AN ABSTRACT OF THE DISSERTATION OF

Gaurav Bajaj for the degree of Doctor of Philosophy in Pharmacy presented on September 16, 2008.

Title: Regulation of NMDA-type Glutamate Receptors and MDR1 by Two Members of the EF-hand Protein Family

Abstract approved ____

Jane E. Ishmael

EF-hand proteins are a conserved family of proteins that are modulated by divalent cations and regulate diverse cellular activities. In the present study we characterized the molecular determinants of myosin regulatory light chain (RLC) interaction with several subunits of the N-methyl-D-aspartate (NMDA)-type glutamate receptor. Myosin RLC is an EF-hand protein that is traditionally considered an integral component of the myosin II complex. We show that this light chain is important for trafficking of NMDA receptors and that this function is likely independent of the myosin II complex. In addition, we studied the interaction of myosin RLC with a second non-myosin target, multi-drug resistance protein 1 (MDR1) also known as P-glycoprotein. Finally, we characterized a previously undescribed calcium-dependent calmodulin binding site on the NMDAR 2A (NR2A) subunit of the NMDA receptor. Calmodulin is structurally-related to myosin RLC and also considered an EF-hand protein.

Myosin II motors are hexameric complexes containing two heavy chains that each bind a pair of light chains: one essential light chain and one RLC. Alternative binding partners have been described for both light chains of myosin II raising the possibility that myosin RLC, like other EF-hand proteins,

may adopt conformations that can be distinguished from conventional myosin RLC-heavy chain interactions. In this study, we mapped the myosin RLC binding site to a 30-37 amino acid region of the C-termini of NR1 and NR2 subunits. Myosin RLC-NMDA receptor subunit interactions could be distinguished from the prototypical interaction of myosin RLC with the neck region of non-muscle myosin II-B heavy chain. NMDA-myosin RLC interactions were maintained in the absence of the fourth EF-hand domain and did not require the addition of magnesium. We report that sequence similarity in the "GxxxR" portion of the incomplete IQ2 motif found in nonmuscle myosin II heavy chain isoforms likely contributes to the recognition of NR2A as a non-myosin target of the myosin RLC. We report that myosin RLC-NR2A interactions likely occur in the Golgi complex and this interaction is important in forward trafficking of NR1/NR2A receptors. We suggest that a role for myosin RLC in protein trafficking in polarized cells is distinct from the typical interaction of myosin RLC as a component of the myosin II complex.

Like glutamate receptor subunits, MDR1 is also a membrane-bound protein expressed in polarized cells. MDR1 is a drug efflux transporter that consists of two homologous halves, each consisting of six membrane-spanning domains plus a nucleotide binding domain linked by an intracellular linker region. The linker region of MDR1 is a determinant of cell surface expression and directly interacts with intracellular cytoskeletal, regulatory, and motor proteins. A previous study documented a direct interaction between myosin RLC and the linker region of a related ABC transporter, bile salt export protein (BSEP), and established a role for myosin in BSEP trafficking. We found that myosin RLC interacts with MDR1 via the amino terminal of the light chain as observed with NMDA receptor subunits. This interaction of myosin RLC with both of its binding partners (MDR1 and NMDA receptor subunits) is decreased upon phosphorylation of specific residues in the amino terminal of the light chain. We used Madin-Darby canine kidney (MDCK) cells stably expressing MDR1 (MDCKII-MDR1) as a model system to study the functional consequences of perturbing the phosphorylation state of myosin RLC in intact

cells. Treatment of polarized MDCKII-MDR1 monolayers with ML-7, a pharmacologic inhibitor of myosin light chain kinase, increased the permeability of [^{3}H]-digoxin (a well-known substrate of MDR1) and decreased apical expression of MDR1 in MDCKII-MDR1 cells.

The combination of NR1 splice variants and NR2 subunits imparts differing physiological and pharmacological properties on NMDA receptor assemblies. The NR2 C-termini of NMDA receptors are approximately 600 amino acids long and the middle region of NR2A C-terminus (NR2A (875-1029)) bears only 29% sequence similarity with the corresponding region in the NR2B subunit. We used a proteomics approach to uncover proteins that may interact with this region from mouse brain homogenates both in the presence and absence of calcium. Calmodulin was found to interact with NR2A (875-1029) in a calcium-dependent manner. The binding affinity of calmodulin for the NR2A subunit was found to be 5.2 ± 2.4 nM, which is comparable to the affinity of a previously described binding site of calmodulin on the NR1 subunit. We found that tryptophan at position W1014 in NR2A C-terminal is critical for interaction with calmodulin. We also confirm in our studies that calmodulin is not a binding partner of the NR2B subunit of the NMDA receptor.

Together our studies provide insight to the interaction of two EF-hand proteins with target proteins. For the first time we provide a functional role of myosin RLC in trafficking of NMDA receptors to the plasma membrane. We also show that myosin RLC can influence cell surface expression of MDR1 and as a consequence alter the transport properties of MDR1 as a drug efflux transporter. This study will contribute to our understanding of the mechanisms that underlie increased expression of MDR proteins associated with drug refractory conditions. In addition, we characterized a novel calmodulin binding site on the NR2A subunit of the NMDA receptor. This previously undescribed calmodulin binding site within the NR2A C-terminus potentially highlights an important distinction between Ca^{2+}/calmodulin regulation of NR2A versus NR2B containing complexes.

©Copyright by Gaurav Bajaj

September 16, 2008

All Rights Reserved

Regulation of NMDA-type Glutamate Receptors and MDR1 by Two Members of the EF-hand Protein Family

by

Gaurav Bajaj

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented September 16, 2008
Commencement June 2009

Doctor of Philosophy dissertation of Gaurav Bajaj presented on September 16, 2008.

APPROVED:

____

Major Professor, representing Pharmacy

____

Dean of the College of Pharmacy

____

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

____

Gaurav Bajaj, Author

This work is dedicated to
my parents

ACKNOWLEDGEMENTS

First and the foremost, I would like to thank God for giving me wisdom and guidance throughout my life.

I would like to express my utmost gratitude to my advisor, Dr. Jane E. Ishmael, for the opportunity to learn and develop my skills as a scientist under her invaluable guidance. I would like to thank her for her financial support, understanding and encouragement throughout my studies. I would like to thank members of my graduate committee - Dr. Mark Leid, Dr. Theresa Filtz, Dr. Kathy Magnusson, and Dr. David Williams for their guidance and encouragement. I would also like to thank Dr. Michael Schimerlik and Dr. Sonia Anderson who were in my committee and retired during the completion of my studies.

I am really grateful to my colleagues - David Amparan, Jing Yang, Andrew Hau, Peter Hsu and Masa Safic for their assistance, friendship and encouragement. I would like to thank members of Leid laboratory particularly - Valerie Peterson, Anand Venkataraman and Dr. Walter Vogel for their assistance and discussions; other members of Leid lab - Dr. Olga Golonzhka, Lingjuan Zhang and Acharawan Khamsiritrakul for their support and friendship. I would like to acknowledge College of Pharmacy for the financial support and thank all people in College of Pharmacy for their encouragement.

In the end I would like to express my deepest appreciation to my family for their constant emotional support, care and love. I thank all my friends for their love and support during my studies; and extend my sincere thanks to all those who have been associated directly and indirectly with my thesis.

CONTRIBUTION OF AUTHORS

Dr. Jane Ishmael designed the research. Gaurav Bajaj performed research. Gaurav Bajaj and Dr. Jane Ishmael analyzed and wrote the manuscripts.

The experiments described in Fig. 2.2, 2.3, 2.4B, 2.5, 2.7, 2.8, 2.9, were performed by Gaurav Bajaj in the laboratory of Dr. Jane Ishmael. Experiments for Fig. 2.1 were performed by Dr. Jane Ishmael in the laboratory of Dr. Mike Schimerlik. Experiments for figures 2.4D and 2.11 were performed by Jing Yang. Dr. Yong Zhang performed the experiments for Fig. 2.6. For figures 2.12 and 2.13 cells were transfected by Andrew Hau, immunostaining was done by Gaurav Bajaj. Dr. Jane Ishmael did the microscopy and Dr. Chrissa Kioussi helped in making figures.

All experiments in chapter 3 were performed by Gaurav Bajaj. Microscopy for figures 3.6 and 3.7 was performed by Anand Venkataraman and Dr. Chrissa Kioussi helped in making figures. Experiments for figures 3.4 and 3.5 were performed by Gaurav Bajaj in the laboratory of Dr. Rosita Proteau with the help of Dr. Ying Fan.

In chapter 4, experiments for figures 4.1, 4.2, 4.5, 4.6, 4.7 and 4.8 were performed by Gaurav Bajaj. Peter Hsu performed experiments for figures 4.3 and 4.4. Experiments for Fig. 4.7 were performed by Gaurav Bajaj in the laboratory of Dr. Mike Schimerlik.

Dr. Mike Schimerlik analyzed the data for the experiments in Fig. 2.1 and Fig. 4.7.

TABLE OF CONTENTS

Page

CHAPTER 1 ……………………………………………………..............................1
Introduction
1.1 Glutamate: an excitatory neurotransmitter……………………….....…2
1.1.1 Glutamate receptors ……………………………………..….2
1.1.2 The Postsynaptic density ……………………………..…..3
1.1.3 Cytoskeletal organization at excitatory synapses ……..….4
1.1.3.1 Myosins motor proteins ………………………..…..5
1.1.3.2 Non-muscle myosin II ……………………….…….6
1.1.4 NMDA receptors ……………………………………….…….7
1.1.5 Membrane topology of NMDA receptors ……………….……9
1.1.6 Assembly of NMDA receptors ……………………………..…10
1.1.7 Trafficking of NMDA receptors ……………………………....12
1.1.8 Regulation of NMDA receptor numbers at synapses ……13
1.1.9 Pharmacology and regulation of NMDA receptors ……...14
1.2 EF-hand calcium binding proteins ..………………………………....16
1.2.1 Myosin light chains ……………………………………..…...17
1.2.2 Calmodulin …………………………………….……………..…18
1.2.2.1 Calcium dependent CaM regulation of NMDA receptors ……………………………….….....…19
1.3 ATP-binding cassette efflux transporters ………………………...…19
1.4 Summary ………………………………………………………...…21
1.5 References ………………………………………………………….40

CHAPTER 2 ………………………………………………………………..…57
NMDA receptor subunits: Non-myosin targets of myosin regulatory light chain

ABSTRACT ……………………………………………………………...…58
INTRODUCTION …………………………………………………………….58
MATERIALS AND METHODS …………………………………………..…61
RESULTS ………………………………………………………………..…68
Smooth muscle and brain isoforms of myosin RLC bind to the NR1 subunit with high affinity……………………………………………..…68
Light chain-NMDA receptor interactions are not dependent upon magnesium …………………………………………………..…69
Myosin RLC interacts with the intracellular membrane-proximal region of the C terminus of NR2 subunits …………………………...…70
Membrane-proximal regions of NR2A and NR2B C termini are not targets for calmodulin ………………………………………......…72
Myosin RLC co-localizes with NMDAR2A in whole cells …………...…73
Can myosin RLC bind to NMDA receptor subunits in the context of a myosin II complex? ..……………………………………..…74

TABLE OF CONTENTS (Continued)

Page

The amino terminal region of myosin RLC is critical for interaction with NMDA receptor subunits 75
Myosin RLC binding to the NMDA receptor is sensitive to the phosphorylation state of the light chain 76
Residues K844 and L845 in the membrane proximal region of NR2A are critical for myosin RLC binding 77
Myosin RLC-NR2A interactions facilitate forward trafficking of NR1/NR2A receptors 78
Myosin RLC co-localizes with endogenous myosin heavy chain following disruption of the RLC target sequence in NR2A 79

DISCUSSION 80
REFERENCES 113

CHAPTER 3 119
MDR1/P-glycoprotein expression and function is sensitive to the phosphorylation state of a myosin II light chain

ABSTRACT 120
INTRODUCTION 120
MATERIALS AND METHODS 123
RESULTS 129
Myosin RLC is a direct binding partner of diverse membrane-bound proteins 129
The amino terminal of myosin II RLC is required for interaction with MDR1 130
The phosphorylation state of myosin RLC is a critical determinant of MDR1-light chain binding 131
Apical-to-basal transport of [^{3}H]digoxin is enhanced by an inhibitor of MLCK 132
Apical expression of MDR1 is decreased by an inhibitor of MLCK 134

DISCUSSION 135
REFERENCES 157

CHAPTER 4 160
Biochemical characterization of a calcium-dependent calmodulin binding site on the NR2A subunit of the NMDA receptor

TABLE OF CONTENTS (Continued)

Page

ABSTRACT ……………………………………………………………………..……161
INTRODUCTION ………………………………………………………………………161
MATERIALS AND METHODS …………………………………………….……164
RESULTS ……………………………………………………………………………168
Proteomic analysis of NR2A (875-1029) binding partners……………168
Calmodulin binds directly to the NR2A C-terminal in a calcium dependent manner ……………………………………………..…169
Calmodulin does not interact with the NR2B C-terminal …………..…169
NR2A C-terminal can pull out CaM from mouse brain Homogenates ……………………………………………………..……170
W1014 in the NR2A C-terminal is critical for interaction with calmodulin …………………………………………………….......…170
Calmodulin binds to the NR2A subunit with high affinity…………..…172

DISCUSSION ……………………………………………………………..……173
REFERENCES …………………………………………………………………192

CHAPTER 5 ……………………………….…………………………………197
CONCLUSION

BIBLIOGRAPHY…………………………………………………………………..202

LIST OF FIGURES

Figure Page

1.1 Schematic representation of an excitatory synapse located on dendritic spines 24
1.2 Schematic representation of the organization of the NMDA receptor signaling complex in a A) neuronal cell B) dendritic spine 25
1.3 Schematic representation of cytoskeletal arrangement in the dendritic shaft and spine.....27
1.4 Schematic representation of non-muscle myosin II 28
1.5 Schematic representation of the membrane topology of NMDA receptor subunits 29
1.6 Schematic representation of A) NR1 splice-variants B) NR2A and NR2B subunits 30
1.7 Schematic representation of NMDA receptor model illustrating: A) important modulatory sites B) excitatory synaptic transmission 32
1.8 Schematic representation of secondary structure of myosin RLC and calmodulin 34
1.9 Myosin RLC interacts with both the NR2A and NR2B subunits of the NMDA receptor 35
2.1 Myosin RLC isoforms enhance fluorescence emission of a fluorescein-labelled peptide corresponding to the C0 region of the NMDAR1 carboxyl tail.....90
2.2 Light chain binding to NMDA receptor subunits does not require added magnesium 92
2.3 Myosin RLC interacts with a short membrane-proximal region of the C-terminal domain of the NMDAR2A subunit 93
2.4 The membrane-proximal C-terminal domain of three major NMDA receptor subunits harbors a binding site for myosin RLC 95
2.5 The membrane-proximal C-terminal domains of NMDAR2A and NMDAR2B subunits do not binding calmodulin 97
2.6 The expression pattern of myosin RLC and NMDAR2A remains congruent in whole cells in the presence of NMDAR1 98
2.7 Myosin RLC does not bind NMDAR2A in the context of either a phosphorylated or non-phosphorylated myosin II subfragment 100
2.8 The interaction of myosin RLC with NMDA receptor target sequences can be distinguished from RLC-heavy chain interactions 102
2.9 Myosin RLC-NMDA receptor interactions are sensitive to phosphorylation by myosin light chain kinase 104

LIST OF FIGURES (Continued)

Figure		Page
2.10	Sequence alignment of putative RLC binding domains in nonmuscle myosin heavy chain isoforms, NMDAR2 subunits and Bile Salt Export Protein	106
2.11	Residues within a tyrosine-based endocytic motif associated with endocytosis of the NMDAR2A subunit are critical for myosin RLC binding	107
2.12	Disruption of the myosin RLC binding site on NMDAR2A leads to a trafficking defect in NR1/NR2A assemblies	109
2.13	Myosin RLC relocates to cellular extensions containing endogenous myosin heavy chain isoforms following disruption of NMDAR2A binding	111
3.1	Myosin RLC binds directly to the human MDR1-linker region	143
3.2	Myosin RLC binds to MDR1-linker region through its amino terminal	145
3.3	Myosin RLC interaction with MDR1-linker region is sensitive to phosphorylation	147
3.4	Permeability of [^{3}H]-digoxin was enhanced in MDCKII-MDR1 cell monolayer within 30 minutes of ML-7 pretreatment.	149
3.5	Comparison of ML-7 and verapamil treatment on permeability (P_e) of [^{3}H]-digoxin in the MDCKII-MDR1 cell monolayer	151
3.6	Myosin light chain colocalizes with MDR1 in MDCKII-MDR1 cells	153
3.7	Treatment with myosin light chain kinase inhibitor, ML-7, decreases apical expression of MDR1 in MDCKII-MDR1 cells	154
4.1	Polyacrylamide gel electrophoresis of proteins from mouse brain homogenate that interact with NR2A (875-1029) in the presence and absence of calcium	179
4.2	Calmodulin binds to the NR2A C-terminal within amino acids 875-1029	181
4.3	CaM interacts with C-termini of NR1 and NR2A but not with NR2B C-terminal	182
4.4	Full-length NR2A and NR1 C-termini can bind to CaM in brain lysate in the presence of calcium	183
4.5	Site directed mutagenesis of six putative calmodulin binding sites found in NR2A (875-1029)	184
4.6	W1014 is critical for binding of CaM to the NR2A C-terminal	186
4.7	Titration of either GST-NR2A (991-1029) or GST-NR1-C0 led to fluorescence quenching of alexa-calmodulin	188
5.1	EF-hand proteins as binding partners of NR2A subunit of NMDA receptor and MDR1	201

LIST OF TABLES

Table Page

1.1 The myosin superfamily ……………………………………………….....37
1.2 The myosin II subfamily ……………………………………………...39

3.1. Transepithelial electrical resistance (TEER) of MDCKII-MDR1 monolayers before and after treatment with ML-7, a myosin light chain kinase inhibitor ………………………………………..………155
3.2. The efflux ratios of [^{3}H]-digoxin in MDCKII-MDR1 cells is decreased in response to ML-7 and verapamil pretreatment …..……156

4.1 Details of CaM peptides found in the band sent for mass spectrometric analysis ………………………………………………..…190
4.2 Amino acid coverage of CaM peptides determined by Mass spectrometric analysis …………………………………… ………191

Regulation of NMDA-type Glutamate Receptors and MDR1 by Two Members of the EF-hand Protein Family

CHAPTER 1

INTRODUCTION

1.1 Glutamate: an excitatory neurotransmitter

Neurons in the mammalian brain communicate with each other through specialized junctions called synapses. Amino acid neurotransmitters present in the presynaptic terminal exert their effect by binding to specific receptors on the neuronal postsynaptic membrane. A neurotransmitter can either 'excite' its neighboring neuron so increasing its activity, or 'inhibit' its neighboring neuron, suppressing its activity. In general, the activity of a neuron depends on the balance between the number of excitatory (depolarization) and inhibitory (hyperpolarization) processes affecting it, and these can occur simultaneously. L-glutamate is the major excitatory neurotransmitter in brain. There are two major inhibitory neurotransmitters in the mammalian central nervous system (CNS). Gamma-amino butyric acid (GABA) is the principal neurotransmitter mediating fast inhibitory synaptic currents in the brain (Kuffler and Edwards, 1958). Glycine is the other inhibitory neurotransmitter especially in the spinal cord (Aprison and Werman, 1965; Hokfelt and Ljungdahl, 1971).

1.1.1 Glutamate receptors

Glutamate receptors are classified into two major groups: (i) ionotropic receptors that are cation-specific, ligand-gated ion channels and (ii) metabotropic or G-protein-coupled receptors. Ionotropic receptors are further classified depending on the exogenous agonist activating these receptors: N-methyl-D-aspartate (NMDA)-type glutamate receptors, and non-NMDA type glutamate receptors that include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Watkins and Evans, 1981). Glutamate receptors are involved in fundamental processes such as neural development and learning and memory (Bliss and Collingridge, 1993; Constantine-Paton et al., 1990). However, excessive activation of glutamate receptors is responsible for the destruction of neural tissue during ischemic stroke (Choi et al., 1988; Lee et al., 1999). In addition, NMDA type glutamate

receptors has an important physiological role and are involved in the etiology of neurological disorders like Alzheimer's (Scarpini et al., 2003), epilepsy (Najm et al., 2004) ,and Parkinson's disease (Hallett and Standaert, 2004; Waxman and Lynch, 2005).

1.1.2 The postsynaptic density

Most excitatory synapses in the central nervous system (CNS) are located on dendritic spines (Peters et al., 1991). Dendritic spines are small membranous protrusions that extend from the surfaces of the dendritic processes of neurons and contain a unique organelle called the postsynaptic density (PSD) (Sheng and Hoogenraad, 2007). The PSD is protein rich and can be visualized by electron microscopy in excitatory synapses due to the presence of numerous protein complexes (Sheng and Hoogenraad, 2007). The PSD is distinct from the endocytic zone (EZ), where clathrin-dependent membrane recycling takes place (Blanpied et al., 2002). In addition to ionotropic glutamate receptors, the PSD contains other ion channels, receptor tyrosine kinases, scaffolding, motor, and cytoskeletal proteins and other signaling molecules (Husi and Grant, 2001; Sheng and Hoogenraad, 2007; Wenthold et al., 2003). A schematic representation of the PSD in the dendritic spine is shown in Fig. 1.1 and Fig. 1.2B. Postsynaptic density-95 (PSD-95) was the first membrane-associated guanylate kinase (MAGUK) protein that was found to bind directly to NMDA receptors (Husi and Grant, 2001). Other proteins from the PSD-95 family that interact with NMDA receptors include PSD-93, synapse associated proteins (SAP-97), and SAP-102 (Kornau et al., 1995; Sans et al., 2003). These proteins are also called PDZ-domain proteins and interact with the PDZ binding domain ES(E/D)V motif present in the C-terminal of NR2 subunits (Kim and Sheng, 2004). SAP-102 is a major MAGUK expressed in neurons during early stages of development and PSD-

95 is believed to be the preferred partner of NMDA receptors at mature synapses (Sans et al., 2000).

1.1.3 Cytoskeletal organization at excitatory synapses

Transport of intracellular organelles and other cargo in glutaminergic neurons is carried out with the help of microtubules and actin filaments (Fig. 1.3). Compared to dendritic shafts (cylindrical portion of the dendrite, directly stemming from the cell body of the neuron) dendritic spines are rich in actin but tend to exclude microtubules and intermediate filaments (Kaech et al., 2001) (Fig. 1.3). Kaech et al. have shown that actin filaments predominate in the dendritic spines, whereas the cytoskeleton of the underlying dendritic shafts consists predominantly of microtubules which are bidirectionally oriented (Kaech et al., 2001). Kinesins, which are microtubule based motors, play an important role in transport of organelles and other cargo proteins in both axons and dendrites (Brady et al., 1985; Saito et al., 1997). Kinesin family member 17 (KIF17), a member of the kinesin superfamily of microtubule motor proteins, indirectly associates with NR2B-containing vesicles (Fig. 1.2A) (Setou et al., 2000). Decreased endogenous expression of KIF-17 using antisense RNA caused decreased expression of NR2B, but not NR2A or NR2C, containing NMDA receptors (Guillaud et al., 2003).

Dendritic spines show dynamic motility and undergo changes in shape during development (Ethell and Pasquale, 2005; Tada and Sheng, 2006). The size, shape, motility and stability of dendritic spines depend on actin which is the primary cytoskeletal element in spines (Sheng and Hoogenraad, 2007). Actin filaments are functionally coupled to actin filaments and depolymerization of actin causes about a 40% decrease in the number of synaptic NMDA and AMPA receptor clusters (Allison et al., 2000; Rosenmund and Westbrook, 1993). Actin is also associated with non-synaptic AMPA receptors as depolymerization of F-actin causes dispersion of AMPA receptor

clusters present in extrasynaptic sites (Allison et al., 2000). Actin filaments can make direct contacts with proteins in PSD (Sheng and Hoogenraad, 2007). Actin based myosin motors are also present in the PSD and are implicated in many important functions. For example, non-muscle myosin IIB is present as a part of the NMDA receptor associated complex purified from the mouse brain (Husi et al., 2000; Jordan et al., 2004; Peng et al., 2004). Myosin II has also been shown to be important for dendritic spine morphology and maintenance of synaptic transmission (Ryu et al., 2006). This regulation of myosin II in spines is mechanistically distinct from actin polymerization/depolymerization (Ryu et al., 2006). We have previously shown that a light chain of myosin II, myosin RLC interacts directly with the C-terminals of NR1 and NR2 subunits (Amparan et al., 2005). Postsynaptic density fractions from the rat brain also show the presence of another myosin motor myosin Va (Walikonis et al., 2000). Many members of the myosin superfamily including class I, II, V, VI and XVIII (Cheng et al., 2006; Collins et al., 2006), have been identified at glutamate synapses but the functional significance of these findings is not yet known.

1.1.3.1 Myosin motor proteins

Myosins are a large family of structurally diverse molecular motors. The myosin superfamily consists of eighteen distinct classes of proteins and are expressed in all eukaryotic cells (Table 1.1) (Berg et al., 2000; Korn, 2000; Sellers, 1999; Yamashita et al., 2000). These molecular motors bind to actin filaments in an ATP-dependent manner. The myosin II subfamily includes skeletal, cardiac, smooth muscle and non-muscle myosin (Sellers, 1999; Sellers, 2000). The regulation of smooth muscle myosins resembles that of non-muscle isoform in that the phosphorylation of myosin RLC is important for actin activation and regulation of myosin ATPase activity (Sellers, 1999). In contrast, cardiac and skeletal myosins are regulated by calcium and the troponin-tropomyosin complex (Weber and Murray, 1973). Non-muscle cells,

such as neurons, express multiple isoforms of myosin II. Non-muscle isoforms of myosin II share many biological properties with skeletal, cardiac and smooth muscle myosin (Sellers, 1999). However, the function and regulation of non-muscle myosin are more similar to smooth muscle myosin rather than striated muscle.

Myosin proteins are often classified as conventional (myosin-II like) or unconventional (all other types) myosins (Cheney and Mooseker, 1992). Conventional myosins possess two enzymatic heads, form bipolar filaments, and have characteristic actin-activated ATPase activity (Warrick and Spudich, 1987). Unconventional myosins are structurally distinct from conventional myosins but possess similar enzymatic properties *in vitro* due to the presence of conserved myosin head domains (Cheney and Mooseker, 1992; Cheney et al., 1993; Korn and Hammer, 1990; Pollard et al., 1991).

1.1.3.2 Non-muscle myosin II

The myosin II complex is composed of six subunits: two myosin heavy chains (MHCs) and two pairs of myosin light chains (MLCs), one of which is associated with each of the MHC head. Each MHC binds one 20kDa regulatory light chain (Myosin RLC) and one 17kDa essential light chain (ELC). As illustrated in Figure 1.4, non-muscle myosin II (NM II) contains a globular head region at the amino terminal and a coiled-coil rod portion at the carboxyl terminal. The myosin heavy chain has two distinct domains: the globular subfragment 1 (S1) head and α-helical rod domain (Fig. 1.4). The S1 head is composed of a motor head domain that harbors the site for actin binding, and the lever arm (or neck domain) that possesses the light chain binding site. The α-helical rod of MHC pairs with a second MHC rod to form a coiled-coil tail domain that include sites of myosin molecule association for filament formation (Sellers, 1999).

Myosin RLC is a substrate of several kinases including Rho kinase, AMP-activated protein kinase (AMP-kinase), and myosin light chain kinase (MLCK) which are known to modulate the extent of RLC phosphorylation at Ser 19 and Thr 18 (Amano et al., 1996; Gallagher et al., 1991). Phosphorylation of myosin II by several different kinases suggests multiple signal transduction pathways regulating its function. Studies with smooth muscle myosin suggest that the phosphorylation of myosin RLC at serine 19 and threonine 18 can modulate myosin activity (Amano et al., 1996; Gallagher et al., 1991). Phosphorylation of myosin RLC increases the actin-activated MgATPase and contractile activity, and filament formation (Sellers, 2000). On the contrary, myosin phosphatase causes dephosphorylation that leads to decreased contractile activity (Ito et al., 2004).

Non-muscle myosin and actin are thought to be important for cell motility, cell adhesion, and cell shape (Cramer, 1999) (Maciver, 1996). Additionally, myosin II motors in non-muscle cells have diverse functions involved in cytoplasmic contractility (Condeelis and Taylor, 1977), cytokinesis (De Lozanne and Spudich, 1987), and neurite outgrowth (Wylie and Chantler, 2001; Wylie et al., 1998). There are three different isoforms of non-muscle myosin II: NM II-A, NM II-B, and NM II-C (Berg et al., 2000; Golomb et al., 2004; Simons et al., 1991). Two isoforms of myosin RLC have been identified in the rat brain (Feinstein et al., 1991; Taubman et al., 1987). In our previous studies, we identified myosin RLC in mouse brain that shares about 97% similarity with the rat brain myosin RLC (Amparan et al., 2005).

1.1.4 NMDA receptors

The NMDA receptor (NR) 1 subunit cDNA was initially cloned from rat brain in 1991 (Moriyoshi et al., 1991). NR2 subunits were cloned in 1992 (Kutsuwada et al., 1992; Meguro et al., 1992) and NR3 subunits in 1995 (Ciabarra et al., 1995). Human cDNAs were subsequently identified that share

considerable sequence identity with their rat counterparts (Karp et al., 1993; Kutsuwada et al., 1992; Planells-Cases et al., 1993). NMDA receptors are permeable to both divalent (calcium) and monovalent cations (sodium and potassium) (Mayer and Westbrook, 1987b). At resting membrane potentials, NMDA receptors are subject to an extracellular magnesium blockade which is relieved only when the neuron is depolarized (Mayer et al., 1984). Unlike other ionotropic glutamate receptors, NMDA receptor activation depends on the simultaneous binding of glutamate and the co-agonist glycine (Johnson and Ascher, 1987).

NMDA receptors are now known to be heteromeric complexes composed of NR1 (ζ for mouse) and NR2 (ε for mouse) subunits (Ishii et al., 1993; Monyer et al., 1992; Moriyoshi et al., 1991). In some cases the NR3 subunit can assemble with NR1 but the role of this receptor in the central nervous system is still unclear (Cull-Candy et al., 2001). However, it is believed that NR3 containing NMDA receptors can modify channel activity (Das et al., 1998; Perez-Otano et al., 2001). The extracellular domain of the NR1 subunit forms a low affinity glycine-binding site while the glutamate-binding site is formed in the NR2 subunits (Furukawa and Gouaux, 2003; Furukawa et al., 2005). Electrophysiological studies show that NMDA receptor activation requires two independent glycine and two independent glutamate binding sites (Benveniste and Mayer, 1991; Clements and Westbrook, 1991). This suggests that functional NMDA receptors are tetrameric (formed as a dimer of dimers) composed of two NR1 and two NR2 subunits (Mayer and Armstrong, 2004; Schorge and Colquhoun, 2003). However, previous studies have also reported the pentameric assembly of NMDA receptors like $(NR1)_3$ $(NR2)_2$ or $(NR1)_2$ $(NR2)_3$ (Blackstone et al., 1992; Brose et al., 1993). Currently, it is widely believed that NMDA receptors have tetrameric assembly with the findings that have supported this model using electron microscopy, X-ray crystallographic studies (Furukawa et al., 2005; Stephenson et al., 2008; Tichelaar et al., 2004; Ulbrich and Isacoff, 2007).

1.1.5 Membrane topology of NMDA receptors

A schematic representation of the membrane topology of all NMDA receptor subunits is shown in Figure 1.5. Ionotropic glutamate receptors consist of four transmembrane domains. The membrane topology of these ionotropic glutamate receptors is distinct from other ligand gated ion channels in that the second transmembrane domain forms a reentrant loop leading to an extracellular N-terminal and an intracellular C-terminal (Bennett and Dingledine, 1995). The NR1 subunit is derived from a single gene and exists in eight different isoforms that are generated by alternative splicing of mRNA (Durand et al., 1992; Nakanishi et al., 1992; Sugihara et al., 1992). The four major C-terminal splice variants of NR1 are: NR1-1, NR1-2, NR1-3 and NR1-4. These splice variants differ by the presence and absence of three C-terminal cassettes: C1, C2 and C2' (Fig. 1.6A). The membrane proximal region C0 is common to all splice variants and is involved in interactions with various cytoskeletal proteins (Wyszynski et al., 1997). Each of these splice variants can exist in two forms depending on the presence (b form) or absence (a form) of an exon cassette in the N-terminal extracellular domain that modifies ion channel activity (Fig. 1.6A) (Sugihara et al., 1992). The NR2 subunit exists in four related subtypes that share greater sequence similarities with each other than NR1 and are derived from four separate genes *NR2A-NR2D* (Ishii et al., 1993; Monyer et al., 1992). Recently, recombinant NR1 and NR3 receptor subunits were shown to co-assemble as tetramers in oocytes but the existence of this tetramer *in vivo* has yet to be proven (Ulbrich and Isacoff, 2007). It was reported that NR1/NR3 subunits formed a novel glycine gated excitatory receptor but the role of NR3 subunits is still unclear (Chatterton et al., 2002; Smothers and Woodward, 2003).

The intracellular C-termini of NMDA receptors are considered important for the functioning and regulation of these ion channels. The C-terminal tails regulate receptor interactions with a variety of cytosolic proteins and are

important for intracellular trafficking and localization of NMDA receptors (Wenthold et al., 2003). Figure 1.2b shows proteins in the PSD that interact with the C-termini of NMDA receptors. Knockout mice lacking either NR1 or NR2B subunit die shortly after birth (Sprengel and Single, 1999). Electrophysiological studies in embryonic cultures from NR2B knockout mice show significantly faster NMDA receptor responses indicating that NR2A can be delivered to the synapse in the absence of the NR2B subunit (Tovar et al., 2000). On the contrary, NR2A knockout mice have slower NMDA receptor kinetics and show reduced long term potentiation (LTP) (Kadotani et al., 1996). NMDA receptors in mice lacking the NR2A C-terminal are absent in the synapse and are present only in the extrasynaptic sites (Steigerwald et al., 2000). A study has shown that the knockout mice that are deficient in the C-terminals of NR2B or NR2C resemble mice that are lacking the whole subunit (Sprengel et al., 1998).

1.1.6 Assembly of NMDA receptors

NMDA receptors are likely formed from co-assembly of two NR1 subunits and two NR2 subunits (Furukawa et al., 2005; Tichelaar et al., 2004; Ulbrich and Isacoff, 2007; Wenthold et al., 2003). NR1-1a is the most abundant NR1 splice variant and is not trafficked to the cell surface when expressed alone due to presence of an endoplasmic reticulum (ER) retention signal in the NR1-C1 cassette (Scott et al., 2001; Standley et al., 2000). ER retention motifs are also present in the C-terminal of NR2 subunits. The NR2B C-terminal contains three ER retention motifs that are identified as: RRR (1110-1112), KRRK (1079-1082) and KKR (1090-1092) (Hawkins et al., 2004). It has also been shown that C-terminal mutants of NR1 and NR2B subunits when expressed alone are still retained in ER (Hawkins et al., 2004; Horak et al., 2008). In addition to the above described ER retention motifs in the C-terminals of NR1 and NR2B, a recent study has shown the presence of

another ER retention motif in the third transmembrane domain of NR1 and NR2B subunits (Horak et al., 2008). Co-expression of NR1 and NR2 subunits masks these ER retention motifs in the third trans-membrane domain of the protein thereby leading to cell surface expression (Horak et al., 2008). Thus it is imperative for NR1 and NR2 subunits to co-assemble in the ER to form functional receptors before they can be trafficked to the plasma membrane.

NMDA receptors trafficking from the ER are also regulated by a “HLFY” motif which is present immediately following the fourth transmembrane domain of both NR2A and NR2B subunits (Hawkins et al., 2004). However, in a recent study it was shown that only three amino acids, “EHL”, are sufficient to overcome ER retention of NMDA receptor subunits (Yang et al., 2007). In addition to the above described motif, an ER export sequence, “TVV”, was also identified in NR1-C2’ cassette that is responsible for the export of NMDA receptors (Mu et al., 2003; Standley et al., 2000).

The NR1 subunit is present in excess relative to NR2 subunits in neurons and NMDA receptor formation is rate-limited by expression of NR2 subunits (Huh and Wenthold, 1999; Stephenson et al., 2008). Unassembled NR1 subunits do not reach the cell surface alone and are instead rapidly degraded (Huh and Wenthold, 1999). NR1/NR2A and NR1/NR2B are the major receptor subtypes that are expressed in the brain. NR2B-containing NMDA receptors are more abundant in the early stages of neuronal development and are present at both synaptic and extra-synaptic sites (Cull-Candy et al., 2001). NR2A containing NMDA receptors are more predominant in the adult brain (Monyer et al., 1994; Sans et al., 2000; Tovar and Westbrook, 1999). NR2B containing NMDA receptors becomes more enriched at extrasynaptic sites in mature synapses (Li et al., 2002; Tovar and Westbrook, 1999). NR1/NR2A/NR2B receptors are also present in the brain and their existence is confirmed by immunoprecipitation experiments from adult mammalian brain (Chazot and Stephenson, 1997). NR1/NR2A or NR1/NR2B receptors have similar conductance but much smaller conductance

was observed for NR1/NR2C and NR1/NR2D receptors (Stern et al., 1992; Wyllie et al., 1996). NMDA receptors containing NR2C and NR2D subunits have shown to have more restricted localization in midbrain and interneuron compared to receptors with NR2A and NR2B subunits and are less sensitive to magnesium blockade (Kuner and Schoepfer, 1996; Monyer et al., 1994). NR2C subunit has also been shown to have a cerebellar distribution (Karavanova et al., 2007; Watanabe et al., 1992)

1.1.7 Trafficking of NMDA receptors

Trafficking of proteins to the neuronal plasma membrane involves the ER, the Golgi apparatus and the trans-Golgi network. After assembly NMDA receptors exit from in the ER, and are forward trafficked to the Golgi network. In neurons the arrangement of the ER and Golgi is different than a normal eukaryotic cell. In addition to the somatic Golgi, the Golgi elements are also localized in the dendritic shafts (Horton and Ehlers, 2004). The smooth ER extends through the neck of many spines where this organelle could traffic synaptic proteins such as AMPA and NMDA receptors (Nusser et al., 1998; Racca et al., 2000). The trafficking of proteins from ER to Golgi follows two branches within the early secretory pathway. The major route whereby trafficking of proteins from ER is directed for long distances to the Golgi apparatus in the cell soma, and the minor route where ER to Golgi trafficking occurs locally in the dendrites (Horton and Ehlers, 2003; Horton and Ehlers, 2004).

After exiting from Golgi, NMDA receptors are targeted to the synapse. Using immunocytochemistry and time-lapse imaging of fluorescently tagged glutamate receptors in cortical cultures, Washbourne et al. have shown the distribution and dynamics of glutamate receptors in cortical neurons before and during synaptogenesis (Washbourne et al., 2002; Washbourne et al., 2004). It was shown that NMDA receptors are present in distinct mobile

transport packets that move along the microtubules and are recruited to the synapses (Washbourne et al., 2002). During synaptogenesis NMDA receptors are transported along the microtubules more rapidly than AMPA receptors (Washbourne et al., 2002). NMDA receptors are associated with several other proteins to form an exocyst complex before they are targeted to the membrane. The major proteins in this complex belong to a family of MAGUKs, and sec8, which is a large complex of proteins essential for the secretory pathway (Fig. 1.2A) (Sans et al., 2003). Specifically Sec8 and m-Pins associate with NR1/NR2B or NR1/NR2A through SAP-102 or PSD-95 to form a NMDA receptor targeting complex (Sans et al., 2005).

1.1.8 Regulation of NMDA receptor numbers at synapses

NMDA receptor endocytosis takes place by a clathrin dependent mechanism. Dendrites possess all the endocytic machinery that is required for endocytosis of the cell surface receptors (Blanpied et al., 2002; Lu et al., 2007; Racz et al., 2004; Yao et al., 2006). Both NR2A and NR2B subunits have been shown to interact with the adaptor protein-2 (AP-2) and are endocytosed in a clathrin dependent manner (Lavezzari et al., 2003; Roche et al., 2001; Vissel et al., 2001). The NR2B C-terminus contains a tyrosine-based AP-2 binding motif near the PSD-95 binding site (Lavezzari et al., 2003). Binding of the PSD-95 family of proteins prevents the interaction of AP-2 with NR2B subunits and thereby its endocytosis (Roche et al., 2001). Furthermore, Fyn-kinase can cause the phosphorylation of the tyrosine binding motif in NR2B thereby preventing its endocytosis and synaptic targeting (Prybylowski et al., 2005). NR2A contains both tyrosine-based (Y842) and dileucine based AP-2 binding motif and the latter is required for the AP-2 mediated endocytosis (Prybylowski et al., 2005). Tyrosine based AP-2 binding motif which is in C-terminus and close to the last transmembrane domain is believed to be present in all NR2 subunits as well as NR1 (Vissel et

al., 2001). Following endocytosis, NMDA receptors can be sorted for either recycling back to the cell surface or directed to intracellular lysosomes for degradation (Scott et al., 2004). It is believed that the membrane proximal C-terminals of NR2 subunits possess the sorting signals for lysosomal degradation while the distal end possess sorting motifs for the recycling of receptors even in the presence of proximal degradation motifs (Scott et al., 2004). The Rab family of GTPases regulates the trafficking of NMDA receptors through both recycling endosomes (Rab11) and trafficking in cargo from trans-Golgi network (Rab8) (Gerges et al., 2004; Park et al., 2004). However, it is not well known where exactly the postsynaptic membrane proteins are first delivered to the cell surface.

1.1.9 Pharmacology and regulation of NMDA receptors

The NMDA receptor complex consists of multiple modulatory sites which can act to inhibit, activate or enhance the function of the receptor (Fig 1.7A). NMDA itself is a commonly used agonist. Besides NMDA, several other dicarboxylic amino acids are also agonists at the NMDA binding site. These include L-glutamate, L-homocysteate, L-aspartate, homoquinolinate, L-homo-cysteinesulfinate, L-cysteinesulfinate, L-cysteate, and quinolinate (Mayer et al., 1992).

NMDA receptors also require the binding of the co-agonist glycine for the opening of the ion channel (Johnson and Ascher, 1987). Both AMPA and NMDA receptors are permeable to monovalent cations (Na^+ and K^+), while NMDA receptors are also permeable to Ca^{2+}. Upon excitation, glutamate is released from the pre-synaptic neuron terminal and activates AMPA-type and NMDA-type ionotropic glutamate receptors. While AMPA receptors contribute with cationic conductance at negative membrane potentials, NMDA receptors exhibit a voltage-dependent block by Mg^{2+} (Collingridge and Watkins, 1994). Agonist binding alone is insufficient to activate the channel as the channel is at hyperpolarized state and the channel pore is blocked by magnesium

(Collingridge and Watkins, 1994). However repetitive stimuli can cause sufficient depolarization to remove the magnesium blockade allowing calcium ions to enter the dendritic spine (Collingridge and Watkins, 1994) (Fig 1.7B).

The pharmacology of the NMDA receptor/channel complex indicates three major possible mechanisms of antagonism. Some of the well-characterized competitive antagonists include: (*R*)-2-amino-5-phosphonopentanoate (D-AP5), D-AP7, (±)-*cis*—4-phosphonomethyl-2-piperidine carboxylic acid (CGS 19755). Several noncompetitive antagonists have been identified that bind to a site within the channel itself are dizocilpine (MK-801), ketamine, phencyclidine (PCP) (Wong et al., 1986). There are also non-competitive antagonists that bind to the glycine binding site: kynurenate, 5,7-dichlorokynurenate (Herrling, 1997). Memantine HCl is an uncompetitive antagonist which is used for treating Alzheimer's disease. In addition there are also binding sites for polyamines and zinc that can modulate the activity of the receptor (Corsi et al., 1996). At physiological concentrations, zinc (Zn^{2+}) significantly inhibits NMDA receptor function (Westbrook and Mayer, 1987). It appears to act as a noncompetitive antagonist whose major site of action lies outside of the channel pore (Mayer and Westbrook, 1987a). It was shown recently that N-terminal domains of NR2A and NR2B forms a Zn^{2+} binding site (Choi and Lipton, 1999; Paoletti et al., 2008; Rachline et al., 2005).

Upon activation of the NMDA receptors, influx of Ca^{2+} triggers many downstream signaling pathways. In the presence of calcium, Ca^{2+}/CaM activates other enzymes like Ca^{2+}/CaM-dependent protein kinase II (CaMKII) that regulate the activity of NMDA receptors (Chung et al., 2004; Grant et al., 2001). In addition to CaMKII other kinases like protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase (MAPK) also regulate the activity of NMDA receptors through serine/threonine phosphorylation (Mammen et al., 1999; Roche et al., 1994). There are many serine/threonine and tyrosine phosphorylation sites in the C-termini of NR1 and NR2 subunits that are targets of kinases and phosphatases (Chen and Roche, 2007; Chen

et al., 1986; Mammen et al., 1999). These kinases regulate the intracellular trafficking and channel properties of NMDA receptors resulting in the changes in synaptic strength (Lee, 2006). PKC phosphorylates NR2A on S1291 and S1312 which potentiates NR2A-containing receptor currents (Grant et al., 2001; Jones and Leonard, 2005). PKC also phosphorylates NR2A on S1416 decreasing the binding of CaMKII (Gardoni et al., 2001). The C-termini of NR1 contains two phosphorylation sites, S890 and S896, that are targets of PKC (Tingley et al., 1997) and one site ,S897, which is a target of PKA (Scott et al., 2001). Phosphorylation of S897 is important for NMDA receptor surface expression, while phosphorylation of S890 disrupts clustering of NR1 subunits (Tingley et al., 1997).

NR2B-containing receptors are also regulated by phosphorylation by PKC at S1303 and S1323 (Liao et al., 2001) and CaMKII at S1480 (Chung et al., 2004). Phosphorylation of S1480 by CaMKII disrupts the PSD-95 interaction with NR2B (Chung et al., 2004). Tyrosine kinases like Src and Fyn are also important modulators of NMDA receptors as NMDA receptor currents are potentiated with increases in tyrosine kinase activity (Wang and Salter, 1994; Wang et al., 1996).

1.2 EF-hand calcium binding proteins

EF-hand Ca^{2+} binding proteins are a conserved family of proteins that contain from two to twelve copies of a basic structural motif that makes the EF-hand domain (Kretsinger and Nockolds, 1973). There are forty-five distinct families of EF-hand domains that had been identified and classified based on their functional and chemical characteristics (Kawasaki et al., 1998). The EF-hand is about thirty amino acids long and consists of an alpha-helix (E), loop, and a second alpha-helix (F) (Kretsinger and Nockolds, 1973).

Many EF-hand proteins are modulated by Ca^{2+} (Grabarek, 2006; Kawasaki et al., 1998; Kretsinger and Nockolds, 1973). Under physiological conditions a Ca^{2+}-ion is usually bound to the loop region of EF-hand proteins

thereby regulating diverse cellular activities like exocytosis, muscle contraction, transcription, cell proliferation and homeostasis (Carafoli and Klee, 1999; Celio et al., 1996; Van Eldik and Watterson, 1998). In quiescent cells, EF-hand proteins exist in apo- or a magnesium-bound form and bind to Ca^{2+} following stimulation. Calcium signals from various physiological responses with the cell are sensed by EF-hand proteins that translate them to cellular responses by undergoing a large conformational change. Light chains of myosin II (myosin RLC and ELC) closely relate to CaM and have been classified under one subfamily, collectively referred to as CTER (Calmodulin, troponin C, essential light chain, and regulatory light chain) (Kawasaki et al., 1998). The focus of our study is the interaction of CaM and myosin RLC with NMDA receptors. Myosin RLC is predicted to harbor one functional N-terminal EF-hand domain that can bind calcium or magnesium ions and three additional non-functional EF-hands (Fig. 1.8) (Houdusse et al., 1996; Rayment et al., 1993). However, all four EF-hand domains in CaM can bind calcium (Fig. 1.8) (Finn et al., 1995; Zhang et al., 1995).

1.2.1 Myosin light chains

Myosin II can reversibly bind to actin filaments and hydrolyze ATP converting chemical energy into mechanical force and movement (Sellers, 2000). The rod domain is responsible for the filament formation (Sellers, 2000). During cellular events such as mitosis and secretion, multiple residues on myosin RLC are phosphorylated. The light chains of myosin II bind to tandem IQ motifs located in the neck region of MHC (Houdusse et al., 1996; Rayment et al., 1993; Xie et al., 1994). The IQ motif consists of 25 amino acids with the consensus sequence IQxxxRGxxxR (Bahler and Rhoads, 2002). The number of IQ motifs varies within the class of myosins and are constant in some classes like myosin II (2 IQ binding motifs) and myosin V (6 IQ motifs) (Herm-Gotz et al., 2002). Structural studies have shown that light chains bound to IQ motifs form a rigid structure that serves as a mechanical

lever (Block, 1996). Binding of light chains to IQ motifs also influences other functional properties. For example, myosin ELC modulates ADP release by binding to the first IQ motif in smooth muscle myosin II (Dominguez et al., 1998). Phosphorylation of myosin RLC near its N-terminal region by MLCK regulates myosin II function by increasing contractile activity and filament formation (Ludowyke et al., 1989; Sellers, 2000; Yamakita et al., 1994). However, dephosphorylation of myosin RLC can lead to decreased contractile activity and inhibition of motor activity (Ito et al., 2004; Trybus et al., 1994).

1.2.2 Calmodulin

Calmodulin (CaM) is a cellular Ca^{2+} sensing molecule that acts as a trigger for many enzymes. CaM also regulates ion channels, cytoskeletal organization, and other events in the cell through its interaction with a diverse group of cellular proteins. CaM is a dumbbell shaped protein and interacts with many proteins both in the presence and absence of calcium. In the presence of Ca^{2+}, CaM interacts with a broad range of proteins that possess a basic amphipathic helix composed of approximately 20-25 amino acids (O'Neil and DeGrado, 1990; Persechini and Kretsinger, 1988).

CaM can also interact with the proteins in a Ca^{2+}-independent manner to perform many cellular functions (Geiser et al., 1991). The IQ motifs where CaM binds in the Ca^{2+}-dependent or independent manner are highly conserved. Neuromodulin and neurogranin are examples for Ca^{2+}-independent interactions of CaM (Baudier et al., 1991; Espreafico et al., 1992; Garcia et al., 1989). The target sequence of 25 amino acid residues (IQ motifs) in the heavy chain of myosin V forms a Ca^{2+}-independent interactions of CaM (Cheney and Mooseker, 1992). However, myosin VI has been shown to possess binding sites for both Ca^{2+}-dependent and Ca^{2+}-independent interactions (Bahloul et al., 2004).

Many attempts have been performed to determine the critical residues required for CaM binding, including comparative analysis of CaM binding

motifs, which are further used for identifying new CaM binding targets (Ataman et al., 2007; Rhoads and Friedberg, 1997). In the present work a Ca^{2+}-dependent CaM binding motif has been characterized in the C-terminal of NR2A subunit of the NMDA receptor.

1.2.2.1 Calcium-dependent CaM regulation of NMDA receptors

Numerous studies have documented the importance of Ca^{2+} entry through NMDA receptors for many downstream signal transduction pathways (Nicoll and Malenka, 1995). Ca^{2+} influx is important for synaptic transmission but excessive Ca^{2+}-influx may lead to excitotoxic cell death (Choi, 1995). CaM in the presence Ca^{2+} can also regulate the activity of NMDA receptors. Direct interaction of Ca^{2+}/CaM has been shown with the C-terminal of the NR1 subunit at two different sites (Ehlers et al., 1996; Zhang et al., 1998). Ehlers et al. proposed a model that the binding of CaM to the low affinity or high affinity site on the NR1 C-terminal can result in inactivation of the NMDA receptor channel in a calcium dependent manner (Ehlers et al., 1996). In 1998, Zhang et al. further showed that inactivation of NMDA receptors by CaM can be prevented by co-expression a region from the cytoskeletal protein α-actinin-2 that was previously known to interact with C0 region of NR1 C-terminal (Zhang et al., 1998). More recently, it was shown that calcium-dependent inactivation of NMDA receptors by Ca^{2+}/CaM is mediated by dimerization of NR1 C-terminal whereby a single Ca^{2+}/CaM binds to two C-termini simultaneously (Wang et al., 2008). Ca^{2+}/CaM thus plays an important role in regulating NMDA receptor activity.

1.3 ATP-binding cassette efflux transporters

Recent evidence suggests that myosin RLC interacts with the linker domain of some ATP-binding cassette (ABC) drug efflux transporters such as bile salt export protein (BSEP), multi-drug resistance (MDR) 1 and MDR2 in

polarized cells (Chan et al., 2005). Chan et al. have also reported that myosin RLC is important for trafficking of BSEP in hepatocytes where it is responsible for the transport of many bile acids (Chan et al., 2005; Gerloff et al., 1998). The identification of myosin RLC as a binding partner of BSEP, MDR1, and MDR2 adds to the list of direct binding partners of light chain. MDR1 is considered a classic ABC drug efflux transporter and expressed in a polarized manner and is located on the apical surface of intestinal epithelial cells and on the luminal side of capillaries in blood brain barrier (BBB). In these polarized cells MDR1 is involved in the physiological redistribution of drugs and other endogenous substances, delivering them out of the cells. Thus the apical distribution and trafficking of MDR1 and other efflux transporters to the plasma membrane is critical.

ABC transporters are efflux transporters that are responsible for the transport of harmful metabolites, endogenous molecules and toxic substances (Loscher and Potschka, 2005; Marchi et al., 2004; Sisodiya and Thom, 2003). These membrane-bound proteins use the energy of ATP hydrolysis for unidirectional transport of molecules across the cell membrane (Gottesman and Pastan, 1993; Sauna and Ambudkar, 2001). Forty-nine ABC-transporter genes have been identified in humans and are classified into 7 subfamilies: ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG. The ABCB subfamily (or MDR-ABC transporters) consists of 11 members, ABCB1 through ABCB11. ABCB1, or MDR1 or P-glycoprotein (Pgp), and ABCB11, or BSEP, are the largest proteins consisting of 1280 amino acids (Dean and Allikmets, 2001; Dean et al., 2001). Both proteins contain two homologous halves each consisting of six transmembrane domains linked by a cytoplasmically located linker region (Chen et al., 1986; Germann, 1996; Hrycyna et al., 1998; Rao et al., 2006). MDR1 is responsible for the efflux of many clinically important drugs, other endogenous and exogenous toxic substances in the BBB, and enterocytes (Potschka et al., 2001; Potschka and Loscher, 2001; Sisodiya et

al., 2002). MDR1 is expressed in brain, intestinal epithelium, liver, kidney, and colon (Fojo et al., 1987; Thiebaut et al., 1987).

Targeting of ABC transporters to the apical membrane in polarized cells is tightly regulated and involves many protein components through different trafficking pathways (Wakabayashi et al., 2006). The linker region of MDR1 transporters contain approximately 75 amino acids and various studies have shown that this region is important for cell surface expression (Chan et al., 2005; Hrycyna et al., 1998; Kolling and Losko, 1997). This region has been shown to interact directly with several intracellular, cytoskeletal, regulatory, and motor proteins in the cell thereby supporting the important role of the linker region in cellular distribution of MDR1 (Chan et al., 2005; Georges, 2007; Ortiz et al., 2004; Wakabayashi et al., 2006).

.1.4 Summary

Proteins that interact with the C-terminus of NMDA receptor subunits are important for regulation and function of the receptor. Previously, it was shown that the actin-binding proteins, α-actinin and spectrin, bind to the NMDA receptor carboxy terminal (Wechsler and Teichberg, 1998; Wyszynski et al., 1998). This interaction was believed to be significant as NMDA receptor function was previously known to be regulated by the polymerization state of the actin cytoskeleton in dendritic spines (Rosenmund and Westbrook, 1993; Wechsler and Teichberg, 1998; Wyszynski et al., 1997). Although α-actinin-2 and spectrin are physically associated with NMDA receptors, it is still not clear how the actin cytoskeleton regulates the trafficking and/or function of NMDA receptors.

Actin-based myosin motors are also present in the dendritic spines (Husi et al., 2000; Ryu et al., 2006). When I joined the Ishmael laboratory a myosin RLC had been identified as a direct binding partner of NR1 C-terminal. This myosin RLC colocalized with the NR1 subunit in dendritic spines of

isolated hippocampal neurons (Amparan et al., 2005). My thesis work began in 2003, when we showed that the interaction of myosin RLC is not limited to the NR1 C-terminal of NMDA receptors, rather myosin RLC is also a binding partner of NR2A and NR2B C-termini Fig 1.9 (Amparan et al., 2005). In Chapter 1, we characterized the interaction of myosin RLC with NMDA receptor subunits focusing on the NR2A subunit which was not considered a target of calmodulin. We also analyzed if myosin RLC interacts with the C-termini of NMDA receptors in the context of the myosin II complex and whether this can form a bridge to link myosin heavy chain to a non-myosin protein to form a ternary complex. The aim was to study how myosin RLC can act as an important regulatory component that may couple NMDA receptors to the actin cytoskeleton to regulate their trafficking and/or function. Various biochemical studies, fluorescence spectroscopy were the main techniques that were used to determine the interaction of myosin RLC with NR2A subunit of NMDA receptor. Transient expression in mammalian cells and immunocytochemistry were performed to determine the functional role of this interaction in trafficking of NMDA receptors.

In 2005, ABC transporters were found to be direct binding partners of myosin RLC (Chan et al., 2005). Myosin RLC is required for trafficking of newly synthesized BSEP to the apical membrane of hepatocytes and is important for the release of BSEP vesicles from the trans-Golgi network (TGN) of hepatocytes (Chan et al., 2005; Wakabayashi et al., 2006). In Chapter 2, we studied the role of myosin RLC in regulation and function of MDR1, a related ABC-transporter, using MDCKII-MDR1 cells as a model system. The transport characteristics of MDR1 were analyzed by using [^{3}H]-digoxin which is a well known MDR1 substrate. We also analyzed the effect of myosin RLC phosphorylation on the trafficking and function of MDR1. This study will provide an insight into the influence of a myosin II motor on MDR1 cell surface expression and function, and thus contribute to our understanding of trafficking mechanisms of myosin RLC-associated proteins. Biochemical studies were

used to determine the interaction of myosin RLC with MDR1-linker region and NR1C-terminal. Role of phosphorylation on function and apical expression of MDR1 was determined using transepithelial transport studies.

Calmodulin is another EF-hand protein which is an important regulator of NMDA receptor function (Ehlers et al., 1996; Wang et al., 2008; Zhang et al., 1998). NMDA receptors are permeable to Ca^{2+} and under normal conditions Ca^{2+} influx through NMDA receptors reduces the activity of NMDA receptors by a process called Ca^{2+}-dependent inactivation (Ehlers et al., 1996). This Ca^{2+}-dependent inactivation of NMDA receptors is mediated by CaM by binding to the NR1 subunit of NMDA receptors but it was also shown that Ca^{2+}-dependent inactivation of NMDA receptors is also NR2 subunit specific (Ehlers et al., 1996; Krupp et al., 2002; Zhang et al., 1998). In Chapter 3, we found that CaM also interacts with the NR2A subunit but not NR2B subunit of NMDA receptors in a Ca^{2+}-dependent manner. Upon analysis we found six putative CaM binding sites within the NR2A C-terminal within the region of NR2A (amino acids 875-1029). We believe this finding is significant as CaM is currently considered only as an NR1-interacting protein. In chapter 3, we used a proteomics approach to determine the interaction of CaM with NR2A C-terminal and site directed mutagenesis was used to analyze the putative CaM binding sites in NR2A C-terminal.

Taken together, the following study will provide an insight to the role of two distinct EF-hand proteins that can interact and regulate the trafficking and function of the NMDA receptor and other proteins, like MDR1, that are directly associated with them.

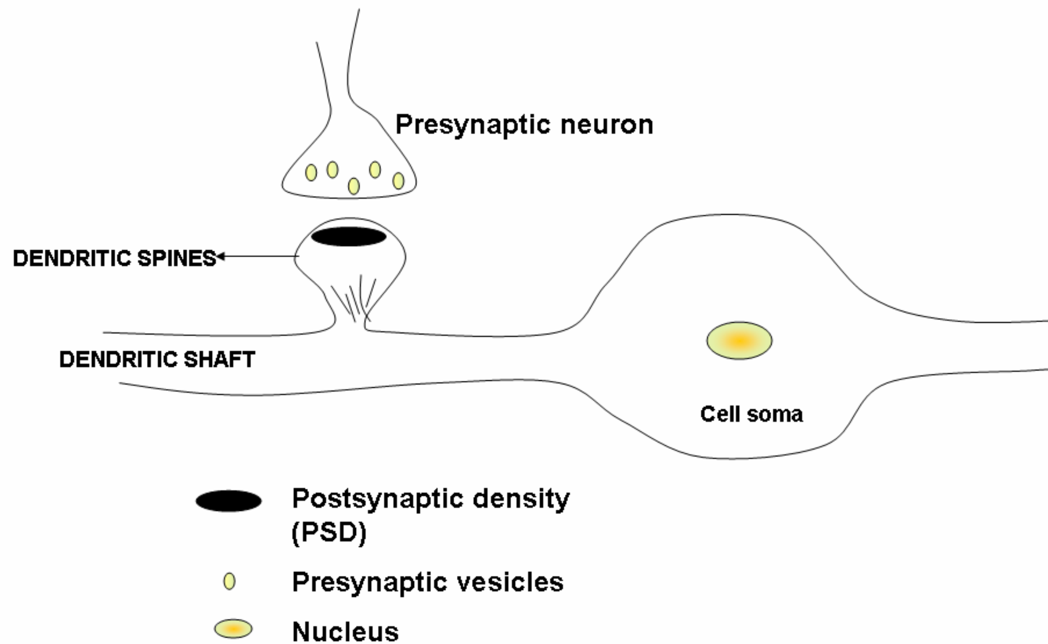
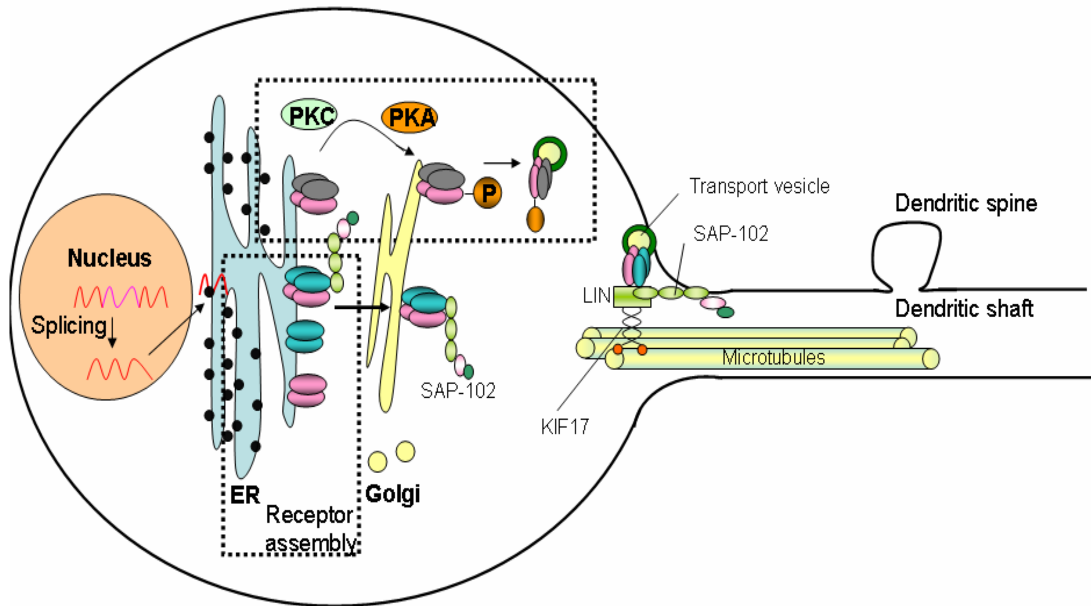


Figure 1.1 Schematic representation of an excitatory synapse located on a dendritic spine. Dendritic spines are small membranous protrusions from the surfaces of the dendritic processes of neurons that contain postsynaptic densities (PSD) and other membrane bound organelles. The PSD is very dense and can be visualized by electron microscopy due to the presence of membrane associated protein complexes.

A)



B)

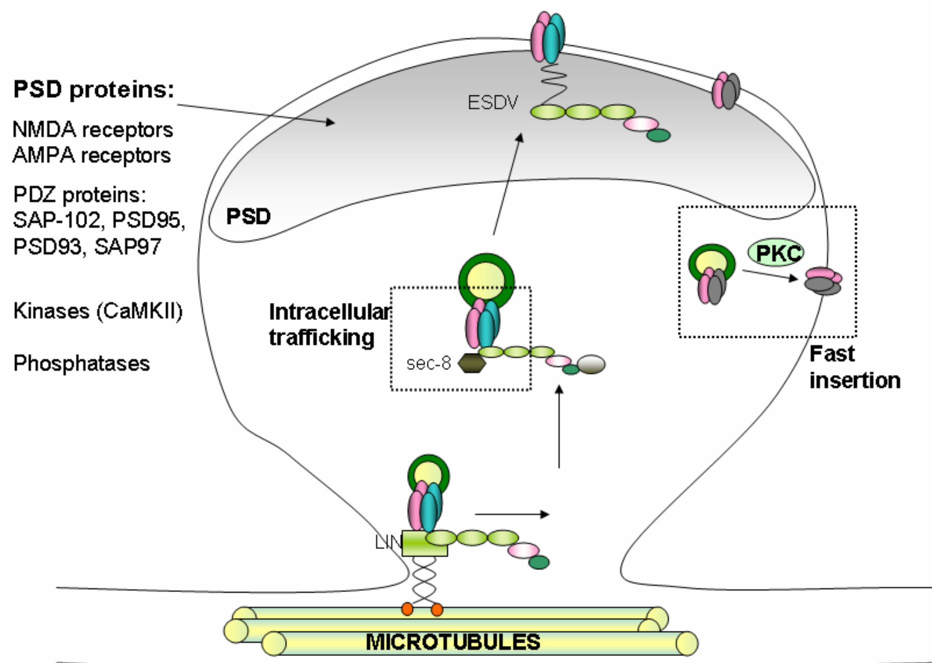


Figure 1.2 Schematic representation of the organization of the NMDA receptor signaling complex in a A) neuronal cell, B) dendritic spine. A) NMDA receptors are assembled into tetramers in the endoplasmic reticulum (ER) of a neuronal cell and proceed to the Golgi network. NMDA receptor transport packets are transported along microtubules from the cell body to synaptic sites in association with synaptic scaffolding proteins (such as PSD-95, SAP-102). SAP-102 links the NR2B subunit to SEC8, a component of exocyst complex, and promotes the delivery of NMDA receptors to the dendritic spines. Kinesin KIF-17 interacts with NR2B subunit through adaptor proteins LIN2, LIN7 and LIN10. KIF17 along with NR2B receptor cargo moves along the microtubules in the dendrites shaft to the synaptic site. Protein kinase C (PKC) and PKA regulates the NMDA receptor trafficking by phosphorylating NR1 and promoting ER export of the NMDA receptors to the plasma membrane. **B)** The postsynaptic dendritic spine contains a specialized structure called the postsynaptic density (PSD) that contains a dense network of proteins. NMDA and AMPA receptors in the PSD associate with scaffold proteins, signaling enzymes like kinases and phosphatases, adhesion molecules, actin binding proteins that regulate their trafficking and function. It is still unknown how receptor cargo from dendritic shaft is transported to the postsynaptic membrane. Adapted from (Lau and Zukin, 2007).

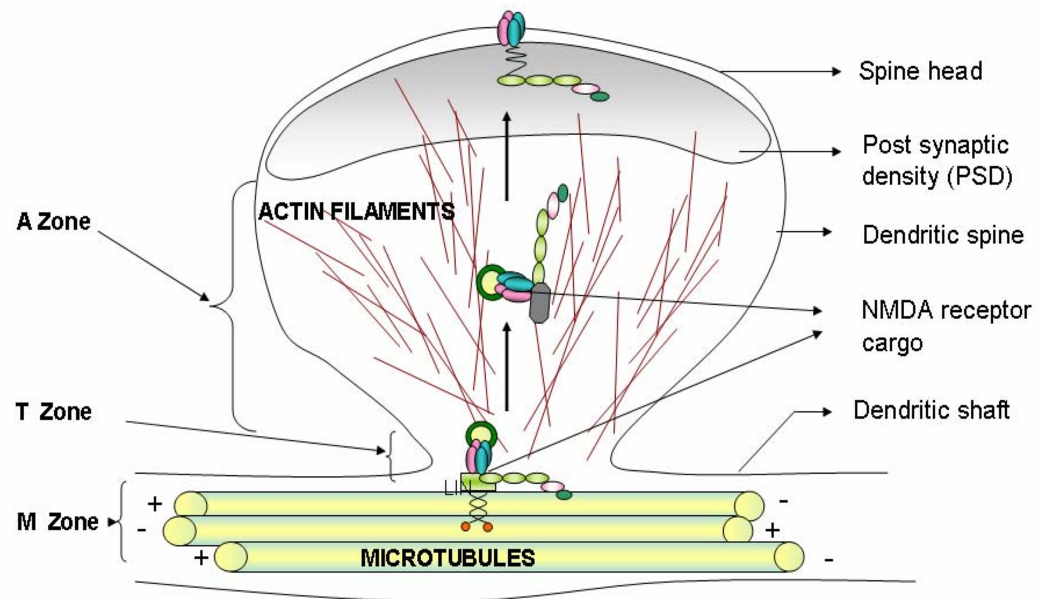


Figure 1.3 Schematic representation of cytoskeletal arrangement in the dendritic shaft and spine.

The cytoskeleton of dendritic spines is composed of actin filaments (red lines) that are extended into the postsynaptic density (PSD). The cytoskeleton in the dendritic shaft consists predominantly of microtubules. The distribution of cytoskeletal filaments in dendrites demarcates three cytoplasmic zones: an M-zone in the dendrite shaft, where microtubules predominate; an A-zone in the dendritic spine, where actin filaments predominate, and a T, or transition, zone. Adapted from (Kaech et al., 2001)

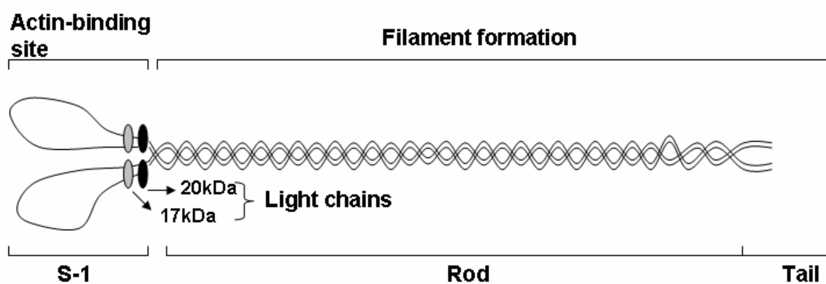


Figure 1.4 Schematic representation of non-muscle myosin II. Myosin II is a hexamer composed of two heavy chains, two 17kDa essential light chains (grey) and two 20kDa regulatory light chains (black). Each heavy chain is composed of a globular amino terminal head containing the ATP and actin binding domains required for motor activity, an intermediate domain that dimerizes to form α-helical coiled coil rod, and a non-helical tail. Adapted from (Conti and Adelstein, 2008)

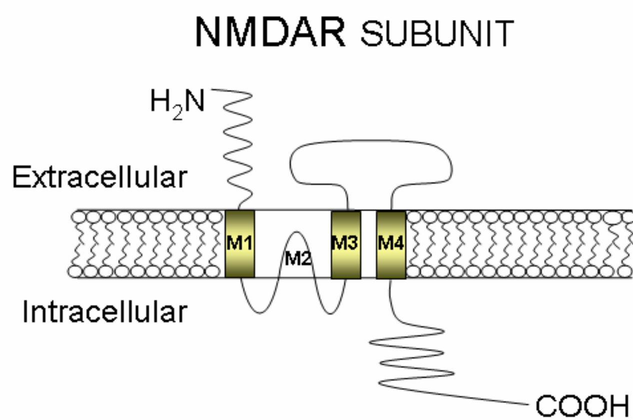
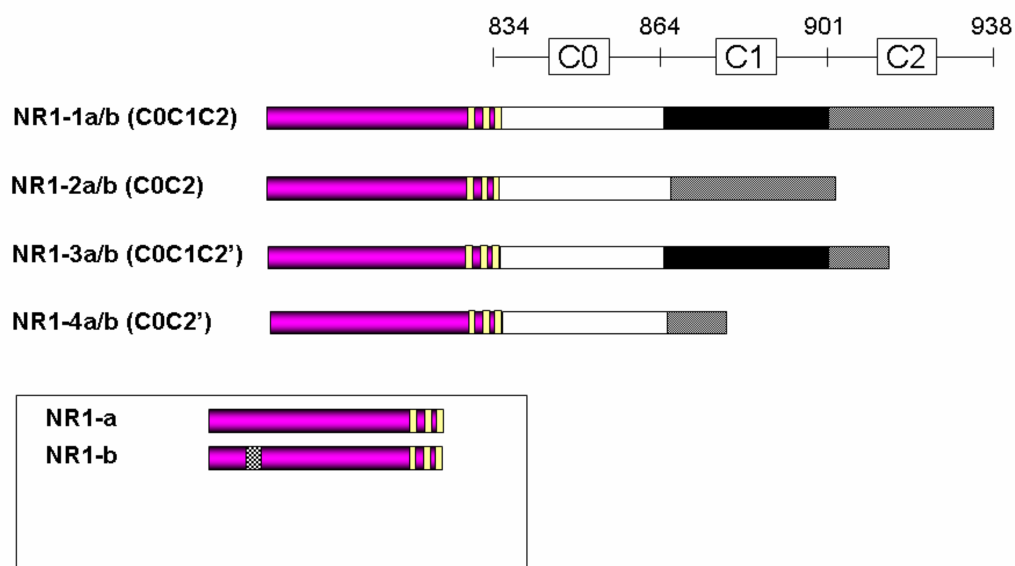


Figure 1.5 Schematic representation of the membrane topology of NMDA receptor subunits. Each subunit consists of an extracellular N-terminal domain with three transmembrane regions (M1, M3, and M4) and one re-entrant loop (M2) that form the channel pore, and an intracellular C-terminal.

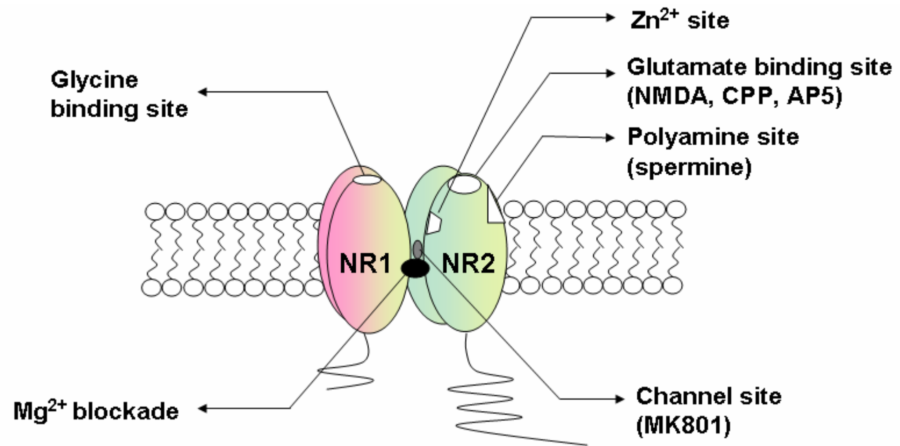
A)



B)

Figure 1.6 Schematic representations of A) NR1 splice-variants and B) NR2A and NR2B subunits. A) NR1 subunit gene undergoes extensive splicing to yield eight different spice variants NR1-1a/b to NR1-4a/b. NR1 'b' forms contain an additional exon, exon 5, in the N-terminal region. Splice variants NR1-1 and NR1-3 both contain C1 cassette. All splice variants contain C0 cassette. C2 cassette in splice variants NR1-3 and NR1-4 is replaced by C2'. **B)** C-terminal tails of the NR2A and NR2B subunit are about 6 times longer than the NR1 subunit. The amino acid numbers shown in the figure are representative of rat NR1 and NR2 subunits.

A)



B)

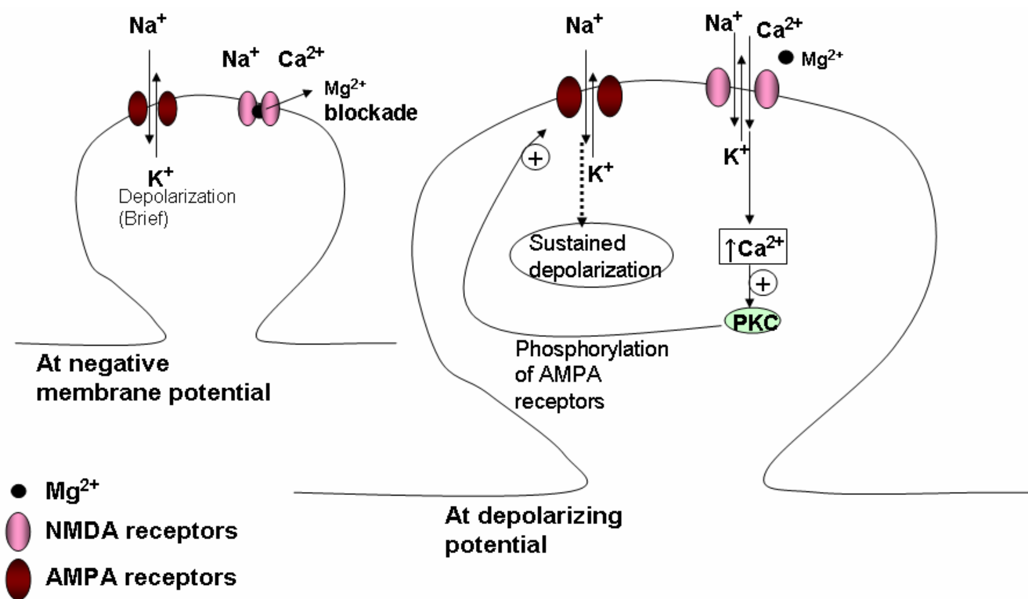


Figure 1.7 Schematic representation of NMDA receptor model illustrating: A) important modulatory sites B) excitatory synaptic transmission. The NMDA receptor is one of the main mediators of excitatory neurotransmission. The binding of both glutamate and glycine activates this receptor. The NMDA receptor can be modulated by a number of antagonists, including competitive antagonists at the glutamate binding sites and non-competitive NMDA receptor channel blockers. Since NMDA receptor activation is dependent on the binding of both glycine and glutamate, the binding of an antagonist at either of these sites prevents activation and opening of the channel. The receptor is a ligand gated ion channel, which permits the movement of calcium, sodium and potassium across the post-synaptic membrane. While AMPA receptors contribute with cationic conductance at negative membrane potentials, NMDA receptors exhibit a voltage dependent block by Mg^{2+}. However repetitive stimuli can cause sufficient depolarization event that removes the magnesium blockade allowing calcium ions to enter the dendritic spine. At depolarized potentials synaptic NMDA receptors also contribute to the conductance after removal of Mg^{2+} blockade leading to increased permeability of Ca^{2+} ions which further activates post synaptic events.

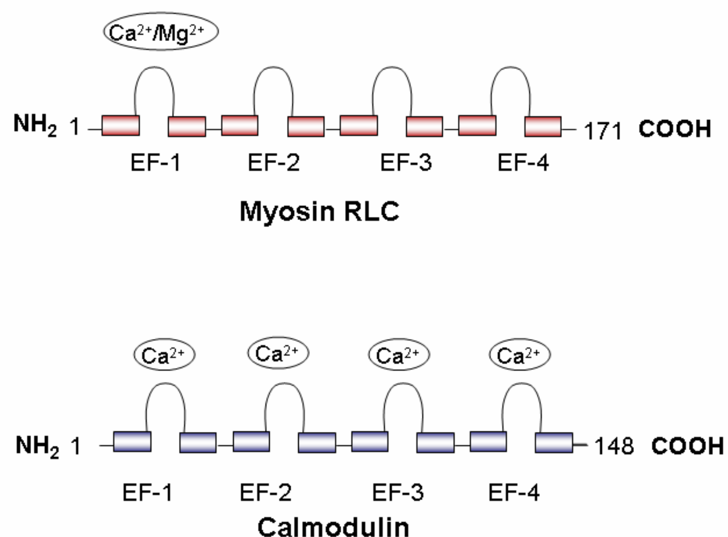


Figure 1.8 Schematic representation of the secondary structure of myosin RLC and calmodulin. Myosin RLC and calmodulin are members of the family of EF-hand calcium binding proteins. Myosin RLCs differ from calmodulin in that they have only one functional EF-hand domain that is thought to bind magnesium rather than calcium. An EF-hand is about thirty amino acids long and consists of an alpha-helix (E), loop region, and a second alpha helix (F).

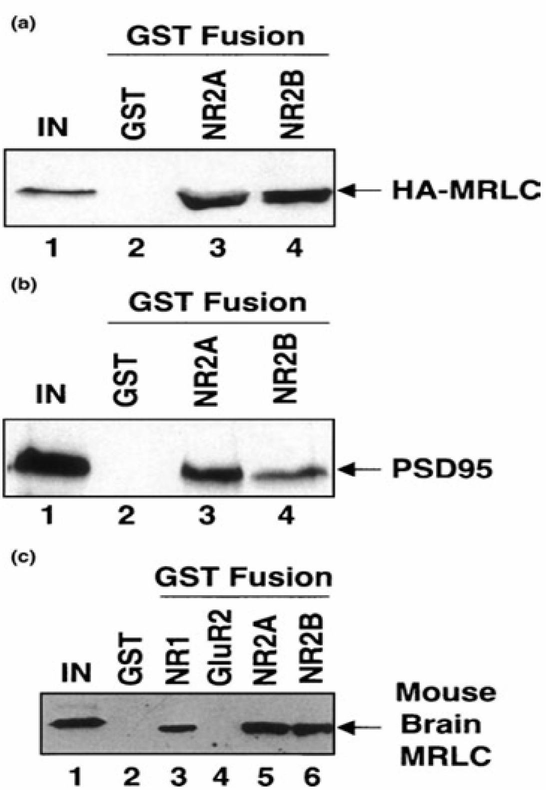

(a)
GST Fusion
IN
GST
NR2A
NR2B
HA-MRLC
1
2
3
4
(b)
GST Fusion
IN
GST
NR2A
NR2B
PSD95
1
2
3
4
(c)
GST Fusion
IN
GST
NR1
GluR2
NR2A
NR2B
Mouse
Brain
MRLC
1
2
3
4
5
6

Figure 1.9 Myosin RLC interacts with both the NR2A and NR2B subunits of the NMDA receptor. (a) Protein–protein interactions between the carboxy tails of NR2A and NR2B NMDA receptor subunits and purified recombinant myosin RLC (HA-MRLC). (b) Protein–protein interactions between the carboxy tails of NR2A and NR2B NMDA receptor subunits and in vitro translated [^{35}S]methionine-labelled PSD-95, a known NR2-interacting protein. (c) The carboxy tails of NR2A and NR2B 'pull out' native myosin RLC (MRLC) from a mouse forebrain extract. For in vitro studies assays were initiated by the addition of purified recombinant HA-MRLC (a) or in vitro translated [^{35}S]methionine-labelled PSD-95 (b) to glutathione–Sepharose beads bound with GST fusion protein or GST alone, and incubated with rotation for 1 h at 4°C. For studies with mouse brain extracts (c), assays were initiated by the addition of soluble brain extract protein (1 mg) and incubated with rotation overnight at 4°C. Immune complexes were detected using an anti-HA antibody (Santa Cruz) to identify recombinant myosin RLC (a), autoradiography to detect in vitro translated [^{35}S]methionine-labelled PSD-95 (b) or an anti-myosin RLC antibody (Amparan and Ishmael unpublished results) to detect native myosin RLC (c). Autoradiographs are representative of four independent experiments. (Reprinted from J. Neurochem. (2005) 92, 349–361; Amparan D, Avram D., Thomas CG, Lindahl MG, Yang J, Bajaj G, and Ishmael JE., Direct interaction of myosin regulatory light chain with the NMDA receptor, with permission from Wiley-Blackwell Publishing).

Myosins	Distinguish characteristics	Function and characteristic feature
Myosin I	First unconventi-onal myosin to be discovered in in *Acanthamoeba* and *Dictyostelium*	Single headed myosin, unable to self-associate into bipolar filaments. Important in the endocytic pathway and can be localized to specialized compartments such as the recycling endosome or lysosome (Barylko et al., 2000).
Myosin II (conventi-onal myosins)	At least one myosin II gene has been identified in all eukaryotic cells except those of plants.	Myosin II is a hexamer with two heavy chains and two pairs of light chains called the essential (ELC) and regulatory light chains (RLC). Myosin II is required for cytokinesis, cell motility, cell polarity/chemotaxis, maintaining cell architecture and development in non-muscle cells. (Sellers, 1999; Sellers, 2000)
Myosin III	Named ninaC (neither inactivation nor after potential C) and was first discovered in Drosophila eye	Monomeric and differs from all other myosins in having an N-terminal kinase domain. Important for localization, termination of photo-transduction and rhabdomere maintenance (Montell and Rubin, 1988).
Myosin IV	Identified in *Acanthamoeba*	Contains a tail with a myosin tail homology Predicted to have a single motor domain, one IQ motif. (Horowitz and Hammer, 1990)
Myosin V	Present in most eukaryotes excluding plants.	Dimeric molecule consisting of conserved motor domains followed by six IQ motifs which bind specific light chains and calmodulin. (Reck-Peterson et al., 2000)
Myosin VI, VII and XV	Myosin VI was first identified in *Drosophila* and subsequently in most animal tissue Myosin VII: in *Drosophila*, *C.elegans*, pig, mouse and humans. Myosin XV has been identified in humans and mice.	These three classes of myosins are associated with genetic deafness disorders in mammals. Mutations in these myosins result in abnormalities in the stereocilia in the sensory cells (Hasson et al., 1997; Hasson and Mooseker, 1997; Sellers, 2000)

Myosins	Distinguish characteristics	Function and characteristic feature
Myosin IX	Identified in rat, human and *C. elegans*	Acts as a negative regulator of Rho kinase (Reinhard et al., 1995)
Myosin X	First identified in frog inner ear and since then in bovine tissue	Three IQ motifs following the motor domain and a tail containing a region of coiled coil, three pleckstrin homology (PH) domains. Function: unknown
Myosin XII	Identified in *C. elegans*	Least conserved myosin known and contains a large tail region with two MyTH4 domains and a short region of coiled coil (Baker and Titus, 1997).
Myosin XIV	Identified in the parasites *Toxoplasma gondii* and *Plasmodium falciparum* (malaria parasite)	Simplest myosins known containing a motor domain, no classic IQ motif and variable length tails. (Hettmann et al., 2000).
Myosin XVI	Identified in humans and mouse.	unknown
Myosin XVIII	Found in Humans	Contains a PDZ-domain and identified from bone marrow stromal cells. Also called Myo18A (Furusawa et al., 2000).

Table 1.1 The myosin superfamily. Approximately 140 members of the myosin superfamily have been grouped into 18 classes based on the sequence of their head domains. The databases now contain complete DNA sequences of approximately 145 different myosin heavy chains from about 40 different species (J. Cope and T. Hodge The Myosin Home Page, http://www.mrclmb.cam.ac.uk/myosin/myos.html,). Traditionally, and largely as a result of extensive biochemical studies of the conventional (class II) myosin, its catalytic activity and regulation have been attributed solely to the heavy chain head domain and light chains associated with the neck domain (Berg et al., 2000; Korn, 2000; Sellers, 2000; Yamashita et al., 2000).

Myosin II (Conventional myosins)	
Vertebrate	
Skeletal muscle myosin Cardiac muscle myosins	Regulated by calcium troponin/tropomyosin complex (Farah and Reinach, 1995; Weber and Murray, 1973)
Smooth muscle Non-muscle myosins	Regulated by phosphorylation of the myosin RLC by a calcium/calmodulin dependent light chain kinase (Bresnick, 1999; Tan et al., 1992)
Non- vertebrate	
Dictyostelium /Acanthamoeba type myosins	Regulated by phosphorylation at distinct sites in their heavy chain and light chain (Tan et al., 1992)

Table 1.2 The myosin II subfamily. The myosin II motors are the conventional myosins and can be divided into three groups or types: 1) skeletal/cardiac muscle (sarcomeric) myosins; 2) Smooth muscle/nonmuscle myosins; 3) *Dictyostelium* /*Acanthamoeba* type myosins. The characteristic feature of these myosins is their helical coiled coil tails that self assemble to form a variety of filament structures (bipolar or side polar filaments) that are essential for their function.

References

Allison DW, Chervin AS, Gelfand VI and Craig AM (2000) Postsynaptic scaffolds of excitatory and inhibitory synapses in hippocampal neurons: maintenance of core components independent of actin filaments and microtubules. *J Neurosci* **20**(12):4545-4554.

Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y and Kaibuchi K (1996) Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *The Journal of biological chemistry* **271**(34):20246-20249.

Amparan D, Avram D, Thomas CG, Lindahl MG, Yang J, Bajaj G and Ishmael JE (2005) Direct interaction of myosin regulatory light chain with the NMDA receptor. *Journal of neurochemistry* **92**(2):349-361.

Aprison MH and Werman R (1965) The distribution of glycine in cat spinal cord and roots. *Life sciences* **4**(21):2075-2083.

Ataman ZA, Gakhar L, Sorensen BR, Hell JW and Shea MA (2007) The NMDA receptor NR1 C1 region bound to calmodulin: structural insights into functional differences between homologous domains. *Structure* **15**(12):1603-1617.

Bahler M and Rhoads A (2002) Calmodulin signaling via the IQ motif. *FEBS letters* **513**(1):107-113.

Bahloul A, Chevreux G, Wells AL, Martin D, Nolt J, Yang Z, Chen LQ, Potier N, Van Dorsselaer A, Rosenfeld S, Houdusse A and Sweeney HL (2004) The unique insert in myosin VI is a structural calcium-calmodulin binding site. *Proceedings of the National Academy of Sciences of the United States of America* **101**(14):4787-4792.

Baker JP and Titus MA (1997) A family of unconventional myosins from the nematode Caenorhabditis elegans. *Journal of molecular biology* **272**(4):523-535.

Barylko B, Binns DD and Albanesi JP (2000) Regulation of the enzymatic and motor activities of myosin I. *Biochimica et biophysica acta* **1496**(1):23-35.

Baudier J, Deloulme JC, Van Dorsselaer A, Black D and Matthes HW (1991) Purification and characterization of a brain-specific protein kinase C substrate, neurogranin (p17). Identification of a consensus amino acid sequence between neurogranin and neuromodulin (GAP43) that corresponds to the protein kinase C phosphorylation site and the calmodulin-binding domain. *The Journal of biological chemistry* **266**(1):229-237.

Bennett JA and Dingledine R (1995) Topology profile for a glutamate receptor: three transmembrane domains and a channel-lining reentrant membrane loop. *Neuron* **14**(2):373-384.

Benveniste M and Mayer ML (1991) Kinetic analysis of antagonist action at N-methyl-D-aspartic acid receptors. Two binding sites each for glutamate and glycine. *Biophysical journal* **59**(3):560-573.

Berg JS, Derfler BH, Pennisi CM, Corey DP and Cheney RE (2000) Myosin-X, a novel myosin with pleckstrin homology domains, associates with regions of dynamic actin. *Journal of cell science* **113 Pt 19**:3439-3451.

Blackstone CD, Moss SJ, Martin LJ, Levey AI, Price DL and Huganir RL (1992) Biochemical characterization and localization of a non-N-methyl-D-aspartate glutamate receptor in rat brain. *Journal of neurochemistry* **58**(3):1118-1126.

Blanpied TA, Scott DB and Ehlers MD (2002) Dynamics and regulation of clathrin coats at specialized endocytic zones of dendrites and spines. *Neuron* **36**(3):435-449.

Bliss TV and Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**(6407):31-39.

Block SM (1996) Fifty ways to love your lever: myosin motors. *Cell* **87**(2):151-157.

Brady ST, Lasek RJ and Allen RD (1985) Video microscopy of fast axonal transport in extruded axoplasm: a new model for study of molecular mechanisms. *Cell motility* **5**(2):81-101.

Bresnick AR (1999) Molecular mechanisms of nonmuscle myosin-II regulation. *Current opinion in cell biology* **11**(1):26-33.

Brose N, Gasic GP, Vetter DE, Sullivan JM and Heinemann SF (1993) Protein chemical characterization and immunocytochemical localization of the NMDA receptor subunit NMDA R1. *The Journal of biological chemistry* **268**(30):22663-22671.

Carafoli E and Klee CB (1999) *Calcium as a cellular regulator*. Oxford University Press, New York.

Celio MR, Pauls TL and Schwaller B (1996) *Guidebook to the calcium-binding proteins*. Sambrook & Tooze Publication at Oxford University Press, Oxford.

Chan W, Calderon G, Swift AL, Moseley J, Li S, Hosoya H, Arias IM and Ortiz DF (2005) Myosin II regulatory light chain is required for trafficking of bile salt export protein to the apical membrane in Madin-Darby canine kidney cells. *The Journal of biological chemistry* **280**(25):23741-23747.

Chatterton JE, Awobuluyi M, Premkumar LS, Takahashi H, Talantova M, Shin Y, Cui J, Tu S, Sevarino KA, Nakanishi N, Tong G, Lipton SA and Zhang D (2002) Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. *Nature* **415**(6873):793-798.

Chazot PL and Stephenson FA (1997) Molecular dissection of native mammalian forebrain NMDA receptors containing the NR1 C2 exon: direct demonstration of NMDA receptors comprising NR1, NR2A, and NR2B subunits within the same complex. *Journal of neurochemistry* **69**(5):2138-2144.

Chen BS and Roche KW (2007) Regulation of NMDA receptors by phosphorylation. *Neuropharmacology* **53**(3):362-368.

Chen CJ, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM and Roninson IB (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47**(3):381-389.

Cheney RE and Mooseker MS (1992) Unconventional myosins. *Current opinion in cell biology* **4**(1):27-35.

Cheney RE, O'Shea MK, Heuser JE, Coelho MV, Wolenski JS, Espreafico EM, Forscher P, Larson RE and Mooseker MS (1993) Brain myosin-V is a two-headed unconventional myosin with motor activity. *Cell* **75**(1):13-23.

Cheng D, Hoogenraad CC, Rush J, Ramm E, Schlager MA, Duong DM, Xu P, Wijayawardana SR, Hanfelt J, Nakagawa T, Sheng M and Peng J (2006) Relative and absolute quantification of postsynaptic density proteome isolated from rat forebrain and cerebellum. *Mol Cell Proteomics* **5**(6):1158-1170.

Choi DW (1995) Calcium: still center-stage in hypoxic-ischemic neuronal death. *Trends Neurosci* **18**(2):58-60.

Choi DW, Koh JY and Peters S (1988) Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. *J Neurosci* **8**(1):185-196.

Choi YB and Lipton SA (1999) Identification and mechanism of action of two histidine residues underlying high-affinity Zn2+ inhibition of the NMDA receptor. *Neuron* **23**(1):171-180.

Chung HJ, Huang YH, Lau LF and Huganir RL (2004) Regulation of the NMDA receptor complex and trafficking by activity-dependent phosphorylation of the NR2B subunit PDZ ligand. *J Neurosci* **24**(45):10248-10259.

Ciabarra AM, Sullivan JM, Gahn LG, Pecht G, Heinemann S and Sevarino KA (1995) Cloning and characterization of chi-1: a developmentally regulated member of a novel class of the ionotropic glutamate receptor family. *J Neurosci* **15**(10):6498-6508.

Clements JD and Westbrook GL (1991) Activation kinetics reveal the number of glutamate and glycine binding sites on the N-methyl-D-aspartate receptor. *Neuron* **7**(4):605-613.

Collingridge GL and Watkins JC (1994) *The NMDA receptor*. Oxford University Press, Oxford ; New York.

Collins MO, Husi H, Yu L, Brandon JM, Anderson CN, Blackstock WP, Choudhary JS and Grant SG (2006) Molecular characterization and comparison of the components and multiprotein complexes in the postsynaptic proteome. *Journal of neurochemistry* **97 Suppl 1**:16-23.

Condeelis JS and Taylor DL (1977) The contractile basis of amoeboid movement. V. The control of gelation, solation, and contraction in extracts from Dictyostelium discoideum. *The Journal of cell biology* **74**(3):901-927.

Constantine-Paton M, Cline HT and Debski E (1990) Patterned activity, synaptic convergence, and the NMDA receptor in developing visual pathways. *Annual review of neuroscience* **13**:129-154.

Conti MA and Adelstein RS (2008) Nonmuscle myosin II moves in new directions. *Journal of cell science* **121**(Pt 1):11-18.

Corsi M, Fina P and Trist DG (1996) Co-agonism in drug-receptor interaction: illustrated by the NMDA receptors. *Trends in pharmacological sciences* **17**(6):220-222.

Cramer LP (1999) Organization and polarity of actin filament networks in cells: implications for the mechanism of myosin-based cell motility. *Biochemical Society symposium* **65**:173-205.

Cull-Candy S, Brickley S and Farrant M (2001) NMDA receptor subunits: diversity, development and disease. *Current opinion in neurobiology* **11**(3):327-335.

Das S, Sasaki YF, Rothe T, Premkumar LS, Takasu M, Crandall JE, Dikkes P, Conner DA, Rayudu PV, Cheung W, Chen HS, Lipton SA and Nakanishi N (1998) Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A. *Nature* **393**(6683):377-381.

De Lozanne A and Spudich JA (1987) Disruption of the Dictyostelium myosin heavy chain gene by homologous recombination. *Science (New York, NY* **236**(4805):1086-1091.

Dean M and Allikmets R (2001) Complete characterization of the human ABC gene family. *Journal of bioenergetics and biomembranes* **33**(6):475-479.

Dean M, Rzhetsky A and Allikmets R (2001) The human ATP-binding cassette (ABC) transporter superfamily. *Genome research* **11**(7):1156-1166.

Dominguez R, Freyzon Y, Trybus KM and Cohen C (1998) Crystal structure of a vertebrate smooth muscle myosin motor domain and its complex with the essential light chain: visualization of the pre-power stroke state. *Cell* **94**(5):559-571.

Durand GM, Gregor P, Zheng X, Bennett MV, Uhl GR and Zukin RS (1992) Cloning of an apparent splice variant of the rat N-methyl-D-aspartate receptor NMDAR1 with altered sensitivity to polyamines and activators of protein kinase C. *Proceedings of the National Academy of Sciences of the United States of America* **89**(19):9359-9363.

Ehlers MD, Zhang S, Bernhadt JP and Huganir RL (1996) Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* **84**(5):745-755.

Espreafico EM, Cheney RE, Matteoli M, Nascimento AA, De Camilli PV, Larson RE and Mooseker MS (1992) Primary structure and cellular localization of chicken brain myosin-V (p190), an unconventional myosin with calmodulin light chains. *The Journal of cell biology* **119**(6):1541-1557.

Ethell IM and Pasquale EB (2005) Molecular mechanisms of dendritic spine development and remodeling. *Progress in neurobiology* **75**(3):161-205.

Farah CS and Reinach FC (1995) The troponin complex and regulation of muscle contraction. *Faseb J* **9**(9):755-767.

Feinstein DL, Durand M and Milner RJ (1991) Expression of myosin regulatory light chains in rat brain: characterization of a novel isoform. *Brain Res Mol Brain Res* **10**(2):97-105.

Finn BE, Evenas J, Drakenberg T, Waltho JP, Thulin E and Forsen S (1995) Calcium-induced structural changes and domain autonomy in calmodulin. *Nature structural biology* **2**(9):777-783.

Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman MM and Pastan I (1987) Expression of a multidrug-resistance gene in human tumors and tissues. *Proceedings of the National Academy of Sciences of the United States of America* **84**(1):265-269.

Furukawa H and Gouaux E (2003) Mechanisms of activation, inhibition and specificity: crystal structures of the NMDA receptor NR1 ligand-binding core. *The EMBO journal* **22**(12):2873-2885.

Furukawa H, Singh SK, Mancusso R and Gouaux E (2005) Subunit arrangement and function in NMDA receptors. *Nature* **438**(7065):185-192.

Furusawa T, Ikawa S, Yanai N and Obinata M (2000) Isolation of a novel PDZ-containing myosin from hematopoietic supportive bone marrow stromal cell lines. *Biochemical and biophysical research communications* **270**(1):67-75.

Gallagher PJ, Herring BP, Griffin SA and Stull JT (1991) Molecular characterization of a mammalian smooth muscle myosin light chain kinase. *The Journal of biological chemistry* **266**(35):23936-23944.

Garcia A, Coudrier E, Carboni J, Anderson J, Vandekerkhove J, Mooseker M, Louvard D and Arpin M (1989) Partial deduced sequence of the 110-kD-calmodulin complex of the avian intestinal microvillus shows that this mechanoenzyme is a member of the myosin I family. *The Journal of cell biology* **109**(6 Pt 1):2895-2903.

Gardoni F, Bellone C, Cattabeni F and Di Luca M (2001) Protein kinase C activation modulates alpha-calmodulin kinase II binding to NR2A subunit of N-methyl-D-aspartate receptor complex. *The Journal of biological chemistry* **276**(10):7609-7613.

Geiser JR, van Tuinen D, Brockerhoff SE, Neff MM and Davis TN (1991) Can calmodulin function without binding calcium? *Cell* **65**(6):949-959.

Georges E (2007) The P-glycoprotein (ABCB1) linker domain encodes high-affinity binding sequences to alpha- and beta-tubulins. *Biochemistry* **46**(25):7337-7342.

Gerges NZ, Backos DS and Esteban JA (2004) Local control of AMPA receptor trafficking at the postsynaptic terminal by a small GTPase of

the Rab family. *The Journal of biological chemistry* **279**(42):43870-43878.

Gerloff T, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, Hofmann AF and Meier PJ (1998) The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. *The Journal of biological chemistry* **273**(16):10046-10050.

Germann UA (1996) P-glycoprotein--a mediator of multidrug resistance in tumour cells. *Eur J Cancer* **32A**(6):927-944.

Golomb E, Ma X, Jana SS, Preston YA, Kawamoto S, Shoham NG, Goldin E, Conti MA, Sellers JR and Adelstein RS (2004) Identification and characterization of nonmuscle myosin II-C, a new member of the myosin II family. *The Journal of biological chemistry* **279**(4):2800-2808.

Gottesman MM and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annual review of biochemistry* **62**:385-427.

Grabarek Z (2006) Structural basis for diversity of the EF-hand calcium-binding proteins. *Journal of molecular biology* **359**(3):509-525.

Grant ER, Guttmann RP, Seifert KM and Lynch DR (2001) A region of the rat N-methyl-D-aspartate receptor 2A subunit that is sufficient for potentiation by phorbol esters. *Neuroscience letters* **310**(1):9-12.

Guillaud L, Setou M and Hirokawa N (2003) KIF17 dynamics and regulation of NR2B trafficking in hippocampal neurons. *J Neurosci* **23**(1):131-140.

Hallett PJ and Standaert DG (2004) Rationale for and use of NMDA receptor antagonists in Parkinson's disease. *Pharmacology & therapeutics* **102**(2):155-174.

Hasson T, Gillespie PG, Garcia JA, MacDonald RB, Zhao Y, Yee AG, Mooseker MS and Corey DP (1997) Unconventional myosins in inner-ear sensory epithelia. *The Journal of cell biology* **137**(6):1287-1307.

Hasson T and Mooseker MS (1997) The growing family of myosin motors and their role in neurons and sensory cells. *Current opinion in neurobiology* **7**(5):615-623.

Hawkins LM, Prybylowski K, Chang K, Moussan C, Stephenson FA and Wenthold RJ (2004) Export from the endoplasmic reticulum of assembled N-methyl-d-aspartic acid receptors is controlled by a motif in the c terminus of the NR2 subunit. *The Journal of biological chemistry* **279**(28):28903-28910.

Herm-Gotz A, Weiss S, Stratmann R, Fujita-Becker S, Ruff C, Meyhofer E, Soldati T, Manstein DJ, Geeves MA and Soldati D (2002) Toxoplasma gondii myosin A and its light chain: a fast, single-headed, plus-end-directed motor. *The EMBO journal* **21**(9):2149-2158.

Herrling PL (1997) *Excitatory amino acids : clinical results with antagonists*. Academic Press, San Diego.

Hettmann C, Herm A, Geiter A, Frank B, Schwarz E, Soldati T and Soldati D (2000) A dibasic motif in the tail of a class XIV apicomplexan myosin is

an essential determinant of plasma membrane localization. *Molecular biology of the cell* **11**(4):1385-1400.

Hokfelt T and Ljungdahl A (1971) Light and electron microscopic autoradiography on spinal cord slices after incubation with labeled glycine. *Brain research* **32**(1):189-194.

Horak M, Chang K and Wenthold RJ (2008) Masking of the endoplasmic reticulum retention signals during assembly of the NMDA receptor. *J Neurosci* **28**(13):3500-3509.

Horowitz JA and Hammer JA, 3rd (1990) A new Acanthamoeba myosin heavy chain. Cloning of the gene and immunological identification of the polypeptide. *The Journal of biological chemistry* **265**(33):20646-20652.

Horton AC and Ehlers MD (2003) Dual modes of endoplasmic reticulum-to-Golgi transport in dendrites revealed by live-cell imaging. *J Neurosci* **23**(15):6188-6199.

Horton AC and Ehlers MD (2004) Secretory trafficking in neuronal dendrites. *Nature cell biology* **6**(7):585-591.

Houdusse A, Silver M and Cohen C (1996) A model of Ca(2+)-free calmodulin binding to unconventional myosins reveals how calmodulin acts as a regulatory switch. *Structure* **4**(12):1475-1490.

Hrycyna CA, Airan LE, Germann UA, Ambudkar SV, Pastan I and Gottesman MM (1998) Structural flexibility of the linker region of human P-glycoprotein permits ATP hydrolysis and drug transport. *Biochemistry* **37**(39):13660-13673.

Huh KH and Wenthold RJ (1999) Turnover analysis of glutamate receptors identifies a rapidly degraded pool of the N-methyl-D-aspartate receptor subunit, NR1, in cultured cerebellar granule cells. *The Journal of biological chemistry* **274**(1):151-157.

Husi H and Grant SG (2001) Isolation of 2000-kDa complexes of N-methyl-D-aspartate receptor and postsynaptic density 95 from mouse brain. *Journal of neurochemistry* **77**(1):281-291.

Husi H, Ward MA, Choudhary JS, Blackstock WP and Grant SG (2000) Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. *Nature neuroscience* **3**(7):661-669.

Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M and et al. (1993) Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. *The Journal of biological chemistry* **268**(4):2836-2843.

Ito M, Nakano T, Erdodi F and Hartshorne DJ (2004) Myosin phosphatase: structure, regulation and function. *Molecular and cellular biochemistry* **259**(1-2):197-209.

Johnson JW and Ascher P (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* **325**(6104):529-531.

Jones ML and Leonard JP (2005) PKC site mutations reveal differential modulation by insulin of NMDA receptors containing NR2A or NR2B subunits. *Journal of neurochemistry* **92**(6):1431-1438.

Jordan BA, Fernholz BD, Boussac M, Xu C, Grigorean G, Ziff EB and Neubert TA (2004) Identification and verification of novel rodent postsynaptic density proteins. *Mol Cell Proteomics* **3**(9):857-871.

Kadotani H, Hirano T, Masugi M, Nakamura K, Nakao K, Katsuki M and Nakanishi S (1996) Motor discoordination results from combined gene disruption of the NMDA receptor NR2A and NR2C subunits, but not from single disruption of the NR2A or NR2C subunit. *J Neurosci* **16**(24):7859-7867.

Kaech S, Parmar H, Roelandse M, Bornmann C and Matus A (2001) Cytoskeletal microdifferentiation: a mechanism for organizing morphological plasticity in dendrites. *Proceedings of the National Academy of Sciences of the United States of America* **98**(13):7086-7092.

Karavanova I, Vasudevan K, Cheng J and Buonanno A (2007) Novel regional and developmental NMDA receptor expression patterns uncovered in NR2C subunit-beta-galactosidase knock-in mice. *Molecular and cellular neurosciences* **34**(3):468-480.

Karp SJ, Masu M, Eki T, Ozawa K and Nakanishi S (1993) Molecular cloning and chromosomal localization of the key subunit of the human N-methyl-D-aspartate receptor. *The Journal of biological chemistry* **268**(5):3728-3733.

Kawasaki H, Nakayama S and Kretsinger RH (1998) Classification and evolution of EF-hand proteins. *Biometals* **11**(4):277-295.

Kim E and Sheng M (2004) PDZ domain proteins of synapses. *Nature reviews* **5**(10):771-781.

Kolling R and Losko S (1997) The linker region of the ABC-transporter Ste6 mediates ubiquitination and fast turnover of the protein. *The EMBO journal* **16**(9):2251-2261.

Korn ED (2000) Coevolution of head, neck, and tail domains of myosin heavy chains. *Proceedings of the National Academy of Sciences of the United States of America* **97**(23):12559-12564.

Korn ED and Hammer JA, 3rd (1990) Myosin I. *Current opinion in cell biology* **2**(1):57-61.

Kornau HC, Schenker LT, Kennedy MB and Seeburg PH (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science (New York, NY* **269**(5231):1737-1740.

Kretsinger RH and Nockolds CE (1973) Carp muscle calcium-binding protein. II. Structure determination and general description. *The Journal of biological chemistry* **248**(9):3313-3326.

Krupp JJ, Vissel B, Thomas CG, Heinemann SF and Westbrook GL (2002) Calcineurin acts via the C-terminus of NR2A to modulate desensitization of NMDA receptors. *Neuropharmacology* **42**(5):593-602.

Kuffler SW and Edwards C (1958) Mechanism of gamma aminobutyric acid (GABA) action and its relation to synaptic inhibition. *Journal of neurophysiology* **21**(6):589-610.

Kuner T and Schoepfer R (1996) Multiple structural elements determine subunit specificity of Mg2+ block in NMDA receptor channels. *J Neurosci* **16**(11):3549-3558.

Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M and et al. (1992) Molecular diversity of the NMDA receptor channel. *Nature* **358**(6381):36-41.

Lau CG and Zukin RS (2007) NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. *Nature reviews* **8**(6):413-426.

Lavezzari G, McCallum J, Lee R and Roche KW (2003) Differential binding of the AP-2 adaptor complex and PSD-95 to the C-terminus of the NMDA receptor subunit NR2B regulates surface expression. *Neuropharmacology* **45**(6):729-737.

Lee HK (2006) Synaptic plasticity and phosphorylation. *Pharmacology & therapeutics* **112**(3):810-832.

Lee JM, Zipfel GJ and Choi DW (1999) The changing landscape of ischaemic brain injury mechanisms. *Nature* **399**(6738 Suppl):A7-14.

Li B, Chen N, Luo T, Otsu Y, Murphy TH and Raymond LA (2002) Differential regulation of synaptic and extra-synaptic NMDA receptors. *Nature neuroscience* **5**(9):833-834.

Liao GY, Wagner DA, Hsu MH and Leonard JP (2001) Evidence for direct protein kinase-C mediated modulation of N-methyl-D-aspartate receptor current. *Molecular pharmacology* **59**(5):960-964.

Loscher W and Potschka H (2005) Drug resistance in brain diseases and the role of drug efflux transporters. *Nature reviews* **6**(8):591-602.

Lu J, Helton TD, Blanpied TA, Racz B, Newpher TM, Weinberg RJ and Ehlers MD (2007) Postsynaptic positioning of endocytic zones and AMPA receptor cycling by physical coupling of dynamin-3 to Homer. *Neuron* **55**(6):874-889.

Ludowyke RI, Peleg I, Beaven MA and Adelstein RS (1989) Antigen-induced secretion of histamine and the phosphorylation of myosin by protein kinase C in rat basophilic leukemia cells. *The Journal of biological chemistry* **264**(21):12492-12501.

Maciver SK (1996) Myosin II function in non-muscle cells. *Bioessays* **18**(3):179-182.

Mammen AL, Kamboj S and Huganir RL (1999) Protein phosphorylation of ligand-gated ion channels. *Methods in enzymology* **294**:353-370.

Marchi N, Hallene KL, Kight KM, Cucullo L, Moddel G, Bingaman W, Dini G, Vezzani A and Janigro D (2004) Significance of MDR1 and multiple drug resistance in refractory human epileptic brain. *BMC medicine* **2**:37.

Mayer ML and Armstrong N (2004) Structure and function of glutamate receptor ion channels. *Annual review of physiology* **66**:161-181.

Mayer ML, Benveniste M, Patneau DK and Vyklicky L, Jr. (1992) Pharmacologic properties of NMDA receptors. *Annals of the New York Academy of Sciences* **648**:194-204.

Mayer ML and Westbrook GL (1987a) Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones. *The Journal of physiology* **394**:501-527.

Mayer ML and Westbrook GL (1987b) The physiology of excitatory amino acids in the vertebrate central nervous system. *Progress in neurobiology* **28**(3):197-276.

Mayer ML, Westbrook GL and Guthrie PB (1984) Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. *Nature* **309**(5965):261-263.

Meguro H, Mori H, Araki K, Kushiya E, Kutsuwada T, Yamazaki M, Kumanishi T, Arakawa M, Sakimura K and Mishina M (1992) Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. *Nature* **357**(6373):70-74.

Montell C and Rubin GM (1988) The Drosophila ninaC locus encodes two photoreceptor cell specific proteins with domains homologous to protein kinases and the myosin heavy chain head. *Cell* **52**(5):757-772.

Monyer H, Burnashev N, Laurie DJ, Sakmann B and Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12**(3):529-540.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B and Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science (New York, NY* **256**(5060):1217-1221.

Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N and Nakanishi S (1991) Molecular cloning and characterization of the rat NMDA receptor. *Nature* **354**(6348):31-37.

Mu Y, Otsuka T, Horton AC, Scott DB and Ehlers MD (2003) Activity-dependent mRNA splicing controls ER export and synaptic delivery of NMDA receptors. *Neuron* **40**(3):581-594.

Najm I, Ying Z, Babb T, Crino PB, Macdonald R, Mathern GW and Spreafico R (2004) Mechanisms of epileptogenicity in cortical dysplasias. *Neurology* **62**(6 Suppl 3):S9-13.

Nakanishi N, Axel R and Shneider NA (1992) Alternative splicing generates functionally distinct N-methyl-D-aspartate receptors. *Proceedings of the National Academy of Sciences of the United States of America* **89**(18):8552-8556.

Nicoll RA and Malenka RC (1995) Contrasting properties of two forms of long-term potentiation in the hippocampus. *Nature* **377**(6545):115-118.

Nusser Z, Lujan R, Laube G, Roberts JD, Molnar E and Somogyi P (1998) Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. *Neuron* **21**(3):545-559.

O'Neil KT and DeGrado WF (1990) How calmodulin binds its targets: sequence independent recognition of amphiphilic alpha-helices. *Trends in biochemical sciences* **15**(2):59-64.

Ortiz DF, Moseley J, Calderon G, Swift AL, Li S and Arias IM (2004) Identification of HAX-1 as a protein that binds bile salt export protein and regulates its abundance in the apical membrane of Madin-Darby canine kidney cells. *The Journal of biological chemistry* **279**(31):32761-32770.

Paoletti P, Vergnano AM, Barbour B and Casado M (2008) Zinc at glutamatergic synapses. *Neuroscience*.

Park M, Penick EC, Edwards JG, Kauer JA and Ehlers MD (2004) Recycling endosomes supply AMPA receptors for LTP. *Science (New York, NY* **305**(5692):1972-1975.

Peng J, Kim MJ, Cheng D, Duong DM, Gygi SP and Sheng M (2004) Semiquantitative proteomic analysis of rat forebrain postsynaptic density fractions by mass spectrometry. *The Journal of biological chemistry* **279**(20):21003-21011.

Perez-Otano I, Schulteis CT, Contractor A, Lipton SA, Trimmer JS, Sucher NJ and Heinemann SF (2001) Assembly with the NR1 subunit is required for surface expression of NR3A-containing NMDA receptors. *J Neurosci* **21**(4):1228-1237.

Persechini A and Kretsinger RH (1988) Toward a model of the calmodulin-myosin light-chain kinase complex: implications for calmodulin function. *Journal of cardiovascular pharmacology* **12 Suppl 5**:S1-12.

Peters A, Palay SL and Webster Hd (1991) *The fine structure of the nervous system : neurons and their supporting cells*. Oxford University Press, New York.

Planells-Cases R, Sun W, Ferrer-Montiel AV and Montal M (1993) Molecular cloning, functional expression, and pharmacological characterization of an N-methyl-D-aspartate receptor subunit from human brain. *Proceedings of the National Academy of Sciences of the United States of America* **90**(11):5057-5061.

Pollard TD, Doberstein SK and Zot HG (1991) Myosin-I. *Annual review of physiology* **53**:653-681.

Potschka H, Fedrowitz M and Loscher W (2001) P-glycoprotein and multidrug resistance-associated protein are involved in the regulation of extracellular levels of the major antiepileptic drug carbamazepine in the brain. *Neuroreport* **12**(16):3557-3560.

Potschka H and Loscher W (2001) In vivo evidence for P-glycoprotein-mediated transport of phenytoin at the blood-brain barrier of rats. *Epilepsia* **42**(10):1231-1240.
Prybylowski K, Chang K, Sans N, Kan L, Vicini S and Wenthold RJ (2005) The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2. *Neuron* **47**(6):845-857.
Racca C, Stephenson FA, Streit P, Roberts JD and Somogyi P (2000) NMDA receptor content of synapses in stratum radiatum of the hippocampal CA1 area. *J Neurosci* **20**(7):2512-2522.
Rachline J, Perin-Dureau F, Le Goff A, Neyton J and Paoletti P (2005) The micromolar zinc-binding domain on the NMDA receptor subunit NR2B. *J Neurosci* **25**(2):308-317.
Racz B, Blanpied TA, Ehlers MD and Weinberg RJ (2004) Lateral organization of endocytic machinery in dendritic spines. *Nature neuroscience* **7**(9):917-918.
Rao PS, Mallya KB, Srivenugopal KS, Balaji KC and Rao US (2006) RNF2 interacts with the linker region of the human P-glycoprotein. *International journal of oncology* **29**(6):1413-1419.
Rayment I, Rypniewski WR, Schmidt-Base K, Smith R, Tomchick DR, Benning MM, Winkelmann DA, Wesenberg G and Holden HM (1993) Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science (New York, NY* **261**(5117):50-58.
Reck-Peterson SL, Provance DW, Jr., Mooseker MS and Mercer JA (2000) Class V myosins. *Biochimica et biophysica acta* **1496**(1):36-51.
Reinhard J, Scheel AA, Diekmann D, Hall A, Ruppert C and Bahler M (1995) A novel type of myosin implicated in signalling by rho family GTPases. *The EMBO journal* **14**(4):697-704.
Rhoads AR and Friedberg F (1997) Sequence motifs for calmodulin recognition. *Faseb J* **11**(5):331-340.
Roche KW, Standley S, McCallum J, Dune Ly C, Ehlers MD and Wenthold RJ (2001) Molecular determinants of NMDA receptor internalization. *Nature neuroscience* **4**(8):794-802.
Roche KW, Tingley WG and Huganir RL (1994) Glutamate receptor phosphorylation and synaptic plasticity. *Current opinion in neurobiology* **4**(3):383-388.
Rosenmund C and Westbrook GL (1993) Calcium-induced actin depolymerization reduces NMDA channel activity. *Neuron* **10**(5):805-814.
Ryu J, Liu L, Wong TP, Wu DC, Burette A, Weinberg R, Wang YT and Sheng M (2006) A critical role for myosin IIb in dendritic spine morphology and synaptic function. *Neuron* **49**(2):175-182.
Saito N, Okada Y, Noda Y, Kinoshita Y, Kondo S and Hirokawa N (1997) KIFC2 is a novel neuron-specific C-terminal type kinesin superfamily

motor for dendritic transport of multivesicular body-like organelles. *Neuron* **18**(3):425-438.

Sans N, Petralia RS, Wang YX, Blahos J, 2nd, Hell JW and Wenthold RJ (2000) A developmental change in NMDA receptor-associated proteins at hippocampal synapses. *J Neurosci* **20**(3):1260-1271.

Sans N, Prybylowski K, Petralia RS, Chang K, Wang YX, Racca C, Vicini S and Wenthold RJ (2003) NMDA receptor trafficking through an interaction between PDZ proteins and the exocyst complex. *Nature cell biology* **5**(6):520-530.

Sans N, Wang PY, Du Q, Petralia RS, Wang YX, Nakka S, Blumer JB, Macara IG and Wenthold RJ (2005) mPins modulates PSD-95 and SAP102 trafficking and influences NMDA receptor surface expression. *Nature cell biology* **7**(12):1179-1190.

Sauna ZE and Ambudkar SV (2001) Characterization of the catalytic cycle of ATP hydrolysis by human P-glycoprotein. The two ATP hydrolysis events in a single catalytic cycle are kinetically similar but affect different functional outcomes. *The Journal of biological chemistry* **276**(15):11653-11661.

Scarpini E, Scheltens P and Feldman H (2003) Treatment of Alzheimer's disease: current status and new perspectives. *Lancet neurology* **2**(9):539-547.

Schorge S and Colquhoun D (2003) Studies of NMDA receptor function and stoichiometry with truncated and tandem subunits. *J Neurosci* **23**(4):1151-1158.

Scott DB, Blanpied TA, Swanson GT, Zhang C and Ehlers MD (2001) An NMDA receptor ER retention signal regulated by phosphorylation and alternative splicing. *J Neurosci* **21**(9):3063-3072.

Scott DB, Michailidis I, Mu Y, Logothetis D and Ehlers MD (2004) Endocytosis and degradative sorting of NMDA receptors by conserved membrane-proximal signals. *J Neurosci* **24**(32):7096-7109.

Sellers JR (1999) *Myosins*. Oxford University Press, Oxford ; New York.

Sellers JR (2000) Myosins: a diverse superfamily. *Biochimica et biophysica acta* **1496**(1):3-22.

Setou M, Nakagawa T, Seog DH and Hirokawa N (2000) Kinesin superfamily motor protein KIF17 and mLin-10 in NMDA receptor-containing vesicle transport. *Science (New York, NY* **288**(5472):1796-1802.

Sheng M and Hoogenraad CC (2007) The postsynaptic architecture of excitatory synapses: a more quantitative view. *Annual review of biochemistry* **76**:823-847.

Simons M, Wang M, McBride OW, Kawamoto S, Yamakawa K, Gdula D, Adelstein RS and Weir L (1991) Human nonmuscle myosin heavy chains are encoded by two genes located on different chromosomes. *Circulation research* **69**(2):530-539.

Sisodiya SM, Lin WR, Harding BN, Squier MV and Thom M (2002) Drug resistance in epilepsy: expression of drug resistance proteins in common causes of refractory epilepsy. *Brain* **125**(Pt 1):22-31.

Sisodiya SM and Thom M (2003) Widespread upregulation of drug-resistance proteins in fatal human status epilepticus. *Epilepsia* **44**(2):261-264.

Smothers CT and Woodward JJ (2003) Effect of the NR3 subunit on ethanol inhibition of recombinant NMDA receptors. *Brain research* **987**(1):117-121.

Sprengel R and Single FN (1999) Mice with genetically modified NMDA and AMPA receptors. *Annals of the New York Academy of Sciences* **868**:494-501.

Sprengel R, Suchanek B, Amico C, Brusa R, Burnashev N, Rozov A, Hvalby O, Jensen V, Paulsen O, Andersen P, Kim JJ, Thompson RF, Sun W, Webster LC, Grant SG, Eilers J, Konnerth A, Li J, McNamara JO and Seeburg PH (1998) Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. *Cell* **92**(2):279-289.

Standley S, Roche KW, McCallum J, Sans N and Wenthold RJ (2000) PDZ domain suppression of an ER retention signal in NMDA receptor NR1 splice variants. *Neuron* **28**(3):887-898.

Steigerwald F, Schulz TW, Schenker LT, Kennedy MB, Seeburg PH and Kohr G (2000) C-Terminal truncation of NR2A subunits impairs synaptic but not extrasynaptic localization of NMDA receptors. *J Neurosci* **20**(12):4573-4581.

Stephenson FA, Cousins SL and Kenny AV (2008) Assembly and forward trafficking of NMDA receptors (Review). *Molecular membrane biology* **25**(4):311-320.

Stern P, Behe P, Schoepfer R and Colquhoun D (1992) Single-channel conductances of NMDA receptors expressed from cloned cDNAs: comparison with native receptors. *Proceedings* **250**(1329):271-277.

Sugihara H, Moriyoshi K, Ishii T, Masu M and Nakanishi S (1992) Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. *Biochemical and biophysical research communications* **185**(3):826-832.

Tada T and Sheng M (2006) Molecular mechanisms of dendritic spine morphogenesis. *Current opinion in neurobiology* **16**(1):95-101.

Tan JL, Ravid S and Spudich JA (1992) Control of nonmuscle myosins by phosphorylation. *Annual review of biochemistry* **61**:721-759.

Taubman MB, Grant JW and Nadal-Ginard B (1987) Cloning and characterization of mammalian myosin regulatory light chain (RLC) cDNA: the RLC gene is expressed in smooth, sarcomeric, and nonmuscle tissues. *The Journal of cell biology* **104**(6):1505-1513.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I and Willingham MC (1987) Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proceedings of the National*

Academy of Sciences of the United States of America **84**(21):7735-7738.

Tichelaar W, Safferling M, Keinanen K, Stark H and Madden DR (2004) The Three-dimensional Structure of an Ionotropic Glutamate Receptor Reveals a Dimer-of-dimers Assembly. *Journal of molecular biology* **344**(2):435-442.

Tingley WG, Ehlers MD, Kameyama K, Doherty C, Ptak JB, Riley CT and Huganir RL (1997) Characterization of protein kinase A and protein kinase C phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. *The Journal of biological chemistry* **272**(8):5157-5166.

Tovar KR, Sprouffske K and Westbrook GL (2000) Fast NMDA receptor-mediated synaptic currents in neurons from mice lacking the epsilon2 (NR2B) subunit. *Journal of neurophysiology* **83**(1):616-620.

Tovar KR and Westbrook GL (1999) The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *J Neurosci* **19**(10):4180-4188.

Trybus KM, Waller GS and Chatman TA (1994) Coupling of ATPase activity and motility in smooth muscle myosin is mediated by the regulatory light chain. *The Journal of cell biology* **124**(6):963-969.

Ulbrich MH and Isacoff EY (2007) Subunit counting in membrane-bound proteins. *Nature methods* **4**(4):319-321.

Van Eldik LJ and Watterson DM (1998) *Calmodulin and signal transduction*. Academic Press, San Diego.

Vissel B, Krupp JJ, Heinemann SF and Westbrook GL (2001) A use-dependent tyrosine dephosphorylation of NMDA receptors is independent of ion flux. *Nature neuroscience* **4**(6):587-596.

Wakabayashi Y, Kipp H and Arias IM (2006) Transporters on demand: intracellular reservoirs and cycling of bile canalicular ABC transporters. *The Journal of biological chemistry* **281**(38):27669-27673.

Walikonis RS, Jensen ON, Mann M, Provance DW, Jr., Mercer JA and Kennedy MB (2000) Identification of proteins in the postsynaptic density fraction by mass spectrometry. *J Neurosci* **20**(11):4069-4080.

Wang C, Wang HG, Xie H and Pitt GS (2008) Ca2+/CaM controls Ca2+-dependent inactivation of NMDA receptors by dimerizing the NR1 C termini. *J Neurosci* **28**(8):1865-1870.

Wang YT and Salter MW (1994) Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature* **369**(6477):233-235.

Wang YT, Yu XM and Salter MW (1996) Ca(2+)-independent reduction of N-methyl-D-aspartate channel activity by protein tyrosine phosphatase. *Proceedings of the National Academy of Sciences of the United States of America* **93**(4):1721-1725.

Warrick HM and Spudich JA (1987) Myosin structure and function in cell motility. *Annual review of cell biology* **3**:379-421.

Washbourne P, Bennett JE and McAllister AK (2002) Rapid recruitment of NMDA receptor transport packets to nascent synapses. *Nature neuroscience* **5**(8):751-759.

Washbourne P, Liu XB, Jones EG and McAllister AK (2004) Cycling of NMDA receptors during trafficking in neurons before synapse formation. *J Neurosci* **24**(38):8253-8264.

Watanabe M, Inoue Y, Sakimura K and Mishina M (1992) Developmental changes in distribution of NMDA receptor channel subunit mRNAs. *Neuroreport* **3**(12):1138-1140.

Watkins JC and Evans RH (1981) Excitatory amino acid transmitters. *Annual review of pharmacology and toxicology* **21**:165-204.

Waxman EA and Lynch DR (2005) N-methyl-D-aspartate receptor subtypes: multiple roles in excitotoxicity and neurological disease. *Neuroscientist* **11**(1):37-49.

Weber A and Murray JM (1973) Molecular control mechanisms in muscle contraction. *Physiological reviews* **53**(3):612-673.

Wechsler A and Teichberg VI (1998) Brain spectrin binding to the NMDA receptor is regulated by phosphorylation, calcium and calmodulin. *The EMBO journal* **17**(14):3931-3939.

Wenthold RJ, Prybylowski K, Standley S, Sans N and Petralia RS (2003) Trafficking of NMDA receptors. *Annual review of pharmacology and toxicology* **43**:335-358.

Westbrook GL and Mayer ML (1987) Micromolar concentrations of Zn2+ antagonize NMDA and GABA responses of hippocampal neurons. *Nature* **328**(6131):640-643.

Wong EH, Kemp JA, Priestley T, Knight AR, Woodruff GN and Iversen LL (1986) The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. *Proceedings of the National Academy of Sciences of the United States of America* **83**(18):7104-7108.

Wylie SR and Chantler PD (2001) Separate but linked functions of conventional myosins modulate adhesion and neurite outgrowth. *Nature cell biology* **3**(1):88-92.

Wylie SR, Wu PJ, Patel H and Chantler PD (1998) A conventional myosin motor drives neurite outgrowth. *Proceedings of the National Academy of Sciences of the United States of America* **95**(22):12967-12972.

Wyllie DJ, Behe P, Nassar M, Schoepfer R and Colquhoun D (1996) Single-channel currents from recombinant NMDA NR1a/NR2D receptors expressed in Xenopus oocytes. *Proceedings* **263**(1373):1079-1086.

Wyszynski M, Kharazia V, Shanghvi R, Rao A, Beggs AH, Craig AM, Weinberg R and Sheng M (1998) Differential regional expression and ultrastructural localization of alpha-actinin-2, a putative NMDA receptor-anchoring protein, in rat brain. *J Neurosci* **18**(4):1383-1392.

Wyszynski M, Lin J, Rao A, Nigh E, Beggs AH, Craig AM and Sheng M (1997) Competitive binding of alpha-actinin and calmodulin to the NMDA receptor. *Nature* **385**(6615):439-442.

Xie X, Harrison DH, Schlichting I, Sweet RM, Kalabokis VN, Szent-Gyorgyi AG and Cohen C (1994) Structure of the regulatory domain of scallop myosin at 2.8 A resolution. *Nature* **368**(6469):306-312.

Yamakita Y, Yamashiro S and Matsumura F (1994) In vivo phosphorylation of regulatory light chain of myosin II during mitosis of cultured cells. *The Journal of cell biology* **124**(1-2):129-137.

Yamashita RA, Sellers JR and Anderson JB (2000) Identification and analysis of the myosin superfamily in Drosophila: a database approach. *Journal of muscle research and cell motility* **21**(6):491-505.

Yang W, Zheng C, Song Q, Yang X, Qiu S, Liu C, Chen Z, Duan S and Luo J (2007) A three amino acid tail following the TM4 region of the N-methyl-D-aspartate receptor (NR) 2 subunits is sufficient to overcome endoplasmic reticulum retention of NR1-1a subunit. *The Journal of biological chemistry* **282**(12):9269-9278.

Yao PJ, Bushlin I and Petralia RS (2006) Partially overlapping distribution of epsin1 and HIP1 at the synapse: analysis by immunoelectron microscopy. *The Journal of comparative neurology* **494**(2):368-379.

Zhang M, Tanaka T and Ikura M (1995) Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. *Nature structural biology* **2**(9):758-767.

Zhang S, Ehlers MD, Bernhardt JP, Su CT and Huganir RL (1998) Calmodulin mediates calcium-dependent inactivation of N-methyl-D-aspartate receptors. *Neuron* **21**(2):443-453.

CHAPTER 2

NMDA RECEPTOR SUBUNITS: NON-MYOSIN TARGETS OF MYOSIN REGULATORY LIGHT CHAIN

Gaurav Bajaj , Yong Zhang , Michael I. Schimerlik , Andrew Hau , Jing Yang , Theresa M. Filtz , Chrissa Kioussi and Jane E. Ishmael

In revision for “The journal of Biological Chemistry”

Abstract

Alternative binding partners have been described for both light chains of myosin II raising the possibility that the regulatory light chain (RLC), like other EF-hand proteins, may adopt conformations that can be distinguished from conventional RLC-heavy chain interactions. We characterized the molecular determinants of RLC binding to two major subunits of the N-methyl-D-aspartate receptor (NR). RLC bound to a 30-37 amino acid carboxyl terminal region of NR1 and NR2 subunits in a manner that could be distinguished from the interaction of RLC with the neck region of nonmuscle myosin II-B (NMII-B) heavy chain; NR-RLC interactions did not require the addition of magnesium, involved the N-terminal domain of the RLC and were maintained in the absence of the fourth EF-hand domain. Equilibrium fluorescence spectroscopy experiments indicate that the affinity of myosin RLC for NR1 is high (30 nM) in the context of the isolated light chain. Focusing on the NR2A subunit, which is not a target of calmodulin, we report that sequence similarity in the "GxxxR" portion of the incomplete IQ2 motif found in nonmuscle myosin II heavy chain isoforms likely contributes to the recognition of NR2A as a non-myosin target of the RLC. Binding to NR subunits was not favored following phosphorylation of RLC by myosin light chain kinase, and in the context of a recombinant NMII-B subfragment one, indicating that if the RLC is already bound to NMII-B it is unlikely to form a bridge between two binding partners. We suggest that a role for the RLC in protein trafficking in polarized cells is distinct from the typical interaction of RLC as a component of the myosin II complex.

Introduction

Regulation and maintenance of glutamate receptor numbers at postsynaptic sites is critical for excitatory neurotransmission in the CNS. Mechanisms that underlie targeting of glutamate receptors to synapses almost certainly involve a regulated series of protein-protein interactions between the receptor and various binding partners. In trying to understand how these

events are temporally and spatially coordinated, the intracellular carboxyl termini of glutamate receptor subunits have attracted much attention as they have been shown to bind directly and indirectly to a number of cytoskeletal and motor proteins, scaffolding proteins, enzymes and other signaling molecules (Wenthold et al., 2003). We have previously demonstrated that three major subunits of the N-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptor bind directly to myosin II regulatory light chain (RLC) (Amparan et al., 2005). Myosin RLC is an accessory light chain of the actin-based motor myosin II, which is a hexameric complex composed of two heavy chains, two RLCs, and two essential light chains (ELC) (Conti and Adelstein, 2008). Myosin II light chains are, like calmodulin, members of the EF-hand family of calcium-binding proteins (Kawasaki et al., 1998). However, the interaction of myosin RLC with NMDA receptor (NR) subunits could be distinguished from the strictly Ca^{2+}-dependent interaction of calmodulin with the NR1 subunit (Amparan et al., 2005).

The light chains of myosin II are integral components of the myosin II complex that bind to tandem IQ motifs located in the neck region of myosin II heavy chain. An IQ motif is approximately twenty-five amino acids long containing the classic consensus sequence IQxxxRGxxxR (Bahler and Rhoads, 2002). Variations in this consensus IQ sequence can however, explain both the light chain binding preference of heavy chains, and also the particular conformation that a light chain can adopt upon binding (Houdusse and Cohen, 1995; Terrak et al., 2002; Terrak et al., 2003). For example, RLCs of myosin II are predicted to adopt an extended conformation when bound to the neck region of myosin II heavy chain (Houdusse and Cohen, 1996; Rayment et al., 1993). However, crystallographic evidence indicates the ELC of *Saccharomyces cerevisae*, Mlc1p, can exist in two distinct conformations: compact and extended depending on the specific IQ sequence of the myosin heavy chain (Terrak et al., 2003). When bound in an extended conformation,

it is suggested that Mlc1p has a free amino terminal that may interact with another binding partner to form a ternary complex with myosin II heavy chain (Terrak et al., 2003). This finding has attracted much attention because ELC in organisms such as *S. cerevisae*, *Schizosaccharomyses pombe* and *Drosophila melanogaster* have several binding partners (Franke et al., 2006). These include class II myosin heavy chains (Myo1p in *S. cerevisae* (Luo et al., 2004; Stevens and Davis, 1998)*;* Myp2 and Myo2 in *S. pombe* (Bezanilla et al., 1997) and *zipper* in *D. melanogaster* (Franke et al., 2006)), myosin V heavy chains (Espindola et al., 2000; Franke et al., 2006), additional unconventional myosins (VI and VIIa in *D. melanogaster* (Franke et al., 2006)), cytoskeletal IQ containing GTPase activating protein (IQGAP)-like proteins (Boyne et al., 2000; Shannon and Li, 2000; Weissbach et al., 1998), and a microtubule-associated protein (Franke et al., 2006).

Myosin RLC has also been reported to interact with a number of proteins other than myosin II heavy chain. In addition to NMDA-type glutamate receptor subunits (Amparan et al., 2005), myosin RLC binds via its amino terminal to the smooth muscle protein calponin (Szymanski and Goyal, 1999). The RLC also binds directly to bile salt export protein (BSEP) and at least two other members of ATP-binding cassette (ABC) family of drug efflux transporters (Chan et al., 2005), as well as a novel ezrin, radixin, moesin (ERM)-like protein, with ubiquitin E3-ligase activity, known as myosin regulatory light chain-interacting protein (MIR in human brain (Bornhauser et al., 2003; Nagano et al., 2006; Olsson et al., 2000; Olsson et al., 1999); Mir in zebrafish (Knowlton et al., 2003; Knowlton and Kelly, 2004)). As it is widely assumed that RLC functions only in the context of a myosin II complex, the existence of these additional binding partners raises the possibility that the RLC could form a ternary complex with myosin II heavy chain and a third non-myosin binding partner (Chan et al., 2005; Olsson et al., 1999). In the present study we therefore undertook a detailed characterization of the interaction of

myosin II RLC with NR1 and NMDAR2A (NR2A) subunits of the NMDA receptor to provide some insight into the molecular determinants of RLC interactions with two non-myosin binding partners. We report that RLC binds to a relatively short membrane-proximal C-terminal region of NR1 and NR2 subunits in a manner that can be clearly distinguished from the interaction of RLC with the neck region of nonmuscle myosin II-B (NMII-B) heavy chain. Equilibrium fluorescence spectroscopy experiments revealed that the affinity of myosin RLC for NR1 is high (30 nM) in the context of the isolated light chain. Binding of the light chain to NMDA receptor subunits was, however, not favoured if the RLC was already in a complex with NMII-B heavy chain. Focusing on the NR2A subunit, which is not a target of calmodulin, we report that sequence homology in the "GxxxR" portion of the incomplete IQ2 motif found in all three nonmuscle myosin II heavy chain isoforms likely contributes to the recognition of NR2 subunits as a non-myosin target of the RLC.

Materials and methods

Plasmid and constructs

Plasmids containing rat NMDAR2A, NMDAR2B and NMDAR1-1a cDNAs have been decribed previously (Amparan et al., 2005). The human MHC-B clone HB5.12/1.21, used to construct MHC (771-843) encoding the neck region of nonmuscle myosin II-B (MHC-B) in pGEX-2T (Amparan et al., 2005), was a kind gift from Dr. R. Adelstein (National Institutes of Health, Bethesda, MA). The membrane-proximal region of the C-terminus of the NMDAR2A subunit (amino acid residues 838-874) in pGEX-6P-3 was the generous gift of Dr. J. Saugstad (Robert S. Dow Neurobiology Laboratories, Portland, OR).

For bacterial expression, NR2A (amino acids 875-1029), NR2A (1030-1464), NR2A (1030-1290), NR2A (1291-1464), NR2A (838-867 known as Δ868), NR2A (838-860 known as Δ861), NR2A (838-854 known as Δ855)

were amplified by PCR and inserted into the bacterial expression vector pGEX-6P-3 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The membrane proximal region of the C-terminus of the NMDAR2B subunit (amino acid residues 839-873) was amplified by PCR and inserted into pGEX-6P-3. Myosin regulatory light chain (1-101), RLC (102-172), RLC (1-129), RLC (61-129) and RLC (61-172) were amplified by PCR from the mouse pACT2/5-6-1 clone (Amparan et al., 2005), and inserted into the bacterial expression vector pET 28a (Novagen, Madison, WI). The construction of full-length myosin RLC (1-172), NMDAR1 (834-938), NR1 (834-863 encoding C0), NR1 (864-938 encoding C1C2) and NR1 (834-855 known as ∆856) has been described previously (Amparan et al., 2005). For expression in mammalian cells, NR2A (1-1028) and RLC (1-172) were amplified by PCR and inserted into *EcoR* I and *Xho* I sites of pECFP-N1 and pEYFP-N1 (Clontech, Palo Alto, CA), respectively. NR1 was excised from the plasmid pTL1/NR1-1a (Ishmael et al., 1996) and inserted into *Hind* III and *Xba* I restriction sites of pCDNA 3.1 (Invitrogen Corporation, Carlsbad, CA). Full-length RLC (1-172) was also inserted into pCDNA 3.1. Site-directed mutagenesis was performed using standard techniques. All sequences were confirmed by the Center for Genome Research and Biocomputing core facility (Oregon State University, Corvallis, OR).

Antibodies

The anti-myosin RLC antibody (αMRLC/3) was raised in rabbits against a unique peptide and has been described previously (Amparan et al., 2005). Antiserum to the phosphorylated form of serine 19 of myosin RLC was also raised in rabbits against a unique phosphopeptide (CRPQRATS-PO3-NVFAM) and affinity purified (αMRLC/P) (Bethyl Laboratories Inc., Montgomery, TX). The anti-GST antibody was a gift of Dr. Mark Leid (Oregon State University, Corvallis, OR). Other primary antibodies used in this study are commercially available and included: anti-calmodulin (Upstate Biotechnology, Lake Placid,

NY), anti-PSD95 (Affinity Bioreagents Inc., Golden, CO), anti-myosin light chain (MLC) (Abcam, Cambridge, MD), anti-T7 (Novagen, Madison, WI), anti-adaptin α (Transduction Laboratories, Newington, NH), anti-Golgi 58K protein (clone 58K-9) and anti-FLAG (both from Sigma, St. Louis, MO), and anti-nonmuscle myosin heavy chain isororms IIA and IIB (both from Covance Research Products, Denver, PA).

Protein purification

Recombinant myosin RLC was expressed in *Escherichia coli* (BL21-Gold(DE3)pLysS; Stratagene, La Jolla, CA), and purified by nickel-chelate chromatography. The poly-histidine tag was removed by thrombin cleavage (Thrombin Clean Cleave Kit; Sigma-Aldritch Corp., St. Louis, MO) followed by dialysis against a buffer containing 20 mM Tris (pH 7.7) and 500 mM NaCl. GST fusion proteins were bacterially expressed, as above, and purified by column chromatography using immobilized glutathione (Pierce, Rockford, IL). The bound GST fusion protein was eluted using a buffer containing 10 mM reduced glutathione and 50 mM Tris-HCl (pH 8.0) followed by dialysis against the buffer containing 50 mM Tris-HCl (pH 8.0). Native smooth muscle myosin light chain (MLC-2) was isolated and purified from washed turkey gizzard myofibrils as previously described (Malencik and Anderson, 1982; Malencik and Anderson, 1988), and was a kind gift from Drs. Sonia Anderson and Dean Malencik (Oregon State University, Corvallis, OR). Smooth muscle myosin light chain kinase (MLCK) was a kind gift from Dr. Christine Cremo (University of Reno, ND). Bovine calmodulin was purchased from Calbiochem, San Diego, CA. Purified FLAG-tagged human nonmuscle IIB subfragment one (NMIIB S1), a truncated myosin heavy chain fragment co-expressed with regulatory and essential myosin light chains in baculovirus (Wang et al., 2003), was a kind gift of Dr. James Sellers (National Institutes of Health, Bethsesda, MA).

Phosphorylation of isolated RLC and nonmuscle IIB subfragment one

Regulatory light chain RLC, either in isolated form or in the context of NMIIB S1, was phosphorylated in the presence of smooth muscle MLCK (Cremo et al., 2001). Isolated RLC or NMIIB S1 was added to a buffer containing 20 mM Tris (pH 7.5), 0.1 mM EGTA, 1 mM DTT, 10 µg/ml MLCK, 10 µg/ml calmodulin, 5 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 1 mM ATP, and incubated at 37°C for 1 hour. Phosphorylation of each sample was verified by polyacrylamide gel electrophoresis (PAGE) in the presence of urea; control samples were nonphosphorylated, or subjected to mock phosphorlylation reactions (lacking MLCK). Samples were heated at 80°C for 2 minutes in urea sample buffer (8 M urea, 33 mM Tris-glycine pH 8.6, 0.17 mM EDTA and bromophenol blue), before loading on NOVEX Tris-Glycine pre-cast gels (Invitrogen, Carlsbad, CA). Proteins were visualized directly by Coomassie blue stain (BioRad Laboratories, Hercules, CA), or were electro-transferred to nitrocellulose membrane (Amersham Biosciences) and detected by immunoblot with either a T7-tag antibody (RLC), αMRLC/P (phosphorylated RLC), anti-FLAG antibody (heavy chain) or anti-MLC1 (ELC) and appropriate horseradish peroxidase-conjugated secondary antibodies (Calbiochem). Immune complexes were revealed using a chemiluminescence assay (Roche, Indianapolis, IN).

Fluorescence spectroscopy

A peptide corresponding to the first thirty amino acids of the carboxyl tail of the NMDAR1 subunit (residues 834 to 863) was labeled with fluorescein on the amino terminal (FITC-EIAYKRHKDARRKQMQLAFAAVNVWRKNLQ-COOH) and purified by reverse phase HPLC (United Biochemical Research, Inc., Seattle, WA). Fluorescence measurements were taken in 2.0 mL of buffer containing 20 mM Tris (pH 7.7), 500 mM NaCl and a final peptide concentration of 300 nM. Purified RLC was added from a concentrated stock solution and allowed to equilibrate in the dark for 5 min prior to fluorescence

determinations. All additions of purified RLC were made sequentially from a single stock solution, and the fluorescence emission was corrected for dilution. All experiments were performed on a SLM 8000C spectrofluorometer (SLM Instruments, Urbana, IL). Data were fit to *equation 1* by weighted nonlinear least squares using the computer program Scientist® (Micromath Inc., St. Louis, MO) when the weighting factors were equal to the square of the reciprocal of the standard deviation for each data point:

Equation 1

$$(1) \qquad F = a + b \left\{ \frac{2 R_0 L_0}{R_0 + L_0 + K + \sqrt{(R_0 + L_0 + K)^2 - 4 R_0 L_0}} \right\} + c L_0$$

In equation (1), F is the measured fluorescence enhancement, *a* the fluorescence observed in the absence of RLC, R_0 the total concentration of fluorescently labeled peptide, L_0 the total concentration of RLC, *K* the dissociation constant for the peptide-protein complex, *b* is the relative fluorescence enhancement of the fluorescent peptide-protein complex, and *c* is a parameter used to fit the background linear fluorescence increase observed as RLC concentration increased.

Fluorescence microscopy, Culture and Transfection of Mammalian Cells

Human embryonic kidney cells (HEK) 293 cells were maintained in 90% DMEM (Mediatech, Herden, VA), 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C under 5% CO_2 in humidified air. The day before transfection, cells (2×10^4) were plated on poly-L-lysine coated glass coverslips in medium supplemented with kynurenic acid (3 mM, Sigma, St. Louis, MO) and DL-AP5 (1 mM Tocis,

Ballwin, MO) (Krupp et al., 2002). Cells were transfected using Fugene 6 (Roche Diagnostics, Indianapolis, IN) and maintained for up to 72 hours. Cells were then washed twice with 1 x PBS, fixed in 3% formaldehyde (20 min.), and mounted on slides with a ProLong® antifade kit (Molecular Probes, Eugene, OR). Confocal images were obtained using a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY) with a Zeiss 63X oil immersion objective. Fluorescence signals were collected using laser excitation at 514 nm for YFP and 458 nm for CFP. LP 530 and BP 470-500 filters were used for detection of YFP and CFP emission as described previously (Zhang et al., 2006). For detection of endogenous K58 Golgi protein and heavy chain isoforms, sections were incubated with appropriate primary antibodies after fixation followed by secondary antibodies conjugated to Alexa 546 or Cy5, respectively.

Glutathione-S-transferase (GST) pull-down assays

Pull-down assays from brain homogenates (extracted in a buffer containing 250 mM NaCl and 10 mM ATP) were conducted as previously described (Amparan et al., 2005). Pull-down assays with recombinant myosin RLC and the carboxyl regions of NR1 or the NMII-B heavy chain neck region have also been described (Amparan et al., 2005), but included the following modifications: (1) assays were initiated with the addition of recombinant RLC (100 nM) and, (2) the incubation time was extended to 2 hours. For studies to determine the interaction of full length and mutant RLC (phosphorylated and non-phosphoryated) with the NR2A subunit, GST fusion proteins or GST alone were incubated in binding buffer (10 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% NP-40 and 10% glycerol), and allowed to proceed for 2 hours at 4°C as described above. Unbound protein was removed by three sequential washes with binding buffer and bound proteins eluted from the beads by boiling in sample buffer. Proteins were separated by sodium dodecyl sulfate (SDS)-PAGE, transferred to nitrocellulose membranes

and processed for immunoblot analysis as described above. Calcium-dependent calmodulin binding to NMDA receptor subunits was assessed in the same way in the absence (binding buffer) or presence of calcium (binding buffer lacking EDTA but including 2 mM $CaCl_2$). The magnesium-dependence of RLC binding was also assessed in the absence and presence of magnesium (lacking EDTA but including 1 mM $MgCl_2$). In all calcium and magnesium studies, the appropriate binding buffer was used in all subsequent washes to remove unbound protein. In light of the apparent magnesium-dependence of RLC binding to the neck region of NMII-B heavy chain, subsequent studies were carried out in the presence of magnesium (1 mM).

Immunopreciptation studies:

NR2A (838-874) fused to GST (150 nM) was purified and mixed with either purified NMIIB S1, NMIIB S1-P, or bacterially expressed myosin RLC (MRLC) in 10mM HEPES containing 0.1% NP-40, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, pH 7.5 at 4°C. Bovine serum albumin was added to a final concentration of 0.5% and proteins were allowed to incubate with gentle rotation for 2 hours at 4°C. Anti-GST (12 μg) was then added to the samples and allowed to incubate with gentle rotation for a further 2 hours at 4°C. Precipitation of immune complexes was facilitated by the addition of a 25 μl aliquot of 50% Protein G-sepharose slurry (Amersham Biosciences) to each tube, incubated with gentle rotation for 2 hours at 4°C. Immune complexes were collected by centrifugation at 3,000 x g for 1 min, followed by three consecutive resuspension/wash and centrifugation steps. Precipitated proteins were eluted from the beads by heating for 2 min in 2 X Laemmli sample buffer. Proteins were separated by sodium dodecyl sulfate (SDS)-PAGE, transferred to nitrocellulose membranes and processed for immunoblot analysis as described above.

Results

Smooth muscle and brain isoforms of myosin RLC bind to the NR1 subunit with high affinity

Although there is considerable sequence homology at the amino acid level amongst RLCs in nonmuscle and smooth muscle tissues, subtle differences have been noted between brain isoforms (Feinstein et al., 1991; Taubman et al., 1987; Wang and Chantler, 1994) and the well-characterized smooth muscle isoform of RLC ((Zavodny et al., 1988); Fig. 2.1A). To determine if these small differences in amino acid sequence were sufficient to influence binding of myosin RLC to the NMDA receptor, we determined binding affinities for the mouse brain isoform previously identified as an NMDA receptor-interacting protein (Accession number AK013776), and the major smooth muscle isoform from chicken gizzard (Accession number P02612) using equilibrium fluorescence spectroscopy. These initial binding studies were conducted at supra-physiological salt concentrations (0.5 M NaCl), to distinguish RLC-NR1 interactions from those, such as calponin (Szymanski and Goyal, 1999), that may be strictly limited to low ionic strength conditions. Functional NMDA receptors differ with respect to their complement of NR2 subunits, but the first thirty amino acid region of the C terminus of NR1 (referred to as C0; Fig. 2.1B) is conserved in all splice variants of NR1 and thus present in all functional NMDA receptors. As we have previously determined that NR1-C0 is necessary and sufficient for myosin RLC binding (Amparan et al., 2005), we used a fluorescein-labeled peptide (F-NR1-C0) corresponding to this entire region for our fluorescence binding studies. The F-NR1-C0 peptide alone exhibited maximum excitation at 493 nm and maximum emission at 520 nm (data not shown). Titration of either purified, recombinant brain (Fig. 2.1C), or smooth muscle light chain, isolated and purified from chicken gizzard (Fig. 2.1D), enhanced the fluorescence emission of F-NR1-C0 by approximately 30%. These data were best fit by a single hyperbolic curve using a non-linear, curve fitting analysis, allowing us to derive

binding affinities of 37 ± 24 nM and 17 ± 2.2 nM for brain and chicken gizzard RLC isoforms (Fig. 2.1C and 1D), respectively. The affinity of chicken gizzard RLC for F-NR1-C0 was not significantly different when the buffer containing half-molar salt was substituted for phosphate-buffered saline (29 nM; data not shown). Given the large degree of sequence homology between brain and smooth muscle isoforms and the fact that both bind to the C0 region of NR1 with comparable affinity, it appears unlikely that myosin RLC-NMDA receptor interactions can be explained by unique characteristics of RLC isoforms present in rodent brain.

Light chain-NMDA receptor interactions are not dependent upon magnesium

As Mg^{2+} is thought to fulfill a key structural requirement for RLC binding to myosin II heavy chain, we compared the magnesium dependence of NR1 and heavy chain interactions. For these studies the full-length C terminus of NR1 fused to GST, and the neck region of nonmuscle myosin II-B heavy chain fused to GST, were expressed in bacteria and examined for interaction with RLC in the presence and absence of Mg^{2+} (Fig. 2.2). Although Mg^{2+} enhanced binding of myosin RLC to both interaction partners, RLC-NR1 interactions appeared to be much less dependent upon Mg^{2+} (Fig. 2.2). Consistent with our previous studies (Amparan et al., 2005), myosin RLC showed a specific interaction with the NR1 C terminus in the absence of added Mg^{2+} (Fig. 2.2, lane 3), whereas no interaction was detected between myosin II heavy chain and RLC under the same conditions (Fig. 2.2, lane 4). These findings suggest that RLC-NMDA receptor complexes may be distinguished from RLC-myosin II heavy chain complexes *in vitro* on the basis of their sensitivity to Mg^{2+}. As our peptide binding studies, carried out in the absence of added Mg^{2+}, revealed no major distinction between RLC isoforms, the basis for this difference likely resides within the target sequence itself.

Taken together, these observations support a role for NMDA receptor subunits as non-myosin targets of the RLC.

Myosin RLC interacts with the intracellular membrane-proximal region of the C terminus of NR2 subunits

We have previously shown that myosin RLC binds directly to the carboxyl-terminal region NR1 and NR2 subunits (Amparan et al., 2005). However the C terminus of NR2 family subunits are roughly six-times longer than those of the NR1 subunit (approximately 600 amino acids versus 90 amino acids). We used a series of deletion mutants (Fig. 2.3A) to map regions of the NR2A cytoplasmic tail required for interaction with myosin RLC. Truncation mutants were expressed in bacteria as GST fusion proteins and immobilized on glutathione-Sepharose beads. Immobilized GST/NMDA receptor fusion proteins and GST alone were then used as affinity matrices to examine interactions with RLC from homogenates derived from mouse forebrain (lower panel of Fig. 2.3B). Immunoblotting with an anti-myosin RLC antibody revealed that native mouse brain RLC interacted strongly and specifically with GST/NR2A (838-874) corresponding to the first 37 amino acids of the NR2A C terminus (lane 5; Fig. 2.3B), but failed to interact with GST/NR2A (875-1029), GST/NR2A (1030-1464) and GST/NR2A (1030-1290) (lanes 6 to 8; Fig. 2.3B). A faint immunoreactive signal was observed corresponding to retention of native myosin RLC on a GST affinity matrix containing amino acids 1291 to 1464 of the NR2A carboxyl tail (lane 9; Fig. 2.3B). RLC binding, however, was not observed with the longer GST/NR2A (1030-1464) mutant, suggesting that the interaction of native RLC with this distal portion of the NR2A subunit is either weak or possibly hindered by the presence of another NR2-interacting protein in mouse brain.

Multiple protein-protein interactions have now been documented at the level of glutamate receptor carboxyl-termini (Wenthold et al., 2003). We therefore tested all GST affinity matrices concurrently for their ability to bind

the known NR2-interacting protein, postsynaptic density protein of 95 KDa (PSD-95). Consistent with the NMDA receptor binding specificity of PSD-95 (Kornau et al., 1995), native mouse brain PSD-95 bound only to those mutants that contained the distal portions of the NR2A carboxyl tail, (GST/NR2A (1030-1464) and GST/NR2A (1291-1464) (see lanes 7 and 9 of upper panel of Fig. 2.3B). PSD-95 binds to a distal four amino acid motif (Kornau et al., 1995), and thus our findings served to validate the use of a GST pull-down assay from mouse brain homogenates to map RLC-NMDA interactions. In agreement with our previous findings (Amparan et al., 2005), myosin RLC bound specifically to a GST fusion protein containing the first thirty amino acids of the C terminus of the NR1 subunit (lane 3; Fig. 2.3B), yet failed to interact with either GST alone (lane 2) or a GST fusion protein containing the distal region (amino acid residues 864-938) of the NR1 C terminus (lane 4). In addition, native PSD-95 was not retained on control or GST/NR1 affinity matrices (lanes 2 to 4 of Fig. 2.3B) indicating that PSD-95 displayed appropriate binding specificity in this assay.

Although NR1 and NR2 subunits of the NMDA receptor generally share low overall sequence homology (Monyer et al., 1992), the membrane-proximal regions of the C terminus of both subtypes contain some conserved motifs that are primarily responsible for trafficking NMDA receptors to endocytic and degradative pathways (Scott et al., 2004; Vissel et al., 2001). We therefore expressed the membrane-proximal region of the NR2B subunit in bacteria as a GST fusion protein (GST/NR2B (839 to 873) to determine if RLC also binds to this region of the NR2B subunit (alignment of all three subunits is shown in Fig. 2.4A). Native myosin RLC interacted strongly and specifically with GST/NR1 (834-863), GST/NR2A (838-874) and GST/NR2B (839-873) (upper panel of Fig. 2.4B, lanes 3, 5 and 6), but failed to interact with the distal region of NR1 (864-938) (Fig. 2.4B, lane 4). Consistent with our previous findings with the NR1 subunit (Amparan et al., 2005), none of the fusion proteins

bound myosin ELC extracted from mouse brain even though this protein was also present in the homogenate (lower panel of Fig. 2.4B). Deletion of the membrane-proximal region of the NR2A C terminus by 7 to 20 amino acids (GST/NR2A∆868 to GST/NR2A∆855; Fig. 2.4C) abolished the interaction of RLC with NR2A (Fig. 2.4D, lanes 6 to 8). Taken together, these data indicate that RLC extracted from mouse brain binds to a membrane-proximal region of the C terminus of three major subunits of the NMDA receptor. The minimal RLC binding site on the NR2A subunit is approximately 37 amino acids, and is not a target for myosin ELC.

Membrane-proximal regions of NR2A and NR2B C termini are not targets for calmodulin

Calmodulin is a resident light chain of some classes of myosin motor. Calmodulin also modulates NMDA receptor function via a direct calcium-dependent interaction with the NR1 subunit (Ehlers et al., 1996; Krupp et al., 1999). We have previously shown that myosin RLC and Ca^{2+}/calmodulin share a common binding site on the membrane proximal region of the NR1 subunit; myosin RLC was displaced by Ca^{2+}/calmodulin *in vitro*, but was unaffected by calmodulin in the absence of calcium (Amparan et al., 2005). To investigate the possibility of nonspecific interactions between EF-hand family proteins and NR2 subunits, we tested the ability of purified calmodulin to bind the membrane-proximal regions of NR2A and NR2B *in vitro*. These regions of the NR1, NR2A and NR2B C termini were expressed in bacteria as GST fusion proteins and examined for interaction with purified calmodulin in the presence and absence of Ca^{2+} (Fig 2.5). Calmodulin bound strongly and specifically to GST/NR1 (834-863) in the presence of Ca^{2+} (compare lanes 3 and 8 in Fig 2.5), yet failed to interact with the equivalent membrane-proximal regions of the NR2A (lanes 4 and 9 of Fig. 2.5) and NR2B C-termini (lanes 5 and 10 of Fig. 2.5). These findings indicate that the first 34-36 amino acids of

the NR2A and NR2B C termini do not harbor recognition sequences for calmodulin.

Myosin RLC co-localizes with NMDAR2A in whole cells

We have previously shown that myosin RLC co-localizes with the NR1 subunit at dendritic sites in mature hippocampal neurons grown in culture (Amparan et al., 2005). Recombinant GFP-tagged myosin RLC also localizes to postsynaptic sites in isolated hippocampal neurons (Zhang et al., 2005). However, as all functional NMDA receptors are assemblies of NR1 and NR2 subunits it is difficult to determine the possible significance of NR2A-RLC interaction in these studies. To provide some insight into the significance of myosin RLC-NR2A interactions in an intact cell, we took advantage of a heterologous expression system. Previous studies have shown that unassembled NR2 subunits are retained in the endoplasmic reticulum of heterologous cells and neurons by a short retention motif in the membrane proximal region of the NR2 C terminus (HLFY highlighted in Fig. 2.4A), and that cell surface expression of NR2 occurs only in the presence of the NR1 subunit (Hawkins et al., 2004). We therefore co-expressed myosin RLC and NR2A in HEK293 cells in the presence and absence of NR1. To avoid a potential second myosin RLC recognition site in the distal part of the C terminus (see Fig 2.3), we used NR2A (1-1028) for these studies as this NR2A deletion mutant has been characterized previously in HEK cells and forms a functional channel with normal physiological characteristics (Vissel et al., 2002). In the present study, expression of functional heteromeric NR1/CFP-NR2A (1-1028) receptors was validated by the omission of glutamate receptor antagonists from the culture medium, which resulted in cell death (data not shown). Confocal fluorescence microscopy of HEK 293 cells transiently expressing either myosin RLC fused to YFP, or NR2A (1-1028) fused to CFP (hereafter known as NR2A) revealed a predominant intracellular distribution of both proteins that could be clearly distinguished from that of YFP or CFP alone

(Fig. 2.6, compare YFP alone in row 1 (panels A to C), with rows 2 (panels D to F) and 3 (panels G to H); CFP alone is not shown). This cytoplasmic distribution was retained in HEK cells co-expressing YFP-RLC and CFP-NR2A (Fig 2.6, panels J and K), as shown in an overlay of YFP and CFP channels (Fig 2.6, panel L). YFP-RLC and CFP-NR2A signals remained congruent in cells transfected with all three plasmids, yet the expression pattern was changed to a more peripheral, membrane localization by the addition of the NR1 subunit (Fig. 2.6, panels M to O). Although these studies do not provide evidence of a direct interaction of myosin RLC with either NMDA receptor subunits, they suggest that a cytoskeletal rearrangement of myosin RLC and NR2A occurs in response to the presence of a functional NR1/NR2A assembly in intact cells.

Can myosin RLC bind to NMDA receptor subunits in the context of a myosin II complex?

To determine if a ternary complex could exist between myosin II heavy chain, the RLC and NMDA receptor subunits, we tested the ability of recombinant NMIIB subfragment 1 (a myosin II heavy chain fragment that is already in a complex with RLC and ELC) to bind to the membrane-proximal region of NR2A. Preliminary analyses indicated that all three components of NMIIB S1 complex (heavy, regulatory and essential light chains) could be identified independently either by Coomassie stain (not shown) or immunoblot analysis (Fig 2.7A). Using an immunoprecipitation strategy we tested the ability of NR2A (838-874) fused to GST to form a complex with (1) nonphosphorylated NMIIB S1 (Fig 2.7B) or, (2) NMIIB S1 in which the RLC had been phosphorylated by MLCK (Fig. 2.7C). No precipitation of either form of NMIIB S1 was seen when NR2A (838-874) was precipitated with an anti-GST antibody. Panels B and C of figure 2.7 show detection of myosin II heavy chain fragments in the input lane only (lane 1, upper panels), whereas control blots indicate that NR2A was precipitated by anti-GST (lanes 5 and 6, lower

panels). An isolated myosin RLC was, however, co-immunoprecipitated with NR2A (838-874) in parallel reactions; figure 2.7 panel D shows positive identification of RLC (lane 6, upper panel) and NR2A (lane 6, lower panel) indicating that these two proteins were able to form a binary complex. Using conditions expected to favor NMDA receptor binding (i.e. physiological salt concentrations in the absence of magnesium), titration of NMIIB S1 (0 -120 nM) produced a 100 % increase in the fluorescence of the F-C0 peptide by 120 nM that was not saturable (data not shown). Taken together these data indicate that if RLC is already bound in the context of a myosin II complex, association with an NMDA receptor target protein likely represents a much lower affinity interaction than the interaction than that measured between NR1 and the isolated RLC.

The amino terminal region of myosin RLC is critical for interaction with NMDA receptor subunits

To determine which regions of myosin RLC are required for interaction with NMDA receptor subunits, we expressed several deletion mutants of RLC in bacteria (Fig 2.8A) and examined them for interaction with either the C terminus of NR1 (Fig. 2.8B), the membrane proximal region of NR2A (Fig. 2.8C), or the neck region of NMHC IIB (Fig. 2.8D) fused to GST, respectively. The RLC mutant corresponding to the first three helix-loop-helix motifs (or EF-hand domains) of the protein (1-129) bound to GST/NR1 and GST/NR2A in a manner that was comparable to that of the full-length (1-172) protein (compare lanes 12 and 18 of panel B, and lanes 9 and 15 of panel C). The RLC mutant lacking the first EF-hand domain (61-172) was also retained, albeit to a lesser extent, on a GST/NR1 affinity matrix (lane 15 of panel B), but was not retained on GST/NR2A (lane 12 of panel C). Further deletion of either EF-hand domains three and four (1-101), or EF-hand domains one and two (102-172) abolished the interaction of myosin RLC with GST/NR1 and GST/NR2A (lanes 3 and 6 of panels B and C, respectively). A mutant consisting of EF-hand

domains two and three also failed to bind to NR1 (lane 6 of panel B). In contrast to the pattern observed with NMDA receptor target sequences, all deletion mutants of myosin RLC failed to bind to NMHC IIB (lanes 3, 6, 9 and 12 of Fig. 2.8D). As shown in Fig. 2.8D (lane 15) only the full-length RLC was retained on the GST affinity matrix comprising the neck region of the myosin IIB heavy chain (771-843). These findings indicate that the interaction of myosin RLC with NMDA receptor subunits may be structurally distinct from the classic RLC-heavy chain interaction. All regions of the light chain appear to be critical for binding to myosin II heavy chain. In contrast, the interaction of myosin RLC with NMDA receptor subunits requires residues in the amino terminal of the light chain and occurs in the absence of the fourth EF-hand domain.

Myosin RLC binding to the NMDA receptor is sensitive to the phosphorylation state of the light chain

The cyclical phosphorylation and dephosphorylation of myosin RLC represents a critical step in the actomyosin cycle of nonmuscle and smooth muscle myosin II motors. To determine if the interaction of myosin RLC with the NMDA receptor is influenced by phosphorylation of the light chain, we tested the ability of NR2A to bind full-length (1-172) and mutant (1-129) RLC phosphorylated by MLCK. By analogy to other RLCs, *Thr* 18 and *Ser* 19 are the likely targets of phosphorylation by MLCK. The extent of phosphorylation was first confirmed by urea-glycerol PAGE of RLCs followed by immunoblot analysis (Fig. 2.9A). Phosphorylated and non-phosphorylated RLCs were then examined for interaction with GST/NR2A (838-874) using an antibody to both forms of the protein (Fig 2.9B). Both the full-length and truncated (1-129) light chains consistently showed reduced binding to NR2A in the phosphorylated state when compared with non-phosphorylated proteins (compare lanes 5 and 6 with lanes 11 and 12, upper panel of Fig. 2.9B) or mock-phosphorylated proteins (data not shown). Binding of full-length and

truncated RLC to NR2A was however, revealed by the use of a phospho-specific antibody (Fig 2.9B, lower panel). These findings indicate that phosphorylation of target residues in the amino terminus of the light chain disrupt binding to NR2A (838-874). This does not rule out the possibility that the light chain can remain bound to NR2A in the phosphorylated state.

Residues K844 and L845 in the membrane proximal region of NR2A are critical for myosin RLC binding

The membrane-proximal regions of NMDA receptor subunits contain two tyrosine-based recognition sequences (Yxxϕ, where ϕ = a hydrophobic residue) for the clathrin-dependent, endocytic adapter protein 2 complex (AP-2) (Scott et al., 2004; Vissel et al., 2001) (see Fig. 2.4A). The neck region of smooth muscle and nonmuscle myosin II heavy chains also contain a similar tyrosine-based sequence in the region of the neck that is considered an incomplete region of the IQ2 sequence (Fig. 2.10). For example residues in positions 8, 10 and 11 of IQ2 of nonmuscle II-B are identical to residues 842, 844 and 845 in the C terminus of NR2A (Fig. 2.10). We therefore used site-directed mutagenesis to target this YxKL motif in NR2A to determine if these residues may be important for myosin RLC binding to the receptor (Fig. 2.11). Mutation of tyrosine residues Y842 and Y868 within NR2A (838-874), either alone or together, produced no change in myosin RLC binding (Fig 2.11B). Myosin RLC binding was lost, however, when the third and fourth positions of the first endocytic motif were also mutated to NR2A (Y842F/K844A/L845A) (Fig. 2.11B). None of the mutations tested affected the ability of the NR2A fusions to retain AP-2, which was detected using an antibody raised to the alpha subunit of the AP-2 complex (data not shown).

These data indicate that specific residues within the first membrane-proximal endocytic motif of NR2A are critical for myosin RLC binding. Sequence similarity between the membrane-proximal region of NR2 subunits and residues beginning at position 8 of the “GxxxR” portion of the incomplete IQ2

motif found in nonmuscle myosin II heavy chain isoforms likely contributes to the recognition of NR2A as a non-myosin target of the RLC. Furthermore, alignment of three nonmuscle myosin II heavy chains with the membrane-proximal regions of NR2A, NR2B, and the linker region of BSEP (as shown in Fig. 2.10) suggests that amino acid residues lying outside the typical IQ2 motif may contribute to recognition of non-myosin target sequences by the RLC.

Myosin RLC-NR2A interactions facilitate forward trafficking of NR1/NR2A receptors

We disrupted amino acid residues 842, 844 and 845 in the context of CFP-NR2A and co-expressed NR2A(Y842F/K844A/L845A) (abbreviated to CFP-ΔNR2A) with NR1 and myosin RLC in HEK 293 cells. Cells expressing the RLC-deficient NR2A showed dramatically delayed expression of membrane–associated CFP-ΔNR2A fluorescence when compared with control cells expressing wild-type NR2A (Fig. 2.12). Twenty-four hours following transfection, CFP-ΔNR2A fluorescence appeared restricted to a discrete intracellular compartment in 73% of transfected cells, compared with the membrane-associated pattern of CFP-NR2A fluorescence anticipated in the presence of NR1 (compare panel A with panels D and G of Fig. 2.12). As the three amino acid tail required for exit of NR1/NR2A assemblies from the ER is intact in CFP-ΔNR2A (Yang et al., 2007), we stained transfected cells with an antibody raised to the Golgi-specific marker protein K58 (panels B and E of Fig. 2.12). Overlay of CFP-ΔNR2A and anti-K58 staining revealed CFP-ΔNR2A restricted to a sub-region of K58 positive staining within the cell (Fig 2.12, panel F). Intracellular CFP-ΔNR2A fluorescence (Fig 2.12, panel G) co-localized with YFP-RLC fluorescence (Fig 2.12, panel H) when observed 20-24 hours post transfection, shown in an overlay of YFP and CFP channels (Fig 2.12, panel I), however YFP-RLC expression was also observed in the absence of CFP-ΔNR2A (Fig 2.12, panel I). When observed beyond 24 hours after transfection, YFP-RLC and CFP-ΔNR2A signals were generally not

congruent (compare panels E and F, plus M and N of Fig. 2.13). Consistent with a trafficking delay, cells expressing NR1/CFP-ΔNR2A receptors showed increased viability in the absence of glutamate receptor antagonists, but were not protected from cell death (cells expressing NR1/CFP-ΔNR2A showed 50 % increase viability at 72 hours post-transfection compared with cells expressing NR1/CFP-NR2A, data not shown). In summary, myosin RLC-NR2A interactions likely occur within the Golgi secretory trafficking pathway. Disruption of RLC binding in intact cells led to altered NR2A expression and resulted in a delay in delivery of NR1/ΔNR2A receptors to the cell membrane.

Myosin RLC co-localizes with endogenous myosin heavy chain following disruption of the RLC target sequence in NR2A

To determine if YFP-RLC fluorescence was associated with a myosin heavy chain, we stained transfected cells with isoform-specific antibodies raised to either NMHC-IIA or NMHC-IIB (Fig. 2.13). It was immediately apparent that both myosin heavy chain antibodies stained cellular extensions that showed neither YFP nor CFP fluorescence (panels A to C and I to K of Fig. 2.13). Overlay of all three channels revealed co-expression of YFP-RLC, CFP-NR2A and a myosin heavy chain isoform in association with the cell membrane, in addition to cellular regions that stained only for NMCH-IIA (panel D, Fig. 2.13) or NMCH-IIB (panel L, Fig. 2.13). This pattern changed however, if cells were transfected with the RLC-deficient mutant CFP-ΔNR2A (panels E to G and M to N of Fig. 2.13). Under these conditions cellular extensions showed YFP-RLC fluorescence that co-localized with NMCH-IIA (panel H, Fig. 13) or NMCH-IIB (panel P, Fig. 2.13), but not CFP-ΔNR2A fluorescence. These data indicate that an isolated RLC can localize to distinct subcellular compartments within an intact cell. Furthermore, the YFP-RLC was not a component of all myosin II complexes in the cell, yet could re-localize to a previously unoccupied cellular compartment if binding to a non-myosin target, such as NR2A, was disrupted.

Discussion

Several unrelated myosin RLC-binding proteins have been reported in polarized epithelial cells and neurons, yet it is unclear how the prototypical myosin II motor complex interacts with these non-myosin targets. The identification of diverse myosin RLC targets raises the possibility that the RLC, like other EF-hand proteins, may adopt target-dependent conformations distinct from the conventional interaction of RLC with myosin II heavy chain. In the present study we investigated the ability of a mouse brain myosin RLC to interact with NMDA-type glutamate receptor subunits. We characterized the molecular determinants that underlie direct binding of myosin II RLC to the membrane proximal C-terminal regions of NR1 and NR2 glutamate receptor subunits. The interaction of RLC with NMDA receptor subunits was fundamentally different from the typical interaction of the light chain with the neck region of myosin II heavy chain; NMDA receptor-RLC interactions did not require the addition of magnesium, were maintained in the absence of the fourth EF-hand domain of the light chain, and were sensitive to RLC phosphorylation by MLCK. Thus, our data support a model whereby the RLC forms a distinct complex with either NR1 or NR2 subunits that essentially serve as the "heavy chain". Finally, subcellular fluorescence microscopy studies reveal co-localization of myosin RLC with NR2 subunits and subsequent re-localization of both proteins in response to NR1 co-expression to form a functional NMDA receptor channel. Myosin RLC-NR2A interactions were functionally significant, inasmuch as cells expressing a RLC-deficient NR2A subunit showed a trafficking defect in the presence of NR1. Together these findings suggest a role for myosin RLC independent of the myosin complex.

We defined a myosin RLC binding site on three NMDA receptor subunits that was restricted to a 30-37 amino acid stretch directly following the fourth hydrophobic domain of eachNR2A subunit. This region is functionally equivalent in NR1 and NR2 subunits in that it contains tyrosine-based

recognition motifs for endocytosis of the receptor (Scott et al., 2004; Vissel et al., 2001). These membrane-proximal regions lack conserved IQ motifs that are normally associated with myosin light chain or apo-calmodulin binding, however the C0 region of the NR1 subunit harbors a non-IQ, atypical CaM binding site that binds Ca^{2+}-CaM as well as myosin RLC. The relatively high affinity (~ 30 nM) of myosin RLC for the C0 region of NR1 was comparable to binding affinities derived previously for Ca^{2+}-CaM binding to C0 *in vitro*, reported as 87 nM (Ehlers et al., 1996) and 21 nM (Krupp et al., 1999) by two different groups. Although apo-calmodulin associates with C0 *in vitro* (Akyol et al., 2004; Amparan et al., 2005), it does so with relatively modest affinity in the absence of calcium (K_D = 2.5 μM) (Akyol et al., 2004). Thus the affinity of myosin RLC for C0, in the absence of calcium, is comparable to the affinity of Ca^{2+}-CaM for this site. Overlapping myosin RLC and CaM binding sites have been described previously in the RLC-interacting protein calponin. Unlike NR1, the membrane-proximal regions of NR2A and NR2B represent RLC target sequences that do not bind apo-CaM, Ca^{2+}-CaM or myosin ELC. Thus the NR2 subunits potentially represent RLC interacting proteins requiring novel RLC structural conformations, and for this reason formed the focus of our study.

Our studies support a role for only three of the four EF hand domains as necessary for RLC binding to NMDA receptor subunits. However, we were unable to isolate a stable, high affinity ternary complex *in vitro* that, in theory, could be formed if the fourth EF hand domain remained free to bind myosin heavy chain. Instead our data support a model whereby the RLC forms a distinct complex with either NR1 or NR2 subunits that interact in lieu of the heavy chain. Such a scenario would not necessarily require a large pool of free light chain as local physiological conditions, for example divalent cation concentration and/or the phosphorylation status of the RLC, may favor transition between one binding partner over another.

Our findings with brain myosin RLC are reminiscent of the *C. cerevisiae* light chain Mlc1p, in that Mlc1p forms independent complexes with myosin II and IQGAP; Mlc1p/Myo1p (myosin II) interactions can be separated from Mlc1p/IQGAP interactions (Boyne et al., 2000). All three proteins can be isolated in a biochemical complex, co-localize in cells during late mitosis, yet Mlc1p does not form a bridge between IQGAP and Myo1p (Boyne et al., 2000). As a working model we propose that RLC forms a distinct complex with NMDA receptor subunits and also with myosin II heavy chain. By analogy to Mlc1p, such an interaction could serve to recruit the NMDA receptor and a myosin II motor sequentially to a specialized subcellular compartment in a neuron. This model takes into account our biochemical characterization of direct NMDA-RLC interactions, as well as the work of others showing that a nonmuscle myosin II-B motor complex is closely associated with the NMDA-type glutamate receptor (Amparan et al., 2005; Husi et al., 2000; Kioussi et al., 2007; Lei et al., 2001; Ryu et al., 2006).

Although several RLC-interacting proteins have been described in the literature (Amparan et al., 2005; Chan et al., 2005; Olsson et al., 1999; Szymanski and Goyal, 1999), there is currently no unifying mechanism to explain how a myosin II RLC could bind simultaneously to a myosin II heavy chain and a second target protein such as MIR, an NMDA receptor subunit or an ABC transporter. It has been proposed that calponin binds F-actin and the phosphorylated form of RLC, in the context of a myosin II complex, to regulate smooth-muscle contractility (Szymanski, 2004). Calcium appears to be a central regulator of this interaction; myosin RLC is phosphorylated by the Ca^{2+}-CaM-dependent enzyme MLCK, and the interaction between F-actin and calponin may be disrupted by Ca^{2+}-CaM (Szymanski, 2004; Szymanski and Goyal, 1999). This is intriguing as NMDA receptor subunits bind directly to myosin RLC, and are also physically “latched” to F-actin at postsynaptic sites by actin-binding proteins such as alpha-actinin-2 and spectrin (Wechsler and

Teichberg, 1998; Wyszynski et al., 1997). The NMDA receptor is a ligand-gated calcium channel and thus some functional parallels can be drawn with the calponin interaction in smooth muscle. Ca^{2+}-CaM competes for myosin RLC and alpha-actinin-2 binding sites on the C0 region of NR1 (Wyszynski et al., 1997). The direct binding of Ca^{2+}-CaM to the C0 region of NR1 results in a reversible calmodulin-dependent inactivation of NMDA receptor channels (Krupp et al., 1999; Zhang et al., 1998), some characteristics of which can be recapitulated in hippocampal neurons by direct manipulation of MLCK (Lei et al., 2001).

Myosin RLC-NMDA receptor interactions were sensitive to the phosphorylation state of the light chain, in that phosphorylation of RLC by MLCK decreased receptor binding relative to the unphosphorylated RLC. These findings are consistent with a role for the amino portion of the light chain as a determinant of NMDA receptor binding, as the expected targets for phosphorylation by MLCK, amino acid residues threonine 18 and serine 19, are located within this region (Conti and Adelstein, 2008). Our ability to detect phosphorylated myosin RLC bound to NMDA receptor targets was not surprising inasmuch as the RLC remains bound to the heavy chain neck throughout the actin myosin ATPase cycle. These studies revealed a potentially important similarity between the interaction of RLC with NMDA receptor subunits and the smooth muscle protein calponin (Szymanski and Goyal, 1999), in that the amino terminal of the light chain is important for binding to both non-myosin targets. In contrast to the reported interaction of calponin with RLC (Szymanski, 2004), the phosphorylated form of RLC was not favored in our studies. Taken together, these protein-protein interaction studies indicate that the RLC adopts a conformation with the NMDA receptor that can be distinguished from the interaction of RLC with its normal binding partner, myosin II heavy chain. The disposition of myosin RLC in binding MIR or ABC transporters such as BSEP, remains to be determined.

Despite some common themes, we saw no evidence for binding of either unphosphorylated or phosphorylated myosin RLC to NR2A in the context of a recombinant NMIIB subfragment 1. These studies were carried out in physiological salt concentrations capable of detecting direct binding of proteins such as PSD-95 or Ca^{2+}-CaM to the receptor. In addition, the fluorescence signal arising from the C0 peptide upon titration of subfragment 1 was not easily saturable, indicating that the affinity of myosin RLC for NR1 in the context of the myosin subfragment would be significantly lower than the nM affinity measured for the isolated light chain. These kinds of studies are, however, technically challenging *in vitro* as the solubility and stability of the myosin II subfragment is also compromised at physiological salt concentrations. Although it is not possible to rule out a transient calponin-like interaction between the receptor and a neuronal myosin II *in vivo*, it seems most likely that binding of a third target protein is not favored once the RLC is already in a complex with myosin II heavy chain.

The current understanding of conventional myosin II structure therefore does not explain how a RLC could bind to two target proteins simultaneously. Structural studies have revealed that the RLC always binds to the neck region of myosin II heavy chain in an open and extended conformation. In fact the N-terminal of RLC binds to a highly conserved tryptophan-rich target sequence that is not actually part of the IQ2 motif, whereas the C-terminal binds to the IQxxxR portion of the incomplete IQ2 located in the myosin heavy chain (Terrak et al., 2003). Site-directed mutagenesis of residues within the NR2A target sequence, however, revealed that the most likely explanation for recognition of NR2 subunits as non-myosin targets was due to sequence homology between NR2 and residues within the neck region of myosin II that are not part of the canonical IQ2 motif. These findings illustrate that a protein need not contain a consensus IQ motifs to be a target of the isolated RLC. This is important as screening for the presence of IQ motifs has been

employed as a strategy for uncovering new binding targets of highly related EF-hand-containing proteins.

It is becoming accepted that myosin ELC has binding partners other than myosin II heavy chain. This viewpoint has been facilitated by clear differences in the spatial and temporal distribution and/or stability of the ELC versus RLC and myosin II heavy chain in organisms such as *Dictyostelium* (Maeda et al., 2000), yeasts *Saccharomyces cerevisae* and *Schizosaccharomyces pombe* (Boyne et al., 2000; Luo et al., 2004; Shannon and Li, 2000), and *Drosophila melanogaster* (Franke et al., 2006). These kinds of studies are more difficult in the mammalian CNS as expression of components of the myosin II complex is under the control of more than one gene, thus making it difficult to track each polypeptide. For example three separate genes encode nonmuscle myosin II heavy chain isoforms (II-A, II-B and II-C) (Conti and Adelstein, 2008), and at least two of these (II-B and II-C) are alternately spliced (Golomb et al., 2004; Takahashi et al., 1992). Several RLC isoforms have also been described in brain (Feinstein et al., 1991; Taubman et al., 1987; Wang and Chantler, 1994), yet it is not clear if particular light chain isoforms are shared by more than one heavy chain or if the pairing of RLC light chain and heavy chain II isoforms is isoform specific. Our present findings indicate that a brain RLC can localize with either myosin II-B or II-A heavy chain isoforms. Differential staining patterns observed with RLC and nonmuscle myosin II-B heavy chain antibodies in brain also indicate that heterogeneity in the composition of myosin II complexes is likely, despite comparable expression of RLC, ELC and NMHC-IIB in mouse cortex, hippocampus and cerebellum (Kioussi et al., 2007). In *D. melangaster,* which has a single gene encoding the ELC (*essential light chain-cytoplasmic*), RLC (*spaghetti squash*) and heavy chain (*zipper*), at least four alternate binding partners for the ELC have been identified (Franke et al., 2006). However, all three components of the myosin II complex appear to be expressed in

relatively constant stochiometric amounts and by far the majority of both light chains is bound to myosin II heavy chain; using an immunodepletion strategy greater than 90% of ELC and greater than 95% of RLC was bound to myosin II heavy chain in wild-type embryos (Franke et al., 2006). These authors conclude that although the majority of both light chains are associated with nonmuscle myosin II heavy chain, pools of free light chain and/or light chain bound to other proteins are present (Franke et al., 2006). These findings illustrate the potential challenges in studying either alternate binding partners or free pools of myosin light chains in the mammalian brain.

The expression pattern of YFP-myosin RLC in HEK cells showed significant overlay with that of the NR2A subunit when expressed either alone or in the context of a heteromeric membrane-bound NR1/NR2A receptor. We used site-directed mutagenesis to disrupt co-localization of NR2A with the light chain and identify the Golgi complex as a specific region within the cell where RLC interactions may be functionally important in forward trafficking of NR1/NR2A receptors. The cytosolic localization of myosin RLC and NR2A in cells is consistent with our previous observation that native myosin RLC is co-expressed with the NR1 subunit in the soma of some neuronal populations in neonatal and adult mice (Kioussi et al., 2007). A punctate, perinuclear staining pattern corresponding to overlapping NR1 and myosin RLC immunoreactivity was clearly observed in the soma of deep cortical neurons and in Purkinje neurons of the cerebellum (Kioussi et al., 2007). Although the exact intracellular compartment remains to be identified, the simplest explanation for the observed expression patterns in cells and neurons is that myosin RLC is closely associated with NMDA receptor subunits early in the secretory pathway before delivery from the Golgi to postsynaptic sites. In the case of the ABC transporter BSEP, a direct binding partner of RLC, it has been proposed that myosin RLC is required for trafficking of newly synthesized BSEP to the apical membrane and/or the release of BSEP vesicles from the

trans-Golgi network (TGN) of hepatocytes (Chan et al., 2005; Wakabayashi et al., 2006). Myosin II binds to the Golgi membrane via the tail domain of the heavy chain, leaving the head region free to interact with F-actin. In this orientation, myosin II is thought to move Golgi-derived vesicles away from the Golgi complex (Fath, 2005). Our finding that nonmuscle myosins IIB and Va are, like NMDA receptor subunits, raft-associated proteins (Ishmael et al., 2007), is consistent with the general hypothesis that affinity for lipid rafts can provide a sorting platform for exit from the TGN in polarized cells (Zegers and Hoekstra, 1998). The specific mechanisms responsible for movement and export of NMDA receptor-containing vesicles from the Golgi are not clear, however microtubule-based motors subsequently play a role in trafficking NMDA receptor transport packets to the vicinity of a new postsynaptic site (Washbourne et al., 2002). The kinesin family member KIF17, has been reported to associate with the NR2B subunit, however NR2B trafficking by this particular molecular motor occurs at the level of the dendritic shaft and KIF17 does not enter the actin-rich dendritic spine (Guillaud et al., 2003). Dendritic spines are largely devoid of microtubules and thus it is unlikely that microtubule-based motors are responsible for movement and trafficking of NMDA receptor complexes within the spine itself. Although proteomic analyses have identified members of the myosin superfamily representing classes I, II, V, VI and XVIII at glutamate synapses (Cheng et al., 2006; Collins et al., 2006), the functional significance of this observation is not yet appreciated.

In the present study, we observed significant re-localization of NR2A and myosin RLC in heterologous cells in the presence of the NR1 subunit. Receptors containing a RLC-deficient NR2A subunit showed a temporal trafficking defect, indicating that more than one mechanism for trafficking NR1/NR2A assemblies in HEK cells. The apparent redistribution of RLC and wild-type NR2A proteins to a membrane-associated domain is consistent with

previous studies in mature hippocampal neurons that show native myosin RLC is located at dendritic spines with NMDA receptors. Green fluorescent protein (GFP)-tagged myosin RLC has also been shown by others to localize to the dendritic spines of transfected hippocampal neurons (Zhang et al., 2005). Our findings in intact cells therefore extend our previous observations in that myosin RLC appears to be associated with the NR2A subunit, a second marker of functional NMDA receptors, as well as the NR1 subunit. By analogy to our current understanding of the role of RLC in BSEP trafficking in polarized epithelial cells (Chan et al., 2005; Wakabayashi et al., 2006), two possible regions of interest emerge as being potential locations for direct and/or indirect interactions between the NMDA receptor, the RLC and a neuronal myosin II motor: (1) an endomembrane compartment within the neuronal soma that is likely associated with the Golgi apparatus, and, (2) a postsynaptic compartment corresponding to the dendritic spine. The direct interaction of a myosin RLC with a non-myosin binding partner can only presently be explained by the ability of RLC to adopt target-dependent conformations that are distinct from the conventional interaction of RLC with myosin II heavy chain.

.

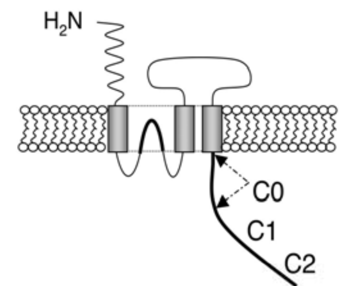
Acknowledgments

The authors thank Dr. Jim Sellers (National Institutes of Health, Bethesda, MD) for his generous gift of recombinant nonmuscle II-B subfragment 1. We also thank Anne-Marie Gerard and Tamara Fraley for assistance with confocal imaging, and Dr. Jeff Greenwood and Dr. Gary Westbrook for helpful discussions. This work was supported by a Beginning Grant-in-Aid award from the American Heart Association, and in part, by Facilities and Services Cores of Grant P30-ES000210 from the National Institute of Environmental Health Sciences. We also thank the Research and Scholarship Fund of the OSU College of Pharmacy for the purchase of the fluorescent NR1 peptide.

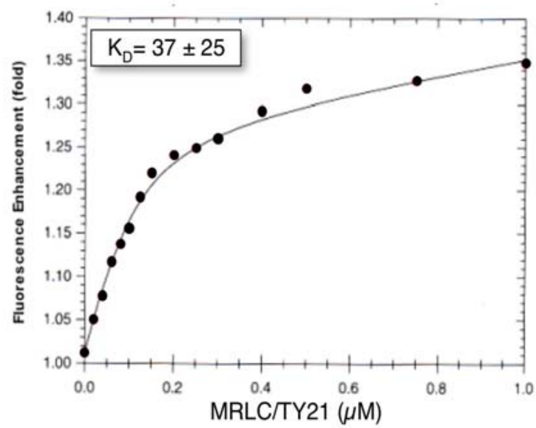
A

```
TY21/MRLC     MSSKRAKTKTTKKRPQRATSNVFAMFDQSQIQEFKEALNMIDQNRDGFIDKEDLHDMLAS 60
MLC-2(L20A)   -------A-----------------------------F----------------------
FY53          ----K--------------------------------L----------------------

TY21/MRLC     MGKNPTDEYLDAMMNEAPGPINFTMFLTMFGEKLNGTDPEDVIRNAFACFDEEAIGTIQE 120
MLC-2(L20A)   ----------EG--S---------------------------------------S-F-H-
FY53          L------A----------------------------------------------T-----
YU63             DQ-GDC---------------------------------------------T-----

TY21/MRLC     DYLRELLTTMGDRFTDEEVDELYREAPIDKKGNFNYIEFTRILKHGAKDKDD  172
MLC-2(L20A)   -H-------------------M--------------V---------------
FY53          ----------------------------------------------------
YU63          ----------------------------------------------------
```

B



C



D

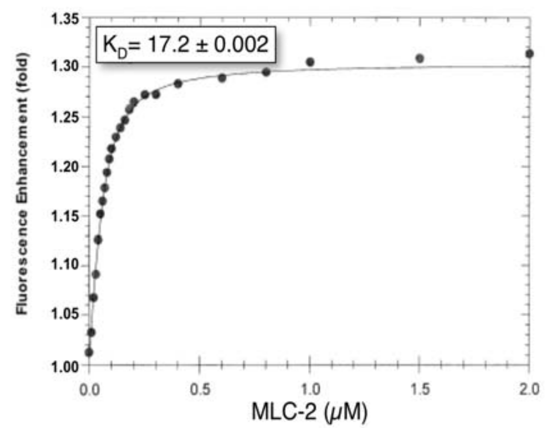


Figure 2.1 Myosin RLC isoforms enhance fluorescence emission of a fluorescein-labelled peptide corresponding to the C0 region of the NMDAR1 carboxyl tail.

A. Amino acid sequence of a myosin regulatory light chain (RLC) isoform previously identified in mouse hippocampus (Accession number AK013776), the major smooth muscle RLC isoform in chicken gizzard (P02612) and a RLC isoform identified from mouse cerebellum (AK005133). AK013776 is identical to the isoform previously identified in mouse brain (MRLC) as an NMDA-receptor interacting protein (Amparan et al., 2005). B. Schematic representation of the NMDAR1 subunit showing the location of C0, C1 and C2 regions. C and D, Titration of either purified, recombinant MRLC (panel C), or purified smooth muscle MLC-2 (panel D) enhanced fluorescence emission by approximately 30%. Data were fit to a single hyperbolic curve using equation *1* to derive binding affinities of 37 ± 25 nM (a = 1.0110 ± 0.0001, b = 2.6 ± 0.9, c = 0.09 ± 0.03, R_0 = 0.10 ± 0.03, r^2 = 0.999) for MRLC (panel C), and 17.2 ± 2.2 nM (a = 0.996 ± 0.004, b = 7.42 ± 0.54, c = 0.029 ± 0.006, R_0 = 0.073 ± 0.004, r^2 = 0.999) for MLC-2 (panel D). Each point represents fluorescence determined in quadruplicate; standard errors lie within the data points.

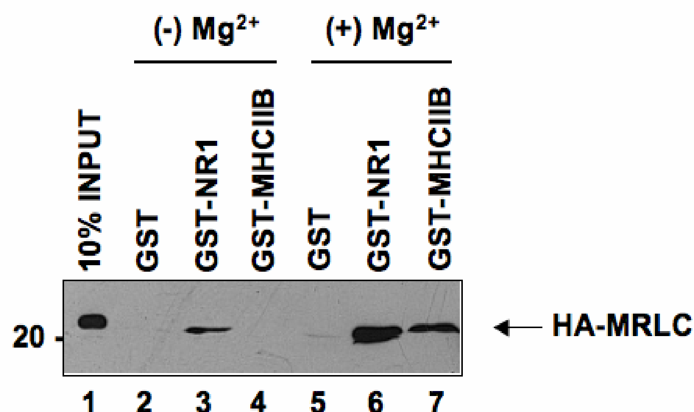
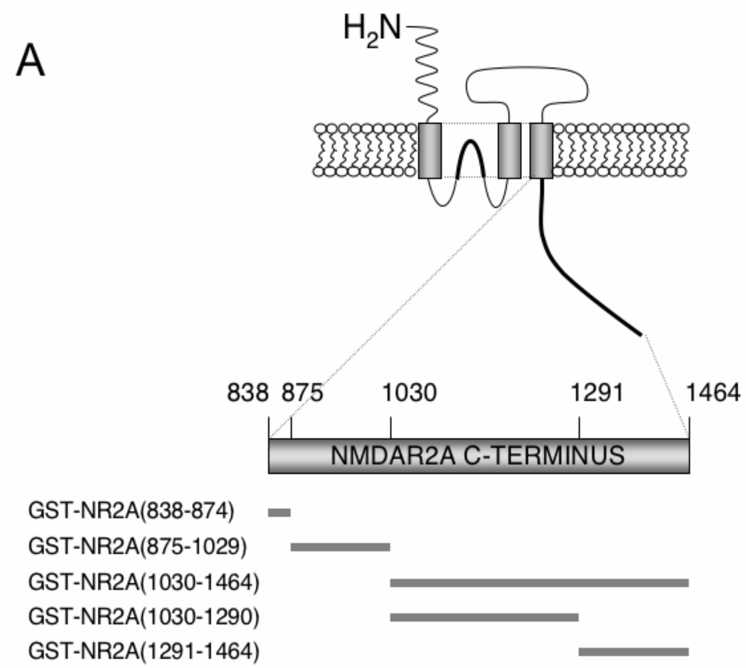


Figure 2.2 Light chain binding to NMDA receptor subunits does not require added magnesium.

A direct comparison of light chain binding to the C-terminus of NR1 and the neck region myosin IIB heavy chain revealed that NR1-RLC interactions are less sensitive to the effects of Mg^{2+}. The carboxyl tail of NR1 (834-938) and the neck region of nonmuscle myosin heavy chain IIB (771-843), each fused to GST, were immobilized on glutathione-Sepharose beads for use in GST pull-down experiments. GST fusion proteins or GST alone were used as affinity matrices to examine interactions with purified, recombinant myosin RLC in the absence (lanes 2,3 and 4) or presence (lanes 5,6 and 7) of 1 mM Mg^{2+}. Assays were initiated by the addition of 300 nM myosin RLC, and incubated with rotation for 2 hr at 4°C. Immune complexes were revealed using an anti-T-7 antibody (Novagen) to detect recombinant myosin RLC. Panel is representative of three independent experiments.


A
H_2N
838
875
1030
1291
1464
NMDAR2A C-TERMINUS
GST-NR2A(838-874)
GST-NR2A(875-1029)
GST-NR2A(1030-1464)
GST-NR2A(1030-1290)
GST-NR2A(1291-1464)

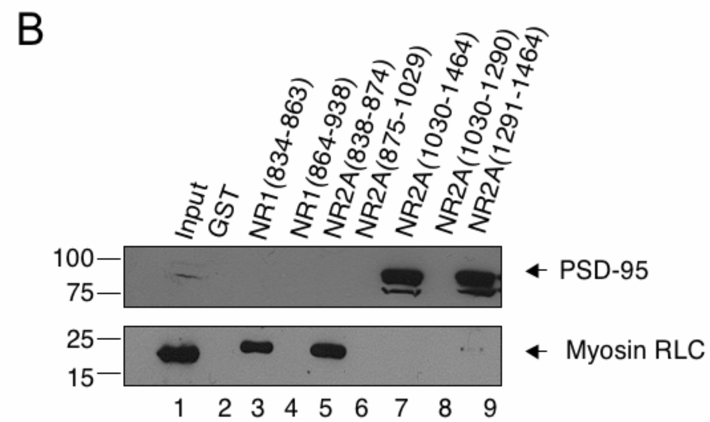

B
Input
GST
NR1(834-863)
NR1(864-938)
NR2A(838-874)
NR2A(875-1029)
NR2A(1030-1464)
NR2A(1030-1290)
NR2A(1291-1464)
100
75
25
15
PSD-95
Myosin RLC
1 2 3 4 5 6 7 8 9

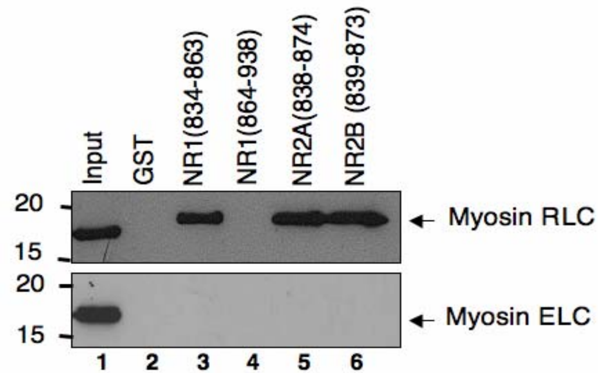
Figure 2.3 Myosin RLC interacts with a short membrane-proximal region of the C-terminal domain of the NMDAR2A subunit.

A. Schematic representation of NMDAR2A C-terminal deletion mutants used in B. B Deletion mutants of NR1 and NR2A C-termini were fused to GST and used as baits to pull down native myosin RLC and a reference NMDA receptor-interacting protein (postsynaptic density protein of 95 kDa (PSD-95)) from mouse forebrain extract. Native myosin RLC was retained on the affinity matrices corresponding to the membrane-proximal region of NR2A (838-874), and the membrane-proximal region of NR1 (834-863). Native PSD-95 was retained on NR2A affinity matrices containing only the extreme distal portion of the NR2A C-terminus, consistent with recognized binding pattern of this protein (lower panel B). GST fusion proteins, or GST alone, were immobilized on glutathione-Sepharose beads and incubated with rotation overnight at 4°C with soluble forebrain extract (1 mg). Immune complexes corresponding to bound proteins were revealed with anti-myosin RLC (Amparan et al., 2005), and anti-PSD95 antibodies (Abcam). Autoradiographs are representative of three independent determinations (from three different animals) using all seven NR mutants fused to GST; various combinations of NR2A mutants fused to GST were tested more frequently.

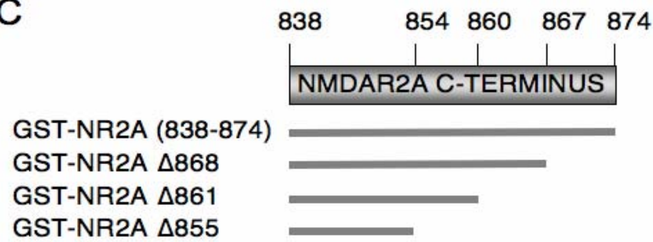
A

NR1 (834-863) EIA**YKRH**KDARRKQMQLAFAAVN**VWRK**NLQ
NR2A (838-874) EHLF**YWKL**RFCFTGVCSDRPGLLFSISRGI**YSCI**HGV
NR2B (839-873) EHLF**YWQF**RHCFMGVCSGKPGMVFSISRGI**YSCI**HGV

B



C



D

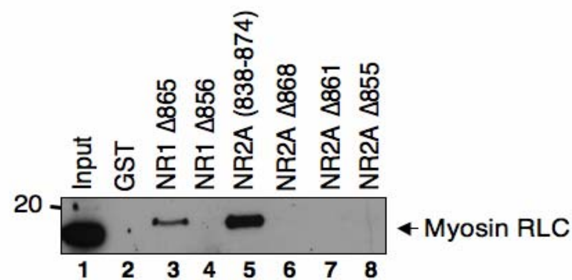


Figure 2.4 The membrane-proximal C-terminal domain of three major NMDA receptor subunits harbors a binding site for myosin RLC.

A. Amino acid alignment of the membrane-proximal regions of NR1, NR2A and NR2B subunits; these regions were fused to GST and are represented in protein-protein interaction studies in B. The location of conserved endocytic motifs in all three subunits is shown in bold typeface, whilst the location of an endoplasmic reticulum retention motif (HLFY) in NR2A and NR2B is shown underlined. B. Membrane proximal regions of NR1 (834-863), NR2A (838-874) and NR2B (839-873) pull down myosin RLC but not a related EF-hand binding protein, myosin essential light chain (ELC), or from a mouse forebrain extract. C. Schematic representation of NR2A C-terminal truncation mutants used in D. D. Amino acids 838 to 874 in NR2A represent a minimal NR2A interaction domain. In panels B and D, NR1 (834-863) corresponding to the C0 domain, NR1Δ856, NR1 (864-863), NR2A (838-864), NR2B (839-873), NR2AΔ868, NR2AΔ861 and NR2AΔ855 were expressed as GST fusion proteins. These GST fusion proteins, or GST alone, were immobilized on glutathione-Sepharose beads and used as baits to pull down myosin light chains from a mouse brain extract. Assays were initiated by the addition of soluble forebrain extract (1 mg) and incubated with rotation overnight at 4°C. Bound proteins were resolved by PAGE and blotted to nitrocellulose; anti-myosin RLC (Amparan et al., 2005) and anti-MLC1 (Abcam) antibodies were used to detect native RLC or ELC, respectively. Panels are representative of between three and five independent experiments from different animals.

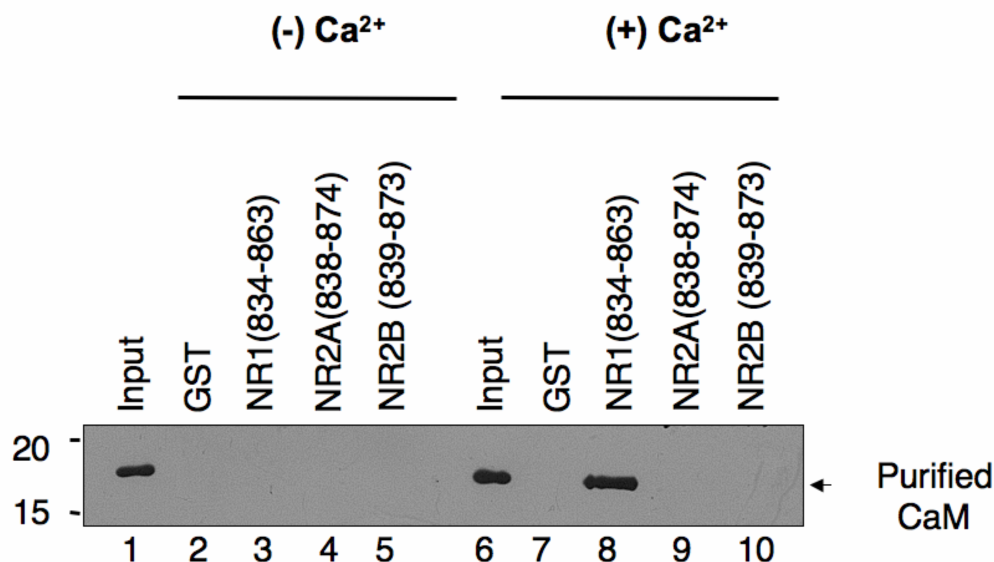


Figure 2.5 The membrane-proximal C-terminal domains of NMDAR2A and NMDAR2B subunits do not binding calmodulin.

Calmodulin is structurally-related to the myosin light chains yet does not bind NR2 subunits. The membrane-proximal region of NR1 was used as a positive control; NR1 (834-863), harbors one of two calmodulin binding sites on the C-terminus of the NR1 subunit that bind calmodulin in a strictly calcium-dependent manner. NR1 (834-863), NR2A (838-874) and NR2B (839-873) were expressed as GST fusion proteins, immobilized on glutathione-Sepharose beads and tested for their ability to bind calmodulin in the absence (lanes 1 to 5), and presence (lanes 6 to 10) of calcium (2 mM). Assays were initiated by the addition of 500 nM calmodulin (Calbiochem) for 2 hours at 4°C in the presence and absence of calcium. Where appropriate, calcium (2 mM) was present throughout the experiment including all wash buffers. Calmodulin bound to GST fusion proteins was resolved by PAGE, and blotted to nitrocellulose. Immune complexes were detected using an anti-calmodulin antibody (Upstate Biotechnology). Panel is representative of three independent experiments.

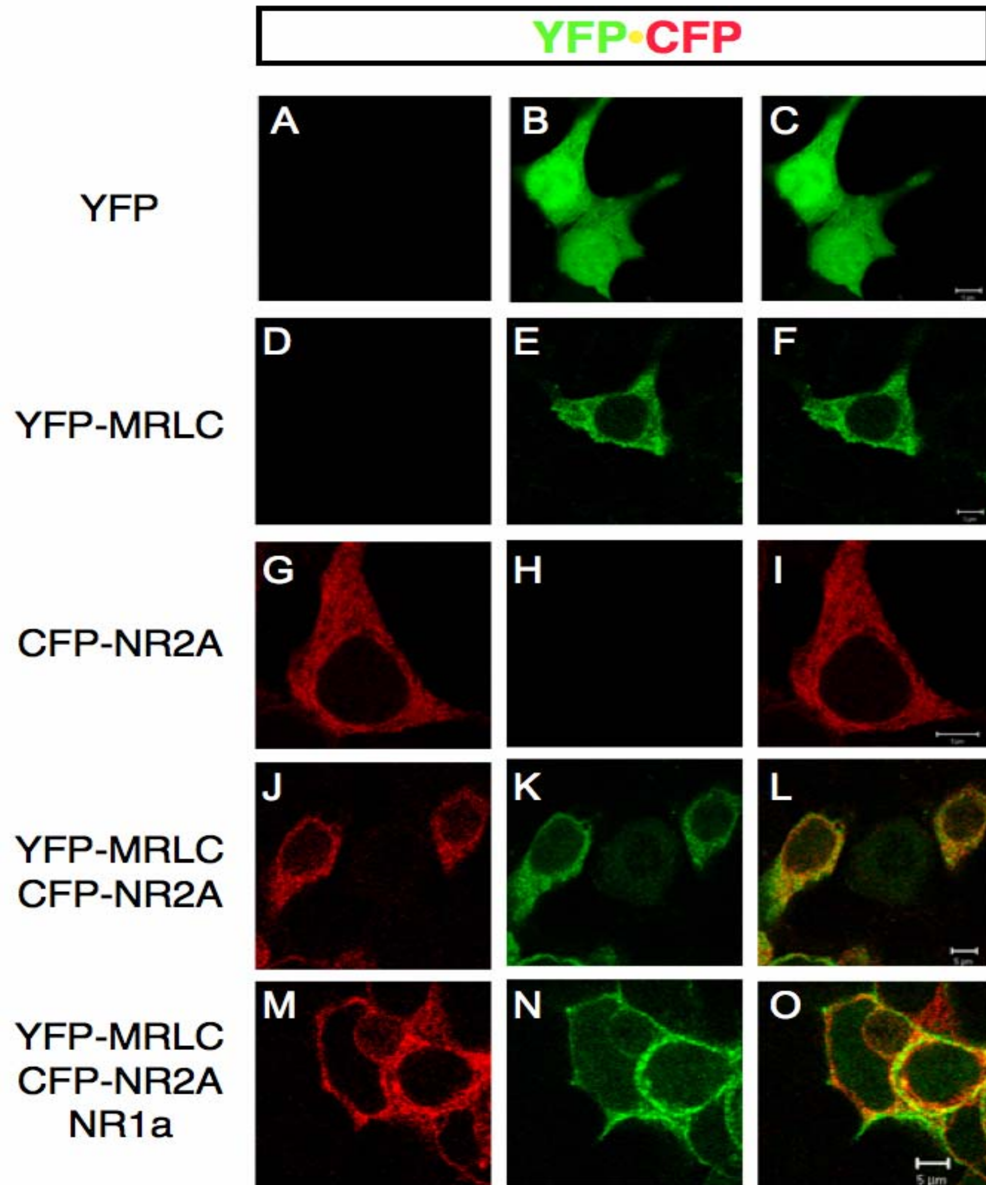

YFP•CFP
A
B
C
YFP
D
E
F
YFP-MRLC
G
H
I
CFP-NR2A
J
K
L
YFP-MRLC
CFP-NR2A
M
N
O
YFP-MRLC
CFP-NR2A
NR1a
5 µm

Figure 2.6 The expression pattern of myosin RLC and NMDAR2A remains congruent in whole cells in the presence of NMDAR1.

Co-expression of a YFP-tagged myosin RLC and a CFP-tagged NR2A subunit in heterologous cells revealed considerable overlay of both proteins (see overlay of YFP and CFP signals in panel L). The expression pattern of YFP-myosin RLC (MRLC-YFP) and CFP-NR2A remained congruent in the presence of the NR1 subunit (see overlay of YFP and CFP signals in panel O; NR1a is untagged), but was redistributed to a membrane-associated domain. HEK293 cells grown on glass coverslips were transiently transfected with: vector only (pEYFP-N1 is shown in row 1), a plasmid encoding full-length myosin RLC (MLC-YFP; row 2), a plasmid encoding NMDAR2A (1028-CFP; row 3), plasmids encoding myosin RLC plus NMDAR2A (MRLC-YFP + CFP-NR2A; row 4) or plasmids encoding myosin RLC, NMDAR2A plus NMDAR1 (MRLC-YFP + CFP-NR2A + NR1a; row 5). Confocal images were captured forty-eight to seventy-two hours after each transfection, using a Zeiss LSM510 confocal microscope with a Zeiss 63× oil immersion objective. Fluorescence signals were collected using laser excitation at 514 nm for YFP and 458 nm for CFP; pseudocolour images were collected with LP 530 and BP 470-500 filters for detection of YFP (column 1) and CFP emission (column 2), respectively. All cells were grown under standard conditions in medium supplemented with kynurenic acid (3 mM) and D,L-AP5 (1 mM) to protect against cytotoxic cell death due to expression of functional recombinant NR1/NR2A receptors. Confocal images are representative of seven independent transfections. Scale bar = 5 μm.

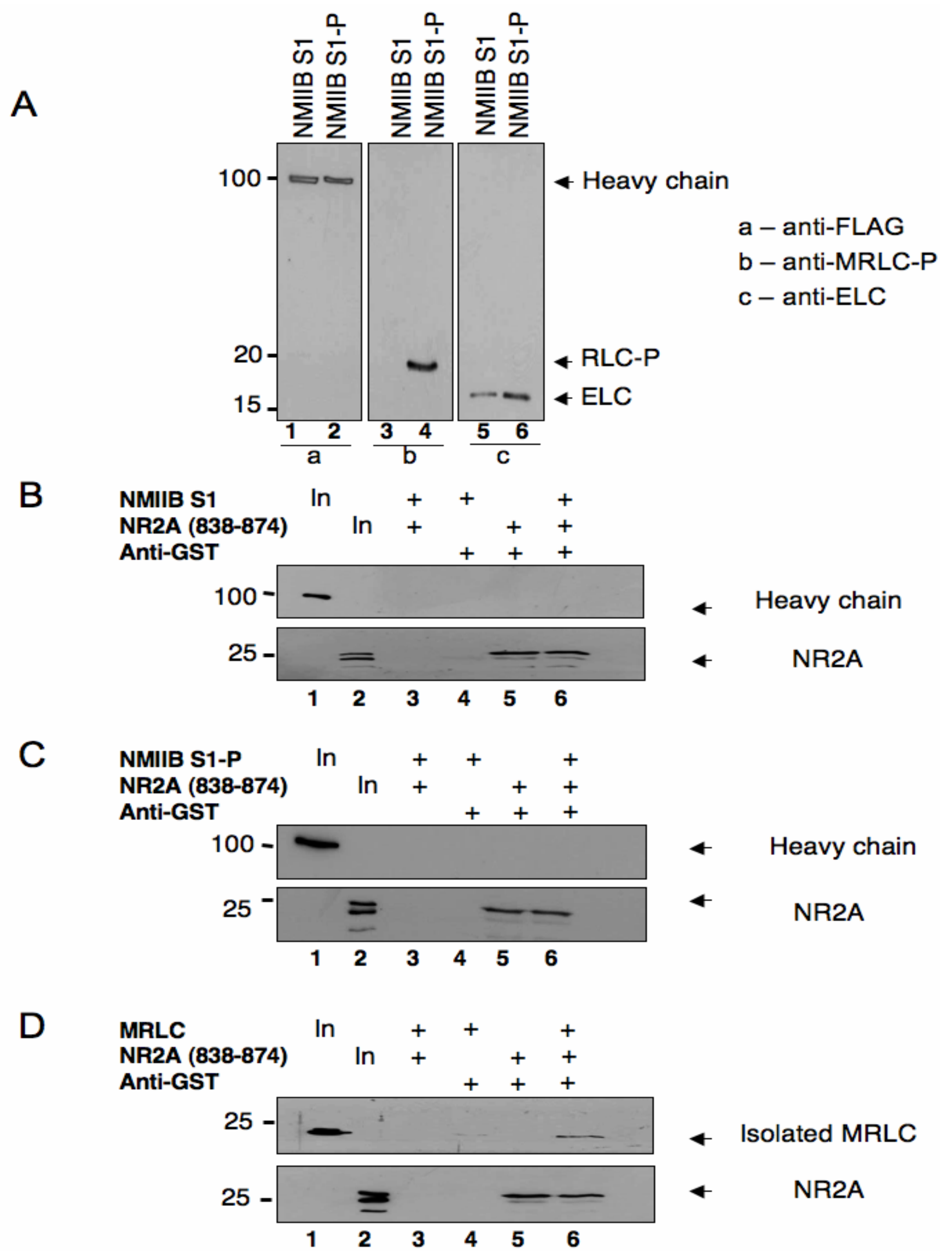

A
NMIIB S1
NMIIB S1-P
NMIIB S1
NMIIB S1-P
NMIIB S1
NMIIB S1-P
100
20
15
Heavy chain
RLC-P
ELC
a – anti-FLAG
b – anti-MRLC-P
c – anti-ELC
1 2 3 4 5 6
a b c
B
NMIIB S1
NR2A (838-874)
Anti-GST
In
100
25
Heavy chain
NR2A
1 2 3 4 5 6
C
NMIIB S1-P
NR2A (838-874)
Anti-GST
In
100
25
Heavy chain
NR2A
1 2 3 4 5 6
D
MRLC
NR2A (838-874)
Anti-GST
In
25
Isolated MRLC
NR2A
1 2 3 4 5 6

Figure 2.7 Myosin RLC does not bind NMDAR2A in the context of either a phosphorylated or non-phosphorylated myosin II subfragment.

A recombinant nonmuscle myosin II-B subfragment 1 (NMIIB S1), consisting of a truncated heavy chain in complex with RLC and ELCs, did not form a ternary complex with NR2A. A. Immunoblot analysis verifying phosphorylation of NMIIB S1 (NMIIB S1-P) by myosin light chain kinase (MLCK). Blots were probed with antibodies to all three components of the myosin II complex, shown are: (a) lanes 1 and 2 detection of heavy chain probed with an anti-FLAG antibody (Sigma), (b) lanes 3 and 4 detection of phosphorylated myosin RLC (RLC-P) probed with a phospho-specific myosin RLC antibody raised in the laboratory, and (c) lanes 5 and 6 detection of myosin ELC (ELC) probed with an anti-MLC1 antibody (Abcam). B. Myosin II-B heavy chain (upper panel) is not precipitated with NR2A (838-874) as a component of a non-phosphorylated NMIIB S1. C. Myosin II-B heavy chain (upper panel) is not precipitated with NR2A (838-874) as a component of MLCK-phosphorylated NMIIB S1. D. Co-immunprecipitation of recombinant myosin RLC (MRLC) with NR2AC-terminal (838-938). For immunoprecipitation studies, purified GST-NR2A (838-874) was incubated with non-phosphorylated NMIIB S1 (B), phosphorylated NMIIB S1 (C) or isolated myosin RLC (D) for 1 hour at 4°C in binding buffer (10mM HEPES pH 7.5, 100mM NaCl, 1mM EDTA, 1mM dithiothreitol, 0.1% NP-40 and 10% glycerol). Protein complexes were then incubated with an anti-GST antibody, and precipitated with protein G-sepharose. Protein complexes were resolved by PAGE, transferred to nitrocellulose and revealed by immunoblot analysis of NR2A and each component of the myosin II complex. The phosphorylation status of NMIIB was determined after each phosphorylation reaction; panels B to D are representative of at least three independent determinations.

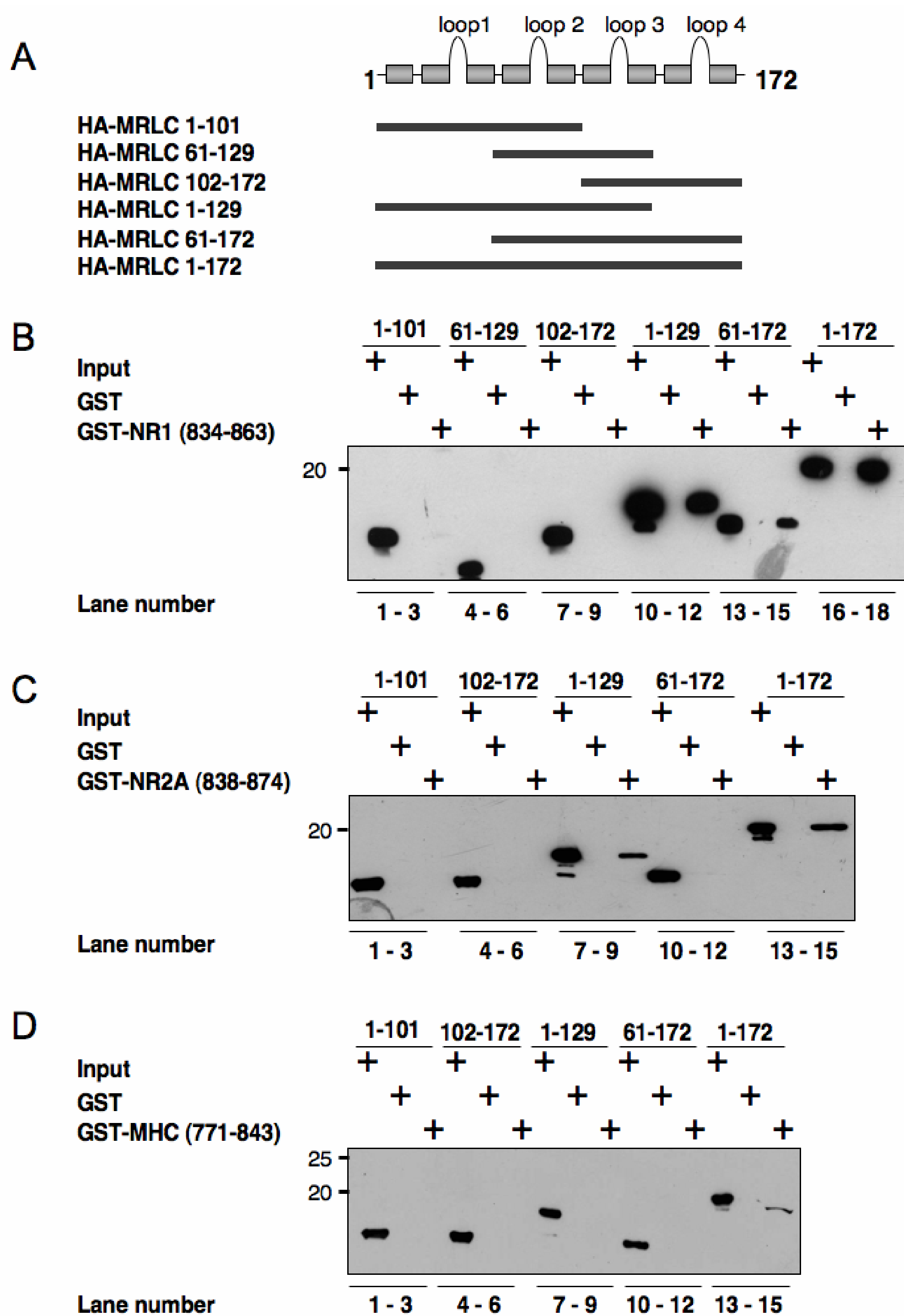

A
loop1
loop 2
loop 3
loop 4
1
172
HA-MRLC 1-101
HA-MRLC 61-129
HA-MRLC 102-172
HA-MRLC 1-129
HA-MRLC 61-172
HA-MRLC 1-172
B
1-101
61-129
102-172
1-129
61-172
1-172
Input
GST
GST-NR1 (834-863)
20
Lane number
1 - 3
4 - 6
7 - 9
10 - 12
13 - 15
16 - 18
C
1-101
102-172
1-129
61-172
1-172
Input
GST
GST-NR2A (838-874)
20
Lane number
1 - 3
4 - 6
7 - 9
10 - 12
13 - 15
D
1-101
102-172
1-129
61-172
1-172
Input
GST
GST-MHC (771-843)
25
20
Lane number
1 - 3
4 - 6
7 - 9
10 - 12
13 - 15

Figure 2.8 The interaction of myosin RLC with NMDA receptor target sequences can be distinguished from RLC-heavy chain interactions.

A. Schematic representation of a series of HA-tagged myosin RLC deletion mutants used in panels B to D. B. A truncated recombinant myosin RLC(1-129) is sufficient for NR1 (834-938) binding (compare lanes 12 and 18). C. The interaction of myosin RLC with NR2A (834-874) is qualitatively similar to that of NR1, inasmuch as myosin RLC(1-129) is sufficient for NR2A binding (compare lanes 9 and 15). D. Full-length myosin RLC is necessary for binding nonmuscle myosin II-B heavy chain (MHC); all deletion mutants of myosin RLC failed to bind the neck region of the heavy chain MHC (771-843). *Method*. Three RLC target sequences, NR1, NR2A and myosin heavy chain, fused to GST were tested for their ability to bind full-length and mutant RLCs. GST fusion proteins, or GST alone, were immobilized on glutathione-Sepharose beads and incubated at 4°C with rotation overnight in the presence of 1 mM magnesium with either full-length (MRLC 1-172), or truncated light chains: MRLC 1-101, MRLC 61-129, MRLC 102-172, MRLC 1-129, MRLC 61-172. Bound proteins were resolved by PAGE, and blotted to nitrocellulose. Immune complexes were revealed by anti-T-7 antibody (Novagen). Panels B to D are representative of three or four (NR2A) independent determinations.

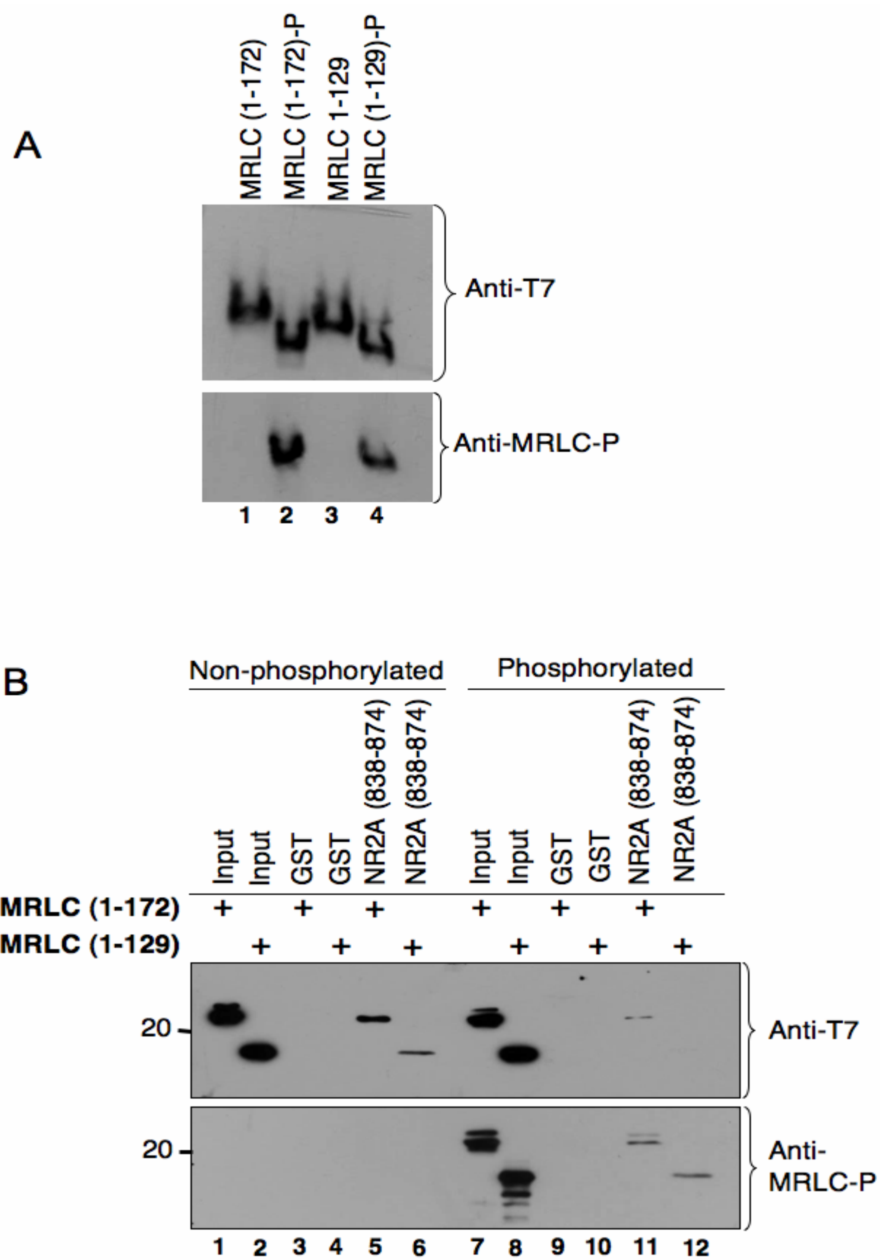

A
MRLC (1-172)
MRLC (1-172)-P
MRLC 1-129
MRLC (1-129)-P
Anti-T7
Anti-MRLC-P
1 2 3 4
B
Non-phosphorylated
Phosphorylated
Input
Input
GST
GST
NR2A (838-874)
NR2A (838-874)
Input
Input
GST
GST
NR2A (838-874)
NR2A (838-874)
MRLC (1-172)
MRLC (1-129)
20
Anti-T7
20
Anti-MRLC-P
1 2 3 4 5 6 7 8 9 10 11 12

Figure 2.9 Myosin RLC-NMDA receptor interactions are sensitive to phosphorylation by myosin light chain kinase.

A. Phosphorylation of recombinant proteins by myosin light chain kinase (MLCK) was verified after each reaction by PAGE in 8M urea followed by Coomassie staining to visualize recombinant proteins. Panel shows representative immunoblot of full-length (1-172) and truncated (1-129) myosin RLC (MRLC), before (lanes 1 and 3) and after (lanes 2 and 4) phosphorylation by MLCK *in vitro*. B. Following phosphorylation of RLC by MLCK, binding of myosin RLC to NR2A (838-874) was decreased relative to the non-phosphorylated state. Purified proteins (MRLC (1-172) and MRLC (1-129)) were phosphorylated in vitro and used in GST pull down experiments. NR2A (838-874) fused to GST, or GST alone, were immobilized on glutathione-Sepharose beads and incubated with non-phosphorylated (lanes 1 to 6) or phosphorylated (lanes 7 to12) forms of the isolated RLC at 4°C with rotation for 2 hours. Bound proteins were resolved by PAGE, transferred to nitrocellulose membranes and probed with an anti-T-7 antibody (Novagen) capable of detecting both forms of the RLC (upper panel), or an anti-phospho-specific antibody raised in the laboratory (anti-MRLC-P; lower panel). Panel B is representative of three independent determinations.

```
Consensus IQ2         IQXXXRGXXXR
Position            BXXBQXXBRGXXBRXXBXXXXXXXXXXXXX

                        1         8
NMHCIIA (809-851) MKVLQRNCAAYLKLRNWQWWRLFTKVKPLLQVSRQEEEMMAKE
NMHCIIB (816-858) LKVLQRNCAAYLKLRHWQWWRVFTKVKPLLQVTRQEEELQAKD
NMHCIIC (833-875) LRVMQRNCAAYLKLRHWQWWRLFTKVKPLLQVTRQDEVLQARA
NR2A (832-874)    LITFIWEHLFYWKLRFCFTGVCSDRPGLLFSISRGIYSCIHGV
NR2B (833-875)    LITFICEHLFYWQFRHCFMGVCSGKPGMVFSISRGIYSCIHGV
BSEP (653-695)    LVTLQSQGDNAHKETSIMGKDATEGGTLERTFSRGSYRDSLRA
```

Figure 2.10 Sequence alignment of putative RLC binding domains in nonmuscle myosin heavy chain isoforms, NMDAR2 subunits and Bile Salt Export Protein.

Amino acid sequences of incomplete IQ2 motifs in human nonmuscle myosin II heavy chain isoforms: II-A (accession number NP_002464), II-B (accession number NP_005955) and II-C (accession number Q7Z406), plus RLC binding motif found in rat NMDAR2A (accession number NP_036705), NMDAR2B (accession numberAAA50554) and Bile Salt Export Protein (BSEP; accession number O70127). A consensus IQ2 motif is shown in black, where B indicates positions usually occupied by hydrophobic amino acids and X is any amino acid. For nonmuscle myosin heavy chain isoforms, the N-terminal of myosin RLC is predicted to bind to a conserved target sequence WQWWRVFTKVPLL for II-B, underlined in the consensus IQ2. Arrows indicate: (1) the location of a tyrosine-based YXXXR motif beginning within the incomplete GXXXR portion of IQ2 that is conserved in all three heavy chains and both NMDA receptor subunits and, (2) the location of an arginine residue that is conserved in all six myosin RLC targets. Standard single amino acid codes are used for all amino acid residues; residues are coloured according to Clustal W (Higgins et al., 1996), whereby red denotes small + hydrophobic (AVFPMILW), blue denotes acidic residues (DE), magenta denotes basic residues (RHK) and green denotes QSTYHCNG.

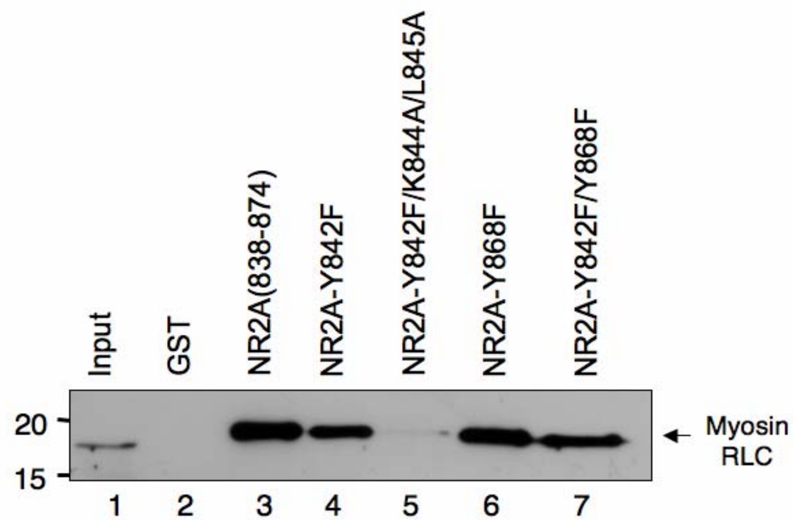

A
NR2A (838-874) EHLFYWKLRFCFTGVCSDRPGLLFSISRGIYSCIHGV
Y842F ----F--------------------------------
Y842F/K844A/L845A ----F-AA------------------------------
Y868F ----F-------------------------F------
Y842F/Y868F ----F-------------------------F------
B
Input
GST
NR2A(838-874)
NR2A-Y842F
NR2A-Y842F/K844A/L845A
NR2A-Y868F
NR2A-Y842F/Y868F
20
15
Myosin RLC
1 2 3 4 5 6 7

Figure 2.11 Residues within a tyrosine-based endocytic motif associated with endocytosis of the NMDAR2A subunit are critical for myosin RLC binding.

A. Schematic representation of site mutants used in panel B. The location of two tyrosine-based endocytic motifs within NR2A (838-874) is shown in bold typeface. The location of residues targeted for site-directed mutagenesis is listed below the wild-type sequence of NR2A. B. Residues K844 and L845 in the membrane proximal region of NR2A are critical for myosin RLC binding. NR2A (838-874), NR2A (838-874)Y842F, NR2A (838-874)Y842F/K844A/L845A, NR2A (838-874)Y868F and NR2A (838-874)Y842F/Y868F were expressed as GST fusion proteins. NR2A GST fusion proteins, or GST alone, were immobilized on glutathione-Sepharose beads and used as baits to pull down myosin light chains from a mouse brain extract. Assays were initiated by the addition of soluble forebrain extract (1 mg) and incubated with rotation overnight at 4°C. Bound proteins was resolved by PAGE and blotted to nitrocellulose. Immune complexes were revealed using an anti-myosin RLC antibody (Amparan et al., 2005). Autoradiographs are representative of three independent determinations (from three different animals) using all five NR mutants fused to GST; various combinations of NR2A mutants fused to GST were tested more frequently.

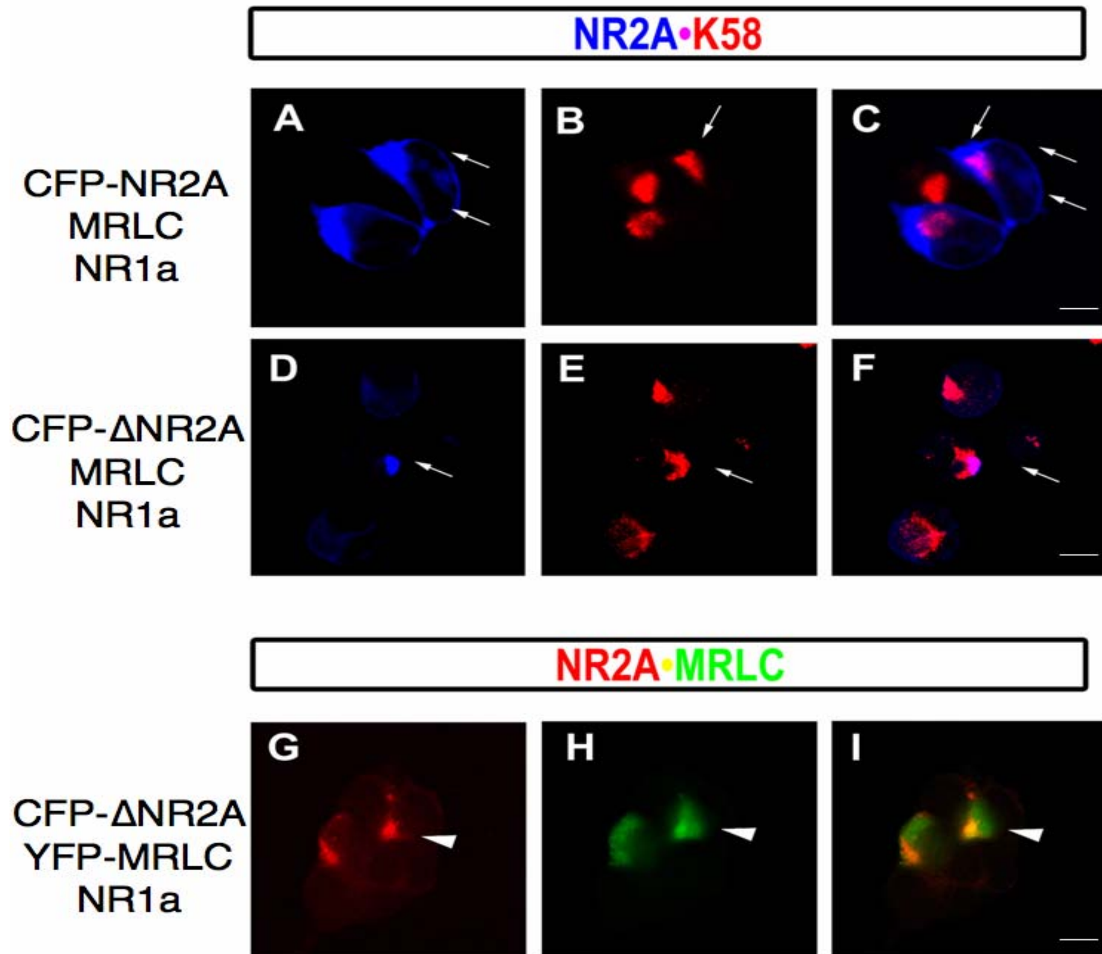

NR2A•K58
A
B
C
CFP-NR2A
MRLC
NR1a
D
E
F
CFP-ΔNR2A
MRLC
NR1a
NR2A•MRLC
G
H
I
CFP-ΔNR2A
YFP-MRLC
NR1a

Figure 2.12 Disruption of the myosin RLC binding site on NMDAR2A leads to a trafficking defect in NR1/NR2A assemblies.

Co-expression of NR1, myosin RLC and CFP-tagged NR2A (row 1, panels A-C) in heterologous cells revealed membrane-associated expression of CFP-NR2A twenty-four hours post transfection (paired arrows in panels A and C). In contrast, the RLC-deficient mutant CFP-ΔNR2A (CFP-NR2A-Y842F/K844A/L845A; row 2, panels D-F) showed a restricted cytosolic expression pattern within a region that was positive for the Golgi-specific marker protein K58 (panels E and F) at twenty-four hours post transfection. The restricted expression of CFP-ΔNR2A was congruent with YFP-myosin RLC (YFP-MRLC) despite the presence of NR1 (row 3, panels G-I) at twenty-four hours post transfection. In addition, a separate zone of YFP-myosin RLC fluorescence is also present (see arrow head panels G-I). *Method.* HEK293 cells grown on glass coverslips were transiently transfected with: plasmids encoding full-length myosin RLC (MRLC), NMDAR1 (NR1a) and either wild-type NMDAR2A (CFP-NR2A; row 1) or NMDAR2A-Y842F/K844A/L845A (CFP-ΔNR2A; row 2) and grown under standard conditions in medium supplemented with kynurenic acid (3 mM) and D,L-AP5 (1 mM). Cells were fixed and counterstained with an anti-K58 antibody (Sigma) to visualize the Golgi complex (rows 1 and 2). Cells were also transfected with plasmids encoding NMDAR1 (NR1a), myosin RLC (YFP-MRLC) and either wild-type NMDAR2A (not shown) or NMDAR2A-Y842F/K844A/L845A (CFP-ΔNR2A; row 3). Confocal images were captured using a Zeiss LSM510 confocal microscope with a Zeiss 63× oil immersion objective. Scale bar = 5 μm.

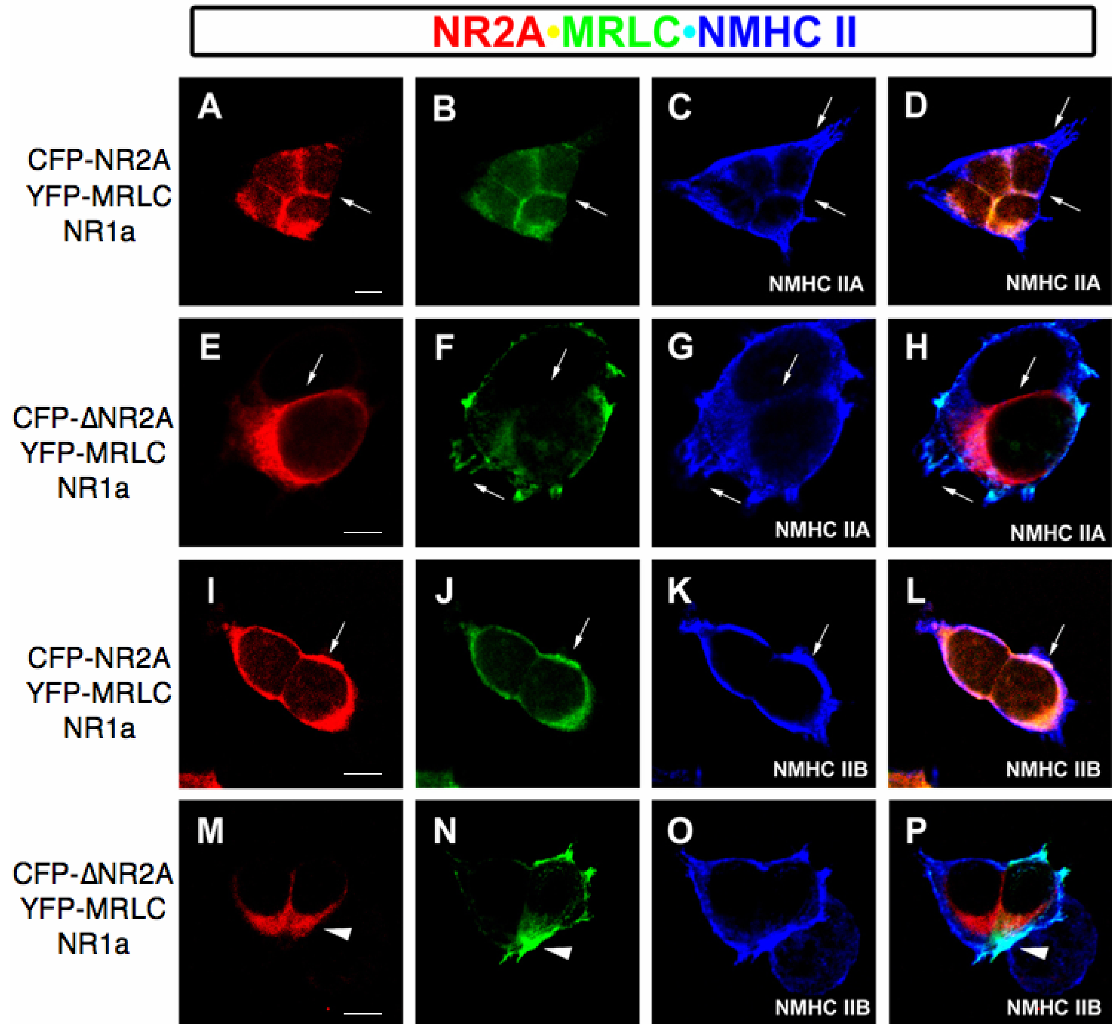

NR2A•MRLC•NMHC II
A
B
C
D
CFP-NR2A
YFP-MRLC
NR1a
NMHC IIA
NMHC IIA
E
F
G
H
CFP-ΔNR2A
YFP-MRLC
NR1a
NMHC IIA
NMHC IIA
I
J
K
L
CFP-NR2A
YFP-MRLC
NR1a
NMHC IIB
NMHC IIB
M
N
O
P
CFP-ΔNR2A
YFP-MRLC
NR1a
NMHC IIB
NMHC IIB

Figure 2.13 Myosin RLC relocates to cellular extensions containing endogenous myosin heavy chain isoforms following disruption of NMDAR2A binding.

In the presence of a RLC-deficient NR2A mutant (CFP-NR2A-Y842F/K844A/L845A), the expression of RLC (YFP-MRLC) changes to a pattern that mimics myosin heavy chain II-A (see regions that appear teal-colored in panel H) or myosin heavy chain II-B (see regions that appear teal-colored in panel P). *Method.* HEK293 cells grown on glass coverslips were transiently transfected with plasmids encoding: either wild-type NMDAR2A (CFP-NR2A; rows 1 and 3) or NMDAR2A-Y842F/K844A/L845A (CFP-ΔNR2A; rows 2 and 4), plus full-length YFP-myosin RLC (YFP-MRLC) and NMDAR1 (NR1a) and grown under standard conditions in medium supplemented with kynurenic acid (3mM) and D,L-AP5 (1mM). Thirty hours following transfection cells were fixed and counterstained with isoform specific antibodies to either nonmuscle myosin heavy chain II-A (rows 1 and 2) or nonmuscle myosin heavy chain II-B (rows 3 and 4) both from Covance. Confocal images were captured using a Zeiss LSM510 confocal microscope with a Zeiss 63× oil immersion objective. Scale bar = 5 μm.

References

Akyol Z, Bartos JA, Merrill MA, Faga LA, Jaren OR, Shea MA and Hell JW (2004) Apo-calmodulin binds with its C-terminal domain to the N-methyl-D-aspartate receptor NR1 C0 region. *J Biol Chem* **279**(3):2166-2175.

Amparan D, Avram D, Thomas CG, Lindahl MG, Yang J, Bajaj G and Ishmael JE (2005) Direct interaction of myosin regulatory light chain with the NMDA receptor. *J Neurochem* **92**:349-361.

Bahler M and Rhoads A (2002) Calmodulin signaling via the IQ motif. *FEBS Lett* **513**(1):107-113.

Bezanilla M, Forsburg SL and Pollard TD (1997) Identification of a second myosin-II in Schizosaccharomyces pombe: Myp2p is conditionally required for cytokinesis. *Mol Biol Cell* **8**(12):2693-2705.

Bornhauser BC, Johansson C and Lindholm D (2003) Functional activities and cellular localization of the ezrin, radixin, moesin (ERM) and RING zinc finger domains in MIR. *FEBS Lett* **553**(1-2):195-199.

Boyne JR, Yosuf HM, Bieganowski P, Brenner C and Price C (2000) Yeast myosin light chain, Mlc1p, interacts with both IQGAP and class II myosin to effect cytokinesis. *J Cell Sci* **113 Pt 24**:4533-4543.

Chan W, Calderon G, Swift AL, Moseley J, Li S, Hosoya H, Arias IM and Ortiz DF (2005) Myosin II regulatory light chain is required for trafficking of bile salt export protein (BSEP) to the apical membrane in MDCK cells. *J Biol Chem* **280**(25):23741-23747.

Cheng D, Hoogenraad CC, Rush J, Ramm E, Schlager MA, Duong DM, Xu P, Wijayawardana SR, Hanfelt J, Nakagawa T, Sheng M and Peng J (2006) Relative and absolute quantification of postsynaptic density proteome isolated from rat forebrain and cerebellum. *Mol Cell Proteomics* **5**(6):1158-1170.

Collins MO, Husi H, Yu L, Brandon JM, Anderson CN, Blackstock WP, Choudhary JS and Grant SG (2006) Molecular characterization and comparison of the components and multiprotein complexes in the postsynaptic proteome. *J Neurochem* **97 Suppl 1**:16-23.

Conti MA and Adelstein RS (2008) Nonmuscle myosin II moves in new directions. *J Cell Sci* **121**(Pt 1):11-18.

Cremo CR, Wang F, Facemyer K and Sellers JR (2001) Phosphorylation-dependent regulation is absent in a nonmuscle heavy meromyosin construct with one complete head and one head lacking the motor domain. *J Biol Chem* **276**(44):41465-41472.

Ehlers MD, Zhang S, Bernhadt JP and Huganir RL (1996) Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* **84**(5):745-755.

Espindola FS, Suter DM, Partata LB, Cao T, Wolenski JS, Cheney RE, King SM and Mooseker MS (2000) The light chain composition of chicken

brain myosin-Va: calmodulin, myosin-II essential light chains, and 8-kDa dynein light chain/PIN. *Cell Motil Cytoskeleton* **47**(4):269-281.
Fath KR (2005) Characterization of myosin-II binding to Golgi stacks in vitro. *Cell Motil Cytoskeleton* **60**(4):222-235.
Feinstein DL, Durand M and Milner RJ (1991) Expression of myosin regulatory light chains in rat brain: characterization of a novel isoform. *Brain Res Mol Brain Res* **10**(2):97-105.
Franke JD, Boury AL, Gerald NJ and Kiehart DP (2006) Native nonmuscle myosin II stability and light chain binding in Drosophila melanogaster. *Cell Motil Cytoskeleton* **63**(10):604-622.
Golomb E, Ma X, Jana SS, Preston YA, Kawamoto S, Shoham NG, Goldin E, Conti MA, Sellers JR and Adelstein RS (2004) Identification and characterization of nonmuscle myosin II-C, a new member of the myosin II family. *J Biol Chem* **279**(4):2800-2808.
Guillaud L, Setou M and Hirokawa N (2003) KIF17 dynamics and regulation of NR2B trafficking in hippocampal neurons. *J Neurosci* **23**(1):131-140.
Hawkins LM, Prybylowski K, Chang K, Moussan C, Stephenson FA and Wenthold RJ (2004) Export from the endoplasmic reticulum of assembled N-methyl-d-aspartic acid receptors is controlled by a motif in the c terminus of the NR2 subunit. *J Biol Chem* **279**(28):28903-28910.
Higgins DG, Thompson JD and Gibson TJ (1996) Using CLUSTAL for multiple sequence alignments. *Methods Enzymol* **266**:383-402.
Houdusse A and Cohen C (1995) Target sequence recognition by the calmodulin superfamily: implications from light chain binding to the regulatory domain of scallop myosin. *Proc Natl Acad Sci U S A* **92**(23):10644-10647.
Houdusse A and Cohen C (1996) Structure of the regulatory domain of scallop myosin at 2 A resolution: implications for regulation. *Structure* **4**(1):21-32.
Husi H, Ward MA, Choudhary JS, Blackstock WP and Grant SG (2000) Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. *Nat Neurosci* **3**(7):661-669.
Ishmael JE, Franklin PH, Murray TF and Leid M (1996) High level expression of the NMDAR1 glutamate receptor subunit in electroporated COS cells. *J Neurochem* **67**(4):1500-1510.
Ishmael JE, Safic M, Amparan D, Vogel WK, Pham T, Marley K, Filtz TM and Maier CS (2007) Nonmuscle myosins II-B and Va are components of detergent-resistant membrane skeletons derived from mouse forebrain. *Brain Res*.
Kawasaki H, Nakayama S and Kretsinger RH (1998) Classification and evolution of EF-hand proteins. *Biometals* **11**(4):277-295.
Kioussi C, Appu M, Lohr CV, Fischer KA, Bajaj G, Leid M and Ishmael JE (2007) Co-expression of myosin II regulatory light chain and the

NMDAR1 subunit in neonatal and adult mouse brain. *Brain Res Bull* **74**(6):439-451.

Knowlton MN, Chan BM and Kelly GM (2003) The zebrafish band 4.1 member Mir is involved in cell movements associated with gastrulation. *Dev Biol* **264**(2):407-429.

Knowlton MN and Kelly GM (2004) Zebrafish mir antagonizes frizzled 7-induced gastrulation defects. *Zebrafish* **1**(2):133-144.

Kornau HC, Schenker LT, Kennedy MB and Seeburg PH (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* **269**(5231):1737-1740.

Krupp JJ, Vissel B, Thomas CG, Heinemann SF and Westbrook GL (1999) Interactions of calmodulin and alpha-actinin with the NR1 subunit modulate Ca2+-dependent inactivation of NMDA receptors. *J Neurosci* **19**(4):1165-1178.

Krupp JJ, Vissel B, Thomas CG, Heinemann SF and Westbrook GL (2002) Calcineurin acts via the C-terminus of NR2A to modulate desensitization of NMDA receptors. *Neuropharmacology* **42**(5):593-602.

Lei S, Czerwinska E, Czerwinski W, Walsh MP and MacDonald JF (2001) Regulation of NMDA receptor activity by F-actin and myosin light chain kinase. *J Neurosci* **21**(21):8464-8472.

Luo J, Vallen EA, Dravis C, Tcheperegine SE, Drees B and Bi E (2004) Identification and functional analysis of the essential and regulatory light chains of the only type II myosin Myo1p in Saccharomyces cerevisiae. *J Cell Biol* **165**(6):843-855.

Maeda M, Kuwayama H, Yokoyama M, Nishio K, Morio T, Urushihara H, Katoh M, Tanaka Y, Saito T, Ochiai H, Takemoto K, Yasukawa H and Takeuchi I (2000) Developmental changes in the spatial expression of genes involved in myosin function in Dictyostelium. *Dev Biol* **223**(1):114-119.

Malencik DA and Anderson SR (1982) Binding of simple peptides, hormones, and neurotransmitters by calmodulin. *Biochemistry* **21**(14):3480-3486.

Malencik DA and Anderson SR (1988) Association of melittin with the isolated myosin light chains. *Biochemistry* **27**(6):1941-1949.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B and Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science* **256**(5060):1217-1221.

Nagano K, Bornhauser BC, Warnasuriya G, Entwistle A, Cramer R, Lindholm D and Naaby-Hansen S (2006) PDGF regulates the actin cytoskeleton through hnRNP-K-mediated activation of the ubiquitin E3-ligase MIR. *EMBO J* **25**(9):1871-1882.

Olsson PA, Bornhauser BC, Korhonen L and Lindholm D (2000) Neuronal expression of the ERM-like protein MIR in rat brain and its localization

to human chromosome 6. *Biochem Biophys Res Commun* **279**(3):879-883.

Olsson PA, Korhonen L, Mercer EA and Lindholm D (1999) MIR is a novel ERM-like protein that interacts with myosin regulatory light chain and inhibits neurite outgrowth. *J Biol Chem* **274**(51):36288-36292.

Rayment I, Rypniewski WR, Schmidt-Base K, Smith R, Tomchick DR, Benning MM, Winkelmann DA, Wesenberg G and Holden HM (1993) Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science* **261**(5117):50-58.

Ryu J, Liu L, Wong TP, Wu DC, Burette A, Weinberg R, Wang YT and Sheng M (2006) A critical role for Myosin IIb in dendritic spine morphology and synaptic function. *Neuron* **49**(2):175-182.

Scott DB, Michailidis I, Mu Y, Logothetis D and Ehlers MD (2004) Endocytosis and degradative sorting of NMDA receptors by conserved membrane-proximal signals. *J Neurosci* **24**(32):7096-7109.

Shannon KB and Li R (2000) A myosin light chain mediates the localization of the budding yeast IQGAP-like protein during contractile ring formation. *Curr Biol* **10**(12):727-730.

Stevens RC and Davis TN (1998) Mlc1p is a light chain for the unconventional myosin Myo2p in Saccharomyces cerevisiae. *J Cell Biol* **142**(3):711-722.

Szymanski PT (2004) Calponin (CaP) as a latch-bridge protein--a new concept in regulation of contractility in smooth muscles. *J Muscle Res Cell Motil* **25**(1):7-19.

Szymanski PT and Goyal RK (1999) Calponin binds to the 20-kilodalton regulatory light chain of myosin. *Biochemistry* **38**(12):3778-3784.

Takahashi M, Kawamoto S and Adelstein RS (1992) Evidence for inserted sequences in the head region of nonmuscle myosin specific to the nervous system. Cloning of the cDNA encoding the myosin heavy chain-B isoform of vertebrate nonmuscle myosin. *J Biol Chem* **267**(25):17864-17871.

Taubman MB, Grant JW and Nadal-Ginard B (1987) Cloning and characterization of mammalian myosin regulatory light chain (RLC) cDNA: the RLC gene is expressed in smooth, sarcomeric, and nonmuscle tissues. *J Cell Biol* **104**(6):1505-1513.

Terrak M, Otterbein LR, Wu G, Palecanda LA, Lu RC and Dominguez R (2002) Crystallization, X-ray characterization and selenomethionine phasing of Mlc1p bound to IQ motifs from myosin V. *Acta Crystallogr D Biol Crystallogr* **58**(Pt 10 Pt 2):1882-1885.

Terrak M, Wu G, Stafford WF, Lu RC and Dominguez R (2003) Two distinct myosin light chain structures are induced by specific variations within the bound IQ motifs-functional implications. *Embo J* **22**(3):362-371.

Vissel B, Krupp JJ, Heinemann SF and Westbrook GL (2001) A use-dependent tyrosine dephosphorylation of NMDA receptors is independent of ion flux. *Nat Neurosci* **4**(6):587-596.

Vissel B, Krupp JJ, Heinemann SF and Westbrook GL (2002) Intracellular domains of NR2 alter calcium-dependent inactivation of N- methyl-D-aspartate receptors. *Mol Pharmacol* **61**(3):595-605.

Wakabayashi Y, Kipp H and Arias IM (2006) Transporters on demand: intracellular reservoirs and cycling of bile canalicular ABC transporters. *J Biol Chem* **281**(38):27669-27673.

Wang F, Kovacs M, Hu A, Limouze J, Harvey EV and Sellers JR (2003) Kinetic mechanism of non-muscle myosin IIB: functional adaptations for tension generation and maintenance. *J Biol Chem* **278**(30):27439-27448.

Wang Y and Chantler PD (1994) Functional analysis of individual brain myosin II isoforms through hybrid formation. *FEBS Lett* **348**(3):244-248.

Washbourne P, Bennett JE and McAllister AK (2002) Rapid recruitment of NMDA receptor transport packets to nascent synapses. *Nat Neurosci* **5**(8):751-759.

Wechsler A and Teichberg VI (1998) Brain spectrin binding to the NMDA receptor is regulated by phosphorylation, calcium and calmodulin. *Embo J* **17**(14):3931-3939.

Weissbach L, Bernards A and Herion DW (1998) Binding of myosin essential light chain to the cytoskeleton-associated protein IQGAP1. *Biochem Biophys Res Commun* **251**(1):269-276.

Wenthold RJ, Prybylowski K, Standley S, Sans N and Petralia RS (2003) Trafficking of nmda receptors. *Annu Rev Pharmacol Toxicol* **43**:335-358.

Wyszynski M, Lin J, Rao A, Nigh E, Beggs AH, Craig AM and Sheng M (1997) Competitive binding of alpha-actinin and calmodulin to the NMDA receptor. *Nature* **385**(6615):439-442.

Yang W, Zheng C, Song Q, Yang X, Qiu S, Liu C, Chen Z, Duan S and Luo J (2007) A three amino acid tail following the TM4 region of the N-methyl-D-aspartate receptor (NR) 2 subunits is sufficient to overcome endoplasmic reticulum retention of NR1-1a subunit. *J Biol Chem* **282**(12):9269-9278.

Zavodny PJ, Petro ME, Kumar CC, Dailey SH, Lonial HK, Narula SK and Leibowitz PJ (1988) The nucleotide sequence of chicken smooth muscle myosin light chain two. *Nucleic Acids Res* **16**(3):1214.

Zegers MM and Hoekstra D (1998) Mechanisms and functional features of polarized membrane traffic in epithelial and hepatic cells. *Biochem J* **336 (Pt 2)**:257-269.

Zhang H, Webb DJ, Asmussen H, Niu S and Horwitz AF (2005) A GIT1/PIX/Rac/PAK signaling module regulates spine morphogenesis and synapse formation through MLC. *J Neurosci* **25**(13):3379-3388.

Zhang S, Ehlers MD, Bernhardt JP, Su CT and Huganir RL (1998) Calmodulin mediates calcium-dependent inactivation of N-methyl-D- aspartate receptors. *Neuron* **21**(2):443-453.

Zhang Y, Vogel WK, McCullar JS, Greenwood JA and Filtz TM (2006) Phospholipase C-beta3 and -beta1 form homodimers, but not heterodimers, through catalytic and carboxyl-terminal domains. *Mol Pharmacol* **70**(3):860-868.

CHAPTER 3

MDR1/P-GLYCOPROTEIN EXPRESSION AND FUNCTION IS SENSITIVE TO THE PHOSPHORYLATION STATE OF A MYOSIN II LIGHT CHAIN

Gaurav Bajaj, Ying Fan, Anand Venkataraman, Rosita R. Rodriguez-Proteau, Chrissa Kioussi and Jane E. Ishmael

In preparation for submission to “Toxicology and Applied Pharmacology”

Abstract

Multiple drug resistant protein 1 (MDR1 or P-glycoprotein) is a member of the family of ATP-binding cassette (ABC) drug efflux transporters localized in the apical membrane of brain capillary endothelial cells. Recent evidence suggests that myosin II regulatory light chain (RLC) is required for trafficking of bile salt export protein (BSEP), an ABC drug efflux transporter, in polarized cells. RLC has also been reported to be localized in endothelial cells of cerebral vasculature and previously shown to interact directly with C terminals of NMDA receptor subunits in the brain. The interaction of RLC with MDR1 linker region is qualitatively similar to the interaction of RLC with NMDA receptor subunits, in that RLC binds directly to the MDR1 linker region via amino terminal of light chain. This interaction is sensitive to the phosphorylation state of RLC as phosphorylation of RLC by myosin light chain kinase (MLCK) resulted in a loss of binding to both MDR1 and NMDAR1 *in vitro*. Using Madin-Darby canine kidney (MDCK) cells stably expressing MDR1 (MDCKII-MDR1) as a model system to study the consequences of disrupting the phosphorylation state of myosin RLC in whole cells, we treated the cells with a pharmacologic inhibitor of MLCK, ML-7. Treatment of polarized MDCKII-MDR1 monolayers with ML-7: (1) increased the permeability of [^{3}H]-digoxin, a well-known substrate of MDR1, and (2) decreased apical expression of MDR1. These preliminary studies provide understanding of factors that may regulate trafficking and cell surface expression of MDR1 in drug refractory epilepsy.

Introduction

Under normal physiological conditions members of the ATP-binding cassette (ABC) family of efflux transporters are responsible for the translocation of a variety of endogenous molecules, ions and potentially harmful metabolites across plasma and endo-membranes (Loscher and Potschka, 2005a; Marchi et al., 2004; Sisodiya et al., 2006; Sisodiya and

Thom, 2003). A subset of these transporters, collectively known as multi-drug resistance (MDR) proteins, has broad substrate specificity that effectively serves as an endogenous protective mechanism against toxic insult to the cell. This property, however, presents a considerable challenge to many drug treatment regimens in that the bioavailability of drugs, that are also substrates for MDR transporters, can be dramatically reduced by the presence of an active efflux mechanism. In the most extreme cases, efflux of multiple classes of structurally diverse pharmaceuticals, coupled with pathological increases in cell surface expression of MDR proteins can render conditions such as cancer, infectious disease and epilepsy resistant to treatment with the drugs that are currently available.

MDR protein 1 (MDR1), also known as P-glycoprotein or ABCB1, is considered the prototypical ABC transporter associated with drug resistance. MDR1 is composed of two homologous halves, each consisting of six membrane-spanning domains plus a nucleotide binding domain, which are linked by an intracellular linker region (Chen et al., 1986; Germann, 1996; Hrycyna et al., 1998; Rao et al., 2006). The linker region of MDR1 contains approximately seventy-five amino acids and is essential to both the transport function and ATPase activity of MDR1 (Hrycyna et al., 1998; Nuti and Rao, 2002). The study of other ABC transporter family members, including MDR1, in a variety of cell types suggests that the linker region may also be a determinant of cell surface expression (Chan et al., 2005; Hrycyna et al., 1998; Kolling and Losko, 1997). The identification of several intracellular cytoskeletal, regulatory and motor proteins as direct binding partners of the linker peptide has continued to support this idea (Chan et al., 2005; Georges, 2007; Ortiz et al., 2004; Rao et al., 2006). For example, linker-interacting proteins such as HAX-1 (a cortactin-binding protein) and myosin II regulatory light chain (RLC) have been implicated in clathrin-mediated endocytosis and apical trafficking of Bile Salt Export Protein (BSEP), respectively (Chan et al.,

2005; Ortiz et al., 2004). Although originally characterized with respect to their role in apical expression of BSEP, yeast two-hybrid and biochemical studies suggest that HAX-1 and myosin RLC may represent components of trafficking pathways that are common to other members of the ABC transporter family. The identification of myosin RLC as a binding partner of BSEP, MDR1 and MDR2 was particularly intriguing as we had independently identified the same myosin II light chain as a direct binding partner of subunits belonging to the N-methyl-D-aspartate (NMDA) family of neuronal glutamate-gated ion channels (Amparan et al., 2005).

Myosin II motors are hexameric complexes that consist of two myosin heavy chains, two essential light chains and two regulatory light chains (Bresnick, 1999). Although traditionally associated with muscle contraction, myosin II is also expressed in non-muscle cells where it contributes to cell motility, adhesion, shape and polarity. In addition, myosin II is one of several myosin motors that have been implicated in short-range, actin-based vesicular trafficking events (Allan et al., 2002; DePina and Langford, 1999; DePina et al., 2007; Ikonen et al., 1997; Musch et al., 1997). Chan and coworkers were the first to link forward trafficking of newly synthesized BSEP to a direct interaction with the RLC of myosin II, thus raising the possibility that the RLC and the myosin II complex performs a similar function with other binding partners. In the present study we used biochemical techniques to compare myosin RLC binding to two unrelated target proteins: the linker region of MDR1 and the C-terminus of the NMDAR1 subunit. We report that binding of myosin RLC to MDR1 occurs via the amino terminal of myosin RLC, and that the interaction of myosin RLC with both binding partners is decreased by phosphorylation of specific residues in the amino terminal of the light chain. Using Madin-Darby canine kidney (MDCK) cells stably expressing MDR1 (MDCKII-MDR1) as a model system, we examined the consequences of disrupting the phosphorylation state of myosin RLC in whole cells. Treatment

of polarized MDCKII-MDR1 monolayers with ML-7, a pharmacologic inhibitor of MLCK, altered both the transport function and apical expression of MDR1. Our findings complement and extend previous studies of the role of myosin RLC in BSEP trafficking, in that the permeability of an MDR1 substrate was enhanced by disrupting myosin II motor function in polarized epithelial cells.

Materials and Methods

Plasmids and constructs

Full-length human MDR1 cDNA was obtained from the American Type Culture Collection (GenBank accession no. M14758). For bacterial expression, a fragment encoding the linker region of MDR1 (amino acid residues 633 to 709) was amplified by PCR and inserted into the expression vector pGEX-6P-3 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Deletion mutants of myosin RLC were prepared from the mouse brain pACT2/5-6-1 clone (Amparan et al., 2005); fragments encoding amino acid residues 1-101, 102-172, 1-129 and 61-172 were amplified by PCR and inserted into the bacterial expression vector pET 28a (Novagen, Madison, WI) to yield MRLC(1-101), MRLC(102-172), MRLC(1-129) and MRLC(61-172), respectively. The construction of full-length myosin RLC (1-172) in pET 28a, and the C-terminus of the NMDAR1 subunits (amino acid residues 834-938) in pGEX-2T have been described previously (Amparan et al., 2005). The C-terminus of the acid-sensing ion channel (ASIC) 2a expressed in pGEX-6P-3 was the generous gift of Dr. Julie Saugstad (Robert S. Dow Neurobiology Laboratories, Portland, OR). All sequences were confirmed by the Center for Genome Research and Biocomputing core facility (Oregon State University, Corvallis, OR).

Antibodies and fluorescent reagents

The anti-myosin RLC antibody (αMRLC/3) used in this study has been described previously (Amparan et al., 2005). The phosphorylated form of myosin RLC was detected using an antibody raised in rabbits against a unique phosphopeptide (CRPQRATS-PO3-NVFAM) targeting serine residue 19 and affinity purified (αMRLC/P) (Bethyl Laboratories Inc., Montgomery, TX). Additional antibodies were purchased from commercial sources and included: anti-MDR1 (anti-ABCB1 MAb/ C219; Axxora, LLC, San Diego, CA, USA), anti-T7 (Novagen, Madison, WI) and horseradish peroxidase-conjugated secondary antibodies for immunoblot analyses (Calbiochem, San Diego, CA). Alexa Fluor 546- and Alexa Fluor 488-conjugated secondary antibodies, and 4',6-diamidino-2-phenylindole (DAPI) stain for fluorescence microscopy were obtained from Molecular Probes, (Eugene, OR).

Chemicals and Reagents

Radiolabelled [^{3}H]-digoxin (23.4 Ci/mmol, >97% purity) was obtained from Amersham, Inc. (Piscataway, NJ, USA). 1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7), verapamil and unlabelled digoxin were purchased from Sigma, Inc. (St Louis, MO, USA). (-)-Blebbistatin, the active enantiomer of (±)-blebbistatin, and calmodulin (for phosphorylation reactions) were purchased from Calbiochem (San Diego, CA, USA). Smooth muscle myosin light chain kinase (MLCK) was a kind gift from Dr. Sonia Anderson (Biochemistry and Biophysics, Oregon State University, Corvallis, OR). All stock solutions were prepared in the appropriate vehicle on the day of the experiment; ML-7 and verapamil stock solutions were prepared in DMSO and 50% ethanol, respectively. In all studies the final concentration of DMSO or ethanol did not exceed 0.1% v/v or 0.5% v/v, respectively.

Purification and phosphorylation of recombinant myosin RLC

Recombinant pET28a/MRLC was expressed in *Escherichia coli* (BL21-Gold(DE3)pLysS; Stratagene (La Jolla, CA, USA), and purified by nickel chelate chromatography. The poly-histidine tag was removed by thrombin

cleavage (Thrombin Clean Cleave Kit; Sigma-Aldrich Corp., St. Louis, MO) followed by dialysis against a buffer containing 20 mM Tris (pH 7.7) and 500 mM NaCl. Purified light chain was phosphorylated in the presence of smooth muscle MLCK. Briefly, purified light chain was added to a buffer containing 20 mm Tris (pH 7.5), 0.1 mM EGTA, 1 mM DTT, 10 µg/ml MLCK, 10 µg/ml calmodulin, 5 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 1 mM ATP, and incubated at 37°C for 1 hour. Phosphorylation of each sample was verified by polyacrylamide gel electrophoresis (PAGE) with control samples (either nonphosphorylated, or mock phosphorlylated lacking MLCK). Samples were heated at 80°C for 2 minutes in urea sample buffer (8 M urea, 33 mM Tris-glycine pH 8.6, 0,17 mM EDTA and bromophenol blue), before loading on NOVEX Tris-Glycine pre-cast gels (Invitrogen, Carlsbad, CA). Protein was visualized directly by Coomassie blue stain (BioRad Laboratories, Hercules, CA), or were electro-transferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) and detected by immunoblot with T7-tag antibody (RLC), αMRLC/P (phosphorylated RLC) and appropriate horseradish peroxidase-conjugated secondary antibodies. Immune complexes were revealed using a chemiluminescence assay (Roche, Indianapolis, IN).

Glutathione-S-transferase (GST) pull-down assays

Regions of MDR1, NMDAR1 or ASIC2a fused to GST were expressed in *E. coli* (BL21-Gold(DE3)pLysS and tested for their ability to interact with either recombinant myosin RLC or native myosin RLC derived from mouse brain (Amparan et al., 2005). Equal amounts of GST alone or GST fusion proteins were immobilized on Glutathione–sepharose beads and prequilibrated in a binding buffer consisting of 10 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% NP-40 and 10% glycerol. For direct protein-protein binding studies, assays were initiated by the addition of either phosphorylated (100 nM) or non-phosphoryated myosin RLC (100 nM). The reaction allowed to proceed for 2 hours at 4°C with gentle rotation.

For pull-down assays from brain homogenates, cortex or hippocampi were pooled from three adult Swiss Webster mice and homogenized in a buffer containing (150 mM NaCl, 25 mM Tris, 5 mM EDTA, 5 mM EGTA, 10 mM ATP, 5 mM DTT). Assays were initiated by the addition of soluble hippocampal or cortical protein (1 mg) and incubated with gentle rotation overnight at 4°C. At the end on the incubation period unbound proteins were removed by three sequential washes with binding buffer. Bound proteins, retained on the beads, were eluted by boiling in sample buffer. These proteins were separated by sodium-dodecyl sulfate (SDS)-PAGE, and transferred to nitrocellulose membranes for immunoblot analyses. Recombinant myosin RLC was detected with either an anti-T7-tag antibody (RLC) or αMRLC/P. Native myosin RLC was detected with αMRLC/3.

Mammalian cell culture

MDCKII-MDR1 cells, a kind gift from Dr. Piet Borst (The Netherlands Cancer Research Institute), were grown in Dulbecco's Modified Eagle medium (DMEM obtained from Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Hyclone; Logan, UT, USA), plus 0.01% penicillin/streptomycin. Cells were maintained at 37°C, with 5% CO_2 and 95% relative humidity under standard conditions. For transport studies and expression analyses, cells were seeded at 3 x 10^5 cells/well onto six-well Transwell® plates with inserts (Corning; Lowell, MA, USA) approximately 7 days prior to use. The cells were allowed to grow to confluency and maintained for a further 3 days to ensure polarization.

Immunofluorescence microscopy

For expression analyses, MDCKII-MDR1 cells were maintained and allowed to polarize on Transwell® inserts as described above either on "sister" plates or exactly as described for transport studies. For imunocytochemistry, each insert was carefully excised with a clean, sharp scalpel and gently washed twice with PBS. Cells were fixed and permeabilized by a sequential

paraformaldhyde-methanol fixation according to the following schedule: 4% paraformaldhyde for 12 minutes at room temperature, gentle washing two times with PBS followed by a 2 minute fixation in ice cold-methanol. Membranes were treated with 0.2 % Triton-X for 5 minutes, washed and blocked with goat serum. This was followed by incubation with anti-MDR1 and/or anti-MRLC for 1 hour at room temperature. Alexa fluor 546 (red) and alexa fluor 488 (green) conjugated to respective secondary antibodies were used. Membranes were then mounted and cells visualized with a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY).

Transepithelial transport studies

MDCKII-MDR1 cells were maintained and allowed to polarize on Transwell® inserts as described above. On the day of the experiment, the medium was removed and the inserts washed three times with transport buffer consisting of Hanks' buffered salt solution (HBSS) with 10 mM HEPES and 25 mM D-glucose. Cells were allowed to equilibrate in transport buffer for 30 min., and the integrity of the cell monolayer was then assessed by measuring transepithelial electrical resistance (TEER) using a World Precision Instrument, (EVOM; Sarasota, FL, USA). The TEER value of each monolayer was determined by subtraction of the resistance of blank inserts and correction for the surface area (4.71 cm^2). Only cultures meeting the criteria of resistance greater than 500 Ω cm^2, indicating formation of tight junctions, were used for subsequent analyses. The affects of pharmacological inhibition of myosin light chain kinase (ML-7), or MDR1 function (verapamil) on: (i) [^{3}H]digoxin transport, or (ii) MDR1 expression was assessed by pre-treating MDCKII-MDR1 monlayers with either drug or the appropriate vehicle control for up to 4 hours before either initiation of transport studies or processing of inserts for immunocytochemistry, respectively. The potential for drug-induced changes in barrier integrity was assessed by calculating TEER values for each insert at various time points before and after drug or vehicle treatment.

Transport studies were conducted with [^{3}H]-labeled digoxin in transport buffer at a pH of 6.8 for the apical (AP) compartment and 7.4 for the basolateral (BL) compartment. Experiments were performed at 37°C in air, 5% CO_2 and 95% relative humidity. The concentration of digoxin used in the experiment was 0.5 μM. In all experiments, [^{3}H]digoxin transport was assessed in both the AP to BL and BL to AP direction. One hundred μl aliquots were taken from the donor and receiver chambers initially and from the receiver chamber at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hours. The entire receiving compartment was replaced with a fresh solution of HBSS after removing aliquots at each time interval. Aliquots collected for each time interval were placed in 0.9 ml scintillation fluid (Cytoscint ES, ICN, Cosa Mesa, CA, USA) and [^{3}H]-activity measured on a Beckman LS 6500 scintillation counter (Palo Alto, CA, USA). The effective permeability coefficients (P_e) of digoxin after every 30 minutes were calculated using the following equation:

$$P_e = V_d \times \Delta\% / A \, . \, \Delta t$$

where P_e is the effective permeability coefficient (cm s^{-1}), V_d is the volume (cm^3) of the donor compartment, A is the surface area of the monolayer (4.71 cm^2), and $\Delta\%/\Delta t$ is the percentage mass transported (s^{-1}).

The apparent permeability (P_{app}) of digoxin after 3 hours of study was calculated using the following equation:

$$P_{app} = dQ/dt \; x \; 1/\,(A \; x \; C_0)$$

where A is the surface area of the monolayer (4.71 cm^2), C_0 is the initial concentration of radiolabelled probe substrate in the donor compartment; and dQ/dt is the slope of the steady-state rate constant. The efflux ratio was determined by dividing P_{app} in the B to A direction by the P_{app} in the A to B direction:

$$\text{Efflux ratio} = \frac{P_{app}\,(B \rightarrow A)}{P_{app}\,(A \rightarrow B)}$$

Statistical analysis of transport data

All values are presented as mean ± standard error mean (SEM). Differences in the apical and basolateral permeability values, TEER reading and efflux ratios of treatment groups were compared with respective control groups using unpaired t-test.

Immunofluorescence microscopy

For expression analyses, MDCKII-MDR1 cells were maintained and allowed to polarize on Transwell® inserts as described above either on "sister" plates or exactly as described for transport studies. For imunocytochemistry, each insert was carefully excised with a clean, sharp scalpel and gently washed twice with PBS. Cells were fixed and permeabilized by a sequential paraformaldhyde-methanol fixation according to the following schedule: 4% paraformaldhyde for 12 minutes at room temperature, gentle washing two times with PBS followed by a 2 minute fixation in ice cold-methanol. Membranes were treated with 0.2 % Triton-X for 5 minutes, washed and blocked with goat serum. This was followed by incubation with anti-MDR1 and/or anti-MRLC for 1 hour at room temperature. Alexa fluor 546 (red) and alexa fluor 488 (green) conjugated to respective secondary antibodies were used. Membranes were then mounted and cells visualized with a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY).

Results

Myosin RLC is a direct binding partner of diverse membrane-bound proteins

Toward the goal of determining if protein-protein interactions between myosin RLC and target proteins represent a common trafficking mechanism in polarized cell types we compared the ability of myosin RLC to bind two target sequences (Fig. 3.1A). For these studies the intracellular linker region of MDR1 (amino acids 633 to 709), the full-length C-terminus of NR1, and the C-

terminus of an unrelated ion channel subunit, acid-sensing ion channel (ASIC) 2a, were expressed in bacteria as GST fusion proteins and immobilized on glutathione-Sepharose beads. Immobilized GST fusion proteins were then used as affinity matrices to examine interactions with either recombinant myosin RLC (Fig. 3.1B, upper panel), or native light chain derived from brain homogenates derived from mouse cortex (Fig. 3.1B, lower panel). Purified myosin RLC bound strongly and specifically to GST fusion proteins containing the MDR1 linker region and NMDAR1 C terminus, yet failed to interact with either GST alone or a GST fusion protein containing an intracellular C-terminus of ASIC2a (lanes 2 and 3, Fig. 3.1B, upper panel). A similar pattern of binding was observed in GST pull-down assays where purified myosin RLC was substituted for tissue homogenates derived from mouse cortex (Fig. 3.1B, lower panel). Immunoblotting with an anti-myosin RLC antibody revealed that mouse brain myosin RLC was retained on GST affinity matrices containing the linker region of MDR1 (lane 4, Fig. 3.1B, lower panel) and the NMDAR1 C terminus (lane 5, Fig. 3.1B, upper panel), yet did not bind to either GST alone (lane 2, Fig. 3.1B, lower panel) or GST-ASIC2a (lane 3, Fig. 3.1B, lower panel). In all studies, MDR1 and NMDAR1 appeared equally effective at binding myosin RLC suggesting that light chain binding to both target sequences is of comparable affinity. These data indicate that myosin RLC is a direct binding partner of at least two integral membrane proteins that are functionally important in the plasma membrane of polarized cell types.

The amino terminal of myosin RLC is required for interaction with MDR1

Myosin RLC has been proposed to couple BSEP in polarized cell types such as hepatocytes (Chan et al., 2005). Towards the goal of determining the structural basis of light chain interactions with ABC transporter family members, we expressed several deletion mutants of myosin RLC in bacteria and examined them for interaction with the linker region of MDR1 (Fig 3.2).

The RLC mutant corresponding to the first three helix-loop-helix motifs (or EF-hand domains) of the protein (1-129) bound to GST/MDR1 in a manner that was comparable to that of the full-length (1-172) protein (compare lanes 9 and 15 of Fig. 3.2, panel B). The RLC mutant lacking the third EF-hand domain (1-101) was also retained, albeit to a lesser extent, on a GST/MDR1 affinity matrix (lane 3 of panel B). Deletion of the first one or two EF-hand domain of the light chain (to yield either 61-172 or 102-172, respectively) dramatically reduced or abolished the interaction of myosin RLC with GST/MDR1 (lanes 6 and 12 of panel B, respectively). Taken together, these findings indicate that amino acid residues in the amino terminal of myosin RLC are critical for binding to MDR1. The first three EF-hand domains are necessary and sufficient for binding; residues between 102 and 129 of the light chain likely contribute to the overall stability of the protein-protein interaction whereas residues 130 to 172 are not part of the MDR1-myosin RLC interface.

The phosphorylation state of myosin RLC is a critical determinant of MDR1-light chain binding

The amino terminal region of myosin RLC is phosphoryated by a specific calmodulin-dependent protein kinase, known as myosin light chain kinase (MLCK), at serine 19 and threonine 18. To determine if myosin RLC-MDR1 interactions are regulated by MLCK-induced phosphorylation, a minimal RLC-interacting domain (residues 1-129) was phosphorylated *in vitro* by MLCK and tested for interaction with: (1) the linker region of MDR1 and the C-terminal region of the NMDAR1 subunit (Fig. 3.3). The extent of phosphorylation was confirmed following all *in vitro* reactions by urea-glycerol PAGE of RLCs followed by immunoblot analysis (Fig. 3.3B). Phosphorylated and non-phosphorylated RLCs were then examined in parallel for interaction with GST/MDR1 (633 to 709) and GST alone (Fig. 3.3C), or GST/NMDAR1 (834 to 863) and GST alone (Fig. 3.3D) using an antibody to both forms of the protein. In the phophorylated state myosin RLC (1-129) binding to either

MDR1 (Fig. 3.3C), or NMDAR1 (Fig. 3.3D), was dramatically reduced when compared with non-phosphorylated RLC (lane 3 and 6, Figs. 3.3C and 3.3D) or mock-phosphorylated protein (data not shown). A loss of binding was confirmed by the use of a phospho-specific antibody that recognizes only the phosphorylated form of the RLC (data not shown). This pattern of binding was also observed when phosphorylated and non-phosphorylated forms of myosin (1-129) were substituted for full-length myosin RLC (data not shown). Taken together these findings indicate that phosphorylation of residues in the amino terminus of the light chain disrupts binding of RLC to targets such as MDR1 and NMDAR1.

Apical-to-basal transport of [^{3}H]digoxin is enhanced by an inhibitor of MLCK

We used MDCK cells stably expressing MDR1 (MDCKII-MDR1) to determine if the phosphorylation status of myosin RLC influences the transport function of MDR1 in an intact cell. Like neurons, MDCK-type epithelial cells polarize and are widely used as a model system to evaluate MDR1-mediated transport of drugs, as well as general mechanisms of protein sorting and trafficking to the plasma membrane. Monolayers of MDCKII-MDR1 cells were grown to confluency on semipermeable supports, and were then allowed to polarize and form electrically tight junctions. To inhibit phosphorylation of myosin RLC we treated cell monolayers with ML-7, a pharmacological inhibitor of MLCK. Prior to initiating transport studies we analyzed the effect of ML-7 treatment on the integrity of MDCKII-MDR1 monolayers. TEER readings were taken for each monolayer before and for up to 4 hours after treatment with ML-7 (3 and 10 μM) or vehicle alone (Table 3.1). Although TEER readings were generally higher in the presence of ML-7, we found no statistically significant difference between TEER readings measured before and after treatment. These results indicate that ML-7 did not adversely impact the integrity of the

MDCKII-MDR1 monolayer, in that electrically resistant tight junctions were maintained in the presence of an inhibitor of MLCK.

MDR1 function was studied by measuring transepithelial transport of $[^3H]$-digoxin, a well-known MDR1 substrate, across MDCKII-MDR1 monolayers. In all studies, cells were pretreated with ML-7 (10 μM) for 4 hours prior to initiation of transport studies. Transport of $[^3H]$-digoxin (1 µCi), in the absence of inhibitor, was subsequently measured as a function of time in the apical-to-basal and basal-to-apical direction for up to 3 hours after ML-7 treatment (Fig. 3.4). We observed a significant increase in apical-to-basal permeability of $[^3H]$-digoxin following ML-7-induced suppression of myosin RLC phosphorylation when compared to vehicle-treated control cells (Fig. 3.4A). This response was most dramatic in the first hour following ML-7 treatment (Fig. 3.4A). In contrast, we observed no significant change in basal-to-apical permeability of $[^3H]$-digoxin following ML-7 treatment (Fig. 3.4B). The significant increase in apical-to-basal $[^3H]$-digoxin apparent permeability (P_{app}) over the 3-hour time frame (Fig. 3.4A) resulted in an apparent decrease in the efflux ratio of $[^3H]$-digoxin in response to ML-7 treatment. The efflux ratio (calculated as P_{app} BL→AP/ P_{app} AP→BL), of $[^3H]$-digoxin decreased from 8.44 to 2.7 after ML-7 treatment when compared to vehicle-treated control cells (Table 3.2). This decrease in the efflux ratio of $[^3H]$-digoxin was comparable to that measured in control studies conducted with verapamil, a prototypical inhibitor of MDR1 function. Pretreatment of cell monolayers with verapamil (100 μM), resulted in a decrease in the efflux ratio of $[^3H]$-digoxin from 6.82 to 3.75 (Table 3.2). However, in contrast to ML-7 treatment, we observed a significant decrease in basal-to-apical permeability of $[^3H]$-digoxin in response to verapamil with no significant change in apical-to-basal permeability. Distinct differences in the characteristics of $[^3H]$-digoxin transport following ML-7 or verapamil pretreatment are highlighted in figure 3.5. Thirty minutes after treatment with ML-7 we observed over a two-fold increase in apical-to-basal effective permeability, with no significant change in

the basal to apical permeability of [^{3}H]-digoxin (Fig. 3.5A). Verapamil, however, induced a thirty-five percent decrease in basal-to-apical effective permeability at thirty minutes with no significant change in the apical-to basal permeability of [^{3}H]-digoxin (Fig. 3.5B). The use of a direct inhibitor of MDR1 function thus serves to validate our transport assays in that the efflux function of MDR1 was inhibited by pretreatment of cells with verapamil. MDR1 function was also inhibited by pharmacologic manipulation of a direct binding partner of MDR1, myosin RLC. Specifically, site-specific suppression of phosphorylation in the amino terminal of endogenous myosin RLC by ML-7 resulted in an alteration in the transport function of MDR1. Together these findings support the hypothesis that MDR1 is closely associated with a light chain of the molecular motor myosin II in intact polarized cells.

Apical expression of MDR1 is decreased by an inhibitor of MLCK

To determine the relative expression patterns of myosin and MDR1 in MDCKII-MDR1 cells, untreated cells were grown to confluency on semipermeable supports, and allowed to form electrically tight junctions as before. Confocal fluorescence microscopy of cells co-stained with anti-myosin RLC and anti-MDR1 antibodies, plus DAPI stain to visualize nuclei, revealed varying degrees of co-localization of MDR1 and myosin RLC throughout the cell. When visualized in cross-section in the X-Z plane (Fig. 3.6), by plotting an average of 27 consecutive confocal X-Y images to construct a Z-projection of the confocal image stack, co-expression of myosin RLC (green channel) and MDR1 (red channel) was particularly evident in the apical portion of the MDCK-MDR1 cells lying above the DAPI-stained nuclei (blue channel) and in lateral sites corresponding to regions of cell-cell contact at presumed gap junctions (see overlay of all three channels in upper image of Fig. 3.6).

Using comparable conditions to those used in our [^{3}H]-digoxin transport studies, we analyzed MDR1 and light chain expression in response to pretreatment with ML-7. We observed little change in the distribution of

myosin RLC in response to inhibition of MLCK; myosin RLC was strongly expressed in apical and lateral portions of MDCKII-MDR1 cells (Fig. 3.7, green channel represents RLC immunoreactivity in XZ-vertical cross section 30 min. and 3 hours after pretreatment). In contrast, when the same stack of X-Y images was collected and analyzed for MDR1 immunoreactivity, MDR1-associated fluorescence was increasingly more difficult to detect in the apical portions of MDCKII-MDR1 cells following ML-7 pretreatment. For example, the upper 7-9 X-Y images of an average stack of 27 images lacked MDR1-associated fluorescence 3 hours after ML-7 pretreatment. When viewed as a XZ-vertical cross section (as shown in Fig. 3.7, red channel), MDR1 expression appeared more flattened towards the DAPI-stained nuclei (Fig. 3.7, blue channels), corresponding to reduced MDR1-assocted fluorescence in the upper third of the image stack. Together these studies indicate that changes in MDR1 cell surface expression likely account for alterations in the transport characteristics of [^{3}H]-digoxin in response to ML-7 pretreatment

Discussion

Sorting and trafficking of MDR proteins to the cell surface likely involves a regulated series of direct and indirect interactions with a number of molecular motors, cytoskeletal and adapter proteins. In the present study we undertook a biochemical characterization of a direct interaction between the intracellular linker region of MDR1 and a light chain of the actin-based motor myosin II. Binding of myosin RLC to MDR1 required the amino terminal domain of the light chain and was inhibited by phosphorylation of the light chain by MLCK. To determine the functional consequences of perturbing the phosphorylation state of myosin RLC in intact cells, we studied the transport properties of [^{3}H]-digoxin following exposure to a specific inhibitor of MLCK. Pretreatment of MDCKII-MDR1 cells with ML-7 caused a significant increase in the apical to basolateral transport of digoxin, thereby reducing the efflux ratio of digoxin in a manner that was comparable to that observed following

pretreatment with verapamil. Rather than acting as a direct inhibitor of MDR-mediated efflux, increased absorption of digoxin was attributed to reduced apical expression of MDR1 in response to ML-7 treatment. These findings indicate that transepithelial transport of digoxin can be modulated indirectly by pharmacological manipulation of a direct binding partner of MDR1. Identification of the full complement of molecular motors that influence trafficking and cell surface expression of MDR proteins will ultimately contribute to our understanding of the mechanisms that underlie enhanced expression of MDR proteins associated with drug refractory conditions.

In a previous study, Chan and co-workers demonstrated that expression of a mutated GFP-tagged myosin RLC in which Thr-18 and Ser-19 residues were changed to alanine (MLC-AA-GFP) resulted in decreased apical expression of BSEP in polarized MDCK cells (Chan et al., 2005). A role for a non-muscle myosin II motor complex in forward trafficking of BSEP was further supported by pulse-chase studies performed in the presence of blebbistatin. Blebbistatin is a selective inhibitor of myosin II that binds to the head region of myosin II heavy chain and effectively blocks the cyclical interaction of myosin II with actin (Kovacs et al., 2004). Blebbistatin reduced apical expression of transiently expressed YFP-tagged BSEP by 72 to 60 % of control-treated cells in 2 to 4 hours, respectively. In accordance with these studies, we find that an inhibitor of MLCK produces a functionally similar response with the related ABC transporter, MDR1. ML-7 is a direct inhibitor of MLCK, a Ca^{2+}/calmodulin-dependent enzyme that phosphorylates myosin RLC at Thr-18 and Ser-19. MLCK-induced phosphorylation of nonmuscle and smooth muscle myosins induces a conformational change in the myosin II complex that allows the head region of the myosin II heavy chain to bind actin. In the present study, we disrupted the ability of endogenous myosin RLC to undergo cyclical phosphorylation and dephosphorylation in MDCK cells stably expressing MDR1. Our biochemical analysis of myosin RLC-MDR1

interactions indicate that MDR1 was likely held in a RLC bound conformation in the presence of ML-7, as site-specific phosphorylation provides a mechanism for dissociation of myosin RLC from its binding partners. Taken together, these studies highlight the importance of two specific amino terminal residues of myosin RLC, Thr-18 and Ser-19, for appropriate localization of the ABC-transporters MDR1 and BSEP in polarized epithelial cells.

Myosin RLC-MDR1 interactions were qualitatively similar to the interaction of myosin RLC with the NMDAR1 subunit of the NMDA receptor, suggesting that the light chain likely adopts a similar conformation when bound to a non-myosin target. Our data shows that myosin RLC interacts with linker domain of MDR1 through its amino terminal. The linker region of MDR1 and the NMDAR1 appear to bind myosin RLC in a similar manner is significant. There are several unrelated proteins that have been shown to interact with myosin RLC in polarized cells and neurons. However, it is still unclear how myosin RLC can interact with other protein targets if it is present as a complex in myosin II. Taken together our results show myosin RLC can form a distinct complex with other proteins and myosin heavy chain neck. These results are in accord with the finding that Mlc1p in yeast forms independent complexes with myosin II and IQGAP (Boyne et al., 2000).

Although on one level binding of glutamate receptors and MDR proteins appear to be unrelated binding partners of myosin RLC, this observation may be of particular relevance to the biological phenomenon of overexpression of MDR proteins in drug-refractory epilepsy. Using in situ hybridization, Karssen et al. have previously shown that MDR1 is expressed by endothelial cells and possibly dentate gyrus neurons (Karssen et al., 2004). Immunohistochemical studies had previously shown the localization of myosin RLC in cerebral vasculature in meningial arteriole and venules (Ishmael et al., 2007). Previous studies on mouse brain also show myosin RLC staining in the granule cell layer of dentate gyrus and cortex region which is co-expressed with NMDAR1

in the soma of neuronal populations in neonatal and adult mice (Kioussi et al., 2007). The finding of a common protein for both NMDA receptors and MDR1 transporter in brain seems important as a recent study shows that increased release of glutamate is associated with seizure induced overexpression of MDR1 in brains of pilocarpine model of rats (Bankstahl et al., 2008).

Our studies show that myosin RLC co-localizes with MDR1 in MDCKII-MDR1 cells grown on semipermeable inserts. Endogenously present myosin RLC and overexpressed MDR1 are enriched in the apical domains of MDCKII-MDR1 cells. The colocalization of MDR1 and myosin RLC takes place mainly in the apical region at tight junctions. The presence of myosin RLC in apical domains of polarized MDCK cells are in support of the data previously shown in MDCK cells with another ABC transporter (Chan et al., 2005). ML-7 treatment decreases this apical distribution of MDR1 in MDCKII-MDR1 cells. Our results are in support of the data published by Chan et al. that non-phosphorylatable myosin RLC mutant, that has two of its phosphorylation sites mutated to alanine at positions 18 and 19, decreases the expression of BSEP in apical region of MDCK cells (Chan et al., 2005). Function of myosin RLC seems to be important for apical membrane localization of ABC proteins as inhibition of myosin RLC prevents BSEP apical membrane localization in MDCK cells (Chan et al., 2005). Different studies show that myosin motors and actin filaments are present in the vesicles transported through golgi and play an important role in their transport (Allan et al., 2002; DePina and Langford, 1999; DePina et al., 2007; Musch et al., 1997). ML-7 is a specific inhibitor of MLCK at 10 μM and prevents the phosphorylation of myosin RLC. Inhibition of MLCK by ML-7 inhibits motility of vesicles on actin filaments at 10 μM concentrations by 31% which was completely inhibited at 200 μM in clam oocytes (DePina and Langford, 1999). ML-7 does not inhibit the formation of actin network and it blocked the vesicles movement by inhibiting myosin II activity (DePina and Langford, 1999). In addition to that intracellular trafficking of MDR1 in HeLa cells (transfected with MDR1) shows its localization in rough

endoplasmic reticulum (RER) and golgi complex before the protein is expressed at the cell surface (Fu et al., 2004). Localization of MDR1 was further confirmed by using in situ localization studies in RER, golgi complex of rat brain tissue and cytoplasmic vesicles of rat brain microvessel endothelial cells (Bendayan et al., 2002; Bendayan et al., 2006). Thus it is possible that ML-7 treatment in our experiments is inhibiting the vesicular transport of MDR1 to cell surface.

We have previously shown in our studies that an isolated myosin RLC can interact with the NMDA receptor subunits through its amino terminal region. After disrupting the amino acid residues that bound to myosin RLC in NR2A subunit, the distribution of NMDA receptors containing mutated NR2A subunit was restricted to the intracellular Golgi compartment compared to cells expressing wild type NR2A. Myosin RLC is associated with NMDA receptor subunits in the early secretory pathway before delivery from Golgi to the plasma membrane. Previous studies with BSEP have reported that myosin RLC is required for trafficking of BSEP to the apical membrane from the trans-Golgi network (TGN) of hepatocytes (Chan et al., 2005; Wakabayashi et al., 2006). Thus it may be possible that the interaction of myosin RLC and MDR1 occurs inside the TGN and pharmacological manipulation of this interaction by ML-7 in MDCKII-MDR1 cells led to decreased apical expression of MDR1. Together these studies indicate the importance of linker domain for possible interactions with cellular proteins and its role in intracellular trafficking.

Transepithelial resistance is an important indicator of barrier functional properties in the cells that are connected by tight junctions. In our experiments, the transepithelial resistance of MDCKII-MDR1 monolayers was maintained after 2 hours of ML-7 treatment and this integrity is maintained even after 4 hours. Phosphorylation of myosin RLC has been shown to play an important role in maintaining the blood brain barrier (BBB) integrity. Ethanol induced oxidative stress activated MLCK that phosphorylates myosin

RLC that diminishes the integrity of brain microvascular endothelial cells (BMVEC) (Haorah et al., 2005). Modification of cytoskeletal organization by ethanol treatment led to loss of BBB integrity and reduced TEER readings in BMVEC cells (Haorah et al., 2005). Fluvastatin, that increased nitric oxide levels, causes barrier enhancing effects in endothelial cell line (ECV304) and glioma cell line (C6) which are mediated through decreased phosphorylation of myosin RLC (Kuhlmann et al., 2006). Compared to previous studies in BMVEC cells our data shows minimal increase in the TEER readings on ML-7 treatment and confirms the barrier stabilization effect on inhibition of phosphorylation of myosin RLC.

The functional relevance of myosin RLC interaction with MDR1 was tested through transport studies. MDR1 function was tested in our experiments by showing decreased efflux ratio of [^{3}H]-digoxin in presence of verapamil which is a well known inhibitor of MDR1. We show in our studies that the permeability of MDR1 substrate [^{3}H]-digoxin increased from the AP to BL side in the presence ML-7 in MDCKII-MDR1 cells. This increase in permeability of [^{3}H]-digoxin may be due to the decreased expression of MDR1 in the apical region of MDCKII-MDR1 cells which is evident from our immunocytochemistry data. It may be possible that the expression of MDR1 on cell membrane is decreased due to inhibition of vesicular transport by treatment with ML-7 and this may cause an increase in permeability from AP to BL side. The change in efflux ratios from 8.44 to 2.7 after ML-7 treatment shows that the permeability of [^{3}H]-digoxin is increased by inhibiting the phosphorylation state of myosin RLC. It has been shown previously that inhibition of myosin II by blebbistatin can decrease the apical expression of BSEP in MDCK cells (Chan et al., 2005). Blebbistatin treatment in our study also cause a decrease in the efflux ratio of [^{3}H]-digoxin but the change in permeability from AP to BL was not significant. This data further confirms and supports the role of myosin in the apical localization of an ABC transporter in MDCK cells.

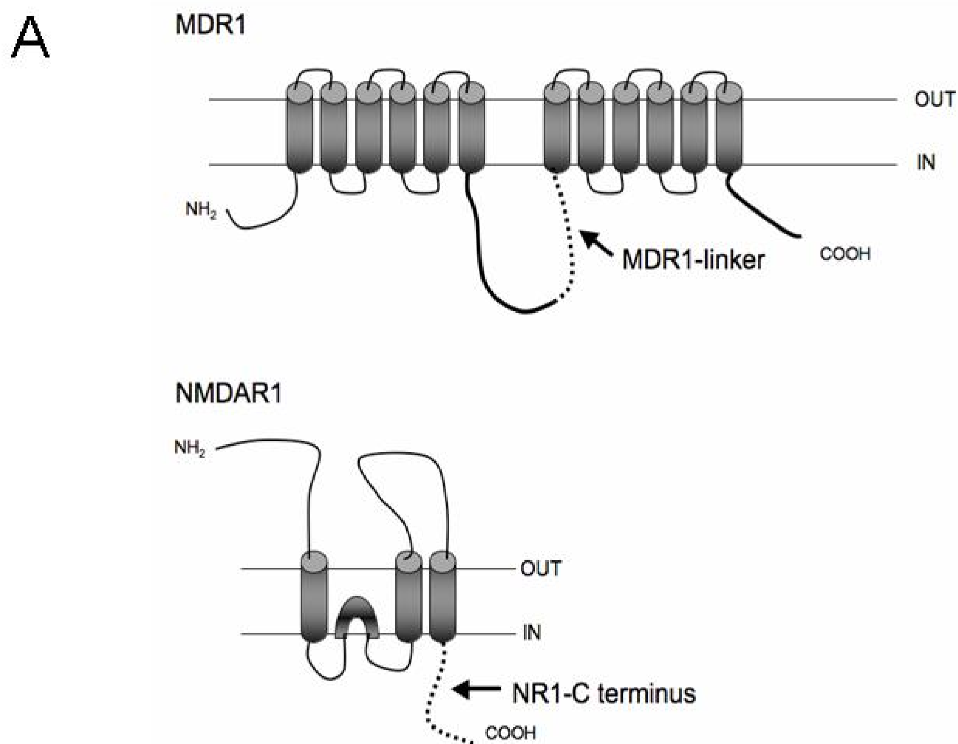
The biological basis of pharmacoresistance in epilepsy is poorly understood. Over-expression of efflux transporters at the BBB prevents AED's including phenytoin, phenobarbital, lamotrigine, felbamate to reach sufficient high concentrations in brain despite adequate plasma levels (Potschka et al., 2002; Potschka and Loscher, 2001). There is now evidence that multiple members of the super family of ATP-binding cassette (ABC) transporters, may be upregulated in fatal status epilepticus (Sisodiya and Thom, 2003) and refractory epilepsy (Marchi et al., 2004; Sisodiya et al., 2006) and recently reviewed in (Loscher and Potschka, 2005b). This study shows an important role of myosin motor which can bind directly with MDR1. Myosin by interacting with intracellular domain of MDR1 may help in the trafficking or localization of the receptors in the cell. We also show that distribution of MDR1 is regulated by the phosphorylation state of myosin RLC which can further affect the functional transport of MDR1 substrates. Future studies will concentrate on the changes in functional transport of antiepileptic drugs.

Acknowledgement

We also thank David Cole for his contribution. We thank Center of genomics research and biotechnology (CGRB) and Environment Health and Science Center (EHSC) at Oregon state university for using confocal microscope facility. This work was supported by an award from the American Association of Colleges of Pharmacy (AACP) New Investigator Program (NIP) to Dr. Jane Ishmael.

.

A



B

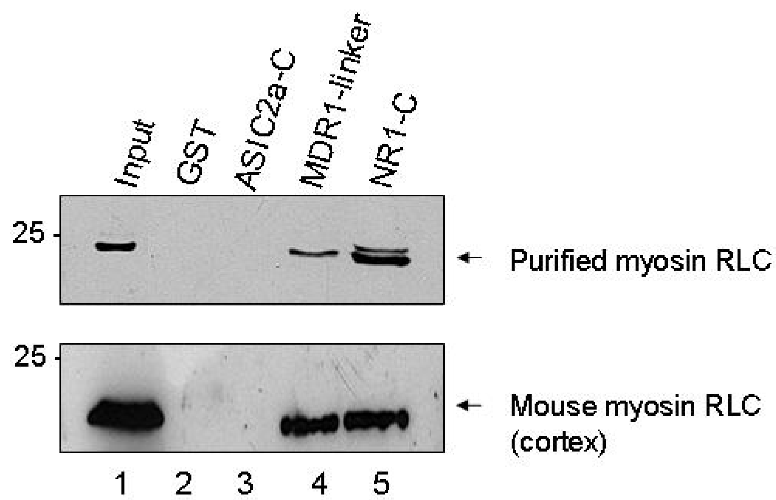
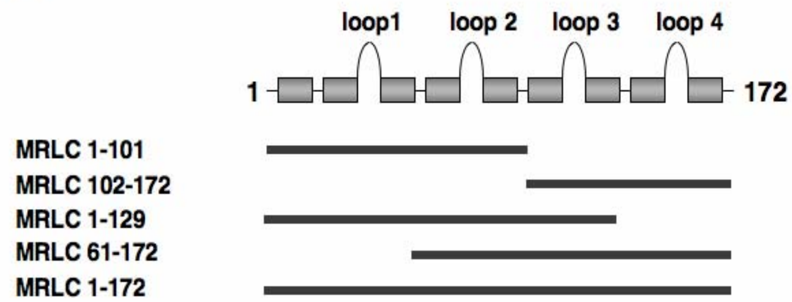


Figure 3.1 Myosin RLC binds directly to the human MDR1-linker region.

A) Schematic representation of the membrane topology of MDR1 and NMDA receptor subunits showing intracellular domains that are binding partners of myosin RLC. **B)** MDR1-linker region, NR1 C-terminal (NR1-C) and acid sensing ion channel (ASIC) 2a C-terminal were bacterially expressed as GST fusion proteins. These GST-fused proteins or GST alone were immobilized on glutathione sepharose beads and used as baits to pull down native myosin RLC from mouse cortex, or treated with purified recombinant myosin RLC and analyzed for the interaction in an immunoblot analysis. Both recombinant and native myosin RLC were retained on the affinity matrices corresponding to MDR1-linker region and NR1 C-terminal (lanes 4 and 5). GST alone and ASIC-2a C-terminal, which has been shown previously not to interact with NR1 subunit of NMDA receptors, were used as negative control in this experiment (lanes 2 and 3). The detection of recombinant myosin RLC was done using anti-T7 antibody and detection of native myosin RLC was done using anti-MRLC antibody, αMRLC/3, raised in the laboratory.


A
loop1
loop 2
loop 3
loop 4
1
172
MRLC 1-101
MRLC 102-172
MRLC 1-129
MRLC 61-172
MRLC 1-172

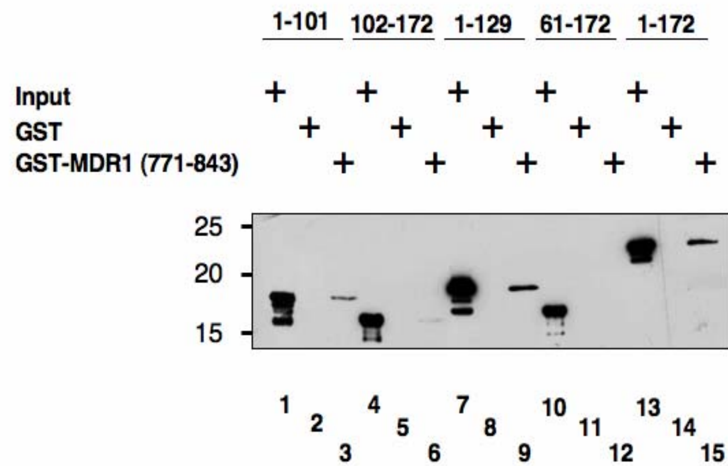
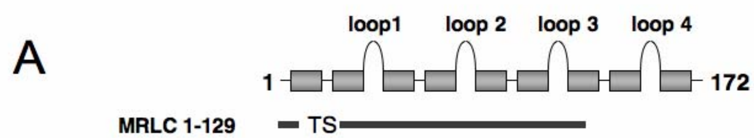

B
1-101
102-172
1-129
61-172
1-172
Input
GST
GST-MDR1 (771-843)
25
20
15
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

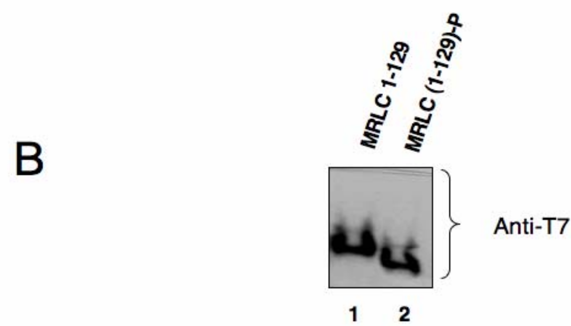
Figure 3.2 Myosin RLC binds to MDR1-linker region through its amino terminal.

A) Schematic representation of a series of myosin RLC deletion mutants used in panel B. **B)** A truncated recombinant myosin RLC (1-129) is sufficient for binding to MDR1-linker region (compare lanes 9 and 15). MDR1 linker region was fused to GST and tested for its ability to bind full-length and mutant RLCs. GST-fused MDR1-linker region, or GST alone were immobilized on glutathione-Sepharose beads and incubated with either full-length (MRLC 1-172), or truncated light chains: MRLC 1-101, MRLC 61-129, MRLC 102-172, MRLC 1-129, MRLC 61-172. Bound proteins were resolved by PAGE, and blotted to nitrocellulose. Immune complexes were revealed by anti-T-7 antibody.

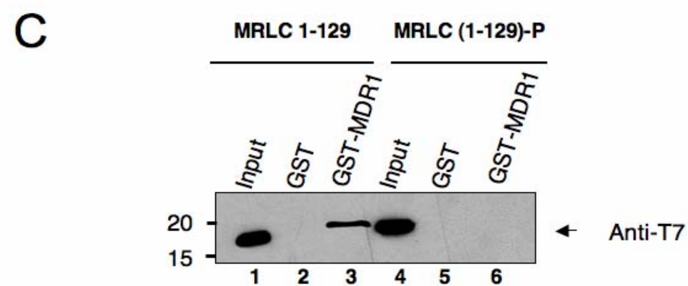
A



B



C



D

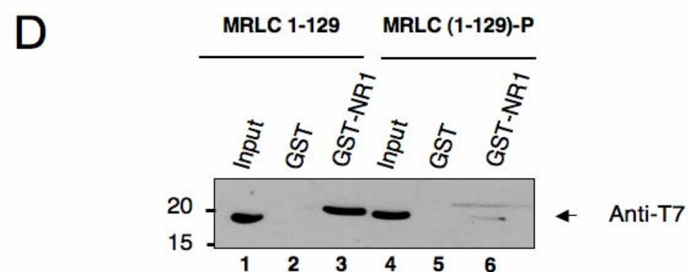
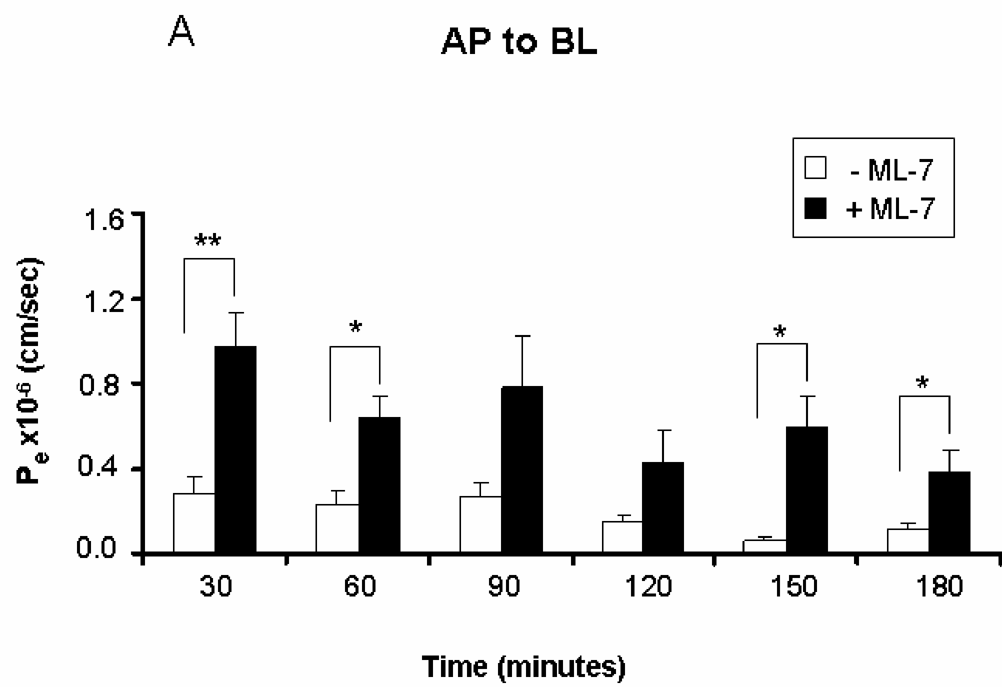


Figure 3.3 Myosin RLC interaction with MDR1-linker region is sensitive to phosphorylation.

A) Schematic representation of the truncated myosin RLC mutant MRLC 1-129 used in the experiment containing first three EF-hand domains. MRLC 1-129 also has two phosphorylation sites: threonine and serine at positions 18 and 19, respectively. **B)** MRLC 1-129 was phosphorylated using myosin light chain kinase (MLCK) and was verified by PAGE using urea gel. Panel shows immunoblot analyses of MRLC 1-129 before (lane 1) and after (lane 2) phosphorylation by MLCK *in vitro*. The gels were transferred on to the nitrocellulose membranes and immunoblots were detected using anti-T7 antibody. **C)** Binding of phosphorylated MRLC 1-129 to MDR1 linker region was lost compared to its interaction with non-phophorylated form. GST-fused MDR1-linker region and GST alone were immobilized on glutathione sepharose beads and were incubated with both non-phosphorylated (MRLC 1-129) and phosphorylated **(**MRLC (1-129)-P**)**. MRLC 1-129 interacts with MDR1-linker region in its non-phosphorylated form and this interaction was lost when MRLC 1-129 was phosphorylated (compare lanes 3 and 6). **D)** Following phosphorylation of MRLC 1-129 by MLCK, binding of MRLC 1-129 to NR1 C-terminal (834-938) was decreased relative to the non-phosphorylated state. GST-fused NR1 C-terminal was incubated with both non-phosphorylated and phosphorylated form of MRLC 1-129 under similar conditions as MDR-linker region. GST-NR1 bound strongly with non-phosphorylated MRLC 1-129 and this interaction was no longer strong seen when MRLC 1-129 was phosphorylated (compare lanes 3 and 6). Bound proteins were resolved by PAGE, transferred to nitrocellulose membranes and probed with an anti-T-7 antibody capable of detecting both forms of the RLC.


A
AP to BL
- ML-7
+ ML-7
1.6
1.2
0.8
0.4
0.0
**
*
*
*
P_e x10^-6 (cm/sec)
30
60
90
120
150
180
Time (minutes)

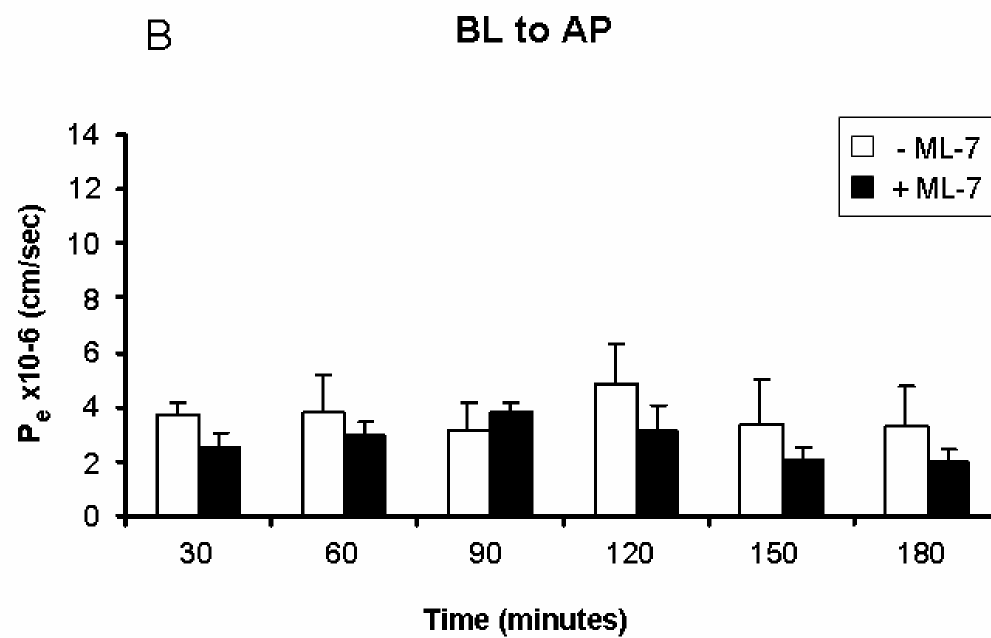
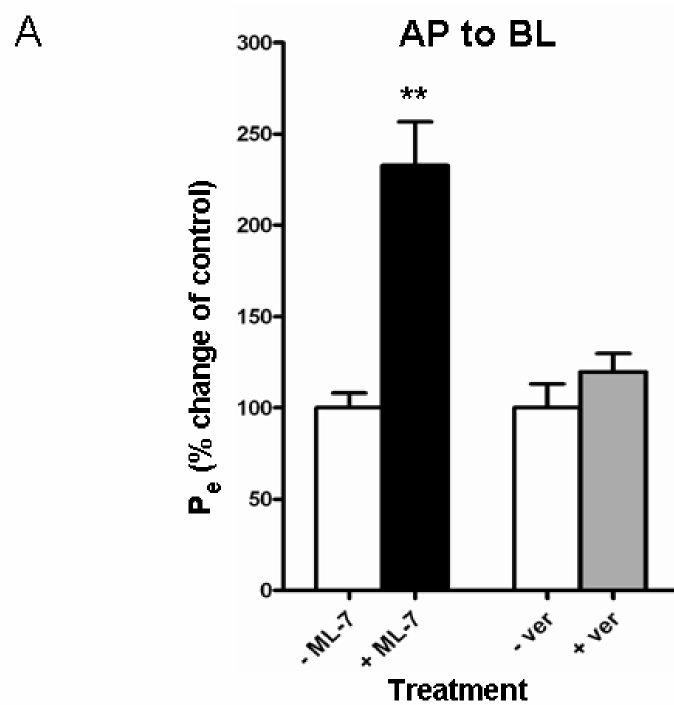

B
BL to AP
- ML-7
+ ML-7
14
12
10
8
6
4
2
0
P_e x10-6 (cm/sec)
30
60
90
120
150
180
Time (minutes)

Figure 3.4 Permeability of [^{3}H]-digoxin was enhanced in MDCKII-MDR1 cell monolayer within 30 minutes of ML-7 pretreatment.

Apical to basolateral transport of [^{3}H]-digoxin was enhanced within 30 minutes following treatment with ML-7, a myosin light chain kinase inhibitor. MDCKII-MDR1 cells were grown on Transwell® inserts and allowed to polarize before they were treated with ML-7 (10 μM) for 4 hours. Both AP→BL **(panel A)** and BL→AP **(panel B)** transport of [^{3}H]-digoxin was determined every 30 minutes for total 180 minutes. Effective permeability (P_e) of [^{3}H]-digoxin was calculated and data is represented as mean ± standard error, n = 3-4. Differences between ML-7 treatment with respective control treatment were analyzed using unpaired t-test for each time interval and the statistical significance is shown as: ** $p < 0.01$, * $p < 0.05$.

A



B

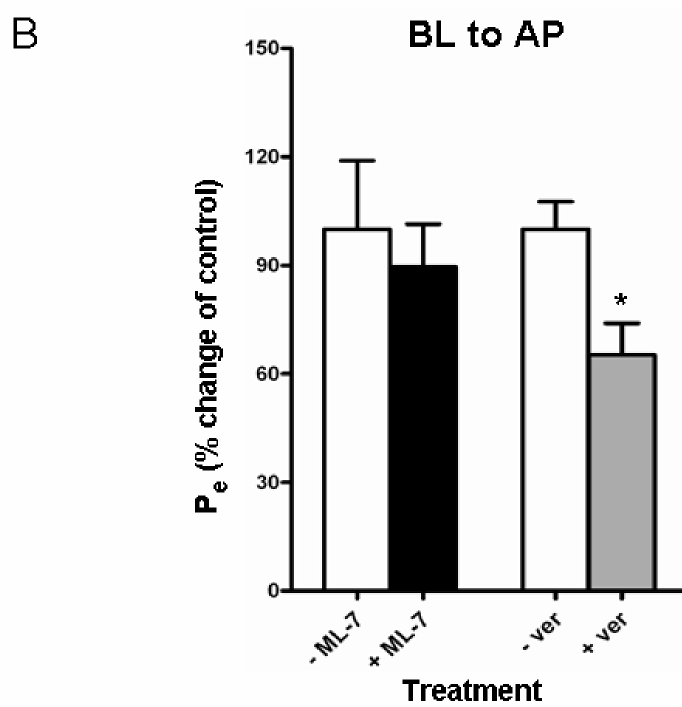


Figure 3.5 Comparison of ML-7 and verapamil treatment on permeability (P_e) of [^{3}H]-digoxin in the MDCKII-MDR1 cell monolayer.

ML-7 treatment in MDCKII-MDR1 cells caused a significant increase in the apical to basolateral permeability of [^{3}H]-digoxin, however no significant change in the basolateral to apical permeability was observed. Verapamil, a well known inhibitor of MDR1, caused a significant thirty five percent decrease in basolateral to apical permeability of [^{3}H]-digoxin. Transport studies were performed on MDCKII-MDR1 monolayers after treating cells with ML-7 (10 μM) and verapamil (100 μM). Both AP→BL **(panel A)** and BL→AP **(panel B)** transport of [^{3}H]-digoxin through MDCKII-MDR1 monolayers were determined after 4 hours of ML-7 or verapamil (ver) treatment. Relative changes in P_e (% of control treatment) after 30 minutes are expressed as means ± S.E.M (n = 3-4). Differences between ML-7 or verapamil treatment with respective control were analyzed using unpaired t-test and the statistical significance is shown as: ** $p < 0.01$, * $p < 0.05$.

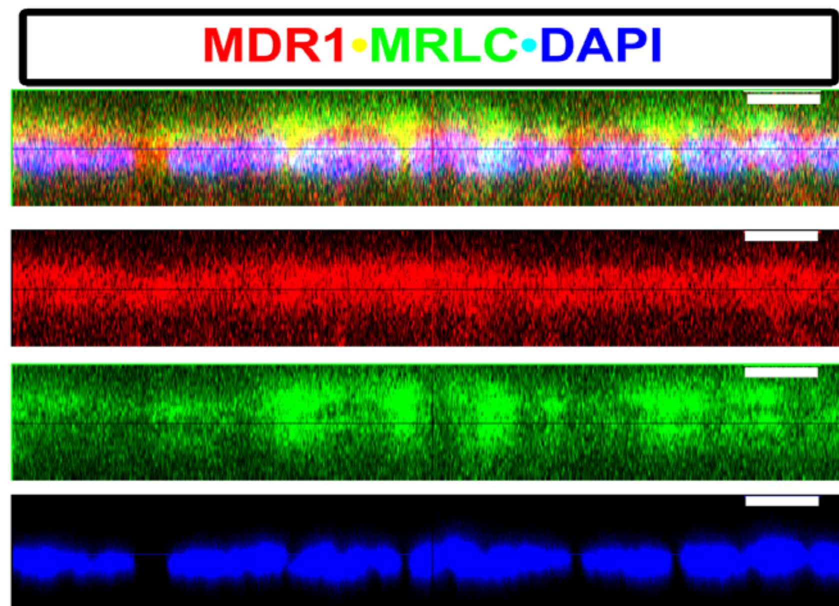


Figure 3.6 Myosin light chain colocalizes with MDR1 in MDCKII-MDR1 cells. Endogenously expressed myosin RLC and overexpressed MDR1 were detected in MDCKII-MDR1 cells and immunofluorescence microscopy of MDCKII-MDR1 cell monolayers revealed that myosin RLC colocalizes with MDR1 in the apical region. MDCKII-MDR1 cells were grown on semipermeable inserts and maintained for 6-7days. Cell monolayers on semipermeable membranes were excised, fixed and stained using anti-MDR1 C219 antibody, and anti-MRLC antibody, αMRLC/3, raised in the laboratory. Cells were analyzed using alexa fluor 546 (red, for MDR1), alexa fluor 488 (green, for MRLC) and DAPI (blue) for nucleus. Figures were visualized in the X-Z plane by plotting 27 consecutive confocal X-Y images. Myosin RLC was strongly expressed in apical and lateral portions (green channel) and MDR1 in the apical portion (red channel) of MDCKII-MDR1 cells. DAPI was used to stain nuclei (blue channel) in these cells. Localization of MRLC and MDR1 was seen on the apical side in polarized MDCKII-MDR1 cells mainly in the tight junctions (yellow, in the first panel). The XZ sections of stained cells were obtained with Zeiss LSM510 confocal microscope.

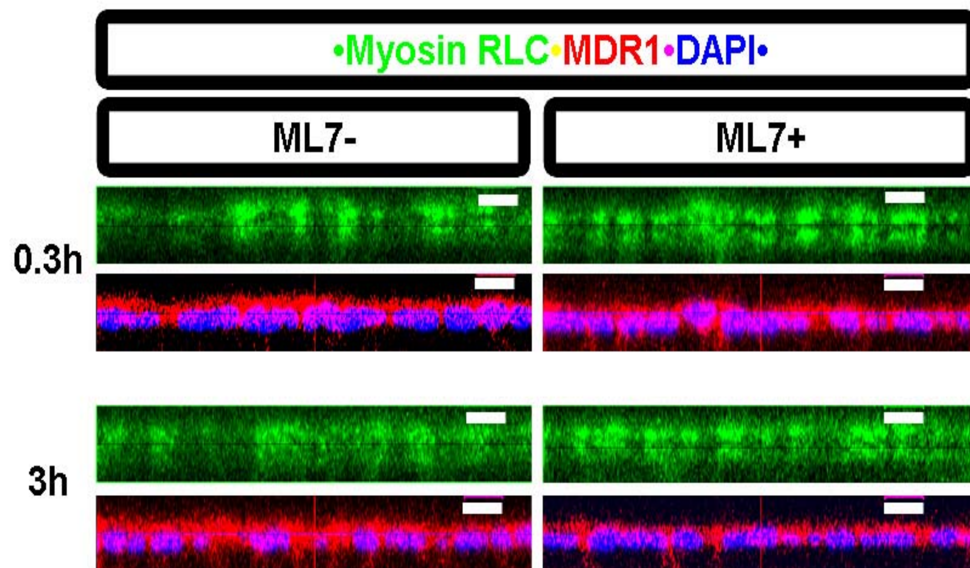


Figure 3.7 Treatment with myosin light chain kinase inhibitor, ML-7, decreases apical expression of MDR1 in MDCKII-MDR1 cells.

MDR1 expression in the apical portions of MDCKII-MDR1 cells was decreased following ML-7 pretreatment. MDCKII-MDR1 cells were grown on semipermeable inserts and maintained for 6-7days. ML-7 (ML-7+) or control (ML7-) treatment was done for 0.3 or 3 hrs and cell monolayers on semipermeable membranes were excised, fixed and stained using anti-MDR1 C219 antibody, and anti-MRLC antibody, αMRLC/3, raised in the laboratory. Cells were analyzed using alexa fluor 546 (red, for MDR1), alexa fluor 488 (green, for MRLC) and DAPI (blue) for nucleus. Myosin RLC was strongly expressed in apical and lateral portions of MDCKII-MDR1 cells (green channel) showed as XZ-verical cross section 30 min. and 3 hours after pretreatment. However, MDR1-associated fluorescence (red channel) was decreased in the apical portions of MDCKII-MDR1 cells following ML-7 pretreatment and appears more flattened with DAPI-stained nuclei (blue channel). The XZ sections of stained cells were obtained with Zeiss LSM510 confocal microscope.

Table 3.1. Transepithelial electrical resistance (TEER) of MDCKII-MDR1 monolayers before and after treatment with ML-7, a myosin light chain kinase inhibitor.

	TEER readings (Ω cm^2)											
	Vehicle				**ML-7 (3 μM)**				**ML-7 (10 μM)**			
	Treatment hours				Treatment hours				Treatment hours			
	0	2	0	4	0	2	0	4	0	2	0	4
Mean	578	568	581	590	577	554	589	601	588	623	588	626
SE	11	18	17	23	8	5	22	8	21	17	18	19
n	4	4	4	4	4	4	4	4	5	5	5	5

The integrity of MDCKII-MDR1 monolayers is not compromised by ML-7 treatment. MDCKII-MDR1 monolayers grown on Transwell® inserts were washed three times with transport buffer consisting of Hanks' buffered salt solution (HBSS) with 10 mM HEPES and 25 mM D-glucose and allowed to equilibrate for 30 min before the addition of test compounds. The integrity of the monolayer was assessed before and after 2 or 4 hour treatment with vehicle or ML-7 (3 or 10 μM). TEER readings (Ω cm^2) were taken in triplicates and averaged for each treatment per experiment. TEER readings calculated are shown as mean ± standard error for the indicated number of separated experiments (n).

Table 3.2. The efflux ratios of [^{3}H]-digoxin in MDCKII-MDR1 cells is decreased in response to ML-7 and verapamil pretreatment.

Treatment	Efflux ratio ($P_{app(BL\rightarrow AP)}$/ $P_{app(AP\rightarrow BL)}$)
Vehicle	8.44 ± 1.09
ML-7 (10μM)	2.7 ± 0.59*
Vehicle	6.82 ± 1.09
Verapamil (100 μM)	3.75 ± 1.29

MDCKII-MDR1 cells were grown on Transwell® inserts and allowed to differentiate before they were treated with ML-7 (10 μM) or Verapamil (100 μM) for 4 hours. Both AP→BL and BL→AP transport of [^{3}H]-digoxin was determined over 180 minutes. Apparent permeability (P_{app}) of [^{3}H]-digoxin was determined and efflux ratio ($P_{app(BL\rightarrow AP)}$/ $P_{app(AP\rightarrow BL)}$)was calculated and data are the mean ± standard error of efflux ratios, n = 3-4. Statistical significance of efflux ratios from vehicle treatment is shown as: *$p<0.01$

References

Allan VJ, Thompson HM and McNiven MA (2002) Motoring around the Golgi. *Nature cell biology* **4**(10):E236-242.

Amparan D, Avram D, Thomas CG, Lindahl MG, Yang J, Bajaj G and Ishmael JE (2005) Direct interaction of myosin regulatory light chain with the NMDA receptor. *Journal of neurochemistry* **92**(2):349-361.

Bankstahl JP, Hoffmann K, Bethmann K and Loscher W (2008) Glutamate is critically involved in seizure-induced overexpression of P-glycoprotein in the brain. *Neuropharmacology* **54**(6):1006-1016.

Bendayan R, Lee G and Bendayan M (2002) Functional expression and localization of P-glycoprotein at the blood brain barrier. *Microscopy research and technique* **57**(5):365-380.

Bendayan R, Ronaldson PT, Gingras D and Bendayan M (2006) In situ localization of P-glycoprotein (ABCB1) in human and rat brain. *J Histochem Cytochem* **54**(10):1159-1167.

Boyne JR, Yosuf HM, Bieganowski P, Brenner C and Price C (2000) Yeast myosin light chain, Mlc1p, interacts with both IQGAP and class II myosin to effect cytokinesis. *Journal of cell science* **113 Pt 24**:4533-4543.

Bresnick AR (1999) Molecular mechanisms of nonmuscle myosin-II regulation. *Current opinion in cell biology* **11**(1):26-33.

Chan W, Calderon G, Swift AL, Moseley J, Li S, Hosoya H, Arias IM and Ortiz DF (2005) Myosin II regulatory light chain is required for trafficking of bile salt export protein to the apical membrane in Madin-Darby canine kidney cells. *The Journal of biological chemistry* **280**(25):23741-23747.

Chen CJ, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM and Roninson IB (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47**(3):381-389.

DePina AS and Langford GM (1999) Vesicle transport: the role of actin filaments and myosin motors. *Microscopy research and technique* **47**(2):93-106.

DePina AS, Wollert T and Langford GM (2007) Membrane associated nonmuscle myosin II functions as a motor for actin-based vesicle transport in clam oocyte extracts. *Cell motility and the cytoskeleton* **64**(10):739-755.

Fu D, Bebawy M, Kable EP and Roufogalis BD (2004) Dynamic and intracellular trafficking of P-glycoprotein-EGFP fusion protein: Implications in multidrug resistance in cancer. *International journal of cancer* **109**(2):174-181.

Georges E (2007) The P-glycoprotein (ABCB1) linker domain encodes high-affinity binding sequences to alpha- and beta-tubulins. *Biochemistry* **46**(25):7337-7342.

Germann UA (1996) P-glycoprotein--a mediator of multidrug resistance in tumour cells. *Eur J Cancer* **32A**(6):927-944.

Haorah J, Knipe B, Leibhart J, Ghorpade A and Persidsky Y (2005) Alcohol-induced oxidative stress in brain endothelial cells causes blood-brain barrier dysfunction. *Journal of leukocyte biology* **78**(6):1223-1232.

Hrycyna CA, Airan LE, Germann UA, Ambudkar SV, Pastan I and Gottesman MM (1998) Structural flexibility of the linker region of human P-glycoprotein permits ATP hydrolysis and drug transport. *Biochemistry* **37**(39):13660-13673.

Ikonen E, de Almeid JB, Fath KR, Burgess DR, Ashman K, Simons K and Stow JL (1997) Myosin II is associated with Golgi membranes: identification of p200 as nonmuscle myosin II on Golgi-derived vesicles. *Journal of cell science* **110 (Pt 18)**:2155-2164.

Ishmael JE, Lohr CV, Fischer K and Kioussi C (2007) Localization of myosin II regulatory light chain in the cerebral vasculature. *Acta Histochemica*.

Karssen AM, Meijer O, Pons D and De Kloet ER (2004) Localization of mRNA expression of P-glycoprotein at the blood-brain barrier and in the hippocampus. *Annals of the New York Academy of Sciences* **1032**:308-311.

Kioussi C, Appu M, Lohr CV, Fischer KA, Bajaj G, Leid M and Ishmael JE (2007) Co-expression of myosin II regulatory light chain and the NMDAR1 subunit in neonatal and adult mouse brain. *Brain research bulletin* **74**(6):439-451.

Kolling R and Losko S (1997) The linker region of the ABC-transporter Ste6 mediates ubiquitination and fast turnover of the protein. *The EMBO journal* **16**(9):2251-2261.

Kovacs M, Toth J, Hetenyi C, Malnasi-Csizmadia A and Sellers JR (2004) Mechanism of blebbistatin inhibition of myosin II. *The Journal of biological chemistry* **279**(34):35557-35563.

Kuhlmann CR, Lessmann V and Luhmann HJ (2006) Fluvastatin stabilizes the blood-brain barrier in vitro by nitric oxide-dependent dephosphorylation of myosin light chains. *Neuropharmacology* **51**(4):907-913.

Loscher W and Potschka H (2005a) Drug resistance in brain diseases and the role of drug efflux transporters. *Nature reviews* **6**(8):591-602.

Loscher W and Potschka H (2005b) Role of drug efflux transporters in the brain for drug disposition and treatment of brain diseases. *Progress in neurobiology* **76**(1):22-76.

Marchi N, Hallene KL, Kight KM, Cucullo L, Moddel G, Bingaman W, Dini G, Vezzani A and Janigro D (2004) Significance of MDR1 and multiple drug resistance in refractory human epileptic brain. *BMC medicine* **2**:37.

Musch A, Cohen D and Rodriguez-Boulan E (1997) Myosin II is involved in the production of constitutive transport vesicles from the TGN. *The Journal of cell biology* **138**(2):291-306.

Nuti SL and Rao US (2002) Proteolytic Cleavage of the Linker Region of the Human P-glycoprotein Modulates Its ATPase Function. *The Journal of biological chemistry* **277**(33):29417-29423.

Ortiz DF, Moseley J, Calderon G, Swift AL, Li S and Arias IM (2004) Identification of HAX-1 as a protein that binds bile salt export protein and regulates its abundance in the apical membrane of Madin-Darby canine kidney cells. *The Journal of biological chemistry* **279**(31):32761-32770.

Potschka H, Fedrowitz M and Loscher W (2002) P-Glycoprotein-mediated efflux of phenobarbital, lamotrigine, and felbamate at the blood-brain barrier: evidence from microdialysis experiments in rats. *Neuroscience letters* **327**(3):173-176.

Potschka H and Loscher W (2001) In vivo evidence for P-glycoprotein-mediated transport of phenytoin at the blood-brain barrier of rats. *Epilepsia* **42**(10):1231-1240.

Rao PS, Mallya KB, Srivenugopal KS, Balaji KC and Rao US (2006) RNF2 interacts with the linker region of the human P-glycoprotein. *International journal of oncology* **29**(6):1413-1419.

Sisodiya SM, Martinian L, Scheffer GL, van der Valk P, Scheper RJ, Harding BN and Thom M (2006) Vascular colocalization of P-glycoprotein, multidrug-resistance associated protein 1, breast cancer resistance protein and major vault protein in human epileptogenic pathologies. *Neuropathology and applied neurobiology* **32**(1):51-63.

Sisodiya SM and Thom M (2003) Widespread upregulation of drug-resistance proteins in fatal human status epilepticus. *Epilepsia* **44**(2):261-264.

Wakabayashi Y, Kipp H and Arias IM (2006) Transporters on demand: intracellular reservoirs and cycling of bile canalicular ABC transporters. *The Journal of biological chemistry* **281**(38):27669-27673.

CHAPTER 4

BIOCHEMICAL CHARACTERIZATION OF A CALCIUM-DEPENDENT CALMODULIN BINDING SITE ON THE NR2A SUBUNIT OF THE NMDA RECEPTOR

Gaurav Bajaj, Michael I. Schimerlik, Peter Hsu and Jane E. Ishmael

In preparation for submission to “Journal of Neurochemistry”

Abstract

Calcium (Ca^{2+}) influx through the N-methyl-D-aspartate (NMDA) receptor activates many signal transduction pathways which are important in mediating excitatory neurotransmission in the brain. However, prolonged activation of NMDA receptors is involved in excitotoxicity underlying cell death associated with ischemic stroke. Previous studies have shown that the identity of the NR2 subunit strongly influences the pharmacological and biophysical properties of the NMDA receptor, for example NR1/NR2A receptors have faster gating kinetics and a higher open probability than NR1/NR2B receptors. The C-termini of NR2A (838-1464) and NR2B (839-1482) subunits are almost identical in number of amino acids but show only 29% sequence similarity in the middle region of NR2A and NR2B C-termini. Using a proteomics approach we identified Ca^{2+}/calmodulin as a binding partner of GST-NR2A (875-1029). We show that calmodulin interacts directly with NR2A C-terminus and we located this calmodulin binding site within amino acids 991-1029 of the NR2A C-terminus. This interaction of calmodulin was found to be specific for NR2A C-terminus and not with NR2B C-terminus. Using site directed mutagenesis we show that tryptophan at position W1014 is critical for this interaction; either W1014A or W1014F resulted in the loss of calmodulin binding. Equilibrium fluorescence spectroscopy studies show that the binding affinity of calmodulin for NR2A (875-1029) is 5.2 ± 2.4 nM. Until now calmodulin is believed to be an interacting partner of NR1 C-terminus that plays an important role in Ca^{2+}-dependent inactivation. In our study, we show a direct interaction of Ca^{2+}/calmodulin with NR2A C-terminus which will provide further information and help us understand the mechanism of Ca^{2+}-dependent inactivation which was found to be NR2 subunit specific.

Introduction

N-methyl-D-aspartate (NMDA)-type glutamate receptors are key components of postsynaptic excitatory synapses. At the postsynaptic

membrane of excitatory synapses, NMDA receptors are bound to different scaffolding and signaling proteins that regulate the process of synaptic transmission (Wenthold et al., 2003). These receptors are permeable to calcium (Ca^{2+}) and plays significant roles in development and in forms of synaptic plasticity that underlie the processes of learning and memory, neuronal development, and some neurological disorders (Hollmann and Heinemann, 1994; McBain and Mayer, 1994).

Ionotropic glutamate receptors are ligand-gated ion channels that mediate the majority of excitatory neurotransmission in the brain (Nakanishi et al., 1994). They are sub-divided according to the ligands selective for these ion-channels: NMDA receptors (activated by NMDA) and non-NMDA receptors (which are sensitive to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate). NMDA receptors are hetero-oligomeric proteins and are believed to be tetrameric complexes assembled from two NR1 and two NR2 subunits (Furukawa et al., 2005; Hollmann and Heinemann, 1994; McBain and Mayer, 1994; Stephenson et al., 2008; Tichelaar et al., 2004; Ulbrich and Isacoff, 2007). The NR1 subunit has eight different splice variants generated by alternative splicing of the NR1 gene (Durand et al., 1992; Nakanishi et al., 1992; Sugihara et al., 1992). The C-terminal of NR1 subunit consists of the membrane proximal C0, C1 and C2 cassettes. The C0 cassette is common to all splice variants. The NR2 subunit family consists of four related subtypes, NR2A-NR2D, each encoded by a different gene (Ishii et al., 1993; Monyer et al., 1992; Moriyoshi et al., 1991). The existence of NR3 subunits have also been reported which consists of two subunits NR3A and NR3B, encoded by different genes but its biological significance is still being investigated (Cull-Candy et al., 2001).

Each subunit of NMDA receptors consists of an extracellular N-terminal domain with three transmembrane regions and one re-entrant loop that form the channel pore, and an intracellular C-terminal domain (Bennett and Dingledine, 1995). The large intracellular carboxyl terminus of NMDA receptor

subunits is important for receptor function as it has been shown that knockout mice lacking the carboxyl terminus of NR2A, NR2B or NR2C resemble mice lacking the whole subunit (Sprengel et al., 1998). NR2A and NR2B subunits are predominant throughout the forebrain, including hippocampus, and C-termini of these two subunits contain almost similar number of amino acids (Sheng et al., 1994). Different proteins that interact with C-terminal of NMDA receptors are important in the mechanism by which NMDA receptors are targeted, anchored at synaptic or extrasynaptic sites (Wenthold et al., 2003). To date, considerable research has been done on the proteins interacting with C-termini of NMDA receptors and many similar proteins have been found to be associated with C-termini of NR2A and NR2B subunits particularly in the proximal and distal region. For example, PSD95, a highly abundant protein in postsynaptic density (PSD), interacts with last four amino acids of both NR2A and NR2B subunits (Kornau et al., 1995). A Ca^{2+}/CaM-dependent protein kinase II (CaMKII) interacts with the C-termini of both NR2A and NR2B subunits in the distal region (Gardoni et al., 2001; Leonard et al., 1999; Strack and Colbran, 1998). NR2A and NR2B subunits of NDMA receptors also interacts with the adaptor protein-2 (AP-2) and are endocytosed in a clathrin dependent manner (Lavezzari et al., 2003; Roche et al., 2001; Vissel et al., 2001). In addition to the above described proteins, we have shown that myosin RLC, a light chain of myosin II, interacts with the membrane proximal first 30-37 amino acids of both NR2A and NR2B subunits. While these findings explain much sequence similarity within C-termini of NR2A and NR2B subunits in the proximal and distal region, only 29% sequence similarity was observed in the middle region of these two subunits.

We used a proteomic approach to determine the proteins interacting with the middle region of NR2A C-terminus (amino acids: 875-1029) from mouse brain homogenates both in the presence and absence of calcium. Using mass-spectrometric analysis we identified peptides corresponding to CaM as binding partners of NR2A (875-1029) in the presence of calcium.

Further studies using purified protein confirmed that this interaction is calcium (Ca^{2+}) dependent and CaM binds directly to the NR2A C-terminal. Based on previous analyses by Rhoads et al., analysis of the NR2A (875-1029) region confirmed six putative CaM binding sites within this region (Rhoads and Friedberg, 1997). Using site-directed mutagenesis, sequential mutants were prepared to remove each putative CaM binding site and we found that tryptophan residue at position 1014 is critical for this interaction. Binding of CaM was further analyzed using truncated mutants of NR2A (875-1029) and CaM binding site in the NR2A C-terminal was confined to the region between amino acids 991-1029. Fluorescence equilibrium studies with alexa-Cam showed a high affinity interaction of CaM with NR2A (991-1029) and a binding affinity of 5.2 ± 2.4 nM. We believe an interaction of CaM with the NR2A C-terminal is significant as CaM is currently considered as a NR1-interacting protein.

Materials and methods

Plasmids and constructs

Plasmids containing rat NMDAR2A and NMDAR2B were generous gifts from Dr. P. Seeburg (Heidelberg, Germany). The plasmid (pN60) containing the rat NMDAR1-1a cDNA was a kind gift of Dr. S. Nakanishi (Kyoto, Japan). The construction of full-length C-terminus of the NR1 (amino acid residues 834-938), NR1 C0 (834-863), NR2A (amino acid residues 838-1464) and NR2B (amino acid residues 839-1482) subunits in pGEX-2T have been described previously (Amparan et al., 2005).

For bacterial expression, NR2A (amino acids 875-1029), NR2A (1030-1464), NR2A (875-962), NR2A (991-1029), NR2A (991-1021), NR2A (1004-1029) were amplified by PCR and inserted into the bacterial expression vector pGEX-6P-3 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). NR2A

subunit (amino acid residues 838-874) in pGEX-6P-3 was the generous gift of Dr. J. Saugstad (Robert S. Dow Neurobiology Laboratories, Portland, OR).

Site-directed mutagenesis of GST-NR2A (875-1029) was carried out using the Stratagene QuikChange II XL site-directed mutagenesis kit to yield: mutant-1 (I942A), mutant-2 (L1013A, I942A), mutant-3 (V998A, L1013A, I942A), mutant-4 (V1000A, V998A, L1013A, I942A), mutant-5 (W1014A, V1000A, V998A, L1013A, I942A), mutant-6 (I904A, W1014A, V1000A, V998A, L1013A, I942A), mutant-7 (W1014A), mutant-8 (I904A), mutant-9 (W1014A, I904A). PCR amplifications were carried out according to the manufacturer's protocol and the primers were ordered based on Zheng et al. (Zheng et al., 2004). All sequences were confirmed by the Center for Genome Research and Biocomputing core facility (Oregon State University, Corvallis, OR).

Antibodies and reagents

The anti-GST antibody was a generous gift of Dr. Mark Leid (Oregon State University, Corvallis, OR). Other primary antibodies used for immunoblot analysis are commercially available and included anti-calmodulin (Upstate Biotechnology, Lake Placid, NY), and anti-PSD95 (Affinity Bioreagents Inc., Golden, CO). Alexa-488 labeled calmodulin was a kind gift from Dr. Sonia Anderson (Department of Biochemistry and Biophysics, Oregon State University).

Glutathione-S-transferase (GST) pull-down assays

For pull-down assays from brain homogenates, mouse forebrain extracts were prepared in a binding buffer containing 10 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% NP-40 and 10% glycerol. For mass spectrometric studies to determine proteins that interact with NR2A (875-1029), GST fusion protein or GST alone were incubated with 1mg of brain homogenate both in the presence and absence of calcium. In all Ca^{2+} studies, the appropriate binding buffer (binding buffer lacking EDTA but

including 1 mM calcium chloride) was used in the reaction and all subsequent washes to remove unbound protein. Samples were allowed to incubate at 4°C overnight, unbound protein was removed by three sequential washes with binding buffer. Bound proteins were eluted from the beads by boiling in sample buffer. Proteins were separated by one dimensional polyacrylamide gel electrophoresis (PAGE) on a 4–12% pre-cast Criterion gel (BioRad Laboratories, Hercules, CA). After electrophoresis, gels were washed three times for 10 min in deionized (DI) water. Proteins were visualized by Bio-safe Coomassie G250 blue stain (BioRad), rinsed and stored overnight in DI water at room temperature. A single band that can be clearly differentiated for the reactions done in absence/presence of calcium was excised from the gel and were sent to the Fred Hutchinson Computational Proteomics Laboratory (Seattle, WA) for analysis. The data from mass spectrometric analysis was processed and filtered using peptide filter parameter, peptide prophet score ≥ 8 (Tables 1 and 2). At this score, peptide identifications with peptide prophet scores less than 0.8 were removed. Mass spectrometry data were searched against a mouse protein database using the software search algorithms COMET.

Pull-down assays with purified bovine CaM (purchased from Calbiochem, San Diego, CA, cat. no.208690) were done as previously described except assays were initiated with the addition of 250 nM recombinant CaM (Amparan et al., 2005). In brief, GST fusion carboxyl regions of NR1 and NR2A, or GST alone were incubated in binding buffer (as described above) for 1 hour at 4°C. Unbound protein was removed by three sequential washes with binding buffer and bound proteins were eluted from the beads by boiling in sample buffer. In all Ca^{2+} studies, the appropriate binding buffer (binding buffer lacking EDTA but including 1 mM calcium chloride) was used in the reaction and all subsequent washes to remove unbound protein. Proteins were separated by SDS-PAGE, transferred to

nitrocellulose membranes and processed for immunoblot analysis using an anti-CaM antibody.

Protein purification

GST-fused NR1-C0, NR2A (991-1029), NR2A (991-1021), and NR2A (1004-1029) were expressed in *Escherichia coli* (BL21-Gold (DE3)pLysS; Stratagene, La Jolla, CA), and purified by column chromatography using immobilized glutathione (Pierce, Rockford, IL). The bound GST fusion protein was eluted using a buffer containing 10mM reduced glutathione and 50 mM Tris-HCl (pH 7.5) followed by dialysis against a buffer containing 50 mM Tris-HCl and 150 mM NaCl (pH 7.5).

Fluorescence spectroscopy

Fluorescence measurements were taken in 300 μl of buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl and a final alexa labeled CaM concentration of 20 nM. Purified GST-NR2A (991-1029) or GST-NR1 C0 was added from a concentrated stock solution: 98 μM (GST-NR2A (991-1029)) and 89 μM (GST-NR1C0). All additions of purified GST-fused proteins were made sequentially from a single stock solution, and the fluorescence emission was corrected for dilution. All experiments were performed on a SLM 8000C spectrofluorometer (SLM Instruments, Urbana, IL).

Data were fit to equation (1) by weighted nonlinear least squares using the computer program Scientist® (Micromath Inc., St. Louis, MO) when the weighting factors were equal to the square of the reciprocal of the standard deviation for each data point:

$$(1) \qquad F = a + b \left\{ \frac{2\, R_0\, L_0}{R_0 + L_0 + K + \sqrt{(R_0 + L_0 + K)^2 - 4\, R_0\, L_0}} \right\} + c\, L_0$$

In equation (1), F is the measured fluorescence enhancement, a the fluorescence observed in the absence of alexa labeled CaM, R_0 the total concentration of fluorescently labeled protein, L_0 the total concentration of alexa-CaM, K the dissociation constant for the alexa CaM-protein complex, b is the relative fluorescence quenching of the fluorescent alexa-CaM-protein complex, and c is a parameter used to fit the background linear fluorescence increase observed as alexa-CaM concentration increased.

Results

Proteomic analysis of NR2A (875-1029) binding partners

To determine which proteins interact with the NR2A (875-1029) (Fig. 4.1A), we used GST-fused NR2A (875-1029) as bait to pull out potential interacting partners with this region from mouse brain homogenate. GST-fused NR2A (875-1029) and GST alone were incubated with mouse brain homogenates both in the presence and absence of calcium (1mM). Proteins that are able to interact with the GST or GST-fused protein were separated using SDS-PAGE and stained with Coomassie blue (Fig. 4.1B). Lane 5 shows GST-NR2A (875-1029) as a control. Lane 6 and 7 show proteins interacting GST-NR2A (875-1029) after incubating with brain homogenate in the absence and presence of calcium, respectively. A prominent band was observed in lane 7 where GST-NR2A (875-1029) was incubated with brain homogenate in the presence of calcium. This band was excised from the gel and sent for mass spectrometric analysis. The data from mass spectrometric analysis was processed and filtered using peptide filter parameter, peptide prophet score ≥ 8 (Tables 1 and 2). A total of 63 peptides were identified that corresponds to calmodulin (CaM). Table 1 also shows the number of unique peptides and their corresponding accession numbers. In total, 9 unique peptides were identified that covered approximately 52% of the amino acid sequence of CaM (Table 2).

Calmodulin binds directly to the NR2A C-terminal in a calcium dependent manner

We validated the mass spectrometric data and binding of CaM to the C-terminus of NR2A subunit of NMDA receptor in a pull-down assay using GST-fusion proteins. Several truncation mutants of the NR2A C-termini were prepared to test if CaM binds to other regions of the NR2A C-terminal. The membrane proximal region of NR2A C-terminal NR2A, (838-874); middle region, NR2A (875-1029); distal region, NR2A (1030-1464); and NR1-C0 (834-864) were bacterially expressed as GST fusion proteins and immobilized on glutathione sepharose beads. GST-fused deletion mutants and GST alone were incubated with purified CaM (250nM) in the presence and absence of calcium. The membrane proximal region of NR1 C-terminus (NR1-C0) was used as a positive control as it has previously been shown to interact with CaM (Ehlers et al., 1996). Immunoblotting analyses with an anti-CaM antibody revealed that CaM binds strongly and specifically to NR2A (875-1029) and NR1-C0 only in the presence of calcium (Fig. 4.2, compare lanes 4 and 6 with 9 and 11). No interactions were observed with GST alone or with the proximal and distal C-terminal mutants of NR2A in the presence of calcium (lanes 7, 8, and 10). Together these data show that CaM binds specifically to the middle region of NR2A within amino acids 875-1029 in a calcium dependent manner.

Calmodulin does not interact with the NR2B C-terminal

To determine if CaM interacts with the equivalent region of the NR2B C-terminal, we expressed full length C-termini of NR1, NR2A and NR2B subunits as GST fusion proteins and examined these regions for interaction with CaM. The GST-fused proteins and GST alone were immobilized on GST beads and incubated with CaM in the presence and absence of calcium. Bound proteins were separated using SDS-PAGE and the presence of CaM was analyzed by immunoblotting using an anti-CaM antibody. CaM was able to interact only

with C-termini of NR1 and NR2A in the presence of calcium (Fig. 4.3, compare lanes 3, 4, and 5 with 8, 9, and 10). No interaction of CaM was observed with the NR2B subunit indicating that the interaction of CaM is specific for the NR1 and NR2A subunits of the NMDA receptors.

NR2A C-terminal can pull out CaM and PSD-95 from mouse brain homogenates

To further confirm the interaction of CaM with the NR2A C-terminus, we used GST fusion proteins as bait to pull out CaM from mouse brain homogenates. We tested the GST affinity matrices concurrently for their ability to bind the known NR2-interacting protein, postsynaptic density protein of 95 kDa (PSD-95). GST alone or GST-fused NR1 and NR2A C-termini were immobilized on glutathione sepharose beads and incubated with mouse brain homogenates both in the presence and absence of calcium. Proteins interacting with GST fusion proteins were separated using SDS-PAGE and CaM was identified using immunoblot analysis. GST-NR1 and NR2A C-termini were able to pull out CaM from mouse brain homogenates in a calcium dependent manner (compare lanes 3, 4 with 7, 8 of lower panel; Fig. 4.4). Consistent with previous data for NMDA receptor binding specificity of PSD-95, the NR2A C-terminus was able to pull out PSD-95 (lanes 4 and 8 of upper panel; Fig. 4.4). However, we noticed a decrease in the binding affinity of PSD-95 with NR2A C-terminus in the presence of calcium. This study further confirms the Ca^{2+}-dependence for the interaction of CaM with NMDA receptor subunits. In addition, native PSD-95 was not retained on control or GST/NR1 affinity matrices (lanes 2 and 6 of Fig. 4 upper panel) indicating that PSD-95 displayed appropriate binding specificity in this assay.

W1014 in the NR2A C-terminal is critical for interaction with calmodulin

Calmodulin binds to specific motifs that have been identified through various sequence homology analyses in different studies (Rhoads and

Friedberg, 1997). We searched for putative CaM binding sites in NR2A (875-1029) that belong to 1-14, 1-8-14, 1-5-8-14, and 1-16 class (Rhoads and Friedberg, 1997). Upon analysis we found five putative Ca^{2+}-dependent CaM binding motifs 1 (1-8-14), 1 (1-10), 2 (1-14), 1 (1-16) and a tryptophan residue (Fig. 4.5A). We used site-directed mutagenesis to disrupt these putative CaM binding sites. A single hydrophobic residue of these putative CaM binding sites was replaced with alanine using site-directed mutagenesis. Sequential mutants were prepared and named mutant-1 through mutant-6 in which the higher mutant has all mutations that are present in the lower one (Fig. 4.5A). All mutants were expressed as GST-fusion proteins and tested for binding with CaM in the presence of calcium. GST alone, GST-fused NR2A (875-1029) and its mutants (mutant-1 through mutant-6) were immobilized on GST beads. Immobilized beads were tested for their interaction with CaM (250nM) in the presence of calcium (1mM). Immunoblotting with anti-CaM antibody indicates that CaM binds to mutant-1, 2, 3, and 4 similarly as with wild type NR2A (875-1029) (lanes 3 to 7; Fig. 4.5B). Compared to NR2A (875-1029), binding of CaM to mutant-5 is decreased while binding of CaM was lost with mutant-6 (lanes 8 and 9; Fig. 4.5B). Together these data indicates that W1014 and I904, which corresponds to the mutations in mutant-5 and mutant-6, are important for interaction with CaM.

We further analyzed the importance W1014 and I904 with regard to CaM binding by mutating them individually in NR2A (875-1029). Individual mutants containing the single mutation at W1014A (mutant-7), I904A (mutant-8), and both (mutant-9) (as shown in Fig. 4.6A) were prepared and expressed as GST fusion proteins and tested for binding with CaM in the presence of calcium. CaM was unable to interact with mutant-7 and mutant-9 which contain the W1014A mutation (lanes 4 and 6, Fig. 4.6B). However, CaM was still able to bind mutant-8 that had a single mutation of I904A (lane 5, Fig. 4.6B). Together this data shows that W1014 is critical for CaM binding to the NR2A C-terminus. Since mutating tryptophan to alanine may be considered

as a severe mutation, we further tested the NR2A (875-1029) mutant in which W1014 is replaced by phenylalanine. The GST-fused NR2A (875-1029) mutant with the W1014F mutation was not able to interact with CaM (data not shown). This further confirmed that tryptophan at position 1014 is critical for this interaction.

<u>Calmodulin binds to the NR2A subunit with high affinity</u>

Analysis of the NR2A (875-1029) region with truncated mutants revealed that amino acids 991-1029 are important for interaction with CaM (data not shown). We used purified GST-NR2A (991-1029) to determine the binding affinity of CaM using equilibrium fluorescence spectroscopy. Alexa-CaM exhibited maximum excitation at 495 nm and maximum emission at 520 nm was used in our experiments (data not shown). GST-NR2A (991-1029) quenched the fluorescence of Alexa488-CaM in a dose-dependent manner (Fig. 4.7A). A plot of fluorescence intensity as a function of GST-NR2A (991-1029) concentration is shown Fig. 4.7A. The concentration of GST-NR2A (991-1029) for a half maximal quenching of Alexa-488CaM fluorescence was found to be 5.2 ± 2.4 nM. To confirm that the quenching of Alexa488-CaM is not due to non-specific effects of the GST-fused protein, we monitored the fluorescence of Alexa488-CaM in the absence of calcium. No change in the fluorescence intensity of Alexa488-CaM was observed in the absence of calcium (data not shown). We also determined the binding affinity of alexa-CaM with GST-NR1-C0 in our experiments. The concentration of GST-NR1C0 for a half maximal quenching of Alexa-488CaM fluorescence was found to be 5.87 ± 5.48 nM (Fig. 4.7B). Our data shows that the binding affinity of CaM-GST-NR2A (991-1029) interaction was found to comparable to the interaction of CaM-GST-NR1C0. This high affinity interaction of CaM with NR2A C-terminus suggest that Ca^{2+}/CaM may bind NR2A subunit at physiologically relevant concentrations

DISCUSSION

CaM has previously been shown to interact with the NR1 C-terminal of NMDA receptors (Ehlers et al., 1996). In the present study we found a novel CaM binding site in the NR2A C-terminal. Using a proteomics approach, we identified CaM as a binding partner of NR2A (875-1029). A total of 63 peptides of CaM were identified out of which 9 were unique and cover about 52% of the CaM sequence. We report that binding of CaM to the NR2A C-terminus is Ca^{2+}-dependent. In our studies, the Ca^{2+}-dependent interaction of CaM with the NR1 C-terminal is in agreement with previous studies done by Ehlers et al. (Ehlers et al., 1996). We also confirm in our studies that CaM is not a binding partner of the NR2B C-terminal.

In contrast to other glutamate receptors, NMDA receptors are highly permeable to Ca^{2+}-ions which activates many downstream signal transduction pathways. The NR2B subunit is predominantly expressed in the cortex and hippocampus at early stages of development, while expression of NR2A subunit increases over time till adulthood (Sheng et al., 1994). It has also been shown that synaptic and extra-synaptic NMDA receptors have distinct subunit compositions and functional roles (Li et al., 2002; Stephenson et al., 2008). It is widely believed that the identity of the NR2 subunit (NR2A-D) strongly influences the pharmacological and biophysical properties of the receptor (Erreger et al., 2004; Erreger et al., 2005; Krupp et al., 1998). NR1/NR2A receptors have faster gating and higher open probability than with NR1/NR2B receptors (Erreger et al., 2005). These data suggest that in addition to developmental and activity-dependent regulation of NR2A and NR2B subunits, NR2 subunits have unique physiological roles.

The activity of NMDA receptors is highly regulated by intracellular Ca^{2+}, kinases, and phosphatases (McBain and Mayer, 1994; Rosenmund et al., 1995; Tong et al., 1995). Multiple proteins like CaMKII, AP2, and PSD-95 (Chung et al., 2004; Grant et al., 2001; Husi and Grant, 2001; Lavezzari et al., 2003; Roche et al., 2001) have been shown to interact with the proximal and

distal regions of NR2A and NR2B C-termini. The C-termini of NR2A (838-1464) and NR2B (839-1482) subunits are almost identical in number of amino acids. In addition, there is about a 38-52% amino acid identity between full length NR2 A-D subunits (Stephenson et al., 2008). We observed only 29% amino acid identity in the middle region of the NR2A (871-1030) and NR2B (871-1028) C-termini where few proteins are known to interact and this formed a basis for our study to identify the proteins that interact with NR2A C-terminal middle region.

In our studies we found that CaM was unable to interact with the NR2B subunit of the NMDA receptors. This data is consistent with the previous studies in which CaM was not co-immunoprecipitated with NR2B subunit (Ehlers et al., 1996). We were able to pull out CaM and PSD-95 from the mouse brain lysate using GST-NR2A full length C-terminal. However, we noticed that the binding of PSD-95 with NR2A C-terminus was decreased in the presence of calcium compared to the PSD-95 binding observed in the NRA C-terminus in the absence of calcium (Fig 4.4). This decrease in binding of PSD-95 with NR2A C-terminus may be either due to less availability of PSD-95 in the presence of Ca^{2+} or due to the competition of PSD-95 with other proteins that may interact in the vicinity of PSD-95 binding site. It has been shown previously that calcium-calmodulin-dependent protein kinase II (CaMKII) phosphorylation modulates PSD-95 binding to NMDA receptors (Gardoni et al., 2006). Thus it may be possible that in the presence of Ca^{2+}/CaM CaMKII gets activated and competes with PSD-95 on the NR2A C-terminus. Also it has been found that PSD-95 can interact with CaM in the presence of other PDZ binding proteins (Fukunaga et al., 2005). Binding of CaM to PSD-95 is the other possible reason for the decreased binding of PSD-95 with the NR2A C-terminal.

We concentrated on the NR2A (875-1029) region of NR2A C-terminus in order to identify the proteins that interact with NR2A C-terminus. The region NR2A (875-1029) is also considered important for modulating desensitization

of NR2A containing NMDA receptors by calcineurin (Krupp et al., 2002). Our finding that CaM is also a binding partner of NR2A C-terminus is important as in many studies CaM or CaM-dependent proteins have been shown to regulate the functional effect of NMDA receptors. Intracellular Ca^{2+} may lead to Ca^{2+}-dependent inactivation of NMDA receptors which was further shown to be mediated by CaM through the NR1 subunit (Ehlers et al., 1996; Krupp et al., 1996; Legendre et al., 1993; Zhang et al., 1998). However, this Ca^{2+}-dependent inactivation of NMDA receptors is subunit specific and is seen only with NR1-1a/NR2A or NR1a/NR2D heteromers (Krupp et al., 1996). Binding of CaM to NR2A C-terminus will provide more insight to the process of Ca^{2+}-dependent inactivation of NMDA receptors which is known to be NR2 subunit specific.

In previous studies the binding affinity of dansyl-CaM with the NR1-C0 peptide was found to be 87 nm (Ehlers et al., 1996). However, different studies report different binding affinity for the interaction of CaM to the NR1-C0 region. For instance, using fluorescein-tagged NR1-C0 peptide Akyol et al. shows a comparable affinity of 80nM while Krupp et al. shows the affinity of 21nM (Akyol et al., 2004; Krupp et al., 1999). We also report a high affinity binding for the interaction CaM with both NR2A C-terminus and NR1-C0 subunit. Our finding that the interaction of CaM with the NR2A C-terminus is of high affinity indicates an important role for this interaction at physiological concentrations of CaM. Moreover, we also show that this interaction is comparable to the interaction of CaM with NR1 C-terminus which has already been shown to be involved in the Ca^{2+} -dependent inactivation of NMDA receptors (Ehlers et al., 1996).

CaM binding motifs have been extensively studied in the past decade to characterize the sequences that interact with CaM both in the presence and absence of Ca^{2+} (Ataman et al., 2007; Rhoads and Friedberg, 1997; Shen et al., 2005; Yap et al., 2000). Proteins that bind CaM in a Ca^{2+}-dependent manner are often characterized by a basic, often amphipathic, helix which

consists of approximately 20 amino acids. In the presence of Ca^{2+}, CaM binds to a specific sequence of 10 to 14 amino acids with hydrophobic amino acids at specific positions (Rhoads and Friedberg, 1997; Yap et al., 2000). According to the data available from previous studies, the Ca^{2+}-dependent binding site of CaM possesses critical hydrophobic residues at positions 1-8-14 or 1-5-10 or 1-16 (Rhoads and Friedberg, 1997; Yap et al., 2000). The CaM binding 1-8-14 motifs have flanking hydrophobic residues at positions 1 and 14 with internal conserved hydrophobic residues occurring at position 8. These binding motifs have previously been recognized in structural analyses of CaM-target peptide interactions (Ikura et al., 1992; Meador et al., 1993). The two CaM binding sites in NR1 C-terminal have not been classified until recently and it was shown that CaM interacts with a new 1-7 binding motif found in the C1-C2 exon cassette (Ataman et al., 2007). However, binding motifs in NR1-C0 indicates no classical Ca^{2+}-dependent CaM motifs and it is believed that tryptophan at position 858 is important for this interaction (Ataman et al., 2007).

We find it surprising in our studies that CaM did not interact with the classical 1-8-14 binding motif that we identified as one of the putative binding site for CaM. Instead, we found that CaM interacts with the binding site that has the only tryptophan residue in NR2A (875-1029). A tryptophan-based motif has previously been shown to bind CaM in caldesmon, an actin binding protein (Graether et al., 1997). On further analysis we found a sequence similarity between the binding site of CaM in NR1-C0 and NR2A C-termini. Similar to the CaM binding site in NR1-C0, we were not able to classify the binding site of CaM in NR2A C-terminus in any of the previous known CaM binding motifs. We believe that the CaM binding site on NR2A C-terminal may introduce a new class for CaM binding site which is present in both NR1 and NR2A C-termini.

In a recent study, it was shown that CaM is involved in inactivation of NMDA receptors and this inactivation is achieved through dimerization of NR1

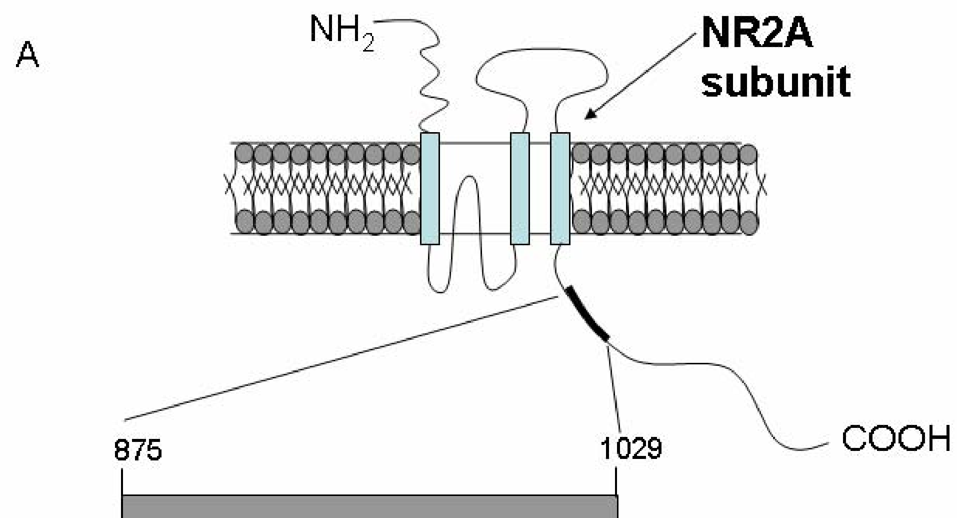
subunits by CaM (Wang et al., 2008). A role of CaM in dimerization has also been implicated in another protein called petunia glutamate decarboxylase (PGD) (Yuan and Vogel, 1998). CaM causes dimerizaton of PDG and the binding site of CaM in C-terminal of PGD has tryptophan and is unusual compared to previous known CaM binding domains (Yuan and Vogel, 1998). After analyzing the sequence of NR1 C-terminal, PGD and NR2A C-terminal, we found a sequence similarity in the CaM binding site in all three proteins which has a tryptophan residue followed by two basic amino acids. Thus it is possible that this interaction of CaM may be involved in dimerization of NR2A subunits through its C-terminal domain.

Previous studies show that NR2A C-terminus is involved in glycine-independent desensitization and is also important for CaM-dependent inactivation (Krupp et al., 1996; Krupp et al., 2002). It was shown that this modulation of glycine-independent desensitization of NMDA receptors by calcineurin was caused by its phosphatase effect on serines at positions 900 and 929 on NR2A C-terminal. However, no direct interaction of calcineurin has been reported with NR2A C-terminus. Since calcineurin is dependent on CaM for activation, the interaction of CaM to the similar region that has shown to be important for modulation of glycine independent desensitization may provide some more insight to this mechanism (Hubbard and Klee, 1987; Krupp et al., 2002).

Together these studies indicate that proteins regulated by Ca^{2+} have an important role to play in regulating NMDA receptors. In this study, we provide a novel CaM binding site in the C-terminus of NR2A subunit of NMDA receptor. This interaction of CaM with the NR2A subunit of NMDA receptors provides a logical basis for investigating the role of NR2A subunit in Ca^{2+}-dependent inactivation of NMDA receptors and dimerization of NR2A subunits. This knowledge may fill a gap in the current understanding of how CaM modulates the function of NMDA receptors.

Acknowledgement

We thank Dr. Phil Gafken and the Computational Proteomics Laboratory at the Fred Hutchinson Cancer Center for mass spectrometric analysis. This work was supported by an award from the American Heart Association, North West affiliate (0060435Z) to Dr. Jane Ishmael. Peter Hsu was supported by an undergraduate research, innovation, scholarship and creativity (URISC) award from Oregon State University.


A
NH2
NR2A
subunit
875
1029
COOH

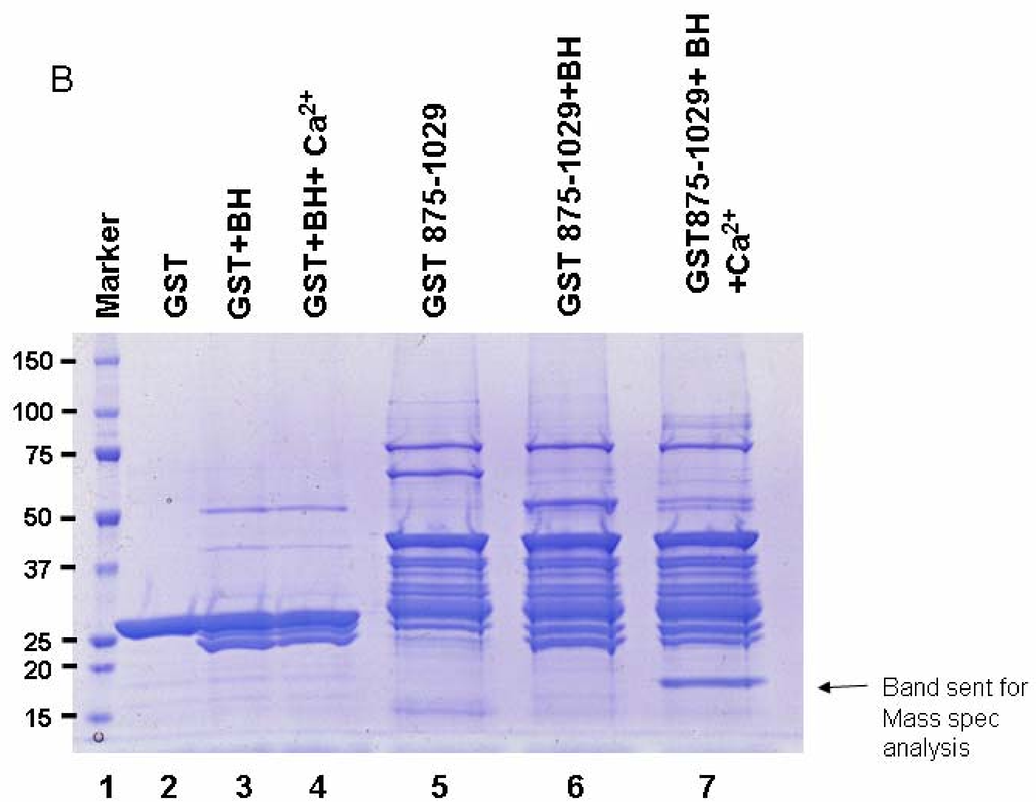

B
Marker
GST
GST+BH
GST+BH+ Ca2+
GST 875-1029
GST 875-1029+BH
GST875-1029+ BH
+Ca2+
150
100
75
50
37
25
20
15
Band sent for
Mass spec
analysis
1
2
3
4
5
6
7

Figure 4.1. Polyacrylamide gel electrophoresis of proteins from mouse brain homogenate that interact with NR2A (875-1029) in the presence and absence of calcium.

A) Schematic representation of C-terminus of NR2A subunit of NMDA receptor showing the middle region (amino acids 875-1029) used in the experiment. **B)** Coomassie blue stain of GST and GST fused NR2A (amino acids 875-1029) incubated in homogenates from mouse forebrain (BH). Proteins were resolved by SDS-PAGE in 4–12% gradient gels and stained to reveal proteins. GST alone and GST fused NR2A (amino acids 875-1029) without incubation with brain homogenates are shown as control (lanes 2 and 5). GST alone and GST fused NR2A (amino acids 875-1029) are shown after incubation with brain homogenates in the absence (lanes 3 and 6) and in the presence (lanes 4 and 7) of calcium. A prominent band observed in lane 7 (~16.7 KDa) was excised and sent for mass spectrometric analysis.

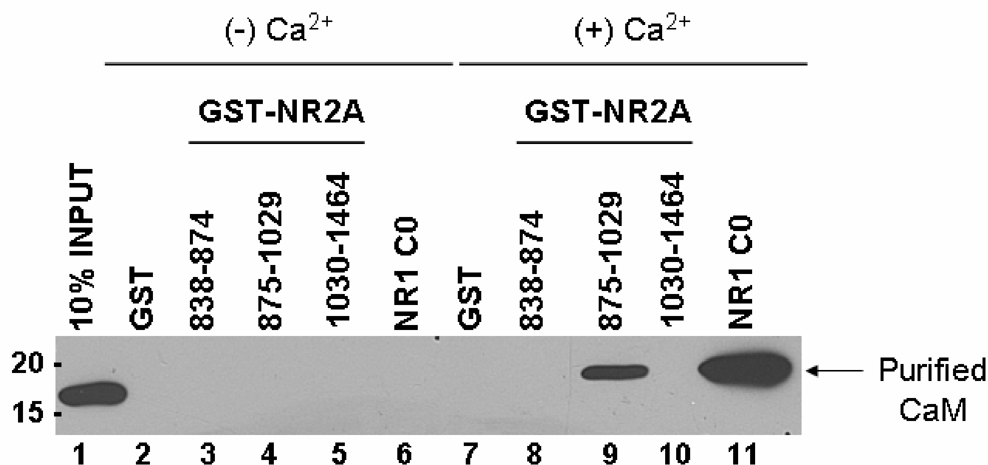


Figure 4.2. Calmodulin binds to the NR2A C-terminal within amino acids 875-1029.

CaM binds to NR2A (875-1029) in the GST pull-down assay. The membrane-proximal regions of NR2A C-terminal, NR2A (838-874); middle region, NR2A (875-1029); distal region, NR2A (1030-1464); and the membrane-proximal region of NR1-C-terminal, NR1-C0 (834-864) were expressed as GST fusion proteins, immobilized on glutathione-Sepharose beads and tested for their ability to bind CaM in the absence (lanes 1 to 6), and presence (lanes 7 to 11) of calcium. Assays were initiated by the addition of 250 nM CaM (Calbiochem) for 2 hours at 4°C in the presence and absence of calcium. Where appropriate, calcium (1 mM) was present throughout the experiment including all wash buffers. Calmodulin bound to GST fusion proteins was resolved by PAGE, and blotted to nitrocellulose. Immune complexes were detected using an anti-calmodulin antibody (Upstate Biotechnology).

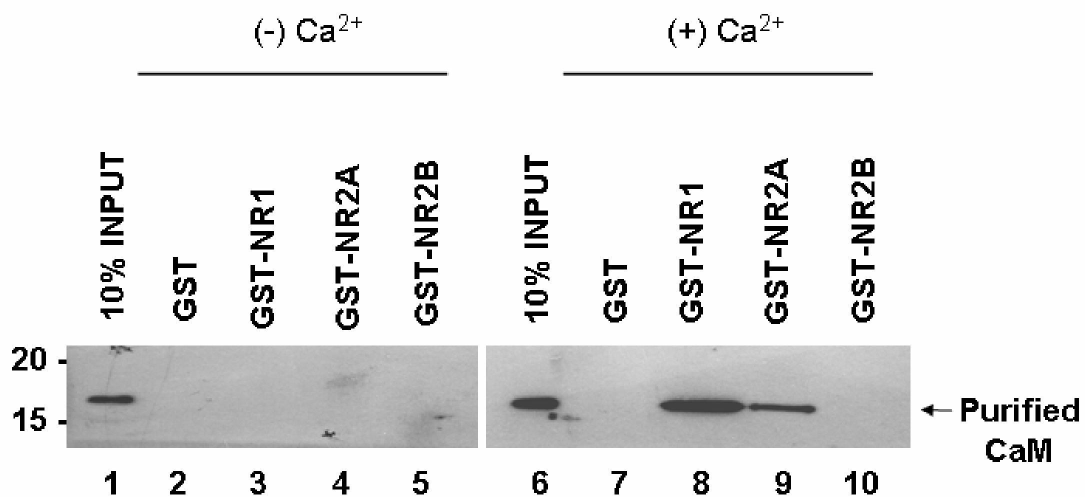


Figure 4.3. CaM interacts with C-termini of NR1 and NR2A but not with NR2B C-terminal

Binding of CaM is specific for NR1 and NR2A C-termini. NR1, NR2A and NR2B C-termini were fused to GST and expressed as GST fusion proteins which were immobilized on glutathione-sepharose beads. Immobilized GST-fusion proteins were tested for their ability to bind CaM both in the absence (lanes 1 to 5) and presence (lanes 6 to 10) of 1 mM calcium. Assays were initiated by the addition of 250 nM calmodulin (Calbiochem) for 2 hours at 4°C in the presence and absence of calcium. Where appropriate, calcium (1 mM) was present throughout the experiment including all wash buffers. Calmodulin bound to GST fusion proteins was resolved by PAGE, and blotted to nitrocellulose. Immune complexes were detected using an anti-calmodulin antibody (Upstate Biotechnology).

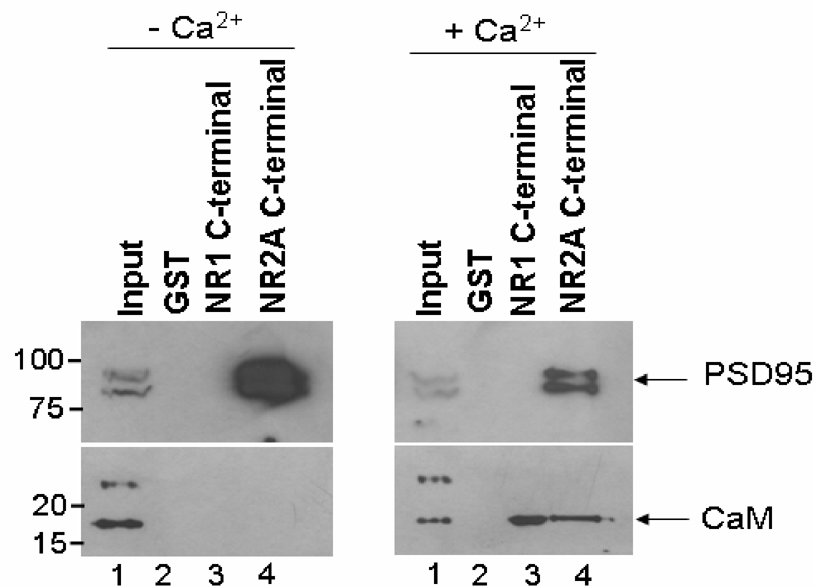
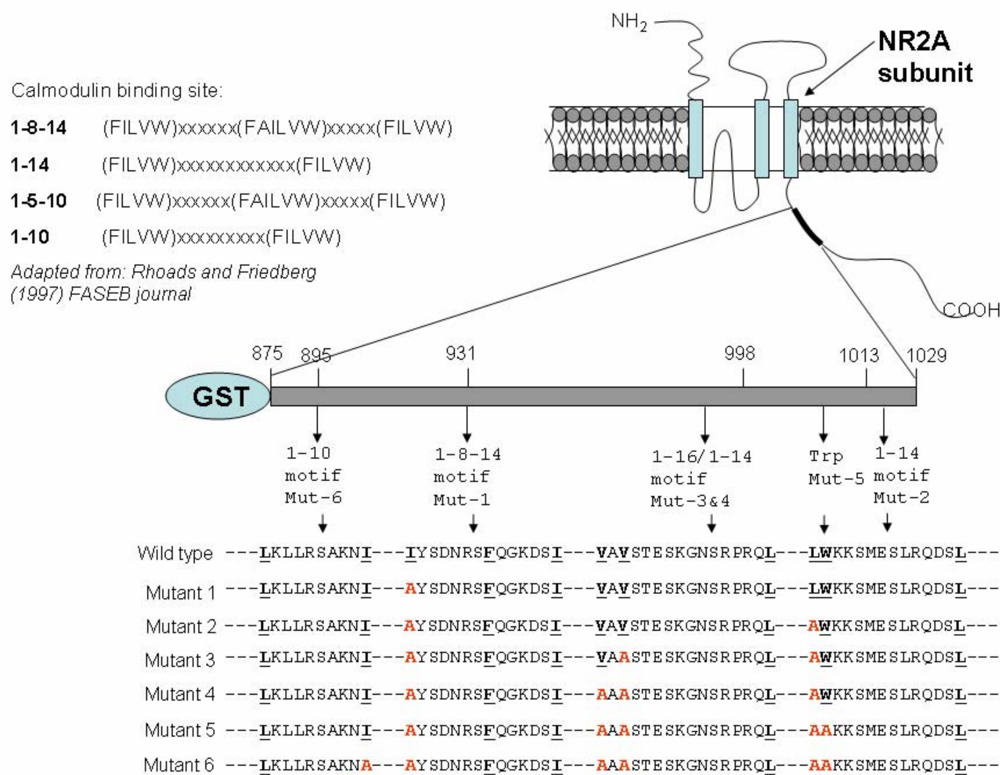


Figure 4.4. Full-length NR2A and NR1 C-termini can bind to CaM in brain lysate in the presence of calcium. NR1 and NR2A C-termini were fused to GST and expressed as GST fusion proteins which were immobilized on glutathione-sepharose beads. GST fusion proteins and GST alone were used as baits to pull down native CaM and a reference NMDA receptor-interacting protein (postsynaptic density protein of 95 kDa (PSD-95)) from a mouse forebrain extract. CaM was retained on the affinity matrices corresponding to the both NR1 and NR2A C-termini (lower panel). Native PSD-95 was retained on NR2A affinity matrices containing only the extreme distal portion of the NR2A C-terminus, consistent with the recognized binding pattern of this protein (upper panel). The immobilized fusion proteins and GST alone were incubated with rotation overnight at 4°C with soluble forebrain extract (1 mg). Calmodulin bound to GST fusion proteins was resolved by SDS-PAGE and blotted to nitrocellulose. Immune complexes corresponding to bound proteins were revealed with anti-CaM (Upstate Biotechnology) and anti-PSD95 antibodies (Abcam).

A



B

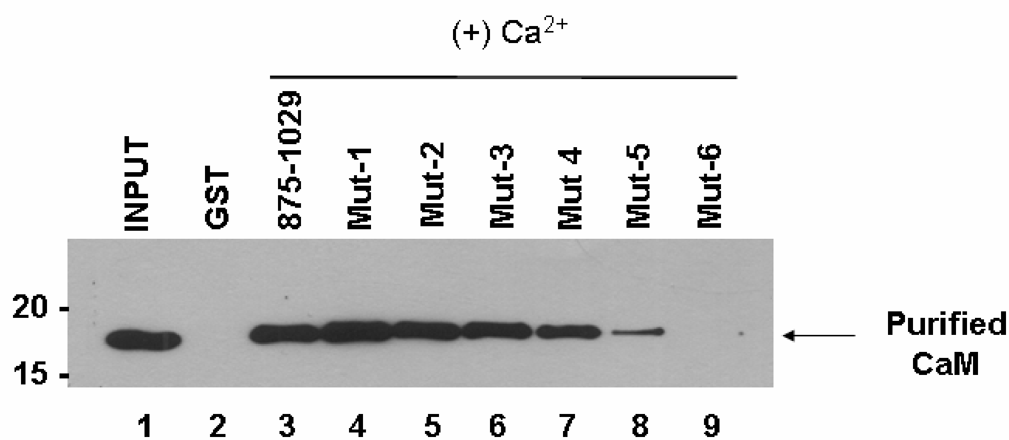


Figure 4.5. Site directed mutagenesis of six putative calmodulin binding sites found in NR2A (875-1029). A) Strategy of site directed mutagenesis for removing putative calmodulin binding sites. Schematic representation of NR2A subunit showing classic CaM binding motifs in NR2A (875-1029) region. Amino acid analyses show 6 classic CaM binding motifs. Hydrophobic residues that bind to CaM were replaced with alanine to remove putative CaM binding sites in NR2A (875-1029). Site-directed mutagenesis was be used to generate these mutants and sequence analysis was done at the Center for Genome Research and Biocomputing lab, Oregon State University. **B)** Sequential removal of six putative CaM binding sites in NR2A (875-1029) led to six mutants named mutant-1 (I942A), mutant-2 (L1013A, I942A), mutant-3 (V998A, L1013A, I942A), mutant-4 (V1000A, V998A, L1013A, I942A), mutant-5 (W1014A, V1000A, V998A, L1013A, I942A), and mutant-6 (I904A, W1014A, V1000A, V998A, L1013A, I942A). Mutant-1 through mutant-6 are sequential mutants that were prepared in the context of the preceding mutants. These mutants were expressed as GST-fusion proteins and tested for binding *in vitro* using recombinant CaM in the presence calcium. CaM binds to mutant-1, 2, 3, and 4 is a similar manner as wild type GST-NR2A (875-1029) (see lanes 3 to 7). CaM binding with mutant-5 is decreased (lane 8) and lost with mutant-6 (lane 9).

A

875 904 1014 1029

GST

Wild type ---LKLLRSAKNI---AYSDNRSFQGKDSI---AAASTESKGNSRPRQL---AWKKSMESLRQDSL---

Mutant 7 ---LKLLRSAKNI---AYSDNRSFQGKDSI---AAASTESKGNSRPRQL---AAKKSMESLRQDSL---

Mutant 8 ---LKLLRSAKNA---AYSDNRSFQGKDSI---AAASTESKGNSRPRQL---AWKKSMESLRQDSL---

Mutant 9 ---LKLLRSAKNA---AYSDNRSFQGKDSI---AAASTESKGNSRPRQL---AAKKSMESLRQDSL---

B

INPUT GST 875-1029 Mut-7 Mut-8 Mut-9

20 -

15 -

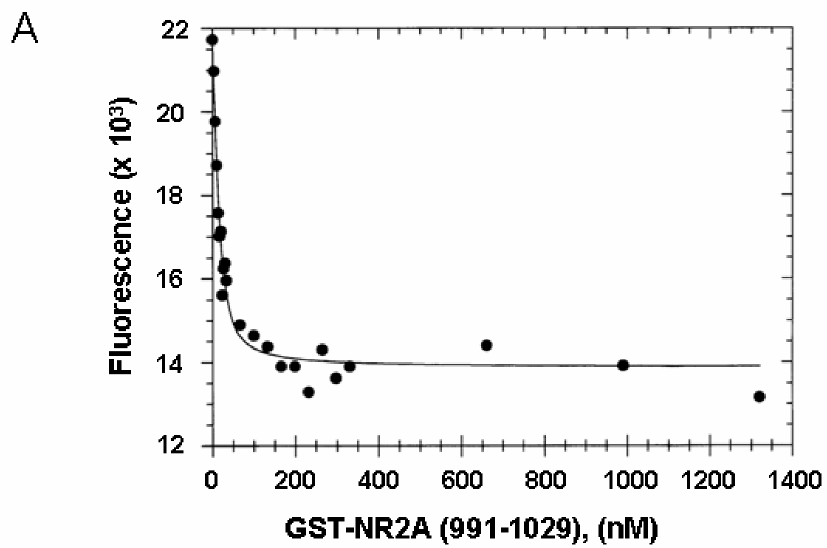
Purified CaM

1 2 3 4 5 6

Figure 4.6. W1014 is critical for binding of CaM to the NR2A C-terminal

A) Schematic representation of the mutants-7,8,and 9 prepared in NR2A (875-1029). **B)** Individual mutants mutant-7 (W1014A), mutant-8 (I904A), and a double mutant, mutant-9, (containing both W1014A and I904A) were prepared, expressed as GST-fusion proteins, and tested for binding with CaM. CaM binds with mutant-8 but this binding is not seen with mutant-7 and mutant-9 (lanes 3-6, lower panel). Assays were initiated by the addition of 250nM calmodulin (Calbiochem) for 1 hour at 4°C in the presence of calcium. Where appropriate, calcium (1 mM) was present throughout the experiment including all wash buffers. Calmodulin bound to GST fusion proteins was resolved by PAGE and blotted to nitrocellulose. Immune complexes were detected using an anti-calmodulin antibody (Upstate Biotechnology).

A



B

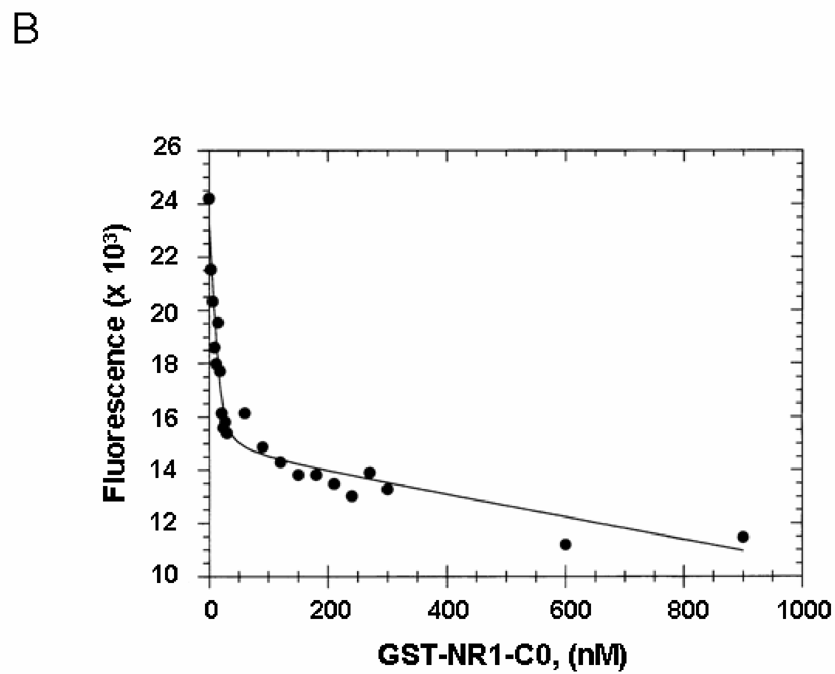


Figure 4.7. Titration of either GST-NR2A (991-1029) or GST-NR1-C0 led to fluorescence quenching of alexa-calmodulin.

Titration of either purified, GST-NR2A (991-1029) (panel A), or purified GST-NR1 C0 (panel B) led to quenching of alexa-calmodulin. Data were fit to a single hyperbolic curve using equation *1* to derive binding affinities of 5.23 $\pm$ 2.4 nM (a = 21602 $\pm$ 367, b = 386 $\pm$ 142, R_0 = 20 $\pm$ 6.56, r^2 = 0.999) for GST-NR2A (991-1029) (panel A), and 5.877 $\pm$ 5.48 nM (a = 22891 $\pm$ 858, b = 502 $\pm$ 375, R_0 = 20 $\pm$ 13.28, r^2 = 0.999) for GST-NR1 C0 (panel B). Each point represents fluorescence determined in triplicate; standard errors lie within the data points.

Table 4.1. Details of CaM peptides found in the band sent for mass spectrometric analysis

Accession number	Name of the protein identified on the gel	Sequence Mass	No. of Peptides	No. of unique peptides
NP_033920 NP_031615 NP_031616	Calmodulin 1 Calmodulin 2 Calmodulin 3	16,852	57	4
NP_081692	Calmodulin-like 3	16,700	6	5

A total of 63 peptides corresponding to calmodulin and 9 unique peptides were found in mass spectrometric analyses. GST alone or GST-fused NR2A (875-1029) were incubated with mouse brain homogenates in presence and absence of calcium. The bound proteins were separated using SDS-PAGE and stained for coomassie blue. A band was specifically identified in the lane where GST-fused NR2A (875-1029) was incubated with mouse brain homogenate in the presence of calcium and the band was sent for mass spectrometric analysis. List of peptides were obtained which were filtered on the bases of Peptide prophet score (≥8).

Table 4.2. Amino acid coverage of CaM peptides determined by mass spectrometric analysis.

```
MADQLTEEQI AEFKEAFSLF DKDGDGTITT KELGTVMRSL GQNPTEAELQ
DMINEVDADG NGTIDFPEFL TMMARKMKDT DSEEEIREAF RVFDKDGNGY
ISAAELRHVM TNLGEKLTDE EVDEMIREAD IDGDGQVNYE EFLQMMTAK
```

Peptide identified in the mass spectrometric analyses covers 52% of amino acid sequence of calmodulin. Amino acid sequence of mouse calmodulin-1 (accession number: NP_033920) is shown in black and amino acid sequence of the peptides that were identified in the mass spectrometric analyses is shown in blue.

References

Akyol Z, Bartos JA, Merrill MA, Faga LA, Jaren OR, Shea MA and Hell JW (2004) Apo-calmodulin binds with its C-terminal domain to the N-methyl-D-aspartate receptor NR1 C0 region. *The Journal of biological chemistry* **279**(3):2166-2175.

Amparan D, Avram D, Thomas CG, Lindahl MG, Yang J, Bajaj G and Ishmael JE (2005) Direct interaction of myosin regulatory light chain with the NMDA receptor. *Journal of neurochemistry* **92**(2):349-361.

Ataman ZA, Gakhar L, Sorensen BR, Hell JW and Shea MA (2007) The NMDA receptor NR1 C1 region bound to calmodulin: structural insights into functional differences between homologous domains. *Structure* **15**(12):1603-1617.

Bennett JA and Dingledine R (1995) Topology profile for a glutamate receptor: three transmembrane domains and a channel-lining reentrant membrane loop. *Neuron* **14**(2):373-384.

Chung HJ, Huang YH, Lau LF and Huganir RL (2004) Regulation of the NMDA receptor complex and trafficking by activity-dependent phosphorylation of the NR2B subunit PDZ ligand. *J Neurosci* **24**(45):10248-10259.

Cull-Candy S, Brickley S and Farrant M (2001) NMDA receptor subunits: diversity, development and disease. *Current opinion in neurobiology* **11**(3):327-335.

Durand GM, Gregor P, Zheng X, Bennett MV, Uhl GR and Zukin RS (1992) Cloning of an apparent splice variant of the rat N-methyl-D-aspartate receptor NMDAR1 with altered sensitivity to polyamines and activators of protein kinase C. *Proceedings of the National Academy of Sciences of the United States of America* **89**(19):9359-9363.

Ehlers MD, Zhang S, Bernhadt JP and Huganir RL (1996) Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* **84**(5):745-755.

Erreger K, Chen PE, Wyllie DJ and Traynelis SF (2004) Glutamate receptor gating. *Critical reviews in neurobiology* **16**(3):187-224.

Erreger K, Dravid SM, Banke TG, Wyllie DJ and Traynelis SF (2005) Subunit-specific gating controls rat NR1/NR2A and NR1/NR2B NMDA channel kinetics and synaptic signalling profiles. *The Journal of physiology* **563**(Pt 2):345-358.

Fukunaga Y, Matsubara M, Nagai R and Miyazawa A (2005) The interaction between PSD-95 and Ca2+/calmodulin is enhanced by PDZ-binding proteins. *Journal of biochemistry* **138**(2):177-182.

Furukawa H, Singh SK, Mancusso R and Gouaux E (2005) Subunit arrangement and function in NMDA receptors. *Nature* **438**(7065):185-192.

Gardoni F, Bellone C, Cattabeni F and Di Luca M (2001) Protein kinase C activation modulates alpha-calmodulin kinase II binding to NR2A

subunit of N-methyl-D-aspartate receptor complex. *The Journal of biological chemistry* **276**(10):7609-7613.

Gardoni F, Polli F, Cattabeni F and Di Luca M (2006) Calcium-calmodulin-dependent protein kinase II phosphorylation modulates PSD-95 binding to NMDA receptors. *The European journal of neuroscience* **24**(10):2694-2704.

Graether SP, Heinonen TY, Raharjo WH, Jin JP and Mak AS (1997) Tryptophan residues in caldesmon are major determinants for calmodulin binding. *Biochemistry* **36**(2):364-369.

Grant ER, Guttmann RP, Seifert KM and Lynch DR (2001) A region of the rat N-methyl-D-aspartate receptor 2A subunit that is sufficient for potentiation by phorbol esters. *Neuroscience letters* **310**(1):9-12.

Hollmann M and Heinemann S (1994) Cloned glutamate receptors. *Annual review of neuroscience* **17**:31-108.

Hubbard MJ and Klee CB (1987) Calmodulin binding by calcineurin. Ligand-induced renaturation of protein immobilized on nitrocellulose. *The Journal of biological chemistry* **262**(31):15062-15070.

Husi H and Grant SG (2001) Isolation of 2000-kDa complexes of N-methyl-D-aspartate receptor and postsynaptic density 95 from mouse brain. *Journal of neurochemistry* **77**(1):281-291.

Ikura M, Clore GM, Gronenborn AM, Zhu G, Klee CB and Bax A (1992) Solution structure of a calmodulin-target peptide complex by multidimensional NMR. *Science (New York, NY* **256**(5057):632-638.

Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M and et al. (1993) Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. *The Journal of biological chemistry* **268**(4):2836-2843.

Kornau HC, Schenker LT, Kennedy MB and Seeburg PH (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science (New York, NY* **269**(5231):1737-1740.

Krupp JJ, Vissel B, Heinemann SF and Westbrook GL (1996) Calcium-dependent inactivation of recombinant N-methyl-D-aspartate receptors is NR2 subunit specific. *Molecular pharmacology* **50**(6):1680-1688.

Krupp JJ, Vissel B, Heinemann SF and Westbrook GL (1998) N-terminal domains in the NR2 subunit control desensitization of NMDA receptors. *Neuron* **20**(2):317-327.

Krupp JJ, Vissel B, Thomas CG, Heinemann SF and Westbrook GL (1999) Interactions of calmodulin and alpha-actinin with the NR1 subunit modulate Ca2+-dependent inactivation of NMDA receptors. *J Neurosci* **19**(4):1165-1178.

Krupp JJ, Vissel B, Thomas CG, Heinemann SF and Westbrook GL (2002) Calcineurin acts via the C-terminus of NR2A to modulate desensitization of NMDA receptors. *Neuropharmacology* **42**(5):593-602.

Lavezzari G, McCallum J, Lee R and Roche KW (2003) Differential binding of the AP-2 adaptor complex and PSD-95 to the C-terminus of the NMDA receptor subunit NR2B regulates surface expression. *Neuropharmacology* **45**(6):729-737.

Legendre P, Rosenmund C and Westbrook GL (1993) Inactivation of NMDA channels in cultured hippocampal neurons by intracellular calcium. *J Neurosci* **13**(2):674-684.

Leonard AS, Lim IA, Hemsworth DE, Horne MC and Hell JW (1999) Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor. *Proceedings of the National Academy of Sciences of the United States of America* **96**(6):3239-3244.

Li B, Chen N, Luo T, Otsu Y, Murphy TH and Raymond LA (2002) Differential regulation of synaptic and extra-synaptic NMDA receptors. *Nature neuroscience* **5**(9):833-834.

McBain CJ and Mayer ML (1994) N-methyl-D-aspartic acid receptor structure and function. *Physiological reviews* **74**(3):723-760.

Meador WE, Means AR and Quiocho FA (1993) Modulation of calmodulin plasticity in molecular recognition on the basis of x-ray structures. *Science (New York, NY* **262**(5140):1718-1721.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B and Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science (New York, NY* **256**(5060):1217-1221.

Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N and Nakanishi S (1991) Molecular cloning and characterization of the rat NMDA receptor. *Nature* **354**(6348):31-37.

Nakanishi N, Axel R and Shneider NA (1992) Alternative splicing generates functionally distinct N-methyl-D-aspartate receptors. *Proceedings of the National Academy of Sciences of the United States of America* **89**(18):8552-8556.

Nakanishi S, Masu M, Bessho Y, Nakajima Y, Hayashi Y and Shigemoto R (1994) Molecular diversity of glutamate receptors and their physiological functions. *Exs* **71**:71-80.

Rhoads AR and Friedberg F (1997) Sequence motifs for calmodulin recognition. *Faseb J* **11**(5):331-340.

Roche KW, Standley S, McCallum J, Dune Ly C, Ehlers MD and Wenthold RJ (2001) Molecular determinants of NMDA receptor internalization. *Nature neuroscience* **4**(8):794-802.

Rosenmund C, Feltz A and Westbrook GL (1995) Calcium-dependent inactivation of synaptic NMDA receptors in hippocampal neurons. *Journal of neurophysiology* **73**(1):427-430.

Shen X, Valencia CA, Szostak JW, Dong B and Liu R (2005) Scanning the human proteome for calmodulin-binding proteins. *Proceedings of the*

National Academy of Sciences of the United States of America **102**(17):5969-5974.

Sheng M, Cummings J, Roldan LA, Jan YN and Jan LY (1994) Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature* **368**(6467):144-147.

Sprengel R, Suchanek B, Amico C, Brusa R, Burnashev N, Rozov A, Hvalby O, Jensen V, Paulsen O, Andersen P, Kim JJ, Thompson RF, Sun W, Webster LC, Grant SG, Eilers J, Konnerth A, Li J, McNamara JO and Seeburg PH (1998) Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. *Cell* **92**(2):279-289.

Stephenson FA, Cousins SL and Kenny AV (2008) Assembly and forward trafficking of NMDA receptors (Review). *Molecular membrane biology* **25**(4):311-320.

Strack S and Colbran RJ (1998) Autophosphorylation-dependent targeting of calcium/ calmodulin-dependent protein kinase II by the NR2B subunit of the N-methyl- D-aspartate receptor. *The Journal of biological chemistry* **273**(33):20689-20692.

Sugihara H, Moriyoshi K, Ishii T, Masu M and Nakanishi S (1992) Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. *Biochemical and biophysical research communications* **185**(3):826-832.

Tichelaar W, Safferling M, Keinanen K, Stark H and Madden DR (2004) The Three-dimensional Structure of an Ionotropic Glutamate Receptor Reveals a Dimer-of-dimers Assembly. *Journal of molecular biology* **344**(2):435-442.

Tong G, Shepherd D and Jahr CE (1995) Synaptic desensitization of NMDA receptors by calcineurin. *Science (New York, NY* **267**(5203):1510-1512.

Ulbrich MH and Isacoff EY (2007) Subunit counting in membrane-bound proteins. *Nature methods* **4**(4):319-321.

Vissel B, Krupp JJ, Heinemann SF and Westbrook GL (2001) A use-dependent tyrosine dephosphorylation of NMDA receptors is independent of ion flux. *Nature neuroscience* **4**(6):587-596.

Wang C, Wang HG, Xie H and Pitt GS (2008) Ca2+/CaM controls Ca2+-dependent inactivation of NMDA receptors by dimerizing the NR1 C termini. *J Neurosci* **28**(8):1865-1870.

Wenthold RJ, Prybylowski K, Standley S, Sans N and Petralia RS (2003) Trafficking of NMDA receptors. *Annual review of pharmacology and toxicology* **43**:335-358.

Yap KL, Kim J, Truong K, Sherman M, Yuan T and Ikura M (2000) Calmodulin target database. *Journal of structural and functional genomics* **1**(1):8-14.

Yuan T and Vogel HJ (1998) Calcium-calmodulin-induced dimerization of the carboxyl-terminal domain from petunia glutamate decarboxylase. A

novel calmodulin-peptide interaction motif. *The Journal of biological chemistry* **273**(46):30328-30335.

Zhang S, Ehlers MD, Bernhardt JP, Su CT and Huganir RL (1998) Calmodulin mediates calcium-dependent inactivation of N-methyl-D-aspartate receptors. *Neuron* **21**(2):443-453.

Zheng L, Baumann U and Reymond JL (2004) An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic acids research* **32**(14):e115.

CHAPTER 5

CONCLUSION

Regulation of NMDA-type Glutamate Receptors and MDR1 by Two Members of the EF-hand Protein Family

In the present study we identified a mechanism for exit of NR1/NR2A containing N-methyl-D-aspartate (NMDA) receptors from the Golgi complex that involves a direct interaction with myosin regulatory light chain (myosin RLC). Myosin RLC is an integral component of the myosin II complex that binds to the tandem IQ motifs located in the neck region of myosin II heavy chain. In our studies we have shown that the isolated light chain is also a binding partner of proteins other than myosin heavy chain such as NMDA receptor subunits and multi-drug resistance protein 1 (MDR1) (Fig. 5.1). By adopting different confirmations when bound to proteins other than myosin heavy chain, myosin RLC may help in trafficking of proteins. We were able to prove in our studies that myosin RLC does not bind to two target sequences simultaneously. Our study provides an important role of the myosin motor where myosin can interact with intracellular domains of NMDA receptors and MDR1 and help in trafficking or localization of receptors in a cell.

An additional important observation in our study is that NMDA receptors are likely non-myosin targets of myosin RLC. Our biochemical studies indicate that myosin RLC forms distinct complexes with NMDA receptor subunits and have an independent role apart from the myosin II complex. The functional significance of the myosin RLC interaction with the NR2A subunit of NMDA receptor was shown by mutating myosin RLC binding site in NR2A. Mammalian cells co-expressing NR1 and the NR2A subunit deficient in RLC binding site showed a defect in NR1/NR2A trafficking. NR2A subunits lacking myosin RLC binding site have restricted cytosolic expression and were identified in a compartment of the Golgi complex. We also noticed that myosin RLC colocalizes with endogenous myosin heavy chain following the disruption of the RLC target sequence in NR2A. This may suggest that myosin RLC may re-localize to a previously unoccupied cellular compartment with myosin heavy chain if its binding to NR2A subunit is disrupted.

Another group identified myosin RLC as a binding partner of the ATP-binding cassette (ABC) family of efflux transporters and established a role for the same light chain in trafficking of bile salt export protein (BSEP). This was an important and timely development in understanding the functional role of myosin RLC when associated with non-myosin target proteins. In our study we analyzed the possibility if myosin RLC binds to MDR1 in a manner that is similar to NMDA-myosin RLC binding.

We report that myosin RLC interacts with a relatively short linker region of MDR1 through its amino terminal and this interaction of myosin RLC with the MDR1-linker region was decreased upon its phosphorylation by MLCK. In addition, the first three EF-hand domains of myosin RLC are important for this interaction. We saw similar results for the interaction of myosin RLC with the NR1 subunit of NMDA receptors which led to the conclusion that the interaction of myosin RLC with MDR1 is qualitatively similar to the interaction of myosin RLC with the NR1 subunit of the NMDA receptor. Using MDCK cells stably expressing MDR1 as a model system, we found that phosphorylation of myosin RLC plays an important role in trafficking and apical localization of MDR1. Phosphorylation of myosin RLC in MDCKII-MDR1 cells was disrupted using ML-7, an inhibitor of MLCK. A functional consequence of disrupting the phosphorylated state of light chain was determined using [^{3}H]-digoxin which is a well known substrate of MDR1. Upon treatment with ML-7, the permeability of [^{3}H]-digoxin was increased from the apical (AP) to basolateral (BL) side which may be due to the decreased expression MDR1 in the apical region of MDCKII-MDR1 cells. This decreased apical expression of MDR1 was further confirmed using immunocytochemistry. Using confocal microscopy, we show that the apical distribution of MDR1 in these cells was decreased after ML-7 treatment, confirming the distribution of MDR1 is regulated by the phosphorylation state of myosin RLC.

Finally, while investigating the specificity of myosin RLC-NMDA receptor interactions, we identified another EF-hand protein, calmodulin (CaM)

that interacts with the NR2A subunit of the NMDA receptor. We report that CaM binds directly to the NR2A C-terminal in a Ca^{2+}-dependent manner. In previous studies, CaM has been shown to interact with NMDA receptors through its NR1 subunit. CaM in the presence of calcium (Ca^{2+}) binds to two distinct sites on the C-terminus of the NR1 subunit of NMDA receptors resulting in inactivation and decreased open probability of these ion channels. Our finding that CaM is also a binding partner of NR2A C-terminus is important as Ca^{2+}-dependent inactivation of NMDA receptors is NR2 subunit specific. In addition, calcineurin, a CaM-dependent protein, has been shown to modulate the desensitization of NMDA receptors by acting on NR2A C-terminal region close to this interaction. We characterized this binding site of CaM in the NR2A C-terminus and found that the CaM binding site in the NR2A C-terminal was located in the region between amino acids 991-1029. Using site directed mutagenesis, we also found that tryptophan residue at position 1014 of NR2A C-terminus is critical for this interaction. Using fluorescence spectroscopy, the binding affinity of CaM for GST NR2A (875-1029) was found to be 5 ± 2.4 nM. Future studies should be concentrated on the physiological relevance for this interaction.

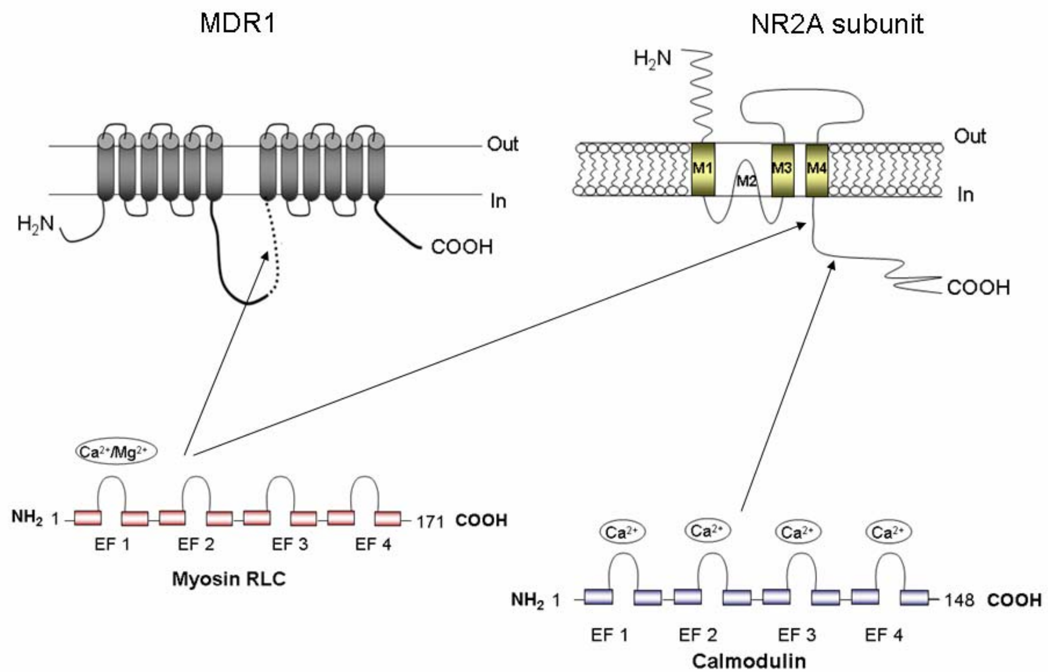


Figure 5.1 EF-hand proteins as binding partners of NR2A subunit of NMDA receptor and MDR1. NR2A subunit of NMDA receptor and MDR1 are two membrane bound proteins. Myosin RLC, an EF-hand protein and a light chain of myosin II, interacts with the intracellular linker region of MDR1 and NR2A C-terminus in a similar manner through first three EF-hand domains. Interaction of myosin RLC with the NR2A subunit facilitates forward trafficking of NMDA receptors containing NR1/NR2A receptors. Also, myosin RLC interaction with MDR1 linker region is important for its function and apical expression in MDCKII-MDR1 cells. Calmodulin, another EF-hand protein, interacts with NR2A C-terminal at a place other that myosin RLC in a calcium dependent manner. Ca^{2+}/calmodulin have been shown to inactivate NMDA receptors by interacting with NR1 subunit. This interaction of calmodulin with NR2A subunit is a novel interaction and is believed to be important for NMDA receptor function which needs further investigation.

BIBLIOGRAPHY

Akyol Z, Bartos JA, Merrill MA, Faga LA, Jaren OR, Shea MA and Hell JW (2004) Apo-calmodulin binds with its C-terminal domain to the N-methyl-D-aspartate receptor NR1 C0 region. *The Journal of biological chemistry* **279**(3):2166-2175.

Allan VJ, Thompson HM and McNiven MA (2002) Motoring around the Golgi. *Nature cell biology* **4**(10):E236-242.

Allison DW, Chervin AS, Gelfand VI and Craig AM (2000) Postsynaptic scaffolds of excitatory and inhibitory synapses in hippocampal neurons: maintenance of core components independent of actin filaments and microtubules. *J Neurosci* **20**(12):4545-4554.

Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y and Kaibuchi K (1996) Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *The Journal of biological chemistry* **271**(34):20246-20249.

Amparan D, Avram D, Thomas CG, Lindahl MG, Yang J, Bajaj G and Ishmael JE (2005) Direct interaction of myosin regulatory light chain with the NMDA receptor. *Journal of neurochemistry* **92**(2):349-361.

Aprison MH and Werman R (1965) The distribution of glycine in cat spinal cord and roots. *Life sciences* **4**(21):2075-2083.

Ataman ZA, Gakhar L, Sorensen BR, Hell JW and Shea MA (2007) The NMDA receptor NR1 C1 region bound to calmodulin: structural insights into functional differences between homologous domains. *Structure* **15**(12):1603-1617.

Bahler M and Rhoads A (2002) Calmodulin signaling via the IQ motif. *FEBS letters* **513**(1):107-113.

Bahloul A, Chevreux G, Wells AL, Martin D, Nolt J, Yang Z, Chen LQ, Potier N, Van Dorsselaer A, Rosenfeld S, Houdusse A and Sweeney HL (2004) The unique insert in myosin VI is a structural calcium-calmodulin binding site. *Proceedings of the National Academy of Sciences of the United States of America* **101**(14):4787-4792.

Baker JP and Titus MA (1997) A family of unconventional myosins from the nematode Caenorhabditis elegans. *Journal of molecular biology* **272**(4):523-535.

Bankstahl JP, Hoffmann K, Bethmann K and Loscher W (2008) Glutamate is critically involved in seizure-induced overexpression of P-glycoprotein in the brain. *Neuropharmacology* **54**(6):1006-1016.

Barylko B, Binns DD and Albanesi JP (2000) Regulation of the enzymatic and motor activities of myosin I. *Biochimica et biophysica acta* **1496**(1):23-35.

Baudier J, Deloulme JC, Van Dorsselaer A, Black D and Matthes HW (1991) Purification and characterization of a brain-specific protein kinase C substrate, neurogranin (p17). Identification of a consensus amino acid

sequence between neurogranin and neuromodulin (GAP43) that corresponds to the protein kinase C phosphorylation site and the calmodulin-binding domain. *The Journal of biological chemistry* **266**(1):229-237.

Bendayan R, Lee G and Bendayan M (2002) Functional expression and localization of P-glycoprotein at the blood brain barrier. *Microscopy research and technique* **57**(5):365-380.

Bendayan R, Ronaldson PT, Gingras D and Bendayan M (2006) In situ localization of P-glycoprotein (ABCB1) in human and rat brain. *J Histochem Cytochem* **54**(10):1159-1167.

Bennett JA and Dingledine R (1995) Topology profile for a glutamate receptor: three transmembrane domains and a channel-lining reentrant membrane loop. *Neuron* **14**(2):373-384.

Benveniste M and Mayer ML (1991) Kinetic analysis of antagonist action at N-methyl-D-aspartic acid receptors. Two binding sites each for glutamate and glycine. *Biophysical journal* **59**(3):560-573.

Berg JS, Derfler BH, Pennisi CM, Corey DP and Cheney RE (2000) Myosin-X, a novel myosin with pleckstrin homology domains, associates with regions of dynamic actin. *Journal of cell science* **113 Pt 19**:3439-3451.

Bezanilla M, Forsburg SL and Pollard TD (1997) Identification of a second myosin-II in Schizosaccharomyces pombe: Myp2p is conditionally required for cytokinesis. *Molecular biology of the cell* **8**(12):2693-2705.

Blackstone CD, Moss SJ, Martin LJ, Levey AI, Price DL and Huganir RL (1992) Biochemical characterization and localization of a non-N-methyl-D-aspartate glutamate receptor in rat brain. *Journal of neurochemistry* **58**(3):1118-1126.

Blanpied TA, Scott DB and Ehlers MD (2002) Dynamics and regulation of clathrin coats at specialized endocytic zones of dendrites and spines. *Neuron* **36**(3):435-449.

Bliss TV and Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**(6407):31-39.

Block SM (1996) Fifty ways to love your lever: myosin motors. *Cell* **87**(2):151-157.

Bornhauser BC, Johansson C and Lindholm D (2003) Functional activities and cellular localization of the ezrin, radixin, moesin (ERM) and RING zinc finger domains in MIR. *FEBS letters* **553**(1-2):195-199.

Boyne JR, Yosuf HM, Bieganowski P, Brenner C and Price C (2000) Yeast myosin light chain, Mlc1p, interacts with both IQGAP and class II myosin to effect cytokinesis. *Journal of cell science* **113 Pt 24**:4533-4543.

Brady ST, Lasek RJ and Allen RD (1985) Video microscopy of fast axonal transport in extruded axoplasm: a new model for study of molecular mechanisms. *Cell motility* **5**(2):81-101.

Bresnick AR (1999) Molecular mechanisms of nonmuscle myosin-II regulation. *Current opinion in cell biology* **11**(1):26-33.

Brose N, Gasic GP, Vetter DE, Sullivan JM and Heinemann SF (1993) Protein chemical characterization and immunocytochemical localization of the NMDA receptor subunit NMDA R1. *The Journal of biological chemistry* **268**(30):22663-22671.

Carafoli E and Klee CB (1999) *Calcium as a cellular regulator*. Oxford University Press, New York.

Celio MR, Pauls TL and Schwaller B (1996) *Guidebook to the calcium-binding proteins*. Sambrook & Tooze Publication at Oxford University Press, Oxford.

Chan W, Calderon G, Swift AL, Moseley J, Li S, Hosoya H, Arias IM and Ortiz DF (2005a) Myosin II regulatory light chain is required for trafficking of bile salt export protein (BSEP) to the apical membrane in MDCK cells. *The Journal of biological chemistry* **280**(25):23741-23747.

Chan W, Calderon G, Swift AL, Moseley J, Li S, Hosoya H, Arias IM and Ortiz DF (2005b) Myosin II regulatory light chain is required for trafficking of bile salt export protein to the apical membrane in Madin-Darby canine kidney cells. *The Journal of biological chemistry* **280**(25):23741-23747.

Chatterton JE, Awobuluyi M, Premkumar LS, Takahashi H, Talantova M, Shin Y, Cui J, Tu S, Sevarino KA, Nakanishi N, Tong G, Lipton SA and Zhang D (2002) Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. *Nature* **415**(6873):793-798.

Chazot PL and Stephenson FA (1997) Molecular dissection of native mammalian forebrain NMDA receptors containing the NR1 C2 exon: direct demonstration of NMDA receptors comprising NR1, NR2A, and NR2B subunits within the same complex. *Journal of neurochemistry* **69**(5):2138-2144.

Chen BS and Roche KW (2007) Regulation of NMDA receptors by phosphorylation. *Neuropharmacology* **53**(3):362-368.

Chen CJ, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM and Roninson IB (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47**(3):381-389.

Cheney RE and Mooseker MS (1992) Unconventional myosins. *Current opinion in cell biology* **4**(1):27-35.

Cheney RE, O'Shea MK, Heuser JE, Coelho MV, Wolenski JS, Espreafico EM, Forscher P, Larson RE and Mooseker MS (1993) Brain myosin-V is a two-headed unconventional myosin with motor activity. *Cell* **75**(1):13-23.

Cheng D, Hoogenraad CC, Rush J, Ramm E, Schlager MA, Duong DM, Xu P, Wijayawardana SR, Hanfelt J, Nakagawa T, Sheng M and Peng J (2006) Relative and absolute quantification of postsynaptic density

proteome isolated from rat forebrain and cerebellum. *Mol Cell Proteomics* **5**(6):1158-1170.

Choi DW (1995) Calcium: still center-stage in hypoxic-ischemic neuronal death. *Trends Neurosci* **18**(2):58-60.

Choi DW, Koh JY and Peters S (1988) Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. *J Neurosci* **8**(1):185-196.

Choi YB and Lipton SA (1999) Identification and mechanism of action of two histidine residues underlying high-affinity Zn2+ inhibition of the NMDA receptor. *Neuron* **23**(1):171-180.

Chung HJ, Huang YH, Lau LF and Huganir RL (2004) Regulation of the NMDA receptor complex and trafficking by activity-dependent phosphorylation of the NR2B subunit PDZ ligand. *J Neurosci* **24**(45):10248-10259.

Ciabarra AM, Sullivan JM, Gahn LG, Pecht G, Heinemann S and Sevarino KA (1995) Cloning and characterization of chi-1: a developmentally regulated member of a novel class of the ionotropic glutamate receptor family. *J Neurosci* **15**(10):6498-6508.

Clements JD and Westbrook GL (1991) Activation kinetics reveal the number of glutamate and glycine binding sites on the N-methyl-D-aspartate receptor. *Neuron* **7**(4):605-613.

Collingridge GL and Watkins JC (1994) *The NMDA receptor*. Oxford University Press, Oxford ; New York.

Collins MO, Husi H, Yu L, Brandon JM, Anderson CN, Blackstock WP, Choudhary JS and Grant SG (2006) Molecular characterization and comparison of the components and multiprotein complexes in the postsynaptic proteome. *Journal of neurochemistry* **97 Suppl 1**:16-23.

Condeelis JS and Taylor DL (1977) The contractile basis of amoeboid movement. V. The control of gelation, solation, and contraction in extracts from Dictyostelium discoideum. *The Journal of cell biology* **74**(3):901-927.

Constantine-Paton M, Cline HT and Debski E (1990) Patterned activity, synaptic convergence, and the NMDA receptor in developing visual pathways. *Annual review of neuroscience* **13**:129-154.

Conti MA and Adelstein RS (2008) Nonmuscle myosin II moves in new directions. *Journal of cell science* **121**(Pt 1):11-18.

Corsi M, Fina P and Trist DG (1996) Co-agonism in drug-receptor interaction: illustrated by the NMDA receptors. *Trends in pharmacological sciences* **17**(6):220-222.

Cramer LP (1999) Organization and polarity of actin filament networks in cells: implications for the mechanism of myosin-based cell motility. *Biochemical Society symposium* **65**:173-205.

Cremo CR, Wang F, Facemyer K and Sellers JR (2001) Phosphorylation-dependent regulation is absent in a nonmuscle heavy meromyosin

construct with one complete head and one head lacking the motor domain. *The Journal of biological chemistry* **276**(44):41465-41472.

Cull-Candy S, Brickley S and Farrant M (2001) NMDA receptor subunits: diversity, development and disease. *Current opinion in neurobiology* **11**(3):327-335.

Das S, Sasaki YF, Rothe T, Premkumar LS, Takasu M, Crandall JE, Dikkes P, Conner DA, Rayudu PV, Cheung W, Chen HS, Lipton SA and Nakanishi N (1998) Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A. *Nature* **393**(6683):377-381.

De Lozanne A and Spudich JA (1987) Disruption of the Dictyostelium myosin heavy chain gene by homologous recombination. *Science (New York, NY* **236**(4805):1086-1091.

Dean M and Allikmets R (2001) Complete characterization of the human ABC gene family. *Journal of bioenergetics and biomembranes* **33**(6):475-479.

Dean M, Rzhetsky A and Allikmets R (2001) The human ATP-binding cassette (ABC) transporter superfamily. *Genome research* **11**(7):1156-1166.

DePina AS and Langford GM (1999) Vesicle transport: the role of actin filaments and myosin motors. *Microscopy research and technique* **47**(2):93-106.

DePina AS, Wollert T and Langford GM (2007) Membrane associated nonmuscle myosin II functions as a motor for actin-based vesicle transport in clam oocyte extracts. *Cell motility and the cytoskeleton* **64**(10):739-755.

Dominguez R, Freyzon Y, Trybus KM and Cohen C (1998) Crystal structure of a vertebrate smooth muscle myosin motor domain and its complex with the essential light chain: visualization of the pre-power stroke state. *Cell* **94**(5):559-571.

Durand GM, Gregor P, Zheng X, Bennett MV, Uhl GR and Zukin RS (1992) Cloning of an apparent splice variant of the rat N-methyl-D-aspartate receptor NMDAR1 with altered sensitivity to polyamines and activators of protein kinase C. *Proceedings of the National Academy of Sciences of the United States of America* **89**(19):9359-9363.

Ehlers MD, Zhang S, Bernhadt JP and Huganir RL (1996) Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* **84**(5):745-755.

Erreger K, Chen PE, Wyllie DJ and Traynelis SF (2004) Glutamate receptor gating. *Critical reviews in neurobiology* **16**(3):187-224.

Erreger K, Dravid SM, Banke TG, Wyllie DJ and Traynelis SF (2005) Subunit-specific gating controls rat NR1/NR2A and NR1/NR2B NMDA channel kinetics and synaptic signalling profiles. *The Journal of physiology* **563**(Pt 2):345-358.

Espindola FS, Suter DM, Partata LB, Cao T, Wolenski JS, Cheney RE, King SM and Mooseker MS (2000) The light chain composition of chicken

brain myosin-Va: calmodulin, myosin-II essential light chains, and 8-kDa dynein light chain/PIN. *Cell motility and the cytoskeleton* **47**(4):269-281.

Espreafico EM, Cheney RE, Matteoli M, Nascimento AA, De Camilli PV, Larson RE and Mooseker MS (1992) Primary structure and cellular localization of chicken brain myosin-V (p190), an unconventional myosin with calmodulin light chains. *The Journal of cell biology* **119**(6):1541-1557.

Ethell IM and Pasquale EB (2005) Molecular mechanisms of dendritic spine development and remodeling. *Progress in neurobiology* **75**(3):161-205.

Farah CS and Reinach FC (1995) The troponin complex and regulation of muscle contraction. *Faseb J* **9**(9):755-767.

Fath KR (2005) Characterization of myosin-II binding to Golgi stacks in vitro. *Cell motility and the cytoskeleton* **60**(4):222-235.

Feinstein DL, Durand M and Milner RJ (1991) Expression of myosin regulatory light chains in rat brain: characterization of a novel isoform. *Brain Res Mol Brain Res* **10**(2):97-105.

Finn BE, Evenas J, Drakenberg T, Waltho JP, Thulin E and Forsen S (1995) Calcium-induced structural changes and domain autonomy in calmodulin. *Nature structural biology* **2**(9):777-783.

Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman MM and Pastan I (1987) Expression of a multidrug-resistance gene in human tumors and tissues. *Proceedings of the National Academy of Sciences of the United States of America* **84**(1):265-269.

Franke JD, Boury AL, Gerald NJ and Kiehart DP (2006) Native nonmuscle myosin II stability and light chain binding in Drosophila melanogaster. *Cell motility and the cytoskeleton* **63**(10):604-622.

Fu D, Bebawy M, Kable EP and Roufogalis BD (2004) Dynamic and intracellular trafficking of P-glycoprotein-EGFP fusion protein: Implications in multidrug resistance in cancer. *International journal of cancer* **109**(2):174-181.

Fukunaga Y, Matsubara M, Nagai R and Miyazawa A (2005) The interaction between PSD-95 and Ca2+/calmodulin is enhanced by PDZ-binding proteins. *Journal of biochemistry* **138**(2):177-182.

Furukawa H and Gouaux E (2003) Mechanisms of activation, inhibition and specificity: crystal structures of the NMDA receptor NR1 ligand-binding core. *The EMBO journal* **22**(12):2873-2885.

Furukawa H, Singh SK, Mancusso R and Gouaux E (2005) Subunit arrangement and function in NMDA receptors. *Nature* **438**(7065):185-192.

Furusawa T, Ikawa S, Yanai N and Obinata M (2000) Isolation of a novel PDZ-containing myosin from hematopoietic supportive bone marrow stromal cell lines. *Biochemical and biophysical research communications* **270**(1):67-75.

Gallagher PJ, Herring BP, Griffin SA and Stull JT (1991) Molecular characterization of a mammalian smooth muscle myosin light chain kinase. *The Journal of biological chemistry* **266**(35):23936-23944.

Garcia A, Coudrier E, Carboni J, Anderson J, Vandekerkhove J, Mooseker M, Louvard D and Arpin M (1989) Partial deduced sequence of the 110-kD-calmodulin complex of the avian intestinal microvillus shows that this mechanoenzyme is a member of the myosin I family. *The Journal of cell biology* **109**(6 Pt 1):2895-2903.

Gardoni F, Bellone C, Cattabeni F and Di Luca M (2001) Protein kinase C activation modulates alpha-calmodulin kinase II binding to NR2A subunit of N-methyl-D-aspartate receptor complex. *The Journal of biological chemistry* **276**(10):7609-7613.

Gardoni F, Polli F, Cattabeni F and Di Luca M (2006) Calcium-calmodulin-dependent protein kinase II phosphorylation modulates PSD-95 binding to NMDA receptors. *The European journal of neuroscience* **24**(10):2694-2704.

Geiser JR, van Tuinen D, Brockerhoff SE, Neff MM and Davis TN (1991) Can calmodulin function without binding calcium? *Cell* **65**(6):949-959.

Georges E (2007) The P-glycoprotein (ABCB1) linker domain encodes high-affinity binding sequences to alpha- and beta-tubulins. *Biochemistry* **46**(25):7337-7342.

Gerges NZ, Backos DS and Esteban JA (2004) Local control of AMPA receptor trafficking at the postsynaptic terminal by a small GTPase of the Rab family. *The Journal of biological chemistry* **279**(42):43870-43878.

Gerloff T, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, Hofmann AF and Meier PJ (1998) The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. *The Journal of biological chemistry* **273**(16):10046-10050.

Germann UA (1996) P-glycoprotein--a mediator of multidrug resistance in tumour cells. *Eur J Cancer* **32A**(6):927-944.

Golomb E, Ma X, Jana SS, Preston YA, Kawamoto S, Shoham NG, Goldin E, Conti MA, Sellers JR and Adelstein RS (2004) Identification and characterization of nonmuscle myosin II-C, a new member of the myosin II family. *The Journal of biological chemistry* **279**(4):2800-2808.

Gottesman MM and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annual review of biochemistry* **62**:385-427.

Grabarek Z (2006) Structural basis for diversity of the EF-hand calcium-binding proteins. *Journal of molecular biology* **359**(3):509-525.

Graether SP, Heinonen TY, Raharjo WH, Jin JP and Mak AS (1997) Tryptophan residues in caldesmon are major determinants for calmodulin binding. *Biochemistry* **36**(2):364-369.

Grant ER, Guttmann RP, Seifert KM and Lynch DR (2001) A region of the rat N-methyl-D-aspartate receptor 2A subunit that is sufficient for potentiation by phorbol esters. *Neuroscience letters* **310**(1):9-12.

Guillaud L, Setou M and Hirokawa N (2003) KIF17 dynamics and regulation of NR2B trafficking in hippocampal neurons. *J Neurosci* **23**(1):131-140.

Hallett PJ and Standaert DG (2004) Rationale for and use of NMDA receptor antagonists in Parkinson's disease. *Pharmacology & therapeutics* **102**(2):155-174.

Haorah J, Knipe B, Leibhart J, Ghorpade A and Persidsky Y (2005) Alcohol-induced oxidative stress in brain endothelial cells causes blood-brain barrier dysfunction. *Journal of leukocyte biology* **78**(6):1223-1232.

Hasson T, Gillespie PG, Garcia JA, MacDonald RB, Zhao Y, Yee AG, Mooseker MS and Corey DP (1997) Unconventional myosins in inner-ear sensory epithelia. *The Journal of cell biology* **137**(6):1287-1307.

Hasson T and Mooseker MS (1997) The growing family of myosin motors and their role in neurons and sensory cells. *Current opinion in neurobiology* **7**(5):615-623.

Hawkins LM, Prybylowski K, Chang K, Moussan C, Stephenson FA and Wenthold RJ (2004) Export from the endoplasmic reticulum of assembled N-methyl-d-aspartic acid receptors is controlled by a motif in the c terminus of the NR2 subunit. *The Journal of biological chemistry* **279**(28):28903-28910.

Herm-Gotz A, Weiss S, Stratmann R, Fujita-Becker S, Ruff C, Meyhofer E, Soldati T, Manstein DJ, Geeves MA and Soldati D (2002) Toxoplasma gondii myosin A and its light chain: a fast, single-headed, plus-end-directed motor. *The EMBO journal* **21**(9):2149-2158.

Herrling PL (1997) *Excitatory amino acids : clinical results with antagonists*. Academic Press, San Diego.

Hettmann C, Herm A, Geiter A, Frank B, Schwarz E, Soldati T and Soldati D (2000) A dibasic motif in the tail of a class XIV apicomplexan myosin is an essential determinant of plasma membrane localization. *Molecular biology of the cell* **11**(4):1385-1400.

Higgins DG, Thompson JD and Gibson TJ (1996) Using CLUSTAL for multiple sequence alignments. *Methods in enzymology* **266**:383-402.

Hokfelt T and Ljungdahl A (1971) Light and electron microscopic autoradiography on spinal cord slices after incubation with labeled glycine. *Brain research* **32**(1):189-194.

Hollmann M and Heinemann S (1994) Cloned glutamate receptors. *Annual review of neuroscience* **17**:31-108.

Horak M, Chang K and Wenthold RJ (2008) Masking of the endoplasmic reticulum retention signals during assembly of the NMDA receptor. *J Neurosci* **28**(13):3500-3509.

Horowitz JA and Hammer JA, 3rd (1990) A new Acanthamoeba myosin heavy chain. Cloning of the gene and immunological identification of the polypeptide. *The Journal of biological chemistry* **265**(33):20646-20652.

Horton AC and Ehlers MD (2003) Dual modes of endoplasmic reticulum-to-Golgi transport in dendrites revealed by live-cell imaging. *J Neurosci* **23**(15):6188-6199.

Horton AC and Ehlers MD (2004) Secretory trafficking in neuronal dendrites. *Nature cell biology* **6**(7):585-591.

Houdusse A and Cohen C (1995) Target sequence recognition by the calmodulin superfamily: implications from light chain binding to the regulatory domain of scallop myosin. *Proceedings of the National Academy of Sciences of the United States of America* **92**(23):10644-10647.

Houdusse A, Silver M and Cohen C (1996) A model of Ca(2+)-free calmodulin binding to unconventional myosins reveals how calmodulin acts as a regulatory switch. *Structure* **4**(12):1475-1490.

Hrycyna CA, Airan LE, Germann UA, Ambudkar SV, Pastan I and Gottesman MM (1998) Structural flexibility of the linker region of human P-glycoprotein permits ATP hydrolysis and drug transport. *Biochemistry* **37**(39):13660-13673.

Hubbard MJ and Klee CB (1987) Calmodulin binding by calcineurin. Ligand-induced renaturation of protein immobilized on nitrocellulose. *The Journal of biological chemistry* **262**(31):15062-15070.

Huh KH and Wenthold RJ (1999) Turnover analysis of glutamate receptors identifies a rapidly degraded pool of the N-methyl-D-aspartate receptor subunit, NR1, in cultured cerebellar granule cells. *The Journal of biological chemistry* **274**(1):151-157.

Husi H and Grant SG (2001) Isolation of 2000-kDa complexes of N-methyl-D-aspartate receptor and postsynaptic density 95 from mouse brain. *Journal of neurochemistry* **77**(1):281-291.

Husi H, Ward MA, Choudhary JS, Blackstock WP and Grant SG (2000) Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. *Nature neuroscience* **3**(7):661-669.

Ikonen E, de Almeid JB, Fath KR, Burgess DR, Ashman K, Simons K and Stow JL (1997) Myosin II is associated with Golgi membranes: identification of p200 as nonmuscle myosin II on Golgi-derived vesicles. *Journal of cell science* **110 (Pt 18)**:2155-2164.

Ikura M, Clore GM, Gronenborn AM, Zhu G, Klee CB and Bax A (1992) Solution structure of a calmodulin-target peptide complex by multidimensional NMR. *Science (New York, NY* **256**(5057):632-638.

Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M and et al. (1993) Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. *The Journal of biological chemistry* **268**(4):2836-2843.

Ishmael JE, Franklin PH, Murray TF and Leid M (1996) High level expression of the NMDAR1 glutamate receptor subunit in electroporated COS cells. *Journal of neurochemistry* **67**(4):1500-1510.

Ishmael JE, Lohr CV, Fischer K and Kioussi C (2007a) Localization of myosin II regulatory light chain in the cerebral vasculature. *Acta Histochem*.

Ishmael JE, Safic M, Amparan D, Vogel WK, Pham T, Marley K, Filtz TM and Maier CS (2007b) Nonmuscle myosins II-B and Va are components of detergent-resistant membrane skeletons derived from mouse forebrain. *Brain research* **1143**:46-59.

Ito M, Nakano T, Erdodi F and Hartshorne DJ (2004) Myosin phosphatase: structure, regulation and function. *Molecular and cellular biochemistry* **259**(1-2):197-209.

Johnson JW and Ascher P (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* **325**(6104):529-531.

Jones ML and Leonard JP (2005) PKC site mutations reveal differential modulation by insulin of NMDA receptors containing NR2A or NR2B subunits. *Journal of neurochemistry* **92**(6):1431-1438.

Jordan BA, Fernholz BD, Boussac M, Xu C, Grigorean G, Ziff EB and Neubert TA (2004) Identification and verification of novel rodent postsynaptic density proteins. *Mol Cell Proteomics* **3**(9):857-871.

Kadotani H, Hirano T, Masugi M, Nakamura K, Nakao K, Katsuki M and Nakanishi S (1996) Motor discoordination results from combined gene disruption of the NMDA receptor NR2A and NR2C subunits, but not from single disruption of the NR2A or NR2C subunit. *J Neurosci* **16**(24):7859-7867.

Kaech S, Parmar H, Roelandse M, Bornmann C and Matus A (2001) Cytoskeletal microdifferentiation: a mechanism for organizing morphological plasticity in dendrites. *Proceedings of the National Academy of Sciences of the United States of America* **98**(13):7086-7092.

Karavanova I, Vasudevan K, Cheng J and Buonanno A (2007) Novel regional and developmental NMDA receptor expression patterns uncovered in NR2C subunit-beta-galactosidase knock-in mice. *Molecular and cellular neurosciences* **34**(3):468-480.

Karp SJ, Masu M, Eki T, Ozawa K and Nakanishi S (1993) Molecular cloning and chromosomal localization of the key subunit of the human N-methyl-D-aspartate receptor. *The Journal of biological chemistry* **268**(5):3728-3733.

Karssen AM, Meijer O, Pons D and De Kloet ER (2004) Localization of mRNA expression of P-glycoprotein at the blood-brain barrier and in the hippocampus. *Annals of the New York Academy of Sciences* **1032**:308-311.

Kawasaki H, Nakayama S and Kretsinger RH (1998) Classification and evolution of EF-hand proteins. *Biometals* **11**(4):277-295.

Kim E and Sheng M (2004) PDZ domain proteins of synapses. *Nature reviews* **5**(10):771-781.

Kioussi C, Appu M, Lohr CV, Fischer KA, Bajaj G, Leid M and Ishmael JE (2007) Co-expression of myosin II regulatory light chain and the NMDAR1 subunit in neonatal and adult mouse brain. *Brain research bulletin* **74**(6):439-451.

Knowlton MN, Chan BM and Kelly GM (2003) The zebrafish band 4.1 member Mir is involved in cell movements associated with gastrulation. *Developmental biology* **264**(2):407-429.

Knowlton MN and Kelly GM (2004) Zebrafish mir antagonizes frizzled 7-induced gastrulation defects. *Zebrafish* **1**(2):133-144.

Kolling R and Losko S (1997) The linker region of the ABC-transporter Ste6 mediates ubiquitination and fast turnover of the protein. *The EMBO journal* **16**(9):2251-2261.

Korn ED (2000) Coevolution of head, neck, and tail domains of myosin heavy chains. *Proceedings of the National Academy of Sciences of the United States of America* **97**(23):12559-12564.

Korn ED and Hammer JA, 3rd (1990) Myosin I. *Current opinion in cell biology* **2**(1):57-61.

Kornau HC, Schenker LT, Kennedy MB and Seeburg PH (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science (New York, NY* **269**(5231):1737-1740.

Kovacs M, Toth J, Hetenyi C, Malnasi-Csizmadia A and Sellers JR (2004) Mechanism of blebbistatin inhibition of myosin II. *The Journal of biological chemistry* **279**(34):35557-35563.

Kretsinger RH and Nockolds CE (1973) Carp muscle calcium-binding protein. II. Structure determination and general description. *The Journal of biological chemistry* **248**(9):3313-3326.

Krupp JJ, Vissel B, Heinemann SF and Westbrook GL (1996) Calcium-dependent inactivation of recombinant N-methyl-D-aspartate receptors is NR2 subunit specific. *Molecular pharmacology* **50**(6):1680-1688.

Krupp JJ, Vissel B, Heinemann SF and Westbrook GL (1998) N-terminal domains in the NR2 subunit control desensitization of NMDA receptors. *Neuron* **20**(2):317-327.

Krupp JJ, Vissel B, Thomas CG, Heinemann SF and Westbrook GL (1999) Interactions of calmodulin and alpha-actinin with the NR1 subunit modulate Ca2+-dependent inactivation of NMDA receptors. *J Neurosci* **19**(4):1165-1178.

Krupp JJ, Vissel B, Thomas CG, Heinemann SF and Westbrook GL (2002) Calcineurin acts via the C-terminus of NR2A to modulate desensitization of NMDA receptors. *Neuropharmacology* **42**(5):593-602.

Kuffler SW and Edwards C (1958) Mechanism of gamma aminobutyric acid (GABA) action and its relation to synaptic inhibition. *Journal of neurophysiology* **21**(6):589-610.

Kuhlmann CR, Lessmann V and Luhmann HJ (2006) Fluvastatin stabilizes the blood-brain barrier in vitro by nitric oxide-dependent dephosphorylation of myosin light chains. *Neuropharmacology* **51**(4):907-913.

Kuner T and Schoepfer R (1996) Multiple structural elements determine subunit specificity of Mg2+ block in NMDA receptor channels. *J Neurosci* **16**(11):3549-3558.

Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M and et al. (1992) Molecular diversity of the NMDA receptor channel. *Nature* **358**(6381):36-41.

Lau CG and Zukin RS (2007) NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. *Nature reviews* **8**(6):413-426.

Lavezzari G, McCallum J, Lee R and Roche KW (2003) Differential binding of the AP-2 adaptor complex and PSD-95 to the C-terminus of the NMDA receptor subunit NR2B regulates surface expression. *Neuropharmacology* **45**(6):729-737.

Lee HK (2006) Synaptic plasticity and phosphorylation. *Pharmacology & therapeutics* **112**(3):810-832.

Lee JM, Zipfel GJ and Choi DW (1999) The changing landscape of ischaemic brain injury mechanisms. *Nature* **399**(6738 Suppl):A7-14.

Legendre P, Rosenmund C and Westbrook GL (1993) Inactivation of NMDA channels in cultured hippocampal neurons by intracellular calcium. *J Neurosci* **13**(2):674-684.

Lei S, Czerwinska E, Czerwinski W, Walsh MP and MacDonald JF (2001) Regulation of NMDA receptor activity by F-actin and myosin light chain kinase. *J Neurosci* **21**(21):8464-8472.

Leonard AS, Lim IA, Hemsworth DE, Horne MC and Hell JW (1999) Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor. *Proceedings of the National Academy of Sciences of the United States of America* **96**(6):3239-3244.

Li B, Chen N, Luo T, Otsu Y, Murphy TH and Raymond LA (2002) Differential regulation of synaptic and extra-synaptic NMDA receptors. *Nature neuroscience* **5**(9):833-834.

Liao GY, Wagner DA, Hsu MH and Leonard JP (2001) Evidence for direct protein kinase-C mediated modulation of N-methyl-D-aspartate receptor current. *Molecular pharmacology* **59**(5):960-964.

Loscher W and Potschka H (2005a) Drug resistance in brain diseases and the role of drug efflux transporters. *Nature reviews* **6**(8):591-602.

Loscher W and Potschka H (2005b) Role of drug efflux transporters in the brain for drug disposition and treatment of brain diseases. *Progress in neurobiology* **76**(1):22-76.

Lu J, Helton TD, Blanpied TA, Racz B, Newpher TM, Weinberg RJ and Ehlers MD (2007) Postsynaptic positioning of endocytic zones and AMPA

receptor cycling by physical coupling of dynamin-3 to Homer. *Neuron* **55**(6):874-889.

Ludowyke RI, Peleg I, Beaven MA and Adelstein RS (1989) Antigen-induced secretion of histamine and the phosphorylation of myosin by protein kinase C in rat basophilic leukemia cells. *The Journal of biological chemistry* **264**(21):12492-12501.

Luo J, Vallen EA, Dravis C, Tcheperegine SE, Drees B and Bi E (2004) Identification and functional analysis of the essential and regulatory light chains of the only type II myosin Myo1p in Saccharomyces cerevisiae. *The Journal of cell biology* **165**(6):843-855.

Maciver SK (1996) Myosin II function in non-muscle cells. *Bioessays* **18**(3):179-182.

Maeda M, Kuwayama H, Yokoyama M, Nishio K, Morio T, Urushihara H, Katoh M, Tanaka Y, Saito T, Ochiai H, Takemoto K, Yasukawa H and Takeuchi I (2000) Developmental changes in the spatial expression of genes involved in myosin function in Dictyostelium. *Developmental biology* **223**(1):114-119.

Malencik DA and Anderson SR (1982) Binding of simple peptides, hormones, and neurotransmitters by calmodulin. *Biochemistry* **21**(14):3480-3486.

Malencik DA and Anderson SR (1988) Association of melittin with the isolated myosin light chains. *Biochemistry* **27**(6):1941-1949.

Mammen AL, Kamboj S and Huganir RL (1999) Protein phosphorylation of ligand-gated ion channels. *Methods in enzymology* **294**:353-370.

Marchi N, Hallene KL, Kight KM, Cucullo L, Moddel G, Bingaman W, Dini G, Vezzani A and Janigro D (2004) Significance of MDR1 and multiple drug resistance in refractory human epileptic brain. *BMC medicine* **2**:37.

Mayer ML and Armstrong N (2004) Structure and function of glutamate receptor ion channels. *Annual review of physiology* **66**:161-181.

Mayer ML, Benveniste M, Patneau DK and Vyklicky L, Jr. (1992) Pharmacologic properties of NMDA receptors. *Annals of the New York Academy of Sciences* **648**:194-204.

Mayer ML and Westbrook GL (1987a) Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones. *The Journal of physiology* **394**:501-527.

Mayer ML and Westbrook GL (1987b) The physiology of excitatory amino acids in the vertebrate central nervous system. *Progress in neurobiology* **28**(3):197-276.

Mayer ML, Westbrook GL and Guthrie PB (1984) Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. *Nature* **309**(5965):261-263.

McBain CJ and Mayer ML (1994) N-methyl-D-aspartic acid receptor structure and function. *Physiological reviews* **74**(3):723-760.

Meador WE, Means AR and Quiocho FA (1993) Modulation of calmodulin plasticity in molecular recognition on the basis of x-ray structures. *Science (New York, NY* **262**(5140):1718-1721.

Meguro H, Mori H, Araki K, Kushiya E, Kutsuwada T, Yamazaki M, Kumanishi T, Arakawa M, Sakimura K and Mishina M (1992) Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. *Nature* **357**(6373):70-74.

Montell C and Rubin GM (1988) The Drosophila ninaC locus encodes two photoreceptor cell specific proteins with domains homologous to protein kinases and the myosin heavy chain head. *Cell* **52**(5):757-772.

Monyer H, Burnashev N, Laurie DJ, Sakmann B and Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12**(3):529-540.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B and Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science (New York, NY* **256**(5060):1217-1221.

Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N and Nakanishi S (1991) Molecular cloning and characterization of the rat NMDA receptor. *Nature* **354**(6348):31-37.

Mu Y, Otsuka T, Horton AC, Scott DB and Ehlers MD (2003) Activity-dependent mRNA splicing controls ER export and synaptic delivery of NMDA receptors. *Neuron* **40**(3):581-594.

Musch A, Cohen D and Rodriguez-Boulan E (1997) Myosin II is involved in the production of constitutive transport vesicles from the TGN. *The Journal of cell biology* **138**(2):291-306.

Nagano K, Bornhauser BC, Warnasuriya G, Entwistle A, Cramer R, Lindholm D and Naaby-Hansen S (2006) PDGF regulates the actin cytoskeleton through hnRNP-K-mediated activation of the ubiquitin E3-ligase MIR. *The EMBO journal* **25**(9):1871-1882.

Najm I, Ying Z, Babb T, Crino PB, Macdonald R, Mathern GW and Spreafico R (2004) Mechanisms of epileptogenicity in cortical dysplasias. *Neurology* **62**(6 Suppl 3):S9-13.

Nakanishi N, Axel R and Shneider NA (1992) Alternative splicing generates functionally distinct N-methyl-D-aspartate receptors. *Proceedings of the National Academy of Sciences of the United States of America* **89**(18):8552-8556.

Nakanishi S, Masu M, Bessho Y, Nakajima Y, Hayashi Y and Shigemoto R (1994) Molecular diversity of glutamate receptors and their physiological functions. *Exs* **71**:71-80.

Nicoll RA and Malenka RC (1995) Contrasting properties of two forms of long-term potentiation in the hippocampus. *Nature* **377**(6545):115-118.

Nusser Z, Lujan R, Laube G, Roberts JD, Molnar E and Somogyi P (1998) Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. *Neuron* **21**(3):545-559.

Nuti SL and Rao US (2002) Proteolytic Cleavage of the Linker Region of the Human P-glycoprotein Modulates Its ATPase Function. *The Journal of biological chemistry* **277**(33):29417-29423.

O'Neil KT and DeGrado WF (1990) How calmodulin binds its targets: sequence independent recognition of amphiphilic alpha-helices. *Trends in biochemical sciences* **15**(2):59-64.

Olsson PA, Bornhauser BC, Korhonen L and Lindholm D (2000) Neuronal expression of the ERM-like protein MIR in rat brain and its localization to human chromosome 6. *Biochemical and biophysical research communications* **279**(3):879-883.

Olsson PA, Korhonen L, Mercer EA and Lindholm D (1999) MIR is a novel ERM-like protein that interacts with myosin regulatory light chain and inhibits neurite outgrowth. *The Journal of biological chemistry* **274**(51):36288-36292.

Ortiz DF, Moseley J, Calderon G, Swift AL, Li S and Arias IM (2004) Identification of HAX-1 as a protein that binds bile salt export protein and regulates its abundance in the apical membrane of Madin-Darby canine kidney cells. *The Journal of biological chemistry* **279**(31):32761-32770.

Paoletti P, Vergnano AM, Barbour B and Casado M (2008) Zinc at glutamatergic synapses. *Neuroscience*.

Park M, Penick EC, Edwards JG, Kauer JA and Ehlers MD (2004) Recycling endosomes supply AMPA receptors for LTP. *Science (New York, NY* **305**(5692):1972-1975.

Peng J, Kim MJ, Cheng D, Duong DM, Gygi SP and Sheng M (2004) Semiquantitative proteomic analysis of rat forebrain postsynaptic density fractions by mass spectrometry. *The Journal of biological chemistry* **279**(20):21003-21011.

Perez-Otano I, Schulteis CT, Contractor A, Lipton SA, Trimmer JS, Sucher NJ and Heinemann SF (2001) Assembly with the NR1 subunit is required for surface expression of NR3A-containing NMDA receptors. *J Neurosci* **21**(4):1228-1237.

Persechini A and Kretsinger RH (1988) Toward a model of the calmodulin-myosin light-chain kinase complex: implications for calmodulin function. *Journal of cardiovascular pharmacology* **12 Suppl 5**:S1-12.

Peters A, Palay SL and Webster Hd (1991) *The fine structure of the nervous system : neurons and their supporting cells*. Oxford University Press, New York.

Planells-Cases R, Sun W, Ferrer-Montiel AV and Montal M (1993) Molecular cloning, functional expression, and pharmacological characterization of an N-methyl-D-aspartate receptor subunit from human brain.

Proceedings of the National Academy of Sciences of the United States of America **90**(11):5057-5061.

Pollard TD, Doberstein SK and Zot HG (1991) Myosin-I. *Annual review of physiology* **53**:653-681.

Potschka H, Fedrowitz M and Loscher W (2001) P-glycoprotein and multidrug resistance-associated protein are involved in the regulation of extracellular levels of the major antiepileptic drug carbamazepine in the brain. *Neuroreport* **12**(16):3557-3560.

Potschka H, Fedrowitz M and Loscher W (2002) P-Glycoprotein-mediated efflux of phenobarbital, lamotrigine, and felbamate at the blood-brain barrier: evidence from microdialysis experiments in rats. *Neuroscience letters* **327**(3):173-176.

Potschka H and Loscher W (2001) In vivo evidence for P-glycoprotein-mediated transport of phenytoin at the blood-brain barrier of rats. *Epilepsia* **42**(10):1231-1240.

Prybylowski K, Chang K, Sans N, Kan L, Vicini S and Wenthold RJ (2005) The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2. *Neuron* **47**(6):845-857.

Racca C, Stephenson FA, Streit P, Roberts JD and Somogyi P (2000) NMDA receptor content of synapses in stratum radiatum of the hippocampal CA1 area. *J Neurosci* **20**(7):2512-2522.

Rachline J, Perin-Dureau F, Le Goff A, Neyton J and Paoletti P (2005) The micromolar zinc-binding domain on the NMDA receptor subunit NR2B. *J Neurosci* **25**(2):308-317.

Racz B, Blanpied TA, Ehlers MD and Weinberg RJ (2004) Lateral organization of endocytic machinery in dendritic spines. *Nature neuroscience* **7**(9):917-918.

Rao PS, Mallya KB, Srivenugopal KS, Balaji KC and Rao US (2006) RNF2 interacts with the linker region of the human P-glycoprotein. *International journal of oncology* **29**(6):1413-1419.

Rayment I, Rypniewski WR, Schmidt-Base K, Smith R, Tomchick DR, Benning MM, Winkelmann DA, Wesenberg G and Holden HM (1993) Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science (New York, NY* **261**(5117):50-58.

Reck-Peterson SL, Provance DW, Jr., Mooseker MS and Mercer JA (2000) Class V myosins. *Biochimica et biophysica acta* **1496**(1):36-51.

Reinhard J, Scheel AA, Diekmann D, Hall A, Ruppert C and Bahler M (1995) A novel type of myosin implicated in signalling by rho family GTPases. *The EMBO journal* **14**(4):697-704.

Rhoads AR and Friedberg F (1997) Sequence motifs for calmodulin recognition. *Faseb J* **11**(5):331-340.

Roche KW, Standley S, McCallum J, Dune Ly C, Ehlers MD and Wenthold RJ (2001) Molecular determinants of NMDA receptor internalization. *Nature neuroscience* **4**(8):794-802.

Roche KW, Tingley WG and Huganir RL (1994) Glutamate receptor phosphorylation and synaptic plasticity. *Current opinion in neurobiology* **4**(3):383-388.

Rosenmund C, Feltz A and Westbrook GL (1995) Calcium-dependent inactivation of synaptic NMDA receptors in hippocampal neurons. *Journal of neurophysiology* **73**(1):427-430.

Rosenmund C and Westbrook GL (1993) Calcium-induced actin depolymerization reduces NMDA channel activity. *Neuron* **10**(5):805-814.

Ryu J, Liu L, Wong TP, Wu DC, Burette A, Weinberg R, Wang YT and Sheng M (2006) A critical role for myosin IIb in dendritic spine morphology and synaptic function. *Neuron* **49**(2):175-182.

Saito N, Okada Y, Noda Y, Kinoshita Y, Kondo S and Hirokawa N (1997) KIFC2 is a novel neuron-specific C-terminal type kinesin superfamily motor for dendritic transport of multivesicular body-like organelles. *Neuron* **18**(3):425-438.

Sans N, Petralia RS, Wang YX, Blahos J, 2nd, Hell JW and Wenthold RJ (2000) A developmental change in NMDA receptor-associated proteins at hippocampal synapses. *J Neurosci* **20**(3):1260-1271.

Sans N, Prybylowski K, Petralia RS, Chang K, Wang YX, Racca C, Vicini S and Wenthold RJ (2003) NMDA receptor trafficking through an interaction between PDZ proteins and the exocyst complex. *Nature cell biology* **5**(6):520-530.

Sans N, Wang PY, Du Q, Petralia RS, Wang YX, Nakka S, Blumer JB, Macara IG and Wenthold RJ (2005) mPins modulates PSD-95 and SAP102 trafficking and influences NMDA receptor surface expression. *Nature cell biology* **7**(12):1179-1190.

Sauna ZE and Ambudkar SV (2001) Characterization of the catalytic cycle of ATP hydrolysis by human P-glycoprotein. The two ATP hydrolysis events in a single catalytic cycle are kinetically similar but affect different functional outcomes. *The Journal of biological chemistry* **276**(15):11653-11661.

Scarpini E, Scheltens P and Feldman H (2003) Treatment of Alzheimer's disease: current status and new perspectives. *Lancet neurology* **2**(9):539-547.

Schorge S and Colquhoun D (2003) Studies of NMDA receptor function and stoichiometry with truncated and tandem subunits. *J Neurosci* **23**(4):1151-1158.

Scott DB, Blanpied TA, Swanson GT, Zhang C and Ehlers MD (2001) An NMDA receptor ER retention signal regulated by phosphorylation and alternative splicing. *J Neurosci* **21**(9):3063-3072.

Scott DB, Michailidis I, Mu Y, Logothetis D and Ehlers MD (2004) Endocytosis and degradative sorting of NMDA receptors by conserved membrane-proximal signals. *J Neurosci* **24**(32):7096-7109.

Sellers JR (1999) *Myosins*. Oxford University Press, Oxford ; New York.

Sellers JR (2000) Myosins: a diverse superfamily. *Biochimica et biophysica acta* **1496**(1):3-22.

Setou M, Nakagawa T, Seog DH and Hirokawa N (2000) Kinesin superfamily motor protein KIF17 and mLin-10 in NMDA receptor-containing vesicle transport. *Science (New York, NY* **288**(5472):1796-1802.

Shannon KB and Li R (2000) A myosin light chain mediates the localization of the budding yeast IQGAP-like protein during contractile ring formation. *Curr Biol* **10**(12):727-730.

Shen X, Valencia CA, Szostak JW, Dong B and Liu R (2005) Scanning the human proteome for calmodulin-binding proteins. *Proceedings of the National Academy of Sciences of the United States of America* **102**(17):5969-5974.

Sheng M, Cummings J, Roldan LA, Jan YN and Jan LY (1994) Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature* **368**(6467):144-147.

Sheng M and Hoogenraad CC (2007) The postsynaptic architecture of excitatory synapses: a more quantitative view. *Annual review of biochemistry* **76**:823-847.

Simons M, Wang M, McBride OW, Kawamoto S, Yamakawa K, Gdula D, Adelstein RS and Weir L (1991) Human nonmuscle myosin heavy chains are encoded by two genes located on different chromosomes. *Circulation research* **69**(2):530-539.

Sisodiya SM, Lin WR, Harding BN, Squier MV and Thom M (2002) Drug resistance in epilepsy: expression of drug resistance proteins in common causes of refractory epilepsy. *Brain* **125**(Pt 1):22-31.

Sisodiya SM, Martinian L, Scheffer GL, van der Valk P, Scheper RJ, Harding BN and Thom M (2006) Vascular colocalization of P-glycoprotein, multidrug-resistance associated protein 1, breast cancer resistance protein and major vault protein in human epileptogenic pathologies. *Neuropathology and applied neurobiology* **32**(1):51-63.

Sisodiya SM and Thom M (2003) Widespread upregulation of drug-resistance proteins in fatal human status epilepticus. *Epilepsia* **44**(2):261-264.

Smothers CT and Woodward JJ (2003) Effect of the NR3 subunit on ethanol inhibition of recombinant NMDA receptors. *Brain research* **987**(1):117-121.

Sprengel R and Single FN (1999) Mice with genetically modified NMDA and AMPA receptors. *Annals of the New York Academy of Sciences* **868**:494-501.

Sprengel R, Suchanek B, Amico C, Brusa R, Burnashev N, Rozov A, Hvalby O, Jensen V, Paulsen O, Andersen P, Kim JJ, Thompson RF, Sun W, Webster LC, Grant SG, Eilers J, Konnerth A, Li J, McNamara JO and Seeburg PH (1998) Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. *Cell* **92**(2):279-289.

Standley S, Roche KW, McCallum J, Sans N and Wenthold RJ (2000) PDZ domain suppression of an ER retention signal in NMDA receptor NR1 splice variants. *Neuron* **28**(3):887-898.

Steigerwald F, Schulz TW, Schenker LT, Kennedy MB, Seeburg PH and Kohr G (2000) C-Terminal truncation of NR2A subunits impairs synaptic but not extrasynaptic localization of NMDA receptors. *J Neurosci* **20**(12):4573-4581.

Stephenson FA, Cousins SL and Kenny AV (2008) Assembly and forward trafficking of NMDA receptors (Review). *Molecular membrane biology* **25**(4):311-320.

Stern P, Behe P, Schoepfer R and Colquhoun D (1992) Single-channel conductances of NMDA receptors expressed from cloned cDNAs: comparison with native receptors. *Proceedings* **250**(1329):271-277.

Stevens RC and Davis TN (1998) Mlc1p is a light chain for the unconventional myosin Myo2p in Saccharomyces cerevisiae. *The Journal of cell biology* **142**(3):711-722.

Strack S and Colbran RJ (1998) Autophosphorylation-dependent targeting of calcium/ calmodulin-dependent protein kinase II by the NR2B subunit of the N-methyl- D-aspartate receptor. *The Journal of biological chemistry* **273**(33):20689-20692.

Sugihara H, Moriyoshi K, Ishii T, Masu M and Nakanishi S (1992) Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. *Biochemical and biophysical research communications* **185**(3):826-832.

Szymanski PT (2004) Calponin (CaP) as a latch-bridge protein--a new concept in regulation of contractility in smooth muscles. *Journal of muscle research and cell motility* **25**(1):7-19.

Tada T and Sheng M (2006) Molecular mechanisms of dendritic spine morphogenesis. *Current opinion in neurobiology* **16**(1):95-101.

Takahashi M, Kawamoto S and Adelstein RS (1992) Evidence for inserted sequences in the head region of nonmuscle myosin specific to the nervous system. Cloning of the cDNA encoding the myosin heavy chain-B isoform of vertebrate nonmuscle myosin. *The Journal of biological chemistry* **267**(25):17864-17871.

Tan JL, Ravid S and Spudich JA (1992) Control of nonmuscle myosins by phosphorylation. *Annual review of biochemistry* **61**:721-759.

Taubman MB, Grant JW and Nadal-Ginard B (1987) Cloning and characterization of mammalian myosin regulatory light chain (RLC) cDNA: the RLC gene is expressed in smooth, sarcomeric, and nonmuscle tissues. *The Journal of cell biology* **104**(6):1505-1513.

Terrak M, Otterbein LR, Wu G, Palecanda LA, Lu RC and Dominguez R (2002) Crystallization, X-ray characterization and selenomethionine phasing of Mlc1p bound to IQ motifs from myosin V. *Acta Crystallogr D Biol Crystallogr* **58**(Pt 10 Pt 2):1882-1885.

Terrak M, Wu G, Stafford WF, Lu RC and Dominguez R (2003) Two distinct myosin light chain structures are induced by specific variations within the bound IQ motifs-functional implications. *The EMBO journal* **22**(3):362-371.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I and Willingham MC (1987) Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proceedings of the National Academy of Sciences of the United States of America* **84**(21):7735-7738.

Tichelaar W, Safferling M, Keinanen K, Stark H and Madden DR (2004) The Three-dimensional Structure of an Ionotropic Glutamate Receptor Reveals a Dimer-of-dimers Assembly. *Journal of molecular biology* **344**(2):435-442.

Tingley WG, Ehlers MD, Kameyama K, Doherty C, Ptak JB, Riley CT and Huganir RL (1997) Characterization of protein kinase A and protein kinase C phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. *The Journal of biological chemistry* **272**(8):5157-5166.

Tong G, Shepherd D and Jahr CE (1995) Synaptic desensitization of NMDA receptors by calcineurin. *Science (New York, NY* **267**(5203):1510-1512.

Tovar KR, Sprouffske K and Westbrook GL (2000) Fast NMDA receptor-mediated synaptic currents in neurons from mice lacking the epsilon2 (NR2B) subunit. *Journal of neurophysiology* **83**(1):616-620.

Tovar KR and Westbrook GL (1999) The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *J Neurosci* **19**(10):4180-4188.

Trybus KM, Waller GS and Chatman TA (1994) Coupling of ATPase activity and motility in smooth muscle myosin is mediated by the regulatory light chain. *The Journal of cell biology* **124**(6):963-969.

Ulbrich MH and Isacoff EY (2007) Subunit counting in membrane-bound proteins. *Nature methods* **4**(4):319-321.

Van Eldik LJ and Watterson DM (1998) *Calmodulin and signal transduction*. Academic Press, San Diego.

Vissel B, Krupp JJ, Heinemann SF and Westbrook GL (2001) A use-dependent tyrosine dephosphorylation of NMDA receptors is independent of ion flux. *Nature neuroscience* **4**(6):587-596.

Vissel B, Krupp JJ, Heinemann SF and Westbrook GL (2002) Intracellular domains of NR2 alter calcium-dependent inactivation of N-methyl-D-aspartate receptors. *Molecular pharmacology* **61**(3):595-605.

Wakabayashi Y, Kipp H and Arias IM (2006) Transporters on demand: intracellular reservoirs and cycling of bile canalicular ABC transporters. *The Journal of biological chemistry* **281**(38):27669-27673.

Walikonis RS, Jensen ON, Mann M, Provance DW, Jr., Mercer JA and Kennedy MB (2000) Identification of proteins in the postsynaptic density fraction by mass spectrometry. *J Neurosci* **20**(11):4069-4080.

Wang C, Wang HG, Xie H and Pitt GS (2008) Ca2+/CaM controls Ca2+-dependent inactivation of NMDA receptors by dimerizing the NR1 C termini. *J Neurosci* **28**(8):1865-1870.

Wang F, Kovacs M, Hu A, Limouze J, Harvey EV and Sellers JR (2003) Kinetic mechanism of non-muscle myosin IIB: functional adaptations for tension generation and maintenance. *The Journal of biological chemistry* **278**(30):27439-27448.

Wang Y and Chantler PD (1994) Functional analysis of individual brain myosin II isoforms through hybrid formation. *FEBS letters* **348**(3):244-248.

Wang YT and Salter MW (1994) Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature* **369**(6477):233-235.

Wang YT, Yu XM and Salter MW (1996) Ca(2+)-independent reduction of N-methyl-D-aspartate channel activity by protein tyrosine phosphatase. *Proceedings of the National Academy of Sciences of the United States of America* **93**(4):1721-1725.

Warrick HM and Spudich JA (1987) Myosin structure and function in cell motility. *Annual review of cell biology* **3**:379-421.

Washbourne P, Bennett JE and McAllister AK (2002) Rapid recruitment of NMDA receptor transport packets to nascent synapses. *Nature neuroscience* **5**(8):751-759.

Washbourne P, Liu XB, Jones EG and McAllister AK (2004) Cycling of NMDA receptors during trafficking in neurons before synapse formation. *J Neurosci* **24**(38):8253-8264.

Watanabe M, Inoue Y, Sakimura K and Mishina M (1992) Developmental changes in distribution of NMDA receptor channel subunit mRNAs. *Neuroreport* **3**(12):1138-1140.

Watkins JC and Evans RH (1981) Excitatory amino acid transmitters. *Annual review of pharmacology and toxicology* **21**:165-204.

Waxman EA and Lynch DR (2005) N-methyl-D-aspartate receptor subtypes: multiple roles in excitotoxicity and neurological disease. *Neuroscientist* **11**(1):37-49.

Weber A and Murray JM (1973) Molecular control mechanisms in muscle contraction. *Physiological reviews* **53**(3):612-673.

Wechsler A and Teichberg VI (1998) Brain spectrin binding to the NMDA receptor is regulated by phosphorylation, calcium and calmodulin. *The EMBO journal* **17**(14):3931-3939.

Weissbach L, Bernards A and Herion DW (1998) Binding of myosin essential light chain to the cytoskeleton-associated protein IQGAP1. *Biochemical and biophysical research communications* **251**(1):269-276.

Wenthold RJ, Prybylowski K, Standley S, Sans N and Petralia RS (2003) Trafficking of NMDA receptors. *Annual review of pharmacology and toxicology* **43**:335-358.

Westbrook GL and Mayer ML (1987) Micromolar concentrations of Zn2+ antagonize NMDA and GABA responses of hippocampal neurons. *Nature* **328**(6131):640-643.

Wong EH, Kemp JA, Priestley T, Knight AR, Woodruff GN and Iversen LL (1986) The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. *Proceedings of the National Academy of Sciences of the United States of America* **83**(18):7104-7108.

Wylie SR and Chantler PD (2001) Separate but linked functions of conventional myosins modulate adhesion and neurite outgrowth. *Nature cell biology* **3**(1):88-92.

Wylie SR, Wu PJ, Patel H and Chantler PD (1998) A conventional myosin motor drives neurite outgrowth. *Proceedings of the National Academy of Sciences of the United States of America* **95**(22):12967-12972.

Wyllie DJ, Behe P, Nassar M, Schoepfer R and Colquhoun D (1996) Single-channel currents from recombinant NMDA NR1a/NR2D receptors expressed in Xenopus oocytes. *Proceedings* **263**(1373):1079-1086.

Wyszynski M, Kharazia V, Shanghvi R, Rao A, Beggs AH, Craig AM, Weinberg R and Sheng M (1998) Differential regional expression and ultrastructural localization of alpha-actinin-2, a putative NMDA receptor-anchoring protein, in rat brain. *J Neurosci* **18**(4):1383-1392.

Wyszynski M, Lin J, Rao A, Nigh E, Beggs AH, Craig AM and Sheng M (1997) Competitive binding of alpha-actinin and calmodulin to the NMDA receptor. *Nature* **385**(6615):439-442.

Xie X, Harrison DH, Schlichting I, Sweet RM, Kalabokis VN, Szent-Gyorgyi AG and Cohen C (1994) Structure of the regulatory domain of scallop myosin at 2.8 A resolution. *Nature* **368**(6469):306-312.

Yamakita Y, Yamashiro S and Matsumura F (1994) In vivo phosphorylation of regulatory light chain of myosin II during mitosis of cultured cells. *The Journal of cell biology* **124**(1-2):129-137.

Yamashita RA, Sellers JR and Anderson JB (2000) Identification and analysis of the myosin superfamily in Drosophila: a database approach. *Journal of muscle research and cell motility* **21**(6):491-505.

Yang W, Zheng C, Song Q, Yang X, Qiu S, Liu C, Chen Z, Duan S and Luo J (2007) A three amino acid tail following the TM4 region of the N-methyl-D-aspartate receptor (NR) 2 subunits is sufficient to overcome endoplasmic reticulum retention of NR1-1a subunit. *The Journal of biological chemistry* **282**(12):9269-9278.

Yao PJ, Bushlin I and Petralia RS (2006) Partially overlapping distribution of epsin1 and HIP1 at the synapse: analysis by immunoelectron microscopy. *The Journal of comparative neurology* **494**(2):368-379.

Yap KL, Kim J, Truong K, Sherman M, Yuan T and Ikura M (2000) Calmodulin target database. *Journal of structural and functional genomics* **1**(1):8-14.

Yuan T and Vogel HJ (1998) Calcium-calmodulin-induced dimerization of the carboxyl-terminal domain from petunia glutamate decarboxylase. A novel calmodulin-peptide interaction motif. *The Journal of biological chemistry* **273**(46):30328-30335.

Zavodny PJ, Petro ME, Kumar CC, Dailey SH, Lonial HK, Narula SK and Leibowitz PJ (1988) The nucleotide sequence of chicken smooth muscle myosin light chain two. *Nucleic acids research* **16**(3):1214.

Zegers MM and Hoekstra D (1998) Mechanisms and functional features of polarized membrane traffic in epithelial and hepatic cells. *The Biochemical journal* **336 (Pt 2)**:257-269.

Zhang H, Webb DJ, Asmussen H, Niu S and Horwitz AF (2005) A GIT1/PIX/Rac/PAK signaling module regulates spine morphogenesis and synapse formation through MLC. *J Neurosci* **25**(13):3379-3388.

Zhang M, Tanaka T and Ikura M (1995) Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. *Nature structural biology* **2**(9):758-767.

Zhang S, Ehlers MD, Bernhardt JP, Su CT and Huganir RL (1998) Calmodulin mediates calcium-dependent inactivation of N-methyl-D-aspartate receptors. *Neuron* **21**(2):443-453.

Zhang Y, Vogel WK, McCullar JS, Greenwood JA and Filtz TM (2006) Phospholipase C-beta3 and -beta1 form homodimers, but not heterodimers, through catalytic and carboxyl-terminal domains. *Molecular pharmacology* **70**(3):860-868.

Zheng L, Baumann U and Reymond JL (2004) An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic acids research* **32**(14):e115.