ANALYSIS of THE MOLECULAR MECHANISMS of BDNF mRNA LOCALIZATION and TRANSLATION in NEURONS

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1 INTRODUCTION

1.1 Biological significance of mRNA sorting

The compartmentalization of RNAs is an efficient and effective mechanism to concentrate specific proteins in defined subcellular compartments and represents an essential step during the development of polarized cells.

The first evidence for a specific subcellular localization of messenger RNAs (mRNAs) was reported in a study demonstrating that the messenger RNA encoding actin was particularly concentrated in the myoplasm of the oocytes of the ascidian Styela placata (Jeffery et al, 1983). A growing body of experimental evidence has contributed to demonstrate that this phenomenon is actually widely diffused in several organisms, from plants, to fungi to mammals, suggesting its strong evolutionary conservation. Moreover, RNA localization is often coupled to local control of protein synthesis, resulting in a finely regulated mechanism for the spatial and temporal regulation of protein expression that has been adopted in many different cell types (Kloc et al, 2002).

The biological significance of mRNA sorting has to be considered in view of the metabolic needs that highly polarized cells like oocytes and neurons have to face, and can be summarized in five points. First, in these cells it is necessary to avoid ectopic expression of proteins in cellular districts where their presence would be otherwise deleterious, like a mislocalization of nanos and oskar in Drosophila’s embryos leading to abnormal development, or an incorrect presence of Myelin Basic Protein (MBP) mRNA in proximal compartments of oligodendrocytes (Gavis et al, 1996; Smith et al, 1992; St Johnston, 1995). Second, thanks to a local accumulation of mRNA a cell can drive a local increase of a particular gene product in defined sudomains, as for β-actin in fibroblasts’ leading edge or bicoid in the anterior portion of Drosophila’s embryos (Berleth et al, 1988; Kloc & Etkin, 1995). Third, it has also been described a regional accumulation of cyclin B mRNA at mitotic spindle (Groisman et al, 2000), as an example of how transcripts can be segregated in particular organelles or subcellular structures. Forth, thanks to regulated RNA sorting, a cell can build up a gradient of one morphogenic substance and therefore influence the expression of several other proteins, as happens for bicoid in Drosophila’s oocytes (Kloc et al, 2002). Fifth, even a new cell lineage can be generated by sequestering transcripts in particular cellular compartments of future daughter cells, as seen during differentiation of neuroblasts or in Drosophila’s oocytes (Kloc et al, 2002). Moreover, RNA
localization can contribute directly to the establishment of the polarization within a cell: in fact, disruption of β-actin mRNA localization at fibroblasts’ leading edge results in the collapse of cellular lamellae and lost of asymmetry, even if the global levels of β-actin mRNA and protein are conserved (Kislauskis et al, 1994). As a consequence, it is easy to imagine the importance of mRNA compartmentalization for the maintenance of polarity in highly specialized cells like neurons.

RNA sorting is also an efficient way to save cellular energy, since synthesis of many proteins is surely a much more energy-consuming process than localization of one or few mRNA molecules that, potentially, may be translated several times (St Johnston, 1995). This is particularly relevant in distal compartments, where proteins need to be transported from hundreds to thousands of microns away from the cell soma. This phenomenon results to be highly energy consuming and quite inefficient since it could take a time longer than the protein half-life. It is therefore easier and more convenient for a neuron to target some transcripts to neurites, opening the possibility of a local regulation of translation. In fact, it is well known that mRNAs encoding for crucial elements involved in synaptic plasticity like CaMKIIα and MAP2 are actively transported in dendrites (Bruckenstein et al, 1990; Burgin et al, 1990; Garner et al, 1988; Kleiman et al, 1990) as well that ribosomes are present at the base and neck of dendritic spines, together with several other translational machinery elements (Steward & Banker, 1992; Steward & Levy, 1982). It is therefore intriguing to speculate that local synaptic transmission could independently modulate protein expression at post-synaptic sites, resulting in a “molecular signature” that confers a unique identity to each synapse depending on the set of mRNA represented and the experienced stimuli.
2 TRANSPORT OF mRNA IN DENDRITES

2.1 First studies

Before 80’s, one of the major dogmas of neurobiology was based on the assumption that the protein synthesis machinery was present only at the level of cell soma, assuming that the proteins required in distal compartments like dendrites and axons would be first synthesized in the perikarion and from here transported at their final destinations (Peters et al, 1976). According to this view, the complex functions and morphological features of such highly polarized cells were exclusively driven by an extremely finely regulated process of protein transport along the cytoskeleton. However, this concept cannot explain many of the key features of central nervous system (CNS) neurons. These cells are able to maintain extremely polarized structures very far from the cell soma, to continuously modify the strength of their connections and respond in a very fast and efficient fashion to several environmental stimuli. If the somatic transport to distal subcellular domains would be the principal route to drive proteins in neurites, it is easy to imagine how this phenomenon wouldn’t meet the constant high metabolic need of neuronal cells. In fact, this would be a high energy consuming process, and, as previously discussed, inefficient, because the whole transport could take a time longer than the mean cargo half-life. Moreover, the very distal synapses would experience serious difficulties in signalling their metabolic need to the cell soma. For this reason, Steward and colleagues hypothesized that RNAs, instead of mature proteins, might be transported at synaptic sites and there locally translated upon demand. This theory was indeed supported by the discovery of polyribosomes under the base of dendritic spines of hippocampal granule cells (Steward & Levy, 1982), followed by the demonstration of the presence of several mRNAs in dendrites of hippocampal neurons (Davis et al, 1987). The relevance of this finding gained further importance a couple of years later, when Merlie and co-workers discovered the presence of the acetylcholine (Ach) receptor mRNA at the neuromuscular junction (Merlie & Sanes, 1985), followed by the discovery of dozens of other transcripts in distal subcellular domains, as the Microtubule Associated Protein2 (MAP2) mRNA in neuronal dendrites (Garner et al, 1988).

Of particular relevance was the characterization of one of the first dendritic targeting elements (DTEs) found in Myelin Basic Protein (MBP) mRNA (Ainger et al, 1997). It has been demonstrated that a small segment of 21 nucleotides, termed hnRNPA2 response element (A2RE) was necessary and
sufficient to drive MPB RNA localization in the myelin compartment. Later, the minimal binding sequence for hnRNPA2 was restricted to an 11 nt fragment, that is shared with many other localized mRNAs as myelin-oligodendrocyte basic protein or the α subunit of Ca\(^{+}\) Calmodulin Kinase II (CaMKII\(\alpha\)), neurogranin (NG), Arc and PKM\(\zeta\) (Burgin et al, 1990; Gao, 1998; Gould et al, 1999; Muslimov et al, 2004). Later on, many other transcripts have been discovered in distal dendrites, as G protein γ subunit (Watson et al, 1994), InsP3 receptor (Furuchi et al, 1993), Brain Derived Neurotrophic Factor (BDNF) and its receptor Tropomyosin Related Kinase (TrKB) (Tongiorgi et al, 1997), β-actin (Eom et al, 2003; Tiruchinapalli et al, 2003), vasopressin (Mohr et al, 2001b), the AMPA receptor subunit GluR2 (Ju et al, 2004) and many others.

In the first pioneer studies on MBP localization in living oligodendrocytes, Ainger and colleagues found out that microinjected MBP mRNA first appears in a dispersed form into the perikaryon, before being packaged in RNA transport granules that anchor to the cytoskeleton to move towards the periphery of the cell (Ainger et al, 1993). In this compartment, however, RNAs appear to be again widely dispersed, probably because some of them maintain the granular structure whilst others unpack to be ready for translation. Moreover, it has been demonstrated that also endogenous MBP RNA is transported along the microtubule network in the form of RNA transporting granules that exhibit different movement velocities, depending on the subcellular compartment (Ainger et al, 1993). These observations where further confirmed in neuronal dendrites a couple of years later by Knowles and coworkers, where mRNA transport particles (mRNPs) were directly labelled through the membrane permeable dye SYTO14 (Knowles et al, 1996). Those particles were found to colocalize with poly(A\(^{+}\))mRNAs, the 60S ribosomal subunit and with eEF1\(\alpha\), suggesting that they actually could represent a potential translational unit. As seen in oligodendrocytes, neuronal RNA granules moved at different rates depending also on the age of the cells analyzed, displaying anterograde and retrograde movements that were completely abolished after treatment with microtubule-disrupting drugs.

The results obtained by Ainger, together with the evidence collected by Knowles and co-workers strongly supported the idea that RNAs might be localized in processes in a tightly regulated and ordered way, thanks to this cellular trafficking system. Many descriptions of RNA granules in neurons have been performed by in situ hybridization techniques, suggesting that many different RNAs could be present at the same time in the same structure (Blichenberg et al, 1999; Knowles et al, 1996). RNA granules can be seen as discrete units in the dendritic compartment, but they are also present in the somata where are less distinguishable because of their high density. Furthermore, it
became increasingly clear that this transporting system is evolutionary conserved since it has been adopted by a very variety of cells, from oocytes (Kloc & Etkin, 1995), to fibroblasts (Sundell & Singer, 1990), budding yeasts (Bertrand et al, 1998) and Drosophila embryos (Ferrandon et al, 1994).

At this point, one crucial question has been raised: is it just a passive diffused transport or is there a specific mechanism to drive RNA localization in the periphery of the cell? Several lines of evidence supported the latter possibility. First, a growing body of evidence described the presence of dendritic targeting elements among many localized mRNA, as in the 3'UTR of CaMKIIα (Blichenberg et al, 2001; Blichenberg et al, 1999; Mori et al, 2000), MBP and MAP2 mRNA (Ainger et al, 1997; Blichenberg et al, 1999), β-actin mRNA (Kislauskis et al, 1994), the non coding BC1 (Muslimov et al, 1997) or in the 5’UTR of VP mRNA (Mohr, 1999; Prakash et al, 1997). Second, synaptic activity and neurotrophin application have demonstrated to be able to modulate the transport of mRNA containing particles (Knowles & Kosik, 1997; Righi et al, 2000; Steward et al, 1998). For example, induction of Long Term Potentiation (LTP) evoked a dramatic increase of CamKIIα transcript into the synaptic compartment (Thomas et al, 1994), as well as Arc mRNA and protein accumulation after local synaptic activation in hippocampal granule cells (Steward et al, 1998). Finally, mRNPs display heterogeneous velocities and directions of movement that are directly related to the activity of motor proteins (kinesins and dyneins) that govern the overall phenomenon of RNA transport along the cytoskeleton (Hirokawa et al, 1998; Hirokawa & Takemura, 2005; Kanai et al, 2004; Setou et al, 2000; Severt et al, 1999). Starting from the first early studies up to the present observations, a complex framework has been put forward of how mRNAs are transported along neurites, involving a plethora of different elements, from RNAs, to trans-acting RNA binding proteins (RBPs), molecular motors and cytoskeletal elements. All these components are essential to guarantee an efficient and effective mechanism for RNA delivery that is at the basis of the fine and fast adaptive neuronal responses required to promote synaptic plasticity and indeed memory and learning.

2.2 Cis-elements and trans-acting factors involved in mRNA trafficking

2.2.1 Cis elements

In many cell types and organisms, RNA localization is a regulated phenomenon mediated by the recognition of cis-targeting elements, present on RNAs, by trans-activating elements, i.e. RNA binding proteins. This interaction gives rise to the RNA transporting granules that anchor to the microtubules and are subsequently driven into specific subcellular compartments. Once granules have reached the final destination, they are either stored into translational silent units or are
promptly translated into proteins upon an adequate stimulus. In this section, the most important cis-acting elements found up to now will be taken into consideration, with particular interest for neuronal targeting elements. In most cases, cis-acting sequences, alternatively called “zipcodes”, localization or targeting elements, are found in the 3'UTR (UTRs). However, few important exceptions have demonstrated the presence of those signals even at the level of the coding region/Open Reading Frame (CDS/ORF) (Severt et al, 1999; Shan et al, 2003) or 5' UTRs (Muslimov et al, 2004; Muslimov et al, 1997; Steward & Banker, 1992). RNA localization elements (RLEs) are quite variable in terms of length, structure, position and type of cognate protein bound, resulting in a wide landscape of combinations that can lead to extremely specific pattern of RNA targeting.

In chicken fibroblasts and myoblasts, β-actin mRNA is accumulated into the leading edge of the cell (Lawrence & Singer, 1986), a localization mediated by a 3'UTR 54nt long “zipcode “ later characterized by Kislauskis (Kislauskis et al, 1994) and known to be recognized by Zipcode Binding Protein 1 (ZBP1) (Ross et al, 1997). The same RLE is active in neuronal cells, where it mediates the distal localization of β-actin transcripts through the recognition of ZBP1 in immature chicken neuronal growth cones and mature rat neurons (Eom et al, 2003; Tiruchinapalli et al, 2003). The usage of same cis and trans acting factors between different cell types and organisms demonstrates how RNA localization is a widespread mechanisms that has been conserved during evolution, thus highlighting its biological importance. However, since β-actin mRNA is restricted in the somata of avian retinal neurons but targeted in neurites of mammalian retinal cells, dendritic localization has to be considered as an evolulutional “conquer” that might have been adopted later in evolution as the complexity of brain structures and functions (i.e learning and memory) were growing (Cristofanilli et al, 2004).

A key molecule involved in synaptic plasticity is the α- subunit of Ca++-Calmodulin Kinase II that is highly enriched in post-synaptic domains, especially after local translation induced by intense neuronal activity (Bagni et al, 2000; Ouyang et al, 1999; Wu et al, 1998). The 3'UTR of its transcript is quite long and complex, and it has been found to contain several non-overlapping dendritic targeting elements (DTEs). Mori and co-workers found two signals, one in the first 28-56 nts of 3'UTR that promotes RNA dendritic sorting (also common to neurogranin mRNA, therefore called CNDLE, CaMKIIα Neurogranin Dendritic Localization Element) that in turn is counteracted by a downstream retention signal that blocks mRNA export in resting conditions, but is relieved after KCl induced depolarization (Mori et al, 2000). On the other hand, Blichenberg and co-workers have characterized
A separate 1200nt long DTE that induces RNA dendritic sorting. Moreover, this complex transcript also contains two hexanucleotide Cytoplasmic Polyadenylation Elements (UUUUAU) (CPEs) that facilitate both mRNA transport and translational activation (Huang et al, 2003). Even if the UTRs contain several localization elements, it also possible to find them at the level of the open reading frame, as the ORF-3'UTR spanning vasopressin DTE (Prakash et al, 1997), or the Y element in CaMKIIα recognized by translin, a DNA/RNA binding protein (Severt et al, 1999). In protein kinase Mζ transcript, one DTE is positioned at the interface between 5'UTR and CDS, and mediates somatic export, while a second 42nt long stem loop sequence is required for a full localization in neurites (Muslimov et al, 2004). The latter features a GA kink turn (K-turn) motif that has been described as a potential docking site for many RNA binding proteins (Klein et al, 2001). Another transcript that contains multiple targeting elements is MAP2, with one DTE recognized by MARTA1 and MARTA2 trans-acting factors that promote mRNA sorting (Blichtenberg et al, 1999; Rehbein et al, 2000; Rehbein et al, 2002), together with a CPE consensus site (Huang et al, 2003), both present in the 3'UTR. However, like BDNF and CamKIIα, MAP2 mRNA displays a sorting sequence in the CDS (A2RE, (Huang et al, 2003). In addition, also MBP mRNA features a bipartite DTE, consisting of a proximal dendritic targeting signal that is necessary and sufficient hnRNPA2 mediated targeting, and a highly structured downstream sequence that is required for mRNA anchoring and stabilization to the myelin compartment (Ainger et al, 1997). It is interesting to note that also the immediately early gene Arc/Arg3.1, that is strongly induced after neuronal activity (Link et al, 1995; Lyford et al, 1995), is predicted to be a hnRNPA2 target due to a homology A2RE sequence in its open reading frame (Huang et al, 2003). However, in a study performed by Kobayashi and colleagues, dendritic targeting of Arc mRNA in hippocampal neurons resulted to be mediated by two signals in its 3'UTR that displayed any sequence correlation to other known dendritic targeting signals (Kobayashi et al, 2005).

It is worth noting that the non coding BC1 RNA displays dendritic as well axonal localization thanks to a 62nt long targeting signal in its 5'UTR (Cheng et al, 1996; Muslimov et al, 1997; Tiedge et al, 1991). Even if much more attention has been paid to dendritically localized mRNAs, there are growing evidences demonstrating the presence in axons and axonal growth cones of few transcripts, like the ones encoding for κ-opioid receptor (KOR) (Bi et al, 2003), tau (Aranda-Abreu et al, 1999; Litman et al, 1993), neurofilament, enolase, olfactory marker protein, neuropeptides, tyrosine hydroxylase and oxytocyn (Jirikowski et al, 1990; Mohr et al, 1991; Mohr et al, 1992; Ressler et al, 1994; Skutella et al, 1994). It is remarkable that those mRNAs are found during early developmental stages and in the axon hillock and growth cones of immature neurons, but are much rarer in mature
cells, suggesting their critical role in promoting and guaranteeing a proper development, maturation and differentiation of neurites (Bassell et al, 1998; Kindler et al, 2005; Mohr et al, 2001a).

In conclusion, from the studies described above it emerges that mRNAs localized in neuronal processes might share common cis-acting elements that are recognized by a similar set of trans-acting factors. On one side, this could represent a strategy to regulate at the same moment different transcripts that are necessary for common cellular functions, on the other, the combination of different targeting signals can exponentially increase the combination of trans-acting factors that can simultaneously bind to (or even compete for) the same transcript, increasing the complexity of subcellular regulation of mRNA transport and translation.

### 2.2.2 Trans acting factors

From their birth in the nucleus to their death, RNAs are constantly bound by a heterogeneous and dynamic set of proteins (trans acting factors) that regulate their metabolism including, maturation of the primary transcript (capping, splicing and polyadenylation), export into the cytoplasm and modulation of subcellular localization, control of mRNA stability/decay and regulation of translation. In this context, trans acting factors (TAFs) are defined as proteins that support RNA metabolism through binding to cognate cis-acting sequences on RNAs (Kindler et al, 2005).

**hnRNPA2**, among the “core” RBPS that accompany mRNAs from the nucleus to the cytoplasm, plays a leading role, especially in neuronal cells where localization of mRNAs represent a key mechanism to post-transcriptionally modulate the expression of several gene product in response to a wide variety of extracellular stimuli. As already mentioned, hnRNPA2 modulates the subcellular targeting of several transcripts bearing a A2RE response elements, like CaMKIIα (Blichenberg et al, 2001), MAP2 (Shan et al, 2003), Arc (Huang et al, 2003) in neurons, MBP in oligodendrocytes (Ainger et al, 1997; Huang et al, 2003; Munro et al, 1999) and GFAP in astrocytes (Carson et al, 2001; Medrano & Steward, 2001). hnRNPA2 binding to A2RE RNAs is necessary for their assembly in transporting granules and for exclusion of non-A2RE RNAs from those transport particles. In fact, a point mutation (A8G) on the cis recognition elements prevents A8G RNAs binding to hnRNPA2 and assembly into granules (Carson et al, 2001). Moreover, the homotypic interaction between hnRNPA2 molecules could provide a driving force for granules assembly, bridging together different mRNAs with common functions, providing a mean for a coordinate expression of gene products in the same subcellular compartment, as described above (Cartegni et al, 1996).
ZBP1, that interacts with β–actin mRNA, belongs to the family of RBPs that contains two RNA recognition domains (RRMs) and four hnRNPK domains (Bassell & Kelic, 2004; Yaniv & Yisraeli, 2002). Mutation of zipcode sequences disrupts neurotrophin induce targeting of β–actin mRNA at growth cones and reduces growth cone motility (Zhang et al, 1999) and reduced accumulation of transcripts at the leading edge of fibroblasts (Ross et al, 1997). The same effects are mimicked by downregulation of ZBP1 expression, while overexpression of ZBP1 target mRNAs leads to an increase density of filopodia in neuronal cells (Eom et al, 2003).

MARTA1 and MARTA2 are two TAFs that bind with high affinity to the dendritic targeting element of MAP2 mRNA (Rehbein et al, 2000; Rehbein et al, 2002), and belong to the FUSE binding protein family of RBPs (FBPs) (Duncan et al, 1994; Min et al, 1997; Rehbein et al, 2002). MARTA1 is the rodent ortholog of human KH-type splicing regulatory protein (KSRP) and belongs to a macromolecular complex involved in neuronal splicing regulation (Gu et al, 2002; Min et al, 1997). On the other side, ZBP2, the avian ortholog of MARTA1 is involved in β–actin mRNA sorting in fibroblasts and neurons (Gu et al, 2002). Moreover, MARTA2/FBP3, in contrast to MARTA1/ZBP2, resides in the somatodendritic compartment and when overexpressed partially co-localizes with recombinant zipcode containing RNAs, while a truncated form severely interferes with MAP2 mRNA dendritic localization (Kindler et al, 2005).

Translin, also known as Testis-Brain RNA Binding Protein (TB-RBP) is a DNA/RNA binding protein able to bind the Y element found in some localized transcripts, like ligatin, CaMKIIα, protamine2 (Chiaruttini et al, 2009; Kwon & Hecht, 1991; Severt et al, 1999). Translin binds to microtubules and interacts in a specific manner with both ssDNA and RNA sequences (Han et al, 1995; Kwon & Hecht, 1991). In testis, Translin is involved in maintenance of the translational suppression state of germ cell mRNAs (Kwon & Hecht, 1991; Kwon & Hecht, 1993) and in mRNA transport along the intracellular bridges of male germ cells (Hecht, 1998; Morales et al, 1998). Translin is therefore a multitasking protein exerting different functions depending on the cell type and the nucleotide sequence bound, resulting in either suppression of protein translation (Kwon & Hecht, 1993), RNA transport (Kobayashi et al, 1998; Severt et al, 1999) or even cell cycle regulation and DNA repair (Castro et al, 2000; Erdemir et al, 2002).

Staufen, is a double strand RNA binding protein that has been identified as a MAP2 interacting factor (Monshausen et al, 2001). In Drosophila’s oocytes and neuroblasts, Staufen is essential for the localization of bicoid, prospero and oskar transcripts and the subsequent determination of body axis.
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(Bashirullah et al, 1998). Two Staufen homologs, Staufen1 and Staufen2 have been found in mammals, with the former expressed in most body tissues and the latter exhibiting a prominent neuronal expression. Both isoforms have been found in neuronal dendrites, associated with mRNA transporting granules and interacting with the microtubule network (Duncan et al, 1994; Monshausen et al, 2001). Even if the cis-element recognized by Staufen is still unknown, it seems to be able to bind any structured RNA in vitro, but its binding specificity in vivo seems to be quite remarkable (Mohr & Richter, 2003). Biochemical fractionation analyses led to the isolation of high density granules containing ribosomal aggregates (Krichevsky & Kosik, 2001) that were also positive for Staufen. A couple of years later, Mallardo and colleagues have purified Staufen form two set of different mRNPs: one was enriched in ribosomes and endoplasmic reticulum markers, whilst the other was devoid of these structures and was enriched in kinesin heavy chain and the non coding BC1 transcript, a known translational repressor (Mallardo et al, 2003). Therefore, it seems that Staufen is present in different RNA transporting granules with different translational competences. The driving force that promotes inclusion in these structures is still not understood, since it could be due to the identity of the mRNA bound by Staufen or its ability to interact with different sets of proteins that in turn characterize silent RNA transporting granules or translationally competent units.

CPEB1-4, or Cytoplasmic Polyadenylation Element Binding proteins 1, 2, 3, 4 constitute a family of 4 related proteins, involved in regulation of both mRNA transport and repression/activation of translation in many different cell types. All CPEBs, in both vertebrates and invertebrates, display a similar structure, mainly composed by two RNA Recognition Motifs (RRMs) and two zinc-finger domains in their carboxy-terminal ends (Huang et al, 2006). However, despite these similarities, a sequence comparison between the RNA-binding regions suggested that CPEB1 has different binding preferences and affinities than CPEB2-4 (Mendez & Richter, 2001). In fact, the canonical CPE is recognized by CPEB1 and not the other family members, that seem rather to bind to secondary U-rich structures (Huang et al, 2006). The CPE response element (UUUUAU), the cis-binding sequence for CPEB1, has been found in many dendritic transcripts (Huang et al, 2003; Wells et al, 2000; Wu et al, 1998) and in Xenopus’ oocytes transcripts as well (Groisman et al, 2000; Hake & Richter, 1994). In Xenopus, a single CPEB binds several mRNAs and contributes to keep them in a translationally dormant state and, together with other factors, becomes essential for the poly(A) tail elongation that is fundamental for translation initiation (Mendez & Richter, 2001). The induction of polyadenylation requires the site specific phosphorylation of CPEB, that in turn recruits the Cleavage and Polyadenylation Specificity Factor (CPSF) and PolyA polymerase (PAP) that finally mediates poly(A) tail elongation and activation of protein synthesis. Under resting conditions, Maskin interacts with
both CPEB and eukaryotic initiation factor 4E (eIF4E), inhibiting translation activation via blockade of eIF4E-eIF4G interaction. However, after CPEB phosphorylation and poly(A) elongation, Maskin dissociates from this complex allowing the small ribosomal subunit to bind to the mRNA and initiate protein synthesis (Mendez & Richter, 2001). A similar mechanisms operates in neurons, where CPEB1, the best known family member, regulates CaMKII translation induced by experience dependent synaptic activity, which triggers a net increase in CaMKIIα polyadenylation, promoting the local translation of this plasticity related transcript (Huang et al, 2002).

**PolyA binding protein (PAP)** is necessary for poly(A) tail stabilization and binds in multiple copies to extended tails increasing translation efficiency through interaction with the initiation factors bound to the 5' of mRNA (Preiss et al, 1998). Interestingly, PAP is able to bind also to sequences different than poly(A) tails, suggesting other function beyond poly(A) tail stabilization. In fact, PAP can exert translational repression on its own transcript by binding to the 5'UTR (Bag & Wu, 1996; de Melo Neto et al, 1995) and also by recognition of an inhibitory element on viral HIV p17Gag mRNA (Afonina et al, 1997).

**ELAV** proteins are the orthologues of Drosophila Embryonic Lethal Abnormal Vision, also known also as Hu paraneoplastic antigens in humans, and play fundamental roles in development, regulation of mRNA stability, splicing and translatability. This family of proteins consists in four members, HuR (ELAVL1), that is widely expressed in all tissues, and HuB (Hel-N1/ELAVL2), HuC (PLE21) and HuD that are all neuronal specific isoforms, with the exception of HuB that is present also in gonads (Dalmau et al, 1992). Each ELAV/Hu displays three classic RRMs, where RRMs 1 and 2 bind to AREs while RRM3 contributes to increase binding affinity and is probably involved in poly(A) tail recognition or protein-protein interactions. In addition to the RRMs, each ELAV/Hu protein presents a unique hinge region that might mediate the back and forth shuttling from the cytoplasm to the nucleus. ELAV/Hus share an overall 90% of sequence identity among the different family members (Okano & Darnell, 1997). These proteins extensively bind to AU an U rich sequences often present in 3'UTRs, preventing degradation and promoting mRNA stabilization (Chen et al, 2002; Chung et al, 1996; Levine et al, 1993; Ma et al, 1996; Myer et al, 1997), competing with many proteins for binding, like hnRNPD (or AUF1) (Barreau et al, 2005; Raineri et al, 2004; Zhang et al, 1993), CUG-triplet RNA binding protein-1 (CUG-BP1) (Carballo et al, 1998; Vlasova et al, 2008), tristetraprolin (TTP) (Carballo et al, 1998). Besides a role into the cytoplasm, these proteins are known to control splicing and polyadenylation of several transcripts (Barreau et al, 2005; Zhu et al, 2006). Moreover, they can regulate translation by direct binding to untranslated regions of target
mRNAs like neurofilament M, Insulin Like Growth Factor I (IGF-IR), p27 and GLUT1 (Antic et al, 1999; Jain et al, 1997; Kullmann et al, 2002; Meng et al, 2005; Millard et al, 2000) or even by relieving the miRNA mediated repression of CAT-1 expression (Bhattacharyya et al, 2006). The broad spectrum of action of Hu proteins, together with their evolutionary conservation among organisms, suggest their prominent role in development and differentiation on nerve cells (Antic & Keene, 1997) so that they are considered among the earliest markers of neuronal differentiation (Marusich et al, 1994; Wakamatsu & Weston, 1997).

**FMRP** (Fragile-X Mental Retardation Protein) is a translational repressor whose misregulation can lead to a severe disorder, the Fragile X syndrome in which FMRP is no more expressed by virtue of an abnormal CGG triplet expansion in its 5’UTR that can lead to transcriptional gene silencing and loss of FMRP protein. The phenotype ranges from mild to severe mental retardation, autistic-like behavior and anxiety (Jacquemont & Taniguchi, 2007). In neurons, FMRP is present in somata, along dendrites and also at the base of dendritic spines as well as in growth cones and axons (Antar et al, 2004; Antar et al, 2006; Feng et al, 1997; Ferrari et al, 2007; Price et al, 2006). FMRP has been frequently found in RNA transporting granules, containing several other partner RBPs as well as non-coding RNAs. As the majority of mRNPs, FMRP containing particles undergo to dynamic changes in their composition depending on the subcellular localization and activation state of the cell (Bagni & Greenough, 2005). In fact, FMRP can be detected either in large mRNPs cosedimenting with polysomes, or in small translationally silent units or even in stalled mRNP complexes (Ashley et al, 1993; Brown et al, 2001). FMRP containing granules travel along dendrites by interaction with the microtubule network and kinesins motor proteins (Antar et al, 2004; Davidovic et al, 2007; Kanai et al, 2004). Moreover, it has recently been demonstrated that FMRP-dependent mRNA transport is modulated by synaptic activity, at least for map1b, CaMKIIα and Sapap mRNA cargoes (Dictenberg et al, 2008). FMRP is able to bind to many neuronal transcripts (reviewed inBagni & Greenough, 2005) either through recognition of a purine G-rich quartet (Darnell et al, 2001; Schaeffer et al, 2001), or the “kissing complex” (Darnell et al., 2005) or even U rich stretches (Chen et al, 2003). In addition, FMRP can indirectly bind to target transcripts through interaction with BC1 non coding RNA or microRNAs (Jin et al, 2004; Zalfa et al, 2005; Zalfa et al, 2003). It is now becoming widely accepted among the scientific community the fact that during transport most mRNAs are kept translationally dormant in order to avoid ectopic protein expression: it is therefore easy to understand why many RBPs involved in transport, like CPEB and FMRP are translational repressors as well. In particular, FMRP is thought to repress translation at multiple steps (i.e. initiation end elongation), since it is able, similarly to CPEB, to interact with an eIF4E binding protein, namely CYFIP1 (Cytoplasmic FMRP
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Interacting Protein1) (Napoli et al, 2008). In resting conditions protein synthesis is repressed because of the inhibited interaction between eIF4E and eIF4G that is necessary to promote translation initiation. However, after synaptic activation, CYFIP1-FMRP complex dissociates from RNA and translation can eventually take place (Napoli et al, 2008). However, it has been recently demonstrated a positive role for FMRP in regulating Super Oxide Dismutase (SOD) mRNA translation via interaction with a novel RNA binding motif called SoSLIP (Sod1 mRNA Stem Loops Interacting with FMRP). This motif features an extremely complex secondary structure consisting in three stem loops that act as enhancers for FMRP dependent translation (Bechara et al, 2009). A recent study by Zalfa and colleagues reported a novel stabilizing function of FMRP with respect of PSD95 and MBP mRNAs (Zalfa et al, 2007; Zhang et al, 2007), that seems to counteract a parallel work of Zang and co-workers, where FMRP was described as a negative modulator of Nfx1 transcript, promoting its decay (Zhang et al, 2007).

From the complex scenery described above, it is evident that even if a cell expresses a limited number of cis-acting signals and trans-acting factors involved in RNA transport, the broad spectrum of signal combinations coupled to the many different functions exerted by a single RBP contribute to create a unique scenario for a finely regulated mRNA localization and expression.

2.3 RNA transporting granules: structure and functions

As already mentioned, RNA transport in the periphery of the cell is mediated by mobile structures called RNA granules, initially discovered in hippocampal neurons through uridine radioactive labeling (Davis et al, 1987). Later, Knowles and colleagues deeply investigated this phenomenon by fluorescently labeling endogenous RNA transport particles in cultured neurons, showing their anterograde and retrograde movements that was consistent with the interaction with the microtubule network (Knowles et al, 1996). Moreover, they were described to contain translational machinery components such as elongation factors or ribosomes. A similar study was also performed in living oligodendrocytes where, through microinjection of fluorescently labeled MBP mRNA, dynamic RNA granules were observed, with heterogeneous sizes and movement behavior (Ainger et al, 1993). These observations strongly suggested that RNA granules are intermediate structures mediating RNA sorting in peripheral subcellular domains, and represented the cornerstone for subsequent studies focused on dissecting their molecular composition. A growing body of evidence supports the idea that RNAs are transported in heterogeneous structures with different compositions and functions: transport particles, RNA granules, translating polysomes, stress granules, processing bodies (P bodies), microRNA/RNA silencing complex particles (Sossin &
In this paragraph we will only discuss the known differences between transport particles and RNA granules.

- **Transport particles**: these structures transport mRNAs but are devoid of ribosomes, and contain (i) mRNAs, (ii) proteins required for translational suppression, (iii) specific RBPs, (iv) adaptors that connect RBPs with molecular motors. One of the best characterized transport particle is the one mediating ASH1 mRNA localization in budding yeasts (Shepard et al, 2003). In neurons, the situation is less defined, since it has been only partially described the composition of RNA transporting particles. It is known that they may contain translational repressors like FMRP as well as many specific RNA binding proteins (Pur alpha, Staufen) and molecular motors (myosin Va, kinesins) (Mallardo et al, 2003; Ohashi et al, 2002). Conversely, many data are lacking about putative candidates for adaptors and anchoring molecules that would connect mRNPs to molecular motors.

- **RNA granules**: RNA granules resemble to transport particles in their composition, except that they do contain ribosomes. In those structures mRNA are still translationally silent despite the presence of ribosomes, and are presumably blocked at the stage of elongation (Anderson & Kedersha, 2006). RNA granules have been first described in oligodendrocytes (Ainger et al, 1993; Barbaresse et al, 1995), and later also in neurons, in the form of amorphous aggregates (Knowles & Kosik, 1997; Krichevsky & Kosik, 2001). The latter were found to contain ribosomal proteins and rRNAs and many elements of the translation machinery like arginyl-tRNA synthase, elongation and initiation factors (eEF1a, eEF2 and eIF2b) and many RNA binding proteins (Barbaresse et al, 1995; Krichevsky & Kosik, 2001). Krichevsky and co-workers have furthermore demonstrated the absence of eIF4E and eIF4G, two fundamental elements to initiate protein synthesis, therefore corroborating the idea that RNA granules are translationally silent units. However, after KCl induced depolarization, RNA granules appear to open to release RNAs and translation factors for their subsequent association with polysomes engaged in protein translation (Krichevsky & Kosik, 2001). Two extensive studies independently performed by Kanai ad Elvira have characterized the protein content of RNA granules based in the association with KIF5 kinesin and on subcellular fractionation from embryonic brain, respectively (Elvira et al, 2006; Kanai et al, 2004). Kanai and colleagues isolated large detergent-insensitive RNA complexes tightly associated with microtubules via KIF5 anchoring, enriched in Arc and CaMKIIα mRNAs, together with at least 42 different RNA binding proteins. Among these, particular attention has been paid to the most abundant ones, as Purα and Purβ, both involved in RNA transport (Kelm et al, 1997), Dead Box Helicases 1 and 3 (DDX1, DDX3) (Godbout & Squire, 1993; Sowden et al, 1995), NonO (Yang et al,
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1993), PSF (polypyrimidine tract binding protein-associated splicing factor) (Chanás-Sacre et al., 1999), TLS (Yang et al., 1998), SYNCRIP (Mizutani et al., 2000), ALY (Bruhn et al., 1997) and hnRNPU

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Table 1. Protein composition of the different RNA particles discussed (from Sossin and DesGroseillers, 2006) ND, not determined

(Kiledjian & Dreyfuss, 1992). Moreover, those granules contained FMRPs, Staufen, eEF1a and two functionally unknown proteins, HSPC117 (Zhang et al, 2000) and CGI-99 (Lai et al, 2000). Systematic silencing induced by siRNAs allowed to identify key components necessary for CamKII\alpha localization, i.e. Pur\alpha, hnRNPU, PSF and Satuten1 (Kanai et al, 2004). On the other hand, the studies of Elvira et al. (2006) and Kanai and colleagues (2004) described only a partially overlapping set of proteins, that could be either due to the techniques adopted in the two studies or the fact that Elvira started from embryonic brain tissue while Kanai used mature cortex. The proteomic analysis made by Elvira led to the identification of many ribosomal (61 out of 64) and microtubule associated proteins, a large set of RNA binding proteins with a prominent enrichment in \beta-actin but not CamKII\alpha mRNA. Notably, many of the identified proteins were previously described in RNA transporting granules, like hnRNPs (hnRNPA1, SYNCRIP, hnRNPD, hnRNPA2/B1) (Kanai et al, 2004; Shan et al, 2003), DDXs (Kanai et al, 2004) and other RNA binding proteins like Staufen, nucleolin and RNG105 (Kanai et al, 2004; Shiina et al, 2005). This study was groundbreaker also for the identification of novel RNA granule associated peptides, like hnRNPC, elf4A, ZBP2, CYFip2, ELAV-like proteins, PABP and many others (Elvira et al, 2006). It is noteworthy that two well-known granule components, like FMRP and Pur\alpha, have not been purified in this second study, probably, as mentioned above, because of technical reasons. However, both studies showed the presence of a
novel protein, CGI-99, whose function is still poorly understood but has been proposed as a candidate for being one of the adaptors necessary for the formation of RNA transporting units (Sossin & DesGroseillers, 2006).

Several groups have studied the dynamics of transport of RNA containing granules in cultured neurons, especially through live imaging techniques (Dynes & Steward, 2007; Huang et al, 2003; Knowles et al, 1996; Tiruchinapalli et al, 2003; Zhang et al, 2001). It has emerged that granules are in most cases stationary, while a minor fraction displaying oscillatory movements, as well as anterograde and retrograde movements with very different velocities, with a mean of 4-6 µm/min (Knowles & Kosik, 1997; Knowles et al, 1996; Muslimov et al, 1997). This heterogeneity could be due to the set of proteins bound to the transcripts, as well as to the nature of molecular motor driving the subcellular translocation. In a study performed by Rook and coworkers, neuronal activity increased the transport of CaMKIIα mRNA, probably due by a shift from the oscillatory population of granules to the anterogradely moving ones (Rook et al, 2000). Moreover, also the translocation of ZBP1 containing particles is influenced by neuronal depolarization and NMDA receptor activation (Tiruchinapalli et al, 2003). As a general rule, microtubule and microfilaments represent a “railway” onto which RNA transporting particles are anchored and allow back and forth long range movements along neurites (Bassell et al, 1994), due to the mixed polarity of microtubules in proximal dendrites (Baas, 1998). In distal dendritic compartment, at the opposite, the cytoskeleton is mainly composed by actin filaments and unipolar microtubules with their plus end directed away from the soma (Burton & Paige, 1981). This peculiar organization may serve to organize and regulate the dynamic short range transport processes that occur at synapses.

Molecular motors employ the energy derived by ATP hydrolysis to drive macromolecular cargoes to and from the periphery of the cell. They can be divided into non conventional myosins, if binding to actin filaments, or in kinesins/dyneins if binding to microtubules. In general, kinesins promote plus end directed movements whilst minus ended transport is governed by dynein motors (Hirokawa & Takemura, 2005). All kinesins have in common a globular motor domain, a microtubule interacting domain and an ATP-binding pocket. Apart these conserved regions, the aminoacidic sequence between members is quite variable, thus explaining the ability of transporting different cargoes, also mediated by interaction with different adaptor proteins (Hirokawa & Takemura, 2005), fig.1).
Fig. 1. Kinesin superfamily and cargoes for dendritic and axonal transport (reproduced from Hirokawa and Takemura, 2005). A) overview on microtubule network and transport machinery organization. Axonal (a) and dendritic (b) specific KIFs and relative cargoes.

In dendrites, Kinesin 17 (KIF17) mediates the anterograde transport of NR2B subunit of AMPA receptors. Interestingly, it has been demonstrated that these two genes are contextually co-regulated, and overexpression of KIF17 in mouse models increases episodic-like memory and spatial learning (Guillaud et al, 2003; Wong et al, 2002). On the other side, it seems that AMPA receptors steer KIF5A,B and C in dendrites through interaction with the adaptor protein GRIP (Setou et al, 2002). The proteomic study performed by Kanai has also demonstrated that KIF5s are able to transport in dendrites huge ribonucleoparticles containing CamKIIα and Arc mRNA that actually represent RNA transporting granules (Gu et al, 2003; Kanai et al, 2004). We have also to remember that molecular motors play important roles not only in dendrites, but in axons as well, where many membranous organelles are sorted from the cell soma to the axonal periphery. These cargoes include ion channels (Garrido et al, 2001; Gu et al, 2003), adhesion molecules (Vogt et al, 1996), synaptic vesicles and even mitochondria (Kanai et al, 2004; Nangaku et al, 1994).
3 mRNA TRANSLATION IN DENDRITES

3.1 Dendritic localization of protein translation machinery

The first ideas about a possible local protein synthesis in neuronal dendrites came from studies performed by Bodian and colleagues describing the presence of ribosomes in distal dendrites (Bodian, 1965). Those findings were later confirmed by electron microscopy studies on central nervous system neurons, supporting the existence of ribosomal aggregates under the base of dendritic spines (also known as SPRC, synapse-associated polyribosome complexes) (Palacios-Pru et al, 1981; Spacek, 1985; Steward & Levy, 1982). Polyribosomes have been found in more than 75% of visual cortical spines and at the base of spines of CA1 and dentate gyrus neurons, but less frequently in spine heads (Spacek, 1985; Steward & Levy, 1982). Further studies have also shown that most of the ribosomes beneath the base of spines are associated with membranous cisterns in a RER-like distribution, whilst the ones inside the spine head are barely associated with such structures (Steward & Reeves, 1988). This peculiar distribution is also dramatically pronounced during denervation-induced synaptogenesis (Steward, 1982; Steward & Fass, 1983) and could then represent a molecular marker for newborn synapses (Steward & Falk, 1986). On the other side, when considering other synapses not associated to spines, either excitatory or inhibitory, ribosomes are often located beneath the post-synaptic membrane (Steward et al, 1996). From this scenario, independently from the specific neuron considered, it clearly emerges that ribosomes are an ubiquitous element of the post-synaptic cytoplasm. Further studies have characterized the synaptic micro-environment, finding also the presence of several other elements belonging to the protein-translation machinery including, transfer RNAs (tRNAs), aminoacyl-tRNA-synthases, elongation and initiation factors (in particular, eEF2 and eIF2β), together with elements of the cotranslational protein sorting machinery like TRAP (a component of the translocation complex in RER membranes), the Signal Recognition Particle (SRP) (Tiedge & Brosius, 1996) and several other markers of rough endoplasmic reticulum like ribophorin I (Torre & Steward, 1996). In the latter work, Torre and Steward have elegantly demonstrated the existence of RER and Golgi Apparatus (GA) associated glycosil-transferase activities in severed dendrites, together with the presence of several RER and GA markers in proximal and distal neurites (Torre & Steward, 1996). Moreover, it has been shown that some membranous formations in proximal dendrites are enriched in α-mannosidase II and β-galactosidase, two Golgi resident enzymes (Eckstein & Shur, 1989; Lopez et al, 1989; Moremen et al, 1991).
A regional specialization of the endoplasmic reticulum is the spine apparatus (SA), a complex organelle composed of stacks of smooth endoplasmic reticulum (SER) tightly packed with densely stained material, almost localized into the spine neck. Even if its function is not fully understood, the spine apparatus seems to be involved in synaptic plasticity through the modulation of local calcium homeostasis and post-translational regulation of protein synthesis (Jedlicka et al., 2008). In fact, synaptopodin deficient mice lacking of a functional SA show deficits in spatial learning (Jedlicka et al., 2008). Interestingly, the spine apparatus has been recently hypothesized to be involved in receptor synthesis and/or trafficking and, more generally, to be part of the secretory pathway for membrane bound proteins (Deller et al., 2007; Steward & Schuman, 2001). Since both AMPA and NMDA receptors have been localized in the SA (Nusser et al., 1998; Racca et al., 2000) and since the latter is also a local reservoir/source of calcium, it is tempting to speculate its fundamental role in synaptic plasticity and therefore memory formation.

An important progress in understanding the mechanisms of regulation of local protein synthesis has been made thanks to a molecular dissection of the putative transduction pathways associated to synaptic plasticity events, in particular the (i) MAPK-Mnk-eIF4E, (ii) p70S6K-S6 ribosomal protein, (iii) PI3-kinase-PDK-Akt - mTOR-4EBP and (iv) eEF2 kinase /CaMKIII-eEF2 pathways (Asaki et al., 2003). All the proteins involved in these cascades have been detected in proximal and distal dendritic compartments, thus indicating that the very same pathways controlling somatic protein translation are also present in the periphery of the cell, that can be therefore fully considered as an independent cellular domain capable of autonomous protein synthesis.

Researchers have been looking for a proof of local protein synthesis in dendrites since late ’70s, when radiolabelled leucine spots were identified in distal hippocampal dendrites after ventricular injection (Kiss, 1977) or with the documented increased rate of protein precursors inclusion during reinervation processes (Fass & Steward, 1983). Later on, (Torre & Steward, 1992) demonstrated the incorporation of $^3$H-leucine in dendrites that were severed from the cell body, in order to rule out any contaminating protein transport from the cell soma. Similarly, transfection of an in vitro transcribed RNA led to the expression of the reporter gene even in dissociated MAP2 positive neurites (Crino & Eberwine, 1996), consistent with the elegant experiments carried by Aakalu and coworkers, showing that in the same situation a dendritically targeted GFP reporter could be expressed after treatment with growth factors (Aakalu et al., 2001). On the other side, several reports certificate that in vitro isolated synaptoneurosomes are able to synthesize proteins as well (Weiler & Greenough, 1991; Weiler & Greenough, 1993), as a proof that synaptic subcellular
compartments do contain all the elements required to autonomously produce functional proteins on demand. This feature is fundamental for the induction and maintenance of long term forms of synaptic plasticity that differ from the short term ones relying only on post-translation modification of pre-existing proteins (Richter & Lorenz, 2002).

3.2 Translational control mechanisms

The contemporary presence of many components of the translation apparatus and post-translation modification machinery suggest that dendrites are actually equipped with key factors necessary to synthesize proteins independently from the cell soma, hence conferring long term translation regulatory competences to each single dendritic microdomain. The consolidation and maintenance of long term memories, but not short term ones, require synthesis of new macromolecules, particularly proteins (Bliss & Collingridge, 1993; Malenka & Nicoll, 1999). At the cellular level, synaptic plasticity is defined as long term modifications in the strength of synaptic transmission in response to different extracellular and environmental stimuli (Gladding et al, 2009). Long Term Potentiation (LTP) and Long Term Depression (LTD) in vertebrates and Short and Long Term Facilitation (STF, LTF) in invertebrates are widely accepted models to measure the strength of synaptic changes that underlie synaptic plasticity (Malenka & Bear, 2004). In particular, LTP, that has been used as a paradigm of learning, shares some cellular and molecular mechanisms characteristic of memory formation (Lynch, 2004; Neves et al, 2008). For example, LTP can be temporarily subdivided into three distinct phases: Early LTP (E-LTP or LTP1) lasting 1-3 hours, that relies on post-translational modification of pre-existing synaptic proteins and is protein synthesis independent, Intermediate LTP (I-LTP or LTP2) that is dependent on new protein but not mRNA production and lasts 1-3 hours. Finally, Late-LTP (L-LTP or LTP3) is the persistent form of LTP (from hours to weeks) and is dependent on both mRNA transcription and protein synthesis (Adams & Dudek, 2005; Raymond, 2007). Likewise, facilitation can be induced in Aplysia sensory neurons by serotonin application, evoking either post-translational modification of pre-existing proteins (STF) or new protein synthesis (LTF) (Kandel, 2001). From this scenario it is intuitive to understand how important is the local control of protein translation, that can deeply influence the strength of synaptic transmission through a regulated temporal and spatial modulation of protein expression. In eukaryotes translation takes place in three different steps: initiation, elongation and termination. Initiation is without any doubt the rate limiting step of the entire process involving at least 12 different initiation factors, and is therefore one of the major targets for translational control. Translation initiation requires (i) formation of the 43S ternary complex, (ii) recognition of the target mRNA to the 43S complex and (iii) formation of the 80S ribosomal complex (fig.2). The first step in
turn needs the arrangement of the so-called ternary complex (eIF2-GTP-tRNA\textsubscript{Met}) that further binds to the 40S ribosomal subunit to form the 43S preinitiation complex (i). The assembly of the ribosome on the mRNA (ii) requires either the recognition of the 5' cap structure (m\textsuperscript{7}GpppX) present on all nuclear transcribed mRNAs or of an internal ribosomal entry site (IRES), a structured element in the 5'UTR that \textit{per se} is able to recruit ribosomes (Sarnow et al, 2005).

Fig.2. Assembly of the translational initiation complex in eukaryotes (reproduced from (Klann & Dever, 2004). For detailed explanation see text.

A key factor involved in cap-dependent translation is eIF4F, composed by eIF4A (an ATP-dependent RNA helicase that unwinds secondary structures), eIF4E (the cap binding protein) and eIF4G (a large scaffold protein that bridges the mRNA to the 43S ribosomal subunit) (Gingras et al, 1999). Once bound to the mRNA, the 43S subunit starts scanning the 5'UTR searching for the AUG initiation codon in a 5'-3' direction, whose selection is affected by several factors (eIF1, eIF2 and eIF3) that prevent joining to the 60 subunit. eIF5, a GTPase activating protein (GAP) promotes eIF2 release through stimulation of eIF2-bound GTP hydrolysis, thus allowing the 60S subunit to join to the small 40S complex (Pestova et al, 2008). At this point, the elongation factors are recruited for the peptide chain elongation. eEF1A is a GTPase needed for the tRNA to entry in the ribosome, while eEF1B is a guanine nucleotide exchange factor (GEF) for eIF1A. eEF2 then promotes the translocation of the ribosome on the mRNA once the peptide bond has been formed. Finally, upon recognition of
the stop codon, termination factors catalyze the release of the polypeptide form the ribosome, allowing a new round of translation to take place.

In general, global translational control mechanisms in most cases involve 5’UTRs whilst mRNA specific regulation requires the recognition of specific cis acting sequences located in 3’UTRs (with some exceptions), as previously discussed. In the next sections we will give a panoramic view of the possible mechanism of translation regulation acting at 3’UTR ends, with particular attention to synaptic implications.

3.2.1 Translational control mechanisms acting at 3’UTR

Many of the translational control mechanism acting at 3’translated regions involve the recognition of cis-acting sequences by specific trans-acting factors that can either promote or repress translation. Notably, many of these regulators are actually RNA binding proteins that, as discussed above, can exert a dual role in transporting and regulating mRNA expression through interaction with 5’UTR binding factors.

- RNA binding protein mediated translational control

A well known mechanism of translational regulation is mediated by CPEB, a sequence specific RNA binding protein that governs both mRNA sorting in dendrites as well as translational repression of dormant mRNAs and activity dependent polyadenylation, that in turn promotes translational activation (Huang et al, 2003; Wu et al, 1998). Translational repression is exerted by the interaction of CPEB with Maskin (or its orthologue neuroguidin) that build a complex with two other additional proteins, the poly(A) polymerase Gld2 and the poly(A) ribonuclease PARN that are hold together with the scaffolding protein symplekin (Barnard et al, 2004). Maskin contains an eIF4E binding domain similar to the eIF4G binding pocket, and therefore acts as a mRNA specific 4EBP, thus inhibiting the assembly of the eIF4F complex and translational initiation (Stebbins-Boaz et al, 1999). Moreover, since in resting conditions the deadenylase is more active than GLd2, it contributes to translational repression by maintenance of a short poly(A) tail (Kim & Richter, 2006). Synaptic activation promotes Aurora A kinase- and CaMKIIα-dependent phosphorylation of CPEB, that in turn promotes the dissociation of maskin and PARN, recruits CPSF and PAP/Gld2 for poly(A) tail elongation and translational activation (Atkins et al, 2004; Huang et al, 2002; Mendez & Richter, 2001). Another example of message specific 4EBP is Cup in Drosophila embryo. Here, Smaug binds to the 3’UTR of unlocalized Nanos mRNA and through interaction with Cup, that sequesters eIF4E, inhibits eIF4F complex formation (Nelson et al, 2004). A very similar mechanism involving Cup represses also oskar
mRNA translation in the anterior pole of Drosophila oocytes, by interaction with the RBP Bruno (Wilhelm et al, 2003).

In neuronal cells, an important translational repressor is FMRP that is thought to regulate the expression of several target mRNAs (Laggerbauer et al, 2001; Li et al, 2001). The exact mechanism through which FMRP regulates protein synthesis is not fully understood, however several different models have been proposed. One posits that FMRP modulates their targets via interaction with the non-coding RNA BC1 (Zalfa et al, 2003). This interaction may either increase FMRP affinity for its target or enhance BC1 dependent eIF4A inhibition, repressing the expression of mRNAs carrying highly structured 5'UTRs (Lin et al, 2008; Wang et al, 2002). Furthermore, it has been recently proposed that FMRP dependent repression relies on the interaction with a novel neuronal 4EBP, CYFIP1/Sra-1 (Cytoplasmic FMRP Interacting Protein 1) (Napoli et al, 2008). Similarly to CPEB/maskin, FMRP/CYFIP interaction blocks translation initiation by competition with eIF4G for eIF4E binding, even if through a non canonical 4E binding domain. CYFIP repression can be relieved after activation of signaling cascades mediated by BDNF or DHPG (Dihydroxyphenylglycine), a phenomenon related to initiation of protein synthesis (Napoli et al, 2008). On the other side, an additional model has been proposed, in which FMRP interferes at post-initiation steps, since it has been found to be associated with functional polyribosomes (Stefani et al, 2004). In particular, phosphorylated FMRP is found in stalled ribosomal fraction, whilst the nonphosphorylated version is tightly associated with actively translating polyribosomes (Ceman et al, 2003). FMRP can be modified by S6K1 on a conserved serine residue that is fundamental for mRNA binding (Narayanan et al, 2008). In both FMRP and S6K1 knock-out mice the levels of SAPAP3, a FMRP target, are increased, suggesting that S6K1 mediated phosphorylation of FMRP is necessary for its inhibitory activity. Many other RBPs seem to be involved in translation repression of transported mRNAs. Among these, the repressor RNG105 helps maintaining transcripts translationally silent during mRNA sorting (Shiina et al, 2005). In addition, hnRNPK and E1 have been described to block protein translation at post-initiation steps. In detail, they bind the differentiation control element (DICE) in the 15-lipoxygenase (LOX) mRNA 3'UTR. This cis acting sequence consist in several tandem repeats of the CU dinucleotide, that, when bound to hnRNPK and E1, prevents the assembly of the 80S ribosomal complex, possibly through interference with some still unidentified initiation factors (Ostareck et al, 2001). Moreover, hnRNPE1 mediates translational silencing also interacting with the hnRNPA2 partner. As already discussed, A2RE containing RNAs are transported in oligodendrocytes and in neuronal dendrites. In order to prevent ectopic protein expression, mRNAs are kept translationally silent during transport. It has recently been discovered that hnRNPA2 can also cooperate with its partner hnRNPE1, inhibiting synthesis of
A2RE RNAs (Kosturko et al, 2006). An intriguing translational control mechanism has been recently reported to involve the large ribosomal subunit protein L13a. Ceruloplasmin synthesis was known to be inhibited after interferon γ treatment through binding of a stem loop structure in its 3’UTR (Mazumder & Fox, 1999). Later, L13a has been identified as the trans-acting factor mediating translational repression (Mazumder et al, 2003). Actually, L13a phosphorylation leads to its dissociation from the ribosome, without affecting global translation rates, but strongly inhibiting the expression of ceruloplasmin mRNA. Even if the exact molecular mechanism is not understood, it has been proposed that circularization of the mRNA through eIF4G-PABP is necessary to bring L13a binding site on 3’UTR in close proximity to the 5’UTR, therefore mediating translational repression (Mazumder et al, 2003).

- **Repression by deadenylation**: another efficient mechanism to repress mRNA translation is the destabilization and removal of the poly(A) tail. As discussed earlier, CPEB is a key element regulating protein synthesis through activity dependent modulation of polyadenylation. PABP, similarly to eIF4E, binds to eIF4G, and stimulates translation initiation. A current model posits that Pumilio, another repressor, binds to specific cis acting sequences in 3’UTRs and forms protein complexes that in turn promote deadenylation and translational silencing (de Moor et al, 2005).

- **Non sense mediated decay (NMD)**: an elegant way to tightly control local protein abundance, especially at synaptic sites is the regulation of mRNA turnover. A well known pathway of mRNA stability control is the non sense mediated decay (NMD). Thanks to this quality control mechanism, aberrant transcripts bearing premature stop codons, and therefore potentially dangerous for the production of truncated protein, are rapidly destroyed (Isken & Maquat, 2007). However, NMD seems to be also used for the modulation of protein expression in a context of wild type mRNAs (Giorgi et al, 2007; Wittmann et al, 2006). NMD is triggered by the recognition of premature stop codons upstream of a splice site, where the exon-junction complex (EJC) is deposited. This complex is composed by a tetrameric protein core (eIF4AIII, Y14, MAGOH and MLN51) and is normally displaced after the first “pioneer” round of protein translation. When a premature stop codon is present, the key NMD factor, UPF1, rapidly leads to the degradation of the transcript (Maquat, 2004). However, any transcript bearing introns in its 3’UTR becomes a natural target for NMD, because the natural stop codon is then recognized as an aberrant stop. Recent work of Giorgi and colleagues has demonstrated that Arc is a natural target for NMD, because of the presence of two introns in its 3’UTR (Giorgi et al, 2007). In cortical dendrites, Arc colocalizes with eIF4AIII, FMRP and Staufen, suggesting that most of the dendritically localized Arc mRNA has not yet undergone the first round of translation. It has been estimated that the mean Arc mRNA half life in cultured neurons is 47 minutes (Hutvagner & Zamore, 2002; Rao et al, 2006; Zeng et al, 2003).
translation dependent degradation of Arc is an extreme but efficient way for regulating local protein abundance, allowing a fine spatio-temporal regulation of mRNA translation. This evidence perfectly fits with the requirement of a sustained Arc mRNA synthesis during LTP consolidation.

### 3.2.2 Translational control by microRNAs

MicroRNAs (miRNAs) are a family of small, 21-22 nucleotide long RNAs that regulate protein expression by binding to 3’UTR of at least 50% of the expressed genes (Bushati & Cohen, 2007). Since the same miRNA can bind to multiple transcripts and because each mRNA can be targeted by several miRNAs, the scenario of possible regulatory combination is extremely huge.

Mammalian miRNAs are encoded by both mono- and polycistronic gene clusters, often found in intronic regions. They are transcribed in the nucleus by RNA polymerase II in the form of long pri-miRNAs that are further processed by the nuclear RNase Drosha, to generate a 70nt long stem-loop pre-miRNA. Pre-miRNAs are subsequently exported into the cytoplasm where are further cleaved by DICER into 21nt long ds miRNAs. Following cleavage, the guide strand is incorporated in the RNA induced silencing complex (RISC) that guides target recognition and therefore gene silencing. The imperfect base pairing between miRNAs and target transcripts represses translation and does not induce target degradation, that is instead triggered by the prefect seed matching of siRNAs (Hutvagner & Zamore, 2002; Zeng et al, 2003). The molecular mechanism by which miRNAs promote translational silencing are multiple: (i) inhibition of translation initiation, (ii) inhibition of translation elongation, (iii) ribosome drop-off, (iv) cotranslational protein degradation and in some cases (v) mRNA degradation (Filipowicz et al, 2008; Jackson & Standart, 2007). miRNAs are widely expressed in many brain regions (Kosik, 2006) and have demonstrated to be critical for normal brain function and development (Davis et al, 2008; Kim et al, 2007; Schaefer et al, 2007). In mammalian hippocampal neurons, it has been recently demonstrated the leading role of miRNA 134 in regulating dendritogenesis and spine development (Schratt et al, 2006). This brain enriched miRNA negatively regulates the expression of Limk1, a protein kinase involved in spine growth and maintenance. BDNF application relieves miRNA 134 mediated repression, promoting Limk1 expression and spine morphogenesis (Schratt et al, 2006). Interestingly, it has been shown that also miRNA132 is a direct target of BDNF regulation through CREB mediated transcription. The authors have identified p250GAP as major target transcript for this miRNA, whose BDNF-dependent expression downregulates p250GAP protein levels, to promote neurite outgrowth (Vo et al, 2005).
miRNA mediated regulation of neuronal functions has been widely conserved during evolution, since also in *Drosophila*'s neuronal cells a local increase in CaMKIIα expression is triggered by a parallel decrease in miRNA mediated repression (Ashraf & Kunes, 2006). This phenomenon seems to be related to a concomitant proteolysis of a RISC complex component, that in turn downregulates the miRNA mediated inhibition on CaMKIIα expression. As discussed above, miRNAs can be stored and transported in dendrites in special transport particles called miRNPs. However, it is not completely clear whether or not miRNAs are already associated to their targets during transport or subsequently bind to the mRNPs once they have reached their final subcellular destination.
4 ROLE OF DENDRITIC mRNAs

4.1 What is the biological significance of mRNA localization and regulated local translation?

As already discussed, learning and memory formation require long lasting synaptic changes that in turn rely on a fine modulation of the protein content of each synapse, depending on the type and number of stimulations experienced. The first theories elaborated to justify how synapse plasticity is generated during learning posed the ability of synapses to send retrograde messages to cell body, so that the somatically synthesized proteins would be transported specifically to the peripheral compartments. According to this view, the “synaptic tagging” theory has been elaborated, assuming that activated synapses are marked with molecular tags that in turn attract new proteins from the soma (Frey & Morris, 1997; Frey & Morris, 1998). As previously mentioned, a process based exclusively on protein transport would not meet the rapid changes needed for a fast neuronal response. Indeed, without excluding the ability of synapses to send retrograde signals as well as the tag theory, it could rather be that the latter also serves as an address for mRNAs to be synaptically targeted. In view of that, the experiments carried out by Schuman and coworkers have strongly supported this idea, demonstrating that lesioned hippocampal slices, where dendrites had been severed from their cell bodies, are still able to express a form of LTP that is induced by BDNF. Moreover, the authors have demonstrated that this event is dependent on local protein synthesis since it is completely abolished after pharmacological treatment with translation inhibitors, suggesting that BDNF acts locally inducing synaptic potentiation through activation of local protein synthesis processes (Kang & Schuman, 1996). On the same line, Huber and colleagues have shown that activation of mGlu receptors induced LTD in severed CA1 stratum radiatum, and that LTD is blocked by protein translation antagonists, confirming the importance of newly synthesized proteins for the establishment of neuronal plasticity (Huber et al, 2000).

Notably, local protein synthesis is essential not only for the induction and maintenance of LTP and LTD, but also for the formation of new spines, that in turn could be a downstream effect of long term neuronal plasticity (Engert & Bonhoeffer, 1999; Trommald et al, 1996). In fact, observations collected by (Steward & Falk, 1986) have reported a significant accumulation of polyribosomes under the base of developing synapses. Accordingly, also after denervation it has been observed a local ribosomal enrichment at the level of newborn synapses (Steward & Fass, 1983).
A putative protein involved in shaping and formation of new spines in FMRP, since in animal models lacking of this protein many developmental abnormalities have been reported, including long, thin and immature spines (Weiler & Greenough, 1999). Another essential actor involved in synaptogenesis and structural changes related to plasticity phenomena is Arc, whose induction is spatially and temporally regulated. In the next section we will deeply discuss about the structural and functional implications of Arc expression with respect to LTP and spine stability. Finally, to support the role of local protein synthesis during LTD, we will provide an example of the consequences of the misregulated expression of FMRP that results in exaggerated hippocampal depression.

4.2 Arc/Arg3.1, actin and LTP consolidation

Among the immediately early genes induced by LTP, a pivotal role is played Arc. Indeed, animals lacking of this important gene show strong deficits in long term potentiation maintenance (Plath et al, 2006). The leading role of Arc during synaptic plasticity can be deduced also by the fact that Arc is rapidly induced in many regions of the brain after stimulation. Newly expressed Arc mRNA is rapidly targeted to dendrites and accumulates nearby to activated synapses (Steward et al, 1998). Moreover, high frequency stimulation induces an enrichment of Arc protein at synapses of dentate granule cells and its localization to the post synaptic density of excitatory synapses (Bramham et al, 2008). In a recent study by (Messaoudi et al, 2007), a time course Arc ODNs injection has demonstrated a specific time window in which Arc expression is required for proper LTP maintenance, in between 2-4 hours after synaptic stimulation. After 2 hours of LTP induction, ODNs injection rapidly reduced Arc mRNA an protein levels, promoted cofilin dephosphorylation and loss of F-actin (Messaoudi et al, 2007). The same treatment at 15 minutes post stimulation did only induce a rapid but reversible inhibition of LTP, accompanied by the same blockade and recovery of Arc expression. This transient effect is consistent with the key feature of early LTP, i.e. protein synthesis mRNA transcription independence. In vivo, an Arc knock out animal model showed severe deficits in LTP consolidation in the dentate gyrus as well as in CA1 region of acute hippocampal slices in vitro (Plath et al, 2006). Taken together, these results support a central role for Arc in LTP: its early expression contributes to LTP induction, while a sustained Arc protein synthesis is necessary for proper LTP consolidation and memory storage.

BDNF-induced LTP in dentate gyrus has been associated with strong upregulation of Arc mRNA and has been shown to be dependent on new mRNA transcription (Messaoudi et al, 2002). Indeed, it has been shown that Arc ODN injection prevents BDNF dependent LTP induction together with cofilin
inhibition of phosphorilation, that, as earlier discussed, promotes F-actin depolimerization and spine shrinkage (Rex et al, 2007). As depicted in figure 3, BDNF signaling is essential to support protein translation. In fact, BDNF can promote mRNA release from transporting granules as well as activation of crucial signaling pathways involved in protein translation, as the PI3K/Akt/mTOR pathway. In this way, BDNF can either promote phosphorylation of inhibitory 4EBPs, or activation of p70S6 kinases, phosphorylation of eIF4E and eEF2K, with a general promotion of mRNA translation. During LTP consolidation, a sustained Arc synthesis is indeed necessary for local actin polymerization and cofilin phosphorylation that result in the well known spine enlargement associated with late phase LTP. Moreover, a prominent role of BDNF in supporting spine remodeling is given by its ability to relief the miRNA134 repression of LIM kinase-1, the major cofilin kinase, that results in an increased phosphorylation of cofilin and subsequent F-actin stabilization (Meng et al, 2002; Schratt et al, 2006). Intriguingly, BDNF might be related also to LTD processes and more generally to homeostatic regulation of synaptic activity, since it promotes Arc expression that has recently been described to be involved in mGluRI dependent LTD AMPAR endocytosis (Waung et al, 2008).

Interestingly, Arc is not only essential during LTP related spine remodeling, but also during mGluR dependent LTD. In fact, Guzowski and coworkers have shown that inhibition of Arc protein expression through antisense oligodeoxynucleotide (ODN) injection before LTD induction impairs the maintenance of long term potentiation and the consolidation of long term memory (Guzowski et al,
Moreover, it has recently been demonstrated that Arc promotes LTD through increased AMPA receptor exocytosis via interaction with dynamin and endophilin 2/3 (Chowdhury et al, 2006; Waung et al, 2008) and its expression is under the control of both eEF2 and FMRP proteins (Park et al, 2008). In particular, there is compelling evidence that Arc translational control relies on eEF2, since in eEF2 kinase knock out slices both Arc expression and mGluR LTD are inhibited. Wild type conditions can be however restored with application of low concentrations of cycloheximide, known to induce eEF2 phosphorylation. Moreover, mGluR activation leads to fast FMRP dephosphorylation, relieving its inhibitory effect on Arc mRNA translation (Antar et al, 2004; Narayanan et al, 2007). Lack of FMRP occurring in Fragile-X syndrome and related animal models, causes a constitutively deregulated Arc overexpression that may, at least in part, account for the exaggerated protein synthesis independent LTD (Park et al, 2008).

**4.3 mGluR LTD, FMRP and regulation of protein translation**

It is widely accepted that BDNF is a key modulator of LTP consolidation and memory formation (Bramham & Messaoudi, 2005). As will be further discussed in greater detail, BDNF is postsynaptically released from or near glutamatergic synapses and locally activates TrkB receptors on both pre and postsynaptic membranes.

By definition, LTD is a long lasting decrease in the efficacy of synaptic transmission (Gladding et al, 2009). This phenomenon was first described as *heterosynaptic depression*, in which depotentiation was observed in a non stimulated pathway after induction of LTP in a separate hippocampal CA1 pathway (Lynch et al, 1977). The same phenomenon was also observed by (Steward & Levy, 1982) in the dentate gyrus, indicating that heterosynaptic LTD may occur in different brain areas. Like LTP, also LTD can be locally evoked in a protein synthesis dependent manner, since it could be still elicited in CA1 dendrites that have been severed from their cell bodies (Huber et al, 2000).

Among the proteins induced by mGluRI activation, a leading role is played by FMRP. It is worth noting that the same kind of stimulation promotes the translation of other dendritically FMRP-interacting mRNAs, like PSD-95, amyloid precursor protein (APP), MAP1B, elongation factor 1a (eEF1α) and Arc (Davidkova & Carroll, 2007; Huang et al, 2005; Park et al, 2008; Todd et al, 2003; Waung et al, 2008; Westmark & Malter, 2007). As a consequence, any alteration of FMRP function would be reflected in synaptic anomalies, as seen for enhanced mGluR dependent LTD and spine structure abnormalities in *Fmr1*KO mice. Additionally, in these mice the M1 muscarinic acetylcholine
(mAch) receptor and mGluR-dependent forms of LTD are enhanced and persist in an independent manner form new protein synthesis (Hou et al, 2006; Nosyreva & Huber, 2006). Based on these results, it has been hypothesized that FMRP normally inhibits the expression of the LTD related proteins, and this inhibition is relieved after mGluR activation that in turn leads to activation of protein synthesis. Therefore, genetic deletion of FMRP leads to a chronic, constitutively active expression of such LTD proteins, resulting in an exaggerated depression which is independent form new protein synthesis. At a molecular level, mGluR activation promotes a decrease in the number of surface AMPA receptors, in particular of GIR1 and GluR2 subunits (Snyder et al, 2001). New protein translation is not required to initiate the process of internalization, but instead is necessary for a long term DHPG dependent decrease in the density of surface AMPARs, indicating the requirement of new “LTD-related” proteins that are involved in AMPARs trafficking and endocytosis (Snyder et al, 2001; Waung et al, 2008). Moreover, in order to maintain a steady state level of surface AMPARs, even if globally reduced, there must be a compensatory mechanism leading to increased exocytosis of GluR subunits, accordingly to a more rapid recycling of AMPARs seen during mGluR dependent LTD (Waung et al, 2008).

Many LTD proteins have been discovered to be regulated at the mRNA level by FMRP. As previously mentioned, Arc is one of the major candidates in both LTP and LTD processes. With respect to the latter, Arc is known to promote AMPAR endocytosis via interaction with the endocytic proteins endophilin 2/3 and dynamin (Chowdhury et al, 2006). Accordingly, Arc KO neurons display a marked impairment in LTD processes and long term decrease of surface mGluR1 and AMPARs (Park et al, 2008; Waung et al, 2008). Interestingly, DHPG-induced mGluR activation led also to specific translation of the striatal-enriched protein tyrosine phosphatases (STEP) that has been implied in LTD related AMPAR endocytosis (Zhang et al, 2008). Inhibition of STEP activity, either by genetic deletion or pharmacological treatments increased AMPAR surface expression and prevented dephosphorylation of GluR2 that in turn would promote its internalization. This second LTD protein may cooperate with Arc in maintaining a sustained AMPAR internalization via Tyr dephosphorylation. However, evidence of altered STEP levels in Fmr1 KO models is still lacking.

A third LTD protein is microtubule associated protein 1B (MAP1B), whose mRNA is a well known FMRP target. MAP1B is actively involved in LTD processes, since DHPG treatment increased its levels in neuronal dendrites, whilst knock down of MAP1B prevented AMPAR internalization (Davidkova & Carroll, 2007). MAP1B has been described to interact with GRIP1, a scaffolding and GluR2 interacting protein (Davidkova & Carroll, 2007; Seog, 2004). It has been therefore proposed...
that increased MAP1B levels seen after DHPG stimulation might lead to an increased association with GRIP1, therefore destabilizing the surface GluR2 expression and therefore promoting their internalization. Moreover, NMDA activation promotes MAP1B expression as well, therefore contributing to the NMDAR dependent LTD and AMPAR endocytosis (Waung & Huber, 2009).

Stimulation of mGluR promotes translation by activating two main downstream signaling cascades, the ERK-MAP and PI3K-mTOR pathways, that in turn lead to the activation of key protein kinases as earlier discussed. In addition to these general stimulation of translation, mGluRs provide also a means for mRNA specific regulation of translation through FMRP. Upon synaptic stimulation, CYFIP1, a cytoplasmic FMRP interacting protein that inhibits translation by sequestering eIF4E, dissociates from its partner allowing the formation of eIF4F complex that in turn promotes translational of target mRNAs like Arc and MAP1B (fig.4). Accordingly, reduced levels of CYFIP1 would promote an increased rate of FMRP targets mRNA translation (Napoli et al, 2008).

Additionally, dephosphorylation of FMRP has been linked to increased association with translating polysomes (Ceman et al, 2003) and rapid translation of some target transcripts like SAPAP3 (Narayanan et al, 2007). It has been proposed that rapid, mGluR induced dephosphorylation of FMRP might facilitate the movement of ribosomes along mRNA, as well as rapid ubiquitination of FMRP leading to its immediate degradation, in order to relief any translational suppression (Bassell & Warren, 2008; Hou et al, 2006).
Summarizing, it emerges how mGluR signaling supports the coordinated expression of functionally related proteins that together act in promoting GluR1 and 2 internalization to reduce surface AMPAR expression. In particular, a pivotal role is covered by Arc, since it seems to be sufficient for the induction of LTD even in the absence of new STEP and MAP1b expression (Waung et al, 2008; Zhang et al, 2008). Moreover, enhanced LTD in Fmr1 KO mice strongly relies on Arc activity, since crossing of Arc KO with Fmr1 KO mouse strains leads to a less marked LTD (Park et al, 2008). In a similar way, Fmr1 KO animals backcrossed with GluR5 +/- animals show a less pronounced exaggeration of mGluR LTD, sustaining the so called “mGluR” theory stating that mGluR5 signaling that promotes AMPAR internalization is abnormally enhanced in Fmr1 KO mice (Dolen et al, 2007; Huber et al, 2002).
Neurotrophins (NTs) are small secreted proteins that play important roles in the development of the nervous system in vertebrates (Chao et al, 2006), whose first member, Nerve Growth Factor (NGF), was discovered during early '50s by Levi Montalcini and Hamburger (Levi-Montalcini et al, 1954). Twenty years later, studies carried by Barde and colleagues led to the isolation and characterization of a second member, called Brain-derived Neurotrophic Factor (BDNF) (Barde et al, 1982). Since then, four other additional neurotrophins have been identified, i.e. neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7). The latter two have been found only in fish (Carballo et al, 1998; Gotz et al, 1994; Lai et al, 1998). Once secreted, NTs can bind to two types of cell surface receptors, the Trk tyrosine kinases and the p75 neurotrophin receptor (p75NTR). Each Trk receptor (Trk A, Trk B and Trk C) is preferentially activated by one or more NTs and is responsible for mediating most cellular responses. More specifically TrkA is activated by NGF, TrkB by BDNF and NT-4/5, and TrkC by NT-3 (figure 5).

5 **BDNF mRNA LOCALIZATION AND FUNCTIONS**

**Fig. 5.** NTs binding to their receptors.

Binding of the NT initiates Trk dimerization, transphosphorylation of tyrosine residues in its cytoplasmic domain and kinase activation (see figure 5). The phosphotyrosine residues function as binding sites for recruiting specific cytoplasmic signalling proteins. These proteins may in turn be activated by phosphorylation. Contrarily to Trk receptors, p75NTR can be activated by the binding with all the four neurotrophins with approximately equal affinity, even if it is lower than Trk receptors. While Trk receptors transmit positive signals, p75NTR transmits both positive and negative signals. The signals generated by the two neurotrophin receptors can either augment or oppose each
other, regulating almost all aspects of neuronal development and function, including precursor proliferation and commitment, cell survival, axon and dendrite growth, membrane trafficking, synapse formation and function, as well as glial differentiation and interactions with neurons. Moreover, recent studies have revealed a diversity of roles for these factors outside the nervous system, most notably in cardiac development, neovascularisation and immune system function (Donovan et al, 2000; Kermani et al, 2005). Activated Trk receptors control three major signaling pathways: the MAP kinase signaling cascade, which promotes neuronal differentiation; the phosphatidylinositol 3-kinase pathway, that promotes survival and growth of neurons and other cells; the phospholipase C-γ pathways that promotes synaptic plasticity (Reichardt, 2006).

5.1 BDNF signaling and gene structure

BDNF protein structure and sequence have been highly conserved during evolution among several species, suggesting its fundamental role in regulating many different cellular functions (Hofer et al, 1990; Rosenthal et al, 1991). BDNF is widely expressed in the central nervous system, especially in cerebral cortex, hippocampus formation and amygdaloidal complex (Ernfors et al, 1990a; Ernfors et al, 1990b; Hofer et al, 1990; Phillips et al, 1990; Wetmore et al, 1990), and its expression increases during development until it reaches a maximal level after birth (Friedman et al, 1991a; Friedman et al, 1991b; Maisonpierre et al, 1990; Schecterson & Bothwell, 1992). Then, the expression of BDNF seems not to decline with age (Katoh-Semba et al, 1998; Lapchak et al, 1993; Narisawa-Saito & Nawa, 1996). However, BDNF has not only been found in the central nervous system, but its mRNA has been observed in numerous other tissues and cell types as colon, intestine, leukocytes, ovary, spleen, thymus, testis, pancreas and blood (Liu et al, 2005; Pruunsild et al, 2007; Rosenfeld et al, 1995). The widespread distribution of BDNF among tissues underlines its importance in a large number of different cellular processes, especially for what concerns neuronal functions. BDNF like the other neurotrophins is a morphoregulatory molecule that supports cell proliferation, cell survival/cell death, differentiation and phenotype maintenance. New evidence indicates that BDNF is also involved in dendritogenesis and axonogenesis (McAllister, 1999), spine formation and maturation, synaptogenesis and moreover neuronal homeostasis, brain plasticity-related process such as memory and learning (Tyler et al, 2002; Yamada et al, 2002) and drug addiction (Ghitza et al, 2009). The regulation of synaptic transmission and plasticity, peculiar for NT-3 and especially BDNF, was reported in a study carried by Lohof and co-workers, demonstrating how such neurotrophins are able to potentiate synaptic transmission in developing neuromuscular junctions (Lohof et al, 1993). Alterations in BDNF expression in specific neuronal subpopulations contribute to the onset of various
pathologies, including depression, epilepsy, and Alzheimer’s-, Huntington’s- and Parkinson’s diseases (Bibel & Barde, 2000; Binder, 2004; Castren, 2004; Cattaneo et al, 2005; Gottschalk et al, 1998; Lu & Chow, 1999; Murer et al, 2001; Russo-Neustadt & Chen, 2005).

BDNF plays a pivotal role in LTP, because of its capability to act at both pre- and post-synaptic level through the interaction with its receptor TrkB (Xu et al, 2000). In fact, when BDNF is released into the inter-synaptic compartment, it induces the release of glutamate from pre-synaptic neurons on one hand, and inhibits the release of the inhibitory neurotransmitter GABA (Frerking et al, 1998; Tanaka et al, 1997) on the other. Moreover, pre-synaptic activation of TrkB receptor seems to contribute to LTP through retrograde nuclear signaling, leading to transcriptional activation in pre-synaptic cell bodies (Gooney et al, 2004). At post-synaptic level, BDNF modulates the sensitivity of NMDA receptors and promotes new protein synthesis of synapse-targeted transcripts and appears to be directly associated with up-regulation, dendritic transport and local protein synthesis of Arc mRNA (Messiaoudi et al, 2002; Rodriguez et al, 2005). From a molecular point of view, BDNF induces the dimerization of TrkB, promoting the trans-phosphorylation of specific residues in the intracellular domain of the receptor (Huang & Reichardt, 2003). This event induces the activation of three different signaling cascades, i.e., the MAP Kinase (Mitogen Activated Protein Kinase), PI3 Kinase (Inositol-3 Phosphate Kinase) pathways and the activation of Phospholipase Cγ (PLCγ) (Kaplan & Miller, 2000) (fig.6). The phosphorylated tyrosine residues provide a docking site for adaptor proteins such as Shc and Frs2, which induce Ras activation. The latter initiates MAP kinase-signaling cascade, having as final target eIF4E that in the phosphorylated form is able to initiate the translation of target transcripts (Bramham & Wells, 2007).

At pre-synaptic level, BDNF-induced activation of MAPK leads to an increase in the release of glutamate, because of the phosphorilation of synapsin, a vesicular protein required for the fusion of vesicles with plasma membrane (Takei et al, 1997; Yamada et al, 2002). On the other hand, the activation of PI3 kinase pathway prevents the phenomenon of “synaptic fatigue”, i.e. the depletion of neurotransmitter-containing vesicles, seen especially after repeated series of high frequency action potentials (Gottschalk et al, 1999). Moreover, a downstream postsynaptic target of PI3K is Akt, a crucial kinase able to phosphorylate mTOR (mammalian Target Of Rapamycin), whose transduction pathway is involved in the regulation of CAP-dependent mRNA translation (Kang & Schuman, 1996; Takei et al, 2001) and represents the principal mechanism through which BDNF is able to induce local protein synthesis (LPS). In fact, even if for the maintenance of LTP new mRNA transcription is required, during the induction phase only protein synthesis form pre-existing mRNAs is needed.
It is thought that this is the critical phase in which de novo synthesis of synaptic proteins is regulated by BDNF signaling, achieving the long-term modifications characteristic of synaptic plasticity (Kang & Schuman, 1996; Yin et al, 2002). The last intracellular pathway initiated by BDNF is the PLCγ cascade, that leads to the post-synaptic phosphorylation of B2 subunit of NMDA receptor (Frerking et al, 1998; Yamada & Nabeshima, 2003) via CaMKIIα. This kinase is then responsible for the BDNF-induced E-LTP that, we remember, requires exclusively post-translational modifications of synaptic proteins (Minichiello et al, 2002). The increase of intracellular Ca++ mediated by PLCγ signaling is able to induce also the activation of CaMKIV and subsequently to the transcription factor CREB (cAMP Responsive Element Binding Protein), thus allowing the transcription of new genes that are required during the consolidation phase of L-LTP (Minichiello et al, 2002). In conclusion, we can state that PLCγ pathway is involved in both BDNF-dependent E-LTP induction (through CaMKIIα) and consolidation of L-LTP through de novo mRNA synthesis under the control of CREB.

All genes encoding neurotrophins have a basically similar structure, but among all discovered neurotrophins the genomic structure of the BDNF gene is unusually complex. Despite the fact that the gene organization and regulation of human and rodent BDNF gene expression have received close attention during the last decade, knowledge of the structural organization of mouse, rat and human BDNF gene has been well characterized only in the last years (Liu et al, 2005; Pruunsild et al,
Each BDNF transcript is composed by multiple alternatively non-coding exons spliced upstream of a common 3’ exon that encompasses the whole coding sequence and the 3’ untranslated regions (3’UTRs). The detailed characterization of the rodents and human BDNF gene has revealed the existence of four exon clusters, each of them regulated by different promoters located upstream of these alternatively spliced exons. These promoters enable a precise regulation of BDNF expression in different cell types or in response to different stimuli. The biological meaning of this complexity has been extensively investigated in our lab in the past years, focusing on a possible spatio-temporal fingerprint for the regulation of BDNF synthesis and localization, as will be discussed in the next paragraphs.

5.2 In vitro and in vivo studies of BDNF mRNA localization

Early in vitro experiments carried out by Crino and Eberwine revealed the presence of BDNF mRNA in dendritic growth cones of developing neurons after 24 hours in culture, whilst TrkB mRNA has been detected only after 48 hours (Crino & Eberwine, 1996). The presence of BDNF and TrkB mRNA in mature dendrites was demonstrated by Tongiorgi et al. (1997) using cultured hippocampal neurons. This study showed that TrkB and BDNF mRNAs are targeted to distal dendrites in an activity dependent manner (Tongiorgi et al, 1997), demonstrating that does not rely on new mRNA synthesis, but rather depends on an accumulation in dendrites of pore-existing mRNAs. Moreover, calcium signalling revealed to be essential for promoting BDNF and TrkB mRNA sorting in dendrites, since application of BAPTA-AM and EGTA, two Ca$^{++}$ chelators, significantly inhibited dendritic localization of these two mRNAs. Similarly, blockade of voltage gated L-type Ca$^{++}$ channels by nifedipine application severely impaired BDNF mRNA sorting (Tongiorgi et al, 1997).

Fig. 7 High potassium increases the dendritic localization of BDNF mRNA in cultured hippocampal neurons (modified from Tongiorgi et al, 1997). A, Whole-cell patch-clamp recordings, under current-clamp conditions, after depolarization with 10 mM KCl. B, In situ hybridization on cultured hippocampal neurons with BDNF riboprobe in resting conditions. C, Depolarization with 10 mM KCl for 3 hr increases the dendritic localization of BDNF mRNA.
During those years, studies suggesting that BDNF might be rapidly synthesized and released in an activity dependent manner (Goodman et al, 1996; Tongiorgi et al, 1997), together with evidence supporting a role for NT-3 in promoting RNA sorting (Knowles & Kosik, 1997), prompted to investigate whether or not BDNF itself might also stimulate its own mRNA transport into dendrites. It was found that BDNF is able to promote the peripheral sorting of its own mRNA in a feedback autocrine-paracrine autoregulatory loop (Righi et al, 2000). Even if the proteins directly involved in this process remain still unknown, it was shown that the PI3K signalling pathway is crucial to promote BDNF mediated mRNA sorting, whilst MAP/ERK and CaMKII activation did not result to be essential (Righi et al, 2000). These results perfectly fit with the notion that BDNF plays a prominent role in potentiating synaptic transmission at both pre- and post synaptic levels. However, an abnormal accumulation of this transcript might be related to pathological disturbances like epilepsy (Binder et al, 2001). Indeed, in vivo animal models of epilepsy displayed a dramatic accumulation of BDNF mRNA in the stratum radiatum of CA1 and CA3 neurons after administration of pro-epileptogenic stimuli like pilocarpine and kainic acid (Tongiorgi et al, 2004). Importantly, acute, intense seizures per se are not sufficient to induce distal BDNF sorting, that is instead triggered by stimulation paradigms able to induce the cellular cascades underlying epileptogenesis. The same stimulation paradigm was applied to study the relative distribution of the five most abundant BDNF splice variants, namely those containing exons I, IIB, IIC, IV and VI (Aid et al, 2007; Chiaruttini et al, 2008; Liu et al, 2006). The finding that after 3 hours of pilocarpine administration only exon IIB/C and VI containing splice variants were present at dendritic level, whilst the other two were still confined in the cell soma suggested the hypothesis that the different splice variants can represent a spatial code and can be expressed in a regulated manner to locally modulate BDNF availability (Chiaruttini et al, 2008). To support this hypothesis, very recent submitted data revealed that some mRNAs are implicated in cell survival, whilst other splice variants are involved in the regional regulation of dendritogenesis and spinogenesis (Baj & Tongiorgi, 2009; Baj and Tongiorgi, personal communication). BDNF mRNA sorting in vivo occurs also during most physiological stimuli, like during developmental plasticity induced by visual experience (Pattabiraman et al, 2005).

Despite this huge body of experimental evidence, however, the molecular mechanism underlying the selective localization of the different splice variants is still poorly understood. Very recently, two important advances have been made for understanding the dynamics if this complex phenomenon. Firstly, An and colleagues demonstrated that the long 3’UTR of BDNF mRNA, but not the short one, is necessary and sufficient for mRNA dendritic targeting, and its truncation completely
abolishes BDNF mRNA targeting in vivo (An et al, 2008). Moreover, the importance of this splice variant has been highlighted by finding that TG mice carrying a constitutive deletion if the long 3’UTR display severe depletion of dendritic BDNF protein that in turn can account for the LTP deficits and spine abnormalities seen in adult, but not young animals (An et al, 2008). However, these authors did not consider the effect of the short 3’UTR nor the potential consequences of neuronal activity regarding the subcellular distribution of BDNF mRNA. In this doctoral thesis, I will show and discuss data supporting a functional role in vitro and in vivo for both short and long 3’UTRs, paying particular attention to the different effects induced by different extracellular stimulation paradigms. In addition, we recently demonstrated that BDNF’s CDS contains an unexpected but essential dendritic targeting element for the correct activity dependent sorting of the transcript in hippocampal dendrites (Chiaruttini et al, 2009). This element, conserved in humans and rodents, is recognized translin, a DNA/RNA binding protein. A frequent human SNP, G196A (or Val66Met) has been frequently associated with neuropsychiatric diseases, possibly because if an impaired activity-dependent secretion of BDNF<sub>Met</sub> (Chen et al, 2004; Egan et al, 2003). We also proposed that such mutation additionally disrupts the BNDF mRNA:translin interaction, depleting dendrites not only of the protein, but also of the fraction of dendritic BDNF mRNA addressed to local translation.
ANALYSIS OF THE MOLECULAR MECHANISMS OF BDNF mRNA LOCALIZATION AND TRANSLATION IN NEURONS

6 AIMS

The subcellular compartmentalization of transcripts represents a milestone for the local regulation of mRNA availability that guarantees a fine spatio-temporal regulation of protein expression fundamental for the development and maintenance of highly polarized cells. In particular, in neuronal cells this phenomenon has gained a broader significance, since it seems to be at the basis of synapse-specific delivery of new mRNAs. This regulated sorting allows the local synthesis of new proteins that are required during plasticity related phenomena, therefore significantly contributing to higher brain functions like memory and learning (Steward & Schuman, 2001).

A growing body of evidence indicates that dendritic sorting of BDNF mRNA represents a crucial event regulating synaptic plasticity (Soule et al, 2006; Tongiorgi et al, 1997). However, little is known about the molecular mechanisms regulating the selective, activity-dependent sorting of BDNF mRNA in dendrites (Righi et al, 2000; Tongiorgi et al, 1997) and how it could effectively influence synaptic activity. In recent studies performed in our and other laboratories it has been demonstrated that some BDNF splice variants differ in their subcellular localization (An et al, 2008; Baj & Tongiorgi, 2009; Chiaruttini et al, 2008; Pattabiraman et al, 2005), indicating that they could actually represent a spatial code to preferentially regulate BDNF expression and consequently to modulate the metabolism of restricted subcellular domains.

To help define the mechanism underlying the regulation of activity-dependent BDNF mRNA subcellular localization and the biological meaning of having two 3’UTRs, we separately investigated the role of the different transcript’s regions searching for specific localization signals and translational regulatory elements. In particular, with both in vitro and in vivo approaches we analyzed:

- the role of 5’UTRs and coding region respect to mRNA traffic regulation;
- the role of both 3’UTR short and long in mRNA sorting;
- the translational regulation exerted by BDNF short and long 3’UTRs.
7 MATERIALS AND METHODS

7.1 Cell cultures

7.1.1 SK-N-BE and PC12 cell lines

SK-N-BE human adrenal neuroblastoma cells were cultured in Dulbecco’s modified Eagle’s medium (D-MEM, Euroclone), 10% fetal bovine serum (FBS, Euroclone), 2 mM L-glutamine, penicillin 100 U/mL, streptomycin 100 µg/mL (Euroclone) at 37°C in a 5% CO2-humified incubator. Likewise, rat pheochromocytoma cells (PC12) were cultured in RPMI medium (Euroclone) supplemented with 10%FBS, 5% donor horse serum (DHS, Euroclone), 20mM HEPES, 2 mM L-glutamine, penicillin 100 U/mL, streptomycin 100 µg/mL (Euroclone) at 37°C in a 5% CO2-humified incubator. Undifferentiated cells were plated onto 6 multiwell plates at 55% of confluence for subsequent transfection assays.

7.1.2 Primary rat hippocampal neurons cultures

Primary hippocampal from postnatal Sprague–Dawley rats were made according to the method of Malgaroli and Tsien (Malgaroli & Tsien, 1992) with slight modifications (Tongiorgi et al, 1997). Hippocampi were dissected from 0 to 1-day-old animals (PO-P1). All the dissections were performed in cold Hank’s balanced salt solution (NaHCO3 4.2 mM (Sigma), Hank’s salt powder 0.952% (Sigma), Hapes 12 mM (Sigma), D-glucose 33 mM (Sigma), Kinurenic acid 2 mM, streptomycin 100 µg/mL (Euroclone)); the dissected tissue was maintained in ice until the end of the extraction. Hippocampi were then digested with 500 μL of 0.25%, tripsin in native MEM (Minimum Essential Medium + Earle’s salts + glutaMAX™ (Euroclone)), for 7 minutes at 37°C, shaking periodically and gently the tube. The digestion was blocked with 4 mL of D-MEM supplemented with 10% heat inactivated Fetal Bovine Serum (FBS, Gibco) and the dissected tissue was centrifuged at 800 rpm for 5 minutes at 8°C. The pellet was resuspended in 5 mL of fresh Neurobasal supplemented with 2% B27 (invitrogen). To isolate single cells, pellets were dissected by mechanically pipetting the suspension in order to get a homogeneous solution. Cells were slowly filtered using a cell strainer (40 μm pore diameter, Sarstedt) to remove impurities and cellular debris. Cells were counted at the microscope using the vital dye Trypan Blue. Cells were seeded onto 12 mm glass coverslips treated with poly-L-ornithyne (100 µg/mL) (Sigma) and Matrigel® (BD Biosciences, diluted 1:50 in Hank’s solution) at the concentration of 100.000 cells per well. After 4-5 hours the medium was replaced with fresh Neurobasal supplemented with 2% B27 (Invitrogen) supplemented with 1mM L-glutamine, penicillin 100 U/mL, streptomycin 100 µg/mL (Euroclone). Medium was changed every two days, and from the
second day in culture additioned of 5 μM cytosine arabinofuranoside (ARA-C, Sigma) to prevent the proliferation of non-neuronal cells. Cells were cultured for 6 days at 37°C in a 5% CO2-humified incubator.

7.2 Animal treatments

Procedures involving animals and their care were carried out in accordance with national (Decreto Legge N116, Gazzetta Ufficiale, suppl 40, 18-2-1992) and international laws and policies (European Community Council Directive 86/609, Oja L 358, 1, December 12, 1987; National Institutes of Health Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996). All efforts were made to minimize animal suffering and to reduce the number of animals. For pilocarpine experiments, animals were treated with 300mg/kg i.p pilocarpine for 3 hours (for in situ hybridization, ISH) or 6 hours (for immunohistochemistry (IHC) analyses) under urethane anaesthesia (100mg/Kg i.p.). Mice were transectionally perfused under anaesthesia with ice-cold 2% (for IHC) or 4% paraformaldehyde (PFA, Sigma) in phosphate buffered saline (PBS, 137mM NaCl, 2.7mM KCl, 8.1mM NaH₂PO₄, 1.47mM KH₂PO₄, pH 7.4). After extraction, brains were left in PFA for other 2 hours, and afterwards cryoprotected in 2% or 4% PFA/20% sucrose at 4°C up to 6 months. Brains were quickly frozen with CO₂ and cut in serial 40μm tick coronal sections with the Histoslide 200R cryomicrotome (Leica). Free floating sections were either processed for non-radioactive fluorescent ISH (FISH) after one week of post-fixation in 4% PFA in PBS or stored at -20°C in antifreeze solution (30% glycerol, 20% PEG supplemented with 0.1% sodium azide) for IHC experiments.

7.3 Chimaeric BDNF-GFP and RSV-MS2hairpin CDS constructs

Total RNA was extracted from whole rat brain using TriZol Reagent (Invitrogen). 1mg of total RNA was then heat denaturated and reverse-transcribed into cDNA with a mix containing 100ng random primers (Roche Diagnostics), 5X first strand buffer (250mM Tris-HCl pH8.3, 375mM KCl, 15mM MgCl₂), 10mM DTT (Invitrogen), 2U/mL RNase inhibitor (Roche Diagnostics), 500mM dNTPs (Promega) and 200U Superscript III (Invitrogen). The reaction was carried out at 42°C for 50 minutes followed by SuperscriptIII inactivation for 15 minutes at 70°C. For CDS-GFP chimaera cloning refer to Chiaruttini et al., 2008 (supplementary methods). PCR protocol was 5 min at 95 °C; 35 cycles of the progression- 1 min 95 °C, 1 min 55 °C, 2 min 72 °C-, 10 min at 72°C. For GFP- 3′UTR short, GFP-3′UTR short mut ELAVup, GFP-3′UTR long, GFP-3′UTR mid and GFP-3′UTR end cloning, PCR were performed with 0.02 U/μL of Phusion Hi-Fidelity DNA polymerase (Finnzymes), 1 μg cDNA, 5X Reaction Buffer, 1.5mM MgCl₂, 200μM dNTPs (Promega) and 500nM of each specific forward and reverse primer (MWG biotech) (see Table2 for details). PCR protocol was 30 sec at 98 °C; 31 cycles of the progression- 10 sec at 98°C, 20 sec at 56 °C, 30 sec (1.5 min for 3′UTR long amplification) at 72 °C,
10min at 72°C. For GFP-3’UTR short mutCPE and GFP-3’UTR short mutELAvdw cloning, an assembly PCR strategy was adopted. Briefly, a first round of PCR was carried out as above described using the following protocol and primer pairs: 30 sec at 98 °C; 31 cycles of the progression- 10 sec at 98°C, 2min at 57.5 °C, 30sec at 72°C. For CPE mutation: Fw 3’short 1-65 and Rev 3’short mut CPE; for ELAvdw mutation: Fw 3’sh 1-65 and Rev ELAVdw mut (see table XXX for sequence details). A second round of PCR (30 sec at 98 °C; 31cycles of the progression- 10 sec at 98°C, 2min at 58 °C, 30sec at 72°C) was performed using 2 µl of CPE and ELAVdw mutant fragments (as forward “megaprimers”) with 3’UTRsh Rev primer, to obtain mutated full length 3’UTR short sequence. PCR fragments were then purified (PCR Cleanup kit, Sigma) and digested with XhoI/SacII (New England Biolabs)(for BDNF CDS) or NotI/HpaI (New England Biolabs)(for 3’UTRs and mutants) restriction enzymes.

| 3’UTR long | Fw Rev | 5’- GGGCGGCCGCTGGATTTATGTTGATAG -3’
| 3’UTR long | Fw Rev | 5’- GCCGGGTAAAC TTACAATAGGCTTTCGATG -3’
| 3’UTR mid | Fw Rev | 5’- ACTAGCGGCGC AGCACATTCTTTCCCTCCTC -3’
| 3’UTR mid | Fw Rev | 5’- GTCACTGAACTATGCACTGATGCTG -3’
| 3’UTR end | Fw Rev | 5’- GTCAGGGTAACTACATGCTGATGTG -3’
| 3’UTR end | Fw Rev | 5’- GTCAGCCGCAATCCTACGGTGATAGGG -3’
| 3’UTR short CPEmut | Fw1-65 Rev1mut Rev2(3’sh) | 5’TGCAGCAGCCGCGCTGGATTTATGTTGATAGTTATGAGACAAAAATTCTAT TTGTATATATACACACAGGG -3’
| 3’UTR short CPEmut | Fw1-65 Rev1mut Rev2(3’sh) | 5’ACATGCAGTTGCCAGATCTTATTCCTTAATATACATGCTGATGG -3’
| 3’UTR short mut ELAVup | Fwmut Rev(3’sh) | 5’TGCAGCAGCCGCGCTGGAGCGCGGTGTTATGATAGTTATGAGACAAAATTCTAT TTGTATATATACACACAGGG -3’
| 3’UTR short mut ELAVup | Fwmut Rev(3’sh) | 5’GTCACTGAACTATGCACTGATGCTG -3’
| 3’UTR short mut ELAVdw | Fw1-65 Revmut Rev2(3’sh) | 5’TGCAGCAGCCGCGCTGGATTTATGTTGATAGTTATGAGACAAAATTCTAT TTGTATATATACACACAGGG -3’
| 3’UTR short mut ELAVdw | Fw1-65 Revmut Rev2(3’sh) | 5’GTCACTGAACTATGCACTGATGCTG -3’
| CDS RSV | Fw Rev | 5’- GTCA AGATCATGACCATCCTTTCTTTAC -3’
| CDS RSV | Fw Rev | 5’- GTCAAGCGGCGCGCTCCCTTTATAGGCTCAG -3’

Table 2. Primer sequences used for reporter cloning.

For RSV-MS2 hairpin CDS chimaera cloning, we used the same standard procedure above adopted, using 500nM of each specific primer pair (see table 2). PCR protocol was 30 sec at 98 °C; 31 cycles of the progression- 10 sec at 98°C, 20sec at 56 °C, 30sec at 72°C and final extension at 72°C for 10min. PCR fragments were purified (PCR Cleanup kit, Sigma) and digested with BglII/NotI restriction enzymes (New England Biolabs).

For each clone, digested fragments were gel purified (Gen Elute gel extraction kit, Sigma) and ligated with 300U of T4DNA ligase (New England Biolabs) overnight at 16°C with properly digested pEGFPN1
vector for GFP chimaeras (Clontech) or RSV-MS2 hairpin vector (kind gift of professor K.Kosik, UCSB, California, USA). Ligase products were then transformed into chemically competent DH5α E.coli bacterial strain. Colonies were screened by PCR and positive clones have been further confirmed by sequencing. Already available clones have been used in this study, in particular ratCDS-GFP and ratΔ(1-202)CDS-GFP and GFP-3’UTR short (cloned by dr.C.Chiaruttini), ratΔ(68-212)CDS-GFP (cloned by dr.G.Baj), human Val66CDS-GFP and Met66CDS-GFP (kind gift of dr.B.Lu, NIH, Bethesda), humanG177A-, human G178A- and human G177A/G196A-CDS-GFP (cloned by dr.Z.Li, JHU, Baltimore), MS2-NLS-GFP by dr.Li-Na Wei (UMMS, Minnesota).

7.4 siRNA production

The target regions of interest were amplified from whole rat brain/testis cDNA, using T7 promoter sequence-containing primers. Each PCR reaction was performed with 200 ng of cDNA, 500nM of each specific fw and rev primer (see table 3 for sequence details), 0.2 µM of dNTPs mix (Promega), 2.5 mM MgCl₂, 10X reaction Buffer, 0.05U/µL of High Fidelity Taq DNA polymerase (Invitrogen). The PCR protocol was 5min at 95 °C; 31 cycles of the progression- 1min at 95°C, 1min at 56 °C, 2min at 72°C- and final extension at 72°C for 10min.

<table>
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<tr>
<th>Target</th>
<th>siRNA primer</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPEB1 Fw T7</td>
<td>5'-GGCTATACGCATCTAGTATAGGgcattgclcagctccagg-3'</td>
<td>414</td>
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<tr>
<td>RevT7</td>
<td>5'-GGCTATACGCATCTAGTATAGGtgagtcagagggctgtag-3'</td>
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<tr>
<td>CPEB2 Fw T7</td>
<td>5'-GGCTATACGCATCTAGTATAGGtggggcaatgctgtggcagag-3'</td>
<td>~ 400</td>
</tr>
<tr>
<td>RevT7</td>
<td>5'-GGCTATACGCATCTAGTATAGGaacagattgtctgtctgg-3'</td>
<td>~ 500</td>
</tr>
<tr>
<td>CPEB 3 Fw T7</td>
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<td>494</td>
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<tr>
<td>RevT7</td>
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</tr>
<tr>
<td>CPEB 4 Fw T7</td>
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<td>471</td>
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<tr>
<td>RevT7</td>
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<tr>
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<tr>
<td>Translin Fw T7</td>
<td>5'-GGCTATACGCATCTAGTATAGGtgagcactggagtttgtgc-3'</td>
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Table 3. Sequences of the primers used for in vitro dsRNA production. Underlined, T7 promoter sequence.

dRNA synthesis was performed using the commercial kit Silencer™ siRNA Cocktail (RNase III) (Ambion) adding, in the following order, 1 µg of purified T7 PCR product, 2 µL of transcription buffer 10X (Roche), 2 µL of ribonucleoside triphosphate mix 100 mM (ATP, CTP, GTP, UTP) (Roche), 2 µL of T7 RNA polymerase (Roche) and H2O DEPC up to the final volume of 20 µL. The reaction was carried out at 37°C for 2 hours and stopped adding the stop solution (20 µL of H2O DEPC, 8 µL of sodium acetate 1.5 M pH 5.2 and 80 µL of 96% cold ethanol). PCR products were precipitated at -20°C and then centrifuged at 14000 rpm for 15 minutes at 8°C. The pellet was washed with of 80% ethanol and incubated for 10 minutes at room temperature. Subsequently, samples were centrifuged (14000 rpm for 5 minutes at 8°C) and resuspended in 50 µL of DEPC-treated H2O. The long dsRNAs were digested for 1 hour at 37°C with RNase III, to obtain pool of short siRNA (1µg of dsRNAs, 5 µL of buffer 10X (Ambion), 1 µL of RNase III (Ambion) and H2O DEPC up to the final volume of 50 µL). The reaction was stopped as previously explained for in vitro transcription. This methodology allows the production of pools of 12–30 bp double-stranded RNAs. The siRNAs were purified by EtOH precipitation and resuspended in 20 µL of H2O DEPC. The siRNAs were checked on 2% agarose gel and quantified using the spectrophotometer.

For FMR1 and FXR2P, specific duplexes were kindly provided by Dr. Maja Castrén (Orionpharma, Helsinki). The efficiency of gene silencing was evaluated by western blotting analysis on siRNA transfected rat PC12 cells.

### 7.5 Lipofectamine transfection

The different vectors and siRNAs were transfected into primary neurons, PC12 or SK-N-BE cells, using lipofectamine 2000™ (Invitrogen). The relative quantity of DNA to lipofectamine was 1µg of DNA (and, if it is the case, 19 nM of each siRNA) to 2µL of lipofectamine for each well of a 24 multiwell slide. The culture medium was replaced 3-6 hours before the transfection with fresh Neaurobasal supplemented with 2%B27. The conditioned medium was filtered (through 0.2 µm pore diameter filters) and diluted 1:1 with fresh neuron medium. The DNA (1 µg) was mixed with 50 µL of
native MEM medium (EUroclone) without serum nor antibiotics and incubated for 5 minutes at room temperature. At the same time, 2 μL of 1mg/ml lipofectamine were mixed with 50 μL of native MEM and incubated at room temperature for 5 minutes. The two solutions were then mixed and incubated for 20 minutes at room temperature, added to the cells and incubated for 1 hour at 37°C, 5% CO2. One hour after transfection the medium was removed and replaced with fresh filtered conditioned medium. For cell lines transfections on 6 well plates, a ratio of 4μg (or 19nM siRNAs) to 10μL of lipofectamine were used in 250μL of native MEM. The protocol was followed as described above except that the mixture was kept in culture until lysate preparation.

### 7.6 Western blot analysis

After transfection the cells were carefully washed twice in PBS. Cells were trypsinized and collected in lysis buffer (25mM TrisHCl (pH 7.5),1mM EDTA, 1mM Spermidin, 1mM PMSF, 1mM iodoacetamide (IAA), 4μg/ml (1mM) Soy Bean Trypsin inhibitor (SBTI), 10μg/ml Turkey Egg White inhibitor (TEWI), 0.75% NP-40). Lysates were centrifuged at 10000 rpm for 15 minutes at 10°C to remove cellular debris, followed by Bradford assay to determine protein concentration. Sample were then boiled and analyzed on 12% polyacrylamide gel electrophoresis (10μg for SK-N-BE and 20μg for PC12 extracts). Proteins were then electroblotted for 1 hour onto nitrocellulose membrane (Schleicher & Schuell). Membranes were saturated for 40 minutes with a solution containing 5% non fat milk in PBST (PBS+0.1% Tween 20) before an overnight incubation at 4°C with either mouse monoclonal anti-GFP (1:4000 in 5% milk/PBST, Sigma), mouse anti-tubulin alpha (Sigma, 1:20000), rabbit anti CPEB-1,-2,-3,-4 (1:500, 1:400, 1:700 and 1:500 respectively, Abcam), rabbit antiELAV-1, -2, mouse anti ELAV-4 (1:500, 1:500, 1:1000 respectively, Abcam), goat anti ELAV-3 (1:1000, Santa Cruz), rabbit anti FMRP (1:1000, Abcam), rabbit anti FXR2 (1:700, Abcam), mouse anti hnRNPA2/B1 (1:1000, Abcam) and rabbit anti translin (1:2000). Membranes were then washed 3X5 minutes in 5% non fat milk in PBST and incubated for 1h at RT with the respective secondary antibody (anti rabbit HRP,1:10000 (Jackson); anti mouse HRP 1:10000 (Pierce); anti goat HRP 1:10000 (Dako); anti mouse AP 1:1500 (Jackson)). For alkaline phosphatase development, membranes were equilibrated in 100mM Tris-HCl, pH 9.5, 50mM MgCl2, 100mM NaCl in presence of 70mg/ml NBT and 50mg/ml BCIP. For HRP development, membranes were incubated for 5 minutes with chemiluminescent substrate (GE Healthcare) before exposure to X-ray films (Kodak).

### 7.7 Non radioactive in situ hybridization

Non radioactive in situ hybridization on wild-type (WT), BDNF$^{lox}$ (courtesy of K.Jones, University of Colorado, Boulder, USA) and G196A knoxk in mice (courtesy of F.Lee, Cornell-Weill School of Medicine, New York, USA) brain sections was made according to the method described by
Tongiorgi et al. (Tongiorgi et al., 1998) with slight modifications. According to this method, free-floating slices were washed twice in PBS/0.1% Tween20 (PBST) at room temperature (RT) for 5 min, quickly washed in H₂O, then permeabilized with 2.3% sodium meta-periodate (Sigma) in H₂O at RT for 5 min and quickly washed again in H₂O. After these steps, the sections were incubated in 1% sodium borohydrate (Sigma) in 100mM Tris-HCl buffer pH 7.5 at RT for 10 min, washed twice in PBST at RT for 5 min. Then the slices were digested with 8µg/ml proteinase K (Roche Diagnostics), for a time empirically determined with each set of slices, generally between 12 min, and washed twice in PBST at RT for 5 min. After digestion the tissue sections were fixed in 4% PFA/PBST at RT for 5 min and washed two times in PBST at RT for 10 min. For in situ hybridization on cell cultures, the cells were fixed 15 min at RT in 4% PFA/PBS, washed in PBST, and permeabilized at -20°C in ethanol absolute for 20 min. To remove the ethanol, cells were rehydrated for 3 min at RT with respectively a solution 50% ethanol/50% PBST, 30% ethanol/70% PBST and finally PBST alone. After rehydration, both slices and cells were prehybridized at 55°C for 90 min in the hybridization mix containing: 20mM Tris-HCl, pH 7.5, 1mM EDTA (Sigma), 1x Denhardt’s solution, 300mM NaCl, 100mM dithiothreitol (DTT, Fluka), 0.5mg/ml salmon sperm DNA (Labtek Eurobio), 0.5mg/ml polyadenylic acid (Sigma), and 50% formamide (Sigma). In situ hybridization was performed overnight at 55°C in the hybridization mix with 10% dextrane sulphate (Sigma) and the digoxigenin riboprobe (50-100ng/ml). High-stringency washes were performed in 2X saline sodium citrate buffer (SSC, 150mM NaCl, 15mM C₆H₅Na₃O₇·2H₂O, pH 7.0) 0.1% Tween20 (SSCT)/50% deionised formamide at 55°C for 30 min, 20 min in 2X SSCT at 55°C and twice in 0.1X SSCT at 60°C for 30 min. Cells hybridized with digoxigenin riboprobe were processed for immunodetection with an anti-DIG Fab fragments coupled to alkaline phosphatase (Roche Diagnostics). Sections were instead incubated overnight at 4°C in presence of the antibody diluted 1:1000 in PBST/10% fetal calf serum (FCS); while cells were incubated at RT in presence of the antibody diluted 1:500 in PBST/10% FCS for 3 hours only. After four washes in PBST for 10 min, in situ staining has been developed at RT with 4-nitroblue tetrazolium 70mg/ml (NBT; Labtek Eurobio) and 5-bromo-4-chloro-3-indolyl-phosphate 50mg/ml (BCIP; Labtek Eurobio) in 100mM Tris-HCl, pH 9.5, 50mM MgCl₂, 100mM NaCl and 1mM Levamisol (Sigma). The time of incubation was empirically determined, generally for slices the reaction was carried out for at maximum 5 hours at room temperature, while cells were developed for about 45 to 60 min at room temperature because of the strong expression of the GFP-chimeras. For fluorescent in situ hybridization, brain sections were stained with fluorescent anti-DIG enhancer set (Roche diagnostics) following manufacturer’s instructions. Sections were then mounted in anti fade mounting solution (Invitrogen) and stored at 4°C in dark.
7.8  **GFP and CDS antisense probes preparation**

To produce anti-sense probes, 1 µg of BamHI-linearized pBSKS-GFP or pGEM ratBDNF CDS were incubated with 2 µL of Transcription Buffer 10x (Roche), 2 µL of DIG-UTP labelling mix (Roche), 2 µL of T7 RNA polymerase (for GFP antisense probes) or SP6 RNA polymerase (for rat BDNF CDS) (Roche), 2U/µL of Rnase inhibitor (Roche) and H₂O DEPC up to 20 µL. Reaction was blocked after 2 hours with 2 µL of EDTA 0.5 M, 2 µL of LiCl 4 M, 75 µL of cold EtOH 100% and precipitated at -20°C. RNA probes were centrifuged at 13000 rpm for 20 minutes at 8 °C. The pellet was resuspended in 50 µL of H₂O DEPC. Alkaline hydrolysis of the probes was performed in order to increase the specificity of ISH and reduce the background signal. One volume of the probes and one of the sodium bicarbonate buffer (40 mM NaHCO₃/ 60 mM Na₂CO₃ pH 10.2) were mixed and incubated for 7 minutes at 60 °C. The reaction was neutralized and stopped adding half volume of HCl 0.1 M. RNA probe quality and integrity was tested through northern blot on nylon membranes before use.

7.9  **Immunohistochemistry on brain sections**

BDNF immunohistochemistry on wt and BDNF<sup>Klox</sup> brain sections were performed on 2% PFA perfused brains cut at 40 µm thick coronal sections. Briefly, slices were washed 3X5min in Tris buffered saline (TBS, 20mM Tris pH 7.5, 150mM NaCl), followed by incubation in 0.3% H₂O₂ in TBS to block the endogenous peroxidase activity. Sections were then washed 3X5 min in TBS before 30min of blocking in 10% FBS in Tris-X (0.75% Bovine Serum Albumin (BSA), 0.3% Triton X-100). After blocking, slices were washed again 3X5 min in Tris-X and incubated with polyclonal anti-BDNF antibody (Santa Cruz, 1:100) for 48hours at 4°C. To remove unspecific primary antibody binding, 3X5 min washes in Tris-Y (0.25%BSA, 0.05% Triton X-100 in TBS) were performed, followed by incubation with anti rabbit HRP secondary antibody (Dako, 1:400) diluted in Tris-Y, for 1hour at room temperature. Slices were the washed the 3X5 min in Tris-Y and again 3X5min in TBS before. Slices were finally developed through Nickel-enhanced diaminobenzidine (DAB) chromogenic reaction (one DAB tablet dissolved in 25mL TBS with 50mg D-glucose with 0.2% Nickel ammonium sulphate) for about 20-30 min at room temperature in dark. All passages, except development, were performed in gentle shaking.

7.10  **Polyadenylation assay (PAT)**

For polyadenylation assay (Salles et al, 1999), 1 µg of total RNA was transcribed in cDNA as previously described, except that 200ng of oligo(dT) anchor primer have been used instead of random primers. Subsequently, 100ng of retrotranscribed single stranded cDNA have been used in a second round of PCR to detect the poly(A) tail extension. The PCR program was 5min at 95 °C; 30 cycles of the progression- 45sec at 95°C, 45sec at 59.5 °C, 1min and 30sec at 72°C and a final
extension at 72°C for 10 min. For 3'UTR short the following pair of primers have been used: Fw 3'UTR short 5'-GGGCGGCCGCTGGATTTATGTTATAG-3', Rev oligo(dT) anchor 5'-GCGAGCTCCGCGGCGCG-T12; for 3'UTR long the following primers have been used: Fw 3'long PAT 2585 T5'-CATGCATGGCGAGGCCT-3', Rev oligo(dT) anchor 5'-GCGAGCTCCGCGGCGCG-T12.

7.11 Live cell imaging

For live cell imaging of RNA trafficking, we took advantage of the MS2 hairpin/MS2-GFP imaging system. Briefly, the RNA of interest was fused to an eight tandem repeat MS2 stem–loop sequence contained in the RSV vector that in turn had to be recognized by the MS2-NLS-GFP chimaeric protein. Primary rat hippocampal neurons at 5 DIV were co-transfected with a GFP-NLS-MS2 vector (kind gift of professor Li-Na Wei, UMMS, Minneapolis, Minnesota) and a RSV-rBDNF CDS vector or a control RSV empty vector (a kind gift of Professor Kenneth Kosik, UCSB, Santa Barbara, California) to a 1:3 ratio. 24 hours after transfection, neurons were observed by live cell imaging using a camera (NIKON AXM 1200). Photos were taken every 20 seconds for 30 minutes, both in control condition and after application of 10mM of KCl to elicit neuronal activity. Images were elaborated and assembled with the stack builder plug-in of Image J 1.34 software (NIH).

7.12 Sequence analysis and RNA folding

An “in silico” analysis was performed on both BDNF coding region and 3'UTR using different alignment annotation softwares and computer predictions starting from known cis-acting elements. Sequences obtained from NCBI (http://www.ncbi.nlm.nih.gov) were analyzed using BioEdit nucleic acid annotation (www.mbio.ncsu.edu/BioEdit/bioedit.html) and Primer Premier software with the support of ClustalW multiple alignment output (www.ch.embnet.org/software/ClustalW.html). Only conserved sites among different species have been taken in consideration in order to minimize false positives. Computer prediction of RNA 3'UTR short and long secondary folding structures was performed using the RNA structure 4.5 software based on the algorithm developed by M. Zuker (Zuker, 2003). Folding predictions were made by using parameters at default settings. Selection of the secondary structures has been carried out based on the most frequent observed ones that displayed the lowest free energy.

7.13 Quantitative imaging and statistic analysis

Non radioactive in situ hybridization was analyzed by viewing stained cultures under bright-field illumination. The positive neurons were acquired by a Nikon AXM1200 digital camera on a Nikon E800 Microscope with interference contrast-equipped lens (60x magnification) and then analyzed with the image analysis program ImageProPlus (Media Cybernetics, Silver Spring, MD). The function “Trace” was used to measure, starting from the base of the dendrites, the maximal distance of
dendritic labeling (MDDL) as done before (Tongiorgi et al, 1997). Dendrites were traced, in a conservative manner, up to the point at which the in situ labeling was clearly distinguishable from the background. The quantitative analysis refers to at least 4 different experiments in which about 150 dendrites were measured in total. The mean of all the measurements are represented in bar graphs (see results) with corresponding standard errors (SE). Statistical data analysis was performed with the Sigma Stat 3.2 software (Systat Software, Inc.). Graph elaborations were done with Sigma Plot 11 software (Systat Software, Inc.). Statistical significance among groups was evaluated performing Kruskal-Wallis one-way ANOVA on ranks, followed by a multiple comparison procedure with Dunn’s method. Images in the thesis were captured with a 40X magnifications for the CA1, CA3 and dentate gyrus (DG), cultured cells images were captured with a 60x objective (oil immersion). Densitometries on positively stained neurons were performed with the “Straighten” plugin of ImageJ 1.42q (NIH). Western blots and PCRs signals were quantified with the same software as well.
8 RESULTS

8.1 Activity dependent targeting of endogenous BDNF mRNA

Several lines of evidence indicate that targeting of BDNF mRNA to dendrites plays a key role in mediating synaptic plasticity (An et al, 2008; Soule et al, 2006; Tongiorgi et al, 1997). As previously discussed, the studies carried by Tongiorgi, Righi and Cattaneo (1997, 2000) demonstrated that synaptic activity, elicited by BDNF signaling and neuronal depolarization, represents the main event leading to increased dendritic sorting of the mRNAs encoding BDNF and its receptor TrkB. These studies have represented the starting point of the present study, focused on understanding the molecular mechanism as well as the stimuli underlying the activity dependent localization of BDNF mRNA in neuronal dendrites.

In a first set of experiments, we sought for other possible stimuli able to induce BDNF mRNA translocation in neurites. To this aim, we used primary rat hippocampal neurons at 6 days in vitro (DIV) treated for three hours with 10mM KCl or 50ng/mL of each neurotrophin (NGF, BDNF, NT-3 and NT-4). After fixation, neurons were subjected to non radioactive in situ hybridization (ISH) (Tongiorgi et al, 1998) using a DIG-labeled antisense riboprobe against the coding region of BDNF mRNA to detect all possible BDNF splice variants. As reported in figure 8, panel A, BDNF mRNA is restricted in the proximal dendritic compartment in resting conditions, but a dramatic accumulation in distal dendrites is seen after 3 hours of depolarization with 10mM KCl. We found that BDNF itself, even at a lesser extent, was able to promote the dendritic delivery of its own mRNA, confirming the data published in 2000 by Righi, Tongiorgi and Cattaneo. Additionally, we found that a dendritic BDNF mRNA localization can be induced also by NT-3, but not NT-4 nor NGF. It was previously reported that NT-3 may promote the extrasomatic transport of RNA granules but the identity of the transported mRNAs was not discovered (Knowles & Kosik, 1997). Hence, BDNF appears to be the first known mRNA whose localization is influenced by NT-3 signaling.

Data have been quantified by manually tracing the Maximal Distance of Dendritic Labeling (MDDL), that is the maximal distance starting from the base of the dendrite at which a signal above the mean background was detected. Only apical dendrites of bona-fide pyramidal neurons identified by morphological criteria, were measured. For each case, more than 100 cells were analyzed. Then, this measure has been normalized to the mean MDDL of BDNF mRNA in control conditions to obtain the “fold increase”. The specificity of the in situ hybridization technique was assessed using a sense...
DIG-labeled riboprobe that, as expected, generated only a negligible background signal (Fig. 8 panel A). We found that KCl induces an increase in the distance reached by BDNF mRNA in dendrites of about 2.83±0.17 fold increase with respect to control conditions, while BDNF and NT-3 induce an increase of 1.59±0.11 and 1.49±0.10 folds, respectively. NGF and NT-4 did not significantly alter BDNF mRNA distribution. In further confirmation of the strict dependence of BDNF mRNA sorting on BDNF and NT-3 signaling, we found that blocking the Trk receptors activity with 30nM of K252a, either 30 minutes before stimulation and throughout the stimulation period completely abolished the activity dependent sorting of BDNF transcripts (Fig. 8, panel C). As observed previously, inhibition of Trk signaling by K252a depressed significantly also BDNF mRNA KCl-dependent dendritic targeting, indicating a contribution of neurotrophins during KCl-induced neuronal depolarization (Righi et al., 2000).

Fig. 8. Activity dependent targeting of endogenous BDNF mRNA in primary hippocampal neurons. A) representative in situ hybridizations showing strong dendritic labeling after KCl, BDNF and NT-3 application. Lower panel, control in situ hybridization using CDS sense probe. Scale bar 10 µm; B) quantification of BDNF mRNA half-life in cultures treated with the transcription inhibitor actinomycin D (5µg/mL) in control and stimulated conditions; C) quantification of the fold increase of BDNF mRNA in dendrites shows a strong upregulation after KCl, BDNF and NT-3 treatment (***, P<0.001, Kruskal-Wallis One Way Anova on Ranks followed by a multiple comparison procedure with Dunn's method).
To rule out the possibility that the increased dendritic localization seen after stimulation was due to increased mRNA stability rather than enhanced transport, we performed semi-quantitative RT-PCRs on total BDNF mRNA extracted from stimulated and unstimulated control neurons treated with the transcription inhibitor actinomycinD. As reported in fig.8 panel B, there is no statistical difference between BDNF mRNA stability in control and stimulated conditions at all the time points considered, arguing for a specific transport dependent mechanism initiated by KCl, BDNF and NT-3. To confirm the biological activity of the neurotrophins used throughout this study, c-fos immunostaining on neuroblastoma SK-N-BE cells was performed. The strong nuclear c-fos labeling seen after KCl and NTs application was indicative of their active biological role, ruling out the possibility that any lack of response was due to inactive neurotrophin signaling (fig. 9).

Fig.9. c-fos immunostaining on stimulated SK-N-BE to demonstrate the biological activity of KCl and neurotrophins. Strong nuclear signaling is detected in most of the labeled cells. Scale bar, 10μm.

### 8.2 Analysis of BDNF mRNA localization signals

Given the high complexity of BDNF gene structure leading to the expression of up to 22 different splice variants, one goal of this PhD study was to dissect the role of each different UTRs with respect to the regulation of BDNF mRNA subcellular localization. Previous studies carried by Cristina Chiaruttini in our laboratory demonstrated that chimaeric rat BDNF-GFP constructs display a different localization in neurons. In particular, rEx1-CDS-GFP and rEx4-CDS-GFP constructs were localized within the first 30% of the dendritic compartment whilst labeling for rEx2B-CDS-GFP, rEx2C-CDS-GFP and rEx6-CDS-GFP extended into distal dendrites. Remarkably, the localization of these
constructs was not affected by extracellular stimuli like 10mM KCl and paralleled that of the corresponding endogenous transcripts in vivo even though they lacked the BDNF 3’ UTR (Chiaruttini et al, 2008). Thus, these findings are consistent with the presence of dendritic targeting signals in exons 2 and 6. Surprisingly, rCDS-GFP alone showed a comparable distal localization expressed as relative dendritic filling (RDF). The relative dendritic filling index (RDF) was determined by the MDDL normalized to the length of the dendrites as determined by MAP2 immunostaining. For the rat CDS, the RDF has resulted to be 69.97±2.38 (P<0.001 vs. GFP). Control constructs had the expected localization and consisted of GFP alone (RDF 32.95±1.60, proximal dendritic localization), GFP-α tubulin (RDF 15.26±1.31, somatic localization) and GFP-α subunit of Ca2+‑calmodulin-dependent protein kinase II (RDF 82.49±6.69, activity-dependent targeting to dendrites) (Mori et al, 2000). This unexpected finding indicates that, conversely, the CDS contains a dendritic targeting signal that is suppressed by exons 1 and 4, and seems to be constitutively active since it is not sensitive, like the exon containing chimaeras, to KCl stimulation. In addition, using the MS2 RNA-time lapse imaging system (for details, see materials and methods), we confirmed in living neurons that the dendritic trafficking of rCDS labeled granules is not affected by KCl treatment (fig. 10). Most MS2-rCDS granules (75%) had anterograde or retrograde movements with average velocity 0.12µm/s ±0.018 (max 0.26 µm/s; min. 0.07 µm/s), while the remaining 25% oscillated around a fixed position. Conversely, MS2-control granules did not display extrasomatic localization, since the control vector did not encode for a dendritic targeting element.

Fig.10 Video time-lapse analysis of living neurons shows no effect of KCl treatment on dendritic trafficking of MS2-GFP-labeled rCDS RNA granules. The plasmid encoding the MS2-binding protein fused with GFP, or the GFP alone (GFP), has a nuclear localization (GFP/MS2), but diffuses in the soma and dendrites cytoplasm when co-transfected with a 6_MS2-BDNF CDS construct (GFP/MS2-CDS). MS2-BDNF CDS (MS-CDS) moving granules (75% of total granules) recorded over 10 min, move at the same speed as those under 10mMKCl depolarization. Oscillatory granules (25% total granules) do not change behavior during 10mindepolarization with 10 mM KCl.
8.2.1 The coding region contains a constitutive dendritic targeting signal

To help identifying the dendritic targeting signal(s) present in the coding region, the rat and human sequences were scanned with BIOEDIT and ClustalW software to search for conserved recognition sequences for RNA binding proteins involved in dendritic RNA targeting. This sequence analysis revealed the presence of a strikingly conserved, bipartite recognition site (rBDNF CDS nucleotides 173–181 and 193–205; Fig. 11A), for the single stranded DNA/RNA binding protein translin, also known as testis brain-RNA binding protein (TB-RBP) (Kwon & Hecht, 1993; Li et al, 2008). These two elements are 70% and 80% identical to the rat Protamine-2 Y' and H' elements, respectively. Of note, the position of the H element within the human BDNF sequence overlaps the location of the G196A (Val66Met) mutation (Freudenberg-Hua et al, 2003) (arrow in Fig. 11A), which has been shown to impair dendritic localization of BDNF and is associated with cognitive impairment and neuropsychiatric disorders (Egan et al, 2003). To determine whether this region is required for dendritic trafficking of the CDS-GFP chimaeric construct, we tested in primary neurons two deletion chimaeras: \(\Delta(68–212)\)-rCDS-GFP and \(\Delta(1–202)\)-rCDS-GFP. Both constructs had somatic expression levels that did not differ from that of GFP and displayed RDF values lower than that of the intact rCDS-GFP sequence (fig. 11), suggesting that these deletions disrupt the constitutive dendritic targeting signal present in the CDS.

Based on these encouraging observations, we proceeded to assess the involvement of translin in dendritic targeting of BDNF mRNA by using an siRNA “cocktail” against translin (si translin), generated by digestion of a 300-bp fragment of translin (Fig. 12B). Si-translin produced 72% down-regulation of endogenous translin mRNA and 73% reduction of translin protein in hippocampal
cultures without interfering with control GAPDH RNA or tubulin protein expression (Fig. 13A and B). Dendritic targeting of both rat and human BDNF CDS-GFP constructs was significantly reduced by si-translin \( (P<0.001; \text{Fig. } 12B) \) leading to RDF values close to that of GFP alone (GFP RDF 32.06±3.12). Similar effects on dendritic targeting of transfected BDNF rCDS-GFP, were obtained by silencing translin with two defined siRNA oligo sequences used either alone or in combination (si690, si744; Fig. 12B), that we found able to silence endogenous translin in rat cortical cultures (fig.13 C).

Fig. 12. Role of translin in BDNF mRNA targeting. (A) Conserved translin binding Y and H elements in human and rat BDNF and mouse Protamine2. (B) Inhibition of the rat and human CDS-GFP RDF values with an siRNA translin mixture (rat \( ***, P < 0.001 \); human \( \delta\delta\delta, P < 0.001 \) vs. control), or two siRNA oligos used either alone or in combination (si690, si744), mutated si744 (si744M) had no effect. Inhibition of CDS-GFP dendritic targeting by si690 and si744 was rescued by co-transfection with siRNA-insensitive human translin. (C) Endogenous BDNF mRNA dendritic targeting up-regulation by KCl (\( \text{***, } P<0.001 \) Ctrl_KCl vs. Ctrl), is inhibited by translin siRNA (\( \text{###, } P < 0.001 \) si-translin KCl vs. Ctrl_KCl), or si690 and si744, but not by scrambled siRNA (NS, not significant). (D) GFP-3_UTRlong displays an activity-dependent targeting to dendrites in response to 3 h 10 mM KCl that is not affected by si-translin. (E) (Upper) trax antibody immunoprecipitates Translin (arrowhead).Coprecipitation of translin is blocked by omission of Trax antibody or preincubation with its antigen peptide. (Lower) RT-PCR analysis of the immunoprecipitated RNA:protein complexes. BDNF mRNA was detected only in the “offered” sample and the IP pellet containing translin. (F) A radiolabelled segment of the human CDS region containing the Y and H elements (nucleotides 176–211) forms two gel-shift bands after incubation with rat brain extracts.
The lower band (arrowhead “Translin”) is “supershifted” with Trax antibody (marked with *). Upper band (small arrow) reflects binding of this probe with an unrelated complex.

In contrast, a scrambled siRNA oligo generated by inserting multiple mismatches into si744 si744M) had no effect on BDNF rCDS-GFP dendritic targeting (Fig. 12B). As human translin contains multiple mismatches with both si690 and si744, we assessed whether it would reverse their inhibitory effects. As expected, cotransfection of human translin with si690 and si744 rescued the constitutive dendritic targeting of the BDNF rCDS-GFP construct (Fig. 12B), confirming that the inhibitory effect of these siRNA oligos is due to silencing endogenous translin.

Fig. 13. Analysis of efficacy and specificity of siRNA- and shRNA-mediated translin knockdown.

(a) Semiquantitive RT-PCR showed that siRNA to translin downregulated translin mRNA by 72% while the signal for the housekeeping gene GAPDH was unaffected. (b) Western blot analysis of hippocampal neurons transfected with si-translin showed a 73% down-regulation of translin protein. (c) The ability of defined shRNA sequences to decrease endogenous expression of translin was tested in primary cortical cultures following infection with lentiviral constructs containing shRNA inserts. Immunoblots show that two sequences, called 690 and 744, reduce translin protein expression. Of note, deletion of translin also reduces trax protein levels. β-actin blot shown as a normalizing control. In contrast, the sequence generated by inserting multiple mismatches into 744, called 744M, did not decrease either translin or trax protein levels. For the values presented in the bar graph, the intensity of the translin bands was adjusted for changes in β-actin levels. Translin levels in cultures treated with either 690 or 744 were significantly reduced (*, P < 0.05).

Taken together, these studies demonstrate that translin mediates a constitutive dendritic targeting signal located in the CDS and this targeting mechanism is conserved in rat and human mRNAs. As the constitutive targeting signal was identified with an artificial truncated BDNF construct, its physiological relevance was still unclear. Accordingly, we examined translin’s role in dendritic targeting of endogenous, full-length BDNF mRNA. To this end, primary hippocampal cell cultures were transfected with si-translin or a control, scrambled, siRNA and then depolarized with 10 mM KCl for 3 h. In accordance with previous studies (Tongiorgi et al, 1997), depolarization induced a strong RDF increase for endogenous full-length BDNF mRNA (from CTRL 29.18 ± 0.74 , to 85.37±2.18 for 10 mM KCl; P<0.001). However, in the presence of si-translin and KCl, the RDF only increased to 40.85 ±1.6 (P<0.001 with respect to KCl alone), while a scrambled siRNA had no significant effect (Fig. 12C).
Similarly, activity-induced dendritic targeting of endogenous BDNF mRNA was abolished by transfecting neurons with si690 and si744 either alone or in combination (Fig. 12C). Of note, si-translin did not block the ability of KCl to induce dendritic trafficking of the long 3' UTR fragment of BDNF mRNA that encodes an electrical activity dependent targeting signal (see paragraph 8.3) (Fig. 12D). Taken together, these results suggest that the constitutive dendritic targeting signal located in the CDS, which depends on translin, also plays a critical role in mediating the inducible targeting of full-length BDNF mRNA into distal dendrites.

In collaboration with prof. Baraban at Johns Hopkins School of Medicine (Baltimore, USA), we were able to demonstrate through co-immunoprecipitation and supershift experiments that translin effectively interacts in vivo with BDNF mRNA together with its partner Trax (Translin associated factor X) (fig. 12E and F). Omission of trax antibody or its preincubation with the antigen peptide blocked immunoprecipitation of both translin and BDNF mRNA. Thus, these results indicate that BDNF mRNA is associated with the translin complex in vivo. To check whether translin binds to the segment of the CDS containing adjacent Y- and H-like elements, gel-shift studies employing forebrain homogenates using this recognition element as probe confirmed that endogenous translin/trax complex binds to this sequence on BDNF mRNA. Moreover, in collaboration with dr. Braiuca and prof. Gardossi at the Department of Pharmacological sciences at the University of Trieste, we created an in silico 3D interaction model between two translin monomers and a 48nt long RNA stretch encompassing the translin recognition site.

Fig. 14. Three-dimensional modeling of translin dimer binding to human BDNF mRNA. (A) Minimal consensus RNA sequence for binding to the translin complex with two critical G residues separated by a poly U spacer of variable length. In human BDNF, spacing of the relevant G residues (G178 and G196, in bold) is constrained by formation of a stem-loop. (B) and (C) Interaction of G178 and G196 and position of the stem-loop on BDNF mRNA with a translin dimer (monomers in two different brown shades). (D) Modeling of the eight BDNF mRNA nucleotides interacting with a translin dimer. (E) Translin amino acids His-90, Arg-21, Glu-89 form three hydrogen bonds with G196 (normal BDNF) but only one with A196 (mutated BDNF, in F). (G) Summary of the nucleotide:amino acid selectivity for each of the four binding sites on translin.
Thanks to this computational analysis, we were able to identify four adjacent binding sites for individual ribonucleotide bases on each translin monomer: the first one is mainly formed by Arg-86, the second one is delimited by His-90, Arg-21, and Glu-89, and the third and fourth sites are less distinct, as they are globally delimited by His-88, Asp 136, and Arg 92 (Fig. 14 B–D and G).

Moreover, computer simulation predicted that the RNA would fold into a 5’-3’ “M” conformation, in which the central part would form a stem loop structure whereas each of the external bars of the M (ascending and descending, respectively) might interact with the four-nucleotide binding site present on each translin monomer (fig. 14A). To test the prediction made by in silico 3D modeling that the G196A SNP impairs binding of the BDNF oligo to the translin/trax complex, our collaborators at Johns Hopkins performed gel-shift competition assays with a wildtype human BDNF oligo spanning nucleotides 176–199 and the G196A version of the same segment. Since the docking simulation also predicts that G178 binds to the highly selective site 2 on the other translin monomer, we also examined the impact of mutating this G residue, as well as its neighbor G177 (Fig. 15A). While 10 nM of wild type or G177U BDNF RNA was sufficient to displace about 85% of a labeled rat protamine-2 oligo probe containing the translin binding site, the mutated G196A or G178U oligos were able to displace only 50% of the protamine-2 probe, as would be expected if these oligos have lower affinity for the complex (Fig. 15A, and B). In agreement with the model, mutating both these key G residues, G178 and G196, almost completely abolished the ability of this oligo to inhibit binding of the protamine-2 probe.

Fig. 15. G178 and G196 mediate binding to translin. (A) Stem-loop structure and the nucleotides (in gray) interacting with translin in the oligo used for these experiments. (B) In competition gel-shift assays, significant loss in inhibitory potency is produced by the G178U and G196A substitutions. The double mutation G178U/G196A markedly decreases its ability to compete with the WT Prm-2 probe. The G177U mutant does not differ from WT, and the inhibitory activity of the 177U/G196A double mutant equals that of G196A mutant.
In contrast, mutating G177 and G196 did not decrease its inhibitory activity below that of the single G196A mutation (Fig. 15 B and C). Taken together, these competition data strongly support the in silico modeling that predicts G178 and G196 to be the crucial residues for binding of BDNF mRNA to translin. These encouraging results prompted us to investigate if the G residues in position 178 and 196 actually represent the critical residues bound by translin. We therefore tested this prediction transfecting primary rat hippocampal neurons with the human BDNF-GFP chimeras containing either the normal G196 allele (Val) or the mutated G196A (Met) version. In addition, we tested whether mutations at G177 and G178 residues would exert similar effects on BDNF mRNA targeting as they do on binding to the translin complex. As predicted, we found that dendritic the G196A form of human BDNF transcripts (hCDS Met-GFP) was markedly reduced compared with the normal allele ($P < 0.001$, Fig. 16B). Consistent with the competition assays, G178U, as well as combined mutation of both G178 and G196, also decreased CDS dendritic localization. Of note, G196A, G178U, and G196A/G178U showed decreases of similar magnitude ($\approx 30\%$ decrease), suggesting that changing either of these key G residues is sufficient to achieve the maximal possible impairment of the translin-mediated dendritic targeting of the CDS-GFP construct (Fig. 16 A and B; $P < 0.001$). In contrast, G177U, which does not impair binding to translin, also did not affect targeting of the human BDNF CDS (Fig. 16B). Of note, si-translin has little further inhibitory effect on targeting of the G196A construct (hCDS Met-GFP), even though it strongly inhibits either rat or human wild-type CDS. (Fig. 16C).

Fig.16. Impact of human G196A mutation on BDNF mRNA targeting. (A) In situ hybridization on hippocampal neurons transfected with human BDNF CDS-GFP with mutations at G177,G178 and G196. Arrows show the MDDL.(Scale bar, 10 $\mu$m.) (B) hCDS-G178U-, hCDS-G196A-, and hCDS-G178U/G196AGFP mRNAs show lower RDF values than hCDS-G196-GFP (***, $P < 0.001$), hCDS-G177U had no effect (NS). (C) Translin silencing impairs dendritic targeting of human BDNF CDS mRNA (***, $P < 0.001$) but has a smaller effect on hCDS Met-GFP (phi symbol, $P < 0.05$ vs. hCDS Met-GFP without siRNA). Error bars, SEM.
The marked difference in dendritic targeting noted between G196 (Val) and G196A (Met) BDNF constructs was not due to their effects on the degree of arborization of transfected neurons, because we did not find any difference in the total length of processes of the in situ positive neurons 24 h posttransfection with mutant or normal human BDNF constructs (hCDS Val- GFP 186.35±2.05 mm; hCDS Met-GFP 184±6.65 µm). As a final demonstration of the importance of translin binding for the correct BDNF mRNA sorting in dendrites, we investigated the impact of the G196A mutation in a knock in animal model carrying this SNP on both BDNF alleles (Chen et al, 2006). BDNF mRNA is targeted to distal dendrites of hippocampal neurons upon stimulation with the proepileptic drug pilocarpine (fig. 17), in agreement with previous studies done in rats (Tongiorgi et al, 2004). Although Val/Val and Met/Met mice showed comparable seizure activity in response to pilocarpine, dendritic targeting of BDNF mRNA in hippocampal neurons was abolished in G196A (Met/Met) mice (fig. 17).

Fig. 17. In situ hybridization for BDNF mRNA on hippocampal sections from wt (Val/Val) and Met/Met knock-in mice. In saline-treated mice, BDNF mRNA is confined to the cell body layers of CA1 (Left) and CA3 (Right) in both wt and Met/Met mice (Upper). Upon pilocarpine treatment for 3 h (PILO), BDNF mRNA labeling is apparent in dendritic regions (stratum radiatum) of CA1 and CA3 neurons in Val/Val mice, but not in Met/Met mutant mice (Lower). (Scale bar, 100 µm.)
Thus, these findings indicate that the inhibitory effect of G196A on dendritic targeting is not limited to the truncated CDS transcript but also applies to endogenous, full-length BDNF mRNA. These important results shed new insights on the physiological implications of this simple but deleterious mutation in human beings affected by obsessive compulsive disorders. It has been proposed that the Val66Met mutation impairs the correct intracellular sorting and secretion of BDNF protein, leading to the phenotypic characters and memory deficits (Bath & Lee, 2006; Chen et al, 2006; Pezawas et al, 2004). Thanks to these results we provide further evidence that there is an upstream deficit at the level of dendritic BDNF mRNA targeting that might account for a reduced distal BDNF protein availability related to the reduction in the complexity of the dendritic arbor induced by this mutation. The work presented here has recently been published in PNAS ("Dendritic trafficking of BDNF mRNA is mediated by translin and blocked by the G196A (Val66Met) mutation" C. Chiaruttini*, A. Vicario*, Z. Li, G. Baj, P. Braiuca, Y. Wu, F. S. Lee, L. Gardossi, J. M. Baraban and E. Tongiorgi, PNAS (2009). *CC and AV are equally contributing as first author)

8.2.2 The 3’UTRs contain inducible signals mediating BDNF mRNA activity dependent targeting

Given the unexpected finding that the coding region of BDNF contains a constitutive dendritic targeting element and none of the 5’UTRs responds to extracellular stimulation, we hypothesized that the two different 3’UTRs of BDNF may contain the activity dependent signals that mediate the KCl- and neurotrophin-dependent BDNF mRNA translocation in dendrites. A recent work highlighted that the long 3’UTR but not the short 3’UTR, is involved in mediating BDNF mRNA dendritic sorting and translation in resting neurons, but did not investigate the effect of neuronal activity with respect to its localization (An et al, 2008). To study more in detail the role of 3’UTRs, primary rat hippocampal neurons were transfected with the GFP-3’UTR short or long chimaeras, and 24h later neurons were stimulated for 3h with either 10mM KCl or 50ng/mL of NT-3 or BDNF. Afterwards, neurons were subjected to standard ISH using a DIG-labeled GFP antisense probe, and the localization of the mRNA in positive transfected cells was measured (fig 18). To rule out any unspecific staining, control ISHs using either GFP sense probes on transfected cells or either antisense probes on non transfected neurons have been performed (fig.18D, right panel). In resting neurons, the short 3’UTR was mainly confined in the proximal dendritic compartment (MDDL 41.56±0.82µm) in accordance with a previous study by An et al. (2008) and similarly to the GFP reporter alone (MDDL 40.34±1.72µm), whose localization is not influenced by neuronal depolarization (MDDL 40.36±1.87µm). However, the short 3’UTR was found to promote a significant increase in reporter mRNA localization in distal
dendrites either after high potassium depolarization (MDDL 80.57±1.94µm, P<0.001 respect to GFP-3’UTR short) or treatment with NT-3 (MDDL 69.14±1.61µm, P<0.001 respect to GFP-3’UTR short) but not with NGF, BNDF or NT-4 (Fig. 18A,B).

![Fig.18. Activity dependent localization of BDNF 3’UTRs. A, D) In situ hybridization on hippocampal neurons transfected with either GFP-3’UTR short or long. Panel D, right side, control in situ hybridizations using GFP sense probe on transfected cells (upper picture) and GFP antisense probe on non transfected cells (lower picture). Arrows indicate MDDL. (Scale bar 10µm) B)10MmKCl and 50ng/mL NT-3 induce a significant increase in mean 3’UTR short MDDL (respectively 80.57±1.94µm and 69.14±1.61µm, ***P<0.001 respect to GFP-3’UTR short). C,F) densitometric analysis of neurons transfected with GFP-3’UTR short (C) or long (F) treated with different agonists. Increased signals along dendrites have been detected in both GFP-3’UTR short and long transfected neurons after KCl/NT-3 and KCl/BDNF application respectively (***, P<0.001 respect to GFP). Data are in accordance with the mean MDDL observed in each case.](image-url)
The broad Trk tyrosine kinase inhibitor K252a abolished the GFP-3’UTRsh reporter dendritic localization in response to NT-3 application (Fig.18B). Similarly, also KCl-induced translocation was significantly impaired, therefore indicating that a NT-3-dependent component plays a significant role during neuronal depolarization (Fig.18B). However, the BDNF-sensitive region remained still unknown, since the short 3’UTR delivery in dendrites was unaffected by this neurotrophin. These initial results prompted us to investigate the regulation of long 3’UTR dendritic targeting. In resting conditions, the localization of the GFP-3’UTR long chimera did not differ significantly from the GFP reporter alone (MDDL 43.14±1.36 µm). However, depolarization induced a strong increase in dendritic localization of this construct mRNA (MDDL 62.44±1.44 µm, P<0.001 respect to GFP-3’UTR long) (fig. 18D, E and F). Furthermore, we found that with 50ng/mL BDNF the dendritic localization of the 3’UTR long reached distances from the soma comparable to those seen after high potassium depolarization (MDDL 61.38±1.92 µm). Accordingly, K252a application significantly abolished both BDNF and KCL dependent mRNA targeting, suggesting that BDNF signaling may largely account for the effect of KCl depolarization on BDNF 3’UTR long mRNA localization (fig.18E). Surprisingly, even if the 3’UTR long does contain the nucleotide sequence of 3’UTR short, it did not result sensitive to NT-3 signaling, arguing for a different mechanism of control of neurotrophin-dependent subcellular localization for these two variants. The same data have been quantified through a densitometric analysis of in situ signal throughout the entire dendritic length of positive neurons, leading to results comparable to those obtained with MDDL measurements (fig. 18C and F).

Taken together, these data provide strong evidence that the inducible signals responsible for the activity dependent localization of BDNF mRNA lie at the level of the two 3’UTR regions. These signals are modulated by neuronal depolarization (KCl) for both regions but are specifically regulated by NT-3 for 3’UTR short and BDNF for 3’UTR long. To confirm these in vitro data accounting for an active role of 3’UTR short in mediating BDNF mRNA sorting, we took advance of a transgenic mouse model lacking of a functional long 3’UTR (Gorski et al, 2003). As shown in figure 19, in both wild type and BDNF<sup>klox</sup> mice, BDNF mRNA is confined in the proximal dendritic compartment of CA1 and CA3 neurons in resting conditions and dendritic staining in resting BDNF<sup>klox</sup> mice was reduced with respect to wt animals as described previously (An et al, 2008). On the basis of similar findings, An and coworkers concluded that only the long 3’UTR results necessary for BDNF mRNA targeting into dendrites (An et al, 2008). However, after treatment with the proepileptic drug pilocarpine for 3h, a dramatic accumulation of dendritic BDNF transcripts was observed in wt as well as BDNF<sup>klox</sup> animals, confirming our finding that under also BDNF mRNA with a short 3’UTR can be transported in dendrites after stimulation (see paragraph 8.2.1) (fig 19, right panel).
Fig. 19. BDNF 3’UTR short is targeted in dendrites. Left panel, proximal BDNF mRNA localization in control conditions in wild type (wt) and transgenic mouse (Klox). A dramatic increase of dendritic BDNF messenger content has been observed after 3 hours of pilocarpine treatment (300mg/kg) in both wildtype and klox animals, confirming the previous in vitro data accounting for an active role of short 3’UTR in mediating BDNF mRNA activity dependent dendritic sorting. Scale bar, 20 µm.

Interestingly, in BDNF\textsuperscript{klox} neurons a punctuate distribution of BDNF messenger was observed, whilst a more uniform one was seen in wild-type animals. These differences may be due to the fact that the long 3’UTR is lacking from distal compartments of BDNF\textsuperscript{klox} neurons and therefore the staining for BDNF mRNA is generally weaker.

8.2.3 The short and long 3’UTRs require a different but partially overlapping set of RNA binding proteins for their inducible localization

Given that the two 3’UTR forms responded to different stimuli, we searched for putative RNA binding proteins that could account for this differential regulation. To this aim, we carried an exhaustive bioinformatic analysis out on both sequences using clustalW and bioedit softwares. We took in consideration only the most conserved sites on BDNF mRNA among different species (Rno, Hsa, Ptr, Mml, Oga, Cpo, Ocu, Sar, Eeu, Cfa, Fca, Eca, Bta, Dno, Laf, Ete, Mdo, Oan, Aca, Gga, Xtr) because they might be more indicative for a conserved biological function. As illustrated in figure 20, the short 3’UTR (1-321) displays two different conserved sites for ELAV proteins (the 5’ most is...
referred as “ELAV up” and the 3’most as “ELAV down”), one for pumilio in addition to one CPEB binding site.

![Fig.20 Putative binding sites for known RNA binding proteins on rat BDNF 3’UTR mRNA following bioinformatic analysis. Only conserved sites among species are shown.](image)

Of note, the short 3’UTR is highly conserved among species, suggesting its critical role in regulating BDNF protein expression. On the other side, even if the long 3’UTR is less conserved, we have identified three conserved “hot spots” (red lines) containing several candidate recognition sequences for RBPs. The first conserved sequence presents many clustered binding sites for ELAV1 (HuR) and has been named “ELAV rich region”. A nearby second conserved sequence of the 3’UTR long is characterized by a G-quartet like structure, known to be recognized by FMRP. Finally, a third region at the end of the long 3’UTR encodes a pattern of cis-elements similar to the one found in the short 3’UTR (i.e. CPEs and ELAV recognition sites). In this study we did not analyze the potential effect of miRNAs on BDNF expression, since it was beyond the principal aim of this project and a recent study already addressed this issue (Mellios et al, 2008).

To test the effective contribution of these proteins with respect to BDNF mRNA targeting, we looked at any alteration in 3’UTR short or long dendritic localization in presence of siRNAs made against each of these RNA binding proteins. Starting from the short 3’UTR, we cotransfected primary neurons with GFP-3’UTR short together with siRNA cocktails against each CPEB protein (siRNA specific for CPEB-1, -2, -3 and -4) or ELAVs separately (siRNAs specific for ELAV-1, -2, -3, and -4). Analysis of siRNA efficacy showed a high degree of reduction in the relative proteins and a high specificity for all siRNA cocktails used (see Fig. 21 for silencing efficacy and Fig. 22 for specificity).
Fig. 21. Analysis of efficacy and specificity of siRNA-mediated knockdown of the RBPs considered in the present study. PC12 rat pheochromocytoma cells were silenced for 36 hours with 19nM of each siRNA mixture. Protein extracts were then subjected to standard western blot with specific antibodies (see materials and methods).

To rule out any possible siRNA-induced off target effect among homologous members of the CPEB and ELAV family, we performed a series of RT-PCR reactions. The results obtained demonstrate that each siRNA cocktail specifically targets only the desired isoform and not the other ones (fig 22).

Fig. 22. Representative PCRs of the silencing effect among CPEB (left) and ELAV (right) family members. No off target effects were observed during silencing of each specific isoform. CPEB3 displayed a very faint mRNA expression in primary neurons but a significant one in rat brain.

As reported in figure 23, panel A, activity-dependent localization of the GFP reporter requires the activation of both CPEB-1 and -2, but not CPEB-3 or -4 (CPEB1 siRNA KCL MDDL 37.16±1.12 μm, CPEB2 siRNA KCL MDDL 37.48±1.63 μm, ****P<0.001 respect to KCl no siRNA; CPEB1 siRNA NT-3 MDDL 37.69±1.09 μm, CPEB2 siRNA NT-3 MDDL 38.96±1.18 μm , ### P<0.001 respect to NT-3 no siRNA). A control, luciferase-specific siRNA has no effect on BDNF mRNA targeting. To further confirm the role of CPEBs, we introduced point mutations to disrupt the CPEB binding site on the short 3’UTR (UUUUUAU→CGAUCG, fig 23B). This mutated chimaeric mRNA completely lost its ability to travel in distal dendrites, confirming the fundamental role of CPEB1 and 2 during activity-dependent mRNA
A comprehensive analysis of the molecular mechanisms of BDNF mRNA localization and translation in neurons. However, it is unclear if CPEB 2 can bind directly to the mRNA since UUUUAU is the consensus sequence for CPEB1, and a specific CPEB2 recognition site has not been described yet (Huang et al., 2006). When analyzing the ELAV family, we found that both ELAV-2 and -4 are required for the correct 3’UTR short KCL- and NT-3-dependent localization (ELAV2 siRNA KCL MDDL 43.57±1.99 µm, ELAV4 siRNA NT-3 MDDL 41.32±1.39 µm, ***P<0.001 respect to KCl no siRNA; ELAV2 siRNA NT-3 MDDL 42.53±1.28 µm, ELAV4 siRNA NT-3 MDDL 41.48±1.14 µm, ***P<0.001 respect to NT-3 no siRNA) (fig 22C). The other two family members, ELAV-1 and -3, did not result involved in BDNF subcellular localization. Involvement of ELAV-2 and -4 proteins is confirmed by the fact that disruption of either the S’ or 3’ ELAV binding sites (AUUUAU→AGCGCG) severely impairs the correct BDNF 3’UTR dendritic targeting. It is conceivable that ELAV-2 and -4 may bind separately to the two cis-elements or either form heterodimers that subsequently may contact the two recognition sites.

Fig. 23. CPEB and ELAV proteins are involved in 3’short activity dependent targeting. A) silencing of CPEB 1 and 2 selectively abolishes KCl and NT-3 induced 3’UTR short localization, whilst CPEB-3 and -4 do not (***, P<0.001 respect to ctrl no siRNA+Kcl, ###, P<0.001 respect to ctrl no siRNA +NT-3). B) A similar impairment in mRNA transport has been registered after selective mutation of the CPE binding site (***, P<0.001 respect to 3’sh wt+KCl, ###, P<0.001 respect to 3’sh wt +NT-3. C) knock down of either ELAV-2 or -4, but not ELAV-1 or -3, abrogates activity induced sorting of the short 3’UTR. The same effect is mimicked by point mutations on both ELAV recognition sites on BDNF 3’UTR short (D) (***, P<0.001 respect to ctrl no siRNA+KCl, ###, P<0.001 respect to ctrl no siRNA +NT-3).
The lack of dendritic localization of the 3′UTR mutants is related to a deficit in transcript transport and not to a decreased stability or expression, since the relative RNA levels of these mutants are comparable to the parental unmutated GFP and wild-type GFP 3′UTR short mRNAs (fig 24). In summary, these results clearly demonstrate that KCl and NT-3 are able to induce distal dendritic localization of BDNF 3′UTR short through a complex mechanism that requires the contextual intervention of CPEB-1, CPEB-2, ELAV-2 and ELAV-4.

After having identified some of the key proteins mediating 3′UTR short dendritic localization, we decided to determine which RBPs are responsible for the KCl- and BDNF-dependent sorting of the long 3′UTR isoform. We cotransfected primary neurons with the GFP-3′UTR long chimaera together with siRNAs specifically directed against CPEB and ELAV. We found that dendritic targeting the long 3′UTR requires only CPEB1 but not CPEB2, CPEB3 or CPEB4 (KCl MDDL 48.84±1.15 µm, *** P<0.001 respect to KCl no siRNA, BDNF MDDL 49.17±1.55 µm, ### P<0.001 respect to BDNF no siRNA) (fig 25 A).

This finding suggest that CPEB2 is specifically required in the context of the short 3′UTR and might potentially confer the responsiveness to NT-3 that characterizes the short but not the long 3′UTR. On the other side, with ELAVs silencing the situation appears more complicated. Indeed, we surprisingly found that ELAV-1, -3 and -4 downregulation promotes a global increase in 3′UTR long targeting even in resting conditions, in particular with siRNAs targeting ELAV1 (ctrl MDDL 59.90±1.31...
μm, ***P<0.001 respect to ctrl no siRNA; KCl MDDL 81.40±1.64 μm, *** P<0.001 respect to KCl no siRNA; BDNFMDDL 78.36±2.13 μm, ### P<0.001 respect to BDNF no siRNA). A similar increase in dendritic targeting of the 3'UTR long targeting found in resting conditions also when siRNAs against the proteins of the Fragile-X proteins (FXRPs) family FMRP1 and FXR2 (Fig 25C). Interestingly, activity- or neurotrophin-dependent dendritic targeting was normal when knocking down both FXRPs (Fig 25C).

Fig. 25 RNA binding proteins involved in the activity dependent sorting of BDNF 3'UTR long. A) siRNAs against CPEB family members show the importance of CPEB1, but not the other isoforms, in mediating KCl and BDNF induced localization. B) siRNA mediated knock down of all ELAV proteins induces either a basal or stimulus depend increase in the mRNA dendritic transport, with the strongest effect seen in absence of ELAV1. C) Similarly, silencing of either FMRP or FXR2P promotes a basal, but not activity induced enhancement of 3'long accumulation in distal dendrites. siRNAs against hnRNPA2, translin or control luciferase don’t induce any significant alteration in BDNF 3'long subcellular localization. D) GFP-3'UTR END displays a constitutive dendritic targeting respect to the full length sequence, and is moreover augmented after KCl or BDNF application, suggesting for the presence of an active DTE. E) The middle fragment (GFP-3'MID) does not contain any obvious DTE nor responds to stimulation (left), but it rather blocks the constitutive targeting of the CDS either in control conditions or after KCl application. This inhibitory activity is instead relieved only with BDNF treatment, implying the existence of a true retention signal whose activity is limited in presence of BDNF. (###P<0.001*P<0.01, ***P<0.001).
Additionally, we tested the role of other two RNA binding proteins, translin and hnRNPA2/B1. The former was used as an internal control since this protein is not involved in the sorting of 3’UTR long. The latter was tested since we found a putative A2RE11 recognition element in the terminal part of the 3’UTR long. However, we did not observe any significant alteration in the extent of BDNF mRNA targeting after hnRNPA2/B1 silencing (Fig 25C). The finding of an increased basal but not activity-dependent BDNF mRNA targeting in absence of FXRP proteins was confirmed in vivo in both FXR2P and FMR1 knockout mouse models sacrificed 3h after injection with pilocarpine (fig 26).

In conclusion, we found a specific set of RBPs able to exert a retention activity under resting conditions to confine BDNF mRNA with the long 3’UTR in the somato-proximal dendritic compartment. Of note, these “inhibitory” proteins display clustered recognition sites in the central region of the 3’UTR long that are very well conserved throughout the evolution (nts 890-1510, see figure 20). In summary, we found that CPEB-1, but not -2, promotes BDNF 3’UTR long sorting in dendrites during neuronal activity and we discovered that the FXRP family is able to block the basal dendritic targeting of BDNF transcript and that the and all four members of ELAV protein are able to control both basal and activity-induced BDNF mRNA dendritic trafficking.

On the basis of these unexpected but exciting results, we speculated that the central region of the 3’UTR long might actually represent a retention signal, able to counteract the constitutive dendritic targeting signal in the coding region of BDNF mRNA mediated by translin. On the other side, if this hypothesis were true, deletion of this region or expression of the terminal 3’UTR tail would display an increased dendritic targeting. To test this hypothesis, we cloned and transfected in primary neurons a construct expressing GFP in fusion with terminal 3’UTR long nucleotides (2339-2790, named GFP-3’UTR END). We found an increased basal targeting with respect to the full length

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**Fig 26.** Enhanced basal BDNF mRNA localization in CA1 and cortical neurons of FMR1 KO mice. Upper panel, increased basal dendritic BDNF mRNA localization in FMR1 KO (TG) CA1 neurons with respect to wt ones. After 3 hours of pilocarpine treatment a similar extent of BDNF mRNA dendritic sorting was observed. A similar enhanced basal targeting has been observed in layer V cortical neurons. Scale bar, 10µm.

In conclusion, we found a specific set of RBPs able to exert a retention activity under resting conditions to confine BDNF mRNA with the long 3’UTR in the somato-proximal dendritic compartment. Of note, these “inhibitory” proteins display clustered recognition sites in the central region of the 3’UTR long that are very well conserved throughout the evolution (nts 890-1510, see figure 20). In summary, we found that CPEB-1, but not -2, promotes BDNF 3’UTR long sorting in dendrites during neuronal activity and we discovered that the FXRP family is able to block the basal dendritic targeting of BDNF transcript and that the and all four members of ELAV protein are able to control both basal and activity-induced BDNF mRNA dendritic trafficking.

On the basis of these unexpected but exciting results, we speculated that the central region of the 3’UTR long might actually represent a retention signal, able to counteract the constitutive dendritic targeting signal in the coding region of BDNF mRNA mediated by translin. On the other side, if this hypothesis were true, deletion of this region or expression of the terminal 3’UTR tail would display an increased dendritic targeting. To test this hypothesis, we cloned and transfected in primary neurons a construct expressing GFP in fusion with terminal 3’UTR long nucleotides (2339-2790, named GFP-3’UTR END). We found an increased basal targeting with respect to the full length
3'UTR long (MDDL 63.96±1.93 µm, ***P<0.001 respect to ctrl 3'UTR long), comparably to what observed for ELAV1 siRNA (fig.25C). At the same time, both KCl and BDNF induced a small but significant increase in transport with respect to 3'UTR long (3'UTR END KCL MDDL 73.41±2.61 µm, ***P<0.001 respect to KCI 3'UTR long; 3'UTR END BDNF MDDL 75.79±1.88µm, ###P<0.001 respect to BDNF 3'UTR long). Taken together, these results strongly suggest that the terminal sequence of BDNF 3'UTR long contains a constitutively active dendritic targeting element whose function can be further modulated by neuronal activity (KCL and BDNF). In addition we found evidence suggesting the presence of an upstream retention element since the full length 3'UTR long is not targeted in dendrites under resting conditions. These speculations prompted us to generate a second chimaera, encompassing nts 849-1533 of BDNF 3'UTR long in fusion with the GFP reported (GFP-3’UTR MID). However, to really understand if the MID region actually represents a retention element, it was necessary to determine if the MID region can counteract the constitutive dendritic targeting signal of the coding region. To this end, we analyzed the subcellular localization of a CDS-GFP-3’UTR MID chimaera in comparison to CDS-GFP known to be constitutively targeted in dendrites (fig 25E). We found that the 3'UTR MID fragments per se does not respond to any stimulus nor contain any DTE, but in the context of the coding region it does block the spontaneous dendritic localization mediated by translin (GFP-3’UTR MID ctrl MDDL 39.65±0.91 µm vs. CDS-GFP ctrl MDDL 57.25±2.06 µm, ***P<0.001). Notably, this retention is not relieved by stimulation with 10mM KCl (GFP-3’UTR-MID KCl MDDL 45.17±1,28µm, ***P<0.001 respect to KCl GFP-CDS) but only by BDNF (GFP-3’UTR-MID BDNF MDDL 61.85±1.63µm). In summary, these findings demonstrate that the region spanning nucleotides 849-1533 of 3’UTR long contains a retention signal able to override both constitutive signals, one located the terminal part of the 3’UTR long and one in the CDS. This retention signal is specifically relieved by BDNF and may account for the proximal BDNF mRNA localization seen under basal physiological conditions and may be missing in FMR1 and FXR2P knock out models causing an exaggerated dendritic accumulation of BDNF mRNA at rest.

8.3 The short and long 3’UTR display a differential translatability that may account for a distinct biological function

During the first part of this study, we found a different combination of stimuli and RBPs that drive a different response and subcellular localization of short and long 3’UTRs. Therefore, we continued our study searching for a physiological significance of this phenomenon. In a recent work, it has been demonstrated that in BDNF<sup>lox/lox</sup> mice, lacking of a functional long 3’UTR, there is a selective depletion of dendritic BDNF in hippocampal neurons, accompanied by a deficit in distal CA1 synapses
LTP, reduced spine head diameter and increased spine density (An et al, 2008). In light of these findings, we speculated that these variants may not only be regulated at the level of mRNA localization, but also from a translational point of view. In fact, it was already known that the short variant is more polyadenylated and potentially more translatable than the long one (Timmusk et al, 1994). Accordingly, we found that the long 3’UTR has a non canonical polyadenylation signal (AAUUAA) that may hypothetically confer a very low polyadenylation efficacy. By performing a standard polyadenylation assay (PAT) on hippocampal neuronal cultures, we confirmed this hypothesis as we found that the short 3’UTR displays a more extended poly(A) tail than the long 3’UTR (fig.27).

Fig.27. Increased poly(A) tail length of short 3’UTR respect to the long isoform. A) Upper panel, representative PAT assay for 3’UTR short tail, showing a long A tail also in resting conditions, slightly increased after KCL treatment. siRNA targeting CPEB1, but not 2, severely interferes with the normal polyadenylation process, as quantified in the lower panel. B) PAT assay on the long 3’UTR confirms a shorter adenosine tail respect to BDNF UTRsh, that is strictly dependent on CPEB1 and requires the activation of CPEB2 as well.

Surprisingly, we found that KCl-induced depolarization increases the poly(A) tail length of the short 3’UTR but not of the long 3’UTR and that both 3’UTRs require CPEB1 for the correct poly(A) extension, whilst CPEB2 seems to have a minor role (fig. 27B). On the basis of these results, we expected the 3’UTR short to be more easily translatable than the long 3’UTR, since the translation efficiency is directly related to poly(A) tail extension (Huang et al, 2003). To test this prediction, we analysed the translatability in SK-N-BE neuroblastoma cells of the same constructs used for the subcellular localization studies in relation to the downstream 3’UTR sequence (fig.28).
In the presence of the short 3’UTR sequence the GFP reporter showed 142% higher protein levels with respect to GFP alone (*, p<0.05) and mutation of the CPE or upstream ELAV binding sites severely impaired translation of the reporter (58% and 4% respectively, *P<0.05 respect to GFP). The relative levels of RNA, evaluated trough semi-quantitative RT-PCR, were constant among constructs, therefore any difference was imputable to a pure translational effect (see fig.24). In the same experiment, we also confirmed the poor translatability of the full length long 3’UTR (27% of GFP alone). However, when only the isolated terminal fragment was considered, we found a striking increase in translatability (+218%; *p<0.05). The intermediate region of the 3’UTR long had a comparable expression with respect to GFP, meaning that it does not contain any enhancing nor inhibitory element active on translation at least under control conditions. We then proceeded to analyze separately the behavior of 3’UTR short and relative mutants in different experimental conditions (fig.29). We started analyzing a set of possible stimuli able to promote an increase of GFP expression driven by 3’UTR short. We tested either 50mMKCl or 50ng/ml NT-3, since they induce mRNA transport, as well as 50ng/ml BDNF, BDNF paired to glutamate and glutamate alone.
Fig. 29. Effect of 3′UTR short on GFP expression in BE cells. A) Wild type 3′UTR short enhances reporter translation after KCl, BDNF and glutamate stimulation. NT-3 showed the greatest effect almost doubling the GFP expression (*P<0.05 respect to wt ctrl). B) CPE binding site mutation causes a marked decrease of reporter translation even in basal conditions. Any applied stimulus is able to restore the expression to basal levels (*P<0.05 respect to CPEmut ctrl). C) ELAVup mutation showed the highest repression, being only partially relieved after NT-3 and glutamate application (*P<0.05 respect to ELAVup mut ctrl). D) ELAVdw mutation blocked the KCl, BDNF and NT-3 increase of reporter translation, that was instead significantly impaired during glutamate mediated responses (*P<0.05 respect to ELAVdw mut ctrl). GFP expression for each construct in any condition was internally normalized to the relative tubulin levels and subsequently to ctrl GFP-3′UTR short wt. Results in stimulated conditions are represented as % of expression respect to their relative basal state (*P<0.05, paired t-Test).

The glutamate stimulus was chosen since CPEB, a putative interactor of 3′UTR short, has been previously described to be activated by glutamate signaling trough NMADR and Aurora Kinase activation. As compared to the basal expression, KCl, BDNF, 20µM glutamate and glutamate+BDNF were able at the same extent to enhance GFP expression under the control of 3′UTR short (respectively +49%, +53%, +64% and +49%, *P<0.05 respect to 3′UTR short GFP control). Notably, NT-3 had the greatest effect, almost doubling the GFP expression (fig. 29A). In order to unravel the possible mechanism/s responsible for this enhancement in GFP translation, we analyzed whether mutation of specific RNA binding protein sites would have any effect. As shown in fig.29B, mutation of CPE binding site had a dramatic effect on 3′UTR short mediated expression (-63% in control...
conditions respect to GFP-3’UTR short). However, all stimuli considered here were still able to enhance, even if at a lesser extent, GFP translation comparably to the basal 3’UTR short. We concluded that CPEB is required for the basal 3’UTRsh driven expression, but is only partially involved in KCl, BDNF, NT3 and glutamate responses, and among these stimuli is more important in mediating NT-3-dependent responses (-5% vs. +114% for CPEmut and 3’sh wt respectively). However, whether this is a direct effect of CPEB1 or 2 or is a secondary effect generated by the lack of interaction with other proteins is still unclear. The construct bearing the most disrupting mutation is the one affecting the upstream ELAV binding site (-78% respect to 3’UTR short wt) that resulted unable to respond to BDNF or KCl, and responded only partially to NT-3 and glutamate (fig 29C). We concluded that KCl- and BDNF-induced responses are dependent on the binding of the ELAV proteins to the upstream ELAV binding site. On the other hand, the partial response observed in absence of the CPEB binding site might be related to a lack of interaction with an ELAV partner. Accordingly, a residual NT-3 and glutamate response (+41% and +26% respect to basal ELAVup mutant) may be accounted by the reduced, but still significant activity of CPEB proteins. Additionally, since ELAVs seem to be involved in repression of miRNA activity (Bhattacharyya et al, 2006), this strong repression might be related to the constitutive activity of miRNAs, since in a bioinformatic study we recently found a very close and conserved miRNA-381 target site. Finally, mutation of the downstream ELAV binding site does not alter significantly the basal GFP expression levels, but it blocks the activity dependent activation of translation, similarly to the CPE mutation. This would further posit for an interaction between CPEB and ELAV family members during activity-dependent activation of BDNF mRNA translation. Surprisingly, mutation of the downstream ELAV site had deleterious effects on glutamate mediated responses, suggesting that this site is not involved in basal 3’UTRsh driven expression but is essential during glutamate stimulation (fig.29D). A possible interpretation is that in absence of the stabilizing activity of ELAVs the mRNA becomes degraded during glutamate excitation, or in alternative, that ELAV proteins may operate by repressing any inhibitory mechanism that would otherwise impede mRNA translation.

In conclusion, the greatest increase in BDNF protein expression depends on NT-3-driven activation of 3’UTR short, that is almost entirely mediated by CPEB1 and by binding of one ELAV isoform to the upstream recognition site. The latter has also revealed to be essential for the KCl- and BDNF-mediated responses, whilst the downstream ELAV binding site resulted to be involved not in basal, but in activation of translation induced by glutamate, since its disruption led to fatal decrease of reporter translatability. In this intricate scenario, it appears that CPEB and ELAVs are differentially able to respond to distinct agonists, but these proteins are influencing each other.
In the next series of experiments we analyzed the translational effect of the long 3'UTR and relative fragments (MID and END) in BE cells to. As reported in figure 30, after transfection of full length 3'UTR long we applied a similar set of agonists: 50mM KCl, 50ng/mL BDNF, or NT-3, 20µM Glu, Glu+BDNF, 50µMDHPG and 30µM NMDA+DHPG. DHPG was used since it is a well known activator of mGluR1 receptors, whose signaling leads to a concomitant activation of FMRP. This stimulation paradigm might therefore help us to elucidate any effect on BDNF mRNA translatability potentially mediated by FMRP.

Fig.30. 3'UTR long effect on GFP expression in BE cells. A) BDNF, but not significantly KCl nor NT-3 slightly enhanced the 3'UTR long mediated GFP expression (**P<0.01). Glutamate (glu), glu+BDNF, DHPG and DHPG+NMDA increasingly depressed the reporter expression in a significant manner respect to control conditions (*P<0.05; **P<0.01). B) GFP-3'long MID showed an increased basal expression respect to wild type, full length (FL) 3'UTR long. Almost all the stimuli applied depressed the translation efficiency of GFP, possibly via an mGluR and NMDAR dependent mechanism (P<0.05 respect to ctrl GFP-3'long MID. C) The terminal 3'UTR long fragment induced an enhanced GFP expression in resting conditions and after KCl or BDNF application. The translation levels were restored to the full length dependent ones after NT-3, glu, glu+BDNF and DHPG+NMDA (**P<0.05). GFP expression for each construct in any condition was internally normalized to the relative tubulin levels and subsequently to ctrl GFP-3'UTR full length. Results in stimulated conditions are represented as % of expression respect to their relative basal state ( *P<0.05; **P<0.01, paired t-Test).
The pairing of NMDA and DHPG was tested to prove potential responses mediated by CPEB (via NMDAR→AuroraA→CPEB) or FMRP (via DHPG). Interestingly, we found a small but significant increase in 3’long dependent GFP expression only with BDNF treatment (+14% respect to ctrl GFP-3’long**P<0.01) and we found a surprising decrease with either Glu, Glu+BDNF, DHPG and DHPG+NMDA (respectively -12%, -24%,-65% and -88% respect to the control, *P<0.05;**P<0.01) (fig.30A). These results suggest that BDNF is not only able to promote specifically the dendritic transport of the long 3’UTR form, but also its translation through a positive feedback loop. Moreover, the depression seen after glutamate application may be indicative of an mGluR activation rather than AMPARs or NMDARs, a result confirmed by the specific action of DHPG that in turn led to a significant overall decrease of reporter expression. Interestingly, the strongest effect seen with concomitant DHPG and NMDA application does not reflect any activation of CPEBs, but could be rather informative of an ongoing process of NMDAR and mGLUR-mediated LTD, that in turn would lead to a severe decrease in BDNF expression. Regarding the expression of the MID region of 3’UTR long, we confirmed its increased expression with respect to the full length 3’UTR (FL 3’UTR), but after stimulus application we found a global decrease of GFP expression comparable to the levels observed with the full 3’UTR long, regardless the stimulus used (fig. 30B). Of note, the MID 3’UTR long is also repressed by DHPG and DHPG+NMDA application (respectively -58%and -68% respect to ctrl GFP-3’long MID, *P<0.05), suggesting that this decrease may be an FMRP-dependent mechanism that acts specifically on this region, since the terminal 3’long fragment is not sensitive to DHPG (fig.30C). On the other side, we noticed that the MID 3’UTR long fragment conferred an enhanced reporter translation in both basal (+56% respect to control GFP-3’long), and either KCl or BDNF application (respectively +62% and +44% compared to control GFP-3’long), mirroring the BDNF and KCl responses observed in the context of the full length 3’UTR long. However, we are not able to find an exhaustive explanation about the apparent inhibitory role played by NT-3 on MID and END 3’long dependent-GFP expression, since this neurotrophin does not repress the full length 3’UTR long mediated translation (fig. 30A).

Finally, we proceeded through immunohistochemistry analysis on brain sections of wildtype and BDNF<sup>Klox</sup> mice, searching for possible differences in BDNF expression after 3h of strong electrical activity induced by the proepileptic drug pilocarpine. As reported in fig. 31, both in wt and BDNF<sup>Klox</sup> hippocampal neurons we detected a clear signal in dendrites, especially in CA1 region. These results strongly suggest that the short 3’UTR is also locally translated in dendrites, and that in absence of the long isoform the 3’UTR short may account for the localization of BDNF protein in distal dendrites. These results are in contrast with the observations of An and coworkers that were limited to animals.
under resting conditions (2008), but are in accordance with the ability of the 3'UTR short to be localized in dendrites and locally translated.

Fig.31. BDNF immunohistochemistry on wt and Klox brain slices shows a comparable localization of the protein. Left panel, BDNF staining in dendrites in control conditions. A strong dendritic labeling has been detected in both wt and klox animals, even if less uniform in CA1 region of Klox neurons. On the other hand, a stronger signal has been detected in dentate granule (dg) cells of transgenic animals (arrow, middle panel). Right panel, BDNF immunostaining after 6 hours of pilocarpine treatment. There is no obvious increase in dendritic BDNF content in both wt and klox CA1 neurons, both showing an evident vacuolization as consequence of epileptic seizures. Respect to resting conditions, there is a dramatic upregulation of BDNF expression in DG granule cells and stratum molecular (arrowheads) in either wt and klox neurons. Lower panel, negative control staining with only secondary HRP-conjugated antibody. Scale bar, 20µm
9 DISCUSSION

9.1 Main findings

In this study we investigated the molecular mechanisms regulating BDNF mRNA subcellular localization and its translation into protein. We analysed the role of the 5’UTRs, the coding region and the two 3’ untranslated regions (3’UTR short and long) and found a different set of proteins controlling mRNA dendritic sorting and, for the 3’UTRs, translation as well. The main findings can be summarized as follows:

1) We identified potassium-induced depolarization, BDNF and NT-3 as the three main stimuli able to induce endogenous BDNF mRNA sorting in neuronal dendrites in vitro.

2) We were able to demonstrate in vitro that none of the 5’UTR sequences considered (exon 1, 2B and 2C, 4 and 6) contains inducible dendritic targeting elements, but rather that the somatic isoforms (those containing exon 1 and 4) encode some still unidentified retention signals preventing their delivery in dendrites.

3) Surprisingly, we found that the driving force mediating constitutive BDNF mRNA targeting, in absence of its 3’UTRs, is mediated by a targeting element localized in the coding region and thanks to a detailed bioinformatic, biochemical and mutational/functional analysis we identified translin (also known as TB-RBP) as the interacting partner with BDNF mRNA at this site. Notably, we found that only two conserved guanosines (G178 and G196, human BDNF numeration) are necessary for a correct protein:RNA interaction. Mutation of either G causes a severe deficit in binding of translin to BDNF mRNA and a blockade of BDNF mRNA dendritic localization in vitro of the same magnitude as that observed in presence of translin targeting siRNAs.

4) We found that a common human single nucleotide polymorphism of BDNF gene, associated with eating and neuropsychiatric disorders (G196A or Val66Met), overlaps with one G of the translin binding site. This SNP had deleterious effects on both in vitro and in vivo BDNF transcript localization, in addition to a previously reported impaired activity-dependent secretion of BDNF<sub>Met</sub> (Chen et al, 2004; Egan et al, 2003). The novelty of our findings resides on the fact that besides an improper BDNF protein secretion there is a much more severe depletion of the dendritic fraction of BDNF mRNA that in normal conditions might potentially modulate and strengthen the force of the synapses experiencing long term plasticity.
5) We found that both short and long 3’UTR regions promote dendritic targeting of a GFP reporter mRNA in response to KCl and selectively to NT-3 (short 3’UTR) or BDNF (long 3’UTR). The latter findings are quite exciting since to date they represent the first description of one mRNA with two 3’UTR variants whose localization is selectively regulated by different sets of stimuli, with strong implications on the local modulation of synaptic plasticity.

6) Thanks to an exhaustive bioinformatic analysis and siRNA-based approach, we have identified two distinct sets of RNA binding proteins regulating BDNF 3’UTR short and long dendritic transport. In particular, we demonstrated that CPEB-1 and -2, together with ELAV-2 and -4 are essential for the NT-3- and KCL-dependent delivery of short 3’UTR splice variants in dendrites. On the other side, the long 3’UTR requires the activation of only CPEB1 for its localization and surprisingly, it contains inhibitory signals mediated by ELAVs (in particular ELAV1) and FXR proteins that modulate its basal and activity dependent sorting.

In light of these unexpected findings, we hypothesized that the long 3’UTR contains at least two separates sets of signals, one positively and one negatively regulating dendritic targeting. Therefore, analyzing the subcellular localization of the last conserved 500 nts of BDNF 3’UTR long, we found both a basal and stimulus dependent enrichment of this mRNA in dendrites, posing for the identification of a “permissive” DTE. These observations closely mirror the effects induced by the absence of either FXRs or ELAV1, that were expected to recognize as very conserved region in the middle of 3’UTR long (nts 849-1533). After isolating this fragment, hosting a retention signal, we demonstrated not only that it does not respond to any stimulus, but also it inhibits the constitutive dendritic localization of the CDS. Moreover, this retention is not relieved by KCl, but only by BDNF, therefore validating our hypothesis about the presence of two different sets of signals at the level of long 3’UTR. In front of these results, we reasoned whether the different stimulus dependent localization of these two 3’UTR variants may also be linked to a different translatability.

7) Using the very same constructs used for the localization studies, we found that the short 3’UTR positively influences the translatability of a GFP reporter that is further enhanced by different stimuli including NT-3. Furthermore, the high translational competence of the short 3’UTR has been validated in vivo, where we were able to detect strong labeling for BDNF protein in dendrites of neurons of a transgenic mouse lacking a of functional long 3’UTR. Additionally, thanks to a fine mutational analysis, we demonstrated that CPEB and ELAV proteins were actively involved in this process.
We also found that the 3’UTR long confers a very poor translatability to the GFP reporter, possibly because of a constitutively poorly active polyadenylation signal and the additional inhibitory effect of internal translation repressor sequences. A detailed analysis of the terminal 500 nucleotides of 3’UTR long sequence (END fragment) allowed us to conclude that it contains signals that positively modulates translation. We concluded that in the context of a full length 3’UTR long, there are at least one inhibitory element accounting for a poor translatability of the 3’UTR long under basal conditions.

9.2 Both 3’UTR short and long contain inducible, activity-dependent localization signals.

This study unravels the dynamics of activity dependent regulation of BDNF mRNA sorting in dendrites. Notably, we found that NT-3 and BDNF mutually control the subcellular localization of 3’UTR short and long respectively, and both of them respond to high potassium-induced depolarization. Even though the long 3’UTR does contain the nucleotide sequence of the short 3’UTR, its localization is not influenced at all by NT-3 signaling. This specificity may be accounted for an NT-3 sensitive element directly or indirectly interacting with the short 3’UTR (potentially CPEB2), whose access to the mRNA in the context of the long 3’UTR is not anymore allowed. As reported in figures 32 and 33 showing the secondary structure of the native 3’UTR short and long respectively, the CPE site at nts 93-98 (Fig. 32; red square) is no more accessible in the context of long 3’UTR folding. Up to date it is unknown if CPEB2 is influencing BDNF mRNA metabolism through direct mRNA contact or through an interaction with CPEB1. Nevertheless, due to the buried corresponding site in 3’UTR long, it is conceivable that CPEB1/2 are no more able to contact this sequence inside the long 3’UTR.
To further confirm the involvement of CPEBs in NT-3-mediated signaling, a recent work by Kundel and coworkers reported the that CPEB-1 mediates the NT-3-dependent beta-catenin mRNA translation in developing hippocampal neurons (Kundel et al, 2009). As reported by Huang and coworkers, CPEB-2, -3 and -4 seem to bind to a completely different sequence than the canonical CPE and may be regulated by p70S6 kinase rather than AuroraA (Huang et al, 2006). Since the latter specifically activates CPEB1 after NMDAR activation, one can hypothesize that KCl stimulation promotes both pre-synaptic neurotransmitter release (e.g. glutamate) leading to post-synaptic CPEB1 activation, and post-synaptic neurotrophin exocytosis (e.g. NT-3), promoting TrK-dependent p70S6K and CPEB2 activation. In this way, CPEB1 and 2 may respectively act as pre- and post-synaptic coincidence detectors modulating the localization and possibly the expression of such an important plasticity mediator like BDNF.

Interestingly, we found that the two ELAV binding sites on short 3’UTR (green circles, nts 4-9 and 148-153) are quite close in terms of secondary structure. This further corroborates the idea of a potential interaction between these two sites that would further explain why the absence of either ELAV-2 or ELAV-4 leads to a severe disruption of 3’UTR short targeting in dendrites. It has been reported in many cases that ELAVs are able to form omo- or hetero-dimers (Gao & Keene, 1996; Kasashima et al, 2002), and these reciprocal protein-protein interactions positively modulate their RNA binding activity. In this way, ELAV2-4 dimerization may allow the formation of RNA transporting granules able to interact with the cytoskeleton for transport and with the translational machinery as well, as suggested by Antic and colleagues (Antic & Keene, 1998; Gao & Keene, 1996).
Additionally, we found two other conserved sites in the 3’UTR short sequence, namely a nanos response element (NRE) and a CUG rich region, that represent respectively, one potential target for pumilio, a translational regulator (Wickens et al, 2002), or for CUG-binding protein, known to regulate splicing, deadenylation, mRNA stability and translation (Ladd et al, 2001; Moraes et al, 2006; Mori et al, 2008; Paillard et al, 2003; Philips et al, 1998; Savkur et al, 2001; Timchenko et al, 1999). Despite the high conservation of those sequences, we did not included pumilio and CUGBPs in the present study, since there are no reports assessing their involvement in mRNA regulation of transport.

On the other side, by looking at the long 3’UTR, the situation is much more complex, also by virtue of the length and complexity of this untranslated region (fig.32). Our observations about the subcellular localization of 3’long led to the identification of only one protein, among the ones considered, able to drive its activity dependent localization. One highly conserved CPEB1 binding site is contained in the terminal part of 3’UTR long, in close proximity to several AU-rich stretches that can be recognized by ELAV proteins. This region revealed to contain a constitutively active DTE mediated by CPEB1, but at the same time we found that this very same region harbors the the binding sites for ELAV proteins that inhibit dendritic sorting of this mRNA (see fig25). This dual role of the terminal region of the 3’UTR long may be explained hypothesizing that in the context of a full length 3’UTR, the ELAVs binding to the terminal segment may cooperate and interact with the two AUUUAU sequences in the short 3’UTR, therefore strengthening their inhibitory effect.
Fig. 33. Secondary structure prediction of long 3′UTR (Mfold algorithm). Red circle, short 3′UTR domain (Red square, CPE binding site; green circles, ELAV binding sites); yellow box, ELAV1 binding sites clusters; green box, mid 3′UTR region, purple circle, FMRP binding site; blue “C”, terminal END region. Of note, the domains containing targeting elements (END) and retention signals (MID) are spatially segregated and are conserved in 3′long structures with different free energy contents (not shown). For comparison, see fig. 20.

This model would be in accordance to what has been observed either after siRNA induced ELAVs silencing and after isolation of the terminal segment, that lacks of all the upstream regulatory sequences. Moreover, An and colleagues reported that a chimaera consisting of the GFP reporter followed by the long 3′UTR except the short sequence displays an enhanced dendritic localization than the full length 3′UTR (An et al, 2008). On the other side, we identified a previously unknown retention signal localized in the middle of the long 3′UTRs, that is able to overcome the constitutive
sorting signal mediated by translin at the level of the CDS. These findings are in accordance with an increased BDNF mRNA basal content in dendrites of FMR1 and FXR2 KO animals in resting conditions and this might also account for an increased susceptibility to epileptic seizures seen in these animals (El Idrissi et al, 2005). Moreover, a very recent study, Sharma and colleagues reported an exaggerated mTOR and PI3K activation in FMR1 KO mice (Sharma et al, 2010) that would therefore explain the PI3K-dependent accumulation of BDNF mRNA in dendrites observed by Tongiorgi and coworkers (Righi et al, 2000). From this scenario, it clearly emerges that the long 3’UTR is controlled by a set of inhibitory signals and proteins that prevent its basal dendritic sorting, thus also contributing to the proximal localization observed for the endogenous transcript. On the other side, the strategy controlling the dynamics of short 3’UTR localization seems to rely on the activation of dormant targeting elements and proteins.

Interestingly, a similar organization of targeting elements has been observed in another dendritically localized transcript, namely CaMKIIα mRNA. In fact, it has been reported the presence of a translin binding site at the level of the coding region that is necessary for its correct sorting in dendrites (Severt et al, 1999), exactly as for BDNF mRNA. Moreover, Mori and colleagues identified two opposing signals at the level of CaMKIIα 3’UTR: a DTE in the first 28-56 nts of 3’UTR, promoting RNA sorting (also known as the CNDLE sequence, see par.2.2.1), that is overridden by a downstream retention signal whose inhibition is relieved after KCl-induced depolarization (Mori et al, 2000). This behavior strongly remembers our findings about a bipartite signal in BDNF 3’UTR long: an active DTE, most likely mediated by CPEB1, and an upstream retention signal normally blocking mRNA sorting, but relieved after BDNF (but not KCl) application, mediated by several RNA-binding proteins of the ELAV and FXRP families. Moreover, in a separate work, Huang and coworkers identified in CaMKII-alpha mRNA two CPE elements facilitating mRNA transport and translational activation (Huang et al, 2002). Through a bioinformatic analysis, we also found two AU-rich regions, many ELAV binding sites and a G-quartet-like structure in BDNF mRNA similarly to CaMKII-alpha (for comparison, see Dictenberg et al, 2008). Finally, Also CaMKII-alpha may express a short or a long 3’UTR, with the latter being much more abundant than the former.

This striking parallelism between BDNF and CaMKIIα targeting elements, their distribution, the RNA binding proteins involved and their activity-depend sorting further supports the “RNA operon theory” (Keene & Tenenbaum, 2002), by which common signals and RNA binding proteins are shared by different mRNAs with common functions (e.g. synaptic plasticity and potentiation) therefore jointly coordinating their post-transcriptional outcome. Hence, it is tempting to speculate that these transcripts which are both involved in the induction and maintenance of synaptic plasticity, and
9.3 Towards a model of BDNF mRNA dendritic targeting mechanism

This study provides compelling evidence that the BDNF coding region common to all BDNF splice variants is sufficient to direct constitutive targeting of BDNF mRNAs to the distal dendritic compartment. Characterization of this signal has demonstrated that it: (i) can be overridden by the 5'UTRs of BDNF mRNA isoforms (exon 1 and 4) that are retained in the cell body and proximal dendrites, (ii) is mediated by translin, and (iii) is blocked by a retention signal mediated by a complex of ELAV and FXRP proteins that likely binds to conserved sequences in the central regions of the 3'UTRlong; iv) we also found that the targeting of full length BDNF mRNA is induced by KCl depolarization, BDNF and NT3 and that the inducible targeting signals that respond to these stimuli are encoded by the both 3'UTRs. Interestingly, the 3'UTR short responds to KCl and NT3 while the 3'UTR long to KCl and BDNF; v) the short 3'UTR contains inducible dendritic targeting signals that respond to KCl and NT-3 via CPEB1 and 2 while the the 3'UTR long contains signals that respond to KCl and BDNF for and via CPEB1. The long 3'UTR contains additional retention signals that are relieved only upon stimulation with BDNF and are mediated by FXR and ELAV proteins that might cooperate with the constitutive signals in the CDS mediated by the translin/trax complex.

From these results it is possible to conclude that dendritic trafficking of BDNF transcripts displays two key features: activity-dependence and transcript selectivity. Although we showed that 3'UTR plays a critical role in mediating activity-dependent trafficking of BDNF transcripts, this segment is unlikely to mediate transcript selectivity because both the short and long forms of the 3'UTR are contained in BDNF transcripts that are retained in the soma/proximal dendrites, as well as those targeted to distal dendrites. Our results indicate that transcript selectivity is mediated by a constitutively active dendritic targeting element (DTE) located in the CDS that can be overridden by signals located in the 5'UTR of transcripts that are retained in the soma/proximal dendrites, but not by those targeted to distal dendrites (Fig. 34). The ability of si-translin to block activity-dependent targeting of both the truncated CDS-GFP construct and endogenous, full-length transcripts, but not the isolated 3’ UTR, indicates that both a translin-dependent DTE in the CDS and a translin-independent DTE in the 3’UTR are required to mediate activity-dependent trafficking of full-length BDNF transcripts. Further confirmation of this model is provided by our finding that the G196A SNP,
which by definition acts selectively on the CDS DTE, blocks pilocarpine-induced dendritic trafficking of BDNF mRNA in vivo.

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**Activity**

**(BDNF, KCl)**

**(NT-3)**

Fig. 34 Proposed model of BDNF mRNAs sorting mechanisms. Multiple 5'UTR sequences with either inhibitory or permissive targeting properties determine transcript-selective sorting. Translocation to dendrites is mediated by constitutive dendritic targeting signals in the CDS that require binding of the translin/trax complex at the G178-G196 residues. The 3'UTR region(s) contain inducible dendritic targeting signals that respond to KCl, BDNF and NT-3 via CPEB1 and 2 for the short 3'UTR and via CPEB1 for the 3'UTR long. The long 3'UTR contains additional retention signals that are relieved only upon stimulation with BDNF and are mediated by FXR and ELAV proteins that might cooperate with the constitutive signals in the CDS mediated by the translin/trax complex.

### 9.4 Short vs. long BDNF 3'UTR: in search for different functions

In a recent work, it has been reported that the long 3'UTR is targeted in dendrites and there it locally regulates BDNF mRNA translation in vitro. On the other side, the short isoform seems not to play any significant role (An et al, 2008). Our results are partially in accordance, but the main findings presented here are based on the analysis of 3'UTRs localization in presence of different stimuli known to mediate BDNF mRNA somatic export. Moreover, our in vitro data accounting for an active role played also by the short 3'UTR are strongly corroborated by in vivo evidences showing that (i) in mice lacking of a functional long 3'UTR there is still a significant sorting of BDNF mRNA after pilocarpine-induced seizures and (ii) comparable levels of dendritic BDNF protein between BDNF<sup>K</sup> and wt animals at 2 months of age. Additional in vitro data demonstrate the positive translational control exerted by the short 3'UTR respect to the long that, similarly to the regulation of its
subcellular localization, is controlled by a set of inhibitory signals that repress its translation in resting conditions.

The same authors, despite normal levels of total BDNF in brain, reported a selective impairment of LTP, but not brief high frequency stimulation (1X100Hz, 1s) in apical dendrites of CA1 BDNF\textsuperscript{lox} neurons, accompanied by an increased spine density and decreased spine head diameter, consistent with a local regulatory effect. This phenotype, reminding the one observed in immature spines, was accounted to a decreased spine pruning, as a consequence of a selective dendritic depletion of BDNF protein. Of note, those differences have been reported only in adult (8-10 weeks) but not juvenile (P21) mice. These abnormalities, together with a significant decrease in activity-regulated exocytosis of BDNF, are consistent with spine network remodeling exerted by 3'UTR long driven-BDNF expression in the adult. In front of the normal electrophysiological and neuroanatomical phenotype seen in young animals, we reasoned whether the short 3'UTR may vicariate or at least compensate the absence of the long isoform during early life stages. From a developmental point of view, several reports on alternative polyadenylation site selection stated a preferential usage of long 3'UTRs vs short ones as development and differentiation progresses (Ji & Tian, 2009; Liu et al, 2007; Sandberg et al, 2008; Wood et al, 2008). This progressive lengthening of 3'UTRs is in accordance with a much more complex network of mRNA regulation, since the number of potential miRNA target sites is increased respect to short 3'UTRs, often accompanied with an increased AU rich content leading to instability and increased efficacy of miRNA targeting (Grimson et al, 2007; Ji & Tian, 2009; Long et al, 2007). Moreover, it is conceivable that sequence lengthening may lead to the introduction of other types of cis-elements that cooperate to finely regulated developmental and differentiation programs. With respect to these observations, and consistent with an abnormal phenotype seen in adult BDNF\textsuperscript{lox} mice, our findings stating (i) short 3'UTR localization in dendrites of BDNF\textsuperscript{lox} mice, (ii) increased translatability conferred by the short variant respect to the long 3'UTR , (iii) increased number of cis-acting elements, potential miRNA binding sites and AU rich regions in the latter, strongly support the idea that the short 3'UTR may exert a leading role in controlling BDNF expression in early development, whilst the long 3'UTR gets involved later as development and differentiation proceed. In fact, spine pruning and shaping is a finely regulated step that coincides with maturation of the neuronal network. On the other side, a strong BDNF expression in early stages driven by the short 3'UTR would support neuronal survival and spine formation, that would eventually be reinforced or removed during maturation as long 3'UTR usage becomes more important. We therefore strongly believe that the biological meaning of the presence of a short and long 3'UTR is not confined to a mere difference in their subcellular localization (soma vs. dendrites), but rather on a exclusive role in modulating neuronal survival and network formation depending on
the developmental stage. To test our hypothesis, we are currently looking at potential differences between 3'UTR short vs. long usage at different stages (P10, P21 and P60). Hence, we expect an increased abundance of 3'short in P10 and P21 mice and an opposing predominance of 3'UTR long in the adult (P60).

It is known since a long time that during development the relative levels of each neurotrophin and respective Trk receptor may vary. In particular, the earliest neurotrophin receptor to be expressed during brain development is that for neurotrophin-3, followed by a later expression of TrkB (Bernd, 2008). The reciprocal interplay between neurotrophins has been investigated, particularly concerning BDNF and NT-3, both in vitro and in vivo systems (Lindholm et al, 1994; McAllister et al, 1997; Patz & Wahle, 2006). Both BDNF and NT-3 have been shown to play important but somehow counteracting effects during development, as opposing effects in regulating cortical dendritic growth (McAllister et al, 1997). Moreover, NT-3 mRNA levels, detected prenatally in visual cortex, dramatically decreases at later stages of development, with an opposing trend for BDNF mRNA (Lein & Shatz, 2000; Patz & Wahle, 2006). This evidence is further supported in rat cerebellar neurons, were an up-regulation of BDNF expression in vitro is accompanied by a significant decrease of NT-3 expression (Condorelli et al, 1998). Moreover, it has been reported that acute NT-3 administration leads to increased BDNF secretion in hippocampal neurons (Canossa et al, 1997; Kruttgen et al, 1998). In light of this large body of evidence and supported by our in vitro data, we propose a model whereby earlier expression of NT-3 might promote both 3'UTR short-containing splice variants localization in distal dendrites and their local translation into protein. At the same time, NT-3 signaling may promote de novo synthesized BDNF secretion, that in turn may lead to both pre- and post-synaptic TrkB activation. As reported in fig.29, 3'UTR driven expression is strongly upregulated by NT-3, but also significantly by BDNF itself. Therefore, as long as NT-3 expression is maintained, it might sustain BDNF targeting, translation and exocytosis through a positive feedback loop that would locally potentiate BDNF action promoting neuronal survival and spine formation. Accordingly, when NT-3 levels decrease during development, the local production of BDNF may in turn promote dendritic targeting of the long isoform, its local translation into protein (see fig.30) and exocytosis. Therefore, accordingly to An and colleagues, only at later stages the local 3'UTR long-dependent production of BDNF would then promote spine pruning and a fine neuronal network remodeling, ongoing with development and differentiation.
9.5 Implications for neuropsychiatric and developmental diseases

Our finding that the G196A SNP blocks dendritic trafficking of BDNF mRNA implies that phenotypic changes induced by this mutation, such as reduced hippocampal dendritic complexity and volume, as well as memory deficits (Bath & Lee, 2006; Pezawas et al, 2004), and susceptibility to mood disorders (Krishnan et al, 2007) may be due to this effect. This finding links defects in dendritic trafficking with defective neuronal development resulting in susceptibility to eating and mood disorders, and reduced memory performance. Recent studies have suggested that this SNP may also impair BDNF protein sorting by disrupting its interaction with sortilin, a vesicular membrane protein implicated in protein trafficking (Chen et al, 2005). Thus, conceivably, the G196A SNP interferes with BDNF processing by disrupting trafficking of both BDNF mRNA and protein through distinct mechanisms.

It is also noteworthy that translin deletion in mice impairs performance in several behavioral paradigms used to assess learning, memory, and anxiety (Stein et al, 2006). However, it is important to emphasize that this deficit in subcellular BDNF mRNA sorting may not account completely for the biological deficits produced by this mutation. On the other hand, it has been found that klox mice lacking BDNF transcripts with the long 3’UTR show deficits in dendritic localization of BDNF mRNA under resting conditions and a marked impairment in long-term synaptic potentiation and Morris water maze performance of these mutant mice (An et al., 2008). However, the lack of deficits in dendritic arborisation in BDNF$klox$ mice argues that the phenotype of these mice is not as severe as that of G196A mice. The reason for this milder phenotype is clearly explained by our compelling demonstration that the 3’UTR short isoform may largely vicariate the 3’UTR long in producing BDNF protein in dendrites. This compensatory mechanism may operate particularly during early development when the 3’UTR short isoform is predominant with respect to the long variant.

In conclusion, these findings indicate that further studies are warranted to assess the role that dendritic trafficking of BDNF mRNA plays in the pathophysiology of neuropsychiatric disorders.
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