigenic and capable of unlimited clonal expansion in vitro (Cossu and Bianco, 2003), and have the capacity of longterm survival, which make them suitable for cell transplantation. Our in vitro results showed in PART I and PART II, demonstrate the beneficial effects of the conditioned media from MABs-BDNF cultures, including promoting cells survival, maintaining neuronal function, and increasing neurogenesis. Thus, transplantation of the MABs-BDNF could be a new therapeutic approach for CNS disorders, such as neurodegenerative diseases, spinal cord injury, epilepsy, ischemic stroke. In these injure-related CNS disorders, MABs are not designed to be used for a cell replacement strategy, because they can not differentiate into neurons. MABs-BDNF graft are expected to home in the damaged brain and function as “minipumps” for local BDNF delivery, where the transgene BDNF and other factors (as detailed in the discussion of PART I and PART II) may favor neuroprotection and neurogenesis. These considerations prompt further investigations to determine whether implantation of MABs-BDNF could be used as a therapy for injure-related CNS disorders.

In this part of the study, we explored the possibility of transplanting MABs-BDNF into the lesion brain induced by status epilepticus (SE). We selected an epilepsy model of temporal lobe epilepsy as our first attempt of MABs transplantation because: (i) our previous studies demonstrated the efficiency of NTFs gene therapy mediated by viral vectors in this model; (ii) BDNF (especially in combination with other NTFs) can prevent or repair seizure-induced damage (Simonato et al., 2006).

Temporal lobe epilepsy (TLE) is among the most frequent types of drug-resistant epilepsy (Diehl and Luders, 2000; Engel, 2001). Individuals affected with TLE typically have comparable clinical description, including an initial precipitating injury (IPI) such as status epilepticus (SE), head trauma, encephalitis or childhood febrile seizures (Harvey et al., 1997; Fisher et al., 1998). There is a pressing need to develop alternative therapeutic approaches that prevent the epileptogenesis that followed IPIs. TLE is frequently associated with hippocampal sclerosis, mainly exemplified by significant neurodegeneration in the dentate hilus (DH), and the CA1 and CA3c subregions (Sloviter, 2005). From this perspective, a new therapy that is efficacious for providing neuroprotection to the hippocampus after the onset of SE may have great significance. Based on a body of evidence from in vitro experiments, including primary hippocampal neuron culture and organotypic hippocampal slice culture, showing that MABs-BDNF can secrete BDNF and other factors to promote hippocampal cell survival and neurongenesis in the dentate gyrus, it seems that transplantation of MABs-BDNF to the hippocampus might provide an efficient restoration of the damaged hippocampus in the TLE model, which is vital for minimizing the incidence of chronic epilepsy.

MABs-BDNF therefore hold great promise for the treatment of TLE. To pursue this aim, we applied the TLE models initiated by prolonged status epilepticus as IPI. After the onset of IPI, we transplanted MABs-BDNF through infusion in the peripheral blood. MABs are expected to home in the damaged hippocampus, settle down, and survive. In the hippocampus, MABs-BDNF are expected to 1) differentiate in situ in endothelial cells and repair microvessel damage, a possible cause and/or consequence seizures (Krizanac-Bengez et al., 2004); 2) at the same time, to act as a reservoir and delivery system for NTFs. Thus, the pilot trial is to trace the infused MABs-BDNF in the brain of TLE animals.

4.2 Methods

Animals

Adult male SD rats (Harlan, Italy), Swiss mice (Morini, Italy), and C57BL mice (Harlan, Italy) were used in this part of study. They weighed around 200-300 g (Rat) or 30-50 g (Mouse) at the start of the experiment. The animals were caged individually. Ambient temperature was maintained at 21 ℃ and humidity at 55% with ad libitum access to food and tap water. Animals were kept on reversed 12/12-h light-dark cycles. All efforts were made to minimize animal suffering. All procedures were carried out under European Community and national laws and policies.

TLE animal models

For rats, 1 mg/kg scopolamine was injected intraperitoneally 30 min before the injection of pilocarpine (300 mg/kg). Scopolamine is used to reduce the peripheral consequences of pilocarpine. The rat's behavior was observed for several hours thereafter. Within the first hour after injection, all animals developed seizures evolving into recurrent generalized convulsions (SE). SE was interrupted 2 h after

onset by administration of diazepam (10 mg/kg i.p.). Animals required special care until they recovered from the acute insult and were closely tended, hydrated and fed.

For mice, the above protocol with pilocarpine was used. A group of mice were induced with 20 mg/kg kainate (i.p.). Mice showed higher mortality than rats. Animal survive 2 hours of status epilepticus received transplantation of MABs-BDNF. The control groups received saline instead of pilocarpine or kainate.

Transplantation procedures

MABs-BDNF were passaged and amplified using the method described in PART I. In brief, cells were washed with sterile DPBS when they grew into 70%-80% confluence on the bottom of the flask. Following the wash, cells were digested with TrypLE Express (GIBCO) for 1 min at room temperature. After centrifuging, cells were resuspended with saline at a density of 1.0×10^7/ml.

In a separate experiment, the cells were pretreated with 1 μg/ml bisbenzimide (Hoechst 33342, Sigma) for 30 min, which provides strong fluorescent staining of cell nuclei by binding to DNA. Hoechst 33342 staining does not impair the viability of the cells. Hoechst 33342 staining seems to be maintained for several generations (at least 8 days in vitro).

Animals were anesthetized with ketamine, and cells were injected via tail vein or left heart ventricle at a dose of 3×10^6 (for rats) or 5×10^5 (for mice).

Cell tracing

As described, MABs-BDNF were constructed with GFP, and exhibited green fluorescence. Therefore, GFP became the tracing marker for the grafted MABs-BDNF. The Hoechst 33342 staining was also used as a marker. In each time point, animals were sacrificed and the brains were put immediately into the cryostat for sectioning. Coronal sections were observed under the microscope to trace the cells marked with GFP or Hoechst 33342. To amplify the GFP signal, we also used immunohistochemistry for GFP. For GFP immunohistochemistry, brains were fixed in the formaline and embedded with paraffin. After routine section procedures, the sections including the dorsal hippocampus received immunohistochemistry for GFP. The procedure of GFP immunohistochemistry was similar to the one described in PART I, except for the replacement of anti-GFP antibody (1:100, Chemicon).

4.3 Results

To exploit the beneficial effects of MABs-BDNF demonstrated in vitro, we tested the transplantation of MABs-BDNF via peripheral blood infusion in an animal model with neuronal loss induced by prolonged generalized seizures. The entity of hippocampal damage was very similar in all animals. We injected the MABs-BDNF into peripheral blood circulation after the establishment of hippocampal damage and before the occurrence of SRSs, according to our protocol

that used for NTFs gene therapy (Paradiso et al., 2009).

After the establishment of damage with prolonged status epilepticus induced by either kainite acid or pilocarpine, MABs-BDNF were infused into the blood circulation and traced in the brain with GFP. Unfortunately, we did not find any clear deposition in the brain of the infused MABs-BDNF using different strains of animal, alternative infusion sites, different numbers of transplanted cells, and a range of injection and examination time points in this model (as indicated in **Table 1**). The hippocampal damage is pronounced in this model, and we expected that MABs-BDNF could home to the damaged site. Thus, we examined extensively the hippocampus using immunohistochemistry for GFP in continuous sections. As shown in **Fig. 8**, no signal was present in the hippocampus. To find out whether the injected MABs-BDNF home in other regions of the brain, we marked the MABs-BDNF with chelated paramagnetic ions and scanned the whole brain after cell transplantation using nuclear magnetic resonance imaging. Still, there was no signal of the injected MABs-BDNF in the brain.

Table 1 Systemic infusions of MABs-BDNF in the TLE model

Animal/Strain	Model	Sample Size	Days Injec.	Dose of cell	Injection Methods	Tracing Marker	Days Exam.
Rat/SD	PILO	2	4 days	3×10^6	Intracardial	GFP	5 days
Rat/SD	PILO	2	4 days	3×10^6	Tail vein	Hoechst	5 days
Rat/SD	PILO	1	4 injections*	3×10^6	Intracardial	Hoechst	10 days
Mouse /Swiss	KA	2	1 day	5×10^5	Intracardial	Hoechst	2 days
Mouse /Swiss	KA	2	1 day	5×10^5	Intracardial	GFP	5 days
Mouse /C57BL	KA	2	1 day	5×10^5	Tail vein	Hoechst	2 days
Mouse /C57BL	PILO	3	2 day	5×10^5	Intracardial	GFP	5 days
Mouse /C57BL	PILO	6	1 day	5×10^5	Intracardial	GFP	2 days

Annotate: Days Injec.**,** intervals between Status Epilepticus (SE) and MABs treatment; Days Exam.**,** intervals between MABs treatment and sacrificing**.** Abbrievation: KA, Kainate; PILO, pilocarpine. * The rat was pretreated with MABs 1 day before PILO-induced SE, and subsequently injected with MABs every 3 days (4 injections).

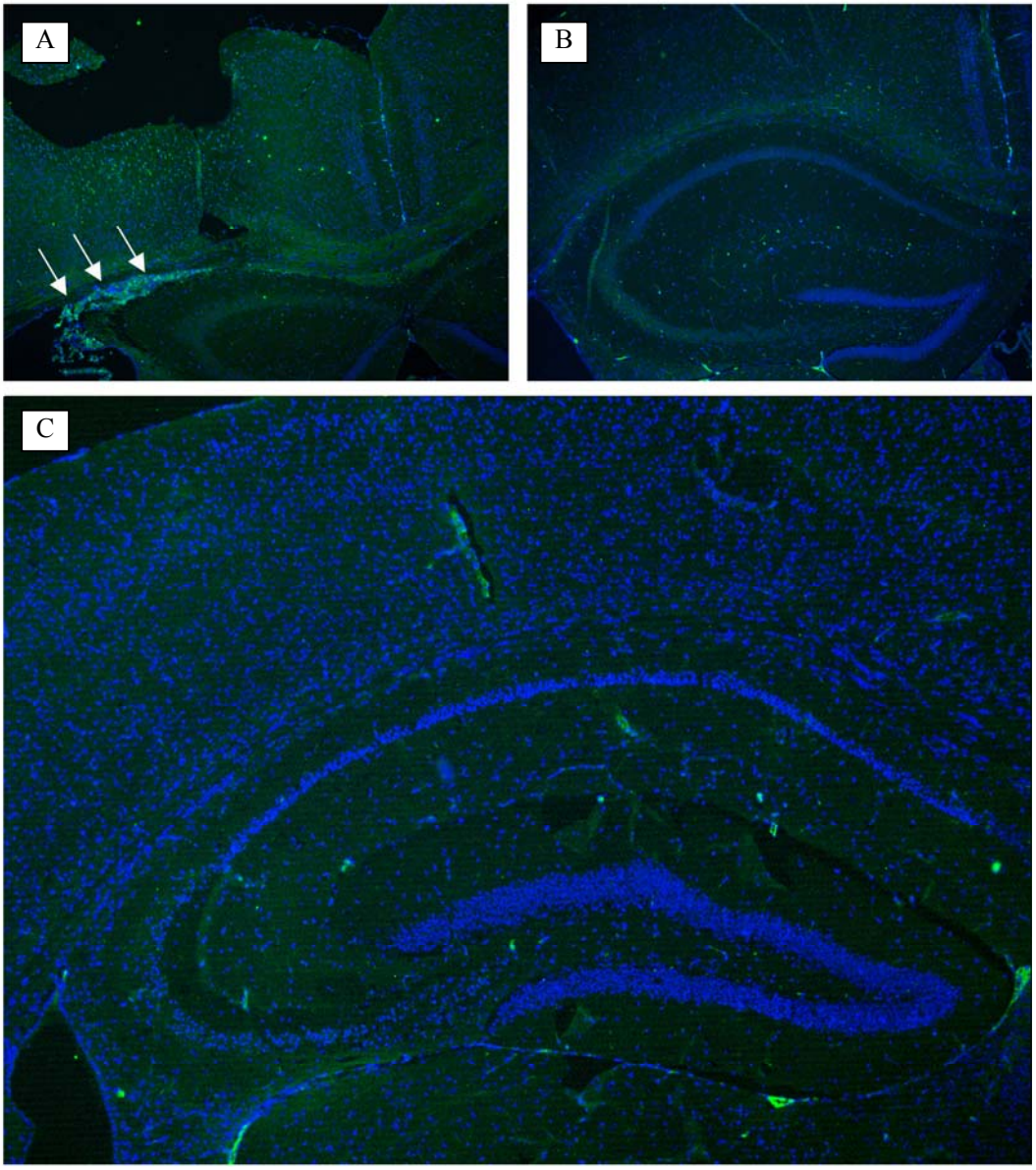

Fig. 8 Cell tracing using immunohistochemistry to detect GFP-positive cells in a pilocarpine induced epilepsy mouse model. The figures illustrate the missing trace of MABs-BDNF deposition in the hippocampus, the main damage area after peripheral infusion. (A) Control in which MABs-BDNF were intracranially injected, in order to ascertain the validity of the method. As indicated by the arrows, the MABs-BDNF were located in the ventricle with green signals. However, we did not find such assured GFP signals in (B) the normal mice, and (C) the mice underwent status epilepticus, after peripheral infusion of MABs-BDNF.

4.4 Discussion

4.4.1 Epilepsy and NTFs

Epileptic seizures can have severe and lasting effects on the architecture of the brain (Diehl and Luders, 2000). Following an epileptogenic insult, a cascade of neurobiological events such as neuronal cell death, enhanced neurogenesis, axonal sprouting, and reactive gliosis occur in distinct but overlapping sequences, which lead to the occurrence of spontaneous seizures and to the diagnosis of epilepsy (McNamara, 1994; Beach et al., 1995). This phenomenon is termed "epileptogenesis". One of the earliest consequences of prolonged seizure activity is the selective neuronal loss observed in specific subfields of the hippocampus (Schwob et al., 1980; Nadler, 1981; Mello et al., 1993; Lynch et al., 1996). NTFs are natural candidates for mediating these pathological events that follow seizure, as they have well-known roles in regulating neuronal birth, death, and the reorganization of connectivity, and more recent data show their functional effects at the synaptic level with distinct modulatory actions at excitatory and inhibitory synapses (Schinder and Poo, 2000).

However, the epileptogenic or antiepileptogenic effects of various NTFs following brain insults like seizures are still being studied. With specific reference to the BDNF, epileptogenic insults have been reported to increase BDNF synthesis and activation of its high affinity receptor, TrkB (Binder et al., 1999a; Binder et al., 2001), indicating an activation of the system during epileptogenesis. The effect of this activation, however, remains unclear. Many studies support the notion that BDNF play a pro-epileptogenic role. In particular, the observation that epileptogenesis is completely abolished in Synapsin-Cre conditional TrkB knockout mice, in which TrkB is ablated in hippocampal granule cells and CA3 pyramidal neurons, argues that TrkB activation plays an essential, and not merely regulatory, role in epileptogenesis (He et al., 2004). Another study showed that intracerebroventricular administration of BDNF accelerates the development of kindling (Xu et al., 2004a). It was also suggested that long-term suppression of BDNF after the SE may be beneficial for diminishing the mossy fiber sprouting as well as the incidence of chronic epilepsy after an IPI (Scharfman et al., 2002b). BDNF may also exert beneficial effects at different times in the natural history of the disease (Palma et al., 2005). Furthermore, our groups previously demonstrated that BDNF favors survival and regeneration of hippocampal neurons damaged by SE (Simonato et al., 2006). In our previous studies, local NTFs (BDNF and FGF2) gene delivery into hippocampus, when administered after brain damage onset, could exert restorative actions via promoting neuronal survival, stimulating proliferation of endogenous neural progenitors and directing them to differentiate along the neuronal lineage, thus interrupting the development of chronic epilepsy with reduced occurrence of spontaneous seizures (Paradiso et al., 2009). Extensive studies testing whether the various conditions (e.g. time, location, concentration, combination etc.) of administration of distinct neurotrophic factors (or the other strategies that maintain higher levels of distinct neurotrophic factors in the brain) after the onset of the SE is effective for preventing epilepsy are needed for further advances in this field.

In general, it is thought that neuronal loss plays a causal role in epileptogenesis and progression and that increased neurogenesis after IPI represents an attempt to repair damage (McNamara, 1994). This concept leads to the development of two strategies: neuroprotective strategy and cell replacement strategy. Neuroprotective strategy aims to salvage, protect and repair neurons in post-SE condition, while cell replacement is to reconstruct the disrupted circuitry via self-repair mechanism of endogenous neural progenitor cells or integration of transplanted stem cells (or neural tissue). An increased focus for developing neuroprotective and/or proneurogenesis interventions is vital for minimizing the incidence of chronic epilepsy (Bonde et al., 2006; Acharya et al., 2008). Transplantation of MABs-BDNF seems likely to be suitable for the strategy according to our in vitro results.

4.4.2 Ineffectiveness of MABs-BDNF systemic infusion in the TLE model

MABs have been reported to home in damage areas like skeletal muscle (Sampaolesi et al., 2006) and myocardial tissue (Galli et al., 2005) after systemic infusion, but have never been tested in CNS. We aimed to exploit the application of MABs in the CNS. In the pilot attempt of MABs-BDNF transplantation using a brain damage model induced by prolonged status epilepticus, we failed to find the deposition of MABs-BDNF after peripheral infusion in blood circulation. The reasons for the absence of MABs-BDNF in the damage brain might be multiple. The homing mechanism of MABs to the damage areas remains unclear, possibly relating with various cytokines and adhesion molecules. In particular, it was shown that a cytokine, high mobility group box 1 (HMGB1), which is released in the extracellular milieu by necrotic and inflammatory cells, induces migration and proliferation of MABs, and promotes their homing to damage area (Palumbo et al., 2004). To date, the released cytokines or inflammatory response in seizure-induced brain damage have not been elucidated. Cells in the CNS that can produce cytokines upon activation include macrophages, microglial cells, astrocytes, and cerebral endothelial cells (de Boer and Breimer, 1998). It was proposed that cytokines produced by these cells after seizures may influence the transport of compounds into the brain by opening the blood-brain barrier (BBB) (de Boer and Breimer, 1998). The BBB is a physical and metabolic barrier which serves to regulate and protect the microenvironment of the brain (Zlokovic, 2008). The tightness of the BBB is a serious hindrance to the entry of both immunocompetent cells and graft cells. Thus, the permeability of the BBB might be one of the factors leading to the failure of deposition of MABs-BDNF from the blood. It is well known that epilepsy or seizures are linked with not only neurodegeneration in several areas of the brain but also with the compromised integrity of the BBB (Wasterlain and Shirasaka, 1994; Jacobs et al., 2000; Armstrong, 2005). The BBB plays an active role in the mediation of neuroimmune responses, either by the production of inflammatory mediators or by expression of adhesion molecules. In in vitro studies, MABs were able to efficiently cross endothelium-coated filters in vitro when the other side held either mature myotubes (because the mature mytubes

secrete sufficient cytokines) or muscle-associated cytokines, such as SDF-1 (Galvez et al., 2006; Palumbo et al., 2007). In addition to cytokines, adhesion molecules also improved migration (Peault et al., 2007). We expected that MABs-BDNF could be attracted by the adhesion molecules or inflammatory mediators and migrate across the endothelial cell monolayer. However, the MABs-BDNF in the blood vessels could not be detained in the epileptic brain, possibly due to the insufficient release of molecules in response to the damage or exclusion by the BBB. Thus, it is important to create a permissible, biocompatible environment that allows for cell infiltration, which is the prerequisite for MABs-BDNF to exert their by-stander effects. Studies revealed that the administration of IL-1, IL-6, TNF-α, and IFN-γ, increase endothelial permeability (Freyer et al., 1999; Rivest, 1999; Schilling and Wahl, 1999), which might be helpful for the MABs-BDNF transplantation in brain. In fact, transfection of mesoangioblasts with L-selectin or α4 integrin increases the cells' migration efficiency across endothelium-coated filters. L-selectin and α4 integrin are not normally expressed by mesangioblasts, but are known to help leukocytes migrate through vessel walls into nearby tissues (Galvez et al., 2006). These strategies, which were demonstrated to improve the homing ability of MABs in a mouse model of muscular dystrophy, might also be applied to improve the MABs-BDNF transplantation in the CNS in future exploration.

Another possible cause for the failure of MABs-BDNF transplantation might be the immune rejection. A common problem with cell transplants is their limited survival and interaction with the host tissue. It is important to enhance survival and integration of donor cells in the host tissue to further advance cell transplantation therapy. During the exploration of MABs-BDNF transplantation, we have tried to avoid allogeneic transplantation by using C57BL mice (the MABs-BDNF was isolated from C57BL embryonic aorta). However, the uncertain brain damage in the model established from C57BL mice confounded the analysis. The type, duration and intensity of seizures relating with brain damage and BBB integrity may be additional confounding factors in this experiment. Moreover, there are still other hurdles to be taken in considerations such as the number of injected cells, the route of administration, the time of transplantation and so on.

4.4.3 Alternative MABs-based NTF delivery strategies

In spite of the ineffectiveness of MABs-BDNF transplantation via peripheral vessels in the TLE model, MABs-BDNF are still promising for future applications, based on the findings in vitro of their markedly beneficial effects on brain tissue. Up to now, the peripheral infusion of the MABs-BDNF did not meet our expectation to target the damaged area in the TLE model. However, the neuroprotective and neurogenic therapy using NTF delivery strategy is efficient in the treatment of the TLE model according to our previous report. Together with the present data showing the beneficial effects of MABs delivered NTFs, further explorations on the transplantation of MABs-BDNF are worth carrying on by changing graft strategy.

Intravenous (i.v.) injection is less invasive, and can be more readily carried out

in the clinic compared with the intracerebral injection. However, it has been estimated that less than 1% of i.v.-injected stem cells reached and survived in the lesioned brain after stroke (Chen et al., 2001b). The local intracerebral transplantation can reduce the number of transplanted cells and target directly the lesioned area, despite the surgical risk. In our case, intracerebral transplantation may be an alternertive route to seed MABs-BDNF in the leasioned brain. Compared with intraparenchymal injection, the intracerebroventricular delivery of cells allows more cells to be delivered, and produces more extensive cell seeding. In a preliminary experiment, the MABs-BDNF were found seeding along the ependyma and surviving for at least 4 weeks after the intracerebroventricular injection of the MABs-BDNF in a normal mouse brain (data not shown). Ependyma is the thin epithelial membrane lining the ventricular system of the brain. The seeded MABs-BDNF might secrete NTFs into the CSF and change the extracellular milieu throughout the brain and spinal cord. The central delivery of NTFs in the CSF may not be suitable for the local NTF delivery strategy for epilepsy, but may represent an alternative means of enabling neuroprotection or neuronal regeneration in diseases such as stroke, trauma etc. If trophic factors prove to be a worthwhile therapeutic strategy, the method of delivery via the MABs intracerebroventricular transplantation will certainly be intestesting. To date, administration of trophic factors has been limited to intraventricular infusions using pumps or cannulae.

To compromise the ineffective homing ability of MABs-BDNF in the TLE model, cell encapsulation technology might be another option. This strategy involves the use of genetically engineered producer cells that secrete a protein with therapeutic potential (Maysinger et al., 1994). The cells are encapsulated initially in an immunoisolating material that makes them suitable for transplantation. The capsules, or bioreactors, permit the release of recombinant proteins like NTFs that may assert their effects in altering the microenvironment. There has been significant progress in the development of encapsulation technologies that comprise devices for both macro- and microencapsulation (Visted et al., 2001). In a macroencapsulation like encapsulated cell biodelivery (ECB) strategy, the MABs-BDNF might be placed in semipermeable capsules and implanted into the targeted area (e.g. hippocampus in the case of TLE), where the cells release the necessary NTFs while the capsule protects the implanted cells against the host immune system (Maysinger et al., 1994). The dose and the duration of treatment can be conveniently controlled in the delivery system. A wide spectrum of cells and tissues has been encapsulated and implanted, both in animals and humans, indicating the general applicability of this approach for both research and medical purposes, including CNS disorders (Visted et al., 2001). For example, transplantation of immortalized cell lines genetically engineered to produce γ-aminobutyric acid(GABA) appears to reduce excitability in chronic epilepsy models (Gernert et al., 2002; Thompson and Suchomelova, 2004). Similar results were also seen after transplantation of encapsulated fibroblasts genetically engineered to produce adenosine (Guttinger et al., 2005).

The MABs-BDNF transplantation via systemic circulation may be tested for treating other injury-related CNS disorders, like neurodegenerative diseases, stroke, trauma etc. Because of the inability of CNS neurons to regenerate injured axons unaided, the vulnerability of injured neurons, and the non-permissive nature of the CNS environment, damage to brain and spinal cord produces devastating

consequences with, until recently, little hope of significant recovery. Using MABs as NTF delivery vehicles offers a new option to treat CNS injury. Trials of cell-based NTF delivery have now been performed in Parkinson's disease (Nakao et al., 2000; Kakishita et al., 2003; Villadiego et al., 2005; McKay et al., 2006), Huntington's disease (Martinez-Serrano and Bjorklund, 1996), spinal cord injury (Lu et al., 2003), stroke (Kameda et al., 2007). MABs-BDNF transplantation might meet the demands of targeted drug delivery strategies for NTFs in these CNS diseases.

As a whole, the strategy of using MABs as a local delivery vehicle of NTFs is promising in many CNS disorders, despite a number of difficulties that need to be overcome. The present transplantation trial is preliminary, and more comprehensive investigations are in program. Much effort for the application of MABs-BDNF will mainly in the fields of improving the homing ability, testing intracranial transplantation, and attempting treatment of injury-related CNS disorders.

5. Conclusion

In conclusion, our study reveal that BDNF exerts a dose-dependant protective effect in the low-density primary hippocampal neuron culture and in the adult organotypic hippocampal slice culture. MABs genetically engineered with BDNF can secrete bioactive BDNF in culture. Our data also demonstrate that treatment with conditioned media derived from MABs-BDNF cultures highly prevents the death of neurons in vitro due to a synergy between the engineered BDNF and other factors produced intrinsically by MABs. Moreover, the MABs-BDNF conditioned media allowed retention of morphologic characteristics and functional connections in adult slice cultures in comparison with the MABs media and the control. Neurogenesis analysis in the cultured slice showed that the combination of BDNF and FGF2 promoted gliosis and neurogenesis, while the MABs-BDNF conditioned media promoted neurogenesis and proliferation of glia cells. The present data indicate that MABs-BDNF may be a potential donor source for cell-based protective and regenerative therapy for injure-related CNS disorders. Although evidence of MABs-BDNF deposition in the brain was not obtained in a first attempt in which the MABs-BDNF were infused via peripheral blood vessels in the TLE model, the beneficial effects of the MABs-BDNF on neurons encourage a more extensive exploration of their therapeutic potential of the MABs-BDNF.

6. Future outlook: opportunities and challenges

In the study, we demonstrate the neuroprotective and neurogenesis effects of exogenous BDNF including BDNF genetically delivered by engineered MABs. These findings raise the possibility that exogenous administration of BDNF might be developed into a strategy for functional preservation and restoration in neurological diseases associated with brain damage. The supplementation of BDNF and other NTFs could be essential to sustain the survival and functional recovery of neurons by modulating the post-injury microenvironment. Ideally, neurotrophin delivery to the nervous system should be target-specific, regionally restricted, chronic, safe, well-tolerated and of sufficient concentration to elicit responses from target neurons. Cell-based NTF delivery may be optimal to meet these criteria. In recent years, implantation of genetically modified cells for supplementation of NTFs has been tried. In most of these paradigms, the cells only functioned as the vehicles for the NTFs secretion. MABs seem to be an attractive alternative cell source for NTF delivery. These cells are able to expand to virtually unlimitedly in vitro, so that they are amenable to genetic modification. Indeed, we found major advantages of BDNF delivery using the MABs engineered with BDNF (MABs-BDNF), which proved very efficient in neuroprotection and neurogenesis. We also demonstrated that it was the coaction of BDNF and MABs inherently produced factors that led to the markedly beneficial effects on neurons. Although further studies are required to identify these factors, our study suggests that the soluble mediators from MABs might play a role in the future as therapeutic agents or in transplantation.

Exploiting stem cells for NTF delivery offers great potential, with cells functioning as biologically active systems to produce specific beneficial factors or to replace lost cells and tissue. Though MABs may not replace lost neurons by themselves according to the present knowledge, the transplantation of MABs-BDNF may stimulate and augment endogenous neural stem cell populations with more neurons being formed through a ‘bystander’ mechanism that is alternative to cell replacement. The endogenous neurogenesis and migration of precursor cells have been reported to replace some lost neurons in brain structures such as striatum, and a significant portion of the new neurons seems to reestablish the connection to the striatum with supplementation of the appropriate NTFs (Arvidsson et al., 2002). The findings that the secreted factors from MABs-BNDF promote neurogenesis are of significant interest for regenerative therapies.

Together with the data showing that the conditioned media from MABs-BDNF greatly improved the survival of neurons in culture, the advantages of transplanting MABs-BDNF in the damaged brain seem to include the ability to target multiple neuroprotective and neuroregenerative mechanisms and the ability to provide a sustained treatment. The transplanted MABs-BDNF may provide an optimal environment for tissue repair in the damaged brain, with low chronic dosing of BDNF and other beneficial factors. MABs-BDNF may migrate to inflammatory or injure sites, produce BDNF and change the environment of nearby neurons, thus improving the bioavailability of the main neurotrophins. This is a potentially novel therapeutic approach which might be used in the injury-related diseases of the CNS in which drug access is limited. However, there are many difficulties before its

clinical application. First, slice culture (from which the data were obtained) cannot duplicate all the features of the brain. Some of these differences may limit the neuroprotective qualities of the BDNF delivered by MABs-BDNF in intact animals compared with brain slices. Second, the homing ability of MABs may probably be lost because of the differences of inflammatory mechanism between CNS and muscle tissue (it was indicated that the inflammatory molecules were one of the homing mechanism of MABs in muscular dystrophy and myocardiac infarction). In this study, we tried to use prolong-seizure induced brain damage as a model to transplant MABs-BDNF through peripheral infusion. The lack of evidence of MABs-BDNF deposition in the brain reminds us that the homing ability of MABs is more complicated than expected. Even if we directly intracranially transplant MABs-BDNF in damage area, there are still some drawbacks which may be encountered in animal experiments including surgical grafting process, non-specificity of the transduced cells, unstable expression of the transgene, disruption of host circuitry, aberrant fiber growth, and the potential for graft rejection or tumor formation. Another problem associated with cells transplantation relates to the quasi-impossibility of retrieving the cells once implanted, a problematic limitation in the case of occurrence of serious adverse effects. This limitation could be corrected by the use of encapsulated cell biodelivery systems or using inducible promoters that allow controlled transgene expression through the intake of a small molecule that can cross the BBB.

Nevertheless, the data presented here suggest that transplantation of MABs-BDNF is an alternative, and possibly more efficacious, therapeutic approach. that may be further developed as a neuroprotective and neurogenic therapy to treat brain diseases caused by neurodegeneration, including multiple sclerosis, epilepsy, stroke, and spinal cord trauma. Another possible exploitation of the beneficial effects of MABs-BDNF might be the combination with biomaterial scaffolds. Biomaterial-based strategies include those where the biomaterial itself has some therapeutic benefit or serves as a delivery vehicle for growth factors and extracellular matrix proteins, with the goal of recruiting host cells or enhancing axonal growth. When used as a delivery vehicle for cells, biomaterials must provide a suitable microenvironment for cell survival, tissue regeneration, and host tissue integration. For example, the development of encapsulated cell biodelivery (ECB) might provide a way for the application of MABs-BDNF. The delivery of cells in suitable biomaterials may lead to greater donor cell survival and thus greater benefit. However, this technique is invasive since it involves the stereotactic surgical injection and delivery of cells into a limited area or into the cerebral ventricles for a more global delivery. In terms of the routes of cell transplantation, an effective global delivery of cells to the brain is almost impossible when treating neurological disorders with diffuse neuropathology, such as Alzheimer's disease, most malignant brain tumors and inherited metabolic disorders. Direct intraparenchymal injection may generally restrict the diffusion of the MABs-BDNF, but restrict the number of the graft cells at the same time. Intraventricular injection to achieve more global delivery results in bihemispheric distribution through the ventricles, but the adverse effects of NTFs might be more serious. Therefore, in the future exploitation of MABs-BDNF cell therapy in the CNS diseases, the routes of transplantation should be seriously considered.

MABs-BDNF so far can be viewed as candidates for cell-based NTF therapy, but there are several obstacles before clinical application. This study is of heuristic

value for future development of cell-based NTF delivery. Using this study as a template, advances can be made. For example, stem cells can be engineered to produce other genes such as transmitters, anti-apoptotic genes. Other cell sources, like neural progenitor cells, or embryonic stem cells, might also be exploited into a neuroprotective or neurogenic agent by genetically engineering them to produce suitable NTFs.

Despite the numerous challenges that exist in the definition of the conditions for a successful treatment, MABs-BDNF hold great promise in the treatment of brain damage.

7. References

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Appendix

(Related articles published or submitted in the 3 years study period)

Appendix I: Page 1-6

- Paradiso B, Marconi P, Zucchini S, Berto E, Binaschi A, Bozac A, Buzzi A, Mazzuferi M, Magri E, Navarro Mora G, Rodi D, **SU T,** Volpi I, Zanetti L, Marzola A, Manservigi R, Fabene PF, Simonato M. Localized delivery of fibroblast growth factor-2 and brain-derived neurotrophic factor reduces spontaneous seizures in an epilepsy model. *Proc Natl Acad Sci U S A*. 2009 Apr 28;106(17):7191-6

Appendix II: Page 7-17

- **SU T**, Cong WD, Long YS, et al., Altered expression of voltage-gated potassium channel 4.2 and voltage-gated potassium channel 4-interacting protein, and changes in intracellular calcium levels following lithium-pilocarpine-induced status epilepticus. *Neuroscience*. 2008 Dec 2;157(3):566-76.

Appendix III: Page 18-32

- Tao Su, Beatrice Paradiso, Anna Binaschi, et al.. By-stander effect on brain tissue of mesoangioblasts producing the neurotrophin BDNF. (Manuscript Submitting)

Localized delivery of fibroblast growth factor–2 and brain-derived neurotrophic factor reduces spontaneous seizures in an epilepsy model

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A loss of neurons is observed in the hippocampus of many patients with epilepsies of temporal lobe origin. It has been hypothesized that damage limitation or repair, for example using neurotrophic factors (NTFs), may prevent the transformation of a normal tissue into epileptic (epileptogenesis). Here, we used viral vectors to locally supplement two NTFs, fibroblast growth factor–2 (FGF-2) and brain-derived neurotrophic factor (BDNF), when epileptogenic damage was already in place. These vectors were first characterized in vitro, where they increased proliferation of neural progenitors and favored their differentiation into neurons, and they were then tested in a model of status epilepticus-induced neurodegeneration and epileptogenesis. When injected in a lesioned hippocampus, FGF-2/BDNF expressing vectors increased neuronogenesis, embanked neuronal damage, and reduced epileptogenesis. It is concluded that reduction of damage reduces epileptogenesis and that supplementing specific NTFs in lesion areas represents a new approach to the therapy of neuronal damage and of its consequences.

epilepsy | gene therapy | neurotrophic factors

Epilepsies originating in the temporal lobe of the brain are the most common in adults. In patients with these diseases, focal pathological abnormalities can be observed, the most prominent of which is a loss of neurons in the hippocampus termed hippocampal sclerosis. These abnormalities develop in a previously healthy tissue, often after an initial "epileptogenic" event that can produce damage (for example, an episode of prolonged, uncontrolled seizures know as status epilepticus [SE]). After a latent period of weeks to years, epileptogenic events my be followed by the occurrence of spontaneous recurrent seizures (SRS), i.e., epilepsy (1). Unfortunately, currently available antiseizure drugs do not prevent this process.

It can be hypothesized that embanking or repairing damage may lead to antiepileptic effects (1). To date, however, the treatment of diseases associated with neuronal death has been restricted to attempts to prevent or limit the damage. More recently, the discovery of neural stem cells disclosed two new approaches: the transplantation of stem cells and the recruitment of endogenous stem cells for generating new neurons by means of extracellular proliferation/differentiation factors (2). In view of all approaches, key extracellular regulators of neuron survival and of stem cell proliferation and differentiation into neurons are the neurotrophic factors (NTFs) (3).

Endogenous NTFs are actually produced after an epileptogenic insult, but they remain insufficient or inadequate for neuroprotection and/or for providing the endogenous stem cells with the proper cues to proliferate, differentiate into neurons, and restore function (4, 5). In fact, damage ensues and a reactive neurogenesis occurs but fails to repair it (1). The aim of this study was to attempt suppressing neuronal damage and preventing the development of epilepsy by means of a local supplementation of specific NTFs within the lesioned, epileptogenic tissue.

To pursue this aim, we generated a viral vector expressing a combination of fibroblast growth factor–2 (FGF-2) and brain-derived neurotrophic factor (BDNF). We chose this combination because: (*i*) both these NTFs have neuroprotective properties; (*ii*) FGF-2 potently induces proliferation of hippocampal progenitors (6, 7); (*iii*) BDNF is required for neuronogenesis in the hippocampus (8–10). We used replication-defective herpes simplex virus–1 (HSV-1) vectors, for multiple reasons (11): they efficiently infect nonreplicating cells such as neurons; they can accommodate large inserts; they do not integrate their genome into the host DNA (that is, there is no mutagenesis risk); they can be transported retrogradely in neurons (therefore, transgene expression can occur in remote areas through nerve terminals afferent to the injection area); and finally, they produce a transient transgene expression: It is desirable to obtain transient expression of NTFs, because NTFs can trigger plastic changes that remain detectable when they are no longer expressed, whereas their long-term expression may be detrimental for brain function (12).

Results and Discussion

Vectors. A schematic representation of the vectors we used is shown in supporting information (SI) Fig. S1. For FGF-2, we used a previously characterized vector, TH-FGF2 (13), in which the transgene was under the control of the human cytomegalovirus (HCMV) immediate-early (IE) promoter. For BDNF, we used a vector, T0-BDNF, in which the transgene was under the control of the ICP0 promoter. The double mutant (TH-FGF2/0-BDNF) was obtained by crossing over TH-FGF2 and T0-BDNF. Promoters were chosen to promote a synergy between the NTFs, making use first of the FGF-2 property of potently inducing proliferation of hippocampal progenitors and then of the BDNF property of inducing their neuronal differentiation: the HCMV IE promoter driving FGF-2 is known to ensure robust, but transient, transgene expression, whereas the ICP0 promoter driving BDNF provides a longer-lasting expression (14).

Author contributions: B.P., P.M., S.Z., A.M., R.M., P.F.F., and M.S. designed research; B.P., P.M., S.Z., E.B., A. Binaschi, A. Bozac, A. Buzzi, M.M., E.M., G.N.M., D.R., T.S., I.V., and L.Z. performed research; B.P., P.M., S.Z., E.B., A. Binaschi, A. Buzzi, M.M., G.N.M., D.R., T.S., A.M., P.F.F., and M.S. analyzed data; and M.S. wrote the paper.

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NEUROSCIENCE

The vector-induced transgene expression and the biological activity of the NTF proteins produced by infected cells were first studied in vitro. The data we obtained (*SI Text* "In vitro Characterization" and Fig. S2) indicate that: (*i*) vectors induce production and release of FGF-2 and BDNF at biologically active levels; and (*ii*) FGF-2 and BDNF supplied together, not individually, potently favor survival, proliferation, and neuronal differentiation of neural progenitors. Therefore, we elected to test the vector expressing FGF-2 and BDNF together in vivo.

The efficiency of infection and the time-course of transgene expression in vivo was examined after a single stereotaxical inoculation of the vector in the hippocampus of adult rats (*SI Text* "Transgene Expression in Vivo" and Fig. S3). The data show that inoculation of TH-FGF2/0-BDNF provides a bilateral, short-term (≈1 week) increase in FGF-2 expression accompanied and followed by a bilateral, slightly longer-lasting (at least 11 days) increase in BDNF expression, and that these events are not associated with significant toxicity.

Effects on SE-Induced Cell Death and Neurogenesis. We tested TH-FGF2/0-BDNF on the pilocarpine SE–induced neurodegeneration and epileptogenesis. In this epilepsy model, an episode of SE produces intense neuronal damage and, after a latent period of 2–3 weeks, SRSs, i.e., epilepsy (15).

In the present experimental series, pilocarpine (300 mg/kg i.p.) rapidly induced a robust convulsive SE (latency, 15 ± 2 minutes) that was interrupted after 2 hours through administration of the anticonvulsant diazepam (10 mg/kg i.p.). Based on behavioral observation and EEG recordings, the severity of SE in the different animals was indistinguishable. This procedure caused damage in several brain areas: in particular (16, 17), hippocampal damage closely resembled human hippocampal sclerosis and was invariably remarkable. Typically, 3 days after pilocarpine administration, Fluoro-Jade C (FJC) staining allowed identification of several degenerating cells in CA1, CA3, and the hilus of the dentate gyrus. A pronounced edema with cell loss was also evident. A three-dimensional structural reconstruction demonstrated a 27.8 ± 3.1% reduction of hippocampal volume 3 days after pilocarpine administration, as compared with the response on control animals ($P < 0.001$, Mann-Whitney U test for unpaired data).

Three days after SE, these lesioned animals were randomly assigned to three groups: one group was injected in one hippocampus with the vector expressing FGF-2 and BDNF; the second group was injected with a control vector; and the third group was not treated at all (no difference was observed between these latter two groups in any of the parameters examined, and therefore they have been pooled together for statistical analysis, and collectively termed "control"). To explore the mitogenic effect of the vectors, animals were treated with bromodeoxyuridine (BrdU, four 50-mg/kg i.p. injections, one every 2 hours) 6 days after SE (i.e., 3 days after vector inoculation, Fig. S4). This time point was chosen because it is both within the peak of SE-induced proliferation of neural stem cells (18) and of vector-induced overexpression of FGF-2 (see above). A subset of animals in each group was killed 14 days after SE (i.e., 11 days after vector inoculation) to examine possible effects on cell survival and neurogenesis, and another subset was killed 28 days after SE (25 days after vector inoculation) to examine tissue morphology. These latter animals also underwent 20 days behavioral and EEG monitoring, to examine the outcome of the treatment (Fig. S4).

Although the bulk of SE-induced damage is in the first few days and, thus, was already in place when we injected the vector, several degenerating cells were still observed 14 days after pilocarpine administration in different hippocampal subfields (CA1, CA3, hilus of the dentate gyrus; Fig. 1*Aa* and *Ac*). Administration of the vector expressing FGF-2 and BDNF did not alter this ongoing cell loss, except for a slight, nonsignificant attenuation in the injected hippocampus (Fig. 1), indicating that, in vivo, the neuroprotective effect of this vector is limited or may require more prolonged or higher-level transgene expression.

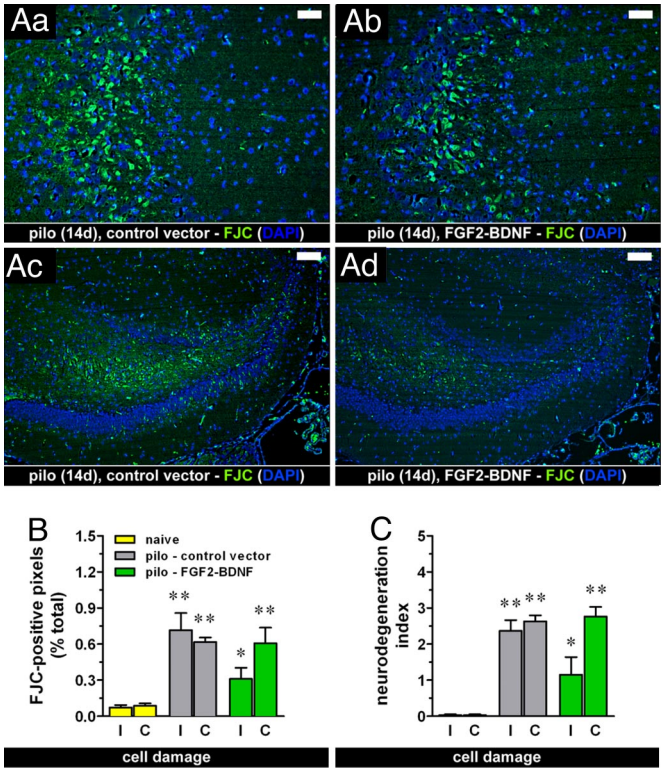

Fig. 1. (*A*) Effect of the treatment with the vector expressing FGF-2 and BDNF together (TH-FGF2/0-BDNF) on SE-induced ongoing damage. Degenerating cells are marked with FJC (green); nuclei marked by DAPI (blue). Fourteen days after pilocarpine (pilo)–induced SE (14d), ongoing hippocampal damage was still detectable in animals injected with the control vector (control), especially in the CA3 area (*Aa*) and in the dentate (*Ac*). This pattern was identical to the pattern observed in untreated animals (not shown). In animals treated with TH-FGF2/0-BDNF (FGF2-BDNF), a reduction, but not the abolishment of damage was observed (*Ab* and *Ad*). Horizontal bars, 25 μm in (*Aa*) and (*Ab*) and 50 μm in (*Ac*) and (*Ad*). (*B*, *C*) Quantification of neurodegeneration. Shown is the percentage of FJC-positive pixels in the hippocampus (*B*) and the degeneration index (*C*), calculated as described in *Materials and Methods*, in naïve rats (yellow bars) and pilocarpine-treated rats, injected with either the control vector (gray bars) or TH-FGF2/0-BDNF (green bars). I, injected (ipsilateral) hippocampus; C, noninjected (contralateral) hippocampus. Data are means ± SE for seven to eight animals per group. $*P < 0.05$, $**P < 0.01$ vs. naïve; Kruskal-Wallis test. No significant difference was observed between pilo – control vector and pilo – FGF2-BDNF.

In contrast to the limited effect on cell death, the effect of the vector expressing FGF-2 and BDNF on neurogenesis was quite remarkable. In the adult hippocampus, neural progenitors are found both in the subgranular zone (SGZ) and in the caudal subventricular zone (SVZ). SE has been reported to increase proliferation of hippocampal progenitors in both areas, but SGZ progenitors differentiate into neurons that, in large part, migrate ectopically to the hilus and SVZ progenitors differentiate into glial, not neuronal, cells: that is, SE-induced neurogenesis is aberrant and may contribute to epileptogenesis (19, 20).

We used pulse-chase BrdU labeling and immunohistochemistry for markers of progenitors to determine the mitogenic and differentiative activity of the NTFs supplemented by the vectors. To explore the mitogenic effect, we counted BrdU-positive cells in the dorsal hippocampus 14 days after pilocarpine. As expected, SE *per se* induced a significant increase in the number of

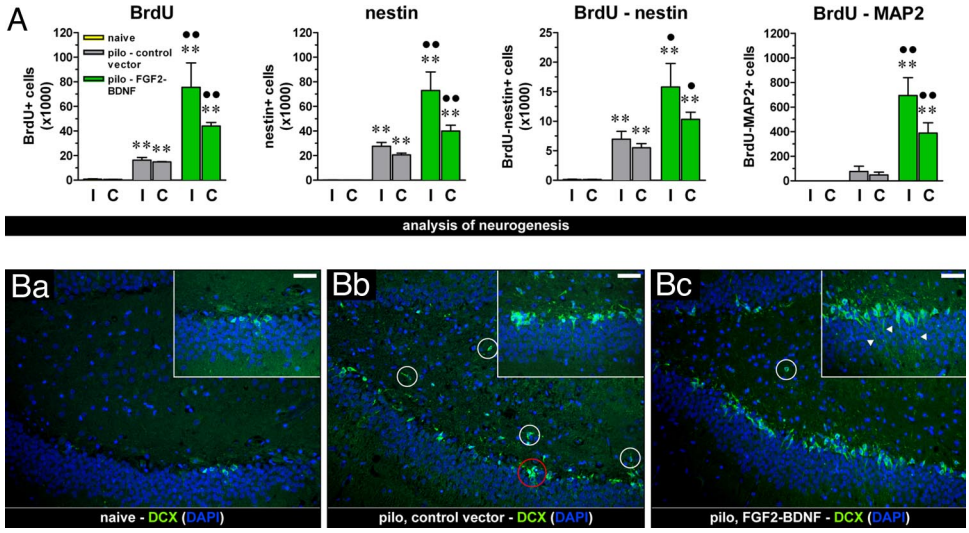

Fig. 2. Effect of the vector expressing FGF-2 and BDNF together on cell proliferation. (*A*) Average number of BrdU-positive, nestin-positive, double-labeled BrdU-positive and nestin-positive, double-labeled BrdU-positive and MAP2abc-positive cells in the dorsal hippocampus of naïve rats (yellow bars) and of pilocarpine-treated rats 11 days after inoculation of control vector (pilo – control vector, gray bars) or of the vector expressing FGF-2 and BDNF (pilo – FGF2-BDNF, green bars) in the right, ipsilateral to inoculation (I) and in the left, contralateral (C) hippocampus. Data are the means ± SE of four to five animals per group. ** $P < 0.01$ vs. naïve; ● $P < 0.05$, ●● $P < 0.01$ vs. pilo – control vector; analysis of variance (ANOVA) and *post hoc* Newman-Keuls test. (*B*) The vector expressing FGF-2 and BDNF together induced proliferation of DCX-positive cells in vivo. Immunofluorescence (dentate gyrus region) for DCX (green) in control (naïve) rats (*Ba*), in pilocarpine-treated rats given the control vector (*Bb*) and in pilocarpine-treated rats given the vector expressing FGF-2 and BDNF (*Bc*). Nuclei are marked by DAPI (blue). It should be noted that DCX-positive cells in the naïve animal dentate gyrus area are located in the subgranular zone and present detectable elongations projecting across the granular layer (*Ba Inset*); pilocarpine-induced SE (both in untreated animals and in animals administered the control vector) caused an increase in DCX-positive cells (Bb), which tended to produce relatively fewer elongations (*Bb*, *inset*), to group into clusters (example in red circle), and to localize ectopically (white circles). Treatment with the double mutant (*Bc*) further increased the number of DCX-positive cells while reducing their aberrant features, i.e., lower numbers of ectopic cells and presence of numerous elongations across the granular layer (arrowheads, *Bc*, *inset*). Horizontal bar, 25 μm.

mitotically active cells (Fig. 2 and Fig. S5). In keeping with previous reports (6, 18, 20), this increase was observed in the dentate gyrus and in the pyramidal gyrus (especially CA1 strata oriens and lacunosum-molecularis, near the SVZ). Inoculation of the control vector did not alter this pattern. In contrast, inoculation of the vector expressing FGF-2 and BDNF produced a further, bilateral, highly significant increase in the number of BrdU-positive cells (Fig. 2*A*), indicating proliferation of progenitors. Notably, an increased number of positive cells was also observed in the ventral hippocampus (Fig. S6).

In both the SGZ and SVZ, a series of differentiation steps have been described: from early, glia-like progenitors (termed type-1 for SGZ and type-B for SVZ), to transiently amplifying cells (type-2 and type-C), to late, migrating cells (type-3 and type-A) (21, 22). Type-1 and type-B cells are positive to GFAP, nestin, and Sry-related HMG box transcription factor (Sox2); type-2 and type-B cells to nestin and Sox2, but not to GFAP; type-3 and type-A cells to doublecortin (DCX) (22, 23). Pilocarpine SE *per se* significantly increased the number of nestin-positive cells in the hippocampus as compared with naïve controls, but treatment with TH-FGF2/0-BDNF dramatically potentiated this effect, more markedly in the injected hippocampus (Fig. 2*A*). Many (≈20%) of these nestin-positive cells were also BrdU-positive, indicating that part of them were actively proliferating at the time of BrdU administration (Fig. 2*A*). Furthermore, a subset of these cells was also GFAP-positive (Fig. S5B), an indication of the presence of early progenitors. Finally, we observed an increased number of Sox2-positive cells in pilocarpine animals treated with TH-FGF2/0-BDNF, 7 days after SE (Fig. S5*D*). These data indicate that SE *per se* causes proliferation of hippocampal early to intermediate progenitors and that vector-mediated supplementation of FGF-2 and BDNF greatly increases this phenomenon.

In the subsequent steps of neurogenesis, progenitors begin to migrate and become DCX-positive. As stated above, this migration is partly aberrant after SE, because many SGZ progenitors do not head toward the granular layer but toward the hilus (19). Therefore, we also analyzed DCX-positive cells. Under control conditions (naïve animals), a limited number of DCX cells, with elongations projecting across the granular layer, was found in the SGZ (Fig. 2*Ba*) and even less so in the SVZ (essentially, just a few, weakly labeled DCX-positive cells in the CA1 stratum oriens). SE *per se* caused a robust increase in the number of DCX-positive cells in the dentate gyrus (Fig. 2*Bb*). However, these cells did not often produce elongations, tended to aggregate into clusters, and were often ectopically located (Fig. 2*Bb*). These observations are consistent with the aberrant aspects of SE-induced neurogenesis (19, 24). A slightly increased number of DCX-positive cells was also observed in the pyramidal gyrus (CA3 and CA1). This pattern was not modified by the administration of the control vector. In contrast, administration of TH-FGF2/0-BDNF in pilocarpine-treated animals caused a greater increase in the number of DCX-positive cells compared with pilocarpine alone or pilocarpine and control vector (Fig. 2*B*). Moreover, it increased the length and number of elongations, reduced cluster formation, and reduced the number of ectopic cells (Fig. 2*Bc*). An increased number of DCX-positive cells was also observed in the pyramidal gyrus, indicating commitment to neuronal differentiation (Fig. S5*E*).

Finally, we checked whether, consistent with our in vitro data, a subset of these new cells were differentiating into neurons and migrating into the appropriate location to possibly restore function. Thus, we performed double-label immunofluorescence with BrdU and MAP2abc, a marker of immature and mature neurons. SE *per se* did not significantly increase the number of BrdU-MAP2–positive cells; in contrast, we found several double-labeled cells in the hippocampus of pilocarpine animals treated with the double mutant (Fig. 2*A*).

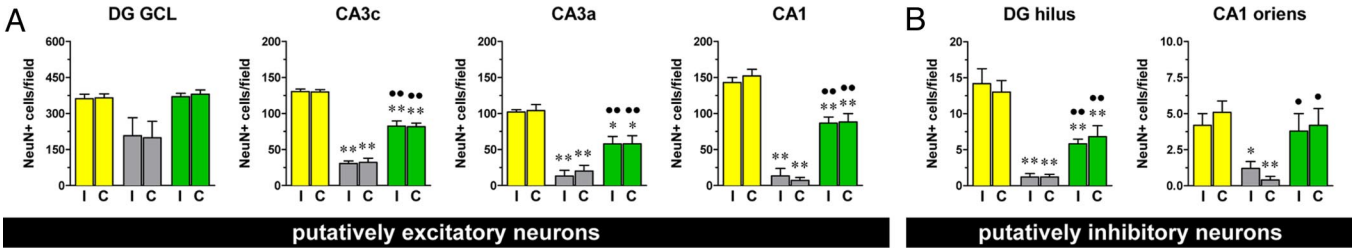

Fig. 3. Neuropathological outcome of the treatment with the vector expressing FGF-2 and BDNF (TH-FGF2/0-BDNF), 28 days after pilocarpine-induced status epilepticus. (*A*) Measure of the density of NeuN-positive, presumably excitatory neurons in selected subareas of the dorsal hippocampus: dentate granule layer (DG GCL), CA3c pyramidal layer (CA3c), CA3a pyramidal layer (CA3a), and CA1 pyramidal layer (CA1). I, injected (ipsilateral) hippocampus; C, noninjected (contralateral) hippocampus. (*B*) Measure of the density of NeuN-positive, presumably inhibitory neurons in selected subareas of the dorsal hippocampus: hilus of the dentate gyrus (DG hilus) and stratum oriens of CA1 (CA1 oriens). Data are the means ± SE of 10–12 animals per group. $*P < 0.05$, $**P < 0.01$ vs. naïve; ● $P < 0.05$; ●● $P < 0.01$ vs. pilo – control vector; ANOVA and *post hoc* Newman-Keuls test.

These data suggest that injection of the vector expressing FGF-2 and BDNF in a SE-damaged hippocampus favors proliferation of early progenitors according to a pattern that, at variance with what was observed in untreated animals, appears to follow the physiological pattern more faithfully, leading to the production of cells that enter the neuronal lineage of differentiation and reducing the aberrant aspects of SE-induced neurogenesis.

Outcome: Pathology and Behavior. To evaluate the outcome of the treatment, we compared the different experimental groups for the morphology of the hippocampus. First: compared with naïve controls, the dorsal hippocampus of pilocarpine animals remained smaller 4 weeks after SE (approximately −30%, like 3 days after SE: see above). Treatment with the double mutant produced a significant increase in hippocampal volume, with a recovery of approximately a third of the loss (Fig. S7*A*).

Importantly, the SE-damaged hippocampus presents a clearly reduced density of neuronal cells, most notably hilar and pyramidal cells, that was not observed in animals treated with the double mutant (Fig. S7). We measured the density of astrocytes and neurons in the hippocampus using GFAP and NeuN immunofluorescence. The density of GFAP-positive cells in the entire hippocampus was significantly increased in pilocarpine-treated animals compared with naïve controls, an indication of reactive astrocytosis (25). In the pilocarpine group of animals treated with the vector expressing FGF-2 and BDNF, the density of GFAP-positive cells decreased slightly in comparison with untreated animals, but was still greater than in naïve animals (Fig. S7*B*). Neuronal density was measured using NeuN. Putatively excitatory dentate gyrus granule cells were not significantly decreased by SE and treatment with the double mutant did not alter the situation (Fig. 3*A*). In contrast, putatively excitatory pyramidal neurons were dramatically decreased in animals that experienced SE, and the double mutant significantly increased their number (Fig. 3*A*). Putatively inhibitory neurons in the dentate hilus and in the stratum oriens of CA1 were even more dramatically decreased in animals that experienced SE; and, again, TH-FGF2/0-BDNF significantly improved the situation (Fig. 3*B*).

These data suggest that treatment with the vector expressing FGF-2 and BDNF partially heals the damage induced by SE, maintaining a good ratio between excitation and inhibition in the circuit. However, some inhibitory cells are present in the pyramidal layer and excitatory cells are present in the hilus (for example, the glutamateric mossy cells). Thus, we deepened the investigation analyzing some of the main populations of GABAergic interneurons, namely parvalbumin, somatostatin, and calbindin. Parvalbumin-containing interneurons exert perisomatic, whereas somatostatin and calbindin exert dendritic inhibition; calbindin also labels a subpopulation of principal, glutamatergic cells, both in the pyramidal and in the granular layer (26). In keeping with previous reports (27–29), we found that the number of parvalbumin-positive cells in epileptic animals was similar to that in controls (Fig. S8*A*), while that of somatostatin-positive cells was halved (Fig. S8*B*). Also, calbindin-positive principal neurons in the pyramidal and granular layer, but not calbindin-positive interneurons in the strata oriens and radiatum and in the dentate gyrus hilus, were significantly decreased in the pilocarpine group (Fig. S8*C*) (30, 31). Treatment with TH-FGF2/0-BDNF effectively corrected these cell losses (Fig. S8).

Avoiding the loss of somatostatin cells may imply maintenance of dendritic inhibition and, therefore, decreased susceptibility to seizures. This leads to the key question: do these beneficial effects ameliorate the outcome in terms of SRSs? We performed behavioral and EEG analyses: animals were video-EEG monitored for 20 days and the occurrence, severity, and duration of SRSs were recorded. As expected, all non vector-injected pilocarpine-treated rats exhibited SRSs beginning ≈2 weeks after SE. On average, we observed in this group four seizures per day (Fig. 4*A*) lasting about 1 minute each and with a mean severity score (32) of 2.5 (Fig. 4*B*). Administration of the control vector did not modify this pattern. In contrast, rats treated with the double mutant displayed a highly significant improvement: (*i*) a subset of animals (two of 11) never developed SRSs during the time frame of observation; (*ii*) the average number of seizures per day in this group was highly significantly reduced compared with the other two groups (Fig. 4*A*); (*iii*) the seizure severity was also significantly decreased (Fig. 4*B*). Thus, the FGF-2 and BDNF-expressing vector, injected in the hippocampus after the establishment of hippocampal sclerosis, provided both significant recovery from damage and significant reduction of SRSs. These results are striking, considering that this epilepsy model is accompanied by extensive damage also in extra-

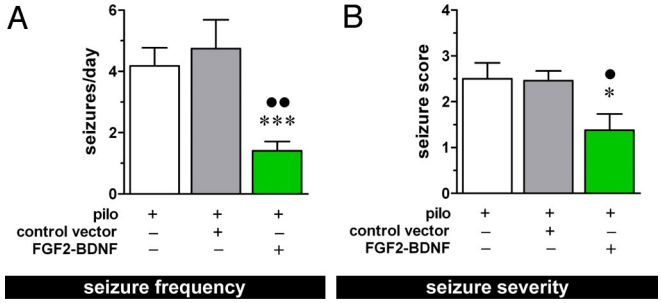

Fig. 4. EEG and behavioral analysis. Average frequency (*A*) and severity (*B*) of spontaneous seizures in the chronic period (14–28 days after pilocarpine-induced SE). Data are the means ± SE for 10–12 animals per group. $*P < 0.05$ and $***P < 0.001$ vs. pilo; ● $P < 0.05$ and ●● $P < 0.01$ vs. pilo – control vector; ANOVA and *post hoc* Newman-Keuls test for (*A*), Kruskal-Wallis test for (*B*).

hippocampal regions (33), such that only a subset of SRSs may actually originate in the hippocampus (34), i.e., where we documented favorable effects by the vector. In other words, SRSs originating in the hippocampus may be essentially abolished by vector treatment.

Finally, to test whether the vector has a truly antiepileptogenic effect and is not merely dampening SRSs (i.e., blocking ictogenesis), we investigated whether the vector expressing FGF-2 and BDNF was effective in controlling seizures when injected in animals that were already experiencing SRSs. To address this question, a group of pilocarpine-treated rats was video-EEG monitored beginning 2 weeks after SE. Three weeks after the first spontaneous seizure, these animals were injected either with the control vector or with TH-FGF2/0-BDNF, and monitoring was continued for 4 more weeks. The frequency, duration, and severity of SRSs were then comparatively analyzed in the 2 weeks preceding vector inoculation and in the third and fourth weeks after inoculation. Inoculation of the FGF-2/BDNF expressing vector did not affect any of the parameters examined (Fig. S9), indicating that supplementation of FGF-2 and BDNF can interfere with epileptogenesis but not with ictogenesis.

In conclusion, the main finding of this study is that supplementation of FGF-2 and BDNF in the hippocampus lesioned by prolonged seizures effectively limits or repairs damage, maintaining a good ratio between excitation and inhibition, and significantly improves the natural history of the disease.

Understanding in depth the mechanistic basis of these effects will require further studies, but the present data provide some initial insights. FGF-2 and BDNF may provide protection against damage or gain of function of partially damaged neurons. Even if the present study did not provide support to this idea, subtle effects on specific cell populations that could not be detected with the techniques that we used cannot be excluded. Nonetheless, another hypothesis, namely, that FGF-2 and BDNF exert effects on neural stem cells and progenitors to generate new neurons, seems more plausible. The phenotype(s) of these new neurons remain uncertain, but we identified one interesting candidate in a subtype of interneurons (somatostatin). GABA-somatostatin interneurons play an important role in dendritic inhibition, and their loss may favor the development of epilepsy (28); thus, the replacement of these neurons may explain the favorable effects of FGF-2 and BDNF supplementation.

Some aspects of the present findings are worthy of note. First, virus injection was performed under conditions compatible with the clinical settings of patient observation (after the epileptogenic insult), conditions that reproduce those that may allow therapeutic intervention. Second, the disease-modifying effect is striking and can be interpreted as anti-epileptogenic, an effect that is not achieved by the currently available therapy. Further studies will be required in both respects, to establish how long the effect lasts and to challenge the hypothesis that it is truly anti-epileptogenic. Last but not least, these findings may have a heuristic value for the many other neurological diseases associated with neuronal damage.

Materials and Methods

A detailed description of all of the methods used in this study is provided in the *SI Text* ''Materials and Methods.''

Animals. Male Sprague-Dawley rats (240–260 g; Harlan Italy) were used for experiments. All procedures were carried out in accordance with guidelines by the European Community and national laws and policies.

Hippocampal Volumetry. Paraffin-embedded brains were cut in successive 10 μm sections across the entire hippocampus and one of every five sections was stained with hematoxylin and eosin. Sections were scanned and digitalized using a Nikon Coolscope. Segmentation and orientation were obtained by Amira TM 4.0 (Mercury Computer Systems), by two researchers blind to the animal groups. Three-dimensional reconstructions were then calculated and averaged using the same software.

HSV Infusion. Under ketamine and xylazine anesthesia, a glass needle connected to a perfusion pump was implanted in the dorsal hippocampus. A total of 1.6×10^6 pfu of vector were injected in a volume of 2 μl at a flow rate of 0.1 μl/min.

FJC Staining. FJC staining was performed on 6-μm paraffin-embedded sections as previously described (35).

Immunohistochemistry and Immunofluorescence. For BrdU immunostaining, cellular DNA was denatured with HCl. The primary antibodies used for immunohistochemistry were as follows: BrdU mouse monoclonal (1:100, Roche Molecular Biochemicals); nestin mouse monoclonal (1:100; Chemicon); MAP2abc mouse monoclonal (1:100; Chemicon); GFAP rabbit polyclonal (1:100; Sigma); parvalbumin mouse monoclonal (1:50; Swant); and calbindin rabbit polyclonal (1:50; Swant). Detection was obtained using the biotin-streptavidin system (Ultra Vision Detection System, Lab Vision Corporation). The reaction product was detected using a 3,3-diamino-benzidinetetrahydrochloride (DAB) substrate kit for peroxidase (Vector Laboratories).

For immunofluorescence, the primary antibodies were: BrdU (rat monoclonal, AbD Serotec) and nestin (mouse monoclonal, Chemicon) 1:10 and 1:10 respectively; BrdU (as above) and MAP2abc (mouse monoclonal, Immunological Sciences) 1:10 and 1:10; nestin (mouse monoclonal, Chemicon) and GFAP (rabbit polyclonal, Sigma) 1:25 and 1:100; NeuN (mouse monoclonal, Chemicon) and GFAP (as above) 1:25 and 1:100; MAP2abc (mouse monoclonal, Immunological Sciences) and GFAP (as above) 1:25 and 1:100; Sox2 (rabbit polyclonal, Immunological Sciences) and GFAP (mouse monoclonal, Immunological Sciences) 1:25 and 1:100; DCX (rabbit polyclonal, Cell Signaling) and nestin (as above) 1:20 and 1:20; DCX (goat polyclonal, Santa Cruz Biotechnology) 1:25; somatostatin-14 (rabbit polyclonal, Peninsula Laboratories) 1:25.

Telemetry. Seizure onset, severity, and duration were assessed by video-EEG monitoring of the animals, performed by means of Phenotyper cages (Noldus Information Technology), using telemetric technology (Dataquest A.R.T. Data Acquisition, Data Sciences International).

Quantification and Statistical Analysis. All experiments were performed strictly under double-blind conditions. The volume of the dorsal hippocampus was calculated using a stereological approach based on the principle of Cavalieri (36). The degree of cell damage was quantified using two approaches: the ''neurodegeneration score'' (37) and a thresholding of digital images (38). Counting of cells positive for the different markers was conducted on one of every 26 sections across the entire dorsal hippocampus (i.e., nine regularly spaced sections per animal). Quantification of GFAP-positive and NeuN-positive cells was performed in frames taken from the various hippocampal subareas. Behavioral alterations and seizure scoring were performed by means of the Observer (Noldus). Seizure detection (frequency, duration and severity), as well as single ictal events ($<$2 seconds) were scored for each individual rat for the whole analysis period (24 h/day for 20 consecutive days).

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ALTERED EXPRESSION OF VOLTAGE-GATED POTASSIUM CHANNEL 4.2 AND VOLTAGE-GATED POTASSIUM CHANNEL 4-INTERACTING PROTEIN, AND CHANGES IN INTRACELLULAR CALCIUM LEVELS FOLLOWING LITHIUM-PILOCARPINE-INDUCED STATUS EPILEPTICUS

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Abstract—The A-type voltage-gated potassium channels (Kv4) have been proved to play a major role as modulators of somatodendritic excitability. Recent studies indicate that neuronal hyperactivity in epilepsy is associated with changes in Kv4. However, the precise regulation of Kv4 in the development of epilepsy and its underlying mechanism remain unclear. In this study, we investigated whether the expression of the Kv4.2 channel and of its major modulator, voltage-dependent potassium channel-interacting protein (KChIP1), is altered following lithium-pilocarpine induced status epilepticus (SE) and the chronic-epilepsy phase in the rat model. We found that Kv4.2 and KChIP1 expression was transiently up-regulated following SE, whereas it was down-regulated during the chronic phase: this was most prominent in the CA1 and CA3 regions. The time-course analysis of the protein expression level showed that the peak Kv4.2 up-regulation was between 6 and 24 h after SE, whereas KChIP1 expression was increased earlier and for a shorter period. The temporo-spatial changes in Kv4.2 were very similar to those of its major modulator KChIP1. We compared the difference in 4-aminopyridine (4-AP)-induced intracellular calcium ($[Ca^{2+}]i$) elevation between model and control brain slices. The results showed that the $[Ca^{2+}]i$ elevation induced by the Kv4 channel blocker 4-AP was aggravated and prolonged in the model slice after SE. The functional relevance of these changes in Ca^{2+} homeostasis and Kv4.2 and KChIP1 expression may be associated with intrinsic neuronal excitability regulation and epileptogenesis. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: pilocarpine, Kv4 potassium channel, KChIP, intracellular calcium concentration, hippocampus, epilepsy.

Status epilepticus (SE) is a common epileptogenic brain injury that can cause acquired epilepsy (AE) (Raza et al., 2001). The recurrent or continuous seizure activity that occurs during SE, is thought to result in structural and functional changes in the brain, thus leading to the development of chronic epilepsy (Sloviter, 1999). Epileptogenesis is the process by which injuries such as SE, stroke, or traumatic brain injury produce short- and long-term plastic changes in the neurons, resulting in spontaneous recurrent seizures (SRS) in previously normal brain tissue (Sun et al., 2001; Brandt et al., 2006; Swartz et al., 2006). These plastic changes have been recognized as the most important determinants of neuronal excitability (Bliss and Collingridge, 1993), and thereby contribute to the development of AE. Selective and coordinate changes in certain voltage- and transmitter-gated ion channels have been found to be involved in this process (Faas et al., 1996; Su et al., 2002; Nishimura et al., 2005).

Fast transient (A-type) voltage-gated potassium channels (Kv) recently attracted special attention because they contribute to controlling the amplitude of back-propagating action potentials and the neuronal action potential firing properties (Hoffman et al., 1997; Korngreen et al., 2005). A-type potassium current (I_A) can be generated by Kv1.4, Kv3.4, or any of the Kv4 family subunits (Kv4.1, Kv4.2, and Kv4.3) (Pongs et al., 1999; Song, 2002). Kv4.2 subunit is one of the key components underlying I_A (Serodio et al., 1996). Changes in the properties of these physiologically important I_A by the transcriptional and posttranscriptional regulation of Kv4.2 could lead to the dysregulation of neuronal excitability, which is a hallmark of epilepsy. Elementary evidence for the role of Kv4 channels in epilepsy is provided by studies that use Kv4 channel blockers, such as 4-aminopyridine (4-AP) (Psarropoulou and Avoli, 1996), or other highly selective blockers (Ebbinghaus et al., 2004), which lead to convulsions in mice (Juhng et al., 1999). Furthermore, altered Kv4.2 expression has been observed in several epilepsy models (Tsaur et al., 1992; Francis et al., 1997; Castro et al., 2001; Bernard et al., 2004). Physiological studies evidenced the relation of decreased Kv4.2 expression with the functional consequences of I_A reduction in CA1 pyramidal cells in pilocarpine-induced epilepsy (Bernard et al., 2004) and in the heterotopic neurons of cortical malformation models that were associated with epilepsy (Castro et al., 2001). In humans, a marked down-regulation of classical I_A was observed in dentate granule cells isolated from patients with lesion-associated temporal lobe epilepsy (TLE) (Beck et al., 1997). Even in the case of idiopathic epilepsy, a truncation mutation of Kv4.2 has been found in a TLE

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Abbreviations: aCSF, artificial cerebrospinal fluid; AE, acquired epilepsy; $[Ca^{2+}]i$, intracellular calcium concentration; CICR, calcium-induced calcium release; DAB, 3,3′-diaminobenzidine; EPSP, excitatory postsynaptic potential; I_A, A-type potassium current; KChIP, voltage-gated potassium channel-interacting protein; Kv, voltage-gated potassium channel; PBS, phosphate-buffered saline; PTZ, pentylenetetrazol; ROI, region of interest; SE, status epilepticus; SRS, spontaneous recurrent seizures; TLE, temporal lobe epilepsy; 4-AP, 4-aminopyridine.

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patient, with attenuated potassium current density on the cell surface (Singh et al., 2006). These data strongly suggest that Kv4.2 is an important determinant of neuronal excitability and is implicated in epilepsy. However, thus far, limited information is available on the dynamic regulation of Kv4.2 and its underlying mechanisms. Recent studies have emphasized the importance of voltage-dependent potassium channel interacting proteins (KChIPs) in regulating the expression levels and physiological properties of Kv4 channels (An et al., 2000; Bahring et al., 2001; Beck et al., 2002). KChIPs are calcium binding proteins that belong to the family of EF-hand neuronal calcium sensor (NCS) proteins (An et al., 2000). KChIPs appear to transduce Ca^{2+} signals to actively altering neuronal membrane excitability by modulating the properties of Kv4 (Nakamura et al., 2001). It is implied that the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) may influence Kv4 channels activities through the KChIP pathway. KChIPs have 4 subtypes, namely, KChIP1-4. KChIP1 is prominently expressed in the brain and is tightly associated with Kv4.2 (Strassle et al., 2005). Currently, information regarding a possible involvement of KChIPs in epilepsy is insubstantial. We were particularly curious about the association of Kv4.2 with KChIP1 and their involvement in short- or long-term plasticity in epileptogenesis. Thus, the present study was undertaken to examine the possible plasticity changes in Kv4.2 and KChIP1 expression after SE, and explore the possible relationship between changes in A-type potassium channels and disturbed intracellular calcium homeostasis.

EXPERIMENTAL PROCEDURES

Animals

Adult female Sprague–Dawley rats (200~230 g) were obtained from the Laboratory Animal Center of Guangdong Province (Guangzhou, China), housed at a constant temperature of 22±2 °C with a 12-h light/dark cycle, and given free access to food and water. Care and handling of the animals were approved by the Ethics Committee for the Use of Experimental Animals of Guangzhou Medical College. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Antibodies and reagents

The anti-Kv4.2 antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA), the anti-KChIP1 antibody was a gift from Dr Z. H. Zhang (University of California, USA), and anti-β-actin was obtained from Cell Signaling Technology (Danvers, MA, USA). Alexa Fluor 568–conjugated cholera toxin subunit B and Texas Red–labeled goat anti-rabbit IgG were obtained from Molecular Probes (Eugene, OR, USA). Pilocarpine, lithium chloride, horseradish peroxidase–conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Sigma-Aldrich (St. Louis, MO, USA). Atropine and diazepam were purchased from Tianjin Pharmaceutical Co. (Tianjin, China). BCA Protein Assay Kit was obtained from Biocolor (Shanghai, China), the whole protein extraction kit was obtained from Active Motif (Carlsbad, CA, USA). The ABC immunohistochemistry kit was purchased from Martianit (Fuzhou, China). Chemiluminescence reagents were obtained from Applygen Technologies Inc. (Beijing, China), and Fluo-3-AM, from Molecular Probes.

Lithium–pilocarpine protocol

Animals were divided into experimental and control groups. All rats administered lithium chloride (3 mEq/kg, i.p.). After 24 h, the experimental group received 1 mg/kg atropine sulfate, followed by the s.c. injection of pilocarpine (30 mg/kg) after 30 min. Atropine was used to reduce the peripheral effects of pilocarpine. Diazepam (10 mg/kg, i.p.) was administered at 1 h after the onset of SE in order to terminate convulsions in the responsive rats. The animals required special care until they recovered from the acute insult, and they were closely tended, hydrated and fed. The rats that did not show seizures of grades over 4–5 (Racine) were allocated to another group. Following SE, the rats underwent a seizure-free period, namely latent period, and then exhibited SRS. The behavior of the Li-Pilo-treated rats that underwent SE was observed daily in the following 5–9 weeks in order to detect SRS. Those rats exhibiting SRS, known as being in chronic epilepsy phase, were sacrificed 50 days after SE The control group received atropine sulfate, but saline was used instead of pilocarpine. The behavioral characteristics of SE were similar to those observed in previous studies (Roch et al., 2002).

Western blot

An adult rat was anesthetized, and its brain was rapidly excised. Fresh brain tissue was homogenized in lysis buffer according to the protocol of the manufacturer (Active Motif). The protein concentration was determined in the supernatant using BCA method, as recommended by the manufacturer (Biocolor, Belfast, UK). To perform Western blot analysis, the supernatant (2 μg/lane) was resolved by SDS-PAGE, and the protein was transferred to PVDF membranes. Membranes were blocked with Tris-buffered (50 mM) saline solution (pH 7.6) with 0.05% Tween containing 5% milk powder at room temperature for 1 h. The membranes were incubated with the primary antibodies and subsequently with the secondary antibodies; they were then incubated with the chemiluminescence reagents. The membranes were exposed to a radiographic film immediately after incubation. To be reprobed with another primary antibody, the membrane was incubated with a stripping buffer (62.5 mmol/L Tris pH 6.8, 2% SDS, and 100 mmol/L dithiothreitol) at 70 °C for 30 min, washed extensively, reblocked with 5% nonfat milk, and reprobed with the other antibody as described above.

Immunohistochemistry

The rats were anesthetized and perfused intracardially with 0.9% saline followed by 4.0% paraformaldehyde. Following perfusion, the brain was excised, postfixed for 16 h, and sectioned on a cryotome at a thickness of 30 μm along the coronal plane. Following a thorough washing in phosphate-buffered saline (PBS; pH 7.4), the free-floating sections were blocked with 3% normal goat serum and incubated overnight at 4 °C with the KChIP1 monoantibody (1:1000 dilution in 1% normal goat serum in PBS containing 0.25% Triton X-100). On the following day, the samples were washed in PBS before being incubated for 1 h with a biotinylated secondary antibody. After washing, the sections were processed with the avidin biotinylated complex (ABC) and the reaction was developed using 3,3′-diaminobenzidine (DAB). The sections were mounted on slides.

Slice preparation and calcium imaging

At 24 h after Li-Pilo-induced SE, 11 rats (six models, five controls) were anesthetized and the brains were excised, placed in ice-cold artificial cerebrospinal fluid (aCSF; 122 mM NaCl, 25 mM $NaHCO_3$, 3.1 mM KCl, 1.8 mM $CaCl_2$, 12 mM $MgSO_4$, 0.4 mM KH_2PO_4, and 10 mM D-glucose, at pH 7.4), and quartered. Transverse brain slices (400 μM), cut on a vibratome (Hitachi, Japan),

were placed in an incubation chamber containing gassed (95% O_2/5% CO_2) aCSF. The slices corresponded to horizontal plates 98–100, as described by Paxinos and Watson (1986). They were transferred to a dish containing aCSF, loaded with the calcium fluorescent probe Fluo-3-AM (5 μmol/L) and incubated at 37 °C for 30 min. Individual slices were washed and transferred to a home-made glass bottom chamber. The slices were gently pressed with a metal web to prevent them from floating in the water. The chamber was placed on the stage of an inverted microscope. The slices were maintained at 37 °C, submerged in aCSF, and continuously gassed with 95% O_2/5% CO_2. Calcium imaging was performed by using a laser scanning confocal system (Leica SP2, Mannheim, Germany). Sequential scanning was initiated immediately after the addition of 10 mM 4-AP in the aCSF, and it was continued for 7 min at 10 s intervals. 4-AP was washed out using aCSF at 2 min after it was added. During image acquisition, the parameters of the confocal system were kept constant.

Data analyses

For immunostaining and Western blot, densitometric analysis for the quantification of the staining strength or the bands was performed using image analysis software (Kontron, Germany). For KChIP1-postive cell counting, cells were quantified at the coronal section levels (between 3.0–4.0 mm posterior to bregma) on coded slides by a researcher who had no knowledge of the treatment received by the animals. Within certain hippocampal subregion, the number of immunoreactive cells was estimated by counting the neurons in which the reaction product was present within a clear and regular-shaped cytoplasmic border. The anatomy of rat brain was identified with the Stereotaxic Atlas of the Rat Brain (Paxinos and Watson (1986)). For $[Ca^{2+}]i$ measurements, five pyramidal cells in the microscopic field were randomly selected. The neuronal calcium transient was determined by drawing a region of interest (ROI) around the cell and averaging the fluorescence intensity within the delimited area. Fluorescence intensity, which represented $[Ca^{2+}]i$, was converted to numerical values and imported to a spreadsheet. A measurement control and real-time data acquisition, and analysis were performed using the confocal system program (Leica SP2). To analyze the amplitude and decay of the calcium transients, the fluorescence traces were expressed as relative fluorescence changes (F/F_0), where F_0 was the background-corrected pre-stimulus fluorescence of the ROI. All data were expressed as mean$\pm$S.D., and the statistical significance was determined by one-way ANOVA or the two-tailed Student's *t*-test.

RESULTS

Changes in the expression of Kv4.2 and KChIP1 following SE

As an initial experiment, we examined the localization of Kv4.2 and KChIP1 in the normal rat brain. The expression patterns of Kv4.2 and KChIP1 in the hippocampus and neocortex resembled those previously reported (Rhodes et al., 2004). In the hippocampus, enriched and punctate Kv4.2-postive signals were detected in the strata oriens and radiatum of CA1–CA3, corresponding to the apical and basal dendrites of pyramidal cells, and in the molecular layer of the dentate gyrus (Fig. 1). In the cerebral cortex, Kv4.2 staining was observed primarily in the somata and dendrites of the prefrontal, lateral orbital, frontal, parietal, and cingulated cortices, particularly within layers V and VI (data not shown). KChIP1-positive signals were mainly detected in the somatodendritic domains. The ensuing quantitative analyses were thus based on the number of KChIP1-stained cells in the somata. In the brain of the controls, cells strongly stained for KChIP1 were observed to be scattered throughout the hippocampus and neocortex. The CA1 region of the hippocampus showed the strongest KChIP1 staining.

As described above, two main phases were observed following Li-Pilo-induced prolonged SE: the seizure-free latent period and the chronic epileptic phase with SRS. To explore the changes in the expression of Kv4.2 and KChIP1 during these two phases, we selected two time points (2 and 50 days) to perform immunohistochemical examinations. As shown in Fig. 1, Kv4.2 staining in the stratum oriens and stratum radiatum of the hippocampus increased 2 days after SE. The increase was most pronounced in the stratum oriens of the CA1 region. However, the intensity of Kv4.2 staining decreased slightly during the chronic phase, i.e. 50 days after SE in this experiment, and reached the statistical significance only in CA1 and CA3 subregions. Intriguingly, as a candidate of Kv4.2 modulator, KChIP1 also showed a similar change in its expression, which increased 2 days after SE and decreased during the chronic phase. However, the increase in KChIP1 staining at 2 days after SE was not as conspicuous as that in Kv4.2 staining; this was confirmed by the KChIP1-positive cell counting analysis (Fig. 1). The underlying reason, as shown in the next experiment (Fig. 2), was that the peak increase in KChIP1 expression reached earlier and lasted less than that of Kv4.2. On the other hand, decreased KChIP1 staining with fewer positive cells was observed 50 days after SE. Cell counting analysis also revealed a significant reduction in the number of KChIP1 immunoreactive cells in the CA1 and CA3 regions of the hippocampus (Fig. 1). No statistically significant change was observed in the other regions of the hippocampus. The cellular distribution and intracellular localization of Kv4.2 and KChIP1 were not found to be altered with SE and were similar to those observed in the control group.

To evaluate the expression levels of Kv4.2 and KChIP1 following SE, Western blot was used to analyze the extracts from bilateral hippocampus and cerebral cortex. Since many rapid and complicated alterations may occur in the intrinsic neuronal properties during the acute phase of seizure-free latency in the Li-Pilo model, the time-course of dynamic regulation of Kv4.2 and KChIP1 expression in the acute phase following SE was examined. The expression level was traced by measuring the relative optical density of each band. Rats that failed to exhibit seizures after Li-Pilo treatment were also investigated. As shown in Fig. 2, the expression level of Kv4.2 was found to increase at 3 h and was significantly higher at 6, 24 and 48 h, with a peak between 6 and 24 h. KChIP1 also showed a significant increase after SE. However, the peak in KChIP1 expression was observed at 3 h, earlier than that in Kv4.2 expression. Subsequently, it gradually reduced, and no significant difference was observed at 48 h. The controls and the rats that failed to exhibit seizures after Li-Pilo injection showed no significant changes, indicating that the elevation in Kv4.2 and KChIP1 expression was seizure dependent. Fig. 3 shows the changes in the expression of

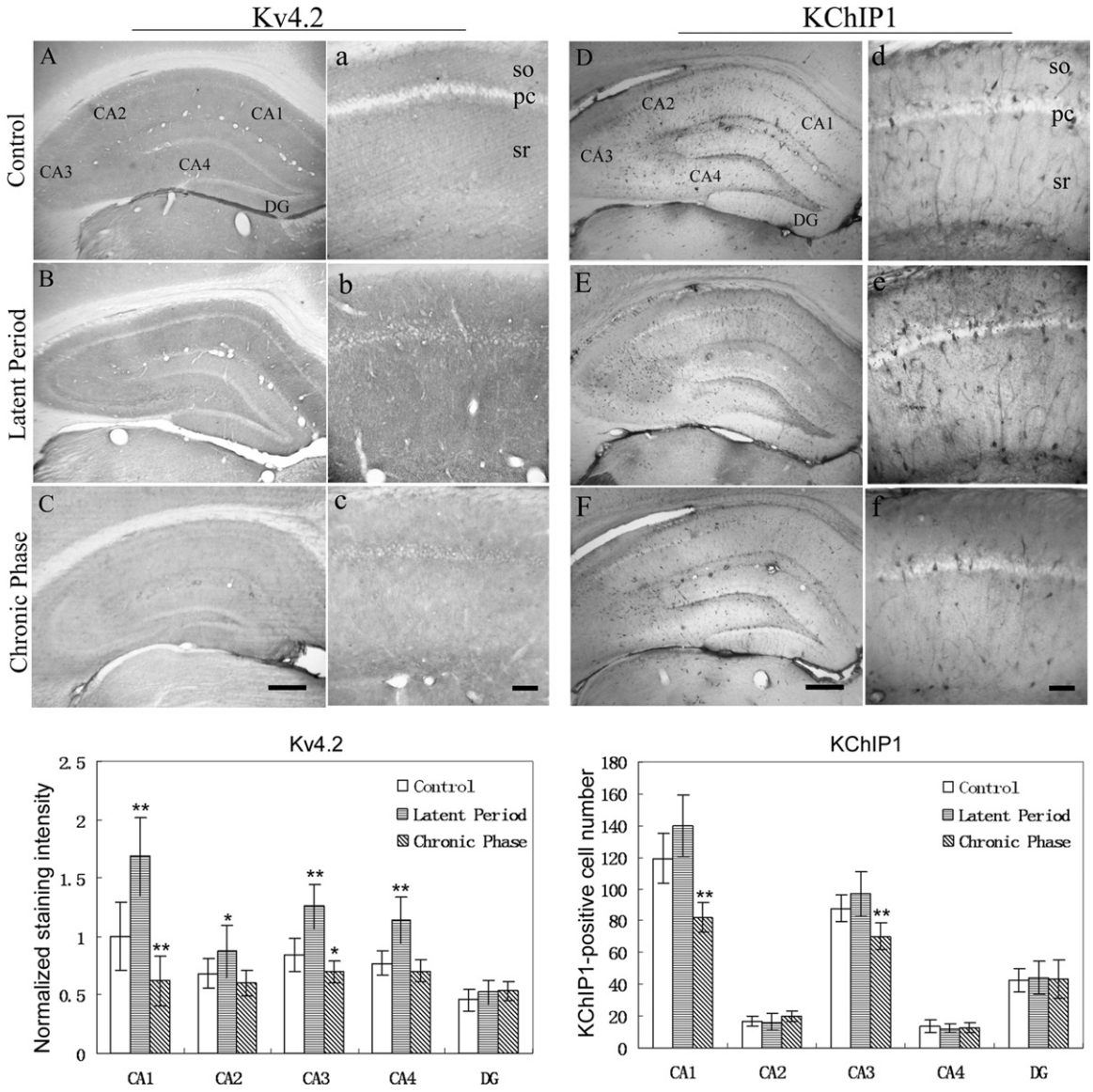

Fig. 1. Expression pattern of Kv4.2 and KChIP1 in the hippocampus in the early latent (2 days) and chronic phase (50 days) of the Li-Pilo model. All sections are stained by DAB. Left images, in a low power view, show the altered intensities of Kv4.2 (A–C) and KChIP1 (D–F) immunoreactive signals in the hippocampus. Right images, in a high power view, highlight the changes (upregulation in latent period and downregulation in chronic phase) in the CA1 region. The bottom graphs show the group data respectively from densitometric measurement of Kv4.2 immunostaining and KChIP1 immunoreactive cell counting. Data are shown as mean±S.D. The asterisks indicate statistically significant differences to control values (** $P<0.01$, * $P<0.05$, *t*-test). $n=8$ for models and $n=12$ for controls. Abbreviations: CA1-4, fields CA1-4 of Ammon's horn; DG, dentate gyrus; so, stratum oriens; pc, pyramidal cell layer; sr, stratum radiatum. Scale bar=1 mm (right); 200 μm (left).

Kv4.2 and KChIP1 in the hippocampus and neocortex during the latent (2 days after SE) and chronic phases (50 days after SE). Generally, the expression levels of Kv4.2 and KChIP1 in the hippocampus were lower than that in the cerebral cortex of the controls. Increased Kv4.2 expression in both the hippocampus and cortex, as well as an increased level of KChIP1 expression in the hippocampus, was observed at 1 day after SE; this is in agreement with the abovementioned results. A slight decrease in the average expression level of Kv4.2 and KChIP1 in the hippocampus and neocortex was observed at 50 days after SE, but no statistical difference was observed. In addition, it appeared that the changes in the hippocampus were more apparent than those in the neocortex for both Kv4.2 and KChIP1.

Difference in $[Ca^{2+}]i$ elevation induced by the potassium channel blocker 4-AP after SE

To characterize the involvement of $[Ca^{2+}]i$ changes during epileptogenesis in this Li-Pilo-induced epilepsy model and explore its possible relationship with A-type potassium channels, we analyzed the dynamics of the changes in $[Ca^{2+}]i$ in the CA1 pyramidal neurons after SE. We chose

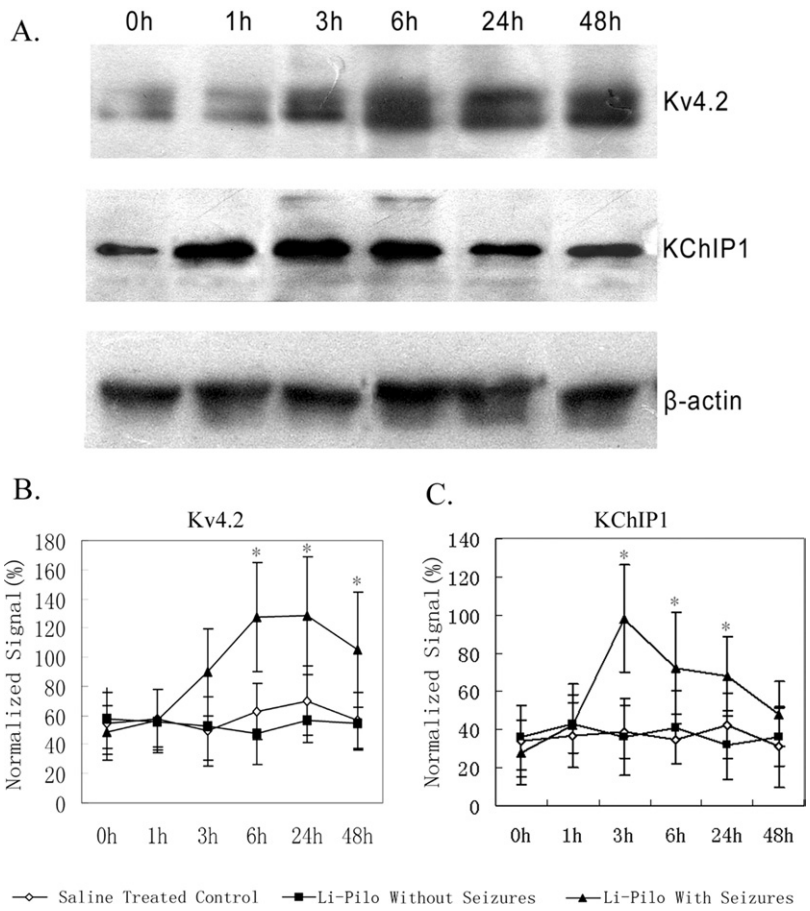

Fig. 2. Dynamics of Kv4.2 and KChIP1 expression in hippocampus following SE. (A) The dynamics of Kv4.2 and KChIP1 expression at different time after seizures. Zero hour refers to the time when rats were administrated diazepam to terminate the generalized seizures. (B, C) Group data from the experiments described in A, values of relative optical density had been normalized and presented as mean±S.D. (n=6). The asterisks indicate statistically significant differences vs. control values (* $P<0.05$). The group of "model without seizures" represents those rats that failed to exhibit SE after Li-Pilo injection.

24 h after SE as the time point for observation, because the expression of Kv4.2 and KChIP1 was significantly increased at that time point. No difference was observed in the resting level of $[Ca^{2+}]i$ in the CA1 pyramidal neurons of the hippocampal slices between Li-Pilo and controls. The exposure of CA1 neurons to 10 mM 4-AP resulted in an abrupt increase in $[Ca^{2+}]i$ (Fig. 4). In the control slices, the 4-AP-induced elevation in $[Ca^{2+}]i$ could be observed in both the soma and dendrites of the pyramidal neurons. Within 1.5 min 4-AP exposure, the $[Ca^{2+}]i$ level reached a plateau, with an average increase of 293±65% from the resting level. The $[Ca^{2+}]i$ then gradually began returning to the resting level gradually with the washing out of 4-AP. In some cases of the models, the elevated $[Ca^{2+}]i$ could not return to the resting level even at the end of the 7-min observation period. Therefore, the decay time for the $[Ca^{2+}]i$ to reach 180% of the resting level was used as an index for performing statistical analyses. The amplitude of the $[Ca^{2+}]i$ elevation and the latency to the peak value were comparatively measured in control and Li-Pilo. As shown in Fig. 4, a sharper and higher elevation in $[Ca^{2+}]i$ was found in Li-Pilo slices, with a peak value of 524±73%. The latency to the peak response was similar in control (88±13.2 s) and Li-Pilo (102±17.5 s). The $[Ca^{2+}]i$ recovery curve and the decay time (93.4±27.3 s vs. 51.1±24.5 s, $P<0.05$) showed that the $[Ca^{2+}]i$ recovery was slower in Li-Pilo, even after 4-AP washout. These data indicate that the pyramidal neurons that underwent SE are more sensitive to 4-AP exposure, and were less capable of restoring the normal $[Ca^{2+}]i$ level.

DISCUSSION

Since I_As that are mediated mainly by Kv4.2 play a crucial role in the regulation of neuronal membrane excitability, the changes of Kv4.2 in relation to epileptic activity have frequently been investigated. The altered expression of Kv4.2 has been inconsistently observed in previous studies. Some investigators have shown that the Kv4.2 mRNA levels were transiently reduced in the granule cell layer of the dentate gyrus following pentylenetetrazol (PTZ)-(Tsaur et al., 1992) or kainate-induced generalized seizures (Francis et al., 1997). Similarly, a recent study showed reduced Kv4.2 expression in the CA1 subregion in a pilocarpine-induced spontaneously epileptic rat (Bernard et al., 2004). Other studies have reported that unaltered

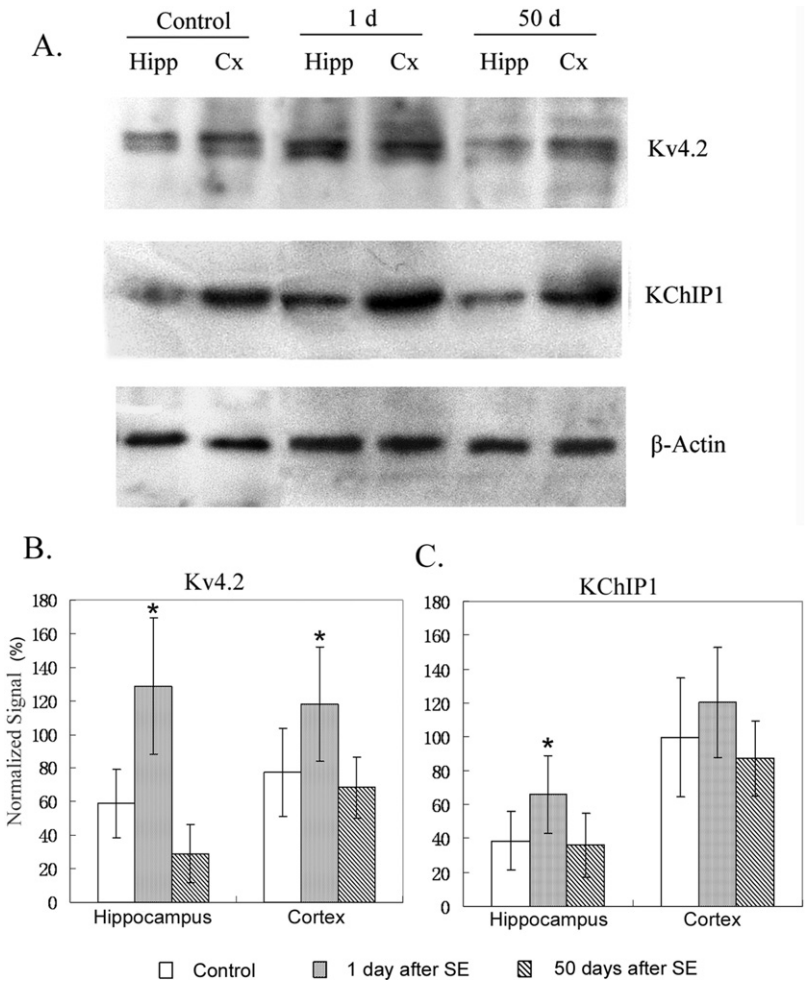

Fig. 3. Expression levels of Kv4.2 and KChIP1 in hippocampus and cerebral cortex in the early latent and the chronic phase. (A) The expression changes in Kv4.2 and KChIP1 in the cerebral cortex are similar but less conspicuous as compared with that in the hippocampus. (B, C) Group data from experiments as described in A with signals normalized to control. * Indicates statistically significant differences between Li-Pilo-treated rats and controls ($n=6$, * $P<0.05$. *t*-test).

Kv4.2 mRNA levels in the dentate gyrus during the chronic phase of pilocarpine-induced epilepsy in rats (Ruschenschmidt et al., 2006). The present data show that the Kv4.2 expression was transiently upregulated at the level of the entire hippocampus during the latent period of Li-Pilo-induced epilepsy in rats; however, it exhibited a reducing trend during the chronic phases in which spontaneous seizures could occur. It is suggested that the alterations in Kv4.2 expression may be a common yet complex process that occurs during the development of epilepsy. The time course of Kv4.2 expression following Li-Pilo-induced prolonged SE has not previously been investigated. In the present study, observation of the dynamic changes in Kv4.2 expression in the hippocampal formation showed that Kv4.2 expression started increasing from 3 h after the seizures, reached a maximum at 6–24 h; then, it began returning to its normal levels. The present data are comparable with that presented in a report on the kainate epilepsy model (Francis et al., 1997), in which the Kv4.2 mRNA expression levels in the dentate granule cell layer decreased 3 h after the seizures, but rebounded to levels greater than those of the controls at 24 h; however, Kv4.2 mRNA expression was significantly decreased in CA3 at the same time point. An increase in Kv4.2 expression was observed in both the studies, but with differences in the substructures. It is possible that different substructures in the brain may be involved, or that different dynamic changes may occur due to different convulsants. Moreover, Kv4.2 is an important regulator of electrical signaling in the brain. Thus, it is not surprising that it exhibits various dynamic regulations in different epilepsy models.

It is reported that the A-type current can influence active discharge properties and the processing of synaptic input by limiting the amplitudes of backpropagating action potentials, decreasing the amplitude of excitatory postsynaptic potentials (EPSPs), and raising the threshold for dendritic spike initiation (Hoffman et al., 1997; Magee and Carruth, 1999); this finally limits the excitability of individual neurons. From these data, the most pronounced effect that is expected following the up-regulation of the Kv4.2 channels as observed here would be the suppression of neuronal excitability in the brain, assuming that the levels of

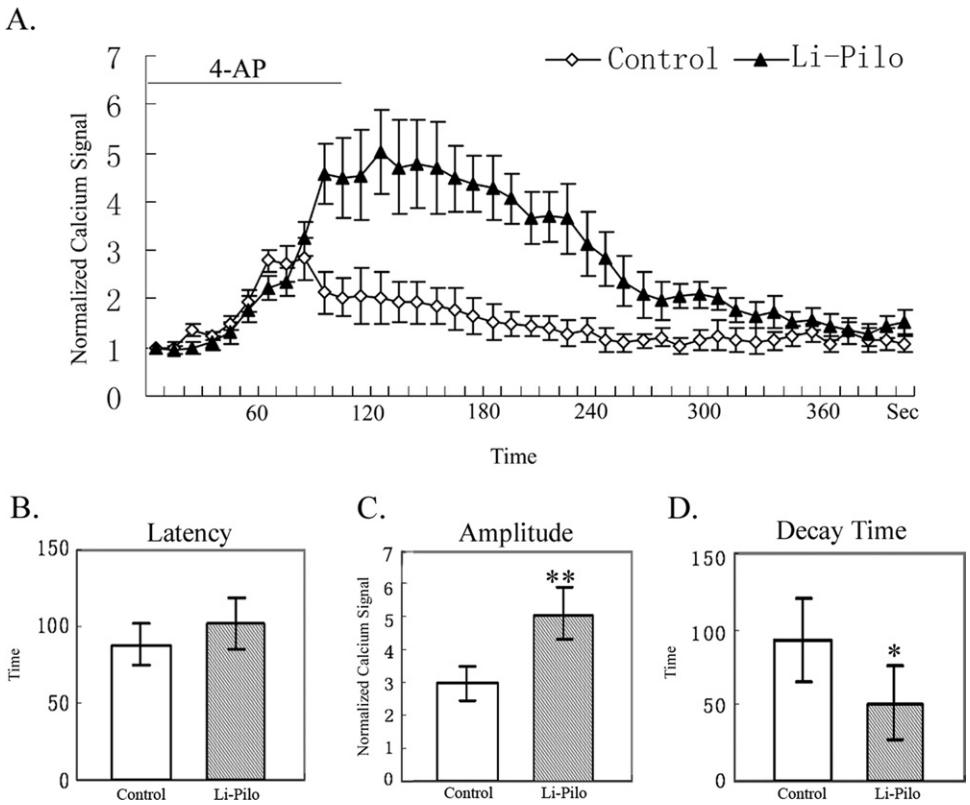

Fig. 4. Alterations in 4-AP-induced $[Ca^{2+}]i$ transients in Li-Pilo rats and controls 24 h after SE. Slices were perfused with Kv4 channel blocker 4-AP for 2 min and then washed out (see the bar in A). $[Ca^{2+}]i$ was measured for 7 min from the time of 4-AP application. (A) The dynamic changes of $[Ca^{2+}]i$. (B–D) The statistical analyses on the amplitude of $[Ca^{2+}]i$ elevation, the latency to the amplitude, and the decay time after 4-AP washout. * Indicates statistically significant differences (** $P<0.01$, * $P<0.05$, *t*-test) between models ($n=6$) and controls ($n=5$).

Kv4 expression reflect the density of the A-type current in the membrane. In our opinion, such suppression would represent a homeostatic mechanism following severe and persistent seizures. The available evidence suggests that some expression changes in various channels serve to stabilize the properties of neural circuits (Stemmler and Koch, 1999; Turrigiano, 1999). For example, the immunoreactivity of KCNQ2, another type of Kv that mediates the M-current, was found to be up-regulated both in both the amygdala kindling model and the spontaneous epilepsy initiated by SE; the up-regulation was only induced by severe generalized seizures (Penschuck et al., 2005). In addition, electroshocks in humans (Krystal et al., 1998; Regenold et al., 1998) and animals (Bolwig et al., 1999) result in a 40–100% increase in the seizure threshold and a 30–90% decrease in the seizure duration; this reflects that generalized seizures possibly induce a suppression in neuronal excitability. Considerable interest has recently been generated in such seizure-induced neuronal excitability suppression by pre-exposure to generalized seizures in order to exert anti-convulsive effects via modulation of neuronal excitability (Andre et al., 2000). Therefore, it is plausible that the up-regulation of Kv4.2 expression would adjust the neuronal excitability to a suppressed state, which might act as a sort of self-protective mechanism following prolonged SE. It is noteworthy that the time of maximal Kv4.2 overexpression was 6–24 h after seizures, when many neurons in the hippocampus are morphologically dying in the model used here. Therefore, the up-regulation of Kv4.2 after SE may be thought to protect neurons from seizure-induced damage by counteracting hyperexcitability.

In agreement with the reduced Kv4.2 expression observed in the CA1 region during the chronic phase of the pilocarpine model (Bernard et al., 2004), our immunostaining, also in the chronic phase, showed that Kv4.2 expression decreased, although the reduction did not reach a value of statistical significance in the protein analysis by Western blot. The lack of significance may be due to the regions selected. In our studies, the Western blot data represent Kv4.2 expression at the level of the whole hippocampal formation. Therefore, it is possible that the reduction might become less considerable when different subregions are included, considering the fact that Kv4.2 expression is highly variable among different subregions of the hippocampus, as we found in the immunostaining. The results of immunohistochemistry which showed that the signal of Kv4.2 immunoreactivity was weaker and the number of KChIP1-positive cells was less in the CA1 and CA3 regions suggested that the expression of Kv4.2 was down-regulated during the chronic phase of the Li-Pilo-induced model. Although not directly tested, such reduction in Kv4.2 expression during the spontaneous seizure period should lead to an increase in the amplitude of the back-

propagating action potentials and boost dendritic EPSPs, thereby producing a general increase in dendritic excitability. This increase in CA1 or CA3 is likely to lower the threshold for seizure initiation in the hippocampus, which is a frequent site of limbic seizures. Therefore, abnormalities in Kv4.2 expression could contribute to the generation of seizures and epilepsy.

The abundance and distribution of Kv4.2 channels are regulated by multiple molecular mechanisms (Birnbaum et al., 2004). First, the gene is activated at the transcriptional level by various stimuli. Second, a large variety of Kv4.2-interacting proteins, including Kvβ, KChIPs, and DPPX, regulate the expression and trafficking of Kv4.2. Third, channel proteins undergo posttranslational modifications, such as glycosylation, methylation, and phosphorylation. Recent data suggest that KChIPs are one of the most important factors that determine the level of the Kv4-encoded current in various excitable cells. Coexpression studies have shown that surface expression of the Kv4.2 protein is highly dependent on the presence of KChIP1 (Shibata et al., 2003). KChIP1 can dramatically increase this surface expression, slow the turnover of the Kv4 protein, slow the inactivation kinetics, and increase the rate of recovery from the inactive state of Kv4 channels (An et al., 2000; Shibata et al., 2003; Jerng et al., 2004). However, the functional contributions of KChIPs to channels in neural circuits are not well understood. Recently, a decrease in KChIP3 expression in the hippocampal tissue was shown in human chronic epilepsy (Hong et al., 2003). Our previous data have also shown that the number of KChIP1-positive cells was increased in an acute PTZ-induced epilepsy model (Su et al., 2006). We therefore hypothesize that the Kv4–KChIP complex may be involved in epilepsy. Indeed, one of the most prominent findings with respect to the changes in Kv4.2 in the present study is the significant change of KChIP1 following SE. Time-course analysis of the elevation in KChIP1 and Kv4.2 expression in the early latent period following SE showed that the altered expression of Kv4.2 precisely followed the change in KChIP1, with the elevation in KChIP1 occurring a little earlier than that in Kv4.2.

Although KChIP1 staining is most intense on the somata of cells that resemble interneurons and Kv4.2 staining is most intense on the dendrites of the pyramidal neurons of all the CA subfields and dentate granular neurons, a considerable level of overlap is observed. A colocalization experiment show that Kv4.2 and KChIP1 are mostly colocalized in the neocortex and less in the hippocampus (data not shown). In the immunohistochemistry experiment, however, we found that the changes in Kv4.2 were marked in CA1 and CA3, but were observed to a lesser extent in the other regions. Interestingly, KChIP1 expression is also selectively up-regulated in the CA1 and CA3 subregions of the hippocampus after SE. These results suggest that the regulation of Kv4.2 and KChIP1 is highly related to the subfield and the cell type. On the other hand, in most other brain regions, like the neocortex, the changes in Kv4.2 and KChIP1 were not as apparent as those observed in the hippocampus; this was shown by the results of both immunohistochemical staining and Western blot analysis. Such subregion-specific regulation of ion channels has also been demonstrated for Kv4.2 channels (Francis et al., 1997) and other voltage- or transmitter-gated ion channels (Becker et al., 2003). All these data suggest that in Li-Pilo-induced epilepsy models, the hippocampus is preferentially affected, as compared with other limbic regions or the cerebral cortex. It is also reported that the neurodegenerative processes initiated by prolonged SE occur prominently in the hippocampus (Roch et al., 2002), particularly in the pyramidal cells in CA3 and CA1 (Peredery et al., 2000). We found a decreased number of KChIP1-immunoreactive cells in CA1 and CA3 as well as a decreased level of the KChIP1 protein in the hippocampus during the chronic period. We cannot exclude the possibility that the decrease in the number of KChIP1-positive cells is a result of neurodegeneration caused by prolonged SE. Considering that KChIP1 is mainly located on GABAergic interneurons, as revealed in our previous data (Su et al., 2006), it is likely that the observed decrease in KChIP1 was associated with the loss of GABAergic neurons, possibly resulting in deficient release of the inhibitory neurotransmitter and, consequently, hyperexcitability during the chronic period. However, we cannot confirm this without additional evidence.

It is well accepted that the expression and trafficking of Kv4 channels are regulated by KChIPs, that act as neuronal calcium sensor. Calcium, which is a major signaling molecule in neurons, regulates a wide variety of cellular processes, including ionic channel permeability, neurotransmitter release, enzyme activity, and gene transcription. Alterations in neuronal calcium homeostasis are believed to play an essential role in the development of epilepsy (Delorenzo et al., 2005). This prompted us to extend our investigation by measuring the $[Ca^{2+}]i$ transient that is induced by the potassium channel blocker 4-AP in the epileptic hippocampal slices, with the aim of improving our understanding of the signaling cascades underlying the up-regulation of KChIP1 and Kv4 expression after SE and the role of calcium homeostasis in epileptogenesis. Although it is known that increased $[Ca^{2+}]i$ can be induced by 4-AP administration in the normal brain (Grimaldi et al., 2001), the effect of 4-AP on the brain after prolonged SE has not yet been studied. Thus, we compared the differences in elevated $[Ca^{2+}]i$, which was preloaded by 4-AP, between the SE slices and the control slices. Statistical analysis revealed that the amplitude of $[Ca^{2+}]i$ elevation in the CA1 pyramidal cells, which was induced by 4-AP, was higher in the epileptic slice than in the control slice. Although several classes of potassium channels are sensitive to 4-AP (Coetzee et al., 1999), Kv4.2 is expressed more strongly in CA1 pyramidal cells (Serodio and Rudy, 1998). Thus, the effect of 4-AP on the CA1 pyramidal cells mainly reflects the blocking of Kv4.2. Based on these observations, one of the causes of the higher elevation of $[Ca^{2+}]i$ induced by 4-AP after SE may be the blockage of Kv4.2 channels in CA1 pyramidal cells. Because Kv4.2 channels expression was up-regulated after SE, as shown by the Western blot and immunohistochemistry experi-

ments, it is reasonable to see an enlarged elevation of $[Ca^{2+}]i$. It is also possible that the calcium influx and release is facilitated by the changes in multiple calcium homeostatic mechanisms after SE, including VSCC, ligand-gated ion channels, Mg^{2+}/Ca^{2+} ATPase, and calcium-induced calcium release (CICR) (Pal et al., 2001; Raza et al., 2001; Parsons et al., 2001).

We have also demonstrated that 4-AP-induced $[Ca^{2+}]i$ elevation is potently prolonged in the epileptic slice. This delayed restoration of $[Ca^{2+}]i$ may be caused by an impairment in neuronal Ca^{2+} homeostatic mechanisms. Several sequestration mechanisms function in the physiological condition, such as the activity of the sarcoendoplasmic reticulum calcium ATPase (SERCA) and IP3-stimulated CICR, that has been demonstrated to undergo chronic changes in epileptic neurons (Pal et al., 2001). Calcium-binding proteins are one of the critical elements that maintain the stability of intracellular calcium (Missiaen et al., 2000). Changes in the expression level and/or in the subcellular localization of Ca^{2+}-binding proteins may affect the regulation of cytosolic calcium-buffering capacity (Baimbridge et al., 1992; Chard et al., 1993). For example, the loss of the Ca^{2+}-binding protein calbindin-D28K impairs Ca^{2+}-buffering in epileptic neurons (Kohr et al., 1991), and it is associated with the development of epilepsy (Hwang et al., 2004). In the present study, the expression of KChIP1, a calcium-binding protein, was also found to be altered during epileptogenesis. Although it is difficult to define the effect of KChIP1 on calcium-buffering capacity by using the present data, the possibility that KChIPs function not only as regulators of Kv4 but also as contributors to calcium buffering cannot be excluded.

Regardless of the mechanisms underlying calcium influx and buffering, the potentiation and prolongation of 4-AP-induced $[Ca^{2+}]i$ elevation may reflect disturbed calcium homeostasis and may be related to abnormal potassium channel expression in the membrane in the hippocampus after prolonged SE, probably with a consequent development of limbic epilepsy. Since the neuronal free $[Ca^{2+}]i$ is highly regulated, any change in $[Ca^{2+}]i$ dynamics may affect neuronal function, including potassium channel expression, trafficking and properties (Adams and Hill, 2004; Jow et al., 2004). KChIPs are calcium binding proteins, sensing the subtle changes in $[Ca^{2+}]i$ and transducing the calcium signals into changes of membrane excitability by modulating Kv4 activity (Burgoyne and Weiss, 2001). Considering the close relationship between Ca^{2+} and the Kv4–KChIPs complex under physiological conditions, it is possible that disturbed Ca^{2+} homeostasis affects the regulation of A-type potassium channels. It is also conceivable that altered Ca^{2+} dynamics trigger pathological changes, such as gene transcription, protein expression, neurogenesis, neuronal sprouting (Delorenzo et al., 2005) and so on, leading to the development of epilepsy; however, at present, it is difficult to determine which of these processes is involved.

In a word, the present study shows that the expression of Kv4.2 and KChIP1 is increased at the early latency stage and decreased during chronic phase of the Li-Pilo epilepsy model. Based on their close modulation relationship and important physiological function on neuronal network excitability, the plastic changes in Kv4.2 and KChIP1 may collaborate to influence the intrinsic neuronal excitability, thereby contributing to the development of epilepsy. The altered transient of $[Ca^{2+}]i$, elicited by the Kv4 channel blocker 4-AP, has been found in the model, and this suggest the disturbed calcium homeostasis, which might be related with Kv4 modulation, involved in epileptogenesis. At the present stage, we were unable to reveal the exact functional significance of altered Kv4.2 and KChIP1 expression or a clear correlation between the calcium signal and A-type channels during epileptogenesis. Further studies in this field will be helpful in understanding the mechanism underlying epileptogenesis.

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By-stander effect on brain tissue of mesoangioblasts producing the neurotrophin BDNF

Long-term maintenance of the structural integrity of organotypic hippocampal slices prepared from the adult brain

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Abstract

Nerve growth factors, especially brain-derived neurotrophic factor (BDNF) are involved in the regulation of neuronal survival and function and, thus, they may be applied to treat neurological diseases associated with neuronal death. One of the major keys for a successful clinical application of neurotrophic factors is the mode of delivery. Stem cell-based gene therapy is a potential delivery option by which cells are engineered to produce neurotrophic factors in vitro and then transplanted to the target area where neurotrophic factors are secreted to exert protective effects on host tissue. To pursue this aim, we generated a genetically modified stem cells (mesoangioblasts) to produce BDNF (MABs-BDNF) and assessed their neuroprotective effects in vitro using organotypic cultures of adult hippocampal slices, in which slices exhibit continuous and extensive neurodegeneration. The viability of the cultured slices was assessed in several ways: fluorescein diacetate (FDA) hydrolysis assay, lactate dehydrogenase (LDH) release assay, immunohistochemistry for MAP2, immunoblot for neurofilament 68, and field potential recording. We found that the engineered MABs maintained transgene expression and secretion of bioactive BDNF in time. Direct exposure of recombinant BDNF to the adult slices resulted in a concentration-dependent protective effect. Surprisingly, the addition of culture supernatant from MABs-BDNF reduced cell death to a much greater extent, while the conditioned media from the control cells (MABs) or an equivalent amount of rBDNF showed smaller beneficial effects. The protective effects of MABs-BDNF could be attenuated by adding either the TrkB receptor blocker K252a or the BDNF scavenger TrkB-IgG. These data indicate that the conditioned media from MABs-BDNF can foster the adult slice culture through secretion of BDNF and other, unknown pro-survival factors produced intrinsically by MABs. In terms of preservation of the basic cellular and connective organization, as visualized by MAP2 immunolabeling and field EPSP, the slices cultured with MABs-BDNF conditioned media better retained their morphologic characteristics and functional connections. These findings demonstrate that MABs-BDNF can be an effective vehicle for BDNF delivery, capable of promoting the survival of adult neurons. Transplantation of the MABs-BDNF might be a potential therapeutic approach to treat neurodegenerative diseases.

Key words: mesoangioblasts, BDNF, organotypic hippocampal slice culture, cell viability, drug delivery

INTRODUCTION

Neurotrophic factors (NTFs) are critical for the survival, development and function of neurons in the mammalian central nervous system (Huang and Reichardt, 2001). Alterations in NTFs or their receptors leading to loss of function can cause neuronal death and contribute to the pathogenesis of neurodegenerative diseases such as Alzheimer disease (Connor et al., 1997), Huntington disease (Zuccato and Cattaneo, 2007), Parkinson disease (Bradford et al., 1999), and multiple sclerosis (De Santi et al., 2009). Many NTFs have been also implicated in diseases associated with damage, like stroke and some forms of epilepsy (Simonato et al., 2006). It is not surprising, therefore, that they received considerable interest as therapeutic agents for neurological diseases (Thoenen and Sendtner, 2002; Simonato et al., 2006).

A particular attention has been devoted to brain derived neurotrophic factor (BDNF). A vast literature shows that BDNF exerts trophic and neuroprotective effects on neurons, but its clinical use is hindered by a short biological half-life and a poor blood-brain barrier permeability (Boado, 2008). Given these difficulties, the attention turned into the development of techniques that allow local BDNF delivery. These may be based on gene therapy (Paradiso et al., 2009) or on cell-mediated approaches.

Stem cell-mediated gene delivery, by which stem cells are genetically modified with NTFs and then transplanted into the brain, is emerging as a strategy to treat chronic neurological disorders and injury-related neural degeneration. This strategy has already been used and validated in several lines of stem cells, including neural (Martinez-Serrano and Bjorklund, 1996; Lu et al., 2003; Kameda et al., 2007), embryonic (Li et al., 2008; Rizvanov et al., 2008; Makar et al., 2009) and mesenchymal stem cells (Harper et al., 2009): when engineered to produce NTFs, these cells were found to ameliorate experimental neurodegenerative diseases. The advantages of using genetically modified stem cells as grafts include the fact that they could exert not only long-term functional integration but also repair capabilities and that they can ensure a continuous and concentrated local supplementation of diffusible therapeutic molecules (like BDNF), reducing non-selective delivery and allowing high treatment efficiencies for long time periods.

These findings prompt the search for alternative cell sources that, unlike neural or embryonic stem cells, may be readily used in the clinics. Here, we explored the feasibility of a new approach for the local supplementation of BDNF in lesioned brain areas. This approach is the use of multipotent, mesodermal stem cells (mesoangioblasts, MABs) that constitutively produce a subset of NTFs (e.g.VEGFB, FGF-2, FGF7) and can be engineered to produce others. MABs are an affordable cellular source. They can be isolated from the perivascular human adult tissue, and are already utilized for regenerative purposes in complex animal models, other than rodents. Their principal feature is the ability to differentiate, under appropriate conditions, in the different mesodermic tissues. The MAB-based therapy is now approaching the clinics (Sampaolesi et al., 2006).

MABs have a high adhesin-dependent migratory capacity and, therefore, can reach perivascular targets. Their chemotactic ability is increased under inflammatory conditions, when the adhesin-integrin system and diapedesis moving are activated (Galvez et al., 2006). Therefore, when peripherally administered, MABs may selectively cross the blood-brain barrier and home in the lesioned areas. These cells have many neuro-ectodermal genes, but they do not differentiate into neurons (Tagliafico et al., 2004). Thus, we do not plan to use them for a restorative engraftment, but to exploit their migratory ability for targeting the lesion area: after infusion in the peripheral blood, MABs are expected to act as a reservoir and delivery system for NTFs.

Aim of this study was to examine the by-stander effects of MABs engineered to produce and secrete BDNF. To pursue this aim, the medium collected from MAB cultures (containing BDNF and other constitutively produced NTFs) has been applied to hippocampal organotypic cultures. The organotypic hippocampal slice culture (OC)

provides an ideal in vitro model system to assess toxic or trophic effects of the test agents since it preserves the morphological and physiological features of the hippocampal neuronal network and allows easy access and precise control of extracellular environment for a short or long period (Stoppini et al., 1991; Kristensen et al., 2001). In general, OCs are prepared from early postnatal animals, not older than postnatal day 10 (P10) (Gahwiler et al., 1997) because, when prepared from adult hippocampal slices, they rapidly undergo neuronal degeneration (Wilhelmi et al., 2002). We elected to employ hippocampal cultures prepared from adult animals (Xiang et al., 2000) because they do not survive well in standard culture media and, thus, may provide a very convincing evidence of the favorable effects of the supplementation of NTFs if they survive when cultured in the MAB medium.

METHODS

MABs-BDNF and cell culture

In order to achieve ex vivo cell-based gene delivery, MABs was transfected with a reporter green fluorescent protein (GFP) and with the human BDNF gene, obtaining a stable cell line (MABs-BDNF) that can constantly express BDNF. The control MABs were only engineered with GFP. These experiments were performed by Raffaella Scardigli and Antonino Cattaneo at the EBRI institute in Rome. The control MABs were only engineered with GFP. The two cell lines were cultured with the ordinary methods, using the following culture medium (here referred to as Medium-1): 88% Dulbecco's modified Eagle's medium, 10% fetal bovine serum，0.5% GlutaMaxII, 22 mM mg/ml glucose, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin (all from Gibco, Invitrogen). After 2 days in culture, the medium was collected and centrifuged for 5 min at 1000 rpm, and then the supernatant was collected. The supernatant consists of the medium containing the soluble substances secreted by MABs, including BDNF in the case of MABs-BDNF. An ELISA Kit (Chemicon) was used to measure the amount of BDNF in the supernatant.

Primary hippocampal neuronal cultures

Primary hippocampal neuronal cultures (PCs) were derived from P0 newborn Swiss mice. Hippocampi were dissected and minced with forceps, and then completely dissociated into a single-cell suspension using trypsin digestion. Isolated hippocampal cells were plated at a low density of approximately $5 \times 10^4 \cdot cm^{-2}$ viable cells in 24-well plates coated with poly-L-lysine (Sigma). Cells were grown in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin 50 U/ml and streptomycin 50 mg/ml. The culture medium was replaced with the different conditioned media (see below) supplemented with 5 μM Ara-C at the day after plating. The cells were maintained at 37°C under a humidified atmosphere of 95% air and 5% CO2.

Organotypic hippocampal slice cultures

Hippocampal organotypic slice cultures (OCs) were prepared as described by Stoppini et al. (Stoppini et al., 1991) with slight modifications. Male Swiss mice (4 weeks old, Morini Co., Italy) were briefly anesthetized by diethyl ether and decapitated. The brains were removed into ice-cold artificial cerebrospinal fluid (aCSF) consisting of (in mM): NaCl 118, KCl 2.5, $MgSO_4$ 3, NaH_2PO_4 1.1, $NaHCO_3$ 26, $CaCl_2$ 1 and glucose 11 (all reagents from Sigma) bubbled with 95% O_2/5% CO_2. Subsequently, 300 μm thick coronal slices were cut with a vibrotome (MA752, Campden Instruments). The hippocampi were dissected in cold, oxygenated Hank's balanced salt solution (Gibco, Invitrogen), transferred onto sterile porous membrane confetti (Millicell, Millipore), and cultured with their standard medium (here referred to as Medium-2) or with the MAB-conditioned media (see below). The incubation conditions were maintained in a humidified 5% CO_2 atmosphere at 37°C. The culture medium was changed the day after preparation and then every 2–3 days for the course of the experiment.

Preparation of the conditioned media

The effects of the MABs delivering BDNF on cell survival were evaluated in several experimental

groups cultured with a special conditioned media. In the groups “MABs” and “MABs-BDNF”, the condition media was composed by the mixture of equal volumes of fresh Medium-1 and of the 2-day-culture supernatant from MABs and MABs-BDNF, respectively. The group called Medium-1 served as a control, being challenged with the fresh Medium-1. Medium-2 is a standard culture medium for OHSC from postnatal animals, consisting of 50% MEM, 25% horse serum, 18% HBSS, 4 mM L-glutamine, 12 mM glucose, 4.5 mM $NaHCO_3$, 20 mM sucrose, 100 U/ml penicillin and 100 mg/ml streptomycin (from Gibco or Sigma). The other conditioned media were based on the above media with different supplementations of reagents, like the recombinant human BDNF (ranged from 0.03 ng/ml to 300 ng/ml, Immunological Sciences), the BDNF antagonist K252a (50 nM, Sigma) or TrkB-IgG (2 μg/ml, R&D Systems), a recombinant tyrosine kinase receptor B (TrkB) engineered as an immunoadhesin to sequester BDNF. Sister slices were randomly assigned to the different groups.

Assessment of viability

The viability of the neurons in primary culture and the slices in organotypic culture was assessed in two ways. First, the fluorescein diacetate (FDA) hydrolysis assay was used to measure enzyme activity in cells. Living cells actively convert the non-fluorescent FDA into the green fluorescent compound "fluorescin", an indication of cell viability. Slices have been incubated with 10 μg/ml FDA (Sigma) for 30 min, then images were captured using an optical microscope (DMRA2, Leica, Germany) and the fluorescence intensity was quantified using the software Image-Pro Plus 6.0 (Media Cybernetics, USA). Second, the lactate dehydrogenase (LDH) release assay was used to measure cell death in vitro. LDH is a stable cytoplasmic enzyme present in all cells, that is rapidly released into the culture medium upon damage of the plasma membrane; thus, LDH leakage to the medium is a marker for cell damage. The culture medium was all collected and LDH leakage was quantified by using a LDH Cytotoxic Test kit (Clontech) according to the manufacturer’s instructions.

Immunohistochemistry

Cultured slices were fixed with 4% paraformaldehyde (PFA) in phosphate buffer solution (PBS) for 1 h at room temperature. After a 48 h incubation with 30% sucrose at 4°C, slices were sectioned to a thickness of 30 μm in a cryostat (CM1510, Leica, Germany). They were then washed in PBS (pH 7.4) and incubated with 0.3% Triton in PBS for 1 h. PBS with 5% BSA and 5% normal goat serum was used to block the slices for 30 min, and then they were incubated overnight with the primary antibodies mouse anti microtubule-associated protein (MAP2abc; 1:50; Immunological Sciences). Finally, they were rinsed with PBS and incubated with the secondary antibody Alexa 488 goat anti mouse IgG (1:100). The slices were mounted onto slides after staining with DAPI (1:1000).

Western blot

To identify the density of the survival neurons within a slice, we performed immunoblot analysis of the neuron-specific marker neurofilament 68. After 14 days in culture, slices were rinsed with ice-cold PBS and then lysed in sample buffer (ECL western blotting kit, Amersham). Aliquots from each sample (15 μg protein/lane) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidine fluoride membranes (Millipore). The blots were blocked in blocking buffer (20 mM Tris-HCl, 137 mM NaCl, and 5% skim milk) for 1 h at room temperature and then treated with anti-neurofilament 68 antibody (diluted 1:500; Sigma) overnight at 4°C. Membranes were washed repeatedly in Tris-buffered saline containing 0.05% Tween 20, and then the HRP-conjugated secondary antibody (diluted 1:20000) was added for 1 h. Immunoreactive bands were detected using enhanced luminol-based chemiluminescence (ECL). The membranes were stripped and then immunoblotting for beta-actin (1:1000, Sigma) performed, as a loading control. Bands were scanned into digital images and analyzed with the software of Image-Pro Plus (Media Cybernetics, USA).

Field potential recording

After 7 days in culture, slices were transferred to a holding chamber for 1 hour at room temperature in aCSF, while continuously aerated with 95% O_2 and 5% CO_2. Slices were then placed in a submerged in vitro recording chamber and perfused with oxygenated aCSF. The temperature in the recording chamber was kept at 36±1°C. Bipolar wire electrodes (tungsten with a tip diameter of 90 µm; WPI Inc.) were used for stimulation of the Schaffer collateral pathway in the CA3 region (70 µs duration rectangular pulses at 0.05 Hz for 120 s). Glass microelectrodes filled with 0.9% NaCl were placed on the CA1 stratum radiatum to record field excitatory postsynaptic potential (fEPSP). A conventional electrophysiological technique of extracellular recordings was employed to identify the maximal response and to adjust the stimulus strength. The stimulus intensity that could repeatedly evoke the maximal synaptically evoked response/excitatory postsynaptic current (up to 900 µA) was used in each slice. Signals were acquired under constant conditions, and off-line processed using the Patchmaster software (HEKA Instruments Inc., Germany).

Statistical analysis

All values have been expressed as the means±S.E.M. The Student's t-test was used when two groups were compared. When three or more groups were compared, analysis of variance (ANOVA) and *post hoc* Fisher's least significant difference (LSD) test or Newman-Keuls test were employed.

RESULTS

Recombinant BDNF exhibits concentration-dependent protective effects on cell survival in primary hippocampal neuronal cultures and in organotypic cultures from adult hippocampal slices

First, we tested if continuous application of recombinant BDNF (rBDNF) in the medium exerted trophic effects on cell survival in low-density primary cultures of hippocampal neurons (PCs) and in organotypic cultures of adult hippocampus slices (OCs). Thus, culture media were supplemented with increasing concentrations of rBDNF, ranging from 0.03 ng/ml to 300 ng/ml.

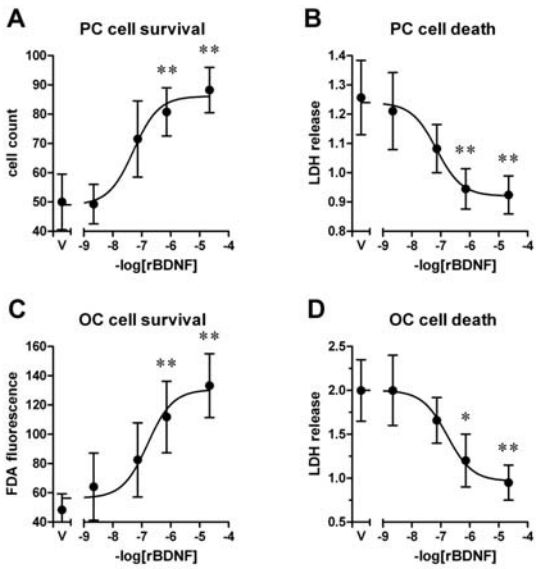

Fig. 1. Recombinant BDNF enhances cell survival in primary hippocampal neuronal cultures (PC, panels **A** and **B**) and in organotypic adult hippocampus slice cultures (OC, panels **C** and **D**) in a concentration-dependant manner. Neurons were cultured with the indicated concentration of BDNF for 7 days, slices for 14 days. Panels **A** and **C** indicate viable cells, as quantified using cell counting for PCs and fluorescence intensity of the marker FDA for OCs. Panels **B** and **D** represent cell death, as estimated measuring the levels of released LDH. Data are expressed as the mean±SEM (n=6). * P<0.05, ** P<0.01 vs. control (0 ng/ml); ANOVA and *post-hoc* Newman-Keuls test.

In PCs, neurons seeded at low density can hardly survive without the support of rBDNF. Quantification of survival, based on cell counting and on LDH release, showed that BDNF produces a significant beneficial effect in a concentration-dependent manner (**Fig. 1A** and **1B**). The maximal effect was reached at a concentration of approximately 10^{-5} M (slightly more than 100 ng/ml) and the EC50 was approximately 10^{-7} M (slightly more than 100 ng/ml). These data are in keeping with previous findings (Cheng and Mattson, 1994; Lindholm et al., 1996).

We also examined the neurotrophic activities of rBDNF in OCs. In the absence of rBDNF supplementation, OCs displayed degeneration aspects (like white spotted cell debris and uneven surface) after 6-8 DIV, that were clearly visible under phase contrast microscopy. Similar to PCs, rBDNF concentration-dependently reduced these signs of degeneration. Viability was quantified using two assays: FDA fluorescence intensity, to identify viable cells; LDH release, to identify the degree of cell death. As shown in **Fig. 1C** and **1D**, BDNF concentration-dependently increased FDA fluorescence, in line with previous observations reporting that BDNF (50-100 ng/ml) enhances the cell tolerance to serum-deprivation of organotypic culture from postnatal slices (Nakagami et al., 1997), and reduced LDH release. These effects were obtained in the same range of concentrations that proved effective for PCs.

Soluble mediators from MABs engineered to produce BDNF highly improve cell survival in

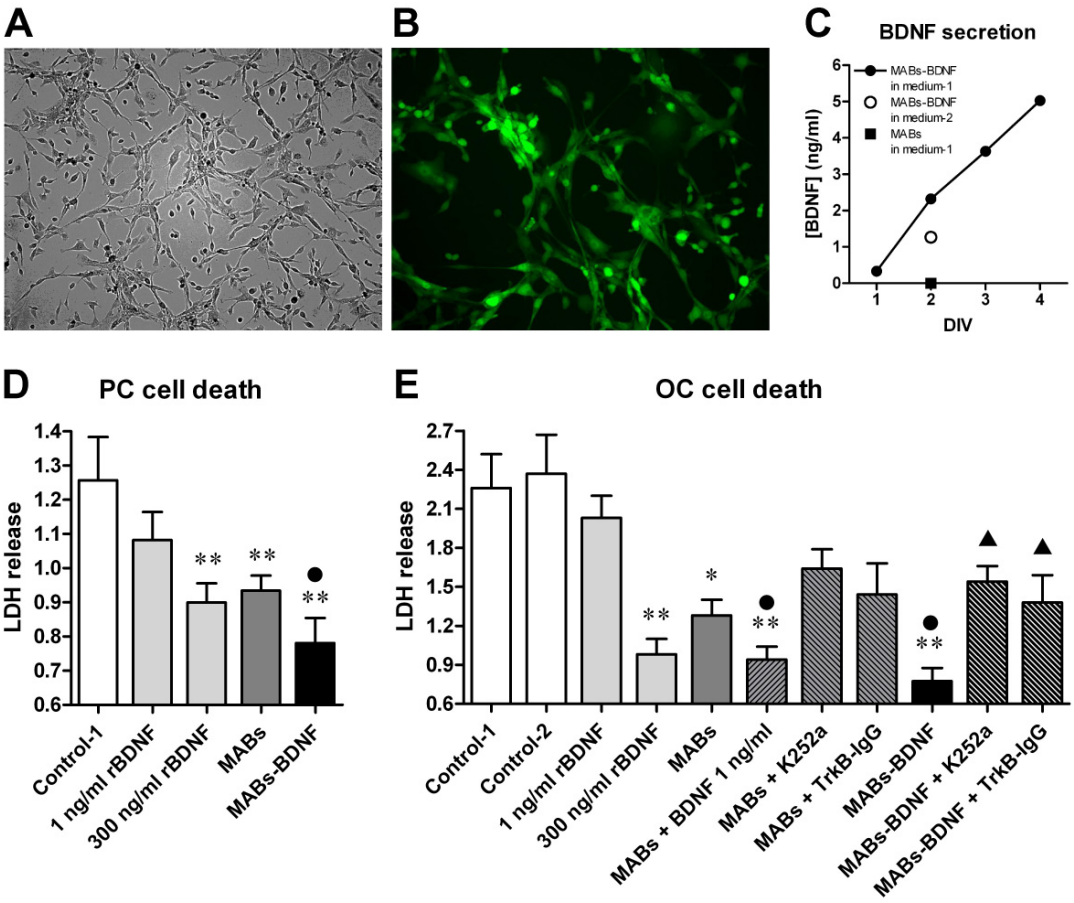

Fig. 2. MABs-BDNF deliver BDNF and enhance cell survival in primary hippocampal neuronal cultures (PC) and in organotypic adult hippocampus slice cultures (OC). (**A**) and (**B**): genetically modified MABs-BDNF proliferate and constantly express GFP. (**C**) MABs-BDNF secrete BDNF. Concentration of BDNF in the medium of MABs and MABs-BDNF at various days in vitro (DIV), as measured using ELISA. MABs-BDNF were cultured in 2 different types of medium (Medium-1 and Medium-2, see text for details) as indicated. (**D**) Effect of different treatment procedures on the viability of hippocampal PCs, as estimated using the LDH release assay. Data are the mean±SEM of 6 replicates in separate experiments. ** P<0.01 vs. Control-1; ● P<0.05 vs. MABs; ANOVA and *post-hoc* LSD test. (**E**) Effect of different treatment procedures on the viability of adult hippocampus OCs, as estimated using the LDH release assay. Data are the mean±SEM of 6 replicates in separate experiments. * P<0.05 and ** P<0.01 vs. Control-1; ● P<0.05 vs. MABs; ▲ P<0.05 vs. MABs-BDNF; ANOVA and *post-hoc* Newman-Keuls test. Control-1 are slices treated with the typical medium for MABs culture (Medium-1). Control-2 are slices treated with the typical medium for postnatal slice culture (Medium-2).

primary hippocampal neuronal cultures and in organotypic cultures from adult hippocampal slices

We engineered a line of MABs (MABs-BDNF) to stably produce and secrete human BDNF and the green florescence protein (GFP) as a tracer. These cells can be cultured long-term in vitro and constantly express BDNF and GFP (**Fig. 2A** and **2B**). The amount of BDNF secreted into the medium (Medium-1) from MABs-BDNF after 2 days in culture was approximately 2 ng/ml, as measured using an ELISA assay (**Fig. 2C**). If MABs-BDNF were cultured with the medium usually employed to culture OCs (Medium-2), the amount of secreted BDNF was approximately halved (**Fig. 2C**). Therefore, although controls were also performed using Medium-2, the conditioned media that we used in all experiments was based on Medium-1: 50% culture supernatant from 2-day-cultured MABs-BDNF and 50% fresh Medium-1. Thus, according to the ELISA measures, this conditioned medium contained approximately 1 ng/ml BDNF, whereas no detectable of BDNF was present in the medium conditioned with control, conventional MABs (**Fig. 2C**).

We first screened the trophic effects of the MABs-BDNF conditioned medium in the PC system. The MABs conditioned media increased the number of surviving neurons by about 80%, a level similar to the effect of 300 ng/ml rBDNF. The effect of the MABs-BDNF conditioned medium was much greater (it enhanced neuronal survival by approximately 170%). We used the LDH assay to measure these effects: as shown in **Fig. 2D**, both 300 ng/ml rBDNF and the MABs medium significantly (and to a similar extent) reduced cell death, but the MABs-BDNF medium had a significantly greater effect. Interestingly, 1 ng/ml rBDNF (i.e. the concentration of BDNF present in the medium) alone did not produce a significant effect on neuronal survival. Together with the observation of a significant effect of the MABs medium, this indicates that other trophic factors are secreted by MABs that can synergize with BDNF to produce neuroprotection.

We then extended and deepened this analysis in OCs. Adult OCs were cultured with different media and, after 14 DIV, cell death was measure using the LDH release assay. As shown in **Fig. 2E**, cell death was remarkable in control media (Medium-1 and Medium-2, see also **Fig. 3A**). As with PCs, the MABs-conditioned medium significantly decreased cell death, to nearly the level of rBDNF 300 ng/ml, and the MABs-BDNF-conditioned medium produced a significantly more pronounced effect. This extra-effect is mediated by the low concentrations (1 ng/ml) of BDNF produced by the MABs-BDNF, because (1) adding 1 ng/ml rBDNF to the MABs medium produced an effect similar to the one produced by the MABs-BDNF medium; (2) the TrkB inhibitor K252a and the BDNF scavenger TrkB-IgG to the medium conditioned by MABs-BDNF abolished the extra effect observed in the MABs-BDNF group, while these two BDNF blockers had no significant influence on the medium conditioned by MABs (**Fig. 2E**). These data support the notion that the pro-survival effect of the MABs-BDNF medium is mediated by a synergy between BDNF and other, yet unidentified, soluble substances constitutively produced by MABs. Accordingly, 1 ng/ml rBDNF alone did not reduce cell death at all, and much higher concentrations (300 ng/ml) were needed to almost reach the effect of the MABs-BDNF conditioned medium.

MABs-BDNF-produced mediators sustain neuronal structural integrity and functional properties in adult organotypic slices

The above findings have been confirmed at the light microscope and using FDA fluorescence, an indicator of viable cells. At the bright field observation, as compared with slices cultures in the control medium (which, as described, display obvious signs of degeneration, **Fig. 3A**), the slices cultured in the medium conditioned on MABs-BDNF remained thicker and preserved their structural organization for a long period, up to 25 days (**Fig. 3C**). Slices cultured in the MABs medium appeared healthier that those cultured in control medium but less healthy that those cultured in the MAB-BDNF medium (**Fig. 3B**). In line with

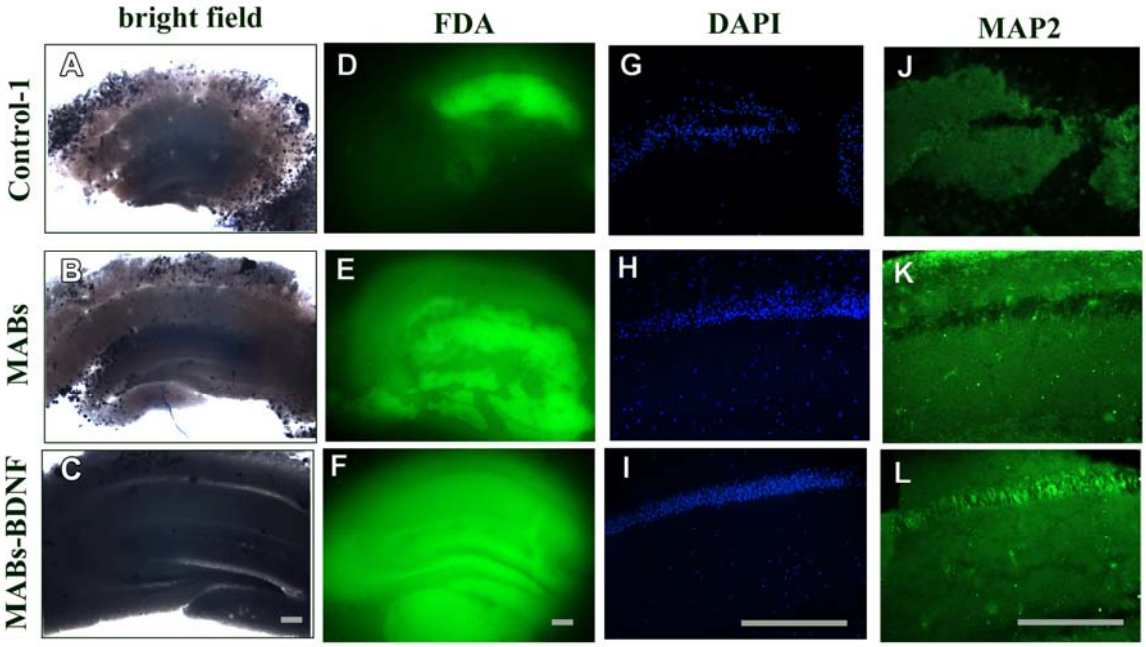

Fig. 3. Morphological evidence of the beneficial effects of the medium conditioned in MABs-BDNF on adult organotypic cultures. Adult slices were cultured for 14 days with the control Medium-1, with medium conditioned in MABs, or with medium conditioned in MABs-BDNF. Note that slices cultured in the MABs-BDNF conditioned medium, compared with the other two groups, better maintain their integrity based on bright field observation (**A-C**), remain more viable based on FDA (**D-E**), DAPI staining (**G-I**) and MAP2 immunohistochemistry (**J-L**). Nuclei in blue in **G-I**, neurons in green in **J-L**. Scale bar: 200 μm.

these findings and paralleling to the high levels of LDH release described above, FDA fluorescence was weak and uneven in OCs cultured with control medium (**Fig. 3D**), indicating that most of the cells are indeed degenerating. OCs cultured with the MABs medium displayed a better labeling quality (**Fig. 3E**), which was further improved in OCs cultured with the MABs-BDNF medium (**Fig. 3F**).

Cell counting, performed by using the DAPI nuclear stain, confirmed that, even if some cell loss was present in slices cultured with MABs-BDNF conditioned medium (**Fig. 3I**), the number of surviving cells was much higher than in the control group (**Fig. 3G**). Slices treated with the medium conditioned in MABs performed a little better that controls, but much worse than those treated with the medium conditioned in MABs-BDNF (**Fig. 3H**).

We also performed other morphological examinations and electrophysiological recordings. For morphological analysis, slices were fixed and immunostained with microtubule-associated protein 2 (MAP2). MAP2 is a cytoskeletal protein primarily found in neuronal dendrites. As shown in **Fig. 3J**, adult slices cultured in the control media for 14 days exhibit extensive not only cell loss, but also a grossly altered structure, with many lacunae in the pyramidal cell layer and just a few remaining, exclusively pyknotic neurons. MAP2 staining was essentially absent in control slices. While the MABs medium only slightly improved this situation (**Fig. 3K**), the one conditioned in MABs-BDNF clearly attenuated the neuronal loss (**Fig. 3L**). The dendritic network and the cytoarchitectonic characteristics were essentially maintained in adult OCs treated with the MABs-BDNF conditioned medium, although signs of neuronal degeneration could still be detected, with loss of neurons, decreased dendrites, and many condensed or swelling pyramidal cells. These signs of degeneration progressively increased in time. Nonetheless, this

morphological analysis further confirms that the

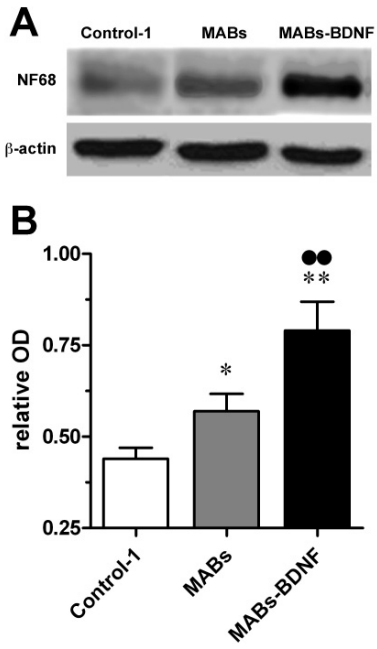

Fig. 4. Density of neurons surviving in adult slices after 14 days in vitro, under the different experimental conditions. Neuronal density was quantified performing Western blot analysis for a neuron-specific marker, neurofilament 68. (**A**) Representative blot. (**B**) Data quantification. The data are mean±SEM of 4 separate experiments. * P<0.05 and ** P<0.01 vs. Control-1; ●● P<0.01 vs. MABs; ANOVA and *post-hoc* LSD test.

medium conditioned in MABs-BDNF enhances neuronal survival and preserves structural organization.

The density of neurons in the slices cultured with the different media was also assessed using Western blot analysis for the neuron-specific marker neurofilament 68. We found that OCs cultured on MABs-BDNF conditioned medium contained more neurofilament 68, thus more neurons, as compared with the slices cultured in the control medium or in medium from conventional MABs (**Fig. 4**). A statistically significant difference was also found between the MABs and the control group, indicating that supplementation of the soluble substances produced by MABs can slightly (but significantly less efficiently than with BDNF) attenuate neuronal loss.

Finally, we performed electrophysiological recordings to demonstrate the viability of the surviving neurons and the persistence of synaptic connections at a functional level. We measured fEPSPs in CA1 pyramidal neurons after stimulation of the Schaffer collaterals. We chose 7 DIV as a checking time point, because adult slices in culture, even those cultured with the MABs-BDNF conditioned medium, display a progressive decrease in the amplitude and stability of the extracellular field potentials in time (data not shown), such that currents could not be reliably measured at 14 DIV, when neurons are still visible using immunofluorescence: this is an indication that, as expected, the functional damage precedes the morphological one. As shown in **Fig. 5A**, adult slices cultured with the typical medium exhibited very small (if any) synaptic response at 7 DIV, while relatively stable fEPSPs could be recorded in those cultured with the medium conditioned in MABs or MABs-BDNF. However, the mean amplitude of the evoked fEPSPs in the MABs-BDNF group was much higher than in the MABs group (**Fig. 5B**). The presence of evoked synaptic currents, albeit lower and less stable than in acute slices or in cultured postnatal slices, in adult OCs cultured with the MABs-BDNF-conditioned medium demonstrates that the neuronal activity and the hippocampal circuitry remain functional by virtue of the beneficial effects of the mediators secreted by MABs-BDNF.

DISCUSSION

Neurodegenerative disease is a condition in which cells of the brain and spinal cord are lost. NTFs are target-derived molecules that prevent neuronal degeneration during development and in adulthood (Blesch, 2006). They offer substantial promise as therapeutic agents in neurological diseases by preventing cell loss and promoting axonal regeneration. There are currently several experimental approaches for the therapy of neurodegenerative diseases, based on modulation of the levels of NTFs in lesion areas (Lindsay, 1994): a pharmacological approach, based on perfusion of NTFs in the brain; a gene therapy approach, based on the development of viral vectors capable of

efficiently delivering trophic factors; a cell therapy approach, aiming at replacing the lost neurons. The pharmacological approach confronts the great challenge of the difficulties in delivering proteins to the CNS (Jones et al., 2001; Boado, 2008). Therefore, the development of targeted drug delivery strategies becomes necessary. Direct localized CNS delivery of NTFs is now achievable by cell-based (ex vivo) or direct (in vivo) gene delivery. Stem cell-based delivery is of particular interest for drug manipulation since stem cells have a great therapeutic potential in repopulating damaged tissues as well as being genetically manipulated and used in cell-based gene therapy (Sonntag and Sanchez-Pernaute, 2006). Here we described a novel stem cell, the MAB, isolated from murine embryonic dorsal aorta, as an attractive candidate for localized cell-based gene delivery.

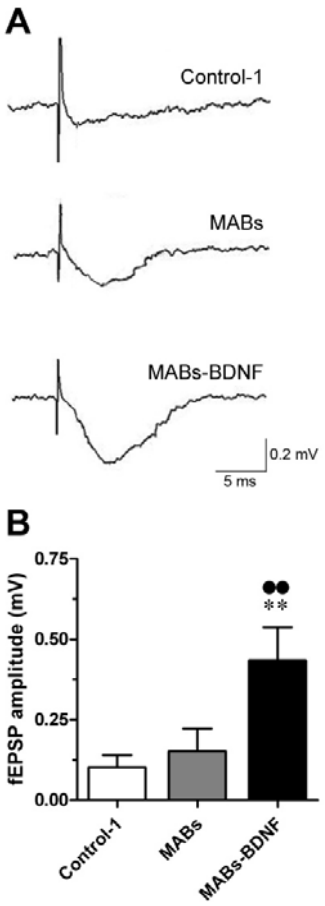

Fig. 5. Maintenance of synaptic connections in adult slices after 7 days in culture with the MABs-BDNF conditioned medium. Synaptic connections were identified by recording evoked fEPSP in CA1 after stimulation of the Schaffer collaterals. (**A**) Representative recordings of the evoked fEPSP in the different groups. (**B**) Peak amplitude of the fEPSPs, measured and statistically analyzed as described in the Materials and Methods. The data are the means±SEM of 5 separate experiments. ** P<0.01 vs. Control-1; ●● P<0.01 vs. MABs; ANOVA and *post-hoc* LSD test.

MABs are mesodermal progenitors associated with vessels, that are regarded as suitable candidates for cell therapy of muscle degenerative diseases (Sampaolesi et al., 2003; Sampaolesi et al., 2006), as well as myocardial infarction (Galli et al., 2005). Owing to their specific homing to the damage areas, MABs could be used for targeted delivery of therapeutic growth factors, like BDNF, in neurodegenerative diseases. Thus, we generated MABs engineered to produce BDNF and validated the possible effects of the mediators secreted by these MABs-BDNF by using primary neuronal cultures and organotypic cultures from adult hippocampal slices as in vitro models of neurodegeneration.

First, we showed that continuous application of the recombinant BDNF provided protective effects on primary neuronal cultures and adult organotypic hippocampal slice cultures (OHSC) in a concentration-dependant manner. The effective dose of the rBDNF is similar those previous reported using primary cultures (Lowenstein and Arsenault, 1996) and organotypic hippocampal slice cultures from postnatal slices (Nakagami et al., 1997). We here used the organotypic hippocampal slice culture from adult mice as a model of neurodegeneration. It is well known that the age of the animal is crucial for the viability of OHSC. OHSC maitained in a typical media always result in rapid and continuous cell death. This spontaneous cell degeneration may be used as a model to screen trophic or neuroprotective substances. There are also other models based on OHSC from postnatal animal, in which cell death is induced by exogenous insults such as neurotoxins (Noraberg et al., 1998), oxygen-glucose deprivation (Wise-Faberowski et al., 2009), and trauma (Morrison et al., 2006). As opposed to these models based on postnatal slices,

the OHSC from adult slices display good regional morphology with less cell reorganization, and resemble the adult brain in gene expression profile, despite the unclear mechanisms of the spontaneous cell death. Consistent results in two independent assays, FDA hydrolysis and LDH release, suggest the feasibility of OHSC as a model of cell degeneration.

Next, we found that MABs-BDNF can be grown extensively in culture and stably secrete BDNF into the culture medium. Although the secreted BDNF is at relatively low concentrations, the conditioned media from the supernatant of MABs-BDNF still exerted a robust protective effect in adult OHSC. According to our concentration-response assay with rBDNF, the concentration of rBDNF equivalent to that secreted by MABs-BDNF (1 ng/ml), and even the highest concentration of rBDNF (300 ng/ml) could not extend cell survival as much as MABs-BDNF, indicating MABs-BDNF produced not only BDNF but also some other, yet unknown, survival factor. Indeed, the conditioned media from control MABs enhanced cell survival in adult OHSC when compared with the control medium. The finding that the MABs-BDNF beneficial effects could be simulated by the combination of MABs and 1 ng/ml of BDNF reinforce the conclusion that MABs-BDNF can produce two pro-survival components: the secreted BDNF, and other unknown soluble substances. It also seems that there is a synergistic effect between BDNF and these unknown factors, because MABs-BDNF increased cell survival to a much higher extent than the equivalent dose of rBDNF or MABs. Synergistic effects between NTFs are common in the nervous system. A proper combination of different NTFs could enhance the trophic abilities and reduce possible side effects. MABs seem to be ideal for delivering BDNF since the delivery was effective at a low dose, which would reduce the side effects in possible, future clinical application. So far, the effective components in the culture supernatant of MABs have not been identified. A micro-array analysis of gene expression revealed that several growth factors are expressed at high level in these cells, including vascular endothelial growth factor B (VEGFB), basic fibroblast growth factor (bFGF), FGF7, platelet-derived growth factor AA (PDGF AA), hepatomaderived growth factor, and stromal derived factor 1 (Galli et al., 2005). Thus, the mechanism of the effect of MABs on adult slice survival is probably multifactorial, relating with various growth factors and chemokines. It is worthy to note that MABs express many neural genes including receptors and transcription factors in spite of their mesodermal origin. Therefore, it is reasonable that they express survival factors for neurons.

Several investigations indicate a pivotal role of BDNF in neuroprotection in different models of neurotoxicity, such as in vivo and in vitro ischaemic neuronal damage (Larsson et al., 1999; Kano et al., 2002), glucose deprivation (Tong and Perez-Polo, 1998) or glutamate-induced neuronal damage in neuronal cultures (Cheng and Mattson, 1994)，kainate acid-induced excitotoxicity in the striatum (Gratacos et al., 2001) and hippocampal kindling (Larmet et al., 1995). In this study, we demonstrated the beneficial effects of rBDNF or MABs delivered BDNF on cell survival in OHSC. These findings suggest that BDNF is an essential constituent of the microenvironment for neuronal survival in the adult hippocampus. It is reported that BDNF administration to cultured slices potentiates presynaptic release (Li et al., 1998) and promotes dendritic and axonal growth (Labelle and Leclerc, 2000). Independently, other studies have provided insights into the effects of BDNF on potentiating excitatory transmission in the cultured slices (Huber et al., 1998; Jiang et al., 2001).

The signaling mechanisms underlying the pro-survival effects of MABs-BDNF appear to involve TrkB activation. The TrkB blocker K252a attenuated the protective effects of MABs-BDNF to an extent similar to MABs. TrkB receptors have long been known to mediate trophic support of adult neurons (Ferrer et al., 1999), and a number of studies show that decreased expression of this receptor is associated with cell loss (Barbacid, 1994).

Based on the morphological analysis and the

immunoblot assay for neuron-specific marker, the evidence supporting the beneficial effects of the conditioned medium from MABs-BDNF on adult OHSC is solid. Moreover, these OHSC also diplayed a better synaptic response with higher peak amplitudes of fEPSP compared to the groups of MABs and control. The facilitated synaptic responses may be ascribed to a better preservation of synaptic connections or to a potentiated synaptic transmission. It is reported that application of BDNF modulates the strength of existing synaptic connections (Huber et al., 1998; Jiang et al., 2001) and acts in the formation of new synaptic contacts within the hippocampal circuit (Lauterborn et al., 2007). In any event, the MABs-BDNF conditioned medium reduced, but did not prevent, cell degeneration in adult OHSC, according to our morphological analysis and electrophysiological recordings. Slices, even those in the MABs-BDNF conditioned medium, exhibited continuous signs of degeneration during culture. Furthermore, the electrophysiological recordings indicate that adult OHSC are not as healthy as the postnatal OHSC, since the fEPSPs in adult slices were hard to evoke and almost disappeared after 10 days in culture (data not shown). In the future, an improved culture method, together with supplementation of the MABs-BDNF conditioned medium might make it possible to achieve truly successful adult OHSC, which is a technical bottleneck for many studies.

In this study, we demonstrate that soluble mediators secreted by the MABs-BDNF have profound survival effects on neurons in adult OHSC. We found a potential merit of using MABs to deliver BDNF, which were these unknown survival factors inherently secreted by MABs. The coaction of BDNF and MABs-produced survival factors led to a higher tolerance to cell death and a better maintenance of the neuronal network, and allowed the delivered BDNF to exert a profound protective effect at a low dose. Besides, MABs possess the other natural advantages for cell-based therapy: they can be expanded to virtually unlimited numbers in vitro; they are amenable to genetic modification; they self-renew and have multipotent capacities; they specifically home to the damage area. These advantages raise the possibility that the MABs-BDNF might be developed into a new cell-based strategy for BDNF delivery into the CNS, thereby promoting neuronal survival and restoring network function in neurological diseases associated with brain damages. Transplantation of MABs-BDNF might become an alternative and possibly more efficacious therapy for brain diseases caused by neurodegeneration, including multiple sclerosis, stroke, epilepsy and trauma. The transplanted MABs-BDNF may protect neurons from damage through a “bystander” mechanism, by which MABs-BDNF chronically secrete low dose BDNF and other survival factors to provide an optimal environment for tissue repair and neuronal survival.

Further studies in the animal models of neurodegenerative diseases are required to identify the therapeutic value of MABs-BDNF transplantation. In addition, there might be other growth factors more suitable than BDNF in view of the different pathophysiological processes in neurodegenerative diseases. Using this study as a template, advances can be made in the way MABs are engineered with other growth factors. Finally, this study may have heuristic value for the exploitation of stem cell-based therapies in terms of cell sources, gene modification, and screening methods.

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