



VCU

Virginia Commonwealth University
VCU Scholars Compass

Theses and Dissertations

Graduate School

2005

The Effect of Traumatic Brain Injury on Expression Levels of Ankyrin-G in the Corpus Callosum and Cerebral Cortex

Andrew S. Vanderveer
Virginia Commonwealth University

Follow this and additional works at: <https://scholarscompass.vcu.edu/etd>



Part of the [Nervous System Commons](#)

© The Author

Downloaded from

<https://scholarscompass.vcu.edu/etd/1538>

This Thesis is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.

**The Effect of Traumatic Brain Injury on Expression Levels of Ankyrin-G
in the Corpus Callosum and Cerebral Cortex**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University

By

Andrew S. Vanderveer
B.S., North Carolina State University, 2002

Director: Thomas M. Reeves, Ph.D.
Associate Professor
Department of Anatomy and Neurobiology

Virginia Commonwealth University
Richmond, Virginia
July, 2005

Acknowledgements

I would like to thank several people for helping me during my two years at VCU. First, my advisor, Dr. Thomas Reeves, helped me in numerous ways with my research project. His guidance, support and overall scientific expertise enabled my project to be a success, and I am very appreciative of his help. I would also like to thank my committee members, Dr. Linda Phillips and Dr. Robert Hamm for their input and support. The 7th floor of the MSB is a very special place because of several people. Nancy Nixon Lee, Raiford Black, Lesley Harris, Dr. Cristina Falo, John Greer and Janna Harris were instrumental in my neurotrauma research and I feel privileged to have worked with each of them. I would also like to thank Dr. George Leichnetz for the wonderful guidance and support that he gave me early and often. Finally I would like to thank my friends and family for believing in me every step of the way.

Table of Contents

List of Tables	v
List of Figures	vi
List of Abbreviations	vii
Abstract	ix
Introduction.....	1
Traumatic Brain Injury: A Public Healthcare Problem	1
Exitotoxicity.....	3
NO/ NOS.....	4
Reactive Oxygen Species.....	4
Inflammation.....	5
Apoptosis	6
Traumatic Axonal Injury.....	7
Ankyrins: structure and isoforms.....	10
The cytoskeleton	16
Ankyrins as multifunctional proteins involved in many cellular pathways.....	20
Sodium Channels	22
The electrogenic pump (Na ⁺ /K ⁺ ATPase)	29
Receptors.....	30
Protein Kinase C (PKC).....	31
Cell Adhesion Molecules (CAM's)	31
Clathrin	32
Potential TBI pathology involving Ankyrin	36
Failure of cellular stabilization	37
Alterations in cell shape.....	39

Alterations to voltage-gated sodium channels (NaV).....	40
Alterations to the electrogenic pump (Na ⁺ /K ⁺ ATPase).....	42
Injury effects related to ankyrin/CAM associations	42
Statement of experimental hypothesis for the current study.....	44
Methods.....	45
Subjects	45
Surgical Preparation.....	45
Injury Device and Procedure	46
Western Blot Analysis of Ankyrin-G	50
Re-probing of PVDF Membranes for Actin Load Controls	53
Statistical Analysis of Western Blot	53
Results.....	55
Discussion.....	66
List of References	82
Vitae.....	94

List of Tables

Table 1: Summary of significant changes in ankyrin-G expression in the corpus callosum and cortex with corresponding statistical values	65
---	----

List of Figures

1. Domain structure of ankyrin isoforms.	14
2. Membrane cytoskeleton structure.....	19
3. Major classes of cell adhesion molecules (CAMs).....	25
4. Model of interaction between ankyrin and β subunit of NaV channels	28
5. Model of clathrin-mediated endocytosis.....	35
6. Fluid percussion device and position of craniotomy	49
7. Western blot analysis of ankyrin-G in the corpus callosum and cerebral cortex.....	60
8. Quantitative changes in ankyrin-G expression within the corpus callosum.....	62
9. Quantitative changes in ankyrin-G expression within the cerebral cortex	64
10. Summary of methods and results from prior study of evoked callosal CAPs	73
11. Present ankyrin-G measurements compared to prior electrophysiology	75
12. Corpus callosum ultrastructure (at 3h) from prior study	78

List of Abbreviations

53BP2- p53-binding protein
AMPA- amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Ank1- Gene encoding ankyrin-R
Ank2- Gene encoding ankyrin-B
Ank3- Gene encoding ankyrin-G
BIAA- Brain Injury Association of America
CAM- Cell Adhesion Molecule
CAP- Compound Action Potential
CD44- Hyaluronate receptor
CDC- Centers for Disease Control and Prevention
COOH- Carboxy Terminus
DAI- Diffuse Axonal Injury
Dank1- Gene encoding drosophila melanogaster ankyrin
Dank2- Gene encoding drosophila melanogaster ankyrin
DNA- Deoxyribonucleic Acid
ELAM- Endothelial Leukocyte Adhesion Molecule
eNOS- Endothelial Nitric Oxide Synthase
ICAM- Intracellular Adhesion Molecule
Ig- Immunoglobulin
IL- Interleukin
iNOS- Inducible Nitric Oxide Synthase
IP3- Inositol 1,4,5-triphosphate
IP3R- Inositol 1,4,5-triphosphate receptor
kD- Kilo Daltons
MAP2- Microtubule-Associated Protein
Mr- Molecular weight
NaV- Voltage-Gated Sodium Channel
NF- Neurofilament
NgCAM- Neuro-glial Cell Adhesion Molecule
NH2- Amino Terminus
NHIS- National Health Interview Survey

NIH- National Institutes of Health
NMDA- N-methyl-D-aspartate
nNOS- Neuronal Nitric Oxide Synthase
NO- Nitric Oxide
NOS- Nitric Oxide Synthase
NrCAM- Neuronal Cell Adhesion Molecule
PKC- Protein Kinase C
ROS- Reactive Oxygen Species
RyR- Ryanodine Receptor
S2- Schneider's Line 2 Cells
TAI- Traumatic Axonal Injury
TBI- Traumatic Brain Injury
TNF α - Tumor Necrosis Factor Alpha
unc44- Gene encoding *Caenorhabditis elegans* ankyrin

Abstract

THE EFFECT OF TRAUMATIC BRAIN INJURY ON EXPRESSION LEVELS OF ANKYRIN-G IN THE CORPUS CALLOSUM AND CEREBRAL CORTEX

Andrew S. Vanderveer, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2005

Director: Thomas M. Reeves, Ph.D.
Associate Professor
Department of Anatomy and Neurobiology

The ankyrins comprise a family of proteins serving as components of the membrane cytoskeleton, and participate in a diverse set of associations with multiple binding partners including the cytoplasmic domains of transporters, ion channels, some classes of receptors, and cell adhesion proteins. Moreover, evidence is accumulating that ankyrin participates in defining functionally distinct subcellular regions. The complex functional and structural roles of ankyrins indicate they are likely to play essential roles in the pathology of traumatic axonal injury. The current study examined changes in ankyrin-G

expression following a moderate central fluid percussion injury administered to adult rats. At 1d, 3d, and 7d postinjury (or following a sham control injury), protein levels of ankyrin-G in the corpus callosum and cerebral cortex were assessed using Western Blot analysis. Three immunopositive bands were identified in both brain regions as 220, 212, and 75 kD forms of ankyrin-G. Time-dependent changes in ankyrin-G were observed in the corpus callosum. At 1d injury-induced elevations were observed in the callosal 220 kD (+147% relative to sham levels) and in the 212 kD (+73%) forms of ankyrin-G, but in both cases the expression decreased to control levels by 3d and 7d. In contrast, the 75 kD form showed moderate increases at 1d postinjury, but was significantly below control levels at 3d (-54%) and at 7d (-41%). Ankyrin-G expression in the cerebral cortex was only slightly affected by the injury, with a significant decrease in the 220 kD form occurring between 1d and 3d. These data suggest that the 220 and 212 kD changes probably represent postinjury proteolytic fragments derived from intact ankyrin-G isoforms of 480 and/or 270 kD, while the 75 kD effects are likely breakdown products of intact 190 kD ankyrin-G. These results were discussed as they relate to prior findings of differential vulnerabilities of callosal myelinated and unmyelinated axons to injury. In this context, the 220,212 kD changes may reflect pathology within myelinated axons, and alterations to the 75 kD form may reflect more persistent pathology affecting unmyelinated callosal fibers.

INTRODUCTION

Traumatic Brain Injury: A Public Healthcare Problem

Among individuals in the United States under the age of 45, injury remains the primary cause of death. Within this group, traumatic brain injury (TBI) is the major cause of mortality and morbidity (Sosin et al, 1989). Estimates of the incidence of TBI vary widely, depending upon the such the sampling methodology, regional variations in reporting, historical trends, and basic definitional issues. Some estimates fail to make a clear distinction between TBI and “head injury”: the latter being a somewhat antiquated term referring to external face and/or scalp injuries, including lacerations, contusions, and fractures which may or may not be associated with TBI. A more accurate definition of TBI has been offered as “...an alteration in brain function manifest as confusion, altered level of consciousness, seizure, coma, or focal sensory or motor neurological deficit resulting from a blunt or penetrating force to the head.” (Bruns and Hauser, 2003, p. 2).

The incidence of TBI in developed countries is usually estimated at about 200 per 100,000 population at risk per year (Bruns and Hauser, 2003). However, this estimate includes only TBI patients admitted to hospitals, and probably systematically underestimates the TBI frequency by undercounting cases which are medically unattended or are handled by nonreporting emergency department facilities.

Prior projects to estimate TBI incidence from the U.S. National Health Interview Survey (NHIS) have suggested a historical trend in this form of injury. Fife (1987) reported an incidence rate of TBI in the U.S., during the period 1977 to 1981, as 825 per 100,000, while Sosin (1996) provided an estimate of 618 per 100,000. This discrepancy in overall incidence may reflect, in part, a decreasing rate of TBI in the United States. By any estimate, TBI remains a serious health concern in the United States, with 52,000 annual deaths, and prevalence estimates of up to 6.5 million affected (NIH, 1999). Across a broad spectrum of head injury conditions and accidents, TBI patients often face a lifelong struggle to regain cognitive and physical functioning.

Epidemiological studies have shown that the types of accidents leading to TBI are strongly associated with the patient's demographics, age, and geographic location. One survey in the United States showed that automobile, motorcycle, and bicycle collisions accounted for approximately half of all traumatic brain injuries (Annegers et al, 1980). This survey also showed that about one-third of TBIs resulted from falls, and 10% from recreational activities. Irrespective of the nature of the accident leading to TBI, the pathophysiological processes set in motion have been the subject of extensive investigation. Most researchers agree that TBI can be described as a biphasic process: with an acute excitotoxic phase of widespread cellular depolarization and necrotic cell death, followed by a more protracted secondary phase characterized by changes in cerebral blood flow, local and systemic inflammation, alterations in oxygen delivery and

metabolism, and apoptotic cell death (for reviews, Hays et al., 1992; Dutton and McCunn, 2003; McIntosh et al, 1998; Leker and Shohami, 2002). Extensive evidence, accumulated over several decades, has identified a number of pathological processes affecting cells following neurotrauma. These are generally death-promoting mechanisms, and include excitotoxicity, nitric oxide-induced neurotoxicity, postinjury generation of deleterious free radical ionic species, inflammation of damaged nervous tissue, and programmed cell death (apoptosis). These aspects of TBI pathology are briefly described below.

Excitotoxicity

A critically important early event in the course of TBI pathology is the uncontrolled and indiscriminant release of excitatory amino acid neurotransmitters, especially glutamate and aspartate (Faden et al, 1989; Katayama et al, 1990). Within minutes after exposure of neurons to high levels of glutamate, NMDA and AMPA receptors are opened to allow the influx of calcium, sodium, and water into injured cells (Young, 1986; Goldberg and Choi, 1993; Obrenovitch and Urenjak, 1997). This cationic influx leads to cytotoxic edema and a disruption of ionic homeostasis associated with a deficit in metabolic energy stores. The resulting elevations of intracellular calcium activates a number of enzymes (lipases, proteases, endonucleases) which may damage cellular proteins, lipids, and DNA, ultimately culminating in cellular death. Experimental treatments which inhibit excitatory amino acid release from presynaptic terminals, or

block the postsynaptic binding of these ligands, have proved neuroprotective in various TBI and ischemia experimental models (Foster et al, 1988; Shimizu et al., 1998; Schielke et al., 1999).

NO / NOS

Nitric oxide (NO) is a gaseous compound with multiple roles in the nervous system, and appears to function as a neurotransmitter at some levels of the nervous system. The compound is produced from arginine by three distinct forms of NO synthase (NOS): neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) (Forstermann et al., 1994). nNOS and eNOS are calcium dependent and constitutively expressed (Forstermann et al., 1998), whereas iNOS is calcium independent and is upregulated under various stressful conditions including trauma (Gahm et al., 2000). Both iNOS and nNOS are upregulated following TBI (Alagarsamy et al., 1998; Wada et al., 1998; Rao et al., 1999; Gahm et al., 2000), and are thought to be destructive due to mitochondrial damage and the production of damaging radicals (Lipton et al., 1993; Rao et al., 1998).

Reactive Oxygen Species

A necessary consequence, of persistently elevated intracellular calcium, is the formation of superoxide anion radicals by the respiratory chain, and by cytosolic enzymes such as xanthine oxidase. This ultimately leads to increased oxidative stress

(Juurlink and Paterson, 1998). The brain is especially vulnerable to oxidative damage for the following reasons: 1. a high rate of oxidative metabolic activity, 2. intensive production of reactive oxygen metabolites, 3. relatively low antioxidant capacity, 4. low repair mechanism activity, 5. non-replicating nature of its neuronal cells, and, 6. a high membrane surface to cytoplasm ratio (Evans, 1993; Reiter, 1995; Leker and Shohami, 2002). Prolonged elevated levels of ROS eventually leads to lipid peroxidation, whereby free radicals destroy adjacent molecules. The outcome of these destructive processes may explain some of the oxidative damage detected after TBI or ischemic injury to the brain. Microdialysis studies have reported the over-production of ROS in the lesioned brain (Lewen and Hillered, 1998). Neutralization of reactive nitrogen or oxygen species may be protective within a window of 3–4 hours postinjury by attenuating oxidative stress and its devastating consequences. Moreover, it has been demonstrated that antioxidants can indeed reduce tissue damage following TBI (Dewitt et al, 1997; Xiong et al., 1997; Fabian et al., 1998).

Inflammation

An additional mechanism of posttraumatic cellular damage involves inflammatory cells in the traumatic region. Inflammatory cytokines including IL-1, IL-6, and TNF α , are activated and secreted as early as 1 hour after TBI (Shohami et al., 1994; Arvin et al., 1996; Fan et al., 1996). These, and related, cytokines induce an inflammatory reaction, functioning as chemoattractants to leukocytes. Metalloproteinases, along with associated

cell adhesion molecules in the extracellular matrix (e.g., ICAM and ELAM), are also expressed early after injury and promote the penetration of leukocytes through the blood brain barrier (Pantoni et al., 1998).

Apoptosis

Contemporary theory in TBI classifies cells as dying of necrosis or apoptosis, and estimates suggest that up to 50% of cellular death is due to programmed (apoptotic) mechanisms (Raghupathi et al., 2000). Intracellular and extracellular signals have both been reported to initiate apoptosis: with intracellular apoptotic signals triggered by mitochondrial dysfunction, and extracellular signals initiated by activation of TNF receptors which recruit caspases and activate death domains (Eldadah et al., 2000).

The pathological cascade of caspase activation entails release of cytochrome c from mitochondria and the subsequent activation of procaspases. This process culminates in the formation of effector caspases (primarily caspase 3) that mobilize endonucleases which breaks down DNA and ultimately lead to cell death (Snider et al., 1999).

Apoptosis is not exclusively an experimental observation, as evidence of active apoptosis has been described in humans following trauma, lending support to the importance of accelerated programmed cell death as a pathological process (Clark et al., 1999).

Traumatic Axonal Injury

Axons in the CNS are subject to all the foregoing TBI pathomechanisms, and axonal damage is a concern in the aftermath of any head injury. A growing body of

evidence indicates that axons represent a particularly vulnerable subcellular compartment. Axonal injury is now recognized as one of the most common pathologies, and a number of studies have reported significant correlations between the extent of axonal damage and the degree of functional deficits following TBI (Adams et al., 1982; Graham et al., 1988; Povlishock, 1992). Research in traumatic axonal injury (TAI) is a topic of especially high interest and activity in the field of head injury. Because the present thesis is concerned primarily with TAI, the following section provides a general discussion of essential findings in this area. In this discussion, an emphasis is given to those research results and concepts which provide a theoretical context for the importance of the axonal cytoskeleton, in general, and the ankyrin molecules, in particular, as key links in the pathological failure of injured axons.

The distinction between “focal” injury and “diffuse” injury has a special relevance to research in TAI, both historically and for the interpretation of current findings. A focal brain injury usually denotes the results of a blow to the head that produces cerebral contusions and/or hematomas (Gennarelli, 1993). In contrast, a diffuse injury may occur without an impact component to the injury, but instead usually results from inertial forces, and is commonly seen clinically after motor vehicle crashes and some falls (Blumbergs et al., 1989; Grady et al., 1993). The inertial forces result from rapid head motions, which deform the white matter and lead to a diffuse injury of axons. Some authors have pointed out that the term “diffuse” is imprecise in the sense that the axonal damage actually presents as a multifocal pathology, particularly affecting midline structures such as the corpus callosum (Smith and Meaney, 2000). Microscopic

examination of diffusely injured white matter reveals numerous swollen and disconnected axons (Povlishock, 1992). Severe cases of diffuse TAI often show white matter tissue tears accompanied by intraparenchymal hemorrhage in central commissures, basal ganglia, and brainstem, and is associated with prolonged postinjury unconsciousness, high mortality, and poor outcome (Ommaya and Gennarelli, 1974; Gennarelli, 1993).

Historically, it was thought that the rapid acceleration and deceleration of the brain during a traumatic injury led to a shearing of axons. This stress was presumed to lead to actual breakage of axons, with each proximal portion retracting and expelling a ball of axoplasm. This accounted for the appearance of the traditional histological features of “retraction balls” observed on autopsy (Strich, 1961; Oppenheimer, 1968). However, more recent evidence has accumulated that does not support this basic concept of axonal disconnection at the time of injury. Instead, contemporary models of TAI conceive of the injury process as a more gradual multiphasic progression (Fitzpatrick et al., 1998; Povlishock, 1992; Erb and Povlishock, 1988; Sheriff et al., 1994). Axonal membranes may undergo damage at the time of the injury, but rather than an immediate disconnection, the course of pathology appears to begin with an initial failure of membrane integrity. Whether due to microporation or changes in transmembrane ionic channels, the early phases of TAI are characterized by a pathological influx of cations, Na^+ and Ca^{2+} , which initiates aberrant cellular depolarization and the mobilization of multiple calcium-dependent processes, such as the activation of proteases which degrade the structural and functional properties of axons. Pathological changes, caused by the depolarization, ionic dysregulation, and a cascade of proteolytic processes, evidently

culminates in axonal disconnection and the appearance of retraction balls. This delayed phase of TAI unfolds over the course of hours to days after an injury, and is referred to as secondary axotomy.

A critically important feature of axons, which relates to this thesis, is the axonal cytoskeleton. This intricate molecular network performs multiple functions in axons: structural support, localization and stabilization of transmembrane proteins such as receptors and ion transporters, extension and movement during neuritogenesis, participation in axoplasmic transport both antero- and retrogradely, and a newly-appreciated intermediary role in some signal transduction pathways (Pumplin and Bloch, 1993; Robtsov and Lopina, 2000; Panicker et al, 2003). TAI appears to elicit a focal axonal abnormality that leads to the failure of axoplasmic transport, and the build-up of organelles and transported material and consequent swelling of the axon (Povlishock, 1992). The disconnection leads to two axonal segments, a proximal portion in continuity with the soma, and a distal axon segment which undergoes Wallerian degeneration (and deafferentation of postsynaptic cellular targets). A primary cause of the impaired axonal transport is damage to the axonal cytoskeleton, a major component of which are neurofilaments (NF). Some NF subtypes contain “sidearm” domains with dimensions which are modulated by their phosphorylation. Accordingly, the diameter of axons is determined, in large part, by the conformation and dimensions of the NF sidearms. An important series of studies has indicated that TAI induces a compaction of NFs, caused by proteolysis of the sidearms (Hall et al., 1995; Povlishock et al., 1997; Maxwell et al., 1997).

In addition to injury-induced NF changes, TAI also appears to impair additional cytoskeletal components, such as microtubules. Microtubules are the primary conveyors of fast axonal transport, and evidence suggests that they are degraded by proteolytic events triggered by intracellular calcium loading after injury (Maxwell and Graham, 1997).

Ankyrins: structure and isoforms.

The ankyrins form a family of proteins that function not merely as simple linker molecules, but are also involved in signal transduction and protein sorting. This molecular family has binding sites for diverse integral membrane proteins, as well as for cytoskeletal proteins. The diversity in this family has been understood, in part, by studies employing molecular cloning, which has identified three genes encoding ankyrins: ANK1, ANK2, and ANK3, which are expressed as alternatively spliced isoforms (Rubtsov and Lopina, 2000). Polypeptides corresponding to these genes are designated as ankyrin-R, ankyrin-B, and ankyrin-G. All three ankyrins that have been cloned and sequenced have a large NH₂ terminal domain responsible for binding to integral membrane proteins, a central domain responsible for binding spectrin and a COOH domain with regulatory functions (Bennett and Gilligan, 1993). However, other ankyrin variants have been reported that do not share this structure. The most thoroughly characterized of these molecules are ankyrin-R with molecular weight (Mr) 206 kD, and ankyrin-B with Mr = 220 kD. These isoforms exhibit a similarity in domain structure

(Bennett, 1992). These isoforms contain three domains: an N-terminal membrane-binding domain, a central spectrin-binding domain, and a C-terminal regulatory domain. Figure 1 summarizes these isoforms, along with information regarding diversity within domain structure, and tissue specificity of ankyrin isoforms.










The membrane-binding domain is formed by 24 tandemly organized repeat motifs ("ankyrin repeats"), each of which is made of 33 amino acids (Bennett, 1992). Ankyrin repeats (ANK repeats) are involved in protein recognition and are found in more than 325 unrelated human proteins, including tankyrase, p53-binding protein (53BP2) among others. (Sedgewich and Smerdon, 1999). The membrane-binding domain is formed by four independently folded subdomains of six repeats each (Michaely and Bennett, 1993), and the entire domain assumes a globular shape.

The spectrin-binding domain is based on three sub-domains (Peters and Lux, 1993). The C-terminal region of this subdomain is neutral. The central region contains mainly basic amino acids. The N-terminal end of this subdomain is enriched in proline and acidic amino acids. This acidic portion is variable, while the neutral and basic regions are highly conserved between ankyrins R and B (Bennett, 1992).

The regulatory domain of ankyrin is highly acidic and is sensitive to proteolysis (Lux et al., 1990; Lambert et al., 1990). This regulatory domain modifies the affinities of the spectrin-binding and membrane-binding domains to their target proteins (Hall et al., 1987).

The various ankyrin isoforms are differentially distributed in various tissues of the body. In human erythrocytes, the major ankyrin is ankyrin-R ($M_r = 206$ kD) (Palek and Lambert (1990). Ankyrin-R is also expressed, although less abundantly, in macrophages, muscle tissue, and endothelial cells (Birkenmeier et al., 1993; Thevananther et al., 1998). Ankyrin-R has been identified in neural cells, specifically in granule and Purkinje cells of the cerebellum, and in some neurons of the spinal cord and hippocampus (Kordeli and Bennett, 1991).

Figure 1. Domain structure of ankyrin isoforms. Information regarding regional tissue levels of ankyrin isoforms and cell type is given for ankyrin-R, ankyrin-B, and ankyrin-G.

Isoforms		Domain Structure
Ankyrin-	(kD)	
-R Expressed in erythrocytes, muscle, macrophages, endothelial cells, cerebellar cells, spinal cord, hippocampus.	206	The major ankyrin in human erythrocytes. 
	186	Alternative splice: results from deletion of 162 AAs from regulatory domain. 
	20-26	Alternative splice: found in skeletal muscle. Contains only regulatory domain. 
-B Found mainly in nervous tissue.	440	Found in embryonic unmyelinated axons & dendrites. 
	220	Organized similar to ankyrin-R. Expressed in cell bodies and dendrites of neurons and in glia. 
-G Expressed in many tissues: epithelial cells, myocytes, hepatocytes, and neurons.	480	Localized in nodes of Ranvier, and initial segments of axons. 
	270	Localized in nodes of Ranvier, and initial segments of axons. 
	190	Abundant in unmyelinated axons. 
	100-120	Expressed in epithelium, muscle, associated with Golgi apparatus. 

Key:



Membrane-binding domain: Formed by 24 ankyrin repeats, organized as globular structure with 4 folded sub-domains of 6 repeats each.



Spectrin-binding domain: Contains 3 sub-domains: acidic N-terminal, basic central, variable C-terminal.



Regulatory domain: Modulates activities of membrane- and spectrin-binding domains. Sensitive to proteolysis.



Extended rod-shaped domain: 220 kD structure inserted between C-terminal and spectrin-binding domains.



Threonine and serine enriched domain.



Unique fragments (30-80 amino acid residues) in non-neural ankyrins.

Ankyrin-B is present primarily in nervous tissue. The large 440 kD form of ankyrin B contains a 220 kD rod-shaped domain inserted between the spectrin-binding and C-terminal domains (Figure 1). This ankyrin isoform is expressed in unmyelinated axons and dendrites in embryonic brain (Kunimoto et al., 1991). The 220 kD isoform of ankyrin-B (which lacks the rod-shaped inclusion) is expressed in glial cells and in neuronal perikarya and dendrites (Otto et al., 1991).

Ankyrin-G is expressed in multiple tissues, being the main form in myocytes, hepatocytes, and neurons (Peters et al., 1995; Rubtsov and Lopina, 2000). The distribution of the isoforms of ankyrin-G is particularly interesting. The 190 kD isoform is abundant in unmyelinated axons, while the 270 and 480 kD isoforms of ankyrin-G are present in the nodes of Ranvier as well as in the initial segments of myelinated axons (Kordeli et al., 1995; Peters et al., 1995). The 480 kD isoform of ankyrin-G contains the extended rod-shaped domain, analogous to that found in ankyrin-B, but also contains a unique 46 kD fragment enriched in serine and threonine inserted between the spectrin-binding domain and the terminal part of the 480 and 270 kD ankyrin-G molecules (Figure 1). It is this serine/threonine component which appears to determine the specific localization of these isoforms, probably mediated by the glycosylation status of the fragment (Kordeli et al., 1995).

The cytoskeleton

The shape of a neuron is a vital and fundamental property, and is primarily maintained by the cytoskeleton. Any manipulation that damages the cytoskeleton, for example a mechanical injury or a pharmacological manipulation, will usually have grave consequences for the cell, and, as discussed below, the consequences will be both structural and functional. Cytoskeletal changes have been studied in models of experimental brain injury. A number of studies have reported postinjury changes in neurofilaments and microtubules, two of the primary cytoskeletal components. A TBI-induced decrease in microtubule-associated protein (MAP2) and a decrease in the amount of neurofilament (NF) protein has been reported (Posmantur et al., 1994; Hicks et al., 1997). Most workers in this area agree that a rapid postinjury influx of calcium is a primary event that leads to cytoskeletal degradation. High intracellular calcium has been shown to induce activation of calpains (calcium-activated proteases) which degrade multiple cytoskeletal components (Newcomb et al., 1997; Zhao et al., 1998).

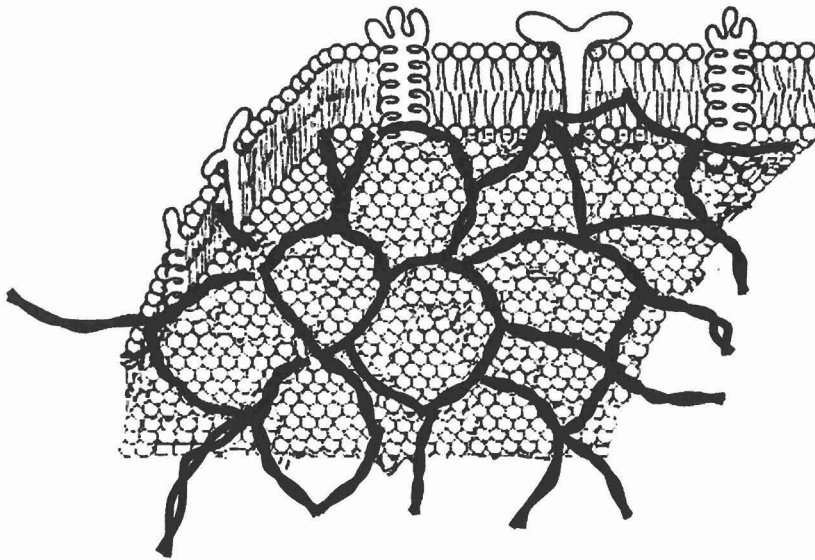
The membrane cytoskeleton. In addition to the neuronal cytoskeleton, comprised of neurofilaments, microtubules and actin microfilaments, there is a separate cytoskeletal component that associates with the plasma membrane. Often referred to as the "membrane cytoskeleton," it is a molecular assembly forming basically a two-dimensional network located on the cytoplasmic surface of cell membranes (Bennett and Gilligan, 1993). The primary component of the membrane skeleton is an arrangement of

spectrin molecules joined at their ends to form five- or six-sided polygons (Fig. 2 A,B). At the corners of the polygons, the spectrin is complexed with short actin filaments. The membrane cytoskeleton is attached to the plasma membrane through ankyrin (Fig. 2 B,C). Ankyrin appears to also provide linkages between the cytoskeleton and the membranes of intracellular organelles.

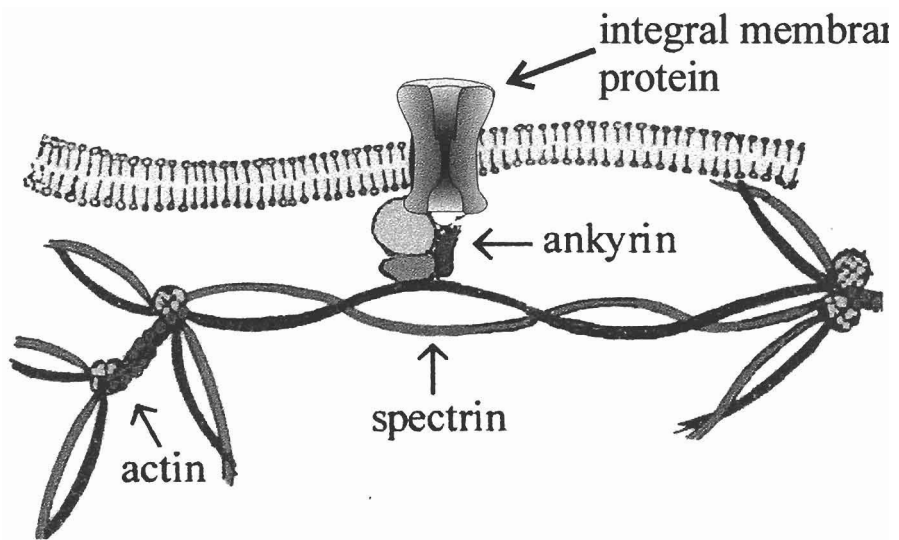
The membrane cytoskeleton has been most extensively investigated in human erythrocytes, both biochemically and ultrastructurally (Bennett, 1990; Pumplin and Bloch, 1993). It was in this cell type that the modular and flexible properties of spectrin, and its binding partners, were first appreciated. Spectrin, and related actin-binding proteins, have a flexible oligomerization property: forming tetramers and larger oligomers. These polymorphic molecular associations lead to a deformability in cytoarchitecture, which is essential for a blood-borne element which must pass through highly tortuous microcapillaries. The membrane cytoskeleton interacts with the general cellular cytoskeleton in highly specified associations. Specifically, spectrin interacts preferentially with neurofilaments, while ankyrin has a binding specificity for microtubules (Bennett, 1990).

Figure 2. Membrane cytoskeleton structure, relationship to plasmalemma, and detail of ankyrin domains. **A.** Interior view of a patch of plasmalemma showing apposition of spectrin/ankyrin polygons to membrane. **B.** Detailed view of membrane cytoskeleton, showing ankyrin as a linker molecule between β -subunit of spectrin and an integral membrane protein (in this case an ion channel). **C.** Enlargement showing presumptive globular domains of ankyrin. [*Source:* adopted from Rubtsov and Lopina, 2000].

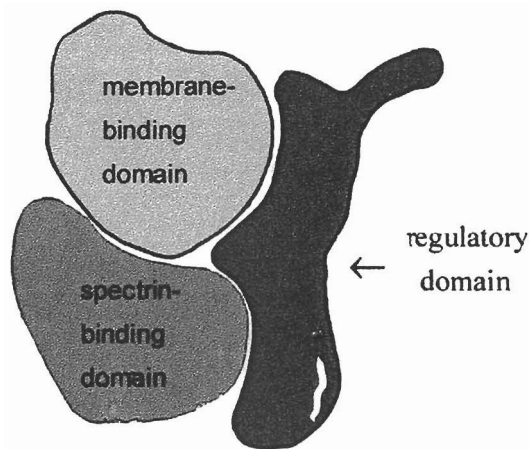
A



B



C



The membrane skeleton also participates with the other cytoskeletal components - microtubules, neurofilaments, and microfilaments -- in preserving cell shape, but serves the specialized function of stabilizing some integral membrane proteins within the lipid bilayer. Ankyrin plays a pivotal role in this aspect of membrane cytoskeletal functioning, and appears to restrict the lateral diffusion of a large, and growing, class of integral membrane proteins. The most thoroughly studied of these associations is the restriction of lateral mobility of the anion exchanger molecule in erythrocytes (the so called "band 3" protein which exchanges bicarbonate for chloride ions) (Michaely and Bennett, 1995a). In this capacity, ankyrin is positioned between the spectrin network and the transmembrane protein. This trimolecular complex arrangement has also been documented for ankyrin associations with sodium channels, the Na^+/K^+ -ATPase electrogenic pump (Baines, 1990), certain classes of receptors, and cell adhesion molecules. Antibodies against ankyrin immunoprecipitate sodium channels in membrane fractions from rat brain, and ankyrin binds to purified sodium channels in a ratio of approximately one ankyrin molecule per sodium channel (Srinivasan et al., 1988).

Ankyrins as multifunctional proteins involved in many cellular pathways

A distinguishing feature of ankyrin, and of its functionality, is the molecule's diverse set of interactions with various membrane proteins. The membrane-binding domain of ankyrin interacts with the cytoplasmic domains of transporters, ion channels, some classes of receptors, and cell adhesion proteins. Evidence is accumulating that

ankyrin participates in defining functionally distinct subcellular regions: spatially segregated "patches" of individual cells are often required to be structurally and functionally distinct. One property of ankyrin which facilitates the formation of subcellular zones is that a single molecule of ankyrin is able to bind several membrane proteins to coordinate the assembly of integral membrane proteins into specialized zones of plasma membrane (Michaely and Bennett, 1995b). An example of such a specialized zone is the node of Ranvier which contains neurofascin (Davis et al., 1993) along with a specialized form of ankyrin (Kordeli et al., 1995), and is enriched in several ankyrin-binding membrane proteins including the Na^+/K^+ -ATPase, voltage gated Na^+ channels, and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Waxman and Ritchie, 1993). The formation of specialized regions, such as the node, may be established during development by early interactions of ankyrin with members of the L1/neurofascin family which may be involved in the localized assembly of various ion channels and transporters.

The mechanism by which ankyrin is capable of establishing linkages, with such a diverse set of membrane proteins, was studied in detail by Michaely and Bennett (1995b). These authors explored the structural basis for diversity in recognition of membrane proteins based on the specialized N-terminal region of the ankyrin molecule which is comprised of a 24 tandemly arranged ANK repeats organized into 4 folded domains of 6 ANK repeats each (Davis and Bennett, 1994). Using a competitive binding methodology, these authors demonstrated that neurofascin interacts with high affinity at two distinct sites on ankyrin formed by different combinations of the ANK repeat subdomains. They described a complex set of competitive interactions that governed the

ankyrin interaction with neurofascin and the $\text{Cl}^-/\text{HCO}_3^-$ ion exchanger, and proposed a model of ankyrin binding. One aspect of the model is shown in Figure 3C, which illustrates how ankyrin may be able to coordinate the formation of neurofascin 'pseudodimers' through the flexible binding combinations of the four ANK subdomains.

The following is a discussion of results that relate to specific binding partners of ankyrin. Some of these studies have focused on neural tissue, for example ion channels in axons. In contrast, other molecular roles for ankyrin have primarily been described in non-neural cells: in several cases T-lymphocytes. However, in the same way that many concepts of structure and function in erythrocyte ankyrin has transferred to an understanding of the neuronal expression of this molecule, there is every reason to expect a high degree of homology between the ankyrin binding partners in neurons and the non-neural cellular models.

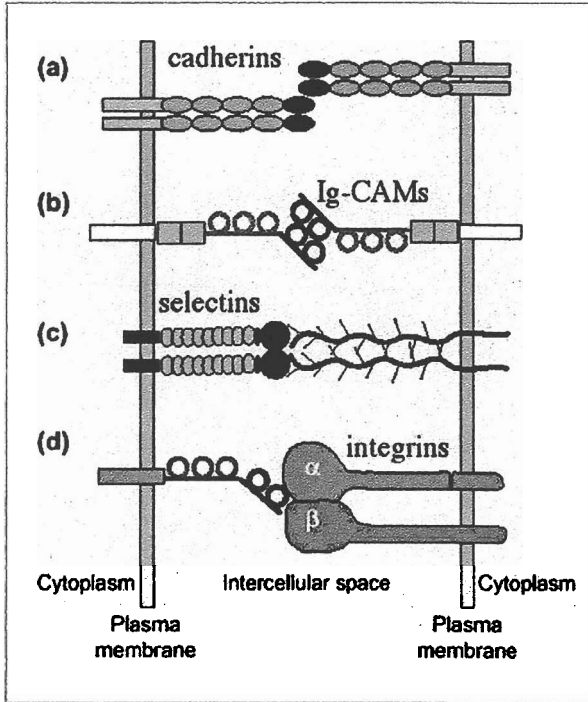
Na-channels. Voltage-dependent sodium channels (NaV) are found throughout the neuraxis: concentrated at the initial segments of axons and at nodes of Ranvier. NaV channels are thought to be uniformly distributed in the axolemma of unmyelinated axons (Pellegrino et al., 1984). It is thought that ankyrins play an important role in the localization and maintenance of NaV channels. Control of the density and localization of NaV channels is a critical aspect of neuronal functioning. This appears to be especially important at nodes of Ranvier where high NaV densities are required for rapid and reliable saltatory conduction. NaV channels from mammalian brain are heterotrimeric

structures composed of a central pore-forming α -subunit and two auxiliary subunits $\beta 1$ and $\beta 2$ (Isom et al., 1994). While the β subunits do not form the ion conducting pore, they do play vital roles in channel gating, mechanisms of voltage-dependent activation and inactivation, and the expression levels of channels on the cell membrane (Patton et al., 1994).

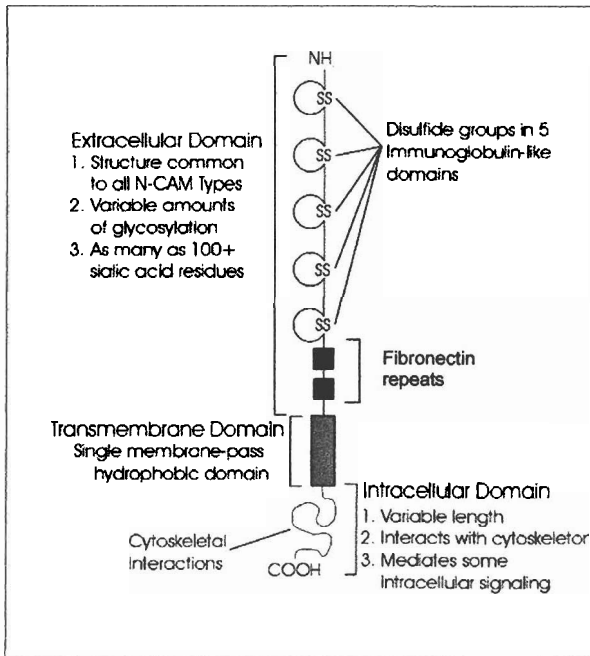
The β subunits contain immunoglobulin (Ig)-like extracellular domains and are members of the Ig superfamily, which includes many cell adhesion molecules (CAMs) (Isom and Catterall, 1996). The β units of NaV have been demonstrated to interact with the extracellular matrix proteins tenascin-C and tenascin-R (Srinivasan et al., 1998; Xiao et al., 1999). A number of studies have suggested that the NaV β subunits function as CAMs: transfected cells expressing β subunits initially bind to tenascin-R substrate (Xiao et al., 1999); purified NaV bind tenascin-C and tenascin-R (Srinivasan et al., 1998). Interestingly, CAMs of the L1 family also bind directly to cytoskeletal elements such as ankyrin (Davis et al., 1993).

Figure 3. Major classes of cell adhesion molecules (CAMs), and details of ankyrin association with Ig-CAMs. **A.** Homophilic and heterophilic interactions of CAMs are shown for the major classes of CAMs. Ig-CAMs and cadherins are shown as typifying homophilic interactions. The Ig-CAMs interact via the extracellular Ig domains. **B.** Details of Ig-CAM showing extracellular Ig domains at N-terminus, adjacent to fibronectin repeats. Cell attachment is via a transmembrane hydrophobic domain. Ankyrin interacts with Ig-CAMs at a highly variable cytoplasmic component is found at the C-terminal region. **C.** Model for the association of neurofascin with ankyrin, as proposed by Michaely and Bennett (1995). The membrane-binding region of ankyrin contains ANK repeats which form two sites with an affinity for neurofascin: one involving the second and third (D2, D3) repeat domain, and the other requiring D3 and D4. This arrangement, involving two potential ankyrin binding configurations, allows ankyrin to form a 'pseudodimer' of neurofascin.

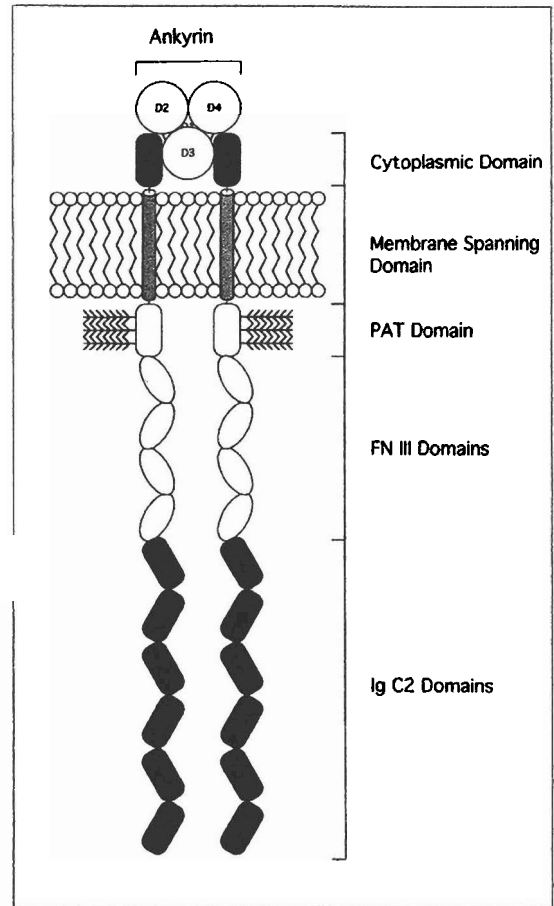
A



B

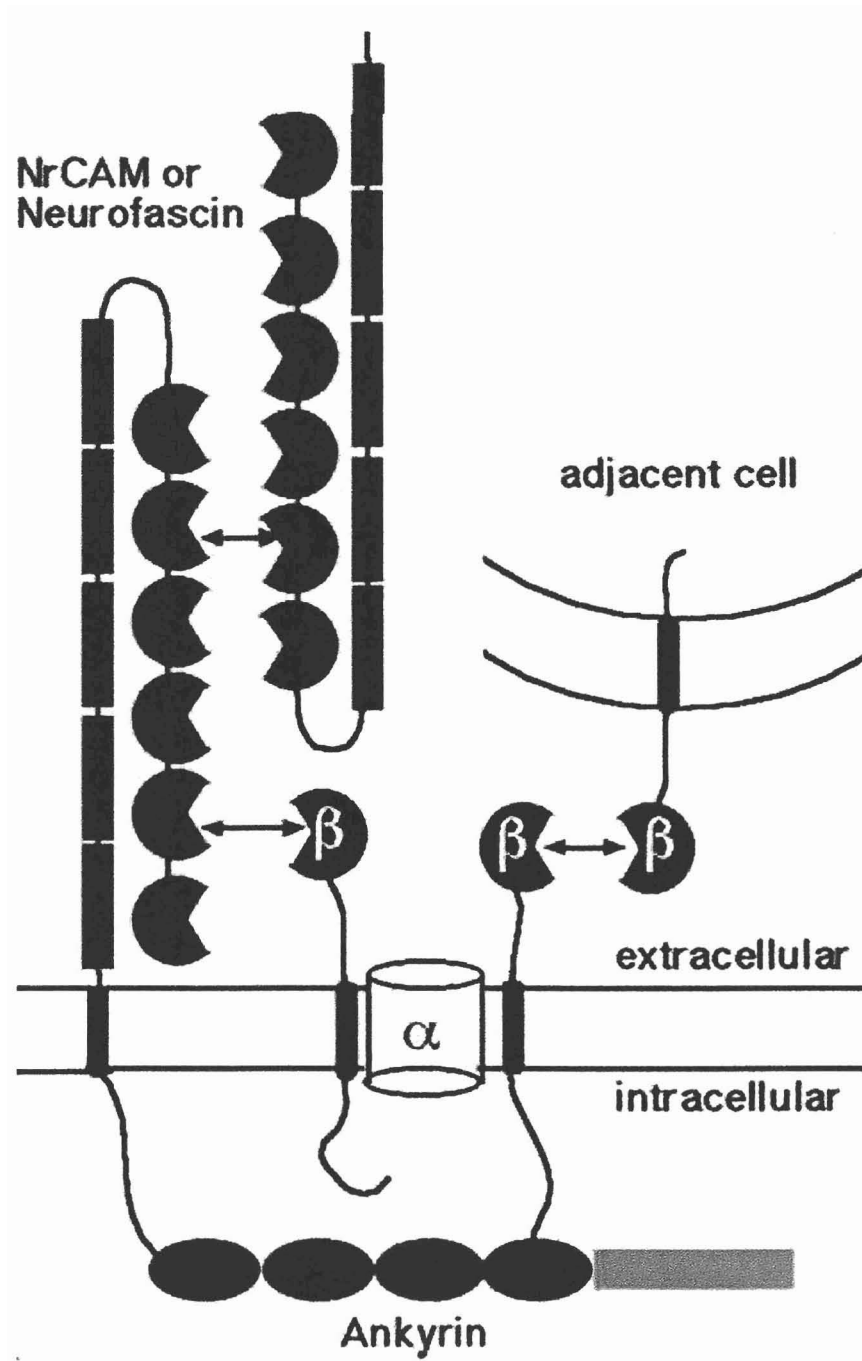


C



Although it is certain that ankyrins associate with NaV, it is not known which specific NaV subunit(s) bind with ankyrin. This issue was addressed by Malhotra et al. (2000), who tested the hypothesis that the β subunits of NaV would interact with ankyrin, because these subunits are structurally and functionally homologous to CAMs of the Ig superfamily. Using cell transfection studies (based on Schneider's line 2 ('S2') cells), it was found that rat brain $\beta 1$ and $\beta 2$ subunits, but not the α subunit, interact in a trans-homophilic fashion, resulting in recruitment of ankyrin to sites of cell-cell contact (Malhotra et al., 2000). An intriguing model was presented, by these authors, which offered two alternative mechanisms for a β subunit / ankyrin linkage: 1) an indirect linkage, whereby β subunits may interact via a cis-heterophilic mechanism with NrCAM or neurofascin; or, 2) a direct linkage, whereby β subunits may bind ankyrin following trans-homophilic interactions with β subunits on adjacent neurons. Malhotra et al. (2000) argued that their results favored the latter (direct) alternative. These two alternatives are illustrated in Figure 4.

Figure 4. Model of interaction between ankyrin and β subunit of NaV channels. Two alternative mechanisms for interaction between NaV β subunit and ankyrins are presented. [left side] β subunits may interact via a cis-heterophilic mechanism with various CAMs (especially NrCAM or neurofascin). The CAMs bind ankyrin via trans-homophilic interactions with the same molecule on an adjacent neuron. This is an indirect linkage between β subunits and ankyrin. [right side] An alternative based on a direct linkage between ankyrin and β subunits is proposed based on trans-homophilic interactions with β subunits on adjacent neurons. [Model based on Malhotra et al., 2000.]



Placing these results in a larger context, it is important to note that other laboratories have reported that ankyrin-G, certain CAMs (neurofascin, NrCAM), and NaV colocalize at the axolemma of the adult node of Ranvier (Davis et al., 1996; Kordeli et al., 1990). These research groups have also shown that during development, clusters of neurofascin and NrCAM are later joined by ankyrin-G and NaV during differentiation of myelinated axons. Accordingly, it may be said that formation of the node of Ranvier results from the fusion of two cluster intermediates. These investigators have proposed that a linkage between ankyrin-G and CAMs (neurofascin, NrCAM) and ion channels permit the CAMs to actually localize NaV in the axolemma (this would correspond to the first alternative ('indirect' linkage) in Figure 4.). In this context, the advantages of the flexible binding capabilities of ankyrin are made apparent; the multivalent membrane binding domain of ankyrin may thus allow an interaction with diverse CAMs, as well as ion channels, to form distinct and functionally heterogeneous membrane complexes.

The electrogenic pump (Na⁺/K⁺-ATPase). Ankyrins bind to Na⁺/K⁺-ATPase with high affinity. This association is mediated by two binding sites on the ankyrin molecule: one localized in the membrane-binding domain, and the other in the spectrin-binding domain (Devarajan et al., 1994). In addition, there are two binding sites for ankyrin on the cytoplasmic domain of the α subunit of Na⁺/K⁺-ATPase (Davis and Bennett, 1990). As knowledge of the ankyrin functionality has increased, an appreciation has emerged for the flexibility enabled by multiple binding sites. Similar to the example of neurofascin dimerization discussed above, multiple steric associations are enabled in the case of Na⁺/K⁺-ATPase as well. The β subunit of Na⁺/K⁺-ATPase has a large extracellular

glycosylated domain, which functions, at least in part, as a cell adhesion molecule, exhibiting recognition elements for adhesion that link cell adhesion with ion transport (Gloor et al., 1990). In this way, Na^+/K^+ -ATPase can simultaneously interact with the cytoskeleton and participate in cell-cell contacts. It has been suggested that ankyrin binding to Na^+/K^+ -ATPase (or the involvement of Na^+/K^+ -ATPase β subunit in cell adhesion) may also affect the activity of this electrogenic pump (Robtsov and Lopina, 2000).

Receptors. Ankyrin binds certain classes of receptors with high affinity and specificity. These include the ryanodine receptor (RyR), the inositol 1,4,5-triphosphate receptor (IP_3R), and the hyaluronate receptor (CD44). Evidence has shown that the cytoskeleton plays an active role in the regulation of RyR-mediated calcium release in response to lymphocyte activation (Bourguignon et al., 1995).

Ankyrins may play at least an indirect role in the modulation of intracellular calcium levels. The IP_3 receptor in brain and lymphocytes binds ankyrin-R, which apparently acts to inhibit IP_3 -induced calcium release (Bourguignon and Jin, 1995). In the context of traumatic brain injury, this may be an important point of vulnerability, if injury significantly reduced the availability of ankyrins to perform this normal inhibition of efflux from intracellular calcium stores.

A trimeric complex has been described involving ankyrin-R, IP_3R and sigma-1 receptors. The sigma-1 receptor, located on endoplasmic reticulum, binds certain

steroids, neuroleptics and psychotropic drugs (Hayashi and Su, 2001). The sigma-1 receptors modulates neurotransmitter release, synaptic activity, and is implicated in learning and memory (Maurice et al., 1998).

Protein kinase C (PKC). PKC, ankyrin, and spectrin, colocalize in lymphocytes. Activation of PKC leads to accumulation of ankyrin, spectrin and the β subunit of PKC in the cytoplasm as a single complex. PKC inhibitors produced a translocation of ankyrin, spectrin and the β subunit of PKC to the plasma membrane (Gregorio et al., 1994).

Cell adhesion molecules (CAMs). CAMs participate in diverse activities involving cell-cell recognition including axon fasciculation, myelination, synaptogenesis, and axonal guidance (Hymniewicz-Jankowska et al., 2002). An illustrative case of the molecular binding of ankyrin and a CAM (neurofascin) was examined in detail above, as the work of Davis and Bennett (1994) was described. In general, ankyrin shows the greatest affinity to the L1 family of CAMs (including L1, neurofascin, Nr-CAM, and Ng-CAM) which are members of the immunoglobulin superfamily. This family of CAMs is involved in neurite outgrowth, growth cone guidance, and the formation of axons. Ankyrin binds with the C-terminus cytoplasmic domain of L1-family molecules. This ankyrin-binding zone is the most conserved region of the CAMs, and does not share homology with any other (known) ankyrin-binding proteins (Davis et al., 1993). Interestingly, cell culture studies have shown that ankyrin was recruited to the sites of

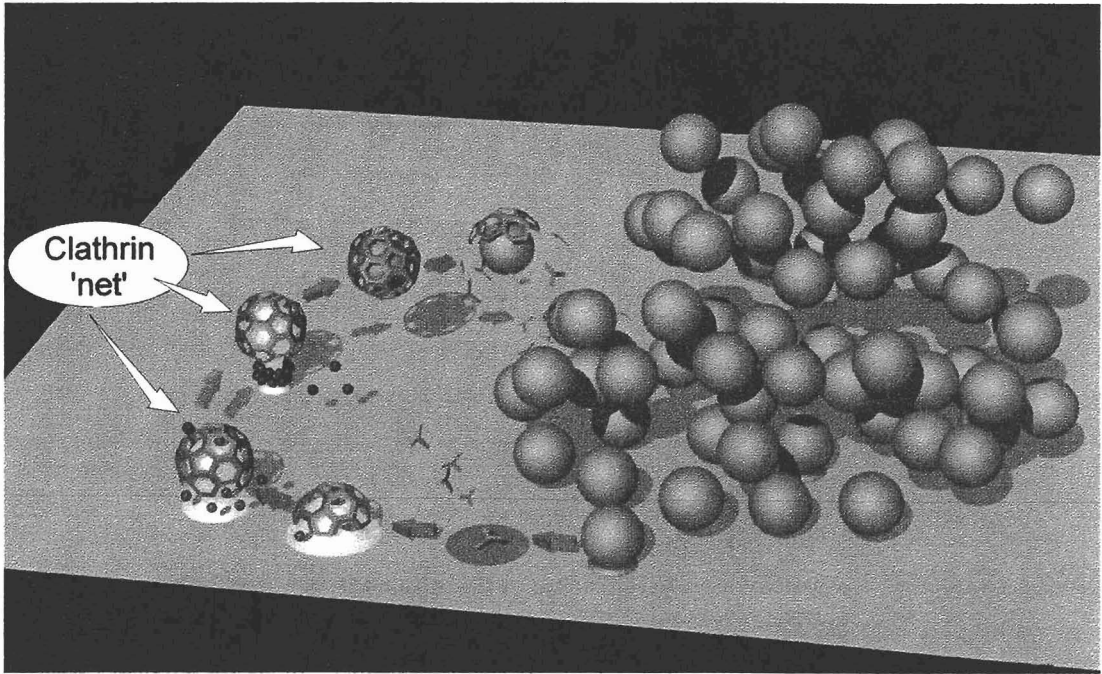
cell-cell contact following CAM-mediated cell adhesion (Dubreuil et al., 1996), indicating an extracellular cue for ankyrin expression and organization.

As mentioned above, the cytoplasmic domain of CAMs is highly conserved, to such an extent, in fact, that a sequence of 9 amino acid residues (EDGSFIGQY) is present in all the L1-CAMs from *C. elegans* to human (Garver et al., 1997). Phosphorylation of tyrosine within the sequence FIGQY inhibits the linkage between ankyrin and neurofascin, and also inhibits the neurofascin mediated cell adhesion. Mutation in the human L1 gene is associated with abnormal development of corpus callosum axons, mental retardation, and motor deficits (Kenwrick et al., 2000). Thus, a modulation of the ankyrin/CAM association may be involved in mobility of extracellular matrix constituents required during plasticity and development.

Clathrin. The study of ankyrin functionality spans approximately a 20 year period, and has seen an evolution from a concept of ankyrin as a simple "linker molecule," to a growing appreciation of the diverse and dynamic aspects of this molecule. When the cell membrane is involved in a function, there appears to be a high probability that ankyrin will play a role. One recent line of study has examined ankyrin's role in endocytosis: the process of vesicular formation at the plasma membrane which uptakes extracellular fluid and substances into vesicles within the cell cytoplasm. Endocytosis also recycles cell membrane. This recycling function is vital to maintain constancy of membrane area and capacitance; for instance, synaptic vesicle fusion during neuronal

synaptic transmission, will add the vesicular membrane to the existing plasma membrane. Continued indefinitely, the process of vesicular fusion would alter the area and shape of the terminal bouton membrane. The regulation of plasmalemmal membrane area is mediated, in part, by endocytosis, which recovers membrane from the plasmalemmal to the vesicular pool. The process of endocytosis involves the recruitment of clathrin to the membrane, which initiates the formation of clathrin 'nets': a latticework which mediates the plasmalemmal invagination during endocytosis and gives shape to the spherical vesicle (Figure 5). There is relatively recent evidence that ankyrins can associate directly with clathrin and participate in clathrin-mediated endocytosis. Michaely and colleagues (1999) reported that the N-terminal domain of the clathrin heavy chain molecule contained a binding site for ankyrin. This is an isolated finding, based on a methodology of microinjection of ankyrin subdomains which inhibited receptor mediated endocytosis of low-density lipoprotein. Accordingly, this aspect of ankyrin function must be considered somewhat speculative, at this point, and future work will determine which ankyrin isoform is the *in vivo* binding partner for clathrin.

Figure 5. Model of clathrin-mediated endocytosis. Interior of plasmalemma is illustrated showing recruitment of clathrin molecules to zone of endocytosis. Clathrin molecules form a network giving shape and strength to endocytosed vesicle. Clathrin has been shown to be associated with cytoskeletal elements. [Source: based on Cowan et al, 2001].



Potential TBI pathology involving ankyrin

The particular focus of this thesis originated with an effort to interpret a set of experimental results showing electrophysiological abnormalities following fluid percussion TBI in adult rats. Specifically, compound action potentials (CAPs) evoked in the corpus callosum of rats are dramatically reduced in amplitude following the injury (Baker et al., 2002; Reeves et al., 2004). While a functional change to axons would not be surprising, given the abundant literature on post-TBI changes to axon morphology, these results were significant for at least two reasons. First, they provided a rodent model in which to explore the electrophysiological aspects of traumatic neuropathology, which had previously been elusive due to the diffuse nature of the axonal injury in lissencephalic rodents. (Signals from occasional scattered injured axons are difficult to detect and quantify). Secondly, the nature of the electrophysiological changes suggested that not all axons were equally impaired: small unmyelinated axons appeared to sustain a more dramatic injury, and exhibited less recovery of function after injury, than was observed for larger myelinated axon. Thus, questions arose regarding what could be the basis for such selective fiber vulnerability following injury.

The observed TBI-induced suppression of evoked callosal CAP amplitude could conceivably result from a large number of possible failures of axon structure and/or function. While the primary ionic event, generating the callosal CAPs, is an influx of sodium through voltage gated NaV channels, this is only the "final common pathway," and proper functioning of NaV is dependent on a complex infrastructure. TBI is known

to alter membrane integrity and the transmembrane bioelectric potential, along with multiple components in the axoplasm, including the cytoskeleton and the spectrin/ankyrin membrane skeleton. The laboratory experiments for this thesis were undertaken as a component of an ongoing investigation of posttraumatic axonal neuropathology, which aims to systematically examine intracellular and extracellular axolemmal molecular domains, and determine their role in the pathological process.

An understanding of a potential pathological role for ankyrin is a key objective in this investigation. During the decade of the 1990's the concept of ankyrin grew from a simple 'linker,' to its current understanding as a multifunctional molecule critical for a diverse set of membrane-associations (both plasmalemmal and organelle membranes), as well as for potential signal transduction functionality through its association with cell adhesion molecules and ion channels. In view of the many roles of ankyrin, it would be predicted that TBI could alter ankyrin, or its binding partners, in multiple ways. This, in fact, is a natural conclusion from an examination of the ankyrin literature. The following discussions of potential TBI effects on ankyrin present potential injury effects to both cellular structure and for function. It should be noted, however, that virtually all injury-induced changes will affect both structure and function.

Failure of cellular stabilization -- Most neurobiologists would agree that one of the functions of the extracellular matrix is to stabilize cells. However, it can also be argued that this function may not be a one-directional effect: the membrane cytoskeleton

probably acts to stabilize extracellular components as well. Ankyrins would be predicted to participate in such a stabilization role. The infrastructure for this appears to be present. Both ankyrin and spectrin are known to interact with proteins of both neurofilaments and microtubules, respectively (Bennett, 1990), and the ankyrin association with Ig CAMs has already been discussed. In at least some domains, connections to the membrane (and membrane skeleton) extend not only into the extracellular space, but also to adjacent cells. At the node of Ranvier, for example, 40-80 nm long filaments have been described that span the gap between the neuronal membrane and the processes of adjacent glial cells (Pumplin and Bloch, 1993).

It is a prediction of this thesis that TBI will alter neuronal ankyrin. This expectation is based on the current understanding that a critical feature of TBI pathobiology is an aberrant degree of cellular depolarization, subsequent to a posttraumatic influx of cations into the cytoplasm. Elevated intracellular calcium activates proteolytic enzymes, and it has previously been demonstrated that, under proteolyzed conditions, ankyrin loses binding to spectrin (Davis and Bennett, 1990).

A TBI-induced damage to ankyrin, and the membrane cytoskeleton, may be predicted to lead to an increase in the mobility of some integral membrane proteins, and probably a diffusion of these away from zones where they are concentrated in the normal state. An *in vitro* model of such a diffusion has been reported. In cultured rat myotubes, large clusters of acetylcholine receptors form at sites of cell-substrate adhesion, and the spectrin/ankyrin membrane cytoskeleton is linked with these clusters. Removal of β

spectrin causes the acetylcholine receptors to diffuse away from the clusters and into other regions of the membrane (Pumplin and Drachman, 1983), and a similar effect has also been reported in erythrocytes.

The association of ankyrin with membrane proteins, and, via CAM molecules with the extracellular matrix, implicates all of these molecules in pathological states where cell spacing is disrupted. To cite a specific example, the intimate functional relationship between myelinating glial cells and axons is reflected in the highly regular apposition of their plasma membranes. These are separated by an extracellular space of approximately 12 to 14 nm and is a parameter which is actively maintained, even under conditions of osmotic change or pathology. This periaxonal space, and the attachment of the myelin sheath to the axon, is disrupted by the action of proteases (Yu and Bunge, 1975), suggesting that it is maintained actively by cell surface proteins. This may have a significance in the context of TBI pathology where proteolysis is a frequently reported cause of cellular morbidity and mortality.

Alterations in cell shape -- A posttraumatic alteration in ankyrin and the membrane cytoskeleton can reasonably be expected to lead to alterations in the general shape of affected cells. A sizeable knowledge-base already exists which documents alterations to axonal diameter subsequent to cytoskeletal collapse, resulting from proteolytic cleavage of neurofilament sidearms, after TBI (Povlishock, 1993; Buki et al., 1999; Povlishock et al., 1999). Although postinjury changes in cell shape can be caused

by other mechanisms, for example osmotic swelling subsequent to intracellular ionic loading, it is likely that failures in the membrane cytoskeleton leads to pathological changes in much the same way as the well-documented alterations to the neurofilament network. Alterations in cell shape, following neurotrauma, which may implicate the membrane cytoskeleton, include focal alterations suggesting localized failure of structural elements. Nakayama and colleagues (2001) developed an in vitro model of axonal injury which subjected PC12 cells to a fluid shear stress. They identified two forms of shape changes: an increase in the terminal diameter of the processes, and 'beading' along the injured portions of the process most directly affected by the shearing force. Interestingly, the authors reported that focal areas of cytoskeletal destruction were associated with the formation of growth cones, indicating a mobilization and plasticity of some cytoskeletal elements concurrent with pathological processes.

Alterations to voltage-gated sodium channels (NaV) -- Extensive evidence points to a NaV-ankyrin linkage, and the binding of ankyrin to the β -subunit of NaV has been discussed above. Pathology affecting the NaV channels is a priority area of investigation for our laboratory, in view of our prior observations of TBI-induced suppression of evoked axonal responses that are dependent on NaV operation (Reeves et al., 2003). Recent evidence has implicated sodium channel pathology as a very early event in TBI, perhaps preceding even intra-axonal calcium loading (Iwata et al., 2004). These authors concluded that the initial pathological depolarization -- induced by excessive cationic

influx -- led to proteolytic damage to NaV channels. This early damage initiates a positive feedback cycle, where the influx of sodium produces some depolarization, and a recruitment of NMDA receptor mediated calcium influx, evolving to accelerated ion influxes and more depolarization. Whether the earliest pathological change involves sodium or calcium movements remains controversial at this date. However, irrespective of the source of the pathological depolarization, the consequent damage to the membrane skeleton is a well established phenomenon. This is reflected in the fact that measurement of calpain-mediated spectrin proteolysis has become a standard marker for the detection of traumatic axonal injury (Buki et al., 1999; Povlishock et al., 1999; Saatman et al., 2003). Additional evidence also converges on the importance of the NaV-ankyrin association in the aftermath of TBI. A clustering of both ankyrin molecules and NaV has been identified at the nodes of Ranvier, and nodes are preferentially vulnerable to trauma, being the primary site of damage during stretch-induced injury to optic nerves (Maxwell et al., 1991).

Genomic deletion experiments have further supported the importance of ankyrin to the normal functioning of NaV. Inactivation of the AnkG gene in the cerebellum prevented the expression of ankyrin-G in cerebellar Purkinje and granule cells (Zhou et al., 1998). In these mutant mice, NaV channels were absent from axon initial segments of granule cell neurons. Purkinje cells exhibited electrophysiological abnormalities, being unable to initiate action potentials and support rapid, repetitive firing. The knockout mice exhibited a progressive ataxia beginning around postnatal day P16 and subsequent loss of Purkinje neurons. To the extent that TBI alters ankyrin, there are

likely to be adverse effects to clustering of NaV channels, and to the normal electrophysiological properties of sodium channel activity.

Alterations to the electrogenic pump (Na^+/K^+ -ATPase) -- The localization of the Na^+/K^+ -ATPase in the plasma membrane is due to its interaction with membrane cytoskeleton elements (Marrs et al., 1993). A pathological compromise of Na^+/K^+ -ATPase functionality has been implicated as important features of TBI pathology (Tavalin et al., 1997). Thus, a potential effect of TBI-induced alteration of ankyrin may be a decrease in the efficiency of the electrogenic pump, and a consequent degradation of the transmembrane bioelectric potential.

Injury effects related to ankyrin/CAM associations -- There are numerous potential mechanisms by which the effects TBI may be expressed in alterations to the molecular complexes formed by ankyrin and CAMs. The Ig-CAMs, in addition to promoting cell adhesion, may participate in activation of intracellular signaling pathways. The excessive neuroexcitation, following TBI, is known to initiate pathological cascades which deleteriously affect intracellular signaling.

The nature of the pathology induced by TBI suggests that alterations to the molecular complexes formed by ankyrin and CAMs may be affected, and the CAMs may play a role in recovery of function as well. Depending on the severity of TBI, some neurons either die or exhibit varying degrees of structural change and functional

impairment. In moderate severities of TBI there may be little, if any, evidence for dead or dying neuronal cell bodies, but even in these cases there is usually some degree of axonal damage with degenerating axons (Povlishock and Christman, 1995; Singleton et al., 2002). Some level of traumatic axonal injury is a concomitant of almost all brain injuries (Povlishock and Christman, 1995). Degenerating axons and terminals necessarily implies a deafferentation of target (postsynaptic) cells, and this is an adequate stimulus to trigger neuroplasticity and sprouting responses. The plasticity triggered by a deafferenting or concussive injury is thought to be a massive recruitment of plasticity mechanisms that are routinely implemented at a low level in normal cellular learning and memory, as well as growth and development. However, if TBI may not only initiate a neuroplastic phase, but may compromise the reactive molecular elements as well. If elements of the membrane cytoskeleton are among the more vulnerable, this may affect the ability of the system to mount an adequate plasticity response for recovery, and could potentially suggest strategies for therapeutic intervention.

Synaptic activity alone leads to structural remodeling of pre-and postsynaptic structures (Korkotian and Segal, 1999; Colicos et al., 2001; Matsuzaki et al., 2004). In comparison to the excitation produced during the course of normal neuronal functioning, any TBI, from mild to severe, will induce a more intense degree of excitation that can reasonably be expected to elicit a correspondingly greater neuroplasticity response. One category of CAM, cadherins, have been shown to be required for activity-induced spine remodeling (Okamura et al., 2004). From a TBI perspective, this is interesting because cadherins assume a stable configuration in the presence of extracellular calcium levels. It

is well documented that TBI leads to a redistribution of calcium, from the extracellular to the intracellular compartments. In comparatively lower calcium, the cadherins may uncouple from their normal cell-cell adhesions, and enter a reactive plasticity phase.

Statement of experimental hypothesis for the current study.

The foregoing discussion of ankyrin, its integral structural role, and its diverse set of binding partners, suggests a multifunction molecule which may be especially vulnerable to TBI. The general hypothesis for this thesis is that moderate fluid percussion TBI, in adult rats, will alter the amount, or the form, of brain ankyrin-G. By form is meant the measurement of injury-related changes in proportions of various ankyrin-G isoforms, or the postinjury changes in ankyrin-G proteolytic fragments or breakdown products.

Specific hypotheses are formulated separately for the cerebral cortex, and for the corpus callosum. Accordingly, evidence will be obtained to address quantities and form of ankyrin-G in each brain region. The selection of these two brain regions will allow a comparison of the relative injury effect(s) in a region which is relatively enriched in axons, as opposed to a region with a parenchymal mix of cell bodies, dendrites, and axons.

METHODS

Subjects

The procedures for this study followed all national guidelines for the care and use of experimental animals, and the experimental protocol was approved by the Medical College of Virginia Animal Research Committee. 28 adult Sprague-Dawley rats (Hilltop Lab Animals, Inc., Scottsdale, PA) weighing between 300-400 grams were used. Animals were housed in individual cages with food and water available ad libitum. The animal colony was maintained at a temperature of 20-22 degrees Celsius with a 12 hour (light)/ 12 hour (dark) cycle.

Surgical Preparation

Each rat was placed in a bell jar anesthesia chamber and anesthetized for four minutes with isoflurane (4% carrier gas of 70% N₂O and 30% O₂). Upon removal from the chamber the top of the head was shaved and the animal was then positioned into a stereotaxic surgical device (David Kopf Instruments, Tujunga, CA). A thermostatically controlled heating pad (Harvard Apparatus, Holliston, MA) was then placed under the animal and set to monitor the temperature, and via feedback control, maintain the body temperature at 37°C during the surgery. Vetropolycin eye ointment was applied to the

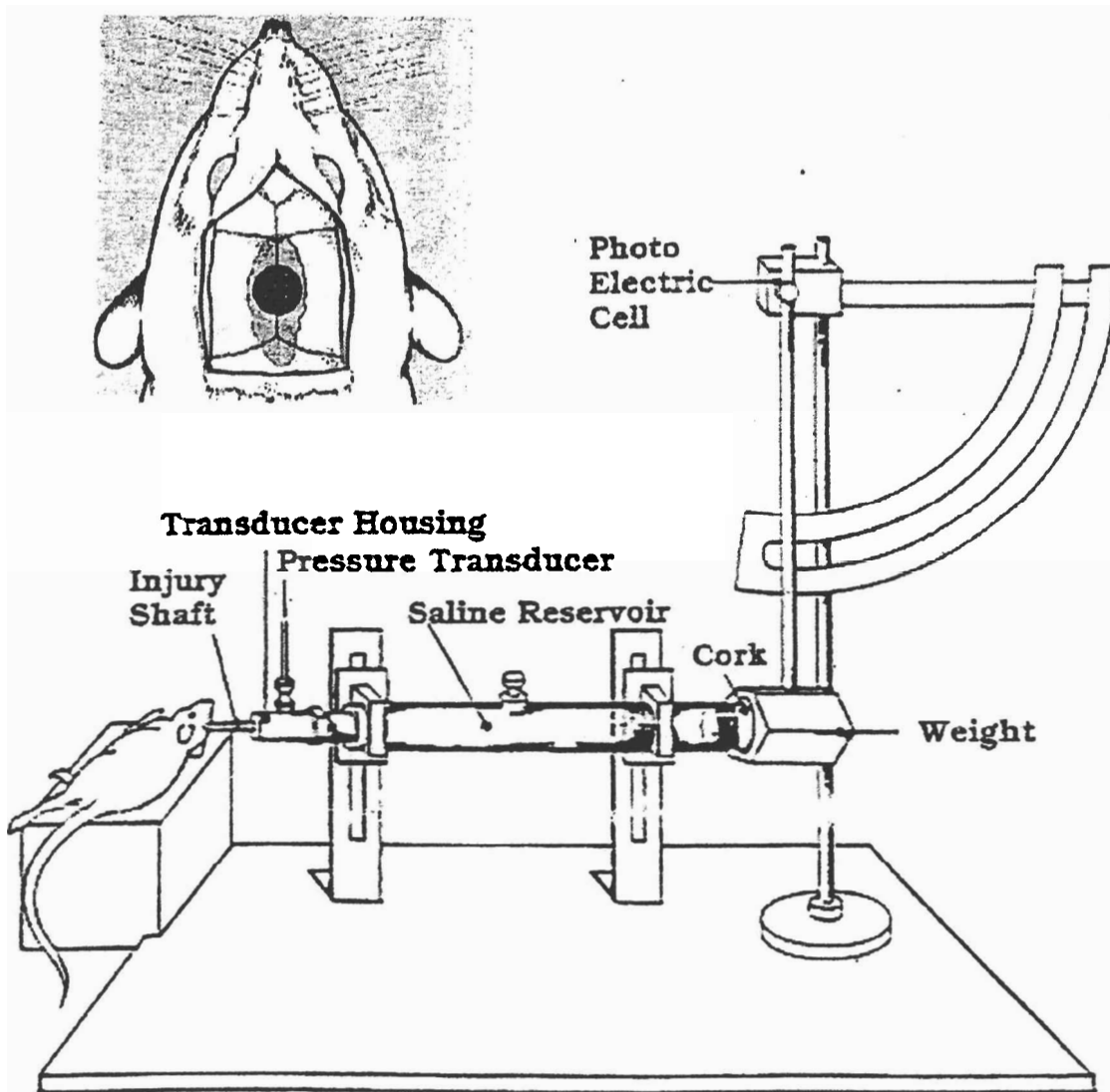
eyes and bacitracin was applied to the shaved region. The anesthesia was adjusted to 2% isoflurane, a midline sagittal incision was made to expose bregma and lambda. Connective tissue and fascia were retracted from the top of the skull, the skull cleaned, dried, and two 1 mm holes were drilled in the right frontal and occipital bones, 1 mm rostral and caudal to bregma and lambda, respectively, for the subsequent insertion of fixation screws. A 4.8 mm circular craniotomy was then made along the sagittal suture midway between lambda and bregma, taking precaution not to damage the underlying dura. A Leur-Loc syringe hub was then cut away from a 20-gauge needle and affixed to the craniotomy site using cyanoacrylate. After confirming the integrity of the seal between the hub and the skull, two fixation screws (round machine screws, 3/16 inch. long) were inserted into the 1 mm holes. Dental acrylic was then applied over the screws and around the hub to provide stability during the induction of the injury. After the dental acrylic had solidified, the skin over the hub was sutured, using polyamide non-absorbable suture. Topical triple antibiotic ointment was applied, and the animal was removed from the anesthesia and monitored in a warmed cage until fully recovered. The animal was then returned to the central housing facility and allowed to recover for 24 hrs.

Injury Device and Procedure

Twenty four hours following injury, each animal was again anesthetized for 4 minutes in a bell jar with 4% isoflurane in 70% N₂O, and 30% O₂. After removal from the jar, the previous incision was quickly reopened to expose the craniotomy site and the male end of a spacing tube was inserted into the hub. The hub-spacer assembly was filled with normal 0.9% saline and the female end of the spacer was inserted in the male

end of the fluid percussion apparatus ensuring that no air bubbles were introduced into the system. Each animal was then injured at a magnitude of 2.00 ± 0.05 atm, corresponding to a brain injury of moderate severity (Dixon et al., 1987). The fluid percussion device used to produce TBI is described in greater detail elsewhere (Dixon et al., 1987), and is shown in Figure 6. Briefly, the device consisted of a 60 x 4.5 cm Plexiglas cylinder filled with distilled water, fitted at one end with a piston mounted on O-rings, and the opposite end contained a pressure transducer (Entran Devices, Inc.; EPN-0300A). The injury was produced by the metal pendulum striking the piston, which injects a small volume of saline into the closed cranial cavity, briefly deforming the brain tissue (20 ms). The resulting pressure pulse was recorded extracranially by the transducer and expressed in atmospheres (atm) of pressure, and recorded on a storage oscilloscope (Tektronix 511: Beaverton, OR). The magnitude of injury was controlled by varying the height from which the pendulum was released. For the current study, the pendulum height was adjusted for a target injury severity in the range 2.0 ± 0.05 atm. Injury preparation and induction were completed prior to recovery from anesthesia. Following injury the animals were monitored for spontaneous respiration and, if necessary, ventilated to ensure adequate postinjury oxygenation until spontaneous respiration was regained. The hub, dental acrylic, and screws were removed en bloc and the midline incision was closed using polyamide non-absorbable suture. The righting response was a complex postural somatomotor function which was temporarily suppressed following TBI, and was used as a measure of the length of trauma induced unconsciousness (Dixon et al., 1987).

Figure 6. Fluid percussion device and position of craniotomy used for central fluid percussion TBI. [Source: Adopted from Dixon et al., 1987]



In addition, the latency to demonstrate withdrawal reflexes elicited by a standard tail pinch and paw pinch was assessed for each injured animal, along with the latency to show reflex responses to a tactile cornea and pinna stimulation. Suppression of the righting reflex was measured as the duration in time after injury required for an animal to right itself three consecutive times after being placed in the supine position. Following recovery of the righting reflex, the animals were placed in a holding cage with a heating pad to ensure maintenance of normothermia and monitored during recovery. For control rats administered sham injuries, all of the above steps were followed minus the release of the pendulum to induce the injury. In addition, the body weight of each animal was monitored and recorded for five days postinjury to ensure equivalent levels of general health.

Western Blot Analysis of Ankyrin-G

Protein levels of ankyrin-G in the corpus callosum and cerebral cortex were assessed using Western Blot analysis. At either 1d (n = 8), 3d (n = 11) or 7d (n = 9) after receiving TBI or sham, the rats were anesthetized with 4% isoflurane for 4 minutes, and decapitated. Their brains were quickly extracted from the skull and placed onto an ice cold dish for dissection. The cerebellum was first removed and the remaining portion of the forebrain was cut into five coronal sections. The two most anterior and posterior brain slices were discarded and the remaining series of slices transferred to a dissecting microscope where sections containing mid-dorsal portions of the corpus callosum were selected for sampling. Corpus callosum tissue was dissected bilaterally from the midline, beginning at a level adjacent to the ventral hippocampal commissure and ending at the

level of the splenium. Cerebral cortical samples were also dissected from these coronal slices, targeting the parieto-temporal regions known to be vulnerable to injury in this model.

Tissue samples were weighed and then immediately homogenized in 10X RIPA Lysis Buffer (Upstate; Lake Placid, NY) containing “Complete” Protease Inhibitor (Roche Diagnostics; Mannheim, Germany), Sodium Dodecyl Sulphate, EGTA and deionized water. The volume of RIPA Lysis Buffer used was equal to 8 times the wet weight (in gm) of harvested tissue. In corpus callosum samples with wet weights of less than 0.02 gm RIPA Lysis Buffer was added in a minimal 125 μ l volume. The homogenates were centrifuged for 20 min at 14,000xg/4° C and the supernatants decanted. A 5 μ l volume from each sample was reserved for protein assay (BioRad Dye Reagent Concentrate; BioRad Laboratories, Hercules, CA) and the remaining supernatant aliquotted and frozen at -80° C. The protein concentration in each sample was then obtained by performing a Bio-Rad protein assay (Bio-Rad Labs, Inc. Hercules, CA). The absorbance of each sample was measured at 595nm, using a UV160U Shimadzu Spectrophotometer. Aliquots containing 30 μ g of protein from each sample were then combined with double deionized water, 6.3 μ l of NuPAGE LDS Sample Buffer and 2.5 μ l of NuPAGE Sample Reducing Agent. Each sample was then heated at 70°C for 10 min, loaded into separate lanes on 3-8% NuPAGE Tris-Acetate gel (NuPAGE Gel, Carlsbad, CA) and electrophoresed using Invitrogen Tris-Acetate Buffer System (60 mins/150 V, 120 mA and 25 W/ room temperature). After the gel run, proteins were transferred to PVDF membrane (0.45 μ m pore size; Invitrogen) according to Invitrogen protocol (30 V,

220 mA, and 17 W for 75 min) using transfer buffer containing methanol (20% methanol for 2 gels, 10% methanol for one gel). Following transfer, the membrane was subjected to two 5 min washes in PBS (50 ml vol; BioRad) on a rotating platform. Post-blotted gels were stained with 0.1% Coomassie Brilliant Blue (Sigma, St. Louis, MO) to check for efficacy of transfer from gel to membrane. Post-blotted membranes were first blocked for 1 hr at room temperature in 0.05% TBST (+ 5% non-fat dry milk). The membranes were then placed in opaque dishes and incubated overnight at 4°C in a 15ml volume of TBST+ 3% non-fat dry milk containing mouse anti-ankyrin-G primary antibody (1:100, Research Diagnostics, Flanders, NJ). On the following day membranes were washed 6X for 5 min in 40 ml of TBST + 3% non-fat dry milk. The membranes were subsequently incubated with peroxidase conjugated affinity purified anti-goat IgG secondary antibody (1:4000, Rockland Laboratories; Gilbertsville, PA) in 40 ml TBST + 0.05% non-fat dry milk for 1 hr, rocking at room temperature. A parallel blot with samples from each run was processed in buffer without the primary antibody. Membranes were washed again 6X 5 min each in 40 ml TBST. Immunoreactive bands were visualized using ECL Western Blotting Analysis System (Amersham Biosciences; Piscataway, NJ) and membranes exposed to Kodak BioMax Film (Eastman Kodak Company; Rochester, NY). Exposure times varied between 1sec and 15 min. Optical density of each band was determined using the Scion Image Analysis System (Scion Corporation; Frederick, MD). Three immunopositive bands migrating at approximately 220, 212 and 75 kD were analyzed by assessing band area (A) and signal density (M) within that area. Relative optical density was calculated as $A \times M$ and injury-induced

effects on ankyrin-G were expressed as percent change from paired sham-injured cases run on the same blot.

Re-probing of PVDF Membranes for Actin Load Controls

The PVDF membranes from each Western blot were subsequently stripped and re-probed using the Western Re-probe protocol (Geno Tech, St. Louis, MO). Each blot was first exposed to methanol for 30 sec, then washed 3X in 40ml of deionized water for 5 min. Blots were then incubated in 40ml of Western Reprobe for 1 hr at room temperature, after which they were washed 3X for 5 min in 0.1% Tween-20 PBS-T at a volume of 40ml per blot. The blots were then blocked for 1 hr at room temperature in PBS-T + 5% non-fat dry milk, and incubated in PBS-T + 3% non-fat dry milk containing anti- β -actin monoclonal antibody (1:3,000, Sigma, St. Louis, MO) at 4°C overnight. The following day each blot was washed 3X for 5 min in a 40 ml volume of PBS-T + 3% non-fat dry milk. After washing in PBS (40 ml volume, 3X, 5 min each wash), the blots were incubated in 40ml of PBS with peroxidase conjugated goat anti-mouse IgG secondary antibody (1:20,000 Rockland Laboratories; Gilbertsville, PA). The blots were then subjected to 6 final washes in PBS (5 min each) and the actin immunopositive band in each gel visualized using the ECL Western Blotting Analysis System, as described above.

Statistical Analysis of Western Blot

The effect of injury on levels of ankyrin expression was evaluated using ANOVA based on a completely randomized factorial design (Keppel, 1991), with two levels of

injury condition (sham injury vs. fluid percussion TBI) and three levels of survival time (1d, 3d, 7d). The potential for 'familywise type I error' was reduced by following the recommendations of Keppel (1991, pp. 247-248). Specifically, only *a priori* planned comparisons were implemented, which were theoretically meaningful within the context of the experiment. Changes in ankyrin due to injury were evaluated using simple effects of injury at each survival interval. Changes in ankyrin that occurred postinjury, in a time-dependent manner, were evaluated using orthogonal contrasts among ankyrin levels at each survival interval following TBI. Contrasts were implemented using MANOVA (SPSS v. 11), using the SPSS syntax recommended by Levine (1991, pp. 80-84). A probability of less than 0.05 was considered statistically significant for all experiments.

RESULTS

Moderate central fluid percussion injuries were administered as indicated above and the atmospheric pressure for each injury recorded. Measurement of reflex and righting recovery were used to identify the length of unconsciousness induced by injury and as a control for injury consistency (Dixon, et al., 1987). As a rule, animals with inappropriate injury pressures or having responses outside a 5-8 min righting time were eliminated from the study.

Overall, Western blot analysis of both corpus callosum and cerebral cortex identified three major immunopositive bands in sham and injured animals. These bands exhibited molecular weights of 220, 212 and 75 kD (Figure 7). Specificity for ankyrin-G was confirmed using parallel lanes processed without primary antibody and equal gel loading checked by subsequent membrane stripping and re-probing with an antibody to β -actin (see again Figure 7). Given that the commercial antibody used in this study recognizes both intact ankyrin-G and proteolytic fragments, it was not unexpected that the major signals detected fell in the range of ankyrin-G breakdown products. Other researchers have similarly observed that the major intact forms of ankyrin-G (480, 270 and 190 kD) are in relatively low abundance within the CNS and sometimes difficult to

extract for analysis (Bouzidi et al., 2002). Indeed, in a subset of blots, a weak signal at 270 kD could be visualized, however, the optical density of these bands was outside the acceptable range for accurate quantification. Therefore, the current study focused on a spatio-temporal analysis of the three major fragments showing strong ankyrin-G signal after TBI. Given the expressed molecular weights of these fragments and their different temporal profiles following injury, it is likely that the 220 and 212 kD are derived from either the 480 or 270 kD intact forms, known to be associated with the nodes of Ranvier in myelinated axons, and that the 75 kD band represents a proteolytic fragment of the 190 kD isoform of ankyrin-G, known to be abundant in unmyelinated axons.

Notably, moderate central fluid percussion altered the expression of each of the three major bands, however, the predominant changes were observed in the corpus callosum (Figure 8), with little or no change seen in the cortical samples (Figure 9). Details of the statistical analyses for sham vs. injury and time-dependent effects of injury in both brain regions are shown in Table 1. When relative optical density was compared between callosal samples from sham and injured groups, time-dependent changes in ankyrin-G expression were detected. At 1d postinjury, all three immunopositive bands showed higher density relative to paired controls. In the case of the 220, 212 kD doublet, this increase (147.1, 73.7 %, respectively) was significant ($p < 0.001$). By contrast, ankyrin-G protein levels showed very different blot patterns at 3 d after injury, where the 220, 212 kD bands did not change relative to sham animals and the 75 kD fragment was reduced by 54.3% ($p = 0.038$). This shift toward selective injury effect within the lower

molecular weight form persisted at the 7 d time point, where the 75 kD band was again reduced (41.3%; $p=0.043$).

Individual day by day comparisons also showed a shift in ankyrin-G expression within the corpus callosum as a function of survival time (see again Figure 8). Note that elevation of the 220, 212 kD ankyrin-G doublet at 1 d was essentially normalized to sham levels by 3 d and remained at control levels until 7 d after injury (see Table 1 for levels of significance in each time point/kD comparison). This temporal pattern suggests that changes in these two ankyrin-G fragments are more closely associated with the early, pathological phases of injury response. When temporal changes in the 75 kD form were analyzed, this fragment presents a very different pattern, exhibiting a marked reduction in expression at later postinjury intervals. The 40-50% reduction of this fragment at 3d and 7d suggests that it may be more closely associated with pathological processes affecting a distinct axon population (see Discussion).

In the present study, injury had very little effect on ankyrin-G protein expression in the cerebral cortex (Figure 9). Again, see Table 1 for details of statistical analyses. While there was a strong trend toward a 1 d rise and 3 d reduction in the 220 kD fragment relative to sham controls, this change failed to reach statistical significance. However, a significant reduction in 220 kD expression was observed when 1 d vs. 3 d protein levels were compared. No cortical effects of injury were detected for either the 212 or 75 kD band at any time point examined. Likewise, no time dependent effects were observed for the latter two fragments. The pattern of cortical ankyrin-G response stands in contrast to that found in the corpus callosum, however, it is important to remember that cortical

gray matter naturally contains a higher percentage of cell bodies and synaptic regions.

Given these regional differences, the present results point to a critical role for ankyrin-G in white matter tract injury after brain trauma.

Figure 7. Western blot analysis of ankyrin-G in the corpus callosum and cerebral cortex.

A. Western blot analysis of ankyrin-G protein expression in the corpus callosum at 1 day (1 d), 3 days (3 d) and 7 days (7 d) following moderate central fluid percussion brain injury. A sham (S) and injured (I) pair showing the three immunopositive fragments of ankyrin-G is illustrated for each time point, with kD identified at the left of the panel. A representative minus primary control is shown on the right and β -actin levels for each lane presented below each lane as a confirmation of equal protein loading.

B. Western blot analysis of ankyrin-G protein expression in the cerebral cortex at 1 day (1 d), 3 days (3 d) and 7 days (7 d) following moderate central fluid percussion brain injury. As in (A) above, representative S and I pairs are shown for each time point (kD for each band indicated at left of panel) and β -actin load controls are presented below each lane.

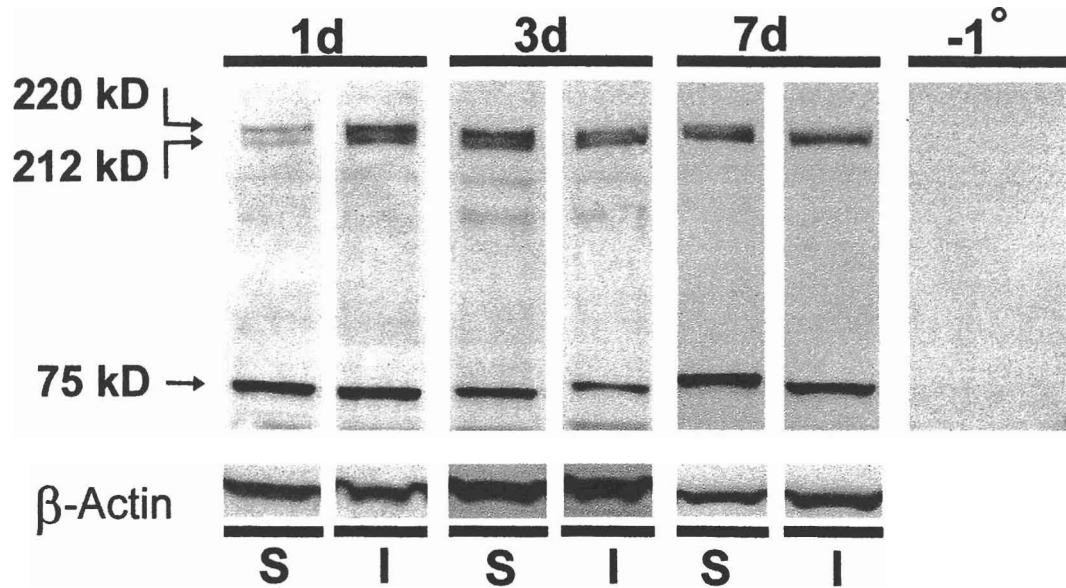
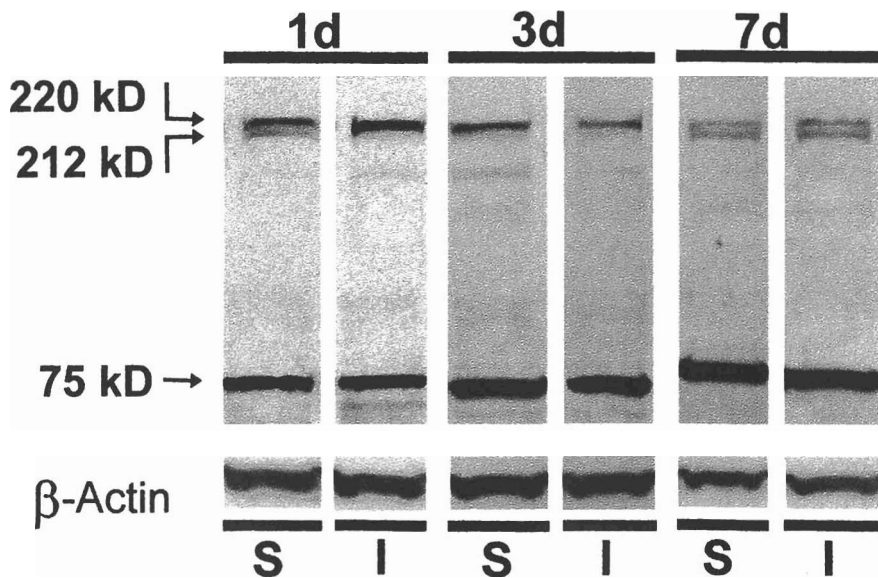
A**Corpus Callosum****B****Cerebral Cortex**

Figure 8. Profile of quantitative changes in ankyrin-G expression within the corpus callosum at 1 day (1 d), 3 days (3 d) and 7 days (7 d) following moderate central fluid percussion brain injury. Ankyrin-G levels are expressed as percent of sham control values (\pm S.E.M.) for each of the immunopositive fragments detected (220, 212 and 75 kD). A significant increase with respect to sham control cases was observed at 1 d in the 220 and 212 kD bands (a- $p < 0.001$; $p = 0.003$, respectively), and a significant decrease was seen in the 75 kD band at both 3 and 7 d postinjury (a- $p = 0.038$; $p = 0.043$, respectively). Time-dependent changes in ankyrin-G expression were found for each fragment (b-see Table 1 for p values).

Ankyrin-G expression in the corpus callosum

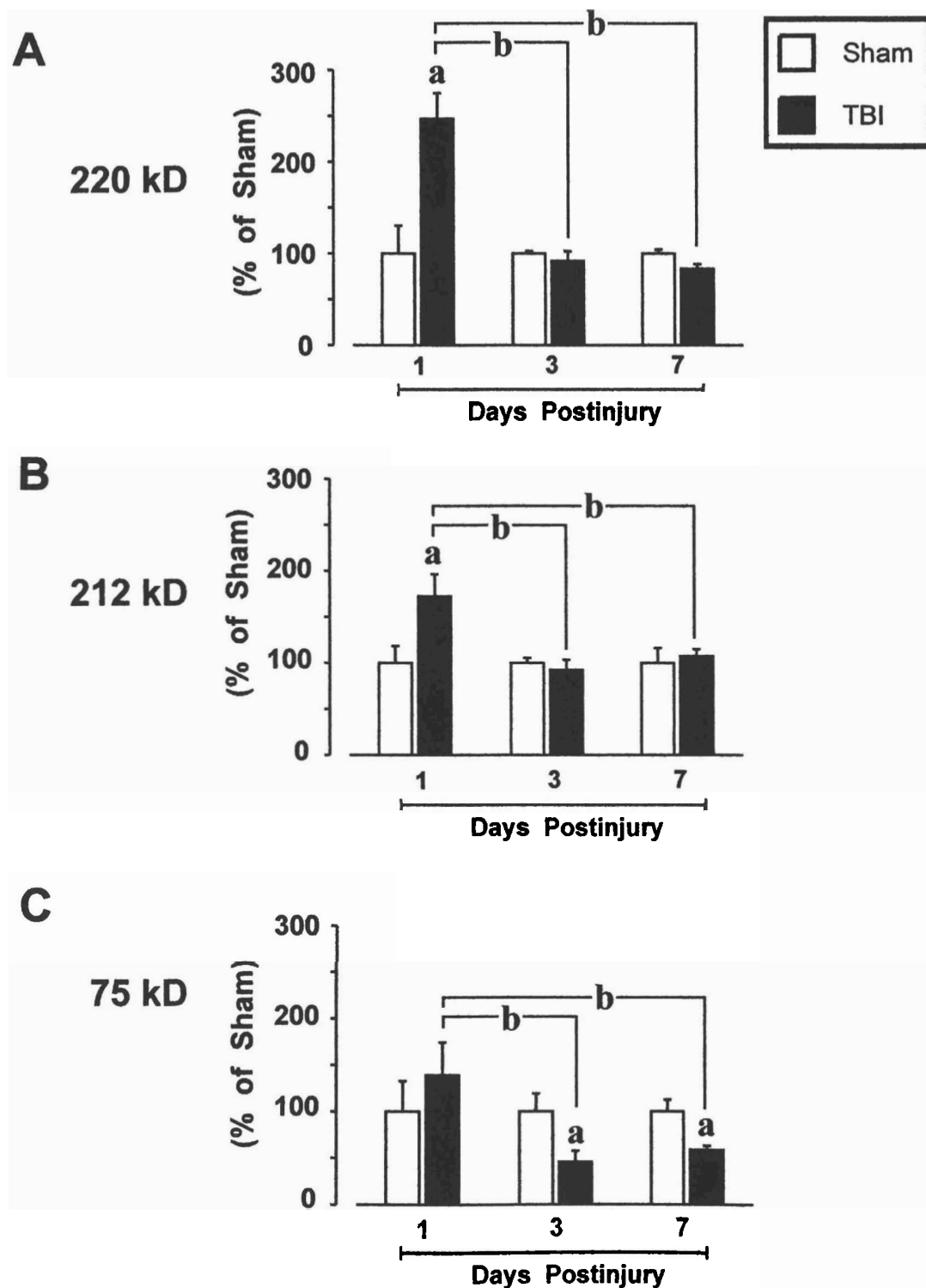


Figure 9. Profile of quantitative changes in ankyrin-G expression within the cerebral cortex at 1 day (1 d), 3 days (3 d) and 7 days (7 d) following moderate central fluid percussion brain injury. Ankyrin-G levels are expressed as percent of sham control values (\pm S.E.M.) for each of the immunopositive fragments detected (220, 212 and 75 kD). No significant change from sham control cases was observed for any of the ankyrin-G bands, however, a trend reflecting 1 d increase and 3 d decrease in the 220 kD fragment was found. These trends supported a significant time-dependent change between 1 and 3 d in the high kD band (b - $p=0.025$).

Ankyrin-G expression in the cerebral cortex

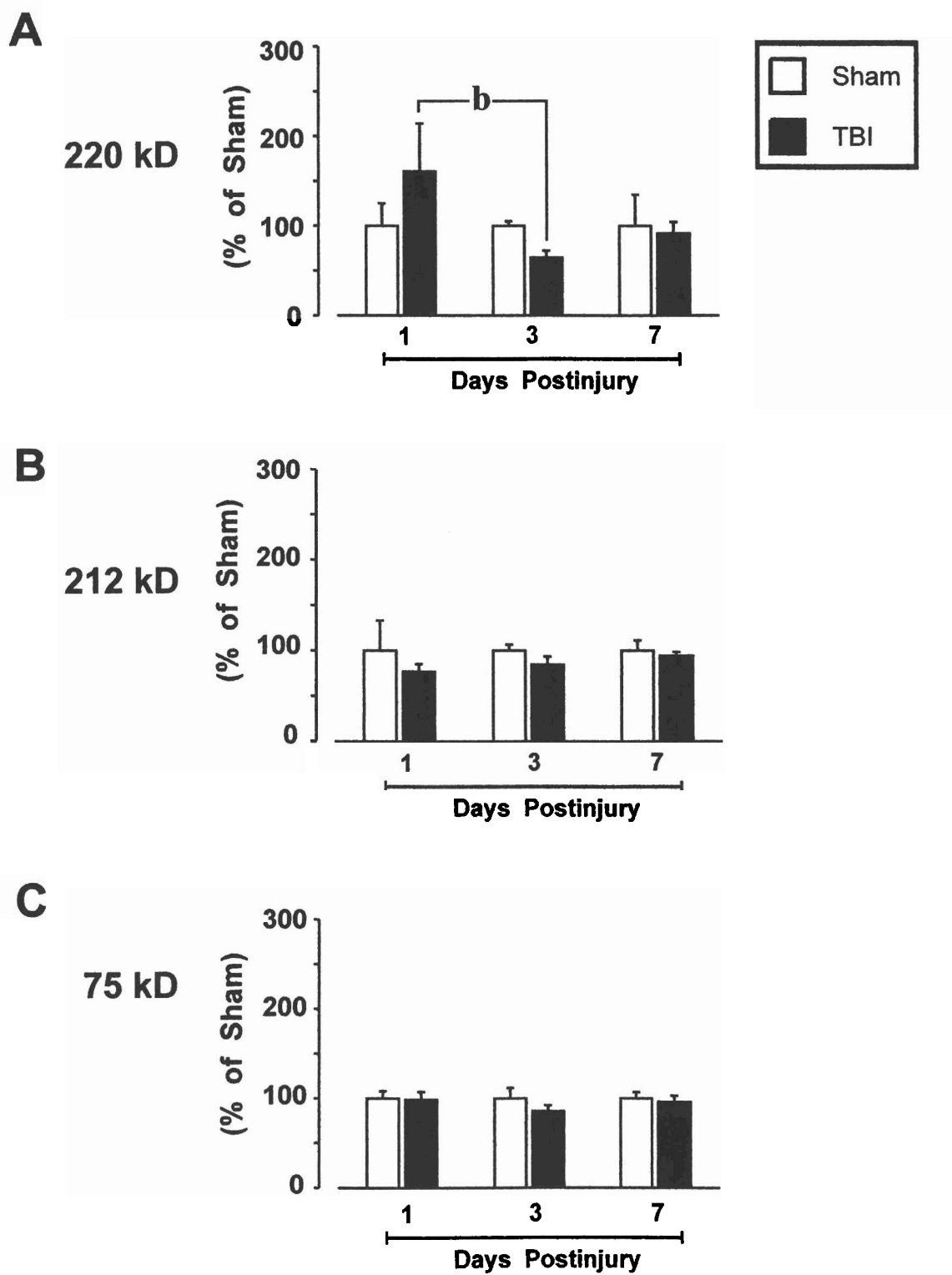


Table 1. Summary of significant changes in ankyrin-G expression in the corpus callosum and cortex with corresponding statistical values

<u>Corpus Callosum</u>				
<u>Comparing sham vs. TBI at each survival interval.</u>				
	<u>Time postinjury</u>	<u>kD</u>	<u>% change from sham</u>	<u>Significance</u>
	1 day	220	+147.1%	$F_{(1,21)} = 22.09; p < 0.001$
		212	+73.7%	$F_{(1,21)} = 11.48; p = 0.003$
	3 days	75	-54.3%	$F_{(1,21)} = 5.02; p = 0.038$
	7 days	75	-41.3%	$F_{(1,21)} = 4.93; p = 0.043$
<u>Time -dependent changes following TBI</u>				
	1d vs. 3d	220 kD	-155.1%	$F_{(1,22)} = 38.84; p < 0.001$
	1d vs. 7d	220 kD	-163.5%	$F_{(1,22)} = 43.20; p < 0.001$
	1d vs. 3d	212 kD	-80.2%	$F_{(1,22)} = 15.76; p = 0.001$
	1d vs. 7d	212 kD	-65.3%	$F_{(1,22)} = 10.47; p = 0.004$
	1d vs. 3d	75 kD	-93.2%	$F_{(1,22)} = 11.44; p = 0.003$
	1d vs. 7d	75 kD	-80.2%	$F_{(1,22)} = 8.47; p = 0.008$
<u>Cerebral Cortex</u>				
<u>Time -dependent changes following TBI</u>				
	1d vs. 3d	220 kD	-96.4%	$F_{(1,18)} = 5.97; p = 0.025$

DISCUSSION

The present study examined TBI-induced changes in ankyrin-G in two brain regions, corpus callosum and cerebral cortex, in sham control rats and in rats which survived for 1d, 3d, or 7d following fluid percussion injury. This approach allowed an examination of injury effects expressed in a region which is highly enriched in axons (callosum) to be contrasted with injury effects in a gray-matter region with more diverse cellular elements (cortex). No prior published studies have measured any forms of ankyrin following fluid percussion TBI. Accordingly, this thesis represent the initial attempt to examine how the multifunctional ankyrin-G molecule is affected by injury.

A general principle in traumatic brain injury is that axons are exquisitely sensitive to the mechanical forces sustained during the injury process. Whether this is due to the tortuous route taken by axons through the neuropil, or to the high membrane-to-cytoplasm ratio in axons, or to other unspecified vulnerabilities, is a matter of debate among neuroscientists. However, the high incidence of axonal pathology seen in TBI did influence the experimental method and design selected for this project. The laboratory where this work was undertaken has previously noted electrophysiological changes

induced in axons by injury (Reeves et al., 2000, 2003, 2004), and more recently has concentrated on functional abnormalities affecting corpus callosum axons in the aftermath of TBI (Reeves et al., 2003; Reeves et al., in press). Within this on-going context, the primary focus of the current project was to pursue a molecular correlate to help understand how callosal axons failed functionally and structurally after TBI. Ankyrin-G was selected, both for its association with voltage-gated sodium channels, relevant to postinjury deficits in callosal axon conduction, and for its role as part of the membrane skeleton which undercoats the axolemma, relevant to postinjury structural degeneration seen in callosal axons. Following a brief summary of the current experimental results, based upon Western blot analysis of ankyrin-G, these data are compared to prior electrophysiological and ultrastructural findings which were obtained using identical injury conditions and severities. Finally, a more general discussion examines how the present data may best be assimilated into the existing theory of traumatic brain pathology.

The anti-ankyrin-G antibody produced measurable immunopositive signals for both brain regions at each postinjury survival time point. In the corpus callosum, Western blot analysis revealed bands corresponding to 220 and 212 kD forms of ankyrin-G which were elevated at 1d postinjury but normalized to control levels at 3d and 7d postinjury. On the gels, these bands appeared as a 'doublet', and given their molecular weights, it is probable that they represent proteolytic fragments derived from the 480 or 270 kD intact forms of ankyrin-G. As discussed below, a comparison with previous studies of corpus callosum electrophysiology and ultrastructure, suggests the postinjury

changes in the 220,212 kD ankyrin reflect posttraumatic pathological processes in the myelinated axons of the corpus callosum. Western blot analysis also revealed a 75 kD band which tended also to rise at 1d, but was significantly below control levels at 3d and 7d postinjury. Given this molecular weight, it is likely that this band represents a proteolytic fragment of the 190 kD isoform of intact ankyrin-G, known to be abundant in unmyelinated axons. On the basis of a comparison with prior electrophysiological and ultrastructural evidence, postinjury alterations in the 75 kD band probably reflects pathological processes primarily occurring in the unmyelinated axons of the corpus callosum. Ankyrin-G immunoreactivity in the cerebral cortex was, in comparison to the corpus callosum, minimally affected by TBI. This supports a predominantly white-matter locus for the ankyrin-G role in fluid percussion TBI.

There are firm theoretical grounds to predict that the ankyrin family of proteins would be altered by TBI, and that changes affecting these molecules would play critical roles in both the pathology and outcome of injury. It is well established that ankyrins are spectrin-binding proteins that associate via ANK repeats with a variety of ion channels/pumps, calcium release channels and cell adhesion molecules. Ankyrins have a physiological role in restricting voltage-gated sodium channels and members of the L1 CAM family of cell adhesion molecules to excitable membranes in the nervous system (e.g., reviewed by Bennett and Chen, 2001), and disturbances to these associations would be expected to lead to functional changes manifested as behavioral deficits and electrophysiological changes.

Prior to the conduct of this project, essentially no information existed which specifically addressed changes in ankyrin following traumatic brain injury. Given the absence of preceding data, measurements of any of several ankyrin isoforms could serve as useful 'entry points' to initiate the investigation of postinjury ankyrin changes. This thesis targeted ankyrin-G for reasons of its strong association with sodium channels, these channels attracting interest as potential substrates underlying postinjury deficits in corpus callosum electrophysiological functioning (Reeves et al., 2004; Reeves et al., in press). Future studies may, for instance, examine injury-induced alterations in ankyrin-B to exploit this isoform's relationship to immature unmyelinated axons, and its association with neurofascin (Davis et al., 1993). However, the study of ankyrin-G is also advantageous because it may serve an organizational role during periods of plasticity, such as the neuroplasticity phase following TBI during which degenerative products are cleared and surviving axons may be repaired. Evidence for an organizational role for ankyrin-G, during periods of growth and plasticity, was provided by Jenkins and Bennett (2001). These authors demonstrated that ankyrin-G played an essential role in coordinating the assembly of Nav 1.6 channels, Beta-IV spectrin, and the L1 cell adhesion molecules at axon initial segments of cerebellar Purkinje cells. Ankyrin-G, in fact, was observed at the initial segments prior to the appearance of the cell adhesion molecules, and none of the remaining molecules (Nav 1.6, spectrin, L1-CAMs) are normally clustered at the initial segments in mice lacking ankyrin-G (Jenkins and Bennett, 2001).

The observation that ankyrin-G coordinates molecular binding at excitable membrane domains, as demonstrated by Jenkins and Bennett (2001), may assist an interpretation of prior findings pertaining to axonal injury in the CNS, as well as data in the present study relating to the modest changes in ankyrin in the cerebral cortex following injury. While postinjury alterations in ankyrin-G were much more dramatic in the corpus callosum than in the cerebral cortex, there was a measurable time dependent shift in the 220 kD form of ankyrin-G occurring between 1d and 3d following injury in the cortex. It is conceivable that the locus of this effect is the initial segments of cells undergoing shifts in distribution and conformation of ankyrin in response to axonal and somatic injury. In such a scenario, ankyrin-G may participate in the reorganization and re-clustering of appropriate molecules which underlies the survival and functional recovery of cortical cells. Other workers have reported that many cortical neurons survive despite TBI-induced axonal disconnection which occurs very near the cell body (Singleton et al., 2002), and reactive shifts in initial segment ankyrin-G may be a determinant in the survival and recovery of these neurons.

The focus of the present project was motivated, in part, by an effort to understand the process by which CNS axons exhibit functional deficits and (sometimes) functional recovery following TBI. Specifically, it was previously shown that compound action potentials (CAPs), evoked through corpus callosum axons, exhibited decreased amplitudes following fluid percussion injury (Baker et al., 2002; Reeves et al., 2004; Reeves et al., in press). An intriguing aspect of these results was that CAPs in myelinated axons showed a postinjury amplitude decrement which subsequently

recovered, while the CAP for unmyelinated axons remained suppressed in amplitude for at least a week postinjury. As an attempt to help understand this differential deficit and recovery pattern, the present investigation examined changes in ankyrin-G, important in this context because it secures ion channels and transporters essential for bioelectric function in axons. To facilitate a discussion of how the present results may relate to the prior electrophysiological findings, Figure 10 summarizes key features of the recording electrode placement, the typical CAP waveform, and illustrates the differential degree of amplitude suppression and recovery in the type fiber types.

Perhaps the most straightforward approach for comparing post-TBI changes in ankyrin with time-dependent changes in evoked CAPs is to graphically compare the two sets of measurements. In Figure 11 the time courses of changes in callosal CAP amplitude have been superimposed on a bar graph showing expression levels of ankyrin at the same postinjury intervals. Data have been normalized, in all sets of measurements, as percentage of the sham control level. Several key features of this comparison should be emphasized. CAPs showed the minimum amplitude (= maximum deficit) at 1d postinjury, coincident with the time when ankyrin levels were most perturbed. We interpret the upsurge of ankyrin 220 and 212 (appearing as the 'doublet' on gels) to represent breakdown of higher isoforms of ankyrin, specifically the 270-kD and 480-kD isoforms of ankyrin-G which are highly localized to myelinated axons. In contrast, the spike at 1d in levels of the 75-kD is likely to represent primarily a breakdown product of the 190-kD ankyrin-G, which has been associated with unmyelinated axons (Rubtsov and Lopina, 2000), although the possibility that some of the 75-kD surge reflects residual

Figure 10. Summary of methods and results from prior study of evoked callosal CAPs.

A. Diagram showing placement of stimulating and recording electrode in corpus callosum of brain slices removed from rat following TBI or sham injury. **B.** Example of evoked CAP biphasic waveform, showing wave components generated by myelinated callosal axons (N1) and by slower unmyelinated axons (N2). **C.** Summary of results from a prior study (Reeves et al., 2004) showing transient depression of CAP amplitude in myelinated fibers, contrasting with persistent depression of unmyelinated CAP amplitude.

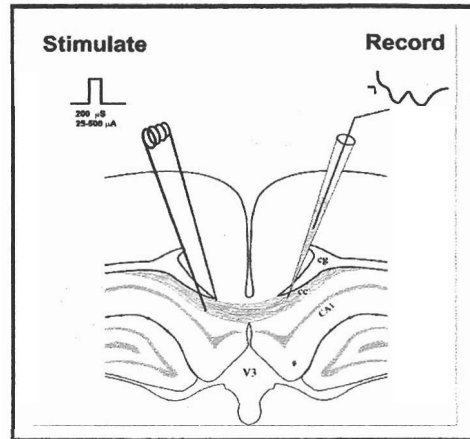
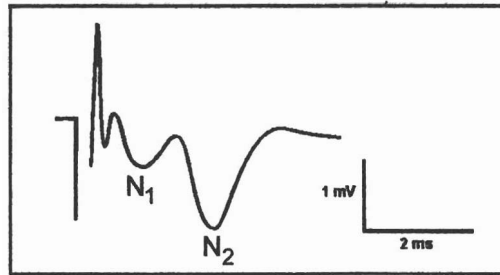
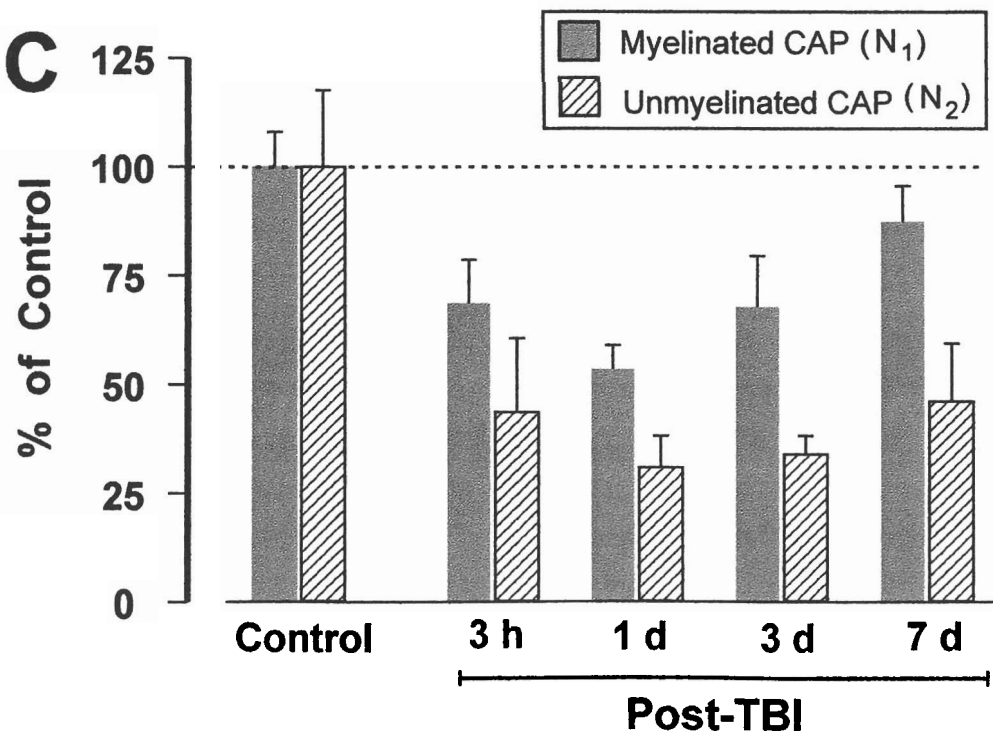
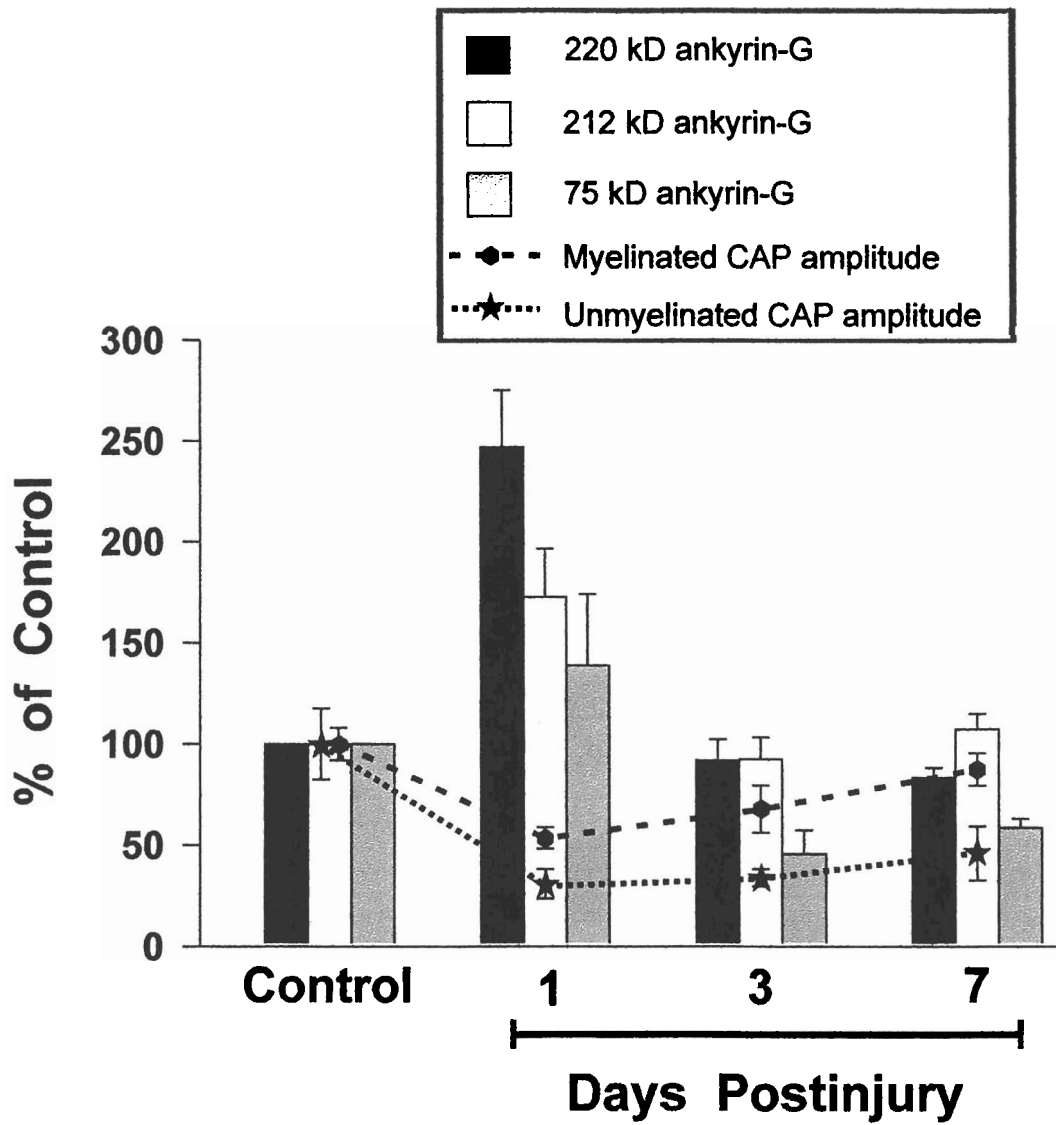
A**B****C**

Figure 11. Comparison of present ankyrin-G expression measurements with prior electrophysiological results. Bars depict levels of corpus callosum ankyrin-G (mean +/- S.E.M.) separately for 220, 212, and 75 kD. Superimposed on the bars is the time course derived from the prior study of amplitudes of callosal evoked CAPs for myelinated axons (black dots) and for unmyelinated axons (black stars). Note that the myelinated CAP data more closely corresponds to postinjury levels of 220,212 ankyrin-G, while the unmyelinated CAP more accurately tracks the levels of 75 kD ankyrin-G.

Comparison of TBI-induced changes in callosal Ankyrin expression and amplitude of evoked compound action potentials

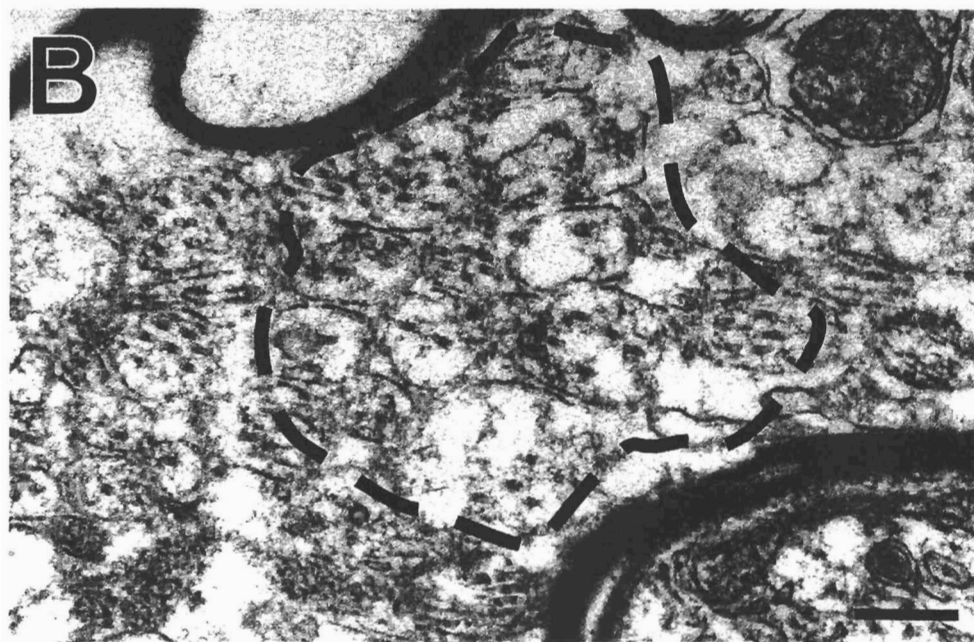
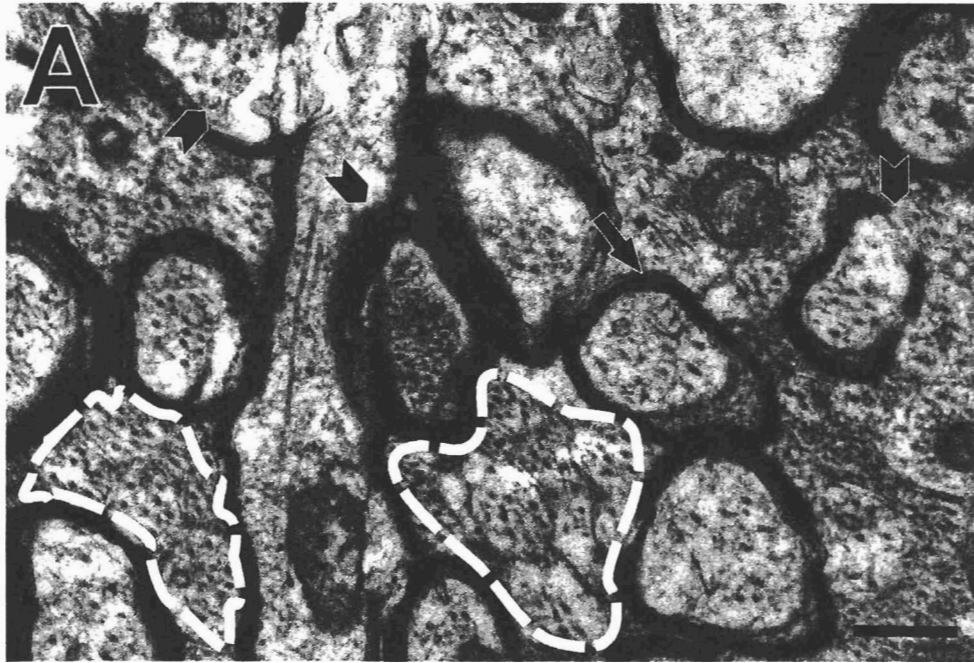


fragments of the 270/480 forms cannot be eliminated. A close examination of the ankyrin and CAP measurements at 3d and 7d also support the interpretation that the recovery of myelinated CAP amplitudes most closely track the 220/212 ankyrin forms, while the persistent suppression of unmyelinated CAP amplitudes match the levels of 75-kD ankyrin at 3d and 7d.

Prior ultrastructural observations of axons in the corpus callosum, following fluid percussion injury, are also relevant for an appreciation of the present changes in ankyrin-G. An example electron micrograph of callosal axons is shown in Figure 12. While this particular sample was taken from an a rat killed at an earlier survival interval (3h) than used in the current study, it serves to illustrate a profound morphological disorganization affecting unmyelinated axons, initiated quite rapidly after injury. Continuing work in this area is suggesting that the numbers of surviving unmyelinated axons progressively decreases in proportion to the number of surviving/recovering myelinated callosal axons. The significant decreases in 75 kD ankyrin-G, observed at 3d and 7d postinjury, may well represent a role for ankyrin-G in the progressive dissolution of cytoarchitecture and ultimate loss of unmyelinated axons.

Figure 12. Ultrastructural profile of corpus callosum axons at 3 h following central fluid percussion brain injury, from prior study (Reeves et al., 2004). Panel (A) illustrates a region with both intact (arrow) and damaged (arrowheads) myelinated fibers. Isolated groups of unmyelinated fibers (outlined in white) exhibit relatively intact morphology. Other regions contain sites of significant unmyelinated fiber degeneration (B), with axolemmal breakdown and cytoskeletal disruption (outlined in black). Bar= 0.5 μm .

Corpus Callosum Fiber Injury



The assimilation of the results of this thesis into the context of existing theories of TBI is made difficult by the multiplicity of roles and binding partners of ankyrin. In addition, because this project is the first to measure ankyrin in the specific pathology of TBI, the interpretation of the present findings cannot directly appeal to prior published reports. There are, however, published reports of ankyrin measurements following ischemia in the brain (Harada et al., 1997) and in the kidney (Woroniecki et al., 2003), although these did not comparably examine ankyrin-G as in the present investigation. In spite of these difficulties, the conclusion is reached here that the present observations accord well with concepts of traumatic neuropathology based upon acute ionic dysregulation that leads to a failure of cytoskeletal components, in general, and alterations in the membrane skeleton, in particular. A well established feature of traumatic axonal neuropathology involves a failure of neurofilament sidearms leading to a compaction of major axonal cytoskeleton components with deleterious results (Hall et al., 1995; Povlishock et al., 1997; Maxwell et al., 1997).

As mentioned in the Introduction to this thesis, a hallmark of traumatic axonal injury is an abnormal cellular depolarization subsequent to the rapid accumulation of cations into the intracellular compartment. Existing knowledge does not yet allow a definitive statement as to whether the aberrant ion fluxes arise from a failure of the bilipid membrane ('microporation') or changes in transmembrane ionic channels. In any event, intracellular loading of Na^+ and Ca^{2+} mobilizes multiple calcium-dependent processes, such as the activation of proteases which degrade the cytoskeleton and

functional properties of axons, culminating, in the worst cases, in axonal disconnection and degeneration.

Postinjury changes to ankyrin-G expression, as observed in this thesis, are consistent with an axonal pathology arising from proteolytic changes to the membrane skeleton, affecting the ankyrin/spectrin subplasmalemmal network and the channel proteins which are attached on their cytoplasmic domains to ankyrin. This conclusion does not, in any way, eliminate other pathological roles involving ankyrin and its diverse associations with other molecules, including various transporters, receptors, and cell adhesion molecules. However, the association of ankyrin-G with voltage-gated sodium channels (NaV) appears to be a fundamental tendency of ankyrin-G, and has been the membrane protein most frequently studied as a linker of ankyrin-G (e.g., Bouzidi et al., 2002; Custer et al., 2003; Garrido et al., 2003; Jenkins and Bennett, 2001, 2002; Malhotra et al., 2002). On a conceptual level, a disturbance of the ankyrin/Nav channel complex would be predicted to lead to functional deficits previously seen in callosal axonal excitability following identical injury conditions (Reeves et al., 2004; Reeves et al., in press), and it is reasonable to expect such a pathology to be expressed as behavioral deficits characteristically seen following these injuries. Sodium channel pathology has been identified as a very early event in TBI, perhaps preceding intraaxonal calcium loading (Iwata et al., 2004), and involving specific proteolytic damage to NaV channels. In addition to the biochemical processes leading to proteolysis of nodal components, the actual mechanical aspects of injury may preferentially stress ankyrin/Nav linkages. In this regard, nodes are selectively vulnerable to trauma, for example, being the primary

site of damage during stretch-induced injury to optic nerves (Maxwell et al., 1991), and it is known that ankyrin-G molecules cluster with Nav channels at nodes of Ranvier.

It is thus the conclusion of this thesis that the initial increases in ankyrin 220,212 -kD forms, seen at 1d postinjury in the corpus callosum, probably represent proteolyzed segments of higher 480,270 -kD ankyrin-G, and are unlikely to represent a *de novo* synthesis process. The subsequent recovery of the callosal 220,212 -kD forms to control levels, seen at 3d and 7d postinjury, agrees well with observations of initial electrophysiological deficits in myelinated callosal axons that moderate during the first postinjury week. The summary conclusion regarding the 75-kD ankyrin-G seen to increase at 1d, and then to fall significantly below control levels at 3d and 7d postinjury, is that this most likely reflects a more deleterious and persistent pathology that affects the unmyelinated fiber population. Such an interpretation is supported by initial ultrastructural evidence that the fluid percussion TBI initiates a pathology in unmyelinated fibers that leads to the total failure of membrane integrity and the subsequent dissolution of a percentage of callosal fibers. The restorative mechanisms whereby the levels of ankyrin return to control levels is beyond the scope of this project. However, the disparity in the status of the 220,212-kD forms vs. the 75-kD form, at 3d and 7d, may also reflect the greater loss of unmyelinated than myelinated axons. Finally, in conclusion regarding observations in the cerebral cortex, the relative lack of TBI-induced changes in ankyrin supports a preferential role of the ankyrin-G molecule in axon-enriched regions, although a modest change observed in the 220-kD form may conceivably reflect pathological events at the initial axonal segments of cortical neurons.

List of References

- Baines AJ (1990) Ankyrin and the node of Ranvier. *Trends Neurosci* 13:119-121.
- Baker AJ, Phan N, Moulton RJ, Fehlings MG, Yucel Y, Zhao M, Liu E, Tian GF (2002) Attenuation of the electrophysiological function of the corpus callosum after fluid percussion injury in the rat. *J Neurotrauma* 19:587-599.
- Bennett V (1990) Spectrin-based membrane skeleton: a multipotential adaptor between plasma membrane and cytoplasm. *Physiol Rev* 70:1029-1065.
- Bennett V (1992) Ankyrins. Adaptors between diverse plasma membrane proteins and the cytoplasm. *J Biol Chem* 267:8703-8706.
- Bennett V, Gilligan DM (1993) The spectrin-based membrane skeleton and micron-scale organization of the plasma membrane. *Annu Rev Cell Biol* 9:27-66.
- Birkenmeier CS, White RA, Peters LL, Hall EJ, Lux SE, Barker JE (1993) Complex patterns of sequence variation and multiple 5' and 3' ends are found among transcripts of the erythroid ankyrin gene. *J Biol Chem* 268:9533-9540.
- Bourguignon LY, Chu A, Jin H, Brandt NR (1995) Ryanodine receptor-ankyrin interaction regulates internal Ca²⁺ release in mouse T-lymphoma cells. *J Biol Chem* 270:17917-17922.
- Bourguignon LY, Jin H (1995) Identification of the ankyrin-binding domain of the mouse T-lymphoma cell inositol 1,4,5-trisphosphate (IP₃) receptor and its role in the regulation of IP₃-mediated internal Ca²⁺ release. *J Biol Chem* 270:7257-7260.
- Bouzidi M, Tricaud N, Giraud P, Kordeli E, Caillol G, Deleuze C, Couraud F, le Alcaraz G (2002) Interaction of the Nav1.2a subunit of the voltage-dependent sodium

channel with nodal ankyrinG: In vitro mapping of the interacting domains and association in synaptosomes. *J Biol Chem* 32: 28996–29004.

Buki A, Siman R, Trojanowski JQ, Povlishock JT (1999) The role of calpain-mediated spectrin proteolysis in traumatically induced axonal injury. *J Neuropathol Exp Neurol* 58:365-375.

Colicos MA, Collins BE, Sailor MJ, Goda Y (2001) Remodeling of synaptic actin induced by photoconductive stimulation. *Cell* 107:605–616.

Cowan WM, Sudhof TC, Stevens CF (2001) *Synapses*. Johns Hopkins University Press, Baltimore, MD.

Custer AW, Kazarinova-Noyes K, Sakurai T, Xu X, Simon W, Grumet M, Shrager P (2003) The role of the ankyrin-binding protein NrCAM in node of ranvier formation. *J Neurosci* 23:10032–10039.

Davis JQ, Bennett V (1990) The anion exchanger and Na⁺K⁺-ATPase interact with distinct sites on ankyrin in in vitro assays. *J Biol Chem* 265:17252-17256.

Davis JQ, Bennett V (1994) Ankyrin binding activity shared by the neurofascin/L1/NrCAM family of nervous system cell adhesion molecules. *J Biol Chem* 269:27163-27166.

Davis JQ, Lambert S, Bennett V (1996) Molecular composition of the node of Ranvier: identification of ankyrin-binding cell adhesion molecules neurofascin (mucin+/third FNIII domain-) and NrCAM at nodal axon segments. *J Cell Biol* 135:1355-1367.

- Davis JQ, McLaughlin T, Bennett V (1993) Ankyrin-binding proteins related to nervous system cell adhesion molecules: Candidates to provide transmembrane and intercellular connections in adult brain. *J Cell Biol*, 121, 121-133.
- Devarajan P, Scaramuzzino DA, Morrow JS (1994) Ankyrin binds to two distinct cytoplasmic domains of Na,K-ATPase alpha subunit. *Proc Natl Acad Sci U S A* 91:2965-2969.
- Dixon CE, Lyeth BG, Povlishock JT, Findling RL, Hamm RJ, Marmarou A, Young HF, Hayes RL (1987) A fluid percussion model of experimental brain injury in the rat. *J Neurosurg* 67:110-119.
- Dubreuil RR, MacVicar G, Dissanayake S, Liu C, Homer D, Hortsch M (1996) Neuroglial-mediated cell adhesion induces assembly of the membrane skeleton at cell contact sites. *J Cell Biol* 133:647-655.
- Garrido JJ, Giraud P, Carlier E, Fernandes R, Moussif A, Fache M-P, Debanne D, Dargent B (2003) A targeting motif involved in sodium channel clustering at the axonal initial segment. *Sci* 300: 2091-2094.
- Garver TD, Ren Q, Tuvia S, Bennett V (1997) Tyrosine phosphorylation at a site highly conserved in the L1 family of cell adhesion molecules abolishes ankyrin binding and increases lateral mobility of neurofascin. *J Cell Biol* 137:703-714.
- Gloor S, Antonicek H, Sweadner KJ, Pagliusi S, Frank R, Moos M, Schachner M (1990) The adhesion molecule on glia (AMOG) is a homologue of the beta subunit of the Na,K-ATPase. *J Cell Biol* 110:165-174.

- Gregorio CC, Repasky EA, Fowler VM, Black JD (1994) Dynamic properties of ankyrin in T lymphocytes: colocalization with spectrin and protein kinase C beta. *J Cell Biol* 125:345-358.
- Hall TG, Bennett V (1987) Regulatory domains of erythrocyte ankyrin. *J Biol Chem* 262:10537-10545.
- Hayashi T, Su TP (2001) Regulating ankyrin dynamics: Roles of sigma-1 receptors. *Proc Natl Acad Sci U S A* 98:491-496.
- Harada K, Fukuda S, Kunimoto M, Yoshida K-I (1997) Distribution of ankyrin isoforms and their proteolysis after ischemia and reperfusion in rat brain. *J Neurochem* 69:371-376.
- Hicks RR, Baldwin DH, Scheff SW (1997) Serum extravasation and cytoskeletal alterations following traumatic brain injury in rats. *Mol Chem Neuropathol* 32:1-16.
- Hryniewicz-Jankowska A, Czogalla A, Bok E, Sikorski AF (2002) Ankyrins, multifunctional proteins involved in many cellular pathways. *Folia Histochem Cytobiol* 40:239-249.
- Isom LL, Catterall WA (1996) Na⁺ channel subunits and Ig domains. *Nature* 383:307-308.
- Isom LL, De Jongh KS, Catterall WA (1994) Auxiliary subunits of voltage-gated ion channels. *Neuron* 12:1183-1194.
- Iwata A, Stys PK, Wolf JA, Chen X-H, Taylor AG, Meaney DF, Smith DH (2004) Traumatic axonal injury induces proteolytic cleavage of the voltage-gated sodium

channels modulated by tetrodotoxin and protease inhibitors. *J Neurosci* 24:4605-5613.

Jenkins SM, Bennett V (2001) Ankyrin-G coordinates assembly of the spectrin-based membrane skeleton, voltage-gated sodium channels, and L1 CAMs at Purkinje neuron initial segments. *J Cell Biol* 155:739-745.

Jenkins SM, Bennett V (2002) Developing nodes of Ranvier are defined by ankyrin-G clustering and are independent of paranodal axoglial adhesion. *PNAS* 99:2303-2308.

Keppel G (1991) *Design and Analysis: A Researcher's Handbook* (3rd ed.). Prentice Hall, New Jersey.

Kenwrick S, Watkins A, De Angelis E (2000) Neural cell recognition molecule L1: relating biological complexity to human disease mutations. *Hum Mol Genet* 9:879-886.

Kordeli E, Bennett V (1991) Distinct ankyrin isoforms at neuron cell bodies and nodes of Ranvier resolved using erythrocyte ankyrin-deficient mice. *J Cell Biol* 114:1243-1259.

Kordeli E, Davis J, Trapp B, Bennett V (1990) An isoform of ankyrin is localized at nodes of Ranvier in myelinated axons of central and peripheral nerves. *J Cell Biol* 110:1341-1352.

Kordeli E, Lambert S, Bennett V (1995) AnkyrinG. A new ankyrin gene with neural-specific isoforms localized at the axonal initial segment and node of Ranvier. *J Biol Chem* 270:2352-2359.

- Korkotian E, Segal M (1999) Release of calcium from stores alters the morphology of dendritic spines in cultured hippocampal neurons. *Proc Natl Acad Sci USA* 96:12068–12072.
- Kunimoto M, Otto E, Bennett V (1991) A new 440-kD isoform is the major ankyrin in neonatal rat brain. *J Cell Biol* 115:1319-1331.
- Lambert S, Yu H, Prchal JT, Lawler J, Ruff P, Speicher D, Cheung MC, Kan YW, Palek J (1990) cDNA sequence for human erythrocyte ankyrin. *Proc Natl Acad Sci U S A*. 87:1730-1734.
- Levine G (1991). *A Guide to SPSS for Analysis of Variance*. Lawrence Erlbaum Associates, Publishers, New Jersey.
- Lux SE, John KM, Bennett V (1990) Analysis of cDNA for human erythrocyte ankyrin indicates a repeated structure with homology to tissue-differentiation and cell-cycle control proteins. *Nature* 344:36-42.
- Malhotra JD, Koopmann MC, Kazen-Gillespie KA, Fettman N, Hortsch M, Isom LL (2002) Structural requirements for interaction of sodium channel beta-1 subunits with ankyrin. *J Biol Chem* 277: 26681–26688.
- Marrs JA, Napolitano EW, Murphy-Erdosh C, Mays RW, Reichardt LF, Nelson WJ (1993) Distinguishing roles of the membrane-cytoskeleton and cadherin mediated cell-cell adhesion in generating different Na⁺,K⁽⁺⁾-ATPase distributions in polarized epithelia. *J Cell Biol* 123:149-164.

- Maurice T, Su TP, Privat A (1998) Sigma1 (sigma 1) receptor agonists and neurosteroids attenuate B25-35-amyloid peptide-induced amnesia in mice through a common mechanism. *Neuroscience* 83:413-428.
- Maxwell WL, Irvine A, Graham J, Adams T, Gennarelli T, Tipperman R, Sturatis M (1991) Focal axonal injury: the early axonal response to stretch. *J Neurocytol* 20:157-164.
- Michaely P, Bennett V (1993) The membrane-binding domain of ankyrin contains four independently folded subdomains, each comprised of six ankyrin repeats. *J Biol Chem* 268:22703-22709.
- Michaely P, Bennett V (1995a) The ANK repeats of erythrocyte ankyrin form two distinct but cooperative binding sites for the erythrocyte anion exchanger. *J Biol Chem* 270:22050-22057.
- Michaely P, Bennett V (1995b) Mechanism for binding site diversity on ankyrin. Comparison of binding sites on ankyrin for neurofascin and the Cl⁻/HCO₃⁻ anion exchanger. *J Biol Chem* 270:31298-31302.
- Michaely P, Kamal A, Anderson RG, Bennett V (1999) A requirement for ankyrin binding to clathrin during coated pit budding. *J Biol Chem* 274:35908-35913.
- Matsuzaki MN, Honkura N, Ellis-Davies GC, Kasai H (2004) Structural basis of long-term potentiation in single dendritic spines. *Nature* 429:761-766.
- Newcomb JK, Kampfl A, Posmantur RM, et al. (1997) Immunohistochemical study of calpain-mediated breakdown products to alpha-spectrin following controlled cortical impact injury in the rat. *J Neurotrauma* 14:369-383.

Okamura K, Tanaka H, Yagita Y, Saeki Y, Taguchi A, Hiraoka Y, Zeng LH, Colman DR, Miki N (2004) Cadherin activity is required for activity-induced spine remodeling. *J Cell Biol* 167:961-972.

Otto E, Kunimoto M, McLaughlin T, Bennett V (1991) Isolation and characterization of cDNAs encoding human brain ankyrins reveal a family of alternatively spliced genes. *J Cell Biol* 114:241-253.

Palek J, Lambert S (1990) Genetics of the red cell membrane skeleton. *Semin Hematol* 27:290-332.

Patton DE, Isom LL, Catterall WA, Goldin AL (1994) The adult rat brain beta 1 subunit modifies activation and inactivation gating of multiple sodium channel alpha subunits. *J Biol Chem* 269:17649-17655.

Pellegrino RG, Spencer PS, Ritchie JM (1984) Sodium channels in the axolemma of unmyelinated axons: a new estimate. *Brain Res* 305:357-360.

Peters LL, John KM, Lu FM, Eicher EM, Higgins A, Yialamas M, Turtzo LC, Otsuka AJ, Lux SE (1995) Ank3 (epithelial ankyrin), a widely distributed new member of the ankyrin gene family and the major ankyrin in kidney, is expressed in alternatively spliced forms, including forms that lack the repeat domain. *J Cell Biol* 130:313-330.

Peters LL, Lux SE (1993) Ankyrins: structure and function in normal cells and hereditary spherocytes. *Semin Hematol* 30:85-118.

- Posmantur R, Hayes RL, Dixon CE, Taft WC (1994) Neurofilament 68 and neurofilament 200 protein levels decrease after traumatic brain injury. *J Neurotrauma* 11:533-545.
- Povlishock JT (1993) Pathobiology of traumatically induced axonal injury in animals and man. *Ann Emerg Med* 22:980-986.
- Povlishock JT, Christman CW (1995) Diffuse axonal injury. In: *The axon* (Waxman SG, Kocsis JD, Stys PK, eds) pp504-529. New York: Oxford UP.
- Povlishock JT, Buki A, Koizumi H, Stone J, Okonkwo DO (1999) Initiating mechanisms involved in the pathobiology of traumatically induced axonal injury and interventions targeted at blunting their progression. *Acta Neurochir Suppl* 73:15-20.
- Pumplin DW, Bloch RJ (1993) The membrane skeleton. *Trends Cell Biol* 3:113-117.
- Pumplin DW, Drachman DB (1983) Myasthenic patients' IgG causes redistribution of acetylcholine receptors: freeze-fracture studies. *J Neurosci* 3:576-584.
- Reeves TM, Kao CQ, Phillips LL, Bullock MR, Povlishock JT (2000) Presynaptic excitability changes following traumatic brain injury in the rat. *J Neurosci Res* 60:370-379.
- Reeves TM, Prins ML, Zhu J-P, Povlishock JT (2003) Matrix metalloproteinase inhibition alters functional and structural correlates of deafferentation-induced sprouting in the dentate gyrus. *J Neurosci* 23:10182-10189.
- Reeves TM, Phillips LL, Walker SA, Povlishock JT (2004) Unmyelinated axons of the corpus callosum show selective functional and structural changes after injury and FK506 treatment. *J Neurotrauma* 21: 1324.

- Reeves TM, Phillips LL, Povlishock JT (in press) Myelinated and unmyelinated axons of the corpus callosum differ in vulnerability and functional recovery following traumatic brain injury. *Exp Neurol*.
- Rubtsov AM, Lopina OD (2000) Ankyrins. *FEBS Lett* 482:1-5.
- Saatman KE, Abai B, Grosvenor A, Vorwerk CK, Smith DH, Meaney DF (2003) Traumatic axonal injury results in biphasic calpain activation and retrograde transport impairment in mice. *J Cereb Blood Flow Metab* 23:34-42.
- Sedgwick SG, Smerdon SJ (1999) The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem Sci* 24:311-316.
- Singleton RH, Zhu J, Stone JR, Povlishock JT (2002) Traumatically induced axotomy adjacent to the soma does not result in acute neuronal death. *J Neurosci* 22:791-802.
- Srinivasan J, Schachner M, Catterall WA (1998) Interaction of voltage-gated sodium channels with the extracellular matrix molecules tenascin-C and tenascin-R. *Proc Natl Acad Sci U S A* 95:15753-15757.
- Srinivasan Y, Elmer L, Davis J, Bennett V, Angelides K (1988) Ankyrin and spectrin associate with voltage-dependent sodium channels in brain. *Nature* 333:177-180.
- Tavalin SJ, Ellis EF, Satin LS (1997) Inhibition of the electrogenic Na pump underlies delayed depolarization of cortical neurons after mechanical injury or glutamate. *J Neurophysiol* 77:632-638.
- Thevananther S, Kolli AH, Devarajan P (1998) Identification of a novel ankyrin isoform (AnkG190) in kidney and lung that associates with the plasma membrane and binds alpha-Na, K-ATPase. *J Biol Chem* 273:23952-23958.

- Waxman SG, Ritchie JM (1993) Molecular dissection of the myelinated axon. *Ann Neurol* 33:121-36.
- Woroniecki R, Ferdinand JR, Morrow JS, Devarajan P (2003) Dissociation of spectrin-ankyrin complex as a basis for loss of Na-K-ATPase polarity after ischemia. *Am J Physiol Renal Physiol* 284: F358–F364.
- Xiao ZC, Ragsdale DS, Malhotra JD, Mattei LN, Braun PE, Schachner M, Isom LL (1999) Tenascin-R is a functional modulator of sodium channel beta subunits. *J Biol Chem* 274:26511-26517.
- Yu RC, Bunge RP (1975) Damage and repair of the peripheral myelin sheath and node of Ranvier after treatment with trypsin. *J Cell Biol* 64:1-14.
- Zhao X, Posmantur R, Kampfl A, et al. (1998) Subcellular localization and duration of u-calpain and m-calpain activity after traumatic brain injury in the rat: a casein zymography study. *J Cereb Blood Flow Metab* 18:161-167.
- Zhou D, Lambert S, Malen PL, Carpenter S, Boland LM, Bennett V (1998) AnkyrinG is required for clustering of voltage-gated Na channels at axon initial segments and for normal action potential firing. *J Cell Biol* 143:1295-1304.

Vita

Andrew Schuil Vanderveer was born in Springfield, Illinois on May 31, 1979, the son of Dr. Craig Andrew Vanderveer and Mrs. Gail Hila Vanderveer. He has two younger siblings, Nathan and Abigail. He attended Charlotte Country Day High School where he was a member of State Championship teams in lacrosse and football. He attended North Carolina State University on a full athletic scholarship, and contributed to the resurgence of the Wolfpack football program. Andy joined the Delta Epsilon Chapter of Sigma Chi in the Spring of 1998 and received a Bachelor of Science in Biology and a minor in Genetics in the year 2002. Upon graduation Andy moved back to Charlotte and worked at Carolinas Medical Center, both as a lab assistant in Surgical Pathology and as a research assistant in the Department of General Surgery. He also worked as an Environmental Field Technician for SCS Field Services and Engineering. In 2003 Andy enrolled in the certificate program at VCU, in the Department of Anatomy and Neurobiology. Upon completion of the certificate, Andy began to pursue a Master's of Science in Anatomy and Neurobiology and finished in the summer of 2005. Andy is now a member of the UNC Chapel Hill School of Medicine class of 2009.