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# Electrophysiological and Morphological Characterization of Potentiated Synapses at the Micro and Nanoscale

**(Settore scientifico-disciplinare BIO/09)**


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*“... a smile lasts just for a while,*
*but in memory it can be eternal...”*

# RIASSUNTO

Col termine generale di “plasticità sinaptica” si intendono tutti i meccanismi che stanno alla base della capacità del sistema nervoso di plasmarsi a seguito della sua maturazione e a fronte di stimoli esterni. Variazioni nella forma e nelle dimensioni oltre che l’instaurazione di nuove sinapsi o l’eliminazione di altre (sinaptogenesi) sono i meccanismi che regolano la plasticità sinaptica. Il sistema nervoso centrale è in grado di mettere in atto fenomeni di plasticità sinaptica in grado di modificarne la struttura e la funzionalità sia a corto che a lungo termine.

Uno dei più studiati meccanismi cellulari alla base della memoria e dell’apprendimento è il potenziamento a lungo termine (Long Term Potentiation – LTP), una forma di plasticità neuronale che porta a un incremento dell’efficienza della trasmissione sinaptica durevole nel tempo.

A livello cellulare, l’LTP aumenta la capacità di due neuroni di comunicare attraverso le sinapsi. Il meccanismo molecolare alla base di tale aumento dell’efficienza della trasmissione sinaptica non è univocamente stabilito, questo in parte è dovuto al fatto che l’LTP è determinato da diversi meccanismi che variano in base alla specie e alla regione del cervello in cui viene indotto.

Una volta innescato, l’LTP conduce a varie modificazioni postsinaptiche, tra cui sintesi di nuovi recettori, nascita di nuove sinapsi (in particolare a livello del recettore glutamatergico NMDA) e cambiamenti a livello delle spine dendritiche (Engert and Bonhoeffer, 1999).

Ragionevolmente, per indurre potenziamento a lungo termine è necessario che la membrana postsinaptica sia depolarizzata nell’intervallo di tempo in cui il terminale presinaptico libera glutammato: la depolarizzazione rimuove il blocco degli ioni magnesio dai recettori NMDA consentendo il passaggio (oltre al sodio e al potassio) anche agli ioni calcio. Il calcio è l'elemento centrale del processo perché, una volta raggiunta una certa concentrazione nella cellula, è in grado di attivare un processo per cui i recettori AMPA presenti nella cellula vengono trasferiti sulla membrana e i recettori già presenti lasciano passare una maggiore quantità di ioni. La sinapsi risulta così rinforzata.

Questa condizione è stata sperimentalmente dimostrata su campioni di fettine di ippocampo usando una stimolazione elettrica (tetanica) (Nishi et al., 2001).

Dopo la stimolazione tetanica, il neurone bersaglio rafforzato dall'LTP è molto più responsivo e produce un aumento dell'ampiezza delle correnti eccitatorie post-sinaptiche (Excitatory Post Synaptic Currents – EPSC) che perdura nel tempo. Questo comportamento trova spiegazione in una modificazione delle spine dendritiche sia nella forma, sia nel numero e dimensione.

L'attività del mio dottorato di ricerca è stata condotta nell'ambito del progetto NanoMosquito, il cui scopo prinicipale consiste nell'indurre fenomeni di plasticità neuronale in cellule dissociate d'ippocampo di ratto e, successivamente, nel caratterizzare le mutazioni funzionali (tramite la tecnica elettrofisiologica del patch-clamping) e morfologiche, in scala micro e nanometrica, utilizzando tecniche quali la microscopia confocale e la microscopia a forza atomica (Atomic Force Microscopy – AFM).

Diverse stimolazioni sono state testate per carcare di capire quali potessero indurre potenziamento della rete. Studi di plasticità vengono condotti in genere su fettine organotipiche, ma queste rendono impossibile studiare i cambiamenti che avvengono a livello delle spine dendritiche con tecniche in scala nanometrica, quali l'AFM.

Diversi protocolli di stimolazione (treni a bassa frequenza, theta burst) sono stati utilizzati in esperimenti a doppio patch (due elettrodi usati in simultanea) su due cellule neuronali vicinali. Questo tipo di stimolazione ha portato però solo a un numero limitato di sinapsi potenziate e per questo motive abbiamo deciso di uitlizzare una particolare forma di plasticità sinaptica che prende il nome di Spike-Timing Dependent Plasticity (STDP).

In questo tipo di plasticità il preciso ordine temporale tra i potenziali d'azione presinaptici e postsinaptici determina i cambiamenti che avverrano a livello della sinapsi stessa; per ottenere un potenziamento a livello del contatto sinaptico, il potenziale d'azione a livello postsinaptico deve seguire la depolarizzazione a livello presinaptico in una finestra temporale che va dai 5 ai 20 millisecondi (Bi and Poo, 1998).

Anche in questo caso, monitorando successivamente l'ampiezza delle EPSCs, solo poche sinpasi andavano incontro a plasticità e il meccanismo che sta alla base di questo deve essere ancora determinato.

Al contrario, il Brain Derived Neurotrophic Factor (BDNF), membro della famiglia delle neurotrofine e abbondantemente espresso nel sistema nervoso centrale (SNC), sta emergendo come un importante mediatore nella sopravvivenza, sviluppo e funzione dei neuroni (Lu, 2003).

Colture embrionali dissociate di ippocampo sono state per la prima volta trattate cronicamente con BDNF promuovendo la formazione di nuove sinapsi, sia a livello eccitatorio che inibitorio, con conseguente aumento dell'attività spontanea dell'intera rete. Il BDNF inoltre si pensa induca modificazioni morfologiche sia nella complessità dell'albero dendritico che nel promuovere la crescita delle terminazioni assonali (Vicario-Abejon et al., 1998).

Registrazioni elettrofisiologiche sono state effettuate per monitare l'attività spontanea della rete: nel dettaglio sono state misurate le EPSC e le IPSC tra neuroni incubati in BDNF e campioni di controllo mentre registrazioni doppie sono state effettuate per confrontare la percentuale di accoppiamento.

Abbiamo così visto come il BDNF rafforzi l'attività sinaptica della rete e aumenti il numero di connessioni sinaptiche eccitatorie.

Registrazioni *paired-pulse* ed esperimenti di imaging con FM1-43 hanno invece dimostrato come il BDNF induca anche delle modificazioni nella probabilità di rilascio vescicolare, in quanto, anche in questo caso l'ampiezza della risposta risulta aumentata nelle colture incubate.

Marcando i neruoni (β-tubulin III) abbiamo visto anche come il BDNF aumenti la sopravvivenza neuronale, sopratutto a carico delle cellule piramidali, riconosciute dalla loro forma.

Inoltre, eseprimenti condottti su cellule transfettate con cds-BDNF hanno confermato ulteriormente i nostril dati su come il BDNF aumenti la trasmissione sinpatica.

La caratteristica comune di tutti questi diversi approcci è stata quella di indurre modifiche funzionali nelle connessioni sinaptiche eccitatorie.

Successivamente l'induzione della plasticità sinaptica, la microscopia a scansione sarà utilizzata per seguire in tempo reale i cambiamenti morfologici delle sinapsi.

# ABSTRACT

The brain is programmed to drive behaviour by exactly wiring the appropriate neuronal circuits. Wiring and rewiring of neuronal circuits widely depends on the orchestrated changes in the strengths of synaptic contacts.

For many years, neuroscientists believed that neurogenesis - the generation of new neurons – and establishment of new neuronal connections was restricted to early brain development (Segal et al, 2005). New findings have challenged this view and currently many neuroscientists believe that the capacity for circuitry rearrangement is maintained throughout life. However the mechanisms that controls plasticity in the adult brain are still not entirely clear.

The connection between neurons is named synapse. The synapse is the most fundamental unit of information transmission in the nervous system. Information storage, including all forms of memory and behavioural adaptation, are believed to come out from changes in neuronal transmission, both in the short-term and the long-term, a property known as synaptic plasticity. Synaptic plasticity is a highly regulated process, refers to all the mechanisms that underlie the ability of the nervous system to adapt to external stimuli. Variations in the shape and size as well as establishment of new synapses or the elimination of others (synaptogenesis) are the mechanisms that regulate synaptic plasticity. Thus, understanding the mechanisms underlying synaptic plasticity may help to apprehend general learning and memory processes.

Changes in synaptic plasticity are achieved by changes in inhibitory or excitatory neurotransmission or both. This thesis deals with the modulation of excitatory neurotransmission. The principal excitatory neurotransmitter in the brain is glutamate. The regulation of glutamate-mediated excitatory neurotransmission has been shown to play a critical role in many aspects of synaptic plasticity.

One of the most studied cellular mechanisms is the long-term potentiation (LTP), a form of synaptic plasticity that leads to an increase in the efficiency of synaptic transmission (Engert et al., 1999).

The induction of LTP is classically achieved by tetanic stimulation but it is also possible to induce chemically a long-term potentiation of the synaptic efficacy, thus enhancing a larger number of synapses compared to electrical stimulation.

The work of this thesis has been conducted in the wider framework of the NanoMosquito project, whose major aim was to combine electrophysiological measurements, scanning probe microscopy (AFM-Atomic Force Microscopy) and fluorescence microscopy in order to develop new generation neurophysiological tool to understand neuronal plasticity at the nanoscale.

Studies of synaptic plasticity are often carried out in slices of hippocampus, but these prevent to study change in nanoscale with a surface-microscopy technique such is AFM: dissociated hippocampal neurons lend themselves well for this purpose.

Understanding in detail the mechanism of action of these processes may be of critical importance not only for a detailed view of memory related processes but also in the case of some diseases: being able to control synaptic plasticity may help to restore a functional connectivity lost, for example, in the case of brain lesions.

The first part of this thesis handles the setting of an electrophysiological stimulation to induce neuronal plasticity, starting from the stimulations trains usually performed in hippocampal slices, such as slow frequency stimulation and theta burst. Long-term synaptic modifications can be induced also by a particular form of synaptic plasticity named Spike-Timing Dependent Plasticity (STDP) where the precise timing and the order of presynaptic and postsynaptic action potentials determine the magnitude and the direction of the changes in synaptic strength (Bi and Poo, 1998). I have tested trains of with a delay of 5, 10 and 20 milliseconds between pre- and postsynaptic neuron. By monitoring the amplitude and frequency of the EPSCs, responses varied from no changes to potentiation but just in a small sample of coupled neurons where we measured a strong increase in the amplitude and frequency of spontaneous EPSCs after the stimulation. The cellular basis that gives rise to the induction of such synaptic modifications remains to be determined.

On the other hand, BDNF ability to mediate activity-dependent modifications in synaptic strength (Bolton et al., 2000; Vicario-Abejón et al., 1998) has recently received considerable attention; in particular the acute BDNF effects on excitatory synapses have been the object of an increasing amount of studies. On the contrary, the role of BDNF in regulating long-lasting changes in synaptic function is comparably less investigated and may have large impact on post injury alteration of synaptic networks and neuronal rescue.

To address this issue, during my PhD, I studied the long-term (chronic) effects of BDNF on AMPA receptor mediated excitatory synaptic transmission and on neuronal survival in vitro.

Dissociated rat (P2-P3) hippocampal cultures were chronically treated (4 days) with BDNF between 4 and 8 days in vitro (DIV). Single and dual patch-clamp recordings in whole-cell configuration were used to monitor spontaneous and evoked post synaptic currents (IPSCs and EPSCs) in hippocampal network grown in culture for 8-10 DIV. Excitatory PSCs (EPSC) were identified by their kinetic (fast decay $\tau$) and pharmacology (CNQX sensitivity). EPSCs recorded from BDNF-treated cultures show a strong increase in their mean frequency and amplitude when compared to controls untreated sister cultures. In the presence of TTX, miniature excitatory PSCs (mEPSCs) in BDNF treated networks still displayed an increase in both frequency and amplitude. In BDNF-treated cultures pair recordings showed an increased probability of finding coupled pairs. Paired pulse (20 Hz) experiments and FM1-43 fluorescence imaging suggested that BDNF treatment increased the probability of release. Immunofluorescence ($\beta$-tubulin III) visualization of neurons allowed to quantify neuronal density and showed that BDNF mediated an increase (40%) in neuronal survival, when compared to controls, together with an increase in the pyramidal neuron/interneuron ratio (0.33 for BDNF, 0.19 for controls). Additionally, neuronal cells were transfected with different BDNF-GFP expressing vectors to gain insights in the specific molecular mechanisms involved in long term BDNF effects on synapses.

However the common feature of all these functional modifications is in the direction of a pronounced potentiation of excitatory synaptic connections.

Subsequently to the induction of synaptic plasticity, scanning probe microscopy would be used to follow in real time morphological changes of synapses undergoing potentiation or neuronal processes development with submicrometrical resolution in all 3 dimensions. Final goal of the entire project, whereof this thesis is the fundamental initial step, will be the development of new paradigms to evaluate and induce synaptic plasticity on specific synapses to govern in a controlled way neuronal outgrowth and synaptogenesis.

# INDEX

# Abbreviations

AFM, Atomic Force Microscopy

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

AP, Action Potential

BDNF, Brain Derived Neurotrophic Factor

BS, Baseline protocol

CNQX, 6-Cyano-7nitroquiloxaline-2,3-dione disodium salt

CNS, Central Nervous System

DIV, *days in vitro*

EPSCs, Excitatory Postsynaptic Currents

GABA, γ-aminobutyric acid

GFP, Green Fluorescence Protein

HFS, High-Frequency Stimulation

IPSCs, Inhibitory Postsynaptic Currents

LTD, Long-Term Depression

LTP, Long-Term Potentiation

NMDA, N-methyl-D-aspartate

PBS, Phosphate buffer solution

PFA, paraformaldehyde

PSC, Post-Synaptic Current

rCDS, rat Coding Sequence

STDP, Spike Timing-dependent Plasticity

STP, Short-Term Plasticity

TBS, Theta-Burst Stimulation

TTX, Tetrodotoxin

# 1 INTRODUCTION

The most fascinating property of the mammalian brain is its remarkable plasticity, which can be translated in the ability of external experience to modify neuronal circuitry and consequently to modify future thought, behavior and feeling.

Thinking simplistically, neuronal activity can remodel the neuronal network by one of three mechanisms:

- by modifying the strength or efficacy of synaptic transmission at pre-existing synapses;
- by eliciting the growth of new synaptic connections or the removal of existing ones;
- by modulating the excitability properties of individual neurons.

Synaptic plasticity refers to all of these mechanisms, and for almost 100 years, changes in the efficacy of synaptic transmission have been proposed to play an important role in the capacity of the brain to translate transient experience into infinite memories that can last for decades (Malenka, 2002).

Long-term changes in synaptic strength have been described for many systems, ranging from the invertebrate neuromuscular junction (NMJ) to the mammalian hippocampus and neocortex (Malenka and Nicoll, 1999; Zucker, 1999), highlighting the idea for a general mechanism.

Correspondingly, it is believed that there are several important consequences of long-term synaptic modifications depending for example whether they occur during distinct phases of neural development (Katz and Shatz, 1996), cortical map formation, and reorganization (Cruikshank and Weinberger, 1996; Buonomano and Merzenich, 1998; Kilgard et al., 2002), alteration of receptive field properties (Fregnac and Shulz, 1999; Froemke et al., 2007), perceptual learning (Gilbert, 1998), and memory encoding and storage (Martin et al., 2000).

## 1.1 Basic principles of neurotransmission

The nervous system is composed of billions of specialized cells called neurons. Efficient communication between these cells is crucial to the normal functioning of the central and peripheral nervous systems.

Extending from the cell body membrane of the neuron is a system of dendritic branches, which has the role of receptor sites for information sent from other neurons and which ultimately enrich neurons of their extraordinary computational power. Information transmission has a domino effect: if the dendrites receive a suitable signal from a neighbouring nerve cell, or from several neighbouring nerve cells, the resting electrical potential of the receptor cell's membrane becomes depolarized. Regenerating itself, this electrical signal travels along the cell's axon, a specialized extension from the soma which ranges from a few hundreds micrometres in some nerve cells (e.g. hippocampal neurons), to over a meter in others (e.g. motoneurons). This wave of depolarization is called an *action potential*.

The process by which this information is communicated is called *synaptic transmission* and the connection between neurons is named *synapse*.

A synapse is an anatomically specialized junction through which cells of the nervous system signal to one another and to non-neuronal cells such as glands or muscles.

The human brain has about 100 billion neurons and 100,000 billion synapses. Most synapses occur between the axon terminals of one neuron and the cell body or dendrites of a second. The neurons conducting information towards synapses are called *presynaptic*, and those conducting information away are *postsynaptic* neurons. Every postsynaptic neuron has thousands of synaptic junctions on the surface of its dendrites or cell body (Fig.1.1).

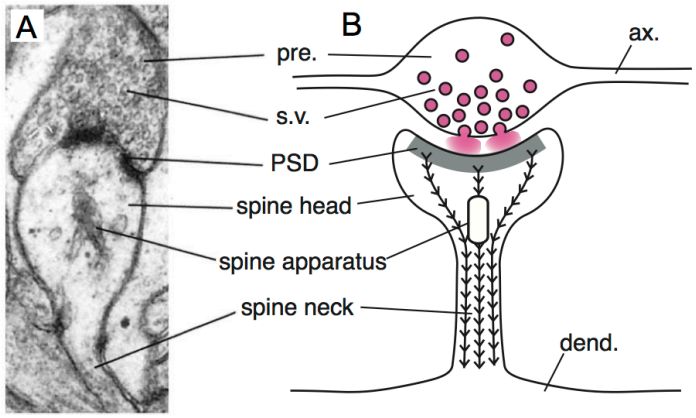


**Figure 1.1:** ***Synapses are structures permitting neurons to electrically or chemically communicate. Synaptic transmission begins with the arrival of an action potential that travels along the axon of a presynaptic neuron; when the action potential reaches the presynaptic terminal, it provokes the release of a small quantity of neurotransmitters, which binds to chemical receptor molecules located in the membrane of the postsynaptic neuron, on the opposite side of the synaptic cleft. In (A): A single spine synapse seen by electron microscopy, and (B) a schematic illustration of a spine structure. The neurotransmitter glutamate (pink) is stored within synaptic vesicles and released into the synaptic cleft where it activates receptors located in the postsynaptic density (PSD). Actin filaments are represented by the barbed lines. ax., axon; pre., presynaptic bouton; dend., shaft of dendrite; s.v., synaptic vesicle (adapted from Matus A., 2000).***

There are two types of synapses either electrical or chemical, although electrical synapses are less common than chemical synapses in the brain. At electrical synapses, transmission occurs by means of current flow through gap junctions which connect the cytoplasm of the pre- and postsynaptic cells. Transmission across these electrical synapses is extremely rapid.

At chemical synapses, small molecules, the neurotransmitters, mediate information transfer between neurons. Before release, the transmitters are stored in small membranous organelles, the synaptic vesicles.

Synaptic transmission begins when a nervous impulse reaches the presynaptic axon, leading to the depolarization of the presynaptic membrane. The voltage gated $Ca^{2+}$ channels open leading to $Ca^{2+}$ ions flowing into the axon terminal and initiating a sequence of events that end with neurotransmitter vesicle release into the synaptic cleft, which separates the transmitting cell from the receiving (postsynaptic) cell. Then, the neurotransmitters bind to specific membrane receptors, the autoreceptors, which can regulate the rate of transmitter release; on the postsynaptic membrane, they bind to receptors.

The activation of receptors mediates the specific ion entry into the post-synapse and generates the response, an excitatory postsynaptic potential (EPSP) or inhibitory postsynaptic potential (IPSP).

There are two major classes of postsynaptic receptors named ionotropic and metabotropic receptors. Ionotropic receptors are directly coupled to ion channels and are responsible for fast chemical transmission. As soon as the neurotransmitter binds to the receptor, the ion channel opens. Common neurotransmitters that mediate signalling of this type are glutamate and acetylcholine, which are usually excitatory neurotransmitters, and glycine and γ-aminobutyric acid (GABA), which are usually inhibitory neurotransmitters. The metabotropic receptors in the postsynaptic membrane, involved in the slow chemical transmission pathway are not directly coupled to ion channels but affect them or alter the level of intracellular second messengers like adenosine 3',5'-cyclic monophosphate (cAMP), $Ca^{2+}$ and diacylglycerol (DAG), through intermediary G-proteins.

If the neurotransmitter would remain bound to the receptor, the postsynaptic cell would be in a state of constant depolarization or hyperpolarization. To avoid this, neurotransmitters are enzymatically degraded or transported back to the presynaptic cell via transporters (reuptake). It is also possible that the transmitter-receptor complex is taken back into the cell via invagination of membrane which pinches off to form a vesicle, a process called internalisation. The vesicles then fuse with the endosome and within it transmitter dissociates from the complex and is transferred to the lysosome for degradation; subsequently, the receptor is recycled to the membrane. The receptor itself can also become desensitized after prolonged exposure to its own transmitter (Shaw et al., 1989).

Much of the basic knowledge about synaptic function was obtained from studies on neuromuscular junction, as well as other easily accessible synapses such as the calyx of Held and the squid giant synapse. Most central synapses, for example within the hippocampus, operate in a similar manner except that the amount of released transmitter at each synapse is usually quite small (Allen and Stevens, 1994).

A neuron that has been excited transmits information to other neurons by generating impulses known as *action potentials*. These signals propagate like waves along the cell's single axon and are converted to chemical signals at synapses.

When a neuron is at rest, its external membrane maintains an electrical potential difference of about –70 mV (the inner surface is negative relative to the outer surface). At rest, the membrane is more permeable to potassium ions than to sodium ions, and it is this condition that governs the resting potential. When the cell is stimulated, the permeability to sodium increases, leading to an influx of positive charges. This influx triggers an impulse, a momentary reversal of the membrane potential. The rising phase is called *depolarization*. After about 1 ms the sodium permeability declines, potassium conductance increases during this *repolarisation* phase and the membrane potential returns to –70 mV.

The level of excitability of this cell depends on the number of synapses active at any one time, and how many are excitatory or inhibitory. In this manner, postsynaptic neurons function as neural integrators, i.e. their output reflects the sum of all the incoming bits of information arriving in the form of excitatory and inhibitory synaptic inputs.

### 1.1.1 Excitatory synapses

An excitatory synapse, when activated, increases the likelihood that the membrane potential will reach threshold and the cell will undergo an action potential. The movement of positive ions into the neuron slightly depolarizes the postsynaptic cell. This potential change, called the *excitatory postsynaptic potential* (EPSP), is a local, passively propagated potential; its only function is to help trigger an action potential. At the hippocampal level, most of the neurons use glutamate as a synaptic transmitter.

Glutamate is the transmitter that controls the majority of excitatory neurotransmission in the mammalian central nervous system (CNS). It is crucial for normal brain functions and plays a key role in the phenomena related to synaptic plasticity. It also has a relevance to clinical neurology as the increase in its concentration caused, for example, by brain damage is toxic to neurons.

Glutamate receptors are clustered into two main families: ionotropic and metabotropic receptors (mGluRs).

Ionotropic receptors have the characteristic to contain in one molecule both the ability to act as a channel and to bind the receptor. Once activated, they allow the rapid ions flow across the neuronal membrane, and are thus responsible for rapid synaptic transmission.

Metabotropic receptors, instead, are able to activate channels through one or more metabolic steps triggered by molecules called G-proteins.

Both types of glutamate receptors have been reported to take part in synaptic plasticity processes.

Three types of ionotropic glutamate receptors have been characterized in the postsynaptic membrane, called by their agonist, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), Kainate and N-methyl-D-aspartate (NMDA) receptors (Hollmann and Heinemann, 1994; Seeburg, 1993).

Most of the studies on synaptic transmission are focused on AMPA and NMDA receptors. These two receptors with different physiological properties often coexist in the same synapses in the hippocampus. The AMPA receptor is the most representative and generates rapid postsynaptic electrical response. The NMDA receptor, normally contributes less to the response, but rather acts as a release system for synaptic plasticity. Both receptors are permeable to $Na^+$ and $K^+$; NMDA receptors are also permeable to $Ca^{2+}$ ions: $Ca^{2+}$ flux through NMDARs is thought to be critical for synaptic plasticity (Seeburg, 1993).

NMDA receptors are characterized by a voltage-dependent $Mg^{2+}$ block (Nowak et al., 1984). Upon binding glutamate, the NMDA receptor allows the influx of both $Na^+$ and $Ca^{2+}$ ions (Ascher and Nowak, 1988). Besides binding of glutamate to the receptor, the inhibitory neurotransmitter glycine is required as coagonist for the NMDA receptor channel to enter the open state (Kleckner and Dinglendine, 1988).

The subunits that make up NMDA receptors have been identified by molecular cloning techniques and have been characterized as NR1, NR2A-NR2D (Fig.1.2).

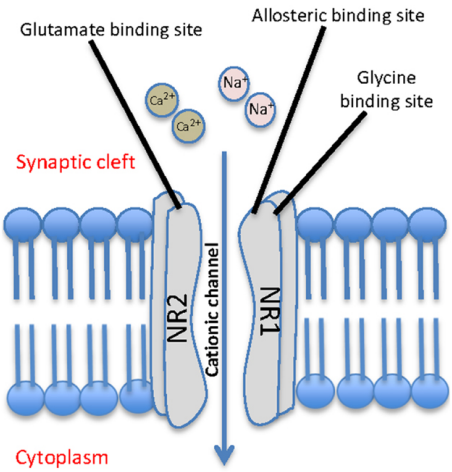


**Figure 1.2:** ***Each NMDA receptor is built from four subunits. Usually it comprises two N1 and either two N2 subunits. Its activation depends on binding of both glutamate and glycine. Upon binding glutamate, the NMDA receptor allows the influx of both Na+ and Ca2+ ions (adapted from Lakhan et al., 2013).***

The combination of the NR1 subunit with at least one of the four NR2 subunits leads to functional NMDA receptors.

Incorporation of different NR1 splice variants into NMDA receptor complexes, influences receptor properties such as its modulation by zinc, polyamines and PKC, as well as binding to intracellular proteins (Durand et al., 1993; Ehlers et al., 1996; Hollmann et al., 1993). The NR2 subunit composition determines biophysical characteristics of the channel such as conductance, mean open time, and sensitivity to $Mg^{2+}$ block (Monyer et al., 1992, Stern et al., 1992).

NMDA receptors are found throughout the brain but above all within the forebrain. The highest levels in the entire brain are found in the CA1 region of the hippocampus. NR1 mRNA is distributed ubiquitously but the four NR2 subunits display distinct regional patterns. NR2A and NR2B are mainly found in the forebrain, NR2C in the cerebellum and NR2D in the thalamus, brain stem and olfactory bulb (Kutsuwada et al., 1992; Monyer et al., 1992, 1994). Since the functional properties of the NMDA receptor depend on coassembly of the four NR2, it is reasonable that spatial patterns of expression of the NR2 genes lead to region specific differences in receptor function.

Recently, an additional NMDA receptor subunit, NR3A, has been identified in mammalian brain (Ciabarra et al., 1995; Sucher et al., 1995). Until now, only few studies have reported evidence for a functional role of this subunit in the brain (Das et al., 1998).

A weak electrical stimulation of presynaptic axons can promote the release of glutamate from the presynaptic terminal and its binding to both AMPA and NMDA postsynaptic receptors. However, under conditions of resting membrane potential, of about –75 mV, AMPA receptors are pre-eminently in the open state. The movement of ions through the NMDA receptors is minimal even when the cell becomes partially depolarized, because of the blockade of the channel by $Mg^{2+}$ ions.

When there is a stimulus with appropriate magnitude or frequency, AMPA receptors are able to depolarize the postsynaptic membrane beyond –35 mV, $Mg^{2+}$ ions are expelled from NMDA receptors ($Mg^{2+}$ block removal) and these are opened in response to glutamate, not only mediating $Na^{+}$ but passing a large amount of $Ca^{2+}$ as well. The increase of postsynaptic intracellular $Ca^{2+}$ concentration via NMDA receptors has been thought to be the key that triggers NMDA-dependent synaptic plasticity (Lynch and Larson, 1983; Malenka and Kauer, 1988). Moreover, there is growing evidence for the existence of glutamate presynaptic receptors. So far, AMPA, Kainate, NMDA and mGluR have all been identified in hippocampus as presynaptic autoreceptors. Those receptors can modulate the transmitter release in different ways and may also contribute to lasting forms of synaptic plasticity (Breukel and Besselsen, 1998; Manahan-Vaughan and Herrero, 1999; Suarez and Solis, 2006).

### 1.1.2 Inhibitory synapses

Activation of an inhibitory synapse produces changes in the postsynaptic cell, which decrease the likelihood that the cell will undergo an action potential. The binding of the transmitter to the receptor sites on the inhibitory postsynaptic membrane increases the permeability to potassium or chloride ions depending on the inhibitory receptor type, but not to sodium. The net effect is an increased negativity (*hyperpolarization*) called an *inhibitory postsynaptic potential* (IPSP).

Compared with glutamate, GABA plays an opposite role. It mediates most of the inhibitory neurotransmission in the brain. Ionotropic GABA receptors are chloride-selective ion channels and two types have been identified, $GABA_A$ and $GABA_C$. These receptors mediate fast synaptic inhibition whereas the $GABA_B$ type receptor is of a metabotropic type and mediates slow inhibition (Johnston, 1996). It also has been found that Long-Term Potentiation (LTP) and Long-Term Depression (LTD) can occur at GABAergic synapses in certain brain areas. In addition, GABA receptors, via their

effect on membrane potential, play crucial roles in controlling the conditions leading to LTP/LTD at glutamatergic synapses (Bernard and Cossart, 2000; Jerusalinsky and Kornisiuk, 1997; Wigström and Gustafsson, 1983, 1985).

Glycine is another type of inhibitory neurotransmitter present in the CNS. Like GABA, it acts through chloride channels and exerts its inhibition by causing hyperpolarization (Torsney and MacDermott, 2005). As mentioned above, glycine also acts on the NMDA receptor, being necessary as a cofactor for glutamatergic activation. Both GABA and glycine, acting at their "own" receptors, may also have excitatory action at early developmental stages.

## 1.2 Synaptic plasticity: a key role in learning and memory

Our brains enable us to observe and interact with the outer world. Synaptic connections between neurons can be modified in response to changes in the environment, for example when we learn.

Plasticity mechanisms play important roles during early development although the demands for synaptic plasticity keep changing during the course of a lifetime. At the first developmental stage of life, neuronal networks are being built in the brain in order to prepare itself for interacting with the outside world after birth. To achieve this, synaptic contacts are being shaped in the absence of post-natal sensory input. In the next phase of life, the newborn has to absorb and process a great deal of new information in short time (parents, language, cultural behaviour), which demands high levels of synaptic plasticity. At the mature stage the need for synaptic plasticity becomes gradually less urgent: a picture of the outside world is made that only in a minor way requires modification.

Changing the strength of connections between neurons is widely assumed to be the mechanism by which memory traces are encoded and stored in the central nervous system.

Since neuronal changes evoked by the stimuli can persist for very long times, virtually for the whole life of the individual, it seems clear that neural plasticity represents the basis of the higher brain functions such as learning and memory or, conversely, that the built-in property of neural plasticity allows experience to shape both functionally and structurally the nervous system (Benfenati F., 2007).

As mentioned above, synaptic strength can be finely tuned over a short or even a long time scale by a combination of factors including previous activity of the network, generation of second messengers, functional changes in pre- and post-synaptic proteins as well as regulation of the expression of genes implicated in growth, survival and synaptic transmission. This results in changes in the efficiency of synaptic transmission, that can last from fraction of seconds to minutes in case of short-term synaptic plasticity (including paired-pulse facilitation or depression, post-tetanic potentiation) to hours, days and months in case of long-term synaptic plasticity (long-term potentiation, long-term depression).

Attention has focused on synapses in the hippocampus because clinical and experimental data have identified it as a critical structure for the process of memory consolidation (Milner, 1972) and spatial memory (O'Keef and Nadel, 1978).

### 1.2.1 Hippocampus

Hippocampus is thought to be the place where memories of experienced events are stored momentarily before being fixed in other cortical regions.

This idea was upheld by the discovery that bilateral hippocampal lesions in a human patient severely impaired memory for events occurring after the time of the lesion (Scoville and Milner, 1957). The hippocampus has widespread connections with other brain regions via the adjacent enthorinal cortex and a nerve bundle called *fimbria*. A famous trisynaptic excitatory pathway within the hippocampus, which involves cells of dentate gyrus, CA3 and CA1 has been commonly used for experimental studies of synaptic plasticity (Amaral and Witter, 1995) (Fig.1.3).

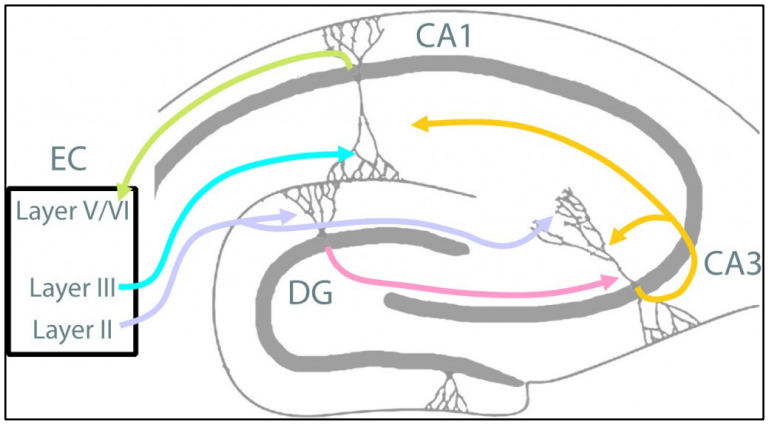


**Figure 1.3:** ***The Hippocampus network: the hippocampus forms a principally uni-directional network, with input from the Enthorinal Cortex (EC (violet line) that forms connections with the Dentate Gyrus (DG) and CA3 pyramidal neurons via the Perforant Path. CA3 neurons also receive input from the DG via the mossy fibres (pink line). They send axons to CA1 pyramidal cells via the Schaeffer Collateral Pathway (yellow line), as well as to CA1 cells in the contralateral hippocampus via the Commissural Pathway. CA1 neurons also receive input directly from the Perforant Path and send axon to the Subiculum. These neuron in turn send the main hippocampal output back to the EC (green line), forming a loop (adapted from: The Hippocampus & The Brain. © 1999 Scientific Learning Corporation)***

The first synaptic connections of the circuit are formed between the enthorinal cortex and the dentate gyrus. The cells in the superficial layers (mainly layer II) of the enorhinal cortex send their axons to the molecular layer of the dentate gyrus and they provide the hippocampus its main glutamatergic input. This pathway is called the *Perforant Pathway*. Collaterals of the same axons form also connections with CA3 pyramidal cells. The second synaptic connections are formed between the dentate gyrus and the CA3. The axons from the granular cells of the dentate gyrus innervate the dendrites of the CA3 pyramidal cells. These innervations are called *mossy fibers*. As in the case of the perforant pathway, also mossy fibers form connections with another cell population, namely the mossy cells of the dentate gyrus and provide feedback excitation back to the granule cells. In the third and last stage of the trisynaptic circuit, the axons of the CA3 pyramidal cells in layers form connections with the dendrites of the CA1 pyramidal cells in layers stratum radiatum and stratum oriens. These axons are called *Schaffer collaterals*, and again, they too branch to form connections with another cell population: the cells of the lateral septum and mammillary bodies. These axons pass through the fimbria/fornix. In this way, the trisynaptic circuit has been closed, but the information that has been processed in the circuit by the principal cells and the interneurons is projected back to the enthorinal cortex by the CA1 pyramidal cell axons,

either directly or via the subiculum. While the input cells to the hippocampus were located in the superficial layers of the entorhinal cortex, the output axons from the hippocampus projects to the deep layers of the enthorinal cortex (Amaral and Witter, 1995). Detailed knowledge of its anatomy and connections together with its highly laminated pattern of inputs and outputs make it an area well suitable for electrophysiological recordings.

### 1.2.2 Long-term and Short-term plasticity

Long-term plasticity is concerned with modifications in the strength of the synapses which occur on a time scale of minutes or more. These long-term synaptic modifications depend on pre- and postsynaptic activity, and they may lead to an increase or a decrease of the synaptic weight. The corresponding processes for these two situations are known as *long-term potentiation* (LTP) and *long-term depression* (LTD).

According to experimental evidences, long-term modifications could occur via two general mechanisms: the alteration of existing synaptic proteins, or the regulation of gene transcription mediated by second messengers (Kandel et al., 2000). This second mechanism can be triggered by protein phosphorylation providing the mechanism for long-lasting memory storage.

Probably, the most relevant implications of long-term synaptic modifications are *learning* and *memory* (discuss further later).

The relation between changes in synaptic strength and memory can be understood by means of the hebbian prescription. In 1943, Donald Hebb established a physiological principle which has been simplified by modern neuroscience as follow: when two interconnected neurons generate action potentials (APs) strongly correlated in time, the synaptic connection between these two neurons is strengthened (Hebb, 1949). In other words, when two neurons communicate via APs very frequently, the synapse that links them becomes stronger. This principle, known as Hebb's rule, states the physiological changes on the synapses that may influence the performance of the brain in later situations.

On the other hand, short-term plasticity (STP) refers to a mechanism in which synaptic efficacy changes in a short time scale (of the order of hundreds to thousands of milliseconds) (Stevens et al., 1995; Markram et al., 1996). The modification it induces

to synaptic efficacy is temporary. Without continued presynaptic activity, the synaptic efficacy will quickly return to its baseline level.

The two mayor mechanisms responsible for short-term plasticity are known as *short-term synaptic depression* (STD) and *short-term synaptic facilitation* (STF).

STD is caused by depletion of neurotransmitter vesicles consumed during the synaptic signalling process at the axon terminal of a presynaptic neuron. In this case, the neuron is not able to efficiently transmit an incoming APs (the synapse is *fatigued*) and the postsynaptic response becomes weaker (Tsodyks and Markram, 1997).

The mechanism of short-term facilitation (STF) is caused, instead, by influx of calcium into the axon terminal after spike generation, which increases the release probability of neurotransmitters.

The mechanism of STD has been found to be involved in several complex behaviours observed in neural system, such as attention (Buia and Tiesinga, 2005), up and down cortical transitions (Pantic et al., 2002; Holcman and Tsodyks, 2006), to name a few.

On the other hand, STF has been the focus of little attention until now, although very recent studies reveal that it could have a role in working memory tasks (Romani et al., 2006) or in slow oscillations (Melamed et al., 2008).

### 1.2.3 Long-term potentiation

The current and most widely accepted model of synaptic plasticity that results in long-term synaptic change and possibly long-term memory encoding involves the experimental models of long-term potentiation and depression (Bear and Malenka, 1994).

Long-term potentiation (LTP) is a persistent increase in synaptic efficacy that follows brief periods of stimulation.

LTP was discovered in 1973 from experiments on organotypic samples (Bliss and Gardner-Medwin, 1973; Bliss and Lømo, 1973). A few seconds of high-frequency electrical stimulation (referred to as a “tetanus” in the following) of the *perforant path* in rabbit hippocampus was found to increase synaptic transmission between the axons of stimulated granule cells and the postsynaptic as revealed by an increase in the size of the field EPSP recorded. This change in synaptic transmission persists for hours, and under certain conditions, for days and weeks and for this reason, this phenomenon has been called LTP.

Mechanisms underlying LTP have been proposed as possible mechanisms underlying memory formation in the brain. This is in part due to the properties LTP displays (Bliss and Lømo, 1973; Nicoll et al., 1988; Bliss and Collingridge, 1993) and supported by the observation that a LTP-like phenomenon can be seen in brains of animals successively learning a behavioural task (Berger, 1984; Moser et al., 1994).

In organotypic hippocampal slices, LTP is characterized by three basic properties: cooperativity (a significant number of presynaptic fibres must be simultaneously activated to evoke LTP), input-specificity (when LTP is evoked at one set of synapses on a postsynaptic cell, neighbouring synapses that were not activated during stimulation, do not show LTP) and associativity (low-intensity stimulation of two pathways or higher intensity stimulation of weak inputs, converging on the same cell, is sufficient for the induction of LTP) (Bliss and Collingridge, 1993).

Several forms of LTP in the hippocampus have been described so far. The most extensively studied form of LTP is mediated by activation of the NMDA receptor, which upon gating $Ca^{2+}$ in the postsynaptic cell activates the processes that lead to LTP expression (Fig. 1.4). However, at certain synapses a sturdy form of LTP can be produced that does not require NMDA receptor activation. In the hippocampus this form of LTP occurs at mossy fibres synapses (Nicoll and Malenka, 1995).

After postsynaptic depolarization and removal of the magnesium block, $Ca^{2+}$-ions entering the postsynaptic cell, lead to a significant increases in intracellular calcium levels (MacDermott et al., 1986). This rise of intracellular calcium is thought to be a critical trigger for LTP induction, considering that the injection of calcium chelators into the postsynaptic cell has been shown to block LTP induction in the hippocampus (Lynch et al., 1983; Baranyi and Szente, 1987). The sudden influx of calcium activates a cascade of intracellular events that are thought to have a key role in the expression of LTP: activation of the protein kinase α-calcium-calmodulin-dependent protein kinase II (CaMKII) is believed to be a crucial component of the molecular mechanisms underlying LTP induction. Subsequently, the CaMKII activation leads to the phosphorylation of existing, and insertion of additional AMPA receptors (AMPARs) in the synaptic membrane, which facilitate postsynaptic response (Malenka and Nicoll, 1999; Manilow and Malenka, 2002).

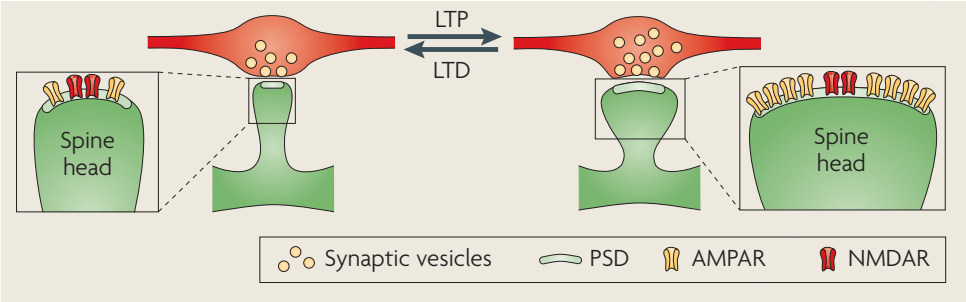


**Figure 1.4:** ***When glutamate is released from the presynaptic terminal and binds to NMDA and AMPA receptors, $Na^+$ and $K^+$ ions will be allowed to pass only through AMPA receptors because of $Mg^{2+}$ blockage in NMDA receptors. When the postsynaptic cell membrane depolarized the NMDA $Mg^{2+}$ block is relieved. Only in this condition $Na^+$, $K^+$, and $Ca^{2+}$ ions can pass through NMDA channels. Calcium acts as a second messenger, activating several intracellular signalling cascades, one of which phosphorylates AMPA receptors, thus facilitating their conductance (more ions enter in the cell). Another intracellular signalling cascade acts to recruit additional AMPA receptors to the postsynaptic membrane, thereby creating a larger postsynaptic dendritic spine with an enhanced synaptic strength. A synapse that has already been strengthened will respond faster to a presynaptic glutamate release, opening a larger number of AMPA receptor channels. This allows NMDA receptors to open sooner and consequently calcium to activate its cascades, so that the neural connection will be activated more rapidly (adapted from Holtmaat, A., & Svoboda, K.,2009).***

Depending on the experimental procedure, LTP can last from several hours to even weeks. The maintenance of LTP is thought to occur via changes in protein synthesis and gene transcription, as well as morphological changes, such as increases in dendritic spine number or the enlargement of existing spines (Malenka and Bear, 2004) (Fig. 1.5).

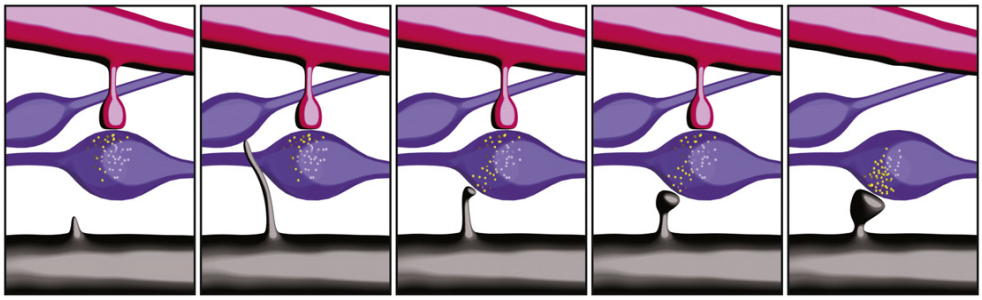

***Figura 1.5:*** ***Schematic drawings of plastic phenomena involving synapses. Images show the formation of a dendritic spine occurring through the initial formation of a filopodia (grey) that contacts an already-present axonal bouton (blue) connected to a dendritic spine (red). This filopodia then forms a synaptic contact, enlarges, increasing its volume to create a multi-synaptic bouton contacting two spines. Eventually the first spine is lost and a single synapse bouton remains with the new spine (adapted from Graham et al., 2008).***

No question concerning LTP has generated more discussion and confusion over the last two decades than the question whether the increase in synaptic strength is due primarily to a pre- or postsynaptic modification. Many authors agree that the most likely postsynaptic change that could cause LTP would be a modification in AMPA receptor

function and/or number because LTP increases the AMPA-receptor mediated currents much more than the NMDA-receptor mediated currents (Kauer et al., 1988): the phosphorylation of the AMPA receptor subunit GluR1 by CaMKII is critically important for the change in AMPA receptor responsiveness (Barria et al., 1997). Furthermore, after the induction of LTP, there is a rapid and selective up-regulation and trafficking of AMPA receptors regulated by CaMKII (Hayashi et al., 2000; Lu et al., 2001). However, it is important to say that these studies were performed in newborn rats (Hayashi et al., 2000; Shi et al., 1999). In mature animals LTP does not alter membrane association of AMPA receptors but leads to a rapid surface expression of NMDA receptors (Grosshans et al., 2002). Besides long-lasting changes on the postsynaptic terminal, recent papers provide additional evidence for a possible role of enhanced transmitter release in LTP (Zakharenko et al., 2001; Tyler and Pozzo-Miller, 2001).

Another aspect of past studies was to identify *electrical* stimulation protocols that induce and simulate the physiological conditions that are believed to occur during the formation of new memories. *Chemical* protocols for inducing LTP (LTPc) also exist and have gained in popularity (Aniksztejn and Ben-Ari, 1995; Otmakhov et al., 2004): in this case, LTPc ensures that a larger proportion of synapses are potentiated, unlike electrical stimulation, which is highly localized and only a small fraction of synapses are activated. In any case, LTP of synaptic transmission is a process that results in a persistent increase in the size of the synaptic component of the evoked response (i.e., recorded from cells or populations of cells), which is a reasonable representation of endogenous processes of memory formation (Albensi et al., 2007).

### 1.2.4 Electrically induced synaptic plasticity

From an experimental point of view, synaptic plasticity can be induced by a plethora of protocols, which can be loosely grouped into two: frequency dependent and pairing dependent (Letzkus et al., 2007). In frequency dependent protocols, the size and magnitude of the change in synaptic strength depends on the frequency of synaptic activation. Low-frequency activation, usually, causes LTD, whereas high-frequency activation typically elicits LTP. On the other hand, pairing-dependent protocols induce plasticity through coincidence of presynaptic activation and postsynaptic depolarization, in a specific form of synaptic plasticity termed spike timing-dependent plasticity (STDP). In contrast with frequency dependent protocols, during STDP the sign and

magnitude of the change in synaptic strength depends on the precise timing of pre- and postsynaptic activation (Letzkus et al., 2007).

### 1.2.4.1 High-frequency stimulation (HFS)

LTP can be typically induced by a so-called high-frequency tetanus, which is a train of 50÷100 stimuli (i.e., square pulses) (Fig. 1.6) at 100 Hz (Bliss and Collingridge, 1993).

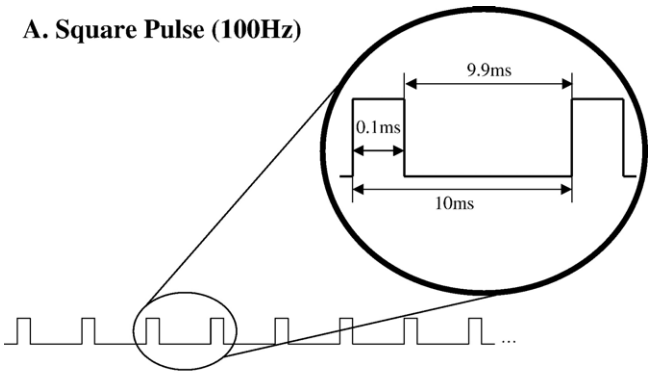


**Figure 1.6:** ***Representation of a 100 Hz square pulse, commonly used for LTP induction. The pulse width= 0.1 ms; interpulse interval= 9.9 ms and the pulse lenght is 10 ms (adapted from Albensi et al., 2007)***

Until now, all LTP protocols to our knowledge use only square pulses. However, it was realized that different trains of 100 Hz electrical stimulation, even if they lead to an increase in long-term synaptic transmission (Bliss and Collingridge, 1993; Bliss and Lømo, 1973), may not be effective in the same way (Albensi et al., 2007). For example, three trains of 100 Hz stimulation (100 pulses for 1 s repeated 3 times with an interval ranging from ~0.5 to 10 s; total of 300 pulses), which some consider to be a strong stimulation (Vertes, 2005), is sufficient for producing so-called late LTP that lasts 3 h or more and involves protein synthesis (Frey et al., 1993; Huang and Kandel, 1994). Whereas, a single 100 Hz train (100 pulses over 1 second), considered a weak stimulation, leads to early LTP (1÷3 h) and is protein synthesis-independent (Vertes, 2005). 100 Hz protocols (i.e., 100 Hz, for 1 second, at baseline stimulation intensity) have been used and are effective for inducing both NMDA receptor-dependent and NMDA receptor-independent forms of LTP.

However standard high frequency stimulation protocols appear inherently different from naturally occurring firing patterns of neurons (Albensi et al., 2007). For example, it is not certain that hippocampal neurons in living animals fire at 100 Hz for one full

second: CA1 hippocampal pyramidal cells typically fire for only 30÷40 ms bursts of three to four spikes (Feder and Ranck, 1973; Kandel and Spencer, 1961).
In response to this criticism, other stimulation protocols, such as theta burst (Graves et al., 1990; Morgan and Teyler, 2001) were developed that appear efficient at eliciting LTP and are physiologically closer to what occurs in the hippocampus during episodes of learning and memory in living animals.

#### 1.2.4.2 Theta-burst stimulation

LTP can also be induced by stimulation protocols that are much more similar to naturally occurring in the hippocampal firing patterns. During animal exploration and learning of a new environment, rat or mouse hippocampal pyramidal neurons fire bursts of action potentials at about five bursts per second, i.e. 5 Hz. This is the hippocampal "theta" rhythm that has been widely described in the literature. One variation of LTP-inducing stimulation that mimics this pattern of firing is referred to as Theta-Frequency Stimulation (TFS), which consists of 30 seconds of single stimuli delivered at 5 Hz. Another variation, Theta-Burst Stimulation (TBS) consists of three trains of stimuli delivered at 20-seconds intervals, each train composed of ten stimulus bursts delivered at 5 Hz, which each bursts consisting of four pulses at 100 Hz. These stimulations, which are based on naturally-occurring firing patterns *in vivo*, lead to LTP in hippocampal slice preparations.
So why theta bursts electrical stimulation protocols are so effective in LTP induction? A primary characteristic of theta bursts protocols is the interburst interval of 200 ms. which corresponds to the time period when inhibitory post-synaptic potentials (IPSPs) are difficult to recruit. This is because the refractory period for IPSPs ranges from 200 to 500 ms, a period longer than the interburst interval. Without IPSP recruitment, repeated stimulation allows for more effective temporal summation of excitatory post-synaptic potentials (EPSPs) (Albensi et al., 2007).
Until recently, the mechanisms that were responsible for theta burst were not fully understood. Thanks to the latest results, data show that theta burst stimulation, in area CA1, like high-frequency stimulation, also involves transcription, translation, and protein kinase A (PKA) activation (Nguyen et al., 1994). Furthermore, calcium-imaging studies involving HFS versus theta burst in CA1 rat hippocampus demonstrate that during theta burst, the mean time to peak of $Ca^{2+}$ signals was significantly longer, and

the mean peak amplitude and area under the $Ca^{2+}$ response were larger than during HFS (Perez et al., 1999). In addition, it has been reported that different calcium sources (e.g., voltage-dependent calcium channels (VDCC), intracellular stores) have different thresholds for activation by theta burst trains (i.e., 10 times 100 Hz bursts [5 pulse/burst] and a 200 ms interburst interval at test pulse intensity) where each calcium source might be involved to the induction of a different form of LTP (Raymond and Redman, 2002).

#### 1.2.4.3 Spike timing-dependent plasticity

Spike timing- dependent plasticity (STDP) is a form of bidirectional plasticity in which the temporal order of pre- and post-synaptic action potentials on a precise (ms) time-scale triggers changes in synaptic strength (Dan and Poo 2004; 2006).

The dependence of synaptic plasticity on temporal order was first described in experiments using different stimulation intensities for ipsi- and contralateral projections from the enthorinal cortex to the dentate gyrus (Levy and Steward, 1983).

In studying the temporal specificity of associative synaptic modification in the hippocampus, Levy and Steward (1983) noted that when a weak and a strong input from enthorinal cortex to the dentate gyrus were activated together, the temporal order of activation was crucial.

A detailed characterization of the timing dependence of STDP showed that most synapses undergo LTP when the postsynaptic action potential follows the synaptic input (positive timing), whereas LTD is usually observed when the postsynaptic action potential precedes the synaptic input (negative timing) (Debanne et al., 1998; Markram et al., 1997; Bi and Poo, 1998) (Fig. 1.7).

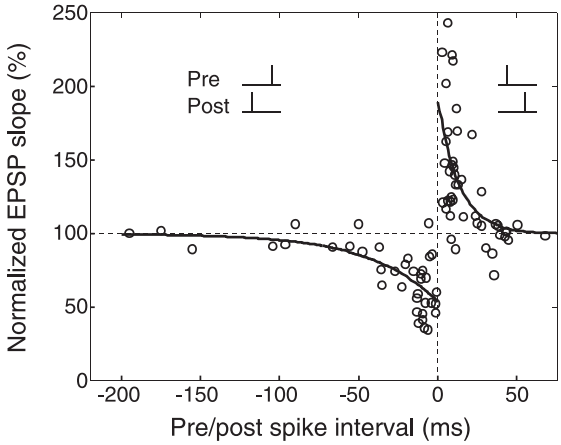


**Figura 1.7***: Critical window for the induction of synaptic potentiation and depression. The percentage change in the EPSC amplitude at 20÷30 min after the repetitive correlated spiking (60 pulses at 1 Hz) was plotted against the spike timing. Synaptic modification induced by repetitively paired pre- and postsynaptic spikes in layer 2/3 of visual cortical slices from the rat. Each symbol represents result from one experiment. Curves are single exponential, least-squares fits of the data. Insets depict the sequence of spiking in the pre- and postsynaptic neurons. Spike timing was defined by the time interval (Δt) between the onset of the EPSP and the peak of the postsynaptic action potential during each cycle of repetitive stimulation, as illustrated by the traces above (adapted from Dan and Poo, 2006).*

In addition, STDP also depends on the subcellular location of inputs in the dendritic tree (Debanne et al., 1998; Du and Poo, 2004; Dudek and Bear, 1992), the firing mode during induction (Dudek and Bear, 1992; Egger et al., 1999) and the generation of dendritic spikes (Engert and Bonhoeffer, 1997; Engert et al., 2002; Feldman, 2000; Fitzsimonds et al., 1997).

In conventional protocols using steady postsynaptic depolarization and hippocampal slices, high-frequency presynaptic stimulation induces LTP and low-frequency stimulation induces LTD, but in STDP low-frequency stimulation can be used to induce both LTP and LTD (Dan and Poo, 2006).

The common model of STDP induction assumes that depolarization associated with back-propagating action potentials triggers plasticity via relief of the voltage-dependent block of the N-methyl-D-aspartate (NMDA) receptor by magnesium ions (Frick et al., 2004; Froemke and Dan, 2002). At positive spike timings, this leads to substantial calcium influx through synaptic NMDA receptors, triggering LTP (Froemke et al., 2005). In contrast, the moderate NMDA receptor-mediated calcium signal evoked by pairings at negative times is thought to trigger LTD (Bliss and Lømo 1973; Fu et al., 2002; Ganguly et al., 2000; Golding et al., 2002). However, LTD induction appears to be more heterogeneous because it has also been reported to depend on NMDA receptor

desensitization (Debanne et al., 1998), voltage-gated calcium channels (Celikel et al., 2004), metabotropic glutamate receptors (Chavez-Noriega et al., 1990; Froemke et al., 2005; Hausser and Mel, 2003) and presynaptic NMDA receptors (Hess and Gustafsson, 1990).

The ~20 ms pre-post window for LTP induction is much shorter than the time constant for the dissociation of glutamate from NMDARs (Vicini et al., 1998). One explanation of this narrow LTP window is the kinetics of $Mg^{2+}$ unblock of the NMDARs. Kampa at al. (2004), measuring the rate of depolarization-induced $Mg^{2+}$ unblock of NMDARs at different times after a brief pulse of glutamate application, found that $Mg^{2+}$ unblock consists of a fast and a slow component, whose relatives amplitude depend on the timing of the depolarization relative to the glutamate pulse, with the fast component preferentially reduced at later times. Thus the postsynaptic spikes, arriving immediately after glutamate binding, are more effective in opening NMDARs, thus sharpening the time window for LTP induction (Dan and Poo, 2006).

### 1.2.5 Chemically induced synaptic plasticity

Despite the great contribution that electrically-induced LTP has provided to the study of phenomena related to synaptic plasticity, this method is applicable only to a fraction of the totality of the available synapses in cultured neurons. For these reasons, other methods have been developed for the induction of LTP to gain the advantage of involving a greater number of synapses. One of these methods is precisely the strengthening of synaptic transmission through the use of compounds that allows both to activate many more synapses but also opens access to biochemical and morphological studies on synaptic plasticity.

There are many ways to induce chemical plasticity and among them chronically or acute bath application has become the favorable method because it is easy to manipulate and would efficiently affect all the cells in the culture.

A diffuse approach to induce long term synaptic strengthening is by the delivery of drugs that lead to an increased production of cAMP (Bolshakov et al., 1997; Brandon et al., 1995). As a result it has been observed a direct activation of transcription/translation processes that underlie the consolidation phase and long-term maintenance of LTP.

Since the postsynaptic $Ca^{2+}$ entry through NMDA receptors has been found to be the key point to induce synaptic plasticity (Collingridge and Herron, 1988; Collingridge and

Kehl, 1983; Cummings and Mulkey, 1996), the NMDA receptor has received much more attention than others from neuroscience research community. NMDA, as a specific agonist of NMDA receptors, has been widely used to induce different forms of synaptic plasticity since long (Asztely and Hanse, 1991; Broutman and Baudry, 2001; Collingridge and Kehl, 1983).

This chemically-induced plasticity likely involves stimulation of both extrasynaptic and synaptic NMDA receptors. The influx of $Ca^{2+}$ produced by the activation of NMDA receptors leads to an increase in intracellular calcium levels, which spreads throughout the neuron and in dendritic spines and can activate calcium-dependent kinases (i.e., CamKII and PKC) as well as phosphatases (i.e., PP2B).

Furthermore, the selective blockade of the NMDA receptor by MK-801 prevents the LTP, confirming the crucial role of synaptic NMDA receptors in inducing NMDA receptor-dependent hippocampal LTP in both cultures and slices.

Given that the long-term changes in synaptic efficacy lead to structural alterations of synapses, the structural changes of synaptic structures may be controlled by the growth activity of Brain Derived Neurotrophic Factor (BDNF).

## 1.3 The Neurotrophins

The development, survival and differentiation of the nervous system is influenced by a family of polypeptides called neurotrophic factors or neurotrophins. The family of neurotrophins consists of four members: the nerve growth factor (NGF; Levi-Montalcini and Booker, 1960), the brain derived neurotrophic factor (BDNF; Leibrock et al., 1989), the neurotrophin-3 (NT3; Hohn et al., 1990) and neurotrophin-4 (NT4; Berkemeier et al., 1961) (Fig. 1.8).

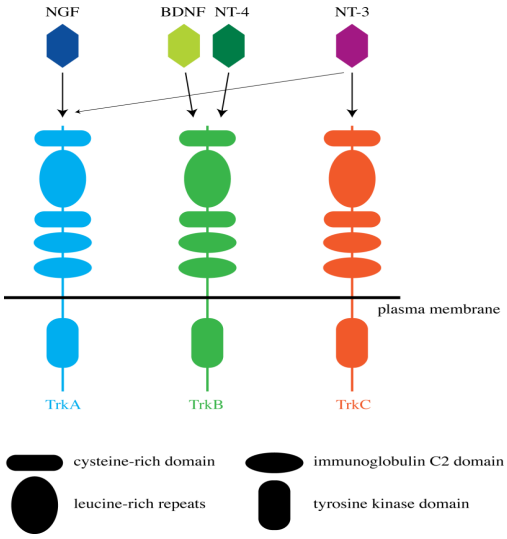


***Figura 1.8: The neurotrophin family consists of four closely related proteins encoded by four different genes that include NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor), NT-3 (neurotrophin-3) and NT-4 (neurotrophin-4). Three Trk receptors encoded by independent genes afford selectivity among neurotrophins with NGF and NT-3 binding to TrkA, BDNF and NT-4 binding to TrkB and NT-3 binding to TrkC. All Trk receptors are composed of extracellular leucine-rich repeats, cysteine-rich domains and a single immunoglobulin C2 domain that ensure proper conformation of the ligand-binding pocket. Neurotrophin binding promotes homodimerization of Trk receptors, which in turn initiates intracellular phosphorylation cascades. The intracellular tyrosine kinase domain common to all Trk receptors is necessary for neurotrophin signaling as it allows transphosphorylation of several key tyrosine residues of the Trk receptor dimer at the origin of pleiotropic neurotrophic function (adapted from Deinhardt and Jeanneteau, 2012).***

In addition to supporting neuronal survival, neurotrophins are important regulators of neuronal growth and morphology, and emerging evidence support their involvement in neuronal plasticity (Aloyz et al., 1999; Schinder and Poo, 2000; Lu, 2003b). In fact, neurotrophins mediated regulation of excitatory and inhibitory signalling as well as changes in neuronal network are fundamental features of learning and memory.

Neurotrophins act via specific tyrosine kinase receptors called tropomyosin-related kinase (Trk)-receptors (Barbacid, 1994; Huang and Reichardt, 2003). Although typically all neurotrophins can interact with several Trk receptors, NGF preferentially binds to TrkA, BDNF and NT-4 to TrkB and NT-3 to TrkC (Fig. 1.8).

The neurotrophin binding to Trk receptor occurs with high affinity and starts several signalling cascades that transport the message to the targets (Patapoutian and Reichardt, 2001). Anyway, the first identified neurotrophin receptor was the p75 that binds all mature neurotrophins with significantly lower affinity than Trk-receptors (Hempstead, 2002; Huang and Reichardt, 2003). Recent results have revealed a putative role of p75

in modifying neurotrophin signalling both independently and in collaboration with Trk receptors (Beattie et al., 2002; Roux and Barker, 2002; Kaplan and Miller, 2003). Neurotrophin signalling appears however to be much more complicated than originally thought, influencing several different functions in the nervous system.

### 1.3.1 Brain Derived Neurotrophic Factor (BDNF)

Brain-derived neurotrophic factor (BDNF) is a member of the neurotophin family consisting of small-secreted protein that plays important roles in the development of the nervous system in vertebrates (Bibel and Barde, 2000; Binder and Scharfman, 2004). As all other neurotrophins, BDNF is produced initially in the form of a precursor proBDNF protein, characterized by a molecular weight (MW) of about 28-32 kDa, which is subsequently cleaved into the mature BDNF protein, 14 kDa of MW (Fig.1.9).

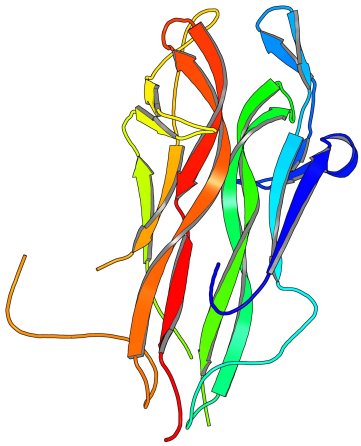

**Figure 1.9:** ***Biological assembly of Brain Derived Neurotrophic Factor from Protein Data Bank (PDB). BDNF crystallographic unit cell measures 5x10x5 nm$^3$.***

It is mainly produced by neurons, but is also synthesized and released by astrocytes under the control of neuronal activity (Juric et al., 2006; Balkowiec and Katz, 2000). For many years, it was believed that BDNF, like other neurotrophins, was secreted from only postsynaptic neurons, acting retrogradely on presynaptic terminals (Wetmore et al., 1991). However, recent studies indicate that BDNF secretion occurs through anterograde transport to the axons with subsequent release to postsynaptic neurons (Kohara et al., 2001; Adachi et al., 2005).

BDNF is abundantly expressed throughout the brain, with the most pronounced expression in the hippocampus, cerebral cortex, thalamic and hypothalamic nuclei and

striatum (Hofer et al., 1990; Nawa et al., 1995; Ivanova and Beyer, 2001). In addition, BDNF is also produced in activated cells of the immune system (Kerchensteiner et al., 1999), endothelial cells (Donovan et al., 2000), in liver and muscle tissue (Cassiman et al., 2001; Matthews et al., 2009).

BDNF mediates its neurotrophic effect through binding to a specific high affinity receptor, the tropomyosin-related kinase B (TrkB), and to the low affinity neurotrophin receptor p75NTR. These receptors are often present on the same cell and modulate two different responses: binding of TrkB promotes neuronal survival and differentiation while ligation of p75NTR frequently promotes apoptosis (Kaplan et al., 2000). Moreover BDNF through the activation of TrkB facilitates hippocampal LTP while pro-neurotrophin interaction with p75NTR facilitates hippocampal LTD (Kaplan et al., 2000).

#### 1.3.1.1 Physiological roles of BDNF

BDNF was first described as a survival factor for certain neuronal populations. Concerning cell cultures of central nervous system neurons, it supports the survival of retinal ganglion neurons (Johnson et al., 1986), basal forebrain cholinergic neurons (Alderson et al., 1990), substantia nigra dopaminergic neurons (Hyman et al., 1991; Knusel et al., 1991), cerebellar granule cells (Segal et al., 1992; Lindholm et al., 1993) and cortical neurons (Ghosh et al., 1994).

Studies involving BDNF deficient mice have been shown its involvement in learning (Linnarsson et al., 1997), obesity (Lyons et al., 1999b), anxiety (Rios et al., 2001) and depression (Chan et al., 2006).

The peripheral effects of BDNF are only barely investigated and indicate that BDNF is involved in neuronal regeneration after nerve injury (Novikova et al., 1997) and in regulating the immune system (Schuhmann et al., 2005; Schenone et al., 1996). However, the effects of BDNF signalling oncell survival can be converted into increase of cell death depending on the intracellular pathways activated (Kim et al., 2002). This is supported by studies showing that excess BDNF intensifies both epileptic seizure and stroke-induced neuronal damage in the brain (Rudge et al., 1998; Gustafsson et al., 2003a). Therefore, BDNF signalling regulated by the expression of different BDNF receptors and the post-translational cleavage of BDNF control the effects of BDNF on neurogenesis, cell survival, maintenance and cell death.

Several studies with either wild type or gene-modified (knock-out or knock-in) mice suggest that BDNF has a prominent effect also on neuronal morphology. It modifies axonal and dendritic growth both *in vitro* and *in vivo*. Danzer and co-workers (2002) observed increased basal dendrite number and apical dendritic branching of dentate granule cells in hippocampal slice cultures transfected with BDNF. BDNF affects also the morphology of other brain areas, such as cortex and neocortical neurons show decreased dendritic complexity both in TrkB knock-out mice and in cell cultures from P0 $TrKB^{-/-}$ mice (Gates et al., 2000; Xu et al., 2000).

Besides controlling neurite growth, BDNF regulates the fine structures of active neurons. It influences the formation, stabilization and maintenance of spines, the major structures where synapses dwell. Consequently, BDNF promotes the formation of both excitatory and inhibitory synapses and increases their maturation (Martinez et al., 1998; Huang et al., 1999; Lu 2003b). BDNF also stabilizes newly formed synapses in response to neuronal activity and it may be able to stabilize synapses even in the absence of neuronal stimulus (for review, see Vicario-Abejon et al., 2002; Miller and Kaplan, 2003). All these results support the idea that BDNF presumably is one of the key players in the formation and stabilization of neuronal connections.

#### 1.3.1.2 BDNF and synaptic transmission

In addition to its trophic effects during brain development, BDNF has been shown to exert acute effects on synaptic transmission and plasticity (Bramham and Messaoudi, 2005; Lu, 2003; Poo, 2001). In particular has been found that BDNF modulates both excitatory and inhibitory transmission, through different mechanism (Bolton et al., 2000; Vicario et al., 1998).

BDNF increases excitatory synaptic transmission through pre and postsynaptic mechanisms (Minichiello, 2009; Waterhouse and Xu, 2009). These effects are mediated primarily by TrkB receptor signalling, localized at the level of synaptic terminals and dendritic spines (Aoki et al., 2000; Cabelli et al., 1996; Drake et al., 1999). The binding of BDNF to its receptor TrkB leads to the initiation of signalling cascades that include activation of the voltage-gated sodium channels and potassium channels but also of glutamate and GABA receptors (Blum et al., 2002; Cheng and Yeh, 2003; Kramar et al., 2004).

Presynaptically, BDNF enhances glutamate release and increases the frequency of mEPSCs in hippocampal neurons (Lessmann and Heumann, 1998; Takei et al., 1998). On the postsynaptic side, BDNF increases NMDA-single channel open probability (Levine et al., 1998; Levine and Kolb, 2000) presumably through tyrosine phosphorylation of the NMDA receptor subunits NR1 and NR2B (Lin et al., 1998; Suen et al., 1997). Altogether, BDNF enhances excitatory synaptic strength by enhancing neurotransmitter release in the synapse.

BDNF enhances neuronal excitability by increasing also expression of voltage-gated $Ca^{2+}$ and $Na^{+}$ channels in the plasma membrane (McAllister et al., 1999).

All of these findings strongly support enhanced excitatory synaptic transmission by BDNF, and suggest possible mechanisms by which BDNF may affect LTP, learning and memory.

Regarding the inhibitory synaptic transmission, BDNF is required for the maturation of inhibitory γ-aminobutyric acid (GABA)ergic synapses and the regulation of interneuron properties in the hippocampus (Berninger et al., 1995: Yamada et al., 2002b). BDNF reduces inhibitory synaptic transmission in hippocampal cell cultures and both evoked and spontaneous GABAergic currents are decreased in hippocampal slices via TrkB receptor (Marty et al., 1996; Rutherford et al., 1997).

Recently, BDNF has been reported to suppress $Cl^{-}$-dependent fast inhibitory transmission by decreasing the expression of $K^{+}/Cl^{-}$ co-transporter KCC2 (Rivera et al., 2002; Wardle and Poo, 2003). This provides a possible mechanism for TrkB-mediated depression in the brain.

#### 1.3.1.3 BDNF and synaptic plasticity

Among members of the neurotrophin family, brain-derived neurotrophic factor (BDNF) stands out for its ability to regulate synaptic plasticity and various cognitive functions of the brain (Woo and Lu, 2006).

Since his discovery, the biological role of BDNF for developing neurons has been shown extensively: BDNF promotes the differentiation and survival of developing neurons *in vivo* and *in vitro* (Bibel and Barde, 2000). Furthermore, BDNF-Trk signalling has been characterized in plenty of brain functions: neuronal cell survival, neurite growth, cell migration, dendritic growth, synapse formation, stabilization and potentiation (Mizui and Kojima, 2013).

Electrophysiological studies have demonstrated that BDNF have a key role in synaptic plasticity (Poo, 2001; Barco et al., 2005). Acute application of mature form of BDNF on hippocampal slices facilitates the early phase of LTP (E-LTP) in the hippocampus (Korte et al., 1995; Figurov at al., 1996; Patterson et al., 1996). Inhibition of BDNF activity, by gene knockout or functional blocking using BDNF antibody or TrkB-immunoglobulin G, attenuates hippocampal E-LTP (Korte et al., 1995; Patterson et al., 1996; Chen et al., 1999; Xu et al., 2000).

In other studies, it was shown that the exposure to BDNF led to axonal branching (Cohen-Cory and Fraser, 1995; Gallo and Letourneau, 1998), dendritic growth (McAllister et al., 1999; Suzuki et al., 2007), and refinement of synapses in an activity-dependent manner (Cabelli et al., 1995). It was also shown that long-term treatment (72 h) of hippocampal slices with BDNF (250 ng/mL) increases synapse number and spine density in apical dendrites of pyramidal neurons in the hippocampus (Tyler and Pozzo-Miller, 2001), suggesting that BDNF acts on different types of spines, depending on spontaneous synaptic transmission.

Kafitz et al. (1999) showed that low concentrations of BDNF causes membrane depolarization of hippocampal, cortical and cerebellar neurons within a few milliseconds, leading to the firing of an action potential. This was a remarkable finding because, until then, only classic neurotransmitters had been found to have such a rapid effect on the membrane potential of neurons. Since then, substantial evidence has accumulated to indicate a critical role for BDNF in LTP induction, not only at hippocampal synapses at both the Schaffer collateral-CA1 synapse (Kang and Schuman, 1995) and in the dentate gyrus (Messaoudi et al., 2002), but also in the visual cortex (Akaneya et al., 1997).

Interestingly, it has been shown that the induction of LTP is associated with the activation of a large number of signalling cascades, including the ones activated by BDNF (Cunha et al., 2010).

Other *in vitro* studies showed that exogenous BDNF promoted the induction of LTP in young hippocampal slices (Figurov et al., 1996), and rapidly enhanced the frequency of miniature excitatory postsynaptic currents in solitary neurons (Taniguchi et al., 2000). Conversely, LTP was attenuated in slices pre-treated with function-blocking BDNF antibodies or the fusion protein TrkB-IgG, a molecular scavenger of endogenous BDNF (Figurov et al., 1996; Kang et al., 1997).

Altogether these results propose the crucial involvement of BDNF/TrkB signalling in synaptic plasticity and possibly in learning and memory.

## 1.4 Hippocampal cultures as neuronal network model

Cultured neural networks are extremely helpful in investigating the mechanisms that underlie synaptic plasticity.

Neuronal networks are computational systems devoted to information processing, composed of physically interconnected homologous neurons able to perform specific physiological functions. Our CNS indeed, thanks to the activity of many complex neural networks, has the ability to perform complex functions, such as sensory perceptions, planning voluntary movements, memory, attention and, of course, consciousness.

How the CNS accomplishes complex functions *via* the activity of neural networks has been intensely investigated for two main reasons:

1) the identification of the mechanisms at the basis of the generation of neural codes may allow the knowledge needed to cure CNS diseases, where presumably such mechanisms are altered (Eidelberg, 2009);
2) the comprehension of rules underlying CNS processes may lead to the development of artificial neuronal network for the improvement of human-made electronic devices and communication networks (Laughlin and Sejnowski, 2003);
3) to increase our knowledge about central nervous system.

Neural networks can obviously be studied *in vivo* to accomplish the "global level rule" (whether the entire brain or brain large regions), through technologies such as the functional magnetic resonance imaging (MRI), which is based on the detection of blood oxygen levels using molecular resonance imaging technology, or positron emission tomography (PET) scans, where a radiolabeled glucose analogue is employed to measure metabolic activity. Although such techniques allow the simultaneous recording of entire brain activity, they are usually limited by low temporal resolution.

On the other hand, the use of *in vitro* models in place of *in vivo* ones presents several advantages, among these, the possibility of investigating neural networks with a better spatio-temporal resolution, namely at the cellular and synaptic levels. However, *in vitro* models display several limitations. First of all, these models allow the simultaneous

investigation of a limited number of neurons kept in an artificial *ex-vivo* environment. Studying neural networks *in vitro* cannot be assumed by any means even close to studying the entire brain behaviour, but it helps enormously in understanding the fundamental rules governing neural circuits behaviour.

Being aware of such limitations, an alternative approach to study the activity of neural networks at high spatio-temporal resolution consists in electrophysiological recordings from acute tissue slices isolated from the brain. Although brain slices are just a little portion of entire CNS, they can be kept alive and functional for hours, and they preserve the same cytoarchitecture observed in the intact animal. In these systems it is possible to perform recordings directly from single neurons by means of intracellular sharp electrode or patch clamp techniques or from small clusters of neurons by extracellular recordings of field potential or multi units. These techniques allow monitoring simultaneously a limited number of neurons and only for the narrow lifespan during the external procedure (Colicos and Syed, 2006).

Also the direct access to investigate and to manipulate (electrically or chemically) individual synapses becomes difficult by using hippocampal acute slices.

The last problem can be overcome thanks of the use of dissociated cultures, of course with the price of an additional step of simplification of the network model.

Neurons dissociated in cell cultures are explanted from their respective areas in the intact brain, and, upon re-growing in vitro, can recapitulate their connectivity patterns remaining viable for weeks or months on appropriate substrates and in the presence of trophic factors (Potter and DeMarse, 2001).

During the dissociated process, the original *in vivo* cytoarchitecture is lost, however, neurons, especially obtained from embryo or neonatal rodents, show an extraordinary ability in re-establishing *ex novo* synaptic connections, which present properties resembling the ones seen *in vivo* (e.g. processes re-growth, interconnection and activity).

Exemplificative is the case of dissociated retinal neurons, which, once grown in the presence of appropriate grown factors, recapitulate during their development in culture waves of activity usually detected in the entire retina (Colicos, 2004).

In addition, as dissociated cells can be placed in close contact with their growth substrate, this provides a unique advantage to develop an entirely new way of

interfacing recording and stimulating devices with neurons and such knowledge might be exploited in the field of brain machine interfaces.

### 1.4.1 Dissociated hippocampal cultures

Dissociated hippocampal cultures are widely used models in neuroscience research, due to two reasons: hippocampus is a well defined brain region, easily identifiable for isolation during dissection and, in this structure, plasticity phenomena as LTP and LTD, presumably involved in synaptic changes underlying memory and learning, occur (Lynch, 2004).

Cultured brain circuits provide an *in vitro* simple model of a neuronal network and offer a variety of investigative levels to answer fundamental questions in neurobiology such as how neurons reconstruct a functional network, how they orient their growth trajectory and recognize the target cells, how they rebuild active synapses, what rules govern such interaction and the nature of the basic cell circuits that comprise the more complex neuron tissue. *In vitro* hippocampal network activity has been assessed mainly via direct electrophysiological recordings (e.g. patch-clamping).

The most obvious advantage of dissociated neuronal cultures is that they make individual living neuronal cells and their synapses easily accessible. Dissociated neurons will grow as a monolayer and the surface-expressed neurotransmitter receptors and other membrane proteins are therefore accessible for both immunocytochemical and biochemical analysis. Hippocampal neurons in dissociated cultures retain their characteristics, from the properties of the transmitter receptors and ion channels they express, to the organisation of their cytoskeletal constituents and the characteristic synapses. This is due to the fact that they are postmitotic and are therefore committed in their differentiation at the time they are introduced into the culture (Molnár E., 2011).

Banker and Cowan (1977) developed a method for culturing dissociated hippocampal neurons. Following trypsinization of isolated rat embryonic hippocampus, cells were seeded at low density on polylysine-treated coverslips in an enriched medium. The isolated neurons rapidly attached to the substrate and initiated process extension (Banker and Cowan, 1977). After a week in culture, a significant proportion of cells resembled normal pyramidal cells with a more-or-less triangular shaped soma, a single dominant dendrite-like process emerging from the apex of the soma, and several “basal dendrites” arising from the opposite pole of the cell. Comparisons of the lengths of

these dendrite-like processes with those of hippocampal cells visualized in brains of animals sacrificed at post-natal day fourth showed that in some cases the rate of process formation *in vitro* approximates that detected *in vivo* and that the general shape of the neurons was remarkably similar to that of immature pyramidal cells (Banker and Cowan, 1979). Morphological characterization by means of scanning electron microscopy (SEM) revealed that dissociated embryonic hippocampal neurons after one week in culture, presented specializations, which were identified as dendrites or axons based on their intracellular structures (Bartlett and Banker, 1984a). After 24 days in vitro, neuronal cell body became larger in diameter, neurites were longer and many ultrastructures connected to neuronal activity, i.e. synaptic vesicles, were visible.

Segal (1983) reported that embryonic dissociated rat hippocampal neurons could be maintained in culture for 4÷6 weeks. During this period, neurons develop a dense network of interconnections and cell feature a shape with morphology similar to that of hippocampal cells *in situ*. In this work, Segal characterized the basic electrophysiological properties of cultured neurons, including input resistance, resting membrane potential, membrane time constant. All these parameters were found comparable to those measured from similar neurons in acute slices.

Dissociated hippocampal neurons were able to generate spontaneously electrical signals (Segal, 1983); both excitatory and inhibitory transmission has been detected.

Cultured hippocampal networks, despite of the variability in the experimental strategies adopted, basically due to the variable ages of animals used for the explant and to the variable cell density used for culturing, usually show a common developmental trend.

Neurons usually initiate to generate single spontaneous APs within the first week *in vitro*. In the following days, the rate of spontaneous activity increases, due to a progressive maturation and enhancement in the synaptic connectivity of the network, however single APs occur still randomly. After the second-third week in culture, APs are progressively synchronized into burst activity (Li et al., 2005), represented by fast series of spikes of decreasing amplitude, with short inter spike intervals (Harris et al., 2001).

A more detailed investigation of synaptic properties and plasticity can be achieved through the dual recordings approach, because it can reveal modifications of synaptic operativity and strength. Recording simultaneously from pairs of hippocampal neurons, Wilcox and co-workers (1994) elicited APs (under CC conditions) in the presynaptic

neurons in a controlled fashion, and evoked excitatory or inhibitory monosynaptic responses (EPSCs and IPSCs, respectively) in the postsynaptic ones (under VC conditions). While excitatory responses were mediated by NMDA or non-NMDA ionotropic glutamate receptors, evoked inhibitory currents were due to the activation of postsynaptic gamma-aminobutyric acid (GABA) receptors.

### 1.4.2 Synaptic plasticity in dissociated neuronal cultures

Dissociated hippocampal cultures have been considered as excellent models to investigate the generation of plastic changes in neural network activity.

Just to mention some of these studies, Bi and Poo (1998) showed that correlated spiking in pre- and postsynaptic cultured neurons induced the expression of persistent potentiation or depression in glutamatergic synapses (Bi and Poo, 1998). Ivenshitz and Segal (2006) demonstrated that long-term plasticity of excitatory and inhibitory transmissions could occur simultaneously, but independently, to modulate the output of neural networks (Ivenshitz and Segal, 2006).

Neuronal cultures were extensively used to understand the formation and development of synapses (Craig et al., 2006) and developmental changes in glutamate receptors (Molnár et al., 2002).

Hippocampal neurons in culture simplify complex circuitry compared to the considerably more complex brain slice preparations (Traynelis et al., 2010).

Using immunocytochemical approaches, these studies have also provided morphological evidence for “silent synapses” that physically contain NMDARs but no AMPARs (reviewed in Molnár E. and Isaac J.T.R., 2002). Nonetheless, the development of reliable procedures for the induction LTP in dissociated cultures proved to be more challenging. Conventional electrophysiological LTP induction protocols involve an intensive but transient activation of a small set of synapses. In contrast, continuous chemical activation of neuronal networks, which involves many more synapses, maximizes the likelihood of detection of molecular and morphological changes in cells involved (MacDonald et al., 2001; Salter, 2001). Over the last ten years various protocols were used for the investigation of NMDAR dependent LTP in primary hippocampal neuronal cultures. These models created new opportunities for the study of LTP-related changes in endogenous native proteins and signalling pathways (Molnár E., 2011).

# 2 AIM OF THE THESIS

Neuronal plasticity is an intriguing property of central nervous systems covering all the mechanisms involved in its capacity to adjust and remodel itself in response to environmental stimuli, experience, skill acquisition and new challenges, including lesions. Neurons continuously rearrange their synaptic circuitry, a feature termed synaptic plasticity. Local and quick changes in the morphology and efficacy of specialized neuronal areas, such as dendritic spines and synapses, are the fundamental key to adaptive remodelling of CNS neuronal networks.

Intrinsic sub-micrometric dimensions and metastability of these neuronal features make their study a real challenge, especially when the interest is in real time investigation.

The work of this thesis has been conducted in the wider framework of the NanoMosquito project, whose major aim was to combine electrophysiological measurements, scanning probe microscopy (AFM – Atomic Force Microscopy) and fluorescence microscopy in a new generation neurophysiological tool to understand neuronal plasticity at the nanoscale. In particular it intends to study neuronal ultrastructures of living, unlabelled, neurons undergoing plastic phenomena, with an unprecedented level of resolution. Sub-nanometric three-dimensional morphological reconstruction and mechanical analysis (and stimulation) by means of AFM would be performed on dendritic synapses and spines. Final goal of the main project would be to simultaneously perform patch clamp electrophysiology and AFM measurements and perturbation on neuronal cells.

It has been shown that scanning probe microscopy can be combined with the patch-clamp technique (Horber et al., 1995; Larmer et al., 1997) but the possibility to join them together in a single nano-tool has never been explored. The fundamental necessity to induce synaptic modifications on a specific synapse formed between a pair of neurons, in order to perform all the future AFM investigations connected to the NanoMosquito project, induced us to evaluate or develop opportune electrical and chemical potentiation protocols. This need represented the starting point aiming this thesis work.

Understanding in detail the mechanisms that underlie synaptic plasticity may be important not only for a detailed view of memory related processes but also in the case

of some diseases: being able to control synaptic plasticity may help to restore a functional connectivity lost, for example, in the case of brain lesions.

Studies of synaptic plasticity are often carried out in slices of hippocampus, an important organ for learning and memory. Unfortunately, acute slices prevent to study changes in the nanoscale, occurring at synaptic level, with a surface-microscopy technique such is the atomic force microscopy: in fact this approach needs a physical "contact" to the structures that one wants to image, consequently, dissociated hippocampal neurons lend themselves well for this purpose.

Therefore, the very first objective was to develop a protocol able, with a high probability of success, to induce long-term synaptic changes localized in well identified (or easy to identify) neurons and synapses. The first part of this thesis handles the setting of a localized electrophysiological stimulation, starting from the stimulation trains usually performed in hippocampal slices and adopting the experimental configuration of the paired recordings, where two mono-synaptically connected neurons are simultaneously patched clamped.

In the second part we induced chemically synaptic plasticity and among the multiple molecular moieties involved in the mechanism of synaptic plasticity, neurotrophin expression and in particular Brain Derived Neurotrophic Factor (BDNF) is found to be of crucial importance (Woo and Lu, 2006).

Since his discovery, the biological role of BDNF for developing neurons has been shown extensively: BDNF has been consistently shown to modify excitatory synaptic transmission and long-term synaptic plasticity in a variety of preparations (Lessmann et al., 1994; Kang and Schuman, 1995). Furthermore, it has been shown the role of BDNF in increasing and modulating spine density (Lebmann et al., 1994).

On the other hand, the role of chronic BDNF exposure in regulating long-lasting changes in synaptic function is comparably less investigated and may play a role on post injury alteration of synaptic networks and neuronal rescue (Lu V.B. et al., 2007; Cho et al., 1997; 1998).

In this work I sized up different electrophysiological protocols in order to evaluate the more suitable for NanoMosquito integration. I found particularly reliable, despite the lost in synapse spatial selectivity, the chemically induced (*via* BDNF) synaptic potentiation. Moreover, we went beyond this result investigating in detail the contribution of a chronic neurotropic treatment on dissociated hippocampal neurons:

this thesis work can be considered a starting point to better understand the mechanisms that underlie neurotrophin related phenomena or pathologies.

To give an example, recent experiments showed that alterations in BDNF level is involved in Alzheimer and Parkinson's disease. Furthermore, an imbalance of this level may contribute to neurodegenerative or psychiatric disorders (Siegel et al., 2000). For this reason, and since the BDNF involved in the sprouting of new synapses, I decided to investigate its role in regulating long-lasting changes in synaptic networks.

# 3 METHODS

## 3.1 Dissociated hippocampal cultures

Primary cell cultures were made from rat hippocampal neurons according to Malgaroli and Tsien (1992), with slight modifications (Tongiorgi et al., 1997). Hippocampi were dissected from 1÷3 days old animals (P1÷P3). Isolation and slicing were performed in a dissection medium containing, amongst other components, 200 µM kinurenic acid (Sigma, St. Louis, MO) and 25 µM D-AP5, D(-)-2-amino-5-phosphopentanoic acid (Tocris Neuramin, Bristol, UK) (see Table 3.1).

**Dissection medium (7.3 pH)**

| Reagent | Concentration |
|---|---|
| Hank's modified solution | 4.76 g |
| $NaHCO_3$ | 4.2 mM |
| D-Glucose | 33 mM |
| Kinurenic acid | 200 µM |
| D-AP5, D(-)-2-amino-5-phosphopentanoic acid | 25 µM |
| Gentamicina | 5 µg/ml |
| BSA | 300 mg |
| $MgSO_4$ | 12 mM |
| HEPES | 12 mM |

**Table 3.1**

Hippocampi were shredded in a sterile environment under a laminar flow hood and their fragments moved to a 15 ml Falcon tube where tissue slices were digested with trypsin (15000 units/mL) in the presence of deoxyribonuclease (1560 units/mL) using a buffered digestion medium (see Table 3.2).

**Digestion medium (7.4 pH)**

| Reagent | Concentration |
|---|---|
| NaCl | 137 mM |
| KCl | 5 mM |
| $Na_2HPO_4$ | 7 mM |
| HEPES | 25 mM |
| D-AP5, D(-)-2-amino-5-phosphopentanoic acid | 25 µM |
| $NaHCO_3$ | 4.2 mM |
| Kinurenic acid | 200 µM |

**Table 3.2**

After an initial manual heating to activate enzymes, cells were maintained 5 minutes at room temperature with the tube sloped to improve the surface to volume ratio. Subsequently they were washed with dissection medium supplemented with trypsin inhibitor (10.000 units/mL) at 4 °C for 10 minutes. The trypsin activation was followed by a wash with the dissection medium, added with DNAase 1248 units/mL. Mechanical dissociation was performed using a Gilson micro-pipette (equipped with a 1 mL tip) until homogeneous cellular dispersion was achieved. Steps in cell preparation procedure were alternated with dissection medium washing.

Cells were recovered and washed by two successive centrifugations at 500 rpm to remove the supernatant, the pellet containing cells was suspended in the incubation medium with fetal bovine serum 10% and plated on rectangular glass coverslips (24×12 $mm^2$) coated with 50 µg/ml polyornithine in 35 mm Nunc petri dishes at a density of 200000±16000 cells/ml (determined using a Petroff-Hauser cell count). Cells were cultured for 8÷10 days in a 37 °C, 5% $CO_2$ humidified incubator, in neuron medium (see Table 3.3). The thin film of poly-L-ornithine on coverslips promotes hippocampal isolated cell adhesion.

**Neuron medium (7.3 pH)**

| Reagent | Concentration (MEM) |
|---|---|
| MEM | 250 mM |
| Glucose | 35 mM |
| Apo-transferrin | 1 mM |
| HEPES | 15 mM |
| Insulin | 48 µM |
| biotina | 3 µM |
| Vitamin B12 | 1 mM |
| Gentamicin | 500 nM |
| BDNF [(1)] | 20 nM |

**Table 3.3** [(1)] BDNF was present only if needed by the specific experiment

Culture medium (neuron medium) was renewed after two days from seeding and hereafter changed every 2 days. Proliferation of non-neural cells was prevented by the addition of 10 µM arabinofuranosyl cytidine (Ara-C) from the second day in culture onward. For the experiments regarding BDNF-treatment, after 4 days *in vitro*, cultures were treated for further 4 days (2 medium renews) with 20 nM BDNF (Sigma) (see Table 3.3).

Hippocampal neurons were then used in our experiments after 8÷10 days in culture.

## 3.2 Electrophysiological recordings

Patch clamping is the main electrophysiological technique used throughout the thesis *via* classic tapered glass pipettes. Patch clamp measurements were performed according to Sackman and Neher paper (Sackman and Neher, 1986). This technique allows measuring the small currents (instrumental noise <1 pA), generated by neuronal cells characterized by small cell soma (<15 µm in diameter), such as hippocampal interneurons.

In all electrophysiological recordings, we used the whole cell configuration (after formation of a stable tight seal between the cell membrane and the glass pipette) by breaking the *patched* membrane applying a moderate negative pressure to the pipette (suction). In whole-cell patching the inner solution of the pipette communicates directly with the intracellular space through an ultra low electrical leakage junction (GΩ seal).

Noiseless and prolonged electrophysiological recordings of neuronal cells were possible thanks to reduced membrane perturbation.

We performed both voltage and current clamp recordings: in the former mode, we controlled the voltage of the cellular membrane (holding potential $V_h$) and we measured trans-membrane currents, in the latter we recorded the variations of membrane potential, which could be modified through current injections.

**Extracellular solution (7.4 pH)**

| Reagent | Concentration |
|---|---|
| NaCl | 150 mM |
| KCl | 4 mM |
| $MgCl_2$ | 1 mM |
| $CaCl_2$ | 2 mM |
| HEPES | 10 mM |
| Glucose | 10 mM |

**Table 3.4**

Recordings were performed from dissociated hippocampal cultures after 8÷10 DIV as previously described (Lovat et al., 2005); briefly, coverslips with cultures were positioned in a Perspex chamber mounted on an inverted microscope (Eclipse TE-200, Nikon, Japan) and continuously superfused with the recording solution (extracellular solution) at room temperature (see Table 3.4 for composition). Neurons were selected for recording depending on the experiment, in blind or based on their size and shape (e.g. triangular soma in the case of pyramidal neurons). Selection was done upon visual identification at 400× magnification under differential interference microscopy (DIC). In the case of pair recording, neurons up to 50±20 μm in soma distance were evaluated for monosynaptic connectivity; this was a limitation induced by the maximum field of view we had in our setup. Patch clamp pipettes were filled with an intracellular solution 390 mOsm in osmolarity (see Table 3.5).

**Intracellular solution (7.3 pH)**

| Reagent | Concentration |
|---|---|
| K-Gluconate | 120 mM |
| KCl | 20 mM |
| EGTA | 10 mM |
| HEPES | 10 mM |
| $MgCl_2$ | 2 mM |
| $Na_2ATP$ | 2 mM |

**Table 3.5**

During LTP-experiments the intracellular solution was EGTA-free to avoid $Ca^{2+}$ ions chelation. Drugs used for pharmacological testing during electrophysiology experiments were bath-applied *via* perfusion; these include 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, Sigma-Aldrich), bicuculline (Sigma-Aldrich), tetrodotoxin (TTX, Alomone, Israel).

Whole-cell patch clamping recordings were performed at room temperature employing patch-pipettes 5÷7 MΩ in bath resistance, under GΩ patch sealing conditions. Either an EPC-7 amplifier (List, Germany) or a Multiclamp 700B (Molecular Devices LLC, US) patch amplifier were used for voltage clamp recordings with the cell voltage clamped to –56 mV holding potential (never corrected for junction potential, that was 14 mV). For current clamp recordings we used an Axoclamp 2B amplifier (Molecular Devices LLC, Axon Instruments, US) or a Multiclamp 700B (Molecular Devices LLC, US) under bridge-balance mode (that was continuously monitored and adjusted). Current and voltage clamp responses were digitized using a Digidata 1322A or a Digidata 1440A (Molecular Devices LLC, US) at 10 KHz sampling frequency using pClamp 10 acquisition software (Molecular Devices LLC, US) and stored for further analysis. Single spontaneous synaptic events were detected taking advantage of the AxoGraph 1.4.4 event analysis software (developed and maintained by Dr. John Clements) on a MacPro workstation (Apple Inc., US).

Capacitance and input resistance were measured in voltage clamp configuration at resting potential (–56÷–60 mV). Voltage clamped cell was stimulated with a 100 ms lasting hyperpolarizing stimulus (5 mV); the area below the response was measured and normalized for voltage transient amplitude to calculate cellular capacitance. Input

resistance was obtained through Ohm's law by measuring the amplitude of the current response generated by the voltage transient.

With regard to the LTP-induction experiments, for assaying synaptic connectivity and to monitor synaptic potentiation, each presynaptic neuron (held in current clamp mode) was stimulated at a low frequency (0.05 Hz before and 0.03 Hz after the stimulation train) by injecting short (4 ms) square current pulses (1 nA). The response was recorded in the postsynaptic neuron, held in voltage clamp mode, 3 min before (10 sweeps for experiment) and 30 min after (60 sweeps for experiment) the application of the potentiation protocol (see Figure 3.1 A and B, BS1 and BS2 respectively, for a summary of baseline protocols use in this thesis work).

Different potentiation protocols were performed trying to induce synaptic plasticity:

- Low frequency stimulation (LFS): 60 pulses (5 ms, 1 nA) at 1 Hz (Fig. 3.1C) (Bi and Poo, 1998);
- Tetanic stimulation: 50 pulses (5 ms, 1 Hz) at 100 Hz (Fig. 3.1D) (Albensi et al., 2007);
- Theta Burst stimulation (TBS): the tetanic stimulation was repeated 4 times (Fig. 3.1E) (Albensi et al, 2007);
- Spike Timing Dependent Plasticity protocols (STDP): 60 pulses (5 ms, 1 nA) at 1 Hz, with a delay between the pre- and postsynaptic spike ranging from 5 to 20 ms (Fig.3.1F) (Bi and Poo, 1998);

During the stimulation trains both cells were held in current clamp mode to allow spiking.

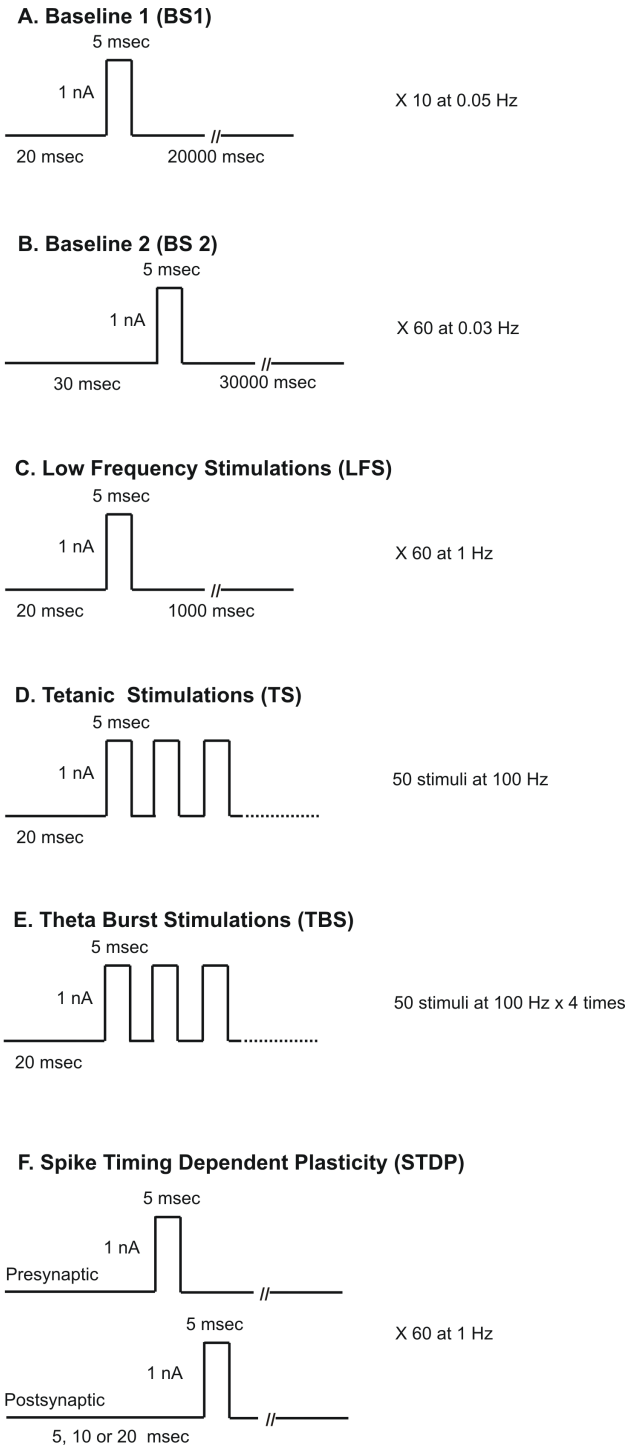


**Figura 3.1:** ***Schematic representation of the baseline and stimulation protocols that were used within this thesis to induce and test synaptic potentiation. (A and B) Baseline protocols used to test synaptic connectivity and to record the synaptic response before and after the stimulation train. The presynaptic neuron was stimulated at 0.05 Hz and 0.03 Hz frequencies before and after the stimulation protocol. (C) The first protocol that we performed: 60 pulses (5 ms, 1 nA) at 1 Hz. (D) Tetanic stimulation corresponding to 50 pulses at 100 Hz. (E) Theta burst stimulation, in which case the tetanic stimulation was repeated 4 times. (F) Spike time dependent plasticity protocol: the postsynaptic neuron was stimulated with a delay time of 5, 10 and 20 ms to the respect of the presynaptic one.***

Monosynaptic connections were recognized by their short latency (<5 ms, Pavlidis et al., 2000), measured between the peak of the evoked action potential (AP) and the onset of the postsynaptic current (PSC) response recorded in voltage clamp.

Paired-pulse recordings were performed to estimate release probability in BDNF-treated neurons compared to control sister cultures, by stimulating the presynaptic cell (held in current clamp) with pairs of action potential (50 ms interval) at 20 Hz frequency.

In addition, in each pair, hyperpolarizing current steps (–0.05 nA; 100 ms) were delivered to the presynaptic cell to check the presence of electrical synapses (Zsiros et al., 2007). According to them, gap junctions can be identified by the presence of currents in the postsynaptic neuron coincident to presynaptic hyperpolarizing stimuli. In all our recordings (n=80 pairs), we detected only a small fraction of gap-junction-coupled neurons (1%).

## 3.3 Immunofluorescence staining

Hippocampal neurons were fixed in 4% paraformaldehyde (PFA) in PBS for 20 min. They were permeabilized by means of 0.1% Triton X-100 detergent and blocked in 1% fetal bovine serum (FBS) in PBS for 30 min to prevent unspecific binding of antibodies. After incubation with rabbit polyclonal antibody against β-tubulin III (1:500 dilution, Sigma Aldrich) for 1 h, they were incubated with the secondary goat anti-rabbit Alexa Fluor 594 (1:500 dilution) and with DAPI (1:200 dilution) for 1 hour. Both secondary antibody and DAPI were obtained from Invitrogen. Fixed cultures were imaged and examined using a Nikon Eclipse Ti-U fluorescence microscope. Neuronal densities were calculated for the central area and for the periphery (distal areas) of the glass coverslip by manual counting after the calibration of the microscope field of view. The shape of the soma and the processes organization and number recognized pyramidal neurons. In particular, there were several distinct types of neurons in dissociated hippocampal cultures as already described by Segal in 1983. The first consisted of small (5÷10 μm) round neurons that tended to aggregate and form large homogenous clusters (Figure 3.2) and appeared to send neurites toward the other, larger cells.

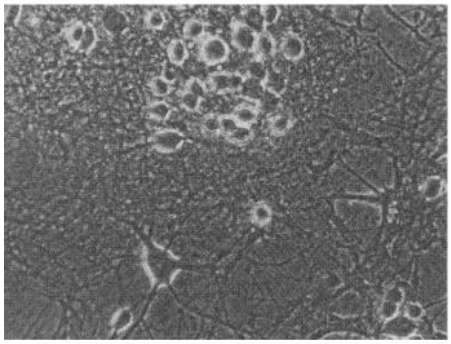

**Figure 3.2:** ***A cluster of small, round neurons present in dissociated cultures (image from: Segal, 1983).***

We can speculate on the basis of their morphology these neurons are dentate granular cells. The larger neurons, 15÷20 μm in diameter, were not aggregated and could be divided on the basis of the characteristic morphology of their soma into pyramidal-shaped cells having one thick process, which tend to bifurcate some 50÷100 μm away from the soma, multipolar cells having more spherical shape and several main neurites without a dominant dendrite, and fusiform cells having an elongated soma and two main opposing neurites, which are smaller than the first two types (see example in Figure 3.3). Most of our recordings were made specifically from pyramidal cells. In fact these cells, apart being extremely easy to identify thanks to their distinct morphology, presented a significant higher ratio in excitatory glutamatergic synapses than others cells.

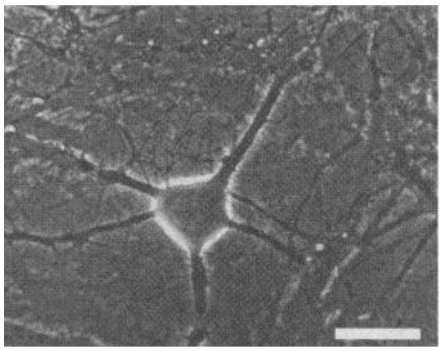

**Figure 3.3:** ***A higher magnification of a pyramidal cell with a thick, apical dendrite and two basal dendrites. Scale bar, 40 μm (image from: Segal, 1983).***

## 3.4 FM1–43 fluorescence imaging

Coverslips with 9÷10 DIV cultured neurons were washed three times with a saline solution containing (in mM): 150 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, pH adjusted to 7.4 with NaOH, and left in 2 mL of the same buffer at room temperature for 10 min. Coverslips were then mounted onto a RC-40LP open bath chamber placed on the stage of an Axiovert 35 inverted microscope (Carl Zeiss International, Oberkochen, Germany) and perfused with saline (1.5 mL/min). Depolarization-dependent staining of synaptic terminals with the styryl dye N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide (FM1–43, Molecular probes, Life Technologies Corporation; Figure 3.4) was obtained by bathing the cells for 120 seconds with 2 mL of saline solution containing 50 mM KCl (46 mM NaCl removed to maintain osmolarity) and 15 μM FM1–43 dye.

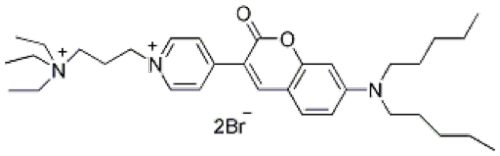


**Figure 3.4:** ***The chemical structure of FM1-43. The molecule is composed of a positively charged head group (left), which prevents the dye from permeating membranes, and a lipophilic tail (right), which ensures dye partitioning into membranes. A double bond aromatic bridge connects these two components: the number of double bonds determining the spectral characteristics (adapted from Hoopman et al., 2012).***

At the end of stimulation the buffer was replaced by 2 mL of normal saline solution containing 15 μM FM1–43 and cells were left to recovery for 10 minutes, to ensure complete recycling of the vesicles (Hoppmann et al., 2012). To wash off non-internalized dye, cells were extensively perfused with dye-free buffer for 5 minutes. At the end of wash period, cells were incubated 10 minutes with saline solution containing 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 μM 2-aminophosphonovaleric acid (2-APV) to prevent network activity altering the rate of FM release. Exocytosis of synaptic vesicles was obtained by perfusing the cells with saline containing 50 mM KCl for 2 minutes, followed by a 2 minutes perfusion with normal saline. Neurons stimulation caused release of dye-containing terminals by exocytosis, which was visualized as a loss of fluorescence. For spontaneous FM1–43

destaining time courses, images were acquired for 5 minutes without any stimulation (see Figure 3.5).

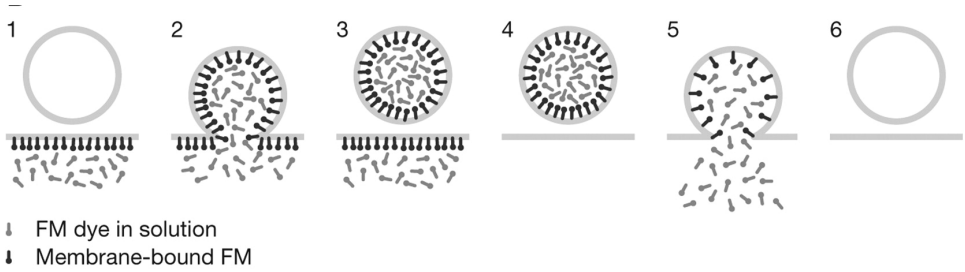


**Figure 3.5:** ***FM dye staining and destaining. The dye was added to the solution bathing. (2) When stimulating the neurons, vesiscles exocytose and come in contact with the dye. (3) Vesicles endocytose and take up dye. (4) The dye is washed from the extracellular solution: it departitions from the plasma membrane but remains trapped in internalized vesicles (staining). (5) The stained preparation is stimulated in the absence of the dye. Vesicles fuse with the membrane and release dye (destaining). (6) Destained vesicles are internalized: a new experiment of staining and destaining can be performed (adapted from Hoopmann et al., 2012).***

Images were continuously acquired by a Till Photonics Till-Imago system, exciting the FM1–43 dye with a 475 nm wavelength generated by a monochromator (Polychrome IV, Till Photonics GMBH, Grafelfing, Germany) and acquiring fluorescence images using a 800×600 pixels CCD camera (CCD Imago type super VGA, Till Photonics; 1 frame/second, 50 ms exposure) interfaced to the TillVision software (Till Photonics). Off-line analysis were performed on image sequences with TillVision software.

Time-dependent fluorescence changes on FM1–43 labeled terminals were obtained by drawing regions of interest (ROIs) around the largest portion of fluorescent spots (typically 6×6 pixels, ~1 $\mu m^2$), corresponding to individual puncta, each visibly separate from its nearest neighbors, and including as little background possible. Data sets were discarded whenever lateral displacement of a vesicle beyond the ROI occurred. The average fluorescence value of a field devoid of cell processes (background) was subtracted from each image in the sequence. A comparison of the brightness of total vesicle pool puncta (raw fluorescence intensity) in BDNF-treated and control cultures before the unloading stimulus gave an estimate of the number of vesicles endocytosed during FM1–43 loading.

To evaluate and compare the time course of synaptic terminals destaining independently on the degree of dye loading, we normalized each image to the maximal fluorescence

intensity, by employing the DeltaF ratio function of the TILLVision software. Briefly, for each image sequence the fluorescence intensity averaged pixel by pixel over the first five images before stimulation (Fav) was used as a reference to normalize all the other images, according to the following equation:

$$\Delta F = \text{scale factor} \times [(F_t - Fav) / (Fav + 1)].$$

The scale factor was set to 1000 and maintained constant for all experiments. Data analysis was performed using Origin Pro 7.5 software (OriginLab Co., US).

## 3.5 Statistical Analysis

All data were compared using parametric and nonparametric statistical tests (Student's *t*-test, Mann-Whitney and Kolmogorov-Smirnov tests) and $P<0.05$ was considered the condition necessary to proof significance. The Kolmogorov-Smirnov two-sample statistical test was, in particular, used to compare the distribution of events' amplitudes between control and BDNF-treated samples. All the values in the text represent mean±SEM, where *n* represents the number of samples (e.g. cells).

# 4 RESULTS

The firsts set of experiments was design to reliably induce synaptic potentiation at glutamate AMPA receptor mediated synapses in cultured neurons. We considered such a "confined induction of plasticity", instrumental in further analysis at the nanoscale. Potentiated synapses has, in fact, to serve for subsequent mechanoelectrical investigations at the dendritic spine level via AFM, and for this reason needs to be localized univocally. To combine a high rate of success in potentiating (mono)synaptic responses in well identified neuronal pairs, has required the setting and testing of original and complex stimulation paradigms, that are illustrated in the following paragraphs.

Simultaneous patch clamp recordings of visually identified pair of mono-synaptically connected neurons were performed from n=70 couples of neurons (from n=38 culture series; Figure 4.1).

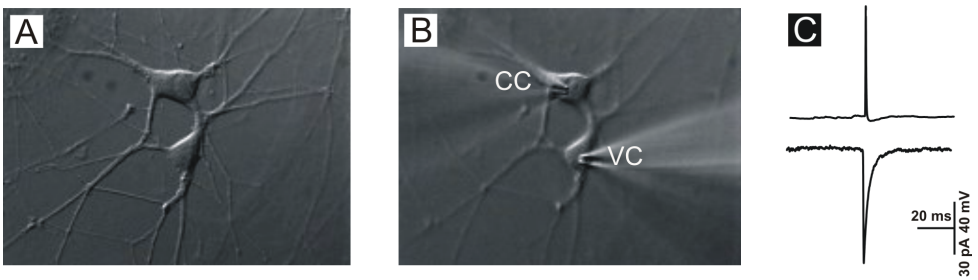


**Figure 4.1:** ***Schematic representation of recording from pairs of hippocampal neurons. (A) Representative image of two coupled pyramidal neurons, at 400× magnification. (B) One of the two-patched cells, the putative presynaptic neuron, was held under current clamp mode at –60 mV, while the postsynaptic neuron was monitored under voltage clamp mode. (C) Monosynaptic connections were recognized by their short latency.***

Samples were characterized by nerve cells uniformly distributed on the entire coverslip surface. That being the case we choose recording fields both located at the periphery or the center of the glass coverslip. Neuronal pairs were on average at 50 ± 20 µm of distance, at 400x magnification and usually at least one of the two cells displayed pyramidal shape. Only cells with soma diameter ranging from 8 to 12 µm were considered. These morphological features improved the chances of recording glutamate AMPA receptor mediated monosynaptic responses (see Cellot et al., 2011 for further details). Paired whole-cell recordings were obtained with the presynaptic cell under

current-clamp mode and the postsynaptic cell was voltage clamped, usually at -56 mV holding potential (not corrected for liquid junction potential, that was calculated to be 14 mV in pClamp software).

Under our culturing conditions the coupling probability in hippocampal cultures (8÷11 DIV) was 40% that is within the range reported for cultures developed under similar growth conditions (Basarsky et al., 1994). Monosynaptic connections were recognized by their short latency (<5 ms; Cellot et al 2011; Pavlidis et al., 2000), measured between the peak of the elicited presynaptic AP and the onset of the postsynaptic current (PSC) response obtained in voltage clamp. We excluded from this study all recordings where clear bi-synaptic connections were present and those where ongoing spontaneous activity was occasionally superimposed to the evoked PSCs.

We identified the different populations of evoked PSCs on the basis of their kinetic properties, reverse potential, and pharmacology (Cellot et al 2011; Galante et al., 2000). In fact, glutamatergic AMPA receptor-mediated PSCs display fast decay (5.4±0.2 ms, n=80) inverted polarity around 0 mV holding potential (n=2) and were further blocked by application of 10 μM CNQX (n=15). GABAergic PSCs display slow decay (25.4±1 ms, n=45) inverted polarity at -40 mV holding potential, which is close to the Nernst value for chloride reverse potential in our experimental conditions (n=35), and were fully abolished by administration of 10 μM bicuculline (n=3).

In our cultures when selecting pairs of neurons with the features reported above, the large majority (80%) of monosynaptically coupled pairs displayed evoked currents mediated by the $GABA_a$ receptors; we usually detected only a minority (20%) of glutamate AMPA receptors -mediated monosynaptic PSCs.

There are discordant reports (Basarsky et al., 1994; Bi and Poo, 1998) on the occurrence of GABAergic or glutamatergic monosynaptic connections in pair recordings in dissociated hippocampal cultures. These discrepancies may involve differences in the culturing procedures, such as the animals' age, the cellular density, or the medium composition; however, our previous results indicated larger occurrence of inhibitory monosynaptic connections when closely located neurons are simultaneously patch clamped (Cellot et al 2011).

Neuronal passive properties were routinely measured in voltage and/or current clamp, and are commonly used as indicators of neuronal healthy conditions. In addition, values of the membrane capacitance, the input resistance and the resting membrane potential,

allow comparing similar classes of mature neurons. In fact, such properties are widely accepted indicators of neuronal functional state and of neuronal developmental stage. In particular, membrane capacitance is an indirect measure of cellular dimension, input resistance is related to the number of leak channels expressed in the neuronal membrane and resting membrane potential is a relatively stable value of trans-membrane voltage, which characterizes neurons when not undergoing any active signals such as action potentials (APs) **(**Kandler, 2000).

Membrane passive properties of the presynaptic and postsynaptic cells are summarized in Table 1.

| | Capacitance (pF) | Input Resistance (MΩ) | Resting Potential (mV) |
|---|---|---|---|
| Presynaptic (n=70) | 66 ± 5 | 554 ± 26 | -49 ±1 |
| Postsynaptic (n=70) | 69 ± 4 | 576 ± 30 | -46 ±1 |

***Table 1***

## 4.1 Inducing synaptic plasticity in pairs of monosynaptically connected neurons: low-frequency stimulation

The first set of protocols that we used to induce synaptic plasticity in AMPA glutamate synapses consisted in a low frequency stimulation paradigm (Bi and Poo, 1998). To characterize the short-term dynamics of synaptic contacts, we delivered repetitive stimulation (60 square pulses, 5 ms in duration at 1 HZ frequency and 1 nA in amplitude) to the presynaptic neuron while both cells were, transiently (see Methods, Figure 3.1C for details) held in current-clamp to allow for fluctuation in membrane voltage and spiking (Bi and Poo, 1998). Synaptic efficacy was assayed before and after the protocol by test stimulation (BS1 and BS2 respectively, refer to Methods, Figure 3.1 A,B for further details) of the presynaptic neuron (in current clamp mode) and postsynaptic neuron (in voltage clamp mode) at a low frequency (0.05 Hz before and 0.03 Hz after, respectively). Monosynaptic PSCs were defined as postsynaptic responses with 5 ms delay (Basarsky et al., 1994).

We measured, before and after the stimulus train, the amplitude of the evoked monosynaptic EPSC and the amplitude and frequency of the heterogeneous spontaneous PSCs recorded from the postsynaptic cell (Figure 4.2).

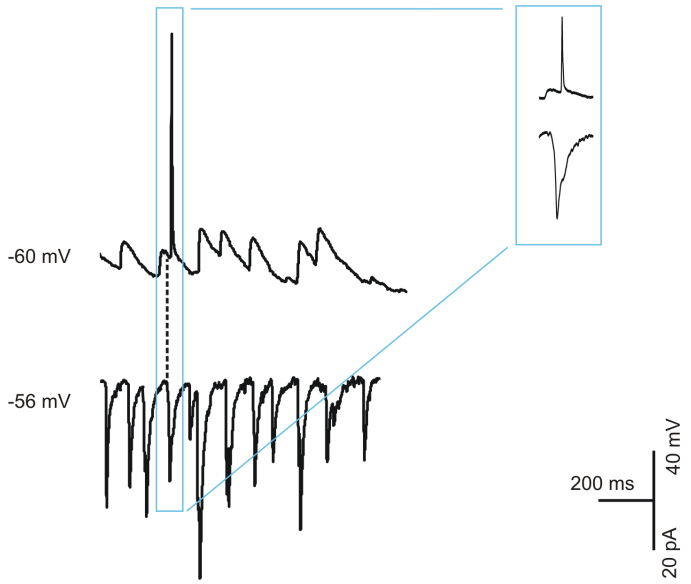


**Figure 4.2:** ***During our experiments, we measured the amplitude of the evoked-EPSC, and the amplitude and frequency of the PSCs recorded in the postsynaptic cell, before and after the stimulation train.***

We performed this stimulation protocol in n=20 pairs of AMPA coupled neurons from 30 different series of cultures. Responses varied from no changes, to potentiation; however we measured a consistent increase in the induction of synaptic potentiation just in a small sample (n=2) of coupled neurons where measurements of the amplitude and frequency of spontaneous PSCs revealed a persistent increase in synaptic efficacy after the repetitive stimulation. In the first couple of potentiated neurons the PSCs amplitude value increased from 3.4±0.9 pA prior to the potentiation protocol to a maximum value of about 133 pA, immediately after the protocol, that decays exponentially in 10 minutes to a steady state value of 60±11 pA, while the frequency value increased from 1.2±0.6 to 3±1.4 Hz (Figure 4.3).

In the second pair PSCs amplitude value were 20±0.9 pA prior the potentiation protocol train and 47±3 pA as steady state after the stimulation (maximum value reached was about 119 pA). on the other hand frequency values were 1.2±0.1 and to 5.5±0.4 Hz, respectively. Regarding evoked EPSCs, in both experiments were not observed significant variations in the responses before and after the potentiation protocol (from 34.2±3.9 pA to 33±2.8 pA for the first couple; form 25.4±1.5 pA to 26.2±1.9 pA for the second couple).

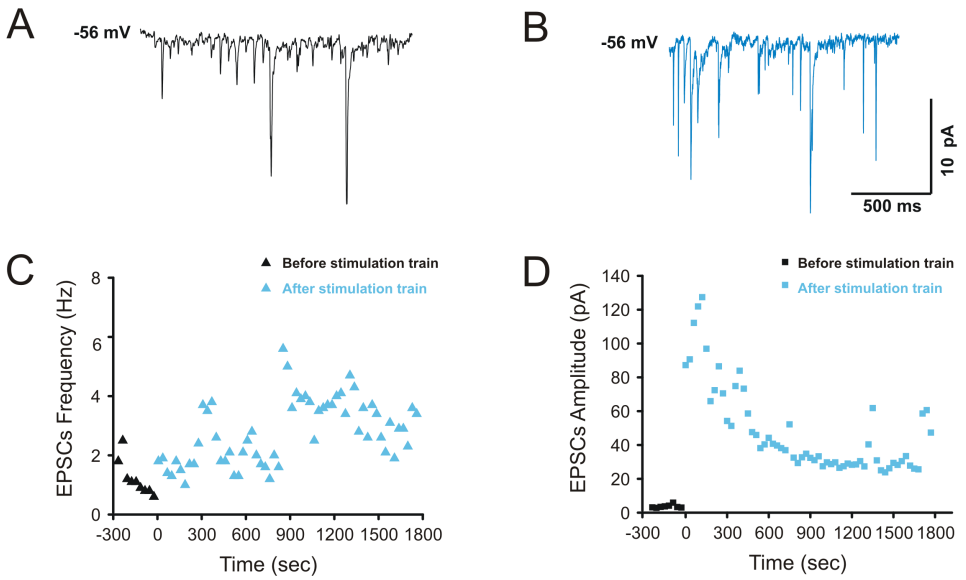


**Figure 4.3:** ***(A-B) Representative tracings before (black) and after (blue) the stimulation protocol. (C-D) Synaptic potentiation induced by repetitive stimulation in n=2 pair of neurons. Data depict the increase in the frequency and in the amplitude of PSCs in neuronal network. Each symbol corresponds to the average value of EPSCs calculated between 2 subsequent baseline pulses (see Methods) before the stimulation protocol (10 black marks) and after (60 blue marks).***

The first, and preliminary, experimental session involved about 150 samples from 30 different series of cultures. Only 30% of cell pairs were monosynaptically coupled (437 couples); only a small subset of them presented excitatory AMPA synapses (~5%, 20 couples)

In a set of experiments, the probability of having failures during repetitive stimulation (lack of unitary PSC within 5 ms from presynaptic AP peak) was quantified within each train. For each AMPA synaptically coupled pair, the percentage of failures was measured in 10 subsequent sweeps as the rate between the total number of postsynaptic failures and total number of presynaptic stimuli. Failures in PSCs were 25% of the total amount of AMPA coupled pairs and were not averaged within the analysis of the short-term plasticity.

Because of this extremely low yield of success in synaptic potentiation we choose to move to a different protocol involving a borrowed-from slices high frequency stimulation. Anyhow, this first attempt was fundamental for us to set up all the experimental framework accounting for both instruments and electrophysiological experimental skills.

## 4.2 Inducing synaptic plasticity in pairs of mono-synaptically connected neurons: THETA burst potentiation

Long-term potentiation (LTP) can be typically induced by a so-called tetanization, that is delivering a 100 Hz train for 1 s total duration at the presynaptic site (Bliss and Collingridge, 1993) or delivering a so-called theta burst stimulation, which consists of delivering short bursts consisting of 4 pulses at 100 Hz at the presynaptic site with 200 ms inter-bursts intervals (Larson et al., 1986).

To induce synaptic potentiation, tetanic stimuli and theta bursts were delivered while cultures were perfused by low $Mg^{2+}$ solution, leading to a partial activation of NMDA glutamate receptors, a suitable condition for LTP induction in the hippocampus (Bliss and Collingridge, 1993).

The first set of experiments try to induce LTP by "standard tetanization" while in a second set of experiments we moved to theta-burst stimulation, i.e. a sequence of n=4 brief 100 Hz trains repeated at "theta frequency", 5 Hz (see Methods, Figure 3.1 D,E for details).

The outcome measures were the evoked monosynaptic EPSCs amplitude, and the spontaneous EPSCs (identified by both kinetic and pharmacology features, see Methods) amplitude and frequency, before and after stimulations. Unfortunately, as summarized in Figure.4.4, we were not able to induce any detectable potentiation in the sampled coupled neurons using neither of the two protocols. In fact we measured any significant differences in terms of evoked EPSCs amplitude or in spontaneous EPSCs amplitude and frequency when comparing activity before and after either the tetanus or the theta burst stimulation.

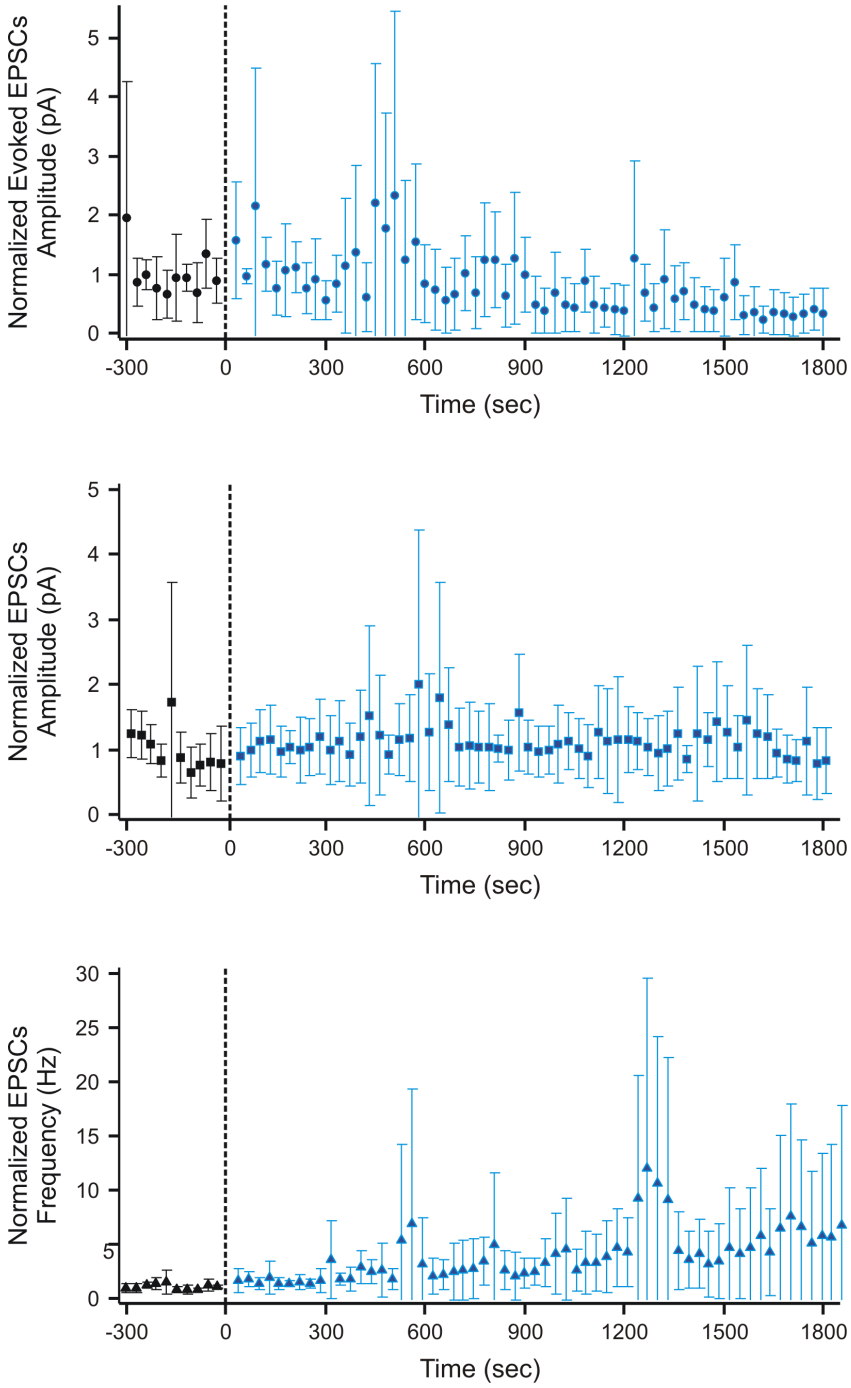


**Figure 4.4:** ***Plots polled data of evoked EPSC amplitude, amplitude and frequency of spontaneous EPSCs before and after the tetanus and theta-burst stimulation. The number of sweeps corresponds to the number of spike pair repetition. No differences were detected after the stimulation (n=5 cells tetanus and n=6 cells theta bursts).***

As noticeable from Figure 4.4, the only changes observed were in terms of variability, as indicated by the different coefficient of variations calculated before ($c_v$ for evoked EPSC amplitude is 2.08 while $c_v$ for spontaneous EPSC amplitude and frequency are 1.72 and 1.49 respectively) and after ($c_v$ for evoked EPSC amplitude is 6.76 while $c_v$ for

spontaneous EPSC amplitude and frequency are now 4.40 and 8.58 respectively) the stimulations. Although these differences tetanization and theta bursts reveal to be non effective protocols to induce LTP in dissociated hippocampal neurons therefore we moved to a more sophisticated potentiation protocol.

## 4.3 Inducing synaptic plasticity in pairs of mono-synaptically connected neurons: Spike-timing Dependent Plasticity

In the last decades, several studies reported that the temporal correlation among pre- and postsynaptic activity is crucial for LTP induction (see for example: Bell et al., 1997; Bi and Poo, 1998; Debanne et al., 1998; Froemke and Dan, 2002; Sourdet and Debanne, 1999; for review, see Dan and Poo, 2004). In addition, a number of works (Bi and Poo, 1998; Nishiyama et al., 2000; Markram et al., 1997) showed that in a variety of *in vitro* preparations, repetitive stimulation with precise pairing of pre-and post-synaptic action potentials (pre/post pairs) led to the induction of long-term synaptic plasticity, either potentiation or depression (LTD). Importantly, the precise control of the pre/post spike timing impact the sign and magnitude of synaptic modification and was called Spike Time Dependent Plasticity (STDP). For a more detailed discussion about STDP paradigm refer to Markram et al., 1997 and Zhang et al., 1998 or see Methods.

The precise timing between the pre and postsynaptic stimulation is fundamental to induce the desired synaptic modifications namely LTP phenomena. According to that to reliably correlate presynaptic and postsynaptic spiking we varied from 5 to 20 ms the time interval between the presynaptic and the postsynaptic stimulations. For both we used the same protocol of repetitive trains (60 1 Hz square pulses 5 ms in duration and 1 nA in amplitude; see Methods Figure 3.1 F for further details; Bi and Poo, 1998).

However, despite the application of this protocol, the paired neurons displaying a genuine potentiation were only a minority (n= 2 out of n=20) (Fig.4.5)

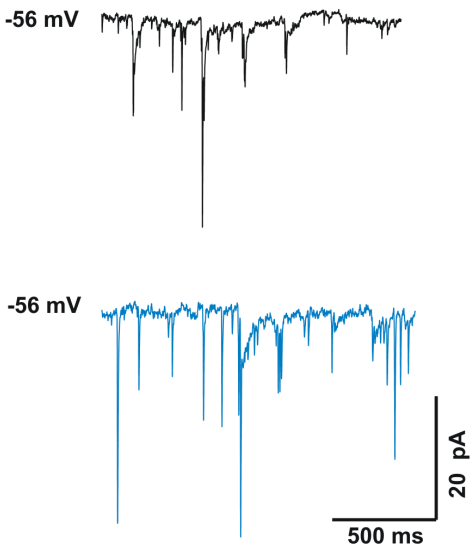


**Figure 4.5:** ***Representative tracings before (black) and after (blue) the stimulation protocol.***

In these 2 cases we monitored the amplitude and the frequency of EPSCs using the same baseline protocol (refer to Methods for details) and, as shown in Figure 4.6, we measured a stable increase in the amplitude and an exponential trend in frequency increase. In particular the pair shown in Figure 5.6 increases its amplitude from 5.5±0.7 pA to a steady state value of about 21.99±1.7 pA after 15 minutes, with a maximum of about 130 pA immediately after the potentiation. On the other hand frequency increased as a power low of time ($f=f_0+A\cdot t^B$) where $f_0$ was 1.02±0.17 Hz and the exponent B was 3.64±0.40.

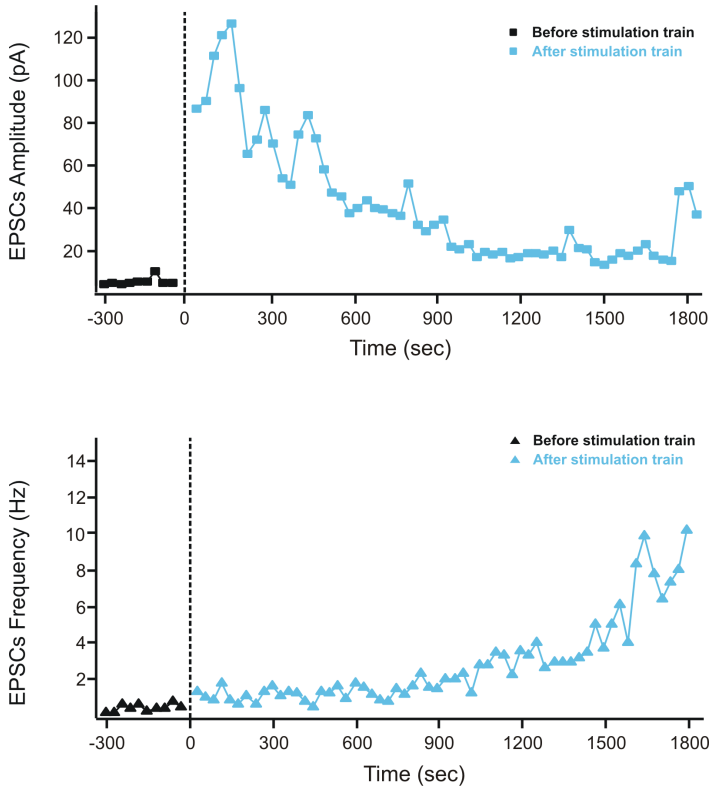


**Figure 4.6:** ***Synaptic potentiation induced by repetitive presynaptic stimulation. Plots refer to a pair of glutamatergic neurons in hippocampal culture.***

We separately analyzed GABAergic synapses in n=5 pairs. GABAergic monosynaptic (<5 ms delay) PSCs were identified based on their kinetic ($\tau$=20±1.2 ms) and their reverse potential (–40 mV; Cellot et al 2011). Repetitive stimulation of the presynaptic and postsynaptic neurons (following exactly the same protocol described previously), did not result in any change in synaptic efficacy.

We conclude that in our culturing conditions to induce selective and localized synaptic potentiation in monosynaptically connected pairs of neruons by means of different paradigms of presynaptic stimulation holds in all cases an unsatisfactory low probability of success. Thus prevents us in the development of the designed experiemental approach to induce synaptic plasticity on a well defined synapse. Here we have to remind this thesis work' aim was to induce a synaptic modification on a localized neuronal junction in order to perform subsequently on it nanomechanical investigations taking advantage of AFM technique. The ability to induce changes only on a specific subset of synapses in the entire network (a prerogative of electrically incuced potentiation) would have given the possibility for a differential approach in such

characterization. In other words, on the same sample, synapses potentiated and not potentiated would have investigated simultaneously.
Unfortunatelly electrical stimulation have not proven to be reliable for this purpose, mainly due to its extremelly low yield.
For this reason we moved to an alternative approach where electrical, synapticaly defined, potentiation was replaced by chemical potentiation. Following this approach we lost the possibility to have only a specific synapse undrgoing plastic modification but all (excitatory) junctions of the neuronal network will be, in principle, subjected to modification.

## 4.4 BDNF highly improve synaptic event frequency and amplitude in treated hippocampal cultures

Following our unsuccessful attempts to selectively govern activity dependent synapse plasticity among pairs of cultured neurons, we decided to move towards a different experimental approach. We focused our attention on brain-derived neurotrophic factor (BDNF) signaling. BDNF, a pro-survival protein, has been involved in development, plasticity, such as LTP promotion and memory formation (Desai et al., 1999; Asztely et al., 2000; Ikegaya et al., 2002; Maffei, 2002). In fact BDNF directly facilitates LTP (Kramar et al., 2004) and is involved in pathways that mediate activity-dependent formation of actin at spines level. Such processes are considered crucial to promote structural changes in dendritic spines, ultimately involved in stabilizing synaptic potentiation (Rex et al., 2007). For such reasons we decided to use BDNF to induce synaptic changes in cultured hippocampal neurons (Carlos V. Melo et al.,2013). Differently from that recent study, where cultures were treated acutely with BDNF (100 ng/ml ≃ 7 nM concentration), we found intriguing to explore the contribution of BDNF chronic exposure to synaptic modifications, given that issues such as the presence of a compensatory BDNF up-regulation in damaged tissue, or the BDNF interplay with chronic inflammation, still need to be investigated (Tonqet et al. al., 2012).
BDNF ability to mediate activity-dependent modifications in synaptic strength has recently received considerable attention (Bolton et al., 2000; Lohof et al., 1993; Vicario-Abejon et al., 1998; Sherwood and Lo, 1999). In particular, acute BDNF effect on excitatory synapses has been the object of an increasing amount of studies. On the

contrary, the role of chronic BDNF exposure in regulating long-lasting changes in synaptic function is comparably less investigated and may play a role on post injury alteration of synaptic networks and neuronal rescue (Lu V.B. et al., 2007; Cho et al., 1997; 1998).

To investigate the contribution of extended exposure to BDNF in regulating the overall excitability of dissociated hippocampal neurons via synaptic potentiation, cultures were treated for 4 days with 20 nM BDNF (named BDNF cultures) and neuronal networks were functionally and morphologically compared to untreated ones (named controls).

By using single cell patch clamp technique (current and voltage clamp configurations) we compared BDNF and control neurons (Fig. 4.7).

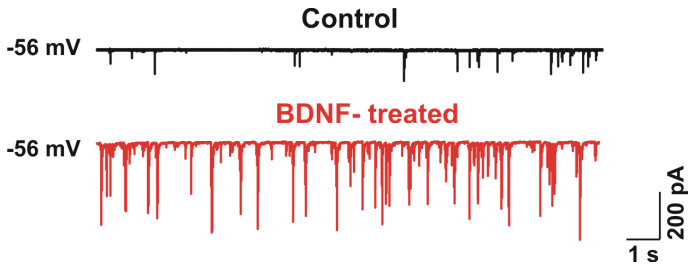


**Figure 4.7:** ***Voltage clamp recordings of spontaneous synaptic activity of control (black trace) and BDNF treated (red trace) cultures. Inward deflections of variable amplitude represent PSCs due to the activation of heterogeneous receptors (AMPA glutamate and $GABA_A$). The increase in frequency and amplitude of spontaneous PSCs in this BDNF-treated neuron when compared to a control one is appreciable.***

First we analyze cell passive membrane properties, the membrane capacitance, the input resistance and the resting membrane potential. On average, cell capacitance was 78 ± 5 pF (n=44, control) and 96 ± 6 pF (n=75, BDNF); cell input resistance was 500 ± 53 MΩ (n= 44 control) and 477 ± 47 MΩ (n=75, BDNF); resting membrane potential -47 ± 1 mV for control cultures (n= 40), and -50 ± 1 mV for BDNF-treated cells (n= 72). Thus, no significant differences (p-value larger than 0.05 in all cases) were detected in the passive properties of cells sampled for recordings from the two groups of cultures.

Spontaneous synaptic activity was assessed by voltage patch-clamp recordings of single neurons to monitor the activity of the networks grown for 8÷10 DIV both under control and BDNF-treated conditions. BDNF treated cultures displayed an increase in PSC amplitudes from 111.9±13.3 pA in controls (n=19) to 157.2±19.1 pA in BDNF cultures (n=26), while frequencies rise from 1.2±0.3 Hz in controls to 2.7±0.3 Hz. Amplitude

and frequency increases were tested in significance using t-test (P<0.001 in both cases) as illustrated in Figure 4.8.

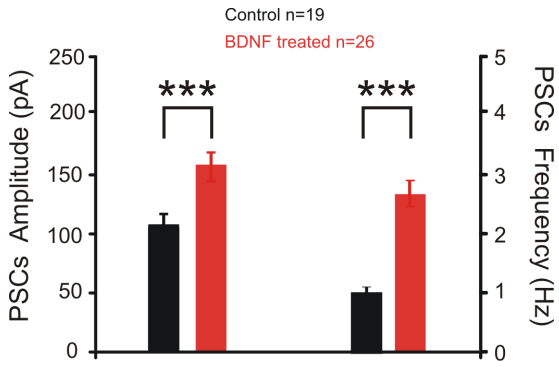


**Figure 4.8:** ***Spontaneous synaptic activity was assessed by voltage patch-clamp recordings of single neurons to monitor the activity of the networks grown for 8÷10 DIV both under control and BDNF-treated conditions. BDNF treated cultures displayed an increase in PSCs amplitude and frequency.***

In cultured hippocampal networks, PSCs belong to two major categories, excitatory and inhibitory, mediated by glutamate AMPA and GABAa receptors activation, respectively (Segal and Barker, 1984; Rothman and Samaie, 1985). In our culturing conditions, under voltage clamp mode at $V_h$ –56 mV holding potential, all PSCs are detected as inward currents, however we can further dissect AMPA receptor mediated from $GABA_A$ receptor mediated PSCs on the basis of their different pharmacology, kinetic properties and reverse potential (Cellot et al., 2011; see Figure 4.9) to investigate whether AMPA receptor and/or $GABA_A$ receptor mediated events contributed to the increased activity.

We identified AMPA receptor mediated PSCs by their fast decay time constant ($\tau$=4±0.2 ms, n= 46; Figure 5.9), these events were abolished by application of CNQX (10 μM) and reversed polarity at 0 mV holding potential (Cellot et al., 2011).

Conversely, GABAergic PSCs were identified by their slow decay ($\tau$ =25.4±1 ms, n=45; Figure 4.9) these events were abolished by bicuculline (10 μM) and inverted polarity at –35 mV holding potential (Cellot et al., 2011).


GABA receptors mediated currents Glutamate receptors mediated currents

**Figure 4.9:** ***Average tracings of spontaneous $GABA_A$ receptor (left) and glutamate receptor mediated PSCs identified on the basis of their decay time: τ=4±0.2 ms, n=46 and τ=25.4±1 ms, n=45, respectively.***

When, specifically, EPSCs were measured from BDNF neurons, we detected a strong increase in event frequency when compared to EPSCs measured from control neurons (frequency in controls 0.88±0.24 Hz, n=24; in BDNF, 2.16±0.31 Hz, n=38 cells; P<0.001 from Student's t-test, refer to plots in Figure 5.10). BDNF also significantly increased the amplitude of EPSCs (amplitude in controls 74.2±1.4 pA, n=18; amplitude in BDNF, 146.4±2 pA, n=28, P<0.001 from Mann-Whitney's test; see also relative cumulative amplitude distribution in Figure 4.11 of n=24 control and n=38 BDNF cells). When similarly analyzing $GABA_A$ receptor mediated PSCs in neurons grown in control and in BDNF conditions, we detected only a slight, not significant (p=0.45) increase in peak amplitude (control IPSC amplitude 152±6 pA, n=19; BDNF IPSC amplitude 182±4 pA, n=26) and frequency (in controls 0.40 ± 0.08 Hz, n=21; in BDNF-treated cultures 0.60±0.08 Hz, n=36, but it was not statistically significant with p=0.19). Quantification of PSCs frequency is summarized in the plots of Figure 4.10 that indicate how BDNF highly strengthen excitatory synaptic event frequency in neuronal hippocampal network.

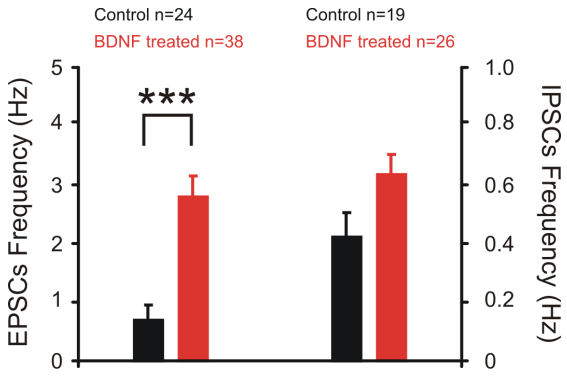


**Figure 4.10:** ***BDNF-treatment increases the EPSCs frequency in comparison to control sister cultures (P<0.001 Student's test). Note in the figure that the scale bar between EPSCs and IPSCs Frequency is different, due to lower level in frequency regarding inhibitory events.***

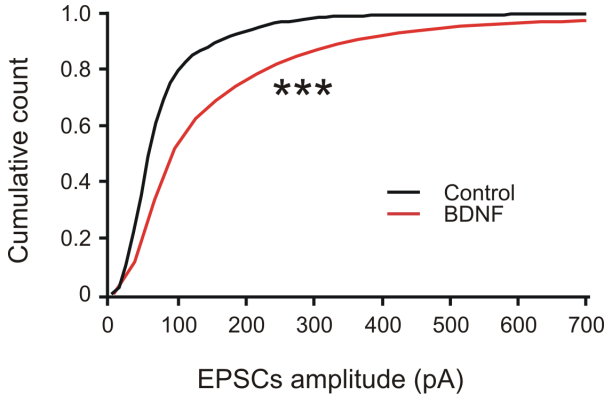


**Figure 4.11:** ***Relative cumulative amplitude distribution plot of control and BDNF EPSCs. Note that the BDNF distribution (red) curve is shifted to the right of the control (blue) distribution one, indicating that BDNF-treated cultures are more likely to have larger EPSCs than control ones. The different probability is statistically significant (Kolmogrov-Smirnov test; P<0.001).***

The increased spontaneous EPSCs frequency and amplitude between control and BDNF networks can emerge from several mechanisms, which can work in combination: increased firing activity, for example, with changes in cell excitability due to prolonged BDNF exposure, or differences in neuronal survival and thus in the global network size. Additionally increased number of synaptic connections, or, at the level of presynaptic changes, an increase in the number of release sites and boutons impinging on each neuron in BDNF neurons could account for the detected changes. An increase in presynaptic vesicle release probability, or increase vesicle content, could equally influence the changes detected as well. Additionally, moving to postsynaptic changes, an increase in postsynaptic receptor number or function.

In out attempt to understand the implication in long term treatment with BDNF of some of these mechanisms we investigated the network size and neuronal density and we recorded miniature EPSCs (mEPSC) in the presence of tetrodotoxin (TTX, 1 μM; Cellot et al 2011). Spontaneous mEPSC may reflect several functional and even structural aspects of the synapses. An emerging view assumes that the size and number of mEPSCs are correlated with, respectively, the physical size of synapses and number of spines, although spine dimension and synaptic responses are not always correlated (Segal, EJN 2010).

## 4.5 BDNF favors neuronal survival

Being BDNF a widely accepted pro-survival protein (Bibel and Barde, 2000) we wonder whether such an activity may influence, during long-term treatments, neuronal survival, and in particular that of glutamatergic pyramidal neurons, thus augmenting BDNF network size when compare to controls.

The relationship between synaptic density and strength has not been studied systematically under controlled conditions. The dissociated culture of primary central neurons provides a convenient test system for analysis of the role of network density on connectivity among neurons.

Neurons were visualized by fluorescent immunostaining for neuronal specific markers (β-tubulin III, see Methods; Figure 4.12) in 9÷10 DIV control and BDNF cultures.

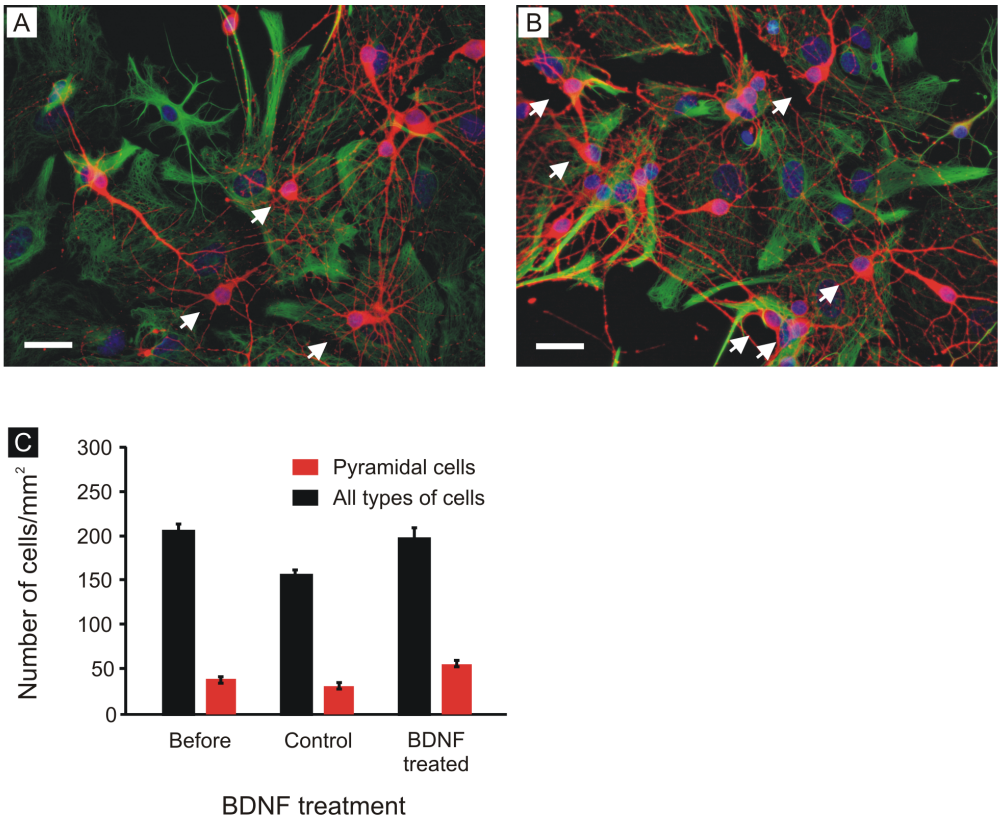


**Figure 4.12:** ***Immunofluorescence (anti β-tubulin III) image taken at 200× magnification to visualize a population of tubulin positive neurons from control (A) and BDND-treated (B) cultures. Healthy morphology and heterogeneous shape and size are noticeable. Similar fields were used to estimate neuronal density (see Methods for details). Note the presence of pyramidal cells identified by their shape (arrow). Scale bar is 10 µm. (C) The plots summarized that BDNF treatment globally sustains neuronal survival and a significant fraction of such increased number of cells is represented by pyramidal cells.***

Neuronal density was calculated as neurons/mm$^2$, starting calculating neuronal density before BDNF-treatment.

This number is substantially lower than originally plated partly because, at the time of plating, it is impossible to separate between the glia and neurons and the latter group of cells is highly sensitive to mechanical insults and many neurons could presumably have died in the process of plating. Altogether, when we counted the number of neurons 2 days after plating, this number was already substantially lower than the cell count at the plating, indicating that the cells do not die over the course of the experiment, but right after plating.

Following this protocol we quantify β-tubulin positive neurons at time points that corresponded to the time before and after BDNF-treatment, and we compared those points between control and in BDNF treated cultures. Cultures at 4 DIV (immediately

before any treatment: normal medium renewal in controls, nurotrophin enriched medium in BDNF cultures) contained 208±75 neurons/mm$^2$. At 9÷10 DIV control neuronal density was of 154±51 neurons/mm$^2$, and that of BDNF was increased to 196±49 neurons/mm$^2$. BDNF cultures showed a significant increase (27%; $p<0.0001$ using Student's t-test) in β-tubulin positive neuron survival when compared to controls (n=4 cultures). To estimate whether this improved survival was involving a specific class of neurons, we identify, according to morphological parameters (dimension and shape of the soma, number or processes, arborization and cell-cell connections; refer to Methods for more details), principal hippocampal neurons and we were able to quantify changes, upon BDNF treatment, in the survival of this class of glutamatergic cells. In control cultures at 9÷10 DIV (same sample as above) not only neuronal density was lower, when compared to BDNF, but the pyramidal neuron/interneuron ratio was 0.19 while it increased to 0.33 in BDNF ones. The plots shown in Figure 4.12 summarize these results.

According to our results, although BDNF treatment globally sustains neuronal survival, a significant fraction of such increased number of cells is represented by pyramidal cells.

These results indicate that the neuronal network size is improved by BDNF, influencing the number of synaptic connections, in addition such improved connections will be unbalanced towards excitatory ones, given the different ratio in pyramidal, commonly excitatory (Segal, 1983), neuronal cells.

## 4.6 BDNF increases synaptic connections

Miniature excitatory postsynaptic currents (mEPSCs) represent the action potential independent release of neurotransmitter (Colomo and Erulkar, 1968) and allow to infer, although indirectly, about the function and structure of synapses. To further characterize the changes in synaptic excitation induced by BDNF, mEPSCs were recorded in the presence of 1 μM TTX (a well-known blocker of voltage gated, fast $Na^+$ channels; Figure 4.13), identified by their fast kinetics (τ=4±0.3 ms) and pharmacologically by application of bicuculline (10 μM). Recorded mEPSCs were then analyzed offline in terms of frequency and amplitude.

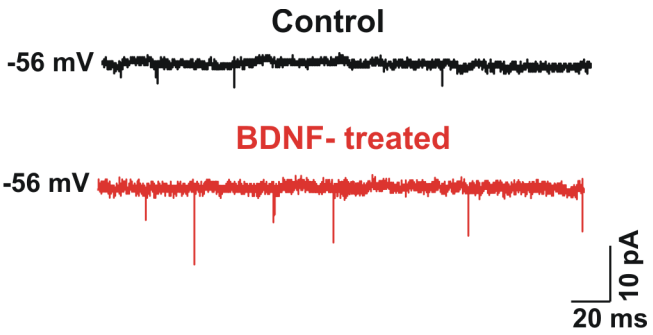


**Figure 4.13:** ***Sample tracings of mEPSCs recorded in control and in BDNF neuron***

Miniature EPSCs amplitude and frequency values are depicted in the plots of Figure 4.14 in control samples (black histogram) and BDNF cultures (red histogram).

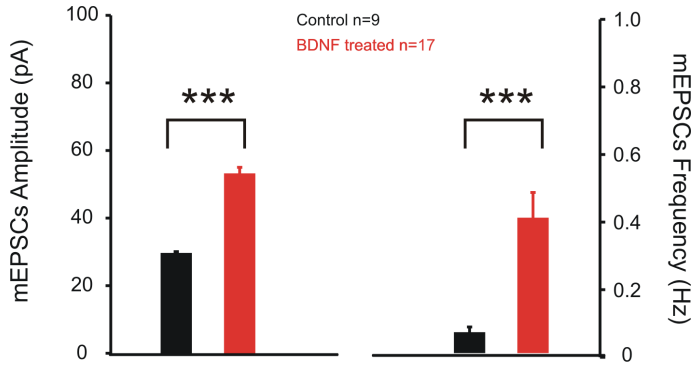


**Figure 4.14:** ***Mean frequency and amplitude of mEPSCs recorded in the two conditions (P<0.001 Student's t-test).***

Notably, BDNF significantly increased both the amplitude and the frequency of mEPSCs (control amplitude and frequency were 30±0.7 pA and 0.06±0.02 Hz respectively, n=9; while BDNF amplitude and frequency risen to 53.4±2.0 pA, and 0.40±0.08 Hz, n=17; P<0.001 calculated by Student's t-test). Relative cumulative amplitude distribution shown in Figure 4.15 (n=9 for controls and n=17 for BDNF cells) were verified in significance *via* Kolmogrov-Smirnov test (P value < 0.001).

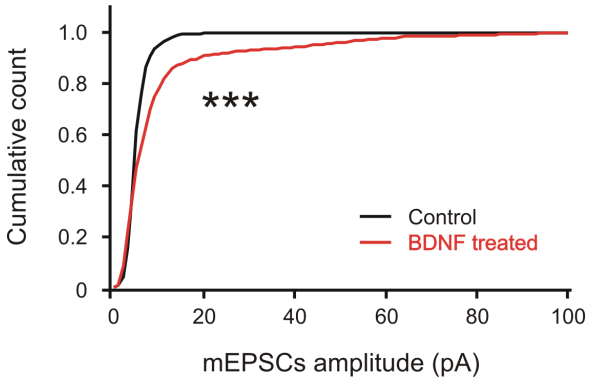


**Figure 4.15:** ***Relative cumulative amplitude distribution plot of control and BDNF EPSCs. Note that the BDNF distribution (blue) curve is shifted to the right of the control (grey) distribution one, indicating that BDNF-treated cultures are more likely to have larger mEPSCs than control ones. The different probability is statistically significant (Kolmogorov-Smirnov test; P<0.001).***

The BDNF selectivity of effects on excitatory, glutamate-mediated synapses was further confirmed by the absence of modulation in mIPSCs frequency and amplitude upon BDNF treatment (n=10). As in the case of mEPSC, miniature IPSC were identified by their kinetics (slower decay: $\tau$=4±0.3 ms) and pharmacologically by application of CNQX (10 μM).

The fact that BDNF changes were replicated at the level of mEPSCs has several mechanistic implications. mEPSCs are thought to correspond to the response that is elicited by a single vesicle of transmitter and mEPSCs frequency is a widely accepted index of the number of active zones/synaptic contacts or of the probability of release at presynaptic terminals, while mEPSCs amplitude is connected to the amount of neurotransmitter receptors expressed at the postsynaptic membrane (Raastad et al., 1992), thus the improvement of neural network activity detected in presence of BDNF seems to be related to modifications occurring at both presynaptic and postsynaptic level.

These results suggest that the neurotrophin enhances the probability of transmitter release and/or the number of releasing sites at the presynaptic terminal and has effects on the sensitivity of the postsynaptic cell to neurotransmitter.

## 4.7 Insights into BDNF chronic effects on synapses: simultaneous pair recordings from monosynaptically coupled neurons

To characterize synapses in cultured neuronal networks, we exploited simultaneous dual patch clamp recordings of mono-synaptically connected neurons.

We perform recordings from hippocampal neurons at 9÷10 DIV. One of the two patched cells, the putative presynaptic neuron, was held under whole cell current clamp mode at –60 mV, and APs were elicited by injecting short (4 ms) square current pulses 1 nA in amplitude (see sketch in Figure 4.16 and refer to Methods for further details about the used protocol). The postsynaptic neuron was simultaneously monitored under whole cell voltage clamp mode, held at –56 holding potential.

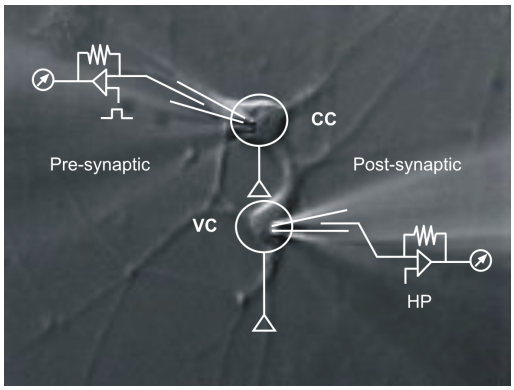


**Figure 4.16:** ***Representative image of two pairs of neurons. The presynaptic cell was held in current clamp mode, while the postsynaptic one in voltage clamp mode.***

We quantified the "rate of connectivity" in neuronal control and in BDNF-treated cultures as the probability of finding mono-synaptically coupled pairs of neurons. We also systematically addressed the presence of gap-junction (electrical synapses) by, in each pair, delivering hyperpolarizing current steps (–0.05 nA impulses; 100 ms in duration; Zsiros et al., 2007) to the presynaptic cell. According to Zsiros gap junctions can be identified by the presence of currents in the postsynaptic neuron coincident to presynaptic hyperpolarizing stimuli. In our recordings we detected only a small fraction of gap-junctions coupled neurons (1% in control, n=26) and 2% in BDNF, n=38) as previously reported in similar cultures (Cellot et al., 2011). Due to the low probability of finding gap-junction-coupled pairs, those detected were not further analyzed.

By pair recordings we further demonstrate the ability of chronic BDNF exposure to increase global synaptic connectivity. In control pair the probability of finding monosynaptic PSC was 20% (n=26 pairs), such a percentage was increased to 70% (n=38 pairs) in BDNF coupled neurons (Fig.4.17).

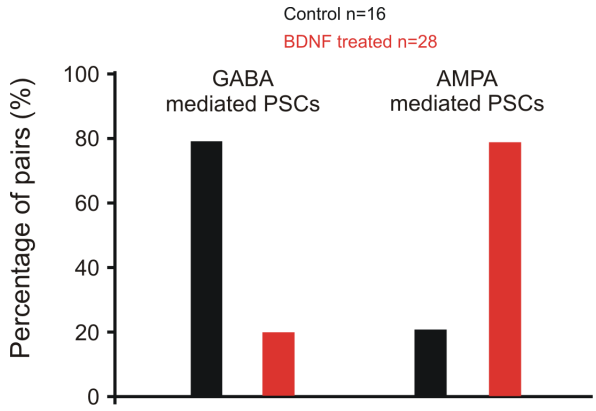


**Figure 4.17:** ***Our results showed that BDNF increases the probability of finding monosynaptic connections. In BDNF-treated cultures the majority of coupled neurons displayed PSCs mediated by AMPA-receptors.***

In addition, in BDNF, the large majority (80%) of mono-synaptically coupled pairs, displayed evoked PSCs (ePSCs) mediated by AMPA receptors (eEPSC) while in control cultures, the 80% of pairs were mediated by $GABA_A$ receptors (Fig.4.17). We identified GABAergic and glutamatergic ePSCs based on the usual criteria: PSCs kinetic properties, pharmacology and reverse potential. In the case of GABAergic ePSCs, they displayed slow decay ($\tau$ =23±0.8 ms, n=16) and were abolished by administration of 10 μM bicuculline; AMPA-mediated ePSCs displayed fast decay ($\tau$ =5±0.6 ms, n=28) and were abolished by application of 10 μM CNQX.

There are discordant evidences (Basarsky et al., 1994; Bi and Poo, 1998; Cellot et al., 2011) reported in literature, concerning the occurrence of GABAergic or glutamatergic monosynaptic connections in pair recordings in dissociated hippocampal cultures. This might be due to the variability in culture preparation, such as the animal age, cellular density, medium composition, etc; however our results confirm that in control conditions, predominant inhibitory monosynaptic connection are detected when tested by pair recordings, and these results are in agreement with previous reports (Segal, 1984; Melnick et al., 1999; Cellot et al., 2011).

The fact that BDNF chronic exposure impact in synaptogenesis is selective on EPSCs (glutamatergic synapses), strengthen the observation, suggested by our data on

spontaneous EPSCs and mEPSCs recordings and on the rate of pyramidal cell survival, that BDNF potentiates excitatory network activity via an increase in the number of cells and of synaptic contacts.

We have to say that, when recording spontaneous activity, the majority of spontaneous PSCs were reported to be glutamatergic in our controls (see Figure 5.19); the higher occurrence of GABAergic monosynaptic connections is apparently due to our experimental setting, which constrains the double recordings to pair of cells located in close proximity within the field of view. As a consequence of that, the large majority of monosynaptically $GABA_A$ mediated connections in control may not imply larger amount of GABA-releasing neurons. Moreover, most evidence indicates that, despite a prevalent glutamatergic innervation of the cultured network, local geometries force to detect GABA *vs.* glutamate monosynaptic connections in control (Cellot et al., 2011).

Thus, the increase brought about by BDNF is not only in the number of pyramidal glutamatergic cells and glutamatergic synapses but also, presumably, in the network geometry, allowing more efficiently to "unmask" AMPA receptor mediated monosynaptic connections.

Regardless the increase in connectivity, and the EPSC/IPSC ratio, our aim in performing pair recordings was to gain insights into presynaptic features, prior and after BDNF chronic treatment.

To this aim, and to estimate the release probability, paired-pulse recordings from couple of mono-synaptically connected neurons were performed. The degree of facilitation or depression of a synaptic connection depends on $p_r$ and can be quantified by the paired-pulse ratio (PPR), which is defined as the amplitude ratio of the second to the first postsynaptic response after stimulating the presynaptic neuron. This ratio is inversely related to the release probability and provides a mean to estimate this parameter (Manabe et al., 1993; Debanne et al., 1996). Two presynaptic spikes are evoked at with short delay and the responses of the postsynaptic cell are measured.

In general, the smaller is the probability of release to the first pulse, the more facilitated is the response to the second pulse. This phenomenon, known as paired-pulse facilitation (PPF), is accounted for by the residual calcium hypothesis, according to which the small fraction of calcium entering the terminal during the first spike increases the probability of transmitter release to a, timely close, second AP (Zucker, 1989). It follows that when a synapse displays low probability of release at the first stimulus,

presynaptic vesicle will not be depleted by the first stimulus, and smaller PSCs will be recorded at the post synaptic site, in addition residual $Ca^{2+}$will trigger higher vesicle release at a close second pulse, leading to a larger, compared to the first, second PSC, thus displaying an increase in PPR (Thomson et al., 1993).

Conversely, when the presynaptic terminal displays high release probability, then the first pulse will deplete the available transmitter, leading to a larger post synaptic PSC and the second pulse will inevitably cause less transmitter to be released, leading to a smaller second PSC and thus a low PPF or even a PPD (paired-pulse depression).

To quantify the paired-pulse ratio, we performed, in each pair of coupled neurons (n=4 control and n=10 BDNF), ten consecutive trials delivering two presynaptic AP (50 ms, apart) at 20 Hz. Paired-pulse modulation was quantified by calculating the ratio between the mean peak amplitude of the second and the first PSC (Fig.4.18).

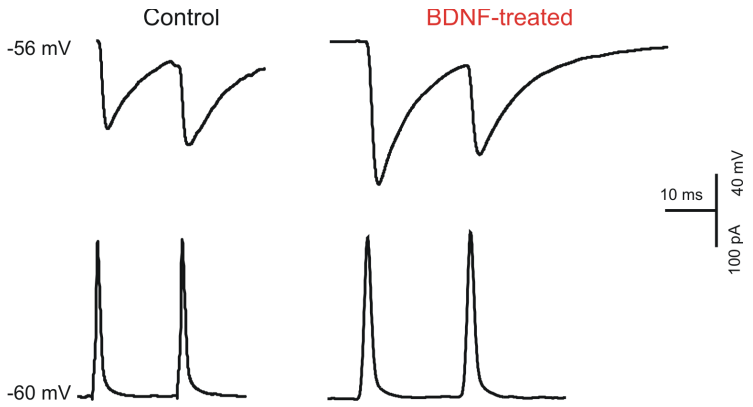


**Figure 4.18:** ***Exemplificative traces of presynaptic stimuli and postsynaptic responses in control and in BDNF-treated cultures. BDNF-treatment induces pair pulse depression, while facilitation is obtained in control cultures.***

Paired pulse activation of BDNF-treated neurons induced depression (0.7±0.06 in pulse ratio; n=10), while in untreated cultures we detected a strong facilitation (3.2±0.3 in pulse ratio; n=4) (Fig.4.19).

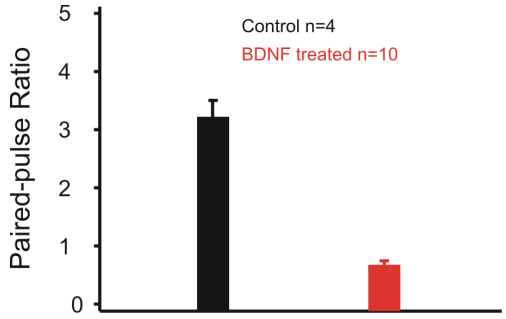


**Figure 4.19:** ***Paired-pulse ratio between control untreated neuronal cultures and BDNF treated ones.***

When we measured the amplitude of the first evoked EPSC we found that in control its amplitude was 45.0±18.2 pA, while in BDNF treated cells it was 94.6±34.6 pA. The corresponding second evoked EPSC was instead 144.2±18.0 pA in controls and 66.2±18.1 in BDNF cells. Regarding decay times, first evoked EPSC was characterized by $\tau$=4.5±0.3 seconds in controls and $\tau$=4.7±0.2 seconds in BDNF. Second evoked EPSC was instead characterized by $\tau$=5.1±2.5 seconds in controls and $\tau$=5.3±1.8 seconds in BDNF samples.

In an attempt to further estimate release from cultured hippocampal neurons, we used the styryl dye FM1-43, that, along with the other dyes of the FM series, has proved to be extremely useful to study the properties of presynaptic vesicle release and recycling (Betz and Bewick, 1982; Ryan et al., 1993; Betz et al., 1996; Murthy et al., 1999). We have used FM1-43 and live imaging wide-field fluorescence microscopy to monitor vesicles release from hippocampal neurons treated or untreated with BDNF. To note is that we could not discriminate between GABAergic and glutamatergic neurons and synapses, but we focused on presynaptic terminal of presumed pyramidal neurons when this was possible.

Presynaptic terminals were loaded with FM1–43 by high (50 mM) $K^+$ loading. Cells were then washed and perfused with a 50 mM $K^+$ and 2 mM $Ca^{2+}$ solution to evoke exocytosis (see Methods, paragraph 3.4 for details). In a different set of experiments in similar cultures we used the intracellular calcium dye Fura-2 AM, to detect depolarization-induced calcium signals. As internal control, under these experimental conditions we confirmed that 50 mM KCl consistently induced calcium transients of comparable amplitude upon repetitive stimulations.

After staining with FM1–43, clusters of presynaptic terminals were visible as bright fluorescence spots (see sequences of images in Figure 4.20: control, above and BDNF,

below, recording fields. A refers to bright fields, B to FM1–43 stained cells before KCl loading, C to same cells at the end of KCl treatment).

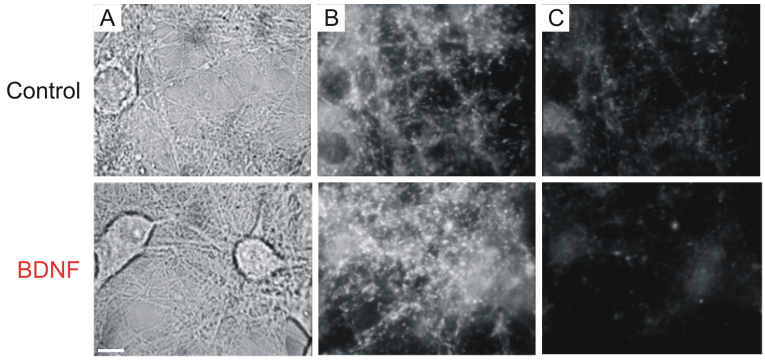


**Figure 4.20:** ***Sequence of images control, above and BDNF, below: sequential FM1-43 staining and destaining. A) Bright field images show the extensive neurite arborization that hippocampal neurons reach at 9÷10 DIV culture. Scale bar represents 10 µm. B) The same fields after staining with FM1-43. The staining protocol consisted of 120 sec exposure to 50mM KCl (see Methods for details). C) Fluorescence images following destaining induced by 50mM KCl. Remaining fluorescence represents non-specific staining.***

The effect of BDNF on the size of recycling pool of synaptic vesicles was evaluated by measuring the raw fluorescent intensities of individual FM1-43 positive terminals ROI (Betz and Bewick, 1982; Figure 4.21). Although this was not normalized per number of neurons, to compare control to BDNF treated cultures we compare similar number of terminals (see Methods) identified as fluorescent spots.

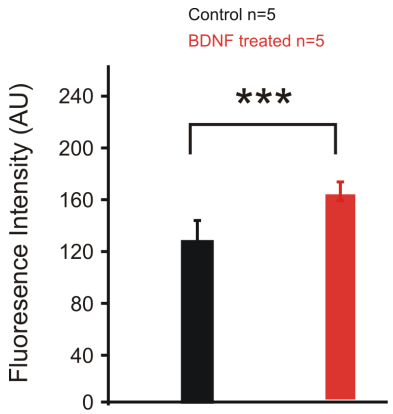


**Figure 4.21:** ***Histograms: BDNF-treated cells showed an increase of fluorescence intensity compared to control cultures (P<0.001, Mann-Whitney test) when loaded with FM1-43. Subsequent KCl treatment induces fluorescence decay due to forced vesicles release (see text).***

It is interesting to note that compared to control cultures, an increase in fluorescence intensity was consistently detected in BDNF neurons ($I^{f}_{CTRL}$=126.7±35.7 arbitrary units

(AU) of fluorescence, mean±SD, n=5 coverslips from 3 independent cultures, 459 terminals; $I^{f}_{BDNF}$=164.6±42.4 AU, n=5 coverslips from 3 independent cultures, 409 terminals, P<0.001, Mann-Whitney test), indicating that long-term treatment with BDNF increases the size of the pool of recycling vesicles. This result, however, differs from what reported by Tyler and co-workers (2006), who evidenced similar fluorescence intensities between control and BDNF treated cultures loaded and de-stained by field stimulation. Such an inconsistency is likely to be due to the different experimental procedures employed to load and de-stain synaptic terminals (electrical stimulation *vs.* high-$K^+$ loading). The protocol of field stimulation employed by Tyler is supposed to selectively load the ready releasable pool of recycling vesicles (Ryan et al., 1995; Rizzoli and Betz, 2004), while the high-$K^+$ loading is thought to label the total amount of recycling pools (Pyle et al., 2000; Harata et al., 2001).

Following FM1–43 loading, we delivered a strong depolarizing chemical stimulus, consisting of a 120 seconds long pulse of KCl 50 mM, to induce destaining in most of the previously labeled terminals. Fluorescence signals reduce to levels comparable to non-synaptic background areas.

To evaluate the effect of long-term exposure of hippocampal neurons to BDNF on the kinetics of FM1–43 release we analyzed exactly these fluorescence de-staining profiles during KCl induced exocytosis. The continuous depolarization caused, in both control and BDNF treated cultures, a biphasic decrease in fluorescence (Figure 4.22).

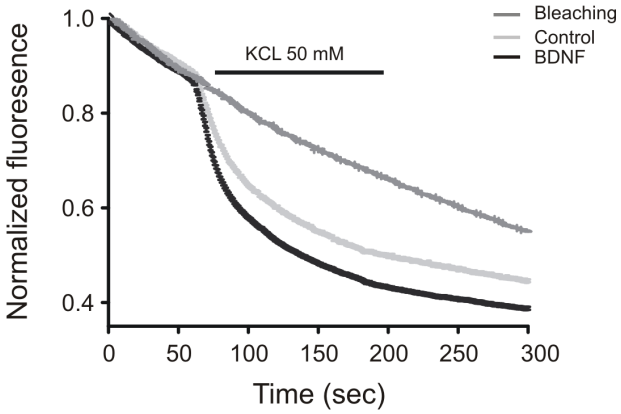


**Figure 4.22:** ***In KCl-stimulated cells, BDNF treatment increased the rate of release from synaptic terminals, compared to control cultures (straight line, mean±SEM from 204 terminals in n=3 independent experiments; CTRL n=459 terminals, BDNF=425 terminals).***

An initial phase of fast fluorescence decay, starting approximately at t=75 seconds, corresponding to the beginning of the KCl chemical stimulation, was replaced within tens of seconds by a more gradual decrease that continued for more than 2 minutes. In parallel experiments, image series captured on FM1–43 stained cells but without any KCl destaining stimulus, produced a baseline reference plot. This kinetic reflects a decrease in fluorescence due to either dye bleaching or non-specific fluctuations in brightness rather than actual de-staining responses due to exocytosis (Figure 4.22, straight dark gray line, mean±SEM from 204 terminals in n=3 independent experiments).

In KCl-stimulated cells, BDNF treatment increased the rate of release from synaptic terminals, compared to control cultures (Figure 23, CTRL n=459 terminals, BDNF=425 terminals).

Despite the differences in the initial, pre de-staining stimulus, fluorescence intensity between controls and BDNF treated cultures, the result in Figure 4.23 is in agreement with what previously reported by Tyler and co-workers in 2006. The researchers saw a similar, two components, decay in fluorescence on FM1–43 marked hippocampal cells' vesicles. Using similar cultures they observed after both acute (3 hours) and chronic (72 hours) neurotrophin treatment a general decay of the fluorescence signal after destaining (electrically induced *via* 1200 AP at 10 Hz) comparable to our trend in Figure 23. In a similar way they were able to identify two classes of events: a "fast" sub-set and a "slow" sub-set, characterized by features very similar to our results highlighted in Figure 4.23.

To better define the basis of the BDNF-induced increased rate of FM–143 release, de-staining profiles of regions of interest (ROIs) corresponding to individual fluorescent spots were examined in both control and BDNF-treated cultures (Figure 4.23). Based on kinetic properties, single de-staining events could be divided into two main populations (see example in Figure 4.23).

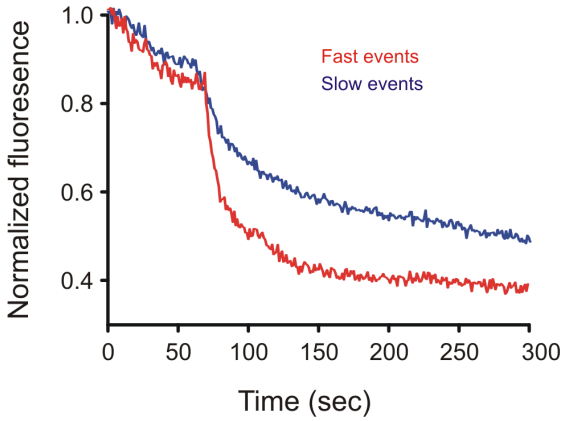


**Figure 4.23:** ***The initial fluorescence drop contribution may be considered due to both a fast (red plot) and a slow (blue plot) subset of events (Tyler et al., 2006).***

We defined "fast" events showing an initial (20 seconds) fluorescence drop fitting an exponential decay with a time constant τ of about 13.27±2.74 seconds (mean±S.D. from 133 terminals of 3 different experiments), and "slow", events with a time constant τ of about 27.87±3.96 seconds (mean±S.D. from 176 terminals of 3 different experiments). The difference between these two classes of events was significative (P<0.001, Mann-Whitney test) (Figure 4.23).

By analyzing the distribution of "fast" and "slow" events in control and BDNF-treated cells we observed that "fast" events prevailed on BDNF-treated cultures, where they represented 57.8 % ± 11.7% of the total population (mean±S.D. from 425 terminals, variation from control "fast" events significative with P< 0.001 by Student's *t*-test), while "slow" events predominated in controls, representing 83.4%±4% of the population (459 terminals, difference from BDNF slow events significative with P<0.001, Student's *t*-test; Figure 4.24).

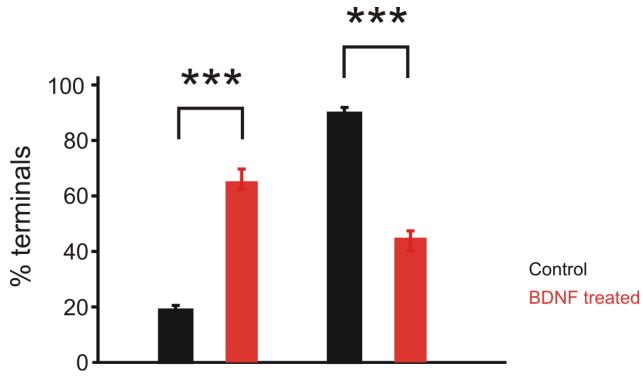


**Figure 4.24:** ***In BDNF-treated cells prevailed fast events (on the left, inside the light gray box), while in control cultures, the large majority displayed slow events (on the right, dark gray box).***

Although the kinetics of styryl dye destaining could account for multiple events, possibly comprising different steps of both exo- and endocytosis (Sara et al., 2005; Richards et al., 2005; Klingauf et al., 1998), and thus could not always be interpreted unambiguously, these results are in agreement with those we obtained with the pair-pulse facilitation, reported in Figure 25, and reinforce the view that long-term treatment of hippocampal neuronal cultures with BDNF increases both the size and the availability of the releasable synaptic vesicle pool.

# 5 DISCUSSION

The human brain is a unique organ composed of 100 billions neurons and 100 trillions of axonal connections between them. Throughout our lives, our brain changes constantly thanks to "*neuronal plasticity*": every thought, perception, or behavioral interaction with the environment is generated by a series of electrical and chemical signals transmitted through connected networks of neurons. Neurons transmit these signals at specialized sites of contact called synapses. In the nervous system, most neurons communicate via chemical synapses by converting electrical signals, in the form of action potentials racing down axons and invading presynaptic boutons, into chemical signals and then back to electrical impulses within the postsynaptic dendrite. Synapses perform this task by releasing neurotransmitters from the presynaptic neuron that bind and activate neurotransmitter-gated ion channels on the postsynaptic cell.

In the last decades, a great interest has focused on the rules governing synaptogenesis, demonstrating the existence of multiple molecules that influence when and where synapses are formed or their specificity and stability. Furthermore, it is widely accepted that also mechanical and electrical instruction strongly contributes to synaptic dynamic plasticity (McNally and Borgens, 2004). Synapse stability, remodeling and elimination are fundamental to learning, memory and cognition in the mature brain (Shahaf and Marom, 2001).

Neuronal activity can vary, increasing or decreasing, and such change may last a few seconds, but also several minutes, hours or even days and electrophysiological recordings can give a measure of this synaptic strength.

The two main physiological mechanisms behind the changes in synaptic strength are long-term potentiation (LTP), which actually increases synaptic strength, and long-term depression (LTD), which instead decreases it.

Understanding in detail the mechanism of action of these processes may be of critical importance not only for a detailed view of memory related processes but also in the case of some diseases: being able to control synaptic plasticity may help to restore a functional connectivity lost, for example, in the case of brain lesions.

Most of the studies on long-term synaptic plasticity have shown that by applying a precise pattern of high electrical stimulation to hippocampal slices it is possible to

experimentally induce the enhancement/depression of synaptic transmission (Bliss and Collingridge, 1993; Pockett et al., 1990) but also the use of chemicals is able to produces in slices the same results (Grey and Burrell, 2008; Jagodzinski and Hess, 2001; Otmakhov et al, 2004).

Synaptic plasticity, induced by electrical or chemical stimulation, share the same molecular pathways (Hanse and Gustaffsonn, 1994; Huang and Malenka, 1993); the difference between the two methods has to be found in the proportion of synapses that are enhanced rather than in the mechanism of induction *per se*.

Hippocampal slices as method for the study of synaptic plasticity has some advantages, in particular, the integrity of the hippocampal cytoarchitecture, reflecting in some way the intact anatomy and functionality of neuronal networks as they are *in vivo*.

However, dissociated neurons in cultures lend themselves well for this purpose due to their versatility. They ensure the activation of a broad population of synapses available in the culture and lend themselves to molecular biology approaches. Furthermore, the most obvious advantage of dissociated neuronal cultures is that it makes individual living cells and their synapses more accessible.

In this work we induced both electrically and chemically synaptic plasticity in cultured hippocampal neurons with a series of stimulation train or by the treatment with brain derived neurotrophic factors (BDNF) in order to analyze by AFM, in an upcoming part of the project, the morphological and mechanical (e.g. stiffness) changes occurring at the dendritic spine level.

It was not technically straightforward to induce plasticity in dissociated hippocampal cultures and it required the setting and testing of particular and complex stimulation paradigms.

The first part of this thesis handles the setting of an electrophysiological stimulation to induce neuronal plasticity at specific synapses in dissociated rat hippocampal cells.

We cultured dissociated cultures from P2÷P3 rats on poly-ornithine coated glass coverslips, and we performed different stimulation protocols to induce a localized synaptic potentiation, started by adapting the electrical stimulation protocols used to induce synaptic plasticity in the organotypic slices.

We first performed protocols comprised repetitive low-frequency (by delivering 60 square pulses at 1 Hz frequency to the presynaptic neuron, while both cells were transiently held in current clamp), or theta burst potentiation (which consists of

delivering short bursts consisting of 4 pulses at 100 Hz). Responses varied from no changes to potentiation but just in a small sample of coupled neurons where we measured a strong increase in the amplitude and frequency of spontaneous PSCs after the stimulation.

During the above study, we noted a consistent failure in the induction of synaptic potentiation: the summary of results from all similar experiments clearly indicated the absence of any synaptic change. The average percentage change in the amplitude and frequency of EPSCs 20÷30 minutes after the repetitive stimulation was not significantly different from that found before the stimulation train.

In a second set of experiments, we focused our attention on a more elegant stimulation: the spike timing dependent plasticity protocols, where the temporal order of pre- and postsynaptic spiking is a crucial parameter for induction of LTP (Baranyi and Feher, 1981; Levy and Steward, 1983). Over the last few decades, a number of groundbreaking studies showed that in hippocampal slices, repetitive stimulation with pairs of pre- and postsynaptic action potentials, with precise pre/post spike timing, led to the induction of long-term synaptic plasticity, controlling both the sign and magnitude of synaptic modifications (Debanne et al., 1994; Bell et al., 1997; Bi and Poo, 1998).

To set the precise timing able to reliably correlate presynaptic and postsynaptic spiking thus potentially inducing synaptic modifications, we varied (from 5 to 20 ms) the time interval between the presynaptic and postsynaptic stimulation.

Unfortunately, in these cultures, we found that only few synaptic connections are susceptible to synaptic potentiation by correlated spiking.

The cellular basis that gives rise to the induction of such synaptic modifications remains to be determined. The huge change in amplitude and frequency seen in few coupled neurons in the current study could be due to insertion of new AMPA receptors at sites already containing AMPA receptors, or it could be due to altered levels of AMPA receptors in the postsynaptic membrane, altered receptors properties, or a combination of these two mechanisms (Liao et al., 2001).

Current evidence suggests that activity-dependent changes in synaptic strength, such as LTP, result at least in part form changes in AMPA receptor-mediated responses (Kauer et al., 1988; Muller et al., 1988; Davies et al., 1989).

An alternative possibility could be due to an increase in the number of functional neuronal junctions, due to the “unsilencing” of “silent” synapses (Kullmann, 1994; Liao

et al., 1995). Several different mechanisms could account for this: the activation of silent presynaptic terminals (Kullmann et al., 1996; Gasparini et al., 2000); an alteration in the amount or kinetics of L-glutamate release such that small responses that previously went undetected become visible (Choi et al., 2000) or the rapid physical insertion of AMPA receptors into synapses that previously lacked these receptors (or where the number of receptors was too low to generate a detectable response).

A third possibility is that a different LTP mechanism also exists in these neurons, such as an increase in single channel conductance (Benke et al., 1998).

However, with this low probability of success the properties of the long-term change in network activity is not easy to understand.

Experiments on synaptic plasticity are often carried out in hippocampal organotypic slices, that presented some different aspects respect to dissociated hippocampal cultures: high three dimensional synaptic density, dendritic arborization, presence of active synaptic vesicles, and astrocytic maturation are a few examples of synaptic maturation in the slice cultures. In addition to structural development, slices cultures have been shown to adopt pattern of gene regulation, protein expression, and synaptic activity of the adult hippocampus presumably missing in dissociated cultures (Bahr, 1995).

Long-term, electrically induced, changes that typically occur in slice cultures were not seen in dissociated cells: in our case just few pairs of neurons shown an increase in synaptic strength. It is possible that the stimulations we performed may not be the most efficient stimulation to evoke a network change, which is governed by multiple factors, such as activation of NMDA receptors and influx of calcium. The long-term enhancement of the global network behavior, and not of the individual synapse, could be associated with this plethora of factors that might result, for example, in differences in gene transcription.

On the other hand, in slices there are probably multiple mechanisms of expression of NMDA receptor-dependent LTP, including both changes in unitary conductance and receptor number (Benke et al., 1998): inappropriate activation of NMDA receptors (which might take place in our cultures) can prevent the induction of NMDA receptor-dependent LTP (Coan et al., 1989).

In addition, considering that the standard protocol for stimulation that normally induces LTP in hippocampal slices had no effects on dissociated hippocampal cultures could result form differences in the postsynaptic molecular machinery underlying synaptic

modifications. For example, both the α isoform of calcium/calmodulin-dependent protein kinase II (CAMK II α) and the $Ca^{2+}$/calmodulin-dependent protein phospahatase β (calcineurin) appear to be absent in the postsynaptic densities of glutamatergic neurons (Stevens et al., 1994; Liu and Jones, 1996 and 1997) in dissociated cultures. A very important aspect that has to be considered working with dissociated cultures is the limited number of synaptic contacts (usually just one) forming between paired neurons, compared with that in the slice system: a single high-efficacy connection observed in dissociated cultures couldn't be analogous to the slices situation of divergent different outputs to a group of synchronously firing postsynaptic cells, whereby propagating signals from various terminals undergoing LTP could be summated (Tao et al., 2000).

Another possibility is that the intervals we tested until now were much shorter than the time constant for the dissociation of glutamate from NMDARs (Vicini et al., 1998). One interpretation of these data is that the amount of LTP could depend not only on the pre/post spike interval but also could imply certain co-operativity of LTP among multiple synaptic sites receiving the same input (Harvey and Svoboda, 2007).

We conclude that in our culturing conditions to induce selective synaptic potentiation by means of presynaptic application of different electrical stimulation on pairs of monosynaptically connected neurons holds, in all cases, an extremelly low probability of success. Thus prevents us to develope and designed an experimental approach able to induce efficently synaptic plasticity only on a well defined and localized synapse.

Following our unsuccessful attempts to selectively govern activity dependent synapse plasticity among pairs of cultured neurons, we decided to move towards a different experimental approach involving, this time, the majority of synapses of the network *via* a chemical stimulation.

Among the multiple molecular effectors involved in the mechanism of synaptic plasticity, neurotrophin expression and in particular, Brain Derived Neurotrohic Factor (BDNF) was found to be of crucial importance. Since this discovery, the biological role of BDNF for developing neurons has been shown extensively: BDNF promotes neuronal cell survival, neurite growth, cell migration, dendritic growth and synapse formation (Mizul and Kojima, 2013).

BDNF has been consistently shown to modify excitatory synaptic transmission and long-term synaptic plasticity in a variety of preparations (Lessmann et al., 1994; Kang and Schuman, 1995; Levine et al., 1995; Figurov et al., 1996). It has been reported to

rapidly enhance excitatory synaptic transmission in hippocampus, visual cortex and other structures (Lohof et al., 1993; LeBmann et al., 1994; Kang and Schuman, 1995; see McAllister et al., 1999 for review).

Recently, Lu and coworkers, in 2007, showed that long-term (5÷6 days) exposure to BDNF, which resembles the time course of nerve injury-induced BDNF elevation, can induce persistent and permanent changes to the dorsal horn network, such as decreased synaptic drive to "tonic" neurons and increased synaptic drive to "delay" neurons.

The proposed mechanism underlying the actions of BDNF includes the immediate and long-term modulation of both pre- and postsynaptic function (Poo, 2001).

BDNF ability to mediate activity-dependent modifications in synaptic strength has recently received considerable attention (Bolton et al., 2000; Lohof et al., 1993; Vicario-Abejon et al., 1998; Sherwood and Lo, 1999). In particular, acute BDNF effects on excitatory synapses have been the object of an increasing amount of studies. On the contrary, the role of chronic BDNF exposure in regulating long-lasting changes in synaptic function is comparably less investigated and may play an important role on post injury alteration of synaptic networks and neuronal rescue (Lu V.B. et al., 2007; Cho et al., 1997 and 1998).

We started to examine the spontaneous synaptic activity on dissociated hippocampal cultures, which could provide an index of possible changes occurred in neuronal network when interfaced to a long (4 DIV) BDNF-treatment. What we discovered was that BDNF-treated cultures displayed a significant increase in glutamatergic peak amplitude (40%) and frequency (more than 100%).

The increase in spontaneous EPSCs amplitude and frequency could be due to different mechanisms involving either the pre- or the post-synaptic level, e.g. increased firing activity, with changes in cell excitability due to prolonged BDNF exposure, increased release probability of neurotransmitter or altered receptors number or clustering, differences in neuronal survival and thus in the global network size. The effect on EPSCs amplitude could result also, at least in part, from an increased postsynaptic responsiveness, via a phosphorylation-dependent pathway (Levine et al., 1995). These larger postsynaptic current may be responsible also for the increase in EPSCs frequency: BDNF activation of TrK tyrosine receptors engages multiple second messenger pathways that may underlie these synaptic effects. This includes activation of many serine-threonine protein kinases (Keegan and Halegoua, 1993; Kaplan and

Stephens, 1994). These kinases may in turn act on postsynaptic neurotransmitter receptors to enhance synaptic currents. For example, a number of studies have shown that the magnitude of glutamate-mediated currents can be increased by activators of cAMP-dependent protein kinase, protein kinase C and calcium/calmodulin-dependent protein kinase (Greengard et al., 1991; Kaplan and Stephens, 1994).

In slice preparations of hippocampus it was reported that BDNF suppressed GABAergic inhibition, but did not significantly change excitatory synaptic transmission (Tanaka et al., 1997; Frerking et al., 1998). This might suggest that the enhancing effect of BDNF on synaptic transmission in slices and dissociated cell cultures preparations might be caused by the suppression of inhibition, although there are a few pieces of evidence against this possibility (Akaneya et al., 1997; Carmignoto et al., 1997).

In order to investigate the implication of BDNF in some of these mechanisms we investigated network size and neuronal density, recording excitatory spontaneous miniature postsynaptic currents (mEPSCs), whose occurrence depends upon the stochastic quantal release of neurotransmitter vesicles [Basarsky et al., 1994]. This may help in clarify whether the observed changes in synaptic transmission have a pre- or post-synaptic origin. Our analysis of mEPSCs resulted efficacious in investigating at least one of the possible mechanism involved in the BDNF-dependent improvement of network activity.

In fact, the increased frequency of mEPSCs strongly suggests an increasing in the number of synaptic contacts. A larger number of AMPA receptors at synaptic sites could account for the increase in mEPSCs amplitude (O'Brien et al., 1998). Recently, rapid insertion of AMPA receptors from potentiated synapses has been proposed to underlie changes in synaptic efficacy (Carroll et al., 1999; Lissin et al., 1999), suggesting that long term action of BDNF, such as those reported here in this thesis, may similarly involve increases in the density of AMPA receptors at synapses (Bolton et al., 2000). BDNF has recently been demonstrated to increase, for example, the expression of AMPA receptor subunit 1 and 2/3 protein in neocortical neurons (Narisawa-Saito et al., 1999). Alternatively, BDNF may induce the accumulation of AMPA receptors at synapses previously devoid of these receptors as has been proposed for "silent" synapses in neonatal hippocampus (Isaac et al., 1995; Liao et al., 1995).

Our results are also consistent with an increase in the amount of glutamate packaged per vesicle (see Reimer et al., 1998, for a review about this mechanism): this accounted for

the increase in mEPSCs frequency in BDNF-treated neurons that is usually associated with presynaptic alterations, such as in probability of release.

BDNF-treatment did not influence the decay time of both EPSCs and mEPSCs suggesting that BDNF did not alter the subunit composition of the NMDA receptors under these conditions because changes in the relative expression of NR2A–NR2D subunits have been shown, for example, to alter NMDA receptor decay time significantly (Carmignoto and Vicini, 1992; Hestrin, 1992).

However, apart from molecular changes and reorganizations, several others mechanisms could be accounted for this increase in excitatory transmission, first of all the increased in the total number of cells and, in particular, in an increased survival of pyramidal cells in respect of control sister cultures.

We asked whether the action of BDNF regulated neuronal survival and we found, by quantifying neuronal density in BDNF-treated neurons compared to control cultures, that BDNF globally increased neuronal survival, especially at pyramidal neurons levels. These findings supported the concept that a long treatment with BDNF strongly increased the excitatory transmission in dissociated cultures of hippocampal neurons.

All these results suggest that the neurotrophin enhances the probability of transmitter release and/or the number of releasing sites at the presynaptic terminals.

We further addressed this issue focusing on the properties of synaptic contacts between pairs of mono-synaptically connected neurons.

In particular, thanks to pair recordings, we characterized an improvement in the probability of finding monosynaptic contacts in BDNF-treated conditions in comparison to control.

Focusing our attention on release probability, we performed paired-pulse and FM1–43 experiments.

Synaptic vesicles are reported to exist in different functional states within the presynaptic terminal, with some vesicles associated with cytoskeletal elements, some one docked at active sites, and others undergoing endocytosis and refilling (Heuser et al., 1979). Vesicles belonging to different pools are released with different kinetic properties and various factors can promote their allocation and fusion with the neural membrane.

It is likely that the amplitude of first ePSCs depends on the “primed” pool of vesicles, that are readily released in relation with the basal probability of release of synapse,

while amplitude of the consecutive ePSCs is more sensible to the characteristic of "back located" pools of vesicles, whose probability of release can be modified by external factors (Debanne et al., 1996), such as the accumulation of $Ca^{2+}$ in presynaptic terminals (residual $Ca^{2+}$).

There is also a correlation between the size of the pool of docked vesicles and the probability of mEPSCs (Murthy et al., 1997). A recent study suggests that synapses with higher rates of spontaneous mEPSCs also possess higher rates of evoked release (Prange and Murphy, 1999). Our results show that long-treatment BDNF increase the number of docked vesicles per terminal, the frequency of mEPSCs and the number of functional connections.

All together, our results show that synapse activation by BDNF cause general change in neuronal network but specifically increases the number of docked vesicles. The increased uptake of FM1–43 by BDNF can be a consequence of enhanced transmitter release because more exocytosis will lead to more endocytosis (Collin et al., 2001).

It is well known that BDNF induces local dendritic instability leading to activity-dependent morphological changes in dendritic spines (Horch and Katz, 2002).

Furthermore, it was previously reported that BDNF converts immature excitatory synapses (silent synapses), containing solely NMDA receptors, into AMPA receptors containing synapses in the developing mouse barrel cortex (Itami et al. 2003). AMPA C-termini (GluR1 and GluR2) critical for their interaction with PDZ proteins were suggested to be important for the unmasking of silent synapses. These results suggest BDNF regulates AMPAR trafficking to the postsynaptic sites.

Some reports suggest that BDNF up-regulates surface AMPA in cultured hippocampal and cortical neurons (Jourdi, 2003; Narisawa-Saito et al., 2002). It is uncertain whether BDNF up-regulates AMPAR translocation to post-synaptic densities in an active spine. The dendritic spine is the site for scaffolding synaptic proteins in most central excitatory synapses (Hering and Sheng, 2001). Rapid delivery of AMPAR to spines upon synaptic NMDAR activation has been demonstrated in hippocampal CA1 neurons (Shi et al., 1999). This effect required calcium/calmodulin-dependent protein kinase II and the PDZ binding domain of GluR1 (Hayashi et al. 2000). In this trafficking process, a subunit specific regulation of AMPAR delivery into the synapses was suggested (Kakegawa et al., 2004; Passafaro and Piech, 2001; Shi et al., 2001).

The role of BDNF in modulating dendritic spines is also confirmed by some works with dissociated cells that showed how BDNF is able to directly handle the assembly/disassembly of actin filaments in filopodia during development. Furthermore, long-term (48÷72 hours) application of BDNF to 11÷12 days *in vitro* hippocampal organotypic cultures increase the mean frequency of mEPSCs recorded from CA1 pyramidal neurons. In the same cell, imaged after whole-cell recording, BDNF increased spine density. These results demonstrate the ability of BDNF to enhance quantal synaptic transmission as well as promote dendritic spine formation as well as the proportion of spines (Tyler and Pozzo-Miller, 2003).

In summary, our experiments have shown that BDNF can have profound effects on the activity of neuronal circuits and that this is possible *via* regulation of excitatory synaptic function. The boosting of neural activity brought about BDNF long term treatment is in terms of both increased amplitude and frequency of spontaneous events and, in particular, at glutamate mediated-level, with a significant increase of both EPSCs and mEPSCs. The analysis of mEPSCs resulted efficacious in investigating at least one of the possible mechanism involved in BDNF-dependent improvement of network activity. The increased frequency and amplitude of mEPSCs strongly suggested an increased number of synaptic contacts in BDNF-treated neurons, all supported by BDNF increase in neuronal survival.

It was previously reported that BDNF increases the probability of transmitter release at excitatory hippocampal CA3÷CA1 synapses, without affecting the number of independent release sites or the quantal amplitude (Lessmann and Heumann, 1998).

Here, using dissociated hippocampal cultures and imaging FM1–43 labeled presynaptic terminals, we observed, first of all, differences in the fluorescence intensity between control and BDNF-treated cultures, suggesting that BDNF modulate the size of the total recycling poll of vesicles after chronic treatment and also an accelerated activity-dependent FM-destaining kinetic, reinforcing the view that long treatment of hippocampal neuronal cultures with BDNF increases both the size and the availability of the releasable synaptic vesicle pool. Whether or not BDNF treatment exerts an effect on the mixing of distinct synaptic vesicles pools in cultured neurons remains to be established but, despite this, BDNF consistently increased the rate of FM-dye destaining modulating the probability of transmitter release. This modulation has been demonstrated to have direct consequences in the expression of a presynaptic component

in LTP-induction (Tyler and Pozzo-Miller, 2001), providing further support to the notion that BDNF exerts its role in hippocampal-dependent learning and memory by modulating fundamental mechanisms of synaptic transmission and plasticity of hippocampal synapses.

# 6 APPENDIX

In this appendix I chose to summarize an additional series of experiments indirectly related to the thesis' main subject. Although representative, these results are preliminary and have to be considered a work in progress. For these reasons I described them in a separate section.

The basic idea of the experiments described in next session is to try to correlate the significant improvement in excitatory synaptic transmission we observed in hippocampal cultures chronically treated with BDNF with a more somatic or dendritic localization of BDNF expression in the cell. Is it in fact well known that BDNF is produced in cells from many transcripts that display distinct subcellular localization, suggesting that spatially restricted effects occur as a function of genetic and physiological regulation.

Overexpressing region specific BDNF-GFP transcripts in cultured hippocampal neurons we want to try to mimic our chronic effects in order to highlight the specific neuronal cell region where its effect takes place. Otherwise we want to address the question if the effect of chronic BDNF treatment is more localized in the somatic region than in dendritic one. For this purpose we chose to compare the effect of three different transcripts on our cultures: one having a distribute effect in the cell (CDS-BDNF-GFP), one a more somatic effect (exon1-BDNF-GFP), and one characterized by a contribution localized to more distal dendrites (exon6-BDNF-GFP) (Baj et al., 2011).

I am currently working on these topics and here I presented preliminary, but promising, results involving only the first of the three transcripts. I hope to conclude this investigation and to clarify several of these issues in the next future.

## 6.1 Investigating the Role of BDNF isoforms-GFP chimeras

It is well-known that BDNF is a neurotrophin with multifaceted functions such as survival, neurite outgrowth, synaptogenesis and synaptic plasticity (Casaccia-Bonnefil et al., 1999; McAllister et al., 1999; Huang and Reichardt, 2001). In addition, BDNF induces dendritic sprouting in the presence of synaptic activity (McAllister et al., 1995; 1996), causes local instability in dendrites (Horch et al., 1999; Horch and Katz, 2002;) and increases spine density and dimension (Tanaka et al., 2008).

Several levels of regulation of BDNF, including proteolytic processing (Lu et al., 2005; Hempstead, 2006) and the use of distinct receptors and signaling cascades (Chao, 2003; Reichardt, 2006), may explain how this neurotrophin exerts so many different functions (Baj et al., 2011). BDNF is produced from many transcripts (Figure 6.1) that display distinct subcellular localization, suggesting that spatial restricted effects occur as a function of genetic and physiological regulation (Baj et al., 2011)

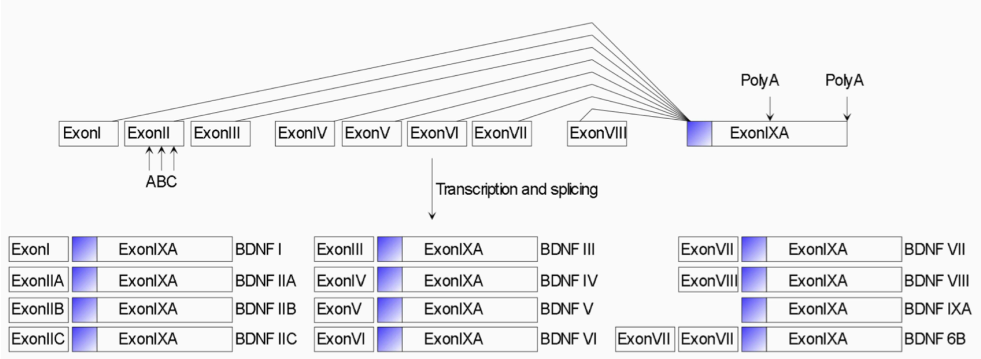


**Figure 6.1:** ***Mouse and rat gene structure and transcripts. Boxes indicate exons. Filled box in exon IXA indicates the coding region of the Bdnf gene. Lines indicate splice variants. Arrows indicate within-exon splice sites and alternative polyadenylation sites. The BDNF gene is transcripted from different promoters, immediately preceding each of the 5' exons (exons i-VIII), so that each full-length transcript contains a unique 5'-exon and a common 3'-exon (exon IXA) that encodes the BDNF protein (adapted from Cunha et al., 2010).***

It has bee demonstrated that the transcript encoding the neurotrophin BDNF is localized in the proximal third of young hippocampal neurons *in vitro* and, in a similar way, the mRNA for its receptor TrkB (Tongiorgi et al., 1997; Righi et al., 2000). Even if these mRNA encode for different proteins with different functions and structures, it is noteworthy to underlie how most of all are involved in synaptic plasticity. It seems that BDNF is able to exert several cellular effects not only by activating different intracellular cascades, but also through the tight regulation of the local availability of the protein itself due to a different regulation of mRNA isoforms localization.

Furthermore, the distinctive subcellular distribution of the transcripts variants could subtend to different local effects of BDNF protein, potentially influencing different aspects of synaptic plasticity, including rearrangements of dendritic complexes and spines numbers and type.

Baj and coworkers, in 2011 proposed a "spatial code hypothesis of BDNF transcripts", in which different BDNF splice variants, through the spatial segregation of their

mRNA, represent a code to direct the protein to either the soma or proximal or distal dendrites (Chiaruttini et al., 2008; Tongiorgi, 2008).

In their work they showed as exon-1 and exon-4 transcripts are mainly localized in the neuron soma, while exon-2 and exon-6 transcripts show a somato-dendritic localization. Thus, splice variants appear to encode spatial localization signals used to preferentially regulate BDNF expression in different subcellular domains (Horch, 2004; Soule et al., 2006) (Figure. 6.2).

In previous works researchers cloned the rat BDNF coding sequence (rCDS) with the green fluorescence protein (GFP) reporter gene, either alone or preceded by one of the five most abundant rat BDNF 5' UTR sequences (rEx1, rEx2B, rEx2C, rEx4 and rEx6) (Aid et al., 2007, Baj et al., 2010).

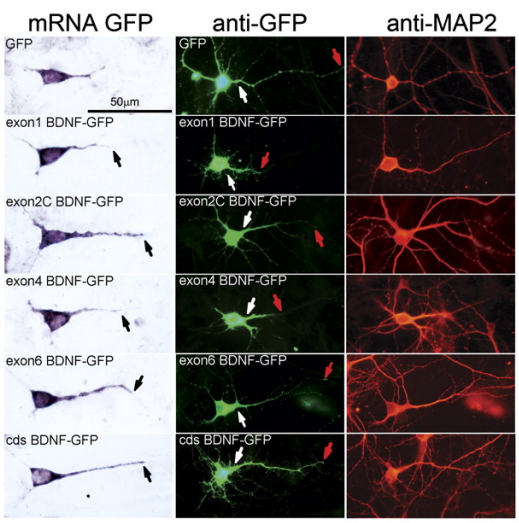


**Figure 6.2:** ***(Left) In situ hybridization on 7 DIV cultures of hippocampal neurons transfected with the cds-BDNF-GFP constructs. (Centre) Fluorescence of BDNF-GFP proteins. (Right) MAP2 staining. Black arrows show the end point of maximal distance of dendritic labelling; while, white and red arrows show respectively the starting and ending points of dendritic BDNF-GFP protein labelling (figure adapted from Baj et al., 2011).***

Starting from these considerations we set a series of experiments was to investigate if the results we obtained on hippocampal neurons with chronic BDNF treatment could be replicated by a specific BDNF construct. For this purpose we investigated the role of the different BDNF-CDS mRNA isoform in modifying the activity of the network in dissociated rat hippocampal cultures.

The BDNF isoforms-GFP chimeras (Figure. 6.3) were prepared by Dr. Gabriele Baj and Dr. Andrea Colliva, former members of Tongiorgi's laboratory (University of Trieste).

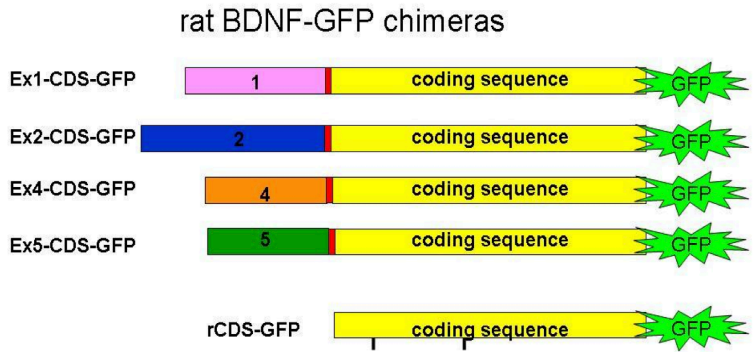


**Figure 6.3:** ***Schematic representation of different BDNF transcripts chimeras and of CDS-GFP alone that was used as control construct. All the transcripts used in the experiments were a courtesy of Tongiorgi's laboratory (University of Trieste).***

To transfected primary neurons with the different constructs we chose the Lipofectamine 2000™ (Life Technology, Invitrogen, Ohki et al., 2001) approach. Cells were transfected with 1 μg of target DNA diluted in 50 μL of MEM medium without serum and antibiotics (in each petri dish). At the same time 2 μL of the Lipofectamine™ solution (1mg/mL) have been dispersed in 50 μL of MEM solution. After 5 minutes the two solutions have been mixed togheter, let for at least 20 minutes stabilizing at RT, diluted to a final concentration of 20 nM and then added to the cellular culture bath for transfection. After 1 hour incubation the Lipofectamine-DNA mixuter has been carefully removed and replaced with our usual culture medium.

Due to the low transfection efficiency of lipofectamine on primary neuronal cultures (<0.01%, Dalby et al., 2004) and considering that the lipofection-technique uses complexes that cross the plasma membrane, partially impairing it was not effortless to perform reliable electrophysiological recordings.

To analyze the amplitude and frequency of both excitatory and inhibitory spontaneous events (identified as described previoulsy, see chapter 4.4) electrophysiological recordings were performed at 9÷10 DIV. Until now, we checked only the CDS-BDNF-GFP and GPF-alone (used as control) on our hippocampal cultures. Neurons (see Figure. 6.4) were recorded after 24 hours from the transfection. Lipofectamine final concentration (20 nM), incubation time (1 hour) and the time interval before experiments (24 hours) were selected in a "guess and check" way in order to optimize both transfection efficacy and cell health/integrity.

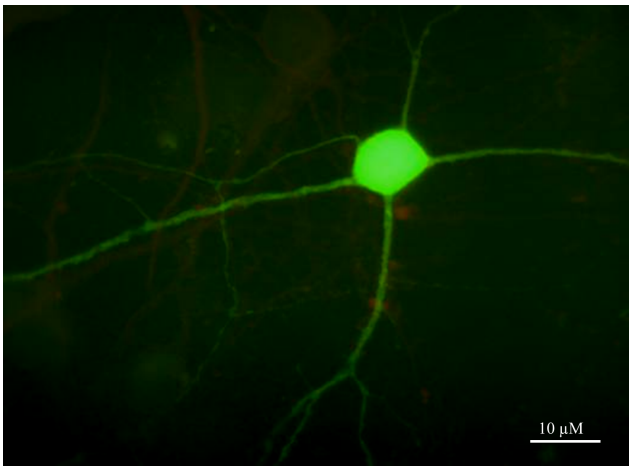


**Figure 6.4:** ***Representative image of a transfected neuron. Electrophysiological recordings were performed only on GFP-transfected neurons after 24 hours from the transfection.***

By using voltage clamp recordings we compared GFP control neurons *vs*. CDS-BDNF-GFP transfected neurons (Fig. 6.5) in order to point out any change induced by the BDNF coding sequence.

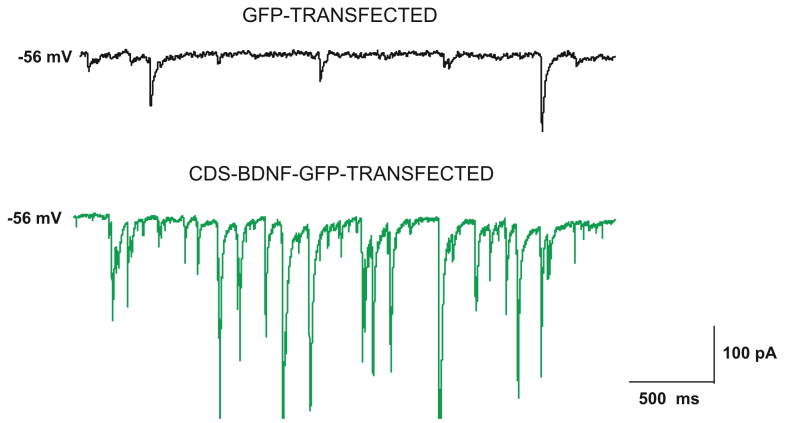


**Figure 6.5:** ***Voltage clamp recordings of spontaneous synaptic activity of GFP-transfected (black trace) and CDS-BDNF-GFP transfected neurons (green trace). Recordings were performed only on transfected neurons (recognizable by the GFP green fluorescence signal). The increase in frequency and amplitude of spontaneous PSCs in these BDNF-transfected neurons when compared to controls is appreciable.***

We started evaluating passive membrane properties: on average, cell capacitance was 66±9 pF (n=6) in control GFP-transfected cultures and 53±6 pF (n=11) in CDS-BDNF-GFP-transfected ones; cell input resistance was 187±46 MΩ (n=6) and 300±84 (n=11) respectively for GFP-only controls and CDS-BDNF-GFP-transfected neurons.

Our first aim was to investigate the effect of CDS-BDNF-GFP transcript on dissociated cultures. This construct was supposed to induce BDNF production in a not localized

fashion that is in the cell body and all proximal and distal compartments of dendrites. When PSCs were recorded from CDS-BDNF-GFP transfected neurons, we detected a strong increase (P<0.001, calculated with Student's *t*-test) both in terms of PSCs amplitude (from 60±11 pA, in GFP-transfected cells, n=6, to 141±15 pA in CDS-BDNF cultures, n=11) and PSCs frequency (from 1.4±0.2 Hz for GFP-cells to 4.3±0.7 Hz for transfected ones, same number of samples of amplitudes). A summary of all results is presented in Figure 6.6.

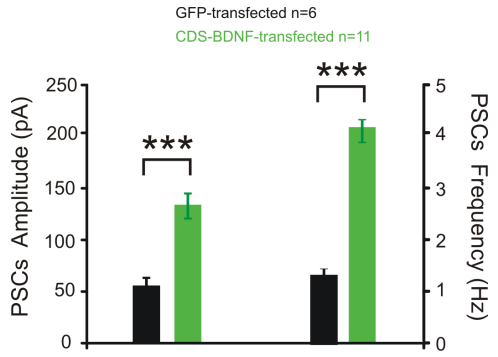


**Figure 6.6:** ***PSCs recorded from CDS-BDNF-GFP transfected neurons shown a significative increase both in terms of PSCs amplitude and frequency (P<0.001 in both cases, calculated with Student's t-test).***

When the two different components of PSCs (inhibitory and excitatory) were analyzed separately, an increased in both $GABA_A$ and glutamate-mediated PSCs was detected in CDS-BDNF transfected neurons. Inhibitory and excitatory events where identified based on their kinetics as previously described.

In particular neurons transfected with a plasmid encoding for CDS-BDNF-GFP shown a significant increase in terms of excitatory and inhibitory amplitude (57.6±15 pA and 83.7±7 pA respectively, n=11; Figure 6.7) and frequency (2.6±0.5 Hz and 1.7±0.3 Hz) in respect to only-GFP expressing neurons' EPSCs and IPSCs amplitude (26.45±3.9 pA and 33.2±8.2 pA) and frequency (1.3±0.2 Hz and 0.2±0.1 Hz, respectively; see plots in Figure 6.7).

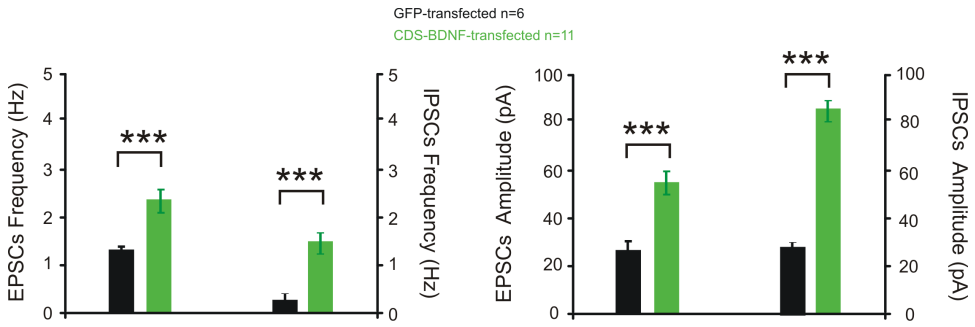


**Figure 6.7:** ***EPSCs and IPSCs recorded from CDS-BDNF-GFP transfected neurons shown a significative increase both in terms of amplitude and frequency (P<0.001 in both cases, calculated with Student's t-test).***

From this set of electrophysiological experiments performed on transfected neurons we confirmed that the BDNF coding region common to all transcripts (represented by the coding sequence CDS-BDNF-GFP transcript, see Baj et al., 2011) is sufficient to induce significative mutations in the synaptic transmission of an hippocampal neurons culture.

From these results it is clear how the specific regulation of single BDNF mRNA transcripts may represent a code to regulate neuronal plasticity. Furthermore, by investigating the effects of the other different transcripts (for example the more somatic exon1-BDNG-GFP transcript and the more dendritic exon6-BDNF-GFP one) it could be possible to better understand the role of this particular neurotrophin in modulating synaptic activity. Moreover, as claimed in the introduction of the chapter, we hope to use these results to better focalize the neuronal region (soma or dendrites) in which a chronic BDNF treatment exerts its effect.

Thus, it is conceivable that the physiological, pathological and pharmacological modulations of different BDNF transcripts could help us to better understand the not fully explored roles of neurotrophins in shaping the central nervous system during normal and pathological morphological rearrangements.

# 7 CONCLUSIONS

The concept of neuronal plasticity covers all the mechanism involved in the capacity of the central nervous system (CNS) to adjust and remodel itself in response to different kind of stimuli. Neuronal cells continuously rearrange their synaptic circuitry, a feature termed synaptic plasticity, and recently was shown that electrical, chemical and mechanical stimulation may significantly contribute to dynamic synaptic plasticity.

Noticeably none of these findings was carried out simultaneously to a real time, high resolution, morphological characterization of the plastic synapse or any investigation about mechanical modifications (e.g. in terms of cytoskeletal reorganization) the potentiated synapse undergoes.

The NanoMosquito project, within framework this thesis work lays, proposes to fill the gap integrating electrophysiological measurements, atomic force microscopy (AFM) and fluorescence microscopy to a new nano-tools with the purpose to study, with a multi-technic approach, neuronal plasticity at the nanoscale. The impact of AFM technology, and therefore of nanotechnology, on neuronal adaption and reorganization, will allow progresses toward the comprehension of plasticity at distinct sub-cellular neuronal sites during network remodeling. The project intends to study neuronal synapses with a sophisticated level of morphological control by means of AFM; to follow dendritic spine and synapse dynamics in real time, network synaptic plasticity has to be induced *in situ* with either patch-clamping technique or chemically.

This thesis work handles precisely with the first part of the project: we tried to induce synaptic changes via both electrical and chemical stimulations in dissociated hippocampal culture, a neuronal culture model suitable for the project purpose having all the cell and its processes easily accessible for further investigations.

Regarding patch-clamping, by applying a series of electrical trains protocols (starting from low-frequency stimulation to theta burst and, finally, by controlling the timing between the pre- and the postsynaptic spike) we succeeded in inducing synaptic potentiation, unfortunately just in few monosynaptically coupled neurons. Understanding the mechanisms that underlie the substantial enhancement in synaptic strength we observed could be extremely useful and intriguing, but the low yield of success we encountered did not allow us to do any speculation about.

Concerning chemical induction of synaptic plasticity, among members of the neurotrophin family, brain-derived neurotrophic factor (BDNF) stands out for its ability to regulate synaptic plasticity and various cognitive functions of the brain (Woo and Lu, 2006): as a consequence of that it was the ideal candidate for our purposes. BDNF driven (chemical) synaptic potentiation lose the ability to spatially confine synaptic potentiation to a single, well determined, neuro-junction location but induces an comprehensive effect in all the existing synapses of the neuronal network.

Since its discovery, the biological role of BDNF in neuronal development has been extensively demonstrated: BDNF promotes the differentiation and survival of developing neurons both *in vivo* and *in vitro* (Bibel and Barde, 2000).

In this thesis, in addiction, I investigated in detail the effects of a long-treatment (4 days chronic treatment) of BDNF on dissociated hippocampal cells by using patch clamp recordings and fluorescence microscopy. Up to the present this work is the first study about such kind of chemical stimulation on dissociated hippocampal cells. What we pointed out was that, the strong increase in both EPSCs and mEPSCs frequency and amplitude, concerned both presynaptic and postsynaptic modifications and, furthermore, BDNF induces changes in synaptic vesicles release probability.

However the common feature of all these functional modifications is in the direction of a pronounced potentiation of excitatory synaptic connections.

As we already mentioned, the end of this thesis work represents the beginning of the next step of NanoMosquito project where, subsequently to the induction of synaptic plasticity, scanning probe microscopy would be used to follow in real time morphological changes of synapses undergoing potentiation or neuronal processes development with submicrometrical resolution in all 3 dimensions. Final goal of the entire project, whereof this thesis is the fundamental initial step, will be the development of new paradigms to evaluate and induce synaptic plasticity on specific synapses to govern in a controlled way neuronal outgrowth and synaptogenesis.

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