“Role of the Neuronal Protein Cap23 in the Maturation and Maintenance of Dendritic Arbors in-vivo.”

INAUGURALDISSERTATION
Inauguraldissertation
zur
Erlangung der Wurde eines Doktors der philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultat
der Universitat Basel

von
Anirban Sadhu
Aus Indien.

Basel 2006

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultat auf Antrag der Herren Prof.Dr.Yves-Alain Barde,Prof. Markus Rueegg,Dr.Pico Caroni und Frau Prof.Isabelle Mansuy.

Basel,den 01 Mar.2006

(Prof.Dr. Yves-Alain Barde)

(Prof.Dr. Hans-Jakob Wirz)
Dekan

ACKNOWLEDGEMENTS

The experiments described in this thesis were conducted at the Friedrich Miescher Institute in the lab of Dr.Pico Caroni. I fondly remember the times there - through thick and thin - with my labmates. In particular, I am thankful to Corinna, Smita and Vincenzo for reinforcing the belief that the end is benedictive, even if the journey is blithe.

The FMI was a wonderful place to work in. That is where I learned the essence of scientific reasoning. That is where I learnt how to formulate scientific questions and look at the bigger picture. I owe this to the lab meetings and the journal clubs. These lessons will remain with me long after the work done itself may fade into obscurity.

Looking back, while I still regret lapses that occurred, and wish I had received more mentoring, I still feel enriched by the learning experience that my stay at the FMI has been. My thanks in this regard particularly go to members of my thesis committee Prof. Yves Barde and Prof. Isabelle Mansuy.

None of this would have been possible but for Rejina's constant presence by me. When both of us now look back on the FMI days with relief, nostalgia and achievement, I cannot help remembering how she made it all possible with her assurances and excel sheets, encouragements, faith and help with the figures.And for saying that eventually all will be well, and pieces will fall into place.

-Anirban Sadhu

Table of Contents

THESIS ABSTRACT

Dendrites in the central nervous system are the postsynaptic counterparts in the neural circuitry, and the principal sites of excitatory synaptic inputs. Little is known about the genetic elements regulating the specification, formation, development, and maintenance of these structures. They are formed early in development, and barring small changes in structure, remain essentially unchanged throughout life. More than 90% of the synapses in the brain are located on the heads and necks of dendritic protuberances called spines. Since synapses are the functional units of brain function, a detailed study of their anatomical and morphological features is important for understanding the functioning of the brain, both in health and disease. But the high density and the structural complexity of neurons, and the small size of the spines in the brain impedes a detailed examination of spine morphology and synaptic structure.

We used a line of transgenic mice expressing membrane targeted GFP (m-GFP) under the Thy-1 promoter to study dendritic morphology. These lines of mice express GFP in a subset of neurons, and lights up their entire arbor, enabling visualization of the proverbial “tree from the forest”. Expression of GFP in the membrane gets rid of all artifacts associated with volume and intensity, and enables visualization of fine structure of dendrites with an unprecedented clarity and resolution. Using deconvolution confocal microscopy, we are able to detect dendritic structures hitherto visible only in electron micrographs, and are able to resolve spines that are below the theoretical limit of resolution of a light microscope. This line of mice has the potential to become an invaluable assay tool for detecting early onset defects in neuropsychiatric disorders, as it is increasingly becoming apparent that changes in synapses (i.e. spines) are the first markers of all neural diseases.

We next used the increased clarity offered by the m-GFP mice to address the role of the neuronal protein Cap23 in the maintenance of dendrites. Cap23 is a

major cortical-cytoskeleton associated and calmodulin protein binding protein that is widely and abundantly expressed during development, maintained in selected brain regions in adults, and re-induced during nerve regeneration. Mice deficient in Cap23 start out having normal dendritic structure and arborization, but subsequently start decreasing in their arborization from around the time corresponding to synapse elimination in the CNS. This decrease in branching is progressive in nature, and correlates with the levels of the protein expressed. Since dendrites are normal to start with, but decrease in their arborization subsequently and in a steady manner, we refer to this novel phenomenon as “Dendritic Atrophy”, and implicate Cap23 in the maintenance of dendrites. The atrophy starts in the higher-order branches and proceeds towards the lower-order ones, and the remaining branches develop ‘complex spines’.

Deficiency of Cap23 leads to the misregulation of a number of important genes in the proteome of the brain, but not in the transcriptome, suggesting the role of Cap23 in regulating dendritic structure by modulating the levels of several important dendritic proteins. Interestingly, in the background of a deficiency of Cap23, the transcriptome of the brain shows the predominant upregulation of a number of non-coding RNAs of unknown function that show important similarities with microRNAs. At least one miRNA (miR-128) is starkly downregulated in Cap23 mutants. This leads to the interesting possibility that Cap23 might be involved in the maintenance of dendrites through miRNA mediated regulation of protein levels. Since defects in dendritic structure and arborization is a hallmark of all neuronal diseases, it becomes interesting to speculate whether aberrations in RNA mediated control is a general mechanism underlying neuropsychiatric diseases in general.

Chapter I:

INTRODUCTION

The mammalian brain is an organ of incredible complexity. According to estimates, a microlitre of brain tissue from the mouse cortex contains approximately 10^5 neurons, 10^9 synapses, and about 10 kilometers of axon. Neurons are the structural and functional building blocks of the brain. They join with each other at junctions called synapses to establish the wiring of the brain.

Imaging methods in the brain - Techniques to visualize neuronal morphology and synapses.

To understand fully the complexity of the nervous system at the molecular, cellular and circuit level, both anatomical and functional studies are needed. These approaches may be extremely diverse, depending on the questions and interests that are addressed with the research. Traditionally, researchers, e.g. of neocortical microcircuits, have studied their function and deduced information on their connectivity (Martin, 2002). Recently, *in-vitro* slice experiments on microstructure and physiology have provided a greater level of detail in the attempt to relate the structure of circuits to their function. This information is critical for understanding the processes that lead to brain dysfunction. Despite this effort, it is clear that we simply do not know enough about the fine anatomical structure of the mammalian brain. This information would also provide a cellular basis for cognitive processes and behavior. To this end, neuroscientists have developed a variety of techniques to visualize projections and connections of interest. Some of these methods, from the traditional use of staining techniques and dyes to more recent reporter based tracers, are briefly outlined here. A particular emphasis has been given to synaptic labeling techniques that are illuminating our view on the nervous system.

Since the fundamental functional unit of the brain is the synapse, it is not surprising that an important aim was to try to visualize synaptic morphology and plasticity. Synaptic junctions are specialized dynamic structures, sites of extensive changes in response to a variety of stimuli. There are two classes of changes (Bailey and Kandel, 1993): changes in the strength of pre-existing synapses without alterations of interneuronal connectivity (Tanzi, 1893), (Martin

et al., 2000)) and changes in circuit connectivity due to formation and elimination of synapses (Cajal, 1893, Dailey and Smith, 1996; Darian-Smith and Gilbert, 1994; Engert and Bonhoeffer, 1999; Harris and Woolsey, 1981; Lendvai et al., 2000; Maletic-Savatic et al., 1999; O'Rourke and Fraser, 1990; Purves and Hadley, 1985; Rajan and Cline, 1998; Toni et al., 1999; Wong et al., 2000). This dynamic view of the nervous system is starting to be widely accepted especially now that imaging techniques capable of visualizing synapses in real time are being further developed.

Staining techniques, dyes (FM-dyes), and reporter-based methods.

Traditionally, ultra structural and immunocytochemical studies gave us a static picture of synapses. Nevertheless, in particular the *Golgi technique* (Golgi, 1873) has had a tremendous impact since its introduction at the beginning of the 20th century and it is still the technique of choice for investigation of samples that are not amenable for molecular genetic approaches (e.g. human samples). Other methods introduced in the 1970s and 1980s include staining for specific transmitters, or cell-type specific molecules, as well as microinjection of a wide range of anterograde and retrograde tracers (e.g. horseradish peroxidase, biocytin, the cytoplasmic lucifer yellow, etc.) (Callahan et al., 1998). With the advent of fluorescent dyes such as the carbocyanine DiI and DiO, it also became possible to look at neuronal morphology in living tissue, thus allowing the possibility of studying the dynamics of neurons and neuronal processes (Honig and Hume, 1989). One application of this approach is the study of synapse morphology, formation and remodeling at the Neuromuscular Junction (NMJ) using dyes that label organelles concentrated at NMJ (Magrassi et al., 1987) (Balice-Gordon and Lichtman, 1990) and the use of lipophilic dyes (e.g. FM-dyes) to investigate synaptic vesicle (SV) trafficking at NMJ *in vitro* (Betz et al., 1992a) and SV dynamics in cultured neurons (Ahmari et al., 2000; Friedman et al., 2000). FM-dyes are based on the internalization of the compound during membrane recycling and represented the approach of choice to investigate activity-

dependent SV dynamics in cultured cells and whole mount preparations for more than a decade. However, although they proved very useful, these techniques have intrinsic limitations. Dye labeling depends on the possibility of identifying and accessing the neurons of interest. For many classes of neurons this is not a feasible task. Some of the dyes present unspecific labeling and background problems and can even interfere with synaptic function (Balice-Gordon, 1997), and, as in the case of FM-dyes, an activity-dependent protocol of staining is necessary.

Since discrete populations of neurons can be defined by patterns of gene expression, reporter-based tracers have greatly increased the ability to label subsets of neurons and their processes. Markers like the *lac*-z product ß-gal have then been used to label specific populations of cells, and in the attempt to label processes a number of ß-gal fusion proteins have been introduced as first attempts in *Drosophila,* and also in mice (Mombaerts et al., 1996).

GFP-based methods.

The discovery of Green Fluorescent Protein (GFP) and its variants ten years ago (Chalfie et al., 1994) is leading to a revolution in cell biology allowing the ability to visualize and quantify molecules and events in living cells with high spatial and temporal resolution. One of the major advantages of this genetically encoded fluorophore is that it can be fused to virtually any protein of interest and thus allow studying its subcellular localization. To exploit the GFP technology for the study of synaptic morphology and dynamics, ways to deliver the proteins in subsets of neurons have been devised. For example, by particle mediated gene transfer (gene gun) or viral-based approaches, GFP fusion proteins can be targeted in specific brain areas (Gan et al., 2000; Haas et al., 2001; Lo et al., 1994; Moriyoshi et al., 1996). Unfortunately these methods are labor intensive, difficult to control, often compromise cell viability extensively, and, e.g. in the case of gene gun, do not specifically label neurons.

To overcome this, genetic methods to express GFP or GFP fusion proteins in particular sets of neurons are available in model systems like *Drosophila* (Edwards et al., 1997), *zebrafish* (Higashijima et al., 2000), *C. elegans* (Nonet, 1999) and in mouse (van den Pol and Ghosh, 1998). In particular, the use of a neuron specific promoter, the Thy.1 expression cassette (Caroni, 1997) to drive the expression of different spectral variants of cytosolic fluorescent proteins**,** proved to be a valuable tool to study neuronal morphology and dynamics in vivo (Feng et al., 2000; Walsh and Lichtman, 2003); (Grutzendler et al., 2002; Trachtenberg et al., 2002). However, the cytosolic nature of the fluorescent proteins could represent a serious problem when detailed morphological analysis is needed. In fact, small compartments like filopodia and tiny protrusions may be poorly represented, as the fluorescent signal is proportional to the amount of fluorophore that diffuses in a particular neuronal structure. This becomes evident in the poor resolution of the fine structure of dendritic spines offered by mice expressing cytosolic GFP (Feng *et al* 2000). Since the fluorophore signal intensity is directly proportional to the amount of the cytoplasm, and thus to the size of the structure, the intensity of the signal goes down as we go from the primary to the tertiary dendrites. Thus, small structures such as dendritic spines and thorny excrescences are not labeled properly. Moreover, due to the uneven levels of the GFP signal, artifacts are invariably introduced in the imaging process.

A solution to this problem is to devise a way so that the fluorescence signal intensity is independent of the volume of the body being imaged. We achieved this by tagging GFP to the membrane anchoring domain (N-terminal domain) of a human membrane anchoring protein called MARCKS. Thus, the GFP signal expresses only along the cell membrane, and is independent of the volume of the body. This leads to uniform signal intensity from all structures, and makes even the smallest details visible without any artifacts. In principle, by this method GFP can be replaced by any other genetically encoded fluorophore, thus making this approach more versatile for simultaneous imaging of pre and post-synaptic terminals. Coupled with deconvolution, membrane GFP allows resolution of

dendritic structures that are at the theoretical limit of resolution of a light microscope. Chapter 1 of this thesis describes the results of using m-GFP to image dendrites, and compares the results with those obtained using cytosolic GFP.

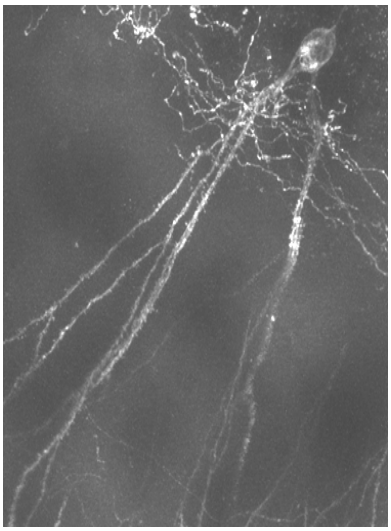
Formation, Stability, and Maintenance of Dendritic Arbors in the Brain.

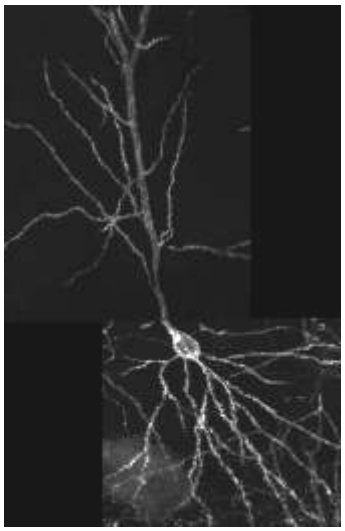
Dendrites are the postsynaptic counterparts in the brain circuitry, specialized for receiving, integrating and processing the vast majority of excitatory synaptic inputs. Though it was long considered that dendrites transmit this information passively to the soma, recent evidence suggests that dendrites act as dynamic integrators of synaptic input (Hausser et al 2000). The transmission of the synaptic signal to the soma is greatly affected by the branching pattern of the dendritic tree, and the striking variation in the dendritic tree morphology in the CNS therefore has an enormous consequence on neuronal information processing. They are the sites where presynaptic terminals release their neurotransmitter cargoes leading to the cascade of events that ultimately lead to the propagation of the action potential. In mammals, dendrites have actin rich protuberances called dendritic spines that are the actual sites of excitatory synapses. Dendritic spines are believed to be an evolutionary adaptation to increase the net surface area available to make synapses. About 10% of total synapses in the brain are inhibitory in nature, and these are formed by direct contact between the presynaptic terminal and the dendritic shaft.

Dendrites come in an enormous variety of shapes and forms. In many cases, the shape of the dendritic arbor can be related to the mode of connectivity between neurons, with the dendrites often ramifying in characteristic spatial domains where they receive specific inputs. The characteristic dendritic branching of specific cell types is so characteristic that specific neuronal subtypes are known and named according to their dendritic branching patterns. Dendrites can essentially be thought of as extensions of the cell body meant to provide increased surface area at much lower cell volumes. It is estimated that 97% of

the surface area of a motor neuron (excluding the axon) is dendritic (Ulfahke & Kellerth 1981). The dendrites have 370,000sq.microns of surface area while occupying only 300,000 cubic microns. To provide an equivalent surface, a spherical body would be 340 microns in diameter and occupy 2,000,000 cubic microns. The fact that 80% of the surface area of proximal dendrites of motor neurons is covered with synapses (Kellerth et al 1979) suggests that this increased surface area is indeed valuable for increasing the number of inputs to a neuron.

Dendrites make relatively local connections compared to axons. Whereas axons can extend to distant targets, sometimes up to a meter or so away from the cell body, dendrites are rarely longer than1-2 mm, even in the largest neurons. The diameter of dendrites is inversely proportional to their degree of branching (viz. primary, secondary, tertiary etc) and to their distance from the cell body.





Neurons are classified structurally according to the types of their dendritic arbors because the architecture of the arbors is remarkably preserved in specific types of neurons. This had led to the view that dendritic arbor formation is governed entirely by cell intrinsic programs. With the advent of more sophisticated analysis, however in the last decade the consensus opinion has shifted more towards the

idea that dendritic structure develops as a part of a continuous dynamic process that balances the effect of cell-intrinsic factors, neuronal activity, neurotrophins, and homeostatic mechanisms. Dendrites develop as a part of the neural circuit, and constant feedback and feedforward mechanisms operate during the development of the circuits to ensure that they will function in the developing and the adult animal. The consensus opinion these days is that dendrites are formed quite early in development and remain essentially stable throughout the rest of the life of the organism. Our knowledge about the formation of the dendritic tree derives from the studies conducted on cortical neuronal cultures (Scott & Luo 2001), the developing tadpole (Cline,HT 2001), and the epidermal neurons of the developing drosophila larva (Grueber *et al* 2003).

Cortical neurons in cultures do not begin to extend a well differentiated dendrite until a day or two after the axon has emerged. Strikingly, the dendrites of neurons plated on cortical slices behave just like endogenous pyramidal neurons, thus arguing in favor of an intrinsic program responsible for dendrite formation. Neurotrophins are known to act through the Trk family of tyrosine kinase receptors to increase the dendritic complexity of pyramidal neurons by increasing total dendritic length, the number of branchpoints, and/or the number of primary dendrites (Baker *et al* 1998, McAllister et al 1995, Niblock et al 2000). The response is rapid, and an increase in complexity is apparent within 24 hrs of neurotrophin exposure. Though the generic effects of the neurotrophin exposure on dendrites are broadly the same, there are minor differences in the way different neurotrophins act on different types of neurons. Recently, BMP-7, a member of the TGF-B family has also been shown to affect growth and branching in cortical neurons (Le Rouxet al 1999). Further, IGF-1 has also been shown to affect dendritic growth and branching in layer 2 cortical neurons (Niblock et al 2000). In contrast to neurotrophins, IGF1 affected both apical and basal dendritic growth, illustrating that the final dendritic complexity of pyramidal neurons is likely to be influenced by the action of multiple neurotrophic factors. The short term dynamics involved in the effects of neurotrophins on dendrites indicate a rapid modulation of cytoskeletal elements by neurotrophin signaling. Out of the major

pathways activated by the Trk receptors, the MAP-kinase pathway and the PI3-Kinase pathways have been implicated in neurite formation in both neuronal cell lines and primary neurons. (Posern et al 2000, Wu et al 2001). It is likely that these signaling pathways influence neuronal morphology by regulating the activity of the Rho family GTPases, which mediate actin cytoskeleton dynamics, and are known to induce rapid dendritic remodeling. Experiments in neuronal cell lines show that NGF can activate the small GTPase Rac1 in a PI3 Kinase dependent manner, and this activation is necessary for neurite elaboration (Kita et al 1998, Posern et al 2000, Yasui et al 2001). Part of the effect of neurotrophins on dendrites may also include the control of expression of structural proteins. It was recently reported that BDNF can upregulate local protein synthesis in dendrites within hours (Aakalu et al 2001).In addition, specific mRNAs for several cytoskeletal proteins are present in dendrites (Kuhl and Skehel 1998). This raises the interesting possibility that local synthesis of structural components is involved in the neurotrophins mediated control of dendritic growth. In addition to neurotrophins, recent studies have implicated Notch signaling in the control of contact dependent inhibition of dendritic growth. For example, in postmitotic neurons there is an inverse correlation between Notch1 expression and total neurite length, and overexpression of constitutively active Notch 1 construct leads to a reduction in total dendrite length (Sestan et al 1999). Expression of constitutively active Notch1 in the hippocampal neurons also leads to an inhibition of neuritic outgrowth. Inhibition of Notch1 signalling by overexpression of dominant negative Notch1 construct or with antisense oligoneucleotide treatment leads to a decrease in dendritic branching in neurons. Taken together, these experiments reveal a positive role of Notch1 in dendritic branching, and a negative role in dendritic and total neurite length.

Recent studies in Drosophila have also led to the identification of genes responsible for the control of dendritic branching and growth. This has been facilitated in part because of the accessibility that the drosophila larval system allows to study the development of the dendrites of the epidermal neurons. Using

the dendritic arborization (*da*) neurons of drosphila as a model system, the homeodomain protein Cut was found to regulate distinct dendritic branching patterns in Drosophila (Grueber et al 2003). The levels of the Cut protein in individual *da* sensory neurons correlates with distinct patterns of terminal dendrites: high Cut in neurons with extensive unbranched terminal protrusions, medium levels in neurons with expansive and complex arbors, and low levels of Cut in neurons with simple dendrites. Downregulation of Cut levels reduces dendrite growth and branching, whereas overexpression of Cut led to a tendency towards greater dendritic branching.

Live imaging of dendritic growth shows that it is a very dynamic process. (Gao et al 1999, Grueber et al 2003, Sugimura et al 2003). Dendrites can branch either via bifurcation of growth cone like tips or through interstitial sprouting of new branches from an existing dendrite branch. These new branches extend and retract to undergo constant remodeling. Only a subset is eventually stabilized. Table 1.1 gives a list of known drosophila mutants and their phenotypes in dendritic morphogenesis.

In contrast to flies, our knowledge of dendritic branching and morphology from mice is rather limited. This is largely because of the relative inaccessibility of the system to detailed non-invasive morphological examination. The extremely high density of neurons in the CNS, and the inadequacy of prevalent methods in labeling fine dendritic structures are additional reasons for this lack of information. However, some recent studies have identified a few genetic elements responsible for control of dendritic branching in mice. Dendritic branching of the barellette cells of the trigeminal principal nuclei have been shown to depend on NMDA receptors (Lee et al 2005). With the reduction of NMDA currents, the postnatal barrel cells retain their membrane and synaptic properties but develop longer dendrites with no orientation preference. This study makes the conclusion that NMDARs control growth of presynaptic terminal arbors and postsynaptic branching, thereby leading to consolidation of synapses and consolidation of pre

Drosophila Mutant	Major Dendritic Phenotype.
Dcdc42	outgrowth and branching
prospero	misrouting
sequoia	excessive dorsal branch outgrowth
tumbleweed	excessive branching
shrub	excessive branching
shrinking violet	reduced lateral branches
flamingo	excessive dorsal branch outgrowth and misrouting
Kakapo(short stop)	reduced lateral branches
enabled	misrouting
yew	misrouting
cut	reduced dendritic branching.
ponderosa	reduced lateral branches

Table 1.- Important Drosophila mutants having defects in dendritic growth and branching.

and post synaptic elements. Consistent with the fact that dendritic abnormalities are a hallmark of neuro-pschyciatric diseases, overexpression of the methyl-CpG-binding protein MeCP2 causes increased branching in cultured cortical neurons (Jugloff et al 2005). Modulators of the actin cytoskeleton like the Rho family of small GTPase have been known for a long time to have an effect on dendritic length and branching. However, the upstream signaling pathways responsible for these effects are still poorly understood. However, Wnt7b is one such upstream molecule that is known to regulate dendrite branching in cultured hippocampal neurons. Wnt7b is known to express in the mouse hippocampus

and its overexpression causes an increase in dendritic branching. This effect is mimicked by the expression of Dishevelled (Dvl), a downstream effector of Wnt7b and is blocked by Sfrp1, a secreted Wnt antagonist. This action of Wnt7b is independent of GSK3B signaling, and goes through Rac and JNK (Rosso et al,2004). A recent study also implicates the non receptor tyrosine kinases Abl and Arg in the maintenance of cortical dendrites in the mouse brain. $Arg^{-/-}$ mice have normal dendrites till the onset of adulthood (21days). Thereafter, there is a progressive loss of dendritic branches leading to a reduction in dendritic arbor size (Kolleske 2005). In another study conducted on cultured neurons, core components of the Origin Recognition Complex (ORC) have been implicated in the regulation of dendritic arbors (Reichardt 2004).

Chapter two of this thesis describes the role of the neuronal protein Cap23 in the maturation and maintenance of dendritic arbors. The use of membrane targeted GFP under the Thy1 promoter allows the dendritic arbors of single CA1 pyramidal cells in the hippocampus to be seen with an unprecedented level of clarity. Using mice that are either heterozygous or null for the Cap23 allele, we show that there is a progressive loss of dendrites in mice that have low levels of the Cap23 protein.

Goal of the Thesis.

The aim of this thesis is to understand the role and mechanism of the neuronal protein Cap23 in regulating synaptic connectivity in the brain. Previous studies using Cap23 deficient mice had shown the presence of abnormalities in the structure of peripheral synapses (NMJ). This led to the hypothesis that Cap23 could have a general role in the maintenance of synapses in the CNS as well. In addition, 2-D gel analysis of proteins expressed in the brain of mice deficient in Cap23 showed the misregulation of a number of proteins important in synapse structure and function (Wacha & Caroni- unpublished results).The experiments detailed in this thesis are designed to investigate this hypothesis.

Towards this aim, we use mice expressing membrane targeted GFP in a subpopulation of neurons. The use of deconvolution confocal microscopy, allows us to visualize a neuron in the entirety of its arbor and its fine structure in great detail. Using this tool, we then follow the arborization of dendrites of CA1 pyramidal cells in mice of different ages deficient in Cap23. We further follow up these observations with transcriptome analysis of the brain of Cap23 deficient mice using microarrays to get possible clues about the role of the protein *in-vivo*. The results of these experiments and the conclusions and inferences thereof form the contents of the chapters II and III and the supplementary materials after that.

Chapter II:

Membrane tagged GFP enables high resolution visualization of synaptic morphology and the fine structure of dendrites.

ABSTRACT

The morphological details of the neuron and the fine structure of the synapse have important functional correlates in the proper functioning of the brain. Morphological abnormalities in the structure of the axon, dendrite, or in the fine structure of spines is indicative of functional abnormalities and pathological states. The ability to resolve better the structure of neurons also makes the nervous system amenable to more methodical scrutiny and study. Methods of visualizing neurons so far suffer from drawbacks of non specificity, invasiveness, lack of resolution etc. Recently, the creation of stable mouse lines expressing soluble GFP (cGFP) driven by the Thy-1 promoter in a subset of their neurons has been reported. Here we present data to show that lines of mice expressing membrane targeted GFP (m-GFP) in a subpopulation of neurons allow the visualization of neuronal structures with an unprecedented level of resolution and clarity. We make comparisons with data from cGFP lines and electron micrographs, and show that this mouse line enables the clear visualization of a subclass of dendritic spines that could not be seen hitherto by conventional light microscopy.

Further, we demonstrate the utility of this tool by quantitating the volumes of single spine heads, and comparing the values with those reported in the literature. On the basis of this, we propose that these lines of membrane tagged GFP are an important new tool with a decisive advantage over others to image fine structure and dynamics of neurons in the CNS.

INTRODUCTION

The mammalian brain, on an average contains about 10^{13} neurons. Each of these form, on an average, about 10,000 synapses with its neighbours, thus making the brain a neuronal circuit of incredible complexity. Added to this are an equal number of non-conducting cells that perform the auxillary functions to make the brain a functioning entity. Neurons vary remarkably with respect to each other with respect to their shapes, sizes, and branching. These differences in appearance have direct bearing on the functional significance of the dendrites.

To understand the brain at the molecular, cellular and circuit level, both structural and functional studies are essential. Of central importance in all these approaches is the ability to visualize a single neuron or a defined class of neurons in their entirety. This is of particular importance in studies of brain circuits where it is of utmost importance to be able to tell apart the tree from the forest. A microlitre volume of the mouse cortex contains approximately 10^5 neurons, 10^9 synapses and about 4 kilometers of axons. To be able to study functional neuronal circuits in such compactly arranged tissue, it is necessary to be able to have a way to label single neurons in a manner such that their entire dendritic and axonal arborization is visible.

The ability to visualize neurons and their fine structures in their entirety is as important in understanding normal brain physiology as it is in understanding its pathology. There is now emerging consensus that almost all neuropathological diseases are ultimately diseases of the synapse. Whether the origin is from the presynaptic side or the postsynaptic side, almost all neuropsychiatric diseases manifest themselves in form of a visible defect in the structure of the dendritic spine, or the dendrite in its entirety. Thus genetically linked neuropsychiatric diseases like Down's syndrome, Rett's syndrome, Fragile -X syndrome- all of these are associated with phenotypes in dendritic structures, notably spines(Huttenlocher,1974;Purpura 1975;Kaufmann And Moser 2000,Broadbelt *et al* 2002,Jones 2004).

Dendritic spines are the target of about 90% of the synapses in the brain. Morphologically and functionally they are very heterogeneous in terms of their morphologies, receptor distribution and calcium influx pathways, though they are classified into a few main types. Subclasses of these spines are upregulated or downregulated in specific neuropsychiatric diseases. Specific morphological types of spines are unique by virtue of the presence of an elaborate spine apparatus or transcriptional machinery at their base. This has important implications for synapse strengthening and in learning and memory in terms of local translation of important transcripts. The proper visualization of dendritic structures is of paramount importance also in electrophysiological studies of the brain. Spines are known to be units for compartmentalization of calcium. The calcium dynamics of spines are known to depend on their dendritic location, and their shapes. Thus it is important to be able to visualize accurately the shape of dendritic spines.

Traditionally, ultrastructural and immunocytochemical studies provided us the first detailed pictures of neurons and synapses. The earliest of the labeling techniques, namely the Golgi impregnation technique was first used in the beginning of the 20th century, and became a very powerful method which is still in use for samples that are not amenable to molecular genetic interventions, for example human brain samples. In this method, treatment with silver salts led to deposition of the metal stochastically on a random subset of few neurons making them visible. Subsequently, other staining methods introduced in the 1970s and the 1980s included immunostaining for specific transmitters, cell-type specific molecules, as well as microinjection of a wide range of anterograde and retrograde tracers (eg. Horseradish peroxidase, biocytin, lucifer yellow etc). However, the main drawback of all these methods was that they did not allow visualization of living tissue, and gave no indication of the dynamic nature of the brain. With the advent of fluorescent lipophilic dyes like DiI and DiO and the FM dyes, it became possible for the first time to visualize neuronal morphology in living tissues in vitro, and raising the possibility of studying the dynamics of neurons and neuronal processes. Using these approaches, elegant studies were

conducted to study synapse morphology, formation and remodeling at the neuromuscular junction (NMJ), and to study synaptic vesicle recycling at the NMJ *in vitro* and in cultured neurons. Though the arrival of these lipophilic dyes heralded a quantum leap in optical imaging techniques they suffered from intrinsic drawbacks. Dye labeling depended on the ability to identify precisely and access the neuron of interest, which was often a problem. In addition to that, there was the persistent problem of non-specific staining and background problems that often interfered with normal tissue function to present serious artifacts. Since discrete populations of neurons can be defined by patterns of gene expression; reporter-based tracers have greatly increased the ability to label subsets of neurons and their processes. Markers like the *lac*-z product ß-gal have been used to label specific populations of cells, and in the attempt to label processes a number of ß-gal fusion proteins have been introduced as first attempts in *Drosophila,* and also in mice (Mombaerts et al., 1996).

The discovery and popularization of the green fluorescent protein and its spectral variants in the 1990s heralded a new era in vital imaging. The great advantage of this genetically encoded fluorophores is that they can be fused to almost any protein or structure of interest to render it visible. Methods like particle mediated gene transfer (gene gun) and viral based approaches were developed to deliver GFP into cells or defined brain regions. Unfortunately these methods were labor intensive, difficult to control, and in many cases compromised cell viability intensively.

Genetic methods to express GFP or GFP fusion proteins in particular sets of neurons are available in model systems like *Drosophila* (Edwards et al., 1997), *zebrafish* (Higashijima et al., 2000), *C. elegans* (Nonet, 1999) and in mouse (van den Pol and Ghosh, 1998). In particular, the use of a neuron specific promoter, the Thy.1 expression cassette (Caroni, 1997) to drive the expression of different spectral variants of cytosolic fluorescent proteins**,** proved to be a valuable tool to study neuronal morphology and dynamics in vivo (Feng et al., 2000; Walsh and Lichtman, 2003); (Grutzendler et al., 2002; Trachtenberg et al., 2002). However,

the cytosolic nature of the fluorescent proteins presents a serious problem when detailed morphological analysis is needed, because small compartments like filopodia and tiny protrusions may be poorly represented, as the fluorescent signal is proportional to the amount of fluorophore that diffuses in a particular neuronal structure. Moreover, a way to monitor in vivo subcellular compartment (e.g. pre-synaptic compartments) dynamics in a non-invasive manner in the mouse is currently lacking.

To address this problem we generated transgenic mice expressing GFP targeted to the membrane in a small subset of all the neurons, and used these mice to study presynaptic terminal dynamics in the hippocampus (De Paola *et al* 2003). We provide evidence that our line of mice expressing membrane targeted GFP(m-GFP) provide a much better system to study fine neuronal structure and dynamics compared to existing models of transgenic line expressing soluble GFP. Using deconvolution microscopy, we show that the m-GFP line enables the visualization of fine dendritic structures that are not visible in the c-GFP mice, and also enables visualization of details with greater resolution. This is the first report on the fine morphological details and dynamics of immature granule cell axons and dendritic spines in a mature network. On the basis of all this, we suggest that mGFP is a superior tool in the study of neuronal plasticity compared to cGFP.

RESULTS.

1. The overexpression of the fusion protein does not cause any detectable phenotype.

The m-GFP expressing transgenic mice had no obvious phenotype, and the expression of these transgenes did not affect neuronal physiology and morphology. The mice were normal in terms of their longevity, exhibited normal breeding pattern and vigor and showed no evidence of coronal lesions. Finally, neurons expressing different levels of the mGFP transgene exhibited undistinguishable morphologies in brain slices (not shown). These data confirm previous reports from the literature that indicate that mGFP and spGFP are suitable markers for the visualization of the neuronal membrane and presynaptic compartments respectively (Callahan et al., 1998; De Paola et al., 2003; Li and Murthy, 2001; Moriyoshi et al., 1996) and validate the use of membrane-targeted GFP markers in transgenic mice.

We also transfected the membrane targeting domains in COS cells and observed no change in cell morphology (Data not shown). The GFP signal localized in a crisp pattern along the cell membrane giving a clear signal. As already reported (Moriyoshi et al., 1996) plasma membrane-targeted GFP does not alter cell morphology and visualizes the whole cell membrane effectively.

2. High resolution imaging of dendritic spine morphology.

We first tested the hypothesis that a plasma membrane-targeted variant of GFP (mGFP) in transgenic tissues reveals details of fine neuronal structures that appear absent in cytosolic GFP (cGFP) expressing cells from transgenic mice (Feng et al., 2000) . Since cGFP is starting to be widely used also for the study of central synapses *in vivo* (Grutzendler et al., 2002; Trachtenberg et al., 2002), we studied this important issue in the context of dendritic spine imaging. We imaged dendritic branches from two of the most intensively studied brain regions: the hippocampus and the cortex. We compared neurons from mice expressing cGFP

(from Feng et al., 2000) obtained from the laboratory of E.Welker with neurons from mGFP expressing mice with confocal microscopy and treatment with deconvolution software. Representative examples are shown in (Fig 1). At low magnification a major difference is evident. cGFP diffuses in apical and basal pyramidal dendrites but, surprisingly, faintly labels the basal dendrites of CA1 and cortical pyramidal neurons. The intensity of the cGFP fluorescence falls of rapidly in distal dendritic structures. m-GFP, in contrast, labels all regions uniformly. At higher magnification spines are visible in cGFP expressing neurons but the fine details of the outlines of spines are completely absent, limiting the use of this tool for spine size measurements and subtype classification. For example, all mushroom-shaped spines appeared to have a globular head in cGFP lines. In contrast mGFP expressing spines revealed the typical diverse shapes (including craggy appearances of mushroom-shaped spines) seen from electron microscopic reconstructions and other membrane labeling techniques (Fiala et al., 2002); Richards, D.A., De Paola, V., Caroni, P., Gähwiler, B.H. and McKinney, R.A., (2003a) and are thus amenable for detailed size measurements and monitoring. While cGFP labeling makes the dendrite appear like a smooth tubular structure, mGFP labeling brings out the irregular nature of the dendritic surface too. Since spine size is closely related to synaptic strength (Nimchinsky et al., 2002) the advantage represented by the mGFP marker for high-resolution analysis of both spine structure and dynamics appears considerable. Moreover, quantitative analysis of spine densities and morphology in mGFP mice is comparable to those from electron microscopy and other membrane staining techniques (Richards, D.A., De Paola, V., Caroni, P., Gähwiler, B.H. and McKinney, R.A., (2003a). We noted the greatest difference between the two markers when the complexity of the spine arrangement along dendritic segments were maximal, as in primary dendritic branches. In fact, in these segments, the bright dendritic shaft fluorescence signal literally covers the numerous spines that emerge from the surface of it, thus limiting the use of this tool for high resolution imaging of complex dendritic regions. When the gain is reduced, several protrusions, with far less fluorescence signal than the one coming from the dendritic shaft, are no longer visible in cGFP dendrites. In contrast, under the

same conditions, mGFP-labeled complex dendritic regions can still be resolved at the level of single filopodia and spines (Fig. 2). To independently and directly test the hypothesis that mGFP labels neuronal structures not labeled by soluble fluorescent markers, we injected soluble fluorescent dye (microruby) in mGFP expressing neurons in organotypic slice cultures in vitro. Figure 2 shows that the cytoplasmic marker fails to label fine structures like spinules, the neck of thin spines and dendritic filopodia (see overlay). Thus, since mGFP expressing spines seem to reveal the known complex outlines of spine heads and necks, whereas cGFP spines do not, these data show that the cGFP marker is unsuitable for high-resolution imaging of dendritic protrusions and indicate that mGFP could be an appropriate alternative.

3. Measurement of spine volumes.

To further test the fact that the spines rendered visible by the mGFP marker are indeed faithful representations of the reality, we compared their morphology with data reported in the literature from serial micrographs (Nimchinsky, Sabatini and Svoboda- Ann.Rev.Physiol. 2002). Morphological examination clarifies that mGFP labeled spines appear in their characteristic polymorphic shapes with craggy outlines as seen in electron micrographs. We further calculated the volume of the spines imaged by us, and then compared their values with analogous values reported in the literature from serial electron microscopic representations (see methods). The reported values for spine heads range from ~ 0.001- 1 cubic micrometres, with the upper end of the range representative of spines with prominent heads. We considered spines (n=10) with prominent heads located at different positions on dendrites taken from neurons in organotypic slice cultures. Figure 3b shows five typical examples. Volume measurements were carried out using the Imaris Topography software. The average value obtained for spine head volume was 1.050 cubic microns, which is in striking similarity with reported values. This proves conclusively that mGFP labels spines precisely and that it is a much more faithful labeling technique compared to cGFP.

DISCUSSION.

Recent developments in imaging techniques have enabled the visualization of synapses in real time leading to the discovery of the structural plasticity of dendritic spines. We developed membrane targeted-GFP transgenic mice with selective expression in many classes of neurons that could be useful in this context and that would have advantages over previously available methods of vital staining. Our fusion protein constructs do not alter the physiology or the morphological appearance of cells in any detectable way. When expressed in cultured cells, they label the entire cell uniformly and do not produce any morpholological aberrations in the cell. Electrophysiologically too, NMJs stained with mGFP appear normal and show the same quantal content as the wild type NMJs.

The superiority of mGFP as a label is in its ability to clearly mark fine structures by being able to crisply label the cell membrane. To establish the fact, we imaged dendritic spines and compared our results to pictures taken in the same manner using mice expressing cGFP under the Thy1 promoter (Feng et al 2000). These mice are the best available tool in the field so far. Comparison of the pictures obtained from the two lines bears out clearly the superiority of the mGFP mice. Consistent with the cell culture experiments, in neurons too mGFP labels all neuronal processes uniformly while cGFP fails to do so. The difference is most striking when fine structures like dendritic spines are imaged. m-GFP expressing lines elaborate the craggy structure of the dendritic shaft and the polymorphic nature of dendritic spines with a resolution comparable to serial electron micrographic reconstructions. In lines that express cGFP, in contrast, the signal does not diffuse into fine structures. Thus, all prominent spines appear with a globular head, while spinules and the very small or thin filopodial spines and spine necks are not rendered visible. This feature of the mGFP makes it a very valuable tool for the study of synapses both in normal and pathological conditions. The information on the outline of spines is invaluable because it reveals the diversity and complexity of synapses. This can be useful in monitoring synapse

remodeling over extended periods of time and in i*n-vitro* electrophysiological studies investigating events at the level of defined synapses like activity dependent synaptic remodeling. Defects in dendritic arborization and spine morphology and number are a hallmark of all neuropsychiatric diseases (Fiala et al 2002). Thus, these lines of mGFP mice have the potential of becoming an invaluable tool as a morphological marker for a visual assay in detecting such diseases.

Values of spine volume obtained from the mGFP lines are in good agreement with values obtained from EM reconstructions, further supporting our claims. Further, to show the intrinsic inability of soluble cytoplasmic markers to label fine structures, we co-label the same mGFP expressing dendrite with a soluble dye and show that certain structures are not labeled.

In conclusion, we present data to show that targeted expression of GFP to the cell membrane allows high resolution non-invasive imaging of synaptic junctions in the CNS both in vivo as well as in vitro. Based on our data and comparisons with cGFP labeled cells, we propose that mGFP is a much more advanced tool for neuroscience research.

MATERIALS AND METHODS.

1. High resolution imaging of dendritic spines.

Mice (three months old) expressing the cytosolic form of YFP and the membrane tagged form of GFP under the Thy-1 promoter were perfused transcardially. Briefly, mice were first perfused with Lactated Ringer's solution (Ringer's solution containing 50% sodium lactate) at $4^{o}C$ and subsequently with a fixative solution containing 4% paraformaldehyde at room temperature. Coronal sections of thickness 40-50 microns were taken in a vibratome.

Confocal images were taken in a Olympus Fluoview microscope (confocal aperture 90-100 microns), and deconvolved using the Huygens deconvolution software. Confocal stacks of thickness 0.15 microns were taken throughout the thickness of the dendrite and reconstructed using the Imaris software to get the three dimensional view.

For measurement of spine volumes, serial confocal sections of 0.15 micron thickness were reconstructed using Imaris, and the volume of the heads of isolated spines was measured using the Imaris Topography software.

Figures.

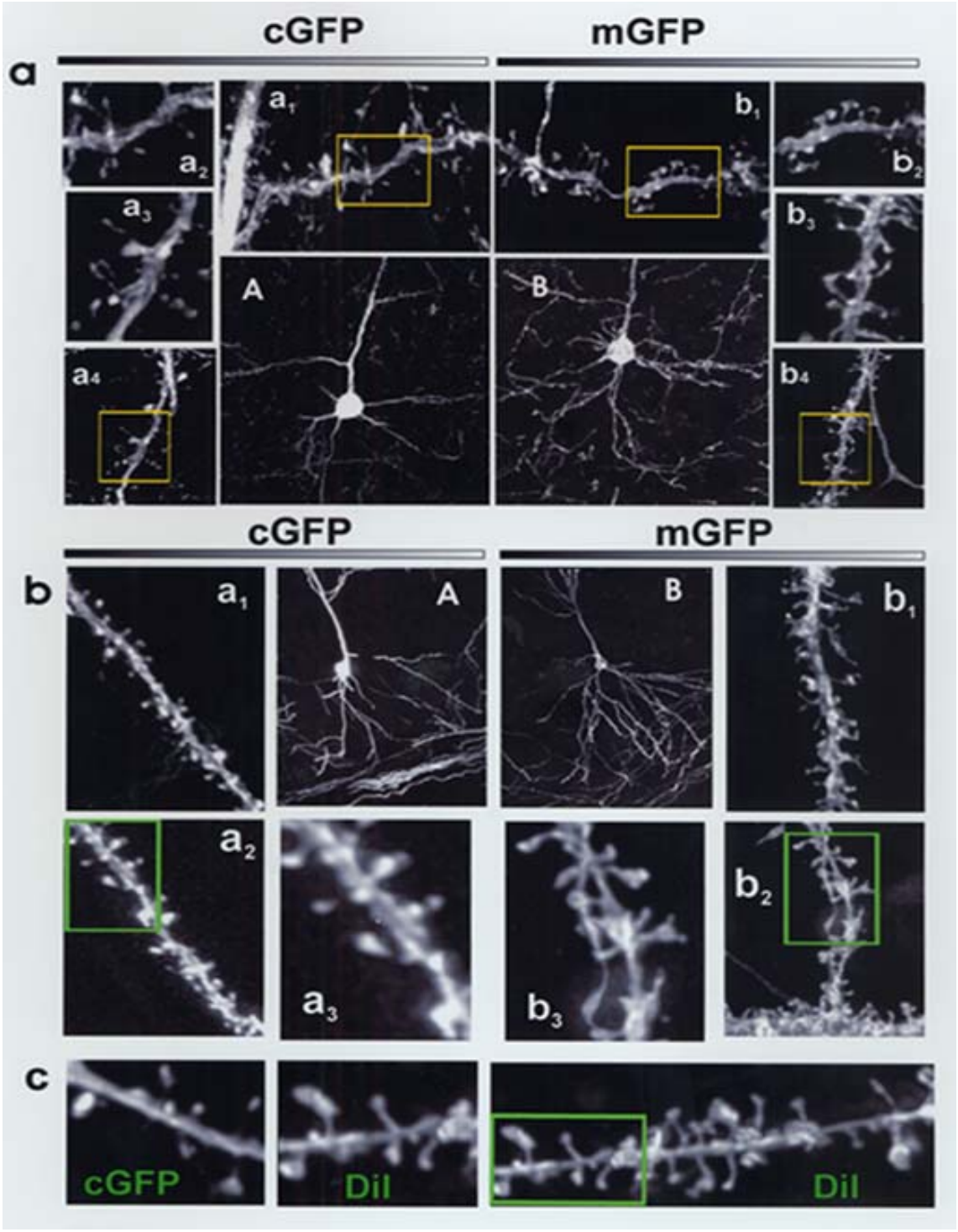


Figure 1: Comparison of clarity between mGFP and cGFP.

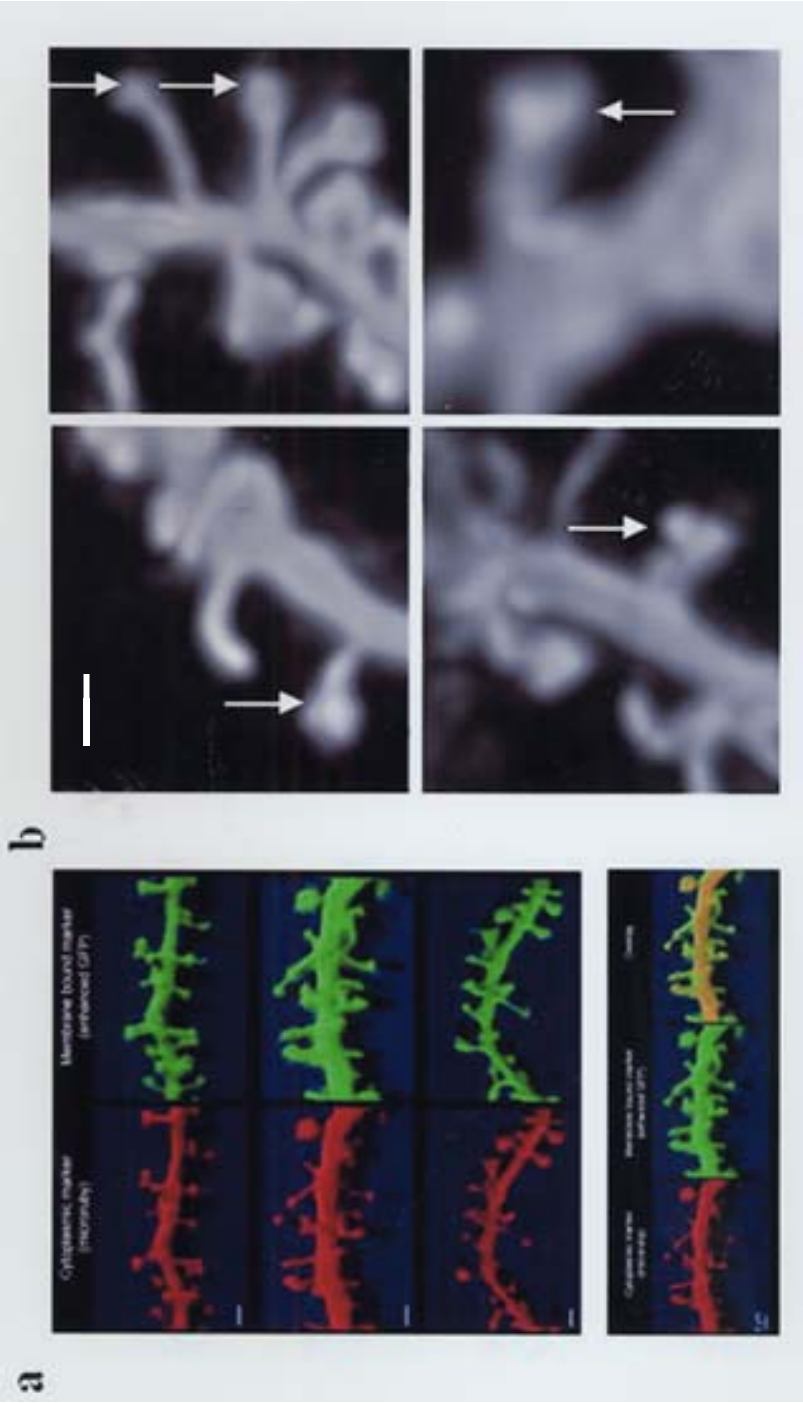


Figure 2: Comparison of the details elucidated by the m-GFP marker and (micro-ruby).

FIGURE LEGENDS.

Figure 1: mGFP expressing spines reveal the known complex outline of spine heads and necks whereas cGFP spines do not.

(**a**) High resolution imaging of cortical dendritic spines in mGFP versus cGFP transgenic lines.

(**A**, **a_1-a_4**) cGFP expressing cortical pyramidal neuron, (**B**, **b_1-b_4**, mGFP). Note the incomplete labeling of basal and apical distal dendrites (**A**) when compared to (**B**, mGFP cortical neuron). The entire dendritic tree and axons are sharper in m-GFP expressing pyramidal neurons (B). **a_1** (cGFP) versus **b_1** (mGFP) represent comparable regions from the apical dendrites. Note at high magnification (100X with 5X digital zoom), spines are not as clearly resolved in the cGFP (**a_2**, corresponding to the yellow rectangles in a_1) as is in case of the m-GFP (**b_2**). In particular, the spine neck is not visible and the finer structures of the head as well as neck are not resolved. **a_3** and **b_3** show the comparison for basal proximal dendrites. The distal region of the basal dendrites, where spine density is higher and the dendritic shaft is thinner, is shown in (**a_4**, cGFP and **b_4**, mGFP). Note the highly irregular structure of the spine neck and the head region in the mGFP example. Note also large number of spines in the m-GFP marker example, not visible with cGFP.

(b) High resolution imaging of hippocampal dendritic spines in mGFP versus cGFP transgenic lines.

(**A**) (cGFP) and (**B**) (mGFP) show the distribution of the cGFP and m-GFP markers in hippocampal pyramidal neurons at low magnification. Note strong cGFP signal only in cell bodies and proximal apical dendrites (top part of panel) and striking faint signal in distal parts of basal dendrites (bottom part of panel). mGFP, in contrast, labels effectively the whole neuronal membrane. At higher magnification (60X) in **A_1** (cGFP) and **B_1** (mGFP) note the more uniform signal around the neuronal membrane in mGFP. **a_1** (cGFP) versus **b_1** (mGFP) represent comparable regions of secondary apical dendritic segments. Note globular appearance of cGFP spine heads and diverse shapes in the mGFP example. This feature is also true for other cells in the hippocampus (data not shown). **a_2**, cGFP, and **b_2**, mGFP, show pictures of primary basal dendrites close

to the cell body. **a_3**, cGFP, and **b_3**, mGFP, show pictures of primary distal basal dendrites. Note here as well the varied shapes in the mGFP example but not in cGFP. **a_4** shows a high magnification view of the insert in **a_3**, whereas **b_4** corresponds to the green insert in **b_3**. Note that the mGFP marker visualizes complex spine regions in great details highlighting its advantage for high-resolution imaging of fine dendritic structure.

Figure 2: Comparison of the details elucidated by the m-GFP marker and (micro-ruby).Panel a shows three dendritic segments imaged for micro-ruby(red channel) and m-GFP(green channel) in the same dendritic segment to show that the m-GFP marker outlines structures nor shown by the cytosolic dye(last panel-overlap)..Note that in the microruby channel certain structural details such spine necks and the heads of spinules are not clearly visible whereas they are visible in the green channel. The overlay in the last panel illustrates this. Panel b shows representative pictures of deconvolved confocal images of four isolated spines(scale bar- 1µ) at high resolution and magnification(100x magnification and 10X digital zoom) used for measuring spine head volumes.

CHAPTER III

A critical role for the neuronal protein CAP23 in the maintenance of dendritic arbors in vivo.

Abstract.

Though a lot is known about genetic elements that regulate the formation of dendrites, very little is known about the genes involved in their maintenance. To understand the role of the neuronal protein Cap23 in the maintenance of dendrites *in-vivo* , mice deficient in Cap23 were obtained in the background of a line expressing membrane targeted GFP under the Thy1 promoter. Imaging the CA1 pyramidal cell dendrites across different ages in mice deficient in Cap 23 led to the observation that these mice start losing dendritic branches from around the onset of adulthood. This phenotype had a definite onset point and was thereafter progressive in nature. This atrophy started from the higher order dendrites(eg. tertiary) and then proceeded towards the primary branches. The branches that survived this atrophy tended to increase in thickness and develop more number of complex spines that ultimately led to conservation in terms of the total synapse numbers. On the basis of these major observations, it seems likely that Cap23 is involved in the maintenance of dendrites *in-vivo*.

INTRODUCTION.

CAP23 is a major cortical cytoskeleton–associated and calmodulin binding protein that is widely and abundantly expressed during development, maintained in selected brain structures in the adult, and re-induced during nerve regeneration (Frey et al 2000). It has homologs in rats (Nap22) and in humans (Basp1). In mice, the Cap23 cDNA is about 1.5kb long, and codes for a 23 kD protein. The protein is known to be enriched in the brain and is known to have a calcium dependent Calmodulin binding activity. The exact result and significance behind Calmodulin is not exactly clear. Cap23 is known to be a substrate of Protein Kinase-C (PKC) *in-vivo*, and Calmodulin is known to inhibit this phosphorylation. The protein localizes to the cell membrane through the myristoylation and palmitoylation motifs present in its N-terminus. Various lines of evidence suggest that Cap23 maybe responsible for formation, maintenance and maturation of synapses by controlling cholesterol dependent membrane dynamics (Frey *et al* 2000, Laux *et al* 2000, Kashihara *et al* 2000). The protein is also thought to bind microtubules, and thus contribute to the maintenance of the integrity of the cytoskeleton. There are also reports that the protein is selectively present in the synaptic vesicles of rat brain (Yamamoto *et al* 1997), on the basis of which it has been implicated in synaptic vesicle recycling. In the peripheral nervous system (PNS), the NMJs of mice lacking Cap23 have abnormal appearances and defects in paralysis induced nerve sprouting (Frey *et al* 2000). At the amino acid level, Cap23 shows a resemblance with the growth associated protein Gap43. transgenic overexpression of Gap43 in mice lacking Cap23 rescues many of the defects associated with Cap23 knockout mice. Along with Gap43 and MARCKS, Cap23 forms a family of proteins that are involved in neurite growth and maintenance of the actin cytoskeleton. These three proteins share a number of important characteristics including the following:

1. Regulated, abundant expression related to contact mediated differentiation, cell surface activity, motility and process outgrowth.
2. Membrane association mediated by palmitoylation or myristoylation.

3. Highly hydrophilic, with markedly acidic isoelectric points, rodlike structures, and a unique amino acid composition.
4. The presence of one unique stretch of 10-28 basic and hydrophobic residues, the effector domain(ED); this domain binds acidic phospholipids, calmodulin and actin filaments in a mutually exclusive manner, and contains the PKC phosphorylation sites.
5. Colocalisation at characteristic patches in the cell membrane.
6. Induction of dynamic actin structures at the surface of transfected cells.

Basp1, the human homolog of Cap23 is known to function as a transcriptional co-suppressor for the Wilm's Tumor suppressor protein WT1(carpenter *et al* 2004). Basp1, by virtue of being a homolog of Cap23 contains an N-terminal myristoylation signal and several potential protein kinase C and casein kinase II recognition sites. The phosphorylation sites are nested within the PEST sequences, which are characteristic of high-turnover proteins. However, in addition to this, BASP1 also contains a nuclear localization signal (NLS) close to the N-terminus that allows it to go to the nucleus and carry out it's transcriptional activity.

Mice having a complete deletion of the Cap23 locus (Knockouts) are sterile, have difficulty in movement (ataxia), lower body weight, smaller overall size and occasional developmental defects in the brain. The knockouts rarely survive beyond three weeks of age. There is a peak in the mortality around P0-P1days of age. Out of the ones that survive, most die around 3 weeks of age. Thus, the availability of viable adults is indeed a rare phenomenon. However, about 1% of mice that do survive till adulthood seem to avert the obvious phenotypes associated with the knockouts and look apparently normal. Cap23 knockouts have abnormal synapses in the neuromuscular junctions (Frey et al 2000) and a defect in paralysis induced nerve sprouting. Mice having only a single allele of Cap23 (Cap23 heterozygotes) have about 30% of the wild type level of the protein. They also show a more subtle phenotype compared to the knockouts. Cap23 heterozygotes survive to adulthood without any obvious defects, are

normal in appearance, and have no problems in movement or breeding. Since they do not have any neonatal mortality, and survive readily till old age, the heterozygotes are much more amenable and readily available for experiments.

This study investigates the effect of the Cap23 deletion in the CNS of Cap23 knockouts and heterozygotes. Crossing the Cap23 mutation in a line of mice (L15) expressing m-GFP in very few neurons in the hippocampus enables the clear visualization of the dendritic architecture of CA1 pyramidal cells and granule cells in the dentate gyrus. Using deconvolution microscopy, we are also able to visualize with great clarity the finer details of the dendritic architecture and the structure of the spines. Using this approach with mice of different ages we have been able to follow the changes that take place in the dendritic tree of the CA1 pyramidal neurons in mice lacking the Cap23 protein.

RESULTS

1. Mice deficient in Cap23 show a progressive dendritic atrophy.

Using the tools described earlier, we looked at hippocampal CA1 pyramidal cells in wild type mice and in mice that are either heterozygous or complete null for Cap 23. The results of analysis of mice of different ages are illustrated in Figure 1. We looked at Cap23 heterozygous mice aged 10 days, 21 days, 30 days, 3months and 1 year respectively (Fig.3). For each time point, at least 3 or more mice of a particular genotype were analyzed and wild type littermates were used as controls. In case of the complete knockouts, one individual mouse aged 5 days, 10 days and 21 days respectively were analyzed (Figure 4). For each genotype and timepoint, at least 50 cells or more (n>/= 50) were analyzed. The result of the entire analysis is summarized in figure 5. We looked at dendritic branching in CA1 pyramidal cells, and quantitated it by counting the number of dendritic branchpoints.

Comparison of the dendritic branching of CA1 pyramidal cells in wild type mice of different ages confirms the known facts about dendritic stability. In wild type mice of all ages starting from 3 weeks onwards, the dendritic branching remains stable and unchanged. This is in conformity with known data that suggests that dendrites are formed quite early in development and then remain essentially unchanged throughout the life of the individual. However, in mice heterozygous for the Cap23 allele, there is a progressive loss in dendritic branching starting from the onset of adulthood. More specifically, though the wild type and the heterozygotes are identical in terms of their dendritic branching at 21 days of age, there is a progressive decrease in the number of dendritic branchpoints since that time in the heterozygotes, though in the wild type mice the dendritic tree essentially remains the same. Due to this progressive atrophy, by the age of 1 year, the number of dendritic branchpoints in the heterozygotes reach a value of about one-thirds of the wild type, leading to a greatly reduced dendritic arborization. Quantitation of the dendritic branchpoints indicates that this atrophy starts from the tertiary dendrites onwards, and proceeds toward the cell body, i.e.

towards the lower order dendrites. As a result, the number of primary dendrites remain the same, but there is a decrease in the number of secondary and tertiary branches. In this context it is worth mentioning that the Cap23 heterozygotes have about a 70% downregulation in the expression of the Cap23 protein compared to the wild type. This therefore indicates that the protein has a dose-dependent effect on dendritic branching and the other phenotypes.

An important observation from figure6 is that there is an apparent increase in dendritic branchpoints between the age of 10 and 21 days. This increase is seen both in the wild type mice as well as in the Cap23 heterozygote mice. It is interesting to note in this context that from other studies, the time period from 10 to 21 days is associated with synapse elimination in mice. This is a time when there is large scale reorganization, rerouting and axonal pruning going on in the mice. We have no clear idea of how this could be related to the increase in the number of dendritic branchpoints, but it is tempting to speculate that there is also some amount of dendritic growth going on that is associated with synapse elimination and subsequent synapse stabilization.

In mice lacking Cap23 (Cap23 Knockouts), the dendritic tree starts off by looking similar to the wild type mice at 5 days of age. At this age, the dendrites are much thinner than at subsequent ages, and are totally devoid of spines. But even by 10 days of age, the number of dendritic branchpoints in the knockouts go down to almost 75% of its initial value, whereas the wild type and the heterozygotes maintain the same complexity of the dendritic tree. Subsequently, between 10 and 21 days (a period coinciding with synapse elimination in the CNS), when there is an increase in dendritic branchpoints in the wild type and the heterozygotes, the Cap23 knockouts fail to follow suit. Instead, they maintain the same complexity of the dendritic tree as at 10 days. It is interesting to note in this context that at 10 days, the knockout has the same complexity of the dendritic tree that the heterozygote has at 1 month of age. Since the knockouts totally lack the Cap23 protein, this confirms the dose dependent effect of Cap23 on the

dendrite branching phenotype. Since the dendritic tree starts out being the same in the wild type, heterozygotes and in the knockouts, this suggests that Cap23 is responsible for the maintenance of dendritic branching in these cells, and not in their formation.

It is important to mention in this context that the decrease in the number of dendritic branchpoints in Cap23 heterozygotes and knockouts with age is seen in the apical as well as the basal dendrites of the CA1 pyramidal cells. It is only for the sake of brevity that the documented examples are only from basal dendrites. The values in figure 6 are cumulative values from apical as well as basal dendrites.

2. Deficiency of Cap23 leads to an increase in the number of complex spines.

As mentioned earlier (fig. 3 and 5), in the Cap23 heterozygotes, the dendritic atrophy phenotype starts after 21 days of age. At 21 days of age the wild type and the Cap23 heterozygotes are similar in appearance in terms of the dendritic branching, appearance of the dendrites and the structure of the spines. But with the onset of dendritic atrophy, the Cap23 heterozygotes start showing morphological changes in the fine structure of the dendrite and the ultrastructure of the spines. Figure 6 illustrates this phenotype. At 21 days of age, both the wild type and the Cap23 heterozygotes have the same average thickness of the dendrites. They also have an abundance of thin spines characteristic of this age, and a few well formed mushroom spines. Importantly, at this age, neither the wild type nor the heterozygotes have any complex spines. But by the time they are fully matured adults, the Cap23 heterozygotes have an abundance of complex spines (Fig. 6), which are conspicuously absent in the wild type mice. For the purpose of this analysis, complex spines are defined as spines that have multiple spine heads arising from a single spine neck. Figure 7 shows a quantitative analysis of this phenotype.

To quantitate this phenotype, we imaged continuous stretches of dendrites starting from the first branching point after the cell body till the end of its length, and counted the total number of spines in that stretch and the number of complex spines(n=10). Figure 7 (left) shows that though the density of spines per 100 microns is the same, the Cap23 heterozygous mice have about 5 times the number of complex spines compared to the wild type mice.

It is important to take note of the fact that simultaneous to the appearance of the complex spines, there is a decrease in the number of dendrites in the heterozygotes (Figure 3). Since spines are known to be stabilized by contact with presynaptic terminals and formation of a synaptic contact(Trachtenberg et al 2003), it is reasonable to assume that the splitting of spines to form complex spines in the Cap23 heterozygotes is a mechanism to keep the total number of synapses in the brain circuit constant. This indeed is the case, as is borne out from figure 7, where the total number of spine terminals in the wild type and the heterozygotes are the same. The same conclusions can be derived from the Quantitation of synapses from electron micrographs (Figure 8). A logical deduction from this observation would imply that to accommodate the larger number of synapses per spine, the dendrites should increase in thickness to provide a greater surface area. This indeed is the case (data not shown). Though the thickness of a dendrite varies significantly along its length, dendrites in adult Cap23 heterozygote mice on an average tend to be thicker than dendrites of equivalent regions in the wild type mice. Taken together, this data implies that lack of Cap23 protein causes dendritic instability leading to a decrease in the number of dendritic branchpoints. In order to keep the number of synapses constant, the remaining dendrites then increase in thickness to gain in surface area, and possess more complex spines. Figure 6 (right) shows the preponderance of multispine boutons and perforated synapses in the Cap23 heterozygous mice. The heterozygotes contain greater number of boutons with more than one postsynaptic contact compared to the wild type mice. Simultaneously, the heterozygotes have fewer boutons making a single postsynaptic contact compared to the wild type mice.

3. Total synapse number is conserved in the absence of Cap23Electron microscopy analysis of the Cap23 neuropil.

At the ultrastructural level, the Cap23 neuropil shows a few interesting phenotypes. These are illustrated in figure 8. Most conspicuous among the phenotypes in the neuropil is the presence of multispine boutons. Compared to the wild type, where on an average, a single presynaptic bouton (identified by the presence of synaptic vesicles) makes a synapse (identified by the presence of a post synaptic density) by contacting a single spine, in the Cap 23 knockout mice, a single presynaptic terminal makes multiple post synaptic contacts. Figure 8 shows a quantitative representation of this phenotype. This observation confirms the assumption that the complex spines that are formed in the Cap23 heterozygotes indeed form synapses with the same presynaptic terminal.In other words, the multiple postsynaptic partners of the same bouton are spine heads belonging to he same complex spine. Since the same presynaptic terminal synapses with multiple postsynaptic partners, a logical assumption from this observation would be that the presynaptic terminal should have a larger surface area compared to that of the wild type neuropil. This indeed is the case, as is borne out of figure 9. In addition, when counted in terms of the post synaptic densities, the total number of synapses in the Cap23-/- neuropil is the same as in the wild type neuropil. This data is in agreement with, and reinforces the data shown in figure 7. Based on measurement of surface areas from electron micrographs, on an average, Cap23-/- mice have larger presynaptic terminals and smaller postsynaptic terminals as compared to their wild type counterparts. Taken together, this implies the presence of compensatory mechanisms that ensure stability in synapse numbers in spite of dendritic instability due to lack of Cap23.

DISCUSSION.

In the background of a deficiency of Cap23, our results indicate that CA1 pyramidal cells in the hippocampus start losing dendritic branches starting from around the onset of adulthood. To begin with however, they have dendritic arborization similar to the wild type mice. Therefore, the role of the Cap23 protein seems to be more likely in the maintenance rather than the formation of dendrites.

The agreement of the data with existing knowledge about dendrites also strengthens the observation and conclusion of this thesis. The prevailing belief so far is that the growth and architecture of dendrites is determined to a large extent by neuron specific intrinsic programs. This conclusion is based largely on experiments conducted on cortical and hippocampal pyramidal cells in culture. Even in the absence of their natural environment, the cultured neurons form a dendritic tree that is largely reminiscent of their normal dendritic arborization. External factors like the presence and the concentration of neurotrophins are thought to only influence and augment the intrinsic 'blueprint' for dendritic patterning. Independent studies on tadpoles (*Xenopus spp.)* and Drosophila also concur with these conclusions.

In the light of these observations, it is not surprising that in the background of a deficiency of Cap23 protein, the neurons start out with a perfectly normal dendritic tree, but cannot maintain it subsequently. This delineates a role for Cap23 in the maintenance of dendrites, as opposed to a role in their formation. Dendrites are known to be formed very early in development and then essentially remain unchanged throughout the life of the individual. Cap23 is therefore, probably not involved in executing the cell-intrinsic blueprint for dendritic patterning. Instead, it is more likely that Cap23 is involved in maintenance of dendritic branching through its role in maintenance of cell structure. The Cap23 protein contains an N-terminal myristoylation and palmitoylation domain through which it is firmly tethered to the cell membrane. It is known to bind to the end of microtubules, and this could lead to mechanical strengthening of the cell cortex

and to enhancing the stability of the cell. Taken together, all this could constitute a mechanism by which Cap23 helps in stabilization and maintenance of the cell cortex of the dendrites. The fact that the severity of the dendritic atrophy phenotype inversely varies with the level of the protein *in-vivo*, further points to a dosage-dependency, a stoichiometric requirement of the protein, and a role of the protein in maintaining structural stability.

The use of membrane targeted GFP enables us to see the entire dendrite arbor in great detail. The L15 line of mice used in this study labels only a few neurons, so there is no overlap between the arbors of neighboring neurons. To quantitate the dendrite atrophy phenotype, we counted the number of dendrite branchpoints in the mice of the different ages. By this criteria, in Cap23 knockout mice and in the heterozygotes, the number of dendritic branchpoints decrease in the apical and basal dendrites of the CA1 pyramidal cells of the hippocampus. This directly implies that the dendritic atrophy leads to a decrease in net dendritic length too. In addition, this also implies that it is the higher order dendrites (viz. tertiary and secondary) are the first ones to disintegrate while the primary dendrites remain intact. Thus, the dendrite atrophy proceeds from tertiary and quaternary dendrites and gradually proceeds to the secondary and primary dendrites. Indeed, only in some severely affected neurons in 1 year old mice we see that the primary dendrites have also been affected.

Recently, another report of an analogous nature has emerged from a study conducted on the $Abl^{-/-}$ knockout mice. Dendrites of cortical neurons in the Abl related gene (Arg) and the $Abl^{-/-}$/ $Arg^{-/-}$ double knockout mice initially develop normally, and are initially indistinguishable from wild type dendrites till postnatal day 21. Subsequently, there is a problem with dendritic branch maintenance, because of which there is a severe dendritic loss subsequently. In this context, it is interesting to note that the time point of 21 days is the same that we observe in our study as well, and whose significance still remains unexplained. Dendritic branch extension, branching and maintenance are known to be modulated by activation of integrin receptors by adhesion to Laminin-1 or Semaphorin7A.

Interestingly, this effect of laminin binding is absent in Arg-/- neurons in culture, leading to the speculative hypothesis that the maintenance of dendrites in the Arg-/- mice is mediated by integrins (Moresco et al 2005). The Abelson (Abl) family of non-receptor tyrosine kinases are known to function by translating information from cell surface receptors into changes in cytoskeletal structure and cell morphology by phosphorylation of cytoskeletal elements.

The Cap23 protein is known to bind to the end of actin filaments, presumably stabilizing them, and therefore stabilizing the cell structure. It is known that Abl family of proteins also have a C-terminal extension containing binding sites for actin and microtubules, which can be directly used to maintain cytoskeletal structure (McWhirter and Wang, 1993; Van Etten et al 1994; Wang et al 2001; Miller et al 2004). So, it is likely that the deficiency of Cap23 leads to weakening of the cytoskeletal structure and thereby leads to a deficiency in the maintenance of dendrites.

Materials and Methods.

1. Imaging and Quantitation of dendritic arbors.

The L15 line of transgenic mice expressing m-GFP under the Thy-1 promoter was used to study dendritic branching. Mice of the desired age were perfused transcardially under anaesthesia and the brains and vibratome sections of 40 micron thickness were taken. For perfusion, the circulatory system was first washed with Lactated Ringer's solution (Ringer's solution containing 50% sodium lactate) and then fixed transcardially with 4% paraformaldehyde. Perfusion was carried out manually using a syringe at roughly 6 ml of liquid per minute. Confocal imaging was done with an Olympus Fluoview microscope. Single confocal stacks were taken at 0.4-0.5 micron intervals using a 60X oil immersion objective and were reconstructed using the Imaris imaging software to look at the entire dendritic tree. Dendritic branching was quantitated by counting the number of branchpoints. Where necessary, 3-D movies made out of the individual confocal stacks were used to quantitate the number of branchpoints.

2.Imaging of fine structure of dendrites and dendritic spines.

The fine structure of the dendrites and the spines was imaged using a Fluoview confocal microscope. Images were taken with a 100X oil immersion objective (N.A=1.4; Confocal aperture 90-100 microns) and digitally magnified 5 times. After acquisition, images were deconvolved using the Huygens deconvolution software. Confocal stacks were taken at 0.15 micron step size, and were collapsed together using the Imaris imaging software. Where necessary, 3-D movies were made using the single stacks to quantitate the spine morphology.

To quantitate the complex spine phenotype, we imaged continuous stretches of dendrites starting from the first branching point after the cell body till the end of its length, and counted the total number of spines in that stretch of 100 microns and also the number of complex spines(n=10).

Figures.

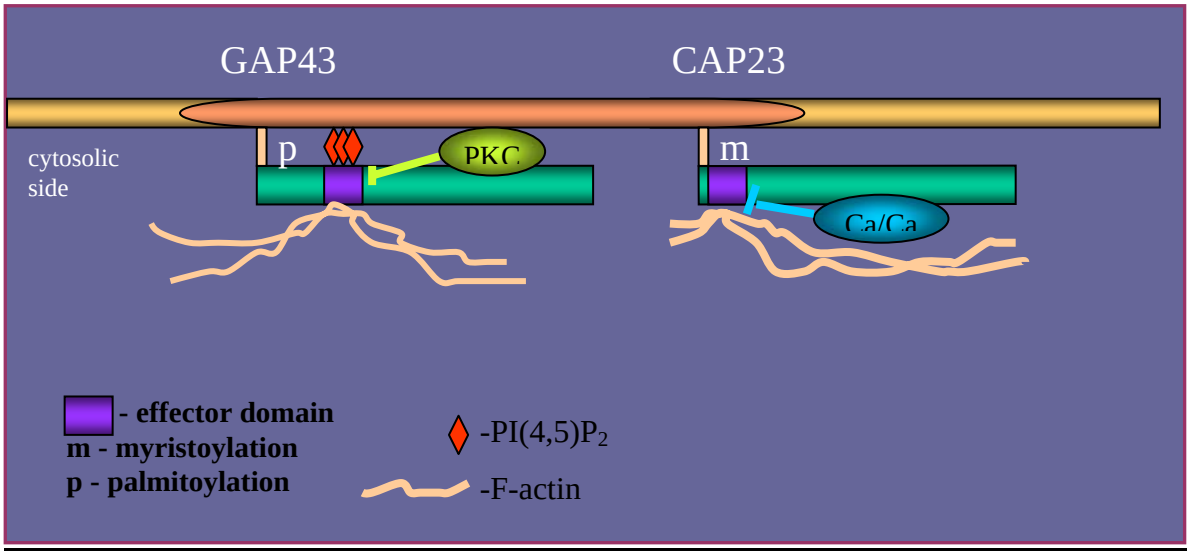


Fig.1- Schematic drawing showing the domain architecture and membrane localization of Cap23 and a closely related member, Gap43.

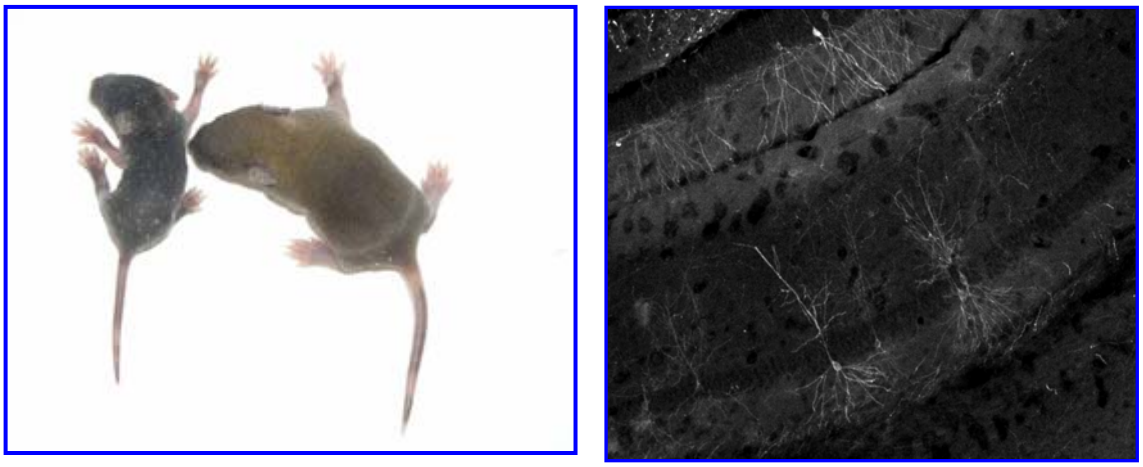


Fig. 2: Figure showing the difference in body size between wild type and heterozygous mice and low magnification overview of the hippocampus of the line of mice used in this study

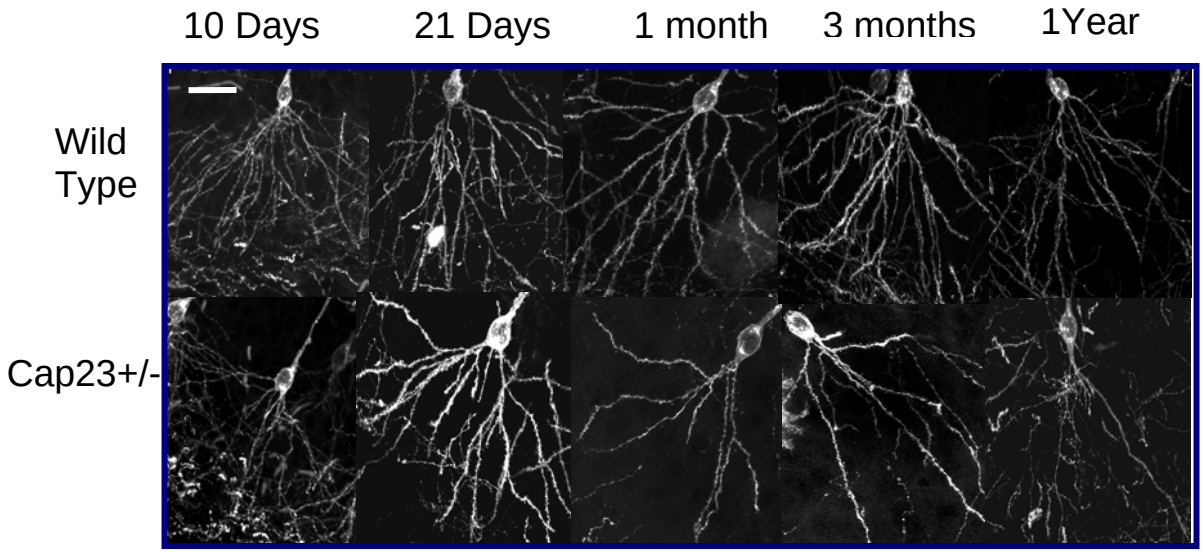


Figure 3: Mice heterozygous for Cap23(lower panel) start out having dendritic arborization identical to wild type mice(Upper panel),but start losing branches progressively starting from around 1 month of age.

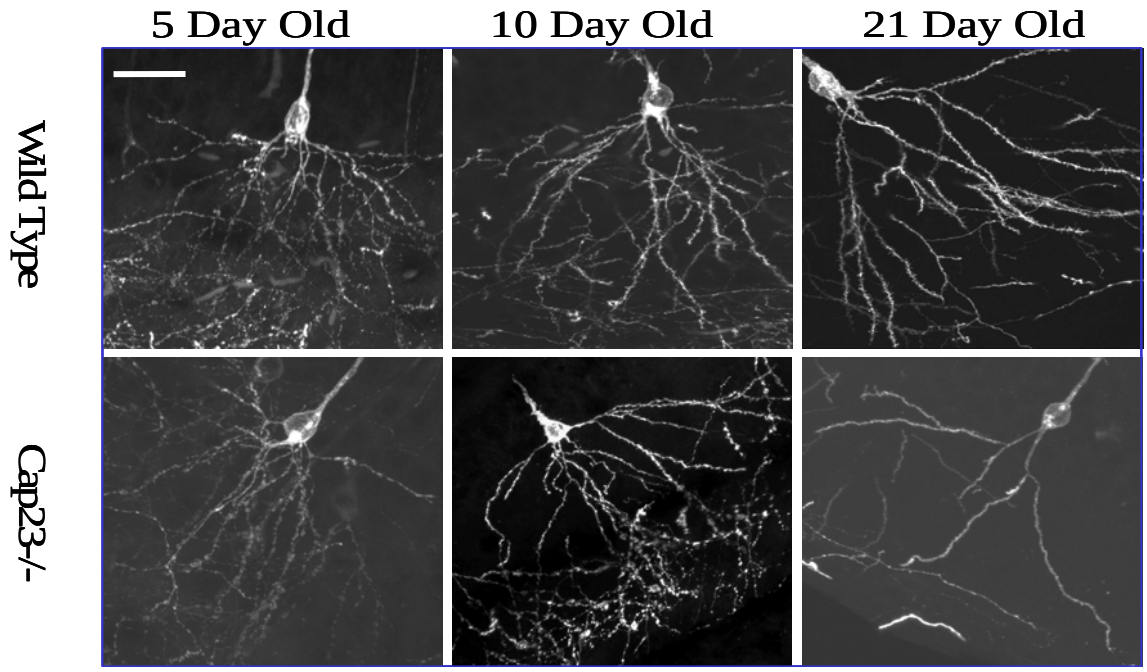
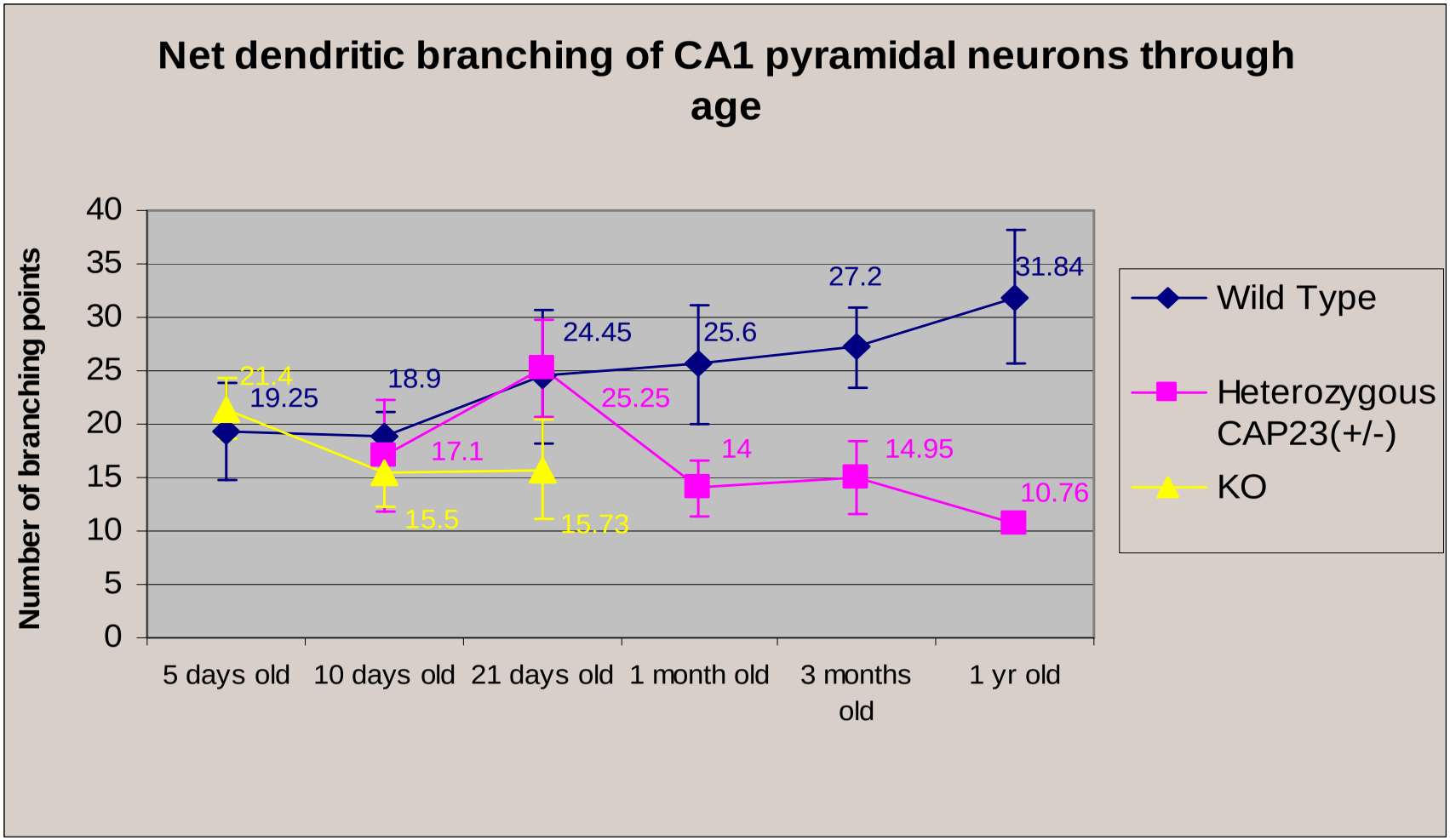


Figure 4: Mice that are null for Cap23 also start out with a dendritic arbor similar to that of the wild type at 5 days of age, but drastically lose their arbor by the time they reach 21 days of age.


Net dendritic branching of CA1 pyramidal neurons through age
Number of branching points
40
35
30
25
20
15
10
5
0
5 days old
10 days old
21 days old
1 month old
3 months old
1 yr old
21.4
19.25
18.9
17.1
15.5
24.45
15.73
25.25
25.6
14
27.2
14.95
31.84
10.76
Wild Type
Heterozygous CAP23(+/-)
KO

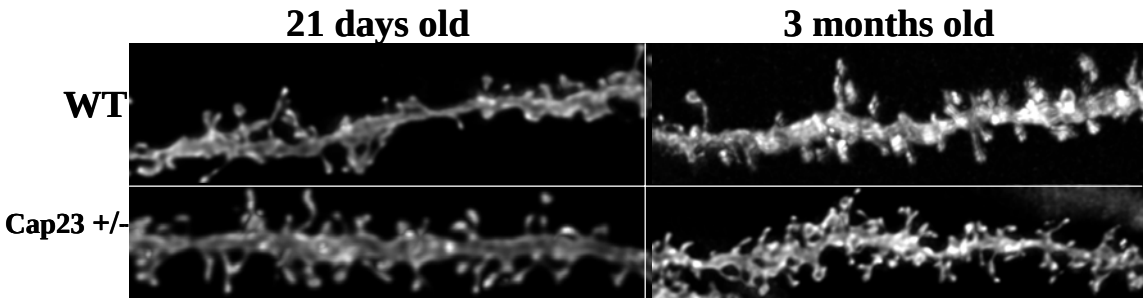
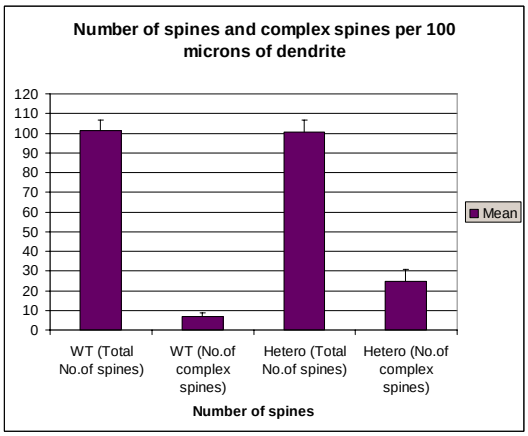


Figure 6: Comparison of the change in the fine structure of dendrites in wild type and Cap23 heterozygous mice that from 21 days to 3 months of age.



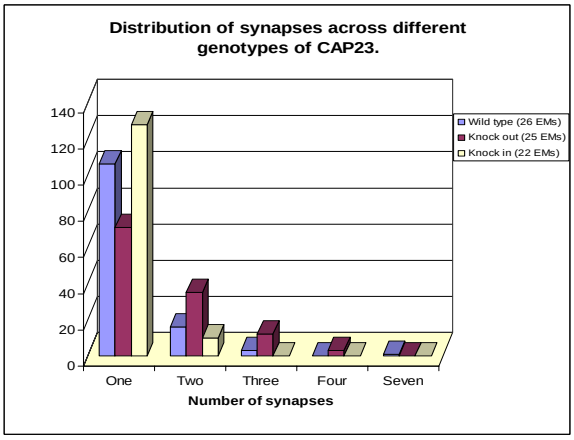


Figure 7: A quantitative representation of the presence of additional complex spines and perforated synapses in the adult Cap23 heterozygotes.

Cap23-/- Wild Type

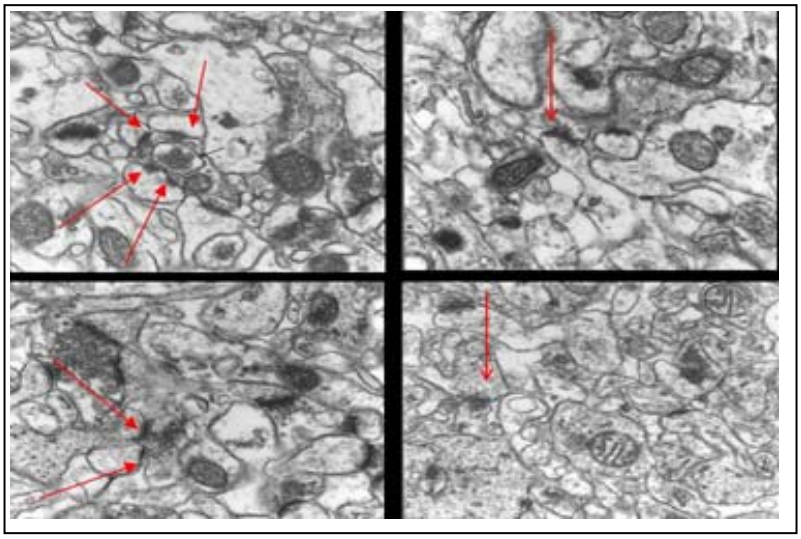


Figure 8: Electron micrographs of the neuropil of Cap23 knockout mice and wild type mice showing the presence of multispine boutons and perforated synapses.

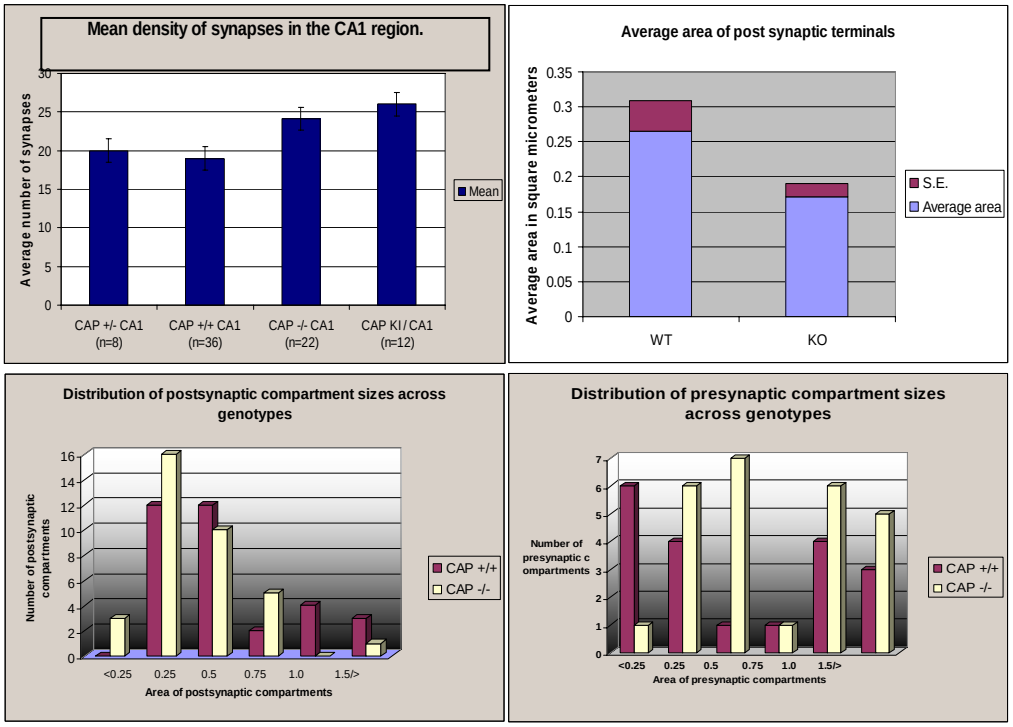


Figure 9: Figure showing conservation of central synapse numbers between wild type mice and mice deficient in Cap23.

FIGURE LEGENDS:

Figure 1. Schematic drawing showing the domain architecture and membrane localization of Cap23 and a closely related member, Gap43. Both the proteins attach to the inner leaflet of the plasmamembrane and are characterized by the presence of an effector domain that is thought to bind to bundles of actin filaments through which it could ensure maintenance of the integrity of the cell cortex. Gap43 has a palmitoylation domain at it's N-terminal through which it attaches to the membrane whereas Cap23 has a myristoylation domain at a similar position. In addition, Gap43 also has $PI(4,5)P_2$ binding sites at its N-terminus.

Figure 2. Figure showing the (A)difference in body size between wild type and knockout mice and(B) low magnification overview of the hippocampus of the line of mice used in this study. Cap23 knockout mice die around birth(P0). The few mice that survive this period have no anatomical abnormalities, but are smaller in size compared to the corresponding wild type littermates. The brain size of these knockout mice(left) are also correspondingly smaller compared to the wild type controls(right). When crossed into the mGFP expressing background, both the wild type and the knockout mice express GFP in a subpopulation of the neurons in the hippocampus. Figure B shows a representative confocal section from the L15 line of mGFP transgenic mice in which CA1 pyramidal cells and granule cells of the hippocampus express GFP. The number of GFP expressing granule cells are always larger than the number of GFP expressing pyramidal cells.

Figure 3. Mice heterozygous for Cap23 start out having dendritic arborization identical to wild type mice but start losing branches progressively starting from around 1 month of age. Wild type mice(top panel) do not undergo any significant change in the number of dendritic branchpoints between 10 days of age to a year

of age. But during this time interval, Cap23 heterozygous mice(Lower panel) start out having similar degree of dendritic branching compared to the wild type mice, but start losing dendritic branches after 21 days of age. It is worthwhile to note that in case of the Cap23 heterozygous mice, following 21 days of age, there is a steady loss in the complexity of the dendritic arbor due to the loss of dendritic branches.

Figure 4. Mice that are null for Cap23(lower panel) also start out with a dendritic arbor similar to that of the wild type (upper panel) at 5 days of age, but drastically lose their arbor by the time they reach 21 days of age. Since the knockout mice do not live beyond 21 days, imaging beyond this point is not possible. The more drastic occurrence of the dendritic atrophy in the knockouts as seen in this confocal sections compared to the heterozygotes indicates the dependence of this phenotype on the levels of the Cap23 protein.

Figure 5. : Graph illustrating the Dendritic Atrophy phenotype in the mice deficient in Cap23 and comparison with wild type mice Wild type mice (dark line), Cap23 heterozygotes (pink line) and Cap23 knockout mice (yellow line) start out having the same degree of complexity of their dendritic arbors early on in their lives.However, while the complexity of the dendritic tree in fact increases a bit in case of the wild type mice by the time they reach 1 year of age,(19.25 to 31.84) the heterozygotes and the knockouts start losing branches leading to a decrease in dendritic branching. The rate of loss of dendritic branches is proportional to the levels of the Cap23 protein expressed. Cap23 knockouts reach the same level of dendritic branching at 21 days of age that is reached by the Cap 23 heterozygotes at 3 months of age.

Figure 6. Mice heterozygous for Cap23(lower panel) are similar to wild type mice(upper panel) at 21 days of age in terms of the types of spines and the dendritic thickness. At this age, in both the wild type as well as the heterozygotes, the dendrites are relatively thin and are characterized by the presence of long thin spines. Also, note the total absence of complex spines in both the wild type and the heterozygote mice at this age. However, as dendritic atrophy proceeds, the surviving dendrites of the heterozygous mice develop more complex spines and tend to become thicker. This phenomenon is totally absent in the dendrites of the wild type mice.

Figure 7. (A)The mice that are heterozygous for Cap23 develop greater number of complex spines by adulthood as compared to the wild type. In adult Cap23 heretozygous mice, the number of complex spines per 100 micrometres of dendritic length is on an average 4-5 times more than that in the wild type mice that are littermates. These complex spines make synaptic contacts with presynaptic terminals that look like perforated synapses in electron micrographs. Electron Micrographs of Cap23 knockout neuropil show the presence of enlarged presynaptic terminals making multiple postsynaptic contacts(B).Whereas most wild type presynaptic terminals synapse with a single postsynaptic terminal, in the knockouts, this ratio is increased.

Figure 8.Electron micrographs of the neuropil of Cap23 knockout mice show the presence of presynaptic terminals making multiple postsynaptic contacts (perforated synapses) (left panel),whereas the neuropil from the wild type controls is devoid of perforated synapses(right panel)

Figure 9. In spite of dendritic atrophy due to reduced levels of Cap23 in the brain, total synapse numbers are conserved in the neuropil of the mice.When

quantitated in terms of the total number of PSDs from the electron micrographs, the density of synapses in the neuropil of the wild type mice as well as the heterozygotes and the knockouts is equal (top left). However, the average cross sectional area of presynaptic terminals in EMs tend to increase in the mice that are Cap23 knockouts(Top right). The two panels at the bottom illustrate that in Cap23 knockouts, most postsynaptic terminals tend to be smaller in sizes(bottom left), whereas most presynaptic terminals in the knockouts tend to be larger in size compared to their wild type controls (bottom right).

SUPPLEMENTARY MATERIAL

MICROARRAY ANALYSIS OF THE TRANSCRIPTOME OF MICE EXPRESSING ALTERED LEVELS OF THE CAP23 PROTEIN.

INTRODUCTION.

Mice that are either heterozygous or knockouts for Cap23 have defects in the formation of central synapses. This is borne out from Electron Microscopy data as well as confocal microscopy analysis of spines and dendrites. A defect in the structure of peripheral synapses is also evident from the morphology of the NMJs (Frey et al 2000). This gives rise to the question as to how exactly Cap 23 controls synaptic function and structure. What are the intermediaries through which CAP 23 acts to exert its effects? Is there a signaling cascade involved?

To answer these questions, it becomes necessary to find the partners through which Cap23 acts. The presence of RNA binding motif (G-Quartet sequence) in the 5'-UTR of the Cap23 mRNA also raised the possibility that the Cap23 mRNA binds other RNAs and has an effect on their translation. Taken together with the fact that the Cap23 protein is found at synapses, this leads to the interesting possibility that Cap23 is involved in the regulation of local translation of a family of mRNAs that are important for the structure and function of the synapse. This would explain how Cap23 exerts its effect on the synapse.

2-D PAGE analysis followed by mass spectrometry shows that in the background of a Cap23 mutation, the levels of many proteins are altered (Wacha and Caroni-Unpublished results). These proteins can be categorized into different groups based on their functions. There are proteins like that are involved in energy metabolism, or signal transduction or cytoskeletal rearrangement. Prominent among these are also proteins like Gelsolin, Drebrin etc that have a role to play in the maintenance of spine structure (Shim and Lubec 2002, Takahashi *et al* 2003). These proteins showed either upregulation or downregulation in the Cap23 heterozygous and knockout mice. More importantly, the extent of misregulation of the protein showed a dose dependent effect. For example, the extent of misregulation of a particular protein in the Cap23 knockout mice was to a higher extent but in the same direction as compared to that in the Cap23 heterozygous mice. This was indicative of a dose dependent effect of Cap23 on the expression of the respective genes. Whether this effect of Cap23 was due to its action at the level of transcription or at the level of translation is not clear. The mouse Cap23

protein does not show the presence of any obvious DNA binding domains through regular bioinformatics tools like Prosite. However, reports in the literature suggest that the human homolog of Cap23, i.e. Basp1 has a DNA binding activity, and acts as the transcriptional co-activator for a gene associated with Wilm's Tumor (Carpenter *et al* 2004). Cap23 and Basp1 show about 70% homology at the protein level. Taken in the context of this report, it is likely that the mouse Cap23 protein may have some cryptic DNA binding domains that allow it to influence transcriptional activity. However, the 5'UTR of the Cap23 mRNA has some consensus RNA binding motifs like the G-quartet sequences. Presence of G-quartet motifs in the FMR1 m-RNA has been linked to its ability to control local translation of the mRNAs that it binds. In fact, Cap23 is one of the known binding partners of the FMR1 mRNA (Darnell *et al* 2001). In this context, the presence of RNA binding motifs in the Cap23 mRNA could suggest a role for Cap23 in translation of transcripts. This would also explain the misregulation of a host of proteins in the background of a Cap23 deletion. To explain the proteome data and to understand the role of Cap23 in detail at the molecular level, we resorted to microarray analysis of the entire transcriptome of the mouse brain of wild type and Cap23 heterozygous mice using total brain RNA as the starting material.

RESULTS

- **Microarray analysis of adult Cap23 heterozygous mice.**

The transcriptome analysis of the Cap23 heterozygous mice was carried out using a total of 4 mice- two each of heterozygotes and two of the wild type. All 4 mice were about 5 months of age, and were littermates. Stringent criteria were used in the selection of the misregulated transcripts, as described later in this chapter. The experiment was repeated twice with independent samples, and the results were consistent between the two experiments.

In the Cap23 heterozygous background, about 100 transcripts are misregulated to an extent of two folds or more compared to the wild type controls. The extent of misregulation of the transcripts ranges from about 20 fold upregulation to

about 12 fold downregulation, as shown in figure 4.1. Most of the misregulated transcripts do not have any obvious relationship with Cap23. In fact, a sizeable portion of the misregulated transcripts code for sequences that are either not annotated, or do not have experimentally verified functions. One of the important findings from the analysis is that most of the transcripts that are misregulated, and all of the highly misregulated transcripts code for non coding RNAs

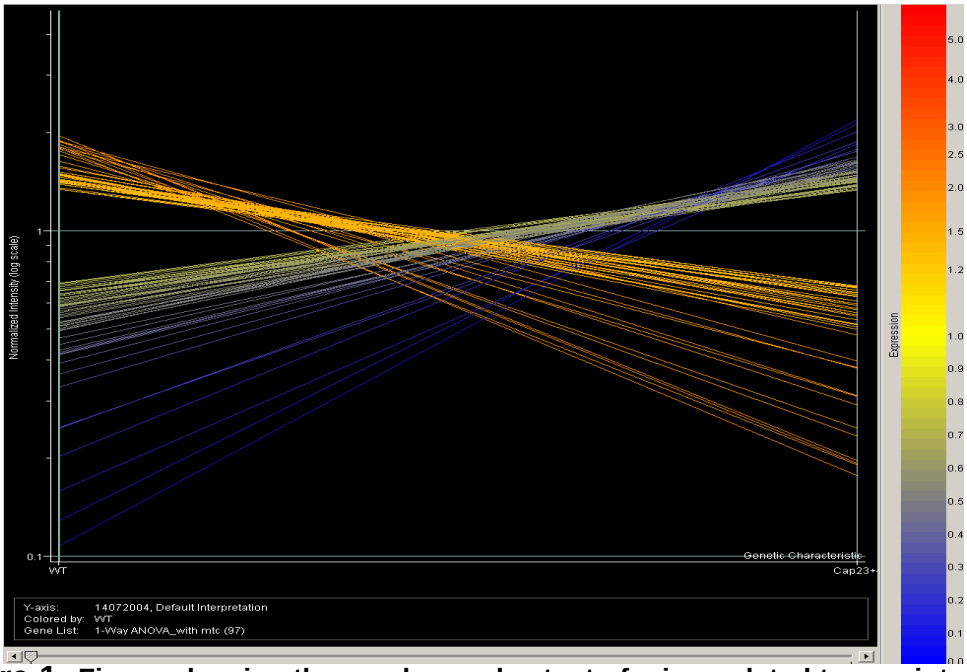


Figure 1: Figure showing the number and extent of misregulated transcripts between adult Cap23 heterozygous mice and their wild type controls. On the left is shown the expression levels in the wild type and on the right the heterozygotes. Each line represents a single transcript, and its color represents its extent of misregulation.

A list of such transcripts is provided in figure 4.2. Non coding RNAs comprise a bulk of the transcriptome of the mammalian brain, and have recently gained increased attention. Though the function of most of them are still unknown, some of these, like the BC1 RNA have been found to be functionally important in regulating local translation in the brain (Zalfa et al 2003). Figure 4.3 lists the misregulated non-coding RNAs and the extent of their misregulation. Perhaps the single most interesting finding from the microarray analysis is that none of the

Fold up or down	Names of transcripts
5 - 4	syndecan 4; Chemokine ligand; Photoreceptor Cadherin
4 - 3	Serum glucocorticoid regulated kinase; Protocadherin beta3; Myelin Basic protein; Cyclin M1
3 - 2	Nuclear factor 1A;Syntaxin 3 ;peptidylglycine monoxygenase Serine protease inhibitor; ATRX mental retardation gene; PIP3 binding protein; RNA binding protein 1(RNP1) ;signal recognition particle 54;RNA binding motif protein 5; CAMKinase 4; Protein tyrosine phosphatase receptor;calcitonin modifying protein;GPCR 49;voltage gated pottassium channel.
2	NK2 transcription factor related locus; Hippocalcin; ADH family A1;Serine threonine kinase.
2-6	Caspase9; protocadherin beta 9; Collagen triple helix protein ,galactosylceramidase; zinc finger protein 162; cysteine rich protein 61.

Figure 2: A list of genes with known functions misregulated in adult Cap23 heterozygotes.

Affymetrix I.D.	Nature	Comments.
1429906_at	Non-coding RNA. 20 Fold Upregulated.	1. Present within introns 2. Has consensus stem loop structure 3.Shows homology with known miRNAs.
1460101_at	Non Coding RNA 16 fold Upregulated.	1. Present in introns 2. Has consensus stem loop structure. 3.Shows homology with known miRNAs.
1456655_at	Non Coding RNA 7 fold Downregulated.	1. Located in introns. 2. Has consensus stem loop structure.
1443621_at	Non Coding RNA\ 11.6 fold Upregulated	1. Located in an intron 2. Shows stem loop structure.
1443772_at	Non Coding RNA 10 fold Downregulated	1. Located in an intron 2.Shows consensus stem loop structure 3. Shows homology with known miRNAs.
1432198_at	Non Coding RNA. 11.3 fold downregulated	1. Shows consensus stem loop structure. 2. Shows homology with known miRNAs.

Figure 3: A list of highly misregulated non-coding RNAs misregulated in adult Cap23 heterozygous mice.

genes misregulated at the protein level (as seen in 2D gels and Westerns) are misregulated at the level of their transcripts. This suggests that the role of Cap23 may be at the level of post-transcriptional regulation of these genes.Taken together with the fact that the highly misregulated transcripts are the non-coding RNAs, it is interesting to speculate that these non coding RNAs could be somehow involved in mediating translation. It could be possible, for example, that these non-coding RNAs are precursor of micro RNAs. Consistent with this possibility, many of these RNAs show the presence of stem-loop structures, and also show homology with known miRNAs, as shown in figure 4.3. When analyzed in details, all these non coding RNAs were found to have many properties in common. All of them localized to non-coding regions of the genome where there were no functional genes in the vicinity. Many of them localized to introns of known genes. The sizes of these transcripts were in the range of about 1kb, and therefore too short to code for functional proteins. None of these transcripts were found to contain any known or predicted ORFs, and no known protein motifs. Almost all of these RNAs had a well formed stem-loop secondary structure reminiscent of miRNA precursors, and at least some of them showed homology with known miRNAs. Figure 4.4 shows one such example.

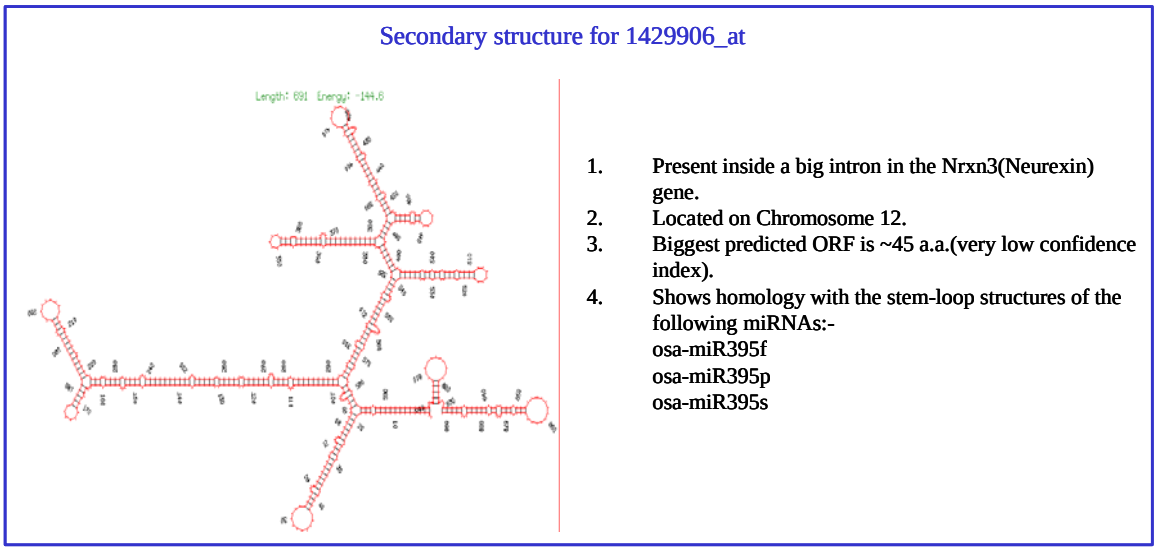


Figure 4: In-silico modeling of one of the non-coding RNA 1429906_at to show the presence of stem-loop structures.

To determine the localization of these misregulated non-coding RNAs, one of them, viz. 1460101_at (Fig.4.3) was cloned in an expression vector containing SP6 and T7 promoters. A RNA probe of 800 base pairs was used for in-situ hybridization in adult brain sections to detect the localization of 1460101_at. Figure 4.5 shows the representative results of the in-situ hybridization.

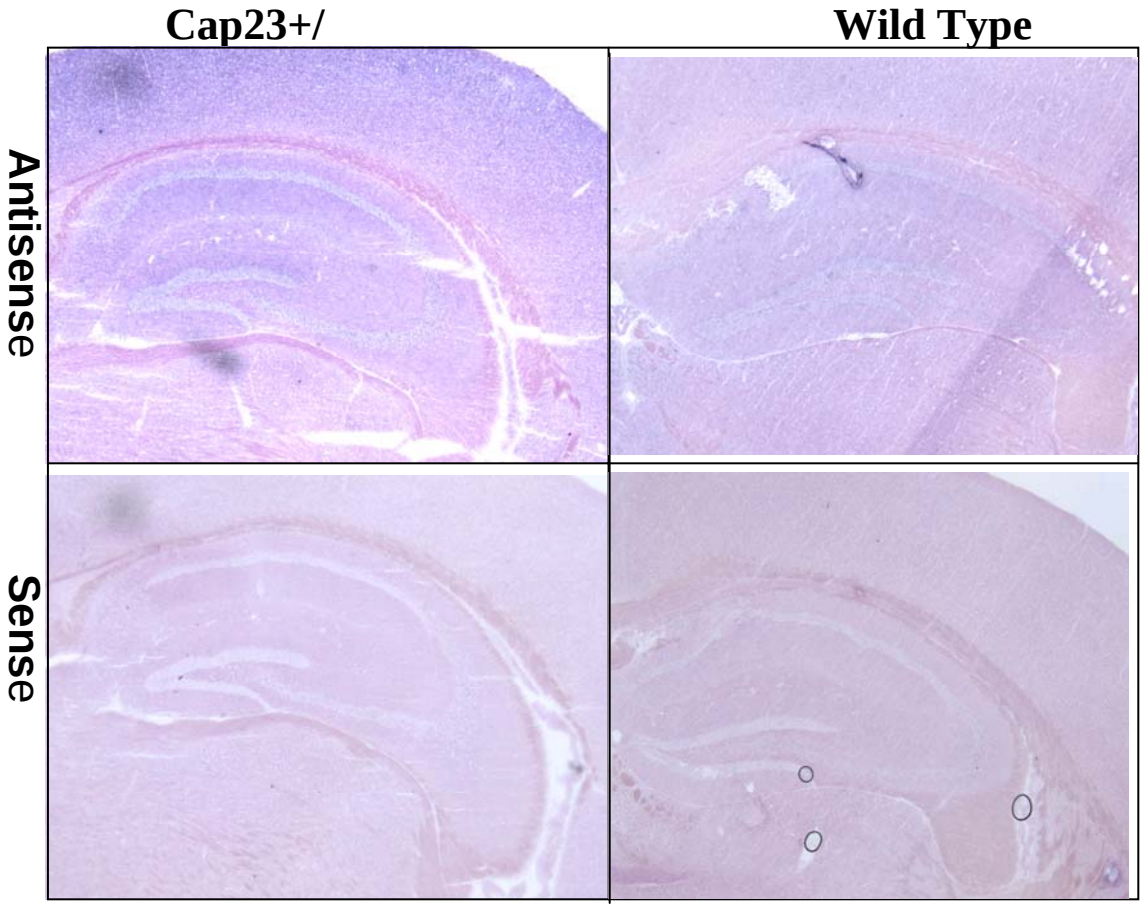


Figure 5: In-situ hybridization to detect the presence of the non-coding RNA 1460101_at in the mouse hippocampus. Both in the wild type and the heterozygotes (top panel), the cell bodies are stained, while the control(lower panel) shows the absence of staining in the cell body layer. There is no detectable difference between the wild type and the heterozygotes.

From figure 4.5 it becomes apparent that both in the wild type and the Cap23 heterozygous mice, the non-coding transcript 1460101_at is localized in the cell bodies of the CA1, CA3 and dentate gyrus. In both the wild type as well as the Cap23 heterozygous mice, the sense probe fails to light up the cell body layers in the hippocampus. However, from this experiment, no conclusive result could be

reached regarding the levels of the transcript between the wild type and the Cap23 heterozygous mice.

Levels of micro RNA miR128 is downregulated in adult Cap23+/- brain.

The misregulation of a number of proteins in the proteome of the Cap23+/- mice hints at the probable involvement of Cap 23 in the regulation of the level of these proteins. The misregulation of a number of non-coding RNAs in the transcriptome of these mice leads to the possibility that these RNAs could be involved in the regulation of the protein levels in a post-transcriptional manner. Non-coding RNAs are believed to be important in regulatory functions in the brain. In recent years, the functions of a number of them have been elucidated. It is also possible that these non-coding RNAs are precursors of micro-RNAs. To check if the levels of any of the known microRNAs are misregulated the Cap23+/- transcriptome; we did northern blot hybridization for the micro RNA miR128 with total brain RNA from Cap23 heterozygous mice and its wild type control. The results of the experiment are illustrated in the blot below.

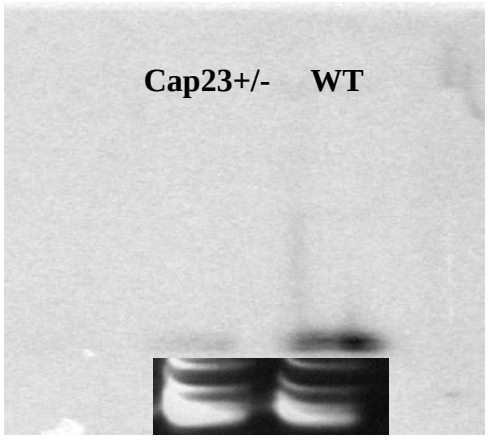


Figure6: Levels of miR128 in the brain of Cap23+/- mice(Left lane) are about 8 times less in wild type mice(right lane).

Out of an average of 3 independent experiments, the microRNAs miR128 was consistently found to be downregulated in the Cap23 heterozygotes.

- **Microarray analysis of young Cap23 heterozygous mice.**

The dendrite atrophy phenotype is present only in the adult Cap23 mice and not in the juveniles. More specifically, the phenotype is present at 1 month of age, but is absent at 20 days of age. Microarray analysis with adult mice showed the misregulation of a class of non-coding RNAs in the Cap23 heterozygotes. To understand whether the misregulation of the non-coding RNAs has a direct bearing on the dendrite atrophy phenotype, and follows the same time course, it was necessary to know what the transcriptome of young Cap23 heterozygous mice looked like.

For this experiment, we took two 20 day old wild type mice and along with their Cap23 heterozygous littermates. At the age of 20 days, the Cap23 heterozygous mice look exactly similar to their wild type counterparts in terms of their dendritic branching and the structure of dendrites. But at the level of the transcriptome, the 20 day old Cap23 heterozygous mice show some important differences when compared to their counterparts at 6 months age.

The most prominent result from the Microarray analysis of the young mice is that there is very little difference wild type and the heterozygous mice. The adult heterozygotes were quite different from the wild types. There was a whole class of prominent transcripts misregulated in the adult heterozygotes. Moreover, the extent of misregulation in the adults was also very prominent. However, in the young mice, the bulk of the misregulated transcripts are only very slightly perturbed, i.e. about 90% of them are misregulated to an extent of only 2-4 fold. Moreover, in the young mice, most of the misregulated transcripts are downregulated rather than upregulated. Out of a total of 263 misregulated transcripts in the young heterozygous mice, only 63 are upregulated and the rest 200 are downregulated, albeit to very low extents as mentioned before. Only 8 transcripts are upregulated 4 fold or above and 16 are downregulated 4 fold or more. Overall, the effects of loss of one allele of Cap23 at 20 days is very subtle at 21 days of age. Worth mentioning in this context is the fact that in spite of this subtle difference between the transcriptome of the juvenile wild type and the

heterozygotes, the single largest group of misregulated transcripts belongs to the family of non-coding RNA. In total, there are 90 transcripts of the non-coding type, 41 of which are upregulated two fold or more, and 52 are downregulated to the same extent. Table 4.6 gives a list of the top 10 misregulated transcripts in the juvenile Cap23 mice.

Affymetrix I.D	Raw Expression. Value WT	Raw Expression Value Cap23+/-	Transcript.	Fold Misregulation
1429310_at	138.3	840.5	Non-coding	6.07
1442812_at	16.5	99.3	APC subunit 5	6.01
1449832_at	55.7	260.2	Non annotated	4.6
1425530_at	45.7	207.2	Syntaxin3	4.53
1456693_at	16.9	79.8	Non Coding	4.72
1451691_a_at	1205.3	2678	Myelin Basic protein	2.2
1415824_at	612.3	101.8	Stearoyl Co-A desaturase.	-6.1
1424454_at	483.4	67.6	Non Annotated.	-7.15
1416986_a_at	278.2	67.3	Pyphosphatase-non-receptor type.substate.1	-4.1

Table 7: A list of transcripts that are most highly misregulated in the 21 day old Cap 23 heterozygotes compared to the respective controls.

At first look, the transcriptome of 20 day old Cap23 mice contains many misregulated transcripts that are important in the nervous system, though the extent of misregulation of these transcripts is small. Table 4.7 gives a list of some

transcripts misregulated in these mice that have known functions in the nervous system.

Affymetrix I.D	Raw Expression value WT	Raw Expression value. Juvenile Cap	Name	Fold Misregulation.
1425530_a_at	45.7	207.2	syntaxin3	4.53
1417752_at	150.5	75.8	Coronin-actin binding	-1.98
1450368_a_at	239.6	117.4	Calcineurin B	-2.04
1421327_at	530.7	258.6	Map2	-2.05
1432004_a_at	100.6	48.66	Dynamin2	-2.05
1417885_at	375.75	177.6	Tau	-2.11
1452638_s_at	233.9	99.7	Dynamin1 like	-2.34
1427754_a_at	947.1	381.0	Dynamin	-2.48
1420506_a_at	385.0	149.8	Syntaxin binding protein1	-2.57
1421862_a_at	196.2	65.4	VAMP1	3

Table 8: A list of the most highly misregulated transcripts in the transcriptome of 20 day old Cap23 heterozygous mice compared to their wild type contols. Note that this list does not contain any non-coding RNAs.

In addition, there are two observations from this experiment that are of seminal importance. The first is that the transcripts for the proteins that are misregulated in the proteome of these mice are not misregulated in the transcriptome. For example, though Drebrin, CamKII etc are misregulated in the 2-D gels made from

protein extracts of the brain of these mice, the levels of these transcripts are fully conserved between the wild type and heterozygotes of the juvenile Cap23 mice. Secondly, none of the non-coding transcripts that are prominently misregulated in the transcriptome of the adult Cap23 mice is misregulated in the juveniles.

Microarray analysis of adult transgenic mice overexpressing Cap23.

All the phenotypes due to the deficiency of Cap23 show a dosage dependent effect. The heterozygotes show a partial (more subtle) phenotype compared to the knockout mice. Even the results of the proteome analysis from the brain preparations show a dosage dependent effect of Cap23.Proteins whose levels are downregulated in the total or partial absence of Cap23 are upregulated in mice that overexpress Cap23. These mice are transgenic mice that have express chick Cap23 under the Thy1 promoter. To understand how the transcriptome of these mice behaves, we used the C11 line of these transgenic mice for Microarray experiments. Two transgenic C11 mice and two non- transgenic littermates (age – 75 days) were used for these experiments. The results were analyzed using the same stringent criteria as before.

Broadly speaking, the transcriptome of the adult C11 mice more closely resembles that of the juvenile Cap23 mice than it resembles the adults. Most prominently, the transcriptome of the Cap23 mice does not show misregulation of any non-coding RNA. In the final analysis of the Microarray data, 200 transcripts were misregulated. While 50 of these were upregulated two fold or more, 150 were downregulated. Only 10 transcripts were upregulated by a factor of 4 fold or more. Out of all the transcripts that were downregulated, only 15 were downregulated by a factor of 4 fold or more. An important and interesting observation in the results is that among the misregulated transcripts, there are many that are important in the immune system and the nervous system. Table

4.8 gives a list of some of the transcripts misregulated in the C11 mice that have known roles in the nervous system.

Name	Raw Score C11	Raw Score C11	Fold Change
Syntaxin3	45.78	341.37	7.45
Spectrin Beta 2	115	257.56	2.23
Cortactin	319.5	156.275	2.04
MARCKS-like protein	3178	1499.265	2.12
Syntaxin binding protein	385	174.88	2.20
Gelsolin	1689	758.08	2.22
VAMP	596.7	267.63	2.22
Dynamin2	100.6	44.755	2.25
Dynamin	947.2	427.40997	2.21

Table 9 : A list of genes with known functions misregulated in C11 mice.

Materials and Methods.

1. Microarray Analysis:

For all the Microarray experiments described here, the Affymetrix mouse array MOE430 was used. This array has about 45,000 known mouse transcripts represented on it. Hybridization was done according to manufacturer's instructions.

Stringent parameters were used for setting the filters on the Microarray analysis. A gene had to reach a raw expression value of at least 50 in at least one of the conditions to be considered in the calculations. Further, the second filter required the change in the normalized expression value to be changed by a factor of a least 2 fold for that transcript to be considered in the analysis. Finally, only those transcripts showing a T-test value of 0.05 or lower, and those passing the multiple testing corrections were finally accepted.

2. RNA preparation.

Total brain RNA was prepared using the Trizol reagent (Invitrogen Corporation) according to the manufacturer's instructions. Briefly, the brain was dissected out of the mice quickly under deep anesthesia. The brain was homogenized manually in a homogenizer in the presence of Trizol. Total RNA obtained in this way was finally dissolved in water to a final concentration of 5 microgram per microlitre.

3. RNA in-situ hybridization.

The genomic fragment corresponding to the Affymetrix transcript 1460101_at was cloned from mouse genomic DNA by PCR, and was cloned into the pCRII TOPO vector using the manufacturer's instructions. The sense and antisense RNA probes were produced through in-vitro transcription using T7 and Sp6 RNA polymerase from the 800bp PCR product. In-situ hybridization was carried out using standard protocols at 56^0C, using 56% formamide. Briefly, frozen brains

were cut on a cryostat (Leica) into 20μm sections and mounted on SuperFrost Plus slides (Fischer scientific), fixed in 4% paraformaldehyde (Sigma) for 30 min, washed briefly with phosphate buffered saline (PBS), followed by washing in 2X SSC and then treated with 0.25% acetic anhydride in 1X triethanolamine for 10 min. Prehybridization was carried out for 3 hours at 56^{o}C in prehybridization mix (50% formamide, 5X SSC, 5X Denhardt's, 250μg ml^{-1} yeast tRNA and 500μg ml^{-1} herring sperm DNA). Hybridization was performed overnight at 56^{o}C in prehybridization mix containing probe. Washing was carried out at 56^{o}C with 5X SSC, 2X SSC, 0.2X SSC for 5 min each and 50% formamide /0.2X SSC for 20 min. followed by successive washing in 0.2X SSC and 1x TE (Tris-EDTA, pH 8) at RT for 5 min. Blocking was carried out with 3% milk powder in 1X TE and then incubated with Anti-Dig-AP-Fab (1:5000) (Roche). Immunological detection was carried out using nitro blue tetrazolium (NBT) and 5-bromo 3-chloro 4-indolyl phosphate (BCIP) (Roche) as substrates in 1X AP buffer (100 mM Tris-HCl, 50 mM $MgCl_2$, 100 mM NaCl, pH 9.5). Blue-purple colour was judged to be a positive signal. Sense controls were included in all experiments as controls for non specific background signals.

4. **Northern Blot analysis for micro RNA.**

For Northern analysis of miRNAs, total RNA was run on 15% polyacrylamide gel along with relevant nucleic acid markers, and then transferred electrically overnight to nylon membrane. Radiolabeled 21mer oligonucleotide probes with 100% sequence homology to the miRNA were hybridized at 37^{0}C in 5x SSPE buffer containing 5x Denhardt’s solution and 250ug/ml of sheared salmon sperm DNA. Washing was done at 37’C in 2x SSC buffer containing 0.1% SDS (two changes). Exposures to films were carried out for different durations and the gel bands were quantitated using a phosphorimager.

CHAPTER V

DISCUSSION and OUTLOOK

Cap23 and Dendritic Instability

The central finding of this thesis is that in mice that are deficient for the Cap23 protein, dendritic arborization starts out normally, but cannot be maintained subsequently. Since all the data on which this observation is based is *in-vivo*, it rules out the possibility that the finding is a result of some artifact of experimental conditions. The loss of dendritic branches correlates with the onset of synapse elimination in the CNS, and continues thereafter. Since this phenotype has a definite onset point, and is progressive in nature, we refer to it as ‘dendritic atrophy’ as opposed to ‘dendritic pruning’.

The agreement of the data with existing knowledge about dendrites also strengthens the observation and conclusion of this thesis. The prevailing belief so far is that the growth and architecture of dendrites is determined to a large extent by neuron specific intrinsic programs. This conclusion is based largely on experiments conducted on cortical and hippocampal pyramidal cells in culture. Even in the absence of their natural environment, the cultured neurons form a dendritic tree that is largely reminiscent of their normal dendritic arborization. External factors like the presence and the concentration of neurotrophins are thought to only influence and augment the intrinsic ‘blueprint’ for dendritic patterning. Independent studies on tadpoles (*Xenopus spp.)* and Drosophila also concur with these conclusions.

In the light of these observations, it is not surprising that in the background of a deficiency of Cap23 protein, the neurons start out with a perfectly normal dendritic tree, but cannot maintain it subsequently. This delineates a role for Cap23 in the maintenance of dendrites, as opposed to a role in their formation. Dendrites are known to be formed very early in development and then essentially remain unchanged throughout the life of the individual. Cap23 is therefore, probably not involved in executing the cell-intrinsic blueprint for dendritic patterning. Instead, it is also likely that Cap23 is involved in maintenance of dendritic branching through its role in maintenance of cell structure. The Cap23

protein contains an N-terminal myristoylation and palmitoylation domain through which it is firmly tethered to the cell membrane, and could lead to mechanical strengthening of the cell cortex and to enhancing the stability of the cell structure. This could constitute a mechanism by which Cap23 helps in stabilization and maintenance of the cell cortex of the dendrites. The fact that the severity of the dendritic atrophy phenotype inversely varies with the level of the protein *in-vivo*, further points to a dosage-dependency, a stoichiometric requirement of the protein, and a role of the protein in maintaining structural stability.

The use of membrane targeted GFP enables us to see the entire dendrite arbor in great detail. The L15 line of mice used in this study labels only a few neurons, so there is no overlap between the arbors of neighboring neurons. To quantitate the dendrite atrophy phenotype, we counted the number of dendrite branchpoints in the mice of the different ages. By this criteria, in Cap23 knockout mice and in the heterozygotes, the number of dendritic branchpoints decrease in the apical and basal dendrites of the CA1 pyramidal cells of the hippocampus. This directly implies that the dendritic atrophy leads to a decrease in net dendritic length too. In addition, this also implies that it is the higher order dendrites (viz. tertiary and secondary) are the first ones to disintegrate while the primary dendrites remain intact. Thus, the dendrite atrophy proceeds from tertiary and quaternary dendrites and gradually proceeds to the secondary and primary dendrites. Indeed, only in some severely affected neurons in 1 year old mice we see that the primary dendrites have also been affected.

Recently, another report of an analogous nature has emerged from a study conducted on the $Abl^{-/-}$ knockout mice. Dendrites of cortical neurons in the Abl related gene (Arg) and the $Abl^{-/-}$/ $Arg^{-/-}$ double knockout mice initially develop normally, and are initially indistinguishable from wild type dendrites till postnatal day 21. Subsequently, there is a problem with dendritic branch maintenance, because of which there is a severe dendritic loss subsequently. In this context, it is interesting to note that the time point of 21 days is the same that we observe in

our study as well, and whose significance still remains unexplained. Dendritic branch extension, branching and maintenance are known to be modulated by activation of integrin receptors by adhesion to Laminin-1 or Semaphorin7A. Interestingly, this effect of laminin binding is absent in Arg-/- neurons in culture, leading to the speculative hypothesis that the maintenance of dendrites in the Arg-/- mice is mediated by integrins (Moresco et al 2005). The fact that the Abelson (Abl) family of non-receptor tyrosine kinases function by translating information from cell surface receptors into changes in cytoskeletal structure and cell morphology by phosphorylation of cytoskeletal elements, can be also used to speculate how Cap23 could be involved in maintenance of dendritic structures. It could be conjectured that reduced responsiveness to adhesive cues comprises the basis of the lack of dendritic branch maintenance in neurons deficient in Cap23.

The Cap23 protein is known to bind to the end of actin filaments, presumably stabilizing them, and therefore stabilizing the cell structure. It is known that Abl family of proteins also have a C-terminal extension containing binding sites for actin and microtubules, which can be directly used to maintain cytoskeletal structure (McWhirter and Wang, 1993; Van Etten et al 1994; Wang et al 2001; Miller et al 2004). So, it is likely that the deficiency of Cap23 leads to weakening of the cytoskeletal structure and thereby leads to a deficiency in the maintenance of dendrites due to abnormal signaling through integrins and the non-receptor tyrosine kinases.

Maintenance of synapse numbers in Cap23 mutants.

Since dendrites are the postsynaptic counterparts in the brain, and provide the substratum for the formation of synapses, a loss of dendrites would imply a decrease of synapse number in the brain. Since synapses are the functional elements of the brain, it is difficult to imagine how the animal would be able to live a normal life in the face of such drastic reduction in synapse number. It might seem surprising that the Cap23 heterozygous seem morphologically normal and

are able to live a healthy life in spite of the severe loss of dendrites in its brain. This anomaly is resolved by analyzing the ultrastructure of the neuropil of these mice. Electron micrographs show the presence of larger presynaptic terminals in the neuropil of the Cap23 knockout mice. The increased surface area offered by these larger presynaptic terminals is covered by multiple postsynaptic terminals, thus increasing the total number of release sites and conserving the total synapse numbers. It is also tempting to speculate that these large presynaptic terminals may have different neurotransmitter release properties, and may have a higher quantal content. Our time course studies of the fine structure of the dendrites offers an insight of how these large preterminals and perforated synapses develop. Before the onset of dendritic atrophy, the dendrites of wild type mice and the Cap23 mutants look similar in terms of the fine structure of the dendrites. But concomitant with the loss of dendrite branches in the mutants, the remaining branches tend to get thicker than the corresponding branches in the wild type controls. This increase serves to increase the net surface area offered by these dendrites. Simultaneously, the dendrites of the heterozygotes and the mutants develop increased number of complex spines. For the purpose of this study, we define complex spines as the spines that have multiple heads. Since each of these spine-heads makes a synapse, this presumably acts as a mechanism for conservation of synapse numbers in the absence of Cap23. In terms of a broader outlook, this reinforces the precept that there are very strong compensatory mechanisms in the brain that work to keep the total number of synapses in the brain at a constant number.

Cap23 and non-coding RNAs.

It is clear from this study that the lack of the Cap23 protein is upstream of the atrophy of dendrites. However, the exact mechanism by which Cap23 controls the maintenance of dendrites is not clear from this study. Cap23 may be directly involved in the maintenance of dendritic branches, or there may be other intermediaries in the process. However, the importance of the study lies in the

fact that this is the first study that clearly shows the importance of one single gene in the maintenance of dendritic arbors of a prominent class of neurons *in-vivo.*

An insight into the molecular mechanism by which Cap23 controls stability of dendrites can be obtained by knowing the *in-vivo* function of Cap23. It is a relatively small protein, and is conspicuous due to the lack of any consensus DNA binding domain. However, reports exist in the literature implicating the human homolog of Cap23, Basp1 as being a transcriptional coactivator for the Wilm's Tumor gene WT1(Carpenter *et al* 2004). Cap23 however has Calcium Calmodulin binding domains, though the downstream events of this binding are not exactly clear. On the contrary, the 3'UTR of the Cap23 transcript has a few interesting sites. Notable among them is the presence of the G-quartet sequence- a sequence of four guanosine residues spaced out in a particular way- that is believed to be a recognition sequence for binding to the Fmr1 protein. The FMRP protein is known to be localized to synapses and is known to control the local translation of a number of transcripts important for synaptic plasticity. Cap23 is a known target for FMRP binding. It is likely that the G-quartet sequence in the 3'UTR of Cap23 is functionally important for the *in-vivo* role of Cap23, though we have no clear idea as to what its mode of action could be.

One of the most important insights into the role of Cap23 *in-vivo* came from 2D-gels done in the lab from whole brain extracts. These gels showed the clear downregulation of a number of proteins in the background of a deficiency of Cap23 (Wacha & Caroni-unpublished results). Moreover, the levels of these proteins showed a dosage dependency on the levels of Cap23, i.e. the extent of misregulation was more in the Cap23 knockouts compared to that in the Cap23 heterozygotes. A large number of these proteins had known roles in maintaining structural and functional viability of the synapse and also of the axon and the dendrites. It was not clear from this data whether the role of Cap23 in regulating these proteins is at the level of transcription or translation. However, the level of

these transcripts in the transcriptome of the adult Cap23 heterozygotes is conserved, it suggests that Cap23 exerts its effect post-transcriptionally. This effect could either be on the translational efficiency of the transcripts, or on their rate of degradation.

One of the prominent features of the transcriptome of the Cap23 heterozygous mice is the upregulation of a group of non-coding RNAs. These RNAs are upregulated only in the transcriptome of the adult mice and not in the transcriptome of the young mice. This correlation with the occurrence of the dendritic atrophy phenotype strongly suggests that these non coding RNAs could have a role to play in the regulation of the proteins and in the occurrence of dendritic atrophy. Non-coding RNAs are assuming increasing importance, especially in the nervous system. According to recent estimates, about 50% of the human genome is thought to code for non-coding RNAs. Most prominent among the non-coding RNAs are the micro RNAs. We have no idea whether the non-coding RNAs upregulated in our screens are miRNA precursors. However, all of the non-coding RNAs that show up in our screens have features similar to miRNA precursors. They have predicted stem loop structures, localize to introns of known genes and even show homology with known miRNAs. In adult Cap23 heterozygote brains, at least one miRNA, Mir128 is known to be massively downregulated. No targets are known for this micro RNA, though from work done elsewhere, it is known to localize to the cell body layers of the pyramidal cells and the dentate gyrus in the hippocampus. Interestingly, in-situ hybridization studies done with one of the most highly upregulated non-coding RNAs(1460101_at) also shows localization of the RNA to the cell body layers of the dentate gyrus and the pyramidal cells in the hippocampus. The changes in the proteome in the background of a deficiency of Cap23 is an important result of key significance. It is not clear from our results as to how Cap23 regulates the expression of these proteins. Nevertheless, the correlation between the time course of occurrence of the non-coding RNAs and the occurrence of the phenotype is indicative of a causal relationship between the two phenomena.

Outlook.

Investigations into the mechanisms regulating growth and maintenance of dendrites are a rather new field. Most of the early work has been done *in-vitro* on neurons in culture. Recently, the relative tractability of the Drosophila system and the availability of the genome data has led to the identification of mutants defective in dendritic arbor formation. Some of these mutants have also been characterized, though in none of these cases, underlying mechanisms have been explored. All of these mutations lead to either decreased or increased arbor formation, indicating that their role is in the growth of dendrites, and not in their maintenance. In mammalian systems, very little is known about mutants defective in dendritogenesis. Only recently, the first report of the Abl and Arg kinases in the maintenance of dendritic arbors in mice was published. Interestingly, they also report a loss of dendritic branches between the time period of 20 to 30 days, indicating that this time interval has a particular physiological significance.

The work reported in this thesis is important because it causally links a mutation in a single gene to the maintenance of dendrites of a prominent class of neurons *in-vivo,* and relates it to changes in the levels of synaptically important proteins that are misregulated in the mutants in a dose dependent manner. A direct causal relationship and the establishment of the chain of events leading from the mutation to the phenotype is missing, and possibilities are rich for further work in that direction. It would be interesting to find out how Cap23 is related to the occurrence of the non-coding RNAs, and whether they, in turn are miRNA precursors. We were unsuccessful so far in replicating the phenotype in organotypic slice cultures in a decisive manner. Achieving that would enable pharmacological interventions to be done to investigate the effect of Cap23 in dendritic arbor maintenance. These experiments would be important in clarifying

whether Cap23 exerts its effect through gene regulation or directly by affecting structure of the cell.

BIBLIOGRAPHY

Aakalu, G., Smith, W. B., Nguyen, N., Jiang, C., and Schuman, E. M. (2001). Dynamic visualization of local protein synthesis in hippocampal neurons. Neuron *30*, 489-502.

Ahmari, S. E., and Smith, S. J. (2002). Knowing a nascent synapse when you see it. Neuron *34*, 333-336.

Aigner, L., Arber, S., Kapfhammer, J. P., Laux, T., Schneider, C., Botteri, F., Brenner, H. R., and Caroni, P. (1995). Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. Cell *83*, 269-278.

Betz, W. J., Mao, F., and Bewick, G. S. (1992b). Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. J Neurosci *12*, 363-375.

Brown, V., Jin, P., Ceman, S., Darnell, J. C., O'Donnell, W. T., Tenenbaum, S. A., Jin, X., Feng, Y., Wilkinson, K. D., Keene, J. D.*, et al.* (2001). Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. Cell *107*, 477-487.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. Science *263*, 802-805.

Cline, H. T. (2001). Dendritic arbor development and synaptogenesis. CurrOpinNeurobiol *11*, 118-126.

Dailey, M. E., and Smith, S. J. (1996). The dynamics of dendritic structure in developing hippocampal slices. J.Neurosci *16*, 2983-2994.

De Paola V, Arber S and Caroni P (2003). AMPA receptors regulate dynamic equilibrium of presynaptic terminals in mature hippocampal networks. Nature Neurosci. 6(5): 491-500.

Dunaevsky, A., Tashiro, A., Majewska, A., Mason, C., and Yuste, R. (1999). Developmental regulation of spine motility in the mammalian central nervous system. Proc. Natl. Acad. Sci. USA *96*, 13438-13443.

Feng, G., Mellor, R. H., Bernstein, M., Keller-Peck, C., Nguyen, Q. T., Wallace, M., Nerbonne, J. M., Lichtman, J. W., and Sanes, J. R. (2000). Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron *28*, 41-51.

Fiala, J. C., Allwardt, B., and Harris, K. M. (2002). Dendritic spines do not split during hippocampal LTP or maturation. Nat Neurosci *5*, 297-298.

Francesca Zalfa, M. G., Beatrice Primerano, Annamaria Moro, Alessandra Di Penta, Surya Reis, Ben Oostra, and Claudia Bagni (2003). The Fragile X Syndrome Protein FMRP Associates with *BC1* RNA and Regulates the Translation of Specific mRNAs at Synapses. Cell, 317-327.

Frey D, L. T., Xu L, Schneider C, Caroni P. (2000). GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. J Cell Biol Jun 26;149(7):1455-72.

Galkin, V. E., Orlova, A., Koleske, A. J., and Egelman, E. H. (2005). The Arg non-receptor tyrosine kinase modifies F-actin structure. J Mol Biol *346*, 565-575.

Grueber, W. B., Jan, L. Y., and Jan, Y. N. (2003). Different levels of the homeodomain protein cut regulate distinct dendrite branching patterns of Drosophila multidendritic neurons. Cell *112*, 805-818.

Grueber, W. B., Ye, B., Moore, A. W., Jan, L. Y., and Jan, Y. N. (2003). Dendrites of distinct classes of Drosophila sensory neurons show different capacities for homotypic repulsion. Curr Biol *13*, 618-626.

Grutzendler, J., Kasthuri, N., and Gan, W. B. (2002). Long-term dendritic spine stability in the adult cortex. Nature *420*, 812-816.

Haas, K., Sin, W. C., Javaherian, A., Li, Z., and Cline, H. T. (2001). Single-cell electroporation for gene transfer in vivo. Neuron *29*, 583-591.

Harris, K. M. (1999b). Structure, development, and plasticity of dendritic spines. Curr Opin Neurobiol *9*, 343-348.

Honig, M. G., and Hume, R. I. (1989). Dil and diO: versatile fluorescent dyes for neuronal labelling and pathway tracing. Trends Neurosci *12*, 333-335, 340-331.

Horch, H. W., Kruttgen, A., Portbury, S. D., and Katz, L. C. (1999). Destabilization of cortical dendrites and spines by BDNF. Neuron *23*, 353-364.

Jennifer C. Darnell., Peng Jin, Victoria Brown, Stephen T. Warren, and Robert B. Darnell. Fragile X Mental Retardation Protein Targets G Quartet mRNAs Important for Neuronal Function. Cell *107*, 489-499.

Jugloff, D. G., Jung, B. P., Purushotham, D., Logan, R., and Eubanks, J. H. (2005). Increased dendritic complexity and axonal length in cultured mouse cortical neurons overexpressing methyl-CpG-binding protein MeCP2. Neurobiol Dis *19*, 18-27.

Kaufmann, W. E., Moser, H. W (2000) Dendritic anomalies in disorders associated with mental retardation. Cereb.Cortex 10:981-991.

Khan, T. K., Yang, B., Thompson, N. L., Maekawa, S., Epand, R. M., and Jacobson, K. (2003). Binding of NAP-22, a calmodulin-binding neuronal protein, to raft-like domains in model membranes. Biochemistry *42*, 4780-4786.

Kirov, S. A., and Harris, K. M. (1999). Dendrites are more spiny on mature hippocampal neurons when synapses are inactivated. Nature Neurosci *2*, 878-883.

Kirov, S. A., Sorra, K. E., and Harris, K. M. (1999). Slices have more synapses than perfusion-fixed hippocampus from both young and mature rats. J.Neurosci *19*, 2876-2886.

Laux,T., Fukami,K, Thelen,M., Golub,T., Frey,D.and Caroni,P.(2000). Gap43,MARCKS, and Cap23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. J.Cell.Biol. 149(7). 1455-72.

Lendvai, B., Stern, E. A., Chen, B., and Svoboda, K. (2000). Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex in vivo. Nature *404*, 876-881.

Lichtman, J. W., and Colman, H. (2000). Synapse elimination and indelible memory. Neuron *25*, 269-278.

Lichtman, J. W., Magrassi, L., and Purves, D. (1987). Visualization of neuromuscular junctions over periods of several months in living mice. J Neurosci *7*, 1215-1222.

Lo, D. C., McAllister, A. K., and Katz, L. C. (1994). Neuronal transfection in brain slices using particle-mediated gene transfer. Neuron *13*, 1263-1268.

Scott, E. K. and Liqun Luo (2001). How do dendrites take their shape? Nature Neuroscience *4*, 359.

Magrassi, L., Purves, D., and Lichtman, J. W. (1987). Fluorescent probes that stain living nerve terminals. J Neurosci *7*, 1207-1214.

Maletic-Savatic, M., Malinow, R., and Svoboda, K. (1999). Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. Science *283*, 1923-1927.

McAllister, A. K., Lo, D. C., and Katz, L. C. (1995). Neurotrophins regulate dendritic growth in developing visual cortex. Neuron *15*, 791-803.

Miller, A. L, Wang,Y, Mooseker, M. S, ,Koleske A. J. (2004):The Abl related gene (Arg) requires it's F-actin microtubule cross-linking activity to regulate lamellipodial dynamics during fibroblast adhesion. J.Cell.Biol. 165:407-419.

Moresco, E. M., Donaldson, S., Williamson, A., and Koleske, A. J. (2005). Integrin-mediated dendrite branch maintenance requires Abelson (Abl) family kinases. J Neurosci *25*, 6105-6118.

Moriyoshi, K., Richards, L. J., Akazawa, C., O'Leary, D. D., and Nakanishi, S. (1996). Labeling neural cells using adenoviral gene transfer of membrane-targeted GFP. Neuron *16*, 255-260.

Moser, M. B., Trommald, M., and Andersen, P. (1994). An increase in dendritic spine density on hippocampal CA1 pyramidal cells following spatial learning in adult rats suggests the formation of new synapses. Proc Natl Acad Sci U S A *91*, 12673-12675.

Niblock, M. M., Brunso-Bechtold, J. K., and Riddle, D. R. (2000). Insulin-like growth factor I stimulates dendritic growth in primary somatosensory cortex. J Neurosci *20*, 4165-4176.

Nimchinsky, E. A., Sabatini, B. L., and Svoboda, K. (2002). Structure and function of dendritic spines. Annu Rev Physiol *64*, 313-353.

Posern, G., Saffrich, R., Ansorge, W., and Feller, S. M. (2000). Rapid lamellipodia formation in nerve growth factor-stimulated PC12 cells is dependent on Rac and PI3K activity. J Cell Physiol *183*, 416-424.

Purves, D., and Hadley, R. D. (1985). Changes in the dendritic branching of adult mammalian neurones revealed by repeated imaging in situ. Nature *315*, 404-406.

Reid, B., Slater, C. R., and Bewick, G. S. (1999). Synaptic vesicle dynamics in rat fast and slow motor nerve terminals. J Neurosci *19*, 2511-2521.

Rosso, S. B., Sussman, D., Wynshaw-Boris, A., and Salinas, P. C. (2005). Wnt signaling through Dishevelled, Rac and JNK regulates dendritic development. Nat Neurosci *8*, 34-42.

Segal, I., Korkotian, I., and Murphy, D. D. (2000). Dendritic spine formation and pruning: common cellular mechanisms? Trends Neurosci *23*, 53-57.

Shim, K. S., and Lubec, G. (2002). Drebrin, a dendritic spine protein, is manifold decreased in brains of patients with Alzheimer's disease and Down syndrome. Neurosci Lett *324*, 209-212.

Smart, F. M., and Halpain, S. (2000). Regulation of dendritic spine stability. Hippocampus *10*, 542-554.

Song, H. J., Ming, G. L., and Poo, M. M. (1997). cAMP-induced switching in turning direction of nerve growth cones. Nature *388*, 275-279.

Takasaki, A., Hayashi, N., Matsubara, M., Yamauchi, E., and Taniguchi, H. (1999). Identification of the calmodulin-binding domain of neuron-specific protein kinase C substrate protein CAP-22/NAP-22. Direct involvement of protein myristoylation in calmodulin-target protein interaction. J Biol Chem *274*, 11848-11853.

Trachtenberg, J. T., Chen, B. E., Knott, G. W., Feng, G., Sanes, J. R., Welker, E., and Svoboda, K. (2002). Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. Nature *420*, 788-794.

van der Pol, A. N., and Ghosh, P. K. (1998). Selective neuronal expression of green fluorescent protein with cytomegalovirus promoter reveals entire neuronal arbor in transgenic mice. J Neurosci *18*, 10640-10651.

VanEtten, R. A., Jackson P. K., Baltimore, D., Sanders M. C., Matsudaira P. T.,Janmey P. A.(1994): The COOH terminus of the c-Abl tyrosine Kinase contains distinct F and G-actin binding domains with bundling activity. J.Cell.Biol. 124: 325-340.

Wang, Y., Miller A. l., Moosker, M. S., Koleske, A. J. (2001)The Abl related gene (Arg) non receptor tyrosine kinaseuses twoF-actin binding domains to bundle F-actin. PNAS98:14865-14870.

.Grueber, W. B., Adrian, B. Y., Jan, L. Y. and Jan, Y. N. (2003). Dendrites of distinct classes of Drosophile sensory neurons show different capacities for homotypic repulsion. Current Biology *13*, 618.

Wiederkehr, A., Staple, J., and Caroni, P. (1997). The motility-associated proteins GAP-43, MARCKS, and CAP-23 share unique targeting and surface activity-inducing properties. Exp Cell Res *236*, 103-116.

Wong, W. T., and Wong, R. O. (2000). Rapid dendritic movements during synapse formation and rearrangement. Curr Opin Neurobiol *10*, 118-124.

Yamamoto, Y., Sokawa, Y., and Maekawa, S. (1997). Biochemical evidence for the presence of NAP-22, a novel acidic calmodulin binding protein, in the synaptic vesicles of rat brain. Neurosci Lett *224*, 127-130.

Yankova, M., Hart, S. A., and Woolley, C. S. (2001). Estrogen increases synaptic connectivity between single presynaptic inputs and multiple postsynaptic CA1 pyramidal cells: a serial electron-microscopic study. Proc Natl Acad Sci U S A *98*, 3525-3530.

Yuste, R., and Bonhoeffer, T. (2001). Morphological changes in dendritic spines associated with long-term synaptic plasticity. AnnuRevNeurosci *24*, 1071-1089.

Yuste, R., and Denk, W. (1995). Dendritic spines as basic functional units of neuronal integration. Nature *375*, 682-684.

Zhai, R. G., Vardinon-Friedman, H., Cases-Langhoff, C., Becker, B., Gundelfinger, E. D., Ziv, N. E., and Garner, C. C. (2001). Assembling the presynaptic active zone: a characterization of an active one precursor vesicle. Neuron *29*, 131-143.

CURRICULUM VITAE

Name: **ANIRBAN SADHU**

Date of birth: 15th April 1976

Profile: Ph.D in Molecular Biology with 7-8 years of research experience in academia and industry,with multicultural work experience.

Address for Correspondence:

FRIEDRICH MEISCHER INSTITUTE
Novartis Research Foundation
Maulbeerstrasse 66
4058, Basel.
Switzerland.

E-mail:
- sadhu@fmi.ch (preferred)
- a_sadhu@hotmail.com

Telephone: **0041-764130934**
Fax: **0041-61-6973976**

Academic Qualifications:

Examination	Board/ University	Year	Marks/ Class/ Remarks
Secondary School (AISSE)	Central Board of Secondary Education (CBSE).	1992	**80.6 %**(First Class- Top 1/8th of all candidates that appeared.)
Higher-Secondary (AISSCE)	Central Board of Secondary Education (CBSE).	1994	**76%**
Bachelor's in Science (Microbiology)	University of Pune, India	1998	First Class
Masters in Science (Molecular Biology)	Tata Institute of Fundamental Research (TIFR). University of Bombay. (One of a class of five).	2001	By thesis- 1st Class. (Original work elucidating the genetic basis of sugar transport across cell membranes.)
2001-2005 Pursuing doctoral research(Ph.D) at the FMI,Novartis, Switzerland . Working towards understanding the structural plasticity in the mammalian brain.			

Employment History:

Organization	Designation	Duration
Friedrich Miescher Institute,Novartis Research Foundation, Basel	**Doktorand**	**September 2001- 2005**
Tata Institute of Fundamental Research (TIFR); Dept. of Atomic Energy;Govt. of India.	**Scientific Assistant'B'.**	**1998- 2001**

Additional Academic Honors and Research Experiences:

1.Stood second out of 300 university level students in the **Exploratory** Science Talent Search Examinationat the University level in 1996-97.

2. Summer Project: Worked as a summer trainee in the **Molecular Biology Research Laboratory (MBRL, University of Pune)** (1996-1997). Familiarised myself with research methodology in this first exposure to advanced research.

3. Master's(M.Sc) Thesis Project : Worked on an independent and original research project aimed at elucidating the molecular and genetic basis of sugar transport using the fission yeast as a model system at the **Tata Institute of Fundamental Research(TIFR)**, India(1998-2001). The work was published in form of a master's thesis and has implications in the understanding of human disorders like diabetes.

4.International Workshop: Chosen to participate in an international workshop on new Theoretical and Practical developements in Yeast Molecular Genetics at the **International Centre for Genetic Engineering and Biotechnology(ICGEB)** at Trieste, Italy(21st to 26th March 1999).

5. International Summer Student's Program: Selected to participate in this prestigious program at the **Weizmann Institute of Science, Rehovot,Israel** in the Department of Neurobiology (June 2000-August 2000). Worked on an independent and original project aimed at purifying and characterizing a protein(Nir2) involved in causing retinal degeneration and blindness.

6. Doctoral research: Presently working in the field of neurobiology at the **Friedrich Meischer Institute(FMI),Novartis** in Basel, Switzerland. The work is aimed at understanding the genetic and physiological basis of the dynamics involved in the maintenance of structural stability in the mammalian brain.

Representations in Important Scientific Events:

1. Selected to participate in the **International Brain Research Organisation**(IBRO) conference on Neurobiology(6th to 13th Dec. 1998; India).

2. Selected to participate and present a poster in the 2000 **Gordon Research Conference** on "Membrane Transporters" in New London,Connecticut.(Offer declined).

3.Selected to participate in the **NASA** 'Exobiology Summer Program' at **NSCORT**,San Diego in June 2000.(Offer Declined.)

4. Presented a poster titled"Studies on sugar transport defective(*std1*) mutants of *S.pombe*" in the International Symposium on Developemental Regulation at the **National Centre for Cell Science** (NCCS), Pune from 18th to 20th February 2000.

5.Selected to participate in the Third Borsellino College on Neurophysics titled"Evolution of intelligent behavior " at the **International Center for Theoretical Physics**(ICTP),Trieste,Italy from 22nd April to 5th March 2001.

6.Selected to participate in the **EMBO-FMI** workshop titled" Organising the brain: Genes, neurons and circuits" at Ascona,Switzerland (Feb 5th to 9th. 2002).

7. Selected to participate in the course on "Mouse Behavioral Analysis" at the **Cold Spring Harbor Laboratory**,New York (23rd June to 11 July 2002).

8. Invited to give a talk on "Plasticity in the Adult Brain" in the Department of Biotechnology,**Indian Institute of Technology**(IIT),Kharagpur in December 2002.

9. Selected to participate in the international conference on "Axon Guidance and Neural Plasticity" at the **Cold Spring Harbor Laboratory**(CSHL), New York(18th to 22nd Sept 2004.)

Grants and Fellowships:

1. Received a travel grant from the "**Sir Ratan Tata Trust**" Mumbai, in June 2000 to travel to attend the International Summer Program at the Weizmann Institute Israel.
2. Recieved a travel grant from the **International Centre for Theoretical Physics** (ICTP) to attend the Third Borsellino College on Neurophysics.
3. Recieved a travel grant from the "**Dorabji Tata Trust**",Mumbai in 2000 for a research related travel to the Weizmann Institute, Israel.
4. Currently a recipient of the **FMI Ph.D Fellowship**.

Organizational and leadership activities:

1. Presently involved in organizing a career guidance conference (http://www.cgc2005.com/) at the university level in collaboration with Novartis,FMI and the University of Basel.(**Draft Proposal**)
2. Presently involved in nominating and inviting speakers for the **FMI student's science colloquia**.
3. Previously been involved in the organisation and the running of debating societies and journal clubs at the university level, and in the counselling of junior students.
4. Have intermittently given popular science talks to senior school and college level students many times in the last three years.