Scuola Internazionale Superiore di Studi Avanzati - Trieste

Use of intracellular antibodies to study the cellular biology of neuronal cells

Thesis submitted for the degree of
"Doctor Philosophiae"
Biophysics sector

Candidate:
Giada Pastore

Supervisors:
Prof. Antonino Cattaneo
Dr. Kevin Ainger

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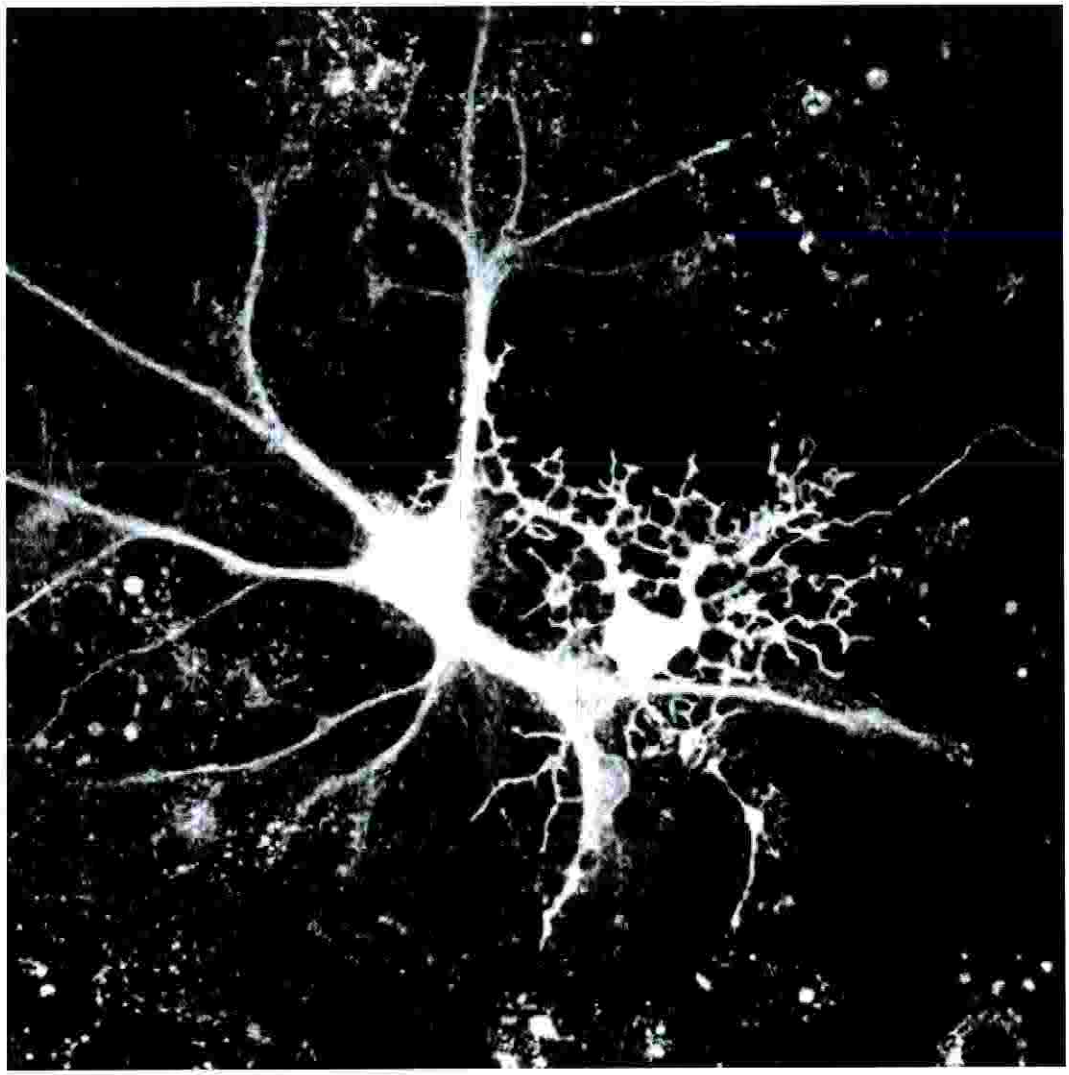

by KA

Agli uomini della mia vita.

To the men in my life.

A mio papa'
che mi ha insegnato, e mi insegna sempre a non mollare.
Anche, soprattutto quando la paura e' l'unica amica
che sembra non volerti abbandonare.

To my father,
who taught me,
and constantly teaches me,
never to give up,
especially when fear seems to be the only constant friend left.

A Lorenzo
che, chi lo sa perche', ha sempre creduto in me
e mi e' sempre stato vicino.
E lo e' anche ora.
Dopo vent'anni.

To Lorenzo,
who, goodness knows why, has always believed in me
and stood by me,
even now.
Twenty years on.

A Sergio,
a mio marito,
e non nascondo l'orgoglio nel dirlo,
che e' stato per me solida e sempre affidabile ancora.
Con tutto il mio amore.

To Sergio,
my husband
– a word that makes me proud –
who has always been firm and reliable for me.
With all my love.

A Kev,
l'unico motivo per il quale sono arrivata fino a qui.
Grande maestro di scienza e di vita,
ma soprattutto grande amico.

To Kev,
the one and only reason why I managed to get this far.
He has been a great teacher of science and life,
but above all a great friend.

A Mauri,
ultimo tesoro che la vita mi ha regalato,
nuovo grande e speciale amico
che ha saputo aiutarmi con la sua eccezionale sensibilita'.

To Mauri,
the most recent gift of fortune,
a new great, special friend,
who has been able to help me with his exceptional sensibility.

Table of contents

Abbreviated names of the plasmid contructs used for protein and RNA targeting

GFP: Enhanced Green Fluorescence Protein (sometimes referred as EGFP).

R4 GFP cyto: α β-Galactosidase scFv (R4) in fusion with GFP and the cytoplasmic protein targeting signal (sometimes referred simply as R4 cyto).

R4 GFP NLS: α β-Galactosidase scFv (R4) in fusion with GFP and the nuclear localization signal.

R4 GFP sec: α β-Galactosidase scFv (R4) in fusion with GFP and the secretory leader sequence.

R4 GFP KDEL: α β-Galactosidase scFv (R4) in fusion with GFP and the KDEL signal for endoplasmi reticulum retention.

A2RE-GFP: GFP carrying the hnRNPA2 Responsive Element (A2RE) for RNA transport.

A2RE-R4 cyto: R4 GFP cyto palsmid carrying the A2RE RNA targeting element.

DTE-GFP: GFP carrying the bases from 1 to 94 of CaMKIIα 3'UTR, the putative Dendritic Targeting Element.

DTE-R4 cyto: R4 GFP cyto palsmid carrying the bases from 1 to 94 of CaMKIIα 3'UTR.

UTR-GFP: GFP carrying the the complete 3'UTR of CaMKIIα .

All the movies described in this thesis can be downloaded at:

http://www.sissa.it/~jada/movies/movies.htm

All the files were saved in DivX format.

Abstract

In this thesis the natural cellular trafficking has been used to target the expression of intracellular antibodies to specific subcellular compartments. Recombinant antibodies in the form of single chain Fab variable fragments (scFvs) were expressed in fusion with GFP and targeted into intracellular organelles of neuronal cells. It was first verified that neurons can correctly target intracellularly expressed scFvs to the nucleus, secretory pathway and endoplasmic reticulum (ER) when fused with an appropriate, nuclear localization sequence (NLS), secretory leader (sec), or KDEL ER retention signal (KDEL). Since neurotrophin receptors are located in the distal dendrites and are the focus of my study, specific mRNA targeting elements were used to reach particular cell compartments such as distal dendrites in neurons. Three different RNA sequences were compared in order to determine the most advantageous conditions for neuronal expression: the hnRNPA2 Responsive Element (A2RE) for transport of Myelin Basic Protein (MBP) mRNA into oligodendrocyte processes, the 3'UTR of Ca^{2+}- calmodulin-dependent protein kinase II α (CaMKIIα) that regulates CaMKIIα mRNA trafficking, and the first 94 nucleotides of the CaMKIIα UTR. The RNA targeting experiments described in this thesis show that it is possible to target an scFv mRNA to dendrites of transfected neurons, and indicates that the A2RE as the most powerful element among the studied sequences. Having validated the protein and RNA targeting of scFvs, these technologies were exploited to interfere with the NGF receptors, TrkA and $p75^{NTR}$. Three different scFvs were selected to reach this goal: α TrkA MNAC as a neutralizing antibody, α TrkA, and α p75 scFvs as intracellular anchors. After an initial characterization of the three scFvs, the efficiency of the KDEL retained scFvs as intracellular anchors was demonstrated in stably transfected C6 cells. The consequences of reducing the number of NGF receptors present on the cell surface was evaluated in PC12 and primary hippocampal neurons after transient transfection. The inhibition of differentiation in PC12 cells, and the inhibition of signal transduction both in PC12 cells and neurons, demonstrate that ER-retained scFvs are able to

interfere effectively with neurotrophins induced signal transduction, confirming that phenotypic knock-out occurred. With the same type of experiments it was also possible to observe, with a completely novel approach, a complex formation between $p75^{NTR}$ and Trk receptors. Finally, the new tools developed were used as a basis for the design of intrabody constructs to create new transgenic mice for the study of Alzheimer's disease.

Chapter 1

Introduction

1.1 mRNA localization

In 1982 Sabatini and colleagues found that myelin fractions from rat brains were highly enriched in Myelin Basic Protein (MPB) mRNAs. This was the first evidence that mRNAs are not restricted to cell bodies [Colman DR et al., 1982]. One year later it was demonstrated that β-actin mRNA accumulates in the myoplasm of Ascidian eggs [Jeffery WB et al., 1983]. Since then improvements in the resolution and sensitivity of *in situ* hybridization techniques, have led to the identification of a large number of localized mRNAs in many different cell types. Despite the increase of knowledge of targeted transcripts, progress in understanding the mechanism of mRNA transport has been comparatively slow, largely because it is a difficult process to analyze. Unlike other intracellular trafficking events, such as nucleo-cytoplasmic transport or protein secretion, the localization of mRNAs does not involve movement between membrane-bound compartments that can be purified by fractionation. Moreover, the specific mRNAs that localize to a particular site represent only a small fraction of the total mRNA in the cell. Although these factors have delayed biochemical analysis, recent work has identified several of the key mRNA-binding proteins and has begun to reveal the cellular mechanism that targets the transcripts to the correct locations. In parallel, genetic screens in both yeast and *Drosophila* have identified *trans*-acting factors required for the localization of mRNAs with essential developmental functions and have contributed significantly to our understanding of how these mRNAs reach their destination.

1.1.2 Biological roles of mRNA localization

One of the primary purpose of mRNA localization could be to target the expression of the protein to a particular region of the cell or to prevent its expression elsewhere. In some cases mRNA localization it might be preferable to protein localization because one mRNA molecule could serve as a template for multiple rounds of translation. In addition, there could be special requirements for high concentrations of proteins that act as determinants to induce specific cell fates, or maybe also for assembly of specific protein complexes in a particular region of the cell. The synthesis of cell-fate determinants must be restricted to defined cytoplasmatic position, as their mislocalization would be disastrous for the cell. Examples of such cell-fate determinants include the product of the genes *bicoid* (*bcd*), *nanos* (*nos*) and *oskar* (*osk*) in *Drosophila* [for review see Placios IM and St.Johnston D, 2001]. It is known that in many invertebrate and in some vertebrates, the primary axis of the organism is defined in the unfertilized egg through the localization of determinants that control the development of the region that inherits them after fertilization. *Drosophila* eggs contain at least six localized transcripts that act in different ways to pattern the anterior-posterior axis of the embryo. The anterior determinant is encoded by *bcd* mRNA that is both necessary and sufficient to define where the anterior structure develops [Berleth T et al., 1988; Driever W et al., 1990]. On the other hand, the first mRNA to reach the posterior of the oocyte is *osk* mRNA that establishes, together with *nos*, the posterior determinant [Ephrussi A and Lehmann R, 1992; Wang C and Lehmann R, 1991].

As in *Drosophila*, localized maternal mRNAs also have important functions in embryonic axis formation in *Xenopus*. At least ten mRNAs have been shown to determine the vegetal pole of the oocyte (for review see Placios and St. Johnston, 2001). One of these, Vg1 mRNA that encodes for a secreted signaling molecule, can affect the patterning of the embryo when injected elsewhere [Melton DA, 1987; Thomsen GH and Melton DA, 1993].

1.1.3 Localized mRNA in polarized cells

Almost all examples where the biological importance of mRNA localization has been demonstrated come from experiments on eggs and early embryos, but many transcripts are asymmetrically distributed in other types of cells. For example mRNA of β-actin is localized to the leading edge of migrating fibroblast of chick embryo where it seems to be polymerized to drive cell movement [Kislauskis EH et al., 1993]. But we can also look at the most spatially complex and highly differentiated mammalian cells: neurons of the central nervous system (CNS). An individual neuron may have several thousand synaptic connections and it is the modification of these synapses that gives rise to the brain's capacity to learn, remember, and even recover function after injury. In these synapses, specific changes need to be sustained by new protein synthesis, but how the selective control of protein delivery is obtained, remains an open issue. Traditionally, it has been thought that new proteins are synthesized in the perikarion and then targeted to the periphery, but now we know that dendrites themselves are also capable of protein synthesis.

The story regarding RNA localization in neurons began with the discovery of synapse-associated polyribosome complexes (SPRCs) [Steward O and Fass B, 1983], clusters of polyribosomes and associated membranous cisterns that are selectively localized beneath postsynaptic sites on dendrites in the CNS. Subsequent studies demonstrated that intact dendrites are able to translate RNA of a targeted reporter gene [Aakalu G et al., 2001; for review see Steward O and Schuman EM, 2001].

As the distribution of various mRNAs were mapped, it was established that most of the neuronal mRNAs could be detected only in the perikarion, sometimes extending for a short distance into proximal dendrites, an area that is considered to have a cytoplasmatic composition similar to the soma. However, a few mRNAs were found to be present in dendrites at relatively high levels [for review see Steward O and Schuman EM, 2001]. In particular, the first mRNA shown to be localised to the dendritic compartment and excluded from axons of CNS neurons,

was the one for the microtubule-associated protein MAP2 [Garner CC et al., 1988]. The mRNA for the α subunit of the Ca^{2+}- calmodulin-dependent protein kinase II (CaMKIIα) was found in hippocampal and cortical dendrites (even in their most distal portion), whereas no dendritic localisation of this mRNA was observed in cerebellar granule cells and in Purkinje cells [Burgin KE et al., 1990]. In contrast, Tau mRNA was found to localize only in the proximal region of the axon [Litman P et al., 1993].

The temporal and spatial regulation of protein translation in neurons may be one important mechanism for neuronal synaptic plasticity and might contribute to the remodelling of individual synapses in response to neuronal activity. We know that neuronal stimulation enhances the dendritic localization of a number of transcripts; for example, depolarization induces an increase in the amount of BDNF and TrkB mRNAs in the dendrites of cultured hippocampal cells [Righi M et al., 2000; Tongiorgi E et al., 1997] and also in *in vivo* experiments [Tongiorgi E et al., 2000]. Long-term potentiation (LTP) induces an accumulation of CaMKIIα and MAP2 mRNAs in hippocampal dendrites *in vivo* [Thomas KL et al., 1994]. In agreement with these results is the observation that the localized stimulation of NMDA receptors in the dentate gyrus causes the selective and rapid appearance of Arc (activity-regulated cytoskeletal-associated protein, an immediate early gene) mRNA in the regions of the dendritic tree that have been stimulated, perhaps by active transport even if not directly activated by the stimulus [Steward O and Worley PF, 2001]. This experiment suggests that activity could regulate the strength of individual synapses by increasing the expression of specific proteins through the targeting of their mRNA. This mechanism may also play a role in neuronal development since β-actin mRNA has been found to localize to dendritic growth cones in response to the chemoattractant neurotrophin-3, possibly contributing to the growth of dendrites toward their targets [Zhang HL et al., 2001].

Another example of mRNA targeting in polarized cells, is the MBP transcript, which is localized in oligodendrocytes. In these cells, MBP mRNA is transported

along microtubules into the oligodendrocyte myelin compartment that generate myelin sheets which surround neuronal axons.

1.1.4 Mechanisms of localization

While mRNA localization has been well documented in many systems, the mechanism that generates it is poorly understood. It must be underlined that the localization phenomenon might be the result of two distinct cellular mechanisms: the mRNA transport is responsible for mRNA delivery, while its static localization, about which little is known, is probably essential when the target destination has been already reached. For example, this kind of "anchoring effect" can be achieved with the aid of a docking protein, able to bind the mRNA at its final destination. It is also possible that a specific region of the cell protects certain mRNAs from degradation occurring in the other parts of the cell.

What is known is that cytoplasmic transport of most localized mRNAs requires a functional cytoskeleton as well as motor proteins that move along cytoskeletal filaments. There are two classes of cytoskeletal networks that have been implicated in the transport of RNA: these are actin microfilaments and microtubules [for review see Kloc M et al., 2002]. In general, the actin network is involved in short-distance transport, while microtubules are responsible for long-distance transport. In each case, a variety of motors have been implicated such as kinesin and dynein molecular motors, that direct trafficking on microtubules, and myosin-based motors for trafficking on actin microfilaments. Both microtubules and microfilaments network have been implicated in mRNA transport [Bassell GJ et al., 1999], although yeast *Ash1* mRNA is the only myosin dependent mRNA targeting identified so far [Long RM et al., 1997]. Microtubule-dependent motors, in particular, the plus-end-directed kinesin motor [Brendza RP et al., 2000], are essential for transport of *osk* mRNA to the posterior end of *Drosophila* oocytes [Glotzer JB et al., 1997]. By contrast, dynein motors, expected to move minus-end-directed *bcd* mRNA, have not yet been identified, while several lines of evidence indicate dynein motor for transport of *wg* mRNA, that encodes for a

secreted factor required for segmentation of *Drosophila* embryo [Wilkie GS and Davis I, 2001].

As for the translocation machinery itself, it seems clear that in some cases mRNA is transported as a part of a larger structure that can be visualized easily in dendrites. These structures, called RNA granules, were observed, by confocal microscopy, when fluorescent MBP mRNA was microinjected into living oligodendrocytes [Ainger K et al., 1993]. The properties of the RNA granule, such as size (≅0.7 μm in diameter) and speed (≅0.2 μm/s) were also calculated, and it was demonstrated that the translocation of granules requires microtubules kinesin motor [Carson JH et al., 1997]. The authors were also able to detect the granules in living oligodendrocytes by labelling total RNA with the fluorescent dye SYTO 12 [Barbarese E et al., 1995]. In addition, immunofluorescence experiments in oligodendrocytes showed that some RNA granules colocalized with components of the protein synthetic machinery such as arginyl-tRNA synthetase, elongation factor 1α (eEF1α), and rRNA, in addition to MBP mRNA. It is not yet clear if transported RNA granules observed in living cells and granules detected with immunofluorescence analysis are part of the same structures. RNA granules were later identified in neurons using a similar RNA dye, SYTO 14 [Knowles RB et al., 1996] and by high resolution *in situ* hybridization [Racca C et al., 1997]. It is now clear that RNA granules are also present in other cell types such as fibroblasts [Sundell CL and Singer RH, 1990], *Drosophila* embryos and oocytes [Ferrandon D et al., 1994] and *Xenopus* oocytes [Forristall C et al., 1995].

Biochemical analysis of other types of granules found in *Drosophila* oocytes has recently yielded information on the presence of at least seven proteins in the *osk* mRNA granules [Wilhelm JE et al., 2000]. The number of examples of mRNA reported to be transported in granules is quickly increasing and include β-actin and Tau transcripts [Aronov S et al., 2002; Gu W et al., 2002].

A hypothetical model for how mRNA localization can be achieved was proposed by Ainger K et al. [1993], according to which, mRNA localization is a multistep process. The first step is the cytoplasmic assembly of ribonucleoprotein particles

(RNP) as a functional complex that can inhibit protein translation. Then, translocation of the complex to its final destination takes place, followed by anchoring to the local cytoskeleton, and translation of the localized mRNAs.

Although mRNAs localization is a complex phenomenon, two aspects of this process are clear. First, the transcript must contain *cis*-acting elements that direct its localization. Second, these elements must be recognized by specifics *trans*-acting factors that link the RNA to one of the transport mechanisms described above.

1.1.5 *Cis*-acting elements

The transport of localized mRNAs to their final destination requires the presence of specific *cis*-acting sequences that mediate the localization. In most cases they are found in the 3' untranslated region (UTR), in analogy to other regions involved in eukaryotic mRNA regulation,). Their apparent lack of similarity at the level of primary and secondary structure means that it is hard to define a common theme. They can be short segments with a defined nucleotide sequence, such as the Vg1 localization element (VLE), a region mapped in the 3' UTR that consists of 340 nucleotides [Mowry KL and Melton DA, 1992] or the β-actin mRNA *cis*-element that comprises a 54 nucleotide long sequence within the 3' UTR [Zhang HL et al., 2001]. *Cis*-acting elements can also be secondary or tertiary structures such as stem loops, where the primary sequence is less important. One example is the *cis*-acting element of *bcd* mRNA which consists of five large stem loops in the 3' UTR [Macdonald PM and Kerr K, 1998]. In a number of cases, localization of transcripts can be mediated by multiple, partially redundant, elements such as for *bcd* and for *nos* mRNAs, which contain four different regions in their 3' UTR that direct localization into *Drosophila* eggs [Gavis ER et al., 1996]. The secondary structure may be also important for the dendritic localization of MAP2 mRNA that carries a Dendritic Localization Element (DTE) in a segment of 650 nucleotides in the 3' UTR [Blichenberg A et al., 1999].

It is known that the mRNA 3' UTRs themselves are involved in translational control and there are evidence that the processes of RNA localization and regulation of translation are linked [for review see Kloc M et al., 2002]. For example, CaMKIIα mRNA contains a Cytoplasmic Polyadenylation Element (CPE) which regulate polyadenylation and translation in *Xenopus*. It was found that two CPE are present in CaMKIIα 3' UTR, and that they are capable of regulating CaMKIIα mRNA polyadenylation and translation in a stimulation-dependent manner [Wu L et al., 1998]. CPE Binding protein (CPEB) is present in hippocampus and cerebellar cortex of mouse and rat brains and it is enriched in the postsynaptic densities. Experiments on visual cortex demonstrated that CaMKIIα mRNA undergoes both polyadenylation and translational activation in response to visual experience, suggesting that CPEB-mediated polyadenylation is a key event in generating long-lasting changes in synaptic activity.

1.1.6 *Trans*-acting factors

Only few *trans*-acting factors involved in mRNA localization and transport have been identified so far, in most cases by biochemically purifying RNA-binding proteins that recognize *cis*-acting elements. However, it is quite possible that other classes of molecules, such as small regulatory RNAs, play a role in this process.

The first RNA-binding protein that was shown to play a role in RNA localization is called Staufen, a double-stranded (ds) RNA-binding protein first found to be essential for anchoring *bcd* mRNA in the anterior cytoplasm of *Drosophila* eggs. Staufen is also required for *osk* mRNA microtubule-dependent transport and for its anchoring and translation, once it has reached the posterior pole [St.Johnston D et al., 1991].

In rat hippocampal neurons, Staufen is found in large RNA-containing granules that are associated with microtubules, and these structures have been observed to move along the dendrites [Kiebler MA et al., 1999]. The RNA in these particles has not been identified, but one candidate is MAP2 mRNA, since Staufen was isolated in a yeast three-hybrid screen for proteins that interact with MAP2 DTE

[Monshausen M et al., 2001]. Recently, another *trans*-acting protein for MAP2 mRNA has been identified and characterized, the rat brain protein MARTA1, a member of the family of Heterogeneous Nuclear Riboproteins (hnRNPs) [Rehbein M et al., 2002].

Xenopus Vera or Vg1RBP, which contains five RNA-binding motifs, is one of the six proteins that bind VLE required for the localization of Vg1 mRNA; Vera has been found to colocalize with Vg1 mRNA and to associate with microtubules and the rough Endoplasmic Reticulum (ER) [Deshler JO et al., 1997]. Vera is 78% identical to ZPB1 (Zipcode Binding Protein), which, together with ZPB2, is involved in the actin-dependent transport of β-actin. ZPB2 is a predominantly nuclear protein that, like several hnRNPs, has a role in the cytoplasmatic localization of β-actin mRNA [Gu W et al., 2002; Zhang HL et al., 2001]. Recently, two ZBP1 domains (the KH domain homologous to hnRNP K protein domain) are found to bind to the β-actin mRNA and to be involved in mRNA localization and granule formation in chicken embryo fibroblast [Farina KL et al., 2003]. Originally described as mRNA-binding proteins with a nuclear function, hRNPs have been shown to play a crucial role in the process of RNA localization. Their association with *cis*-acting elements of localized mRNAs suggests that the first step in producing a transport particle occurs in the nucleus and could provide important insights into the mechanism that distinguishes localized and non-localized transcripts.

1.1.7 Ca^{2+}- calmodulin-dependent protein kinase II

CaMKII is a multimeric enzyme consisting of 8 to 12 identical subunits. The α and β isoforms are exclusively expressed in the brain, whereas the γ and δ isoforms of CaMKII are ubiquitously expressed [Tobimatsu T and Fujisawa, H 1989]. In the brain CaMKII is involved in the signal transduction of forebrain neurons and is one of the major components of postsynaptic densities. It plays an essential role in synaptic plasticity mechanisms such as LTP. In particular, it is

involved in the late phase LTP and in memory consolidation [Miller S et al., 2002; Silva AJ et al., 1992].

In situ hybridization studies in hippocampal sections have shown that the mRNA for CaMKII alpha subunit is localized in neuronal dendrites and excluded from axons [Burgin KE et al., 1990]. Recently, it has also been demonstrated that CaMKIIα can be synthesized locally in dendrites and that its translation is increased by neuron activation [Aakalu G et al., 2001].

Many studies have tried to clarify which are the *cis*-acting RNA elements that regulate CaMKIIα mRNA trafficking. A transgene carrying the CaMKIIα 3'UTR and β-Galactosidase as a reporter gene, is dendritically localized, suggesting that the localization signal is present in the 3'UTR of the mRNA [Mayford M et al., 1996]. This result was confirmed, but without giving a definitive answer, by Miller and colleagues, who disrupted the CaMKIIα mRNA transcript localization by deleting its 3'UTR in transgenic mice [Miller S et al., 2002]. However, other studies clearly indicate that the transport process might be regulated in a more complicated fashion. In fact, three studies on primary cultures of rat hippocampal neurons tried to map the DTE specific sub-regions in the 3'UTR that can produce dendritic localization of a reporter mRNA, but showed contrasting results. Two of these works were conducted in a similar way, using transfected cultured hippocampal neurons with both full-length and deletions of the CaMKIIα 3'UTR in fusion with Green Fluorescence Protein (GFP) as a reporter gene [Blichenberg A et al., 2001; Mori Y et al., 2000]. These transfected neurons were analyzed by *in situ* hybridization in order to determine the percentage of transfected cells with a dentritic localization of the reporter gene mRNAs. The results obtained by Blichenberg and colleagues with the full-length 3'UTR demonstrated a dendritic distribution of mRNA in 30-35% of transfected cells with a staining between 26 and 35 μm from the cell body, in accordance with similar work performed with a different reporter gene [Rook MS et al., 2000]; in contrast Mori and colleagues found a dendritic mRNA localization in only 6% of transfected cells, suggesting that the full-length 3'UTR in resting neurons is not sufficient for the mRNA

transport. Different results were also obtained trying to identify the specific DTE. The former group defined the DTE in the region between position 1481 and 2708 [Blichenberg A et al., 2001], whereas the latter group showed that the first 94 bases are both necessary and sufficient to direct CaMKIIα mRNA into the dendrites [Mori Y et al., 2000]. These contrasting results suggest that one or more inhibitory *cis*-elements must be present together with the DTE and that the inhibitory effect can be blocked by a long-lasting stimulation of synaptic activity.

1.1.8 Myelin basic protein

Mature oligodendrocytes express MBP, a protein that functions in the compaction of myelin. MBP is translated and immediately incorporated into the myelin sheath that wraps around the axons. This was demonstrated, by Benjamins and Morell about 25 years ago. They found that newly synthesised MBP was immediately incorporated in myelin sheaths in rat oligodendrocytes, suggesting that MBP mRNA was already present at the myelin compartment [Benjamins JA and Morell P, 1978]. These observations were made by rat intracranial injections of radioactive methionine and consequent detection of labelled MBP and ProteoLipid Protein (PLP) a major component of myelin into the myelin fractions. Starting from this work, it was then calculated the time interval between the injection of radioactive methionine and the appearance of the proteins: radioactive MBP was found in the myelin compartment just 2 minutes after the injection, whereas PLP was detected only after a lag of 30 minutes [Colman DR et al., 1982]. Still, the first direct demonstration of intracellular movement of mRNA in living cells was provided more than ten years later by injecting labeled MPB mRNA in oligodendrocytes and direct visualization of mRNA movement [Ainger K et al., 1993]. Subsequent studies have demonstrated that MBP mRNA moves along the microtubules within granules, with the aid of kinesin as a molecular motor [Carson JH et al., 1997].

Experiments based on microinjection of various deletion constructs of MBP mRNA have delineated the *cis*-acting elements necessary and sufficient to target

MBP RNA to the myelin compartment [Ainger K et al., 1997]. These elements have been called the hnRNPA2 Responsive Element (A2RE) and the RNA Localization Signal (RLS).

The A2RE, formerly known as the RNA Transport Signal (RTS), is a bipartite sequence of 21 nucleotides found in the MBP 3' UTR mRNA, that is required and sufficient for transport of the transcript along the processes. Extensive mutational analyses have shown that only the first 11 nucleotides are sufficient to bind its cognate *trans*-acting protein, the hnRNPA2, and mediate MBP RNA trafficking [Munro TP et al., 1999]. Studies of A2RE/hnRNPA2 molecular interactions have identified two distinct RNA binding sites contained within the amino-terminal half of the protein, one A2RE-specific and the other one a non-specific RNA binding domain [Shan J et al., 2000]. Interestingly, the Human Immunodeficiency Virus (HIV) type 1 genome also contains two functional A2RE-like sequences, which bind hnRNPA2, and confer transport of HIV RNAs in oligodendrocytes. Therefore, HIV-1 could exploit the A2RE/hnRNPA2 pathway to transport its mRNAs encoding for proteins that are incorporated into the virions [Mouland AJ et al., 2001].

The RLS, also found in the 3' UTR, is required for localization of the MBP transcript. When it is deleted, the mRNA is transported into granules along the processes, but does not localize to the myelin compartment. It has a predicted secondary structure which is conserved in different species and its postulated function is to anchor MBP mRNA to the myelin compartment [Ainger K et al., 1997].

A summary of some targeted mRNA and their *cis*- and *trans*-acting factors is reported in Table 1.1

TABLE 1.1 mRNAs LOCALIZATION IN NEURAL CELLS

CELL/RNA	Localization	Mechanism	*cis*-acting elements	*trans*-acting factors	References
NEURONS					
MAP2	soma, dendrites	microtubules-dependent	DTE (3'UTR-640 bp)	Staufen? MARTA1	Blichenberg A et al., 1999; Rehbein M et al., 2002
Tau	axons	microtubule-dependent	ALS (3'UTR)	unknown	Aronov S et al., 2001
β-actin	growth cones	microtubule-dependent	Zipcode (3'UTR-54 bp)	ZBP1/ZBP2	Zhang HL et al., 2001;
CaMKIIα	dendrites	unknown	3'UTR	unknown	Mayford M et al., 1996; Miller S et al., 2002
Arc	soma, dendrites	unknown	unknown	unknown	Steward O and Worley PF 2001
BDNF	dendrites	unknown	unknown	unknown	Tongiorgi E et al., 1997
TrkB	dendrites	unknown	unknown	unknown	Tongiorgi E et al., 1997
OLIGODENDROCYTES					
MBP	myelin compartment	microtubules-dependent	A2RE	hnRNPA2	Shan J at al., 2000

1.2 Intracellular protein trafficking

Eukaryotic cells have a very complex organization which is subdivided into functionally distinct membrane-bound compartments. Each compartment contains its own distinct set of enzymes and other specialized molecules. Since all the proteins (except for those synthesized into the mitochondria) originate on ribosomes in the cytoplasm, it is vital for a cell to have a very well organized system able to direct the intracellular protein traffic in order to convey products to the correct intracellular compartment. Figure 1.1 shows a map of the biosynthetic protein traffic that starts with proteins synthesis on a ribosome and terminates to the final destination.

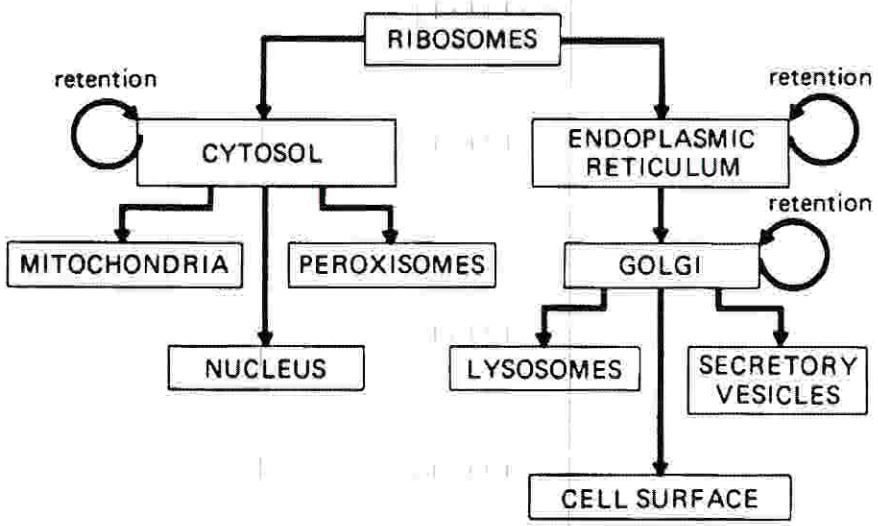

Fig. 1.1 A simplified "road map" of biosynthetic protein traffic.
[Alberts B et al., 1994]

This highly organized system is achieved by the expression of specific amino acid targeting sequences present at the N- or C-terminus of proteins. Major advances have been made in our understanding of these signals. Many proteins host short and conserved amino acids motifs, often separate from other functional parts of

the molecule, that are involved in their intracellular targeting. These sequences can determine the spatial location of a protein, as well as its folding, post-translational modification, and degradation. The conservation of these motifs across evolution varies: in some cases they are highly conserved, while in others substitutions of few amino acids are tolerated. Most often these targeting motifs are autonomous and dominant and confer the ability to independently specify targeting to a reporter protein.

1.2.1 Sorting in the secretory pathway

The first protein targeting sequence to be characterized was the leader sequence, an amphipathic α-helix which is usually found at the N-terminus of nascent secretory and membrane proteins that allows them to enter the Endoplasmic Reticulum (ER) [Blobel G and Sabatini DD, 1971]. The leader sequence is guided to the ER membrane by the Signal Recognition Particle (SRP) complex that cycles between ER and cytoplasm. Once it reaches the ER membrane, SRP binds the SRP receptor, docking the nascent protein and the ribosome to the membrane and leading the insertion of the signal peptide into the translocation channel [Pool MR et al., 2002]. The presence or absence of the leader sequences is thought to determine the first protein decision after leaving the nucleus: translation by membrane bound versus cytoplasmic ribosomes, the secretory pathway versus the cytosol.

Proteins destined for secretion, for the plasma membrane or membranous organelles enter the secretory pathway in the ER and move through the Golgi complex that occupies a key position in the secretory membrane system of the cell. Morphologically, it is difficult to define the boundary between the ER and the Golgi complex due to the dynamic nature of the two compartments, but one can define the ER-Golgi Intermediate Compartment (ERGIC) also called the *cis*-Golgi Network (CGN), the cisternae of the Golgi Stack (GS) and the *trans*-Golgi Network (TGN) [Hauri HP and Schweizer A, 1992; Mollenhauer HH and Morre DJ, 1991]. The CGN is the site where ER-derived proteins are received, and

where vesicles involved in the retrograde transport bud from the Golgi back to the ER. This retrograde flow serves to maintain the surface area of the ER despite the extensive membrane outflow into the secretory pathway [Wieland FT et al., 1987], to return escaped ER resident proteins [Pelham HR, 1995], and to recycle membrane machinery involved in ER to Golgi transport [Lewis MJ and Pelham HR, 1996]. As a protein moves through the ER membrane and the Golgi stack, it is modified by processing enzymes, such as numerous glycosyltransferases and glycosidases. These enzymes are generally not evenly distributed between Golgi cisternae, but they are organized in the order in which they act on their substrate. In this manner a protein can be exposed to an ordered range of processing steps [Gleeson PA, 1998]. Then, upon reaching the TGN, the protein is sorted and packaged into post-Golgi transport intermediate vesicles that direct them to the plasma membrane, into secretory storage granules and into various compartments of the endosomal/lysosomal system. Until the last few years it was widely believed that anterograde movement of proteins is achieved only by transport vesicles that bud from one cisternae and fuse with the next [Rothman JE and Wieland FT, 1996; Schekman R and Orci L, 1996]. However, other models for vesicular transport have been recently proposed. One of these, of particular interest, is reviewed by Sprong H et al., [2001; see also Holthuis JC et al., 2001]. These authors started from the consideration that a membrane lipid concentration gradient is present along the ER and Golgi stacks, and that the Golgi acts as a filter between the glycerolipid-rich ER and the sphingolipid-rich plasma membrane. In fact, sphingolipids synthesis takes place primarily in the Golgi and it is spatially separated from glycerolipid production in the ER. Due to the mechanism of slow cisternal migration towards the *trans*-side, cisternal concurrent maturation and to the strong intrinsic tendency of sphingolipids to segregate from unsatured glycerolipids, this gradient is raised and maintained. This gradient is also preserved by the continuous renewal of membranes and by the fact that vesicles budding back to the ER are enriched of glycerolipids. It was previously shown that there are preferential interactions between lipids and transmembrane proteins with the best matching hydrophobic length, hence is a

potential mechanism for protein sorting in the Golgi [Munro S, 1998]. These interactions could be determined by the length of the transmembrane domains, since Golgi-resident proteins have transmembrane domains which are, on average, five residues shorter than those of plasma membrane proteins [Bretscher MS and Munro S, 1993]. The physical-chemical properties of the sphingolipid-glycerolipid gradient together with the preferential association of membrane protein to subdomains of the Golgi, explain the forces driving *cis* to *trans* protein transport through the Golgi complex.

Once packaged in the TGN, transport vesicles containing their cargo must find the correct acceptor membrane with which to fuse. A class of cytoplasmically oriented membrane proteins called SNAREs plays a key role in the targeting and fusion of vesicles. Both vesicles and target membrane have their own unique SNAREs: v-SNAREs, localized in the transport vesicles, and t-SNAREs present in the target membranes. Their interaction is thought to mediate the process of fusion. The mechanisms and components involved in vesicular transport have been extensively reviewed [Machamer CE, 1996].

Vesicle-mediated secretion utilizes three classes of secretory vesicles. The first class is the Constitutive Secretory Vesicle (CSV). In polarized cells such as neurons at least two different types of CSVs coexist: CSVs that deliver proteins to the axonal domains of the plasma membrane, and CSVs for delivering to the dendritic domains. The second class is the secretory granule (in neurons, dense core vesicles), which mediates the stimulus-dependent release of stored proteins. The third class is the synaptic vesicle of neurons that mediate the release of neurotransmitters. While the first two classes derive from the TGN, synaptic vesicles derive from the early endosome [Huttner WB et al., 1995].

Many molecular steps remain to be elucidated on the regulation of intracellular trafficking in the exocytic pathway. However, a lot of sorting signals have been characterized, which, when fused with reporter proteins, are able to specify the protein final destination.

The KDEL retention signal

Maintenance of the complex architecture of the secretory pathway relies on the correct localization of newly synthesized proteins. Proteins located in the organelles of this pathway have to be maintained at their proper position, despite the continuous flow of membrane to the cell surface. Resident proteins of the ER and Golgi, which are essential for the folding, assembly and maturation of secretory and membrane proteins, are subjected to retrograde transport that allows them to escape exocytic transport. A conserved carboxyl-terminal sequence, called KDEL (single letter amino acid code), is necessary and sufficient to their retention in the ER [Munro S and Pelham HR, 1987]. Proteins carrying the KDEL signal can acquire Golgi-specific carbohydrate modifications indicating that they reach a post-ER compartment from which they are continuously retrieved [Pelham HR, 1988]. This retrieval is achieved by the KDEL-receptor, a membrane protein localized in the Golgi apparatus characterized by seven highly conserved hydrophobic segments spanning the membrane [Scheel AA and Pelham HR, 1996]. The hydrophilic binding site of the KDEL-receptor is formed by conserved residues of the transmembrane domains [Scheel AA and Pelham HR, 1998]. ER proteins carring the KDEL signal bind to the KDEL-receptor and enter a retrograde pathway which delivers them back to the ER. Here, the complex dissociate to release the KDEL proteins and to free the receptors for further rounds of retrieval [Pelham HR, 1990].

It is believed that the sorting of KDEL proteins is controlled by the pH difference between the Golgi and the ER (approximately 0.5 pH units) [Wilson DW et al., 1993] and that KDEL protein binding to the KDEL-receptor induces retrograde transport of the complex via a signal transduction mechanism able to span across the membrane [Lewis MJ and Pelham HR, 1992]. Neither of these two mechanisms has been well clarified.

1.2.2 Sorting in the cytosol

In the absence of the leader sequence, the nascent polypeptide is translated by cytoplasmic ribosomes in the cytosol, from which proteins have access to a

number of different intracellular locations. Many motifs have been identified, which specify the targeting from the cytosol to different organelles. In some cases (e.g. nucleus, cytoplasmic face of the plasma membrane), proteins have a direct access to the compartment, while in others (for instance mitochondrial proteins) their translocation through intracellular membranes is required.

Nuclear targeting

The exchange of macromolecules between the cytoplasm and the cell nucleus is a vital process in eukaryotic cells, because of the spatial segregation of DNA replication and RNA biogenesis in the nucleus, and protein synthesis in the cytoplasm. This traffic is mediated by soluble transport receptors that shuttle through the nuclear pore complex, an aggregate of large proteins that form aqueous channels across the nuclear membrane [Vasu SK and Forbes DJ, 2001]. Small molecules can pass from one compartment to the other simply by passive diffusion, while proteins larger than 45 kDa require an active carrier-mediated process and must contain a Nuclear Localization Signal (NLS), recognized by the transport receptors. These transport receptors are members of a conserved family of homologous proteins (importins or exportins) collectively known as karyopherines [Wozniak RW et al., 1998]. Each karyopherin specifically recognizes a set of NLSs either directly or indirectly, with or without the help of adaptors molecules. The importin-protein complex forms in the cytoplasm, enters the nucleus where it dissociates, and the importin carrier recycles back to the cytoplasm [Conti E and Izaurralde E, 2001]. A crucial role in this process is played by Ran, a Ras-related GTPase. Ran binds to GTP and GDP, and its nucleotide-bound state is regulated by several cofactors. The conversion of RanGDP into RanGTP requires the guanine nucleotide exchange factor (RanGEF or RCC1) [Bischoff FR and Ponstingl H, 1991], that is tightly bound to the chromatin in the nucleus, while the GTP hydrolysis is triggered by the RanGTPase protein-1 (RanGAP-1) and co-stimulated by the Ran-binding protein-1 and -2 (RanBP1/2), present mainly in the cytoplasm [Lounsbury KM and Macara IG, 1997]. As a result of this distribution, there is a concentration gradient of RanGTP across the nuclear pore complex: low in the cytoplasm and high in the

nucleus [Izaurralde E et al., 1997]. In this way importins can bind their cargo in the cytosol and release it upon binding to RanGTP in the nucleus, while exportins act the other way round. Following transport of a cargo molecule in one direction, both karyopherins and their adaptors must be recycled to allow another round of transport to occur.

The first NLS to be characterized and studied was a sequence derived from the large T antigen of the SV40 virus, able to confer a nuclear localization to a large number of proteins [Kalderon D et al., 1984]. The NLSs are generally short stretches of amino acids that are not cleaved during transport [Agutter PS and Prochnow D, 1994]. They appear either in the form of a single stretch of residues (consensus: $(K/R)_{4-6}$) or as two smaller clusters separated by a dozen amino acids (consensus: $(K/R)_2\ X_{10-12}\ (K/R)_3$) [Christophe D et al., 2000]. In the latter case the length of the spacer can modulate the targeting efficiency. In addition, targeting efficiency can be modulated by phosphorylation of the flanking regions, or by the presence of multiple copies of NLSs [Jans DA and Hubner S, 1996]. This implies that NLSs conformation and hydrophobicity may be important to control nuclear import and export.

Mitochondrial targeting

The majority of mitochondrial proteins are encoded in the nucleus of the cell, synthesized in the cytoplasm and then targeted to mitochondria. The remaining proteins are encoded by the mitochondrial genome. Most nuclear-encoded mitochondrial proteins contain an amino-terminal extension of the protein (the presequence, usually 20-50 residues), called Mitochondrial Targeting Signal (MTS), which is sufficient to direct them into the mitochondria and which is cleaved upon import into the mitochondria [Horwich AL et al., 1985; Hurt EC et al., 1985]. MTSs can direct non-mitochondrial proteins through both the outer and inner mitochondrial membranes into the matrix, showing that they contain all the information for targeting and membrane traslocation. MTSs do not show significant sequence homology, but they have characteristic physical-chemical properties. They are enriched in positively charged, hydroxylated and

hydrophobic residues, and can form an amphiphilic α-helix [von Heijne G, 1986; von Heijne G et al., 1989].

The protein import machinery of the mitochondrial membranes contains a series of proteins, called translocases, that successively interact with the presequence of a protein in transit. The translocases of the outer and inner membranes are called TOM and TIM (Translocase of the Outer/Inner Membrane) complexes respectively. They bind the MTS and form a channel through the two membranes [reviewed by Voos W et al., 1999]. The translocation through the outer membrane channel seems to be dependent on interactions between the MTS and the highly negative charged domains of Tom22, a protein of the complex, that follow the transport along the channel. Translocation across the inner membrane utilizes the energy of the membrane potential – negative on the matrix side – and the hydrolysis of ATP.

1.2.3 Other intracellular targeting signals

There are many other ways by which a cell can localize its products in a regulated manner. For example, digestive enzymes are targeted into lysosomes where they can degrade intracellular macromolecules as well as particles taken up from the outside by endocytosis. Other enzymes, utilized in a variety of oxidative reactions, must be targeted into the peroxisomes to carry out their functions.

In addition to carrying signals that determine their location, proteins also possess signals that determine their life-time. Cellular proteins are subjected to continuous turnover; multiple pathways of protein degradation operate within the cell. Numerous amino acid sequences responsible for this high turnover have been characterized. Regulated proteolysis is a pathway that removes misfolded proteins, but also a regulatory system, which allows the level of a given protein to be rapidly controlled. One example is the degradation of RNase A through its import into lysosomes. The sequence KFERQ (aa 7-11 of the protein) has been found to be the necessary signal for the uptake into lysosomes [Dice JF, 1990]. The exact sequence is only present in proteins of the RNase A family, but there

are many other proteins containing sequences of similar charge and hydrophobicity. This signal is recognized by a specific receptor, the lysosomal membrane glycoprotein LPG96, that mediates the selective protein import [Cuervo AM and Dice JF, 1996].

A non-lysosomal proteolytic system, with a high degree of specificity towards its substrates, is the so called ubiquitin-proteasome pathway. Briefly, this pathway involves two successive steps: tagging of the substrate by covalent attachment of multiple ubiquitin molecules, and degradation of the tagged protein by the 26S highly conserved proteasome complex which degrades proteins to yield small peptides and free ubiquitin in an ATP-dependent fashion. There are specific signals within cellular proteins that are recognized by several ubiquitinating enzymes and that can be switched on or off (for instance by phosphorylation), thus regulating rapid protein degradation [for review see for example Glickman MH, 2000].

Hydrophilic stretches of about 12 amino acids enriched in proline (P), glutamate (E), serine (S), and threonine (T), the so called PEST sequence, represents a constitutive signal for rapid protein degradation [Rogers S et al., 1986]. The mechanism by which PEST sequences act has not been fully elucidated yet, but the involvement of proteasome degradation has been suggested [Rechsteiner M and Rogers SW, 1996]. Phosphorylation of PEST sequence can lead to a regulated degradation showing that the ubiquitin-proteasome pathway and other intracellular protein degradation mechanisms must be finely controlled.

1.3 Neurotrophins and their receptors

Neurotrophins constitute a small family of structurally related trophic proteins that affect essentially all biological aspects of vertebrate neurons, including their survival, shape, differentiation and function [for review see Huang EJ and Reichardt LF, 2001].

In mammals the known neurotrophins are: Nerve Growth Factor (NGF) [Levi-Montalcini R, 1966; Levi-Montalcini R, 1987], Brain-Derived Neurotrophic Factor (BDNF) [Barde YA et al., 1982], Neurotrophin-3 (NT-3) [Hohn A et al., 1990] and Neurotrophin-4/5 (NT-4/5) [Berkemeier LR et al., 1991; Hallbook F et al., 1991]. The NT-6 and NT-7 genes have only been identified in fish, and do not appear to have mammalian homologous [Gotz R et al., 1994; Nilsson AS et al., 1998]. Neurotrophins are secreted proteins and share many functional properties with classical neurotransmitters; for example, neurotrophins are released at synapses and are required for activity-dependent forms of synaptic plasticity [reviewed by Poo MM, 2001]. Recently, it has also been found that BDNF is able to open sodium-ion channels, inducing membrane depolarization within milliseconds [Blum R et al., 2002].

Neurotrophins are small homodimeric polypeptides, of approximately 120 aminoacid long, sharing approximately 50% sequence identity [Hallbook F et al., 1991]. The structure of the NGF monomer consists of 3 antiparallel β-strands forming a large flat surface that, in the dimer, is covered by the corresponding surface of the second monomer [Holland DR et al., 1994]. Is is thought that all neurotrophins possess a similar dimer arrangement, as demonstrated by the crystal structure of the BDNF/NT-3 heterodimer [Robinson RC et al., 1995].

The broad spectrum of biological activities exerted by the neurotrophins results from their ability to bind and activate two kinds of structurally unrelated receptors, the p75 neurotrophin receptor ($p75^{NTR}$), a member of the Tumor Necrosis Factor Receptor (TNFR) superfamily, and the three members of the Tropomyosin-related Kinase (Trk) receptor family of tyrosin kinases TrkA, TrkB and TrkC [Kaplan DR et al., 1991]. The three members of the Trk receptor family

selectively bind different neurotrophins [Ip NY et al., 1993]: TrkA binds NGF as its preferred ligand; TrkB binds BDNF and NT-4/5 with apparently equal affinity; and TrkC is specific for NT-3. Some limited cross-reactivity of NT-3 with TrkA and TrkB has also been reported. Interaction of neurotrophins with $p75^{NTR}$ is important for modulating the specific activation of the Trk receptors [Benedetti M et al., 1993; Bibel M et al., 1999; Vesa J et al., 2000]. The Trk receptors contain a combination of cell-adhesion motifs in their extracellular domain, with three tandem leucine-rich domains flanked by two cysteine-rich clusters and, in the more membrane-proximal region, an immunoglobuline-like domain [Urfer R et al., 1998]. The cytoplasmic region contains a catalytic tyrosine-kinase domain. Binding of the neurotrophin ligands to these receptors leads to receptor dimerization, tyrosine auto-phosphorylation [Jing S et al., 1992], and kinase activation.

The $p75^{NTR}$ has a large extracellular structure that contains four negatively charged cysteine-rich repeated modules, the ligand binding domain, a transmembrane domain and a cytoplasmic part, which is highly conserved among species and devoid of any homology with protein kinases. The intracellular domain of the $p75^{NTR}$ contains a so called "death domain" involved in signalling pathways modulating apoptosis [Liepinsh E et al., 1997].

Neurotrophin receptors are differentially distributed throughout the CNS and Peripheral Nervous System (PNS). During development, expression of TrkA is limited to sensory and sympathetic neurons in the PNS and cholinergic neurons of the basal forebrain [Holtzman DM et al., 1992; Martin-Zanca D et al., 1990], although a recent work has reported the presence of this receptor in cultured hippocampal neurons [Culmsee C et al., 2002]. More extensive CNS expression is found for TrkB and TrkC [Barbacid M, 1994] in the cerebellum, the hippocampus and the cerebral cortex. On the other hand, $p75^{NTR}$ has a much wider distribution and is expressed in a variety of cell types in the CNS [Bothwell M, 1991], in sympathetic and sensory neurons in the PNS [Schatteman GC et al., 1993], and also in non-neuronal tissue.

In addition to their specific patterns of expression in the adult CNS, neurotrophins and their receptors are developmentally regulated and during development some neurons switch their neurotrophin sensitivity [Buchman VL and Davies AM, 1993; Davies AM, 1997]. These observations suggest a broad function for neurotrophins and their receptors in supporting neuronal growth, differentiation and synaptogenesis during development, in addition to their activity in neuronal survival and synaptic plasticity.

1.3.1 Neurotrophins signal transduction

Neurotrophin binding to Trk receptors results in receptor dimerization and kinase activation. There are 10 conserved tyrosines in the cytoplasmic domain, three of which are in the autoregulatory loop of the kinase domain. Phosphorylation of these residues further activates the kinase [Cunningham ME and Greene LA, 1998]. Phosphorylation of the other tyrosines promotes signaling by creating docking sites for adaptor molecules that couple the receptors to the intracellular signaling cascade. In particular, phosphorylated tyrosine 490 mediates the interaction with the adaptor protein Shc [Dikic I et al., 1995], while tyrosine 785 leads to the activation of Phospholipase C γ subunit (PLC-γ). Five of the remaining conserved tyrosines contribute to NGF-induced neurite outgrowth [Inagaki N et al., 1995].

Three main signaling cascades are activated by Trk receptors substrates [Kaplan D and Miller FD, 2000; Patapoutian A and Reichardt LF, 2001]. First, the activation of the Ras/MEK/MAPK pathway, that results from the formation of a variety of adaptor molecules complexes including Sch protein. Second, the association with the receptor and activation of PI-3K [Holgado-Madruga M et al., 1997; Yamada M et al., 1997], that is especially implicated in neuronal survival via the activation of the serine/threonine kinase Akt (protein kinase B) [Crowder RJ and Freeman RS, 1998]. Third, the activation of PLC-γ regulates intracellular Ca^{2+} levels and Protein Kinase C (PKC) mediated cleavage of the substrate PIP_2. This pathway

seems to play an important role in neurotrophin-mediated neurotrophin release [Canossa M et al., 1997].

In general, tyrosine phosphorylation of membrane receptors results in downstream stimulation of cellular proteins responsible for activating the gene transcriptional machinery that controls growth, migration, cell shape, and survival of neurons. The system shows a remarkable redundancy allowing the regulation of multiple responses to stimuli depending on the cellular context.

In figure 1.2 an outline of the main signalling pathways through Trk receptors.

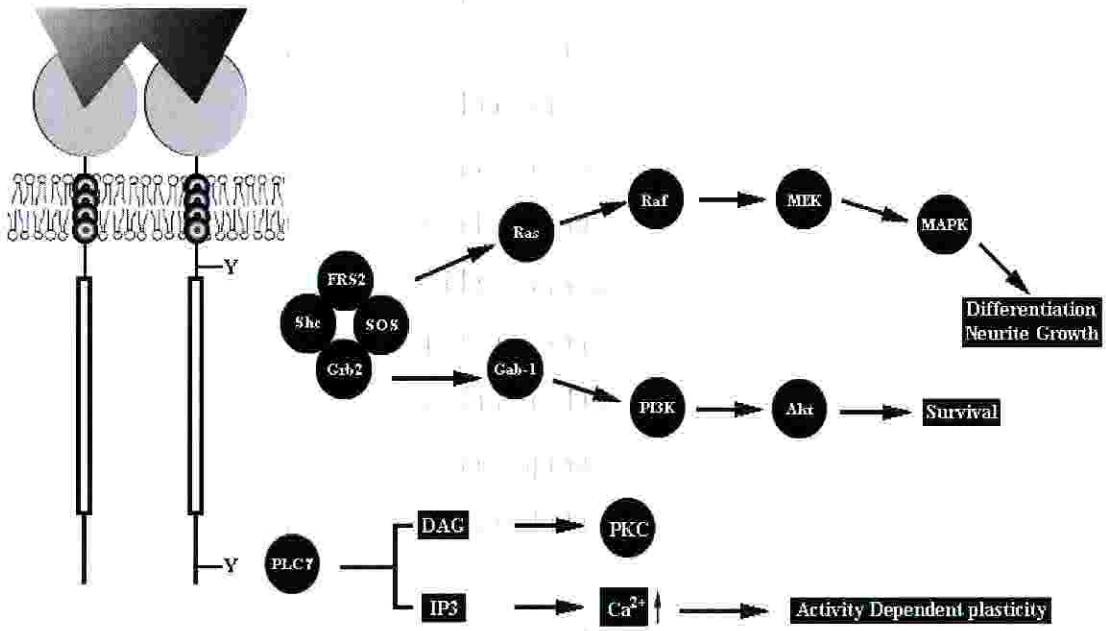

Fig 1.2 Main signalling pathways through Trk receptors.
[Adapted from Bibel M and Barde YA, 2000]

DAG diacylglycerol;
FRS-2 fibroblast growth factor substrate 2;
IP_3 inositol-1,4,5-triphosphate;
MAPK mitogen activated protein kinase;
MEK MAPK kinase;
PIP_2 phosphtidylinositol-4,5-biphosphate;
PKC protein kinase C;
PLC phospholipase C;
PI-3K phosphatidylinositol-3-OH kinase;
SOS son of Sevenless

The other neurotrophin receptor, $p75^{NTR}$, has several important roles in the nervous system. Because of its many distinct functions in diverse tissues, it has been hypothesized that ligand binding may activate distinct intracellular signalling pathways, depending on the cellular context of the receptor. Numerous adaptor proteins that bind $p75^{NTR}$ have been reported (for a definition of the acronyms see figure 1.3 legend). Three of them, NRIF [Casademunt E et al., 1999], NADE [Mukai J et al., 2000] and NRAGE [Salehi AH et al., 2000], contribute to apoptosis in cell lines or are correlated with neurotrophin-dependent cell death. Each protein binds to separate sequences in the cytoplasmic domain of the receptor. Other protein including RhoA GTPase [Yamashita T et al., 1999] and SC-1 [Chittka A and Chao MV, 1999], exert non-apoptotic activities such as neurite elongation and cell cycle arrest. The use of similar signalling pathways between $p75^{NTR}$ and other TNFR family members has suggested to evaluate whether receptor proximal events are also shared: it has been found that TNF receptor-associated factors (TRAFs) can bind to $p75^{NTR}$ and regulate NFκB activation [Khursigara G et al., 2001; Ye X et al., 1999] through which $p75^{NTR}$ and TrK receptors signal transduction pathway may be functionally linked. In addition, Rac GTPase has been identified as a novel effector of $p75^{NTR}$-mediated JNK activation in primary oligodendrocytes, although direct interactions between $p75^{NTR}$ and Rac have not yet been demonstrated [Harrington AW et al., 2002; for recent reviews, see Hempstead, 2002; Dechant and Barde, 2002; Roux and Barker 2002]. Among the most obviously relevant proteins interacting with $p75^{NTR}$ are the three Trk receptors. Both receptor types are often expressed by the same cells, and form a complex that can be immunoprecipitated [Bibel M et al., 1999]. The Ankyrin Repeats Membrane Spanning (ARMS) molecule may serve to bridge $p75^{NTR}$ with Trk receptors as an integral part of the complex [Kong H et al., 2001]. This interaction is functional in several relevant ways; receptor association leads to high-affinity neurotrophin binding [Hempstead BL et al., 1991; Horton A et al., 1997], increases ligand discrimination by the Trk receptors, (especially important in the case of TrkA and TrkB which bind more than one neurotrophin

[Benedetti M et al., 1993; Bibel M et al., 1999]), and allows an interaction between the signalling pathways triggered by both receptors.

Although the complexity of the biological action of $p75^{NTR}$ has not yet been comprehensively explained, the recent identification of novel intracellular partner, suggests that this receptor uses different neurotrophin ligands in distinct cell types, during different developmental stages or following injury.

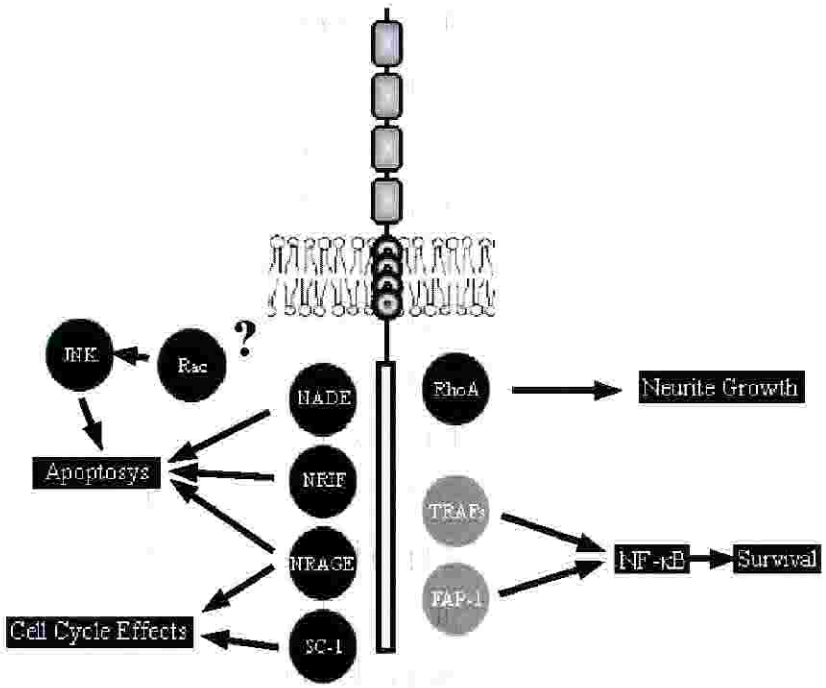

Fig. 1.3 $p75^{NTR}$ recruits cytoplasmic interactors to signal that mediate different biological functions.
[Adapted from Bibel M and Barde YA, 2000]

NRIF	neurotrophin receptor-interacting factor
NADE	$p75^{NTR}$-associated cell death executor
NRAGE	neurotrophin receptor interacting melanoma associated antigen homolog
SC-1	Schwann cell factor 1
TRAFs	TNF receptor-associated factors
NFκB	nuclear factor κB
JNK	c-jun N-terminal kinase

1.4 Intracellular immunization

The virtually unlimited repertoire of antibodies and their specific high affinity binding properties can be exploited as a source of specific and selective reagents and probes for scientific and applicative purpose, such as identification, purification, and functional manipulation of specific proteins. The advent of monoclonal antibodies (mAbs) represents a milestone in the field of immunology [Kohler G and Milstein C, 1975]. Antibodies, or products derived from them by chemical or genetic engineering methods, are extremely useful since they can be easily delivered to a biological system. Antibodies have been used for a long time to inactivate the function of a target protein by injection *in vivo* [Levi-Montalcini R and Angeletti PU, 1966] or by intracellular microinjection [Graessmann A et al., 1980; Morgan DO and Roth RA, 1988]. For example, microinjection of purified mAb anti p21 Ras causes the inhibition of NGF-induced neurite formation and extension in pheochromocytoma (PC12) cells [Hagag N et al., 1986]. However, injected antibodies can interfere with cytosolic or nuclear proteins, but do not have access to proteins which are in the membrane compartments like the ER, Golgi or mitochondria. Furthermore only short-term and fast biological responses can be studied, since antibodies are rapidly degraded.

The availability of hybridoma cells lines secreting predefined antibodies allows the rearrangement of the genes that encode for that specific antibody and provides unlimited amount of the antibody in a purified form. Vast repertoires of these genes are now even more accessible thanks to the phage display technology [McCafferty J et al., 1990]. Using this technology, any antibody can be expressed on the surface of a phage particle by cloning the antibody gene into the phage genome in fusion with a coat protein. The resulting particle can be easily purified by a number of cycles of affinity selection. This technique not only allows the isolation of antibodies against virtually any antigen in a relative simply *in vitro* procedure, but it is also the simplest way to clone human antibodies. In fact, the

major advantage of phage display is that the phenotype and genotype of the antibody are selected together because both are included into the phage particle.

The ectopic expression of genes encoding specific antibodies in a wide variety of non-lymphoid biological systems could be used to interfere with the corresponding protein [Cattaneo A and Neuberger MS, 1987]. The basic idea is to redirect antibodies to the place and at the time where the target protein is expressed. Antibodies can be ectopically expressed in cells that do not normally express them as secretory proteins, to interfere with extracellular antigens, or as intracellular proteins, targeted to different compartments, to neutralize intracellular gene products. The intracellular immunization approach is based on the idea that the antibody can be targeted towards intracellular sites when equipped with the suitable targeting signals, by taking advantage of the great wealth of information accumulated on the different ways in which proteins, but also their mRNAs, are specifically targeted inside the cell.

1.4.1 Engineering antibodies

Immunoglobulins consist of four polypeptides chains, two Heavy (H) and two Light (L) chains, held together by disulfide bonds and non-covalent interactions. The immunoglobulins chains are composed of domains with similar structure, each consisting of two layers of β-sheets surrounding an internal space filled with hydrophobic amino acid side chain, with terminal exposed loops. Domains are termed either Constant (C) or Variable (V) on the basis of the degree of sequence variation among different antibodies. There are three hypervariable-exposed loops at the top of the VH and VL domains that together form the binding site of the antibody. These loops vary in length as well as in sequence and are also known as Complementarity Determining Regions (CDRs) due to their dominant role in determining shape and specificity of the binding site.

Digestion of immunoglobulins with the proteolytic enzyme papain divides the antibody molecule in two identical antigen-binding fragments, called Fabs, and a fragment containing most of the constant domains, called Fc (see Fig. 1.4), which

performs functions that are neither needed nor necessary for intracellular immunization. Even though the heavy and light chains can functionally associate even in the rather hostile cytosolic environment [Biocca S et al., 1990], the whole antibody format is only to be recommended for extracellular immunization experiments. Antibody engineering methods have produced a number of simpler antibody forms that have been successfully used for intracellular immunization such as the Fabs fragments and the Single-Chain Fv (Fab variable) fragments (scFvs) [e.g. Biocca S et al., 1993]. The Fv fragment is the smallest fragment obtained by association of the VL and VH. Because of the weak hydrophobic interactions between these two domains, a covalent link (usually a peptide linker of 15-20 amino acids) was added to obtain a stable molecule [Bird RE et al., 1988]. The resulting small molecule, the scFV, has high-affinity binding capacity and minimal assembly requirements and can be easily directed to subcellular compartments with the utilization of well characterized protein signal sequences (see chapter 1.2).

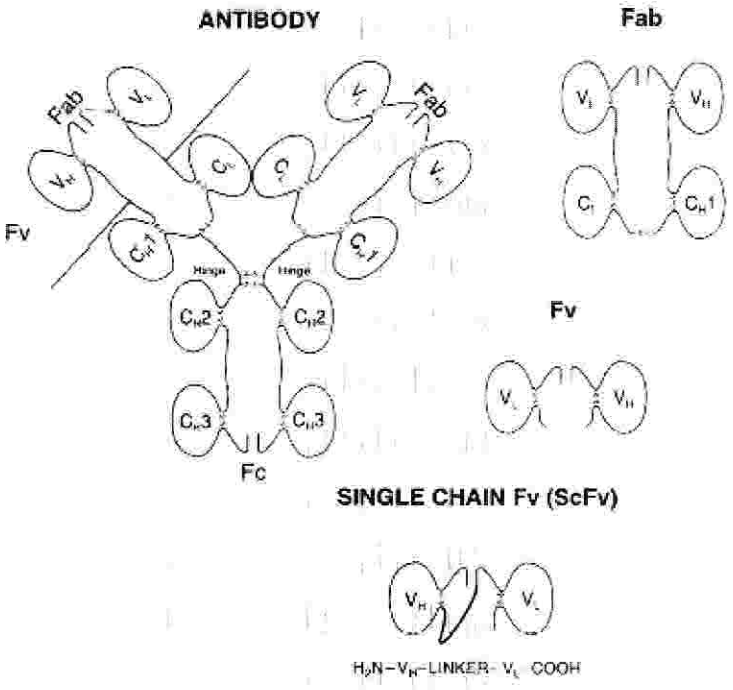

Fig 1.4: Antibodies and antibody domains

Antibody fragments can fold correctly in the oxidizing environment of the ER where they can also interact with specific chaperones. In contrast, when they are expressed in the cytoplasm, the reducing conditions hinder the formation of the intra-domain disulfide bounds and the molecule is not folded properly. Some exceptions have been described [Proba K et al., 1998], and, unfortunately, there is not a general rule which can reliably predict how antibodies will fold in the cytoplasm. A strategy for selection of cytoplasmic intracellular antibodies *in vivo* using the two-hybrid system has recently been developed [Visintin M et al., 1999]. The Intracellular Antibody Capture Technology (IACT) can be applied not only to select for specific antibodies from a pool, but also for the *de novo* selection of a panel of functional intracellular antibodies against several different proteins on the basis of their capacity to bind the antigen in the cytoplasmic environment [Visintin M et al., 2002].

The property of dominant and autonomous targeting sequences to confer a new localization to a reporter protein has been exploited to redirect individual antibodies to different intracellular compartments. In principle, a large number of sorting signals can be used for antibody targeting. Table 1.2 summarizes the signals used for antibody targeting to different intracellular compartments [Biocca S and Cattaneo A, 1995; Persic L et al., 1997a].

TABLE 1.2 PEPTIDE SIGNALS USED FOR ANTIBODIES TARGETING

COMPARTMENT	SIGNAL	REFERENCE
Secretory pathway	Hydrophobic leader sequence at the N-terminus: **MGWSCIILFLVATATGVHSQ** (for example)	naturally occurring
Cytoplasm	Hydrophilic leader sequences at N-terminus: **MGWSCKRRSSEETATAGVHSQ** or no leader	e.g. Biocca S et al., 1994
Nucleus	NLSs at the N-terminus or C-terminus: **MGWSCPKKKRKVGGGTATVHSQ**	e.g. Strube RW and Chen SY, 2002
Mitochondrion	Presequence of mitochondrial proteins at N-terminus: **MSVLTPLLLRGLTGSARRLPVPRAK**	Biocca S et al., 1995
ER lumen	Leader sequence and **SEKDEL** sequence at C-terminus	e.g. Marasco WA et al., 1993

1.4.2 Phenotypic knock-out

"Phenotypic Knock-Out" (pKO) indicates the possibility to inactivate the function of a target protein without impairing its gene. This can be done using specific intracellular antibodies that can generate the functional knock-out of the protein of interest. This is a powerful and valuable tool to investigate the *in vivo* functions of a given protein in both basic research and in the development of therapeutic agents [for review see Marasco WA and Dana Jones S, 1998]. The pKO can be achieved in many different ways. For example, it is possible to redirect proteins from their usual cellular compartment to other sites where they are unable to carry on their function. Proteins can also be targeted to degradative cellular

compartments. We can block enzyme functions by occluding its active site. It is also possible to prevent macromolecular interaction by obstructing the binding site of a receptor. For the last two examples of pKO the antibody or antibody fragment is required to recognize and bind specifically to the active or binding sites of the target protein. These are called "neutralizing antibodies", they act as dominant negative inhibitors, and can be use both intracellularly and extracellularly. Neutralizing antibodies have been used to inhibit the function of a variety of proteins, above all viral and oncogenic proteins. The inhibition of meiotic maturation in *Xenopus* oocytes was one of the first examples of pKO. The oocytes inhibition was achieved by neutralizing the activity of p21 Ras protein with the cytoplasmic expression of an anti Ras scFv [Biocca S et al., 1994]. Using cytoplasmic neutralizing scFv it was also possible to block the replication of HIV-1 [Duan L et al., 1994] and to prevent its infection *in vitro* by inhibiting the activity of HIV reverse transcriptase [Maciejewski JP et al., 1995]. Recently, HIV-1 replication has also been blocked with an scFv that binds a subunit of the virus elongation factor [Bai J et al., 2003]. Intracellular immunization is a potential method for cancer therapy, not only by expressing antibodies in the cytoplasm [Jean D and Bar-Eli M, 2001; Strube RW and Chen SY, 2002], but also by directing antibodies into the nucleus in order to interfere with nuclear proteins involved in tumor proliferation [Cochet O et al., 1999; Kasono K et al., 2000].

When an antibody is engineered to reach the secretory pathway, it can easily interfere with the activity of membrane protein receptors by blocking ligand-specific interactions. This can be obtain either by antibody-receptor binding directly within the secretory pathway, or by extracellular antigen recognition. This was done to inhibit the function of the epidermal growth factor receptor [Beerli RR et al., 1994] and the NGF receptor [Ruberti F et al., 2000]. In this work Cattaneo's group expressed an anti NGF neutralizing monoclonal antibody (αD11) [Cattaneo A et al., 1988; Ruberti F et al., 1993] as two transgenes in mice in order to form the intact antibody [Ruberti F et al., 2000]. A very interesting aspect in this work is that aged αD11 transgenic mice show neurodegenerative

pathology strictly related to Alzheimer's disease, making this animals the most comprehensive model for this severe neurodegenerative disease [Capsoni S et al., 2000].

If it can be very difficult to obtain a given antibody able to correctly fold into the cytoplasm (a problem now solved with the IACT), more difficult is to obtain a correctly folded neutralizing antibody that recognize exactly specific epitopes of the target protein. For this reasons other approaches have been developed, such as the redirection of target proteins from their usual surrounding using antibodies that do not necessarily have to be neutralizing. The major application of this kind of approach is to prevent the cell surface expression of receptors using antibodies as "intracellular anchors". A similar strategy is used by viruses to prevent appearance of host membrane proteins, such as histocompatibility complexes, by blocking their export [Burgert HG and Kvist S, 1985; Crise B et al., 1990]. Using intracellular antibodies, this block can be achieved with the KDEL signal. Re-engineered antibodies, which are expressed in fusion with the KDEL signals, are synthesized along the secretory pathway, but remain trapped in the ER. They show high level of expression, low turnover rates, efficient folding and disulfide bound formation, probably because of the presence of chaperon proteins. Finally, the tubular architecture of this organelle maximizes the chance of interaction between a resident antibody and its target. A clear example of this approach was the abrogation of cell surface expression of interleukine-2 receptor with a scFv KDEL [Richardson JH et al., 1995]. This technique was also used to develop therapeutic tools against HIV by preventing the appearance of both T cell HIV receptors [BouHamdan M et al., 2001; Steinberger P et al., 2000], and viral proteins [Zhou P et al., 1998]. Another interesting application of this kind of pKO was recently studied: the retention in the ER of the major histocompatibility complex was suggested as a strategy to inhibit rejection after allografts [Mhashilkar AM et al., 2002].

Other re-location approaches with non-neutralizing antibodies could be developed, such as the recruitment of cytoplasmic proteins into the nucleus and *vice-versa*. An experiment moving in this direction has already given good results

[Visintin M et al., 2002]. In this work, CHO cells co-transfected with DNA encoding both for a cytoplasmatic Tau protein and a nuclear targeted anti-Tau scFv, show a predominant nuclear staining for Tau antigen, suggesting a redirection of Tau protein into the nucleus.

In order to neutralize the activity of a specific protein it may be possible to target the antibody-protein complex to the degradation pathway. Then the so called "suicide antibodies" could be engineered to cause the concomitant transport to a degradative compartment and degradation of the bound antigen.

The existing knowledge on RNA localization may be exploited to create other powerful pKO tools for polarized cells. In fact, it could be possible to target mRNA of engineered antibodies to, for example, neuronal dendrites. There, they can be expressed, bind their specific antigen and, because of the presence of protein targeting signal, redirect themselves together with the antigen, into different cellular compartment such as dendritic ER or mitochondria. In this way perhaps it may be possible to redirect a dendritic protein into the nucleus and so prevent its activity at the post-synaptic site. On the other hand it could be also possible to exploit this "double targeting" in case of a slightly cytotossical scFvs. In fact the protein targeting signal could be used to localize expressed scFvs to the subcellular site of interest while providing RNA targeting element that direct scFvs mRNA localization to an other intracellular compartment. In this way the ultimate effect should allow to reduce the concentration of toxic scFvs on the site of interest while preserving its ability to reach it. Moreover the combination of RNA and protein targeting is a powerful technique also for cellular biology studies of extremely complex cell types like neurons.

Aim of the work

The aim of the present work is to deeply investigate the powers and limitations of the intracellular antibody technology in particular when used in combination with specific intracellular targeting elements.

The general idea is to verify the possibility to exploit the natural cellular trafficking (see movie **A**) to exert a fine control on the localization of the recombinant intrabodies of interest. In this way an antibody in form of single chain Fab variable fragments (scFvs) could be used to selectively interfere with the function of specific proteins that, in our work, are the neurotrophins receptors. Ideally this would allow the opening of a wide range of new possibilities for the phenotypic knock out approach.

In order to move the first steps in this direction it was necessary to verify if intracellularly expressed scFvs could indeed reach the compartment where they are targeted. To do that, recombinant scFvs in fusion with Green Fluorescent Protein (GFP) where expressed in combination with different targeting signals and their fate within the cell was followed.

In the general theme of neurotrophin receptor study; the possibility to use specific mRNA targeting elements it has been also explored in order to reach particular cell compartments such as distal dendrites in neurons.

Specific scFvs that recognize the neurotrophins receptors $p75^{NTR}$ and TrkA were analysed for their functionality after a targeted expression and, in particular, for their ability to alter the trafficking of the two receptors towards the membrane. In this way the presence on cell surface of the two receptors was modified and the functional consequence on receptor signalling in different neuronal cell types was studied. With the same experiment it was also possible to observe, from a completely novel point of view, the probable complex formation between $p75^{NTR}$ and TrkB receptors.

Finally, the new tools developed were used as a basis for a further development of intrabody constructs to create new transgenic mice for the study of Alzheimer's disease based on the brilliant results of the αD11 transgenic mice recently obtained by Capsoni and colleagues in the Cattaneo lab.

Chapter 2

Methods

2.1 Plasmids and scFvs targeting signals

A brief description of all the DNA constructs used for several steps of this work.

2.1.1 Constructs for protein targeting

Anti β-Gal (R4) scFv was obtained by subcloning the NcoI-NotI fragment from pPM163R4 (kindly provided by P. Martineau) [Martineau P et al., 1998], into the ScFvExpress plasmids [Persic L et al., 1997a] with nuclear (NLS), KDEL, cytoplasmic (cyto) and secretory (sec) targeting signals. The NcoI site of sec and KDEL plasmid had been previously moved from the original position downstream the secretory leader sequence.

The scFvs α p75 (#19E) and α TrkA (#29), selected from Vaughan library [Vaughan TJ et al., 1996], were subcloned into ScFvExpress vectors carrying the secretory and KDEL signals using the restriction sites NcoI and NotI.

MNAC (#13), cloned into the phagemid vector pDAN3 [Cattaneo A et al., 1999], was subcloned by PCR in ScFvExpress sec and KDEL vectors with BssHII and NotI (primers used: MVL for and MVH back, see chapter 2.1.6). The correct DNA sequence of MNAC after the subcloning was verified by automated analysis with Global IR^2 LI-COR DNA Sequencer. The Sequitherm EXCEL II Long-Read DNA Sequencing Kit-LC with 2 pmoles of IRD-800 modified oligonucleotide primers, to allow laser detection at 800 nm, was used for the enzymatic reaction. The sequencing gel was analyzed with e-Seq software (LI-COR). In general, 1 ng of template DNA was necessary for each reaction, while the reactions were performed following the standard protocol provided with the sequencing kit. All the DNA sequences analyzed for this work were made with the same sequencer, software and kit.

ScFvs GFP tagged vectors were prepared by subcloning EGFP from pEGFP-N1 (Clontech) by PCR amplification (primers: GFP for and GFP back) into ScFvExpress plasmids, as N-terminal fusion protein of scFvs, with NotI-NotI restriction sites. The correct cloning direction was determined first by PCR with a suitable combination of oligonucleotides (inside and outside the fragment encoding GFP), and then by DNA sequence analysis.

Anti p21Ras scFv B5 has been previously cloned into ScFvExpress plasmids [Persic L et al., 1999a]. The N-terminal fused GFP was subcloned as described above. Anti p21Ras scFv HH2 has been previously cloned into pEGFP-N1 (Clontech) [Lener M et al., 2000].

2.1.2 Constructs for RNA targeting

GFP targeting vectors were prepared starting from pEGFP-N1 plasmid (Clontech). A linker was cloned at the 5' of GFP sequence between restriction sites BsrGI and XbaI by the aid of two complementary oligonucleotides (GXB for and GXB back). The inserted linker contains the unique restriction sites XbaI, BssHII, AscI, SpeI and BstEII, while the original restriction sites XbaI was not recreated with the oligos design. The resulted plasmid was called pGXB.

The A2RE targeting signal was cloned into pGXB plasmid between sites AscI-SpeI with the aid of partially overlapping oligonucleotides (A2RE for and A2RE back). The clone obtained was then analyzed by automated sequencer.

The 3'UTR sequence of CaMKIIα was extracted and purified from pMM281 [Mayford M et al., 1995; kindly provided by Prof. Mayford] using AscI-SpeI restriction sites, where the AscI site was created, by Klenow nucleotides insertion, from the original NarI site. The obtained fragment (3310 bp) was ligated into the pGXB plasmid with the same restriction sites. More than 1500 bp of the UTR were sequenced after cloning into pEGFP-N1.

The 94 bp DTE fragment of 3'UTR CaMKIIα was purified from pMM281 using AscI-EcoRI restriction sites GXB plasmid was digested with AscI-SpeI restriction enzymes. EcoRI sticky end of DTE fragment and SpeI sticky end of GXB plasmid

were blunted by Klenow nucleotides insertion in order to perform a AscI/AscI-SpeI/EcoRI ligation. The full length DTE was sequenced after cloning.

R4 GFP cyto plasmids carrying A2RE and DTE RNA targeting elements were prepared as described:

> The fragment R4 GFP cyto, starting from the end of the promoter and ending just before the poly adenylation signal, was purified with the restriction sites PmlI (blunt end) and XbaI.

> GFP sequence was removed from A2RE and DTE plasmids by SmaI (blunt end) and XbaI restriction sites.

> The fragment was ligated into DTE and A2RE plasmid lacking the GFP (PmlI/SmaI-XbaI/XbaI) obtaining DTE and A2RE R4 GFP plasmids which were sequenced.

2.1.3 Constructs for transgenic mice

The VH and VK backbone plasmids, with the human Cγ1 gene and the human Cκ gene, [Persic L et al., 1997b] were used to subclone DNA of whole antibodies.

VH and VL chains of MNAC were amplified with oligonucleotides carrying respectively BssHII-BstEII and SacI-BglII restriction sites (Ab MVH for and Ab MVH back; Ab MVL for and Ab MVL back). VH and VL chains were subcloned into the respective plasmids obtaining the chimeric antibody mouse/human.

The VH chain of anti NP antibody was subcloned from pSV-HSμ1 [Mason JO et al., 1988] into VH backbone plasmid by PCR using oligonucleotides carrying BssHII-BstEII restriction sites (NPVH for and Ab NPVH back). The VL chain of α NP antibody was subcloned from pSV-HSλ1 [Cattaneo A and Neuberger MS, 1987] into VL backbone plasmid by PCR using oligonucleotides carrying ApaLI-XhoI restriction sites (Ab NPVL for and Ab NPVL back).

The α NP scFv was constructed by subcloning NP antibody VH and VL chains into pDAN3 plasmid [Sblattero D and Bradbury A, 2000] in order to add the scFv linker present in pDAN3. VH and VL chains were amplified using oligonucleotides carrying respectively BssHII-SalI and XhoI-NheI restriction sites

(NPVH for, NPVH pDAN back, NPVL pDAN for, NPVL pDAN back). The resulting α NP scFv was subcloned by PCR with oligonucleotides carrying BssHII-NotI restriction sites (NPVH for and NPVL back) into the ScFvExpress plasmids sec and KDEL that had exchanged the EF-BOS promoter with the CMV promoter by Dr. Lidija Persic (CMVmut.-ScFvExpress plasmids).

The α TrkA (#29) and α p75 (#19E) scFvs were subcloned from ScFvExpress into CMVmut.-ScFvExpress KDEL plasmid by ligation using the BssHII-NotI restriction sites.

MNAC scFv was subcloned from ScFvExpress into CMVmut.-ScFvExpress sec and KDEL plasmids by ligation using the BssHII-NotI restriction sites.

The α D11 scFv, previously cloned into the ScFvExpress plasmid by Dr. Sonia Covaceuszach, was subcloned into CMVmut.-ScFvExpress plasmid sec by ligation using the BssHII-NotI restriction sites.

2.1.4 DNA extraction from mice tails

The specificity of the designed primers for screening selection of transgenic mice was tested by PCR using genomic mice DNA extract as templates.

DNA was extracted as follows:

Tails were placed it into a 1.5 ml microcentrifuge tube containing 500µl of extraction buffer (50 mM Tris-HCl, pH 8; 100 mM EDTA; 100 mM NaCl; 1% SDS; 600 µg/ml proteinase K). Tubes were incubated overnight at 55°C with periodic agitation. Five hundred µl phenol-chloroform 1:1 (v/v) were added to each tube and the tubes were incubated at RT for 20 minutes in agitation. Samples were centrifuged at 12.000 rpm (5 min, RT) and the upper phase was recovered. One ml 100% ethanol was added. The precipitate was recovered, placed in a fresh tube and resuspended in 500 µl of water.

2.1.5 Molecular biology

Standard for molecular biological techniques were used as described in Sambrook J et al. [1989].

Enzymes for DNA modification were obtained from Boehringer Mannheim, Gibco BRL, New England Biolabs, Pharmcia, Promega, and Stratagene.

MIDI Plasmid preparations were made with Nucleobond kits. MINI plasmid preparations were made with SIGMA kit. DNA purification and extraction from gel were made with PCR Purification kit (QIAGEN) and Gel Extraction kit (QIAGEN).

To produce DNA for cloning the high fidelity Pfu polymerase (NEB) was used, for other reasons (analytic or fingerprinting) the RedTaq polymerase (SIGMA) was used. All other routine molecular biology reagents were from Sigma, Merck, or Calbiochem.

In figure 2.1 the maps of all the plasmids used in this work are shown.

Fig 2.1 Plasmid maps

Panel **A**: General map of ScFvExpress. In the expression cassette the four different protein targeting signals used: cytoplasmic (cyto) secretory (sec), endoplasmic reticulum (KDEL) and nuclear (NLS). The scFvs constructs for transgenic mice were made with CMVmut ScFvExpress in which the EF-BOS promoter was substituted with the CMV promoter.
Panel **B**: pEGFP-N1 (Clontech). This plasmid was the starting point for GFP RNA targeting vectors and the source of GFP DNA for tag cloning.
Panel **C**: pBluescript II SK (Stratagen). The plasmid for GFP RNA riboprobe *in vitro* transcription.
Panel **D**: pMM281 [Mayford M et al., 1995]. The plasmid source of CaMKIIα 3'UTR and DTE.
Panels **E** and **F**: VH and VK express plasmid used for whole antibodies transgenic mice constructs.

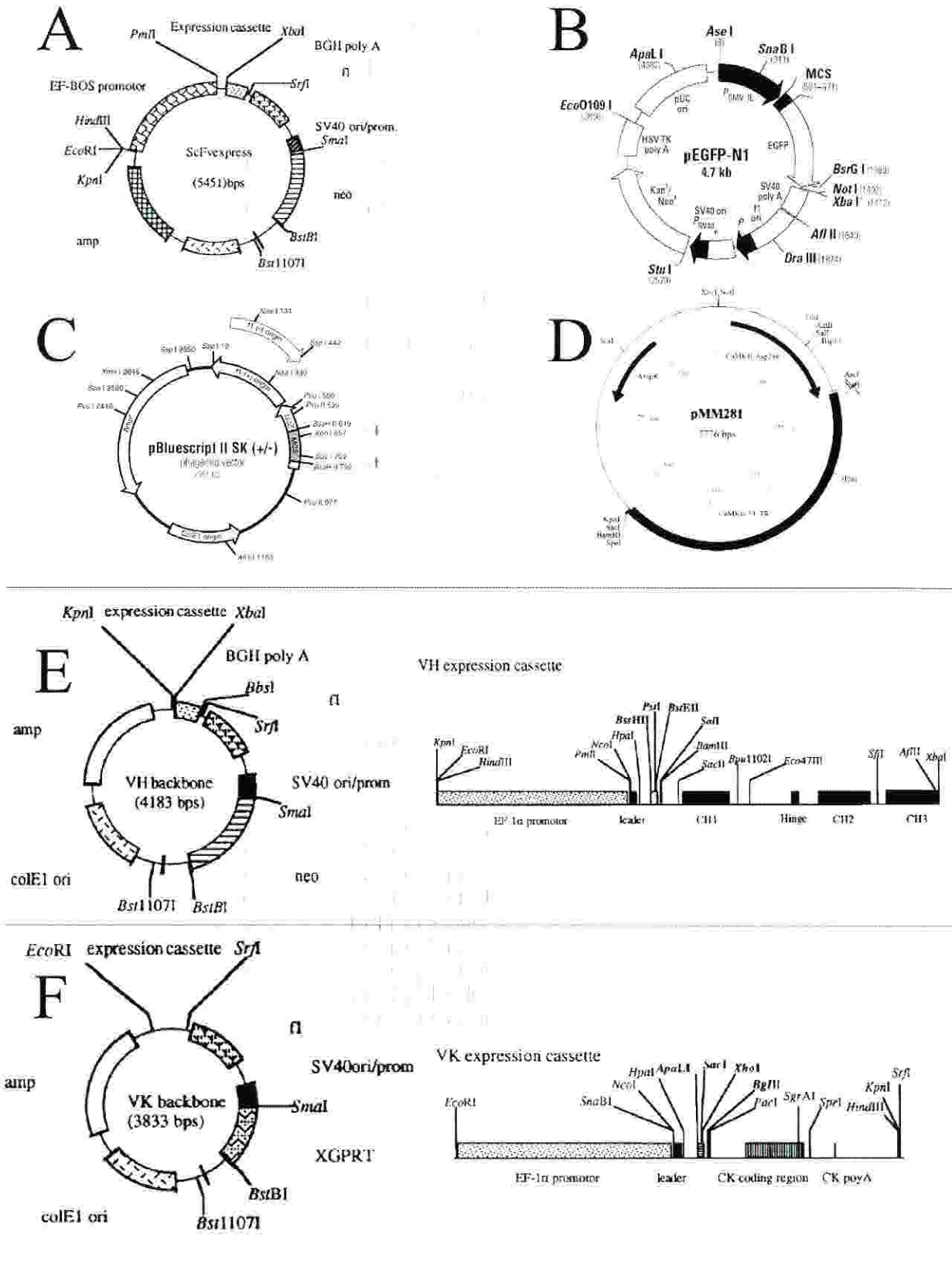

Fig 2.1 Plasmid maps

2.1.6 List of primers

Below you can find the complete list of oligonucleotides used for subcloning GFP, VL and VH chains of antibodies, and to construct plasmids for RNA targeting.

The restriction sites are in bold while the annealing sequences are underlined.

MVL for

BssHII
CTA AAT T **GC GCG C** AC GGA TCC GAT ATT GTT CTC TCC

MVH back

NotI
GAA ATT A **GC GGC CGC** TGA GGA GAC GGT GAC TG

GFP for

NotI
GAT CCA CCG **GCG GCC GC** C ATG GTG AGC

GFP back

NotI
AGA GTC GCG G **GC GGC CGC** CTT GTA CAG C

GXB for

GTA CAA GTA ATC TAG AGG CGC GCC GGT AAC CAC TAG TA
Containing the restriction sites **XbaI, BssHII, AscI, BsrFI, BstEII** and **SpeI**

GXB back

CTA GTA CTA GTG GTT ACC GGC GCG CCT CTA GAT TAC TT

A2RE for

*AscI SpeI°
CGC GCC GCC AAG GAG CCA GAG AGC ATG **A**

A2RE back

°SpeI AscI*
CTA GT C ATG CTC TCT GGC TCC TTG GC **G G**

Ab MVH for

BssHII
CTA AAT T **GC GCG C** TC GAG GTG AAG CTG GTG G

Ab MVH back

BstEII
GAA ATT **GGT GAC C** CC TGA GGA GAC GGT GAC TG

Ab MVL for

SacI
CAT TTA **GAG CTC** GGA TCC GAT ATT GTT CTC TCC

Ab MVL back

BglII

CAT TTA **AGA TCT** ATT CTA CTC ACG ACG TTT GAT TTC CAG CTT GG

NPVH for

BssHII

C ACA G **GC GCG C** AG GTC CAA CTG CAG CAG CC

Ab NPVH back

BstEII

GGA CAG **GGT GAC C** CC TGA GGA GAC TGT GAG AGT GG

Ab NPVL for

ApaLI

C GAT ATC **GTG CAC** CAG GCT GTT GTG ACT CAG G

Ab NPVL back

XhoI

TTT GAT **CTC GAG** GCC TAG GAC AGT CAG TTT GG

NPVH pDAN back

SalI

GA ACC GCT G **GT CGA C** CC TGA GGA GAC TGT GAG AGT GG

NPVL pDAN for

XhoI

C GAT AT **C TCG AG** C CAG GCT GTT GTG ACT CAG G

NPVL pDAN back

NheI

TTT GAT **GCT AGC** GCC TAG GAC AGT CAG TTT GG

NPVL back

NotI

AAC AAG A **GC GGC CGC** GCC TAG GAC AGT CAG TTT GG

2.2 Cell cultures

COS [Gluzman Y, 1981], NIH 3T3 [Jainchill JL et al., 1969] and C6 [Benda P et al., 1968] cells were grown in DMEM + 10% Fetal Calf Serum (FCS).

PC12 cells [Greene LA and Tischler AS, 1976] were cultured in RPMI 1640 medium, with 5% FCS, and 10% heat-inactivated horse serum. Priming of PC12 cells was performed for 1 week in the same medium containing 50 ng/ml NGF (from mouse, Alomone Labs) on collagen-coated Petri dishes. Cells were then washed with serum-free medium and re-plated in RPMI medium with 1% horse serum. NGF-induced neurite outgrowth was scored after 48 hours.

Primary hippocampal neurons were kindly prepared by Dr. Massimo Righi according to the method of Malgaroli A and Tsien RW [1992], with slight modifications [Righi M et al., 2000]. Cells were cultured for 1 week before transfections, in Minimum Essential Medium (MEM) with Earle's salts and Glutamax I with 5% dialyzed FCS, 7 mg/ml D-glucose, 3.6 mg/ml HEPES, 0.1 μg/ml biotin, 1.5 μg/ml vitamin B12, 30 μg/ml insulin, and 100 μg/ml bovine transferrin. Proliferation of non-neural cells was prevented by adding 2.5-5 μM cytosine β-D-arabinofuranoside on the second day in culture.

All the media, the sera, and the additives described are from GIBCO-BRL (Invitrogen).

2.3 Transfections

Primary hippocampal neurons in culture (1 week), 3T3, and C6 cells were transfected with scFvs DNA by the Calcium/Phosphate (Ca/PO_4) protocol. COS cells were transfected with the DEAE protocol. PC12 cells were transfected with the Biolistic System (BIORAD).

For the stabilized C6 clones, a "killing" titration assay was performed on untransfected cells with Geneticin antibiotic (G418; SIGMA). The minimum concentration able to kill all the cells in a 35mm dish was found to be 1 μg/ml. The day after transfection C6 cells were incubated with G418 1 μg/ml. The medium was changed every three days until resistant clones were visible. Clones were isolated by picking and transferring them into new dishes with a sterilized cotton swab. The antibiotic selective pressure on clones was maintained for all the time necessary for the subsequent analysis.

2.3.1 Ca/PO_4 transfections

One day before transfection 10^5 cells per 35mm Petri dish were plated in 2ml culture medium. After one wash with serum-free medium, cells were incubated at least 1 hour in 1.5 ml serum-free medium (saving the conditioned medium). At least 2 μg DNA were dissolved in TE (Tris HCl 10mM, EDTA 0.1 mM, pH 7.6) or H_2O, in a final volume of 42 μl. Eighteen μl 1 M $CaCl_2$ were added to DNA solution. The DNA/$CaCl_2$ solution was mixed with 60 μl HeBS 2X (250 mM NaCl, 0.5 mM Na_2HPO_4, 1 mM NaH_2PO_4, 50 mM Hepes, pH 7.07) slowly while agitating, and left for 45 min. at least, at RT. The precipitates were gently dropped into the dishes. After 2 hours of incubation at 37°C, cells were washed three times with serum-free culture medium. Cells were fed with 1:1 conditioned medium: fresh medium.

2.3.2 COS cells transfection with DEAE

The day before transfection 10^5cells were plated into 35mm Petri dishes and fed with 2 ml DMEM + 10% FCS. Five μg DNA were added to 0.45 ml DMEM. Then 50 μl DEAE-dextran (5 mg/ml; Pharmacia) were added and mixed by vortex. DMEM/DNA/DEAE solution (0.5 ml) was added to the empty culture dishes after one wash with DMEM. Cells were incubated 30 min at 37°C and the medium mixed every 5-10 min. Two point five ml of 100 mM chloroquine in DMEM + 10% FCS were added, and cells were incubated at 37°C for 2 hours and 30 min. After three washes with DMEM, cells were fed with DMEM + 10% FCS.

2.3.3 PC12 transfection with the Biolistic System

PC12 cells, primed for 1 week with NGF, were used for the transfections. 30 μl of 60 mg/ml gold particles (diameter: 1μm) were coated with 3-5μg plasmids as described by manufacturers instructions. Petri dishes with cells were placed at a distance of 3 cm from the source of particles inside the chamber. After transfection, medium was replaced and cells were cultured again. After 24 hours, cells were re-plated in the presence of NGF.

2.4 Immunofluorescence

This method was used to detect expression of scFvs within cells (when not tagged with GFP), to visualize the presence of c-fos, egr1/zif and activated MAPK proteins, and to determine the expression of MAP2 protein in mature neurons.

Cells, plated on poly lysine-coated glass coverslips, were transfected according to the previous protocol. Cells were washed once with PBS and then fixed with 4 % paraformaldehyde in PBS for 15-20 min at RT. After three washes with PBS, cells were permeabilized with 0.1% Igepal CA 630 (SIGMA) in PBS for 5 min at RT, and then incubated with 10% FCS in PBS (blocking solution) for at least 20 min. Coverslips were incubated with the primary antibody diluted in blocking solution, at RT, for at least 40 min. After three washes with the blocking solution, cells were incubated with the secondary antibody diluted in blocking solution, at RT, for at least 30 min. After three washes with PBS, the coverslips were mounted with glycerol: PBS (pH 8.6) 9:1, containing 2.5% (w/v) 1,4-Diazabicyclo[2,2,2]octane (DABCO) (SIGMA) to prevent fluorescence fading. Coverslips were sealed with nail polish.

Primary antibodies and dilutions used for immunofluorescent assays:

- Mouse anti-MAP2 (Chemicon) 1:1000
- Rabbit anti-c-fos (Calbiochem) 1:1000
- Rabbit anti-egr1/zif (Santa Cruz Biotechnology) 1:1000
- Mouse monoclonal anti- activated MAPKinase (clone MAPK-YT, Sigma) 1:1000
- Mouse monoclonal anti-Myc Tag 9E10 supernatant [Chan S et al. 1987] 1:1

Secondary antibodies and dilutions used for immunofluorescent assays:

- Anti-mouse Alexa fluor 594 (Molecular Probes) 1:500
- Anti-rabbit biotinylated (Vector) 1:200
- Anti-mouse biotinylated (Vector) 1:200
- ExtrAvidin-TRITC (SIGMA) 1:500 in 10 mM HEPES, 0.15 M NaCl, pH 8.2
- Anti-mouse FITC (Molecular Probes) 1:500

2.5 Protein targeting detection

For confocal microscopy observation, cells were placed in GPS buffer* and observed at RT with LUMPlan/FI/IR 60x/0.90 water immersion objective and Olympus BX51WI confocal microscope with argon (488 nm) and helium neon (543 nm) lasers (Meles Griot).

Wide-field observations were performed with Zeiss Axioskop 2 PLUS microscope. Photos were acquired with a CCD camera (Magnafire, Optronics).

PC12 cells activation and differentiation, and neurons activation were analyzed with a Zeiss Axioplan microscope. Images were acquired with a Nikon Coolpix 990 digital camera.

*GPS buffer pH 7.00

120 mM NaCl
5 mM KCl
1 mM CaCl2
1 mM MgCl2
25 mM Glucose
20 mM PIPES

2.5.1 Fluorescent probes

All the fluorescent probes used for revealing subcellular compartment are from Molecular Probes. Mito-Tracker red CMXRos and Fluorescent Nissl stain (Neuro Trace 530/615 red fluorescent Nissl stain) were detected by confocal microscopy, while ER-Tracker Blue-White DPX was detected by wide-field microscopy.

Mito-Tracker

Cells plated on coverslips were incubated 30 min at 37°C with 167 nM (final concentration; dilution 1:6000) of Mito-Tracker directly into the cell medium. Cells were washed three times with MEM and incubated in MEM + 10% FCS (GIBCO, Life Technology) 30 min at 37°C. Analysis by confocal microscopy was performed on living cells maintained in MEM.

ER-Tracker

Cells plated on coverslips were incubated 30 min at 37°C with 50 nM (final concentration; dilution 1:20.000) of ER-Tracker directly into cell medium. Then

cells were washed three times with MEM and coverslips mounted with 90% glycerol/DABCO (SIGMA). Microscope detection was done immediately after the slides preparation because, in any case, the ER-Tracker fluorescence signal would not last more than 7-10 hours.

Fluorescent Nissl staining

Cells were fixed with 4% paraformaldehyde in PBS and permeabilized as described below:

- 15 min of incubation at -20°C in 100% Ethanol
- 5 min of incubation at RT in 50% Ethanol
- 5 min of incubation at RT in 30% Ethanol

Cells were washed three times with PBS and incubated at RT with 2.5 nM (final concentration; dilution 1:2000) of Fluorescent Nissl staining in PBS.

After three washes with PBS cells were rocked 10 min in PBST (0.1% Tween20, PBS).

After other three washes in PBS cells were left over night at 4°C in PBS and then were mounted with 90% glycerol/DABCO (SIGMA).

2.5.2 Block of secretory pathway and microtubules movement

Block of secretory pathway from *trans*-Golgi to the plasma membrane was obtained in living transfected cells by 1 hour of incubation at 18°C into a water-bath. One day after transfection cells on coverslips were placed into a 6-well plate with culture medium. Plate floated on water-bath. Immediately after incubation cells were fixed with 4% paraformaldehyde in PBS and mounted with 90% glycerol/DABCO (SIGMA).

In order to block ER intracellular typical movement, Nocodazol, known to perturb microtubules depolymerization and so intracellular movements along microtubules, was used. One day after transfection cell were incubated for not more than 1 hour at 37°C with a final concentration of 10 μg/ml of Nocodazol (SIGMA) directly into cell medium. Cells were maintained in medium with

Nocodazol during the analysis by confocal microscopy. After the recording cells were placed in fresh medium and the day after it was verified if cells were still alive.

2.6 *In situ* hybridization

In order to detect GFP and R4 GFP mRNAs transport on transfected hippocampal neurons, *in situ* hybridization was performed 24 hours after transfection according to Tongiorgi E et al., [1998]. Neurons were fixed 15 min at RT with 4% paraformaldehyde in PBS, washed with PBS and permeabilized with 100% ethanol for 15 min at -20°C. After rehydratation with decreasing ethanol concentrations in PBS at RT, cells were prehybridized by incubation in moist chamber at 55°C for 1 hour with the hybridization solution (20 mM Tris/HCl pH 7.5, 1 mM EDTA, 1x Denhardt's solution*, 300 mM NaCl, 100 mM DTT, 0.5 mg/ml Salmon sperm DNA (SIGMA), 0.5 mg/ml polyadenylic acid (SIGMA) and 50% formamide). *In situ* hybridization was carried overnight in moist chamber at 55°C with the hybridization solution after the addition of 10% dextran sulphate (SIGMA) and 100 ng/ml (final concentration) of digoxigenin labeled riboprobe (see chapter 2.6.1).

After hybridization cells were washed twice in 2x Saline Sodium Citrate solution, 0,1% Tween20 (SSCT) with 50% of deionized 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.

*Denhardt's solution

For 500 ml of solution 50x:
5 g Ficoll (SIGMA)
5 g Polyvinylpyrrolidone (SIGMA)
5 g BSA (SIGMA)
Fill to 500 ml with deionized water and filter.

2.6.1 Riboprobe

The DNA encoding for GFP was previously subcloned from pEGFP-N1 (Clontech) into pBluescript II SK (Strategen) by Dott. Francesca Paoletti.

About 30 µg of Bluescript-EGFP plasmid were linearized with BamHI, then purified with phenol/ chloroform precipitation and dissolved in 30 µl of diethylpirocarbonate-treated (DEPC) H_2O. 20 µg of purified DNA was used for *in vitro* transcription for the synthesis of the digoxigenin labeled riboprobe with T7 polymerase and the DIG-RNA labeling kit from Boehringer. After digestion with

DNase RNase free, the riboprobe was purified with RNeasy mini Kit (QIAGEN), resuspended in 40 µl of H_2O DEPC, aliquoted, and stored at -80°C.

2.6.2 Immunofluorescent detection

Cells hybridized with digoxigenin labeled riboprobe were incubated 20 min with a suitable volume of blocking solution (10% FCS in PBS). Then cells were incubated at least two hours at RT with a monoclonal anti-DIG (mouse ascite fluid, SIGMA) diluted 1:1000 in blocking solution (10% FCS in PBS). After three washes with blocking solution, cell were incubated at least 45 min with a goat anti-mouse antibody (DAKO) diluted 1:200 in blocking solution. After other three washes cells were incubated with an anti-goat antibody Cy3 conjugated (SIGMA) diluted 1:150. Coverslips were then washed in PBS and mounted with 90% glycerol/DABCO (SIGMA).

mRNA granules detection

Granules of mRNA were detected in cultured hippocampal neurons with the nucleic acids staining SYTO 61 red fluorescent dye (Molecular Probes).

Living cells were incubated with a final concentration of 100 nM of SYTO 61 (dilution: 1:50.000) in GPS* buffer with 5% of dialyzed FCS at least for 15 min. Cells were maintained in GPS buffer for subsequent analysis by confocal microscopy.

2.6.2 Enzymatic detection

Cells hybridized with digoxigenin labeled riboprobe were incubated 20 min with a suitable volume of blocking solution (10% FCS in PBS). Then cells were incubated at least two hours at RT with anti-DIG $F(ab)_2$ fragments alkaline phosphatase conjugated (Roche), diluted 1:500 in blocking solution. After four washes in PBST for 10 min, cells were incubated in developing buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, 1 mM Levamisol) for 5 min. Finally enzymatic detection was performed by incubation in a cromogenic solution composed by the developing buffer with 4-nitro blue-tetrazolium (NBT, SIGMA),

and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, SIGMA). The reaction was carried out overnight at 4°C and was stopped by rinsing cells with PBS. Coverslips were then washed twice with PBS and mounted in 90% glycerol/PBS. When the detection for the presence of MAP2 protein was carried out together with riboprobe immunodetection, 1 hour of incubation with mouse anti-MAP2 antibody (Chemicon) diluted 1:200 in blocking solution followed by 45 min of incubation with anti-mouse HRP conjugated (DAKO; 1:1000 in blocking solution), were performed just after anti-DIG antibody incubation. Detection of HRP antibody was done with diaminobenzidine (DAB) staining. Cells were first incubated with 0.4 mg/ml of DAB solution (in PBS containing 0.5 μg/ml of glucose oxidase) for 5 min, washed with PBS and then developed with AP cromogenic substrate.

2.6.3 Statistical analysis

Statistics on PC12 neurite outgrowth was performed as described.

Fluorescence of transfected cells was analyzed with a Zeiss Axioplan microscope and images were acquired with Nikon Coolpix 990 digital camera. Only transfected cells with neurites length of one cell body diameter were considered as positive cells for neurite outgrowth. Data were collected from at least three independent experiments. Data were indicated as percentage of transfected cells that showed neurite outgrowth, and were statistically analyzed two by two with the t-Student's test (assuming unequal variance). The two samples were considered different when the likelihood probability resulting from the test was below 0,05.

Staining for activated neurons and PC12 cells was analyzed with a Zeiss Axioplan microscope and images were acquired with Nikon Coolpix 990 digital camera. Data were collected from at least three independent experiments, were indicated as percentage of transfected cells that showed immunofluorescence activation, and were statistically analyzed two by two with the t-Student's test

(assuming unequal variance). Again, the two samples were considered different when the likelihood probability was below 0,05.

Statistics on RNA transport was performed as described.

Non-radioactive *in situ* hybridization was analyzed by viewing stained transfected neurons under bright field illumination with a Zeiss Axioskop 2 PLUS microscope. Stained neurons were acquired with a CCD camera (Magnafire, Optronics) and digitized with the image analysis program Image Pro Express 4.0. The "trace" tool function was used to measure, starting from the center of the cell, the maximal distance of dendritic labeling. Dendrites were traced up to the point at which the *in situ* labeling was clearly distinguishable from the background. The analysis of measurements was done by considering data from at least three independent experiments. The average measurement of each experiment was used to calculate the final averages and standard deviations. The final averages of different experiments were statistically analyzed two by two with the t-Student's test (assuming unequal variance). The two samples were considered different when the likelihood probability was below 0,05.

For a detailed description of RNA transport distributions analysis and for the definition of RANGE, see chapter 3.2.3.

All the described measurements were analyzed in a blind manner.

2.7 ScFvs expression and purification in *E. Coli*

Bacterial clones carrying plasmids for α p75 and α TrkA were first incubated shaking over night at 30°C in 2xTY Amp with 5% glucose and then, once diluted 1:100 in 2xTY Amp with 0.5% glucose, grown at 37°C to reach an OD_{600nm} of 0.7. To induce expression of each scFv, Isopropyl-β-D-thiogalactopyranoside (IPTG) 0.5 mM was added and left at 30°C for 5 hours. Then the bacteria were pelleted by centrifugation at 3500 rpm for 15 min 4°C.

For a periplasmic extraction, pellets were resuspended in 1/40 of total growth volume of PPB buffer (200 mg/ml sucrose, 1 mM EDTA, 30 mM Tris-HCl pH 8) and incubated in ice for 20 min. After centrifugation at 5000 rpm for 15 min at 4°C to separate the supernatant (osmotic shock preparation) the pellets were resuspended in the same volume of Mg_2SO_4 5 mM and incubated in ice for 20 min. Then both the osmotic shock preparation and periplasmic preparation were spun for 10 min at 13000 rpm at 4°C, pooled together and dialyzed over night at 4°C against PBS using Spectra-Por Membrane with 12/14K of molecular cut off (Spectrum).

2.7.1 ScFvs purification by metal ion affinity chromatography

To purified scFvs from bacterial periplasm was performed a metal ion affinity chromatography exploiting the polyhistidine purification tag fused at the C terminus of each scFv.

To the dialyzed bacterial periplasmic preparation NaCl was added to a final concentration of 300 mM.

Periplasmic extract was then mixed with 1ml of Ni NTA sepharose (QIAGEN) and washed twice by PBS.

After batch incubation at 4°C for 2 hours rotating, the resin was spun for 10 min at 800 rpm and washed in batch with 50 ml of WB1 buffer (50 mM Na Phosphate pH 8, 250 mM NaCl).

Resin was then packed in a chromatography column and extensively washed with WB1 and, in second instance, with WB2 buffer (50 mM Na Phosphate pH 8, 500 mM NaCl).

To remove non-specific binders the column was successively washed with low concentration of imidazole contained in EL1 buffer (WB2 buffer + 15 mM imidazole).

Finally elution took place by competition with high concentration of imidazole using EL2 buffer (WB2 buffer + 100 mM imidazole).

All the collected fractions were pooled, dialyzed over night at 4°C across a Spectra-Por Membrane with 12/14K of molecular cut off (Spectrum) against PBS.

The relative quantities of purified scFvs were estimated using Coomassie Blue staining of discontinuous 12% SDS PAGE.

2.8 Cell extracts

In order to obtain the pool of cellular protein for western blot or ELISA assays, cultured cells were washed two times with PBS and incubated 20 min with 200 µl (for a 35mm dish) of lyses buffer [50 mM Tris-HCl pH 7.4; 2 mM $CaCl_2$; 1% Igepal CA 630 (SIGMA) and Complete, EDTA-free (Roche) cocktail of protease inhibitors].

Cells were then scraped and centrifuged (2 min, 13.000 RPM).

Pellet was discarded and the supernatant was stored at -20°C

2.9 ELISA assays

ELISA detection assays was used to verify scFvs expression in transformed bacteria and transfected cells, and to determine scFvs binding to the plated antigen in cell extracts and supernatants of transfected cells.

96 well plates were coated with 100 µl per well of the antigen diluted in coating buffer (50 mM Na carbonate pH 9.6) at a final concentration of ~7 mg/ml and left over night at 4°C in a moist chamber.

As antigen TrkA and p75 immunoadhesins (TrkA and $p75^{NTR}$ extracellular domain with constant chains of camel IgG expressed by Baculovirus system), kindly purified by Dr. Sonia Covaceuszach, and NP31-BSA (Biosearch Technologies) were used.

After 3 washes with PBST (PBS Tween20 0.1%) and 3 washes with PBS, plates were blocked by 200 µl of PBS / 4% non fat dry milk (MPBS 4%) for 1 hour at RT.

After 3 washes with PBST and 3 washes with PBS 50 µl of MPBS 8%, 50 µl of each bacterial extract, cell lysate or supernatant were added per well and incubated 2 hours at RT.

Then plates were washed 3 times with PBST and 3 times with PBS and incubated with the primary antibody diluted in MPBS 4%, for 1 hour at RT.

After the same washing procedure of the previous step the secondary antibody, horseradish peroxidase (HRP) conjugated, diluted in MPBS 3% was added and incubated for 1 hour at RT.

Finally, after 3 washes with PBST and 3 washes with PBS, 70 µl of substrate solution (diluted TMB; TECNA) were added.

After few minutes at RT in the dark the reaction was blocked adding an equal volume of 0.1 M H_2SO_4 and the intensity of each colorimetric signal was measured at 450 nm by an ELISA Reader (Spectra).

Primary antibodies and dilutions used for ELISA assays:

- Mouse monoclonal anti-Myc Tag 9E10 supernatant [Chan S et al. 1987] 1:20
- Mouse monoclonal anti-TrkA MNAC13 [Cattaneo A et al., 1999] (purified by Dr. Sonia Covaceuszach) 1:1000
- Mouse sera, kindly provide by Dr. Simona Capsoni, from immunized mice with NP-BSA (Biosearch Technologies) 1:10
- Rabbit anti-human κ chain (Pierce) 1:1000
- Goat anti-human γ chain (Pierce) 1:1000

Secondary antibodies and dilutions used for ELISA assays:

- Anti-mouse HRP (DAKO) 1:1000
- Anti-rabbit HRP (DAKO) 1:1000
- Anti-goat HRP (DAKO) 1:1000

2.10 Western blot analysis

In order to evaluate the presence of specific proteins (scFvs, $p75^{NTR}$, and tubulin) in cell extract or supernatant of transfected cell, western blot analyses were performed. About 15 µl of cell extracts or supernatants were loaded on discontinuous 12%SDS PAGE, separated by electrophoresis and transferred onto nitrocellulose filters (HybondECL, Amersham) by electroblotting (1 hour, 100 mA per gel).

Blotted membranes were blocked by MPBS (PBS, 4% non fat dry milk) for 1 hour at RT and then incubated with the primary antibody over night at 4°C.

After 3 washes by PBST (PBS, Tween20 0.1%) and three washes with PBS the membranes were incubated for 1 hour at RT with the secondary antibody.

Finally after extensive washings by PBST and by PBS, electrochemiluminescence (ECL) system (Amersham) was employed to detect the presence of the proteins of interest.

Primary antibodies and dilutions used for western blots:

- Mouse monoclonal anti-Myc Tag 9E10 supernatant [Chan S et al. 1987] 1:1
- Mouse monoclonal anti-$p75^{NTR}$ MC192 [Chandler CE et al., 1984] ascite fluid 1:250
- Rat monoclonal anti-tubulin YL 1/2 supernatant [Kilmartin JV et al., 1982] 1:200

Secondary antibodies and dilutions used for western blots:

- Anti-mouse HRP (DAKO) 1:1000
- Anti-rat HRP (DAKO) 1:1000

2.11 Flow cytometry analysis

To prepare a cell suspension for sorting in a Fluorescence-Activated Cell Sorter (FACS, Becton Dickinson), cells (about 1 million for each analysis) were detached from dishes by incubation with EDTA. They were then centrifuged (5 min 1000 RPM), resuspended in 1 ml of blocking solution (PBS + 3% BSA) and incubated for 10 min. Membrane proteins immunofluorescent detection was performed by 1 hour of incubation with primary antibodies (diluted in blocking solution), 45 min of incubation with secondary biotinylated antibodies (diluted in blocking solution), and 15 min of incubation with Avidine FITC (diluted in 10 mM HEPES, 0.15 M NaCl, pH8.2).

Washes between each step of incubation were performed by two centrifugations of cells (5 min, 2000 RPM) through 1 ml of undiluted FCS that was placed below the cells by gently injecting it on the bottom of the tube. Immunolabeled cells were then resuspended in 1 ml of PBS for the analysis.

Primary antibodies and dilutions used for FACS analysis:

- Mouse monoclonal anti-$p75^{NTR}$ MC192 [Chandler CE et al., 1984] ascite fluid 1:250
- Rat anti-NGF α D11 [Cattaneo A et al., 1988] (purified by Dr. Sonia Covaceuszach) 1:500

Secondary antibodies and dilutions used for FACS analysis:

- Anti-mouse biotinylated (DAKO) 1:1000
- Anti-rat biotinylated (DAKO) 1:1000
- Streptavidin-FITC (Pierce) 1:500

2.12 Hippocampal neurons and PC12 cells bioassays

After primed PC12 transfection, complete fresh medium was added and cells were cultured for 24 hours at 37°C. Cells were then re-plated on polylisine-coated glass coverslips and incubated for additional 48 hours in the presence of NGF (20 ng/ml). Cells were then fixed in 4% paraformaldehyde in PBS and mounted in 90% glycerol in PBS for neurite outgrowth assay.

For activated MAPK detection, unprimed cells were transfected and, after 48hours, incubated with NGF (50 ng/ml) for 10 min. Then cells were fixed and, after immunostaining, mounted in 90% glycerol in PBS.

For c-fos and egr1/zif detection, 48 hours after transfection, unprimed cells were incubated with NGF (50 ng/ml) for 3 hours, fixed and, after immunostaining, mounted in 90% glycerol in PBS.

Hippocampal neuron activation was started 48 after neuron transfection. Cells were incubated with 50 ng/ml of BDNF for 10 minor 3 hours, fixed and analyzed by immunostaining, for the presence of MAPK activated, c-fos and egr1/zif.

Chapter 3

Results

3.1 Targeting scFvs to specific intracellular compartments

In this work the possibility to use intracellular antibodies to selectively interfere with the functions of neurotrophin receptor activity was tested. Therefore, tools and techniques have been developed to express functional scFvs in cells and to target them into specific organelles. In order to visualize the ability of the chosen targeting signals to correctly direct proteins to intracellular destinations in neuronal cells, the fluorescence of GFP fusion proteins was exploited. In this way, GFP-scFvs were followed to their final destination, determined by different targeting signals. Plasmids were prepared, as described in chapter 2.1, containing the DNA encoding for an anti β-Galactosidase scFv (R4), which has no corresponding intracellular antigen in eukaryotic cells, in fusion with GFP. R4 scFv was previously selected from the naive Vaughan combinatorial human antibody scFv library [Vaughan TJ et al., 1996] for its ability to correctly fold into the cytoplasm [Martineau P et al., 1998]. R4 GFP was cloned into ScFvExpress plasmids [Persic L et al., 1997a] carrying the protein targeting signals for the cytoplasm (cyto) the secretory pathway (sec), the endoplasmic reticulum (KDEL), and the nucleus (NLS). Primary cultured hippocampal neurons were transfected with each of the plasmids and, after one day of expression, the intracellular distribution of GFP scFvs was analyzed by confocal microscopy. In order to determine if the transfected cells were indeed neurons, they were immunolabeled for the Microtubule Associated Protein (MAP2), a marker for mature neurons, as shown in figure 3.1 (in red). Figures 3.1 A and D show the results for R4 containing the cyto and NLS signals respectively. R4 GFP cyto transfected cells show an extensive and uniform distribution of GFP (figure 3.1A).

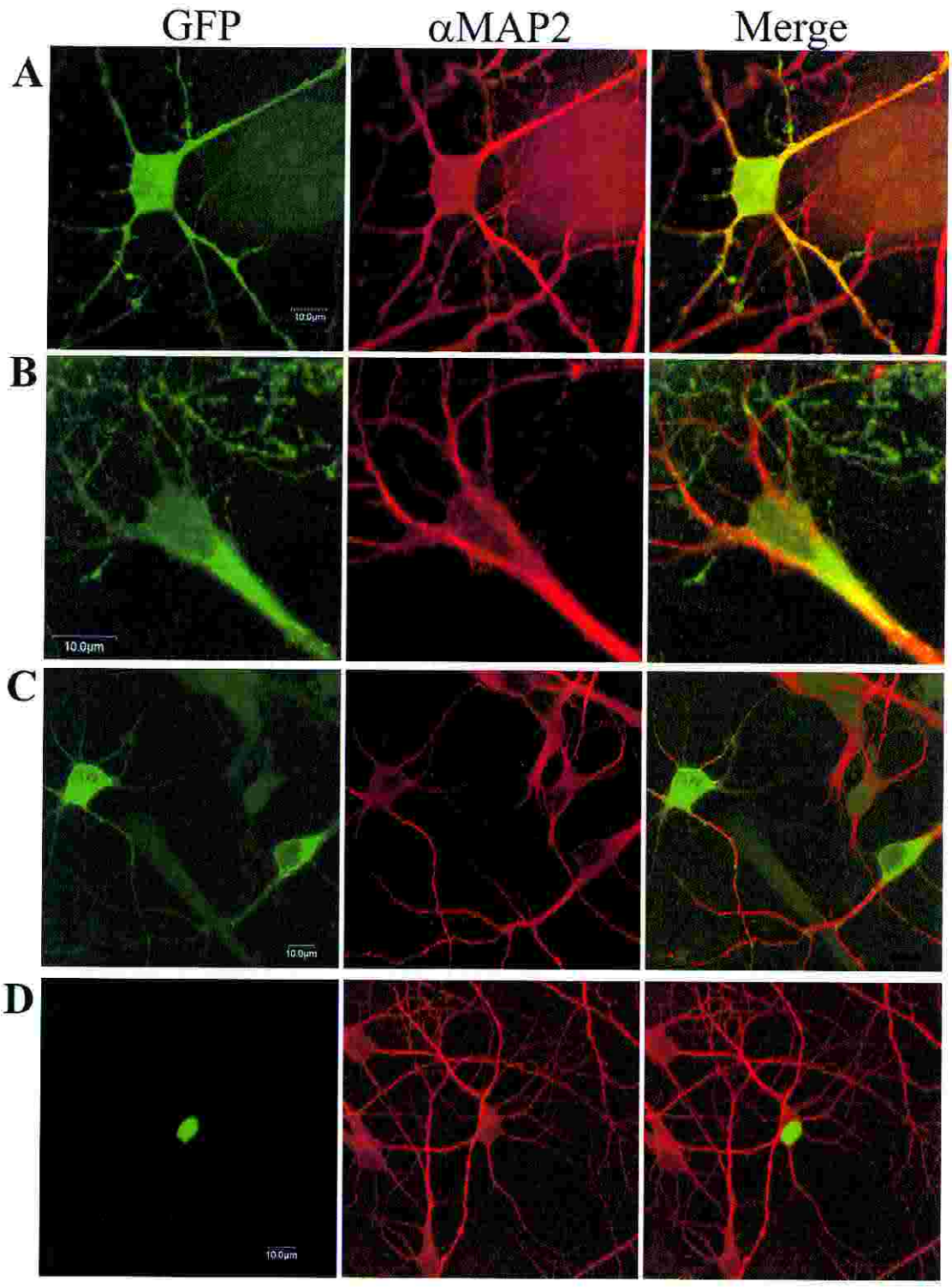

Fig. 3.1 ScFvs targeting in neurons

Transfected hippocampal neurons with anti β–Gal (R4) scFv express plasmids and different targeting signals fused with EGFP. Neurons were stained in immunofluorescence with anti MAP2 antibody (secondary antibody Alexa 594 anti mouse). GFP and MAP2 were detected by confocal microscopy. All the pictures are projections of Z-series. (**A**) cytoplasmic signal (Cyto); (**B**) secretory signal (Sec); (**C**) endoplasmic reticulum signal (KDEL); (**D**) nuclear signal (NLS). Scale bars: 10μm.

The protein diffuses into processes and can also enter the nucleus. Figure 3.1 D clearly shows that the presence of NLS targets R4 GFP NLS to the nucleus. This is even better appreciated when the transfected cell is counterstained for MAP2. No other fluorescence in the whole cell is detectable. Concerning the sec (B) and KDEL (C) signals, it can be seen no fluorescence in the nucleus and, for R4 GFP sec scFv, also no fluorescence in the processes. Cells transfected with R4 GFP KDEL scFv show dendritic fluorescence also in distal dendrites with a characteristic discontinuity and granularity in the same way as in the cell body. The same discontinuous pattern of distribution is found in the cell body of R4 GFP sec transfected cell. Even if this fluorescence pattern seems compatible with the secretory pathway and ER localization, it seemed necessary to confirm these results with more specific assays.

3.1.1 Sec and KDEL protein localization signals

It is known that cells incubation at different temperatures specifically blocks some steps of the secretory pathway. [Kuismanen E and Saraste J 1989]. In fact when cells are incubated for a suitable time at 15°C, the transport of proteins from ER to Golgi apparatus is blocked, while incubation at 18°-20°C causes the block of protein transport only from the distal part of Golgi to the plasma membrane, resulting in an expansion of the *trans*-Golgi due to the accumulation of proteins within the cisternae. Therefore a cell expressing GFP inside the secretory pathway and incubated at 18°C should show a redistribution of fluorescence from the secretory pathway to the *trans*-Golgi. This kind of redistribution must not occur in cells expressing GFP in the cytoplasm or inside the ER, thus this kind of cells provide a good negative control for the experiment. This approach has been chosen to verify the presence of R4 GFP sec protein in the secretory pathway. In contrast to neurons, fibroblast cells present some advantages, such as their flatness and their resistance at difference in temperature, that allow easy cellular biology investigations, leading to a high spatial resolution. For this reason NIH 3T3 murine cell line [Jainchill JL et al., 1969] was selected for this kind of experiments. 3T3 cells were transfected with R4 GFP plasmids carrying the cyto,

KDEL and sec signals. One day after transfection, cells were incubated at 18°C for 1 hour, fixed and analyzed by confocal microscopy. The results are showed in picture 3.2. Cytoplasmic GFP (3.2 A) shows a uniform distribution before and after the treatment both within the cytoplasm and in the nucleus. There is a slight increase in fluorescence in the nucleus with respect to the cytoplasm in cell incubated at low temperature. Since only the ratio of cytoplasmatic versus nuclear fluorescence can be really appreciated from picture, and since at low temperature protein synthesis is slowed down, the increase of nuclear fluorescence may be due only to a decrease of cytoplasmic fluorescence. Moreover it is to be said that not all the analyzed cells showed the described effect. Cells transfected with R4 GFP KDEL (3.2 B) show a discrete and vesicular fluorescence distribution, not present in the nucleus, in agreement with ER localization, that do not undergo any redistribution after the treatment. On the contrary, after low temperature incubation, GFP fluorescence of R4 GFP sec transfected cells redistribute into a large green structure close to the nucleus, as in picture 3.2 C, and into small vesicular elements, while cells maintained at 37°C show a vesicular fluorescence all over the cell, more concentrated just near the nucleus. The perinuclear structure is the *trans*-Golgi apparatus where GFP accumulate at low temperature while the secretory pathway is blocked. Further experiments have then demonstrated that proteins with the sec signal are successfully secreted into the cell medium (see chapter 3.3.2).

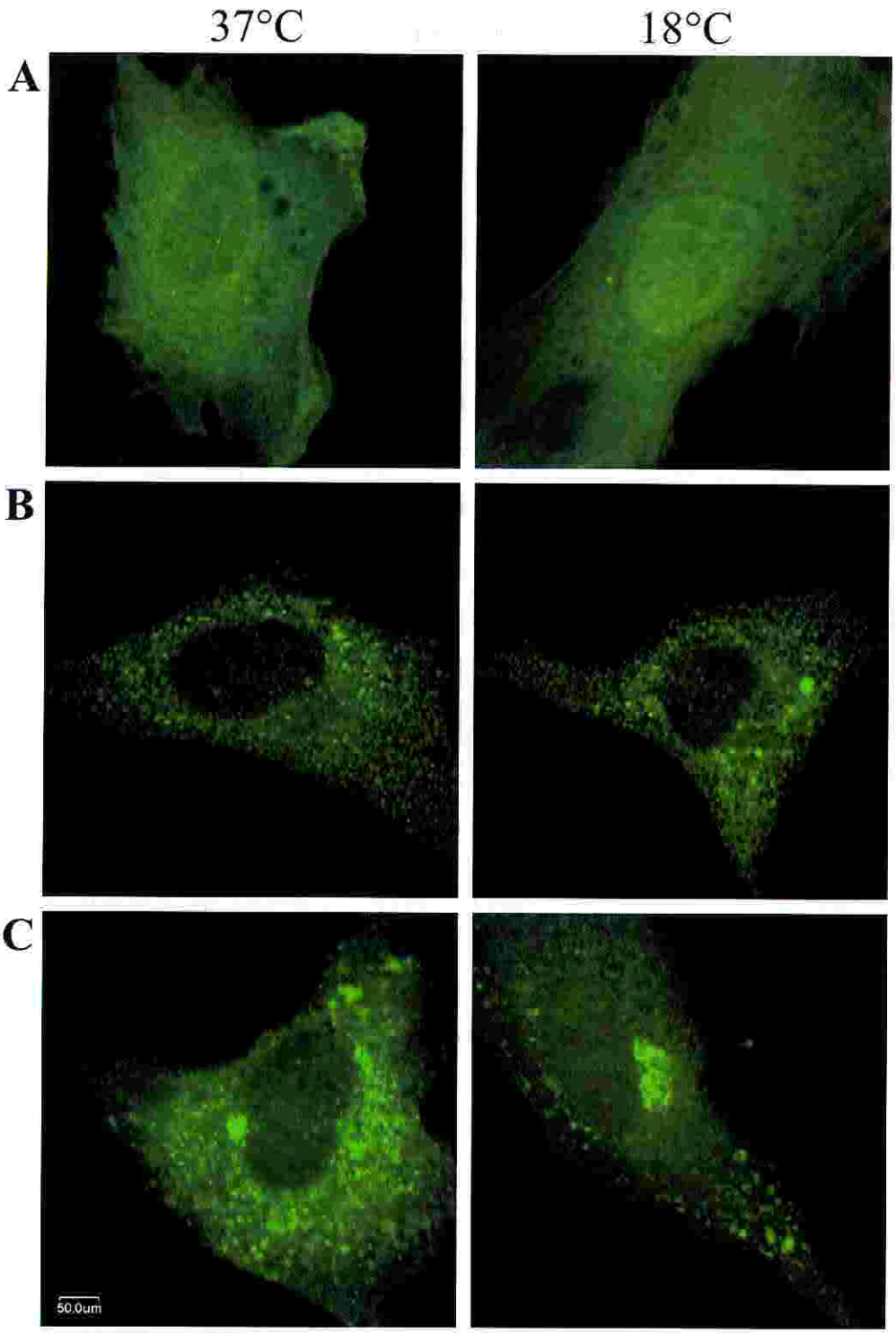

Fig. 3.2 Block of protein transport in the secretory pathway.

3T3 cells were transfected with R4 GFP carrying the cytoplasmic (**A**), KDEL (**B**) and secretory (**C**) signals. One day after transfection cells were incubated for 1h at 18°C in order to block the distal stage of protein transport, from *trans*-Golgi to plasma membrane. No difference in GFP distribution is visible in treated cells transfected with cyto and KDEL scFvs, respect to cells left at 37°C. Cells transfected with R4 GFP sec show, at 18°C, a redistribution of GFP into *trans*-Golgi cisternae and vesicular elements.

GFP fluorescence was detected with confocal microscope. All the pictures are projections of Z-series. Scale bar in panel C: 50μm.

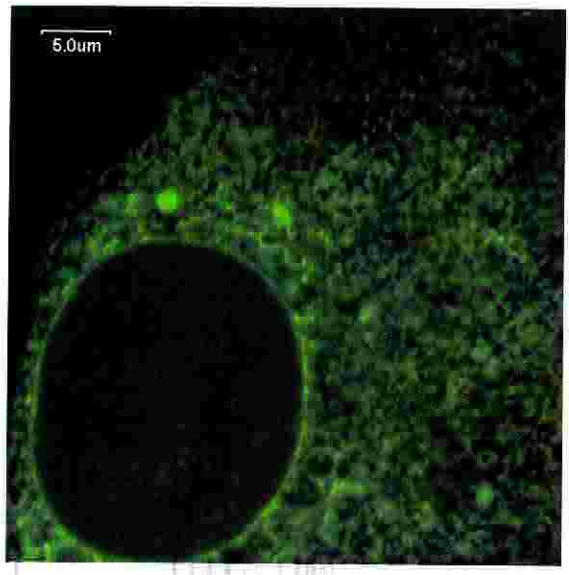

Fig. 3.3 Endoplasmic reticulum of 3T3 cells

A 3T3 cell transfected with R4 GFP carrying the KDEL retention signal. GFP fluorescence is clearly visible in tubular structures of the endoplasmic reticulum. The picture is an equatorial single-frame taken on confocal microscope. Scale bar: 5µm.

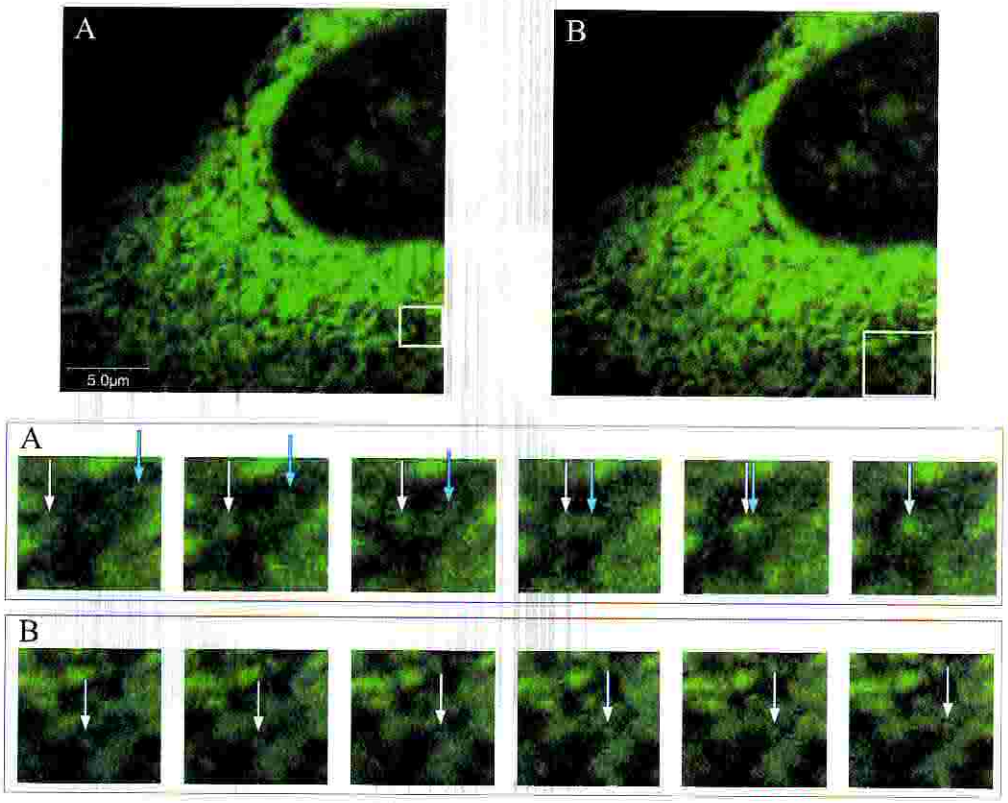

Fig. 3.B Movement of 3T3 endoplasmic reticulum

In picture **A** and **B**: a single frame of 3T3 cell transfected with R4 GFP KDEL scFv from movie B. The corresponding panels in the bottom are a time series of single frames showing the detail of the white boxes at higher magnification. Arrows indicate different structures of ER that were moving during the recording. The time lapse between each frame is about 3 sec. Recording was taken on confocal microscope. Scale bar in panel A: 5 µm

Different experiments have been developed to determine the exact ER localization of scFv GFP KDEL in transfected cells. In picture 3.3 it is shown a 3T3 cell transfected with R4 GFP KDEL plasmid, in which tubular and continuous structures, that range from the nucleus membrane towards the cellular membrane, are fluorescent for the presence of GFP. The nucleus remains completely unstained, which indicates that no freely diffusible GFP is present. The distribution of fluorescence is totally consistent with ER distribution.

Since the ER is strictly linked with microtubules and it moves following polymerization and depolymerization of microtubules [Terasaki M et al., 1986] and by the microtubules-based motors kinesin and dynein [reviewed in Cole NB and Lippincott-Schwartz J, 1995], it was decided to investigate also this typical movement of ER in living cells. **Movie B** is a time series of a single confocal scan showing a living 3T3 cell expressing R4 GFP KDEL protein; it is possible to see the movement of ER tubular structures. In picture 3.B a time series of single frames from movie B is shown: it is possible to follow three different particles of GFP marked ER membrane that were moving during 18 seconds of recording. In order to exclude the possibility that this kind of movement was due to mitochondria, that indeed have a tubular structure, transfected cells were stained with a specific red dye for mitochondria (MITO-tracker) that accumulates in mitochondria [e.g. Suzuki H et al., 2000]. In **movie C** there is the merged result of a time series taken form R4 GFP KDEL expressing cells stained with MITO-tracker, while in figure 3.C a single frame from movie C is pictured. It is easy to see that the two tubular structures have different morphology, that they occupy different spaces inside the cell and that the two fluorescence signals (red for mitochondria and green for GFP in the ER) do not colocalize.

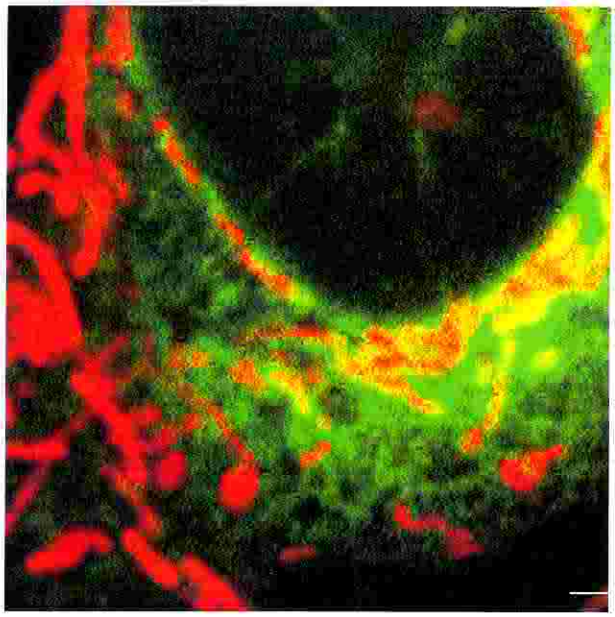

Fig. 3.C Mitochondria and endoplasmic reticulum of 3T3 cells
A single frame of a 3T3 cell transfected with R4 GFP KDEL scFv and stained in red with MITO-tracker (by Molecular Probes) from movie C. The picture is the merge result of two-channel recording on confocal microscope. Even if both ER and mitochondria show tubular structures moving inside the cell, their shape is clearly different and they do not colocalize. Scale bar: 5μm.

To demonstrate the correct localization into the ER in neurons, cells that express receptors for neurotrophins, the key point of this work, were studied. Cultured hippocampal neurons transfected with R4 GFP KDEL were fixed and stained with ER-tracker, a lipophilic probe that specifically binds ER [e.g. Grimaldi M et al., 1999]. In figure 3.4 pictures taken on wide-field microscope of the same transfected neuron are shown. In the first panel it is possible to see the green fluorescence of GFP with the same granular distribution into the cell body and along dendrites as described in picture 3.1. In the second panel is represented the blue staining of ER-tracker that show a perinuclear and granular fluorescence distribution very similar to the green signal, and present also within the dendrites and absent into the nucleus. The last panel is the merge of the two previous pictures where the colocalization pattern is clear (in cyan). The right part of the picture shows a detail of the cell at higher magnification in which at least five details of the punctuate ER structure can be easily seen that are both green and blue and result cyan in the merged panel.

The Nissl substance was described 100 years ago as a marker of neuronal cells. What it is known and widely accepted about the Nissl substance is that it is composed by a large amount of ribosomes in the ER, more precisely the rough ER. RER is identified by the presence of ribosomes that reflect the high protein synthesis level in neurons [Nievel JG and Cumings JN, 1967]. It was decided to exploit the new developed fluorescent Nissl staining in order to have information both about localization of KDEL proteins and about the nature of Nissl substance. The conventional Cresyl Violet coloration for Nissl substance was used to verify that the fluorescent dye and the traditional staining showed the same pattern of distribution. Even if the pattern seemed the same, the very low contrast of Cresyl Violet did not allow a high definition microscopy study. Neurons transfected with R4 GFP KDEL and cyto, were fixed and stained with the red fluorescent Nissl stain [Quinn B et al., 1995]. In picture 3.5 it is possible to see a granular distribution of Nissl staining of a neuronal dendrite very similar to the one of R4 GFP KDEL (3.5A), while R4 GFP cyto transfected dendrites show a more continuous and homogeneous distribution (3.5B). In the merged picture A it is shown that R4 GFP KDEL and fluorescent Nissl stain have a similar but not identical patterns; in fact not all the fluorescence appears yellow, due to the imperfect colocalization, but there are some zones exclusively green. Therefore it can be speculated that R4 GFP is retained not only in rough ER (containing ribosomes), but also in the smooth ER without ribosomes and thus not stained with the fluorescent Nissl stain.

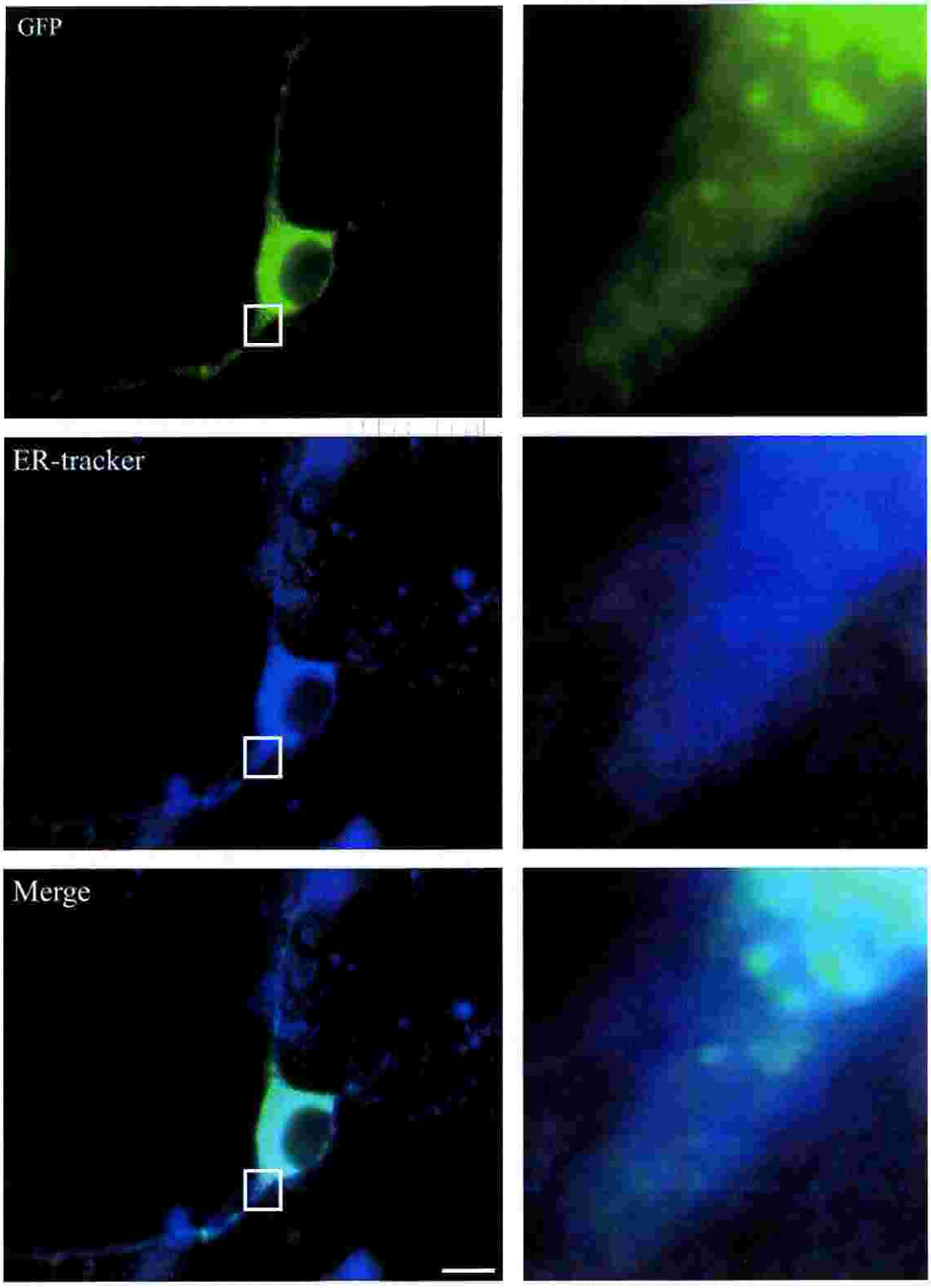

Fig. 3.4 Endoplasmic reticulum of hippocampal neurons
A hippocampal neuron transfected with R4 GFP carrying the KDEL retention signal. To verify that GFP fluorescence corresponds to the ER, cells were stained with a specific dye for the ER (ER-tracker, by Molecular Probes). In the merged picture is possible to see the colocalization of GFP (green fluorescence) and ER-tracker (blue fluorescence). The panels on right part of picture show the detail of the white boxes at higher magnification. It is possible to see the granular distribution of the two staining with a perfect colocalization. Pictures were taken on a wide field microscope. Scale bar: 10μm.

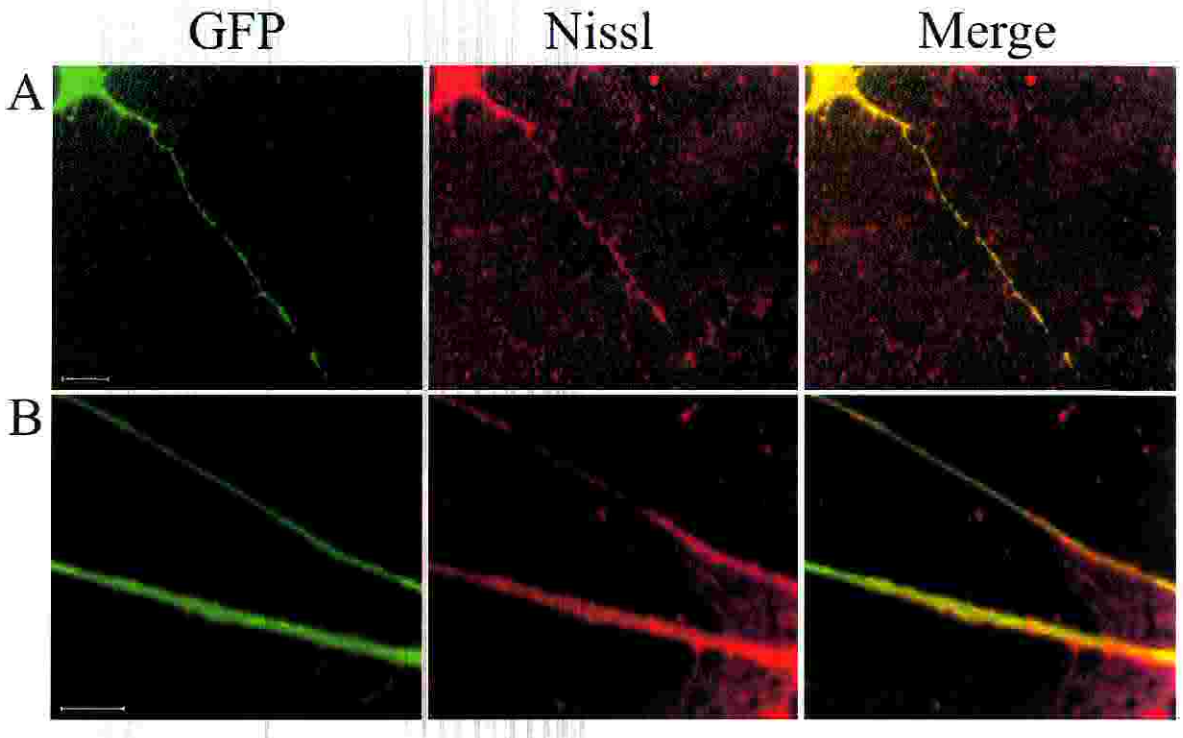

Fig. 3.5 Transfected neurons stained with Nissl stain
Hippocampal neurons were transfected with R4 GFP KDEL (**A**) and with R4 GFP cyto (**B**) scFvs. One day after the transfections cells were stained with a fluorescent Nissl stain (Neuro trace red by Molecular Probes). Unlike cells transfected with the R4 GFP cyto, R4 GFP KDEL florescence show a discontinuous pattern of distribution similar to the one of Nissl fluorescence, as seen in the merge column. All the pictures are projections of Z-series taken on confocal microscope. Scale bars: 10μm

The movement of neuronal ER, linked to microtubules movement, was also exploited to verify the presence of the GFP within ER membranes. As shown in **movie D**, a neuron transfected with the KDEL protein reveals traffic of fluorescent structures in the same way as expected for ER of a living cell. This traffic can be easily followed in picture 3.D were are displayed four single frames from movie D, lasting in total 20 seconds, in which a fluorescent ER structure were moving along a dendrite. After 1 hour of incubation with Nocodazol, known to disrupt microtubules and so intracellular movements along microtubules [Terasaki M et al., 1986], that traffic was completely lost (see **movie E**) confirming from another point of view that the KDEL retention signal indeed traps proteins into the ER. In picture 3.E (C, D and E) are shown three different

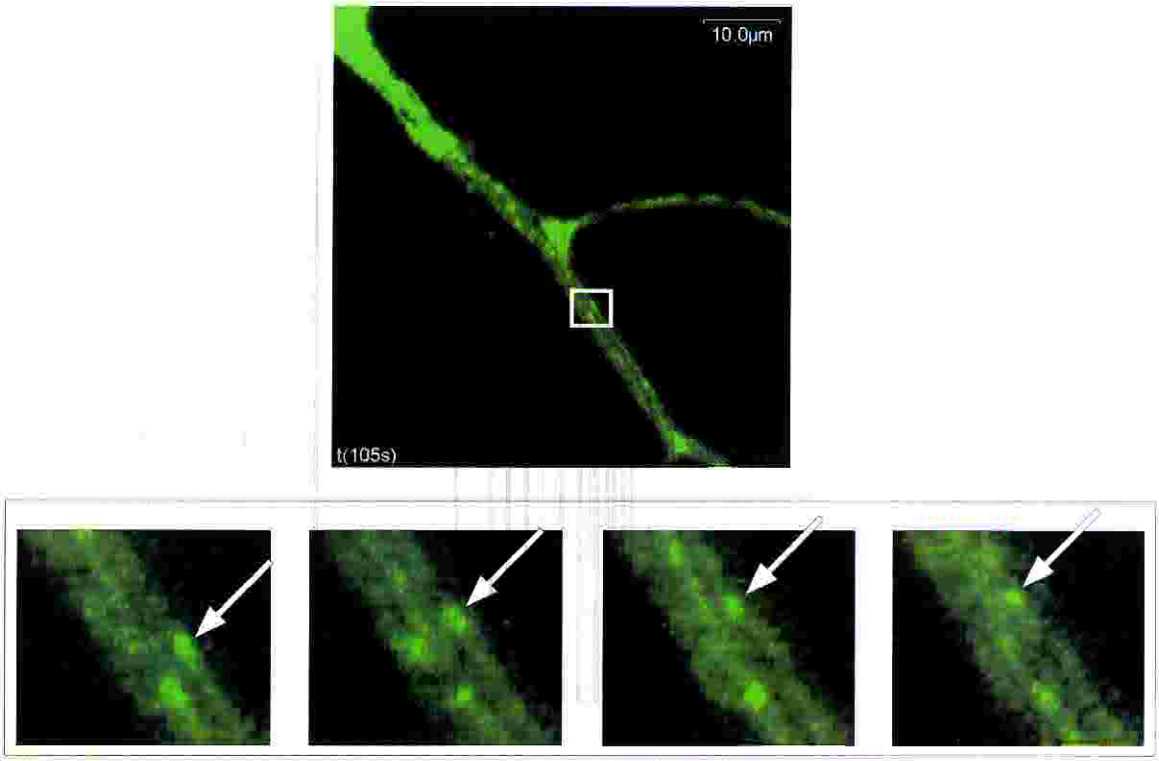

Fig 3.D Movement of endoplasmic reticulum from a hippocampal neuron
A single frame of a neuron transfected with R4 GFP KDEL from movie D. The panel in the bottom is a time series of single frames showing the detail of the white box at higher magnification. Arrows indicate ER membranes that were moving during the recording. The time lapse between each frame is 5 sec. from second 105 to second 120. Recording was taken on confocal microscope.
Scale bar: 10 µm.

frames form movie E in which no ER movement is detectable even after 2 minutes of recording. In the same picture (3.E B) it is also possible to see that the studied neuron was still alive at the moment of the time series indicating that the lack of movement was not due to cell death. The day after the experiment, neurons treated with Nocodazol were alive and healthy.

In conclusion, in the first part of this project, it has been demonstrated that scFvs can be correctly targeted into subcellular compartments of interest and that when expressed in fusion with GFP, they can be easily visualized both in fixed and in living cells.

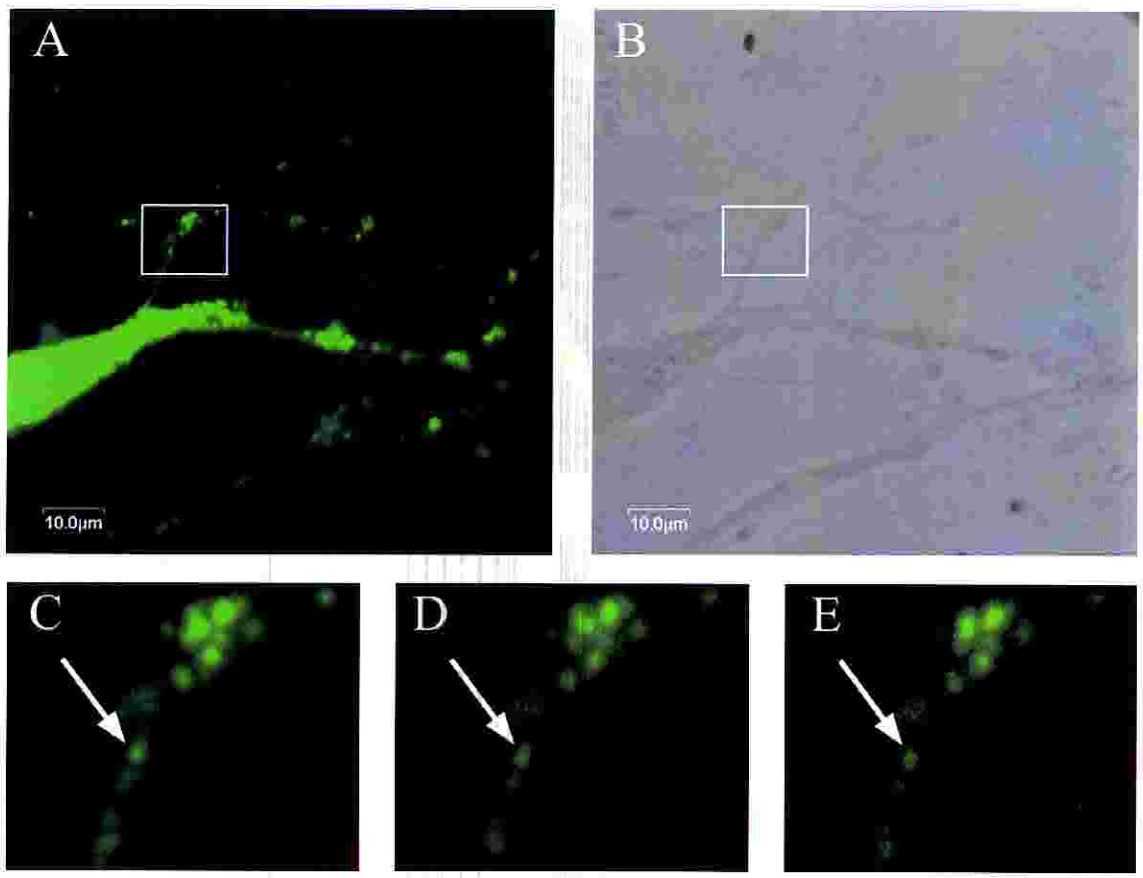

Fig. 3.E Blocking of ER movement after Nocodazol incubation

Blocking of ER movement after Nocodazol incubation

In panel **A**: a single frame of a neuron transfected with R4 GFP KDEL from movie E.

In panel **B**: same frame as in A taken with Differential Interference Contrast (DIC) showing that the filmed dendrite was still alive after Nocodazol treatment.

In panels **C**, **D** and **E**: a time series of single frames showing the detail of the white box at higher magnification. Arrows indicate GFP marked ER membranes during the recording on confocal microscope. Frame C was taken at time 0, frame D at the 60th second, and frame E at 120th second.

Scale bar: 10µm.

3.2 ScFvs mRNA localization

Since the ultimate aim of this work is to study particular proteins expressed in neuronal cells, the neurotrophin receptors, and since neurons are highly polarized with dendrites of hundred of microns, another targeting approach was exploited. In fact the known protein targeting signals alone may not be sufficient to allow a protein to reach the very distal parts of dendrites. On the other hand the simple diffusion is probably not sufficient for proteins with a great molecular weight, the observations for GFP, that is able to diffuse until distal dendrites, is probably not extendable to bigger proteins. For this reason it has been tried to investigate closely the mRNA targeting in neuronal cells, in order to develop tools that can combine protein and RNA targeting for future prospects in intrabody technologies. To reach this goal it was taken in account what it is known about the RNA targeting elements and it has been chosen to work with the well characterized A2RE targeting element for MBP protein [Munro TP et al., 1999] and with the controversial 3'UTR of the CaMKIIα protein (see chapter 1.1.7). Concerning the A2RE, known to work in oligodendrocytes cells, its targeting ability in neurons has not been demonstrated. For CaMKIIα 3'UTR it was tried to verify its targeting ability and to understand something more about its Dendritic Targeting Element (DTE). Hence plasmids for the expression of GFP were constructed carrying, as targeting elements, the 21 bases A2RE, the complete 3'UTR of CaMKIIα (from now on called simply UTR), and one of its putative DTE made of the bases from 1 to 94 of CaMKIIα 3'UTR. Cultured hippocampal neurons were transiently transfected with GFP carrying UTR, DTE and A2RE targeting signals. The first result obtained was that the small molecular weight of GFP allows its rapid and wide diffusion into the cell, until the very distal part of dendrites, even without RNA targeting element, just 5 hours after the transfection. For this reason it was clear that there was no possibility to exploit the fluorescence of GFP for our studies. Therefore it was decided to focus the experiments on the effect of targeting elements on mRNA dendritic distribution and not on protein distribution. Transfected neurons were thus analyzed by *in situ* hybridization in

order to measure how far in dendrites the mRNAs can be targeted by the RNA localization elements of interest.

3.2.1 mRNA in neurons is present in granules

First of all the *in situ* assay on transfected neurons was set up using antisense GFP RNA conjugated with digoxigenin (DIG) as a probe and then detected with enzymatic reaction of anti DIG antibody Alkaline Phosphatase (AP) conjugated. Neurons transfected with A2RE-GFP DNA were analyzed after one day of expression. The results are shown in picture 3.6. It could be evaluated that the efficiency of this kind of transfection was pretty high as from picture 3.6 A, in which is visible a large percentage of positive cells for the presence of GFP mRNA. The *in situ* procedure demonstrated to be reliable as can be seen from the very low background together with a high positive signal of transfected cells. This is more appreciable in picture 3.6 B in which are depicted two neurons, one transfected and the other non transfected. The positive signal of the transfected neuron is extremely high and the presence of GFP mRNA is detected also in distal dendrites meaning that RNA was transported. In the same neuron are also visible some dendritic mRNAs local concentration characteristic of transported mRNA. However it is also known that mRNA is transported as a part of a larger structure, called granules, that can be visualized in dendrites (see chapter 1.1.4) but they do not seems to be visible with *in situ* assay detected by enzymatic reaction. For this reason it was decided to exploit a fluorescent *in situ* hybridization in order to observe granules of mRNA by confocal microscopy. Neurons and 3T3 cell were transfected both with GFP and A2RE-GFP plasmids and the presence of GFP mRNA was detected with a fluorescent (Cy3) antibody. The granular distribution of GFP RNA was immediately clear both in 3T3 (picture 3.7 A and B) and in neurons (picture 3.7 C). Figures 3.7 A and B are single equatorial frames of the 3T3 transfected cells in which is clearly visible the unstained nucleus and a negative non transfected cell, while picture 3.7 C show a single frame of a neuron, taken just above the nucleus, in which mRNA granules are present also in

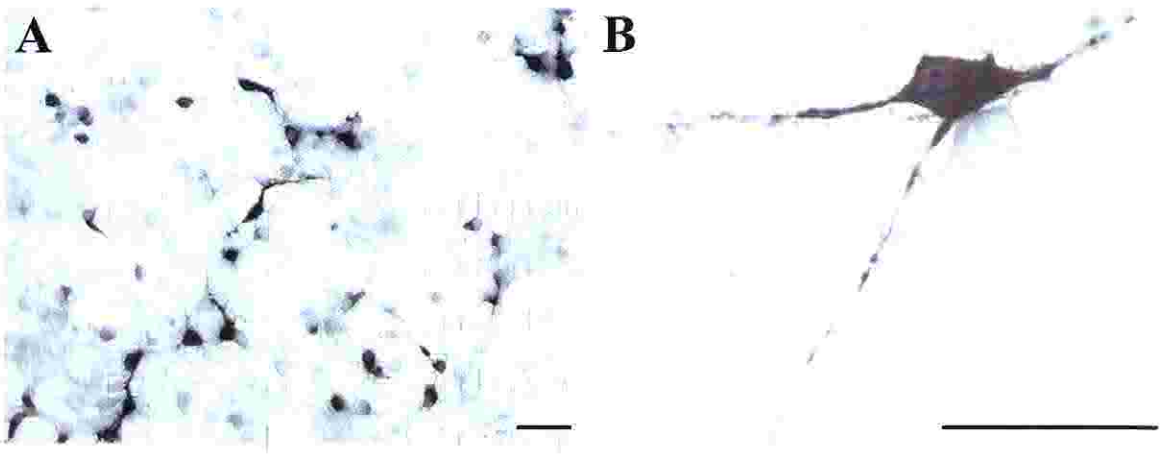

Fig. 3.6 *In situ* hybridization of transfected neurons

Pattern of mRNA distribution in transfected hippocampal neurons. Detection of GFP mRNA was done with a DIG labeled probe and an anti DIG antibody, AP conjugated, stained with NBT/BCIP. In panel **A** a 10x magnification of neurons transfected with GFP fused with A2RE RNA targeting signal; it is possible to see the dendritic staining and evaluate the efficiency of culture transfection. In panel **B** a neuron transfected as in A at higher magnification (40x); here it is possible to see that the staining reaches the more distal part of dendrites. The punctuate staining distribution is due to spots of mRNAs accumulation.

Scale bars: 50μm

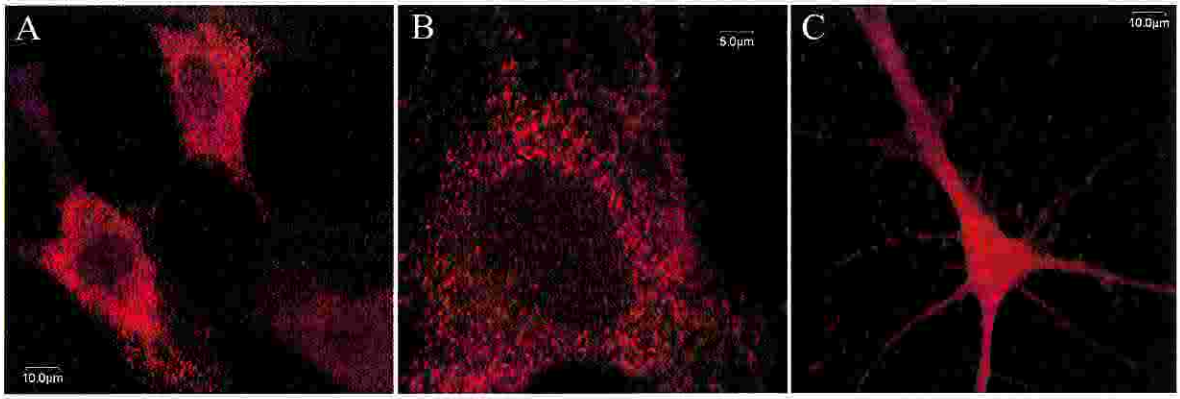

Fig. 3.7 Granules of GFP mRNA

In panel **A** and at higher magnification in **B**, 3T3 cells transfected with plasmid carrying GFP alone.

In panel **C** a hippocampal neuron transfected with GFP DNA carrying the A2RE RNA targeting signal. One day after the transfection an *in situ* Hybridization was performed on transfected cells using a GFP RNA DIG labeled probe detected by immunofluorescence. The GFP green fluorescence was completely lost after the treatment. It is clear that the mRNA of GFP has a granular distribution both in 3T3 and neuronal transfected cells. A and B picture are single equatorial frames of the cell. C is a single frame taken just above the nucleus.

dendrites. From this result it seems clear that transported (A2RE-GFP) and not transported (GFP) RNAs are both arranged in granules. But what about the total amount of granules in a neuronal cell? Transported granules of mRNAs were previously seen, with the aid of SYTO dyes (specific for nucleic acids), in different cell types such as oligodendrocytes [Barbarese E et al., 1995] with SYTO 12, and neurons [Knowles RB et al., 1996] with SYTO 14. In the same it was decided to stain neurons exploiting SYTO 61 red dye in order to visualize mRNA trafficking in a living cell. Moreover, in this way the SYTO 61 specificity for mRNA granules was demonstrated for the first time. The choice of the 61 dye was made considering that it stains with higher contrast and less background respect to 12 and 14 dyes. Furthermore it emits at wavelengths very distant from the typical autofluorescent cell emissions. On **movie F** it is possible to see a time series of neuronal dendrites stained as described, in which numerous mRNA granules move back and forth towards the cell body. In picture 3.E there is a time series of single frames from movie E showing a transported granule of mRNA followed during the recording on confocal microscope. The cell bodies of illustrated cells were over stained for the large amount of mRNA granules that hence were not resolved. Structures with more tubular shapes are clearly mitochondria, also stained for the presence of their own DNA and RNAs. This result, besides giving information about the total unspecific granules, indicated that the used preparations of hippocampal neurons were suitable for experiments of RNA targeting since those neurons were actively transporting their own mRNAs.

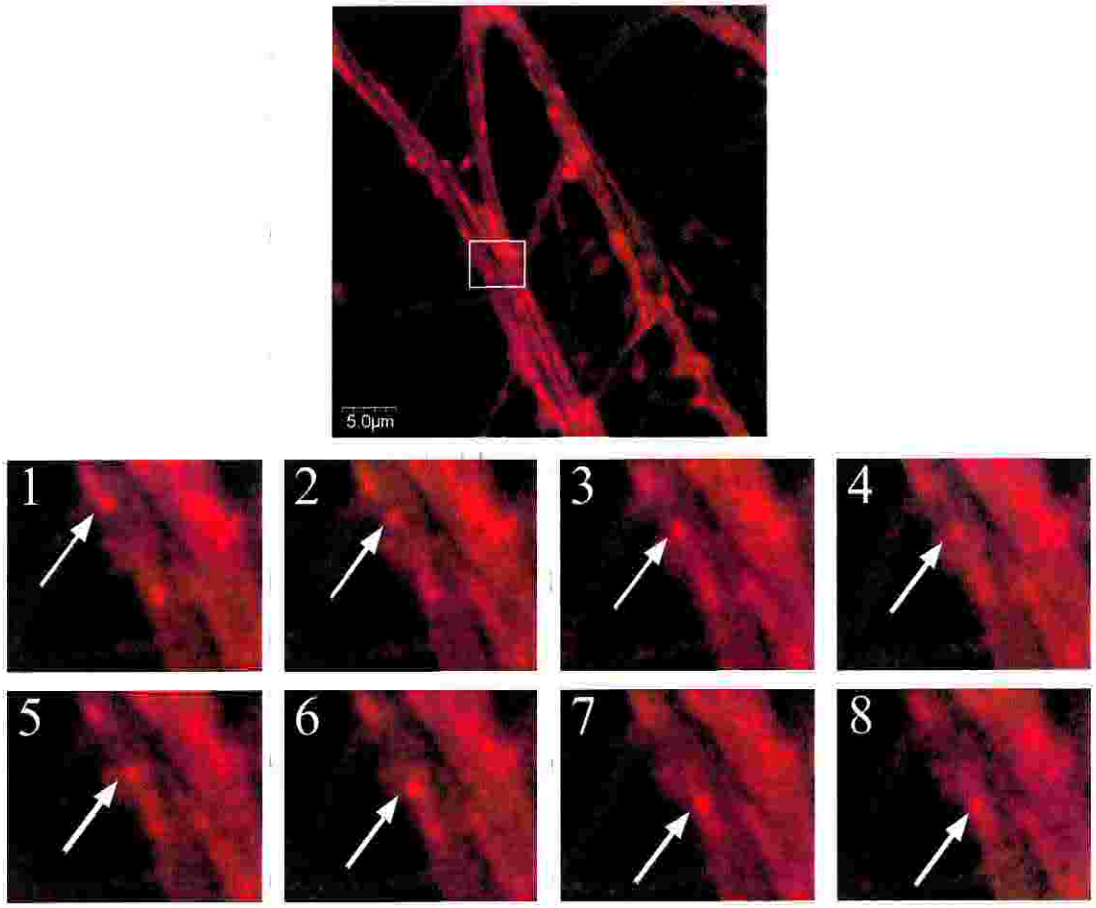

Fig. 3.F Movement of mRNA granules inside dendrites
The picture is a single frame from movie F of cultured hippocampal neurons stained with SYTO 61, specific for nucleic acids.
In panels **1** to **8**: a time series of single frames showing the detail of the white box at higher magnification. Arrows indicate a transported granule of mRNA followed during the recording on confocal microscope.
The time lapse between each frame is 3 sec. from second 123 to second 147.
Scale bar: 5 µm.

3.2.2 GFP mRNA targeting in neurons

As deducible comparing picture 3.6 and 3.7, the length of dendrites stained with *in situ* assays is more appreciable when carried out with the enzymatic reaction. In fact, respect to fluorescent *in situ*, the staining is clearer, with no background and high contrast, and the enzymatic reaction can amplify the signal thanks to the cromogenic substrate deposits. For these reasons targeted GFP RNA detection was performed with AP antibody.

To perform the RNA targeting studies, plasmids containing GFP alone and with the targeting elements at the 3' were used to transfect hippocampal neurons. One day after the transfection neurons were analyzed by *in situ* hybridization. In figure 3.8 A the results of *in situ* assay of neurons transfected with DTE-GFP are shown, it is clearly visible the distal dendritic staining and the characteristic varicosities probably made of cluster of granules of dendritic mRNA. In the same picture there are also visible the remains of a dead transfected cell, a rare occurrence subsequent to transfection. In figure 3.8 B neurons transfected with R4 GFP cyto, that does not carry any RNA targeting element, are pictured. Transfected neurons did not show any dendritic staining. It was then confirmed that the transported RNA were localized in dendrites and not axons by a immunocytochemical staining of MAP2 protein, present only in dendrites, with diaminobenzidine (DAB) just before the AP development (data not shown).

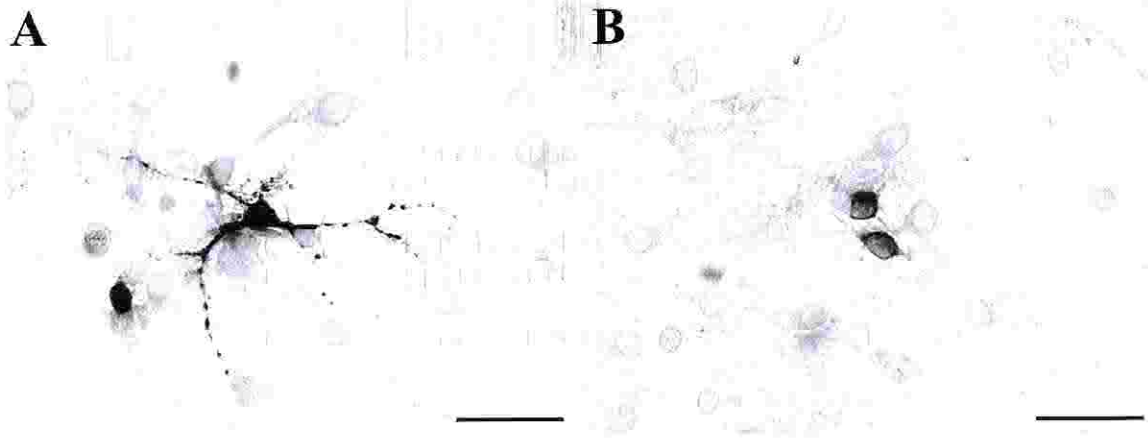

Fig. 3.8 *In situ* hybridization of transfected neurons for statistical analysis
Different pattern of mRNA distribution in transfected hippocampal neurons. Detection of GFP mRNA was done with a DIG labeled probe and an anti DIG antibody, AP conjugated, stained with NBT/BCIP.
In panel **A**: hippocampal neurons transfected with GFP carrying the DTE of CaMKIIα mRNA. Cluster of granules of dendritic mRNA are clearly visible. The GFP mRNA can reach also the distal part of the processes.
In panel **B**: same cell line transfected with R4 GFP cyto. No dendritic staining is detectable. Picture with this magnification (20x) were used to perform the measurements and the subsequent statistics.
Scale bars: 50μm

Measures of dendritic staining, and therefore how far in dendrites GFP mRNA could be transported, were carried out with the aid of Image Pro Express software. More than 100 cells per each transfection were analyzed and, for all the constructs, at least three transfections were performed. Each measure represents the distance in μm between the furthest stained part of a dendrite and the center of the cell body. Each visible dendrite was measured for the analyzed cells. All the measures were taken from pictures taken at the same magnification as in 3.8 A and B. For cells with no dendritic staining, such as picture 3.8 B, a cellular radius was recorded. The averages of each transfection were put together in order to have a final average and a standard deviation among the different experiments that are plotted collectively in picture 3.9.

Hippocampal neurons were initially transfected only with GFP carrying the three RNA targeting elements A2RE, DTE and UTR. Statistical analysis on measured length of dendritic staining are reported in picture 3.9 showing that GFP RNA carrying the A2RE element is significantly more distal than the GFP control, that DTE-GFP dendritic staining, is not significantly different than the negative control, and that UTR-GFP is present only in proximal dendrites For this reason, this simple analysis allows only to draw conclusions for the A2RE targeting signal, already known to work in oligodendrocytes: it is clearly able to promote a distal dendritic mRNA localization also in hippocampal neurons. With these preliminary results nothing can be concluded about the UTR-GFP. However it can be speculated that the full-length UTR may be functional only in stimulated neurons for the presence of an inhibitory *cis*-element [Mori Y et al., 2000; Rook MS et al., 2000], or that it may not be functional at all in our neurons preparations, or finally that there may be a threshold in RNA size above which mRNAs can diffuse in dendrites, therefore the very highly molecular weighted UTR-GFP needed an appropriate size-match control. In fact it is possible that mRNAs with high molecular weight can be kept into the cell body and that small mRNAs can scatter through processes by diffusion. It was found in oligodendrocytes that microinjected RNAs smaller than ~500 bases can diffuse out of the cell body into

the myelin compartment [Ainger K, et al 1997]. For this reason assays on RNA transport had to be conducted with appropriate size-match controls. In figure 3.9

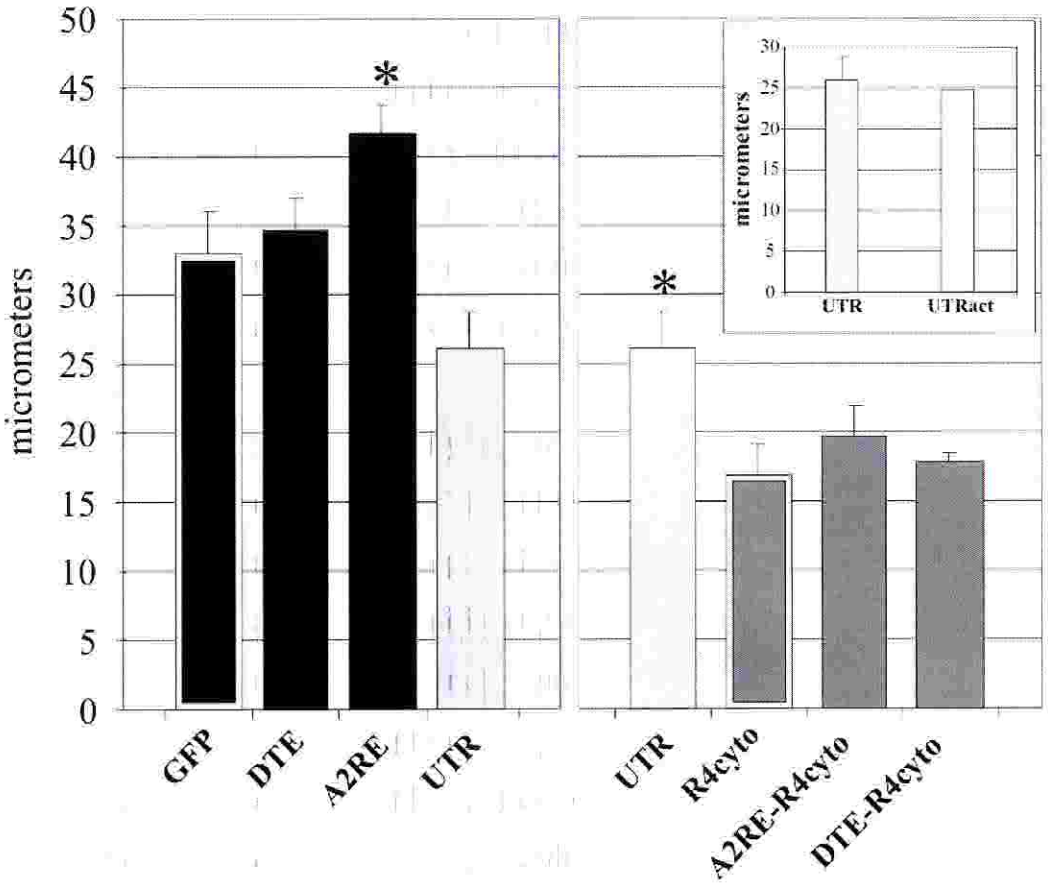

Fig. 3.9 Statistical analysis on RNA targeting in hippocampal neurons

Left part of the panel: neurons were transfected with GFP and with plasmids carrying GFP and the following RNA targeting elements: UTR, DTE and A2RE. RNA of transfected neurons was detected as described in picture 3.8 and the length of dendritic staining was measured (μm in y axis). RNA carrying the A2RE element is significantly more present in dendrites ($p \leq 0,05$, with the asterisk) respect to GFP RNA used as a negative control.

The data concerning RNA with UTR were compared with their natural negative control, the R4 GFP cyto which RNA length is about 1400 base pairs.

Right part of the panel: neurons were transfected with R4 GFP cyto and with plasmids carrying R4 GFP cyto and the specific RNA targeting elements: DTE and A2RE. None of them is significantly more distal respect to R4 GFP cyto negative control.

The UTR-carrying RNA is significantly more present in dendrites respect to R4 GFP cyto negative control ($p \leq 0,05$ with the asterisk).

In the square: comparison between cells transfected with UTR-GFP with or without depolarization. Further depolarization of the cells did not change the dendritic distribution of RNA carrying the UTR targeting signal.

(left part) it is possible to see that GFP RNA, thought as a negative control, was found more than 30μm far from the center of the cell body. From these results it seemed to result that approximately 700 bases, the length of GFP RNA, was the threshold RNA length beneath which a given RNA can passively diffuse and reach also the distal parts of the neuron. This observation is basically in accordance with the experiments of RNA microinjection [Ainger K, et al 1997]. As a result it was decided to use cells transfected with GFP only as negative controls for cells transfected with A2RE-GFP (~721 bases) and DTE-GFP (~821 bases). For UTR-GFP, a nearly 4 Kbases long RNA, GFP in fusion with R4 GFP cyto (without any targeting signal) was used as a negative control, since it is about 1,5 Kbases long, sufficiently more than the putative threshold. In the light of these considerations, comparing the results of UTR-GFP with R4 GFP cyto as a negative control, the conclusion turns out to be considerably different. The UTR resulted, as shown in picture 3.9 (right part), to be able to target the GFP RNA more than 25μm far in neuron dendrites respect to 15 μm of R4 GFP cyto control, roughly the average measure of a cellular radius.

When it was decided to test also the hypothesis of activation of neurons (three hours of incubation with KCl 10mM) in order to increase the transport of RNA as was described for the 3'UTR of CaMKIIα, it was found that neurons were in some way already activated. This result came from immunofluorescent assays aimed to detect the presence of intermediate early genes c-fos and zif/egr1 in the nucleus both in transfected neurons incubated with KCl and not. In picture 3.10 it is possible to see two neurons, one transfected with UTR-GFP, and the other one non transfected, that were not incubated with KCl and so were not activated (panel A). The non transfected neuron show, after immunofluorescence assay, a strong nuclear signal due to the presence of c-fos protein (panel B). This means that the neuron was activated, and that this kind of activation was not the consequence of transfection since the transfected ones are not c-fos positive. One possible explanation may come from transfection procedure. It could be possible that Ca/PO_4-DNA precipitates used for the transfections cause cellular stress able

to alter neuron resting state and to activate them. A further confirmation to this appeared when dendritic staining after *in situ* assay was measured. As shown in the inset of picture 3.9 no difference is detectable regarding GFP RNA transport in activated and non activated neurons.

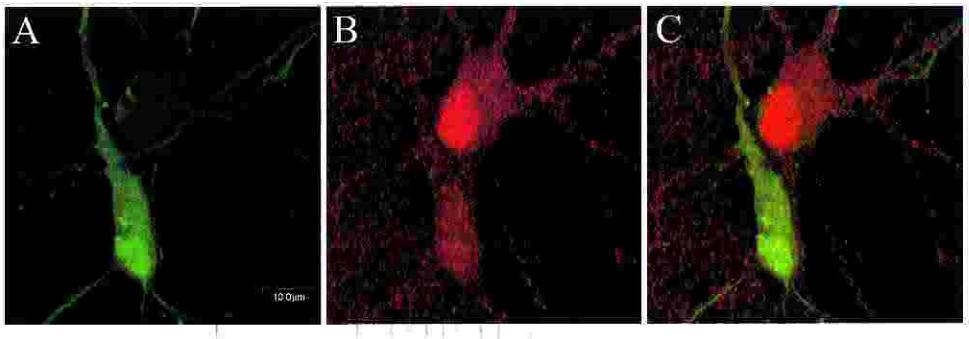

Fig 3.10 Neuron activation due to transfection procedure
Cultured hippocampal neurons transfected with UTR-GFP without any standard activation procedure. In panel **A**: a transfected neuron. In panel **B**: immunodetection of c-fos nuclear protein of the untransfected neuron. In panel **C**: merged picture. The activation was not due to transfection itself, but to transfection protocol. Scale bar: 10µm.

At this point it can be concluded that the presence of a *cis*-acting element into CaMKIIα 3'UTR, able to target a reporter RNA into dendrites, may be confirmed from more convincing results with a size match control, but results obtained with DTE-GFP seems to indicate that the *cis*-acting element is doubtfully present inside the first 94 bases (the DTE).

3.2.3 ScFvs mRNA targeting in neurons

To confirm the results obtained with GFP RNA, GFP in fusion with R4 cyto scFv was used as a reporter RNA. Neurons were transfected with A2RE-R4 cyto, DTE-R4 cyto and, as a negative control, with R4 GFP cyto lacking any RNA targeting signal. Transfected neurons were stained and measured as described before. The results, showed in picture 3.9 (right part), are clearly comparable with the previous, obtained with GFP alone. From this results it is also clear that the extent

of R4 GFP mRNA transport shows a great decrease respect to the transport of GFP alone. This could be due to some unrevealed sequences in R4 GFP RNA that inhibit the transport or, more likely, it could be the result of the R4 GFP mRNAs high molecular weight (length about 1400 bases). The last hypothesis is in accordance with the result of R4 GFP cyto mRNA that is clearly restricted to the cell body and does not show diffusion (Fig. 3.9 right part). If what hypothesized is true it would confirm the threshold length of diffusible RNAs that seems to be comprised between ~700 bases and ~1400 base.

On the other hand, results of figure 3.9 showed also that neither A2RE-R4 cyto nor DTE-R4 cyto mRNAs transports are significantly different from the control. In order to understand this lack of significance and to better describe the real situation, and to see how the collected data were distributed in detail, the same data were visualized with a different approach. In fact RNA transport measurements is a very complex assay for the presence, in neurons cultures, of several different cell types at different cell cycle and developmental stages. This cells multiplicity is clearly involved in RNA transport properties and hence the average and standard deviation of measurements are not so informative. Additional information may be obtained by displaying the maximum extent of GFP mRNAs within 5 μm intervals for the dendritic measured population. Therefore the total numbers of measures of dendritic staining by *in situ* were clustered into defined intervals of 5μm for each RNA targeting element. In this way, it was obtained the real number of dendrites that showed the presence of GFP mRNA as far as the specified extent. These numbers were then normalized in order to compare data from the different trials resulting in a distribution of extent of staining expressed as percentage of dendrites. In figure 3.11 there is an example of this statistical approach. In panel A the distribution, obtained as described, of the data from neurons transfected with A2RE-GFP is shown. Each bar corresponds to the percentage of dendrites with a length of staining within a specific interval. In panel B there is the distribution obtained from transfections with GFP alone. To compare the two distributions they were superimposed as

shown in panel C. Here it is easier to see the spatial localization of the two distributions that are clearly separated: in the left part, until the 25 μm, the majority of non superimposed bars are brown and came form GFP distribution, while from 25 to 105 μm the prevalence is of the gray color (A2RE distribution).

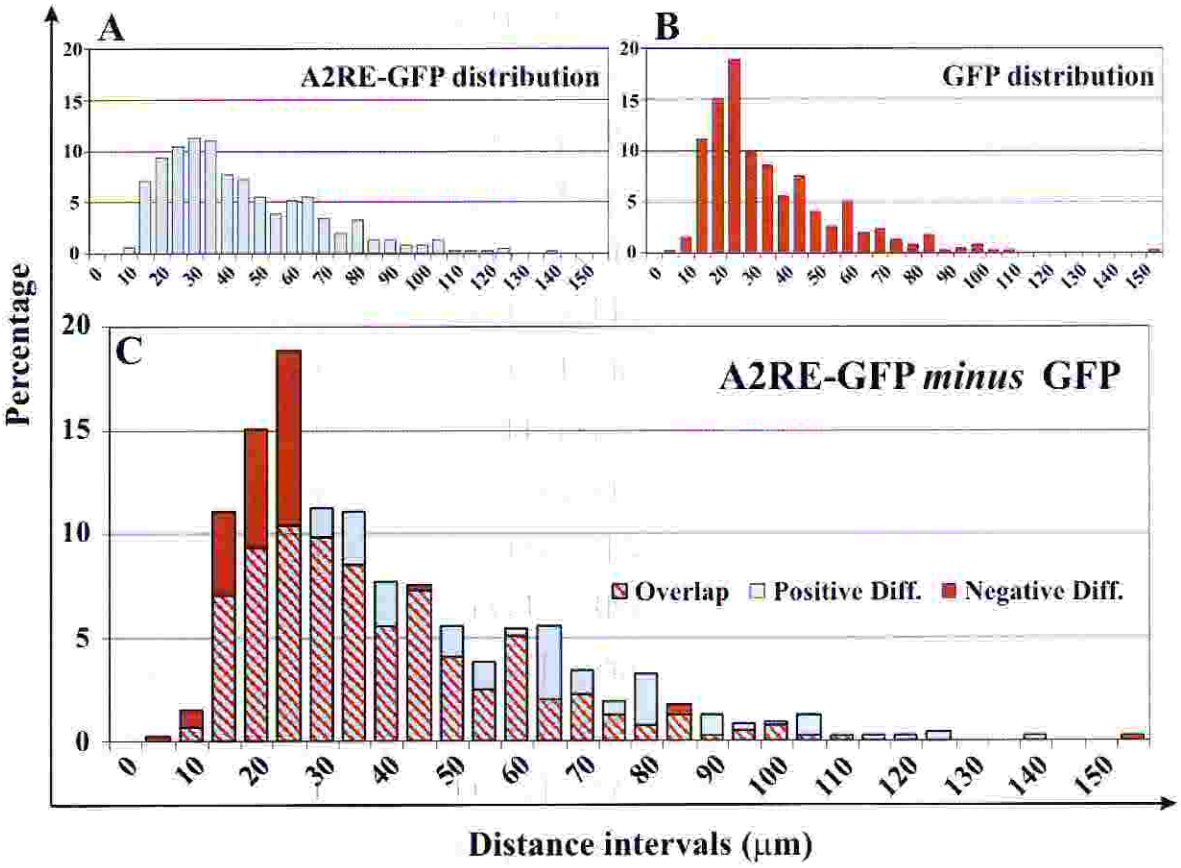

Fig. 3.11 Distribution of the extent of mRNAs detected in transfected neurons
Distributions obtained clustering measured extents of mRNAs into 5μm intervals. Each bar corresponds to the percentage (y axis) of transfected dendrites that were measured, with *in situ* assays, at a distance included inside the specific interval (x axis)
Panel **A**: distribution obtained from data of neurons transfected with A2RE-GFP, in gray.
Panel **B**: distribution obtained from data of neurons transfected with GFP alone, in brown.
Panel **C**: superimposition of the two previous distributions.

In this way a subtraction between A2RE distribution and GFP distribution, was calculated: A2RE percentages minus GFP percentages. Hence in graphic C, the gray corresponds to a positive value. This means that, for the specific length interval, there are more measures coming from A2RE transfected dendrites than

Considering this graphic as a subtraction between A2RE distribution and GFP distribution (A2RE-GFP), the gray part of each bar corresponds to a positive percentage result (A2RE-GFP=positive number), the brown part to a negative percentage result (A2RE-GFP=negative number), and the double colored part to the overlapping fraction of distributions (A2RE-GFP=0).GFP transfected dendrites. In a similar way, the brown bar portions correspond to a negative result of the subtraction A2RE minus GFP, indicating, for the specific length interval, a higher staining percentage of GFP distribution. The part of bars with doubled colored diagonals represents the overlapped data of the two distributions.

In order to accentuate differences and remove similarity the same data were plotted in a different way, more easy to visualize (figure 3.12). Panel 3.12 A corresponds to panel 3.11 C. The negative bars come from negative values of the subtraction (brown part of graphic 3.11 C), while positive values of the subtraction (gray in graphic 3.11 C) are the positive bars. Here we can see a segregation of bars in the negative part of the Cartesian plan (in the left part) until the interval of 25 μm, and a majority of positive bars in the right part of the panel that are distributed into a well defined range from 30 to 105 μm. It is also possible to evaluate two ranges within which values are distributed. This range is narrow for the negative values, and wide and well distributed for the positive ones. With this kind of approach it is easier to evaluate the actual abilities of the A2RE element, in this case, to target the RNA of GFP. In fact measured dendritic RNAs from cells transfected with GFP alone are more present in the first part of the graphic, until a measured distance of 25 μm, while from 30 μm to 105 μm the vast majority of data comes from cell transfected with A2RE. Above the limit of 105 μm the number of counts and the computed percentage differences are apparently too small to claim any significance. Thus for the results from this targeted mRNA we have observed a RANGE interval between 30 to 105 μm. The introduction of the RANGE concept seems an effective way to describe the transport behavior of a given mRNA, therefore it was developed a more rigorous way to define the RANGE interval values, in order to apply a constant criterion to all the targeted

mRNAs analyzed. More in detail the RANGE values calculation was performed by applying the simple equation defined below. To simplify the description of the method proposed, the distance intervals shown in figure 3.12 will be referred as "bins". The RANGE interval corresponds to the first contiguous intervals of bins where all the bins fulfill the following condition:

$$\mathbf{F = pD(n)+pD(n-1)+pD(n+1) \geq 1\%}$$

where the expression pD(x) stands for the value of the percentage Difference (pD) for the x bin. In this way the stochastic fluctuations that are generated by the experimental errors are compensated for each bin with the aid of the neighboring values. With this criterion the computed RANGE interval for the A2RE-GFP mRNA results included between 35 and 110 μm. It was decided to focus the attention on values of F ≥ 1% in order to maintain as much information as possible and to highlight the complexity of information coming from our data. The RANGE intervals with F ≥ 2%, 3% and 4% were also calculated and are reported below.

RANGE (RNG) intervals with different F (the values are μm)

	F ≥ 1%	**F ≥ 2%;**	**F ≥ 3%;**	**F ≥ 4%;**
A2RE-GFP	35 ≥ RNG ≥ 110	35 ≥ RNG ≥ 85	35 ≥ RNG ≥ 85	35 ≥ RNG ≥ 75
DTE-GFP	30 ≥ RNG ≥ 55	30 ≥ RNG ≥ 50	30 ≥ RNG ≥ 50	30 ≥ RNG ≥ 45
A2RE-R4cyto	20 ≥ RNG ≥ 45	20 ≥ RNG ≥ 40	20 ≥ RNG ≥ 35	20 ≥ RNG ≥ 30
DTE-R4cyto	20 ≥ RNG ≥ 45	20 ≥ RNG ≥ 35	20 ≥ RNG ≥ 35	20 ≥ RNG ≥ 30

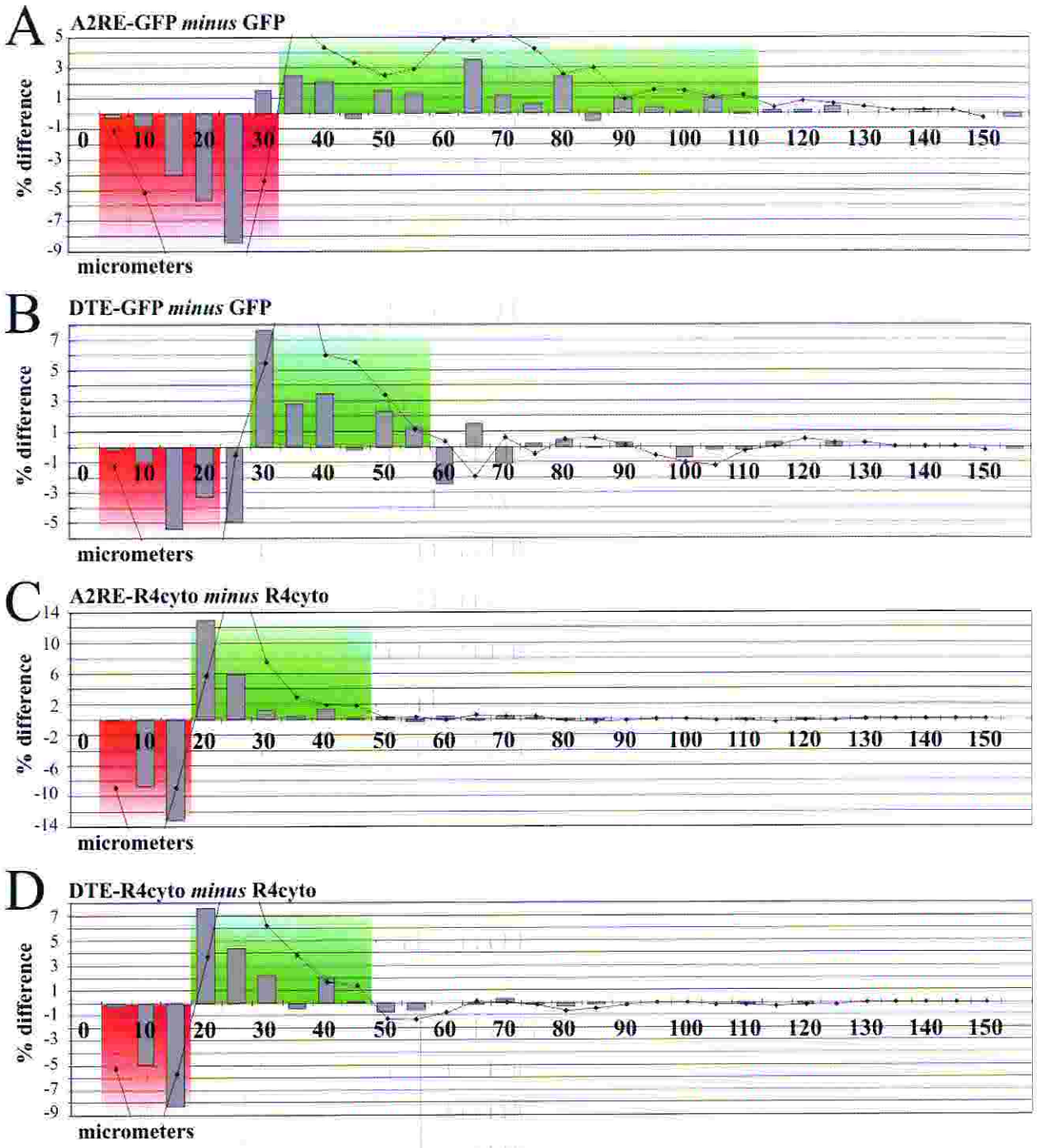

Fig. 3.12 Detailed dendritic length staining distribution comparison.
The percentage of dendritic mRNA distributions A2RE-GFP (panel **A**) and DTE-GFP (panel **B**) are plotted against the distributions of GFP alone, while A2RE-R4cyto (panel **C**) and DTE-R4cyto (panel **D**) are plotted against R4 GFP cyto. The results represent the differences in the distributions between the targeted RNAs and their untargeted counterparts. The distributions obtained for the targeted RNAs were subtracted to their counterparts control RNAs: A2RE-GFPand DTE-GFP minus GFP alone, DTE-R4cyto and A2RE-R4cyto minus R4 GFP cyto.
Green area: RANGE interval with $F \geq 1\%$; Red area: RANGE interval with $F \leq -1\%$; (for a definition of RANGE and F function see the text)

The more interesting result came from this kind of approach and from RANGE interval calculation applied to the targeting of DTE mRNAs. In fact from picture 3.9 it seemed that DTE does not target effectively mRNA into dendrites, as its measured average transport distance did not significantly differ from negative controls. Observing graphics 3.12 B and D it can be seen that the DTE fused RNAs is more present in the right part of panels beginning from 30 μm both for DTE-GFP (3.12 B) and DTE-R4 cyto (3.12 D). Calculating the RANGE interval from DTE-GFP distribution, which is included between 30 and 55 μm, it can be seen that this value is considerably lower than the RANGE from A2RE-GFP distribution. Different is the conclusion that can be made comparing the computed RANGE interval for DTE-R4 cyto (20μm ≥ RANGE ≥ 45μm) and for A2RE-R4 cyto (20μm ≥ RANGE ≥ 45μm) that resulted equal, suggesting that the DTE seems as powerful as A2RE. These consideration taken together can lead to conclude that the DTE targeting element is not efficient as A2RE, but indicate that it is without a doubt a targeting element. Since from previous results it has been verified the function of 3'UTR of CaMKIIα, this new result indicate that the CaMKIIα dendritic targeting element, may be present in the first 94 bases of its 3'UTR and that it is involved in RNA targeting in activated neurons.

The same conclusions can be drawn for the A2RE element. In fact, when put in fusion with R4 GFP, it seemed not to be able to target RNA as well as when fused with the GFP only. But, examining graphic 3.12 C and the resulting RANGE interval, it can be concluded quite strongly that it is a good RNA targeting element in activated neurons (even if initially found to work in oligodendrocytes) not only in fusion with GFP but also with R4 GFP. Results from RANGE intervals calculated in a more stringent way (with F ≥ 2%, 3% and 4%) do not differ significantly from the first one confirming the conclusions just drown. This different statistical approach not only gave a real idea of the complexity of the problem studied, but also allowed to obtain new and interesting results absolutely not appreciable only considering the average and the standard deviation of the data shown in figure 3.9.

To summarize the results obtained studying different protein and RNA targeting signals it can be concluded that scFvs can be successfully targeted in the intracellular compartment of interest by means of traditional protein targeting signals, and that, to reach the distal part of dendrites, the RNA targeting seems to be a promising approach. Moreover it has been introduced a new method to calculate the targeting ability of different *cis*-acting elements which allow to establish, in a semi-quantitative way, when and how a given mRNA is actively transported notwithstanding the experimental errors and the limitation of the experimental procedures that cannot be overcome by elementary statistical analysis.

3.3 Function of targeted scFvs

Once established the possibility to intracellularly target scFvs, it was necessary to verify if they can maintain their ability to recognize and bind their specific antigen.

With this work it is wanted to investigate the possibility to use scFvs to interfere with the function of neurotrophin receptors, in particular $p75^{NTR}$ and TrkA receptors. Therefore three specific scFvs were chosen to go on with the study: α TrkA and MNAC both recognizing and binding specifically to TrkA receptor, and α p75 used against $p75^{NTR}$. MNAC scFv was previously characterized [Cattaneo A et al., 1999]. It is known to recognize the extracellular portion of TrkA receptor and to neutralize the binding and activation of TrkA by NGF. For this work it was cloned into ScFvExpress plasmids carrying the sec and KDEL protein targeting signals as described in chapter 2.1.

α p75 and α TrkA were previously selected from Vaughan library for their ability to bind the extracellular part of the two receptors; hence they needed, first of all, to be characterized.

3.3.1 Characterization of α p75 and α TrkA

The phagemids containing the DNA encoding for α p75 and α TrkA, were used to express the two scFvs in *E.Coli.* After expression they were purified by affinity chromatography as described in chapter 2.7.1. The purification yield was estimated by SDS-PAGE and their ability to bind with the substrate was tested by ELISA assay. In figure 3.13 it is shown the pattern obtained using sequential dilutions of the purified protein against both $p75^{NTR}$ and TrkA immunoadhesins (as coating antigen) in ELISA assay. The different O.D. values of the two curves were due to a different starting concentration of the purified scFvs. Purified α p75 was more concentrated respect to α TrkA, as estimated by SDS-PAGE.

These purified scFvs were used later on as positive control in the ELISA and western blot experiments.

Analysis of scFvs DNA sequences revealed that both α p75 and α TrkA VH chains belong to VH3 family while α p75 contains a VL3 family chain and α TrkA a VL chain of the Vκ1 subgroup [Chothia C et al., 1992: Tomlinson IM et al., 1995].

Both the scFvs were then cloned into sec and KDEL ScFvExpress plasmids as done for MNAC.

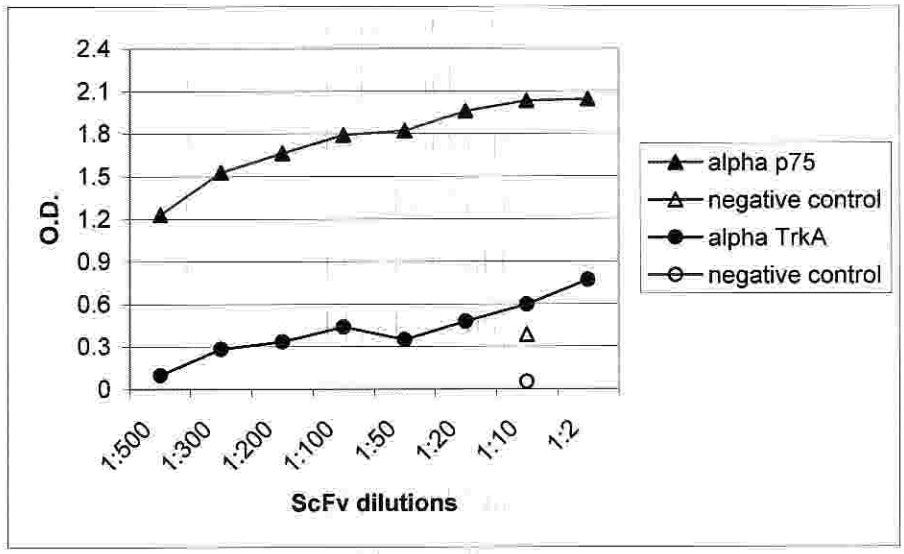

Fig. 3.13 Curves obtained with ELISA assay of purified α p75^{NTR} and α TrkA ScFv.
ELISA assays obtained with different concentrations of the purified scFvs
The triangles (▲) show the results for α p75 purifications; the circles (●) for α TrkA.
Coating: TrkA and p75^{NTR} immunoadhesins for ● and for ▲ respectively.
Primary antibody: 9E10 anti myc tag.
Secondary antibody: a mouse HRP conjugated.
Detection method: enzymatic reaction of TMB.
Negative control: only secondary antibody at dilution 1:10.
x axis: the ratio of scFv dilutions.
y axis: the value of Optical Density measured at 450nm (O.D.).

3.3.2 ScFvs expression in eukaryotic cells

In order to verify that scFvs can recognize their specific antigen once they reach the intracellular compartment of interest, α p75, α TrkA and MNAC were expressed in COS cells [Gluzman Y, 1981] As expression vectors, ScFvExpress plasmids with sec and KDEL signals were used for transient transfections.

COS cells were transiently transfected with the three scFvs both with the sec and the KDEL signals. ScFvs expression was detected by immunofluorescence using a specific antibody against the myc tag, expressed in fusion at the N-terminus of proteins to easily detect their expression. Cells culture supernatant and lysates of transfected cells were analyzed then by western blot. Secreted and retained proteins were detected with the anti myc-tag antibody.

To verify scFvs function ELISA assays were performed using the same supernatants and cells lysates. In this way the ability of expressed and targeted scFvs to bind their specific antigen (that is, $p75^{NTR}$ and TrkA immunoadhesins) was tested. Supernatant and lysate preparations of non transfected cells were used as a negative control. The results are showed in figure 3.14, it is easy to verify the binding of expressed scFvs to the antigens, confirming a correct folding after the targeting. Differences in measured optical density among results of the same scFv are due to differences in proteins concentration. As expected, a greater amount of proteins is present in the supernatant of sec scFvs transfected cells, while KDEL scFvs were detected mainly in cell lysates. This result confirms the ability of the KDEL signal to block a secretory protein within the ER; for this reason it is found more abundantly in cell extracts. The presence of KDEL proteins in the supernatant, visible in figure 3.14, may be due both to the molecules escaping from the ER and to death of transfected cells. Results of the western blot analysis described before were consistent with ELISA assays.

In conclusion the cloned scFvs constructs maintain their function in *in vitro* assays after expression and targeting in COS cells; and were considered suitable for the subsequent phenotypic knock-out of the neurotrophin receptors experiments.

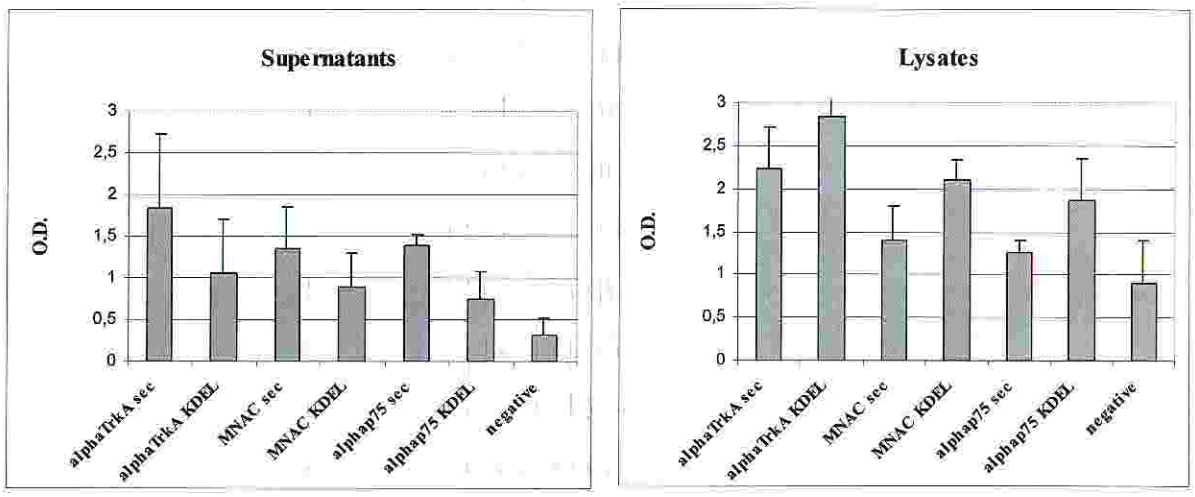

Fig. 3.14 ELISA of supernatants and lysates of transfected COS cells

ELISA assay obtained with supernatants and lysates (diluted 1:2) of COS cells transfected with α p75 and α TrkA both in fusion with secretory and KDEL signals. The bars represent the mean optical density value; error bars are the standard deviations among three different experiments.

A larger amount of protein is present in supernatants of sec scFvs and in lysates of KDEL scFvs, suggesting a correct targeting. Both scFvs are able to recognize the antigen indicating a correct folding.

ELISA assay was performed as described in picture 3.13.

Negative control: untransfected COS cells

y axis: the value of Optical Density measured at 450nm (O.D.).

3.4 Alteration of neurotrophin receptors trafficking towards membrane

At this point it seemed possible to anchor NGF receptors inside the cells by the binding of anti-receptor scFvs expressed with the retention signal KDEL. In this way the antibody fragment (in this case α p75) could bind with its antigen ($p75^{NTR}$) in the secretory pathway and prevent the receptor appearance on the membrane surface by sequestering it within the ER. Such trapped $p75^{NTR}$ receptors should no longer activate the intracellular signal transduction triggered by NGF. To verify this working hypothesis, a stable transfection of scFvs was performed on C6 rat glioma cells [Benda P et al., 1968] that constitutively express $p75^{NTR}$ and TrkB receptors and not TrkA [Colangelo AM et al., 1994; Pantazis NJ et al., 2000]. The α p75 scFv, in fusion with the KDEL retention signal were used for the transfections. The level of scFvs expression on the selected stabilized clones was preliminary tested by immunofluorescence as described in chapter 3.3.2. Subsequently the presence of the scFv inside the cells, while retained into the ER, was detected by western blot of clone lysates. Figure 3.15 shows the results of western blot of 8 different C6 clones. Only 5 out of 8 showed expression of α p75 indicating that the others had maintained the gene for the antibiotic resistance while they had lost DNA encoding for the scFv. Lysates of the 5 positive clones were afterwards analyzed by ELISA assay for their ability to bind the antigen. The results, showed in picture 3.16, were consistent with the previous western blot for clones L, N, P and Q concerning the amount of expressed scFvs. The ELISA negative controls were protein extracts from C6 stabilized clones expressing α TrkA scFv.

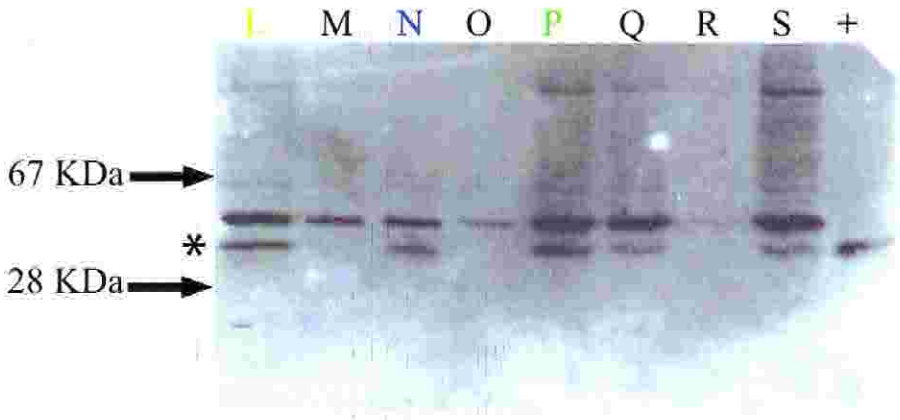

Fig. 3.15 Western blot of C6 clones expressing αp75 KDEL.
10 μl of undiluted lysates were loaded into the gel and detected with 9E10 (primary antibody) and anti mouse HRP conjugated (secondary antibody) revealed by chemiluminescent ECL. The band corresponding to α p75 is marked with an asterisk. Other bands at higher molecular weight are non-specific 9E10 binding. While for R clone the protein extract is too low to be detected, clones M and O clearly show no α p75 expression. The positive control is the scFv purified from *E. Coli.* (α p75 MW ≅ 32 kDa).

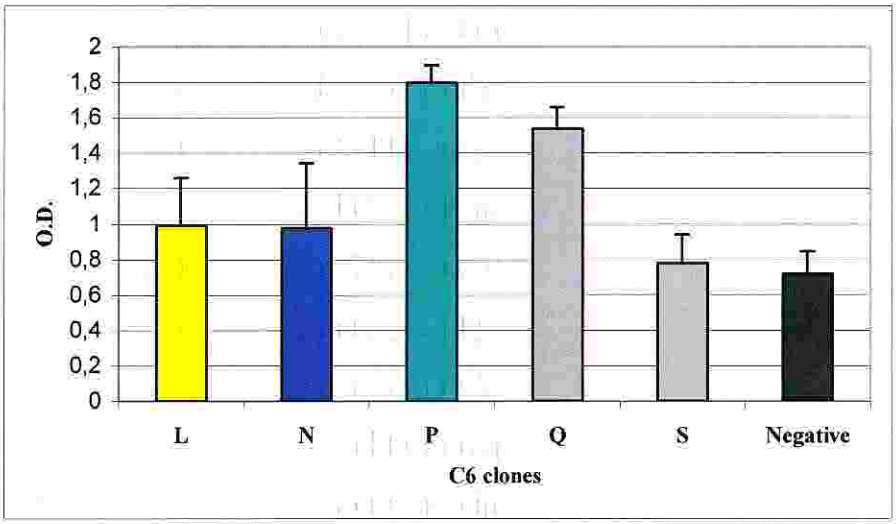

Fig. 3.16 ELISA assay on lysates of C6 clones expressing αp75 KDEL
The ELISA assay was performed as described in figure 3.13 with protein extracts from the different clones diluted 1:2. The bars correspond to the optical density average of three experiments; error bars are the standard deviations among the different experiments. For clones L, N, P and Q the differences in O.D. are due to different protein amount as shown in figure 3.15.
Negative control: protein extract form the C6 stable clone expressing α TrkA KDEL.

3.4.1 *In vivo* flow cytometer analysis

Since C6 glioma cells constitutively express $p75^{NTR}$, the ability of α p75 to prevent receptor appearance on the membrane surface was tested. Fluorescence-Activated Cell Sorter (FACS) analysis was performed. The flow cytometer is an instrument able to analyze the fluorescence of every single cell in suspension by measuring their fluorescence emission one by one while passing through a narrow opening. By labeling with a fluorescent antibody the $p75^{NTR}$ present on cell surface, it was possible to establish the relative amount of membrane receptor on each cell by measuring the fluorescence intensity. C6 untransfected cells and C6 clones were labeled by immunofluorescence *in vivo* with an anti $p75^{NTR}$ (MC 192) and, as negative control, with a non relevant antibody (αD11 anti NGF). A total of 10.000 cells per experiment were analyzed with the flow cytometer by measuring the fluorescence intensity. In figure 3.17 the results for the positive and negative controls are shown. It is possible to see the fluorescence intensity distribution due to the constitutive amount of $p75^{NTR}$ present on cell surface of untreated C6 cells (positive control), and the background fluorescence due to cell autofluorescence and non-specific antibody binding (negative controls).

In the same way three of the selected clones, L, N and P, were analyzed with the flow cytometer after *in vivo* immunofluorescence. As shown in figure 3.18, the surface fluorescence of all the three clones showed a significant decrease respect to untransfected cells, indicating the presence of a lower amount of $p75^{NTR}$ on the cell surface.

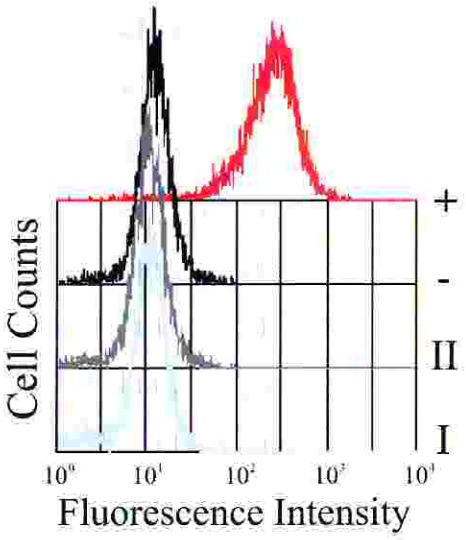

Fig. 3.17 FACS analysis controls

C6 untransfected cells were labeled with MC192 (anti $p75^{NTR}$) primary antibody, α mouse biotinylated secondary antibody and avidine FITC for the positive control (+, in red). As negative control the same cells were immunostained with a non relevant antibody, anti NGF αD11 (–).
Fluorescence background: cells treated with only secondary antibody (**II**) Autofluorescence: untreated cells (**I**). x axis: fluorescence intensity (FL1-H at 488 nm); y axis: number of events (10.000 per each experiment)

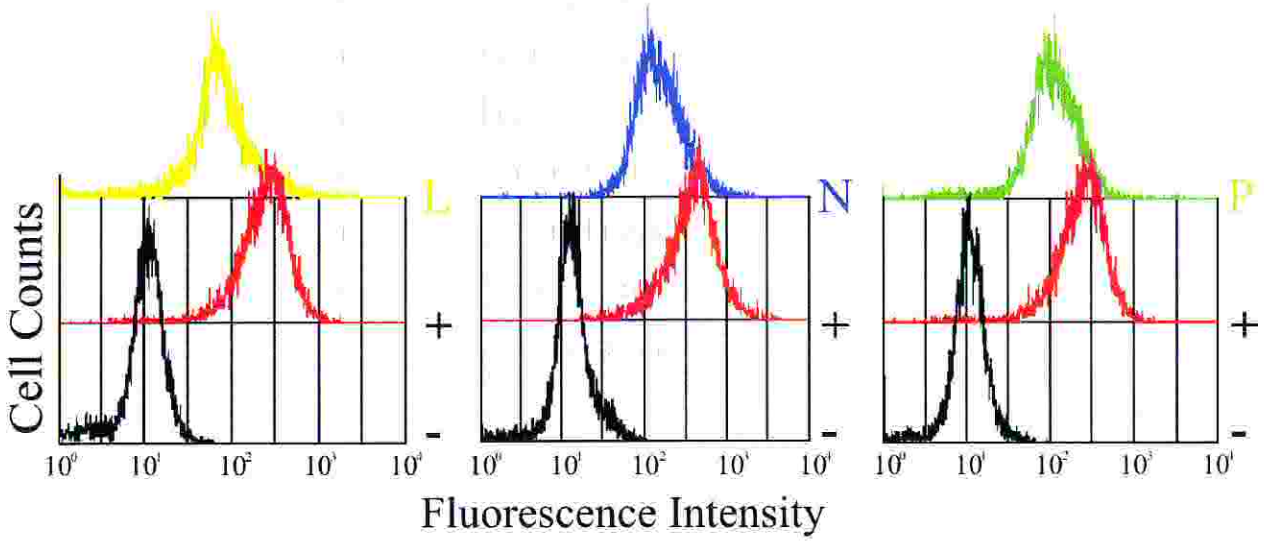

Fig. 3.18 FACS analysis on C6 clones

C6 stable transfected clones L, N and P were immunostained as described in figure 3.17.
Negative controls (–): clones L, N and P treated with αD11 primary antibody.
Positives controls (+): untransfected cells
A decrease of fluorescence respect to the positive control can be seen for all the clones due to a reduced amount of $p75^{NTR}$ receptor on cell surface.
x axis: fluorescence intensity (FL1-H at 488 nm)
y axis: number of events (10.000 per each experiment)

To verify that the selected clones did not express by themselves, for other reasons, a low amount of $p75^{NTR}$, two different western blot were performed with protein extracts: one against $p75^{NTR}$, with MC 192 antibody (fig 3.19 A), and the other using YL 1/2, an antibody that recognize the constitutively expressed protein tubulin (fig 3.19 B). This second western blot was used to evaluate the yield of the protein extraction procedure, using tubulin as a constitutively expressed reference protein. In figure 3.19 it is possible to see that all the clones as well as C6 untransfected cells express a comparable amount of $p75^{NTR}$ and also that the receptor is not degraded. This result confirmed that the lower amount of membrane fluorescence in FACS analysis, due to the decreased presence of $p75^{NTR}$ in analyzed C6 clones, was the result of the KDEL scFvs anchoring effect. In conclusion at this point of the work it has been demonstrated that a scFv, correctly retained in the ER by KDEL signal, is able to bind its antigen in the secretory pathway and that its binding can effectively block the translocation of a membrane protein on cell surface.

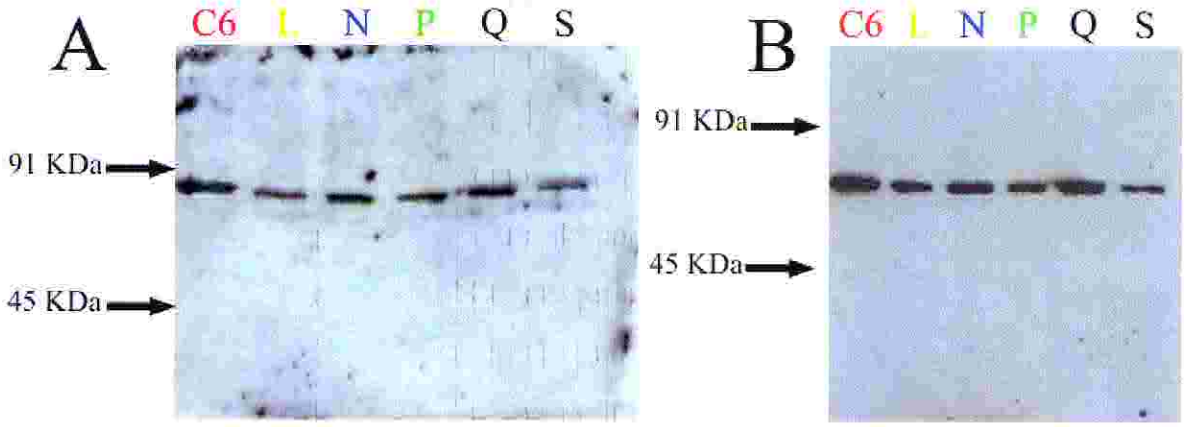

Fig. 3.19 Western blot of $p75^{NTR}$ and tubulin of untransfected and C6 clones

A: Western blot analysis to verify the $p75^{NTR}$ level of expression in C6 clones. 15 µl of undiluted cell extracts were loaded into the gel.

Primary antibody: MC 192

Secondary antibody: anti mouse HRP conjugated

B: Western blot analysis of the constitutively expressed protein tubulin C6 clones as a normalization control. 15 µl of undiluted cell extracts were loaded into the gel.

Primary antibody: YL 1/2

Secondary antibody: anti rat HRP conjugated

C6: untransfected cells

L, N, P, and S: αp75 stable transfections

Detection method: chemiluminescence (ECL)

From the intensity of the band it is possible to see that the amount of $p75^{NTR}$ does not change between C6 untransfected and stable transfected cells.

($p75^{NTR}$ MW ≅ 75kDa)

(Tubulin MW ≅ 55kDa)

3.5 Neurotrophin receptors phenotypic knock-out

The next question that had to be answered was if the alteration of NGF receptors presence on cell surface has some consequences on receptor signaling, or better, what are the consequences in transfected cells. In other words, can be scFvs used to block or reduce the function of membrane proteins?

To answer this question PC12 pheochromocytoma cells [Greene LA and Tischler AS, 1976] were transiently transfected with α p75, α TrkA and MNAC scFvs, all of them in fusion with GFP and with the KDEL retention signal. This cell line constitutively expresses both $p75^{NTR}$ and TrkA receptors and is known to activate and differentiate in neuronal cells in response to NGF incubations through the two receptors. Markers of activation are, among others, the increase of intermediate early genes c-fos and zif/egr1 in the nucleus and phosphorylation of MAPK a cytoplasmic protein downstream in NGF signal transduction cascade. On the other hand differentiation consists of a drastic change in morphology: the growth of dendritic extensions. These easily measurable changes were used to understand if transfections with anti-receptor scFvs are sufficient to produce significant alterations in NGF signal transduction pathway.

3.5.1 ScFvs inhibition of NGF-induced neurite outgrowth in PC12 cells

Transient transfections of PC12 cells were performed with Biolistic System (BIORAD, for details see chapter 2.3.3) with α p75 α TrkA and MNAC KDEL scFvs in fusion with GFP. PC12 cells were primed for 1 week with NGF, transiently transfected and, after 24 hours, re-plated in the presence of NGF. After 48 hour cells were fixed and analyzed. Neurite outgrowth was evaluated in cells expressing GFP scFvs and were considered differentiated when they showed dendritic extensions as long as one cell diameter or more. It will be referred as "differentiation" the visible changes in cell morphology knowing that actually primed cells have just differentiated. The percentage of differentiated cells was then calculated respect to the total number of transfected cells. R4 GFP with the KDEL signal was used to transfect PC12 in order to obtain a well-matched

negative control. Results are showed in picture 3.20 in which it can be distinguished between cells with and without neurite outgrowth. Cells transfected with α p75, α TrkA and MNAC scFvs (3.20 C, A and B) show the characteristic globular shape of undifferentiated PC12 cells, while the negative control (3.20 D) has a completely different morphology with clear neurite outgrowth. The percentage of transfected cells showing neurite outgrowth for the R4 negative control was found around 66%. Measuring in the same way the percentage of differentiation in transfected cells with α p75, α TrkA and MNAC scFvs, a strong and significantly ($p \leq 0,05$) reduction in the percentage of cells with neurite outgrowths was found (34%, 27%, and 18% of neurite outgrowth respectively). In graphic 3.20 E are schematized the results from all the experiments and the reduction in differentiation is clearly visible.

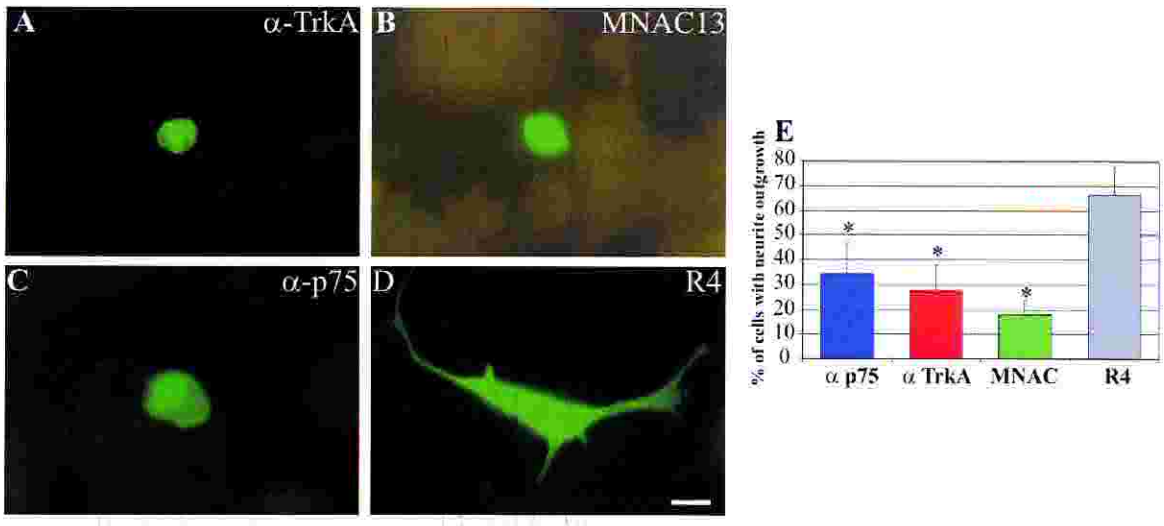

Fig. 3.20 ScFvs inhibition of NGF-induced neurite outgrowth in PC12 cells
PC12 cells were transfected with α TrkA (**A**), MNAC (**B**) and α p75 (**C**) scFvs-GFP KDEL and treated with NGF. Cells do not show neurite outgrowth, respect to cells transfected with R4 GFP KDEL (**D**) used as negative control.
In panel **E:** quantification of the percentage of transfected cells that show differentiation. Bars in indicate the mean percentage among at least three experiments. Error bars represent the standard deviations.
(∗): significant difference ($p \leq 0,05$) between scFvs transfected cells and the negative control.
Scale bar (shown in H): 10μm.

It seems clear that expression into ER of anti NGF receptors scFvs, leads to an inhibition of PC12 NGF induced differentiation, while expression into the same cellular compartment of a non relevant scFv does not affect cells neurite outgrowth.

To confirm this result it was decided to perform the same set of experiments transfecting PC12 cells with two other scFvs. It was chosen the well characterized anti p21Ras protein HH2 scFv, known to bind Ras protein when expressed into the cytoplasm and to neutralize its activity, and to inhibit 3T3 cell proliferation [Lener M et al., 2000]. HH2 scFv was selected from Vaughan library using purified p21Ras protein as a solid phase selector, and cloned in fusion with GFP into ScFvExpress plasmid cyto. Ras is a protein involved in signal transduction of Trk receptors, therefore, if its function is neutralized, differentiation of PC12 cells will be inhibited as in the previously described experiments. In this way it was possible to take advantage of a positive internal control for phenotypic knock-out experiments. At the same way, it was decided to exploit the neutralizing activity of an scFv against the same protein: the previously purified [Persic L et al., 1999] and characterized [Lener M et al., 2000] B5 anti p21 Ras. B5 was selected from a large phage display scFv library, against the peptides 57 to 76 and was cloned into ScFvExpress plasmid cyto in fusion with GFP.

PC12 cells were transfected, as described for the previous experiments, after one week of priming. The percentage of differentiated cells was calculated after 48 hours of NGF incubation. In this case an scFv expressed in the cytoplasm as negative control had to be used, therefore anti R4 GFP cyto was used together with GFP. The results, illustrated in picture 3.21, were very similar to the previous. PC12 cells transfected with anti Ras scFvs showed reduction in differentiation (Fig. 3.21 A and B) respect to the negative controls (Fig. 3.21 C and D). As schematized in graphic 3.21 E, 55% of cells transfected with R4 GFP cyto, and 60% of GFP transfected cells exhibited neurite outgrowth. On the contrary cells expressing both anti Ras scFvs showed only 25% of differentiated

cells, demonstrating a strong and significant ($p \leq 0,05$) reduction in percentage of differentiation.

In conclusion it was assessed the possibility to inhibit PC12 growth of dendritic extension induced by NGF by inactivation of NGF receptors, trapping them into the ER, and by knocking out proteins involved in its signal transduction.

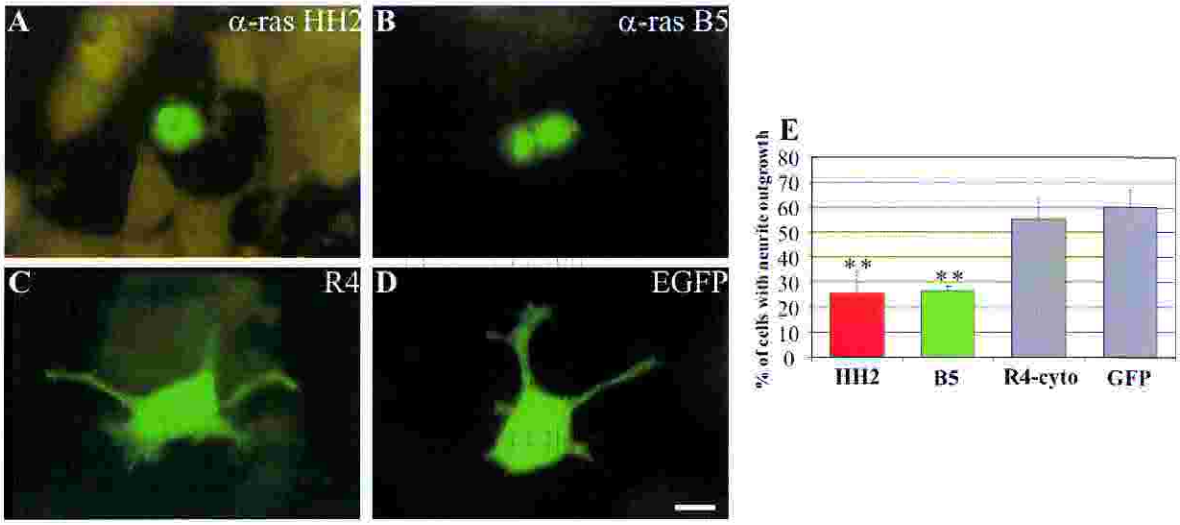

Fig. 3.21 Internal control of scFvs inhibition of NGF-induced neurite outgrowth in PC12 cells
PC12 cells were transfected with two anti Ras GFP scFvs (HH2 in **A** and B5 in **B**) with the cytoplasmic targeting signal in order to have a positive internal control for PC12 inhibition of neurite outgrowth.
Anti Ras transfected cells do not show neurite outgrowth. Cytoplasmatic EGFP and non relevant scFv R4 GFP cyto and GFP (**D** and **C**) were used as negative controls.
In panel **E**: quantification of the percentage of transfected cells that show differentiation respect to the two negative controls. Bars in indicate the mean percentage among at least three experiments. Error bars represent the standard deviations.
(**):significant difference ($p \leq 0,05$) between scFvs transfected cells and the negative control.
Scale bar in D: 10μm

3.5.2 ScFvs inhibition of NGF-induced activation in PC12 cells

PC12 incubation with NGF provokes, in addition of differentiation, activation of cells detectable by an increase of intermediate early genes c-fos and zif/egr1 into the nucleus and of cytoplasmic MAPK phosphorylation. These three proteins were therefore used as marker of activations in a series of experiments performed

as previously described. To detect the activated reporters, immunofluorescence assays were carried out using specific antibodies against c-fos and zif/egr1 proteins and against MAPK specifically in its activated state. Cells were transfected with the KDEL GFP plasmids carrying α TrkA, α p75, MNAC and R4 as a negative control. Transfected cells were identified by GFP expression and were then analyzed for the presence of intermediate early genes in the nucleus and for activated MAPK. In this way the percentage of activated cells among all transfected cells was calculated. It resulted, as described in table 3.1, that the three scFvs utilized were able to inhibit also PC12 activation, with a difference, respect to the negative control, statistically significant in many cases. It is also remarkable that the decreased level of the three activation markers in cell transfected with α p75 scFv is not so strong as for the other scFvs even if significantly different from the control. This could be an interesting result since it is not known if MAPK protein phosphorylation is linked to the signal transduction $p75^{NTR}$ pathway and whether the phosphorylation is a direct consequence of NGF/$p75^{NTR}$ interaction or not. In the same way these considerations can be valid also about c-fos and zif/egr1, since they are both proteins located extremely far downstream in the signal transduction pathway.

The results obtained from PC12 transfected with the two anti Ras scFvs, one of them indeed functional as a neutralizing scFv confirmed the validity of our approach. As described in table 3.2, the reduction in percentage of cells activated and transfected, respect to the negative controls (in this case R4 GFP cyto and GFP), was more evident even if not statistically significant in all the measured situations. In this and in other cases the lack of significance was probably due to a too low number of analyzed cells. In any case, the possibility to block the function of a cytoplasmic protein with a neutralizing intracellularly expressed scFv was confirmed by the results of transfection with the two anti Ras scFvs. On the other hand the reported results about the functional block of membrane proteins by they retention into the ER with specific scFvs, strongly support the feasibility of this approach.

Intracellular scFvs	Targeting sequence	% of activated cells		
		act MAPK	c-fos	egr1/zif
α TrkA	KDEL	39,5*	38*	41.5*
MNAC		32*	37*	36*
α p75		46.3*	41.7	40.3*
α β–gal R4		61.5	56.2	55

Table 3.1 ScFvs inhibition of NGF-induced activation in PC12 cells

Cells transfected as described in picture 3.20 were analyzed for the activation of MAPK and expression of c-fos and egr1/zif. MNAC, α TrkA, and α p75scFvs transfected cells showed a strong reduction in the percentage of NGF-activated cells with respect to R4 scFv negative controls. Activation was detected in immunofluorescence with three markers of activation: NGF-increased MAPK phosphorylation or c-fos and egr1/zif expression.

Numbers indicate the mean percentage of transfected cells with increased MAPK phosphorylation or c-fos and egr1/zif expression.

Primary antibodies: anti c-fos, anti egr1/zif, anti MAPK activated

Secondary antibodies: anti rabbit and anti mouse biotinylated; avidin TRITC

(*): significant difference respect R4 negative controls (P≤0.05).

Intracellular scFvs	Targeting sequence	% of activated cells		
		act MAPK	c-fos	egr1/zif
α ras HH2	Cytoplasm	29.3**	28**	36**
α ras B5		42**	36.7**	25.7**
α β–gal R4		57.8	56	52.2
GFP		65	56	57.3

Table 3.2 Internal control of scFvs inhibition of NGF-induced activation in PC12 cells

Cells transfected as described in picture 3.21 were analyzed for the activation of MAPK and expression of c-fos and egr1/zif in order to have a positive internal control for PC12 inhibition of activation. Anti Ras scFvs transfected cells showed a strong reduction in the percentage of NGF-activated cells with respect to R4 GFP and GFP negative controls.

Activation was detected in immunofluorescence with three markers of activation: NGF-increased MAPK phosphorylation or c-fos and egr1/zif expression.

Numbers indicate the mean percentage of transfected cells with increased MAPK phosphorylation or c-fos and egr1/zif expression.

Antibodies used: as described in Table 3.1

(**): significant difference respect R4 GFP and GFP negative controls (P≤0.05).

3.5.3 ScFvs inhibition of BDNF-induced signal transduction in hippocampal neurons

Hippocampal neurons in culture maintain the expression of their neurotrophin receptors as well as their ability to be activated by BDNF/TrkB interactions. In fact BDNF can induce, in these primary cultures, a cascade of receptor-triggered intracellular events, which mediate its diverse biological actions. As for PC12 cells the markers of activation are, among others, the phosphorylation of MAPK protein and the increased expression of c-fos and zif/egr1 into the nucleus.

The possibility to inhibit NGF signal transduction by blocking the activity of p21Ras protein in PC12 cell with specific scFvs was just described. Ras, together with MAPK, c-fos and zif/egr1, is a protein shared by all Trk receptors in the signal transduction cascade. Therefore the neutralizing ability of the two described anti Ras scFvs was exploited in hippocampal neurons to verify the power of phenotypic knock-out technique also in primary cultures.

The experiments were conducted in the same way as for PC12 cells. Neurons were transfected with HH2 and B5 GFP anti Ras scFvs and, as negative controls, with the non relevant anti R4 GFP scFv and with GFP alone. All of them were expressed into the cytoplasm. In order to avoid some neuron activation due to transfection procedure cellular stress (see chapter 3.2.3), cells were let express for 48 hours for a complete recovery. Markers of neuronal activation were detected, after 3 hours of BDNF incubation, with specific antibodies as previously described. The results are shown in figure 3.22. In panel 3.22 A and B there are the immunostaining pictures of untransfected neurons before (A) and after (B) BDNF treatment in which it is possible to see the increment of c-fos nuclear protein because of the activation. Hippocampal neurons expressing the two anti-Ras scFvs (Figure 3.22 C and 3E) do not show an increase in c-fos expression, following BDNF stimulation (Figure 3.22 D and 3.22 F, arrows indicate the scFv transfected cells). The difference is clear when c-fos immunostaining of transfected cells is compared to the one of neighboring untransfected cells. The increase in presence of nuclear c-fos is clearly visible in neurons transfected with

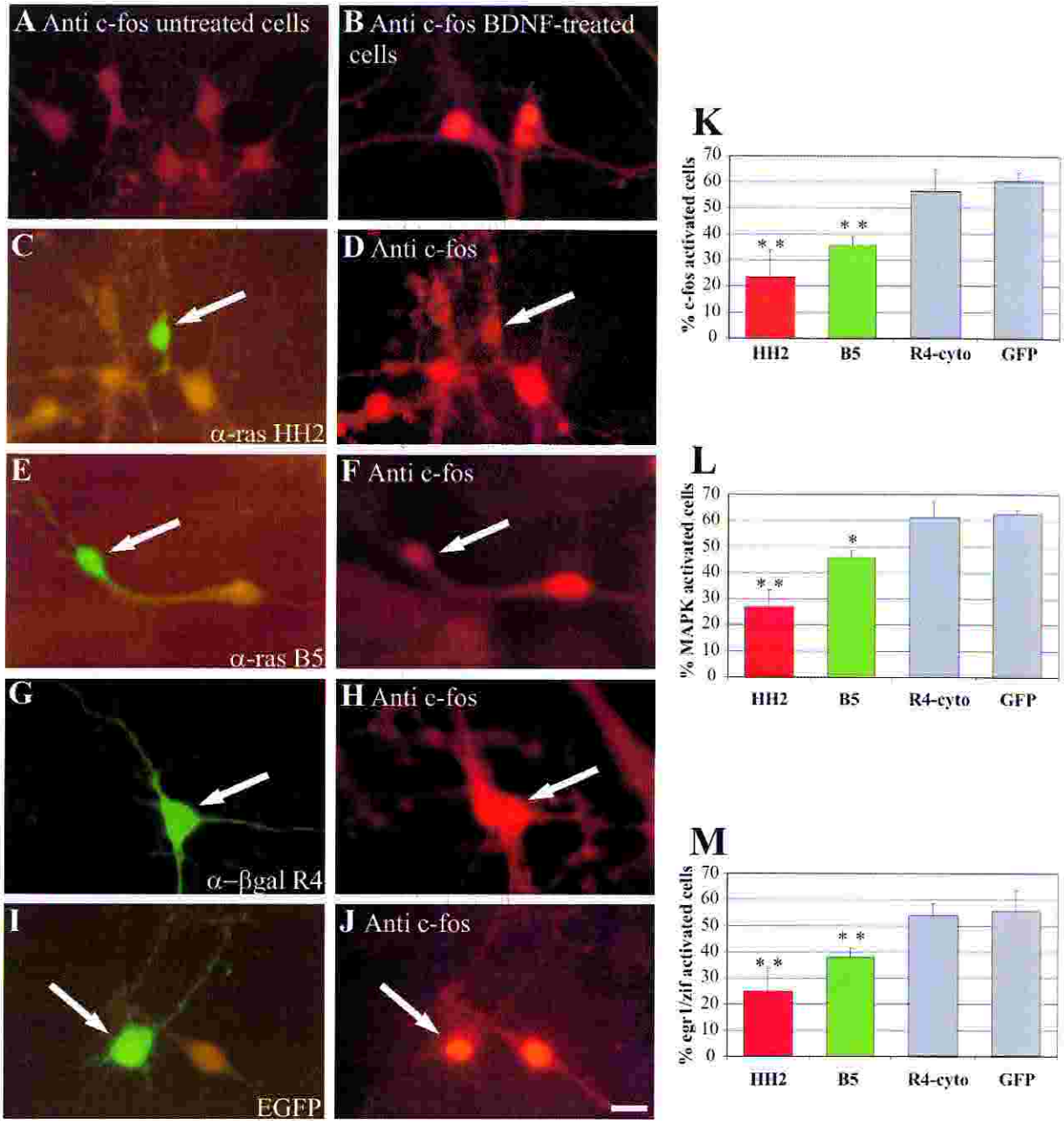

Fig. 3.22 Inhibition of BDNF-induced c-fos, egr1/zif expression and MAPK activation in neurons
Increased nuclear c-fos immunostaining in BDNF-stimulated neurons (**B**) respect to not stimulated cells (**A**). Hippocampal neurons were transfected with the two anti-Ras scFvs (**C** and **E**). Both scFvs transfected cells do not show an increase in c-fos staining (**D** and **F**) as observed for neighboring cells. The two controls, R4 scFv and GFP transfected cells (**G** and **I**), show an increase in c-fos immunostaining comparable with the increase observed for non transfected cells (**H** and **J**). Scale bar (shown in J): 15μm.
In panel **K**, **M** and **L**: percentage of transfected cells that showed increased c-fos, egr1/zif expression and MAPK activation respectively. Bars indicate the mean percentage of at least three experiments. Error bars represent standard deviations. The percentage of activated cells, cells that showed increase in expression of c-fos, egr1/zif and activation of MAPK, is significantly lower than the GFP and R4 GFP controls. Antibodies used: as described in Table 3.1
(**):significant difference ($p \leq 0,05$) between scFvs transfected cells and the negative controls.
(*):significant difference ($p \leq 0,05$) between B5 scFvs transfected cells and GFP negative control.

the controls R4 GFP (3.22 G and H) and GFP (3.22 I and J) that do not show differences respect to neighboring untransfected cells. In graphic 3.22 K there are the quantifications of these results in which it can be appreciated that the scFvs-dependent decreased presence of c-fos protein is statistically significant. Similar results were obtained when it was investigated the increase of zif/egr1 protein (3.22 M) and MAPK phosphorylation (3.22 L).

Taken together, these results demonstrate that both HH2 and B5 anti Ras scFvs expressed in primary cultured neuronal cells are able to interfere effectively with BDNF induced signal transduction, confirming that phenotypic knock-out is a effective technique.

3.6 $p75^{NTR}$ and Trk receptors interactions

It is now widely accepted that Trk and $p75^{NTR}$ receptors form, when expressed by the same cell, a complex that can be immunoprecipitated [Bibel M et al., 1999], and that other proteins can be involved in the formation of this complex [Kong H et al., 2001]. The results obtained with α p75 transfection suggested to investigate about the Trk/$p75^{NTR}$ receptors complex from another point of view. In fact the inhibition of activation induced in PC12 cells by α p75 scFv was not so strong such the one induced by the two α TrkA scFvs. The difference, as discussed, could be due to the fact that the markers for activation utilized (act. MAPK, c-fos and zif/egr1) are probably not directly part of the $p75^{NTR}$ main signal transduction cascade. Considering a probable TrkA/$p75^{NTR}$ complex in PC12 cells, another intriguing explanation emerges. If the TrkA/$p75^{NTR}$ complex forms passing through the secretory pathway, it may be possible that while α p75 blocks its counterpart into the ER, TrkA receptor could be consequently trapped. The inhibition of the activation in α p75 transfected PC12 cells could therefore due to a block of NGF/TrkA-dependent activation, as in α TrkA transfected cells. The minor efficiency in phenotypic knock-out could derive from the fact that not all receptors form the complex inside the secretory pathway.

3.6.1 Trk/$p75^{NTR}$ complex in C6 cells

The above hypothesis was confirmed by the results obtained with a C6 clone stably transfected with α TrkA scFv in fusion with KDEL retention signals. The selection of this clone, that constitutively expresses α TrkA scFv, was initially meant to provide a negative control for the α p75 C6 clones experiments (see chapter 3.4), since C6 cells do not express TrkA receptor [Colangelo AM et al., 1994]. When the α TrkA C6 clone was analyzed by FACS, in order to see the amount of $p75^{NTR}$ on cell surface, the results were unexpected. In fact, as shown in figure 3.23, α TrkA KDEL expressed in C6 cells seems to slightly block the appearance of $p75^{NTR}$ on membrane. The reduction in fluorescence is lower for

the α TrkA clone with respect to all the α p75 clones but anyway noticeable (see also picture3.18). To explain this behavior some considerations need to be done. It is known that C6 cells express, together with $p75^{NTR}$ also TrkB receptor [Pantazis NJ et al., 2000]. Since these receptors all share a similar structure it is possible that α TrkA scFv cross-reacts also with the other Trk family receptors. The possible complex formation into secretory pathway between $p75^{NTR}$ and Trk receptors may allow TrkB C6 receptors, that are retained into the ER through α TrkA scFv, could block also $p75^{NTR}$ translocation, explaining its reduced presence on the membrane surface.

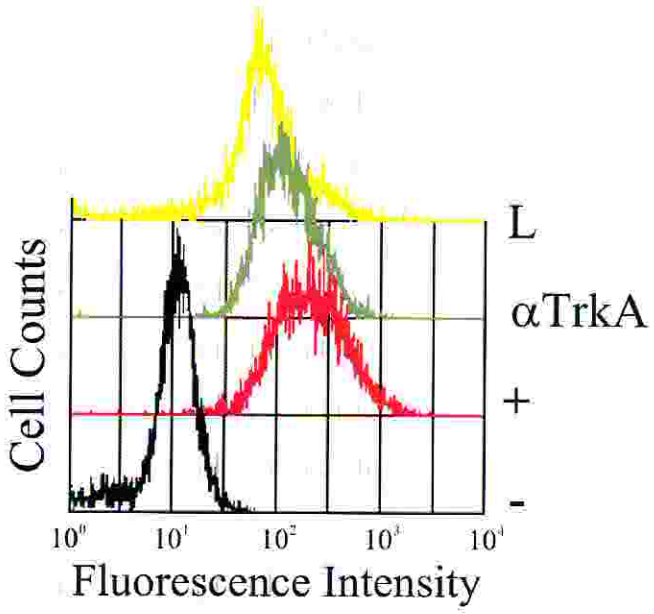

Fig. 3.23 FACS analysis of C6 clone expressing α TrkA
The α TrkA KDEL C6 clone was immunostained as described in figure 3.16 and analyzed by FACS. The result was compared with data of clone L (yellow trace), positive (+) and negative (–) control.
x axis: fluorescence intensity (FL1-H at 488 nm)
y axis: number of events (10.000 per each experiment)

These results, even if intriguing, left FACS analyses on C6 transfected cells without a proper negative control that is very important, since a scFv trapped into the ER can in some way interfere with protein translocation towards the membrane surface.

3.6.2 TrkB/p75^{NTR} complex in hippocampal neurons

Other confirmations on the existence of p75^{NTR} and Trk complexes came from experiments conducted with hippocampal neurons that express both TrkB and p75^{NTR} receptors. Furthermore the receptors complex formation into the secretory pathway suggests the possibility to inhibit the BDNF induced activation of TrkB receptor by blocking the traffic of p75^{NTR}. Thus, the efficacy of BDNF signaling was tested in hippocampal neurons expressing α p75 scFv retained in the ER. Neurons were transfected with α p75 GFP KDEL and with R4 GFP KDEL as a negative control. After BDNF stimulation the activation of neurons was measured as previously described. All the results are reported in picture 3.24. Neurons expressing α p75 (3.24 A, E and I) do not show phosphorylation of MAPK (3.24 B), or increasing expression of c-fos and zif/egr1 nuclear proteins (3.24 F and J) in contrast with their neighboring untransfected cells. Clear evidences of activation (3.24 D, H and L) are instead visible in neurons transfected with R4 GFP scFv (3.24 C, G and K). The overall measured percentages are plotted in graphic 3.24 M, showing a limited but significant reduction in the number of BDNF activated cells when transfected with α p75 scFv.

Taken together the results reported in chapters 3.5 and 3.7 not only demonstrate that the phenotypic knock-out using scFvs is a powerful technique usable with proteins of different cellular compartments and with several type of cells, but also that it can be used to obtain a lot of interesting information about the studied protein.

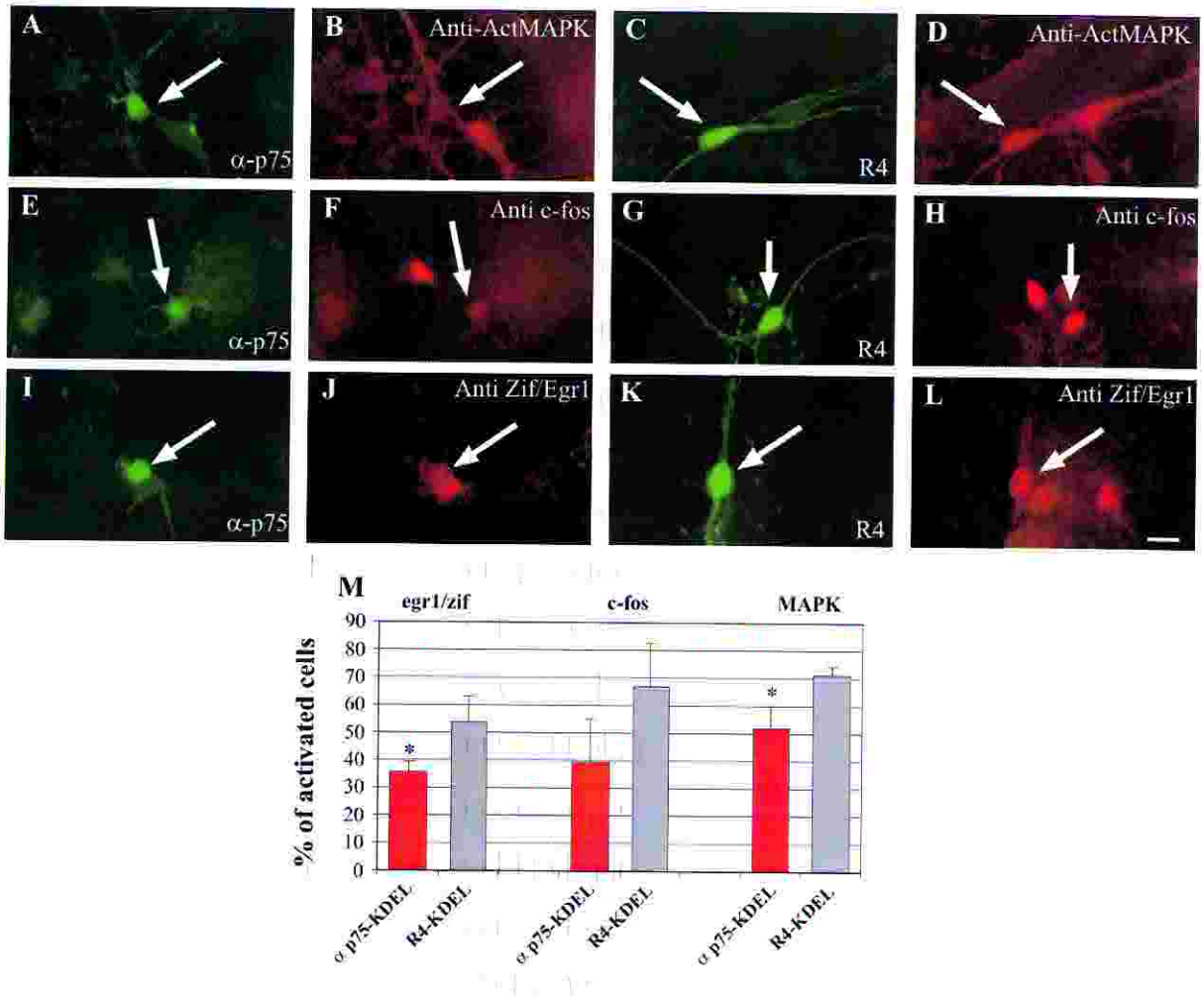

Fig. 3.24 αp75 KDEL scFv inhibition of neurons activation

Hippocampal neurons were transfected with α p75 and R4 GFP scFvs carrying the KDEL retention signal. α p75 scFv transfected cells (A, E and I arrows) do not show an increase in activated MAPK, c-fos and egr1/zif staining (B, F and J), as observed for neighboring cells. R4 GFP transfected cells (C, G and K) show activation comparable with the activation observed in non transfected cells (D, H and L).

Scale bar (shown in F): 15μm.

The graphic in panel M represent the quantification of the percentage of transfected cells that showed increased markers expression. Bars indicate the mean percentage among at least three experiments, while the error bars represent the standard deviations. The decrease in α p75 transfected neurons was probably due to a complex of $p75^{NTR}$ and TrkB receptors.

(∗):significant difference ($p \leq 0,05$) between αp75 transfected cells and R4 GFP negative control.

3.7 Design and construction of new transgenes

Recently the phenotypic knock-out (pKO) technique led to new and very interesting results. In fact the adult transgenic mouse expressing a secreted antibody against NGF (α D11), able to interfere with NGF/TrkA interactions, [Ruberti F et al., 2000], showed Alzheimer-like neurodegeneration [for more details of mice phenotype see Capsoni S et al., 2000]. The production of this mouse (the AD11 mouse) is a very important result for several reasons, first of all for the pharmacological implications. In fact the availability of an animal model for Alzheimer disease is essential to test new drugs as possible therapy. Moreover AD11 mouse can be seen as a new approach towards the understanding of the causes of Alzheimer disease. For this reason it was decided to prepare DNA constructs of the antibodies characterized in this work in order to block NGF/TrkA interactions and verify if, when used as transgenes, could lead to Alzheimer-like phenotype in adult transgenic mice. These constructs were specifically designed to allow the production of several new transgenic mice lines. To produce the AD11 transgenic mouse the variable regions of light and heavy chains of α D11 antibody were linked to human κ and γ 1 chains and placed under control of the human cytomegalovirus (CMV) promoter. Mice expressing functional α D11 antibodies were obtained by crossing mice lines expressing the heavy chain with mice expressing the light chain.

The first approach developed is aimed to reproduce a similar phenotype to the AD11 mice, by blocking the NGF/TrkA interactions using the neutralizing properties of MNAC antibody. In this way it was thought to mimic the effect of α D11 blocking the TrkA receptor instead of NGF itself. Therefore it was decided to prepare DNA constructs from MNAC in the same way as done for AD11, obtaining the chimeric human/mice antibody under control of CMV. To provide a suitable negative control it was chosen to obtain similar constructs with α NP antibody that recognize the antigen 4-hydroxy-3-nitrophenacetyl. These new constructs were then analyzed for their possibility to be expressed in eukaryotic cells. COS cells were co-transfected with DNA of the heavy and light chain and

the expression of the whole functional antibody were detected by immunofluorescence and by ELISA assay of cellular supernatants. The results of ELISA are schematized in picture 3.25 were it is clear that both the antibodies can be expressed, folded and secreted in a functional form. The results obtained with cell transfected only with the heavy or the light chain, used as negative controls, allow to verify that the antibodies are able to recognize the antigens only when both the light and heavy chain are expressed and secreted together.

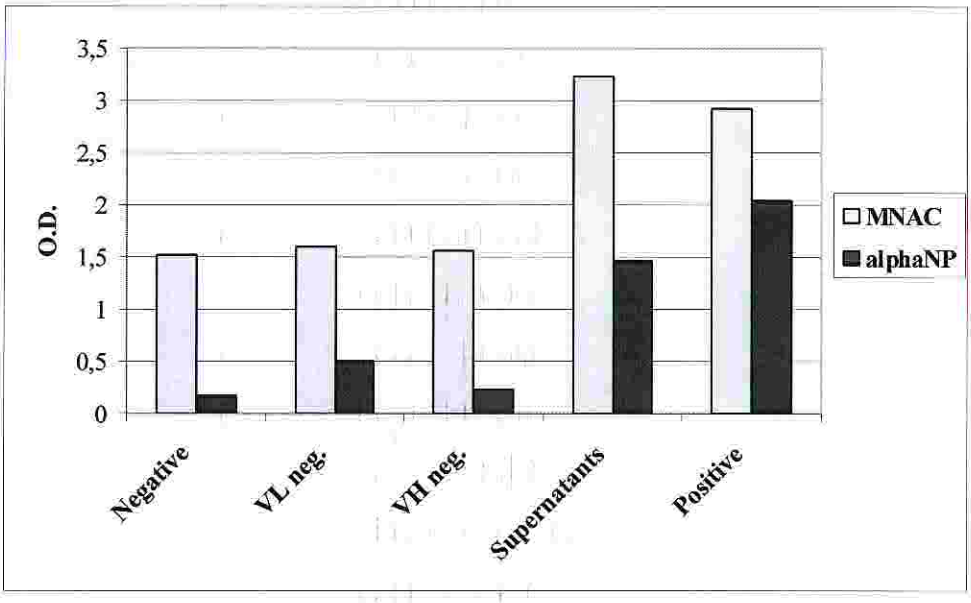

Fig. 3.25 ELISA of supernatants of co-transfected COS cells

COS cells were co-transfected with plasmids carrying the light and the heavy chains of the MNAC and anti NP chimeric antibodies. The supernatants of transfected cells were analyzed in ELISA assay. The antibodies are able to recognize their antigen only when both the light and heavy chain are expressed and secreted together.
Coating: NP-BSA; TrkA immunoadhesin
Negative controls: supernatants of untransfected cells.
Primary antibody: supernatants of transfected cells.
Secondary antibody: anti human γ 1 chain.
Tertiary antibody: anti goat HRP.
Light and heavy chains negative controls: cell transfected either with heavy or light chain. Anti γ 1 human chain and anti κ human chains respectively were used as primary antibodies.
Positive control MNAC: MNAC monoclonal antibody purified as primary antibody. Anti mouse HRP as secondary antibody.
Positive control α NP: sera from immunized mice as primary antibody. Anti mouse HRP as secondary antibody.
Detection method: enzymatic reaction of TMB.

Since the use of simpler form of antibodies, such as scFvs fragments, offers several advantages with respect to the expression of the whole antibody, it was decided to prepare the DNA of these two antibodies also in the form of secreted scFvs. Variable light and heavy chains of MNAC and α NP were linked and cloned into vectors carrying the CMV promoter and the secretory protein signal. These DNA were analyzed in COS cells as described before by ELISA and immunofluorescence (data not shown).

The introduction of scFvs to prepare transgenic mice allows the possibility to exploit the scFvs with KDEL signals to perform other pKO approaches made possible by the work described in this thesis by applications on animals models. Therefore α TrkA, α p75, and α NP, as negative control, scFvs were cloned in vectors containing CMV promoter and the KDEL retention signal in order to inhibit the TrkA and $p75^{NTR}$ signal transduction pathway by their retention within the ER. All the DNA constructs prepared for future transgenic mice are summarized in picture 3.26.

The antibody DNAs prepared as described, were cut away from the plasmid obtaining a fragment starting from CMV promoter and ending with the polyadenilation signal. The purified fragments are now ready to be inserted within genome of mice.

In order to have good tools for mice screening for the presence of the transgenes, a pair of primers were designed for each construct. These primers were used in PCR using genomic mice DNA extract as templates in order to verify their specificity and thus the possibility to use them for screening procedure. In picture 3.27 are shown two examples of PCR results: the amplifications of α NP heavy and light chain constructs. The other primer couples gave all comparable results (data not shown). The primers resulted suitable since they can trigger amplification of antibody DNA without amplifying any other unspecific DNA sequences present in the mice genome.

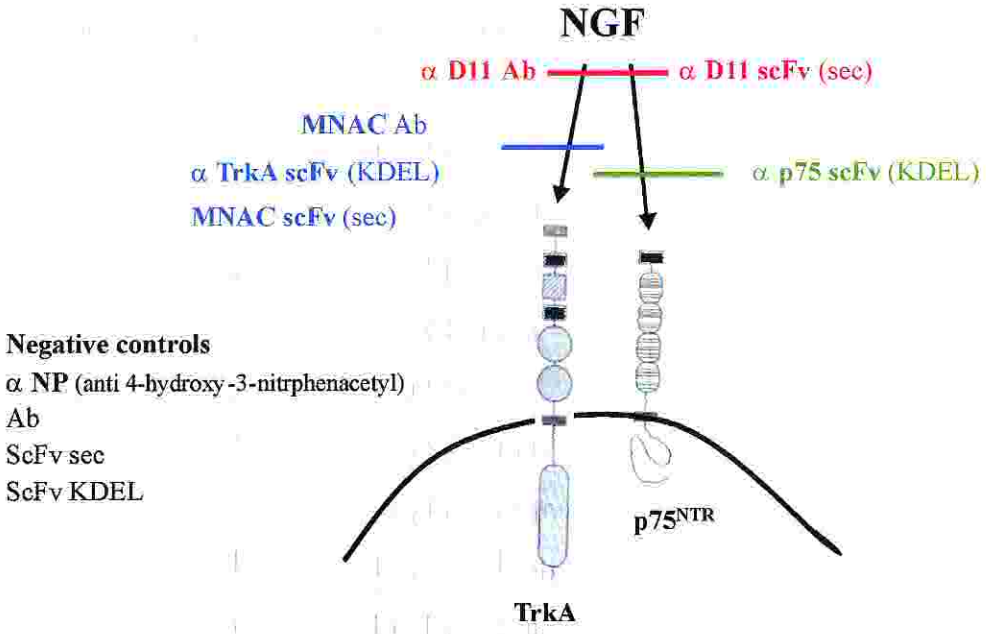

Fig. 3.26 Constructs for transgenic mice

Schematic picture that shows all the constructs prepared as transgenes. In addition to αD11 antibody (Ab; light and heavy chains) [Ruberti F et al., 2000] it was decided to use the scFv fragment of αD11 in order to block NGF/TrkA interactions. In the same way the secreted neutralizing Ab MNAC was thought to block this interaction both in form of a complete antibody and in form of a scFv sec fragment. NGF/TrkA interactions may be also prevented by blocking the TrkA receptor within the ER with α TrkA scFv KDEL. The same can be done to inhibit the signal transduction of $p75^{NTR}$ with α p75 KDEL scFv. As a negative control α NP antibody has been chosen and prepared in form of secreted Ab and scFv fragment both with sec and KDEL signals.

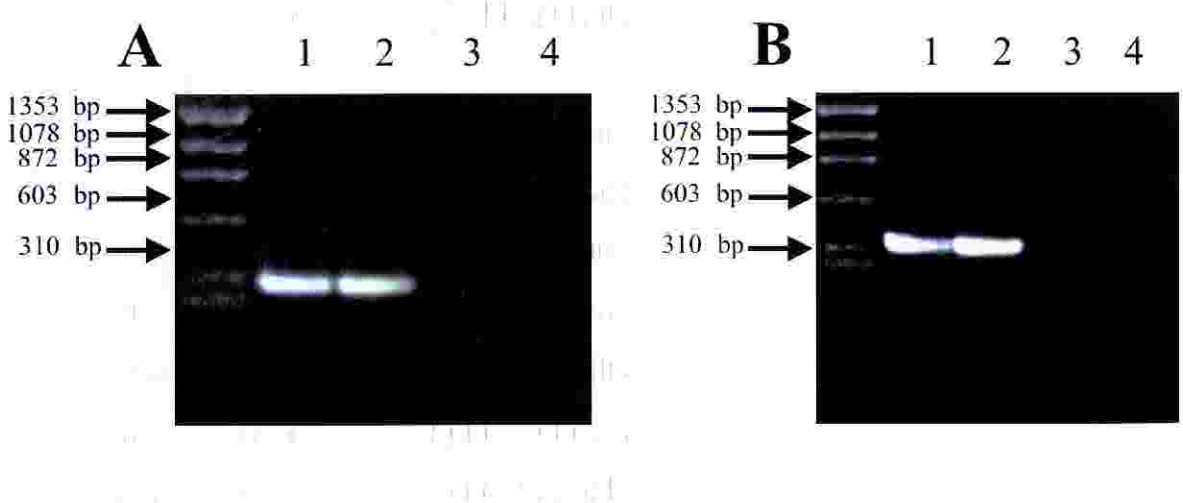

Fig. 3.27 PCR analysis to detect the presence of the transgenes

The designed primers were used to amplify the antibody DNA and were tested primers specificity using genomic mice DNA extracts as templates.

A: αNP VL chain PCR

B: αNP VH chain PCR

1) Ab DNA cut from vector **2**) Entire vector **3**) genomic DNA extracted from mice tail **4**) negative control

Chapter 4

Discussion

4.1 ScFv targeting in neuronal cells

The phenotypic knock-out (pKO) is a helpful technique to inhibit or study the function of a given protein by intracellular expression of scFvs. To exploit, in an efficient way, the pKO it may be advantageous to target scFvs to the subcellular compartment of interest by expressing them in combination with specific intracellular targeting signals. First of all it was necessary to verify that the expressed scFvs could indeed reach the compartment where they were targeted in order to find the antigen of interest.

An anti β-Galactosidase (R4), an scFv known to correctly fold when expressed intracellularly [Martineau P et al., 1998], was expressed in fusion with GFP and targeted to different compartments inside the cell. GFP allowed facile visualization of scFv fate after the expression within living and in fixed cells, and did not have an apparent effect on intracellular sorting. The R4 scFv was put in fusion with GFP and with four different protein targeting signals: cytoplasmic (cyto), nuclear (NLS), endoplasmic reticulum (KDEL) and secretory pathway (sec), by the aid of the ScFvExpress plasmids [Persic L et al., 1997a].

The nuclear distribution of an NLS scFv has already been demonstrated in COS cells by immunofluorescence [Persic L et al., 1997a]; here the NLS localization signal is shown to target R4 GFP in living neuronal cells by direct visualization of the fluorescent tag. In fact, neurons transfected with R4 GFP NLS showed GFP fluorescence restricted to the nucleus. No other fluorescence was identified inside the cell indicating that all detectable GFP was correctly transported within the nucleus. It is likely that the time required to import nascent polypeptides is less than the time needed by the R4 GFP NLS to fold and become fluorescent.

In the same way it was demonstrated, by direct visualization of GFP tagged R4, the cytoplasmic scFv distribution in neurons. Cytoplasmic scFv distribution was previously observed in COS cells by immunofluorescent assays [Persic L et al., 1997a]. The patterns obtained by expressing scFvs in the cytoplasm were complex. Together with a diffuse cytoplasmic distribution, granular and"doughnut-like" distributions due to aggregates of scFvs were also found. It is known that the solubility of cytoplasmic scFvs is very heterogeneous and that the formation of aggregates is a frequent event independent of the presence of the antigen [Cattaneo A and Biocca S, 1999; Cardinale A et al., 2001]. It has been demonstrated that α p21Ras, an scFv selected to recognize and bind p21Ras protein, showed aggregate formation when expressed in the cytoplasm. Nevertheless, α p21Ras remained able to bind its antigen, since Ras protein was found within the aggregates [Cardinale A et al., 1998]. This antigen sequestration by the α p21Ras scFv led to an efficient inhibition of Ras function. The aggregate structures have been found to be highly similar to aggresomes [Kopito RR, 2000] and the scFv was ubiquitinated and degraded [Cardinale A et al., 2001]. The recent Intracellular Antibody Capture Technology (IACT) [Visintin M et al., 2002] is a yeast two-hybrid approach, which addresses the problem. In fact, IACT is a genetic screen *in vivo* for scFvs that correctly fold in the cytoplasm and that can be transported into the nucleus for the transactivation of the reporter gene.

Since targeted scFvs in neurons have not been previously observed, R4 GFP cyto was used, because it does not form any kind of aggregate in other cell types studied. R4 GFP with the cytoplasmic signal was found throughout the cell with a homogeneous distribution. No clusters of scFv aggregation were found. In this way it was demonstrated that neuronal intracellular environment is not sufficient *per se* to alter the pattern of scFv expression.

When GFP alone is expressed in cells, it is present in the cytoplasm, but accumulates in the nucleus. This is likely due to the small molecular weight of GFP, which should be able to freely diffuse into the nucleus, since it is less than the 40kD threshold [Izaurralde E et al., 1997]. Neurons and fibroblasts transfected

with R4 GFP cyto show a bright diffuse fluorescence all over the cell body, and a less marked fluorescence within the nucleus, as compared to GFP alone. The difference between the amount of GFP and R4 GFP cyto in the nucleus is most likely due to the increase in size (27kD vs. 54kD), but may also be due to specific interactions that trap GFP inside the nucleus. It is possible that the presence of the scFv hinders the crossing of the nuclear pore by the R4 GFP, or that some R4 sequences may have affinity for cytoplasmic structures. Nevertheless it appears that the cytoplasmic R4 GFP is freely diffusible, since it was uniformly distributed in the cell body and was detectible even in the most distal part of dendrites.

Until now the secretion of scFvs and their retention within the ER were mainly studied from a biochemical point of view. Some studies were conducted using metabolic pulse/chase labeling to follow expression and secretion of newly synthesized scFvs [e.g. Jost CR et al., 1994]. In this way it was demonstrated that, after 2 hours from the pulse labeling, an scFv with a secretory leader sequence was present in the culture supernatant, while a KDEL scFv remained within the cell. Experiments by ELISA with solid-phase bound antigen, performed using both the culture supernatants and cell extracts, showed the presence of scFvs inside the cell (KDEL) or in the culture medium (sec). In addition, the fact that the scFvs were able to interact specifically with the antigen showed that some correct folding of the scFvs had occurred [Biocca S et al., 1995]. In this thesis, the ability to reach the extracellular environment and to correctly bind the specific antigen after secretion was shown for all the scFvs used with a secretory leader sequence by ELISA experiments. The same kind of experiments demonstrated that KDEL scFvs were retained in the cells and, if extracted, possessed the ability to recognize the antigen.

In addition to this evidence, within this thesis further investigations have been conducted in order to demonstrate, with cellular biology methodologies, the correct scFv localization. Colocalization experiments and specific alterations of organelle function were exploited to confirm the working hypothesis.

The presence of the scFv sec within the secretory pathway was examined by exploiting the block at 18°C of the exit from the *trans*-Golgi network. Traffic of expressed R4 GFP sec towards the plasma membrane was blocked by incubations at 18°C leading to a redistribution of the fluorescence. In this way the accumulation of R4 GFP sec within the *trans*-Golgi network was verified, as expected for a secreted protein, whereas no similar redistribution was seen for the R4 GFP cyto or R4 GFP KDEL. This evidence together with the expression pattern showed by R4 GFP sec expressing neurons (a perinuclear and vesicular GFP distribution) and to the results from ELISA experiments, demonstrates the correct localization and folding of a secreted scFv. The possibility to use an scFv sec to bind, for example, the extracellular domain of membrane proteins, or molecules located outside the cell was here confirmed.

Concerning the expression and localization of KDEL scFvs, the colocalization in transfected neurons of R4 GFP KDEL scFv and a specific dye for the ER was demonstrated. Furthermore, it was established that the local distribution of R4 GFP KDEL changes following the same pattern and time scale as known for ER. This particular movement within neurons was sensitive to nocodazole as are all the structures that depend on microtubules and microtubules-motors [e.g. Waterman-Storer CM and Salmon ED, 1998]. The data collected regarding the spatial distribution, the dynamics and the pharmacological sensitivity of dynamics of R4 GFP KDEL, are consistent with an ER distribution of the scFv. A new fluorescent Nissl staining was also exploited. Although no precise information exists on the nature of Nissl substance, it is widely accepted that Nissl bodies correspond to ribosomes associated with the endoplasmic reticulum (together referred to as rough ER, RER) more present in neurons, because of their high synthetic activity [Nievel JG and Cumings JN, 1967]. From colocalization experiments of the expressed R4 GFP KDEL and the red fluorescent Nissl staining it can be easily seen that all the structures stained in red also contain GFP. There are some structures clearly containing GFP but not labeled with Nissl stain. Considering green structures as general ER marked by GFP and red structures as RER, one must conclude that R4 GFP KDEL accumulates both in RER and in

smooth ER (SER, without ribosomes). For the first time, the precise localization of an scFv KDEL was determined. The principle use foreseen for ER-trapped scFvs in pKO studies is to block the appearance of membrane proteins on cell surface. The subcellular compartment in which this scFv-antigen recognition occurs is mostly likely the RER, where the membrane proteins are synthesized. The presence of a large amount of scFv KDEL within the SER results in lower concentration of scFvs in the RER, and, therefore, in a reduced probability of finding and binding an antigen of interest present within RER. If the local concentration of scFv is a critical step of pKO, a lower effectiveness of pKO would be predicted. Actually, the high expression level of scFvs with strong promoters such as CMV and EF-BOS is enough to overcome this problem. Moreover, the presence of the scFv in the SER raises the possibility of blocking membrane proteins that are normally located within the SER. Since the KDEL receptor is present in the *cis*-Golgi membranes, it should be also possible to reach and inhibit the function of *cis*-Golgi located proteins, or proteins that recirculate from Golgi to ER.

The results on ER targeting presented, taken together with the evidence that expressed and extracted KDEL scFvs from transfected cells can bind the specific antigen, demonstrate that a given scFv can be expressed, targeted and trapped within the ER of neurons where it may fold correctly and maintain its ability to bind the antigen of interest.

The experiments on visualization of intracellularly targeted R4 GFP KDEL in neurons leads to some interesting considerations. In fact, cells transfected with R4 GFP KDEL showed a clear and strong fluorescence until the very distal tip of dendrites. Since with previous experiments it has been demonstrated that the R4 GFP KDEL localized within the ER, it can be concluded that a large amount of ER is present along all the dendrites. The existence of some SER and RER membranes within the dendrites has been previously demonstrated. In particular, dendritic SER membranes were visualized by electron microscopy [Cooney JR et al., 2002]; serial ultra-thin section of hippocampal neurons were used to compute a three dimensional reconstruction of the membrane structures within dendrites. In

this way the dendritic presence of a continuous SER was clearly outlined. On the other hand presence of discontinuous RER in proximal dendrites was detected by immunofluorescence for specific RER proteins in magnocellular and spinal cord neurons [Ma D and Morris JF, 2002; Gardiol A et al., 1999]. The activity of glycosyltransferase (mannose), characteristic of the RER within proximal hippocampal dendrites, was visualized [Torre ER and Steward O, 1996]. All these results were confirmed by fluorescent detection of R4 GFP KDEL. In the past synapse-associated polyribosomes complexes (SPRCs), that are positioned beneath post-synaptic sites and in rare clusters within the dendritic shaft, were described [reviewed by Steward O and Schuman EM, 2001]. SPRCs are membranous organelles with a RER-like configuration. The fluorescence of R4 GFP KDEL did not permit visualization of that kind of structure, likely because R4 GFP KDEL labels a large and often continuous structure which includes both the RER and SER. In fact, the fluorescent Nissl staining of the RER appears discontinuous and similar to the RER markers that were used in the publications cited above, except for the greater extent of labeling by both R4 GFP KDEL and Nissl stain in the distal dendrites in this thesis. The time-lapse series of images of GFP marked ER in living neurons illustrate the microtubule-associated movement of membranes in dendrites, as in movie D. *In situ* hybridization of neurons transfected with R4 GFP KDEL (data not shown) clearly demonstrate that the mRNA of R4 GFP KDEL restricted to the cell body. Since the protein is inside the ER and present within distal dendrites, while the RNA remains in the cell body, it seems plausible that ER membranes containing GFP can move through the dendrite shaft. The very noticeable movement reported in movie D confirms this hypothesis. The R4 GFP KDEL can be a useful tool to study the dynamics and regulation of dendritic ER transport. In particular it is possible to interfere with ER function by incubation with brefeldin A, which blocks protein transport from ER to Golgi, and with nocodazole, which interferes with microtubules associated movement. Following the recovery of R4 GFP KDEL transfected cells after brefeldin A and nocodazole treatment, it should be possible to visualize the re-establishment of the entire dendritic ER network.

These considerations are strictly correlated with the possibility of protein synthesis within dendritic processes. There is some evidence of protein synthesis in isolated dendrites [Aakalu G et al., 2001] and glycosylation of proteins, both in intact neurons and separated dendrites, has been already shown [Torre ER and Steward O, 1996]. In addition to the protein synthetic machinery needed for cytoplasmic proteins, the synthesis of membrane and secretory proteins in dendrites requires ER and Golgi components for protein processing and sorting.

Unexpectedly, neurons transfected with R4 GFP sec were heavily labeled only in the proximal dendrite. The extensive dendritic ER labeled by R4 GFP KDEL was not evident. Unlike R4 GFP KDEL, R4 GFP sec does not accumulate in the ER. The total ER staining by R4 GFP sec is a balance between the rate of synthesis and folding of GFP and the rate of export to the Golgi apparatus. It is possible that a high rate of cytoplasmic protein synthesis and secretion did not allow the detection of Golgi apparatus or vesicular compartments within the small dendritic compartment by confocal microscopy. With a specific block of the secretory pathway, not so damaging for neurons as incubation at 18°C, R4 GFP sec may accumulate within the Golgi. It is formally possible that a *cis*-acting RNA targeting sequence, absent from R4 GFP sec, is required for dendritic synthesis of secreted or membrane proteins.

There are many theoretical advantages to local protein synthesis in dendrites of neurons. It could be a mechanism exploited to quickly produce proteins for local synapse-specific modification in cases of long-term modification and long-lasting plasticity, or in response to synaptic activity. Since the ER also represents the principal intracellular calcium store, the presence of a dynamic ER at sub-synaptic sites raise the possibility that the protein synthetic machinery and a major secondary messenger of neuronal signal transduction are coordinately regulated.

4.2 mRNA targeting in neuronal cells

The principal targets of this work are neurons that can extend their processes for hundreds of microns. The known protein targeting signals alone may not be sufficient to allow an scFv to reach the very distal parts of dendrites. In particular, diffusion is probably not sufficient for proteins with a high molecular weight. The observations that GFP and R4 GFP are able to diffuse until the distal dendrites are probably not extendable to other proteins. Therefore, the use of specific mRNA targeting elements has been explored in order to reach particular subcellular compartments, such as distal dendrites in neurons. Several reasons justify the choice to explore and to set up the mRNA targeting technique. A specific dendritic targeting element could be used to concentrate the scFv in an area of interest. In this way any eventual toxicity provoked by the expression of scFv all over the cell could be reduced. Moreover, a more profound knowledge about the sorting and targeting of mRNA may lead to more effective strategies for targeting scFvs by combining RNA and protein targeting elements.

Three different RNA sequences were compared in order to determine the most advantageous conditions for neuronal expression: the hnRNPA2 Responsive Element (A2RE) for transport of Myelin Basic Protein (MBP) mRNA into oligodendrocyte processes [Ainger K et al., 1997; Munro TP et al., 1999]; the 3'UTR of Ca^{2+}- calmodulin-dependent protein kinase II α (CaMKIIα) that regulates CaMKIIα mRNA trafficking [Mayford M et al., 1996; Miller S et al., 2002]; and the first 94 nucleotides of the CaMKIIα UTR [Mori Y et al., 2000], which is referred to, in this thesis, as the dendritic targeting element (DTE).

The A2RE was identified as a 21-nucleotide sequence in the 3'UTR of MBP mRNA essential for transport of the transcript into oligodendrocytes processes. It is well characterized and small enough to be easily handled for subcloning strategies. Moreover, it was demonstrated in oligodendrocytes, neuroblastoma B104 and CHO cells [Kwon S et al., 1999], that the A2RE functions as a translational enhancer. The experiments in this thesis on transfected neurons are entirely consistent with these considerations, since the GFP detected in A2RE-

GFP or A2RE-R4 cyto transfected cells is present in noticeably greater amounts. For these reasons the A2RE was chosen for the first trials on RNA targeting in neurons. In this way it was also possible to examine whether an oligodendrocyte RNA transport element can function in neurons.

The experiments used to define the A2RE were conducted by microinjection of RNAs into cultured oligodendrocytes [Ainger K et al., 1997]. In this thesis a different approach was attempted in order to verify if the RNA targeting element was able to dendritically localize mRNAs in neurons. The A2RE was placed at 3' of a reporter gene, GFP, and of the R4 GFP cyto. Neurons were transfected with the constructs and the presence of the mRNA within dendrites was detected by *in situ* hybridization. The amount of RNA present within dendrites was calculated by measuring the extent of dendritic staining of transfected neurons. The average measurement of each experiment was compared with the same construct without any RNA targeting element. Comparison of the mRNA of GFP in dendrites with the mRNA of A2RE-GFP demonstrated the effectiveness of A2RE as a targeting element in neurons. In fact A2RE-GFP mRNA is present significantly more distally within dendrites with respect to GFP alone. When the extent of dendritic staining of A2RE-R4 cyto mRNA was compared with the same construct without the targeting element, no effect of the A2RE was evident. The mRNAs of both constructs were restricted to the most proximal part of dendrites and no significant differences were detectable between R4 GFP with or without the targeting element. The system used for these experiments, hippocampal neurons in culture, is extremely complex. In the same preparation a lot of different cell types are present at different developmental stages and at different steps of the cell cycle. Since the statistical distributions of the extent of dendritic staining were, as expected, not gaussian distributions, the calculated averages and standard deviations are not sufficient to describe the RNA transport in a mixed cell population. For these reasons the distributions of the extent of dendritic staining by transfected GFP mRNA were displayed graphically. The difference between the distribution of GFP mRNA with or without the RNA targeting element and the calculation of the RANGE (for a definition see chapter 3.2.3) clearly demonstrate

that the mRNA of A2RE-R4 cyto is present more distally within the dendritic shaft with respect to the mRNA of R4 GFP cyto. In fact, the RANGE interval of the A2RE-R4 cyto distribution minus the R4 GFP cyto distribution was positive and included between 20 and 45 μm meaning that the mRNA of A2RE-R4 cyto is present more distally than the mRNA of R4 GFP cyto. This result, coming from a different data representation, is consistent with the significant difference between the average measures of dendritic A2RE-GFP mRNA with respect to GFP mRNA. For these reasons it can be concluded that the A2RE RNA targeting element is actually able to target mRNA in transfected cultured neurons and therefore it can be an useful tool for specifically target scFvs in dendrites.

The reduced extent of dendritic staining for mRNAs containing the R4 sequence (GFP vs. R4 GFP cyto, and A2RE-GFP vs. A2RE-R4 cyto) still remains an open question. In this thesis a direct linkage was proposed between the diffusion of mRNAs within dendrites and the length of the mRNAs. From the results shown the presence of a threshold between 700 and 1400 nucleotides has been proposed, below which an mRNA can passively diffuse and reach the dendrites. Furthermore the presence of a specific sequence within R4 able to retain the mRNA within the perikaryon is possible. It is formally possible that a specific sequence able to target mRNAs is present within the GFP transcript. This hypothetical sequence would explain the large amount of GFP mRNA in distal dendrites. None of the hypotheses discussed above have been tested in this thesis. However, the simplest explanation for the collected data is the existence of a diffusion threshold for mRNAs. Further experiments must be performed with transcripts of variable length to verify the threshold effect, and with different fragments of R4 transcripts in order to validate or reject the hypothesis of a retention sequence within R4 mRNA. With the *in situ* hybridization experiments it was not possible to gain any information about the transport dynamics, or on RNA granule formation, or the possibility of a connection between the putative threshold length and granule formation. In fact it was only possible to collect information about the static situation at the moment when the cells were fixed.

The second RNA targeting element chosen for this thesis was the 3'UTR of CaMKIIα. Despite its cumbersome length (more than 3000 nucleotides), its function has been clearly demonstrated in neurons in culture and in *in vivo* studies conducted on transgenic mice [Mayford M et al., 1996]. The possibility to subclone and to handle such a large piece of DNA was clearly demonstrated by cloning the 3' UTR at the 3' of the GFP. However a smaller part of the 3'UTR, the DTE, was also added to the GFP and R4 GFP cyto plasmids, in order to take advantage of its shorter length. Furthermore, this was an occasion to address the open question about the location of the RNA transport element within the 3'UTR of CaMKIIα. In fact there are different opinions regarding the RNA transport element of CaMKIIα 3'UTR; Blichenberg A et al. [2000] suggested that the bases between position 1481 and 2708 of the 3'UTR are essential, whereas Mori Y et al. [2000] showed that first 94 bases are both necessary and sufficient. Nevertheless no *in vivo* evidence conducted on transgenic mice exists to support any of the above observations. The putative DTE of Mori Y et al. [2000] was placed 3' of the GFP and R4 GFP cyto sequences. Neurons were transfected and the average length of dendritic staining after *in situ* hybridization was measured as described. The results showed a more dendritic presence of DTE-GFP with respect to the control GFP, even if the difference between the two averages was not statistically significant. The same conclusion can be drawn for DTE-R4 cyto and the control R4 GFP cyto. Moreover, the difference between the presence and absence of R4 was dramatic; when the R4 sequence was present the transport down the dendrites was restricted in some manner. This case is also consistent with the A2RE-containing constructs, and the proposed effect of the length threshold. Comparing the average length of dendritic staining of the DTE-R4 cyto with the R4 GFP cyto and of the DTE-GFP with the GFP, no statistically significant differences could be revealed. Again the RANGE interval calculation was required to extract further information. The computed intervals clearly demonstrated, in case of both GFP and R4 GFP cyto as reporter genes, that the DTE-containing mRNAs are more distal in dendrites. In the case of DTE-GFP the RANGE interval was restricted in

the more proximal part of dendrites, if compared with the A2RE-GFP RANGE interval. On the contrary the RANGE interval for DTE-R4 cyto was nearly identical even if calculated with F ≥ 1%, 2%, 3% and 4% (for a definition of the F function see chapter 3.2.3). Therefore, the first 94 nucleotides of CaMKIIα 3'UTR are very likely to contain a functional dendritic targeting sequence.

By merely examining the average length of dendritic staining for UTR-GFP, it seems that the full length 3'UTR was not able to transport the mRNA in dendrites when compared to the GFP control. If the threshold length effect discussed until now is taken into consideration, the most suitable negative control present in this study for UTR-GFP is the R4 GFP cyto without RNA targeting elements. It is the longest transcript available, even if it is much shorter (~ 1500 nucleotides) than the UTR-GFP (a little less than 4000 nucleotides). When the average length of dendritic staining by UTR-GFP mRNA within dendrites was compared with the average length from R4 GFP cyto transfected cells, the effective mRNA transport induced by the UTR signal became clear. In fact the average length of measured dendrites transfected with UTR-GFP is significant higher than the control.

One or more inhibitory *cis*-elements in the UTR have been proposed to be regulated by neuronal synaptic activity and to reside between bases 95 and 724 [Blichenberg A et al., 2000]. In order to verify if the results with UTR-GFP have been influenced by the presence of an activity dependent element, the transfected neurons were activated using depolarization with KCl after the transfection. Neuronal activation was assayed by immunodetection of c-fos and zif proteins in the nucleus, or activated MAPK within the cytoplasm. Surprisingly, resting transfected neurons and transfected neurons depolarized by KCl showed no differences in the pattern of activation with the markers assayed. Apparently, the transfection procedure alters the resting state of the cultured neurons, such that KCl induced depolarization does not result in an additional increase in neuronal activation. In light of this, it is not surprising that the transport of UTR-GFP mRNA remained unaltered in transfected neurons incubated with KCl. The possible presence of an inhibitory *cis*-element can not be addressed without

complete control over the level of activation of the neurons during the period of RNA transport. The alteration in the neuronal activation state due to the transfection procedure may be avoided by letting the cells rest for a longer period of time after the transfection. Similarly, experiments may be performed by blocking mRNA transcription for the time necessary to let the neurons re-reach their resting state. After the removal of the transcriptional inhibitor, the mRNAs would be synthesized and targeted at their final destination. Otherwise, specific vectors for eukaryotic expression with inducible promoters may allow temporal control of transcription of the reporter gene. Nonetheless, the short extent of dendritic staining of UTR-GFP with respect to DTE-GFP and GFP transcripts is more likely due to the greater length of the UTR-GFP mRNA.

In conclusion, the RNA targeting experiments described in this thesis confirm the possibility to target an scFv mRNA to dendrites of transfected neurons. Moreover, the function of a RNA targeting sequence specific for oligodendrocytes, the A2RE, has been demonstrated also in neurons. In accordance with Mori Y et al., [2000], the results here reported suggest the presence of a functional DTE within the first 94 bases of CaMKIIα 3'UTR. The DTE is a small and easily handled targeting element, which directs the dendritic localization of scFvs.

4.3 Function of targeted scFvs

Having validated the protein targeting of scFvs, these technologies were used to interfere with the NGF receptors, TrkA and $p75^{NTR}$. Our research group is particularly interested in the relation between the NGF signal transduction pathway and the development of Alzheimer disease. In fact, the induction of an Alzheimer-like phenotype by disrupting the interaction between NGF and its receptors has been previously demonstrated in transgenic mice. To further investigate the etiology of the disease model, the selective impairment of either TrkA, or $p75^{NTR}$ would be of great interest. This goal should be possible by utilizing scFvs directed against these two receptors, either as neutralizing antibodies (with the sec protein targeting signal), or as intracellular anchors (with the KDEL retention signal).

Three different scFvs were selected for this strategy: MNAC as a neutralizing antibody, α TrkA and α p75 as intracellular anchors. While MNAC is known to prevent NGF/TrkA binding and has been well characterized [Cattaneo A et al., 1999], the other two scFvs required further characterization. Their expression and purification in *E. Coli* allowed the production of functional scFvs able to selectively bind the specific antigens.

Furthermore, the ELISA experiments performed with the three scFvs expressed in transfected COS cells, demonstrate that both the sec and KDEL constructs allowed the targeting of functional antibodies for all the three scFvs.

4.4 Alteration of membrane receptor trafficking

The next step of the work was focused on the putative ability of the KDEL constructs to effectively block the appearance of the neurotrophin receptors on the cell surface. The decision to focus on the anchoring mechanisms, as opposed to the neutralizing approach, was made for a several reasons. First of all, for the validation of a new technology, the advantages of the KDEL retention signal are particularly relevant. In fact, the KDEL exploitation does not require the selection of neutralizing antibodies, making the strategy easier to apply. Therefore, a larger panel of suitable scFvs was available for use as intracellular anchors, exactly because of the difficulties of a neutralizing antibody selection.

Furthermore, demonstration of the effective function of a secreted scFv *in vitro* with the goal of creating a transgenic mouse, is a rather questionable approach. Indeed all the available *in vitro* assays are clearly unable to simulate the extracellular environment of a living animal, making these kinds of experiments less informative for secreted proteins. On the other hand, an scFv retained within the ER is probably less influenced by the *in vitro* culture conditions. Finally the use of secreted antibodies in transgenic mice is already an established approach that does not need any further *in vitro* validation.

The effectiveness of the anchoring function of a KDEL scFv was assessed by the aid of C6 cells stably transfected with α p75 KDEL scFv. Since those cells are known to express $p75^{NTR}$ and TrkB [Pantazis NJ et al., 2000], but not TrkA [Colangelo AM et al., 1994], the α TrkAs were not expected to provide significant results. After verifying the intracellular scFv expression of the selected clones, the presence of $p75^{NTR}$ on the cell surface was measured by FACS. Indeed a diminished amount of the receptor was observed in α p75 KDEL scFv clones with respect to the controls, while the overall $p75^{NTR}$ production remained comparable in both cases.

Having demonstrated the efficiency of the KDEL retained scFvs as intracellular anchors, new approaches were developed to test the effect of α TrkA, MNAC and α p75 scFvs on the NGF signal transduction pathway.

4.5 Biological consequences of receptor and signal transduction protein phenotypic knock-out

PC12 cells were chosen to carry out functional studies of scFv expression, since this cell line express both of the target receptor proteins, TrkA and $p75^{NTR}$. Furthermore their well known response upon binding NGF made this cell type the ideal subject to assay the ability of the selected scFvs to interfere with the neurotrophin transduction pathway.

NGF primed PC12 cells were transfected and re-plated in presence of NGF. The responsiveness to NGF was evaluated morphologically by counting the cells that showed neurite outgrowth. Although the primed PC12 had already undergone differentiation, in this thesis those cells that were able to re-gain their differentiated morphology were considered as "differentiated". It is known that both TrkA and $p75^{NTR}$ play an important role in the neurite outgrowth response to NGF making this assay appropriate for all the anti-receptor scFvs tested. In fact, the collected data clearly show that the expression of the anti-neurotrophin receptor scFvs combined with their retention in the ER leads to a strong inhibition of NGF-induced differentiation. Furthermore, the inhibition of Ras, a protein involved in the signal transduction of Trk receptors, was obtained with the cytoplasmic expression of a neutralizing scFv and led to comparable results. In this way the congruence of the effects of the anti Ras scFv and anti-receptor scFvs provided a strong confirmation of the specificity of the pKO using a morphological assay.

The activation state of some of the proteins involved in the NGF signal transduction pathway was assayed in order to evaluate the intracellular effects of these scFvs. Both short and long term NGF dependent responses were examined. MAPK switches to an active state within 10 minutes after NGF application and was used as a short term activation marker. On the other hand, the intermediate early genes c-fos and zif/egr1 show an increased nuclear concentration three hours after the NGF stimulation and were used as long term activation markers. The c-fos and zif/egr1 nuclear concentration is influenced by a large number of stimuli;

their alteration as a result of the transfection-induced stress has been demonstrated in this thesis (see chapters 3.2.2 and 4.2). For this reason the NGF application was performed two days after the transfections allowing the cells to recover from the transfection and return to the resting state. This simple expedient permitted the use of the transduction signal reporters c-fos and zif/egr1, that were specifically selected among the last steps of the transduction pathway, in order to highlight the signal amplification. The observed activation of c-fos and zif/egr1 was indeed the result of the NGF application and not of some other factors, since all the reporters were negative in transfected cells not treated with NGF two days after the transfection. The cytoplasmic expression of the neutralizing anti Ras scFv led to decreased activation of MAPK and reduced amounts of nuclear c-fos and zif/egr1. Most importantly, a similar reduction of all three signal transduction reporters was observed when the TrkA receptor was impaired by the binding of either of the anti TrkA KDEL scFvs.

Similar results were obtained using the α p75 KDEL scFv, even if the level of inhibition was slightly lower for all the three reporters. Indeed the remarkable and unexpected result is the lack of activation of MAPK by NGF application, which occurs when the amount of $p75^{NTR}$ at the plasma membrane is reduced. Since there is no evidence supporting the direct effect of the NGF/$p75^{NTR}$ binding on MAPK activation, it appears that the retention of $p75^{NTR}$ in the ER is somehow able to inhibit the signal transduction pathway specific for the Trk receptors. Considering that it is now well accepted that Trk and $p75^{NTR}$ receptors form a complex stable enough to be immunoprecipitated [Bibel M et al., 1999], one possible explanation of the anomalous α p75 dependent down regulation of MAPK may be that the trapping of $p75^{NTR}$ in the ER also leads to the retention of TrkA receptors by formation of a α p75/$p75^{NTR}$ /TrkA complex in the ER. This idea is supported by the other unexpected result (presented in chapter 3.6.1) of the FACS analysis of the α TrkA C6 clones. The clones stably expressing α TrkA showed a reduction of the appearance of $p75^{NTR}$ on the cell surface. Since C6 cells were reported to express TrkB, but not TrkA receptors, no effect on the

appearance of $p75^{NTR}$ on the cell surface was expected. It is possible that the α TrkA scFv cross-reacts with TrkB, since the Trk receptors all share a similar structure. If this is the case, the α TrkA may be able to trap TrkB receptor in the ER of the C6 cells. In the same way as the retention of TrkA is caused by the trapping of its partner $p75^{NTR}$ in PC12 cells, it is possible that in C6 cells the ER retention of TrkB prevents the appearance of $p75^{NTR}$ on the cell surface.

In order to confirm of this hypothesis a different cellular context was exploited. Primary hippocampal neurons constitutively express TrkB and $p75^{NTR}$, making it possible to verify the interference ability of α p75 KDEL scFv with the BDNF signal transduction pathway. First of all the system was validated with the positive control for the neurotrophin signal transduction, the neutralizing scFv anti Ras. Neurons were transfected with anti Ras and its ability to down regulate the BDNF signal transduction pathway was tested by detection of MAPK activation and c-fos and zif/egr1. The results obtained showed a significant down regulation of the three markers with respect to the R4 GFP cyto negative control, thus reproducing the results obtained in PC12 cells. This validated the use of primary hippocampal neurons for the trials with α p75 KDEL scFv. The retention of $p75^{NTR}$ in the ER by α p75 KDEL scFv greatly reduced the nuclear appearance of c-fos and zif/egr1. The most significant result is the lack of MAPK activation, which supports the hypothesis of the retention of a Trk/$p75^{NTR}$ complex in the ER. The functional trials of the KDEL scFvs reported in this thesis suggest that the ER retention signal is suitable for preventing the appearance of membrane proteins on cell surface, thus providing a handy tool to inhibit plasma membrane protein function without the need of a neutralizing scFv. Furthermore, the evidence collected suggests that it may be possible to interfere with the action of entire protein complexes by means of an scFv selected against any single protein of the complex. This adds a powerful tool for functional analysis to the pKO technology.

4.6 RNA interference and phenotypic knock-out

Recently a new field for post-transcriptional gene silencing has been studied and developed: double strand (ds) RNA interference, or RNAi. This new technique, together with gene knock-out and pKO, is a powerful tool for functional genomic studies. The first hint that dsRNAs could inhibit gene function was found by Guo S and Kemphues KJ [1995], who were trying to block the expression of the *Caenorhabditis elegans par-1* gene with antisense RNAs. Surprisingly, the *par-1* null phenotype was induced by injection of both antisense RNA, or a mixture of sense and antisense RNA. This result was confirmed and explained three years later by Mello and colleagues [Fire A et al., 1998], who were the first to intentionally inject dsRNA into *C. elegans*. This injection resulted in a much more efficient silencing of a gene than injection of either sense or antisense strands alone. Furthermore only few molecules of dsRNA were sufficient to trigger the gene silencing, not only throughout the whole animal, but also in the first generation of progeny. Several biochemical and genetic studies on this phenomenon have shown that it is the same as the previously characterized Post-Transcriptional Gene Silencing (PTGS), that naturally occurs in a variety of organisms including plant, protozoa, and *Drosophila* and is considered a feature of nearly all eukaryotes [for reviews see Hammond SM et al., 2001; Sharp PA and Zamore PD, 2000].

The mechanism that underlies RNAi is now quite well understood. A long dsRNA (typically > 200 nucleotides) can be introduced into a cell by microinjection, transfection or virus infection. Upon introduction, the dsRNA enters a cellular pathway, commonly referred as RNAi pathway, which is restricted to the cytoplasm [Zeng Y and Cullen BR, 2002]. The long dsRNA is first processed into 21-23 nucleotide dsRNAs with symmetric ~2 nucleotide 3' overhangs, which are referred to as small interfering RNAs (siRNAs) [Zamore PD et al., 2000]. The enzyme involved in this process belongs to the RNaseIII ribonuclease family and is called Dicer. Then, the siRNAs duplex assembles into to a nuclease complex known as RNA-Induced Silencing Complex (RISC). There is a strict requirement

for the siRNA to be 5' phosphorylated to enter into RISC [Nykanen A et al., 2001]. The duplex siRNAs are unwound leaving the antisense strand to guide RISC to its homologous target mRNA. siRNAs bind to complementary mRNA molecules with Watson-Crick base pairing triggering the endonucleolytic cleavage of the homologous transcript [for a recent review see Hannon GJ, 2002]. The target mRNA is cleaved at a single site in the center of the duplex region, 10 nucleotides from the 5' end of the siRNA [Elbashir SM et al., 2001b].

The long dsRNAs allow the inhibition of gene expression by the presence of multiple siRNA sequences able to bind the target mRNA. Unfortunately, this approach is not useful for mammalian cells, since the introduction of dsRNA longer than 30 nucleotides induces an interferon response in the same way as viral infections. Interferon triggers degradation of mRNAs and a general inhibition of mRNA translation [for a recent review of interferon action and induction see Samuel CE, 2001].

With the knowledge that RNAi can be induced in mammalian cells directly by siRNAs without inducing the interferon response [Elbashir SM et al., 2001a], many researchers are beginning to use RNAi as a biomolecular interfering tool in human, mouse and other mammalian cultured cells by siRNA transfection [e.g. Lee NS et al., 2002; Wianny F and Zernicka-Goetz M, 2000]. The siRNA transfection can be obtained by microinjection, by the aid of lipid-based reagents, by plasmid vector mediation or by retroviral vector mediated siRNA expression [for a recent review see Dykxhoorn DM et al 2003].

Why study and develop protein knock-out techniques when a powerful tool such as RNAi can be exploited?

RNAi has some clear advantages such as the few collateral effects, the specificity for the target gene, the possibility to cross cellular barriers and to work at a distance from the injection. Hence, RNAi is proving to be a robust, versatile and inexpensive technique for controlling gene expression in mammalian cells and for the analysis of gene function in mammals. However, this kind of approach is linked to a series of limitations that make protein knock-out techniques preferable in some cases. First of all, the crucial design of the sequence of dsRNA is all but

straightforward; the rules that govern efficient siRNA-directed gene silencing remain undefined. In fact, choosing a functional siRNA is still an empirical process. Several groups have proposed a set of guidelines to search for siRNAs that could potentially silence gene expression. Some sequence motifs seem consistent with effective siRNA-directed silencing; the regions should not contain repetitive sequences and intronic sequences must be avoided [Martinez J et al., 2002]; some sequence peculiarity, such as the percentage of G/C content that has to be between 30 and 70%, and the presence of single nucleotides stretches can heavily affect siRNA activity. Several different trials are usually performed before the right sequence can be found. Numerous siRNA must be synthesized for a single gene, and the efficiency of each must be validated to ensure that the chosen siRNA targets a single gene. In order to avoid disrupting the activity of other mRNAs, the selected sequences must be specific for the RNA of interest and not present in any other mRNA in the cell. This can be analyzed by sequence alignment with the aid of public nucleic acids sequences databases available. Nevertheless, for this kind of experiments, it is always very complicated to provide a convincing negative control. On the contrary the pKO approach, coupled with the latest antibody selection technologies such as IACT, offers a mature strategy characterized by reliability and standardization of the procedures. In principle pKO should even allow interference with proteins of unknown sequences, which are present in a complex containing a well-characterized protein partner, as explained above (see chapter 4.5). The function of neurotrophin receptor complexes may be investigated in this way, since anti-receptor scFvs can block the activity not only of the receptors themselves, but also of the other proteins of the membrane receptor complex. For example, by trapping the TrkA receptor, the function of the Transient Receptor Potential (TRP) ion channel V1, which is known to be strictly associated with the TrkA receptor in sensory neurons [Chuang HH et al., 2001] might be altered. Acting on TrkB, it might be possible to interfere with the sodium channel Na_v 1.9, that forms a complex with the receptor [Blum R et al., 2002].

Unlike fungi [Cogoni C et al., 1999], nematodes [Dalmay T et al., 2000] and plants [Sijen T et al., 2001], which can replicate siRNAs, there is no indication of siRNA replication in mammals. Therefore, siRNA-directed silencing by transfection is limited by its transient nature. As siRNAs are relatively resistant to degradation, the transient nature of knock-down is determined by the rate of cell growth and the dilution of the siRNA. The other crucial factor that determines the amount and the duration of the siRNA-mediated knock down is the half-life of the target protein. The onset of any phenotype due to siRNA-mediated knock down will be delayed in the case of a very stable protein. Moreover, if one mRNA molecule escapes from RNAi degradation pathway, many active proteins can be synthesized, especially if the mRNA is characterized by a long half-life. Obviously this unpredictable feature of the siRNA approach is not a concern when using pKO.

Even though the RNAi approach has been successfully used to inhibit specific isoforms of a protein [e.g. Kisielow M et al., 2002], RNAi can never discriminate between conformational changes of the same protein, even in the cases of huge changes as those observed for the prion protein. Furthermore, post-translational modifications cannot be targeted. It may be nearly impossible to selectively block a protein with a single aminoacid mutation. On the other hand, pKO targets the very final gene products, providing a selective knocking down tool for proteins that differ in conformation, post transcriptional modifications, or a single aminoacid. However, the greatest advantage of the pKO over the other knocking down strategies resides in its intrinsic flexibility. Unlike pKO, the all or none nature of RNAi is its biggest limitation. The use of antibody proteins as interfering agents allows fine tuning of the effect in many ways. In principle, it is possible to modulate the level, time, mechanism and place of the specific inhibition. A set of scFvs that differ in the affinity for the antigen binding and, hence, that interact with different strength on the intracellular target, can be selected from the beginning. The concentration of the scFv can be regulated in the subcellular compartment of interest by means of targeting signals, effectively increasing or reducing the local concentration as needed at the site of interest.

This should also lead to a reduction of perturbation at other intracellular sites. In the future, the use of inducible promoters should allow facile control of the timing of the inhibitory effect. The interfering mechanism itself shows a great level of flexibility. Antibodies can be used as neutralizing tools to directly inhibit a specific protein function when used to block the active site, or can be used to prevent the appearance of the target protein in its functional compartment. It is also possible to divert the protein of interest to another compartment of the cell, effectively preventing the antigen from exerting its particular function. For example, an scFv bound with its antigen was redirected from the cytoplasm to the nucleus [Visintin M et al., 2002]. When using redirection of the antigen as a strategy, the timing of scFv folding and antigen recognition with respect to transport dynamics is crucial. The balance should be in favor of the binding versus the transport. Specific signals may be exploited to redirect scFvs together with the antigen toward degradation pathway effectors, such as proteosomes and lysosomes. Perhaps protein degradation can be prevented by selecting scFvs against sites recognized by proteolytic enzymes, leaving the protein able to exert its function. Finally, the work presented in this thesis strongly supports application of fine control over the intracellular site where an scFv functions, a very important feature when the target cells have an extremely polarized morphology like neurons. The use of well suited, or even a combination of targeting signals, may allow specific interference with a protein only in a subcellular compartment, or even in subdomains of selected organelles. For example, a ubiquitous protein may be easily inhibited only inside mitochondria by means of a neutralizing antibody expressed in fusion with a mitochondrial targeting signal. In this case the inhibitory effect of the scFv would be restricted inside the compartment of interest, leaving the target protein able to exert its function in the rest of the cell environment. In the neurotrophin receptors the flexibility of pKO is particularly useful since a number of different strategies may help in discriminating among the many different tasks each receptor performs. Membrane proteins can be manipulated by scFvs in several ways. For example, the KDEL signal provides a way to prevent receptor appearance on the

plasmalemma along with tightly bound partners. On the other hand, a neutralizing secreted scFv could bind the extracellular domain of the antigen. An scFv may be designed with a transmembrane domain in order to gain a higher local concentration with respect to a secreted antibody. In many cases the use of these different approaches might help in unraveling the complex receptor mechanisms. For example, the $p75^{NTR}$ extracellular domain is cleaved by metalloproteinases [DiStefano P et al., 1993]. A membrane bound scFv might be informative about the hypothetical function of the released extracellular fragment by retaining the fragment at the plasmalemma. The γ-secretase cleaves $p75^{NTR}$ in its intracellular domain, which is released and may function in the nucleus as a transcriptional modifier [Schecterson L et al., 2002]. Recently, a targeting sequence able to anchor proteins on the cytoplasmic side of the membrane has been identified [Borgese N et al., 2003]. This signal can be coupled to an scFv in order to inhibit the release from the membrane of the γ-secretase cleaved cytoplasmic portion of $p75^{NTR}$. Another strategy would be to target the same scFv to the nucleus, in order to block the transcriptional activity of the cytoplasmic portion of $p75^{NTR}$, but not to interfere with the receptors remaining at the membrane. Another possible approach is to design scFvs localized specifically to the lipid rafts in the same way as glycosyl phosphatidylinositol (GPI) anchored proteins are targeted by the C-terminal GPI signal peptide [Udenfriend S and Kodukula K, 1995]. Lipid rafts represent specific sites within the membrane where the intracellular partners of receptors concentrate [Simons K and Ikonen E, 1997]. Hence, lipid rafts work as signal transduction platforms and are sites where activated receptors are thought to group within active clusters [Tsui-Pierchala BA et al., 2002]. In fact, activated TrkA and $p75^{NTR}$ accumulates in rafts [Huang CS et al., 1999; Higuchi H et al., 2003]. GPI-anchored scFvs might accumulate in lipid rafts and, hence, might inhibit the TrkA signal transduction pathway by interacting preferably with the activated form of TrkA, but not preventing its interaction with NGF.

For all these reasons, the potential antagonism between pKO and RNAi is not a problem, even if RNAi is confirmed as a useful knocking down methodology in

the future. In fact, the pKO approach shows its full potential in many situations that spread far beyond the simple silencing of a protein considered as an effecter of only one precise function. In the same way as single proteins show a variety of functions and fine regulatory mechanisms, pKO with specific protein or RNA targeting tools can be used in a very wide range of approaches with a minimal perturbation of the cells normal life.

4.7 The design of new constructs for transgenic mice: combining protein and RNA targeting signals

In the Cattaneo research group application of the pKO technique in transgenic mice led to novel results. The adult AD11 transgenic mouse expresses the secreted whole antibody α D11, which is known to interfere with the NGF/TrkA interaction [Ruberti F et al., 2000], and shows Alzheimer-like neurodegeneration [Capsoni S et al., 2000]. Although these results represent a real breakthrough in understanding Alzheimer's disease (AD), some additional constructs were prepared in this thesis to further clarify the biology of this disease model. Even if these mice are the best available AD animal model, there are still some open questions about how the transgene α D11 causes the AD phenotype. Three distinct factors, or a combination thereof, may be responsible for the observed phenotype. Obviously, one critical factor is the ability of α D11 to block the interaction between NGF and its receptors. This might not be the only cause, especially since α D11 is expressed as a chimeric antibody. The presence of the human portion of α D11 (CL, CH1, 2, and 3) may produce some effects due to its recognition by the murine immune system as non-self. In addition, the Fc fragment might induce an inflammatory response [Mellman I et al., 1988]. For these reasons negative controls were designed to elucidate the results of the AD11 mouse and to examine the roles of the three possible causes of the phenotype. The variable light and heavy chains of α NP, an antibody that recognizes the antigen 4-hydroxy-3-nitrophenacetyl, were put in fusion with the antibody human constant regions. The aim of this construct was to produce transgenic mice similar to the α D11 strain, but with a secreted antibody unable to bind any antigen in the mouse. Observing the phenotype of the resulting α NP mice should help understand possible effects of the ectopic expression of a complete and chimeric antibody. Another concern was the effect of expressing a human Fc fragment in mouse. The use of an scFv should abolish inflammatory side effects induced by the human Fc tail when the antibody is bound to the antigen. Moreover, the use of

antibodies in the form of scFvs should provide additional advantages. The shorter sequences of scFvs are more easily handled during subcloning procedures and generation of transgenics. Finally, in this way two transgenic strains are not required, i.e. one for each antibody chain (light and heavy). Two scFv constructs were prepared; one for the expression of the secreted scFv form of α D11, and the other to provide a suitable negative control, the secreted α NP scFv.

In spite of the aforementioned considerations, the block of the interaction between NGF and its known receptors, TrkA and $p75^{NTR}$, is likely to be the principle cause of the AD-like phenotype in the AD11 mice. The tools developed in this thesis represent the ideal means to further investigate the validity of this hypothesis. The constructs designed to block the activity of each receptor separately were prepared and validated. A transgenic with the MNAC chimeric human/mice antibody may produce a phenocopy of the AD11 mouse. Since a complete antibody might provoke nonspecific responses *per se*, the MNAC sec scFv construct was also prepared. Both these transgenes should block the binding of NGF to TrkA.

The work discussed in this thesis details the efficacy of the KDEL signal as a pKO tool for membrane proteins and the usefulness of α TrkA and α p75 scFvs. In order to exploit this new pKO strategy and to verify if the NGF/$p75^{NTR}$ interaction is involved in the AD phenotype development, the α TrkA KDEL, the α p75 KDEL, and the α NP KDEL scFv constructs were prepared for the generation of transgenic lines.

It is plausible that the production of all these mice strains will provide new and informative results. It is impossible to try to foresee the effects of these constructs; in some cases the transgenic embryos may have embryonic, or lethal phenotypes. Only after the transgenic lines are produced and an accurate evaluation of the phenotypes is performed, the final outcome will hopefully provide a strong impulse to new research approaches.

The new technologies developed in this thesis are, however, opening new fields for research. Even if the field of protein targeting has reached a remarkably mature stage, the same is not true for the field of mRNA transport and targeting.

A more profound knowledge of mRNA transport will be necessary when combining protein and RNA targeting. In this new use of protein targeting with mRNA transport, the fine regulation and the hierarchy that this arrangement of multiple signals obeys is not yet clear. In this thesis a precise study was done only with the single targeting signals; the same has to be performed in this new field. However, using the same tools characterized in this thesis, the already known experimental limitations would rise again. In particular, it would not be possible to observe directly the initial events of protein targeting within cells, because of the large amount of diffusion by GFP. Moreover, measuring the mRNA presence in dendrites with *in situ* hybridization assays, all the problems related to the mRNA length and diffusion threshold will have to be addressed. The same is true also for the hypothesized presence of a specific sequence, able to block the transport until proximal dendrites, present into R4 mRNA.

Preliminary results on combined protein and mRNA targeting were obtained during this thesis. Two double targeting constructs were tested in hippocampal neurons for the presence of mRNA within dendrites: the A2RE-R4 KDEL and the A2RE-R4 sec. In both cases the results suggest that the A2RE is dominant and directs dendritic transport in the presence of both protein targeting signals. The results obtained for the two constructs were entirely consistent with the transport characteristics of A2RE-R4 cyto transcript. Similar results were previously obtained in oligodendrocytes for a microinjected RNA encoding a transmembrane protein with an A2RE [Ainger K et al., 1997]. In this thesis the hypothesis of the presence of the complete machinery for protein synthesis and secretion within distal dendrites, including ribosomes, SER, RER, and Golgi apparatus, was discussed. Even if there are some strong indications, it is not yet certain that protein synthesis occurs in distal dendrites. On the other hand, it is not known whether protein synthesis of transported mRNAs is initiated in the perikaryon, or if it is inhibited until the mRNAs reach its final destination. In the first case, a double targeting might be used to eliminate any scFv produced along the mRNA route inside the cell. In particular, it might be possible to use this strategy to neutralize a protein, which is present in the perikaryon and the distal dendrites,

specifically in distal dendrites. This might be achieved by exploiting the A2RE-scFv NLS construct and sequestering any scFv synthesized in the cell body within the nucleus. The rate of folding and antigen binding would have to be slow enough to allow the transport before the binding occurs.

In the case that protein synthesis can be restricted to the distal dendrites, it would be simple to target an scFv mRNA within distal dendrites without provoking any perturbation in cell body due to the presence of the scFv. Once in the periphery the scFv could be translated and targeted within the ER, in the secretory pathway or into mitochondria. Neutralizing antibodies would thus be able to inhibit their antigen function only in distal subcellular compartments. It would also be possible to exploit non-neutralizing antibodies as sequestering agents. Inducing the expression of an scFv in fusion with a NLS in the distal dendrites may allow the antibody to carry the antigen in its new destination, the nucleus. In this case the rate of folding and antigen binding needs to be quick enough to allow the binding before the diffusion and transport of the scFvs.

In order to implement this ambitious technique some problems need to be addressed. First of all a tool to verify with reasonable precision where the translation starts and is completed would help enormously. A step in this direction has already been done in the Cattaneo lab with the synthesis of a fluorescent marker of actively synthesizing ribosomes, APTS cycloheximide. This will allow a better understanding of the relationship between RNA transport and translation, knowledge essential for the development of reliable double targeting of scFvs. Additionally, direct measurements of antigen-antibody binding in discrete subcellular locations with Fluorescence Correlation Spectroscopy (FCS) [e.g. Schwille P 2001] would help in formulating more efficient pKO strategies. The application of a more profound understanding of cell biological mechanisms can only enrich the future of the pKO strategy. New insights into cell biology and new disease models will undoubtedly emerge, especially since the pKO strategy is not limited to proteins, but can be used for any kind of antigen recognized by an antibody.

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"Forget about it" is like if you agree with someone, you know,
like "Raquel Welsh is one great piece of ass forget about it."
But then, if you disagree,
like "A Lincoln is better than a Cadillac?
Forget about it!" you know?
But then, it's also like if something's the greatest thing in the world,
like Mingrio's Peppers, "forget about it."
But it's also like saying "Go to hell!" too.
Like, you know, like "Hey Paulie, you got a one inch pecker?"
and Paulie says, "Forget about it!"
Sometimes it just means forget about it.
Donnie Brasco