

**Cytoskeletal dynamics in and traumatic injury of
cerebellar and hippocampal neurons**

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**Cytoskeletal dynamics in and traumatic injury of
cerebellar and hippocampal neurons**

Cytoskeletele dynamiek en traumatische letsel van
cerebellaire en hippocampale zenuwcellen

Proefschrift

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Chapter 3.1: Stepanova T, Slemmer J, Hoogenraad CC, Lansbergen G, Dortland B, De Zeeuw CI, Grosveld F, van Cappellen G, Akhmanova A, Galjart N (2003) Visualization of microtubule growth in cultured neurons via the use of EB3-GFP (end-binding protein 3-green fluorescent protein). J Neurosci 23: 2655-2664.

science, religion
are not incompatible—
the more that i learn

about cellular
and molecular bases
of life the more i

believe in the soul—
attempting to reduce the
human form to a

mere collection of
genes only demonstrates our
divine instructions

for my parents...

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CHAPTER 1

General Introduction

1.1 Scope of this thesis

This thesis addresses two separate, yet overlapping, physiological processes, namely traumatic brain injury (TBI) and microtubule (MT) dynamics, primarily through the use of cultured cells derived from embryonic mice. The difficulties which arise through the experimental study of these processes, either in brain slices or in the whole animal, can be partially circumvented by using various *in vitro* systems to tease out the factors contributing to, and the mechanisms behind, these complex phenomena. Here, focus is given to cell cultures derived from two different brain regions, the hippocampus and the cerebellum, reviewed in this Chapter. In addition, the Introduction describes cell culture protocols, and neuronal cytoarchitecture in the hippocampus and the cerebellum, before relating these findings to glutamate-mediated cell death, particularly in cerebellar Purkinje cells (PCs). The effects of “excitotoxicity” are identified in these cells at the level of the dendritic spine and the MTs of the neuronal cytoskeleton. Alterations in both cultured PCs and hippocampal neurons are also described in relation to traumatic injury and to Williams Syndrome (WS), a rare genetic disorder. This initial portion provides a theoretical framework for the experimental studies contained in the successive chapters. In **Chapter 2**, cultured cells are injured using an *in vitro* model of stretch-induced injury. With this model, the cellular sequelae of trauma are examined in hippocampal and cerebellar cells. Several questions are asked: 1) What are the effects of increasing degrees of stretch on these cells? 2) What are the similarities and differences between neurons and glia in response to stretch injury? 3) What are the similarities and differences between hippocampal and cerebellar cells in response to stretch injury? 4) What is the relationship between TBI and glutamate-mediated excitotoxicity? and 5) What are the potential correlates among stretch injury *in vitro*, experimental injury to animals *in vivo*, and clinical TBI? **Chapter 3** is concerned with the activity of MT plus-end binding proteins, namely end-binding protein 3 (EB3) and cytoplasmic linker protein of 115 kDa (CLIP-115), in neurons. First, cultured hippocampal and cerebellar cells are made to express EB3 tagged with green fluorescent protein (GFP) in order to image and quantify the activity of MTs in neurons and glia. Second, hippocampal brain slices from adult CLIP-115 homozygous (knockout, or KO) mice are examined in order to quantify the cellular effects of this genetic deletion. Several questions are asked: 1) What is the role of plus-end binding proteins in neurons, especially as compared to glia or to other non-neuronal cells? 2) What are the similarities and differences in the velocities of growing MTs in hippocampal neurons and glia, and in cerebellar PCs and glia? 3) What happens to MT growth velocities, and other dynamic processes, when cultured cells derived from CLIP-115 KO mice are made to express EB3-GFP? 4) What are the effects of the deletion of CLIP-115 on dendritic and spine morphologies in the adult mouse hippocampus? and 5) What are the implications of altered MT dynamics for patients with WS? Lastly, **Chapter 4** presents concluding remarks and implications of the present data. Here future studies are highlighted that utilize *in vitro* preparations for the combined study of cytoskeletal dynamics and traumatic injury.

1.2 Cytoarchitecture

1.2.1 Cultured embryonic neurons and glia

Many experimental techniques derive benefit from the use of dissociated cell cultures. A major advantage of culture systems over *in vivo* experimentation in the whole animal is that a minimum number of animals are used to generate multiple dishes or plates of cells. Cultured cells also

have certain advantages over organotypic slice cultures—in that they are easier to generate and grow—and acute, brain slice preparations—in that they can live for many days, even weeks. For example, cultured hippocampal cells are a traditional way of examining neuronal function, as these cells are easy to grow and maintain *in vitro*, and mature quickly with acceptable neuronal morphology (axons, dendrites and soma). In Chapter 3.1, young hippocampal neurons [2-6 days *in vitro* (DIV)] were transfected with EB3—an MT-associated protein (MAP) that specifically attaches to growing, plus-ends of MTs—tagged with GFP. In this way, living individual neurons, and individual growing MTs inside these neurons, could be visualized and imaged using confocal microscopy. EB3-GFP was then expressed in cerebellar PCs, as these cells produce morphologically distinct regions upon maturity *in vitro* (<10 DIV). Cerebellar cell cultures, especially cultures enriched in PCs, are rarely if ever used to study the two phenomena covered in this thesis, as PCs are often difficult to maintain *in vitro*. In our hands, previously published protocols did not produce a large enough number of viable PCs. Therefore, a unique culturing procedure was developed (described in detail in the Methods section of Chapter 2.1); mouse hippocampal cells (for all chapters other than Chapter 3.1 and 3.2) were generated using the same protocol. This procedure has subsequently been successfully used to culture mouse cortical cells. Figure 1 depicts prototypical cultured PCs stained for calbindin-D28K, a calcium-binding protein specific to PCs in cerebellar cultures, at increasing DIV. These cells display large and complex dendritic arborizations that are clearly distinguishable from axons as early as 10 DIV, thus allowing for precise analysis of intracellular processes.

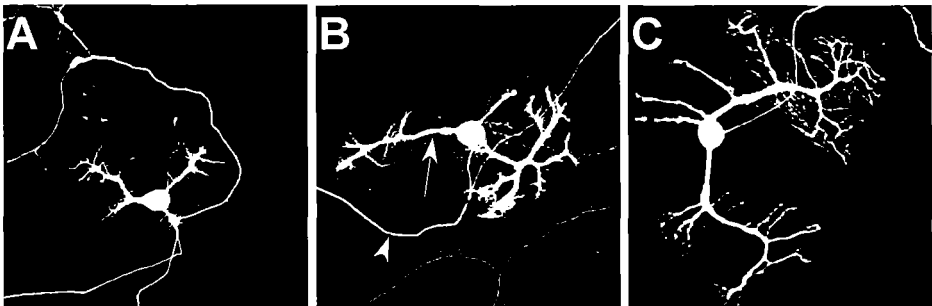


Figure 1. Purkinje cells (PCs) stained for calbindin-D28K at increasing days *in vitro* (DIV). The development of a unique cell culturing protocol enabled the growth and maintenance of cerebellar PCs with recognizable morphology. An arrow in B indicates a dendritic branch, whereas an arrowhead indicates the axon. **A:** 10 DIV. **B:** 16 DIV. **C:** 23 DIV.

There are many protocols available for the generation of cultured neurons and glia, and although the details differ widely (for example, the type of culture media used), several basic features remain the same. The first portion of all protocols requires the surgical isolation of a region or regions of interest. For our embryonic cultures, pregnant female mice are killed by rapid cervical dislocation. The embryos (at embryonic day 18) are surgically removed from the mother, and placed immediately on ice to minimize suffering, as embryos and newborn pups can survive asphyxiation by CO₂ for long periods of time. The embryonic sacs are cut open to expose the embryos. The brains are then surgically removed from the skulls and are placed in ice-cold Ca²⁺/Mg²⁺-free Hank's balanced saline solution (HBSS). Brain regions of interest (e.g. cortex, hippocampus, and/or cerebellum) are removed under a dissecting microscope. Tissue is collected in a 15-ml tube containing fresh, ice-cold HBSS and spun in a centrifuge to collect the pieces at the bottom of the tube, a step often referred to as “washing” the tissue. (Cell cultures can be very

vulnerable to fungal and bacterial infections; therefore, it is important to continually wash the tissue in fresh, clean media to reduce this potentiality.) The tissue is then treated with trypsin, an enzyme that is often used in culturing protocols in order to separate the bonds that hold individual cells together. This step is performed at 37°C in order to activate the trypsin. After approximately 13-15 min of trypsinization, the cells are centrifuged, the supernatant is removed, and then the cells are washed with serum-containing growth media in order to inactivate the trypsin. The tissue, however, still remains in rather large pieces, and the cells are repeatedly taken up into and forced out of a plastic pipette, or “triturated”. Eventually, the cells become dissociated from each other, and form a somewhat homogenous cell suspension. This suspension is then forced through a sterilized microfilter to break up any last clumps of cells. The suspension is diluted in an appropriate volume of serum-containing media, forming a completely homogenous mixture, and is then immediately aliquoted onto culture plates or dishes to ensure that each culture well receives an identical number of dissociated cells. In general, it is crucial to keep brains, media and surgical instruments sterile throughout the dissection, and to complete the entire procedure as quickly as possible to minimize the amount of handling of the cells, which has been shown to increase cell death.

Several key decisions must be made, however, in order to generate successful, healthy cultures for *in vitro* experimentation. We have used cultures derived from embryonic mice for our experiments. It is possible to use cultures derived from rat, as well as cultures derived from neonatal animals. Embryonic cultures have several benefits: first, because minimal animal suffering is one of our major aims, killing embryos is less invasive than killing neonatal pups; and second, both neurons and glia continue to grow and reproduce more easily after they are dissociated and plated. Our growth media, like most others, contains serum so that the glia can grow and multiply during the initial DIV. In our hands, an abundant monolayer of glia, upon which neurons tend to grow, appears to be crucial for the generation of healthy PCs. As glia grow and proliferate, they produce harmful toxic substances which accumulate in the media, and therefore, at 2 DIV, and then two times per week, half of the media is removed, and is replaced with serum-free culture media in order to reduce further proliferation of glia (see Chapter 2.1). In this way, there is always a small amount of serum remaining in each well; many other culturing protocols deprive cells of serum after a few DIV. To conclude, although some trial-and-error is to be expected when instigating a cell culture system, previously published protocols do offer an abundance of suggestions and tips.

One of the most impressive feats performed by molecular geneticists in recent years concerns the generation of mutant animals, often mice, in which a gene of interest is either added to (transgenic mouse), or made ineffective in (KO mouse), the animal’s genome. In Chapter 2.4, cultures were created from transgenic mice that possess an enhanced yellow fluorescent protein (EYFP) that is sensitive to changes in intracellular Cl⁻ in subsets of neurons. One of the initial events in neurons after TBI is a large influx of Cl⁻, which not only causes cell swelling, but also alters the delicate homeostasis within the cell. Short applications of glutamate led to membrane depolarization, causing Cl⁻ to enter hippocampal neurons via specific Cl⁻ channels. Cultures derived from these mice highlight the role of Cl⁻ in glutamate-mediated excitotoxicity. In addition, a large portion of this thesis focuses on mice in which the *Cyln2* gene has been deleted (Chapters 3.2 and 3.3). This gene codes for the cytoplasmic linker protein, CLIP-115, which, like EB3, associates specifically with growing MT plus-ends (see section 1.5.6). The use of cell cultures from CLIP-115 KO mice and from their wild-type littermates enables the study of two distinct populations of neurons and glia, to the point of determining the role of a single protein, and the deletion thereof, in the regulation of MT dynamics.

1.2.2 *In vitro* systems and experimentation

As mentioned above, various systems of *in vitro* experimentation have been created with the aim of breaking down complex physiological phenomena into smaller, and hopefully more simple, pieces. For example, several of the following chapters (Chapters 2.1, 2.2 and 2.3) concern the study of traumatic injury by subjecting hippocampal and cerebellar cell cultures to an *in vitro* model of stretch-induced mechanical injury (see Figure 2). This model, which was developed in the laboratory of E. F. Ellis in 1995, simulates a major component of TBI, that being tensile tissue strain, or stretch. These acceleration-deceleration stresses are similar to those incurred during motor vehicle accidents (Schreiber *et al.*, 1995), and sport-related concussions (Powell and Barber-Foss, 1999). Other models have also been developed in order to study traumatic injury *in vitro* (for review, see Morrison *et al.*, 1998). The uniaxial stretch model for axonal injury, for example, allows the careful study of alterations in axonal morphology and intracellular dynamics after injury (Pfister *et al.*, 2003). Although data gathered from *in vivo* studies of single and repetitive insults may be advantageous, in that changes in animal behavior post-injury can be measured and correlated to cell damage, these studies can only postulate the underlying mechanisms of cognitive impairment at the cellular level after trauma. Thus, it is important to supplement *in vivo* data with well-designed *in vitro* studies.

Our model offers the opportunity to study cellular mechanisms of trauma that were otherwise unable to be studied *in vivo* due to technical constraints. Previous studies have validated this model by demonstrating that *in vitro* stretch injury produces many of the post-traumatic responses observed after *in vivo* TBI, including intracellular lesions to mitochondria, Golgi, and cytoskeletal elements in astrocytes and neurons (Ellis *et al.*, 1995; McKinney *et al.*, 1996); increases in astrocytic and neuronal membrane permeability (Pettus *et al.*, 1994; Ellis *et al.*, 1995; McKinney *et al.*, 1996; Rzigalinski *et al.*, 1997); increased total cell Ca^{2+} in astrocytes (Hovda *et al.*, 1992; Fineman *et al.*, 1993; Rzigalinski *et al.*, 1997); phospholipase activation (Lamb *et al.*, 1997); and free radical formation (McKinney *et al.*, 1996; Lamb *et al.*, 1997).

Later studies have identified novel alterations after traumatic injury that have not been previously demonstrated *in vivo* because of technical limitations or other reasons: a transient stretch-induced delayed neuronal depolarization (Tavalin *et al.*, 1995, 1997); a stretch-induced

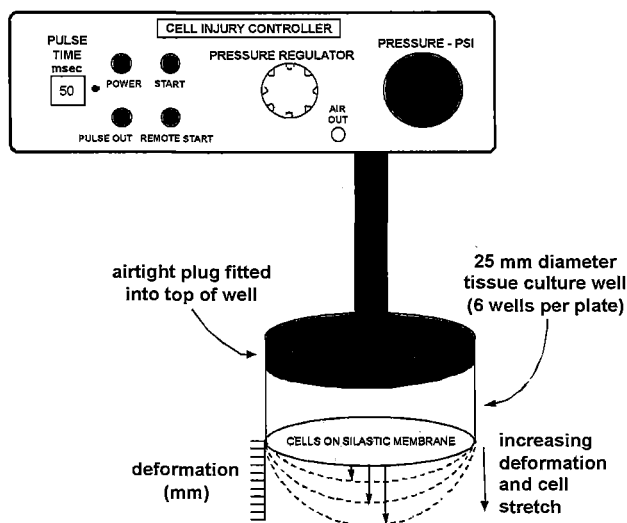


Figure 2. *In vitro* model of stretch-induced injury. Cells are grown on Silastic tissue culture wells to confluency, and are then subjected to a controlled amount of stretch by delivery of an air pressure pulse of precise magnitude and duration. Variations in the pressure pulse produce varying deformations of the Silastic membrane, and therefore, cell stretch. Schematic is not drawn to scale.

reduction in the magnesium blockade of *N*-methyl-D-aspartate (NMDA) receptors (Zhang *et al.*, 1996); a reduced α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor desensitization in a subset of neurons after stretch injury, leading to increased responsiveness and possible over-activation of AMPA receptors (Goforth *et al.*, 1999); alterations in Ca^{2+} stores and Ca^{2+} -mediated signaling pathways (Weber *et al.*, 1999, 2001, 2002); modulation of NMDA receptors and subsequent activation of Ca^{2+} /calmodulin kinase II (CaMKII), producing a long-lasting reduction of AMPA receptor desensitization (Goforth *et al.*, 2004); and an increase in γ -aminobutyric acid (GABA_A) receptor function (Kao *et al.*, 2004).

The response to trauma may vary between different cell types in the brain, and *in vivo* models are often limited with regards to dissecting the differential responses of a heterogeneous cell population. To this end, cell death and damage after increasing levels of mechanical injury were quickly, yet reproducibly, measured in hippocampal and cerebellar cells using a live-dead fluorescent assay, a clear advantage of *in vitro* models over *in vivo* injury to rodents. Also, the release of specific proteins into the culture media, namely neuron specific enolase (NSE) and glial S-100 β protein, two commonly measured clinical markers of CNS damage, comprised an interesting component of these studies. For example, hippocampal cultures subjected to a single mild injury released significant amounts of NSE and S-100 β protein 24 hr later, and levels of these proteins after repeated mild injury were significantly elevated as compared to a single injury. Because cell cultures can be generated from very specific brain regions (limited only to one's surgical expertise), the release of proteins such as S-100 β , which, in animals and in humans, is often measured in CSF or blood, can be traced to their specific brain region of origin. Indeed, we found that cerebellar cell cultures (Chapter 2.3), subjected to similar injury paradigms, presented similar patterns of cell damage and death, but vastly different patterns of NSE and S-100 β release compared to hippocampal cultures.

1.2.3 Hippocampus: organization and function

The hippocampus is one of the major components of the limbic system, along with the amygdala, the cingulate gyrus and the prefrontal cortex. The hippocampus proper is divided into four fields, CA1-4 (CA stands for cornu ammonis), and, with the exception of the CA4, the neuronal patterning is the same: a single layer of soma from pyramidal cell neurons whose dendrites protrude and extend in one direction. Anatomically, this structure can be conceived as a "rolled-over" portion of the cerebral cortex that becomes simpler with each rollover, with the CA1-3 regions being capped off by the dentate gyrus (DG; see Figure 3). Interestingly, neurons in these different CA regions can also be categorized by their sensitivities to various pathologies. For example, neurons in CA3 are more densely packed, making them more likely foci of epileptic events, whereas neurons in CA1 are more loosely packed, and have been shown to be more sensitive to certain pathologies such as ischemia. [For example, patient R.B. suffered an ischemic episode, leading to amnesia; post-mortem evaluation indicated no brain abnormalities except for damage to the CA1 (Zola-Morgan *et al.*, 1986).] The intrinsic circuitry of the hippocampus moves in one direction: entorhinal cortex $\rightarrow$ perforant fiber pathway $\rightarrow$ DG $\rightarrow$ CA3 $\rightarrow$ CA1 $\rightarrow$ subiculum $\rightarrow$ entorhinal cortex (see Figure 4). This form of synaptic flow is unusual in cortical structures, and has been shown to be very important in the consolidation of memories. One of the most important portions of this pathway, in which CA1 neurons receive excitatory input from the axons, or Schaffer collaterals, of CA3 pyramidal neurons, is described in detail in Chapter 4. In brief, this connective pathway represents a key form of hippocampal long-term potentiation (LTP), a potential physiological candidate underlying learning and memory. LTP was "discovered" by Bliss and Lomo (1973) in hippocampal slice preparations, and many subsequent

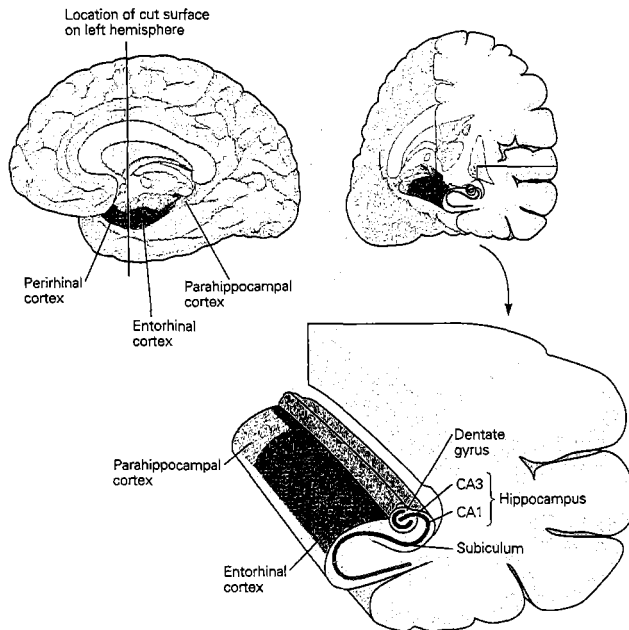


Figure 3. Anatomical organization of the hippocampal formation. Medial (left) and ventral (right) surfaces depicting key components of the medial temporal lobe important for memory storage. Enlargement (below) shows the location of the CA1 and CA3 hippocampal regions, the dentate gyrus, and the entorhinal cortex (from Kandel *et al.*, 2000).

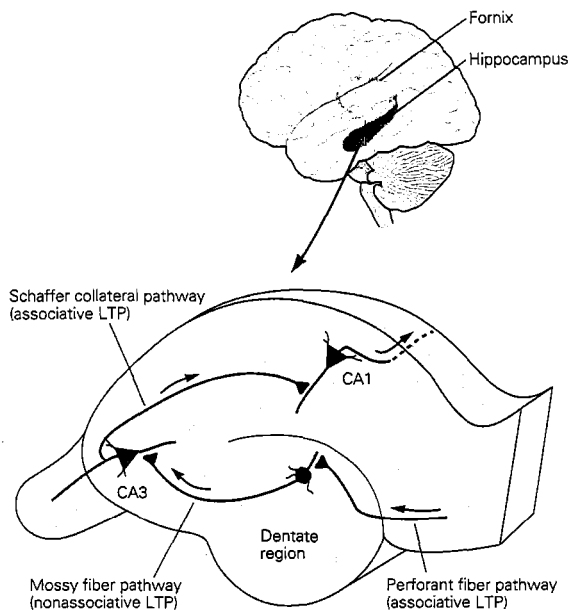


Figure 4. Afferent pathways in the hippocampus. The perforant fiber pathway from the entorhinal cortex forms excitatory connections with granule cells in the dentate gyrus. The axons of these cells in turn form connections with CA3 pyramidal neurons. Their axons comprise the Schaffer collateral pathway via connections with CA1 pyramidal neurons (from Kandel *et al.*, 2000).

studies have attempted to locate the causal link between LTP and learning. The link between the hippocampus and memory processes, however, has been well established, and the experiments performed in Chapters 2.1 and 2.2 reflect that fact, as changes in hippocampal function after stretch injury could begin to explain the cognitive difficulties seen after human TBI. Because the embryonic mouse hippocampus is already a very small structure, the hippocampal cultures utilized in the following chapters contain all types of hippocampal neurons. The data presented in Chapter 3.3, however, were gathered from whole brain slices; in this way, the CA1 and DG regions could be more easily dissected out and analyzed microscopically.

1.2.4 Cerebellum: organization and function

The main functions of the cerebellum include the fine-tuning of motor coordination and posture from input received via most of the major sensory and motor systems. [Recent evidence implicates the cerebellum with certain cognitive functions, such as the planning of movements (for review, see Thach, 1996).] Although damage to the cerebellum can lead to motor discoordination, or ataxia, and difficulties in the initiation and preparation of movement, classical studies using lesioned animals (for example, see Rolando 1809, 1823; and Luciani 1891, 1915) indicated that the cerebellum does not generate, but rather regulates, normal movement. These early experiments also indicated that the cerebellum must have adaptive functions, as movements could become more coordinated after multiple repetitions (Flourens, 1842; Luciani, 1891). Eccles *et al.* (1967) demonstrated that the neurons of the cerebellum are arranged in a regular pattern of repeating units or modules. Later studies determined that lesions to specific cerebellar regions resulted in specific motor impairments, indicating that the cerebellum is arranged in distinct functional units with their own precise connections (Groenewegen and Voogd, 1977; Groenewegen *et al.*, 1979). Further investigations into this precise circuitry have indicated that synaptic transmission among modules represents a cellular basis for cerebellar motor learning, a concept discussed in more detail in Chapter 4 (for reviews, see Raymond *et al.*, 1996; Ito, 2001; Carey and Lisberger, 2002).

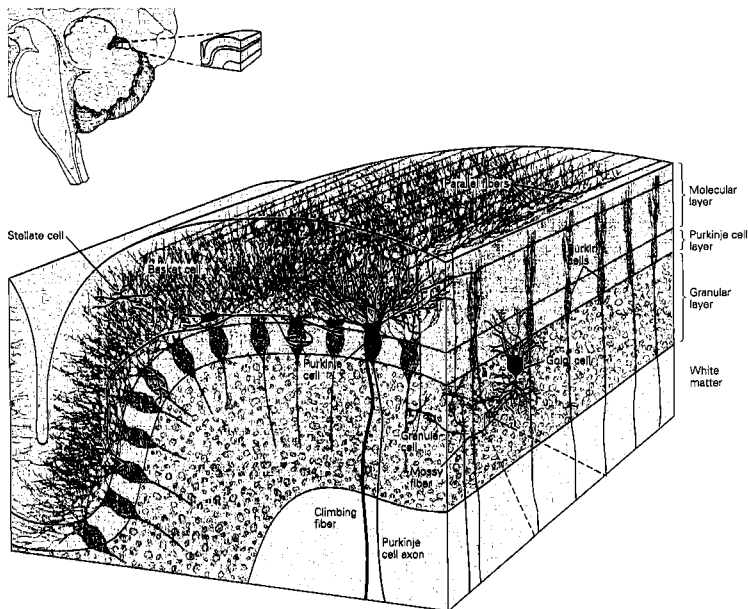


Figure 5. Anatomical organization of the cerebellar cortex. Enlargement (below) of a vertical section (above) of the cerebellar cortex in both longitudinal and transverse planes. Note that the granule cells are found in the granular layer, the Purkinje cell (PC) soma are found in the molecular layer, and that PC dendrites are located in the molecular layer (from Kandel *et al.*, 2000).

This intrinsic cellular circuitry, as shown in Figure 5, has direct consequences on the final output. The cerebellum contains five neuronal cell types, the last of which (PCs) will be described later in great detail. Granule cells represent the only excitatory cell type; their axons are referred to as parallel fibers (PFs). These cells, found in the so-called granule layer, are the most abundant neuronal type in the cerebellum, and comprise a largely homogenous population. Golgi, stellate and basket cells are all inhibitory neurons; their key function is the inhibition of PCs. The net effect of all cerebellar operations is a change in the activity level of PCs, and thus, the deep cerebellar nuclei (DCN).

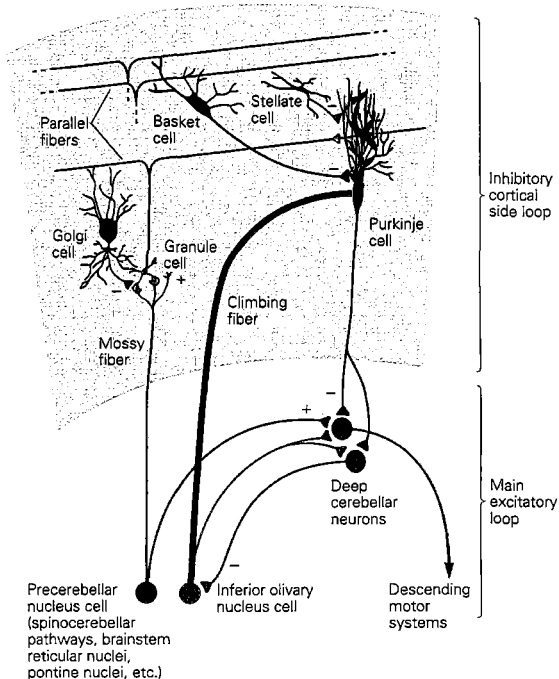


Figure 6. Synaptic organization in the cerebellar cortex. A single Purkinje cell (PC) receives two forms of excitatory input: from a single climbing fiber (arising from the inferior olive), and from numerous parallel fibers (the bifurcating axons of granule cells). PCs also receive direct inhibitory input from basket and stellate cells, and indirect inhibition from Golgi cells via their connections with granule cells (from Kandel *et al.*, 2000).

1.2.5 Cerebellar Purkinje cells

PCs are found in the cerebellar cortex where they receive two types of excitatory synaptic inputs. The first is from climbing fibers (CFs), arising from the inferior olive (IO). Figure 6 depicts the intricate connections among cerebellar neurons, particularly those between CFs and PCs. CFs wrap themselves around the soma and proximal dendrites of PCs and convey somatosensory, visual and cerebral cortical information. The second type of excitatory input is from numerous PFs, the axons of granule cells, also in the cerebellar cortex. The dendrites of granule cells receive their afferent projections from mossy fibers, which are also excitatory. The axons of PCs project to the DCN, and, because they represent the only output cell of the cerebellar cortex, PCs play a vital role in normal cerebellar function. Therefore, both MT dynamics (Chapters 3.1 and 3.2) and the effects of stretch injury (Chapter 2.3) were studied in these cells.

Although PCs may be innervated by multiple CFs during development, they receive input from a single CF in adulthood. Despite being innervated by only a single CF, there are approximately 1,500 CF-PC synaptic contacts (Strata and Rossi, 1998), which are characterized

by a high probability of release (Dittman and Regehr, 1998). CFs primarily contact the more proximal portions of the PC dendritic tree, while PFs form synaptic contacts at the spines of the distal portion of the PC dendrites. The distal dendrites of PCs receive input from upwards of 100,000 PFs. Neurotransmission at both CF-PC and PF-PC synapses is mediated by the excitatory amino acid, glutamate.

Interestingly, PCs are highly susceptible to a variety of pathological conditions that may involve glutamate-mediated “excitotoxicity”, a term coined to describe an excessive release of glutamate, and a subsequent over-activation of neurons via excitatory amino acid (NMDA, AMPA, and kainate) receptors, leading to cellular damage. Glutamate-mediated excitotoxicity is believed to play a large role in several pathological processes, such as TBI, ischemia, and neurodegenerative disorders like Alzheimer’s, Huntington’s and Parkinson’s disease (Farooqui and Horrocks, 1994; Weber, 2004). The fact that each CF innervates one PC with hundreds of synapses not only provides a secure synaptic connection, but the synchronous release of glutamate at these synapses can put PCs at risk for excitotoxic damage. In addition, the vast number of glutamatergic PF-PC synapses also represents a risk of excessive glutamate neurotransmission. Indeed, a very recent review (Sarna and Hawkes, 2003) highlights the susceptibility of PCs to a variety of pathological conditions that may involve excitotoxic components. Given the unique role of PCs as the only output cell from the cerebellar cortex, understanding the reasons underlying their susceptibility to excitotoxicity is important for developing possible treatment strategies for various disease states.

Here the mechanisms underlying glutamate-mediated death in PCs are discussed in detail. To this end, the types of glutamate receptors in the CNS, and general mechanisms of excitotoxicity in neurons and specifically in PCs, are reviewed. The roles of glutamate transporters and calcium-binding proteins in PCs, and of Bergmann glia (BG), are also examined. Attention is then given to glutamate-induced cytoskeletal alterations in dendrites and dendritic spines, again first in neurons in general and in PCs in specific. Neuronal MTs and their associated proteins are reviewed in detail before discussing data gathered on the interactions between cytoskeletal and post-synaptic proteins in PCs. The complex interactions among PCs, the pre-synaptic input from PFs and CFs, and the BG, can be appreciated by referring to Figure 7 throughout this introduction.

1.3 Glutamate and excitotoxicity

1.3.1 Types of glutamate receptors in the CNS

Glutamate activates a variety of functionally distinct receptors in the CNS (for reviews, see Ozawa *et al.*, 1998, and Coutinho and Knöpfel, 2002). These include ionotropic receptors, such as NMDA, AMPA and kainate receptors, as well as metabotropic glutamate receptors (mGluRs). The ionotropic receptors are composed of various subunits that form membrane channels, allowing a flux of ions into the cell from the extracellular space. NMDA receptors (which consist of the subunits NR1, NR2A-D and NR3A-B) are well distributed in the brain, and activation of this receptor subtype causes an influx of Ca^{2+} into cells. AMPA (GluR1-4 subunits) and kainate (GluR5-7; KA1 and 2 subunits) receptor activation leads primarily to Na^+ influx, although some types of these receptors are also permeable to Ca^{2+} depending on their subunit combination. AMPA receptor activation can also indirectly lead to Ca^{2+} influx by causing membrane depolarization which activates voltage-gated Ca^{2+} channels (VGCCs), as well as by increasing the activation of NMDA receptors, which are sensitive to the membrane potential of the cell.

There are also several types of mGluRs, divided into three groups, which are activated directly by binding of glutamate (for review, see Coutinho and Knöpfel, 2002). Group I mGluRs (mGluR1 and mGluR5) are linked through G-proteins which are coupled to phospholipase C (PLC). PLC cleaves phosphatidylinositol biphosphate (PIP_2) from the cell membrane, producing diacylglycerol (DAG), which activates the enzyme protein kinase C (PKC), and inositol 1,4,5-trisphosphate (IP_3), which binds to IP_3 receptors on intracellular Ca^{2+} stores located on the endoplasmic reticulum (ER), resulting in a release of Ca^{2+} from the stores and an elevation of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$). In many types of neurons, the depletion of Ca^{2+} stores also stimulates influx of extracellular Ca^{2+} through channels on the plasma membrane, a process

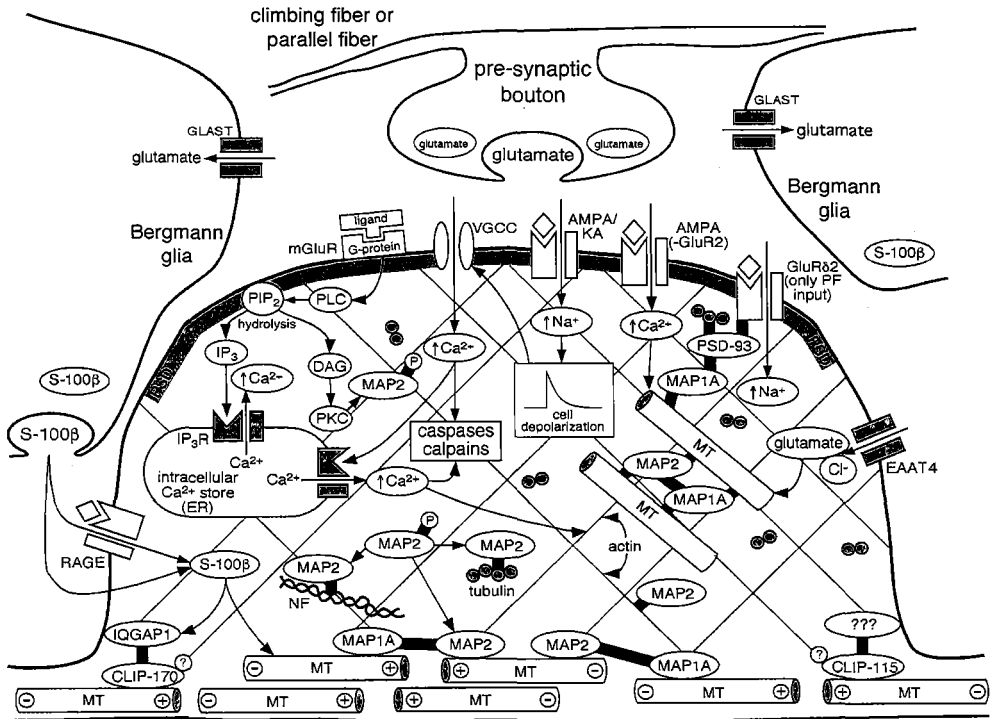


Figure 7. Physiological and pathological glutamate-mediated signaling in a Purkinje cell spine. Glutamate released from a pre-synaptic bouton from either a climbing fiber or a parallel fiber into the synaptic cleft initiates several pathways in Purkinje cells. Involvement of Bergmann glia is also shown, as these cells can take up glutamate via GLAST, and can release S-100 β . A putative scaffold protein that associates with CLIP-115 (similar to the association between IQGAP1 and CLIP-170) is indicated by question marks. Putative contact points between CLIP-115 and actin, and between CLIP-170 and actin, are also indicated by question marks. See text for further explanations. Abbreviations: AMPA/KA: Na^+ -permeable AMPA/KA receptor; AMPA (-GluR2): Ca^{2+} -permeable AMPA receptor; CLIP-115: cytoplasmic linker protein of 115 kDa; CLIP-170: cytoplasmic linker protein of 170 kDa; DAG: diacylglycerol; EAAT4: excitatory amino acid (glutamate) and chloride co-transporter; GLAST: glutamate-aspartate transporter; GluR δ 2: PC-specific glutamate receptor subtype which receives input from parallel fibers (PFs) only; IP_3 : inositol-(1,4,5)-trisphosphate; IP_3R : IP_3 receptor; IQGAP1: (calmodulin-binding) IQ (motifs) Ras GTPase-activating protein 1; MAP1A: microtubule associated protein 1A; MAP2: microtubule associated protein 2; MAP2 with P: phosphorylated MAP2; MT: microtubule; NF: neurofilament; PIP_2 : phosphatidylinositol biphosphate; mGluR: metabotropic glutamate receptor; PLC: phospholipase C; PKC: protein kinase C; PSD: post-synaptic density; PSD-93: post-synaptic density protein 93; RAGE: receptor for advanced glycation end-products; VGCC: voltage-gated calcium channel.

termed “capacitative Ca^{2+} influx” (Bouron, 2000; Weber *et al.*, 2001; Baba *et al.*, 2003). Group II (mGluR2 and 3) and III (mGluR4 and 6-8) mGluRs are also G-protein-linked receptors that inhibit adenylate cyclase, which leads to a decrease in the second messenger, cAMP. In general, activation of group II and III mGluRs leads to a decrease in $[\text{Ca}^{2+}]_i$ (Coutinho and Knöpfel, 2002). mGluRs can also have other types of modulatory effects on membrane ion channels. For example, some studies have shown that activation of group I mGluRs can enhance the elevation of $[\text{Ca}^{2+}]_i$ mediated by NMDA receptors (Bruno *et al.*, 1995; Rahman and Neuman, 1996; Pisani *et al.*, 2001).

1.3.2 General mechanisms of glutamate-mediated excitotoxicity

Glutamate excitotoxicity occurs primarily in two phases: first, a rapid phase in which excessive glutamate receptor activation leads to increased $[\text{Na}^+]_i$, $[\text{Cl}^-]_i$ and $[\text{H}_2\text{O}]_i$, inducing cell swelling (Rothman, 1985); and second, a more delayed phase in which increased Ca^{2+} influx and release of Ca^{2+} from intracellular stores leads to activation of Ca^{2+} -dependent enzymes, inducing degradative and apoptotic cell death pathways (Trump and Berezesky, 1995). These phases have been well characterized in a wide range of *in vivo* and *in vitro* preparations, in both cortical and hippocampal neurons. In general, excitotoxicity is linked to over-activation of NMDA receptors in most areas of the brain, for example, in the hippocampus, and in particular, in the CA1 region (Rosenmund and Westbrook, 1993; Arias *et al.*, 1997; Blaabjerg *et al.*, 2001). In cortical cells, NMDA activation and Ca^{2+} influx couples directly to cell death pathways involving excess nitric oxide (NO) production from neuronal nitric oxide synthase (nNOS) (Sattler *et al.*, 1999; Aarts *et al.*, 2002).

Because an excessive elevation of $[\text{Ca}^{2+}]_i$ can be highly detrimental, neurons have developed several mechanisms with which to maintain $[\text{Ca}^{2+}]_i$ homeostasis. Levels of $[\text{Ca}^{2+}]_i$ can change due to VGCCs, receptor-operated channels, and Ca^{2+} stores in the ER, as shown in Figure 7, as well as to Ca^{2+} efflux (Kostyuk and Verkhratsky, 1994; Miller, 1991) via ionic pumps in the plasma membrane (Weber, 2004; see Figure 8). Although these homeostatic mechanisms are normally able to maintain $[\text{Ca}^{2+}]_i$ at low levels (usually about 100 nM in neurons, as compared to an extracellular concentration of approximately 1 mM), they are often incapable of preventing the large increases in $[\text{Ca}^{2+}]_i$ associated with glutamate excitotoxicity or TBI.

Although PCs express various NMDA receptor subunits, they apparently do not express functional NMDA receptors in mature animals (Llano *et al.* 1991; Kataoka and Ohmori, 1996). NMDA receptors represent the primary means by which Ca^{2+} enters the cell in “classic” hippocampal and cortical models of excitotoxicity. Therefore, the pathways that lead to PC death after exposure to a high concentration of glutamate may not be the same as for other types of neurons. If glutamate-mediated death in PCs is also linked to elevated levels of $[\text{Ca}^{2+}]_i$, then we must look to the other receptors expressed in PCs for answers. AMPA receptors represent the major class of glutamatergic ionotropic receptors in PCs, which are expressed at both CF (Zhang *et al.*, 1990) and PF synapses (Elias *et al.*, 1993). Upon activation, there is an influx of Na^+ and a subsequent depolarization of the membrane, causing an influx of Ca^{2+} through VGCCs. (AMPA receptors can also gate Ca^{2+} , as described below). The mGluR types 1, 3, 4 and 7 have been reported in PCs (for review, see Knöpfel and Grandes, 2002). PCs also express GluR δ 2 receptors, which are located in the dendritic spines of PF-PC synapses (Takayama *et al.*, 1996). The GluR δ 2 subunit is uniquely expressed in PC spines from early development, where it stabilizes synapses between PCs and PFs, but not between PCs and CFs (Kurihara *et al.*, 1997). The deletion of GluR δ 2 in mice leads to the persistence of multiple CF innervations, a loss of PC-PF synapses, and impaired coordination (Kashiwabuchi *et al.*, 1995). Kurihara *et al.*

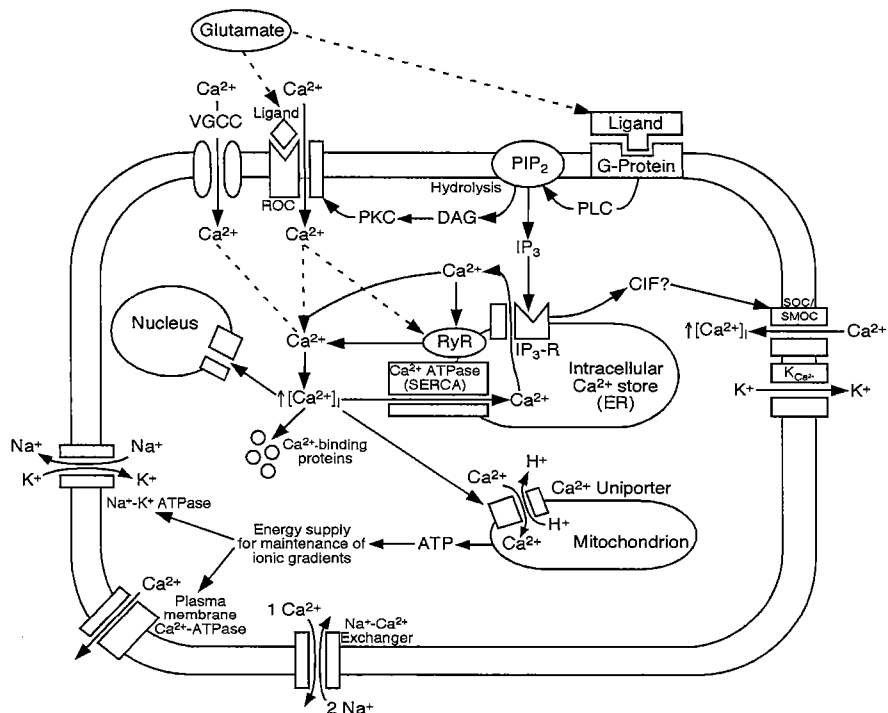


Figure 8. Glutamate-mediated increases in $[Ca^{2+}]_i$. Glutamate activates ionotropic receptors (i.e. AMPA and NMDA receptors) on neurons which can cause an increase in $[Ca^{2+}]_i$. Glutamate also activates metabotropic receptors (group I mGluRs) which produce IP₃ and cause the release of calcium from internal stores on the endoplasmic reticulum (ER). Activation of mGluRs also causes influx of calcium through store-operated/ second messenger-operated channels (SOCs/SMOCs), which further increases $[Ca^{2+}]_i$, a process termed “capacitative calcium influx”. Abbreviations: CIF, calcium influx factor; DAG, diacylglycerol; IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; PIP₂, phosphatidyl inositol bisphosphate; PKC, protein kinase C; PLC, phospholipase C; ROC, receptor-operated channel; RyR, ryanodine receptor; SERCA, sarcoplasmic-endoplasmic reticulum Ca²⁺-ATPase; VGCC, voltage-gated calcium channel (from Weber, 2004).

(1997) investigated the role of GluRδ2 in early development, and found that, whereas 98-99% of PC spines form synapses with PFs in wild-type mice, that number is reduced to almost half in GluRδ2 mutant mice. They postulated that signaling downstream normally triggers the switch in CF innervation from multiple to a single fiber per PC. Although glutamate receptors in PCs are critical in maintaining normal synaptic neurotransmission, excitotoxic levels of glutamate can alter PC morphology and function, leading to altered cerebellar output.

1.3.3 Glutamate action on Purkinje cells

The release of glutamate into the synaptic cleft by single-shock activation of PFs or CFs causes a depolarization of the PC membrane, which is mediated by AMPA receptor activation (Eilers *et al.*, 1995; Schmolesky *et al.*, 2002). The repetitive stimulation of PFs appears to activate two signaling pathways in PCs: 1) a rapid influx of Ca^{2+} through VGCCs caused by the depolarization of the membrane by AMPA receptor activation; and 2) a delayed release of Ca^{2+} from intracellular stores caused by the production of IP₃ by mGluR activation (Finch and Augustine, 1998). CNQX, an AMPA receptor antagonist, is able to block the initial, but not the late, rise in Ca^{2+} . Llano *et al.*

(1991) have also shown that glutamate causes a large but transient increase in dendritic $[Ca^{2+}]_i$ in both Ca^{2+} -containing and Ca^{2+} -free media, indicating that part of the increase in $[Ca^{2+}]_i$ is attributable to release from intracellular Ca^{2+} stores.

Recently it has been shown that mGluR antagonists have little effect on the glutamate-induced production of IP_3 in cultured PCs, but that the AMPA receptor antagonist CNQX could block most of the glutamate-mediated IP_3 production (Okubo *et al.*, 2001). In this preparation, AMPA receptor activation, and the resultant depolarization and activation of VGCCs, is specifically required for IP_3 production. These results were corroborated in acute cerebellar slices, in which electrical stimulation of CFs induced IP_3 production. However, only PF and CF co-activation would produce sufficient IP_3 to activate IP_3 receptors, leading to an increase in $[Ca^{2+}]_i$ in the spines of PCs (Okubo *et al.*, 2001). In another study, the depletion of ER Ca^{2+} stores instigated the docking of the ER to the plasma membrane, where IP_3 receptors could open store-operated Ca^{2+} channels (Ma *et al.*, 2000), similar to a capacitative mechanism reported in other neuronal types (Bouron, 2000; Weber *et al.*, 2001; Baba *et al.*, 2003). Under certain experimental conditions, activation of PFs or CFs produces an mGluR-mediated slow membrane conductance (Tempia *et al.*, 2001; Dzubay and Otis, 2002). Recently, it has been demonstrated that mGluR1 activation is coupled directly to activation of TRPC1 membrane cation channels at PF synapses, which allow additional influx of Ca^{2+} into PCs (Kim *et al.*, 2003). This TRPC1 channel appears to be responsible for the mGluR-mediated slow membrane potential conductance at PF-PC synapses. Therefore, glutamate receptor activation appears to stimulate a wide variety of mechanisms, which can lead to Na^+ and Ca^{2+} elevation in PCs.

1.4 Mechanisms of excitotoxicity in Purkinje cells

In addition to studies conducted using cortical or hippocampal neurons, cerebellar granule neurons have also been extensively used as models of excitotoxicity, as these cells possess viable NMDA receptors (for review, see Contestabile, 2002). In contrast, Brorson *et al.* (1994) found that activation of non-NMDA receptors was sufficient to cause excitotoxic death in cultured PCs independent of Na^+ (and thus independent of activation of VGCCs after depolarization) but was dependent on direct Ca^{2+} permeation, apparently through non-NMDA receptors. Kainate application in Ca^{2+} -free solution slightly attenuated cell death, but the removal of Na^+ did not block kainate-induced toxicity. CNQX, NOS inhibitors, and calpain inhibitors, however, were effective in reducing PC death without blocking kainate-induced Ca^{2+} influx. Therefore, Ca^{2+} -mediated toxicity appeared to be dependent on activation of degradative enzymes, such as phospholipases, endonucleases and proteases, as reported for other types of cells (Weber, 2004). Further studies in cerebellar cultures found that PCs displayed different reactions to excitotoxic agonists. Whereas Ca^{2+} -dependent toxicity via AMPA application was selective for PCs as compared to other cerebellar neurons, PCs were obviously spared from NMDA-mediated toxicity (Brorson *et al.*, 1995). Neuritic beading caused by kainate application has also been found at regions with large, localized increases in $[Ca^{2+}]_i$, and in close proximity to synaptic contact points (Bindokas and Miller, 1995). Increased $[Ca^{2+}]_i$ hindered the restoration of normal $[Na^+]_i$, leading to both localized swelling (i.e. beads) and the activation of Ca^{2+} -dependent degradative enzymes (Bindokas and Miller, 1995). An example of a beaded axon in a cultured PC can be seen in Figure 2B in the Discussion. Other previous reports have also theorized that the excitotoxic degeneration of PCs is caused by an exacerbated increase in $[Ca^{2+}]_i$, leading to activation of calcium-dependent enzymes (Choi and Rothman, 1990; Schmidt-Kastner and Freund, 1991; for review, see Weber, 2004).

1.4.1 Mutant rodent models

Several transgenic and mutant rodent models have shed further light on the mechanisms of glutamate-mediated PC death (for extensive review, see Sarna and Hawkes, 2003). For example, the spastic *Han-Wistar* mutant rat model has proven useful in studying some cause and effect relationships in PC degeneration. The original characterization of these mutants indicated that this rat might constitute a model for excitotoxicity (Cohen *et al.*, 1991). These rats display glutamate dysfunction in both the hippocampus and the cerebellum, and by P60, there is a reduction in PCs of approximately 30% (Nisim *et al.*, 1999). Non-NMDA receptor antagonists, such as CNQX, provided neuroprotection in a dose-dependent manner by attenuating PC loss. Several intrinsic factors could lead to ataxia and, ultimately, to the premature death that occur in these mutants. Most importantly, these rats express very low levels of the GluR2 subunit (Margulies *et al.*, 1993), and decreased GluR2 makes AMPA receptors permeable to Ca^{2+} (Carriedo *et al.*, 1998). This subunit is developmentally down-regulated, leading to Ca^{2+} influx, and allowing levels of $[\text{Ca}^{2+}]_i$ similar to that after NMDA receptor activation. Also, experiments in *Xenopus* oocytes injected with mutant *Han-Wistar* rat cerebellar mRNA elicited significantly enhanced responses to glutamate or to kainate (Cohen *et al.*, 1991). Taken together, these findings implicate glutamate-mediated toxicity, which leads to high Ca^{2+} influx, as a causal factor for PC death in this model.

In another study, *Han-Wistar* rats were given injections of the non-competitive NMDA receptor antagonists, MK-801 and ketamine, and their neuroprotective abilities were measured in both the hippocampus and the cerebellum (Brunson *et al.*, 2001). Both compounds, but more importantly MK-801, which has a much higher affinity for the NMDA receptor than ketamine, were able to attenuate degeneration in the hippocampus, likely by binding to the NMDA receptor and reducing the influx of Ca^{2+} into the cell. Interestingly, these compounds also reduced PC death. The ability of these compounds to provide neuroprotection to PCs, however, requires a more complex explanation and cannot be exerted through the same mechanism as in the hippocampus, considering that PCs do not express functional NMDA receptors. Therefore, a more indirect mechanism of NMDA receptor antagonism must be involved, such as a decrease in pre-synaptic glutamate release. Similar findings have been demonstrated *in vitro*, where application of MK-801 increased PC survival (Mount *et al.*, 1993). In an extensive study, Tolbert and Clark (2000) studied the effects of ablation of the IO in normal and *Shaker* mutant rats, which exhibit a hereditary form of ataxia. *Shaker* rats display a predictable pattern (both spatially and temporally) of PC degeneration, starting at around the seventh week, and lasting for a period of six to eight weeks. The authors hypothesized that IO ablation in mutant rats would rescue PCs that were destined to die. Although ablation had no effect on PCs in normal rats, ablation surprisingly accelerated PC loss in mutant rats, indicating that the *Shaker* phenotype may be due to the loss of functional CF-PC synapses. CFs can influence gene expression in PCs via synaptically-elevated levels of $[\text{Ca}^{2+}]_i$ (Nieto-Bona *et al.*, 1995). The loss of normal CF contact in turn may have caused the loss of a trophic signal necessary for PC survival. Taken together, all of these studies in mutant rats indicate that PCs are vulnerable to glutamate-mediated excitotoxicity even downstream of the initial action of glutamate.

Two mutant mice complement the PC degeneration seen in the *Shaker* rat. First, the ataxic *Lurcher* mouse is characterized anatomically by atrophic PC dendritic trees, with branches ending in “stubs”, and overall reductions in size of more than 60% as compared to controls (Caddy and Herrup, 1990; see Figure 9B). The *Lurcher* phenotype is caused by a mutation in the GluR δ 2, leading to specific PC apoptosis. As mentioned above in section 1.3.2, GluR δ 2 was shown to stabilize spines in order to form mature synapses between PCs and PFs (Kurihara *et al.*, 1997). The altered GluR δ 2 in these mice makes PCs more susceptible to excitotoxic mechanisms

by creating a persistent state of membrane depolarization, leading to increased $[Ca^{2+}]$, (Zuo *et al.*, 1997). Second, the Purkinje cell degeneration (pcd) mouse also presents a clear pattern of PC degeneration, but on a much earlier time scale than the *Shaker* rat. These mice lose almost all PCs between P15 and P45 (Landis and Mullen, 1978), apparently via the upregulation of several cell death effector genes, such as *c-fos* and *junB* (Gillardon *et al.*, 1995).

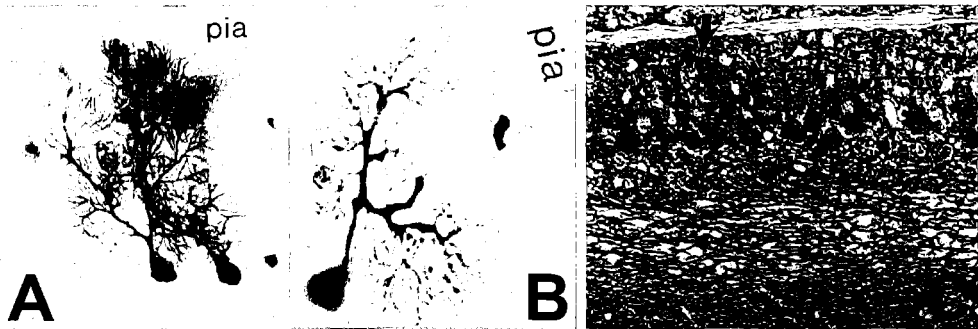


Figure 9. Purkinje cell (PC) dysmorphology. A and B: The ataxic *Lurcher* mouse is characterized anatomically by atrophic PC dendritic trees, with branches ending in “stubs”, and overall reductions in size of more than 60% (B) as compared to controls (A; from Caddy and Herrup, 1990). C: The ablation of astrocytes causes PC dendrites to display an aberrant morphology: the so-called “weeping willow” appearance is indicated by an arrow (from Delaney *et al.*, 1996).

1.4.2 Glutamate transporters and glia

Once glutamate is released from pre-synaptic terminals into the synaptic cleft, and exerts its actions on postsynaptic receptors, it needs to be removed from the synaptic cleft by glutamate transporters, which are located on PCs themselves, as well as on the nearby glia. Disturbances to transporters could lead to elevated levels of glutamate and excitotoxic mechanisms. The PC-specific EAAT4 transporter is located largely on post-synaptic spines found in parasagittal zebrin II(+) stripes in the cerebellum, which may make EAAT4-containing PCs more capable of handling excitotoxic levels of glutamate (Yamada *et al.*, 1996; Nagao *et al.*, 1997; Welsh *et al.*, 2002). EAAT4 has been shown to take up glutamate together with Cl^- (Fairman *et al.*, 1995). The findings of Dehnes *et al.* (1998) and others (Fairman *et al.*, 1995; Barbour *et al.*, 1994; Tong and Jahr, 1994; Takahashi *et al.*, 1996; Otis *et al.*, 1997) corroborate the proposal that glutamate transporters, including EAAT4, may do more than simply remove glutamate from the extracellular space—they may also serve as glutamate-gated chloride channels and assist in the regulation of synaptic transmission. Therefore, alterations in EAAT4 could have adverse effects on normal PC physiology as well as make PCs more susceptible to excitotoxic damage (Takahashi *et al.*, 1996).

Ruiz and Ortega (1995) identified a Na^+ -dependent glutamate-aspartate transporter (GLAST) specific to BG in the cerebellum, which is responsible for the majority of glutamate uptake (Lopez-Bayghen *et al.*, 2003). Many studies indicate an important interaction between PCs and GLAST-expressing BG. Immunogold localization has identified that the vast majority of EAAT4 is found on PC spine membranes that are contacted by BG (Dehnes *et al.*, 1998). BG fibers almost completely seal PC synapses (Grosche *et al.*, 1999) and GLAST has been localized to the portions of BG membranes facing excitatory PC synapses (Chaudhry *et al.*, 1995). In normal PCs, postnatal developmental processes subsequently increase the need for GLAST expression in BG; the rapid creation of elaborate dendritic arborizations and the formation of glutamatergic

PF-PC synapses coincide with the up-regulation of GLAST in BG (Shibata *et al.* 1996; Takacs and Hamori 1994; Kurihara, *et al.* 1997). There are higher levels of GLAST expression in BG which are associated with PCs versus BG not associated with PCs in the cerebella of wild-type and mutant *reeler* and *weaver* mice; when comparing these populations of BG, however, the level of GLAST in BG from mutant cerebella was significantly reduced as compared to controls (Fukaya *et al.*, 1999).

Importantly, BG have Ca^{2+} -permeable AMPA receptors. Because of the important interaction between BG and PCs, aberrant BG could have detrimental implications for PC synaptic functions (for review, see Watanabe, 2002). For example, alterations in the GluR2 subunit of AMPA receptors in BG could have adverse effects on PCs. Inserting a GluR2 subunit into BG, making them Ca^{2+} -impermeable, has a distinct effect on BG morphology, resulting in deficient ensheathing of PC synapses (Iino *et al.*, 2001). This retraction of BG fibers after the insertion of GluR2 subunits increases the distance between GLAST and PC synapses, thus reducing the ability of the transporter to take up glutamate from the synaptic cleft. PCs would therefore become more vulnerable to elevated glutamate levels. Interactions between cerebellar neurons and glia may have surprising ramifications for neuropathological disease. Interestingly, Sjöbeck and Englund (2001) noted gliosis in the molecular layer, and a significantly reduced mean PC density in the cerebellar vermis, of Alzheimer's patients. In fact, decreased glutamate transporter activity and increased excitotoxicity has been linked to the synaptic damage seen in Alzheimer's disease (Masliah *et al.*, 1996).

Glia can also contribute to glutamate toxicity in PCs in other indirect ways. For example, mGluR and AMPA receptor activation lead to $[\text{Ca}^{2+}]_i$ oscillations in cultured astrocytes, causing them to release glutamate (Pasti *et al.*, 2001). Glutamate release from astrocytes can have similar kinetic and exocytotic characteristics as neuronal transmitter release, and can elevate $[\text{Ca}^{2+}]_i$ repeatedly in neurons (Pasti *et al.*, 2001). The electrical stimulation of PFs raised $[\text{Ca}^{2+}]_i$ in BG, which Grosche *et al.* (1999) identified as Ca^{2+} microdomains in BG that ensheathed PC spines. These microdomains are reminiscent of dendritic spines, in that they are independent from each other and from the parent BG fiber (Grosche *et al.*, 2002), indicating that cerebellar glia may not form an integrated network similar to that seen in the hippocampus. Alterations in BG, however, could affect their ability to maintain local, autonomous pools of $[\text{Ca}^{2+}]_i$. Indeed, conditional ablation of astrocytes in the cerebellum can have direct effects on PCs (Delaney *et al.*, 1996). Although there was no reduction in PC number, ablation of astrocytes caused PC dendrites to display an aberrant morphology (the so-called "weeping willow" appearance; see Figure 9C). There was also a significant loss of granule cells in this study, so the abnormal development of PCs could also be due to this finding, as the maturation of granule cells is also required for normal growth and elaboration of PC dendrites (Caddy and Herrup, 1990). In either case, BG are key players in the clearance of extracellular glutamate, and thus in the maintenance of normal cerebellar function.

1.4.3 Role of calcium-binding proteins

As already described, previous reports have theorized that the excitotoxic degeneration of PCs is caused by an exacerbated increase in $[\text{Ca}^{2+}]_i$ to such an extent that Ca^{2+} -dependent enzymes are activated (Choi and Rothman, 1990; Schmidt-Kastner and Freund, 1991; Trump and Berezsky, 1995). PCs have a unique presence of Ca^{2+} -binding proteins (CBPs) in the cerebellum, which include high expression levels of the two Ca^{2+} buffers, calbindin-D28K and parvalbumin. The fact that calbindin comprises 15% of total protein in PCs (Baimbridge *et al.*, 1982) has led to the hypothesis that it plays a crucial role in PC function, presumably by buffering large Ca^{2+} -influxes

induced by PF and CF activation, and thereby maintaining normal Ca^{2+} homeostasis. Further evidence for this suggestion stems from the findings that a decrease of CBPs *in vitro* is correlated with PC death and dysfunction, and there are also decreased amounts of CBPs in spinocerebellar ataxia (Vig *et al.*, 2001). Although PCs are rich in calbindin, the ability of this protein to buffer large amounts of Ca^{2+} does not always appear to reduce death in these cells. Similar findings have demonstrated the insufficient buffering capabilities of calretinin and parvalbumin in other cerebellar cell types (Schwaller *et al.*, 2002). Single CF stimulation does not appear to saturate parvalbumin and calbindin in PCs (Schmidt *et al.*, 2003), repetitive stimulation, however, may saturate these proteins. This could also occur when there are excess levels of glutamate in the synaptic cleft under certain pathological circumstances (i.e., ischemia or TBI). Indeed, the extracellular level of glutamate is known to be elevated after *in vivo* TBI (Faden *et al.*, 1989; Palmer *et al.*, 1994).

Several studies have been conducted using KO mice lacking various CBPs (for review, see Schwaller *et al.*, 2002). Mice deficient in calbindin present an interesting profile, in that they display normal development without concurrent up-regulation of other CBPs, such as parvalbumin and calretinin, and yet they suffer from severe ataxia when presented with complex coordination tests (Airaksinen *et al.*, 1997). Further investigations using confocal imaging revealed that the post-synaptic dendritic Ca^{2+} transients were altered in these mutant mice. Airaksinen *et al.* (1997) concluded that calbindin rapidly buffers $[\text{Ca}^{2+}]_i$ with a high affinity during the initial phase of Ca^{2+} influx, and that this protein could be involved in signaling pathways which affect cerebellar movement control. Further studies by Barski *et al.* (2003) in PC-specific calbindin KO mice indicated that Ca^{2+} transients had increased amplitudes and faster decay rates. These mice also display impaired motor functioning, again suggesting that calbindin may play an important role in pathways determining proper motor coordination. Sayer *et al.* (2000) have also postulated that calbindin may not act solely as a Ca^{2+} buffer, but may have additional roles, such as signal transduction to the nucleus. In addition, calbindin and parvalbumin may also serve to regulate Ca^{2+} dynamics that are used as temporal and spatial signals in neurons, similar to calmodulin (Braun and Schulman, 1995).

The ability of a single dendritic spine to make morphological modifications in response to synaptic input allows each spine to isolate the synaptic signal, and any local increases in $[\text{Ca}^{2+}]_i$ from other spines and from the parent dendrite. Harris and Stevens (1988) described the intimate relationship between spine morphology and the size of the post-synaptic density (PSD) in PCs. The PSD structure is located below the post-synaptic membrane in dendritic spines, and contains specialized proteins that assist in the membrane targeting of receptors (for review, see Kennedy, 1997). They found that PC spines with large heads and large PSDs had shorter necks than spines with smaller heads and PSDs. Spine volume overall was proportional to the volume of the ER, which occupied a large portion of both spine and neck cytoplasm (Harris and Stevens, 1988). A larger ER could increase the uptake capacity of intracellular Ca^{2+} stores. Spine size was also proportional to PSD size and to the number of associated pre-synaptic vesicles (Harris and Stevens, 1988). Indeed, anatomical differences have also been noted in studies of KO mice. Whereas dendritic spines in PCs from parvalbumin knockout mice were indistinguishable from controls, spines from calbindin knockout mice had slightly longer necks, and spines from double KO animals had longer, thinner spine necks and heads that were doubled in size (Vecellio *et al.*, 2000). Therefore, quantitative alterations in spine morphology, such as in neck thickness, neck length, head size and PSD size, can be conceived of as compensatory mechanisms to account for the lack of sufficient Ca^{2+} -buffering proteins (Vecellio *et al.*, 2000).

1.4.4 Alterations in dendritic spines

Glutamate exerts its effects primarily at dendritic spines, the contact points between PCs and CFs or PFs. Spines are a dynamic component of the neuronal cytoskeleton that form, grow, mature and shrink in response to synaptic and intracellular signals. In PCs, dendritic spines are dramatically affected by the release of pre-synaptic glutamate, and their ability to gather and disseminate reliable information to the soma, and then to the axon, is crucial for normal cerebellar function. Thus, it is not surprising that PC spines contain a broad variety of synaptic proteins and cytoskeletal elements, some of which are either PC-specific in the cerebellum, such as calbindin, or are very highly expressed in PCs, such as MAP1A. A large portion of the studies regarding spine dynamics have been conducted in cell types residing outside of the cerebellum; therefore, we must rely on much of this data when making inferences about these processes in PCs. In the following sections, normal synaptic and spine physiology in hippocampal/cortical neurons and in PCs are described and compared, and the relationship between glutamate excitotoxicity and its effects on cytoskeletal elements, such as MTs, MAPs and actin, are discussed.

1.5 Cytoskeletal elements in neurons

1.5.1 Actin and neurofilaments

The neuronal cytoskeleton is comprised of three main elements—actin, neurofilaments (NFs) and MTs—each playing specific roles during development and after cellular maturity (Figure 10). All three cytoskeletal types form linear filaments composed of protein subunits that undergo assembly and disassembly in the cell, and in the cases of MTs and actin, often with amazing rapidity. Actin and NFs are described here; MTs, the largest-diameter fibers, are discussed

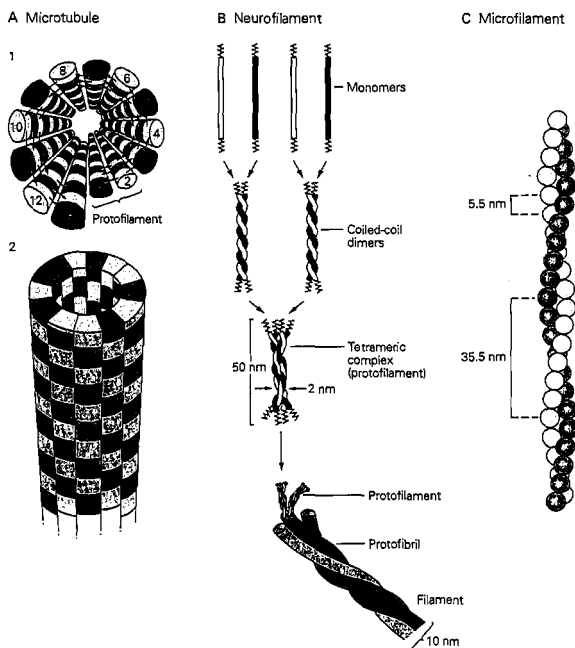


Figure 10. Cytoskeletal elements in neurons. **A:** Protofilaments are formed out of α - and β -tubulin heterodimers. Microtubules are composed of thirteen protofilaments that form a tube-like structure. **B:** Neurofilaments are rope-like structures that are initially formed from monomers that twist together to form dimers. Two dimers coil to form a protofilament, two protofilaments in turn comprise a protofibril, and three protofibrils twist to form a single neurofilament. **C:** An actin microfilament is composed of two strands of globular (G) actin molecules (from Kandel *et al.*, 2000).

extensively in the following sections. Actin microfilaments (Figure 10 and also Figure 11A) are the smallest-diameter fibers (approximately 7 nm). Individual globular actin (G-actin) proteins link together to form a monomeric strand; the twisting of two strands forms an actin filament—filamentous actin, or F-actin. Like MTs, actin is a polar structure with a faster growing plus-end and a slower growing minus-end, and can serve as tracks for intracellular organelles and proteins. Actin filaments give a neuron shape and form, assist in the anchoring of proteins and receptors to the cell membrane, and, along with their array of specific binding proteins, form a complicated network. Their highly dynamic nature makes actin a key player in neurite outgrowth and retraction. NFs almost always exist in a totally polymerized state in neurons, making them much less dynamic structures than actin or MTs. As shown in Figure 10B, monomeric strands form coiled-coiled dimers, which in turn combine to form tetrameric complexes called protofilaments. Two protofilaments combine to become a protofibril, and the combination of three protofibrils creates a single NF with a diameter about 10 nm. NFs are composed of three proteins, each with a differing molecular weight: NF68, NF 150 and NF200; alterations in the last of these proteins is frequently monitored after TBI (see below). NFs are found in high quantities in axons, and compounded with their more stable nature, they provide strength to neurons; for example, they have been shown to act as cytoskeletal spacers, thereby determining axonal diameter (Lee and Cleveland, 1994).

1.5.2 Microtubules and MAPs

The organization of MTs differs greatly between neurons and non-neuronal cells (see Figure 11), but their composition remains the same. Protofilaments are formed by heterodimers composed of α - and β -tubulin subunits; thirteen protofilaments in turn form a single MT with a diameter of approximately 25 nm (Figure 10). MTs are highly dynamic and polarized structures. In non-neuronal cells, almost all MT minus-ends are attached to the centrosome or MT organizing center (MTOC), leaving the plus-ends free in the cytoplasm to assemble and disassemble. In neurons, however, very few MTs are bound to the centrosome or the MTOC. MTs lend structural stability to cells; in neuronal axons and dendrites, they also provide tracks for intracellular transport. Their intrinsic dynamic instability allows cells to adapt rapidly to changing needs, for example, increased demand for organelles at axon terminals. Although both plus- and minus-ends are now able to polymerize and depolymerize, MT plus-ends tend to grow and shrink faster than minus-ends. MT organization differs between axons and dendrites: in axons, MTs are oriented with their minus-ends towards the soma, and their plus-ends towards the axon terminal in mature cells (or towards the growth cones in developing neurons); in dendrites, MT plus-ends are oriented in both directions (Baas *et al.*, 1988). These data, originally gathered in hippocampal neurons, have been reconfirmed in PCs (Chapter 3.1).

In order to maintain cytoskeletal structure and thus synaptic efficacy, PC spines contain a wide range of MAPs that serve to stabilize polymerized tubulin. One MAP that has received considerable attention is MAP2, a dendrite-specific MAP. Several studies have highlighted the importance of MAP2 in neuronal, and in PC, survival and function. Decreased MAP2 and abnormalities of MAP2 in PCs leads to beading, or blebbing, and destabilization of dendritic processes, resulting in abnormal functioning. In particular, PCs display a loss of viable synapses due to reabsorption of post-synaptic spines (Abdel-Rahman *et al.*, 2001). Studies by Matus and colleagues have demonstrated that, although MAP2 accumulates distally within the growing dendritic tree, there is no analogous accumulation of tubulin, suggesting that both the distribution and the function of MAP2 may be related to factors other than its binding to MTs (Matus *et al.*, 1990). As both MAP2 and a related protein, MAP1, are dendrite-specific in PCs, these proteins

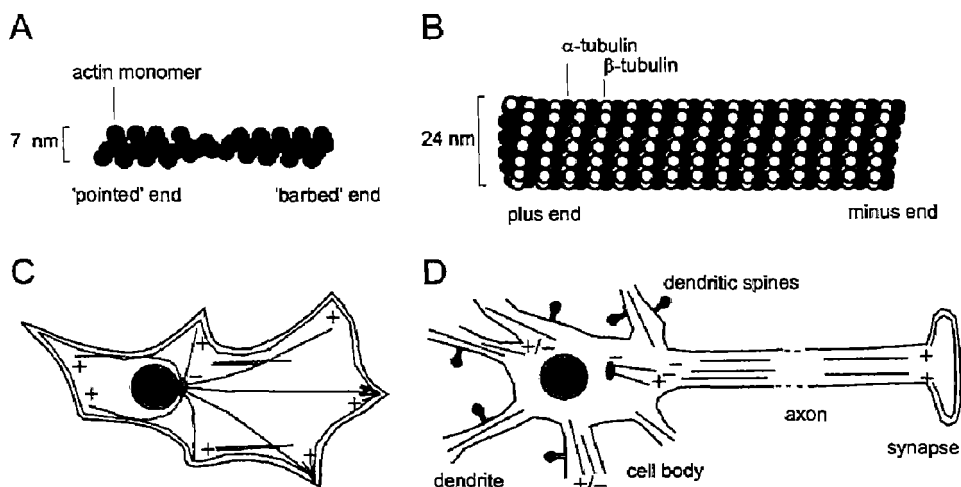


Figure 11. Microtubule organization in neuronal and non-neuronal cells. A: Actin microfilament (as also shown in Figure 10C) is a polarized structure, with a faster-growing, barbed plus-end, and a slow-growing, pointed minus-end. B: Microtubules are also polarized structures, with plus- and minus-ends. Protofilaments are formed out of α - and β -tubulin heterodimers, thirteen of which are arranged to form a single polymer. C: In fibroblasts (and other non-neuronal cells, such as glia), microtubule minus-ends are nucleated and stabilized at the centrosome close to the nucleus, while plus-ends grow into the cytoplasm towards the cell periphery. Actin filaments are shown at the periphery. D: In contrast, few microtubules are embedded in neurons, but form bundles in axons and dendrites. Their orientation differs between axons and dendrites: in axons, microtubules are predominantly oriented with their minus-ends pointed towards the soma and with their plus-ends pointed towards the growth cone or axon terminal; in dendrites, they are oriented in both directions. Actin is shown in dendritic spines and in the growth cone (from Hoogenraad *et al.*, 2004).

may play a role in establishing neuronal polarity by specifying dendritic and axonal routes of transport from the cell body (Matus *et al.*, 1981). Bernhardt and Matus (1982) suggested that the synthesis of MAP2 in PCs could be a prerequisite for the formation of dendritic MTs and thus for the stabilization of growing dendrites. Indeed, Garner *et al.* (1988) found that MAP2 mRNA was located in dendrites, allowing neurons to initiate protein synthesis based on local demands (e.g. during synaptic plasticity). Interestingly, PC degradation—without concurrent cell death—was correlated with decreased MAP2 expression in the *nervous mouse* model, though it was not possible to draw a causal relationship between these two events (Brion *et al.*, 1988).

1.5.3 Glutamate-induced MT disruptions

Glutamate-induced $[Ca^{2+}]_i$ elevations have been shown to promote disassembly of established MTs and inhibit the assembly of new MTs via intracellular pathways (see Figure 7). In hippocampal neurons, short applications of glutamate (4 hr) caused dendritic outgrowth, but longer applications (12 hr) instigated dendritic retraction, as well as fewer MTs in growth cones and more MTs in shafts (Wilson and Keith, 1998). In addition, high Ca^{2+} (millimolar concentrations) causes MT depolymerization *in vitro* (Schliwa *et al.*, 1981). High Ca^{2+} after glutamate application may initiate a variety of downstream signaling pathways, resulting in MT growth or retraction. Ca^{2+} -induced activation of calpain has been shown to inhibit MT growth (Johnson *et al.*, 1991), whereas activation of calmodulin/CaMKII leads to phosphorylation of MAPs and neurite outgrowth (Diéz-Guerra and Avila, 1995). Bigot *et al.* (1991) found that MAP2, which exhibited

diffuse staining in unstimulated cultures, became bound to MTs following the administration of a variety of excitatory amino acids (e.g., NMDA and AMPA); MAP2-bound MTs were also more resistant to depolymerization by nocodazole. Ca^{2+} imaging, utilizing the indicator dye fura-2, indicated that hippocampal neurons pretreated with taxol (a MT stabilizing agent) had a significantly attenuated $[\text{Ca}^{2+}]_i$ response. This finding indicated that MT depolymerization played a role in the glutamate-induced elevation of $[\text{Ca}^{2+}]_i$ in untreated neurons (Furukawa and Mattson, 1995). Taxol did not reduce Ca^{2+} influx through NMDA receptors, whereas it did suppress influx through AMPA receptors directly, as $[\text{Ca}^{2+}]_i$ levels did not differ between taxol-treated and untreated neurons after a depolarizing dose of KCl. Colchicine, a selective MT disruptor, caused a significant decrease in cell survival, a situation that was markedly improved by pretreatment with taxol (Furukawa and Mattson, 1995). Taxol, already used extensively in the treatment of cancer, exerts its effects through the stabilization of MTs, thereby halting the natural process of cell division. The fact that MTs may contribute to the mechanisms behind excitotoxicity merited further investigation into the usefulness of taxol and related compounds in treating pathological conditions such as ischemia and TBI. Prolonged administration of taxol, however, has been shown to lead to neuropathies in the peripheral nervous system (for reviews, see Quasthoff and Hartung, 2002, and Peltier and Russell, 2002). To that end, Adlard *et al.* (2000) administered taxol to rats after traumatic injury *in vivo*, and measured outcome at 1 and 4 days post-injury. They found that the use of taxol led to MAP2 preservation and to increased MT polymerization and stabilization around the site of injury at 1 day, but not at 4 days, post-injury. The data indicated that taxol might have detrimentally prolonged the response of the affected neurons to the trauma due to their subsequent inability to institute a reactive or plastic cytoskeletal reorganization (Adlard *et al.*, 2000).

It was previously assumed that cytoskeletal alterations, caused by agents that raised $[\text{Ca}^{2+}]_i$, such as glutamate, were detrimental to cell survival. It appears, however, that various members of the neuronal cytoskeleton react differently to elevated levels of $[\text{Ca}^{2+}]_i$. Recent data indicate that actin depolymerizing agents, such as cytochalasin D, can protect hippocampal neurons against excitotoxic damage by reducing Ca^{2+} influx through both NMDA receptors and VGCCs (Johnson and Byerly, 1993; Rosenmund and Westbrook, 1993; Furukawa and Mattson, 1995; Furukawa *et al.*, 1995). F-actin is highly localized in spines as compared with dendritic shafts. Halpain *et al.* (1998) studied the early effects of NMDA and glutamate application on dendritic spines in cultured hippocampal neurons. Brief applications of either agonist caused a loss of spines and a reorganization of spine actin, but not a loss of synaptic markers. Blocking NMDA receptors with D-APV or MK-801 prohibited the loss of F-actin punctae. The influx of Ca^{2+} through many types of glutamate receptors led to spine actin destabilization; Ca^{2+} influx through mGluRs, through L-type Ca^{2+} channels or after depolarization, however, had no effect on actin. Thus, specific pathways of Ca^{2+} influx are required to destabilize actin. Jasplakinolide, an actin-stabilizing compound, prevented the loss of F-actin punctae when co-applied with NMDA, and Halpain *et al.* (1998) postulated that the specific activation of NMDA receptors stimulated calcineurin, a Ca^{2+} -dependent phosphatase abundant in neurons, which may in turn regulate F-actin stability. Indeed, calcineurin was shown to label both axonal and dendritic growth cones in immature cells, but its expression shifted to neuritic processes in mature cells (Ferreira *et al.*, 1993). Its ability to regulate the phosphorylation of the axon-specific tau was crucial for the determination of neuronal polarity. Dendritic MAP2 can also be dephosphorylated by calcineurin (Goto *et al.*, 1985). In this state, MAP2 would display a decreased affinity for MTs.

In a similar study, Allison *et al.* (1998) studied the contribution of AMPA receptors to actin depolymerization, a phenomenon with more relevance to PC spine dynamics. They found that F-actin in hippocampal neurons was resistant to depolymerization by cytochalasin D, but

not to latrunculin A; application of the latter compound resulted in spine collapse but not total synapse failure, as only 40% of synapses lost AMPA receptors and NMDA receptor clusters. Thus, one major function of dendritic spines may be to sequester AMPA receptors, but they are less tightly attached to the cytoskeleton and the PSD than NMDA receptors (Allison *et al.*, 1998). AMPA receptor activation by glutamate was shown to inhibit actin dynamics, leading to firmer, rounder, and therefore, more stable spines, without the involvement of NMDA receptors (Fischer *et al.*, 2000). The blockade of spine motility by AMPA receptor activation is dependent on depolarization and the resultant influx of Na^+ into the cell. Blocking voltage-gated Na^+ channels, however, did not prevent spine rounding and stability, indicating that glutamate-induced AMPA activation specifically provides the required Na^+ influx (Fischer *et al.*, 2000).

MT or MAP2 alterations have profound effects on neuronal growth and survival. For example, Paula-Barbosa and Tavares (1985) found that the number of MTs in rats fed alcohol for one month was significantly reduced compared to controls, a trend which continued for rats fed alcohol for much longer periods. Previous data had indicated that rat PC dendritic trees are morphologically altered after alcohol consumption (Tavares *et al.*, 1983), which the authors correlated with a loss of MTs (Paula-Barbosa and Tavares, 1985). In the hippocampus, injections of both dihydrokainate (a glutamate transport inhibitor) and kainate induced alterations in MAP2, likely due to excitotoxic activation of NMDA and non-NMDA (AMPA and kainate) receptors (Arias *et al.*, 1997). Initially, there was a loss of MAP2 immunoreactivity at 3 hr post-injection, then a redistribution of MAP2 from dendrites to soma at 12 hr, and finally a decrease or total loss of MAP2 at 24 and 48 hr. This late-phase protein loss was likely due to the activation of calpains or other calcium-dependent proteases (Siman *et al.*, 1989; Johnson *et al.*, 1991). Similarly, almost all of the MAP2 staining in hippocampal dendrites disappeared in slice cultures exposed to the mGluR agonist, *trans*-ACPD, as compared to control slices; many of those dendrites that did remain demonstrated clear signs of degeneration (Blaabjerg *et al.*, 2001). The neurotoxic effects of millimolar doses of *trans*-ACPD appeared to result from activation of NMDA receptors, as co-application of *trans*-ACPD with MK-801 abolished this effect. The co-application of NBQX, an AMPA receptor antagonist, however, did not attenuate the effects of *trans*-ACPD.

Although reports of MTs, and associated proteins such as MAP2, in spines have been contradictory (Bernhardt and Matus, 1984; Kaech *et al.*, 1997), marked amounts of free tubulin have been found in PSD fractions (van Rossum *et al.*, 1999) and van Rossum and Hanisch (1999) have provided a model of glutamatergic spine dynamics involving both MTs and MAPs (see Figure 12). In the hippocampus, NMDA receptor activation, and subsequent Ca^{2+} influx, may cause both the dissolution of the actin framework, and the polymerization of MTs, stabilized by MAPs. Although Ca^{2+} has been shown to cause MT disassembly *in vitro* (for review, see Donato, 2001), it is possible that the rich abundance of CBPs in spines could minimize the destabilizing effect of Ca^{2+} . (Indeed, NMDA receptor activation leads to increased MT stability by modulating the phosphorylation state of MAP2). These MTs could serve as temporary tracks for the transport of newly synthesized proteins into nascent spines. The fact that MTs have been difficult to locate in spines (for example, Westrum *et al.*, 1980; see Figure 13) may be due to their transient nature in these cellular structures (van Rossum and Hanisch, 1999).

1.5.4 Interactions among different cytoskeletal elements

Mounting evidence indicates that various cytoskeletal networks are tightly connected (for a recent review, see Dehmelt and Halpain, 2004). For example, MAP2 has been shown to bind to actin (Selden and Pollard, 1983) and to NFs (Aamodt and Williams, 1984). A proposed model for growth cones depicts MTs growing along actin bundles with MAPs, such as MAP1B and

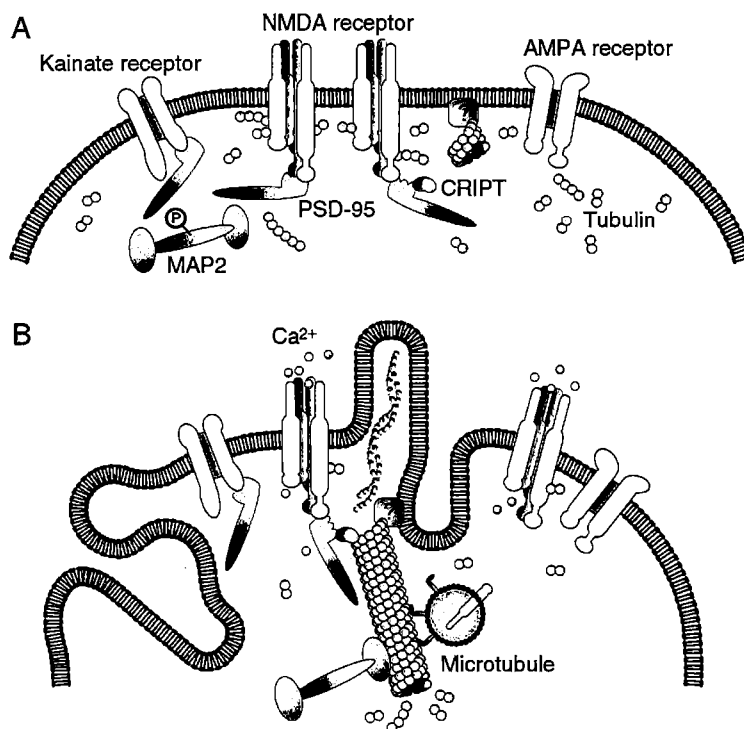


Figure 12. Putative tubulin dynamics in dendritic spines. **A:** The postsynaptic density is highly enriched with tubulin. A direct link between tubulin dimers and the NMDA receptor is depicted. **B:** The dissolution of the microfilament network causes local concentrations of tubulin dimers to increase dramatically, which allow the initiation of tubulin polymerization and the formation of microtubules (MTs). The newly formed MTs may then be further stabilized by the high-affinity binding of dephosphorylated microtubule-associated proteins (MAPs). MTs may also serve as a support to guide the vesicular transport of newly synthesized proteins to activated spines. An as yet unidentified membrane-spanning protein (?) could serve as an anchor for the new microtubule. Abbreviations: CRIPT, cysteine-rich interactor of PDZ three; MAP2, microtubule-associated protein 2 (from van Rossum and Hanisch, 1999).



Figure 13. Microtubule in a dendritic spine. A microtubule (*t*) from a 33-day-old rat is shown surrounded by sacs (*s*) in a dendritic spine. The point of contact between the microtubule and the post-synaptic density is indicated by an arrowhead. Scale bar = 0.25 μm (from Westrum *et al.*, 1980).

MAP2, acting as linking proteins (Dehmelt and Halpain, 2004). The relationship between MAP1A and MAP2 may result in the elaborate network of cross-bridges that exist among dendritic MTs (Shiomura and Hirokawa, 1987). The MT and actin cytoskeletons may also work in concert to transport receptors and other key components to the synapse. Washbourne *et al.* (2002) demonstrated that packets of NMDA receptors were transported along dendritic MTs in cortical neurons. Application of vincristine, a MT depolymerizing agent, abolished NMDA receptor transport, whereas latrunculin A, an actin-depolymerizing drug, had no effect. Imaging experiments showed that these NMDA receptor packets were transported via MTs to contact sites, either between growth cone filopodia and a dendritic shaft, or between a dendritic shaft and an axon, where they remained (Washbourne *et al.*, 2002). Sergé *et al.* (2003) demonstrated that mGluR5 receptors can bind to MTs (though evidence was lacking for either direct or MAP-associated binding), and that the MTs are transported by actin. Similarly, Kim and Lisman (2001) demonstrated that AMPA receptors were transported first via MTs, and then directed from dendritic shafts to spines by actin.

Furukawa *et al.* (1995) found that glutamate did not reduce phalloidin staining of actin in hippocampal cultures incubated in Ca^{2+} -free media, indicating that Ca^{2+} influx is crucial for the actin-depolymerizing action of glutamate. The application of cytochalasins decreased Ca^{2+} influx through NMDA receptors and VGCCs. Actin depolymerization protected neurons against excitotoxic damage, suggesting that the protective mechanism involves suppression of Ca^{2+} influx through plasma membrane channels. MT depolymerization, however, does not suppress increased $[\text{Ca}^{2+}]_i$ in neurons. Although Ca^{2+} can cause a selective loss of F-actin (Halpain *et al.*, 1998), local increases in Ca^{2+} in PC dendritic spines are critical for cell signaling. Acute NMDA or glutamate application caused dendritic spine collapse in cultured hippocampal neurons. PSD proteins and NMDA receptors remained intact on the dendritic surface, however, and the fact that the dendrite would continue to receive synaptic activation, and therefore increased levels of $[\text{Ca}^{2+}]_i$, could make the neuron as a whole more vulnerable to a second excitotoxic event (Halpain *et al.*, 1998). Proteins like espin, whose ability to bundle actin is not inhibited by Ca^{2+} , can help PC spines retain their shape, and thus their function as isolators of Ca^{2+} (Sekerova *et al.*, 2003).

1.5.5 Post-synaptic proteins in Purkinje cells

As mentioned above in section 1.4.3, spine morphology and PSD size are tightly coupled in PCs (Harris and Stevens, 1988). PCs were shown to contain PSD-93 (Brenman *et al.*, 1996; Kim *et al.*, 1996), a relative of the well-characterized PSD-95 protein, at both the PSD, where PCs receive excitatory input from PFs, and along dendritic MTs (Brenman *et al.*, 1998). PSD proteins may assist in synaptic efficacy by maintaining the proximity between receptors and signaling enzymes (van Rossum and Hanisch, 1999; see Figure 12). PSD-93 highly co-localized with MAP1A in both soma and proximal dendrites in PCs (Brenman *et al.*, 1998). Pedrotti *et al.* (1994) demonstrated that MAP1A was also able to bind to actin and NFs. PSD-93 was shown to link nNOS with membrane-bound receptors (Brenman *et al.*, 1996). PSD-93 was not required for either the development or the function of PC-PF synapses, but may serve as a link between postsynaptic receptors in PC spines and signaling pathways (McGee *et al.*, 2001). Indeed, PSD-93 was shown to cluster GluR δ 2, as PSD-95 clustered NMDA receptors (Roche *et al.*, 1999). GluR δ 2 was found at both PC-PF and PC-CF synapses in early development, but only at PC-PF synapses in adult animals (Roche *et al.*, 1999). Hirai (2000) demonstrated that these receptors are bound to the actin cytoskeleton. Actin-disrupting agents decreased the number of immunoreactive receptor clusters, indicating that cytoskeletal alterations could have profound effects on GluR δ 2 localization and function.

The fact that PSD-93 localizes to PC dendritic MTs, as well as associates with MAP1A, may give evidence of a putative tubulin-based cytoskeleton in spines. Indeed, other MAPs have been implicated in linking cytoskeletal elements with synaptic proteins. For example, CRIPT (cysteine-rich interactor of PDZ three) was shown to interact with both NMDA receptors and MTs, and the co-expression of CRIPT and PSD-95 in COS7 cells caused PSD-95 to be redistributed to MTs. CRIPT also recruited PSD-93 in a similar fashion (Niethammer *et al.*, 1998). CRIPT may bind to MTs and PSD-95 independently, and mediate the interaction of MTs and PSD-95, as a truncated form of CRIPT, which could not bind with PSD-95, could still organize tubulin into MTs. Like PSD-93, CRIPT was also expressed in the somatodendritic region of PCs (Niethammer *et al.*, 1998). The authors postulated that CRIPT could link PSD-95 to a form of tubulin or MTs in dendritic spines.

1.5.6 CLIP-115 and Williams Syndrome

Brain specific CLIP-115, like its more ubiquitous relative, CLIP-170 (Pierre *et al.*, 1992), was also shown to reorganize MTs in a similar fashion to CRIPT (De Zeeuw *et al.*, 1997). CLIPs and other related proteins (e.g. EB1 and EB3), collectively referred to as +TIPs, interact specifically with MTs in a process called “treadmilling”. As shown in Figure 14, +TIP molecules bind to tubulin dimers in such a way that they appear to move along the length of a growing MT by binding to its polymerizing plus-end. Individual +TIP molecules then fall off behind the region of growth. CLIP-170, the original member of the CLIP family, was found to bind endocytotic vesicles to MTs (Pierre *et al.*, 1992). Interestingly, it has been shown to participate in the coordination of the actin and MT cytoskeletons in growth cones (Fukata *et al.*, 2002). Originally, CLIP-115 was hypothesized to be responsible for both the transport and the localization of dendritic lamellar bodies (DLBs), structures that the authors hypothesized may be a special form of intracellular

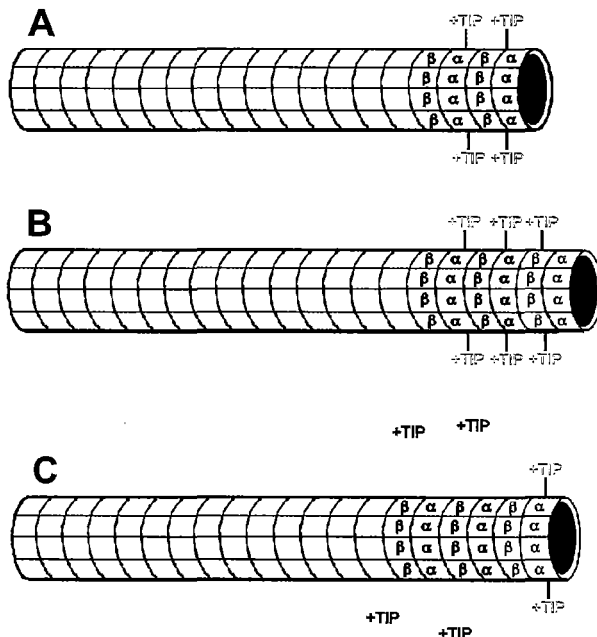


Figure 14. Plus-end binding proteins, or +TIPs, bind to microtubule (MT) plus-ends. +TIPs, such as CLIP-115 and EB3, associate with growing MT plus-ends in a process called “treadmilling”. **A:** A MT is composed of tubulin dimers with α and β subunits. +TIP molecules bind to these dimers. **B:** The +TIPs appear to move along the length of a growing MT by binding to its polymerizing plus-end. **C:** Individual +TIP molecules then fall off behind the region of growth.

Ca²⁺ store (De Zeeuw *et al.*, 1997). Although nocodazole-induced MT depolymerization disrupted the translocation of DLBs, more recent data have indicated CLIP-115 does not colocalize with these structures (Hoogenraad *et al.*, 2002). CLIP-115 was expressed, however, in MAP2-positive dendrites in cultured hippocampal cells and in cultured PCs (Stepanova *et al.*, 2003).

The haploinsufficiency for the *CYLN2* gene in humans could partially account for the marked neurodevelopmental deficits seen in WS, a rare neurodevelopmental disorder caused by the hemizygous deletion of approximately 20 genes (or 1.6 Mb) on chromosome band 7q11.23 (Hoogenraad *et al.*, 1998; Francke, 1999; Osborne *et al.*, 1996). The genes located within the Williams Syndrome Critical Region (WSCR) are shown in Figure 15. The targeted deletion of *Cyln2*, the gene that codes for CLIP-115 in the mouse, resulted in anatomical and behavioral phenotypes reminiscent of human WS (Hoogenraad *et al.*, 2002). In addition, CLIP-115 KO mice exhibited hippocampal and cerebellar dysfunction, possibly due to altered MT-dependent intracellular transport (Hoogenraad *et al.*, 2002).

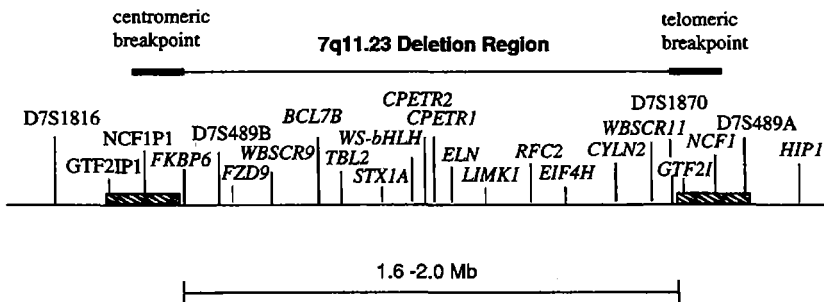


Figure 15. Genetic deletions located within the Williams Syndrome Critical Region (WSCR). The hemizygous deletion of *CYLN2*, and approximately 20 other genes, on chromosome 7 causes Williams Syndrome (WS), a very rare (1:20,000 births) disorder with a complex phenotypical profile. *CYLN2* codes for CLIP-115, a protein that associates with growing microtubule plus-ends (from Francke, 1999).

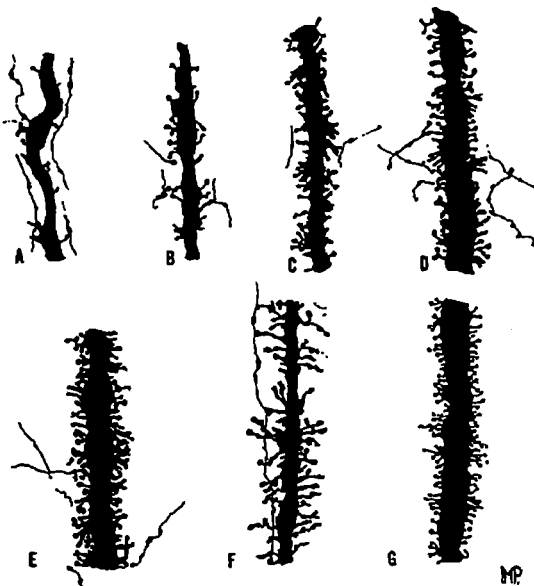


Figure 16. Dendritic and spine dysmorphology in Patau and Down Syndromes. Drawings were made from Golgi preparations derived from layer V pyramidal neurons in the motor cortex. A-E: Dendritic development in normal brain. A: Fifth gestational month. B: Seventh gestational period. C: Neonatal period. D: Second postnatal month. E: Eighth postnatal month. Note the progressive increase in spine density and associated with reduced spine lengths. F: Newborn with Patau Syndrome. Note that spines are not only sparse, but are longer than expected for a neonate. G: Eighteen-month-old infant with Down Syndrome. Note that these spines are shorter and thinner, rather than longer as in F (from Kauffmann and Moser, 2000).

The fact that MT regulation is essential to cell development and plasticity (Mattson, 1999; Baas, 2002) makes CLIP-115 a prime candidate for mediating some of the deficits seen in WS. Deletions within the WSCR result in a unique phenotype with associated mental retardation (MR). Neuronal cytoarchitectonic analysis for several genetic disorders with MR, including WS, has provided substantial support for the theory that a major factor underlying MR is dendritic abnormalities. In fact, MT organization has been investigated in several genetic disorders associated with MR, implicating aberrant MT network organization in the development of dendritic abnormalities (Bodick *et al.*, 1982; see Figure 16). While similar morphological abnormalities may exist in WS, the effects of this disorder on dendritic and spine morphology are not well understood (Kaufmann and Moser, 2000). Indeed, little recent data has been gathered on the relationship between MTs and MR.

1.6 Traumatic brain injury

TBI continues to be a leading cause of death in Western industrialized nations (Jennett, 1996), producing staggering numbers of hospitalizations per year. In 1999, the National Institutes of Health in the United States compiled an alarming set of statistics on the occurrence of TBI, the cognitive and physical ramifications after TBI, and the current lack of viable prevention and treatment options. Although positive outcome after moderate or severe TBI is often unlikely, many studies, both clinical and *in vivo*, continue to focus on the treatment of these forms of TBI, leaving mild traumatic brain injury (MTBI) often overlooked. *In vivo* studies have shown that cell death and tissue damage, commonly found after moderate and severe TBI, are not apparent after MTBI (DeFord *et al.*, 2002; Dixon *et al.*, 1991; Scheff *et al.*, 1997). Patients who have suffered an MTBI, however, often complain of persistent cognitive deficits. Indeed, Thurman and Guerrero (1999) believe that the 51% decrease in hospitalizations in the United States for TBI between 1980 and 1995 can be attributed to the troubling fact that more TBI cases are being classified as MTBIs, and are being treated on an outpatient basis.

1.6.1 Traumatic injury of hippocampal cells

Patients who survive a TBI must continue to function despite any ensuing memory or learning dysfunction; therefore, cellular damage to the hippocampus has received particular attention. As seen in human TBI cases, injured animals have demonstrated deficits in memory and learning that correlated to the level of *in vivo* injury (Hamm *et al.*, 1992; Hicks *et al.*, 1993). Lowenstein *et al.* (1992) found that an increased degree of hippocampal impact correlated with increased hippocampal damage, possibly providing a link between brain injury and resultant disorders such as memory loss and epilepsy. The CA1 region has been found to be especially vulnerable to TBI (Jenkins *et al.*, 1989) and to ischemia (Martone *et al.*, 2000). Cell death and tissue damage are commonly found after moderate and severe TBI, but not necessary after MTBI; animals subjected to models of MTBI, however, show resultant cellular dysfunction and memory deficits (DeFord *et al.*, 2002; Dixon *et al.*, 1991; Kanayama, *et al.*, 1996; Lyeth *et al.*, 1990; Scheff *et al.*, 1997; Uryu *et al.*, 2002).

As previously mentioned, the extracellular level of glutamate is elevated after *in vivo* TBI (Faden *et al.*, 1989; Palmer *et al.*, 1994). Glutamate-mediated excitotoxicity, a critical component in the study of TBI, also has profound effects on the cytoskeletal elements of neurons. Experimental models *in vivo* have indicated that axonal injury can lead to abnormal intracellular transport and MT and NF organization (for reviews, see Maxwell *et al.*, 1997, and Povlishock and

Christman, 1995). More recent studies have examined dendritic MAP2 alterations after *in vivo* TBI to the cortex and hippocampus. Taft *et al.* (1992) subjected rats to moderate TBI, and MAP2 levels were quantified at 3 hr post-injury. They found significantly reduced hippocampal, but not cortical, MAP2 levels, indicating that these MAP2 deficits were region-specific. Therefore, the degradation of MAP2 could lead to functional deficits induced by TBI (Taft *et al.*, 1992). Similar MAP2 deficits were found by Hicks *et al.* (1995) after longer survival times (up to 7 days). They noted reductions in MAP2 immunoreactivity that correlated well with neuronal degeneration. Interestingly, blocking glutamate receptors with kynurenate was shown to attenuate the loss of MAP2, suggesting that, at least in cortical and hippocampal TBI, glutamate-mediated alterations in cytoskeletal organization can lead to cellular dysfunction. Both of these studies subjected rats to a single injury, whereas Kanayama *et al.* (1996) examined changes in MAP2 and NF200—a high-weight (200 kDa) NF found in axons—after repeated MTBI (rMTBI). Rats that received seven mild injuries (spaced 24 hr apart) showed abnormal accumulation of both MAP2 and NF200 one week after the final injury. Surprisingly, the accumulation of these proteins was further increased at one month after injury, indicating that intracellular transport was altered in both axons and dendrites following rMTBI.

Previous data has indicated that the loss of MAP2 and NFs after moderate injury corresponded with increased calpain activation (Saatman *et al.*, 1996a). Similarly, Pike *et al.* (2000) found that stretch injury *in vitro* caused calpain and caspase-3 activation, and subsequent necrotic and apoptotic cell death in septo-hippocampal cell cultures. Calpains are activated by increased Ca^{2+} due to elevated glutamate after injury (see Figure 8); changes in Ca^{2+} flux (Fineman *et al.*, 1993) and decreased extracellular Ca^{2+} levels (Nilsson *et al.*, 1993) have been reported even after MTBI *in vivo*. The administration of calpain inhibitors after moderate injury *in vivo* attenuated the loss of cortical cytoskeletal proteins (Posmantur *et al.*, 1997) and certain behavioral deficits (Saatman *et al.*, 1996b). Later, Saatman *et al.* (1998) compared MAP2 and NF levels in the cortex and hippocampus after single mild or moderate TBI. They noted loss of both proteins at 6 hr after moderate injury, and a delayed loss at 7 days after mild injury, suggesting that a potential “time window” after MTBI may allow for effective treatment strategies.

1.6.2 Traumatic injury of cerebellar cells

The number of clinical and experimental studies that have described cerebellar damage after TBI remains small. For example, Soto-Ares *et al.* (2001) found cerebellar atrophy in children after severe TBI, possibly due to reduced synaptic input via the cortico-ponto-cerebellar pathway. A unique Japanese study determined that a majority of snowboarding-related incidents caused severe TBI to the back of the head (referred to by the authors as the “occiput”), often resulting in a loss of consciousness and diffuse brain damage (Nakaguchi and Tsutsumi, 2002). Even though cerebellar TBI has rarely been studied, the brain stem (which contains the IO) is affected by trauma to the back of the head. Because cerebellar PCs receive synaptic input from CFs arising from the IO, alterations to the brain stem could have direct consequences for the cerebellar cortex.

Indeed, PCs have been shown to be particularly vulnerable to TBI, after either a cerebral or a direct cerebellar insult *in vivo* (see Figure 17). Fukuda *et al.* (1996) subjected rats to cerebral injury, and found profound PC loss and microglial activation in the cerebellum by 3–7 days post-injury. The reactive microglia corresponded to the zebrin stripes created by the dendritic trees of PCs. The authors theorized that the microglia were activated by apoptotic signals derived from dying PCs, and that the zebrin-like stripes were caused by excitotoxic mechanisms derived from excess activation of CFs and PFs within these stripes. Allen and Chase (2001) subjected

rats to direct cerebellar injury, and found that severe, but not mild, TBI caused significant motor deficits. This level of injury also induced the expression of heat shock protein (HSP) 27 in PCs, BG and neurons of the DCN, whereas mild TBI induced HSP expression in PCs and BG only. HSPs, including HSP70/72 (Chen *et al.*, 1996) and HSP27 (Landry *et al.*, 1989), have been shown to provide neuroprotection against stress and trauma, although their mechanism of action in these pathologies remains largely unknown. The fact that HSP27 expression mimicked the improvements in motor performance suggests that this protein may indicate cellular responses to damage and repair (Allen and Chase, 2001). Ai and Baker (2002) found pre-synaptic hyperexcitability in mossy fibers and PFs in rats after direct cerebellar injury, leading to extensive PC death at 7 days post-injury. Like Fukuda *et al.* (1996), they postulated that excitotoxic mechanisms, derived from the extremely high number of synaptic contacts between PFs and PCs, caused the massive PC death.

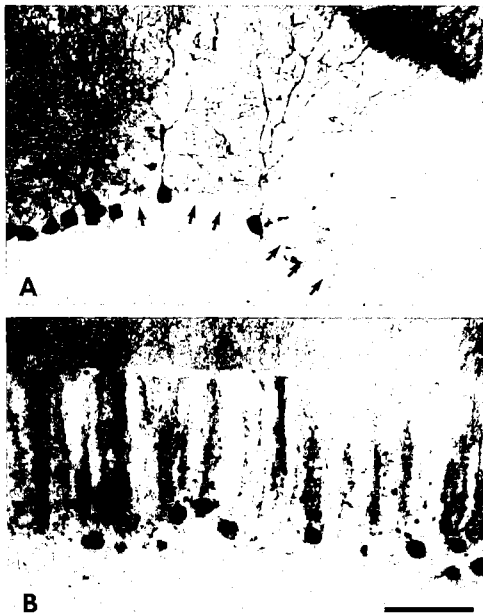


Figure 17. Degenerating Purkinje cells (PCs) after traumatic injury in the rat. Immunolocalization of PEP-19, a PC-specific protein, indicates the massive loss of PC soma (arrowheads) and degeneration of processes at 7 days post-injury. **A:** Sagittal section. **B:** Coronal section. Scale bar = 80 μ m (from Fukuda *et al.*, 1996).

Glutamate-mediated excitotoxicity after injury may also be related to cerebellar BG. Watase *et al.* (1998) subjected GLAST mutant mice to cold-induced cerebellar injury. They postulated that the significantly larger edema in these mice was caused by the inability of the mutant BG to reuptake glutamate from the extracellular space. GLAST may therefore be critical in preventing excitotoxic cerebellar damage to PCs after injury. Similar findings have been reported in rats treated with the psychoactive compound ibogaine (O'Hearn and Molliver, 1997). Ibogaine treatment caused PC degeneration in parasagittal bands, which corresponded with regions of activated microglia. Harmaline, which also causes neuronal hyperactivity in the IO, caused a similar pattern of degeneration, indicating that these two compounds likely act on IO neurons with similar mechanisms (O'Hearn and Molliver, 1997). The chemical ablation of the IO, however, spared PCs and did not cause microglial activation. Interestingly, both ibogaine and harmaline caused a loss of MAP2 and calbindin concomitantly with a loss of PCs that degenerated in parasagittal stripes (O'Hearn and Molliver, 1993). The findings from these studies suggest that similar mechanisms of action may contribute to PC death after TBI.

CHAPTER 2

Traumatic injury in cultured neurons

CHAPTER 2.1

**Repeated mild injury causes cumulative damage
to hippocampal cells**

Abstract

An interesting hypothesis in the study of neurotrauma is that repeated traumatic brain injury may result in cumulative damage to cells of the brain. However, post-injury sequelae are difficult to address at the cellular level *in vivo*. Therefore, it is necessary to complement these studies with experiments conducted *in vitro*. In this report, the effects of single and repeated traumatic injury *in vitro* were investigated in cultured mouse hippocampal cells using a well-characterized model of stretch-induced injury. Cell damage was assessed by the level of propidium iodide (PrI) uptake and retention of fluorescein diacetate (FDA). Uninjured control wells displayed minimal PrI uptake and high levels of FDA retention. Mild, moderate, and severe levels of stretch caused increasing amounts of PrI uptake respectively, when measured at 15 min and 24 hr post-injury, indicating increased cellular damage with increasing amounts of stretch. For repeated injury studies, cultures received a second injury one hour after the initial insult. Repeated mild injury caused a slight increase in PrI uptake versus single injury at 15 min and 24 hr post-injury, which was evident primarily in glial cells. However, the neurites of neurons in cultures that received repeated insults showed signs of damage not evident after a single mild injury. The release of neuron specific enolase (NSE) and S-100 β protein, two common clinical markers of CNS damage, was also measured following the repeated injuries paradigm. When measured at six hours post-injury, both NSE and S-100 β were found to be elevated after repeated mild injuries when compared to the single injury group.

These results suggest that cells of the hippocampus may be susceptible to cumulative damage following repeated mild traumatic insults. Both glial cells and neurons appear to exhibit increased signs of damage after repetitive injury. To our knowledge, this study represents the first report on the effects of repeated mechanical insults on specific cells of the brain using an *in vitro* model system. The biochemical pathways of cellular degradation following repeated mild injuries may differ considerably from those that are activated by a single mild insult. Therefore, we hope to use this model in order to investigate secondary pathways of cellular damage after repeated mild traumatic injury and as a rapid and economical means of screening possibilities for treatment strategies, including pharmaceutical intervention.

Introduction

Traumatic brain injury (TBI) continues to be a leading cause of death in Western industrialized nations (Jennett, 1996). In the United Kingdom, it is estimated that 200-300 per 100,000 people are hospitalized each year due to TBI (McGregor and Pentland, 1997). In 1999, a National Institutes of Health Consensus Development Panel in the United States compiled an alarming set of statistics on the occurrence of TBI, the cognitive and physical ramifications after TBI, and the current lack of viable prevention and treatment options. For example, as many as 6.5 million individuals may be living with the consequences of TBI in the United States alone. One poorly understood aspect of TBI is mild traumatic brain injury (MTBI). Thurman and Guerrero (1999) found a 51% decrease in hospitalizations in the United States for TBI between 1980 and 1995. They believe that changing admission practices in hospitals, spurned by a fixed number of beds and insurance practices, are excluding many cases of MTBI. Unfortunately, MTBI is difficult to properly diagnose, and the fact that more medical cases are being treated as outpatient rather than inpatient is troubling (Macciocchi *et al.*, 1993; Robinson, 1996; Thurman and Guerrero, 1999). Similar to human TBI cases, injured animals have demonstrated deficits in memory and learning that correlated to the level of *in vivo* injury (Hamm *et al.*, 1992; Hicks *et al.*, 1993). *In vivo* studies

have shown that cell death and tissue damage, commonly found after moderate and severe TBI, is not apparent in MTBI (DeFord *et al.*, 2002; Dixon *et al.*, 1991; Scheff *et al.*, 1997). Though MTBI may not necessarily produce cell death (DeFord *et al.*, 2002; Kanayama *et al.*, 1996), animal models show resultant cellular dysfunction and memory deficits (DeFord *et al.*, 2002; Kanayama, *et al.*, 1996; Lyeth *et al.*, 1990; Uryu *et al.*, 2002).

An emerging hypothesis in the study of neurotrauma is that repeated mild traumatic brain injury (rMTBI) may cause cumulative damage to the brain, which could ultimately result in memory and learning dysfunction. Recurrent brain injuries are not uncommon in many sports (Kelly and Rosenberg, 1997). Some sports include blows to the head (contact sports) or with the head as part of the accepted way of playing the game (headers in soccer). The most frequent brain injury in sports is concussion and accumulation of these MTBIs. In contact sports, like boxing and martial arts, cumulative concussion is common and inevitable because victory is direct by rendering the opponent unconscious by a grade three concussion. In collision sports, like soccer, ice hockey, rugby and American football, a high frequency of cumulative concussion, due to collisions between players, have been documented (Bailes, 1998). In American football, for example, players with repeated concussions perform worse on neuropsychological tests than those with either a single concussion or no history of concussion (Collins *et al.*, 1999). Moreover, in soccer, a mechanism for potential brain injury is repetitive heading of the ball, a unique aspect of soccer (Matser *et al.*, 2001). Studies have shown that soccer players, who regularly headed the ball, had more brain concussions, and demonstrated impairment on multiple neuropsychological tests when compared to matched controls (Matser *et al.*, 1998, 1999). These forces on the brain may be mild, but their accumulation may be similar to other medical problems caused by repeated low-level activity, such as lung cancer from smoking cigarettes (Babbs, 2000). Even in non-collision sports, such as horse back riding, biking, skiing, ice skating, and skateboarding, cumulative concussions are often reported as a result of collision and falls. Most of cumulative concussion results in deterioration of planning and memory capacity (Matser *et al.*, 2001), the most common symptom of chronic TBI in athletes.

Damage to the hippocampus following TBI has received particular attention. Lowenstein *et al.* (1992) found that an increased degree of impact correlated with increased hippocampal damage. They theorized that this damage could provide a link between brain injury and resultant disorders such as memory loss and epilepsy. Jenkins *et al.* (1989) subjected rats to either a single or double insult paradigm; after seven days, only those animals that received the double insult had significant neural loss, specifically in hippocampal area CA1. Although there was no axonal injury in any animals when slices were examined at the light microscopic level, a definite threshold of damage was crossed by a double injury that was not crossed by a single injury. It has been suggested that post-injury sequelae may be different between single and repeated brain trauma (Olsson *et al.*, 1971). Therefore, our objective was to better characterize the cellular degradation and dysfunction that occurs after single and rMTBI using cultured mouse hippocampal cells.

Although few studies have dealt with the issue of rMTBI, recent reports have examined repeated injury in the whole animal (DeFord *et al.*, 2002; Kanayama *et al.*, 1996; Laurer *et al.*, 2001; Uryu *et al.*, 2002). *In vivo* studies of repetitive insults are advantageous, in that changes in animal behavior post-injury can be measured and correlated to cell damage. These studies, however, can only postulate the underlying mechanisms of cognitive impairment at the cellular level following rMTBI. Thus, it is important to supplement *in vivo* with well-designed *in vitro* studies. In the current study, we used and established an *in vitro* model of mechanical injury (Ellis *et al.*, 1995; Weber *et al.*, 1999) to better address rMTBI-related cell damage. This model replicates the acceleration-deceleration stresses incurred during motor vehicle accidents (Schreiber *et al.*,

1995), and sport-related concussions (Powell and Barber-Foss, 1999). We used two fluorometric dyes that indicate cell viability and damage as has been previously reported (McKinney *et al.*, 1996; Pike *et al.*, 2000; Weber *et al.*, 1999; Zhao *et al.*, 2000). We also measured the release of two commonly-used clinical markers of brain damage following cardiac surgery (Ali *et al.*, 2000) and TBI: neuron specific enolase (NSE) (Berger *et al.*, 2002; Herrmann *et al.*, 2001; Persson *et al.*, 1987; Pleines *et al.*, 2001; Woertgen *et al.*, 2001) and S-100 β protein (Berger *et al.*, 2002; Herrmann *et al.*, 2001; Persson *et al.*, 1987; Pleines *et al.*, 2001; Raabe and Seifert, 1999; Romner *et al.*, 2000; Waterloo *et al.*, 1997). By using a combination of these approaches, we are able to make comparisons to clinical data and to directly investigate the underlying cellular damage that may contribute to findings described at the behavioral level. This model therefore provides an *in vitro* correlate to what is described following repeated injury experimentally *in vivo* and in the human clinical situation.

Methods

Cell Culture

Primary hippocampal cultures were prepared from E18 FVB/N mouse embryos. Embryonic cultures were used in order to optimize neuronal and glial growth. Pregnant female mice were killed by rapid cervical dislocation and the embryonic sacs were surgically removed. Embryonic mouse brains were dissected out of the skulls and placed on ice in 15 ml ice-cold Hank's balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA) supplemented with gentamicin (10 μ g/ml; Sigma, St. Louis, MO, USA). The hippocampi were separated from the cortices and placed in 10 ml of fresh HBSS. The tissue was washed at 1000 rpm at 4°C for 3 min; the supernatant was removed, 10 ml fresh HBSS was added, and the tissue was washed again. The supernatant was discarded, and 2 ml 0.1% Trypsin-EDTA (Invitrogen) in HBSS was added, supplemented with 8 ml fresh HBSS. The tissue was incubated at 37°C for 15 min, followed by centrifugation at 1000 rpm at 4°C for 3 min. The supernatant was removed and the tissue was washed with 10 ml growth media (Basal Medium Eagles [BME; Invitrogen] containing 10% horse serum [Invitrogen], 10 μ g/ml gentamicin, 0.5% glucose [Sigma], 1 mM sodium pyruvate [Sigma] and 1 % N2 supplements [Invitrogen]) at 1200 rpm at 4°C for 5 min. The cells were triturated with 4 ml fresh growth media using a 10-ml plastic pipette until all large aggregates were dissociated, followed by further trituration with the same pipette equipped with a 100 μ l plastic pipette tip. The suspension was filtered through a 70- μ m nylon cell strainer, the cells were counted, and the suspension was diluted with growth media to a concentration of 500,000 cells/ml. Cells were plated in 1 ml aliquots onto collagen-coated six-well FlexPlates (FlexCell, Hillsborough, NC, USA) coated overnight with poly-L-ornithine (500 μ g/ml; Sigma). Cultures were maintained in a humidified incubator (5% CO₂, 37°C). Neuronally enhanced cultures were obtained by replacing half of the media at 2, 6 and 9 days *in vitro* (DIV) with serum-free culture media (BME containing 10 μ g/ml gentamicin, 0.5% glucose, 1 mM sodium pyruvate, 1% N2 supplement and 2% B27 supplement [Invitrogen]). Glia formed a confluent monolayer that adhered to the membrane substrate. Anti-MAP-2 immunostaining was initially performed on cultures to verify the existence of morphologically distinct neurons. MAP-2 staining revealed neuronal phenotypes growing in the upper layer of cultures that adhered to the underlying glial layer, which is consistent with previous reports in hippocampal cultures (Pike *et al.*, 2000; Zhao *et al.*, 2000). Cells were used for experiments within 9-13 DIV.

Cell Injury

Primary mouse hippocampal cultures were injured using a model 94A Cell Injury Controller (Bioengineering Facility, Virginia Commonwealth University, Richmond, VA, USA) as previously described (Ellis *et al.*, 1995; see Figure 2 in General Introduction). In brief, the Silastic membrane of the FlexPlate well is rapidly and transiently deformed by a 50-ms pulse of compressed nitrogen, which deforms the Silastic membrane and adherent cells to varying degrees controlled by pulse pressure. The extent of cell injury—produced by deforming the Silastic membrane on which the cells are grown—is dependent on the degree of deformation, or stretch. Based on previous work (Ellis *et al.*, 1995), three levels of cell injury were chosen (5.5 mm, 6.5 mm and 7.5 mm deformations), and defined as mild, moderate, and severe, respectively. These degrees of membrane deformation result in a biaxial strain or stretch of 31, 38, and 54%, respectively. This range of cell stretch has been shown to be relevant to what would occur in humans after rotational acceleration/deceleration injury (Schreiber *et al.*, 1995). Uninjured control wells were contained in the same FlexPlates as injured wells, and thus underwent the same manipulations, except that they did not receive rapid deformation of the Silastic membrane.

Cell Viability

Cell injury was assessed using the two dyes fluorescein diacetate (FDA; Sigma) and propidium iodide (PrI; Sigma) as previously reported (McKinney *et al.*, 1996; Pike *et al.*, 2000; Weber *et al.*, 1999; Zhao *et al.*, 2000). FDA is known to stain healthy, viable cells and fluoresces green, while PrI cannot pass through intact cellular membranes. If membranes are damaged, however, cells lose their ability to retain FDA. In addition, PrI will enter cells and stain the nucleus, resulting in a bright red fluorescence. Stock solutions of FDA (20 mg FDA/ml acetone) and PrI (5 mg PrI/ml PBS) were created, from which a working solution was prepared (10 μ l FDA stock and 3 μ l PrI stock, diluted in 10 ml PBS). The culture media was removed from the well and replaced with 1 ml PBS and 200 μ l FDA/PrI working solution. The working solution was added to the culture well immediately after injury, or the injured cells were returned to the incubator and the solution was added immediately before measurement (for experiments conducted at 1 hr and 24 hr). The cells were stained for 3 minutes at room temperature, and the PBS and stain were removed. A 15-mm glass coverslip was centered over the stained cells, and images were captured using red (TX) and green (Fitc) filters on a Leica DMRBE fluorescence microscope, equipped with a Hamamatsu C4880 CCD camera. FDA and PrI images were taken separately, pseudocolored, and overlaid. Images were adjusted for contrast and brightness using Adobe Photoshop 4.0 (Adobe Systems, San Jose, CA, USA). Five sequential 100 $\times$ images were counted and averaged per well. All images were taken from the center portion of the well, as this region was previously shown to receive equal impact from the cell injury controller (Ellis *et al.*, 1995). All PrI and FDA cell counting was performed blind. Data is expressed as the percentage of total cell number that stained positively for PrI. In control cultures, PrI staining was low, accounting for approximately 5% of total cell number, consistent with previous reports (Zhao *et al.*, 2000).

NSE and S-100 β Assays

NSE and S-100 β protein levels were measured in culture media at 6 and 24 hr post-injury using lumino-immunometric (LIA-mat) assay kits (Sangtec Medical, Bromma, Sweden). These kits have been used previously to detect NSE and S-100 β levels in experimental animals and human patients (Herrmann *et al.*, 2001; Romner *et al.*, 2000; Woertgen *et al.*, 2001). NSE is found in

the cytoplasm of neurons, concentrations of which can be measured in CSF and serum, once it has been released from the cytoplasm by cell damage. S-100 β is a calcium-binding protein found in astrocytes and Schwann cells (Herrmann *et al.*, 2001). All experiments were completed using cultures at 9-10 DIV. Following the assays, the remaining culture media was removed, and the cells adhering to the Silastic membrane were solubilized with 1 ml 0.1% NaOH to determine protein concentration. Total protein count was determined by the BDA assay (Pierce, Rockford, IL, USA). Final results are expressed as nanograms of NSE or S-100 β released into the culture media per milligram of protein. NSE levels were determined in three separate culture preparations while the S-100 β assay was performed on four separate culture preparations.

Data Analysis

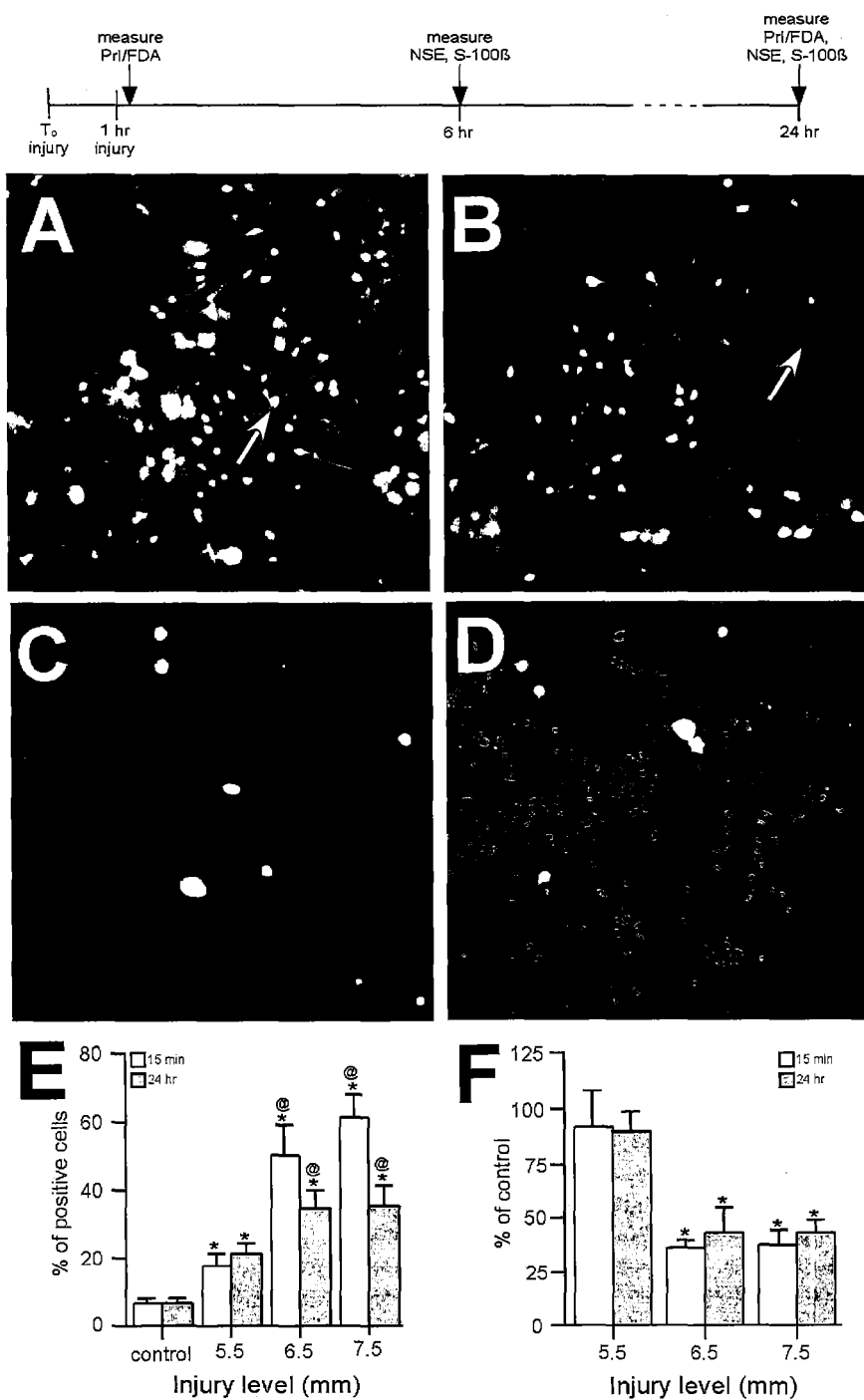
The data were analyzed using the statistical program GB Stat (Dynamic Microsystems, Silver Spring, MD, USA). Data were computed as means $\pm$ standard error of the mean (SEM) values. Statistical significance was established by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test. Data are considered significant at $p < .05$.

Results

Increased level of stretch injury correlates to increased PrI uptake

Prototypical images of control and injured mouse hippocampal cultures at 15 min post-injury are presented in Figures 1A-1D. The microscope we used required that FDA and PrI images be taken separately, pseudocolored, and overlaid; color images can be seen in the original article (Slemmer *et al.*, 2002). The focal plane was chosen to be the neuronal layer, and thus the underlying glial monolayer appears slightly blurred. A Timeline is given at the top of Figure 1 for the sake of clarity. Figure 1A depicts uninjured cultured cells with a high retention of FDA and normal cellular morphology; the same was true for control cultures at 24 hr (images not shown). There is little PrI staining, indicating that most cells are healthy and viable, with intact cell membranes. Fifteen minutes after injury, however, cultures demonstrated an increasing amount of PrI staining, the majority of which occurred in the underlying layer, suggesting that it was primarily glial in origin (images at 24 hr not shown). The increased staining was minimal after mild injury (5.5 mm; Figure 1B) where FDA retention appears unchanged, especially in the neuronal layer. Note that some gaps have appeared in the monolayer. Moderate injury (6.5 mm; Figure 1C) created increased PrI staining in the glial layer and "beaded" neurites in the neuronal layer. However, the neuronal somata appear intact after injury. Moderate injury also caused detachment of some cells from the Silastic membrane, which resulted in a decrease in total cell number measured at 15 min post-injury. After severe injury (7.5 mm; Figure 1D), most cells are stained with PrI, possibly in both neuronal and glial layers. As with moderate injury, severe stretch led to an even greater detachment of cells from the membrane at 15 min post-injury, evident by a decrease in total cell number and the empty gaps not found in control cultures.

A summary of the effects of increasing levels of stretch magnitude on PrI uptake in hippocampal cultures at both 15 min and 24 hr post-injury is represented in Figure 1E. At 15 min post-injury, the number of cells stained with PrI increased with increasing levels of stretch ($n = 3-5$). At 24 hr post-injury, the amount of PrI positive cells was elevated versus matched controls at all levels of injury ($n = 3-5$). Moderate and severe levels of stretch exhibited a decrease in PrI stained cells versus 15 min post-injury. There was no change in the total cell number at 24



hr versus 15 min after injury suggesting that the decrease in the percentage of injured cells was not due to glial proliferation. Therefore, it appears that many glial cells regain their capacity to exclude PrI by 24 hr post-injury.

In addition to the total number of injured cells after injury we also investigated the effects of injury on the number of neuronal cell phenotypes, summarized in Figure 1F ($n = 3-5$). FDA-stained neuronal cells growing in the upper layer of the cultures were identified with morphology consistent with that of MAP-2 immunostaining as described in Methods. Cell density in the cultures was consistent, as there was no significant difference between the number of neuronal cell phenotypes in uninjured control images between 15 min and 24 hr, or from different culture preparations. There was no change in the number of neuronal cells after mild (5.5 mm) injury, indicating minimal, if any, neuronal death, or of cells lifting from the membrane. Following moderate and severe injury, however, the number of neuronal cells was decreased dramatically, indicating either a loss of FDA retention or lifting of the neurons from the cell membrane. The latter explanation is more likely, considering the fact that cell lifting from the membrane was evident at these levels of injury as described above. There was no further change in neuronal cell phenotypes at 24 hr versus 15 min, suggesting no further increase in neuronal cell death or of cells lifting from the membrane.

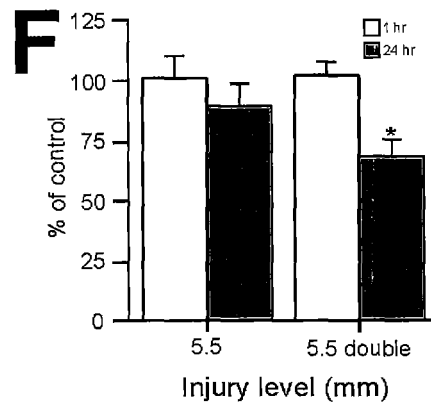
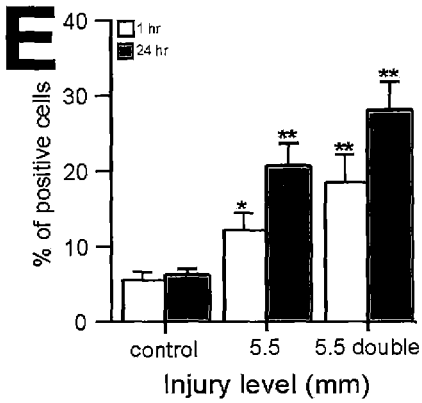
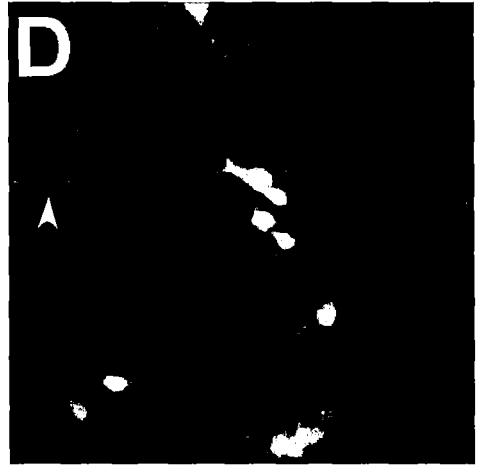
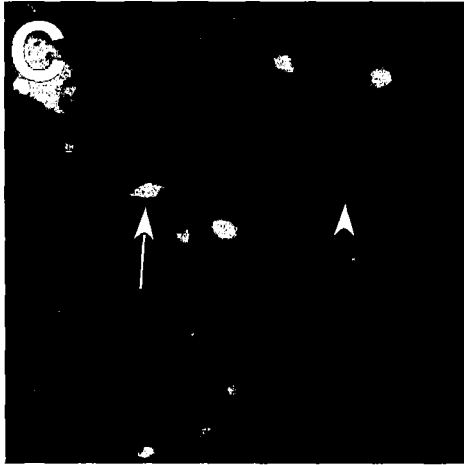
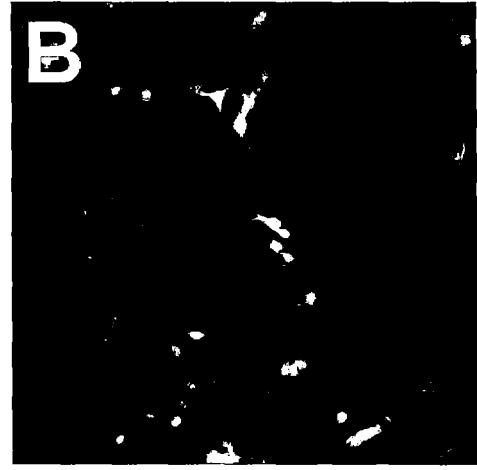
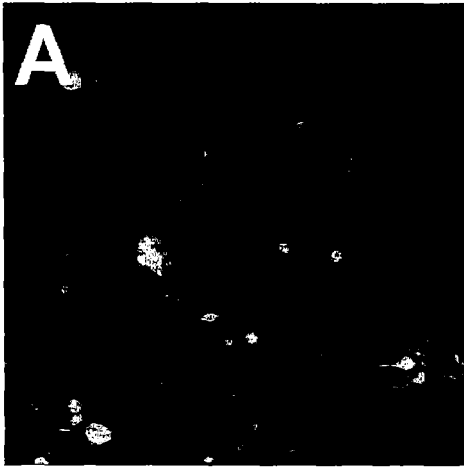
Repetitive mild stretch injury cause cumulative cell damage

In this set of experiments, cultures that received a double insult were injured one hour after the initial injury. Cultures that received a single insult were evaluated at 1 hr post-injury, while cultures receiving repeated insults were evaluated immediately after the second insult. Cultures were also evaluated at 24 hr post-injury (or 23 hr after the second insult; see Timeline shown in Figure 1).

Prototypical images of mouse hippocampal cells that received either one or two stretch injuries are presented in Figures 2A-2D. As shown in Figure 2A, PrI uptake following mild injury (5.5 mm) at 1 hr post-injury was similar to that seen at 15 min post-injury (see Figure 1B), while a double insult increased PrI uptake (Figure 2B). In cultures that received two mild injuries many of the neurons showed beaded neurites as previously seen only after higher levels of injury (see Figure 1C). The marked difference between single and double mild (5.5 mm) injury can be better seen in Figure 2C and 2D, respectively.

On opposing page:

Figure 1. Effect of stretch injury on hippocampal cultures. FDA and PrI images were taken separately and overlaid. Experimental measurements were conducted at the indicated time points (see Timeline at top of figure). **A:** Control, uninjured cultures demonstrate retention of FDA and minimal PrI uptake, indicating healthy, viable cells. An arrow indicates a neuron stained with FDA. **B-D:** Fifteen minutes post injury. **B:** mild injury, 5.5 mm deformation (an arrow indicates a nucleus, likely from a glial cell, stained with PrI); **C:** moderate injury, 6.5 mm deformation; **D:** severe injury, 7.5 mm deformation. Cultures demonstrate an increased amount of PrI staining, the majority of which occurred in the underlying monolayer, suggesting that it was glial in origin. Note that the neuronal somata appear intact following injury, however neuronal processes appear beaded at the higher injury levels, as shown in C. Magnification A-D: 100 $\times$. **E:** PrI uptake is positively correlated to the degree of stretch. Data shown is at 15 min and 24 hr post-injury. Uptake of PrI increased with mild (5.5 mm; $n = 5$), moderate (6.5 mm; $n = 3$) and severe (7.5 mm; $n = 4$) levels of stretch at 15 min post-injury versus control ($n = 5$). At 24 hr, PrI levels remained elevated at all levels of injury (5.5 mm, $n = 5$; 6.5 mm, $n = 5$; 7.5 mm, $n = 3$) versus control ($n = 5$). Moderate and severe levels of stretch caused a decrease in total cell number at 15 min and 24 hr post-injury due to detachment of cells from the membrane that did not occur after mild injury. $*p < .01$ vs. control. $@p < .05$ vs. 5.5 mm injury. **F:** Neuronal cell phenotypes in injured hippocampal cultures. Data is shown at 15 min and 24 hr post-injury. Mild injury caused only a slight reduction, while moderate and severe levels of stretch caused a significant reduction, in neuronal phenotypes at 15 min and 24 hr post-injury. $*p < .05$ vs. control, n is same as Figure 1E.



The effects of repeated insults on cultured hippocampal cells are summarized in Figure 2E. At both 1 hr and 24 hr post-injury, PrI uptake was significant after a single mild (5.5 mm) insult versus matched controls ($n = 7$ for all conditions). In addition, there was an increase in PrI positive cells following a double mild (5.5 mm) insult as compared to a single injury. Although a significant reduction in neuronal cell phenotypes was not evident initially after double injury, as shown in Figure 2F, we found a significant decrease of neuronal cell phenotypes of $32 \pm 6\%$ versus controls at 24 hr following a double insult ($n = 7$ for all conditions). This suggests a reduction in neuronal FDA retention or detachment of some neurons from the membrane, which could be indicative of a delayed neuronal injury or death.

Release of NSE and S-100 β after single and repeated mild injuries

We initially measured NSE and S-100 β release at 1 hr after injury. In the case of NSE, there was no elevation of release following any injury level versus control (data not shown). For S-100 β we did not find reportable levels of this protein after any degree of stretch. We then measured NSE and S-100 β release at 6 and 24 hr post-injury, consistent with time points previously reported *in vivo* (Woertgen *et al.*, 2001). Figure 3A summarizes the release of NSE into the culture media in nanograms per milligram of protein. NSE release was significant versus controls after both single and double 5.5 mm injury; the release of NSE after double insults was also significant versus a single injury at 6 hr post-injury. NSE levels remained elevated 24 hr after both single and repeated injuries ($n = 5$ for all conditions).

The release of S-100 β after injury is depicted in Figure 3B. Unlike NSE, in which there were detectable levels of protein in culture media in the majority of uninjured control wells, we found detectable levels of S-100 β (at least 0.2 ng/ml) in only 31% of control wells. However after injury, there were detectable levels of S-100 β in the culture media of 65% of wells. Similar to previous studies (Romner *et al.*, 2000), for the purpose of statistical analysis, we report S-100 β levels after injury from culture wells that are S-100 β positive (at least 0.2 μ g/L). At 6 hr after injury there was a significant increase in release of S-100 β after repeated mild injury versus single injury. At 24 hr post-injury, S-100 β levels were still elevated; however, the level of S-100 β following repeated injury was not significant versus single injury ($p = .08$) due to an increased SEM ($n = 8$ for all conditions).

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Figure 2. Effect of repeated stretch injury on hippocampal cultures. Cultures that received a single insult were evaluated at 1 hr post-injury, while cultures receiving repeated injuries were evaluated immediately after the second insult (see Timeline in Figure 1). FDA and PrI images were taken separately and overlaid. **A:** PrI uptake following mild injury at 1 hr post-injury was similar to that at 15 min post-injury (see Figure 1B). **B:** A double mild insult increased PrI uptake. Note that many cells have beaded neurites that resemble cultures that received a higher level of injury (see Figure 1C). Magnification A and B: 100 $\times$. **C and D:** Enlargements of A and B, respectively. An arrow in C indicates a neuron stained with FDA, whereas an arrowhead indicates a nucleus stained with PrI. An arrowhead in D indicates a beaded neurite. Magnification: 200 $\times$. **E:** PrI uptake following repeated stretch injury. Data shown is at 1 hr and 24 hr post-injury. $*p < .05$ vs. control, $**p < .01$ vs. control, $n = 7$ for all conditions. **F:** Neuronal cell phenotypes after repeated injury of hippocampal cultures. Data is shown at 1 hr and 24 hr post-injury. There was a significant reduction in neuronal phenotypes after 5.5 mm double injury at 24 hr. $*p < .05$ vs. control, $n = 7$ for all conditions.

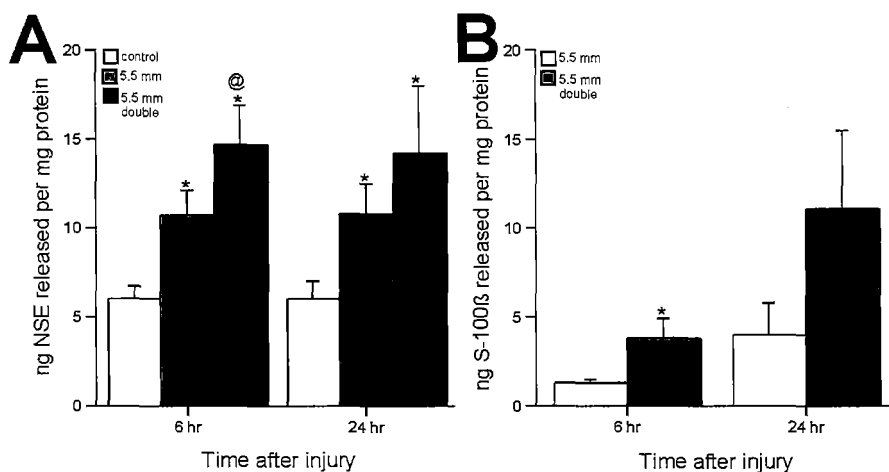


Figure 3. Release of NSE and S-100 β following single and repeated mild injury. **A:** NSE release is shown for control wells, after 5.5 mm single injury, and after 5.5 mm double injury at both 6 hr and 24 hr post-injury. NSE is represented as nanograms released into the culture media per milligram of protein in the culture well. * $p < .05$ vs. control, [@] $p < .05$ vs. 5.5 mm single insult, $n = 5$ for all conditions. **B:** S-100 β release is shown for 5.5 mm single injury and 5.5 mm double injury at both 6 hr and 24 hr post-injury. S-100 β is represented as nanograms released into the culture media per milligram of protein in the culture well. * $p < .05$ vs. 5.5 mm single insult, $n = 8$ for all conditions.

Discussion

Cognitive impairment is one of the most devastating deficits after moderate and severe TBI and greatly influences the quality of life of survivors. Deficits in cognitive ability are also seen after MTBI (McAllister, 1992). Not only has experimental and clinical data demonstrated that the hippocampus plays a critical role in learning and memory, studies also indicate that the hippocampus is uniquely vulnerable to injury following even mild brain trauma (Lowenstein *et al.*, 1992; Lyeth *et al.*, 1990). An emerging hypothesis in the study of neurotrauma is that rMTBI may cause cumulative damage to the brain, and in the absence of cell death, is believed to contribute to ensuing cognitive deficits. The investigation of rMTBI is in its infancy, and while *in vivo* models provide essential information regarding behavior, and pathological and physiological sequelae on macroscopic and microscopic levels, they cannot easily address questions concerning dysfunction at the cellular and sub-cellular levels. Important *in vitro* studies are required to compliment *in vivo* research in order to better understand the cellular mechanisms that contribute to rMTBI-related sublethal cellular dysfunction. In the current report we used an *in vitro* model of stretch-induced injury (Ellis *et al.*, 1995) to examine cellular events following rMTBI.

One aim of the present study was to successfully characterize a model of stretch injury (Ellis *et al.*, 1995) in mouse hippocampal cells that can be reliably used to investigate the pathology of repeated injury at the cellular level. We first demonstrated that cultured mouse hippocampal cells respond to stretch injury in a similar manner to rat cortical (McKinney *et al.*, 1996; Weber *et al.*, 1999) and rat septo-hippocampal cultures (Pike *et al.*, 2000). As with these other reports, we found that an increase in PrI uptake was a reliable indicator of injury severity. Pike *et al.* (2000) found similar differences between mild and moderate/severe degrees of stretch in rat septo-hippocampal cultures. At these higher injury levels, cultured cells had a tendency to detach from the cell membrane, to develop beaded neurites, and to have a reduced number of

neuronal phenotypes. Also similar to previous studies (McKinney *et al.*, 1996; Weber *et al.*, 1999), we found a reduction in PrI positive cells 24 hr following moderate and severe levels of injury compared to 15 min post-injury, suggesting that there may have been glial proliferation after injury which would decrease the percentage of cells that would stain positively for PrI. However, FDA staining indicated that there was no change in total cell number between 15 min and 24 hr post-injury. Therefore, the most likely explanation for this finding is that injured glial cells may have gained the ability to repair their cellular membranes by 24 hr after injury, which has been previously suggested by other investigators (Ellis *et al.*, 1995; Rzigalinski *et al.*, 1997).

To our knowledge, this is the first study to investigate repetitive mechanical injury on cells of the brain using an *in vitro* system of trauma. There were few examples of *in vivo* reports in the literature upon which to determine the injury time points used in the current study; therefore we injured cells 1 hr after an initial insult similar to a previously reported double insult paradigm (Jenkins *et al.*, 1989). Single and repeated mild injury caused a significant amount of PrI uptake at 1 hr and at 24 hr post-injury, which appeared to be present primarily in the glial layer, suggesting no immediate, overt damage to neuronal membranes. In addition, repeated insults caused a slight increase in PrI uptake versus a single insult. Although the majority of neurons after repeated insults had normal-appearing somata, neurites appeared beaded and damaged, a finding that was not observed following a single mild insult. The damaged neurites following repeated injury were similar to dendritic abnormalities observed in hippocampal neurons following moderate TBI *in vivo* (Folkerts *et al.*, 1998), and they strongly resembled those observed after higher levels of stretch magnitude. These results suggest that there is cumulative damage to cultured hippocampal neurons following repetitive injury.

In agreement with this evidence of sublethal cellular damage, we found that NSE levels were elevated after repeated insults versus a single insult 6 hr after injury, a time point in which peak levels of NSE have been reported following TBI *in vivo* (Woertgen *et al.*, 2001). Levels of NSE remained elevated versus uninjured controls 24 hr after injury. This finding is clinically relevant as elevated levels of NSE can persist for several hours or days after MTBI and increased NSE levels are often correlated with neuropsychological dysfunction (Herrmann *et al.*, 2001). Also similar to clinical reports (Ali *et al.*, 2000; Berger *et al.*, 2002; Herrmann *et al.*, 2001; Pleines *et al.*, 2001; Raabe and Seifert, 1999; Waterloo *et al.*, 1997) we found elevated levels of S-100 β following injury *in vitro*. Levels of S-100 β increased after repeated injury compared to single mild insults. S-100 β can also remain elevated for several days post-injury *in vivo* (Pleines *et al.*, 2001). Although the mechanisms for elevated S-100 β are unknown following TBI, increased S-100 β levels in peripheral blood after trauma may be indicative of damage to the blood-brain barrier or could indicate the activation of secondary damage pathways (Herrmann *et al.*, 2001; Raabe and Seifert, 1999). In addition, S-100 β stimulates glial proliferation (Reeves *et al.*, 1994), which could possibly lead to brain swelling after trauma. However, it is also possible that S-100 β is playing somewhat of a protective role. For example, S-100 β may be an important mediator of glia-neuronal interactions, and it has been shown to stimulate neurite extension (Reeves *et al.*, 1994). Therefore, glial cells may release S-100 β in an attempt to save, or to repair, dying or damaged neurons.

Collectively, the data suggests sublethal cellular damage to the majority of hippocampal cells. This is an important finding in light of the fact that following TBI *in vivo*, memory function can be impaired in the absence of hippocampal cell death (Lyeth *et al.*, 1990). Although neuronal death was not readily apparent following single or repeated mild injury, we cannot rule out the possibility that at least some of the neurons following repeated injury go on to die based on the finding that there was a reduction in neuronal phenotypes 24 post-injury. Other types of traumatic insults, such as elevated glutamate exposure, trophic factor withdrawal, or ischemia, can cause

fragmented and beaded neurites, reportedly due to apoptotic caspase activation (Mattson and Duan, 1999; Mattson *et al.*, 2000; Zhao *et al.*, 2000). This could eventually lead to withdrawal or degeneration of neurites, which could then cause the neurons to lift from the membrane. Also, neurites can become fragmented and beaded under necrotic conditions and plasma membranes can become compromised. Therefore, lifting of neurons from the membrane or a delayed uptake of PrI could be responsible for the reduction in neuronal phenotypes 24 hr following repeated injury. However, further detailed studies of cell death potential, whether apoptotic or necrotic, need to be carried out in this model. It should also be noted that, as currently used, there is no hypoxia superimposed on the cells. As ischemia is often a major contributor to cellular damage following TBI, another important set of experiments in the future will be to investigate single or repeated mechanical injury in addition to a secondarily produced ischemic insult. This may lead to further findings elucidating interactions between mechanical and ischemic damage at the cellular level.

Repeated brain injuries occur in a number of populations. Perhaps the most relevant population is that of athletes. Collision sports such as American football, ice hockey, soccer, rugby and boxing present a population of athletes of all ages who encounter rMTBI. In soccer players, for example, the number of concussions was inversely related to the performance on several neuropsychological tests (Matser *et al.*, 1999); these athletes, when compared to matched controls from non-contact sports, had lower scores on memory, planning and visuosperceptual tests (Matser *et al.*, 1998). In addition, repeated MTBI can increase the susceptibility to chronic TBI as well as neurodegenerative diseases (Jordan, 2000; Uryu *et al.*, 2002). For example, there is established evidence of the development of dementia pugilistica in professional boxers (Jordan, 2000). The reasons for these findings could start to be unraveled by using a combination of studies at the cellular and whole animal level.

As with human MTBI, the occurrence of hemorrhage or blood-brain barrier compromise in animal models following MTBI or rMTBI is controversial. Clinical studies describe MTBI patients who present no signs of hemorrhage but still perform poorly on neuropsychological examinations. In fact, it is the MTBI patient population that presents with few or any signs of medical complications (e.g. hemorrhage on CT scan) yet demonstrates cognitive deficits that have the medical and scientific community puzzled (Macciocchi *et al.*, 1993). In the presence or absence of blood-brain barrier compromise, cytoskeletal alteration has been reported following MTBI and rMTBI (Dixon *et al.*, 1991; Folkerts *et al.*, 1998; Jenkins *et al.*, 1989; Kanayama *et al.*, 1996; Laurer *et al.*, 2000; Saatman *et al.*, 1998). In addition, it appears that the effects of a second MTBI may not be additive but synergistic (DeFord *et al.*, 2002; Kanayama *et al.*, 1996; Laurer *et al.*, 2001; Uryu *et al.*, 2002).

In conclusion, our overall results suggest that repeated mild injury causes increased amounts of cellular damage when compared to single insults of the same magnitude. It is possible that pathways of cellular degradation differ between single and multiple TBI. The stretch-injury model used in our study, as well as similar models that cause secondary damage (Adamchik *et al.*, 2000), could provide a relatively fast and economical method for investigating secondary pathways of damage or for screening potential pharmacological treatments for rMTBI. Also, because we have now characterized this model in mouse hippocampal cells, it is possible to investigate the effects of injury on cultures from various transgenic mice in the hopes of gaining further information in regards to possible treatment strategies. Although acute TBI has received much more attention, both clinically and experimentally, an increasing amount of studies are currently being conducted on repetitive TBI. We are optimistic that better prevention and intervention treatments for rMTBI will soon be devised based on both *in vivo* and *in vitro* experimentation.

CHAPTER 2.2

**The extent of damage following repeated injury
to hippocampal cells is dependent on the
severity of insult and inter-injury interval**

Abstract

Recent evidence suggests that repeated mild traumatic brain injury may result in cumulative damage. We investigated cell damage and death in hippocampal cultures following different paradigms of repeated mechanical trauma *in vitro* by measuring propidium iodide (PrI) uptake, release of neuron specific enolase (NSE) and glial S-100 β protein, and by performing neuronal counts. Cultures that received two mild injuries (31% stretch), either 1 hr or 24 hr apart, displayed different profiles of PrI uptake and S-100 β release, although neuronal loss and NSE release was similar in both paradigms. When cells received a sub-threshold, low-level stretch (10%) repeated several times, they eventually stained with PrI. Cultures receiving 10% stretch 24 hr before a mild insult, released less S-100 β protein compared to a mild insult alone, suggesting a preconditioning effect. Lastly, exogenous S-100 β applied to injured cultures decreased PrI uptake, implying a possible protective role for S-100 β following trauma to hippocampal cells.

Introduction

Traumatic brain injury (TBI) continues to be a leading cause of death in Western industrialized nations (Jennett, 1996), and in survivors, the cognitive impairment observed after TBI greatly affects quality of life. It is estimated that as many as 6.5 million individuals may be living with the consequences of TBI in the United States alone (NIH Consensus Development Panel on Rehabilitation of Persons with Traumatic Brain Injury, 1999). Similar to moderate and severe TBI, cognitive deficits have often been reported after mild TBI (MTBI; McAllister, 1992), and an emerging hypothesis is that repeated MTBI (rMTBI) may cause cumulative damage to the brain, ultimately resulting in memory and learning dysfunction. The study of rMTBI is important for understanding the pathological sequelae in individuals that may receive several insults to the head, such as child abuse victims and athletes.

The hippocampus appears to be particularly vulnerable to injury following MTBI (Lyeth *et al.*, 1990; Lowenstein *et al.*, 1992). Studies *in vivo* have found that neither cell death nor tissue damage, though commonly found after moderate and severe TBI, are apparent after MTBI; these animals, however, demonstrate cellular dysfunction and memory deficits (Lyeth *et al.*, 1990; Dixon *et al.*, 1991; Kanayama, *et al.*, 1996; Scheff *et al.*, 1997; DeFord *et al.*, 2002; Uryu *et al.*, 2002). Post-injury consequences involving the hippocampus may differ between single and repeated brain trauma (Olsson *et al.*, 1971). To this end, we have previously studied the effects of rMTBI on cultured hippocampal cells using an *in vitro* model of stretch-induced injury, and found that cells which received two mild injuries one hour apart displayed damage similar to a single, moderate level of injury (Slemmer *et al.*, 2002). We also found subsequent increases in neuron specific enolase (NSE) and glial S-100 β protein, two markers of brain injury commonly employed in the clinic, following repeated mild trauma *in vitro* (Slemmer *et al.*, 2002). In order to investigate further the effects of repeated injury to the hippocampus at the cellular level, we varied the amount of time between mild injuries, as well as the duration of recovery time after injury before measures of cell death and dysfunction. We also analyzed the effects of repeating a sub-threshold level of injury—a level of stretch producing no cell death or obvious cell damage when administered once—as well as the effects of sub-threshold injury on the subsequent effects of a mild injury, often referred to as “preconditioning”. Here we report that the extent of cell damage following repeated injury to hippocampal cells is dependent on the severity of the insult and the inter-injury interval, and that reactions to repeated injury differ between neurons and glia. We also find that S-100 β protein released after injury, may not only be a good marker of cell

damage, but that it can affect neighboring glia and neurons once released. This model of repeated injury at the cellular level *in vitro* could prove useful for unraveling the mechanisms underlying differences between single and repeated injury *in vivo*.

Materials and Methods

Hippocampal cell cultures

Primary hippocampal cell cultures were prepared from E18 wild-type FVB/N mouse embryos as previously described (Slemmer *et al.*, 2002). Briefly, dissociated hippocampi were diluted in serum-containing media [Basal Medium Eagles (GIBCO, Grand Island, NY) containing 10% horse serum (GIBCO), 10 μ g/ml gentamicin (Sigma, St. Louis, MO), 0.5% glucose (Sigma), 1 mM sodium pyruvate (GIBCO) and 1% N2 supplements (GIBCO)] to a concentration of 500,000 cells per ml; cells were then plated in 1 ml aliquots onto collagen-coated six-well FlexPlates (FlexCell, Hillsborough, NC) coated overnight with poly-L-ornithine (500 μ g/ml; Sigma). All cultures were maintained in a humidified incubator (5% CO₂, 37°C). Neuronally enhanced cultures were obtained by replacing half of the media two days after plating, and then twice per week, with serum-free media containing 2% B27 supplements (GIBCO). Glia formed a confluent monolayer that adhered to the membrane substrate, while neurons grew in the upper layer of cultures that adhered to the underlying glial layer.

Cell injury

Cultured cells were injured using a model 94A Cell Injury Controller (Bioengineering Facility, Virginia Commonwealth University, Richmond, VA) as previously described (Ellis *et al.*, 1995; Slemmer *et al.*, 2002, 2004a). In brief, the Silastic membrane of the FlexPlate well was rapidly and transiently deformed by a 50-ms pulse of compressed nitrogen, which deformed the Silastic membrane and adherent cells to varying degrees controlled by pulse pressure. The extent of cell injury—produced by deforming the Silastic membrane on which the cells are grown—was dependent on the degree of deformation, or stretch. Previous work conducted using rat cortical neurons and glia (Ellis *et al.*, 1995; McKinney *et al.*, 1996; Weber *et al.*, 1999) and mouse hippocampal neurons (Slemmer *et al.*, 2002) have characterized three levels of cell stretch (5.5 mm, 6.5 mm and 7.5 mm deformations), defined as mild, moderate, and severe injury, which result in membrane deformation and biaxial strain or stretch of 31, 38 and 54%, respectively. This range of cell stretch has been shown to be relevant to what would occur in humans after rotational acceleration/deceleration injury (Schreiber *et al.*, 1995). In the present report, cells received one or more injuries of either 5.5 mm (31% stretch; mild injury) or 3.4 mm (10% stretch; sub-threshold injury) membrane deformation, at various time intervals (see Timelines in Figures 1 and 6). Uninjured control wells were contained in the same FlexPlates as injured wells, and thus underwent the same manipulations, except that they did not receive rapid deformation of the Silastic membrane.

Cell viability

Cell injury was assessed in cultured cells using the two dyes fluorescein diacetate (FDA; Sigma) and propidium iodide (PI; Sigma) as previously described (Slemmer *et al.*, 2002, 2004a). Images were captured using Texas Red and FITC (green) filters on a Leica DMRBE fluorescence

microscope, equipped with a Hamamatsu C4880 CCD camera. FDA and PrI images were taken separately, pseudocolored, and overlaid. Cells in five contiguous $\times 100$ images were counted and averaged per well. All images were taken from the center portion of the well, as this region was previously shown to receive equal impact from the cell injury controller (Ellis *et al.*, 1995). All PrI and FDA cell counting was performed blind. In control cultures, PrI staining was low, accounting for less than 5% of total cell number, consistent with previous reports (Zhao *et al.*, 2000; Slemmer *et al.*, 2002, 2004a). Final results are expressed as percentage of control values. All experiments were completed using at least three separate culture preparations at 9-10 DIV.

NSE and S-100 β assays

Neuron specific enolase (NSE) and S-100 β protein levels were measured in culture media (adjusted to 500 μ l several hours before injury) using lumino-immunometric (LIA-mat) assay kits (Sangtec Medical, Bromma, Sweden) as previously described (Slemmer *et al.*, 2002, 2004a). Following the assays, the cells adhering to the Silastic membrane were solubilized with 1 ml 0.1% NaOH to determine protein concentration. Total protein count was determined by the bicinchoninic assay (BCA; Pierce, Rockford, IL). Final results are expressed as nanograms of NSE or S-100 β released into the culture media per milligram of protein. All experiments were completed using at least three separate culture preparations at 10 DIV.

Immunohistochemistry and neuronal cell counts

Immunohistochemistry was performed on control and injured hippocampal cells as previously described (Slemmer *et al.*, 2004a). Briefly, cultures were fixed with 4% paraformaldehyde (all steps conducted at room temperature), permeabilized with 0.2% Triton X-100, and then incubated with primaries antibodies [anti-microtubule-associated protein 2 (MAP2, 1:300; Sigma)] for 1 hr, followed by incubation with secondary antibodies (Alexa-594-conjugated goat-anti-mouse, 1:300; Molecular Probes, Eugene, OR) for 1 hr. Cultures were dehydrated with ethanol and mounted with DAPI for nuclear staining. Images were captured using a Leica DMRBE microscope (see Cell Viability). MAP2-positive neurons were counted in five contiguous fields at a magnification of $\times 100$.

Exogenous S-100 β application

We first calculated the average release of S-100 β (nanograms per milliliter of culture media) after a single 5.5 mm injury to be approximately 0.5 ng/ml. We therefore added this amount of S-100 β , a lower concentration (0.1 ng/ml), or a higher concentration (1 ng/ml) to cultures that received either a mild (5.5 mm) injury, or remained uninjured. Aliquots (10 μ l) of exogenous S-100 β (to produce a final concentration of 0.1, 0.5 or 1 ng/ml, and dissolved in Milli-Q water; Sigma) were added to 1 ml fresh culture media, and the cells were returned to the incubator for 24 hr. In order to quantify cell damage and death, cultures were first incubated with PrI (see Cell viability above) before being fixed and stained against MAP2 and mounted with DAPI (see Immunohistochemistry above). All experiments were completed using at least three culture preparations at 9-10 DIV.

Western blots

Samples (groups of two wells from control or injured conditions) were collected in 250 μ l of triple detergent homogenization buffer (20 mM HEPES, 1 mM EDTA, 2 mM EGTA, 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% deoxycholic acid, pH 7.6; supplemented with protease inhibitor cocktail and 1 mM DTT) at 10–12 DIV. Equal amounts of protein (20 μ g) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% acrylamide gel) for 1 hr at 130V, and were transferred to PVDF membranes for 45 min at 12V. Blots were blocked in 5% non-fat milk in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) for 1 hr and incubated overnight with primary antibody (anti-S-100 β ; Sigma) diluted 1:5000 in 0.5% milk in TBST. After multiple washes in TBST, the blots were incubated in appropriate secondary antibody (horseradish-peroxidase-labeled rabbit-anti-mouse) diluted 1:3000 in 3% milk in TBST. Membranes were washed in TBST followed by visualization using SuperSignal West Dura Extended Duration Substrate (Pierce), and imaged using a Kodak Image Station 440CF. Protein quantification was performed using accompanying Kodak software.

Data analysis

The data were analyzed using the statistical program GB Stat (Dynamic Microsystems, Silver Spring, MD). Data were computed as mean $\pm$ standard error of the mean (SEM) values. Statistical significance was established by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test. Data are considered significant at $p < 0.05$.

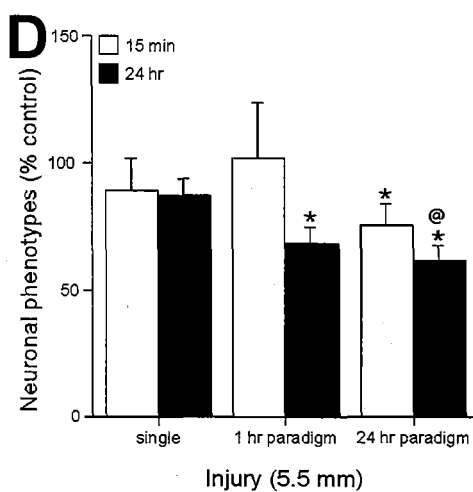
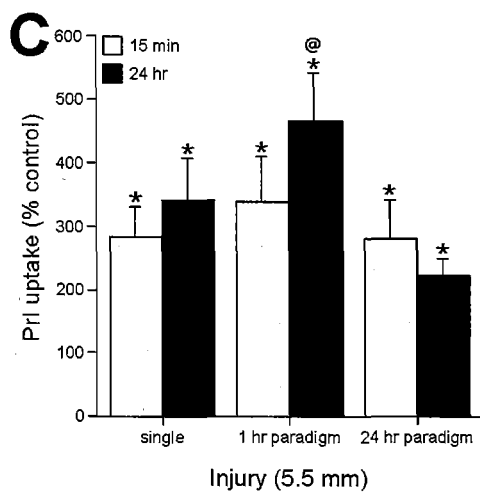
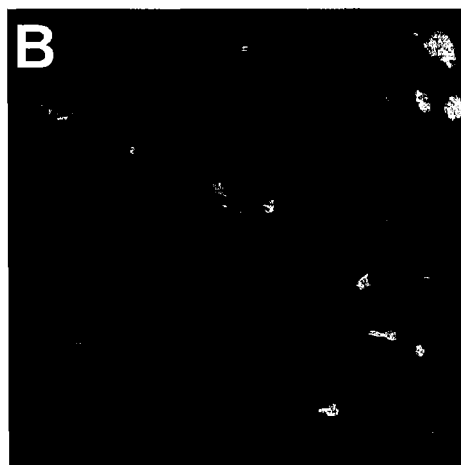
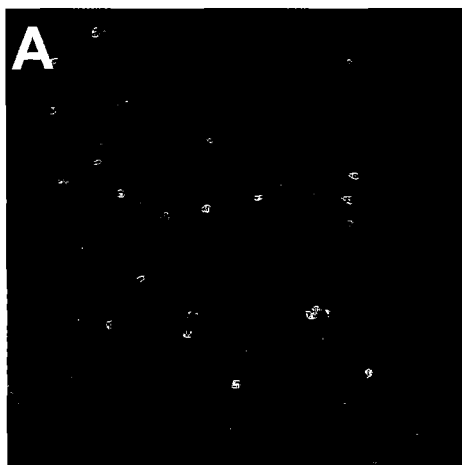
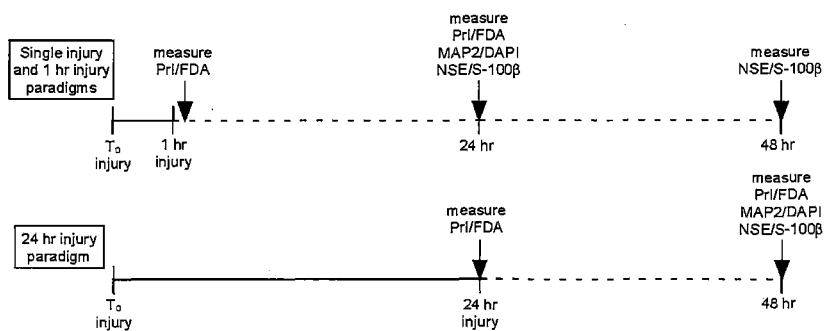
Results

The effect of repeated mild stretch injury depends on the inter-injury interval

The effect of repeated mild injury on cultured hippocampal cells was initially characterized using FDA and PrI. FDA is known to stain healthy, viable cells and fluoresces green, while PrI cannot pass through intact cellular membranes. If membranes are damaged, however, cells lose their ability to retain FDA. In addition, PrI will enter cells and stain the nucleus, resulting in a bright red fluorescence. Cultures received either a single, or a double mild injury (5.5 mm deformation, 31% stretch), 1 or 24 hr apart (for clarity, refer to the Timeline at the top of Figure 1). PrI uptake and the number of neuronal phenotypes (indicated by FDA counts) were assessed at either 15 min or 24 hr after the second injury. Figures 1A–B depict representative images of a control, uninjured culture, and cultured cells 15 min after a single mild injury, respectively; color images can be

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Figure 1. Effect of single and repeated stretch injury on cell viability in hippocampal cultures. Cultures received either one or two mild injuries (5.5 mm deformation, 31% stretch), 1 or 24 hr apart. FDA/PrI was assessed either 15 min or 24 hr after the second injury (see Timeline at top of figure). **A:** Representative image of control, uninjured cultures, demonstrates good retention of FDA and minimal PrI uptake, indicating healthy, viable cells. **B:** Representative image after injury (15 min after single 5.5 mm injury) shows an increase in PrI and a disruption of the glial monolayer. Magnification A and B: $\times 100$. **C:** PrI uptake (indicative of cell membrane damage) is significantly increased at all injury levels tested, at 15 min and 24 hr after injury. $*p < 0.05$ vs. control; $@p < 0.05$ vs. 24 hr time point of 24 hr repeated injury paradigm. $n = 7-8$. **D:** The number of neuronal cell phenotypes (indicated by FDA counts) decreases significantly at 24 hr in both injury paradigms, and at 15 min after the 24 hr paradigm. $*p < 0.05$ vs. control; $@p < 0.05$ vs. 24 hr after single injury. n is same as C.



seen in the original article (Slemmer and Weber, in press). Similar to what we have previously reported, stretch injury shows an increase in PrI uptake, and a disruption of the glial monolayer (Slemmer *et al.*, 2002). Indeed, the number of PrI-positive cells is significantly increased at all injury levels tested, at 15 min and 24 hr after injury (Figure 1C). The majority of this PrI staining appeared to occur in the underlying glia monolayer. PrI uptake increased with time following single injury and the 1 hr repeated injury paradigm. However, following the 24 hr repeated injury paradigm there was a decrease in PrI-positive cells, suggesting the possibility of glial membrane repair. The number of neuronal cell phenotypes (indicated by FDA counts) was unaffected after a single insult, but decreased significantly 15 min post-injury in the 24 hr paradigm and at 24 hr in both repeated injury paradigms.

Stretch injury causes reductions in hippocampal neurons and in total cell counts

In our initial study of stretch-injured hippocampal cells, we began to separate the effects of injury to neurons and glia in our mixed culture preparation using FDA retention and NSE release as markers of neuronal death and damage, and PrI uptake and S-100 β release as markers of glial damage (Slemmer *et al.*, 2002). For our investigation of cerebellar cell cultures, however, we also used immunohistochemical markers to track the progress of cell damage and death after injury (Slemmer *et al.*, 2004a). We found that our FDA neuronal counts were very comparable to the number of MAP2-positive neurons before and after injury. Here we show representative images of MAP2-positive neurons and DAPI-labeled nuclei in a control, uninjured culture (Figure 2A), and in cultured cells after injury (Figure 2B; 24 hr after the second insult in the 24 hr paradigm). These images clearly show neurons with smooth, dendritic processes, and an abundance of underlying glia. Both a single injury and the 1 hr repeated injury paradigm cause a significant decrease in total cell number, as indicated by DAPI-stained nuclei at 24 hr post-injury (Fig. 2C), and there is an apparent recovery in the 24 hr repeated injury paradigm, suggesting a proliferation of glia. In contrast to this glial response, the number of MAP2 neurons is again unaffected by single injury, reduced after the 1 hr injury paradigm, and significantly decreased following the 24 hr paradigm (Figure 2D). Although, there is significant neuronal loss following the 24 hr paradigm, surviving neurons demonstrate no obvious morphological abnormalities, such as beading of neurites (compare Figure 2B to uninjured culture in Figure 2A), which we have previously found after a second injury in the 1 hr paradigm (Slemmer *et al.*, 2002).

The release of NSE and S-100 β is elevated after repeated stretch injury

The data shown in Figures 1 and 2 suggest that neurons undergo a delayed, but not an immediate death, as reported previously (Slemmer *et al.*, 2002, 2004a). In order to further corroborate this evidence, we measured the release of NSE into the culture media after single and repeated mild injury (see Timeline in Figure 1 for clarity). Significant NSE release was observed at 24 and 48 hr in both the 1 hr and 24 hr repeated injury paradigms, but not following a single mild injury (Figure 3A). No NSE was detected in culture media when measured at 1 hr after injury, suggesting a more delayed release. In contrast to the release of NSE, release of S-100 β from glia was elevated after a single injury, as well as after both repeated injury paradigms. There was a most notable increase in S-100 β release after 24 hr in the 1 hr injury paradigm which decreased by 48 hr (Figure 3B).

NSE and S-100 β were measured using luminometric assay kits for which small samples of culture media were collected and tested. We also collected control and injured cells from the Silastic membrane and processed them for Western blots. As shown in the representative Western blot in Figure 3C, the amount of intracellular S-100 β protein appears to be low in uninjured

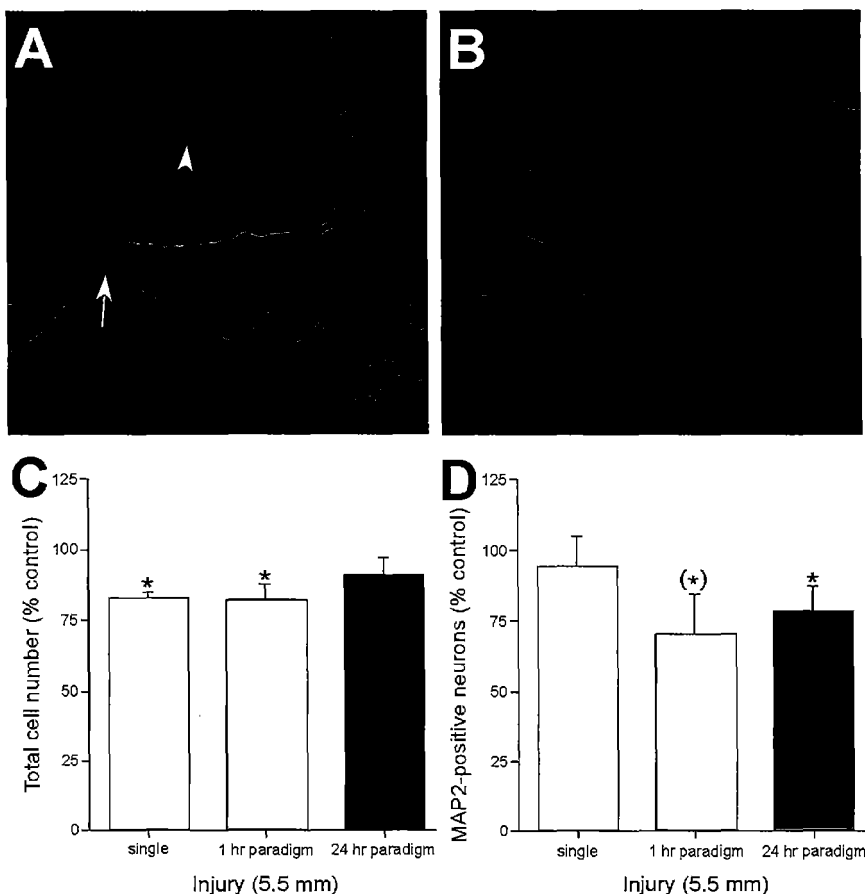


Figure 2. Effect of repeated stretch injury on hippocampal neurons and total cell count. A and B: Representative images of MAP2-positive neurons and DAPI-labeled nuclei demonstrate intact soma and smooth dendrites. A: Control well. A MAP2-labeled soma is indicated by an arrow; a DAPI-positive nucleus from a glia cell by an arrowhead. B: Injured well 24 hr post-injury in the 24 hr repeated injury paradigm. See Timeline in Figure 1 for explanation of injury paradigms. Magnification A and B: $\times 160$. C: Both a single injury and the 1 hr injury paradigm cause a significant decrease in total cell number when measured 24 hr post-injury. $*p < 0.05$ vs. control. $n = 4-7$. D: Although the number of MAP2 neurons is reduced [near significance (*) = 0.057] after the 1 hr injury paradigm, the number of neurons is significantly decreased after the 24 hr paradigm (24 hr after the second insult). $*p < 0.05$ vs. control. n is same as C.

control cells, and is elevated after injury, again most notably after repeated mild injury in the 1 hr injury paradigm. Quantification revealed slightly elevated intracellular S-100 β after single injury ($112 \pm 5.60\%$ of control; $n = 3$) and the 24 hr repeated injury paradigm ($119 \pm 3.14\%$; $n = 3$), but that the highest amount of intracellular S-100 β was indeed after the 1 hr repeated injury paradigm ($149 \pm 12.9\%$ of control; $n = 3$). This apparent increase in intracellular protein implies that surviving glia actively produced S-100 β , and that not all S-100 β protein is simply released from injured cells.

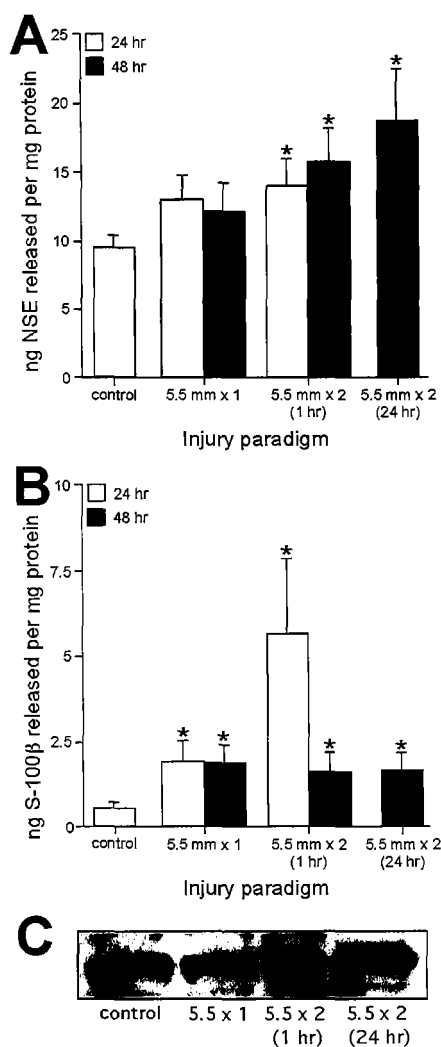


Figure 3. Release of NSE and S-100 β following stretch injury. See Timeline in Figure 1 for explanation of injury paradigms. **A:** There is a significant release of NSE at 24 and 48 hr in the 1 hr injury paradigm, and in the 24 hr injury paradigm. $*p < 0.05$ vs. control. $n = 7-9$. **B:** There is a significant release of S-100 β at all time points after injury, most notably after 24 hr in the 1 hr injury paradigm. $*p < 0.05$ vs. control. n is same as A. **C:** Representative Western blot obtained from uninjured hippocampal cultures or from cultures that received either a single 5.5 mm injury, or two 5.5 mm injuries (1 or 24 hr apart).

Low-level stretch causes damage only after multiple injuries

As previously shown, a level of stretch which produces cell damage when administered a single time, will produce cumulative damage if repeated in short duration. It was unclear if a sub-threshold level of mechanical injury, that is a level of stretch that produces no overt cell death or morphological damage when administered a single time, would cause cell damage when repeated. In order to test this, we first measured PrI uptake and FDA neuronal counts after a low-level (10%) stretch *in vitro*. Cultures subjected to a single 3.4 mm stretch injury, demonstrated no uptake in PrI or loss of neurons when measured at 15 min and 24 hr after the insult (Figures 4A-B). This suggested that this low level of stretch was indeed sub-threshold to produce cellular injury following a mechanical insult. When a 3.4 mm deformation was repeated using the 1 hr

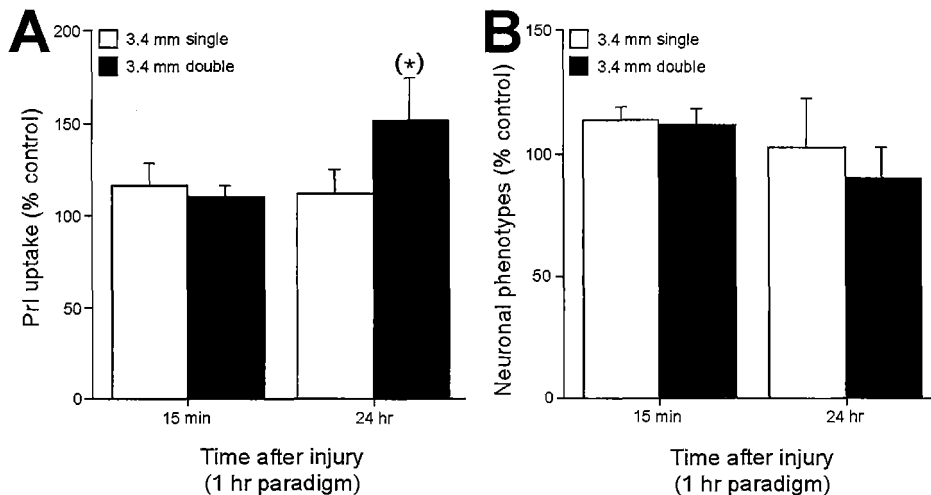


Figure 4. Effect of low-level stretch on cultured hippocampal cells. See Timeline in Figure 1 for explanation of injury paradigms. **A:** Although low-level stretch (10%) has no significant effect on PrI uptake, at 24 hr after double 3.4 mm injury PrI uptake does near significance [(*) = 0.075]. **B:** Low-level stretch also has no significant effect on the number of neuronal phenotypes (indicated by FDA counts). $n = 7-9$ for all experiments.

repeated injury paradigm, there was no significant uptake of PrI or loss of neurons (Figures 4A-B), unlike the cumulative damage observed after repeated 5.5 mm deformation. Although there was a trend towards increased PrI uptake at 24 hr after repeated 3.4 mm deformation, this was not significant ($p = 0.075$ vs. control).

We next evaluated the effect of multiple (up to six) low-level stretch injuries with a much shorter inter-injury interval in order to reduce recovery time of cells after receiving a mechanical insult. Stretch injury (3.4 mm) was administered 2 min apart, and measurements of cell viability were performed. The number of PrI-positive cells does significantly increase, but only after five or six injuries (Figure 5A). Similarly, the number of neuronal phenotypes is significantly reduced after four, five or six consecutive injuries (Figure 5B). In order to quantify further the effects of multiple low-level stretch injuries on hippocampal neurons and glia, we measured the release of NSE and S-100 β in control cells, and in cultures that received either one or six consecutive injuries. As shown in Figures 5C-D, there is a significant release of NSE, but not of S-100 β , after six consecutive injuries.

Preconditioning has differential effects on hippocampal neurons and glia

There has been much conflicting evidence either validating or repudiating the effects of “preconditioning” the brain with a low-level injury, which protects against the effects of a more severe injury at a later time. To evaluate a possible preconditioning effect with mechanical injury, hippocampal cultures received either a low-level stretch (3.4 mm deformation) followed by a mild injury, or a mild injury only (for clarity, see the Timeline at the top of Figure 6). We then evaluated the cultures on a variety of parameters. As shown in Figure 6A, there is a significant increase in PrI uptake following preconditioning to an extent similar to that seen after a mild injury alone. Preconditioning does not affect the number of neuronal phenotypes after mild injury (Figure 6B). Also, NSE release is not elevated above control after either a mild injury alone or

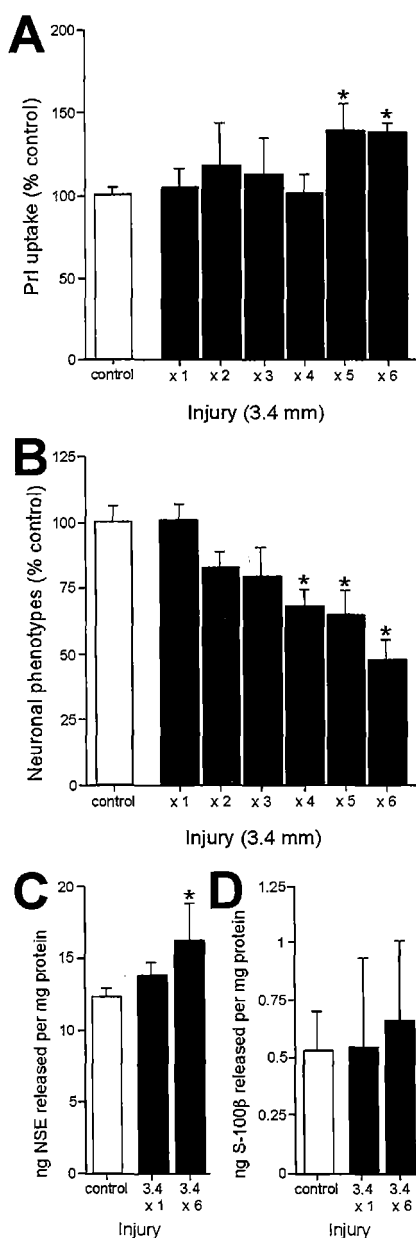


Figure 5. Effect of multiple, low-level stretch injuries on cultured hippocampal cells. A: Prl uptake is significantly increased only after 5 and 6 low-level stretch injuries (10%). $*p < 0.05$ vs. control. $n = 4-6$. **B:** Repeated low-level stretch injury significantly reduces the number of neuronal cell phenotypes (indicated by FDA counts) after 4, 5 or 6 consecutive injuries. $*p < 0.05$ vs. control. n is same as A. **C:** There is a significant release of NSE after 6 consecutive low-level injuries when measured at 24 hr. $*p < 0.05$ vs. control. $n = 6-7$. **D:** Low-level stretch (up to 6 consecutive injuries) has no effect on the release of S-100 β at 24 post-injury. $n = 7$.

preconditioning (Figure 6C). The data in Figure 6D, however, indicate that preconditioning does have a marked effect on hippocampal glia, as the significant release of S-100 β after a mild injury alone is attenuated by preconditioning.

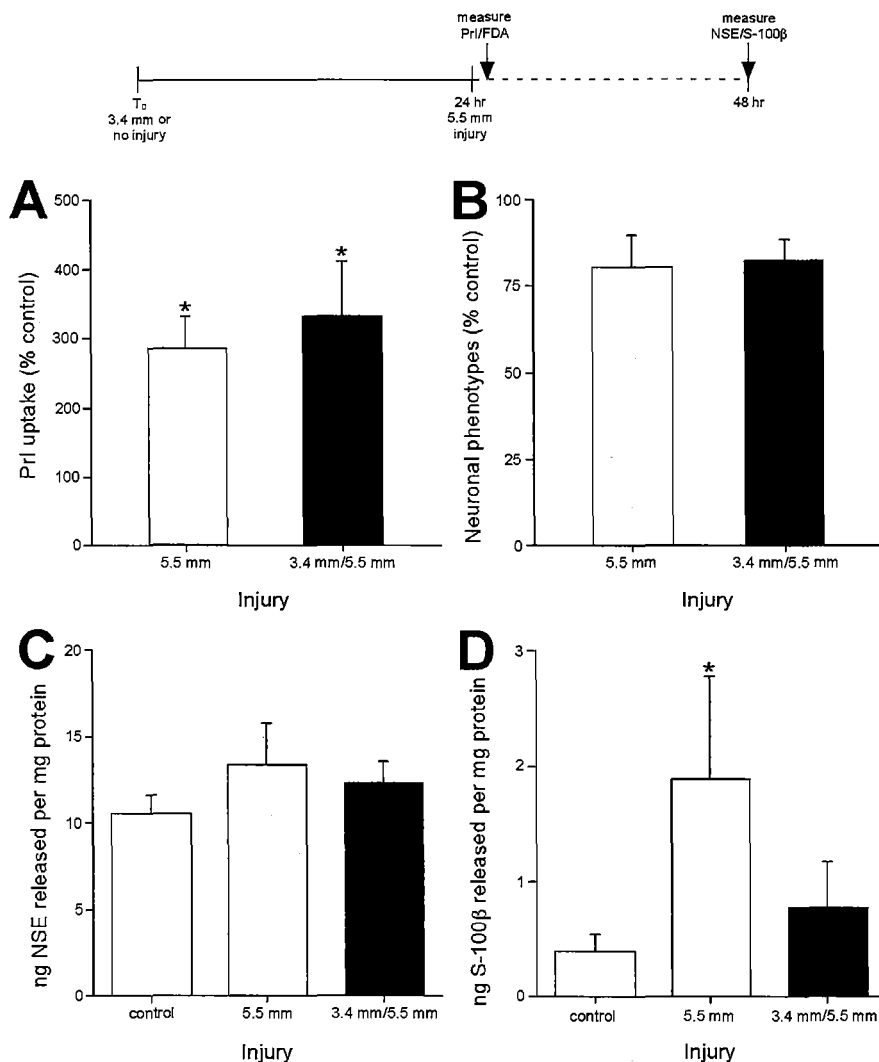


Figure 6. Effect of preconditioning on cultured hippocampal cells. Cultures received either a 3.4 mm deformation or no stretch, followed by a 5.5 mm deformation 24 hr later, referred to as “preconditioning” (see Timeline at top of figure). **A:** PrI uptake increases to a similar extent following preconditioning as after 5.5 mm injury alone. **B:** Preconditioning does not affect the number of neuronal phenotypes (indicated by FDA counts) compared to 5.5 mm injury alone. **C:** NSE release is not elevated above control after either 5.5 mm injury alone or preconditioning. **D:** The significant release of S-100β after 5.5 mm injury is attenuated by preconditioning. * $p < 0.05$ vs. control. $n = 6$ for all experiments.

Exogenous S-100β application on injured hippocampal cells

Although both clinical and experimental reports indicate elevated levels of S-100β after TBI (Rothoerl *et al.*, 2000; Herrmann *et al.*, 2001), the reasons for this release are unclear. For example, S-100β may be released from glia purely due to membrane damage or it may be released actively, which could adversely affect neurons or protect them from secondary insults.

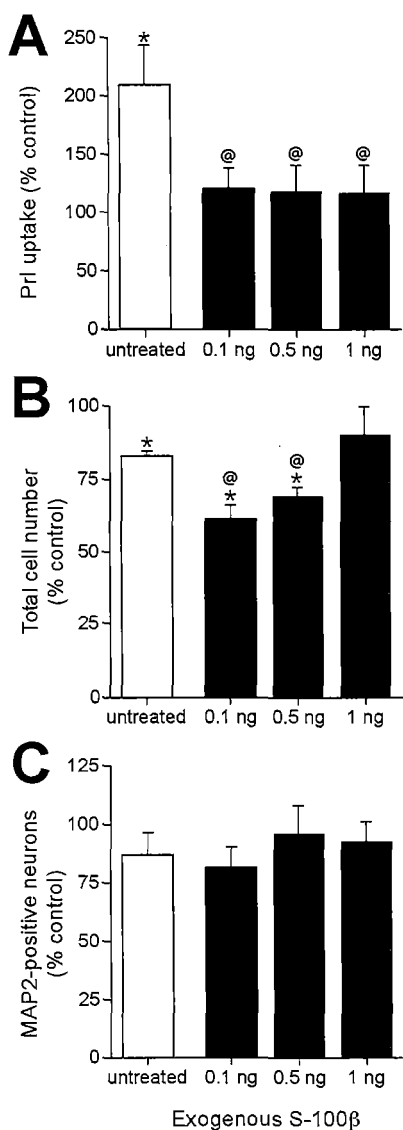


Figure 7. Effect of exogenous S-100 β application on injured hippocampal cells. All cultures were injured with 5.5 mm deformation and either left untreated or they received S-100 β (0.1, 0.5 or 1.0 ng/ml) immediately after injury. Measurements of PrI uptake, total cell number, and MAP-2 positive neurons were taken 24 hr post-injury. **A:** PrI uptake is significantly increased above control in untreated injured cells, but not in cultures that received exogenous S-100 β . * p < 0.01 vs. control (uninjured); @ p < 0.05 vs. untreated. **B:** There is a significant decrease in total cell number in untreated injured cells as compared to control, and in injured cells that received 0.1 or 0.5 ng S-100 β . * p < 0.05 vs. control (uninjured); @ p < 0.05 vs. untreated. **C:** The number of MAP-2 positive neurons in untreated cultures and cultures that received S-100 β is not significantly affected at 24 hr post-injury. n = 5-8 for all experiments.

To elucidate the effects of S-100 β on hippocampal cells following trauma we treated cultures that received a single mild injury with different amounts of exogenous S-100 β and measured various parameters at 24 hr post-application. Treatment of uninjured cultures with 0.1, 0.5 or 1.0 ng/ml of S-100 β caused no change in PrI uptake or MAP2-positive neurons. However, 1 ng/ml of S-100 β increased total cell number to $134 \pm 20.2\%$ of control at 24 hr, but this finding was not statistically significant (p = 0.094; n = 5; data not shown). Consistent with the previous data from the PrI/FDA cell viability assays (Figure 1), Figure 7A shows that the number of PrI-positive cells is significantly increased in untreated injured cells. However, injured cells that received 0.1, 0.5 or 1 ng/ml of S-100 β immediately after injury had reduced PrI uptake when measured 24 hr

post-injury. There is a significant decrease as compared to control in total cell number in untreated injured cells, and in injured cells that received 0.1 or 0.5 ng S-100 β (Figure 7B). However, total cell number had recovered to control values in cultures treated with 1 ng/ml S-100 β . Lastly, the number of MAP2-positive neurons was not significantly affected by any concentration of S-100 β (Figure 7C).

Discussion

Although acute moderate and severe TBI have received considerable attention, current studies have highlighted the prevalence of MTBI and rMTBI in large populations of patients (McAllister, 1992; Thurman and Guerrero, 1999), and several recent experimental studies of rMTBI *in vivo* have also been conducted (Kanayama *et al.*, 1996; Laurer *et al.*, 2001; DeFord *et al.*, 2002; Uryu *et al.*, 2002). To complement the findings from humans and *in vivo* experimentation, we have previously investigated the effects of repeated trauma on hippocampal cells by producing stretch-induced injury *in vitro* (Slemmer *et al.*, 2002). We found a significant increase in the uptake of PrI by 15 min post-injury when mild injuries were administered 1 hr apart, which appeared to occur primarily in the glia. There was a significant decrease in the number of FDA-retaining neurons after double injury at 24 hr post-injury, indicating that the neurons experienced a form of delayed, but not immediate, death. This initial study indicated that glia and neurons might react differently to stretch injury *in vitro*. We have recently tested this hypothesis using cerebellar cell cultures, and found that cerebellar BG, PCs, and non-Purkinje neurons (such as granule cells) followed varying patterns of degradation and death following mild and moderate injury (Slemmer *et al.*, 2004a).

In the present report, we extend the findings of the aforementioned studies using cultured hippocampal cells, injured according to a variety of paradigms (shown in the Timelines at the top of Figures 1 and 6). PrI staining assessed cell membrane damage, and viable neurons were calculated by counting FDA-positive neuronal phenotypes and MAP2-positive cells. In order to further differentiate between the effects of trauma on neurons and glia, we also measured the release of NSE and S-100 β , which are often elevated in the serum and CSF of patients that have suffered a TBI (Herrmann *et al.*, 2001). Therefore, we have more closely investigated the response of different cell types to repetitive injury, and the role that S-100 β may play in the injured brain. Overall, our results suggest that cumulative damage to hippocampal cells following repeated injury is dependent on the degree of the injury, as well as the time between the injuries. Cultures that received a second mild injury, at either 1 or 24 hr after the first injury, demonstrated more cell damage than a single mild injury alone. Although neuronal loss was similar in both repeated injury paradigms, the data suggest that glial cells react differently to these injury regimens. For example, PrI counts decreased after the 24 hr paradigm, whereas they increased after the 1 hr paradigm, a phenomenon not completely accounted for by an increase in total cell number. As previously noted, most of this PrI uptake appeared to occur in the underlying glial monolayer. This data suggests that the decrease in PrI uptake at later time points may indicate that glia repair their membranes. Therefore, it is important to note that the immediate uptake of PrI may indicate membrane damage, whereas the delayed uptake may more closely mark cell death. Also, a much higher amount of S-100 β protein was released after the 1 hr paradigm compared to other injury regimens. Interestingly, we found that cerebellar Bergmann glia released significant levels of S-100 β by 1 hr post-injury, and reached peak levels of release 10-fold higher than that of hippocampal glia, unlike levels of released NSE, which were similar between glia types (Slemmer *et al.*, 2002, 2004a). Similar findings were reported by Pinto *et al.* (2000) among

hippocampal, cerebral cortical and cerebellar glia. Because S-100 β increases intracellularly (as shown by Western blots) at 24 hr after the second injury in the 1 hr paradigm, our results suggest that S-100 β levels are actively increased by glia, and are not purely a marker of cellular membrane damage.

We also found that a sub-threshold level of injury (3.4 mm; 10% stretch), which normally causes no overt cellular damage, can eventually cause damage when repeated several times within short periods. After several consecutive injuries there was marked cell damage as indicated by PrI/FDA and following six consecutive injuries, there was a significant release of NSE at 24 hr, but not a concurrent significant release of S-100 β . Although direct comparisons to the clinical situation are difficult, these types of repetitive low-level mechanical stress may be similar to the types of insults received by certain athletes, such as boxers, and hockey and soccer players (Jordan, 2000; Wennberg and Tator, 2003). Indeed, Webbe and Ochs (2003) reported that soccer players that regularly hit the ball with their heads experience deficits in neurocognitive functioning, especially those players with moderate-to-high heading frequency shortly before testing; similar, though more robust findings, were demonstrated by Matser *et al.* (1998, 1999).

Several studies have indicated that an initial, very mild injury to either cultured cells or the brain itself may provide some protection from a second, more severe insult, a finding that has been termed “preconditioning”. Ischemic preconditioning, in particular, has been well described (for review, see Schaller and Graf, 2002). In the present report, we subjected hippocampal cells to a novel form of mechanical preconditioning, at least to glia. A single 3.4 mm deformation appeared to precondition glial cells when it was administered 24 hr prior to a mild (5.5 mm) injury, as observed with the significant decrease in released S-100 β . Interestingly, there was no concurrent reduction in the release of NSE, again indicating differential responses between neurons and glia. In contrast, Arundine *et al.* (2003) reported an increased vulnerability to glutamate challenge in cultured cortical neurons following sub-lethal mechanical stretch. These cultures, however, contained more than 85% neurons, and the potential role that glia could play in protecting neurons from damage is therefore difficult to assess. Allen *et al.* (2000) studied the effects of preconditioning *in vivo*, and found that the administration of rMTBI before a severe TBI significantly attenuated the motor deficits seen after severe TBI alone. An extensive assessment of cognitive and motor function after injury *in vivo* demonstrated that rMTBI may not serve to protect, or precondition, the brain against further insults, but may instead make it more vulnerable to a second insult, especially if the second injury falls within 24 hr of the first (Laurer *et al.*, 2001). Although the authors state that the effects of a second MTBI may not be additive, but synergistic, the idea that the brain can recover from a first injury, given a sufficient amount of time, is appealing. Our *in vitro* model can provide a reliable system in which to study the mechanisms underlying the preconditioning phenomenon.

Because neurons and glia seem to react differently to injury depending on the injury regimen, this *in vitro* model also represents a possible way to investigate the underlying mechanisms contributing to these observations, which could lead to a greater understanding of rMTBI *in vivo*. Our data pointed towards an important role of S-100 β after injury *in vitro*. For example, S-100 β was dramatically elevated following repeated injuries 1 hr apart, but was not as elevated when injuries were administered 24 hr apart. Also, there was a clear reduction in released S-100 β when cells were preconditioned. Although this protein is released into CSF and serum following TBI, its exact function following TBI is yet unknown, as it has been shown to have both neurotoxic and neuroprotective effects. S-100 β may be released purely as a marker of glial membrane damage, it could have adverse effects on neurons, or it may be actively released in an attempt to repair dying or damaged neurons. For example, S-100 β has been shown to stimulate neurite extension (Reeves *et al.*, 1994). However, exogenous application of S-100 β has also

been shown to stimulate large increases in intracellular free Ca^{2+} in cultured neurons, potentially resulting in Ca^{2+} levels commonly associated with glutamate-mediated excitotoxicity (Barger and Van Eldik, 1992). S-100 β may also be an important mediator of glia-neuronal interactions, as it can influence the induction of hippocampal long-term potentiation (Gerlai *et al.*, 1995; Nishiyama *et al.*, 2002). We have previously reported that exogenous application of S-100 β to uninjured cerebellar cultures, at the same concentration that is released in injured cultures, causes a selective loss of Purkinje neurons (Slemmer *et al.*, 2004a). In hippocampal cultures, we discovered different effects. When S-100 β was added to uninjured hippocampal cultures there was no effect. However, when S-100 β was added to cells immediately after injury, there was an obvious reduction in PrI uptake, suggesting a protective effect. This finding is in agreement with a recent study *in vivo* in which infusion of S-100 β following TBI to rats improved cognitive performance (Kleindienst *et al.*, 2004). Since the majority of PrI staining is apparently derived from glia our results suggest that S-100 β may have a beneficial effect on these cells within a certain concentration range. Also, based on our previous results in cerebellar cultures, these new findings suggest that cells from different brain regions may react differently to various amounts of S-100 β released after trauma. The data presented in the current report indicate that the mechanisms underlying S-100 β release after injury could possibly be manipulated to devise novel treatment strategies for clinical TBI.

CHAPTER 2.3

**Cell death, glial protein alterations and elevated S-100 β release
in cerebellar cell cultures following mechanically induced trauma**

Abstract

Recent studies *in vivo* have shown that cells of the cerebellum, and in particular Purkinje cells (PCs), are susceptible to damage following traumatic brain injury (TBI). In order to investigate more closely the effects of TBI at the cellular level, we subjected cerebellar cell cultures to injury using an *in vitro* model of stretch-induced mechanical trauma, and found increased cell damage and neuronal loss with increasing levels of injury and time post-injury. The release of neuron specific enolase and S-100 β were also elevated after injury. Compared to our previous findings in hippocampal cells, S-100 β levels were much higher in cerebellar cultures after injury, suggesting that cells from different brain regions show variable responses to mechanical trauma. Lastly, the addition of exogenous S-100 β to uninjured cerebellar cells caused no overt change in cell viability or overall neuronal number; there were, however, fewer calbindin-positive PCs, similar to findings after stretch injury.

Introduction

Patients who have suffered from traumatic brain injury (TBI) often complain of cognitive difficulties after injury, even when the damage was shown to be mild or non-existent by CT scans. The experimental study of TBI, both *in vivo* and *in vitro*, has therefore traditionally focused upon cortical and hippocampal regions of the brain, as the majority of human TBI cases report damage in these areas. The cerebellum can also be damaged after TBI, for instance after falls and automobile accidents; this region of the brain, however, has received little attention in evaluating the mechanisms of TBI. Fukuda *et al.* (1996) subjected rats to cerebral injury, and found profound Purkinje cell (PC) loss and microglial activation in the cerebellum by 3-7 days post-injury. The reactive microglia corresponded to the zebrin stripes created by the dendritic trees of PCs. They theorized that the microglia were activated by apoptotic signals derived from dying PCs, and that the zebrin-like stripes were caused by excitotoxic mechanisms leading from climbing fibers and parallel fibers (PFs) to the PCs.

More recent studies have subjected rats to direct cerebellar injury. Although a mild weight drop injury did not alter motor performance as compared to uninjured animals, performance was significantly impaired for up to 20 days after a more severe injury (Allen and Chase, 2001). Similarly, Ai and Baker (2002) found presynaptic hyperexcitability in mossy fibers and PFs in rats after fluid percussion injury, leading to extensive PC death at 7 days post-injury. Like Fukuda *et al.* (1996), they postulated that excitotoxic mechanisms, derived from the extremely high number of synaptic contacts between PFs and PCs, caused the massive PC death.

In order to investigate further the effects of mild and moderate trauma on the cerebellum at the cellular level, we used an *in vitro* model of stretch-induced injury that has been characterized previously in rat cortical neurons and glia (Ellis *et al.*, 1995; McKinney *et al.*, 1996; Weber *et al.*, 1999), in rat septo-hippocampal cultures (Pike *et al.*, 2000) and in mouse hippocampal neurons and glia (Slemmer *et al.*, 2002). This model of *in vitro* injury has been validated by demonstrating that it produces many of the post-traumatic responses observed *in vivo*, such as damage to mitochondria and cytoskeletal elements in astrocytes and neurons (Ahmed *et al.*, 2000; Ellis *et al.*, 1995); increased astrocyte and neuronal membrane permeability (Ellis *et al.*, 1995; McKinney *et al.*, 1996; Weber *et al.*, 1999); activation of phospholipases (Floyd *et al.*, 2001; Lamb *et al.*, 1997; Weber *et al.*, 2001); increased free radical formation (Lamb *et al.*, 1997; McKinney *et al.*, 1996); and decreased levels of cellular ATP (Ahmed *et al.*, 2000). We conducted several experiments that we have previously performed in hippocampal cells (Slemmer *et al.*, 2002) using cerebellar cell

cultures: measurement of cerebellar cell death, and specifically neuronal death, using a live cell viability assay; and the release of two clinical markers of cell damage, neuron specific enolase (NSE) and S-100 β protein, into the culture media after injury. Neuronal (especially PC) and glial death was further investigated by performing immunohistochemistry on injured cells. Lastly, we applied exogenous S-100 β to uninjured cerebellar cells to evaluate cell damage and death without mechanical injury. Our results also point to the high susceptibility of PCs to mechanical and chemical damage.

Methods

Cerebellar cell cultures

Primary cerebellar cell cultures were prepared from E18 wild-type FVB/N mouse embryos as previously described (Slemmer *et al.*, 2002; Stepanova *et al.*, 2003). Briefly, dissociated cerebella were diluted in serum-containing media [Basal Medium Eagles (GIBCO, Grand Island, NY) containing 10% horse serum (GIBCO), 10 μ g/ml gentamicin (Sigma, St. Louis, MO), 0.5% glucose (Sigma), 1 mM sodium pyruvate (GIBCO) and 1% N2 supplements (GIBCO)] to a concentration of 1×10^6 cells per ml; cells were then plated in 1 ml aliquots onto collagen-coated six-well FlexPlates (FlexCell, Hillsborough, NC) or 4-well chambered coverglasses (NalgeNunc, Naperville, IL), coated overnight with poly-L-ornithine (500 μ g/ml; Sigma). All cultures were maintained in a humidified incubator (5% CO₂, 37°C). Neuronally enhanced cultures were obtained by replacing half of the media two days after plating, and then twice per week, with serum-free media containing 2% B27 supplements (GIBCO).

Cell Injury

Cultured cells were injured using a model 94A Cell Injury Controller (Bioengineering Facility, Virginia Commonwealth University, Richmond, VA) as previously described (Ellis *et al.*, 1995; Slemmer *et al.*, 2002). In brief, the Silastic membrane of the FlexPlate well was rapidly and transiently deformed by a 50-ms pulse of compressed nitrogen, which deformed the Silastic membrane and adherent cells to varying degrees controlled by pulse pressure. The extent of cell injury—produced by deforming the Silastic membrane on which the cells are grown—was dependent on the degree of deformation, or stretch. Previously work conducted using rat cortical neurons and glia (Ellis *et al.*, 1995; McKinney *et al.*, 1996; Weber *et al.*, 1999) and mouse hippocampal neurons (Slemmer *et al.*, 2002) have characterized three levels of cell stretch (5.5 mm, 6.5 mm and 7.5 mm deformations), defined as mild, moderate, and severe injury, which result in membrane deformation and biaxial strain or stretch of 31, 38 and 54%, respectively. This range of cell stretch has been shown to be relevant to what would occur in humans after rotational acceleration/deceleration injury (Schreiber *et al.*, 1995). We have previously shown that severe injury (7.5 mm deformation) to hippocampal cells results in a high level of cell death and a large number of neurons detaching from the Silastic membrane (Slemmer *et al.*, 2002); therefore, in the present report, cells were subjected to mild (5.5 mm deformation) or moderate injury (6.5 mm deformation) only. Uninjured control wells were contained in the same FlexPlates as injured wells, and thus underwent the same manipulations, except that they did not receive rapid deformation of the Silastic membrane.

Cell Viability

Cell injury was assessed in cultured cells using the two dyes fluorescein diacetate (FDA; Sigma) and propidium iodide (PrI; Sigma) as previously described (Slemmer *et al.*, 2002). Images were captured using Texas Red and FITC (green) filters on a Leica DMRBE fluorescence microscope, equipped with a Hamamatsu C4880 CCD camera. FDA and PrI images were taken separately, pseudocolored, and overlaid. Cells in five contiguous 100× images were counted and averaged per well. All images were taken from the center portion of the well, as this region was previously shown to receive equal impact from the cell injury controller (Ellis *et al.*, 1995). All PrI and FDA cell counting was performed blind. Data was expressed as the total cell number that stained positively for PrI. In control cultures, PrI staining was low, accounting for less than 5% of total cell number, consistent with previous reports (Zhao *et al.*, 2000; Slemmer *et al.*, 2002). Final results were expressed as percentage of control values. All experiments were completed using at least three separate culture preparations at 13-15 DIV.

NSE and S-100β Assays

Neuron specific enolase (NSE) and S-100β protein levels were measured in culture media (adjusted to 500 μl several hours before injury) at 1 and 24 hr post-injury using lumino-immunometric (LLA-mat) assay kits (Sangtec Medical, Bromma, Sweden) as previously described (Slemmer *et al.*, 2002). Following the assays, the cells adhering to the Silastic membrane were solubilized with 1 ml 0.1% NaOH to determine protein concentration. Total protein count was determined by the bicinchoninic assay (BCA; Pierce, Rockford, IL). Final results are expressed as nanograms of NSE or S-100β released into the culture media per milligram of protein. All experiments were completed using at least three separate culture preparations at 14-16 DIV.

Immunohistochemistry and neuronal cell counts

Cultured cerebellar cells received either a mild (5.5 mm) or a moderate (6.5 mm) injury, or remained uninjured; after either 15 min or 24 hr post-injury, the cultures were washed with PBS and fixed for 20 min with 4% paraformaldehyde (all steps conducted at room temperature). The cultures were permeabilized with 0.2% Triton X-100 for 10 min, and then incubated with blocking solution (BS; 2% BSA, 2% fetal calf serum, 0.2% fish skin gelatin in PBS) for 30 min. Anti-calbindin-D28K (1:900; Sigma), anti-microtubule associated protein 2 (MAP2; 1:300; Sigma), anti-glutamate-aspartate transporter (GLAST; 1:400; Abcam, Cambridge, UK), anti-S-100β (1:300; Sigma) or anti-glial acidic fibrillary protein (GFAP; 1:300; Dakopatts, Glostrup, Denmark) were diluted in 10% BS (in PBS); cultures were incubated in primary antibodies for 1 hr, followed by incubation with secondary antibodies (Alexa 488-conjugated goat-anti-rabbit, 1:300; Alexa-594-conjugated goat-anti-mouse, 1:300; both Molecular Probes, Eugene, OR) diluted in 10% BS for 1 hr. Cultures were dehydrated with 70% followed by 100% ethanol. Images were captured using a Leica DMRBE microscope (see Cell Viability). MAP2-positive neurons were counted in five contiguous fields at a magnification of 160×. Calbindin-D28K-positive PCs were counted in nine contiguous fields at a magnification of 64×.

Exogenous S-100β application

Dissociated cerebellar cells were plated onto 4-well chambered coverglasses. We calculated the average release of S-100β (nanograms per milliliter) after mild injury (1 and 24 hr) and

moderate injury (1 hr). Aliquots (10 μ l) of exogenous S-100 β (350 ng/ml dissolved in Milli-Q water; Sigma) or 10 μ l Milli-Q water (for controls) were added to 1 ml fresh culture media, and the cells were returned to the incubator for 1 or 24 hr. The cultures were then either 1) stained with FDA and PrI and visualized live as described above in Cell Viability or 2) fixed and stained against several primary antibodies (anti-MAP2 and anti-calbindin-D28K) as discussed above in Immunohistochemistry. All experiments were completed using at least two culture preparations at 13-15 DIV.

Data Analysis

The data were analyzed using the statistical program GB Stat (Dynamic Microsystems, Silver Spring, MD). Data were computed as mean $\pm$ standard error of the mean (SEM) values. Statistical significance was established by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test. Data are considered significant at $p < .05$.

Results

Characterization of cell viability in cerebellar cell cultures

Previous studies in our lab (Slemmer *et al.*, 2002) and others (Ellis *et al.*, 1995; Pike *et al.*, 2000) have measured cell viability after *in vitro* stretch injury using the two dyes propidium iodide (PrI) and fluorescein diacetate (FDA). FDA has been shown to stain healthy, viable cells and fluoresces green, while PrI cannot pass through intact cellular membranes. If membranes are damaged, however, cells lose their ability to retain FDA; PrI will then enter cells and stain the nucleus, resulting in a bright red fluorescence. Cultured cerebellar cells were subjected to either mild (5.5 mm) or moderate (6.5 mm) stretch injury, and PrI-positive cells and FDA-positive neurons were counted at both 15 min and 24 hr post-injury. Prototypical images of control (uninjured) cerebellar cells, and cells at 15 min after a moderate (6.5 mm) injury, are shown in Figures 1A and 1B, respectively; color images can be seen in the original article (Slemmer *et al.*, 2004a). There is increased PrI uptake in Figure 1B, as well as a disruption of the glial layer; it is still possible to recognize morphologically distinct neurons, however. Similar to the studies named above, we found that PrI uptake was significantly increased after both degrees of stretch after 15 min (Figure 1C). At 24 hr, however, there was a decrease in PrI uptake in both injury levels; although PrI uptake remained significant after moderate injury, the amount of PrI-positive cells was greatly decreased, and uptake had even returned to control levels after mild injury. This delayed decrease in PrI uptake after both mild and moderate injury could have been due to either injured cells lifting from the Silastic culture membrane, or to cellular (likely glial) repair. In cerebellar cultures, as well as in hippocampal cultures (Slemmer *et al.*, 2002), we saw that the vast majority of PrI staining came from the glial layer.

The number of FDA-positive neurons dropped significantly 24 hr after mild injury, and both 15 min and 24 hr after moderate injury (Figure 1D). The fact that there were fewer neurons labeled with FDA at 24 hr post-injury suggests that either there were more unhealthy neurons that could no longer retain FDA, that many had died, or that the cells lifted away from the Silastic membrane.

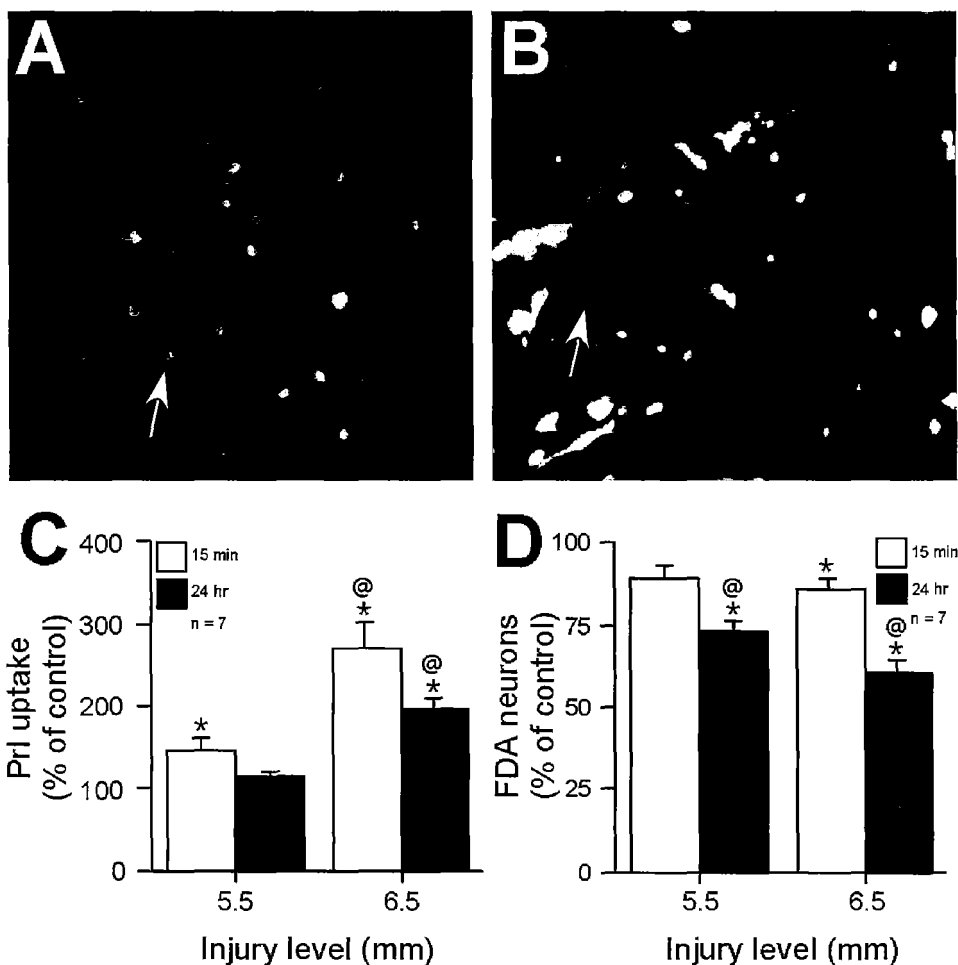


Figure 1. Effect of stretch injury on cell viability of cerebellar cultures. FDA and PrI images were taken separately and overlaid. **A:** Control, uninjured cultures demonstrate retention of FDA and minimal PrI uptake, indicating healthy, viable cells. An arrow indicates a neuron stained with FDA. **B:** Fifteen minutes after a moderate (6.5 mm) injury. There is an increased amount of PrI staining, the majority of which occurred in the underlying monolayer, suggesting that it was glial in origin (arrowhead). Note that the neuronal somata appear intact following injury. Magnification A and B: 100 $\times$. **C:** PrI uptake is positively correlated to the degree of stretch injury. Data is shown at 15 min and 24 hr post-injury. Control values at 15 min and 24 hr were not significantly different. PrI uptake after injury is represented as percent of control values. * $p < .05$ vs. control, @ $p < .05$ vs. 5.5 mm injury, $n = 7$. **D:** Neuronal cell phenotypes in injured cerebellar cultures. Data is shown at 15 min and 24 hr post-injury. Control values at 15 min and 24 hr were not significantly different. Neuronal counts after injury are represented as percent of control values. * $p < .05$ vs. control, @ $p < .05$ vs. 15 min at same injury level, n is same as Figure 1C.

Release of NSE and S-100 β after stretch injury

In order to characterize further the damage caused by mild and moderate stretch injury, we measured the release of two clinical markers of cell damage, neuron specific enolase (NSE) and S-100 β protein into cell culture media. NSE is found in the cytoplasm of neurons, concentrations

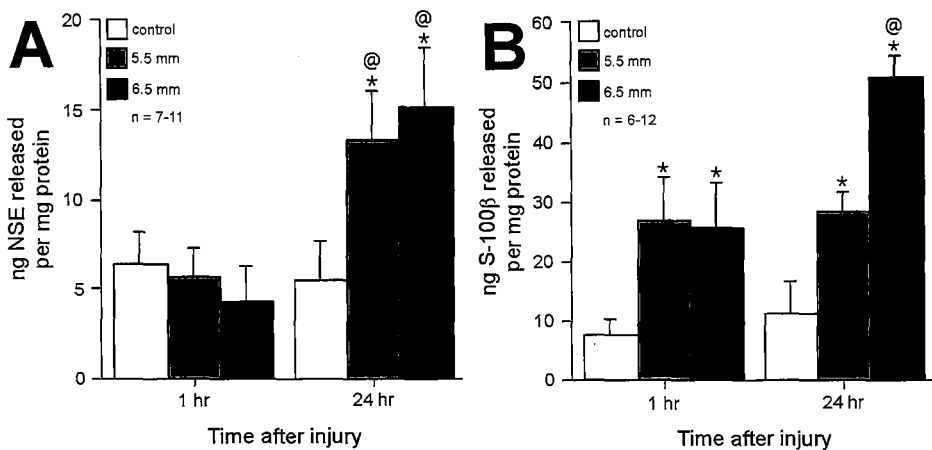


Figure 2. Release of NSE and S-100 β after stretch injury. **A:** NSE release is shown for control wells, and after mild and moderate injury, at 1 and 24 hr post-injury. NSE is represented as nanograms released into the culture media per milligram of protein in the culture well. * $p < .05$ vs. control, @ $p < .05$ vs. same injury level at 1 hr time point, $n = 11$ for control wells, $n = 7-8$ for injured wells. **B:** S-100 β release is shown for control wells, and after mild and moderate injury, at 1 and 24 hr post-injury. S-100 β is represented as nanograms released into the culture media per milligram of protein in the culture well. * $p < .05$ vs. control, @ $p < .05$ vs. 5.5 mm injury at 24 hr, $n = 12$ for control wells, $n = 6$ for injured wells.

of which can be measured in CSF and serum, once it has been released from the cytoplasm by cell damage (Herrmann *et al.*, 2001; Woertgen *et al.*, 2001). The release of NSE into culture media at 1 and 24 hr post-injury is shown in Figure 2A. The control values at these time points were not significantly different from each other and were thus pooled for the purposes of data analysis. There was a significant release of NSE after both mild and moderate injuries at 24 hr, which was also significant compared to the same injury level at 1 hr.

We then turned our attention to the release of S-100 β , a calcium-binding protein found in astrocytes and Schwann cells (Persson *et al.*, 1987; Herrmann *et al.*, 2001). The release of S-100 β at 1 and 24 hr into culture media is depicted in Figure 2B; control values were again pooled for data analysis. In this case, unlike hippocampal glia (Slemmer *et al.*, 2002), the release of S-100 β by cerebellar glia was significantly increased at all time points after both mild and moderate injury, with a most striking increase at 24 hr post-moderate injury. Unlike NSE, S-100 β levels were significantly elevated by 1 hr post-injury.

Neuronal dysfunction and death after injury

We further analyzed the amount of neuronal damage and death after injury by staining cerebellar cultures against anti-MAP2, an antibody specific for the dendrites of all neurons, and against anti-calbindin-D28K, an antibody specific for PCs. Anti-MAP2 and anti-calbindin staining did colocalize in PCs; in our cultures, however, PCs accounted for between 5 and 10% of total neurons, and MAP2 staining in PCs was faint (data not shown). Thus, we assumed that the neuronal counts of MAP2-positive neurons, although likely including PCs, were more indicative of changes in the number of non-PCs. Neuronal counts revealed significant decreases in the number of neurons after both mild and moderate injury at 24 hour post-injury (Figure 3A). The decreasing number of neurons seen in this graph was comparable to that seen in live cell cultures stained with FDA (see Figure 1D). Prototypical images of cerebellar neurons after mild and moderate injury are

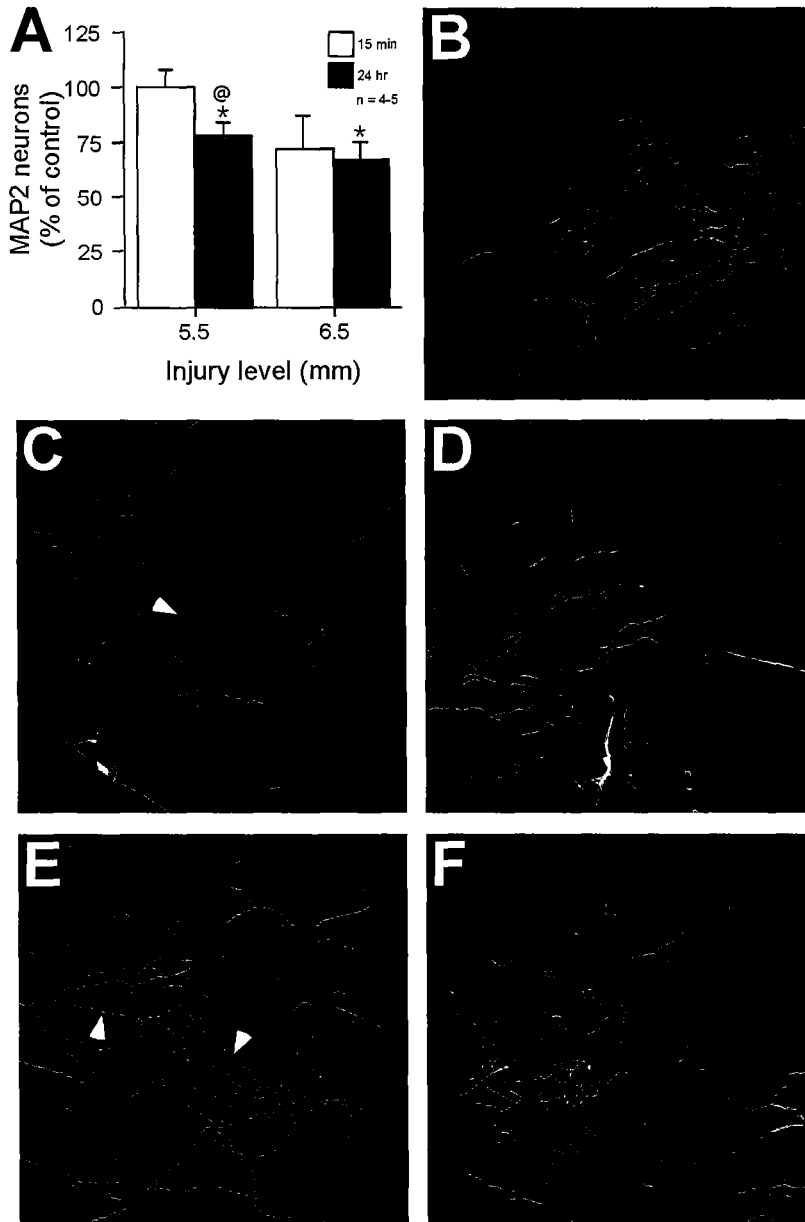


Figure 3. MAP2-positive cerebellar neurons after stretch injury. **A:** The number of cerebellar neurons is shown after mild and moderate injury, at both 15 min and 24 hr post-injury. Control neuron counts at 15 min and 24 hr were not significantly different; data after injury are represented as percent of control values. * $p < .05$ vs. control, @ $p < .05$ vs. 5.5 mm injury at 15 min, $n = 4-5$. **B-F:** Prototypical images of anti-MAP2 neurons and anti-GFAP-positive cerebellar glia; magnification: 100x. **B:** Control well. Uninjured cerebellar neurons have intact soma and smooth dendrites. **C and D:** mild injury (5.5 mm) at 15 min and 24 hr post-injury, respectively. Note that some dendrites have become beaded at 15 min (arrowheads). **E and F:** Moderate injury (6.5 mm) at 15 min and 24 hr post-injury, respectively. There is increased beading (arrowheads) at 15 min after a moderate injury as compared to a mild injury in C. At 24 hr post-injury, in both D and F, the beading has disappeared.

shown in Figures 3B-3F. Control neurons have round soma and smooth dendritic processes; the morphology of these neurons is quite different from that of PCs (see Figure 4B). Fifteen minutes after moderate injury, and to a lesser degree after mild injury, the dendrites are no longer smooth but have become beaded. Note, however, that by 24 hr post-injury, the dendritic beading has disappeared.

The number of PCs stained against anti-calbindin-D28K was significantly reduced after moderate injury, at both 15 min and 24 hr post-injury. (Figure 4A). For purposes of comparison to other neuronal types, a prototypical image of an injured PC is shown in Figure 4B.

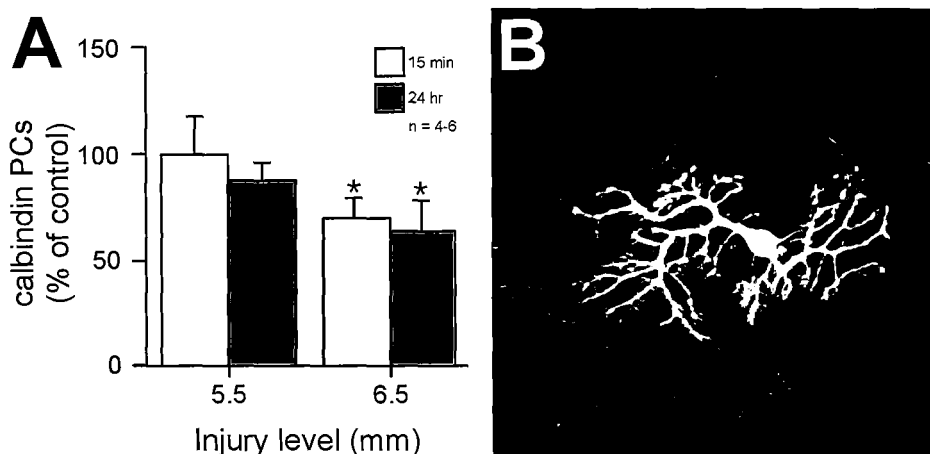


Figure 4. Calbindin-positive Purkinje neurons (PCs) after stretch injury. **A:** The number of PCs is shown after mild and moderate injury, at both 15 min and 24 hr post-injury. Control PC counts at 15 min and 24 hr were not significantly different; data after injury are represented as percent of control values. * $p < .05$ vs. control, $n = 4-6$. **B:** Pseudocolored image of a control PC stained against calbindin-D28K; magnification: 160 $\times$.

Glial alterations in cerebellar cultures after injury

During the completion of the FDA and PrI cell viability experiments, we noted that the gaps on the cell membrane at 15 min post-injury (see Figure 1B) were almost completely absent after 24 hr. We postulated that the filling of these gaps was due to reactive gliosis, similar to that shown previously after stretch injury of septo-hippocampal cultures (Pike *et al.*, 2000). Initial immunohistochemistry on injured cerebellar cells using anti-MAP2 was accompanied by anti-GFAP staining for glia (see Figures 3B-3F). Note that the strong fluorescence seen in uninjured glia is abolished at 15 min post-injury, especially after a moderate injury, and that the fluorescence level has returned almost to normal at 24 hr after injury. We then completed additional experiments using an antibody against the glutamate-aspartate transporter (GLAST) that is highly specific for Bergmann glia (BG). These glia are vital in the developing cerebellum, where they act to transport nascent PCs to the Purkinje cell layer; they later ensheath mature PCs (for review, see Yamada and Watanabe, 2002). By costaining cerebellar cultures with anti-GLAST and anti-S-100 β , we were able to identify that most of the S-100 β -positive glia were in fact BG (Figure 5B), similar to previous findings that showed that BG stain strongly for S-100 β (Lossi *et al.*, 1995). Pseudocolored images, which clearly show changes in antibody staining, can be seen in the original article (Slemmer *et al.*, 2004a). At 15 min post-injury, there was a decrease in the fluorescence intensity of both S-100 β and GLAST; although the fluorescence level of S-100 β

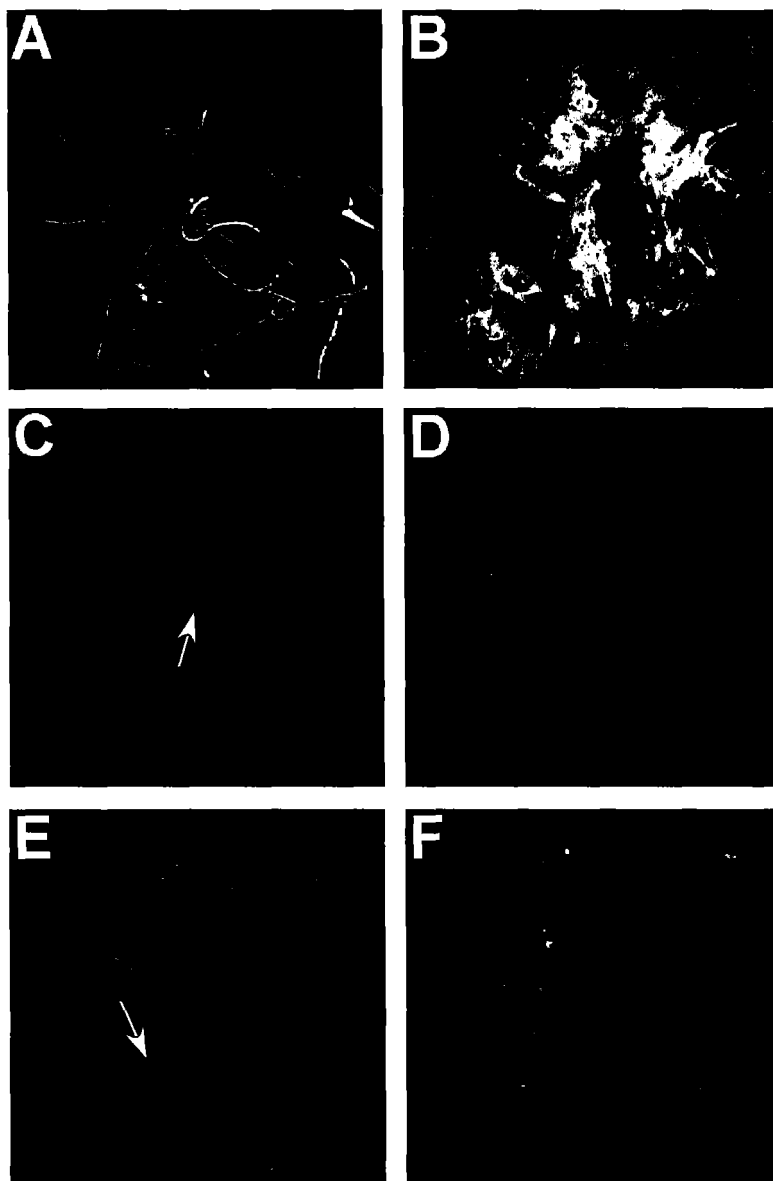


Figure 5. Colocalization of glial markers in cerebellar cultures. **A:** The co-localization of anti-GFAP- and anti-S-100 β -positive glia is depicted here for comparison purposes. **B-F:** The colocalization of anti-GLAST (Bergmann glia) and anti-S-100 β -positive glia is shown in control cultures, and after mild and moderate injury, at both 15 min and 24 hr post-injury. **B:** Control well; note that only glia are visible using two glia-specific antibodies. There is a high level of colocalization between GLAST and S-100 β antibody staining, as compared to colocalization in **A**, indicating that most S-100 β -releasing glia are Bergmann glia. **C:** At 15 min after a mild injury, there is a marked reduction in fluorescence, as well as gaps in the cell membrane (arrow). **D:** At 24 hr after a mild injury, there are no gaps on the membrane, likely due to reactive gliosis after injury. **E:** At 15 min after a moderate injury, there is also reduced antibody fluorescence, as well as large gaps on the Silastic membrane (arrow). **F:** At 24 hr after a moderate injury, similar to that seen at 24 hr after a mild injury, the gaps on the membrane have again filled by reactive gliosis. Anti-GLAST staining, however, is disturbed. Magnification of all pictures: 160 $\times$.

appears to have partially recovered by 24 hr, the level of GLAST remains markedly decreased (Figures 5C-5F). Note that the colocalization of anti-GFAP and anti-S-100 β (uninjured control cells shown) was less specific (Figure 5A).

Effects of exogenous S-100 β application to cerebellar cells

Considerable controversy surrounds the dual ability of S-100 β to have both beneficial and detrimental effects. For example, S-100 β has been shown to be neurodegenerative in conditions such as Alzheimer's disease and Down's syndrome (Whitaker-Azmitia *et al.*, 1997; Sheng *et al.*, 2000), and yet neuroprotective against glutamate and staurosporine-induced damage (Ahlemeyer *et al.*, 2000) as well as glucose deprivation (Barger *et al.*, 1995). We hypothesized that the application of exogenous S-100 β to control cultures (without concurrent mechanical injury) would elucidate its effect on cerebellar cell survival. We calculated the average release of S-100 β

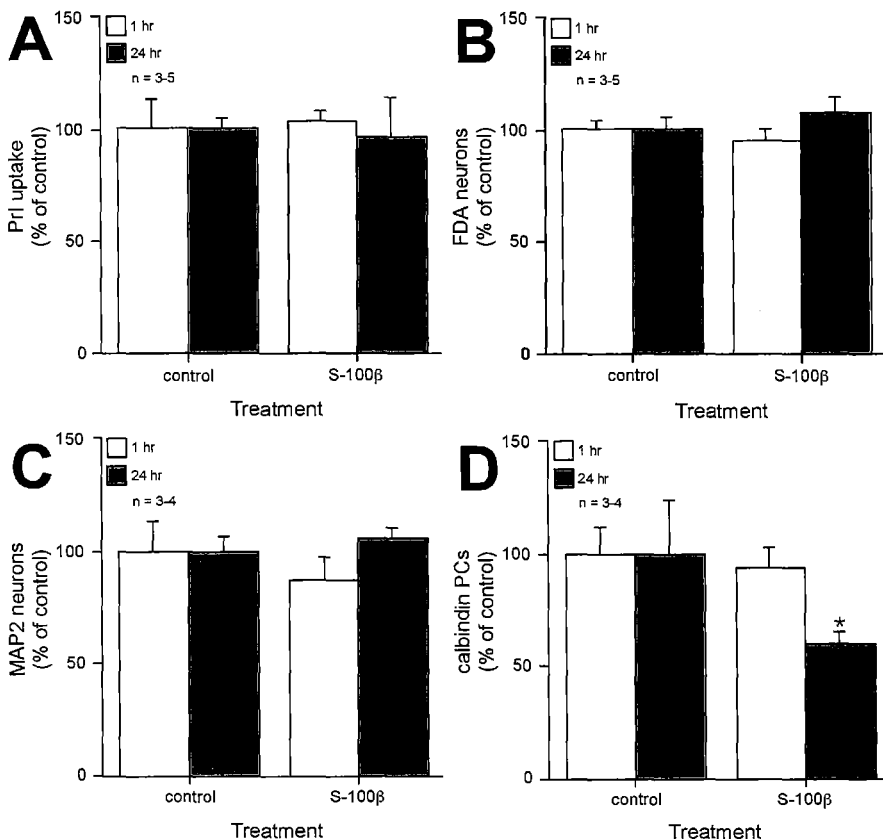


Figure 6. Exogenous S-100 β application in cerebellar cultures results only in delayed PC death. All data is shown at 1 and 24 hr post-application, and is expressed as percentage of control. **A:** There is no change in PrI uptake after S-100 β application (see Figure 1C for comparison). $n = 3-5$. **B:** There is no change in the number of FDA-positive neurons after S-100 β application (see Figure 1D for comparison). n is same as Figure 6A. **C:** There is no change in the number of MAP2-positive cerebellar neurons after S-100 β application (see Figure 3A for comparison). $n = 3-4$. **D:** The number of calbindin-positive PCs remains unchanged at 1 hr post-application, but is significantly decreased at 24 hr, indicating a delayed loss of PCs (see Figure 4A for comparison). * $p < .05$ vs. control, n is same as Figure 6C.

after injury by our cerebellar glia to be approximately 25 ng per mg cellular protein (or 3.5 ng/ml) and added this concentration of S-100 β to control cultures. Cell damage and death were evaluated at 1 and 24 hr post-application by: 1) PrI and FDA uptake in live cell cultures (Figures 6A and 6B, respectively), and 2) immunohistochemistry and cell counting of MAP2-positive neurons and calbindin-D28K-positive PCs in fixed cells (Figures 6C and 6D, respectively). The data in Figure 6 were thus comparable to data collected after stretch injury in cerebellar cells. Our data in Figure 1C and 1D indicated that increasing levels of stretch injury caused increasing amounts of PrI uptake, as well as a decrease in the number of FDA-retaining neurons, respectively. In control cultures, without stretch injury, however, the application of exogenous S-100 β caused neither significant overall cell damage or death (indicated by PrI uptake; Figure 6A), nor a significant reduction in the number of FDA-positive neurons (Figure 6B), nor a significant decrease in the number of neurons stained against anti-MAP2 (Figure 6C). Surprisingly, however, the number of calbindin-positive PCs was significantly reduced 24 hr after exogenous S-100 β application (Figure 6D).

Discussion

Although the vast majority of experimental TBI research focuses upon its effects on the cerebral cortex and the hippocampus, recent reports have demonstrated that the cells of the cerebellum, and particularly PCs, are susceptible to damage (Fukuda *et al.*, 1996; Ai and Baker, 2002). In the present report, we demonstrate the effects of stretch injury *in vitro* on cultured mouse cerebellar cells.

Increased magnitude of mechanical stretch injury causes increased cell damage and death

Following moderate stretch injury, PrI uptake was dramatically increased versus a mild injury. Although there was a higher amount of neuronal loss after moderate as compared to mild injury, this difference was much less evident than that of PrI uptake between the two injury levels. In addition, NSE release was similar after both mild and moderate injury. These results suggest that PrI staining primarily reflected glial damage. This finding is consistent with the fact that most of the PrI staining appeared to be in the underlying glial monolayer, similar to our previous results in hippocampal cell cultures (Slemmer *et al.*, 2002). However, PCs in particular seemed to be more susceptible to moderate as compared to mild injury.

S-100 β is a reliable marker of cerebellar cell damage

Although clinical data differ on the predictive value of NSE and S-100 β in either CSF or serum of TBI patients as markers of brain damage (for review, see Rothermundt *et al.*, 2003), our previous work using cultured hippocampal cells (Slemmer *et al.*, 2002) demonstrated that both proteins were efficient indicators of cell damage *in vitro*. The increase in PrI uptake and the significant reductions in FDA-positive neurons led us to hypothesize that NSE and S-100 β levels should also be increased after injury. We found that NSE was elevated at 24 hr post-injury while S-100 β was elevated by 1 hr post-injury. The fact that there was no change in the level of NSE release at 1 hr after injury was a further indication of delayed neuronal death. Although NSE levels were significantly elevated in our cultures at 24 hr post-injury, our data indicate that S-100 β may be a better indicator of cerebellar damage than NSE, especially if the damage is assessed before this 24 hr time point. Similarly, Pleines *et al.* (2001) found that, after clinical TBI, the level of S-100 β

is a better indicator of damage, while NSE better indicates the degree of inflammation. Also, the relative amount of S-100 β released by cerebellar glia was several-fold higher than what we have previously reported for injured hippocampal glia (Slemmer *et al.*, 2002). This is similar to a report by Pinto *et al.* (2000) in which serum-deprived astrocyte cultures from rat cerebellum released much higher concentrations of S-100 β than either hippocampal or cortical astrocyte cultures. These data suggest that the S-100 β measured in CSF or serum following TBI in patients may be derived to a greater extent from the cerebellum than from other brain regions. All things considered, S-100 β appeared to be an excellent indicator of injury in cerebellar cultures. We felt that a further investigation into cellular damage at both the neuronal and the glial layers was merited.

S-100 β and Bergmann glia

It is possible that the high S-100 β release is caused by the cerebellum-specific BG. During development, the release of S-100 β by glia has been shown to initiate neurite outgrowth (Azmitia *et al.*, 1990). Because BG play such a critical role in cerebellar development, they may already have higher levels of S-100 β . Further investigation here is warranted. Figures 5C-5F depict the colocalization of anti-GLAST and anti-S-100 β after mild or moderate injury, at 15 min and 24 hr post-injury. Again, similar to that seen in anti-GFAP-stained glia, there is a marked decrease in the fluorescence levels of both anti-GLAST and anti-S-100 β after injury. Gaps in the cell membrane can be seen at 15 min post-injury at both injury levels (Figures 5C and 5E). At 24 hr after a mild injury, the gaps have been sufficiently filled by reactive gliosis (as shown by anti-GFAP staining in Figure 3), suggesting that the surviving glia have replicated; also, anti-S-100 β , but not anti-GLAST fluorescence, has returned to control levels. Anti-GLAST fluorescence has increased as compared to 15 min post-injury, however. The fact that GLAST expression has not completely recovered could indicate that the transporter itself has been downregulated after injury or that the epitope for the antibody has somehow been altered by the mechanical insult.

Secondary damage to cerebellar neurons

The uptake of PrI by 15 min after injury suggests that the membrane disruption occurred as a direct result of mechanical damage caused by stretch injury alone. The delayed neuronal loss and the release of NSE, however, also suggested that secondary mechanisms might contribute to neuronal damage and death. One possible contributor to secondary damage is S-100 β protein. S-100 β acts as a microtubule disruptor (for review, see Donato, 1991), and thus an instigator of neuritic outgrowth, a necessary element in developing neurons (Azmitia *et al.*, 1990). It has also been shown to be involved in neurodegeneration in Alzheimer's disease and Down's syndrome (Whitaker-Azmitia *et al.*, 1997; Sheng *et al.*, 2000), and yet neuroprotective against excitotoxic damage and metabolic insults (Ahlmeyer *et al.*, 2000; Barger *et al.*, 1995). Neuronal counts of MAP2-positive neurons were not decreased after exogenous S-100 β application, indicating that these neurons were able to withstand the destabilizing effects of S-100 β . PCs, however, were susceptible to exogenous S-100 β ; it is possible that there was some direct form of S-100 β -mediated toxicity that caused PC death or that the PCs retracted their neuritic processes and lifted from the substrate. Consistent with this latter hypothesis, early studies conducted by Ramón y Cajal on traumatically-injured cats showed that PCs developed highly retracted dendritic trees as early as 2 days post-injury (Cajal, 1933, but also see Azmitia *et al.*, 2002). It is also possible that excess S-100 β could alter the composition or synthesis of calbindin in such a way to make it unrecognizable to the calbindin antibody, similar to what may have happened to GLAST after

injury. In light of this statement, however, we did note that all PCs that did stain for calbindin (in both injured and control conditions) exhibited equal levels of calbindin fluorescence (data not shown).

Stretch injury has been shown to cause the release of glutamate from astrocytes using this *in vitro* model (Ahmed *et al.*, 2002). Therefore, another secondary mechanism that could contribute to cerebellar neuronal death is glutamate excitotoxicity (for review, see Choi, 1996). For example, we describe beading of neurites at 15 min post-injury in MAP2-positive neurons (Figure 4). We also made this observation in hippocampal neurons that had received a moderate level of injury or repeated mild injuries (Slemmer *et al.*, 2002). Although it is possible that this beading could be due to mechanical damage caused by the injury model itself, it is unlikely for two reasons. One, dendritic beading, sometimes referred to as blebbing, has been frequently shown after *in vivo* injury in animals in areas that were not directly affected by the impact (Folkerts *et al.*, 1998); and two, we have observed beading in both cerebellar and hippocampal cells derived from transgenic mice expressing enhanced yellow fluorescent protein (EYFP; Metzger *et al.*, 2002) after bath application of glutamate (Slemmer *et al.*, 2004b). Zhao *et al.* (2000) also reported beading in septo-hippocampal cell cultures treated with glutamate. What is interesting is that this phenomenon seemed to be reversible in MAP2-positive cerebellar neurons; however, the underlying mechanisms need to be further elucidated.

PCs have been shown to be particularly vulnerable to glutamate excitotoxicity (O'Hearn and Molliver, 1997). In two studies that evaluated PC loss after either a cerebral or a cerebellar injury, both groups hypothesized that the massive PC death was due to excitotoxic stimulation of PC dendrites at the PF-PC synapses (Fukuda *et al.*, 1996; Ai and Baker, 2002). This excitotoxicity may be related to the cerebellar BG. For example, Watase *et al.* (1998) subjected GLAST mutant mice to cold-induced cerebellar injury. They postulated that the significantly larger edema in these mice was caused by the inability of the mutant BG to reuptake glutamate from the extracellular space. If GLAST is critical in preventing excitotoxic cerebellar damage after injury, the reduction we see in GLAST fluorescence after stretch injury could be indicative of faulty glutamate transport, causing excitotoxic levels of glutamate to accumulate in the extracellular space. Therefore, any neuronal death not caused by the mechanical injury itself could be caused by glutamate excitotoxicity. Because PCs lack NMDA receptors, these cells would undergo some form of non-NMDA mediated excitotoxicity. These possibilities need to be further addressed in cerebellar cultures *in vitro*.

CHAPTER 2.4

Glutamate-induced elevations in intracellular chloride concentration in dissociated hippocampal cultures

Abstract

The homeostasis of intracellular Cl^- concentration ($[\text{Cl}^-]_i$) is critical for neuronal function, including γ -aminobutyric acid (GABA)ergic synaptic transmission. Here, we investigated activity-dependent changes in $[\text{Cl}^-]_i$ using a transgenetically expressed Cl^- -sensitive enhanced yellow-fluorescent protein (EYFP) in cultures of mouse hippocampal neurons. Application of glutamate (100 μM for 3 min) in a bath perfusion to cell cultures of various days *in vitro* (DIV) revealed a decrease in EYFP fluorescence. The EYFP signal increased in amplitude with increasing DIV, reaching a maximal response after 7 DIV. Glutamate application resulted in a slight neuronal acidification. Although EYFP fluorescence is sensitive to pH, EYFP signals were virtually abolished in Cl^- -free solution, demonstrating that the EYFP signal represented an increase in $[\text{Cl}^-]_i$. Similar to glutamate, a rise in $[\text{Cl}^-]_i$ was also induced by specific ionotropic glutamate receptor agonists and by increasing extracellular $[\text{K}^+]$, indicating that an increase in driving force for Cl^- suffices to increase $[\text{Cl}^-]_i$. To elucidate the membrane mechanisms mediating the Cl^- influx, a series of blockers of ion channels and transporters were tested. The glutamate-induced increase in $[\text{Cl}^-]_i$ was resistant to furosemide, bumetanide and 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS), was reduced by bicuculline to about 80% of control responses, and was antagonized by niflumic acid (NFA) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB). We conclude that membrane depolarization increases $[\text{Cl}^-]_i$ via several pathways involving NFA- and NPPB-sensitive anion channels and GABA_A receptors, but not through furosemide-, bumetanide-, or DIDS-sensitive Cl^- transporters. The present study highlights the vulnerability of $[\text{Cl}^-]_i$ homeostasis after membrane depolarization in neurons.

Introduction

The homeostasis of intracellular Cl^- concentration ($[\text{Cl}^-]_i$) is a critical determinant of the function of γ -aminobutyric acid (GABA)ergic synaptic transmission in the mammalian brain. Neuronal $[\text{Cl}^-]_i$ has been found to be higher during development than in adult brain; therefore, in immature cells, GABAergic synaptic transmission is depolarizing (Rivera *et al.*, 1999; Ganguly *et al.*, 2001). In mature neurons, however, a switch can occur in GABAergic activity from inhibitory to depolarizing actions during sustained activation of GABA_A receptors (Barker and Ransom, 1978; Alger and Nicoll, 1979) due to an accumulation of Cl^- and a collapse of the Cl^- gradient. Importantly, alterations in Cl^- -homeostasis have been implicated in several pathophysiological processes, such as ischemia, excitotoxicity and mechanical trauma (van den Pol *et al.*, 1996; Inglefield and Schwartz-Bloom, 1998a; Schwartz-Bloom and Sah, 2001; Rothman, 1985; Grondahl *et al.*, 1998).

Despite the importance of $[\text{Cl}^-]_i$, direct measurements of $[\text{Cl}^-]_i$ in neurons have been sparse and mainly limited by available experimental approaches. For example, Cl^- -sensitive electrodes (Neild and Thomas, 1973) can only be used in large cells and offer limited selectivity, whereas optical methods offer the potential for spatially-resolved measurements of $[\text{Cl}^-]_i$ but are limited by adequate Cl^- indicator dyes (Schwartz-Bloom, 1999). The discovery that some yellow variants of *Aequorea* fluorescent protein exhibit a Cl^- -sensitive fluorescence has provided a new class of highly biocompatible Cl^- -sensitive dyes (Wachter and Remington, 1999; Kuner and Augustine, 2000; Jayaraman *et al.*, 2000; Galletta *et al.*, 2001). Genetically encodable fluorescent probes for $[\text{Cl}^-]_i$ overcome several of the limitations suffered by organic dyes such as phototoxicity and invasive staining methods (Kuner and Augustine, 2000). Recently, our lab has created transgenic mice expressing a Cl^- -sensitive EYFP under the control of a voltage-gated potassium channel

promoter, pKv3.1 (Metzger *et al.*, 2002). In our initial characterization of these mice, we found that depolarization, caused by increased extracellular K^+ concentration ($[K^+]_o$) or glutamate application, decreased EYFP fluorescence in cerebellar granule cells. The interpretation of these signals were confounded, however, by the fact that EYFP fluorescence is not only an effective reporter of changes in $[Cl^-]_i$ (Galiotta *et al.*, 2001) but is also sensitive to changes in pH (Elslinger *et al.*, 1999). Indeed, cerebellar granule cells showed a significant acidification during glutamate application (Metzger *et al.*, 2002). In the present report, we readdress this issue using cultured hippocampal neurons derived from EYFP-expressing mice. Cultured cells permit a faster and more-controlled change of the composition of the external solution; they also display much less tissue auto-fluorescence than acute brain slices. Cultured EYFP-expressing neurons exhibited large single-cell fluorescence levels, and we demonstrate that $[Cl^-]_i$ changes dramatically after bath application of glutamate or high K^+ .

Materials and Methods

Cell cultures

Primary hippocampal cell cultures were prepared from E17-18 mouse embryos from either wild-type ICR mice or transgenic mice expressing EYFP in hippocampal neurons (line #27, Metzger *et al.*, 2002), as previously described (Slemmer *et al.*, 2002). Briefly, pregnant female mice were killed by rapid cervical dislocation, and the embryos were surgically removed. The hippocampi were dissected out, and were treated with 0.1% Trypsin-EDTA (Gibco, Grand Island, NY) for 13-15 min at 37°C. Dissociated hippocampal cells were diluted in serum-containing media [Basal Medium Eagles (Gibco) containing 10% horse serum, 10 μ g/ml gentamicin (Sigma), 0.5% glucose, 1 mM sodium pyruvate (Gibco) and 1% N2 supplements (Gibco)] to a concentration of 500,000 cells per ml. The cells were plated in 500- μ l aliquots onto 9- mm-square glass coverslips in 24-well plates coated overnight with poly-L-ornithine (500 μ g/ml; Sigma). Cultures were maintained in a humidified incubator (5% CO_2 , 37°C). Neuronally enhanced cultures were obtained by replacing half of the media two days after plating, and then twice per week, with serum-free culture media containing 2% B27 supplements (Gibco).

Drugs

Stock solutions were prepared in H_2O , and final concentrations were prepared in artificial cerebral spinal fluid (ACSF): glutamate (Sigma); glycine (Sigma); *N*-methyl-D-aspartate (NMDA; Tocris Cookson, Bristol, UK); α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA; Tocris); dihydroxyphenylglycine (DHPG; Tocris); DL-2-amino-5-phosphonopentanoic acid (D-APV; Tocris); 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; Tocris); bicuculline methochloride (Tocris); and 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS; Sigma). Furosemide (100 μ M; Sigma) and bumetanide (100 μ M; Sigma) were prepared directly in ACSF. Solution with elevated $[K^+]_o$ (30 and 90 mM) was prepared by mixing ACSF with a 145 mM KCl stock solution. Stock solutions of 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; Sigma) were dissolved in approximately equal portions of water and NaOH, and final concentrations (50 and 200 μ M) were prepared in ACSF. Niflumic acid (NFA; Sigma) was prepared using dimethyl sulfoxide (DMSO; final DMSO concentration 0.5% v/v) as an initial solvent, and the final concentration (100 μ M) was prepared in ACSF. Nigericin (Sigma) was first dissolved in ethanol, and the final concentration (10 μ M) was prepared directly in PIPES-

buffered salt solution (PIPES-BS; see below). Tributyltin chloride (10 μ M; Sigma) was added directly to PIPES-BS.

Imaging and drug application

Coverslips were placed in a bath chamber and perfused with ACSF (in mM: NaCl, 118; KCl, 3; MgCl₂, 1; CaCl₂, 1.5; NaH₂PO₄, 1; NaHCO₃, 25; D-glucose, 10; gassed with 95% O₂/5% CO₂; pH 7.4). For Cl⁻-free solutions, all Cl⁻ salts were substituted by corresponding gluconate salts. Cultured cells [between 2 and 17 days *in vitro* (DIV)] were viewed through a 40x water immersion objective using a cooled CCD camera (Sensicam, PCO, Germany) at 23–27°C. EYFP fluorescence was measured in the somatic region of neurons at 500 nm excitation and 530 $\pm$ 15 nm emission wavelengths. After an initial baseline period, glutamate (100 μ M, supplemented with 10 μ M glycine in order to saturate the glycine-binding site of NMDA receptors) was applied for 3 min, and was allowed to wash out until fluorescence levels returned to baseline. The application of 10 μ M glycine alone caused no change in fluorescence levels. In all subsequent descriptions, glutamate application will imply the addition of glycine. In experiments in which other drugs were used, drugs were applied following washout of glutamate, and were also allowed to wash out fully. Finally, glutamate was reapplied in order to measure the recovery of the cells. The initial response to glutamate was considered as the control glutamate response in each cell. Data in bar graphs are presented as a percentage of this control response. It should be noted that NPPB, at its final concentration, created a yellow solution, which produced a shift in fluorescence levels. Therefore, the fluorescence level in the presence of NPPB alone was used as the baseline for this set of experiments (see Figure 5). Images were captured using Axon Workbench software (Axon Instruments, Union City, CA), and analyzed using Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD). Statistical significance was established using Student's t-test, and confirmed with ANOVA where appropriate; *n* refers to the number of cultures used in each experiment.

pH measurements

Experiments with the pH indicator SNARF-1 (Molecular Probes, Eugene, OR) were performed in hippocampal cultures prepared from wild-type ICR mice. Cultured cells (between 13 and 17 DIV) were loaded for 30 minutes with 4 μ M SNARF dissolved in HEPES-based saline (HBS; in mM: NaCl, 150; KCl, 4; MgCl₂, 1; D-glucose, 5; CaCl₂, 2; HEPES, 5; pH 7.4) at 37°C (using DMSO as an initial solvent at a final concentration of 0.2% v/v), and viewed through a 40x water immersion objective lens using a cooled CCD camera (PCO) at 23–25°C. Calibration of the SNARF signals was employed by changing the bath solution to a 25 mM HEPES-buffered calibration solution (pH 7 or pH 8) containing the H⁺/K⁺ ionophore, nigericin (10 μ M; Sigma), and (in mM) NaCl 10; KCl, 115; MgCl₂, 1; D-glucose, 5 (Negulescu and Machen, 1990). Glutamate (100 μ M) was bath-applied for 3 min in ACSF as described above. SNARF fluorescence was excited at 488 nm and imaged at 524 nm wavelengths. Images were captured using TILLvisION software (T.I.L.L. Photonics, GmbH, Germany), and analyzed using Image-Pro Plus software.

pH and Cl⁻ sensitivity of EYFP

EYFP fluorescence changes were measured in PIPES-BS buffers (containing 0 or 80 mM Cl⁻, at either pH 7.0 or 7.2) in the presence of nigericin (10 μ M) and the Cl⁻ ionophore, tributyltin chloride (10 μ M), by mixing 126 mM Cl⁻-containing PIPES-BS (in mM: NaCl, 10; KCl, 115; MgCl₂, 1;

D-glucose, 5; supplemented with 25 mM PIPES) at either pH 7.0 or 7.2, with Cl⁻-free PIPES-BS (by substitution of the corresponding gluconate salts) at either pH 7.0 or pH 7.2. Images were captured using Axon Workbench software and analyzed using Image-Pro Plus software.

Results

Expression of EYFP in hippocampal cell cultures at different DIV

The soma and processes of cultured hippocampal neurons displayed a bright, EYFP fluorescence as early as 2 DIV (Figure 1A). At later stages of culturing (e.g. 10 DIV, Figure 1B), pyramidal-shaped neurons, as well as multipolar cells can be, at least tentatively, identified as principal cells and interneurons consistent with the expression pattern observed in intact tissue from the EYFP transgenic mice (Metzger *et al.*, 2002). Single cells were sufficiently bright for the monitoring of local changes in fluorescence for an extended period of time with little bleaching. Figures 1C and D portray fluorescent signals induced by glutamate (100 μ M) in the soma of cultured neurons at 12 DIV. Several cells are depicted in the fluorescence image (Figure 1C) and all cells responded with a marked decrease in fluorescence upon glutamate application (Figure 1D). To assess the developmental profile of glutamate-induced changes in EYFP fluorescence, neurons of various DIV were subjected to bath-applied glutamate in ACSF. Glutamate application induced a decrease in EYFP fluorescence in neurons at 3-17 DIV (Figure 1E). Analysis of the data revealed that the response to glutamate increased between 3 and 7 DIV, and that glutamate-induced fluorescence changes were significantly larger between 7 and 8 DIV ($n > 5$ independent experiments for each DIV; $p < 0.001$). Maximal responses were obtained after 7 DIV, consistent with the developmental profile of trans-membrane Cl⁻ gradients in hippocampal neurons (Rivera *et al.*, 1999; Ganguly *et al.*, 2001). Thus, we used cultures at 8-12 DIV in all subsequent experiments.

Glutamate-induced changes in EYFP fluorescence represent increases in [Cl⁻]_i

EYFP fluorescence is dependent on [Cl⁻]_i and [pH]_i (Kneen *et al.*, 1998; Kuner and Augustine, 2000; Jayaraman *et al.*, 2000; Elsiger *et al.*, 1999; Galletta *et al.*, 2001). Both [Cl⁻]_i and [pH]_i are modulated when neurons are excited by glutamate (Metzger *et al.*, 2002; Wang *et al.*, 1994; Schwartz-Bloom, 1999). Furthermore, [Cl⁻]_i and [pH]_i are physiologically coupled to each other (Ahmed and Connor, 1980). To investigate whether the decrease in EYFP fluorescence was caused predominantly by an decrease in [Cl⁻]_i or by an intracellular acidification, responses to glutamate were recorded first in control and then in Cl⁻-free solutions. The removal of Cl⁻ virtually abolished the EYFP responses to glutamate application (to $1.92 \pm 1.63\%$ of controls; $n = 12$; Figure 2A). Cultured hippocampal neurons, however, are known to respond with an acidification to depolarization and/or to glutamate (Wang *et al.*, 1994). To investigate the amount of acidification caused by glutamate application, we loaded cultures prepared from non-transgenic mice with the pH indicator SNARF-1. As illustrated in Figure 2B, glutamate application induced an acidification of approximately 0.2 pH units (0.196 ± 0.013 pH units; $n = 5$). From the pH sensitivity of EYFP ($pK_a = 6.1$ at 15 mM Cl⁻; Metzger *et al.*, 2002), a shift of intracellular pH from 7.2 to 7.0 translates into less than 5% change in fluorescence. Therefore, the small acidification observed during application of glutamate does not account for the large change in EYFP fluorescence. As an additional control and for calibration of the EYFP signals, we bathed cells in a calibration solution containing 115 mM K⁺ and ionophores to equilibrate extracellular and intracellular ion concentrations. Variation of [Cl⁻] from 0 mM to 80 mM (at pH

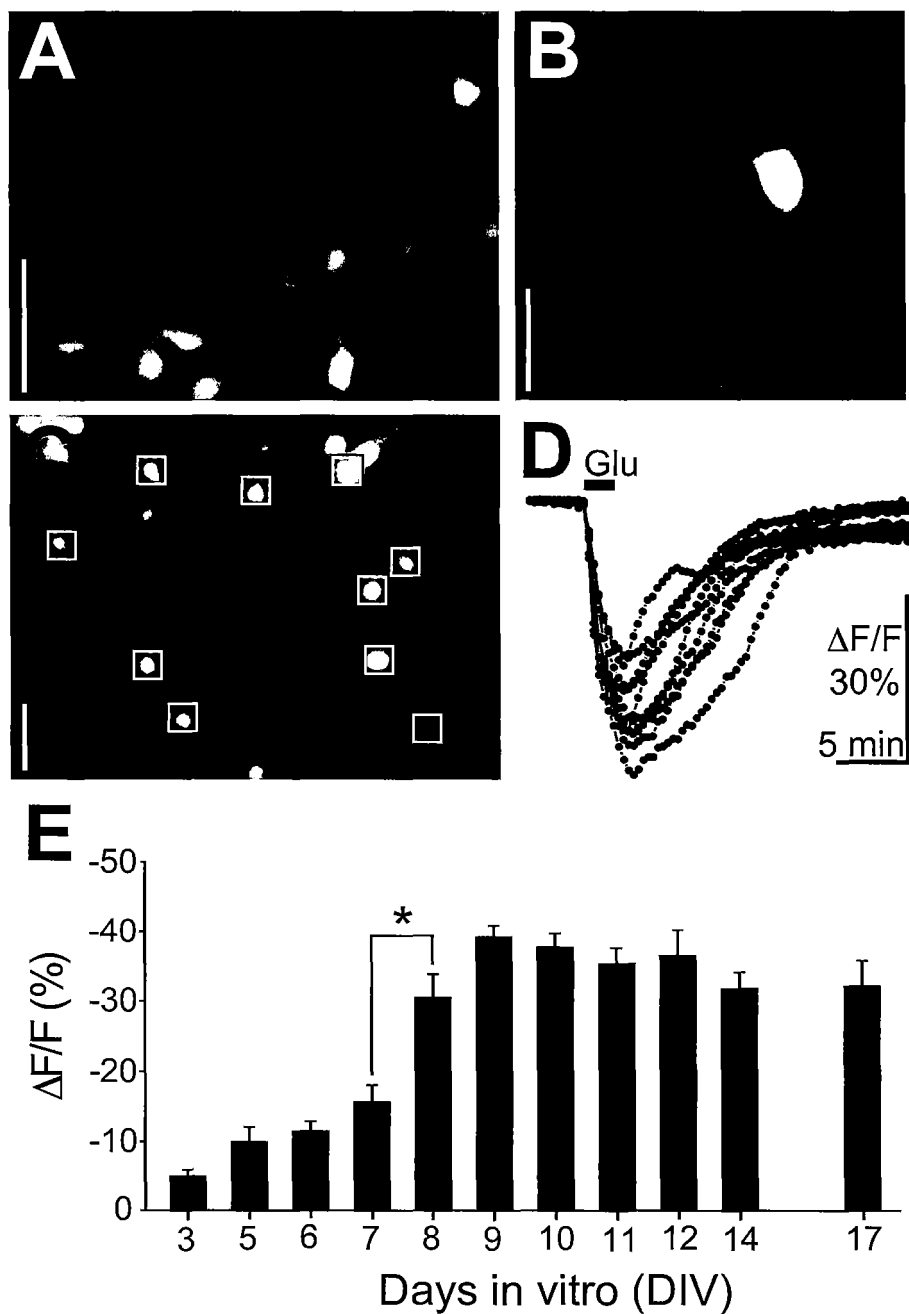


Figure 1. Expression and response to glutamate application of EYFP in hippocampal cell cultures at different days *in vitro* (DIV). A–C: Fluorescence images from hippocampal cultures at 2 days DIV (A), 10 DIV (B) and 12 DIV (C). Note that the neuronal soma display a bright fluorescence whereas glial cells are not fluorescent. Scale bars in A–C represent 50 μ m. D: Fluorescence signals induced by application of glutamate (Glu; 100 μ M) from regions of interest shown in C. E: Summary of responses to glutamate (100 μ M) in neurons from 3 to 17 DIV. Data are shown as mean values $\pm$ SEM. * $p < 0.001$; $n > 5$ independent experiments for each DIV.

7.2) decreased EYFP fluorescence by $21.4 \pm 2.2\%$ ($n = 8$) while changing pH from 7.2 to 7.0 had almost no effect on EYFP fluorescence ($-0.48 \pm 0.88\%$; $n = 6$; Figure 2C). The sensitivity of EYFP to Cl^- increases with a decrease in pH (Jayaraman *et al.*, 2000; Kuner and Augustine, 2000) and therefore, we also varied Cl^- along with pH. A combined 80 mM Cl^- and 0.2 pH units step decreased EYFP fluorescence by $28.0 \pm 3.3\%$ ($n = 5$). The size of this latter response is close to that induced by glutamate. Taken together, the control experiments using SNARF, and the lack of responses to glutamate in Cl^- -free solution, demonstrate that the glutamate-induced decrease in EYFP fluorescence represents an increase in $[\text{Cl}^-]_i$ amounting to roughly 80 mM.

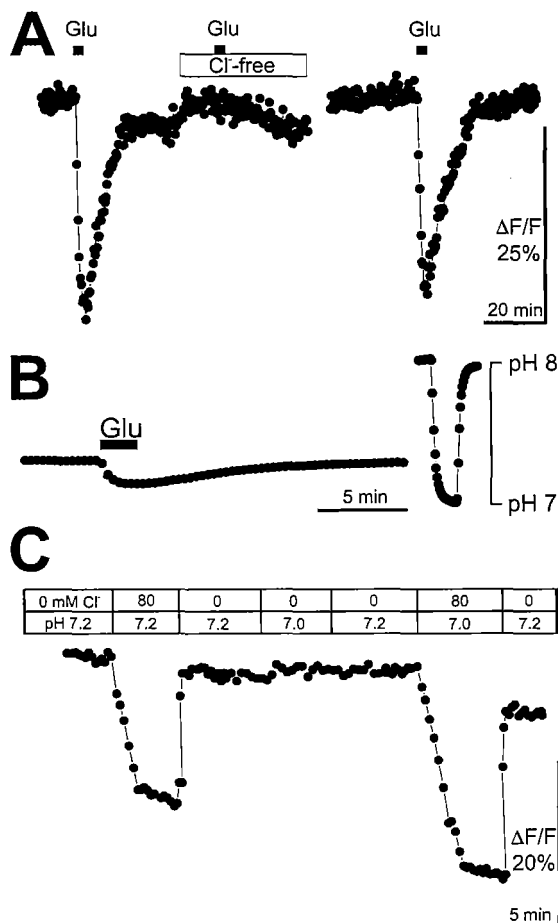


Figure 2. Glutamate-induced EYFP responses are mainly due to decreases in $[\text{Cl}^-]_i$. **A:** Representative fluorescence signals from an EYFP-expressing neuron induced by glutamate (Glu; 100 μM), first in normal (Cl^- -containing) ACSF, then in Cl^- -free ACSF (indicated by labeled rectangle), and finally after return to control ACSF. **B:** Representative pH signals from a non-transgenic neuron induced by glutamate (Glu; 100 μM) in ACSF and loaded with the pH indicator SNARF (left trace). After recovery from the glutamate application, the superfusate was switched to pH calibration HBS (high K^+ , 10 μM nigericin), at pH 8, then to HBS at pH 7, and finally returned to pH 8 (calibration; right trace). Note the intracellular acidification (~ 0.2 pH units) during glutamate application. **C:** Representative fluorescence signals from an EYFP-expressing neuron induced by switching the pH/ Cl^- calibration superfusate (indicated by labeled rectangles) from 0 mM Cl^- (pH 7.2) to 80 mM Cl^- (pH 7.2) for assessment of Cl^- sensitivity, from 0 mM Cl^- (pH 7.2) to 0 mM Cl^- (pH 7.0) for pH sensitivity, and from 0 mM Cl^- (pH 7.2) to 80 mM Cl^- (pH 7.0) for the combined Cl^- and pH response. The pH/ Cl^- calibration solution contained 115 mM KCl, 10 μM nigericin and 10 μM tributyltin chloride.

Glutamate-mediated increases in $[Cl^-]_i$ are due to depolarization via ionotropic receptors

Glutamate activates a variety of receptors on hippocampal neurons, including NMDA and AMPA ionotropic receptors (for review, see Ozawa *et al.*, 1998), as well as metabotropic glutamate receptors (mGluRs; for review, see Coutinho and Knöpfel). In order to investigate the profile of glutamate receptor activation that leads to elevated $[Cl^-]_i$, we applied NMDA (100 μ M), AMPA (100 μ M) or the Group 1 mGluR agonist, DHPG (100 μ M) to cell cultures. The application of NMDA or AMPA caused a large decrease in fluorescence (NMDA: $62.7 \pm 14.4\%$ of control glutamate response; AMPA: $36.6 \pm 4.4\%$ of glutamate response), and their combined amplitudes accounted for the glutamate-mediated fluorescence change (Figures 3A and 3C). The response to glutamate was also antagonized by a cocktail of the NMDA receptor antagonist, D-APV, and the

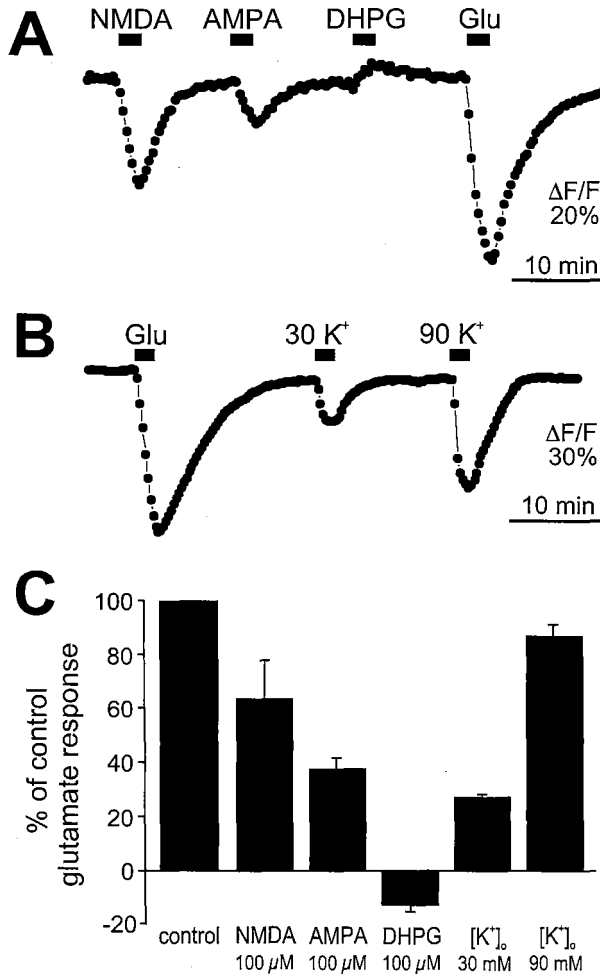


Figure 3. Glutamate receptor agonists and high K^+ depolarization cause increases in $[Cl^-]_i$. **A:** Representative fluorescence signals induced by applications of NMDA (100 μ M), AMPA (100 μ M), DHPG (100 μ M) and glutamate (Glu; 100 μ M). **B:** Representative fluorescence signals induced by applications of glutamate (Glu; 100 μ M), and two concentrations of $[K^+]_o$ (30 mM and 90 mM). **C:** Summary of responses to glutamate elicited in the presence of glutamate receptor agonists, and to elevated $[K^+]_o$, expressed as the percentage of the control glutamate response. Data are shown as mean values $\pm$ SEM.

AMPA receptor antagonist, NBQX (data not shown), further suggesting that ionotropic receptors are responsible for the glutamate-mediated $[Cl^-]_i$ increase. DHPG did not cause a decrease in fluorescence ($-12.2 \pm 2.6\%$), but instead a slight increase (Figures 3A and 3C), suggesting that mGluRs do not contribute to increases in $[Cl^-]_i$ after glutamate exposure. Since activation of NMDA and AMPA receptors leads to a depolarization of the neuronal membrane, it is possible that this cellular depolarization is the driving force for the increase in $[Cl^-]_i$. To investigate this possibility, we applied elevated extracellular concentrations of K^+ ($[K^+]_o = 30$ and 90 mM) to cells (Figures 3B and C). Increasing $[K^+]_o$ to 30 mM produced a decrease in fluorescence that amounted to $25.8 \pm 0.9\%$ ($n = 3$) of the control response to glutamate, while 90 mM $[K^+]_o$ caused a massive decrease in fluorescence, amounting to $86 \pm 4.4\%$ ($n = 6$) of the glutamate response (Figure 3C). These results suggest that depolarization caused by glutamate provides the driving force for the Cl^- gradient and the resulting increase in $[Cl^-]_i$. Consistent with this finding, the response to 90 mM $[K^+]_o$ was three times larger than that of 30 mM $[K^+]_o$. We next sought to elucidate the membrane mechanisms mediating the Cl^- influx.

GABA_A receptor blockade reduces but does not abolish glutamate-induced increases in $[Cl^-]_i$

Glutamate application activates hippocampal interneurons, which in turn release GABA and activate GABA_A-receptor operated Cl^- channels. Activated GABA_A receptors might therefore provide the conductance that, together with the increased driving force, causes the Cl^- influx. We thus tested bicuculline (40 μ M), a specific GABA_A-receptor antagonist, and found that bicuculline reduced the glutamate-induced increases in $[Cl^-]_i$ to $80.4 \pm 8.7\%$ of control glutamate responses ($n = 28$; $p < 0.05$) (Figures 4A and 4C). Although 40 μ M bicuculline is a maximally-effective inhibitory concentration, the small effect (20% reduction) led us to also test the effects of antagonists of Cl^- co-transporters: furosemide (100 μ M), an inhibitor of the neuron-specific K^+ - Cl^- co-transporter (KCC2) and the Na^+ - K^+ - $2Cl^-$ -co-transporter (NKCC1), and bumetanide (100 μ M), an more selective inhibitor of NKCC1 (Payne *et al.*, 2003; Yamada *et al.*, 2001; Kakazu *et al.*, 2000; Rivera *et al.*, 1999). As shown in Figure 4D, both furosemide ($93.9 \pm 7.2\%$ of glutamate response; $n = 4$) and bumetanide ($102.5 \pm 16\%$ of glutamate response; $n = 22$) were ineffective. The anion exchange inhibitor 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid, DIDS (500 μ M), also had no effect on glutamate-induced increases in $[Cl^-]_i$ ($95.3 \pm 1.0\%$ of glutamate response; $n = 4$; Figure 4D). These data indicate that GABA_A receptors, but not Cl^- co-transporters, contribute to increased $[Cl^-]_i$.

Involvement of other anion channels

The influx of Cl^- through GABA_A receptors, however, does not account for the total increase in $[Cl^-]_i$; therefore, other types of channels must play a role. To this end, we tested the effects of the anion channel blockers, NPPB and NFA (Van Damme *et al.*, 2003), on the glutamate response. NFA (100 μ M) significantly reduced the decrease in EYFP fluorescence ($71.3 \pm 8.4\%$ of control glutamate response; $n = 7$; $p < 0.05$) (Figure 5C). NPPB significantly reduced both the glutamate-induced (50 μ M: $50.3 \pm 2.9\%$ of glutamate response; 200 μ M: $12.6 \pm 2.8\%$ of glutamate response; $n = 3-5$; $p < 0.01$) and the 90 mM $[K^+]_o$ -induced (50 μ M: $12.1 \pm 2.2\%$ of glutamate response; 200 μ M: $5.5 \pm 2.5\%$ of glutamate response; $n = 2-3$; $p < 0.01$) changes in EYFP fluorescence in a dose-dependent manner (Figures 5A and 5B). Our data suggests that NPPB-sensitive Cl^- channels comprise the largest contributors to $[Cl^-]_i$ increases induced by glutamate application.

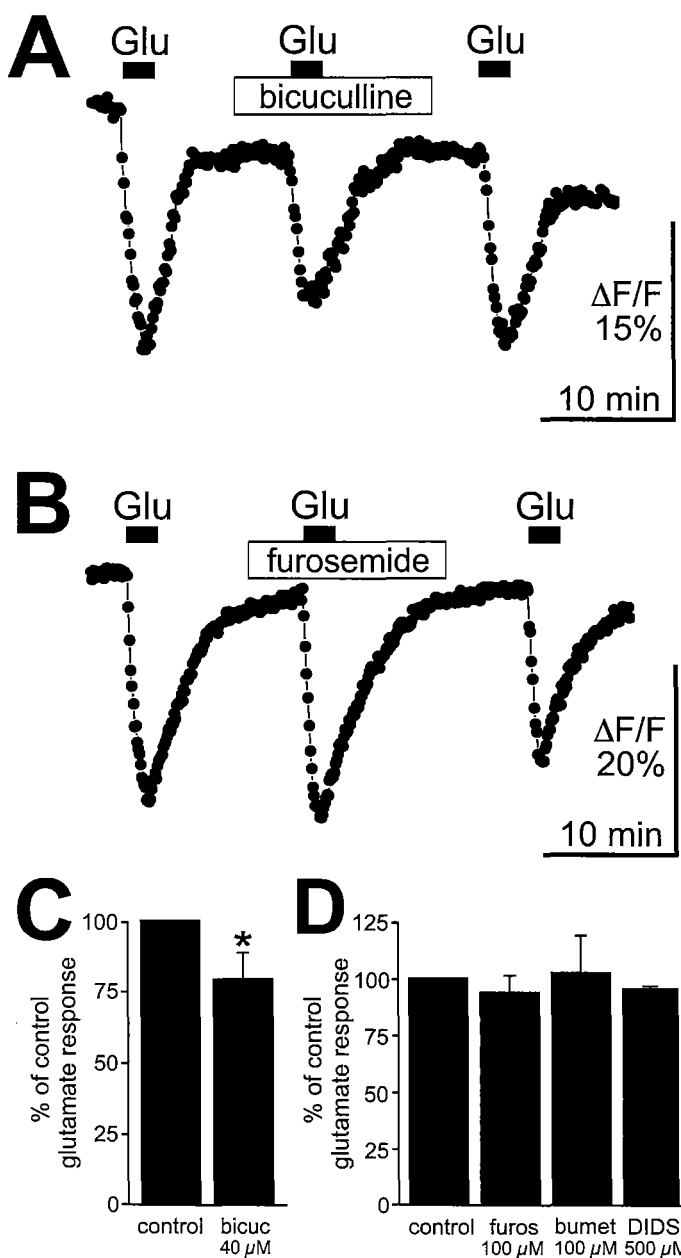


Figure 4. Contribution of GABA_A receptors and Cl⁻ transporters to glutamate-induced [Cl⁻]_i elevations. A and B: Representative fluorescence signals induced by glutamate (Glu; 100 μM) alone, and in the presence of bicuculline [40 μM , (A)] or furosemide [100 μM , (B)]; drug application is indicated by labeled rectangles. **C:** Summary of response to glutamate elicited in the presence of bicuculline (bicuc; $n = 28$; $*p < 0.05$) expressed as the percentage of the control glutamate response. **D:** Summary of responses to glutamate elicited in the presence of furosemide (furos; $n = 4$), bumetanide (bumet; 100 μM ; $n = 22$) or 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS; 500 μM ; $n = 4$) expressed as the percentage of the control glutamate response. Data are shown as mean values $\pm$ SEM.

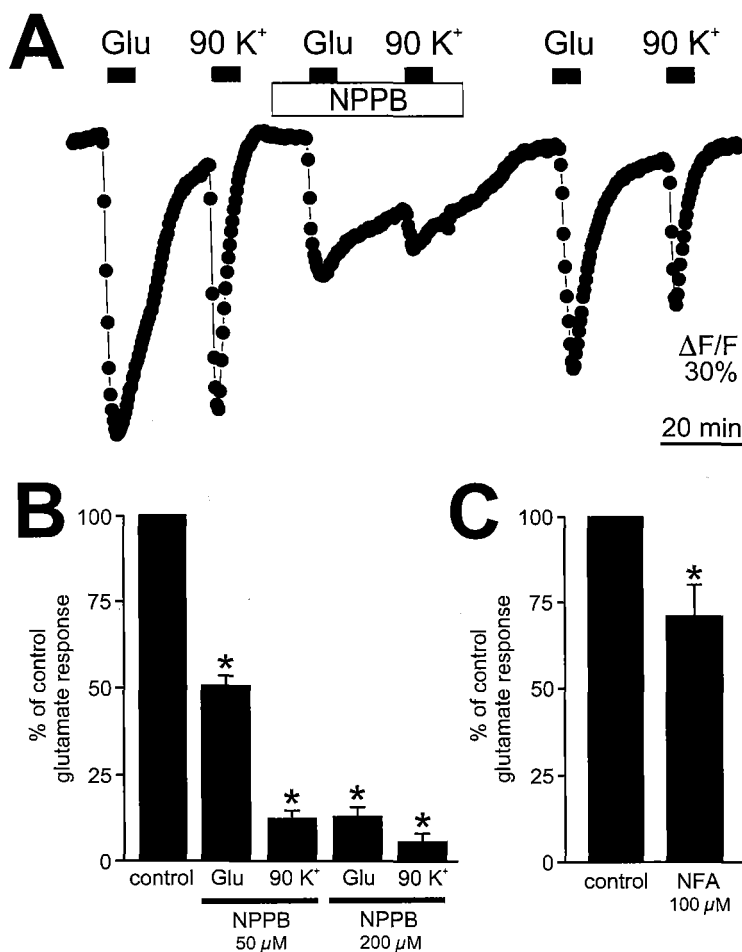


Figure 5. Glutamate- and high $[K^+]_o$ -induced $[Cl^-]_i$ elevations are antagonized by NPPB. **A:** Representative fluorescence signals induced by glutamate (Glu; 100 μ M) and 90 mM $[K^+]_o$ in normal ACSF, followed by glutamate and elevated $[K^+]_o$ responses in the presence of 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; 50 μ M; indicated by labeled rectangle) in ACSF, and wash out responses. **B:** Summary of responses to glutamate elicited by glutamate and elevated $[K^+]_o$ in the presence of NPPB (50 and 200 μ M; * p < 0.01, n = 2-5) expressed as the percentage of the control glutamate response. **C:** Summary of response to glutamate elicited in the presence of niflumic acid (NFA; 100 μ M; n = 5) expressed as the percentage of the control glutamate response. Data are shown as mean values $\pm$ SEM.

Discussion

We established primary hippocampal cell cultures from transgenic mice that express EYFP under the regulatory sequences of the potassium channel Kv3.1, and investigated the effect of glutamate application on $[Cl^-]_i$ by exploiting the Cl^- sensitivity of EYFP. We first discuss the opportunities provided by EYFP-expressing cell cultures, and then the mechanisms behind and implications of the observed glutamate-induced elevation in $[Cl^-]_i$.

Expression of Kv3.1 potassium channel protein increases during the first two postnatal weeks (Weiser *et al.*, 1994; Perney *et al.*, 1992) and it was therefore not clear if significant EYFP expression would be observed at early stages in culturing. We observed bright neuronal EYFP fluorescence as early as 2 DIV in cultures prepared from embryonic hippocampal and cerebellar tissue (cerebellar data not shown). Notably, we have found that neurons from other transgenic mouse types [(e.g. L7-GFP); Zhang *et al.*, 2001] were fluorescent in slices but not in culture (J. E. Slemmer, unpublished observations). Because EYFP causes little disturbance to cells, it can serve as an excellent neuron-specific marker (Zhang *et al.*, 2002) with clear advantages over more traditional methods of live-cell staining. This system is ideal for assessing neuronal function in several cells simultaneously, for example in studies of intercellular communication, or measuring differential responses of neurons to traumatic injury *in vitro* (van den Pol *et al.*, 1996; Weber *et al.*, 2002). In addition, we monitored fluorescent cells for several hours, and live recording could be extended to days using an incubator mounted on the microscope. Thus, cultures prepared from these mice could also be used for long-term examinations of neuronal function under both physiological and pathological conditions, including measurements of neuronal excitability following synaptic plasticity protocols, or changes induced by ischemic or mechanical insults.

Mechanisms of glutamate-induced elevation in $[Cl^-]_i$

During the initial characterization of our pKv3.1/EYFP transgenic mice, we found that high K^+ or glutamate stimulation produced a significant acidification along with an increase in $[Cl^-]_i$ in cerebellar granule cells (Metzger *et al.*, 2002). In our hands, the application of glutamate (100 μM) for 3 min induced a decrease in pH of approximately 0.2 pH units. This acidification differs slightly from the findings of Wang *et al.* (1994), who reported a decrease of approximately 0.4 pH units after glutamate (10 μM) for 3 min, and from Hartley and Dubinsky (1993), who reported a larger decrease in pH (approximately 0.52 pH units) after glutamate (500 μM) for 5 min. Methodological differences may account for these discrepancies in intracellular acidification after glutamate application; for example, we measured pH changes in ACSF, whereas Wang *et al.* (1994) and Hartley and Dubinsky (1993) used HBS solutions. Measurements in ACSF would allow for more stable regulation of pH (Schwiening and Boron, 1994). In order to elucidate the membrane mechanisms underlying the glutamate-induced elevation in $[Cl^-]_i$, we tested several blockers of Cl^- co-transporters: furosemide (100 μM), a blocker of KCC2 and NKCC1; bumetanide (100 μM), a more selective blocker of NKCC1 (Yamada *et al.*, 2001; Kakazu *et al.*, 2000; Rivera *et al.*, 1999); and DIDS, a broad spectrum inhibitor of anion transport (Sheldon and Church, 2002). None of these compounds had a significant effect on the glutamate-induced elevation in $[Cl^-]_i$, indicating that Cl^- influx through KCC2 and NKCC1 do not contribute to the glutamate-induced increases in $[Cl^-]_i$. In contrast to our findings, Zeevalk *et al.* (1989) saw reductions in Cl^- -dependent, glutamate-induced edema when retinal cells were treated with 1 mM furosemide; however, their results could have been confounded by the high dose of drug used (Beck *et al.*, 2003). The reason why Zeevalk *et al.* (1989) found DIDS (600 μM) effective, whereas we did not (at 500 μM) is unclear. We used neurons at 8-12 DIV in the present study, which is mid-range between the two groups (7-8 DIV and 14-15 DIV) used by Beck *et al.* (2003), who demonstrated that the long-term effects of either oxygen-glucose deprivation or glutamate application on NKCC1 could be blocked by bumetanide. In the present study, we applied glutamate (or agonists such as NMDA) for 3 min; the cells were apparently able to recover from the transitory, large increases in $[Cl^-]_i$.

We did find that depolarization of the cells by ionotropic glutamate receptor agonists (NMDA or AMPA), or directly by increasing $[K^+]_o$, replicated the effect of glutamate-application on $[Cl^-]_i$, indicating that depolarization of the hippocampal neurons sufficed to significantly elevate $[Cl^-]_i$. Thus, an increase in Cl^- driving force promotes Cl^- influx through Cl^- channels. To find out which Cl^- channels contribute to the influx we tested the GABA_A receptor antagonist bicuculline on the glutamate-induced Cl^- signal. We found that only a minor component of the glutamate-induced elevation of $[Cl^-]_i$ was blocked by bicuculline, suggesting that bicuculline-sensitive GABA_A receptors contribute to, but are not the major route, of Cl^- entry. Similar to our findings, Inglefield and Schwartz-Bloom (1998b) found that activation of glutamate receptors in hippocampal slices led to an increase in $[Cl^-]_i$ that was primarily due to mechanisms other than GABA_A receptor activation. We found that the majority of glutamate-mediated Cl^- influx appeared to be through anion channels other than GABA_A receptors, as increases in $[Cl^-]_i$ were sensitive to the anion channel blockers, NFA and NPPB. Although NFA (100 μ M) blocked roughly 30% of the glutamate-mediated Cl^- influx, NPPB blocked almost all of the Cl^- influx at a high concentration (200 μ M). This latter finding was surprising; Van Damme *et al.* (2003), however, have also reported that depolarization caused by AMPA receptor stimulation in cultured spinal neurons causes Cl^- influx that occurs predominately through NFA- and NPPB-sensitive anion channels.

The current results suggest that depolarization of neurons by glutamate, or by an elevated $[K^+]_o$, can increase $[Cl^-]_i$ to levels as high as 80 mM. The excitotoxic effects of glutamate in hippocampal neurons are well characterized (Rothman, 1985; Skaper *et al.*, 2001). It has long been suggested that Cl^- entry may play a role in neuronal excitotoxicity, and that a reduction in Cl^- entry may be able to produce a protective effect (Rothman, 1985; Grondahl *et al.*, 1998). This hypothesis could be important for the study of pathologies that are believed to involve alterations in $[Cl^-]_i$ homeostasis, such as ischemia and trauma (Beck *et al.*, 2003; van den Pol *et al.*, 1996; Inglefield and Schwartz-Bloom, 1998a; Schwartz-Bloom and Sah, 2001; Grondahl *et al.*, 1998; Yamada *et al.*, 2001). The present observations provide both a framework and a system for the careful study of the mechanisms and consequences of pathological Cl^- influx in further detail.

CHAPTER 3

Microtubule plus-end binding proteins in neurons

CHAPTER 3.1

**Visualization of microtubule growth in cultured neurons
via the use of EB3-GFP
(end-binding protein 3-green fluorescent protein)**

Abstract

Several microtubule (MT) binding proteins, including CLIP-170, CLIP-115 and EB1, have been shown to specifically associate with the ends of growing MTs in non-neuronal cells, thereby regulating MT dynamics and the binding of microtubules to protein complexes, organelles and membranes. When fused to GFP, these proteins, which are collectively called +TIPs (plus-end tracking proteins), also serve as powerful markers for visualizing MT growth events. Here, we demonstrate that endogenous +TIPs are present at distal ends of MTs in fixed neurons. Using EB3-GFP as a marker of microtubule growth in live cells we subsequently analyze microtubule dynamics in neurons. Our results indicate that MTs grow slower in neurons than in glia and COS-1 cells. The average speed and length of EB3-GFP movements is comparable in cell bodies, dendrites, axons and growth cones. In the proximal region of differentiated dendrites approximately 65% of EB3-GFP movements are directed towards the distal end, whereas 35% are directed towards the cell body. In more distal dendritic regions and in axons most EB3-GFP dots move towards the growth cone. This difference in directionality of EB3-GFP movements in dendrites and axons reflects the highly specific MT organization in neurons. Together these results suggest that local MT polymerization contributes to the formation of the MT network in all neuronal compartments. We propose that similar mechanisms underlie the specific association of CLIPs and EB1-related proteins with the ends of growing MTs in non-neuronal and neuronal cells.

Introduction

Neurons are signaling cells with a unique polarized composition, which is paralleled by a highly specific microtubule (MT) network organization. For example, MTs are organized non-uniformly in differentiated dendrites, with dynamic plus-ends pointing both distally and towards the cell body (Baas *et al.*, 1988). In axons, on the other hand, MTs are uni-directionally organized, with all plus-ends pointing towards the growth cone. This organization is different from that of other cell types, in which minus ends of MTs are, for example, often embedded in the MT organizing centre (MTOC), while plus-ends explore the cytoplasm. In view of these differences and the fact that the distance between cell body and neuronal periphery can be quite extensive, it is logical to assume that the localization and transport of membrane bounded organelles over MT tracks, the formation and maintenance of the neuronal MT cytoskeleton itself, as well as the transport of cytoskeletal elements, might be organized in a unique fashion.

The dynamic properties of MTs are regulated by a large number of cellular factors, including MT associated proteins (MAPs) and MT motors. Recently, a novel type of MT binding has attracted considerable interest, both with respect to the regulation of MT dynamics and intracellular membrane transport over MTs. Live imaging studies have shown that an increasing number of MT regulatory proteins from various organisms specifically associates with the distal ends of growing MTs. This characteristic behavior was first documented for CLIP-170 (Perez *et al.*, 1999); it was explained by a mechanism called “treadmilling”, or “plus-end tracking” and MT plus-end binding proteins that show this behavior have since been called “plus-end tracking proteins”, or +TIPs (Schuyler and Pellman, 2001). Treadmilling involves the incorporation of +TIPs into growing MTs ends, either by copolymerization with tubulin, or by recognition of a specific feature of the MT distal end, and subsequent release from an older, more proximal part of the MT. The kinetics of association and release may be regulated by posttranslational modifications of the +TIP, for example phosphorylation (Rickard and Kreis, 1991; Hoogenraad

et al., 2000; Tirnauer *et al.*, 2002; Vaughan *et al.*, 2002). In addition to CLIP-170, CLIP-115 and CLIP-associated proteins, or CLASPs (Akhmanova *et al.*, 2001), LIS1 (Han *et al.*, 2001; Coquelle *et al.*, 2002), EB1 and its associated protein APC (Mimori-Kiyosue *et al.*, 2000a, 2000b), as well as components of the dynein/dynactin motor complex (Han *et al.*, 2001; Vaughan *et al.*, 2002), appear to undergo treadmilling behavior. Besides their role in the regulation of MT dynamic events, some of these +TIPs may also exert an influence on intracellular transport of vesicular cargo via their control over MT tip association of the dynein/dynactin complex (Valetti *et al.*, 1999; Vaughan *et al.*, 1999).

All live imaging studies have so far documented the behavior of +TIPs, fused to GFP (GFP+TIPs), in non-neuronal cells. Since the growth rates of MTs, as measured with various GFP+TIPs, are similar to values obtained after injecting fluorescently labeled tubulin (for example, see Komarova *et al.*, 2002a), the dynamic behavior of MTs is not significantly altered by low expression levels of the +TIPs. Thus, GFP+TIPs appear excellent tools for studying MT growth dynamics in living cells. Using these markers in neurons we have addressed the important question whether changes in the MT network during neuronal differentiation are reflected by changes in MT polymerization dynamics in different neuronal compartments. We first show that endogenous CLIPs and EB1-related proteins are present at the distal ends of neuronal MTs. Using EB3-GFP as a marker, we subsequently highlight polymerizing MTs in two different types of cultured neurons. Our results support the conclusion that local MT polymerization events occur throughout neuronal differentiation. We propose that similar mechanisms control the association of +TIPs with MT distal ends in neurons and non-neuronal cells.

Materials and methods

GFP-fusion constructs

The *GFP-CLIP115* and *-170* constructs have been described (Perez *et al.*, 1999; Hoogenraad *et al.*, 2000; Akhmanova *et al.*, 2001). Mutant GFP-CLIP170 (called GFP-CLIP170 Δ Hind), lacking the C-terminal domain and part of the coiled-coil region of rat CLIP-170, was generated by Hind III/BamHI digestion of GFP-CLIP170 in the pEGFP vector, removal of the insert containing the 3' CLIP-170 sequence and religation of blunted ends. For cloning of *EB1*, *EB2* and *EB3* cDNAs, gene-specific primers were designed, with restriction sites for in-frame cloning after PCR amplification into *pEGFP-N1* (Clontech). Primer sequences were based on the following accession numbers: AW106491 (Image clone 2225780, mouse *EB1*); AW231083 (Image clone 2644901, mouse *EB2*, or *RPI*); AA2892 (Imageclone 714028; human *EB3* cDNA). Western blot analysis of COS-1 cells transfected with the different cDNAs (Hoogenraad *et al.*, 2000), shows that all EB-GFP fusions are produced with an expected molecular weight (Figure 1).

Cell culture and protein expression

COS 1 cells were cultured and transfected as described previously (Akhmanova *et al.*, 2001). Mouse hippocampal neurons were isolated from E17 FVB/N embryos and cultured based on published procedures (Dotti *et al.*, 1988; de Hoop *et al.*, 1998). Briefly, hippocampi were dissected from fetal brains, treated with 0.25 % trypsin for 15 min at 37°C, washed in Ca²⁺/Mg²⁺-free Hank's balanced saline solution (HBSS) and dissociated by repeated passage through a constricted Pasteur pipette. Hippocampal neurons were electroporated using a BioRad Gene Pulser at 850 V, 25 mF, and 200 Ohm (time constant: 0.8) immediately after isolation by adding

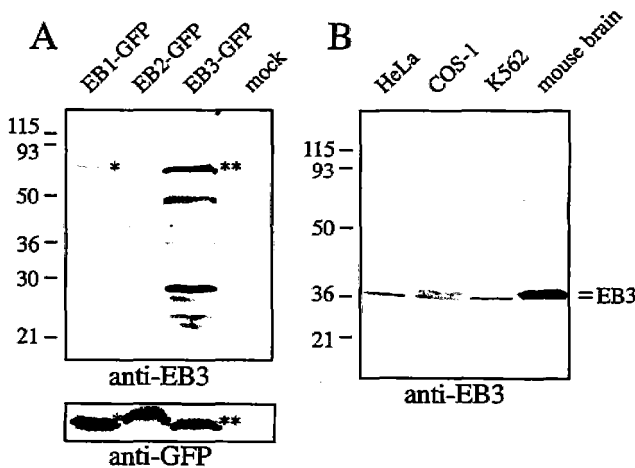


Figure 1. Characterization of anti-EB3 antiserum. Novel anti-EB3 antibodies were raised in rabbit against GST-EB3 and characterized on western blot. **A:** COS-1 cells were mock transfected, or transfected with EB1, -2 and -3, fused to GFP. The lower panel (western blot with anti-GFP antiserum) demonstrates that each fusion protein is expressed at a similar level. The upper panel (western blot with the novel anti-EB3 antiserum) shows that anti-EB3 antibodies recognize full length EB3-GFP (marked by a double asterisk). EB1-GFP (marked by single asterisk) is also weakly recognized, whereas EB2-GFP is not. **B:** Equal amounts of whole cell protein extract from HeLa, COS-1 and K562 cells and from mouse brain are analyzed. Two isoforms of EB3, which are more abundantly expressed in brain extracts, are recognized by the novel anti-EB3 antiserum.

highly purified plasmid DNA (25 µg; EndoFree Maxi Prep; Qiagen, Germany) to the suspension in HBSS (0.4 ml). Neurons were subsequently plated on 22 mm, poly-L-lysine coated coverslips. Plating medium contained 2 mM sodium butyrate for 17–24 hr, to enhance expression of CMV-promoter driven genes. After this time, the coverslips with the neuronal cultures were placed upside down in 3.5 cm plastic dishes, containing N2 medium (de Hoop *et al.*, 1998), conditioned by a confluent monolayer of astroglial cells. Small paraffin droplets on the coverslips prevented the neurons from making contact with the glia.

Mouse Purkinje cell (PC) neurons were isolated from E18 FVB/N embryos as previously described with minor modifications (Slemmer *et al.*, 2002). Briefly, dissociated cerebella were diluted in serum-containing media [Basal Medium Eagles (Invitrogen) containing 10% horse serum (Invitrogen), 10 µg/ml gentamicin (Sigma), 0.5% glucose (Sigma), 1 mM sodium pyruvate (Invitrogen) and 1% N2 supplements (Invitrogen)] to a concentration of 1×10^6 cells per ml; cells were then plated in 1 ml aliquots onto 4-well chambered coverglasses (NalgeNunc, Naperville, IL), coated overnight with poly-L-ornithine (500 µg/ml; Sigma). All cultures were maintained in a humidified incubator (5% CO₂, 37°C). Neuronally enhanced cultures were obtained by replacing half of the media two days after plating, and then twice per week, with serum-free media containing 2% B27 supplements (Invitrogen).

To express foreign proteins in PCs, we used Semliki Forest virus (SFV)- mediated gene delivery (Ehrengruber *et al.*, 1999; Lundstrom *et al.*, 2001a, 2001b). The EB3-GFP construct was cloned into the pSFV2 vector, according to the manufacturer's instructions (GIBCO). Constructs were packaged into SFV replicons, using co-electroporation of helper and vector RNA into baby hamster kidney (BHK)-21 cells. Cultured PCs (or hippocampal neurons) were infected between 10 and 17 DIV by addition of SFV infectious replicons (1–5 µl) to the cultures.

Antibodies and immunocytochemistry

Cultured neurons were washed in PBS and fixed first in freshly prepared -20°C methanol supplemented with 1 mM EGTA, followed by a 2% paraformaldehyde fixation at room temperature (20 min for each step). Antibody incubation and washing steps have been described,

as have the CLIP-115 and -170 specific antisera (#2221; #2238 and #2360) (Hoogenraad *et al.*, 2000; Coquelle *et al.*, 2002). A combination of these sera was used (diluted 1:1000). Novel polyclonal antibodies to EB3 (#02-1005-07) were raised in rabbit, as previously described (Hoogenraad *et al.*, 2000), using GST-EB3 as antigen. Monoclonal antibodies against EB1 (Transduction Laboratories), actin (Chemicon), acetylated alpha-tubulin, tyrosine alpha-tubulin and beta-tubulin (Sigma) were used in a dilution of 1:100. FITC-conjugated goat-anti-rabbit (1:100, Nordic Laboratories) and Alexa 594-conjugated goat anti-mouse (1:500, Molecular Probes) were used as secondary antibodies. Signals were captured as described previously (Hoogenraad *et al.*, 2000; Akhmanova *et al.*, 2001), using either a Leica DMRBE microscope, with a 100× oil immersion lens (NA 1.3) and a Hamamatsu C4880 camera, or a Zeiss LSM510 confocal microscope with a 63× oil immersion lens (NA 1.4). EB3 and EB1 fluorescent intensities at plus-ends were analyzed using Image Pro Plus V 4.5 (Media Cybernetics, Silver Spring, MD). After background subtraction, a threshold was placed on the red fluorescent channel to select the plus-ends. The red and green intensities were then measured at the same spot. Small spots (less than 0.1 μm^2) and large spots (more than 1 μm^2) were discarded from the analysis. The confocal images representing EB3-tubulin co-staining (Figures 2H and 2I) were reconstructed with a Quick Maximum Likelihood Estimation (Huygens2 Pro, Scientific Volume Imaging, The Netherlands) based on a theoretical point spread function. Different color channels were analyzed with different point spread functions. After analysis, the channels were merged again.

Live-cell imaging and analysis of GFP movements

Cells were analyzed at 37°C on a Zeiss 510 confocal laser scanning microscope (LSM510) as described previously (Akhmanova *et al.*, 2001). In most experiments the optical slice (Z-dimension) was set to 1 μm . Other settings used (e.g. laser intensity and gain value) differed slightly in the various experiments and were adapted to obtain optimal signal-to-noise ratios. Nocodazole (Sigma) and taxol (Molecular Probes) were added at 0.1 or 10 μM final concentrations. Images of GFP+TIP movements in transfected cells were acquired every 1-3.5 seconds. Image capture time was typically less than one second in COS-1 cells. In contrast, capture times for neuronal imaging were in the range of 1-3.5 seconds, due to the lower signals present at MT tips in these cells. Images were recorded and movies were assembled using LSM 510 software. (The original movie files can be viewed at www.eur.nl/fgg/ch1/galjart, or by accessing the website of The Journal of Neuroscience.)

Distances traveled by GFP+TIP dashes were measured in different neuronal areas. The velocity of the different +TIP dashes was calculated by dividing distances traveled by time spent traveling. We only included dashes that could be followed for at least three consecutive frames. To measure the percentage of anterograde EB3-GFP movements (i.e. movements towards the distal end of neurons) and retrograde movements (i.e. towards the soma), we measured fluorescent displacements occurring within one complete time-lapse movie within selected areas of the neuron (i.e. in different neuronal compartments). In this analysis, all movements were included, irrespective of how many consecutive frames they lasted. Each fluorescent movement within the selected area was also traced to determine the average number of consecutive frames that we could follow displacements. Percentages of forward and backward movement are either expressed relative to total number of fluorescent movements observed, or relative to the number of fluorescent MT distal ends (see Table 2). Since the values obtained do not differ considerably, this analysis suggests that the average duration of forward and backward displacements is similar.

Results

+TIP localization in cultured neurons

Several +TIPs have been shown to localize to the ends of a subset of MTs in fixed cultured cells, in agreement with their plus-end tracking behavior in live cells. We wanted to analyze whether a similar MT distal end localization is present in neurons, as this has not yet been described. We focused our analysis on CLIP-115 and CLIP-170, and on EB1 and EB3. Both CLIP-115 (De Zeeuw *et al.*, 1997) and EB3 (Nakagawa *et al.*, 2000) mRNAs are enriched in the central nervous system, making the analysis of the distribution pattern of these proteins particularly interesting. Antibodies against CLIP-115 and -170 have been previously described (Hoogenraad *et al.*, 2000) and a commercial monoclonal antiserum against EB1 is available. To obtain EB3 localization data, we raised a novel polyclonal antiserum in rabbit. When tested on western blot, this anti-EB3 antiserum specifically recognizes EB3-GFP in transfected COS-1 cells (Figure 1A, lane 3). Transfected EB1-GFP (Figure 1A, lane 1) is also weakly recognized, whereas EB2-GFP (Figure 1A, lane 2) is not detected. In extracts from different cell lines and from mouse brain, the novel anti-EB3 antiserum mainly recognizes two closely migrating EB3 isoforms of approximately 36 kDa (Figure 1B), which are enriched in the brain extract compared to the cell line lysates and likely represent the two splice forms of EB3 that have previously been described (Su and Qi, 2001). Together these results indicate that the novel antiserum is specific for EB3.

To determine whether endogenous CLIPs associate with the ends of MTs in neurons, we incubated fixed mouse hippocampal cell cultures with antibodies against CLIP-115 or -170. Labeling with the individual CLIP antibodies, however, failed to demonstrate a clear plus-end staining pattern in neurons, in contrast to the bright staining observed in neighboring glial cells (data not shown). We therefore attempted staining of neurons with a cocktail of the different antibodies. This labeling procedure reveals cytoplasmic labeling, most intensely in cell bodies, in addition to a clear staining of comet-like dashes in cell bodies, dendrites, axons and growth cones (Figure 2, arrows highlight examples of this staining). Double labeling with anti-tubulin antibodies identified these dashes as MT distal ends (data not shown). Although these data indicate that endogenous CLIPs associate with MT plus-ends in cultured hippocampal neurons, the labeling is substantially weaker than in non-neuronal cell types, suggesting that less protein is present per MT tip in neurons. This may be the main reason why plus-end staining of CLIP-115 in cultured neurons was not observed previously (De Zeeuw *et al.*, 1997).

To analyze whether EB-related proteins also associate with MT distal ends in neuronal cultures, we incubated fixed cells with a mixture of monoclonal antibodies against EB1 and the new polyclonal anti-EB3 antiserum (Figures 2E-2G). In these experiments, co-localization is observed of EB1 (Figure 2E) and EB3 (Figure 2F) on comet-like dashes in glia and in neurons (see merge in Figure 2G). As EB1 is a verified marker of growing MT ends in non-neuronal cells (Mimori-Kiyosue *et al.*, 2000b), these data suggest that the same holds true for EB3. Quantification of EB1 and EB3 fluorescent intensity on comets reveals that EB1 signal intensity [expressed in arbitrary units (AU) per comet] is similar in glia and neurons (i.e. ~0.95 AU/comet), whereas EB3 signal increases from approximately 0.5 AU in glia to 1 AU in neurons (>1000 comets measured in 3 glia and 4 neurons). Although this quantification does not reveal the absolute levels of EB1 or EB3 in neurons and glia, the data suggest that, in neurons, more EB3 is associated per MT distal end than in glia.

Co-staining of EB3 antiserum with monoclonal anti-beta-tubulin antibodies reveals that, in those cases where individual MTs can be distinguished within the dense MT network, the EB3-positive dashes are always located at the ends of MTs, both in neurons (Figure 2H) and glia

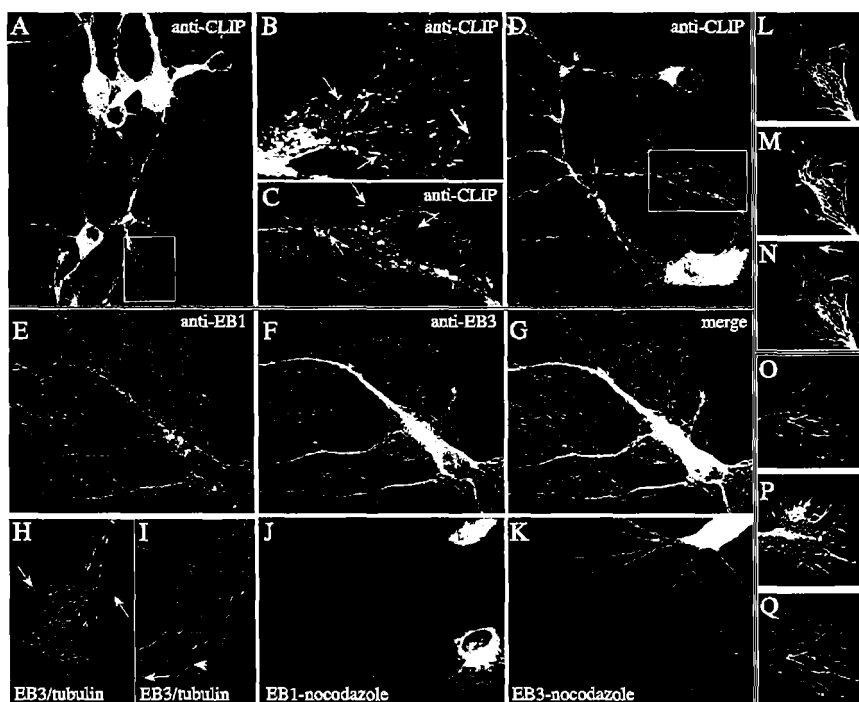


Figure 2. +TIPs in hippocampal neurons. A-K: Hippocampal neurons at 2-6 DIV were fixed and incubated with a cocktail of antibodies recognizing both CLIP-115 and CLIP-170 (A-D), with an antibody mixture recognizing EB1 and EB3 (E-G), with a mixture recognizing EB3 and beta-tubulin (H and I), with EB1 (J) or EB3 (K) antibodies. Panels B (growth cone) and C (neurites) are magnifications of the rectangles in A and D, respectively. Examples of comet-like CLIP dashes are indicated by arrows. In panel G the merged image of EB1 (E) and EB3 (F) staining is shown. Clear comet-like labeling is visible with both antibodies in the neuron and in the two glial cells. In panels H (neuronal growth cone) and I (cell periphery of a glial cell) deconvoluted confocal images are used to clearly demonstrate EB3 localization to MT distal ends. The large arrows in H and I indicate clear examples of such EB3 localization. The small arrow in I points towards a MT plus-end that is not labeled by EB3. Given that the other end of this small MT is stained, we presume that this end represents a MT minus end. The cell periphery of the glial cell in I is particular in that it contains several of such "free" MTs. J and K: Nocodazole was added to the culture medium prior to fixation of the cells. Note that the typical comet-like staining of EB1 and EB3 has vanished after this brief treatment. L-Q: Hippocampal neurons, transfected with EB3-GFP, were fixed 3-4 days after plating and stained with cytoskeletal markers. Costaining of EB3-GFP (L) in a growth cone with antibodies against tyrosinated alpha-tubulin (M) reveal that the GFP signals are located at the tip of MTs (the arrow indicates an example of such a tip in the merged image in N). Costaining of EB3-GFP (O) in a transfected neuron with antibodies against actin, followed by Alexa 594-conjugated secondary antibody incubation (P), reveals that EB3-GFP positive MT plus-ends are present in the growth cone and are sometimes detected in actin-rich filopodial extensions (see merged image in Q).

(Figure 2I). In the latter example, some of the visible MTs are not embedded in the MTOC. On these MTs, EB3 staining is only observed at one of the two ends, which presumably represents the growing end.

Nocodazole and taxol are drugs which interfere with MT dynamics in opposing manners (for review, see Downing, 2000). Nocodazole acts by sequestering tubulin dimers, eventually causing collapse of the MT network, while taxol stabilizes the MT network by specifically binding to MTs. Application of low amounts of both drugs completely abolishes binding of +TIPs to the ends of growing MTs (Perez *et al.*, 1999; Mimori-Kiyosue *et al.*, 2000b; Akhmanova *et*

al., 2001), in spite of the fact that MTs are still present and MT-based motors are active. Addition of low concentrations of these drugs to hippocampal cultures leaves the MT network intact, yet both EB1- and EB3-positive comets disappear (Figures 2J and 2K, and data not shown). Taken together, our results suggest that in neurons, like in non-neuronal cells, CLIPs and EB-related proteins specifically associate with the growing ends of MTs.

Characterization of GFP+TIP behavior in transfected COS-1 cells

Since endogenous +TIP localization (and by implication, function) is conserved in non-neuronal and neuronal cultures, we next compared GFP+TIP behavior, focusing again on CLIPs and EB1-related proteins. Based on published results, we inserted the GFP tag at the N-terminus of the CLIPs, and at the C-terminus of the EB1-related proteins (Figure 3). In the case of GFP-CLIP170, its dynamic behavior has been described in several reports (Perez *et al.*, 1999; Akhmanova *et al.*, 2001; Komarova *et al.*, 2002a), whereas, in the case of EB1-GFP, experiments have documented its dynamic behavior in live *Xenopus* A6 cells and human neuroblastoma N2A cells (Mimori-Kiyosue *et al.*, 2000b; Morrison *et al.*, 2002). Live imaging studies, however, have yet to be reported for GFP-CLIP115, EB2-GFP or EB3-GFP. We therefore first compared the behavior of all of these proteins in transfected COS-1 cells, which were analyzed under a confocal microscope 20-48 hr after transfection (Figure 3).

For live cell imaging, we only studied cells with low expression levels of the GFP fusions, but even under these conditions, EB2-GFP binds all along MTs and has no preference for MT distal ends (data not shown). The other GFP+TIPs, however, all move in comet-like dashes, mainly from the presumptive MTOC to the cell periphery, consistent with their preferential association with the growing ends of MTs (see Movies 1 and 2 for GFP-CLIP115 and EB3-GFP behavior, respectively; the behavior of the other fusion proteins has been published previously

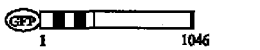
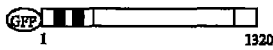
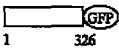
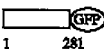
Protein structure	GFP-fusions	Velocities in non-neuronal cells ($\mu\text{m}/\text{sec}$)	
		published	present study
	GFP-CLIP 115	—	0.48 $\pm$ 0.07 (n=84)
	GFP-CLIP 170	0.21 $\pm$ 0.05 (n=59) ¹ 0.24 $\pm$ 0.04 (n=41) ² 0.27 $\pm$ 0.1 (n=110) ³	0.48 $\pm$ 0.08 (n=74)
	EB1-GFP	0.23 $\pm$ 0.05 (n=89) ⁴ 0.2 $\pm$ 0.02 (n=22) ⁵	0.50 $\pm$ 0.05 (n=58)
	EB2-GFP	—	No plus end labelling
	EB3-GFP	—	0.49 $\pm$ 0.03 (n=49)

Figure 3. Velocities of GFP+TIP fusion proteins in non-neuronal cells. COS-1 cells, expressing the indicated GFP+TIP fusion proteins, were monitored at 37°C on a Zeiss LSM510 confocal microscope and analyzed for GFP+TIP velocities. Only cells expressing low levels of the fusion proteins were investigated. For comparison, previously published values are indicated [1: Perez *et al.*, (1999); 2: Akhmanova *et al.*, (2001); 3: Komarova *et al.*, (2002); 4: Mimori-Kiyosue *et al.*, (2000) 5: Morrison *et al.*, (2002)]. MT binding domains (black bars) and coiled-coil regions (grey bars) are indicated in the CLIPs. The MT binding motif has not been clearly identified in EB1-related proteins.

and is not shown here). Co-staining with tubulin antibodies in fixed cells reveals that these comets represent MT distal ends, and low doses of nocodazole or taxol abolish GFP+TIPs staining at MT ends in transfected COS-1 cells (data not shown). We conclude that, of the five GFP-markers analyzed, four (GFP-CLIP115 and -170, EB1- and EB3-GFP) faithfully label the ends of growing MTs in COS-1 cells.

The rates of GFP+TIP movement reported here (Figure 3) are higher than values published elsewhere (Perez *et al.*, 1999; Mimori-Kiyosue *et al.*, 2000b; Akhmanova *et al.*, 2001; Komarova *et al.*, 2002a; Morrison *et al.*, 2002; Vaughan *et al.*, 2002). This might be due to the different cell types used in some of the studies. In addition, we found that slight differences in temperature and/or in culture medium conditions affect these speeds (Figure 3, compare GFP-CLIP170 value in Akhmanova *et al.*, 2001, with current value). A similar result has been obtained using GFP-p150^{Glued} as a plus-end marker (Vaughan *et al.*, 2002). Interestingly, the measured velocities of the different GFP fusion proteins are quite similar in transfected COS-1 cells in the current experiments (Figure 3), in spite of the reportedly different functions of CLIPs and EB1-related proteins (Komarova *et al.*, 2002b; Rogers *et al.*, 2002; Tirnauer *et al.*, 2002). These results indicate that, at low levels of over-expression, these GFP fusion proteins do not affect MT growth dynamics to a great extent.

GFP+TIP expression in mouse hippocampal neurons

Transfection of hippocampal neurons with GFP-CLIP115, -CLIP-170, and EB1- and EB3-GFP yields comet-like GFP dashes in cells expressing low amounts of these proteins (Figures 2L and 2O, and data not shown). These dashes are present in all neuronal compartments, indicating that growing MTs are located throughout neurons. Importantly, the distributions of the different GFP+TIP fusion proteins are similar to that observed with the antibodies. Consistent with these data, we found that the MT plus-end signal, as detected with EB3-GFP, is brighter than that detected with the other +TIP fusion proteins (data not shown).

EB1-GFP and GFP-CLIP115 distribute along MTs at higher expression levels (data not shown). At these levels GFP-CLIP170 aggregates in patches (data not shown), similar to its behavior in transfected non-neuronal cells (Pierre *et al.*, 1994). We therefore concentrated on EB3-GFP for our further analysis, since this fusion protein remains associated with MT distal ends unless expressed at very high levels. Double labeling studies in the growth cone with antibodies against tyrosinated (unmodified) alpha-tubulin reveal that EB3-GFP is located at distal segments of a subset of MTs (Figures 2L-2N, one example of MT plus-end labeling by EB3-GFP is indicated with an arrow). We also visualized EB3-GFP dashes and actin simultaneously, using anti-actin antibodies. Both EB3-GFP positive dashes (Figure 2O) and actin-rich extensions (Figure 2P) are clearly detected. Most, but not all, of the filopodial extensions in growth cones are devoid of EB3-GFP positive comet-like dashes (Figure 2Q). These data indicate not only that EB3-GFP is a faithful marker of MT plus-ends, but they also suggest that low-level expression of the fusion protein does not influence neuronal growth cone formation to a great extent. Comparison of movements of EB3-GFP dashes in transfected COS-1 cells reveals no aberrant effect of the expressed fusion protein on MT growth rates with respect to other, established MT plus-end markers (Figure 3). These data together suggest that EB3-GFP is the best suitable fusion protein for live studies of MT dynamics in transfected neurons.

To investigate growing neuronal MTs, hippocampal neurons were electroporated with EB3-GFP, and live neurons were examined with the confocal microscope 2-6 days later. Many moving fluorescent dashes are observed in these cells in all neuronal compartments, i.e. cell bodies, neurites and growth cones (see Movie 3). Still images of EB3-GFP movements from

Movie 3 are depicted in Figure 4. Application of nocodazole and taxol abolish the EB3-GFP dashes in live neurons (data not shown), again suggesting that EB3-GFP specifically associates with the ends of growing MTs.

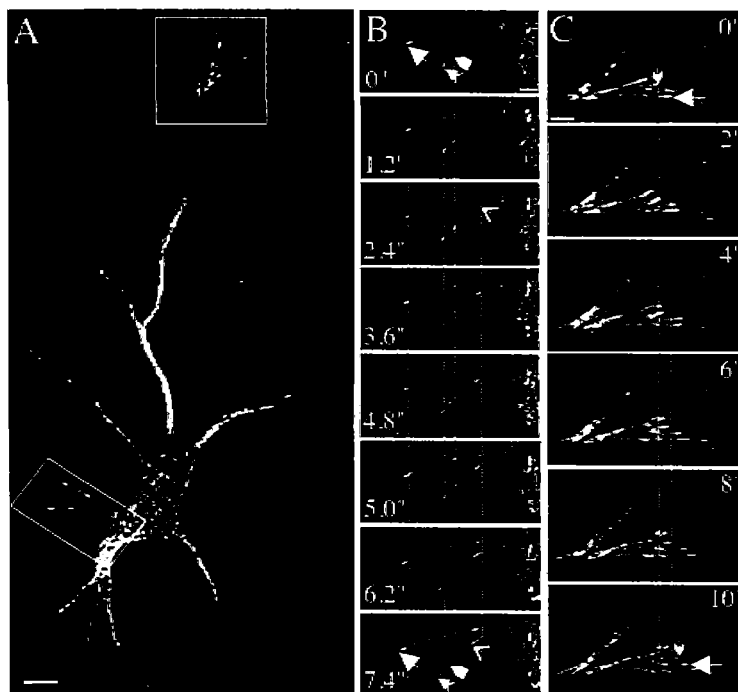


Figure 4. EB3-GFP localization in transfected hippocampal neurons. Hippocampal neurons were transfected with EB3-GFP and live cells were analyzed at 3–4 DIV by confocal microscopy in a 37°C chamber. EB3-GFP comets are seen to move in all neuronal compartments. This figure contains images of Movie 3, shown in the supplemental data section. The rectangles in panel A represent the areas, magnified in panels B (cell body and dendrites) and C (growth cone). Images in B were acquired every 1.2 s; in C, every 2 s. Arrows and arrowheads in these panels indicate beginning and end position of a selected number of EB3-GFP dashes. Stippled lines help distinguish the movements of these dashes. The lifetime of the individual dashes differs, but each moves with an average velocity of approximately 0.2 $\mu\text{m/s}$. Scale bars in A: 10 μm ; B: 5 μm ; C: 2 μm .

Table 1. Forward and backward movement of EB3-GFP in neurons

Compartment	Dashes			EB3-GFP displacements			Displacements per dash ^d	
	n ^a	% f ^b	% b ^c	n	% f	% b	f	b
Hippocampal cultures								
Proximal neurite	26	65	35	119	71	29	4.9	3.9
Distal neurite	15	87	13	55	87	12	3.7	3.5
Distal axon	9	100	0	37	100	0	4.1	
Distal axon ^e	47	83	17	137	83	17	2.9	2.9
Growth cone	7	100	0	28	100	0	4	
PC cultures								
Proximal dendrites	54	65	35	145	64	36	2.6	2.7
Distal dendrites	51	75	25	151	74	26	2.9	3
Axon 1	20	100	0	65	100	0	3.25	
Axon 2	13	85	15	40	83	17	3	3.5

^aNumber (n) of dashes observed.

^bPercentage (%) of forward (f) moving dashes.

^cPercentage (%) of backward (b) moving dashes.

^dEB3-GFP displacements per dash were calculated by dividing the number of displacements by the number of dashes. With an average acquisition time of 2.5 sec/frame and average velocity of 0.22 $\mu\text{m/sec}$, the average displacement per dash is $\sim 1.65 \mu\text{m}$.

^eDistal axon near growth cone.

Many of the EB3-GFP positive fluorescent stretches in the neuronal cell body appear to move randomly (see Movie 3), consistent with results using fixed neurons (Figure 2). Upon entry into neurites, however, MT growth becomes restricted to the long axis and is therefore directional. Within neurites, some comets touch the plasma membrane and then disappear, whereas other dashes move alongside the membrane for a number of frames. Most of the dashes move from the cell body towards the distal neurites (anterograde growth); retrograde movement (i.e. back to the cell body), however, is also observed. Retrograde MT growth constitutes approximately 35% of the total moving dashes or frames in proximal neurites of transfected hippocampal neurons (Table 1). Retrograde movements generally decline in more distal parts of the neurons (Table 1).

Behavior of EB3-GFP in differentiated Purkinje cell neurons

As an alternative to the hippocampal system we studied cerebellar Purkinje cells (PCs), as these neurons possess morphologically distinct dendritic compartments compared to hippocampal neurons. Using immunostaining against calbindin-D28K, a marker specific for PCs in cerebellar cultures, we confirmed that healthy, viable cells, with good dendritic arborizations and distinguishable axons are detectable as early as 10 DIV (data not shown). Staining with a cocktail of the three anti-CLIP antibodies indicates that these proteins are present in the cell bodies and dendrites of PCs (Figures 5A and 5B). In peripheral dendrites, the labeling pattern resembles the decoration of MT ends that we observed in other cell cultures (Figure 5B, see arrowheads). MT plus-end staining is also detected with EB antibodies (data not shown).

We used the Semliki Forest virus (SFV) vehicle (Lundstrom *et al.*, 2001b) to deliver EB3-GFP to highly differentiated PCs in culture. With this infection system, live imaging studies of EB3-GFP movements are optimal approximately 5-8 hr post-infection, since at later time points expression of the fusion protein becomes too high and MTs are completely bundled. At 5-8 hr post-infection we observe bidirectional EB3-GFP movements in PC dendrites (Figure 5D and Table 1; see also Movies 4 and 5), while in axons most, but not all, movements are unidirectional (Figure 5C and Table 1). A similar movement is detected in axons of infected hippocampal neurons of 10 DIV (see Movie 6). These results indicate that the directionality of EB3-GFP movements actually reflects the organization of the MT cytoskeleton in the different neuronal compartments (Baas *et al.*, 1988, 1989).

Atypical behavior of EB3-GFP in neurons

While most of the EB3-GFP comets move in either the anterograde or retrograde directions, and in growth cones, some dashes appear to extend into filopodia-like extensions, on rare occasions we observed pausing and/or oscillating GFP-positive dashes (for example, see some of the EB3-GFP dashes in the growth cone in Movie 3). In these cases, we cannot determine whether EB3-GFP is on a pausing, shrinking or growing MT end (which is pulled backward), since both anterograde and retrograde movements of MTs have been observed in growth cones (Dent *et al.*, 1999).

Studies with SFV-mediated EB3-GFP delivery revealed movement of the fusion protein in highly dynamic hippocampal axons (see Movie 7), with a morphology strikingly similar to that of the recently described retracting axon (He *et al.*, 2002). Some of the EB3-GFP dashes at the end of the axonal shaft of these typical axons move on curved tracks, which might represent curved and bent MTs. In addition, rapid EB3-GFP excursions into filopodia-like protrusions are observed. In most instances EB3-GFP signal disappears upon collapse of the filopodium. We conclude that, even under conditions that resemble axonal retraction, EB3-GFP dashes (and by implication MT growth) persist.

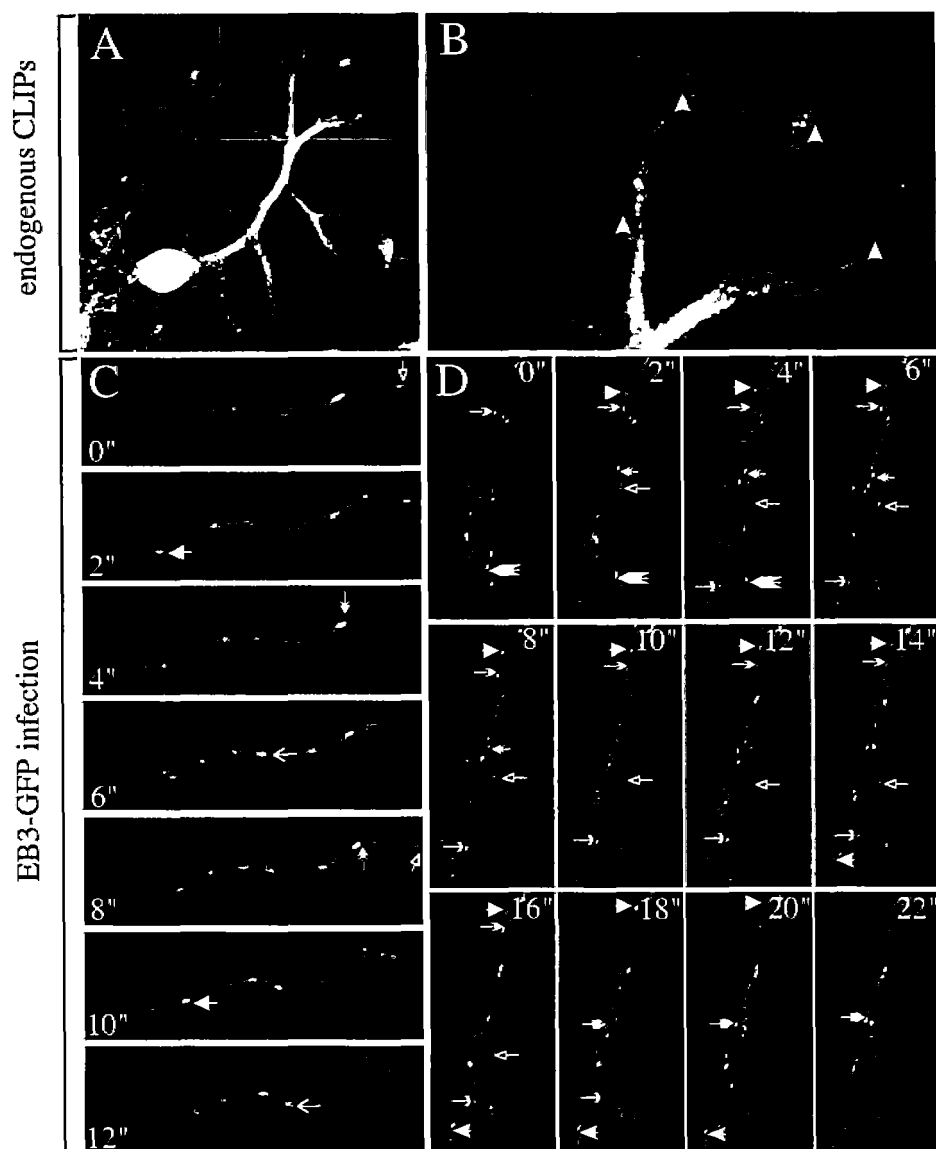


Figure 5. CLIP and EB3-GFP localizations in Purkinje cells. A and B: PCs at 10-17 DIV were fixed and incubated with a cocktail of antibodies recognizing both CLIP-115 and CLIP-170. A: The distinct initial dendritic arborization of a PC is clearly visible, indicating CLIP accumulation in this region. B: A comet-like labeling is visible in the more peripheral parts of the dendrite (examples of comet-like dashes are indicated by arrowheads). C and D: PCs at 10-17 DIV were infected with SFV-EB3-GFP and cells were analyzed 5-8 hrs post-infection by confocal microscopy in a 37°C chamber. C: Movements of EB3-GFP dashes in an axon are shown (each dash is indicated by a distinct arrow). Images were acquired every 2 s. Anterograde movement of EB3-GFP dashes towards the growth cone is towards the right. D: Images (derived from Movie 4) demonstrate bi-directional movement of EB3-GFP dashes in dendrites (each dash is indicated by a distinct arrow).

Analysis of EB3-GFP movements

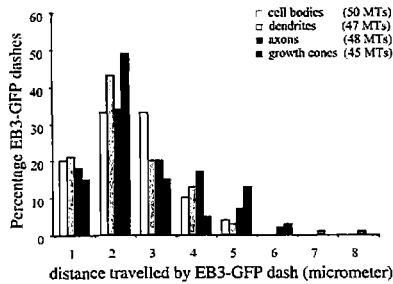
EB3-GFP dashes in neurons are on average observed for 2-5 frames, or displacements (see average number of displacements per EB3-GFP dash in Table 1). The average number of displacements per dash is similar for forward and backward movements. For quantification of the speed of EB3-GFP movements (and thus MT growth rates), we only included dashes that could be clearly followed for three frames or more. In electroporated hippocampal neurons, the distances traveled by these EB3-GFP dashes are comparable, irrespective of the compartment analyzed (Figure 6A). In these neurons, approximately 30-50% of the dashes can be followed for 2 μm (Figure 6A). Taken together, the data in Table 1 and Figure 6A indicate that under, normal culture conditions, EB3-GFP movements are similar in the different neuronal compartments.

Velocities of EB3-GFP movements were calculated in electroporated hippocampal neurons and glial cells of 2-6 DIV, in infected, differentiated PCs and neighboring glial cells of >10 DIV, and in the three examples of retracting hippocampal axons (10 DIV) that we observed (Table 2). As a control for the values of MT growth dynamics in neurons obtained with EB3-GFP, we electroporated neurons with YFP-tubulin, but could not locate transfected neurons with a recognizable fluorescent MT array (data not shown). We therefore electroporated neurons with EB1-GFP (see Movie 8) and compared its behavior to that of EB3-GFP. Since EB1 and EB3 are highly similar proteins and might have similar effects on the MT network, we also wanted to transfect neurons with a reliable plus-end marker that is structurally unrelated to EB3. For this we chose a mutant form of GFP-CLIP-170 (GFP-CLIP170 Δ Hind), that lacks the C-terminal, metal-binding motif, as studies in non-neuronal cells have shown that this domain is responsible for the formation of patches of over-expressed protein at the cell periphery (Pierre *et al.*, 1994). When expressed in neurons, GFP-CLIP170 Δ Hind does not aggregate in patches as does the full-length GFP-CLIP170, but instead moves in comet-like dashes (see Movie 9).

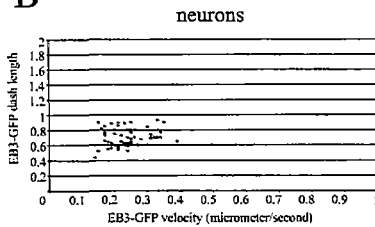
The measurements show that the average velocity of EB3-GFP movement is very similar in electroporated hippocampal neurons, infected PCs and in the single Golgi neuron taken along for evaluation (Table 2). In addition, the velocities of EB1-GFP ($0.22 \pm 0.07 \mu\text{m/s}$; $n = 39$ dashes) and GFP-CLIP170 Δ Hind ($0.25 \pm 0.05 \mu\text{m/s}$; $n = 5$ dashes) in electroporated hippocampal neurons are also comparable. EB3-GFP speeds do not vary remarkably within different neuronal compartments and there is no significant difference in average speed between comets moving in opposite directions (Table 2). The velocities measured in neurons are approximately half the speed of movement of EB3-GFP labeled plus-ends in COS-1 cells (compare Figure 3 and Table 1). One concern is that slight differences in the system set up and/or culture conditions might cause variations in EB3-GFP velocities in different cell types. We therefore measured velocities in neurons and glia, cultured in the same chamber. EB3-GFP speeds in glia are comparable to the values derived from COS-1 cells (Table 1). These data indicate that MT growth rates are generally lower in neurons, compared to glia and COS-1 cells. However, in the three retracting axons the average velocity of EB3-GFP dashes is significantly increased compared to normal values (Table 1; $p < 0.001$), yet it is still significantly lower than values measured in glia (Table 1; $p < 0.001$).

In the original studies on GFP-CLIP170 (Perez *et al.*, 1999) and EB1-GFP (Mimori-Kiyosue *et al.*, 2000b), the authors plotted the length of an individual comet, versus the speed of movement of that particular dash. A stochastic association and dissociation behavior was detected for both GFP fusion proteins, leading to variation in the length of the individual dashes over time. Still, an increased speed of the dash generally was associated with an increased length of the comet tail. We measured average lengths and corresponding speeds of a large number of the

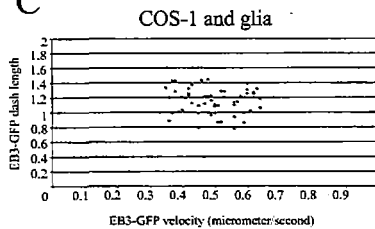
A



B



C



D

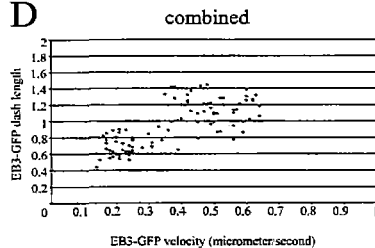


Figure 6. Distances traveled by EB3-GFP dashes in transfected neurons. **A:** Hippocampal neurons were transfected with EB3-GFP and cells were analyzed at 3-4 DIV by confocal microscopy in a 37°C chamber. The distance that individual EB3-GFP dashes could be followed was measured in different neuronal compartments (cell bodies, dendrites, axons and growth cones). The total number of individual MTs counted is indicated. In all compartments the distance traveled by EB3-GFP dashes is comparable. **B-D:** The average speed and length of a selected number of EB3-GFP dashes, derived from different measurements in neurons (**B**), COS-1 cells and glia (**C**) and the combined data (**D**) are plotted.

dashes used for the calculations in Table 1 (Figures 6B-6D). These data suggest that an increased speed of movement, as observed in the population of EB3-GFP dashes derived from COS-1 cells and glia, correlates with an increased length of EB3-GFP staining of a MT. The average speed of the selected population of COS-1 and glial dashes is $0.49 \pm 0.35 \mu\text{m/s}$, which corresponds to an average length of $1.14 \pm 0.16 \mu\text{m}$. For the neuronal dashes, these values are $0.23 \pm 0.25 \mu\text{m}$ and $0.70 \pm 0.11 \mu\text{m}$, respectively.

Table 2. Velocity of EB3-GFP dashes in neurons and glia

Neuronal compartment	Average velocity ^a (number of dashes measured)
Hippocampal cultures	
Cell body	0.22 ± 0.06 (64)
Neurites	0.22 ± 0.04 (52)
Neurite forward	0.22 ± 0.06 (91)
Neurite backward	0.21 ± 0.08 (20)
Growth cone	0.20 ± 0.07 (35)
Glia	0.43 ± 0.11 (63)
Retracting axons ^b	0.35 ± 0.06 (37)
PC cultures	
Dendrite forward	0.24 ± 0.07 (60)
Dendrite backward	0.26 ± 0.07 (21)
Axon forward	0.21 ± 0.04 (75)
Axon backward	0.24 ± 0.04 (8)
Cell body	0.22 ± 0.05 (20)
Golgi cell	0.28 ± 0.05 (12)
Glia	0.44 ± 0.11 (42)

^aμm/sec ± SD.^bEB3-GFP velocity derived from infected hippocampal cultures.

Discussion

Here we have investigated the behavior of the distal ends of growing MTs in cultured hippocampal and PCs by following the movement of GFP-tagged to EB3. Our conclusions are based on the assumption that EB3-GFP reliably marks the tips of growing MTs without changing MT growth parameters. Several observations support these statements. First, we show that endogenous EB3 co-localizes with the verified plus-end marker EB1 at MT distal ends in cultured hippocampal neurons and glia. The MT plus-end association of EB3 is further confirmed using anti-tubulin antibodies. In contrast to the other +TIPs analyzed, EB3 accumulates preferentially at neuronal plus-ends with respect to glial cells and no other intracellular structure is detected in neurons by the novel anti-EB3 antibodies. The MT plus-end association of EB3 is sensitive to nocodazole and taxol, drugs which perturb MT dynamics and which have been shown to abolish MT plus-end localization of several +TIPs (Perez *et al.*, 1999; Mimori-Kiyosue *et al.*, 2000b; Akhmanova *et al.*, 2001). Co-staining of EB3-GFP and tubulin in fixed neurons and COS-1 cells suggests that the comet-like GFP signals are located at the ends of MTs, in line with the endogenous EB3 localization. Addition of taxol and nocodazole abolishes EB3-GFP localization to MT distal ends, similar to the effect on endogenous EB3 and EB1. Furthermore, EB3-GFP, EB1-GFP and mutant GFP-CLIP170 show very similar behavior in transfected hippocampal neurons, suggesting that MT growth rates are not affected by low-level expression of these proteins. In a recent study, MT growth rates were measured in the same cell (or cytoplasm) that had been injected with fluorescent tubulin and that expressed GFP-CLIP170 (Komarova *et al.*, 2002a). MT growth rates (~0.30 μm/s) were similar using both fluorescent proteins, validating the use of +TIPs as markers for MT growth. Finally, a short report has recently appeared describing the behavior of EB1-GFP in a neuroblastoma cell line (Morrison *et al.*, 2002). Although this study is less detailed than our report, the properties and behavior of EB1-GFP in cells with neuronal features are consistent with our results in cultured hippocampal neurons and PCs.

The average distance traveled by an EB3-GFP dash is not directly comparable to the average length of growth of an individual MT. For example, two consecutive and nearby EB3-

GFP growth events that we record as separate may in fact represent a growth-pause-(or slow growth)-growth event of one individual MT. Moreover, as we used a confocal imaging system for our analysis, in the neuronal cell body some of the plus-ends may move in and out of focus, since the thickness of the optical slice is approximately 1 μm . Thus, we may not capture a complete growth spurt of an individual MT in the cell body. Note that in both examples mentioned, the EB3-GFP derived results underestimate the true value of the average length of an MT growth event. It is therefore likely that the latter are higher than the average distance traveled by EB3-GFP, which, based on the data in Table 1 and Figure 6A, we estimate to be approximately 1-2 μm . The average length of MTs in young hippocampal neurons is approximately 4 μm , both in minor processes and in an early axon (Yu and Baas, 1994). Although these studies were carried out in rat embryonic neurons, they compare well with our data, suggesting that the movement of EB3-GFP reflects the growth of MTs in differentiating neurons.

Except in the case of retracting axons, we find that the speed of growing MTs and the average duration of growth events, as measured by the movement of EB3-GFP, are similar in all neuronal compartments, i.e. cell bodies, dendrites, axons and growth cones. The graphs in Figures 6B-6D indicate a correlation between length of distal MT plus-end staining and speed of EB3-GFP movement, and do not suggest the existence of more than one type of EB3-GFP movement. For example, if a significant proportion of EB3-GFP would associate with shrinking MTs, one would expect two populations of dashes to come forward in the graph in Figure 6B, since MT shrinking rates are higher than MT polymerization rates (Komarova *et al.*, 2002a). Similarly, if molecular motors transported a significant population of EB3-GFP molecules, we would also expect to see this as a separate population in the graph, but we do not. We therefore propose that similar mechanisms underlie the dynamic association of EB3-GFP with MTs in neurons and non-neuronal cells.

We observed anterograde and retrograde movements of EB3-GFP in both hippocampal neurons of 2-6 DIV and differentiated PCs. Most bi-directional EB3-GFP movements were detected in proximal dendrites, while in axons the vast majority of dashes moved towards the growth cone (with the noted exceptions). The directionality of EB3-GFP dash movement agrees with observations in rodent hippocampal neurons, in which the differentiation of the axonal and dendritic compartments is accompanied by changes in the polarity of MTs, such that in (proximal) dendrites, MT polarity is mixed, with approximately half of the plus-ends distal to the cell body, while in axons, all MTs have a plus-end distal orientation (Baas *et al.*, 1988, 1989). This is further evidence for a preferential association of EB3-GFP with the ends of growing MTs. Taken together, our data suggest that local MT polymerization occurs throughout neurons, irrespective of MT organization. These polymerization events may contribute to the formation of the MT network. However, as EB3-GFP is only a marker for growing MT plus-ends, our analysis does not address the issue of movement of MT polymer in axons and dendrites, or what percentage it constitutes of the MT assembly process (Baas, 1997; Chang *et al.*, 1999; Terada *et al.*, 2000).

In young hippocampal axons, there is a shift towards shorter and longer MTs and the total content of MTs in the early axon increases about ten-fold, compared to minor processes (Yu and Baas, 1994). As the axon grows further, MT mass increases even more. We do not find an obvious increase in the amount of EB3-GFP dashes in axons compared to minor processes in electroporated hippocampal neurons of 2-6 DIV. This suggests that preferential MT stabilization in axons, compared to dendrites, and not increased MT polymerization frequencies, account for the increase in total MT polymer. It has indeed been found that the proportion of stable, nocodazole-resistant, MTs in axons is more than twice as high than that in dendrites (Baas *et al.*, 1991) and that preferential stabilization of MTs occurs in the proximal part of axons (Baas *et al.*, 1993). In addition, the labile MT proportion in axons is found as distinct domains at the plus-ends

of stable MTs (Baas and Black, 1990) and it has been shown that the plus-ends of stable MTs serve as the sole nucleating structures for MTs in the axon (Baas and Ahmad, 1992). These data suggest that MT polymerization can occur on existing MTs in axons.

Using SFV-mediated EB3-GFP expression, we were able to document MT growth events in highly differentiated PCs and hippocampal neurons; we also recorded EB3-GFP movements in three retracting hippocampal axons. Consistent with the result that MT polymer mass does not diminish significantly in these axons (He *et al.*, 2002), we detected EB3-GFP excursions into the growth cones of these axons, one of which was in the process of collapsing during the recording session (see Movie 7, supplemental information). Interestingly, MT growth rates are significantly higher in the three retracting axons that we observed, compared to MT growth rates under more static conditions. These increased MT growth rates must be balanced in order to maintain MT mass. We therefore hypothesize that MT dynamics are affected under conditions of axonal retraction, which may contribute to the curving and bending of MTs observed in these axons.

MT growth rates are in part determined by the concentration of free tubulin present in the cytoplasm of a cell. In neurons, the balance of MTs versus free tubulin may be tilted in favor of the MTs, such that less cytoplasmic tubulin is present than in non-neuronal cells. This may explain the lower neuronal MT growth rates we have observed. Another remarkable feat is the altered distribution of +TIPs at MT distal ends in cultured neurons versus non-neuronal cells. We have recently shown that reduction in the levels of another +TIPS, CLIP-115, leads to neuronal dysfunction (Hoogenraad *et al.*, 2002). The development of EB3-GFP as a tool for measuring MT growth rates will allow us to determine in more detail what the consequences are of a deletion of CLIP-115, as well as that of other +TIPs, on MT growth dynamics in mammalian neurons. This will be particularly interesting in view of the recent report that low dose application of taxol and nocodazole abolish the sensitivity to extracellular guidance cues of growth cones from embryonic *Xenopus* spinal neurons, whereas focal applications of these drugs cause growth cone turning without addition of the cues (Buck and Zheng, 2002).

CHAPTER 3.2

Alterations in microtubule dynamics in cultured hippocampal and cerebellar Purkinje neurons derived from CLIP-115 and CLIP-170 knockout mice

Chapter 3.2: Stepanova T, Miedema M, Slemmer JE, De Zeeuw CI, Akhmanova A, Galjart N. Alterations in microtubule dynamics in cultured hippocampal and cerebellar Purkinje neurons from CLIP-115 and CLIP-170 knockout mice. *Manuscript in preparation.*

Abstract

CLIP-115 and CLIP-170 are two related microtubule (MT) binding proteins involved in the regulation of MT dynamics and the action of the dynein/dynactin motor complex. In humans, the gene *CYLN2*, which encodes for CLIP-115, is located within a region that is hemizygously deleted in patients with Williams Syndrome (WS), a rare neurodevelopmental disorder. In mice, haploinsufficiency for the *Cyln2* gene mimics features of WS, including a mild growth deficiency, brain abnormalities, hippocampal dysfunctioning and particular deficits in motor coordination. CLIP-170 deficient mice also show behavioral problems, indicating that CLIP-115 and CLIP-170 play important and unique roles in neuronal functioning. Recent studies in Chinese hamster ovary (CHO) cells have shown that these proteins have redundant roles as rescue factors, acting in the cytoplasm to prevent MTs from shrinking completely back to the centrosome. Here we show that MT dynamics are also affected in cultured hippocampal neurons and cerebellar Purkinje cells (PCs), but not in glia, derived from single CLIP-115 or CLIP-170 knockout (KO) mice. In addition, MT growth rates are increased by ~1.5 fold, and the length of MT growth events is greater in hippocampal neurons from CLIP KO mice. Further study of CLIP-115 KO PCs revealed an increased number of MTs in proximal dendrites and reduced axonal lengths, as compared to wild-type cells. We suggest that the lack of either one of the CLIPs results in an altered MT balance in neurons. A higher free tubulin concentration could explain the observed increase in MT growth rates. Thus, CLIP-115 and CLIP-170 have non-redundant roles in the regulation of MT dynamics in cultured neurons, implicating MT defects in the pathogenesis of WS.

Introduction

Microtubules (MTs) are one of the three structures that constitute the neuronal cytoskeleton. MTs are essential for cell division, cell migration, vesicle transport and cell polarity (Gundersen, 2002). They are constructed from heterodimeric α - and β - tubulin subunits, which form protofilaments in a head-to-tail arrangement; thirteen protofilaments compose a polar tubular structure. Each MT has a faster-growing plus-end, and a slower-growing minus-end. In fibroblast-like cells, MTs are nucleated at the MT-organizing center (MTOC), which contains γ -tubulin and associated proteins. In most cases, minus-ends remain embedded to the MTOC, while plus-ends grow into the cytoplasmic space. Thus, with MT minus-ends bound to the MTOC, MT dynamics are determined mostly at plus ends, which can undergo rapid bouts of subunit addition, followed by depolymerization. For example, in Chinese hamster ovary (CHO) cells, it has been determined that MTs grow persistently to the cellular membrane (Komarova *et al.*, 2002a). Once the MTs reach this physical barrier, they significantly slow their growth velocity (Dogterom and Yurke, 1997), thus highly increasing their chances of undergoing a catastrophe, i.e. switching from growth to shrinkage. MT dynamics in neurons have been shown to be quite different. Although MTs are present throughout the whole cell (including axons, dendrites and growth cones), very few are attached to the centrosome found in the cell body. The majority of MTs are either released from the MTOC and tightly packed in the axons and dendrites, or are locally synthesized. In dendrites, MTs have a bi-directional orientation, with about half of the MT plus-ends directed towards the cell body, and the other half directed to the proximal part of the neuron (Baas *et al.*, 1988). In contrast, axonal MTs are uniformly oriented, with virtually all plus-ends pointing towards the growth cone (Baas *et al.*, 1988); precisely how the MT network reorganizes itself within neurons and how MT minus ends are stabilized remains unclear (Hirokawa *et al.*, 1997; Baas and Brown, 1997).

Recent data have revealed a heterogeneous population of MT plus-end binding or tracking proteins (+TIPs) that accumulate mainly at the distal ends of polymerizing MTs (Galjart and Perez, 2003), making them ideal candidates for the regulation of MT dynamics. These proteins include: EB1, EB3, APC, CLASP2, LIS1, CLIP-170, CLIP 115, and the dynactin complex (Komarova *et al.*, 2002a; Mimori-Kiyosue *et al.*, 2000a, 2000b; Stepanova *et al.*, 2003; Akhmanova *et al.*, 2001; Coquelle *et al.*, 2002; Perez *et al.*, 1999; Hoogenraad *et al.*, 2000; Vaughan *et al.*, 1999). The protein tip1p—the CLIP-170 homologue from yeast—has been shown to be involved in regulation of MT dynamics. In wild-type fission yeast, MTs that touch the cell cortex near the middle of the cell do not undergo catastrophe, but continue to grow and curve along the cortex toward the cell tip. When tip1p is absent, however, MTs undergo catastrophe (Brunner and Nurse, 2000), resulting in shorter MTs. The fact that the frequency of catastrophe after the deletion of tip1p increases in specific cell regions suggests that tip1p could be an anti-catastrophe factor which delays MT catastrophe until MTs have grown into cortical regions of cellular poles (Brunner and Nurse, 2000).

Since tip1p is the fission yeast homolog of CLIP-170 (notably, the tip1p deletion mutant that is able to rescue MTs in central regions of the fission yeast cell resembles CLIP-115), it was interesting to find out whether CLIPs can have a similar influence on MT dynamics, i.e. as anti-catastrophe factors, in mammalian cells. As mentioned above, it was shown that in CHO cells MTs grow almost continuously from the centrosome to the cell periphery. Once in the periphery, MTs become especially dynamic and frequent rescues prevent them from complete shrinkage to the centrosome (Komarova *et al.*, 2002b). In order to keep MT growth persistent, CHO cells have low catastrophe frequency in the cytoplasm and high rescue frequency and rapid growth velocity. Studies of MT dynamics in mammalian cells suggest that CLIP-170 and CLIP-115 may play a similar role as tip1p in fission yeast (Komarova *et al.*, 2002b). By removing both CLIPs from MT ends, using a dominant negative CLIP-170 Δ head mutant, it was shown that MT rescue frequency decreased seven fold, resulting in MTs shortening all the way back to the centrosome. The other parameters of dynamic instability such as velocities of MT growth or shortening and catastrophe frequency were not changed. These studies suggest that CLIPs act as rescue factors, or that they recruit a factor, which provokes MT rescue (Komarova *et al.*, 2002b). Interestingly, rescue in the cytoplasm is restored by expression of the head domain of the CLIPs, which contains a conserved MT binding motif. Since both CLIPs contain such motifs, they are in principle redundant factors with respect to promoting MT rescue. In fission yeast, it was shown that a region including this motif (the CAP-GLY domain) is sufficient for interaction of tip1p with mal3p (Busch and Brunner, 2004). It is possible that the overexpression of the MT binding motif of the CLIPs in CHO cells recruits EB1 to shrinking MTs and induces rescue.

Williams Syndrome (WS) is a rare neurodevelopmental disorder which is caused by the hemizygous deletion of approximately 20 genes (or 1.6 Mb on chromosome band 7q11.23; Francke, 1999), including *CYLN2*, the gene which codes for CLIP-115 (Hoogenraad *et al.*, 1998; Osborne *et al.*, 1996). In mice, *Cyln2* is located at the telomeric end of chromosome 5 (Tassabehji *et al.*, 1999) in an area orthologous to human chromosome 7q11.23 (Valero *et al.*, 2000). We have shown that the deletion of the *Cyln2* gene in mice partially mimics some of the features of WS patients, in particular a mild growth deficiency, and neuronal dysfunction, including hippocampal deficiency and particular deficits in motor coordination (Hoogenraad *et al.*, 2002). Importantly, these deficits were also observed in heterozygous *Cyln2* mice, indicating that haploinsufficiency for *CYLN2* is linked to neurodevelopmental features of WS (Hoogenraad *et al.*, 2002).

We have recently generated CLIP-170 knockout (KO) mice, which show no obvious phenotype, other than severely decreased fertility in male mice and mild behavioral abnormalities (Akhmanova *et al.*, manuscript in preparation). In order to understand how reduced levels of

CLIP-115 may contribute to the neurological phenotype of WS, we studied MT dynamics in neurons and glia from CLIP-115 and CLIP-170 deficient mice. Studies on primary fibroblasts cultured from CLIP-115 KO mice revealed that the deletion of CLIP-115 did not significantly alter MT dynamics. This corresponds well with previous data gathered from CHO cells, in which CLIP-115 and CLIP-170 have redundant functions with respect to MT dynamics. Here we measured MT growth rates in cultured hippocampal neurons and glia, and in cerebellar Purkinje cells (PCs) and glia, from single CLIP KO mice. We also quantified the number of MTs (via intensity of β -tubulin antibody staining) in cultured neurons, and axonal and neuritic lengths in PCs and hippocampal neurons, respectively, from CLIP-115 KO and wild-type mice.

Materials and Methods

Cell cultures

The generation of CLIP-115 KO mice has been previously described (Hoogenraad *et al.*, 2002). The generation of CLIP-170 KO mice will be described elsewhere in detail (Akhmanova *et al.*, manuscript in preparation). Mouse hippocampal neurons, and cultures enriched in cerebellar PCs, were obtained from embryos (day E17.5-18) from timed matings of homozygous male and female CLIP-115, or CLIP-170, KO mice as previously described (Stepanova *et al.*, 2003). Wild-type cultures were obtained from C57/bl6 or FVB/N mice.

Antibodies and immunocytochemistry

Cell cultures were fixed with methanol/paraformaldehyde and stained with different antibodies [CLIP-115 and -170 specific antisera (2221; 2238 and 2360); EB3 rabbit polyclonal antibodies (02-1005-07); monoclonal antibodies against EB1 (Transduction Laboratories); pan-EB specific antisera; MAP2 polyclonal antibodies (Jackson Immunolabs); calbindin-D28K polyclonal antibodies (Jackson); actin (Chemicon); acetylated α -tubulin, and tyrosine α -tubulin and β -tubulin (Sigma)] as described previously (Stepanova *et al.*, 2003; Slemmer *et al.*, 2004a). FITC-conjugated goat-anti-rabbit (Nordic Laboratories), Alexa 488-conjugated goat-anti-rabbit, Alexa 594-conjugated goat-anti-mouse and Alexa 594-conjugated goat-anti-rat (Molecular Probes) were used as secondary antibodies. For β -tubulin intensity quantification, hippocampal neurons were co-stained with MAP2, and PCs were co-stained with calbindin. Signals were captured as described previously (Stepanova *et al.*, 2003; Slemmer *et al.*, 2004a). Images were acquired using either a Leica DMRBE microscope, with a 16x, a 64x or a 100x oil immersion lens (NA 1.3) and a Hamamatsu C4880 camera, or a Zeiss LSM510 confocal microscope with a 63x oil immersion lens (NA 1.4).

Live-cell imaging and calculation of MT growth parameters

Cultures were infected with EB3-GFP to visualize MT growth rates. This fusion protein was chosen not only because it marks well the tips of growing MTs, but also because its presence in a neuron at low expression levels does not appear to disturb neuronal growth and differentiation in culture. We used Semliki Forest Virus (SFV) particle-mediated infection to introduce EB3-GFP into hippocampal neurons of 6-8 days *in vitro* (DIV) and into PCs of more than 14 DIV (Stepanova *et al.*, 2003). Infected neurons were analyzed at 37°C on a Zeiss LSM510 confocal laser-scanning microscope as described previously (Stepanova *et al.*, 2003). Images of GFP-TIP

movements in infected cells were acquired every 1-3 sec. Images were recorded and movies were assembled using accompanying LSM 510 software.

After recording a time-lapse movie, we visually inspected and included only those EB3-GFP dashes or comets that could be followed for at least three consecutive frames. All measurements were performed on different areas of cultured neurons. The velocities of the EB3-GFP dashes were calculated by dividing the distance traveled by a dash by the time it spent traveling. The average duration of MT growth events were estimated in neurons from both KO and wild-type mice.

Quantification of β -tubulin intensities

Confocal images of fixed hippocampal neurons and glia, and of cerebellar PCs and glia derived from CLIP-115 KO and wild-type mice, were analyzed using the LSM Reader plug-in for ImageJ (NIH Software). Tubulin intensities of dendritic regions proximal to the soma of hippocampal neurons and cerebellar PCs were quantified by first drawing a region of interest (ROI) in the MAP2 or calbindin image to ensure precise neuronal labeling. The tubulin intensity was then divided by the surface area (in μm^2) of the ROI. Tubulin ratios were then divided by the mean ratio of the corresponding glia to serve as an internal control (i.e. KO hippocampal neurons were divided by KO hippocampal glia).

Quantification of axonal and neuritic length

Images of fixed hippocampal neurons and cerebellar PCs derived from CLIP-115 KO and wild-type mice were taken at $\times 16$. Neuritic length (both total length of all branches and length of main neurite) for hippocampal neurons, and axon length (both total length of all branches and length of main axon) for PCs were measured using the NeuronJ plug-in for ImageJ. This program allows for a precise tracking of specific neuronal compartments.

Results

Immunocytochemistry on fixed preparations of CLIP deficient neurons

We have previously demonstrated that several +TIPs localize not only to the distal ends of MTs in fixed preparations, but that they can also be seen as fluorescent dashes in living neurons (Stepanova *et al.*, 2003). The latter are likely to represent the plus-ends of growing MTs. A recent report fully supports our data, demonstrating movement of MT plus-ends in the axonal shaft of cultured embryonic *Xenopus* neurons expressing GFP-EB1 (Ma *et al.*, 2004). Immunostaining of cultured neurons from single CLIP-115 and CLIP-170 KO mice with polyclonal antibodies against either CLIP reveals a lack of specific MT plus-end staining (data not shown). In contrast, co-staining with anti- β -tubulin and anti-EB antibodies reveals that MT plus-ends are present throughout all neuronal compartments of each KO genotype. Prototypical images of a CLIP-115 KO cerebellar glial cell and hippocampal neuron, stained against EB3 and β -tubulin, are shown in Figure 1. Removal of either of the respective CLIPs does not cause any obvious morphological alteration in cultured neurons (data not shown).

Interestingly, an enhanced accumulation of CLIP-170 and dynactin was seen at MT ends of primary fibroblasts from CLIP-115 deficient mice (Hoogenraad *et al.*, 2002), suggesting that CLIP-115 and CLIP-170 normally compete for binding to MT plus ends. It was suggested that, in

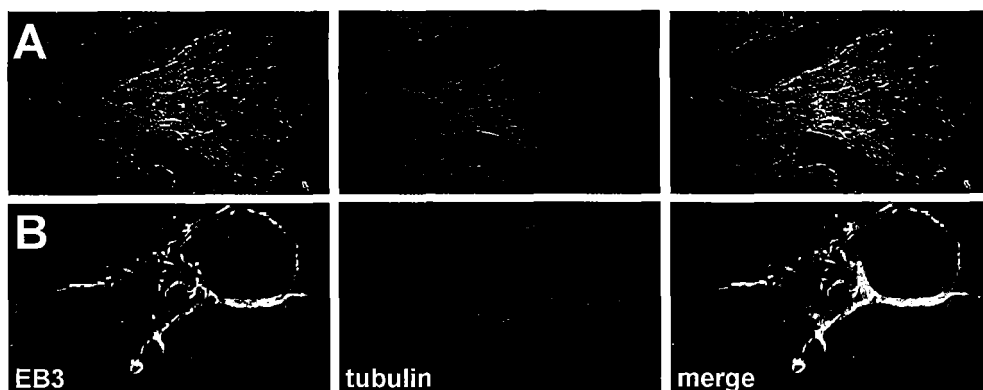


Figure 1. EB3 and β -tubulin labeling in cells derived from CLIP-115 knockout mice. A: Cerebellar glia cell labeled with EB3 and β -tubulin antibodies. Note the clear plus-end labeling in the merged image. B: Hippocampal neuron stained with same antibodies as in A.

normal fibroblast-like cells, the cytoplasmic concentration of each CLIP is in great excess of the total amount of available plus-end binding sites, so that when one CLIP is absent, the other one can occupy more binding sites. We were interested whether a competition occurs between +TIPs for plus end binding in neurons. Data obtained from the immunocytochemical experiments, however, did not demonstrate any accumulation of CLIPs, dynactin or EBs on the distal ends of neuronal MTs in primary cultures from KO mice (data not shown).

MT growth rates in CLIP deficient neurons

Because CLIPs may compete for binding to MT plus-ends (Hoogenraad *et al.*, 2002) and play redundant roles as rescue factors in MT dynamics in CHO cells (Komarova *et al.*, 2002b), we studied MT dynamics, specifically in terms of growth velocity, in neurons and glia derived from CLIP-115 and CLIP-170 KO mice. We expressed the fusion protein EB3-GFP as a marker for growing MT ends in cultured hippocampal neurons. Strikingly, we found a significant increase ($p < 0.0001$) of ~ 1.5 fold in MT growth velocities in neurons derived from either the CLIP-115 or the CLIP-170 KO mice, as compared to wild-type mice (see Table 1). Three observations strongly suggest that this increase reflects a physiological change in MT dynamics in the CLIP-deficient neurons. First, the increase is measured in KO neurons only, as MT growth rates in glia from the same culture dish are comparable to those in glia from wild-type mice. Second, we tested whether differences in the genetic background of different mouse strains influenced MT growth rates, but found that wild-type neurons and glia from FVB/N mice had virtually identical values as those from C57/bl6 mice (Table 1). Third, MT growth rates in glia and wild-type neurons are identical to our previous report (Stepanova *et al.*, 2003). Interestingly, EB3-GFP velocities in PCs from CLIP-115 KO mice demonstrated the same increase as in infected hippocampal neurons, suggesting a pan-neuronal effect of the lack of CLIPs.

Average growth velocities are calculated by dividing the distance that one EB3-GFP dash travels by the time one can follow this dash, and then by taking the average of multiple dashes in a number of cells (Table 1). When these two parameters are plotted against each other (Figure 3A), two features stand out. First, in the KO neurons, many of the fluorescent dashes can be followed for longer time periods than in wild-type neurons, indicating that a substantial

	hippocampal neurons	Purkinje cells	glia
wild-type (FVB)	0.22 ± 0.05 (n = 88, 14 cells)	0.23 ± 0.05 (n = 58, 9 cells)	0.43 ± 0.11 (n = 63, 10 cells)
wild-type (C57/bl6)	0.23 ± 0.03 (n = 54, 9 cells)	N.D.	0.41 ± 0.02 (n = 26, 5 cells)
CLIP-115 knockout	0.32 ± 0.04 (n = 81, 15 cells)	0.31 ± 0.03 (n = 15, 5 cells)	0.41 ± 0.05 (n = 44, 8 cells)
CLIP-170 knockout	0.33 ± 0.05 (n = 88, 11 cells)	N.D.	0.42 ± 0.07 (n = 64, 13 cells)

Table 1. MT growth rates in cultured neurons and glia. Average velocities ($\mu\text{m/s}$) of EB3-GFP fusion protein in neurons and glial cells from wild-type and both knockouts of CLIP mice. Cells expressing EB3-GFP were monitored at 37°C on Zeiss LSM 510 confocal microscope and velocities were analyzed. n = total number of MTs. N.D. = not determined. Data are expressed as means and standard deviation of the mean.

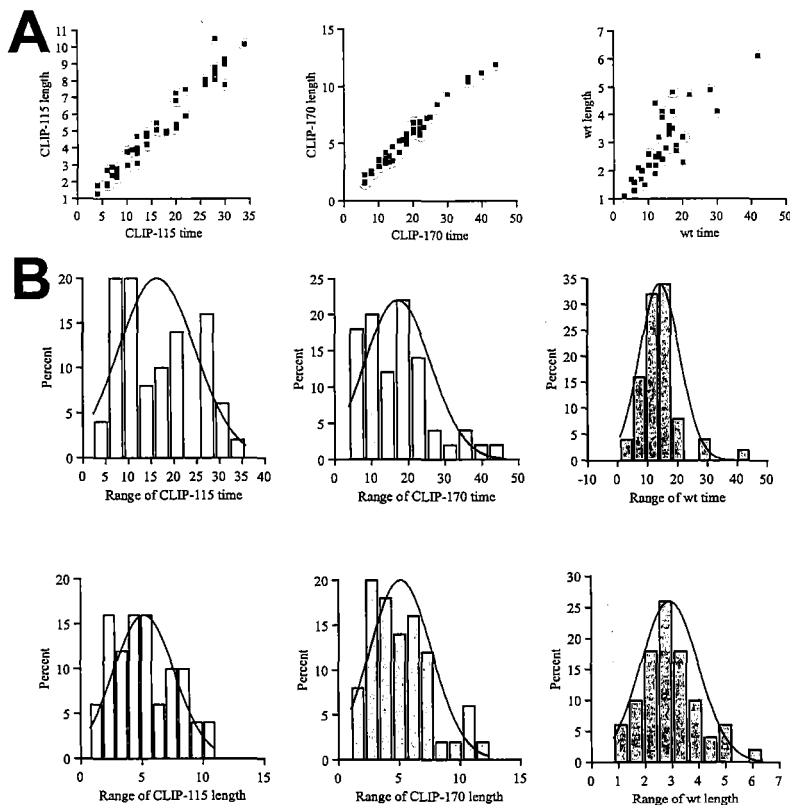


Figure 2. Comparison of MT growth events in neurons of different genotypes. Hippocampal neurons expressing EB3-GFP were analyzed by confocal microscopy in a 37°C chamber. The distances that individual EB3-GFP dashes could be followed were measured, independent of neuronal compartment, in neurons from wild-type and knockout mice. **A:** The correlation between distance of movement (plotted as length in μm ; y-axis) and the duration of movement (plotted as time in s; x-axis). **B:** The duration of growth events (plotted as “range of time”) and their distance (plotted as “range of length”) are represented in a histogram of distribution versus probability.

fraction of MT growth events persist in KO cells. Second, irrespective of the genotype of neurons, there is a good correlation between distance of movement (plotted as length, y-axis) and the duration of movement (plotted as time, x-axis), indicating that all dashes move with a similar speed, including those dashes that can be followed for longer periods of time. When duration (plotted as “range of time”) and distance (plotted as “range of length”) of EB3-GFP dashes are represented in a histogram of distribution versus probability (Figure 3B), the difference between the narrow range in wild-type neurons, and the much wider range in KO cells, is clear. This is reflected by the “normal distributions” of time (see curves in upper panel of Figure 3B), which have a similar midpoint peak in the three genotypes, but are much broader in CLIP-deficient cells. Interestingly, in CLIP-115 neurons, two peaks appear to be present with a midpoint at ~9 seconds and at ~25 seconds.

β-tubulin intensities and axonal length in CLIP-115 deficient neurons and glia

In order to determine intracellular changes in the amount of tubulin in hippocampal neurons and PCs, mean β-tubulin antibody staining was measured in proximal dendrites. Regions of interest of similar size were drawn in corresponding glia. Table 2 shows data gathered from neurons and glia from CLIP-115 KO and wild-type mice. There are no significant differences between KO and wild-type hippocampal neurons, or between KO and wild-type glia derived from either the hippocampus or the cerebellum. There was, however, a significant difference between KO and wild-type PCs ($p < 0.05$), indicating increased MT number in KO proximal dendrites.

	wild-type	CLIP-115 knockout
hippocampal glia	1.05 ± 0.10 ($n = 10$)	1.11 ± 0.17 ($n = 10$)
hippocampal neurons	2.95 ± 0.41 ($n = 10$)	2.38 ± 0.23 ($n = 9$)
cerebellar glia	1.87 ± 0.21 ($n = 10$)	1.51 ± 0.26 ($n = 10$)
Purkinje cells	1.08 ± 0.05 ($n = 10$)	$1.54 \pm 0.15 *$ ($n = 10$)

Table 2. Tubulin intensities in cultured neurons and glia from wild-type and CLIP-115 knockout mice. Ratios of mean tubulin intensity per surface area (μm^2) are given for hippocampal neurons and glia and for cerebellar Purkinje cell (PCs) neurons and glia. $*p < 0.05$ compared to wild-type cells. n = total number of cells analyzed. Data are expressed as means and standard error of the mean.

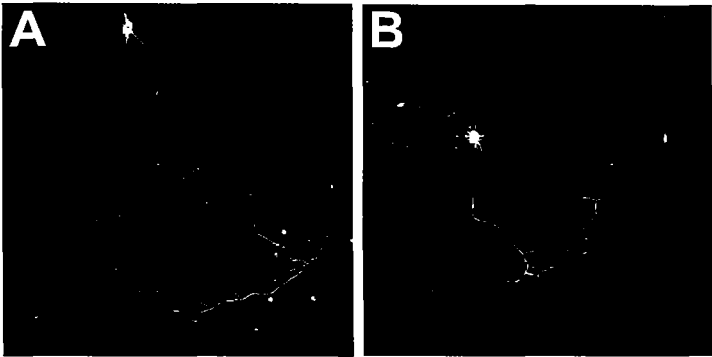


Figure 3. Axonal length in CLIP-115 knockout (KO) and wild-type Purkinje cell (PC) neurons. A: CLIP-115 wild-type PC. B: CLIP-115 KO PC. Note that both the main axon and the total axonal length (main axon plus its branches) are shorter in B as compared to A.

In order to examine further the deletion of CLIP-115 on MT dynamics, we measured axonal and neuritic lengths in PCs and hippocampal neurons, respectively. We found that CLIP-115 KO PCs had significantly shorter main axons ($p < 0.05$; $n = 16$) than wild-type cells; total axonal length (the main axon plus its branches) was also significantly less in KO cells ($p < 0.05$; $n = 16$). Prototypical images of KO and wild-type PCs are shown in Figure 3. In contrast, neither measurement of neuritic length was found to be significant between KO and wild-type hippocampal neurons.

Discussion

Because both CLIP-115 and CLIP-170 may play important, potentially redundant, roles in MT dynamics, and because CLIP-115 KO mice have an interesting neurological phenotype related to WS (Hoogenraad *et al.*, 2002), we studied MT dynamics in neurons from both single CLIP KO animals. Importantly, cultured hippocampal neurons and cerebellar PCs from single CLIP KO animals appeared to develop and differentiate normally, showing no obvious morphological differences as compared to wild-type neurons.

It was shown previously that velocities of MT growth in normal neurons ($\sim 0.20 \mu\text{m/s}$) do not vary in different compartments of the cell (Stepanova *et al.*, 2003). MT growth behavior in retracting axons during growth cone collapse, however, represents a notable exception to normal MT dynamics in neurons (Stepanova *et al.*, 2003). We observed intensive MT growth in retracting axons and, surprisingly, we found that the MT growth rates were significantly higher compared to parameters from “static” axons. During axonal retraction, MTs undergo both reconfiguration and depolymerization in addition to growth. Since the growth rate of MTs is dependent on the concentration of free tubulin molecules in the cytoplasm, the balance between free cytoplasmic tubulin and the polymeric form is one of the important parameters for MT growth rate and persistence. If there is more MT depolymerization in a retracting growth cone than elsewhere in the neuron, then this local increase of cytoplasmic tubulin concentration could lead to enhanced MT polymerization velocities in retracting axons. We propose that an altered MT-tubulin balance also underlies the change in MT growth rates in neurons from CLIP-deficient mice.

Interestingly, we found that CLIP-115 KO PCs displayed significantly shorter axons than wild-type cells. According to Komarova *et al.* (2002b), MTs are more prone to depolymerize and collapse in cells lacking the stabilizing MAP, CLIP-170. In addition, CLIP-170 interacts with IQGAP1 in order to mediate between the MT and actin cytoskeletons in growth cones by capturing MT plus-ends (Fukata *et al.*, 2002; also see Figure 3 in Chapter 4). Thus, it is possible that CLIP-115 plays a similar stabilizing role in PC axons, and the deletion of CLIP-115 could limit overall axonal growth by reducing the efficiency of MT-actin cooperation. In other words, faster MT growth velocities do not necessarily imply longer, stable MTs: even though growth rates are significantly faster in KO cells, the lack of CLIP-115 could instigate not only more catastrophes, but faster MT retraction rates. Proximal dendrites from KO PCs had higher tubulin intensities than wild-type cells, indicating a larger number of MTs in these structures. In contrast, overall MT length in KO PC axons may be considerably shorter, as the lack of CLIP-115 would increase the probability of shrinking. Indeed, the deletion of tip1p, which was shown to resemble CLIP-115, in fission yeast, resulted in shorter MTs (Brunner and Nurse, 2000).

The intrinsic differences between axonal and dendritic MTs may determine the differential effects of the deletion of CLIP-115 on MT growth and structure in KO PCs. Although CLIP-115 is ubiquitous in PCs, the deletion of this protein may have different effects between axons and dendrites. Indeed, MAP2, a dendrite-specific MAP in neurons, may play a role in

establishing neuronal polarity by specifying dendritic and axonal routes of transport from the cell body (Bernhardt and Matus, 1984). The fact that differences were found in PCs and not in hippocampal neurons makes this argument compelling. We did not find any significant differences between CLIP-115 KO and wild-type hippocampal neurons, even though MT growth rates in KO cells were enhanced. This may be due in part to the fact that accurate measurements of neuritic length were more difficult in hippocampal cultures. Both MAP2 and β -tubulin antibody staining presented a high background levels, whereas calbindin antibody staining is very specific for PCs, giving a clear distinction between cell and background as can be seen in Figure 3. In any case, although not significant, β -tubulin intensities—and therefore MT quantities—were slightly higher in wild-type hippocampal neurons as compared to KO cells (Table 2), again indicating that PCs may possess unique MT properties.

Studies in CHO cells have shown that CLIPs play a role as rescue factors in the cytoplasm, where they mediate the transition from shrinkage to growth (Komarova *et al.*, 2002b). Consequently, in normal CHO cells, only a short part of the MT will depolymerize before being rescued by CLIP (and interacting factors). The removal of both CLIPs from MT tips in CHO cells leads to the complete depolymerization of MTs back to the MTOC (Komarova *et al.*, 2002b). It is possible that in CLIP KO neurons an increased, persistent MT depolymerization also occurs which enlarges the cytoplasmic pool of free tubulin and results in the increase of polymerization speed of MTs. Because the chance of a catastrophe is inversely correlated to MT growth velocity (Dogterom and Yurke, 1997), an increased MT growth velocity should be accompanied by increased time and length of MT growth events, that is, if no barriers, such as membranes, are encountered in the growth spurt. This is indeed what our data showed: increased MT growth rates are accompanied by longer growth spurts in neurons from the CLIP-deficient mice. MT growth velocities in glial cells of KO animals were not altered as compared to wild-type glia. These data correspond to our results involving MT growth rates in fibroblasts, where the lack of CLIP-115 did not affect MT growth velocities (Hoogenraad *et al.*, 2002). We also did not detect different growth rates in primary fibroblasts from CLIP-170 KO mice (data not shown). Thus, fibroblasts and glial cells act in a similar manner when a single CLIP is deleted.

If CLIP-115 and CLIP-170 are redundant MT rescue factors in CHO cells (Komarova *et al.*, 2002b), why does the removal of one of the CLIPs influence MT growth velocities specifically in neuronal cells, but not in fibroblast-like cells? Several explanations, which are not mutually exclusive, could account for this fact. First, the types and amounts of +TIPs greatly vary in glia, fibroblasts and hippocampal neurons. CLIPs appear to be more abundant in glia than in neurons, whereas the reverse is true for EB3 (Stepanova *et al.*, 2003). EB1 is equally expressed in glia and hippocampal neurons. Interestingly, differentiated PCs contain absolutely no EB1, but express EB3 instead (data not shown). A higher level of CLIPs in glia and fibroblasts indicates that the absence of one CLIP can be compensated for by the other. In neurons, the levels of each CLIP may be too low to allow for compensation. If this is the case, neuronal MTs that begin to depolymerize from their plus-ends are not rescued frequently enough, leading to an increased level of free tubulin.

The second explanation concerns the way MTs are organized in different cell types. In dividing cells, interphase MTs are anchored at the centrosome by minus-ends and their plus-ends are directed to the cell periphery. Thus, MT dynamics is regulated at the plus-end by dynamic instability (one could also state that the MTOC is a major regulator of MT dynamics by eliminating the minus-end pathway). However, in many terminally differentiated cells, such as neurons (Baas *et al.*, 1988) or epithelial cells (Mays *et al.*, 1994), MTs are not bound to the centrosome but are distributed in the cytoplasm, including axons and dendrites. It is not yet clear how MTs elongate in these latter compartments, or how neurons maintain total MT mass, an amount that increases

tremendously during development. In our previous work, we observed events of MT growth in all neuronal compartments and at different stages of development with velocities and number of MT growth events being similar and not depending on stage of differentiation (Stepanova *et al.*, 2003). We proposed that local MT polymerization takes place throughout neuronal cells. During development, the stabilization of MTs in particular compartments may cause the accumulation in total MT polymer (Baas *et al.*, 1993). It has been shown that MT nucleation in neurites occurs exclusively on already-existing, stable MTs (Baas and Ahmad, 1992). If shrinking MTs are rescued less frequently in CLIP-deficient neurons, they shrink further back and eventually eliminate part of the pool of MT nucleation sites in axons and dendrites, thus leading to a decreased amount of MTs and, consequently, increased tubulin levels. This, in turn, results in higher MT growth rates and increased growth duration times. Finally, it is possible that the minus-end pathway also plays a role in sustaining normal MT-tubulin ratios in neurons by providing free tubulin through the depolymerization from selective minus-ends. If this pathway is altered in CLIP-deficient neurons, it might also contribute to increased tubulin levels. If the compensation theory is correct, then the removal of both CLIPs in fibroblasts is expected to lead to increased MT growth rates. CHO cells lacking both CLIPs from MT plus ends, however, showed no differences in MT growth velocity (Komarova *et al.*, 2002b). We hypothesize that, in these cells, more frequent formation of new MTs at the centrosome occurs, so that the MT balance is maintained. In neurons, this form of compensation cannot occur, because most MT synthesis does not originate at the MTOC.

CHAPTER 3.3

Alterations in hippocampal dendritic and spine morphology in the *Cyln2* knockout mouse: a model of Williams Syndrome

Chapter 3.3: Marrone DF, Slemmer JE, LeBoutillier JC, Pearson V, Haasdijk ED, Petit TL, De Zeeuw C
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Abstract

Williams Syndrome (WS) is a rare neurodevelopmental disorder caused by the hemizygous deletion of approximately 20 genes on chromosome band 7q11.23, a region containing the *CLYN2* gene, which encodes the cytoplasmic linker protein CLIP-115. Deletions within this region result in a unique phenotype with associated mental retardation (MR). Neuronal cytoarchitectonic analysis for several genetic disorders with MR, including WS, has provided substantial support for the theory that a major factor underlying MR is dendritic abnormalities. CLIP-115 is a brain-specific protein that associates with growing microtubule plus-ends; knockout (KO) mice lacking the *Cyln2* gene show strong hippocampal dysfunction when subjected to behavioral and electrophysiological tests. We investigated dendritic and spine morphology of CA1 pyramidal and dentate granule cells using the rapid-Golgi technique in KO mice and wild-type littermates. CA1 pyramidal cells from KO mice displayed increased apical and basilar dendritic lengths; Sholl analysis indicated that this deletion was correlated with an increase in dendritic material in CA1 neurons and increased branching complexity in the apical region. Increased spine densities were found in outer apical CA1 dendrites and in both the medial and distal region of dentate granule cells. Region-dependent differences in spine morphology—for example, an increased number of thin spines in KO mice—were observed in CA1 region, and corroborated by electron microscopy. Lastly, the ratio of synaptophysin to MAP2 intensity in CA1 dendrites indicated fewer synapses in KO mice. These changes may present a neural substrate for the unique behavioral phenotype associated with WS.

Introduction

Williams Syndrome (WS) is a rare (1 in 20,000 births) neurodevelopmental disorder first identified in 1961 (Williams *et al.*, 1961). The genetic basis of WS is associated with erroneous recombination events leading to chromosomal deletions within a 1.6 Mb region of chromosome 7 (7q11.23). This region, often referred to as the Williams Syndrome Critical Region (WSCR), has been shown to encode up to 20 genes (Francke, 1999). Deletions within this critical region result in a unique phenotype (McKusick, 1988; Bellugi *et al.*, 2000), characterized by a relative preservation of language and face-processing skills alongside profound deficits in visuospatial abilities and mental retardation (MR).

The *CYLN2* gene encodes the cytoplasmic linker protein CLIP-115, which associates with the plus-ends of neuronal microtubules (MTs) primarily in the hippocampus, the amygdala, and the cerebellum (De Zeeuw *et al.*, 1997; Hoogenraad *et al.*, 2000; Stepanova *et al.*, 2003). The fact that MT regulation is essential to cell development and plasticity (Mattson, 1999; Baas, 2002) makes the CLIP family of proteins a prime candidate for mediating the deficits seen in WS. In fact, MT organization has been investigated in several genetic disorders associated with MR, implicating aberrant MT network organization in the development of dendritic abnormalities (Bodick *et al.*, 1982). The deletion of *CLYN2* may cause alterations in overall MT organization, thus influencing dendritic and spine morphology. While similar morphological abnormalities may exist in WS, the effects of this disorder on dendritic and spine morphology are not well understood (Kaufmann and Moser, 2000).

We have previously generated a knockout (KO) mouse lacking the *Cyln2* gene (Hoogenraad *et al.*, 2002). This targeted deletion resulted in anatomical and behavioral phenotypes reminiscent of WS, including increased ventricular volume and decreased callosal volume, similar to those observed with MRI investigations in WS patients (Schmitt *et al.*, 2001;

Tomaiuolo *et al.*, 2002). In addition, KO mice exhibited poor contextual fear conditioning and normal cued fear conditioning, indicating that the deletion of *Cyln2* specifically affects hippocampal-dependent performance (Hoogenraad *et al.*, 2002). Again, this resembles the typical cognitive profile of WS patients (McKusick, 1988; Bellugi *et al.*, 2000). In addition, KO mice demonstrated hippocampal and cerebellar dysfunction, possibly due to altered MT-dependent intracellular transport (Hoogenraad *et al.*, 2002).

CLIP-115 and other related proteins (e.g. CLIP-170, EB1 and EB3), which interact specifically with growing MT plus-ends, could play a vital role in actin-MT interaction, as the haploinsufficiency for the *CYLN2* gene in humans could partially account for the marked neurodevelopmental deficits seen in William's syndrome (Franke, 1999; Hoogenraad *et al.*, 1998). Indeed, CLIP-170, a related protein, has been shown to participate in the coordination of the actin and MT cytoskeletons in growth cones (Fukata *et al.*, 2002). In the present study, we analyzed dendritic arborization, spine density, and spine morphology in hippocampal CA1 pyramidal and dentate granule cells using the rapid-Golgi technique, and examined individual spines at the electron microscopic (EM) level. Lastly, synaptophysin, a protein found in synaptic vesicles, and microtubule-associated protein 2 (MAP2) intensities were measured in CA1 dendrites in whole brain slices in order to quantify better the amount of viable synapses.

Materials and Methods

Rapid-Golgi technique

Three *Cyln2* KO mice (see Hoogenraad *et al.*, 2002) and three *Cyln2* wild-type littermates (age: 5 months) were sacrificed, and dissected brains were fixed with 2% paraformaldehyde supplemented with 0.2% glutaraldehyde in PBS buffer. Complete right hemispheres were immersed in 1% osmium dichromate (24°C) for five days [left hemispheres were used for Electron Microscopy and Immunohistochemistry; see below]. Hemispheres were blotted, placed into 0.75% silver nitrate for 24 hr, and dehydrated through a graded series of alcohol, ending in a 1:1 diethyl ether/ethanol solution, followed by 1:1 low-viscosity nitrocellulose/ether mixture for 3 days. The tissue was then embedded in this mixture overnight, and serial 100- μ m-thick coronal sections were taken using a sliding microtome.

Ten, fully impregnated cells (see Figure 1A) were blindly selected at random from both the CA1 region and the dentate gyrus (DG) from each animal, and were drawn with a camera lucida (312.5 $\times$) using a Lietz Orthoplan microscope. Dendritic lengths were measured from the perikarya center to the most distal dendritic point, parallel to the long axis. Maximal dendritic widths were measured perpendicular to this axis. The length and width of apical and basilar dendritic fields of CA1 pyramidal cells were measured separately, while single measurements were made for DG cells.

Several indicators of dendritic arborization were also employed. The Sholl (1953) technique (see Figure 1B) was utilized by overlaying the drawing with a series of concentric circles (20 μ m apart) and recording the number of dendritic processes intersecting each circle to a maximal distance of either 360 μ m (CA1 apical dendrites) or 240 μ m (CA1 basilar and granule cell dendrites). In addition, branching order was calculated for both terminal and non-terminal dendrites in all cells. The primary apical dendrite was assigned a branch order of zero; dendritic processes originating from that dendrite are first order branches; subsequent bifurcations are second order; and so forth (Uylings *et al.*, 1986). Dendritic spine density and morphology were analyzed at 1000 $\times$ under oil immersion. The total number of dendritic spines

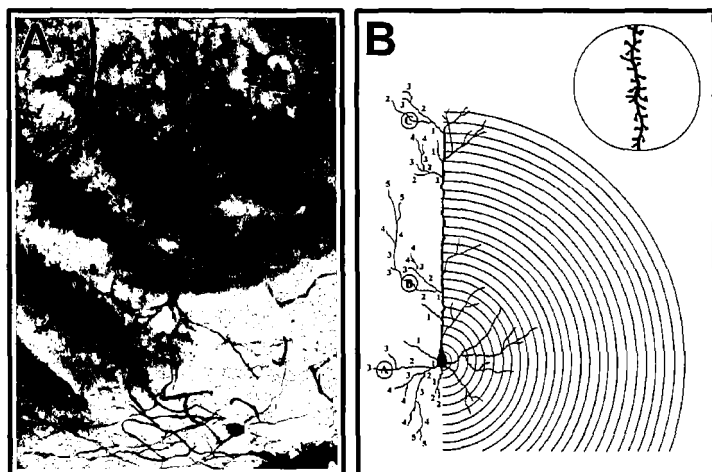


Figure 1. Sholl analysis permits quantification of dendritic material. **A:** Prototypical image of CA1 pyramidal neuron processed with the Rapid-Golgi technique. **B:** Schematic of Sholl (1953) analysis in which a Camera Lucida drawing is overlaid with a series of concentric circles (20 μm apart) and the number of dendritic processes intersecting each circle is quantified.

in a random 25- μm -long segment was determined for the basilar, mid-apical, and outer-apical tufts of the CA1 dendritic fields. Similar measurements of spine density were made for dentate granule cells on dendritic regions proximal, medial, and distal to the soma. Regions of dendrites selected for spine analysis were straight, within the same z-plane, and did not contain additional dendritic branches. Spine morphology was classified as stubby, crook, thin, mushroom, gemule or branched (Fiala and Harris, 1999; see Figure 4A). Data were analyzed by one-way ANOVA and considered significant at $p < 0.05$.

Electron Microscopy

Hippocampal areas were dissected out, and 200- μm -thin sections were generated using a Vibratome. Sections were post-fixed in 1% osmium tetroxide, stained with 1% uranyl acetate, dehydrated and embedded in Durcupan. Ultrathin sections were cut, contrasted with uranyl acetate and lead citrate, and analyzed using a Philips CM100 electron microscope. Samples were visually scanned for dendritic spines, and images were taken blind.

Immunohistochemistry

Whole brain slices (40 μm) were generated using a sliding microtome with cryostatic adaptations. Sections were blocked in normal goat serum, incubated with primary antibodies [mouse anti-synaptophysin (1:200; Sigma, St. Louis, MO) and rabbit anti-MAP2 (1:1000; Chemicon, Temecula, CA)], followed by incubation with secondary antibodies [Alexa 594 goat-anti-mouse (1:50; Molecular Probes, Eugene, OR) and Fitc 488 goat-anti-rabbit (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA)]. Samples were imaged blind, and images of CA1 region were taken at 64 $\times$. Synaptophysin and MAP2 intensities were measured using ImageJ software (NIH, USA). Data were analyzed by one-way ANOVA and considered significant at $p < 0.05$.

Results

Deletion of CLIP-115 results in aberrant hippocampal dendrites

The rapid-Golgi technique was employed to evaluate quantitative differences in dendrites and spines between KO and wild-type mice. Global analysis indicated that apical dendrites in KO mice were significantly longer than in wild-type littermates ($523.95 \mu\text{m} \pm 27.85$, and $442.22 \mu\text{m} \pm 22.03$, respectively; $p < 0.03$), and that basilar dendritic lengths were also greater in KOs ($304.64 \mu\text{m} \pm 10.52$, and $253.91 \mu\text{m} \pm 6.96$, respectively; $p < 0.01$; see Figure 2). No differences were found in the length or width of DG cells, or in the widths of CA1 dendritic fields (data not shown). In addition to dendritic size, significant differences were seen in the dendritic complexity in the apical dendrites of wild-types and KOs. Although analysis of branching order revealed no significant differences in either apical or basilar dendrites (data not shown), Sholl analysis of CA1 dendrites revealed greater dendritic material present in KO animals at more distal regions of both apical and basal dendrites (see Figure 3). These results suggest that the deletion of *Cyln2* results in longer reorganized dendrites without changes in net branching complexity. DG cells showed no differences in branching (data not shown).

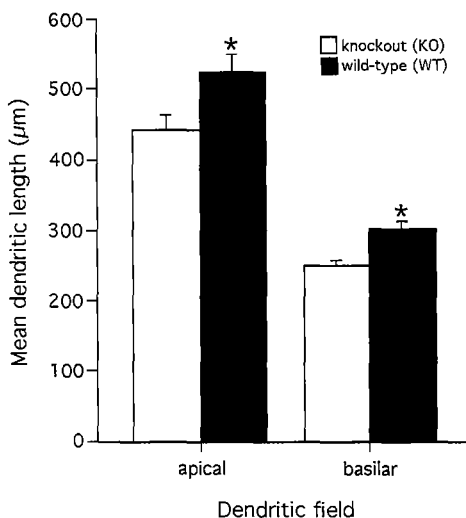


Figure 2. Quantification of dendritic arborizations in CA1 pyramidal cells. The lengths of both apical and basilar dendritic fields are significantly longer in pyramidal cells derived from knockout (KO) mice as compared to cells from wild-type controls.

Deletion of CLIP-115 results in altered hippocampal spine morphology

Although basilar dendrites from both KO and wild-type mice did not display significant differences in spine densities, several differences in spine morphology were noted. In KO mice, fewer spines displayed stubby ($p < 0.01$) or gemule ($p < 0.01$) morphologies as compared to wild-type littermates (see Figure 4A). In addition, significantly more thin spines ($p < 0.02$) were recorded in KO animals. Again, although no differences in spine densities were found within the mid-apical dendritic area, KO mice had more thin spines ($p < 0.04$) as compared to wild-type animals. In the outer apical tufts of CA1 pyramidal cells, KO mice displayed greater overall spine density ($p < 0.01$), as well as a greater proportion of thin spines ($p < 0.01$). In contrast, the number of stubby spines in KOs was decreased ($p < 0.04$) as compared to wild-type littermates.

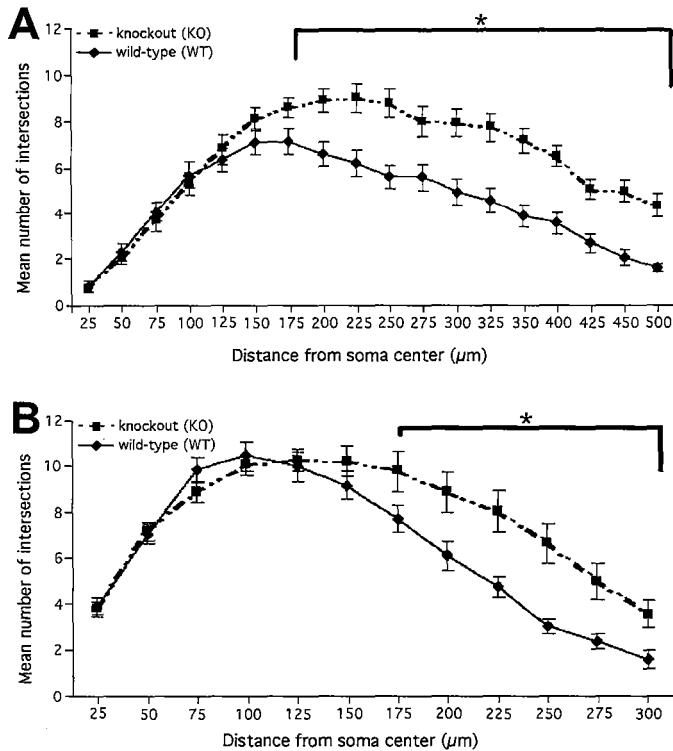


Figure 3. Quantification of dendritic material in CA1 pyramidal cells. Sholl analysis indicates that pyramidal cells derived from knockout (KO) mice have greater dendritic material in both apical (A) and basilar (B) regions as compared to cells from wild-type controls.

DG cells of KO mice also displayed localized differences in spine density and morphology. Increased spine densities were found in KO mice as compared to wild-type littermates in both medial (46.37 ± 0.69 , and 43.83 ± 0.96 respectively; $p < 0.04$) and distal (44.67 ± 0.63 , and 41.20 ± 0.82 , respectively; $p < 0.01$) dendrites. Although not significantly different, KO mice also exhibited an increased proportion of thin spines at distal dendrites when compared to wild-type animals (30.20 ± 0.832 , and 26.60 ± 0.70 , respectively).

Deletion of CLIP-115 results in decreased number of synapses

Our initial EM investigations demonstrated a possible qualitative difference in the number of synapses between KO and wild-type animals. EM analysis corroborated that the number of thin spines in CA1 region was increased in KO mice (Figures 4B and 4C). Although spines were relatively easily to locate and identify in all samples, spines with recognizable synapses were much more difficult to find in samples from KO mice. We therefore investigated the intensities of synaptophysin and MAP2 in the dendrites of CA1 pyramidal cells in whole brain slices. The ratio of synaptophysin intensity to MAP2 intensity was significantly increased in wild-type (2.08 ± 0.09) as compared to KO animals (1.64 ± 0.11 ; $p < 0.05$). Although not significant, trends indicated that synaptophysin intensities decreased, and MAP2 intensities increased, in KO animals.

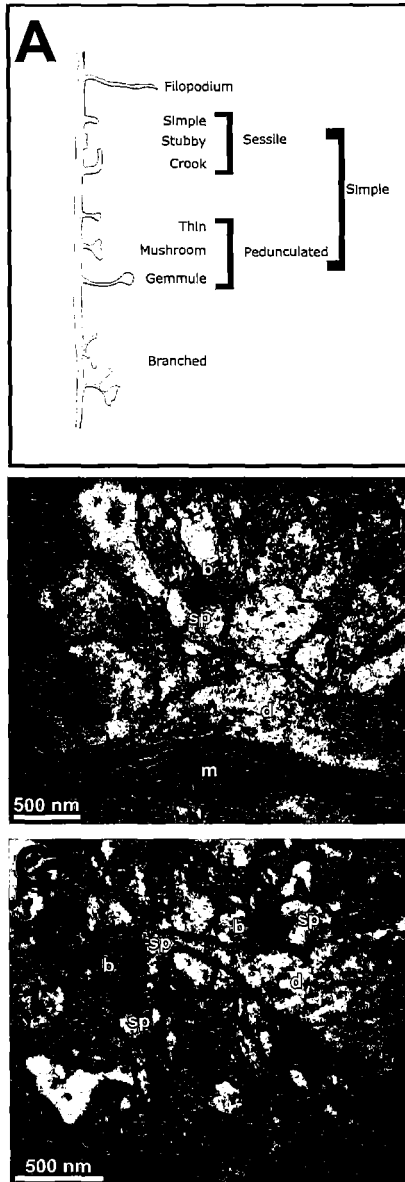


Figure 4. Aberrant spine morphology in knockout (KO) mice. **A:** Prototypical spine morphologies as classified by Fiala and Harris (1999): stubby, crook, thin, mushroom, gemmule or branched. **B:** Dendritic spine from CA1 region in wild-type (WT) animal. **C:** Dendritic spines from CA1 region in KO animal. Note that the KO spines display thinner necks, even as two spines contact the same pre-synaptic bouton. Abbreviations: sp (spine); b (pre-synaptic bouton); d (dendrite); m (mitochondria).

Discussion

The mouse provides an effective model system to investigate the genetic basis of WS due to the highly conserved nature of the WSCR between the mouse and human genomes (Valero *et al.*, 2000). In a comprehensive study, Hoogenraad *et al.* (2002) characterized KO mice lacking *CYLN2*, the gene encoding CLIP-115, as well as their heterozygous and wild-type littermates. They found that the KO mice compared well with several aspects of human WS, including

anatomical differences such as a growth deficiency, larger ventricles, and a smaller corpus callosum. Importantly, patients with WS score poorly on spatial cognition tests—in accordance, the KO mice also displayed strong hippocampal dysfunction when subjected to behavioral and electrophysiological tests.

The data presented here demonstrate that deletion of the *Cyln2* gene in mice results in changes in both hippocampal cell size and complexity. Pyramidal neurons from the CA1 region were increased in length (both apical and basilar), while maximal widths remained unchanged. These differences in cell size were found to accompany increases in the dendritic complexity as indicated by both Scholl analysis and branching order. Previous imaging, however, indicated that deletion of the *Cyln2* gene did not affect hippocampal volume or surface area (Hoogenraad *et al.*, 2002). Thus, it is likely that further investigations will reveal decreased cell density in these mutants, as neuronal packing density and dendritic arborization are inversely correlated (Raymond *et al.*, 1996b; Kemper and Bauman, 1998). Indeed, whole brain slices from KO mice did indicate that the amount of MAP2 in CA1 dendrites was increased as compared to wild-type littermates. These findings may provide a novel anatomical substrate for the profound hippocampal deficits and preserved language that characterize this genetic disorder.

In addition, the changes observed in dendritic morphology following *Cyln2* deletion are consistent with the hypothesis that the morphological changes associated with MR are correlated with changes in MT organization (Bodick *et al.*, 1982; Purpura *et al.*, 1982). Targeted gene knockout and protein fusion experiments (De Zeeuw *et al.*, 1997; Hoogenraad *et al.*, 2000, 2002; Stepanova *et al.*, 2003) suggest a degree of competition between CLIP-115 and CLIP-170 for association with the distal ends of growing MTs. Over-expression of CLIP-170 has been correlated with longitudinal extension of cytoplasmic processes and thus the aggregation of CLIP-170 at the distal ends of MT may be important in the projection of dendrites. In the absence of competition from CLIP-115, CLIP-170 may be able to bind to a greater proportion of sites without protein upregulation, since intracellular concentrations of CLIP-170 exceed the availability of MT binding sites (Hoogenraad *et al.*, 2002). These results suggest that the deletion of CLIP-115 is associated with increased cell length in both the apical and basilar dendritic fields of CA1 pyramidal cells. If CLIP-170 is involved in longitudinal extension as previously suggested (Hoogenraad *et al.*, 2000), the increase in CLIP-170 binding to MTs (resulting from the loss of competition from CLIP-115) would provide a possible pathway through which deletion of *Cyln2* could result in the increased cell length. However, previous results (Hoogenraad *et al.*, 2000, 2002) indicate that increased binding of CLIP-170 does not increase the rate of cytoplasmic extension; therefore the increased cell growth reported here may be due to dysfunction of systems regulating the degree of dendritic projection. Hoogenraad *et al.* (2004) postulated that the lack of CLIP-115 could alter MT network dynamics and impair vesicle transport. Thus, we propose that, although the increase in cell length observed in the KO mice may be mediated by increased CLIP-170 binding to growing MTs, the mechanisms of this change in MT organization remain unknown.

There are few data concerning neuroanatomical changes in the brains of WS patients as compared to the brains of patients with Down (DS), Rett (RS) and Fragile-X syndromes (Kaufmann and Moser, 2000). Dendritic dysmorphology has been clearly demonstrated in these latter syndromes, with most of the studies focusing on hippocampal pyramidal neurons. Moser (1995) compiled data gathered on dendritic abnormalities in genetic disorders with concurrent MR, and found that several cytoarchitectural features, namely reductions in dendritic length and spine dysgenesis, remained unknown in WS. Kaufmann and Moser (2000) reported that, in patients with MR, dendritic spines were found to be more sparse, and longer and thinner, as compared to controls of the same age.

Alterations in spine morphology, and thus potential cellular causes underlying the behavioral deficits in the *Cyln2* mice, have not been investigated to date. A recent study has characterized abnormal hippocampal spine morphology in mice lacking a different gene found in the WSCR, LIM kinase 1 (LIMK1), an enzyme shown to be associated with the actin cytoskeleton in spines (Meng *et al.*, 2002). They found that the deletion of LIMK1 caused altered spine shape, specifically that wild-type spines had thin necks whereas KO spines had thick necks. KO mice also displayed increased hippocampal long-term potentiation (LTP). Spine analysis in our CLIP-115 KO mice indicated precisely opposite findings: spines from KO mice had thinner necks than wild-type spines, and Hoogenraad *et al.* (2002) reported significantly decreased levels of LTP in KO mice. Dendritic spines are a dynamic component of the neuronal signaling apparatus that form, grow, mature and shrink in response to synaptic and intracellular signals. The ability of a single dendritic spine to make morphological modifications in response to synaptic input allows each spine to isolate the synaptic signal from other spines and from the parent dendrite. Indeed, the fact that spines serve to isolate high levels of Ca^{2+} from the remainder of the neuron indicates that they may serve a neuroprotective role (Segal, 1995). Although spines could be effectively analyzed using the rapid-Golgi technique and EM, the number of spines in CA1 does not necessarily reflect the number of viable synapses. *CYLN2* deletion greatly affected learning and memory performance in KO mice as compared to wild-type littermates (Hoogenraad *et al.*, 2002). We found that the ratio of synaptophysin intensity to MAP2 intensity was significantly decreased in KO mice, indicating a decreased number of synapses in these mice. The decreased levels of LTP seen in these mice could also be explained by the concurrent reduction in viable synapses.

WS presents a unique opportunity to examine a complex phenotype under a well-defined genotype. Thus, although the number of WS patients is very small, the study of WS as a model of genotype-phenotype interactions can prove beneficial for the comprehension of many similar diseases. Here we present further evidence for an anatomical profile that may provide a neural substrate for the unique cognitive profile observed in WS. This study was conducted using three KO and three wild-type mice; the fact that clear morphological differences were found between these small groups only reinforces the existence of a cellular phenotype. The creation of *LIMK1/Cyln2* double-knockout mice could better indicate the role of the cytoskeleton in WS (Hoogenraad *et al.*, 2004). In addition, our findings add weight to the claim made by Meng *et al.* (2002) that investigations of spine dysmorphology in tissue derived from patients with WS are greatly needed.

CHAPTER 4

General Discussion and Future Directions

In recent years, improved understanding of the harmonious interplay among a multitude of cellular processes has been partly derived from experiments using cultured neurons and glia. In addition, the generation of transgenic and knockout (KO) mice has greatly assisted in untangling the webs of mystery that still reside in many corners of cellular and molecular neuroscience. It is now possible, for example, to investigate the effects of glutamate application or traumatic injury on cultures from genetically modified mice in the hopes of gaining further information in regards to possible treatment strategies.

In **Chapter 1**, the two main foci of this thesis, namely traumatic brain injury (TBI) and microtubule (MT) dynamics, were outlined and described in detail. In addition, lists of pertinent questions were presented, which were then answered in Chapters 2 and 3. In **Chapter 2**, various effects of stretch-induced injury to cultured hippocampal and cerebellar cells were clearly delineated. We found that increasing degrees of stretch caused increasing degrees of cellular damage and death, and that two mild injuries could cause cumulative damage to hippocampal cells. Neurons and glia reacted differently to injury—specifically, to different degrees of stretch, to various injury paradigms, and to preconditioning—as measured by PrI uptake, and by the release of NSE and S-100 β . These experiments led to an in-depth exploration of the role of S-100 β after injury, and we found that, although both hippocampal and cerebellar glia exhibited increased PrI uptake after injury and regenerative properties, they displayed vastly different levels of S-100 β release. Cerebellar Purkinje cells (PCs) were also examined in detail, especially in reference to their unique reaction to increased levels of S-100 β . Glutamate-mediated excitotoxicity has been implicated as a vital component of clinical TBI. We saw that glutamate, either released after injury or applied directly to cultured cells, could have severe implications for cell survival, and that the pathways initiated by glutamate overlap those pathways influenced by S-100 β . Interestingly, both glutamate and S-100 β were found to have deleterious effects on the neuronal cytoskeleton. In summary, we found that, although stretch injury *in vitro* is not a substitute for experimental injury to animals *in vivo*, the experiments outlined in Chapter 2 have wide implications for the study and the treatment of clinical TBI.

In **Chapter 3**, the activity of MT plus-end binding proteins, namely EB3 and CLIP-115, were examined in both hippocampal and cerebellar cells. Several experiments required either the transfection of hippocampal cells, or the infection of cerebellar cells, with EB3-GFP. We found that this protein labeled MT plus-ends in neurons, similar to glia and to other non-neuronal cells, that the orientation of MTs in neurons depended on the neuronal compartment (axon, dendrite or soma), and that the growth of MTs changed as cultured neurons differentiated and matured. The velocities of growing MTs—as measured by EB3-GFP dashes—were significantly faster in glia and in other non-neuronal cells as compared to velocities in neurons. Interestingly, EB3-GFP growth velocities were found to be significantly faster in both hippocampal neurons and cerebellar PCs derived from CLIP-115 homozygous KO mice, as compared to their wild-type controls. Further investigations using hippocampal brain slices from adult animals revealed that the deletion of CLIP-115 affected dendritic and spine morphologies, as well as synapse formation. Taken together, the experiments outlined in Chapter 3 indicate that alterations in MT dynamics have deleterious effects on neuronal function and morphology that could represent a partial explanation for the unique neural deficits seen in patients with Williams Syndrome (WS).

Answers in science always seem to pose additional questions. In the following sections, several issues are discussed, drawing upon data presented in this thesis: 1) What are the roles of intracellular chloride in hippocampal cell death? 2) What are the possible links between S-100 β and glutamate-mediated excitotoxicity, especially in relation to cerebellar PCs and Bergmann glia (BG)? 3) What are the roles of MT-associated proteins (MAPs), such as CLIP-115, between the actin and MT cytoskeletons? and 4) What are the potential roles played by dendritic spines?

4.1 Cultured cells as monitors of neuronal function

Several of the previous chapters have utilized cultured cells that form a confluent monolayer, making these preparations convenient for the study of normal (and disrupted) neuronal function. Many cellular processes, for example MT dynamics, are difficult to witness in slice preparations, as it becomes challenging to distinguish one neuron or glial cell from its neighbors. An early paper by Rothman (1985) quantified the survival of cultured hippocampal neurons (by counting the number of cells which lysed) after the application of glutamate, NMDA or kainate—excitatory compounds that would normally cause a large influx of Ca^{2+} into the cell, and initiate cell death pathways (see Figures 7 and 8 in Chapter 1). Interestingly, these compounds were toxic even after the removal of Ca^{2+} , but not after the removal of Cl^- from the bath solution. In Rothman's own words, "These experiments suggest that the pathophysiology of amino acid neurotoxicity may be rather straightforward" (1985). The number of papers in the past twenty years dealing with the mechanisms underlying excitotoxicity, and the potential pharmacological interventions to treat it, however, would attest to the contrary. In Chapter 2.4, hippocampal cultures derived from transgenic mice expressing a pH- and Cl^- -sensitive variant of EYFP allowed for precise monitoring of $[\text{Cl}^-]_i$ levels, a feat that was difficult to perform in brain slices (Metzger *et al.*, 2002). Our data indicated that EYFP, in cultured cells, was sensitive only to changes in $[\text{Cl}^-]_i$. The application of glutamate, or other excitatory amino acids such as NMDA, for 3 min in ACSF resulted in an increase in $[\text{Cl}^-]_i$ that we were able to measure as a decrease in fluorescence. The cells were then returned to normal ACSF and the drug was allowed to wash out. In later experiments, we applied a variety of Cl^- -containing solutions to EYFP-expressing cells in order to quantify the amount of Cl^- that enters a neuron after glutamate application. The cells were first stabilized in a Cl^- -free solution that contained ionophores to equilibrate intracellular and extracellular ion concentrations. A second solution high in Cl^- (80 mM) produced a marked, and expected, decrease in EYFP fluorescence as $[\text{Cl}^-]_i$ increased. To properly complete our measurements, only those cells that survived the entire experiment were included in our analysis (see Figure 2 in Chapter 2.4). All cells survived the initial 0 mM solution, but lysed upon application of 80 mM Cl^- . In contrast, the number of cells that lysed after the application of glutamate, or other amino acids, was negligible or none, indicating the concentrations of these compounds, and the durations which they were applied, were not toxic. The decrease in fluorescence after glutamate application or after 80 mM Cl^- was found to be almost identical. We demonstrated that the increase in $[\text{Cl}^-]_i$ was due to Cl^- entry via certain channels after membrane depolarization. Therefore, even though we carefully calibrated the amount of Cl^- which entered our cells, the means of entry (via channels after normal membrane depolarization versus small holes in the membrane after ionophore application) indicates that neurons may be able to handle a high Cl^- load under normal circumstances. After stretch injury or TBI, however, mechanical stresses on the cells of the brain cause membrane damage, which is how PrI is able to enter the cell and label the nucleus. Clearly, membrane damage can cause disruptions of Cl^- homeostasis, leading to cell death, a feat which brief applications of glutamate were not able to perform.

Another case-in-point: glutamate-mediated excitotoxicity is a complex phenomenon following a variety of pathologies. In Chapters 1 and 2.3, considerable attention was given to PC death following TBI and stretch injury *in vitro*. Many publications in this field have stressed the importance of Ca^{2+} influx and changes in $[\text{Ca}^{2+}]_i$ (for review, see Weber, 2004)—indeed, Rothman later reported that neuronal degeneration is caused by an exacerbated increase in $[\text{Ca}^{2+}]_i$, leading to activation of calcium-dependent enzymes (Choi and Rothman, 1990), and not by "passive chloride conductance" (Rothman, 1985). Clearly, excitotoxicity is a complicated process in which many ions, receptors and enzymes play a concerted role terminating in cellular dysfunction

or death. This interplay, however, can be more easily studied in cultured cells. As mature PCs lack viable NMDA receptors, alternate mechanisms of Ca^{2+} influx needed to be identified. The persistent interest in $[\text{Ca}^{2+}]_i$ dynamics, and their long-term effects on excitotoxicity, has steered the field away from studying changes in $[\text{Cl}^-]_i$ and its acute effects. The identification of a PC-specific EAAT4 transporter located predominantly on dendritic spines (Yamada *et al.*, 1996; Nagao *et al.*, 1997; Welsh *et al.*, 2002), however, has led to the speculation that $[\text{Cl}^-]_i$ dynamics may play a role in PC excitotoxicity, as this transporter takes up glutamate together with Cl^- (Fairman *et al.*, 1995). Unfortunately, PCs do not express the Kv3.1 receptor subtype (Metzger *et al.*, 2002), and therefore, we cannot measure changes in $[\text{Cl}^-]_i$ using cultures derived from our EYFP transgenic mice. Kataoka *et al.* (1997) demonstrated that Ca^{2+} activates cytosolic phospholipase A_2 (cPLA $_2$), which in turn cleaves phospholipids from the membrane, liberating arachidonic acid (AA). The free AA facilitated Cl^- conductances, and thus glutamate entry, via EAAT4 for more than 20 min, a process that was shown to be independent of protein kinases such as PKC and CaMKII. This facilitation may depress subsequent synaptic transmission in PCs, indicated by decreased amplitudes of excitatory post-synaptic potentials (EPSPs), as well as constitute another way in which PCs protect themselves from excitotoxicity. The existence of EAAT4 offers Cl^- a role in the chronic stages of glutamate-mediated excitotoxicity in PCs. Future experiments will be able to identify the role of Cl^- in PCs using cell cultures derived from an alternate type of transgenic mouse which also contains a Cl^- -sensitive variant of EYFP.

4.2 S-100 β and glutamate-mediated excitotoxicity

Clinical and experimental studies have repeatedly confirmed the fact that S-100 β levels are elevated in blood and CSF after TBI. What have been difficult to confirm, either in animals or humans, are the reasons underlying the release of S-100 β from glia, and the potential assistance or harm it may do to neurons. S-100 β is a calcium-binding protein found in astrocytes and Schwann cells, and clinical studies (for example, see Herrmann *et al.*, 2001) have reported elevated levels of S-100 β following TBI. Although the mechanisms for elevated S-100 β following TBI are unknown, increased S-100 β levels in peripheral blood after trauma may be indicative of damage to the blood-brain barrier or could indicate the activation of secondary damage pathways (Herrmann *et al.*, 2001; Raabe and Seifert, 1999). S-100 β stimulates glial proliferation (Reeves *et al.*, 1994), which could possibly lead to brain swelling after trauma. However, it is also possible that S-100 β is playing somewhat of a protective role. For example, S-100 β may be an important mediator of glia-neuronal interactions, and it has been shown to stimulate neurite extension (Reeves *et al.*, 1994). Therefore, glial cells may release S-100 β in an attempt to save, or to repair, dying or damaged neurons. The cerebellum has been shown to contain high amounts of S-100 β , specifically in BG (Lossi *et al.*, 1995; see Figure 7 in Chapter 1). Because BG play such a critical role in cerebellar development, they may require high levels of S-100 β for their intercellular functions.

Both glutamate-mediated excitotoxicity (Folkerts *et al.*, 1998; Kanayama *et al.*, 1996; Saatman *et al.*, 1998) and glial S-100 β protein have been shown to have profound effects on the cytoskeletal elements of neurons. Therefore, S-100 β could be another possible contributor to secondary damage after TBI and glutamate-induced excitotoxicity. S-100 β has been increasingly linked to a variety of neuronal processes (for review, see Donato, 1991); for example, S-100 β disrupts MTs, and has been shown to instigate neuritic outgrowth, a necessary element in developing neurons (Azmitia *et al.*, 1990). Excessive glial S-100 β release after TBI, however, may prove detrimental to mature neurons. Decades ago, Ramón y Cajal (1913-14, 1933)

described a “neurotrophic factor” found in Schwann cells, and “neurobiones”, living entities which formed a “conductive pathway in the protoplasm, which Azmitia (2002) proposed matched the properties of S-100 β and MTs, respectively. Indeed, S-100 β has been shown to cause Ca²⁺-dependent depolymerization of MTs in a dose-dependent manner (Sorci *et al.*, 2000). Barger and Van Eldik (1992) demonstrated that exogenous S-100 β stimulated large increases in [Ca²⁺]_i in cultured neurons; the authors suggested that abnormal amounts of S-100 β could result in levels of [Ca²⁺]_i commonly associated with excitotoxicity. Donato (1991, 2001) has reviewed extensively the effects of S-100 β on MT assembly and disassembly. At acidic (6.7) pH, S-100 β inhibited MT assembly in the presence of micromolar levels of [Ca²⁺]_i; at basic (7.5) pH, however, Ca²⁺ was no longer required for S-100 β to affect assembly/disassembly (Donato, 1985, 1988). Experiments using *in vitro* MT assays indicated that S-100 β binds to unassembled tubulin, rendering it incapable of forming MTs (Donato *et al.*, 1989). Thus, S-100 β may interact with MTs—in effect, limiting their proper assembly—to the extent that cells begin to retract their neuritic processes; this effect would be notably increased with elevated levels of [Ca²⁺]_i after traumatic injury. Consistent with this hypothesis, early studies conducted by Cajal on traumatically-injured cats showed that PCs developed highly retracted dendritic trees as early as two days post-injury (Cajal, 1933; see also Azmitia, 2002; Figure 1).

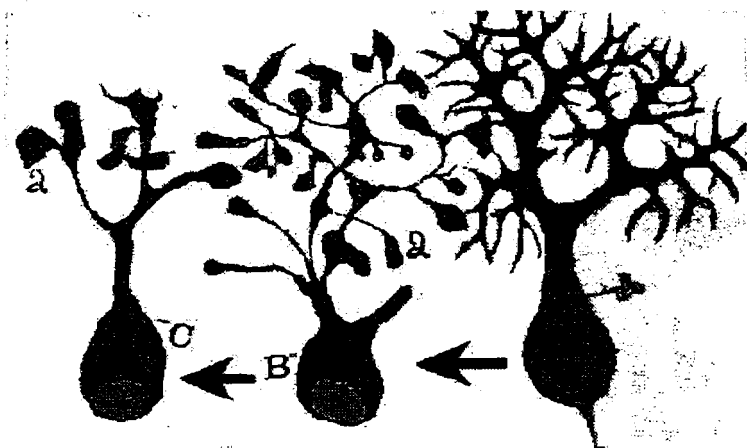


Figure 1. Purkinje cell of cat of twenty-five [days], sacrificed two days after traumatic lesion. A: Normal. B and C: Cells whose retracted dendrites terminate in reticulated clubs (from Cajal, 1933).

Mouse models have demonstrated the importance of S-100 β for glial-neuronal communication. For example, the deletion of S-100 β enhanced LTP in hippocampal neurons (Nishiyama *et al.*, 2002), whereas the over-expression of S-100 β impaired hippocampal LTP (Gerlai *et al.*, 1995). Also, the addition of exogenous S-100 β reduced the level of LTP in S-100 β KO mice to the level of LTP in wild-type mice, indicating that S-100 β , normally released by astrocytes, has a direct effect on the surrounding neurons (Nishiyama *et al.*, 2002). Cerebellar astrocytes from S-100 β KO mice had larger Ca²⁺ transients in response to KCl or caffeine as compared to controls, whereas transients were normal in granule cells. The reduced ability of mutant astrocytes to buffer Ca²⁺ could affect intercellular communication between BG and neurons through aberrant Ca²⁺ waves (Xiong *et al.*, 2000). Although S-100 β is found in control BG, and not in PCs (Xiong *et al.*, 2000), PCs have been shown to selectively take up antibodies to S-100 β (Karpiak and Mahadik, 1987). S-100 β has been shown to bind to RAGE (receptor for

advanced glycation end-products) with nanomolar affinity (Hofmann *et al.*, 1999), though it may exert its effects on neurons via other means (i.e. direct entry into cells; Azmitia and Whitaker-Azmitia, 1997).

We found that S-100 β levels were significantly elevated after hippocampal injury *in vitro* (Chapter 2.1). We also saw a significant release of S-100 β by cerebellar glia after *in vitro* injury (Chapter 2.3), the relative amount of which was several-fold higher than what we had previously reported for hippocampal glia. Thus, we applied exogenous S-100 β to control (uninjured) cerebellar cultures, and found that the number of PCs (but not the number of MAP2-positive neurons) was significantly reduced at 24 hr post-application (Chapter 2.3). Because PCs were also susceptible to mechanical stretch injury, it was possible that there was some direct form of S-100 β -mediated toxicity that caused significant PC death. S-100 β may have interacted with MTs in PCs, causing these cells to retract their neuritic processes and lift from the substrate. Indeed, stretch injury caused a reduction in both MAP2-positive neurons and in calbindin-positive PCs following mechanical injury. Figure 2 depicts prototypical images of both control and injured PCs in which axonal beading and dendritic degeneration are readily apparent. We also noted

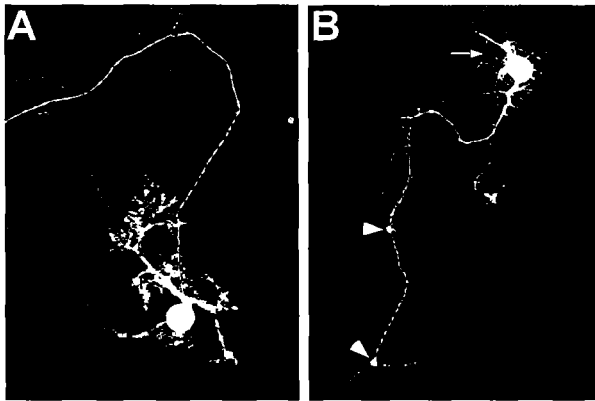


Figure 2. Axonal beading and dendritic retraction after *in vitro* stretch injury in cultured Purkinje cells (PCs) stained against calbindin-D28K. A: Control PC showing a full dendritic arbor and long axon. B: Injured PC with a beaded axon (beads indicated by arrowheads) and a retracted dendritic tree (arrow).

reduced levels of GLAST antibody staining after *in vitro* injury in cerebellar cultures. Although the reasons for the reduced GLAST expression were unclear (e.g., the transporter itself has been down-regulated after injury or is physically altered in some manner), reduced GLAST activity would have marked effects on the function and survival of PCs, as described above. In addition, stretch injury *in vitro* has been previously shown to cause the release of glutamate from astrocytes (Ahmed *et al.*, 2002). Therefore, glutamate levels are elevated after injury, in addition to a lower capacity to clear it from the extracellular space. This correlated well with *in vivo* findings. For example, Watase *et al.* (1998) subjected GLAST mutant mice to cold-induced cerebellar injury. They postulated that the significantly larger edema in these mice was caused by the inability of mutant BG to reuptake glutamate from the extracellular space. Indeed, cortical protein levels of GLAST were significantly reduced for up to 3 days after controlled cortical impact brain injury *in vivo* before returning to control levels at 7 days post-injury (Rao *et al.*, 1998). If GLAST is critical in preventing excitotoxic damage after injury, as Rao *et al.* (1998) claimed, then the reduction we observed in GLAST fluorescence after stretch injury in cultured cerebellar cells could be indicative of faulty glutamate transport, causing excitotoxic levels of glutamate to accumulate in the extracellular space.

It is clear that understanding the mechanisms underlying glutamate-mediated excitotoxicity in PCs cannot be accomplished simply by extrapolating data gathered in other brain

regions, partially because PCs lack functional NMDA receptors. PCs are highly susceptible to a variety of pathologies with an excitotoxic component, such as TBI, and many of these conditions display cytoskeletal alterations. Although physiological levels of intracellular free Ca^{2+} initiate vital second-messenger signaling pathways in PCs, excessive Ca^{2+} influx can detrimentally alter dendritic spine morphology via interactions with the neuronal cytoskeleton, and thus can perturb normal synaptic function. PCs possess various CBPs, such as calbindin and parvalbumin, and glutamate transporters, such as EAAT4, in order to prevent glutamate from exerting deleterious effects. BG are gaining recognition as key players in the clearance of extracellular glutamate; these cells are also high in S-100 β , a protein with both neurodegenerative and neuroprotective abilities. The ability of Ca^{2+} to instigate a variety of acute and downstream signaling pathways has been well documented, and many treatment strategies have focused upon reducing Ca^{2+} influx. The influence of BG and S-100 β , however, has yet to be fully comprehended or appreciated. Although S-100 β has been shown to play a neuroprotective role at times—indeed, we found that exogenous S-100 β may protect hippocampal neurons from stretch injury (Chapter 2.2)—the high release of S-100 β after TBI could be initiating a series of cell death pathways similar to glutamate. We hope that future experiments will clarify the interaction among PCs, BG and S-100 β , thus providing a new model with which to test novel treatment strategies, especially considering the number of excitotoxic animal models that show selective PC degeneration. In addition, future experimentation will demonstrate the pervasive influences of S-100 β on neurodegenerative diseases such as Alzheimer's.

4.3 MAPs, MTs and Ca^{2+}

Many MAPs play key roles in the maintenance of the neuronal cytoskeleton. Proteins such as MAP2 and MAP1 in dendrites, and tau in axons, participate in tubulin polymerization and the stabilization of MTs, leading to the establishment of neuronal polarity. The absence of certain MAPs can have critical repercussions. For example, MAP1B is highly expressed in embryonic neurons and glia, with levels greatly decreasing shortly after birth, except in cells with high levels of plasticity such as adult PCs (Schoenfeld *et al.*, 1989). Edelman *et al.* (1996) generated mice with a targeted mutation in the MAP1B gene, and found that homozygous mice could not survive the loss of MAP1B beyond day E9. In heterozygous animals, they reported a lack of MAP1B staining in PCs without a concurrent change in overall PC number. MAP1A and MAP2 labeling revealed that heterozygotic PC dendrites were truncated and had less branching. Because these PCs still had dendrites, MAP1B did not appear to be required for the formation of dendrites, but MAP1B was necessary for normal dendritic function (Edelman *et al.*, 1996).

Alterations in MAPs, particularly in MAP2, have been extensively investigated following excitotoxicity and TBI. The administration of ibogaine and harmaline to rats caused a loss of MAP2 in PCs concomitantly with a loss of PC soma that degenerated in parasagittal stripes (O'Hearn and Molliver, 1993). These substances acted at the IO, causing excitotoxic levels of glutamate to be released by CFs at CF-PC synapses, and thus a high increase in $[\text{Ca}^{2+}]_i$. Ca^{2+} presents an interesting intracellular profile with regards to MAP2. Normal synaptic transmission in neurons requires low amounts of $[\text{Ca}^{2+}]_i$. High amounts of Ca^{2+} after glutamate application, however, may initiate a variety of downstream signaling pathways, resulting in MT growth or retraction. Ca^{2+} -induced activation of calpain has been shown to inhibit MT growth (Johnson *et al.*, 1991), whereas Ca^{2+} -induced activation of calmodulin/CaMKII leads to the phosphorylation of MAPs and neurite outgrowth (Diéz-Guerra and Avila, 1995). Similarly, the stimulation of cultured spinal cord (Bigot and Hunt, 1991) or cortical neurons (Bigot *et al.*, 1991)

with glutamate resulted in a reorganization of the MT cytoskeleton, namely, the relocation of MAP2 into a network of bundles in soma and proximal neurites. This effect did not lead to neuronal damage, however, possibly indicating that the level of glutamate was sub-toxic. MTs that were depolymerized with nocodazole appeared to lose MAP2 immunoreactivity, but glutamate stimulation increased MT resistance to depolymerization (Bigot and Hunt, 1991). Interestingly, double labeling experiments indicated that MAP2 was now associated with NFs, acting as a cross-linker between the MT and NF cytoskeletons to reinstate stability. MAP2 can be dephosphorylated by calcineurin, a Ca^{2+} -dependent phosphatase abundant in neurons (Goto *et al.*, 1985). In this state, MAP2 would display decreased affinity for MTs. On the other hand, NMDA receptor activation leads to increased MT stability by modulating the phosphorylation state of MAP2 (van Rossum and Hanisch, 1999). Thus, the method of Ca^{2+} entry, and not just the presence of $[\text{Ca}^{2+}]_i$ in the cell, may determine its effect on MAP2.

Similar to MAP2, CLIP-170 has been postulated to act as a mediator between the actin and MT cytoskeletons. Fukata *et al.* (2002) demonstrated that the coordination of actin and MT cytoskeletons is required for neuritic outgrowth (see Figure 3). Specifically, the dynamic reorganization of actin filaments is induced by Rac1 and Cdc42, members of the Rho family of GTPases, at the growth cone. Because MTs are oriented with their plus-ends towards the growth cone, activated Rac1 and Cdc42 could determine the sites where these plus-ends are captured through IQGAP1 and CLIP-170, thus leading to MT polarization. An alternative pathway involving APC and EB1 could also exist (Fukata *et al.*, 2002). What remains unclear is whether the related proteins CLIP-115 and EB3 can function in a similar manner, and whether the ability of +TIP proteins to mediate between the actin and MT cytoskeletons continues in mature cells,

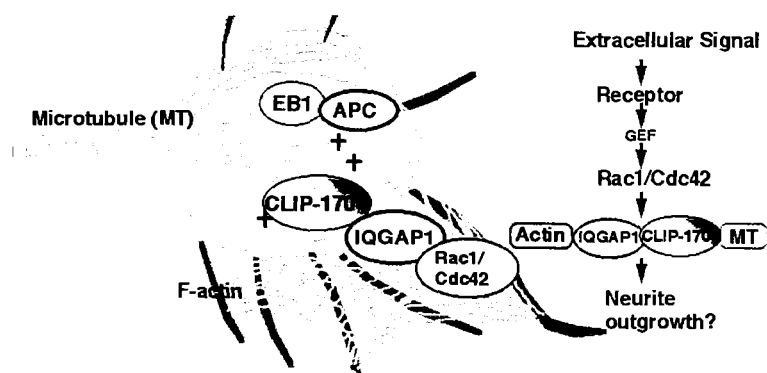


Figure 3. The coordination of actin and MT cytoskeletons is required for neuritic outgrowth. The dynamic reorganization of actin filaments is induced by Rac1 and Cdc42 at the growth cone. MTs are preferentially projected towards the growth cone. Activated Rac1 and Cdc42 determine the sites where MT plus-ends are captured via IQGAP1 and CLIP-170, leading to neurite outgrowth (from Fukata *et al.*, 2002).

i.e. at the level of the dendritic spines. The data presented in the Chapter 3, however, would indicate that the deletion of CLIP-115 in cultured cells and in brain slices has a detrimental effect on neurite length and on synapse formation. Interestingly, S-100 β has been shown to regulate IQGAP1, a scaffold protein that localizes complexes involved in actin- and MT-based functions to the plasma membrane (Mbele *et al.*, 2002). The interaction of S-100 β and IQGAP1 is regulated by both Ca^{2+} and Zn^{2+} ; the presence of Zn^{2+} caused S-100 β to have an affinity for Ca^{2+} within a physiological range (Mbele *et al.*, 2002). Normal amounts of Ca^{2+} would allow for normal activity of S-100 β ; glutamate-mediated Ca^{2+} influx during excitotoxicity, however, could over-

activate S-100 β , which in turn could alter the ability of CLIP-170 or IQGAP1 to bind to MTs, and thus perturb actin-MT interactions at the spine level. Although S-100 β is not an excitatory amino acid, these reports suggest that it can have actions similar to glutamate in PCs, such as a reorganization of the cytoskeleton in dendrites and spines. Such findings could have implications not only for TBI, but also for other types of pathologies that involve excitotoxic mechanisms in PCs. Future studies will focus on the effects of S-100 β on MT-associated processes and on normal electrophysiological parameters in cultured PCs. The ability of antisense S-100 β to block the deleterious effects of traumatic injury *in vitro* will also be assessed by quantifying the velocity of MTs in PCs infected with EB3-GFP.

4.4 Spines as neuroprotective mechanisms

Cellular models of learning and memory have been proposed for both the cerebellum and the hippocampus, the brain regions described in this thesis. Here we discuss one form of LTP (the connections between CA3 and CA1 neurons) in the hippocampus, and both LTP and long-term depression (LTD) in the cerebellum (the connections between PFs or CFs, and PCs). Both LTP and LTD occur at the spine level, and both require certain levels of Ca²⁺ for proper signal transmission (for reviews, see Franks and Sejnowski, 2002; Poncer, 2003; Nicoll, 2003; Lynch, 2004). Unlike LTD in PCs, hippocampal LTP requires NMDA receptors for proper induction. These receptors are not needed for the maintenance of LTP, which occurs at the AMPA receptor. As in glutamate-mediated excitotoxicity, the precise entry of Ca²⁺ into the cell is important: Ca²⁺ entry via depolarization is not sufficient for the induction of LTP. Rather, Ca²⁺ must enter via NMDA receptors in order to maintain Ca²⁺ influx at close proximity to the synapse and the machinery located within the post-synaptic spine. LTP can be considered as a strengthening of synaptic transmission between two cells. Pyramidal neurons in the CA1 receive excitatory input from CA3 axons, evoking an EPSP. If a CA1 neuron receives repetitive stimulation, the size of the EPSP will increase, indicating that synaptic transmission has been potentiated. This potentiation can last for days, even weeks. Several conditions are critical for the proper induction of hippocampal LTP: 1) the post-synaptic cell must be depolarized at the time that the cell receives pre-synaptic input; and 2) Ca influx must occur at the synapse. Ca influx leads to the activation of two post-synaptic kinases, PKC and CaMKII, which may themselves initiate several signaling pathways: 1) phosphorylation of AMPA receptors leading to larger ionic conductance of these receptors; 2) insertion of additional AMPA receptors into the membrane; and 3) instigation of new dendritic spines and subsequently new synapses.

If LTP is a strengthening of synaptic transmission, then LTD can be conceived of as its counterpart, a weakening of synaptic transmission (for reviews, see Ito, 2001; Hartell, 2002; Daoudal and Debanne, 2003). Whereas strengthening in LTP occurs following the co-activation of the pre- and post-synaptic neurons, weakening in LTD occurs following the activation of the pre-synaptic neuron in the absence of strong activation of the post-synaptic neuron. Again, Ca²⁺ plays a vital role. If the amount of [Ca²⁺]_i in the post-synaptic neuron is only slightly elevated when the pre-synaptic neuron is activated, then the enzymes that are activated will be protein phosphatases rather than protein kinases. It is hypothesized that, during LTD, AMPA receptors are thus dephosphorylated, and that AMPA receptors may be removed from the membrane. Recent evidence has shown that lower [Ca²⁺]_i leads to cerebellar LTP, whereas higher [Ca²⁺]_i leads to cerebellar LTD (Lev-Ram *et al.*, 2003; Coesmans *et al.*, 2004); the opposite effect has been found in the hippocampus (Mizuno *et al.*, 2001). The differential effects of Ca²⁺ in the induction of LTP versus LTD are reminiscent of its differential effects on MTs and MAP2.

Normal glutamate neurotransmission appears to stabilize dendritic spines; what happens to spines during glutamate excitotoxicity, however, is not completely known. It is possible that these highly stable spines could be continually activated by glutamate, leading to continued ion influx. The F-actin found in spines has been shown to be particularly resistant to depolymerizing drugs (Allison *et al.*, 1998), which could also be resistant to the effects of Ca^{2+} . NMDA receptor activation, which was shown to stabilize MTs, may cause the dissolution of the actin framework (van Rossum and Hanisch, 1999), instigating spine dysmorphology or collapse. The fact that spines serve to isolate a high level of Ca^{2+} from the remainder of the neuron indicates that they may serve a neuroprotective role (Segal, 1995). Because collapsing spines after NMDA or glutamate application may make the neuron more vulnerable to a second excitotoxic event (Halpain *et al.*, 1998), alterations in spine morphology may constitute compensatory mechanisms (for example, see Vecellio *et al.*, 2000). Dendritic spines in PCs may also play a neuroprotective role in other ways. For example, reductions in the synaptic strength of the PF and CF inputs, as measured by post-synaptic currents in PCs, are well described. In Marr-Albus-Ito models of cerebellar function, LTD at PF-PC synapses provides a cellular substrate of some forms of cerebellar motor learning (Marr, 1969; Albus, 1971; Ito, 1984). PF-LTD can be induced by simultaneous activation of PFs and CFs at low frequencies (Ito *et al.*, 1984). LTD can also be obtained at the CF-PC synapse following tetanization of the CF alone (Hansel and Linden, 2000). CF-LTD is accompanied by a long-term reduction in the amplitude of complex spike-associated Ca^{2+} transients in PCs (Weber *et al.*, 2003). It is possible that a reduction in CF-evoked Ca^{2+} signaling in PCs may provide a neuroprotective mechanism. This idea has previously been suggested for PF-LTD (De Schutter, 1995; Llinás *et al.*, 1997). This hypothesis is even more attractive at the CF input as it has been shown that prolonged periods of CF firing at elevated frequencies can lead to PC death (O'Hearn and Molliver, 1997), despite the high Ca^{2+} buffering capacity of these cells (Fierro and Llano, 1996). Although speculative, a long-term reduction in Ca^{2+} signals may provide a mechanism by which PCs adapt in order to handle a potentially high and toxic Ca^{2+} load. Another protective mechanism in PC spines may be the presence of EAAT4 as described above.

A similar theory could be constructed for the neuroprotective properties of dendritic spines in WS. As over 20 genes are normally deleted in WS, the construction of mouse models in which only one gene is deleted allows for the analysis of a specific gene on neuronal function. It was previously demonstrated that the deletion of *Cyln2*, the gene encoding CLIP-115, generated mice whose behavioral profile matched well that of WS patients (Hoogenraad *et al.*, 2002). These mice also displayed attenuated LTP. In contrast, Meng *et al.* (2002) found that the deletion of *LIMK1*, the gene encoding LIM kinase 1, generated mice with enhanced LTP. Meng *et al.* (2002) also reported that the deletion of LIMK1 caused altered spine shape, specifically KO spines had significantly thicker necks than wild-type spines. Spine analysis in our CLIP-115 KO mice indicated precisely opposite findings: hippocampal spines from KO mice had thinner necks than wild-type spines (Chapter 3.3). Both of these proteins associate with the cytoskeleton: the fact that CLIP-115 is a MAP, and LIMK1 is an actin-associated protein, may partially explain why their deletion results in opposite spine dysmorphology. Unfortunately, insufficient data exists in post-mortem samples derived from WS patients. Kaufmann and Moser (2000) propose that spine dysgenesis in human syndromes with concurrent MR could represent a reduction in the number of excitatory synapses. Indeed, we saw a reduced number of viable synapses in CLIP-115 KO mice. What remains to be investigated are whether hippocampal neurons derived from CLIP-115 KO mice have altered reactions to normal and excitotoxic amounts of glutamate. Future experimentation will focus on alterations in synaptic plasticity as demonstrated by electrophysiological methods, and on subsequent cell damage and death after traumatic injury *in vitro*.

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Abbreviations

AA	arachidonic acid
ACSF	artificial cerebral spinal fluid
AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
BG	Bergmann glia
$[Ca^{2+}]_i$	intracellular free calcium
CaMKII	calcium/calmodulin kinase II
CBP	Ca^{2+} -binding protein
CF	climbing fiber
$[Cl^-]_i$	intracellular chloride
Cl^-	chloride ion
CLIP-115	cytoplasmic linker protein of 115 kDa
CLIP-170	cytoplasmic linker protein of 170 kDa
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
cPLA ₂	cytosolic phospholipase A ₂
CSF	cerebrospinal fluid
DAG	diacylglycerol
D-APV	DL-2-amino-5-phosphonopentanoic acid
DCN	deep cerebellar nuclei
DIDS	4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid
DIV	days <i>in vitro</i>
DHPG	dihydroxyphenylglycine
DLB	dendritic lamellar body
DMSO	dimethyl sulfoxide
EAAT	excitatory amino acid transporter
EPSP	excitatory post-synaptic potential
ER	endoplasmic reticulum
EYFP	enhanced yellow fluorescent protein
FDA	fluorescein diacetate
GABA	γ -aminobutyric acid
GFAP	glial acidic fibrillary protein
GLAST	glutamate-aspartate transporter
HBS	HEPES-buffered saline
HBSS	Hank's balanced saline solution
HSP	heat shock protein
IO	inferior olive
IP ₃	inositol-(1,4,5)-trisphosphate
KO	knockout (animal)
LTD	long-term depression
LTP	long-term potentiation
MAP	microtubule-associated protein
mGluR	metabotropic glutamate receptor
MR	mental retardation
MT	microtubule
MTBI	mild traumatic brain injury
MTOC	microtubule organizing center

NBQX	2,3-dioxo-6-nitro-7-sulfamoylbenzo(f)quinoxaline
NF	neurofilament
NFA	niflumic acid
NMDA	<i>N</i> -methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NPPB	5-nitro-2-(3-phenylpropylamino)benzoic acid
NSE	neuron specific enolase
PC	Purkinje cell
PF	parallel fiber
PIP ₂	phosphatidylinositol bisphosphate
PIPES-BS	PIPES-buffered saline
PKC	protein kinase C
PLC	phospholipase C
PrI	propidium iodide
PSD	post-synaptic density
RAGE	receptor for advanced glycation end-products
rMTBI	repeated mild traumatic brain injury
SEM	standard error of the mean
TBI	traumatic brain injury
<i>trans</i> -ACPD	<i>trans</i> -(±)-1-amino-1,3-cyclopentanedicarboxylic acid
VGCC	voltage-gated calcium channel
WS	Williams Syndrome
WSCR	Williams Syndrome Critical Region

Summary

Cultured neurons provide a useful tool for investigating various cellular processes, and when complemented with data gathered from brain slices and *in vivo* experimentation, the information they can provide about neuronal function becomes greatly compelling. Information from two seemingly separate topics, namely traumatic brain injury (TBI) and cytoskeletal elements such as microtubules (MTs), can benefit from the insights of each other, a concept outlined in **Chapter 1: General Introduction**. In this initial portion, cell culture protocols, and neuronal cytoarchitecture in the hippocampus and the cerebellum, were discussed before relating these findings to glutamate-mediated cell death, particularly in cerebellar Purkinje cells (PCs). The effects of excitotoxicity were identified in these cells at the level of the dendritic spine and the MTs of the neuronal cytoskeleton. Alterations in both cultured PCs and hippocampal neurons were examined in relation to traumatic injury and to Williams Syndrome, a rare genetic disorder.

Chapter 2 began with data gathered from traumatically injured hippocampal neurons and glia derived from embryonic mice. Although TBI is a leading cause of death worldwide, the repercussions of mild TBI (MTBI), which often is not fatal, are less understood and investigated. Cultured cells were injured using an *in vitro* model of stretch-induced mechanical injury. Specifically, **Chapter 2.1** concerned the effects of repeated MTBI, and how it compares with single, or more severe, TBI. The results indicated that repeated mild injury caused cumulative damage to both hippocampal neurons and glia. Initial experiments performed in Chapter 2.1 validated this model—which had previously been used with cells cultured from neonatal rat pups—for use with embryonic mice. Clinical relevance was found not only in the degree of cell death after repeated MTBI, but in the measurement of two markers of damage, neuron specific enolase (NSE) and glial S-100 β protein.

A few unanswered questions remained from the initial study of MTBI in hippocampal cultures. **Chapter 2.2** readdressed the effects of repeated MTBI by subjecting cells to a variety of injury paradigms, and by again measuring the degree of cell damage and death, and the release of NSE and S-100 β . The data indicated that degree of damage following repeated injury was dependent on the severity of the insult and on the inter-injury interval. Repeated injury especially affects two subpopulations of people, namely athletes and victims of child abuse, and the data presented in this chapter indicated not only that neurons and glia reacted differently to the same injury regimen, but that increasing the latency between repeated injuries had a major effect on cell survival.

These findings prompted further study of traumatic injury *in vitro* using cultured cerebellar neurons and glia, found in **Chapter 2.3**. A particular focus was placed on PCs, as these cells represent the only output of the cerebellar cortex. As found in hippocampal cultures, differences became apparent between neurons and glia, and levels of released NSE and S-100 β were again measured after injury. The results indicated that *in vitro* injury caused not only cell death but also glial protein alterations and elevated S-100 β release. Data gathered here provide interesting links among glutamate, cerebellar Bergmann glia (BG), S-100 β and MTs (see Figure 7 in Chapter 1). Importantly, S-100 β appears to have differential effects on hippocampal neurons and PCs. The insights gained by the data presented in this chapter have relevance for the study of cerebellar TBI, and of cerebellar dysfunction, as a whole.

Chapter 2 closes with a study of the glutamate-induced elevations in intracellular chloride concentration in hippocampal cultures, **Chapter 2.4**. Cultures were created from transgenic mice that possess a yellow fluorescent protein that is sensitive to changes in intracellular chloride in subsets of neurons. Although previous attention has focused on the effects of glutamate on increasing intracellular Ca²⁺, as reviewed extensively in Chapter 1, one of the initial events in

neurons after TBI is a large influx of chloride, which not only causes cell swelling but alters the delicate homeostasis within the cell. By performing careful pharmacological analysis, it appeared that short applications of glutamate led to membrane depolarization, causing chloride to enter hippocampal neurons via specific chloride channels. Findings presented in this chapter indicated that further use of cultures derived from these transgenic mice would be greatly helpful in the study of TBI *in vitro*.

Chapter 3 is concerned with the activity of MT plus-end binding proteins, namely EB3 (end-binding protein 3) and CLIP-115 (cytoplasmic linker protein of 115 kDa), in neurons. **Chapter 3.1** demonstrated how MT dynamics could be imaged and studied via the use of EB3 tagged to GFP (green fluorescent protein). MTs are dynamic, polar structures that orient themselves in the axons and dendrites of neurons in specific ways to aid in intracellular transport. Specifically, MTs in axons are oriented with their growing and shrinking plus ends towards the axon terminal (or growth cone), and their minus ends towards the cell body, whereas MTs in dendrites are organized in both directions. The cultured hippocampal neurons and cerebellar PCs used in Chapter 3.1 indicated that MTs grow at similar rate in both hippocampal neurons and PCs, that MT orientation in axons and dendrites differs between immature and mature neurons, and that neurons and glia display markedly different MT growth rates.

Several of the experiments described in Chapter 3.1 were repeated in **Chapter 3.2** using cultured hippocampal neurons and cerebellar PCs either from embryonic CLIP-115 or CLIP-170 homozygous (knockout) mice. The significantly increased velocities of the EB3-GFP dashes in knockout neurons indicated that the deletion of either of these proteins has a clear effect on MT dynamics and growth. Interestingly, velocities in glia from knockout mice were similar to those seen in wild-type (control) glia. In addition, β -tubulin intensities in PC dendrites from CLIP-115 knockout mice were significantly increased as compared to wild-type PCs. The data presented here indicate that the loss of one of the CLIP protein results in altered MT balance in neurons.

CYLN2, the gene coding for CLIP-115, as well as approximately 20 other genes, have been shown to be hemizygotously deleted in people with Williams Syndrome, a genetic disorder that produces a unique behavioral profile. By generating mice homozygous for CLIP-115, the specific effects of this protein on cellular and brain function were determined. The data presented in **Chapter 3.3** were gathered from hippocampal brain slices obtained from adult homozygous (knockout) CLIP-115 mice and from their wild-type littermates. Pyramidal neurons from the CA1 region of the hippocampus were analyzed in terms of dendritic length and dendritic material. Particular attention was paid to the dendritic spines, as these contain the vast majority of the synapses, the contact points between neurons. Slices were processed for the Golgi stain, a procedure widely used by over a hundred years ago by Ramón y Cajal to label only select neurons out of the entire population, as well as for electron microscopy and for fluorescent microscopy. Analysis indicated that the deletion of CLIP-115 had significant effects on neurite length, spine shape, and synapse number. Previous studies demonstrated that CLIP-115 knockout mice have specific difficulties with learning and memory; the data presented in Chapter 3.3 indicate that morphological alterations could play a key role in these deficits.

Finally, **Chapter 4** provides a discussion of the preceding chapters in this thesis, what has been learned, and what neuronal mysteries need yet to be unraveled. First, the role of intracellular chloride in hippocampal cell death was examined beyond the conclusions presented in Chapter 2.4, especially in PCs. Second, although high levels of S-100 β were found to be released after TBI in Chapters 2.1, 2.2 and 2.3, its exact role in neuronal cell death remains unclear. Here possible links between S-100 β and glutamate-mediated excitotoxicity are examined, with particular attention paid to cerebellar PCs and BG. Third, critical roles were found for MTs and for microtubule-associated proteins (MAPs) in Chapters 2 and 3; here evidence indicating

that MAPs such as CLIP-115 and CLIP-170 may mediate between actin and MT cytoskeletons is discussed. Lastly, dendritic spines modulate their shape due to a variety of cues, including synaptic activation and glutamate-mediated excitotoxic stimuli; the deletion of CLIP-115 in Chapter 3.3 also caused spine dysmorphology. Data gathered in Chapters 2 and 3 are presented in the light that spines may serve neuroprotective functions.

Samenvatting

Neuronenkweken zijn voor onderzoek van uiteenlopende celprocessen een nuttig hulpmiddel. Informatie over de werking van zenuwcellen die aan de hand van dergelijke kweken is verkregen wordt beduidend interessanter, wanneer deze wordt aangevuld met gegevens op basis van onderzoek aan hersenplakjes en uit experimenten *in vivo*. In die zin kan informatie over traumatisch hersenletsel (*traumatic brain injury*, hierna TBI) een nuttige aanvulling vormen op informatie over bouwstenen van het cytoskelet zoals microtubuli (hierna MT's) en omgekeerd, ook al staan deze twee onderwerpen ogenschijnlijk los van elkaar. In **Hoofdstuk 1: Algemene Inleiding** is dit uiteengezet. In dit eerste hoofdstuk kwamen celkweekprotocollen aan bod en werd de celarchitectuur van neuronen in de hippocampus en de kleine hersenen besproken, alvorens deze bevindingen in verband te brengen met glutamaat-gemedieerde celsterfte, in het bijzonder het afsterven van Purkinje-cellen in de kleine hersenen. Effecten van "excitotoxiciteit" werden bij deze cellen vastgesteld op het niveau van de dendriet-*spine* en de microtubuli van het neuronale cytoskelet. Zowel bij gekweekte Purkinje-cellen als bij hippocampus-neuronen werden opgetreden veranderingen onderzocht in hun relatie tot traumatisch letsel en het zgn. Williams-syndroom, een zeldzame erfelijke aandoening.

Hoofdstuk 2 begon met gegevens over door verwonding beschadigde hippocampusneuronen en bindweefselcellen van muizenembryo's. Terwijl TBI wereldwijd een veel voorkomende doodsoorzaak is, zijn de gevolgen van de milde vorm van TBI (*mild TBI*, hierna MTBI), die meestal niet dodelijk is, minder vaak onderzocht en minder bekend. Gebruikmakend van een in-vitromodel werd gekweekte cellen mechanisch letsel toegebracht door oprekking. **Paragraaf 2.1** ging dieper in op de effecten van herhaald MTBI en in hoeverre dat verschilt van, of overeenkomt met, eenmalig MTBI of het ernstigere TBI. De resultaten duiden erop dat zowel hippocampusneuronen als bindweefselcellen cumulatieve schade overhouden aan herhaald toegebracht licht letsel. De eerste reeks experimenten, waarvan in Paragraaf 2.1 verslag wordt gedaan, bevestigden de geldigheid van het gebruikte model, dat eerder al was toegepast op celkweken van pasgeboren ratten, bij toepassing op muizenembryo's. Klinische relevantie bleek niet alleen uit de omvang van de celsterfte na herhaald MTBI, maar ook uit metingen van twee indicatoren voor beschadiging of *markers*, te weten neuron-specifieke enolase (NSE) en het bindweefsel eiwit S-100 β .

Het eerste onderzoek naar MTBI in hippocampuskweken liet nog enkele vragen onbeantwoord. **Paragraaf 2.2** beschouwde de gevolgen van herhaald MBTI nogmaals door cellen een scala van letselschema's te laten ondergaan. Opnieuw werden de mate van beschadiging van de cel, celsterfte en de afscheiding van NSE en S-100 β gemeten. De verkregen gegevens duiden erop dat de mate van beschadiging na herhaald toegebracht letsel afhankelijk is van de ernst van de verwonding en van de tijd, verstreken tussen twee verwondingen. Meervoudig letsel treft met name twee groepen, te weten sportlieden en slachtoffers van kindermishandeling. De gegevens uit deze paragraaf duiden er niet alleen op, dat neuronen en bindweefselcellen verschillend reageren op eenzelfde verwondingsregime, maar ook dat het verruimen van de latentieperiode tussen opeenvolgende verwondingen van grote invloed is op de overlevingskansen van de cel.

Deze bevindingen gaven aanleiding tot verder onderzoek aan traumatisch letsel *in vitro* met behulp van kweken van cerebellaire neuronen en bindweefsel. Dit onderzoek is beschreven in **Paragraaf 2.3**. Bijzondere aandacht werd geschonken aan Purkinjecellen, omdat dit celtype als enige verantwoordelijk is voor de output van de kleine-hersenschors. Zoals reeds was waargenomen in kweken van de hippocampus, traden ook hier verschillen aan de dag tussen neuronen en bindweefselcellen. Opnieuw werd telkens na het toebrengen van letsel de afscheiding van NSE en S-100 β gemeten. De resultaten duiden erop, dat *in vitro* toegebracht letsel niet alleen

celstefte tot gevolg heeft, maar daarnaast ook tot veranderingen in de bindweefseiwitten en een verhoogde afscheiding van S-100 β leidt. De hier bijeengebrachte gegevens laten interessante verbanden zien tussen glutamaat, cerebellaire Bergmann-glia (BG), S-100 β en MT's (zie figuur 3 in Hoofdstuk 1). Van belang is, dat S-100 β verschillende uitwerkingen lijkt te hebben op hippocampusneuronen en Purkinjecellen. De inzichten verworven op basis van de gegevens uit deze paragraaf zijn van belang voor het onderzoek aan cerebellair TBI en dysfunctie van de kleine hersenen in het algemeen.

Hoofdstuk 2 besluit met een onderzoek naar toenames in de intracellulaire chlorideconcentratie bij kweken van hippocampuscellen, beschreven in **Paragraaf 2.4**. De kweken werden geprepareerd uit transgene muizen die beschikten over een geel fluorescerend eiwit dat gevoelig is voor veranderingen van het chloridepeil in bepaalde typen neuron. Hoewel tot dan toe de aandacht voornamelijk uitging naar door glutamaat veroorzaakte concentratietoenames van intracellulair Ca²⁺, zoals uitgebreid is besproken in Hoofdstuk 1, is een van de eerste verschijnselen die optreden in neuron na TBI een grote toename van de chlorideconcentratie. Dit veroorzaakt niet alleen een zwelling van de cel, maar wijzigt bovendien het broze homeostatische evenwicht in het celvocht. Nauwkeurige farmacologische analyse liet zien dat kortstondige toediening van glutamaat leidt tot depolarisatie van het celmembraan. Hierdoor kan chloride de hippocampusneuron binnen dringen via speciale chloridekanalen. De bevindingen van deze paragraaf lieten verder zien dat gebruik van celkweken uit transgene muizen zeer nuttig kan zijn bij de studie van TBI *in vitro*.

Hoofdstuk 3 beschouwt de werking van eiwitten die zich binden aan het plus-uiteinde van het MT, namelijk EB3 (*end-binding protein 3*) en CLIP-115 (*cytoplasmic linker protein of 115 kDa*) in zenuwcellen. **Paragraaf 3.1** liet zien hoe de dynamica van MT in beeld kunnen worden gebracht en worden bestudeerd door gebruikmaking van EB3 dat met GFT (*green fluorescent protein*) is gemerkt (*ge-tagged*). MT's zijn beweeglijke, polaire structuren die binnen de axonen en dendrieten van het neuron een bepaalde ligging aannemen om het intracellulaire transport te vergemakkelijken. In het axon liggen de MT's met het groeiende en krimpende positief geladen uiteinde naar de groeikegel van het axon toegekeerd en met het min-geladen uiteinde naar het cellichaam toe. In de dendriet liggen ze in beide richtingen. Bij de gekweekte hippocampusneuronen en cerebellaire Purkinjecellen uit Paragraaf 3.1 lieten de onderzochte MT's in beide typen cellen een vergelijkbaar groeitempo zien. Verder bleek dat de ligging van de MT's in axonen en dendrieten van volgroeide zenuwcellen verschilt van die in onvolgroeide, en dat het groeitempo van MT's in bindweefselcellen aanmerkelijk verschilt van dat in neuron.

Verscheidene experimenten uit Paragraaf 3.1 werden nog een keer uitgevoerd, nu met gebruikmaking van gekweekte hippocampusneuronen en cerebellaire Purkinjecellen afkomstig van CLIP-115 of CLIP-170 homozygote (*knockout*-) muizenembryo's, en beschreven in **Paragraaf 3.2**. De aanmerkelijk toegenomen snelheden van de EB3-GFP "strepen" in de knockout-neuronen duiden erop dat het wegnemen van een van de twee eiwitten effect heeft op de dynamiek van MT's en hun groei. Interessant is, dat de overeenkomstige snelheden in het bindweefsel van knockout-muizen vergelijkbaar waren met die, waargenomen bij het bindweefsel van het "wilde" type (de controlegroep). Bovendien was in de PC-dendrieten van CLIP-115 knockout-muizen de hoeveelheid b-tubuline aanzienlijk hoger vergeleken met wilde-type PC's. De besproken gegevens duiden erop dat verlies van een van de twee CLIP-eiwitten veranderingen in het neuronale MT-evenwicht tot gevolg heeft.

Het is bekend dat bij patienten met het Williams-syndroom het *CYLN-2*, het gen dat codeert voor de aanwezigheid van CLIP-115, evenals een 20-tal andere genen, hemizygoot ontbreken. Het Williams-syndroom is een erfelijke aandoening die tot een uniek gedragsprofiel leidt. Door voor CLIP-115 homozygote muizen te fokken, kon de specifieke invloed van dit

eiwit op het functioneren van de cel en de hersenen worden vastgesteld. De gegevens die in **Paragraaf 3.3** worden gepresenteerd werden verkregen uit hippocampusplakjes van volwassen homozygote (knockout-) muizen met CLIP-115, en van hun wilde-type nestgenoten. Onderzocht werden vervolgens de pyramide neuronen uit het gebied CA1 van de hippocampus, en wel op dendrietlengte en –samenstelling. Bijzondere aandacht ging daarbij uit naar de dendriet-*spines*, omdat die verreweg het meerendeel van de synapsen, de contactpunten tussen neuronen, bevatten. De plakjes werden bewerkt volgens de Golgi-methode, die honderd jaar geleden al door Ramon y Cajal werd toegepast om uit een populatie neuronen alleen selecte exemplaren te merken, en deze te prepareren voor onderzoek met een fluorescentie- of elektronenmicroscop. De analyses wezen uit dat verwijdering van CLIP-115 een beduidend effect heeft op de lengte van het neuriet, de vorm van de *spine*, en het aantal synapsen. Voorgaande onderzoeken hadden al aangetoond dat CLIP-115 knockout-muizen leer- en geheugenproblemen hebben. De gegevens uit Paragraaf 3.3. duiden erop dat morfologische veranderingen wel eens een sleutelrol zouden kunnen spelen bij deze tekortkomingen.

Afsluitend besprak **Hoofdstuk 4** de voorgaande hoofdstukken in hun onderlinge samenhang: welke inzichten zijn er verworven, en welke geheimen van het neuron zullen ontraadseld moeten worden? Allereerst werd de rol van intracellulair chloride bij hippocampuscelsterfte wat nader verkend dan in Paragraaf 2.4. was gebeurd, in het bijzonder bij Purkinjecellen. Vervolgens bleek, dat de precieze rol van S-100 β bij neuronale celsterfte nog onvoldoende duidelijk is, ook al werden in de Paragrafen 2.1., 2.2., en 2.3. hoge concentraties van het eiwit aangetoond na TBI. Daarom is in dit hoofdstuk de mogelijkheid van een verband tussen S-100 β en glutamaat-gemedieerde excitotoxiciteit onderzocht, in het bijzonder bij cerebellaire PC's en BG. Verder was in de Hoofdstukken 2 en 3 al gevonden dat MT's en met microtubuli samenhangende eiwitten (*microtubule associated proteins*, hierna MAP's) een kritieke rol spelen. Hier werden de bevindingen besproken die duiden op een mogelijk medierende functie van MAP's zoals CLIP-115 en CLIP-170 tussen actine en het cytoskelet van de MT's. Tot slot bleek dat een veelheid van factoren aanleiding kunnen geven tot aanpassingen in de vorm van de dendriet-*spine*, waaronder synaptische activering en glutamaat-gemedieerde excitotoxische stimuli. Ook het uitwissen van CLIP-115 als besproken in Paragraaf 3.3. leidde tot vervorming van de *spine*. De Hoofdstukken 2 en 3 behandelen de onderzoeksgegevens uitgaand van het vermoeden dat *spines* mogelijk een rol spelen bij de bescherming van het neuron.

List of publications

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Curriculum vitae

The author of this thesis was born on 22 October 1975 in Franconia, Pennsylvania, in the United States of America. In 1993, she graduated from Souderton Area High School, in Souderton, Pennsylvania, and in 1997, she graduated magna cum laude from the University of Richmond, in Richmond, Virginia, with a Bachelor of Arts (BA) degree in psychology, and minor degrees in Studio Art, Art History and Classical Studies. For her senior honors thesis project (1996-97), she studied the effects of clozapine, an atypical antipsychotic medication, on phencyclidine (PCP) using two-lever drug discrimination in rats at the Medical College of Virginia (MCV), also in Richmond. She was employed at MCV as a laboratory technician after graduation until early 2000, where she studied the interactions between nicotine and cocaine using receptor-binding assays, and the interactions between nicotine and bupropion, an antidepressant drug, using transfected *Xenopus* oocytes. She then moved with her future husband, John Weber, to the Netherlands where she began the projects described in this thesis in the Department of Anatomy, now the Department of Neuroscience, at the Erasmus Medical Center in Rotterdam. Since August 2000 she has lived with her husband, and sometimes her cats, Newton and Noelle, in Gouda.

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brancusi

i watch you from afar
and repeat the words that come out of your mouth.
my mind melds with yours
in case you forget the things you meant to say.

the white light filters through your hair,
and when you move into its glare you shine.
but you always shimmer and shine—
i can see the heat of your body in the dark like an ember's glow.

you go forward and yet you come back,
and realize that i am never far behind.
we did not start this journey together, now you cannot finish it alone:
like the stage and the podium,
i am your pedestal
but perhaps as much of the piece as the sculpture itself.

