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LOCAL SUPPLEMENTATION OF FGF-2 AND
BDNF IN THE EPILEPTOGENIC HIPPOCAMPUS.
EFFECTS AND DELIVERY STRATEGIES.

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RIASSUNTO

Tra le sindromi epilettiche, l'epilessia del lobo temporale (TLE) è sicuramente la forma più frequente nell'adulto. Si manifesta in seguito ad un danno cerebrale (infezione virale, ictus, trauma, neoplasie...), capace di innescare una cascata di eventi che culminano nella comparsa di crisi epilettiche spontanee, in molti casi difficilmente controllabili con la normale farmacoterapia. Il periodo che intercorre fra l'insulto iniziale e lo sviluppo di crisi spontanee viene definito "epilettogenesi". Le alterazioni cellulari e tissutali che avvengono durante questa fase interessano principalmente la regione ippocampale e includono: neurodegenerazione, neurogenesi, neuroinfiammazione e gliosi reattiva, angiogenesi e riorganizzazione dei circuiti cerebrali.

Recentemente è stato dimostrato che la supplementazione di fattori neurotrofici (NTFs), quali FGF-2 (fibroblast growth factor-2) e BDNF (brain derived neurotrophic factor), produce effetti anti-epilettogenici riducendo la morte neuronale, favorendo una neurogenesi non aberrante e ristabilendo un corretto equilibrio tra circuiti inibitori ed eccitatori.

Nel principale lavoro riportato in questa tesi, abbiamo voluto osservare se tale trattamento è in grado di influire anche sui processi neuroinfiammatori. Per lo studio è stato utilizzato il modello sperimentale della pilocarpina, la cui somministrazione è in grado di indurre nell'animale uno stato epilettico (SE) e quindi una lesione epilettogena. Dopo tre giorni dallo SE, vettori virali erpetici esprimenti FGF-2 e BDNF sono stati inoculati nell'ippocampo.

Dopo 4, 11 e 25 giorni dal trattamento (DAI), gli animali sono stati sacrificati e i loro cervelli prelevati per analizzare l'espressione di tre marcatori dell'infiammazione: IL-1 β , GFAP (marker di astrocitosi), Ox42 (marker di microgliosi). I risultati ottenuti dimostrano una riduzione particolarmente marcata di IL-1 β , evidente già dopo 4 giorni dall'inoculazione del vettore virale, e un'attenuazione più ritardata, ma pur sempre significativa, degli altri due markers studiati.

Lo sprouting delle mossy fibers è un'altra caratteristica aberrante del tessuto ippocampale epilettico: gli assoni delle cellule dei granuli formano sinapsi eccitatorie con cellule normalmente non innervate, formando un circuito che potrebbe contribuire all'ipereccitabilità tissutale. I risultati ottenuti dimostrano che il trattamento con i fattori neurotrofici è in grado di ridurre lo sprouting aberrante delle fibre nervose, in un modo che correla con l'attenuazione del danno cellulare.

Studi comportamentali paralleli hanno inoltre evidenziato la capacità del trattamento di ridurre la frequenza e la gravità delle crisi spontanee che, in questo modello, insorgono dopo circa 21 giorni dallo stato epilettico.

Nonostante i promettenti risultati, l'applicabilità clinica dei fattori neurotrofici è limitata dalla scelta di una corretta via di somministrazione. Negli esperimenti riportati in questa tesi sono stati impiegati vettori virali erpetici, defettivi nella replicazione ed esprimenti i due NTFs. Tuttavia, la loro tossicità residua li rende inadeguati a essere applicati all'uomo. Cellule staminali modificate per esprimere i geni di interesse, tra cui i mesangioblasti (MABs), hanno dimostrato *in vitro* la capacità di promuovere la differenziazione, la sopravvivenza e la funzionalità neuronale. Non ultima, la capacità di localizzarsi nella sede cerebrale danneggiata, quando somministrati per via sistemica, rende queste cellule delle valide alternative a trattamenti più invasivi.

Sebbene siano richiesti ulteriori studi, i risultati raccolti in questa tesi rappresentano un importante contributo alla comprensione delle molteplici proprietà dei NTFs. Inoltre, la caratterizzazione di una via di somministrazione alternativa e maggiormente applicabile può avvicinare la terapia genica con fattori neurotrofici all'utilizzo clinico in numerose patologie neurodegenerative.

ABSTRACT

Among the epileptic syndromes, temporal lobe epilepsy (TLE) is the most common form in adults. It is the consequence of a brain damage (viral infection, stroke, trauma, cancer ...), capable of triggering a cascade of events culminating in the appearance of spontaneous seizures that are, in many cases, difficult to control with the usual drug therapy. The period that elapses between the initial insult and the development of spontaneous recurrent seizures (SRSs) is defined "epileptogenesis". The cellular and tissue changes that occur during this phase mainly interest the hippocampal region and include: neurodegeneration, neurogenesis, neuroinflammation and reactive gliosis, angiogenesis, and reorganization of brain circuits.

Recently, it was shown that supplementation of neurotrophic factors (NTFs), such as FGF-2 (fibroblast growth factor-2) and BDNF (brain derived neurotrophic factor), has anti-epileptogenic effects by reducing neuronal death, favoring a correct neurogenesis and restoring a proper balance between excitatory and inhibitory circuits.

In the main study reported in this thesis, we examined if this treatment can also affect neuroinflammatory processes. We used the pilocarpine model, in which an episode of status epilepticus (SE) is followed by an epileptogenic lesion. After three days, herpes viral vectors expressing FGF-2 and BDNF were injected in the hippocampus.

Four, 11 and 25 days after treatment (DAI), animals were sacrificed and their brains removed to analyze the expression of three markers of inflammation: IL-1 β , GFAP (a marker of astrocytosis), Ox42 (marker of microglia). The results show a very marked reduction of IL-1 β expression, evident as early as 4 days after inoculation of the viral vector, and delayed, but significant, attenuation of the other two markers.

The sprouting of mossy fibers is another characteristic of the epileptic hippocampal tissue, in which the axons of granule cells form excitatory synapses with cells not usually innervated, forming a circuit that may favour hyperexcitability. The results show that treatment with neurotrophic factors reduce aberrant sprouting of nerve fibers, in a way that correlates with the attenuation of cellular damage.

Parallel behavioral studies have also highlighted the ability of the treatment to reduce the frequency and severity of SRSs that, in this model, begin to occur about 21 days after status epilepticus.

Despite the promising results, clinical applicability of neurotrophic factors is limited by the choice of an appropriate route of administration. In the experiments reported in this thesis, herpes viral vectors have been used. These vectors were replication defective and engineered to express the two NTFs. However, their residual toxicity makes them unsuitable for human application. Stem cells modified to express genes of interest, including mesangioblasts (MABs), have demonstrated, *in vitro*, the ability to promote differentiation, survival and neuronal function. Last but not least, the ability to localize in the damaged site when systemically administered makes these cells viable alternatives to more invasive treatments.

Although further investigations are required, the results collected in this thesis are an important contribution to the understanding of the multiple effects of NTFs. In addition, the characterization of an alternative and more applicable route of administration renders gene therapy with neurotrophic factor more applicable for the treatment of several neurodegenerative diseases.

GENERAL STRUCTURE OF THE THESIS

Several histological features characterize an epileptic brain tissue. They include loss of neuronal cells, neurogenesis and reorganization of hippocampal neuronal circuits, like *sprouting of mossy fibers (MFS)*. Epileptic tissue also presents strong neuroinflammation with an important reactive astrogliosis and microgliosis and expression of several pro-inflammatory cytokines.

Recently, it has been demonstrated that the local over expression of neurotrophic factors (NTFs), such as FGF-2 (*fibroblast growth factor-2*) and BDNF (*brain derived neurotrophic factor*), has antiepileptogenic effects ([Paradiso et al. 2009](#)).

This thesis is organized in two chapters. Works described in the first chapter, investigate the possibility that these anti-epileptogenic effects of NTFs might involve anti-inflammatory mechanisms and affect sprouting of mossy fibers.

The second chapter is a brief *excursus* on the routes of administration suitable for driving a localized, safe and non-invasive supplementation of NTFs. In particular, it focuses on a new promising approach based on stem-cells gene therapy that employs mesoangioblasts.

Finally, an “*addendum*” collects two other works. The first is a study, conducted in collaboration with an Italian pharmaceutical company, on the evaluation of the central side effects of new generation phosphodiesterase 4 inhibitors. The second deals with the analysis of *in vitro* compatibility of materials used in lab on a chip (LOAC) production. Biocompatibility was analyzed on cells growing in suspension and on hippocampal primary cultures, the latter obtained following a protocol optimized in our laboratory.

CHAPTER I

*The history of epilepsy can be summarized as
4.000 years of ignorance, superstition and stigma,
followed by 100 years of knowledge, superstition and stigma.*

(Kale R.)

1. Epilepsy

1.1 History and definition.

About epilepsy has been spoken and written for over 4.000 years. Characteristics of the illness such as the forced cry, the loss of consciousness, the fall, the twitching and the foaming at the mouth, have suggested, for several centuries, an association with possession of the spirits or prophetic experiences.

Symptoms are mentioned in the Bible in the Gospel of Mark, wherein Christ expels demon from a young person suffering from seizures: *“Teacher, I brought you my son, who is possessed by a spirit that has robbed him of speech. Whenever it seizes him, it throws him to the ground. He foams at the mouth, gnashes his teeth, and becomes rigid. I asked your disciples to drive the spirit out, but they could not.”* (Mark 9:14-29).

Through the centuries, many misconceptions about this condition have been conveyed based mainly on the particular era or in particular part of the world.

Disagreeing with the idea that epilepsy is a curse or a visionary power, in the 4th century B.C., Hippocrates, in his “On Sacred Disease”, stated “ *...it appears to me to be nowise more divine nor more sacred than other diseases, but has a natural cause from the originates like other affections. ... And this notion of its divinity is kept up by their inability to comprehend it and the simplicity of the mode by which it is cured, for men are freed from it by purifications and incantations.*”

Although the Greek physician recognized that epilepsy was a brain disorder, false ideas continued to exist. In 1494, two Dominican friars, under papal authority, wrote a handbook on witch-hunting, the “Malleus Maleficarum”, in which they said that one of the ways of identifying a witch was by the presence of seizures. This book guided a wave of persecution and torture, which caused the deaths of more than 200.000 women.

And misinterpretation continued for many years: in the early 19th century, people who had critical epilepsy and people with psychiatric disorders were cared for in asylums, but the two groups were kept separated because seizures were thought to be contagious.

For the first, clinically correct, definition of epilepsy is necessary to wait for the 1870, when the neurologist John Hughlings Jackson defined a seizure as *“an occasional, excessive and disorderly*

discharge of nerve tissue on muscles". He also recognized that seizures can alter consciousness, sensation, and behaviour.

Even if several headways have been made to define epilepsy, some prejudices remain still now. Recently, in nineties, some U.S. states had laws forbidding people with epilepsy to marry or become parents, and some states permitted sterilization.

Today is well known that epilepsy is not one condition, but a diverse family of disorders, having in common an abnormally increased predisposition to seizures. For the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE), to define the illness is necessary to satisfy three elements: "*Epilepsy is a disorder of the brain characterized by (1) an enduring predisposition to generate epileptic seizures and by (2) neurobiological, cognitive, psychological and social consequences. The definition of epilepsy requires also the occurrence (3) of at least one epileptic seizure*" defined, the latter, as "*a transient occurrence of signs and symptoms due to abnormal, excessive or synchronous neuronal activity in brain*" (Fisher et al., 2005).

Unfortunately, the stigma associated to epilepsy has still a great influence on the education and social life of patients and quite often leads to isolation, restrictions and/or overprotection. For these and other reasons (i.e. economic, legislative...) epilepsy remains a central point in the scientific community.

1.2 Epidemiology.

Epilepsy is one of the most common chronic neurological conditions. About 1% of the world's population is affected by epilepsy and up to 5% may have a single seizure at some time in their lives. Incidence and prevalence studies are critical to provide measures of frequency and therefore the burden of disease, and allow for proper planning of services.

Prevalence is an estimate of the number of people with epilepsy in a given population at a specified time (point prevalence), or during a defined time interval (period prevalence). In most countries worldwide, the prevalence of active epilepsy ranges from 4 to 10/1000.

The incidence, instead, is the number of new cases per year. The incidence of epilepsy ranges from 40 to 70/100.000 in most developed countries and is nearly double in developing countries. These

discrepancies are due to the higher risk of experiencing a condition which can lead to permanent brain damage like meningitis, malaria, pre and perinatal complications and malnutrition (from [Epilepsy in the WHO European Region: Fostering Epilepsy Care in Europe, 2011](#)).

The age distribution of the incidence of epileptic seizures is bimodal, with two peaks of frequency in childhood (in 50-60% of patients, epilepsy begins before the age of 16) and seniority. In Western countries there is evidence of a decreasing incidence in children, with a simultaneous increase in elderly, related to improved life expectancy associated with an increased risk for causes of epilepsy common in old age ([Hauser, 1992](#); [Stephen and Brodie, 2002](#)).

Differences in incidence rates in males and females are not statistically significant. There is no evidence of racial predilection, even if the incidence is significantly higher in the lower socioeconomic classes. ([Sridharan, 2002](#)).

1.3 Classification of epileptic seizures.

In 1981 ILAE divided seizures into three typologies, with subtypes of each: partial, with seizures involving only part of the brain; generalized, with seizures involving both hemispheres, and unclassifiable seizures.

This relatively easy classification appeared soon inadequate to respond to the vast heterogeneity of seizure types. For this reason a supplement to the previous classification was proposed in 1989 in which appears, for the first time, the new term of “epileptic syndrome”: “*An epileptic syndrome is defined as a disorder characterized by a cluster of signs and symptoms occurring together*”.

According to this system, epileptic syndromes are divided into four broad groups: localization-related that involves one or more distinct parts of the brain, generalized that involves both hemispheres at the same time, undetermined whether localized or generalized and, finally, special syndromes.

Within the localized and generalized groups, there are further subdivisions into idiopathic, symptomatic or cryptogenic. In idiopathic epilepsies, causes are unknown but genetic factors provoking, for example, channelopathies, are presumed to have a major causative role in the development of seizures. In symptomatic epilepsies there is an identifiable lesion in the brain that triggers seizures. The term “cryptogenic” has been recently substituted with “presumed

symptomatic epilepsy” in which some brain pathology causing epilepsy is presumed to exist, but has not been identified using current techniques (Engel, 2006).

1.4 Treatment.

The most common therapeutic approach of epilepsy is with antiepileptic drugs (AEDs). During the last 20 years there has been a dramatic increase in the therapeutic options available (**Table 1**).

Antiepileptic medicines	Year of introduction
Phenobarbital	1912
Phenytoin	1939
Ethosuximide	1955
Primidone	1960
Carbamazepine	1965
Valproate	1970
Vigabatrin	1989 serious side effects, use restricted
Oxcarbazepine	1990
Lamotrigin	1991
Gabapentin	1994
Felbamate	1994 serious side effects, use restricted
Topiramate	1995
Tiagabine	1996 marketed at a very low scale
Levetiracetam	2000
Pregabalin	2005
Zonisamide	2007
Lacosamide (Vimpat)	2008
Eslicarbazepine acetate	2009

Table 1: principal antiepileptic drugs currently available.

The success rate of both the older and newer drugs is similar and leads to seizures freedom for up to 70% of patients, even if, the newer drugs, may have less side effects and lead to a better quality of life. Moreover, new promising compounds are actually in clinical trials, they include Brivaracetam (UCB; phase III), Carisbamide (Johnson and Johnson; phase III), Retigabine (Valeant Pharmaceutical and GSK; phase III) and Valroceamide (Tera/Acorda; phase III).

Unfortunately all these drugs are only anticonvulsivant: they act on symptoms reducing recurrent seizures, but their mechanisms of action do not influence the causes of epilepsy. In some cases, monotherapy is sufficient to contain seizures but, more often, a cocktail of different drugs is necessary. The choice among different AEDs available depends on causes, history, evolution of pathology and crisis and, especially for polytherapy, possible pharmacological interactions. Despite the accessibility to a wide range of molecules, between 30 and 40 % of patients with epilepsy continue to have seizures that are not adequately controlled by pharmacotherapy (Kwan and Brodie, 2000).

A solution, in these cases, might come from surgery, with the resection of epileptic area. To be applied, it requires not only a history of intractable epilepsy, but also a focal and circumscribed lesion and a very low risk to develop neurosurgical or cognitive damages. When applied, surgery reaches 90% of success. In a study of surgical versus pharmacological treatment in poorly controlled temporal lobe epilepsy, has been report that, after one year, 64% of the patients operated, but only 8% of those medically-treated, are seizures free (Wiebe et al.; 2001).

In patients refractory to drug therapy and/or in which surgery could not be applied or results ineffective, a valid alternative may be the Vagus Nerve Stimulation (VNS). It consists of regular, mild pulses of electrical energy to the brain via the vagus nerve. These pulses are supplied by a device placed under the skin on the chest wall, connected with a wire to the vagus nerve in the neck. Little is known about the mechanism of action, but a significant reduction in frequency, intensity and duration of seizures, especially when associated to normal pharmacological treatment, has been demonstrated.

Based on dietary approach, ketogenic diet is indicated as an additional treatment in children with drug-resistant epilepsy. It consists of a diet high in fat and low in carbohydrates and proteins. Under these conditions, the liver converts fat into fatty acids and ketone bodies that, passing into the brain, replace glucose as an energy source. Elevated levels of ketone bodies in the blood, a state known as ketosis, lead to a reduction in the frequency of epileptic seizures. Even if side effects are less important than those of several drugs, patients treated for a long time with ketogenic diet may experience kidney stones, high cholesterol levels in blood, dehydration, bone fractures, slowed growth or weight gain and hypovitaminosis that require a careful control by physician. Although the mechanisms underlying anti epileptic effects are unknown, ketogenic diet has reported peaks of 90% of reduction in seizures frequency (Neal et al.; 2008).

Over the years, great progresses have been made in the treatment of epilepsy. With continued cooperation among clinicians, geneticists and basic scientists, will be possible to achieve the goal of curing epilepsy, or preferably, preventing the process that lead to the acclaimed illness.

Among epileptic syndromes, temporal lobe epilepsy (TLE) is the most common serious neurological condition in adulthood, probably affecting at least 20% of all patients with epilepsy.

1.5 Temporal Lobe Epilepsy.

TLE was defined in 1985 by the ILAE as a condition characterized by recurrent seizures originating from the medial or lateral temporal lobe. Medial temporal lobe epilepsy (MTLE) arises in the hippocampus, parahippocampal gyrus and amygdale, lateral temporal lobe epilepsy (LTLE) arises in the neocortex on the outer surface of the temporal lobe of the brain.

Seizures may involve only one or both lobes, giving rise to simple (without loss of consciousness) partial, complex (associated with loss of consciousness) partial or secondarily generalized seizures. About 40% to 80% of people with TLE also perform repetitive, automatic movements (called automatisms), such as lip smacking and rubbing the hands together. As seizures usually involve areas of the limbic system which control emotions and memory, some individuals may have problems with memory, especially if seizures have occurred for more than 5 years. However these memory problems are almost never severe. Seizures occur after an initial insult like an infection, stroke or trauma, vascular malformation or prolonged febrile seizures; a genetic cause is less frequent. Between the initial insult and the onset of the crisis, a so called latent period characterized by the absence of seizures, occurs. During this period, changes in structure and physiology of the brain tissue (“epileptogenesis”) happen.

The most common lesional abnormality identified in patients with TLE is the Hippocampal Sclerosis (HS) (Babb and Brown; 1987). It is characterized by severe loss of the principal neurones associated with widening of the granule cell layer of the dentate gyrus, termed granule cell dispersion (GCD), which is observed in about 40-50% of surgical temporal lobe specimens (Houser et al., 1990 b; Lurton et al., 1998; El Bahh et al., 1999; Thom et al., 2002; Blümke et al. 2002).

The other molecular, cellular and plastic alterations in hardened and sclerotic hippocampus will be described in detail further on.

In spite of the large diffusion, the study of epilepsy can not be performed on humans due to several reasons: ethical issues, unavailability of controls and high costs of human research. Besides, TLE is the most common form of drug-refractory epilepsy. Studies on human specimens are affected by several bias because of patients go toward surgery when pharmacotherapy fails. In these conditions is, thus, difficult to discriminate between the effects of the illness and those of a prolonged therapy. This is the reason for whom, to study epilepsy, animal models are of central importance.

1.6 Experimental models of TLE.

An animal model for a pathology could be isomorphic, if duplicates the disorder but not the underlying aetiology, or predictive, if it does not resemble the human disorder but allows prediction about it or its response. In the case of epilepsy, several models (i.e. electrical, chemical, genetics...) are available; they are resumed in **table 2**.

In vitro models	cell culture models		
	single nerve cells acutely dissociated from animal and human brain		
	in vitro isolated guinea pig brain		
In vivo models	chemical models of epilepsy	GABA	Pentylentetrazole Bicuculline Picrotoxin Glutamic Acid Decarboxylase (GAD) inhibitors Beta carbolines and convulsant benzodiazepine Ro 5-3663 GHB (gamma-hydroxy-butyrate)
		Excitatory Amino-Acid	Kainic acid Quisqualic acid/alfa-amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic acid N-Methyl-D-Aspartic acid (NMDA) Homocysteine, homocysteic acid
		Acetylcholine related substances	Pilocarpine, and litium-pilocarpine Organophosphorus compounds
		Other drugs	Strychnine Aminophylline Insulin induced hypoglycemia Ay-9944 THIP (4,5,6,7 tetrahydroxyisoxazolo (4,5,c) pyridine 3-ol)

		Inhalants	Fluorothyl
		Topical application	Metals (cobalt, zinc, antimony, alumina cream, iron Antibiotic (penicillins and cephalosporins) Tetanus toxin
	Electrical models	electroshock seizures local electrical stimulation electrical kindling self sustaining status epilepticus by Perforant Path stimulation self sustaining status epilepticus by Amygdala stimulation focal status epilepticus by perforant path stimulation in anesthetized rats continuous hippocampal stimulation	
	Lesion models of epilepsy	cortical freeze lesion model antiproliferative agents (5-azacytidine, methyl-mercury, nitrosureas and carmustine) Methylazoxymethanol acetate (MAM) model In-Utero irradiation as a model of cortical dysplasia Hypoxia-induced seizures and hypoxic encephalopathy in neonatal period Lateral fluid percussion brain injury Chronic Partial Cortical Isolation model Head Trauma: haemorrhage-Iron Deposit Stroke	
	Others	Complex febrile seizures – experimental model in immature rodents Infection induced seizures Rasmussen’s encephalitis model	
	Genetic models	Absence model	GAERS rats WAG/Rij rats
		Spike wave	Tottering lethargic ducky stargazer SWE Mocha2j coloboma
		Convulsion	Dilute lethal jimpy jittery megencephaly quaking staggerer torpid veritint waddler wabbler-lethal weaver writter
		Convulsions evoked by sensory stimuli	frings lurcher

Table 2: in vitro, in vivo and genetic experimental models of epilepsy.

For TLE, the pilocarpine model is a highly isomorphic model, described for the first time in 1983 by Turski and colleagues.

In this model, administration of pilocarpine in rats induces a status epilepticus (SE) characterized by tonic-clonic generalized seizures, followed by a latent period of seizures free behavior and by a chronic period, with the occurrence of spontaneous recurrent seizures (SRSs).

In detail: within 5 minutes from the injection, animals begin to be motionless, display oro-facial movements, salivation, eye blinking, twitching of vibrissae and yawning. Discontinuous seizures are observed at 30 minutes and they last up to 90-150 minutes, until limbic motor seizures with intense salivation, rearing, upper extremity clonus and falling, begin (Turski et al., 1983). Around 60% of the animals treated, successfully develop SE (Cavalheiro et al., 1991). All these behavioral changes are correlated to an high voltage, fast electroencephalographic (EEG) activity that appear to originate in the hippocampus and to propagate to the amygdala and neocortex (Turski et al., 1983). SE spontaneously remits within 12 hours after pilocarpine administration (Cavalheiro, 1995), then the animals enter post-ictal coma, lasting 1-2 days (Turski et al., 1983). The dose of alkaloid that reproduces the syndrome in rats is 300-400 mg/Kg. With these concentrations the mortality rate, at the time of the injection, has been reported to be around 30-40% (Turski et al., 1983; Cavalheiro et al., 1991; Liu et al., 1994), even if it can be reduced by stopping SE with anticonvulsant drugs such as diazepam. Our observations demonstrate that, after a three hours SE, animals experience some occasional, self-limiting generalized seizures of less than 1 min duration for 1-3 days (Mazzuferi et al., 2010).

The latent period starts after status epilepticus recover. Its duration varies in function of the dose of pilocarpine (Liu et al., 1994), length of SE (Lemos and Cavalheiro, 1995; Fujikawa, 1996, Biagini et al., 2006, Goffin et al., 2007), strain and age of the animal (Biagini et al., 2006; Goffin et al., 2007). Cavalheiro and collaborators (1991) defined a mean time interval of 14.8 days. During the latent phase, tissue rearrangements related to epileptogenesis occur (Dalby and Mody, 2001; Pitkänen and Sutula, 2002).

Chronic period follows epileptogenesis and, as previously mentioned, is characterized by the appearance of spontaneous recurrent seizures. Gravity of seizures can be established by a score system developed in 1972 by Racine and recently revised (Veliskova, 2006). It is reported in **Table 3**. As schematized, it uses numbers from 1 to 6 to define seizure classes: the firsts three are representative of partial seizures, from 4 to 6 of generalized ones. SRSs begin as partial seizures and become secondary generalized. The recurrence of seizures is almost regular throughout the lifetime

of the animal appearing in a clustered way, in a cycle peaking every 5-8 days or more (Goffin et al., 2007; Arida et al., 1999). Seizures frequency is higher during the diurnal period (Arida et al., 1999), with an incidence of 67% (Arida et al., 1999; Goffin et al., 2007). In 90% of the cases, EEG trace is characterized by a cerebral activity that starts from the hippocampus and spreads to the neocortex, usually lasts less than 60 seconds (Cavalheiro et al., 1991).

<i>Classes</i>	<i>Features</i>	
<i>1</i>	Staring and mouth clonus	<i>Partial</i>
<i>2</i>	Automatisms	
<i>3</i>	Monolateral forelimb clonus	
<i>4</i>	Bilateral forelimb clonus	<i>Generalized</i>
<i>5</i>	Bilateral forelimb clonus with rearing and falling	
<i>6</i>	Tonic-clonic seizures	

Table 3: modify Racine's scale. Scores from 1 to 3 are representative of partial seizures, the last three of generalized ones.

Pilocarpine exerts its effects by binding M1 muscarinic subtype receptors, resulting in alteration in Ca^{2+} and K^{+} currents (Segal, 1988). The high concentration of intracellular Ca^{2+} promotes the release of glutamate from presynaptic termini that, in turn, provokes the SE. Once activated, seizures are subsequently maintained by activation of NMDA receptors. Glutamate, acting on AMPA/KA receptors, allows the entrance of Na^{+} and Ca^{2+} into the cells and, as consequence, the Mg^{2+} , which blockades the NMDA receptor, is removed. In this way it can be activated by glutamate, allowing the entrance of more Ca^{2+} into the postsynaptic cells and inducing excitotoxic effects and cell death.

Recent observations have shown that activation of cholinergic neurons may not be the only factor triggering pilocarpine SE. Marchi and collaborators (2007 a, b) suggested that pilocarpine induces an early focal damage to blood-brain barrier (BBB) in regions highly sensitized to cholinergic agonists, that may contribute to the development of seizures, facilitating the entrance into the brain of blood-borne factors (e.g. K^{+}). Moreover, peripheral activation of the immune system has been hypothesized, since high levels of serum IL-1 β have been found after injection of pilocarpine. High concentration of the pro-inflammatory cytokine is known to cause sudden rapid changes in excitability of both inhibitory and excitatory neurons (Plata-Salamán and Ffrench-Mullen, 1992; Sawada et al., 1992; Yang et al., 2005).

As reviewed by [Curia et al. \(2008\)](#), age, strain, gender, dose, association with other drugs to reduce mortality or collateral effects, are variables that have to be carefully controlled to obtain a reliable model of the pathology. The lack of a standardized protocol has led some investigators to criticize this model. However it continues to be used in many laboratories, including our, because very easy, rapid and shows a high homology with human disease, involving the same mechanisms and brain areas and showing the same pattern of responsiveness to AEDs seen in TLE patients.

2. Epileptogenesis

2.1 Definition and peculiarities.

The term epileptogenesis refers to the phenomenon in which various kinds of brain insults (e.g. traumatic brain injury, stroke, infection, febrile seizure...) trigger a cascade of events that eventually culminate in the occurrence of spontaneous seizures. The period required for this long-lasting transformation of the brain, may vary from weeks to years. Epileptogenesis can occur in various ways, generally divided into genetic and acquired mechanisms. Genetic influence is supposed to be strongest in idiopathic epilepsies, whereas mechanisms of circuitry reorganization, after a brain insult, are most extensive in the acquired symptomatic ones. It should be noted that these mechanisms are not separate realities but, in some cases, functional consequences of brain injury can depend on genetic background (**Fig. 1**) (Pitkänen and Lukasiuk, 2009).

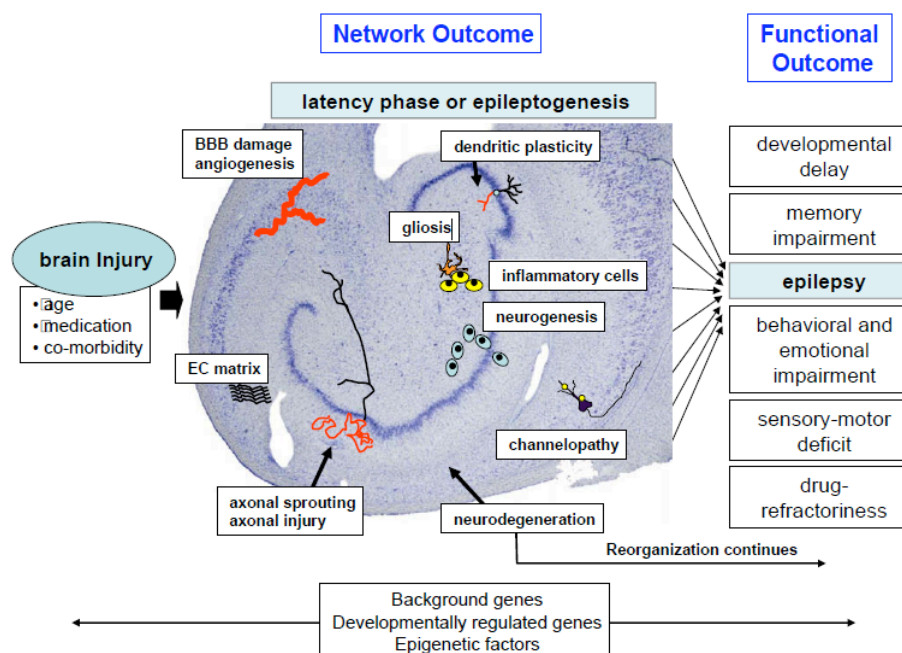


Figure 1: cellular alterations occurring during the epileptogenic process. From Pitkänen and Lukasiuk, 2009.

2.2 Genetic and epigenetic mechanisms of epileptogenesis.

Over 13 genes associated with human epilepsy have been identified so far and at least 33 single gene mutations in mice have been linked to an epileptic phenotype.

Benign familial neonatal convulsion (BFNC), generalized epilepsy with febrile seizures plus (GEFS+) and autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) are three idiopathic diseases in which gene mutations codify for mutated voltage-gated or ligand-gated channels.

In particular, in BFNC, mutations were identified in genes for potassium (K) channels (Singh et al., 1998; Biervert et al., 1998) named KCNQ2 and KCNQ3. These mutations are responsible of a loss of function that leads to a decrease in the size of the potassium current. It has been suggested that, even a moderate reduction (20-25%) of function, may be associated with epilepsy (Schroeder et al., 1998).

GEFS+ is associated with a point mutation in the gene coding for the β -subunit of a voltage-gated sodium channel (SCN1B) and, as found more recently, in the $\alpha 1$ -sodium channel subunit (SCNA1A) (Wallace et al., 1998; Escayg et al., 2000). *In vitro* studies suggest that the mutation results in defective inactivation of the sodium channel, which could lead to failure to limit the sustained repetitive firing of a depolarized neuron (McNamara, 1999).

ADNFLE begins clinically in childhood and patients have brief, nocturnal seizures with motor features. Mutations affect genes codifying for nicotinic cholinergic receptors (Steinlein et al., 1997; Phillips et al. 1998) and result in decreased Ca^{2+} flux through the receptor, which may lead to a reduction in the amount of GABA released from presynaptic terminals, and trigger a seizure by synaptic disinhibition (Kuryatov et al., 1997).

Pathologies described up to here, are characterized by complex inheritance patterns, in which several loci have been identified, but the underlying genetic defects remain to be determined.

PME (progressive myoclonus epilepsy) is a group of rare single-gene epilepsies characterized by myoclonus, generalized tonic-clonic seizures and progressive neurological dysfunction mainly in the form of dementia and ataxia (Berkovic et al., 1986). Among these, Unverricht-Lundborg Disease (ULD) and Lafora Disease (LD) are the most well characterized. Specific genetic mutations are associated with a deficit of two proteins, cystatinB and laforin respectively, that result in epilepsy (Acharya, 2002).

Some other changes in genes occur without directly affecting DNA sequence but by chemical modification of DNA or chromatin, such as DNA methylation and alterations in methylation or acetylation status of histones. These mechanisms are termed *epigenetic*. Seizure or SE-induced histone modifications have been reported for promoters of a number of genes, including those involved in neuronal plasticity such as c-fos, c-jun and CREB (Tsankova et al., 2004; Sng et al., 2005; Sng et al., 2006). Deacetylation of histones at the GluR2 promoter leads to its decreased expression, resulting in enhanced epileptogenesis (Sanchez et al., 2001). Involvement of epigenetic mechanisms have been sustained by the discovery that some AEDs can influence gene expression and, consequently, cellular metabolism (Pitkänen and Lukasiuk, 2009).

As reported by Pitkänen and Lukasiuk, apart from lead to cellular and/or circuitry alterations and brain pathologies associated with epilepsy, these mutations can significantly modify the severity, influence the development or maintain changes associated with postinjury epileptogenic process (Fig. 1).

2.3 Acquired postinjury mechanisms.

In addition to SE, various other types of brain insults such as traumatic brain injury (TBI), stroke and tumors can trigger the epileptogenic progression. Although cellular alterations may be different in different patients, certain features are common and include neurodegeneration, neurogenesis, gliosis, invasion of inflammatory cells and neuroinflammation, axonal sprouting, dendritic plasticity, angiogenesis and changes in extracellular matrix. These alterations are accompanied by a variety of molecular changes that lead, in addition to epilepsy, to a variety of functional impairments such as developmental delay, memory, emotional and behavioural impairment and somatomotor decline (Pitkänen and Lukasiuk, 2009) (Fig. 1).

Neurodegeneration. This is probably the best described change occurring in epileptogenesis. Areas with neuronal damage typically include hippocampus, but the damage also extends to extrahippocampal regions, such as the entorhinal and pyriform cortices or the amygdale. The extend of neuronal damage is directly influenced by the severity of seizures (Ben-Ari and Dudek, 2010).

The normal hippocampus consists of subfields, from CA₁ to CA₄ and the dentate gyrus. The primary neurons of the Cornu Ammonis are the pyramidal cells while those of the dentate gyrus are granule cells. The axons of granule cells are called mossy fibers and these are normally not seen in the inner molecular (IML, supragranular) layer where the dendrites of granule cells are present. The

hippocampal circuitry consists of a trisynaptic excitatory pathway from the entorhinal cortex to the dentate granule cells, which project to the CA₃ pyramidal neurons via mossy fibers, and from there to the CA₁ region through Schaffer collaterals. There are local circuits in each region with excitatory and inhibitory interneurons (**Fig. 2**).

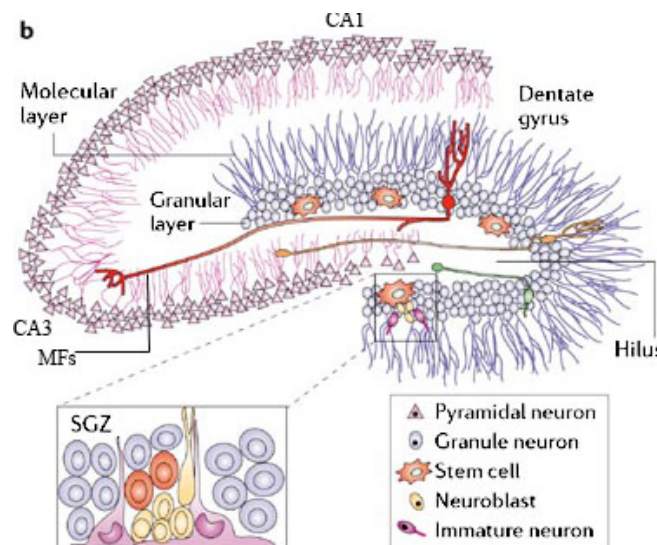


Figure 2: schematic representation of hippocampal circuitry. MFs: mossy fibers.

In an epileptic hippocampus, several degenerating cells are identified in CA₁ and CA₃ pyramidal cell layer and hilus, with milder damage in CA₂ pyramidal layer and granule cells. This pattern is also reproduced in several animal model, including pilocarpine model: pronounced cell loss in these regions, accompanied by oedema, is seen 3 days after the injection of the alkaloid (Paradiso et al., 2009). Several lines of evidence suggest that neuronal death affects specific GABAergic interneuron and glutamatergic neurons. These result, respectively, into permanent reduction (but not complete failure) of inhibitory drive to principal neurons and formation of new excitatory glutamatergic circuits by many of the remaining neurons. Synergistically, these two events increase the propensity for further seizures (Ben-Ari and Dudek, 2010).

The reduced inhibition has been also explained by the “dormant basket cell (DBC) hypothesis” (Sloviter, 1991). In contrast to the loss of GABAergic interneurons, the author found that certain inhibitory cells (basket cells) are more resistant to seizure-induced death. Normally, they receive

excitatory input by mossy cells and provide inhibition to granule cells. According to this hypothesis, seizures cause death of mossy cells, resulting in lack of the normal excitatory input to the basket cells which, although preserved, cannot provide feedback inhibition to granule cells and remain in a dormant state. Other authors have attempted to test the DBC hypothesis but, in general, the results were not supportive. However, the complexity of the hippocampal circuitry makes it difficult to fully test or reject this theory.

Whatever the cellular phenotype involved, cell death is a substantial histological feature of epileptic tissue and its alleviation reduced behavioural consequences after SE (Brandt et al., 2003; Paradiso et al., 2009). Nevertheless, it was not sufficient to prevent epileptogenesis and appearance of SRS, probably due to limits of the therapeutical approaches used. However, these results underlie that neuroprotective treatments have an important role in enhancement of recovery.

Neurogenesis (for details see Kuruba R et al., 2009). Neurogenesis is a process of generation of new neurons in the central nervous system through division of neural stem cells (NSCs) and neuronal differentiation of newly born cells. Although most neurogenesis occurs during initial development, certain regions of the brain maintain neurogenesis throughout life. These include the subgranular zone (SGZ) of dentate gyrus (DG) (Fig. 2) and the subventricular zone lining the lateral ventricles. A great fraction of these new cells differentiate into granule cells of the DG and incorporate into the functional hippocampal circuitry through establishment of granule cell-specific afferent and efferent synaptic contacts. Physical exercise, exposure to an enriched environment or pathological stimuli positively enhance neurogenesis. Studies in animal models clearly reveal that, at early time points after an initial precipitating injury such as acute seizures or SE, increased hippocampal neurogenesis occurs. This is consistent with studies on tissues from pediatric patients in the early phase of TLE (Blümcke et al., 2001). It is believed that the release of mitogenic factors from dying neurons and reactive glia, or that augmented levels of Neuropeptide Y (NPY) found after acute seizures, probably increase the proliferation of NSCs. It is also likely that amplified neuronal activity during and after seizures can directly influence the production of new neurons (Deisseroth et al., 2004).

As indicated in the previous paragraph, increased DG neurogenesis, after acute seizures, is associated with anomalous migration of a fraction of newly born granule cells into the dentate hilus and/or the dentate molecular layer (Houser, 1990b; Parent et al., 1997; Parent et al., 2006; Scharfman et al., 2007a; Scharfman et al., 2007b). Displaced new neurons establish atypical

connectivity with excitatory terminals (e.g. mossy fibres) (Pierce et al., 2005) and exhibit spontaneous bursts of action potential (Scharfman et al., 2000), promoting aberrant circuitry development which contribute to the evolution of an initial-seizure-induced hippocampal injury into chronic epilepsy (see ahead). The precise reasons for anomalous migration of newly born granule cells are still being examined. A recent study supports the involvement of reelin, a migration guidance cue, supposed to promote appropriate migration of newly born neurons into the GCL. It is expressed by interneurons that are typically lost in TLE. Reelin deficiency, after acute seizures, is then thought to contribute to ectopic chain migration and aberrant integration of newborn cells into the dentate hilus (Gong et al., 2007). A contribute may derive also from an aberrant glial scaffold (probably due to gliosis), that may guide new neurons into the hilus and not toward the molecular layer, as happen in naïve animals (Shapiro et al., 2005).

Generally, production of new neurons occurs, both in animals and in humans, during the first few weeks after the seizures episode (Parent et al., 1997) and returns to baseline levels by about two months after the initial episode (Jessberger et al., 2007). Decreased hippocampal neurogenesis is likely to be linked, at least partially, to learning and memory deficit and depressive behaviour observed in TLE and to persistence of seizures.

Actually, decreased neurogenesis is a consequence of dramatic lowering in the neuronal differentiation of newly born cells rather than decreased production of new cells or substantial diminution in numbers of NSCs. (Kralic et al., 2005; Hattiangady et al., 2006). This is probably related to the presence of an unfavorable hippocampal microenvironment, typified by depletion of the concentration of several factors (e.g. FGF-2, IGF-1 and BDNF) that promote neurogenesis. In accordance with this hypothesis, implementation of these factors may ease neuronal differentiation of NSCs and other various impairments associated to chronic TLE. This is exactly the goal reached by our laboratory, by viral-vector mediated implementation of FGF-2 and BDNF (Paradiso et al., 2009).

Dendritic plasticity and changes in extracellular matrix. Postinjury tissue remodelling is also characterized by the loss of dendritic spines, changes in their morphology and reduction of branches. These alterations could affect the availability of various receptor types as well as their stoichiometry and, thus, compromise the information flow from afferent input. Another important change in dendritic plasticity is correlated with neurogenesis. In epileptic conditions, newly generated granule cells, present hilar basal dendrites (HBDs) that persist in the mature cells

integrating into synaptic circuits and, being furnished with spines, probably contributing to additional recurrent excitatory circuits (Spigelman et al., 1998; Ribak et al., 2000). Moreover, in epileptic rats, HBDs are significantly longer and form a dense plexus in the hilus as compared to control animals in which the majority of dendritic processes from new-born cells are orientated along the SGZ-GL border (Shapiro et al., 2005). Alterations in postinjury remodelling of neuronal circuits are accompanied by changes in the extracellular matrix (ECM). A large number of enzymes contribute to ECM degradation and rearrangement, in particular the tissue-type and the urokinase plasminogen activator (tPA and uPA), their inhibitors TIMP-1 and -2, metalloproteinases appear to be involved (Lukasiuk and Pitkänen 2004; Lukasiuk et al., 2006; Lukasiuk et al., 2003).

Up to here, only a little part of the changes that occur during epileptogenesis. Neuroinflammation and gliosis, angiogenesis and damage to the BBB, mossy fibres sprouting represent other important features. This modifications will be described in detail further on, in separate paragraphs, referring to the works conduct during these years.

Each cellular alteration that occurs during epileptogenesis, involves alterations in synthesis and release of numerous substances including neurotransmitters, chemical mediators and proteins. Among the latter, of particular interest are the neurotrophic factors (NTFs).

3. Neurotrophic factors

Involvement of NTFs in epilepsy have earned particular interest in scientific community because of their dual aspect: a pro- (“bad”) and/or an anti- (“good”) epileptic effect. In fact, some NTFs seem to favor epileptogenesis, other, due to their trophic effects, oppose these processes, still other can exert both positive and negative effects or are devoid of any consequence. Among them, two proteins have demonstrated an important role in the pathology of epilepsy: the neurotrophin BDNF (brain derived neurotrophic factor) and FGF-2 (fibroblast growth factor-2).

3.1 Family of neurotrophins.

Some proteins secreted in the brain play a crucial role in the control of neuronal numbers and of dendritic growth. The best studied group is a family of structurally related molecules termed neurotrophins. The first neurotrophin was identified by the Nobel-prize Rita Levi-Montalcini in 1966 and named “the” nerve growth factor (NGF). To date, five member of the family have been identified: BDNF, NGF and neurotrophins (NT) 3, 4/5 and 6, the latter found only in fishes (Götz et al., 1994). Initially produced as proneurotrophins (MW~30 kDa), they are cleaved into the mature protein by prohormone convertases, such as furin (Barde, 1990; Ibáñez, 1998). Mature molecules are noncovalently-linked homodimers, with molecular weight of about 28 kDa. In common, they have very basic isoelectric points, a somewhat unusual property for secreted proteins, which may serve the purpose of limiting their range of action. With the exception of NT4/5, neurotrophin sequences are highly conserved in mammals.

Based on their three dimensional structure (**Fig. 3**), neurotrophins are classified as part of the Cysteine Knot Superfamily due to a distinct structure, formed by cystein residues, involved in a double loop formed by two disulphide bonds penetrated by a third disulphide bond, known as the cysteine knot (McDonald et al., 1991). The N- and C-termini are highly variable in both sequence and structure. In particular, the high variability in the N-terminus region is thought to be involved in receptor binding specificity (Kullander et al., 1997).

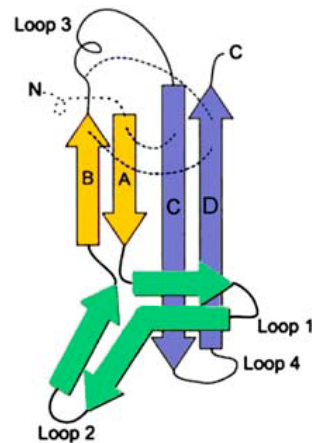


Figure 3: the neurotrophin molecule. Dashed lines represent the three disulfide bonds of the cysteine knot. The N terminus is disordered in the unbound structures and is shown by a dashed line. From Butte et al., 2001.

Neurotrophins bind to two different classes of transmembrane receptor proteins, the tropomyosin receptor kinase (Trk) and the neurotrophin receptor p75^{NTR} (**Fig. 4**). A selective binding to these different receptors permits the transduction of very different signals. It is not excluded a direct interaction that allows fine tuning and cross talk.

Trk. The tropomyosin kinase receptors are transmembrane glycoproteins of ~140 kDa. In mammals, three *trk* genes, codifying for the three different proteins TrkA, TrkB and TrkC, have been identified. A high, but not absolute, binding specificity has been seen among neurotrophins: NGF is the preferred ligand for TrkA, BDNF and NT4/5 for TrkB, NT3 for TrkC, even if the latter is also a ligand for TrkA and TrkB (Barbacid, 1994). As schematized in **figure 4**, five distinct motifs are recognized in the structure of these receptors (Schneider and Schweiger, 1991), but the most important for the interaction with ligands is an Ig-like domain of the extracellular portion, called Ig2 domain (Urfer et al., 1995; Urfer et al., 1998).

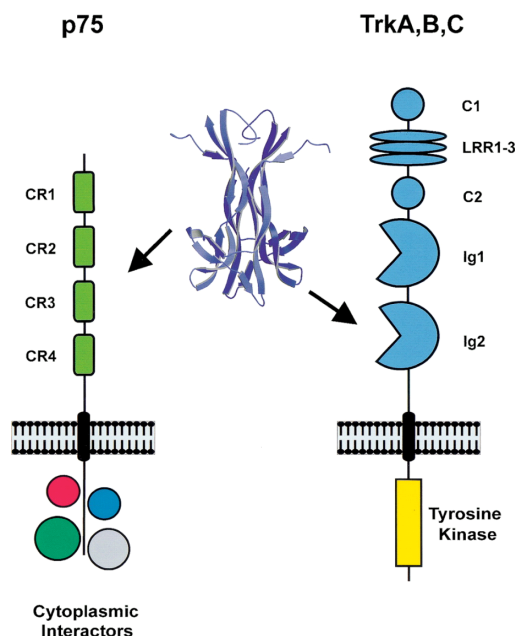


Figure 4: schematic representation of the p75 and Trk receptors. From Bibel and Barde, 2000.

Interaction with ligands induces dimerization of the receptor (Jing et al., 1992) that results in the phosphorylation of specific tyrosine residues, located in the juxtamembrane region and in the C terminus. This leads to an open conformation of the receptor and to the access of substrates to the kinase (Fig. 5).

Two complexes of adapter molecules bind to the tyrosine residue located in the juxtamembrane region: the Shc/Grb2/SOS and the FRS2/SHP-2/Grb2/SOS complex. The recent finding of Shc analogs (N-Shc, Sck; Nakamura et al., 1998) raises the question of recruitment of different Shcs that may be specific for TrkA, TrkB, or TrkC. Three main signalling cascades are activated by the Trk receptors and their substrates. The Ras/Raf/MEK/MAPK pathway induces the differentiation of neurons and neurite growth while the PKB/AKT pathway mediates the survival functions of the neurotrophins. Finally, the phosphorylated tyrosine in the C terminus recruits phospholipase C- γ (PLC γ) which, in turn, catalyses the cleavage of the substrate PIP₂ to DAG and IP₃, with DAG inducing activation of PKC and IP₃ leading to release of Ca²⁺ from internal stores. The latter pathway seems to play an important role in neurotrophin-mediated neurotrophin release (Canossa et al., 1997) and in synaptic plasticity. It has also been reported that the PLC- γ track regulates the neuron-specific intermediate filament protein, peripherin (Loeb et al., 1994).

The first two pathways are interconnected with each other since activation of the MAPK pathway is mediated by FRS2, through the recruitment of the tyrosine phosphatase SHP-2/Grb2/SOS complex,

and also by phospho-Shc, via the Grb2-SOS complex. Other adapter molecules seem to form complexes with Grb2/SOS, for example rAPS, SH2-B (Qian et al., 1998) and SNT (Stephens et al., 1994), making possible to amplify and diversify receptor-mediated signals.

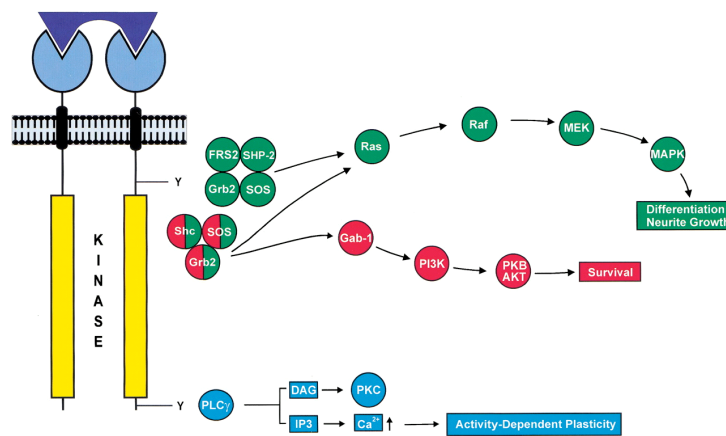


Figure 5: signalling through the Trk receptors; the main pathways. From Dawbarn and Allen, 2003.

Splice variants have been described for all three Trk receptors and include deletions in the extracellular domain and intracellular truncations or inserts. Some of these modifications were found to influence ligand specificity, as demonstrated by the fact that a TrkB splice variant, lacking exon9 in the extracellular domain, shows decreased interaction with NT4/5 and NT3 (Strohmaier, 1996), and a TrkA variant, with an increased specificity for NGF and a decreased specificity for NT3, has been described (Clary and Reichardt, 1994). Too little is known about the localization and the role of these variants, however they are often discussed as dominant negative modulators of Trk signalling (Eide et al., 1996; Ninkina et al., 1996).

p75^{NTR}. Codified by a gene on the human chromosome 17 (17q21-22) (Huebner et al., 1986), it is a transmembrane glycoprotein receptor of ~75 kDa. It is characterized by four cysteine (CR1-CR4) repeats (Fig. 4) in the extracellular domain, and a palmitoylated conserved cysteine residue in the intracellular juxtamembrane region (Barker et al., 1994). Related to proteins of the tumor necrosis factor (TNFR) superfamily, binding to this receptor has been shown to affect cell survival (Barret and Bartlett, 1994), axonal outgrowth (Dechant and Barde, 2002) and to result in activation of NF- κ B (Carter et al., 1996). It does not exhibit any intrinsic catalytic activity, so the signal is a

consequence of an association with, or dissociation from, cytoplasmic interactors. As represented in **figure 6**, they include NADE (p75^{NTR} associated cell death executor), NRIF (neurotrophin receptor-interacting factor) and NRAGE (neurotrophin receptor-interacting MAGE homolog -MAGE is a protein family expressed in cancer tissues-) that mediate NGF-dependent apoptosis in sympathetic neuron precursor cells (Salehi et al., 2000). NRIF and NRAGE also lead to cell cycle arrest, as well as SC-1 (Schwann cell factor-1), whereas the GTPase RhoA modulates neurite growth.

Several variants of the p75^{NTR} receptor have been found, such as soluble forms and truncated proteins lacking in the ability to bind neurotrophins (Dechant and Barde, 1997). The precise role is unknown, but their presence may be functionally relevant.

Neurotrophin receptor expression is highly regulated. In the central nervous system (CNS), few neurons express TrkA (mostly the basal forebrain cholinergic neurons) whereas TrkB is widely expressed, explaining the multitude of actions of BDNF. TrkC is typically expressed in the early phases of development (Tessarollo et al., 1993).

Also the expression profile of P75^{NTR} is highly regulated, showing a down-regulation during postnatal development (Bothwell, 1995) and a rapid induction after injury, such as nerve lesion or seizures (Roux et al., 1999). This confirms the clear link between p75 receptor and cell death in pathological situations.

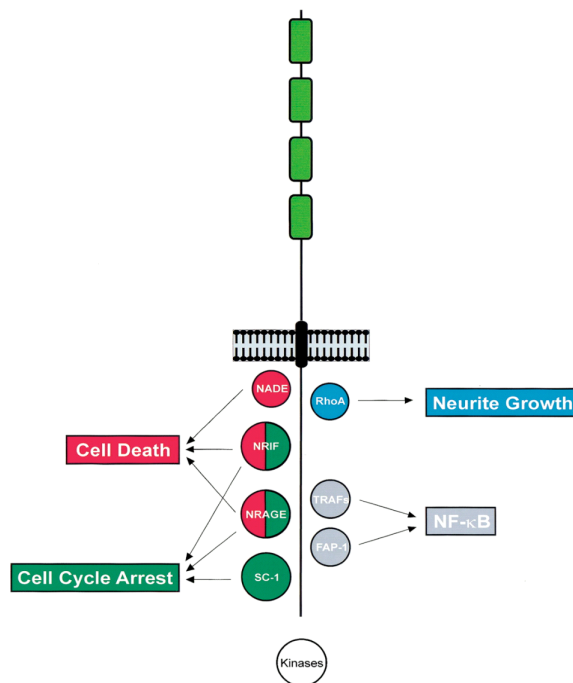


Figure 6: $P75^{\text{NTR}}$ interactors involved in transducing cell death. From Dawbarn and Allen; 2003.

Remarkably, Trk receptors and $p75^{\text{NTR}}$ are often co-expressed by the same cell and form a complex that can be immunoprecipitated after solubilization (Bibel et al., 1999). Both the intra- and the extracellular domains participate in this interaction, which is also dependent on the state of phosphorylation of the Trk receptors (Bibel et al., 1999). Receptor association leads to high-affinity neurotrophin binding, which is crucial in view of the limiting amounts of neurotrophins in vivo (Hempstead et al., 1991; Benedetti et al., 1993; Davies et al., 1993; Barker and Shooter, 1994; Lee et al., 1994; Mahadeo et al., 1994; Horton et al., 1997). This association may also increase ligand discrimination by the Trk receptors, important in the case of TrkA and TrkB that bind more than one neurotrophin (Benedetti et al., 1993; Bibel et al., 1999). Finally, the proximity of $p75^{\text{NTR}}$ and Trk receptors in the membrane allows the signalling pathways, triggered by both receptors, to interact.

An interesting and neuron-specific aspect of neurotrophin is the retrograde transport of signals from axon terminals back to the cell body of neurons, with a process that seems to involve the internalization of the ligand-receptor complex (Bhattacharyya et al., 1997; Riccio et al., 1997) probably via signalling endosomes (Grimes et al., 1996).

BDNF. Among neurotrophins, BDNF is the most abundantly expressed in the CNS. The gene for human BDNF has been mapped to chromosome 11p. It contains eight exons encoding for the 5'

UTR region and a 3' exon which contains the coding sequence and the 3'UTR region. Alternative splicing gives rise to at least 20 different transcripts, all expressed in brain at different levels and in restricted neuronal population. Only the transcript containing exon IV has been found in heart and lung (Binder et al., 2001). Like many other growth factors and hormones, BDNF is generated from a precursor protein (pro-BDNF) cleaved by a protease in the mature form. In addition to the mature form, pro-BDNF is also biologically active.

BDNF mRNA has a widespread distribution in the central nervous system, including limbic forebrain, neocortex and, more than all, hippocampus (Lindvall et al., 1994). In this region it is expressed prevalently in granule cells, pyramidal cells and some hilar GABAergic neurons. BDNF protein immunoreactivity also appears preferentially in cell bodies and axons, compared to dendrites. Both physiologic, such as light and osmotic stimuli, physical exercise and the estrus cycle, and pathologic stimuli, enhance BDNF mRNA. Several evidences support a critical role of this neurotrophin in the pathogenesis of different diseases including epilepsy. After seizures, an increased BDNF synthesis and TrkB receptor activation is seen in granule cells of the hippocampus, both in humans (Mathern et al., 1997) and rodents (Binder et al., 2001). Many studies correlate these phenomena with proepileptogenic effects. Kokaia and collaborators (1995) reported a greater than twofold reduction in the rate of kindling development in BDNF heterozygous (+/-) mice, whereas kindling is completely abolish in absence of TrkB receptors (He et al., 2002). Moreover, application of NTFs, including BDNF, has been shown to potentiate synaptic transmission *in vitro* (Lohof et al., 1993) and *in vivo* (Messaoudi et al., 1998); BDNF also enhances glutamatergic transmission (Lohof et al., 1993) and seems to reduce inhibitory synaptic transmission (Tanaka et al., 1997). The mechanism underlying synaptic potentiation remains unclear but may involve the facilitation of transmitter release or effects on ion channels and/or conductances. Also pro-BDNF, acting via p75^{NTR}, could lead to seizure-induced apoptosis. As summarized by Binder et al. (2001), these alterations are responsible for a state of hyperexcitability that, in turn, could create a positive feedback loop in which increased activity results in upregulated levels of BDNF and of activation of TrkB.

However, other experimental evidence suggests an opposite, anti-epileptogenic, effect. For instance, BDNF is known to modulate the expression of neurotransmitters and neuropeptides, including neuropeptide Y. NPY is thought to inhibit seizure generation and is interesting to note that both kindling and kainate-induced seizures increase NPY immunoreactivity with a distribution that is strikingly similar to phospho-Trk immunoreactivity. This suggests that BDNF-induced Trk

activation could lead to NPY upregulation, which might subsequently limit excitability (Larmet et al., 1995). Contrary to the results published by Tanaka in 1997, a work of Palma and collaborators (2005) demonstrates that BDNF amplifies GABA currents and prevents their run-down in *Xenopus* oocytes expressing GABA_A receptors transplanted from surgically removed specimens of human epileptic brains. Moreover, mature BDNF favours survival or regeneration of hippocampal neurons damaged by SE (Simonato et al., 2006).

Several mechanisms have been proposed to explain the contrasting effects of BDNF. The same neurotrophin may induce different effects depending on splice variants, cellular site of action (dendritic localization is associated with the potentiation of active synaptic contacts and somatic distribution with cell survival and differentiation of neuronal precursors), local synthesis of the pro- and mature forms, and opposing effects resulting from the binding to TrkB or p75^{NTR} (Simonato et al., 2006). Finally, different effects of neurotrophins may also depend on the duration of exposure, as demonstrated by the fact that chronic intrahippocampal infusion of BDNF inhibits the development of hippocampal kindling and reduces the duration of electrographic seizures (Larmet et al., 1995). Hence, acute BDNF could enhance synaptic transmission and neuronal excitability, whereas chronic treatment could promote survival and induce growth and morphological changes of synapses (Lu, 2004). Acute responses are mediated by cAMP activation (Li et al., 1999), whereas the reduced responsiveness, seen after long-term exposition, is probably due to TrkB receptors downregulation (Frank et al., 1996).

3.2 FGF-2.

Basic fibroblast growth factor, or fibroblast growth factor-2 (bFGF-2 or FGF-2), is a neurotrophic factor belonging to a family of 22 structurally related proteins (Itoh and Ornitz, 2004). It is a single-chain polypeptide composed of 146 amino acids, with a core region consisting of 120-130 aa, ordered into 12 antiparallel β -strands flanked by divergent amino and carboxyl termini. In common with other members of the family, FGF-2 owns a positively charged heparin-binding region that allows a high affinity binding to heparansulfate proteoglycans (HSPGs) sited in the extracellular matrix (ECM) and on the cell surface in the vicinity of the FGF receptors (FGFRs). The latter is required for high-affinity interactions with receptors, the former favours local storage of FGFs. Distinct isoforms of FGF-2 are described: the high molecular weight (HMW) forms, ranging from 20 to 24kDa, are predominantly localized in the nucleus, whereas the low molecular weight form (LMW, 18kDa) resides in the cytoplasm (Florkiewicz et al., 1991; Quarto et al., 1991; Sørensen et al., 2006).

FGF-2 can interact with four high-affinity tyrosine-kinase receptors (FGFR 1-4) and one low-affinity cell surface receptor (Klint and Claesson-Welsh, 1999; Reuss and von Bohlen und Halbach, 2003). FGFRs present, in the extracellular portion, three immunoglobulin-like domains (D1-D3) and an acidic, serine-rich sequence in the linker between D1 and D2, called “the acid box”. The D2-D3 fragment is the ligand binding domain, whereas the D1 domain and the acid box have a role in receptor autoinhibition. Activation of these receptors leads to a phosphorylation cascade resulting in the activation of MAPK, IP3, and PKC pathways.

FGFRs isoforms are generated by exon skipping eliminating the D1 domain and/or acid box, others originate as a result of alternative splicing in the second half of the D3 domain of FGFR1-3, that yields to 'b' (FGFR1b, -2b and -3b) and 'c' (FGFR1c, -2c and -3c) isoforms, rendering distinct FGF binding specificities for the various FGFs.

With regard to biological properties, several *in vitro* studies confirm that FGF-2 regulates proliferation, survival and differentiation of neural stem cells (NSC). In particular, NSC can self-renew in response to it (Temple, 2001) and, through a mechanism depending on its concentration, differentiate into neurons (Palmer et al., 1999) or oligodendrocytes (Qian et al., 1997).

In situ hybridization and immunohistochemistry studies conducted in normal rats (Emoto et al., 1989; Bugra et al., 1994; Gall et al., 1994; Gómez-Pinilla et al., 1995; Simonato et al., 1998) and mice (Zucchini et al., 2008) show a widespread distribution of FGF-2 in glial cells of cortex and thalamus and a dense (in CA₂ pyramidal layer) to moderate (in CA₁, CA₃ pyramidal layer and DG granular layer) expression in hippocampal neurons. FGFR1 is the most expressed receptor subtype in the hippocampus (Ohkubo et al., 2004) and its distribution reflects the pattern of the ligand (Zucchini et al., 2008).

Several lines of evidence suggest a link between FGF-2 and epilepsy. As for BDNF, seizures increase mRNA and protein levels (Jankowsky and Patterson, 2001) and upregulate FGFR1 receptors (Gómez-Pinilla et al., 1995). Moreover, transgenic mice overexpressing FGF-2 display an increased susceptibility (in terms of augmented severity, lethality and reduced latency) to kainate-induced seizures (Zucchini et al., 2008). This is probably correlated with the morphogenetic properties of FGF-2, responsible of a latent hyperexcitability. *In vitro* studies confirm an involvement of FGF-2 in axonal branching of hippocampal and cortical neurons (Aoyagi et al., 1994; Patel and McNamara, 1995; Szebenyi et al., 2001) and in the production of excitatory synapses (Li et al., 2002). The latter property was confirmed *in vivo* by Zucchini and collaborators (2008): the overexpression of FGF-2 increases the density of glutamatergic synapses and the excitatory input in the hippocampal CA₁ area.

In the same study, however, mice overexpressing FGF-2 display, especially in the areas of overexpression, a significant reduction of seizure-induced necrosis and apoptosis. In agreement with these results, chronic i.c.v. infusion of low dose FGF-2 does not affect kainate seizures, but improves behavioral recovery and reduces hippocampal damage (Liu et al., 1993; Liu and Holmes, 1997) and, when coinjected with kainate in the hippocampus, FGF-2 prevents neuronal loss (Tretter et al., 2000). Several mechanisms have been proposed to explain these neuroprotective actions, for example Ca^{2+} -dependent inactivation of NMDA receptors (Boxer et al., 1999) and/or induction of ActivinA (ActA) (Mattson, 2000; Tretter et al., 2000 Zucchini et al., 2008).

Based on these and other evidences, as for BDNF, FGF-2 can also exert multiple, contrasting biological effects. Mechanisms proposed to explain these contrasting effects may include the specific activation of FGF receptors or distinct properties of the HMW and LMW isoforms (Zucchini et al., 2008).

3.3 Therapeutical application of NTFs.

The positive effects described above render NTFs very attractive candidates for therapeutic use in neurological disorders. A greater understanding of the mechanisms underlying contrasting effects of NTFs is crucial for this aim, even though other important difficulties must be overcome to allow their clinical application. They have been summarized by Simonato and colleagues (2006) and are reported in the table below (Table 4).

<i>Challenges for the use of NTFs for the treatment of epilepsy</i>
Definition of timing of the treatment (with reference to the natural history of the disease)
Therapeutic goals (prevention of epileptogenesis vs treatment of diagnosed epilepsy)
Use of single NTF or of combination of NTFs (with reference to the existence of synergies between NTFs that might be used to reach specific therapeutic goals)
Route of administration and delivery strategy
Purity of the NTF (pro- vs mature form)
NTF concentration at the site of action (with reference to the existence of high- and low- affinity receptors)
Representation of receptors at the site of action
Duration of treatment (with reference to acute and chronic effects, to possible receptor internalization with loss of effect and to possible detrimental effects for long-term treatments)

Table 4: crucial points that should to be addressed for the application of NTFs to the treatment of epilepsy. Adapted from Simonato et al., 2006.

Among these, the choice of the appropriate route of administration and delivery strategy deserves special attention (**see also chapter II**). Several attempts have been made by peripheral, intrathecal or intracerebroventricular administration of NTFs, but all of them failed. Despite failures, these studies focused the attention to the importance of a prolonged and targeted release of NTFs to the damaged area: an excessive availability in the healthy brain tissue could, in fact, produce undesired or even detrimental effects.

Another important point concerns the possibility of achieving therapeutic goals by exploiting the synergy between different NTFs, as demonstrated *in vitro* and *in vivo* for FGF-2 and CNTF (Song and Ghosh 2004; Marconi et al. 2005) and for FGF-2 and BDNF (Paradiso et al., 2009).

In particular, in the latter study, only the supplementation of the two NTFs together potently favored survival, proliferation and neuronal differentiation of neural progenitors *in vitro*.

However, the most relevant results reported in this paper were obtained *in vivo*, by administering NTFs in rat hippocampus damaged by pilocarpine-induced SE.

Seizures are known to stimulate neurogenesis but newborn cells tend to aggregate into clusters assuming an ectopic localization in the dentate gyrus of the hippocampus. These cells aberrantly integrate into the local circuitry, receive excess excitatory input, exhibit abnormal bursting and are recruited during spontaneous seizures (Sharfmann et al., 2002). Supplementation of FGF-2 and BDNF greatly increases proliferation of hippocampal progenitors and favours their neuronal differentiation reducing aberrant features. In addition, it improves the number of putatively excitatory and inhibitory neurons, both dramatically decreased in the hippocampus of rats that experienced SE. Thus, treatment with FGF-2 and BDNF partially heals the damage induced by SE and maintains a good ratio between excitation and inhibition in the circuit (Paradiso et al., 2009). The effectiveness of BDNF and FGF-2 was also reported by Rao and collaborators in 2006. They demonstrated that grafts of fetal CA₃ cells, enriched with the two neurotrophic factors, exhibit robust integration, prolonged survival and that most of the cells implanted differentiate into CA₃ pyramidal neurons, restoring, at least in part, the damaged circuitry of the epileptic brain and preventing epileptogenesis.

The delivery strategy is of considerable importance for the application of NTFs to the treatment of pathological disorders. Interesting is the approach used by Marconi (2005) and then by Paradiso (2009) to achieve a localized and prolonged delivery of the two NTFs in the brain of animals. They employed herpes simplex virus-based (HSV) vectors engineered to express multiple NTFs (**Fig. 7**).

Several reasons drove the authors to choose this approach such as the ability to infect neuronal cells with no mutagenesis risks, and to produce a transient gene expression (**see chapter II**).

The importance of the study of Paradiso and colleagues (2009), is the demonstration that the addition of specific NTFs in injured areas represents a new approach to the therapy of neuronal damage and of its consequences.

The good results obtained from this research have strongly encouraged us to continue the studies on this topic, analyzing the influence of the two NTFs on different other specific aspects of epileptogenesis: neuroinflammation and sprouting of mossy fibers.

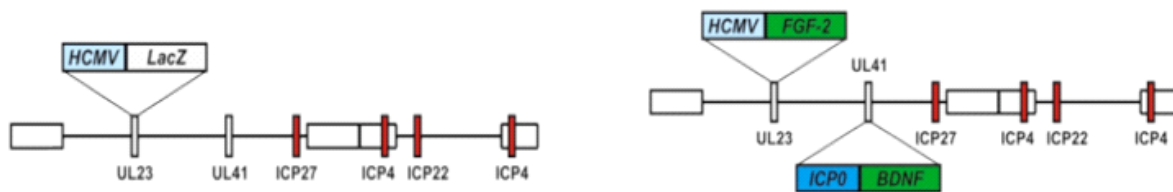


Figure 7: schematic representation of control (left) and double mutant (right) vectors.

4. Influence of NTFs on neuroinflammation and gliosis.

(see the published work: Hippocampal FGF-2 and BDNF overexpression attenuates epileptogenesis-associated neuroinflammation and reduces spontaneous recurrent seizures. Roberta Bovolenta, Silvia Zucchini, Beatrice Paradiso, Donata Rodi, Flavia Merigo, Graciela Navarro Mora, Francesco Osculati, Elena Berto, Peggy Marconi, Andrea Marzola, Paolo F Fabene, Michele Simonato. J Neuroinfl 2010, 7:81)

Historically, the brain has been considered an “immunoprivileged site” because of the presence of the blood-brain barrier, the lack of a conventional lymphatic drainage and an apparently low traffic of monocytes and lymphocytes. However, it is becoming clear that immune and inflammatory reactions do occur in the CNS, either intrinsically from the brain itself or acquired from systemic circulation through a damaged BBB.

Several lines of evidence demonstrate a link between the activation of inflammatory pathways and neurodegenerative diseases, including epilepsy. Interestingly, inflammatory reactions occur not only in epilepsy disorders characterized by an inflammatory pathophysiology, but also in TLE or in tuberous sclerosis, raising the possibility that inflammation may be a common factor contributing or predisposing to the occurrence of seizures and cell death, in various forms of epilepsy with different etiologies. Increased markers of inflammation have been found in plasma and cerebrospinal fluid after recent tonic-clonic seizures and in brain tissue obtained from patients surgically treated for drug-resistant epilepsies (Crespel et al., 2002; Peltola et al., 2000; Peltola et al., 2002).

Experimental studies confirm the human pattern. Significant upregulation of members of toll-like receptors (TLRs) family and of proinflammatory cytokines were seen in animal models after SE (Turrin et al., 2004; De Simoni et al., 2000). Specifically, IL-6, TNF- α and IL-1 β mRNA and protein levels increase early and decline to control values by one week after SE, with the exception of IL-1 β that is detected for several weeks in cells with glial morphology (De Simoni et al., 2000, Ravizza et al., 2008). The fact that monocytes/macrophages, but scarce granulocytes, B- and T-lymphocytes and NK cells, persist during the latent and the chronic period in the pilocarpine model, suggests that specific inflammatory pathways are chronically activated and may contribute to the etiopathogenesis of TLE (Ravizza et al., 2008). To support the link between inflammation and epilepsy, administration of anti-inflammatory drugs, steroids or immunoglobulins has anticonvulsive effects in humans and experimental models of epilepsy.

However, the role of neuroinflammation is controversial. Some authors consider immune reactions in CNS a protective, adaptive and beneficial endogenous response similar to the classic response to

infection. This is supported by the evidence that released cytokines can induce the synthesis of growth factors that can promote repair of the CNS (e.g. NGF, CNTF and insulin-like growth factor) (Elkabes et al., 1996) or stimulate antioxidant pathways (Wilde et al., 2000). Moreover, activated glial cells can operate as scavengers, removing potentially harmful debris.

Despite the different opinions, it is increasingly clear that the final outcome of inflammation on cell function and survival is highly dependent on the extent to which cytokines are produced, the length of time the tissue is exposed to inflammation and the balance between the neurotrophic and inflammatory factors produced by the competent cells. About the duration of inflammatory process, it is plausible that beneficial effects derive from acute exposition, while detrimental outcomes are due to chronic persistence of an inflammatory milieu in the brain.

As previously mentioned, audiogenic or chemically- and electrically- induced seizures increase levels of proinflammatory cytokines in the rodent brain. Among these, the long-lasting persistence of IL-1 β after the first insult suggests a critical role in the development of epilepsy. To support this, multiple intracerebroventricular injections of IL-1 receptor antagonist (IL-1Ra) significantly decrease the severity of behavioural convulsions and reduce TNF- α content in the hippocampus after electrically-induced SE (De Simoni et al., 2000). Synthesized as an inactive precursor of 31kDa, it becomes activated after cleavage by the intracellular interleukin-1 β converting enzyme (ICE) and acts by binding to IL-1 type-I receptors (IL-1RI) located on the plasma membrane of astrocytes (Pinteaux et al., 2002, Ravizza et al., 2008) and pyramidal neurons (Ravizza et al., 2008). Activation of receptors results in association with an accessory protein (IL-1RAcP) and recruitment of the adaptor protein MyD88 by the intracellular domain and leads to the activation of a signalling pathway including MAP kinases and nuclear factor kappa B (NF κ B) (O'Neill and Greene, 1998). IL-1RI receptors are supposed to mediate the proictogenic properties of the cytokine. It has been found that, on hippocampal pyramidal neurons, IL-1RI colocalize with NMDA receptor and its activation increases NMDA receptor-mediated Ca²⁺ influx, promoting excitotoxicity (Viviani et al., 2003).

Moreover, IL-1 β increases astrocytic release of glutamate via TNF- α (Bezzi et al., 2001) or nitric oxide (NO) (Hewett et al., 1994; Casamenti et al., 1999) production, inhibits GABA-mediated neurotransmission (Zeise et al., 1997; Wang et al., 2000) and can decrease the threshold for seizure induction (Dubè et al., 2005; Heida and Pittman, 2005). Taken together, all these data suggest that IL-1 β contributes to neuronal hyperexcitability and, thus, to seizure generation.

Production of cytokine(s) is mediated by glial cells after their activation. With particular reference to TLE, double-labelled immunohistochemical analysis showed, during the acute phases of SE, a strong IL-1 β immunoreactivity in both microglia and astrocytes whereas, in the epileptogenic and chronic phase of pathology, only astrocytes were reported to release the cytokine (Ravizza et al., 2008). Activation of glial cells, known as “reactive gliosis”, is triggered by the initial brain injury and, apart from leading to the synthesis of inflammatory molecules, is responsible for important changes in morphology and functional features of the cells involved.

Microglia cells derive from circulating monocytes or precursor cells in the monocyte-macrophage lineage, that originate in the bone marrow and invade the developing brain during the embryonic, fetal or perinatal stages. In the adult, healthy brain, these cells are found as “resting microglia”, characterized by a small cell body with fine, ramified processes and low expression of surface antigens. After acute injury they react within few hours, assuming a rod-like morphology devoid of branching processes and expressing MHC class II antigens and other numerous surface molecules necessary for antigen presentation. Once activated, they proliferate and accumulate at the site of injury where they phagocytose damaged cells and debris, release soluble factors and provide recruitment of immune peripheral cells (Nguyen et al., 2002). Accumulation of microglia is seen in brain areas associated with loss of neuronal cells. It has been found that media from microglia exposed to dying neurons induces subsequent neuronal death (Pais et al., 2008). The exact mechanism is not fully understood but seems involve the release of reactive oxygen species (ROS) via activation of NADPH oxidase. This may lead to increased internal zinc and potassium concentration, resulting in neuronal apoptosis (Knoch et al., 2008).

Signals for microglial activation may derive from cytosol or membrane of dying neurons, high levels of ATP or extracellular [K⁺] exceeding normal ranges. The released cytokines can drive neurotoxic cascades that in turn recruit more microglia. It has been demonstrated that all purinergic receptors (P₂X and P₂Y) are upregulated in the hippocampus at least until 48h after SE. Among these, P₂X₇ support the release of IL-1 β from microglia and, indirectly, the expression and release of other inflammatory factors. The observation that both receptors and cytokines are up regulated after SE suggests the occurrence of a positive feedback mechanisms exacerbating the activation process (Avignone et al., 2008). Other inflammatory molecules secreted by activated microglia include chemokines, NO, arachidonic acid, prostaglandins D₂, E₂ and F₂ α , tromboxane B₂ and leukotriene B₄ (Minghetti and Levi, 1998). It should be noted that microglia can also produce a

number of neuroprotective substances in response to injury, such as anti-inflammatory cytokines and neurotrophic factors, including IL-10 and IL-1 α , NGF and transforming growth factor β (TGF- β), that promote tissue repair, wound healing and extracellular matrix remodelling (Colton, 2009).

Gliosis associated to epileptogenesis involves also astrocytes. As for microglia activation, unbalanced neurotransmitters such as glutamate and noradrenalin, high levels of ATP, ROS species, molecules associated with neurodegeneration or with metabolic toxicity (e.g. NH $_4^+$), can trigger reactive astrogliosis and, eventually, glial scar formation. Also, the same cytokines initially secreted by microglia can initiate or modulate activation and proliferation of such cells. This is suggested by the fact that astrocytes express receptors for cytokines, especially IL-1 β , and each appears to fulfil a different functional role inducing a specific pattern of gene expression (John et al., 2001). The main feature of astrogliosis is cell hypertrophy, with enhanced expression of cell-type-specific glial fibrillary acidic protein (GFAP).

In the normal activity of the brain (see the reviews: Sofroniew and Vinters, 2010 and de Lanerolle et al., 2010), astrocytes exert essential functions in maintaining the fluid, ion, pH of the synaptic interstitial fluid and play direct roles in synaptic transmission through regulation of availability of synaptically active molecules including glutamate, purines (ATP and adenosine), GABA and D-serine. Astrogliosis is not an all- or none response but it is a finely graded continuum of progressive changes in molecular expression, cellular hypertrophy and, in severe cases, proliferation and scar formation. Changes have the potential to alter cell activities both through gain and loss of functions that can impact both beneficially and detrimentally on surrounding neural and non-neuronal cells. With particular reference to epilepsy, astrocytes in sclerotic hippocampi show down-regulated expression of glutamate transporter molecules EAAT1 and 2 (in rodents termed GLAST and GLT-1) (Proper et al., 2002; Mathern, 1999), probably mediated by IL-1 β (Ye et al., 1996), and aquaporin 4 (AQP4) on the perivascular membrane. This causes a reduced removal of glutamate and altered water homeostasis.

In normal conditions, once captured by astrocytes, glutamate is converted into glutamine by glutamine synthetase then transported to neurons and resynthesised to glutamate. In the epileptic tissue, a down-regulation of the enzyme leads to accumulation of the transmitter in astrocytes and in the extracellular space (Eid et al., 2004). Moreover, the GABA transporter GAT3, normally weakly expressed, is increased in sclerotic hippocampus, resulting into an excessive removal of the neurotransmitter during the ictal state (Lee et al., 2006; During and Spencer, 1993). Ion currents also appear altered in reactive astrocytes. In healthy cells, balance between K $^+$ and Na $^+$ channels

densities is important to maintain homeostasis in the brain. While enhancement of Na⁺ current was not fully confirmed (Bordey and Sontheimer, 1998; Bordey and Spencer, 2004), a clearly reduced expression of functional potassium (Kir) channels is responsible of impaired K⁺ removal in the sclerotic tissue (Kivi et al., 2000; Hinterkeuser et al., 2000; Schroder et al., 2000). Upregulation of astrocyte L-type Ca²⁺ channels was seen both in the kainate model of epilepsy and in specimens of TLE patients (Westenbroek et al., 1998; Djamshidian et al., 2002), suggesting enhanced glial uptake of the ion in the lesioned CNS; enhanced [Ca²⁺]_i is also associated with the release of glutamate. All together, these alterations can provide an excitatory drive underlying seizures disorders, suggesting a prominent contribution of astroglyosis to epileptogenesis.

Changes in astrocyte morphology, physiology and release of cytokines have been demonstrated to influence integrity of the BBB. In particular, IL-1β has been reported to affect permeability properties (Ferrari et al., 2004) probably via disruption of the tight-junctions (del Maschio et al., 1996) or production of NO and metalloproteinases in endothelial cells. Cytokines may also increase the expression of selectins and adhesion molecules (like E- and P-selectin, ICAM-1, VCAM-1) (Vezzani and Granata, 2005) on endothelial and epithelial cells, resulting in leukocyte extravasation (Fabene et al., 2008; Fabene et al., 2010). BBB leakage has been implicated in the induction of seizures and in the progression to epilepsy, exposing astrocytes and neurons to blood albumin and potassium ions, respectively (Seiffert et al., 2004; Ivens et al., 2007; van Vliet et al., 2007; Marchi et al. 2007 a).

As for microgliosis, astrogliosis can also exert positive effects deriving from release of anti-inflammatory molecules, activation of antioxidant mechanisms and scar formation, necessary to repair wound and delimit damaged tissue. For these reasons, complete inhibition of inflammation is not likely to be a useful therapeutic approach. It should be directed at more specific aspects or should prevent a prolonged exposure of brain parenchyma to a harmful environment. Very little is known regarding the possible modulation of these phenomena by NTFs. However, several findings suggesting an involvement of BDNF after inflammatory challenges led us to test their possible anti-inflammatory properties.

SHORT REPORT**Open Access**

Hippocampal FGF-2 and BDNF overexpression attenuates epileptogenesis-associated neuroinflammation and reduces spontaneous recurrent seizures

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Abstract

Under certain experimental conditions, neurotrophic factors may reduce epileptogenesis. We have previously reported that local, intrahippocampal supplementation of fibroblast growth factor-2 (FGF-2) and brain-derived neurotrophic factor (BDNF) increases neurogenesis, reduces neuronal loss, and reduces the occurrence of spontaneous seizures in a model of damage-associated epilepsy. Here, we asked if these possibly anti-epileptogenic effects might involve anti-inflammatory mechanisms. Thus, we used a Herpes-based vector to supplement FGF-2 and BDNF in rat hippocampus after pilocarpine-induced status epilepticus that established an epileptogenic lesion. This model causes intense neuroinflammation, especially in the phase that precedes the occurrence of spontaneous seizures. The supplementation of FGF-2 and BDNF attenuated various parameters of inflammation, including astrogliosis, microglia and IL-1 β expression. The effect appeared to be most prominent on IL-1 β , whose expression was almost completely prevented. Further studies will be needed to elucidate the molecular mechanism(s) for these effects, and for that on IL-1 β in particular. Nonetheless, the concept that neurotrophic factors affect neuroinflammation *in vivo* may be highly relevant for the understanding of the epileptogenic process.

Findings

Many acquired epilepsies have an identifiable cause, such as head trauma, an episode of status epilepticus (SE), stroke, or brain infection [1]. It is thought that these insults set in motion a cascade of neurobiological events that, in time, will lead to the occurrence of spontaneous seizures and to the diagnosis of epilepsy. This phenomenon is termed “epileptogenesis”. Conventional “antiepileptic” agents may exert symptomatic effects on seizures but do not interfere with epileptogenic processes [2].

In principle, understanding the molecular mechanisms underlying the cellular alterations occurring during epileptogenesis (which include cell death, axonal and dendritic plasticity, neurogenesis, neuroinflammation and

functional alterations in ion channel and synaptic properties) should allow development of effective agents. In this respect, neurotrophic factors (NTFs) appear to be strong candidates, because an extensive literature demonstrates their involvement in many of the cellular alterations associated with epileptogenesis [3]: not only do their trophic effects suggest involvement in cell death, neurogenesis and axonal sprouting, but they also exert functional effects at the synaptic level, with distinct modulatory actions at excitatory and inhibitory synapses [4]. In fact, we have recently reported that viral vector-mediated supplementation of two NTFs, namely fibroblast growth factor-2 (FGF-2) and brain-derived neurotrophic factor (BDNF), in a lesioned hippocampus favors “good” neurogenesis, repairs neuronal damage and may prevent epileptogenesis [5].

Recently, central nervous system (CNS) inflammation associated with blood-brain barrier (BBB) leakage has been implicated in the progression to epilepsy [6-9], and

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the pro-epileptogenic role of neuroinflammation has received great attention [10-13]. However, it is still unknown if NTFs may modulate these phenomena.

The aim of this study was to analyze if NTFs-induced anti-epileptogenic effects may involve anti-inflammatory mechanisms. We used a previously generated viral vector [5] to supplement FGF-2 and BDNF in the hippocampus after pilocarpine-induced SE that established an epileptogenic lesion. We decided to start investigating IL-1 β expression and glial activation in controls and inoculated animals. An increase in inflammatory cytokines, IL-1 β in particular, has been reported in the CNS and plasma in experimental models of seizures and in clinical cases of epilepsy [14]. Astrocytic and microglial activation elicited by pilocarpine-induced SE could underlie epileptogenic mechanisms [15,16] and BBB alterations [17]. A detailed description of the methods employed in this study is provided as Additional file 1.

In the pilocarpine model, an episode of SE produces, after a latent period of a few days, spontaneous recurrent seizures (SRSs), i.e. epilepsy. Pilocarpine (300 mg/kg i.p.) rapidly induced a robust convulsive SE (latency: 18 \pm 2 min), which was interrupted after 2 h by administering the anticonvulsant diazepam (10 mg/kg i.p.). Based on behavioral observation and EEG recordings, the severity of SE in different animals was indistinguishable. This procedure caused damage in limbic and extra-limbic brain areas: in particular, hippocampal damage and was invariably remarkable [5]. SRSs began to occur 11 \pm 1 days after SE.

Three days after SE, i.e. during latency, these lesioned animals were randomly assigned to 3 groups: one group was injected in one hippocampus with the vector expressing FGF-2 and BDNF (TH-FGF2/0-BDNF), another was injected with a control vector and the third was not treated at all (no difference was observed between these latter 2 groups in the parameters examined in this and in previous [5] studies and, therefore, they have been pooled together for statistical analysis). TH-FGF2/0-BDNF provides a short-term (about a week) increase in FGF-2 expression accompanied and followed by a slightly longer-lasting (at least 11 days) increase in BDNF expression, in the absence of significant toxicity [5]. As expected on the basis of the ability of HSV to enter nerve terminals and to be retrogradely transported, reaching connected areas, transgene expression was bilateral, even if more robust at the site of injection for the first few days [5].

Animals were killed 7, 14 or 28 days after SE (i.e. 4, 11 or 25 days after vector inoculation) to examine possible effects on neuroinflammation. Continuous video-EEG monitoring for the occurrence, severity and duration of seizures was performed starting 7 days after SE. As previously reported [5], vector-injected rats exhibited

Table 1 EEG and behavioral analysis

	seizures/day	seizure score	seizure duration (s)
pilo - control vector	3.9 \pm 0.6	2.4 \pm 0.2	55 \pm 15
pilo - FGF2-BDNF	1.2 \pm 0.3**	1.5 \pm 0.3*	63 \pm 16

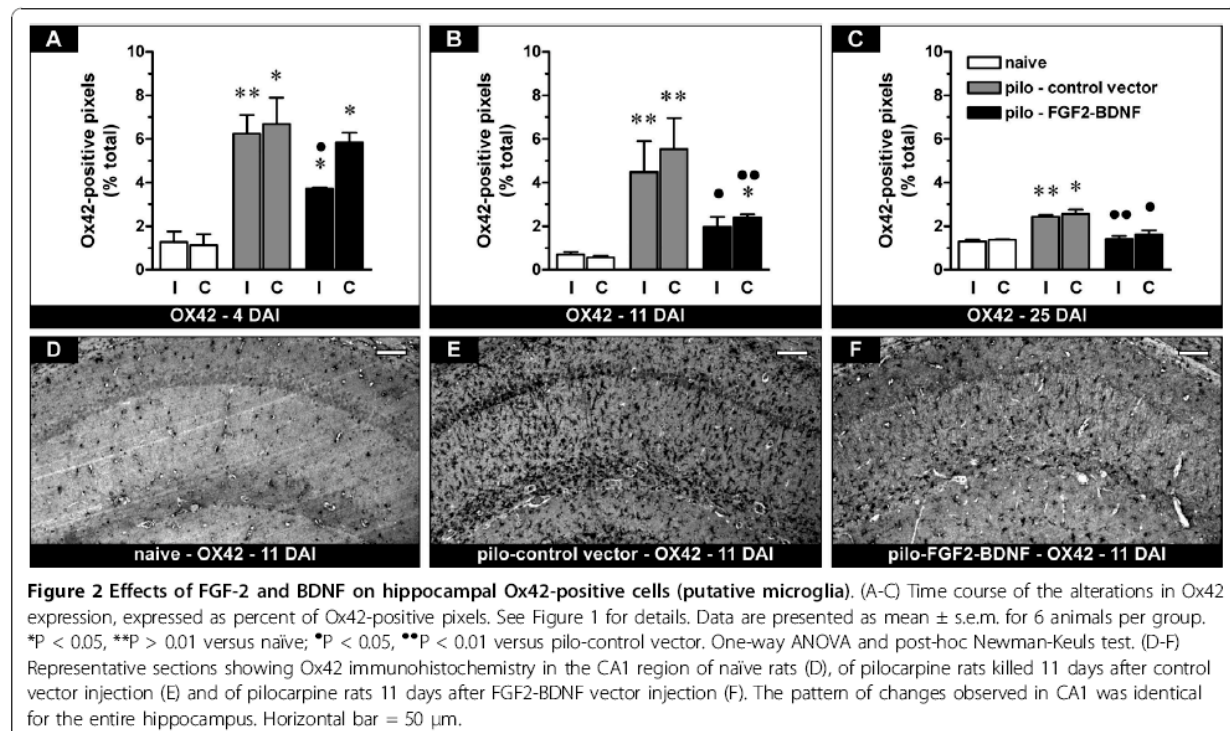
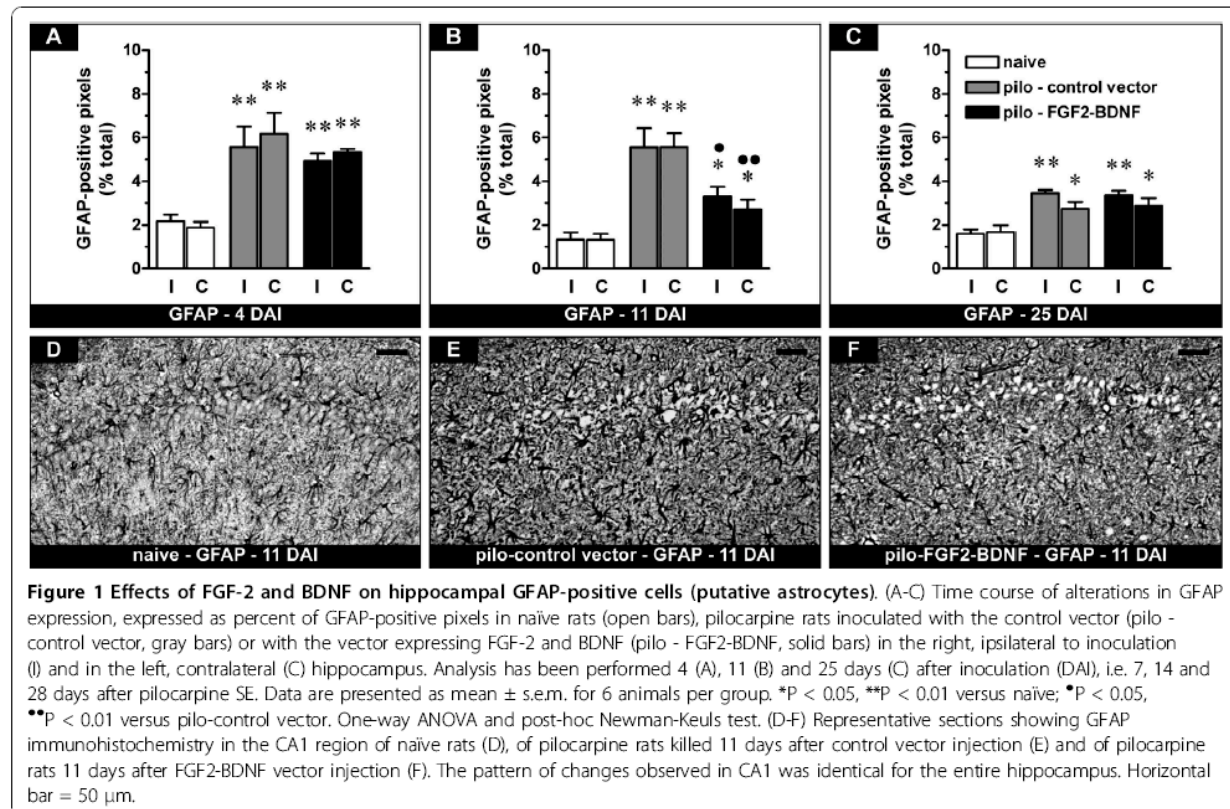
Average frequency, severity and duration of spontaneous seizures in the chronic period (14 -28 days after pilocarpine-induced SE, vectors injected 4 days after SE). Data are the means \pm s.e.m. for 6 animals per group. **P < 0.01 vs. pilo-control vector; Student's t test for unpaired data. *P < 0.05 vs. pilo-control vector; Mann-Whitney U test.

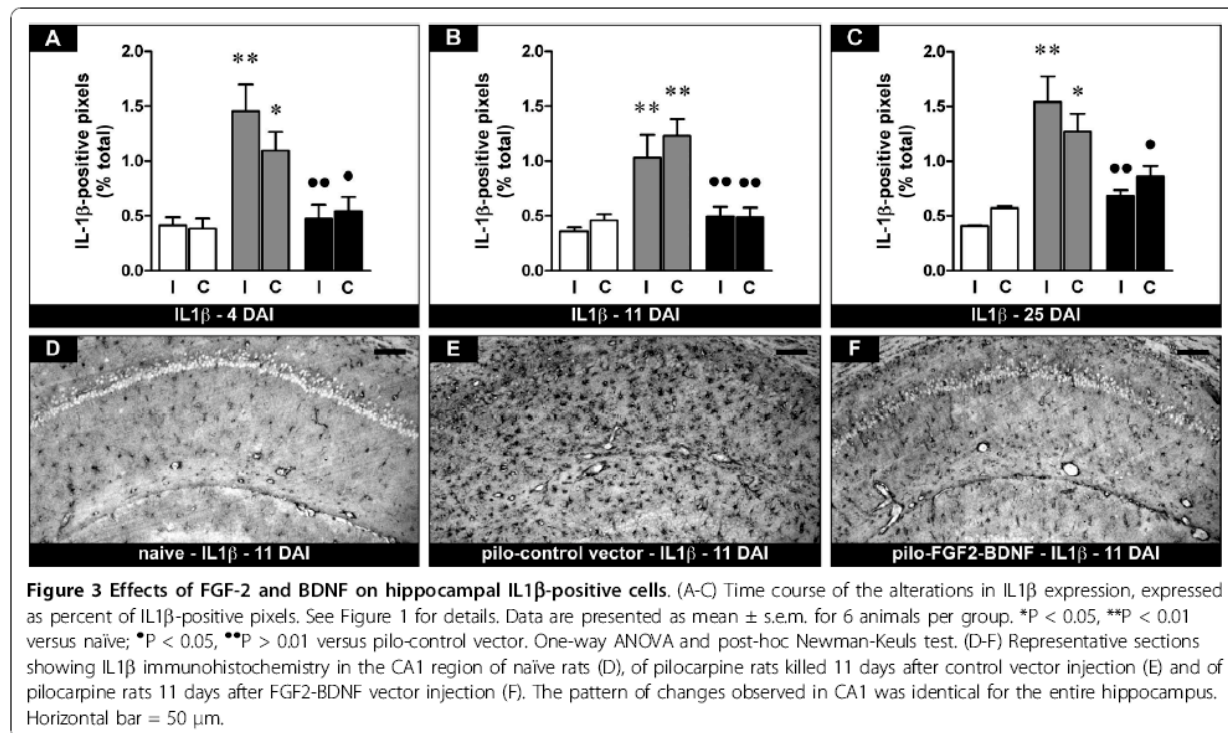
significantly less spontaneous seizures/day compared to untreated or control vector-injected animals (Table 1). These seizures were also significantly less severe in vector-treated rats although, on average, they had the same duration in both groups (Table 1).

The density of GFAP-positive cells in the hippocampus was significantly increased in pilocarpine-treated animals compared with naïve controls, an indication of reactive astrocytosis. In agreement with previous studies [18], this phenomenon was maximal during latency (7 days after SE) and then gradually decreased with time (Figure 1). In pilocarpine animals treated with TH-FGF2/0-BDNF, the density of GFAP-positive cells initially increased to about the same level as in those treated with the control vector (Figure 1A), but then decreased more rapidly, becoming significantly lower 14 days after SE (i.e. 11 days after vector inoculation; Figure 1B and 1E-F). However, this did not lead to a complete normalization of astrocytosis, but only to an acceleration of the process: 28 days after SE (25 days after vector inoculation) the expression of GFAP was identical in the two groups (Figure 1C).

We then measured the density of microglial cells using Ox42 immunohistochemistry. Similar to GFAP-positive cells, the density of Ox42-positive cells in the hippocampus was significantly increased in pilocarpine-treated animals compared with naïve controls, an indication of reactive microgliosis, and this phenomenon was maximal during latency (7 days after SE), then gradually decreased in time (Figure 2). In pilocarpine animals treated with TH-FGF2/0-BDNF, the density of Ox42-positive cells was initially reduced only in the injected hippocampus (Figure 2A), then bilaterally (Figure 2B and 2E-F), paralleling high level transgene expression [5]. Twenty-eight days after SE it was at baseline levels (Figure 2C).

In agreement with previous reports [19], IL-1 β expression dramatically increased in pilocarpine-treated animals compared with naïve controls, in a long-lasting manner (Figure 3). In pilocarpine animals treated with the vector expressing FGF-2 and BDNF, this increase in IL-1 β expression was prevented (Figure 3). The observation of a bilateral effect even at the earliest time-point,





when contralateral transgene expression is still relatively low [5], indicates that low-level FGF-2 and BDNF supplementation may be sufficient to prevent IL-1 β expression. Compared with pilocarpine animals, no change in any of the investigated parameters was observed in TH-FGF2/0-BDNF-treated animals in areas where transgene expression did not occur, such as neocortex (data not shown).

This study suggests that NTFs may affect neuroinflammation *in vivo*: the local, viral vector-mediated, supplementation of FGF-2 and BDNF in a lesioned, epileptogenic hippocampus attenuates various parameters of inflammation, including astrogliosis, microcytosis and IL-1 β expression. The effect appears to be most prominent on IL-1 β , whose expression is almost completely prevented, even at earliest time-point and for relatively low-level transgene expression.

Neuroinflammation appears to be highly implicated in epileptogenesis. Leukocyte-endothelium interaction may result in leukocyte extravasation and BBB permeabilization, favoring the induction of spontaneous seizures [10,13,20,21]. Integrin activation and BBB leakage are thought to exert a proepileptic role by modulating the release of cytokines and chemokines, which are known to modulate intercellular signaling within the CNS [22-25]. Very little is known regarding the possible modulation of these phenomena by NTFs. However, BDNF is upregulated in rodents after inflammatory challenges

[26]. Also, BDNF and its receptor *trkB* are expressed in macrophages [27] and play autocrine and paracrine roles in the modulation of regeneration and angiogenesis following nerve injury [28]. Moreover, recent data suggest that pro-BDNF (the BDNF precursor) is a suppressing factor for macrophage migration and infiltration and may play a detrimental role after spinal cord injury [29].

CNS inflammation is associated with BBB breakdown, and BBB leakage has been implicated both in the induction of seizures and in the progression to epilepsy [6-9]. In addition, BBB opening leads by itself to epileptiform activity, mediated by exposure of astrocytes and neuronal cells to blood albumin and potassium ions, respectively [6,7]. In support of the results obtained from animal models, it has been shown that BBB disruption by intra-arterial injection of mannitol in human patients suffering from cerebral lymphoma induces focal motor seizures [9]. NTFs may be involved in preserving BBB integrity: intracerebroventricular administration of BDNF has been reported to significantly reduce BBB breakdown, brain edema formation, and cell/tissue injury [30]; moreover, FGF-2 also plays an important role in the regulation of BBB permeability *in vivo* [31].

BBB leakage, however, peaks about 4 days after pilocarpine-induced SE [32], while significant FGF-2 and BDNF overexpression is achieved only a few days after vector injection, i.e. about a week after SE [5,33]. Thus,

effects on BBB may only partially explain the observed reduction in neuroinflammatory signs. One alternative hypothesis is that FGF-2 and BDNF primarily reduce IL-1 β expression, thereby attenuating astrocytosis and microgliosis [34,35]. This hypothesis is in line with the temporal sequence of the effects, wherein IL-1 β effects appear earlier than those of GFAP and Ox42. Remarkably, the effect on IL-1 β is observed before the occurrence of SRSs and, thus, cannot be secondary to their reduction.

In conclusion, the present study provides first evidence that NTF overexpression might modulate the brain inflammatory response that follows pilocarpine-induced SE. This effect may underlie the observed reduction in SRS frequency and, therefore, may represent an interesting therapeutic approach. Its molecular mechanism(s) will be explored in the future. Nonetheless, the concept that NTFs affect neuroinflammation *in vivo* may be highly relevant for the understanding of the epileptogenic process.

Additional material

Additional file 1: Additional Material (Bovolenta et al.: Hippocampal FGF-2 and BDNF Overexpression Attenuates Epileptogenesis-Associated Neuroinflammation and Reduces Spontaneous Recurrent Seizures)

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Authors' contributions

PFF and MS conceived and designed the study; RB, SZ, BP, DR, FM, GNM, FO, EB, PM and AM performed and analyzed the experiments. RB, PFF and MS drafted the manuscript. All authors have read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Additional Material

(Bovolenta et al.: *Hippocampal FGF-2 and BDNF Overexpression Attenuates Epileptogenesis-Associated Neuroinflammation and Reduces Spontaneous Recurrent Seizures*)

Methods**Vectors**

Vectors were prepared as previously described [1,2]. A plasmid (pB410-BDNF) was constructed by introduction of the rat *bdnf* cDNA (1127 pb) [3] from pBluscript-BDNF into the HSV flank sequences of the previously described pB41 plasmid [4]. The *bdnf* cDNA was inserted, under the transcriptional control of the HSV-1 IE ICP0 promoter, into the *Xba*I sites of the pB41 plasmid, between the two UL41 HSV fragments [HSV genomic positions 90.145–91.631 and 92.230–93.858 [4]. pB410-BDNF was then recombined within the genome of the T0-LacZ viral vector using the previously described Pac-facilitated LacZ substitution method [4]. T0-LacZ is a replication-defective HSV-1 viral vector with the backbone of TH-LacZ (deletion of the three IE ICP4, ICP27, and ICP22; [5]), with the cDNA encoding LacZ inserted in the UL41 locus. The production of recombinant viruses was carried out using the standard calcium phosphate transfection procedure with 5 µg of viral DNA and 1 µg of linear pB410-BDNF. Transfection and isolation of the recombinant virus was performed in 7b cells, as previously described [4]. The recombinant virus T0-BDNF, containing the *bdnf* cDNA in the UL41 locus, was identified by isolation of a clear plaque phenotype after X-gal staining. This virus was purified by three rounds of limiting dilution and the presence of the transgene was verified by Southern blot analysis. Viral stocks of T0-BDNF were prepared and titrated using 7b cells.

The vector TH-FGF2/0-BDNF, containing FGF-2 in the *tk* (thymidine kinase) locus and BDNF in the UL41 locus, was created by genetically crossing the vectors TH-FGF2 [5] and T0-BDNF. 7b cells, plated in 60-mm Petri dishes, were infected with TH-FGF2 and T0-BDNF at a

MOI of 3.0 and harvested 18 hours postinfection. The mixture of viruses derived from the co-infection was titrated, and the viral vector containing both genes was isolated by Southern blot screening. The TH-FGF2/0-BDNF virus was purified by three rounds of limiting dilution and expression of the transgenes confirmed by Western blot analysis. Purity of the vector was tested by verifying the absence of contaminating FGF-2 and BDNF proteins in each viral stock.

Animals

Male Sprague-Dawley rats (240–260 g; Harlan Italy) were used for all experiments. Animals were housed under standard conditions: constant temperature (22–24°C) and humidity (55–65%), 12 h dark-light cycle, free access to food and water. All efforts were made to minimize animal suffering. All procedures were carried out in accordance with guidelines by the European Community and national laws and policies (authorization from the Italian Ministry of Health n. 83/2009-B).

Pilocarpine was administered i.p. (300 mg/kg) 30 min after methyl-scopolamine (1 mg/kg s.c.), to minimize peripheral cholinergic effect. 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 hours after onset by administration of diazepam (10 mg/kg i.p.).

Three days after pilocarpine SE, under ketamine (87 mg/kg i.p.) and xylazine (13 mg/kg i.p.) anesthesia, a borosilicate glass needle connected to a perfusion pump was implanted in the right dorsal hippocampus (coordinates: 1.5 mm lateral and 1.7 mm posterior to bregma, 3.0 mm deep from dura). A total of 1.6×10^6 pfu of vector were injected in a volume of 2 μ l at a flow rate of 0.1 μ l/min [2]. Trypan blue (at the nontoxic concentration of 0.01%) was added to the vector solution to allow precise identification of the injection site [6]. Animals were sacrificed 4, 11, or 25 days after vector injection. Controls were control vector-injected or naïve rats.

Immunohistochemistry

Rats were sacrificed by decapitation after an anesthetic overdose. Their brains were rapidly removed, immersed in 10% formalin and then paraffin embedded. Successive 6 μm sections were cut across the entire dorsal hippocampus (233 sections, corresponding to approximately 1.4 mm; plates 39-46 [7]) and mounted onto polarized slides (Superfrost slides, Diapath). One every 58 of these sections (5 sections per animal) was stained for the markers below. Sections were dewaxed, rehydrated and unmasked using a commercially available kit (Unmasker, Diapath), according to the manufacturer's instructions. For activated microglia and astrocytes, we employed the Dako Cytomation EnVision[®] + Dual Link System-HRP (DAB+) kit. After washing in PBS 1 \times , sections were incubated for 10 min, at room temperature, with Endogenous Enzyme Block to quench endogenous peroxidase activity. Subsequently, they were incubated with the primary antibody (rabbit polyclonal anti CD11 b/c, clone Ox42, 1:200 dilution, Novus Biologicals; or rabbit polyclonal anti-GFAP, 1:200, Sigma) at room temperature. After 30 minutes, slices were rinsed twice with PBS 1 \times and incubated for another 30 min with Labeled Polymer-HRP [Dako Cytomation EnVision[®] + Dual Link System-HRP (DAB+)]. Staining was completed by a 3 min incubation with 3,3'-diaminobenzidine (DAB) substrated-chromogen, resulting in a brown staining of the antigen-antibody complex. Finally, sections were mounted using a water-based mounting medium (Shur Mount[™], TBS).

For IL1 β , sections were unmasked as described above and, after incubation in H₂O₂ 0.3% for 15 min at room temperature, rapidly rinsed in distilled water and washed again in PBS. They were then incubated for 10 min with Ultra V Block (Ultra Vision Detection System; Lab Vision Corporation) at room temperature, to block nonspecific background. After overnight incubation at 4°C, in humid atmosphere, with the primary antibody (goat polyclonal anti-IL1 β , 1:200; Santa Cruz Biotechnology, Inc.), sections were rinsed in PBS 1 \times and incubated at room temperature for 2 h with HRPO Swine anti-goat IgG (H+L) human/mouse adsorbed (Cedarlane Laboratories). The reaction product was detected using DAB (ImmPACT DAB, Vector Laboratories, Inc). Finally,

sections were washed in PBS and mounted using Shur Mount™ (TBS). The specificity of immunolabeling was verified in all experiments by controls in which the primary antibody was omitted.

Image analysis was conducted using a Leica microscope (DMRA2, Leica). The expression levels of GFAP, Ox42 and IL1 β were measured using a thresholding approach [8] by investigators that were blind for the group to which the rats belonged. Images of the hippocampus were captured using a Leica DFC300FX camera and transformed into gray levels. Using Photoshop CS2 (version 9.0.2), the mean \pm standard deviation gray level was calculated in visually identified positive cells. The hippocampus was then cut out, and positive pixels identified by thresholding at the gray level corresponding to the mean plus two standard deviations. Using this approach, only those pixels that were significantly above background were selected. Data have been expressed as percent of positive pixels over total pixels in the selected area. This method has been validated by comparing data with those obtained counting GFAP-positive cells. As stated above, 4 regularly spaced sections have been examined for each animal. The mean percent of positive pixels was calculated in these 4 sections and used for statistical analysis. Statistical analysis was conducted using one-way ANOVA and post-hoc the Newman-Keuls test.

Telemetry EEG and behavioral analysis

Together with vector injection (3 days after SE), animals were implanted i.p. with a TA11CTA-F40 telemetry transmitter (Data Science International, DSI, USA) with subcutaneous electrodes leading to the sub-dura mater above the parietal cortex. Silicon coating of all the leads was peeled back to expose approximately 5 mm of the helical steel lead. The tip of negative and positive leads created an angle of approximately 90 degrees. These radiotelemetry devices continuously sense, process and transmit information from the animal to a data storing system. Seizures were assessed by video-monitoring of the animals, performed by means of Phenotyper cages (Noldus Information

Technology, the Netherlands) and an acquisition system using telemetric technology (Dataquest® A.R.T. Data Acquisition 4.3 for telemetry systems, DSI). Behavioral analysis related to homecage exploration were automatically detected and calculated by the Ethovision XT system (Noldus), whereas behavioral alteration and convulsion scorings were performed by the means of the Observer (Noldus), a semi-automatized system for behavioral recording. The MPEG4 Encoder and The Observer XT, which connected directly with the cages, were the systems used to acquire video data. The telemetric and video system synchronized data were acquired simultaneously by a time code signal (corresponding to The Observer PC clock), which was sent continuously during acquisition from The Observer PC to the Dataquest ART PC. This time code was then recognized by the Observer software, allowing the synchronization of the videos with the physiological telemetric data. We recorded 24 h/day for 21 consecutive days, beginning 7 days after SE.

Seizures have been defined here as previously described [9,10]. The term “seizure” was used to indicate any electrically recorded seizure (EEG seizure), both non-convulsive or convulsive. Seizures were categorized as paroxysmal activity of high frequency (>5 Hz) lasting for more than 20 s and characterized by a 3-fold amplitude increment over baseline. Seizure severity was scored using the scale of Racine [11]: 1, chewing or mouth and facial movements; 2, head nodding; 3, forelimb clonus; 4, generalized seizure with rearing; 5, generalized seizure with rearing and falling. The behaviors during non-convulsive seizures were those of class 1 or 2. Seizure detection was performed both visually and by the means of the Observer XT (Noldus), a semi-automatized system for behavioral recording. All EEG recordings were examined for artifacts, and all seizures were confirmed by visual inspection. Seizure detection and single ictal events (<2 sec) were scored for each individual rat for the whole analysis period. Analysis was performed by two independent investigators that were blind for the group to which the rats belonged. In case of differential evaluation, data were reviewed together to reach a consensus.

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5. Influence of NTFs on mossy fibres sprouting

(see the published work: **Localized overexpression of FGF-2 and BDNF in hippocampus reduces mossy fiber sprouting and spontaneous seizures up to four weeks after pilocarpine-induced status epilepticus.** Beatrice Paradiso, Silvia Zucchini, Tao Su, Roberta Bovolenta, Elena Berto, Peggy Marconi, Andrea Marzola, Graciela Navarro Mora, Paolo F. Fabene, and Michele Simonato. *Epilepsia* 2011, ahead of print)

Mossy fibres sprouting is the form of axonal plasticity most widely studied in human and experimental epilepsies. It refers to the synaptic reorganization of axons of the glutamatergic granule cells, so that they project into the inner molecular layer of the dentate gyrus and make contact with granule cell dendrites, resulting in a recurrent excitatory circuit which may lead to seizures (Sutula et al., 1989; Represa., 1993).

Molecular profiling data reveal that MFS may result from seizures triggering a cascade of gene expression including immediate early genes (e.g. c-fos and c-jun), genes coding neurotrophic factors and proteins involved in rearrangement of extracellular matrix, such as the axonal growth-associated proteins GAP-43 (Elliott et al., 2003; Bendotti et al., 1993).

Because MFS is typically seen in sclerotic hippocampi with extensive neuronal loss, it may correlate with the death of susceptible neurons normally innervated by the mossy fibres. In this way, axons of the surviving neurons fill up the vacated synapses (Houser et al., 1990 a; Jiao et al., 2007). Sprouting has been found to affect also the axons of pyramidal cells in the CA₁ subfield (Cavazos et al.; 2004). Experimental studies indicate that MFS occurs before spontaneous seizures and is maintained for the lifetime of the epileptic animal (Nissinen et al., 2001). Interestingly, these newly generated synapses act, in part, via kainate receptors, unlike naïve synapses that, instead, operate via AMPA receptors. Therefore, seizures themselves may kill the susceptible neurons and set up a vicious cycle where seizures cause neuronal death, which leads to MFS, which in turn leads to more seizures (Epsztein et al., 2005; Ben-Ari et al., 2008).

Several experimental data demonstrate that mossy fibres make contact also with GABAergic neurons. Two forms of synaptic reorganization, involving inhibitory neurons, have been proposed. First, sprouted collaterals of the granule cells have been hypothesized to reinnervate dormant basket cells, restoring normal inhibitory responses, instead of setting up an excitatory circuit (Sloviter, 1992). The second hypothesis derives from the observation that mossy fibres also form synapses with GABAergic interneurons, raising the possibility that MFS may promote inhibition of granule cells through a disynaptic pathway (Ribak and Peterson, 1991). For these and other reasons, the functional role of mossy fibres sprouting in epileptogenesis and ictogenesis remains controversial.

However, sprouting affecting inhibitory cells fails to preserve the brain from hyperexcitability, rather, the opposite typically occurs leading to spontaneous recurrent seizures. Still, the theory proposed by Sloviter on the reactivation of inhibitory circuits might explain the absence of seizures during the initial (latent) phase of epileptogenesis. At later stages, however, progressive degeneration in the IML might create contacts with granule cells that, in addition to selective loss of specific interneurons, may constitute a substrate for recurrent excitation. In this context, the connections with inhibitory interneurons, that are still observed (Buckmaster and Dudek, 1997; Wenzel et al., 2000), may provide compensatory processes counteracting epileptogenic mechanisms.

Moreover, as previously mentioned, the persistent hilar basal dendrites of newly born neurons also receive synaptic input from mossy fibres (Ribak et al. 2000), thus contributing to the formation of a hyperexcitatory network.

Regarding the influence of NTFs on MFS, the interesting work of Rao and collaborators (2006) reports that, after SE, attraction of mossy fibres that were directed to the IML back to the CA₃ region, using CA₃ fetal cells enriched with BDNF and FGF-2, reduces behavioural seizure frequency for several months.

These evidences are in line with our results.

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FULL-LENGTH ORIGINAL RESEARCH

Localized overexpression of FGF-2 and BDNF in hippocampus reduces mossy fiber sprouting and spontaneous seizures up to 4 weeks after pilocarpine-induced status epilepticus

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SUMMARY

Purpose: We have recently reported that viral vector-mediated supplementation of fibroblast growth factor-2 (FGF-2) and brain-derived neurotrophic factor (BDNF) in a lesioned, epileptogenic rat hippocampus limits neuronal damage, favors neurogenesis, and reduces spontaneous recurrent seizures. To test if this treatment can also prevent hippocampal circuit reorganization, we examined here its effect on mossy fiber sprouting, the best studied form of axonal plasticity in epilepsy.

Methods: A herpes-based vector expressing FGF-2 and BDNF was injected into the rat hippocampus 3 days after an epileptogenic insult (pilocarpine-induced status epilepticus). Continuous video-electroencephalography (EEG) monitoring was initiated 7 days after status epilepticus, and animals were sacrificed at 28 days for analysis of cell

loss (measured using NeuN immunofluorescence) and mossy fiber sprouting (measured using dynorphin A immunohistochemistry).

Key Findings: The vector expressing FGF-2 and BDNF decreased in parallel mossy fiber sprouting and the frequency and severity of spontaneous seizures. The effect on sprouting correlated strictly with the cell loss in the terminal fields of physiologic mossy fiber innervation (mossy cells in the dentate gyrus hilus and CA3 pyramidal neurons).

Significance: These data suggest that the supplementation of FGF-2 and BDNF in an epileptogenic hippocampus may prevent epileptogenesis by decreasing neuronal loss and mossy fiber sprouting, that is, reducing some forms of circuit reorganization.

KEY WORDS: Status epilepticus, Neurotrophic factors, Mossy fiber sprouting, Dynorphin.

Current “antiepileptic” agents may suppress seizures, but they do not prevent the transformation of a normal brain in epileptics (epileptogenesis). To design effective therapeutic approaches, the key question is which events during epileptogenesis lead to circuit reorganization, hyperexcitability, and spontaneous recurrent seizure (SRS). Identified candidates include neuronal loss in the hippocampus (hippocampal sclerosis, HS), mossy fiber sprouting (MFS, the growth of aberrant collaterals of granule cell axons into the dentate gyrus inner molecular layer), and increased neurogenesis (Pitkanen & Sutula, 2002).

Neurotrophic factors (NTFs) interfere with these events and may lead to prevention of epileptogenesis (Simonato et al., 2006): We recently reported that viral vector-mediated supplementation of fibroblast growth factor-2 (FGF-2) and brain-derived neurotrophic factor (BDNF) in a lesioned, epileptogenic rat hippocampus limits neuronal damage, favors neurogenesis, and reduces SRS (Paradiso et al., 2009). To test if this treatment can also prevent hippocampal circuit reorganization, we examined its effect on MFS.

The viral vector approach was chosen to ensure localized delivery of NTFs. Local (rather than systemic) administration of NTFs is thought to be essential for obtaining therapeutic results (Simonato et al., 2006). We employed replication-defective herpes simplex type 1 (HSV-1) vectors because (Simonato et al., 2000): (1) they efficiently infect nonreplicating cells such as neurons; (2) they can accommodate large inserts and allow transfection of multiple genes simultaneously; (3) they do not integrate

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1 their genome into the host DNA (no mutagenesis risk); (4)
 2 they can be transported retrogradely in neurons (therefore,
 3 transgene expression can occur in remote areas through the
 4 nerve terminals afferent to the injection area); and (5) they
 5 produce a transient transgene expression (it is desirable to
 6 obtain transient expression of NTFs because NTFs can trig-
 7 ger plastic changes that will remain detectable when they
 8 are no longer expressed, whereas their long-term expression
 9 may be detrimental for brain function).

11 METHODS

13 Vector construction

14 Vectors have been constructed as described in detail pre-
 15 viously (Marconi et al., 2005). First of all, based on a pub-
 16 lished method (Marconi et al., 1999), we prepared a control
 17 vector (TH-LacZ) and an FGF-2-expressing vector
 18 (TH-FGF2). TH-FGF2 contained the sequence of the rat
 19 ovarian FGF-2 cDNA (A. Baird, The Whittier Institute, La
 20 Jolla, CA, U.S.A.) placed downstream of the human cyto-
 21 megalovirus (HCMV) immediate-early (IE) promoter and
 22 5' to the polyadenylation sequence from the bovine growth
 23 hormone. TH-LacZ contained the *Escherichia coli* LacZ
 24 gene also driven by the HCMV IE promoter. All expression
 25 cassettes were inserted into the thymidine kinase (*tk*) locus
 26 of a triple-deleted recombinant HSV-1 vector lacking three
 27 different IE genes (ICP4, ICP27, which are essential for
 28 viral replication, and ICP22), using the previously described
 29 *PacI* recombination method (Marconi et al., 1999). This
 30 virus provides low cytotoxicity and a high level of transgene
 31 expression (Krisky et al., 1998; Marconi et al., 1999).

32 A plasmid (pB410-BDNF) was constructed by introduc-
 33 tion of the rat *bdnf* cDNA (1,127 pb) (Maisonpierre et al.,
 34 1991) from pBluscript-BDNF into the HSV flank sequences
 35 of the previously described pB41 plasmid (Krisky et al.,
 36 1997). The *bdnf* cDNA was inserted, under the transcrip-
 37 tional control of the HSV-1 IE ICP0 promoter, into the XbaI
 38 sites of the pB41 plasmid, between the two UL41 HSV frag-
 39 ments [HSV genomic positions 90.145–91.631 and 92.230–
 40 93.858 (Krisky et al., 1997)]. pB410-BDNF was then
 41 recombined within the genome of the T0-LacZ viral vector
 42 using the previously described Pac-facilitated LacZ substi-
 43 tution method (Krisky et al., 1997). T0-LacZ is a replica-
 44 tion-defective HSV-1 viral vector with the backbone of
 45 TH-LacZ, with the cDNA encoding LacZ inserted in the
 46 UL41 locus.

47 The production of recombinant viruses was carried out
 48 using a standard calcium phosphate transfection procedure
 49 with 5 μ g of viral DNA and 1 μ g of linear pB410-BDNF.
 50 Transfection and isolation of the recombinant virus was per-
 51 formed in 7b cells, as described previously (Marconi et al.,
 52 1996; Krisky et al., 1997). The recombinant virus
 53 T0-BDNF, containing the *bdnf* cDNA, was identified by
 54 isolation of clear plaque phenotype after X-gal staining.
 55 This virus was purified by three rounds of limiting dilution

and the presence of the transgene was verified by Southern
 blot analysis. Viral stocks of T0-BDNF were prepared and
 titrated using 7b cells (Marconi et al., 1996).

Finally, the vector TH-FGF2/0-BDNF, containing FGF-2
 in the *tk* locus and BDNF in the UL41 locus, was created by
 genetically crossing the vectors TH-FGF2 and T0-BDNF.
 7b cells, plated in 60-mm Petri dishes, were infected with
 TH-FGF2 and T0-BDNF at a multiplicity of infection
 (MOI) of 3.0 and harvested 18 h postinfection. The mixture
 of viruses derived from the coinfection was titrated, and the
 viral vector containing both genes was isolated by Southern
 blot screening. The TH-FGF2/0-BDNF virus was purified
 by three rounds of limiting dilution and expression of the
 transgenes confirmed by western blot analysis.

We have previously shown that, when injected in one dor-
 sal hippocampus, this vector is retrogradely transported to
 connected regions (in particular to the contralateral hippo-
 campus), providing a bilateral, short-term (about a week)
 increase in FGF-2 expression accompanied and followed by
 a bilateral, slightly longer-lasting (at least 11 days) increase
 in BDNF expression, in the absence of significant toxicity
 (Paradiso et al., 2009). Purity of the vector was tested by
 verifying the absence of contaminating FGF-2 and BDNF
 proteins in each viral stock.

Animals

Male Sprague-Dawley rats (240–260 g; Harlan Italy) **3**
 were used for all experiments. Animals were housed under
 standard conditions: constant temperature (22–24°C) and
 humidity (55–65%), 12 h dark–light cycle, and free access
 to food and water. All efforts were made to minimize animal
 suffering. Procedures involving animals and their care were
 carried out in accordance with European Community and
 national laws and policies (authorization from the Italian
 Ministry for Health, 83/2009-B).

Pilocarpine was administered (300 mg/kg, i.p.) 30 min
 after methyl-scopolamine (1 mg/kg, s.c.), to minimize
 peripheral cholinergic effect. Within the first hour after pilo-
 carpine injection, all animals developed seizures evolving
 into recurrent generalized convulsions (status epilepticus,
 SE). SE was interrupted 2 h after onset by administration of
 diazepam (10 mg/kg, i.p.).

Vectors [control (TH-LacZ) or double mutant
 (TH-FGF2/0-BDNF)] were injected 3 days after pilocarpine.
 Under ketamine (87 mg/kg, i.p.) and xylazine (13 mg/kg,
 i.p.) anesthesia, a borosilicate glass needle connected to a
 perfusion pump was implanted in the right dorsal hippocam-
 pus (coordinates: 1.5 mm lateral and 1.7 mm posterior to
 bregma, 3.0 mm deep from dura). A total of 1.6×10^6 pla-
 que-forming units (pfu) of vector were injected in a volume
 of 2 μ l at a flow rate of 0.1 μ l/min. Trypan blue (at the non-
 toxic concentration of 0.01%) was added to the vector solu-
 tion to allow precise identification of the injection site in
 the subsequent histologic analysis (Parent et al., 2002).
 Animals were killed 25 days after vector injection (i.e.,

28 days after pilocarpine SE). At this time point, transgene expression was undetectable (see preceding text) and all untreated animals were experiencing SRSs (see Results). Furthermore, a robust MFS is expected to be observable 28 days after pilocarpine (Mello et al., 1993).

Telemetry EEG and behavioral analysis

Together with vector injection (3 days after SE), animals were implanted intraperitoneally with a TA11CTA-F40 telemetry transmitter (Data Science International, U.S.A.) with subcutaneous electrodes leading to the sub-dura mater above the parietal cortex. Seizures were assessed by video-monitoring of the animals, performed by means of Phenotyper cages (Noldus Information Technology, The Netherlands) and an acquisition system using telemetric technology (Dataquest[®] A.R.T. Data Acquisition 4.3 for telemetry systems; Data Science). Behavioral analysis related to home cage exploration was automatically detected and calculated by the Ethovision XT system (Noldus). We recorded 24 h/day for 21 consecutive days, beginning 7 days after SE.

Seizures have been defined here as in Williams et al. (2009). The term "seizure" was used to indicate any electrically recorded seizure (EEG seizure), both nonconvulsive and convulsive. Seizures were categorized as paroxysmal activity of high frequency (>5 Hz) lasting for >20 s and characterized by a threefold amplitude increment over baseline. Seizure severity was scored using the scale of Racine (1972): 1, chewing or mouth and facial movements; 2, head nodding; 3, forelimb clonus; 4, generalized seizure with rearing; 5, generalized seizure with rearing and falling. The behaviors during nonconvulsive seizures were those of class 1 or 2. Seizure detection was performed both visually and by the means of the Observer XT (Noldus), a semi-automatized system for behavioral recording. All EEG recordings were examined for artifacts, and all seizures were confirmed by visual inspection. Seizure detection and single ictal events (<2 s) were scored for each individual rat for the entire analysis period. Analysis was performed by two independent investigators who were blinded to the group to which the rats belonged. In case of differential evaluation, data were reviewed together to reach a consensus.

Dynorphin-A 1–17 immunohistochemistry

Rats were killed by decapitation after an anesthetic overdose, following a 3 h period during which no seizure was observed. Their brains were rapidly removed, immersed in 10% formalin, and then paraffin-embedded. Successive coronal sections (6 μ m thick) were cut across the entire dorsal hippocampus (232 sections) and mounted onto polarized slides (Superfrost slides; Diapath, Italy). One of every 58 of these sections (four per animal) was immunostained for dynorphin.

Sections were dewaxed (two washes in xylol for 10 min, 5 min in ethanol 100%, 5 min in ethanol 95%) and then

rehydrated in distilled water for 5 min and in phosphate-buffered saline (PBS) 1 \times for 10 min. Antigens were unmasked using a commercially available kit (Unmasker Diapath), according to the manufacturer's instructions. After an incubation in H₂O₂ 0.3% for 15 min at room temperature, sections were rapidly rinsed in distilled water, washed in PBS, and incubated with Ultra V Block (Ultra Vision Detection System; Lab Vision Corporation, U.S.A.) for 5 min at room temperature to block nonspecific background staining, and then overnight at 4°C in humid atmosphere with the primary antibody for dynorphin (anti-dynorphin A 1–17, rabbit polyclonal; 1:25 dilution; U.S. Biological, U.S.A.). Incubation with biotinylated goat anti-polyvalent serum and with streptavidin peroxidase was conducted as described earlier. The reaction product was detected as a brown substrate using 3,3'-diamino-benzidinetetrahydrochloride (DAB). Finally, sections were washed three times in PBS 1 \times (5 min each), counterstained with hematoxylin for 30 s, and washed again in water. Coverslips were mounted using Shur/Mount[™] (TBS, U.S.A.). The specificity of immunolabeling was verified in all experiments by controls in which the primary antibody was omitted.

Images were captured using a 20 \times objective (microscope Leica DMRA2; Leica Italia) with a Leica DFC300 FX video camera, and analyzed in a blinded manner by two investigators. They were transformed into gray levels, and the average gray level was calculated in the middle and inner molecular layers (MML and IML) of the central part of the dentate gyrus upper blade. Because the border between IML and MML is difficult to identify, especially in naive animals, the molecular layer was divided in three parts of equal size (inner, middle, and outer third), and measures were made in the central part of the middle third for the MML, and in the central part of the inner third for the IML. MFS was expressed as percent of signal in the MML (gray level in IML/gray level in MML \times 100). The data obtained from the four sections analyzed per animal were averaged, obtaining a single estimate for each animal.

NeuN immunofluorescence

Sections were dewaxed, rehydrated, and unmasked as described above. After washing in PBS, they were incubated with Triton (0.3% in PBS 1 \times , room temperature 10 min), washed twice in PBS 1 \times , and incubated with 5% BSA and 5% serum of the species in which the secondary antibody was produced, for 30 min. They were then incubated with the NeuN and glial fibrillary acid protein (GFAP) primary antibodies overnight at 4°C, as follows: NeuN (mouse monoclonal, Millipore/Chemi-Con, U.S.A.) and GFAP (rabbit polyclonal; Sigma, U.S.A.) 1:25 and 1:100, respectively. After 5-min rinses in PBS, sections were incubated with Triton (as above, 30 min), washed in PBS, and incubated with the goat anti-mouse, Cy[™]2 (Cyanine)-conjugated, secondary antibody (1:50 dilution; Jackson ImmunoResearch, U.S.A.) for the mouse NeuN primary

antibody, or with a goat anti-rabbit, Texas red-conjugated, secondary antibody (1:100; Jackson ImmunoResearch) for the GFAP rabbit primary antibody, at room temperature for 3.5 h. After staining, sections were washed in PBS, counterstained with 0.0001% 4',6-diamidino-2-phenylindole (DAPI) for 15 min, and washed again. Coverslips were mounted using anti-fading Gel/Mount (Biomedica Corp., Foster City, CA, U.S.A.).

GFAP was used to ensure that NeuN-positive nuclei did not belong to astrocytes. Indeed, under the experimental conditions we employed, no NeuN-positive cell was also GFAP-positive (Fig. S1), arguing that labeling was essentially neuron-specific. The number of NeuN-positive cells was counted in of the hilus of the dentate gyrus and in the CA3c and CA3ab pyramidal layer, in sections adjacent to those employed for dynorphin immunohistochemistry. These regions are defined as in Houser et al. (1990): Hilar neurons are those situated between the granule layer and the CA3 pyramidal neurons remaining between the boundaries of the dentate gyrus (inner CA3 field or CA3c); the third region is the continuation of CA3 beyond the dentate gyrus (outer CA3 or CA3ab). Images were captured with a 20 \times objective (as above) and analyzed in a blinded manner by two investigators, using the program Leica FW 4000. As stated previously, four regularly spaced sections have been examined per each animal (progressive section numbers: 1, 59, 117, and 175). An estimate of the total NeuN-positive cells in the regions under examination was obtained by multiplying the number of NeuN-positive cells in each section by 58 and summing the four resulting counts. Shrinkage of the hippocampus, observed after pilocarpine SE, reduced the thickness but not the length of the dorsal hippocampus and thus did not alter this quantification (Paradiso et al., 2009). Data obtained from the multiple sections examined for each rat were averaged to obtain a single estimate for each animal.

RESULTS

Video-EEG

Pilocarpine (300 mg/kg, i.p.) rapidly induced a robust convulsive SE (latency: 19 ± 2 min), which was interrupted after 2 h using 10 mg/kg, i.p., diazepam. For 1–2 days after SE, the animals experienced some occasional, self-limiting generalized seizures (less than 1 min duration), and then entered a latency state in which they were apparently well (Mazzuferi et al., 2010). Three days after SE, animals were randomly assigned to three groups: one group ($n = 8$) was injected in the dorsal hippocampus with TH-FGF2/0-BDNF, another with a control vector ($n = 5$), and the third group was not treated at all ($n = 3$); no difference in any of the parameters analyzed in this study was observed between these latter two groups that, therefore, have been pooled together for statistical analysis. At this time, severe neuronal damage was invariably observed in the hippocampus

(Mazzuferi et al., 2010). This time point was chosen because it is in the latency period that precedes the occurrence of SRSs, that is, it models the situation in which therapeutic intervention may be applied in patients that underwent an epileptogenic insult to prevent epilepsy development.

Beginning 7 days after SE, animals were continuously video-EEG monitored for the occurrence, severity, and duration of seizures. The first spontaneous seizure occurred 10 ± 1 days after SE. Thereafter, animals continued to experience SRSs. In the time window between day 14 and day 28 after SE, untreated or control vector-injected animals had an average of 5.3 ± 1.2 seizures per day, lasting about 1 min each, and with a mean severity score of 2.4 ± 0.2 (Fig. 1). Compared with these animals, in keeping with previous observations (Paradiso et al., 2009), rats injected with TH-FGF2/0-BDNF exhibited significantly less frequent and less severe SRSs (Fig. 1).

Mossy fiber sprouting

Animals were killed 28 days after pilocarpine SE (i.e., at the end of the monitoring period) to examine MFS. Within this period, a robust MFS is expected to develop (Mello et al., 1993). To directly compare the degree of MFS with the loss in cells that they normally innervate, we elected to employ dynorphin immunohistochemistry rather than the more conventional Timm staining, because this alternative approach allows the use of adjacent sections from tissue blocks processed in identical manner for studying both MFS

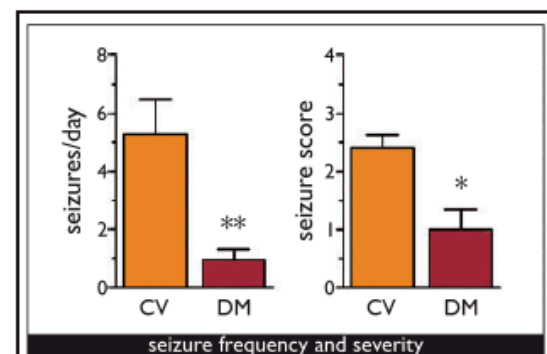


Figure 1. EEG and behavioral analysis. Average frequency (A) and severity (B) of spontaneous seizures in the chronic period (14–28 days after pilocarpine induced-SE). Data are the mean \pm standard error (SE) of eight animals per group. CV, control vector (animals untreated or injected with the control vector); DM, double mutant (animals injected with the vector expressing FGF-2 and BDNF). * $p < 0.05$ and ** $p < 0.01$ versus CV; Mann-Whitney U-test for (A), Student's t-test for unpaired data for (B).
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Attenuation of Mossy Fiber Sprouting

and cell loss. In the hippocampus, dynorphin A is found only in the mossy fibers and, thus, dynorphin-like immunoreactivity (dyn-LI) is observed in the IML only in the presence of MFS. As previously demonstrated, dyn-LI in the hippocampus faithfully overlaps Timm staining in control and epileptic human and rat specimens (Houser et al., 1990; Cavazos et al., 2003). In particular, a band of dyn-LI, which is not detectable in nonepileptic humans and naive animals, can be observed in the IML of epileptic hippocampi (Houser et al., 1990; Cavazos et al., 2003). Here, we confirmed these findings and, further, we observed a significantly lower dyn-LI in the IML of animals treated with TH-FGF2/

0-BDNF (i.e., animals with reduced SRSs) than in the untreated (or control vector-treated) group (Fig. 2A,B).

Finally, we examined if MFS correlated with cell loss in the terminal fields of physiologic mossy fiber innervation (mossy cells in the dentate gyrus hilus and CA3 pyramidal neurons). The number of neurons in these areas (measured using NeuN immunofluorescence) dramatically decreased in animals that experienced SE, but TH-FGF2/0-BDNF significantly improved the situation (Fig. 2C). Cell loss in these areas highly correlated with the degree of MFS in the IML, measured using dyn-LI (Fig. 2D). Similar results were obtained when separately correlating dyn-LI in the IML

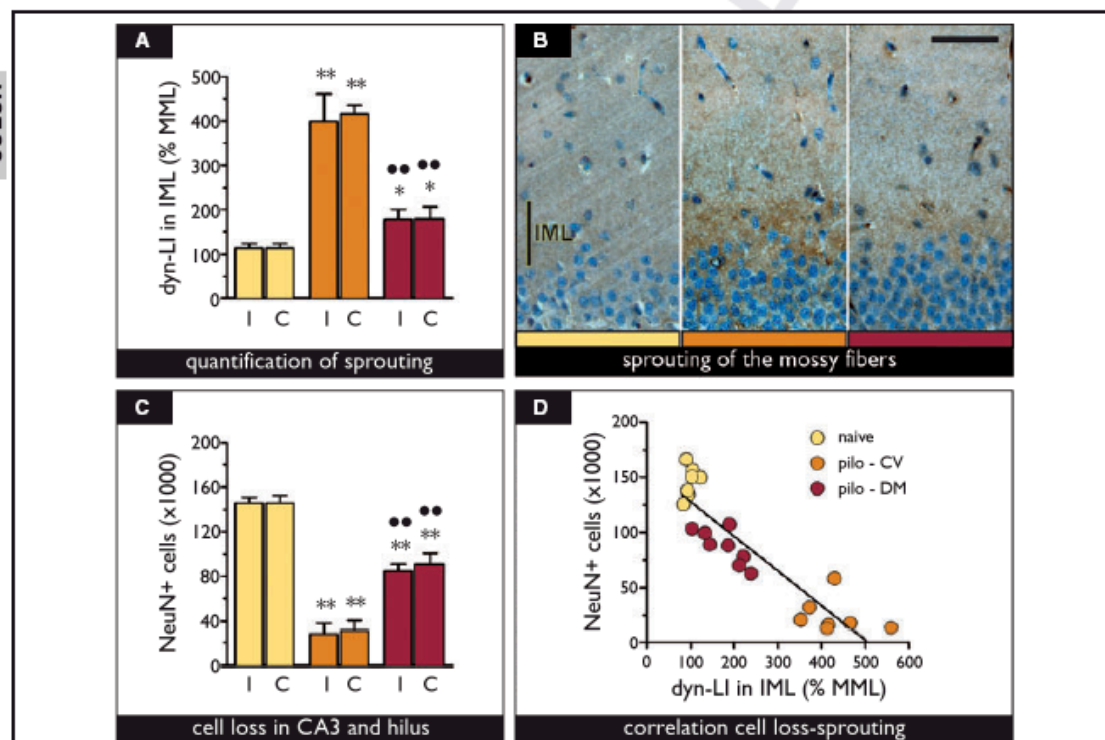
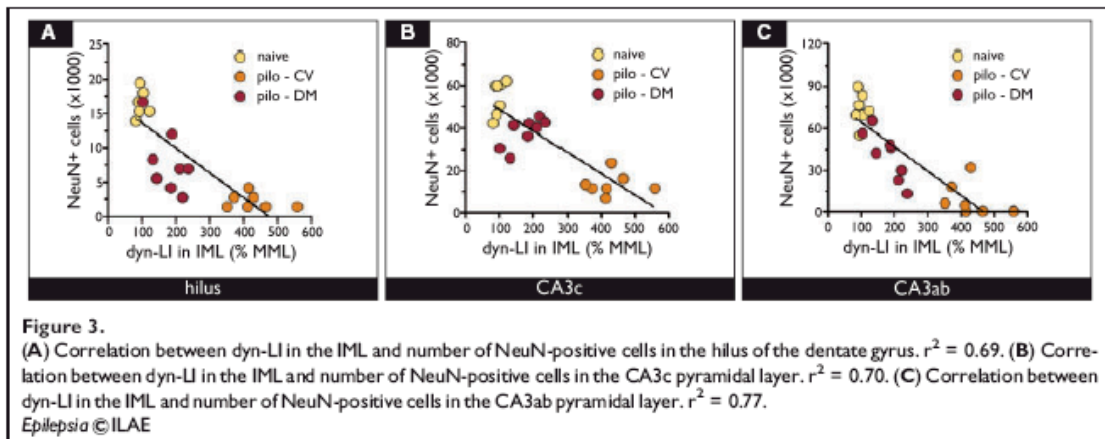


Figure 2. Effect of the vector expressing FGF-2 and BDNF on MFS. (A) Density of dyn-LI in the IML, expressed as percent of the signal in the MML. Yellow bars: naive rats; orange bars: pilocarpine rats, 25 days after inoculation of control vector; red bars: pilocarpine rats, 25 days after inoculation of the vector expressing FGF-2 and BDNF. I: injected (ipsilateral) hippocampus; C: noninjected (contralateral) hippocampus. Data are the mean \pm standard error (SE) of 7–8 animals per group. * $p < 0.05$, ** $p < 0.01$ versus naive; •• $p < 0.01$ versus pilo-control vector; ANOVA and post-hoc Newman-Keuls test. (B) Dyn-LI in the dentate gyrus molecular layer. Panels are color-labeled as in (A). Horizontal bar = 75 μ m. Note the dense labeling in the IML in the central panel, and the robust attenuation following inoculation of the double mutant (right panel). (C) NeuN-positive cells in the CA3 pyramidal layer and in the hilus of the dentate gyrus. Labeling as in (A). Data are the mean \pm SE of the same animals employed in (A). * $p < 0.05$, ** $p < 0.01$ versus naive; • $p < 0.05$; •• $p < 0.01$ versus pilo-control vector; ANOVA and post-hoc Newman-Keuls test. (D) Correlation between data in (A) and in (C). $r^2 = 0.82$.

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with cell loss in the hilus or in the CA3c and CA3ab pyramidal layers (Fig. 3).

DISCUSSION

MFS is the best-studied form of axonal plasticity in epilepsy, even if its functional outcome is still controversial. MFS has been observed in many experimental models (Cross & Cavazos, 2009; Nadler, 2009), and also in the hippocampus of surgical patients with various forms of epilepsy (Houser et al., 1990; Mathern et al., 1996). Its extent may correlate with the loss of cells normally innervated by the mossy fibers, hilar interneurons, and CA3 pyramidal neurons (Houser et al., 1990; Jiao & Nadler, 2007). It has been hypothesized that, at least in the initial phases of epileptogenesis, the prevalent target of sprouted mossy fibers may be inhibitory basket cell interneurons, which would provide a homeostatic compensatory mechanism for restoration of inhibition (Sloviter, 1992; Cavazos et al., 2003; Cross & Cavazos, 2009). At later stages of disease progression, however, terminal degeneration in the IML might lead to the creation of contacts onto dendrites and somata of granule cells, providing a substrate for recurrent excitation (Tauck & Nadler, 1985; Wuarin & Dudek, 1996; Buckmaster et al., 2002; Cavazos et al., 2003; Scharfman et al., 2003), even if some contacts with interneurons remain observable (Buckmaster & Dudek, 1997; Wenzel et al., 2000). In the epileptic hippocampus, granule cells have been found to produce basal dendrites that extend into the hilus, an additional target for recurrent mossy fiber synapses (Ribak et al., 2000). Furthermore, many granule cells born during epileptogenesis and presenting aberrant features, such as basal dendrites and ectopic migration in the hilus (Parent et al., 2007), receive innervation from other granule cells and contribute to the formation of a reverberating network (Nadler, 2009).

We have previously reported that viral vector-mediated supplementation of FGF-2 and BDNF in the hippocampus during epileptogenesis reduces damage and aberrant aspects of neurogenesis (Paradiso et al., 2009). We report here that MFS in the IML (as measured using dyn-LI) is also reduced, and that this phenomenon highly correlates with the preservation of neurons in the CA3 pyramidal layer and in the hilus, that is, of cells in the innervation territories of mossy fibers. Therefore, in line with other reports (Rao et al., 2006), preservation of damaged cells, maybe together with a more efficient (less aberrant) neurogenesis, can prevent circuit alterations and reduce occurrence of SRSs.

Based on these data, it can be suggested that the supplementation of NTFs in an epileptogenic hippocampus may prevent epileptogenesis in HS-associated epilepsies. Because experimental studies indicate that MFS is maintained for life (Nissinen et al., 2001), it can be speculated that supplementation of FGF-2 and BDNF exerts a truly antiepileptogenic, and not merely disease-modifying, effect. Further studies with longer lasting video-EEG monitoring will be needed to verify this hypothesis.

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DISCLOSURE

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. NeuN (A, green) and GFAP (B, red) immunofluorescence (dentate gyrus region) in a pilocarpine rat killed 28 days after SE. A pilocarpine rat was chosen as an example because of the intense astrocytosis, which allows better appreciation of GFAP-positive cells. (C) DAPI staining (blue). (D) Merged. Note that NeuN-positive cells are not GFAP-positive.

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CHAPTER II

1. NTFs routs of administration.

*(based on the submitted work: **By-stander effect on brain tissue of mesoangioblasts producing neurotrophins.** Tao Su, Raffaella Scardigli, Luisa Fasulo, Beatrice Paradiso, Mario Barbieri, Anna Binaschi, Roberta Bovolenta, Silvia Zucchini, Giulio Cossu, Antonino Cattaneo, and Michele Simonato)*

1.1 Generalities.

As stated above, the choice of the route of administration represents a key factor for the therapeutic application of NTFs, since inadequate methods might be responsible for the clinical failure of many candidate trophic factors. An appropriate system of supplementation should ensure a correct amount of trophic factor to the effected sites, avoiding widespread diffusion to extra-target receptors, responsible of unwanted effects. In fact, if the supplied amount is too small, it may not be sufficient to produce the required effects; if it is too large, it may interfere with neuronal plasticity and cause deleterious side effects. Moreover, high levels of neurotrophic factors may cause downregulation of the receptors, thus blocking any possible beneficial response (Frank et al., 1996; Knusel et al., 1997; Sommerfeld et al., 2000). The clinical applicability of NTFs is made difficult by their pharmacological properties, such as the relative instability with a serum half-life of minutes or less, their restricted capability to cross the BBB and their poor oral bioavailability (Poduslo and Curran, 1996; Pardridge, 2002). It is probably due to these reasons that subcutaneous and intrathecal BDNF administrations reported little clinical success (Ochs et al., 2000).

To overcome the problem to bypass the BBB, intracerebroventricular (i.c.v.) or intracranial administration of neurotrophins have been tried (Cirulli et al., 2000; Seiger et al., 1993; Eriksson Jonhagen et al., 1998), but with unsatisfactory outcomes. Other attempts have been made using NTFs analogues that penetrate the blood brain barrier, or molecules that activate Trk receptors in the absence of neurotrophins, acting via, for example, G-protein coupled receptors (GPCRs) (Skaper, 2008) and resulting in neuroprotective pathways. However, systemic application of these molecules is not yet applicable and requires further studies to investigate their pharmacological and pharmacokinetics properties.

Several other strategies of local administration have been described in preclinical studies. They include implantation of encapsulated NTFs in microspheres (Maysinger et al., 1994) or implantation of transfected or encapsulated cells secreting NTFs (Frim et al., 1994; Lindner et al., 1995). These methods are currently under study.

1.2 Viral vectors-based administration.

Works reported in the previous chapter describe an alternative route of administration of exogenous NTFs, based on harmless herpes viral-vectors, engineered to carry the genes of interest into the cells (Marconi et al., 2005; Paradiso et al., 2009).

This approach offers the advantages of bypassing the BBB, achieving high levels of NTF at the therapeutic site, reducing systemic exposure and avoiding unwanted side effects. Several reasons directed the choice to herpes viruses. First of all, they efficiently infect nonreplicating cells such as neurons, can accommodate large inserts, do not integrate their genome into the host DNA thus avoiding mutagenesis risk, 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 (Paradiso et al., 2009).

Despite these vectors are replication-defective, lacking the three immediate-early genes ICP4, ICP27 and ICP22, some degree of residual toxicity has been found for some cell types (Marconi et al., 1996). Although this does not appear to be long-lasting nor to heavily affect neurons (Krisky et al., 1998), it renders viral vectors inadequate to be injected in humans.

1.3 Stem cell-based administration.

To drive a localized delivery of NTFs in the CNS of patients, a valid alternative to viral vectors is represented by stem cells genetically modified to express the genes of interest.

As known, stem cells are unspecialized cells capable of renewing themselves through cell division, sometimes after long periods of inactivity and, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions.

In particular, the ability to self-renew renders these cells particularly apt to mediate gene therapy because it permits to reduce, if not avoid, repeated administration. Moreover, proliferating properties can be exploited to repopulate damaged tissues. Stem cells naturally produce a rich variety of bioactive molecules (i.e. growth factors, cytokines) that may facilitate the survival of the graft and favour regeneration and neuroprotection, probably even interacting and synergizing with the proteins coded by the transgenes.

Gene therapy based on stem cells-driven supplementation of NTFs has been tested in some neurodegenerative disorders by using different cell lines including neural (Lu et al., 2003), haematopoietic (Rizvanov et al., 2008), osteoblasts (Yudoh and Nishioka, 2004), fibroblasts

(Stromberg et al., 1990) and mesenchymal stem cells (Harper et al., 2009), obtaining promising results.

However, clinical application is seriously limited by the fact that this approach requires invasive procedures: stem cells must be directly implanted in the damaged tissue. Fortunately, some types of stem cells, express on their surface, adhesion molecules that confer them migratory capacity and the ability to cross the vessel wall, reaching perivascular targets. This is the case of mesoangioblasts (MABs).

Mesoangioblasts (MABs). Firstly isolated from explants of dorsal aorta or other embryonic or juvenile postnatal vessels (De Angelis et al., 1999; Cossu and Bianco, 2003), MABs involved in the development of the vasculature. These stem cells probably derive from a primitive luminal angioblast, hence the name “mesoangioblast”. 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). The regenerative properties of MABs have been confirmed in different animal models and are expected to be applied in some clinical trials (Tedesco et al., 2010). Interestingly, they express different neural genes such as GPRC5B, which is expressed in brain and spinal cord, Tm4sf2, which has been implicated in activity-dependent brain plasticity, and others. This suggests a role in the development of the nervous system even if they do not differentiate into neurons (Tagliafico et al. 2004).

But the more interesting feature of these cells is the ability to cross the vessel wall and reach perivascular targets, making possible a peripheral administration. Being this property amplified under inflammatory conditions, MABs may selectively cross the BBB and home into lesion brain areas. Moreover, mesoangioblasts *per se* express cytokines, chemokines and their receptors, supporting a key role in tissue regeneration and first inflammatory response to damage (Tagliafico et al., 2004). If engineered to express NTFs, selective recruitment in neuroinflamed sites may reduce adverse effects due to undesired accumulation in areas where they are not required.

The possibility to avoiding invasive surgery procedures certainly renders MABs good candidates for cell-based gene therapy. However, other features deserve attention: they are non-tumorigenic and stable cell lines capable of unlimited clonal expansion *in vitro* and with the capacity of longterm survival. As revealed by micro-array analysis, they express high levels of neurotrophic

factors, like VEGF B, FGF-2, FGF-7, PDGF AA and others ([Galli et al., 2005](#)) that may amplify functions of exogenous, transfected genes. Not least, they permit autologous transplants: they can be isolated from biopsic tissues and then transplanted in the same donor, reducing rejection risks.

These characteristics prompted us to investigate the possible employment of MABs as “more ethically correct” vectors for NTFs-based therapy.

By-stander effect on brain tissue of mesoangioblasts producing neurotrophins

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Running title:

Mesoangioblasts to supplement neurotrophins

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Summary

Neurotrophic factors are involved in the regulation of neuronal survival and function and, thus, may be used to treat neurological diseases associated with neuronal death. A major hurdle for their clinical application is the mode of delivery. We describe here a new strategy, based on the use of progenitor cells called mesoangioblasts (MABs). MABs can be isolated from mammalian post-natal mesoderm tissues and, because of a high adhesion-dependent migratory capacity, they can reach perivascular targets, especially in damaged areas. Whether this occurs in the lesioned brain after peripheral administration is still unknown. We generated genetically modified MABs producing the neurotrophins Nerve Growth Factor (MABs-NGF) or Brain-Derived Neurotrophic Factor (MABs-BDNF) and assessed their by-stander effects *in vitro* using PC12 cells, primary cultures and organotypic cultures of adult hippocampal slices. PC12 cells were induced to differentiation by a medium conditioned by MABs-NGF, while a MABs-BDNF-conditioned medium increased viability of cultured neurons and slices. Slices cultured with MABs-BDNF medium also better retained their morphologic characteristics and functional connections, all these effects being abolished by the TrkB kinase blocker K252a or the BDNF scavenger TrkB-IgG. Interestingly, the amount of BDNF released by MABs-BDNF produced greater effects than an identical amount of recombinant BDNF, suggesting that other neurotrophic factors produced by MABs synergize with BDNF. Thus, MABs can be an effective vehicle for neurotrophic factor delivery, promoting differentiation, survival and functionality of neurons. We also explored the capacity of MABs to localize in a lesioned brain when peripherally administered. After *in vivo* (transcardial or intranasal) injection, GFP-positive MABs reached the brain in AD11 (a model of Alzheimer's disease) but not in healthy mice. Importantly, MABs

were found near the basal forebrain cholinergic neurons, a neuronal population that is selectively vulnerable and hit in Alzheimer's disease. In summary, the MABs hold distinct advantages over other, currently evaluated, approaches for neurotrophin delivery in the CNS, including synergy of MAB-produced neurotrophic factors with the neurotrophins and homing into damaged areas of the brain. Thus, they represent a conceptually novel, promising therapeutic approach to treat neurodegenerative diseases.

Keywords: mesoangioblast; neurotrophin; neurodegeneration; stem cells.

Abbreviations: AAV = adeno-associated virus; aCSF = artificial cerebrospinal fluid; AD = Alzheimer disease; BBB = blood-brain barrier; BDNF = brain derived neurotrophic factor; BFCN = basal forebrain cholinergic neuron; DAPI = 4',6-diamidino-2-phenylindole; DIV = days in vitro; DMEM = Dulbecco's modified Eagle's medium; FBS = fetal bovine serum; FDA = fluorescein diacetate; fEPSP = field excitatory postsynaptic potential; FGF = fibroblast growth factor; GFP = green fluorescent protein; HBSS = Hank's balanced salt solution; HMGB1 = high mobility group box 1; LDH = lactate dehydrogenase; MAB = mesoangioblast; MAP = microtubule-associated protein; NF68 = neurofilament 68; NGF = nerve growth factor; NTF = neurotrophic factor; OC = organotypic cultures; PC = primary neuronal cultures; PDGF AA = platelet-derived growth factor AA; VEGF B = vascular endothelial growth factor B.

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 and of diseases associated with neuronal damage (Connor et al., 1997; Bradford et al., 1999; Capsoni et al., 2000; Simonato et al., 2006; Zuccato and Cattaneo, 2007; De Santi et al., 2009). Not surprisingly, therefore, they received considerable interest as therapeutic agents (Thoenen and Sendtner, 2002; Simonato et al., 2006; Cattaneo et al., 2008).

A particular attention has been devoted to the neurotrophins nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF). A vast literature shows that NGF and BDNF exert trophic and neuroprotective effects on neurons, but their clinical use is hindered by a short biological half-life, a poor blood-brain barrier (BBB) permeability and by side effects due to their pleiotropic systemic actions (Boado, 2008, Thoenen and Sendtner, 2002). Given these difficulties, the attention turned into the development of techniques that allow local delivery, based on gene therapy (Tuszynski, 2007; 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 promising strategy. This strategy has already been validated for several lines of stem cells, including neural (Martinez-Serrano and Bjorklund, 1997; Lu et al., 2003; Kameda et al., 2007), embryonic (Rizvanov et al., 2008; Makar et al., 2009) and mesenchymal stem cells (Harper et al., 2009; Sasaki et al., 2009): when engineered to produce NTFs, these cells were found to ameliorate experimental neurodegenerative diseases or injuries. 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 NGF or BDNF), reducing non-selective delivery and allowing high treatment efficiencies for long time periods. However, the downside of these approaches is that they require an invasive delivery route to the target location in the nervous system, a problem that severely limits their potential for human therapy.

These considerations prompt the search for alternative cell sources that display more favorable homing properties, avoiding the necessity of a direct injection into the brain. Here, we explored the feasibility of a new approach: the use of multipotent, mesodermal progenitor cells (mesoangioblasts, MABs) that constitutively produce a subset of NTFs (e.g. vascular endothelial growth factor B, VEGF B; fibroblast growth factor-2, FGF-2; FGF-7; platelet-derived growth factor AA, PDGF) and can be engineered to produce others. MABs are an affordable cellular source since they can be isolated from small biopsies of post-natal human skeletal muscle, and have already been utilized for regenerative purposes in large animal models, besides rodents. More importantly, a phase I/II cell therapy trial with donor MABs for patients affected by Duchenne Muscular Dystrophy is planned for year 2011 (Tedesco et al., 2010).

MABs have a high, adhesion-dependent, migratory capacity and the ability to cross the vessel wall and reach perivascular targets, a property not demonstrated so far for mesenchymal stem cells. Their chemotactic ability is increased under inflammatory conditions, when the adhesion-integrin system and diapedesis moving are activated (Galvez et al., 2006). When peripherally administered, therefore, MABs may selectively cross the BBB and home into lesioned brain parenchyma. These cells express several neuro-ectodermal genes, but they do not differentiate into neurons (Tagliafico et al., 2004). Thus, they may not be used for a restorative

engraftment. Instead, MABs are expected to act as a reservoir and delivery system for NTFs.

Aim of this study was to engineer MABs to produce and secrete NGF or BDNF and to examine their by-stander effects. Thus, the medium collected from MAB cultures (containing either NGF or BDNF, plus other constitutively produced NTFs) has been applied to PC12 cells, primary neuronal cultures (PC) and organotypic cultures (OC) of adult hippocampal slices. Next, we explored the capacity of peripherally administered MABs to home in a lesioned brain *in vivo*, when transcardially or intranasally injected in a rodent model of Alzheimer disease (AD; Ruberti et al., 2000).

Materials and Methods

Details on all methods employed in this study are provided as supplementary material.

Transfection of cells and neurotrophin quantification

Green fluorescent protein (GFP) expressing MABs (Minasi et al., 2002), grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS), have been stably transfected with the expression vectors ScFv-preproNGF or ScFv-preproBDNF using Lipofectamine (Invitrogen, CA, USA), according to manufacturer's instructions. Upon G418 selection, single clones of stable transfectants have been cultured with the ordinary methods, using the following culture medium (here referred to as Medium-1): 88% DMEM, 10% FBS, 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, CA, USA). The production of exogenous neurotrophins was analyzed by a “two-site sandwich” ELISA (Covaceuszach et al., 2008), performed on the medium collected from NGF- and BDNF-producing MABs.

Assessment of NGF biological activity

PC12 cells. PC12 cells (Greene and Tischler, 1976) have been cultured in RPMI (Invitrogen, CA, USA) plus 10% FBS. Upon washing in serum-free medium, 4×10^5 PC12 cells were plated in 35 mm collagen-treated Petri dishes, in the following different conditions: alone, in co-culture with the same amount of MABs-NGF, in the presence of 5-day-culture supernatant from MABs and MABs-NGF, or in the presence of recombinant NGF (50 ng/ml). PC12 cells were maintained in culture for 7 days, replacing fresh medium every 2 days. Neuronal differentiation was evaluated by the presence of neurite processes in the cultures and confirmed by β -tubulin immunofluorescence.

Assessment of BDNF biological activity

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 5×10^4 cm⁻² viable cells in 24-well plates coated with poly-L-lysine (Sigma, St Luis, MO, USA). Cells were grown in DMEM supplemented with 10% 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 μ M Ara-C at the day after plating.

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, MgSO₄ 3, NaH₂PO₄ 1.1, NaHCO₃ 26, CaCl₂ 1 and glucose 11 (all reagents from Sigma) bubbled with 95% O₂/5% CO₂. Subsequently, 300 μ m thick coronal slices were cut with a vibrotome (MA752, Campden Instruments Ltd, UK). The hippocampi were dissected in cold, oxygenated Hank's balanced salt solution (HBSS, Gibco, Invitrogen), transferred onto sterile porous membrane confetti (Millicell, Millipore, MA, USA), and cultured with their standard medium (here referred to as Medium-2) or with the MAB-conditioned media (see below). The culture medium was changed the day after preparation and then every 2 days for the course of the experiment.

Viability. Viability was assessed using the fluorescein diacetate (FDA) hydrolysis and the lactate dehydrogenase (LDH) release assay.

Immunocytochemistry. Cultured slices were fixed with 4% PFA in 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 μ m in a cryostat (CM1510, Leica, Germany). The primary antibody was mouse anti-microtubule-associated protein (MAP2abc; 1:50; Immunological Sciences, Italy), and the secondary antibody was Alexa 488 goat anti mouse IgG (1:100, Invitrogen, CA, USA). The slices were mounted onto slides after staining with 4',6-diamidino-2-phenylindole (DAPI, 1:1000). For zif7268, hippocampal neurons were incubated for 3 hr at RT with rabbit anti-zif antibody (Egr-1; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 in 10% FCS in PBS, followed by fluorescein anti-rabbit IgG antibody (Invitrogen, CA, USA).

Western blot. To estimate the density of the surviving neurons within a slice, we performed immunoblot analysis of the neuron-specific marker neurofilament 68 (anti-neurofilament 68 antibody; Sigma).

Field potential recording. Bipolar wire electrodes were used for stimulation and glass microelectrodes filled with 0.9% NaCl were used to record field excitatory postsynaptic potential (fEPSP). Signals were acquired under constant conditions, and off-line processed using the Patchmaster software (HEKA Instruments Inc., Germany).

In vivo analysis

Intranasal and intracardiac delivery of MABs. MAB intranasal delivery was performed on 6 months old AD11 mice following a procedure previously described for the intranasal delivery of neurotrophins (Capsoni et al., 2002). For intracardiac delivery, upon anaesthetization, animals received 5×10^5 MABs resuspended in 150 μ l of PBS containing 2% sodium fluorescein as systemic delivery tracer. In both procedures, control AD11 mice were treated with PBS. Animals were sacrificed at different time points (24 h, 1 week, 1 month) after cells administration and olfactory epithelium and brains were analyzed for the presence of GFP positive cells by immunohistochemistry.

Immunocytochemistry. Immunocytochemistry was performed on olfactory epithelium and brains. The primary antibody was anti-GFP (1:500; Invitrogen, CA, USA), the secondary antibody was Alexafluor-488 conjugated anti-rabbit (1:500; Invitrogen, CA, USA). Confocal analysis was carried out on a Nikon Eclipse 90i microscope (Nikon, USA).

Results

Selection and characterization of mesoangioblast cells expressing NGF and BDNF

Two DNA constructs were generated, driving the expression of murine pre-proNGF and of rat pre-proBDNF. The pre-proNGF construct contains the cDNA encoding the short form (27 kDa) of the NGF precursor (isolated from the mouse submaxillary gland), whereas the pre-proBDNF construct contains the cDNA coding for the BDNF precursor (isolated from the rat brain). These constructs are based on the scFv-express vector (Persic et al., 1997) yielding the expression vectors ScFv-preproNGF and ScFv-preproBDNF, respectively. In order to evaluate the appropriate expression and targeting to secretion of the pro-neurotrophins driven by the constructs, COS cells were transiently transfected with ScFv-preproNGF or ScFv-preproBDNF. NGF and BDNF expression and secretion in the COS medium were assessed 72 h after transfection using ELISA. The average concentration of NGF was estimated to be approximately 80 ng/ml, that of BDNF approximately 190 ng/ml (data not shown).

GFP-expressing MABs (Minasi et al., 2002) were stably transfected with either ScFv-preproNGF or ScFv-preproBDNF DNA. Clonal selection was performed with G418 and limited dilution. Among the different clones analyzed by ELISA for neurotrophin production, two were selected: clone F10, producing the highest concentration of NGF after 4 days in culture (Supplementary Fig. 1A), and clone A9, producing the highest concentration of BDNF after 15 days in culture (Supplementary Fig. 1C). These clones were then further characterized for the kinetic of NGF and BDNF secretion. For this purpose, conditioned medium was collected from F10 cultures kept at confluence ($3.5 \times 10^4/\text{cm}^2$) 24, 48, 72 and 96 h (4 days) in the case of NGF; in

the case of BDNF expressing MABs (clone A9) mitomycin-treated cells were kept for 8, 11, 14 and 15 days. Within these timeframes, the maximal concentration of NGF was reached after 4 days of culture (Supplementary Fig. 1B), and the one of BDNF after 15 days (Supplementary Fig. 1D). BDNF secretion was very low in the first week. The reason(s) for the different kinetic of NGF and BDNF secretion are presently under investigation. The estimated rate of accumulation was 36 ng/ml/day/ 10^6 cells for NGF, and 30 ng/ml/day/ 10^6 cells for BDNF, starting from the 8th day in culture.

Assessment of NGF biological activity

The biological activity of NGF was evaluated using the PC12 cell differentiation bioassay (Greene and Tischler, 1976). PC12 cells were challenged with the conditioned medium from NGF producing MABs (clone F10) kept in culture for 5 days (containing approximately 80 ng/ml NGF, based on ELISA), diluted 1:1 with growing medium. In alternative, PC12 cells were co-cultured with F10 cells in a 1:1 ratio. The medium was replaced every other day, and PC12 cell differentiation was assessed by the presence of neurite elongations and the expression of the neuronal marker β -tubulin after 7 days. As shown in Fig. 1, both F10 conditioned medium and co-culture with F10 cells induced efficient neuronal differentiation of PC12 cells similar to what observed upon the addition of recombinant NGF (50 ng/ml). Neither the conditioned medium from control, untransfected MABs, nor co-culture with these cells, induced PC12 differentiation.

Assessment of BDNF biological activity

The biological activity of BDNF was evaluated in several ways. First, we measured the nuclear accumulation of zif/268 in hippocampal primary cultures (PCs). Zif/268 (Egr1) is a zinc finger

protein acting as transcription factor, whose nuclear translocation is commonly used to test the biological activity of BDNF (Righi et al., 2000). PCs were challenged for 3 h with the conditioned medium from MABs-BDNF (A9 clone), collected after 15 days of culture and containing approximately 87 ng/ml BDNF. Immunofluorescence revealed that the nuclear accumulation of zif/268 was increased upon addition of A9 conditioned medium (Fig. 2D), indistinguishable from what observed upon addition of 50 ng/ml recombinant BDNF (rBDNF; Fig. 2B), as compared with untreated neurons (Fig. 2A) and neurons treated with medium conditioned from control MABs (Fig. 2C).

Second, we tested if continuous application of rBDNF in the medium exerted trophic effects on cell survival in low-density 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. 3A and 3B). The maximal effect was estimated to be reached at a concentration of approximately 10^{-8} M (corresponding to 270 ng/ml) and the EC50 was approximately 3×10^{-11} M (slightly less than 1 ng/ml). These data are in keeping with previous findings (Cheng and Mattson, 1994; Lindholm et al., 1996).

We then examined the neurotrophic activities of rBDNF in OCs. OCs provide an ideal *in vitro* model system to assess toxic or trophic effects since they preserve the morphological and physiological features of the hippocampal neuronal network and allow easy access and precise control of extracellular environment for a long period (Stoppini et al., 1991; Kristensen et al., 2001). In general, OCs are prepared from early postnatal animals (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) exactly for this reason: they do not survive well in standard culture media and, thus, may provide a very convincing evidence for the favorable effects of the supplementation of NTFs if they survive when cultured in the MAB medium (in this sense, OCs represent an *in vitro* model of neurodegeneration).

In the absence of rBDNF, OCs displayed degeneration aspects (like white spotted cell debris and uneven surface) after 6-8 days *in vitro* (DIV), which were clearly visible under phase contrast microscopy (see Fig. 5A). Similar to PCs, rBDNF concentration-dependently reduced these signs of degeneration. Viability was quantified using FDA fluorescence, to identify viable cells, and LDH release, to estimate the degree of cell death. As shown in Fig. 3C and 3D, BDNF concentration-dependently increased FDA fluorescence and reduced LDH release, in line with previous observations reporting that BDNF enhances the cell tolerance to serum deprivation of organotypic cultures from postnatal slices (Nakagami et al., 1997). These effects were obtained in the same range of concentrations that proved effective for PCs.

As stated above, the amount of BDNF secreted into the medium (Medium-1) from MABs-BDNF after 2 days in culture was very low: approximately 2 ng/ml, as measured using ELISA (Fig. 4C). If MABs-BDNF were cultured with the medium usually employed to culture OCs (Medium-2), the amount of secreted BDNF was approximately halved (Fig. 4C). 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. This conditioned medium contained approximately 1 ng/ml BDNF, whereas no detectable of BDNF was present in the medium conditioned with

control MABs (Fig. 4C).

We first screened the trophic effects of the MABs-BDNF conditioned medium in the PC system. The medium conditioned in control MABs increased the number of surviving neurons by about 80%, similar to the effect of 300 ng/ml rBDNF (a maximally effective concentration). 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. 4D, 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 MABs-BDNF medium) alone did not produce any 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 the different media and, after 14 DIV, cell death was measured using the LDH release assay. As shown in Fig. 4E, cell death was remarkable in control media (Medium-1 and Medium-2, see also Fig. 5A). 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) adding 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. 4E).

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 approach the effect of the MABs-BDNF conditioned medium.

These findings have been confirmed using light microscopy and FDA fluorescence, an indicator of viable cells. At the bright field observation, as compared with slices cultured in the control medium (these slices, as described, display obvious signs of degeneration, Fig. 5A), 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. 5C). Slices cultured in the MABs medium appeared healthier than those cultured in control medium but less healthy than those cultured in the MABs-BDNF medium (Fig. 5B). In line with these findings and paralleling the high levels of LDH release described above, FDA fluorescence was weak and uneven in OCs cultured with control medium (Fig. 5D), indicating that most of the cells are indeed degenerating. OCs cultured with the MABs medium displayed a better labeling quality (Fig. 5E), which was further improved in OCs cultured with the MABs-BDNF medium (Fig. 5F).

Cell counting, performed by using the DAPI nuclear staining, confirmed that, even if some cell loss was present in slices cultured with MABs-BDNF conditioned medium (Fig. 5I), the number of surviving cells was much higher than in the control group (Fig. 5G). Slices treated with the medium conditioned in MABs performed a little better than controls, but much worse than those treated with the medium conditioned in MABs-BDNF (Fig. 5H).

Other morphological examinations and electrophysiological recordings were performed. For morphological analysis, slices were fixed and immunostained with MAP2, a cytoskeletal protein

primarily found in neuronal dendrites. As shown in Fig. 5J, 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 medium only slightly improved this situation (Fig. 5K), the MABs-BDNF conditioned one clearly attenuated neuronal loss (Fig. 5L). Furthermore, the dendritic network and the cytoarchitectonic characteristics were essentially maintained in adult OCs treated with the MABs-BDNF conditioned medium, even if some signs of neuronal degeneration could be detected (loss of neurons, decreased dendrites, condensed or swelling pyramidal cells). These signs of degeneration progressively increased in time. This morphological analysis further confirms that the medium conditioned in MABs-BDNF enhances neuronal survival and delays the alterations in structural organization.

The density of neurons in OCs was also assessed using Western blot analysis for the neuron-specific marker neurofilament 68 (NF68). We found that OCs cultured on MABs-BDNF conditioned medium contained more NF68, thus more neurons, as compared with slices cultured in the control medium or in medium from conventional MABs (Supplementary Fig. 2). A statistically significant difference was also found between MABs and control group, indicating that supplementation of the soluble substances produced by MABs can slightly 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. Thus, fEPSPs in CA1 pyramidal neurons have been measured after stimulation of the Schaffer collaterals. We chose 7 DIV as a check 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 currents could not be reliably measured at 14 DIV, when neurons are still visible using immunofluorescence: as expected, the functional damage precedes the morphological one. As shown in Fig. 6A, 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. 6B). The presence of evoked synaptic responses, albeit lower and less stable than in acute slices or in cultured postnatal slices, demonstrates that the neuronal activity and the hippocampal circuitry remain functional by virtue of the beneficial effects of the mediators secreted by MABs-BDNF.

***In vivo* implant of MABs in the AD11 mouse model: intranasal and systemic delivery**

It has been demonstrated that MABs can cross the endothelial walls and migrate towards the site of inflammation of certain tissues, such as a dystrophic muscle, where they can stably integrate and participate in tissue repairing (Cossu and Bianco, 2003). Thus, the extra-vascular migration properties of MABs could be used for the delivery of secreted diffusible factors, such as neurotrophins, across the BBB. As an important first step in this direction, we studied the homing properties of MABs into the brain after intranasal and systemic delivery.

The intranasal route was shown to be a rapid, effective and non invasive way to deliver NGF and BDNF neurotrophins to the brain at pharmacologically effective doses in animal models of neurodegenerative diseases (Capsoni et al., 2002; De Rosa et al., 2005; Capsoni et al., 2009). While highly effective, the intranasal delivery of neurotrophins to the brain requires repeated injections, at daily intervals (Capsoni et al., 2002). In principle, neurotrophin-secreting MABs

may be implanted on the olfactory epithelium, constituting a local and continuous source of neurotrophins to the brain, through a single “one shot” injection of cells, avoiding the need for repeated protein injections.

To verify the feasibility of this approach, we intranasally delivered MABs to 6 months old anti-NGF transgenic AD11 mice, a well-characterized animal model of AD neurodegeneration (Ruberti et al., 2000; Capsoni et al., 2000). We analyzed the olfactory epithelium of transplanted mice for the presence of MABs at different time points after cell administration, i.e. 24 h, 1 week and 1 month, by examining live GFP cellular expression. GFP-positive cells (isolated or grouped in clusters) were detected in the host nasal septum, both underneath (Fig. 7A-C) and integrated in the olfactory epithelium (Fig. 7D-F), 24 h after implantation. The specificity of live GFP signal was confirmed by indirect immunofluorescence to GFP, which revealed positive cells only in MABs-transplanted animals (red signals in Fig. 7B,E and merged staining Fig. 7C,F), and not in control, D16-transplanted mice (Fig. 7G-I). No GFP-positive cell was found at longer time intervals. The short persistence of intranasally delivered MABs might be due to the rapid turnover and cellular recycling of the olfactory epithelium (Cummings et al., 2000) and/or to a direct transport of MABs from the nasal compartment into the brain (Danielyan et al., 2009). In order to explore this latter hypothesis, we analyzed the olfactory bulbs of the same injected mice for the presence of GFP-positive cells at longer time points. No migrating MABs were found in this district (data not shown).

We also tested MABs ability to cross cerebral vessels and migrate into the brain after systemic delivery. Thus, intracardiac administration of 5×10^5 MABs was performed and the brains were analyzed at different time points (24 h, 1 week and 1 month) for the presence of GFP positive cells. At the earlier time points (24 h and 1 week) very few transplanted cells were

detected (data not shown), whereas several GFP positive MABs were found in the basal forebrain around the brain ventricles at 1 month (green signal in Fig. 8A and 8D). These GFP positive cells do not express the neuronal marker NeuN (red signal in Fig. 8B and 8E and arrows in 8F), indicating that are exogenous MABs and not endogenous neurons. No GFP-positive/NeuN-negative cell was found in control animals (Fig. 8G-I).

Discussion

Main findings

In this study, we generated MABs that can stably secrete the neurotrophins NGF or BDNF, and found that media conditioned from the culture supernatant of these cells exert trophic effects *in vitro*: PC12 cells are induced to differentiation by a MABs-NGF-conditioned medium, while viability and function of cultured neurons and brain slices are increased by a MABs-BDNF-conditioned medium. MABs-BDNF effects are TrkB receptor-dependent, being abolished by the receptor blocker K252a or by the BDNF scavenger TrkB-IgG. Thus, MABs produce robust by-stander effects on the nervous tissue, that we characterized using endpoints (neuronal differentiation, neuroprotection, function) that are relevant for neurodegenerative diseases (or, more in general, neurological diseases associated with damage).

We also began to explore the capacity of MABs to home in a lesioned brain when peripherally administered. After *in vivo* (transcardial or intranasal) injection, GFP-positive MABs do indeed reach the brain in AD11 mice (an AD neurodegeneration mouse model) but not in

healthy mice. Importantly, after peripheral administration MABs were found in brain areas proximal to the basal forebrain, i.e. close to the cholinergic neurons (basal forebrain cholinergic neurons, BFCNs) that are selectively vulnerable and hit in AD (Bartus et al., 1982). This enrichment of MABs near the BFCNs may be relevant in a therapeutic prospect, since a decreased neurotrophic support of NGF to these neurons has been associated with AD (Phelps, 1989; Cattaneo et al., 2008).

Therapeutic efficacy of NGF and BDNF in neurodegeneration

A large body of evidence shows the essential role played by NGFs in the adult CNS. In particular, BFCNs depend on NGF for their function and maintenance of cholinergic phenotype (Gnahn et al., 1983; Seiler and Schwab, 1984). BFCNs express NGF receptors (Hefti et al., 1986; Holtzman et al., 1992), retrogradely transport NGF from their cortical projections up to their cell bodies (Ferguson et al., 1991) and respond to administration of exogenous NGF increasing cholinergic markers (Gnahn et al., 1983; Mobley et al., 1986). Moreover, NGF can prevent BFCNs death or atrophy (Hefti et al., 1986; Williams et al., 1986; Kromer, 1987; Tuszynski et al., 1990).

A pivotal protective role of NGF in neurodegeneration has also been shown in AD patients. Clinical trials are currently underway using intracerebral delivery systems that by-pass the BBB. For example, a phase 1 clinical trial used stereotaxic intracerebral transplants of autologous fibroblasts engineered to produce NGF, achieving a consistent reduction in the progression of cognitive decline (Tuszynski et al., 2005). Promising results from another Phase 1 clinical trial, evaluating the safety and efficacy of stereotactic intracerebral injections of adeno-associated virus (AAV) harboring the NGF gene have recently been reported (Mandel, 2010). Unfortunately, an invasive surgical step is required for these approaches. One alternative

may be the delivery of NGF through the olfactory route, a less invasive approach that proved capable to achieve pharmacologically relevant NGF concentrations in the brain of animals (Capsoni et al., 2002; De Rosa et al., 2005). In one study (De Rosa et al., 2005), nasal NGF delivery reverted the neurodegenerative phenotype in an AD model, in the absence of appreciable side effects. The drawback of this approach is that it requires repeated injections for a prolonged period.

BDNF is also known to exert neuroprotective effects. For example, exogenous BDNF has been reported to increase survival of neurons in cultures, while reduced survival was found after addition of anti-BDNF antibodies and in BDNF knockout mice (Lowenstein and Arsenault, 1996; Matsumoto et al., 2003). BDNF appears to be an essential constituent of the microenvironment for neuronal survival in the adult hippocampus. It potentiates presynaptic release and excitatory transmission (Huber et al., 1998; Li et al., 1998; Jiang et al., 2001), and promotes dendritic and axonal growth (Labelle and Leclerc, 2000). Moreover, several investigations indicate a pivotal role of BDNF in neuroprotection in different *in vivo* models of disease, including ischemia (Larsson et al., 1999; Kano et al., 2002) and seizures (Paradiso et al., 2009). The signaling mechanisms underlying the pro-survival effects of BDNF appear to involve TrkB activation. 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). Coherently with these data, we demonstrated here the beneficial effects of rBDNF or MABs-delivered BDNF on cell survival in primary neuronal and in adult slice cultures, effects that were TrkB receptor-dependent. We also observed that MABs-delivered BDNF facilitates synaptic responses, an effect that may be ascribed to a better preservation of synaptic connection or to a potentiation of synaptic transmission. It has been reported that BDNF

modulates the strength of existing synaptic connections (Huber et al., 1998; Jiang et al., 2001) and favors the formation of new synaptic contacts within the hippocampal circuits (Lauterborn et al., 2007).

Similarly to NGF, clinical trials have been also undertaken with BDNF (both peripherally administered and delivered directly in the CNS) for the treatment of neurological diseases (Thoenen and Sendtner, 2002; Simonato et al., 2006). Unfortunately, data have been negative thus far.

Synergy between MAB-produced trophic factors

We found that the amount of BDNF released by MABs-BDNF produced greater effects than the identical amount of rBDNF, suggesting either that the specific activity of MAB-released BDNF is higher than that of rBDNF (possibly due to a better folding of BDNF released by MABs), or that other neurotrophic factors produced by MABs synergize with BDNF. A micro-array analysis of gene expression in MABs revealed that several growth factors are expressed at high level in these cells, including VEGF B, FGF-2, FGF-7, PDGF AA and others (Galli et al., 2005). Although it remains unknown which of these NTFs may exert neuroprotective effects, it seems very likely that they will interact and possibly synergize with BDNF.

Regardless of the underlying mechanisms, the markedly beneficial effects of MABs-delivered NTFs on neurons and brain tissue suggest that exogenous, MAB-mediated, administration of BDNF or NGF (maybe synergizing with other MAB-produced NTFs) might be developed into a strategy for functional preservation and restoration in neurological diseases associated with brain damage. In this prospect, a very attractive property of MABs is their potential to reach the lesioned tissue when peripherally administered, because this would

represent a net advantage over other, currently tested delivery strategies. 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 is this the case for the brain?

Are MABs capable to home in the lesioned brain?

We examined two possible routes of peripheral administration and the homing in the brain in a murine AD model. First, we used the intranasal delivery route. As described above, there is evidence of therapeutic efficacy of NTF delivered through the olfactory route in animal models (De Rosa et al; 2005; Capsoni et al., 2009). In the absence of a local lesion of the mucosa (olfactory epithelium), MABs were detected in proximity of the epithelium 24 h after injection, but not after longer intervals. Local homing and survival may be impaired because of the fast rate of epithelium turnover. Moreover, the persistence of MABs may be reduced in the absence of a chemical or enzymatic lesion of the mucosa (Cummings et al., 2000; Danielyan et al., 2009), that might facilitate access of cells to the brain (Danielyan et al., 2009).

Furthermore, we evaluated the efficacy of the intracardiac injection. This approach was successful, since cells were detected in the brain of AD mice, but not of normal, healthy mice, even 1 month after injection. The homing mechanism of MABs to the damaged areas remains unclear, but may depend on 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 in damage area (Palumbo et al., 2004). Interestingly, HMGB1 expression is increased in the basal forebrain of 6 months old AD11 mice (D'Onofrio et al., 2009), the same age at which we performed MAB transplantation. The negative result obtained after shorter time

intervals may be related to the dispersion of the injected cells in the capillary filter of the whole body, while homing, clonal proliferation and maintenance may be exclusively possible in a favorable environment, with recruitment by inflammation “flag” markers, as in the case of neuroinflammation linked to the AD-like phenotype (D'Onofrio et al., 2009). Importantly, cytokines can be produced by different brain cell populations in response to many other pathological insults, for example by seizures (de Boer and Breimer, 1998), which prompts the speculation that MABs may be recruited at the neuroinflammation site, homing selectively in the different lesion areas characteristic of different pathologies.

Homing of MABs might be improved by cytokines and adhesion molecules. 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). Adhesion molecules, like L-selectin or $\alpha 4$ integrin, may also improve migration (Peault et al., 2007). These strategies, which were demonstrated to improve the homing ability of MABs in a mouse model of muscular dystrophy, might also be used to improve homing in the brain.

Conclusions: potential advantages of MABs as a delivery strategy

The supplementation of NTFs can sustain the survival and functional recovery of neurons by modulating the post-injury microenvironment. This study provides evidence that peripherally administered MABs home in the diseased (AD) brain, close to regions that are relevant for the pathology. We need to verify if this is paramount property, using models of different diseases with different lesion areas.

NTF delivery to the nervous system should be target-specific and regionally restricted, prolonged (although not necessarily lasting for life), safe, well tolerated and sufficiently robust to

elicit responses from target neurons. MAB-based NTF delivery may be optimal to meet these criteria. MABs possess the advantages of all cell-based therapies: they can be expanded to virtually unlimited numbers *in vitro*; they are amenable to genetic modification; they have self-renewal and multipotent capacities. Moreover, (1) they may be used for autologous transplants (can be prepared from biopsies from the patient himself); (2) they may be peripherally administered (no need for surgery); (3) they may selectively home in the lesioned area (recruitment at the neuroinflamed site), with no undesired accumulation of NTF in areas that do not need them (reduced side effects); (4) at the site of accumulation, they may act as efficient bioreactors producing robust by-stander effects.

These advantages raise the possibility that the MABs might be developed into a new cell-based strategy for NTF delivery into the CNS, promoting neuronal survival and restoring network function in neurological diseases associated with brain damage.

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Figure Legends

Figure 1. Effects of NGF produced by MABs on PC12 differentiation. PC12 differentiation in the presence of recombinant NGF (**B, E, H, K**), of conditioned medium from MABs-NGF (**C, I**) or when co-cultured with MABs-NGF (**F, L**) is visualized by the presence of neurites (**B-C, E-F**) and confirmed by immunofluorescence for the expression of β -tubulin (Tuj1, red immunostaining in **H-I, K-L**). As negative control, PC12 cells have been cultivated in growing medium without NGF (**A, G**) or co-cultured with control MABs (**D, J**).

Figure 2. Effects of BDNF on zif/268 nuclear accumulation in primary rat hippocampal neurons. High levels of zif/268 immunostaining are concentrated in the nuclei of cells incubated with recombinant BDNF (arrows in **B**) or conditioned medium from A9 BDNF-producing MABs (arrows in **D**), whereas low level of zif/268 immunostaining is present in the nuclei of hippocampal neurons treated with growing medium (**A**) or medium conditioned from control, D16 MABs (**C**).

Figure 3. 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 means \pm s.e. (n=6). * $P < 0.05$, ** $P < 0.01$ vs. control (0 ng/ml); ANOVA and *post-hoc*

Newman-Keuls test.

Figure 4. 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 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 means \pm s.e. of 6 separate experiments. ** $P < 0.01$ vs. Control-1; ● $P < 0.05$ vs. MABs; ANOVA and *post-hoc* Newman-Keuls test. (E) Effect of different treatment procedures on the viability of adult hippocampus OCs, as estimated using the LDH release assay. Data are the means \pm s.e. of 6 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).

Figure 5. 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), and 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: 1 mm.

Figure 6. 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 \pm s.e. of 5 separate experiments. ** $P < 0.01$ vs. Control-1; ●● $P < 0.01$ vs. MABs; ANOVA and *post-hoc* Newman-Keuls test.

Figure 7. Intranasal delivery of MABs-NGF. 72 hrs after intranasal administration, GFP positive cells are visible around (A-C) or integrated (E-F) in the olfactory epithelium as clusters or single cells (arrow and arrowheads, respectively, in A, B, D and E). Specificity of live GFP signal (green in A and D) is confirmed by anti-GFP immunostaining (red in B and E) and merged signal in C and F (arrows). As negative control, olfactory epithelium from untreated AD11 mice show neither live GFP (faint green signal in G and I) nor anti-GFP immunostaining (faint red signal in H and I). Nuclei staining is revealed by DAPI (blue in A-B, D-E, G-H).

Figure 8. MABs homing in the brain upon intracardiac administration. Transplanted MABs (live GFP expression in A, D, C, F) are visible around the ventricles, and do not express the neuronal markers NeuN (red signal in B, C, E and arrows in F). (D-F) 20 \times magnification of the insets in A-C, respectively. No positive GFP exogenous cells are present in the brain of control untreated animals (G and I), stained for NeuN expression (red in H and I).

Supplementary Figure 1. Quantification of NGF and BDNF production in MABs. **(A-B)** Quantification of recombinant NGF produced by MABs-NGF by double site ELISA. **(A)** NGF concentrations (ng/ml) in the supernatant of 3 independent clones identify clone F10 as the best producer. **(B)** Time-course of NGF production in the supernatant from clone F10. **(C-D)** BDNF concentrations (ng/ml) in the supernatant of 4 independent clones **(C)** and time-course of BDNF production in the supernatant from clone A9, the best producer **(D)**.

Supplementary Figure 2. 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 means \pm s.e. of 4 separate experiments. * $P < 0.05$ and ** $P < 0.01$ vs. Control-1; ●● $P < 0.01$ vs. MABs; ANOVA and *post-hoc* Newman-Keuls test.

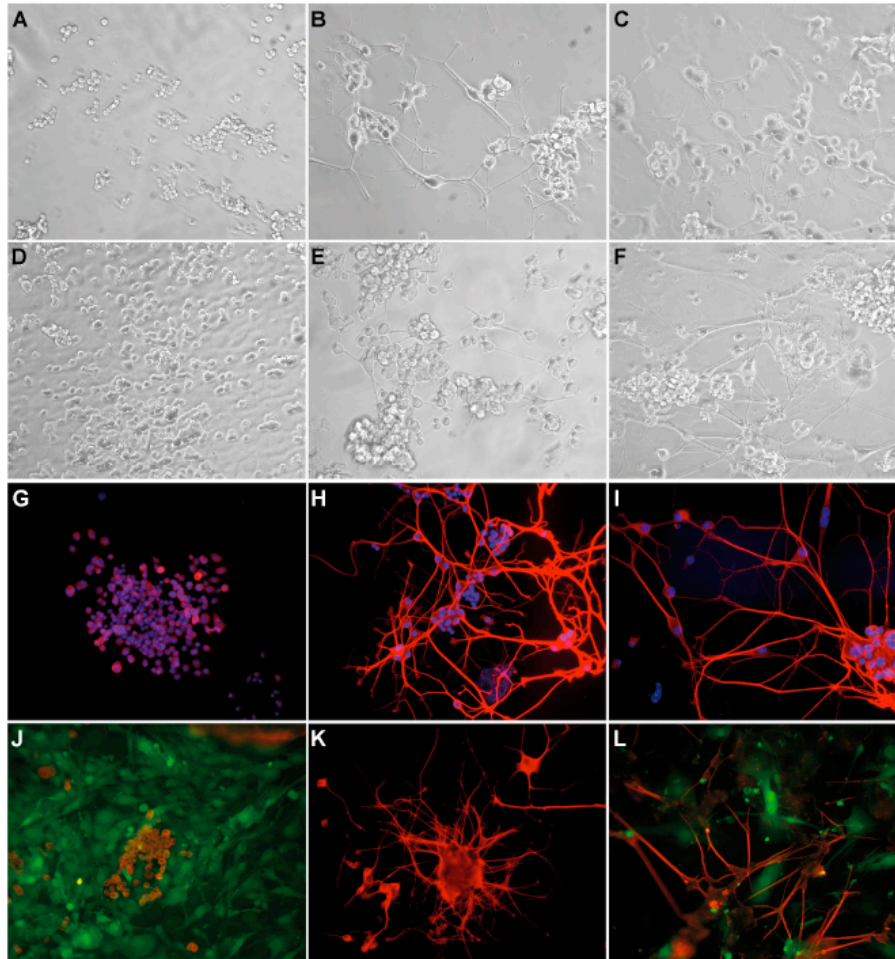


Figure 1. Effects of NGF produced by MABs on PC12 differentiation. PC12 differentiation in the presence of recombinant NGF (**B, E, H, K**), of conditioned medium from MABs-NGF (**C, I**) or when co-cultured with MABs-NGF (**F, L**) is visualized by the presence of neurites (**B-C, E-F**) and confirmed by immunofluorescence for the expression of β -tubulin (Tuj1, red immunostaining in **H-I, K-L**). As negative control, PC12 cells have been cultivated in growing medium without NGF (**A, G**) or co-cultured with control MABs (**D, J**). 197x209 mm (300 x 300 DPI).

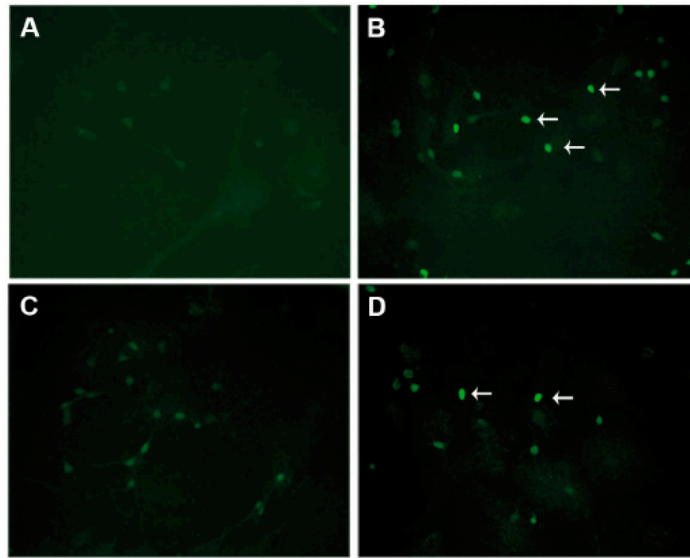


Figure 2. Effects of BDNF on zif/268 nuclear accumulation in primary rat hippocampal neurons. High levels of zif/268 immunostaining are concentrated in the nuclei of cells incubated with recombinant BDNF (arrows in **B**) or conditioned medium from A9 BDNF-producing MABs (arrows in **D**), whereas low level of zif/268 immunostaining is present in the nuclei of hippocampal neurons treated with growing medium (**A**) or medium conditioned from control, D16 MABs (**C**). 99x80mm (300 x 300 DPI)

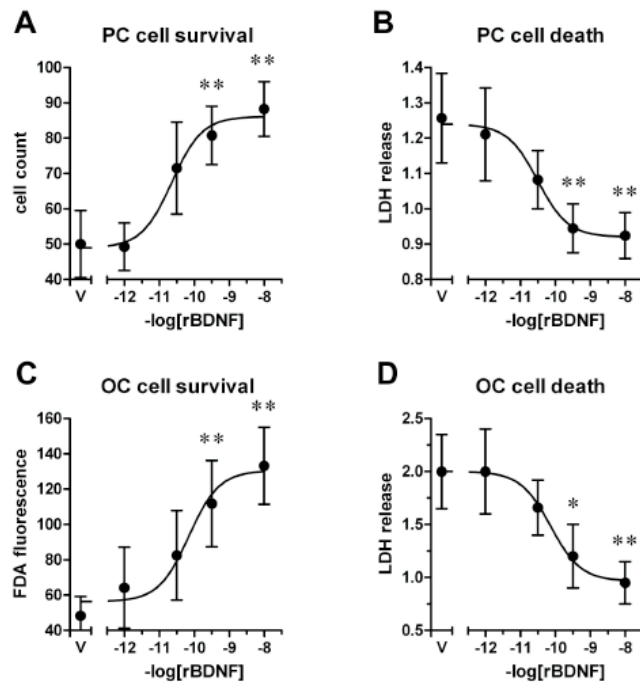


Figure 3. Recombinant BDNF enhances cell survival in primary hippocampal neuronal cultures (PC, panels A and B) and in organotypic adult hippocampus slice cultures (OC, panels C and D) in a concentration-dependant manner. Neurons were cultured with the indicated concentration of BDNF for 7 days, slices 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 means \pm s.e. (n=6). * $P < 0.05$, ** $P < 0.01$ vs. control (0 ng/ml); ANOVA and post-hoc Newman-Keuls test. 168x170mm (300 x 300 DPI).

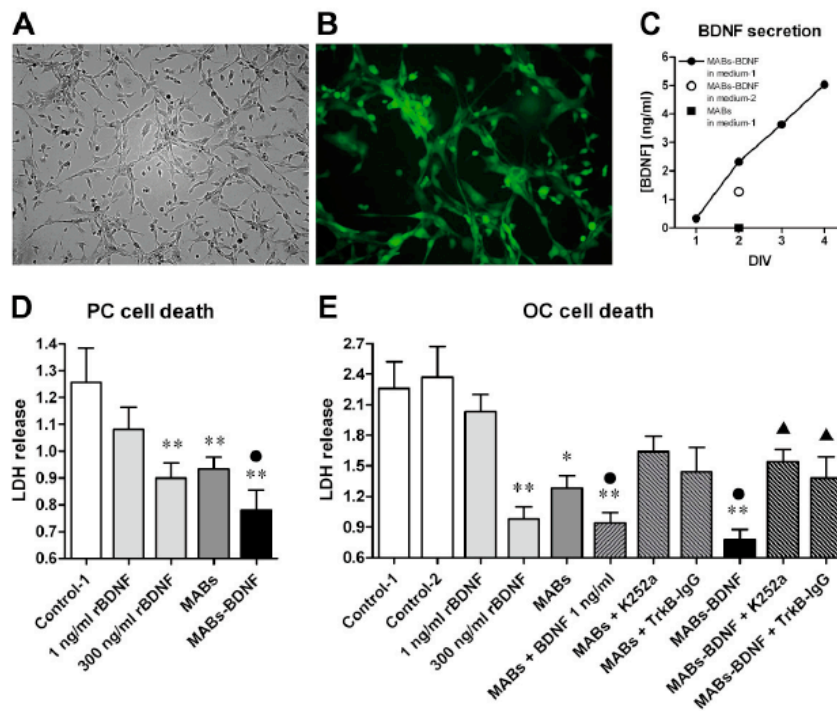


Figure 4. MABs-BDNF deliver BDNF and enhance cell survival in primary hippocampal neuronal cultures (PC) and in organotypic adult hippocampus slice cultures (OC). (A) and (B): genetically modified MABs-BDNF proliferate and express GFP. (C) MABs-BDNF secrete BDNF. Concentration of BDNF in the medium of 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 means \pm s.e. of 6 separate experiments. ** $P < 0.01$ vs. Control-1; $v P < 0.05$ vs. MABs; ANOVA and post-hoc Newman-Keuls test. (E) Effect of different treatment procedures on the viability of adult hippocampus OCs, as estimated using the LDH release assay. Data are the means \pm s.e. of 6 separate experiments. * $P < 0.05$ and ** $P < 0.01$ vs. Control-1; $v P < 0.05$ vs. MABs; $\square P < 0.05$ 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). 214x172mm (150 x 150 DPI).

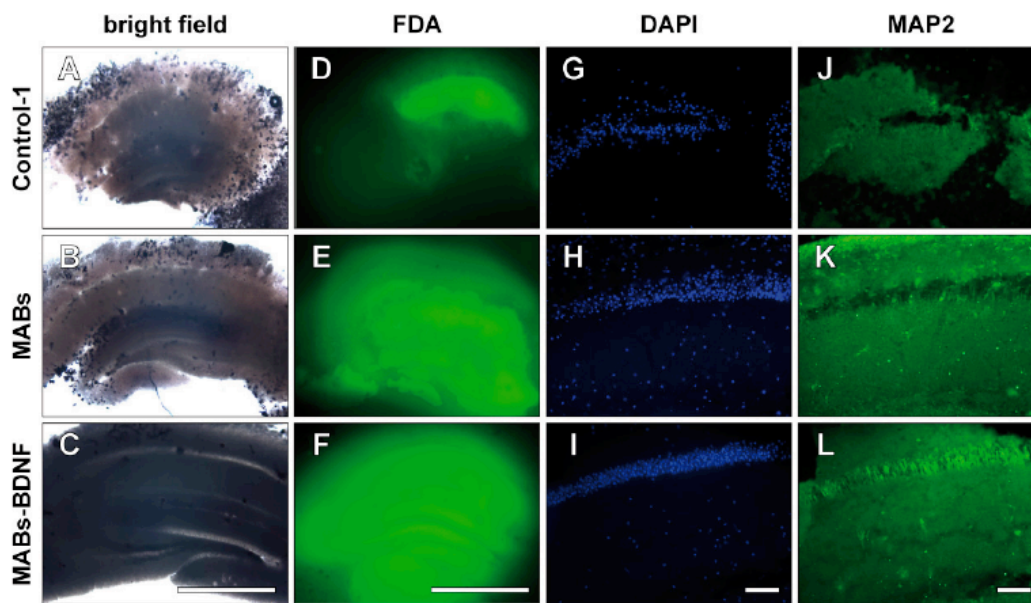


Figure 5. Morphological evidence of the beneficial effects of the medium conditioned in MABs-BDNF on adult organotypic cultures. Adult slices were cultured for 14 days with the control Medium-1, with medium conditioned in MABs, or with medium conditioned in MABs-BDNF. Note that slices cultured in the MABs-BDNF conditioned medium, compared with the other two groups, better maintain their integrity based on bright field observation (A-C), and 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: 1 mm. 216x126mm (150 x 150 DPI).

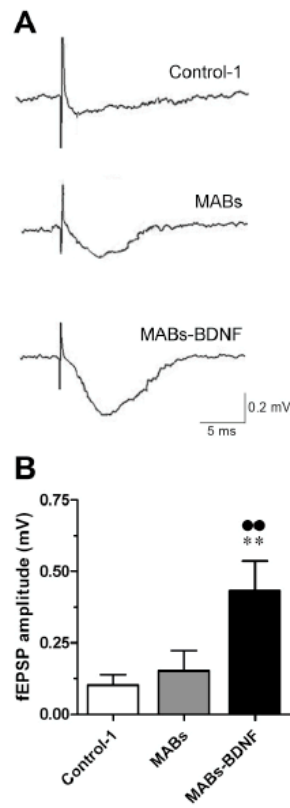


Figure 6. Maintenance of synaptic connections in adult slices after 7 days in culture with the MABs-BDNF conditioned medium. Synaptic connections were identified by recording evoked fEPSP in CA1 after stimulation of the Schaffer collaterals. (A) Representative recordings of the evoked fEPSP in the different groups. (B) Peak amplitude of the fEPSPs, measured and statistically analyzed as described in the Materials and Methods. The data are the means \pm s.e. of 5 separate experiments. ** $P < 0.01$ vs. Control-1; 44 $P < 0.01$ vs. MABs; ANOVA and post-hoc Newman-Keuls test. 77x196mm (150 x 150 DPI).

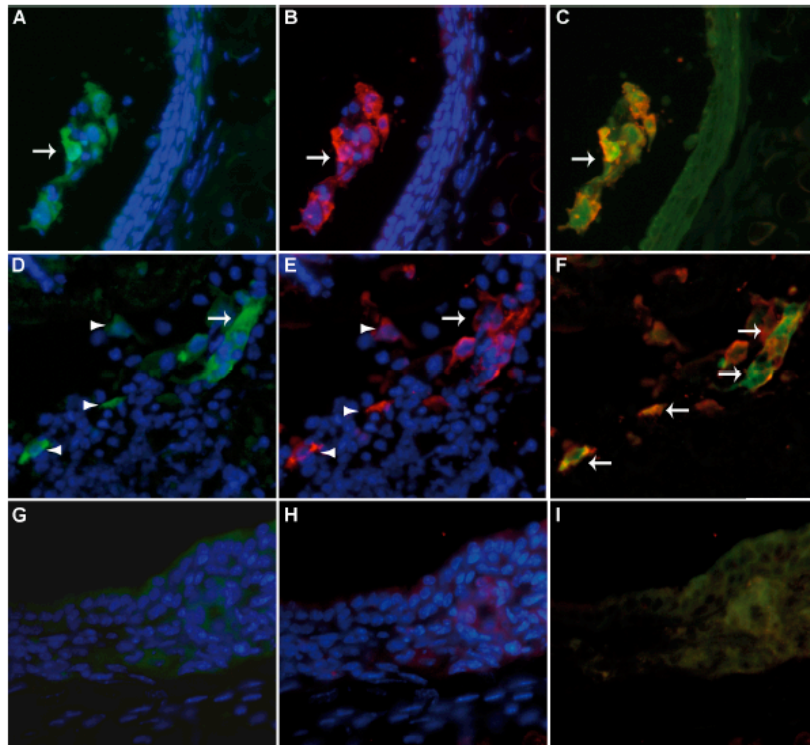


Figure 7. Intranasal delivery of MABs-NGF. 72 hrs after intranasal administration, GFP positive cells are visible around (A-C) or integrated (E-F) in the olfactory epithelium as clusters or single cells (arrow and arrowheads, respectively, in A, B, D and E). Specificity of live GFP signal (green in A and D) is confirmed by anti-GFP immunostaining (red in B and E) and merged signal in C and F (arrows). As negative control, olfactory epithelium from untreated AD11 mice show neither live GFP (faint green signal in G and I) nor anti-GFP immunostaining (faint red signal in H and I). Nuclei staining is revealed by DAPI (blue in A-B, D-E, G-H). 211x193mm (300 x 300 DPI).

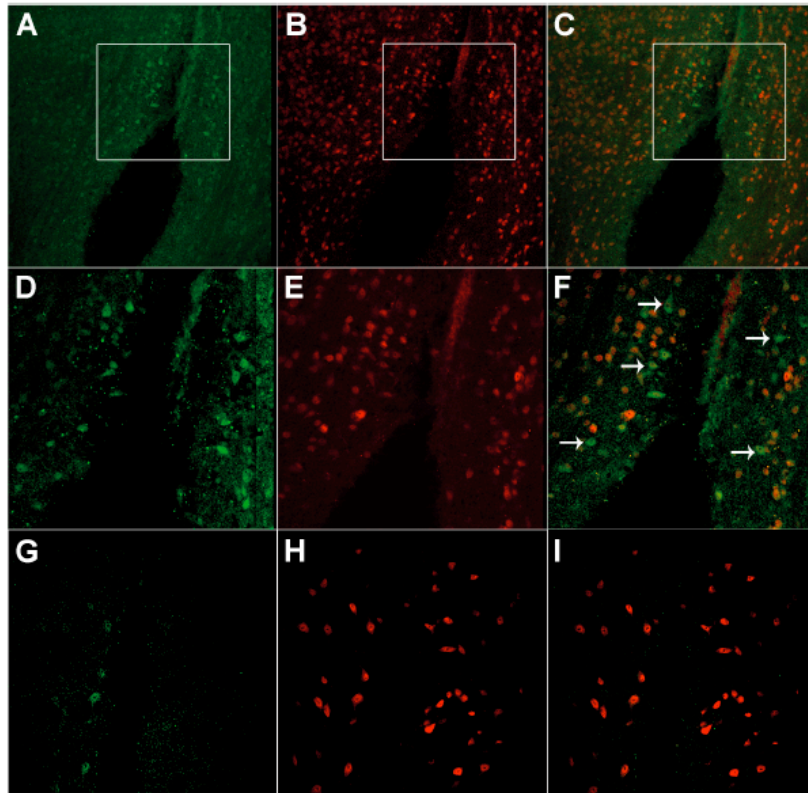
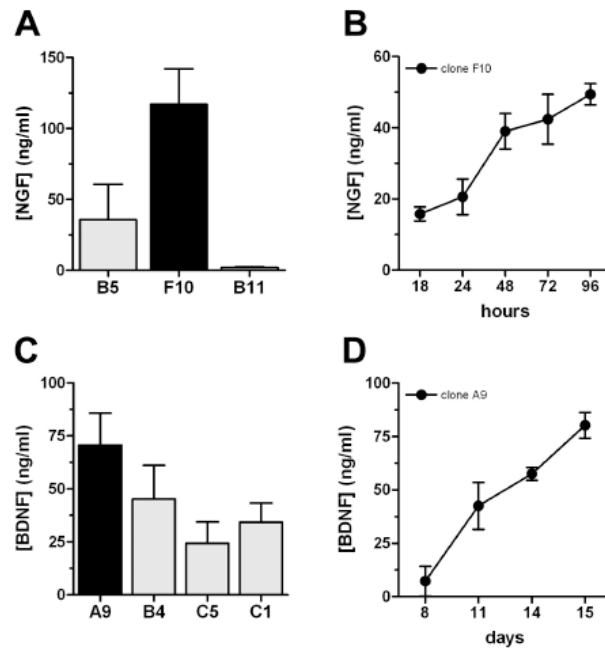
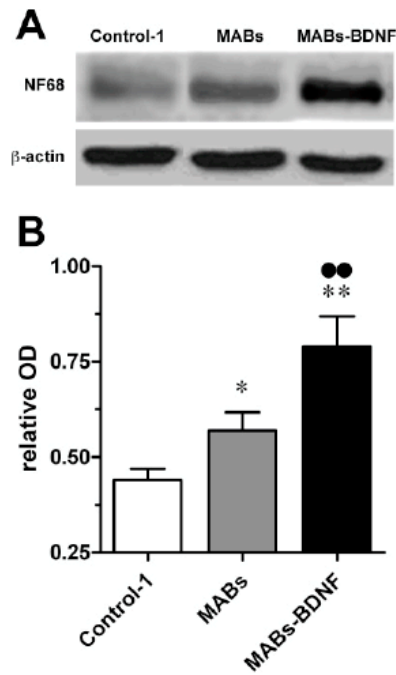


Figure 8. MABs homing in the brain upon intracardiac administration. Transplanted MABs (live GFP expression in A, D, C, F) are visible around the ventricles, and do not express the neuronal markers NeuN (red signal in B, C, E and arrows in F). (D-F) 20× magnification of the insets in A-C, respectively. No positive GFP exogenous cells are present in the brain of control untreated animals (G and I), stained for NeuN expression (red in H and I). 192x189mm (300 x 300 DPI).



Supplementary Figure 1. Quantification of NGF and BDNF production in MABs. (A-B) Quantification of recombinant NGF produced by MABs-NGF by double site ELISA. (A) NGF concentrations (ng/ml) in the supernatant of 3 independent clones identify clone F10 as the best producer. (B) Time-course of NGF production in the supernatant from clone F10. (C-D) BDNF concentrations (ng/ml) in the supernatant of 4 independent clones (C) and time-course of BDNF production in the supernatant from clone A9, the best producer (D). 160x159mm (300 x 300 DPI).



Supplementary Figure 2. 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 means \pm s.e. of 4 separate experiments. * $P < 0.05$ and ** $P < 0.01$ vs. Control-1; vv $P < 0.01$ vs. MABs; ANOVA and post-hoc Newman-Keuls test.

79x128mm (150 x 150 DPI).

By-stander effect on brain tissue of mesoangioblasts producing neurotrophins

Tao Su, Raffaella Scardigli, Luisa Fasulo, Beatrice Paradiso, Mario Barbieri, Anna Binaschi, Roberta Bovolenta, Silvia Zucchini, Giulio Cossu, Antonino Cattaneo, and Michele Simonato

Supplemental material:

Materials and Methods

Expression vectors

Murine pre-proNGF and rat pre-proBDNF cDNAs have been PCR amplified from pTrcHisA-preproNGF (Paoletti et al., 2009) and pBK-CMV-preproBDNF (Benedetti, 2001, PhD thesis, SISSA Trieste), respectively, using the following XhoI-5' and NotI-3' primers:

5'primer-GTCGACCTCGAGGTAATGTCCATGTTGTTCTACACTCTG and

3'primer-AGTCGGCTG-CGGCCGCTCAGCCTCTTCTTGTAGCCTTCCT for NGF;

5'primer-GTCGACCTCGAGAT-GACCATCCTTTTCCTTACTATG and

3'primer-AGTCGGCTGCGGCCGCCTATCTTCCCC-TTTTAATGGTCAG for BDNF. The

PCR reaction has been performed with proofreading Pfu DNA polymerase (Promega, WI, USA) using the following conditions: 95°C, 1 min; 55°C, 1 min; 72°C, 1 min, for 25 cycles.

Amplified preproNGF and preproBDNF cDNAs have been sequenced with the CEQ DTCS Quick Start Kit (Beckman Coulter Inc., CA, USA) and then Xho/NotI cloned into the ScFv

express eukariotic vector (Persic et al., 1997), to produce ScFv-preproNGF and ScFv-preproBDNF constructs, respectively.

Transfection of cells and neurotrophin quantification

Five $\times 10^5$ COS cells, grown for 1 day in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS), were transiently transfected with ScFv-preproNGF or ScFv-preproBDNF using Fugene, according to the manufacturer's instructions (Roche, Basel, CH). NGF and BDNF expression and secretion were evaluated on the conditioned medium 72 hours after transfection.

Green fluorescent protein (GFP) expressing MABs (Minasi et al., 2002), grown in DMEM plus 10% FBS, have been stably transfected with ScFv-preproNGF or ScFv-preproBDNF using Lipofectamine (Invitrogen, CA, USA), according to manufacturer's instructions. Upon G418 selection, single clones of stable transfectants have been cultured with the ordinary methods, using the following culture medium (here referred to as Medium-1): 88% DMEM, 10% FBS, 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, CA, USA). The production of exogenous neurotrophins was analyzed by a "two-site sandwich" ELISA (Covaceuszach et al., 2008), performed on the conditioned medium collected from NGF- and BDNF-producing MABs. The medium was collected and centrifuged for 5 min at 230 g and then the supernatant was collected. The supernatant consists of the medium containing the soluble substances secreted by MABs, including NGF in the case of MABs-NGF and BDNF in the case of MABs-BDNF. NGF and BDNF "coating" were performed using anti NGF mAb α D11 (1 ng/ml) and IgG-TrkB immunoadhesin (5 ng/ml) (Righi et al., 2000), respectively. The following antibodies were used

for ELISA: rabbit polyclonal anti-mNGF (30 μ g/ml, Sigma, St Luis, MO, USA); chick polyclonal anti-hBDNF (1 μ g/ml, Promega, WI, USA); biotinylated anti-rabbit secondary antibody (1:1000, Vector Laboratories, Inc., CA, USA); biotinylated anti-chick secondary antibody (1:1000, Promega, WI, USA). As standards, recombinant 2.5S mNGF and hBDNF (Alomone Labs Ltd, Jerusalem, Israel) were used. Immunoreactivity was visualized with the ABC kit (Vector Laboratories, Inc., CA, USA) using tetramethylbenzidine (TMB) as substrate. Data are reported as average of at least 2 independent experiments, each performed in duplicates.

Assessment of NGF biological activity

PC12 cells. PC12 cells (Greene and Tischler, 1976) have been cultured in RPMI (Invitrogen, CA, USA) plus 10% FBS. Upon washing in serum-free medium, 4×10^5 PC12 cells were plated in 35 mm collagen-treated Petri dishes, in the following different conditions: alone, in co-culture with the same amount of MABs-NGF, in the presence of 5-day-culture supernatant from MABs and MABs-NGF, or in the presence of recombinant NGF (50 ng/ml). PC12 cells were maintained in culture for 7 days, replacing fresh medium every 2 days. Neuronal differentiation was evaluated by the presence of neurite processes in the cultures and confirmed by indirect immunofluorescence using the anti- β -tubulin antibody TuJ1 (Covance, New Jersey, USA).

For β -tubulin and GFP expression in PC12/MABs co-culture, cells were fixed in 4% paraformaldehyde (PFA), washed briefly in phosphate buffered solution (PBS) and permeabilized with 0.1% Triton in PBS. After blocking in 10% normal goat serum (NGS), cells were stained with mouse anti- β -tubulin antibody TuJ1 (1:250, Covance, New Jersey, USA) and rabbit anti-GFP antibody (1:500; Invitrogen, CA, USA), followed by Alexafluor-594 conjugated anti-mouse and Alexafluor-488 conjugated anti-rabbit antibodies (1:500; Invitrogen, CA, USA).

Assessment of BDNF biological activity

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 5×10^4 cm⁻² viable cells in 24-well plates coated with poly-L-lysine (Sigma, St Luis, MO, USA). Cells were grown in DMEM supplemented with 10% 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 μ M 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, MgSO₄ 3, NaH₂PO₄ 1.1, NaHCO₃ 26, CaCl₂ 1 and glucose 11 (all reagents from Sigma) bubbled with 95% O₂/5% CO₂. Subsequently, 300 μ m thick coronal slices were cut with a vibrotome (MA752, Campden Instruments Ltd, UK). The hippocampi were dissected in cold, oxygenated Hank's balanced salt solution (HBSS, Gibco, Invitrogen), transferred onto sterile porous membrane confetti (Millicell, Millipore, MA, USA), 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% CO₂ atmosphere at 37°C. The culture medium was changed the day after preparation and then every 2 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 conditioned media were composed by the mixture of equal volumes of fresh MABs growing medium (Medium-1, see above) 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 growing medium. Medium-2 is a standard culture medium for OCs from postnatal animals, consisting of 50% DMEM, 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 (Sigma, St Luis, MO, USA). The other conditioned media were based on the above media with different supplementations of reagents, like the recombinant human BDNF (0.03 to 300 ng/ml, Immunological Sciences), the BDNF antagonist K252a (50 nM, Sigma) or TrkB-IgG (2 µg/ml, R&D Systems Inc., MN, USA), a recombinant tyrosine kinase receptor B (TrkB) engineered as an immunoadhesin to sequester BDNF. Sister slices were randomly assigned to the different groups.

Viability. The viability of PCs and OCs was assessed in two ways. First, the fluorescein diacetate (FDA) hydrolysis assay was used to measure enzyme activity in cells. 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. The culture medium was all collected and LDH leakage was quantified by using a LDH Cytotoxic Test kit (Clontech, CA, USA) according to the manufacturer’s instructions.

Immunocytochemistry. Cultured slices were fixed with 4% PFA in 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% NGS was used to block the slices for 30 min, and then they were incubated overnight with the primary antibody mouse anti-microtubule-associated protein (MAP2abc; 1:50; Immunological Sciences, Italy). Finally, they were rinsed with PBS and incubated with the secondary antibody Alexa 488 goat anti mouse IgG (1:100, Invitrogen, CA, USA). The slices were mounted onto slides after staining with 4',6-diamidino-2-phenylindole (DAPI, 1:1000). For zif7268, hippocampal neurons were incubated for 3 hr at RT with rabbit anti-zif antibody (Egr-1; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 in 10% FCS in PBS, followed by fluorescein anti-rabbit IgG antibody (Invitrogen, CA, USA).

Western blot. To estimate the density of the surviving 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, GE Healthcare, USA). Aliquots from each sample (15 µg protein/lane) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene 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 horse radish peroxidase-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, St Louis, MO, USA) 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% O₂ and 5% CO₂. 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., FL, USA) were used for stimulation of the Schaffer collateral pathway in the CA3 region. 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 for extracellular recordings was employed to identify the maximal response and to adjust the stimulus strength. The stimulus intensity (70 μs duration rectangular pulses at 40 V) that repeatedly evoked the maximal synaptically evoked response/excitatory postsynaptic potential was used in each slice. Signals were acquired under constant conditions, and off-line processed using the Patchmaster software (HEKA Instruments Inc., Germany).

In vivo analysis

Intranasal and intracardiac delivery of MABs. MAB intranasal delivery was performed on 6 months old AD11 mice following a procedure previously described for the intranasal delivery of neurotrophins (Capsoni et al., 2002). Prior to cell administration, animals were anaesthetized with 2,2,2-tribromethanol (Sigma, St Luis, MO, USA) as previously described (De Rosa et al., 2005). After anesthesia, mice were laid on their back, with the head in upright position, and 10⁶ cells in 100 μl of PBS were administered intranasally, 3 μl at a time, alternating the nostrils, with a lapse of

2 min between each administration, for a total of 14 times. During the procedure, nostrils were always kept open. For intracardiac delivery, upon anaesthetization, animals received 5×10^5 MABs resuspended in 150 μ l of PBS containing 2% sodium fluorescein as systemic delivery tracer. In both procedures, control AD11 mice were treated with PBS. Animals were sacrificed at different time points (24 h, 1 week, 1 month) after cells administration and olfactory epithelium and brains were analyzed for the presence of GFP positive cells by immunohistochemistry.

Immunocytochemistry. Immunocytochemistry for GFP was performed on olfactory epithelium and brains from AD11 mice and age-matched control mice. Briefly, mice were anesthetized as above and perfused with ice-cold PBS followed by 4% PFA/PBS. The olfactory epithelium was dissected from the nose and processed for paraffin inclusion, while brains were post-fixed in the same PFA solution and cryoprotected in 30% sucrose. After microtome and cryostat sectioning, olfactory epithelium and coronal brain slices from whole brains were processed for immunohistochemistry using the anti-GFP antibody (1:500; Invitrogen, CA, USA), followed by Alexafluor-488 conjugated anti-rabbit antibody (1:500; Invitrogen, CA, USA). Confocal analysis was carried out on a Nikon Eclipse 90i microscope (Nikon, USA).

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For Peer Review

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OVERALL CONCLUSIONS AND FUTURE PERSPECTIVES

Ineffectiveness of common AEDs could be attributed to the inability to arrest molecular and plastic modifications that occur after a brain injury and that lead a healthy cerebral tissue to become epileptic. An alternative to the common therapeutic approaches may derive from gene-therapy, through the local supplementation of NTFs. Among NTFs, BDNF and FGF-2 have shown anti-epileptogenic properties, favouring neurogenesis and reducing cell degeneration.

Epileptogenesis, however, is an extraordinarily complex event characterized by neuroinflammation, gliosis and reorganization of circuitries. The primary aim of the works presented in this thesis is to implement the knowledges on NTFs properties, evaluating their possibility to influence these processes.

Neuroinflammation is a well known feature of the epileptic brain, whose chronic persistence is considered to be harmful. Supplementation of BDNF and FGF-2 in a lesioned brain causes a long-lasting attenuation of various parameters of neuroinflammation such as microcytosis, astrocytosis and expression of IL-1 β . In particular, the effects on the cytokine are particularly prominent, being its synthesis almost completely prevented, even at earliest time point after SE. The mechanism(s) of this anti-inflammatory action of NTFs is unknown. We can hypothesized an involvement in the preservation of BBB integrity or an influence in the synthesis of proinflammatory cytokines, in a direct or indirect way (for example through TNF- α synthesis). This may, in turn, reverberate on astrocytes and microglia reducing their expression, according to the temporal sequences of the observed effects.

Mossy fibers sprouting is also affected by local NTFs treatment, in a way that correlates with the preservation from cell damage.

Taken together, these data may explain the reduction in frequency and severity of spontaneous recurrent seizures that animals experience during the chronic phase of the illness.

We are fully conscious that, in both cases, no answer on the mechanisms of action have been provided in this thesis. This was out of the purposes of our studies. However, is in our intent to investigate them in the future, through *in vitro* systems such as microglia or astrocyte cell cultures.

We strongly believe that these results will add a significant insight into the characterization and comprehension of the wide properties of NTFs.

Finally, an additional step has been provided from the study of routes of administration useful in conveying the neurotrophic factors to the brain. Genetically modified viral vectors could be a valid approach, if not for their residual toxicity which prevents their application in humans. Mesoangioblasts (MABs) are progenitor stem cells that can be modified to express genes of interest. *In vitro* characterization of MABs-NGF and MABs-BDNF has shown a real efficacy in promoting differentiation, survival and functionality of neurons and, not secondary, the ability to localize in a lesioned brain when peripherally administered in an animal model of Alzheimer's disease.

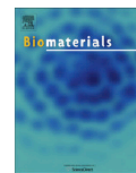
Obviously, further studies are needed to better characterize MABs and to evaluate their usefulness in different pathological contexts. However, we are confident that our results can contribute to move NTFs-gene therapy closer to clinical application.

ADDENDUM



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Biomaterials

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The biocompatibility of materials used in printed circuit board technologies with respect to primary neuronal and K562 cells

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ABSTRACT

Printed circuit board (PCB) technology can be used for producing lab-on-a-chip (LOAC) devices. PCBs are characterized by low production costs and large-scale development, both essential elements in the frame of disposable applications. LOAC platforms have been employed not only for diagnostic and/or analytical purposes, but also for identification and isolation of eukaryotic cells, including cancer and stem cells. Accordingly, the compatibility of the employed materials with the biological system under analysis is critical for the development of LOAC devices to be proposed for efficient and safe cell isolation. In this study, we analyzed the *in-vitro* compatibility of a large set of materials and surface treatments used for LOAC development and evaluation with quasi-standard PCB processes. Biocompatibility was analyzed on hippocampal primary cells (a model of attached cell cultures), in comparison with the reference K562 cell line (a model of cells growing in suspension). We demonstrate here that some of the materials under study alter survival, organization, morphology and adhesion capacity of hippocampal cells, and inhibit growth and differentiation of K562 cells. Nonetheless, a subset of the materials tested did not negatively affect these functions, thus demonstrating that PCB technology, with some limitations, is suitable for the realization of LOAC devices well compatible at least with these preparations.

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1. Introduction

The development of lab-on-a-chip (LOAC) devices for biomedical applications, including manipulation of single cells, is an exciting new research field requiring strict collaboration between electronic engineers and molecular/cellular biologists [1–9]. In the process of LOAC designing and manufacturing, one crucial issue is the choice of biomaterials. The development of modern

biomaterials is more critical for LOAC designed for identification and isolation of eukaryotic cells (like cancer and stem cells) than for diagnostic or analytical applications; for such applications, LOAC platforms have to guarantee isolation of biologically active cells whose growth capacity, differentiation potential and biological properties in general should not be altered by the LOAC-based manipulation. Hence, materials with good biocompatibility need to be used for these applications, even if the eukaryotic cells to be isolated and manipulated are expected to be exposed for a short length of time [10].

In the past few years, printed circuit board (PCB) technology has reached a resolution of tens of micrometers, which is enough for many microfluidics applications [10–13]. The main limitation of this approach in biomedical applications is the biocompatibility of the constituent materials. Recently the range of materials for PCB technologies has been extended. Many have already been developed for

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flex circuits and rapidly become an industrial standard. Cost-effective technologies have also been proposed by introducing additional preparatory steps [14,15] or by testing corrosion-resistant materials (like aluminum) in standard PCB processes [16]. The use of these “board technologies” is particularly relevant to the development of microtiter plates with sensing and actuating features that allow fast, parallel manipulation and analysis of biological samples [17–19]. The adaptation of standard PCB processes, which ensure low production costs and large-scale development, may here boost progress in the design of many disposable applications.

This paper proposes a method for screening LOAC production materials for PCB-related biocompatibility with respect to primary eukaryotic cells growing attached to culture plates [20]. The results have been compared with a standard cell line growing in suspension and undergoing differentiation in a reproducible fashion [21–24].

The first model system, hippocampal primary cell cultures, is obtained from 18–19 day rat embryos. These cell cultures have the characteristics of adhering to the substrate and of growing in monolayers when plated at low density. Their composition is restricted to only two cellular phenotypes (astrocytes and neurons) [20]. These characteristics allow them to be used as a model for investigating modifications in density, organization, phenotype ratio during growth and maturation, as well as survival and maintenance of morphology in time. The second model system (K562 cells) has been developed from a patient suffering from chronic myelogenous leukemia during a blast crisis [21]. K562 cells grow in suspension and undergo erythroid differentiation when cultured with a variety of stimuli of different chemical structure, origin and action mechanism, including drugs like angelicin [22], tallimustine [23], rapamycin [24] and mithramycin [25]. These characteristics allow one to use these cells as a model for investigating modifications in cell growth and differentiation.

2. Materials and methods

2.1. Materials

As a rule, resin-coated copper (RCC) and glass fiber-filled epoxy films are used in standard PCB manufacturing processes. These materials are not applicable to an LOAC device due (1) to non-biocompatibility of the copper (Cu) and (2) to difficulty in processing the fiber-filled epoxies by laser structuring of materials as required for microwell realization. To deal with the first problem, we tested alternative metal layers or combinations of Cu with alternative metals, including aluminum (Al), palladium (Pd), nickel (Ni) and gold (Au). To deal with the second problem, flexible substrates based on polyimide and pyralux, a b-stage modified acrylic adhesive, were considered as an alternative to FR-4 (flame retardant 4), an epoxy resin commonly used for the construction of rigid PCBs. B-stage means an intermediate stage in the cure reaction of a thermosetting resin, at which the material is not fully cured and can be further processed. Upon completion of processing, including lamination for PCB manufacturing, the polymer becomes fully cured. In the present article, materials will be referred to as *uncured* (the material as delivered), *b-stage* (intermediate stage of the curing process) and *cured* (after complete processing). In the frame of this study, we tested pyralux, polyimide (PI) as bondplay or coverlay materials (from DuPont), transparent polyurethane film 4201 (TPU; from Epurex Films GmbH & Co.KG), printable epoxy (PEP, SEMICOAT 513E; from Shin-Etsu Chemical Co), aramid fiber-filled epoxy F161 (AFEP; from Hexcel) and die-attached film (DAF, Adwill LE5000; from Lintec Corporation) for their biocompatibility and for their processability as dielectric layers. All the polymers listed above are commercially available materials. Thus their detailed chemical description is the property of the suppliers.

For realization of the final LOAC device, some additional materials are needed: (a) materials for hydrophobic/hydrophilic surface modification and (b) adhesive tapes for adding special features to the device (for example a membrane). In this study, we tested the biocompatibility of the following surface treatment materials: 1-octadecanethiol C18 (ODT), certonal FC732 and chemlease 41-90 (from Chemtrend LP). Finally, we also tested the following adhesive tapes: CMC 15581 (from CMC Klebtechnik GmbH), Tesa 4983 and Tesa 4985.

A list of all materials analyzed in this study, with the corresponding abbreviations, is shown in Table 1.

2.1.1. Metals

Cu is the standard metal in a PCB but, due to its high chemical instability in an aqueous environment, it cannot be used in bio-devices. To overcome this

Table 1
Materials analyzed in the study.

Group	Material	Abbreviation	Treatment
Metals	Aluminum	Al	
	Gold over nickel over copper	Ni–Au	
	Gold over palladium over nickel over copper	Pd–Au	
	Copper	Cu	
	Palladium over copper	Pd	
Surface treatments	Gold over nickel over copper ODT covered	Ni–Au ODT covered	
	Gold over palladium over nickel over copper ODT covered	Pd–Au ODT covered	
	Copper ODT covered	Cu ODT covered	
	Palladium over copper ODT covered	Pd ODT covered	
	Certonal FC732	Certonal FC732	
	Chemlease 41-90	Chemlease 41-90	
Dielectrics	Aramid fiber-filled epoxies F161 cured	AFEP	Cured; b-stage
	Die-attached film	DAF	
	Printable epoxy	PEP	b-stage
	Polyimide	PI	
	Transparent polyurethane film 4201	TPU	Processed; unprocessed
	Pyralux LF0300	Pyralux	Cured; b-stage
Adhesive tapes	CMC 15581	CMC 15581	
	Tesa 4983	Tesa 4983	
	Tesa 4985	Tesa 4985	

limitation, Cu was tested with various different metal cover layers. Pd, Ni–Au and Pd–Au were applied by an electroless process to a copper substrate [17]. Electroless plating is an auto-catalytic chemical technique used to deposit a layer of ions (e.g. Ni or Pd) on a solid substrate [18]. Au and Pd were always used in combination with Ni, because otherwise they do not attach to Cu. Additionally, Al (cheap and widely available as a foil) was tested to replace copper in the PCB process.

2.1.2. Dielectrics

PI is used in the electronics industry for flexible PCBs or as a high-temperature adhesive. Pyralux LF0300 (tested thickness: 76 μm) is a b-stage acrylic adhesive, available in a wide range of thicknesses and in different forms. TPUs are widely used as flexible and rigid foams, durable elastomers and high performance adhesives and sealants. They are also available as films, which can be applied in the lamination process. AFEP may be applied as an alternative to the standard glass-filled epoxies used for rigid substrates, because microwell drilling by laser ablation is not possible for glass fiber-filled materials. Before processing, this film is also at a b-stage. PEP resins are liquid polymers used as adhesives or encapsulants in microelectronics. They can be applied by screen or stencil printing over large areas and can also be used in a lamination process. Finally, DAF is a polymer film (thickness 120 μm) consisting of a thermosetting and UV curable resin. This commercially available film could also be used for large-area lamination of different layers, as is needed in the case of a multilayer PCB.

2.1.3. Surface treatments

The surface modification is critical in the case of electrodes, because it is important to change the surface hydrophobic/hydrophilic properties while preserving electrical and thermal conductance. The interaction of alkanethiols with metal surfaces has attracted considerable interest because of the tendency of the thiolate species to form self-assembled monolayers (SAMs) on metals. ODT is a well known SAM [27,28] that allows one to tune the hydrophobicity of Au, Pd and Cu. Moreover, ODT passivates the metal with a protective mono-atomic layer, which can be bypassed by increasing the frequency of the electrical signal. The ODT SAM also presents good thermal stability up to 50 $^{\circ}\text{C}$ [29].

Certonal FC732 and Chemlease 41-90 molding release agents were also tested for biocompatibility as a hydrophobic coating for metals and dielectrics.

2.1.4. Adhesive tapes

Transfer tapes can be used for cold bonding. They are double-sided tapes, available with several thicknesses and adhesives. The acrylic adhesive transfer films

Tesa 4985 and Tesa 4983 (Tesa tape, USA) and CMC 15581 (CMC technical tapes, Germany) were selected for this study.

2.1.5. Poly-dimethylsiloxane

Finally, poly-dimethylsiloxane (PDMS) was chosen as the reference material and was utilized for sample preparation for biological testing (see next section). PDMS is extensively used for fabrication of microfluidic devices [30]. It is manufactured in multiple viscosities, which allow thicknesses from microns to millimeters. It is hydrophobic and chemically inert toward most reagents.

2.2. Preparation of materials for biological testing

The materials under test were prepared one by one with the same process parameters as used to fabricate the PCB LOAC. They were cut in samples and embedded in standard multiwell plates for cell culture (Costar – Corning, USA) in which biological experiments for biocompatibility had been carried out. A detailed description of the preparation of the various materials is provided below.

2.2.1. Metals

Pd, Ni–Au and Pd–Au were applied on Cu substrates by electroless deposition. A chemical bath was used to deposit Pd and Au on Cu substrates. The Cu substrates were cleaned in 0.5% HCl for 30 s. For Pd, Ni was used as the adhesion layer (15 min at 90 °C) followed by Pd activation (1 min at 55 °C) and Pd deposition (30 min at 60 °C). For Ni–Au, Ni was used as the adhesion layer (15 min at 90 °C) followed by Au activation (1 min at 55 °C) and Au deposition (30 min at 50 °C). Finally, for Pd–Au, Ni was used as the adhesion layer (15 min at 90 °C) followed by Pd activation (1 min at 55 °C), Pd deposition (30 min at 60 °C) and Au deposition (30 min at 50 °C, flash gold). The resulting layer thicknesses are 5 µm Ni, 100–300 µm Pd and 60–80 µm Au.

2.2.2. Foil materials

The foils of materials were cut in round samples by laser machining: 5.7 mm in diameter for 96-well and 15 mm for 24-well. A blue foil was used to cover the material during cutting, to prevent surface contamination due to particles of melted material. Samples were embedded in the well with a droplet of PDMS. PDMS was partially dried for 5 min at 45 °C after dispensing. The round samples were deposited by a vacuum tip (EFD, USA) applying a light pressure. The protocol for dispensing, heating and sample deposition was repeated for each row of the multiwell plate. Finally, PDMS was cured at 45 °C for 24 h.

Al (thickness 18 µm), Cu (136 µm) and Pt (100 µm) were bought in foils and cut without any further process.

Pyrulux (DuPont, USA), AFEP (HEXCEL, California) and TPU (Epurex Films – Bayer MaterialsScience, Germany) are processed in thermal steps during PCB fabrication. They were tested as uncured material, both in the delivery and in the cured state, after processing. Thermosetting materials such as pyralux or epoxy resins are either liquid or solid at room temperature and consist of a resin and a hardener. To reach their final properties, these materials must be cured, i.e. they must be held at “curing temperature”, so that the polymer reaches low viscosity and the reactive groups of resin and hardener can form the stable network. This curing process can take from a few seconds to several hours and is finished when a high percentage (>99.5%) of all possible reactive groups have reacted. Due to the difficulty of guaranteeing 100% curing of the internal layers during PCB fabrication, pyralux and AFEP were tested both in the uncured and in the cured state. TPU, although it is a thermoplastic material, was tested before and after its thermal cycle (i.e. as delivered or after processing). A Lauffer LC40/2E HAT Vacuum PCB-Laminating Press System (Germany) was employed to laminate these materials. Lamination processes were adapted as recommended by the supplier in the datasheets accompanying each material. PEP (SEMICOAT513E, Shin-Etsu Chemical Co. LTD., Japan) requires up to 150 °C to be cured (60 min at 100 °C + 90 min at 150 °C). This temperature is not compatible with polystyrene-made multiwell plates, whose glass transition temperature is 95 °C. To overcome this limitation a film of epoxy, 100 µm thick, was patterned onto a support covered with cured doubling-silicone for dental copy (SUPERIUM Dubliersilikon, Weber Dental, Germany), then cured, peeled out as a foil and cut by laser. The hydrophobic capacity of the doubling-silicone prevented the epoxy film from sticking to the support during curing. DAF was only tested in the delivered b-stage.

2.2.3. Certonal, Chemlease and PDMS

Certonal FC732 was deposited, filling the well to half of its volume for 5 min, then rinsed with water and dried with a nitrogen flux at room temperature. A thin layer of Chemlease 41–90 was applied on an Al foil –used as a support–, tempered for 20 min at 150 °C on hotplate and cut in samples. PDMS (Sylgard 184, DowCorning, USA) was prepared as follows: the two components of Sylgard 184 were mixed at a ratio of 1:10 for 5 min and degassed for 30 min at 0.1 bar (4 min was required to reach 0.1 bar). It was then cured in the well at 45 °C overnight.

2.2.4. ODT

Au, Pd and Cu surfaces were hydrophobically functionalized with ODT (Sigma–Aldrich, USA). ODT 1 mm was prepared in ethanol. After deposition, the samples were rinsed with ethanol and dried in a stream of high purity nitrogen.

2.2.5. Adhesive tapes

The acrylic adhesive tapes Tesa 4985, Tesa 4983, and CMC 15581 were prepared in multiwells. The tapes, protected on both sides by cover paper, were laser cut in samples, cleaned with deionized water and dried in a stream of nitrogen.

2.2.6. Sterilization

All materials were UV-light sterilized before challenge.

2.3. Cell cultures used for biomaterial tests

2.3.1. Hippocampal primary cultures

Hippocampal cells were obtained from 18–19 day old rat embryos. Briefly, hippocampi were dissected in a modified Hank's balanced salt solution (HBSS, Sigma) containing Hepes 10 µl/ml (Sigma, USA), sodium pyruvate 10 µl/ml (Gibco) and penicillin/streptomycin 2 µl/ml (Sigma); cells were released from tissue in HBSS solution with trypsin 0.025% (Sigma) for 10 min at 37 °C. Enzyme activity was stopped by adding 2% fetal bovine serum (FBS, Gibco). Cell separation was performed by passing the solution through a Pasteur pipette. The mix was centrifuged for 10 min at 45g, the supernatant was discarded and the cell pellet resuspended in a modified Neurobasal medium (Gibco) containing Hepes 10 µl/ml, sodium pyruvate 10 µl/ml, penicillin/streptomycin 2 µl/ml and L-glutamine 2.5 µl/ml (Sigma) after passing it through a 25 gauge needle and a 70 µm cell strainer. Finally, cell density was determined using a hemocytometer and set at 1.2×10^6 cells/ml. The cells obtained (500 µl per well), supplemented with 10% FBS, were then exposed to the various different materials for 1 h at 37 °C. The choice of exposing cells for 1 h relates to the time that the biological material is expected to remain in the LOAC device; in no instance is this expected to exceed 20–30 min. The challenge was always performed in duplicate.

After incubation, wells were scraped and washed in 500 µl fresh medium. Scraping was chosen instead of enzymatic dissociation because, after only 1-h incubation (the one together with the material) the level of adhesion is limited and scraping causes negligible damage. In contrast, we found that trypsin and other enzymes cause more pronounced (sometimes toxic) alterations (MM, unpublished observations). The resulting cell suspension was collected in a 5 ml tube. After 10 min centrifugation at 45g, the supernatant was discarded and the pellet re-suspended in a constant volume, the same as used to obtain a $1-2 \times 10^5$ cells/well under control conditions (cells not exposed to any material). Finally, 500 µl was plated on sterilized glass coverslips coated with an adhesion substrate (poly-L-ornithine 1 mg/ml and laminin 10 µg/ml, Sigma) and kept in the incubator at 37 °C for 24 h. The following day, the medium was changed with a fresh, serum-free neurobasal medium supplemented with 20 µl/ml B27 (Gibco). Experiments were performed both on immature (5 days *in vitro*) cells and at a later stage, by which they are expected to have developed into mature cells (9–10 days *in vitro*).

It should be emphasized that in this protocol materials are only in contact with serum for 1 h. Long exposure to serum (24 h or more) may alter the material's properties. As stated above, 1 h is sufficient to model the operational situation in the LOAC. Furthermore, the same batch of serum was employed in all experiments to avoid batch-to-batch variability and, 24 h after transfer to wells, the serum was withdrawn from the medium. To make sure that the presence of serum was not affecting results, experiments in serum-free conditions were conducted in K562 cells (below).

2.3.2. K562 cells

Human erythroleukemia K562 cells [22] were cultured in a humidified atmosphere at 5% CO₂ in RPMI (Roswell Park Memorial Institute) 1640 medium (Sigma) supplemented with 10% FBS (Celbio, Italy), 2 mM L-glutamine (Sigma), 50 units/ml penicillin (Sigma) and 50 µg/ml streptomycin (Sigma).

The same considerations as to the use of serum described above for primary neuronal cultures also apply here. However, to exclude possible interference on the results by serum, a series of experiments were also conducted under serum-free conditions.

2.4. Biological testing of biomaterials

2.4.1. Analysis of hippocampal culture response after exposure to the different materials

Immunofluorescence was carried out on 4% paraformaldehyde (PFA) fixed cells, using (1) a neuronal marker, the microtubule associated protein 2abc (MAP2abc), detected by a specific antibody (anti-MAP2abc mouse monoclonal, 1:500 dilution, Chemicon, USA) revealed using an anti-mouse secondary antibody conjugated with a green fluorochrome (Alexa Fluor[®]488-conjugated secondary goat anti-mouse antibody, 1:500 dilution, Invitrogen); (2) an astrocyte marker, glial fibrillary acid protein (GFAP), detected by a specific antibody (anti-GFAP rabbit polyclonal, 1:100 dilution, Sigma) revealed using a red fluorochrome (Alexa-Fluor[®]594-conjugated affine pure goat anti-rabbit antibody, 1:500 dilution, Invitrogen). To allow precise measurement of the cell density, nuclei were also stained, using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 100 ng/ml, Sigma). Finally, coverslips were mounted on slides using an aqueous antifading gel mount (Gelmount Biomedica, BioOptica, Italy). In order to assay mortality, cells were exposed to a propidium

iodide solution (Sigma): 5 µg/ml for 10 min at 37 °C in the dark. They were then fixed in 4% PFA and treated with DAPI for 3 min. Coverslips were finally mounted on slides (as above). Labeled cells were examined using a fluorescent microscope system (CTR MIC) and FW4000 software (Leica, Germany).

Image analysis was performed using the "ImageJ" software (Image Processing and Analysis in Java), a public domain Java image processing program inspired by NIH Image. The mortality rate, density, phenotype (neurons to astrocytes ratio) and organization (cluster aggregation) were evaluated by three operators under double-blind conditions. At least 5 different fields (464 × 346 pixels) randomly taken from at least 3 different wells were analyzed for each material. The percentage of dead cells (mortality rate) was quantified by counting the ratio between propidium iodide-positive (dead) and DAPI-positive (all) cells in fields captured at a magnification of 200×. Cell density was estimated counting the total number of DAPI-positive cells in fields taken at a magnification of 100×. The phenotype was the ratio between MAP2abc-positive (neurons) and GFAP-positive (astrocytes) cells, calculated in fields taken at a magnification of 100×. Culture organization was quantified using DAPI, counting the number of clusters in fields taken at a magnification of 100×; clusters were defined as aggregates of a minimum of 5 cells that were less than 5 µm apart from each other. Data are means ± SE of the 5 determinations in the 5 fields examined for each parameter. Statistical analysis was performed using ANOVA and post-hoc Dunnett's multiple comparison test.

2.4.2. Analysis of proliferation and differentiation of K562 cells

After pulse incubation (60 min) with the various materials, K562 cells were seeded at 30,000 cells/ml. Cell growth (proliferation) was studied by determining the cell number/ml after 4 days of *in vitro* cell culture using a ZF Coulter Counter (Coulter Electronics, USA). In order to determine the effects of the materials on erythroid differentiation, K562 cells were treated with 25 nM mithramycin, a powerful inducer of erythroid differentiation [25,26]. The proportion of benzidine-positive cells (which contain hemoglobin, an index of erythroid differentiation) was determined after 6 days in culture using a solution containing 0.2% benzidine in 5 M glacial acetic acid (10% H₂O₂), as previously described [24].

3. Results

3.1. Effects of materials on hippocampal cell cultures

DAF and AFEP b-stage strongly modified cell adhesion: cells did not attach to the coated glass coverslips after challenge with these materials and, 24 h later, all cells were still in suspension. Hence these plates were discarded and the materials were not included in the following tests. Representative images of the best and worst culture situations after exposure to materials are shown in Fig. 1 (with respect to mortality, cell density, phenotype, organization and general morphology) and in Fig. 2 (overall evaluation). The scores and statistical analysis for the different materials are reported in Fig. 3.

Analysis of the effects of materials on cell vitality was performed using the propidium iodide test, a well-known mortality assay [31,32]. Results using this test show good compatibility for nearly all materials, with the exception of PEP b-stage, which displayed a high percentage of cell death (Figs. 1A and B, 3A).

Total cell density was quantified as the number of DAPI-positive nuclei (Figs. 1C and D, 3B). As expected, Cu was found to affect this parameter, an indication of its toxicity. When covered with other metals, however, Cu did not produce any negative effect. Al did not affect cell density at all. Interestingly, when metals were covered with ODT (for use as surface treatments) they tended to produce a decrease in cell density, with the relevant exception of Ni–Au ODT. Other surface treatments did not produce negative effects (Chemlease 41–90 actually increased density). With the exceptions of PEP b-stage and, obviously, of DAF and AFEP b-stage (which could not be evaluated because they did not allow adhesion – see above), most of the dielectrics did not affect culture density. Finally, only Tesa 4985 produced a significant decrease in the number of cells.

This primary cell culture is characterized by two main cell phenotypes: neurons and astrocytes. It is well known that, during the first days in culture (immature stage) neurons are the predominant cell type; in time, the number of replicating astrocytes increases, while the number of non-replicating neurons

remains essentially stable. In particular, the protocol employed in this study was set to obtain a high ratio between neurons and astrocytes. Some of the materials analyzed affected this ratio to a considerable degree (Figs. 1E and F, 3C). Among metals, only Al and Ni–Au appeared to be completely safe on this parameter. In general, surface treatments did not appear to significantly alter the culture phenotype, with the exception of Pd–Au ODT and (most prominently) Cu ODT. Four dielectrics (namely PEP b-stage, PI, TPU processed and TPU unprocessed) again did not affect the phenotype. Finally, none of the adhesives modified this parameter.

Cell organization was evaluated as the cell culture capacity to organize into a monolayer network without formation of clusters. In neuronal primary cultures formation of clusters is regarded as a sign of suffering. During normal maturation, cells produce many different factors, some necessary for survival and others maintaining the organization essential for survival and function. Clustering is a sign of culture suffering that could lead to cell detachment and death. Not surprisingly, a "normal" tendency to form clusters is observed in controls after several days in culture. These data are shown in Fig. 1G and H and in Fig. 3D. Metals did not alter this parameter; the apparent decrease in cluster formation with Cu is actually caused by the extensive cell loss (see evaluation of cell density above), in that the number of cells in the dish was simply too small to form clusters. The only surface treatments that increased clusters were Ni–Au ODT and Certonal FC732. By contrast, many dielectrics produced a significant effect on this parameter: AFEP cured, PEP b-stage, pyralux cured and pyralux b-stage. Bearing in mind that DAF and AFEP b-stage could not be evaluated, only PI and TPU (both processed and unprocessed) appear to be safe. Finally, among adhesives only Tesa 4983 increased cluster formation.

The morphology of the cells and of the entire culture, evaluated mainly as the number of contacts (synapses) that were established, is clearly a parameter to prove the good health of a culture. In this respect, we did not observe any clear difference between the control cultures and cultures kept in contact with the various different materials, with the exception of Cu, Cu ODT, Pd ODT and Tesa 4985 (Fig. 1I and J).

Overall, the materials that scored positive on all parameters (and that could therefore be recommended for use) are as follows: Al and Ni–Au among metals, Chemlease 41–90 among surface treatments, PI and TPU (both processed and unprocessed) among dielectrics, CMC 15581 among adhesives.

3.2. Effects of materials on *in vitro* proliferation and erythroid differentiation of K562 cells

K562 cell proliferation was estimated by determining the cell number/ml after 4 days of *in vitro* cell culture using a ZF Coulter Counter. The results are summarized in Fig. 4A. Only Pd–Au and AFEP b-stage displayed a significant inhibitory activity.

Analysis of the effects of the materials on erythroid differentiation by K562 cells was performed by treating the cells with mithramycin, a powerful inducer of erythroid differentiation. After 6 days culturing with mithramycin, the proportion of benzidine positive (erythroid, hemoglobin-containing cells) was analyzed using the benzidine-test. The results are reported in Fig. 4B. Among metals, expectedly, Cu impaired differentiation. Again, Cu ODT was the only surface treatment that proved unsafe. Among dielectrics, AFEP b-stage (in a very dramatic manner) and TPU processed (to a much lesser extent) affected differentiation. Finally, no adhesive produced a significant effect on this parameter.

As described above (see Materials and methods) long exposures to serum (24 h or more) may alter the material's properties but, in our protocol, materials were in contact with serum only for 1 h. In any event, we verified that this brief exposure to serum was not

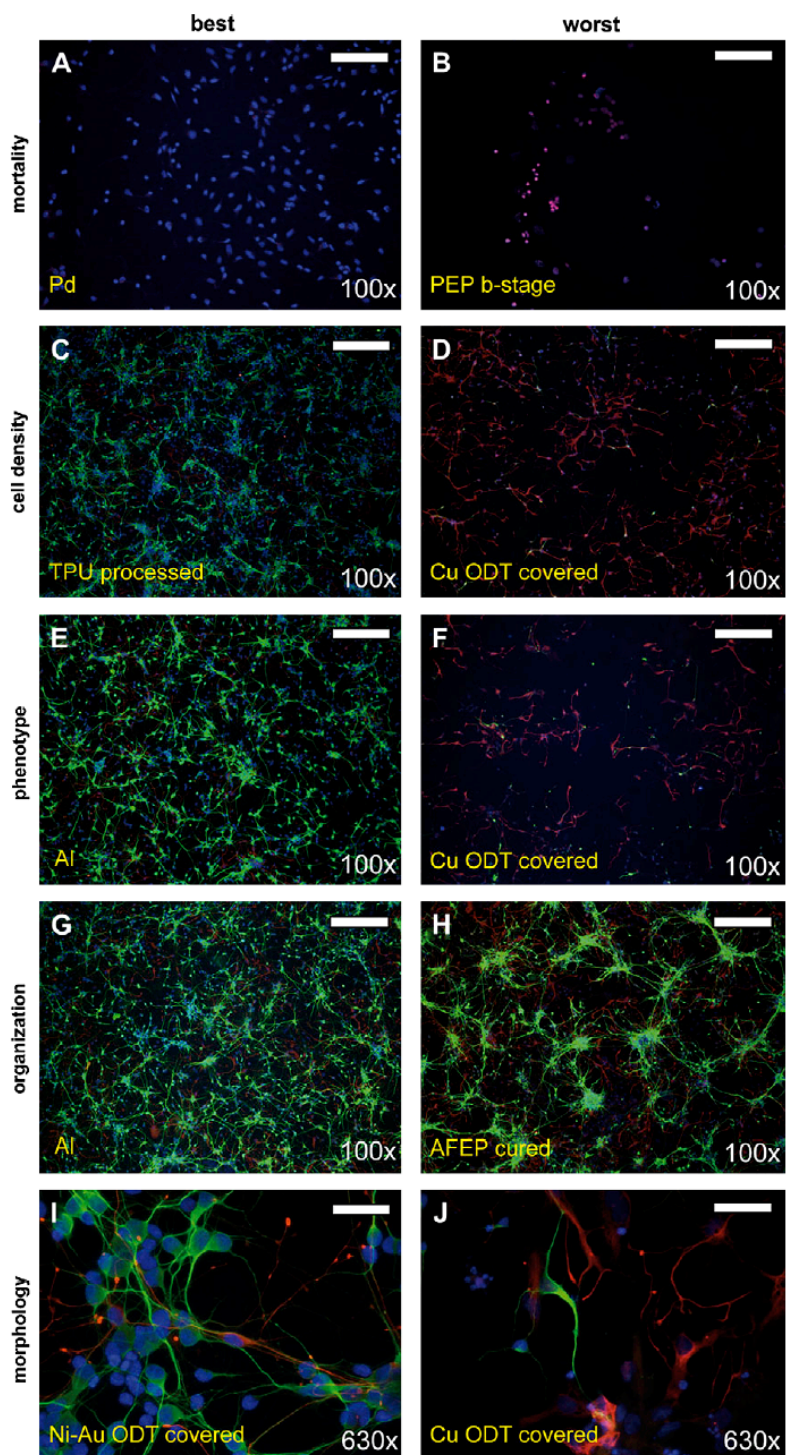


Fig. 1. Examples of best and worst materials, based on the different parameters analyzed in hippocampal primary cell cultures. All cultures were 5 days old. Propidium iodide test displays in red the dying nuclei (A and B). Other nuclei are labeled in blue using DAPI. Immunofluorescence shows MAP2abc-positive neurons in green, GFAP-positive astrocytes in red and DAPI-labeled nuclei in blue (C–J). Horizontal bar = 50 μ m in A to H, 15 μ m in I and J.

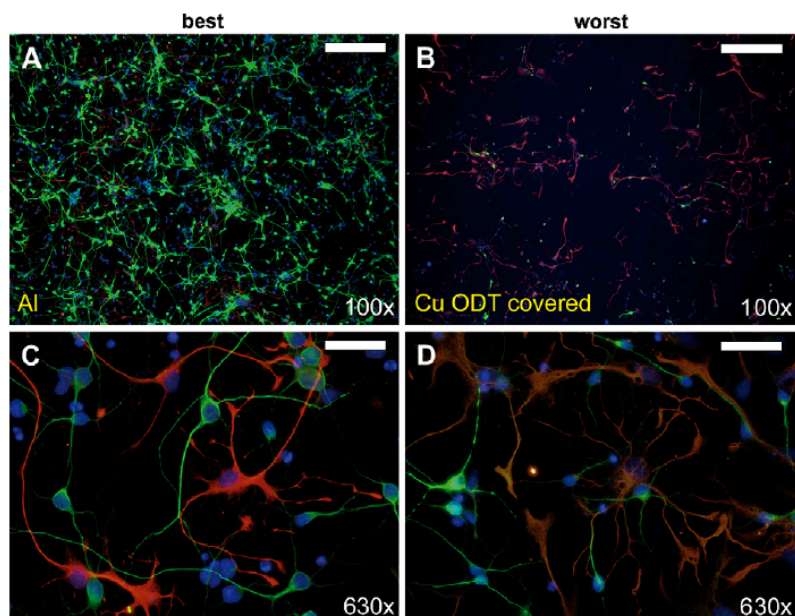


Fig. 2. Overall best and the worst materials based on all the different parameters analyzed in hippocampal primary cell cultures. Hippocampal cell culture growth for 5 days after 1 h exposure to Al (A and C) or to Cu ODT covered (B and D). Immunofluorescence shows MAP2abc-positive neurons in green, GFAP-positive astrocytes in red and DAPI-labeled nuclei in blue. Horizontal bar = 50 μ m in A and B, 15 μ m in C and D.

affecting results by conducting another series of experiments in which K562 cells were exposed to materials in the presence and absence of serum, side-by-side in adjacent wells. We chose to analyze two materials from each group: the one performing best and the one performing worst in the parameters employed in this study. In no instance, significant differences in the proliferation and differentiation of K562 cells were observed when in the presence or in the absence of serum (Fig. 5).

4. Discussion

Precise analysis of the biological effects of materials used to construct LOAC platforms with PCB technologies for cell manipulation is a necessary pre-requisite for designing possible applications of such devices to biomedicine and biotechnology. Materials with good biocompatibility need to be used for these applications, even if the eukaryotic cells to be isolated and manipulated are expected to be exposed for a short length of time [10]. Note, therefore, that the term “biocompatibility” is used here with reference to *in vitro* interactions between materials and cells and that, under such conditions, the material performs best if it does not produce any harm or change in cell function (the situation may be different *in vivo* for materials employed for tissue engineering, gene or cell delivery, and other applications in which the material is expected to perform a function [33]).

In this paper, we assessed the effects of various different candidate “biomaterials” on cell growth and expression of differentiated biological functions. To this end, we comparatively analyzed the effects on two very different experimental model systems: the first, hippocampal primary cultures of neuronal cells and astrocytes; the second, an *in vitro* established cell line capable of undergoing erythroid differentiation.

Taking into account all the parameters we considered on hippocampal cultures (namely cell adhesion, vitality, density, organization, differentiation and morphology), only a subset of

materials proved completely safe: Al and Ni–Au among metals; Chemlease 41-90 among surface treatments; PI and TPU (both processed and unprocessed) among dielectrics; CMC 15581 among adhesives. In contrast, other materials appear to be highly toxic: Cu (as expected) among metals; Cu ODT covered among surface treatments; AFEP b-stage, DAF, PEP b-stage and (to a somewhat lesser extent) AFEP cured and pyralux b-stage among dielectrics. The other tested materials scored well in some parameters, less well in others and, thus, appear to be usable with some caution.

The other model system we employed (K562 cells) allows one to evaluate parameters (namely proliferation and differentiation) that cannot be observed in hippocampal cultures, because neurons are terminal elements that cannot proliferate or differentiate. With reference to these other parameters, most materials appear to be safe. The only ones that scored very poorly were Cu and AFEP b-stage. Interestingly, these materials were found to be toxic even for neurons and should therefore always be avoided for LOAC construction for any kind of application. Some other materials did not score perfectly well in K562 cells: Cu ODT covered among surface treatments and TPU processed among dielectrics. These should be added to the above list of materials that may be employed but only with caution.

One interesting discrepancy between hippocampal cultures and K562 cells concerns DAF, which was highly toxic in the former and perfectly safe in the latter. It may be hypothesized that the toxicity of DAF is limited to cell adhesion, which prevents *in vitro* survival of neurons but does not affect K562 cells, which remain suspended. If this is the case, then one might speculate that certain materials may only be employed in selected instances, depending on the specific characteristics of the biological preparation to be applied to the LOAC.

One comment should be made on the exposure time of cells to substrates. Within the frame of this work, a 1-h exposure was considered as the representative worst case situation for most LOAC used to analyze and manipulate biological samples. This allowed us to identify a subset of the materials tested which is suitable for the

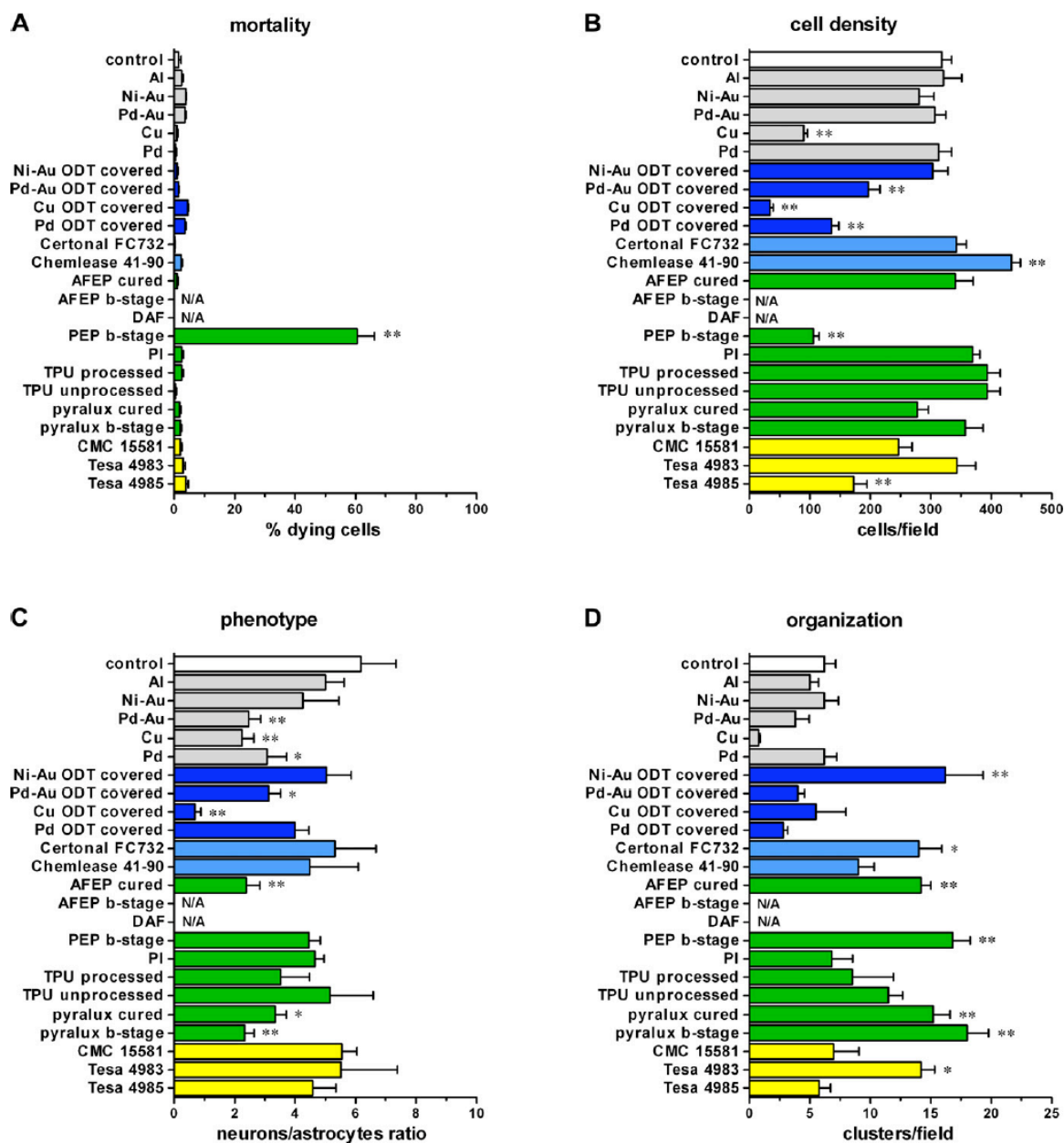


Fig. 3. Effects of different materials on mortality (A), cell density (B), phenotype (C) and organization (D) of rat hippocampal cells exposed for 1 h to the tested materials and then kept in culture for additional 5 days. Metals in gray, surface treatments in blue, dielectrics in green and adhesives in yellow. Data are the means \pm SE of 5 determinations in 5 fields examined for each parameter. * $P < 0.05$, ** $P < 0.01$, ANOVA and post-hoc Dunnett's multiple comparison test. N/A: not applicable (after exposure to these materials, cells became incapable to attach to the substrate).

production of LOAC devices based on PCB technology. However, other potential applications of LOAC platforms, such as on-chip cell and tissue culturing [34,35], will require further tests with more prolonged exposure times.

The set of materials identified as completely safe is sufficient to establish a process flow for the production of devices where cells can be manipulated and handled through properly designed

microfluidic structures and by means of dielectrophoretic forces resulting from non-uniform electric fields. The standard technology adopted for producing flexible PCBs can then be tuned, making use of materials which do not affect cell viability and functional characteristics. In particular, flexible circuits using PI as the substrate and Cu covered by Ni-Au as the metal can be produced using standard technologies and have demonstrated

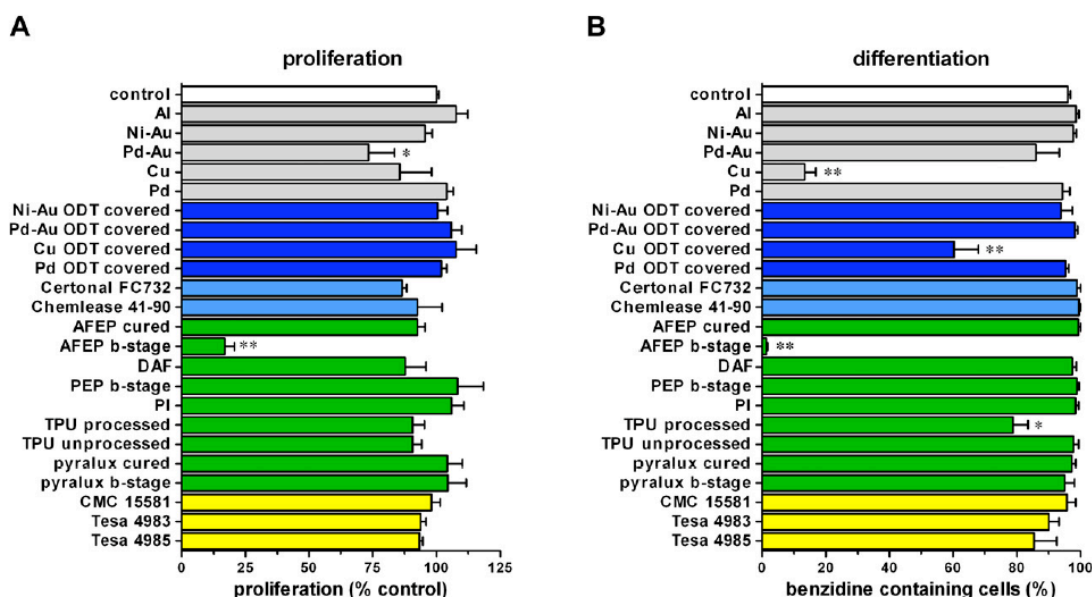


Fig. 4. Effect of different materials on *in vitro* growth of K562 cells. Cells were cultured in the absence (control) or in the presence of the different materials for 1 h, then transferred to standard cell culture conditions. (A) Proliferation (cell number/ml) determined after 4 days of culture. (B) Effects on mitramycin-induced erythroid differentiation of K562 cells. The percentage of benzidine-positive cells was determined after 6 days of cell culture. Data are the means \pm SE of 5 determinations for each parameter. * $P < 0.05$, ** $P < 0.01$, ANOVA and post-hoc Dunnett's multiple comparison test.

their applicability to the production of microfluidic structures [36,37], PCB devices [38] and biosensors, achieving cell manipulation at single-cell level [39]. The adoption of Al as the metal layer would not require any additional metallization process, as is required in the case of Cu, but this is not a standard material for the PCB process. Nonetheless, it was shown to be suitable for the

fabrication of platforms for cell handling, analysis and manipulation [40].

The apparent safety of Chemlease 41-90 makes it a good candidate for integration in the PCB process flow. The availability of a treatment of this kind is of great importance for the fabrication of microfluidic structures where the confinement of fluids to specific

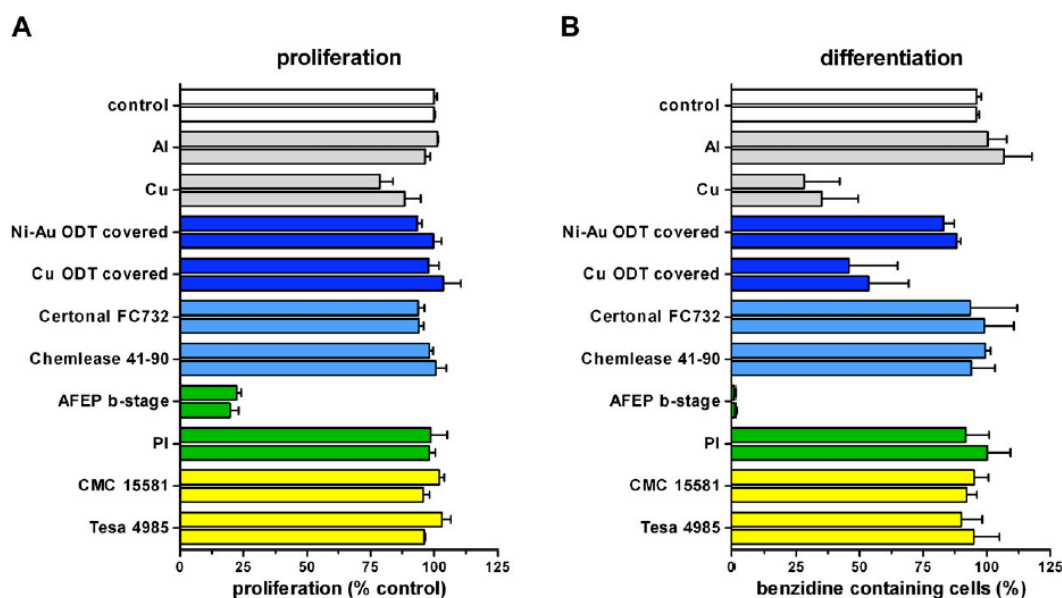


Fig. 5. Effect of materials on *in vitro* growth of K562 cells. Cells were cultured in the presence of the different materials for 1 h, either in the absence (top bar in the pair) or in the presence (bottom bar in the pair) of serum, and then transferred to standard cell culture conditions. (A) Proliferation (cell number/ml), determined after 4 days of culture. (B) Effects on mitramycin-induced erythroid differentiation. The percentage of benzidine-positive cells was determined after 6 days of cell culture. Data are the means \pm SE of 4 determinations for each parameter. No significant difference between absence and presence of serum.

regions of a device can be achieved by selective application of a hydrophobic coating.

Among materials which partially affected cell function, ODT applied on Ni–Au requires particular consideration. ODT, and more in general thiols, are important treatments widely used for the functionalization of surfaces through creation of self-assembled monolayers (SAMs). Thiols are typically bonded to gold surfaces and find application in the construction of biosensors and biomolecular electronic devices, as they provide a method for transducing the molecular properties of a biological sample into electrical signals [41]. The results of our work show that, in the case of adherent cell lines, utilization of ODT applied to Ni–Au electrodes calls for special care since it could affect cell organization (as happens to hippocampal cells). Nonetheless, in the case of a non-adherent cell line such as K562, it did not show any effect on either proliferation or differentiation.

5. Conclusions

Analysis of the two experimental cell systems, one growing in suspension, the other growing attached to well plates, brings a convergent conclusion as to the effects of materials employed in the construction of LOAC platforms based on a low-cost PCB technology. Screening of a total of 23 materials (5 metals, 6 employed in surface treatments, 9 dielectrics and 3 adhesives) led to identification of a subset of materials in each group that appear to be safe with respect to all the parameters of cell vitality and function that we analyzed: Al and Ni–Au among metals; Chemlease 41–90 among surface treatments; PI and TPU unprocessed among dielectrics; CMC 15581 among adhesives. Our analysis also demonstrates that different biological functions may be differentially affected by the same material. Thus, some materials that do not appear to be perfectly safe in all respects, may still be employed for applications in which the biological function(s) that they compromise is (are) not required. The positive biocompatibility results obtained for a subset of the materials tested suggest that technologies based on these materials, such as flexible PCB technology, can be considered promising for the development of biological platforms. The feasibility of PCB-based fabrication processes allowing for development of low-cost and large-area devices is demonstrated by the design and manufacturing of platforms based on the use of PI as the dielectric and Ni–Au or Al as the metal [20,39]. This technology forms a key step and a practical demonstration towards development of a disposable system for single cell handling [39].

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Appendix

Figure with essential color discrimination. All of the figures in this article have parts that are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.10.025.

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Effect of Phosphodiesterase 4 (PDE4) Inhibitors on c-fos Expression in the Forebrain

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INTRODUCTION

The clinical efficacy of rolipram and other PDE4 inhibitors has been hindered by their ability to cross the blood-brain barrier (BBB) and subsequently produce nausea and vomiting at therapeutic plasma concentrations (Krause and Kuhne, 1988; Burnouf and Pruniaux, 2002).

Therefore, the object of this research was to identify PDE4 inhibitors that do not cross the BBB or do not produce central effects.

To pursue this aim, we employed the activation of c-fos expression in the forebrain as readout. In fact, it has been reported that the reference compound Rolipram can acutely induce expression of the proto-oncogene c-fos in the cortex and in the accumbens (Svenningsson et al., 1995; Thompson et al., 2004; Bureau et al., 2006).

METHODS

Animals. Sprague-Dawley rats (300g) have been used for all experiments. Animals were kept under standard conditions: constant temperature (22-24°C) and humidity (55-65%), 12 h dark-light cycle, free access to food and water. Procedures involving animals were carried out in accordance with European Community and national laws and policies.

Identification of the time course of PDE4 inhibitor-induced c-fos expression. In a first set of experiments, rats have been killed 30, 60 and 120 min after rolipram administration (3 mol/kg i.v.) and their brains frozen and processed for c-fos in situ hybridization as described below. These experiments allowed identification of the optimal time-point for analysis. In all in situ hybridization experiments we used, as reference standard, pilocarpine treated rats (dose: 300 mg/kg, i.p); this drug is well known to induce strong c-fos activation 30 minutes after injection.

Effect of PDE4 inhibitors on *c-fos* expression. In a second set of experiments, rats have been killed at the appropriate time-point (30 min) after i.v. administration of the following PDE4 inhibitors: rolipram, roflumilast and a new test compound, refer to as compound “X”. All compounds were administered at the dose of 3 mol/kg. Again, rat brains have been frozen and processed for *c-fos* in situ hybridization as described below.

In situ hybridization. In situ hybridization experiments have been performed as previously described (Simonato et al., 1996; Barbieri et al., 2003). Briefly, antisense and sense *c-fos* riboprobes have been prepared from a full-length cDNA insert (kindly supplied by Dr. T. Curran). The full-length cDNA inserts are cloned in two SP65 plasmids containing the SP6 promoter and a single Xho1 restriction site. Plasmids have therefore been linearized with Xho1 and transcribed with SP6 RNA polymerase. Riboprobes have been obtained by running the transcription assays in the presence of α [³³P]-rUTP (New England Nuclear Perkin Elmer, Boston, MA, USA), and hydrolyzed to fragments of approximately 200 base pairs with sodium carbonate at 60°C.

Twenty μ m coronal sections were cut at 3 brain levels: accumbens (plate 11); somato-sensory cortex, anterior hypothalamus, paraventricular hypothalamic nucleus, basolateral amygdala, (plate 18), area postrema and nucleus of the solitary tract (plate 42, Paxinos et al., 1982). Sections were thaw-mounted onto polylysine coated slides, fixed in paraformaldehyde 4%, soaked in phosphate buffered saline (PBS) for 10 min at room temperature, then rinsed in a graded ethanol series and dried. Sections were then stored at -70°C until use. Immediately before in situ hybridization, they were pretreated with proteinase K (10 μ g/ml in Tris-EDTA buffer) for 10 min at 37°C and with acetic anhydride 0.25% v/v for 10 min at room temperature.

The in situ hybridization mixture contained 50% deionized formamide, 0.6 M NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 20 mM Tris (2x NTE), 5x Denhart's solution, 100 μ g/ml ssDNA, 100 μ g/ml tRNA, 0.05% sodium pyrophosphate and 60 ng/ml [³³P]-riboprobe. Sections were incubated overnight at 52°C with 40 μ l hybridization mixture. Following hybridization, they

were rinsed twice for 10 min in 4x saline-sodium citrate (SSC: 0.6 M NaCl, 0.06 M citric acid) and treated with RNAase A (20 µg/ml) for 30 min at 37°C. Sections were then rinsed in 1x SSC for 10 min, 0.1x SSC at 52°C for 30 min, 0.1x SSC at room temperature for 10 min, dehydrated through an ethanol series, and apposed to Kodak BioMax MR film (Kodak Italia, Milano, Italy) for 30-40 days. Films were developed in Kodak D-19 (5 min) and fixed in Kodak rapid fixer (5 min). The mean total optical density within an area of interest has been calculated by multiple sampling of that area in 3 sections taken from each animal using a digital analysis system (RBR Altair, Firenze, Italy). Background (non-specific) hybridization has been estimated using other sections incubated with the sense probe, and subtracted from the total optical density. Finally, data were expressed as percent of the average values obtained in controls (saline-treated animals).

Preparation of tissues for immunohistochemistry and immunofluorescence. To evaluate Fos protein expression, animals were killed after 60 minutes from the i.v. injection of the following substances: rolipram, roflumilast and the new compound X. This time-point was chosen because it is the peak of Fos protein expression when the peak of its mRNA is at 30 min (Dragunow and Faull, 1989). As in the previous experiment, all compounds were dissolved in a solution of PEG200 (80%) and water (20%) and subsequently administered at the dose of 3 mol/kg. After removal, brains were maintained in a 10% formalin solution for 24 h, then embedded in paraffin.

c-Fos immunohistochemistry. Immunohistochemistry has been performed on paraffin embedded coronal sections (6 µm thick), mounted onto polarized slides (Superfrost slides, Diapath). Sections were dewaxed, rehydrated and unmasked. After washing in PBS 1x, they were incubated for 10 min, at room temperature, with Endogenous Enzyme Block [Dako Cytomation EnVision® + Dual Link System-HRP (DAB+)] to quench endogenous peroxydase activity. Subsequently, they were incubated with the primary anti-Fos antibody (rabbit polyclonal, 1:200, Immunological Sciences) at room temperature. After 30 minutes, slices were rinsed twice with PBS 1x and incubated for another 30 min with Labeled Polymer-HRP [Dako Cytomation EnVision® + Dual

Link System-HRP (DAB+)]. Staining was completed by a 3 min incubation with 3,3'-diaminobenzidine (DAB) substrated-chromogen, resulting in a brown staining of the antigen-antibody complex. Finally, sections were counterstained with hematoxylin for 2 min and, after several washes, mounted using a water-based mounting medium (Shur Mount™, TBS).

Double-label immunofluorescence. Sections were dewaxed, rehydrated and unmasked. To block non-specific background, they were incubated with Ultra V Block (Ultra Vision Detection System; Lab Vision Corporation) for 5 minutes at room temperature. To block any background due to endogenous biotins, specimens were incubated with an avidin and then with a biotin solution (Avidin/Biotin Blocking kit, Zymed Laboratories Inc.), 10 min each. Then they were rinsed in PBS 1x and incubated with the primary antibodies overnight, in a humid atmosphere at 4°C: anti c-Fos (rabbit polyclonal, 1:100, Immunological Sciences) and anti-GFAP (mouse monoclonal, 1:100, Chemicon) or anti-MAP2abc (mouse monoclonal, 1:100, Immunological Sciences). The following day, sections were first incubated for 10 minutes with Biotinilated Goat Anti-Polyvalent (Lab Vision Corporation), rinsed in PBS and then incubated with secondary antibodies for 3 h: Alexa-594 streptavidin conjugated (1:200, Invitrogen) for c-Fos and Alexa-488 goat anti-mouse (1:500, Invitrogen) for GFAP or MAP2, respectively to label astrocytes or neurons. Finally, sections were washed, counterstained with 0,0001% DAPI for 15 min and washed again. Coverslips were mounted using Gel Mount water-based (Electron Microscopy Sciences). The specificity of immunolabeling has been verified by controls in which the primary antibodies were omitted. It must be noted that the use of very thin (6 µm), paraffin-embedded sections offers the advantages of very high resolution, of clear identification of double-stained cells (in that no overlap of multiple cell layers can occur), and of precise anatomical localization.

Intracerebroventricular (i.c.v.) injections. To test if the lack of effects of some compounds was due to inability to cross the BBB or inability to produce central effects, we administered them directly in the brain ventricles. Therefore, a guide cannula was implanted in a

group of animals. Rats were deeply anesthetized with ketamine/xilazine (87/13 mg/Kg), positioned in a Narishige stereotaxic apparatus and maintained under isoflurane flow for the duration of surgery. A sagittal incision has been made in the scalp and a hole has been drilled for placement of a guide cannula into a lateral cerebral ventricle (coordinates: AP: -0,2 mm; L: 1,5 mm from bregma). The animals were allowed to recover for one week and drugs were administered by means of a stainless-steel injector 0.5 mm longer than the guide cannula, so that its tip protruded into the ventricle. The accuracy of the injection coordinates has been verified by dye (Tripan blue) infusion at the end of each experiment.

Administration protocol. Rats have been killed 1 h after i.c.v. administration of 5 μ l of substances dissolved in DMSO. For dose/response curve of roflumilast, this substance was administered at the following doses: 2, 6, 12, 18, 36 μ g. On the bases of the results of this experiment, the dose of 36 μ g was chosen for testing the compound X. After removal, brains have been fixed in 10% formalin for 24 h, and then embedded in paraffin wax. Immunohistochemistry was performed as described above.

Quantification and statistical analysis. Histological analysis was conducted using a Leica microscope (DMRA2, Leica). The number of c-fos positive cells in each area of interest was quantified using a thresholding approach. Images of sections across the area of interest were captured using a Leica DFC300FX camera and transformed into gray levels. Using Photoshop CS2 (version 9.0.2), the mean \pm standard deviation gray level was calculated in visually identified Fos-positive nuclei. The area of interest was then cut out, and Fos-positive pixels were identified by thresholding at the gray level corresponding to the mean plus two standard deviations. Using this approach, only those pixels that were significantly above background (that is, Fos-positive) were selected. The percent of Fos-positive pixels over total pixels in the selected area was calculated in 2 sections taken from each animal, and then data were then expressed as percent of the average values obtained in controls (vehicle-treated animals).

RESULTS

Time-course of rolipram-induced *c-fos* expression. In the first set of experiments we defined the time-course of *c-fos* expression induced by rolipram. In keeping with previous reports, we found that the *c-fos* gene is not constitutively expressed in the normal brain (**Figure 1**, control). Rolipram (3 mol/kg, i.v.) induces *c-fos* expression in many brain areas; we studied the time-course of this effect in the somato-sensory cortex (layers I-II and V-VI), basolateral amygdala, ventromedial hypothalamus (plate 20). Peak induction of *c-fos* mRNA expression was observed 30 min after injection of the drug in all areas examined (**Figure 2**).

Comparative analysis of the effect of rolipram with the effects of other PDE4 inhibitors. Next, we comparatively examined the effect of rolipram on *c-fos* mRNA levels with those of other PDE4 inhibitors: roflumilast and substance X. All compounds were administered i.v. and the animals were killed at the time-point of peak rolipram-induced *c-fos* expression (30 min). In this experiment, we extended the observation and the measurements of *c-fos*-mRNA expression in other brain areas, including the paraventricular nucleus of the hypothalamus and the anterior hypothalamus. The rationale for this choice is that: (a) PDE4-labeled neurons were observed in these nuclei (Cherry and Davis, 1999; Bureau et al., 2006); (b) lithium produces emesis and nausea (Verbalis et al., 1987) and, in animals exposed to lithium, *c-fos* is found in a number of structures, including the amygdala, the area postrema/nucleus of the solitary tract (AP/NST), and the PVN (Gu et al., 1993).

As shown in **Figure 3**, rolipram (as described above) produced a robust increase in *c-fos* expression that resulted significant in all the brain areas considered and particularly in the paraventricular nucleus. Roflumilast produced an overall increase in *c-fos* expression, which reached statistical significance in the hypothalamus (paraventricular nucleus and anterior hypothalamus). The new compound X was devoid of any effect on *c-fos* mRNA levels in any of the

brain areas examined (**Figure 3**).

Analysis of *c-fos* activation induced by an alternative vehicle. The vehicle used to dissolve all substances in the above experiments was composed by 80% PEG200 and 20% water. I.v. Injection of this solution did not induce *c-fos* expression in the brain (**Figure 1**, control). In an attempt to evaluate the effects of higher doses of the substances under investigation (that could not be dissolved in the described vehicle), we studied an alternative vehicle composed by 40% PEG200, 25% ethanol and 35% saline. We found that this solution *per se* induces a robust activation of *c-fos* expression, as compared with the original vehicle, probably due to stress, pain or the elevated percentage of ethanol. In any event, this alternative vehicle could not be employed for the study.

Fos protein expression. As for Fos protein expression, rolipram was the only compound capable to induce significant activation in all areas examined (**Figure 4** and **Figure 5**). In particular, rolipram induced a highly significant ($P < 0.01$) increase in protein levels in the accumbens, anterior hypothalamus and in the paraventricular nucleus. These data are in substantial agreement with those obtained with the analysis of *c-fos* mRNA (above), except for the effect on the accumbens, that was much less pronounced on mRNA than on protein level (increased half-life of the protein?).

As compared with rolipram, roflumilast induced a smaller and spatially restricted increase in Fos protein expression, which was significant only in the accumbens ($P < 0.01$) and nearly significant in the anterior hypothalamus and in the paraventricular nucleus. Again, these data are in fairly good agreement with those on the mRNA, even if some discrepancies were observed in the areas where the changes reached statistical significance (compare **Figures 3** and **5**). As shown in **Figure 5**, the new PDE4 inhibitor did not produce any significant effect in the analyzed brain areas.

Identification of the phenotype of Fos-expressing cells. The *c-fos* gene encodes the nuclear protein Fos, which is considered a marker of activity for a variety of neuronal populations, being expressed only in neurons but not in astrocytes (Sagar et al., 1988; Harris, 1998). Using double label immunofluorescence, we confirmed that the protein is only expressed in neurons

(Figure 6).

Fos protein expression after i.c.v. administration. To identify an i.c.v. dose suitable for comparison between the different compounds, we attempted to generate a dose-response curve for roflumilast. This compound was injected at doses ranging between 2 and 36 g. Although we failed to observe a plateau effect up to this maximal dosage, we could nonetheless demonstrate that 36 g were capable of inducing significant effects in nearly all brain areas (Figure 7).

Next, we compared the effects of 36 g roflumilast with those of an equal dose of rolipram and of the test compound. As shown in Figure 8, rolipram produced effects more pronounced than roflumilast (which, incidentally, was less effective in this set of experiments than in the previous one, the dose-response curve). The new compound did not produce significant effects in any brain area, indicating that its possible inability to cross the BBB is not a critical factor.

CONCLUSIONS

The test compound did not demonstrate any effect even when administered i.c.v., at doses sufficient to produce robust Fos expression with rolipram and, to a lower level, with roflumilast. This indicates that a possible inability to cross the BBB is not a critical factor. Finally, double-label immunofluorescence experiments confirm the neuronal localization of PDE4 inhibitor-induced Fos protein.

FIGURES

Figure 1

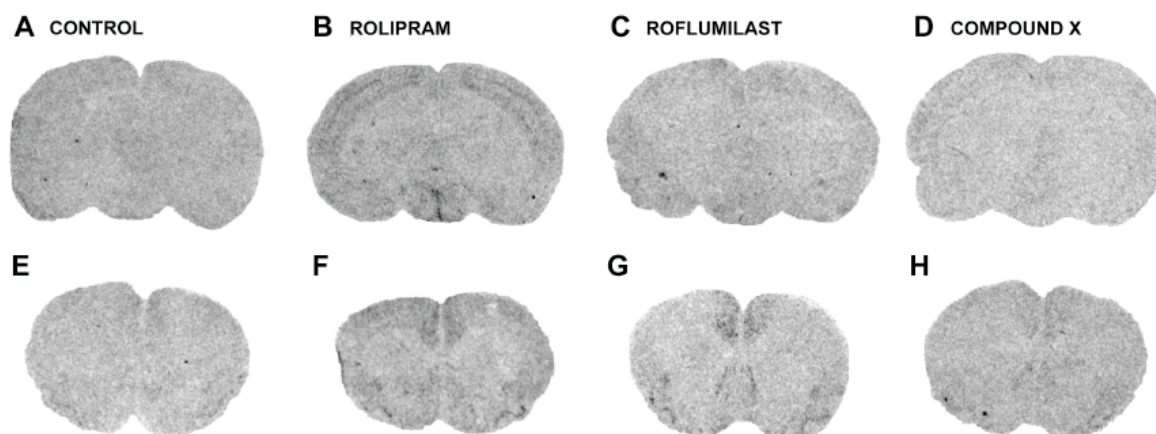


Figure 1. Anatomical distribution of c-fos mRNA expression in control rats and in rats treated with different PDE4 inhibitors, as indicated, and killed 30 min after i.v. injection of the drug. Shown are autoradiograms of representative coronal brain sections at the levels of the nucleus accumbens (plate 11, panels E-H) and of the somato-sensory cortex, basolateral amygdala and anterior hypothalamus (plate 18, panels A-D; Paxinos et al., 1982), exhibiting total hybridization of the [33P]-labeled probe. These representative sections will not fully correlate with the mean changes in c-fos mRNA levels shown in Figures 2 and 3, because of slight differences in gene expression in the 3-6 animals of each group.

Figure 2.

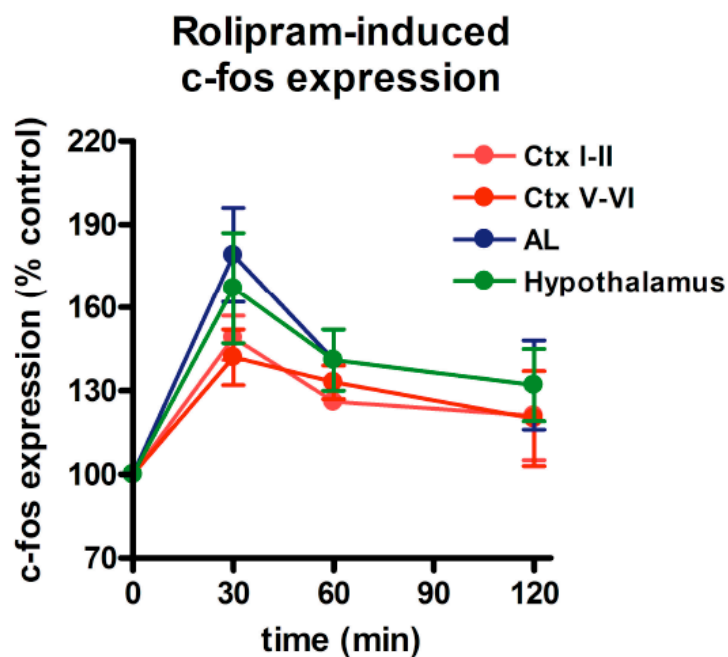


Figure 2. Time-course of the effect of rolipram (3 $\mu\text{mol/kg}$, i.v.) on c-fos mRNA levels in the rat somato-sensory cortex (layers I-II and V-VI), basolateral amigdala and anterior hypothalamus. Shown are the optical density values for autoradiographic signal produced by c-fos hybridization, expressed as percent of control (mean mRNA levels in control rats = 100%). Data are the mean \pm s.e. of 3-6 independent experiments (i.e. of 3-6 animals per group).

Figure 3.

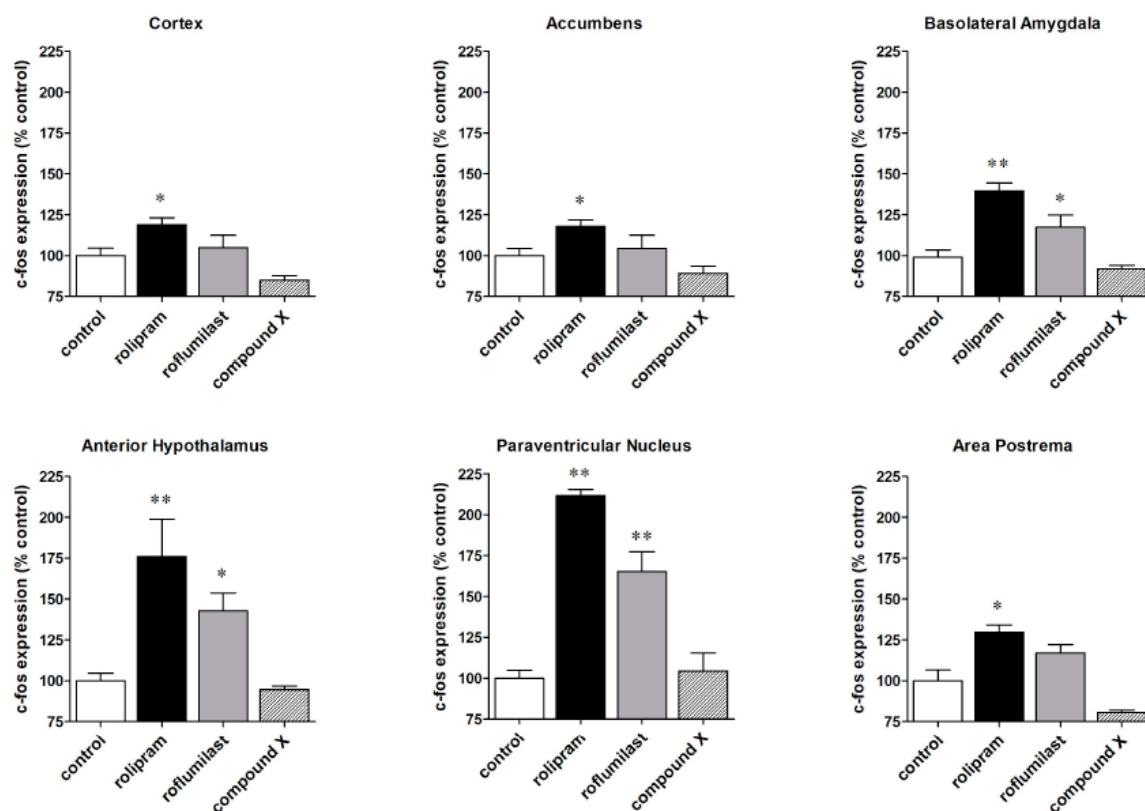


Figure 3. Comparative analysis of the effects of five different PDE4 inhibitors (administered 30 min before animal sacrifice) on c-fos mRNA expression in the rat, somato-sensory cortex (layers I-II and V-VI), nucleus accumbens, basolateral amigdala, anterior hypothalamus, paraventricular hypothalamic nuclei, and area postrema. Shown are the optical density values for autoradiographic signal produced by c-fos hybridization, expressed as percent of control (mean mRNA levels in control, vehicle-injected rats = 100%). Data are the mean \pm s.e. of 6 independent experiments (i.e. of 6 animals per group). * $P < 0.05$, ** $P < 0.01$ vs. control; Kruskal-Wallis test.

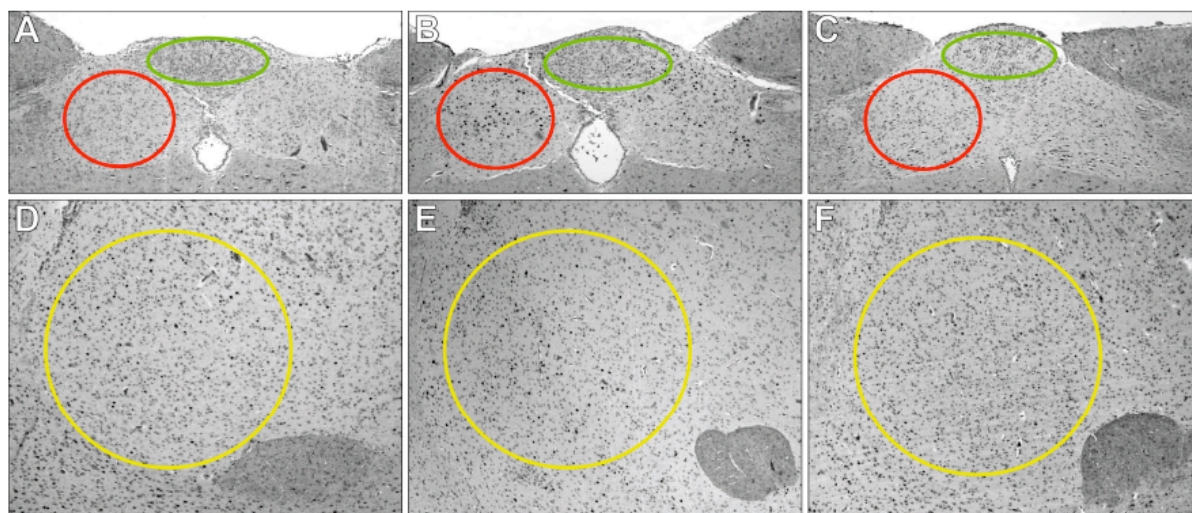
Figure 4.

Figure 4. Distribution of Fos protein expression in a control rat (A,D) and in animals killed 60 min after i.v. injection of rolipram (B,E) or compound X (C,F). Shown are representative Fos-immunostained coronal sections at the levels of the area postrema (green circles), of the nucleus of the solitary tract (red circles, A-C; plate 42, Paxinos et al., 1982) and of the nucleus accumbens (yellow circles, D-F; plate 11, Paxinos et al., 1982).

Figure 5.

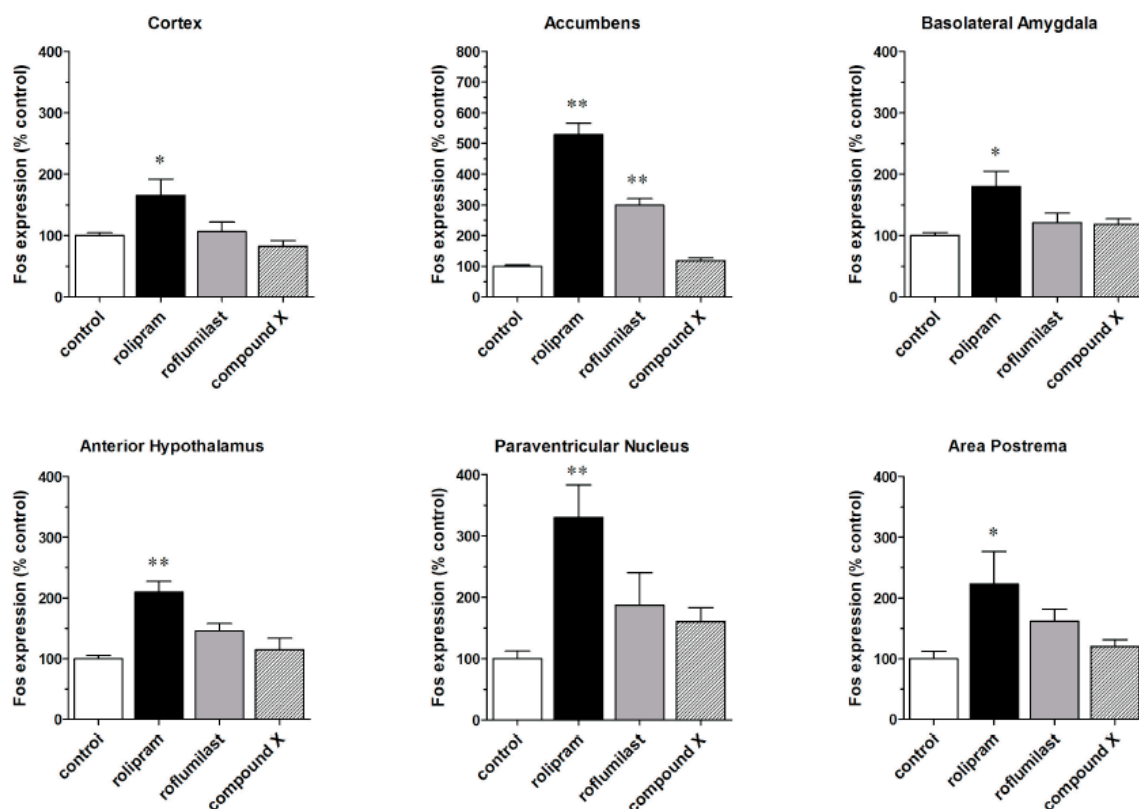


Figure 5. Comparative analysis of the effects of five different PDE4 inhibitors (administered 60 min before animal sacrifice) on Fos protein expression in the rat somato-sensory cortex, nucleus accumbens, basolateral amigdala, anterior hypothalamus, paraventricular hypothalamic nucleus, and area postrema. Shown is a quantification of Fos immunostaining performed as described in the Methods section. Data are the mean \pm s.e. of 4 independent experiments (i.e. of 4 animals per group). * $P < 0.05$, ** $P < 0.01$ vs. control; Kruskal-Wallis test.

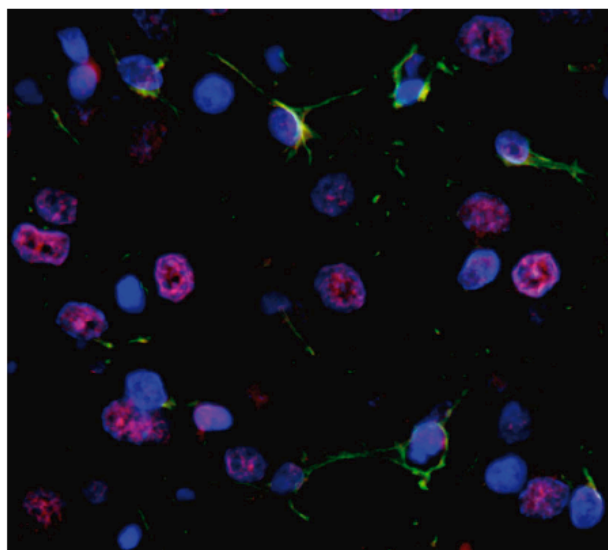
Figure 6.

Figure 6. Immunohistochemical analysis of a representative section (PVN) obtained from a rat treated with rolipram. Fos protein in red (Alexa-594 streptavidin conjugated), GFAP (an astrocytic marker) in green (Alexa-488) and nuclei in blue (DAPI). Fos is found in non-astrocytic cells. Double-label immunohistochemistry with Fos and MAP2 (a marker of neurons) yielded complementary results: all Fos-positive cells were neurons (data not shown).

Figure 7.

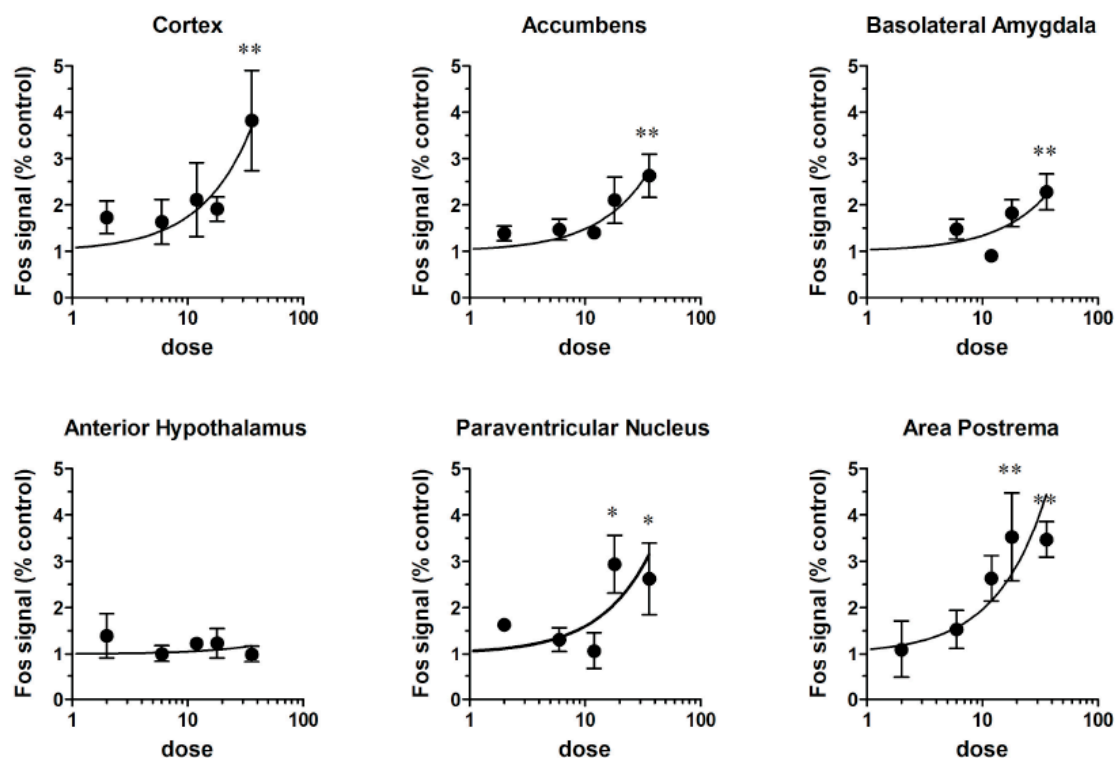


Figure 7. Dose-response of the effect of i.c.v. administered roflumilast on Fos protein expression in the rat somato-sensory cortex, nucleus accumbens, basolateral amigdala, anterior hypothalamus, paraventricular hypothalamic nucleus, and area postrema. Shown is a quantification of Fos immunostaining performed as described in the Methods section. Data are the mean \pm s.e. of 4 independent experiments (i.e. of 4 animals per group). * $P < 0.05$, ** $P < 0.01$ vs. control; Kruskal-Wallis test.

Figure 8.

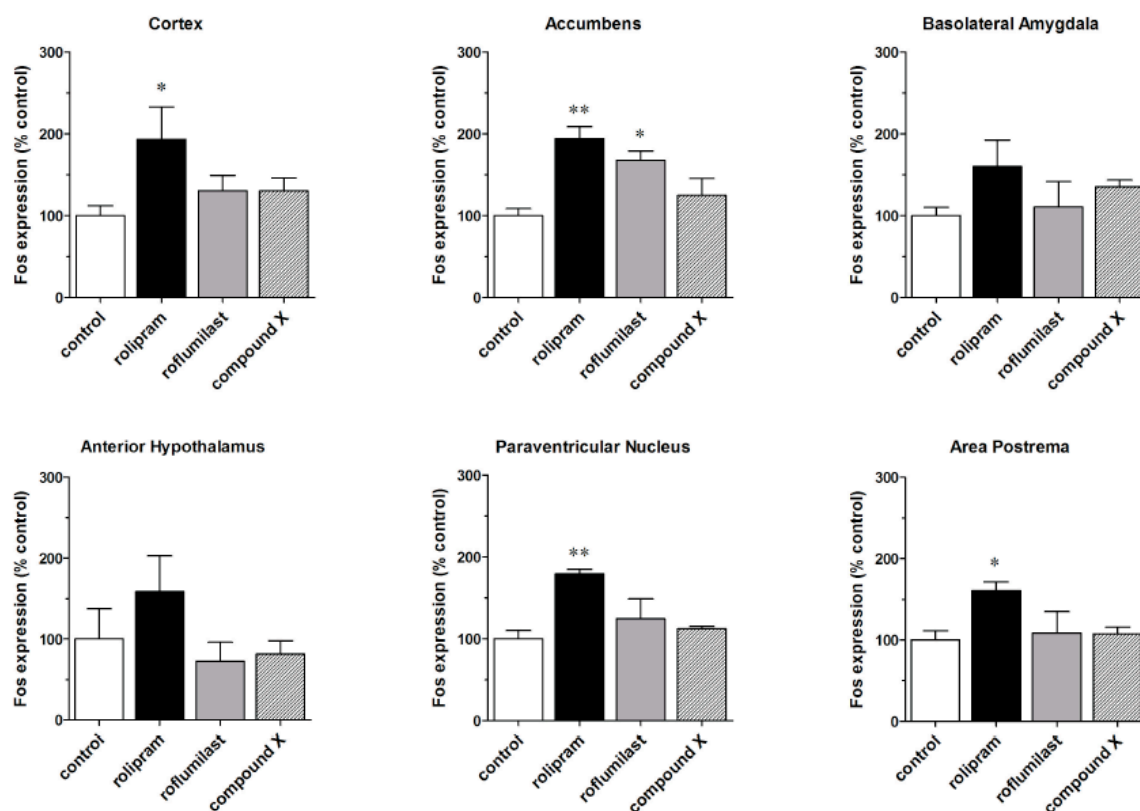


Figure 8. Comparative analysis of the effects of five different PDE4 inhibitors (administered i.c.v.) on Fos protein expression in the rat somato-sensory cortex, nucleus accumbens, basolateral amygdala, anterior hypothalamus, paraventricular hypothalamic nucleus, and area postrema. Shown is a quantification of Fos immunostaining performed as described in the Methods section. Data are the mean \pm s.e. of 4 independent experiments (i.e. of 4 animals per group). * $P < 0.05$, ** $P < 0.01$ vs. control; Kruskal-Wallis test.

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